
BIOMEDICAL VIBRATIONAL SPECTROSCOPY

Edited By

Peter Lasch
Janina Kneipp

 **WILEY-
INTERSCIENCE**

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PREFACE

The interdisciplinary field of biomedical vibrational spectroscopy comprises a growing body of methods that support the development of practical applications in microbiology, cytology, histology, and clinical chemistry. This is not only due to the advantages inherent to vibrational spectroscopic methods, but also a result of the spectacular technological progress seen in the last 15 years. As rapid photonic techniques, infrared (IR) and Raman spectroscopy provide objective information on molecular structure and composition of the samples under investigation. The ease of sample preparation and the speed of the measurement with collection times in the range of seconds or minutes qualify both methods for the operator-independent, cost-efficient and nondestructive characterization of a sample's biochemistry. Therefore, they offer great promise for *in vivo* and *ex vivo* biomedical diagnosis. Furthermore, the rapid development of both vibrational spectroscopic techniques has benefited considerably from the technological progress and scientific breakthroughs, in particular in the fields of light sources, multichannel detector technology, nanotechnology, and optics in general. As in many other technology-driven fields, these developments have been additionally triggered by advances in computer science and information technology.

The contributions in this book provide an overview of state-of-the-art experimental methods and applications of IR and Raman spectroscopy in biomedicine. The first part of this volume contains chapters on established technical concepts and experimental approaches and their applications in biomedical diagnostics and clinical chemistry. In an introductory contribution, D. Naumann provides his view of the field and discusses the nature of the spectroscopic information, technical options, and the perspectives of vibrational spectroscopic methods in microbiology and biomedical diagnostics. The chapter by H. M. Heise reviews technical solutions of IR and Raman spectroscopic applications for clinical chemistry and pathology *in vitro*, *in situ*, and *in vivo*.

In vibrational spectroscopic studies of histological and cytological specimens, the combination of spectroscopy with microscopy is particularly useful, because it enables localized biochemical characterization of cells or tissues. D. L. Wetzel discusses various applications of IR microspectroscopy and IR imaging and reviews important instrumental means for their realization, such as ultra-bright synchrotron light sources and focal plane array detectors. R. A. Shaw et al. provide a chapter on the utilization of IR spectroscopy of biofluids in clinical chemistry and illustrate how the method can be employed for disease diagnosis. The potential of Raman spectroscopy for the characterization of body fluids *ex vivo* and *in vivo* is demonstrated by D. Rohleder and W. Petrich.

The capabilities of Raman microspectroscopy for studies of cells and tissues was demonstrated more than a decade ago. Meanwhile, owing to the progress in instrumentation and the availability of high-quality commercial Raman microscopes, Raman spectroscopy-based diagnostic tools are being developed. In the chapter by M. J. Romeo et al., both IR and Raman microspectroscopy are employed to characterize cells and tissues with high spatial resolution.

In the second part of the book, attractive new vibrational spectroscopic techniques with high potential for biomedical applications are presented. While some of these methods are still in the phase of maturation, others demonstrate their immediate applicability to diagnostic problems or to the elucidation of pathophysiological mechanisms. The possibilities

of exciting Raman scattering in resonance with an electronic transition in the sample molecule and the resulting signal enhancement are discussed in two chapters for the example of heme groups in cells: H.-J. van Manen et al. introduce us to spectroscopy and spectral imaging of heme proteins in leukocytes and discuss experimental concepts and limitations. A contribution by B. R. Wood and D. McNaughton reviews resonant Raman spectroscopy in red blood cells and heme molecules in the solid state and its application in malaria research. Improvements in the analytical sensitivity of the inherently inefficient Raman scattering process can also be achieved by coherent anti-Stokes Raman scattering (CARS). As demonstrated by O. Burkacky and A. Zumbusch, CARS has evolved into a sensitive microscopic method that provides a great amount of chemical structure information from cells and other samples.

The favorable properties of localized surface plasmons and the utilization of nanostructures supporting them are another means of improving both the Raman scattering cross sections and the lateral resolution. The first can be employed to construct sensors for metabolites as is shown by O. Lyandres et al., who used surface-enhanced Raman scattering (SERS) for the detection of glucose, lactate, and other analytes from plasma. The group employed multivariate analysis of SERS data for quantitative biosensing *in vivo*. SERS microspectroscopic experiments at nanometer-scale lateral precision in cells are reported by J. Kneipp et al., who studied the endosomal system of cultured cells by this method.

Another direction of current research is the combination of different methods for optical diagnosis. In the chapter by L.-P. Choo-Smith et al., a combination of optical coherence tomography and Raman spectroscopy is demonstrated for the detection of caries. A number of experimental methods have also been proposed to overcome the diffraction limit of far-field IR microspectroscopy. A. Dazzi explains in his contribution a photothermal method that directly measures the expansion of a tiny sample due to IR absorption, and he illustrates its applicability for IR imaging of individual virus particles inside bacterial cells.

As the experimental tools for IR and Raman studies become established and new ones are developed, proofs of their usefulness in medical diagnostics are gaining more and more importance. Likewise, enormous amounts of spectral data require appropriate concepts and specific tools for data analysis. In the third part of this book, we therefore discuss fundamental aspects of study design and present adequate concepts for the analysis of vibrational spectra as multivariate data. W. Petrich has contributed a chapter that exemplifies how clinical study concepts can be realized in practice. It is also demonstrated how multivariate spectral analysis is applied for quantification of analytes from body fluids and for disease pattern recognition (classification). A. Kohler, W. Martens, and co-workers present a multiblock analysis method that can be employed to analyze and interpret several data sets from one type of biological sample. Although multivariate methods proved very valuable for the analysis of vibrational spectra, the strength of biomedical vibrational spectroscopy is greatly enhanced when the univariate molecular structure information is incorporated into the mindset for data analysis. The interplay of univariate and multivariate concepts of spectral analysis is demonstrated in the chapter by G. Zhang et al. These authors present examples of spectral imaging of skin and bone.

We are grateful to all authors who have shared their experience and knowledge in this book.

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JANINA KNEIPP

Berlin, September 2007

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VIBRATIONAL SPECTROSCOPY IN MICROBIOLOGY AND MEDICAL DIAGNOSTICS

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Infrared (IR) and Raman spectroscopy are relatively old spectroscopic modalities that provide pictures of the molecular vibrations performed by molecules. Since the early experiments of Herschel, who more than 200 years ago discovered heat transporting radiation beyond the range of visible light, it took some 80 years until the first IR spectrum of an organic liquid was obtained. Since then, IR spectroscopy developed into the “workhorse” of vibrational spectroscopy in fundamental science and the industries, while Raman spectroscopy, discovered only in 1928, was initially restricted to a few laboratories in the academic area. Infrared and Raman spectroscopy, though fundamentally different in experimental design and physical background, give complementary information on molecular vibrations and should ideally be used together to attain access to the totality of all vibrational modes of a given molecule.

It has been only for the last two or three decades that both types of vibrational spectroscopy have been used systematically for the more complex building blocks of biological systems or even intact cells, tissues, and biological fluids. These scientific endeavors were facilitated by technological innovations such as the advent of Fourier transform (FT)-IR spectrometers, powerful low-cost lasers in the near-IR region, sensitive detector systems, and rapid low-cost computers, which favored new developments such as focal plane array detectors for true IR imaging systems or surface-enhanced Raman techniques based on nanostructured materials as optically active elements.

The progress achieved and the practical applications realized until now have definitely disproved the notion that IR or Raman spectroscopy are “old-fashioned technologies” useful only for pure systems and relatively small molecules. It has been convincingly proven that

IR and Raman spectra of cells, tissues, or biofluids encode sufficient spectral information to distinguish between different cell types, tissue structures, and biofluids and even to detect changes in these biological materials induced by pathological processes.

1.1 VIBRATIONAL SPECTRA IN BIOMEDICINE PROVIDE FINGERPRINT-LIKE SIGNATURES OF BIOLOGICAL STRUCTURES

A rationale behind the belief that vibrational spectroscopy may be useful to diagnose diseases or pathologies in individuals is that disease processes must, generally speaking, be accompanied by changes in the chemistry/biochemistry of cells, tissues, organs, or body fluids, and vibrational spectroscopy is indeed ideally suited for sensitive detection of such changes as a diagnostic technique. It has furthermore been anticipated that these changes should be detectable also before morphological and systemic manifestation allow clinical diagnosis by conventional methods. Given the fact that sample preparation and measurement are very simple and collection times are in the range of seconds or minutes, IR and Raman spectroscopy should be ideal modalities to establish very rapid nonsubjective and cost-effective tools for early diagnosis of disease processes in individuals.

Biomedical IR and Raman spectroscopy probe biological samples in a way that the active vibrational modes of all constituents present in the mixture are observed in a single experiment, resulting in very complex spectra with broad and superimposed spectral features throughout the whole spectral range. Thus, in contrast to fluorescence spectra, obtained from a biological material labeled with some fluorescing dye, common IR and Raman spectra of intact cells, tissues, or body fluids cannot provide information on a single or even a few specific compounds present. Instead, the spectra provide spectroscopic fingerprints of the total chemical and biochemical composition of the material under study. This situation inevitably results from the fact that the complex superposition of the characteristic IR absorption or Raman signals of all constituents in biomaterials (nucleic acids, proteins, carbohydrates, lipids, and other low molecular compounds, etc.) are observed simultaneously, thereby producing spectral features that encode a vast amount of information potentially useful for bidiagnostic purposes. One peculiarity of vibrational spectroscopy is that it provides information not only on the composition of complex biological material but also on structural states of the molecules under study, since certain bands are sensitive, for example, to the secondary structure in proteins, while others report on the state of order of the membranes or the conformation of the nucleic acid structures. In this sense the total information content in vibrational spectra of biological materials is enormous. One can possibly say that there are presently no other techniques available that can provide such a huge amount of information in one single experiment. On the other hand, this fact severely limits assignments of experimentally observed bands to single discrete structures and qualifies the techniques mainly as fingerprinting methods, though the assignment of spectral bands has improved significantly in the last two centuries due to, for example, spectral resolution enhancement and “spectral feature extraction” capabilities that allow us to more efficiently visualize and resolve specific, hidden bands from the complex spectral signatures.

The nature of information obtained in biomedical vibrational spectroscopy is represented best by the notion of “spectral fingerprints.” Thus, the analysis of these spectral signatures by evaluating peak intensities, frequencies, or half-widths of a few bands that can by some means be resolved will fail in most cases. Moreover, taking into account that thousands of spectra have to be analyzed at a given time, the availability of intelligent data

evaluation concepts is a virtual necessity that should ideally include efficient data pretreatment algorithms such as quality testing, normalization, filtering, and adequate multivariate statistical techniques to achieve data reduction and finally the classification of patterns. With such methods, even hundreds of thousands of spectra – as is the case in spectroscopic imaging – can be analyzed.

Vibrational spectra of cells, tissues, and biofluids are obviously the expression of the sum of cellular chemistry/biochemistry and structure. Therefore they provide an “OMICS”-like view of the total chemical/biochemical status of the samples and give a snapshot on cell division, differentiation, growth and metabolism. In this view, vibrational spectroscopic techniques provide information on phenotypes and mirror transcriptional and translational up- and down-regulation processes and post-translational modifications. In a strict sense, vibrational spectroscopies as applied to biofluids, cells, or tissues are, however, not typical metabolomic techniques. Their advantage is possibly that in some way the totality of all chemical/biochemical changes including those in the pool of nucleic acids, proteins, or low molecular metabolic compounds are reflected in the spectra, constituting a technique that cannot easily be assigned to one of the known “OMICS” disciplines in life science such as genomics, transcriptomics, proteomics, or metabolomics. But, as do the common “OMICS” methods, they deal with complex systems in their entirety and with the simultaneous analysis of many biological individuals or objects rather than a single property of a single gene or metabolic product. In many cases the situation might be similar to global metabolic fingerprinting, but one has to bear in mind that the basis of changes observed does not necessarily have to be purely metabolic. This definition qualifies vibrational spectroscopies as explorative and rapid analysis techniques par excellence, which can be used to diagnose disease or dysfunction via spectral biomarkers that change as indicators of the presence of a particular disease or in response to drug intervention, environmental stress, or genetic modification. When nothing or little is known about an observed phenomenon, vibrational spectroscopy may provide a first hint for further, possibly more specific investigations. This is particularly the case when changing systems, whether it is a cell suspension of synchronized cells or cells treated with some specific drug are measured time-dependently. Such experiments can, however, be done with vibrational spectroscopic techniques in a few minutes compared to serial measurements using, for example, fluorescence labels, testing many genes or separating and analyzing proteins or metabolites from complex mixtures. Therefore, the fundamental fingerprinting nature of vibrational spectra of complex biological samples is a big advantage. It is, however, a disadvantage at the same time, since comprehensive understanding of these spectra is desirable but not achievable in most cases.

1.2 DIFFERENT TECHNICAL OPTIONS TO OBTAIN THE SPECTRAL INFORMATION

The most important step forward in biomedical vibrational spectroscopy within the last two decades is certainly the coupling of spectrometers to light microscopes to obtain spectral information from single cells or to achieve spatial resolution in tissue analysis in a way that is familiar to biologists or pathologists. Since then the technological progress has been enormous and high-quality IR and Raman microscopes are available on the market, which can be used to image tissues and single cells and even analyze subcellular compartments. Raman imaging systems that do not rely on spectral point-by-point mapping are not yet on the market, thus precluding Raman imaging under clinical constraints. Notwithstanding,

tissue or subcellular imaging by various different Raman microspectroscopic modalities provides a wealth of biological information not available by other techniques. Today, focal plane array detectors for mid-IR imaging allow rapid segmentation of histological structures without any tissue staining and to image larger cells. Using focal plane array systems, pioneering applications have been published on IR imaging of various soft and hard tissues and a vast number of pathologies. Infrared synchrotron radiation sources coupled with IR microscopes allowed the analysis of single living cells growing in culture with unprecedented high signal-to-noise ratio and reproducibility, opening up the possibility to perform strict difference spectroscopic investigations on viable cells – for example, after treatment with drugs or other chemicals.

Other technical developments such as fiber-optic probes have dramatically increased the possibilities to use Raman spectroscopy as a diagnostic biomedical tool. Fiber-optic applications useful for *in vivo* applications have made greatest progress in Raman spectroscopy, since the production of Raman compatible fiber probes can be based on materials already developed for fiber-based telecommunications or fiber-based chemical sensors. Compared to this situation, optical halide fibers necessary for mid-IR spectroscopy are only available for a few laboratories apart from the detrimental fact that IR radiation has too small penetration depths and problems with strong water absorptions to be useful for *in vivo* experiments.

SERS is a very sensitive Raman modality that can detect and characterize extremely small amounts of nucleic acids, proteins, or virus particles and can also characterize biomolecular events in subcellular compartments. The attractiveness of SERS relies on detection limits close to immunoassay sensitivities with femtomolar detection of, for example, prostate-specific antigen. Tip-enhanced Raman spectroscopy (TERS), another SERS modality, combines SERS spectroscopy with scanning probe technologies and provides lateral resolutions of around 20 nm and thus provides the possibility to study the surface chemistry and structure or composition of cell membranes and cell walls.

Many scientists have realized that IR spectroscopy has great potentials as a fingerprinting technique, useful for the very rapid diagnosis of disease or dysfunction in humans and animals with high-throughput screening capabilities. At present, however, IR and Raman spectroscopy seem to be best developed in microbiology and clinical chemistry, and first dedicated systems for use under practical conditions are already on the market; also, the development of vibrational spectroscopy based diagnostics for *in vivo* glucose screening is near to practical translation. It has also been recognized that vibrational spectroscopies are simple and economical techniques to screen for changes in cells or body fluids in response to drug-based intervention, environmental stress, or genetic modifications in organisms. The results obtained with bone, cartilage, and dental tissues are impressive, and the possibility of practical applications developed for clinical or other medical settings seems to be obvious. The FT-IR imaging data obtained on colon, prostate, or brain cancer are also significant and could be good candidates for translation to routine applications using benchtop IR imaging system as the technical platform.

1.3 THE NEED FOR AND BENEFIT FROM DATA EVALUATION

The necessity to use multivariate pattern recognition methodologies when dealing with spectral data of complex biomedical materials has been realized by the spectroscopic community more than 20 years ago. Among the first who recognized this problem were scientists working with IR spectroscopic data of intact microorganisms. While

univariate statistical analysis considered only a single property of a given selection of microbial species (e.g., a single intensity or frequency value at a given wavenumber or peak), multivariate statistical methods allowed the evaluation of several, if not all, properties of the spectra at the same time. Only in this way the interrelations between the sample properties and the spectra could be figured out. This learning process has been facilitated at that early time by the need to handle thousands of measurements on hundreds of different microbial species and strains, to evaluate these data systematically for spectral similarity, and to exchange data between different laboratories.

Out of the large number of pattern recognition techniques that are presently used for, or have been adapted to, vibrational spectroscopic data, *factor analysis techniques* like principal component analysis (PCA) and hierarchical clustering analysis (HCA) or *classification methodologies* such as artificial neural nets (ANN), support vector machines (SVM), and linear discriminant analysis (LDA) have experienced broad acceptance. Factor analysis is frequently used to achieve data reduction and the classification of patterns in large data sets, and hierarchical clustering (a so-called unsupervised or data-driven classification method) also attempts to find intrinsic similarity structures within the data sets without the need for any *a priori* class assignment, while ANN analysis as a supervised or concept-driven classification technique needs the class assignment of each individual object from the beginning. Partitioning of the whole data set into a so-called training and internal validation data subset is needed to train the system for optimal performance. It took some years by the spectroscopic community to learn that only independent data sets from ideally blinded samples should be used to objectively test the performance and robustness of the classifier and to evaluate the accuracy of the established models.

Meanwhile, nearly the whole arsenal of multivariate bioinformatic techniques is used, and multivariate statistical analysis of spectroscopic data constitutes an own discipline within the scientific area of biomedical spectroscopy. As for any other scientific discipline, these methods not only can be used to evaluate given data sets, but also allow completely new problem solutions to be addressed. New applications arose, for example, when it was realized that determining the covariance between different large data matrices obtained from the same sample populations with fundamentally different techniques is not only a challenge *per se*, but also provides insight into the interlink between biological structures. One of these new applications recently published was the use of genetic algorithms in combination with partial least-square regression (PLSR) analysis to correlate genes selected from gene expression profiles obtained by microarray technologies to metabolic markers from spectral data sets measured from the same samples by IR spectroscopy. The analysis of covariance patterns in these very complex mixed data sets helped to rapidly recognize and visualize the interrelationships and trends in a developing and changing biological system that is not easily achieved by any other means.

1.4 PERSPECTIVES OF BIOMEDICAL VIBRATIONAL SPECTROSCOPY

Despite all the fascinating potential and technological developments and the vast amount of exciting research papers in the literature, progress toward factual translation of vibrational spectroscopic techniques to practical applications is less evident. Moreover, the present situation of a multiplicity of different vibrational spectroscopic modalities, which are viewed by the nonspecialists as competing technologies, is possibly confusing.

The use of IR and Raman spectroscopy for microbial characterization and identification is presently the best developed and most frequent application of biomedical vibrational

spectroscopy. It is especially remarkable that both spectroscopies are applied in microbiological laboratories not only for research purposes but also for routine analysis, for example, in the food industry for microbiological quality control to guide adequate production measures. This situation has greatly been promoted by dedicated high-throughput IR and Raman instrumentation available now on the market. New avenues of microbiological applications can be expected from the use of IR or Raman microscopes, whether it will be for (a) the microspectroscopic analysis of microcolonies to speed up identification of microorganisms and analyze mixed populations of cells or (b) the identification of single cells directly from environmental samples. The combination of IR focal plane array detectors and microarray printing technologies may contribute to make microbiological IR analysis an extremely rapid, cost-effective and unprecedented high-throughput technology for microbiological analyses. This technology may not only help to scale down the number of cells needed for analysis, to investigate mixed cultures, and to perform population analyses, but also help to detect light-microscopic and spectroscopic features simultaneously, with the prospect of a fully automated IR microscopic system combining detection, enumeration, and identification of microorganisms in one single instrument. One particular aspect of vibrational spectroscopy in microbiology which constitutes its attractiveness is the possibility to achieve subspecies differentiation and the ability to analyze all kind of cells that can be grown in culture. No other technique is currently available that can trace microbiological contaminations in food microbiology or perform epidemiological investigation in clinical settings similarly quickly and easily. It is interesting to note that this potential is currently evaluated in several laboratories and that dedicated instrumentations are being designed for microbial subspecies differentiations in collaboration with industrial partners.

It is the author's personal belief that best perspectives for practical applications will arise in those fields where the various vibrational spectroscopic modalities are used as "coupled" techniques – for example, in the form of spectroscopy and microscopy, microspectroscopy and nanoparticles, spectroscopy and optical fibers, or spectroscopy and optical tweezers. In the case of microbiology, to give an example, this will not only allow us to scale down the number of cells needed for analysis to a few or even only a single cell to perform, for example, population analyses in complex habitats, but also allow to detect light microscopic and spectroscopic features of cells simultaneously, which is impossible for other techniques presently in use.

Immense future applications in cell biology, virology, and microbiology may arise from the use of Raman spectroscopy with optical tweezers. Raman tweezers is a relatively new technology that couples Raman spectroscopy with optical tweezers that are already routinely used for the noninvasive manipulation of biological particles to achieve previously impossible sample control. This combination represents a new category of application and may become a modality for flow cytometry to identify cells on the basis of intrinsic molecular properties instead of the particles' size, shape, or fluorescence.

Noninvasive methods to image single live cells are resonance Raman scattering (RRS) and coherent anti-Stokes Raman scattering (CARS) microscopy, which provide intrinsic molecular-vibration-based contrast with a sensitivity that is orders of magnitude higher than conventional Raman microscopy. CARS technology has recently been used to track lipid metabolism in live cells and may become a significant tool in environmental and medical microbiology.

SERS will most probably gain greatest attention reaching far beyond the relatively small community of vibrational spectroscopists, since it may provide biological information that is not available by any other means. SERS used with biocompatible gold nanoparticles incorporated as sensors by cells holds great promise to sensitively and specifically test

molecules in selected subcellular compartments in femtoliter-scaled volumes. This Raman spectroscopic modality could greatly benefit from the fact that defined SERS-active nanoparticles are routinely available and already used along with fluorescence techniques or electron microscopy in cell biology.

The development of technologies for subwavelength spectroscopy of cells and tissues is presently a major point of interest, and different approaches are being evaluated by several groups. The coupling of atomic force microscopy (AFM) with SERS, the so-called tip-enhanced Raman spectroscopy (TERS), seems to be very promising. The possibility to obtain compositional and structural information at a nanoscale level is the most attractive aspect of this new methodology and could provoke as much attention as AFM did some 20 years ago. Also, the coupling of IR lasers with AFM technology, which can probe in a photothermal deflection near-field experiment the local transient deformation induced by an IR pulsed laser tuned to different absorbing wavelengths, may be developed into a microscopic technique that yields chemical contrast at lateral resolutions not accessible by any IR far-field optical technique.

The use of Raman fiber-optic probes may open new avenues for routine *in vivo* use in clinical settings, since the high specificity of Raman spectroscopy can be combined with the possibility of immediate visualization. For practical applications, such fibers will most reasonably be used in multimodal fashion with other optical techniques such as light scattering, optical coherence tomography, or fluorescence spectroscopy, since wide-field Raman imaging still needs to be developed. Further technological progress will be necessary, because fiber-optic technologies are not routinely compatible with existing endoscopic technologies and because of fundamental physical limitations. Though no technical advances are in sight that could allow retrieval of spectra from several centimeters below the tissue surface, very efficient *in vivo* skin analyses based on confocal Raman spectroscopy are already on the market and in practical use.

Bench-top instrumentation for routine IR imaging of diseased tissue sections is available. The vast amount of applications so far published clearly prove that segmentation of histological structures is possible without any staining, and the identification of cancerous lesions within tissues may be achieved in an objective way using extensive reference data bases. Possibly, the *x*th publication of data showing that vibrational spectroscopic imaging can identify pathologies in tissues is not only lacking novelty hereafter, but even counterproductive. To push biomedical vibrational spectroscopy forward, multicenter clinical trials focusing on selected clinical indications are needed to attract the attention of the clinicians and to establish sensitivity and specificity parameters under practical constraints. At present, however, the following questions remain: Who could conduct such trials? Which relevant cancer types or clinical samples (fresh patient biopsies or archive material) should be used? Which technological platforms should be used?

The use of vibrational spectroscopy together with accepted genomic or metabolomic methods such as DNA/RNA microarrays or mass spectroscopies can be of profit when data sets obtained by fundamentally different experimental techniques from the same selection of samples are combined to analyze the covariance patterns in these complex data blocks. The combined analysis, for example, of gene expression and biomolecular response data to external stress factors in microorganisms would help to close the gap between different disciplines, since they can inherently only be done in cooperation between groups that are able to professionally deal with complex technologies. The results of such joint efforts would immediately be recognized by a much broader range of scientists and potential users of the new vibrational spectroscopic techniques.

Obviously, no killer application has been found yet that could pave the way for further steps forward and that cannot be done with any other type of technology. Although vibrational spectroscopy may be superior to competing methods in some cases, no major application could be found to date that can be done in no other way or which is so much superior to replace present technologies in practical use. The scientific community in biomedical vibrational spectroscopy is perhaps at a turning point where practical applications must arise. It will probably not be easy to invest such a high amount of enthusiasm, money, and time for another 10 or 15 years. Indeed, it will instead become more difficult to attract funding for this scientific field, unless significant progress will be made in the transfer of basic science to important practical applications accepted by biologists or clinicians. The gap between enthusiasm and optimism on the one side and the necessity to significantly contribute to the present practical needs of the medical or biological community on the other side must be closed. It should also be clear that series of nice publications will not be enough to close this gap. What must be paramount are joint efforts that combine experience, manpower, and budgets of several groups to bring selected applications to practical applications and patents to the industries.

A similarly important point is the necessity to define standards to exchange data and compare reproducibility levels between the groups and to establish criteria, for example, how sensitivity and specificity values are determined for objective evaluation of spectral data. Without the definition of standards, protocols, and quality control measures, the value of large amounts of data will be rapidly lost after completion of the primary research and increase the probability of reinventing the wheel. This will be critical for the successful development and maturation of an emerging technology like vibrational spectroscopy.

BIOMEDICAL VIBRATIONAL SPECTROSCOPY – TECHNICAL ADVANCES

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2.1 INTRODUCTION

In recent years, vibrational spectroscopy has been extremely successful and versatile for condensed and gaseous phase analysis due to a plethora of measurement techniques and more affordable spectrometers; and still many growing areas can be listed, for which biomedical applications are published. The spectral range covers the short-wave near-infrared (NIR) down to the far-infrared. A few instrumental aspects will only be mentioned in the introduction, but relevant references are provided, enabling the reader to familiarize himself with those areas through the literature cited.

The lowest frequency range has recently attracted many researchers when the so-called terahertz radiation, spanning the spectral interval between the microwave and the infrared (IR) region of the electromagnetic spectrum, found new rapidly expanding applications in biology and biomedicine. In particular, the spectroscopy of compounds such as proteins, enzymes, biological membranes, or whole cells has been carried out using laboratory-scale terahertz sources. Water absorption dominates spectroscopy and imaging of soft tissues, but the technology could play a role in diagnosing skin diseases. Despite this, there are advantages of terahertz methods that make it attractive for pharmaceutical and clinical applications. Besides low-frequency bond vibrations, also hydrogen-bonding stretches, torsions, and crystalline phonon vibrations can be assigned to this spectral range, interesting enough for crystalline conformation and polymorphism studies; see also the review by Pickwell and Wallace.¹ Most applications use terahertz radiation generated by short-pulse solid-state lasers.

Other lasers have been mandatory for Raman spectroscopy, and the use of sensitive charge-coupled device (CCD) detectors has made dispersive Raman spectral acquisition much more rapid. Such Raman spectrometers typically use holographic diffraction gratings and efficient edge or notch filters due to advances in thin-film technology to achieve a high degree of laser rejection. For biological and medical samples for which problems with fluorescence exist, Fourier transform (FT)–Raman techniques using NIR lasers (785 nm diodes, 1064 nm Nd:YAG) have been routinely applied, although also dispersive multichannel instrumentation is in use, even for 1064 nm excitation. Other lasers are often used when enhanced Raman signals are to be observed from microscopic objects such as for optically trapped erythrocytes (488.0, 514.5, and 568.2 nm for excitation of the heme moiety).² Wavelengths in the deep ultraviolet (UV) – for example, of 229 nm – are used to enhance aromatic amino acids, while the wavelength of 257 nm leads to a predominant enhancement of Raman bands of nucleic acids.³

For the NIR region, diode lasers operated at room temperature have been exhaustively used for gas analysis within the last decades. From the permanently increasing noninvasive ¹³C-breath tests for the investigation of metabolic processes, the urea breath test for diagnosing a *Helicobacter pylori* infection, causing in some people peptic ulcers and even cancer in its worst case, is the most prominent, for which diode lasers have been used for isotope-selective measurements. Many recent developments go beyond gastroenterological applications.⁴ An apparatus recently developed for breath analysis and based on photo-acoustic spectroscopy, using a wavelength-modulated distributed feedback (DFB) diode laser and taking advantage of the acoustic resonances of the sample cell, allows sensitive measurements with detection limits for ¹³CO₂ of a few parts per million (ppm).⁵ Alternatively, nondispersive IR spectrometric devices have routinely been used for such diagnostics.⁶

Furthermore, several applications of mid-infrared (MIR) quantum cascade lasers (QCL), aimed at monitoring blood glucose, have recently been reported with the claim of allowing a miniaturization of a device to the point where personal use of a wearable instrument may be realized.^{7,8} The feasibility of the simultaneous quantification of two different compounds measured at two wavelengths using dual QCL absorption spectroscopy has been reported by Schaden et al.⁹ However, miniaturized devices have yet not been advanced to the size of portable instrumentation, despite the promises made for QCL technology or NIR tunable lasers.¹⁰

A further glimpse is caught of important and interesting, but not routinely applied, measurement techniques. In the past, the theoretical basis for using vibrational spectroscopy as a tool for structure analysis has been well established. As an example, the conformation of biological molecules such as peptides, proteins, nucleic acids, and carbohydrates can be detailed, much opposed to the view of IR and Raman spectroscopy being low-resolution techniques that cannot compete with nuclear magnetic resonance (NMR) or X-ray crystallography. For clarifying this partiality, a recent comprehensive review by Schweitzer-Stenner¹¹ discussed peptide and protein structures elucidated by vibrational spectroscopy.

In this context, vibrational optical activity (VOA) is another area that must be mentioned.^{12,13} It is composed of two areas, vibrational circular dichroism (VCD), providing the difference in the IR absorbance of a chiral molecule for left versus right circularly polarized radiation, and Raman optical activity (ROA), which is the corresponding difference for Raman scattering. Routinely, VCD spectra are measured with Fourier transform–infrared (FT-IR) instruments with commercial spectrometers

available since 1997, which are now used worldwide in research laboratories.¹⁴ Later in 2003, instrumentation for ROA measurements has also become commercially available. The research group of Nafie and Freedman is interested also in extending VCD and ROA into new areas such as NIR VCD of overtones and combination bands,¹⁵ NIR excited ROA, and surface-enhanced ROA and VCD techniques. Enhancement factors of many orders of magnitude have been observed in hot spots with high-surface plasmon fields, enabling even single molecule detection, thus adding an additional level of chiral sensitivity to this method of structural analysis.

In the following, measurement techniques for clinical chemistry analysis will be discussed in more detail, for which biofluids such as whole blood, serum, dialysates, urine, and others, but also solid samples like gallstones and urinary calculi, must be listed. Reagent-free vibrational spectroscopy can provide quantitative results for the specimen composition or can furnish the physician with information on the etiopathology of the patient. Furthermore, pathology assisting and supporting vibrational techniques, either for biopsies or *in vivo* diagnosis, are illustrated. Finally, *in vivo* monitoring of pivotal metabolic parameters and the redox status of important proteins based on near-infrared spectroscopy (NIRS) will be reviewed.

2.2 MEASUREMENT TECHNIQUES FOR CLINICAL CHEMISTRY

2.2.1 Analysis of Liquid Samples

Molecular spectroscopy has brought much progress for medical diagnostics, and particularly the marriage of vibrational spectroscopy with clinical chemistry will enable the implementation into point-of-care analytics for patient monitoring. In the past, this area was reviewed extensively,^{16–18} but several novel techniques have been developed since then and the most interesting applications will be explicated.

Biofluid analysis has several aspects because there is the measurement of liquid aqueous samples involved by using attenuated total reflection (ATR) and transmission spectroscopy with a goal of such instrumentation being developed for routine analyzers. First applications of IR spectroscopy for substrate analysis in whole blood and blood plasma were reported about 20 years ago.^{19,20} Among the different options, discrete blood sampling with subsequent sample preparation has been chosen for many glucose assays. Whereas for MIR spectroscopy the ATR technique or transmission measurements have been used for the analysis of liquid body fluids, exclusively transmission measurements were carried out when NIR or even short-wave NIR spectroscopy were exploited.²¹ However, when simulating the scattering in biological tissue, also diffuse reflectance measurements have been carried out with intralipid solutions spiked with glucose.²² Further details on diffuse reflectance measurements for tissue analysis are given in the *in vivo* spectroscopy section.

Some MIR spectral signatures of different biomolecules are displayed in Figure 2.1 from transmission measurements of crystalline powders using the KBr pellet technique and spectra obtained by transmission and ATR measurements of aqueous solutions, which serve for their quantitative analysis in body fluids. One of the ATR measurements has been carried out using a flow-through micro-Circle cell, which contains a pin-like ZnSe crystal with cones at its ends for optimal radiation coupling (inner volume ~30 μ L). Owing to the several inner reflections, the transmission equivalent optical sample path length is larger when compared with the spectral absorbance resulting from two internal reflections in a diamond

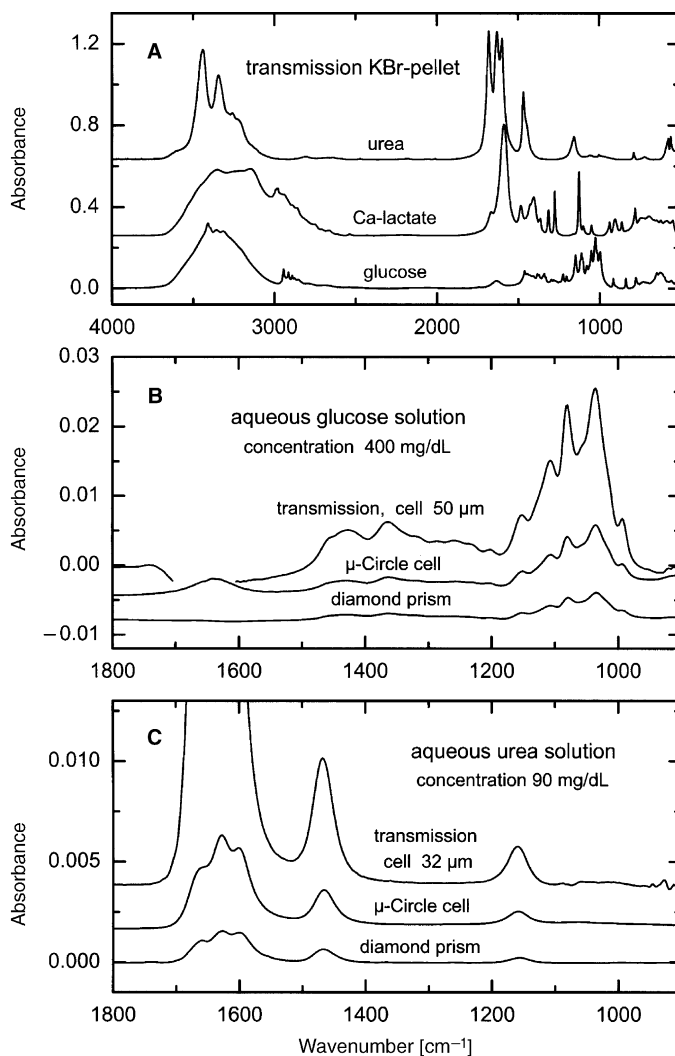


Figure 2.1. Infrared spectra of biologically relevant substances. **(A)** spectra measured in transmission using crystalline powders and the KBr pellet technique. **(B, C)** Aqueous glucose and urea solution spectra measured in transmission, using a micro-Circle cell with a ZnSe crystal (several internal reflections) and a diamond microprism with two internal reflections at 45°, respectively. The water absorbance from the solvent had been compensated by background measurements using a water-filled cell.

prism at 45° (see also Figure 2.2). For this fiber-optic probe with a microprism as ATR-sensor element, both fibers – that for illumination and the other for waveguiding to the MCT detector – were of the same square cross section to fill the diamond prism base of 1.5 mm × 0.75 mm completely.

Other accessories such as a horizontal diamond ATR cell with three internal reflections (DurasampleII, SensIR) have been used for continuous fermentation monitoring²³ or whole blood measurements.²⁴ Transmission micro-cells have been fabricated with inner volumes of less than 1 μL.²⁵ Best quantification can be achieved by using the MIR

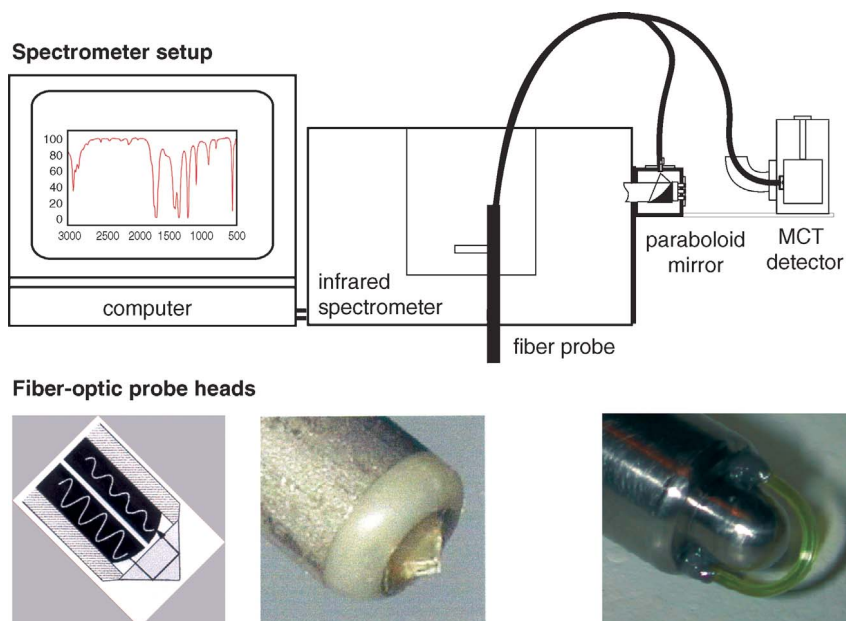


Figure 2.2. Experimental setup with fiber coupling to an FT-IR spectrometer with two different remote-sensing probes. Fiber-coupled micro-diamond prism with schematics is shown on the left, while fiber-only probe using a cross-section silver halide fiber of $750 \times 750 \mu\text{m}^2$ is shown on the right.

spectral features of the fingerprint region, apart from the long-wave NIR region above 4000 cm^{-1} , showing characteristic combination bands. A recent example for the use of fiber-optic NIR transreflectance probes in monitoring industrial bioprocesses was given by Roychoudhury et al.²⁶

Recent advances in microfluidic technology can aid the continuous monitoring applications of vibrational spectroscopy. The employment of IR spectroscopy in combination with microfluidics for serum and other biofluids has been reported by Fabian et al.²⁷ We reported similar applications for glucose^{28–30} and urea²⁵ using microliter sample volumes. Another important field is continuous monitoring of the patient physiological conditions using, for example, the combination of IR spectroscopy – with a FT-IR mini-spectrometer involved – and microdialysis. This preparation step simplifies the sample matrix significantly because only low molecular mass compounds, owing to the dialysis process, are continuously harvested, but excluding higher concentrated proteins. An optimal spectral signal-to-noise ratio for reaching clinically relevant detection limits can be achieved by transmission spectroscopy using room-temperature-operated pyroelectric detectors. The other option was to approach the patient using MIR fiber-optic probes, which require liquid-nitrogen-cooled MCT photodetectors, but these are not acceptable for clinical routine analysis.³¹ As a consequence, we employed a fluidic system for transporting the sample into a microcell that can be housed in the sample compartment of a conventional FT-IR spectrometer.

Further developments led to an automatic bedside IR system coupled to a subcutaneously implanted microdialysis catheter in combination with microfluidics for quasi-continuous interstitial glucose measurements and aimed at critically ill patients. Reports on the instrument prototype, its *in vitro* performance, and application on healthy volunteers have been recently published.^{29,30} An innovative aspect is that, owing to the multicomponent assay capability, the microdialysis recovery rate can be simultaneously determined using a marker substance

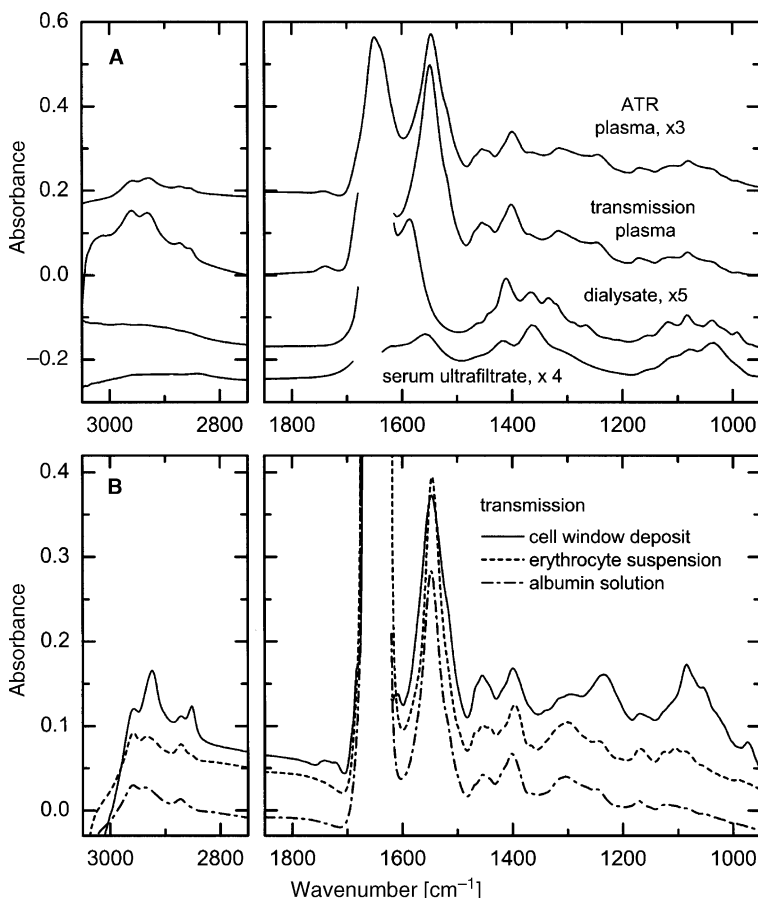


Figure 2.3. (A) Absorbance spectra obtained for EDTA blood plasma using a micro-Circle cell and a transmission micro-cell of 32 μm pathlength, respectively; the same cell was used for the measurement of the dialysate of EDTA blood plasma and a serum ultrafiltrate. (B) Absorbance spectra of an erythrocyte suspension and an aqueous albumin solution measured by the transmission micro cell and cellular deposit of leukocytes onto the CaF_2 windows during continuous measurement of whole blood spiked with EDTA as anticoagulant (all spectra are compensated by water absorbance.)

(acetate) in the perfusate, so that accurate estimations of the interstitial substrate and metabolite concentrations can be achieved. In Figure 2.3, different spectra of blood plasma and corresponding dialysate samples are presented using the different measurement techniques mentioned. Furthermore, the fingerprint spectra enable the characterization of blood components – for example, albumin in solution or cellular components (erythrocytes and leukocytes). Further novel aspects on the quantification of biofluids using MIR spectroscopy will be detailed in the following chapter.

Raman spectroscopy is often competing with IR spectroscopy, and much progress can be reported for the last few years.¹⁸ Serum samples and ultrafiltrates have been quantitatively investigated by Rohleder et al.³² for various serum constituents such as glucose, triglycerides, urea, total protein, cholesterol, high- and low-density lipoproteins, and uric acid using a Kaiser Optical Holospec spectrometer with 785 nm wavelength for Raman excitation. Ultrafiltration actually could reduce the fluorescence background efficiently for improving the relative coefficient of variation for glucose and urea by a factor of two compared to serum measurements. A comparison of the results using MIR dry-film measurements from 3 μL of

serum samples that were pipetted onto a 96-well silicon sample carrier dedicated for transmission measurements (Bruker Matrix HTS-XT spectrometer), with those from Raman spectral recordings of the corresponding liquid samples, was presented by Rohleder et al.³³ For further details, see also Chapter 5 in this volume.

For increasing the sensitivity for the detection of low-concentration analytes, surface-enhanced Raman spectroscopy (SERS) has been advanced significantly, so that also sensors for glucose monitoring in biofluids are under development. The concepts underlying the optimization of such analytical methods have been detailed by Haynes et al.,³⁴ and particular emphasis has to be placed on the optimal relationship between surface roughness described by its nanostructure and the laser excitation wavelength. Special film-over-nanosphere surfaces were used in combination with a portable inexpensive Raman spectrometer. An important aspect was the immobilization of a biocompatible partition layer, self-assembled on the SERS substrate for advantageously concentrating the analyte of interest for further reducing the detection limits. Measurements were successfully carried out for the physiologically relevant concentration interval even in the presence of serum albumin. Further progress for *in vivo* glucose monitoring with a subcutaneous rat-implanted SERS substrate that was functionalized with a two-component self-assembled monolayer was reported by the same group.³⁵ For further details, see Chapter 10 in this volume.

2.2.2 Dry-Film and Solid Sample Analysis

Coming back to the IR measurements, the often-favored option for biofluid analysis – that is, the dry-film measurement technique with a previous evaporation of the biosolvent water – is further illustrated. It can lead to much larger signals when compared with straightforward biofluid analysis, but an inhomogeneous film preparation may limit the photometric accuracy. For most of the reported publications, fluid volumes of a few microliters have been utilized for the sample preparation, either with additional dilution or with internal standard addition. In Figure 2.4 the spectra from 100 nL sample volumes, albumin solutions spiked with glucose and human microdialysates, are shown using a fiber-optic microprobe with an inverted u-bent uncladded silver halide fiber ($\text{AgBr}_{1-x}\text{Cl}_x$ with a refractive index of 2.2), similar to the probe shown in Figure 2.2, but fabricated from a circular cross-section fiber of 750 μm outer diameter and coupled directly to an MCT detector. Such a probe, when utilized for ATR measurements of solids, can render a spatial resolution of around 20 μm by touching the fiber part with the greatest curvature and using the evanescent field of the IR radiation.³⁶ More applications of such an ATR microprobe will be presented in the following pathology section. The dry-film measurement results show impressively the achievable sensitivity.

For minimal-invasive diagnostic tests with reduced pain for the patients, the volume of body fluids when accessed through the skin (e.g., by finger pricking or skin micro-perforation) needs to be rather small, so that even nanoliters have been tested for quantification. The aim of our studies was to reduce the necessary body fluid volumes to 100 nL or less, thus competing well with currently commercially available, electro-chemistry based glucose meters, but at no costs for consumables by applying a reagent-less spectroscopic assay for glucose quantification.

Experiments were carried out with a single reflection, planar micro-ATR accessory (Golden Gate from Specac). For demonstrating the limits of our spectroscopic approach, quantification results for microsamples of dry-film sera have recently been reported, either undiluted or 10 times diluted by distilled water, with original physiological glucose concentrations between 50 and 600 mg/dL. The samples were prepared by micro-spotting by using either a microliter syringe (80 nL) or an automatic micro-dispenser for 1 and 8 nL

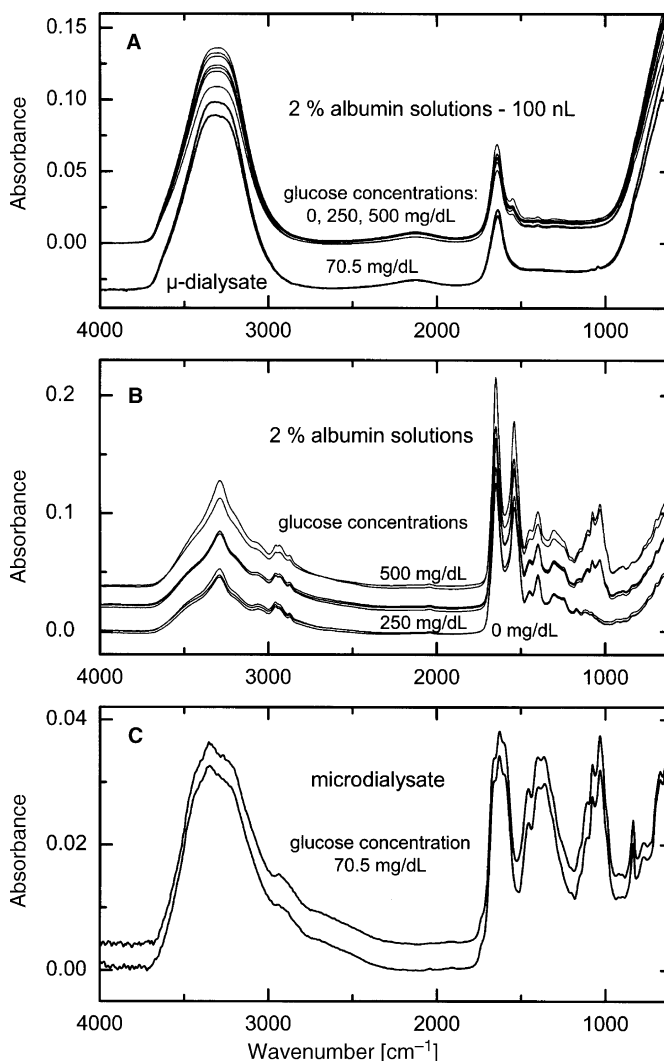


Figure 2.4. (A) ATR spectra of aqueous biofluids (sample volume 100 nL), measured by an inverted u-shaped fiber loop immediately after deposition (16 scans, spectral resolution 8 cm⁻¹). (B, C) Corresponding dry-film measurements of albumin solutions and microdialysates containing different glucose concentrations with repeat measurements, respectively.

sample volumes. For the latter samples, the optimum standard error of prediction (SEP) as obtained from multivariate Partial Least-Squares (PLS) calibration models was 11.2 mg/dL (coefficient of variation 4%).³⁷

The reproducibility of the dry-film technique using the ATR method was higher when compared with simple transmission measurements on silicon carriers, which can be traced back to the inhomogeneous film layer that is formed during the water evaporation. Dry-film roughness surmounting the penetration depth of the evanescent field is not contributing to the integral sample absorbance. For distributing the liquid sample in a more homogeneous manner, also roughened polyethylene films (disposable IR cards) have been employed, but measurement reproducibility could not reach the ATR method performance. In Figure 2.5B,

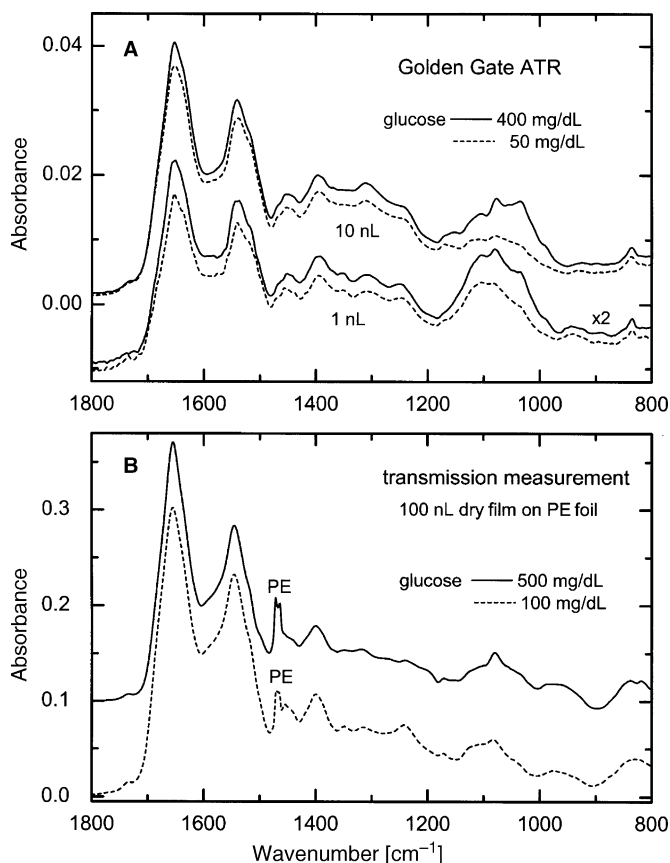


Figure 2.5. (A) ATR spectra of dry-film serum samples with different glucose concentrations. Sample volumes were 10 and 1 nL, respectively (prior to deposition, the samples had been 10-fold diluted). (B) Serum samples spread out onto a polyethylene foil with roughened surface and dried down (spectral resolution 8 cm^{-1} , 16 scans).

exemplary spectra of 100 nL serum samples spiked with different glucose concentrations are shown (artifact bands marked by PE result from incomplete compensation of the intense methylene deformation band of the polyethylene carrier film).

Another application of dried samples will be presented. It is known that cancer is caused by a series of mutations altering the transcription and replication process due to the effect from carcinogens and reactive oxygen species. These structural disorders have their manifestation in the vibrational bands of various deoxyribonucleic acid (DNA) functional groups, such as NH_2 , PO_2^- , and CO , and can therefore be measured by vibrational spectroscopy. IR spectroscopy has been used for nucleic acid studies since the early work of the Frasers 50 years ago. Besides measurements in solution, dry-film techniques have dominated the research. Nucleic acid vibrations stem from different parts of the macromolecules which have been sketched in the spectrum shown in Figure 2.6A. For the interpretation and identification of alterations in the nucleotide bases and phosphodiester-deoxyribose backbone of the DNA extracted from tumors and normal tissue, we have to refer to a recent publication.³⁸ Some spectral results, showing also the reproducibility of such measurements, are presented that were obtained from $40\text{ }\mu\text{g}$ of DNA prepared on 96-well silicon plates and measured in diffuse reflectance using a

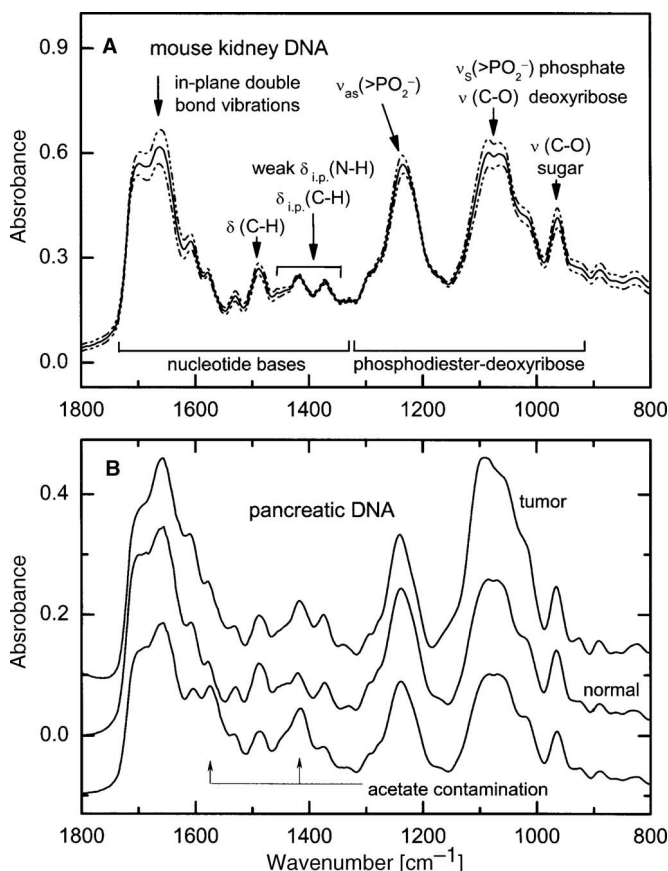


Figure 2.6. (A) Vibration bands in the IR spectrum of mouse kidney DNA with assignment to relevant substructures. The spectral reproducibility as measured for 40 μ g samples by diffuse reflectance, after drying down onto a 96-well silicon sample carrier from an aqueous solution, is shown by the 95% confidence interval given by the dashed curves. (B) Population mean spectra for pancreatic DNA extracted from cancer and normal tissue, respectively. The lowest trace is the spectrum of acetate contaminated DNA from healthy tissue, resulting from the preparation.

high-throughput extension (HTS-XT) accessory from Bruker Optics (Ettlingen, Germany) (Figure 2.6A). Differences can be manifested easily for the population mean spectra that were obtained for normal pancreatic tissue and cancer (Figure 2.6B). Another research group has used transmission microscopy for detecting DNA changes – for example, in ovarian, prostate, and breast cancer. After DNA extraction and drying an aqueous solution (200 nL) on a BaF₂ plate, usually a crater-like dry sample is generated with the ring having the substance of interest concentrated, which can be measured by a microscope spectrometer.³⁹ Owing to the sample film inhomogeneities, normalization of the spectral data as a basis for classification of cancer-related changes in the DNA is of utmost importance. In addition to the inherent structural variations, also contamination from the preparation steps must be avoided (see also Figure 2.6B, lowest trace).

In this context, sensitivity enhancements by up to two orders of magnitude can be achieved by surface-enhanced infrared absorption (SEIRA) spectroscopy when compared with conventional techniques using properties of nano-structured metal surfaces and special

surface modification techniques. Recent applications of DNA and nucleic acid adsorption to gold surfaces, the development of sensitive immunoassays, or protein – protein interactions have been reviewed by Ataka and Heberly.⁴⁰ Studying the functionality of proteins – in particular by difference spectroscopy – could provide a wealth of molecular substructural information, here obtained, for example, for protein monolayers. Owing to the growing interest in biotechnology, biosensors for DNA or proteins are in the spotlight with optical arrangements employing most often so-called metal underlayers (metal-island film). Transmission, ATR, and external reflection can be applied for so-prepared specimens.

The complementary technique to SEIRA is surface-enhanced Raman spectroscopy (SERS) – an example was given above – which takes advantage of the enormous enhancement factors when compared to conventional Raman techniques. A special variety is tip-enhanced Raman scattering (TERS), which is a type of near-field optical microscopy using the concentration of the required metal film “roughness” at the apex of a scanning probe tip. An application of this technique for investigating the spectra of DNA pyrimidine bases has recently been reported by Rasmussen and Deckert.⁴¹ A comparison with standard SERS and Raman measurements of nucleotides and pure bases has been provided. The potential of TERS – combining SERS with atomic force microscopy – for nanometer-sized structural analysis is discussed below.

The earliest applications of vibrational spectroscopy for clinical chemistry actually started with the analysis of urinary calculi,⁴² for which IR spectroscopy in combination with the KBr pellet technique was optimal for investigating their chemistry.⁴³ This technique is even applicable for microsamples.⁴⁴ Such solid samples have most recently been investigated by IR and Raman microscopy, elucidating the growth history of such specimens and providing insights into the etiology. In a recent publication, reflection/absorption IR microscopy was employed for obtaining qualitative information about the composition of the mineralized materials embedded in kidney tissue within a survey, while ATR was used for collecting best-quality spectra.⁴⁵

The formation of gallstones is another complication that is still poorly understood. Gallstones are made up of different compositions displaying various colors that arise from the main compounds such as cholesterol and bilirubin, but also several lipids or calcium carbonate. A study performed a decade ago compared different vibrational techniques, IR microscopy including photoacoustic techniques, and FT-Raman spectroscopy. Of the vibrational techniques studied, photoacoustic spectroscopy proved the most suited to the classification of gallstones due to the minimal sample preparation required.⁴⁶ The protein content within the insoluble material of gallstones treated with various solvents was studied by Liu et al.⁴⁷ Further FT-IR studies to elucidate the pathogenesis of gallstones were performed, for example, by Kleiner et al.⁴⁸ by using KBr disks or stone powder only when employing a horizontal ATR accessory.

2.3 MEASUREMENT TECHNIQUES FOR PATHOLOGY

Vibrational pathology – that is, the study of tissue biopsies and cells without staining methods – is another area with a rapidly increasing number of publications. Single-detector microscopes are still available for routine applications, but focal plane detectors became much more affordable compared to the time when they were first introduced. Another field attracting much recent attention is the use of synchrotron radiation for ultimate microanalysis. Raman microscopy has been known for not suffering from the low spatial resolution limits as due to diffraction effects and experienced for IR microscopy,

because much shorter wavelengths can be used for excitation of the Raman effect, so that single-cell analysis can be readily approached. The ultimate spatial resolution can be achieved either by near-field techniques using a scanning near-field infrared microscope (SNIM),⁴⁹ or by SERS and related techniques (TERS) for the enhancement of the Raman signal.

2.3.1 Mid-Infrared Dermatology Applications

The skin is the largest organ of the body and has attracted many vibrational spectroscopists for structural and chemical investigations. Recent developments in MIR fibers enabled us to construct flexible fiber-optic probes for the measurement of small biosamples using the ATR technique⁵⁰ (see Figure 2.2). The material used is extruded microcrystalline silver halide, but also chalcogenide fibers have been applied for biochemical sensing.⁵¹ Special probes – in particular, for skin analysis – were fabricated from fibers of square cross section for recording reproducible spectra of high signal-to-noise ratio. Applications include the measurement of the *Stratum corneum* within dermatology studies for the assessment of pathological abnormalities or the use of bovine udder skin as a human skin substitute.⁵² Enhanced spectra recorded with flattened silver halide fiber probes were reported by Bindig et al.⁵³ The u-bent fiber-optic microprobe – with circular cross-sectioned fibers – was mentioned already when discussing dry-film samples; such a device is attractive, since a microdomain analysis is possible by replacing expensive IR microscopes with the ATR measurement option. Different types of accessories including a u-shaped fiber probe and a diamond microprism-coupled sensor were utilized in a recent study to illustrate their endoscopy potential for tumor diagnostics.⁵⁴

The epidermis contains a stratified squamous epithelium with important skin barrier functions. Within the epidermis, a differentiation process leads to a skeleton of cornified cells saturated with lipids and packed with keratin macrofibrils. The outer horny layer, consisting mainly of keratin, is the critical component for its function as a barrier. The lipids of the stratum corneum are primarily ceramides, cholesterol, and free fatty acids (see also lowest trace in Figure 2.7A). As yet, there is no biological or functional explanation for the heterogeneity that exists among the several keratin varieties (see Figure 2.7B, which shows spectra of various skin surfaces after cleansing and six times of tape-stripping). The largest differences in the stratum corneum spectra can be found for the C–C and C–O stretching region around 1000 cm^{-1} . We found that the outmost layer lipid concentration is significantly increased; but after application of adhesive tape-stripping for the removal of a few corneocyte layers, it is much reduced. Such barrier disintegration as manifested by fiber-ATR spectroscopy has also drastic consequences for the diffusion of oxygen with oxyhemoglobin formation through the skin as studied for the isolated perfused bovine udder skin by using visible-NIR diffuse reflectance spectroscopy.⁵⁵

Other applications include the characterization of skin samples including penetration studies of vitamins and constituents of pharmaceutical or cosmetic cream formulations. The combination of ATR-measurements and adhesive tape-stripping provides us with a tool for depth profiling within the upper epidermis. The removal of superficial skin lipids by tape stripping can be controlled by MIR spectroscopy (Figure 2.8). Phase inversion temperature (P.I.T.) emulsions are stable emulsions used for the formulation of cosmetic products (see Figure 2.8B). The low penetration depth of the evanescent radiation field makes the fiber-optic probe ideally suited for the analysis and quantification of corneocytes stripped off by adhesive tape and sticking to the tape surface.

A different technique was exploited for the spectra shown in Figure 2.9. For the skin surface measurement, a diffuse reflection accessory was employed, which uses a light-pipe

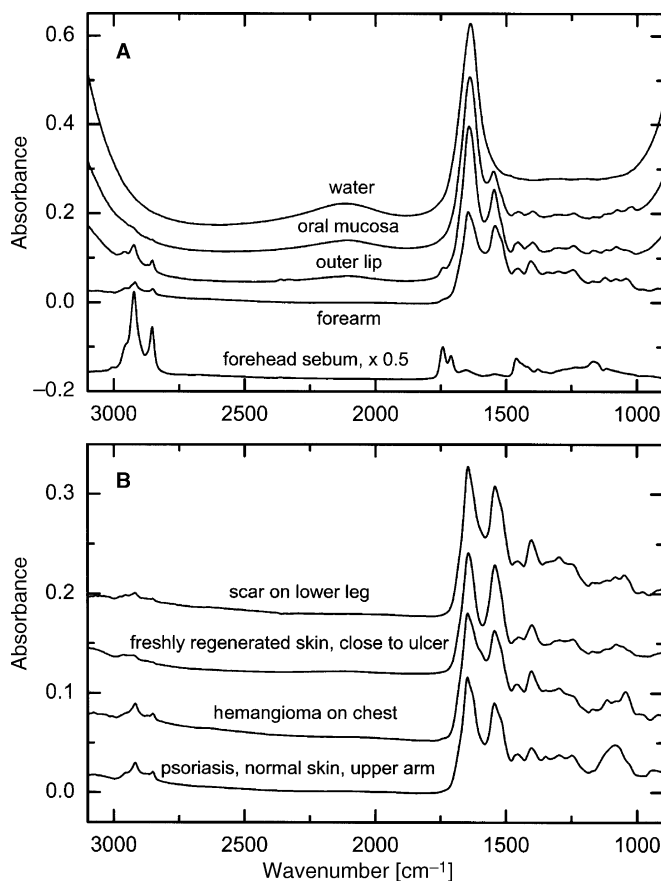


Figure 2.7. (A) Fiber ATR spectra from various skin tissues and of forehead sebum. For clarity and illustrating the different skin water content, also the spectrum of liquid water is shown. (B) Absorbance spectra from various skin abnormalities by measuring the upper stratum corneum layer after cleansing and tape-stripping for efficient skin lipid removal.

for sample illumination and a rotational ellipsoidal mirror for the collection of diffusely back-scattered radiation.⁵⁷ The dispersion features that arise from the Fresnel reflection at the air–skin interface can be transformed by a Kramers–Kronig transformation to give normal absorbance spectra (compare with mid-trace spectrum shown in Figure 2.10A).

Further examples of the possibilities of IR spectroscopy, especially for the analysis of microsamples, are given in Figure 2.10, by which spectra are compared that were recorded in transmission using a micro KBr-pellet, an ATR microscope (model AutoIMAGE, equipped with a multimode micro-ATR objective and Ge crystal from Perkin–Elmer), and a fiber-optic microprobe. Natural dermis samples, as existing for leather, are mainly composed of collagen (cf. the spectra shown in Figure 2.10B), which was found preserved in the skin of an ancient moor-mummified corpse (“Roter Franz”); for details, see Ref. 58.

Raman spectroscopy also possesses a great potential for dermatology applications. An informative review on medical applications had been given by Choo-Smith et al.⁵⁹ that also pointed at skin studies. The diagnosis and monitoring of skin cancer based on vibrational spectroscopy and the different measurement techniques have been described in detail by Skrebova Eikje et al.,⁶⁰ so that only a few special applications and their highlights will be

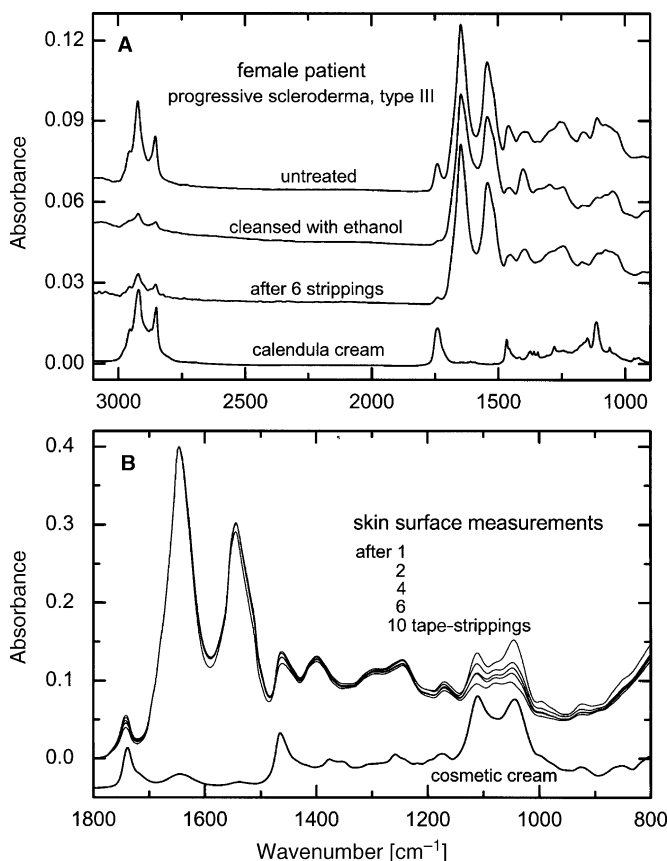


Figure 2.8. (A) ATR spectra from skin without and after cleansing and tape-stripping of the horny layer surface with pharmaceutical cream prepared as dry film. (B) Skin surface measurements with subsequent tape-stripping for depth profiling in the stratum corneum after topical application of a P.I.T. emulsion (for details, see also text). The lowest trace is the spectrum of the pure cosmetic cream, again prepared as dry film.

reported. The noninvasive nature of Raman spectroscopy for skin analysis must be stressed, and an example of using blue and green laser lines for the detection of carotinoids such as lycopene and β -carotene in skin was recently published.⁶¹ The opportunities for *in vivo* confocal Raman microscopy with an axial resolution of 5 μm have been impressively demonstrated by Caspers et al.,^{62,63} Furthermore, the axial resolution uncertainties in confocal Raman microscopy have been addressed.⁶⁴ Recently, the imaging of intact pigskin up to a depth of 70 μm has demonstrated impressively the delineation of specific skin regions.⁶⁵

2.3.2 Other Tissue Applications

Vibrational spectroscopy and imaging can support the difficult *in vitro* and *in vivo* diagnostics of biochemical changes at the cellular level. Applications can be found for classification of microorganisms such as bacteria and yeasts, cell-line research, or pathology. The measurement techniques can be distinguished between single-point analysis, mapping by a single detector or a linear array, and imaging using a focal plane detector with

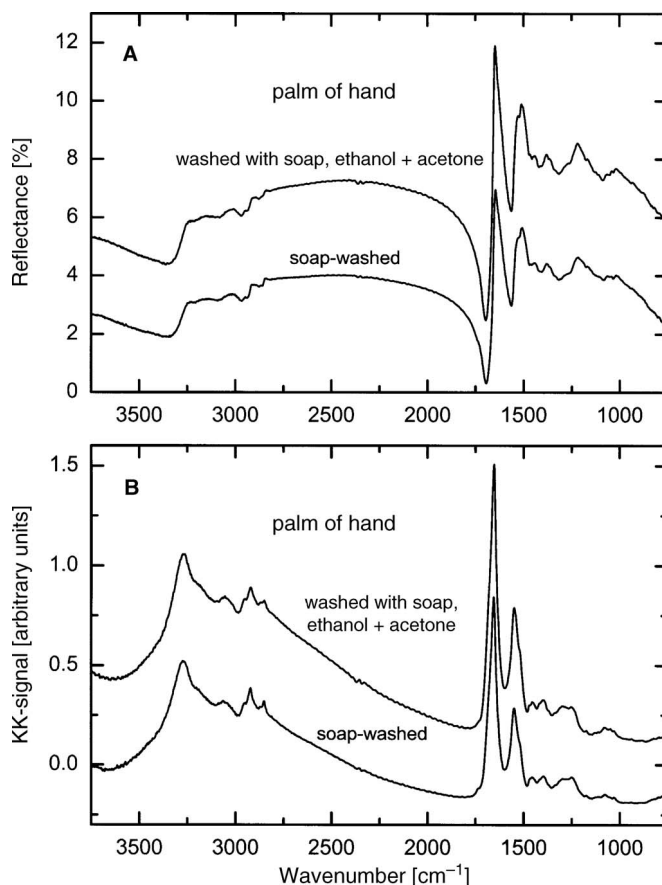


Figure 2.9. (A) Diffuse reflectance spectra of superficial skin from the palm of the hand. (B) After Kramers–Kronig transformation of the reflectance spectra with the result of calculating absorbance equivalent spectra.

up to 256×256 MCT elements.⁶⁶ Microscopy based on various techniques will be further presented in the following chapters, but an overview is allowed. Single-spot analysis using an IR or Raman microscope is nowadays a routine method, which has been applied for various biopsies in our laboratory.⁶⁷ In Figure 2.11, two examples are given for illustrating the techniques. In part A, a collagen sample spectrum, recorded in transmission using a diamond anvil cell, is contrasted with that of a microtomed dermis biopsy after subtraction of the paraffin component, used for embedding the tissue, and chemical identification is straightforward. In part B, another dermis sample, which contained traces of a silicone rubber that was used as implant material for wrinkle removal, is studied by ATR microscopy. Astonishingly, negative absorption bands are produced, which can be explained by an existing air gap between the ATR Ge crystal and the silicone sample. By such an arrangement, actually a phase shift is faced for the interface of air and the optically dense sample, which is not taken care by the routinely applied Merz phase correction (a power spectrum will show only positive bands).

For achieving improved spatial resolution by pushing it to the limits allowed by diffraction, the use of synchrotron sources has often been advocated due to the much higher brightness compared with conventional infrared sources. A comparison has recently been

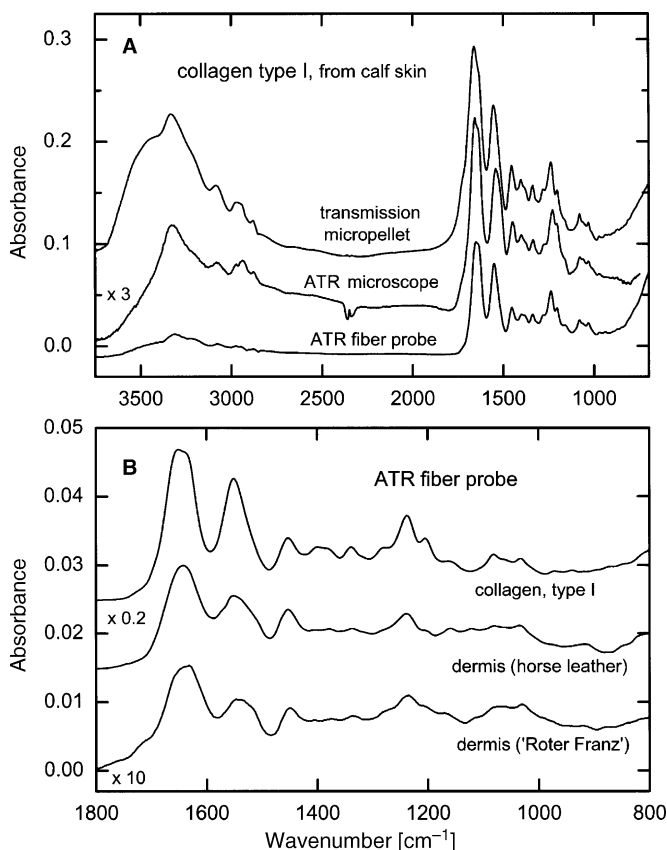


Figure 2.10. (A) Absorbance spectra of microsamples from collagen measured in transmission using the KBr micropellet technique, recorded by an ATR microscope with Ge internal reflection element and by an ATR probe with a u-bent silver halide fiber, respectively. (B) Absorbance spectra measured from different dermis samples (the leather had been treated by an organic tanning agent; lowest trace is from bog preserved skin of a mummy); for comparison, again a spectrum of a pure collagen sample, also measured by an ATR fiber-optic microprobe, is presented.

provided by Diem et al.⁶⁸ Further comparison, dealing also with focal plane array (FPA) detectors versus point detectors, has been detailed by Miller and Smith.⁶⁹ A comprehensive report on the use of synchrotrons as radiation source for IR microscopy has been published by Dumas and Miller.⁷⁰ An interesting review on the spatial resolution in microspectroscopic imaging of tissues has been published by Lasch and Naumann.⁷¹ As demonstrated by these authors, 3D-Fourier self-deconvolution can be successfully applied for spatial resolution enhancement in tissue images. However, as pointed out by Chan et al.⁷² a similar spatial resolution can be achieved also without a synchrotron source, instead using a micro-ATR technique in combination with a FPA detector. By such an arrangement, the chemical imaging of the cross section of a hair showing its core (i.e., the medulla with a diameter of $\sim 5\text{--}10\ \mu\text{m}$) was made possible. The use of a high index of refraction material in combination with a linear array detector – with a pixel size at the sample of $1.6\ \mu\text{m}$ through the $4\times$ magnification provided by the Ge internal reflection element – has been applied by Patterson and Havrilla⁷³ for imaging (e.g., latent human fingerprints). However, recent imaging results on the single cell level by Steller et al.,⁷⁴ obtained with an FPA and based on

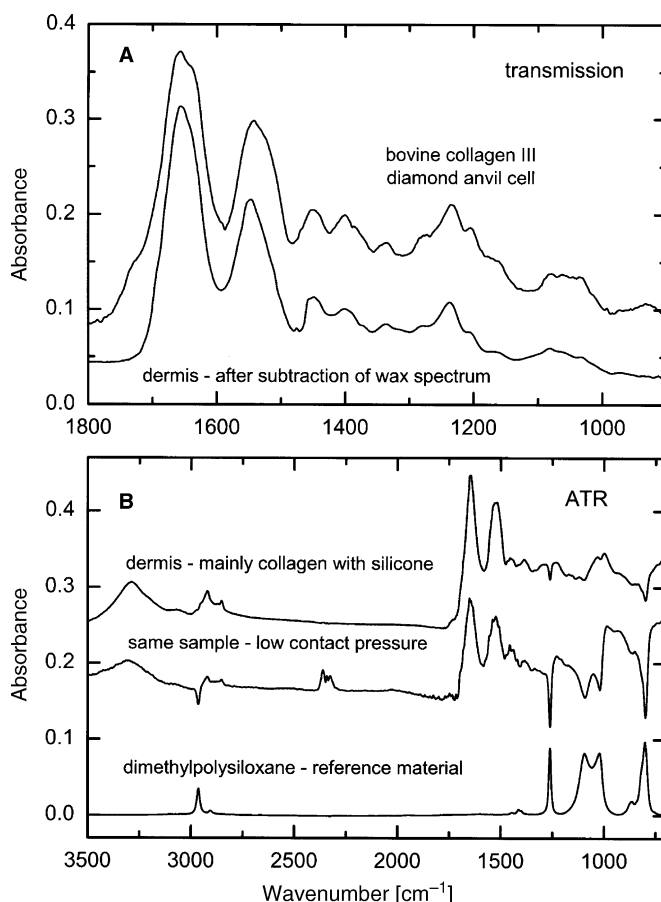


Figure 2.11. (A) Absorbance spectra from transmission measurements. Upper trace: Pure collagen measured in a diamond anvil cell. Lower trace: Microtomed skin on a NaCl crystal using a conventional IR microscope with a $100 \times 100 \mu\text{m}^2$ aperture). (B) Spectral artifacts produced from phase shifts originating from incomplete contact of the biopsy sample to the ATR microelement (air gap); for comparison, also a spectrum of the silicone rubber reference material is shown.

transmission spectroscopy of $10 \mu\text{m}$ -thick microtomed tissue samples of squamous cell carcinoma of the uterine cervix, are extremely impressive when evaluated with fuzzy C-means clustering. In this context, the recent book on imaging technology using multi-channel detectors, edited by Bhargava and Levin,⁷⁵ must be mentioned.

A few remarks will also be allowed for techniques used in Raman microscopy. Since wavelengths for Raman excitation can be much shorter than the wavelengths within the MIR spectral region, also the spatial resolution can be higher than found for IR microscopy. Using the 532 nm radiation from a frequency-doubled Nd:YAG laser, Raman mapping experiments on single yeast cells have been carried out by Röscher et al.⁷⁶ with submicron spatial resolution. Ultraviolet resonance Raman spectra, when studying nucleic acids and protein composition, have been recorded for a special bacterium providing evidence on its growth.⁷⁷ The bacterial growth was monitored by UV resonance Raman spectroscopy. In this context, the pioneering papers by Wu et al.^{78,79} must be mentioned. Single-cell imaging has been carried out by several groups, and one publication from Otto and co-workers⁸⁰ can be listed for exemplifying the opportunities offered by confocal Raman microscopy. By using

nanoparticles, co-workers from the same group succeeded in combining two different optical microscopy techniques on the same cell – that is, Raman and fluorescence microscopy.⁸¹ For replacing existing fluorescent labels, which are usually employed to light up under the microscope, they used “quantum dot” nanoparticles, opening exciting new possibilities for cellular imaging. As another mean for signal enhancement, TERS has also been applied by the authors for bacterial surface membrane studies, reaching a spatial resolution down to a few tens of nanometers.^{77,82} Infrared spectroscopic mapping with nanometer-scale spatial resolution can be done by scattering near-field optical microscopy (s-SNOM) to determine IR “fingerprint” spectra of even viruses and other nanoscale objects.⁴⁹

2.4 MEASUREMENT TECHNIQUES FOR *IN VIVO* SPECTROSCOPY

2.4.1 Instrumental Aspects and Skin Analysis

NIR spectroscopy has several advantages, but certainly also a few disadvantages compared to MIR techniques (lower information content of NIR spectra with regard to structural analysis). It has frequently been applied for biofluid analysis by using transmission quartz cells of millimeter sample thickness, but also dried films have been suggested for measurement. The other positive aspect is the use of fiber-optics that can be used, for example, for remote sensing applications in the operation theater for on-site tissue diagnostics for deciding the question of “cancerous or healthy tissue.” The other essentially important application is noninvasive diagnostics using diffuse reflectance spectroscopy of skin, but photoacoustic techniques have also been presented.

NIR spectroscopy (NIRS) has been partitioned into spectroscopy with intervals of the shortwave (14,700–9000 cm^{-1}) and long wave NIR (9000–4000 cm^{-1}). At short NIR wavelengths, the absorption bands of heme proteins (hemoglobin, myoglobin, and oxyderivatives) and cytochromes of the tissue dominate the spectra and provide information concerning tissue blood flow and oxygen saturation and consumption. The long-wavelength NIR absorptions arise from combinations and overtones of vibrations involving C–H, N–H, and O–H groups and thus render valuable information concerning the chemical composition of tissues – when not limited by the dominating water absorption of biosamples. Thus, any alteration in the composition of the tissue can be detected and used for diagnostic purposes.

As pointed out, NIRS as a simple and inexpensive method can be used for noninvasive or minimally invasive diagnostic applications. For such purpose, different accessories are employed based on either fiber-optics with special fiber arrangements for illumination and detection or exploiting the advantages of special high-throughput mirror optics (for their schematics, see Figure 2.12), which does not suffer from the limitation of the transparency window due to broad MIR quartz fiber absorption. Photon penetration depths may be varied for the latter accessory using a rotational ellipsoidal mirror for the efficient collection of the backscattered radiation by choosing different aperture sizes to alter the field of view of the accessory detector. A comprehensive review on fiber optic probes with different fiber arrangements for optical diffuse reflectance, Raman spectroscopy, and fluorescence, describing also side-looking probes, diffuser tips, and refocusing optics, has been given by Utzinger and Richards-Kortum.⁸³ Several accessories for diffuse reflectance spectroscopy have been constructed – for example, mainly bifurcated fiber-optic probes containing fiber bundles with a random or ordered distribution for illumination and detection.

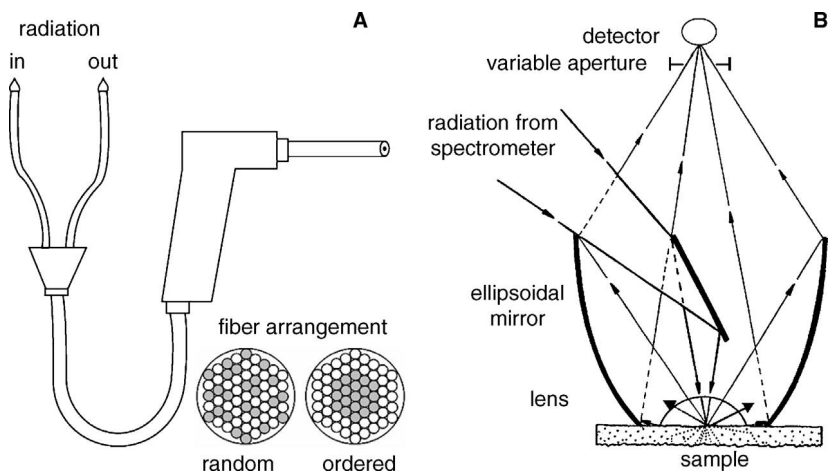


Figure 2.12. Different accessories for measuring diffuse reflectance NIR tissue spectra. (A) Fiber-optic probes with different arrangement of illuminating and radiation collecting quartz fibers (diameter of the whole fiber bundle was 4 mm). (B) Mirror optics for illumination and photon collection based on a rotational ellipsoidal mirror.

Noninvasive near-IR diagnostics show a promising potential for patients, and particularly *in vivo* skin tissue pathology or noninvasive blood glucose assays cannot be left out; for a review on the latter subject, see Ref. 84. To obtain quantitative information on various analytes in blood or tissue, such as glucose and other metabolites, noninvasive transcutaneous spectroscopic measurements of different skin tissues have been proposed. However, according to the optical properties of skin, the diffuse reflection technique can be favorably used, supported also by the spectral information content compared to the short-wave NIR otherwise required.

A special probe with an optimized fiber arrangement (one central fiber for detection and 12 surrounding source fibers for illumination, positioned with a gap of 0.65 mm, so that photons can reach the capillary plexus of the upper dermis) was promisingly employed for noninvasive blood glucose monitoring by Maruo et al.⁸⁵ However, mirror optics have also been employed. As shown in Figure 2.13A, spectra from muscle tissue as phantom were recorded by using different accessories – fiber- and mirror-based optics – with varying sample thickness and the specimens backed by a gold-coated diffuse reflecting substrate. The spectra are also shedding light on the maximum wavelength-dependent average probing depths into the tissue, providing the absorbance fingerprints for the development of gentle medical diagnostic methods. These differences in probing depth can be explained by the different numerical aperture seen by the two accessories.

Spectra of several skin areas have been recorded by means of such accessories, aimed at our application for diabetes screening, which is based on the detection of epidermal and dermal skin changes due to alterations in collagen structure and protein glycation observable in diabetics with poor carbohydrate metabolism stabilization.⁸⁶ The resulting skin spectra can be very different, depending also on skin-probe contact and scattering within the horny layer (Figure 2.13B, lower traces). The intensities of the water absorption bands noticed in the NIR region also provide an estimate of the mean photon path within the skin tissue, when rated against a transmission cell measurement of water. Interestingly, the fiber-optic probe can look deep enough to observe the doublet feature of the subcutaneous fatty tissue of the earlobe below 6000 cm^{-1} .

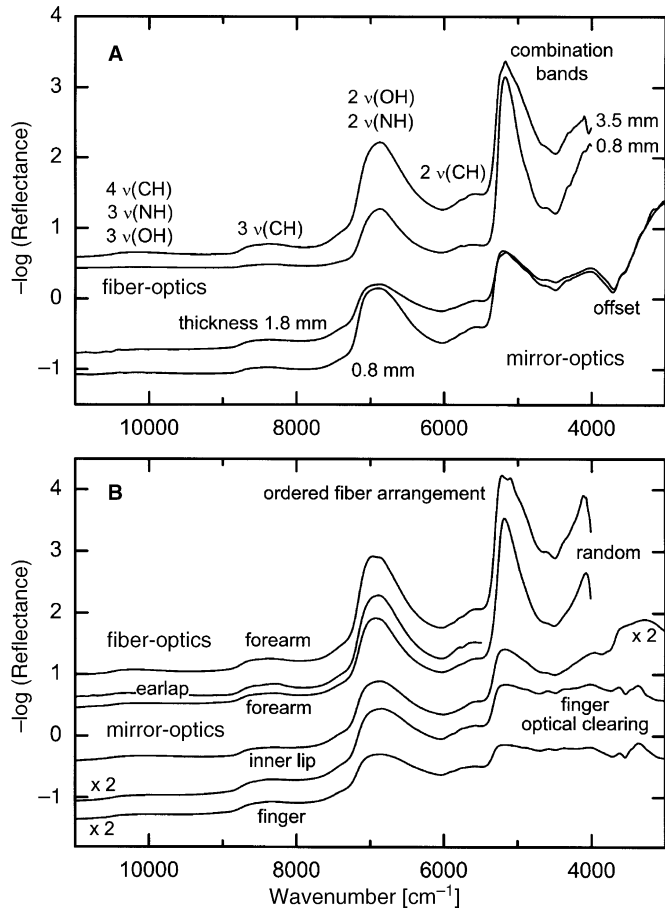


Figure 2.13. (A) Diffuse reflectance spectra of muscle tissue measured at different layer thickness (the samples were backed with a gold-coated diffusely reflecting substrate) recorded by using different NIR accessories. (B) Diffuse reflectance spectra of various skin sites using the same accessories (for optical clearing to reduce surface scattering from the horny layer of the epidermis and to improve the optical contact of the finger tip to the mirror accessory immersion lens, a perfluorated organic solvent was applied).

2.4.2 Soft Tissue Characterization by NIR Spectroscopy

Biopsy followed by pathological assessment is the gold standard and common approach to diagnose cancer. However, it is a time-consuming method and based on the pathologist’s expertise. This was the reason for studying the potential of NIRS for *in situ* pathology during surgery.⁸⁷ The latter paper also provides the literature for the following applications. In previous studies, the application of CH-overtone band information for the detection of human pancreatic and colorectal cancer has been suggested and discussed. In Figure 2.14, its application for both types of cancer is illustrated by highlighting the spectral differences between pancreatic and colorectal cancer tissues, which can be exploited by using different pattern recognition methods. A competing technique is certainly Raman spectroscopy using 1064 nm excitation wavelength and multichannel detection, for which also fiber-optic probes can be employed – for example, for an *in situ* diagnosis of lung cancer.⁸⁸

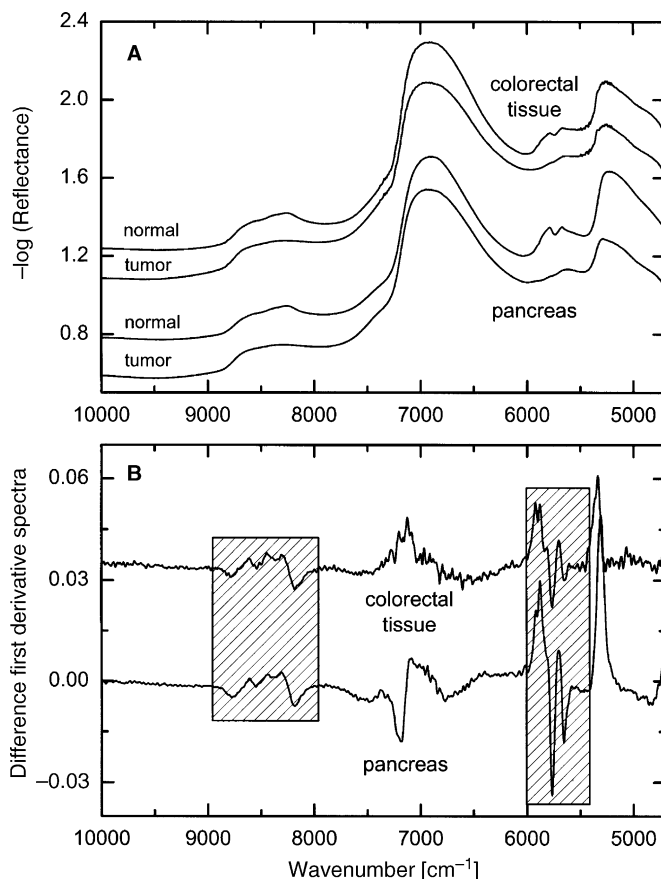


Figure 2.14. (A) Mean NIR spectra of colorectal and pancreatic tissue from the different classes of the sample populations studied (the artifact of diminished band intensities around 5000 cm^{-1} is due to the low transmittance of the quartz fiber-optic probe). (B) Differences between cancer and normal tissue spectra for pancreas and colorectal tissue, respectively; the raw spectra had been preprocessed by calculating first derivatives based on Savitzky–Golay convolution and subsequent vector normalization (shaded spectral intervals were used for organ-specific classification using, for example, linear discriminant analysis).

NIRS studies on animal models were mainly restricted to physiological aspects like tumor vascularity or tumor oxygen dynamics. NIRS studies on brain, muscle, mammary, lung, and prostate cancers in rats and mice reported altered vasculature, oxygen dynamics, and oxy-/deoxyhemoglobin concentrations in tumor tissues. Applications for photodynamic therapy (PDT), photothermal therapy, vascular modifying agents, and antiangiogenic therapy have also been reported. However, most of the NIRS studies reported on human tissues are from breast cancer. A key future development will be novel compounds that target cancers and fluoresce in the NIR window to enhance *in vivo* tumor-normal tissue ratios, affording also biochemical specificity with the potential for effective photodynamic anticancer therapies.⁸⁹ Other studies include cervix, skin, prostate, brain, pancreas, and colorectal tissues, but even further applications could be listed. Quantitative chemical information of breast, based on the absorption signatures of oxy- and deoxyhemoglobin, water, and lipids, has also been reported.

Furthermore, in a large study on skin neoplasms, McIntosh et al.⁹⁰ reported that the spectra of the different types of neoplasms exhibited differences in the regions with bands assignable to deoxyhemoglobin, oxyhemoglobin, water, proteins, and lipids, but differences in a single spectral region were insufficient to allow differentiation between all of the lesion groups.

2.4.3 Near-Infrared Spectroscopy for Imaging and Tomography

In the past 20 years, optical methods have been continuously improved for imaging and tomography applications, owing to their complete noninvasiveness and the use of nonionizing radiation. Several techniques based on the propagation of the radiation in turbid media were developed and applied for tissue diagnostics and/or monitoring of diseases or disease-related processes. For wavelengths between 600 and 1300 nm, the so-called therapeutic window, opportunities exist for measurements of intact body tissue. Monitoring tissue physiology with regard to blood and tissue oxygenation, the respiratory status, or ischemic damage can be assessed.¹⁷ NIRS has been used in functional imaging of the brain, with its regional oxygenation relative to its functionality and monitoring of muscle oxidative metabolism. Another application is breast cancer screening (optical mammography), for which also changes in the hemoglobin oxygenation state have been exploited using inexpensive continuous-wave (CW)-diode lasers with optimal wavelength matching.⁹¹ A recent review on diffuse optical imaging for cancer diagnosis has been given by Xu and Povoski.⁹² Rapid NIR diffuse tomography for hemodynamic imaging was presented by Piao and Pogue,⁹³ by which a low-coherence wideband radiation source was employed in combination with an imaging array consisting of eight sources and the same number of detection channels for realizing a sampling frequency of 5 Hz.

There are three fundamental types of optical techniques using NIR spectroscopy: continuous-intensity, frequency-domain, and time-resolved measurements. In the CW mode, continuous infrared radiation generated by a light emitting diode (LED) or a laser with a specific wavelength is applied to a biological sample. The changes in intensity of the radiation leaving the tissue surface are measured and correlated to changes in concentration of the major tissue chromophores such as hemoglobin, myoglobin, and water based on their specific absorption spectra. To obtain quantitative measurements, a complex model of photon migration in the tissue is necessary such as the knowledge of several parameters like the differential path-length factor. Multichannel continuous-wave (CW) imaging systems have recently been realized and used to generate images of the human brain or muscle in order to produce maps of brain or muscle oxygenation. Unfortunately, only few instruments are commercially available which are expensive or not approved by international standard institutions.

Frequency-domain instruments transmit inside the tissue an intensity-modulated laser beam at megahertz frequencies and measure intensity and phase shift of the backscattered radiation; for details, see also Ref. 94. By processing these parameters, it is possible to calculate absorption and scattering coefficients of the medium and the concentrations of chromophores. *Time-resolved* instruments measure the temporal response of the tissue to an ultrashort (picosecond) laser pulse; for more information, see Ref. 95. A single-photon counting detector records individual photons leaving the tissue and measures the time of flight (TOF) relative to a reference pulse. Absorption and scattering coefficients can be calculated by the TOF information using a radiation transport model.

Recently, optical technologies have emerged as a means for cardiovascular applications – that is, assessing the regional cardiac blood and tissue oxygenation in arrested and

beating isolated porcine hearts.^{96,97} The technique applied uses an NIR-sensitive charge-coupled device (CCD) array camera for two-dimensional image acquisition with a variable wavelength optical filter based on liquid crystal tunable filter (LCTF) technology to acquire images at each of a sequence of wavelengths. Isolated pig hearts were perfused using the Langerdoff method with whole blood and imaged by using the camera. Individual image acquisition was triggered by the electrocardiogram (ECG) signal to ensure that all images were recorded in the same heartbeat cycle phase. Applications of this technique to blood and tissue oxygenation certainly capitalize on the relatively effective penetration of NIR radiation into tissue (a few centimeters),⁹⁸ as compared to visible light with a few millimeters only, and the different near-IR absorbance spectra of oxygenated and deoxygenated hemoglobin, myoglobin, and water. Since the former compounds have nearly identical absorption spectra in the VIS/NIR spectral range, a separate quantification of the chromophores and their oxygenated species is difficult.⁹⁹

2.5 CONCLUDING REMARKS

Early changes in the homeostasis of living organisms have their basis in the biochemistry of the cells and tissues, which can be followed by vibrational spectroscopy, thus providing immense information on the metabolism, proteome, and genome of the living system. There is an extremely wide range of applications from breath analysis with parts-per-million concentration detection up to the analysis of biofluids and solid specimens with enormous diversity and inhomogeneity, for which sensitive analytical methods, preferably reagent-free and multianalyte-capable, are required. The analysis of integral tissue biopsies can be easily performed at the microscopic cellular level, for which even more efficient instruments have been lately developed, reaching the “diagnostic result” in much shorter times due to the technology progress observed in photonics and computers.

Another goal of vibrational spectroscopic technology is to develop noninvasive medical devices and techniques for gentle diagnostics to improve prospects for disease prevention, screening, early diagnosis, and better treatment leading to improved prognosis for the patient. Progress in biology, medicine, and health care largely depends on the advances in our ability to collect information from the analysis of gaseous metabolites, biofluids, and tissues, whether on the microscopic or macroscopic scale of biosamples from the whole body. The practical applicability of such instrumentation certainly depends on the successful collaboration between clinicians and spectroscopists.

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BIOMEDICAL APPLICATIONS OF INFRARED MICROSCOPY AND IMAGING BY VARIOUS MEANS

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3.1 INTRODUCTION

Excellent spatial resolution while maintaining spectral resolution enables localized chemical analysis *in situ* from normal and pathological tissues. These analyses are possible at the cellular and subcellular levels and reveal molecular differences in composition that can be correlated with histological changes between diseased and normal tissues. In either case the local concentrations of these chemical differences are often within the detection limits even when their concentration within the whole-tissue homogenate is below detection limits. Furthermore, the ability to retain spatial information can be used to identify chemical features that are present in structures or lesion sites that differ from the surrounding tissue. For example, plaque formation in Alzheimer brain tissue may be located by classic histological techniques, but the molecular structural changes of the plaque that differ from the surrounding tissue were revealed from the localized infrared spectroscopic response.¹ Figure 3.1 shows (a) Alzheimer plaque and (b) diseased white matter compared to normal white matter.² Analysis of plaque or other structures via nonmicrospectroscopic techniques requires greater effort and is often more complicated. Analytical chemists typically spend more time and effort in extraction, chromatographic separation, and various other concentration or purification steps than they do in the actual spectroscopic or other determination procedure. Thus, chemical analysis of localized pathology *in situ* is enhanced by state-of-the-art spatially resolved infrared microspectroscopy (IMS) and chemical imaging.

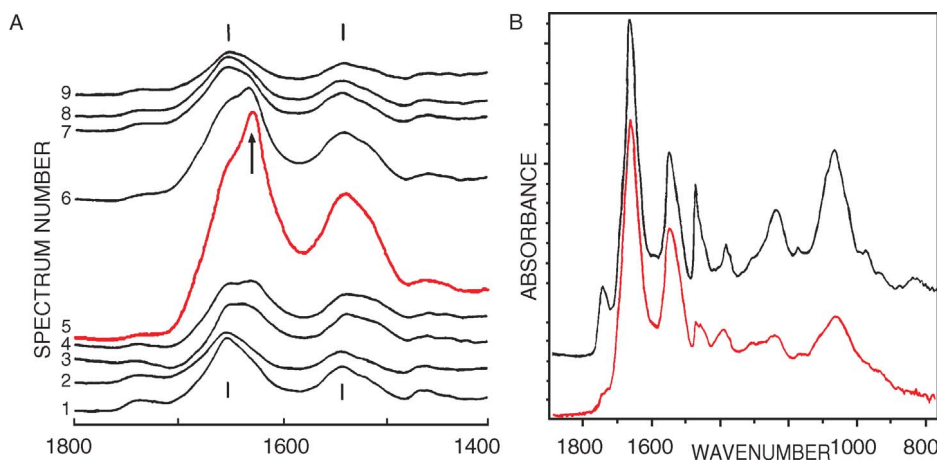


Figure 3.1. (A) Spectrum of Alzheimer plaque (β -sheet structure in red) and spectra of adjacent tissue of Alzheimer victim brain (Adapted from Ref. 1). (B) Spectrum of diseased or damaged brain white matter (bottom) in contrast to normal white matter (top). (With permission of *Biophysical Journal* and *Applied Spectroscopy Reviews*).

Vibrational microspectroscopy is readily available on a day-to-day basis in research laboratories and is used as a tool to study the mechanism of diseases in a variety of conditions.

In plant research, pathological conditions may arise from genetic alteration or environmental stress as well as attack from diseases, pests, or microorganisms. Baseline data from the control tissue of normal plants provides a comparison. In the area of plant breeding, genetic or environmental growth conditions and resistance to disease are factors of concern, but the response to post-harvest processing and the quality for end use also receive attention from the analytical testing step of plant research.

In medical research involving study of the mechanisms of diseases, animal models are commonly employed. Histological comparisons of normal and diseased tissues reveal the localization of pathology. Because of the spatial resolution of IMS and the chemical concentrations that occur in plaques, lesions, or other pathological manifestations, separation may be achieved by simply selecting the region in the microscopic field of view and excluding other parts in the field from contributing to the spectrum by image plane masking. When microscopic functional group maps or chemical images are produced, interpretation of the spectra of select pixels reveals the chemistry of those targeted areas.

Because none of the findings reported in this monograph would have been possible without spectacular instrument development, a list of spectroscopic “tools” is included in the introduction in Table 3.1 that are subsequently discussed in a separate section. Note that at the time of this printing, with the exception of certain specialized instruments, either single-detector or focal plane array instruments are available from a number of different manufacturers, and most array instruments now have the option of deflecting the beam to a single detector for point-and-shoot spectrum acquisition. Table 3.2 highlights some developmental milestones.

TABLE 3.1. Spectroscopic Tools for Biomedical Spectroscopy

1986: Infrared (IR) microscope accessory (with dedicated detector and dual image plane masks) optically interfaced to FT-IR spectrometer. (CC) Pittcon
1989: Integrated IR microscope/IR spectrometer with improved optical efficiency and mechanical stability with autogain, mapping capability, and dual image plane masks. (CC) Microbeam Analysis Society Meeting, Ashville, NC
1990: Designer paired IR microscope/FT-IR spectrometer combination with features of integrated system
1998: Infinity-corrected front-surface matched objective and condenser optics. Confocal operation with a double-pass, single, digitally controlled, image plane mask and image capture programmed mapping. (CC)
2000: Portable FT-IR with video image selected, small-spot diamond internal reflection operation. (CC)
2002: Miniature FT-IR spectrometer accessory for research grade microscope. (CC)
Step scan FT-IR with MCT or InGaAs rectangular focal plane array. (B)
Second-generation, rapid-readout-processing, FPA eliminated step scan. (B)
Linear pushbroom 16 MCT element FPA. (PE)
Near-IR imaging system with 320×256 pixel FPA in series with LCTF spectrometer available in InGaAs up to 1700 nm or TE-Cooled InSb in the 1100 to 2400 nm range.
A near-IR version of the pushbroom FPA and other FT-IR systems is available with change of beamsplitter, source and detector.
Near-IR fiber-optic catheter for artery wall analysis. (K)
Far-IR is available using an FT-IR microspectrometer with a He-cooled Cu bolometer detector and a quartz beam splitter.

CC designates instrument origin with a “**Connecticut Connection**” initially with SpectraTech Inc. (Stamford, CT/Shelton, CT) subsequently with Nicolet Instrument prior to Thermo Electron acquisition. The second-generation “**Connecticut Connection**” origin was SensIR™ Technologies, and its successor in Danbury CT, Smiths Detection. **B** stands for **Bethesda**, MD, National Institutes of Health, Laboratory of Ira Levine. **PE** stands for Perkin-Elmer, Shelton, CT. **K** stands for Kentucky, Lexington.³

TABLE 3.2. Milestone First-Time Events with Long-Term Impact

Introduced research-grade infrared microscope with confocal projected image plane masking 1986. Patented by Messerschmidt ^a and Sting, SpectraTech, Stamford, CT in 1989.
FT-IR microscope interfaced to synchrotron beam at National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY. Reffner ^b , Williams, Carr, September 12, 1993 Upton, NY.
First focal plane array InSb camera detector interfaced to a step scan FT-IR. NIH and Proctor & Gamble, Cincinnati, OH, Marcott and Lewis ^c , June 20, 1994.

^a Williams Wright Award, Robert Messerschmidt Pittcon Chicago 1996.

^b Williams Wright Award, John Reffner Pittcon New Orleans 2000.

^c Williams Wright Award, E. Neil Lewis Pittcon Chicago 2004.

3.2 SPECIMEN SOURCES, EXPERIMENTAL SCHEMES, AND OPTICAL SUBSTRATES

Laboratory research may entail use of an animal model of a human disease. Discovery or development of the essential animal model is a major step that enables design of future controlled experiments to study the mechanism of a particular disease; for example, the twitcher mouse has a genetic deficiency in myelin that mimics a white-matter human brain disease. Also among ApoE—/— knockout mice, the males are susceptible to induced

aneurysm formation. Once a suitable model is developed, studies are carried out to monitor responses to select diets, medications, enzymes, or other stimulation. At various stages of the treatment, the responses may be compared to tissue of a control animal.

The clinical setting provides specimens from ultrasound-guided needle biopsies for breast and other tissues or exfoliated cervical cells as obtained for the conventional Pap test. The clinical pathology laboratory provides specimens from surgical procedures or postmortem tissue from autopsy cases. Tissue banks of postmortem human tissue are maintained to provide a source of specimens for research. Research hospitals with a large patient load provide the opportunity to accumulate tissues that represent the disease being studied, and research-minded clinicians encounter pathological conditions that represent useful material for scientific investigation. In nearly all cases the histological tests serve as a parallel approach. Chemical selectivity of stain is based on the binding affinity to various types of molecules in the tissue. Localized deposition of chromophores or fluorophores results in the characteristic histological microscopic image. The texture and shape of microscopic objects provide valuable information but require subjective judgment.

The experimental scheme used in the research laboratory on animal models involves procuring model animals and programming the treatment and analytical testing by the day within the gestation cycle. Control animals are included in the treatment and testing.

Routinely frozen sections of tissue for infrared (IR) transmission are thaw-mounted onto nonhygroscopic CaF_2 or BaF_2 . The specimen is frozen to a specimen mount with Tissue Tech (OCT). Other mounting materials commonly used for histology on glass slides are avoided because of their spectral absorption. Paraffin sectioning followed by standard toluene treatment dissolves the paraffin but also removes virtually all lipids contained in the specimen.

For IR reflection absorption operation, mirrored slides or IR reflecting low n glass slides are used. IR reflecting glass slides are a low-cost substitute for BaF_2 discs, but because the radiation traverses the specimen twice, the specimen must be half as thick, typically $4\text{ }\mu\text{m}$. Operation in the reflection mode is through a beamsplitter, reducing the signal by half. In both modes, translucency of the tissue is essential to minimize scatter loss.

Specimen substrates introduce chromatic aberration. Windows such as BaF_2 create a nonlinear wavelength-dependent focus that increases at lower wavenumbers. In the spectral region, the IR focus is not coincident with the visible focus. This loss of focus results in loss of signal and spatial resolution is also compromised. This affects mapping for detailed probing. Since the magnitude of chromatic aberration depends on path length through the window, 1 mm-thick BaF_2 windows are less of a problem than the more rugged 2 mm-thick windows. The wavelength-dependent focus may be minimized by using a AgCl or diamond window as described in a later section of this chapter. Manmade diamond windows of usable dimensions are now a practical reality. When the standard BaF_2 window is used, maintaining the focus at the frequency of the band being investigated is possible when a preview scanning function is used to tweak the focus while observing the intensity at the chosen frequency. This procedure is routinely used at synchrotron IR microspectroscopy installations when confocal optics are being used to achieve maximum spatial resolution.

3.3 APPLICATIONS

3.3.1 IMS of Biological Materials in General

A great deal of effort has gone into spectroscopic detection of precancerous or cancerous tissue within biopsies from a variety of human tissues. The end goal in most of these cases

has been to develop clinical diagnostic methods for early cancer detection. On these projects, spectroscopists typically work in partnership with clinical personnel who provide healthy and cancerous tissue accompanied by histological results. An early series of articles by Chiriboga et al.⁴ may be found via one of their post-2000 articles. Other researchers with multiple entries in the area include Dukor,⁵ McNaughton and Wood,⁶ Naumann and Lasch,⁷ Schultz,⁸ Mantsch and McCrae,⁹ and their co-workers. Encouraging results have been reported regarding spectroscopic differences between known diseased and healthy tissue. However, an ambitious blind study of cancer detection from spectroscopic data of breast biopsies involving six million spectra failed, reportedly because the spectral cancer detection criteria developed was based on a limited population of known cases.¹⁰ Therefore, caution and refinement of IR cancer detection schemes is required before clinical use can be adopted on a routine basis. However, progress via data processing schemes is reported in later sections of this chapter.

Fabian et al.¹¹ reported on IR microspectroscopic imaging of benign breast tumor tissue sections. Baseline data were produced with the IR spectra of major tissue constituents observed within benign breast tumor tissue sections. These include the epithelium of a fibroadenoma, connective tissue, adipose tissue, and milk secretion ducts. This work suggests that IMS allows the differentiation between benign and malignant tumor types located in breast ducts.

Research on diseases of the bone, bone development, and the mineralization process has been extensive. Mapping of diamond-sawed, thin bone sections outward from the center of an osteon that is high in protein reveals phosphated bone and ultimately carbonated older bone showing stages of bone development. Studies of female monkey and canine bones from animals with their ovaries removed gave insight into the effects of osteoporosis and osteoarthritis. Mendelsohn, Boskey, and co-workers¹² are long-term bone researchers who use IMS and IMS imaging. Also Miller,^{13,14} working with scientists from Albert Einstein College of Medicine and University of Wisconsin, is an ongoing contributor in the bone research field. Gallstones, teeth, and other materials were examined by Wentrup-Byrne, Paluszkiwicz, and co-workers.¹⁵ Foreign substances in the body were identified by Kalasinsky using IMS.¹⁶ Arterial walls (normal and pathological) have been studied spectroscopically via near-IR fiber-optic catheters by Dempsey, Lodder, and co-workers.¹⁷

In a sequence of investigations by Miller using synchrotron IMS on studies of bone, three new reports have appeared. Miller and co-workers including representative members of the Rutgers bone team¹⁸ studied the phosphate vibration. Huang et al.¹⁹ studied the *in situ* chemistry of osteoporosis. More recently, Ruppel et al.²⁰ studied microdamaged bone and undamaged bone in terms of the localized chemistry.

Single wheat-cell mapping and analysis was initially reported by the author in 1992 using a conventional source,²¹ and subsequent mapping using a synchrotron source produced sharper boundaries²² (see Fig. 3.2). Living cells, dying cells, and cells undergoing mitosis have been mapped by Jamin, Dumas, and co-workers²³ using the maximum spatial resolution possible with synchrotron IMS. Figure 3.3 shows images of a mitotic cell at two wavelengths. Wetzel and Williams, using the synchrotron source at NSLS beamline U2B with longitudinally sectioned human hair, provided detection of drug metabolites localized to a 5.5 μm spot equivalent to less than 22.5 min of head hair growth as shown in Fig. 3.4.²⁴ Kreplak et al.²⁵ profiled lipids across cross sections of hair using synchrotron IMS. The chemical distribution was profiled to show the chemical content of the medulla, the cortex, and the cuticle. In this study, specimens were obtained from pigmented and nonpigmented Caucasian and Afro-American hair. Gough et al.²⁶ reported synchrotron IMS analyses of scar tissue resulting from cardiac surgery with and without postsurgical preventative treatments intended to limit scar

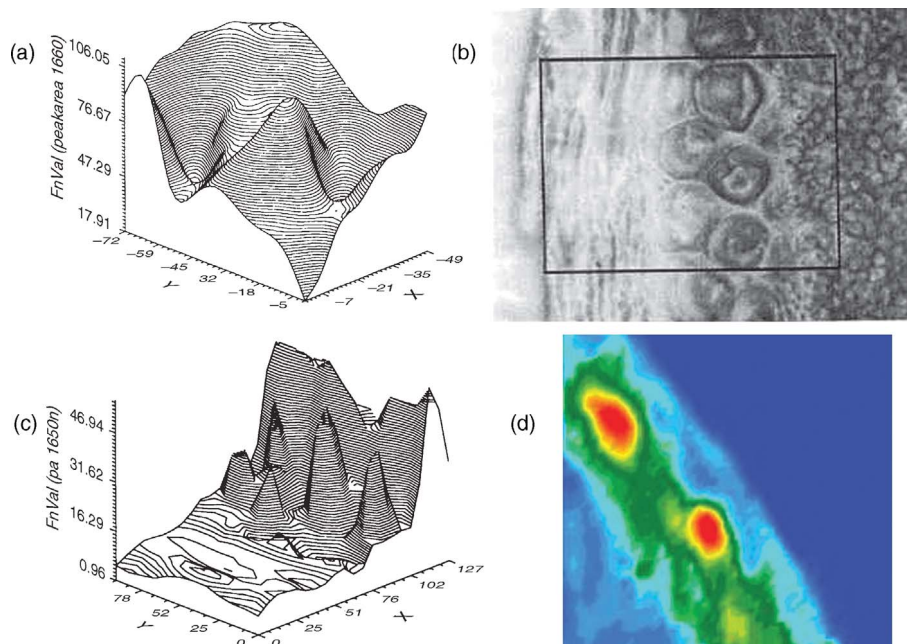


Figure 3.2. Image of single wheat aleurone cell, 1992, from confocal ($6\text{ cm}^{-1} \times 7\text{ cm}^{-1}$) global sourced IR μs^{TM} at KSU. Three-dimensional image showing two cells and partial cell corresponding to photomicrograph with synchrotron IMS (IR μs^{TM}) and confocal operation. Note improved spatial resolution. False color image shows aleurone cells from FPA system. (With permission of *Cellular and Molecular Biology and Vibrational Spectroscopy*).

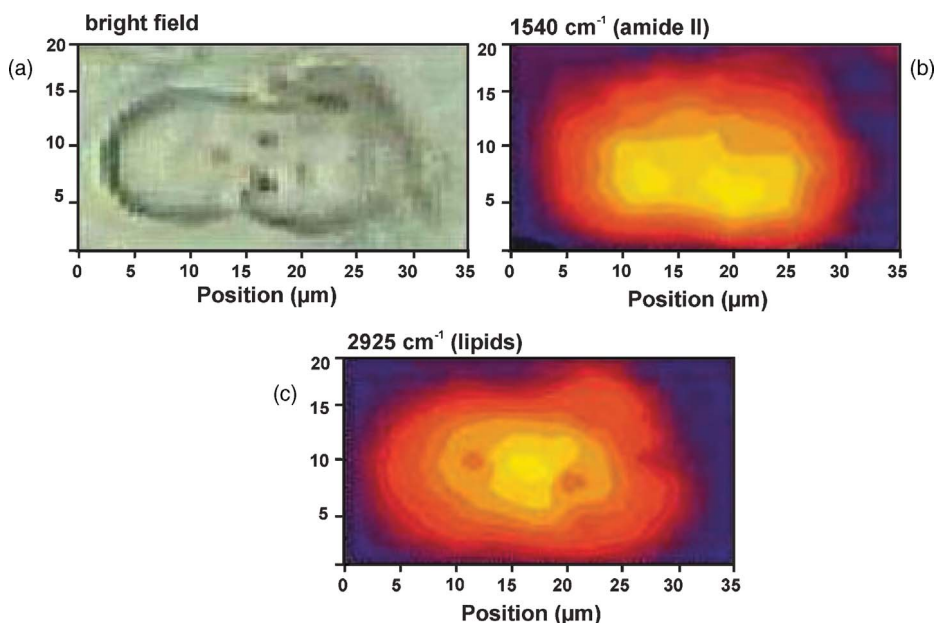


Figure 3.3. (a) Photomicrograph of a cell undergoing mitosis. (b and c) Images of amide II band at 1540 cm^{-1} and the CH_2 stretch at 2925 cm^{-1} , respectively. Note forming nuclei in image c. (Adapted from Ref. 23 with permission of the National Academy of Science.)

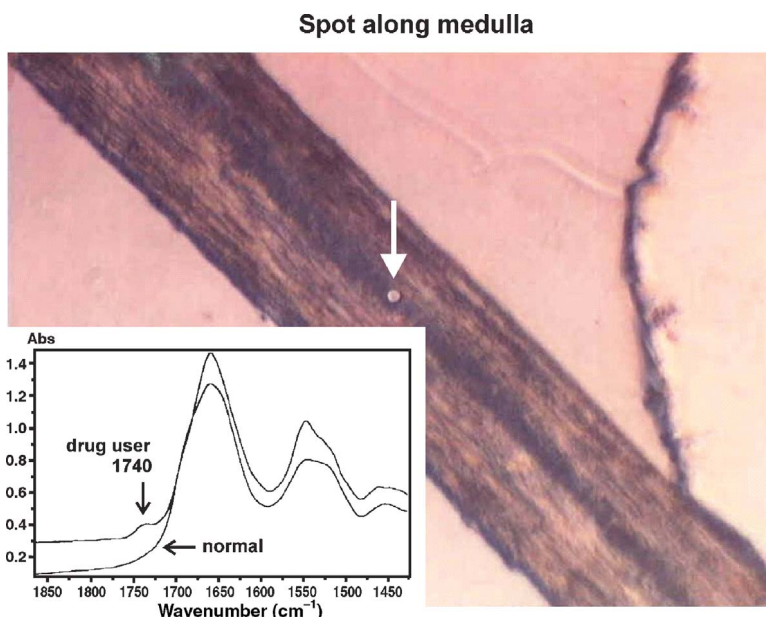


Figure 3.4. Photomicrograph of longitudinal section of human hair representing 1 day's growth. Carbonyl in one spectrum is from a 5.5 μm spot representing 22.5 min in the life of drug user. (From Ref. 24 with permission of *American Institute Physics*).

formation. In the plant research area, scientists from the Carnegie Institute on the campus of Stanford University, working with *Arabidopsis* plants (whose genome has already been sequenced), used IMS to examine the spectra of cell walls of mutants for comparison to those from the cell walls of the wild-type parent plant material. The effects of enzymes upon cell walls reported by Sorenson et al. have been studied by IMS.²⁷ Dubois et al.²⁸ used near-IR chemical imaging to identify bacteria. Space does not permit reference to other excellent biological applications, and the reader is referred to a previous book chapter by the author with 159 references,²⁹ a review,² and other articles.^{30,31} Another recent article targeting industrial chemists describes applications in the materials and forensic sciences.³²

3.3.2 Applications of IMS to Grains and Brains

Application of IMS and IMS imaging to research in the area of grains and brains is featured to exemplify applications of biospectroscopy through a microscope. In our first published report of brain spectra,³³ we examined the white matter, gray matter, and basal ganglia of rat cerebrum sections from multiple animals. The spectra of the white matter was distinguished by carbonyl (1740 cm^{-1}) of lipids along with C–H stretching and bending vibrational bands at 2927 cm^{-1} and 1469 cm^{-1} , respectively. The high C–H to carbonyl ratio and the presence of carbohydrate (HO–C–H) at 1085 cm^{-1} was explained by the presence of galactocerebroside in the white matter. The 1235 cm^{-1} band was attributed to the presence of phospholipids. Subsequent studies of white-matter diseases or damage to white matter from reactive oxygen species have usually revealed a reduction in the bands characteristic of white matter. In tissue regions containing a high population of nuclei, the DNA contributes not only to the amide I and II of the nucleic acids at 1650 cm^{-1} and 1550 cm^{-1} , but also to the phosphate and ribose at 1235 cm^{-1} and 1085 cm^{-1} , respectively.

Selected peak areas were averaged from at least 900 spectra of the three layers of adult rat cerebellum tissue from several animals given 30–40% D₂O in drinking water, for 5.5 weeks. This proved to be a convenient opportunity to study brain metabolism with a mass isotope instead of radioisotopes³⁴ (Refer to Fig. 3.5 for results.) In twitcher mice (a model of globoid cell leukodystrophy),³⁵ differences (Fig. 3.6) were reported by LeVine and Wetzel in WM and GM lipid band intensities. In rat brain containing extravasated blood³⁶ and in multiple sclerosis (human) tissue,³⁷ reported by LeVine and Wetzel, the distinguishing spectral features characteristic of white matter were markedly altered and the altered chemical features were used to address the pathological mechanisms (see Fig. 3.7). The spectrum obtained where no blood was present is that of normal WM. In the penumbral area, major destruction of the lipid has occurred; and where the blood is present, amide bands are higher and the carbonyl is only slightly visible.

3.3.3 Experiments with Retina Tissue

At the medical school site we have been characterizing the *in situ* spectroscopic features of retina tissue of the rat. This layered and highly ordered structure occurring in nature is readily studied by IMS. Replicate spectra of individual layers were used by LeVine et al.³⁸

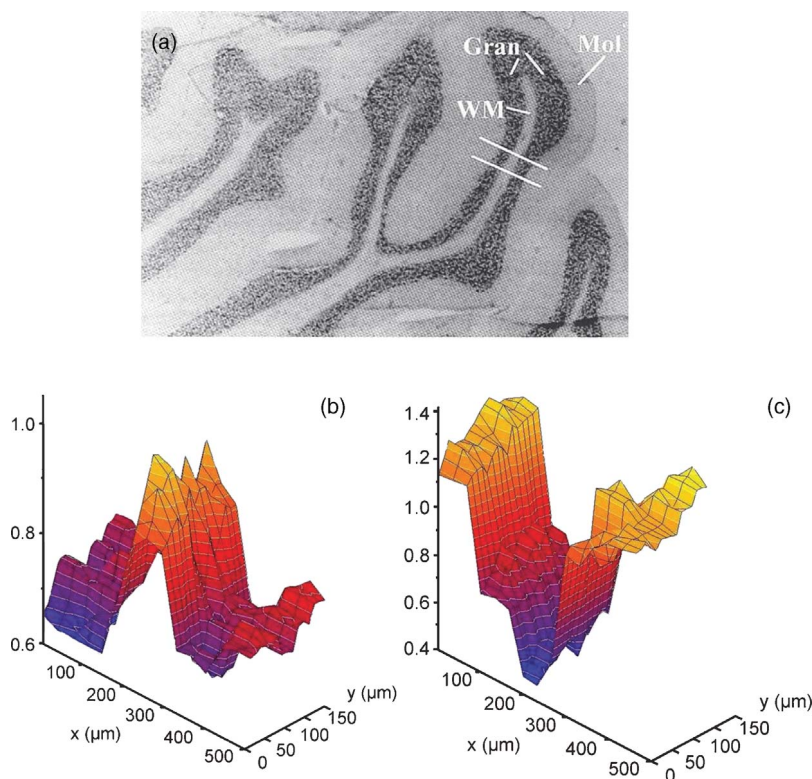


Figure 3.5. (a) Photomicrograph of rat cerebellum from adult rat fed 30% D₂O 5.5 weeks in drinking water (from Ref. 34). Note white matter (WM), granular cell layers (Gran), and molecular cell layer (Mol). (b) False color three-dimensional figure (yellow represents high) shows the distribution of CD highest in WM. (c) Three-dimensional figure showing the CD/CH ratio indicating that the relative uptake was great in MOL. (With permission of *Cellular and Molecular Biology and Biophotonics*.)

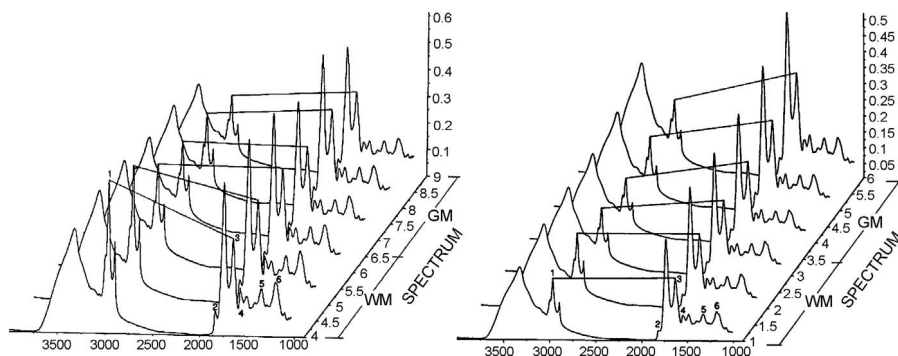


Figure 3.6. Spectra taken from line map of cerebrum white matter (WM) into gray matter (GM). (Left) Normal mouse with prominent lipid bands 2927 cm^{-1} and 1740 cm^{-1} in the WM spectra. (Right) WM diseased brain with little chemical distinction between GM and WM spectra (From Ref. 31 with permission of *Applied Spectroscopy Reviews*).

to provide statistically acceptable baseline data for both pigmented and albino retinas. Chemical compositional differences were observed between various layers of the retina. Retina layers (Fig. 3.8, photomicrograph) listed from the pigment inward are outer segments (OS), inner segments (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), and inner plexiform layer (IPL). In normal animals, the outer segments had striking absorbance values for $\text{C}=\text{C}-\text{H}$ and carbonyl functional groups. The presence of the lipid docosahexaenoic acid (with six conjugated double bonds), which was identified by comparison to a spectrum of the pure compound, was localized to the outer segments.³⁸ Spectra of individual retina layers revealed the distribution by functional groups.

In contrast to the outer segments, the outer nuclear cell layer had relatively low levels of $\text{C}=\text{C}-\text{H}$ and carbonyl groups, but high concentrations of $\text{H}-\text{C}-\text{OH}$ and $\text{P}=\text{O}$, which are likely due to the carbohydrate/phosphate backbone of DNA. In albino retinas, the levels of $\text{C}=\text{C}-\text{H}$ and carbonyl groups were reduced in the outer segments compared to that observed in normal outer segments, indicating that light-induced oxidative damage resulted in diminished levels of docosahexaenoic acid.

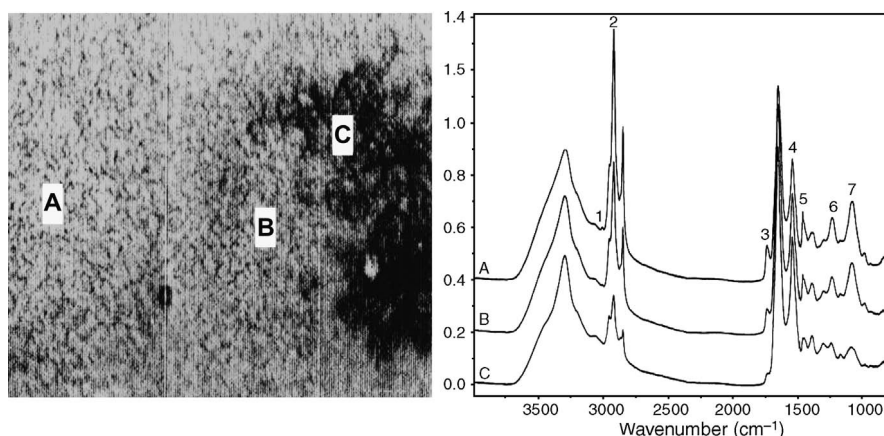


Figure 3.7. Photomicrograph of gray matter with extravasated blood. Lipid bands 2927 cm^{-1} and 1740 cm^{-1} are highest at A away from the blood. Spectrum C (bottom) has these bands reduced from destruction via oxidation. (From Ref. 36 with permission of *American Journal of Pathology*.)

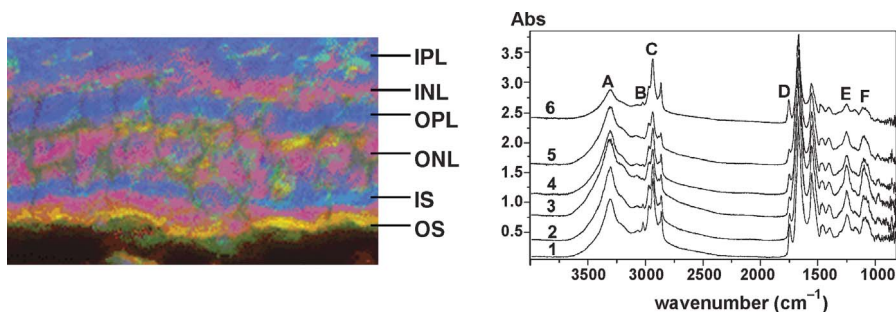


Figure 3.8. (Left) Photomicrograph from differential interference contrast (DIC) image of unstained retina tissue. From the pigment (*bottom*), successive layers are shown inward. (Right) Spectra from individual layers. Note: OS (outer segments) layer is rich in lipid where lipid chain length, branching, and glycolipids are inferred by comparing contributions of C=O, CH₃ stretch, CH₂ stretch, and H–C–OH groups. The amount of unsaturation is in evidence from the CH absorption band at 3015 cm⁻¹ on the carbon that is attached to the C=C bond. Note the ONL (nuclear cell layer) where the nuclei are responsible for the strong P=O band at 1235 cm⁻¹. (From Refs. 31 and 38 with permission of *Science* and *Biochimica et Biophysica Acta*)

In another experiment by Homan et al.³⁹, the application of ferrous sulfate, which causes oxidative tissue damage to the eye, resulted in degradation of lipids in the photoreceptor (outer segments) layer in comparison to controls where only saline was injected. It was also noted that injection of saline alone stimulated metabolism and was accompanied by spectral changes in comparison to controls with no injection. Synchrotron IMS retina mapping by Wetzel and Williams⁴⁰ using small focal image plane masking and small step size showed chemical detail along and across layers.

Recent previously unpublished experiments with rat retinas have involved baseline metabolic studies with a ContinuumTM (Nicolet, Madison, WI) microspectrometer equipped with an auxiliary custom narrow band, liquid nitrogen-cooled, MCT detector with a 50 μm × 50 μm element size. This custom-built MCT detector has a maximum response at 5 μm (instead of 12.5 μm), which is sensitive to the CD stretching vibration in the 2150 cm⁻¹ region and includes the ND and OD absorptions in the 2500 cm⁻¹ region.⁴¹ This narrow-band detector is virtually blind in the fingerprint region of the infrared spectrum, thus reducing the noise. The ContinuumTM with the customized narrow-band detector in our laboratory provides lower detection limits for deuterated species found in tissue. Locally or systemically injected deuterated compounds diluted by circulation are more readily detected and measured with this enhanced IMS instrument.

Pigmented rats given 35% D₂O in their drinking water were used to obtain spectra. At 1-week intervals, multiple spectra were obtained for individual layers. Using the CD-sensitized narrow-band instrument, a measurable amount of metabolically deuterated compounds was found in retina tissue after only 1 week. In the outer segments, the incorporation of deuterium increased up to 3 weeks and then plateaued thereafter.⁴² Subsequent studies examined the effect of photostimulation over a 14 day period by subjecting animals to 1 hour of xenon 3 Hz strobe lighting daily. Photostimulation resulted in an increase in concentration of ND in all layers, but not CD, compared to normal lighting. (See Fig. 3.9 for the effect of photostimulation and the ND formation by layer over 5 weeks).

IMS retinal research in progress is concerned with molecular orientation within individual retina layers and, in particular, in the photoreceptors of the outer segments layers. Electron photomicrographs of rat retinas show the physical ordered structure of individual outer photoreceptor membrane segments made up of successive individual

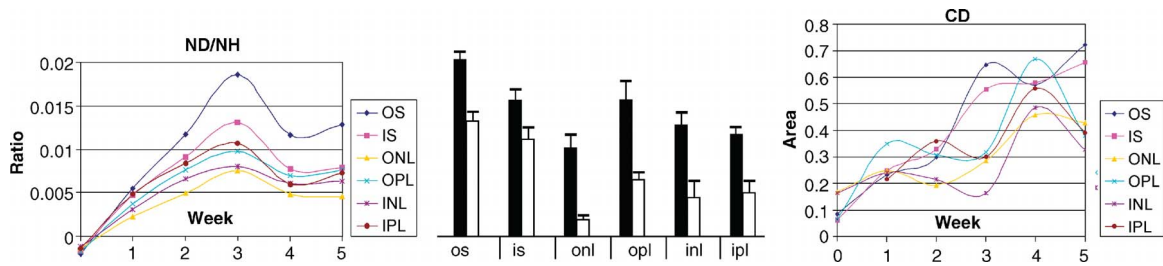


Figure 3.9. (Left) Relative amount of deuterium uptake as ND/NH for each of six layers at weeks 1–5 of D₂O consumption with exposure to strobe light. Note that in all cases a maximum was reached at 3 weeks. The bar graph shows the CD population in solid bars and the CH portion in open bars. (Right) Relative CD composition for each layer for weeks 1–5. Each layer of each retina was analyzed individually. (Original data from the KSU microbeam molecular spectroscopy laboratory.)

photoreceptor membrane disks. Polarized IMS at orthogonal orientations of the polarizer produces spectra from which dichroic ratios for specific functional groups can be calculated. This technique is used to reveal molecular orientation within the photoreceptor segment. Insufficient SNR in a stock IMS instrument, due to attenuation from the polarizer and scattering from the specimen at the aperture size required by dimensions of the outer segments layer, resulted in failure to obtain adequate data. Preliminary experiments by Reffner, Wetzel, and Radel (unpublished) at a series of polarizer orientation angles with synchrotron IMS has indicated a distinct angular dependence of dichroic ratios (and thus molecular orientation) for select organic functional groups within the photoreceptor disks of the outer segments. Figure 3.10 shows typical differences in dichroism at two different orientation angles. This may constitute (to the best of our knowledge) the first successful, relatively nonintrusive, *in situ* polarization IR study of photoreceptors. This series of studies devoted to the retina illustrates the utility of IMS on just one important neurological tissue. Prior classical studies of photoreceptor membranes were performed elsewhere with

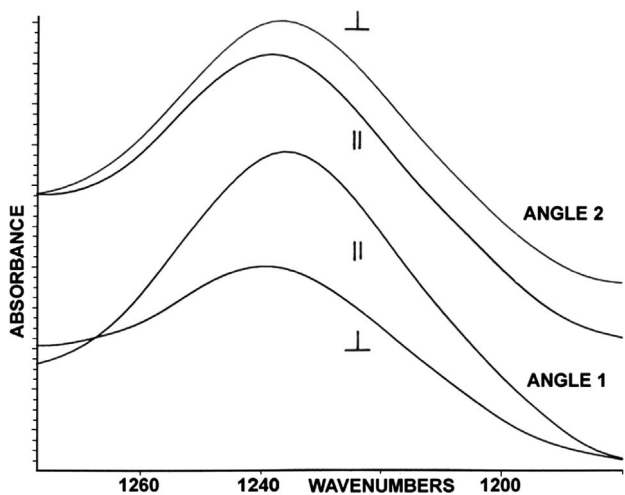


Figure 3.10. The top pair of spectra have similar responses for both perpendicular and parallel polarization, showing little dichroism. At a different angle of polarization, dichroism is observed on the bottom pair of spectra for the protein bands. (These data are original from the KSU microbeam molecular spectroscopy laboratory.)

polarization IR on a macro scale. Working with homogenates of 100–160 bovine retina outer segments, deposited as a layer on an infrared window, polarization effects were observed. However, the spatial relationship of the segments was not maintained.

3.3.4 Heart

Ongoing research in the laboratory of Lodder at the University of Kentucky has been concerned with aneurysm formation and atherosclerosis. This activity has included near-IR monitoring during surgery in connection with the medical school, using a near-IR (InSb) camera mounted at least 1 meter above the wound for sterile requirements. Also, as a result of work at Kentucky, a fiber-optic catheter connected to a near-IR spectrometer was introduced.³ That catheter has been evaluated by the U.S. Food and Drug Administration for the past 9 years, and a commercial product is scheduled for introduction in 2008. With this device, it is possible to analyze arterial walls including those of the aorta for lipid content as evidence of plaque formation. Aneurysm formation is thought to be preceded by conversion of elastin to collagen I. Additionally, some of the collagen III is also converted to collagen I. Another purpose for *in vivo* testing by way of the optical catheter is detection of enhanced concentration of collagen I relative to elastin. An article by Urbas et al.⁴³ describes the use of near-IR spectrometry of the ApoE^{−/−} mouse to reveal collagen I/elastin ratios. In these studies, infusion of the enzyme angiotensin II (Ang II) into the subcutaneous space of mice was done in doses ranging from 500 to 1000 ng kg^{−1} min^{−1} for 7–28 days. These were used as models of abdominal aortic aneurysm (AAA) development. This study showed that near-IR spectrometry and principal component regression (PCR) can be used to obtain the collagen/elastin ratio and to determine the Ang II dose in a mouse aorta.

The vulnerability of male ApoE^{−/−} knockout mice to AAA formation upon administering the enzyme Ang II was established from previous work at the University of Kentucky by Cassis, coauthor of ref.⁴³ A synchrotron IMS experiment with ApoE^{−/−} knockout mice aorta tissues was performed to look for enhanced collagen/elastin ratios in the mid-IR region of the spectrum in localized portions of the aorta wall. A vulnerable portion of the wall would be a region where the collagen I buildup occurred at the expense of elastin. Whereas previous near-IR data obtained *in vivo* via catheter had a limited spatial resolution (due to the size of the probe) and the motion of the subject from heartbeat and respiration was limited, high spatial resolution was possible with mid-IR frozen sections. Frozen sections of aorta from an ApoE^{−/−} mouse to which Ang II had been administered provided the opportunity to examine infrared absorption bands in the fundamental vibrational part of the spectrum in regions along the cell wall of the aorta.

The section examined was from a male mouse; 40% of male mice show aneurysm formation. Spots to probe were selected from video images of the tissue on the microspectrometer stage. In addition, mapping produced images that enabled a detailed search for the vulnerable portions of tissue that may contain enhanced collagen/elastin ratios. This particular aorta specimen did not show a developed aneurysm. However, differences in the collagen/elastin ratio were determined from the spectroscopic responses at different regions along the aorta wall.⁴⁴ One section of the aorta that was opposite a weak spot in the wall showed that in the healthy aorta wall, the intima of the inside contains lipid evidenced by the functional group map of the 1740 cm^{−1} baseline-corrected peak areas. Similarly, the adventitia on the outside of the aorta wall was defined by a higher population image of the 1085 cm^{−1} baseline-corrected peak areas. Figure 3.11 shows maps of two functional groups, along with a textbook drawing representative of this section of the aorta.

In another study by Wetzel and Lodder,⁴⁶ aorta sections were probed from frozen sections of LDL^{−/−} receptor-deficient mice on a C57BL/6 background. AAA tissues were

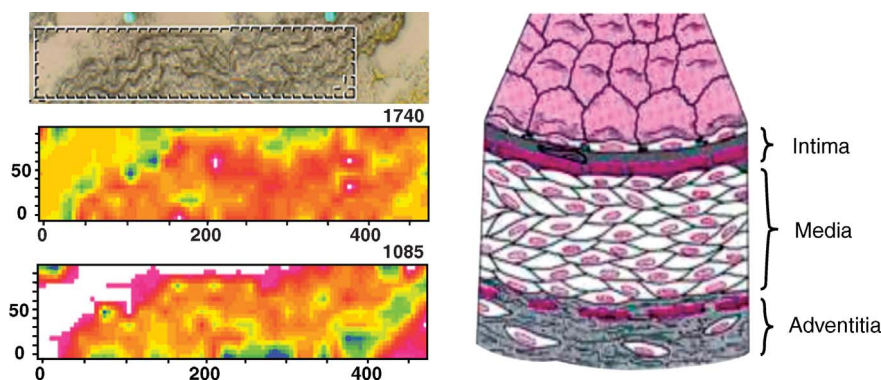


Figure 3.11. (Top left) Photomicrograph of unstained aorta wall. The image from 1740 cm^{-1} baseline adjusted peak areas clearly defines the intima (inside of aorta wall). (Lower left) Image that chemically defines the adventia [from Ref. 44]. (Right) Textbook drawing for clarification [from Ref. 45 with permission of W. B. Saunders].

mapped extensively with confocal operation of a ContinuumTM microspectrometer on a synchrotron IR beamline. Contiguous sections thaw-mounted on IR reflecting glass microscope slides provided selection of the desired stage of aneurysm formation as well as intact aorta tissue in a close proximity to the developed aneurysm. A healthy section of the aorta, which preceded by $50\text{ }\mu\text{m}$ the region that showed an aneurysm, provided the opportunity to image portions of the aorta wall prior to aneurysm formation. The tenth $5\text{ }\mu\text{m}$ -thick section, separated by $50\text{ }\mu\text{m}$ from the intact aorta wall, provided a complete breakthrough of the wall and left in its place the formation of a large aneurysm. Although the section with the aneurysm was too large to map in single experiment, a mosaic made up of several maps was produced. In this way, the detail from a small image plane mask size and small step sizes retained the spatial resolution. The mosaic of these smaller mapped sections produced a large image that retained spatially resolved detail. An example of this is shown in Fig. 3.12.

Of the many people who experience a sudden cardiac event (sudden cardiac death), a large portion have no prior symptoms. One potential *in vivo* spectroscopic technique, the diagnosis of pathological condition that underlies sudden cardiac events, involves use of a near-IR spectroscopic catheter. This device has been previously discussed. To substantiate

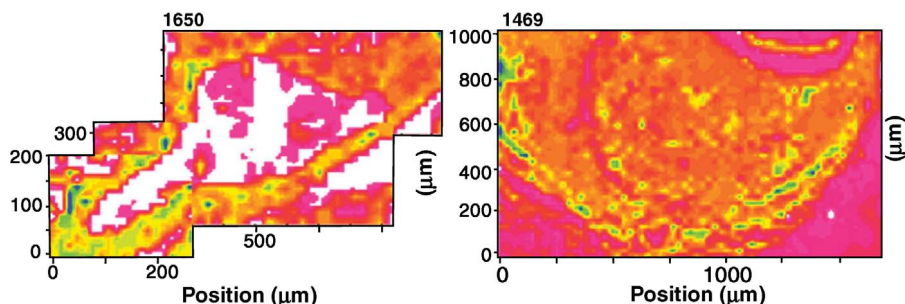


Figure 3.12. (Left) Mosaic image at 1650 cm^{-1} baseline-adjusted peak area from several maps of the same aorta. At the position between 12 and 3 o'clock the aorta wall has been broken and aneurysm formation has taken place. Mapping of this enables study of the localized chemical changes that just precede formation of an aneurysm. (Right) Map of a large aneurysm observed at 1469 cm^{-1} , representing lipid distribution (From Ref. 46 with permission.)

the validity of the near-IR catheter, the most vulnerable region of the aorta needed to be examined. The most vulnerable narrow region is at the shoulder of the thin-cap fibroatheroma. A thin-cap fibroatheroma is a rupture-prone plaque. The shoulder of the cap (where the cap meets the vessel wall) is most vulnerable to rupture because mechanical stress at this point weakens the collagen and elastin fibers. Postmortem human tissue was used for synchrotron IR microspectroscopic analysis of collagen I, collagen III, and elastin on the shoulders of the human thin-cap fibroatheromas.⁴⁷ From the microscopic video image of the rather large human coronary, locations on the fibrous cap were selected from which to obtain IR spectra. An adjacent stained section was also used to help identify the region of interest. Control images were obtained in a different part of the same section away from the fibrous cap. Representative IR spectra were obtained for the region identified. Spectra of lyophilized standards of collagen I, collagen III, and elastin were obtained to allow comparison. Spectra of standard mixtures of collagen I, collagen III, and elastin were used for mean-centered correlation analysis. Separate images of the collagen I distribution, collagen III distribution, and elastin distributions were produced for the same region of the fibrous cap.⁴⁷

This preliminary study had some important limitations. A single patient served as the source of 80,000 spectra collected from 24 coronary sections, limiting the observable variation in the data set. The lack of detailed histological data for the sample and lack of clinical history from the patient prevents association of spectra with specific tissue pathologies and comparison of pathology. Most importantly, the exact location of any rupture (culprit lesion) was not uncovered in the tissue sections examined. For this reason the exact nature of the gradients within 10 μm of any tear in the fibrous cap could not be determined. However, the fact that gradients in collagen/elastin similar to those observed in AAAs did exist in the vicinity of a plaque rupture suggests that a similar mechanism of protein degradation may be responsible in both disease states. Thus an increase in collagen I at the expense of collagen III (and possibly the elastin) might serve as a marker of plaques needing an immediate intervention.

A study was carried out in 2006 that involved examining aortas of ApoE $^{-/-}$ knockout mice on three different diets. The results of this study are of special interest to cardiac patients who are also diabetic. The aortas of adult mice fed for 15 weeks on three different diets (normal, drug, and sucrose) were studied.⁴⁸ With the mouse on a normal diet, the aorta was examined and examination of the video image showed very little evidence of spongy material along the aorta wall. When areas with potential lipid deposition were examined, essentially no lipid was found.

The opposite result was observed for aorta sections from an animal on a sucrose diet. After the feeding period, approximately two-thirds of the inner aorta wall had strips of a foamy nature distributed along it. Probing these foamy regions produced spectra with very large amounts of lipid, as evidenced by the carbonyl band at 1740 cm^{-1} and by a very large CH_2 band at 2927 cm^{-1} relative to the NH stretching band of protein at 3300 cm^{-1} . Mapping of these spongy regions showed considerable heterogeneity in the lipid content even within the same foamy region. In contrast, the adjacent aorta wall tissue showed absolutely no carbonyl band at 1740 cm^{-1} or very much of a CH_2 band at 2927 cm^{-1} . Instead, very prominent protein amide I and II bands appeared at 1650 cm^{-1} and 1550 cm^{-1} , respectively. Also, an NH stretch at 3300 cm^{-1} was prevalent.

One purpose of this study was to show the effectiveness of substituting a particular drug, synthetically prepared, in place of sucrose. The result is that although there were some minor depositions of foamy material distributed around the inside of the aorta, there was no comparison of the deposition on these aortas with those of the animals fed sucrose.

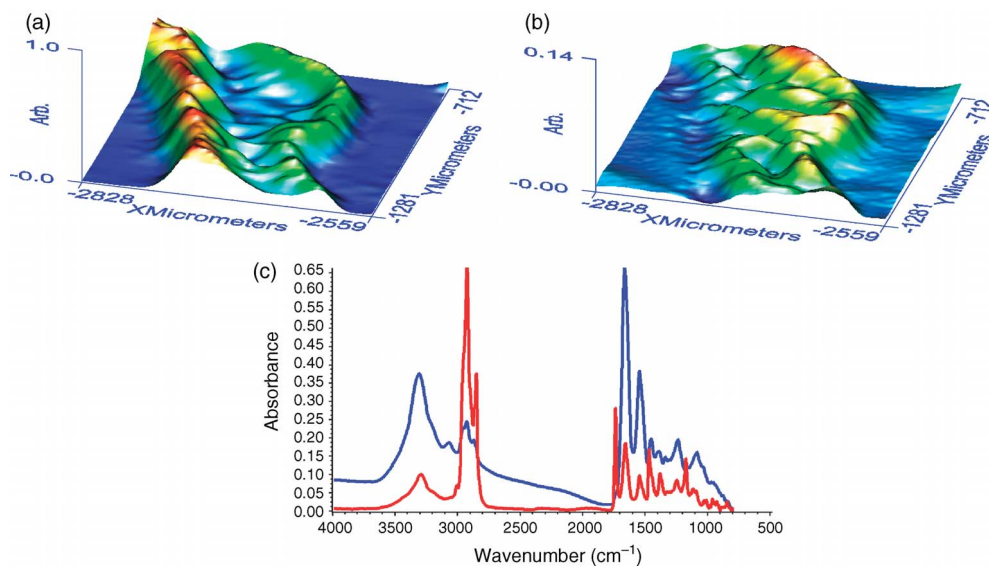


Figure 3.13. (a) Image highlighting the protein of an aorta cell wall. (b) Image highlighting the lipid of spongy material adhering to the aorta wall. (c) Spectra (blue) wall and (red) spongy are from corresponding highlighted parts, respectively. The tissue was from an ApoE^{-/-} knockout mouse that was fed a sucrose diet.

Figure 3.13 shows spectra obtained from a large foamy area and from the corresponding aorta wall in the same tissue.⁴⁸

Infrared imaging of compositional changes in inflammatory cardiomyopathy was reported by Wang et al.⁴⁹ This was a cooperative effort between the German universities Humboldt-Universität zu Berlin and Eberhard Karls Universität Tübingen with Brookhaven National Laboratory. This work addressed the condition commonly referred to as “heart failure.” It is associated with the pathophysiological state in which the heart is unable to pump blood at the required rate. In this work, the lipid/protein ratio and the collagen deposition were monitored. It was shown that when collagen content increased, the lipid/protein ratio decreased. A mouse model was used for this work. Two different immune responses were noted in the affected immunocompetent host. The resistant host recovered from myocarditis, whereas the permissive host developed cardiac disease.

3.3.5 Cervical Cancer

Cervical cancer detection is dependent on classification of individual cells as cancerous, precancerous, or benign. For years the objective of researchers in this area has been to get an objective spectroscopic procedure that would evaluate Pap smears based on relatively small differences in the mid-IR spectrum in the fingerprint region, particularly at frequencies below 1400 cm^{-1} . The results based on classical spectroscopic interpretation had been mixed and in some respects disappointing. More recent experimentation since 2003 has proven successful based on chemometric treatment of the data. Romeo et al.⁵⁰ performed a textbook experiment of attempting to differentiate spectroscopic cells from two origins that are very similar to visual inspection under the microscope. Human oral mucosa cells and canine cervical cells were compared. In this work, spectra of 60 individual oral mucosa cells from one donor were obtained and compared. Spectra of 320 oral mucosa cells averaged separately from each of

five donors were treated with PCA and displayed in a scatter plot of PC2 versus PC1 to look for any spectral variance between donors. Second derivative spectra of the same data set were also compared on a plot of PC3 versus PC2. Plots of 1000 human oral mucosa and canine cervical cancer cells were done with PC3 versus PC2 and PC4 versus PC3. In each plot the cluster of human oral mucosa cells was totally separated from the cluster of canine cervical cells. In a PC4 plot versus PC3 for a $1800\text{--}800\text{ cm}^{-1}$ second derivative, when cervical cells from estrus dogs were added to cells from non-estrus dogs in the mixture, the cluster for the canine sample remained intact. Results from this textbook experiment show that information regarding cell type, level of maturity, and stated disease may be determined when PCA treatment of IMS data is applied. Discriminant analysis is then possible and leads the way to an objective computer algorithm approach to dealing with the classification of cervical cells. In the past, without application of PCA, it was not reliable.

In another article by Wood et al.⁵¹, which cites 49 references, spectral mapping of cervical transformation zone and dysplastic squamous epithelium summarizes much of the previous work from both the New York group and the Monash University group in Australia. Squamous and glandular cervical epithelium of the cervical transformation zone were obtained and analyzed by multivariate unsupervised hierarchical cluster methods. The resulting clusters were correlated to corresponding stained histopathological features in the tissue sections. It was reported that multivariate statistical analysis of FT-IR spectra collected for tissue sections permit an unsupervised method of distinguishing tissue types and differentiating between normal and diseased tissue. The amide I and II region (1740 cm^{-1} – 1470 cm^{-1}) was found to be an important window in the spectrum to examine. In this case an unsupervised rather than diagnostic algorithm was used. Using the hierarchical clustering in combination with FT-IR microspectroscopy provides detail of the spectral signatures of individual cells and shows potential as a diagnostic tool for cervical cancer. Important background for this work was a 1999 cover article in *Applied Spectroscopy* by Diem et al. entitled “Infrared spectroscopy of cells and tissues: shining light onto a novel subject.” More recently, in 2006, Matthäus et al.⁵² applied Raman microspectroscopic imaging as an alternative to IR. Other work on single cells was reported by Falkowski et al.⁵³

3.3.6 Microspectroscopy of Cells or Subcellular Tissue

Single-cell mapping and mapping on a subcellular level was explored by Lasch et al.⁵⁴ For this work, large-size ($100\text{ }\mu\text{m}$ by $100\text{ }\mu\text{m}$) oral mucous cells were chosen, not only for their size, but because they are a defined stage of the cell cycle (G0). The mapping procedure distinguished subcellular features and spectra from each of these areas within this cell were obtained and examined. Chemical heterogeneity in cell death was reported by Jamin et al.⁵⁵ This involved the study of single apoptotic and necrotic cells. Changes in the methylene region 2900 cm^{-1} – 2800 cm^{-1} and in the region 1234 cm^{-1} – 1044 cm^{-1} were documented. These were documented for different stages in the death of the cells. Oral mucosa cells were the subject of a study by Romeo et al.⁵⁶ in which a slurry of cells was passed through an IR beam. The objective of this study was to show the cause of variance in the spectroscopic results based on light scattering as the cells passed through the beam. Mohlenhoff et al.⁵⁷ studied Mie-type scattering human cells. Other references to single cell studies include Boydston-White et al.⁵⁸ Diem et al.,⁵⁹ and Romeo et al.⁶⁰ In 2006, the Raman microspectroscopy article by Matthäus et al.⁵² on single human cells was the *Applied Spectroscopy* cover article. Tfayli et al.,⁶¹ in the laboratory of Manfait, were concerned with absorption and permeability of a substance on the plasma membrane of particular cells. This

has to do with the administration of a drug to the cells for a chemotherapeutic procedure. Drug resistance remains one of the primary causes of suboptimal outcomes in cancer therapy. Greater detail may be obtained in other chapters of this volume that are written by workers in the cancer field.

3.3.7 Skin

The delivery of drugs by way of a patch depends on permeation of the drug through the skin. Methods for quantitative determination of drug localized in the skin are the focus of an article by Touitou et al.⁶² More recently, synchrotron IMS was used to study transdermal drug delivery by Cotte et al.⁶³ In this case, perdeuterated palmitic acid and myristic acid were applied to pig ears. Mapping of cross sections of the skin with high spatial resolution clearly defined the penetration boundary within the skin. In general, the penetration distinguished between that of the stratum corneum from the epidermis and the dermis. A comparison was done from FT-IR on extracted lipids or ATR FT-IR and the current study involving synchrotron IMS. In the latter case, the population was determined from the stratum corneum, the epidermis, and the dermis. This was in comparison to the other two methods that were topical to just the stratum corneum.⁶³ In a third, more recent study, pig skin was also used as the substrate and interpretations of spectra obtained from the stratum corneum were discussed by Mendelsohn et al.⁶⁴ Light microscopy was also used to define the areas in the cross section of the permeated tissue. Lipid conformational changes were also observed in the penetrated material. Adenocarcinoma specimens were imaged, and cluster analysis was used to enhance interpretation of the different areas within the image. It was reported that the use of clustering algorithms dramatically increased the information content of the IR images. Among the cluster imaging methods, Ward's algorithm was considered the best method in terms of tissue structure differentiation. This was a joint effort of scientists from the Robert-Koch-Institut, Max-Delbrück Center, and Humboldt-Universität zu Berlin (all in Berlin, Germany), as well as from Hunter College in New York. In this work, hierarchical clustering, fuzzy *C*-mean clustering, and *k*-mean clustering were compared.⁶⁵

Recently, Xiao et al.⁶⁶ at Rutgers University used a perdeuterated long acyl-chain compound to test the penetration into pig skin. This study was concerned with the aggregational state of permeating vesicles and with the molecular structural change in the exogenous and endogenous lipid components.

Postmortem human skin from a several-thousand-year-old Egyptian mummy in the Museum of France was studied by Cotte et al. using a Perkin–Elmer Spectrum Spotlight focal plane array instrument for mapping. The same specimens were also analyzed by Dumas using the confocal Continuum™ instrument installed on beamline U10B of the National Synchrotron Light Source. With $3\text{ }\mu\text{m} \times 3\text{ }\mu\text{m}$ image plane masking, enhanced spatial resolution resulted and heterogeneous detail was revealed. One dark spot in this image proved to be palmitic acid. In another area, both the acid and the ester groups were present.⁶⁷ This is a classic example of the spatial resolution capability of the confocal microspectrometer/synchrotron combination versus the unmasked FPA instrument.

3.3.8 Alzheimer's Disease, Prion Infection, Secondary Protein Structure

Alzheimer's plaque was studied by IR spectroscopy to reveal the beta-amyloid deposition. The first experiment involving enhanced spatial resolution was performed at the synchrotron

in 1996.¹ In that mapping procedure, the shift of the amide I band maximum to approximately 1630 cm^{-1} from the alpha helix at 1658 cm^{-1} in adjacent pixels, outside of the plaque area, provided a marked contrast. Since that early experiment, there has been a flurry of activity in the study of Alzheimer's plaque. Especially in the last 6 years, experiments have proven quite fruitful. In particular, researchers from Brookhaven National Laboratory and the Universities of Chicago and of British Columbia used synchrotron-based IR and X-ray imaging to show accumulation of Cu and Zn, co-localized with deposits in Alzheimer's disease (AD). Previous AD studies showed association with elevated levels of Fe, Cu, and Zn in the brain. In this study, high spatial resolution was achieved with confocal operation of the ContinuumTM using a $10\text{ }\mu\text{m} \times 10\text{ }\mu\text{m}$ image plane mask and $10\text{ }\mu\text{m}$ step sizes for the data accumulation. In this work the peak intensity was measured at 1625 cm^{-1} (beta sheet) and 1655 cm^{-1} (alpha helix) at each pixel location. A single-point linear baseline at 1800 cm^{-1} was used to correct for the decaying synchrotron beam intensity over time. X-ray fluorescence was done at the synchrotron beamline X26A on the very same specimen mounted on a substrate that was suitable for both IR and X-ray analysis. As standards, one protein known to be 100% alpha helix and another known to be 100% beta sheet were used to produce spectra for comparison. The synchrotron X-ray fluorescence spectrum in the plaque region showed dramatic increases in the Cu and Zn fluorescence intensity, particularly in the center of the plaque. After the elevated regions of metal were identified, correlations were generated to determine how well the metal co-localizes in the tissue. A correlation ($R = 0.97$) resulting for Cu and Zn was reported by Miller et al.⁶⁸

A recent article by Miklossy et al.⁶⁹ on beta-amyloid deposition and Alzheimer's-type changes induced by *Borrelia* spirochetes involved a joint effort of 10 scientists from Canada, Switzerland, Australia, and the United States. In this study *in vitro*, the mammalian glial and neural cells, the neuronal cells, the *Borrelia burgdorferi* spirochetes, and the inflammatory bacterial lipopolysaccharides (following 2–8 weeks of exposure) had induced morphological changes that were analogous to the amyloid deposits of AD brain. The study results reinforced previous observations with spirochetes that can induce a host reaction similar to that seen in AD. Results indicated that bacteria and/or the degradation products may enhance a cascade of events leading to amyloid deposition in AD.

3.3.8.1 Prions. Kneipp et al. of the Robert Koch-Institut⁷⁰ used a hamster brain to detect pathological molecular alterations in a scrapie infection by IMS. Purkinje cells and epithelial cells were among those mapped in the hamster brain. The midsagittal cerebellar section of the hamster brains were used for this study. Cluster analysis was used in the region 3040 cm^{-1} – 2980 cm^{-1} of both the normal and the infected stratum moleculare and the substantia alba. The spectral region 1800 – 1500 cm^{-1} was also compared. Various clustering data treatments were used. Infrared spectroscopy in combination with microscopy yielded spatially resolved information on unstained collembolan thin sections of brain samples that allow generation of maps with high image contrast. The assignment of spectral features to a specific anatomical location was possible using multivariate pattern recognition techniques and permitted the precise correlation of IR characteristics of identical regions in the scrapie-infected and the control hamster brain. Kneipp et al.⁷¹ studied *in situ* protein structure changes in prion-infected tissue.

Subsequent work from the Robert Koch-Institut by Lasch et al.⁷² involved bovine spongiform encephalopathy (BSE) from serum. Artificial neural network (ANN) analysis was used. The study yielded a set of spectra for teaching a classification algorithm. When the

teaching process was finished, the classifier was challenged by an independent validation data set. After selection of the most discriminative spectral information, pattern recognition techniques were utilized for classification. The optimum ANN structure was challenged by a blinded validation set. Upon unblinding the test set, a relatively small number of false positives and false negatives were found from more than 600 samples. The result of this study was proof of principle of spectroscopy as a new diagnostic tool for diagnosis of BSE infection from serum. Classification accuracies were 93.5%. This could lead toward a fully automated objective analytical tool, the antemortem diagnosis of BSE and possibly other diseases. Subsequent to this study, the application of ANN to microspectroscopic imaging has been discussed by Lasch et al.⁷³

Bambery et al.⁷⁴ from the Monash group imaged glioblastoma multiforme. Other collaborative work between Robert Koch-Institut and the National Synchrotron Light source was reported by Kneipp et al.⁷⁵ This work also involved scrapie-infected cells and recombinant prion protein. Synchrotron IMS has been applied to study of prion-infected nervous tissue by Kretlow et al.⁷⁶ Spectroscopic data obtained was compared with immunohistochemistry and X-ray fluorescence techniques. Although the average spectral differences between control and diseased spectra were small, they were consistent. The data suggested that synchrotron IMS is capable of detecting a misfolded prion protein *in situ* without the necessity of immunosaline or purification procedures.

Models of helical peptides and beta-sheet models were used to generate spectra from full quantum mechanical calculations. They show separate individual physical contributions to oscillator coupling by Kubelka et al.⁷⁷ The strength of these parameter-free nonempirical approaches is that the multitude of such contributions to the vibrational properties is not adjusted into a few empirical parameters. Solvent effects and other interaction are not accounted for with this theoretical work.

Work with cancer cells has been ongoing in the laboratory of Diem. Romeo et al.⁶⁰ initially reported on IMS on individual human cervical cancer (HeLa) cells. In this work, single-cell spectra were recorded in reflection/absorption or transmission modes. Both the IlluminatIR™ infrared microspectrometer (SensIR, Inc./Smiths Detection Danbury, CT) and a Spectrum One Spotlight 300 (Perkin–Elmer, LLC Shelton, CT) were used. This work demonstrated the feasibility of collecting high-quality mid-IR data of large individual human cells without the use of synchrotron IMS. A subsequent study addressed the Mie scattering of human cells that gives rise to deviation from Beers law. This was reported by Mohlenhoff et al.⁵⁷

Boydston-White et al.⁵⁸ extended the study of HeLa cells by microspectroscopy using the focal plane array Spectrum One Spotlight 300 Perkin–Elmer instrument. In this study, changes in the spectrum of a single proliferating cell were recorded including maturation, differentiation, and development. This study investigated the spectral changes due to the drastic biochemical and morphological changes occurring as a consequence of cell proliferation. Spectra were recorded at 3, 8, and 11 at 18 hours post mitosis. The results were compared to immunostaining and fluorescence microscopy.

A comparison of FT-IR spectra of individual cells acquired using synchrotron and conventional sources was done by Diem et al.⁵⁹ These investigators reported that for both types of instrumentation there has been a tremendous improvement in instrumental results over the past 5 years. Early synchrotron results reported with a $3\ \mu\text{m} \times 3\ \mu\text{m}$ image plane mask were diffraction-limited; and as one would expect, they suffered at low wavenumbers. The authors report that performance of the third generation of the focal plane array instruments approaches that of the synchrotron-based systems at a fraction of the cost. The authors concluded that the gap between the synchrotron IMS and the

later-generation global source FPA instruments has narrowed, but they acknowledge that there is still a gap.

Boskey and Mendelsohn⁷⁸ used IR spectroscopy to characterize mineralized tissues such as those found in bone, teeth, and calcified cartilage, as well as those formed through pathological processes such as atherosclerotic plaque, kidney stones, salivary stones, and other pathologic deposits. In most cases, collagen represents a major organic contribution to the mineralized tissue. This work emphasized the possibility for characterizing the mineral and matrix in pathologic calcifications and in bone diseases.

A recent *Applied Spectroscopy* cover article by Matthäus et al.⁵² from the Laboratory of Diem reported the first Raman and IR microspectral imaging as mitotic cells. These are the first reports on Raman and IR microspectroscopic images of human cells at different stages of mitosis. Inherent protein and DNA spectral markers were used and no stains were required. It is not feasible to adequately summarize those reports in this chapter. However, the authors found that both Raman and IR intensities depended on the overall chromatin density variation among individual subphases of mitosis.

3.3.9 Protein Structure

Much of biological IMS has been focused on the study of proteins. Even small changes or differences observed in the spectra induce speculation in regard to the protein structure. Several reviews have appeared on the subject. The most recent one by Schweitzer-Stener⁷⁹ (entitled “Advances in Vibrational Spectroscopy as a Sensitive Probe of Peptide and Protein Structure: A Critical Review”) has 82 references. Another post-2000 review entitled “What Vibrations Tell us about Protein,” by Barth and Zscherp,⁸⁰ cites 266 references. These more recent reviews add to the perspective of early articles by Jackson and Mantsch^{81,82} and by Dong et al.⁸³

3.3.10 Medicine

An article by Mantsch et al.,⁸⁴ presented the broad connection of vibrational spectroscopy and medicine. Sixty-four references were cited, mostly dealing with cancer. Images were included from postoperative skin flaps which serve as a model of reconstructive surgery. An earlier article by Jackson et al.⁸⁵ cited 53 references to establish the connection between infrared spectroscopy and medicine.

3.3.11 Nonmammalian Biological Tissue

Studies on grain chemical microstructure and other plant material in our laboratory at Kansas State University and at NSLS include mapping of cross sections of different grains and oilseeds across the boundaries of different botanical parts.²² FPA false color images of cells (Fig. 3.2) in wheat⁸⁶ and synchrotron IMS images of corn sections⁸⁷ reveal molecular distinctions between adjacent tissues. From the chemical distinction between botanical parts, the presence of different parts can be detected among the mixtures produced from physical separation by dry milling.⁸⁸ IMS chemical analysis enables the prediction of digestibility of grasses by ruminant livestock. These are but a few applications. Many others may be found in an earlier book chapter that includes both mammalian and plant materials.²⁹

3.4 INSTRUMENTAL MEANS OF BIOMEDICAL IMS

3.4.1 Instrumental Progress

IMS and imaging is an analytical chemical field driven by instrument development and sensor technology. Originally, IR microscopes were merely accessories to FT-IR spectrometers. A dedicated small-area detector was included as an option with these accessory microscopes introduced in the late 1980s and early 1990s, which improved the IR sensitivity. In response to needs of the material sciences, the IR microscope was developed as a peripheral to conventional FT-IR instruments. The research-quality microscope, introduced in 1986 and patented in 1989, which was equipped with front surface optics (instead of refractive optics), opened up the capability of microscopic examination of select areas of a specimen by use of projected image plane masks that restrict the collection of IR spectra to small spatially resolved targets in the microscopic field. This was described by Messerschmidt and Sting.⁸⁹ Figure 3.14a illustrates the optical scheme for dual remote projected image plane masks and the progression of IR microspectrometer development. The targeted transmission through the IR microscope made IMS possible, and contamination of the spectra by surrounding material was avoided. Accessory IR microscopes (Fig. 3.14b) added to spectrometers were subsequently replaced by second-generation systems comprising both an IR microscope and interferometer bench designed with regard

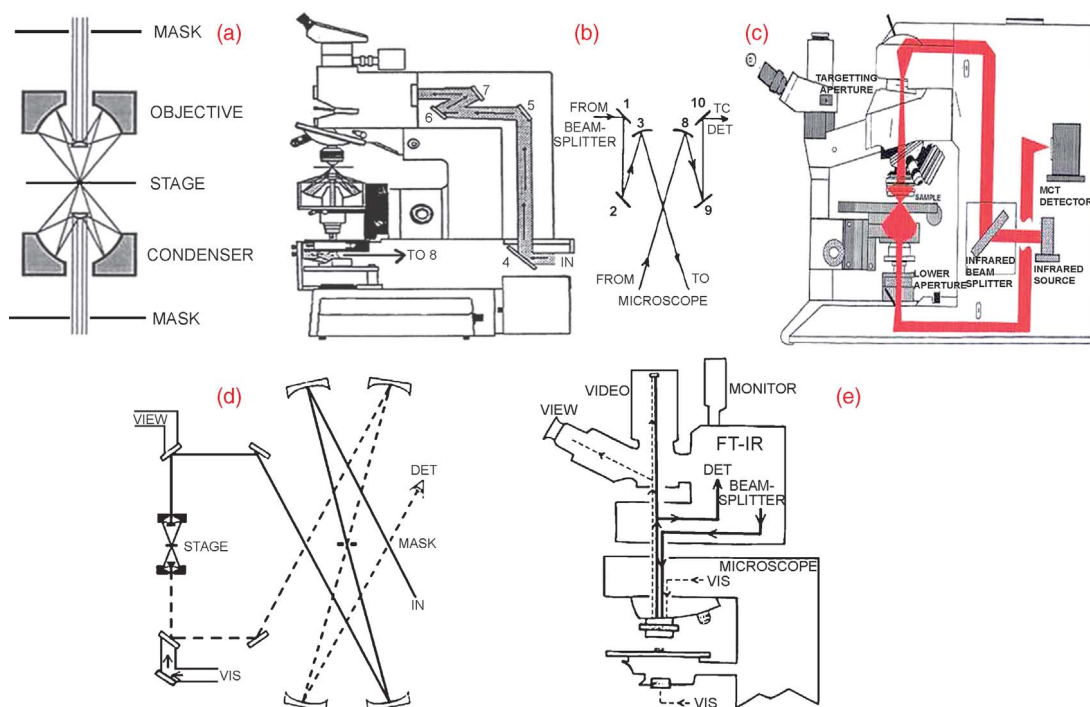


Figure 3.14. Stage of IMS instrument development: (a) Schwartzschild objective and condenser mirror lenses with image plane masks before and after. (b) Peripheral scope with interface. (c) Integrated IR microscope/spectrometer. (d) Infinity-corrected dual confocal IR microscope. (e) Peripheral IR spectrometer for microscope.

to mutual compatibility. The first of these was a 1990 single-unit, optically efficient, integrated instrument (Fig. 3.14c).

Progress in the development of single-detector dedicated instruments reached a high point⁹⁰ with the 1999 introduction of the first infinity-corrected dual confocal IR microscope (Fig. 3.14d). Use of infinity-corrected mirror lenses allows the placing of a polarizer and a pair of Wollaston prisms in the beam before and after the specimen to provide differential interference contrast (DIC) of the unstained tissue for viewing with visible light prior to IR analysis. This optical arrangement also permits the placing of an IR polarizer directly before the specimen instead of after the beamsplitter for obtaining polarized spectra. Customization of IR microscopes for small target sizes has been done with the use of dual $32\times$ Schwarzschild mirror lenses and replacement of the commonly used $250\text{ }\mu\text{m}$ $250\text{ }\mu\text{m}$ size liquid-nitrogen-cooled MCT detector with a $50\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$ element. The $32\times$ matched objective and condenser allow a small masked projection at the image plane, and the small-area detector is filled by the microbeam cross section. Dichroic mirrors allow viewing while scanning in real time. Mapping capability with a motorized stage is enhanced with automatic gain control and coordinate programming from video capture images. Instrumental factors are discussed by Reffner.⁹¹

A more recent entry into the field of IMS is a very compact FT-IR spectrometer (Fig. 3.14e) that converts a research-quality microscope of any of the major brands into an IR microspectrometer.^{92,93} Individual human cervical cancer (HeLa) cells were analyzed with the IlluminatIRTM infrared microspectrometer by investigators in Diem's laboratory⁶⁰ as previously reported in Applications 3.8. Once the mini FT-IR spectrometer has been installed, the conversion from a light microscope to an IR microspectrometer is accomplished by simply rotating the nose piece of the microscope to a position where a Schwarzschild front-surface mirror lens moves into position instead of the microscope's conventional refractive optic. A near-IR video image of the specimen is used to select the area of interest. The IlluminatIRTM, introduced by SensIR Technologies, Danbury, CT (now a product of Smiths Detection), performs IR scanning in the reflection absorption mode. Its confocal operation employs $10\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ image plane masking.

3.4.2 Introduction of Synchrotron IMS

The ideal illumination for IMS is to concentrate the maximum flux of radiation on the minimum-sized target. This was achieved on November 20, 1993, by optically interfacing an IR microspectrometer to a beamline at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory (BNL) in Upton, NY and reported by Carr, Reffner, and Williams.⁹⁴ Shortly after the first successful synchrotron IMS experiments were done, the author had the privilege and opportunity January 29, 1994 to take advantage of this facility and operated routinely with either $12\text{ }\mu\text{m} \times 12\text{ }\mu\text{m}$ or $6\text{ }\mu\text{m} \times 6\text{ }\mu\text{m}$, masking before and after the stage. Spectra of single cells within the primary root of wheat sections were recorded in a $6\text{ }\mu\text{m} \times 6\text{ }\mu\text{m}$ image plane masking confocal operation. Coaddition of only 16 or 32 scans produced excellent spectra with no smoothing and with spatial resolution limited only by diffraction.

Mapping of a single wheat aleurone cell with the same model instrument in 1992 at Kansas State University with a global source required coaddition of 256 scans and smoothing. The distinct advantages of the synchrotron radiation are threefold, including brightness, absence of thermal noise, and nondivergence of the beam. Brightness of the synchrotron is calculated to be 1000-fold greater than a global; however, because of some of the auxiliary optics used, a 1000-fold signal enhancement was not realized. The absence of

thermal noise with synchrotron radiation, combined with its enhanced brightness, yields a great increase in SNR in comparison to that of a global source. Perhaps the most important feature is that synchrotron radiation is highly directional. Such is the nature of relativistically emitted radiation proceeding from bunches of electrons so accelerated that they approach the speed of light traveling within the storage ring of a synchrotron under high vacuum.

The striking findings on the nondivergence of the synchrotron radiation in the microscope optics was demonstrated by Reffner in 1993 on the very first day that the temporary experimental setup was used. He observed that of the total radiation passing through the IR microscope's reflection optics with no image plane masking, 85% went through a 12.5 μm pinhole placed in the beam on the microscope stage. Image plane masking of the synchrotron radiation does not reduce the signal so severely as a divergent thermal source usually does. In fact, one early series of microspectroscopy experiments was done at the beamline without the use of an image plane mask. At NSLS, electrons from an electron gun are accelerated in a linear accelerator to ~ 80 MeV. Further acceleration in a booster ring raises their energy to ~ 1000 MeV. At 4 to 5 h intervals, accelerated electrons from the booster ring are injected into the vacuum ultraviolet (VUV) storage ring to maintain the high-energy electron population required to sustain the desired useful photon flux. At each of the eight bending magnets, there are beamline ports from which photons are emitted. At IR beamlines, photons exit the high-vacuum region of the storage ring through a diamond window into a nitrogen-purged mirror box then through an evacuated flight tube to the microspectrometer. The design of synchrotron IR beamlines, including the six IR beamlines at NSLS, is discussed in detail by Carr et al.⁹⁵ There are 18 operating IR beamlines at synchrotrons worldwide (most have microspectrometers), and 17 more are planned. Existing and planned synchrotrons include the following: Brookhaven, NY; Berkeley, CA; Madison, WI; Baton Rouge, LA; Gaithersburg, MD (USA); Berlin, Karlsruhe, Dortmund (Germany); Daresbury (UK); Paris (France); Lund (Sweden); Rome (Italy); Hsinchu (Taiwan); Osaki, Nishi Harima, Hyogo (Japan); Campinas (Brazil); Shanghai, Hefei (PR China); Korat (Thailand); Saskatoon (Canada); Victoria (Australia).

3.4.3 Focal Plane Array Instruments

Instruments designed for imaging include an IR microscope with a liquid-nitrogen-cooled focal plane array (FPA) camera equipped with MCT photovoltaic detectors in a 64×64 array were used in the original configuration with a step-scan interferometer.⁹⁶ The MCT 64×64 FPAs were originally designed for anti-tank missiles for one-time use en route to the target destination. More recently, nonmilitary FPAs have been developed that were designed specifically for instrumental and for long-term multiple usage. The step-scan approach includes a short time lag between each successive step in order for the electronic backlash to settle down. FPA imaging was undoubtedly a major advance in instrumentation for producing rapid chemical contrasts in the microscopic field of view. Referred to as "fast imaging," FPA systems were used successfully, for example, by Snively and Koenig⁹⁷ to study the kinetics of mixing of polymers. With initial FPA imaging systems, however, the quality of the spectral data was not equivalent to that obtained with a single-detector FT-IR microspectrometer. Operating mostly at 16 cm^{-1} spectral resolution with inadequate signal-to-noise ratio (SNR) limited the sensitivity of minor constituents, which adversely affected the detection limits of the original instruments.⁹⁸

3.4.4 Improvements of FPA Instruments

Since commercialization of FPA imaging systems by at least four companies, significant advances have been made. Most of these advances have been initiated by continued research in the NIH Laboratory of Chemical Physics at Bethesda, MD. As creative data acquisition schemes have been introduced, software written, and optical refinements made by these researchers,^{99,100} instrument manufacturers have incorporated many of these improvements into commercial step scan FPA systems. This has narrowed the spectroscopic quality gap between single-detector and array instruments. A number of factors have been responsible for the significant increase of SNR per pixel of the arrays. The duty cycle was increased by using the recovery time of the step-scan backlash for correcting the nonlinearity and offset of the data and applying gain ranging in that interim. Scan time efficiency also increased by simultaneous data readout in parallel with the interferometer return sweep reset. The use of frame averaging doubled the SNR, and reduction of data acquisition time made it possible to coadd more spectra in the same time period. The trade-off encountered in the design and use of large-format FPA detectors for FT-IR imaging is discussed by Bhargava and Levin.¹⁰⁰ Median corrected mean data filtering was suggested to avoid detector spikes and maintain accuracy. A better cold filter also was added to reduce thermal noise of the detector.

Coincident with the improved step-scan FPA instruments, rapid scan array instruments had been introduced that avoid the necessity of using a step-scan FT-IR spectrometer. A “staggered scanning” scheme that results in summation of multiple undersampled sweeps was introduced at NIH that allowed the modulation frequency to operate independent of the detector. To achieve rapid scan, the MCT optical detector elements that constitute the array use a photoconductive mechanism instead of the photovoltaic process. Pixel size choice is limited to two optical adjustments imposed on the physical size of each tightly positioned detector element in the array.

In general, the rapid scan arrays are limited to many fewer pixels of data acquisition at one time. One example of this is a 16-element pushbroom linear array; that is, 16 elements are in a line, which constitutes the pushbroom. As the microscope stage moves underneath the pushbroom, a 16-element swath is mapped across the specimen. Repeating this procedure and constructing a mosaic produces excellent images. Commercial rapid scanning instruments of this type are currently offered by at least two vendors. One system uses 32 elements. With an array an orthogonalized Graham–Schmidt function of the IR radiation intensity image may be substituted for visible (brightfield) microscopic images traditionally used on single-detector IR microscopes. This approach simplifies the optical design.¹⁰¹

Perkin–Elmer introduced an instrument called the “spotlight” in the fall of 2001 at the Detroit FACSS meeting. This instrument uses a 16-element pushbroom array. BioRad (now Varian) offered rapid scanning on its 32×32 and 64×64 arrays; but for larger arrays (256×256), they retained the step-scan approach. Another feature that has been introduced in at least three commercial instruments allows the option of interposing a mirror before the array to send the beam to a single detector. In such a configuration, either high-quality FT-IR microspectroscopy can be accomplished or rapid images can be produced. The relative merits of the pushbroom and rectangular FPAs have been discussed.¹⁰⁰

3.4.5 Optical Enhancements for IMS

Substrates used for IMS classically have involved a 1 or 2 mm-thick, 13 mm-diameter BaF_2 disk. These IR windows unfortunately are readily scratched, easily broken, and expensive.

Moreover, they cause the IR focus at lower wavenumbers to deviate from the visible focus. For transmission, 1 mm-thick, 5 mm-diameter synthetic diamond windows avoid the dispersive effect of BaF₂ and maintain focus across the spectrum. Figures 3.15a and 3.15b from Wetzel¹⁰² illustrate noise reduction at low wavenumbers with (a) diamond and (b) spot focus with BaF₂.

In the absorption–reflection–absorption mode, IR reflecting glass slides similar to building construction glass allow the spectrum to be scanned or imaged in the reflection mode subsequent to viewing the region to be selected for analysis with transmitted visible light. The IR reflecting glass microscope slides are inexpensive compared to BaF₂ but are nine times the cost of ordinary microscope slides.

In IMS, the brightfield view of unstained tissue does not always allow the operator to find boundaries between adjacent parts such as different layers. This problem is solved with differential interference contrast (DIC) using a polarizer and Wollesten prisms before the objective and after the condenser while viewing the specimen on the stage. This became possible after the first infinity-corrected front-surface optics instrument was introduced in 1998. An example is shown in Fig. 3.8 or on cover art.³¹

Spectroscopic detection limits in IMS are usually dependent on the SNR of the system. Administering a chemical compound deuterated at a particular carbon atom to conduct metabolism studies has physiological limitations. Topical application may be within IMS detection limits, but systemic application diluted by circulation presents a serious challenge for an instrument with a stock MCT detector, even with a 50 $\mu\text{m} \times 50 \mu\text{m}$ element. This

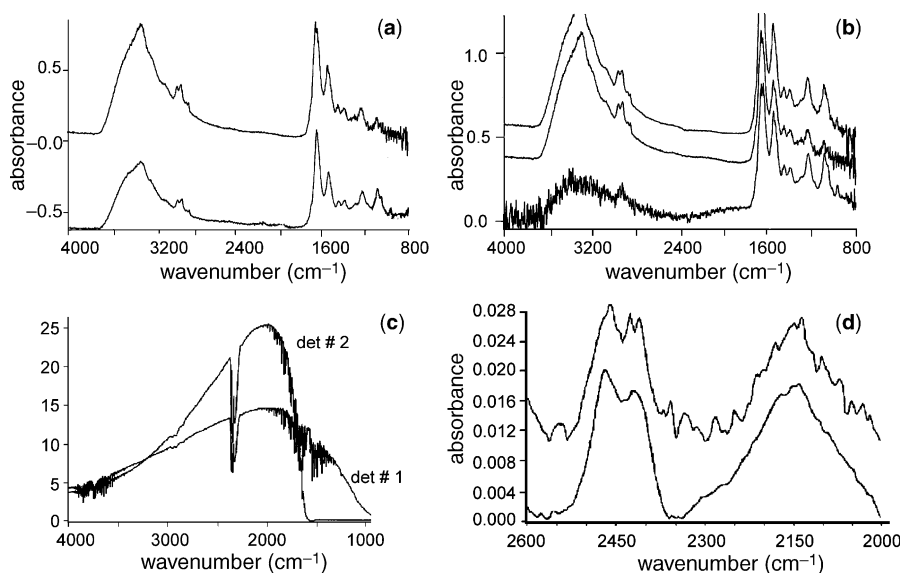


Figure 3.15. (a) Graph showing reduced noise with the diamond at low wavenumbers (*bottom*) versus BaF₂ (*top*). (b) Graph showing reduced noise below 1400 cm⁻¹ when a spot focus is used with BaF₂ window. Note also that the spot focus provides narrow reduction. (c) Graph showing a high response for the custom detector (det #2) and a sudden drop-off. This detector enhances SNR versus a stock MCT (det #1). (d) Graph showing less noise for the spectrum taken with the diamond window (*bottom*) versus the stock detector that is less sensitive to the region of interest (*top*). (From Ref. 102 with permission.)

problem was solved with a made-to-order, custom, narrow-band detector optimized for the CD stretching band. Note in Figs. 3.15c and 3.15d the strong response of a custom, narrow-band detector and the sharp cutoff at long wavelengths that sensitize the instrument and reduce noise in the spectrum for ND, OD, and CD stretching vibrations, thereby lowering their detection limits.¹⁰³

3.4.6 IMS Imaging with FPA Versus Confocal Synchrotron

For a Pittcon 2005 symposium on “Microspectroscopic Characterization of Materials Using Synchrotron Radiation,” invited speaker Paul Dumas performed a head-to-head comparison of an offline FPA versus synchrotron IMS by imaging the same specimen.¹⁰⁴ At first glance the spectra obtained for the two instruments looked alike. A $6.2\text{ }\mu\text{m} \times 6.2\text{ }\mu\text{m}$ aperture was imposed by the area of each of 16 elements in the linear array and by the internal mirror lenses of the instrument used with the array. Confocal $6.0\text{ }\mu\text{m} \times 6.0\text{ }\mu\text{m}$ image plane masking was used with the sync-IMS. The acquisition time for the FPA was 55 min, compared to 3.3 h with the synchrotron experiment. The synchrotron spectra were slightly noisier below 1000 cm^{-1} . Image contrast was dramatically improved in the synchrotron confocal experiment that showed spots within spots. The origin of the image contrast advantage was evident from the resulting spectra. Overlaid spectra from the synchrotron versus global obtained with the same confocal image plane mask of $6.0\text{ }\mu\text{m} \times 6.0\text{ }\mu\text{m}$ yielded a noise-free 32 scan coadded spectrum in 16 s versus a relatively noisy 1000 scan coadded spectrum in 500 s. The origin of high image contrast was credited to confocal operation of the FT-IR microspectrometer installed on NSLS beamline U10B at BNL.

Confocal operation is not an option for single-detector use of the Spectra Tech./Nicolet/ThermoElectron Continuum™ designed by John Reffner and engineered by Steve Vogel and Greg Ressler. It has a double-pass, single-image plane mask. (Incoming light first goes through the mask before being focused by the Schwarzschild mirror lens onto the specimen, and after the condenser Schwarzschild mirror lens it is redirected through the same image plane mask before impinging on the detector.) The properly designated confocal term has been previously referred to as “double aperturing” or by the trademarked term Redundant Aperturing™ so applied by Robert Messerschmidt, who designed the Spectra Tech IR PLAN™ infrared microscope accessory that was introduced in 1986 and patented.⁸⁹ Empirically, the spatial resolution of confocal operation was tested with a conventional IR microspectrometer by placing a material that had a distinct spectrum at various distances from the designated target spot of the projected image plane mask. The distance from the spot at which the specimen spectrum became influenced (contaminated) with spectral features from the foreign material outside of the target enlarged the effective spot size. Reffner designed test substrates (BaF_2 for transmission and mirrored slides with deposited photoresist patterns) and reported the results with a confocal conventional source microspectrometer.¹⁰⁵

Carr approached the confocal application theoretically by calculating the emission pattern for both confocal and non mask operation. Figure 3.16 shows the calculated patterns. The nonconfocal operation is encountered with an FPA scheme such as that of the Perkin-Elmer spotlight and FPA models by Bruker, Varian, and ThermoElectron. Carr also reported empirical spatial resolution limits using data obtained on the synchrotron-illuminated instrument obtained with the previously described photoresist patterns and the familiar USAF 1951 pattern. In reference to imaging, the spatial resolution issue was summarized by

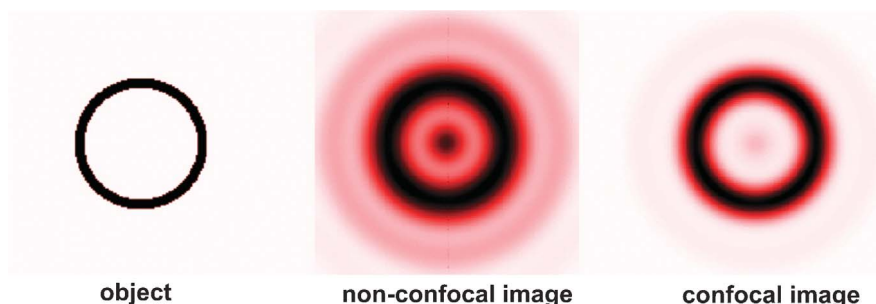


Figure 3.16. (Left) Dimensions of an actual object. (Center) Calculated emission image from nonconfocal operation. (Right) Calculated emission image representing confocal operation. Note the much-improved spatial resolution with a confocal arrangement. (From Ref. 106 with permission of *Review of Scientific Instruments*.)

Carr.¹⁰⁶ Lasch and Naumann¹⁰⁷ reviewed the relative spatial resolution performance applied to both tissue specimens and test patterns.

Working around the diffraction limit to spatial resolution was demonstrated by Reffner et al.¹⁰⁸ in early synchrotron usage where he revealed the spectrum of a $2\text{ }\mu\text{m}$ -thick layer of photographic film by using a $6\text{ }\mu\text{m} \times 6\text{ }\mu\text{m}$ image plane mask in a line map across sequential layers in $1\text{-}\mu\text{m}$ steps. By subtracting the spectrum obtained from the first step that included the unknown thin layer from the spectrum taken predominantly from the unknown thin layer, the spectrum and identity of the unknown layer of a competitor's film was revealed. This author and co-workers¹⁰⁹ recently used $1\text{ }\mu\text{m}$ steps in both the x and y directions to produce images within a $10\text{ }\mu\text{m}$ domain of an ORMOSIL (copolymerized organic/silicate) film shown in Fig. 3.17.

Dumas and co-workers,^{110,111} with $2\text{ }\mu\text{m}$ steps of a $3\text{ }\mu\text{m} \times 3\text{ }\mu\text{m}$ confocal image plane mask of a human-hair cross section, clearly revealed a difference in the lipids of the cuticle as well as features within the cortex and produced secondary protein structural differences within the medulla. In Fig. 3.18, note the enhanced spatial resolution of the center image that enabled protein and lipid analysis in the cortex, respectively, in images to the right of center. Anyone having doubts need only to consult Miller and Dumas¹¹⁰ or Dumas and Miller¹¹² to have this matter clarified.

Alzheimer plaque has been extensively studied by IMS using a synchrotron source, by Choo, Miller, and others, but for the first time within a localized part of the plaque, creatine was reported by Gough and co-workers¹¹³ This recent discovery, based on improved spatial resolution, is reminiscent of a discovery in the past century that was dependent on "spectral resolution." Tantalum specimens separated chemically that were subjected to atomic emission spectroscopy with improved spectral resolution revealed new spectral lines that had not been previously observed in the atomic spectrum of tantalum. As a result, a new chemically similar element was discovered. It was named niobium because in Greek mythology, Niobi was the daughter of Tantalum. In a 2005 discovery, Alzheimer's plaque, characterized by beta-amyloid protein, was found to host creatine. This was not observed in prior experiments with lower spatial resolution. Previously, synchrotron XRF had shown co-occurrence of calcium localized within the plaque. Both of these observations were possible only from synchrotron experiments. The latter involved the same specimen being analyzed for the molecular content at the vacuum ultraviolet (VUV) ring and for fluorescence at the X-ray ring.¹¹⁴

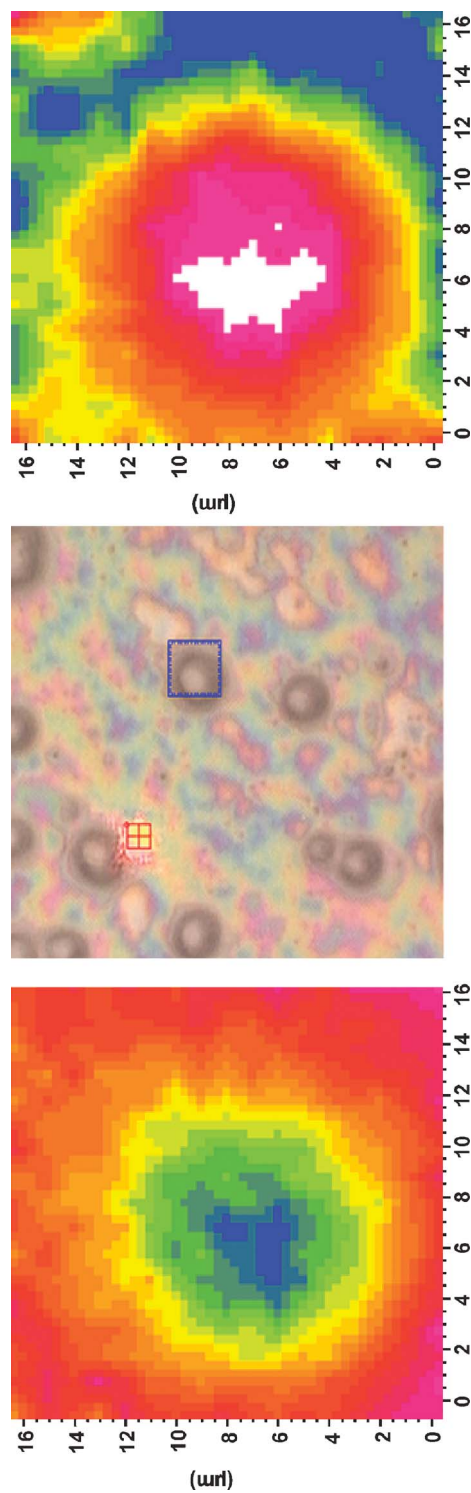


Figure 3.17. Maps (Left and Right) made with the synchrotron instrument in a confocal configuration, and 1- μ m steps that provided detail below the detection limit. **(Left)** Image of the inorganic SiO stretching vibration. **(Right)** Distribution of organic material, as the functional group map of CH₂ at 2927 cm⁻¹ in the ORMOSIL. Both images are of the same domain shown in the box on the photomicrograph (Center). Without this particular confocal synchrotron operation, it was not possible to see the chemical distribution within such a small target. (From Ref. 109 with permission of *Vibrational Spectroscopy*).

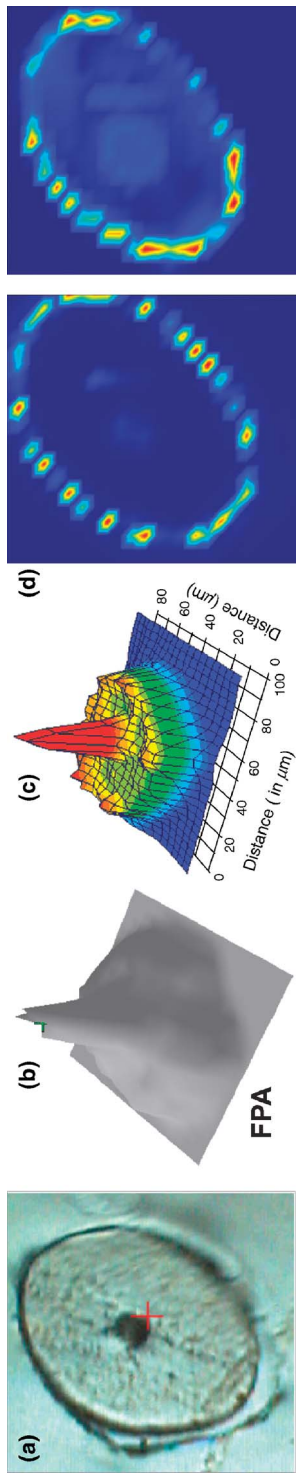


Figure 3.18. (a) Photomicrograph of a transverse section of a human hair. (b) Image from a focal plane array instrument. (c) Image produced with confocal operation of synchrotron IMS. Note the detail in the center image. The red portion in the center is the medulla, and the ridge around the outside is the cortex. (d) Protein and lipid in the ridge of the cortex are analyzed on the adjacent respective false color images left to right, respectively. With such detail from the small target, even the type of protein and lipid present can be analyzed. (From Ref. 110 with permission.)

3.4.7 Near-IR Imaging

Near-IR imaging is readily available commercially with FPA detectors and either a liquid crystal tunable filter (LCTF) or a Fourier transform (FT) spectrometer. The former instrument of Spectral Dimensions/Malvern (Columbia, MD) employs a stage illuminated by four long-wavelength tungsten lamps in which the diffusely reflected light from the specimen is captured by a refractive lens and transmitted through the LCTF to focus on a photovoltaic detector array of either InGaAs or InSb. The InGaAs operates from 1100 nm to 1700 nm. The TE-cooled InSb as used in this system operates in the 1400 to 2400 nm range. Light striking the highly polished mounting plate is specularly reflected away from the lens. For a 320×256 pixel array, approximately 82,000 spectra are collected. An intensity threshold is used to delete pixels with only stray light. The image from only the diffusely reflected specimens remains. The resulting image produces contrast from $\log 1/R$ functional group maps and selects principal component analysis (PCA) factors pixel by pixel. Imaging software ISys™ is used to collect data, process data, and provide contrast. Each generation of processing is designated as a new image cube.

The Perkin–Elmer Spectrum Spotlight mid-IR instrument described previously is available in a near-IR version. In this version the optical geometry and the pushbroom linear array data acquisition scheme is retained. In the near-IR version the detector array, interferometer, beamsplitter, and source are substituted to produce FT-NIR FPA imaging using the P-E spotlight software.

Compared to sharp, strong, fundamental vibrational bands in the mid-IR, the combination and overtone bands in the near-IR spectra are not as intense, sharp, or selective. However, their characteristic of reduced absorption allows penetration below the surface that reveals spectra of hidden material. Subsurface polychromatic contrast uncovers hidden heterogeneity of optical features such as refractive index or density. Near-IR chemical imaging is, in fact, less intrusive and provides a method for nondestructive analysis due to deeper penetration of the shorter wavelengths. Near-IR imaging is presently well established as a useful tool in the pharmaceutical industry, where pure chemical active ingredients that have distinct spectral features are distributed in an incipient matrix that usually has relatively bland broadband features. In such a case, the identity location and relative amount is readily imaged. Uniformity of dosage and dosage per tablet or by lot can be found. For naturally occurring biological materials, identity, location, and relative amount of their constituents may also be found, usually with more data processing. Nondestructive sensitive testing for germination in seeds has recently been reported¹¹⁵ (see Fig. 3.19a). Subsurface probing enables us to detect the developing embryo at earlier stages than were possible by any previous analytical means. Previously, in Europe, mammalian tissue was imaged and analyzed by near-IR to detect the presence of animal protein (i.e., bovine meat and bone meal) in ruminant feeds to avoid transfer of mad cow disease.

3.4.8 Near-Field Synchrotron Near-IR Microspectroscopy

In light microscopy, one way to increase resolution beyond the diffraction limit is the use of near-field optics. Diffraction limitation results when the wavelength of light used is longer than the separation of two points that are resolved as individual objects. In such cases, the process of diffraction degrades and mixes the radiation into a blur. The definition of near field implies that either the light source or the detector is no more distant from the specimen than the wavelength of light directed onto the specimen.

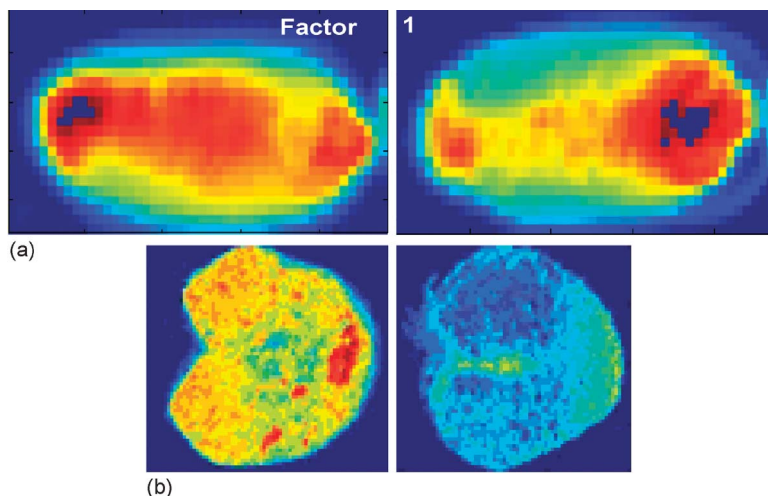


Figure 3.19. (a) Images produced in the near-IR part of the spectrum of whole intact seed. The near-IR radiation has penetrated, and the seed on the right shows the presence of a developing embryo at early stages nondestructively. (b) Cross sections of two different wheat kernels, waxy wheat on the left and non-waxy wheat on the right, show the distinction at a specific wavelength in the near-IR region. (From Ref. 115 with permission of *Vibrational Spectroscopy*.)

Near-field scanning optical microscopy (NSOM) is a well-established technique utilizing the near-field effect for achieving subwavelength spatial resolution. A subwavelength aperture is employed and is maintained at a distance less than half a wavelength from the surface of the sample. In NSOM, the size of the aperture results in an optical throughput reduction by as much as five orders of magnitude with loss of SNR requiring long acquisition times. A synchrotron's high-energy broad-band emission has been deemed an ideal source. The advance concept and design were presented at ICAVS-3.¹¹⁶

In August 2006, the first successful NSOM connection to a synchrotron beamline was established. A proof of concept design, fabricated in-house at the University of Kentucky, was transported to the NSLS/BNL vacuum ultraviolet storage ring, where it was installed on developmental IR beamline U10A. Interfacing optics under vacuum were designed and provided by G. Lawrence Carr and Randy Smith of NSLS. The NSOM instrument contributed by Clay Harris and Robert Lodder was specifically a near-IR version consisting of a gold-coated, pulled optical communication fiber (5 μm core, 50 μm cladding) as the aperture (< 50 nm). An optical glass lens was employed to focus light from the collimated beam into the fiber. The sample rested upon a three-axis stage (20 nm step size) with a single-element InGaAs detector for transmission spectra. In place of an FT-IR, a molecular filter, custom designed for the analyte of interest, was placed in the path of the incident synchrotron beam. With this design, the analyte concentration becomes a function of the detector voltage. The experimental Synchrotron Near-IR NSOM has undergone testing and clearly identifies 50 nm-diameter wires spaced approximately 500 nm on center at a rate of 0.1 s/pixel. Improvements to the system are underway by the University of Kentucky analytical spectroscopy group in collaboration with BNL beamline scientists. Reports in preparation by Harris and Lodder¹¹⁷ will provide experimental detail.

3.4.9 FPA IMS Installation on a Synchrotron Beamline

The theoretical design for adaptation of a commercial FPA mid-infrared spectrometer to a synchrotron beamline was introduced by Carr, Chubar, and Dumas.¹¹⁸ Obtaining real-time images was the primary objective; maximizing spatial resolution with an available commercial instrument was also a goal. Note the advanced design in Fig. 3.20. In order to reach this objective, it was important to fill the FPA with radiation from the synchrotron beam. It also was necessary to make special modifications at the synchrotron facility where the microscope was to be interfaced in order to accommodate the optical scheme. Experimentally, a Bruker FPA Vertex 70 FT-IR and Hyperion™ 3000 imaging microspectrometer were interfaced to beamline U10A as a temporary installation. Because of space limitation in the instrument, a 15×0.57 NA optic was used for the condenser. The objective was 74×0.65 NA. Because the width of the synchrotron beam of each beamline radiating from the bending magnets was 15 milliradians, it was necessary to input multiples of these segments along the horizontal axis into the optic. During this time, activity on the “borrowed” beamlines was temporarily interrupted to allow performing this particular experiment for the duration of 1 week in June 2005. A proof of principle of the operation was established by spectroscopists from NSLS and Bruker Optics with the use of latex microspheres as test samples and with a very thin section of bone that had been sawed with a diamond saw. Spatial resolution was excellent with the $74 \times$ objective (purchased for this experiment) in combination with the FPA of the Hyperion™ instrument. A practical limitation of the system was that only specimens of limited thickness could be analyzed. Complete images were produced very rapidly, nearly in real time. Reports of this advanced experiment are in preparation by Carr.¹¹⁹ A practical test at the ANKA synchrotron IR beamline using rudimentary optical components that were available on hand was reported by Moss et al.¹²⁰

3.4.10 Near-IR Optical Catheter with Fiber Optics

An optical catheter with fiber-optic connections to a near-IR spectrometer, introduced nearly 9 years ago, enables analysis of interior arterial walls *in vivo*.³ This device is used to

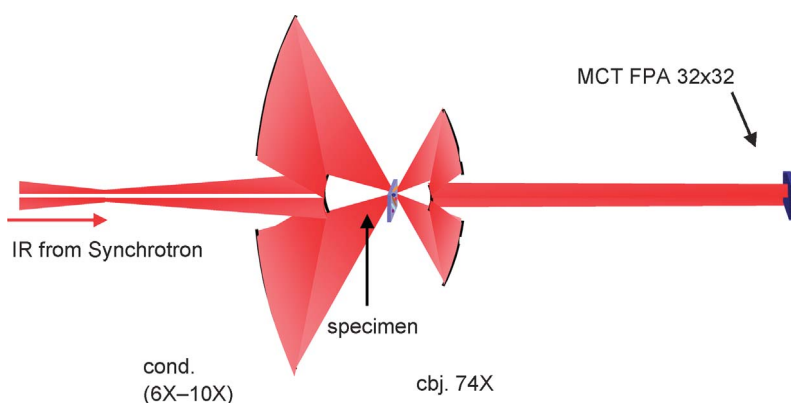


Figure 3.20. Diagram of theoretical design in anticipation of interfacing a commercial FPA IMS to a synchrotron beamline. Synchrotron radiation coming from the left is collected in the $10 \times$ condenser. Several 15 m-rad beamlines are combined to fill the collection optics of the condenser. A $74 \times$ objective closely matched the active area of a 32×32 MCT FPA. (From Ref. 118 with permission of Blackwell.)

detect the level of lipids on the arterial walls, as well as the relative amounts of collagen I in comparison to elastin or collagen III. After the testing procedure over the last 9 years, a commercial product of this nature is scheduled for 2008 release.

3.4.11 State-of-the-Art Synchrotron IMS

In a very recent review by Miller and Dumas¹¹⁰ with 82 references, the subject of chemical imaging of biological tissue in synchrotron IR light is discussed. Space does not permit comment on this extensive review article within this chapter. Other recent synchrotron articles by Dumas et al.¹²¹ and Miller et al.¹²² include synchrotron microspectroscopy from the mid-infrared through the far-infrared regions. Miller and Smith¹²³ compared synchrotron versus global and point detectors versus FPA. Carr¹⁰⁶ previously reported on the resolution limits for IMS explored with synchrotron radiation.

Dumas and Miller¹²⁴ also addressed the use of synchrotron IMS in biological and biomedical investigations. In this report, the edge radiation versus the bending magnet radiation of synchrotrons was discussed. Spectra were compared from a synchrotron source (with an aperture of $3\text{ }\mu\text{m} \times 3\text{ }\mu\text{m}$ and coaddition of 32 scans) versus spectra from an internal global source with an aperture of $6\text{ }\mu\text{m} \times 6\text{ }\mu\text{m}$ and 1000 scans coadded. Various examples were shown for different biological tissues. The superior ability of the synchrotron with a small aperture size and a small step size used in a confocal optical configuration was made clear. In one dramatic case, the protein beta to alpha peak height ratios were imaged (Fig. 3.18) for the ridge of the cortex of a transverse section of human hair.

3.5 COMMENT

Microscopy and spectroscopy are two of the oldest experimental tools widely applicable to the study of nature. Their combination in IMS results in an analytical technology more powerful than the sum of the two. It has expanded from an analytical niche to an important weapon in the analytical chemist's arsenal, and we can expect its wider use in scientific research, general analysis, and routine applications.

ACKNOWLEDGMENTS

It is gratifying to consider the biomedical microspectroscopic strides described in this volume using the current capability that was technologically driven. The scientific curiosity, risk taking, instrument building, ingenuity, and the entrepreneurial spirit within small private companies enabled the “**Connecticut Connection**” referred to in Tables 3.1 and 3.2. Larger instrument companies subsequently contributed their resources. Contributions of the “**Bethesda Connection**” cannot be overemphasized. It is the long dedication of the NIH Chemical Physics lab group with a cadre of talented and productive workers that led to modern focal plane array imaging as we know it. The author is indebted to Spectra Tech, who responded to his need for automated mapping by building a microprocessor-controlled microscope stage and writing software to remove baseline effects that allowed his presentation of functional group maps. The author acknowledges the editor of *Spectroscopy*, who published our first *in situ* rat brain mapping article as an accelerated item after a more traditional journal sat on the manuscript for a year. The author thanks Emily Bonwell for assembling 80 manuscripts from the literature on short order, of which 60 were read and 40

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ACRONYMS AND TRADEMARKS

ANN	artificial neural network
ATR	attenuated total reflection
BNL	Brookhaven National Laboratory
BSE	bovine spongiform encephalopathy
CA	cluster analysis
Continuum™	registered trademark of Spectra-Tech., Inc., Shelton, CT
DIC	differential interference contrast
DSP	digital signal processing
FEL	free electron laser
FFT	fast Fourier transform
FPA	focal plane array
FT-IR	Fourier transform infrared
Hyperion™	Registered trademark of Bruker Optics, Billerica, MA
IlluminatIR™	Registered trademark of Smiths Detection, Danbury, CT
LCTF	liquid crystal tunable filter
MCT	mercury cadmium telluride
NA	numerical aperture
NIH	National Institutes of Health
NSLS	National Synchrotron Light Source
NSOM	near-field scanning optical microscopy
PCA	principal component analysis
SNR	signal-to-noise ratio
Spotlight™	Registered trademark of Perkin Elmer, Shelton, CT
VUV	vacuum ultraviolet

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INFRARED SPECTROSCOPY OF BIOFLUIDS IN CLINICAL CHEMISTRY AND MEDICAL DIAGNOSTICS

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4.1 INTRODUCTION

Modern health care routinely relies upon radiation as a diagnostic probe. Indeed, nearly the full spectrum is exploited in various applications; radio waves and magnetic fields combine to reveal magnetic resonance (MR) images, ultraviolet (UV) and visible radiation are used to detect clinical chemistry reaction end points, and X-rays lie at the heart of both imaging and therapeutic modalities.

Infrared (IR) spectroscopic measurements carry a great deal of information that is potentially useful in the clinical/medical environment.^{1–9} While short-wavelength near-IR spectroscopy has been exploited as a means to monitor tissue oxygenation and hydration, via the absorptions of hemoglobin, deoxyhemoglobin, and water,¹⁰ the aim of the present chapter is to summarize emerging applications based upon mid-IR spectroscopy and, in particular, to describe the recovery of diagnostic and analytical information via mid-IR spectroscopy of biological fluids.

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Clinical applications fall into two categories. One is quantitative analysis; for example, several key components may be quantified simultaneously from the spectrum of a few microliters of serum. The methodologies and benefits of this analytical approach will be outlined. Perhaps more intriguing is the prospect of algorithms that yield medical diagnoses directly from the mid-IR spectroscopic fingerprint. To the extent that disease affects key cellular pathways in characteristic ways, biofluid composition may be altered in a fashion characteristic of that disease; the resultant metabolic fingerprint may be captured directly as a spectroscopic fingerprint. Several applications illustrate the potential of diagnostic metabolic profiling in clinical applications. Those summarized herein will include both human and veterinary applications, along with certain applications of Raman spectroscopy (which is covered separately in this Handbook) as appropriate to complement these applications.

The applicability of IR spectroscopy would be broadened substantially by addressing a single limiting factor, namely the modest sensitivity. While this may be achieved in principle by increasing the optical pathlength, even dehydrated samples soon become essentially opaque due to the strong absorptions of the most concentrated compounds (e.g., serum protein and urine urea), limiting sensitivity to lower concentration species that would otherwise contribute to these spectra. Microfluidics offers a potential solution to this problem, namely the “laminar fluid diffusion interface” (LFDI).

LFDI preconditioning manipulates the relative concentrations of biofluid components based on relative differences among their diffusion coefficients. By using active-flow, pressure-driven technology, biofluid concentrations may be manipulated to achieve very useful goals. For example, LFDI-processed serum specimens depleted of protein, or urine specimens depleted of urea, provide the opportunity for spectroscopic characterization of other species of relatively low or high molecular weight, respectively, that are otherwise masked by these very concentrated compounds. As a consequence, this technology holds out the promise to improve sensitivity in both analytical and metabolic profiling applications of IR spectroscopy, without compromising any of the advantages of IR spectroscopy in these applications. Proof-of-concept studies illustrating this potential are included here.

The chapter concludes with a brief synopsis of the state of the art, and with suggestions regarding what the future might hold as the methods and technologies evolve and adapt to play clinically useful roles.

4.2 VIBRATIONAL SPECTROSCOPY OF BIOFLUIDS

Spectroscopy of biological fluids may be accomplished by any of several experimental arrangements, differing according to the way in which the IR radiation interacts with the sample (transmission versus ATR measurements), in the wavelength range (near-IR versus mid-IR), and whether the sample is dehydrated prior to measurement.^{8,9} ATR spectroscopy carries the advantage of reproducibility in optical pathlength – a criterion that can be difficult to achieve in transmission measurements at the very short pathlengths ($\sim 10\ \mu\text{m}$) required for mid-IR spectroscopy of aqueous samples. Near-IR spectroscopy offers the advantage of convenient sample handling and inexpensive optical materials; very reproducible transmission spectroscopy measurements are straightforward in glass cells of pathlength $\sim 0.5\ \text{mm}$. The compromise is that the technique generally requires larger sample volumes ($\sim 100\ \mu\text{L}$ or more) than is the case for mid-IR spectroscopy (as little as $2\ \mu\text{L}$). Finally, drying the sample is an attractive option from two perspectives in

particular. First, it eliminates the very strong water absorptions that otherwise degrade or obscure other absorptions coincident with them. Second, transmission spectroscopy of dry films is much easier to automate^{11,12} than the counterpart measurements for liquid specimens, particularly at the very short pathlengths required for biological liquids.

4.3 QUANTIFICATION (REGRESSION) AND DIAGNOSTIC (CLASSIFICATION) APPROACHES

In order to be useful, a clinical instrument must produce more than an IR spectrum. The raw measurement must be converted to provide either an analyte level or a diagnostic classification as output to the clinical user. The development and validation of algorithms to accomplish these goals lies at the heart of clinical method development. These aspects are covered in some detail elsewhere in this Handbook, and they have been reviewed previously in the context of medical applications.⁴⁻⁹ We therefore summarize only the essential and relevant features here.

Both analytical and diagnostic methods are developed using the spectra of well-characterized samples, for which the analytical levels and/or diagnostic information of interest are reliably available. These ideally represent the full range of variability that would be encountered in the population targeted for testing. For quantitative, analytical test development, this means that the sample set should not only span the range of concentrations anticipated for the analyte of interest, but must also incorporate the full range of variability that accrues from other factors. For diagnostic test development, the same broad considerations apply; each diagnostic condition of interest must be represented by enough samples that the true diagnostic signal (if any) may be faithfully distinguished from and recovered in the presence of all possible background variability.

Once an appropriate set of samples is recruited, and their spectra measured and preprocessed appropriately, regression or classification methods are typically exploited to generate quantification (analytical) or classification (diagnostic) algorithms. These algorithms are typically trained by using a random selection of about two-thirds of the available samples, and the resulting diagnostic or analytical methods are tested by their ability to faithfully reproduce the true diagnoses or analytical levels of interest for the remaining one-third. So-called “unsupervised” classification methods have occasionally proven adequate to discover diagnostically relevant information, but these methods are generally viewed as being more useful for exploratory analysis.

In practice, partial least-squares (PLS) regression is the approach most commonly adopted to develop quantitative analytical spectroscopic methods.¹³ The same algorithm may also be exploited for diagnostic applications, in which case the spectra are assigned dummy values representing the corresponding diagnostic classes. Many other options exist, however, with neural networks and discriminant analysis among the most commonly used. Regardless of the particular method chosen, the investigator must confront an issue endemic to the development of spectroscopy-based diagnostic tests, namely the fact that the number of spectroscopic data points is inevitably much larger than the number of samples available for test development. Great care must therefore be taken to carefully evaluate seemingly successful trials, to rule out those that may be built upon illusory structures within truly random spectroscopic variations. To guard against that possibility, spectra are generally preprocessed to reduce their dimensionality to a number much smaller than the number of training samples. Possibilities include principal components

analysis (PCA) and region selection algorithms that reexpress spectra respectively as a set of PCA factors or as the set of absorption intensities within a discrete set of wavenumber ranges. The new challenge is then to discover which principal components, or which wavenumber ranges, carry genuine diagnostic information. The advantage of the latter approach is that the wavenumber ranges may in principal be interpreted to suggest the biochemical basis for test success.

4.4 QUANTITATIVE BIOFLUID ANALYSIS

Quantitative blood, serum, and urine assays lie at the core of diagnostic medicine. The prospect of carrying out some of these assays by IR spectroscopy is attractive from several perspectives; no reagents are required, the method is always linear throughout the physiological concentration range, several analyses are available simultaneously from a single IR spectrum, and very little sample (microliters) is required. The utility of this approach is illustrated here by an overview of IR-based blood, urine, and amniotic fluid assays.

4.4.1 Blood, Serum, and Plasma

The chemical analyses collectively referred to as “blood” tests are in fact almost never carried out on blood. Instead, they are generally carried out using serum or plasma samples, both of which are obtained by centrifugation of blood to remove cellular materials. The difference between the two samples is in how the clotting process is handled. An anti-clotting agent such as heparin or EDTA may be added to the blood sample; the supernatant that remains upon centrifugation is then referred to as “plasma.” A second approach is to draw a blood sample with no anti-clotting agent, centrifuge the cells to the bottom of the collection tube, and allow the clotting process to proceed – perhaps even adding a clotting agent to accelerate the process. The clear liquid that remains is then referred to as “serum.”

Serum and plasma may be used interchangeably in most routine clinical chemistry tests, and most clinical analytical instruments are equally accurate for both types of sample. IR spectroscopic assay development trials have therefore been carried out using both plasma and serum. The central finding of early trials was that at least six serum/plasma analytes may be quantified by IR spectroscopy, those being total protein, albumin, total cholesterol, triglycerides, glucose, and urea. Exploratory studies on whole blood have generally been restricted to glucose assay development, with absorptions by cellular materials conspiring against much broader applicability.

4.4.1.1 Mid-IR Serum and Plasma Assays. The first systematic evaluation of the potential for multianalyte plasma analysis made use of attenuated total reflectance (ATR) spectroscopy, in particular the “CIRCLE” cell (Harrick Scientific, Pleasantville, NY), to ensure reproducibility in optical pathlength.^{14,15} The protocol paid particular attention to the need for scrupulous cleaning of the ATR element between samples. With that protocol in place, the study yielded reasonably accurate assays for total protein, total cholesterol, triglycerides, urea, and glucose. The attempt to further provide an IR-based assay for uric acid proved less successful, due entirely to the relatively low concentration of this analyte ($\sim 2\text{--}8$ mg/dL, as compared to a minimum concentration of ~ 20 mg/dL for urea, the most dilute of the other four target analytes).

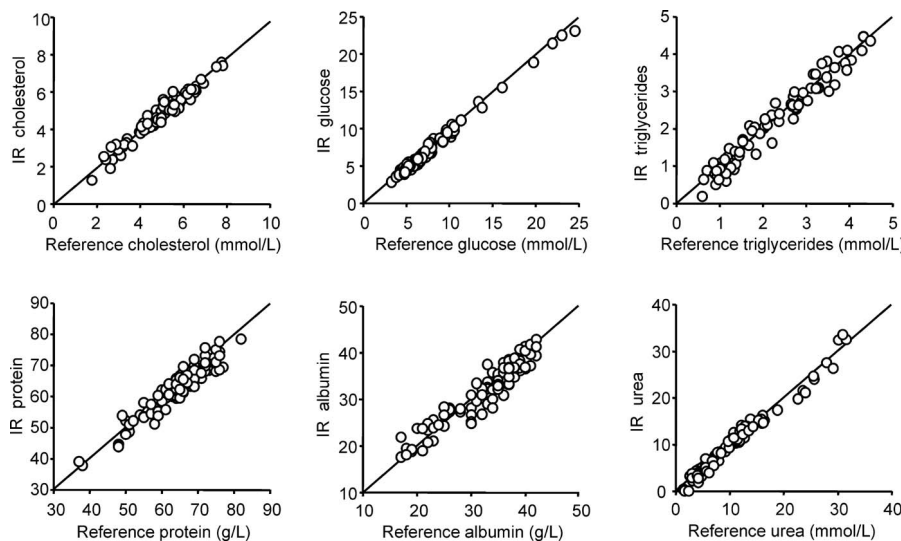


Figure 4.1. Scatterplots summarizing the performance of mid-IR-based assays for six serum analytes. Serum samples were diluted 50:50 with aqueous potassium thiocyanate (4 g/L) and dried to films for spectroscopic measurements; the spectra were then normalized to the 2060 SCN^- absorption prior to PLS algorithm development. Two hundred samples were used to train the PLS algorithms. The scatterplots shown here are for an independent test set of 100 samples. See Ref. 16 for further details.

A comprehensive proof-of-concept study based upon transmission spectroscopy of dried serum films¹⁶ demonstrated good analytical accuracy for albumin, total protein, urea, total cholesterol, triglycerides, and glucose (Fig. 4.1). Since the serum aliquots were spread on barium fluoride windows, possible imprecision was anticipated in manually spreading the sample to within a ~ 1 mm perimeter of the window's edge. To permit compensation for this impression, all samples were diluted 50:50 in aqueous 4 g/L potassium thiocyanate; the strong SCN^- absorption at $\sim 2060\text{ cm}^{-1}$ served as the basis for subsequent spectral normalization. Attempts to quantify uric acid and creatinine were not successful, due to their relatively low concentration ranges. A more recent study further demonstrated that LDL cholesterol and HDL cholesterol may be quantified separately based upon IR spectroscopy of dried serum films.¹⁷

The routine clinical implementation of any method based upon spectroscopy of dried films is unlikely as long as it requires expensive optical materials (a single BaF_2 disk typically costs $\sim \$50$ U.S.) and relies upon a liquid-nitrogen-cooled detector—as was the case for the studies outlined above. Recent innovations in sampling technology have circumvented these concerns. The barium fluoride window may be replaced by a silicon wafer; not only is this material inexpensive, it can be easily cut to fit various holders. For example, a wafer may be cut to the same dimensions as a 96-well microtiter plate, with an adhesive plastic mask with 96 5 mm circular apertures affixed to the wafer (Fig. 4.2). This arrangement not only makes the silicon wafer compatible with standard ELISA liquid handling systems to dispense clinical samples, but also interfaces cleanly with the high-throughput sampling (HTS) accessory, devised by Bruker Optics (Billerica, MA.) to permit automated spectroscopy of materials within the wells of 96-well microtiter plates. In tandem with a spectrometer incorporating a room-temperature DTGS detector, this arrangement has proven very useful for spectroscopy of dry biofluid films.^{11,12} For example,

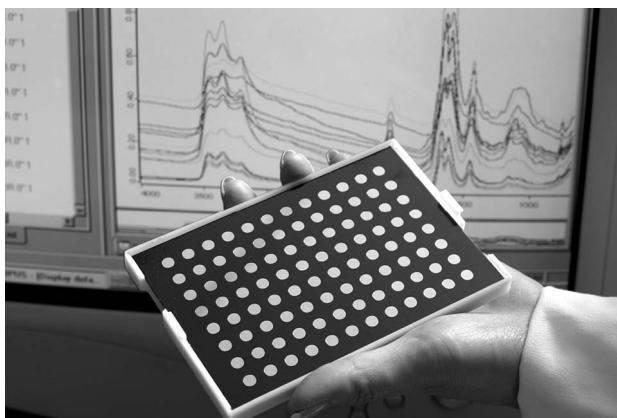


Figure 4.2. A silicon wafer, masked by an adhesive plastic with 96 apertures (5 mm diameter), for mid-IR spectroscopy of dry biofluid films. This device, in conjunction with appropriate spectroscopic hardware (e.g., the Bruker HTS accessory), permits automated, sequential acquisition of 95 spectra (the 96th well is typically left clean, and it is used to acquire a suitable background single-beam spectrum). See Refs. 11,12.

this approach was exploited to develop an assay for apolipoprotein B (“apoB”),¹⁸ which is considered to be a cardiovascular risk marker by virtue of the fact that one molecule is found within each LDL particle. Indeed, as a surrogate of the atherogenic particle count, it has been argued persuasively that apoB is a better indicator of cardiovascular risk than the LDL cholesterol test.¹⁹

One question that inevitably arises in developing quantification methods is which spectral regions are necessary, and the choice can have practical consequences. For example, two reports have illustrated that mid-IR spectra restricted to the X–H stretching region (Fig. 4.3) can provide the basis for reasonably accurate quantitative and diagnostic methods.^{20,21} This finding opens the door to the possibility of using ordinary glass as the substrate for dry biofluid films, with obvious practical benefits including the low cost and durability.

4.4.1.2 Near-IR Serum and Plasma Assays. Two groups have comprehensively illustrated the potential for near-IR spectroscopy in multianalyte serum analysis, showing good accuracy for total protein, albumin, total cholesterol, urea, glucose, and triglycerides.^{22–24} The main difference between the two set of investigations is that one used a rapid-scanning spectrometer (Foss NIRSystems, Laurel, Maryland) with a lead sulfide detector,^{22,23} while the other used an FT–near-IR instrument (Nicolet, Madison, WI) with a liquid-nitrogen-cooled indium antimonide detector.²⁴ Additionally, the pathlength was 0.5 mm and 2.5 mm for the two studies respectively, the latter chosen to optimize signal-to-noise in the combination region 4000–5000 cm^{-1} . Representative scatterplots are reproduced in Fig. 4.4, which includes the results of an attempt to quantify lactate. That attempt proved unsuccessful, certainly due in part to its relatively low concentration and likely due also to the nondescript nature of the lactate near-IR spectroscopic fingerprint.

Apart from the instrumentation, the main practical feature distinguishing near-IR from mid-IR spectroscopy is the sample size, which is typically 200–300 μL for near-IR as compared to <10 μL for mid-IR. More recently, however, it has become clear that the comparative richness of mid-IR spectra can confer additional advantages. For example, an

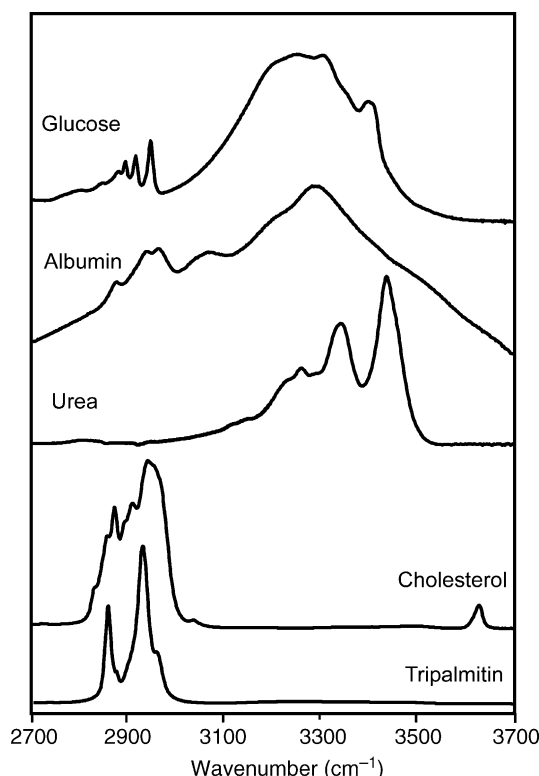


Figure 4.3. Mid-IR spectra of five serum target analytes in the X-H stretching region. The unique spectroscopic fingerprints within this region alone can provide the basis for accurate quantification of these analytes, opening the door to the possibility of using ordinary glass (which is opaque at wavenumbers below $\sim 2000\text{ cm}^{-1}$) as the substrate for mid-IR spectroscopic measurements of dry films. (Reproduced from Ref. 21 with permission from the Society for Applied Spectroscopy.)

attempt to quantify both HDL and LDL cholesterol by near-IR spectroscopy yielded a reasonably accurate method for LDL cholesterol but failed completely for HDL cholesterol.²⁵

4.4.2 Whole Blood

Several groups have developed IR-based assays for glucose based upon spectroscopy of whole blood samples, motivated largely by the prospect of using this technology for diabetes screening/monitoring either *ex vivo* or *in vivo*. Mid-IR investigations^{26–32} have typically yielded assays with standard errors of prediction of approximately 0.6 mmol/L, independent of the experimental details (e.g. ATR vs. transmission, liquid vs. dry film). One study further showed that urea can be quantified with reasonable accuracy based upon spectroscopy of dry blood films.³⁰

4.4.3 Urine

The most common routine urine tests are for creatinine and protein, each of which serves as a probe of kidney function. Creatinine may be quantified by either near-IR spectroscopy of the

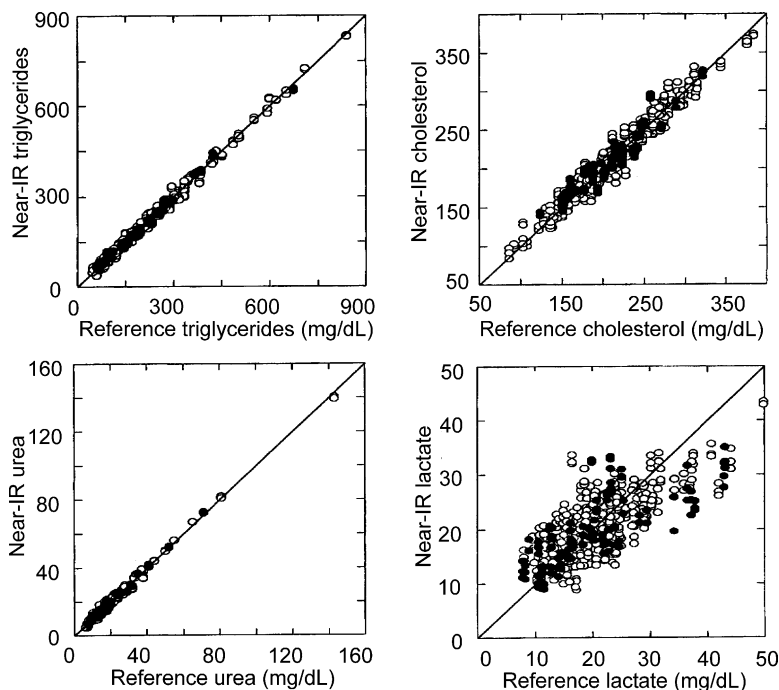


Figure 4.4. Scatterplots summarizing the performance of near-IR-based assays for four serum analytes (see Ref. 24). The relatively poor performance for lactate is due to its relatively low concentration and also likely due in part to the lack of clearly distinctive absorptions within the near-IR fingerprint. (Reproduced from Ref. 24 with permission from Elsevier.)

native fluid³³ or by mid-IR spectroscopy of dry films³⁴ (Fig. 4.5), while protein poses a more difficult challenge. Protein is typically present in urine at a concentration of 100 mg/L or less. Elevation above this level is significant, and protein dipstick tests in present use for routine screening typically have a cutoff of 300 mg/L. To achieve this goal, the IR-based method should achieve an RMS error (RMSE) of ~ 100 mg/L as compared to the “true” values; this in turn converts to a detection threshold (defined as $3 \times \text{RMSE}$) of 300 mg/L. This threshold can be met by a PLS quantification method, but only by calibrating the method with samples restricted to the concentration range 0–1000 mg/L; with this approach, an RMSE of 100 mg/L can be achieved by either near-IR or mid-IR spectroscopy.^{33,34}

Additional urine components that have proven well-suited for IR spectroscopy-based quantification include urea,^{33,34} uric acid, sulfate, and phosphate.³⁵

4.4.4 Amniotic Fluid

Amniotic fluid is the fluid that surrounds the developing fetus. It contains genetic material that provides the basis for prenatal genetic testing. It also contains pulmonary surfactants that are produced by the developing fetal lungs. Amniotic fluid tests that quantify these surfactant levels are carried out to assess the potential for the fetus to thrive outside the womb, should premature delivery be required for the sake of the mother’s health in high-risk pregnancies. One way to carry out this assay is a thin-layer chromatography procedure that requires several hours’ work by a trained technician to

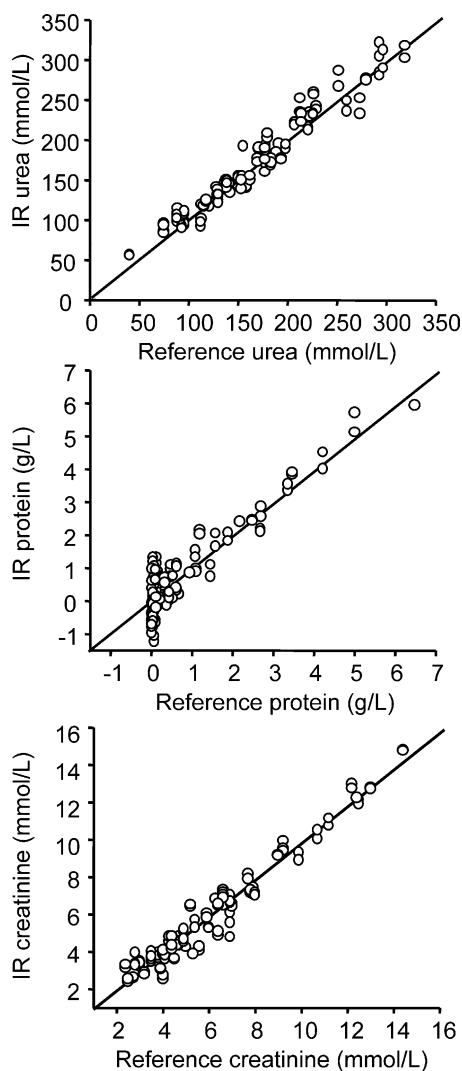


Figure 4.5. Scatterplots summarizing the performance of mid-IR-based assays (test sets only) for three urine analytes. (Reproduced from Ref. 34 with permission from the American Association of Clinical Chemistry.)

produce the ratio of lecithin to sphingomyelin – the “L/S ratio.” A second way is to use a fluorescence depolarization measurement to determine the surfactant-to-albumin (S/A) ratio. A commercial version of this assay is provided by Abbott for implementation on their TDx fluorescence depolarization analyzer. The main drawback of this assay is its expense, and certain common contaminants such as blood and meconium can degrade the accuracy of this assay.

Mid-IR spectroscopy provides the basis to accurately quantify the relevant amniotic fluid surfactants. A series of reports have illustrated good correlation between IR-based assays and both the L/S and S/A ratios^{36,37} (Fig. 4.6). The same measurement further provides the basis to accurately quantify glucose and lactate levels, which have been suggested to have utility as markers of infection and fetal hypoxia.³⁸ The very low incremental cost of testing, the simplicity of the measurement, and good analytical accuracy all conspire to make the prospect for IR-based fetal lung maturity testing an attractive one.

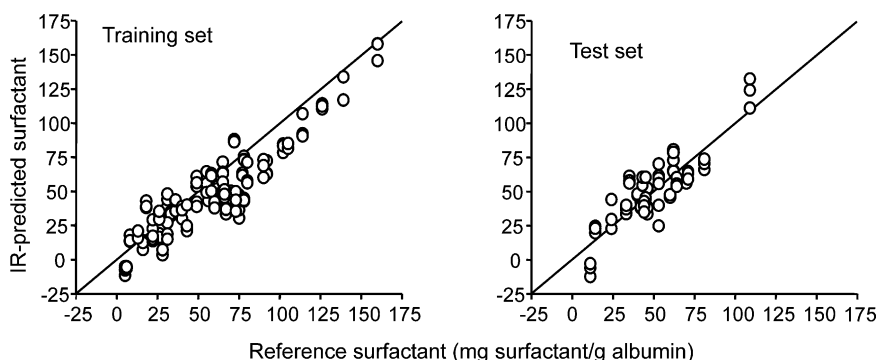


Figure 4.6. Scatterplots summarizing the performance of a mid-IR-based assay for amniotic fluid lung surfactant. Low values suggest fetal lung immaturity, as well as that the fetus would not thrive outside the womb without surfactant therapy. (Reproduced from Ref. 37 with permission from Elsevier.)

4.5 DIAGNOSTIC BIOFLUID TESTS

The clinical chemistry laboratory provides quantitative information that is then interpreted to suggest or support the ultimate diagnosis. For example, serum glucose and troponin concentrations have clear causal relationships with the medical conditions – diabetes and cardiac infarction—that they signify. Elevated levels dictate a clear course of action, and untold lives and lifestyles are saved routinely by application of these tests. How do we discover new disease markers? Classical, hypothesis-driven research provides one avenue; within this paradigm, new possibilities are suggested by considering the basic mechanisms underlying disease initiation and progression. This is no longer the only option. The advent of analytical techniques having broad sensitivity to a range of constituents, along with the development of sophisticated bioinformatic tools, has spawned a new approach wherein the marker is discovered first, and the mechanistic interpretation then follows from interpretation of that marker. Genomics and proteomics provide the most familiar examples in use today of this inverted discovery paradigm.

While the “proteome” and the “genome” have provided fertile ground for the discovery of new diagnostic tests, there is a growing recognition that the “metabolome” can provide the foundation for test development in the same spirit. To the extent that disease affects key cellular pathways in characteristic ways, biofluid (serum/urine) composition may be altered in a fashion characteristic of that disease. The resultant metabolic fingerprint (“metabolome”) may be captured directly as a spectroscopic fingerprint. For example, the IR spectra of serum and urine reflect the biochemical composition of those fluids. While some of the features within the spectroscopic fingerprint may reflect known disease markers, the real significance of the metabolic fingerprinting approach lies in the potential to discover markers or groups of markers that have not previously been recognized as such. The tests described below are all built upon diagnostic spectral patterns that implicitly reflect the presence of such markers.

4.5.1 Arthritis

Arthritis is defined literally as inflammation of the joint [from Greek *arthro-* (joint) + *-itis*, (inflammation)], and it is therefore reasonable to imagine that synovial fluid within joints

might provide the basis to diagnose arthritis. This fluid is a plasma dialysate and is therefore similar in composition to plasma. A key difference lies in the presence of hyaluronate, a glycosaminoglycan that lubricates the joint. While synovial fluid analysis has long been suspected to be of potential use for the diagnosis and staging of arthritis, success has been largely limited to the diagnosis of gout and infection via the detection of crystals and bacteria respectively. The vast majority of the nearly 100 arthritis variants cannot be distinguished on the basis of routine synovial fluid tests.

Infrared spectroscopy of the affected joint provides the basis for the differential diagnosis of rheumatoid arthritis, osteoarthritis, and spondyloarthropathy – the three broad diagnostic categories within which nearly all arthritis variants are found – and distinguishes all of these from the aspirates of “normal” (i.e., nonarthritic) joints.^{39,40} Diagnostic classification of either near-IR³⁹ or mid-IR⁴⁰ spectra reproduced the true diagnoses with an overall classification accuracy of better than 90%. It is interesting to note that the study based upon mid-IR spectra of dry films exploited only the absorptions corresponding to C–H stretching modes, within the range 2800–3100 cm⁻¹. Separate mid-IR^{20,21} and Raman⁴¹ studies have since validated the notion that this spectral range, although not generally viewed as part of the “fingerprint” region, is quite information-rich and appropriate for analytical and/or diagnostic test development and implementation.

4.5.2 Serum Tests

Two examples serve both to illustrate the potential of mid-IR spectroscopy in diagnostic application and to shed light on the potential benefits of sample pretreatment in broadening the applicability of the method.

4.5.2.1 Diagnostic Serum Test for Rheumatoid Arthritis. Encouraged by success in diagnostic spectroscopy of synovial fluid, a more recent study explored the possibility that rheumatoid arthritis might promote distinctive patterns within the mid-IR spectrum of dried serum samples. Figure 4.7 illustrates the serum class average spectra for rheumatoid arthritis patients ($N = 94$) and healthy controls ($N = 97$), and it also shows the difference between these spectra.⁴² The fact that the mean spectra are distinguishable encourages the idea that individual samples/cases might be susceptible to IR-based diagnosis using a suitable algorithm. Such an algorithm was developed by using a genetic algorithm optimal region selection (GA_ORS) approach⁴³ to identify a set of eight diagnostic spectral subregions. Highlighted in Fig. 4.7, these subregions provide the basis to reduce the dimensionality of the spectra from ~ 1600 (the number of data points spanning the spectral range 800–4000 cm⁻¹) to 8 (the set of integrated intensities within the eight optimal spectral regions). Linear discriminant analysis of the compressed data set then yielded a diagnostic classifier with an overall accuracy well over 80%, along with a sensitivity and specificity of 84% and 88%, respectively, for an independent test set.

4.5.2.2 Diagnostic Serum Test for Diabetes. Diabetes is an attractive target to assess the validity and accuracy of spectroscopy-based diagnostic testing. Serum glucose levels are known to change with the onset of diabetes, and serum cholesterol and triglycerides can also be affected by concurrent metabolic disorders. However, the casual (non-fasting) serum glucose level is not sensitive or specific enough to reliably distinguish diabetics from non-diabetics, even in tandem with the lipid tests, and the diagnostic power is further diminished if the diabetic population is undergoing therapy. The question of interest is, then, can a single IR spectroscopic measurement for a non-fasting serum sample provide

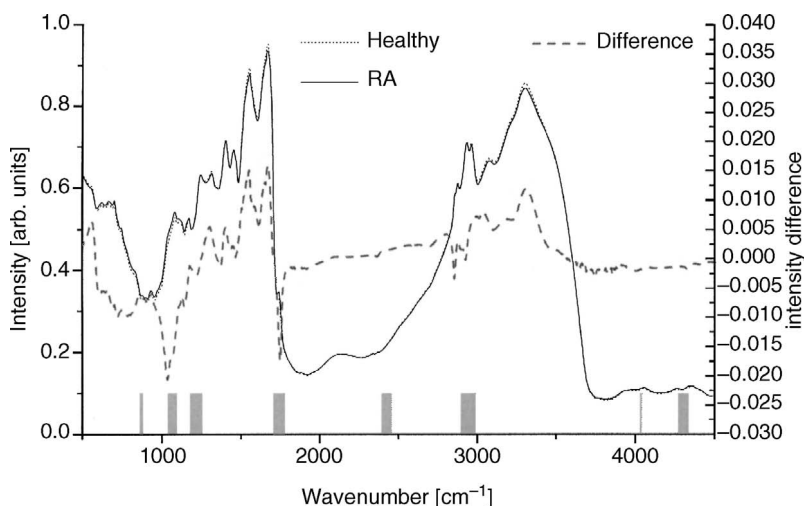


Figure 4.7. Class averages of dried serum mid-IR spectra for 94 rheumatoid arthritis patients and 94 controls, along with the difference between them (note the 15-fold expansion along the absorbance axis for the difference spectrum). The shaded vertical bars highlight the spectral regions that provided the basis for diagnostic classification. (Reproduced from Ref. 42 with permission from Elsevier.)

for more accurate diagnosis than is available from the glucose (and lipid) levels for the same sample? To address this question, a proof-of-concept study sought spectroscopic patterns distinguishing the spectra for 42 healthy volunteers from the counterpart spectra for 40 type 1 diabetics and from 40 type 2 diabetics.^{44–46} Although the differences between class average spectra proved to be quite subtle (Fig. 4.8), an optimal region selection algorithm, in conjunction with either linear discriminant analysis or regularized discriminant analysis, yielded useful diagnostic classifiers. Sensitivities and specificities of $\sim 80\%$ were achieved in the pairwise discrimination of the diabetic data sets from the set of control spectra. Most significantly, that approach proved capable of correctly categorizing the diabetic samples

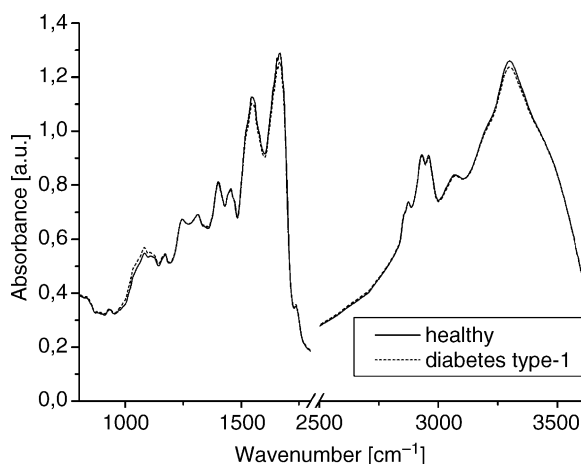


Figure 4.8. Class averages of dried serum mid-IR spectra for 40 type 1 diabetics and 42 controls. (Reproduced from Ref. 45 with permission from Elsevier.)

for which none of the three conventional serum assays (glucose, cholesterol, and triglycerides) were outside normal limits. That work was extended to demonstrate that the same technique might apply for the detection of “metabolic syndrome” – a cluster of risk factors believed to precede the onset of diabetes.^{46,47}

4.5.3 Diagnosis of β -Thalassemia

Literally translated as “anemia of the sea” – so-named due to its prevalence among natives of the Mediterranean countries – the thalassemias comprise a group of genetic disorders of hemoglobin synthesis involving mutations that reduce or abolish α - or β -globin hemoglobin chain synthesis. The hallmark of β -thalassemia is an excess of α -chains due to quantitative defects in the β -globin chain; unbound α -chains denature and precipitate, shortening the lives of red blood cells.

An investigation has been undertaken to assess the potential of mid-IR spectroscopy to diagnose this and possibly other hemoglobin disorders.⁴⁸ The mean spectrum for red blood cell hemolysates from β -thalassemia patients was clearly different from the counterpart spectrum for controls. The most obvious significant difference was the protein amide I region (Fig. 4.9), where bands signifying protein β -structure were stronger and α -helix bands weaker for the thalassemia as compared to control samples. Both unsupervised cluster analysis and supervised classification (GA_ORIS⁴³) proved successful in classifying the individual spectra for 91 samples (56 β -thalassemia and 35 controls); the supervised approach yielded perfect classification of 122 training spectra (61 samples run in duplicate)

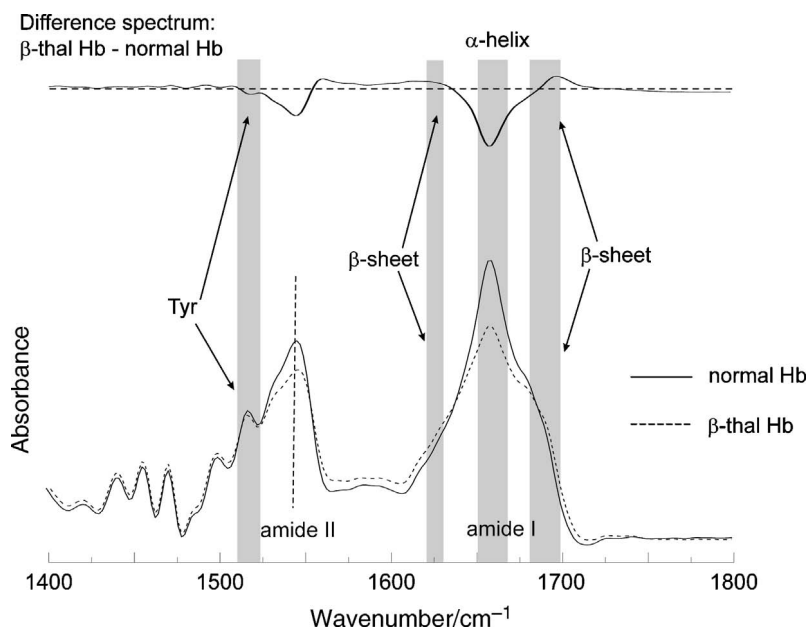


Figure 4.9. Class average mid-IR spectra for hemolyzed blood samples from β -thalassemia victims ($N=56$) and controls ($N=35$), as well as the difference between them. The most significant differences are amide I bands signifying lower α -helical and higher β -sheet structure content in β -thalassemia as compared to control hemoglobin samples. (Reproduced from Ref. 45 with permission from the American Association of Clinical Chemistry.)

and only a single misclassification among the 60 spectra (30 samples) in the test set. Cluster analysis provided useful insights for various choices of spectral region. Most surprising was the good separation of clusters based upon a single absorption – that of the –SH stretching vibration at $\sim 2553\text{ cm}^{-1}$ originating with the α -104 cysteine residue.

4.6 VETERINARY APPLICATIONS

4.6.1 Diagnosis of Prion Diseases

The European “mad cow disease” epidemic brought worldwide attention to a set of diseases collectively known as transmissible spongiform encephalopathies (TSEs), also termed “prion diseases” because of the central role of the prion protein. Diagnosis can be very difficult, and it is usually confirmed only by postmortem examination of brain tissue. Although it would be enormously helpful in livestock screening, no serum test is yet available for this disease. There is therefore significant and widespread interest in a series of studies suggesting that the disease may be diagnosed based upon IR spectroscopy of serum.

The first relevant manuscript appeared in 2002, and it reported nearly perfect accuracy (sensitivity and specificity of 97% and 100%) in distinguishing scrapie-infected hamsters from their control counterparts postmortem.⁴⁹ The test was built upon the mid-IR spectra for serum samples dried onto a zinc selenide substrate, combining a spectral feature selection approach with artificial neural network analysis. The model development was particularly thorough: The 312 total samples (146 scrapie-infected and 166 control) were split into three sets – a training set ($N = 89$), a validation set ($N = 39$), and a test set of 184 samples. The extraordinarily high diagnostic accuracy was maintained for the test set, and this clear success has since prompted several more recent studies. The first follow-up study confirmed that the same approach works for the antemortem diagnosis of late-stage bovine spongiform encephalopathy (mad cow disease), again with sensitivity and specificity better than 95% despite the fact that many of the control animals carried unrelated bacterial or viral infections.⁵⁰ The same approach for the same disease was further validated by a separate study that made use of an automated system to dispense samples onto disposable silicon substrates.⁵¹ That study also included three alternative classification strategies (linear discriminant analysis, “robust” linear discriminant analysis, and support vector machine), which yielded a combined classifier marginally superior that produced by the artificial neural network or support vector machine alone. More recently, the same data have been subject to further analysis using a hierarchical scheme that performs equally well.⁵²

The natural question that arises with success in postmortem and late stage antemortem diagnosis is whether the serum test would be capable of detecting disease in its early, preclinical stage. This question has been addressed through the use of a Syrian hamster model with serum samples acquired at 70, 100, 130, and ~ 160 days post-infection. While accurate diagnosis proved not to be possible using the spectra for samples at 70 days post-infection, samples taken at 100 days post-infection (or later) – still well within the preclinical stage – did provide the basis for diagnosis.⁵³

Finally, the possibility of using liquid rather than dehydrated samples has been explored.⁵⁴ By making use of a custom-built liquid cell and liquid handling system, spectra were acquired for 115 BSE-positive and 108 BSE-negative cow liquid serum samples. Neural network analysis revealed sensitivity and specificity comparable to earlier studies, confirming that diagnostic information content is not compromised by using liquid rather than dehydrated samples.

This series of studies provides a very strong foundation for some general conclusions about spectroscopy-based diagnostic testing, or “metabolic profiling.” In particular, as the authors rightly point out, the spectroscopic measurements certainly do not include any contribution from the prion protein believed to be the causative agent of TSEs. A reasonable alternative explanation is that the disease promotes characteristic changes in metabolic pathways, which translate in turn to characteristic IR spectroscopic patterns. Indeed, evidence in support of this hypothesis has been presented in the form of NMR spectra for sera from scrapie-infected sheep.⁵⁵ In particular, the concentrations of citrate, lactate, and hydroxybutyrate were all found to correlate with the presence of scrapie infection, and the combined concentrations for these three metabolites were sufficient to correctly categorize all but one of 27 serum samples from controls ($N=9$), preclinical scrapie-infected animals ($N=9$), and animals showing clinical signs of scrapie-infection ($N=9$).

4.6.2 Equine Joint Disease

Horses are susceptible to a variety of joint diseases that can interfere with both their development and their performance. For example, lameness accounts for nearly 70% of days lost in training among racehorses. A simple test to diagnose and monitor joint health would provide the basis to improve the management of these horses and other performance animals, possibly avoiding or managing the stresses that cause progression from subclinical disease to clinical problems. Although it is caused by joint stress, traumatic arthritis is more than just a physical derangement. In addition to inflammation of the joint, various enzymes released from the joint lining destroy tissue within that joint.

A proof-of-concept investigation has demonstrated that traumatic arthritis may be diagnosed based upon spectroscopy of synovial fluid from the affected joint.⁵⁶ Diagnostic spectral subregions were identified by a two-step procedure; the first step was to identify those spectral regions within which the intensities differ most significantly for diseased as compared to normal joints, and the second step was to identify the subset of these regions that provides for the most accurate diagnostic LDA classification. Synovial fluid from affected joints proved to be distinguishable from control counterpart samples on the basis of three spectral regions within the normalized second-derivative mid-IR spectra (Fig. 4.10).

A second investigation focused on a developmental disease, osteochondrosis, that has an incidence rate of 10–30% in horses.⁵⁷ The disease manifests itself through the failure of growing bones to ossify, resulting in the development of intraarticular lesions. While the standard of care is orthopedic assessment and radiography, the time and cost involved prohibit screening of asymptomatic horses that may nevertheless have a subclinical disease. Various synovial fluid and serum assays can provide insights into the pathogenesis of the disease and assist in diagnosis; however, they are expensive and there is no single test suitable for routine screening and diagnosis. These observations highlight the need for a rapid screening test, and preliminary indications are that such a test can be developed based upon mid-IR spectroscopy of synovial fluid – a diagnostic algorithm was developed⁵⁷ following the same procedure as that described above in the context of traumatic arthritis. While the overall classification success rate was modest, approaching 80% in cross-validation, the success rate was highest for the young horses – ages 2 and younger – for which the test would be most useful. Indeed, the authors conclude that further study with larger sample size using age, gender, and breed-matched controls is warranted to further validate the clinical value of the IR-based diagnostic method.

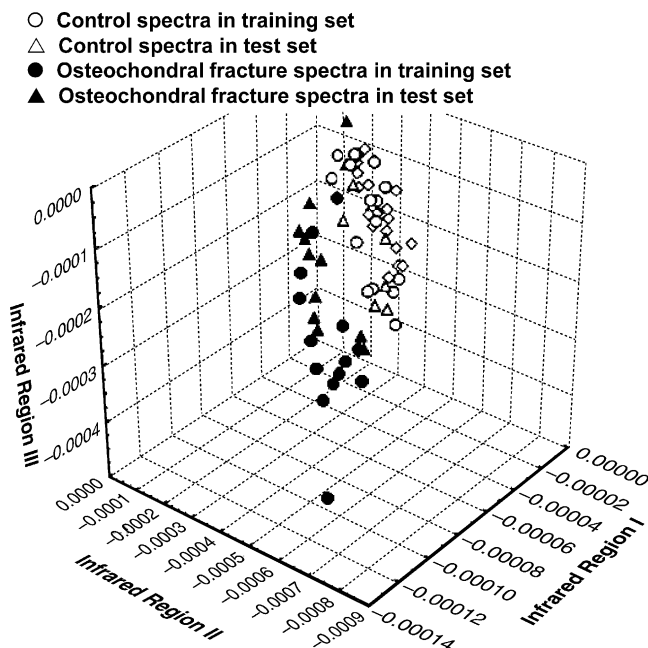


Figure 4.10. Scatterplot highlighting the successful diagnostic classification of equine synovial fluid samples, distinguishing those with traumatic arthritis (osteochondral fracture) from controls on the basis of three mid-IR spectroscopic regions. Each spectrum is represented by the triplet of integrated intensities in the regions $1245\text{--}1257\text{ cm}^{-1}$ (IR region I), $1681\text{--}1684\text{ cm}^{-1}$ (IR region II), and $1691\text{--}1694\text{ cm}^{-1}$ (IR region III) for the second-derivative spectrum. (Reproduced from Ref. 56 with permission from the American Veterinary Medical Association.)

4.6.3 Failure of Passive Transfer of Immunity

Newborn foals have little or no protection from infective agents, and they acquire that protection only through the uptake, through the gut, of immunoglobulins from maternal colostrum (“mother’s milk”). There is only a narrow window of opportunity; the foal must nurse within 24 h of birth, and even that window is closing hour by hour. A significant fraction of foals – estimates range from 5% to 20% depending upon the breed – do not succeed in acquiring immunity in this way, either because they fail to nurse early enough, because the colostrum is too low in immunoglobulins to be of benefit, or because the uptake of immunoglobulins through the gut is unsuccessful. This failure of passive transfer (FPT) is asymptomatic in and of itself, but does leave the foal susceptible to infection. It is therefore common practice to screen newborn foals for FPT, which is defined as a serum immunoglobulin G (IgG) level of less than 400 mg/dL, and to treat immunodeficient animals with IgG-rich serum. While various diagnostic tests exist, the majority are compromised by either poor sensitivity or poor specificity, and the gold standard test – a radial immunodiffusion assay – is too expensive and too slow to consider for routine screening purposes. The prospects for an IR-based assay have therefore been assessed with the aim of providing an accurate test that is rapid enough for routine use.

Two possibilities presented themselves in developing an IR-based test.⁵⁸ One was to treat the FPT status as a dichotomous variable and to develop a test that categorizes spectra/specimens as FPT ⁺ve (IgG < 400 mg/dL) or FPT [−]ve. The second was to provide a

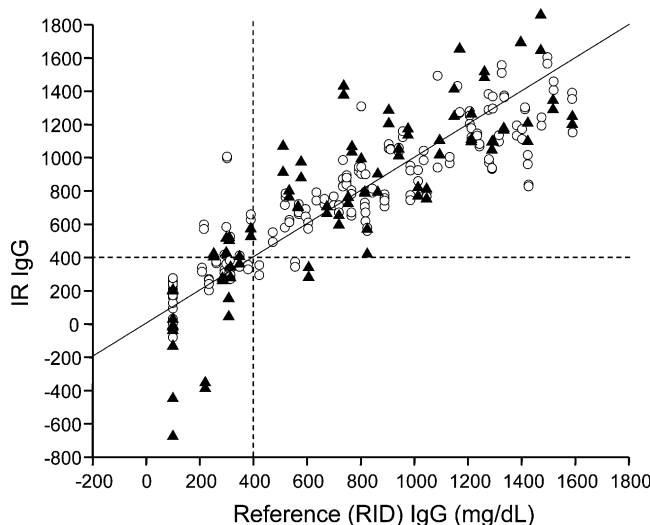


Figure 4.11. Scatterplot summarizing the performance of a mid-IR-based assay for serum immunoglobulin G in newborn equine foals. The dashed lines indicate the 400 mg/dL cutoff for “failure of passive transfer of immunity,” signifying the need for IgG supplementation. (Reproduced from Ref. 58 with permission from the American College of Veterinary Internal Medicine.

quantitative assay for IgG, using the RID-measured values as the gold standard. In the former case, a classifier trained with 92 samples (184 spectra) using the GA_ORs algorithm⁴³ provided sensitivity and specificity of 93% and 97%, respectively, for an independent validation set of 102 samples (204 spectra). The quantitative assay was also accurate enough to distinguish FPT⁺ from FPT⁻ samples with approximately the same level of accuracy (Fig. 4.11), while further providing quantitative information that is not available from many FPT test kits in routine use today. This success has paved the way for the development of further tests of immune function in the neonates of humans and other animals.

4.7 MICROFLUIDICS AND IR SPECTROSCOPY OF BIOFLUIDS

The examples above highlight both the capabilities and limitations of analytical/diagnostic IR spectroscopy of biofluids. While these applications highlight the wide range of disorders that may be detected and characterized by IR spectroscopy, there is a natural desire within both the research and clinical communities to expand and refine these capabilities. The examples of rheumatoid arthritis and diabetes diagnosis illustrate how subtle diagnostic IR signals can be in comparison to the nondiagnostic background spectrum (see Figs. 4.7 and 4.8). One possibility that offers great potential to enhance diagnostic and analytical sensitivity, by reducing the relative concentration of nondiagnostic components, is a microfluidic pre-conditioning technique incorporating a laminar fluid diffusion interface (LFDI).^{59,60}

4.7.1 A Problem

The strength of IR spectroscopy in biomedical applications arises from the unique spectroscopic fingerprints differentiating the various biomolecules, combined with the

fact that in principle all of the constituents contribute simultaneously to the measured spectrum. The potential weakness is that the sensitivity is constrained by the amount of material that may be dried to a film. Although they contribute in principle, the spectroscopic signatures from species of lower concentration may lie below the noise level of the measurement. Of course, drying larger volumes may compensate for this, but beyond a certain limit the film becomes essentially opaque to IR light due to absorption by the most concentrated compounds. This in turn prevents the spectroscopic detection of components below a certain concentration threshold. It is relevant to note here that parallel investigations using Raman spectroscopy have generally not reached the accuracy provided by IR spectroscopy,⁶¹ although other technical features certainly make quantitative Raman spectroscopy an interesting and worthwhile pursuit.

Both the analytical accuracy and the diagnostic sensitivity of biofluid IR spectroscopy would be substantially improved if it were possible to selectively remove the high concentration compounds. The absorption patterns for lower concentration components could then be exploited by drying larger volumes to films for measurement, effectively enhancing the concentration of these components. LFDI preconditioning offers that possibility, opening the door to analytical and diagnostic tests that could not otherwise be contemplated.

4.7.2 A Solution

LFDI preconditioning changes the relative concentrations of fluid components according to relative differences among their diffusion coefficients. The technique is conceptually simple; the fluid of interest flows through a microchannel adjacent to (in contact with) a parallel stream of a different fluid (e.g., water). The small scale ensures laminar flow; while the streams do not mix (there is no turbulence), diffusion does occur across the boundary between them (Fig. 4.12). For serum and water streams flowing in parallel, for example, low-molecular-weight serum constituents diffuse relatively quickly into the adjacent water stream. The original serum sample is thereby partly depleted of low-molecular-weight species, and the exiting water stream (“receiver stream output”) now carries low-molecular-weight species that have migrated across the boundary

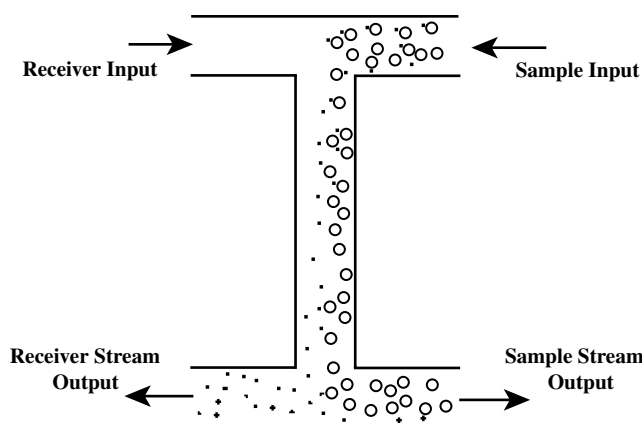


Figure 4.12. Schematic illustration of the laminar fluid diffusion interface, demonstrating the separation of low-molecular-weight components (rapid diffusion into receiver stream output) from comparatively high-molecular-weight components that remain in the sample stream output. (Reproduced from Ref. 60 with permission from Elsevier.)

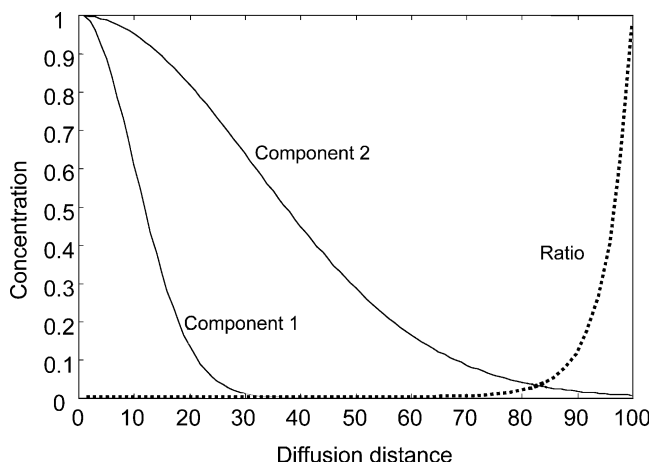


Figure 4.13. Receiver stream concentration versus distance from the LFDI diffusion interface (Fig. 4.12) for two components with diffusion coefficients differing by a factor of 10, along with the ratio of concentrations for the same two components. All units are schematic only, to illustrate qualitatively the exponential increase in the relative concentration of low-molecular-weight (fast diffusion) compounds with distance from the diffusion interface at a given point in time. The profiles do change with time, of course, and the flow speeds (relative and absolute) must be optimized to achieve concentration profiles optimal for the application at hand (see also Fig. 4.16).

(Fig. 4.13). The two exiting streams may be separated, and IR spectra may be acquired for both. The only alternative that accomplishes a similar goal is ultrafiltration. However, separation based upon ultrafiltration excludes certain analytes altogether (there is a defined molecular-weight cut off), and the serum components that are filtered out are unavailable for further use. The method has nevertheless been evaluated for use in preconditioning biofluids for Raman spectroscopy, with promising results.⁶²

The flexibility inherent to the LFDI approach is well illustrated by the different strategies for serum and urine processing. In the case of serum, the LFDI operating conditions may be optimized to maximize the transfer of low-molecular-weight constituents into the receiver stream while simultaneously minimizing the migration of albumin and higher-molecular-weight proteins. IR spectroscopy of the receiver stream output may then recover signatures from components that would otherwise be inaccessible due to very strong serum protein absorptions. On the other hand, diagnostic IR spectroscopy of urine would be optimized by removing urea, a nondiagnostic constituent of comparatively lower-molecular-weight and very high concentration that otherwise masks the contributions of lower abundance species. To accomplish this, the LFDI operating conditions are set to maximize the migration of urea into the receiver stream output, and the (urea depleted) sample stream output may then be harvested for IR spectroscopy.

4.7.3 Proof-of-Concept Studies

Initial experiments used a LFDI technology that relies upon gravity to feed the sample and receiver fluids through the diffusion interface channel.⁶³ Those experiments demonstrated qualitatively that the desired effect was indeed evident in the IR spectra of LFDI processed streams. For example, an aqueous solution with urea and albumin at concentrations of

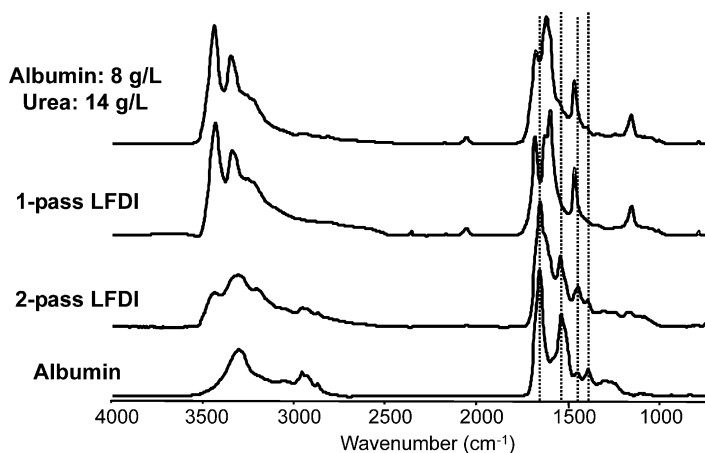


Figure 4.14. Mid-IR spectra of films dried from an aqueous albumin/urea mixture (top) and the sample stream outputs (see Fig. 4.12) from two successive LFDI passes. The loss of urea and the recovery of albumin are highlighted by comparison of the 2-pass LFDI spectrum with the spectrum of pure albumin. (Reproduced from Ref. 63 with permission from the Society for Applied Spectroscopy.)

15 g/L and 8 g/L, respectively, showed a spectrum dominated by urea absorptions (Fig. 4.14). Consecutive passages of the sample stream output through the LFDI card almost completely eliminated the urea from the sample stream output, as evidenced by comparing the relevant spectrum with that for pure albumin (Fig. 4.14).

A more recent proof-of-concept study exploited LFDI technology that actively controls both the absolute and relative flow rates of sample and receiver streams (Micronics Inc., Redmond, WA).⁶⁴ Using “off-the shelf” pressure-driven LFDI cards, spectra were acquired for a set of serum samples and for their counterpart LFDI processed receiver output streams. IR-based creatinine quantification methods were developed independently for the two spectral data sets, with this analyte targeted specifically because its concentration range falls just below the range suitable for IR-based quantification. Figure 4.15 illustrates the clear and

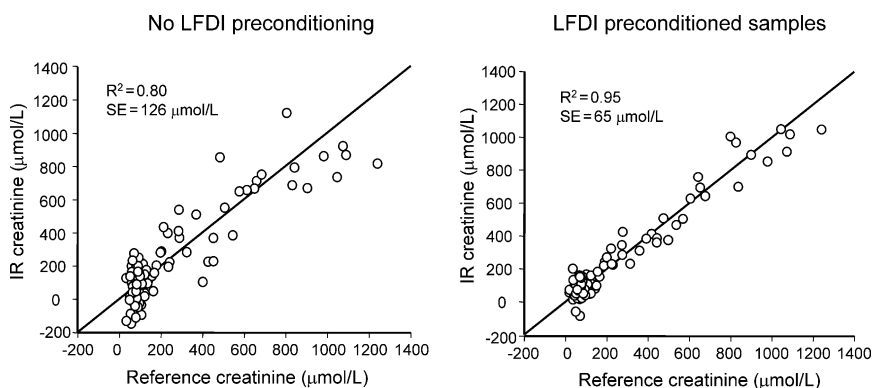


Figure 4.15. Comparison of analytical accuracy (SE stands for standard error of cross-validation) for mid-IR-based serum creatinine assays with and without LFDI sample preconditioning. (Reproduced from Ref. 64 with permission from the Institution of Engineering and Technology.)

compact LFDI card with dimensions comparable to that of a credit card. Processes taking place in a microsystem suitable for IR spectroscopy might include sample introduction, sample clean-up of foreign particulate matter, replacement of the blood centrifugation process (for applications that primarily target serum metabolites), mixing of any required internal standard, analyte concentration enhancement, and controlled deposition of dried micro-films for spectroscopic analysis. Integrated with ever-smaller spectroscopic instrumentation, it is easy to envisage a point-of-care platform suitable for routine use even in developing countries where resources for conventional testing are scarce or absent.

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RAMAN SPECTROSCOPY OF BIOFLUIDS

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5.1 INTRODUCTION

Raman spectroscopy has often been considered inferior to mid-infrared spectroscopy with regard to its ability to quantify analytes in biofluids due to its small signal strength (differential cross section $\sim 10^{-28}$ cm²/sr) and the fact that the Raman spectrum is frequently obscured by a much more intense fluorescent light background. However, since water constitutes the basic solvent of biofluids, Raman spectroscopy benefits from the low absorption coefficient of water in and around the visible region of the spectrum compared to the high absorption coefficient of water in the mid-infrared spectral range: The absorption coefficient of water is decreased by up to seven orders of magnitude when using light of wavelengths λ between ~ 0.3 μ m and 1 μ m [i.e., ultraviolet (UV), visible or near-infrared (NIR) light] for Raman spectroscopy compared to light in the mid-infrared region with wavelengths between 2.5 μ m and 25 μ m.

In addition, the application of light in the UV to NIR spectral region allows for the use of readily available, low-cost optical components for both the manipulation and the guidance of light. For example, since glass fiber optics are biocompatible and well-established in medical applications, even the development of a miniaturized fiber-optic sensor for *in vivo* measurement can be realized more easily than in the MIR region.

This chapter is intended to provide a brief overview of the Raman spectroscopy of biofluids and to elaborate on the two major problems of Raman spectroscopy: the high fluorescent light background and the low signal-to-noise ratio (SNR). It describes the influence of these two principal limitations on the prediction accuracy of analyte concentration in biofluids and introduces techniques to overcome these limitations. Examples for Raman spectroscopic investigations of body fluids are given.

5.2 BACKGROUND FLUORESCENCE

One major drawback in the Raman spectroscopy of biomolecules is the competition between the Raman effect and the (preferentially resonant) absorption of a photon followed by relaxation of the electronically excited molecular state via fluorescence. For large molecules with extended π -electron systems, light (particularly in the UV spectral region) is prone to excite the molecule and to populate the excited electronic state. Excited molecules can then relax via emission of fluorescence photons. While the Raman process carries specific information about the vibrational states of the molecule, fluorescence is less specific to a molecule in a mixture due to its broad emission lines. Biofluids often exhibit a high content of fluorescing molecules such that they frequently generate a spectrally broad background of large amplitude below the Raman spectrum. Depending on, for example, the protein content this background in general exceeds the desired Raman signal by several orders of magnitude due to the high fluorescence differential cross section on the order of 10^{-12} – 10^{-15} cm²/sr.

Different methods for the reduction of fluorescence background are presented in the following sections.

5.2.1 NIR Raman Spectroscopy

Since the process of fluorescence requires that molecules be excited to an electronic state and since this excitation in biomolecules often requires UV or visible light, fluorescent light emission decreases over several orders of magnitude with increasing excitation wavelength. As an example, the autofluorescence of serum is shown in Fig. 5.1 for excitation wavelengths ranging from 310 nm to 450 nm. The emission of fluorescent light is seen to decrease with increasing excitation wavelength. Schrader et al.¹ pointed out this important role of the excitation wavelength for the investigation of biological samples and suggested that NIR excitation could provide a means of emphasizing Raman scattering over fluorescence emission. Unfortunately, the application of laser light in the NIR spectral range not only very effectively reduces fluorescence but also does the same for Raman scattered light emission since Raman scattering cross section is proportional to λ^{-4} .

Therefore, NIR Raman spectroscopy is particularly suited to cases in which a large Raman signal is to be expected due to the high concentration of the substance under investigation or if enhancing mechanisms are applied, particularly surface-enhanced Raman scattering (SERS). Other methods may be more suitable for the investigation of biofluids with low analyte concentrations.

5.2.2 Background Reduction by Ultrafiltration

Since fluorophores are frequently part of or attached to proteins, the depletion of large molecules by means of ultrafiltration reduces the background very effectively. For example, ultrafiltration of serum may readily be performed by centrifugation through a membrane with

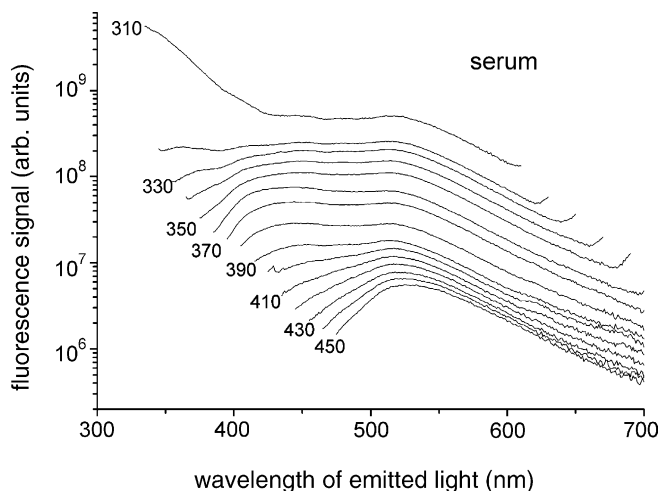


Figure 5.1. Fluorescence of serum as a function of the wavelength of the fluorescent light. The various curves are taken at different excitation wavelengths ranging from 310 to 450 nm. (Ref. 31 by permission)

a cutoff weight as low as 10 kDa, leaving an almost clear and colorless liquid.^{2,3} We used a Fluorolog 3–22 spectrometer (HORIBA Jobin Yvon GmbH) to measure the fluorescent light spectra of serum and serum ultrafiltrate at various excitation wavelengths. The results were normalized to the lamp spectrum and the sensitivity of the photomultiplier tube.

Investigation of the corresponding fluorescence maps of serum (Fig. 5.2 left) and serum ultrafiltrate (Fig. 5.2 right) reveals a significant decrease in fluorescent light emission.

In the UV region, fluorescence can thus be reduced by a factor of more than 100 using ultrafiltration. In the visible region the reduction factor ranges from 1 to 20. For example, the region of excitation at a wavelength of 500 nm and emission at a wavelength of 550 nm (corresponds to a Raman shift of about 1000 cm^{-1}) seems to be well-suited for Raman measurements of ultrafiltrate with visible light.

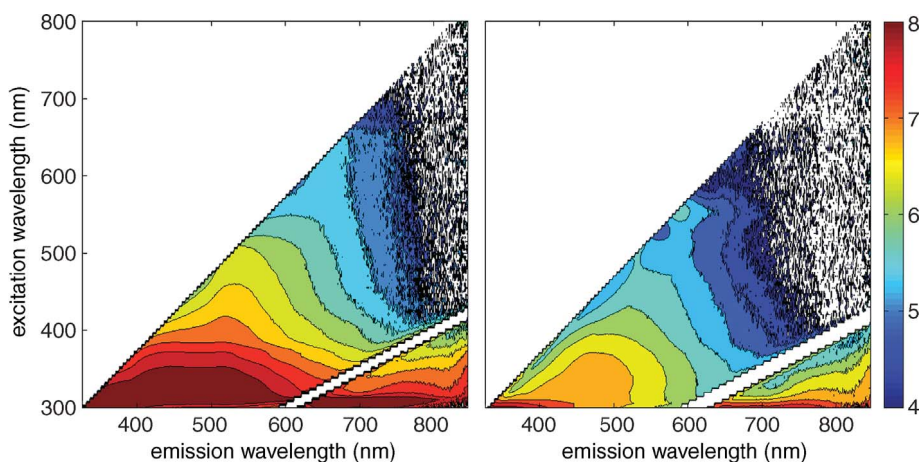


Figure 5.2. False color fluorescence map of serum before (left) and after (right) ultrafiltration using a membrane with a cutoff weight of 10 kDa. The fluorescence signal is given on a logarithmic scale ranging from 10^8 counts/s (dark red) to below 10^4 counts/s (dark blue).

5.2.3 Mathematical Reduction of Fluorescent Light Background

Apart from these physical methods of background reduction in the Raman spectrum, a useful data pretreatment for Raman spectra generally includes a mathematical subtraction of background before multivariate data analysis. For example, Lieber et al.⁴ implemented an iterative fitting function to the local minima in the given Raman spectrum using a fifth-order polynomial.

Figure 5.3 shows a Raman spectrum of serum and serum ultrafiltrate (a) without and (b) with background subtraction following the background subtraction procedure of Lieber et al.⁴

Background subtraction with a fifth-order polynomial reduces background to almost zero and leaves the leaner Raman peaks unchanged. In contrast to ultrafiltration, peaks corresponding to Raman scattering by proteins are still present in the spectra of native serum samples after the mathematical subtraction of fluorescent light background, such that the samples' protein contents can still be evaluated quantitatively in the native serum spectra. As in the case of ultrafiltration, the band at $\sim 1630\text{ cm}^{-1}$ is due to Raman scattering of water and the intensity below this peak can be used to normalize different spectra, particularly in cases in which the concentration of the remaining analytes is low.

In order to illustrate the beneficial role of mathematical background subtraction, we analyzed the spectra of human sera with and without mathematical background correction. The partial least-square (PLS) analysis resulted in a prediction accuracy for the concentration of glucose of 24.8 mg/dL [as measured by the root-mean-square error of prediction

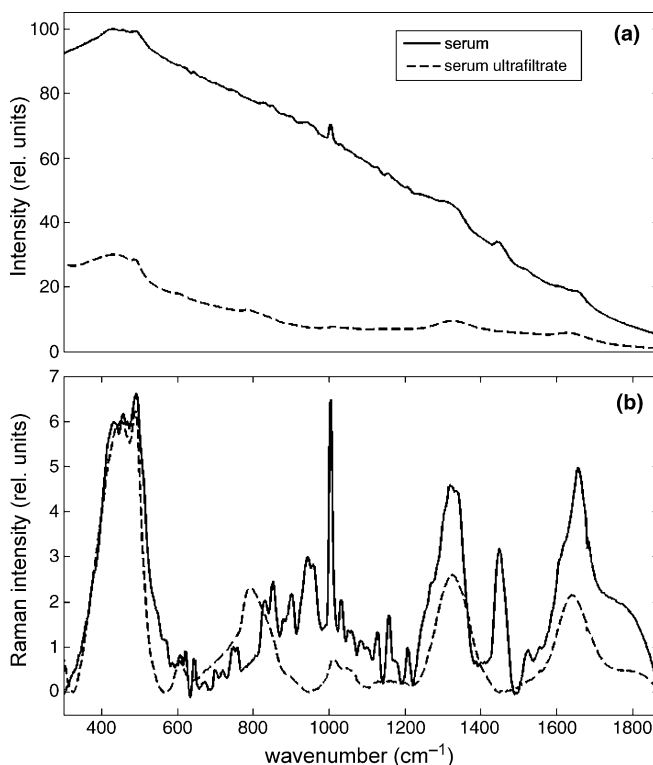


Figure 5.3. Raman spectra of serum (solid line) and serum ultrafiltrate (dashed line) (a) before and (b) after mathematical background reduction. (Adapted from Ref. 2 by permission)

(RMSEP); see Section 5.5] for the raw spectra of serum samples. This value decreased to 17.1 mg/dL after background subtraction whereby the number of parameters needed to model the data set (“latent variables”) was reduced from 15 to 10, respectively.

One fundamental drawback of the mathematical background correction, however, is the relatively high level of noise since the detector noise is proportional to the square root of the overall light intensity (i.e., including the fluorescent background). The noise is therefore high for the spectrum of native serum and cannot be reduced by subtraction of a noise-free function. In contrast, ultrafiltration reduces the fluorescent background signal and therefore reduces the total noise in the spectra. Consequently, we found that the RMSEP is reduced to 6.8 mg/dL (9 latent variables) for serum ultrafiltrate.

5.2.4 Specialized Methods of Raman Spectroscopy

Further methods of emphasizing the Raman signal over the fluorescent background are available for the analysis of biofluids, such as time-resolved Raman spectroscopy, coherent anti-Stokes Raman spectroscopy (CARS), surface-enhanced Raman spectroscopy (SERS), and UV-resonance Raman spectroscopy.

Raman scattering occurs within 10^{-12} to 10^{-15} s after the sample has been exposed to laser light, whereas fluorescence on average requires 10^{-9} s for relaxation, depending on the molecular states involved in the relaxation process. This time difference can be exploited in time-resolved Raman spectroscopy by using pulsed laser excitation with a triggered detection window that cuts off signals later than $\sim 10^{-11}$ s after excitation.

UV-resonance Raman spectroscopy benefits from the enhancement of the Raman effect when using light that matches a molecular absorption resonance. In biomedical samples it has been observed that the Raman spectra of molecules are frequently strongly enhanced such that their spectral signatures may even exceed the strong contribution from background fluorescence.

Furthermore, the enhancement of the Raman signal in the vicinity of dedicated silver- or gold-coated surfaces or particles (surface-enhanced Raman spectroscopy, SERS) has been applied to emphasize the Raman signal over the fluorescent light background in the context of biomedical samples. Finally, the observation of Raman scattering at a coherently modulated molecular ensemble (coherent anti-Stokes Raman spectroscopy, CARS) is a further option for improving the Raman-to-fluorescence ratio for the analysis of biofluids. Dou et al. have compared various Raman schemes on the example of urine analysis (see Section 5.7).

Since many of these methods require sophisticated and expensive equipment, however, we chose to focus the manuscript on “classical” Raman spectroscopy.

5.3 THE PUTATIVE DRAWBACK OF A LOW SIGNAL-TO-NOISE RATIO

Raman spectroscopy has long been considered inferior to the mid-IR spectroscopy of body fluids due to the extremely small scattering cross section and, thus, the low signal-to-noise ratio in Raman spectra. MIR spectroscopy shows an SNR of approximately 1000 within the main absorption bands, while the corresponding SNR is at least one order of magnitude smaller for the usual spectroscopy of Raman-scattered photons.

However, in the quantification of analyte concentrations, noise is not the only limiting factor for prediction accuracy: The reproducibility may also be of profound importance, since it describes how precisely the measurement instrument can repeatedly acquire

identical spectra when using the same sample and the same preparation steps. An investigation of these influences shows that the signal-to-reproducibility ratio S/R is more limiting to accuracy in MIR spectroscopy than the signal-to-noise ratio.⁵

To investigate the influence of spectral noise on the prediction accuracy of glucose in serum in the context of Raman as well as MIR spectroscopy, spectra of serum samples from 247 different donors were evaluated. Raman spectra, MIR spectra, and glucose concentrations of the 247 samples were subjected to multivariate data analysis with the goal of predicting the glucose concentration based on the spectroscopy data. The evaluation criterion for prediction accuracy has been the so-called root-mean-square error of prediction (RMSEP). Further details of the study are given in Section 5.4.

In the investigation on noise we calculated the RMSEP as a function of the SNR of the spectra. For this purpose, different levels of Gaussian noise were artificially added to the spectra prior to the multivariate data analysis. The total noise in the spectrum, consisting of the artificially added and the intrinsically present noise, was derived from the spectra by calculating

$$\sigma = \frac{1}{n} \sum_k \sqrt{\frac{1}{2b} \sum_{l=-b}^{l=b-1} [Y(k_l) - Y(k_{l+1})]^2}$$

where n is the number of points in the spectra, the binning parameter b was chosen to equal 5, and Y denotes the difference between any individual curve and the average spectrum of the 247 spectra (i.e., the spectral residuum of the overall data set). $Y(k_l) - Y(k_{l+1})$ is therefore the difference between the residua at two adjacent wavenumbers.

Figure 5.4 shows the results of this investigation. For Raman spectroscopy, the SNR amounts to ~ 80 without any additional noise and the analysis of the corresponding spectra

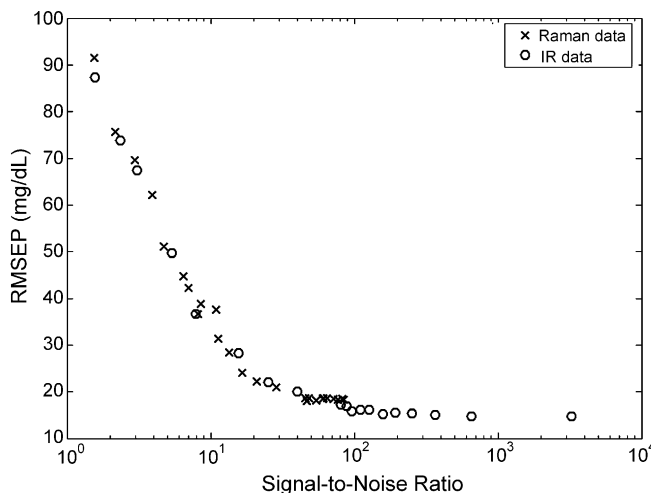


Figure 5.4. Prediction accuracy (expressed as root-mean-square error of prediction, RMSEP) as a function of the signal-to-noise ratio for (○) MIR and (×) Raman spectroscopy. The signal-to-noise ratio is artificially decreased by imposing additional noise onto the recorded raw spectra prior to analysis. The RMSEP stays constant for both spectroscopic methods when decreasing the signal-to-noise ratio down to values as low as ~ 40 , which in turn shows that the maximum accuracy is not yet limited by noise, particularly in the case of IR spectroscopy.

yields a RMSEP of 18.4 mg/dL. This RMSEP remains almost constant down to an SNR of 40 and then rapidly increases to 91.5 mg/dL at SNR = 1.5. For MIR spectroscopy, SNRs as high as 3250 are achieved in a comparative measurement. The RMSEP amounts to 14.7 mg/dL. At SNR = 96 the RMSEP amounts to 15.9 mg/dL and is increasing with decreasing SNR as fast as for Raman spectroscopy.

As a result, we experience that the advantage of high SNR in MIR spectroscopy does not inevitably induce good prediction accuracy in form of a low RMSEP. In reality, MIR and Raman spectroscopy behave almost the same when comparing different noise levels up to SNR = 100 and prediction accuracy of MIR spectroscopy does not improve significantly for higher SNRs.

5.4 SPECTROSCOPY OF BLOOD AND ITS DERIVATES

The analysis of whole blood by means of Raman spectroscopy is hampered by the high content of fluorophores, by laser-induced changes to the sample due to heating via the absorption of light by hemoglobin, by Mie scattering of light due to the blood's cellular components, and by the complexity of the Raman spectra. Nonetheless, Berger et al.⁶ succeeded in the quantification of glucose, cholesterol, triglycerides, urea, albumin, and total protein in whole blood. Enejder et al.⁷ were also able to quantify hemoglobin and haematocrit in a later study. An in-depth investigation of the hemoglobin signatures in red blood cells is described by Wood and McNaughton.⁸ The wavelength dependence of the Raman spectra of whole blood was investigated by Sato et al.⁹

Complexity, fluorescence background, heating, and in particular the background variations due to the elastic scattering of light are strongly reduced if the fluids under investigation have been depleted from the cellular components prior to spectroscopy. If one is interested in the coagulation cascade, blood plasma is an appropriate substance for investigation. Indeed, thrombotic microangiopathies form one focus of Raman spectroscopy of plasma. Popp and co-workers¹⁰ used UV-resonance Raman spectroscopy to enhance signals of the aromatic amino acids and proteins, such that spectral differences were identified between plasma samples originating from healthy volunteers and samples originating from patients suffering from thrombotic microangiopathies. Surface-enhanced Raman spectroscopy was employed to determine the glucose concentration in bovine plasma¹¹ with the long-term goal of providing means for minimally invasive, continuous glucose sensing *in vivo* (see also Section 5.6).

When whole blood or plasma samples are requested, anti-coagulants like EDTA, citrate, or heparin are usually added when drawing the blood in order to avoid coagulation. Since there is no clear preference among all of these anticoagulants in day-to-day clinical use and since the concentration of the anti-coagulant varies slightly in routine practice, it is advantageous to avoid the usage of such substances. The need for anti-coagulants is obsolete when using serum, which is obtained from whole blood by simple centrifuging. Serum is also the most simple blood derivative in terms of the complexity of vibrational spectra. A direct comparison between plasma and serum in terms of the capability of (anti-Stokes) Raman spectroscopy to quantify glucose was described in an early experiment by Dou et al.¹² Berger et al.¹³ have found an approximately threefold improvement in the quantification accuracy of analytes in serum compared to whole blood. Results in the clinically relevant range were also reported by Qu et al.³ Further Raman studies of blood and its derivatives are targeted towards thyroid-stimulating hormone¹⁴ and ischemia.^{15,16}

5.5 IN VITRO RAMAN SPECTROSCOPY OF SERUM FOR LABORATORY DIAGNOSTICS: A CASE STUDY

The spectroscopic analysis of blood or blood derivatives such as serum has the potential to serve as a standard tool in clinical laboratory analysis since it is fast and reagent-free and allows for quantification of several analytes with a single measurement.^{17,18}

In one of the largest studies on the Raman spectroscopic analysis of serum, the Raman spectra of serum samples originating from 247 donors were investigated with the aim of predicting the concentration of total protein, cholesterol, high- and low-density lipoproteins, triglycerides, glucose, urea, and uric acid.²

Blood samples were collected from 238 healthy donors and 9 persons suffering from diabetes mellitus. Serum was isolated by means of a centrifuge. The serum samples were partitioned into multiple aliquots of 3 mL each and stored at -80°C .

Glucose has frequently served as a benchmark for assessing the capabilities of biomedical vibrational spectroscopy in the context of clinical laboratory analysis. Hence, in order to cover glucose concentrations outside the physiological range, 80 randomly chosen samples were artificially spiked with glucose prior to partitioning, filtration, and storage.

One of the aliquots of each sample was used to determine the concentrations of total protein, glucose, urea, uric acid, triglycerides, cholesterol, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol by clinical chemistry (see Table 5.1).

A second aliquot was subjected to Raman spectroscopy using standard quartz cuvettes in a Holospec *f/1.8i* Raman spectrometer (Kaiser Optical Systems). For each sample, 12 spectra of 25 s each were acquired over the course of 5 min using a laser wavelength of 785 nm and a laser power of 200 mW inside the cuvette. The Stokes-shifted spectra were recorded and background corrected in the range from 300 to 1870 cm^{-1} by a fifth-order polynomial. Afterwards the wavenumber range from 300 to 1500 cm^{-1} was subjected to partial least-squares (PLS) analysis.

A third aliquot was ultrafiltrated by centrifugation through a membrane with a cutoff at 10 kDa. Raman spectra of the ultrafiltrate were collected by accumulation of five spectra of

TABLE 5.1. Mean Concentration, Maximum and Minimum Concentrations, and Standard Deviation σ for the Different Analytes Determined by the Reference Method Together with the Coefficient of Variation (%CV) Derived from Repeated Measurements

Analyte	Mean (mg/dL)	Min (mg/dL)	Max (mg/dL)	σ (mg/dL)	Test Method	%CV
Total protein	7008	6100	8100	376	Colorimetry assay	0.95
Glucose	154	42	423	103	Enzymatic UV test	1.7
Urea	31	15	56	7	Kinetic UV assay	3.4
Uric acid	5.3	2.5	9.0	1.3	Enzymatic colorimetric test	1.7
Cholesterol	133	37	392	38	CHOD-PAP	1.7
Triglycerides	198	119	338	68	GPO-PAP	1.8
HDL cholesterol	54	14	99	14	Enzymatic colorimetric test	1.85
LDL cholesterol ^a	118	47	249	34	—	—

^a LDL cholesterol was determined using the Friedewald formula.

1 min each with the above setup. Before subjection to PLS analysis, the spectra were normalized to the area under the water Raman band at 1630 cm^{-1} .

After the preprocessing of spectra, all data (laboratory data and spectra) of the 247 donors were divided randomly into a teaching set of $N_{\text{teach}} = 148$ donors' data and a set of $N_{\text{val}} = 99$ donors' data for independent validation.

Teaching the PLS algorithm was carried out on the basis of the spectra of N_{teach} samples. Afterwards, the final algorithm was applied to a blinded, independent validation set. The validation set was subsequently unblinded, and the quality of the quantitative analysis was assessed by calculating the RMSEP.

The raw spectra of serum and serum ultrafiltrate as well as the background-corrected spectrum are shown in Fig. 5.3. In the serum's spectrum the water Raman band at 1630 cm^{-1} is overlaid by the Amide I band at about 1653 cm^{-1} . The peaks at about 1000 cm^{-1} and at $829/851\text{ cm}^{-1}$ are caused by phenylalanine and tyrosine, respectively. These peaks are not found in serum ultrafiltrate since phenylalanine and tyrosine are mainly bound in proteins that are depleted in ultrafiltration.

After PLS analysis and the spectroscopy-based prediction of analyte concentrations for the independent validation set, the reference data were unblinded. In Fig. 5.5 the predicted concentrations of glucose in serum and serum ultrafiltrate are compared to the reference concentrations as determined by analytical chemistry.

A significant increase in prediction accuracy by ultrafiltration can be seen.

Results for glucose and the other analytes are expressed in terms of RMSEP and are summarized in Table 5.2.

Comparing the data given in Tables 5.1 and 5.2, the relative RMSEP typically amounts to $\sim 10\%$ of the mean concentration of the donors' data. For many of the analytes the square of Pearson's correlation coefficient R^2 is higher than or equal to 0.8. In contrast, low values of R^2 are found in those cases, in which the concentration of the analyte is very low as in the case of urea (mean concentration: 31 mg/dL). While the prediction accuracy of serum analytes like uric acid is only moderate in the native fluid, it can be improved by

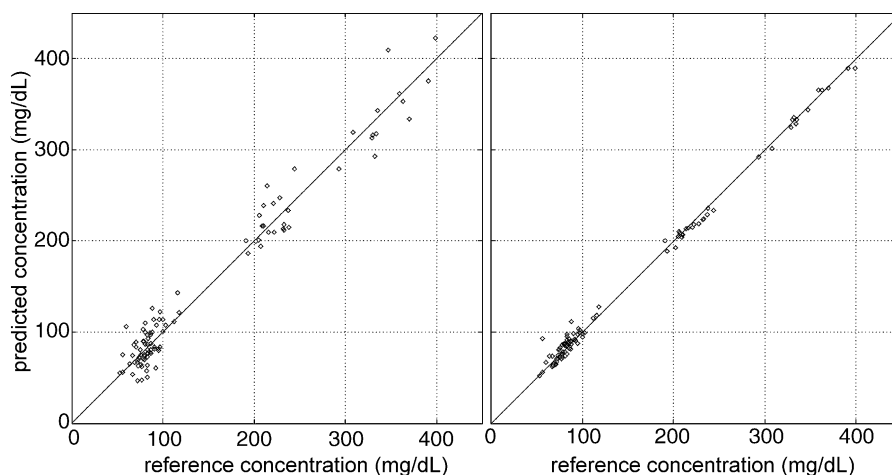


Figure 5.5. The concentration of glucose as predicted by Raman spectroscopy is plotted as a function of the reference concentration that is derived from the chemical analysis for the case of serum (**left**) and serum ultrafiltrate (**right**). The measurement was performed with the Raman laboratory setup. (From Ref. 2 by permission)

TABLE 5.2. Prediction Accuracies for Different Analytes in Serum and Serum Ultrafiltrate are Expressed in Terms of the Root-Mean-Square Error of Prediction (RMSEP)^a

Analyte	Number of LVs	Serum RMSEP (mg/dL)	R^2	Number of LVs	Ultrafiltrate RMSEP (mg/dL)	R^2
Total protein	10	176	0.803	—	—	—
Glucose	10	17.1	0.971	9	6.8	0.996
Urea	12	4.4	0.384	11	2.1	0.917
Uric acid	12	1.08	0.635	9	0.81	0.644
Cholesterol	12	11.5	0.905	—	—	—
Triglycerides	15	20.7	0.906	—	—	—
HDL cholesterol	10	11.0	0.492	—	—	—
LDL cholesterol	14	15.7	0.795	—	—	—

^aThe number of latent variables (LV) may serve as an indication for the dimensionality which is required by the PLS algorithm to model the data appropriately. R^2 denotes the square of Pearson's correlation coefficient. The spectra underlying these data were measured using the Raman laboratory setup.

ultrafiltration. Depletion of the heavy molecules by means of ultrafiltration allows for a reduction of the RMSEP by more than a factor of 2 for urea and glucose and by $\sim 30\%$ for uric acid.

In this study, we have thus demonstrated the possibility of predicting the concentrations of analytes in serum and serum ultrafiltrate by means of Raman spectroscopy with accuracies comparable to MIR or NIR absorption spectroscopy (see Table 5.3).

It is legitimate to ask the question of the minimum detectable analyte concentration in serum samples. As an initial step toward answering this question the RMSEP is shown as a function of the mean concentration in Fig. 5.6. The investigated parameters show a relative RMSEP between 3% and $\sim 20\%$ with the tendency that more abundant molecules can be

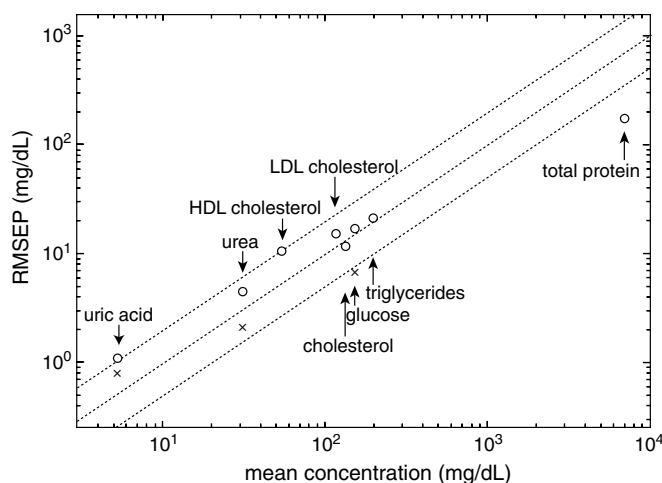


Figure 5.6. Prediction accuracies (expressed in terms of the root-mean-square error of prediction, RMSEP) for the eight analytes under investigation as a function of their average concentration in serum (○) and serum ultrafiltrate (×) measured with the laboratory Raman setup. The dashed lines indicate relative accuracies of 5%, 10%, and 20%. (Adapted from Ref. 17 by permission)

TABLE 5.3. Prediction Accuracies (in units of mg/dL) for Different Analytes in Serum by Mid-IR or Near-IR Absorption Spectroscopy for Comparison

	MIR Spectroscopy of Dried Films			NIR Transmission Spectroscopy
	Shaw et al. [19]	Petrich et al. [20]	Rohleder et al. [2]	(Hazen et al. [21])
Number of validation samples	82–103	24	99	40
Total protein	310	—	176	230
Glucose	27	16	17.1	23.3
Urea	7.2	—	4.4	1.3
Cholesterol	11.2	15	11.5	12.1
Triglycerides	23.6	13	20.7	10.4
Uric acid	—	—	1.1	—
HDL cholesterol	—	—	11.0	—
LDL cholesterol	—	—	15.7	—

quantified with a better relative accuracy and that less abundant molecules tend to deliver larger values for RMSEP. Considering the average molecular weights of the analytes under investigation, the quantification of parameters appears to be limited to concentrations above ~ 0.1 mmol/L.

Although these results cannot compete with accuracies reached by enzymatic testing in analytical chemistry, the measurement delivers an accuracy that is acceptable for many of the medical questions under investigation, whereby Raman spectroscopy offers the advantage that it is faster and reagent-free and provides a panel of parameters with a single measurement. In passing, we would like to note that even a retrospective analysis and therefore, for example, the retrospective investigation of early parameter changes in the progression of a disease may be performed since the spectra can readily be stored for later analysis.

5.6 RAMAN SPECTROSCOPY OF BODY FLUIDS *IN VIVO*

The reagent-free nature of the measurement is an important advantage of Raman spectroscopic analysis. Since the use of chemically active reagents is avoided, the technology appears advantageous with respect to studying body fluids *in vivo*. We investigated this possibility by means of a miniaturized fiber-optic sensor.

In a first step, different fiber-optic designs were simulated using the software ASAP (BRO Inc.) and the most promising and producible concept was realized.²² Miniaturization was only carried out down to an outer diameter of the sensor head of 1.5 mm to guarantee sufficient signal and to allow for relatively easy production of investigational sensor heads.

The sensor concept realized in this study consisted of a fiber bundle of seven fibers in a 6-around-1 geometry, using the inner fiber for excitation and the outer fibers for detection (see Fig. 5.7). The outer fibers were grinded under an angle of 30° with respect to the inner fibers' facets in order to produce a reflection surface oriented to the inner fiber. Thus, the analyte space is formed by the end facet of the inner fiber as well as by the sides of the outer fibers.

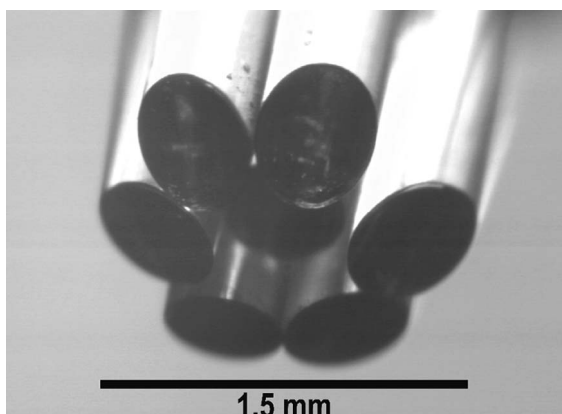


Figure 5.7. Fiber-optical sensor head used in the work toward *in vivo* sensing of body fluids. Excitation light is transported to the detection volume via the central fiber, and the six surrounding fibers serve as detection light guide. (Adapted from Ref. 22.)

After initial measurements had successfully demonstrated the capability of this scheme to quantify glucose even in the presence of other biomedically relevant compounds,²² Raman spectroscopy was applied to the same set of serum and serum ultrafiltrate samples as introduced in Section 5.5, whereby the usual sensing chamber of Holospec *f*/1.8i spectrometer system with sensor head and lens was replaced with the miniaturized fiber-optic sensor head. The accumulation of Raman spectra differed slightly from the parameter settings given in Section 5.5 since the spectra measured with the fiber-optic sensor showed a higher background. Therefore, 200 spectra of each serum sample were accumulated over the course of 1.5 s each (integration time: 5 min). Five spectra were acquired from each serum ultrafiltrate with 60 s integration time per spectrum. The analysis of the concentrations of the analytes under investigation was performed analogously to the study described in Section 5.5.

Figure 5.8 shows the results for glucose in serum (left) and serum ultrafiltrate (right). The corresponding RMSEP were 32.4 mg/dL for glucose in serum and 15.9 mg/dL in ultrafiltrate. The values for the other substances are summarized in Table 5.4.

TABLE 5.4. Prediction Accuracies for Different Analytes in Serum and Serum Ultrafiltrate Expressed in Terms of the Root-Mean-Square Error of Prediction (RMSEP).^a

Analyte	Serum			Ultrafiltrate		
	Number of LVs	RMSEP (mg/dL)	R^2	Number of LVs	RMSEP (mg/dL)	R^2
Total protein	15	190	0.786	—	—	—
Glucose	11	32.4	0.900	9	15.9	0.975
Urea	10	5.0	0.540	10	2.1	0.915
Uric acid	18	1.12	0.335	10	1.23	0.136
Cholesterol	6	20.7	0.696	—	—	—
Triglycerides	14	33.0	0.739	—	—	—
HDL cholesterol	5	12.8	0.274	—	—	—
LDL cholesterol	14	21.4	0.624	—	—	—

^a The number of latent variables (LV) may serve as an indication for the dimensionality that is required by the PLS algorithm to model the data appropriately. R^2 denotes the square of Pearson's correlation coefficient. The spectra underlying this data were measured using the fiber-optic sensor head.

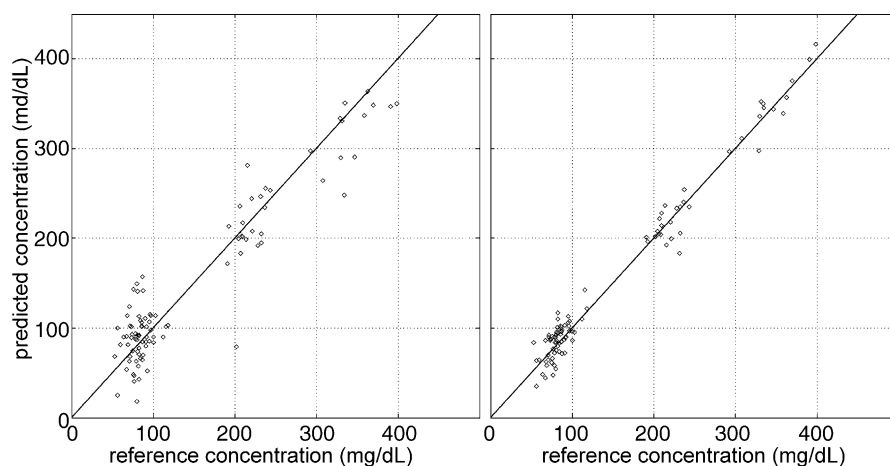


Figure 5.8. The concentration of glucose as predicted by Raman spectroscopy is plotted as a function of the reference concentration that is derived from the chemical analysis for the case of serum (left) and serum ultrafiltrate (right). The measurement was performed using the fiber-optic sensor head shown in Fig. 5.7.

The RMSEP for the different analytes is larger than the results given in Table 5.2. For glucose, the square of Pearson's correlation coefficient amounts to 0.975 after ultrafiltration and the corresponding RMSEP of about 16 mg/dL is approximately as good as is requested from commercially available, hand-held glucose meters.

Although the experiments reported here were carried out *in vitro*, the results indicate that our fiber-based, miniaturized Raman sensor may provide an appropriate concept for the reagent-free, continuous detection of the concentration of glucose *in vivo* in the subcutaneous interstitial fluid.

5.7 RAMAN SPECTROSCOPY OF OTHER BODY FLUIDS

In vivo detection of the concentration of glucose (and other metabolites) is the goal of Raman spectroscopy of aqueous humor, too, similar to the aims of the approach described in Section 5.6. Initial experiments were carried out *in vitro* by Wicksted et al.²³ Lambert et al.²⁴ found that a trade-off is conceivable between medically acceptable energies of the deposited laser light and the prediction accuracy. However, further work is needed to assess the correlation between the concentrations of glucose in blood versus aqueous humor.

In terms of clinical laboratory diagnostics, the quantification of creatinine, glucose, acetone, and urea in urine was performed by means of various methods, among which are the Raman spectroscopy using a liquid core optical fiber and the anti-Stokes Raman spectroscopy of urine samples inside a normal glass cuvette.^{25–30}

An overview of some of the results of Raman spectroscopy of urine samples is given in Table 5.5. In some of the cases, the prediction errors are much smaller than the physiological range such that the application of Raman spectroscopy to the analytical quantification of urine analytes becomes conceivable. However, it has to be noted that a single urine sample had been spiked with the analyte in question for most of the experiments listed in Table 5.5.

TABLE 5.5. Prediction Accuracies for the Quantification of Analytes in Urine Expressed in Terms of the Root-Mean-Square Error of Cross-validation (RMSECV) or the Signal Level at which the Signal-to-Noise Ratio Equals 2^a

Analyte	Physiological Range	λ (nm)	RMSECV	d.l.	Unit	Ref.
Glucose	<15	980		31	mg/dL	[25]
		514		32		[26]
		820		41		[27]
Urea	900–3000	514		20	mg/dL	[26]
		820		490		[27]
Creatinine	90–300	830	<5		mg/dL	[28]
		830	4,9			[29]
		820		150		[27]
Acetone		980		8	mg/dL	[25]
		514		1,5		[26]
		820		40		[27]
Ethanol	<25 [*]	785	3,3		mmol/L	[30]

^aThe asterisk marks the toxic level of ethanol in human serum. d.l. stands for detection level.

Spiking a single urine sample with different concentrations of the analyte constitutes a simplified case since the matrix and therefore the Raman background of all other compounds in urine stays constant. In a practical application, however, this background will strongly vary. McMurdy and Berger²⁹ have thus performed a study that is closer to commercial use since they used the unaltered urine samples of a multi-patient population. Similarly, the group of Wilson³⁰ used samples collected from 21 individuals.

5.8 SUMMARY

It has been shown that Raman spectroscopy offers the possibility to analyze body fluids such as whole blood, plasma, serum, aqueous humor, or urine in a quantitative manner by means of multivariate data analysis. Correcting for the fluorescent light background, normalization, and standardization are examples of appropriate data pretreatment. The prediction accuracies achieved are comparable to the results obtained with mid-infrared spectroscopy. Furthermore, ultrafiltration was shown to provide an appropriate means to reduce the fluorescent light background such that the prediction accuracy is even further improved for small molecules such as glucose or urea. Given these *in vitro* results, Raman spectroscopy may even have the potential to allow for the continuous quantification of analytes in body fluids *in vivo* by means of a miniaturized fiber-optic sensor head.

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VIBRATIONAL MICROSPECTROSCOPY OF CELLS AND TISSUES

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6.1 INTRODUCTION

The field of vibrational spectroscopy is advancing at a rapid pace, particularly in the areas of infrared (IR) and Raman microspectroscopy, with the last decade seeing the introduction of faster, more sensitive instruments that are capable of producing spectra of high signal-to-noise ratio. Probably the fastest growing branch of vibrational spectroscopy is in the application of biomedical diagnostics. Biospectroscopy, as it has been termed, was first investigated in the late 1980s and early 1990s. The pioneering work in this field was for the identification of bacteria,^{1–4} employing multivariate methods to differentiate between strains. These first efforts at successfully applying vibrational spectroscopy to biological systems set the stage for the study of exfoliated cells in particular exfoliated cervical cells, with the goal of developing a spectroscopic method to replace the traditional Papanicolaou smear. The premise that biochemical changes occurring in cells undergoing transformation from normal to cancerous will precede morphological changes⁵ further drove the initiative of spectroscopy as a possible tool for the diagnosis of cervical cancer.

The initial work in the application of infrared (IR) spectroscopy in the detection of cervical cancer was undertaken by Wong et al.⁶ After collecting IR spectra of exfoliated cervical cells from women with normal or dysplastic cytology several spectral differences between normal and malignant cells were noted.^{7,8} Issues arising from these initial studies will be discussed in more detail in Section 6.3.1.

Over the last decade a multitude of cancers and disease states have been investigated spectroscopically for potential diagnostic applications. These include the study of

colon,^{9–12} cervical,^{6–8,13–21} lung,^{22,23} breast,²⁴ and liver²⁵ cancers, as well as leukemia.^{26–28} Studies have also focused on spectroscopic differentiation of connective, normal, and hyperplastic glandular prostate tissue,^{29,30} normal versus cirrhotic liver lobules,²⁹ endothelial from surrounding tissue,²⁹ different tissue types that constitute the normal lining of the colon,³¹ and newly formed and old bone tissue.³² Distinction between normal, dysplastic, and neoplastic cervical tissue,^{17,21} normal and neoplastic prostate tissue,^{33–35} normal and neoplastic colon,^{12,35} gliomas and nonmalignant brain tumors,³⁶ metastatic cancer in lymph nodes,^{37,38} and a few others have been reported. A vibrational spectroscopic method has been used in clinical trials in Europe³⁹ for the *in vivo* diagnosis of esophageal cancer, precancer (dysplasia), and chronic inflammation caused by prolonged acid reflux. These methods have been validated by statistical analyses, and they have shown sensitivities and specificities around 95%.

The main motivation driving the research of vibrational spectroscopy in the biomedical field is the eventual development of instrumentation and methodology with which to serve as adjuncts to conventional cytological and histopathological diagnosis, both in the laboratory and in the operating room.

6.2 INFRARED HISTOPATHOLOGY: IR MICROSCOPIC MAPPING OF TISSUES

6.2.1 Introduction

In both histopathology and cytology, biopsies are considered to be the gold standard. However, this gold standard is not infallible. Diagnosis of disease through cytological smears and/or biopsies is a subjective method and relies on human judgment, with the pathologist diagnosing a given sample through visual pattern recognition of subjective morphologic criteria and comparison against a database stored in the pathologist's mind. As such, this leads to issues with sensitivity, specificity, and reproducibility.^{40,41} Infrared microspectroscopy (IR-MSP), on the other hand, measures the composition of cells and tissue in terms of biochemical constituents. Since IR spectroscopy monitors the inherent signature of cellular components, staining is not required and diagnosis is based on the quantitative and objective pattern recognition methods of multivariate statistics.

In order for the field of spectral diagnosis to be successful, one must keep in mind several key aspects involving the design and implementation of this work. The two most important components relate to (a) the samples chosen for the development of the diagnostic algorithm (cf. Section 6.2.3) (b) and the number of the spectra used for algorithm development.

For the development of a diagnostic algorithm based on artificial neural networks (ANN), it is imperative that the algorithm be trained on as many examples of different tumors as possible, including benign tumors. In order for diagnostic algorithms (particularly artificial neural networks) to be robust, it is essential that they be trained and validated on spectra that represent the inherent variability of biological specimens, incorporating a broad range of the disease characteristics. It is also imperative for the success of the developed algorithm that independent testing be performed, in order to objectively gauge the specificity and sensitivity.

Infrared microspectroscopy, multivariate analysis, and artificial neural networks were combined in order to test and compare with classical medical diagnostics. Using supervised methods for spectral data processing, in conjunction with supervised machine-learning

algorithms, more objective diagnostic tools can be created compared to the conventional procedures that are burdened with inherent subjectivity.

6.2.2 Infrared Substrates

The ultimate aim of IR spectral mapping is the eventual incorporation of this technique into a clinical setting for the purpose of cancer detection and diagnosis. Therefore, it is imperative that substrates be made available which are cost-effective for large-scale use, such as the case for clinical histopathology.

The development of “low e slides” (Kevley Technologies, Chesterfield, OH) offers an affordable (~US\$2 per slide) alternative to traditional IR substrates such as BaF₂ or CaF₂, which can cost more than US\$200 per substrate. The Kevley slides are of dimensions similar to those of traditional glass microscope slides and are composed of glass that is coated with a thin layer of Ag/SnO₂. The slides are chemically inert and nearly transparent to visible light, however, mid-IR radiation is almost completely reflected. Transparency under visible light enables staining of the tissue sections post IR spectral collection and allows traditional microscopic examination by histopathology.

6.2.3 Sample Preparation

The choice of samples for the development of diagnostic algorithms is crucial. Samples must cover a wide range of different stages of the disease, including their variants, as well as examples of metastatic tissue, if appropriate to the type of cancer under investigation. It is essential to obtain tissue samples from different patients for the development of robust diagnostics, and the spectral database from which the algorithm is trained must have unequivocal histopathological diagnoses; it is imperative that the errors in traditional pathological diagnoses not be transferred into the diagnostic algorithm.

For each sample, several adjacent tissue sections were cut (6 μm thick) with a microtome from paraffin-embedded tissue blocks. One tissue section was mounted on a “low e slide” for IR analysis. The other tissue sections were mounted on regular glass microscope slides for histopathological diagnosis. After mounting, tissue sections were deparaffinized and rehydrated by a standard washing procedure, as described previously.¹⁸ One of the tissue sections was stained with hematoxylin/eosin (H&E) for an initial pathological examination. This examination formed the basis for the initial mapping of the tissue section, as the histopathologist marked regions of abnormality on the slide. Given that a tissue section, particularly from the thyroid lobe, can be as large as 3 cm in length, preliminary tissue sections were only mapped from the regions of interest. This facilitates faster cluster analysis and drastically reduces the time required for spectral data collection. This also enabled the collection of a larger number of different tissue sections.

6.2.4 Instrumentation and Data Acquisition

Infrared spectral maps of tissue sections were collected via a Perkin–Elmer Spectrum One/Spotlight 300 Infrared Spectrometer (Perkin–Elmer Corp., Sheldon, CT). This fully integrated IR microspectrometer incorporates a 16 × 1 element (400 μm × 25 μm) mercury cadmium telluride (MCT) array detector for mapping applications. The instrument provides 1 : 1 or 4 : 1 mapping on the detector, resulting in a nominal resolution of 25 or 6.25 μm, respectively. Visual image collection via a charge-coupled device (CCD) camera is

completely integrated with the microscope stage motion and IR spectral data acquisition. The visible images are collected under white light LED illumination, and they are “quilted” together to give pictures of arbitrary size and aspect ratio. The desired regions for the IR maps are selected from these visual images.

Tissue sections were mapped at 25 μm spatial resolution and either 4 or 8 cm^{-1} spectral resolution. IR spectral maps were collected in “transflection,” where the beam passes through the sample, is reflected by the IR substrate, and passes through the sample again before being collected by the detector.

After the necessary spectral maps were collected from the tissue section, it was stained with H&E. This ensured that the histopathological examination and diagnosis of the tissue and the cluster maps were from the same tissue section.

An IR spectral map, also known as a spectral hypercube, for a tissue section measuring 5 mm $\times$ 5 mm consists of $\sim 40,000$ individual IR spectra. At 25 μm spatial resolution and 8 cm^{-1} spectral resolution, data collection time is approximately 14 min.

6.2.5 IR Mapping: Spectral Preprocessing and Analysis Methods

The output of spectral mapping, or imaging if a focal plane array detector is employed, is a hyperspectral image or hypercube. Hyperspectral images contain a vast amount of data and are best visualized in three dimensions. In the X – Y plane, the hypercube gives the spatial information. For each pixel in this plane, there exists a complete spectrum (the third dimension), which reflects the chemical composition of the sample in question.

Spectral hypercubes were imported into CytospecTM software (www.cytospec.com) as binary FSM (the native Perkin–Elmer spectral map) files. Spectra were converted from transmittance into absorbance, followed by a quality test to remove spectra from the map where there was no or very thin sample. In order to baseline correct and enhance the subtle differences between the spectra of tissues, a Savitzky–Golay second derivative with a 13 point smoothing window was applied to the hypercube. The spectral region was reduced to 1800–950 cm^{-1} , and the data were vector-normalized to eradicate spectral differences due to sample thickness.

Due to the volume and complexity of IR and Raman spectra and maps, it is necessary to employ objective, multivariate methods to analyze the data and draw conclusions about variability and trends.

6.2.5.1 Hierarchical Cluster Analysis. Following data preprocessing the spectral hypercubes, representing the IR spectral information of the tissue sections, were classified via hierarchical cluster analysis (HCA). The necessity of this step is obviated by the size of the spectral maps collected, usually on the order of 40,000 IR spectra per map. Because it imparts a level of subjectivity, it is impossible and undesirable to analyze these spectra visually. The spectral differences between normal and diseased tissues are often so subtle that they cannot be differentiated by eye, unless second derivatives are employed to enhance the differences. HCA is sensitive to these subtle differences, and the results discussed in Section 6.2.6 indicate that there is a strong correlation of clusters based on tissue type and disease state, as confirmed by histopathology. This provides strong evidence that the observed spectral changes are the result of biochemical differences between tissue types and disease states. The strength of IR-MSP coupled with HCA has also been previously demonstrated in the diagnosis of many disease states including colon carcinoma,³¹ cervical cancer,²¹ and scrapie.⁴²

Hierarchical cluster analysis is an unsupervised multivariate statistical method. Unsupervised analysis methods require no initial input as to the nature of the data set.

D-values clustering algorithm and Ward's linkage algorithm enabled a uniform basis of hierarchical cluster analysis^{5,43} for all maps. The decision on how many clusters were necessary to accurately describe the tissue section was determined such that the HCA pseudo-color map matched the histopathological diagnosis of the stained slide. The output of HCA is a pseudo-color map, where each pixel represents one IR spectrum. Pixels of the same color indicate spectra that exhibit the most similarity to one another.

Although HCA is an excellent method for preliminary identification of abnormalities in cells and tissues, because it is computationally demanding and the results are nontransferable, it is an unsuitable method for analyzing and diagnosing an unknown data set. HCA is based on the similarity of spectra within a given data set, and it is difficult to apply absolutely on unknown data. Therefore, HCA is employed for determining the clusters of spectra to be used to train a supervised diagnostic algorithm, such as an ANN. The development of successful ANNs in the pharmaceutical and biospectroscopy fields based on analyses of IR spectra have been reported.^{44–46}

6.2.5.2 Artificial Neural Networks. Single data files from each cluster were exported in DAT file format and served as inputs for subsequent artificial neural network analysis.

The inputs for the neural network discussed in Section 6.2.6 were derived from a random selection of IR spectra from clusters identified by histopathological correlation as being representative of breast metastatic carcinoma, as well as representative spectra from normal tissue types present in lymph nodes. The outputs of the network were a desired diagnosis and tissue type, corresponding to the different clusters or classes used for training.

Artificial neural network classification and feature selection were performed with NeuroDeveloper 2.5 (Synthon GmbH, Heidelberg, Germany). Data for each lymph node tissue type were split into three sets. The training data set was used to establish network parameters that provided the best possible classification. The validation data set was used to optimize generalization performance of the network. The test data set served to confirm that a given network had sufficiently broad generalization power to serve reliably as a diagnostic algorithm. Depending on the number of available spectra, the general scheme was to have one-fifth of the data pool used for training and validation (split 80% and 20%, respectively), with the remaining spectra used for testing purposes.

The application of a spectral feature selection algorithm reduced the complexity and dimensionality of the classifier. This also improved the quality of the classification model and removed the high redundancy of information across the entire spectral range. Spectral feature selection was based on the calculation of the covariance of the spectral data points. Ranked in descending order according to the covariance measurement, the best selected features were used as inputs for the ANN classifier. Generally, the 120 data points with the highest covariance were made available for the neural network.

Three-layer, feed-forward networks with 5–120 input neurons, 4–20 hidden nodes, and 4–7 output nodes were tested. Resilient back-propagation (Rprop)⁴⁷ was used as the learning algorithm. Tested Rprop parameters were in the following range: $\Delta_0 = 0.075$ – 0.1 , $\Delta_{\max} = 30$ – 50 , and $\alpha = 4$ – 5 . The training process was stopped when errors of training and validation data sets converged.

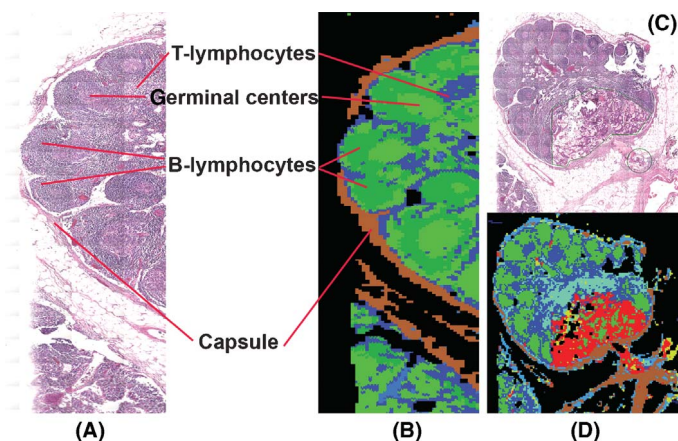


Figure 6.1. (A) Visual image of an H&E-stained lymph node section (without metastasis). (B) resulting pseudo-color map. (C) H&E-stained tissue section of an entire lymph node, cancerous areas are indicated in green. (D) Resulting pseudo-color map.

6.2.6 Results and Discussion

It is important that a diagnostic technique is not only able to identify a malignancy, but is also able to identify the type of malignancy. Many cancers are asymptomatic, and often the first indication that a problem exists is when a lymph node is infiltrated. Figure 6.1 illustrates the sensitivity of IR-MSP and multivariate statistics through the ability of this technique to reproduce tissue architecture and morphology. In panel A an H&E-stained section of a lymph node is shown. We can clearly see the secondary follicles containing germinal centers, and the fibrous capsule and some fatty tissue surrounding the node. Panel B is the pseudo-color map generated from the section in panel A (the section was stained after IR data collection). The spectral clusters clearly show the architecture of the lymph node, reproducing the morphology of the secondary follicles containing the germinal centers. In fact, IR-MSP is such a sensitive technique that it is able to differentiate proliferating B-lymphocytes (germinal center, light green) from nonactive B-lymphocytes (dark green). The capsule is shown in brown and the T-lymphocytes are shown in blue. Panel C shows a stained visual image of a lymph node containing metastatic colon adenocarcinoma, circled in green. The corresponding eight cluster pseudo-color map (panel D) clearly shows the differentiation of colon metastases (red). The node capsule and connective tissue are shown in brown, and B-lymphocytes are represented by the green clusters.

Figure 6.2, panels A and B illustrate another example of the detection of metastatic colon cancer in lymph nodes. Panel A shows the stained visual image of a lymph node containing metastatic colon adenocarcinoma, circled in black. The resulting pseudo-color map (panel B) clearly differentiates the adenocarcinoma (red and burnt orange). The differentiation of this metastases into two distinct clusters again demonstrates the sensitivity of this technique, since we are able to discriminate between the glandular (red) and stromal (burnt orange) components of the cancer. Panels C and D in Fig. 6.2 give an example of an axillary lymph node containing a large metastatic breast cancer infiltration, seen as pink staining in the upper half of the image in panel C. The corresponding pseudo-color map (panel D) reproduces the node architecture and discriminates the breast metastasis from the normal lymph node tissue, with further differentiation of the metastatic tumor into the stromal (burnt orange) and glandular (red) components.

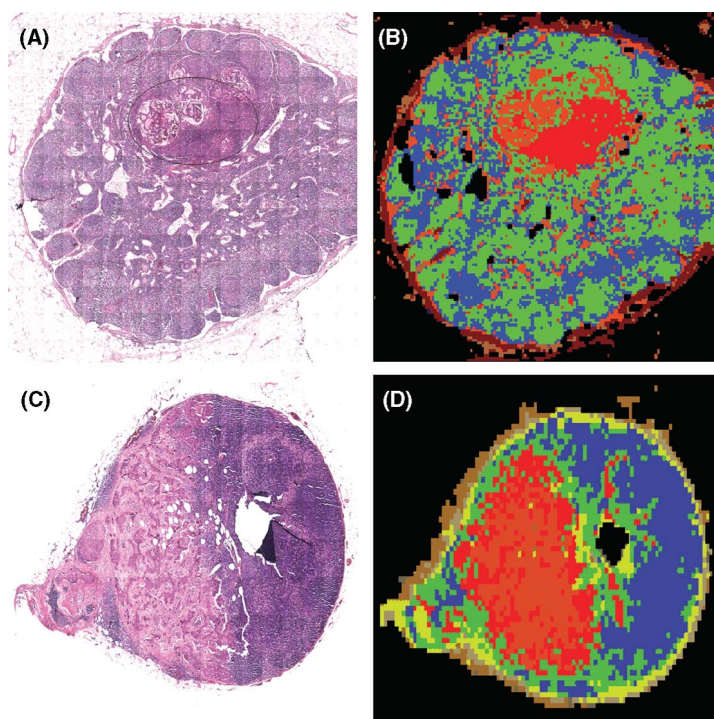


Figure 6.2. (A) Visual image and (B) corresponding pseudo-color map of a lymph node with metastatic colon adenocarcinoma. (C) Visual image and (D) corresponding pseudo-color map of an axillary lymph node with metastatic breast cancer.

The IR spectral map resulting from the breast axillary lymph node shown in Fig. 6.2, panel D, was used to develop an ANN for the detection of metastatic breast cancer in lymph nodes. The total number of spectra in this spectral map was 16,045. These spectra were divided via HCA and histopathology into four classes: fat and capsule (1765), cancer (6255), macrophages (3315), and lymphocytes (4710). The resulting diagnostic algorithm, Fig. 6.3, shows a strong correlation with both the pseudo-color map and the histopathological diagnosis. Ninety-five percent of all spectra were assigned to a given class membership; and of this, 93% were assigned to the correct class.

The real test of the diagnostic power of an ANN involves the testing of the developed network on spectra that were not involved in the training and validation steps. The diagnostic ANN discussed previously was applied to lymph node sections, also containing breast metastatic cancer, previously unseen by the network. Before testing, the algorithm was modified slightly and retrained on the original spectra to give only two output classes: The three normal classes (fat and capsule, macrophages, and lymphocytes) were grouped as one output, resulting in an algorithm that gives a diagnosis as either normal or abnormal. Panels A and C in Fig. 6.4 display the resulting ANN images of two lymph node tissue sections containing breast metastases. Red pixels represent malignant cells, and normal tissue is shown in blue. Panels B and D show the H&E tissue sections for comparison. Cancerous cells exhibit a different staining pattern to normal cells and is one of the diagnostic features of histopathology, along with nuclear/cytoplasm ratio and morphological features. Cancerous cells tend to stain a lighter color than normal tissue, and this is

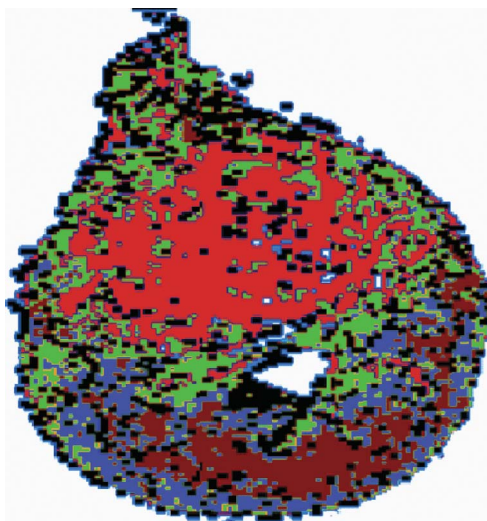


Figure 6.3. ANN diagnostic image showing breast metastasis (red).

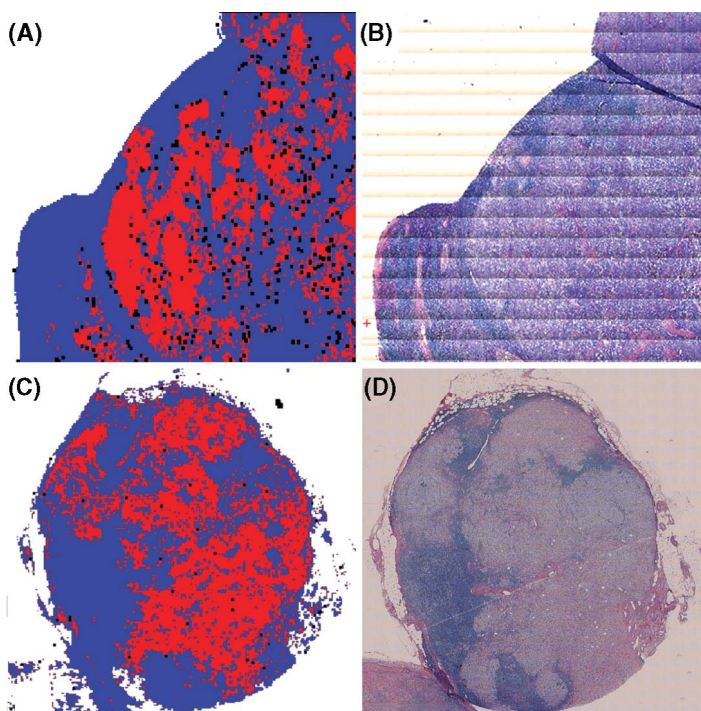


Figure 6.4. (A, C) ANN diagnostic images of lymph node tissue infiltrated with metastatic breast carcinoma (red). (B, D) Corresponding H&E-stained tissue sections shown to give an indication of the correlation between the ANN and histopathology.

manifested in panels B and D as pink. Normal cells tend to stain a darker color, observed here as purple/blue. There is a very strong correlation between the diagnostic algorithm and the histopathological diagnosis. With this approach, it is difficult to give an indication of the specificity and sensitivity of the algorithm, which would require diagnosis of every pixel. This work continues as part of a major collaboration with the research group of J. Rieppo in Finland, who have recorded IR spectral maps of over 500 tissue sections containing breast carcinoma.

IR-MSP has been successfully applied to the diagnosis of various types of thyroid carcinoma both in the thyroid gland and as metastases in lymph nodes. In the late 1990s and early 2000s, fine needle aspirate (FNA) samples from patients presenting with thyroid neoplasms were analyzed using IR-MSP and multivariate statistical analyses such as HCA and linear discriminant analysis (LDA).^{48–50} LDA was able to differentiate malignant from normal IR spectra taken from cell pellets and FNA fluid. Infrared maps of tissue sections from thyroid samples were also collected during these studies, and univariate methods were employed to show differentiation between malignant and normal tissues.

Another study⁵¹ investigating IR spectroscopy of thyroid tissue biopsies also reported reproducible spectral differences between the IR spectra of follicular adenoma (a benign lesion) and normal thyroid tissue. This study employed correlation chemical maps to highlight these differences. The results presented in this section incorporate the use of multivariate analytical and diagnostic tools for the differentiation of benign and malignant changes occurring in the thyroid.

The lymph node shown in Fig. 6.5 is completely dominated with metastatic thyroid tumor, with only small areas of normal lymph node tissue observed along the bottom and right section of the node. Within the metastatic region of the section in panel A, we can clearly observe what are known as cystic cavities. The metastatic thyroid cells try to reproduce the morphology of the thyroid follicles, resulting in the appearance of these cystic cavities. The red box in panel A, was drawn by the histopathologist to indicate where the IR spectral map should be collected. Panel B illustrates the enlarged region from which the IR spectral map was obtained.

Panel C shows the resultant pseudo-color map from HCA. Only three clusters were needed to differentiate the metastatic tissue from the normal lymph node tissue. The assignments of the clusters are as follows: The red cluster represents metastatic thyroid tumor; the green and blue clusters represent normal lymph tissue. The separation of the normal lymph tissue into two clusters indicates the detection and differentiation of

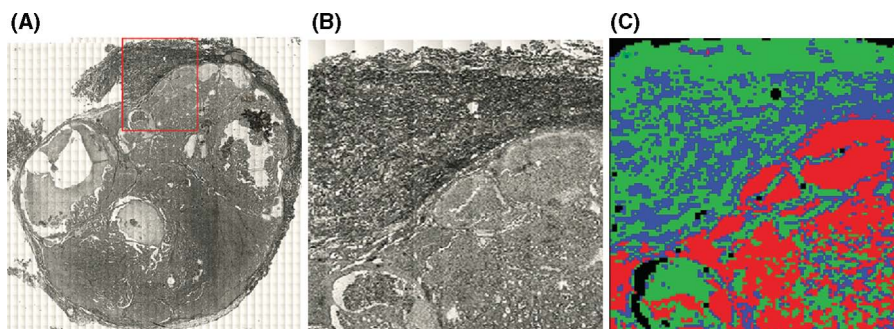


Figure 6.5. (A) Visual image of unstained lymph node; red box indicates sampling area. (B) Expanded visual image of the sampling area. (C) Pseudo-color map of resulting IR image, three clusters.

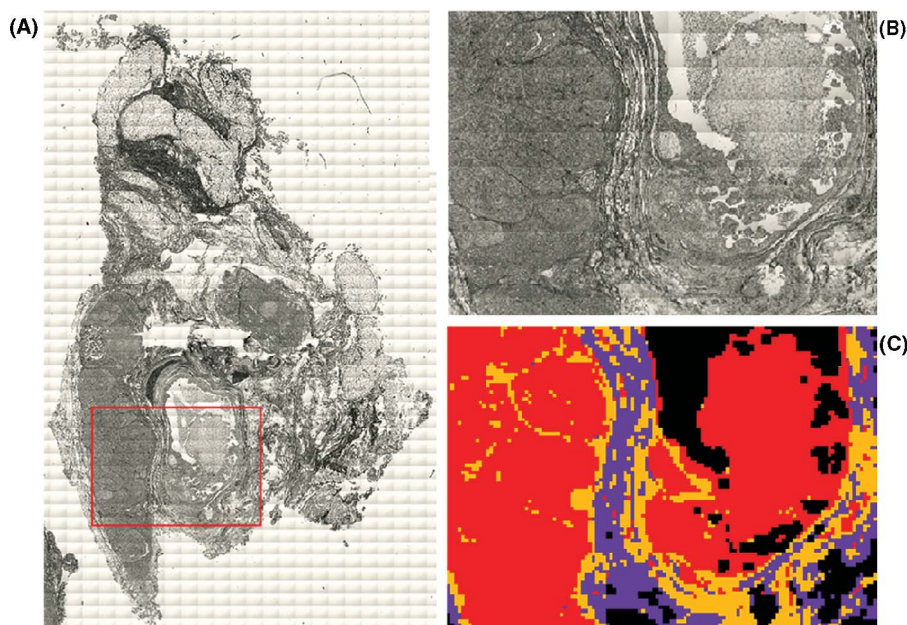


Figure 6.6. (A) Visual image of unstained lymph node, red box indicates sampling area. (B) Expanded visual image of red box. (C) Pseudo-color map of resulting IR image, three clusters.

B-lymphocytes and T-lymphocytes, once again demonstrating the discriminating power and sensitivity of IR-MSP and multivariate statistics.

Figure 6.6 illustrates a lymph node infiltrated with metastatic thyroid tumor, indicated by the cystic cavity seen in the right half of the red box. The red box in panel A indicates the area that was sampled for IR-MSP. Panel B illustrates the enlarged section of this region. The resulting three-cluster pseudo-color map is shown in panel C.

The results of the pseudo-color map for this lymph node present an important finding for the advantages of IR-MSP for optical diagnosis and eventual implementation into the medical community. When this cluster map was initially presented to a histopathologist for correlation with histopathological results, the initial comments were that IR microspectroscopy was not able to differentiate the metastatic thyroid tumor on the right and the normal lymph tissue of the left (panel B). Curious as to the reason why this occurred, the histopathologist reexamined the adjacent H&E stained tissue section of this node. This more detailed inspection showed that the left side of panel B was actually metastatic thyroid tumor. This lymph node shows two patterns of metastatic tumor: aggregated type on the right (where it attempts to mimic thyroid structure) and disaggregated type with individual tumor cells on the left. With this in mind, the cluster assignments for the pseudo-color map in panel C are as follows: The red cluster represents metastatic thyroid tumor, the purple cluster represents fibrosis, and the orange cluster represents secretions from the cystic cavity.

This example demonstrates that IR-MSP detects chemical variations between normal and cancerous cells, even if no architectural changes are exhibited. IR-MSP was able to differentiate cancer even though the histopathologist was initially unable to detect it.

We turn now to a discussion of the preliminary results from IR spectral mapping of thin tissue sections of thyroid glands. The four spectral maps, represented in Fig. 6.7 as pseudo-color maps, were taken from four different thyroid glands each exhibiting a different

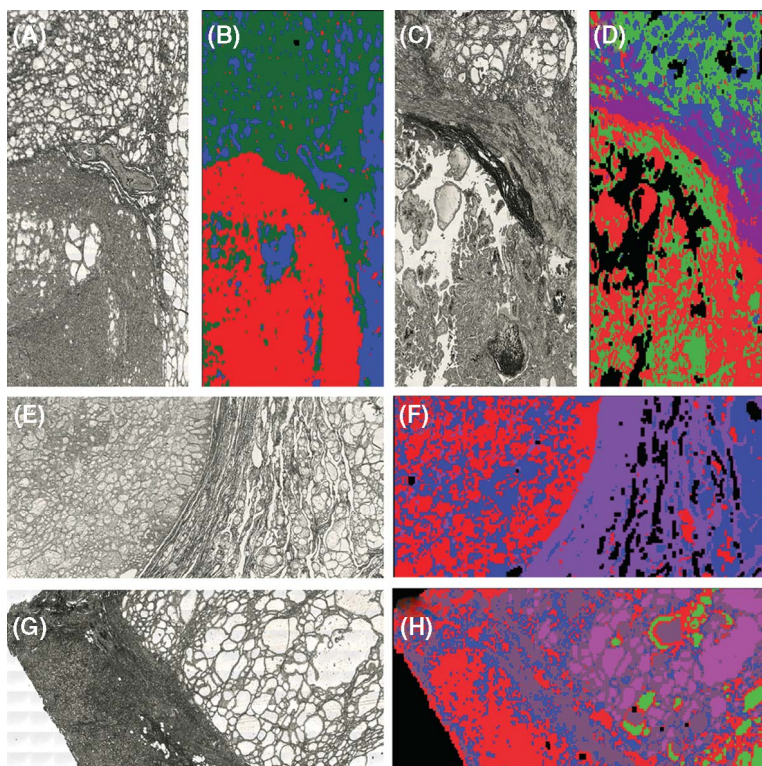


Figure 6.7. (A) Visual image and (B) pseudo-color map showing Hürthle cell adenoma. (C) Visual image and (D) pseudo-color map showing PTC. (E) Visual image and (F) pseudo-color map showing FVPTC. (G) Visual image and (H) pseudo-color map showing anaplastic thyroid carcinoma.

type of thyroid cancer. In each pseudo-color map the cancer is given in red, purple represents fibrosis, and the other colors normal thyroid tissue. The pseudo-color maps show (from left to right) Hürthle cell adenoma, papillary thyroid carcinoma (PTC), follicular variant papillary thyroid carcinoma (FVPTC), and anaplastic thyroid carcinoma.

The cluster maps presented in this section are evidence that IR-MSP and HCA are sensitive methods for the differentiation of thyroid tumors in thyroid glands and in lymph nodes. However, it is critical that we can utilize the sensitivity of IR-MSP to determine if there are significant spectral differences between the different types of thyroid benign and malignant nodules.

Figure 6.8 shows the mean cluster, second derivative spectra from six tissue sections and clearly shows the spectral differences that exist between the different types of thyroid carcinoma.

The IR spectra were carefully examined for the presence of dispersion artifacts (*vide infra*) by plotting the frequency maps of the amide I band. Spectra exhibiting a strong dispersive component gave rise to second-derivative spectra in which the intensities of the derivative bands at 1700 cm^{-1} and 1650 cm^{-1} were almost identical. The ratio of these bands of the spectra presented here do not exhibit this pattern; therefore we are convinced that the shifts in the amide I band are real and in fact due to different biochemical processes occurring in the cells as a function of cancer. These subtle differences are currently being

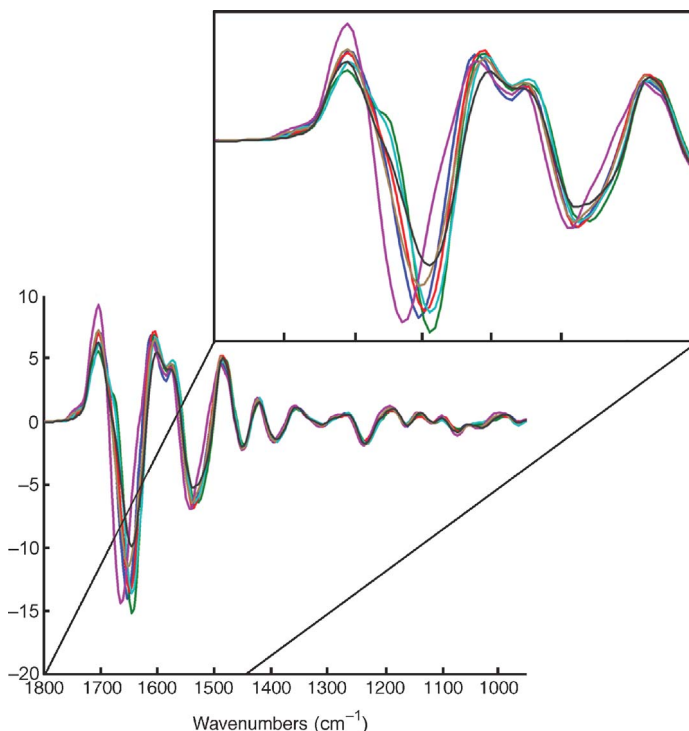


Figure 6.8. Mean cluster spectra of thyroid cancer from six different tissue sections. Blue denotes metastatic FVPTC in lymph node, green denotes metastatic PTC in lymph node, magenta denotes

exploited for the development of ANN algorithms for the detection and identification of thyroid carcinoma.

6.2.6.1 Correcting the Dispersion Artifact. IR-MSP spectra are sometimes contaminated by a dispersion artifact. The causes of this artifact and our attempts to address the problem have been detailed previously.^{38,52} One way to reduce the impact of the dispersion artifact is simply to restrict the spectral range and omit the region in which the artifact is the strongest. The pseudo-color maps in Figs. 6.1 and 6.2, for example, were generated from the spectral region 1580–950 cm^{-1} . By removing the amide I band from the spectral map, the correlation matrix is not dominated by the amide I band shift characteristic of the artifact and therefore the clusters formed result from biochemical changes within the cells rather than being a reflection of the degree of dispersion present in the spectral map.

The presence of this artifact in IR spectra is undesirable due to the peak shifts and changing spectral band ratios overriding the subtle spectral changes that occur within biological samples such as tissue and cells. In spectral maps heavily dominated by the presence of dispersion artifacts, the amide I band can be shifted by as much as 30 wavenumbers. Cluster analysis, which bases the assignment of spectra into a given class based on the correlation between the individual spectra within the hypercube, will be dominated by this shift in the amide I band. Clusters will likely be based on this shift, and to a lesser extent the amide I/II intensity ratio, rather than on the subtle differences between

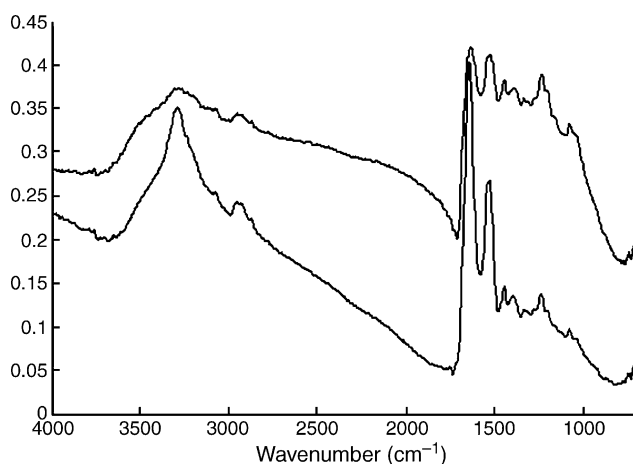


Figure 6.9. (Upper) IR spectra exhibiting a strong dispersion artifact. (Lower) IR spectrum corrected with spectral un-mixing.

normal and diseased cells that arise due the biochemical processes of cancer mechanisms and proliferation. Thus, the removal of this artifact is of prime importance for the analysis of spectral data.

Presently we are investigating methods to reduce the dispersion artifact by a technique known as spectral un-mixing.⁵³ Spectral un-mixing searches for the pure component spectrum in a spectral map or hypercube. The spectrum corresponding to the dispersive component is then subtracted from all the spectra in the hypercube, thus removing the artifact. Preliminary results of this un-mixing are given in Fig. 6.9, which shows a representative example of uncorrected and corrected spectra. The upper IR spectrum shows a strong dispersive component. This is reflected in the position of the amide I band (1633 cm^{-1}), the amide I/II band ratio, and the sharp dip around 1750 cm^{-1} . The lower IR spectrum resulted from spectral un-mixing. Note the position of the amide I band (1646 cm^{-1}) and the corrected amide I/II band ratio. The spectral un-mixing algorithm was tested on a small section of an IR spectral map, and the resulting cluster maps (not shown) give more distinct clusters and better correlation with the architectural morphology of the tissue. It is believed that this algorithm can be routinely applied to IR spectral maps as a means of overcoming the dispersion artifact.

6.3 VIBRATIONAL CYTOLOGY: IR AND RAMAN SPECTROSCOPY OF EUKARYOTIC CELLS

6.3.1 Introduction

The application of IR-MSP to cytology has seen less progress than for that of histopathology. This is due to several reasons. First of all, the inherent heterogeneity of biological samples and particularly single cells makes it difficult to develop a methodology without first understanding the nature of this variability. Second, it is often difficult to obtain cellular specimens from hospitals; IRB committees are more lenient in granting approval for the use of archived tissue sections, but less willing to approve studies involving exfoliated cells or thin needles aspirates. Furthermore, oftentimes the sample treatment used in standard

cytology, such as the use of ThinPrep solution for the processing of cervical smears, renders the sample unsuitable for spectroscopic analysis as methanol alters the structure of the proteins, manifested as a splitting of the amide I band. Third, it is essential to collaborate with a team of cytologists. In order to develop diagnostic algorithms for cytology, it is necessary to obtain an accurate diagnosis of all cells, both in terms of cell type and disease state, in the training, validation, and testing phases of algorithm development. Given that hundreds and ideally thousands of cells are required for development of a robust algorithm, individual diagnosis becomes tedious, time-consuming, and beyond the expertise of most spectroscopists. Another alternative is to develop a diagnostic algorithm based on tissue sections and apply these to cytological samples. However, this approach remains in the development stage in several laboratories, so no comment can be made at this time as to the success of this approach.

IR-MSP is a powerful technique that has the potential for automatic and objective diagnosis of individual cells. However, before this technique can be routinely applied to cytology, one must first understand the underlying nature of biological heterogeneity and spectral variance exhibited by individual cells.

The initial IR cytological work was undertaken in the early 1990s for the study of human cervical cells, as discussed in Section 6.1. This sample type does not readily lend itself to the understanding of spectral variability in normal and diseased cells because cervical smears are often contaminated by erythrocytes, lymphatic cells, cervical mucosa, and bacteria.^{17,19,54}

The initial experiments involved in development of the spectroscopic “Pap” smear were inherently flawed due to the use of cell pellets rather than individual cells. The subtle differences between individual cell types were averaged, and important information was lost. The spectral differences observed with IR spectroscopy could only be attributed to large changes in the total sample, rather than changes occurring as a result of the few abnormal cells generally present in cervical smears.⁵⁵ With the advent of faster and more sensitive spectrometers, however, it has become possible to investigate biological specimens on an individual cell basis.

The discrimination between the infrared spectra of normal and malignant cervical cells reported by Wong et al.^{6,7} was not ideal because results were based on visual inspection of the spectra and the use of peak ratio comparison. Visual inspection of IR spectra introduces subjective bias, and the technique of peak ratio measurements is insensitive to interference from extraneous factors and subtle differences between spectra.¹⁵ Differences in the thickness of the sample can also contribute to peak ratio bias; and if these are to be used as criteria for discrimination, sample thickness must be compensated for.⁵⁵

Due to the inherent variability in biological samples discrimination between IR spectra of cervical specimens requires the use of robust and sensitive methods. These methods must be able to model for nonlinearities arising from various sources including sample processing errors, baseline shifts, patient-to-patient variations, and the presence of nondiagnostic debris.¹⁵ Methods also need to be sensitive to the presence of “outlier” spectra which may result from specimens containing blood, mucus, or other nondiagnostic debris.

Infrared spectroscopy has been coupled to various multivariate statistical techniques to create effective models and classification tools in the investigation of cervical cancer.^{14–16,54} However, the discrimination of normal and malignant cells in these early studies, based solely on the glycogen region, was of questionable value given the inherent amount of variation seen in the glycogen levels of normal squamous epithelium.^{17,56}

The cytological work undertaken in this laboratory is focused on understanding the heterogeneity within cells and the associated spectral variability. We have applied both IR

and Raman microscopy for the investigation of the spectral changes associated with cells during the cell division cycle. Exfoliated cells, which are generally quiescent, were investigated to understand the spectral effects of nuclear to cytoplasm ratio and nuclear size. We believe that these factors are the main contributors to spectral variability within a given cell type.

Our other spectral cytology projects are centered around lymphoma and bladder cancer diagnosis.

While it is unlikely that Raman spectroscopy will be applied routinely for cytological diagnosis, the higher spatial resolution and confocality of Raman instruments enable biospectroscopists to investigate cellular processes in normal and diseased cells on a subcellular level.

6.3.2 Sample Preparation

This chapter will concentrate solely on our research involving spectral investigations of single, exfoliated cells or cultured cells. The initial drive for the study of cultured cells arose from the need for an objective method for the diagnosis of cervical cancer, discussed previously. Present screening methods for cervical cancer are wrought with inaccuracies. It is difficult to acquire cervical smears, the nature of the sample is extremely heterogeneous, and correlation with cytology is very difficult. Instead, we have concentrated on the investigation of readily available cultured and exfoliated cells with which to determine and understand the spectral variance of biological heterogeneity.

6.3.2.1 Cultured Cells. For cell culture studies we utilized HeLa cells, a commercially available (ATCC, Manhasset, VA) adherent cell line (CCI-2) of cervical adenocarcinoma cells. These cells were grown in 75 cm³ culture flasks (Fisher Scientific) or directly onto sterile substrates (either CaF₂ or “low e slides”) with 20 mL of Dulbecco’s Modified Eagle’s medium (DMEM, ATCC) and 10% Fetal Bovine Serum (FBS, ATCC). To prevent contamination from bacteria, 2.5 µg/mL of amphotericin B (ATCC) and 100 IU/mL penicillin/streptomycin (ATCC) were added to the medium. Cells were then incubated at 37 °C and 5% CO₂. It is advantageous to grow cells directly onto the substrate because it circumvents the treatment of cells with trypsin, which significantly changes the morphology of the cells and introduces spectral artifacts while enabling controlled cell synchronization.

In order to track the spectral changes of the cell cycle, it was necessary to synchronize the cells. This was performed using a technique known as mitotic shake-off, which takes advantage of the fact that during mitosis adherent cells round up and lose their attachment to the substrate and can be removed from the culture flask wall. HeLa cells were grown to 50% confluence in culture flasks as discussed previously; the culture medium was removed and 20 mL of phosphate buffered solution (PBS) was added to the flask. The flask was then gently vortexed for 45 s to remove poorly adherent and dead cells. The PBS was discarded and replaced with 15 mL of DMEM/10% FBS and incubated for 1 h. The flasks were again vortexed for 45 s the medium was collected, and centrifuged (5 min, 600 g), and the resultant cellular pellet was resuspended in 1.5 mL of fresh medium.

The cellular suspension was then pipetted into 100 mm × 100 mm sterile Petri dishes (Fisher Scientific) containing sterile “low e slides,” growth medium, and antibiotics, as described previously.

In order to correlate IR spectra with cell cycle events, it was necessary to apply immunohistochemical staining to the cells. The specific stains and protocols have been

described previously.⁵⁷ Slides were removed from the incubator, hourly, over a time course of 30 h to capture cells throughout the entire cell division cycle.

Once a slide was removed from the incubator, it was formalin-fixed, rinsed in PBS, buffered saline solution (BSS), deionized water, and then air-dried. IR spectra were collected from pairs of cells in close proximity to one another, indicating a mitotic cell that had survived the shake-off procedure and had undergone cell division. The spectral aperture was chosen to straddle the entire cell, and a second spectrum was collected over the area of the nucleus.

After spectral data were obtained, fluorescence microscopy was utilized in order to determine the cell cycle phase of each of the slides over the time course.

6.3.2.2 Exfoliated Cells: Oral Mucosa Cells. Readily accessible, normal squamous epithelial cells were required in order to establish spectral variability: Oral mucosa cells and canine cervical cells were utilized for this study. Oral mucosa (buccal) cells were harvested from volunteer graduate and undergraduate students in the department. Prior to sample collection, the mouth was rinsed with mouthwash. The inside of the cheek was then gently swiped with a sterile cotton swab. The swabs were gently agitated in a solution of BSS to collect the cells. Exfoliated cells were then centrifuged (5 min, 600 g), the supernatant was removed, and the cellular pellet was re-suspended in BSS and vortexed. Squamous epithelial cells from the oral mucosa are typically between 60 and 100 μm in diameter.

Exfoliated cells were then deposited onto IR substrates using the Cytospin (Thermo, Waltham, MA, USA) system. The Cytospin instrument uses centrifugal force to deposit the cells in a sparse monolayer while wicking away the suspension medium, resulting in cellular deposits with cells well separated from their nearest neighbors. This technique is preferable to smearing the swab or brush directly onto the substrate, which typically produces thick clumps of cells that are not suited for either visual or spectroscopic analysis. With the Cytospin method, about 0.5 mL of the cell suspension is pipetted into a special funnel, which is attached to a layer of wicking paper with a 5 mm hole. The funnel is clamped to the substrate. The funnel assembly is then placed in the Cytospin system and spun at 1200 rpm for 10 min. The cellular suspension is forced onto the substrate and the suspension medium is wicked away, leaving an air-dried cellular deposit 5 mm in diameter and typically containing about 10^3 cells. Kevley slides, discussed in Section 6.2.2, were utilized for IR cytology.

6.3.2.3 Exfoliated Cells: Canine Cervical Cells. Canine cervical cells were collected in collaboration with a local veterinarian. The reproductive organs, including the cervix, are routinely removed during spaying. Following surgical removal, the organs were immediately placed in BSS, refrigerated, and transported to our laboratory. Cervical cells were collected from the cervix using a miniature dental brush. The dental brushes were gently agitated in a solution of BSS to remove the exfoliated cells. Canine cervical cells were then processed as per the oral mucosa protocol described previously. Epithelial cells from the canine cervix range from 30 to 50 μm in diameter.

6.3.2.4 Exfoliated Cells: Bladder Cells. Voided urine samples were collected using a “clean catch” methodology, whereby only midstream urine is captured. This procedure helps eliminate contamination from bacterial flora that is often present in washings collected from the distal urethra. In addition, specimens were only collected from male donors to alleviate possible contamination from vaginal or perineal squamous epithelial cells.⁵⁸ In this study, multiple samples were collected from four volunteers over a

period of 3 months. To avoid degenerative cellular changes from proteolytic enzymes and bacterial cytolysins, specimens were immediately prepared for spectroscopic analysis. Cells were recovered from the urine using a membrane filtration technique. By use of a standard Luer-lock syringe (30 cc), urine was passed through a nylon net filter (11 μm pore size, 47 mm diameter; Millipore, Billerica, MA, USA) that was held within a polypropylene holder (47 mm diameter, Millipore, Billerica, MA, USA). This device allows diagnostic cells to be captured onto the nylon filter and allows the excess urine to be discarded. The nylon filter was placed directly into a centrifuge tube (50 mL) that contained 20 mL of buffered formalin (10% formaldehyde in water) solution. Cellular material was shaken from the filter, by vortex, and the resulting solution was left for 20 min, permitting the fixation and further preservation of the cells. The filter was removed using forceps, and the remaining specimen was centrifuged (600 g for 25 min) to concentrate the diagnostic cells. After centrifugation the supernatant was decanted, leaving approximately 1 mL of solution at the bottom of the tube that was subsequently vortexed to allow re-suspension of the cells. Each specimen was split into two individual samples, with approximately 0.5 mL of the cell suspension being placed into two separate Cytospin funnels and then centrifuged (1200 rpm, 10 min) onto “low e slides.” All prepared samples were stored in a desiccator until spectroscopic analysis could be undertaken.

After spectroscopic analysis, sample slides were stained using traditional Papanicolaou stains and standard cytological protocols⁵⁹ within our laboratory. Cells that were examined in the analysis were then relocated upon the slide, and visual images were captured at high magnification (40 $\times$) to allow cytological diagnosis.

6.3.2.5 Lymphoma Cells. Lymphoma cells were obtained from a research lab at Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY). Anaplastic large T-cell lymphoma cells (HA 1) were cultured from a patient in the clinic. Peripheral blood was donated from a volunteer at MSKCC, and normal T-cell lymphocytes were isolated using a procedure known as magnetic activated cell sorting (MACS).⁶⁰

Since lymphoma cells are nonadherent, the cultured HA 1 cells did not require the use of chemicals such as trypsin for spectroscopic preparation. Both cell types were transported refrigerated to our laboratory in 15 cc centrifuge tubes containing growth medium (DPMI and FBS). The tubes were centrifuged for 5 min, the supernatant was removed and replaced with BSS, and the cellular pellet was re-suspended. This process was repeated twice to ensure that all traces of the growth medium had been removed. Cells were then prepared for IR-MSP with Cytospin, following the protocol previously outlined.

6.3.3 Instrumentation and Data Acquisition

6.3.3.1 Infrared. Infrared spectra were collected using the Perkin–Elmer Spectrum One/Spotlight 300 system described in Section 6.2.4. Spectra were collected in single point mode.

6.3.3.2 Raman: Jobin Yvon. For the collection of Raman maps for the study of the cell division cycle a JY-Labram Microscope (Jobin Yvon Inc., Edison, NJ) was employed. The instrument, constructed around an Olympus BX30 microscope, incorporates a 300 mm focal length spectrograph with 600 or 1800 lines/mm gratings. Excitation is provided by a 632.8 nm HeNe laser, which provides about 10 mW of power at the sample. A 100 $\times$ objective was used to focus the laser, and Raman scattered photons were detected by a charge-coupled device (CCD) detector. Raman maps were collected at 1 cm^{-1} spectral

resolution and 1 μm spatial resolution. Raman maps were collected at 30 or 60 s exposure times per pixel with a fixed grating position. The average data acquisition time for a single cell map was 15 h.

6.3.3.3 Raman: WITec. For the investigation of subcellular organelles and cell dimensionality, Raman spectra and maps were acquired using a WITec, Inc. (Ulm, Germany) Model CRM 2000 Confocal Raman Microscope. Excitation (~ 30 mW each at 488, 514.5, or 632.8 nm) is provided by an HeNe or an air-cooled Ar ion laser (Melles Griot, Model 05-LHP-928 and 532, respectively). Laser radiation is coupled into the Zeiss microscope through a wavelength-specific single-mode optical fiber. The incident beam is collimated via an achromatic lens and passes through a holographic band-pass filter, before it is focused onto the sample through the microscope objective. A Nikon Fluor ($60\times/1.00$ NA, WD = 2.0 mm) water immersion or a Nikon Plan ($100\times/0.90$ NA, WD = 0.26 m) objective was used in the studies reported.

Raman backscattered radiation is collected through the microscope objective, passing through a holographic edge filter and detected by a 1024×128 pixel CCD camera.

The sample is located on a piezoelectrically driven microscope scan stage with X–Y resolution of ~ 3 nm and Z resolution of ~ 0.3 nm.

6.3.3.4 Data Acquisition. The number of coadded interferograms varied from 64 to 256, depending on the cell type being investigated. Likewise, the size of the spectral aperture varied, depending on the size of the cell. For the studies involving HeLa cells, exfoliated oral mucosa and canine cervical cells and lymphoma cells, the aperture size was set to straddle to entire cell. For the study of exfoliated bladder cells, the aperture was set to 25 μm . All spectra were collected with 4 cm^{-1} spectral resolution.

6.3.4 Data Preprocessing and Analysis

All data preprocessing and subsequent statistical analysis were carried out in MATLAB 7.2 (Mathworks, Natick, MA, USA) using routines and algorithms developed “in house.”

6.3.4.1 IR Spectra of Cell Cycle Studies. Following data collection, spectra were baseline-corrected, min–max normalized to the amide I band, smoothed using a Savitzky Golay smoothing function, truncated to the spectral range $1800\text{--}900\text{ cm}^{-1}$, and second derivatives calculated. Spectra from each time point slide were averaged in order to produce an averaged spectrum to reflect the post-mitosis event.

6.3.4.2 Raman Maps of Cells. Prior to cluster analysis, as described in Section 6.2.5, Raman spectra were vector-normalized in order minimize spectral differences due to sample thickness.

6.3.4.3 IR Spectra of Exfoliated and Lymphoma Cells. All IR spectral data was uniformly pretreated before undergoing further multivariate analysis. Spectra were converted to second derivatives (13- or 15-point smoothing) since distorting Mie scattering effects upon the baseline were evident in some spectra. The wavenumber region was reduced to the biological “fingerprint” region (between 1800 and 950 cm^{-1}). Finally, to negate intensity differences caused by irregularities in cell density, spectra were uniformly vector-normalized.

The small aperture size (20 μm) required to collect spectra of lymphoma cells and lymphocytes resulted in spectra with lower signal-to-noise than typical exfoliated cells. Second derivatives with a 29-point smoothing window were calculated and the spectral region was reduced (1800–1342 cm^{-1}) to remove noisy features enhanced through the calculation of derivatives.

6.3.4.4 Principal Component Analysis. Principal component analysis (PCA) is one of the most widely used multivariate statistical techniques for the extraction and interpretation of information from multivariate data.⁶¹ The aim of PCA is to reduce a large number of variables down to a small number of summary variables, or principal components (PCs), that explain the majority of the variance in the data. All PCs are orthogonal, and each successive component expresses decreasing amounts of variation, with most of the variation explained by the first few components. This enables the multidimensional data to be represented in two or three dimensions, which are easily visualized. The technique works by transforming the original variables onto a new set of axes in the direction of the greatest variation in the data.

The PCs are linear combinations of the original variables, which are fitted in the least-squares sense through the points in measurement space. These new variables usually result in a reduction of variables from the original set and often can be correlated with physical or chemical factors.⁶²

Variables that correlate highly with a particular PC give meaning to that component. The relative magnitudes of the elements in the eigenvectors for a particular PC indicate the relative contribution of the corresponding variable to the variance of that PC. The first PC usually has large correlations with all the variables and is essentially a weighted average of the standardized variable scores. The PC scores for any pair of PCs can be plotted. The reasons for doing this include checking for outlying observations, searching for clusters, and, in general, understanding the structure of the data.⁶³

6.3.5 Results and Discussion

6.3.5.1 IR and Raman Investigation of the Cell Division Cycle. Cancer cells are characterized by rapid proliferation and poor differentiation. In order to study the spectral differences between active and nonactive cells, it was necessary to track spectral changes occurring throughout the cell cycle of HeLa cells. The division cycle of a cell is a complex process in which the “parent” cell mass is doubled during duplication of DNA and subcellular organelles. Eukaryotic cells generally reside in what is known as the G0 or quiescent phase until an appropriate stimulus is received. For actively dividing eukaryotic cells, the division cycle consists of four main phases, which for cells grown *in vitro* usually lasts 24 h, and will be briefly outlined below.

1. G1 phase: This is the first resting (gap) phase after completion of mitosis.
2. S phase: DNA synthesis produces an exact copy of the chromosomal DNA.
3. G2 phase: This is a second resting (gap) phase.
4. M phase: The mitotic phase is where the cell physically divides. Mitosis is further divided into prophase, metaphase, anaphase, and telophase. During prophase, the replicated chromosomes undergo extensive condensation, the mitotic spindle begins to form, and the nuclear envelope breaks down. The two sets of chromosomes align along the “equator” of the parent cell during metaphase. In anaphase, daughter

chromosomes migrate from the equatorial metaphase plate toward each of the mitotic spindles. Finally, in telophase, two sets of chromosomes have formed at the mitotic spindle, a nuclear envelope forms around each chromosomal set to form two distinct nuclei, and cytokinesis results in cell division and the formation of two daughter cells.

While it is not possible to visually distinguish unstained cells in interphase (G0, S, or G2), it is possible with microscopy to determine cells in mitosis.

The complementary techniques of both Raman and IR microspectroscopy were utilized to understand the biochemical and therefore spectral changes occurring in cells as they traverse the cell division cycle. Due to the inherent spatial resolution limits imposed through the diffraction limit, subcellular spectral information is difficult to obtain in the IR. Since confocal Raman spectroscopy does not suffer from these same limitations, it is possible to monitor cellular processes throughout the four subphases of mitosis.

Pre- (BrDU) and post-staining of the cultured HeLa cells enabled, through fluorescence microscopy, identification of the division cycle phase of each cell from which IR and Raman data were collected.^{57,64} Cells were unambiguously categorized into G1, S, or G2 and each of the four subphases of mitosis.

For the IR spectra taken during the 30-h post-mitotic shake-off experiment, only minor spectral differences were observed between cells in interphase. These were manifested in the position of the amide II band and a small wavenumber shift in the phospholipid peak (1738 cm^{-1}) between cells in G1 and cells in G2 or S.

When the spectral aperture was set to straddle only the dimensions of the nucleus, the spectral differences between the cells were more pronounced. Larger shifts in the position of the amide I band indicate different protein structures^{65,66} found in the nucleus at different time points throughout the cell cycle.^{67,68} The nuclear spectra exhibit broader amide II bands and a distinct low-frequency shoulder ($\sim 1500\text{ cm}^{-1}$). DNA peaks (1238 and 970 cm^{-1}) are more pronounced in the spectra of cells during the S phase.

PCA was applied to the average cell nuclei spectrum from each time point, and grouped into cell division phase based on the fluorescence pattern of the stained cells. Figure 6.10 represents the scores plots of IR cell nuclei spectra, reduced to the amide I

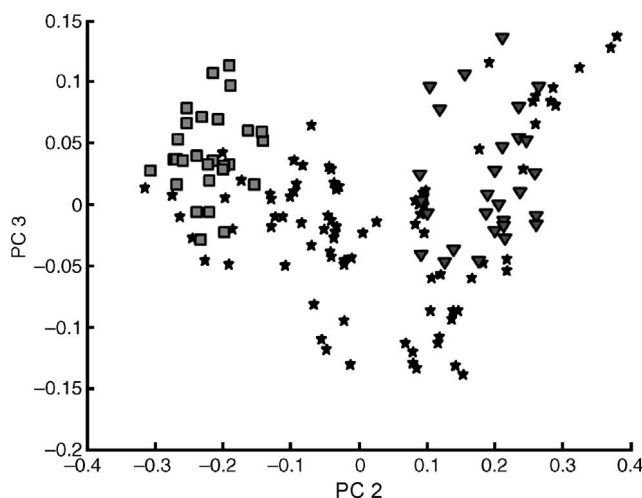


Figure 6.10. PC 2 versus PC 3 scores plots of G1 (squares), S (stars), and G2 (triangles) phase cell spectra. Spectra were reduced to the amide I region ($1700\text{--}1600\text{ cm}^{-1}$).

region ($1700\text{--}1600\text{ cm}^{-1}$), in the three stages of interphase: G1, S, and G2. The scores plot in Fig. 6.10 shows a marked separation between cells in G1 (represented by squares) and G2 (represented by triangles). Cells in G1 synthesize proteins for DNA replication, while cells in G2 are synthesizing proteins in preparation for mitosis and cell division. This explains the peak shift noted in the amide region of the spectra. Furthermore, cells in G2 contain twice as much DNA as those in G1, which may account for band broadening and other differences noted in the amide region, where DNA also has absorption bands.^{69,70} However, there is some overlap within both groups from the S phase (stars), which exhibits marked spectral variability. Cellular processes are not discrete events, so it would be expected that there would be some overlap as the cells traverse through the cycle.

Raman microspectroscopy was employed to determine the thickness and nuclear distribution of the HeLa cells. The purpose of this experiment was to validate our hypothesis that (a) the cytoplasm of a cell is very thin compared to the nucleus and often does not contribute significantly to the IR spectral pattern and (b) the nuclear/cytoplasm ratio plays a key role in the variability noted in the IR spectra of individual cells. Figure 6.11 shows an X–Y (panel A) Raman map and an X–Z (panel C) Raman map of an individual HeLa cell grown directly onto a CaF_2 substrate. Corresponding pseudo-color maps ($1800\text{--}1200\text{ cm}^{-1}$) are given in panels B and D. The thickness (in the Z plane) of the cytoplasm of the HeLa cell measured $1\text{--}3\text{ }\mu\text{m}$, whereas the nucleus of the same cell, seen as the blue cluster in panels B and D, measured $6\text{ }\mu\text{m}$. It is hardly surprising then that gross cell division features of spectra recorded over the entire dimensions of the cell were not observed. A cytoplasm of this thickness is below the limit of detection of the IR instrument, and as such the spectra

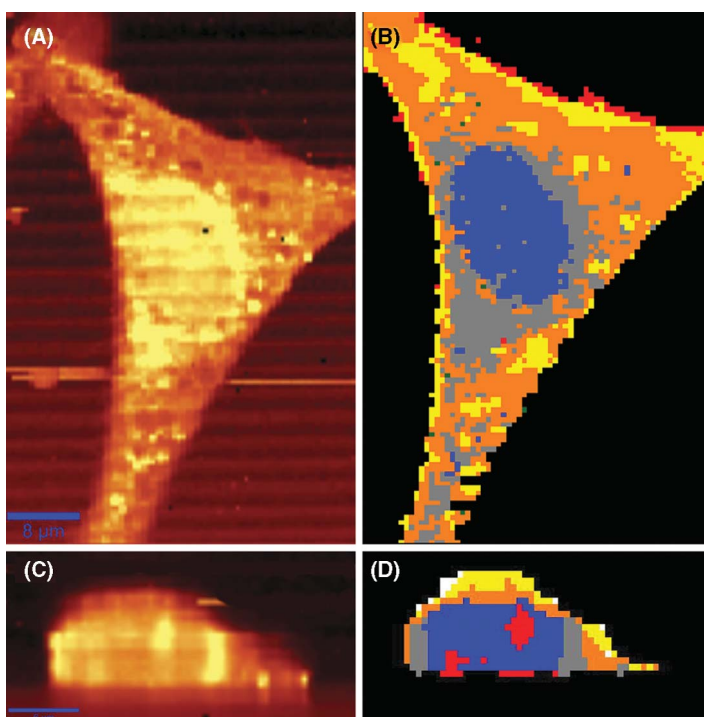


Figure 6.11. (A) Raman X–Y map of a HeLa cell, grown onto a CaF_2 substrate. Blue bar equals $8\text{ }\mu\text{m}$. (B) Corresponding pseudo-color map. (C) Raman X–Z map of the same HeLa cell. Blue bar equals $6\text{ }\mu\text{m}$. (D) Corresponding pseudo-color map.

observed have been averaged out over a large area. This contrasts to the spectral features observed throughout the division cycle when the spectral aperture was set to match the size of the nucleus. The implications of cell thickness and nuclear size will be discussed in more detail later in Section 6.3.5.2.

Raman maps of four individual mitotic HeLa cells, each captured during one of the four subphases, were collected. Each Raman map required 12–24 h of acquisition time. Figure 6.12 shows HeLa cells during the four subphases of mitosis. The left column of images depicts the cells, in culture, as seen through an inverted phase contrast microscope. The second column shows the distribution of DNA within each cell, exhibited as an intensity map of the nucleic acid band at 785 cm^{-1} . The third column illustrates the distribution of protein within each cell, as seen by the intensity map of the amide I band (1655 cm^{-1}). The fourth column shows the fluorescence image of DAPI-stained cells,⁶⁴ demonstrating the

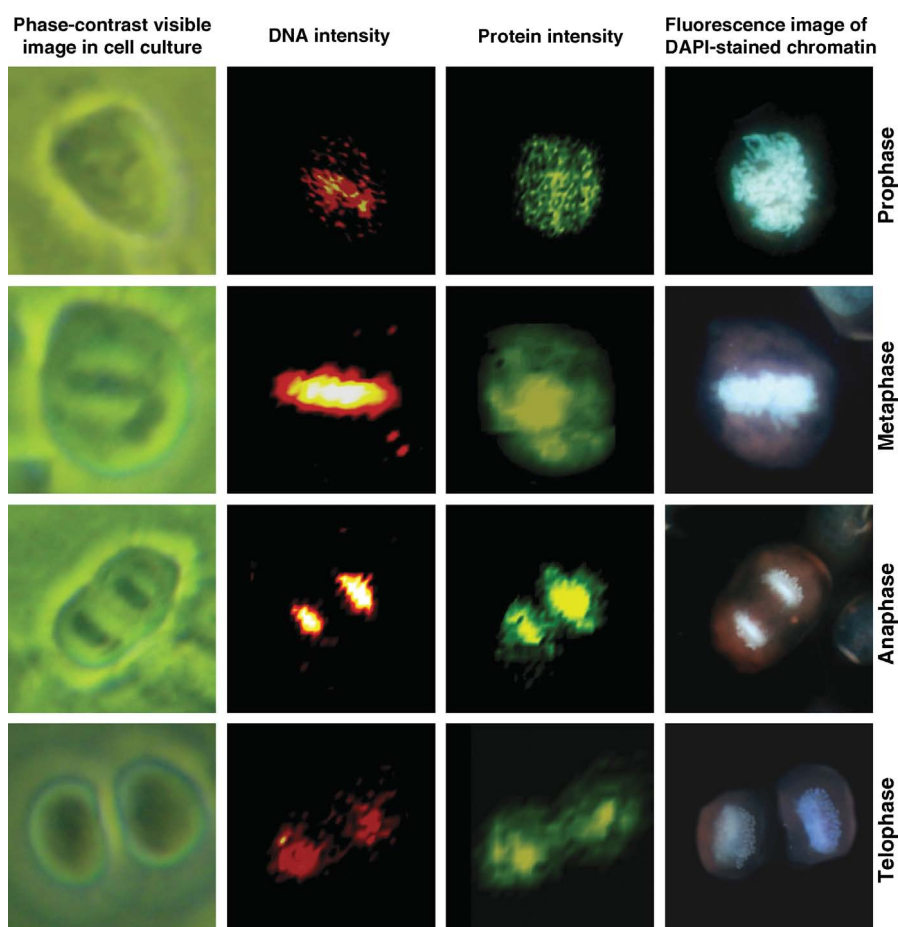


Figure 6.12. Photomicrographs and Raman and fluorescence (DAPI stain) images of HeLa cells during various phases of mitosis, specifically (from top to bottom) prophase, metaphase, anaphase, and late telophase. From left to right the columns show phase-contrast images of live cells in culture, Raman scattering intensity plots for the DNA scattering intensities (white represents highest intensity), Raman scattering intensity plots for the protein scattering intensities (yellow represents highest intensity), and fluorescence images of DAPI-stained cells.

density and distribution of the condensed chromatin within each cell. Note the excellent correlation between the spectral nucleic information (acquired without the use of stains) and the stained fluorescence images, indicating that Raman spectroscopy can be utilized for the visualization of DNA condensation during mitosis. Raman microscopy also provides an indication of the degree of chromatin condensation, with the highly condensed DNA/histone complexes manifested in metaphase and anaphase as an increase in the intensity of the relevant DNA and protein bands. The spatial resolution limitations imposed by the diffraction limit render IR spectroscopy unsuitable for chromatin profiling.⁶⁴

6.3.5.2 IR Spectroscopy of Exfoliated Cells. One of the main issues arising from IR cytology is the inherent heterogeneity of cells. Biological samples exhibit spectral variability both within a given cell type and between donors. Although several studies^{43,71} have shown that differences between normal and diseased cells and tissues are greater than the small variations occurring within a cell type, it is important that these variations be investigated and understood. Figure 6.13 shows the raw IR spectra of individual oral mucosa cells collected from one volunteer, along with the mean spectrum and standard deviations from the mean. Large variations in the overall intensities of the spectra were observed over the entire spectral range, as well as broad, undulating features from 2600 to 1800 cm^{-1} . Distinct spectral variations in the spectral region 1400–950 cm^{-1} were also noted.

The broad, undulating features have been attributed to Mie scattering of the cell nuclei,^{72,73} which becomes pronounced when the spectral aperture straddles the nucleus (Fig. 6.14). When the aperture is set to straddle the entire cell, the Mie scattering and other spectral features arising from DNA and proteins are averaged over a larger area and are observed with lower amplitude.

The overall intensity variations are likely to arise due to variations in the thickness of the cell, as discussed previously, and differences in the nucleus/cytoplasm (N/C) ratio. In dried epithelial cells, the cytoplasm is very thin ($\sim 2\text{--}3\ \mu\text{m}$) and does not strongly contribute to the overall spectral features of the cell. The nucleus ($3\text{--}15\ \mu\text{m}$), on the

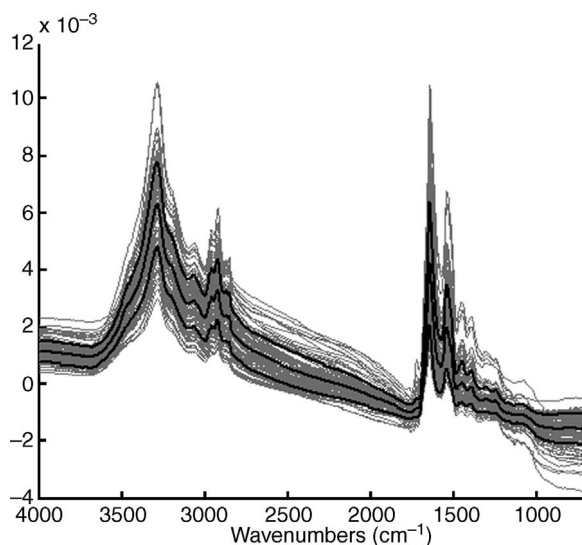


Figure 6.13. Raw IR absorption spectra of individual oral mucosa cells, collected from one volunteer. Black spectra represent the mean and standard deviation of the sample population.

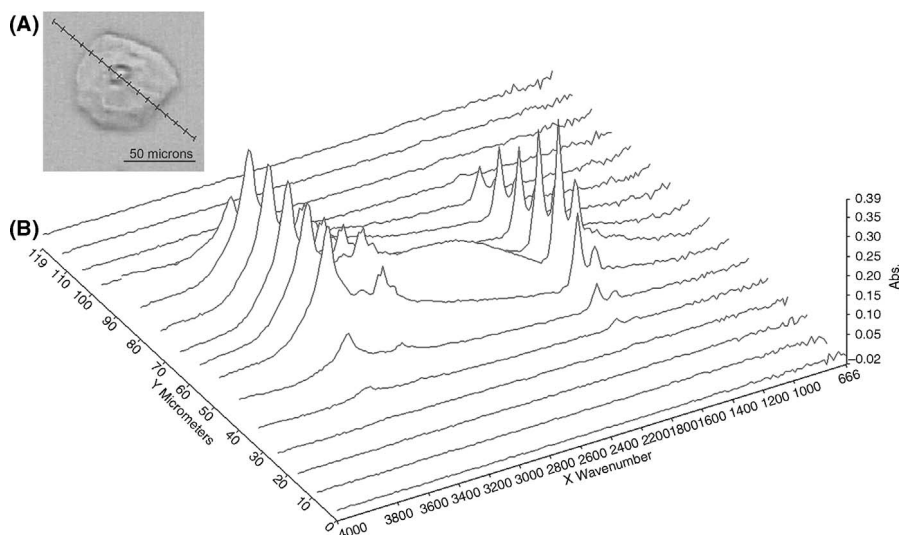


Figure 6.14. (A) Photomicrograph of an unstained oral mucosa cell, with positions indicating the locations of spectral data acquisition. (B) Three-dimensional plot of IR spectra obtained at the positions indicated.

other hand, maintains a more spherical shape and a higher protein contribution. It is estimated⁷¹ that the cell nucleus contributes 20% of the total protein signal even though it accounts for about 2.5% of the total cell volume. Therefore, variations in the size of the cell nucleus, most notably as the nucleus becomes pyknotic,⁷⁴ will strongly influence the total absorption intensity.

A pyknotic nucleus in a superficial cell, which has reached maturity and is therefore tightly compacted, is IR “opaque,”^{17,72} and spectral features of nucleic acids observed from these cells are a result of cytoplasmic RNA rather than nuclear DNA contributions. DNA in the nucleus has an optical density too high to allow transmission of IR radiation.²⁸

Pyknosis and possibly necrosis are believed to be responsible for the spectral variation noted in the low-frequency region below 1400 cm^{-1} ; these processes continue to be investigated in our laboratory.

Even though most cytological samples exhibit large spectral heterogeneity, statistical techniques such as PCA and HCA emphasize common features and deemphasize irrelevant variations in the spectra. To this end, spectral preprocessing is vital to remove baseline artifacts such as Mie scattering, to remove the influence of irrelevant spectral variation both within and between patients, and to emphasize the important spectral range in terms of diagnosis.

It is an essential step in the development of spectral cytology to determine that the developed methodologies are robust and able to distinguish normal from diseased cells while ignoring the inherent spectral variability that exists within one cell type and between different donors.

In order to test this, a comparison was made between the oral mucosa cells and canine cervical cells. Both cells are squamous epithelial cells devoid of glycogen and morphologically very similar. In total, seven sets of oral mucosa cells (427 spectra) and five sets of canine cervical cells (560 spectra) were preprocessed and imported into the principal

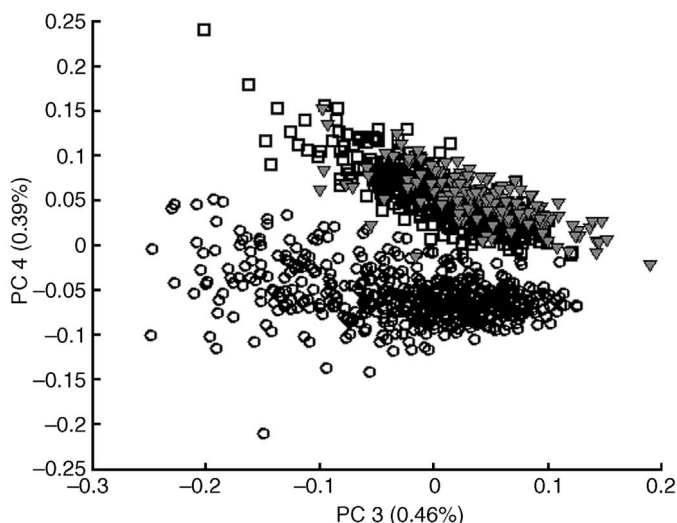


Figure 6.15. PC 3 versus PC 4 scores plot of over 1000 human oral mucosa (squares) and canine cervical (circles and triangles) cells.

component program. The resulting PC 3 versus PC 4 scores plot (Fig. 6.15) shows an interesting pattern. The majority of cervical cell spectra (circles) are differentiated from the oral mucosa cells (squares) except for the cervical cell spectra represented by triangles. Curious as to why these cervical cells have spectral characteristics closer to those of oral mucosa cells than do the majority of cervical cells, we investigated the reproductive history of the dogs in the data set. It was discovered that the animal whose cell spectra (represented by triangles) clustered with the oral mucosa cells was in estrus at the time the spaying procedure was performed.

Exfoliated oral mucosa cells are mature (superficial) squamous epithelial cells, typically with a diameter between 50 and 60 μm . For non-estrus dogs, cervical cells are immature and are generally intermediate squamous epithelial cells.⁷⁵ These cells are significantly smaller, typically 25–35 μm in diameter. However, during estrus, hormonal stimulation causes maturation of the squamous epithelium from intermediate to superficial. This process results in enlargement of the cells to sizes similar to those observed in the oral mucosa. As the size of the cell varies, so too does the N/C ratio. As mentioned previously, changes in the N/C ratio significantly impact spectral characteristics, and it is believed that this is the contributing factor in the differentiation observed between estrus and non-estrus cell spectra and between non-estrus and oral mucosa cell spectra.

6.3.5.3 IR Spectroscopy of Bladder Cells. The data set presented represents a small portion of our overall collected spectral library and comprises data from four different male donors. Only spectra with a firm cytological diagnosis have been included in this analysis. These include both squamous epithelial cells from the distal portion of the urethra and transitional (urothelial) epithelial cells that can originate from the renal calyces, renal pelvis, ureter, bladder, and urethra.⁷⁶

Urine sediments collected from healthy individuals for conventional urinalysis exhibit very small numbers of transitional (urothelial) epithelial cells.⁵⁸ Our spectroscopic studies

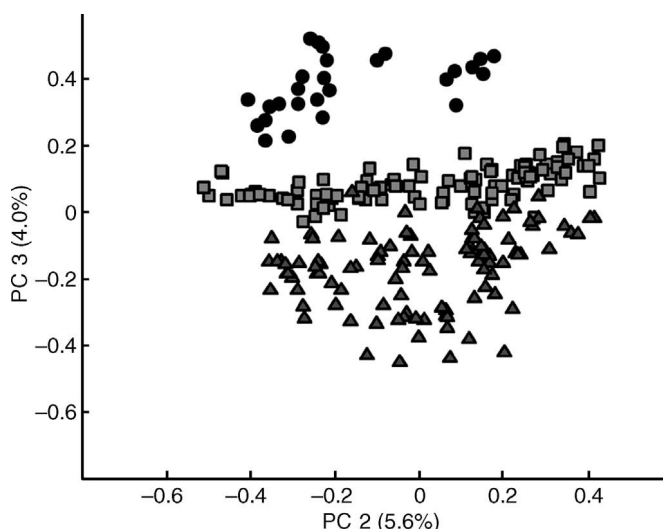


Figure 6.16. PC 2 versus PC 3 scores plot of urothelial cells (circles) and squamous epithelial cells (triangles: glycogen-rich, squares: glycogen-absent) from the voided urine of four male volunteers.

confirm this observation, having encountered a relatively small number of urothelial cells from our healthy male donors. A large variation in the overall amplitude of spectra is also observed. Absorbance values recorded for the amide I band vary in intensity from 0.05 to 0.8 OD units. We believe that these variations are largely due to deviations in both cell thickness and the N/C ratio within the spatial area sampled.⁷¹ Spectra recorded from urothelial cells notably display particularly large absorbance intensities. This type of cell can range in size from ~ 10 to $40\ \mu\text{m}$ ^{58,76} and typically feature large nuclei. We hypothesize that this observed intensity difference for urothelial cells is caused by both an increased cell thickness and a strong nuclear contribution to the spectra. Confocal Raman depth profiles (not shown) collected from both squamous and urothelial epithelial cells provide strong evidence to verify such assumptions. Figure 6.16 displays the PC 2 versus PC 3 scores plot of the normal epithelial cell types found in voided urine, separated into three clusters. The cluster of circles is representative of urothelial cells; note the excellent separation from the squamous cells, indicating a substantial spectral difference among these cells. Furthermore, the glycogen-rich (squares) and glycogen-absent (triangles) squamous cells are also clearly differentiated. Initial results are extremely promising, and we are currently cataloguing the diagnosis of many thousands of recorded cell spectra to serve as inputs for supervised pattern recognition (ANN), with the ultimate goal of developing a spectral database and subsequent diagnostic algorithm for the detection of bladder cancer.

6.3.5.4 IR Spectroscopy of Lymphoma Cells. Once the origin of the spectral heterogeneity in a normal population was understood, the combination of IR microspectroscopy and multivariate statistical methods was applied to normal versus cancerous cells. For this aspect of our investigation, we used lymphoma cells.

Principal component analysis was performed on the IR spectra of individual HA 1 and T-lymphocyte cells. A PC 2 versus PC 3 plot (Fig. 6.17) indicates a strong separation between the normal (triangles) and cancerous (squares) cells.

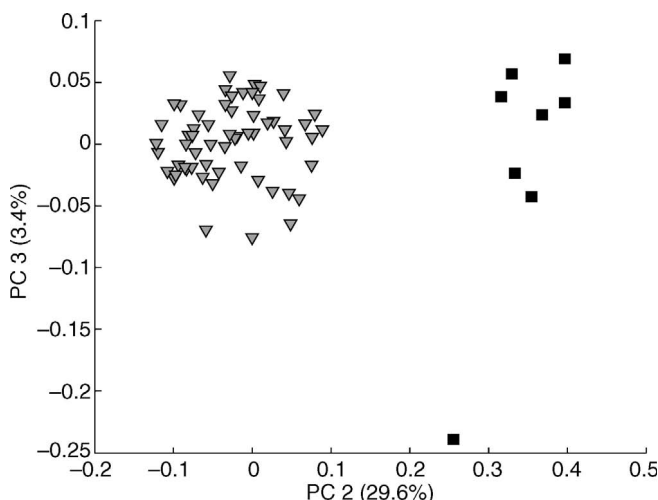


Figure 6.17. PC 2 versus PC 3 scores plot of HA 1 lymphoma cells (triangles) and normal T-lymphocytes from a healthy volunteer (squares).

When comparing IR spectra of cells obtained directly from the body with cells that have been cultured, one has to question if the spectral differences are due to the disease state or due to the treatment of the cells – that is, growth medium, multiple propagations, and so on. The cultured cells received from MSKCC were initially cultured less than 12 months before we analyzed them and were regularly tested to ensure that the integrity of the cell line was maintained. The cells were not treated in any way to ensure proliferation and propagation; being cancerous cells, they naturally possess the genetic mutation that allows unchecked proliferation.

In a similar, independent study,⁷⁷ Raman spectroscopy was utilized for the comparison of peripheral blood mononuclear cells (PMBC) with cultured Jurkat T cells, a readily available lymphoma cell line. PCA was again employed and a good separation between the two groups of cells was noted, with a scores plot almost identical to that shown in Fig. 6.17.

Although the data set is comparatively small, the strong separation and, for the most part, tight grouping within one cell type indicates that this technique may have important implications for the screening of peripheral blood and bone marrow stem cells, to ensure that (a) the sample is cancer-free and (b) contamination is not introduced to immunocompromised patients.

6.4 CONCLUDING REMARKS

IR-MSP and Raman microscopy, coupled with powerful multivariate statistical techniques such as HCA and ANNs, have the potential to discriminate the subtle spectral differences between cell types, disease states, and cellular processes. The inherent heterogeneity within biological cells and tissue can be explained through examination of the N/C ratio, cell

division cycle, and cell thickness. Despite these spectral variations, we are able to extract meaningful algorithms for cancer diagnosis.

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RESONANCE RAMAN MICROSPECTROSCOPY AND IMAGING OF HEMOPROTEINS IN SINGLE LEUKOCYTES

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7.1 HEMOPROTEINS

Iron–porphyrin complexes, known as hemes, are arguably the most versatile redox centers in biology.¹ As prosthetic groups bound covalently or noncovalently to proteins and enzymes, hemes are widespread among living systems. The biological significance of heme-containing proteins (also called hemoproteins) is illustrated by the diversity of functions that they exhibit. These include (hemoprotein examples are given in parentheses):

- Oxygen transport (hemoglobin in erythrocytes) and storage (myoglobin in muscle tissue)
- Electron transport in cellular respiration (cytochromes in mitochondria)
- Enzymatic degradation (decomposition of toxic peroxides by catalases, metabolism of drugs by cytochrome P450 enzymes)
- Innate immunity (superoxide-producing flavocytochrome b_{558} and perhalogenation by peroxidases in leukocytes)

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In addition to being a prosthetic group for a wide range of redox-active proteins, free heme has direct regulatory roles in various molecular and cellular processes such as gene transcription and translation, protein translocation and assembly, and cell differentiation and proliferation.^{2–4} Importantly, since elevated levels of free heme induce proinflammatory effects and cause oxidative stress and tissue damage, the free heme pool must be tightly controlled. The enzyme heme oxygenase (HO) is the rate-limiting factor in heme breakdown. Besides degrading heme for iron recycling, this enzyme has important regulatory properties and is involved in many physiological and pathophysiological processes. Alzheimer's disease, atherosclerosis, cancer, diabetes, hypertension, and sepsis, among other diseases/conditions, have all been associated with increased HO-1 expression levels.⁴

The characterization and investigation of hemes and hemoproteins is facilitated to a large extent by the distinct molecular structure and properties of hemes. The most common natural heme chromophores (heme *a*, heme *b*, and heme *c*) differ in their pattern of β -pyrrolic substituents on the porphyrin macrocycle. Proteins containing these hemes can be distinguished in UV-visible absorption spectra by the position of the lowest energy absorption band in the reduced state – for example, at ~ 605 nm (cytochromes *a*), ~ 565 nm (cytochromes *b*), and ~ 550 nm (cytochromes *c*). In addition to absorption spectroscopy, other spectroscopic techniques that are commonly used to study hemoproteins include nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), resonance Raman (RR), and magnetic circular dichroism (CD) spectroscopy.⁵ Of these, RR spectroscopy is particularly well-suited to probe both the structural and electronic properties of hemes and to shed light on the environment of heme groups (e.g., iron coordination number and ligand types) embedded in hemoproteins. Since the first reported RR spectra of hemoproteins (hemoglobin^{6,7} and cytochrome *c*^{8,9}) in 1972, a large body of literature has dealt with the structural characterization of metalloporphyrins and heme prosthetic groups in proteins using RR spectroscopy. Results from such studies have contributed enormously to our current understanding of the molecular mechanisms that underlie hemoprotein function. Overviews discussing the use of RR spectroscopy to investigate metalloporphyrins and hemoproteins are amply available.^{10–12}

7.2 RAMAN MICROSPECTROSCOPY

Traditionally, RR spectroscopy studies on hemoproteins have been performed in bulk – that is, in microliter-to-milliliter volumes of concentrated hemoprotein solutions. The combination of instrument limitations, resulting in a low photon efficiency of the optical detection system, and the low Raman scattering cross-section of biological molecules has necessitated the use of large sample volumes and long signal integration times (tens to thousands of seconds) in many reported cases. However, in the late 1980s, developments in optical components such as filters, dispersion systems, and detectors had advanced to such an extent that Raman *micro*spectroscopy became feasible. In this technique, a Raman spectrometer is integrated with an optical microscope in order to obtain spectra inside a sample with a lateral spatial resolution that is diffraction-limited (~ 0.25 μ m when using a laser excitation wavelength of ~ 500 nm and a microscope objective with a high numerical aperture (NA), typically 1.2 for water-immersion and 1.4 for oil-immersion objectives). By placing a confocal pinhole of appropriate diameter (typically 25–100 μ m) in the optical detection path to reduce out-of-focus Raman-scattered photons,¹³ an axial resolution along the optical axis of a few micrometers can be achieved.

By combining a high-NA objective, a small-diameter confocal pinhole, and an excitation wavelength of 660 nm, we reported the first nonresonant Raman spectra from subfemtoliter ($<1\ \mu\text{m}^3$) volumes inside single living cells in 1990.¹⁴ Since then, continuous improvements in instrumentation, particularly in high-throughput spectrographs and high-sensitivity charge-coupled devices (CCDs), have boosted the sensitivity of Raman microspectroscopy setups to a level where high-quality spectra of intracellular volumes can now be recorded within a few seconds or less. These developments have sparked considerable commercial interest, and confocal Raman microscopes with excellent sensitivity specifications have become available from several companies.¹⁵ From the increasing number of research reports detailing the benefits of Raman microscopy in cell biology and optical diagnosis, it is apparent that the importance of Raman microscopy in the life sciences will only increase in the coming years.

7.3 OUTLINE OF THIS CHAPTER

In this chapter, we will discuss the characterization of hemoproteins in single leukocytes by resonance Raman microspectroscopy and imaging. Specifically, we will focus on RR studies, at 413.1-nm excitation, of flavocytochrome b_{558} and myeloperoxidase in neutrophilic granulocytes, which are cells that are responsible for the destruction of microorganisms that invade the human body. Flavocytochrome b_{558} and myeloperoxidase are both hemoproteins that are crucial in the innate immune response of neutrophilic granulocytes by virtue of their ability to produce reactive oxygen species that contribute to microbial killing.

Section 7.4 of this chapter provides a brief overview of the confocal Raman instrumentation, the different modes of Raman data collection, and the various spectral data analysis techniques that we employ for RR studies on neutrophilic granulocytes.

A short discussion on the role of flavocytochrome b_{558} in phagocytes is given in Section 7.5. This discussion, which serves as biological background, is followed by a review of the RR microspectroscopy results, obtained during the last decade, on flavocytochrome b_{558} in both quiescent and stimulated neutrophils. We will show that RR spectra of these cells are exquisitely sensitive to the heme iron redox state of flavocytochrome b_{558} , allowing the activity of this enzyme to be established in single living cells without the addition of any probe.

In Section 7.6 we will discuss how the distribution of flavocytochrome b_{558} in neutrophils, along with changes in this distribution that occur when neutrophils phagocytose (internalize) particles, can be visualized by RR imaging/mapping experiments on single cells.

It is well known that high-power laser excitation light, especially in the UV wavelength range, has detrimental effects on living cells. Besides photobleaching, which leads to a loss of signal and is often a serious problem in fluorescence microscopy and RR microscopy, illumination of cells with high doses of UV-visible light may also disturb the overall intracellular redox balance, thereby affecting cellular physiology and leading to oxidative stress. Section 7.7 ends this chapter by discussing our RR experiments on fixed and live neutrophils aimed at investigating photoinduced effects of 413.1-nm excitation light on photobleaching of the flavocytochrome b_{558} Raman signal and on neutrophil autofluorescence, which is a useful indicator of cellular redox homeostasis. We will place our results in the context of reported studies on cellular autofluorescence and photodamage at UV excitation wavelengths.

7.4 INSTRUMENTATION AND SPECTRAL DATA ANALYSIS

7.4.1 The Confocal Resonance Raman Microscope

As mentioned in Section 7.2, Raman microspectroscopy can be performed on cells or other samples by integrating a Raman spectrometer with an optical microscope. Compared to bulk spectroscopy, the confined excitation light in microspectroscopy, focused by a high numerical aperture microscope objective to a diffraction-limited spot, offers the advantage of high spatial resolution and allows samples of micrometer dimensions (such as cells) or submicroliter volumes to be investigated.

A schematic outline of the home-built confocal Raman setup that has been used in our laboratories for RR experiments on neutrophilic granulocytes is given in Fig. 7.1. The following components of this setup have been critical for performing RR microscopy on single leukocytes and for obtaining high-quality RR signals from hemoproteins in these cells:

- A high-power Kr^+ gas laser (Innova 90 K; Coherent, Inc., Santa Clara, CA, USA) providing ~ 50 mW output of 413.1 nm excitation light. This wavelength is ideal for exciting flavocytochrome b_{558} and MPO in their strong Soret absorption bands of 413 and 428 nm,¹⁶ respectively, which results in a large resonance enhancement of the Raman signal of these hemoproteins.

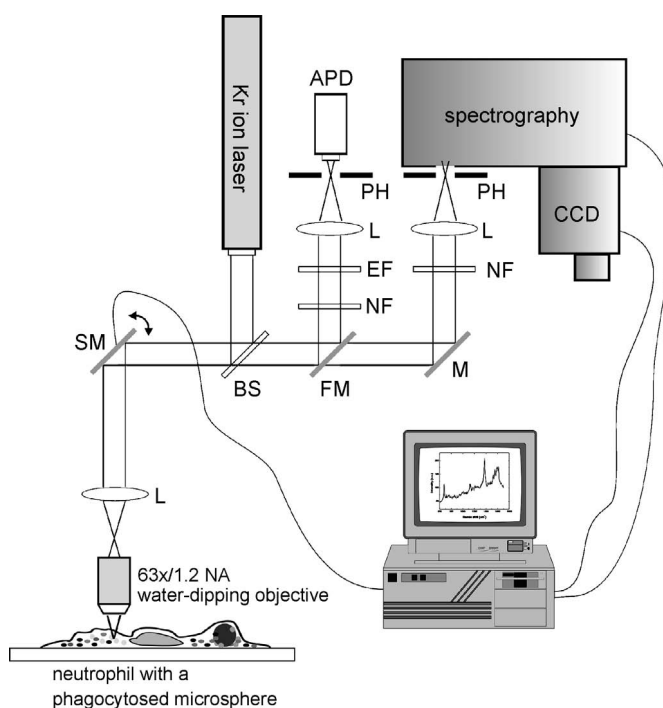


Figure 7.1. Schematic representation of the confocal resonance Raman microscopy setup used to perform RR microspectroscopy and imaging experiments on leukocytes. Abbreviations: BS, beamsplitter (30% reflection, 70% transmission at 413.1 nm); SM, scanning mirror; L, lens; FM, flippable mirror; M, mirror; NF, notch filter; EF, emission filter; PH, pinhole; APD, avalanche photodiode; CCD, charge-coupled device. For further descriptions of individual components, see the text and Refs. 17,60,61,64.

- A scanning mirror (Leica Lasertechnik GmbH, Heidelberg, Germany) to scan the laser excitation spot over cells that have been adhered to an uncoated or poly-L-lysine-coated CaF_2 disk.
- A water-dipping microscope objective (Plan Neofluar, $63\times/1.2$ NA; Carl Zeiss, Jena, Germany) to focus the excitation light to a diffraction-limited spot of $0.32\text{ }\mu\text{m}$ ($1/e^2$ diameter of the laser beam waist under the objective, according to a Gaussian beam approximation).^{17,18}
- A holographic notch filter (Kaiser Optical Systems, Inc., Ann Arbor, MI, USA) to suppress reflected and Rayleigh-scattered laser light.
- An exchangeable pinhole (diameter $25\text{--}100\text{ }\mu\text{m}$) at the entrance of the spectrograph to reduce out-of-focus contributions to the Raman signal and to limit the intracellular detection volume to 0.45 fL in the case of a $25\text{ }\mu\text{m}$ pinhole.¹⁷
- A spectrograph (HR460; Jobin-Yvon, Paris, France) containing a blazed holographic grating with 1200 grooves/mm to disperse the Raman-scattered light.
- A back-illuminated, liquid-nitrogen-cooled CCD detector (LN/CCD 1100 PB/VI-SAR; Princeton Instruments, Inc., Trenton, NJ, USA) with 1100×330 pixels of $24\times 24\text{ }\mu\text{m}^2$ that is placed in the focal plane of the spectrograph exit port, providing a spectral resolution of $2.1\text{ cm}^{-1}/\text{pixel}$.

As shown in Fig. 7.1, we have extended the confocal Raman setup with an additional detection branch for confocal fluorescence microscopy. This branch uses a separate notch filter, an emission filter (a long-pass 435 nm colored-glass cutoff filter [03 FCG 061; Melles Griot, Carlsbad, CA, USA] in case of 413.1 nm excitation), a pinhole (diameter $25\text{ }\mu\text{m}$), and an avalanche photodiode (APD) for the sensitive detection of fluorescence emission from the sample under investigation. A flippable mirror is used to switch between Raman/CCD and fluorescence/APD detection modes. As will be discussed in Section 7.7, we have used the fluorescence detection mode to investigate autofluorescence from neutrophilic granulocytes at 413.1-nm excitation.

The scanning mirror and APD data acquisition on the setup shown in Fig. 7.1 is controlled by software written in LabVIEW (National Instruments Corporation, Austin, TX, USA). Data acquisition with the CCD is controlled by WinSpec software (Princeton Instruments, Inc., Trenton, NJ, USA).

7.4.2 RR Data Collection Modes

The confocal Raman setup described in Section 7.4.1 can be operated in three different data collection modes, allowing us to perform the following types of experiments on cells:

- *Raman point microspectroscopy*, where the focused laser beam is parked in a location of interest and a Raman spectrum from a diffraction-limited spot is acquired.
- *Raman scanning microspectroscopy*, where the focused laser beam is scanned either once or continuously across a region of interest (e.g., a full cell or an intracellular region) and a single Raman spectrum, representing the total Raman signal from the scanned area, is recorded. Spatial information is lost in this collection mode.

- *Raman microscopy* (or *Raman spectral imaging*), where the focused laser beam is scanned across a region of interest and a Raman spectrum is acquired at every image pixel. From the 3D data matrix (one spectral and two spatial dimensions) that is obtained in this way, Raman images can be constructed by integrating Raman bands of interest and plotting the integrated intensities as a function of pixel position (see Section 7.6).

7.4.3 Processing and Analysis of Raman Data Sets

The acquisition of a series of Raman spectra, either in the point or scanning microspectroscopy mode or in the spectral imaging mode, results in large data matrices that often need to be analyzed globally for particular features of interest and changes in these features across the data set. Nowadays, the use of powerful analysis techniques is crucial to extract the required information from multidimensional data sets, especially in diagnostic applications of vibrational spectroscopy. Because other chapters of this book are devoted to this topic, we will restrict ourselves in this subsection to a concise summary of the techniques that we use to construct and analyze Raman spectral images.

After acquisition of Raman spectra, which are wavenumber-calibrated on a daily basis in a separate experiment with toluene, we import spectral data sets into MATLAB (The MathWorks, Natick, MA, USA) and generally perform the following corrections:

- The constant CCD offset, measured by taking the mean value (in the spectral dimension) of a spectrum recorded with no excitation light and zero accumulation time, is subtracted from all data set entries.
- Every spectrum is corrected for the total frequency-dependent throughput of the Raman microscope, by means of a similar procedure as reported before.^{19,20} Because optical components and the CCD chip have a transmission or detection efficiency that is dependent on the optical frequency, recorded spectra must be calibrated for the optical response profile of the entire instrument. This is usually done with the aid of a light source with a known spectrum, such as a tungsten lamp at a known temperature.
- After truncation of spectra to the ~ 400 to 1800 cm^{-1} region, spectral noise is reduced by singular value decomposition (SVD). SVD is a powerful analytical technique for determining the number of significant spectral species that contribute to a spectral data set,²¹ and it works effectively as a noise-suppressing tool in Raman imaging. For further details on how to apply SVD to Raman microscopy data, see Refs. [17,22].

After noise filtering by SVD, Raman images are constructed by plotting the integrated intensity of a band of interest as a function of image pixel position. Band intensities are usually background-corrected.

Finally, we routinely apply hierarchical cluster analysis (HCA) to Raman microscopy data in order to construct multivariate cluster maps of cells in which image pixels with high spectral similarities are grouped together in clusters that are visualized by separate colors (see Fig. 7.6B). Further details about the application of HCA to Raman imaging data can be found elsewhere.²⁰

7.5 RESONANCE RAMAN MICROSPECTROSCOPY ON NEUTROPHILIC GRANULOCYTES

7.5.1 Introduction: Flavocytochrome b_{558} and the Phagocyte NADPH Oxidase

Phagocytic leukocytes such as neutrophils and macrophages constitute the first line of cellular defense against microorganisms that invade the human body. Neutrophils leave the bloodstream and migrate to infected sites, where they recognize and internalize microbes by a process termed phagocytosis.²³ Together with macrophages, they are critical in the innate immune response due to their capacity to kill and degrade internalized pathogens inside phagolysosomes.²⁴ Degradation of microbes occurs by exposing them to high concentrations of reactive oxygen species (ROS) and microbicidal peptides and proteases. The release of these destructive agents in the phagosome is mediated by the enzyme NADPH oxidase.^{25–29} The flavohemoprotein $gp91^{phox}$ (for glycoprotein of 91 kDa; $phox$ = phagocyte oxidase) is the catalytic subunit of this enzyme. It forms a heterodimer called flavocytochrome b_{558} with the membrane protein $p22^{phox}$. In addition, the cytosolic subunits $p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and Rac2 are also critical for NADPH oxidase activity. Upon stimulation of neutrophils, which occurs during and after phagocytosis, these cytosolic subunits translocate and bind to the membrane-bound flavocytochrome b_{558} . The activated NADPH oxidase complex now transports electrons through $gp91^{phox}$ from the cytosolic substrate NADPH, via one FAD and two membrane-embedded heme b prosthetic groups, to oxygen at the extracellular side of the plasma or phagosomal membrane (Fig. 7.2). This results in the production of large amounts of superoxide (O_2^-) – that is, about 100 nmol/min per 10^7 cells.³⁰ It is known that superoxide-derived ROS are involved in bacterial killing, and it has recently been proposed that cation influx, which accompanies electron release into the phagosomal lumen, activates proteases from the proteoglycan matrix of granules that have been released into the phagosome. Apparently, this dual role of the phagocytic NADPH oxidase is necessary for efficient bacterial killing.^{31,32}

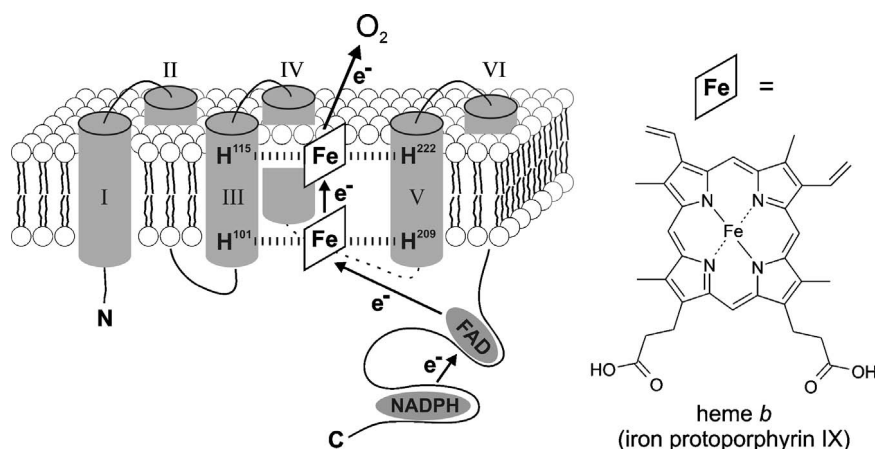


Figure 7.2. Topological model of the phagocyte flavocytochrome b_{558} . Electron transport from NADPH to FAD, two hemes, and then to oxygen, which occurs upon NADPH oxidase activation, is shown with arrows. Coordination of four histidine residues in transmembrane helices III and V (helices are indicated with Roman numerals) to heme b iron centers is shown with dashed lines. The molecular structure of heme b is depicted on the right.

Because it is such a complex and important enzyme in host defense, the phagocyte NADPH oxidase has been investigated extensively by a large community of researchers from diverse areas such as biochemistry, biophysics, cell biology, hematology, and immunology. A large body of review literature provides overviews and discussions of many aspects of NADPH oxidase.^{25–29} Current research in this field is mainly focused on identifying the molecular interactions between NADPH oxidase subunits that are critically involved in (the dynamics of) oxidase activity and regulation in living cells.

7.5.2 Chronic Granulomatous Disease

In 1967 it was discovered that neutrophils from patients with the clinical syndrome called “fatal chronic granulomatous disease (CGD) of childhood,” which had first been described in 1959,³³ lacked the typical “respiratory burst” that occurs upon stimulation of healthy neutrophils.^{34,35} The term “respiratory burst” refers to the metabolic changes that are associated with neutrophil stimulation, including a burst in cellular oxygen consumption (already known since 1933),³⁶ increased glucose oxidation via the hexose monophosphate shunt,³⁷ and the production of superoxide³⁸ and superoxide-derived ROS. The oxidase associated with this respiratory burst was identified as a *b*-type cytochrome in 1978^{39,40} and shown to be missing or defective in X-linked CGD (with the genetic defect on the X chromosome) and normally present but nonfunctional in most autosomal recessive forms of CGD.⁴¹ It is now known that the majority (~70%) of CGD cases is caused by mutations in the *CYBB* gene on the X chromosome (encoding gp91^{phox}), leading to the absence of flavocytochrome *b*₅₅₈ expression. However, there are 16 known *CYBB* mutations in which normal amounts of a nonfunctional flavocytochrome *b*₅₅₈ are expressed.⁴² These loss-of-function mutations have been instrumental in providing insight into the molecular mechanisms of NADPH oxidase activation and regulation, because they enable regions in flavocytochrome *b*₅₅₈ that are involved in the binding of heme, NADPH, FAD, or cytosolic subunits to be identified.^{26,43}

Because their neutrophils cannot produce superoxide and fail to adequately destroy invading microorganisms, CGD patients suffer from recurrent and often life-threatening bacterial and fungal infections and need to be hospitalized frequently. Impressively, a recent clinical trial showed the functional reconstitution of flavocytochrome *b*₅₅₈ in two X-linked CGD patients by gene therapy,⁴⁴ providing exciting prospects for effective treatment of CGD.

7.5.3 Bulk Spectroscopy on Flavocytochrome *b*₅₅₈

Absorption spectroscopy and electron paramagnetic resonance (EPR) spectroscopy were among the early biophysical techniques used to characterize the heme prosthetic groups in flavocytochrome *b*₅₅₈. Whereas absorption spectroscopy was employed to identify this cytochrome as a *b*-type cytochrome^{39,40} and is still widely used today to detect the presence of flavocytochrome *b*₅₅₈ in neutrophils and other leukocytes, EPR data on neutrophil membranes or partly purified flavocytochrome *b*₅₅₈ have shown that the heme groups are in a low-spin hexacoordinated configuration in both ferric (Fe³⁺) and ferrous (Fe²⁺) heme iron oxidation states,^{45–47} indicating that electron transfer to oxygen in activated flavocytochrome *b*₅₅₈ does not occur via direct heme iron–oxygen coordination. Instead, involvement of the heme edge rather than the iron center in superoxide formation has been suggested.^{27,48}

Early resonance Raman (RR) spectroscopy data on concentrated neutrophil suspensions and isolated neutrophil membranes at low temperature (90 K) also revealed the presence of low-spin, six-coordinate hemes in both oxidation states of flavocytochrome b_{558} .⁴⁹ Comparison with synthetic models led the authors to suggest that two histidine ligands are coordinated to the heme iron centers. This was confirmed by later RR measurements on purified flavocytochrome b_{558} at both cryogenic (25 K)⁵⁰ and room (293 K)⁵¹ temperature. More recently, by introducing point substitutions in histidine residues that lie within transmembrane domains (see Fig. 7.2), it was shown that histidines 101, 115, 209, and 222 are critical for heme incorporation and biosynthetic maturation of flavocytochrome b_{558} .⁵² These four histidines involved in axial ligation are strictly conserved among all NADPH oxidase (Nox) family members.⁵³

7.5.4 Single-Cell RR Microspectroscopy on Leukocytes

As outlined in Section 7.2, a big advantage of Raman microspectroscopy over bulk Raman spectroscopy is that vibrational information can be obtained from subfemtoliter volumes inside a sample, allowing Raman images of cells to be recorded with high spatial resolution. This is made possible by strongly focusing the excitation light through a microscope objective and by using a confocal pinhole in the detection path.

Having demonstrated the feasibility of confocal nonresonant Raman microspectroscopy at 660-nm excitation on human granulocytes in 1990–1991,^{14,54} we reported an RR microspectroscopy study of human eosinophilic granulocytes in 1994.⁵⁵ Eosinophils are involved in the nonspecific host defense against parasites and are modulators of innate and adaptive immunity.⁵⁶ At 413.1 nm excitation, the RR spectrum from these cells is dominated by contributions from eosinophil peroxidase (EPO), a very abundant hemoprotein that plays a role in parasite destruction by producing microbicidal hypohalites from peroxide. EPO contains a ferric protoporphyrin IX prosthetic group in the resting state and displays a Soret absorption maximum at 412 nm. In contrast to the *low-spin*, six-coordinate heme groups found in flavocytochrome b_{558} (see Section 7.5.3), RR data on EPO inside eosinophils indicates that the heme is *high-spin*, six-coordinate in both ferric and ferrous oxidation states.^{17,55,57} Based on comparison with other peroxidases (e.g., lactoperoxidase),^{58,59} it was suggested that in the resting state, histidine and water serve as proximal and distal ligands for the EPO heme group, respectively.

We reported the first RR spectra from the cytoplasm of living neutrophils in 1998.^{60,61} By recording spectra from living normal, myeloperoxidase-deficient, and flavocytochrome b_{558} -deficient neutrophils, as well as from isolated specific granules (containing flavocytochrome b_{558}) and azurophilic granules (containing MPO), we found that at 413.1 nm excitation the RR signal of these cells originates predominantly from flavocytochrome b_{558} , with a minor contribution from myeloperoxidase. The strong Soret absorption bands in the resting state of flavocytochrome b_{558} and MPO at 413 nm and 428 nm, respectively, cause the Raman scattering intensity of these enzymes to be enhanced by resonance at 413.1 nm excitation. Figure 7.3A (spectrum 1) shows a representative RR spectrum taken from a single paraformaldehyde-fixed neutrophil.

Strong porphyrin modes observed at 677 (ν_7), 1130 ($\nu_6 + \nu_8$), 1375 (ν_4), 1521, 1552, and 1588 cm^{-1} , and the vinyl stretching mode at 1614 cm^{-1} correspond closely to the reported RR bands from similar measurements in living neutrophils,^{60,61} indicating that paraformaldehyde fixation does not alter the RR properties of the heme chromophores buried inside flavocytochrome b_{558} and MPO. This is an important conclusion with respect

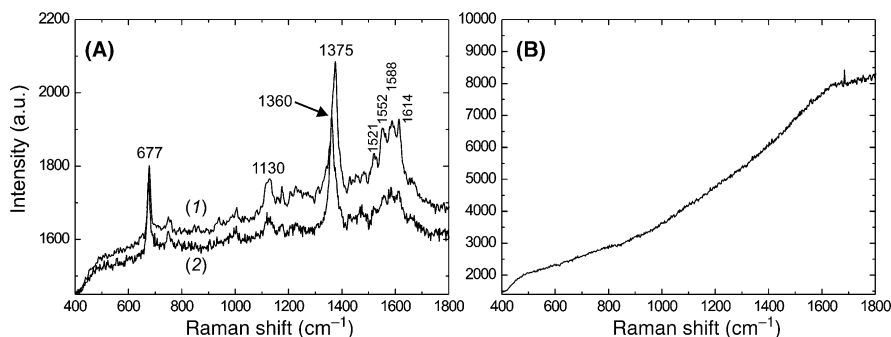


Figure 7.3. (A) RR spectra from a single untreated (1) and dithionite-reduced (2) neutrophil. These spectra were acquired in the scanning microspectroscopy mode (see Section 7.4.2). Excitation power 1 mW at 413.1 nm, 30 s accumulation time per spectrum. For band assignments, see Ref. 60. (B) Fluorescence spectrum of 4 μ M FAD (in water) in the 400 to 1800 cm^{-1} spectral region, showing that FAD fluorescence may contribute to the background in RR spectra from neutrophils. Excitation power 4.6 mW at 413.1 nm, 100 s accumulation time.

to RR imaging experiments, which have so far been performed on paraformaldehyde-fixed neutrophils (see Section 7.6).

The addition of excess sodium dithionite to intact neutrophils, which is known to completely reduce the heme groups in flavocytochrome b_{558} and MPO to their ferrous (Fe^{2+}) state,^{62–64} leads to a large spectral shift from 1375 to 1360 cm^{-1} in the ν_4 oxidation-state marker band (Fig. 7.3A, spectrum 2). In addition to this spectral shift, a reduced background is also observed after dithionite treatment (Fig. 7.3A). Dithionite also reduces flavin groups in flavoproteins, including flavin adenine dinucleotide (FAD) in flavocytochrome b_{558} ,^{65,66} which converts these moieties from an oxidized fluorescent state to a reduced state that is much less fluorescent. We therefore tentatively assign the background in the RR spectra from neutrophils to flavin/flavoprotein autofluorescence. The fluorescence spectrum of oxidized FAD recorded at 413.1 nm excitation and in the same spectral region as the RR spectra from neutrophils supports this assignment (Fig. 7.3B).

The fact that heme ferric and ferrous redox states in flavocytochrome b_{558} and MPO can be distinguished with RR spectroscopy implies that activation of NADPH oxidase, which is known to involve a (transient) reduction of the flavocytochrome b_{558} heme groups during the redox cycle,²⁷ might be detected inside living neutrophils by RR spectroscopy. Stimulation of neutrophils with phorbol 12-myristate 13-acetate (PMA) or by phagocytosis of *E. coli* bacteria, two powerful activators of the NADPH oxidase, indeed led to changes in the RR spectra that could be assigned to a partial reduction of both flavocytochrome b_{558} and MPO.^{61,64} Control experiments with flavocytochrome b_{558} -deficient neutrophils from a CGD patient failed to show a reduction of intracellular MPO, strongly suggesting that the observed changes in the RR spectra from normal cells upon stimulation are due to NADPH oxidase activation. Using p67^{phox}-deficient CGD neutrophils, we have recently provided conclusive evidence for this hypothesis.⁶⁷ Neutrophils lacking p67^{phox} have normal expression levels of flavocytochrome b_{558} and MPO and display resting and dithionite-reduced RR spectra that are identical to those from quiescent and dithionite-reduced wild-type neutrophils, respectively. However, p67^{phox}-deficient cells produce very little if any superoxide upon stimulation with NADPH oxidase activators.^{68,69} Figure 7.4 shows that p67^{phox}-deficient neutrophils that have phagocytosed polystyrene microspheres do not show any changes in their stimulated-minus-resting RR spectra (Fig. 7.4a), recorded in scanning

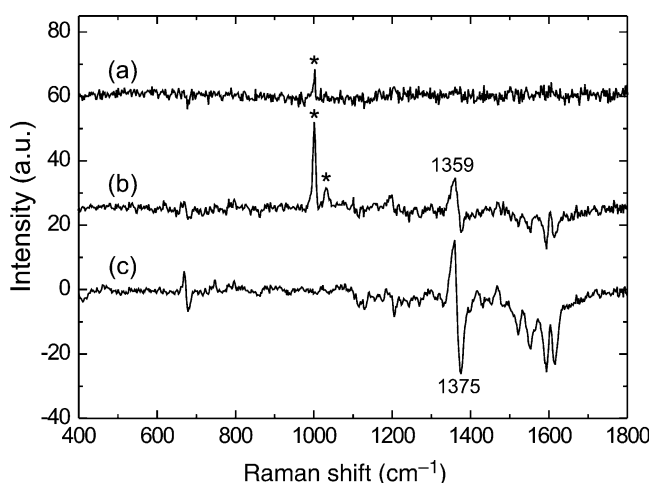


Figure 7.4. RR microspectroscopy shows that activation of neutrophilic granulocytes by phagocytosis of polystyrene microspheres leads to a partial reduction of the heme iron redox centers in flavocytochrome b_{558} and MPO. Shown are RR difference spectra, taken in scanning microspectroscopy mode, of stimulated minus resting p67^{phox}-deficient (a) and wild-type (b, c) neutrophils. In spectra a and b, cells were stimulated by allowing them to phagocytose polystyrene microspheres (bands marked with asterisks); in spectrum c, cells were treated with sodium dithionite to fully reduce heme iron redox centers. Spectra a and b have been shifted along the ordinate for clarity. For measurement conditions, see Ref. 67.

microspectroscopy mode from a region around the phagosome, whereas corresponding RR spectra from phagocytosing wild-type neutrophils clearly indicate a partial reduction of flavocytochrome b_{558} and MPO (Fig. 7.4b). When compared to the full reduction of these hemoproteins with dithionite (Fig. 7.4c), it is estimated that stimulation of neutrophils by phagocytosis results in $\sim 40\%$ reduction of flavocytochrome b_{558} /MPO. We have previously reported similar values using absorption spectroscopy and RR microspectroscopy on neutrophils stimulated with PMA.⁶⁴ However, such high flavocytochrome b_{558} reduction levels are more consistent with literature values reported for anaerobic ($\sim 50\%$ reduction)^{70–72} than for aerobic ($\sim 10\%$ reduction)^{65,73,74} NADPH oxidase activation studies. The difference between the levels of flavocytochrome b_{558} reduction under anaerobic and aerobic conditions is caused by the fact that in the absence of oxygen as a terminal electron acceptor, electrons will accumulate on the heme moieties. Consistent with this, it has been shown that the *rate* of heme reduction and superoxide production is approximately 1000 times higher in aerobic than in anaerobic experiments.^{70,72} Although our RR experiments on neutrophils were performed under aerobic conditions, the discrepancy between the level of flavocytochrome b_{558} reduction in our experiments and in the biochemical literature is likely to reflect differences in sample constitution (intact, live cells in our experiments versus cell-free, detergent-solubilized flavocytochrome b_{558} in the literature), temperature, or other factors.

In conclusion, our RR microspectroscopy results on live neutrophils prove that the flavocytochrome b_{558} hemes are part of the electron transport chain of NADPH oxidase and that electron transfer to heme is impaired in p67^{phox}-deficient CGD neutrophils, which is consistent with previous biochemical, cell-free experiments suggesting that p67^{phox} is critical in regulating electron transfer from NADPH to FAD.⁷⁵ The great advantage of being

able to detect the activity of flavocytochrome b_{558} in intact, individual live cells in a label-free manner will make RR microspectroscopy the technique of choice for studying the presence or absence of electron transport across flavocytochrome b_{558} in those CGD neutrophils where the expression level of flavocytochrome b_{558} is normal.²⁶

7.5.5 Single-Cell RR Microspectroscopy on PLB-985 Cells

As mentioned before, studies with cells from CGD patients have contributed significantly to our understanding of the phagocyte NADPH oxidase – in particular, those investigations in which mutations leading to expression of normal amounts of nonfunctional NADPH oxidase subunits were used. However, due to the scarcity of CGD patients with such mutations and the fact that neutrophils are terminally differentiated cells that are refractory to genetic manipulation, the possibilities for detailed microscopy studies of NADPH oxidase activation and regulation in living CGD neutrophils are limited. The development of the PLB-985 myeloid leukemic cell line as a model cell line for neutrophils has overcome these limitations. In PLB-985 cells, which can be cultured in the laboratory and induced to differentiate into mature neutrophil-like cells, the gene for flavocytochrome b_{558} has been knocked out, resulting in an X-CGD phenotype.⁷⁶ In addition, these cells can be transfected with DNA coding for wild-type or CGD-mutant flavocytochrome b_{558} .^{76,77} CGD-like cells can thus be cultured and studied without having to rely on frequent blood donations from patients.

We have recently begun to characterize PLB-985 cells by RR microspectroscopy at 413.1 nm excitation. As a prelude to future RR studies on CGD-like PLB-985 cells in which mutant forms of flavocytochrome b_{558} are expressed, we are first investigating wild-type PLB-985 cells. In the undifferentiated form, which is the promyelocytic state in which these cells are propagated, PLB-985 cells are already actively synthesizing MPO⁷⁸ but their expression of flavocytochrome b_{558} is low. During differentiation to the mature granulocyte state, expression of flavocytochrome b_{558} increases to a similar total level as in blood neutrophils.⁷⁹

Figure 7.5 shows the average RR spectrum of live, undifferentiated PLB-985 cells adhered to a CaF₂ slide, recorded at 1 mW of 413.1 nm excitation in scanning microspectroscopy mode.

Comparing this spectrum with RR spectra from neutrophils shown in Figure 3 and reported before,⁶⁰ it is clear that the major bands at 344, 411, 674, 748, 1130, 1361, 1373, 1487, 1583, and 1618 cm⁻¹ in the RR spectrum of PLB-985 cells (Fig. 7.5) originate from flavocytochrome b_{558} or MPO. The ratio of the integrated intensity of bands in the 1500- to 1690 cm⁻¹ spectral region (region 2 in Fig. 7.5) and the intensity of the 1300- to 1420 cm⁻¹ region (region 1), which is a crude indicator for the MPO/flavocytochrome b_{558} ratio, is 1.68 for PLB-985 cells and 1.43 for neutrophils, indicating that the MPO/flavocytochrome b_{558} ratio is higher in undifferentiated PLB-985 cells than in mature neutrophils.⁸⁰ This is expected and relates to the different differentiation stages of these cell types. In an absorption spectroscopy study using HL-60 promyelocytic cells, of which the PLB-985 cells are a subclone, it was also found that the ratio of MPO/flavocytochrome b_{558} in undifferentiated HL-60 cells is much higher than in differentiated HL-60 cells and in mature neutrophils.⁸¹

The preliminary RR results on hemoproteins in PLB-985 cells shown here suggest that the RR detection of flavocytochrome b_{558} in a cell system other than neutrophils is feasible. We therefore expect that RR microspectroscopy on PLB-985 cells expressing mutant forms of flavocytochrome b_{558} , obtained by genetic engineering, will facilitate research into the electron transport chain of NADPH oxidase and thus into the causes of chronic granulomatous disease.

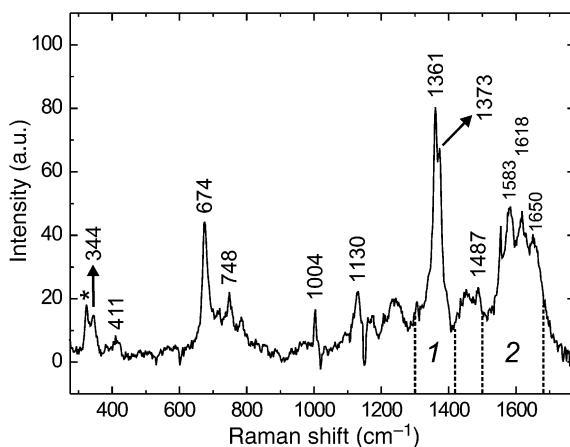


Figure 7.5. Average RR spectrum (background-corrected) of 48 undifferentiated PLB-985 cells, recorded in scanning microspectroscopy mode (60 s per spectrum) at 1 mW of 413.1 nm excitation. The band marked with an asterisk at 323 cm^{-1} is from the CaF_2 substrate. The sharp dips at 1019 and 1149 cm^{-1} are from defects in the CCD chip.

7.6 RESONANCE RAMAN MICROSCOPY ON NEUTROPHILIC GRANULOCYTES

7.6.1 Single-Cell Raman Microscopy

So far, we have discussed the detection of flavocytochrome b_{558} presence and activity in individual neutrophils by RR microspectroscopy. However, by scanning the focused laser beam across a cell and recording a full Raman spectrum at each position, a *Raman image* can be obtained by plotting the intensity of a Raman band of choice as a function of image pixel position, thereby providing spatial information on the presence of intracellular constituents. Due to the narrow width of Raman bands, multiplexed imaging on cells is in general much easier in Raman microscopy than in fluorescence microscopy. In multicolor fluorescence microscopy, dyes must be carefully chosen in order to avoid spectral overlap, although the commercial availability of spectral imaging systems in combination with linear unmixing routines has largely overcome these restrictions.⁸²

In the last 5 years, we have developed sensitive beam-scanning confocal Raman microscopy by constructing the setup depicted in Fig. 7.1 (discussed in Section 7.4.1), and we have demonstrated single-cell, high-resolution *nonresonant* Raman mapping at 647.1 nm excitation of DNA, proteins, and lipids in peripheral blood lymphocytes,⁸³ eye lens epithelial cells,⁸³ apoptotic HeLa cells,²² and phagocytosing neutrophils,⁸⁴ and the degradation of biodegradable microspheres inside macrophages.⁸⁵ Applications of (confocal) Raman imaging on cells or tissues reported by other groups include the characterization of breast duct epithelia⁸⁶ and bronchial tissue,⁸⁷ the identification of organelles and vesicles⁸⁸ and stress-induced subcellular changes in lung fibroblast cells,⁸⁹ and the mapping of oxygenated/deoxygenated hemoglobin and aggregated heme in erythrocytes.⁹⁰ Overviews discussing various aspects of Raman spectral imaging, such as recent advances in instrumentation, different types of image acquisition, data processing techniques, and biological applications are available.^{91–94}

Recently, we have combined confocal Raman and two-photon excitation fluorescence microscopy on cells⁹⁵ and also integrated Raman and scanning electron microscopy to study bone matrix constituents.⁹⁶ Correlative microscopy, such as the combination of Raman microscopy with other microscopy modalities (e.g., fluorescence, scanning probe, and electron microscopy), is a new development that increases the information content of microscopy data recorded from a sample and promises to be valuable for investigating biological specimens.

7.6.2 Mapping Flavocytochrome b_{558} Distributions in Neutrophils

Early microscopy studies based on immunocytochemistry in combination with electron and fluorescence microscopy, together with biochemical analysis of subcellular fractions, have shown that $\sim 80\%$ of the total flavocytochrome b_{558} pool is present in specific granules and secretory vesicles in unstimulated neutrophils (reviewed in Ref. [67]). Upon activation of neutrophils with soluble or particulate stimuli, many of these granules translocate to and fuse with the plasma and/or phagosomal membrane, leading to the insertion of flavocytochrome b_{558} into these membranes. Subsequent activation of NADPH oxidase then results in the release of superoxide into the extracellular environment or the phagosomal lumen.

Having demonstrated the possibility of detecting flavocytochrome b_{558} in resting and activated neutrophils using RR point or scanning microspectroscopy (see Section 7.5.4), we undertook RR spectral imaging experiments to visualize the intracellular distribution of flavocytochrome b_{558} in quiescent and phagocytosing cells. After careful optimization of the RR imaging conditions in terms of excitation power, accumulation time per pixel, and step size of the diffraction-limited spot, it was found that reproducible RR images of flavocytochrome b_{558} in neutrophils could be obtained by employment of 1 mW of 413.1 nm excitation power, 1-s accumulation time per pixel, and 0.4 to 0.5 μm step size.^{17,97} Higher excitation powers and smaller step sizes led to pronounced photobleaching of the flavocytochrome b_{558} RR signal. It should be noted that the step size used in our RR imaging experiments does not satisfy the Nyquist criterium for optimal image pixel sampling,⁹⁸ whereas in nonresonant Raman imaging this criterion can be met without losing Raman signal due to photobleaching.^{22,84,95} A more detailed discussion of photobleaching of the flavocytochrome b_{558} signal under a variety of conditions is provided in Section 7.7. We also point out that Raman microscopy experiments on neutrophils are performed on paraformaldehyde-fixed cells, since the high motility of these cells on the substrate is not compatible with the ~ 17 min exposure time presently required in a mapping experiment.

Figure 7.6A shows an RR image of the flavocytochrome b_{558} distribution in a resting neutrophil. This image was obtained by plotting the intensity of the 1360 cm^{-1} band of dithionite-reduced flavocytochrome b_{558} as a function of position. The corresponding hierarchical cluster analysis image of the same RR data set is shown in Fig. 7.6B. From the average RR spectra extracted from the different clusters (not shown), we assign the blue cluster to flavocytochrome b_{558} -rich regions, the magenta cluster to flavocytochrome b_{558} -poor regions and the cytoplasm, and the green/yellow clusters to the nucleus (nuclei in mature neutrophils are multilobe-shaped). The small size and relative proximity of specific granules and secretory vesicles (diameter 100–300 nm),^{99,100} in which the majority of flavocytochrome b_{558} resides, precludes the observation of individual granules/vesicles in RR images of neutrophils. In contrast, lipid droplets of $\sim 1\text{ }\mu\text{m}$ diameter can easily be visualized in neutrophils with nonresonant Raman microscopy.⁸⁴

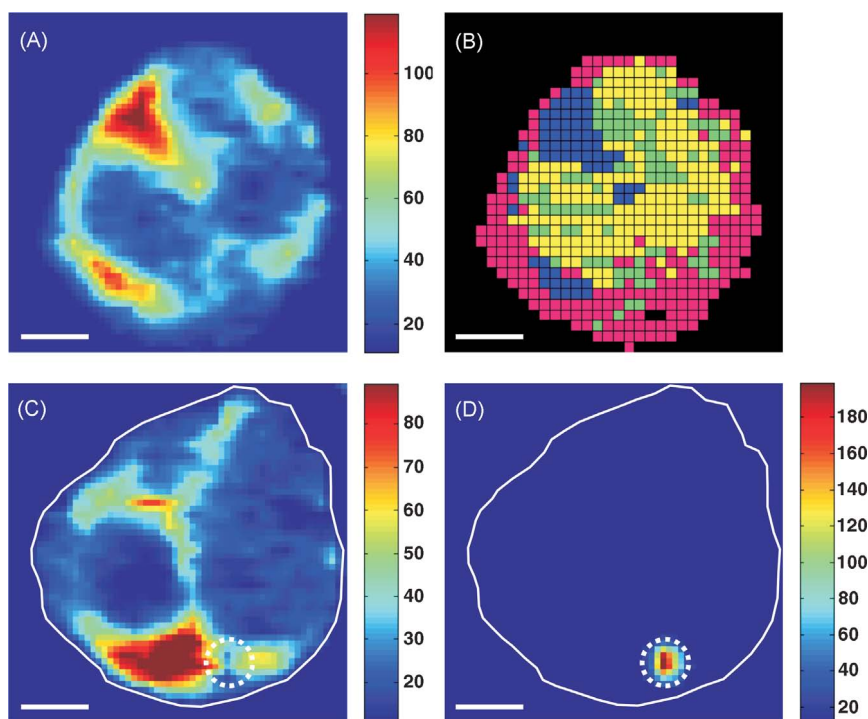


Figure 7.6. (A) RR microscopy image of the flavocytochrome b_{558} distribution in a resting neutrophil. (B) Hierarchical cluster analysis (HCA) image corresponding to image A. (C, D) RR images of the flavocytochrome b_{558} (C) and polystyrene (D) distribution in a neutrophil that has phagocytosed a PS microsphere. Images A and C were constructed in the 1360 cm^{-1} RR band of dithionite-reduced flavocytochrome b_{558} , whereas image D was constructed in the 1000 cm^{-1} Raman band of polystyrene. Dashed circles show the location of the PS microsphere, and the cell border in C and D is outlined in white. Image acquisition was performed using 1 mW of 413.1-nm excitation power, 32×32 image pixels, and 1 s accumulation time per pixel. Scale bars represent $3\text{ }\mu\text{m}$.

After the uptake of polystyrene microspheres by neutrophils, part of the flavocytochrome b_{558} pool can be found in close proximity to the ingested particle, as shown by the RR images in Figs. 7.6 (see also Refs. [17,67,97]). The visualization of both the phagocytosed particle (using the strong Raman band of polystyrene at 1000 cm^{-1}) and flavocytochrome b_{558} from a single recorded data set is an example of the multiplexed imaging possibilities of Raman microscopy. Recently, we also showed by RR imaging that the translocation of flavocytochrome b_{558} to an internalized microsphere is not impaired in p67^{phox} -deficient CGD neutrophils,⁶⁷ which is expected based on the different signaling pathways that regulate granule translocation to the phagosome, including flavocytochrome b_{558} translocation, and NADPH oxidase activation (involving p67^{phox}). Finally, we have exploited the strong RR signal of eosinophil peroxidase (EPO) in eosinophils⁵⁵ to visualize the EPO distribution in the cytoplasm of eosinophils.¹⁷ The RR signal of EPO in eosinophils is much more intense than the flavocytochrome b_{558} /MPO signal in neutrophils, so imaging of EPO can be performed at lower excitation power and/or faster speed than flavocytochrome b_{558} imaging.

To conclude this section, Raman microscopy experiments in the last few years have demonstrated that high-resolution cellular imaging based on resonance Raman microscopy enables the label-free visualization of the intracellular distribution of flavocytochrome b_{558} in neutrophils and EPO in eosinophils, two crucial enzymes in leukocyte innate immunity. We expect that RR microscopy will continue to be a versatile technique in CGD research and that it also has the potential to become a new tool in the characterization of *nonphagocyte flavocytochrome b_{558} homologs* found in a wide variety of cell types and tissues and implicated in, for example, redox signaling, host defense, regulation of gene expression, and cell differentiation.¹⁰¹

7.7 PHOTBLEACHING AND LIGHT-INDUCED CELL DAMAGE IN RESONANCE RAMAN MICROSCOPY

7.7.1 Introduction

The low signal intensities in spontaneous, nonresonant Raman microscopy on single cells, which are due to the very small Raman scattering cross sections of biological macromolecules such as DNA, proteins, and carbohydrates, necessitates the use of high laser excitation powers (~ 10 – 100 mW) to obtain high-quality spectra and to avoid very long data acquisition times. This inevitably raises questions about the response of living cells, and to a lesser extent fixed cells, to such high illumination doses in terms of photobleaching of the Raman signal and inflicted cellular photodamage. Some reports about vibrational microspectroscopy and microscopy on cells, including coherent anti-Stokes Raman scattering (CARS) microscopy, have therefore included data and discussions concerning the presence or absence of photoinduced effects in (the vibrational spectra or images of) cells.^{102–105} We have previously investigated the effect of high laser powers on the reproducibility of nonresonant Raman spectra recorded from living and fixed cells and on cell viability.^{22,106} Sample degradation was found to occur with 514.5 nm excitation light (using a dose of $2.5 \text{ J}/\mu\text{m}^2$) but not with 660 nm (at a dose of $30 \text{ J}/\mu\text{m}^2$).¹⁰⁶ Photochemical reactions were suggested to be responsible for light-induced degradation processes. In a nonresonant Raman imaging study on fixed cells, we showed that imaging can be performed at 100 mW 647.1 nm excitation in combination with a pixel accumulation time of 1 s (i.e., a dose of $523 \text{ mJ}/\mu\text{m}^2$) without loss of Raman signal.²² As a rule of thumb, it can be stated that popular excitation wavelengths for nonresonant Raman microscopy on cells, such as 632.8 nm (He–Ne laser), 647.1 nm (Kr^+ laser), and 785 nm (diode laser), can be used at rather high power (up to ~ 100 – 150 mW) on most cell types without excessive cell degradation or loss of Raman signal, unless the sample contains chromophores with high extinction coefficients at these wavelengths.

The situation is quite different for resonance Raman microscopy and imaging on cells. Here, as in fluorescence spectroscopy, intracellular chromophores such as hemes and carotenoids^{107–109} are excited to a higher electronic level by absorption of excitation photons. Because photobleaching generally occurs from reactions involving the electronically excited state, it is expected that photobleaching of the Raman signal is more prominent in resonance than in nonresonance Raman spectroscopy. Indeed, we observed photobleaching of the flavocytochrome b_{558} RR signal in neutrophils already at a dose of $12.8 \text{ mJ}/\mu\text{m}^2$, as described in Section 7.7.2. In addition, the UV–visible wavelengths that are commonly used in RR spectroscopy will cause an increased contribution of cellular autofluorescence as background to the RR signal in comparison to spectra obtained under

nonresonant Raman conditions. Wavelengths between 350 and 500 nm, which are suited to excite many available fluorophores used in cell biology, are known to cause cellular autofluorescence from, predominantly, porphyrins and redox cofactors such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and flavins.^{110,111} Although autofluorescence may be a nuisance in fluorescence microscopy of fluorophore-labeled cells and tissues, it can also be used to reveal fascinating spatiotemporal dynamics of metabolites in living cells^{112,113} and as a powerful tool in medical diagnosis.¹¹⁴

In the following sections, we will discuss the photobleaching of the flavocytochrome b_{558} RR signal in neutrophils under a variety of conditions. Moreover, as the RR spectra of these cells also provide information about their autofluorescence, the changes in autofluorescence during illumination at 413.1 nm will be included in our discussion.

7.7.2 Photobleaching of the Flavocytochrome b_{558} RR Signal

As mentioned in Section 7.6.2, we regularly use a step size of 0.4–0.5 μm during RR imaging on neutrophils in order to prevent excessive photobleaching of the flavocytochrome b_{558} Raman signal. To investigate photobleaching more systematically, we recorded 10 consecutive RR point spectra (each at a 12.8 $\text{mJ}/\mu\text{m}^2$ dose) from a single location in the cytoplasm of fixed neutrophils and integrated the intensity of the 1375 cm^{-1} band of flavocytochrome b_{558} as a function of total illumination dose.¹⁷ The flavocytochrome b_{558} RR signal, after background correction, was found to decay in a monoexponential fashion under three different illumination conditions, and the autofluorescence background decreased quickly to a stable baseline level in these three series. The monoexponential decay means that RR images of flavocytochrome b_{558} are not distorted by photobleaching, because the relative decrease in signal will be the same independent of flavocytochrome b_{558} concentration.

We have recently studied the autofluorescence of *fixed* neutrophils at 413.1-nm excitation in more detail by fluorescence microscopy, using the fluorescence detection branch of the confocal Raman setup (see Fig. 7.1 and Section 7.4.1). At 10 μW excitation power, which is 100-fold less compared to RR experiments, sufficient autofluorescence signal is detected to obtain good-quality images such as the one shown in Fig. 7.7A. Next, fluorescence timetraces were obtained from selected positions in the cell. Average time-traces of the high-intensity points marked 1 and low-intensity points marked 2 in Fig. 7.7A are shown in Fig. 7.7B. Curve-fitting analysis revealed that the two autofluorescence traces in Fig. 7.7B could be fitted with biexponential decay curves [i.e., $F = F_0 + A_{\text{fast}} \exp(-k_{\text{fast}} \cdot t) + A_{\text{slow}} \exp(-k_{\text{slow}} \cdot t)$]¹¹⁵ containing a fast-decaying and a slow-decaying component, with ratios of decay constants $k_{\text{fast}}/k_{\text{slow}} = 9.5$ for trace 1 and 11.7 for trace 2, and ratios of preexponential factors $A_{\text{fast}}/A_{\text{slow}} = 1.35$ for trace 1 and 1.52 for trace 2. These results indicate that the autofluorescence of neutrophils at 413.1-nm excitation, which likely originates mostly from flavins (e.g., FAD) as discussed in Section 7.5.4 and in the literature,^{116,117} can be empirically described by a two-component model with varying decay rate constants throughout the cell.

We have recently also investigated the effect of 413.1-nm illumination at varying doses (between 26 and 64 $\mu\text{J}/\mu\text{m}^2$) on *living* neutrophils by recording 50 consecutive RR spectra from the same cell in scanning microspectroscopy mode using different acquisition times per full scan (= per RR spectrum). The variation in the background-corrected intensity of the flavocytochrome b_{558} RR band centered at 1375 cm^{-1} as a function of spectrum number depends on the acquisition time per scan, as shown in Fig. 7.7C. The general trend is that the RR signal decreases with increasing spectrum number. Similar to fixed cells, this effect is

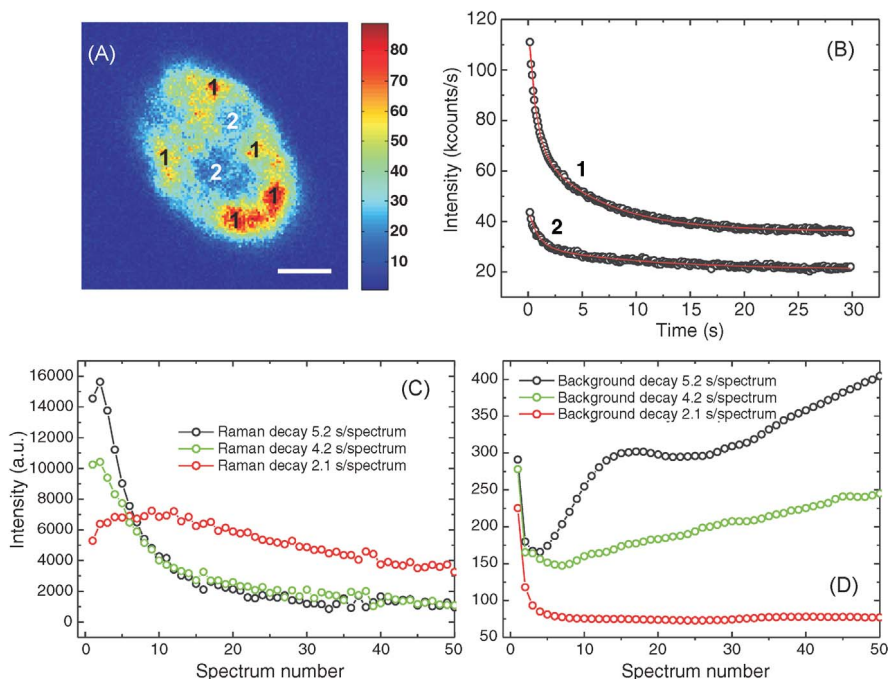


Figure 7.7. (A) Representative autofluorescence image of a neutrophilic granulocyte. This image was recorded using an avalanche photodiode in the fluorescence detection branch of the confocal Raman setup, as described in Section 7.4.1. Image acquisition conditions: excitation power $10\ \mu\text{W}$ at $413.1\ \text{nm}$, 128×128 pixels, $0.25\ \text{ms/pixel}$. The scale bar represents $3\ \mu\text{m}$. (B) Average autofluorescence timetraces from the high-intensity (marked “1”) and low-intensity (marked “2”) regions indicated in A. Experimental data points are shown in black, and biexponential decay curve fits are shown in red. (C) Excitation dose-dependent decay of the flavocytochrome b_{558} Raman signal upon repeated scanning of the same cell. RR measurements were performed on freshly isolated neutrophils adhered to a CaF_2 disk. Spectra (50 per cell) were recorded in the scanning microspectroscopy detection mode (see Section 7.4.2), using different signal accumulation times per spectrum (as shown in the legend). Flavocytochrome b_{558} Raman intensities, obtained by integrating the band centered at $1375\ \text{cm}^{-1}$, were corrected for the highly varying background (see part D). (D) Excitation dose-dependent photobleaching and light-induced autofluorescence upon repeated scanning of the same cell. The same data set as used in part C was employed, except that the 1850 - to $2200\ \text{cm}^{-1}$ spectral region, devoid in RR signals, was integrated to calculate the autofluorescence background.

due to photobleaching and, as expected, most pronounced for the longest acquisition time per scan (highest illumination dose) and least pronounced for the shortest scan time (lowest dose). One interesting feature of the three curves shown in Fig. 7.7C is that the RR intensities *increase* during the first few scans and start to decrease afterwards. Although at present we have no solid explanation for this behavior, it may be that cell and/or granule movement in the beginning of the experiment influences the intensity of flavocytochrome b_{558} and MPO RR signals.

From the same recorded RR data set that was used to construct the curves in Fig. 7.7C, we also determined the variation in the autofluorescence background of the

RR spectra as a function of spectrum number. This was done by integrating the $1850\text{--}2200\text{ cm}^{-1}$ spectral region where there is no RR contribution to the signal. Figure 7.7D shows an initial rapid decrease in autofluorescence background for the three different scan times, which was also observed in the RR experiments on fixed cells described above. However, after ~ 4 scans the background starts to increase again for the 5.2 s/scan and 4.2-s/scan cases but not for the 2.1 s/scan measurements. Clearly, there is no correlation between the variations of the flavocytochrome b_{558} RR (Fig. 7.7C) and the background (Fig. 7.7D) signals, which confirms our assignment of the background signal to autofluorescence. The increase in fluorescence background, which apparently occurs after crossing a threshold in total illumination dose, distinguishes living from fixed cells in RR spectra. Photoinduced cell damage caused by UV-visible and near-infrared illumination, which are used in single-photon and multiphoton fluorescence microscopy, respectively, has been investigated thoroughly in living cells.^{118,119} Prominent light-induced effects include ROS production,¹²⁰ disturbance of the cellular redox state,¹²¹ and direct and indirect (e.g., ROS-induced) damage to DNA.¹²² Impaired reproduction, oxidative stress, and apoptosis are among the cellular consequences of these detrimental processes. Moreover, an increase in cellular autofluorescence upon exposure to UVA light has been reported.^{123–125} In a study on Chinese hamster ovary (CHO) cells, König et al.¹²⁴ found that exposure of cells to 365 nm global illumination led to a fast decrease in autofluorescence, followed by a slower fourfold fluorescence increase in ~ 10 min. They point out that monitoring of NAD(P)H autofluorescence at 365 nm excitation may provide information on light-induced disturbance of metabolism. We speculate that, in a similar way, the flavin autofluorescence increase that we observe upon repeated scanning at 413.1-nm excitation (Fig. 7.7D) is a response of the living neutrophils to the inflicted photodamage.

We conclude this section by providing the following guidelines that are based on the results of nonresonance and resonance Raman degradation studies on cells:

- At excitation wavelengths above ~ 630 nm, relatively high powers (~ 100 mW) and doses ($\sim 100\text{--}500$ mJ) of focused laser light may be employed on cells without suffering from decreases in nonresonance Raman signal. However, it is still recommended to perform and report degradation studies for every new type of Raman experiment on cells that is planned. If maintaining cells alive is of importance, cell viability tests after laser irradiation – for example, by means of the trypan blue exclusion assay – should be carried out.
- At excitation wavelengths below 600 nm, such as the common laser lines at 488, 514, 532, and 568 nm, it is difficult (if not impossible) to completely avoid photodamage and cell damage under the conditions that should provide high-quality nonresonance Raman signals – that is, excitation powers $>5\text{--}10$ mW. Moreover, background signal owing to autofluorescence will start to contribute to the detected Raman signal at these excitation wavelengths.
- At 413.1-nm excitation, as used in the resonance Raman experiments discussed in this chapter, both photoinduced bleaching of the RR signal and cellular damage play a role. To maximize the RR signal from fixed and living cells, recording conditions must be carefully optimized in terms of excitation power, pixel acquisition time, and, in scanning microspectroscopy or imaging mode, step size. In the case of flavocytochrome b_{558} /MPO in neutrophils and PLB-985 cells, autofluorescence contributes to, but does not overwhelm, the RR signal.

7.8 CONCLUDING REMARKS

In this chapter, we have shown that both the activity and distribution of flavocytochrome b_{558} in neutrophilic granulocytes can be detected in a label-free manner using single-cell resonance Raman microspectroscopy and imaging at 413.1 nm excitation. Despite potential problems such as photobleaching and cell damage that are commonly associated with UV radiation, it is possible to obtain high-quality RR spectra from neutrophils by careful optimization of the recording conditions. We have also demonstrated, for the first time, that flavocytochrome b_{558} and myeloperoxidase can be detected by RR microspectroscopy in a cell type other than neutrophils – that is, in PLB-985 cells. These cells can be cultured *in vitro* and induced to differentiate into neutrophil-like cells. Because flavocytochrome b_{558} mutants that are found in chronic granulomatous disease can be stably expressed in PLB-985 cells, RR microspectroscopy experiments on such genetically engineered cells will shed new light on the molecular defects that occur in some CGD types.

With the recent discovery that other flavohemoprotein-containing NADPH oxidase family members are widely distributed in a range of cell types and tissues, albeit at lower concentrations than flavocytochrome b_{558} in neutrophils, it is likely that the importance of RR microspectroscopy and imaging as a label-free biophysical technique to characterize hemoproteins in their natural environment will only increase.

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RESONANT RAMAN SCATTERING OF HEME MOLECULES IN CELLS AND IN THE SOLID STATE

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8.1 INTRODUCTION

Iron porphyrins are the prosthetic groups of heme proteins and constitute an important class of biological molecules that behave as enzymes catalyzing a variety of vital oxidation–reduction reactions in many biological systems. Porphyrins and metalloporphyrins continue to be widely investigated as models that mimic functions of heme proteins, heme enzymes, and supramolecular assemblies for nanowire energy transfer systems that mimic chlorophyll in green plants and photosynthetic bacteria. The therapeutic benefits of porphyrins in photodynamic therapy are only just being realized, and applications for these molecules as antineoplastic agents and antimalarials are now under investigation. Therefore, it is not surprising that the isolation, structural study, characterization, spectroscopic investigation, and modeling of heme compounds for both natural and synthetic heme systems will be the subject of fresh research for many years.

The inherent symmetry and electronic structure of heme molecules gives rise to configuration interaction and vibronic mixing between electronic transitions, producing a multiplicity of interesting resonance effects that can be examined by tuning through the excitation wavelengths from the ultraviolet (UV) to near-infrared (near-IR) wavelengths. Significant and useful signal-to-noise ratio spectra can be achieved when chromophoric complexes are electronically excited by the frequency of the incident photon. The resonance Raman (RR) effect occurs when the incident laser light frequency is in the vicinity of an

electronic absorption band of a chromophore. Under these conditions the intensities of certain Raman bands may be enhanced by factors of 10^3 to 10^5 .

The advent of back-illuminated CCDs optimized to the near-IR region along with quality diode lasers working at wavelengths from 750 to 870 nm enabled Raman exploration into the near-IR yielding spectacular results. Astonishing resonant enhancement was observed at 780 and 830 nm for hemozoin (malaria pigment) in functional erythrocytes infected with *Plasmodium falciparum*. This “excitonic” enhancement was also observed in the iron protoporphyrin IX complexes hemin Fe(III)PPIXCl and hematin (Fe(III)PPIXOH) but more dramatically in hemozoin and its synthetic analogue β -hematin [Fe(III)PPIX]₂.^{1–3}

In this chapter we will convey our central findings investigating the enhancement at near-IR excitation wavelengths for heme molecules in the solid state. In order to present a mostly self-contained chapter, we commence with a brief introduction to resonance Raman scattering before discussing the resonance Raman spectroscopy of protoporphyrin IX in the solid state using near-IR excitation. Spectra showing an intriguing enhancement phenomenon observed at near-IR wavelengths for iron protoporphyrins including hemin, hematin, and β -hematin are presented, and possible mechanisms to explain the enhancement based on the Albrecht formulism⁴ and excitonic coupling are hypothesized. Finally, the application of the technology in malaria research is highlighted and the chapter is summarized.

8.2 ELECTRONIC STRUCTURE OF HEME MOIETIES

Hemoglobin absorbance spectra are dominated by intense $\pi \rightarrow \pi^*$ transitions, known as the Soret (or B state) between 400 and 500 nm and the α and β (Q_v or Q_0) bands at about 530 and 575 nm, respectively, from the ligated porphyrin moiety in oxygenated hemoglobin. The large conjugated system of the porphyrin and the relative small energy gap between its valence and conduction band (~ 2 eV) enables metalloporphyrins to absorb light strongly in the visible and near-UV region of the spectrum.⁵ Gouterman’s four-orbital model, illustrated in Fig. 8.1, is commonly used to describe the electronic wavefunctions for the lowest excited states of metalloporphyrins (the B and Q states).⁶ The high-energy Soret band and low-energy quasi-forbidden Q states result from the configuration interaction between electron excitations from a nearly degenerate pair of π orbitals (a_{1u} , a_{2u}) into a doubly degenerate pair of unoccupied π^* orbitals (e_g).⁶ The B and Q states constitute in-phase and out-of-phase combinations of the $\pi \rightarrow \pi^*$ excitations with approximately equal transition dipole moments. The filled orbitals are similar in energy; consequently, there is a large configuration interaction between the orbital excitations $a_{1u} \rightarrow e_g$ and $a_{2u} \rightarrow e_g$. In the case of the Soret band the two transition dipoles add together, giving the intense band observed at ~ 400 nm, while for the weaker Q_0 band the transition dipoles almost cancel one another out. The remainder of the intensity from the Q_0 band is regained through vibronic mixing with the Soret band resulting in the Q_v side-band, which is an envelope of vibrational bands built on the side of the Q_0 band.⁵ The intense Raman spectra obtained from metalloporphyrin complexes have been interpreted based on vibronically induced scattering from the B (Soret) or Q states from the porphyrin macrocycle.⁵

Exciting into the near-IR region of heme moieties provides an avenue to explore resonance Raman enhancement associated with charge-transfer and d–d electronic transitions of heme moieties. Oxyhemoglobin exhibits a very broad band centered at 925 nm in solution absorption spectra. MCD and CD measurements show this band to be composed of four separate bands designated by Eaton et al.⁷ as bands I to IV. Extended Huckel calculations on the ferrous porphine complexed to imidazole and oxygen indicate that while the d_{xy} orbital remains relatively pure both the d_{xz} and d_{yz} are strongly mixed with oxygen π_g

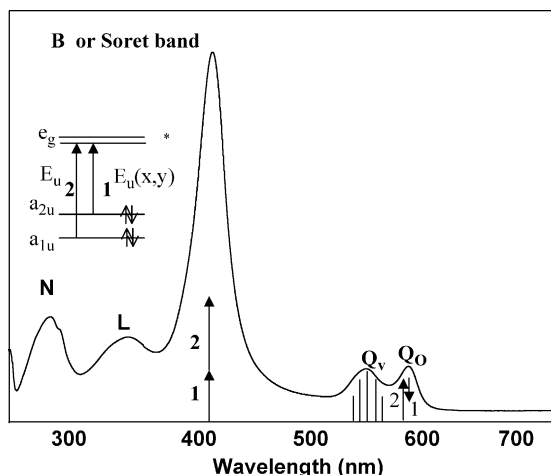


Figure 8.1. UV-visible absorption spectrum of oxygenated hemoglobin within red blood cells showing the Gouterman four-orbital, which explains the origin of the Soret (B) band and Q bands.

orbitals, resulting in four closely spaced molecular orbitals.⁸ Based on circular dichroism, spectroscopy bands I and II, which appear at 1300 and 1150 nm, have been assigned to the magnetic-dipole-allowed transitions $d_{yz} + O_2(\pi_g) \rightarrow d_{xz} + O_2(\pi_g)$ and $d_{x^2-y^2} \rightarrow d_{xz} + O_2(\pi_g)$, respectively. Based on curve-fitting MCD spectral data of oxyhemoglobin, bands III (x polarized) and IV (y, z polarized), which appear at 980 and 780 nm, were assigned to $a_{2u}(\pi) \rightarrow d_{xz} + O_2(\pi_g)$ and $a_{1u}(\pi) \rightarrow d_{xz} + O_2(\pi_g)$, respectively. In the case of deoxygenated hemoglobin, Eaton et al.⁸ assigned a z-polarized band appearing at 917 nm to the $d_{xz} \rightarrow e_g(\pi^*)$ transition and designated it as band I. A band at 813 nm, designated band II, was assigned to the mostly z-polarized transition $d_{xz} \rightarrow d_{z^2}$. A very weak charge transfer band, centered at 758 nm and designated band III, is observed deoxygenated hemoglobin and deoxymyoglobin. This band is more than a 1000 times less intense than the Soret band⁹ and is quite narrow, and models that fit the line shape are reported to exhibit only weak coupling to a low-frequency vibrational mode.¹⁰ Band III has attracted considerable attention due to its sensitivity to protein conformational changes in response to bond breaking and recombination.⁹ The position of band III is assumed to be related to the out-of-plane position of the Fe. Based on the B-term magnetic circular dichroism (MCD) spectrum, band III is assigned to the $a_{1u}(\pi) \rightarrow e_g(d_{yz})$ transition.⁷

High-spin ferric complexes such as hemin and β -hematin have an electronic ground state consisting of orbitally nondegenerate spin sextet $^6A_{1g}$. Based on extended Huckel theoretical calculations performed by Zerner et al.¹¹ on ferric high-spin porphine complexes including hemin and hematin, four charge-transfer transitions have been predicted. These involve promotions from the top four filled porphyrin π orbitals into the degenerate d_{xz} , d_{yz} iron orbitals.¹² Polarized single-crystal absorption spectra showed that all four bands were x, y-polarized, therefore indicating that the transitions are degenerate.¹² MCD analysis of aquomethemoglobin indicated that a broad near-IR band with an absorption maxima at 1040 nm contains two degenerate transitions at 1100 nm and 800 nm, which were also resolved in the fluoride complex and assigned to $a_{2u}(\pi) \rightarrow d_{xz}$, d_{yz} and $a_{1u}(\pi) \rightarrow d_{xz}$, d_{yz} , respectively.⁸ UV-Vis spectra of tetraphenylporphyrin Fe(III)Cl recorded under vacuum on a thin-layer optical cell clearly show an unassigned band at 870 nm.¹³ Eaton and

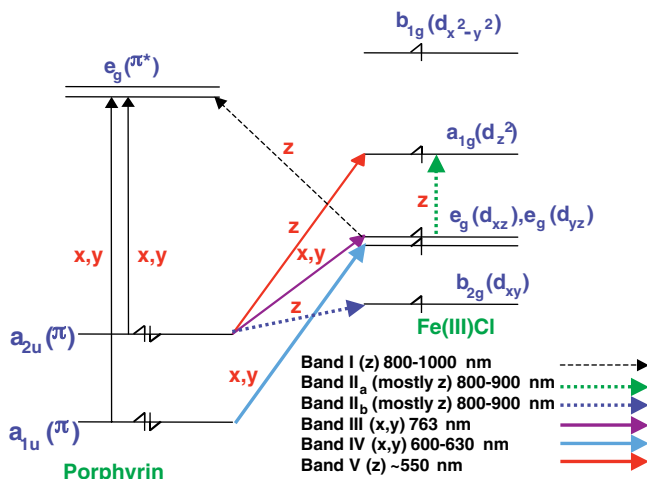


Figure 8.2. A qualitative molecular orbital diagram for the allowed transitions involving the Fe(III) and porphyrin orbitals for hemin based on the calculations by Zerner et al.¹¹ Characteristic bands (I–IV) are indicated, along with their respective polarizations.

Hochstrasser¹² identified a z-polarized band at 695 nm in ferricytochrome *c* and assigned it to the $a_{2u}(\pi) \rightarrow d_{z^2}$ transition. Based on symmetry considerations, the $a_{2u}(\pi) \rightarrow d_{z^2}$ is allowed but the $a_{1u}, a_{2u}(\pi) \rightarrow d_{x^2-y^2}$ pair are not.

Charge transfer can also take place from occupied d orbitals to vacant porphyrin $e_g(\pi^*)$ orbitals. Although this process is parity forbidden, the restriction can be removed for hemes without an inversion center because of mixing in metal p-orbital character.¹⁴ Figure 8.2 shows a qualitative molecular orbital diagram for the allowed transitions involving the Fe (III) and porphyrin orbitals for hemin based on the calculations of Zerner et al.,¹¹ Until now, no z-polarized transition has been reported for high-spin ferric hemes in the 800 to 1000 nm region; however, such z-polarized transitions have been predicted using a semiempirical quantum chemical INDO/ROHF/CI method.¹⁵

8.3 RESONANCE RAMAN SPECTROSCOPY

The basic Raman theory is well known and well described,^{16–20} and the basic processes are summarized in Figure 8.3. For a description of the resonance Raman effect the reader is referred the excellent texts by Spiro,⁵ Smith and Dent,²⁰ and Chang.¹⁶ The intensity of nonresonance Raman scattering is defined by the following equation:

$$I = C l \alpha^2 \nu^4 \quad (8.1)$$

where C is a constant that incorporates Planck's constant and the speed of light, I is the laser power, ν is the frequency of the laser, and α is the polarizability of the electrons in the molecule. Experimentally, we can adjust the power and frequency of our laser to alter the intensity of the Raman scattering.

Raman intensities can be greatly enhanced by excitation with wavelengths that closely approach an electronic absorption peak of an analyte. The early work of Tang and Albrecht²¹ provided insight into the relationship between resonance Raman intensities

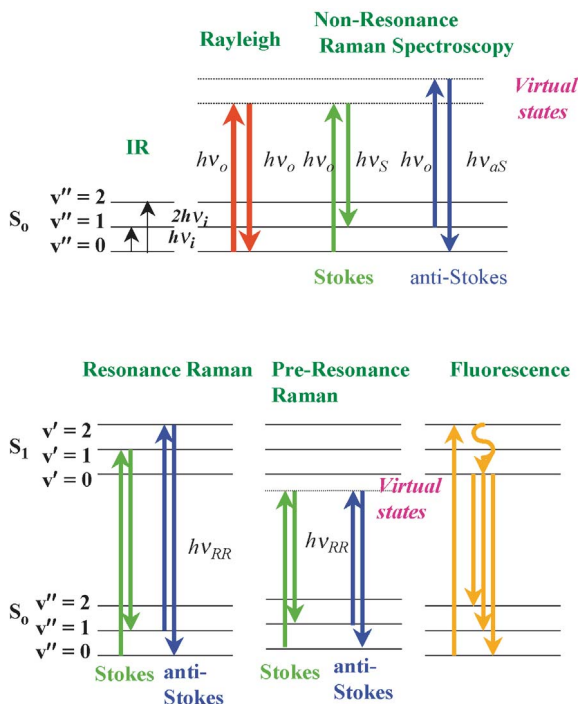


Figure 8.3. Top: Qualitative energy level diagram showing the processes of IR absorption (black arrow) and scattering including Rayleigh (red arrows), Stokes (Green arrows), anti-Stokes (blue arrows), and fluorescence (orange arrows). **Bottom:** Resonance and pre-resonance Raman scattering for Stokes and anti-Stokes scattering.

and excited-state structure. However, a theoretical description of resonant Raman scattering is complex at even the simplest level of approximation because there is no direct relationship between the intensities of a single Raman spectrum and the excited-state structure. The traditional approach to light dispersion is the sum-over-states approach derived by Kramers and Heisenberg²² and Dirac²³ from second-order time dependent perturbation theory. An alternative theory is the time-dependent approach developed by Hizhnykov and Tehver²⁴ and Heller,²⁵ which overcomes the computational difficulties encountered in the sum-over-states approach. Another theoretical approach is to use transform methods, which predict excited-state displacement from experimental data.^{25–27} These formulations exploit the relationship between the optical absorption cross section and the resonance Raman amplitude to obtain a general theoretical Raman excitation profile in terms of the measured absorption spectrum.²⁸ The reader is referred to the explanation by Myers and Mathies²⁸ or McHale¹⁸ for a general descriptions of both the time-dependent method and transform theory.

The two important terms that are derived from the sum-over-states approach are the A term and B term:

$$A = (\mu_e)^2 \frac{1}{\hbar} \sum_v \frac{\langle j|v\rangle\langle v|i\rangle}{\Delta v_v + i\Gamma_v}, \quad B = \mu_e \left(\frac{\partial \mu_e}{\partial Q} \right) \frac{1}{\hbar} \sum_v \frac{\langle j|Q|v\rangle\langle v|i\rangle + \langle j|v\rangle\langle v|Q|i\rangle}{\Delta v_v + i\Gamma_v} \quad (8.2)$$

where $|i\rangle$ and $|j\rangle$ are the initial and final vibrational wave functions of the ground electronic state, Q represents the normal coordinate operator, $|v\rangle$ represents the intermediate vibrational wave function in the resonant excited state, $\Delta\nu_v$ is the difference between the frequency of laser excitation wavelength and the frequency of the vibration in the excited state v , Γ_v is the half-width of the vibrational wave function for the excited state and represents the life time of the excited state, and $\hbar$ is Plank's constant. The A term is dependent on the square of the transition dipole moment μ_e and Franck–Condon integral products. We can see from the equation that as $\Delta\nu_v$ approaches zero, which occurs when the frequency of the laser matches the energy required to put the molecule into the vibronic excited state, the denominator of the first term reduces to Γ_v , which is a small energy correction factor for the lifetime of the molecule in the excited state. Thus under resonant conditions the denominator becomes very small, leading to the first term of the Taylor's expansion becoming very large, increasing polarizability, and giving much greater Raman scattering. To obtain intense scattering, the transition should start from a region of high electron density in the ground state and go to a state where the wave function is such that, once populated, would also contain significant electron density. This is referred to as overlap because of the vertical nature of the transitions.²⁰ The contribution of the B term to the resonance scattering depends on the extent the normal coordinate perturbs the electronic transition moment.²⁹ Type B modes mix the resonant electronic transition to one of higher energy. In the B -term scattering, two excited states are mixed through the coordinate operator Q . Consequently, any displacement in the molecule results in a geometry change that is needed to remix the electronic states to obtain new molecular states.²⁰ Thus if two transitions are close together in the visible region, like the Q and B (Soret) bands in hemoglobin, then the coordinate operator will help mix these two transitions together. B -term enhancement is only strong from the zero and first vibronic states of the excited state, whereas there is no restriction on the excited vibronic states giving rise to A -term enhancement; hence overtones are allowed by this mechanism.²⁰ A schematic diagram showing the two scattering mechanisms is depicted in Figure 8.4.

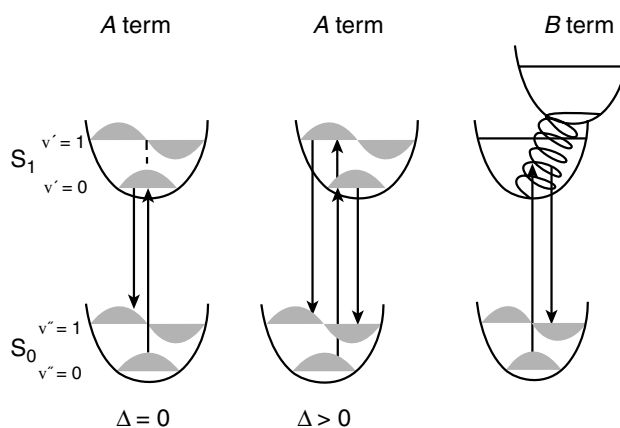


Figure 8.4. A schematic diagram showing type A (Frank–Condon) and type B (vibronic coupling) scattering mechanisms. In type A scattering the band intensity is dependent on the maximum overlap between the ground and excited vibronic states as a function of nuclear displacement, giving rise to enhanced totally symmetric modes. In type B scattering a low-frequency vibrational mode couples two nearby excited-state electronic transitions giving rise to enhanced non-totally symmetric modes.

Type A vibrational modes are expected to be totally symmetric A_{1g} (for D_{4h}), while type B modes may have any symmetry depending on the direct product of the two electronic transition representations – for example, B_{1g} , B_{2g} , and A_{2g} for D_{4h} . In the case of hemes, type A scattering is normally observed when exciting in the Soret (or *B*) band region (~ 400 nm), while type B scattering is usually observed when exciting in the Q-band region (500–600 nm).

8.4 RESONANCE RAMAN SPECTROSCOPY OF HEMES IN CELLS AND THE SOLID STATE

8.4.1 Raman Methods for Single Crystal and Cell Analysis

Raman experiments on dilute solutions using near-IR excitation wavelengths often have a poor signal-to-noise ratio because of the wavelength dependence of intensity [Eq. 8.1]. Consequently, high powers are required which can result in sample heating and thermal degradation. Spinning cells or flow through cells are often incorporated to avoid heating from constant laser exposure, but such devices will always attenuate the scattered photons to some degree. One way to avoid these problems in the near-IR is to work in the solid state using a water immersion objective in a temperature-controlled unit. This serves the dual purpose of reducing sample heating and improving the signal-to-noise ratio because there is no attenuating surface between the microscope objective and sample. The high numerical aperture of the water immersion objective enables efficient collection of scattered photons from a highly spatially resolved target area. Another advantage is that the excitation wavelengths in the near-IR do not stimulate fluorescence, which can swamp Raman signals. While many RR studies have interrogated hemes with UV–Vis excitation wavelengths, fewer studies have applied near-IR excitation wavelengths. With extremely sensitive CCD optimized for the near-IR and efficient photon collecting optics, this region is now becoming accessible.

To obtain Raman spectra of single cells, subcellular structures, and single crystals, it is necessary to couple a microscope with a fully integrated bench-top dispersive Raman spectrometer. Raman spectra of red blood cells and heme solids can be recorded using a range of laser sources from the UV to near-IR. In the near-IR individual, diode lasers can be used to produce a variety of laser wavelengths including 780 and 830 nm, while Ti:sapphire tunable lasers can generate 675- to 1100-nm wavelengths. Heme solids can be very sensitive to laser power, and they readily melt and photodissociate even at near-IR wavelengths with low power (less than 3 mW). It is possible to perform measurements on heme solids under water by using a water-immersion objective and depositing the crystals on aluminium-coated or quartz Petri dish and then using the microscope to selectively target single crystals. This approach is also useful for recording spectra of single living cells in physiological environments and also in avoiding heating effects in sensitive samples.^{30–35} The cells can be affixed to the bottom of the Petri dish with poly-L-lysine or Celltak (Collaborative Biomedical Products, Bedford, MA). The Petri dish is then placed in a temperature-controlled unit purposely built to fit on a microscope stage for temperature control.³⁰ The unit can be sealed with a lid with an opening for the water immersion objective along with insertions for gas exchange, temperature, pH, and oxygen probes.³⁰ Cells suspended in growth media (~ 10 cm³) are transferred to the Petri dish and allowed to settle for 10 min to adhere to the bottom. Individual cells can then be targeted with the water immersion objective with the high refractive index of water, when compared with air, resulting in a

higher spatial resolution. Alternatively, single cells can be trapped with a laser field (laser tweezers). Xie et al.³⁶ were the first to record a spectrum of a single red blood cell in saline using a laser tweezing device. In this experiment a low-power diode laser (785 nm) was used to both trap and record a spectrum of the cell. The trapping required 2 mW of power while the spectrum was recorded using 20 mW of power.³⁶ Another method for analyzing single cells is to place cells in 50 μ L of buffer on a microscope slide coated in poly-L-lysine and target single cells with a water-immersion objective.³⁷ Ramser et al.³⁷ have reported changes in the erythrocyte membrane of cells suspended in a droplet of PBS and affixed to a glass slide. They attributed these changes to a surface-induced effect mitigated by the poly-L-lysine. By keeping the cells under physiological conditions in a Petri dish, we find that the majority of erythrocytes retain their discoidal morphology with no evidence of Heinz bodies (inclusions within RBCs composed of denatured hemoglobin) or perturbation in the exposed cell membrane. It is important to perform a laser power study to assess the effects of localized heating and photodissociation effects.^{30,38} This can be achieved using a series of neutral density filters ranging from 0.1% to 100% attenuation if the laser power itself is not easily attenuated. Modern instruments are equipped with an integrated interactive video camera and a motorized stage to enable the user to map a population of cells by simply “mouse clicking” the targets. Thus, repeated acquisitions and population data can be obtained with ease using this configuration.

8.4.2 Resonance Raman Spectroscopy of Hemes in Living Cells

The molecular dynamics associated with the allosteric transition in hemoglobin have been extensively studied^{39–42}; however, fewer studies have focused on monitoring heme environments within single functional cells. Raman techniques to probe single blood cells were pioneered in the 1990s by Puppels, Greve, and co-workers.^{43,44} Their studies focused primarily on lymphocytes^{44,45} and granulocytes^{44,46,47} and identified heme moieties including eosinophil peroxidase (EPO) and myeloperoxidase (MPO) in eosinophils and neutrophils, respectively,⁴⁴ confirming the high-spin six-coordinated structure of these hemes. Otto et al.⁴⁷ applied resonance Raman spectroscopy to investigate redox changes in cytochrome b_{558} and MPO in neutrophilic granulocytes upon mitogenic activation with phorbol-12-myristate-13-acetate (PMA) and found that the redox state of both molecules changed from the oxidized to the reduced state, indicative of NADPH oxidase activity. Resonance Raman spectra recorded neutrophils from patients with granulomatous disease, which are deficient in cytochrome b_{558} but not MPO; and MPO-deficient cells, which are deficient in MPO but not cytochrome b_{558} , showed a change from the oxidized to reduced state when activated with *E. coli*.⁴⁷ Activation of cytochrome b_{558} -deficient cells with PMA, on the other hand, did not result in a reduction in MPO, indicating the inability to form oxygen metabolites through the NADPH oxidase complex.⁴⁸ The activation of cytochrome b_{558} -deficient neutrophils with *E. coli* resulting in the reduction of MPO indicates either that the superoxide anion may have been produced by the bacteria or that the granulocytes have an alternative path for generating superoxide anions.⁴⁷ Using confocal Raman resonance microscopy, Van Manen et al.^{49,50} detected the distribution of EPO and cytochrome b_{558} in eosinophils and neutrophils, respectively (refer to Chapter 7). Recently, Kneipp et al.⁵¹ successfully deposited nanoparticles via colloidal suspensions into live whole cells and obtained surface-enhanced Raman (SER) spectra of the cellular components that are SER spectroscopically active (refer to Chapter 11).

Strong highly resolved bands have been reported in red blood cells when using 488, 514, 568, 633, and 785 nm, and clear spectral differences have been observed between oxygenated and deoxygenated cells.^{31–35,52} Most notable were changes in the “core-size” or “spin-state” marker band region between 1650 and 1500 cm^{-1} between the two states. Other differences were observed in the methine deformation region between 1250 and 1200 cm^{-1} . A series of Raman studies on single functional red blood cells utilizing a water immersion objective to record spectra and images of cells in growth media and other physiological solutions were undertaken by Wood and colleagues and Ramser and colleagues since 2001.^{2,30–35,37,53} In a very recent study,⁵² 785 nm excitation was applied to monitor red blood cells during four oxygenation/deoxygenation cycles over a 2 hour period. The high-quality spectra exhibited a mixture of A_{1g} , A_{2g} , B_{1g} , B_{2g} , E_u , and vinyl modes. The large database consisting of 210 spectra from the four cycles was analyzed with Principal Component Analysis (PCA). The PC1 loadings plot provided exquisite detail on bands associated with the oxygenated and deoxygenated states. The enhancement of a band at 567 cm^{-1} , observed in the spectra of oxygenated cells and corresponding PC1 loadings plot, was assigned to the Fe-O_2 stretching mode,⁵⁴ while a band appearing at 419 cm^{-1} was assigned to the Fe-O-O bending mode based on previous studies.^{55,56} Figure 8.5 shows the evolution of these modes over one oxygenation–deoxygenation cycle from spectra recorded every 10 s. For deoxygenated red blood cells the enhancement of B_{1g} modes at 785 nm excitation is consistent with vibronic coupling between band III and the Soret transition. In the case of oxygenated cells the enhancement of iron-axial out-of-plane modes and non-totally symmetric modes is consistent with enhancement into the y, z-polarized transition $a_{1u}(\pi) \rightarrow d_{xz} + O_2(\pi_g)$ centered at 785 nm. The enhancement of non-totally symmetric B_{1g} modes in oxygenated cells is indicative of vibronic coupling between band IV and the Soret band.⁵²

These studies demonstrated the feasibility of applying the Raman technique to monitor oxidation–reduction processes in single red blood cells, provided that laser power, heating effects, and substrate-induced effects could be minimized or preferably eliminated.

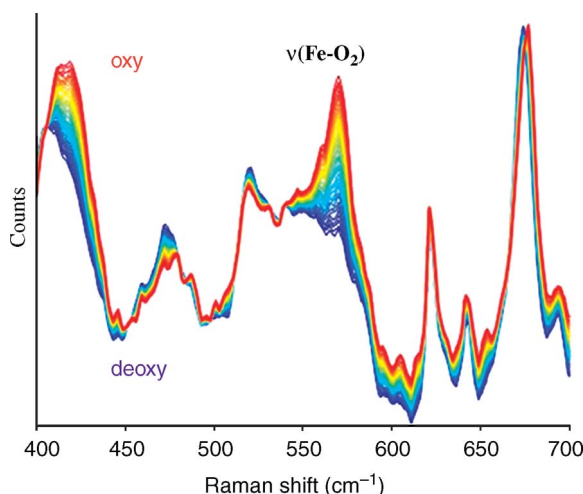


Figure 8.5. A series of spectra from a functional red blood in the low-wavenumber region (400–700 cm^{-1}) recorded every 10 s going from the deoxygenated state to the oxygenated state using 785 nm excitation showing the evolution of the $\nu_s(\text{Fe-O-O})$ vibration.

8.5 RESONANCE RAMAN OF HEME DERIVATIVES USING NEAR-INFRARED EXCITATION IN THE SOLID STATE

8.5.1 Resonance Raman Spectroscopy of Protoporphyrin IX

To begin our discussion on the resonance Raman spectroscopy of heme molecules excited with near-IR laser lines, we investigate the resonant enhancement of iron protoporphyrin IX (FePPIX), the key prosthetic group for a variety of hemoproteins such as hemoglobin, myoglobin, cytochromes, catalases, and peroxidases. Hematin (FePPIX-OH), which is the monomeric pre-cursor to the hydrogen bonded dimer β -hematin ($[\text{FePPIX}]_2$), can be readily synthesized from hemin (FePPIX-Cl). A schematic structure of iron protoporphyrin IX is presented in Fig. 8.6 along with a structure of hemin based on X-ray crystallography.⁵⁷

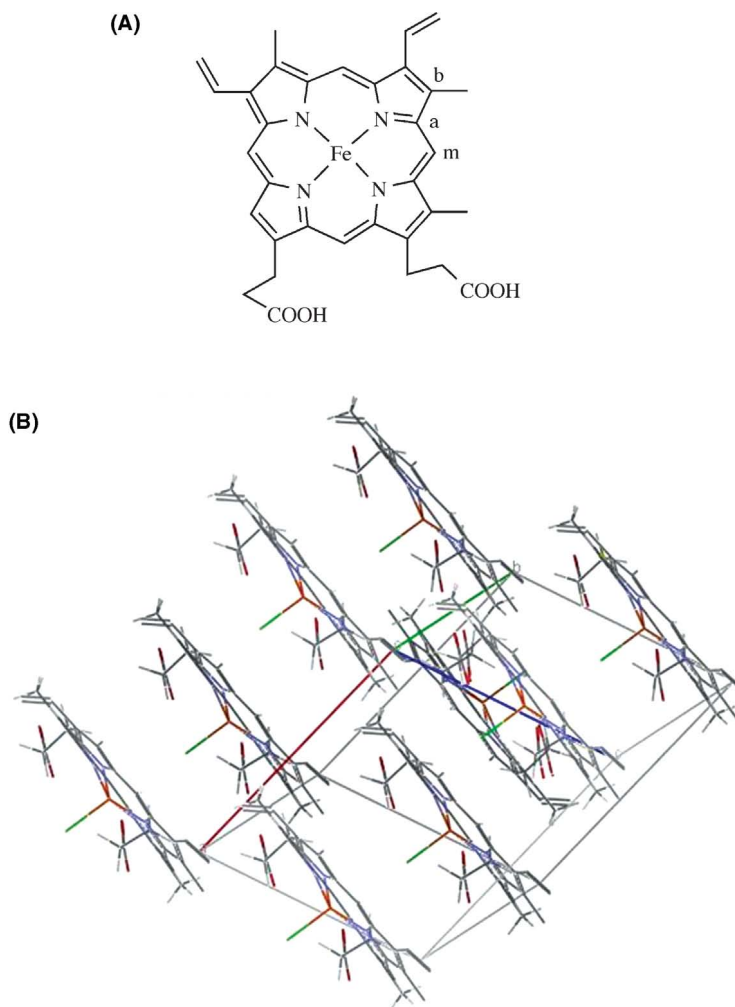


Figure 8.6. (A) Structure of iron protoporphyrin. (B) Structure of hemin based on X-ray crystallographic data. (From Ref. 57, reprinted with permission.)

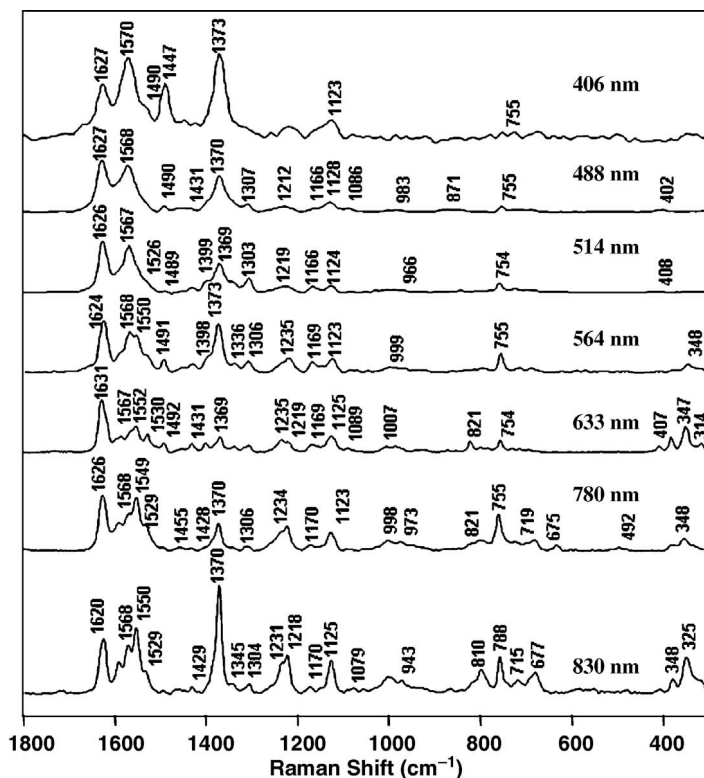


Figure 8.7. Raman spectra of hemin recorded using a variety of laser excitation wavelengths and normalized to ν_{10} . (Reprinted with permission of the American Chemical Society, Copyright 2005.¹)

Figure 8.7 depicts a series of Raman spectra of single hemin crystals for a range of excitation wavelengths. The band assignments and symmetry terms are provided in Table 8.1. Excitation into the Soret band (~ 400 nm) of hemin, hematin, and β -hematin using the 406 nm laser line produces a classic type A (Franck–Condon) scattering pattern showing the enhancement of several totally symmetric modes. These include bands at 1570, 1490, and 1373 cm^{-1} assigned to ν_2 , ν_3 , and ν_4 , all of A_{1g} symmetry. The totally symmetric modes ν_2 , ν_3 , and ν_4 are also enhanced using 488 nm excitation. These modes would be in pre-resonance with the Soret band at ~ 400 nm, and so the enhancement is also typical of a type A scattering. Excitation at 514 nm, which is close to the 515 to 521 nm Q_V band, resulted in a decrease in relative intensity of the totally symmetric modes ν_2 , ν_3 , and ν_4 . Bands at 1431, 1303, and $1170\text{--}1166\text{ cm}^{-1}$ in hemin were slightly more pronounced at this wavelength compared to 488 nm. Exciting at 564 nm, which is close to the 550 nm Q_0 band, results in dramatically enhanced ν_2 , ν_3 , and ν_4 bands of A_{1g} symmetry. Other enhanced bands include $1551\text{--}1550$ and 755 cm^{-1} assigned to ν_{11} and ν_{16} of B_{1g} symmetry. Pyrrole-breathing and deformation modes ($800\text{--}600\text{ cm}^{-1}$) along with out-of-plane modes ($400\text{--}300\text{ cm}^{-1}$) are also enhanced when exciting with 564 nm compared to 514 and 488 nm excitation wavelengths. Consequently, the spectrum has many features in common with the 406 nm spectrum. Excitation with 633 nm results in a dramatic decrease in the intensity of the ν_4 band along with increases in bands at 1552 ($\nu_{11}\text{-}B_{1g}$) and 1567 cm^{-1} ($\nu_2\text{-}A_{1g}$). However, the relative intensity of bands at 1235 ($\nu_{13}\text{-}B_{1g}$), 1125 ($\nu_{22}\text{-}A_{2g}$), 821 cm^{-1} and

TABLE 8.1. Band Assignments, Local Coordinates, Relative Band Intensities, and Symmetry Terms for b-Hematin

406 nm	488 nm	514 nm	568 nm	632.8 nm	780 nm	830 nm	1064 nm	Assignment	Symmetry term	Local coordinate
Absent	Absent	Absent	1751(w)	1750(vw)	Absent	1742(w)	1738(vw)	C=O		v(C=O)
1730(vw)	Absent	Absent	1727(w)	1727(w)	Absent	Absent	Absent	C=O		v(C=O)
Absent	Absent	Absent	1655(m)		1655(vw)	1655(m)	1655(vw)		B _{1g}	Unassigned
1628	1632	1639	1627	1628	1623	1621	1623	v ₁₀	E _u	v(C _α C _m) _{asym}
Absent	Absent	1585(s)	1588(sh)	1581(sh)	1589(sh)	1588(sh)	Absent	v ₃₇	A _{1g}	v(C _α C _m) _{asym}
1571(vs)	1574(s)	1571(s)	1567(s)	1570(s)	1572(s)	1570(s)	1571(m)	v ₂		v(C _β C _β)
Absent	Absent	Absent	1551(s)	1551(s)	1552(vs)	1550(s)	1549(s)	v ₁₁	B _{1g}	v(C _β C _β)
1530(sh)	Absent	Absent	1530(sh)	1528(vw)	1530(sh)	1530(sh)	Absent	v ₃₈	E _u	v(C _β C _β)
1491(vs)	1492(m)	1492(w)	1497(w)	1497(w)	1497(w)	1497(vw)	Absent	v ₃	A _{1g}	v(C _α C _m) _{sym}
1429(w)	1432(w)	1432(w)	1430(w)	1430(m)	1430(vw)	1430(vw)	1429(vw)	v ₄₀	E _u	v(C _α C _m) _{sym}
Absent	1399(w)	1399(w)	1398(m)	1398(m)	1398(sh)	Absent	Absent	v ₂₉	B _{2g}	v(pyrrole quarter-ring)
1372(vs)	1371(s)	1372(m)	1376(vs)	1376(m)	1377(vs)	1375(vs)	1376(m)	v ₄	A _{1g}	v(pyrrole half-ring) _{sym}
1340(sh)	1340(m)	1340(w)	1338(w)	1338(w)	1338(w)	1340(w)	1338(w)	v ₄₁	E _u	v(pyrrole half-ring) _{sym}
1304(vw)	1307(w)	1306(w)	1309(w)	1308(w)	1308(w)	1304(vw)	1310(vw)	v ₂₁	A _{2g}	δ(C _m H)
Absent	Absent	1240(vw)	1240(m)	1240(m)	1241(s)	1236(s)	1235(w)	v ₄₂	E _u	δ(C _m H)
1227	1223(w)	1226(w)	1220(m)	1219(m)	1219(s)	1218(s)	1219(m)	v ₁₃	B _{1g}	δ(C _m H)
Absent	Absent	Absent	1202(w)	1210(w)	1215(sh)	1201(m)	Absent	v ₅ + v ₁₈		δ(C _m H)
1170(vw)	1173(w)	1170(w)	1169(m)	1170(m)	1171(w)	1170(w)	Absent	v ₃₀	B _{2g}	v(pyrrole half-ring) _{asym}
Absent	Absent	Absent	1145(m)	1145(m)	1145(w)	Absent	1149(vw)	v ₁₄	B _{1g}	half-ring _{asym}
1127(w)	1124(w)	1122(w)	1122(m)	1120(m)	1121(m)	1124(s)	1124(m)	v ₂₂	A _{2g}	v(C _β C ₁) _{sym}
Absent	Absent	Absent	1091(vw)	1092(w)	1090(vw)	Absent	Absent			v(C _α N)
1002(vw)	1003(w)	1000(w)	1001(w)	1003(w)	1002(w)	998(m)	1003(vw)	v ₄₅	E _u	γ(=C _β H ₂) _{sym}
Absent	Absent	972(w)	975(w)	971(w)	975(m)	972(m)	Absent	v ₄₆	E _u	v(C _β -methyl stretch)
Absent	Absent	Absent	940(vw)	941(vw)	940(vw)	943(w)	Absent	v ₃₂	B _{2g}	δ(pyrrole deform) _{asym}
Absent	Absent	Absent	822(vw)	822(w)	821(w)	820(w)	Absent	γ ₁₀	B _{1u}	γ(C _α H=)
Absent	Absent	Absent	792(m)	797(w)	796(m)	795(m)	Absent	v ₆	A _{1g}	γ(C _m H)
										v(pyrrole breathing)

(continued)

406 nm	488 nm	514 nm	568 nm	632.8 nm	780 nm	830 nm	1064 nm	Assignment	Symmetry term	Local coordinate
755(vw)	755(w)	755(w)	753(m)	754(s)	753(s)	750(m)	755(w)	ν_{15}	B_{1g}	$\nu(\text{pyrrole breathing})$
Absent	Absent	Absent	729(w)	725(w)	725(vw)	725(w)	Absent	γ_5	A_{2u}	$\text{Pyrrole fold}_{\text{sym}}$
Absent	Absent	Absent	712(w)	710(vw)	710(w)	710(m)	Absent	γ_{15}	B_{2u}	$\text{Pyrrole fold}_{\text{asym}}$
Absent	Absent	Absent	512(vw)	514(w)	Absent	Absent	510(vw)			$\nu(\text{Fe-O})$
Absent	676(vw)	676(vw)	676(vw)	679(vw)	678(w)	678(m)	Absent	ν_7	A_{1g}	$\delta(\text{pyr deform})_{\text{sym}}$
413	Absent	Absent	409(vw)	406(vw)	406(vw)	Absent	Absent			$\delta(C_\alpha C_\beta C_m)$
380	Absent	Absent	379(m)	376(m)	378(w)	378(sh)	Absent			
Absent	Absent	Absent	365(sh)	369(vw)	365(vw)	367(s)	369(w)	γ_6	A_{2u}	Pyrrole tilt
344	Absent	Absent	345(m)	345(vw)	345(w)	348(vw)	344(w)	ν_8	A_{1g}	$\nu(\text{Fe-N})$
Absent	Absent	Absent	323(w)	323(w)	321(w)	324(vw)	Absent	ν_{51}	E_u	$\delta(C_\beta-C_l)$

a Band intensities are defined as vw, very weak; w, weak; m, medium; s, strong; vs, very strong; ν , in-plane stretch; γ , out-of-plane stretch; δ , deformation mode.

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low wavenumber modes at 407, 377, 347, and 314 cm^{-1} all increase when exciting at this wavelength. The increase in intensity of the bands that characterize the A_{2g} modes is consistent with scattering observed in the Q-band excitation region where normally type B or Hertzberg–Teller scattering is the dominant mechanism. The type A (or Franck–Condon) scattering term is not significant at excitation wavelengths close to the Q bands; consequently, the 1570 and 1370 cm^{-1} bands appear diminished. It is interesting to note the increase in intensity of the low wavenumber modes (500–300 cm^{-1}) compared to the spectra acquired using 488 and 514 nm excitation wavelengths. As mentioned above, these modes are generally attributed to out-of-plane porphyrin and axial modes.⁵⁸

The above observations are consistent with both resonant Raman theory and numerous resonant Raman studies performed on solutions of metalloporphyrins. However, at longer excitation wavelengths, well away from the major electronic transitions, we observe the most extraordinary enhancement in the solid state. The spectra recorded using 780 nm and particularly 830 nm show a dramatic increase in the relative intensity of many bands compared to spectra recorded using the other excitation wavelengths. In particular, bands characteristic of totally symmetric A_{1g} modes including 1570, 1371, 795, 677(ν_6), and 344 cm^{-1} along with bands at 1552, 1220, and 755 cm^{-1} associated with B_{1g} modes become dramatically enhanced relative to ν_{10} at 1620 cm^{-1} in hematin, and a similar enhancement profile is observed in hemin.

A resonance Raman enhancement profile of the totally symmetric modes ν_4 and ν_2 with respect to the normalization band ν_{10} is also shown in Fig. 8.8 for hemin and β -hematin. At 830 nm excitation the intensity of the ν_4 band at 1371 cm^{-1} dominates the spectrum of hematin and also the β -hematin dimer. A number of low wavenumber modes are also dramatically enhanced using 780- and 830-nm excitation wavelengths including bands at 821, 796, 752, 722, 710 and 678 cm^{-1} . FT-Raman spectra recorded of hematin using 1064 nm excitation show a dramatic reduction in the intensity of most of the totally symmetric modes including ν_4 because the 1064 nm excitation wavelength must be well away from the electronic transition responsible for the enhancement of these modes at 780 and 830 nm excitation. In the case of hemin, excitation at 564, 780 and 830 nm resulted in

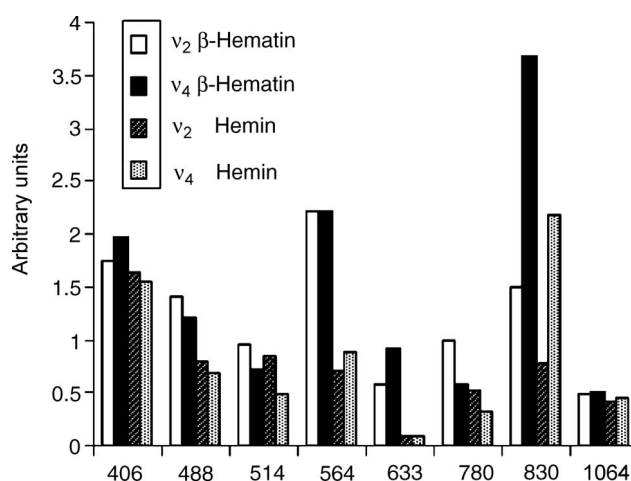


Figure 8.8. Raman intensity as a function of wavelength of β -hematin and hemin for the totally symmetric modes ν_4 and ν_2 . (Reprinted with permission of the American Chemical Society, Copyright 2005.¹)

dramatic enhancement of A_{1g} and moderate enhancement of some B_{1g} modes. A similar enhancement profile was also observed for the hematin (Fe(III) hydroxyprotoporphyrin IX).

8.5.2 Mechanism of Enhancement of Totally Symmetric Modes at Near-IR Excitation Wavelengths

The appearance of dramatically enhanced totally symmetric modes at near-IR excitation wavelengths is indeed interesting and one can only hypothesize as to a possible mechanism until more model compounds are investigated.

RR studies of hemes in the near infrared are few and most have focused on heme proteins. Franzen et al.⁹ reported a complete resonance excitation profile from 740 to 780 nm and demonstrated that non-totally symmetric modes of B_{1g} symmetry are enhanced in the vicinity of a very weak charge transfer band centered at 758 nm and designated band III.⁷ This band is observed in deoxyhemoglobin and deoxymyoglobin and was assigned by Eaton et al.⁷ to the $a_{2u}(\pi) \rightarrow d_{yz}$ transition. This band is quite narrow and models that fit the line shape are reported to exhibit only weak coupling to a low-frequency vibrational mode.¹⁰ Based on symmetry arguments, Franzen et al.⁹ explained the enhancement by adopting a vibronic coupling model and not by a Franck–Condon mechanism. In this model the charge transfer transition is vibronically coupled to the Soret band through a low-frequency vibrational mode of B_{1g} symmetry, giving rise to enhanced B_{1g} modes.

The enhancement observed at near-IR excitation wavelengths in hemin, hematin, and β -hematin is a mechanism different from that described by Franzen et al.⁹ for deoxymyoglobin and deoxyhemoglobin because in the former a combination of totally symmetric and non-totally symmetric modes are observed enhanced as opposed to only non-totally symmetric modes in the latter. In terms of local coordinates, it is interesting to note that the majority of bands enhanced in hematin include out-of-plane vibrations, pyrrole folds, pyrrole breathing modes, and the Fe–N local coordinate. Because of the C_{4v} effective symmetry, most of these modes involve electronic displacement mainly along the z -axis of the porphyrin, thus inducing charge displacement along this axis, which can theoretically result in band enhancement if in the vicinity of a z -polarized transition. In the case of hemin and β -hematin, a small broad electronic transition is observed at 867 nm, which is assigned the z -polarized charge transfer transition $d_{xz} \rightarrow e_g(\pi^*)$ and designated band I.^{1,8} Figure 8.2 shows a qualitative energy level diagram of the allowed transitions for a high-spin ferric heme including band I (dashed line). It is important to note that the enhancement relative to ν_{10} of the totally symmetric modes at near-IR excitation wavelengths is much greater than that observed in the Soret band using 406-nm excitation. This, along with the fact that the enhancement of A_{1g} modes, is greater in dimeric β -hematin compared to hemin and hematin indicates an additional mechanism is at work.

Based on symmetry arguments and also UV–Vis spectroscopy monitoring the acidification of hemin to β -hematin which shows a red shifting of the Soret, Q, and band I during the formation of β -hematin, we hypothesize that an exciton mechanism is contributing to the enhancement.

Excitons in molecular crystals and aggregates have been extensively studied over the previous decade because of the importance in elucidating the functionality of photo-pigments in photosynthesis.⁵⁹ Resonance Raman (RR) spectra of porphyrin arrays in which the porphyrins are directly linked at the meso position have undergone intense study recently. For excellent recent reviews of this work, refer to.^{60,61} The close proximity of adjacent porphyrins (0.83 nm) orthogonally oriented in the arrays manifests in exceptionally strong excitonic

interactions along the axis defined by the *meso,meso*-linkages and negligible interactions along the orthogonal axis.⁶⁰ Exciton transfer can occur from electronic interaction, either by direct overlap of the π orbitals of the chromophores or mediated by the orbitals of the intervening bridge, enabling electron exchange between individual chromophores to occur.⁶² The excitonic coupling breaks the degeneracy of the B excited state, giving rise to two Soret bands.

RR spectra of the *meso,meso*-linked arrays recorded in the B-state excitation range exhibit a complex and unusual scattering pattern. Bhuiyan et al.⁶³ noted several striking features about the spectra: (1) the observation of only polarized (A_{1g} for D_{4h}) and anomalously polarized modes (A_{2g} for D_{4h}) in the RR spectrum, (2) the enhancement of anomalously polarized vibrations with B-state excitation, and (3) the large differential enhancement of symmetric versus non-totally symmetric vibrations with excitation across the B-state absorptions.⁶³ Bhuiyan et al. surmised that these scattering characteristics were due to the effects of symmetry lowering. The asymmetric *meso* substitution pattern inherent to the *meso,meso*-linked arrays contributes to symmetry lowering in both the ground and excited electronic states.⁶³ The strong uniaxial excitonic interactions make an additional contribution to symmetry lowering in the excited state(s) promoting novel Franck–Condon and vibronic scattering mechanisms in the B state(s) of the arrays.⁶³ Collectively, the studies of the *meso,meso*-linked arrays provide insight into the type of RR scattering that might be anticipated for other types of systems that exhibit strong excitonic interactions among the constituent chromophores.

Akins et al.^{64,65} reported that in highly conjugated systems such as cyanine dyes and porphyrin arrays, other enhancement effects can also be significant. One such important mechanism is aggregated enhanced Raman scattering (AERS),^{64,65} where bands can become enhanced through excitonic interactions between neighboring chromophores. In this case, energy in the form of an exciton is either transferred via covalent linkages between chromophores or directly through space via overlapping π orbitals, resulting in the enhancement of particular vibrational modes. The enhancement of vibrational modes can be explained in terms of an increased-size effect and near-resonance terms in the polarizability.⁶⁶ Excitonic coupling will essentially split the electronic states into a broad band of states with different geometries, energies, and oscillator strengths. The Raman intensities for a particular wavelength will then reflect the extent of the excitonic coupling.

It is hypothesized that the enhancement observed at 830 nm in a variety of heme molecules (manuscript in preparation) results from an exciton coupling mechanism that implicates the charge-transfer transition-centered ~ 860 -nm transition known as band I. In this scenario the electronic symmetric component of the near-IR photon couples the excited states of the charge-transfer transitions, resulting in a superposition of states increasing the contributions to the Franck–Condon integrals. Arguments to support this assessment are as follows:

1. The strong enhancement of A_{1g} modes, out-of-plane modes, and some pyrrole-breathing and deformation normal modes is indicative of a z -polarization transition that is a characteristic of band I.
2. Band I has an excited electronic state configuration that corresponds to the direct product $E \times E = A_1 + A_2 + B_1 + B_2$ (under C_{4v}), which cannot mix with the excited state configuration of the Soret transition. Thus, band I cannot vibronically couple to the Soret band. Consequently, one would expect totally symmetric A_1 modes (A_1 in C_{4v}) to be enhanced as predicted by the Albrecht formalism.

3. The stacking of hemes should result in strong excitonic interactions for z -polarized transitions as verified by the intensity of the A_{1g} modes in β -hematin compared to hemin at near-IR excitation wavelengths.
4. The red shifting of the of the Soret band, Q bands, and charge-transfer band during the acidification of hemin to form the dimer β -hematin is indicative of a splitting of degenerate states, which is a characteristic of excitonic coupling.
5. Nonlinear power dependence plot for the totally symmetric modes ν_4 and ν_2 for hemin and β -hematin is characteristic of two photon excitonic mechanisms.

8.6 APPLICATION TO MALARIA RESEARCH

Regardless of the mechanisms involved, the ability to record micro-Raman spectra of heme systems in live systems is possible through the use of the long-wavelength excitation lasers. This provides a powerful tool to follow molecular changes such as ligand exchange, oxidation, and spin-state change as well as aggregation in living cells both normal and diseased. One important area of disease research that can benefit from this Raman ability is malaria, where the heme chemistry in the malarial parasite food vacuole can be monitored. Of particular interest are hemozoin, the malarial pigment, and its synthetic analogue β -hematin.

8.6.1 Resonance Raman Spectroscopy of β -Hematin and Hemozoin

X-ray crystallographic determination of the structure of hemozoin and its synthetic analogue β -hematin have proven to be difficult because of the small needle-like crystals ($\sim 2\ \mu\text{m}$) that result from the synthesis of β -hematin or the isolation of hemozoin from infected red blood cells. Powder diffraction data obtained with synchrotron radiation indicated that β -hematin consists of dimers formed through reciprocal iron-carboxylate bonds to one of the propionate side chains and that the dimers are linked by hydrogen bonds to form chains.⁶⁷ Figure 8.9 shows a schematic structure of β -hematin based on the powder diffraction data. Hemozoin (malaria pigment) is spectroscopically identical to β -hematin at all available Raman excitation wavelengths.² The dramatic enhancement of totally symmetric modes at near-IR excitation wavelengths is even greater for β -hematin than for hemin or hematin.

This enhancement has been used both to record spectra and to image the hemozoin pigment inside a living cell.² In the imaging experiment an optical band-pass filter is placed in line to select the band pass of interest, the whole sample area is irradiated, and the scattered light is collected on the 2D CCD array. A false color image is then constructed from the Raman count at each pixel. Figure 8.10 shows a photomicrograph of a malaria-infected cell in the late trophozoite phase and the corresponding 633 nm excitation spectrum of hemoglobin and β -hematin.

The spectra of hemozoin and hemoglobin were recorded from the same cell under phosphate-buffered saline by targeting the pigment and surrounding area, respectively. The spectrum of β -hematin, was also recorded under PBS and is essentially identical to the spectrum of hemozoin within the cell. This confirms that the structure of hemozoin within functional erythrocytes is identical to that of β -hematin. The spectrum of hemoglobin can be easily discerned from the spectrum of hemozoin especially in the spin-state marker band

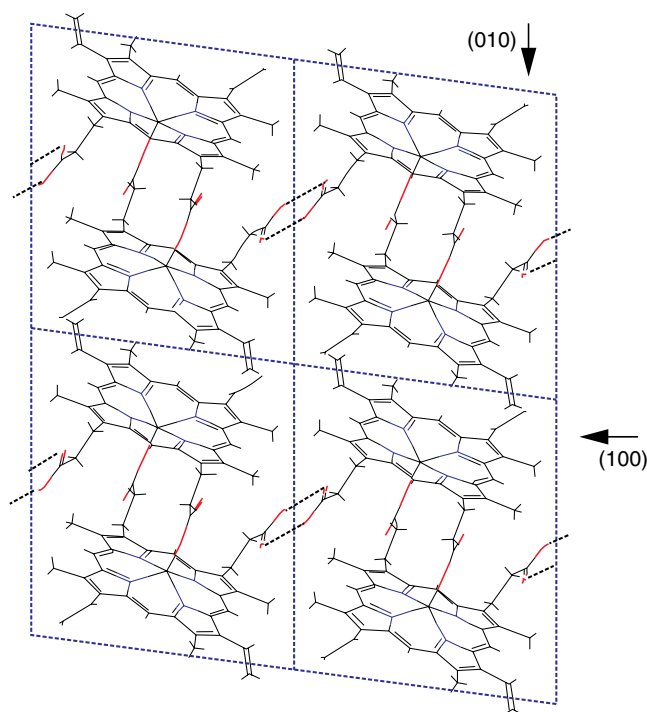


Figure 8.9. Structure and packing arrangement of β -hematin (synthetic malaria pigment) viewed along the c -axis. Some (h, k, l) planes are indicated. (Reprinted with permission of the American Chemical Society, Copyright 2002.)

region $1650\text{--}1500\text{ cm}^{-1}$. Figure 8.11 shows a photomicrograph of malaria-infected cell in the late trophozoite phase and the corresponding 780 nm excitation Raman image of the 1374 cm^{-1} band showing the food vacuole containing hemozoin. As a consequence of the long excitation wavelength and a laser beam defocused over the whole cell, the cell does not lyse during this exposure and the spectra show no chemical changes post-irradiation. The Raman spectra of β -hematin and hematin are also compared with the spectrum of hemozoin from the imaged cell. The spectra of hemozoin and β -hematin are again essentially identical at this wavelength. The spectrum of hematin differs from the dimeric analogues mainly in terms of band enhancement. In particular, bands at $1571, 1376, 1241\text{--}1240, 974, 944, 821, 796, 710, 678\text{ cm}^{-1}$ are dramatically enhanced in the spectra of hemozoin and β -hematin when compared to the spectrum of hematin.

8.6.2 Near IR Raman Excitation for Investigating the Mechanisms of Hemozoin Formation within the Parasite

There is great debate in the literature about the mechanism by which the parasite produces hemozoin in a physiological environment. It has been suggested that hemozoin formation is a spontaneous process occurring in the acidic environment of the food vacuole.^{68,69} However, until recently, synthesis of β -hematin had only been achieved under nonphysiological conditions involving high concentrations of acetic acid, high temperatures, and

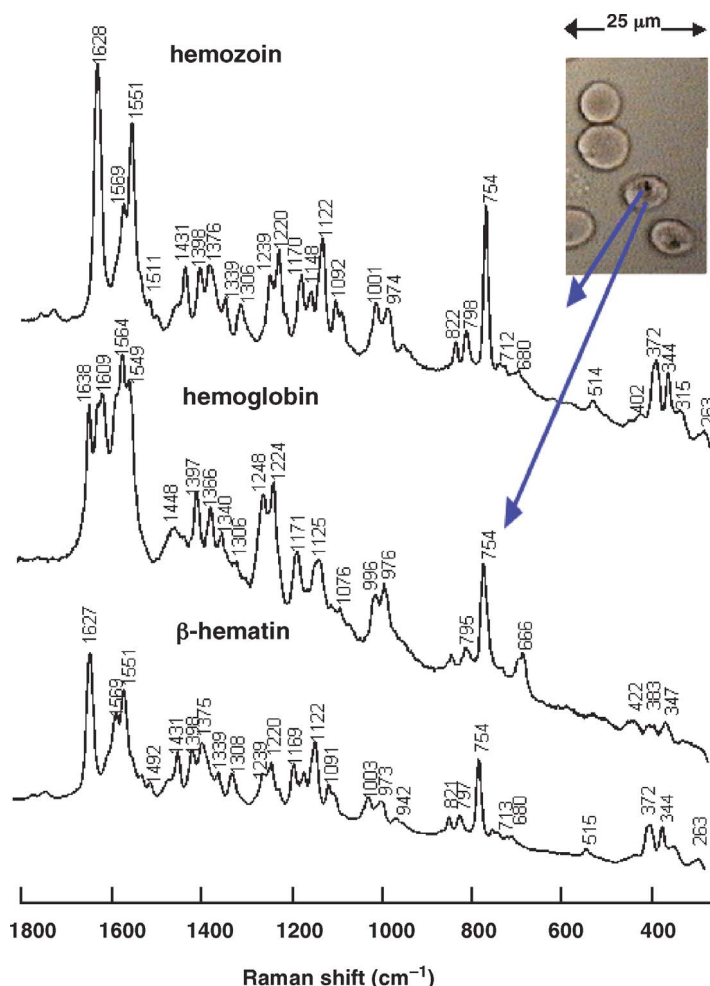


Figure 8.10. Photomicrograph of *P. falciparum*-infected erythrocytes (late trophozoite stage) showing the food vacuoles containing hemozoin. The arrows indicate the laser targets, namely the food vacuole and the surrounding hemoglobin. The corresponding spectra are compared with synthesized β -hematin. (Reprinted with permission of Federation of the European Biochemical Societies.²)

lengthy incubation times.⁷⁰ The most strongly supported models are the lipid-mediated hypothesis⁷¹ or nucleation by histidine-rich protein 2 (HRP2).⁷² Using a combination of resonance Raman spectroscopy, electronic absorption, and EPR, Choi and colleagues⁷³ demonstrated the formation of a stable six-coordinate, low-spin PfHRP-2 ferric heme complex that appeared to be a primary step in hemozoin formation. However, it was found that HRP2 is mainly localized in the erythrocyte cytoplasm of the infected red cell and only a small fraction is located in the parasite food vacuole where hemozoin crystals are located.⁷³ Moreover, *P. falciparum* clones lacking both HRP2 and HRP3 are reported to form hemozoin regardless,^{74,75} and finally HRP2 homologues are not known to be involved in hemozoin formation in other organisms.

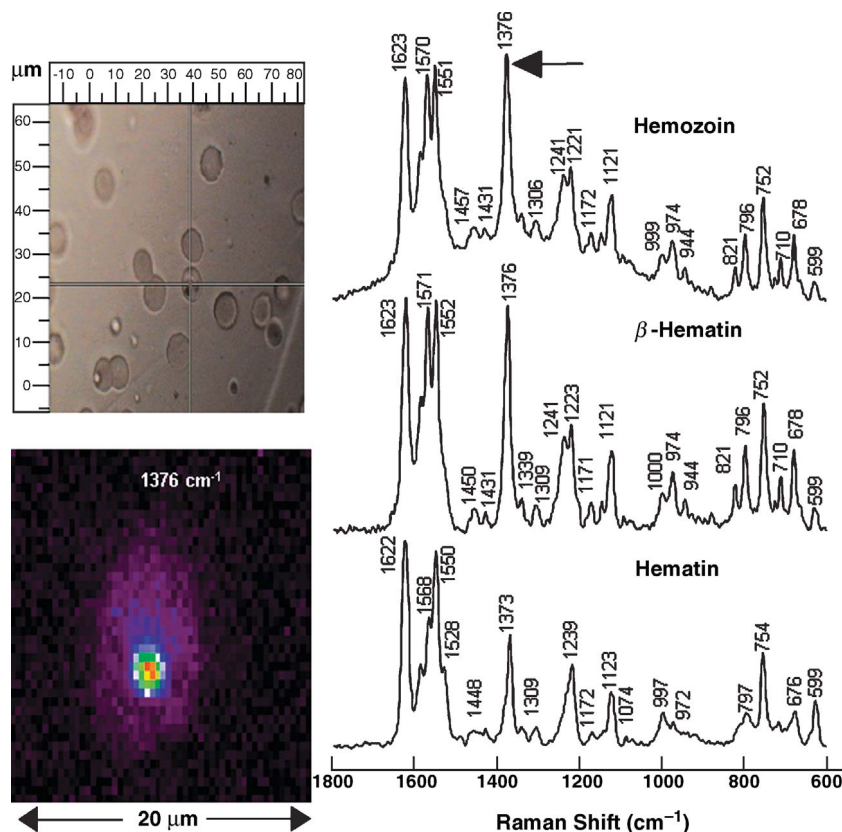


Figure 8.11. Photomicrograph and corresponding Raman image of the 1374-cm^{-1} band clearly showing the parasite's food vacuole along with spectra of hemozoin, β -hematin, and hematin all acquired using 780-nm excitation. The arrow highlights the totally symmetric mode ν_4 at 1374 cm^{-1} . (Reprinted with permission of Federation of the European Biochemical Societies.²)

Lipids have also been implicated in the formation of hemozoin within the food vacuole. In the presence of monooleoylglycerol (MOG), the β -hematin formation rate increases dramatically under physiological conditions.⁷⁶ Neutral lipid bodies composed of di- and triacylglycerols were shown to be associated with the food vacuole of *P. falciparum* and implicated in hemozoin formation.⁷⁷ In addition, the formation of hemozoin in *S. mansoni* is localized in lipid droplets, while in *R. prolixus* the formation occurs in perimicrovillar membranes.⁷⁸ Until now, experimental rates of β -hematin formation brought about by either HRP2 or lipids were far too slow to account for hemozoin formation *in vitro*.

Recently, Egan et al.⁷⁹ reported that β -hematin spontaneously self-assembles near or at octanol–water, pentanol–water, and lipid–water interfaces at physiological temperature and pH. Micro-resonance Raman spectra recorded at the interface using 785 nm excitation confirmed the presence of β -hematin at, or immediately adjacent to, the interface under these conditions. Molecular dynamics simulations indicate that in the absence of water, Fe (III)PPIX has a propensity to spontaneously form an intermolecular precursor to the hemozoin dimer (Figure 8.12). These recent findings demonstrate that hemozoin formation is highly efficient even without a protein catalyst or initiator and explain the role of lipids in hemozoin formation.⁷⁹

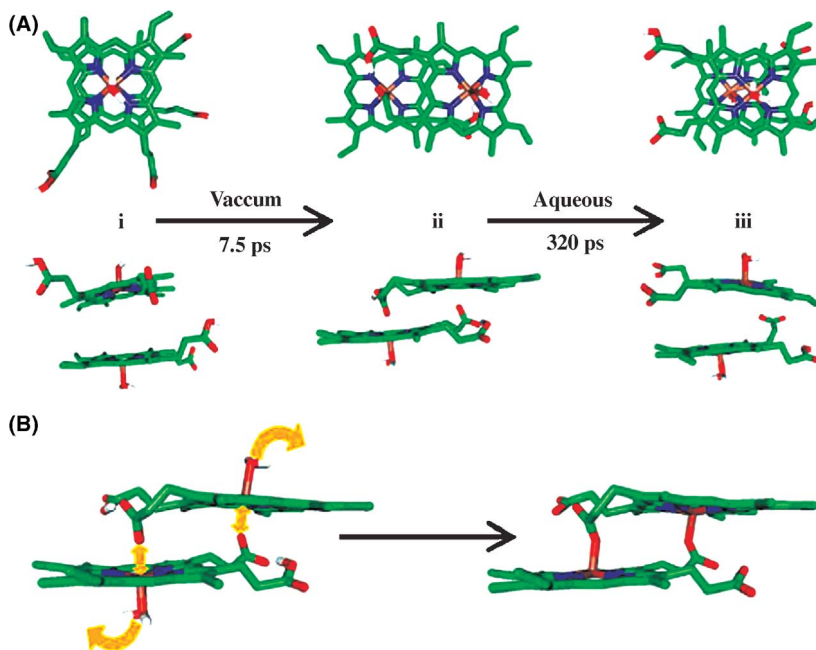


Figure 8.12. (A) A molecular dynamics simulation of the interaction of two $\text{H}_2\text{O-Fe(III)PPIX}$ molecules for the protonation state expected for the pH in the food vacuole. The dynamics were started with the molecules placed in a back-to-back conformation with the propionate and propionic acid groups extended and far apart (i). In vacuum, these two molecules rapidly form the β -hematin precursor (ii). Enlargement of (B), which shows that bond formation of the propionate groups with Fe(III) and release of H_2O is all that is required to convert this precursor to the β -hematin dimer. (iii) When the dynamics were performed in a cube of H_2O starting from the β -hematin precursor, the propionate groups quickly moved away from the Fe(III) centers to interact with the solvent molecules. (Reprinted with permission of Federation of the European Biochemical Societies.⁷⁹)

8.6.3 Resonance Raman Spectroscopy in Anti-Malarial Research

Powder crystal data indicates that chloroquine absorbs onto the β -hematin – and, by analogy, hemozoin absorbs onto surface – resulting in inhibition of further heme aggregation.⁸⁰ Sullivan et al.⁸¹ showed (^3H) chloroquine located on the hemozoin pigment crystals situated in the food vacuole and suggested that quinolines cap the formation of hemozoin. Buller et al.⁸⁰ proposed a model for how some quinoline drugs can cap the fast-growing corrugated surface (001) of the β -hematin. In this model the quinoline aromatic ring is interleaved between porphyrin rings within a crevice at the corrugated (001) surface, and further bonded by a salt bridge between the exocyclic amine and a surface-exposed propionate group, as well as by various Coulombic interactions.⁸⁰ The second model proposes inhibition involving noncovalent binding of chloroquine to the μ -oxo dimer.^{82,83} In this model the drug–dimer complex is envisaged as “capping” the formation of hemozoin, resulting in a higher concentration of toxic free heme in the food vacuole and hence membrane lysis.^{84,85} Electronic absorption spectroscopy reveals distinct changes in the

UV-vis spectrum of Fe(III)PPIX upon interaction with chloroquine and other quinolines, with shifts of bands in the Soret region ~ 400 nm, disappearing Q bands (500–600 nm), shifting charge transfer bands (600–700 nm), and intensity changes reported.⁸⁶ A decrease in the intensities of bands in the Soret region upon CQ binding Fe(III)PPIX has been interpreted as a result of excitonic coupling giving rise to band splitting. Excitonic coupling results if the transition dipoles of the chloroquine and Fe(III)PPIX molecules are correctly aligned. The decrease in intensity of bands from one molecule appears to result from induced dipole moments in the other molecule, essentially the effect of dispersion forces on the transition dipole moment.^{86,87} The strong hypochromism observed upon Fe(III)PPIX and chloroquine complex formation indicates that the molecular planes are stacked approximately parallel to each other and in close proximity.^{86,88}

Resonance Raman spectroscopy has been applied to monitor interactions of antimalarial drugs with hematin and β -hematin. Resonance Raman difference spectra of complexes between metalloporphyrins and chloroquine, viologen dication, and substituted phenanthrolines have been reported by Shelnett.^{89–92} These studies indicated that the binding involves electronic interactions. The viologen dication was found to act as a π - π donor and is also stabilized by strong electrostatic interactions, a situation that has been suggested to occur also with chloroquine. Polarization-resolved RR spectra of hematin and its complex with chloroquine were recorded at 514 nm in order to monitor the binding of the drug to the heme structure.⁹³ Small wavenumber shifts (>2 cm⁻¹) were reported, indicating a noncovalent interaction in the electronic ground state of the drug-target complex.⁹³

Surface-enhanced Raman spectroscopy (SERS) has been applied to monitor hematin-chloroquine (CQ) and hematin-mefloquine (MQ) adsorbed on Au particles. Specific changes in the relative intensities and band positions for hematin-Au and MQ-Au SERS systems were observed. The strongest band at 1532 cm⁻¹ being characteristic for both species remains unshifted, whereas the intensity of the oxidation state marker band at 1369 cm⁻¹ of hematin is significantly increased relative to that at 759 cm⁻¹. Small increases by 2–3 cm⁻¹ were observed in modes mostly associated with the quinoline stretching vibrations, where the aliphatic chain of CQ, or the pyrimidine moiety in the case of MQ, were less involved in interaction upon adsorption on Au particles.⁹⁴

A recent X-ray powder diffraction based study by Solomonov et al.⁹⁵ presented some evidence that quinoline additives reduce crystal mosaic domain size along the needle axis. Coherent grazing exit X-ray diffraction indicated that the mosaic domains are smaller and less structurally stable than in the pure crystals. IR-ATR and Raman spectra show changes that can be attributed to molecular-based differences from modification of surface and bulk propionic acid groups, following additive binding and a molecular rearrangement in the environment of the bulk sites poisoned by occluded quinoline. Averages of micro-Raman spectra of β -pure hematin crystals and β -hematin grown in the presence of 10% quinine and chloroquine show minor variation, mainly in band widths and relative intensities, in the 1800 to 1200 cm⁻¹ region when using 780 nm excitation. Bands at 1584 cm⁻¹ (assigned to $\nu(\text{C}_\alpha\text{C}_m)$ of E_u symmetry), 1566 cm⁻¹ (assigned to $\nu(\text{C}_\beta\text{C}_\beta)$ of A_{1g} symmetry also known as ν_2), and 1372 cm⁻¹ (assigned to $\nu(\text{pyrrole half-ring})$ of A_{1g} symmetry also known as ν_4) appear more intense in the spectra of β -hematin compared to its drug-affected counterparts. Additionally, the band at 1544 cm⁻¹ assigned to $\nu(\text{C}_\beta\text{C}_\beta)$ of B_{1g} (known as ν_{11}) appears less intense in pure β -hematin. These studies demonstrate the potential of resonance Raman spectroscopy in monitoring antimalarial binding to heme target sites.

8.7 SUMMARY

Resonance Raman spectroscopy in the near-IR provides new avenues to explore the electronic and molecular structure of heme derivatives. The extraordinary enhancement of totally symmetric modes observed for some heme derivatives when applying near-IR excitation is explained through a Franck–Condon scattering mechanism invoking exciton coupling of the excited state and not a vibronic coupling mechanism. A small *z*-polarized transition centered between 800–900 nm and designated band I is implicated in the scattering mechanism. The symmetric electronic component of the near-IR photon excitonically couples the *z*-polarized charge transfer transition, causing appreciable Franck–Condon overlap in the vibronic excited states. The combination of a Raman microscope and a purpose-designed unit enabling water immersion is ideal for analyzing both heme solids and single cells. The application of the Raman technique in malaria research shows great promise, especially for monitoring drug interactions in β -hematin and directly within the infected cell. The insertion of gold nanoparticles, either as colloidal suspensions or as individual or groups of nanoparticles, into live cells will evolve the field further. Recently, Kneipp et al.⁵¹ successfully deposited nanoparticles via colloidal suspensions into live whole cells and obtained SER spectra of the cellular components that are SERS-active. One can envisage the application of this technology in monitoring SERS signals to study the drug–cell interaction.

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COHERENT ANTI-STOKES RAMAN SCATTERING (CARS) MICROSCOPY

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9.1 INTRODUCTION

Infrared absorption and Raman scattering are attractive processes for generating contrast in microscopy applications. In both cases, however, specific problems make a broad application difficult. In IR microscopy, the long wavelength of the excitation light leads to a comparatively poor spatial resolution, on the one hand due to the diffraction-limited focusing and on the other hand because only reflective optics with relatively small numerical apertures can be employed. In addition, the broad water absorption makes investigations of aqueous solutions, and therefore of most biological samples, difficult. Spontaneous Raman scattering microscopy, by contrast, allows working with excitation light in the visible spectral range but suffers from the low Raman scattering cross sections. This causes long acquisition times and requires high laser powers (typically tens of milliwatts) that are not tolerated by many samples. Furthermore, sample autofluorescence that is observed in most specimens makes the detection of the weak Raman signals difficult and limits the range of possible applications, especially in biological investigations. Here, we want to present coherent anti-Stokes Raman scattering (CARS) microscopy as a method for vibrational microscopy which is not impeded by the limits of the techniques described above.

CARS as a spectroscopic technique has first been described by P. D. Maker and R. W. Terhune in 1965.¹ Since then, CARS spectroscopy has become an important tool for chemical analysis in the condensed and gas phase. Its use in microscopy has first been proposed in the early 1980s by Duncan et al.² but only the introduction of collinear excitation and the use of NIR excitation by Zumbusch et al.,³ initiated the extraordinary fast development of CARS microscopy during the last decade. The range of demonstrated applications meanwhile ranges from studies of material scientific samples⁴ to the investigation of lipid-trafficking in single living cells and even live animals.^{5,6}

In the following chapter we will present the theoretical basics and the main experimental approaches realized to date. This will be complemented by some examples demonstrating the power of CARS microscopy.

9.2 THEORETICAL CONSIDERATIONS

Formally, CARS is described as a four-wave mixing. The sample is excited by a pump (ω_p), a Stokes (ω_{st}), and a probe pulse. These three electromagnetic fields interact to yield anti-Stokes emission at a fourth frequency (ω_{as}). The experiments are usually performed in a frequency degenerate manner, such that the pump laser also provides the probe field. Energy conservation dictates the relation $\omega_{as} = 2\omega_p - \omega_{st}$, which leads to the excitation scheme depicted in Fig. 9.1.

Microscopic contrast is generated by using the resonance enhancement of the CARS process: If the frequency difference $\omega_p - \omega_{st}$ coincides with a vibrational transition frequency of sample molecules, a strongly enhanced signal is observed. Thus scanning the sample at a given resonance frequency can be used to determine the spatial distribution of molecules with a vibrational transition at this frequency. It is important to note that the detected anti-Stokes frequency is higher than all excitation frequencies. Therefore a separation of the signal can be achieved by simple spectral filtering. For the same reason, any (auto-)fluorescence from the probe can easily be separated from the CARS signal.

The intensity of the CARS signal has a nonlinear dependence on the excitation intensity and is given by

$$I_{\text{CARS}} \propto |\chi_{\text{CARS}}^{(3)}|^2 I_p^2 I_{st}$$

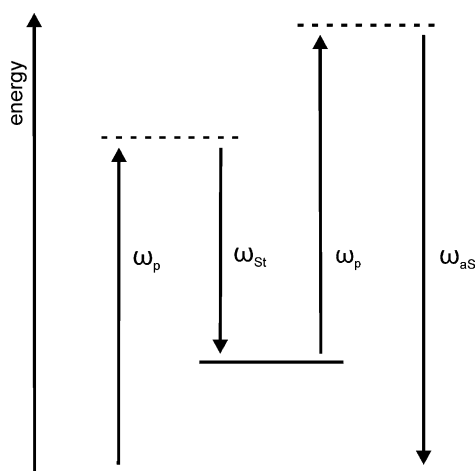


Figure 9.1. Energy level diagram of the CARS process.

where $\chi_{\text{CARS}}^{(3)}$ is the third-order nonlinear susceptibility and I_p and I_{St} are the intensities of the pump and Stokes lasers, respectively.

$\chi_{\text{CARS}}^{(3)}$ can be decomposed into a resonant and a nonresonant part.

$$\chi_{\text{CARS}}^{(3)} = \chi_r^{(3)} + \chi_{\text{nr}}^{(3)}$$

The nonresonant part is a real number and is virtually independent of vibrational resonances of the sample. In the absence of electronic resonances, it leads to a frequency-independent background.

The resonant part, $\chi_r^{(3)}$, is given by

$$\chi_r^{(3)} = \sum_j \frac{A_j}{\delta_j - i\Gamma_j}$$

It is a complex sum over all vibrational resonances j involved, with the detuning from the vibrational frequency $\delta = \Omega_j - (\omega_p - \omega_{\text{St}})$, oscillator strengths A_j , and linewidths Γ_j . $\chi_r^{(3)}$ is maximized when $\delta = 0$, which means that $(\omega_p - \omega_{\text{St}})$ is tuned to a molecular vibration Ω_j in the sample.

One can further decompose the resonant susceptibility into a real and an imaginary part. The real part has a dispersive lineshape, while the imaginary part, which parallels the spontaneous Raman line, has a Lorentzian shape (see Fig. 9.2a).

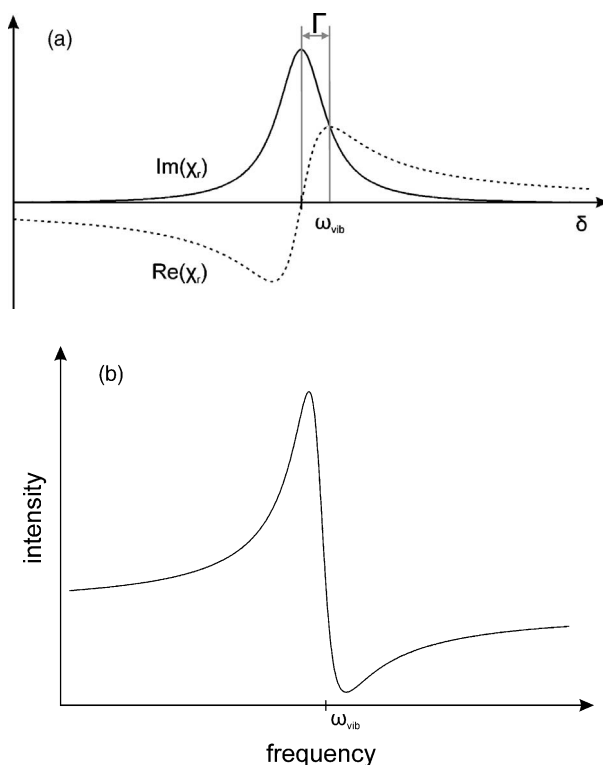


Figure 9.2. (a) The real and imaginary part of $\chi_r^{(3)}$. Γ is the Raman linewidth and δ is the detuning of the vibrational frequency ω_{vib} . (b) Resulting typical shape of a CARS band. Note the shift of the maximum compared to ω_{vib} and the dip in intensity at the high energy side of the band.

Taking all this into account, the overall intensity can now be written as

$$I_{\text{CARS}} \propto |\chi_r^{(3)} + \chi_{\text{nr}}^{(3)}|^2 = \left(\chi_{\text{nr}}^{(3)}\right)^2 + 2\chi_{\text{nr}}^{(3)}\text{Re}\{\chi_r^{(3)}\} + \text{Re}\{\chi_r^{(3)}\}^2 + \text{Im}\{\chi_r^{(3)}\}^2 \quad (9.1)$$

From this brief theoretical considerations, two points emerge, which are noteworthy in comparison to spontaneous Raman scattering: First, the CARS signal itself shows a complicated dependence on the number of scatterers, which is reflected in the material property $\chi^{(3)}$. For high concentrations of a molecular species, however, the quadratic terms in Eq. (9.1) dominate and consequently the intensity dependence is quadratic. For species in low concentrations, the linear term is larger, which leads to a linear intensity dependence. Second, due to the complex nature of $\chi_r^{(3)}$ and the interference between the resonant and the nonresonant parts, the maximum of a CARS band is slightly shifted to lower energies with respect to the corresponding spontaneous Raman band and shows a more or less pronounced dip at the high-energy side of the band (see Fig. 9.2b).

9.3 CARS MICROSCOPY

CARS is a coherent process. This means that in order to generate a strong signal, the phases of the contributing electromagnetic waves have to be matched within the excitation volume. Momentum conservation arguments lead to the phase-matching condition $|\Delta k|l = \pi$ with the interaction length l , the wave vector mismatch $\Delta k = k_{\text{aS}} - 2k_{\text{p}} - k_{\text{St}}$, and the wave vectors k_{aS} , k_{p} , and k_{St} of the anti-Stokes, the pump, and the Stokes fields, respectively. For CARS spectroscopy in condensed phase, this phase-matching condition is commonly fulfilled by choosing an angle between the excitation beams. In doing so, dispersion effects that are relevant since the excitation beams and the signal have three different frequencies can be compensated (see Fig. 9.3).

A similar scheme has been proposed in the first CARS microscopy experiments. Spatial separation of the excitation beams for the phase-matching angle selection according to the scheme depicted in Fig. 9.3, however, means that only small numerical apertures can be realized in microscopy. This, in turn, leads to poor spatial resolution. While phase matching in this manner has been used initially,² later experiments have indeed shown that this scheme is not suitable for high-spatial-resolution microscopy.⁷ The demonstration that CARS microscopy with tight focusing using high-numerical-aperture lenses and collinear excitation leads to very strong CARS signals³ thus brought an important breakthrough for CARS microscopy. It turns out that under these tight focusing conditions, phase matching is provided by the large cone of angles for the k -vectors of the excitation beams and the very small excitation volume.⁸ The small excitation volume emerging from this excitation scheme, along with the nonlinear intensity dependence of the excitation process, offers the additional attractive property of a very good spatial

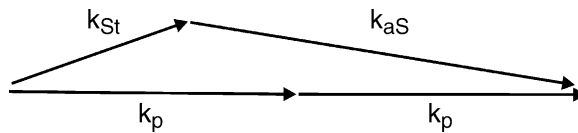


Figure 9.3. The phase-matching condition

resolution in the z -direction – that is, along the optical axis of the microscope. For this latter reason and similar to two-photon fluorescence microscopy,⁹ CARS microscopy has an inherent three-dimensional imaging capability without the need to insert a confocal pinhole into the detection path.

9.4 SUPPRESSION OF THE NONRESONANT BACKGROUND

From the discussion of the theoretical basis of CARS microscopy, it is obvious that CARS microscopy, is not a zero-background detection technique. Instead, if no special techniques are employed, the resonant signal is always accompanied by a nonresonant background signal that reduces the detection limit significantly. Especially for the high-quality imaging of biological samples, a nonresonant background suppression is mandatory. For this reason, the recent technical development of CARS microscopy has been coined by the introduction of several approaches to reduce the nonresonant background. A second important factor with respect to applications of CARS microscopy concerns the improvement of the molecular selectivity. For obtaining information on a specific vibration ω_{vib} , the excitation wavelength is tuned to $\omega_{\text{vib}} = \omega_{\text{p}} - \omega_{\text{st}}$. The unambiguous identification of a molecular species on the basis of its vibrational spectrum, however, often requires the simultaneous monitoring of several vibrational bands. In order to observe different vibrations in one sample using the CARS microscopy scheme described above, it is necessary to change the excitation wavelength. Especially for complex samples with several vibrations to be followed, a manual detuning is tedious and parallel recoding schemes are needed. Exemplary experimental approaches to meet both ends are discussed in the following.

In general, all those approaches can be divided into three groups. In the first, only one CARS wavelength is investigated, preferably using a small-spectral-bandwidth-pulsed picosecond laser system. Here, it is crucial to reduce the nonresonant background very efficiently in order to achieve high signal-to-background ratios. The second group uses a combination of spectrally broad and narrow laser pulses. In this multiplex approach, a broad CARS spectrum is dispersed and detected using a CCD camera. The nonresonant signal can be additionally measured and subtracted. In the third group of experiments, finally, two different vibrational frequencies are simultaneously monitored in order to detect the CARS signal at two different positions. This can be used to either reduce the nonresonant background by measuring its contribution to the signal or to increase the molecular selectivity by simultaneously checking two vibrations of the species of interest.

9.4.1 Ps-Laser Systems and One CARS Wavelength Detection

In the year 2001, two new approaches to suppress the nonresonant background were introduced by the group of Sunney Xie. The first exploits the fact that the polarization properties between the resonant and the nonresonant CARS signal are different.¹⁰ Such an approach had already been employed in CARS spectroscopy before.¹¹ Proper alignment of the polarization of the excitation beams and an analyzer in the detection pathway allow for an efficient suppression of the nonresonant background. At the same time, however, also the resonant signal is severely attenuated. The second approach uses a different detection geometry. In this so-called epi-detection the sample is excited and the signals are collected by the same objective.¹² A detailed analysis shows that the magnitude of the backscattered signal has a strong dependence on the diameter of the scattering sample feature. While the (nonresonant) background from the solvent or the matrix material exhibits a strong

destructive interference in the backward direction and is thus mostly forward-directed, features with diameters below or equal to the wavelength of the excitation light are efficiently backscattered. For small features, epi-detection therefore leads to a good suppression of the solvent signal, even if the quantitative interpretation of the resonant signal is not possible.

9.4.2 Recording of a CARS Spectrum: Multiplex CARS and Spectral Focusing

A combination of a spectrally broad and a spectrally narrow pulse gives access to the simultaneous detection of several vibrations in a broad spectral region (see Fig. 9.4). Apart from an increased sensitivity, this method also allows us to turn the existence of the nonresonant background into an advantage. This can be seen from Eq. (9.1): The cross-term $2\chi_{\text{nr}}^{(3)} \text{Re}\{\chi_r^{(3)}\}$ amplifies the resonant signal in a heterodyne fashion. Of course, it is still necessary to determine the nonresonant background in order to quantify the resonant signal. This, however is easily achieved by measuring the signal of, for example, a glass cover slip. Multiplex CARS thus provides a straightforward access to the quantitative recording of CARS microspectroscopic data with background reduction.

It should, however, be noted that the interpretation of a vibrational CARS spectrum can become very difficult. As has been explained above, the coherent nature of the CARS signal leads to a complex shape even for an isolated vibrational resonance. In congested spectral regions with several vibrational resonances, the interpretation of the CARS spectra necessitates the application of advanced techniques of spectral decomposition in order to correctly assign the contribution of the different resonances.¹³ From what has been said above, it is obvious that multiplex CARS microscopy requires two synchronized laser sources, one with a broad spectrum preferentially from a femtosecond laser source, the other spectrally tight from a picosecond laser source. While there have been great advances in laser development recently, the synchronization of two ultrafast lasers still is a formidable task. Broad spectral pulse can also be obtained by using a photonic fiber.¹⁴ Here only one

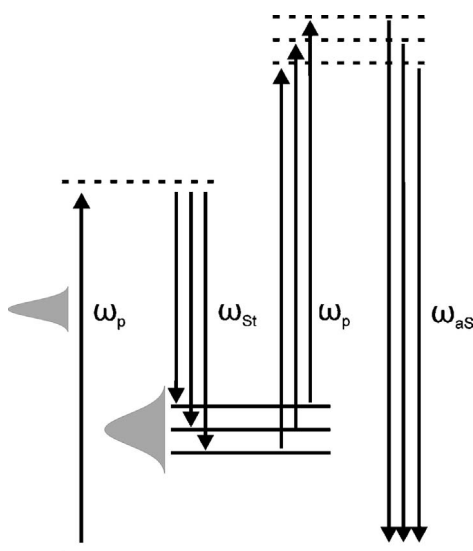


Figure 9.4. Scheme of a multiplex CARS excitation. Note the spectrally broad Stokes pulse with ω_{St} and the spectrally narrow pump pulse with ω_p .

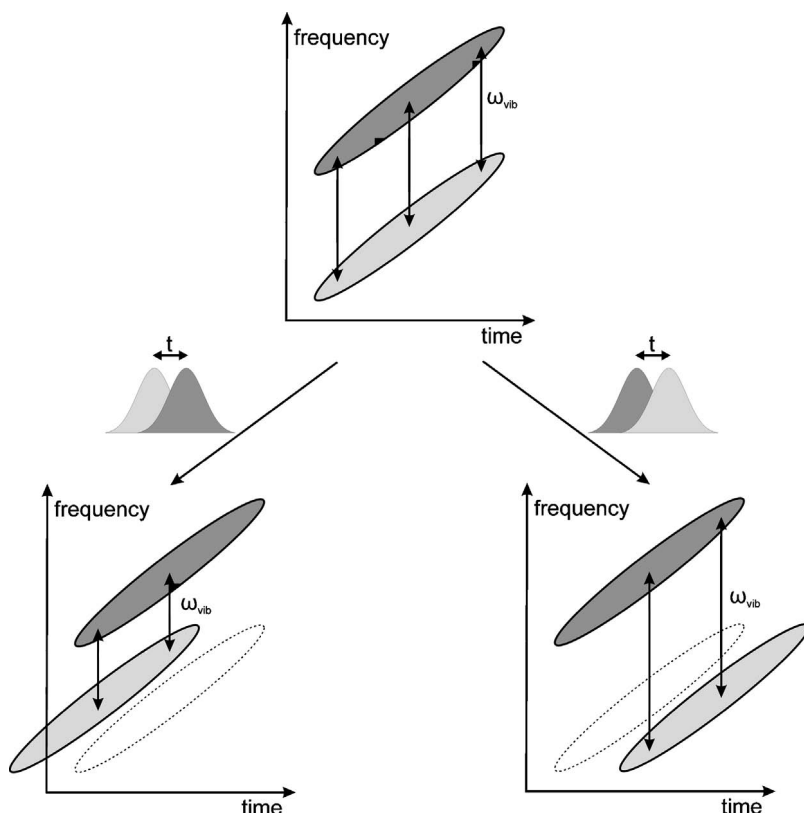


Figure 9.5. Principle of spectral focusing in CARS. Shifting the chirped pump and Stokes pulses in time results in a different vibrational frequency ω_{vib} addressed.

laser source is needed and pump and Stokes pulses are automatically synchronized. To date, the achievable laser powers from such systems in a narrow spectral region are, however, quite low.

A different approach is to use a single femtosecond laser source and to stretch these pulses in time.^{15,16} These chirped pulses can be used in combination with a femtosecond pulse or one more chirped femtosecond pulse in order to obtain CARS spectra. For this purpose, the two excitation pulses are temporally delayed with respect to each other and the CARS signal is recorded for every delay (see Fig. 9.5). The temporal detuning, which is achieved much easier than spectral tuning of an ultrafast laser, can then be easily converted into spectral information. This technique of spectral focusing (see Fig. 9.6) makes it possible to record spectra with very high resolution, since the latter is only determined by the amount of chirp employed.¹⁵

9.4.3 Excitation with Three Different Beams

A recent approach to CARS microscopy uses a reference signal generated outside the probe and mixes it with the signal from the probe. This method is called heterodyne CARS.¹⁷ The reference signal is generated in a substrate free of vibrational resonances in the desired region. It has the same wavelength as the anti-Stokes signal generated in the probe.

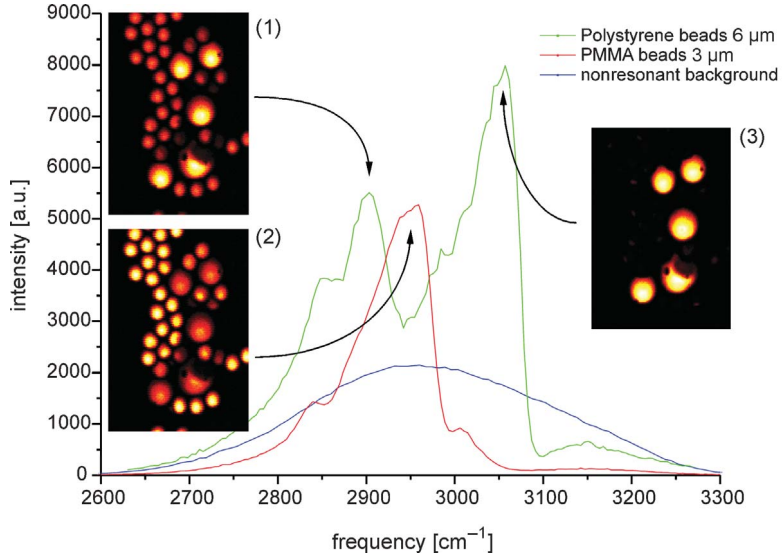


Figure 9.6. Spectral focusing CARS demonstrated on a mixture of polystyrene (6- μm diameter) and PMMA (3 μm diameter) beads. Both have an aliphatic band at 2910 cm^{-1} and at 2950 cm^{-1} , respectively. Only polystyrene, however, has an aromatic band at about 3050 cm^{-1} . The images were taken at three different frequencies: (1) the aliphatic vibration of polystyrene, (2) the aliphatic vibration of PMMA, and (3) the aromatic vibration of polystyrene. Note the different intensity levels in the images, especially in image (3), where there is almost no signal from the PMMA beads.

By mixing the two signals the overall signal can be written as

$$S = |E_{\text{LO}}|^2 + |E_{\text{as}}|^2 + 2E_{\text{LO}}E_{\text{p}}^2E_{\text{St}} \left[\left(\chi_{\text{nr}}^{(3)} + \text{Re}\{\chi_{\text{r}}^{(3)}\} \right) \cos \Phi + \left(\text{Im}\{\chi_{\text{r}}^{(3)}\} \right) \sin \Phi \right] \quad (9.2)$$

where Φ is the phase difference between E_{LO} and E_{as} . The real and imaginary parts have different dependencies on Φ . Therefore, by varying Φ , one can detect the real part ($\Phi = 0^\circ$) or the imaginary part ($\Phi = 90^\circ$), assuming that the homodyne terms $|E_{\text{LO}}|^2$ and $|E_{\text{as}}|^2$ are relatively small. Because only resonant signals contribute to the term $\text{Im}\{\chi_{\text{r}}^{(3)}\}$, a signal free of nonresonant background can be detected. Furthermore, $\text{Im}\{\chi_{\text{r}}^{(3)}\}$ is linearly proportional to the amount of scatterers and can be used for quantitative measurements. The main drawback of this method is that the phase Φ has to be maintained with a phase modulator and later the signal has to be demodulated again. This results in longer acquisition times. In addition, differences in the refractive index within the probe result in artifacts in the image.¹⁸

To circumvent these problems, the nonresonant signal can also be generated inside the probe. For this purpose, two pump beams ω_{p1} and ω_{p2} that are combined with the Stokes beam ω_{St} are needed. The wavelength $\omega_{\text{p1}} - \omega_{\text{St}}$ is tuned to the desired resonance and $\omega_{\text{p2}} - \omega_{\text{St}}$ is tuned to a region free of any resonances. In sum, a mixture of a resonant and nonresonant signal ω_{as1} and a purely nonresonant signal ω_{as2} is detected. The nonresonant signal then can be subtracted, which results in a purely resonant signal.

Two experimental approaches of this type have been demonstrated in CARS microscopy. On the one hand the so-called frequency modulation or FM-CARS,¹⁸ and on the other hand a method called dual-frequency CARS.¹⁹

In FM-CARS, two different lasers are used as separate pump pulses. Either one of the beams is used to excite the sample with a Pockels cell acting as a fast switch between the beams. The detector then detects ω_{AS1} or ω_{AS2} , depending on the Pockels cell setting. If one beam samples a resonance and the other the zero signal crossing at the high-energy side of the CARS spectrum, then the frequency modulation is efficiently translated into an amplitude modulation. The main disadvantage of this method consists in the necessity to select an isolated resonance in order to generate a large amplitude modulation. This problem can be avoided by performing simultaneous measurements with two pump frequencies. This method can be employed to either simultaneously probe two Raman transitions or reduce the nonresonant background. Furthermore, any intensity fluctuation in the excitation beams occur in both channels and are canceled out.

9.5 APPLICATIONS TO BIOLOGY

During the last years, several publications have demonstrated the application of CARS microscopy to biological problems. In this type of application, all advantages offered by CARS microscopy can be exploited: (i) Contrast is generated without the need to label the sample, (ii) no photobleaching is observed, and (iii) the contrast generation is chemically sensitive.

9.5.1 Lipids

To date, most biological applications have dealt with the investigation of lipids. Lipids play an important role as components in biological membranes, as energy storage molecules, and as messengers in cellular communication. Lipids are ideal first targets for biological applications of CARS microscopy, since they are comparatively easy to detect in cells. This is primarily due to their high local concentration and their high CH stretching vibration signal. Furthermore, the CH stretching vibration is well-separated from other resonances. Strong signals are also observed for the C–D stretching vibration. Using deuterated compounds opens a wide range of applications in lipid research but also in other areas. While this reintroduces a type of labeling, the big difference compared to labeling with fluorophores consists in the similarity of the physiological properties of deuterated and nondeuterated compounds and the absence of photobleaching. For this reason, CD labeling has found use in many CARS experiments.^{6,20,21} As has already been pointed out, the detection of lipids is one of the most advanced applications of CARS microscopy. There have been investigations on the orientation of lipids,²² quantitative measurements,^{21,23} and measurements on single lipid bilayers.²⁴

In Fig. 9.7 a HeLa cell is shown. The picture has been acquired at the asymmetric CH_2 stretching vibration (2845 cm^{-1}) using spectrally focused CARS microscopy.^{15,19} The lipid vesicles inside the cell are clearly visible. Note also the profile along the white line.

In Fig. 9.8 also a HeLa cell is shown, this time the image is recorded at 2582 cm^{-1} . This corresponds to the region of the SH stretching vibration, which can be found for example in the tripeptide Glutathion. One can see a clear difference compared to the picture at the CH_2 vibration. The rather big lipid vesicles are no longer visible. By contrast, small vesicles can be clearly seen, the contrast of which could be due to SH stretching vibrations.

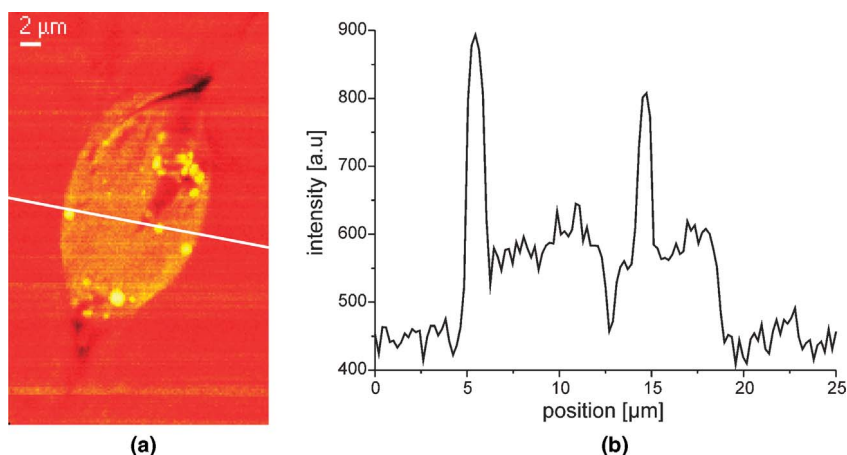


Figure 9.7. (a) An image of a HeLa cell at the asymmetric CH₂ stretching vibration (2845 cm⁻¹). Note the bright lipid vesicles. (b) Intensity along the white line in image a.

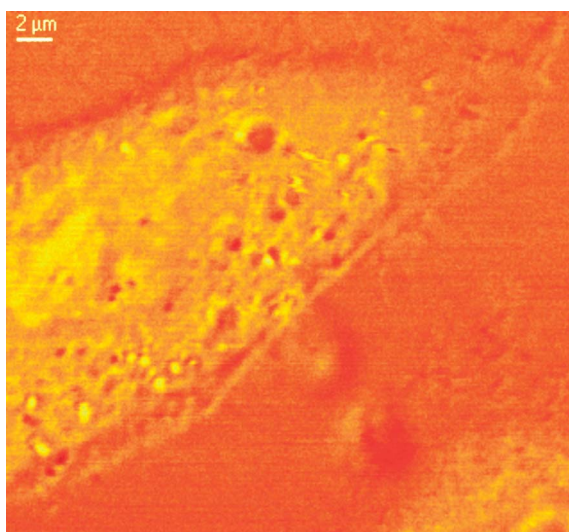


Figure 9.8. HeLa cell imaged in the region of the SH stretching vibration (2582 cm⁻¹).

9.6 OUTLOOK

In this Chapter, we have presented CARS microscopy as a new nonlinear optical microscopy technique. It allows the imaging of unlabeled samples with high sensitivity and chemical specificity, since contrast generation is based on the vibrational spectra of the sample molecules. In contrast to fluorescence microscopy, however, CARS microscopy is not a zero-background technique. The suppression of the nonresonant background signal has indeed been the greatest challenge in making CARS microscopy attractive to a broader audience. It has been successfully tackled in many different ways during the last couple of years. As a result, first applications of CARS microscopy mainly in investigations of biological samples are now starting to appear. With the rapid development of the field

during recent years in mind, it is easy to foresee that this is just the beginning of an exciting new branch of vibrational microscopy. CARS microscopy clearly has the potential to complement established other microscopy techniques in the life sciences, mainly fluorescence microscopy, in problems where these cannot be applied.

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SURFACE-ENHANCED RAMAN SENSORS FOR METABOLIC ANALYTES

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10.1 BACKGROUND

10.1.1 Introduction

In recent years, advances in material science, nanotechnology, and bioengineering have led to a rapid development of novel applications in clinical diagnostics and approaches to interrogate complex biological systems. In particular, vibrational spectroscopy has been utilized for studies ranging from cellular properties to protein structure to direct detection of bioanalytes *in vivo*. Previously, surface-enhanced Raman spectroscopy (SERS) was not widely used for bioanalysis due to several factors: structural similarity of many important molecules, spectral complexity of biological media, placement of molecules within a few nanometers of a nanostructured metal surface, and biocompatibility of the SERS-active surface. While the use of SERS labels provides a successful detection modality,^{1–3} direct detection of bioanalytes *in situ* remains a formidable challenge. This chapter describes a system that overcomes many of the obstacles related to using SERS to sense metabolically relevant analytes such as glucose and lactate. Determination of blood glucose concentration is the most common analytical measurements performed by diabetics in the United States. It is essential to monitor glucose levels for diabetes patients to avoid hyper/hypoglycemia and to mitigate the secondary health risks associated with prolonged buildup of sugar in the vasculature. Likewise, lactate is an important biomarker of anaerobic glycolysis, which occurs under conditions of decreased tissue oxygenation. In a clinical setting, it could be used to monitor drug toxicity and hypovolemic left heart failure, as well as

exertion and athletic performance.⁴ To detect glucose and lactate, we start with a simplified *in vitro* scheme to examine fundamental properties of the sensor such as reversibility, stability, and temporal response. Then, we demonstrate quantitative detection of both analytes in a biological environment (i.e., bovine plasma). Finally, we show the successful quantitative detection of glucose *in vivo* by implanting a SERS substrate into the interstitial space of a rat.

10.1.2 Indirect Detection

Currently, the most successful methods for glucose and lactate measurement are indirect enzymatic assays based on glucose oxidase and lactate oxidase.^{4–8} Most common commercial glucometers known as “fingersticks” utilize electrochemical detection of the hydrogen peroxide produced by the enzymatic oxidation of glucose. Although they are widely used, “fingersticks” are painful and result in infrequent, intermittent measurements. Another example of a device that also utilizes electrochemical detection of glucose is the GlucoWatch, which extracts interstitial fluid by applying voltage to the skin (iontophoresis). While it does not require blood samples, the GlucoWatch is still quite uncomfortable and requires numerous calibrations with the fingerstick.^{9,10} Given the industrial success of the enzymatic approach, due primarily to the protein’s inherent amenability to biological environments and high specificity, many research groups have attempted to utilize glucose-sensitive enzymes in innovative, nonelectrochemical techniques. For example, fluorescence resonance energy transfer (FRET) has been used to detect glucose with fluorescently labelled concanavalin A and dextran.¹¹ Glucose binds to concanavalin A replacing the dextran and separating the FRET pair. As a result, the fluorescently labeled dextran exhibits stronger fluorescence in response to glucose. Another example of enzymatic glucose detection involves the use of a glucose sensitive microcantilever.¹² A microfabricated cantilever surface was functionalized with glucose oxidase, causing the cantilever to bend due to surface stress changes caused by the binding and oxidation of glucose. All enzymatic sensors experience similar limitations because they rely on the same core chemistry – the protein-mediated binding of glucose or lactate. Proteins have inherently finite stability, particularly in terms of enzyme turnover lifetime, leading to a need to replenish the protein. Such sensors are also sensitive to interferences – for example, similar monosaccharides, uric acid, acetaminophen, or dissolved oxygen.^{6,13} The enzyme activity is also known to be temperature- and pH-dependent, factors frequently beyond experimental control in real-world systems. Furthermore, similar to existing methods these techniques require extraction of blood or interstitial fluid. In addition, the measurements are performed on large laboratory instruments and would not make feasible portable devices.

Others have mimicked the specificity of proteins for monosaccharides by using artificial receptors such as boronic acids,^{14,15} molecularly imprinted polymers (MIPs),^{16,17} or other molecules that can bind glucose¹⁸ and transduce fluctuations in analyte concentrations into analytical measurements. These chemistries have been featured in numerous indirect detection methods including diffraction spectroscopy,^{15,19} fluorescence,^{11,14,20–22} and colorimetric UV–visible spectroscopy.^{23,24} The reported techniques have all been used to detect glucose with varying degrees of success. The boronic acid and imprinted polymer sensors lack sensitivity to glucose as compared to other sugars.^{18,20} Many of the boronic acids exhibit optimal binding of glucose at a pH outside the physiological range.²⁵ MIPs also have low selectivity, due to the structural specificity of the “glucose-shaped” binding cavity.¹⁸ Such indirect methods offer a potentially unlimited number of sensitive detection

modalities, including signal multiplication. However, they have many possible sources of error, particularly from competing species. Therefore, it is desirable to be able to directly measure the concentration of glucose and other analytes.

10.1.3 Direct Detection

The direct detection of glucose and lactate has been a more difficult analytical problem than the indirect methods detailed above. While these analytes can be directly assayed by a variety of laboratory techniques (mass spectrometry, chromatography, etc.),^{26,27} the number of methods amenable to clinical or personal use is limited. The majority of the direct glucose and lactate detection techniques are optical due to the reagentless, nondestructive, and rapid nature of spectroscopic analysis. Polarimetry has been used to measure glucose concentration based on the rotation of light in aqueous humor of the eye.^{28,29} Unfortunately, other rotationally active species in aqueous humor, such as ascorbate and albumin, can interfere with the changes caused by glucose. Furthermore, corneal birefringence complicates such polarimetric measurements. Vibrational spectroscopies enable quantitative, molecularly specific identification of analytes based on their unique spectroscopic “fingerprints.” Researchers are actively pursuing a variety of infrared and near-infrared (NIR) absorbance^{30–38} and Raman spectroscopies for glucose and lactate detection.^{38–48} For example, several groups have recently published results demonstrating both sensitive and accurate glucose measurements using infrared absorbance. Unfortunately, the acquisition of high-quality spectra requires high powers delivered at the sample and extensive collection times. Current NIR results are notable, but the portable applications of this technique are constrained by the instrumental size, cost, and power. Raman spectroscopy applied to glucose detection also requires high powers and long acquisition times due to the low Raman scattering cross section of glucose, $5.6 \times 10^{-30} \text{ cm}^2 \text{ molecule}^{-1} \text{ sr}^{-1}$.⁴⁹ This is particularly important when interrogating complex biological mixtures. Intrinsic fluorescence or strong scattering from molecules such as hemoglobin may overwhelm the signal from glucose or lactate. Detection in less complicated media such as interstitial fluid, tears, or the aqueous humor of the eye could potentially ameliorate the difficulties inherent in interpreting spectra with many interfering analytes.⁵⁰ However, this approach is complicated by the low levels and the temporal delay of glucose concentration in secondary fluid as compared to blood.^{51,52} The higher signal intensity gained from the surface-enhanced Raman scattering phenomenon overcomes the limitations imposed by weak signal strength, enabling the use of lower power and shorter acquisition times.⁵³

10.1.4 SERS Multianalyte Detection

In an effort to show that glucose detection is feasible using SERS, Van Duyne and co-workers have developed a sensing platform that not only is capable of detecting glucose, but can also serve as a general detection system for various metabolically relevant analytes and disease biomarkers. It is designed to take advantage of the optical properties and morphology of noble metal nanostructures. Surface-enhanced Raman scattering is observed when molecules are within a few nanometers of such a surface. The collective oscillation of free electrons on the metal surface excited by incident light causes an enhanced electric field, known as the localized surface plasmon resonance (LSPR), which results in the scattering intensity increasing by a factor of 10^6 to 10^8 .^{54,55} In special cases, enhancement factors can be as high as 10^{15} .^{56,57} The surface-enhanced Raman scattering effect is strongest on silver, but is also observed on gold and copper. The enhancement is optimized

when the excitation wavelength matches the oscillation frequency of the electrons at the surface.⁵⁸ Thus, careful control of the roughened metal surface structure is important in achieving consistent enhancements. Molecules confined within the decay length of the electromagnetic field will exhibit surface enhancement even if they are not chemisorbed to the surface. For the SERS-active surfaces used in the work described below, the decay length, d_{10} , at which the intensity decreases by a factor of 10, was calculated to be 2.8 nm.⁵⁹

Electromagnetic enhancement in SERS is due to the locally amplified fields from the LSPR. The peak wavelength of the LSPR depends on several factors such as material, size, shape, spacing, and dielectric environment of the metal nanostructure.^{60–63} Surface-enhanced Raman spectroscopy is typically used to investigate monolayers of molecules deposited or adsorbed to the metal surface. The spectral location of the LSPR significantly affects the resulting surface-enhanced Raman (SER) spectrum. In fact, SERS intensity is optimized when the laser excitation is of slightly shorter wavelength than the LSPR maximum.⁵⁸ As a result, both excitation and scattered photons are optimally enhanced, the sensitivity is increased, and lower detection limits can be reached.

SERS substrates used for glucose and lactate detection are known as film over nanospheres, or FON (Fig. 10.1A). LSPR position tuning can be achieved by varying the diameter of the spheres used to fabricate the mask.⁶⁴ Optimizing the LSPR maximum wavelength and matching it to the laser wavelength will enable optimal sensing. A silver FON (AgFON) surface is functionalized with a self-assembled monolayer (SAM) to increase the interactions between the surface and the analyte (Fig. 10.1B). In initial studies, straight-chain alkanethiols were found to be the most effective in partitioning glucose.⁴⁵ In

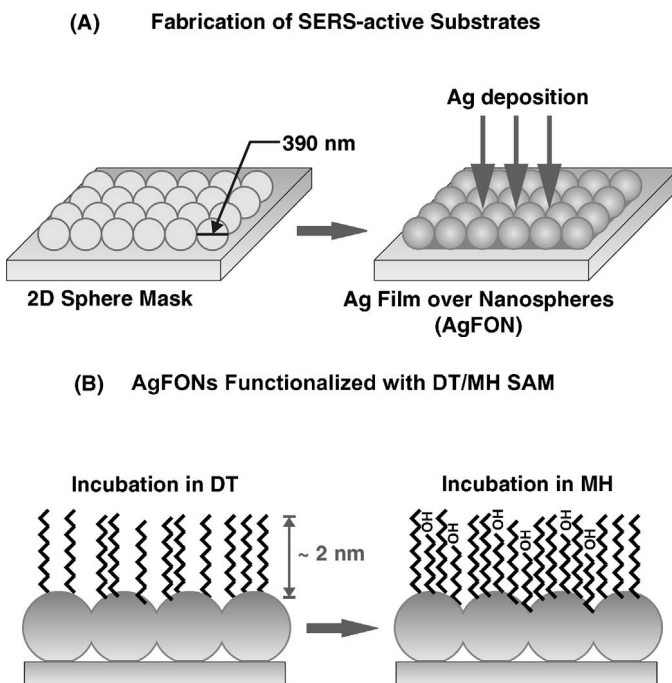


Figure 10.1. (A) Schematic illustrating AgFON fabrication. (B) Formation of the DT/MH self-assembled monolayer on the AgFON surface.

particular, 1-decanethiol ($\text{HS}(\text{CH}_2)_9\text{CH}_3$) was used, which results in a monolayer on silver that is ~ 1.9 nm thick, within the decay length, d_{10} , of the electromagnetic field at the surface. As glucose partitions into the SAM, surface-enhanced Raman scattering is observed.

Glucose partitioned into the SAM from an 80% ethanol–20% water solution, and spectra were acquired from dried samples after incubation in the glucose solution. Further developments in SERS glucose sensing resulted in modification of the SAM to include an ethylene glycol terminal group ($\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_3\text{OH}$), referred to as EG3, to improve biocompatibility of the sensor and eliminate fouling by proteins.⁴⁶ Spectra were acquired with saline glucose solution flowing through the cell. Furthermore, gold FONs were evaluated, resulting in improved stability and predictive accuracy of the sensors.⁶⁵ Also, gold surfaces exhibit strong SERS enhancement at longer excitation wavelengths, which is more appropriate for future *in vivo* application of the sensor due to decreased tissue absorption and scattering in the NIR wavelength region.

The most recent work in the development of the SERS sensor has focused on optimization of the SAM and combating the synthetic challenges associated with EG3 terminated alkanethiols. The latest SERS glucose sensor consists of an AgFON functionalized with a dual component SAM. The SAM is comprised of decanethiol ($\text{HS}(\text{CH}_2)_9\text{CH}_3$) and mercaptohexanol ($\text{HS}(\text{CH}_2)_6\text{OH}$), which provide an appropriate balance between hydrophilic and hydrophobic interactions of the SAM with the analyte and is well-suited for partitioning of small metabolic analytes in aqueous solutions.⁶⁶ We have shown that the sensor is reversible, is stable in bovine plasma, and responds rapidly to changes in concentration. After successfully demonstrating quantitative detection of glucose in biological milieu such as bovine plasma, the sensor further was used to demonstrate *in vivo* detection of glucose in rats.⁶⁷ Simultaneously, the dual component SAM was used to detect lactate *in vitro*.⁶⁸ Many of the challenges associated with the use of SERS for bioanalysis have been overcome in the sensing platform discussed in this chapter. Although small analytes such as glucose and lactate exhibit some spectral overlap, they can easily be identified by several vibrational bands unique to each analyte. Furthermore, the SAM accomplishes several essential tasks: (1) It reversibly partitions the molecule of interest and brings it close to the roughened metal surface within the decay length of the enhancing electromagnetic field, (2) it prevents numerous macromolecules from getting to the surface thus reducing spectral complexity of the signal from biological media, and (3) it serves as a chemically tailored barrier between the environment and the sensor preventing protein adsorption and enhancing biocompatibility. Due to the extensive library of SAM components and their functionalities, and other methods to modify the SERS-active substrates, the sensor can be optimized to detect an unlimited number of analytes simultaneously. Multiplexing SAMs that target different molecules can be used to create a SERS device for rapid identification and quantification of biomolecules in acute clinical situations as well as reliable routine measurements for millions of patients.

10.2 EXPERIMENTAL SETUP

10.2.1 AgFON Substrate Preparation

Copper disks 18 mm in diameter were utilized as substrates for glucose and lactate detection. They were cleaned and pretreated as described previously.⁶⁶ Approximately 10 μL of the nanosphere suspension was drop-coated onto each copper substrate

and allowed to dry in ambient conditions. The substrates were then mounted into an electron beam deposition system for metal deposition (Kurt J. Lesker, Clairton, PA). Silver metal films ($d_m = 200$ nm) were deposited over the sphere masks on the substrates. The resulting Ag films over nanosphere (AgFON) substrates were stored as described previously.^{64,66}

For glucose detection, AgFON surfaces were functionalized with the self-assembled monolayer (SAM) composed of decanethiol (DT) and mercaptohexanol (MH). The AgFON were first incubated in 1 mM DT in ethanol for 45 min and then transferred to 1 mM MH in ethanol for at least 12 h. The SAM-functionalized surfaces were then mounted into a small-volume flow cell for SERS measurements.

10.2.2 SERS Apparatus

A titanium–sapphire laser (CW Ti: Sa, model 3900, Spectra Physics, Mountain View, CA) pumped by a frequency-doubled Nd:YAG laser, λ_{ex} 532 nm (model Millennia Vs, Spectra Physics), was used to generate λ_{ex} of 785 nm as described previously.^{64,66} The setup consisted of laser line and high-pass filters for the corresponding wavelengths used. Furthermore, a lens was used to focus the beam onto the sample, and a collection lens was used to focus scattered light onto the entrance slit of the single-grating monochromator (Acton Research Scientific, Trenton, NJ). For basic studies of reversibility, stability, and time response of the sensor, 532 nm excitation was used. For all *in vivo* studies, as well as *in vitro* studies with biological environment such as plasma, 785 nm excitation was used to minimize autofluorescence of proteins.^{69,70} For the detection of glucose and lactate, a small-volume flow cell was used to control the external environment of the AgFON surfaces.

10.2.3 Quantitative Multivariate Analysis

All data processing was performed using MATLAB (MathWorks, Inc., Natick, MA) and PLS_Toolbox (Eigenvector Research, Inc., Manson, WA). The chemometric analysis is described in detail in previous publications.^{66,67} Prior to processing, the spectra were smoothed and filtered. The slowly varying background, commonly seen in SERS experiments, was removed by subtracting a fourth-order polynomial fit. This method greatly reduced varying background levels with minimum effect on the SERS peaks. The chemometric analysis was performed using the partial least-squares (PLS) method and leave-one-out (LOO) cross-validation algorithm. PLS is an inverse calibration method and does not require *a priori* knowledge of all the components in the system.⁷¹ In general form, the regression vector, $\mathbf{b}$, is determined based on the spectra matrix, $\mathbf{R}$, and concentration of the training set, c :

$$c = \mathbf{R}\mathbf{b}$$

The regression coefficients can be estimated by the following equation:

$$\hat{\mathbf{b}} = (\mathbf{R}^T\mathbf{R})^{-1}\mathbf{R}^T c$$

To predict the concentration of an unknown sample, r_{unk} , the estimated regression vector, $\hat{\mathbf{b}}$, is used:

$$\hat{c} = r_{unk}\hat{\mathbf{b}}$$

To calibrate the SERS sensor, a training set of spectra was acquired. The training set consisted of solutions spectra with concentrations in the physiological range (10–450 mg/dL) for glucose and the clinically relevant range (6–240 mg/dL) for lactate. To demonstrate quantitative detection, an independent validation set of spectra was acquired and the resulting regression vector was used to predict concentrations. Finally, the root-mean-squared error of calibration (RMSEC) and prediction (RMSEP) were calculated to gauge the accuracy of the model and the prediction.

10.2.4 Time Constant Analysis

To examine temporal response of the sensor, the changes in intensity of the following Raman bands were observed: 1462 cm^{-1} CH_2 bend and 860 cm^{-1} C-CO_2^- stretch for glucose and lactate, respectively.^{72,73} The variations in intensity were due to step changes in concentration from 0 to 100 mM and back down to 0 mM for both analytes. The data were processed using PeakFit 4.12 software (Systat Software Inc, Richmond, CA). To remove the varying background in SER spectra, a fourth-order polynomial was subtracted from the baseline using MATLAB software. The spectra were further preprocessed in PeakFit with linear best-fit baseline correction and Savitsky–Golay smoothing. The amplitude of the Raman bands was obtained by fitting the data to the superposition of the Lorentzian amplitude line shapes. The data were then iteratively fit to an exponential curve to minimize the residuals to determine the time constants for partitioning and departitioning.

10.2.5 Animal Experiments

The surgical procedure and animal experiments are described in detail in previous publications.⁶⁷ Briefly, Sprague–Dawley rats were anesthetized with pentobarbital with an initial dose of 50 mg/kg. The rats were kept under anesthetic by hourly administration of pentobarbital at 25 mg/kg. After the anesthetic had taken effect, the surgical areas were prepared by removal of hair and cleaning. Then, the femoral vein was cannulated using PE 50 tubing for glucose injections. The carotid artery was cannulated with PE 90 tubing for blood glucose measurements with FDA-qualified home medical equipment (One Touch II Meter, Lifescan, Inc.). A tracheotomy was performed to enable the attachment of a ventilator to aid respiration. The incisions were shut with surgical clips. The rat was thermally stabilized by an electric heating pad throughout the course of the surgery and experiment. A metal frame containing a glass window was placed along the midline of the rat's back. A circular incision was made to allow the positioning of a DT/MH-functionalized AgFON sensor subcutaneously such that the substrate was in contact with the interstitial fluid and optically addressable through the window. The animal was then gently positioned into a heated holder in the conventional sample position on a lab-scale Raman spectroscopy system (Fig. 10.2).

The SER spectra were acquired through the optical window using a Ti:sapphire laser. Glucose was varied in the rat through intermittent intravenous infusion for 3 h. An infusion of glucose was delivered over 5–10 min, at a concentration of 1 g/mL in sterile phosphate-buffered saline via the femoral cannula. A droplet of blood was drawn from the rat, the glucose level was measured with the One Touch II glucometer, and corresponding SERS measurements were taken. The data were collected and analyzed by the PLS-LOO method described above.

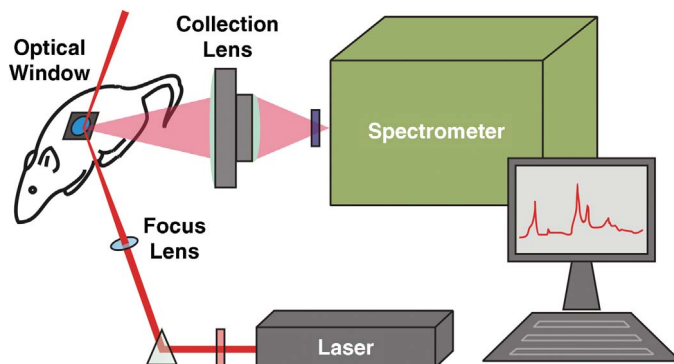


Figure 10.2. Schematic of the animal experimental setup. A rat with a surgically implanted sensor and optical window was integrated into a conventional laboratory Raman spectroscopy system consisting of a Ti:sapphire laser ($\lambda_{\text{ex}} = 785 \text{ nm}$), band-pass and long-pass filters, and steering and collection optics.

10.3 RESULTS AND DISCUSSION

10.3.1 Reversibility of the DT/MH AgFON for Multiple Analytes

In order for the DT/MH sensing platform to sense multiple analytes, it is important for the sensor to demonstrate complete partitioning and departitioning of both analytes. The reversibility experiments for glucose and lactate are outlined below.

We have successfully demonstrated the reversibility of the DT/MH SAM for glucose sensing.⁶⁶ Aqueous glucose solutions with 0 and 100 mM concentrations (pH ~ 7) were alternately introduced into a flow cell containing the DT/MH-modified AgFON in 20 min intervals without flushing the sensor between measurements. The inset in Fig. 10.3 shows the first three step changes in glucose concentration introduced into the sensor. Figure 10.3A shows the spectrum of a saturated aqueous glucose solution. The peaks at 1462, 1365, 1268, 1126, 915, and 850 cm^{-1} correspond to crystalline glucose peaks in the normal Raman spectrum of glucose.⁷⁴ The spectrum obtained in step 2 was subtracted from the spectrum obtained in step 1 and the resulting difference spectrum is shown in Fig. 10.3B. This difference spectrum demonstrates partitioning with glucose peaks at 1461, 1371, 1269, 1131, 916, and 864 cm^{-1} . Literature has shown that SERS bands can shift up to 25 cm^{-1} compared to normal Raman bands of the same compound.⁷⁵ Thus, the features in the difference spectra shown in Fig. 10.3B correspond to the glucose peaks in the normal Raman spectrum of glucose (Fig. 10.3A). The result of subtracting the spectrum obtained in step 3 from that in step 1 demonstrates departitioning. The resulting difference spectrum is shown in Fig. 10.3C, and absence of glucose spectral features clearly demonstrates departitioning. The spectra were normalized by using the nitrate peak at 1053 cm^{-1} as an internal standard to minimize power fluctuations. The difference spectra indicate that glucose can successfully partition and departition into the DT/MH-modified AgFON sensor platform, and thus they clearly demonstrate the reversibility of this sensor for glucose sensing.

Experiments were also conducted to demonstrate the reversibility of the DT/MH AgFON sensor platform for measuring lactate.⁶⁸ Analogous to the experiments done for glucose, 0 mM and 100 mM lactate in water (pH ~ 5) were alternately introduced into a flow cell containing the DT/MH-modified AgFON in 20 min intervals. The inset (Fig. 10.4)

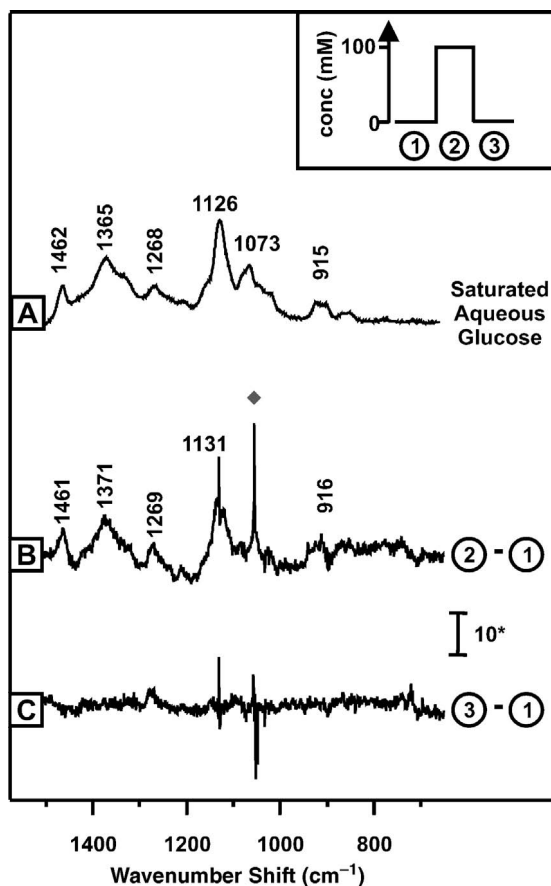


Figure 10.3. Reversible detection of glucose with SERS using the DT/MH-functionalized AgFON sensor. The inset shows the sequence of pulsing steps with varying glucose concentrations. **(A)** The reference normal Raman spectrum of glucose. **(B)** Difference spectrum (2 – 1) with Raman bands matching those in part A demonstrates successful glucose partitioning. **(C)** Difference spectrum (3 – 1) with no features demonstrates complete departitioning of glucose. An asterisk (*) denotes analog-to-digital units $\text{mW}^{-1}\text{s}^{-1}$. A diamond (♦) denotes subtraction residuals of the nitrate internal standard band at 1053 cm^{-1} . $\lambda_{\text{ex}} = 532\text{ nm}$, laser power = 10 mW, $t = 20\text{ min}$, $\text{pH} \sim 7$.

shows the first three steps of the experiment with alternating concentrations of lactate. The spectrum of a 0.8 M aqueous lactate solution is shown in Fig. 10.4A with lactate features at $1457, 1420, 1368, 1321, 1276, 1127, 1090, 1048, 934$, and 866 cm^{-1} .

Figure 10.4B shows the difference between spectra acquired in step 1 and step 2 demonstrating partitioning of lactate with Raman bands at $1463, 1422, 1272, 1134, 1094, 1051, 936$, and 868 cm^{-1} . These features correspond to the lactate peaks in the normal Raman spectrum of lactate (Fig. 10.4A). Figure 10.4C shows the difference spectrum of step 3 and step 1 demonstrating departitioning. The absence of lactate spectral features clearly demonstrates the successful departitioning of lactate. Figure 10.4B and 4C thus demonstrate that lactate can successfully partition and departition into the DT/MH AgFON sensor platform and thus clearly demonstrate the reversibility of this sensor for lactate. Overall, these experiments demonstrate that the DT/MH AgFON sensor can reversibly partition both glucose and lactate completely.

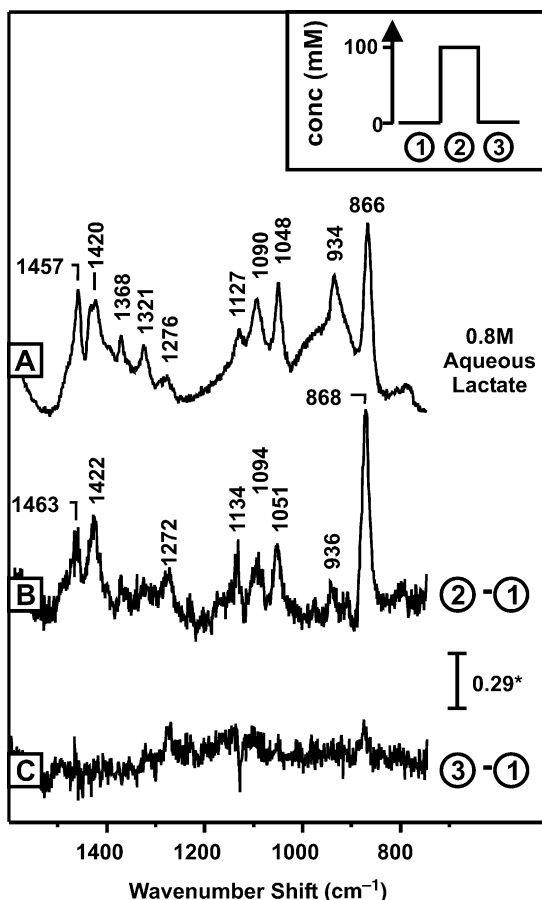


Figure 10.4. Reversible detection of lactate with SERS using DT/MH functionalized AgFON sensor. The inset shows the sequence of pulsing steps with varying lactate concentrations. **(A)** The reference normal Raman spectrum of lactate. **(B)** Difference spectrum (2 – 1) with Raman bands matching those in part A demonstrates successful lactate partitioning. **(C)** Difference spectrum (3 – 1) with no features demonstrates complete departitioning of lactate. $\lambda_{\text{ex}} = 532 \text{ nm}$, laser power = 10 mW, $t = 2 \text{ min}$, $\text{pH} \sim 5$, An asterisk (*) denotes analog-to-digital units $\cdot \text{mW}^{-1} \cdot \text{s}^{-1}$.

10.3.2 Stability

The target stability time for an implanted sensor is at least 3 days.⁷⁶ This is desirable because implantable devices such as insulin pumps are replaced every 3 days. In our previous work, we demonstrated that AgFON substrates functionalized with DT/MH were stable for at least 3 days in phosphate-buffered saline by electrochemical and SERS measurements.⁶⁵ Herein, we demonstrate the stability of the DT/MH-functionalized AgFON surface for 10 days in bovine plasma (Fig. 10.5).

SER spectra were acquired every 24 h from three different samples and three spots on each sample ($\lambda_{\text{ex}} = 785 \text{ nm}$, $t = 2 \text{ min}$). The inset (Fig. 10.5) shows the DT/MH spectrum acquired on day 2. Figure 10.5 shows the mean amplitude of the 1119 cm^{-1} peak for DT/MH on the AgFON for each day over a period of 10 days. The 1119 cm^{-1} band corresponds to the symmetric stretching of the C–C bond.⁷⁷ Only a 2% change in

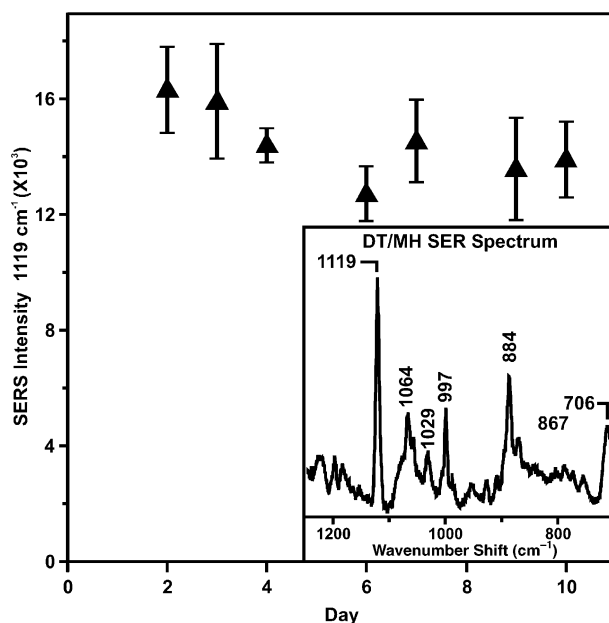


Figure 10.5. Stability of the DT/MH-functionalized FON. The inset shows an SER spectrum of DT/MH-functionalized FON (day 2). Time course of intensity of 1119 cm^{-1} peak is plotted as a function of time. Signal intensities stayed stable over a 10-day period with STDV = 1216 counts. $\lambda_{\text{ex}} = 785\text{ nm}$, laser power = 55 mW, $t = 2\text{ min}$.

intensity of the 1119 cm^{-1} peak was observed from the first day to the last day, with a standard deviation (STDV) of 1216 counts. This indicates that the intensity did not vary significantly over the 10-day period. The small change in intensity can be attributed to the rearrangement of the SAM during the incubation in bovine plasma.⁷⁸ The stability of the 1119 cm^{-1} peak intensity indicates that the DT/MH SAM was intact and well-ordered over a period of 10 days, making this SAM-functionalized surface a viable candidate for an implantable sensor.

10.3.3 Quantitative Detection

The DT/MH-modified AgFON must also demonstrate successful quantitative detection of various concentrations of the corresponding analytes in the physiological ranges. The experiments that demonstrate successful quantitative detection for glucose and lactate are outlined below.

An ideal glucose sensor must be able to detect glucose in the clinically relevant range 10–450 mg/dL (0.56–25 mM), under physiological pH, and in complex media. In order to demonstrate that the DT/MH-functionalized AgFON sensor is capable of detecting glucose successfully, the sensor was placed in a flow cell and incubated for 2 min with various concentrations of glucose (10–450 mg/dL) in filtered bovine plasma. Bovine plasma was used to simulate the *in vivo* environment that the sensor will be exposed to when it is implanted under the skin. Multiple samples and multiple spots were used to collect SER spectra with a near-infrared laser source. A calibration model was constructed using 92 independent spectral measurements of known glucose concentrations using partial least-squares leave-one-out (PLS-LOO) analysis with seven latent variables. A validation model

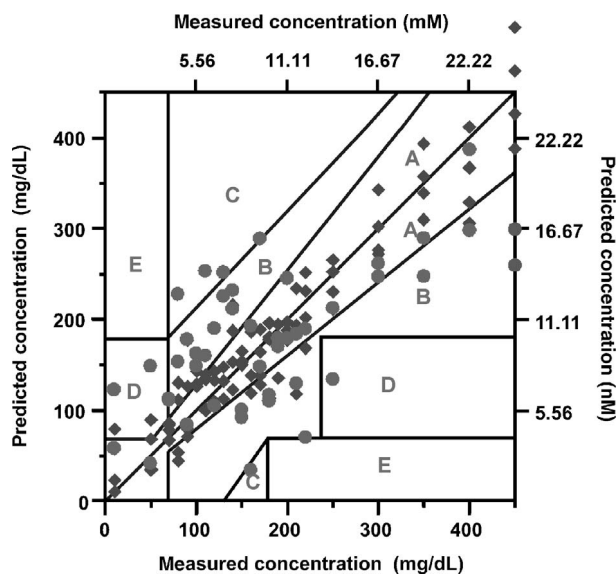


Figure 10.6. Glucose calibration (♦) and validation (●) data plotted on a Clarke error grid. The data were collected over a period of 2 days using three substrates and multiple spots in bovine plasma. PLS calibration plot was constructed using 92 data points. The validation plot was constructed using 46 data points taken over a range of glucose concentrations (10–450 mg/dL) with RMSEC = 34.3 mg/dL (1.9 mM) and RMSEP = 83.16 mg/dL (4.62 mM). $\lambda_{\text{ex}} = 785 \text{ nm}$, laser power = 10–30 mW, $t = 2 \text{ min}$. (Reproduced with permission from Ref. 66. Copyright 2005 *Analytical Chemistry*.)

was then used to test how well this sensor predicted unknown concentrations. The results of the calibration and validation are presented on a Clarke error grid (Fig. 10.6).

The Clarke error grid is the standard used for judging the predictive capability of glucose in the clinically relevant concentration range (0–450 mg/dL).⁷⁹ The grid is divided into five zones, and predictions within these zones lead to the following: (A) clinically correct measurement and treatment, (B) benign errors or no treatment, (C) incorrect measurements leading to overcorrection of acceptable glucose levels, (D) dangerous failure to detect and treat, and (E) treatments that further aggravate abnormal glucose levels. Data points that fall in the A and B range indicate acceptable values. Values outside of this range indicate potential failure to detect blood glucose levels properly, which can result in erroneous and even fatal diagnosis.

The chemometric analysis resulted in a root mean square error of calibration (RMSEC) of 34.3 mg/dL (1.9 mM) and a root mean square error of prediction (RMSEP) of 83.16 mg/dL (4.62 mM).⁶⁶ The data on the Clarke error grid demonstrate that 98% of the calibration data and 85% of the validation data fall in the A and B region. These values indicate that the DT/MH functionalized AgFON sensor is capable of making accurate glucose measurement predictions in an environment similar to that experienced *in vivo*.

We also evaluated the accuracy of this sensor for quantitative lactate detection in the clinically relevant range 6–240 mg/dL (0.5–22 mM), in phosphate buffered saline (PBS).⁶⁸ Lactate concentrations ranging from 6 to 240 mg/dL were randomly introduced into a flow cell, and SER spectra were collected from multiple spots using a visible laser source ($\lambda_{\text{ex}} = 532 \text{ nm}$, $P = 7.5\text{--}12 \text{ mW}$, $t = 2 \text{ min}$). A calibration model consisting of 50 independent spectral measurements was constructed using PLS-LOO with seven latent variables. A

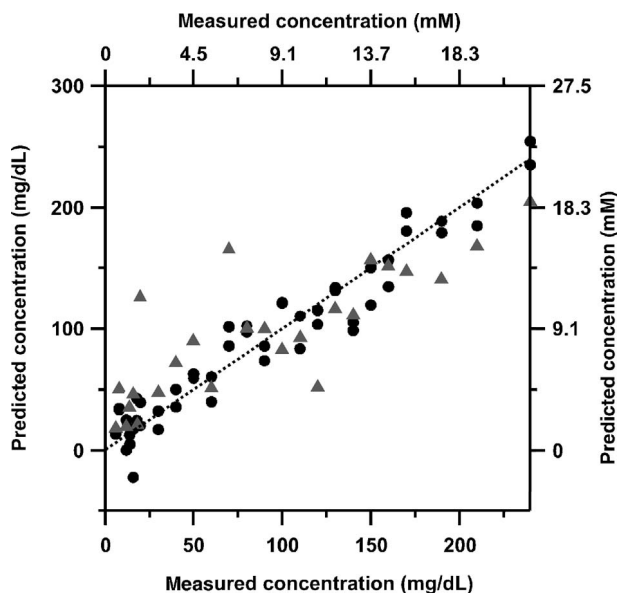


Figure 10.7. Lactate calibration (●) and validation (Δ) data plotted on a Clarke error grid. The data were collected from multiple spots on one substrate in phosphate buffered saline (PBS). The PLS calibration plot was constructed using 50 data points. The validation plot was constructed using 25 data points taken over a range of lactate concentrations (6–240 mg/dL) with RMSEC = 17.8 mg/dL (1.6 mM) and RMSEP = 39.6 mg/dL (3.6 mM). $\lambda_{\text{ex}} = 785 \text{ nm}$, laser power = 7–12 mW, $t = 2 \text{ min}$.

validation model was constructed using 25 data points. The analysis resulted in an RMSEC of 17.8 mg/dL (1.6 mM) and an RMSEP of 39.6 mg/dL (3.6 mM) (Fig. 10.7). These results indicate that lactate can also successfully be measured with the DT/MH-functionalized sensor and bring us one step closer to designing a multiple analyte sensor.

10.3.4 Time Response

Rapid temporal response is an essential characteristic of a viable sensor. The real-time response of the DT/MH functionalized surface to glucose and lactate was examined in a system with bovine plasma and water, respectively. To evaluate the real-time response of the sensor, the intensity of the representative Raman bands was plotted as a function of time and the data points were fitted to exponential curves to estimate the time for partitioning and departitioning.

For the glucose time response, a DT/MH-functionalized AgFON was placed in bovine plasma for $\sim 5 \text{ h}$. The AgFON surface was then placed in a flow cell. SER spectra were collected continuously for 10 min with a 15 s exposure time for each spectrum. To observe partitioning, 50 mM glucose solution in bovine plasma was injected at $t = 0$. At $t = 225 \text{ s}$, the flow cell was rinsed with bovine plasma to evaluate the departitioning of glucose. An excitation wavelength of 785 nm was used to reduce autofluorescence caused by proteins.^{69,70} The amplitude of the 1462 cm^{-1} was then plotted versus time as shown in Fig. 10.8A to evaluate the partitioning dynamics of glucose. The time required for the intensity of the 1462 cm^{-1} band to increase by $1/e$ of the initial value was estimated from the exponential curve to be 28 s. Likewise, the time needed for the intensity at 1462 cm^{-1} to

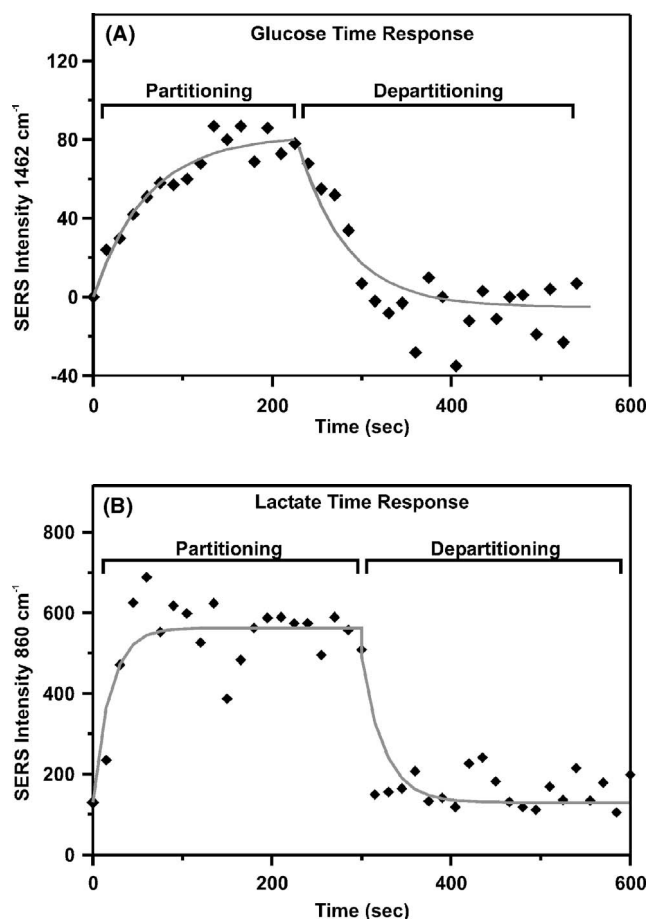


Figure 10.8. Real-time SERS response to a step change in concentration of analyte. The time required for partitioning and departitioning of both glucose and lactate was estimated to be under 30 s. **(A)** 50 mM glucose solution in bovine plasma was injected into the flow cell at $t = 0$ s, and rinsed at $t = 225$ s. $\lambda_{\text{ex}} = 785$ nm, laser power = 100 mW, $t = 15$ s. **(B)** 100 mM aqueous lactate solution was injected into the flow cell at $t = 0$ s, and flushed with water at $t = 315$ s. $\lambda_{\text{ex}} = 532$ nm, laser power 10.6 mW, $t = 15$ s.

decrease by a factor of $1/e$ of the maximum value was determined to be 25 s. The amplitude of the 1462 cm^{-1} peak was obtained by fitting the data to the superposition of three Lorentzian line shapes using PeakFit. While these experiments provide a rough estimate of sensor time response, they demonstrate that partitioning and departitioning occur rapidly, making the SERS-based glucose sensor a potential candidate for implantable, continuous sensing.

A similar experiment was conducted to examine the time course of lactate partitioning and departitioning in an aqueous system. SER spectra were collected continuously for 10 min with 15 s exposure for each spectrum using 532 nm excitation wavelength. At $t = 0$, 100 mM aqueous lactate solution was injected into the flow cell to observe partitioning. To initiate departitioning, pure water was rapidly infused in the flow cell. The partitioning dynamics of lactate were evaluated by examining the intensity of the Raman band at 860 cm^{-1} . An exponential curve fitted to the data shown in Fig.10.8B indicates that partitioning and departitioning occur rapidly, both in less than 30 s.⁶⁸

10.3.5 *In vivo* Glucose Detection

Finally, we successfully demonstrate glucose detection *in vivo*. Figure 10.9 shows the glucose concentration variation in the rat measured using SERS and a standard electrochemical glucometer, One Touch II, as a function of time. Protein autofluorescence is not a significant concern at 785 nm excitation, because the typical SERS spectra of a DT/MH SAM prior to implantation and *in vivo* show only minor differences, attributable to the changes in the environment.⁶⁷

Both the standard glucometer and the SERS-based measurements effectively tracked the time course of glucose level variations. A sharp rise in glucose concentration is detected by both techniques after glucose is injected. Figure 10.10 plots the time-independent data on the Clarke error grid to more precisely gauge the performance of the SERS measurement system.

The Clarke error grid was developed as a convenient and modality-independent means to compare the accuracy and performance of glucose sensors in the clinically relevant range. Figure 10.10 shows a representative Clarke error grid analysis of glucose concentrations from a single animal. The 26 measurements were taken from a single spot on the implanted DT/MH-functionalized AgFON surface. The 21 data points were used to construct the training set, which was calibrated with the commercial One Touch II glucometer. The validation set utilized the remaining five measurements as independent data points. The sensor had relatively low error (RMSEC = 7.46 mg/dL (0.41 mM) and RMSEP = 53.42 mg/dL (2.97 mM)). These data compare favorably with our previous *in vitro* results,⁶⁶ as well as with those of other optically based glucose measurements.^{42,80–82} The results are also consistent with existing detection methods, which have instrument-dependent coefficients of variation of 0.96–26.9% (0.096–2.69 mM, 1.75–49 mg/dL at 10 mM).^{83,84}

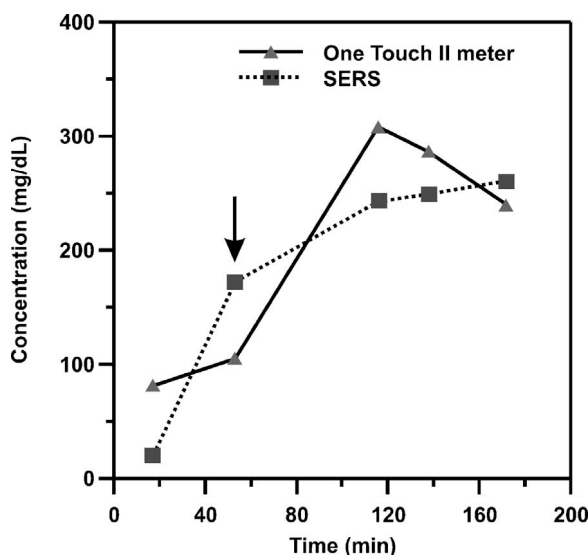


Figure 10.9. Time course of the *in vivo* glucose measurement. Triangles ($\blacktriangle$) are measurements made using One Touch II blood glucose meter, and squares ($\blacksquare$) are measurements made using the SERS sensor. Glucose infusion was started at ~ 1 h, as indicated by the arrow. $\lambda_{\text{ex}} = 785$ nm, laser power = 50 mW, $t = 2$ min.

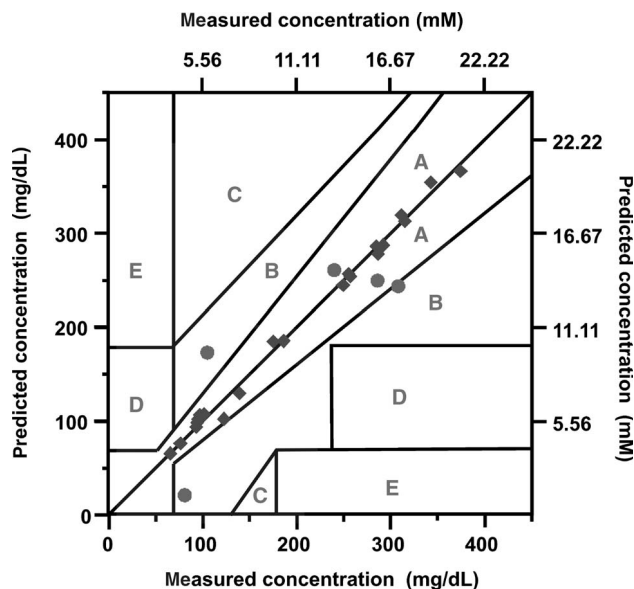


Figure 10.10. *In vivo* glucose calibration (♦) and validation (●) are plotted on a Clarke error grid. Representative data from a single sensor and a single spot on a DT/MH-functionalized AgFON surface, *in vivo*. The calibration set was constructed using 21 data points correlated with the commercial glucometer. The validation set utilized five data points with RMSEC = 7.46 mg/dL (0.41 mM) and RMSEP = 53.42 mg/dL (2.97 mM). $\lambda_{\text{ex}} = 785 \text{ nm}$, laser power = 50 mW, $t = 2 \text{ min}$. Reproduced with permission from Ref. 67. (Copyright 2005 *Analytical Chemistry*.)

10.4 CONCLUSION

We have successfully demonstrated that we can overcome the obstacles usually associated with SERS for biological sensing. We have targeted analytes such as glucose and lactate, which are involved in diagnosis and treatment of metabolic disorders as well as acute trauma. In brief, SERS is a highly sensitive and selective method that can be utilized for rapid quantitative detection of both analytes. We have also validated reversibility and stability of SERS sensors, indicating that they are well-suited to detect fluctuations in concentration levels continuously over a period of several days. Finally, we demonstrated for the first time the use of SERS for quantitative *in vivo* glucose detection in rats. Future work will focus on developing a sensor for simultaneous detection of glucose, lactate, and other potentially important biomarkers. Further animal work will be conducted to achieve sensing of glucose and other analytes and elucidate interactions between the SERS sensor and the biological environment.

ACKNOWLEDGMENTS

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SURFACE-ENHANCED RAMAN SCATTERING FOR INVESTIGATIONS OF EUKARYOTIC CELLS

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11.1 MOTIVATION: SERS AND CELL STUDIES

As shown by many different examples, Raman spectroscopy is a promising method for studies in tissues and cells. The advantages of Raman scattering experiments over infrared absorption studies for *in vivo* measurements are obvious not only in potential diagnostic procedures, since a Raman method as opposed to IR (for example) enables use of fiber-optic probes, but in particular in microspectroscopic experiments with individual cells: Compared with the situation in a mid-infrared microscope, the diffraction-limited lateral resolution in Raman experiments, which is on the order of the diameter of the laser spot, permits investigations of subcellular structures other than nucleus and cytosol. Although oftentimes suggested, Raman investigations of living cells are not necessarily complication-free. To retain viability of the cells, the intensity of the laser used to excite the Raman scattering in small probe volumes has to be limited. Excitation of the Raman scattering for *in vivo* studies is ideally achieved with a laser operating in the NIR, as photodamage and autofluorescence are decreased compared to excitation with visible wavelengths. However, with excitation in the

near-infrared, the weak effect of Raman scattering is even less efficient due to the nonlinear dependency of Raman intensity on frequency ($I(\nu) \sim \nu^4$). In addition, Raman detection in cells usually faces low analyte concentrations. All these circumstances lead to relatively poor Raman signals from live cells, so that the acquisition of meaningful spectra with a reasonable signal-to-noise ratio usually requires acquisition times on the order of several minutes or longer. This precludes regular Raman studies of dynamic processes in individual cells such as trafficking, transport, metabolic activity, and even maturation or death. As was shown by several groups and is also discussed in other chapters of this volume, Raman signatures of selected molecules in cells such as heme prosthetic groups can be greatly enhanced when the excitation laser wavelength matches an electronic transition in that specific molecule.¹⁻⁶ However, due to even more stringent requirements regarding maximum excitation intensities under resonance conditions (see the discussion of Van Manen et al.), resonant Raman experiments are not the ideal solution for live cell studies, and although the selectivity for specific molecules and chromophores is very useful for studies of those particular molecules, it does not allow a general fingerprinting of cellular chemistry.

In addition to the challenges posed by Raman probing in live cells, current cell biology research has been generating a strong need for better optical labels. Although state-of-the-art fluorescence labels using dyes and quantum dots provide high sensitivity, the information they can deliver on chemical composition or molecular structure is very limited, as is their photostability and/or the stability of their spectral signatures.^{7,8} Therefore, improving optical labels regarding sensitivity, specificity, molecular structural information content, and spatial localization in cells has become an important subject.

Since its observation more than three decades ago by Jeanmaire and Van Duyne and by Albrecht and Creighton,^{9,10} surface-enhanced Raman scattering (SERS) has been gaining popularity in analytical and physical chemistry and very recently also in the biomedical field.¹¹⁻¹⁴ The favorable optical properties of noble metal nanostructures, based on their surface plasmon polaritons, provide the key effect for the observation of SERS. As described in detail in a large body of literature, nanostructures from gold or silver are responsible for significant improvements of the Raman signals from the molecules in their nanometer-scaled environment.¹⁵⁻¹⁸

Utilization of SERS in live cell studies has been getting more and more attention in recent years. Most work conducted so far has been focusing on the detection of extracellular molecules in cells such as dye molecules introduced with silver particles¹⁹⁻²¹ or drugs adsorbed onto the cellular membrane.^{22,23} In these cases, high concentrations of the foreign molecules or silver particles or both were used and no SERS signal was detected from the cells. The feasibility of measuring SERS spectra from live cells without any label molecules was first demonstrated with the cell line HT29.²⁴ Since then, our efforts have been directed toward development of SERS into a method for systematic label-free studies of cells and tissues²⁵⁻²⁷ and the construction of SERS labels that can also deliver information on cellular chemistry.²⁸

SERS substrates for *in vivo* use have to meet a number of requirements: They have to be mobile inside cells; at the same time, they must enable targeting of cellular compartments. As has been demonstrated, several different methods allow us to accomplish precise positioning of a SERS probe, such as the utilization of sharp probe tips and atomic force technique or the combination with electron microscopy.^{29,30} However, these approaches are difficult or impossible to carry out *in vivo*. In addition to being controllable with respect to topology, uptake, and retention characteristics of the respective organelle, cell, organ or organism, the biocompatibility of the nanostructures has to be assured. The material should be inert and, if possible, not influence its surroundings. Due to its universal biocompatibility, gold is ideal for optical probing in biology. For decades, gold nanoparticles have been used by cell biologists

because of their favorable physical and chemical properties. Delivery of nanoparticles into the cellular interior, as well as routing of the particles or targeting of cellular compartments, can be achieved in a number of different ways, depending on the nature of the experiment or practical application, but also on the type of cell line and physicochemical particle parameters, such as size, shape, and surface functionalization.^{31–35}

As will be demonstrated here, we have been able to show that nanoparticulate structures from gold, while fulfilling the requirements of cellular systems, also enable ultrasensitive SERS probing of intracellular biochemistry. In this chapter we will discuss (i) probing of cellular biochemistry with improved Raman signatures in the local optical fields of noble metal nanoparticles and (ii) stable, sensitive labeling of morphological substructures and cellular compartments *in vivo* by SERS. We will also demonstrate that optical labels can be constructed that are capable of delivering molecular information from their targets.

11.2 PROBING INTRINSIC CELLULAR CHEMISTRY

11.2.1 Application of SERS Nanosensors to the Endosomal Compartment

The endosomal compartment is an important cellular organelle. Its network of vesicles and membranous tubes extends throughout the cytoplasm and is involved in uptake, transport, and sorting of materials taken up by the cell. In this section we will demonstrate investigation of endosomes by SERS. In general, endosomes form at the cellular membrane when the cell takes up material from the exterior. They undergo a maturation process, during which their molecular makeup changes over time and the pH gradually decreases and drops dramatically in the latest stage, the lysosome.³⁶ Molecular studies of endosomes have so far not been possible without fractionation, purification (i.e., *in vitro* approaches)^{37,38} or the use of very few selected molecular labels *in situ*.³⁹

We generated gold nanoparticles for our experiment by a reduction technique as reported previously.⁴⁰ The nanoparticles were characterized by absorption spectra and transmission electron microscopy. They displayed a size variation between 30 and 50 nm. The electron micrograph in Fig. 11.1A shows that these gold nanoparticles were stable in cell culture medium (DMEM with 10% fetal calf serum (FCS)). This observation is in agreement with other recent reports and with the well-known stabilization effects of serum proteins, the major constituents of FCS (e.g., bovine serum albumin) in experiments with cells and gold nanoparticles.^{41–43}

For probing of the cellular substructures involved in vesicular transport in the cells, the process of endocytotic delivery was used, since the SERS nanosensors are taken up in the same fashion as the material that is usually endocytosed. Non-phagocytic cells can internalize structures of less than 1 μm in size, with clathrin-mediated endocytosis and transport into late endosomes and lysosomes occurring for sizes up to 200 nm and highest efficiency for particles of several tens of nanometers.^{32,43,44} During the process of endocytosis, a part of the cellular membrane undergoes invagination, thereby enclosing a gold nanoparticle that is contained in the cell culture medium. In the cellular interior, the particle is therefore surrounded by a membrane vesicle and enclosed in the endosomal system. The transmission electron micrograph (Fig. 11.1B) shows a snapshot of this enclosure process as we observed it in an epithelial cell.

Figure 11.1C displays a schematic of our delivery experiment. Briefly, cells of the cell lines IRPT (immortalized rat renal proximal tubule) cells and J774 (mouse macrophages) were grown in DMEM culture medium with 10% fetal bovine serum, split and grown on

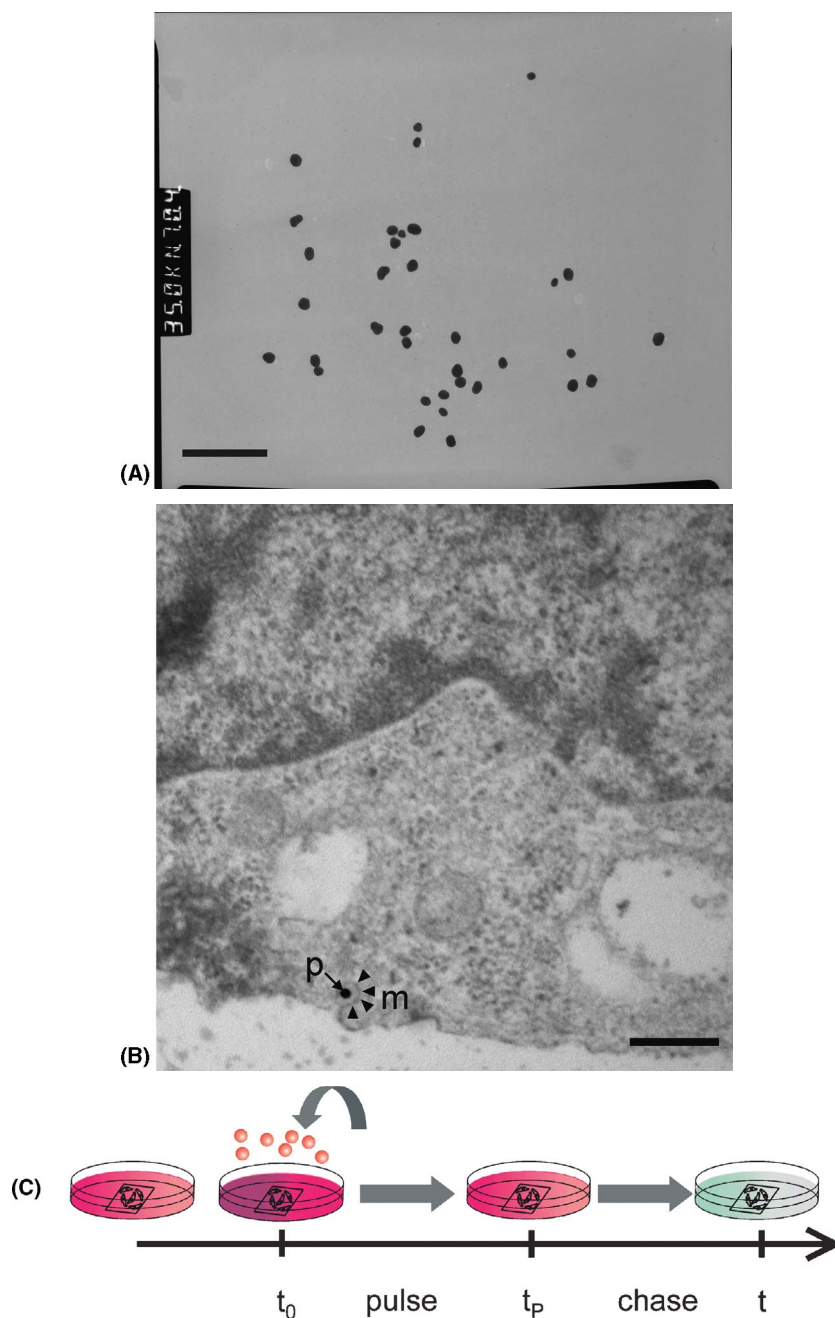


Figure 11.1. (A) Transmission electron micrograph of gold nanoparticles immersed in cell culture medium (DMEM with 10% FCS). Scale bar: 500 nm. (B) Transmission electron micrograph showing the endocytotic uptake of an individual gold nanoparticle by an IRPT cell. The cell membrane (arrowheads labeled m) encloses the particle (arrow labeled p), thereby forming a vesicle in the cytoplasm. Scale bar: 500 nm. (C) Schematic of particle delivery into the endosomal system. Gold nanoparticles were applied with the cell culture medium at time t_0 as a pulse of duration t_p , followed by incubation in medium without nanoparticles for different times t , at which the Raman experiments were carried out in PBS buffer or cells were fixed for TEM studies.

flamed cover slips in multi-well plates to confluence. The gold nanoparticles were delivered into the cells by fluid-phase uptake using the following procedure: At time t_0 , the culture medium was removed and replaced by culture medium containing gold nanoparticles at a concentration of 10^{-12} M. Incubation with the particles with the two different cell lines was performed for 30 min and 60 min, respectively (time t_p in Fig. 11.1C). The gold pulse was followed by a chase in standard medium without gold nanoparticles, terminated at a number of different time periods t ranging from 5 min to 30 h (starting from t_0). In this way, with the termination of the pulse phase, no new particle uptake could take place, and sampling of cells was based on the same amount of gold nanoparticles in the cells at all time points after the beginning of the chase period. The pulsed incubation also ensured that the endosomes containing the gold nanoparticles at a specific time point exhibit a similar stage of maturation. At termination of the incubation period (time t), the cells were washed thoroughly with DPBS buffer containing calcium and magnesium and kept at 4 °C until they were studied by Raman spectroscopy or processed for transmission electron microscopy.

11.2.2 Intracellular Particle Properties and Localization

There is compelling evidence that high SERS enhancement levels are mainly associated with enhanced local optical fields.^{45,46} This implies that the SERS enhancement factor strongly depends on the morphology of the nanoparticles. So far, the highest SERS enhancement factors have been obtained exploiting extremely high field enhancement on aggregates or clusters formed by individual silver or gold nanoparticles 20–60 nm in size.⁴⁷ Experiments and theory have shown that such clusters can vary in size, ranging from dimers^{48,49} to fractal structures.^{50–52} It is likely that when gold nanoparticles enter complex biological systems that provide an environment as diverse and varying over time as the endosomal compartment does, they could assemble in small clusters. As will be shown in the next section, the signal strength of the SERS can give information on the SERS enhancement factor, and thereby allows us to monitor changes in the morphology of the gold nanostructures, such as the formation of aggregates. To find out about the fate of the particles inside the cells, to verify their endosomal localization and to learn more about the particle properties once they are moving through the different endosomal maturation stages, visualization of the particles and the cells was achieved by transmission electron microscopy.

In the IRPT cells, the electron micrographs (Fig. 11.2) suggested ultrastructural differences for the locations of the gold nanoparticles at the different time points. After ~15 min incubation with the particles, the data indicate association of individual nanoparticles with microvilli of the apical membrane. Later, after 30 min, individual gold nanoparticles could be observed associated with the plasma membrane or in small vesicles at the periphery of the cells (Fig. 11.2A, B), while the micrographs obtained from later time points indicate their presence in larger, multivesicular endosomes (Fig. 11.2E–H), partly close to, but not inside, the Golgi complex (Fig. 11.2F). At $t = 60$ min, some of the structures resemble those found for $t = 120$ min (e.g., Fig. 11.2D), but many particles appear also in smaller profiles of more irregular outlines (e.g., Fig. 11.2C). After overnight incubation, the gold nanoparticles are contained in spherical bodies, which, considering the length of the incubation time (~30 h) and the fact that all the nanoparticles accumulate there rather than passing on to again other structures, appear to be lysosomes. The ultrastructural differences in these endosome types suggest variation in their chemical makeup, which was confirmed by Raman spectroscopy.

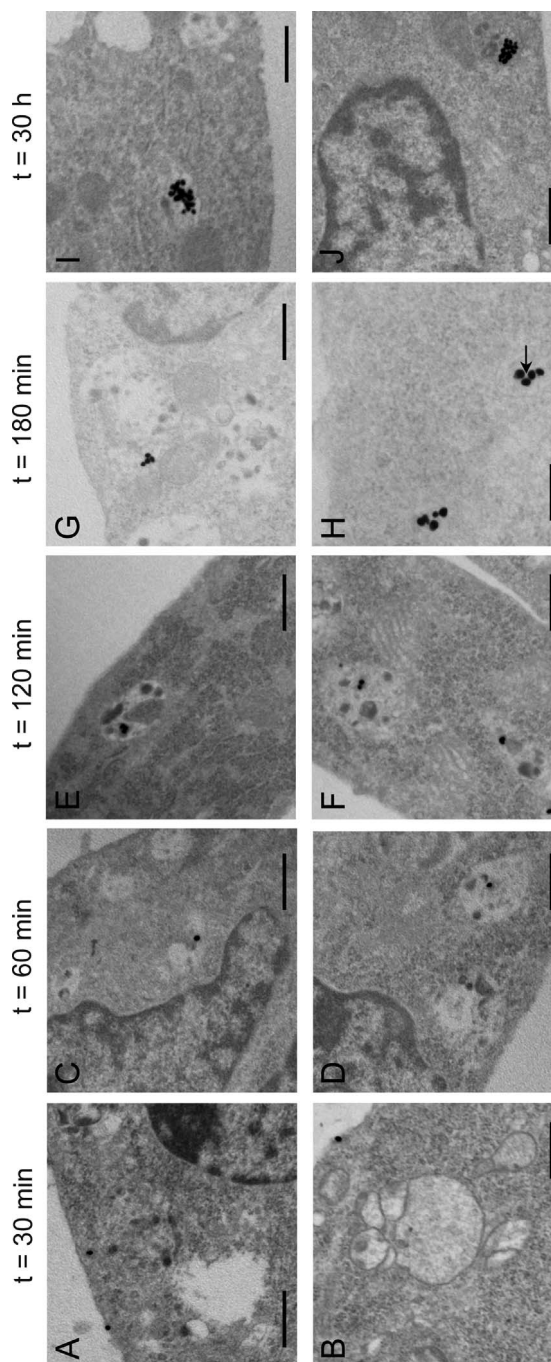


Figure 11.2. Transmission electron micrographs of IRPT cells at different incubation time with the particles (see also Fig. 11.1C). The gold nanoparticles are visible in the cells as black, electron-dense spots. The size of the nanoaggregates varies with incubation time. Nanoclusters of 2–3 particles are forming after 120 min (E, F), those of 4–6 particles are forming after 180 min (G, H), and larger lysosomal nanoaggregates are forming during overnight incubation (I, J) of the cells. The interparticle distance after 180 min is greater than in the other time points, likely due to the enclosure of the particles in multivesicular structures (arrow indicating interparticle space). TEM images were recorded at 80 kV with a Jeol 1011 electron microscope. Scale bars: 500 nm (all except H). Scale bar H: 250 nm. (Reprinted with permission from Ref. 27. Copyright 2006 American Chemical Society.)

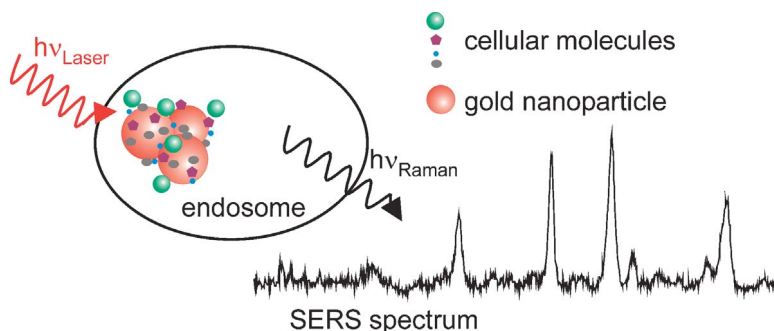


Figure 11.3. Principle of the SERS measurements inside the endosomal compartment. During excitation with laser light in the near-infrared ($h\nu_{\text{Laser}}$), the gold nanoparticles and nanoaggregates provide enhanced local optical fields in their nanometer-scaled vicinity, leading to surface-enhanced Raman scattering (SERS) signals from the molecules in their immediate environment. (Reprinted with permission from Ref. 27. Copyright 2005 American Chemical Society.)

11.2.3 Spectral Information from Endosomes

After washing the cells in buffer (PBS), they were examined by Raman microspectroscopy directly in the same buffer solution. During these measurements, Raman spectra were collected from individual cells in an x - y raster scan point-by-point in a predefined grid. Through use of excitation intensities $<3 \times 10^5 \text{ W cm}^{-2}$ and near-infrared excitation (786 nm), possible changes in the live cells due to laser illumination could be avoided. As we saw in control experiments with cells incubated in medium without gold nanoparticles, the applied low excitation intensity and 1 s collection time per spectrum does not enable acquisition of normal Raman spectra from the cells. As is illustrated in Fig. 11.3, only those spots where gold nanoparticles are present yield a SERS spectrum. Thereby, we selectively probe the immediate vicinity of the particles, in this case the endosomes containing them.

The SERS spectra provide us with two types of information: The first observable is the spectral signature of the SERS, which contains information on the nanometer-scaled molecular (chemical) environment of the gold nanoparticles. The second parameter, as mentioned before, is the signal strength of the SERS, which allows us to monitor changes in the morphology of the gold nanostructures such as the formation of aggregates.

In general, at all time points, a scan over a cell yields different SERS spectra at different positions in the cell (Fig. 11.4). The typical spectral fingerprints underwent drastic time-dependent changes, indicating alterations in the chemical nanoenvironment of the particles over time. Although each of the SERS scans performed at a specific time yields a variety of spectra, distinct signatures could be identified at a higher probability for each time point. These signatures can therefore be assumed to represent the environments of the majority of the gold nanoparticles for a specific time. Assignments of the typical bands observed in the spectra are given in Table 11.1. As can be seen, similar to other Raman and FT-IR experiments of biomaterials, a mixture of molecules and molecular groups is detected: The spectral fingerprints contain contributions from proteins, carbohydrates, lipids, and nucleotides.

When comparing the endosomal SERS signatures obtained from the two cell lines IRPT and J774, very different typical spectra can be found. In particular, apart from spectra that are similar to those found in the IRPT cells, a distinct spectral signature appears at all

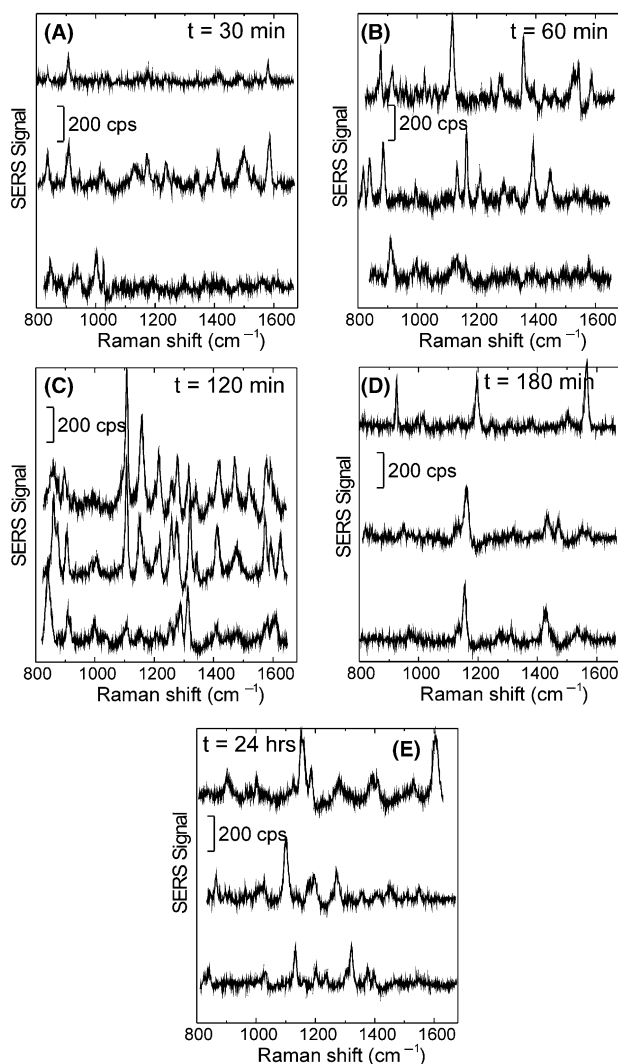


Figure 11.4. Typical surface-enhanced Raman scattering (SERS) spectra from cells of the epithelial cell line IRPT after incubation with gold nanoparticles, excited with $<3 \times 10^5 \text{ W/cm}^2$ at 785 nm, collection time 1 s. The incubation time, including a 30 min nanoparticle pulse, is indicated in each panel. The spectra were acquired from living cells in phosphate-buffered saline by raster scanning over individual cells using a Raman microspectroscopic setup. At the used excitation intensity and collection times, no regular Raman spectra can be observed; only spectra from positions where gold nanoparticles are present display a Raman (SERS) signal. The spectral information reflects the molecular composition in the nanometer vicinity of the gold nanostructures—that is, of their immediate endosomal environment. The positions of the bands are labeled (please see Table 11.1 for an assignment of the molecular groups) molecules that contribute to the Raman signals. Abbreviation: cps, counts per second. (Reprinted with permission from Ref. 27. Copyright 2006 American Chemical Society.)

TABLE 11.1. Raman Frequencies Observed in Endosome SERS Spectra of Cell Lines IRPT and J774 and their Tentative Assignments to the Classes of Molecules and/or Vibrational Modes

Raman Shift (cm ⁻¹)	Tentative Assignment ^{a,b}
815	Phosphate: $\nu(\text{OPO})$
827, 850	Proteins, Tyr: $\delta(\text{CCH})$ Aliphatic, Tyr (ring)
862	Ribose: $\nu(\text{CC})$, ring breathing, $\nu(\text{COC})$
899	Ribose-phosphate; saccharides
906	Amino acids
927	Proline: ring $\nu(\text{CC})$
998	Proteins: amide III'
1004	Phe, ring breathing
1099	Phosphate: $\nu(\text{PO}_2^-)$, $\nu(\text{CC})$, $\nu(\text{COC})$, glycosidic link
1106	Proteins, $\nu(\text{CN})$
1118	Proteins: $\nu(\text{CN})$
1133	Proline
1154	$\nu(\text{CC}, \text{CN})$, $\rho(\text{CH}_3)$
1188, 1194, 1204	Nucleotides: base $\nu(\text{CN})$, Tyr, Phe
1214, 1240, 1254	T, C, A, ring ν .
1254, 1274, 1286	Proteins, lipids: amide III/ $\delta(\text{CH}_2, \text{CH}_3)$
1313	A/proteins: ring $\nu/\gamma_{\text{T}}(\text{CH}_2, \text{CH}_3)$
1338, 1358	Proteins: $\gamma_{\text{T}}(\text{CH}_2, \text{CH}_3)$, $\gamma_{\text{W}}(\text{CH}_2, \text{CH}_3)$
1384	Nucleotides, proteins, lipids, $\delta(\text{CH}_3)$ sym.
1414	Amino acids, $\delta(\text{CH}_3)$ asym., $\nu(\text{COO}^-)$
1427	A, G
1448, 1474	Lipids, proteins: $\delta(\text{CH}_2, \text{CH}_3)$
1505, 1518, 1532, 1578	A, C, G
1548, 1563	Proteins: amide II
1582, 1593, 1605	Proteins, Phe, Tyr
1625	Nucleotides, lipids, proteins, $\nu(\text{C}=\text{C})$ olefinic

^a Based on refs. 66–70.

^b Abbreviations: ν , stretching, δ , deformation, ρ , rocking, γ_{T} , twisting, γ_{W} , wagging, sym., symmetrical, asym., asymmetrical, Tyr, tyrosine, Phe, phenylalanine, A, adenine, T, thymine, C, cytosine, G, guanine.

time points in the spectra of cell line J774 (Fig. 11.5), with exception of the lysosomal stage (22 h incubation). Almost all bands in this spectrum are characteristic of adenosine phosphate and show contributions from all constituents of this nucleotide (adenine, ribose, and phosphate; see band assignments in Fig. 11.5).⁵³ A comparison with data of pure AMP and ATP suggests a prevailing contribution from AMP, since triphosphate and diphosphate markers are absent. From this “AMP/ATP fingerprint” we conclude that we are detecting molecular species involved in the generation of a specific endosomal milieu: The key players in the acidification and pH regulation of endosomes include an ATP-dependent proton pump and a Na-K-ATPase.^{36,54} Furthermore, the endosome acidification profile can be modified by cyclic AMP.⁵⁵ Why is this signature observed in the endosomes of the J774 macrophages but absent from the spectra of the epithelial IRPT cells? Very likely, the composition of endosomes is cell type-dependent, because they are closely associated with specific cellular functions, even if the cells grow in the same culture medium and take up the same type of material. The specific task of macrophages to engulf and digest foreign materials implies a different regulation of the pH and other parameters of their endosomal environment that reflects in the spectra.

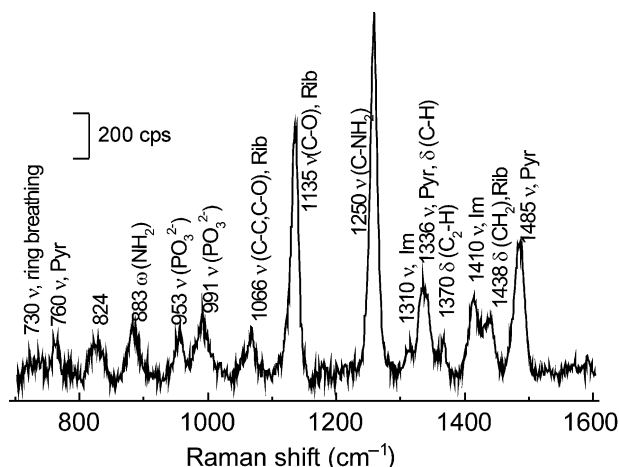


Figure 11.5. One of the typical SERS fingerprints collected from J774 macrophage cells after incubation with gold nanoparticles, excitation wavelength $<3 \times 10^5 \text{ W/cm}^2$ at 785 nm, collection time 1 s. This type of spectrum was found in all endosomal stages except the lysosomal. It was identified as SERS spectrum of AMP and/or ATP. Band assignments based on Ref. 53 are indicated. Abbreviations: cps, counts per second, v, stretching mode, ω , wagging mode, δ , bending mode, Rib, ribose, Pyr, pyrimidine, Im, imidazol.

In addition to changes of the spectral signatures, the overall strength of the SERS signals increased when the particles remained in the cells for longer periods of time. Per time point, ~ 500 – 800 spectra were analyzed. As was discussed in the previous paragraph, the positions and number of bands in the spectra changed over time. As a measure of signal enhancement, the number of bands in the spectra that display 90–100% of the maximum signal level of each data set (time point) were determined. After a 30-min incubation time, these were 2–8 bands, which increased to 4–11 bands after 60 min. At $t = 120$ min, the electron micrographs indicate formation of small aggregates. At this time point, absolute signal levels are significantly increased, and the portion of SERS spectra exhibiting 90–100% of the maximum signal contain 13–19 characteristic bands. They display an abundance of different spectral contributions that were not observed for the other time points (for examples, see Fig. 11.4C). Interestingly, at later time points (180-min incubation) the number of spectral features is again decreased while the intensities of the SERS signals remain constant. At this time point, only 3–6 bands of the 90–100% highest signal level are observed in the SERS spectra. These findings are confirmed also by the spectra of the macrophage cell line J774. Spectra measured after 120 min (Fig. 11.4C) exhibit the strongest signals. As indicated by the electron micrographs, the improvement in signal strengths after 120 min (Fig. 11.4C) correlates with the formation of small gold aggregates, mostly dimers and trimers (Fig. 11.2E, F). This is in agreement with theoretical estimates showing that extremely high SERS enhancement can exist for two gold nanospheres in close proximity.⁴⁹ As evidenced by the electron micrographs, at later time points (180 min and onward) the nanoaggregates contain more than four particles (Fig. 11.2G–J). The interparticle distances in nanoparticle accumulations after 180 min (typical example shown in Fig. 11.2H) are greater than in the dimers and trimers (e.g., Fig. 11.2E, F). This is probably due to inclusion of particles in multivesicular profiles, which prevents the particles from coming into very close proximity (see arrow in Fig. 11.2H). The increasing distances between the particles in the growing aggregates are different from the particle systems

studied *in vitro*, and they would result in less optimized conditions for the electromagnetic enhancement factor that critically depends on interparticle distance.^{49,56,57} Our experimental results strongly suggest that the observed changes in the SERS signal strengths over time in the cells are due to changes in the SERS enhancement level. Based on the similar SERS enhancement pattern, we conclude that the two different cell types (macrophages and epithelial cells) are very similar with respect to uptake and trafficking of gold nanoparticles.

11.3 SERS-BASED OPTICAL LABELS FOR LIVE CELL STUDIES

11.3.1 Spectroscopic Properties of Dye-Based SERS Labels

Raman scattering is a structure-specific method that yields a unique spectrum composed of several narrow spectral lines. As a result, even when molecules are very similar and would show a very similar fluorescence spectrum, their SERS spectra will differ.⁵⁸ When optical labels are to be constructed, this is a major advantage, since a variety of chemically similar labels can be constructed for multiplex probing. Optical labels based on SERS signals of reporter molecules attached to gold and silver nanoparticles instead of fluorescence tags have successfully been demonstrated.^{59–61} Recently developed SERS probes for bioassays provide high spectral specificity, multiplex capabilities, and photostability.^{62,63} Moreover, aside from providing the specific SERS spectrum of a reporter dye, noble metal nanostructures can also enhance the Raman signatures of their environment and in this way enable sensitive chemical probing inside biological structures, such as inside living cells.^{24,28,64,65}

In the following we studied silver and gold nanoclusters and some dyes that are commonly used in biological studies for their potential use in intracellular SERS hybrid probes.

The preparation process used for the production of silver and gold colloidal solutions⁴⁰ results in isolated metal nanoparticles and small clusters consisting of 3–10 nanoparticles. The dyes Methylene blue, Hoechst 33342, and Indocyanine green (ICG) were prepared in 10^{-5} – 10^{-7} M stock solutions in water or, for applications in cells, in 5 mg/mL aqueous solution of human serum albumin (HSA). These stock solutions were added to the aqueous solution of silver and gold nanoclusters for final concentrations between 10^{-7} and 10^{-10} M. For testing the SERS probes, SERS spectra were measured from aqueous solutions (25 μ L droplets) of silver and gold nanoclusters loaded with different concentrations of the reporter dyes. Raman spectra were acquired with a microspectroscopic setup using different wavelengths (680, 786, and 830 nm). A 60 $\times$ microscope water immersion objective was used to focus the excitation laser to a probed volume of $\sim$ 50 fL. The same microscope objective was also used to collect the Raman scattered light. A single-stage spectrograph with holographic edge or notch filters in front of the entrance slit and a liquid-nitrogen-cooled CCD detector were used for spectral dispersion and collection of the scattered light.

Figure 11.6 displays SERS spectra measured from two biocompatible dyes, Methylene blue and Hoechst 33342, attached to silver nanoaggregates. Both dyes possess very specific SERS spectra with several narrow lines. SERS experiments performed on silver and gold nanoaggregates at NIR excitation show that nanoclusters of both metals exhibit comparably good SERS enhancement factors. The idea of creating an optical label based on these SERS signals has several advantages over other labels. As mentioned, the characteristic,

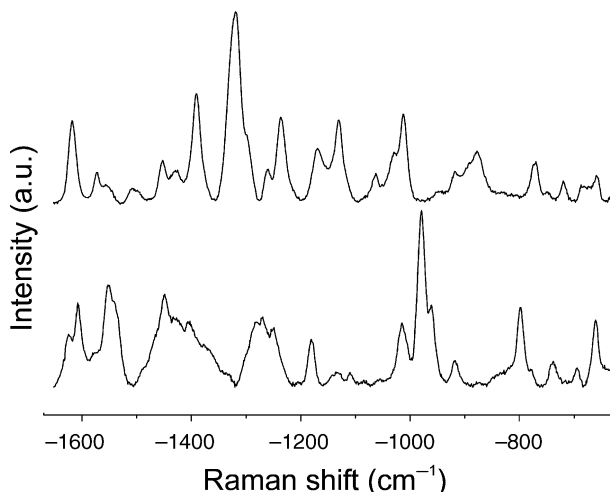


Figure 11.6. SERS spectra of the biocompatible dyes Methylene blue (*upper trace*) and Hoechst 33342 (*lower trace*) on silver nanoaggregates. The signals were collected from ~ 5000 molecules in the probed volume, 3 mW 830 nm excitation, collection time 1 s. SERS spectra are shown after fluorescence background correction. (Reprinted with permission from Ref. 28. Copyright 2005 American Chemical Society.)

fingerprint-like pattern of many narrow Raman bands of the label molecule, rather than, for example, one broad fluorescence band, provides a high specificity, which has been used in multiplexing experiments unprecedented so far by fluorescence studies.⁶¹ Most importantly, the near-infrared excitation intensity is not in resonance with the electronic transitions of the dyes. This prevents photobleaching and results in a high stability of the label and hence of the signal.

11.3.2 Indocyanine Green SERS Labels in a Cell Culture

For studies in cells, we constructed a SERS probe from two biocompatible components, the dye Indocyanine green (ICG) and gold nanoparticles, and introduced it into cultured cells.

The SERS nanoprobe consisting of 10^{-7} M ICG complexed with HSA on 60-nm gold nanoparticles was delivered into cells of a metastatic Dunning R3327 rat prostate carcinoma line (MLL) (donated by Dr. W. Heston, Memorial Sloan-Kettering Cancer Center, New York, NY). After overnight incubation, the endocytosed nanoparticles must have been transferred to lysosomes. In accordance with this assumption, we found gold accumulations in the range of 100–1000 nm in lysosomes by light and electron microscopy after incubation for 20 h and longer. The presence of gold particles inside the cells could also be verified by the appearance of SERS signals collected from the cells after washing in PBS buffer. Microscope inspection provided evidence that the cells were dividing after incubation with the ICG-SERS probes. While incubated with the nanosensors, the cells were visibly growing, and no evidence of cell death was found. After a 20 h incubation with the diluted medium, a slightly lower density was observed when compared with control cells growing in undiluted medium, probably resulting from the dilution of nutrients. Controls in

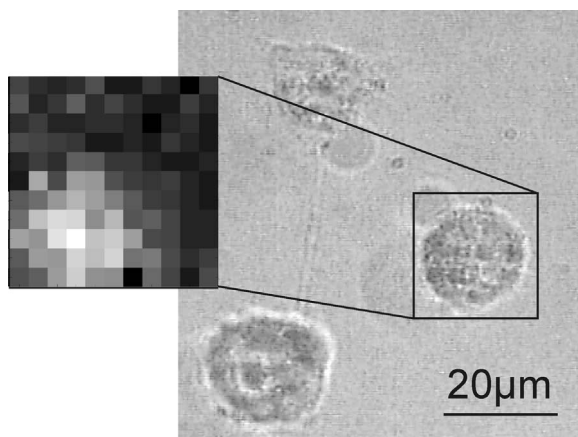


Figure 11.7. Spectral map of ICG in a cell based on the product of two SERS lines of ICG (at 1147 cm^{-1} and 945 cm^{-1}). Intensities are shown in gray scale (highest value in white). A photomicrograph of the cell, indicating the mapped area, is shown for comparison.

diluted medium with ICG/NaCl and NaCl-diluted culture medium showed growth rates similar to those incubated with the gold particles and with the ICG-SERS nanoprobe.

As described in the previous section, Raman spectra were acquired from the living cells in the physiological environment (PBS buffer) using laser intensities of $\sim 2\text{ mW}$ in accumulation times of 1 s and less. All experiments on the cell culture were carried out at 830 nm excitation.

Figure 11.7 shows the detection and imaging of a SERS label made from gold nanoparticles and ICG in a single live cell. The probes can be localized inside the cells by image reconstruction based on their specific spectral information. Images were reconstructed from Raman data collected by raster scanning over the cells in a defined step width. As our data indicate (Fig. 11.8A), the SERS spectrum of ICG consists of more than 10 characteristic bands distributed over a broad frequency range. When imaging the label, this offers the advantage that spectral correlation methods can be used to enhance the contrast between the label and the cellular background. The spectral map in Fig. 11.7 is based on the product of two ICG SERS lines at 1147 cm^{-1} and 945 cm^{-1} . In addition to the SERS signals of the reporter ICG, at these positions, also the Raman signatures from the cellular components in the immediate surroundings of the gold nanostructures experience surface enhancement. This enables sensitive chemical probing of the particles' vicinity in very short times.

Figure 11.8 illustrates molecular probing of the cellular chemistry with the ICG-SERS hybrid probe. Trace A in Fig. 11.8 displays an ICG-SERS spectrum measured from a nanosensor in the physiological medium, and trace B is an example for a spectrum measured inside a cell. It shows the SERS signature of the reporter ICG along with SERS lines that originate from the cellular surroundings of the gold nanoparticles. After subtracting the SERS signal from the reporter ICG (trace A), trace C displays the SERS spectrum of the cellular components. The Raman lines in spectrum C can be assigned to vibrations characteristic for protein and nucleotide molecular groups, such as C–H deformation/bending modes at 1450 cm^{-1} , C–N deformation at 1166 and 1229 cm^{-1} , possible contributions from Phe and Tyr $\sim 1207\text{ cm}^{-1}$, as well as cytosine and adenosine ring vibrations and/or protein amide II contributions around 1540 cm^{-1} (tentative assignments after Refs. 66–70).

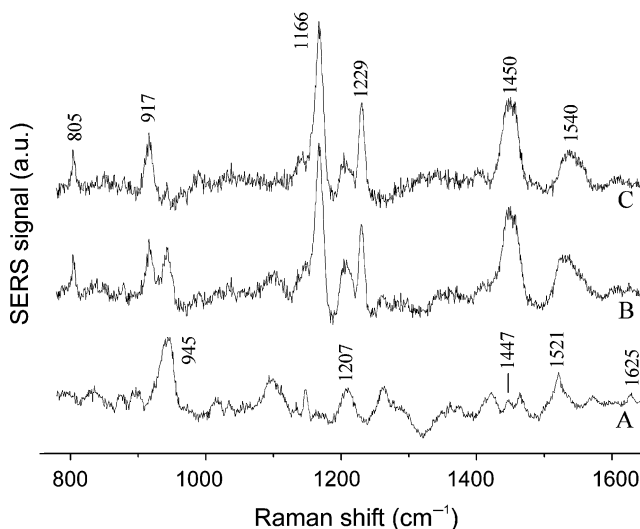


Figure 11.8. SERS spectra measured inside living cells incubated with ICG on colloidal gold: SERS spectrum of ICG (A), SERS spectrum of ICG and cell environment (B), difference spectrum A–B displaying SERS features of cell components (C). For the assignment of the bands see Table 11.1.

The great variety of spectral contributions is in accord with the results of measurements with pure gold nanoparticles without the use of the ICG reporter. As discussed in the above paragraphs, many different types of biomolecules are found in the lysosomal compartment; therefore, many different SERS signatures originate from the nanosensors' environment. Such a variety of combinations of spectral features can be assessed best by multivariate methods.

11.4 CONCLUSIONS AND OUTLOOK

To conclude, we have shown here the application of gold nanoparticles for SERS studies in live cells. SERS takes place in the local optical fields of metal nanostructures and is therefore restricted to the immediate vicinity of the gold nanoparticles. Thereby, SERS probes enable the acquisition of Raman signals not only at high sensitivity but also from nanometer scaled volumes. This local confinement of the SERS effect has several advantages over regular Raman experiments: For SERS studies in cells, SERS nanoprobe can be positioned at discrete locations (e.g., in a specific cellular compartment), and the spectral information is obtained only from the nanoenvironment of these probes and hence that particular compartment. This is different from the spectral information collected in other Raman microspectroscopic experiments, where all positions in a whole cell are probed. It also means that the maximum lateral resolution in such a Raman experiment is no longer limited by the excitation wavelength; instead, it is influenced by the metal nanostructure that is used to provide the enhancement.

In this chapter we have also demonstrated a new type of optical label based on SERS that is stable, specific, and biocompatible and that can be used for applications in live cells. As reporter molecules to be attached to gold nanoaggregates, we chose dyes known and approved in biological and medical applications as fluorescence markers. However, instead of using the broad and relatively nonspecific fluorescence signals of these dyes, here we rely

on their specific (surface enhanced) Raman signature. The SERS nanosensors can be detected and imaged based on the unique spectroscopic signature of the SERS signal of a reporter molecule attached to the gold nanostructure. In addition to its own detection by the characteristic SERS spectrum of the reporter, the probes deliver surface-enhanced Raman signatures of the cell components in their vicinity. This provides the capability of ultrasensitive chemical characterization of nanometer-scaled units in single live cells. Due to the large effective Raman scattering cross section, SERS probes fulfill the requirements of dynamic *in vivo* systems: the use of very low laser powers and very short data acquisition times.

With or without additional label molecules, SERS nanosensors will enable targeted molecular probing of subcellular structures and allow us to get a better understanding of molecular phenomena *in situ* and *in vivo* that cannot be addressed otherwise. The ability of localized probing by SERS could be used to study several simultaneous events in a cell by directing different SERS hybrid probes to different locations in a cell in one experiment.

The size of the nanoprobe determines their delivery into cells and subcellular structures, but also SERS enhancement, confinement of the local fields, and spatial confinement of the collected information. Therefore, further research in this area is currently directed toward influencing the physicochemical and spectroscopic properties of noble metal nanoparticles inside cells and physiological media. The current proof-of-principle that cellular chemistry can be probed very selectively by means of intracellular SERS nanosensors is being followed by systematic SERS studies of well-characterized model systems, such as isolated cell compartments and compounds, which are used to decipher the spectral information from the cells.

The concept of using vibrational spectroscopic methods along with nanoprobe and their local optical fields provides far-reaching perspectives for our understanding of cellular processes on the molecular level. In particular, the ability to detect local heterogeneity in large biomolecules and molecular structure in small subcellular units will be useful in future clinical diagnostic and therapeutic applications.

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COMBINING OPTICAL COHERENCE TOMOGRAPHY AND RAMAN SPECTROSCOPY FOR INVESTIGATING DENTAL AND OTHER MINERALIZED TISSUES

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12.1 INTRODUCTION

Mineralized tissues of the human body are known as the hard or calcified tissues, with the two major groups being the bones and teeth. Bones play a skeletal role and are regarded as structural materials with the ability to resist bending, compression, and fracturing. The teeth are the primary agents for cutting, grinding, and mixing of food consumed. Therefore they need to withstand a significant amount of force. Based on the functions that these hard tissues perform, it is no surprise that disease in mineralized tissues generally manifests as a loss of structural integrity. While the origins are biochemical, overt cues to the presence of disease appear as changes in morphology and/or strength of the tissue. To better understand how the biochemical changes translate into structural alterations leading to diseased states, it is useful to first explore the fundamental biochemical building blocks of mineralized tissues. The biochemistry reveals which vibrational spectral peaks are to be expected from mineralized tissues. The crystalline nature of hard tissues with repeating compositional patterns suggest that polarized Raman spectroscopic approaches can be used to investigate structure as well. The structural aspects of mineralized tissues also determine the optical properties that can be explored with the technique of optical coherence tomography (OCT) to reveal information on morphological structure and integrity.

In this chapter, we will not provide a full review on the application of Raman spectroscopy for mineralized tissues because this topic has been well-covered previously in recent reviews.^{1,2} Rather, we would like to focus on the novel application of using

polarized Raman spectroscopy in combination with OCT for the characterization of mineralized tissues. Our chapter will discuss this potential, its advantages, and its strengths as well as demonstrate the value of this multimodal approach in detecting early caries lesions in human teeth. These studies lay the foundation for the development of appropriate tools for potential clinical application.

12.1.1 Biochemical Composition of Bones and Teeth

Human bone is composed of a rigid organic matrix strengthened with calcium salts, namely calcium hydroxyapatite. The organic matrix is dominated by 90–95% collagen fibers within a mucopolysaccharide-rich semisolid gel known as ground substance.^{3,4} It is these collagen fibers that provide bone with its powerful tensile strength. Bones are made hard by the precipitation of calcium phosphate crystals within the matrix. These inorganic crystals provide the bone with compressional strength. The inorganic crystals lie adjacent to and are bound to the collagen fibers, with the fibers and crystals overlapping like bricks in a wall. This combination of an organic (30%) and inorganic (70%) matrix gives bones the structural strength that approaches that of reinforced concrete while still being lightweight and not brittle.^{3,4}

Human dental enamel has a highly mineralized crystalline structure containing 95–98% inorganic matter by weight. Hydroxyapatite, in the form of crystalline lattices, is the largest mineral constituent and is present in 90–92% by volume. Other minerals and trace elements are present in smaller amounts. The remaining constituents of tooth enamel are an organic content of about 1–2%, with water accounting for 4% by weight. These other components total approximately 6% by volume.⁵ This is in contrast to the composition of human dentin, which is approximately 75% inorganic material, 20% organic material that is primarily collagen, and 5% water and other materials. It is therefore less mineralized and softer than enamel but still harder than bone.⁵

Enamel is the hardest substance of the human body; however, it is a very brittle but rigid structure with high elastic modulus and low tensile strength.⁵ These properties suit its function of cutting through and grinding up food. The enamel makes up the crown portion of the tooth. Dentin, although a hard tissue, is somewhat flexible, which helps support the nonresilient enamel. Dentin makes up the largest proportion of the tooth, extending the length of the tooth. A third dental hard tissue is cementum, which covers the anatomic roots of teeth. Cementum is softer than dentin and consists of a 45–50% inorganic material by weight and 50–55% organic matter and water by weight. The main organic component in cementum is again collagen with some protein polysaccharides. Our studies involving early dental caries have mainly focused on those forming on the crown, thereby affecting the enamel. As such, the majority of our discussion will focus on the enamel.

Surveying the components that make up mineralized tissues, the common theme that emerges is that the inorganic hydroxyapatite is the fundamental building block and principal mineral. Hydroxyapatite is composed of calcium and phosphate with chemical formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. For biological tissues, the major form is carbonated hydroxyapatite where PO_4^{3-} or OH^- (Type B or Type A, respectively) can be substituted by CO_3^{2-} with the dominant form being Type B. Traces of other minerals such as magnesium, sodium, potassium, fluoride (especially in enamel), and chloride are also found in varying proportions depending on tissue type. Enamel contains less carbonate than dentin, with dentin having less carbonate than bone. Carbonated hydroxyapatite crystals are shaped like long flat plates in bone,³ while in dental tissues the crystals are arranged into bundles to form rods

or prisms with intercrystalline spaces between rods to allow diffusion of ions as in the case of dental caries development.⁵

12.1.2 Characterizing Diseases of Mineralized Tissues

Adverse changes in the biochemical composition of mineralized tissues can result in weakening the scaffolding underlying them and disrupting structural integrities, resulting in disease. Some examples of bone diseases include a disruption in the amount of calcium accumulation in the bone matrix, leading to a deficient state known as rickets in children and osteomalacia in adults. A mutation in the gene that codes for collagen results in osteogenesis imperfecta (brittle bone disease). With osteoporosis, both the matrix and mineral are lost, leading to reduced bone mass, reduced strength, and increased incidence of bone fractures.

Dental caries is the clinical term for tooth decay and arises from destruction of the tooth structure by acid formed by bacteria found in dental plaque. The acid leads to demineralization as ions are dissolved to create a porous and weak structure which eventually crumbles to form dental cavities. Dental caries is a chronic infectious disease that is experienced by 45% of school children, and 94% of adults have experienced this infection at some point in their life.⁶ This condition is the most common chronic disease in childhood. The current methods available for the detection of dental caries do not have the sensitivity, specificity, or ability to account for the dynamic process of demineralization–remineralization.^{6,7} A study comparing methods of diagnosis for early (noncavitated) lesions found that the combination of visual inspection and radiographs gave a sensitivity of 49% and a specificity of 87%.⁸ Moreover, this translates into half of the teeth with early caries going undiagnosed while 13% of teeth diagnosed with caries and thought to be in need of restoration were in fact caries-free.⁸ As such, dental clinicians are forced to evaluate a dynamic process as a dichotomous measure of the presence or absence of disease using subjective clinical criteria such as color, “softness,” resistance to removal, and the use of tools such as the sharp explorer and dental radiographs, which are not necessarily effective in detecting early caries lesions.^{6,7} Early untreated caries can progress to dental cavities, resulting in pain, suffering, and the necessity for more costly surgical procedures (e.g., drilling and filling) that undermine tooth structure. Therefore, there is a need to develop more refined diagnostic tools that would be able to identify early time points and clinically stage the presence, activity, and severity of dental caries at different sites. With such tools, a treatment strategy employing nonsurgical methods such as fluoride, antimicrobials, and sealants can be employed. Such nonsurgical preventive methods represent the next era in dental care.⁶ To address the need for more refined diagnostic aids with improved sensitivity and specificity, various optical methods have recently been explored. These include direct digital radiography, digital imaging fiber-optic transillumination, electroconductivity measurements, quantitative light-induced fluorescence, and laser fluorescence.^{6,9} For the most part, specificity values of the emerging diagnostic methods were higher than sensitivity values with overall a greater proportion of false-negative readings.¹⁰ Therefore, despite the potential of these various technologies, at present, none of them adequately fulfill the requirement for more sensitive methods.

The early dental caries lesion (incipient caries) is noncavitated and limited to the outer enamel surface. This caries type presents as a visible “white spot” when the tooth is air-dried and from histological studies is found to consist of four layered regions.^{11,12} The deepest region, the translucent zone, is the advancing front of the enamel lesion and appears structureless when examined with polarized light. The next deepest zone (the dark zone)

does not transmit polarized light (also known as positively birefringent) because it consists of many tiny pores. Next is the body of the lesion, the largest region, with a large pore volume (25–50% porosity) with interprismatic areas and cross-striations. Lastly, the enamel surface of the incipient lesion is intact and well-mineralized. Compared with the subsurface, the surface zone ($\sim 30\text{ }\mu\text{m}$ thick) contains more fluoride, less water, less carbonate, is more highly mineralized and the enamel crystals are often larger and oriented differently from those below.¹² Such properties render the enamel surface more resistant to acid attack. This surface layer is also partly formed by remineralization in which dissolved ions (calcium and phosphate) originating from the subsurface region are deposited into the surface layer. The original crystalline framework of the enamel rods serves as a nucleating agent for remineralization. Depending on the extent of demineralization, the enamel caries can extend from a depth of $\sim 100\text{--}250\text{ }\mu\text{m}$ (for incipient caries) to entirely through the enamel, at which point the cavitated lesion ($\sim 1.5\text{ mm}$ deep) has extended into the underlying dentin.¹³

12.1.3 Raman Spectroscopy and OCT of Mineralized Tissues

The biochemical and notable structural changes in disease states suggest that techniques that are sensitive for revealing such alterations have potential in characterizing and shedding light on understanding these diseases. Invasive investigations rely on histology and immunohistochemistry to understand and measure the relationship between mineralized tissue biochemistry and structure. On the other hand, we propose the use of Raman spectroscopy and optical coherence tomography (OCT), techniques with potential to be developed for noninvasive or minimally invasive purposes. These techniques can lead to the development of novel detection methods for *in vivo* screening and/or monitoring disease progression.

Raman spectroscopy coupled with optical coherence tomography offer nondestructive alternatives that can be applied *in vivo*. The rich biochemical information available from Raman spectroscopy can be used to characterize both the organic and inorganic constituents of mineralized tissue. Since polarization techniques exploit properties of optical anisotropy to reveal information about structure and composition of materials, the ordered structure of mineralized tissues, in particular the enamel, should be sensitive to polarized light. This attribute and the large number of phosphate groups in apatite suggest that polarization-coupled imaging and polarization spectroscopic techniques offer potential for differentiating healthy from diseased tissues. Through polarized Raman spectroscopy, information is gained on the molecular structure and/or orientation of calcified tissues. Optical coherence tomography provides high-resolution surface and subsurface images that can be used to survey the tissue for structural alterations in the crystalline matrix. Combined, these techniques can provide a detailed analysis of mineralized tissues.

12.2 OPTICAL COHERENCE TOMOGRAPHY

Optical coherence tomography is a noninvasive technique that provides high-resolution depth imaging of near-surface tissue structures. This technique is based on the coherent cross-correlation detection of the interference fringe intensity of backscattered light.^{14,15} Similar to ultrasound in operation but offering an order of magnitude of higher spatial resolution, OCT provides morphological images with $1\text{--}20\text{ }\mu\text{m}$ resolution (depending on source type) to depths of $\sim 2\text{--}3\text{ mm}$.^{14,16} OCT has developed tremendously since its possible

applications in medicine and biology were first demonstrated more than a decade ago.¹⁷ In combination with confocal microscopy, OCT has been proven to be a very efficient method for simultaneously measuring the thicknesses and refractive indices in multilayered structures^{18,19} or coating thickness and concentricity of optical fibers.²⁰ Studies have also shown that, due to the sensitivity that characterizes interferometric measurements of weakly backscattering structures, OCT can produce high-quality images of the eye and other transparent tissues.^{21,22} This technology has been applied to image retinal tissue of glaucoma patients^{23,24} and has been applied quite successfully to examine nontransparent media such as hard tissue in the oral cavity,¹⁴ arterial,^{25,26} and intestinal tissues.²⁷ OCT is being developed for cancer detection^{28,29} and vulnerable atherosclerotic plaque assessment.³⁰ This technique is well-suited for detecting changes in optical scattering and tissue birefringence properties as well as alterations of the refractive index.^{15,31,32}

12.2.1 Principles of OCT

The instrumental core of an OCT system is a Michelson interferometer and a low-coherence light source. OCT measurements are based on recording the demodulated patterns generated by the interference between the beam reflected from the reference mirror and the beam that travels in the sample arm that is backscattered by the sample of interest. In most of the available OCT systems, the reference mirror is moving in order to generate a depth scan (also known as an A-scan) of the sample. The interference signal is captured by a detector, and the time-of-flight information is subsequently obtained through the processing electronics and computer data acquisition system. Detailed descriptions of the theoretical principles governing OCT imaging as well as the general instrumentation involved in this technique are beyond the scope of this chapter and are available from various publications.^{17,33–36}

To illustrate the kinds of morphological information attainable from OCT, a two-dimensional (2D) image of the enamel of a sound tooth is shown in Fig. 12.1A. This image was obtained by assembling adjacent OCT depth scans. An example of an A-scan, the one corresponding to the 40th depth line from this image, is shown in Fig. 12.1B. Each depth line corresponds to one position of the focused beam on the tooth surface, and it is a record of the light backscattered from locations within the sample starting from the surface up to the point within the tissue where the signal becomes undistinguishable from noise. Moving the focused spot along the tooth surface and acquiring an A-scan for each position generates the 2D image. The noisy profile presented by the single depth-line shown in Fig. 12.1B is characteristic of all OCT images. This noise presents itself as a spotty pattern in the 2D image, as seen in panel A, and the noise has two sources. One is the random thermal noise arising from the electronics of the OCT scanner. The other source is the speckle noise generated by multiple scattering events of light inside the tooth matrix that forces part of it to experience a change in its travel distance relative to the initial ballistic path. For an accurate estimation of the light propagation properties in such a highly scattering environment, it is necessary to quench some of the noise plaguing OCT measurements.

Our OCT images were acquired with a Humphrey Revision A OCT scanner (Humphrey Systems, Dublin, CA, USA). The source is light emitted by a super-luminescent diode with the central wavelength at 850 nm and a measured coherence length of $\sim 15 \mu\text{m}$ (fullwidth at half-maximum, FWHM). In the reference arm, a rapid-scanning optical delay line with a longitudinal scan range of 3 mm in air modulates the optical field. A 16-bit A/D converter digitizes the interference signals. Each OCT image consists of 100 A-scans, each with a

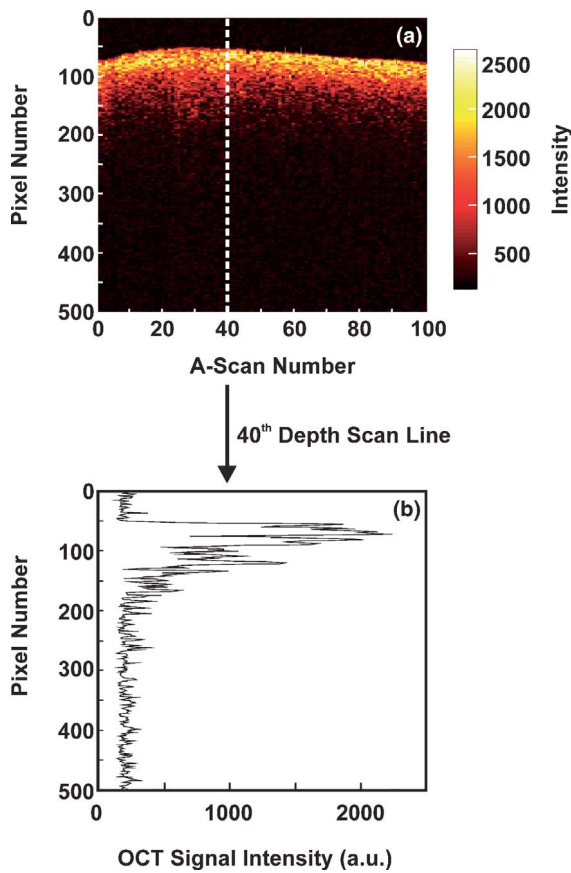


Figure 12.1. (a) Representative 2D OCT image at 850 nm of sound tooth enamel. (b) the A-scan (or depth-scan) that corresponds to the 40th depth scan line shown as the dotted line in the 2D image.

depth of 500 pixels and a measured axial resolution of 5.5 $\mu\text{m}/\text{pixel}$ in the scanning direction.

12.2.2 OCT of Mineralized Tissues Other than Dental Tissues

Several recent studies in the literature have demonstrated the utility of OCT for examining hard tissues. For example, OCT has been used for the assessment of articular cartilage where the loss of joint cartilage is a hallmark of osteoarthritis. For these studies, determining the interface between the bone and cartilage has been important in determining the cartilage width.^{37–39} Other studies have used OCT to examine the temporal bone in the cochlea of the ear for potential use to diagnose inner- and middle-ear pathologies.^{16,40} The mineral bone density of equine cortical bone samples were examined before and after demineralization in acid and demonstrated a linear correlation between the optical scattering coefficient and bone mineral density.⁴¹ The collagen structure of intervertebral disks has been studied with polarization-sensitive OCT.⁴² Overall, because OCT has an image resolution that nearly approaches that of conventional histological methods, along with the fact that it can be fiber-optic-based, it can be integrated into existing endoscopic-based technologies for potential clinical utility in providing information regarding pathologies.

12.2.3 OCT of Dental Tissues

Dental enamel is the most radiopaque material in the body, which confounds the detection of micro-fissures (the precursor of caries) by radiographic methods. However, in the near-infrared spectral region, the hydroxyapatite mineralization in the enamel is reasonably transparent and is also birefringent. The transparency and polarization characteristics of tooth enamel is ideally suited for the detection of surface micro-fissures.⁴³ In recent years, optical coherence tomography has been developed for dental applications such as caries detection and periodontal diseases.^{14,15,32,43–49} These studies have demonstrated the potential of using this technique for *in vivo* imaging of intra-oral tissues. The OCT images obtained were able to delineate structural components of the gingival tissue (e.g., sulcus, epithelium, connective tissue layer) as well as hard tissue structures (e.g., enamel, dentin, dentinoenamel junction). A low coherent light source with a center wavelength in the near-infrared region allows penetration into the sample under investigation. Common OCT center wavelengths are 850 nm and 1310 nm. The axial resolution of OCT increases as the center wavelength decreases and worsens with the square of the FWHM of the source. However, scattering decreases with increasing wavelength such that light at 850 nm is scattered more strongly than at 1310 nm. OCT of teeth using these different wavelengths results in different image presentations. At 850 nm, sound enamel scatters most of the back-reflected light within the first 100 μm while the carious region with reduced mineral matrix allows for deeper penetration of light into the tooth. At 1310 nm, the light penetrates sound enamel to a millimeter depth or more while the demineralization of the carious region greatly increases scattering, preventing light from penetrating past the lesion zone. The OCT depth images shown in Fig. 12.2 were acquired with our in-house OCT system (developed at the National Research Council Canada – Industrial Materials Institute) operating at 1310 nm and clearly demonstrates morphology such as the enamel and dentinoenamel junction. However, with some of the earlier methods reported in the literature, the penetration depth is limited and the acquisition times long. Despite the imaging quality, it is not always trivial to resolve the different structural features, especially in caries detection. One approach involves improvements using polarization-sensitive OCT (PS-OCT)^{43,49,50} with near-infrared excitation, which takes advantage of the birefringent

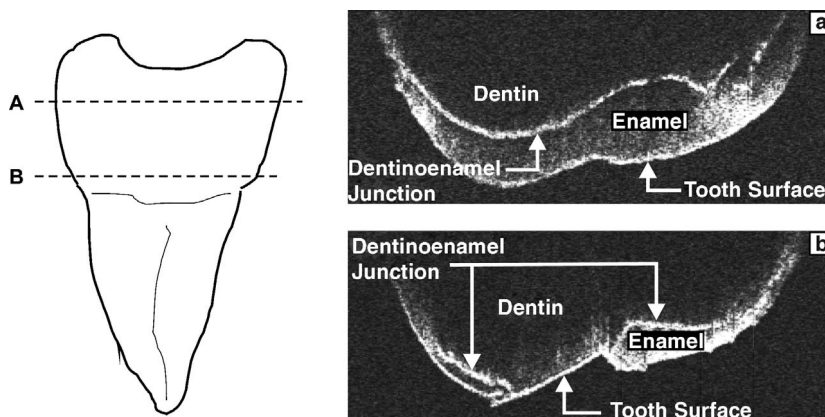


Figure 12.2. (Left) Diagrammatic sketch of a human premolar. (Right) The corresponding OCT depth images obtained along scan lines A and B. The variation of enamel thickness at each scan level as well as the enamel and dentinoenamel junction are clearly delineated.

properties of enamel mineral and allows deeper light penetration.³² PS-OCT technology has recently been used to examine caries lesions and has been shown to detect approximal and occlusal caries on extracted teeth.^{32,49,50} In addition to providing a measure of the intensity of backscattered light as a function of depth, PS-OCT also provides an assessment of the polarization state of the backscattered light relative to depth. The method involves the acquisition of the two orthogonal linearly polarized OCT images either simultaneously⁵¹ or sequentially.⁴³ When the sample under investigation exhibits birefringence properties, such as tooth enamel, then PS-OCT images may provide enhanced structural information. In Jones et al.,⁵¹ linearly polarized light is focused on the tooth sample and the back-reflected light is combined with circular polarized light from the reference mirror. A polarizing beamsplitter divides the signal into parallel and perpendicular components prior to detection. Parallel backscattering OCT images of both sound and carious enamel are similar to traditional OCT images at 1310 nm. The parallel PS-OCT images of sound enamel exhibits a backscattering at the outer surface and to at least 500 μm beneath the surface, while the carious region had a penetration of less than 200 μm . The perpendicular PS-OCT images of sound enamel have a weak specular reflection from the air-tooth interface and a weak backscattering from within the enamel. This is in sharp contrast to the carious region, which demonstrates a strong reflection from the air-tooth interface region and little penetration past the outer surface. The extreme difference in the perpendicular PS-OCT images of sound and carious enamel could be used to differentiate the state of the enamel surface; however, it gives little information as to the depth of the carious region when investigated without parallel PS-OCT or traditional OCT images.

OCT images, acquired with an 850 nm near-infrared source, from a sound surface and from a carious lesion are depicted in Fig. 12.3. The images present the lateral scan position versus the imaging depth with higher intensity correlating with greater light backscattering. In the image of the sound tooth (Fig. 12.3A), an intense light backscattering is observed at the tooth surface. This represents the scattering of the light due to the change of refractive index as the light transitions from air to the tooth enamel surface. For the sound surface, beyond the initial first few microns, the light backscattering rapidly decays with no further changes in intensity deeper into the enamel. This image suggests that the surface is intact with no structural defects, increased porosity, or loss of mineral structure. In contrast to the image of sound enamel surface, the image (Fig. 12.3B) taken of a carious site (Fig. 12.4A) portrays diffuse scattering in a triangular-shaped zone immediately below the surface. Once again, there is intense light backscattering at the tooth surface, indicating that the incipient lesion has an intact surface. The diffuse scattering intensity in the region below the surface is due to the occurrence of multiple scattering and indicative of an area of higher porosity within an otherwise dense enamel structure. This suggests that demineralization has occurred below the intact surface, indicating early dental caries formation. Similar imaging results were recently reported using polarization-sensitive optical coherence tomography (PS-OCT) on natural interproximal lesions.^{32,49} Based on the OCT image, it is estimated that the depth of the lesion is approximately 360 μm deep. This was determined by taking the axial position at which the backscattered intensity has dropped below a predefined threshold level – for example, three standard deviations above the noise level. The triangular-shaped region below the surface, along with this depth estimation, is consistent with histological studies in the literature that have shown a similar triangular-shaped lesion body typical of caries lesions (Fig. 12.4B).^{11–13} Comparison of our OCT depth estimation with gold-standard destructive methods for determining lesion depth such as histology (Fig. 12.4B) and microradiography analyses yield depths of 312 μm and 338 μm , respectively, indicating that the results are comparable. OCT is therefore able to provide morphological information

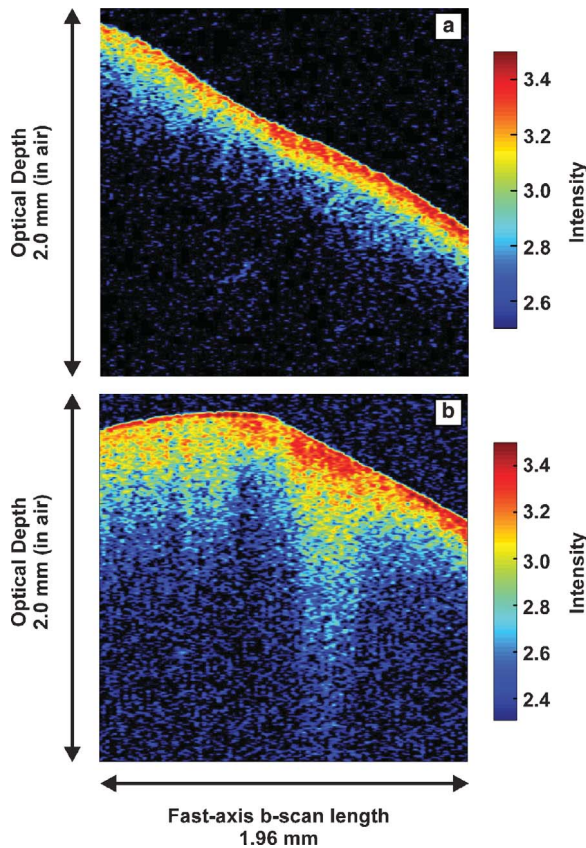


Figure 12.3. Representative false-color OCT images of lateral scan length as a function of depth from (a) a sound enamel tooth surface and (b) a carious enamel lesion. Significant light backscattering with depth is observed at carious regions.

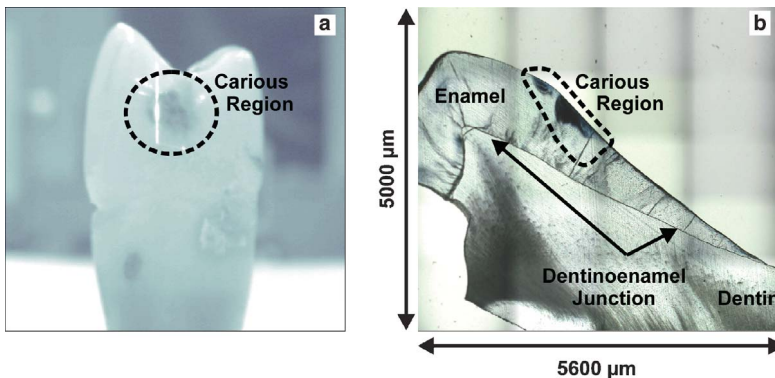


Figure 12.4. (a) Photograph of an extracted human premolar with a carious region. The bright vertical line is a representative reflection of the OCT laser B-scan line. (b) Photomicrograph of a 100 µm thin slice of tooth sectioned with a diamond saw. Histological features such as the enamel crown, underlying dentin, the dentinoenamel junction, and a carious region are clearly observed.

of near-surface tissue structures and defects and is particularly sensitive to changes in refractive index as the light interacts with the sample. It is therefore a good first approach for examining tooth samples to screen for early dental caries, estimating the lesion depth and extent of tooth surface demineralization. From a clinical perspective, lesion depth is useful for determining the extent of caries activity and in aiding the decision either to restore or to promote remineralization. In addition, measurement of lesion depth at regular intervals can be used to demonstrate whether the caries process has been arrested and to monitor remineralization.

An alternative to measuring lesion depth, another useful parameter for differentiating sound enamel from demineralized tissue, is the optical light attenuation coefficient (μ_t). Electronic and speckle noise quenching has to be achieved in order to obtain a smooth profile that characterizes the light penetration into enamel. In the case of an OCT image of enamel tissue, each A-scan is acquired at a different spot on the surface such that the distribution of the scattering aggregates changes from one OCT depth line to another. Changing the distribution of scattering centers induces variations in the speckle pattern from one independent depth-line to another. To compensate for the specific curvature of the tooth surface, all single A-scans from a 2D image were aligned using the reflection rise generated by the air–enamel surface and then added. Adding the profiles of single A-lines, each with its own particular speckle pattern, results in a smooth compounded profile with the speckle generated by multiple scattering of light and the noise generated by random electronic and thermal variations in the OCT detection system significantly reduced. Representative profiles similar to the curves shown in Fig. 12.5, smooth when compared to individual A-scans (see Fig. 12.1B), are obtained after compounding. An attenuation coefficient can be calculated for each compounded profile by fitting the curve with a Beer–Lambert-type function:

$$I(z) = \exp(-2\mu_t z) \quad (12.1)$$

where $I(z)$ represents the OCT signal intensity at an optical distance z beneath the tooth surface and μ_t is the attenuation coefficient, the only fitting parameter used. Each profile from Fig. 12.5 is normalized with respect to the intensity of light back-reflected at the air–enamel interface. The distance of optical penetration of light into enamel is shown along the horizontal axis and the exponential fitting is performed up to a depth where the signal-to-noise ratio becomes 1.5:1. One set of profiles corresponds to early carious regions, while the other set shows light penetration into sound tissue. From the compounded OCT depth profiles, it is evident that the two types of enamel, sound and demineralized, have different light propagation characteristics as detected by OCT. The compounded profiles corresponding to sound enamel are shown in solid lines, while the ones corresponding to carious enamel are shown with dashed lines. All the attenuation coefficients corresponding to profiles obtained for sound enamel are contained within an area bounded by the exponential curves corresponding to the lowest and highest attenuation coefficients found for sound enamel. There is a similar zone shown for the carious cases, also bounded by the exponentials for the highest and lowest attenuation values from this group. The boundaries for attenuation coefficients of sound and demineralized enamel cases are shown in order to demonstrate the variation of the calculated attenuation values among the studied samples. This spread encountered in the attenuation coefficients of teeth acquired from various human subjects could be due to the biological heterogeneity and different clinical histories of the samples. The overall trend is that light attenuation in healthy sound enamel is stronger than the attenuation occurring in the region of the lesion. Moreover, there is no overlap between the attenuation coefficient values of sound enamel and demineralized enamel. This

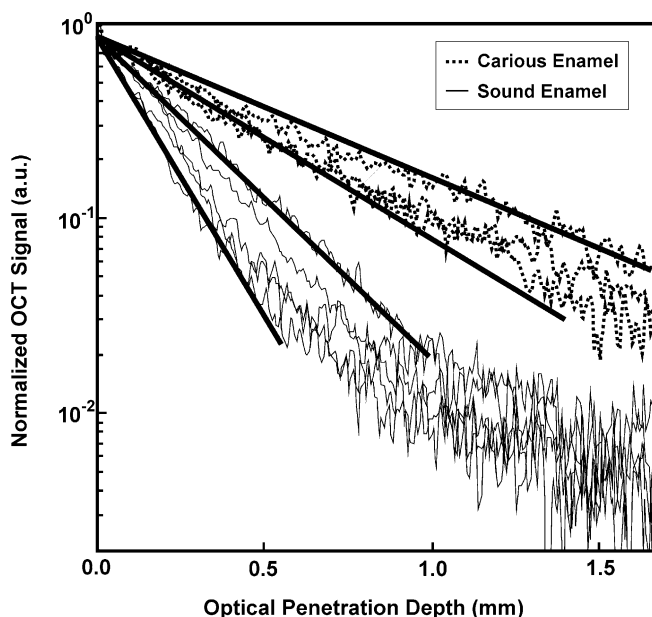


Figure 12.5. Representative sets of OCT compounded attenuation profiles corresponding to regions of sound enamel (solid lines) and regions of carious enamel (dotted lines). The upper and lower limits of the attenuation coefficients are shown with the heavy lines to illustrate the variability of the coefficients within each group with no overlap between the two regions.

difference is reflective of the tooth demineralization and density loss that occurs in carious regions.

12.3 RAMAN SPECTROSCOPY OF MINERALIZED TISSUES

Raman spectroscopy is a vibrational spectroscopic technique that provides details on the biochemical composition, molecular structure, and molecular interaction in cells and tissues. Unlike the complementary technique of mid-infrared spectroscopy, where water poses a problem by having broad bands masking regions of interest, Raman spectroscopy allows measurements of hydrated samples. In particular, highly specific biochemical information (e.g., about proteins, lipids, carbohydrates, and nucleic acids) can be obtained. With mineralized tissues, information about the tooth's inorganic (e.g., hydroxyapatite) and organic (e.g., collagen) composition as well as the mineral orientation can be obtained. Our Raman spectra acquired from human dentin and enamel (Fig. 12.6) indicate that peaks from carbonated hydroxyapatite dominate the spectra, with characteristic peaks from collagen apparent in the dentin spectrum but absent in the enamel spectrum. This observation corresponds well with the different biochemical composition of these two dental tissues. In recent years, Raman spectroscopy has emerged as a potential tool for biomedical diagnostics, with research studies ranging from early cancer detection, delineation of tumor borders, atherosclerotic plaque characterization, and rapid identification of pathogenic microorganisms. A few of these topics are covered in other chapters of this book and have been recently reviewed.^{53–55} It is believed that in disease, changes in the underlying

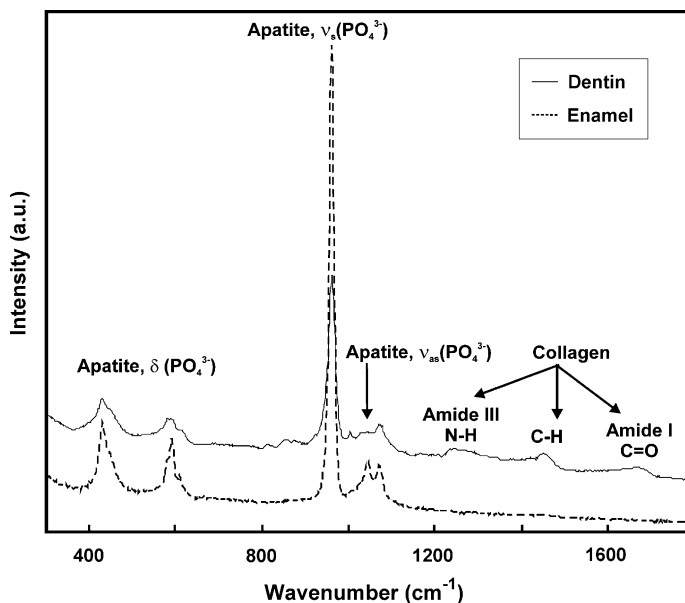


Figure 12.6. Representative Raman spectra of dentin (solid line) and enamel (dashed line) from an extracted human tooth. Spectra are offset for clarity. Assignments of the dominant spectral features are shown. The spectra are dominated by peaks from hydroxyapatite with the dentin spectrum also displaying features arising from collagen, the dominant protein within dentin.

biochemistry of tissues often occur before clinical manifestations are observed. Thus vibrational spectroscopic methods are well-suited for early detection of disease. Through the use of optical fibers, *in vivo* Raman spectroscopic studies are potentially feasible^{53,56,57} for disease detection and monitoring.

Biomedical applications of Raman spectroscopy for investigating mineralized tissues has been ongoing for almost a decade.¹ The majority of the studies are derived from Michael Morris' research group at the University of Michigan and his collaborators at the University of Michigan where they have focused on examining bone and diseases related to bone. The numerous studies range from understanding bone biomechanics, mechanisms of tissue mineralization, and noninvasive spectroscopic assessment of bone quality. For the most part, the phosphate ν_1 band at 960 cm^{-1} , the carbonate peak at 1070 cm^{-1} , and the amide I band at 1665 cm^{-1} have been found to be important in compositional and structural studies of bone. Furthermore, the amide bands can be indicators of protein conformation. Using principal component-based factor analysis to extract out components of interest, Raman imaging has been utilized to study mature and newly formed bone.⁵⁸ Studies of murine calvarial osteoblastic cultures reveal the formation of mineral components that are a lightly carbonated apatite, suggesting that carbonation is an indicator of bone maturity.⁵⁹ Mechanical loading of bone tissue revealed shifts in the frequency of the amide III and amide I bands, suggesting protein structural changes due to rupturing of collagen crosslinks from shear forces of loading.^{60–62} Shifts in the phosphate ν_1 band are observed in damaged bone, possibly due to strain on the mineral lattice upon cracking and damage.⁶³ Aside from examining spectral peak shifts, calculations of the carbonate:phosphate ($\text{CO}_3^{2-} \nu_1$ at

1070 cm^{-1} ; PO_4^{3-} ν_1 at 957 cm^{-1}) and mineral:matrix (PO_4^{3-} at 957 cm^{-1} , amide I at 1665 cm^{-1}) ratios, as commonly performed with FTIR spectra of mineralized tissues,^{64,65} reveal information on the extent of carbonate incorporation in the hydroxyapatite lattice and the extent of mineralization, respectively.^{66,67} Changes in these ratios provide an indication of the mineralization state.⁶⁸ Most recently, this group and their collaborators have demonstrated the feasibility of using Kerr-gated time-resolved Raman spectroscopy to depth profile and obtain typical spectra of bone from samples where bone tissue is beneath a surface of polystyrene⁶⁹ and from rat forelimb carpus with the skin intact.⁷⁰ This latter study shows that major spectral bands associated with the mineral and organic phases of bone were observed in spectra collected through the skin with differences discernible between healthy and disease samples.⁷⁰ Lastly, the first noninvasive Raman spectra of human bone *in vivo* were obtained using fiber optic probes and the approach of spatially offset Raman spectroscopy whereby Raman spectra are collected at set distances away from the illumination point.⁷¹ These are the first transcutaneous Raman measurements of mineralized tissues which previously have focused on biopsy samples and opens up the potential of Raman spectroscopy for noninvasive disease detection and diagnosis of mineralized tissues using fiber-optic probes.

12.3.1 Raman Spectroscopy of Dental Tissues

Raman spectroscopy has previously been applied to dental problems but mainly from the focus of using microspectroscopy to characterize dentin and enamel structures and the adhesive interfaces upon using different resin and bonding agents.^{54,72–74} These various studies have shown that Raman spectra of tooth enamel and dentin exhibit peaks characteristic of the inorganic and organic components of teeth as well as quantitative chemical information of the adhesive interface. Studies using polarized Raman spectroscopy to examine the fundamental structural characteristics of tooth enamel crystallites have detected differences due to crystal orientation.^{75,76} The group of Hill and Petrou have applied Raman spectroscopy for dental caries characterization.^{77,78} In an initial study, spectral differences were observed between sound and carious regions of a tooth where the carious regions demonstrated a broad-band fluorescence background of unknown origin. In a follow-up study, these same authors used a fiber-optic probe to acquire intra-oral Raman spectra. Again spectra from the carious regions displayed a broad-band background that obscured the sharper Raman scattered peaks associated with the phosphate groups of the apatitic matrix. The studies employed laser excitation at 1064 nm and subsequently 785 nm laser excitation. While the pioneering work of Hill and Petrou demonstrates the feasibility of intra-oral Raman spectroscopic assessment of carious lesions, their method largely exploited the difference in the fluorescence between sound and carious regions of the tooth. Similar approaches have recently been reported.^{79,80} Focusing on the fluorescence background is fraught with some of the same confounding sources of variation (deposits of calculus, stains, and trapped food particles) as other fluorescence based methods of diagnosis. Using a laser excitation wavelength slightly longer than 785 nm (e.g. 830 nm), and with recent advances in optical filter design, the fluorescence background from teeth can be attenuated to the point where the Raman scattered light can be detected with minimal interference. Therefore, unlike other emerging techniques, our Raman spectroscopic approach uses spectral peaks specific to the (bio)chemical properties of tooth mineralization, and not the fluorescence background that is influenced by staining or organic matter.

12.3.2 Conventional Raman Spectroscopy of Sound and Carious Enamel

Our Raman spectra of extracted human tooth samples are typically acquired on a LabRamHR confocal Raman microspectrometer (HORIBA Jobin-Yvon, Edison, NJ, USA) operating with near-infrared (NIR) laser excitation at 830 nm (Lynx TEC-100 or Tiger TEC-500, Sacher Lasertechnik GmbH, Marburg, Germany). Figure 12.7 illustrates the optical layout for both nonpolarized and polarized Raman spectroscopic measurements with the polarization optics labeled as optional (O) components. The Raman microspectrometer consists of an Olympus BX41 microscope equipped with a motorized XYZ stage, a spectrograph with 300 lines/mm grating, and an air-cooled CCD detector optimized for the NIR region. Detailed instrumentation and experimental parameters as well as spectral background subtraction methods have been described in our previous papers.^{52,81} LabSpec (ver. 4.12) software accompanying the LabRamHR system was used for spectrometer control and data acquisition. For Raman microspectroscopy, unsectioned tooth samples were placed lying on a microscope slide with the surfaces to be studied positioned approximately orthogonal (i.e., 90°) to the laser beam in a backscattering sampling geometry. This sampling geometry is termed the normal excitation/detection sampling mode according to Tsuda and Arends.⁷⁶

Using Raman microspectroscopy to characterize sound enamel and natural carious lesions, we found that spectral changes were clearly observed in phosphate (PO_4^{3-}) vibrations arising from hydroxyapatite of mineralized tooth tissue (Fig. 12.8). An examination of the spectra from sound and carious enamel reveals differences only in the relative band intensities of various Raman bands, with no evidence of any new bands, band shifts, or

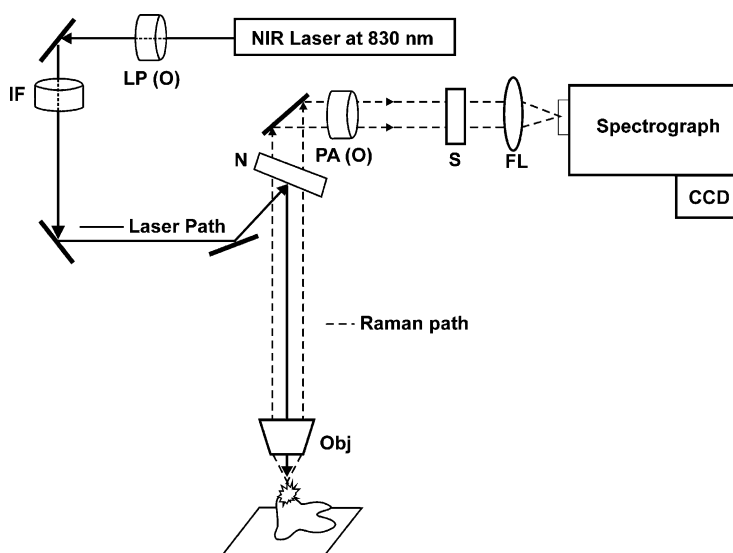


Figure 12.7. Schematic diagram of the layout of a Raman microspectroscopic system illustrating the configuration for nonpolarized and polarized measurements. Shown are the laser excitation (solid line) and Raman signal detection (dashed line) paths for acquiring spectra of tooth samples in a backscattering sampling geometry. Polarization optics are labeled as optional (O) elements. LP, linear polarizer; IF, interference filter; N, notch filter; Obj, microscope objective; PA, polarization analyzer; S, polarization scrambler; FL, focusing lens.

disappearance of bands. The symmetric stretching vibration (ν_1 mode) of phosphate (PO_4^{3-}) at 959 cm^{-1} dominates both sound and carious enamel spectra. The peak position is characteristic of carbonated biological apatite compared to synthetic hydroxyapatite with peak maximum typically at 962 cm^{-1} . Surveying various ratios of PO_4^{3-} ν_2 , ν_4 , ν_3 (431 cm^{-1} , 590 cm^{-1} , 1043 cm^{-1} , respectively) vibrations against the ν_1 vibration (959 cm^{-1}) showed consistent increases in caries lesions compared with sound enamel. Figure 12.9 depicts (A) a photomicrograph of an enamel surface containing a carious lesion and (B) the intensity ratios of selected bands plotted against sampling locations. The carious lesion appears as a white spot on the image with the marked triangles representing various Raman sampling locations. The intensity ratio plot shows enhanced $\sim 431\text{ cm}^{-1}$, $\sim 590\text{ cm}^{-1}$ and $\sim 1043\text{ cm}^{-1}$ bands relative to the 959 cm^{-1} band at the carious lesion, whereas the other bands do not show significant intensity changes. This result demonstrates a method of contrasting sound and carious enamel based on relative Raman signal intensity

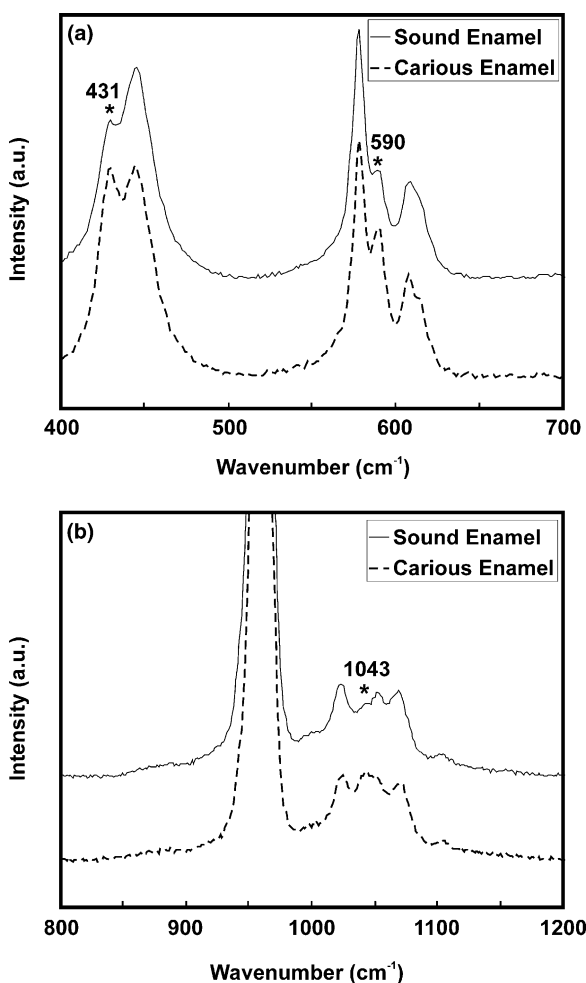


Figure 12.8. Representative Raman spectra, acquired with a Raman microscope, in the spectral regions of (a) $400\text{--}700\text{ cm}^{-1}$ and (b) $800\text{--}1200\text{ cm}^{-1}$ of sound enamel (solid lines) and carious enamel (dashed lines). Spectra are offset for clarity, and the prominent differences between the spectra are marked with asterisks and the peak positions labeled.

ratios of various PO_4^{3-} vibrations. Despite these changes, such intensity differences require spectra of high signal-to-noise ratios and prolonged measurement times that would limit the applicability of this method for clinical use in dental patients.

In subsequent studies, we observed that the relative peak intensities of Raman spectra of sound enamel were different when the measurements were obtained from the cut surface of longitudinally sectioned samples compared with those obtained from the surface of unsectioned samples (Fig. 12.10). Closer examination revealed that the same three bands (431 cm^{-1} , 590 cm^{-1} , and 1043 cm^{-1}) showing changes on carious lesions are also the bands showing large intensity variations, depending on measurement geometry. Such differences are believed to arise from the relative orientation of enamel rods to the laser beam direction. In sound enamel, the majority of the enamel rods are highly ordered within the enamel layer with their *c*-axis (i.e., long axis) oriented orthogonal to the tooth surface. Therefore, the spectroscopic changes detected on carious enamel may be caused by orientational changes of enamel crystallites in caries. In order to further explore this idea,

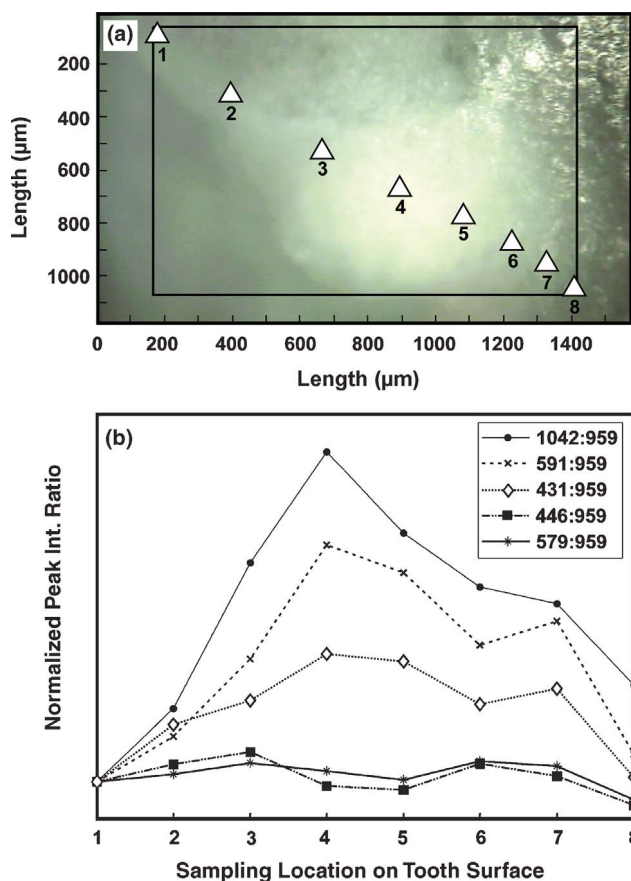


Figure 12.9. (a) Photomicrograph of a human tooth enamel surface containing a carious lesion (“white spot”). The triangular markers represent the sampling locations of a series of spectra acquired along a line spanning from the sound enamel, across the carious region and back to sound enamel again. (b) Peak intensity ratio plot of various phosphate peaks relative to the 959 cm^{-1} peak intensity derived from the spectra acquired in part A.

polarized Raman spectroscopic measurements were conducted on sound and carious enamel.

12.3.3 Polarized Raman Spectroscopy of Sound and Carious Enamel

Polarized Raman spectroscopy is known to be useful for characterizing the molecular orientation of polymers and protein structures and has been used previously to study the orientation of tooth enamel rods of sound enamel.^{75,76} We recently reported various studies in which we used polarized Raman spectroscopy to examine tooth samples more closely.⁸¹ Using NIR linear polarizers in the excitation and detection paths (see Fig. 12.7), both the laser beam and the Raman scattered photons can be independently polarized. Representative parallel-polarized and cross-polarized Raman spectra of sound and carious enamel are shown in Fig. 12.11. Polarization dependence in the Raman spectra of sound enamel is clearly evident with bands at 590 cm^{-1} , 959 cm^{-1} , 1069 cm^{-1} , and 1104 cm^{-1} . Most noticeable is the dramatic intensity difference of the 959 cm^{-1} band between the parallel- and cross-polarized spectra. In both cases, there was a decrease in the intensity of the main hydroxyapatite phosphate peak at $\sim 959\text{ cm}^{-1}$, our so-called “marker peak”. However, the reduction was consistently more significant for sound enamel compared with carious enamel. From the integrated areas under this peak, we can calculate the depolarization ratio (ρ) or polarization anisotropy (A) of this particular Raman band using the following equations:

$$\rho_{959} = \frac{I_{959(\perp)}}{I_{959(\parallel)}} \quad (12.2)$$

$$A_{959} = \frac{(I_{959(\parallel)} - I_{959(\perp)})}{(I_{959(\parallel)} + 2I_{959(\perp)})} \quad (12.3)$$

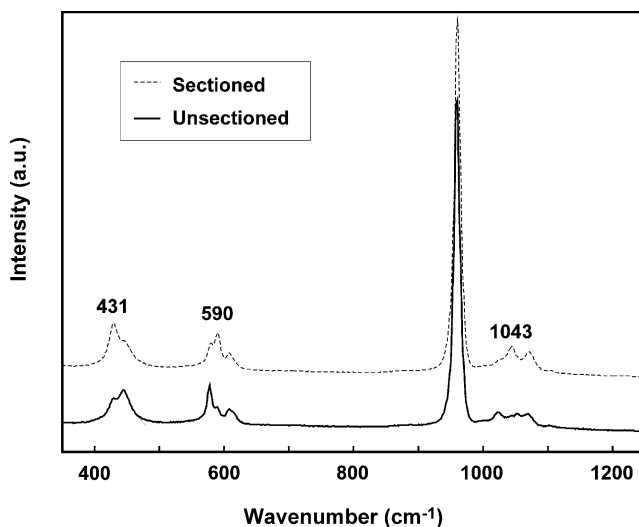


Figure 12.10. Representative Raman microspectroscopic spectra of sound human tooth enamel acquired from an unsectioned tooth (solid line) known as the normal excitation/detection sampling geometry, along with the cut surface of a sectioned tooth (dashed line) known as the transverse excitation/detection sampling configuration. Spectra are offset for clarity. Labeled peaks show major intensity differences between the two sampling modes.

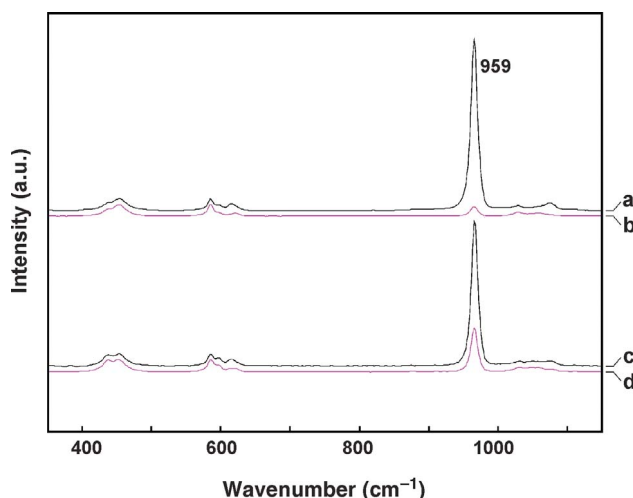


Figure 12.11. Representative polarized Raman spectra of sound enamel (a, b) and carious enamel (c, d) when measured with parallel-polarized (a, c: black traces) and cross-polarized (b, d: red traces) configuration. Spectra are offset for clarity. Significant changes in intensity of the 959 cm^{-1} peak are observed with cross-polarized measurements.

where $I_{959(\perp)}$ and $I_{959(\parallel)}$ are the integrated peak intensities of the 959 cm^{-1} peak detected with the polarization analyzer (PA in Fig. 12.7) oriented perpendicular to ($\perp$) and parallel to ($\parallel$) the polarization direction of the incident linearly polarized laser light, respectively. Figure 12.12 shows a photomicrograph of a tooth surface and the corresponding Raman depolarization ratio map illustrating an example of using p_{959} to detect early carious lesions. The photomicrograph acquired under white light side-illumination shows two early carious lesions appearing as darker shadows when compared to neighboring sound enamel. This result clearly demonstrates that polarized Raman spectroscopy is sensitive to the biochemical and underlying structural changes occurring with acid dissolution of the minerals underlying the surface.

It is known that for randomly oriented molecules, the depolarization ratio (ρ) is mainly dependent upon vibrational symmetry; however, in solid samples, when the molecular

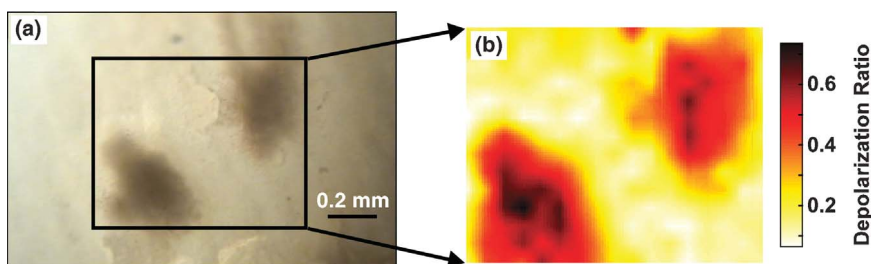


Figure 12.12. (a) Photomicrograph of a tooth surface acquired with side-illumination of white light showing two carious lesions as dark shadows. The area enclosed by the solid black line was mapped by polarized Raman microspectroscopy. (b) The depolarization ratio intensity map of the 959 cm^{-1} peak corresponding to the spectra acquired from the region outlined. The regions of high depolarization ratios (see color bar) match the locations of the carious lesions.

orientation is known relative to the polarization of the laser beam's electric field, the depolarization ratio (ρ) can be strongly influenced by molecular alignment. Since the majority of enamel rods in healthy enamel are highly aligned with preferred orientations, the different ρ_{959} or A_{959} obtained for sound and carious enamel could be indicative of orientational changes and/or scrambling of enamel rods caused by caries activity. It is also possible that increased light scattering within carious lesions (a porous structure) further scrambles the polarization states of the Raman scattered photons generated in the lesion, resulting in a lower polarization anisotropy. Therefore we proposed that the spectral changes observed in the polarized Raman spectra can be attributed to a combination of effects from increased scattering, structural alterations of the enamel crystallite morphology and/or orientation, and changes to the carbonated hydroxyapatite molecular structure as a result of the demineralization process. Relying on the parallel and cross-polarization intensities of the prominent 959 cm^{-1} peak would allow shorter acquisition times enabling this technology to be adopted into a clinical tool.

12.4 TOWARDS CLINICAL DENTAL RELEVANCE

12.4.1 Raman Spectroscopy with Fiber Optics

In our discussion so far, we have demonstrated that carious enamel can be distinguished from sound enamel using the intensity variation of certain hydroxyapatite Raman bands in an *ex vivo* microspectroscopic study. In order to advance this technology toward *in vivo* use, it is essential to demonstrate that similar results can be obtained using fiber-optic-based probes. For this study, we investigated the possibility of detecting the same spectral difference between sound and carious enamel using a commercially available Raman fiber optic probe (InPhotonics, Norwood, MA, USA). Experimental details have been described previously.⁵² With this commercially available probe, conventional (i.e., nonpolarized) Raman studies were performed. The overall spectral pattern of the fiber probe spectra resembles that acquired from the Raman microspectrometer, but with a slightly higher luminescence background. Comparing spectra of sound enamel and carious lesion, similar increases in Raman peak intensities at 431 cm^{-1} , 590 cm^{-1} , and 1043 cm^{-1} were detected as with nonpolarized Raman microspectroscopy. Although the spectral change is similar to that observed in the microspectroscopic data, the degree of changes is less pronounced in the case of fiber probe measurements. This difference likely results from a larger sampling depth obtained by the fiber probe. This depth indicates that the region beyond the caries lesion and into the healthy enamel layer is also being measured in the Raman spectra. As such, any spectral contribution from the caries lesion is gradually diminished in the overall spectrum resulting in slightly reduced spectral discrimination between sound and carious enamel. These fiber-optic measurements suggests that a clinically useful fiber-optic probe should have a pseudo-confocal arrangement (e.g., larger numerical aperture lens at the probe tip and a narrower collection fiber diameter) where the measurement volume more closely approximates that of an early surface carious lesion. Based on the success of the fiber probe study, we are moving on to studies where the fiber-optic probe is used to collect polarized Raman spectra. We are currently investigating the feasibility of simultaneous collection of both parallel- and cross-polarized Raman spectra of teeth using polarizing beamsplitter optics with fiber-optic cables.⁸² Preliminary data show promising results, and the system is currently under further testing with sound and carious tooth samples.

12.4.2 Statistics and Classification Analyses

Unlike measurements on pure biochemicals/biomolecules, the data can greatly vary when exploring biological samples due to the natural heterogeneity that can exist within the complex sample as well as between samples/individuals. The precision, or the agreement between measurements, has important consequences in the ability to use a measure as a biomarker. If precision of the measurement is low, there is decreased ability to differentiate the diseased and healthy populations. Thus an increased sample size is required for a clinical trial aimed at establishing the validity of such an unreliable marker. Information on the precision of the measurement is useful in planning clinical trials. Two conditions of precision, the repeatability and reproducibility conditions, are particularly important in establishing a marker for early dental caries. To establish the clinical utility of a marker, it is necessary to demonstrate that these measurements can be acquired with good repeatability (i.e., successive measurements within the same tooth) and reproducibility (i.e., successive measurements between different tooth samples). In so doing, these new methods can be validated to support their incorporation into clinical trials and eventually into routine clinical use.

In a recent study, the reproducibility and repeatability of the total attenuation coefficient at 850 nm as measured by OCT and the Raman depolarization ratio of the $\sim 959\text{ cm}^{-1}$ phosphate band, measured from *ex vivo* sound enamel, were evaluated.⁸³ With a mean attenuation coefficient of 2.25 mm^{-1} , the estimated 95% confidence intervals for repeatability and reproducibility were 1.85–2.65 and 1.15–3.35, respectively, for sound enamel. The depolarization ratio values were a mean of 0.171 with 95% confidence intervals for repeatability and reproducibility of 0.051–0.291 and 0.0–0.381, respectively for sound enamel. Overall for sound enamel, these parameters (total attenuation coefficient and depolarization ratio) can be measured with good reproducibility and repeatability.

Both the depolarization ratio of the 959 cm^{-1} phosphate band in the Raman spectrum of enamel as well as the total attenuation coefficient of enamel at 850 nm are consistent measures of mineralization showing reasonably good repeatability and reproducibility in normal enamel. These measures also are sensitive to the nascent demineralization associated with early caries formation. In a sample population of 20 teeth, where matched OCT and Raman measurements were made on sound enamel (sound = 39) and areas of early carious lesions (caries = 21), both the μ_t of enamel at 850 nm (μ_{t850}) and ρ of the 959 cm^{-1} phosphate band (ρ_{959}) display statistically significant differences ($p < 0.05$) when comparing sound enamel to caries using repeated measures analysis of variance (ANOVA) (Fig. 12.13). While encouraging, this strong statistical association between early caries formation and a change in μ_t of enamel at 850 nm and ρ of the 959 cm^{-1} phosphate band does not necessarily imply that these measures are useful diagnostic markers for distinguishing sound from carious enamel. The validity of using these OCT and Raman measurements as diagnostic markers for early caries is better summarized by determining the sensitivity and specificity of these measurements in detecting and distinguishing sound from carious enamel.

For continuous markers such as μ_{t850} and ρ_{959} , threshold values that separate the two classes of enamel need to be established before the sensitivity and specificity of the markers can be determined. The receiver operating characteristic (ROC) curve plots the trade-off in sensitivity (sens) and specificity (spec) of a continuous diagnostic marker as a function of decision threshold.⁸⁴ The ROC curve is a plot of the fraction of samples correctly predicted to be caries (TPF: true-positive fraction) versus the fraction of samples incorrectly predicted to be caries (FPF: false-positive fraction) as the decision threshold for the marker is varied between 0 and ∞ . The ROC curves using μ_{t850} and ρ_{959} are superimposed in Fig. 12.14. The area under the curve (AUC) is a general indicator of the predictive power of a marker for

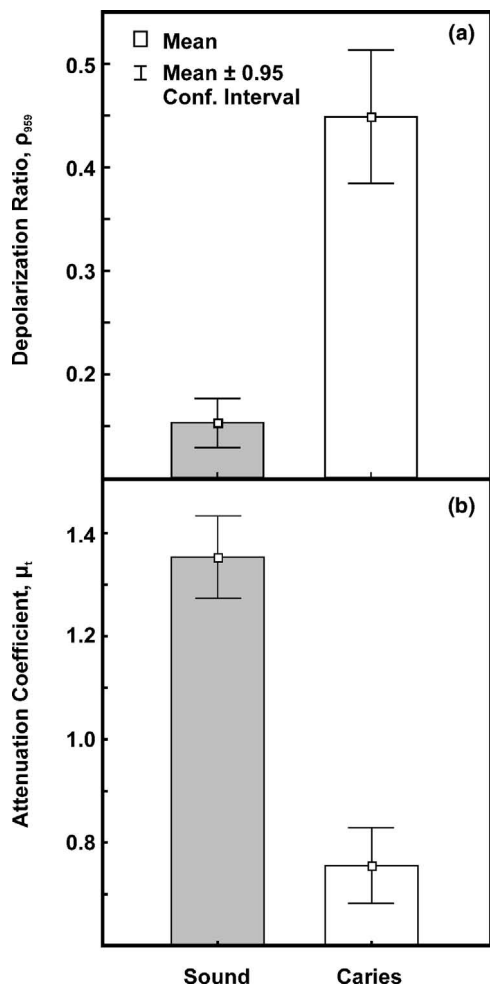


Figure 12.13. Bar graphs of the mean and 95% confidence intervals of (a) the depolarization ratio, ρ_{959} , and (b) the attenuation coefficient, μ_t , from sound enamel (shaded bars) and carious enamel (clear bars). A sample population of 20 human teeth was used with matched OCT and Raman measurements from 39 sound regions and 21 carious lesions. The difference between sound and carious regions are statistically significant ($p < 0.05$) using repeated measures ANOVA.

classifying cases. An AUC of 1 indicates a perfect classifier, while an AUC of 0.5 is expected for a random guess classifier. Using the μ_{t850} as a marker for caries, the ROC curve has an AUC of 0.979, which shows the excellent ranking characteristics of this classifier. On the same database of 60 matched measurements, the classifier based on the measured ρ_{959} has slightly poorer ranking characteristics, AUC = 0.968. The method by Hanley and McNeil for comparing AUC for ROC curves derived from the same cases indicates that this small difference in AUC between the ROC curves is significantly different at the 95% confidence limit.⁸⁵

This cursory examination would suggest that the optical attenuation coefficient at 850 nm would be able to simultaneously convey reasonably high diagnostic sensitivity and specificity for early caries detection by using the threshold that represented the apex of the ROC curve. Selecting $\mu_{t850} > 1.08 \text{ mm}^{-1}$ as the decision threshold provides point estimates of sens = 95.2% and spec = 91.7% for detecting and distinguishing early carious lesions. Using the Wilson interval for univariate binomial confidence interval estimation, 95% confidence intervals of [77–99%] and [70–93%] were obtained for the sensitivity and specificity, respectively. While the upper limits would provide suitable diagnostic performance, a diagnostic test with more than a 10% false-positive or false-negative fraction

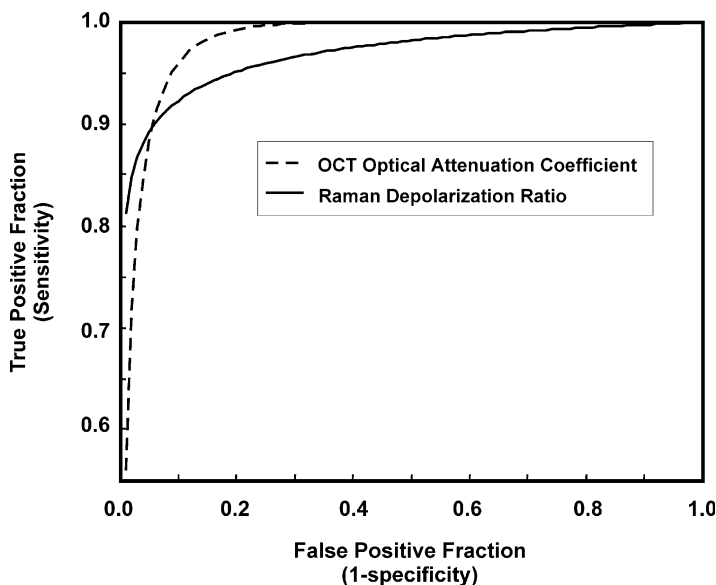


Figure 12.14. Receiver operating characteristic curves with the false-positive fraction (i.e., one-specificity) against the true-positive fraction (sensitivity) for the OCT at 850 nm optical attenuation coefficient (dashed line) and the Raman depolarization ratio of the 959 cm^{-1} peak (solid line).

would be clinically useless. Based on the database of samples available, the measured optical attenuation coefficient measured at 850 nm by OCT may not provide clinically adequate sensitivity and specificity.

Similarly, the depolarization ratio of the 959 cm^{-1} phosphate band may not have the combined sensitivity and specificity for a clinically useful diagnostic marker for dental caries. At a threshold of $\rho_{959} = 0.3$, the univariate 95% confidence intervals for sensitivity and specificity are [71–97%] and [87–99%], respectively.

Closer examination of the ROC curves provides a technically more challenging but more robust diagnostic procedure. Using rapid scanning OCT as a screening tool, regions of the enamel with anomalous light-scattering properties are selected for further polarized Raman spectroscopic measurements. As a preliminary screen of the surface of the tooth, the decision threshold for μ_{850} would be set to the point of maximum sensitivity (TPF) with the maximum practically tolerable false positive fraction. For example, setting a threshold $\mu_{850} = 1.15\text{ mm}^{-1}$, regions with a higher attenuation would trigger the acquisition of a polarized Raman spectroscopic (PRS) measurement. This threshold would yield a false-positive rate of around 18%. Areas flagged by OCT as suspicious lesions with high sensitivity but lower specificity would be subjected to a PRS examination to eliminate false positives by choosing a ρ_{959} threshold that conveys high specificity. At low FPF or high specificity, the ROC curve for the depolarization parameter crosses over the corresponding ROC curve for the OCT attenuation coefficient. Thus at a threshold that conveys high specificity, for example $\rho = 0.4$ (spec = 100%, sens = 65%), the PRS parameter will provide a higher sensitivity than the OCT parameter at the corresponding threshold that provides a similar specificity ($\mu_t > 0.8\text{ mm}^{-1}$, spec = 100%, sens = 57%). The combined use of OCT and PRS in this sequential fashion will improve the data stream rate of the instrumentation using the rapid scanning capability of OCT to survey the surface of the tooth but also improves the overall sensitivity and specificity of the diagnostic test for early caries

detection by using PRS as a confirmatory test on suspicious lesions. In the limited sample set available, the combined use of OCT and PRS was able to correctly classify all cases.

12.5 CONCLUSIONS: OUR MULTI MODAL APPROACH FOR EVALUATING EARLY DENTAL CARIES

We have recently explored the use of two techniques as potential methods for the early detection of dental caries. Our approach combines the imaging technology of optical coherence tomography (OCT) with the technique of Raman spectroscopy (RS).^{52,86} OCT provides morphological detail of tooth surface structure, whereas Raman spectroscopy furnishes information specific to the underlying biochemical changes of the caries process. Both techniques are based on scattering of light. Therefore similar to other emerging techniques for caries detection, the properties of the scattering of light within sound or porous carious regions are being explored. With fluorescence-based techniques, there are a limited number of intrinsic fluorophores to provide diagnostic information without the addition of external dyes. In contrast, Raman spectroscopy can provide information not only about bacterial porphyrins leached into carious regions, but also on the primary mineral matrix and thus about the state of demineralization or remineralization of the tooth. This information is gathered without the need to add extrinsic agents. Polarized Raman spectroscopy provides information on the composition, crystallinity, and orientation of the mineral matrix, all of which are affected in caries formation or remineralization. Meanwhile OCT, which is sensitive to changes in refractive index within the sample, can locate and measure the extent and depth of the carious lesions. In combining OCT with Raman spectroscopy, the biochemical specificity will provide information important in helping resolve the various structural features observed with OCT imaging. Current dental X-rays have a resolution of 50 μm and superimpose the entire three-dimensional tooth structure onto a two-dimensional film. Clearly therefore, the 1–20 μm resolution achievable with OCT and the use of nonionizing radiation are advantages of using this method over other techniques for disease screening and detection.

By obtaining quantitative values for the attenuation of OCT signal in enamel, a distinction could be made between sound and demineralized tissue. The attenuation coefficient obtained from OCT images has potential as an objective parameter for assessing the health of enamel tissue. Furthermore, we have uncovered the utility of using polarized Raman spectroscopy that takes advantage of differences in the tooth's birefringent properties to reproducibly distinguish between sound and carious regions.^{81,87} This multimodal method provides quantitative physical measures rather than a dichotomous variable and therefore can be used to monitor caries progression longitudinally, assess the success of remineralization treatments, and be used as a screening and patient monitoring tool. Combining OCT and polarized Raman spectroscopy offers the potential to convey both high specificity and sensitivity in assessing tooth surface demineralization and remineralization.

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SUB-100-NANOMETER INFRARED SPECTROSCOPY AND IMAGING BASED ON A NEAR-FIELD PHOTOTHERMAL TECHNIQUE ("PTIR")

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13.1 INTRODUCTION

The simultaneous use of infrared (IR) spectroscopy and microscopy is of particular interest since the spectral distribution of the absorption of mid-IR vibrational frequencies is characteristic of each molecular species or its own IR signature. This allows chemical imaging by mapping IR absorption at different wavelengths of a given sample. The use of the high brightness of synchrotron radiation, instead of blackbody sources, already permitted us to reach the ultimate far-field resolution.¹ However, improving the lateral resolution beyond the diffraction limit of 5 μm is critical for most samples of interest in polymers and life sciences. For example, the size of most living cells is comparable to the wavelength in the spectral region of interest for chemical mapping (3–20 μm). Various IR near-field techniques have been developed; among them, the "apertureless" configuration^{2,3} seemed promising. Most of these techniques have used continuous-wave (CW) lasers (e.g., CO₂ lasers). However, the lack of tunability of CW lasers makes the interpretation of the images difficult and restricts greatly its applicability. Several pulsed infrared lasers, particularly free-electron lasers (FELs), offer tunability and are employed in near-field experiments. The apertureless technique is then operating differently than with CW lasers, and various near-field techniques are employed.^{4–7}

CLIO is an infrared-free electron laser facility⁸ operating between 3 and 150 μm , thus covering the entire mid-IR region of interest. Initially, at CLIO,⁹ near-field spectra were recorded with the infrared FEL using the PSTM (photon scanning tunneling microscope) configuration,^{4,10} but with a poor spatial resolution. Indeed, in all optical methods, the characterization of very small samples requires measurement of very tiny absorption. This

means that the transmitted light changes are so small that detection becomes practically more and more difficult as the desired resolution, which is linked to the size of the optical fiber tip, is increased. In addition, theoretical studies¹¹ were performed and showed that the intensity distribution of the evanescent field differs noticeably from the spatial distribution of the imaginary part of the index of refraction. The biggest problem with near-field optical measurements is that they combine the scattering effect coming from the topography (real part of the refractive index) and the absorption variation coming from the sample (imaginary part of the refractive index).

In order to succeed in local spectroscopy, other methods needed to be envisioned. Methods measuring IR absorption directly, such as photoacoustical and photothermal techniques, rather than in transmission, seem more adapted. Recently, we have explored the possibility of working with the photothermal deflection beam (PTDB) effect.¹² The PTDB microscope measures the deflection of a visible laser induced by irradiation of the sample. However, spatial resolution is limited by the visible laser spot size and the need to have a reflecting surface.

The method that is described here is based on a patented photothermal method that we call PTIR (photothermal-induced resonance). We monitor the thermal expansion caused by the absorption of light from a pulsed IR laser. The mechanical sensor is the cantilever of an atomic force microscope (AFM), which goes through its own resonances at each instantaneous sample expansion. The detection of these resonances gives a signal corresponding to the surface deformation, thus a signal related to the absorption only. Indeed, we have to use a short duration excitation and a fast measurement in order to avoid thermal diffusion that would forbid the measurement or, at least, degrade the spatial resolution. We will present the principle of the PTIR method and some results obtained with biological samples to underline the breakthrough potential of this IR high-resolution spectromicroscopy technique.

13.2 AFMIR: PHOTOTHERMAL-INDUCED RESONANCE EXPERIMENT

The PTIR setup installed in our laboratory is an AFMIR^{13,14} (atomic force microscope in infrared range), since we use an atomic force microscope (AFM) to detect the thermal expansion (Fig. 13.1).

We use a commercial AFM (Veeco Explorer) to measure the topography of the sample and also to record the thermal expansion. The sample is usually deposited on a prism made of a material transparent in the mid-IR range, like zinc selenide (ZnSe), silicon, or germanium. The angle of the prism is chosen to be propagative in the sample and evanescent in the air. This setup is similar to the optical near-field microscope PSTM^{15,16} and allows uniform illumination of the sample without illuminating the surrounding air and the tip. In a measurement, the tip of the AFM is placed on the surface of the sample in contact. At each incident pulse of the laser, the rapid expansion induced by the IR absorption of the sample serves as an impulse input to the cantilever that oscillates to its resonance frequencies. These oscillations are recorded by an oscilloscope and analyzed with a fast Fourier transform (FFT) real-time algorithm.

13.2.1 Sample Illumination

Most of the samples of interest have a subwavelength size and react with the incident beam similar to a hole in a dark screen; that is, they produce scattering. As a consequence, only a small part of the incident light goes through the sample (less than 0.1% of the incident light

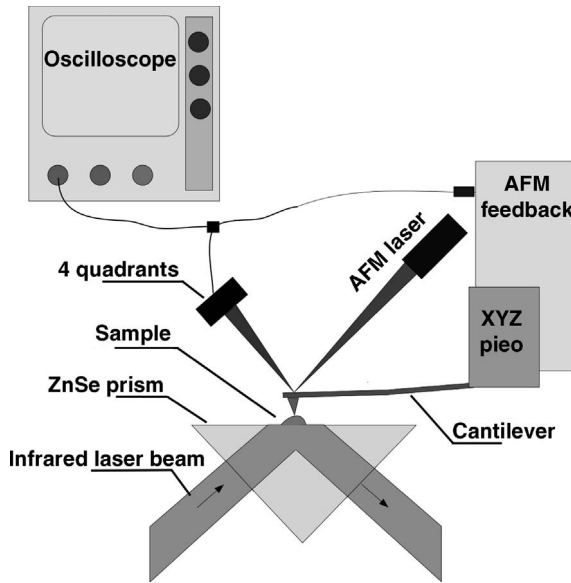


Figure 13.1. AFMIR: PTIR experimental setup.

for a size below $\lambda/2$). Only modeling can predict the transmitted and absorbed light intensity. The effect is not avoidable and is linked to the size and the refractive index of the sample (transmission decreases with sample size). This shows that it is not straightforward to estimate or measure the absorption of one single subwavelength object, since the illumination depends on its size. With a purely optical method, it is very difficult to distinguish these two effects. With the PTIR method, only absorption is measured, since the transmission itself gives no signal.

Another drawback of the setup is due to the angle of incidence. If the sample is too thick, the distribution of the light is not uniform and this effect will disturb the spatial measurement of the sample. To minimize this illumination effect, the best is to study only thin samples. We have modeled the influence of the illumination effect as a function of the size and thickness of the sample. We define the ratio r as the ratio of the maximum and the minimum of the electric field values inside the sample, assuming that the refractive index of organic material is around 1.7 at $6\text{ }\mu\text{m}$ wavelength. This ratio will give us a tool to estimate how asymmetric the light distribution is inside the sample, with $r=1$ corresponding to homogeneous illumination. Table 13.1 summarizes the behavior of the parameter r as a function of sample size and thickness.

TABLE 13.1. Behavior of the Ratio r as a Function of the Size and Thickness of the Sample

Size	Thickness				
	$\lambda/60$	$\lambda/30$	$\lambda/15$	$\lambda/10$	$\lambda/7$
$\lambda/12$	1.077	1.154	1.346	1.492	1.636
$\lambda/6$	1.082	1.171	1.317	1.438	1.533
$\lambda/4$	1.109	1.193	1.423	1.594	1.692
$\lambda/3$	1.124	1.255	1.542	1.834	2.115

If we choose $r_{\min}$ equal to 1.2 as the minimum ratio to obtain a good light uniformity inside the object, we need to have a thickness smaller than $\lambda/15$ – that is, 400 nm at 6 μm wavelength.

We can see that the smallest r value is always obtained at minimal sample thickness. The asymmetry seems to be controlled only by the thickness. This result confirms what Carminati and Greffet¹⁷ have shown about the electric field distribution in near-fields. The near-field distribution of light is homogeneous if the product between the thickness and the difference between sample dielectric constant and surrounding media is constant. In our case the phenomenon is similar, the index is constant, and we note that the distribution of the light inside is also dominated by the value of the thickness. The sample size has a small influence on the asymmetry, but it determines where the maximum of the electric field takes place inside the sample. These results confirm that if we want to employ near-field experiments, we either have to study very small objects that are not perturbed by light confinement, or have to estimate the light distribution by performing a numerical simulation.

13.2.2 Absorption and Thermal Expansion

Considering that the electromagnetic field is uniform inside the sample, the absorbed energy E_{abs} is directly proportional to the imaginary part of the refractive index of the material (for weak absorption). The increase of temperature ΔT is related to the sample heat capacity:

$$\Delta T = \frac{E_{\text{abs}}}{C_p} = \frac{E_{\text{abs}}}{c_p \rho V} \quad (13.1)$$

with c_p being the heat capacity, ρ the density, and V the absorbing volume.

The time evolution of the temperature inside the sample is controlled by the diffusion phenomenon. However, the time of increase is linked to the duration of illumination and, as we use pulsed laser (10^{-6} s to 10^{-8} s), this time is often shorter than the time needed to dissipate the heat. The relaxation time is defined by the time to get $1/e$ of the maximum of temperature. We have developed a numerical model (done by COMSOL, based on a finite element analysis) to estimate this value for organic material polymethylmethacrylate (PMMA) coefficient with a spherical shape of radius R . The time of relaxation seems directly related to the radius R of the sample with a square dependency (fit with 1.987) (Fig. 13.2). The cooling is mainly due to the substrate.

The relaxation time for samples with sizes around 1 μm (like bacteria) is around 10^{-6} s, and for sample with sizes around 100 nm (e.g., viruses) it is 10^{-8} s. This shows that in fact the heating is quickly dissipated by diffusion, quite different from usual thinking about thermal effects.

The increase of temperature will induce a stress inside the object that will create a displacement of matter. The stress can be written in a simple way for an isotropic object:

$$\sigma = \alpha E \Delta T \quad (13.2)$$

where α is the thermal expansion coefficient and E is the Young modulus of the object.

The displacement of the object is directly proportional to the thermal expansion coefficient:

$$\alpha \Delta T = \frac{u}{R} \quad (13.3)$$

where u is the displacement and R is the size of the object.

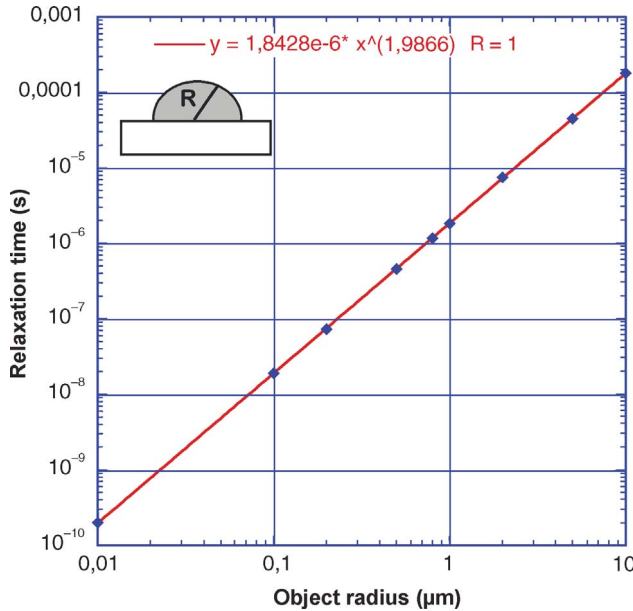


Figure 13.2. Relaxation time of a hemispherical polymethylmethacrylate (PMMA) object in function of its radius.

For example, for a bacterium of 1-μm radius and a ΔT increase of 10 K, the displacement u is 1 nm (taking the thermal expansion coefficient to 10^{-4}). This value is well within the vertical sensitivity of an AFM (a few fractions of nanometers). In practice, due to the resonance of the cantilever, the observed displacement and thus the sensitivity will be greatly enhanced, as will be seen in the next section.

13.2.3 Thermal Expansion Detection

As we have described in the description of the setup, to detect the thermal expansion we bring the tip of the AFM cantilever in direct contact with the sample. The speed of the expansion, which is related to the duration t_0 of the pulsed laser, is faster than the typical time response of the cantilever in contact (T_0 around 10^{-5} s). Therefore, the thermal expansion appears to the cantilever like a delta function giving to its tip an initial vertical displacement and speed. The cantilever will then tend to oscillate at its resonance frequencies. In these conditions the cantilever oscillations in contact resonance can be modeled like a mass–spring system with damping for each mode. If we only consider the first oscillation mode (fundamental), we can write the movement equation of the cantilever for this case:

$$\frac{d^2z}{dt^2} + \beta \frac{dz}{dt} + \omega_0^2 z = 0 \quad (13.4)$$

where z is the deflection of the cantilever (measured by the optical four-quadrants detector), β is the damping, and $\omega_0 = 2\pi/T_0$ is the frequency of the oscillation.

The initial conditions used to solve Equation 13.4 are imposed by the fast expansion. As the tip is in contact mode and the expansion time is shorter than $(1/\omega_0)$, the initial conditions can be expressed as

$$z(t=0) = z_0 = v_0 t_0, \quad \text{with } v_0 = \frac{dz}{dt}_{t=0} \quad (13.5)$$

where v_0 is the speed of the cantilever induced by the expansion and t_0 is the duration of the laser pulse.

The solution of Equation (13.4) considering the initial conditions and small damping ($\beta \ll \omega_0$) can be expressed by

$$z(t) = \left(\frac{v_0}{\omega_0} + \frac{\beta}{2\omega_0} v_0 t_0 \right) e^{-\frac{\beta}{2}t} \sin(\omega_0 t) + v_0 t_0 e^{-\frac{\beta}{2}t} \cos(\omega_0 t) \quad (13.6)$$

Since $T_0 \gg t_0$, the terms $v_0 t_0$ are negligible and the equation reduces to

$$z(t) = \frac{v_0}{\omega_0} e^{-\frac{\beta}{2}t} \sin(\omega_0 t) \quad (13.7)$$

Therefore, the initial displacement is negligible and the system behaves as having experienced a shock and received an initial momentum.

If we consider that the damping is sufficiently small to let the cantilever oscillate several times, we can approximate that the first maximum of the oscillations $z_{\max}$ is

$$z_{\max} = \frac{v_0}{\omega_0} = \frac{z_0}{\omega_0 t_0} \quad (13.8)$$

The cantilever is sensitive to the force applied to the tip, so the corresponding force F induced by the thermal expansion is proportional to the cantilever spring constant k . Formula (13.8) can be rewritten as

$$z_{\max} = \frac{F}{\omega_0 t_0 k} \quad (13.9)$$

The force induced by thermal expansion can be expressed as a function of the sample stress under the tip and the surface of contact between the tip and the sample:

$$F = \sigma \pi a^2 \quad (13.10)$$

where a is the contact radius and σ is the thermal stress 13.2.

By replacing Eqs. (13.10), (13.2) and (13.1) into Eq. (13.9), we finally obtain the expression of the oscillations maximum detected by the AFM as a function of the absorbed energy:

$$z_{\max} = \frac{\alpha \pi a^2 E}{\omega_0 t_0 k} \Delta T = \frac{\alpha \pi a^2 E}{\omega_0 t_0 k c_p \rho V} E_{\text{abs}} \quad (13.11)$$

This expression of the amplitude that could be transmitted to the AFM shows that the maximum amplitude of the oscillation is directly proportional to the absorbed energy (linear dependency). With this mechanical detection of the thermal expansion we are able to deduce the absorption of the sample whatever its size.

The detection sensitivity will be limited by the AFM sensitivity. Let us consider the previous example of an organic material; the typical values are $\alpha = 10^{-4}$, $a = 3$ nm, $E = 1$ GPa, $k = 0.1$ N/m, $t_0 = 0.5 \times 10^{-7}$ s, $\omega_0 = 2\pi \times 50 \times 10^3$ rad s $^{-1}$.

This yields a $z_{\max}$ equal 18 nm for an increase of only 10 K, much larger than the object expansion (1 nm). This oscillation value is easy to detect by an AFM microscope. The AFM is usually able to detect displacements of about 0.1 nm for example; but if we estimate that by applying a Fourier analysis to the oscillations, we can reach 100 pm at least. This makes our device able to detect a thermal expansion induced by an increase as small as 5 mK. In practice, we have to take care in order not to burn the samples, which means that the typical temperature increase is several 10 K for micrometer-size objects. These calculations show that the PTIR technique is highly sensitive and able to measure the absorption of micrometer- and nanometer-scale samples.

13.2.4 Sensitivity of Cantilever Contact Modes

As we have seen in the previous section, the oscillations in contact are really sensitive to the fast thermal expansion. However, this simple model does not provide the shape of the cantilever oscillations, and we need to completely model the cantilever to better understand how the oscillations take place.

Usually we use V-shape commercial cantilevers. To model the eigenmodes, we have measured precisely the dimension of each cantilever. The values of the density (3860 kg/m^3) and the Young modulus (110 GPa) are deduced from the spring constant and resonance frequency of the single arm cantilever given by the manufacturer. The calculations of the eigenmodes are done by the finite element software COMSOL. The two cantilever arms are encased and the tip position is fixed in the z direction (normal to the cantilever) in order to take into account that the tip remains always in contact with the surface or the sample.

Figure 13.3 shows the four first modes of the cantilever. The fundamental mode (mode 0) corresponds to a frequency of 55 kHz and the two arms of the V-shape are

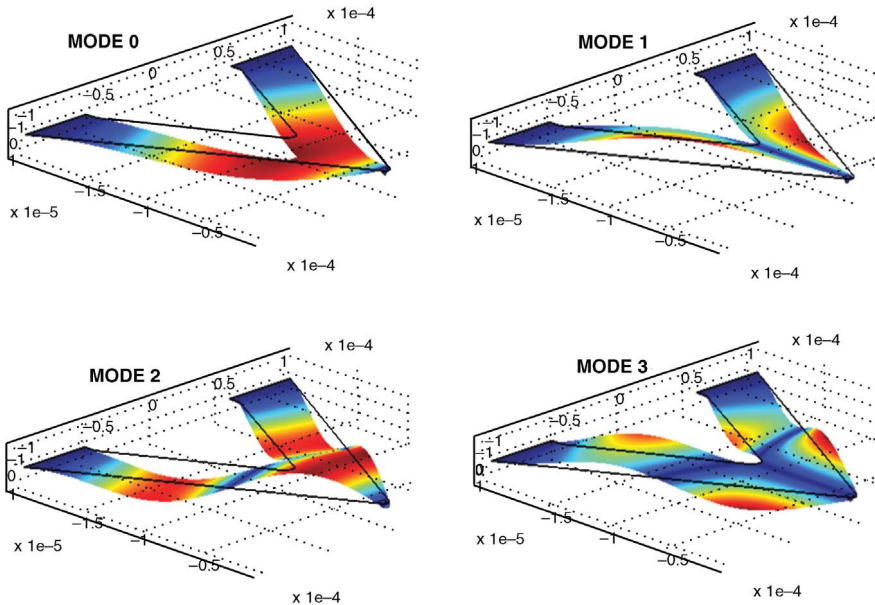


Figure 13.3. Shape of the first four modes (55.2 kHz, 90.2 kHz, 181 kHz, 236.5 kHz) of the V-shape cantilever. The stress is represented in rainbow color scale, from blue (min) to red (max), and the strain is magnified to be seen. The black lines correspond to the cantilever static shape.

oscillating in phase, but, due to the V-shape, the deformation is not perfectly sinusoidal. The stress is represented in color (red for maximum and blue for zero strain) and the strain is exaggerated to be observable. Mode number 1 has a frequency of 90.2 kHz, and we can notice that the two arms oscillations are in opposite phase. The following modes exhibit the same behavior: Even modes are symmetrical (arms in phase) and odd are anti-symmetrical (arms in opposite phase). This modal sensitivity is due to V-shape of the cantilever. Therefore, the even modes are expected to be excited by a normal displacement of the sample (z direction), and the odd modes are likely to be sensitive to lateral deformation (y direction).

13.3 EXPERIMENTAL ILLUSTRATION OF THE PHOTOTHERMAL TECHNIQUE

The test sample that we have chosen to illustrate the characteristic of the AFMIR experiment is the bacterium *E. coli*. This is because the size of these objects are well-calibrated (2–6 μm long, 1 μm wide, 500 nm high), and also the absorption bands are known¹⁸ and assigned – for example, amide I at 1650 cm^{-1} , amide II at 1550 cm^{-1} , and DNA (phosphate backbone) at 1080 cm^{-1} . To prepare the surface of the prism, a large amount of bacteria are centrifuged and washed with distilled water to eliminate their nutritive environment; after that, a suspension of bacteria is deposited and heated at 30°C to slowly evaporate the water. The final concentration of bacteria on the surface is typically 10–20 bacteria in $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$. Using this method, we obtain intact bacteria, which keep their original shape. The absorption bands in the mid-IR are still present even if the bacteria are dried.

13.3.1 Cantilever Temporal Response

After a fast AFM scan and after having chosen an appropriately shaped bacterium (which has not been damaged by the drying), we recorded the electrical signal of the four-quadrant detector for each laser pulse, under different configurations to check the behavior of the cantilever.

Case 1. The AFM tip is positioned on the bacterium and the laser wavelength is centered on the amide I – band. We observe oscillations (Fig. 13.4a) with 30 nm of amplitude at the beginning and decreases within a time constant close to 200 μs (equivalent to a temperature increase of 16 K, assuming that the thermal coefficient is similar to PMMA). The periods of these oscillations are around 16 μs , effectively greater than the predicted time scale of the relaxation for a sample of this size (faster than 1 μs). Under these conditions, the observed oscillations are evidently oscillations of the cantilever, as calculated above. The decay time is directly related to the damping of the cantilever in contact with the bacterium surface and depends strongly on the friction effect. We also notice that the oscillations are centered around the zero value. This value corresponds to an applied static force of 10 nN (300 nm deflection) useful to make the AFM scan. The amplitude of oscillations is always smaller than the static deflection of the cantilever, allowing us to be sure that the tip stays in contact with the bacterium. This shows us that it is better to always look at the induced amplitude in order to keep the tip in contact and avoid a pull-off effect perturbing the measurement. The best way to work is to stay in the linear response of the cantilever.

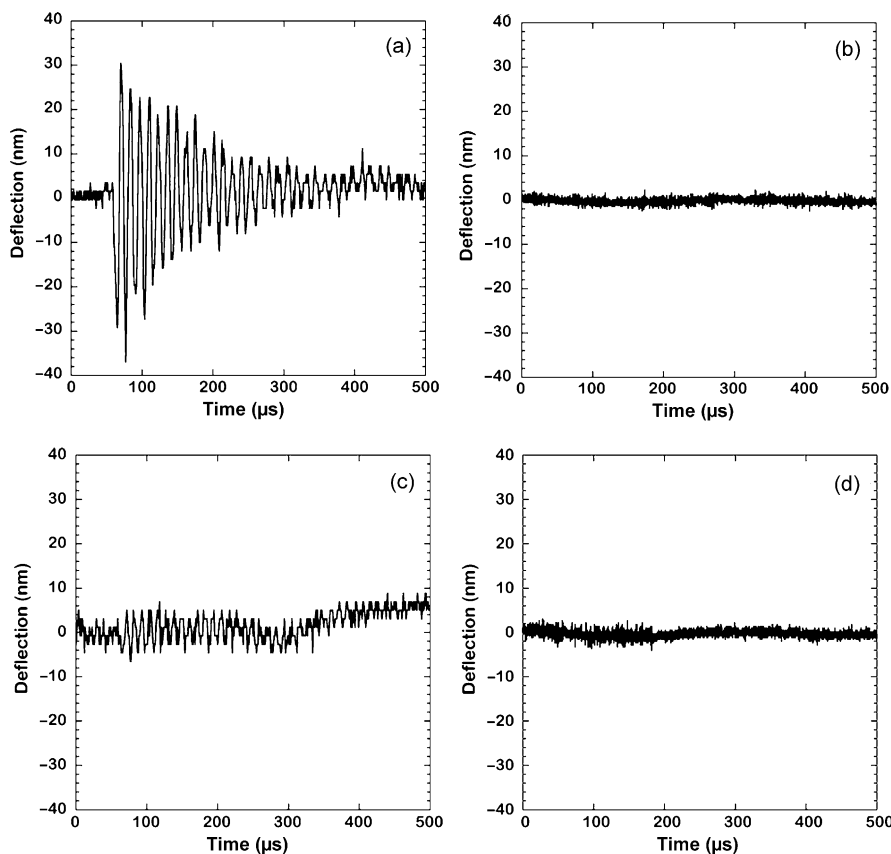


Figure 13.4. Temporal deflection of the cantilever for various cases: (a) the tip is placed on the bacterium, and for an input laser wavenumber $\nu = 1650 \text{ cm}^{-1}$ corresponding to the amide I absorption. (b) Same as (a), but when the tip is out the bacterium. (c) The tip is placed on the bacterium, but for wavelength out of absorption bands ($\nu = 1800 \text{ cm}^{-1}$). (d) The tip is placed out of the bacterium, and the wavelength is out of absorption bands ($\nu = 1800 \text{ cm}^{-1}$).

Case 2. In order to be sure that the signal is not due to direct heating of the tip, a control measurement was done on a clean part of the surface of the ZnSe prism. The relevant signal is displayed in Fig. 13.4b and shows only noise. This proves that the cantilever oscillations are only excited by the bacterium.

Case 3. To test whether the expansion of the bacterium is really related to the absorption, we have tuned the laser wavelength to the amide I band beginning at $\nu = 1800 \text{ cm}^{-1}$ (Fig. 13.4c). The signal shows us a small signal not more than 3 nm, which is in good agreement with the relatively small absorption of the bacterium.

Case 4. In the same way we have also tested that the signal outside the bacterium (on the ZnSe prism) is still zero (Fig. 13.4d).

All these experiments have shown that the oscillations recorded by the four-quadrant detector correspond to the cantilever deflection excited by the photothermal expansion of the bacterium. As we have demonstrated in our theory, we have also seen that the amplitude of the cantilever oscillations is proportional to the absorption. To quantify these oscillations, the best way is to perform a Fourier analysis of this temporal signal.

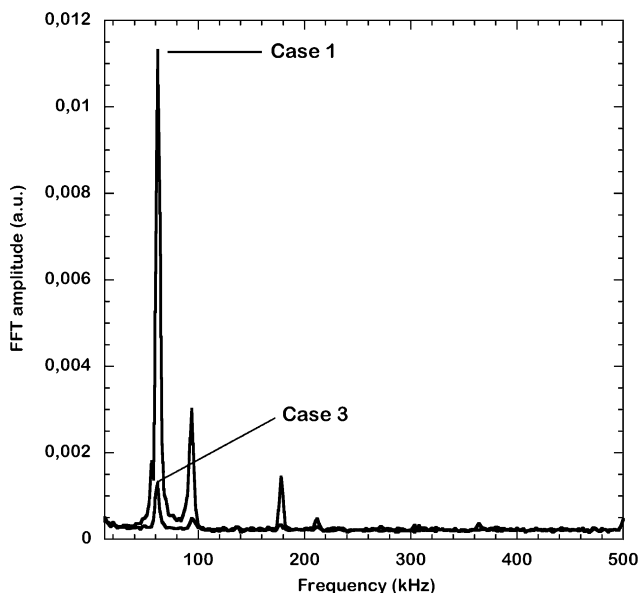


Figure 13.5. Fast Fourier transform (FFT) amplitude (power spectrum density) of the cantilever deflection signals, which are displayed in Figs. 13.4a and 13.4c.

13.3.2 Fourier Analysis

Fast Fourier transform (FFT) analysis is a powerful tool for analyzing the frequency composition of an arbitrary signal and to obtain their relative amplitudes. We use the FFT power density spectrum directly on the oscilloscope to get this analysis in real time. The corresponding FFT signal of Cases 1 and 3 is represented in Fig. 13.5.

FFT analysis reveals that the cantilever oscillations are composed of four modes for the two cases. The frequency modes keep the same values even if absorption changes, which is convenient for studying their evolution. Just by recording the maximum of the fundamental mode of the cantilever, we are able to describe the behavior of the absorption of the bacterium when we fix the position of the tip. This example shows us also that the fundamental mode seems to be the most intense here and contains the majority of the energy coming from the expansion.

Advantages of recording the full FFT spectrum are to analyze frequency shifts and to perform mapping with different modes. This shift analysis has allowed us to point out an effect due to the topography. With a tip positioned in the middle of the cell, we obtain three frequencies: 60 kHz, 180 kHz, and 372 kHz (these modes are not exactly the same as in Fig. 13.5, since the cantilever is different). When positioned near the edge of the bacterium, there are six frequencies with three new ones appearing (89 kHz, 210 kHz, 385 kHz). The three frequencies appearing only near the edge of the bacterium are odd modes of cantilever in contact. The odd modes cannot be excited when the tip is in the middle of the cell since the expansion at this position is purely vertical. When the tip is near the border, the expansion, assuming that is normal to the surface, possesses a horizontal component, and all modes are excited. The frequency values are quite similar to the values calculated by the finite element approach described in Section 13.2.4.

In addition, it appears that the frequencies of the vibration modes vary when making a scan along one line across the bacterium. This effect is also related to the geometry of the surface and depends on the curvature of the sample. It is due to the variation, with the slope, of the friction forces experienced by the tip. Preliminary calculations showed that frequency shifts by about 10% can be attributed to this effect. It follows that when recording the intensity of a mode during a mapping, these shifts have to be taken into account. Also, it has been observed, by looking at the sign of the real part of the signal Fourier transform, that the direction of the laser probe deviation was reversed from one side of the cell to the other, but only for the odd modes: this is consistent with thermal expansion of the sample, leading to a reversed horizontal displacement and a phase difference of π in the odd modes of oscillation.

13.3.3 Ultra-Local Spectroscopy

Localized spectroscopy corresponds to a measurement of the absorption coefficient versus wavelength at a particular point of the sample. The high resolution and sensitivity of the AFM allows us to place the tip on the sample with a transverse accuracy better than 10 nm. The wavelength spectra are obtained by recording the FFT power density spectra of the cantilever deflection (Section 13.3.2), as a function of wavelength. Figure 13.6a shows an example for an *E. coli* bacterium. The vertical axis corresponds to vibration frequencies of the cantilever, and the horizontal axis corresponds to the laser wave-number. In this example, we clearly observe a main efficient resonant frequency at 66 kHz, along with two smaller components at 98 kHz and 190 kHz. By selecting the main component at 66 kHz, we obtain the “PTIR spectrum of absorption” of the bacterium, which is displayed in Fig. 13.6b. This spectrum fits very well a standard FTIR measurement of *E. coli* bacteria, obtained by drying a small quantity of a concentrated solution of bacteria. The absorption bands (amide I, II, III and the DNA) and their relative amplitude are well-reproduced. The most spectacular observation when comparing these curves is that the PTIR spectrum was obtained by collecting the data only from a very small part of one single bacterium (corresponding to the area of contact with the tip that is about 10 nm^2).

13.3.4 Chemical Mapping Using Different Cantilever Modes

For this study, we have first localized one bacterium by AFM scan. The FEL laser is tuned to the maximum of the amide I band (1653 cm^{-1}). We then recorded the variation of the power spectrum amplitude of the deflection signal while the tip scans the surface. As described in the previous section, we also recorded simultaneously the topography of the surface. The results are very reproducible from one bacterium to another. A mapping takes typically 20 min for a 150×150 pixels picture.

Figure 13.7a displays the topography of the surface, where we see the bacterium thickness distribution. Figure 13.7b shows the amplitude distribution of the first even mode (fundamental) of the cantilever on the surface. The two images are very similar. Therefore it appears that the PTIR signal is proportional to the thickness of the sample, as expected for a spectrally homogeneous sample, which is the case for *E. coli* bacterium. We have also verified that the other even modes yield the similar results.¹⁹ The size of the bacterium on the PTIR image (absorption) is very close to that on the AFM (topography). This means that the bacterium expansion is really small (about a few percent), leading the spatial resolution to be better than 100 nm.

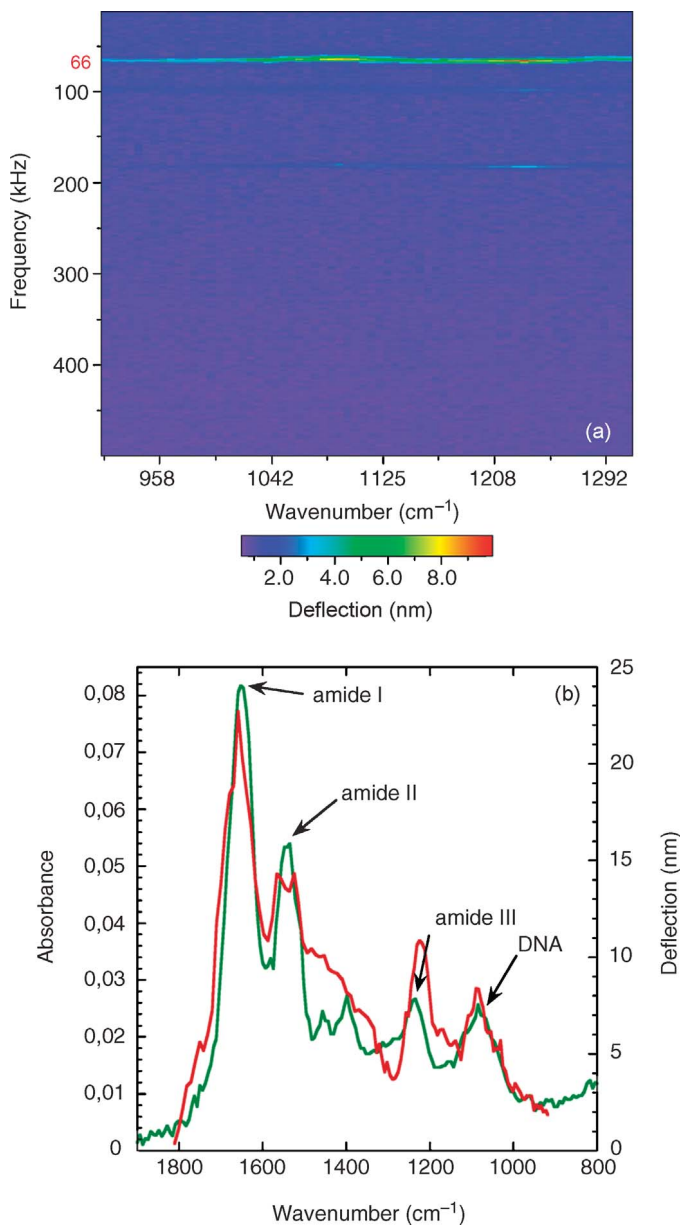


Figure 13.6. (a) Three-dimensional plot of the FFT power density spectrum of the cantilever signal, as a function of the input laser wavenumber and of the vibration frequencies of the cantilever. This measurement has been recorded with a load force on the tip of 5 nN. (b) Comparison of the FT-IR spectrum and the PTIR spectrum of *E. coli*. Red curves represent the PTIR amplitude of the 66-kHz vibration component of the cantilever. The green curve represents the FT-IR spectrum.

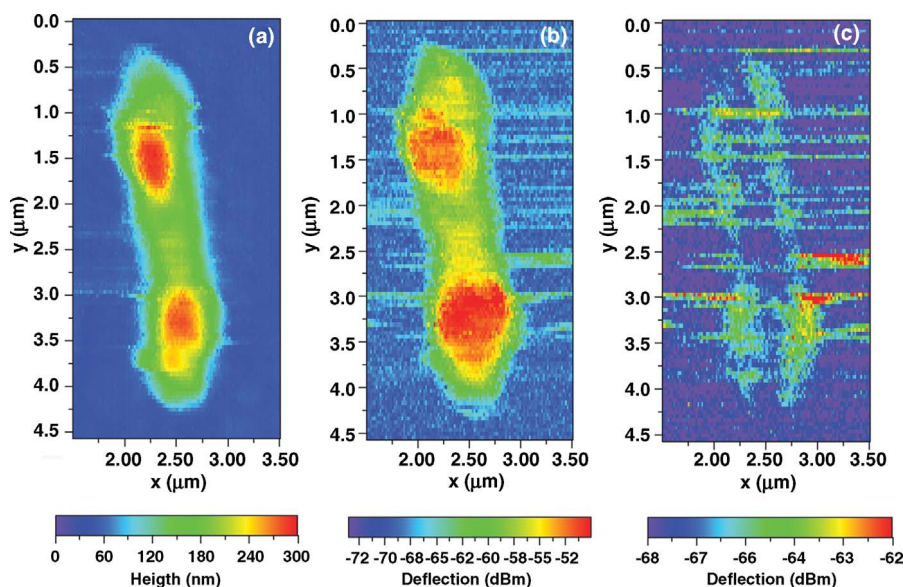


Figure 13.7. Chemical mapping of a single bacterium using the amide I band. (a) AFM topography of the bacterium. (b) Amide I chemical mapping corresponding to the cantilever fundamental mode (sensitive to normal expansion). (c) Amide I chemical mapping corresponding to the cantilever mode 1 (sensitive to lateral expansion).

Figure 13.7c corresponds to the amplitude of the first odd frequency (mode 1). We have verified that other odd modes give the same mapping. This mode is sensitive to the lateral expansion, so that the signal is preponderant only at the edge of the bacterium and equals zero in the middle of the bacterium. This explains why we had obtained similar type of image in our previous work,¹⁴ since we had not yet noticed the influence of the parity of cantilever modes.

In both images of Fig. 13.7, we verified that the deflection is somewhat more intense in the right border than in the left according to the incidence of the beam (discussed in Section 13.2.1). We can notice some signal also on the surface away from the bacterium. This can be explained by the fact that when we perform the first scan to localize the bacterium, some pieces of the biological material could be peeled off by the tip and spread on the prism. This effect can also be observed in the topographic picture (Fig. 13.7a) as small horizontal lines around the bacterium. Rather than representing noise, this effect demonstrates the sensitivity of the device, with a very small amount of biological material being sufficient to give a measurable deflection signal.

13.4 APPLICATIONS: BIOLOGICAL STUDIES

We have started to apply the PTIR technique to various problems such as the study of quantum dots in semiconductors and single-cell imaging. We will discuss only the latter here. The work was done in collaboration with various groups specialized in biophysics or biochemistry.

13.4.1 Study of *Candida albicans**

Candida albicans is an opportunistic fungal pathogen found in the normal gastrointestinal flora of most healthy humans. However, in certain conditions (immunodepressed patients in oncohematology, organ transplantation, ICU patients, medical devices), *Candida albicans* can be responsible for severe infections^{20,21} and is the most reported species in candidiasis. The particularity of *Candida albicans* is its morphogenesis – that is, its ability to grow in three different shapes: the blastospore morphology with a round shape (classical in unicellular yeast); the long-shaped form, usually called hyphae or filament shape, and the broad filament form, referred to as pseudo-hyphae. The hyphal form is considered the most virulent²² form and is responsible for the cell adhesion and invasion. The spectral differences of these different shapes have been studied.²³ Still, FTIR spectroscopy performed with bench-top instruments requires a large number of cells to obtain a good spectrum. In addition, if one wants to study hyphae, the risk of recording mixed signals coming from blastospores as well could perturb the results. It seems therefore important to devise techniques that could be used to study single isolated hyphae. The spatial resolution of bench-top instruments with global sources does not allow such measurements, and even synchrotron IR sources do not meet the necessary resolution for such sizes. The goal of this study was to assess the feasibility of AFMIR to investigate single hyphae, to highlight spectral differences around 1000 cm^{-1} of the yeast in its hyphal morphology, and to monitor variations in glycogen distribution, if any, along a whole single filament.

The blastospores and hyphae were deposited on a prism surface and dried at ambient temperature. The localization of *Candida* was established by the AFM.

The first studies of the blastospores and filaments showed no noticeable spectral differences (Fig. 13.8). The noise recorded on the PTIR spectrum is directly linked to the intensity stability of the laser. This noise reduces the spectral resolution to 10 cm^{-1} , making it difficult to detect minor spectral changes smaller than about 1%.

In case of the hyphae, the spectra of the beginning and the end of the filament (Fig. 13.8a) are also similar and do not reveal either any polarization effect.²⁴ Of greater interest is the distribution of glycogen, so we obtained a PTIR map at its characteristic absorption at 1070 cm^{-1} (Fig. 13.8b). We clearly see that the image is very similar to the topography picture, and no area with a higher concentration of glycogen is found. Because this compound is mainly in the *Candida* cell wall, it is possible that the corresponding absorption signal is so dominant that the PTIR will not be able to detect an absorbing signal coming from inside. The most conclusive result of this study is the high spatial resolution obtained with PTIR imaging of an object with an overall size smaller than a wavelength.

During our investigations about shape discrimination, we found in our culture a pseudo-hyphae form exhibiting a spectral effect. The band around 970 cm^{-1} seems to be more prominent at one position than at others. To verify this effect, we mapped the *Candida* at two different wavelengths corresponding to glycogen (1070 cm^{-1}) and the mannans²³ (970 cm^{-1}). The mappings clearly exhibit a nonuniform spatial concentration of mannans (Fig. 13.9c). The chemical mapping of glycogen (Fig. 13.9b) is not uniform either. However, because the sample is thick compared to the wavelength (about $\lambda/6$), the illumination effect is not negligible (Section 13.2.1), and the hot spots in the mapping are linked to lightening inside the sample. Assuming that the concentration of glycogen is uniform, we can consider

* Collaboration with unite MéDIAN, CNRS UMR6142 UFR Pharmacie, Université de Reims, France.

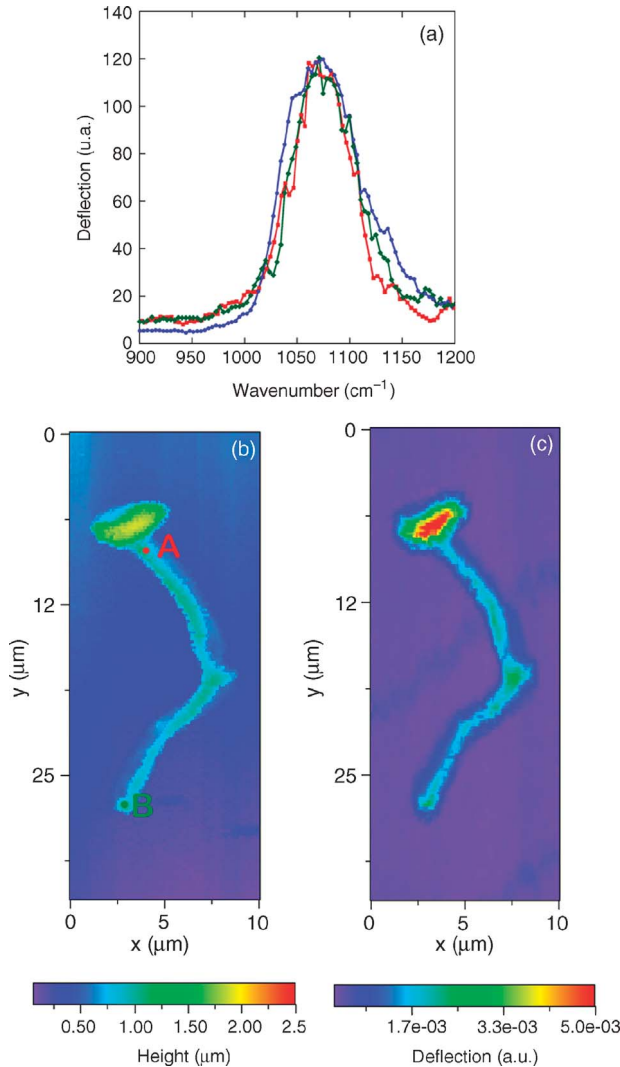


Figure 13.8. (a) PTIR spectrum of different forms of *Candida albicans*: blastospore (blue); beginning of the hyphae (red, see point A of Fig. 13.8b) and the hyphae extremity in point B (green). (b) AFM topography of the hyphae. (c) Glycogen (1070 cm^{-1}) chemical mapping of the hyphae.

this mapping as the illumination reference. Therefore, the mannans inhomogeneities seem real and show that the mannans are distributed only around the borders and, principally, near the top of the *C. albicans* hyphae. The variability of the different forms of the *C. albicans* is such that it is difficult to reproduce this experiment on several equivalent cells. Perhaps this particular distribution of mannans is a stress response or similar, because these *Candida* strains had been stored under unusual conditions (one month in water at 5°C). Although the reproducibility and the understanding of these results are still questionable, these initial data show that the PTIR technique is an effective tool in IR cell imaging at the submicrometer scale.

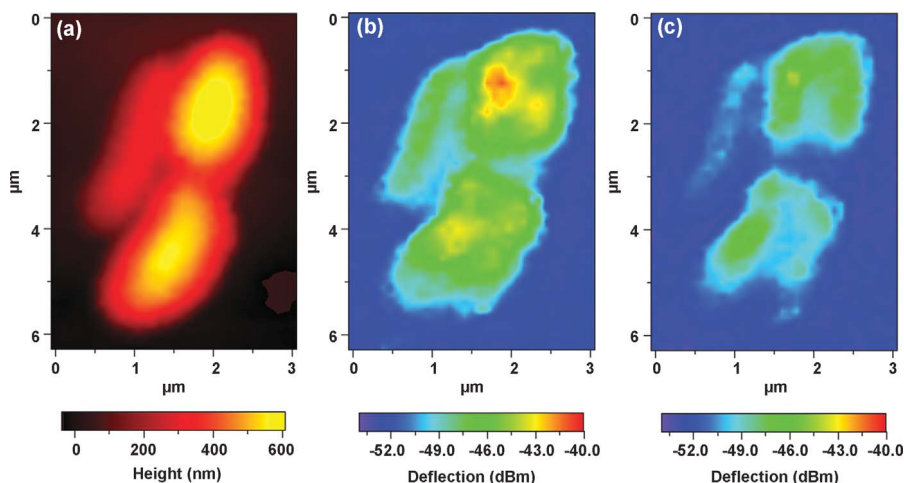


Figure 13.9. (a) AFM topography of pseudo-hyphae *Candida*. (b) Glycogen (1070 cm^{-1}) chemical mapping. (c) Mannans (970 cm^{-1}) chemical mapping.

13.4.2 Study of Bacteriophage T5*

The bacteriophage T5 is one of the best characterized viruses. Over the last 20 years the chemical composition and the developmental cycle of this virus have been extensively studied. The interest of using PTIR to detect such small objects and to follow its evolution when infecting bacteria appears to be great, since it should be possible to track it in real time. Before reaching this final step, we have tested whether our technique was able to detect the virus inside and outside of the bacterium.

The sample was composed of *Escherichia coli* bacteria and phage T5. Bacteriophages T5 st(0) were produced from *Escherichia coli* F and purified.²⁵ The final concentration of the phage stock was evaluated to 1.8×10^{13} infecting phages/mL. Bacteria *E. coli* F were grown in LB medium to the exponential growth phase (3×10^8 cells/mL) and infected by phages with an average multiplicity of 60. The infection was stopped 20 min after the beginning by adding chloramphenicol at a final concentration of $50\text{ }\mu\text{g/mL}$. Chloramphenicol is an antibiotic inhibiting bacterial protein synthesis, and consequently, blocking the phage replication. Infected bacteria were centrifuged and the pellet was washed three times in pure water and resuspended to a final concentration of approximately 1.5×10^8 cells/mL. We have studied two different types of samples: phages alone and bacteria infected by bacteriophages. A drop of the solution was deposited on the ZnSe prism and dried at room temperature. For the infected bacteria, as the infection was stopped by chloramphenicol addition after only 20 min, various stages of the virus development can be found inside the cells.²⁶

When the droplet of phage solution evaporates, one expects the viruses to have preserved their structure and their DNA inside their protein envelope (capsid). To verify this, we have studied the surface of the prism at two different wavenumbers: 1650 cm^{-1} (amide I) characterizing the proteins of the capsid and 1080 cm^{-1} , which is the maximum of the DNA phosphate band. Figures 13.10a and 13.10b show the topography and the

* Collaboration with Laboratoire de Physique des Solides, Universite Paris-Sud, France.

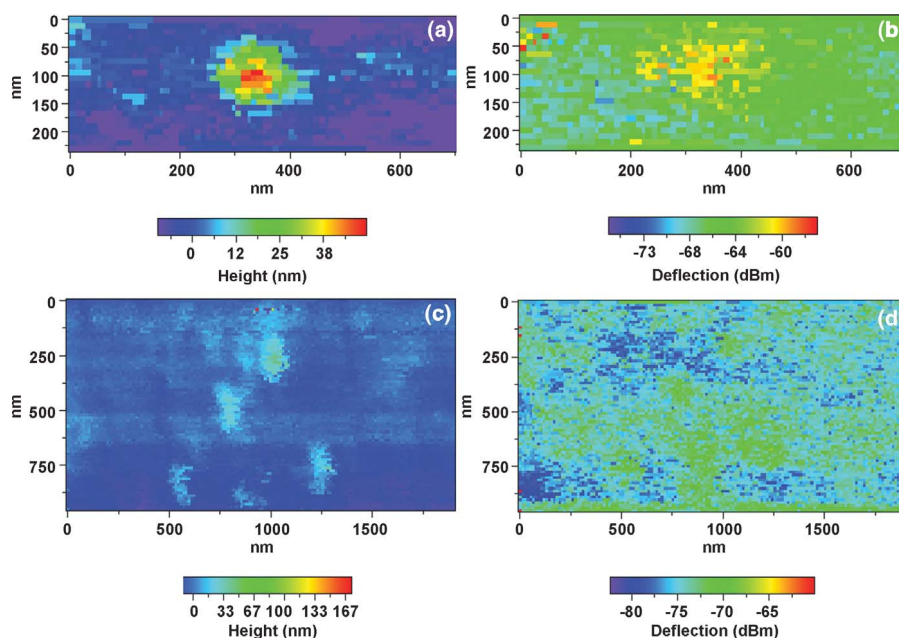


Figure 13.10. (a) AFM topography of a single T5 virus. (b) Amide I (1650 cm^{-1}) chemical mapping. (c) AFM topography of a group of T5 virus. (d) PO_2^- (1080 cm^{-1}) chemical mapping.

corresponding chemical mapping of a single virus, recorded for the wavelength of 1650 cm^{-1} . This wavelength is situated in the absorption band of proteins (amide I).

We can see that the absorption signal of the phage (Fig. 13.10b) corresponds to its topography. There is no detectable lateral expansion due to the heating. Preliminary calculations indicate that this expansion should not be larger than 1 nm. The signal magnitude is weak, because proteins constitute only a small fraction of the phage head, which is mainly constituted of DNA (about 70% of the phage mass). However, the contrast with the background (+6 dB) is sufficient to identify unambiguously the virus, showing that this technique is really sensitive even for such a small entity. Its size appears to be only slightly larger than their usual size (90 nm), demonstrating the excellent lateral detectability of the AFMIR (<50 nm here). As in AFM topography, this value is determined by the convolution of tip curvature (50 nm) with the object.

Topography and chemical mapping at 1080 cm^{-1} of several isolated viruses are presented in Figs. 13.10c and 13.10d. In this case, the PTIR image is very diffuse compared to the topography. These results indicate that part of the phages have certainly been damaged and have lost their DNA when the droplet was dried, leading to weak and extended images. This demonstrates that PTIR certainly brings complementary chemical composition information, when compared to pure AFM topography.

The most interesting aspect for us is to be able to detect viruses inside bacteria. To localize phages inside bacteria, we have recorded a series of chemical mappings centered on the DNA absorption band. It is based on the fact that the virus is mainly constituted of highly concentrated DNA. When a bacteriophage T5 infects *Escherichia coli*, it first binds on the cell membrane and then injects its DNA through the membrane into the bacterium. This injection of the DNA is followed by a full degradation of the host genome and by the synthesis of many copies of the proteins and DNA composing the phage. Phage capsids are

assembled and progressively filled with DNA. At the end of the infection, the host cell contains about a hundred viruses that are liberated by bacteria lysis. When the sample is dried, bacteriophages are not damaged and do not lose their DNA due to the cellular environment, which better preserves their integrity. Therefore, due to the high DNA concentration, the peak around 1080 cm^{-1} is expected to appear more intense at the location of the phages. Mappings of different bacteria correspond generally to different states of infection. The most common situation²⁶ consists in phages “being built” – that is, not all capsids being filled yet with DNA. We illustrate here three infection states: uninfected bacteria or in the first step of infection (empty capsids), partially invaded, and largely invaded with mature phages.

Figure 13.11 describes the topography (left) and the corresponding chemical mapping (right) at 1080 cm^{-1} , for these three states of infection.²⁷ The color bar of the chemical mapping pictures has been adapted to have comparable contrast for each state.

The upper map shows that the DNA distribution of a noninfected cell is homogeneous inside the bacteria. In fact, the DNA of a bacterium is spread all inside the bacteria and is not concentrated in a particular region.

The middle figure shows the most advanced state of infection, where we can observe a significant increase of the PTIR signal, compared to the topography, in some areas of the bacterium, and a decrease in other parts. The topography picture exhibits also a wing at the right side of the main part of the cell. This small part could be a piece of the bacterium membrane. Indeed, the final step infection tends to weaken the cell membrane (lysis) and the drying may have finally destroyed it, due to the induced pressures. In practice, each time we detected an advanced infection state, the shape of the bacterium was not perfectly cylindrical and exhibited a lot of deformations and turgidities. This PTIR image exhibits also a set of three virus particles located inside the small part. In this case, the phages are spatially resolved, contrary to the aggregates that can be seen in the other part of the cell.

A state of partial infection is represented in the lower panel of Fig. 13.11. The AFMIR image exhibits one beautiful hot spot perhaps due to the presence of a single filled phage. Such a result is rather rare, since the development of the infection is quite fast and most bacteria contain at least dozens of viruses.

On the topography image (Fig. 13.11a,e), one can see viruses on the prism surface that are not present in the PTIR image. We can hypothesize that this lack of signal could correspond to empty phages that had expelled their DNA like in the previous study of isolated phages on the prism.

We have performed the local spectroscopy of the DNA band of infected and noninfected cells (Fig. 13.12): These spectra, normalized to the bacterium thickness determined by topography, show that the absorption is much larger when filled phages are present, as expected. It appears also that these spectra fit well with the DNA band obtained by an FTIR spectrometer on a thick layer of bacteria. This illustrates how the PTIR enables ultra local spectroscopy on a nanometric sample.

To look at the potential resolution of buried samples, we have proceeded a new scan of the single-virus-infected bacterium (compare Fig. 13.11f). Figure 13.13 represents the topography (a) and the corresponding PO_2^- chemical mapping (b).

The virus diameter is around 200 nm, which is noticeably larger than those measured previously. In this case, the resolution is determined by the deformation of the bacteria induced by the thermal expansion of the virus and not by the convolution of the tip. A simulation of the thermal expansion calculated by COMSOL is displayed in Fig. 13.14. The resulting apparent size is close to the observed one, even though the virus is not

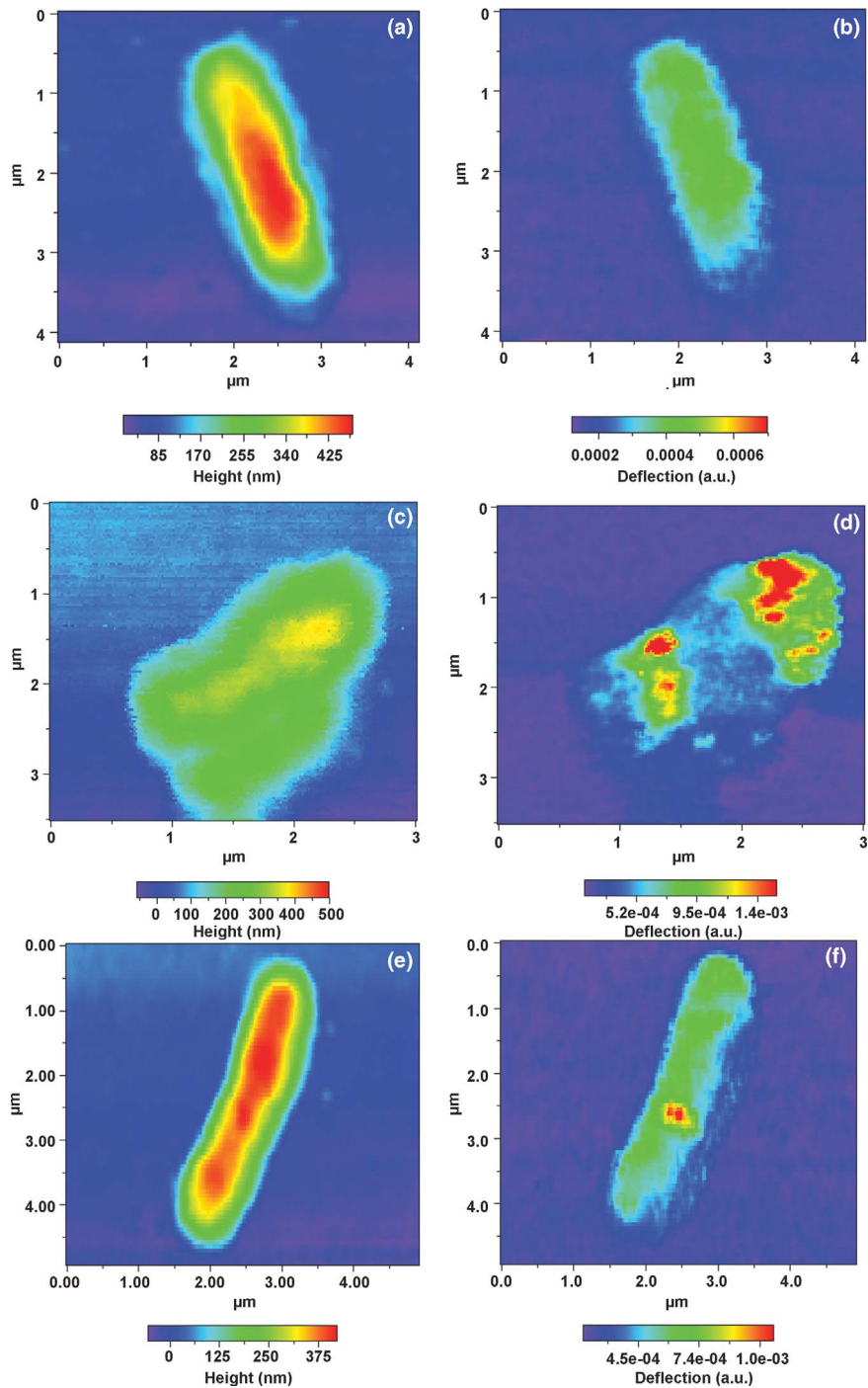


Figure 13.11. (a, c, e) AFM topography of three different infection states of *Escherichia coli* bacteria by the bacteriophage T5. (b, d, f) Corresponding AFMIR images obtained at 1080 cm^{-1} .

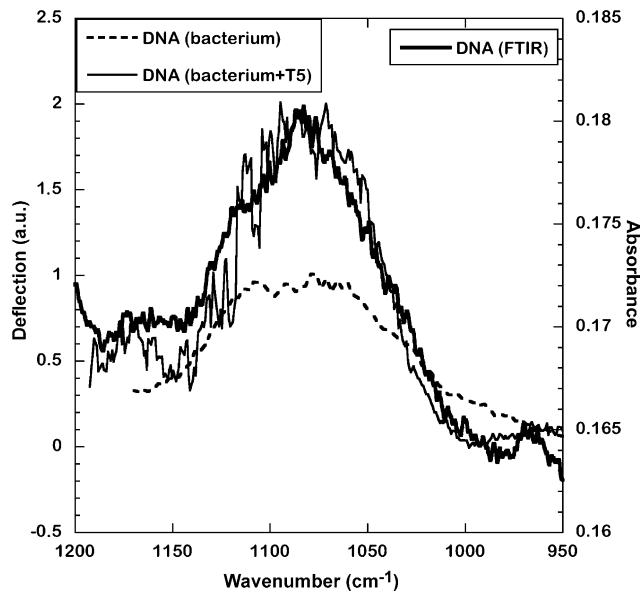


Figure 13.12. Comparison of PTIR spectra of an infected (solid line) and noninfected (dashed line) region of a cell. These curves have been normalized to the thickness of each region (measured by topography). An FTIR spectrum of an assembly of bacteria is shown for comparison (black line).

buried very deep (only a few nanometers from the surface). This example illustrates that the PTIR spatial resolution is related not only to the quality of the AFM tip but also to the thermomechanical coefficient of the studied materials and to the geometry of the sample. To define the resolution, we have detected two virus particles close to one another inside the bacterium and have shown the capacity of PTIR to separate them.

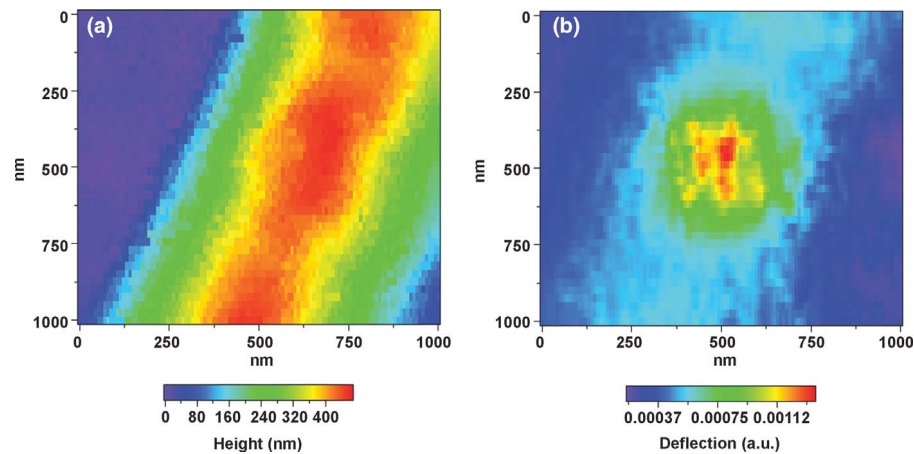


Figure 13.13. (a) AFM zoom of the single filled phage bacterium (Fig. 13.11e). (b) corresponding PO_2^- chemical mapping.

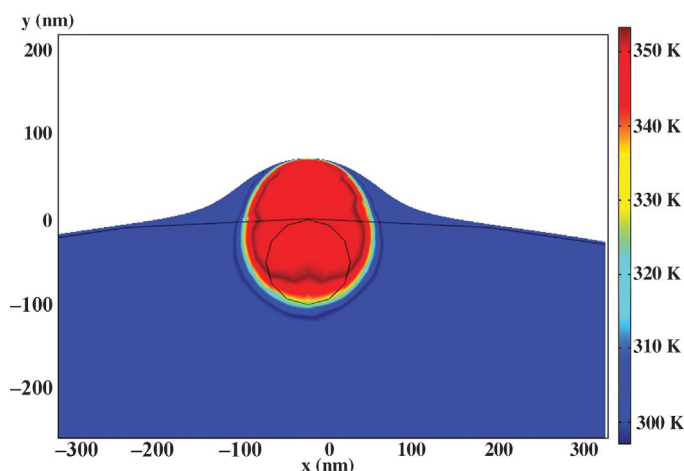


Figure 13.14. Simulation (COMSOL) of the bacterium deformation in the vicinity of the irradiated phage. The color scaling indicates the distribution of the temperature (red equals 350 K, blue equals 300 K). For clarity, the induced strain has been magnified by a factor of 100. Black lines indicate the nondeformed shape of the virus.

13.5 CONCLUSION AND PERSPECTIVES

PTIR is a promising new technique for sub-100-nm IR spectroscopy and imaging that is applicable to a whole range of samples with a resolution improvement of over $50\times$ compared with conventional IR microscopes. We have shown its potential by a few examples on cell imaging. We have also demonstrated that the measurement of the thermal expansion is proportional to the absorption. PTIR resolution is linked to the radius of the tip if the sample is isolated on the surface (convolution effect) and to mechanical and geometrical properties of the sample when the object is buried. The sensitivity and lateral resolution of PTIR is obviously reduced for objects located below the surface. This property is directly linked to the near-field aspect of PTIR technique and is unavoidable. Nevertheless, the capacity of our technique to image buried objects such as viruses inside cells through their IR signature is new and demonstrates the high potential of the PTIR technique for biological studies. The great advantage of IR mapping is to be nondestructive and to avoid the use of specific probes as in fluorescence.

The setup having shown its potential is now proposed to the scientific community and CLIO FEL beam time is available, subject to acceptance of proposals by a scientific program committee.

In the future we will develop PTIR in liquid in order to be able to study living cells and to enable work in real biological environment. In parallel, with a group at IEF (Institut d'Electronique Fondamentale, Universite Paris-Sud, France) we have recently obtained results on quantum dots at room temperature²⁸ and we will also adapt a PTIR for low temperature.

ACKNOWLEDGMENTS

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FROM STUDY DESIGN TO DATA ANALYSIS

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The recent progress in infrared (IR) and Raman spectrometers, mathematical modeling procedures, and powerful computers has enabled the fast exploration of various fields in biomedical vibrational spectroscopy, such as the investigation of tissue sections by means of hyperspectral imaging. Software tools for calculating complex algorithms were developed with particular emphasis on the ease of use, such that other issues like study design and sample acquisition have become more prominent among the remaining challenges for many of the ongoing research activities. At the same time, biomedical vibrational spectroscopy has advanced from pure research to day-to-day application in biology and medicine at a high pace (see, e.g., Ref. [1–3]). In the light of this rapidly changing and highly interdisciplinary environment, this chapter tries to list some common challenges along the path from biomedical study design to the analysis of vibrational spectra and to illustrate prior experiences. The intention is to summarize some of the main procedures that have been established over the past years in terms of preparing, performing, and analyzing investigations in biomedical vibrational spectroscopy.

These issues will be exemplified by investigations from our lab. However, I would like to point out that identifying the hurdles, learning from various experiences, and trying to establish “standard” procedures was a process that evolved from the whole community during debates and discussions.

14.1 ASPECTS IN THE DESIGN OF CLINICALLY RELEVANT STUDIES IN BIOMEDICAL VIBRATIONAL SPECTROSCOPY

14.1.1 Biomedical Relevance

Due to the highly interdisciplinary character of applied vibrational spectroscopy, the biomedical relevance of a study is ranked differently depending on the scientific background of that individual who is asking the question. Since this chapter is concerned with the biomedical application of vibrational spectroscopy, the goal of any investigation herein should originate from a biological or medical interest. In this sense, the scope of any planned experiment needs to be outlined with the potential benefits of the expected results from a biomedical perspective.

For example, it is important to be able to quantify glucose in serum in a laboratory setting in order to show that the sensitivity and reproducibility of IR spectroscopy are sufficiently high to quantify this metabolite at physiological concentrations. It also makes sense to use such an experiment as the starting point for the quantitative analysis of further serum compounds.^{4–9} In addition, the quantification of glucose is important from a medical standpoint, in particular for people with diabetes. However, the quantification of glucose alone can readily be performed in standard clinical chemistry analyzers, and it is one of the easiest and cheapest parameters to be measured. Furthermore, when considering people with diabetes, it may not be very appealing to suggest replacing small, handheld glucose meters and test strips with a mid-IR spectrometer for *in vitro* testing.

Similarly, the quantification of homogentisic acid in urine is an interesting problem from a methodological perspective. If, however, one targets the quantification of this concentration in babies' urine in order to help simplify the diagnosis of alkaptonuria in newborns – a genetic disorder leading to intense joint pain, decreased mobility, and potentially further complications in adults – a physician might not benefit from this achievement from an application point of view, since elevated levels of homogentisic acid lead to a discoloring of the urine, which is readily detected visually (“black diaper disease”).

If vibrational spectra are directly linked to diseases rather than biochemical compounds (a procedure that we dubbed “Diagnostic Pattern Recognition (DPR)”), the quest for medical relevance becomes even harsher. For example, we performed a study in which we compared spectra of sera originating from persons suffering from rheumatoid arthritis with those originating from healthy volunteers. This study was important to show the potential of the method and to obtain indications as to whether an early detection of the disease or a staging may appear feasible with the simple hypothesis that if we can identify the starting point (“healthy”) and an endpoint (“rheumatoid arthritis”), we may perhaps be able to quantify the parameter space in between. In our experiments, we were able to demonstrate the classification of spectra into the groups rheumatoid arthritis versus healthy with an accuracy of 86%, and in this sense we have succeeded in providing a basis for further investigations.¹⁰ On the contrary, by no means have we supplied a marketable tool for the detection of rheumatoid arthritis since the medical query would hardly be its distinction from healthy. In this sense, the next step toward medical relevance would, for example, be either (a) an investigation concerning the potential to classify different stages of rheumatoid arthritis or (b) a study to differentiate between rheumatoid arthritis and osteoarthritis on the basis of a blood test.

These examples may illustrate that it is worthwhile asking precisely what the outcome of the investigation should be. If the study is meant to illustrate a potential or to provide a first step toward application, then the above examples are well-suited as an illustration. On the

other hand, if the goal of a study is to supply a tool for, for example, day-to-day laboratory diagnostics suitable for helping the medical community to diagnose a disease, the study design needs to consider the task in detail by asking questions such as the following: What is the present diagnostic procedure? To what extent will biomedical vibrational spectroscopy improve the present method? What is the reference group? Which level of accuracy is required? What are the confounding factors? Will the diagnostic test lead to a therapeutic consequence?

14.1.2 Reference Methods

In terms of the reference method, the investigator is usually stuck between two extremes:

On the one hand, an application example may be chosen for which well-established and generally recognized reference methods of high precision and accuracy are available. The vibrational spectra can then be readily evaluated without having to consider substantial error contributions from the reference method. In this case, however, it is important to point out the advantages of any vibrational spectroscopy method over the present diagnostic procedure such as time-to-result or costs.

If, on the other hand, vibrational spectroscopy is introduced to a completely novel diagnostic procedure, then it may be difficult to validate the results of vibrational spectroscopy since the reference methods may be unsatisfactory. For example, should a marker for a predisposition for a particular type of cancer be discovered by vibrational spectroscopy, the prognostic aspect of this marker can only be validated in a longitudinal study – that is, by tracking the study participants' health status over a long period of time. Unfortunately, these studies are in general both time-consuming and cost-intensive.

In most of the cases the investigation is somewhere in between these two extremes. In our study on rheumatoid arthritis, for example, a well-defined list of diagnostic criteria is available,¹¹ which, however, includes some cumbersome and expensive medical examinations. A blood-based, sensitive and specific diagnostic laboratory test would simplify and accelerate the diagnostic process as well as possibly serving as a patient-specific, longitudinal marker for the evolvement of the disease – and, maybe, even as an early indicator for forthcoming acute phases.

14.1.3 Estimating the Required Number of Samples

In the language of biometrics a key problem of biomedical studies is posed by the quest for generalization – that is, by asking whether the sample cohort in the planned experiment is sufficiently large to allow for general statements such as the capability to help diagnose a particular disease, to quantify a substance, or to identify a particular microorganism. This issue may be phrased as: “How many samples will be needed such that the spectral signatures of a planned experiment are unlikely to be caused merely by random fluctuations/noise?” Although it might seem odd to ask this question, it turns out that some biomedical investigations should not even be started because the sample cohort required to confirm or to object a hypothesis is far too large to be accessed within a realistic time – or budget.

The required number of samples will generally depend on the specific task of biomedical vibrational spectroscopy. Most of the investigations in biomedical vibrational spectroscopy can be assigned to one of the following three categories:

14.1.3.1 Identification. Frequently, vibrational spectroscopy is used to elucidate the type of molecule that is participating in a process under investigation. Figure 14.1 shows

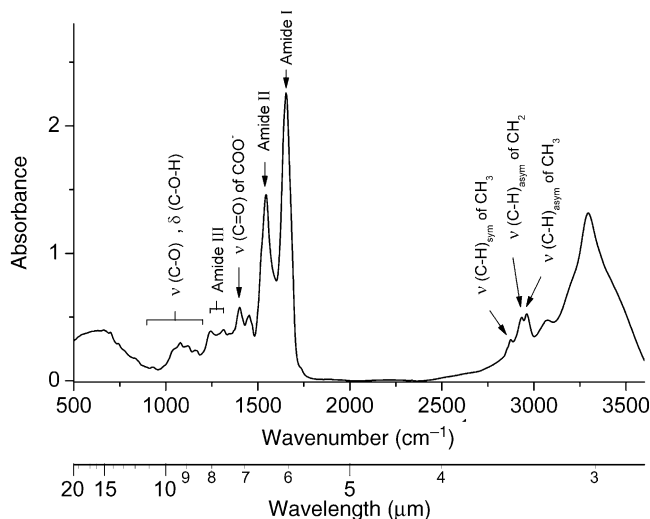


Figure 14.1. Mid-IR spectrum of dried serum and the assignment of major vibrational modes. (From Ref. 15 by permission.)

an illustrative example of the coarse assignment of vibrational signatures to particular vibration modes for a serum sample. These experiments are mostly part of explorative research and the number of samples may be very low since statistical significance is likely to be less important than a study design that provides fundamental cross-checks, a counter-experiment, or other tests to demonstrate consistency. The spectroscopic identification of a molecule may serve as an initial step and, once a substance has been identified, there may be biochemical methods to subsequently study the properties and role of this molecule in a biomedical process.

14.1.3.2 Quantitative Analysis. Quantitative analysis is aimed at determining of the concentration of a substance of interest. If an established reference method is available, the testing hypothesis might be phrased as follows: “Is the spectroscopy-based method significantly different from the reference method?” In this case, the minimum number of required samples may readily be estimated before the actual experiment, since the information content of the multivariate spectra is first broken down into a univariate number – that is, the concentration. A univariate testing procedure can then be inverted to allow for the estimation of the required number of samples (see, e.g., Ref. 12). Note that the estimation requires some prior insight into the expected outcome – for example, the expected standard deviations.

14.1.3.3 Classification. Sometimes the key interest is to classify samples into different categories. For example, a prospective clinical study was carried out in which the causative pathogens of bloodstream infections in hospitalized patients were identified.¹³ In classification problems the difference between two classes will in general not manifest itself in one parameter (e.g., a mean signal within a certain wavenumber interval) but instead appear in a combination of parameters. Hence, one would need to estimate the number of samples on a multivariate basis, but there is currently no simple and general solution to this problem. Instead, the classification may be separated into two steps, analogous to the case of quantification. In a first step, a method needs to be found which is able to perform the

TABLE 14.1. Examples for the Ratios Between the Number of Teaching Samples N_{teach} and the Number of Parameters N_{para} used in a Multivariate Calibration as Derived in Various Studies^a

Study	Method	N_{teach}	N_{para}	Ratio	N_{val}			Ref.
Classification						SE	SP	
Rheumatoid arthritis $\Leftrightarrow$ “healthy”	R-LDA	258	8	32.2	126	84	88	10
Metabolic syndrome $\Leftrightarrow$ “healthy”	R-LDA	127	8	15.9	101	80	82	18
BSE positive $\Leftrightarrow$ BSE negative	PCA/LDA	481	36	13.4	160	82	93	14
	R-LDA	481	15	32.1	160	80	88	
	ANN	481	19	25.3	160	93	93	
	SVM	481	100	4.8	160	88	99	
Quantification						Rel. RMSEP		
Glucose	PLS	148	10	14.8	99	9.5		15
Total protein	PLS	148	3	49.3	99	4.7		
Triglycerides	PLS	148	19	7.8	99	9.1		

^aFor completeness, the results of validation (using N_{val} samples) are also listed whereby sensitivity (SE), specificity (SP), and the relative root mean square error of prediction (rel. RMSEP) in units of percent serve as a measure for the quality of the analysis (R-LDA, robust linear discriminant analysis; PCA, principal component analysis; LDA, linear discriminant analysis; ANN, artificial neural network; SVM, support vector machine; PLS, partial least square). Note that, for simplicity, the listed ratios refer to the total number of samples rather than the number of samples per class for all of the mentioned classification examples.

classification with the outcome that a sample belongs, for example, to “class 1” or “class 2.” In this case, the problem can again be reduced to a univariate (in this case even binary) measure. In a second step the number of required samples may then be readily estimated from a binominal distribution.

On the one hand, the minimum number of required samples is in general thus not easy to determine *a priori*, particularly in classification problems, because it is rarely known how large the spectroscopic differences between two classes will be and how many parameters will be needed to reliably model these differences.

On the other hand, both our experience (see Table 14.1) and that of others suggests that for initial studies one should choose at least approximately five times more samples than the expected numbers of parameters in the case of IR and Raman spectra. For example, in our study to distinguish between serum samples originating from cattle with bovine spongiform encephalopathy (BSE) and those from BSE-negative cattle,¹⁴ we used 15 spectral regions during training a robust linear discriminant analysis on 481 samples (126 BSE-positive, 355 BSE-negative). In this example, the blinded validation of unknown samples led to the correct classification of 135 out of 160 blinded samples by means of the robust linear discriminant analysis. This example may one day offer a method to test live animals for the BSE (the present Western blot reference method requires brain tissue and, hence, is only applicable as a postmortem test).

Beyond this rule of thumb, one may also choose to evaluate the appropriateness of the number of samples *a posteriori* (see Section 14.3.2).

14.1.4 Confounding Factors (Covariates)

It is difficult to assign a spectral difference to one particular compound (particularly in the classification of biomedical vibrational spectra) such that any identified difference may arise for the a variety of reasons – including those that are not directly related to the matter under investigation, such as system drift during the measurement, sample storage, age of the

sample donor, and so on. Such confounding factors can be known *a priori* or might be hidden covariates. In the above study, for example, we observed small but detectable differences depending on the origin of the sample.

14.1.5 Correlations

Biologically relevant molecules exhibit their specific vibrations at multiple frequencies such that their spectroscopic signatures generally occur at multiple wavelengths. As a consequence, the peaks in a vibrational spectrum are partly correlated. On the one hand, this correlation can help to identify the signature of a molecule under investigation, but, on the other hand, it possibly also leads to an overestimation of the number of free parameters in the case of quantification or classification. As an example, the autocorrelations within a set of Raman spectra originating from 247 serum samples are shown in Fig. 14.2. Regions of high correlation are expressed in forms of Pearson's correlation coefficient [1; strong correlation (red); 0, no correlation (green); -1 ; strong anticorrelation (blue)]. The

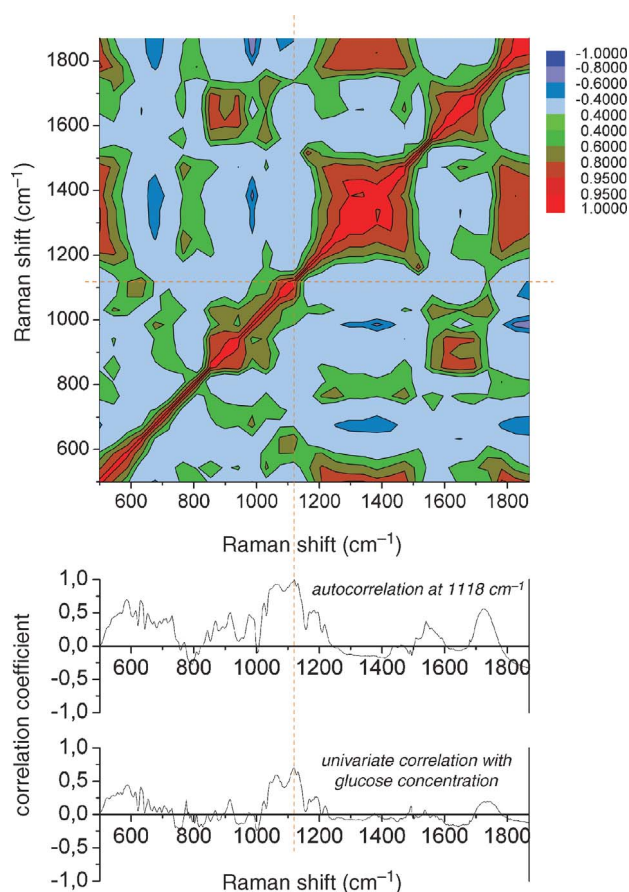


Figure 14.2. Pearson's correlation coefficient as derived from the autocorrelation of (background-corrected, normalized) Raman spectra of serum samples originating from 247 volunteers. Highly correlated ($r=1$), uncorrelated ($r=0$) and anti-correlated data ($r=-1$) are marked in red, green, and blue, respectively. The lower two graphs show the autocorrelation of the peak at 1118 cm^{-1} and the correlation between the Raman spectral signatures and the concentration of glucose.

Raman signal at 1118 cm^{-1} , for example, is highly correlated to the signal at 1063 cm^{-1} (correlation coefficient $r=0.92$). Both are known to represent Raman-active vibrations of glucose. Indeed, the comparison of this fact with the univariate correlation between the Raman spectra and the concentrations of glucose suggests that glucose is the metabolite that is responsible for the autocorrelation within the Raman spectra.

In the quantitative analysis as well as in classification problems, there may also be intrinsic correlations specific to the reference diagnostics, which require additional consideration when interpreting the spectroscopic results. For example, the concentration of low-density lipoprotein (LDL) in serum is derived from the concentrations of cholesterol, triglycerides, and high-density lipoproteins (HDL) based on the Friedewald formula, whereby cholesterol provides the dominant contribution. Consequently, we found a high correlation (square of the correlation coefficient $r^2=0.86$) between the concentration of LDL and the concentration of cholesterol. In these cases it is important to provide cross-checks on whether the spectroscopy-based prediction of LDL is simply a rescaled quantification of the concentration of cholesterol or whether it truly is an independent measure for LDL. For example, one may wish to compare the prediction accuracies of cases with high reference concentration of cholesterol and low reference concentration of LDL to the average prediction accuracy of the remaining samples. Furthermore, a comparison of the principal components necessary to quantify LDL in a principal component regression may elucidate such dependencies. In our experiments we were able to quantify each of the four parameters, but we also performed additional tests concerning the correlation of the reference values.¹⁵

14.2 THE ROLE OF NOISE AND REPRODUCIBILITY IN THE RAW SPECTRA

When the calibration of wavelengths and signal strengths has been performed, following (for example) the procedures outlined in Ref. 16, the signal-to-noise ratio is frequently taken as the measure for the quality of the spectra. The impact of the signal-to-noise ratio on the prediction error is illustrated in Fig. 14.3 based on a study of the mid-IR spectra of serum samples originating from 247 volunteers (see also below) in which we artificially imposed

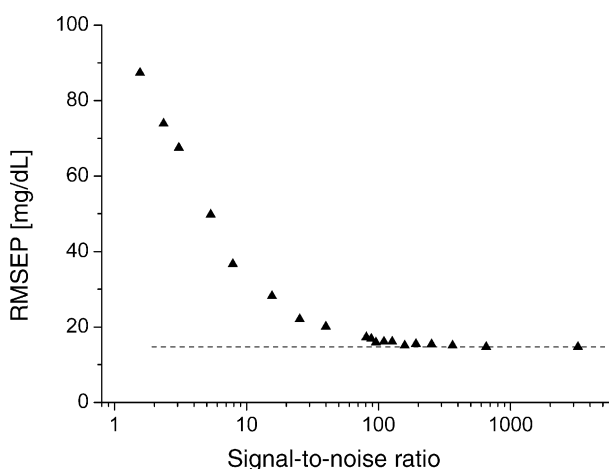


Figure 14.3. Root-mean-square error of prediction (RMSEP) for the mid-IR-based quantification of glucose in sera as a function of the signal-to-noise ratio, whereby various noise intensities had been artificially superimposed to the original spectra prior to analysis.

additional noise onto the spectra after the data acquisition. It can clearly be seen that additional noise hardly affected the quality of the quantitative analysis even when 10 times the original noise was added to the spectra. Hence, there must be another reason for the limited accuracy of the IR spectra in this example.

In our studies on the mid-IR spectroscopy of dried films of serum, we were able to demonstrate that the reproducibility of the spectra was of predominant importance. In turn, it is therefore crucial to verify that any potential spectral differences in a study on biomedical vibrational spectroscopy are not feigned by the lack of reproducibility.

We investigated the reproducibility of mid-IR spectra of dried films of different aliquots of the same bovine serum sample over the course of 4 weeks.¹⁷ For analysis purposes, it is assumed that the signal observed is the sum of a “true” signal (in this case the mid-IR absorbance), a random noise term, and a systematic error, which varies from measurement to measurement. A slow system drift constitutes an example for the latter error contribution. Here, reproducibility was defined as the ratio between the “true” signal and the root-mean-square systematic error. The results of this comparison (Fig. 14.4) show that the reproducibility is strongly reduced in almost all spectral regions under investigation if one proceeds from immediately adjacent measurements to comparing measurements performed over the course of 3 days. The reproducibility reaches a value around 50 when comparing the measurements over the course of 4 weeks.

On the one hand, additional factors such as the system-to-system variation will contribute to a further reduction in the reproducibility if vibrational spectroscopy is considered within the framework of routine application in a biomedical laboratory.

On the other hand, the reproducibility may be improved by appropriate counter-measures such as triplicate measurements, referencing, or advanced methods for baseline

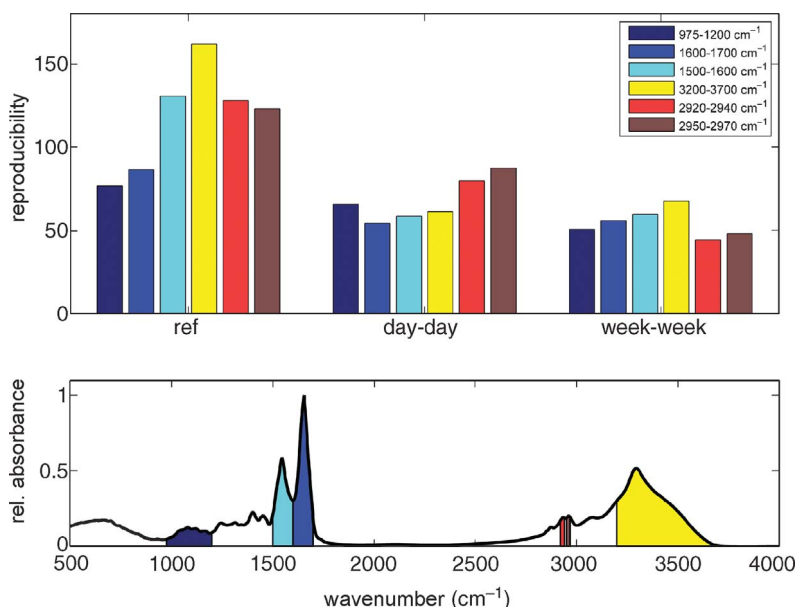


Figure 14.4. Reproducibility of repeated measurements using multiple aliquots of a single serum sample. Here, reproducibility is defined as the ratio between the mid-IR signal and the standard deviation among the repeated measurements. The spectroscopy has been performed over the course of 4 weeks. The intervals used for analysis are indicated in the lower graph. (From Ref. 17 by permission.)

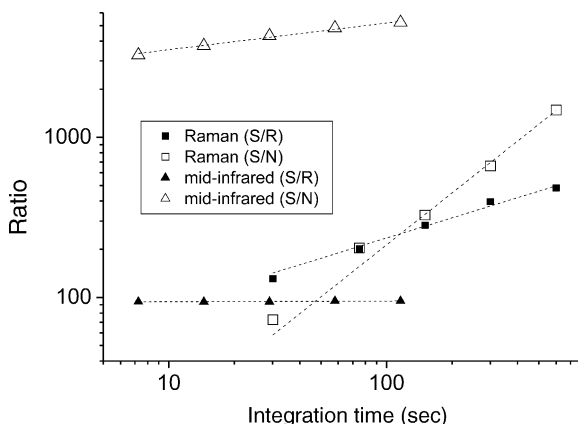


Figure 14.5. Ratios between signal and noise (unfilled symbols) and signal and reproducibility (filled symbols) for Raman (triangles) and IR (squares) spectroscopy. In these experiments, mid-IR spectroscopy of dried films of serum is clearly limited by reproducibility rather than noise, whereas Raman spectroscopy of liquid serum samples is dominated by noise for integration times below 100 s.

correction and normalization. In cases in which minute differences are sought among very similar spectra, we proposed and implemented a simultaneous baseline correction and referencing algorithm, in which the (wavenumber-dependent) comparison with a template spectrum immediately reveals major differences. In passing, I would like to note that the algorithm parameters may also serve as an online quality check for the individual spectra.¹⁷

Reproducibility and noise were monitored as a function of integration time for the case of mid-IR and Raman spectra (Fig. 14.5) with the result that – at least in the cases examined – reproducibility limits accuracy rather than noise in almost all of the investigated range of integration times.

14.3 SAFEGUARDING THE ANALYSIS OF DATA AND ITS INTERPRETATION

After the raw data have been recorded, various methods of data preprocessing may be applied such as

- *baseline correction*
subtracting a linear background signal and, possibly, also higher-order terms of a polynomial
- *background subtraction*
a higher-order polynomial (mostly fifth order) is subtracted in order to compensate for the fluorescent background in Raman spectroscopy
- *normalization*
scaling the maximum peak amplitude or the area under the curve to a constant value by multiplying with a scaling factor
- *differentiation*
frequently, the first-order derivative of the spectrum is used, which converts the spectrum's maxima and minima to zero-crossings in the derivative such that (for example) peak positions can readily be located. Sometimes, even second-order

derivatives may be advantageous when identifying differences in the curvature of two spectra. However, there is a trade off between the order of the derivative and the signal-to-noise of the derived curves.

When data preprocessing has been completed and consistency checks performed, an unsupervised analysis such as the cluster analysis often helps to find (desired and undesired) dependencies in the data set. Note that in the case of classification (e.g., by means of cluster analysis), the choice of the evaluation interval already constitutes a “supervision” per se, which in the extreme case may even allow for finding regions within the data set which happen to perform the desired subdivision among the spectra, regardless of actual biomedical differences among the samples. Therefore any selection of wavelength intervals must be motivated by a hypothesis that is independent of the data, whereas data-driven wavelength selection needs to be avoided for unsupervised analysis.

Supervised analysis strategies such as linear discriminant analysis, artificial neural networks, or partial least-square regression exploit additional information beyond the vibrational spectra. This additional information may cover, for example, a disease state of a sample donor or the concentration of an analyte.

14.3.1 Overfitting

Once a supervised analysis method has been established in a given data set, a measure of the quality of the teaching process is needed. Frequently, the whole data set will be reprocessed through the final algorithm and will be evaluated either by means of the classification accuracy in classification problems or in forms of the regression coefficient or the root-mean-square error of calibration (RMSEC) in the case of quantitative analysis. While these numbers may serve as a first hint toward the quality of the analysis, this procedure tends to be overoptimistic since the same data had been used for teaching the algorithm and, thus, to find the optimum model and parameters in the first place. One may wish to illustrate this fact on an exaggerated example: If, for example, 100 data points (e.g., absorbance values from the spectra of 100 samples taken at particular wavelengths) are used to establish an algorithm, which itself uses 100 free parameters (e.g., the weighting coefficients for wavenumber regions, principal components, input neurons etc.), it is not astonishing that one will readily find a set of numbers which nicely fit all the data – and it will then not be surprising that inserting the 100 original samples into the equations will give superior accuracy. With this example in mind, it is crucial to identify that part of spectral information which is specific to the particular distinction or quantification of interest and to avoid using the remaining spectral information.

One method to safeguard this identification and to optimize the algorithm is to withhold one part of the data and to train the algorithm on the remaining part only. Since biomedical vibrational spectroscopy is frequently limited by the availability and number of well-characterized samples, it is conceivable to withhold just one single sample and to train the algorithm on all the other samples. The spectrum of the single sample can be processed through the algorithm after training and a classification or concentration prediction is obtained for this individual sample. In a next step, another single sample is used, new coefficients of the algorithm are generated, this other sample is evaluated and so on, until finally all of the samples have served as a single sample. This procedure is known as leave-one-out cross-validation, and the associated error is called root-mean-square error of cross-validation (RMSECV) in the case of quantitative analysis.

An even better method to avoid adapting the algorithm to random correlations is to split the data set into two larger parts, one for teaching and one for an independent, blinded

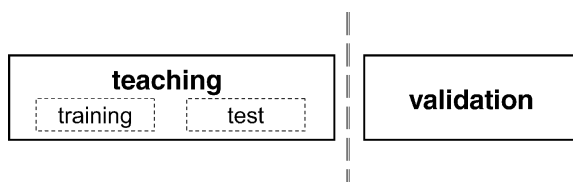


Figure 14.6. It is recommended to split the data set into a teaching set and an independent validation set, whereby the latter needs to be kept blinded until the teaching has been finalized. When teaching the algorithm, the teaching set may be split into a training set and a test set. It is important to note that the validation set is not included in the teaching process at any point during training including the feature reduction process (e.g., the selection the optimum spectral regions).

validation (Fig. 14.6). It is important that the independent validation set remain completely untouched until the teaching is complete.

Again, teaching can be performed by dividing the teaching data into two subsets: one for training and one for testing. The algorithm is then trained on the training set, and its quality is assessed using the test set. When an “optimum” algorithm has been found, it is legitimate to choose another split within the teaching set and to verify prior findings using the new training and test set. Multiple repetitions of this procedure will then provide a valuable “optimum” classification strategy, and the corresponding “optimum” algorithm is finalized subsequently by using the whole training data set. When the overall training process has been finished, the optimum algorithm is applied to the independent validation set in a blinded manner without any changes to the algorithm whatsoever. Finally, the validation data is unblinded and the quality of the classification is calculated without any further adjustments. In the case of quantitative analysis, for example, the difference between the values predicted by the algorithm and reference values may be calculated for the validation data set in forms of the root-mean-square error of prediction (RMSEP).

Note that the term “test set” is sometimes used for the independent validation set and vice versa in the literature on multivariate data analysis. However, since the definition given above (see Fig. 14.6) is consistent with the terminology used in medical diagnostics, I have adopted the above definition throughout this chapter.

In our study on quantitative analysis of serum samples by means of mid-IR spectroscopy¹⁵ the RMSEC, RMSECV, and RMSEP were investigated as a function of the number of parameters (in this case the number of latent variables *LV* used for partial least-square regression). An example of the quantification of glucose in the mid-IR spectra of serum samples is given in Fig. 14.7a. As expected, the value of RMSEC decreases as the number of latent variables increases. Within the teaching set, we identified a minimum of RMSECV for 15 *LV*, whereby the resulting distribution of concentrations predicted with the leave-one-out method is not different from those for 9–31 latent variables on a statistically significant level (*F*-test, $\alpha = 0.05$). After the teaching had been finalized for *LV* = 15 the partial least-squares algorithm was applied to the blinded validation set. The validation was subsequently unblinded and the differences between the predicted concentrations and the reference concentrations were calculated, resulting in an RMSEP = 14.7 mg/dL.

This example nicely shows that, on the one hand, low error contributions may be obtained numerically by referring only to the RMSEC. On the other hand, the low values are misleading since they represent a particular fit to the teaching data, including random correlations at wavenumbers, which are not generally representative for the concentration of glucose but rather represent a fit to the noise – that is, overfitting. The leave-one-out

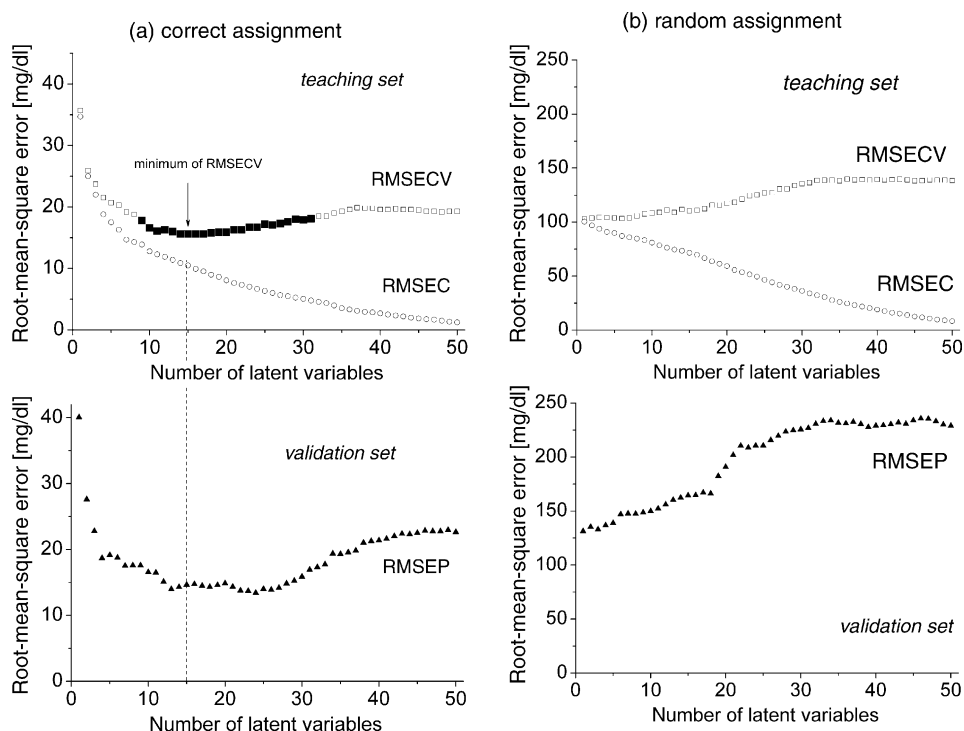


Figure 14.7. The root-mean-square error of calibration (RMSEC), leave-one-out cross-validation (RMSECV), and prediction (RMSEP) are plotted as a function of the number of latent variables LV used for the quantification of glucose in mid-IR spectra. While the RMSEC continues to decrease with increasing LV regardless of (a) a correct or (b) an (intentional) random assignment between samples and concentrations in the teaching set, the values of RMSECV and RMSEP indicate the lack of appropriate modelling for the random assignment. (Figure 14.7 a is from Ref. 15 by permission.)

cross-validation constitutes a much better estimate for the achievable accuracy, since overfitting leads to an increase in RMSECV for $LV \gg 15$. It simultaneously provides a good method to estimate the dimensionality of the system in forms of the number of latent variables. Blinded validation represents the best way of estimating accuracy, since it reflects the conditions within an application environment in which there is no additional *a priori* knowledge about the expected data.

The effectiveness of the blinded validation is illustrated on the same data set (Fig. 14.7b), whereby the assignments between spectra and glucose concentrations have been randomized. While the RMSEC decreases for increasing values of LV , the increase in RMSECV and RMSEP nicely shows the (intentionally) insufficient generalization capabilities of the trained models.

14.3.2 Retrospective Analysis of the Size of the Teaching Set

In an attempt to estimate the required number of samples retrospectively, the error contributions were investigated for different sizes as well as selections of subensembles within the teaching set. The results are depicted in Fig. 14.8 for the above data set (Fig. 14.7a). The value of RMSEP was calculated for the same validation set as above

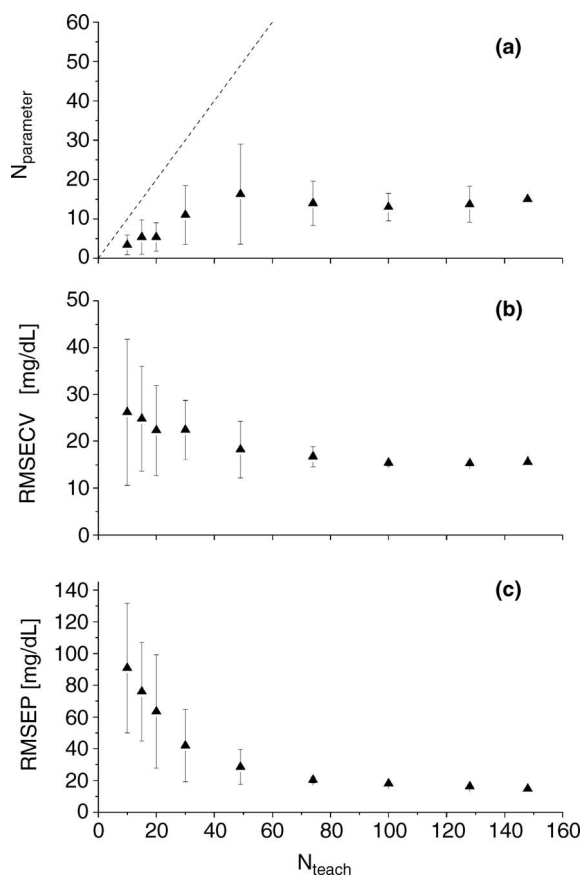


Figure 14.8. (a) The optimum number of latent variables ($N_{\text{parameter}}$), (b) the root-mean-square error of leave-one-out cross-validation (RMSECV), and (c) the root-mean-square error of prediction (RMSEP) are displayed as a function of the size of the teaching set (N_{teach} : number of teaching samples) for the quantification of glucose by means of mid-IR spectroscopy. The dashed line in part a indicates the extreme case of $N_{\text{parameter}} = N_{\text{teach}}$.

but based on different teaching subsets, whereby the numbers and choices of samples within the teaching set had been varied.

If the optimum number of latent variables is derived from the minimum of RMSECV, the dimensionality of the PLS calibration LV is shown to increase with an increasing number of teaching samples up to $N_{\text{teach}} \sim 50$ (Fig. 14.8a). For a lower number of teaching samples, the mean accuracy remains moderate. In addition, the values of RMSECV and RMSEP strongly vary, depending on the particular choice of the teaching subset, and this variation is indicative for a poor modeling of the overall data due to small numbers of teaching samples (Fig. 14.8b, and c). For larger sizes of the teaching set, both the RMSECV and the RMSEP level out at a low value and they stay approximately constant for $N_{\text{teach}} > 60$. Interestingly, we find that small values of RMSECV result in high values of RMSEP and vice versa as illustrated in Fig. 14.9.

In this example, the root-mean-square error of prediction is much larger than 14.7 mg/dL if the ratio between the number of teaching samples and the average number of parameters LV is less than ~ 5 (Fig. 14.10). For $N_{\text{teach}}/\text{LV} > 5$ the variation in RMSEP for different

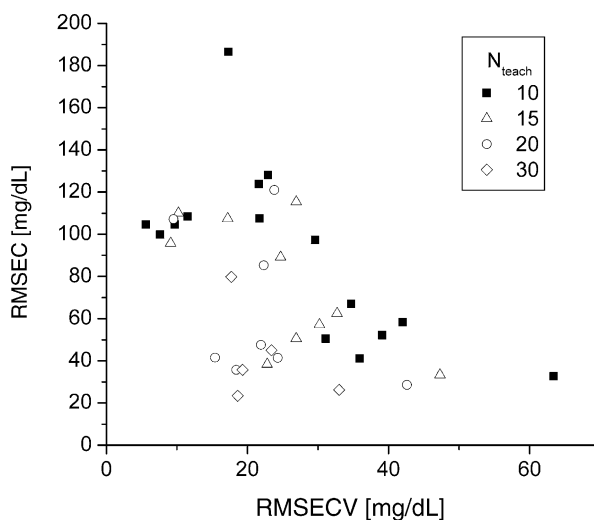


Figure 14.9. RMSEP of the independent validation set of constant size ($N_{\text{val}} = 98$) as a function of the RMSECV for the various teaching subsets using $N_{\text{teach}} = 10$ (filled squares), 15 (triangle), 20 (circle), and 30 (diamond) for the quantification of glucose by means of mid-IR spectroscopy. The RMSEP as well as the RMSECV improve with increasing numbers of teaching samples. For the particular choices of small teaching subsets ($N_{\text{teach}} = 10$, squares), a seemingly good training (i.e., a low value of RMSECV) tends to result in a higher prediction error RMSEP.

selections of teaching data is less than 10% and its value approximates the RMSEP for $N_{\text{teach}}/\text{LV} = 10$. In this way the results of this particular example agrees well with prior findings of classification problems in that for each class at least ~ 5 times more samples than parameters should be used in linear models. Of course, the factor of five between the number of teaching samples and the number of parameters should be considered as a

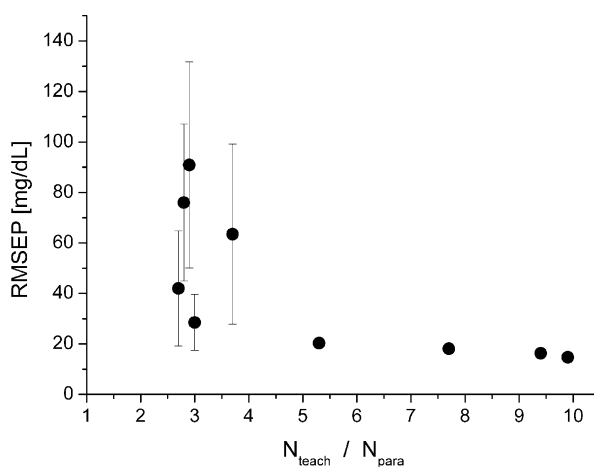


Figure 14.10. RMSEP as a function of the ratio between the number of teaching samples N_{teach} and the number of parameters N_{para} (in this case the number of latent variables LV) as derived from the data in Fig. 14.8.

coarse estimate only. It is interesting, however, that this coarse estimate appears to be valid for quantification problems as well as classification problems.

14.3.3 Results of the Analysis

Biomedical vibrational spectroscopy strongly depends on the interaction between various disciplines such as biology, medicine, chemistry, physics, or mathematics. This interaction requires interdisciplinary communication skills and preferentially also a common nomenclature. However, the particular terms in each field of expertise may neither be generally known nor easy to understand.

As far as the presentation of the results of analysis is concerned, we have experienced that it helps to focus the description of results onto the perspective of application – that is, the biological or medical terminology. For example, the variation of a quantity derived from the repetitive measurements of the same sample (set) is named precision in the quantitative analysis, while any mean deviation (bias) between the spectroscopic test result and the reference test is enumerated in terms of accuracy.

Similarly, in classification problems we tend to state the fraction of samples correctly identified as “positive” as sensitivity. Analogously, the specificity enumerates the fraction of samples correctly classified as “negative.” Confidence intervals should be given with these values. Sensitivity and specificity are, of course, interrelated depending on the threshold value, at which a sample is classified as “positive” or “negative.” For example, if elevated levels of a spectral feature are indicative for a certain disease, a low threshold value for the feature amplitude will lead to the inclusion of most or even all of the diseased people in the group “correctly assigned to positive.” In other words, the sensitivity will be very high. However, many of those people assigned to the positive group by spectroscopy will not truly suffer from the particular disease in this example of a low threshold value. This fact corresponds to a high rate of cases which are falsely classified as positive, and

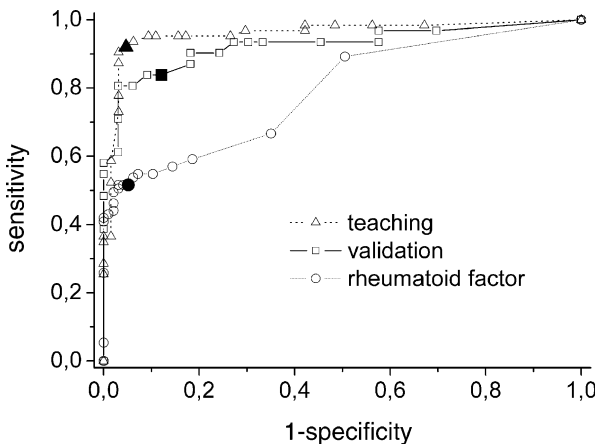


Figure 14.11. Receiver operator characteristics (ROC) for a study on the classification of serum samples originating from healthy volunteers and from patients suffering from rheumatoid arthritis. The ROC curve of the rheumatoid factor of the same study population is shown for comparison. The unfilled symbols represent the pairs of sensitivity and specificity taken at different threshold values. The unfilled symbols correspond to the threshold values of 0.5 for the DPR score and 14 IU/mL for the serum rheumatoid factor.

consequently it corresponds to a lack of those cases which are correctly assigned to “negative,” such that the specificity will be very low. Hence, sensitivity should not be stated without spelling out the specificity and vice versa. In medical diagnostics, these pairs of numbers are frequently plotted (x axis: sensitivity; y axis, 1-specificity) in forms of the receiver operator characteristics (ROC), a graph that originates from data transfer rates in electronic engineering. An example is given in Fig. 14.11 for the rheumatoid arthritis study.¹⁰ Ideally, one would wish to achieve 100% sensitivity and 100% specificity, which would correspond to the upper left corner of the ROC plot in Fig. 14.11. From the data it can be seen that the DPR method approaches this corner much more closely than the rheumatoid factor, which is known to provide only limited sensitivity. A threshold value of 0.5 for the DPR score leads to a sensitivity of 84% and a specificity of 88% for the validation set and to 93% and 95% for the teaching set, respectively. Setting the threshold value of the rheumatoid factor to 14 IU/mL results in a sensitivity of 53% and a specificity of 95% (closed symbols in Fig. 14.11). The rheumatoid factor provides a good example in which the lowering of the threshold concentration increases sensitivity – while at the same time, the specificity in fact starts to strongly deteriorate.

From a practical standpoint it is convenient, however, to combine the pairs of sensitivity and specificity to a single number, and it is the area under the ROC curve (AUC) which is therefore often used to quantify the quality of a medical marker.

14.4 CONCLUSION

Biomedical vibrational spectroscopy requires the interdisciplinary cooperation between biology, medicine, mathematics, biostatistics, computer sciences, physics, chemistry, and engineering. Given the diversity of disciplines on the one hand, it is not easy to satisfy the expectations of each of the disciplines. On the other hand, studies that consider biomedical relevance as well as spectroscopic technology and evaluation algorithms are most beneficial in advancing biomedical vibrational spectroscopy from the research labs to application. A common understanding of the “do’s and don’ts” has evolved during the last few years. This contribution is an attempt to summarize certain aspects of these joint efforts.

Ideally, a study starts by providing a thorough definition of the biological/medical query and the outline of an experiment, which explicitly aims at answering this particular question (study design). On the other hand, researchers in biomedical vibrational spectroscopy often find themselves in a situation in which access to a given number of samples may be obtained. These samples have been collected under a different study protocol with some given covariates and, possibly, even with a completely different goal. In this case, the many caveats of simply “running the samples through the spectroscopy” have to be considered carefully prior to any experiment. Consideration of biomedical relevance, appropriate reference methods, the minimum number of samples, confounding factors, and conceivable correlations are examples of these caveats.

During spectroscopy, it may turn out that the acquired spectra lack reproducibility – that is, the repeated measurement of the same sample yields variations at a scale equal to or larger than the expected variations associated with the issue under investigation. This fact is particularly relevant for *in vivo* measurements, in which the physiology of the skin, for example, may strongly differ from site to site and also depend on temperature and hydration. Hence, it is important to provide an independent measure of reproducibility.

Modern data analysis tools may deliver questionable results for very small data sets – that is in cases prone to overfitting. Therefore, methods and parameters of the data analysis

process have to be selected carefully, and consistency has to be checked. The use of a truly independent validation set has proven to be a meaningful tool for interrogating the outcome of any supervised analysis, such that an independent validation has become almost a prerequisite in biomedical vibrational spectroscopy.

Finally, it has to be kept in mind that the medical doctor and/or biologist will be the “customer” in most of the cases. Hence, findings need to be elucidated in the language of medicine and biology, such as sensitivity, specificity, or ROC curves.

Biomedical vibrational spectroscopy has tremendously advanced from research to application during the last few years. Despite this high pace, researchers from all fields of this interdisciplinary effort have created a common understanding of the prerequisites for this advancement. It is this joint effort which will enable biomedical vibrational spectroscopy to finally cover the entire path from academic research to commercialization.

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INTERPRETING SEVERAL TYPES OF MEASUREMENTS IN BIOSCIENCE

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15.1 INTRODUCTION TO THE ANALYSIS OF SEVERAL DATA SETS

Due to the rapid development of high-throughput instruments in biomedicine during recent years, a mismatch between the large amount of data produced and the capacity to analyze these data has arisen. Often single instruments are producing several hundreds up to several hundred thousands of variables of a given type for each sample. The rapid instrumentation development also allows collecting different types of variables from a given set of samples.

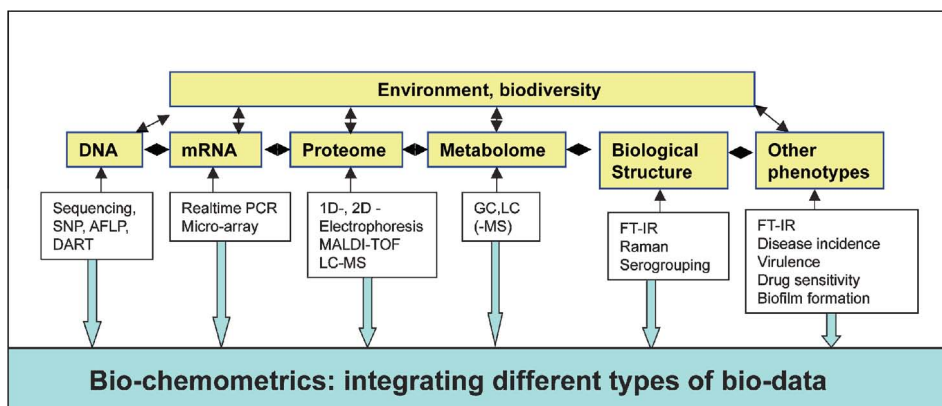


Figure 15.1. Illustration of how in systems biology data are obtained along the causal chain from genotype to phenotype. The obtained data are typical multiblock data; that is, different measurement principles are applied to the same N samples resulting in many multivariate data blocks.

This is illustrated in Fig. 15.1 for functional genomics: Biological systems can nowadays be studied by high-throughput instrumentation at all levels along the causal chain from genotype to phenotype. Examples for the different methodologies are as follows: (a) sequencing or single nucleotide polymorphism (SNP) technologies to study DNA diversity, (b) micro-array techniques to study gene expression, (c) mass-spectroscopic MALDI-TOF or electrophoresis to obtain proteome data, and (d) different biospectroscopic and chromatographic techniques to study the metabolome, other phenotype characteristics such as drug resistance, biological structure and so on, as well as available background information about the samples at hand.

The use of vibrational biospectroscopic techniques for these studies will gain in importance due to the ease of their use, their high specificity for latent phenotype information, and consequently their high potential for screening. When all these different levels of information (or at least some of them) are obtained for the same samples, covariations and interactions between the different stages along the causal chain can be studied, leading to in-depth insight into the underlying patterns of variation and mechanisms of control. In the future the amount of collected data will be so large that professional databases will be needed to deal with it. However, there is also a need for adequate data analysis tools to link the different data sets.

Since most biological systems are very complex, and since many modern measurement techniques yield multichannel data, multivariate data modeling is useful. This chapter outlines some relatively new multivariate methods that help the researcher to handle multiblock situations. A typical multiblock situation is illustrated in Fig. 15.2: Data from several blocks $b = 1, \dots, B$ (different measurement types and/or subsets of instrument channels from a given type) have been obtained from the same set of samples, $i = 1, \dots, N$. Within each block b a certain set of variables $k = 1, \dots, K_b$ has been measured, resulting in an $N \times K_b$ data table (matrix block) X^b , $b = 1, \dots, B$. All measurements are performed for the same N samples. By sorting the N samples in the same order in every block, the necessary row correspondence is achieved.

The present chapter focuses on one family of multivariate data modeling methods, based on so-called bilinear modeling. These provide data-driven (rather than theory-driven) mathematical descriptions of the main content of whole data tables. When the data from only one single block are to be screened, the one-block technique of principal component

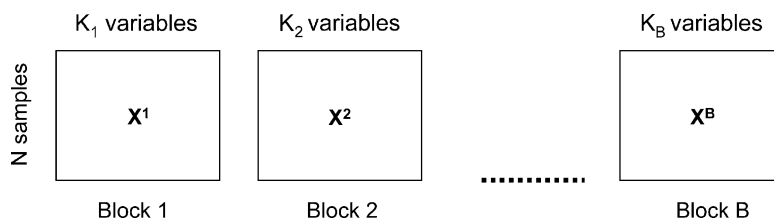


Figure 15.2. Illustration of a typical multiblock situation. Every block (square) is representing one data matrix, where rows are referring to samples and columns to variables. The number of rows N is the same for every data block, and the number of columns $K_1, \dots, K_B$ is in general different for every block.

analysis (PCA) is useful. For relating two different blocks to each other (e.g., for quantitative multivariate calibration), the technique of partial least squares regression (PLSR) is a versatile tool. When extended to the simultaneous analysis of several blocks $\mathbf{X}^b$, $b = 1, \dots, B$ as presented in Fig. 15.2, it is called a multiblock analysis. The scope of the multiblock analysis depends on the application, but in biospectroscopy the following general objectives may be stated: (a) to find the main underlying patterns of covariation that are common for several or all of the blocks, (b) to measure how strongly these covariant patterns are represented in each block and how they relate the blocks predictively to each other, and (c) to visualize how already known bands contribute to the covariance patterns.

The mathematical modeling philosophy here is “soft” – more or less purely data-driven: Contrary to more classical mechanistic mathematical modeling, a minimum of causal beliefs are imposed. Contrary to more classical statistical modeling, a minimum of distributional assumptions and independence requirements are imposed. The idea is to “let the data talk for themselves.”

But the user’s background knowledge is still important – in the planning of experiments, in the preprocessing of data, and in the interpretation of the results. Hence, while this chapter outlines some mathematical data modeling methods, the reader should also note the importance of nonmathematical aspects – in particular, graphical inspection of the input data – of intermediate steps and of the final output. Data-driven modeling can only work if the data are informative. Therefore, it is essential to plan the experiments well, so as to ensure sufficient variations among the samples, along with sufficient precision and relevance among the variables.¹

Before mathematical modeling, the raw input data should be inspected, at least superficially, for gross errors. During the mathematical modeling the statistical results should be inspected critically in light of background knowledge. After the modeling, the conclusions should be checked, for quality assurance of the data analytical process and for easy communication to others, by plotting those input variables that the modeling has shown to be most important.

Data preprocessing is an important mathematical aspect of data modeling that will not be treated much in this chapter. For example, measured transmission spectra T are usually filtered to reduce noise and are transformed into, for example, absorbance $= \log(1/T)$ to ensure linear response to chemical sample composition according to Beer’s Law. Moreover, it is often useful to distinguish physical effects from chemical effects during preprocessing – for example, separating multiplicative light scattering and sample thickness from additive baseline effects and chemical light absorbances. This can be attained, for example, by taking spectral derivatives, followed by so-called standard normal variate (SNV) filtering or the more model-based preprocessing, the extended multiplicative signal correction (EMSC).^{2–5}

The simplest multivariate analysis is to perform PCA on every single block X^b . This approach will only reveal structures within each block; it will not in general reveal common structures between the blocks. However, by visually comparing score plots for the separate PCA block models, the researcher may identify some common variation pattern visually. In order to develop systematically the logic and algorithms of the multiblock analysis and the tools for visualization, we will start in Section 15.2 by introducing the nonlinear iterative partial least-squares (NIPALS) algorithm for PCA. This is historically how the originally psychometric PCA method was introduced into chemometrics.⁶ Once this simple algorithm is understood – in terms of how the model elements (scores, loadings, correlation loadings and residuals) are obtained – it is easier to understand the plots that we will use extensively for finding correlated interpretable signals in the different blocks.

In Section 15.3 we will introduce the PLSR for the analysis of two data blocks. This will bridge to the multiblock method “multiblock principal component analysis” that will be introduced in Section 15.4. In Section 15.5 we will give a short overview over other multiblock approaches.

Sections 15.2–15.3 are organized in the following way: Every section starts by introducing the respective method. The process of data analysis and the NIPALS algorithm is then presented schematically in a frame, followed by an example illustrating this process. A reader who does not want to go into mathematical details may ignore the frames.

Example. An example from microbiology will be used for illustration: *Listeria monocytogenes* is a food-borne bacterium that may cause serious infections.⁷ Most outbreaks of *L. monocytogenes* have been associated with foods that are stored at low temperature and are consumed without heat treatment. Since the market for ready-to-eat products is increasing, this bacterium has been in focus during the recent years. It has been suggested to control the growth of *L. monocytogenes* in food by adding antimicrobial peptides called bacteriocins.^{8,9} Such peptides are produced by lactic acid bacteria and are generally recognized as safe to consume. The mechanism of action of bacteriocins is being investigated, and it is yet not fully understood for any of the different classes of bacteriocins. Studies point toward the presence of a specific molecule in the cell membrane of the target bacterium necessary for the bacteriocin to perform its activity (pore forming and dissipation of the proton motive force).^{10–12} Other influencing factors on the mechanism behind *L. monocytogenes* susceptibility toward bacteriocins seem to be membrane fluidity, cell wall density, and charge of membrane or cell wall.¹³ Knowledge of the mechanism of action is important to be able to elucidate and thereby prevent development of resistance toward such an agent.

In a previous study, 200 different *L. monocytogenes* strains were exposed to the bacteriocin sakacin P, and it was found that the susceptibility toward this bacteriocin varied between the strains (determined by using a bioassay with *Listeria ivanovii* as indicator and purified sakacin P as standard). Surprisingly, the strains formed two groups according to their susceptibility level, separated by a gap when sorted as shown in Fig. 15.3.¹⁴

Such a gap has not been reported for other bacteriocins, and the biochemical and genetic basis behind this grouping of *L. monocytogenes* is not known. Other studies – also related to properties of the cell wall/cell membrane, investigating zero-type and the ability to grow in the presence of an antibacterial agent, benzalkonium chloride (BC), and the bacteriocin nisin – have been performed and altogether have generated large data sets for these strains.

More generic analyses – as genetic fingerprinting [amplified fragment-length polymorphism (AFLP)], spectroscopic fingerprinting (FT-IR and RAMAN), and protein profiling (SDS-PAGE) – have also been performed.^{15–17} For AFLP and FT-IR analysis a subset of 88 strains was analyzed: The 20 strains closest to the sakacin susceptibility gap

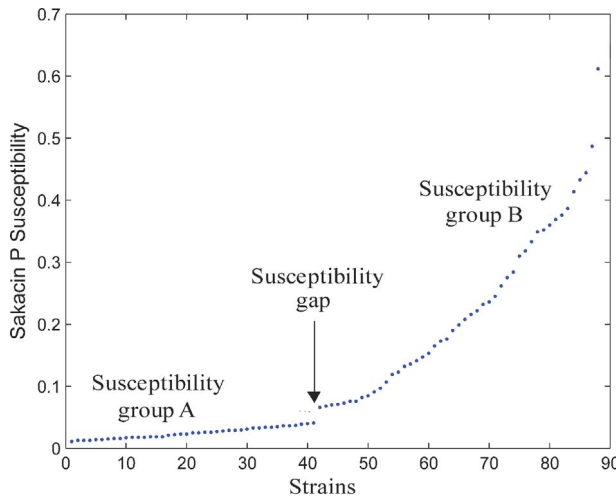


Figure 15.3. Susceptibility level of the 88 *Listeria monocytogenes* strains according to Ref. 14. The strains are sorted with respect to susceptibility.

(10 on each side) and 68 strains covering the whole susceptibility range were chosen (including the 20 most extreme samples with respect to susceptibility – that is, the 10 with lowest and the 10 with highest susceptibility). By the collected data three different types of data according to Fig. 15.1 are represented: “DNA” (AFLP), “Biological Structure” (FT-IR, Raman, SDS-PAGE, sero grouping) and “Other Phenotypes” [susceptibility to sakacin P, nisin, and the antibacterial agent benzalkonium chloride (BC)]. Integrating and exploiting of these data sets in combination will give further information about the background behind the *L. monocytogenes* strain to strain variation in general and especially the variation in susceptibility to bacteriocins. The latter may also lead to further understanding of the mechanism of action of bacteriocins. In this chapter, AFLP data (DNA), FT-IR data, and serotyping (biological structure) and susceptibility to sakacin P, nisin, and benzalkonium chloride (other phenotypes) will be used to illustrate the methods presented for the analysis of several data sets simultaneously.

15.2 PRINCIPAL COMPONENT ANALYSIS OF ONE DATA TABLE

By PCA the user can get an overview of the main patterns of covariation within a data table. PCA may be regarded as “the mother of all multivariate modeling methods,” because it is so simple and so powerful.

For illustration of PCA, consider an input data table consisting of N (e.g., 100) samples (rows, “objects,” e.g., bacterial strains), chosen as interesting and expected to be related in some way, and K (e.g., 2000) input variables (columns, “descriptors,” e.g., wavenumber channels), chosen as possibly informative for these samples, and scaled so that they are expected to have approximately the same noise level: The $N \times K$ data Table $\mathbf{X} = [\mathbf{x}_1, \mathbf{x}_2, \dots, \mathbf{x}_k, \dots, \mathbf{x}_K]$ with columns $\mathbf{x}_k = [x_{1,k}, x_{2,k}, \dots, x_{i,k}, \dots, x_{N,k}]'$, for example, $N = 100$ samples (rows) and $K = 2000$ input variables (descriptors, e.g., wavenumber channels) – is approximated by a mean profile $\bar{\mathbf{x}}$ (symbolized by the bar above $\mathbf{x}$) plus a mean-centered table of variations around this mean: $\mathbf{X} = \bar{\mathbf{x}} + \mathbf{X}_0$. Systematic patterns of covariations in $\mathbf{X}_0$ are now to be searched for in terms of so-called principal components (PCs). The process is

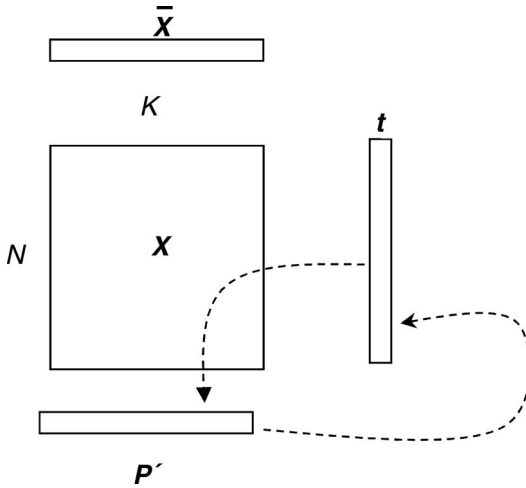


Figure 15.4. Illustration of the NIPALS algorithm (see Frame 1) and the relation between scores $\mathbf{t}$ and loadings $\mathbf{p}$ for PCA.

illustrated in Fig. 15.4; for simplicity, only one such PC is shown, along with arrows that outline how it is obtained from the data.

Each PC is represented by a column vector $\mathbf{t}$ and a row vector $\mathbf{p}'$. The score vector $\mathbf{t} = [t_1, t_2, \dots, t_N]'$ is a “supervariable” whose “concentrations” in the N samples is a linear combination (i.e., a weighted sum) of the mean-centered X -variables. The transposed loading vector $\mathbf{p}' = [p_1, p_2, \dots, p_K]$ characterizes this “supervariable” in terms of the K individual input variables $\mathbf{x}_1, \mathbf{x}_2, \dots, \mathbf{x}_K$. Loading vector $\mathbf{p}$ may be thought of as the average difference spectrum between those samples that have high levels $\mathbf{t}$ and those that have low levels $\mathbf{t}$. The vector pair $\mathbf{t}$ and $\mathbf{p}$ is found by a search process (see below). The outer vector product of the score and loading vector pair, $\mathbf{t}\mathbf{p}'$, defines the contribution of this PC to the modeling of data table $\mathbf{X}$. Hence, using only one PC, the input data $\mathbf{X}$ are approximated by the rank-one “bilinear model” $\mathbf{X} = \bar{\mathbf{x}} + \mathbf{t}\mathbf{p}'_1 + \mathbf{X}_{(1)}$, where $\mathbf{X}_{(1)}$ is the residual after one PC. If the data table $\mathbf{X}$ contains A different types of systematic variation patterns, these are picked up by a sequence of A PCs numbered $a = 1, 2, \dots, A$, each with less importance than the previous ones, plus the unmodeled residual:

$$\mathbf{X} = \bar{\mathbf{x}} + \mathbf{t}_1\mathbf{p}'_1 + \mathbf{t}_2\mathbf{p}'_2 + \dots + \mathbf{t}_a\mathbf{p}'_a + \dots + \mathbf{t}_A\mathbf{p}'_A + \mathbf{X}_{(A)}$$

Several equivalent algorithms exist for extracting the PCA scores and loadings. The NIPALS estimation algorithm is illustrated by the arrows in Fig. 15.4. “ $\mathbf{X}$ ” in the figure symbolizes the information in the $N \times K$ input data table that remains after extracting the $a - 1$ previous PCs, $\mathbf{X}_{(a-1)} = \mathbf{X} - \bar{\mathbf{x}} - \mathbf{t}_1\mathbf{p}'_1 - \mathbf{t}_2\mathbf{p}'_2 - \dots - \mathbf{t}_{a-1}\mathbf{p}'_{a-1}$. This is the variation available for finding $\mathbf{t}$ and $\mathbf{p}$ for the next PC, # a . Hence, for the first PC ($a = 1$), “ $\mathbf{X}$ ” represents the mean-centered variables “ $\mathbf{X}$ ” = $\mathbf{X}_{(0)} = \mathbf{X} - \bar{\mathbf{x}}$. The NIPALS search algorithm is started by choosing an arbitrary score vector $\mathbf{t}$ – for example, N random numbers, or (more preferably) the column in $\mathbf{X}$ with highest remaining variation. The K columns in data matrix $\mathbf{X}$ are projected on the score vector $\mathbf{t}$ in order to calculate the K elements in loading vector $\mathbf{p}$. In other words, from the approximation model for each input variable, $\mathbf{x}_k \approx \mathbf{t}p_k$, each column k in the matrix $\mathbf{X}$ is regressed “vertically” on $\mathbf{t}$ by least-squares regression to estimate the regression coefficient p_k , for $k = 1, 2, \dots, K$. To stabilize the iterative procedure, the loading vector $\mathbf{p}$ is normalized to have a sum of squares of 1. Then, from the approximation model for each input sample, $\mathbf{x}_i \approx t_i\mathbf{p}'$, each of the N rows in matrix $\mathbf{X}$ is conversely regressed “horizontally” on $\mathbf{p}'$ in order to obtain an updated estimate of the N

elements in score vector $\mathbf{t}$. This iterative search procedure is performed until $\mathbf{t}$ and $\mathbf{p}$ change no more.

It has been shown that the NIPALS algorithm for extracting the PCA component # a is equivalent to finding the main variation in $\mathbf{X}$ by diagonalizing the covariance matrix of “ $\mathbf{X}$ ” = $\mathbf{X}_{(a-1)}$. Consequently, the loading vector $\mathbf{p}$, found by the NIPALS algorithm of the matrix “ $\mathbf{X}$,” refers to the first principal component of the residual data matrix $\mathbf{X}_{(a-1)}$. In other words, the loading vector $\mathbf{p}$ defines the direction in the variable space that maximizes the variation of all objects of $\mathbf{X}$. The score vector $\mathbf{t}$ shows the realization of this PC in the individual samples. Once the first PC has been estimated, the effect of its parameters $\mathbf{t}_1 = \mathbf{t}$, $\mathbf{p}_1 = \mathbf{p}$ is subtracted: $\mathbf{X}_{(1)} = \mathbf{X}_{(0)} - \mathbf{t}_1 \mathbf{p}_1'$. The second principal component is then calculated by the NIPALS algorithm applied to the residual matrix “ $\mathbf{X}$ ” = $\mathbf{X}^{(1)}$, yielding $\mathbf{t}_2 = \mathbf{t}$, $\mathbf{p}_2 = \mathbf{p}$. From $\mathbf{X}_{(2)} = \mathbf{X}_{(1)} - \mathbf{t}_2 \mathbf{p}_2'$, the residual “ $\mathbf{X}$ ” = $\mathbf{X}_{(2)}$ is defined and the third PC is searched for, and so on.

The calculation of the residual by subtracting the effect of the consecutive components is called deflation. For PCA there is only one possibility to deflate (described above). For the analysis of two data blocks by partial least-squares regression or several data blocks by multiblock PCA, there are several possibilities for deflation. The deflation procedure is a very important step since it defines orthogonality properties of loadings and scores. In PCA both score vectors and loading vectors are orthogonal, leading to the very special situation that the components are independent in both variable space and sample space. Mathematically, each consecutive PCA component $\mathbf{t}_a$, $a = 1, 2, \dots, A$, is defined as the first eigenvector of the residual covariance between the variables in $\mathbf{X}$.

In the following, index $a = 1, 2, \dots, A$ represents PC #, just like $k = 1, 2, \dots, K$ represents input variable # and $i = 1, 2, \dots, N$ represents sample #. After A estimation and deflation steps, A principal components have been obtained. The score vectors for the A components can be summarized by the score matrix $\mathbf{T}_A = [\mathbf{t}_1, \mathbf{t}_2, \dots, \mathbf{t}_a, \dots, \mathbf{t}_A]$, and equivalently their loading vectors can be summarized by the matrix of loadings $\mathbf{P}_A = [\mathbf{p}_1, \mathbf{p}_2, \dots, \mathbf{p}_a, \dots, \mathbf{p}_A]$.

A versatile concept that will be used throughout this chapter for visualization of the main bilinear structures found (e.g., Fig. 15.5b) is the so-called correlation loading plot. In the correlation loading plot the correlations between variables and scores are plotted. More precisely, let $\mathbf{x}_k$ be a variable (k th column of $\mathbf{X}$) and $\mathbf{t}_m, \mathbf{t}_n$ the score vectors of two PCs, say $m = 1$ and $n = 2$. For the correlation plot, the correlation coefficients r_{km} and r_{kn} relating $\mathbf{x}_k$ to $\mathbf{t}_m$ and $\mathbf{t}_n$ are computed, and the variable $\mathbf{x}_k$ is represented as a point with coordinates $[r_{km}, r_{kn}]$. This is done for every variable $k = 1, 2, \dots, K$ in $\mathbf{X}$. But if there are many variables, the plot may become “crowded,” and only the most salient variables are named explicitly. An important point is that, due to the orthogonality between scores, the norm of the vector $[r_{km}, r_{kn}]$ is by necessity less or equal to 1. It is thus useful to draw, on the correlation plot, a circle of radius equal to 1, with the center at the origin of the graph. A position near the circle in this plot indicates that it is possible to exactly predict $\mathbf{x}_k$ from the scores $\mathbf{t}_m, \mathbf{t}_n$. On the other hand, a variable positioned close to the origin of the graph is not predictable from the studied pair of scores, and it plays almost no role in the “construction” of these scores. Moreover, if two variables $\mathbf{x}_k, \mathbf{x}_j$ are close together on the correlation plot for $\mathbf{t}_1, \mathbf{t}_2$, and also close to the perimeter of the circle, they are strongly positively correlated, while they are strongly negatively correlated if they are found at the opposite perimeter.

Example. The score plot of the principal component analysis of the FT-IR spectra of the set of 88 *Listeria monocytogenes* strains ($N = 88$) as described above and in Ref. [15] is

FRAME 1
Analysis of a Single Data Table by Principal Component Analysis (PCA)

The PCA Process

- Select a set of samples and a set of variables.
- Scale each variable in order to obtain approximately compatible variation ranges, and form data table $\mathbf{X}$ (rows = samples, columns = variables).
- Compute and subtract the mean sample: $\mathbf{X}_0 = \mathbf{X} - \bar{\mathbf{x}}$.
- Model data table $\mathbf{X}_0$ as sum of a few trustworthy principal components (PCs) $a = 1, 2, \dots, A$ plus residual after A PCs, $\mathbf{X}_{(A)}$:

$$\mathbf{X}_0 = \mathbf{t}_1 \mathbf{p}'_1 + \mathbf{t}_2 \mathbf{p}'_2 + \dots + \mathbf{t}_a \mathbf{p}'_a + \dots + \mathbf{t}_A \mathbf{p}'_A + \mathbf{X}_{(A)}$$

that is,

$$\mathbf{X} = \bar{\mathbf{x}} + \mathbf{T}_A \mathbf{P}'_A + \mathbf{X}_{(A)}$$

- First, compute a higher number of PCs (e.g., 10) than necessary. Then determine the optimal number of PCs to trust, A (e.g., 3), by cross-validation, noise assessments, and so on, and leave the remaining PCs $A + 1, A + 2, \dots$ as unmodeled residual $\mathbf{X}_{(A)}$. Study the A PCs graphically: Look for covariation patterns, clusters, and so on. Inspect residual $\mathbf{X}_{(A)}$ for possible outliers, and check the effect of reanalyzing without them.

The parameters $\mathbf{t}$ and $\mathbf{p}$ can be estimated for one PC at a time—for example, by the NIPALS algorithm. For component $\#a$, the NIPALS algorithm searches for a new PC to approximate the residuals after the previous $a - 1$ PCs, “ $\mathbf{X}$ ” = $\mathbf{X}_{(a-1)}$, by the following steps:

- Choose a start score $\mathbf{t}$.
- Iterate the following steps until convergence:
 - Loading vector $\mathbf{p} = \frac{\mathbf{X}'\mathbf{t}}{\mathbf{t}'\mathbf{t}}$.
 - Normalize $\mathbf{p}$ to length 1 by $\mathbf{p} = \frac{\mathbf{p}}{\sqrt{\mathbf{p}'\mathbf{p}}}$.
 - Score vector $\mathbf{t} = \mathbf{X}\mathbf{p}$, or equivalently, $\mathbf{t} = \frac{\mathbf{X}\mathbf{p}}{\mathbf{p}'\mathbf{p}}$.
 - Check convergence—that is, if $\mathbf{t}$ and $\mathbf{p}$ no longer change.

accounts for the second largest variation, and so on. The first two principal components were found to account for the most part of the variation between the 88 samples in this spectral region (62.5% and 21.4%, respectively; total 83.9%). Figure 15.5a shows their sample score configuration ($\mathbf{t}_1$ versus $\mathbf{t}_2$). The samples are found to show three distinct clusters, here named FT-IR groups 1 (red), 2 (blue), and 3 (green), respectively. The samples are labeled according to their sakacin-susceptibility and serotype: (A) and (B) mean the sample has a susceptibility value below or above the gap in Fig. 15.3, respectively; (0) and (1) mean the samples have serotype $1/2$ and 4, respectively. Fig. 15.3 shows that the susceptibility of these strains is widely spread over a whole susceptibility range. The three FT-IR groups separate more or less clearly with respect to serotype, and to some extent also with respect to sakacin sensitivity.

In Fig. 15.5b the corresponding correlation loading plot for these first two PCA components is shown. Only wavenumbers that were referring to band maximum or -minimum positions in the FT-IR spectrum loadings are plotted. Since band positions may shift slightly from sample to sample, there are in general several wavenumber positions that

are referring to the same chemical bond in Fig. 15.5b. The figure shows the correlation between variables and PCs. Variables that are close to one (outer circle) are well-explained by these first two PCs (near 100% variance explained). The inner circle corresponds to 50% explained variance. The two plots should be read together, with directions from the origin named like the handle directions of a watch: Horizontally along PC1, FT-IR group 3 in Fig. 15.5a is situated primarily at around “3 o’clock” (spread between 2 and 5 o’clock). Consequently, the corresponding X variables around “3 o’clock” in Fig. 15.5b (981, 982, ..., 813 cm^{-1}) are higher than average in FT-IR group 3, but lower than average in the other FT-IR groups. Conversely, X variables around “9 o’clock” in Fig. 15.5b (949, 798, ..., 828 cm^{-1}) are higher than average in group 1 and lower than average in group 3. At about “12 o’clock” the plots show that FT-IR group 2 has higher-than-average absorbance (at 1027, ...) and lower-than-average absorbances at the opposite perimeter, 879, 880, 881 882, 1100, 1101, 1102 cm^{-1} .

The example has shown that PCA can be used to explore a single data table for dominant latent structures, that is, systematic patterns of covariation – and to relate both samples (bacteria strains) and variables (FT-IR wavenumbers) to these latent structures. The visual inspection of the loading and score plot allowed us to discover unexpected clustering of samples and to interpret their differences in terms of the variables. However, the way that background information was brought in was only qualitative (letters A and B, numbers 1 and 0 in Fig. 15.5a; wavenumbers in Fig. 15.5b). In order to relate, for example, FT-IR spectra and different susceptibility values to each other quantitatively, a two-block method must be used.

15.3 SIMULTANEOUS ANALYSIS OF TWO DATA BLOCKS BY PARTIAL LEAST-SQUARES REGRESSION (PLSR)

For the analysis of relationships between two data blocks $\mathbf{X}$ and $\mathbf{Y}$, many different methods may be used. The partial least squares regression is one useful method. It owes its versatility to a combination of two aspects – bilinear approximation and linear regression: On one hand, just like PCA, it extracts a few (A) bilinear latent variables or PLS components (PCs) $\mathbf{T} = [\mathbf{t}_1, \mathbf{t}_2, \dots, \mathbf{t}_A]$ from the “regressor” matrix $\mathbf{X}$: $\mathbf{T} = f(\mathbf{X})$. These X -scores are used for modeling each variable in both $\mathbf{X}$ and $\mathbf{Y}$: $\mathbf{X} \approx \mathbf{T}_A \mathbf{P}'_A$, $\mathbf{Y} \approx \mathbf{T}_A \mathbf{Q}'_A$. On the other hand, just like traditional multiple linear regression (MLR), it employs a linear regression model with a predictive direction $\mathbf{Y} \approx \mathbf{X} \mathbf{B}_A$, but with regression parameters $\mathbf{B}_A$ estimated via the A -dimensional bilinear PLSR model. In analogy to the single-block PCA model, the two-block PLSR model with A components may be written $\mathbf{X} = \bar{\mathbf{x}} + \mathbf{T}_A \mathbf{p}'_A + \mathbf{X}'_{(A)}$, $\mathbf{Y} = \bar{\mathbf{y}} + \mathbf{T}_A \mathbf{q}'_A + \mathbf{Y}'_{(A)}$. In contrast to PCA of $\mathbf{X}$ alone, the MLR-like aspect of PLSR ensures that the PCs $\mathbf{T}_A = [\mathbf{t}_1, \mathbf{t}_2, \dots, \mathbf{t}_A]$ from “regressors” $\mathbf{X}$ are relevant also for “regressands” $\mathbf{Y}$. In contrast to traditional full-rank MLR, the low-rank PCA-like aspect of PLSR ensures that graphical overview is attained and that the rank- A regression model is statistically stabilized against measurement errors in block $\mathbf{X}$ and/or $\mathbf{Y}$, even for data sets with highly inter-correlated input variables such as biospectroscopy wavenumber channels. These aspects make PLSR useful for multivariate calibration.¹⁹ In the present multiblock setting, the way these two aspects are combined algorithmically makes PLSR particularly informative because it facilitates graphical overview of the reliable and relevant relationships in rather complex data sets. This will be demonstrated below.

The PLSR model parameters may be estimated in several different, but equivalent ways. In simple words, the PLS components find and reveal the main covariations within

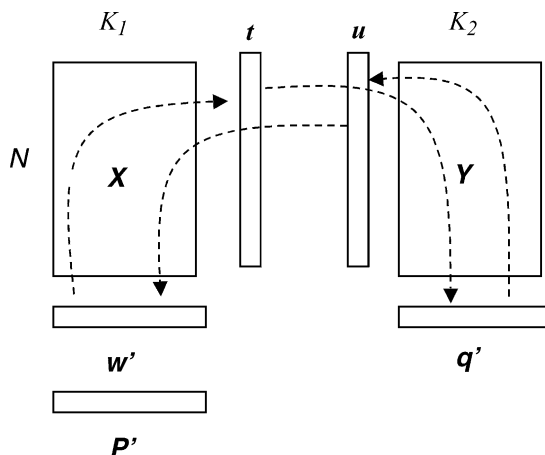


Figure 15.6. Illustration of the NIPALS algorithm (see Frame 2) and the relation between scores and loadings for PLSR.

and between blocks X and Y . Mathematically, each consecutive PLS component is defined by the first eigenvector of the residual covariance between X and Y .

The meaning of the PLSR model parameters may be easier to understand by seeing how the iterative NIPALS algorithm now works; this is outlined in Fig. 15.6 and described in Frame 2.

Just like in PCA, the NIPALS algorithm is employed to estimate the parameters (loadings and scores), for one PLSR PC at a time: The effect of an obtained PC is removed from the input data X and Y by deflation and the same process started again for the next PC. Each PC t_a , $a = 1, 2, \dots, A$ is obtained from block X like in PCA, but now using block Y for guidance. The NIPALS algorithm seeks to approximate the covariation that remains after removal of the $a - 1$ previous PCs, within and between the input matrices X and Y . Hence, input data for PC # a are the residual matrices after removal of the $a - 1$ previous PCs, $X_{(a-1)}$ and $Y_{(a-1)}$, named “ X ” and “ Y ” here, for simplicity: The search algorithm is started by choosing an arbitrary score vector u – for example, the column in Y with maximum remaining variation. Based on the auxiliary approximation model $X \approx uw'$, X is regressed columnwise on the Y -scores u in order to obtain an estimate of the X -loading weight vector w . Then, based on the approximation model $X \approx tw'$, X is projected row-wise on the X -loading weights w in order to calculate the X -scores t . This can be considered as one local “PCA” NIPALS step in X . However, since PLSR is a two-block method, the next NIPALS step is performed in the matrix Y : Based on the approximation model, $Y \approx tq'$, Y is regressed columnwise on the X -scores t in order to estimate the Y -loadings q . From the auxiliary model $Y \approx uq'$, Y is regressed on the Y -loadings q to update the estimate of the Y -scores u . As in PCA, this iterative NIPALS procedure of estimating is repeated until convergence, yielding X -loading weight vector w , X -score vector t , Y -loadings q , and auxiliary Y -scores u .

For a given PLSR PC, the NIPALS search algorithm in itself is completely symmetric in X and Y . Accordingly, at convergence the obtained result is completely symmetric: A “sample” score vector t and a corresponding “variable” vector w are obtained for X and a “sample” score vector u , and a corresponding “variable” vector q are obtained for Y . As in PCA, subsequent loading and score vectors are obtained by deflating the matrices X and Y with respect to the previous component. In PLSR there are two score vectors available for deflation, the score vector t of the matrix X and the score vector u of the matrix Y . In order to ensure that Y can be predicted from X , the

FRAME 2**Analysis of Two Data Tables by Partial Least-Squares Regression (PLSR)**

The PLSR Data Modeling Process

- Select a set of samples and two sets of variables: X variables and Y variables.
- Scale each variable to ensure compatible error levels within the X variables and within the Y variables, and form data tables $\mathbf{X}$ and $\mathbf{Y}$ (rows = samples, columns = variables).
- Compute and subtract the mean sample: $\mathbf{X}_{(0)} = \mathbf{X} - \bar{\mathbf{x}}$, $\mathbf{Y}_{(0)} = \mathbf{Y} - \bar{\mathbf{y}}$.
- Model data tables $\mathbf{X}_{(0)}$ and $\mathbf{Y}_{(0)}$ as sum of a few (A) trustworthy PLS components as weighted linear combinations of the X variables, plus residuals $\mathbf{X}_{(A)}$ and $\mathbf{Y}_{(A)}$. Determine A , the number of PCs to trust.
- The parameters may then be collected in matrices of X -loading weights $\mathbf{W}_A$, X -scores $\mathbf{T}_A$, and X and Y loadings $\mathbf{P}_A$ and $\mathbf{Q}_A$. The obtained model may be written in many equivalent ways; for example,

$$\begin{aligned}\mathbf{V}_A &= \mathbf{W}_A (\mathbf{P}'_A \mathbf{W}_A)^{-1} \\ \mathbf{T}_A &= (\mathbf{X} - \bar{\mathbf{x}}) \mathbf{V}_A \\ \mathbf{X} &= \bar{\mathbf{x}} + \mathbf{T}_A \mathbf{P}'_A + \mathbf{X}_{(A)} \\ \mathbf{Y} &= \bar{\mathbf{y}} + \mathbf{T}_A \mathbf{Q}'_A + \mathbf{Y}_{(A)}\end{aligned}$$

that is,

$$\mathbf{Y} = \mathbf{b}_{0,A} + \mathbf{X} \mathbf{B}_A + \mathbf{Y}_{(A)}$$

where

$$\mathbf{B}_A = \mathbf{V}_A \mathbf{Q}'_A \quad \text{and} \quad \mathbf{b}_{0,A} = \bar{\mathbf{y}} - \bar{\mathbf{x}} \mathbf{B}_A$$

- Study the obtained parameters and residuals graphically. Look for covariation patterns, clusters; reanalyze without outliers.

The parameters of each PC can be estimated from the residuals after the previous $a - 1$ PCs, $\mathbf{X} = \mathbf{X}_{(a-1)}$ and $\mathbf{Y} = \mathbf{Y}_{(a-1)}$, by the NIPALS algorithm, one PLS component at a time. The PLSR NIPALS algorithm for component # a consists of the following steps:

- Choose an arbitrary start score vector $\mathbf{u}$.
- $\mathbf{w} = \mathbf{X}' \mathbf{u} / \mathbf{u}' \mathbf{u}$.
- Normalize $\mathbf{w}$ to length one by $\tilde{\mathbf{w}} = \frac{\mathbf{w}}{\sqrt{\mathbf{w}' \mathbf{w}}}$.
- $\mathbf{t} = \mathbf{X} \tilde{\mathbf{w}}$.
- $\mathbf{q} = \mathbf{Y}' \mathbf{t} / \mathbf{t}' \mathbf{t}$.
- $\mathbf{u} = \mathbf{Y} \mathbf{q} / \mathbf{q}' \mathbf{q}$.
- Iterate until convergence.

Once converged, update $\mathbf{Y}$ by the deflation:

- $\mathbf{Y} = \mathbf{Y} - \mathbf{t} \mathbf{q}'$.

Project also $\mathbf{X}$ on $\mathbf{t}$, to find the x -loading vector $\mathbf{p}$, and update $\mathbf{X}$:

- $\mathbf{p} = \mathbf{X}' \mathbf{t} / \mathbf{t}' \mathbf{t}$.
- $\mathbf{X} = \mathbf{X} - \mathbf{t} \mathbf{p}'$.

Increment PC counter: $a = a + 1$, and repeat.

deflation is performed asymmetrically in PLSR: The X -scores are defined as weighted combinations of the residuals of the X variables: $\mathbf{t} = \mathbf{X}_{(a-1)}\mathbf{w}$. These X -scores are used for approximating both $\mathbf{X}$ and $\mathbf{Y}$: $\mathbf{Y}_{(a-1)} \approx \mathbf{t}\mathbf{q}'$, $\mathbf{X}_{(a-1)} \approx \mathbf{t}\mathbf{p}'$. The Y -loadings $\mathbf{q}$ were already estimated in the NIPALS process above. But the X -loadings $\mathbf{p}$ have to be estimated in a final step, based on the approximation model $\mathbf{X}_{(a-1)} \approx \mathbf{t}\mathbf{p}'$, by regression of each variable in $\mathbf{X}_{(a-1)}$ on $\mathbf{t}$.

The deflation is then performed as $\mathbf{X}_{(a)} = \mathbf{X}_{(a-1)} - \mathbf{t}\mathbf{p}'$ and $\mathbf{Y}_{(a)} = \mathbf{Y}_{(a-1)} - \mathbf{t}\mathbf{q}'$. Then, the next PC is obtained after incrementing the PC counter a , by the same NIPALS process. When sufficiently many PCs, $a = 1, 2, \dots, A$, have been estimated, their parameters are collected in matrices: $\mathbf{W}_A = [\mathbf{w}_1, \mathbf{w}_2, \dots, \mathbf{w}_A]$, $\mathbf{T}_A = [\mathbf{t}_1, \mathbf{t}_2, \dots, \mathbf{t}_A]$, $\mathbf{Q}_A = [\mathbf{q}_1, \mathbf{q}_2, \dots, \mathbf{q}_A]$, $\mathbf{P}_A = [\mathbf{p}_1, \mathbf{p}_2, \dots, \mathbf{p}_A]$. The approximation models with A PCs may thus be written $\mathbf{X} = \bar{\mathbf{x}} + \mathbf{T}_A\mathbf{P}_A + \mathbf{X}_{(A)}$, $\mathbf{Y} = \bar{\mathbf{y}} + \mathbf{T}_A\mathbf{Q}_A + \mathbf{Y}_{(A)}$. More details about this estimation process are given in Frame 2. It is shown that the A -dimensional bilinear model may be summarized by the equivalent linear regression model $\mathbf{Y} = \mathbf{b}_{0,A} + \mathbf{X}\mathbf{B}_A + \mathbf{Y}_{(A)}$. Thus, in samples with known values of X variables, the Y variables can be predicted – not only for the N “calibration samples” initially available for estimating the model parameters $\mathbf{b}_{0,A}$ and $\mathbf{B}_A$, but also later on, for new samples of the same general kind.

An important aspect of data-driven modeling like PCA and PLSR is to find the optimal number of PCs to trust, A . If too few PCs are used, information is lost, because important structures in the data are left unmodeled and instead treated as residual “noise.” This is called “underfitting” or “oversimplification”. If too many PCs are used, compared to the reliable information content of the data, the results become statistically unstable and difficult to interpret, because incidental errors are given the same attention as real effects. This is called “overfitting” or “overparameterization.” The optimal number of PCs of course depends on the number of patterns, known or unknown – desired or undesired – that vary independently in the system being studied. But, more unexpectedly perhaps, it also depends on the quality and quantity of data available: With precise, high-resolution observations from lots of samples with different patterns of covariation, all the patterns might be picked up by the data-driven modeling. But with imprecise observations from only a few samples that only vary in a few ways, only a few of the true, underlying patterns of co-variation can be picked up by the modeling process. Therefore, like all data-driven modeling, PLSR calls for aggressive experimental planning and humble model interpretation.

In PLSR it is relatively easy to determine the optimal number of PCs, using a resampling procedure such as cross-validation: The modeling process is repeated over and over again, each time keeping part of the sample set as a “secret” test set. The prediction errors $\mathbf{Y}_{(A)} = \mathbf{Y} - (\mathbf{b}_{0,A} + \mathbf{X}\mathbf{B}_A)$ in the “secret test samples” decrease until the optimal number of components, $A = A_{\text{Opt}}$, is reached, and then increase again upon overfitting.

The precision of the obtained model parameters, e.g., $\mathbf{B}_{A\text{Opt}}$, may be assessed by the related methods of jack-knifing or boot-strapping: Small elements in $\mathbf{B}_{A\text{Opt}}$ that vary strongly when re-estimated with some samples kept “secret” are deemed unreliable (“not significantly different from zero”).²⁰

Although PLSR is best known for its ability to establish stable predictive models, which is due to the fact that often only few latent variables are needed for obtaining good predictions, it has been used quite extensively during the recent years in biospectroscopy to study underlying covariance structures in two data sets. It has been used to study covariance structures in mRNA microarray data and FT-IR data of *Campylobacter jejuni* for the investigation of stress responses^{21,22} and to study the relation of protein denaturation and water mobility in meat by relating FT-IR microspectroscopic and low field ^1H NMR

Cross-validation showed that primarily the first two PLS PCs contributed with predictive ability for the Y variables. Hence, these $A = 2$ first PCs are presented here. The X -scores $\mathbf{t}$ are shown for the first and the second PLS component in Fig. 15.7a, with symbols and color coding as in Fig. 15.5a. The score pattern in Fig. 15.7a is similar to the PCA score pattern in Fig. 15.5a, but with some variations. In Fig. 15.7b the corresponding PLSR correlation loading plot is shown: The correlations of both the X and Y -variables with the first and second PLSR score vector, $\mathbf{t}_1$ and $\mathbf{t}_2$, are plotted along the abscissa and ordinate, respectively. The interpretation of both the score plot and the loading plot is done just as explained for PCA. FT-IR group 1 is seen to be strongly positively correlated to serotype 4 and, for example, 949 cm^{-1} at about “8 o’clock.” FT-IR group 2 at “11 o’clock” is strongly correlated with 1027 cm^{-1} and strongly anticorrelated with, for example, 881, 882, 883, and $1100, 1101, 1102\text{ cm}^{-1}$. Sakacin P-group B is somewhat associated with FT-IR group 3 (about 4 o’clock), which is seen to have higher-than-average absorbance at, for example, $980, 981, 982\text{ cm}^{-1}$ and $934, 935\text{ cm}^{-1}$. The bands at 835 and 980 cm^{-1} can be attributed to pyranose rings,²⁶ and Ref. [15] suggested that the variation in susceptibility toward sakacin P is connected to variations in the cell wall, probably to variations in pyranose.

15.4 SIMULTANEOUS ANALYSIS OF SEVERAL DATA BLOCKS BY MULTIBLOCK PCA

As an example for multiblock methods, we would like to discuss multiblock principal component analysis (MPCA), which is a very intuitive extension of PCA toward the analysis of many data sets. MPCA is actually a family of methods, all concerned with simultaneous modeling of several data matrices, but differing with respect to properties of the latent variables. The version discussed in this chapter appears to be especially useful for the treatment of multiblock data including spectroscopy data^{27,28}: The obtained block loadings (loadings referring to the K_b variables in each block matrix $\mathbf{X}^b$, $b = 1, 2, \dots, B$) are independent. Thus, the biochemical interpretation of every block loading will be independent from other loadings referring to the same block. As in PCA and in PLSR, MPCA extracts latent variables (block components, defined by block scores $\mathbf{T}_A^b = [\mathbf{t}_1^b, \mathbf{t}_2^b, \dots, \mathbf{t}_A^b]$ and block loadings $\mathbf{P}_A^b = [\mathbf{p}_1^b, \mathbf{p}_2^b, \dots, \mathbf{p}_A^b]$) for A PCs in matrix $b = 1, 2, \dots, B$. These may be used for bilinear modeling of each variable in each of matrix: $\mathbf{X}^b \approx \bar{\mathbf{x}} + \mathbf{T}_A^b \mathbf{P}_A^b + \mathbf{X}_{(A)}^b$, where the $\mathbf{X}_{(A)}^b$ are the block’s residuals after A PCs. In addition to the block components, also global latent variables $\mathbf{t}_T$ are calculated for each PC to reflect a consensus between all blocks $\mathbf{X}^b$. MPCA has been introduced by Ref. [29], and the corresponding NIPALS algorithm for CPCA is illustrated in Fig. 15.8.

As in PCA and PLSR, loadings and scores are estimated by a version of the NIPALS algorithm. The effect of every estimated component is then subtracted by a deflation step. The NIPALS algorithm is again applied on the deflated matrix, and so on.

The NIPALS algorithm for a given PC is started by choosing an arbitrary score vector, this time an arbitrary estimate of the so-called global score vector $\mathbf{t}_T$ – that is, a score vector of sample properties common to all blocks $b = 1, 2, \dots, B$. From $\mathbf{t}_T$ we obtain block loading vectors $\mathbf{p}^b$ by regressing every block $\mathbf{X}^b$ columnwise on $\mathbf{t}_T$ in order to estimate the block loading vectors $\mathbf{p}^b$. By projecting every block $\mathbf{X}^b$ row-wise onto its own loading, $\mathbf{p}^b$, the set of B block score vectors $\mathbf{t}^b$ are then obtained and collected in the matrix

$$\mathbf{D} = \begin{bmatrix} \mathbf{t}^1 & \mathbf{t}^2 & \dots & \mathbf{t}^B \end{bmatrix}$$

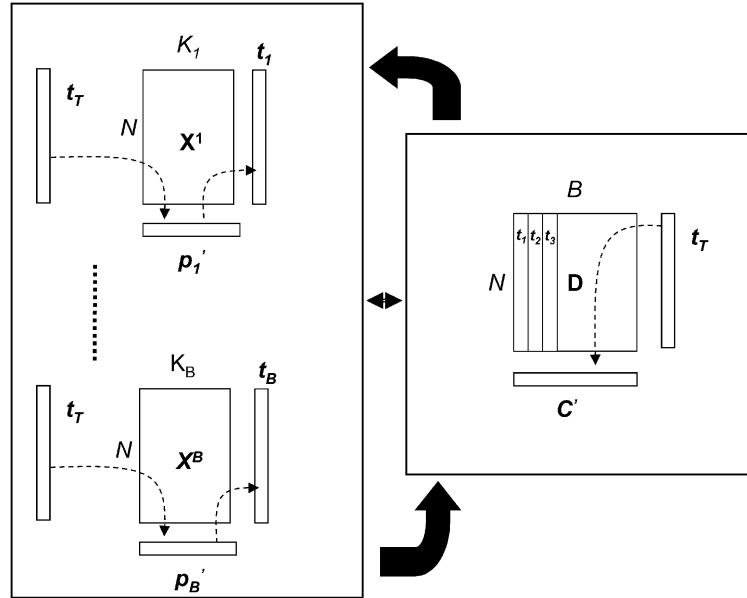


Figure 15.8. Illustration of the NIPALS algorithm (see Frame 3) and the relation between block scores, block loadings, and global scores for MPCA.

Based on the “inner” model $\mathbf{D} \approx \mathbf{t}_T \mathbf{c}_T'$, $\mathbf{D}$ is projected onto $\mathbf{t}_T$ in order to obtain the loading weights $\mathbf{c}_T$. Subsequently, $\mathbf{D}'$ is projected onto $\mathbf{c}_T$, and the next estimate for the global score vector $\mathbf{t}_T$ is obtained. Now the block loadings $\mathbf{p}^b$ can be updated, etc. This is repeated until convergence.

In Frame 3 and in Fig. 15.8, it can be seen that the algorithm can be divided into a part where block properties are calculated (Part II) and a part where global properties are considered (Part III).

In MPCA there are several possibilities for deflating the data blocks $\mathbf{X}^b$ – that is, for subtracting components between two NIPALS runs. The most common procedure is to deflate with respect to the variation expressed by the global scores $\mathbf{t}_T^{29,30}$:

$$\mathbf{X}_a^b = \mathbf{X}_{a-1}^b - \mathbf{t}_T \mathbf{p}_a^{b'}$$

The advantage of this is that the subtracted variation with respect to the common variation is expressed by the global scores $\mathbf{t}_T$ for this PC. The A global scores are collected in matrix $\mathbf{T}_T = [\mathbf{t}_{1,T}, \mathbf{t}_{2,T}, \dots, \mathbf{t}_{a,T}, \dots, \mathbf{t}_{A,T}]$. This deflation procedure also allows reconstructing the global matrix $\mathbf{X} = [\mathbf{X}^1, \mathbf{X}^2, \dots, \mathbf{X}^B]$ by $\mathbf{X} = \mathbf{T}_T \mathbf{P}' + \mathbf{E}$, where $\mathbf{P}$ the matrix of concatenated block loadings is $\mathbf{P}' = [\mathbf{P}_A^1, \mathbf{P}_A^2, \dots, \mathbf{P}_A^B, \dots, \mathbf{P}_A^B]$, where $\mathbf{p}_k$ are the global loadings obtained by concatenating the block loadings in the same blockorder as $\mathbf{X} = [\mathbf{X}^1, \mathbf{X}^2, \dots, \mathbf{X}^B]$. In this chapter, another possible way for deflation is considered, the deflation on normalized block loadings:

$$\mathbf{X}_{(1)}^b = \mathbf{X}^b - \mathbf{t}_1^b \tilde{\mathbf{p}}_1^{b'}$$

where $\tilde{\mathbf{p}}_1^b$ is a copy of the block loading, scaled to length 1. This deflation procedure entails a very specific property, namely that the obtained block loadings $\mathbf{p}_b$ are orthogonal. This ensures that there is no correlation between the loadings of different PCs and that different components therefore are independent in interpretative information. After the calculation of

FRAME 3**Analysis of Several Single Data Tables by Multiblock Principal Component Analysis (MPCA)****The MPCA Process**

- Select a set of samples and several sets of variables for this sample set (rows = samples, columns = variables).
- Scale the variables to comparable noise levels, and form data tables $\mathbf{X}^b$, $b = 1, 2, \dots, B$.
- Order the sample in every block $\mathbf{X}^b$ (variable set) in the same way (row-to-row correspondence between blocks).
- Compute and subtract the mean sample: $\mathbf{X}^{b,0} = \mathbf{X}^b - \bar{\mathbf{x}}^b$ for every block.
- Scale the blocks $\mathbf{X}^b$ in order to give the blocks comparable weights (e.g., many variables: lower block weight; particularly important variables: higher block weight).
- Model the total data table $\mathbf{X} = [\mathbf{X}^1, \mathbf{X}^2 \dots \mathbf{X}^B]$ as sum of a few trustworthy principal components $a = 1, 2, \dots, A$ plus residual:

$$\mathbf{X} = \bar{\mathbf{x}} + \left[\mathbf{T}_A^1 \tilde{\mathbf{P}}_A^{1'}, \mathbf{T}_A^2 \tilde{\mathbf{P}}_A^{2'}, \dots, \mathbf{T}_A^b \tilde{\mathbf{P}}_A^{b'}, \dots, \mathbf{T}_A^B \tilde{\mathbf{P}}_A^{B'} \right] + \mathbf{X}_{(A)}$$

where

$$\mathbf{T}_A^b = (\mathbf{X}^b - \bar{\mathbf{x}}^b) \tilde{\mathbf{P}}_A^{b'}$$

Determination of optimal number of PCs, outlier detection, and so on, can be performed as in PCA.

The parameters can be estimated, for example, by the NIPALS algorithm, one component at a time. Given residuals after the previous $a - 1$ PCs, the NIPALS algorithm for component # a consists of the following steps:

Part I. Initialization

- Choose an arbitrary start global score vector $\mathbf{t}_T$.

Part II. Computation of block scores and block loadings for each block $b = 1, 2, \dots, B$:

- $\mathbf{p}^b = \frac{\mathbf{X}^b \mathbf{t}_T}{\mathbf{t}_T' \mathbf{t}_T}$.
- Normalize $\mathbf{p}^b$ to length 1 by $\tilde{\mathbf{p}}^b = \frac{\mathbf{p}^b}{\sqrt{\mathbf{p}^{b'} \mathbf{p}^b}}$.
- $\mathbf{t}^b = \mathbf{X}^b \tilde{\mathbf{p}}^b$.

Part III. Computation of global scores and global loadings

- $\mathbf{D} = [\mathbf{t}^1 \quad \mathbf{t}^2 \quad \dots \quad \mathbf{t}^B]$.
- $\mathbf{c}_T = \frac{\mathbf{D}' \mathbf{t}_T}{\mathbf{t}_T' \mathbf{t}_T}$.
- Normalize $\mathbf{c}_T$ to length 1 by $\tilde{\mathbf{c}}_T = \frac{\mathbf{c}_T}{\sqrt{\mathbf{c}_T' \mathbf{c}_T}}$.
- $\mathbf{t}_T = \mathbf{D} \tilde{\mathbf{c}}_T$.
- Repeat until convergence.

A components (and thus after A deflation steps), we obtain therefore the linear model of the global matrix $\mathbf{X}$:

$$\mathbf{X} = \bar{\mathbf{x}} + \left[\mathbf{T}_A^1 \tilde{\mathbf{P}}_A^{1'}, \mathbf{T}_A^2 \tilde{\mathbf{P}}_A^{2'}, \dots, \mathbf{T}_A^b \tilde{\mathbf{P}}_A^{b'}, \dots, \mathbf{T}_A^B \tilde{\mathbf{P}}_A^{B'} \right]' + \mathbf{X}_{(A)}$$

where $\mathbf{X} = [\mathbf{X}^1, \mathbf{X}^2, \dots, \mathbf{X}^b, \dots, \mathbf{X}^B]$ is the total data matrix consisting of all data blocks concatenated, the matrices $\mathbf{T}_A^b$ contain A block score vectors, and the matrices $\tilde{\mathbf{P}}_A^b$ contain A normalized block loading vectors for block $b = 1 \dots B$, while vector $\bar{\mathbf{x}}$ represents the mean of each variable, and $\mathbf{X}_{(A)}$ represents the residuals. The advantage of this model is that it provides more detail on the sample configurations within individual blocks, while all block models still relate to the same latent structures (since the block loadings are the same as the global loadings, except for a trivial rescaling to length 1).

Example: For the MPCA illustration, three different ranges of the FT-IR spectra (720–1200 cm⁻¹, fingerprint region and polysaccharide region; 1500–1700 cm⁻¹, protein region; 2800–3000 cm⁻¹, fatty acid regions) were used in order to form three different blocks (preprocessing of FT-IR data was performed as before). In addition, AFLP data and phenotype data were used as two further block matrices. MPCA was performed on the obtained five blocks. The phenotype data block was “passified” (scaled down by a very small number after block standardization) in order to avoid that the phenotype data influence the MPCA model. The advantage of this is that, although the phenotype is not contributing to the model, the phenotype variation can be compared to the remaining four blocks, in its block score space and in the scale-free common correlation loading plot. The AFLP data showed saturation effects in some variable regions. A region without saturation containing 1701 variables was used for the data analysis.

In Fig. 15.9a the score plots (first and second component) for the five different blocks and for the global scores are shown. The symbols, numbers, and color coding are as in Fig. 15.5a. It can be seen that the AFLP data reveal a grouping of the samples/variation pattern similar to that of the FT-IR data in the spectral range 720–1200 cm⁻¹: The AFLP data show three different groups that are clearly separated with respect to serotype and sakacin P susceptibility. In the FT-IR, again three groups are visible (color-coded as FT-IR groups 1, 2, and 3), which are defined by serotype and to some extent by sakacin P susceptibility. The AFLP data and the FT-IR data (720–1200 cm⁻¹) show similar variation patterns, although some of the A and B samples have different FT-IR (720–1200 cm⁻¹) group affiliation. The other spectral regions show some tendencies with respect to sakacin susceptibility, but the separation according to group affiliation (sakacin P group, serotype) is by far not clear. The phenotype block, although down-scaled, shows distinct groups in the score plot, similar to those of FT-IR (720–1200 cm⁻¹). The global scores, combining information from AFLP and the three FT-IR regions (but not the phenotype block), show some grouping, but not nearly as clearly as the AFLP, FT-IR (720–1200 cm⁻¹), and the phenotype blocks.

In Fig. 15.9b the correlation loading plot is shown, revealing relations between three of the data blocks used for MPCA (black: FT-IR range 720–1200 cm⁻¹; green: AFLP data; and blue: reference data) and the global scores $\mathbf{T}$. In general, variables from all data blocks can be shown in the correlation loading plot. For the sake of clarity, only a selection of FT-IR variables is plotted in Fig. 15.9b. It can be seen that some AFLP variables have high correlations with sakacin P susceptibility and FT-IR groups, while others have high correlations with serotype 4. Interpretation of correlation between FT-IR variables and phenotypes are as in the previous sections.

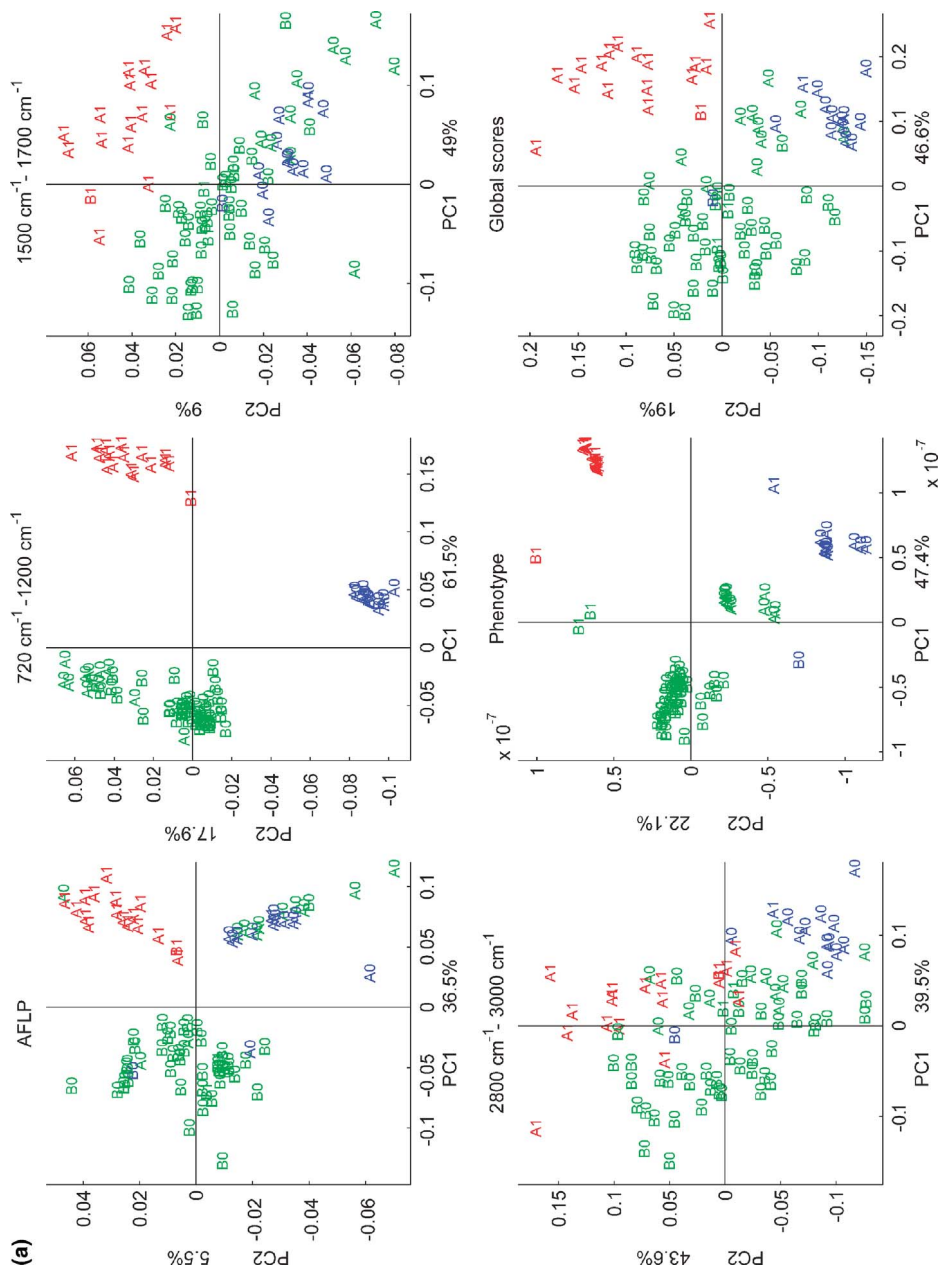


Figure 15.9. Multiblock principal component analysis using AFLP data, different spectral FT-IR regions, and phenotype data as blocks. (a) Score plots. (b) Correlation loading plot. Color coding is according to Fig. 15.3.

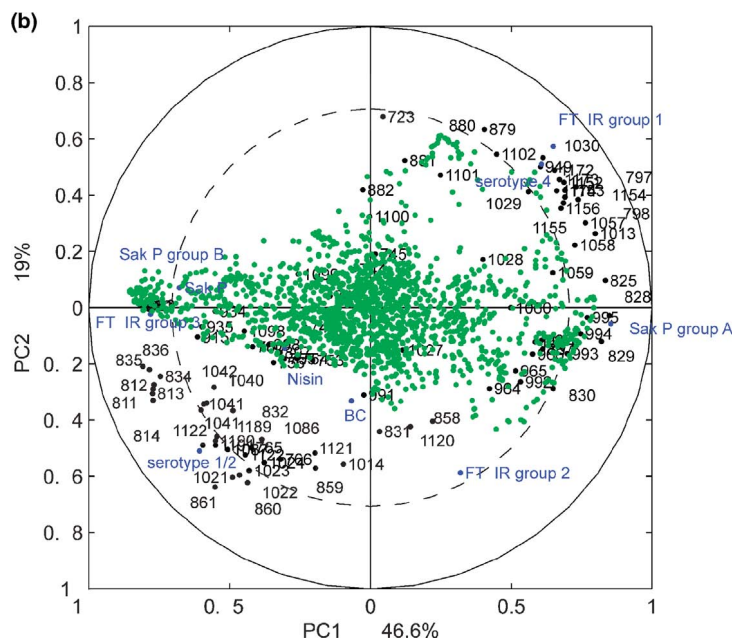


Figure 15.9. (Continued)

A general difference between using PCA on every single block and using a multiblock approach is that a multiblock approach is always seeking a “consensus” in the sense that the same variation is appearing in the same components in every block. This makes it possible to compare score results (and other results) directly between the blocks. An important measure for how strong covariation is represented in every block is the explained variance of the block scores. This is very apparent in the block score plot for the FT-IR region from 2800–3000 cm^{-1} : The second component has a much higher explained variance (43.6%) than the first component (39.5%). A PCA of this block is changing the order of the axes. The variation with respect to sakacin P and FT-IR group is dominating the “consensus” variation (global scores).

Another comment concerns a difference between MPCA and PLSR as discussed in the previous section: While the multiblock model is influenced equally strong by all not “passified” blocks, in PLSR often the X -matrix dominates.

15.5 ALTERNATIVE MULTIBLOCK METHODS

Multiblock data analysis has been in focus of intensive research during the last three decades, and many different methods have been discussed in the literature. As a consequence, the reader may be at a loss to decide which method is best suited for a problem. The reader should also be aware of the fact that several methods that are intrinsically similar can be found in the literature under different names or different acronyms. There are also methods of analysis which basically consist of minor variations of standard methods aimed at speeding the computation time or better adapting to a particular domain of interest.

Just like for one- or two-block data analysis, the multiblock methods are usually either of the clustering type, which search for groups of samples that resemble each other, or of the contrasting type (regression or subspace methods – for example, those discussed in this chapter: PCA, PLSR, MPCA), which search for patterns of systematic differences between samples. The multiblock contrasting methods may be divided into three different groups: (1) methods that maximize the correlation between block scores, (2) methods that maximize a common variation pattern, and (3) predictive methods that establish causal models between blocks. In the following we discuss a few examples for each of these three groups:

1. Canonical correlation analysis (CCA) is a method that maximizes the correlation between two blocks of variables and was initially introduced by Hotelling^{31,32} into psychometrics. CCA maximizes correlations between block scores and has found many applications in social sciences. Several generalizations of canonical correlation analysis have been proposed for handling situations with more than two blocks of variables; see Refs. 33 and 34 for an overview. Maximizing correlations between blocks scores has certainly disadvantages for biospectroscopic data, where the number of variables is often much higher than the number of samples in every block: Spurious correlations may be found that are not represented very strongly by common underlying variation patterns.
2. MPCA is a method that maximizes a common variation pattern, as we have seen above. Many closely related methods are discussed in literature: In Generalized Procrustes Analysis (GPA),³⁵ different variation patterns in the different blocks are matched by rotation and scaling. The common structure of the blocks is then investigated by a PCA of the rotated and scaled variation patterns. STATIS³⁶ is an alternative method where different variation patterns are matched by rotations and scaling as in GPA, but the common variation pattern is calculated in a different way. INDSCAL³⁷ and Common Components and Specific Weights Analysis (CCSA)³⁸ weaken the constraint of using one scaling for each block to different scaling factors (weights) for every dimension and block.
3. PLS methods allow the scientist to impose structural relationships between blocks. An example of structural models was already discussed in Section 15.3 (PLSR), where a data matrix $\mathbf{Y}$ was predicted from another data matrix $\mathbf{X}$ via PCs $\mathbf{T}$. There are many extensions of PLSR – for example, multiblock PLSR, where several blocks are used as $\mathbf{X} = [\mathbf{X}^1, \mathbf{X}^2, \dots, \mathbf{X}^B]$ to predict a data matrix $\mathbf{Y}$.^{30,39} Another important subgroup of methods for establishing structural equation models are the PLS path models.^{40–42} They allow the construction of “predictive networks” between data blocks, where, for example, “feedforward” and “feedback” mechanisms as currently present in systems biology can be built in.

As a conclusion, we can state that most of the methods outlined herein aim at unveiling the hidden patterns and relationships between and within blocks. They also provide important visualizations tools to achieve this purpose. Current research is focused on setting up a general overview of the various methods in order to assess how they relate to each other. Another important issue concerns the validation of the models. As discussed in the context of PLSR, the validation encompasses the assessment of the stability of the model and the uncertainty about the estimated parameters. Validation also consists in setting up a hypothesis testing framework in order to assess the relevance of the patterns revealed by the strategy of analysis.

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INTERPLAY OF UNIVARIATE AND MULTIVARIATE ANALYSIS IN VIBRATIONAL MICROSCOPIC IMAGING OF MINERALIZED TISSUE AND SKIN

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16.1 INTRODUCTION

The rapidly developing technologies of Raman and infrared (IR) microspectroscopic imaging provide spatially resolved molecular structure information from tissues. Reviews have recently appeared (*Biochim. Biophys. Acta*, July 2006, Vol. 1758 and *Anal. Bioanal. Chem.* March 2007, Vol. 387). Unlike other optical imaging approaches, which provide at most limited spectral information from each pixel, vibrational spectroscopy-based imaging provides vast amounts of spectral data, since a full vibrational spectrum is obtained from each pixel. In typical applications from this laboratory, 10,000–15,000 complete mid-IR spectra or several hundred Raman spectra are routinely acquired from skin or bone samples and processed to produce meaningful images. Analysis of such quantities of data presents several formidable challenges:

1. How can we efficiently extract information from tissues using methods that permit the rapid characterization of pathological conditions or other medically useful diagnostics?
2. How can we evaluate the limits of these technologies? For example, is it possible to diagnose disease states at an earlier stage than can currently be seen by pathologists? If so, how can a rational choice for patient sampling be initiated (since presumably there are only limited or no symptoms manifest)?

3. How can we demonstrate the advantages and novel possible applications of these technologies compared with other methods (imaging or otherwise, currently in use)?
4. How can we disseminate this technology in the most convenient, straightforward, and rapid way to the medical community?
5. How we can standardize and compare information acquired from different laboratories?

Practitioners in this field fully recognize these challenges, and it is noted that other imaging technologies, especially those based on NMR, have successfully responded to issues similar to those posed above. The current volume depicts the wide variety of multivariate approaches currently in use to analyze IR or Raman imaging data to address the first and second issues. The fourth and fifth issues are just beginning to be considered, with no concerted plan of attack within the field.

The current chapter is intended to address the third issue noted above. In doing so, we provide a counterpoint to other articles in this volume. In our opinion, a major advantage of vibrational microscopic imaging is the molecular structure information inherent in the original data. If the construction of images is the only goal of vibrational imaging experiments, then the utility of the vibrational microspectroscopy approach is limited, since the images are generated more slowly and are of poorer quality than those acquired from, for example, fluorescence spectroscopy.

IR images are generally restricted to two dimensions with a spatial resolution of about $10\text{ }\mu\text{m}$, since spectra are acquired in the transmission or “transflection” (through a “low-E” slide) modes without the possibility of acquiring axially resolved spatial information. A major advantage of Raman imaging is the ability of the method to generate confocal images from intact tissues. However, the weakness of the signals generally precludes the routine generation of 3D images, because the time required for data collection is too long. Consequently, Raman images reported to date are mostly confined to lines or planes. In addition, the $\sim 1\text{ }\mu\text{m}$ spatial resolution achievable in Raman experiments is significantly worse than fluorescence-based approaches ($\sim 0.2\text{ }\mu\text{m}$) and may in practice be further degraded due to optical effects along the lines discussed by Overall.^{1,2} The magnitude of resolution degradation is difficult to ascertain in intact tissue such as skin, which possesses variable refractive index discontinuities.^{3,4}

It thus seems obvious that a major reason for acquiring IR and Raman images is the ability of the spectral information inherent in the data to provide a unique molecular structure-based characterization of the tissue. The goal of the current chapter is to demonstrate how spatially resolved vibrational spectra coupled with the availability of spectra–structure correlations, may lead to useful medical, biochemical, and pharmacological information. The applications chosen are mainly from the imaging of skin and bone as performed at Rutgers University, with the ongoing collaboration of the Hospital for Special Surgery in New York City. Our approach emphasizes the interplay between univariate analysis (most useful for examination of molecular structure changes in tissue) and multivariate techniques (factor analysis) for image generation. The applications include IR-based molecular characterization of a relatively uncommon form of osteoporosis,⁵ confocal Raman measurements of the permeation and biochemical transformation of exogenous molecules in skin,⁴ and some preliminary data on wound healing in skin.

16.2 IR MICROSCOPIC CHARACTERIZATION OF AN UNUSUAL FORM OF OSTEOPOROSIS

16.2.1 Brief Overview: Bone Structure and Function

Bone tissue imparts strength and rigidity to the musculoskeletal system of vertebrates while also acting as a storage location for calcium and phosphate ions. Collagen (mostly Type I) provides bone with elasticity and serves as a template for mineral deposition, while the mineral phase of bone is a version of hydroxyapatite (HA) highly substituted with magnesium, carbonate, and acid phosphate in the lattice structure.

A photomicrograph of a long bone is depicted in Fig. 16.1; regions of interest are marked. Cortical (solid) bone on the outside forms the shaft of the long bone. Trabecular bone provides supporting strength to the ends of the weight-bearing bone. In osteoporosis, the greatest proportion of bone loss occurs in trabecular bone. Remodeling of bone, ongoing during life, causes the structural anatomy to be altered in response to organism requirements.

16.2.2 IR Spectral Data from Trabecular Bone

Typical IR spectra from trabecular bone from a human iliac crest biopsy are presented in Fig. 16.2. Prior to IR spectral examination, the tissue is embedded in polymethylmethacrylate (PMMA) and microtomed to a thickness of $\sim 5\text{ }\mu\text{m}$. Spectra were collected with a 64×64 array detector; thus 4096 spectra were collected from this specimen. Forty-five spectra from a line crossing trabecular bone are depicted in the figure. The main spectral features assigned to particular vibrational modes are noted. The determination of univariate spectra–structure correlations and their sensitivity to particular facets of structure in mineralized tissue are listed in Table 16.1. Also included in the table are the sensitivities

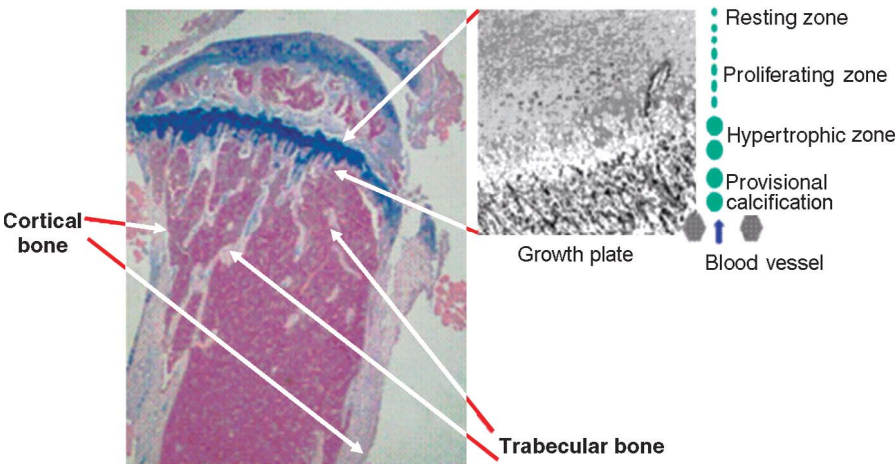


Figure 16.1. Left: Photomicrograph of a rat femur stained with H&E. Typical areas of the two major regions of interest for the current chapter, namely trabecular and cortical bone, are marked. Right: Schematic depiction of the growth plate, demonstrating the rapid spatial variation in bone architecture typical of long bones. (Courtesy of Professor Adele Boskey, Hospital for Special Surgery, New York.).

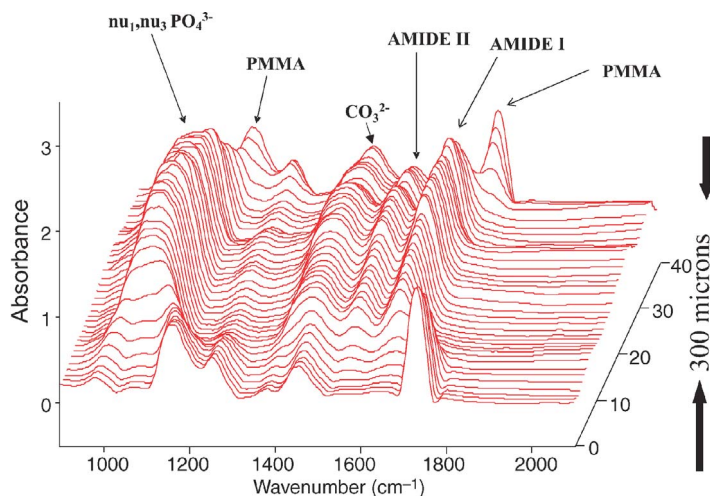


Figure 16.2. Forty-five mid-IR spectra generated as part of an imaging data set. The original set consisted of 4096 spectra. The spectra shown are from a single line across the cortical bone area of a human iliac crest biopsy section. The embedding medium, PMMA, gives a strong characteristic absorbance near 1720 cm⁻¹. The important IR spectral features arising from the hydroxyapatite (HA) and collagen constituents of bone are labeled with their vibrational assignments. It is evident that polymethylmethacrylate (PMMA) does not penetrate significantly into the mineralized area.

of particular parameters to biomedical processes of interest. The references cited provide the primary sources in which the correlations were established.

16.2.3 Osteoporosis

Osteoporosis is generally characterized by reduced bone strength predisposing the patient to increased risk of fracture (Osteoporosis Prevention, Diagnosis and Therapy. NIH Consensus Statement 2000, Vol. 17, pp. 1–45). The competence of bone and concomitant fracture risk to the patient are dependent on bone mass, architecture, and material quality. Two types of osteoporosis are commonly encountered. High-turnover (Type I) osteoporosis is typically

TABLE 16.1. Molecular Structural Information from IR Images of Mineralized Tissue

Molecular property	Spectral origin	Biomedical use	Reference
Identity of mineral phase	From the entire mineral spectrum	Calcium deposits	31
Amount of mineral	Integrated area of PO ₄ ³⁻ v ₁ ,v ₃		
Amount of protein	Integrated area of amide I band		
Mineral/matrix	Tracks “ash weight” measurements	Osteoporosis	
Orientation of collagen	Polarized FTIRM measurements	Osteoarthritis	32
Proteoglycan	Sugar vibrations	Cartilage disease	32,33
Mineral crystallinity/maturity	Shape changes in PO ₄ ³⁻ contour	Osteoporosis	34,35
Amount of carbonate	CO ₃ ²⁻ bands	bone maturity	36
Type of carbonate substitution	CO ₃ ²⁻ v ₂ shift in OH ⁻ vs. PO ₄ ³⁻ site		37,38
Lipids	Methylene modes	Wound healing	
Collagen cross-links	I(1660/1690) in the amide I contour	New form of osteoporosis	5,39

observed in postmenopausal women and is characterized by increased resorptive surface, higher-than-normal numbers of osteoclasts, and normal osteoblastic activity. Low-turnover (Type II) osteoporosis is age-related and displays normal or decreased osteoclastic resorption sites.

Our working hypothesis generated after many years of experiments is that alterations in mineral and matrix (protein, mostly collagen) quantity and quality, in addition to changes in architecture and geometry, account for the loss of bone strength in osteoporosis. Importantly, broad distributions of mineral content, mineral crystal size, and collagen cross-links are present in healthy bone, and these (spatial) distributions are altered (generally reduced in width) in patients with osteoporosis. This spatial variation is deemed essential for providing the correct balance of strength and flexibility in the tissue.

Vibrational spectroscopic imaging can quantitatively map altered spatial distributions in both the mineral and the matrix constituents of bone, and it is the molecular structure information inherent in IR and Raman data which provides this diagnostic ability. The relevance of this hypothesis is depicted in Fig. 16.3, where the mineral/matrix ratio determined from univariate analysis of IR microscopy data is depicted for several different spectral lines across trabecular bone from normal, high turnover (HTOP), and low-turnover (LTOP) osteoporotic patients. It is emphasized that the ordinate scale in each section of the figure is the same. The observed line-to-line variation in the mineral/matrix ratio in normal tissue is striking and has been substantiated in many samples. In contrast, the variation in this ratio for the pathological samples is markedly less. Thus, this metric determined by IR imaging provides a powerful diagnostic for osteoporosis based on the directly measured variation in a molecular property. The approach is depicted below for an unusual form of osteoporosis.

16.2.4 Application to an Unusual Form of Osteoporosis

We recently reported⁵ an interesting application of IR imaging to a form of osteoporosis appearing in a population of women less than 40 years of age with normal bone mineral density and serum biochemistry, who nevertheless suffered multiple spontaneous fractures – that is, possessed one of the major symptoms of osteoporosis. Because the bone mineral density of this sample population was normal as measured both by traditional bone density determinations and by IR studies of mineral/matrix ratio in biopsies, the molecular origin of the pathological condition had to be sought elsewhere. Thus IR imaging data from biopsies taken from these patients were compared with patients diagnosed with either high- or low-turnover osteoporosis.

The spectral parameter most important (Table 16.1) for characterizing the differences among these three pathological states (LTOP, HTOP, and spontaneous fracturing) and normal bone was the spatial variation in the nonreducible:reducible collagen cross-link ratio (see Table 16.1) at bone-forming trabecular surfaces. This quantity is monitored from the univariate I (1660)/(1690) intensity ratio within the bone amide I contour. This ratio was measured in bone-forming surfaces in 2 to 4 μm -thick sections from human iliac crest biopsies. The values obtained were compared with previously published analyses of trabecular bone from normal, nonosteoporotic subjects.

Typical data are presented in Fig. 16.4. The spatial variation in the collagen cross-link ratio in the first 50 μm from the edge of bone-forming surfaces for nonosteoporotic subjects shows a monotonic increase across the tissue. In contrast, LTOP patient biopsies reveal a rapid initial increase from the bone-forming surface followed by a leveling off in the measured parameter. Significantly, the data from the spontaneously fracturing population

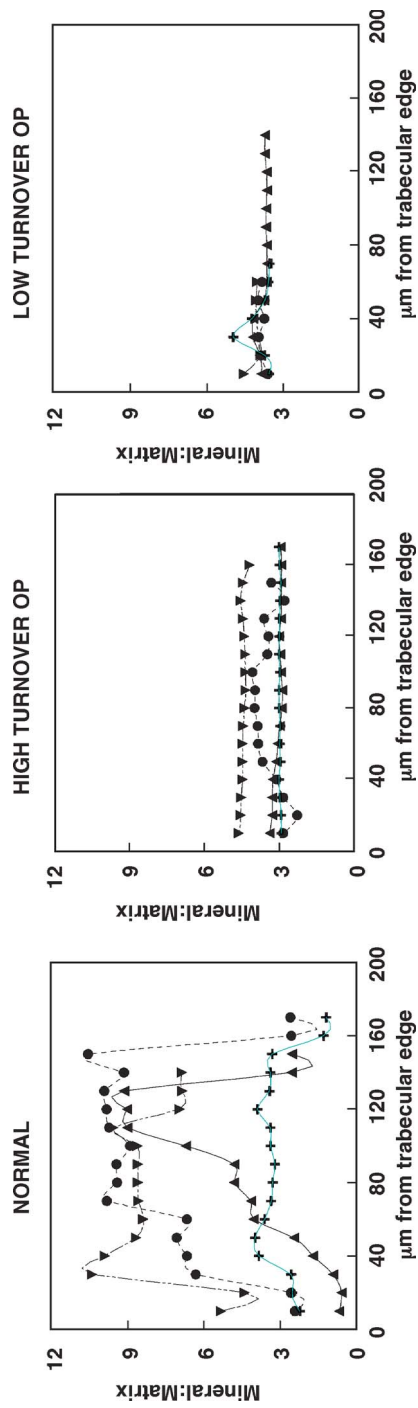


Figure 16.3. Spatial variation in the mineral/matrix ratio across bone-forming trabecular surfaces as a function of anatomical distance from the outer edge of the osteoid for normal, high-turnover, and low-turnover osteoporosis patients as marked.

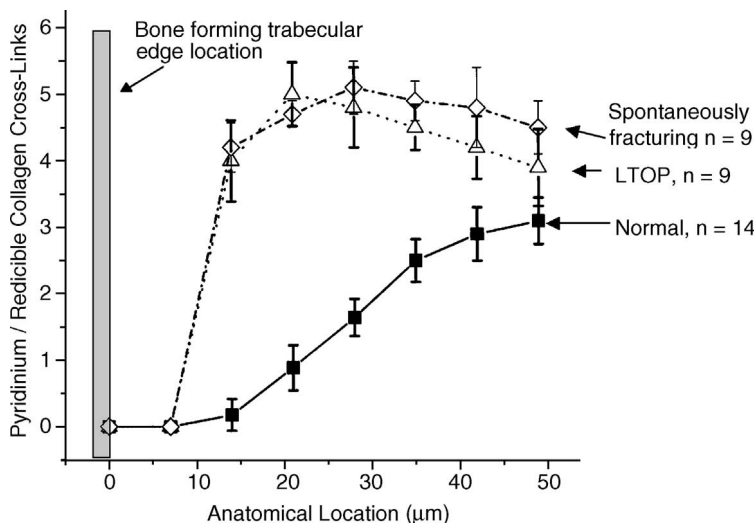


Figure 16.4. Comparison of the spatial variation in the pyridinium/reducible collagen cross-link ratio between the normal (filled squares), low turnover osteoporotic patients (LTOP) (unfilled triangles), and premenopausal spontaneously fracturing (unfilled diamonds) patient groups. (Reprinted from Ref. 5, Fig. 2, with permission.)

are statistically identical to the LTOP data, and both differ from the normal population. It is concluded that the origin of the pathological state arises from abnormal spatial distribution of collagen cross-links. Further progress in understanding this disease state will require implementation of molecular biology approaches to determine if the disease is genetic in origin. The experiment provides a clear demonstration of the gains that accrue from molecular-level understanding of the IR spectral data and from the determination of their sensitivity to elements of changed molecular structure.

16.3 APPLICATIONS TO THE EPIDERMIS

16.3.1 Brief Overview: Skin Structure and Function

A photomicrograph and schematic drawing of a skin section is depicted in Fig. 16.5. The main barrier to permeability resides in the outermost layer of the epidermis, the stratum corneum (SC), which also serves to maintain water homeostasis. This thin ($\sim 10\text{--}20\text{ }\mu\text{m}$) superficial region has a biphasic structure consisting of anucleated, keratin-rich corneocytes embedded in a highly ordered, lamellar lipid network. Underlying the SC is the viable epidermis, a major function of which is to generate the SC. The principal cell of the epidermis is the keratinocyte, which differentiates as it migrates toward the SC. The thickness of the epidermis ranges from 50 to 150 μm . Finally, the underlying layer, the dermis, consists of tough connective tissue and a variety of specialized structures. Collagen comprises $\sim 75\%$ of the dry weight of the dermis. The thickness of the dermis ranges from 0.6 to 3 mm.

As a preliminary to vibrational microscopic imaging studies, our laboratory has undertaken a variety of bulk-phase IR spectroscopic studies of the phase behavior of standard three-component models (ceramide/cholesterol/fatty acids) for the SC lipid phase.^{6,7} This has provided insight into the organization of the SC lamellar phases. In addition, the

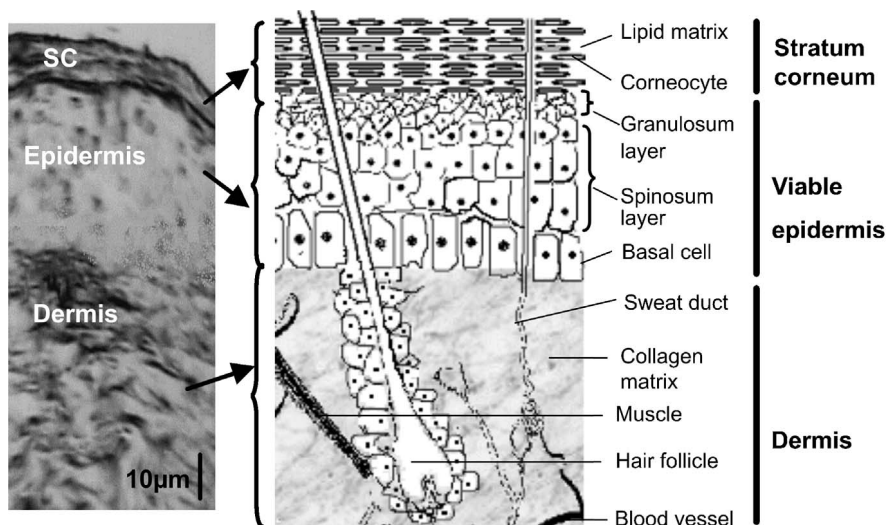


Figure 16.5. **Left:** Optical micrograph of an $\sim 5\mu\text{m}$ thick unstained pigskin section depicting the stratum corneum (SC), underlying epidermis, and dermis regions. **Right:** Schematic cross-section of the same regions in skin with anatomical features and subregions labeled.

applicability of spectra–structure correlations to skin tissue, along with the ability to detect spectral signals from exogenous constituents, opens up new possibilities for vibrational microscopic imaging experiments relevant to pharmacology, physiology, and biochemistry.^{4,8,9} In this chapter, we provide four examples depicting the interplay of vibrational microscopic imaging with spectra–structure correlations.

16.3.2 A Classical Example: Phase Transitions in Intact Stratum Corneum

A basic premise of this chapter is that it is feasible to design experiments on intact tissues which provide important structural information based on results gained from the spectroscopy of small molecules. Toward this end, four decades of seminal studies by Snyder and co-workers have produced a detailed understanding of relationships between alkane phases and their vibrational spectra.^{10–13} These correlations have been shown to carry over to ceramides and phospholipid assemblies.^{7,14} Some relevant correlations are summarized in Table 16.2.

As noted in the table, several methylene chain vibrations are sensitive to various aspects of molecular conformation and interchain interactions. The methylene stretching frequencies monitor both lipid conformational order and acyl chain packing. The sensitivity to conformational order (trans–gauche isomerization in the chains) is well known. However, although the sensitivity of these modes to acyl-chain packing is not as widely utilized, this aspect nevertheless is important for studies of ordered domain formation and solid–solid phase transitions that occur in the ceramide constituents of the SC.^{7,15} For the symmetric CH_2 stretching frequency ($\nu_{\text{sym}} \text{CH}_2$), which ranges from 2847 to 2855 cm^{-1} , the band position distinguishes packing from conformational effects. Packing geometry alterations (usually arising from solid–solid phase transitions) are inferred if cooperative changes in $\nu_{\text{sym}} \text{CH}_2$ appear below about 2850 cm^{-1} , while the introduction of gauche rotamers into the acyl chains causes $\nu_{\text{sym}} \text{CH}_2$ to exceed this value. Because this mode is both intense and not

TABLE 16.2. Lipid IR Frequencies for Acyl-Chain Order and Packing

Vibrational mode	Frequency (cm ⁻¹)	Sensitivity
CH ₂ symmetric stretch	2847–2855	The frequency position monitors chain conformational order and packing.
CH ₂ asymmetric stretch	2915–2924	The asymmetric stretching modes are less sensitive to packing changes.
CH ₂ scissoring	1462, 1473	The splitting reveals the presence of orthorhombic perpendicular packing.
	1468	Usually hexagonal chain packing.
	1473	Triclinic chain packing.
CH ₂ rocking	720, 730	The splitting reveals the presence of orthorhombic perpendicular packing.

overlapped by other vibrational modes in the IR spectrum of skin, it has been very useful for conformational and imaging studies of skin.

The sensitivity of several IR parameters to structural transitions in isolated human SC is demonstrated in Fig. 16.6. In part A of the figure, the CH₂ symmetric stretching frequency is seen to increase cooperatively over the temperature range 70°C–90°C, thereby reflecting the major order–disorder transition from hexagonally packed to disordered chains in the SC lipids. This transition is analogous to the widely studied gel → liquid crystal phase transition in phospholipids. In addition, a second transition accompanied by a small frequency increase of ~0.5 cm⁻¹ is observed at about 20–40°C, and it arises from a solid–solid (orthorhombic → hexagonal) phase transformation. Similar observations have been reported using (cryo-) electron diffraction studies.¹⁶

As noted in Table 16.2, the CH₂ scissoring modes are very sensitive to solid–solid phase transitions. Spectral data for this region as a function of temperature is shown in Fig. 16.6C, while the temperature dependence of the frequency of the band components are shown in Fig. 16.6B. The occurrence of orthorhombic phases at low temperatures is revealed by the splitting of the scissoring modes to produce a doublet with components at ~1473 and 1463 cm⁻¹. As the temperature increases, the two outer-frequency branches approach each other to the point where the splitting collapses at the orthorhombic → hexagonal transition. This temperature coincides with the transition detected in the CH₂ stretching vibration. The central branch in the spectra, at temperatures <40°C, represents lipids not present in orthorhombic phases.

As is evident from the data in Fig. 16.6A–C, the ordered lipids in the stratum corneum may be effectively studied by IR spectroscopy. We have applied the correlations and ideas noted above in published work on the recovery of orthorhombic phases following thermal perturbation of the skin barrier and on the imaging of conformational disorder in both the exogenous and endogenous methylene chains during liposome permeation.^{9,17,18} Experimental details can be found in the above-mentioned reports.

16.3.3 Characterization of Intact Skin Regions by Factor Analysis

As noted above, vibrational spectroscopic images of skin usually include hundreds to thousands of spectra with large variable features from one to another due to skin heterogeneities. Thus a multivariate approach is required to condense the information into a small set of dimensions with a minimum loss of information. We have found principal component analysis (PCA) and factor analysis to be useful for this purpose.

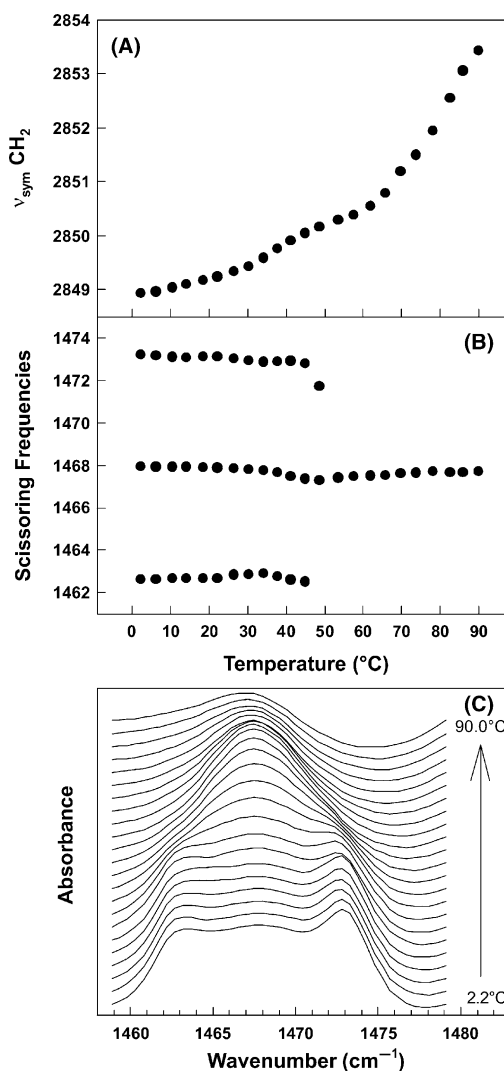


Figure 16.6. Temperature-induced changes in IR parameters (A&B) and spectra (C) evaluating lipid acyl-chain packing and conformation in isolated human stratum corneum (SC). Transmission IR spectra were acquired from SC isolated using a standard trypsin digestion protocol. **(A)** CH_2 symmetric stretching frequency response to temperature displaying two transitions as described in the text. **(B)** CH_2 scissoring frequencies displaying the temperature-induced orthorhombic to hexagonal acyl-chain packing transition as determined from the spectra shown in **(C)** displaying the 1459 to 1479 cm^{-1} spectral region. Spectra are offset from bottom to top in 4 °C increments as temperature is increased.

PCA produces loadings that represent directions of maximum uncorrelated variance, ranked in terms of the percentage of variance they explain, but the loadings do not resemble spectra. Factor analysis seeks transformations of the PCA loadings to “true” absorption spectra to explain the data. Compared to PCA, factor analysis offers a major advantage. Loadings generated by factor analysis resemble real spectra (although not of pure components) for which spectra–structure relationships may exist. If factor analysis is carried out over a limited spectral region (e.g., the C–D stretching region, if exogenous deuterated lipids are employed), then the spectral origin of the peaks within the loading is well-determined.

The benefits of using both factor analysis and existing spectra–structure correlations to characterize skin structure is demonstrated by the following examples.

An IR image acquired from a microtomed pigskin section sliced ($\sim 5\ \mu\text{m}$ thick) perpendicular to the SC with approximate dimensions of $290\ \mu\text{m} \times 250\ \mu\text{m}$ (Fig. 16.7A) was analyzed by multivariate factor analysis performed in three separate spectral regions: $1140\text{--}1476\ \text{cm}^{-1}$, $1476\text{--}1700\ \text{cm}^{-1}$, and $2832\text{--}3600\ \text{cm}^{-1}$. Three factor loadings from each spectral region are shown in Fig. 16.7B (i–iii). Representative score images corresponding to the three factor loadings in the spectral region $1480\text{--}1700\ \text{cm}^{-1}$ [Fig. 16.7B (ii)] are presented in Fig. 16.7C. The score images clearly delineate the stratum corneum (i), viable

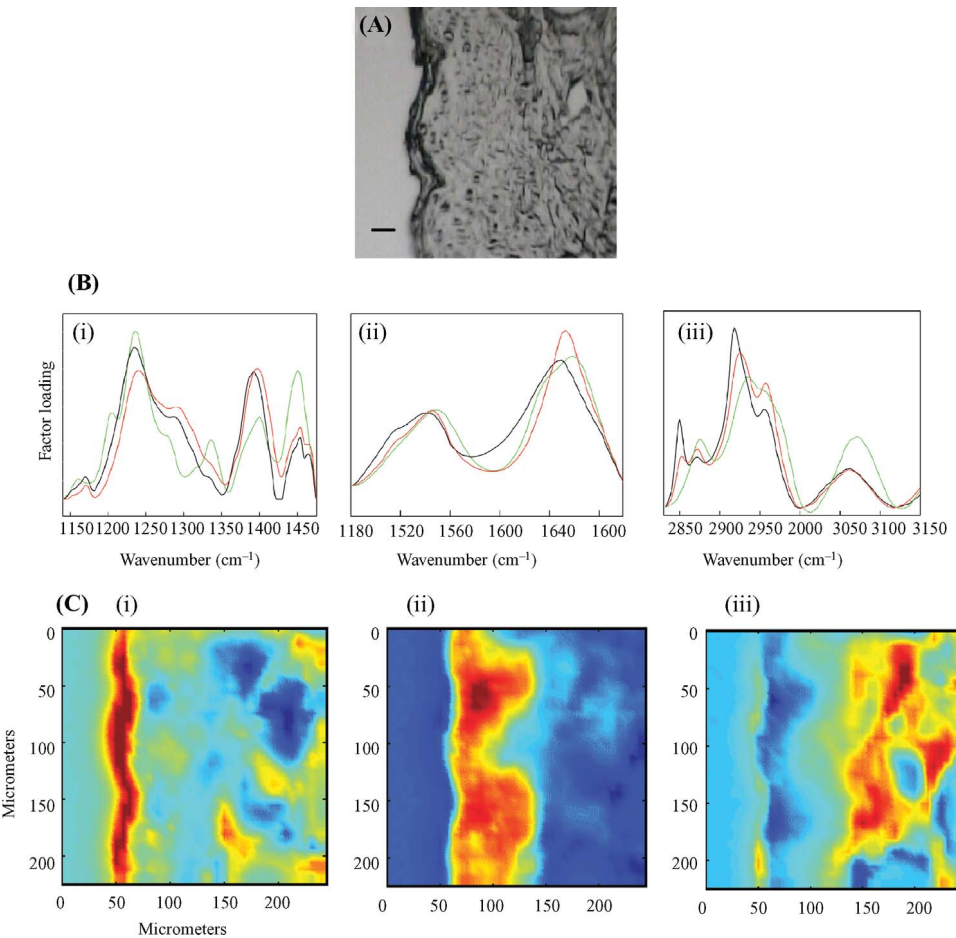


Figure 16.7. (A) Optical micrograph of an $\sim 5\ \mu\text{m}$ thick pigskin section (bar = $20\ \mu\text{m}$) from which IR spectra were acquired. The intact skin was frozen with liquid N_2 and microtomed perpendicular to the stratum corneum surface. IR imaging was conducted using a Perkin–Elmer Spotlight system with a $6.25\ \mu\text{m} \times 6.25\ \mu\text{m}$ detector pixel size. (B) Three distinct factor loadings generated by the ISys score segregation algorithm (Malvern Instruments) in separate spectral regions: (i) $1140\text{--}1476\ \text{cm}^{-1}$, (ii) $1476\text{--}1700\ \text{cm}^{-1}$, and (iii) $2800\text{--}3600\ \text{cm}^{-1}$. The factor loadings depict spectral features of stratum corneum (black), epidermis (red), and dermis (green) as determined by factor score spatial distribution. (C) Factor score images corresponding to the three factor loadings shown in B (ii) clearly differentiate the (i) stratum corneum, (ii) epidermis, and (iii) dermis. Red corresponds to the highest factor score, while blue corresponds to the lowest.

epidermis (ii), and dermis (iii). Score images of factors from the remaining two spectral regions show essentially the same results (not shown); that is, they map to the three skin regions. Correspondingly, the factor loadings contain spectral features specific to each skin region. Briefly, the stratum corneum is rich in an ordered, lamellar lipid phase composed of sphingolipid-derived ceramides, fatty acids, and cholesterol. The factor loading that maps to the stratum corneum region (Fig. 16.7B (iii), black) presents strong lipid CH_2 stretching bands at 2848 cm^{-1} and 2920 cm^{-1} . Our knowledge of the spectra-structure correlations (Fig. 16.6) allows us to confirm the ordered nature of the lipid. The Amide I band (1648 cm^{-1}) in the SC is broadened and shifts slightly to lower wavenumbers compared to the Amide I band assigned to the epidermal region (1650 cm^{-1}) due to ceramide contributions to the contour. Furthermore, the dermis displays a set of spectral features totally different from that of the epidermis since the predominant proteins in the two regions differ—that is, collagen in the dermis and keratin in the epidermis.

One main advantage of IR microscopic imaging is its ability to sample larger areas than Raman microscopy in a relatively short time period with high spectral quality. It is a powerful technique for characterizing relatively large-scale skin features – for example, delineating skin regions, locating skin appendages (hair follicles, sweat ducts), and tracking exogenous agent permeation (see Ref. 17 for a recent review). In contrast, due to its higher spatial resolution, Raman imaging is suitable for the characterization of localized biochemical processes, in addition to defining skin regions or components in a confocal manner without skin sectioning.

Confocal Raman spectra were acquired for image analysis from an untreated piece of pigskin in a plane perpendicular to the skin surface ($\sim 42\text{ }\mu\text{m}$ deep $\times$ $38\text{ }\mu\text{m}$ wide). Factor analysis was performed in the 600 to 800 cm^{-1} spectral region. Four factors are shown in Fig. 16.8A, and corresponding score images are depicted in Fig. 16.8B. In the loadings, the bands at 607 cm^{-1} and 700 cm^{-1} are assigned to cholesterol.¹⁹ Generally, there are relatively higher levels of cholesterol present in the stratum corneum compared to the underlying epidermis. Therefore, factor 2, which is clearly derived from the stratum corneum region, possesses relatively stronger cholesterol bands than factor 1, which has a high score in the viable epidermis. The score image of factor 4, with the most intense cholesterol bands, reveals several small, localized pockets in the stratum corneum. In addition, the cholesterol band near 607 cm^{-1} shifts $\sim 1\text{ cm}^{-1}$ between the pockets (factor 4) and the stratum corneum (factor 2), suggestive of changes in molecular structure, hydrogen bonding, or the hydrophobicity/hydrophilicity of the local environment. Finally, factor 3 reveals a band at 785 cm^{-1} thought to arise from cytosine in DNA.^{20,21} The score distribution for factor 3 displays another set of discrete pockets, localized to the viable epidermis. The identification of DNA reveals the location of cell nuclei in the epidermis. Factor analysis conducted over the 800 to 1140 cm^{-1} region discloses an additional DNA band, the phosphodiester mode at 1090 cm^{-1} (not shown), which is localized to the same regions in the epidermis.²²

16.3.4 Prodrug–Drug Interconversion in Skin

One strategy employed in drug formulation for transdermal delivery makes use of prodrugs. Prodrugs are designed by modifying the physical and chemical properties of the active form of the molecule to meet a particular purpose such as enhancing permeability in skin. Once in the skin, the prodrug is then enzymatically or chemically converted to the active compound at a particular site or within the targeted tissue. Evaluation of the success of the strategy requires techniques that directly detect the drug and the prodrug species along with the spatial distribution of each within tissue in a nonperturbing fashion. The following example

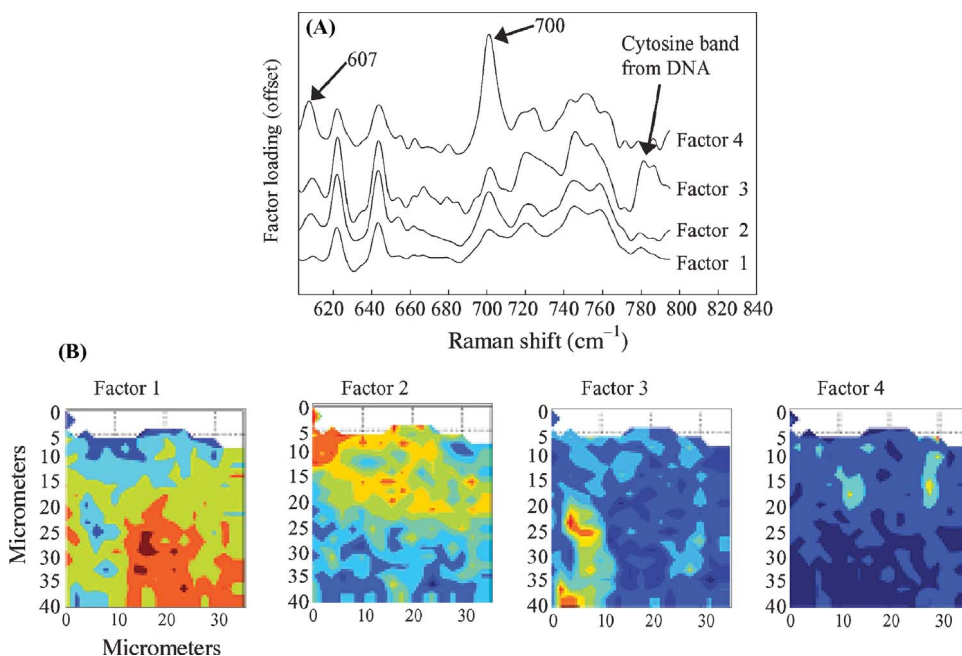


Figure 16.8. The microanatomy of intact pigskin as characterized by factor analysis conducted on confocal Raman spectra. Intact pigskin sections (~3 mm thick) were placed into a milled brass cell with the stratum corneum (SC) side up and sealed with a glass coverslip. A plane of Raman spectra was acquired (Kaiser Optical Systems Raman Microprobe) using a 2 μm step size and a 100 $\times$ oil immersion objective. **(A)** Four distinct factor loadings generated in the 600 to 800 cm^{-1} spectral region by the lSys score segregation algorithm (see caption to Fig. 16.7) with peaks of interest noted. **(B)** Corresponding score images depict the microanatomy of skin by assigning factor 1 to the viable epidermis, factor 2 to the SC, factor 3 to cell nuclei, and factor 4 to localized cholesterol pockets as described in text. Red corresponds to the highest factor score, while blue corresponds to the lowest.

demonstrates the feasibility of utilizing confocal Raman microscopy to monitor this process.

5-Fluorouracil (5FU), a systemic anticancer drug, is also used to treat cancerous or precancerous conditions in skin including solar keratoses, actinic keratosis, superficial basal cell carcinoma, and Bowen's disease.^{23,24} The delivery of 5FU in conventional topical preparations has been suboptimal and limited by its poor solubility in skin. A variety of prodrug forms of 5FU have been prepared, one of which, 1-ethyloxycarbonyl-5FU (Pro-5FU), provided a 25-fold increase in transdermal delivery.²⁵ The molecular structures are shown in Fig. 16.9B.

Significant differences in the Raman spectra of pro-5FU and 5FU in aqueous solution can be observed in the low-frequency region shown in Fig. 16.9A. Several bands likely arising from ring vibrations are diagnostic for each molecule, illustrating the potential for *in situ* differentiation of these molecules in the confocal Raman experiment. A spectrum of untreated pigskin and solid 5FU are also included in Fig. 16.9A. For permeation experiments, a suspension of pro-5FU in isopropyl myristate (IPM) was applied to the SC of intact pigskin samples held at either 22°C or 34°C for 20 h, after which excess prodrug was removed by gentle rinsing with IPM. Subsequently, Raman spectra were acquired at increasing depths beginning at the skin surface using 5 μm increments. For further details

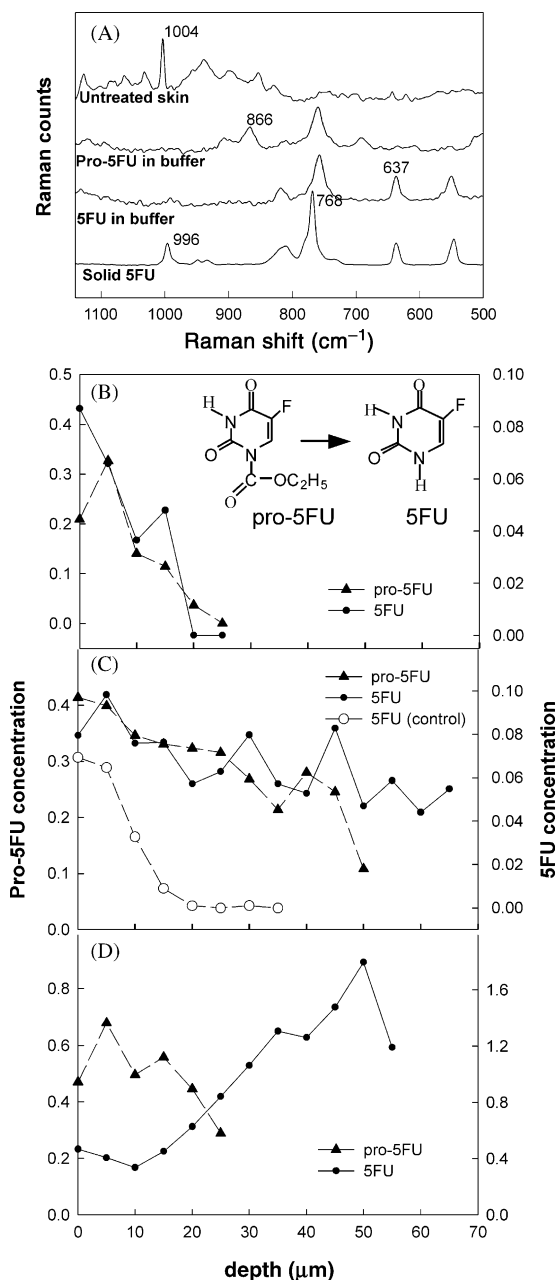


Figure 16.9. Permeation and hydrolysis of pro-5FU and 5FU in skin. **(A)** Raman spectra (500 to 1140-cm⁻¹ region) of controls, from top to bottom: Untreated pigskin spectrum acquired at ~6 μm beneath the stratum corneum (SC) surface, spectra of Pro-5FU (35 mM) and 5FU (22 mM) solutions, and spectrum of 5FU powder. Raman spectra of the treated skin were acquired using the same parameters as described in the caption to Fig. 16.8 except that the step size was 5 μm. The relative concentrations of Pro-5FU and 5FU were calculated as described in text. **(B)** Relative concentrations of Pro-5FU and 5FU as a function of depth in skin for treated samples incubated at 22 °C. **(C, D)** Relative concentrations of Pro-5FU and 5FU as a function of depth in skin for treated samples incubated at 34 °C. The molecular structures of 1-ethoxycarbonyl-5FU (pro-5FU) and 5FU are shown in the inset to part B.

on experimental procedures refer to Ref. 4. The relative concentrations of pro-5FU and 5FU were each characterized by obtaining the ratio of the integrated area of particular bands (866 cm^{-1} and 637 cm^{-1} , respectively) to the area of the skin phenylalanine band at 1003 cm^{-1} . Relative concentrations as a function of depth in skin are shown for samples incubated at 22°C and 34°C in Fig. 16.9B–D. A control experiment is also shown for a skin sample incubated at 34°C in Fig. 16.9C, in which a 5FU suspension was applied on the skin surface. Based on the results, several comments can be made. First, regardless of incubation temperature, the application of pro-5FU results in the observation of 5FU within skin, revealing that esterase activity or chemical hydrolysis has taken place *in situ*. Variability in the concentration profiles shows that hydrolysis does not take place uniformly in skin. Second, pro-5FU delivers 5FU to a markedly greater depth than the direct application of 5FU (compare 5FU and 5FU control in Fig. 16.9C). Third, a striking difference in permeation of both pro-5FU and 5FU is observed between the two incubation temperatures (compare Figs. 16.9B and 16.9C). This is likely to be partially due to the direct effect of temperature on diffusion. Permeation of both species at 22°C is limited to the SC, whereas both are distributed throughout the SC and viable epidermis at 34°C . The increase in permeation may also be due to temperature-induced alterations in the SC barrier. Since the lipid in the standard model of the SC (corneocytes embedded in a continuous lipid-enriched matrix) is thought to be primarily responsible for barrier function, we speculate that temperature-dependent changes in SC lipids are partially responsible for the increased permeation. As shown above, a temperature-induced solid–solid phase transition (orthorhombic to hexagonal acyl chain packing) occurs in the lipids of isolated human SC at $20\text{--}40^\circ\text{C}$. The transition is also known to occur, albeit over a slightly lower temperature range, in porcine SC, most likely due to slight differences in porcine SC lipid composition [Ref. 4 (supplemental figures)]. The loosening in acyl-chain packing may be responsible for the increase in the observed permeability.

The analysis of confocal Raman lines showed a few with a remarkably high 5FU concentration in some areas beneath the skin surface where skin was incubated at the higher temperature. The relative pro-5FU and 5FU concentrations in one of these samples is presented in Fig. 16.9D. At a depth of $\sim 35\text{ }\mu\text{m}$ beneath the skin surface, 5FU in the solid state was identified by sharp Raman marker bands at 768 cm^{-1} and 996 cm^{-1} as shown in the bottom spectrum in Fig. 16.9A. We speculate that a relatively high level of pro-5FU was hydrolyzed in this localized area and 5FU crystallized within the skin.

16.3.5 Vibrational Microscopic Imaging of Wound Healing in Skin

The ability of vibrational microscopic imaging to characterize different types of biological materials and their distribution in tissue has opened up a wide range of biomedical research possibilities. We present previously unreported feasibility studies where vibrational microscopic imaging is used to characterize the spatial, temporal, and molecular structure information from wounds created in human skin tissue in an effort to study healing processes.

When skin is wounded, it is obviously urgent for the body to reestablish the barrier function of the SC to prevent bacterial infection and moisture loss from tissues. The healing of wounds in skin is a complicated biological process involving events such as cell migration and proliferation which have to be coordinated in time and space.^{26–28} The ability of vibrational imaging technology to capture compositional and structural aspects of the wound healing process at different stages will improve our understanding of the healing process and of alterations in the sequence of events caused by the application of external agents.

A 100 to 200 μm -deep wound of $\sim 2\text{ mm}$ diameter was created on the surface of samples of Caucasian human skin tissue explant cultures ($\sim 1\text{ mm}$ thick) using a punch biopsy technique. The wound is smaller than the size of the tissue sample so that both wounded and nonwounded areas are accessible to spectroscopic investigation on each sample. The skin tissue samples are supported on a nutrient buffer matrix and held at 37°C . The viability of the skin cells was thereby maintained for periods of up to one week. A 60 μm -deep, 250 μm -wide depth profile map (Raman image plane) has been collected crossing from the nonwounded into the wounded skin area to demonstrate the power of multivariate statistics to distinguish different components between the two regions. The punch biopsy was performed two days prior to spectral acquisition. Factor analysis of several spectral regions has been conducted to delineate the structure and composition of the skin tissue in the vicinity of and within the wound site. We begin with the CH stretching region as a demonstration of how the spectroscopic approach may be applied in wound healing studies. Four factors have been identified in the 2800 to 3060 cm^{-1} region, and the factor loadings along with score images are shown in Figs. 16.10A and 16.10B, respectively.

The nonwounded area is on the left side of the image, with the wound edge situated at $\sim 80\text{ }\mu\text{m}$ from the left edge. As noted previously, the extracted factors may be assumed to mimic the spectra from which they are derived. Factor loading 1 reveals a strong characteristic lipid symmetric stretching ($\nu_s\text{CH}_2$) band at 2851 cm^{-1} . This factor, along with others extracted from different spectral regions that have the same score distribution (not shown), have spectral features consistent with the assignment of this element to body fat contamination. The other three factors shown in Fig. 16.10 demonstrate marked differences in their loading plots and score images. Factor loading 4 has a strong sharp band at 2879 cm^{-1} , which is assigned to the asymmetric CH_2 stretching mode ($\nu_a\text{CH}_2$) of highly ordered lipid. The score image provides good evidence that factor 4 describes the stratum corneum of the nonwounded part of the skin tissue. The position of the $\nu_s\text{CH}_3$ at 2927 cm^{-1} in factor 3 and 4 is $\sim 13\text{ cm}^{-1}$ lower than that of factor 2 ($\sim 2940\text{ cm}^{-1}$). This difference may serve as a marker for the types of protein (keratin in epidermis and collagen in the dermis) present in the skin tissue. The score images show that these three factors are separated into three different spatial region, with factors 3 and 4 highlighting the nonwounded area while factor 2 highlights the wounded area. Based on the score image, it is likely that factor 3 arises from the nonwounded viable epidermis. The punch biopsy removed the epidermal tissue in the wounded area, so it is reasonable that epidermal tissue can only be found in the nonwounded area during the early stages of the healing process. Epidermis in the nonwounded area contains keratinocytes with high levels of keratin while the wounded areas are mostly collagen. Similar $\nu_s\text{CH}_3$ band positions are observed in Raman spectra of isolated epidermal collagen and keratin (not shown).

Figure 16.11 displays the results from factor analysis in the 800 to 1020 cm^{-1} spectral region of the same Raman data set described above. Factor loadings 1 and 2 show the typical “doublet of doublets” spectral feature characteristic of collagen. Collagen contains a relatively large proportion of proline and hydroxyproline amino acid side chains,²⁹ and the strong bands at 936 and 855 cm^{-1} arise from these side chains and also from $\nu(\text{C}-\text{C})$ of the collagen backbone.³⁰ Thus, it is clear that factors 1 and 2 describe the collagen component, possibly two different types of collagen, and the corresponding score images indicate that collagen is located in the wounded region. On the other hand, the relatively strong phenylalanine band at 1003 cm^{-1} of factor loading 3 maps to the nonwounded epidermal region on the left side of the image. It is clear that by using Raman spectroscopic measurements, together with factor analysis, it is possible to identify wounded and

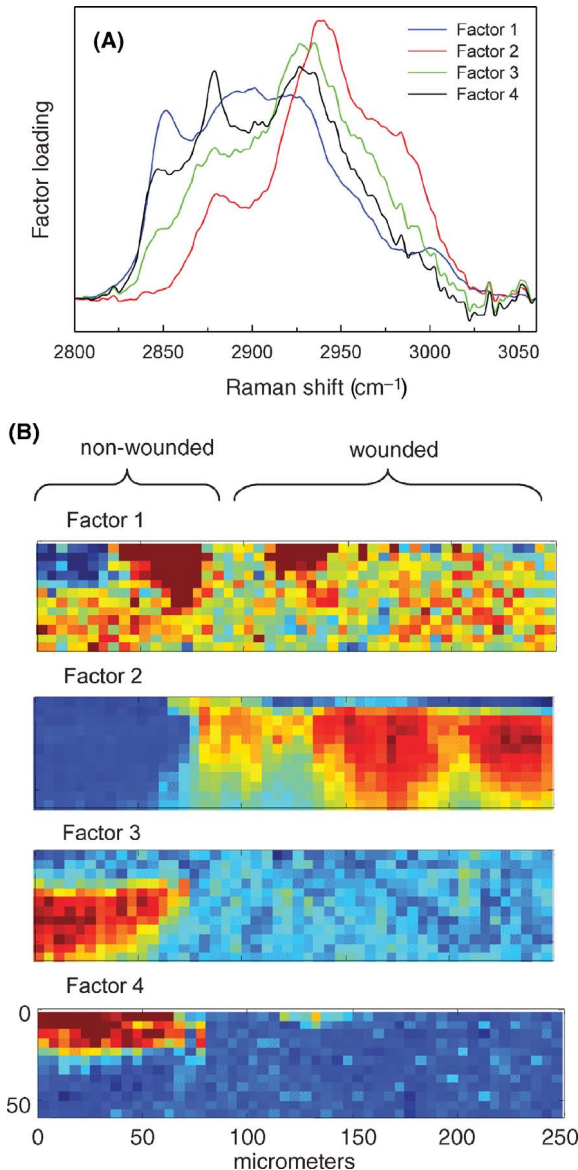


Figure 16.10. Factor analysis based on confocal Raman microscopy of wounded human skin two days after a punch biopsy. Experimental and computational parameters are the same as described in the caption to Fig. 16.8, using a 5 μm step size. **(A)** Four distinct factors extracted from the 2800 to 3060 cm^{-1} region. **(B)** Factor score images (red corresponds to the highest score and blue to the lowest) that delineate the wounded from nonwounded areas, including subregions within each (see text).

nonwounded areas and study both temporal and spatial variations in major skin constituents during wound healing in human skin.

FT-IR imaging has been undertaken to complement the confocal Raman microscopic results. Microtomed skin cross sections were prepared for IR imaging (two days after the punch biopsy) by freezing the skin tissue in liquid N_2 followed by microtoming at -30°C

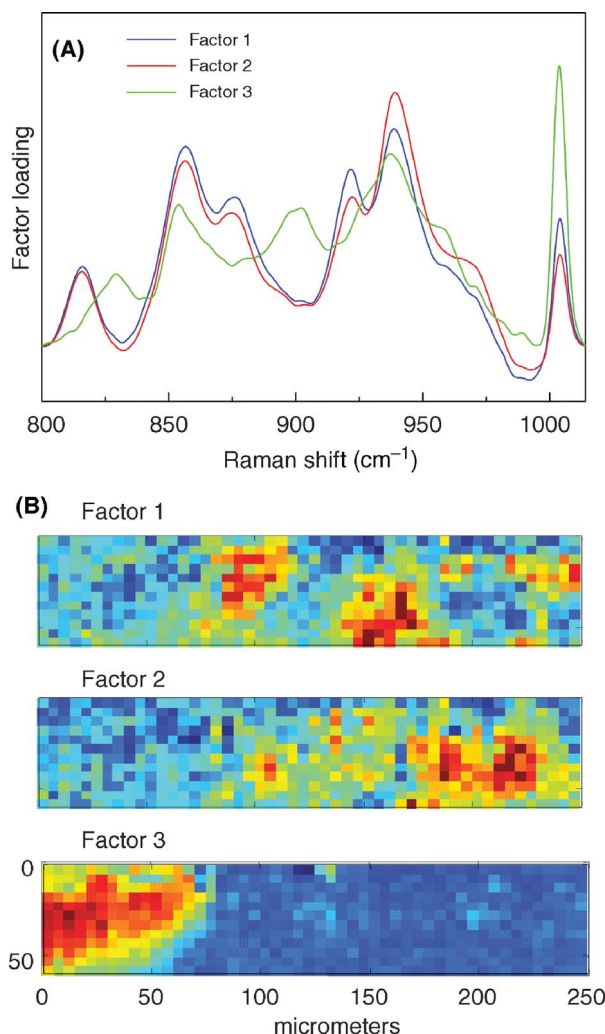


Figure 16.11. Factor analysis based on confocal Raman microscopy of wounded human skin two days after a punch biopsy. Experimental and computational parameters are the same as described in the caption to Fig. 16.8, using a $5\text{ }\mu\text{m}$ step size. **(A)** Three distinct factors extracted from the 800 to 1015 cm^{-1} region. **(B)** Factor score images (red corresponds to the highest score, while blue corresponds to the lowest) that delineate the wounded from nonwounded areas, including subregions within each (see text).

into 7 to $8\text{ }\mu\text{m}$ -thick slices.⁹ The microtomed sections were transferred to CaF_2 windows for IR imaging. Although the sample must be microtomed into thin slices for the IR transmission measurements, a larger area can be measured with a shorter acquisition time compared with Raman, and the sampling depth is limited only by the size of the tissue. The same data analysis approach has been applied to the FT-IR data as to the Raman; typical results are shown in Fig. 16.12. The nonwounded area in this case is situated on the right-hand side of the image, while the wounded area is on the left. Four factors have been extracted from the data. Factor loadings 3 and 4 show an intense lipid band at ~ 2850 ($\nu_{\text{sym}}(\text{CH}_2)$) and $\sim 2920\text{ cm}^{-1}$ ($\nu_{\text{asym}}(\text{CH}_2)$), suggesting a high concentration of lipid in the

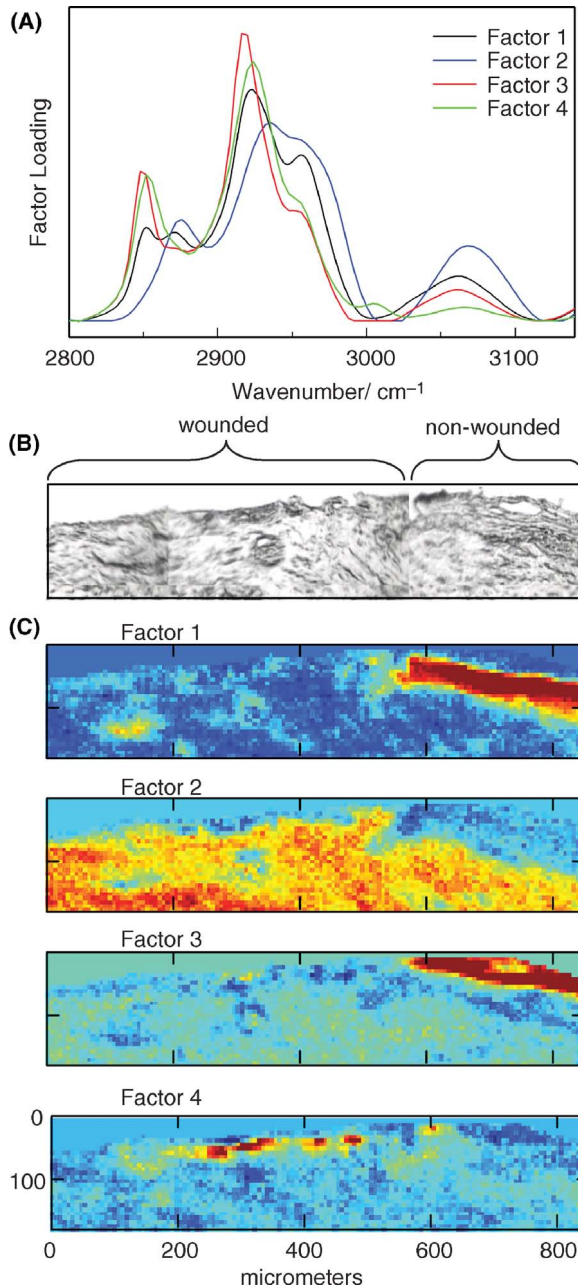


Figure 16.12. Factor analysis based on IR imaging of an $\sim 8\text{ }\mu\text{m}$ -thick human skin section comprising wounded and nonwounded areas two days after a punch biopsy. Experimental and computational parameters are the same as described in the caption to Fig. 16.7. **(A)** Four distinct factors extracted from the 2800 to 3140 cm^{-1} region. **(B)** Optical image of the skin section. **(C)** Factor score images (red corresponds to the highest score, while blue corresponds to the lowest) that delineate the wounded from nonwounded areas, including subregions within each (see text).

red area shown in the corresponding score plots. However, both $\nu(\text{CH}_2)$ band positions of factor loading 3 are slightly lower than those of factor loading 4. The band positions can be used to differentiate the ordered lipid in the stratum corneum from less-ordered lipid based on the shift to lower frequency ($\sim 4 \text{ cm}^{-1}$) of this band (see Table 16.2). Furthermore, a weak band at $\sim 3005 \text{ cm}^{-1}$ is observed in factor 4, which has been assigned to the $=\text{C}-\text{H}$ vibration from unsaturated hydrocarbon chains. Taken together, these results suggest that factor 4 describes lipid or fat contamination from the surgery (also detected in the confocal Raman study), while factor 3 has a high score image in the stratum corneum region of the nonwounded part of the skin. Below the stratum corneum lies the viable epidermis, which is described by factor 1. Factor loading 1 shows relatively strong $\nu(\text{CH}_3)$ bands at 2872 cm^{-1} and 2956 cm^{-1} , characteristic of protein methyl groups in the viable epidermis. Finally, factor loading 2 lacks the lipid bands but shows strong $\nu(\text{CH}_3)$ bands of collagen at 2874 cm^{-1} , 2930 cm^{-1} , and 2972 cm^{-1} , which suggests that factor score 2 presents the distribution of collagen in the skin tissue.

16.4 CONCLUDING REMARKS

The data presented above for both mineralized tissue and skin demonstrate the versatility of vibrational imaging for addressing a wide variety of medical, biochemical, and pharmacological issues. Although the images alone provide important spatial information, the strength of the technology for bioanalytical purposes is greatly enhanced when molecular structure information is incorporated into the mindset for data analysis. The altered cross-linking patterns of bone collagen that characterize the unusual form of osteoporosis in Section 16.2.4 provides a good illustration. Without the availability of the relationship between collagen spectra and the extent of cross-linking, identification of the origin of the disease could not have been made.

A molecular and structure-based rationale for understanding images constructed from IR and Raman data arises from the availability of spectra–structure information. For example, knowledge of the relationship between the IR methylene stretching frequencies and chain conformational order in lipids provides an interpretation of the spatial distribution (score image) of factors extracted from the CH stretching region. Based on this correlation, it is easy to envision that the effects of permeation enhancers and other exogenous materials on the physical barrier properties of the ordered lipids in the SC can be determined.

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