FOURIER TRANSFORM INFRARED VIBRATIONAL SPECTROSCOPIC IMAGING: Integrating Microscopy and Molecular Recognition

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Abstract The recent development of Fourier transform infrared (FTIR) spectroscopic imaging has enhanced our capability to examine, on a microscopic scale, the spatial distribution of vibrational spectroscopic signatures of materials spanning the physical and biomedical disciplines. Recent activity in this emerging area has concentrated on instrumentation development, theoretical analyses to provide guidelines for imaging practice, novel data processing algorithms, and the introduction of the technique to new fields. To illustrate the impact and promise of this spectroscopic imaging methodology, we present fundamental principles of the technique in the context of FTIR spectroscopy and review new applications in various venues ranging from the physical chemistry of macromolecular systems to the detection of human disease.

OVERVIEW

The vast array of vibrational spectroscopic techniques provide exquisite tools for probing the structural, dynamical, and functional properties of wide varieties of molecular systems and aggregates. Coupling interferometry, spectral data transformations, and detailed molecular databases, Fourier transform infrared (FTIR) spectroscopy has become ubiquitous within the physical, analytical, and biochemical disciplines. Raman spectroscopy, a complementary vibrational spectroscopic technique for molecular analyses, continues to benefit enormously from, for example, advances in laser and array detector developments. Because Raman microscopy (1), in particular, utilizes extensively components commonly employed for optical microscopy, the maturation of Raman microspectroscopy, in general,
has preceded that of its infrared (IR) spectroscopic counterpart, even though the signal arising from Raman scattering cross sections is typically six orders of magnitude smaller than that arising from IR absorptivities. Although attempts have been made for over 50 years to harness the capabilities of IR spectroscopy for determining the spatial distributions of chemical species in microsamples, significant progress in this area has accelerated within the past two decades. With a goal to record digital images analogous to those obtained by optical microscopy, the present accessibility of implementing vibrational IR spectroscopic signatures to provide meaningful chemical image contrast has opened new application areas in which a visualization of the spatial distribution of chemical species now allows structural and dynamical considerations to be applied to previously intractable materials. Because the recent availability of IR-sensitive multichannel array detectors provided a major step toward realizing this advantage, their introduction has clearly changed the practice of IR spectroscopy in various fields in a fundamental manner.

We review the rationale for the development of IR imaging, discuss instrumentation and implementation approaches, summarize illustrative applications from several diverse disciplines, and finally, present a perspective regarding the future of this technological advance. Because Raman microscopy instrumentation and applications have been recently reviewed (1), this area of vibrational spectroscopy in the context of imaging will not be discussed further.

FTIR Imaging: A New Avenue for Infrared Spectroscopy

The basic approach in implementing an interferometer to obtain spatially resolved IR spectra requires the detected radiation to define a specific region of a sample. This spatial localization is accomplished in two optical configurations; namely, by restricting radiation at the sample plane, the simplest approach, or by segmenting the transmitted radiation beam at the detection plane. For examining specific sample areas, an optical condensing unit is required to utilize effectively the broad radiation beam produced by the interferometer. In this respect, an IR microscope serves the dual purposes of providing both a means for performing visible microscopy, as well as serving as a condensing unit for IR microscopy. Early attempts at a practical implementation of FTIR microscopy followed a two-step process (2), which is still performed widely in many laboratories, and is based upon the principle of restricting radiation at the sample plane. First, sample regions were identified by subjective, visual inspection using an optical microscope. An opaque mask with an aperture of controlled size was subsequently employed to physically restrict radiation absorption to the sample area of interest. Spectra were then acquired by measuring the attenuation of incident radiation using a single-element detector. The entire field of view was then examined by sequentially rastering the microscope stage to record spectra from various points within the sample area. Because this process required precise positioning and consumed lengthy periods of time, initial research efforts were limited either to an examination of a single point in the sample or to the analysis of limited areas of few samples. The availability over time of sensitive single
element detectors, coupled with the increased throughput inherent in Fourier transform spectroscopy, improved the speed and accuracy of the technique. Faster and cheaper computers incorporated into the spectroscopic assembly provided the ability to position precisely the sample, to control optimally the spectrometer, and to yield faster data processing. With these advances in place by the early 1990s, large sample areas could then be examined through the use of apertures and by moving the sample in a prescribed manner. Because spectra are obtained from neighboring sample points, one at a time, this technique is referred to as mapping and has been commonly applied, for example, in materials science (3) and biology (4, 5).

Mapping suffers from several drawbacks in realizing the goal of a fast imaging analogue to optical microscopy (6). Sample areas are restricted usually to greater than $10 \times 10 \mu m^2$ to allow a sufficient throughput of radiation, although diffraction and stray light effects may still compromise the spatial fidelity of the acquired data. Two apertures (redundant aperturing)—one above and one below the sample—are introduced to minimize the effects of stray light; however, this decreases the radiation throughput and, hence, the spectral quality of the recorded data. To regain reasonable spectral data quality in this optical configuration, either impractically long experimental acquisition times or orders of magnitude brighter sources as, for example, a synchrotron, are required. To date, mapping instrumentation remains a continuous scan interferometer coupled to an IR microscope incorporating redundant apertures. Mapping an area a fraction of a millimeter takes tens of hours with a spatial resolution no better than a $15 \mu m \times 15 \mu m$ area. In most cases this leads to maps containing typically little resemblance to the sample’s morphological features. Higher spatial resolution, however, may be attained by combining the microscope system with a synchrotron source or with specialized near-field apertures (7). Near-field approaches (8, 9), although promising, are not currently common in laboratory settings and remain slow for mapping large sample areas. Despite the chemical specificity of IR spectroscopy, mapping techniques are not popular tools for extended sample area characterizations, particularly when many samples are to be assessed on a routine basis.

The second approach, a recent implementation, toward achieving spatially resolved IR spectroscopy involves the segmentation of radiation at the detection plane. In contrast to mapping measurements in which a small sample area is illuminated, the entire field of view of the sample is illuminated. Contributions from different sample areas are then separated by an array of IR sensitive detection elements in the manner of imaging with charge-coupled device (CCD) instruments for optical microscopy. These IR multichannel detectors, termed focal plane array (FPA) detectors, declassified from defense programs over the past decade, are relatively new to the spectroscopic community. FPAs consist of many small, individual detectors laid out in a grid pattern. The optical setup of an imaging instrument is similar to that of a mapping system. However, no apertures are necessary as the microscope images the sample plane directly onto the detector array. Each individual detector (pixel) in the array is capable of simultaneously collecting data from a specific sample region within the field of view. By coupling the multichannel
detection of FPA detectors with the spectral multiplexing advantage of FTIR interferometry, an entire sample field of view is spectroscopically imaged in a time period comparable to acquiring conventionally a single spectrum. Depending on the detection array and collection parameters, thousands of moderate resolution spectra can be acquired at near diffraction-limited spatial resolution in minutes. In the simultaneous collection of data from the entire sample area, the acquisition of individual spectra renders “snapshots” or images corresponding to each spectral wavelength. This technique is termed FTIR imaging, as it incorporates both the spectral multiplexing advantage of Fourier transform spectroscopy and the multichannel detection advantage commonly available in optical imaging.

**INSTRUMENTATION**

Common configurations for implementing microimaging with FTIR spectrometers consist of three basic elements: an interferometer, an IR microscope, and an IR-sensitive multichannel detector. The schematic of a typical FTIR microspectroscopy system, as shown in Figure 1, interfaces an interferometer to an IR microscope, yielding a specified spot size at the sample plane. The optical train then allows the IR radiation, which samples specific areas at the focal plane of the microscope, to impinge upon the detector. Synchronization between the various components, signal recording, and the numerical transformations necessary to visualize data are accomplished using an attached computer.

![Figure 1](image.png)  
*Figure 1* The configuration of a Fourier transform infrared (FTIR) spectroscopic imaging device consists of an interferometer coupled to a microscope and IR-sensitive focal plane array detector. When a conventional, single-element detector is employed (right), the microscope is necessarily equipped with apertures and a precision stage. For multichannel detection (left), no apertures are required.
Michelson Interferometer

The Michelson interferometer, employed for most microspectroscopy studies, recombines two beams split from a single, broadband source to provide intensity interferences in the spectral domain. The resultant intensity at the detector consists of a component that is invariant of the path difference between a static and a moving mirror. This DC component of the detected signal is a constant value providing a signal offset. The component of the light intensity from the source that changes with the mirror retardation, $\delta$, termed the AC signal component, yields the spectral profile and is given by

$$ I(\delta) = 0.5I(\bar{\nu}) \cos(2\pi \delta \bar{\nu}), $$

where $\bar{\nu}$ is the spectral wavelength. This sinusoidally modulated component of radiation that reaches the detector represents the interferogram, the relevant observable for spectroscopic measurements. When the source is a continuum, the net interference pattern is represented by the integral,

$$ I(\delta) = \int_{-\infty}^{+\infty} B(\bar{\nu}) \cos(2\pi \delta \bar{\nu}) d\bar{\nu}. $$

Using the above equation, a spectral profile is completely specified by the selected signal as a function of a measured intensity at a known optical retardation. A spectral profile is then computed by measuring the interferogram from zero retardation to an infinitely long retardation as a function of infinitesimally small increments of mirror displacements. Computational limitations combined with instrumental considerations require a finite number of discrete measurements, or sampling of the interferogram, over a finite number of mirror retardations. This practical consideration dictates that the sampling interval determines the measured spectral range, whereas the finite number of retardation steps that are implemented defines the spectral resolution of the data. Because the mirror movement proceeds either continuously or in discrete steps to achieve a specified optical retardation, two classes of interferometers are applicable for IR imaging. We now describe the basic characteristics of each type of system.

CONTINUOUS SCAN INTERFEROMETER

In a common implementation of the interferometric process, the moving mirror is scanned at a constant velocity $v$. The rate of change (twice) of the optical retardation, termed the optical velocity, combined with the expression for the acquired interferogram, reduces to

$$ I(t) = \int_{-\infty}^{+\infty} B(\bar{\nu}) \cos(4\pi vt \bar{\nu}) d\bar{\nu}, $$

where $t$ is the time after zero retardation. When the sinusoidal variation of the interference pattern of a single wavenumber is compared to a standard expression
for a sinusoidal wave, \( \cos(2\pi f t) \), the characteristic frequency of the signal from that wavenumber can be determined. This characteristic frequency of the interferogram, termed the Fourier frequency, is given by \( f_\nu = 2v_\nu \). The specific detectivity of AC-coupled IR detectors depends on the Fourier frequency and provides a route to improved detection characteristics for the commonly employed HgCdTe (MCT) detectors.\(^2\) Figure 2 shows a schematic representation for mirror retardation as a function of time for a continuous scanning interferometry.

**STEP-SCAN INTERFEROMETER** In step-scan interferometers, as shown schematically in Figure 2b, the mirror retardation is quickly changed to a desired value and held constant (14–16). After completing measurements at this mirror retardation, the interferometer is stepped rapidly to the next retardation. In this manner, the interferogram is built point-by-point by recording the detector signal at discrete mirror retardations rather than discrete time intervals. One interferometer mirror may be moved while the other is held at a constant position, or the two interferometer mirrors may be moved in tandem (17) to yield a constant retardation. Although an intensity profile at the detector as a function of retardation may be acquired in this manner, two considerations dictate the step-scan method of interferogram acquisition. First, the mirror, or mirrors, require time to stabilize. Inertia and time constants of the control loop determine the response and, ultimately, the stabilization time required to achieve an optical retardation. This aspect defines, in part, the overall time required for an experiment. Second, the additional control mechanisms increase the cost and complexity of the step-scan instrument. Although the step-scan approach was favored in early FTIR configurations because of the availability of slow detectors, this mode is less favored for routine spectroscopic analyses with the modern advent of fast detectors and electronics. Virtually all FTIR spectrometers used for routine measurements incorporate single-element detectors and continuously scanning, rather than step-scanning, interferometers.

The utility of the step-scan interferometer justifies, however, the additional expense in certain cases. In particular, the retardation in a step-scan modality is decoupled from the time domain. A desired retardation can be held constant for
any length of time. This allows ample time for signal detection unlike the rapid scan system in which the detector response must be rapid enough to sample the fast changing mirror retardations without introducing significant sampling errors. Currently, step-scan interferometry is commercially available in research grade instruments and is employed generally in monitoring repeatable, transient events using sensitive, single element detectors in which the mirror retardation is held constant while a molecular event is recorded as a function of time. The retardation is then changed, and the event under study is again triggered. In this manner, the temporal evolution of signal from a transient molecular reorganization (∼10 ns time resolution) can be determined. This procedure forms the basis of time-resolved FTIR spectroscopy and has been widely employed over the past decade (15, 16).

INTERFEROMETRY IN IMAGING MODALITIES Unlike conventionally employed single-element detectors, the large number of elements in an FPA detector requires orders of magnitude larger data handling capacity. In particular, the development of IR spectroscopic imaging technology has generally matched during the past decade existing computer capacity. Although greater computational power is now available, data handling remains a major factor dictating the mode of data acquisition. For every interferogram element, the signal is integrated in the FPA, transferred to a computer, and stored digitally. This process is shown schematically for the step-scan implementation in Figure 3. With the many available detectors defined by the FPA, the time period required for data readout and storage (approximately milliseconds) exceeds significantly the actual signal collection time (approximately tens of microseconds). The first and, presently, the most widely used approach to FTIR microimaging spectrometers incorporates the step-scan interferometer. The ability to utilize a constant mirror retardation over extended time periods, which is afforded by the step-scan system, provides suitable time periods for signal averaging and for computer storage of the acquired data. An initial, small time delay prior to data acquisition allows for mirror stabilization at each interferometer step. The data acquisition and readout formats in the first generation imaging instruments were either spatially sequential (rolling-mode) or simultaneously detected (snap-shot) across the array. In either case, due to detector architecture, the array detectors introduced a large ratio of frame time-to-integration time (>100:1). The integration time, the number of coadded frames, and the number of interferometer retardation steps, which determines the desired spectral resolution, govern the overall time required for an experiment. Because signal integration affects the quality of the data, efforts are made to increase the ratio of the integration time to the total data acquisition time.

An imaging configuration utilizing a continuously scanning (18) interferometer has been proposed for detector arrays. Because a large number of detector elements generally precludes regular rapid scanning velocities owing to slow readout rates, many instrumental configurations employ a continuously scanning interferometer at slow speeds. Some manufacturers employ fast step-scan modes in which the
Figure 3  Data acquisition in an imaging configuration. The data collection process for imaging using a step-scan spectrometer and a focal plane array detector: The retardation is varied quickly (stepped) and held constant to allow detection and construction of the interferogram point-by-point. For each spectrometer step of time, $t_s$, there is a time delay, $t_d$, before collection is initiated to allow the spectrometer to stabilize. A number of frames are coadded at each step, each in time $t_f$. For each frame, the signal is integrated for a fraction of the frame time, $t_I$, and all the frames are read, averaged, and stored in time, $t_r$. (Adapted from Reference 38.)

mirror is partially stabilized to achieve the same retardation error as in continuous scan spectroscopy; this approach is termed slow-scan. Although slow-scan approaches appear to be similar to rapid-scan methods, they are unable to utilize the advantage afforded by coupled Fourier frequencies, as in rapid scanning. With the advent of faster FPA detectors, the utility of these slow-scan methods is limited and of little consequence in acquiring high signal-to-noise ratio (SNR) data. A generalized data acquisition scheme that permits true rapid-scan data acquisition for large arrays or higher mirror speed acquisitions from any size FPA detector has been proposed (19). The evolution of faster detectors will allow the full realization of the advantages of interferometry for large FPA detectors in the near future. Presently, only a small linear array detector achieves the rapid scan advantage, although attempts at employing larger, faster readout FPA detectors are on-going in both our laboratory and that of others. Compared to step-scan approaches, rapid scan data collection allows fast interferogram acquisition, as there is no requirement for mirror stabilization; however, the image stored per resolution element contains increased noise levels. For most FPA detectors available today, however, the motion of the moving mirror in a continuous scan mode does not allow the coaddition of frames per interferometer retardation step. The limiting SNR
achievable because of these variations in mirror retardation is given by

\[ \text{SNR}_{\text{max}} = \frac{4}{\Delta l \bar{\nu}_{\text{max}}}, \]

where \( \Delta l \) is the variation in mirror position and \( \bar{\nu}_{\text{max}} \) is the maximum wavenumber measured by the collection process. The positional stability for research grade spectrometers is typically on the order of 0.1 nm. Hence, over the mid-IR range, the limiting SNR determined by the spectrometer is \( \sim 10^7 \). Continuous scan spectrometers have also been used for data collection using an FPA (18). The achievable SNR is limited by the positional error \( \sim 10^{-25} \) nm of the moving mirror, although continuous scan interferometers offer a faster collection route compared to step-scan spectrometers. Although, random FPA noise is dominant, the error arising from the deviation in mirror position during frame collection is considered the next largest noise contributor. Hence, attempts at improving the SNR by in-scan coaddition may be frustrated by mirror positional errors. Faster detectors and detectors that can record at precise times will provide for in-scan coaddition in the near future. The primary advantage of continuous scan interferometry today lies in significantly reducing instrumentation costs compared to step-scan units and in achieving more efficient data collection because mirror stabilization times are eliminated.

**Infrared Microscopes**

IR microscopes (20) are similar to visible light microscopes but do not employ glass refractive elements because of the opaqueness of glass to IR light of wavelengths longer than \( \sim 5 \) µm. In contrast to conventional IR microscopes, which were designed for maximum light throughput, homogeneous illumination over the field of view is desirable for imaging microscopes. Other than this fine distinction, there is no difference between conventional and current IR microscope design. A diffuser (21), or diverging lens system, may be incorporated in the beam path to increase the spot size and/or to impart greater spatial homogeneity. A variety of mirrors are used to divert the beam from the spectrometer onto the FPA. In the transmission mode of sampling, shown in Figure 1, Cassegranian optics are used to focus light on the sample plane. A separate visible light source and associated optics are used to obtain standard optical microscopic images. Typically, the field of view for the visible path is greater than the IR beam path to allow precise sample positioning. An important difference between imaging microscopes and mapping systems is that the optical and IR paths need not be either parfocal or collinear. The image quality of the IR bright field image is sufficient for independently focusing the sample. A CCD camera is usually placed at the end of the optical train to digitally record visible images. A series of beam splitters or swing mirrors may be used to simultaneously or alternately view either the visible light image or IR bright field image. The similarities between the IR microscopy platform and an optical microscopy configuration provide hybrid design opportunities. An obvious
combination would involve both conventional optical visualization and spectroscopic imaging characterizations, which is achieved by incorporating an interferometer into standard optical microscope assemblies (22). Other instruments capable of hybrid analyses are slowly being made available.4

Detectors and Imaging

The type of detector selected for spatially resolved imaging measurements critically determines the speed of data acquisition, the quality of the recorded data, and the ultimate quality of the resulting chemical image. In particular, multichannel detectors dictate various approaches to data acquisition, along with implications with respect to the resulting SNRs of the recorded images. Descriptions of various detector classes with their theoretical performance parameters are presented below.

MAPPING WITH SINGLE-ELEMENT DETECTORS Because the design for imaging instruments evolved from that of conventional single-point microscopy approaches, we first review briefly conventional microspectroscopy instrumentation consisting of single-element detectors. More detailed descriptions of the technique are available elsewhere (25). FTIR spectroscopy became commercially widespread approximately three decades ago. Within ten years the technique was applied to microscopy, primarily because the advantages of radiation throughput, spectral reproducibility, and time averaging afforded by the method allowed the examination of small, spatial regions. In addition, the development of stable, sensitive, fast-response cryogenic detectors, which were introduced in the 1980s, allowed high fidelity measurements of spectral intensities (26). Today, these systems and their advanced versions are employed for microscopic IR analyses in several thousand laboratories around the world.

In single-detector element microspectroscopic instrumentation (27), modulated radiation from a rapid-scan interferometer is diverted to a set of optics that condense light to a prescribed spatial area. Using opaque apertures of controlled size to restrict illumination by the IR beam, well-defined spatial areas can be imaged. The aperture assemblies are fabricated from carbon black coated metal, although apertures fabricated from IR-absorbing glasses used in later microscope designs allow visible sample images to be obtained while recording IR spectra. To uniquely identify the sample area to be spectroscopically examined, a corresponding white light, optical image is also acquired with the parfocal and collinear IR and optical beam paths. Although this instrumentation is appropriate for studying small impurities or defects in microgram samples, the approach is of limited utility in obtaining quantitative information from large, heterogeneous samples. For mapping extended sample areas, an automated, programmable microscope stage capable of precise movements must be employed to maintain registration between the sample point spectroscopically observed and the resultant acquired spectrum.

Radiation transmitted through an aperture results in the formation of a diffraction pattern that allows light from outside the masked region to impinge upon the
detector. This stray light may compromise spectral content as far as 40 µm away from the sample point of interest (28). This problem is circumvented by employing a second aperture in tandem to further reject radiation. Although the radiation point spread function (a multiplicative property) narrows, a theoretically higher resolution capability is achieved with, unfortunately, a further loss of signal. Therefore, the spectral SNR degrades, necessitating larger acquisition times to improve data quality. Alternatively, large apertures are required for permitting greater radiation throughput, which then results in a trade-off between the time required for accumulating data and a desired spatial resolution. Thus, spatial fidelity and spectral quality are intimately linked in single element systems incorporating apertures; a balance between the two is required to maximize the efficacy of the sampling protocol.

For a conventional wide-beam single-element detector in an FTIR spectrometer incorporating a Michelson interferometer, the SNR achieved in time, $t$, is represented by (29)

$$\text{SNR} = \frac{U_0(T) \Theta \Delta \tilde{\nu} \xi}{\text{NEP} \theta^{1/2}}.$$ 5.

where $U_0(T)$ is the spectral energy density at a given wavenumber, $\tilde{\nu}$; the Noise Equivalent Power (NEP) is the ratio of the square root of the detector area, $A_D$, to the measure of its sensitivity, $D^*$ (specific detectivity); $\Theta$ is the throughput; $\Delta \tilde{\nu}$ represents the spectral resolution; and $\xi$ is the spectrometer efficiency. This relation is a measure of the ratio of signal to the root-mean-square (rms) noise for single-beam spectra. For examining the SNR for FTIR microspectroscopic measurements, using a single-element detector and apertures to delineate areas from which spectra are obtained, a multiplicative correction factor, $\delta$, was proposed to yield the modified equation (20),

$$\text{SNR} = \frac{U_0(T) \Theta \Delta \tilde{\nu} \xi}{\text{NEP} \delta t^{1/2}}.$$ 6.

IR microscopes that employ apertures for mapping are designed after consideration of the largest aperture that may be employed during the course of experiments. Typically, the largest apertures are approximately on the order of 100 × 100 µm$^2$. Thus, the spot size at the sample plane allowed by the optics is fixed at a larger spot size (hundreds of micrometers in diameter) to account for all aperture sizes that a user may conceivably utilize. With the effective area available for light throughput determined by the area of the aperture opening, the flexibility to employ large aperture sizes implies that the efficiency of light utilization is anywhere from 0.25% to 100%.

**Raster Scan Imaging with Linear Array Detectors** Although single-element microspectroscopy provides the capability for obtaining spectra from small spatial regions, poor SNR characteristics, diffraction effects, and stray light issues resulting from the use of apertures, as discussed above, limits the applicability
of these methods. Further, the point-mapping approach results in large collection times and poor utilization of the hundreds of micrometers diameter spot sizes produced at the focal plane. An approach to resolve many of these issues has recently been implemented (30) in which, as an improvement over single-element detectors, a linear array detector is employed to image an area corresponding to a rectangular spatial sample region. Unfortunately, the imaged area may not be sufficiently representative of the distribution of chemical species in the sample, and, hence, a technique to image larger areas is required. Thus, the linear array is moved precisely to sequentially image a selected, relatively large spatial region of the sample. This is referred to as push-broom mapping or raster scanning. The process is conceptually similar to point mapping but takes advantage of the multiple channels of detection. Imaging a large sample area is immediately faster by a factor of $m$, for a linear array containing $m$ detector elements.

The small number of detectors comprising the linear array allows fast readout times and, with the interferometer being operated in a continuous scan mode, instrumentation costs decrease over the use of a step-scan interferometer. By combining a small multichannel detector with rapid-scan spectroscopy to provide mirror scanning in a favorable Fourier frequency regime and by utilizing frequency domain filtering, higher SNR data can be obtained. There is little difference in the theoretical expression for the SNR of a single pixel in an array detection system when compared to a mapping system without an aperture. Though detectors for point-by-point mapping are typically 100–250 µm in size, modern array detector pixels are sized in the tens of micrometers. Hence, a primary advantage arises from the detector size in the theoretical SNR. Recalling that the SNR is proportional to the inverse of the square root of the area of the detector, we note that a decrease of an order of magnitude in the dimensions of the detector provides a sizeable advantage. Because employing a linear array eliminates the need for apertures, a secondary advantage arises from not decreasing radiation throughput. In contrast to geometrical apertures, the nominal spatial resolution for array detectors is determined by the system’s optics. In a recent commercial implementation,5 detectors are 25 µm in size with the instrument being operated at either a 1:1 magnification or 4:1 magnification to provide a 25 µm or 6.25 µm spatial resolution. In another commercial development utilizing array detectors, manufacturers operate on the principle that the true resolution is wavelength limited with the nominal spatial resolution for their instrument being less (∼19 µm).6 These magnification ratios can be readily achieved by employing available aberration-free IR optics. However, the nominal spatial resolution must not be mistaken for the true achievable spatial resolution; that is, the actual spatial resolution is determined by the optics, sample, and detection choice configured for the system. Though the resolution is limited for single-aperture systems in mapping instruments by diffraction effects, the pixels in an array detector can be thought of as virtual apertures that are subject to the same optical principles. Pixel purity is wavelength dependent, and a detailed treatment must include sample effects. The only physical method currently available to achieve limited spatial resolution across the entire spectrum in
conventional microscopy configurations is to employ two apertures in the confocal sense (7).

The configuration of the instrumentation incorporating array detection is similar to that required for single-element IR microscopy, but several important changes must be made. First, no apertures are required. Second, the spot size is optimally matched to the size of the detector array. Analogous to the point-mapping approach, the optical and IR paths are required to be collinear and parfocal. A visible image is usually referenced to acquire IR data. Further, a precision stage that reproducibly steps in small spatial increments is required for any mapping larger than that achieved by a single row of detectors. Once the sample is manually positioned, a visual image can be constructed by using the visible light camera and by moving the sample stage. The sample area from which IR spectroscopic data is to be determined is therefore delineated and spectroscopic acquisition may then be initiated.

GLOBAL IMAGING WITH TWO-DIMENSIONAL ARRAY DETECTORS

The state-of-the-art in FTIR microspectroscopic instrumentation is the combination of an FPA detector and an interferometer (12). FPA detectors consist of thousands of individual detectors placed in a two-dimensional pattern. Compared to a linear array, the increase in the number of detectors increases the multichannel advantage; for example, a $p \times p$ pixel FPA detector provides a $p^2$ time savings compared to a single-element detector and a $p^2/m$ time saving compared to a linear array detector, where $m$ is the number of detector elements in the linear array detector. For a $256 \times 256$ element detector compared to the single-element case, the advantage is a factor of 65,536; in comparison to the 16-element detector, the multichannel advantage is a factor of 4096. An FPA matched to the characteristics of the optical system is capable of imaging the entire field of view afforded by the optics and of utilizing a large fraction of the IR radiation spot size at the plane of the sample. Among the available various IR microspectroscopic methods, FPA-based global imaging represents the most versatile approach for recording spectral intensities over large sample areas.

The typical configuration of an FTIR microimaging system interfaces an interferometer to an IR microscope, yielding a specified spot size at the sample plane. This spot, which is typically hundreds of micrometers in size, is magnified by the optical train and imaged onto an FPA that is tens of millimeters in diameter. The spatial resolution is thus controlled both by the optics that determine magnification and the size of an individual detector element on the FPA. Although the nominal spatial resolution is determined by the optics and the detector, the actual resolution limit usually depends upon the diffraction limit of the spectral wavelengths of interest incident upon the sample. An interferometer coupled to an FPA detector provides spatially resolved images across a wide field of view in data collection times comparable to the time required to record a single spectrum at a single spatial location using a single-element detector. This enormous advantage in acquisition time allows both spatially and spectrally specific analyses. For example, a single spectrum from a small sample region, comparable to a single FPA pixel, may be
examined or a spectroscopic signal from a spectral feature representative of the entire field of view can be imaged. Due to the considerable reduction in experimental recording times both the imaging of large areas of static samples (32) and the examination of dynamic processes is feasible and rapid.

The individual detector sizes comprising the FPA grid are typically tens of microns with the total detector chip being a few mm in size. Several types of IR-sensitive materials have been incorporated onto detector arrays; indium antimonide (InSb) and mercury-cadmium-telluride, HgCdTe (MCT), silicon arsenide (Si:As) (33), and barium strontium titanium (BST) (34). Mid-IR imaging using MCT FPAs (35) has been the most popular in terms of number of studies owing to its availability at a moderate cost and, most importantly, ability to access the IR fingerprint region of the spectrum. The other detectors have specific advantages: near-IR arrays present advantages in terms of less stringent sample preparation and high optical fidelity; Si:As detectors provide a wide spectral range, whereas BST arrays do not require cooling. The mass production of arrays used strictly for thermal imaging is expected to spur developments and result in lower costs for arrays applied specifically to IR imaging. Three types of detectors used in our laboratories, along with their typical properties, are listed in Table 1.

Most arrays used in the mid-IR region are hybrid array systems consisting of material sensitive enough to the IR radiation wavelength bandpass to be detected. Thin, IR-sensitive material is mated to an underlying substrate either by an indium “bump” bond or by epitaxial growth. The underlying substrate is typically a material that has capable readout technology (for example, a silicon CCD); the bonding interface simply acts as an electrical contact between the detector element and the pixel readout circuitry, which is placed beneath. Currently, the size and sophistication of hybrid IR-sensitive devices typically lag behind that of CCD devices. Most hybrid devices require cooling for higher sensitivity and necessarily operate at

<table>
<thead>
<tr>
<th>Property</th>
<th>Array</th>
<th>Small format MCT</th>
<th>Medium format MCT</th>
<th>Small format InSb</th>
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<tr>
<td>Pixels (x,y)</td>
<td></td>
<td>4096 (64 x 64)</td>
<td>65,536 (256 x 256)</td>
<td>16,384 (128 x 128)</td>
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<td>Spectral range (µm)</td>
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<tr>
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<td>∼800 × 800</td>
<td>∼400 × 400</td>
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<tr>
<td>Typical data set sizea (KB)</td>
<td></td>
<td>16</td>
<td>256</td>
<td>64</td>
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</table>

aFor one spectral data point using the entire array.
liquid nitrogen temperatures to prevent the thermally generated electrical flux from overwhelming the generated spectral signals. The most common format in current use is the 64 × 64 pixel MCT detector. A medium-format (256 × 256 pixels) array is also available in some laboratories. Very-large-format cooled arrays are still in developmental stages. It is expected that hybrid arrays approaching 1024 × 1024 pixels will be available soon. It has also been reported that a proposal for the Next Generation Space Telescope (NGST) calls for a 2048 × 2048 element MCT array (36). Hybrid arrays operating at low temperatures are expensive to produce and maintain and may be unwieldy for some applications. For bump-bonded arrays, the repeated thermal cycling of cooling to low temperatures and then warming causes debonding and edge delamination leading to inoperable pixels. Hence, efforts have been made to use uncooled arrays for FTIR imaging.

One report (34) using an uncooled BST array for mid-IR imaging employed a standard spectrometer-microscope configuration. The BST array has a high pixel density, lower fabrication, and lower installation cost compared to the MCT arrays used commonly. These arrays could be helpful in applications where flexibility and low maintenance is desirable. Given the larger formats that are available and the generally constant costs associated with manufacturing and assembling a BST chip and its detector body, the cost per pixel of a BST array is approximately 30 times less than that of a typical 64 × 64 MCT detector. Moreover, BST arrays are sensitive to longer wavelengths than MCT arrays (∼2–14 µm versus ∼2–11 µm), which is useful in examining the IR fingerprint region of many compounds. However, such arrays typically lack the sensitivity of the MCT arrays at low temperatures, which is the primary argument against their widespread use. Thus, several alternatives are clearly available to the practitioner interested in IR spectroscopic imaging techniques. Trade-offs in cost, size, flexibility, sensitivity, and data collection speed typically must be considered before selecting a specific array for an imaging application.

Because the FPAs used for spectroscopic imaging today were originally developed for military purposes to image heat radiated over broad spectral ranges, they have been optimized for that function. Further detector modifications required for optimal microscopic imaging, however, include the incorporation of a bandpass filter at the outer face while the inner housing contains a cold shield close to the detection array, as shown in Figure 1. The cold shield (37) and filter work in tandem to reject both stray radiation and radiation outside the wavelengths of interest. The bandpass filter can be changed to precisely cover only the desired range for data collection, thereby eliminating fold-over perturbations that may occur during data acquisition. Depending on the level of throughput in the system, the variation in integration time for the FPA can be changed to maximize the signal on a case-by-case basis. Further fine tuning can be carried out by adjusting the electronic amplification of the signal before processing by applying a multiplicative gain. Thus, the hardware is optimized for the highest signal levels and minimum noise levels. These adjustments, however, are usually preliminary and cannot be changed during the data acquisition process. Several approaches
incorporating dynamic changes to these parameters (vide infra) to obtain data at higher spectral fidelity have been proposed based on an understanding of the theoretical expression of the instrumentation performance.

In extending the SNR analysis from a single-element detector to an imaging FPA detector, the theoretical expression for the SNR of a single pixel on the FPA was proposed (21) as

$$\text{SNR} = \frac{0.12\pi A \left( 1 - \sqrt{1 - (\text{NA})^2} \right) U(T) \Delta \nu D^*}{\sqrt{\lambda D} t_{1/2}},$$

where NA is the numerical aperture and A the area of the sample imaged onto the pixel. The correction due to apertures is not required and the efficiencies were taken to be commonly encountered values. The minimum collection time to obtain one interferogram data cube is given by $t = n_s [t_d + n_f t_f + t_r]$, where $t_f$ is the time required to acquire each frame. The collection time not only depends on the number of spectrometer steps (a function of spectral resolution and range), as expected, but also on the camera electronics and the number of frames used to obtain the average frame data. The frame rate $(1/t_f)$ is typically determined by signal and dynamic range considerations. The readout time is primarily determined by system electronics. Although the collection time is the time required to obtain an interferogram, the photon flux detection time (i.e., staring time) is the signal integration time, $t_s$, which is included in the theoretical expression via a data acquisition efficiency factor of

$$\text{SNR} = \frac{U(T) \Theta \Delta \nu \xi}{\text{NEP}} \left[ \frac{n_f t_f}{t_d + n_f t_f + t_r} \right]^{1/2},$$

where $\xi$ incorporates the combined efficiency of the spectrometer, beam diverting optics, and microscope optical elements.

The scaling of the SNR with number of coadded frames for a given experimental time is not a simple one. The effect of changing the number of frames on the SNR in a step-scan configuration is given by an acquisition ratio, $\varepsilon_C = \left[ \frac{n_f t_f}{t_d + n_f t_f + t_r} \right]^{1/2}$, which scales as $\sqrt{n_f}$ in the limit that few frames are added and approaches a constant value when many are added. The efficiency can never be larger than that dictated by the system electronics, that is, the square root of the ratio of integration time to frame time, which is termed the limiting acquisition ratio. The acquisition ratio is also a measure of the efficiency of the data acquisition protocol and can be used as a stand-alone comparison metric for different protocols. The first generation of imaging systems employed detectors with $(t_f/t_s)$ ratios of $\sim 0.01$ but newer detectors are fast approaching ratios of 0.99. Further, continuous scan instruments do not require either a delay time or a readout time; hence, the additional SNR loss due to detector inefficiency is gradually disappearing. The deviation from predicted values for coadding more than a few tens of frames in the step-scan configuration (21) is also well-known and arises from low frequency components of FPA noise.
The two different collection strategies that yield high SNR data, namely frame coaddition and image coaddition, may be combined to achieve a net higher SNR than that by simply using either one or the other method. In theory, if there is unlimited time available to obtain data, the SNR determined by instrumental limitations (stability and data capacity considerations) is obtained. In practice, the time available for an experiment is limited, and the highest possible SNR is desired. Conversely, a specified SNR may be required for obtaining data in the shortest possible time. Hence, a plot of SNR achievable against experimental time for different collection strategies, termed characteristic plots, has been proposed to determine the optimal operating point (38). Although the idea was proposed for step-scan data acquisition, the principle is valid for in-scan coaddition in a continuous scan acquisition mode, as well.

Several approaches have been suggested to increase the SNR of imaging data based on the theoretical understanding of the SNR expression by changing the data acquisition configuration or by changing the parameters of the FPA during data acquisition. One example of the former approach is the instrumental proposal of a staggered step-scan method, whereas an example of the latter method is gain ranging. The fraction of frame time used for integration and readout, determined by the array characteristics, is usually fixed either by the manufacturer or by dynamic range considerations. Because the time required by the spectrometer to achieve a specific retardation is assumed to be fixed, there is little flexibility for further improvement in the data collection process. A new method, the staggered step-scan approach, however, proposes increasing the acquisition ratio, $\varepsilon_c$, through a more efficient data collection protocol (39). The usual step-scan approach results in significant dead times. During the interferometer mirror(s) stepping and stabilization stages, the FPA is idle, whereas during the FPA readout and storage stages, the interferometer remains idle. Though seemingly interdependent and sequential, these two events (interferometer stepping and FPA readout) are actually independent processes, and the time required for the two steps may be combined into one step using an external synchronization clock to achieve higher efficiency. It was proposed that while the interferometer is performing the step process, the array is read and stored. Thus, the larger of $t_d$ and $t_r$ determines the total dead time for the process.

In the case of gain ranging, the amplification of the signal prior to digitization reduces noise in Fourier transformed spectra (40). FPA detectors have markedly different electronic characteristics compared to single-element detectors. Hence, the gain ranging concept, as associated with conventional single-element detectors, cannot be applied directly to FPA detectors (vide infra). Second, because each pixel in the FPA is a unique detector in its own right, both small differences in manufacturing processes and the spatial position of a pixel on the array with respect to the intensity profile of the incident beam lead, invariably, to pixel-to-pixel variations in their characteristics. Hence, the gain ranging procedure must account for the unique response functions of the FPA detector elements, as well as the (spatial) heterogeneity of that response. The theoretical limits of the SNR for
an FTIR spectrometer can be improved by selectively increasing the amplitude of the response by a multiplicative gain factor for certain regions of the interferogram (40). Similarly, for FPA detection, an increased gain factor applied to the entire interferogram would ordinarily exceed the saturation limit of the ADC at the centerburst. Hence, data around the centerburst are collected at a lower gain in comparison to the rest of the interferogram. The selectively modified portions of the interferograms are then renormalized by the inverse of the gain factor before Fourier transformation to wavenumber space. The gain ranging process results in a multiplicative improvement factor in SNR at a pixel and is given by a sum of amplification weights for every point in the interferogram, namely (41),

\[
S_I = \left[ \left( \frac{2r_p + 1}{n_p} \right) + \left( \frac{1}{R_g} \right)^2 \left( 1 - \frac{2r_p + 1}{n_p} \right) \right]^{-1/2}.
\]

where \( S_I \) is the ratio of the SNR of the interferogram collected using gain ranging to the SNR of the interferogram collected without gain ranging for the same detector under equivalent data acquisition conditions. \( R_g \) is the ratio of the amplification factor for the signal at the high-gain setting to the signal at a low-gain setting; \( r_p \) is the number of points on either side of the centerburst collected at a lower gain; and \( n_p \) is the total number of data points in the interferogram. Because the SNR of single-beam and absorbance spectra are proportional to the SNRs of their interferograms, the above expression is a measure of the improvement for all spectral data when comparing the two data sets. Equation 9 can also be written in the original notation of Hirschfeld’s proposal of gain ranging (40) as,

\[
S_I = \left[ \left( \frac{2r_p + 1}{n_p} \right) + \left( \frac{1}{R_g} \right)^2 \left( 1 - \frac{2r_p + 1}{n_p} \right) \right]^{-1/2}.
\]

where, compared to our method, \( d = \left( \frac{n_p}{2r_p + 1} \right) - 1 \), and \( n = 2 \). Upon application of the principle, the benefits of gain ranging were limited by the nonlinear noise characteristics with increasing gain of the miniaturized amplifiers in the FPA. Hence, the expression was modified to incorporate the unique effects of noise as a weighted factor similar to that of the gain factor,

\[
S_I = \left[ \left( \frac{2r_p + 1}{n_p} \right) + \left( \frac{N_{D,g}}{R_g} \right)^2 \left( 1 - \frac{2r_p + 1}{n_p} \right) \right]^{-1/2}.
\]

The expression above explicitly provides for the examination of variation of \( S_I \) with the number of points comprising the gain ranging radius, \( r_p \). The optimal conditions for gain ranging were discussed with the procedure being successfully implemented in an FTIR step-scan interferometer-microscope assembly incorporating FPA detection for imaging sections of biological tissue. However, these concepts are also relevant to continuously-scanning imaging spectrometers, where
the SNR of data collected per interferogram cycle is typically much lower. Routine use of the procedure does not involve any increase in data acquisition times and involves only a negligible increase in computational time, and provides a SNR of \(\sim 45\%\) greater than that obtained without gain ranging.

Efforts to improve the SNR of imaging data can be divided into three categories: hardware modifications, data acquisition protocol modifications, and post-acquisition data processing techniques. Hardware modifications have included the use of bandpass filters to restrict incident radiation to bandwidths of interest, diffusers to reduce spatial inhomogeneity of intensity distribution (21), and the use of an optimally-sized cold shield to reject thermal background and unmodulated radiation (37). We anticipate that major improvements in detector technology, spurred by a better understanding of the SNR expression, will lead to higher fidelity imaging in the near future. Because numerous data acquisition strategies and data analysis algorithms are currently being developed, activity in instrumentation development for IR imaging will be vigorous in the foreseeable future. In particular, attempts to obtain faster imaging through increases in array data readout electronics should significantly increase the performance of the next generation of FPA detectors. Although improved data acquisition techniques have included time-averaging data cubes (21), pseudo coaddition (42), and staggered step-scan collection (39), post-acquisition noise reduction has been achieved by within-image coaddition (42), interferogram median filtering (43), and mathematical methods of noise reduction (44, 45). Though all three routes to high fidelity imaging are important and have enjoyed varying degrees of success, any method that requires neither expensive hardware modifications nor involves major changes in collection parameters is most relevant to practitioners of the technique. Methods that provide an improvement in the SNR of the collected data without increasing the data acquisition time are particularly attractive.

SAMPLING TECHNIQUES, ADVANCED DATA COLLECTION METHODS, AND NUMERICAL PROCESSING APPROACHES

Sampling Techniques

A variety of sampling techniques have been developed for IR spectroscopy. This extensive knowledge base has been utilized in spectroscopic imaging experiments. Transmission was the first technique used for imaging and is one of the most popular techniques used to date. Typically, thin sections or films are laid on or sandwiched between substrates that transmit IR radiation. Reflection techniques are used for samples deposited on IR-reflecting substrates. Hot stages (46) and other accessories can be added, as with any microscope, to control the sample environment as desired. Three sampling techniques require further attention: attenuated total reflection (ATR) imaging presents significant potential for increasing spatial resolution and for sampling thick samples; gradient sample imaging allows for sample or property
variations in one dimension; and array sample imaging allows for variations in two dimensions. Relatively straightforward transmission methods have traditionally provided the best SNRs and means of quantitative examination of vibrational modes, as dictated by the Beer-Lambert law. For most mid-IR vibrational bands, however, the thickness of the sample in the transmission configuration is limited to a few micrometers, requiring sample microtoming into thin sections. Diamond anvil cells, used in transmission, may be employed to study thicker materials and present a ready route to examine samples under pressure (S.W. Huffman & I.W. Levin, unpublished data). Because pressure may alter the molecular or spatial structure of the sample, this technique must be employed carefully. Though transmission and reflection modes are straightforward in their analysis, ATR sampling techniques for imaging require additional description.

ATR IMAGING In an ATR mode, the sampling volume is determined by a high refractive index material (the ATR crystal) used to reflect totally light incident at the sample and crystal interface. The electric field of the propagating radiation in the crystal penetrates into the region of the contiguous sample, which is of lower refractive index, through an exponentially decaying evanescent wave. The depth of penetration is provided in terms of the decay characteristics of the evanescent field and is a function of the incident angle, refractive indices of the crystal and the sample, and the radiation wavelength. By detecting the sample attenuation as a function of wavelength, an ATR-IR spectrum is obtained. Conventionally, the path length is maximized by multiple internal reflections in the crystals, but a single reflection is employed for imaging to preserve spatial specificity. Thus, typical attenuation path lengths depend on the penetration of the evanescent wave and range from several tenths of a micrometer to several micrometers. For strongly absorbing materials, this limited path length within the sample allows linear absorption for most vibrational modes in the mid-IR region. Therefore, the ATR-IR sampling technique requires minimal sample preparation prior to imaging.

Although the basic principle of the sampling methods is the same, several different ATR-IR accessories have been employed in a variety of configurations to yield images of different size and spatial resolution. Two modes of imaging using an ATR crystal, in particular, have become popular. In the first mode, lower resolution images have been acquired from larger samples using a “macrochamber,” which is essentially an inverted pyramid ATR accessory (48). Because this configuration enables data acquisition from large samples without extensive preparation, it has often been termed the large sample mode. In a diametrically opposite approach, a micro-ATR-IR objective with a germanium crystal as the ATR material and an IR microscope has been employed. In addition to sampling advantages, ATR imaging affords the attractive possibility of achieving enhanced spatial resolution, as the crystal acts also as a beam condensing unit. This configuration can provide improved lateral resolution compared to transmission imaging (49). Using this approach, individual blood cells (49) and polymeric structures (50) were imaged at increased spatial resolution over transmission methods.
The ATR technique is versatile not only in its optical configuration and in the types of samples that can be analyzed, but also in the manner in which samples are prepared. A recent report (52) has described the production of samples on an ATR accessory, followed by measurements of changing chemical profiles (53). Typically, it is difficult to achieve good contact between the sample and the crystal, and pressure, limited by the strength of the crystal, must be applied to solid samples to achieve good contact. The issue of contact reproducibility has also been investigated and can be enhanced by the introduction of a noninteracting liquid (54). Because materials to be imaged are typically heterogeneous, attempts to enhance contact between the sample and the ATR crystal may result in a distortion of the sample microstructure. The challenge is then to maintain the native microstructure while enhancing contact with the application of tolerable force. We anticipate that the technique is ideally suited to examinations in process control and in the biomedical analyses of intact tissue, where a minor deformation of tissue does not compromise its structural integrity. Unfortunately, the resolution enhancement offered by ATR microimaging is not sufficient to analyze polymer blends or polymer-polymer interfaces in which important compositional variations occur at below the 1 µm scale.

Data Acquisition of Dynamic Processes

TIME-RESOLVED FTIR SPECTROSCOPIC IMAGING

Although the FPA multichannel detection advantage in FTIR spectroscopic imaging allows spatially resolved spectral measurements from extended sample areas, readout of the large number of detection channels precludes the examination of many dynamic processes as they necessitate longer data acquisition times in comparison to single-channel detectors. For repeatable, reversible events, time-resolved spectroscopy (TRS) in an imaging modality has recently been introduced (55) to access noninvasively, at the ∼10 ms timescale, observations of dynamical processes in heterogeneous materials whose characteristic times are of the order of hundreds of milliseconds (56). An extensive time-resolved (TR) literature in terms of conventional spectroscopic instrumentation, theory, and techniques exists for examining the dynamics of repetitive events (57–60). In particular, TR-FTIR spectroscopy involving stable step-scan (16) interferometry (61, 62) has been utilized to monitor dynamic phenomena in wide-ranging systems, including the dynamics of biological molecules, reaction and binding mechanisms, rheo-optics of polymeric materials, and laser emissions. The reorientation dynamics of liquid crystalline materials responding to externally applied electric fields also is an area of considerable activity (63–68).

Polymer dispersed liquid crystals (PDLCs) (69, 70) are well-suited systems for demonstrating specifically the capabilities of TR IR spectroscopic imaging (55). Having a wide variety of applications in the electro-optical industry, these systems consist of liquid-crystalline (LC) domains dispersed in an isotropic polymeric matrix. The average orientation director of any dispersed domain is determined by the local liquid-crystal orientations of the constituent molecules. Upon application of
an electrical potential, all LC directors experience a rotational torque reorienting the molecule along the electric field; hence, for light incident along the direction of the applied field, the refractive index of the liquid crystal matches the ordinary refractive index of the polymer, resulting in a transparent composite. TR-FTIR techniques applied to PDLC switching studies (71–73) are particularly attractive as they allow a correlation of electro-optical properties with specific molecular motions. In employing conventional vibrational spectroscopic techniques to study molecular dynamics, the chemical selectivity is usually high, but the spatial specificity tends to be low or nonexistent. Thus, studies of PDLCs using TR-FTIR spectroscopic techniques have either examined the average behavior of the composite (71, 73) or have consisted of describing a few discrete points across the entire field of view of the composite (72). The latter study, for example, obtained the response sequential responses of the droplet bulk region and the matrix using a procedure involving a point-mapping spectrometer equipped with a microscope and apertures. Point-mapping methods, however, would require days to characterize a 100 $\times$ 100 $\mu$m$^2$ area, virtually negating attempts toward recording simultaneously both spatially and temporally resolved data. Hence, IR spectroscopic imaging presents a unique opportunity to study in situ the dynamics of systems that contain spatially distinct domains. An implementation has been presented in which an FTIR imaging system allows simultaneous spectral, microscopic spatial, and millisecond temporal characterizations of the molecular dynamics in a PDLC (55). In addition to the usual effects of confinement, liquid-crystal motion within domains was found to be influenced by the relaxation dynamics of the polymer. Differences could be clearly observed in the boundary-rich pixels compared to those in the dispersion bulk and the matrix.

The time resolution in TR imaging approaches, as with many other TR spectroscopic approaches, is generally governed by the rate of data acquisition. For the FPA detector, this rate is determined by the frame rate. Due to the large readout requirements, the time resolution constraint generally defers the study of faster dynamical events until appropriate array detectors become available. A close examination reveals, however, that the origin of the lengthy time interval between signal sampling by the FPA, or low frame rate, arises from the extended time required to read intensity values for every pixel. A schematic diagram of the typical data acquisition process for IR FPA detectors is shown in Figure 4b. The frame time is largely composed of a readout time during which accumulated detector signals are transferred to digital storage while the integration time is usually small. A generalized implementation of TR spectroscopic imaging that overcomes the limitations imposed by the frame rate and, instead, allows temporal resolutions to be determined by the integration time alone was recently presented (74). For current FPA detectors, this represents a potential improvement of up to two orders in magnitude compared to previous approaches to TRS imaging. In this alternative implementation, the moving interferometer mirror is stepped rapidly and held at a specific position resulting in a constant optical retardation. The mirror is sequentially moved to achieve successive retardations, as shown in Figure 4a, for
Figure 4  The interferogram is obtained, in the step-scan mode, by observing the magnitude of the signal at a constant retardation. In step-scan, time-resolved spectroscopy cyclic events are excited by a stimulus and the intensity of the interferogram, as a function of time, is recorded for every optical retardation. The characteristic profile of fast events may be measured by stimulating multiple times to obtain a composite profile that contains a sufficient number of measurements to reproduce accurately the response.

recording the interferogram one resolution element at a time. At every optical retardation, molecular reorientations are initiated in the composite with the resulting IR intensity being recorded as a function of time over the field of view of the array detector. As a result, a series of interferograms, corresponding to a specific spatial area on the sample and to specified intervals of time with respect to the initiation of the dynamic event, are obtained. These interferograms are then reorganized into imaging data sets registered to a specific time period in the lifetime of the event.
FTIR SPECTROSCOPIC IMAGING FOR KINETICS  As opposed to TR imaging for repeatable events, transient events can only be monitored by fast imaging approaches. With current detection systems, the transient process must be several seconds long, almost three orders of magnitude longer than one that is amenable to TR imaging analyses. The concept of following the kinetics of processes with spatial visualization has been developed for monitoring diffusion (75, 76), curing state, kinetics (77–79; R. Bhargava, W. Noobut & J.L. Koenig, unpublished data; R. Bhargava, S.W. Huffman, I.W. Levin & S.Q. Wang, unpublished data), crystallization, and lipid biophysics (82). The implementation is straightforward: Data is continuously acquired for a specified period of time and the resulting spectral data sets can be employed to extract temporal profiles for revealing the evolution of structural changes and sample chemistry. The fastest rate of change that can be monitored is suggested to be one that barely changes the information content of a pixel in the time that data is acquired. For one-dimensional diffusion, for example, the maximum rate of diffusion that can be accurately measured can be related to the time required for data acquisition as

\[ t_e \sim \frac{d^2}{D}, \]

where \( t_e \) is the time required to acquire a single spectral data set, \( d \) is the spatial resolution, and \( D \) the diffusion coefficient. The dissolution of polymers by liquids, which has applications ranging from drug delivery to microchip fabrication, has been a particularly fruitful area of study with FTIR imaging. Because continuous scan spectrometer-based imaging systems are typically faster at collecting data compared to step-scan-based systems, they offer an ideal route to the acquisition of kinetics data. An ensemble of sequentially collected data sets may then be coadded to yield low noise, time-averaged data in which the advantage regarding data collection speed is lost but higher SNR data are obtained. This is in contrast to the measurement of reversible dynamics using the TR approach (above), in which the SNR of data can be increased by coaddition.

Spectral Data Processing

PRELIMINARY SPECTRAL PROCESSING  Although interferograms are acquired, chemical identification or quantification using IR vibrational signals, however, are usually conducted using absorbance spectra. Hence, fundamental operations include Fourier transformation of the interferogram for every pixel, pixel-by-pixel ratioing of the resulting single-beam spectrum with a similarly acquired background single-beam spectrum, and, finally, baseline corrections of the absorbance spectra. Digital water vapor subtraction may be conducted to remove the effects of changing atmospheric conditions and to enhance the visualization of small spectral features in the sample. These operations are widely practiced in conventional spectroscopy. Some synergy may be derived from the large numbers of spectra in an imaging configuration. For example, the average of several spectra may be employed to
obtain phase correction information. Further, faster Fourier transformation may be facilitated by compact data handling; although these advantages are relatively small, the computation of absorbance spectra from interferogram space represents a major effort.

A major difference between all other forms of FTIR spectroscopy and FTIR spectroscopic imaging is the extremely large volumes of data that are to be manipulated in the latter mode of data acquisition. Although mapping data sets typically involve several megabytes of data, a user may acquire tens of gigabytes of data per hour using, for example, a $256 \times 256$ element FPA. Human comprehension of such a large volume of data in its entirety is not only impossible, but also undesirable. Hence, the most common operation is to reduce the data in some manner to allow compact visualization of desired features. For example, spectra may be extracted from specific sample regions or simple spectral intensity visualizations in the sample plane may be afforded by univariate plotting of absorbance. Because the information content of the data generally decreases as the complexity of the representation decreases, the best data extraction methods cannot be determined a priori but are implemented on a case-by-case basis. The most common representations either visualize the two-dimensional distribution of the intensity of a specific vibrational mode over the field of view, project a limited number of spectra from any desired sample region or microdomain, or present statistical distributions of a chemical species. Often, several data analysis approaches are employed in tandem to provide an effective visualization of the distribution of chemical species. In general, some combination of both spatial and spectral specificity is usually required. A preliminary data analysis is usually followed by specific data extraction procedures ranging, for example, from the relatively simple extraction of one-dimensional profiles for diffusing species’ measurements to complex multivariate analysis for determining tissue pathology.

NOISE REDUCTION Another data processing facet not commonly encountered in conventional IR spectroscopy is the necessity to reduce noise in the data set. The low SNR in FTIR images arises from the inherent limit of measuring a small radiation flux, from the characteristics of the FPA and from the choice of data acquisition schemes. Although efforts to improve FPAs for spectroscopic imaging have been suggested and advances are constantly being made, the modification of data acquisition methods is an area of considerable attention (discussed previously) that is often coupled to detailed mathematical and spectral analyses. For example, spectral coaddition in imaging systems is not straightforward. A common data acquisition approach has been to coadd as many frames as possible during the available experimental time. Though this approach results in significant gains, as expected, the SNR, as a function of coadded frames, achieves a plateau (21). Thus, after the coaddition of a limited number of frames, the benefits are relatively minor compared to a large expenditure in experimental time. Toward increasing the fidelity of the data, the coaddition of complete image data sets was shown to be effective; an optimum sampling strategy was derived by combining both frame
and image data set coaddition. Though image coaddition results in improved SNR characteristics, the process involves a $2n^2$ increase in collection time for a SNR improvement factor of $n$.

The above data acquisition approach rules out image coaddition as a technique for increasing the SNR either for real-time imaging of rapidly changing systems or for the routine examination of large numbers of samples. Hence, a low noise single-beam background ratioed to a single sample single-beam image was shown to exhibit lower noise characteristics, allowing for imaging measurements of dynamic systems (42). This method, termed pseudo coaddition, is also limited in achievable benefits, as noise from the sample’s single beam tends to dominate the resultant spectra. Spectra from sample areas with the same true absorbance can be coadded to yield low noise average spectra that is similar to conventional spectral coaddition. However, the approach does not allow a corresponding improvement in image quality and relies on the investigator estimating similar spatial areas.

The limitations of the data acquisition approach to obtaining low noise spectra have spurred interest in purely mathematical methods of noise reduction. The Minimum Noise Fraction (MNF) transform has been suggested as an alternate pathway toward obtaining higher fidelity images after data acquisition (44). The transformation and inverse transformation after eliminating components owing to noise is computationally intensive, but does not result in loss of image content or affect image collection times. The gain in the SNR depends on the SNR characteristics of the original data and the number of pixels involved in computation of the data covariance. Noise was reduced by a factor greater than 5 in a typical application when the noise in the initial data is sufficiently low. Similar methods based on any transformation that separates noise and data factors may be employed. Although these approaches are excellent avenues toward increasing image quality and spectral visualization, confidence is limited in the resulting spectral data by the unknown loss of information convoluted within noise-dominated factors.

Data obtained from a single-element detector TR-FTIR microscopic analysis that has been assembled into a map usually have poor resemblance to a visible microscopy image. The relatively coarse spatial resolution and small number of resolution elements over the field of view in the mapping experiments give the resulting presentation a highly pixelated appearance. One of the major benefits of FTIR imaging is higher spatial resolution achievable along with the high density of sample points. An image resembling a visible microscopy image is usually the result. After an image is obtained, visualization of the desired information may be enhanced in a variety of ways. Therefore, data processing directly in the image domain, in contrast to operations in the spectral domain that affect image quality, becomes increasingly important. Spectral operations change image appearance; for example, ratios of peaks may be used to correct for thickness variations and also to enhance contrast. Contrast may also be enhanced by multiplying images by themselves or using a variety of filters. Mathematical means are also found useful to visualize various chemical components, to determine the structure of noise, and
to obtain low noise images. Techniques may then be employed for obtaining the best contrasting visualization or highest sensitivity. Because these techniques do not affect spectral quality, they are useful for emphasizing certain features of an image. Image appearance may change with the manner of processing, thus careful interpretation of pseudo color images, using a color bar, is recommended.

Image Segmentation and Classifications

REAL-TIME VISUALIZATIONS Although actual data acquisition may require relatively short time periods, the extraction of absorbance information from a data set takes significantly longer. For example, a kinetics experiment records 10 time slices of an event in \(\sim 2\) min, but Fourier transformation and background corrections require almost 20 min before results can be accessed in terms of absorbance data or associated image intensity. Though an approach to rapid processing may be to Fourier transform a small region around the centerburst and to examine the resulting low resolution spectral profile, another attractive approach to image segmentation is to utilize a portion of the acquired interferogram that precludes Fourier transformation and results in a reduction in the dimensionality of the data set. One processing route involves a Gram-Schmidt (G-S) vector orthogonalization (83) and has been previously employed in the interferogram domain for hybrid spectroscopic techniques (84), multivariate data processing (85), and MRI (magnetic resonance imaging) image analyses (86). Although data orthogonalization is a primary step in many chemometric analyses involving more complex computations, the G-S orthogonalization process for data is a particularly simple and direct approach that provides a powerful data analysis tool in itself. For IR absorption spectroscopy, a basis set consisting of background interferograms is established before initiating the experiment. This basis set is then used to detect the presence of IR radiation absorbing species in real-time by comparing the sample interferograms to the basis set. Although the theory underlying the G-S orthogonalization process is well documented (83), additional considerations in an imaging context are required. Consider a series of interferograms in which each interferogram, \(I_k\), consists of \(n\) sampling points each at optical retardations \(\delta_m\) such that \(m \leq n\) with some value \(h\) such that \(\delta_h = 0\) and \(h \leq n\). Alternately, each interferogram can be represented as a scalar associated with a unit vector of \(n\) dimensions, \(i_k\), given by

\[
i_k = \frac{I_k}{\sqrt{I_k^T I_k}},
\]

where the denominator is simply the scalar magnitude of \(I_k\). Any orthonormal vector to \(i_k\), \(i_j\), is then given by

\[
i_j = \frac{I_j - (I_j^T I_k) i_k}{\sqrt{[I_j - (i_j^T I_k) i_k]^T [I_j - (i_j^T I_k) i_k]}}.
\]
In this manner, \( p \) vectors may be employed to form a basis set of orthonormal (linearly independent) vectors given by

\[
i_p = \frac{I_p - \sum_{k=1}^{p-1} (i^T_k I_p) i_k}{\sqrt{\left(I_p - \sum_{k=1}^{p-1} (i^T_k I_p) i_k\right)^T \left(I_p - \sum_{k=1}^{p-1} (i^T_k I_p) i_k\right)}}.
\]

Any given interferogram, \( I_s \), may now be referenced to \( i_p \) to provide an orthogonal vector, \( I_{s-p} \), given by \( I_{s-p} = I_p - \sum_{k=1}^{p} (i^T_k I_s) i_k \). The magnitude of \( I_{s-p} \) is the measure of radiation reduction caused by absorption or other sampling effects in the interferogram. Considerable discussion exists on the use of an appropriate region, or window, of the interferogram for calculating the G-S basis set and intensity (87). In particular, the size and position of this window may be critical to the predictive performance of the technique (87, 88) and is usually chosen away from the centerburst (84, 88). In imaging data sets, the region of the interferogram close to the centerburst is also indicative of the transmitted intensity over the entire spectral bandwidth. Because transmitted intensity depends both on the total sample absorption and scattering (89), inclusion of the centerburst provides additional contrast (90). It was suggested that the centerburst may be excluded when routinely performing G-S orthogonalization on heterogeneous samples in which chemical differences are to be determined and only including it when any difference is critical to the task, as for example, in quality control applications.

In a G-S orthogonalization of FTIR imaging data (91) the number of operations (84) and time for computation was calculated. The number of computations to form the basis is \( 2D(2p + 1) + 1 \), whereas the number of operations for subsequent orthogonalizations is \( D(4p - 1) \), where \( D \) is the number of dimensions or vector elements and \( p \) the number of vectors. Using a fast Fourier transform algorithm for a discretely sampled interferogram of \( N \) elements, the number of corresponding operations is of the order of \( 2N \log_2 N \). For a 1024-element interferogram, a common size for imaging data set in which 30 points are employed for calculating the basis and orthogonal vectors from single files, the relative number of computations are reduced from ~20,480 to 331. This ratio of number of computations is typically of the order of 1%–5%, resulting in considerable computational time savings. G-S vector orthogonalization produces useful visualizations for both static and dynamic spectroscopic imaging data. In addition to being faster than the usual processing methods, it requires no a priori knowledge of the constituents, and, finally, does not require sample components having unique and distinct spectral bands. The quantitative correlation of G-S intensities to concentration or absorbance may, however, be complicated by the presence of multiple components and the large absorbance and optical effects that are convoluted within the interferogram. Hence, the images have more information content than simple brightfield microscopy (edge scattering), but less specific information compared to appropriately processed imaging data in terms of absorbance distributions. Due to advantages
in its speed to reach visualization and its high information content, this approach is suitable for routinely visualizing images during data acquisition and for use in quality and process control applications.

ADVANCED NUMERICAL ALGORITHMS Imaging data are generally amenable to both chemometric approaches for spectral processing and image analysis routines for morphological analyses. The link between spectral and image data is critical, as any change in either domain affects the other. Typically, hybrid approaches incorporating both spectral and spatial components are employed to extract and display information from the data in a sequential algorithm. For example, cluster analyses of spectra in a data set are conducted to differentiate pixels with similar spectra from other self-similar groups. The assignment of pixels to a group may then be employed to visualize structure in the image based on group, rather than absorbance. Algorithms that employ both spectral and spatial data to provide information may be considered hybrid approaches to spectroscopic analyses and often involve specific applications. In general, advanced algorithms consist of a spectral preprocessing step including spectral quality checks for every pixel, a spectral preprocessing step applying standard spectral operations such as baseline correction, a selection of the spectral regions to be employed, a chemometric algorithm in which spectra are matched to “standards” using supervised or unsupervised methods, and, finally, an optimized display of the resulting image. Spectral chemometrics is at the core of the algorithm and may arise from a variety of classical spectroscopic analyses (92–94) or pattern matching approaches (95). The use of both unsupervised, for example, clustering methods (96–98), and supervised discriminant analysis (99, 100) have become popular in classifications and image segmentation. The basic ideas underlying these approaches are discussed in the literature; we will discuss here two issues that are pertinent to examining spectroscopic imaging data.

Imaging approaches provide unique algorithm training and validation opportunities, as data sets consisting of thousands to millions of spectra are readily available. We have recently proposed the use of large numbers of spectral observations in devising an algorithm for automated tissue classifications (R. Bhargava, S.M. Hewitt, D.C. Fernandez, & I.W. Levin, unpublished data). This approach was tested extensively to provide performance metrics in the form of receiver operating characteristic (ROC) curves (102). Although including spectra from large numbers of samples is the typical norm in calibration and validation processes of a multivariate analysis, due consideration must be applied to the number of distinct chemical groups, their relative numbers of spectra in a given sample, and the total numbers of spectral contributions from each sample. In contrast to conventional spectroscopic analyses, measures of robust calibration and extensive validation at the spectral, sample and data set levels are more complicated. Conventional leave-one-out approaches to calibration and validation will generally not be applicable. Instead, chemometricians must devise both data analyses routines and objective result evaluations. The latter can be greatly aided by input from statisticians. Though the evaluation of spectral classification is considerably complicated by large numbers of spectra, the visualization of spectral segmentation is greatly
facilitated. For example, we show the segmentation of sebaceous glands in a skin sample using a small number of spectral metrics in Figure 5. The brightfield microscopy image of a skin sample is compared to the IR absorbance image from the same sample area (top and bottom, left) in the Figure. Using the absorbance intensity, the nontissue-bearing pixels (coded black) are segmented from the tissue-containing ones (coded white) in the image identifying tissue (top, middle). By successively employing measures of specific molecular vibrations within the tissue, a component of the sample can be isolated. In this case, the visualization of spectral operations for classification is greatly facilitated by the imaging modality.

Typically, mathematically intensive approaches yielding high quality information in exquisite detail are employed for spectral analyses. For example, the IR spectral response of tissue is complex and necessitates mathematical techniques for information extraction. Typical approaches have reported excellent information extraction using methods that require 1–2 s per spectrum due to the need for spectral normalization, baseline correction, and/or derivative calculation followed by classification. In an imaging modality containing 10,000 spectra, for example, the same mathematical analysis would require over three hours. The mismatch between the time required for spectral acquisition and data analyses can be alleviated by devoting more computation resources or by providing faster data analyses strategies. Both routes are attractive owing to the increasingly large computational power available at continuously decreasing costs. We have recently reported the development and application of a very fast algorithm utilizing machine learning and a quadratic discriminant formulation for spectral pattern classification of tissues.

Figure 5  An unstained sample of human skin tissue is spectroscopically imaged and segmented using an automated numerical analysis procedure. The brightfield microscopy image (top, left) of the sample demonstrates several different structures. The Amide I absorbance intensity (bottom, left) can be employed to differentiate tissue from empty space (top, middle). The further inclusion of specific absorbance modes helps to segment finally (bottom, right) the sebaceous gland components within the sample. The accuracy of the segmentation can be monitored for the entire image at the same time. The bar to the right indicates the gray scale code for absorbance and probabilities for spectral images and classification visualizations, respectively.
FTIR SPECTROSCOPIC IMAGING

(D.C. Fernandez, R. Bhargava, S.M. Hewitt, I.W. Levin, submitted). Classification of tissue was accomplished in the reported study on an average of \( \sim 1 \) ms per spectrum. Thus 10,000 spectra could be analyzed in less than 20 s in comparison to a conventional approach, requiring several hours. Because computation power has increased rapidly in the past three years, the analyses reported using classical methods would likely require now only several tens of minutes.

BIOMEDICAL APPLICATIONS

The application of FTIR imaging to biologics represents an area in which the potential of the technique has become readily apparent, particularly because the opportunity exists for examining tissue specimens in the diagnosis of disease and the monitoring of disease progression. The integration of the noninvasive nature of FTIR spectroscopy with an ability to obtain spatially resolved chemical and structural information presents the pathologist, for example, with an important tool for high throughput screening of spectroscopic images using tissue microarrays.

One of the first demonstrations of FTIR imaging of biological samples involved lipid [C(16)-lysophosphatidylcholine] aggregates isolated in a KBr disk (104). Since then, applications have become widespread, spanning areas invoking, for example, human and animal tissue samples to cell ensembles and even foodgrains. A selection of illustrative studies are discussed below.

Brain Tissue

Monkey cerebellum was among the first of many different classes of biological tissue examined using FTIR imaging. Thin slices of a monkey brain section showed the distribution of common tissue constituents. Images indicating a greater proportion of lipids in white matter relative to gray matter were observed. A detailed study of primate brain tissue (105) in the mid-near-IR regions (7000–1818 cm\(^{-1}\)) demonstrated the capability to collect images from a wide field of view at spatial resolutions higher than with most IR imaging studies. Well-corrected refractive optics could be employed in the near-IR spectral range for better image quality. However, absorption is much weaker in this region than in the mid-IR. Hence, several statistical analysis methodologies were suggested. These included scatter plots, histograms, and profile matching. Rat cerebellum Purkinje cells have been shown to strongly influence the motor coordination and memory processes. Neurotoxic effects of an antineoplastic drug [cytarabine (Ara-C)] on the Purkinje cells was visualized using FTIR imaging of brain sections in model studies (106, 107). Enhanced contrast was observed in the IR brightfield images in contrast to the visible microscopic images of the tissue sections, perhaps due to the tissue’s intrinsic packaging properties. With respect to chemical images, plots of lipid-to-protein vibrational peak ratios display concentration gradients, allowing interpretation in terms of subtle structural changes. The higher protein packing density of the Purkinje layer leads to greater fractions of disordered lipid acyl chains. Both biochemical concentration changes and spatially resolved structural
Figure 6  Infrared spectroscopic images of thin cerebellar sections from a control and Ara-C-treated animal. (A) Spectroscopic image showing the spatial distribution of lipid and protein in a rat treated with saline. (B) Spectroscopic image showing the spatial distribution of lipid and protein in a drug-treated rat. (C) Spectroscopic image depicting the distribution of phosphatidylcholine in a cerebellar section from a drug-treated animal. The image is derived from the intensity of a vibrational absorption band centered at approximately 3060 cm\(^{-1}\), assigned to the methyl (CH\(_3\)) stretching vibration of the lipid choline headgroup (107).

differences were monitored. Neuropathologic effects of a genetic lipid storage disease, Niemann-Pick type C (NPC), were investigated by examining sections of normal and diseased mice (108). The image in Figure 6a allows differential cellular layers to be readily identified. Distinctions between diseased and control samples are discovered based on spectral data and not the use of external stains (Figure 6b). Statistical analyses provides a numerical confirmation of these differences. In particular, lipid depletion was found in diseased samples in comparison to control tissue sections. The chemical changes observed were consistent with significant demyelination within the cerebellum of the NPC mouse.

Bone Tissue

IR spectroscopy has been used for several years to characterize mineralized structures in living organisms (notably, bone). Until the late 1980s, however, the experimental approach had been to grind the bone for single-element detection analysis. This sample “preparation” clearly destroyed local structures, precluding an understanding of molecular variations caused by disease. With the application of spatially resolved FTIR spectroscopy (109), the process was made minimally invasive while maintaining sample integrity. Routine investigations were limited to single-point spectra from regions identified using optical microscopy, resulting in an extended time period required to map the complete field of sample view. With the application of FPA equipped spectrometers to mineralized biological samples (110), spatial variations of several quantities are easily monitored. In particular, hydroxyapatite and protein distributions (Figure 7, see color insert) are readily obtained. Human iliac crest biopsies were examined to correlate FTIR
imaging chemical composition data and morphologic conclusions with optical observations, point-by-point microspectroscopic data, and known developmental processes in bone (111). A gradient in mineral level increased from the middle of the osteon toward the outside. Such observations are consistent with the models for development in Haversian systems. Protein content monitored using the Amide I contour \((1620–1680 \text{ cm}^{-1})\) showed a corresponding gradient. The osteoid, a non-mineralized (but high protein content) region, could be readily detected. An index of crystallinity/maturity was determined using the phosphate \(v_1, v_3\) contour, which was found to increase with distance from osteonal center. Point-mapping experiments compared favorably to the contemporary, high density pixel imaging data. An earlier publication (112) also reported bone (canine jaw) sections imaged using mid-IR detection. Observations of the type seen in the study mentioned above were noted in that case too. Bone sections and polymethyl methacrylate (PMMA) embedding medium could be readily distinguished. “Mineral content” was analyzed using the phosphate peaks. Osteon centers were found to contain less mineral than peripheral areas as in Haversian systems. The organic matrix contribution from the Amide I stretching vibration also corresponded to the bone morphology.

In another study (113), images from normal osteoporotic human iliac crest biopsies were compared to those from normal sections. From the spectral data, two spectral parameters were used to analyze the samples. The first monitors the extent of mineral (hydroxyapatite) formation in the tissue using a mineral/protein ratio (ratio of the integrated areas of the phosphate contour and the Amide I peak). The second parameter monitors the size/perfection of the crystals (ratio of absorbance at 1030 cm\(^{-1}\) to 1020 cm\(^{-1}\)). From a statistical analysis employing histograms, it was found that the average mineral levels in the osteoporotic samples were considerably reduced compared to the normal samples. In the diseased state, the crystal size and perfection was substantially enhanced. A similar analysis was applied to examine the effects of estrogen therapy on fracture healing in rat femurs. Healing of fractured bones in the presence and absence of therapy was studied. Mineral content per unit matrix in the treated samples was higher. The crystal size/perfection was also elevated for the treated samples. Several sites along the fracture contained a disproportionately large protein content and were speculated to be the site for formation of new mineral. Cellular activity was also revealed at the site of the fracture by indications of cell membrane fatty acids. These studies clearly demonstrate the efficacy of using FTIR imaging to detect and monitor pathological changes in bone.

**Breast Tissue**

Breast implants consisting of a silicone elastomer shell filled with saline or silicone gel material are used extensively for breast enhancement procedures. More than 1 million women in America are estimated to have silicone breast implants. The silicone gel may leak from the implant due either to material failure because of aging or to a rupture in the casing leading to complications including capsular contracture, calcification, and some connective tissue disorders. To assess histopathological changes, it is first necessary to confirm the presence of silicone
gel from the implant in the tissue. The presence of gel material could be readily detected (114) in a tissue section using FTIR spectroscopic imaging. An image of the tissue section clearly showing the inclusion can be seen in Figure 8. Si-CH₃ characteristic vibrations were used to provide chemical contrast between the tissue and silicone gel inclusions as small as ∼10 µm. Inclusions could be found even in cases where optical microscopy contrast was poor. Dacron [a commercial name for poly(ethylene terephthalate)] fixative patch threads could be readily discerned in another sample. The technique is also capable of rapid analysis within minutes of sectioning the tissue. Hence, it has potential as a powerful aid in real-time analysis for surgical decisions.

Imaging and point-mapping approaches were compared in the recent analysis of a sample of breast tissue demonstrating adverse pathologic conditions (115). The authors observed that their recorded spectra from an FPA were of

![Figure 8](image_url)  
**Figure 8** Infrared (IR) spectroscopic images and spectra of human breast implant capsular tissue. Vacuoles in the sample appear consistently as nonabsorbing black features in all images. (a) An IR image at 2963 cm⁻¹ highlighting the presence of silicone gel inclusions in the breast tissue. Black indicates an absence of plotted signal, whereas progressively lighter shades of gray indicate higher signal. (b) IR spectrum from one pixel inside one of the inclusions. (c) IR image of the CH₂ and CH₃ stretching modes of lipid and paraffin. Inclusions are dark in this image as silicone gel does not absorb at 2927 cm⁻¹. (d) IR image at 3350 cm⁻¹ detailing the homogeneous distribution of protein throughout the tissue sample. (e) IR absorption spectrum from a pixel in the tissue. The lipid/paraffin CH₂ and CH₃ stretching modes give rise to the peaks in the 2850–2950 cm⁻¹ range, whereas the broad feature centered around 3350 cm⁻¹ is characteristic of the Amide I stretching vibration for proteins (114).
poorer quality (lower SNR) than those recorded using a point-mapping approach through an aperture of 30 µm. The collection of the FPA spectra, however, required significantly less time (4096 single pixel spectra in 5 min versus 256 point spectra in approximately 4 h) and provided wavelength-limited spatial resolution. By comparing the spatial distribution of intensities at 1084 cm$^{-1}$, which is generally ascribed to nuclear material (DNA, RNA), a prominent ovoid area was determined to match the Hematoxylin and Eosin (H&E) stained tumor area from the point-mapping data but a finer visualization was afforded by imaging. The study also reported detecting a microarea of tissue containing only a few nuclei using the imaging approach, which were not detected by classical point microscopy. Because breast tumor cells are $\sim$10 µm in diameter, the differential signal was overwhelmed in the point-mapping data by the spectral contributions of other components surrounding the cells. The study clearly demonstrated that the conventional point-mapping approach can fail to detect a small number of malignant cells owing to its poor resolution capabilities. Although the report presents a recognition of the capabilities of IR imaging in breast pathology, there are no reports, to date, of extensive spectral measurements or automated classifications of large numbers of breast tissue samples.

Esophageal Tissue

Adenocarcinoma of the esophagus is a prevalent and growing concern in the western world with the annual incidence of cancer within Barrett’s esophagus reportedly between 0.5%–1.0% per year (116). Survival rates of $\sim$35% and 10% at 1 and 5 years, respectively (117), are dramatically improved with early detection (118). The typical procedure in population surveillance is the extraction of biopsies from suspicious lesions. This “biopsy surveillance” results in a large number of samples requiring evaluation. Consequently, spectroscopic (119) and imaging approaches are being increasingly employed to determine their utility in clinical activities related to these cancers. We spectroscopically imaged frozen esophageal tissue biopsies (Figure 9, see color insert) to determine the utility of IR spectral monitoring. A contiguous microtomed section of the biopsy was stained using conventional H&E stains for pathologic evaluation. The cell maturation and increased glycogen storage is represented by clear vacuoles as the cells mature from deep within tissue toward the lumen (bottom to top in the stained image). The corresponding absorbance images of a sequential, unstained section demonstrate differences between the absorbance characteristic of glycogen and of protein. The variability in tissue composition is dominated largely by cell maturation, masking differences between the spectra of benign and malignant epithelial cells. Conventional point-vibrational spectra are likely to be influenced more by cell maturation than malignancy; hence, an imaging approach should prove useful.

A disruption in the glycogen gradient is a characteristic of esophageal tumors. Because this gradient is also identifiable in tissue that has been stained in the pathologist’s laboratory, stained tissue may also be employed for IR spectral image analyses in this instance (Figure 10, see color insert). The importance of imaging in
this example is underscored by the observation of potentially diagnostic molecular gradients in addition to the usual spectral visualization and analysis approach. Although no application has been reported that employs spectroscopically derived morphological information as input to a diagnostic protocol, we believe that this concept could be explored with the accessibility of spectroscopic data determined across extended sample regions.

Skin Tissue

Human skin can be considered a multilayered system with various functional structures dispersed within. The structural organization of skin consists of numerous units that have characteristic dimensions in the 10–500 µm range, ideal for studies using FTIR spectroscopic imaging. We have previously reported the potential of FTIR spectroscopic imaging for human skin (120), in which the spectral characteristics of benign skin lesions were compared to lesions from patients suffering from Birt-Hogg-Dube syndrome. Figure 11 (see color insert) shows the multilayer structure using various vibrational modes to provide contrast for an unstained section of the skin sample. A detailed assessment of the spectroscopic characteristics of skin components has recently been reported (121) using pig skin as an example. Stratum corneum, epidermis, and dermis are clearly visible in the images based upon lipid- and protein-specific vibrational modes. The upper stratum corneum was noted to consist of large areas of lipids and aggregated corneocytes, while some segmentation was also observed for the deeper stratum corneum layers. Differences in the stratum corneum organization were noted to be contrary to the simple “bricks and mortar” model. Further, the presence and distribution of components from a “sun cream” lotion could be detected; differences in their spatial distributions were based on the specific formulation employed in the production process.

The distribution of exogenous molecules in transverse skin biopsies has also been measured in other studies (122). Two penetration enhancers, dimethylsulphoxide (DMSO) and propylene glycol, were monitored using spectroscopic imaging and their distribution quantitatively analyzed. In addition to revealing the protein and lipid distributions, the spatial distribution of triglyceride and protein were monitored as a function of distance from the skin surface. Both penetrants employed in the study were found to be coincident with the protein constituents of the skin. The rapid determination of the spatial distribution of both exogenous and endogenous components demonstrates the potential of applying this method to study the mechanism and kinetics of molecular transport within tissue.

Prostate Tissue

Prostatic adenocarcinoma, originating within the epithelial regions of the prostate gland, is prevalent in the western world. In the United States, for example, almost 180,000 men are diagnosed with prostate cancer per year and nearly 29,000 people a year die from the malignancy. Early and accurate detection would clearly impact the treatment of this disease. Using FTIR imaging, we have attempted to discern chemical markers of prostatic adenocarcinoma, as opposed to the usual
examination of morphological changes by a trained pathologist. The chemical specificity of FTIR spectroscopy is particularly useful in the study of this disease as it can be combined with the spatial selectivity afforded by imaging when analyzing tissue. For prostatic adenocarcinoma, it is particularly important to assess spectral changes in small (5–20 µm wide), localized regions around prostatic ducts, or lumens. A comparison of a stained visible image, as employed for conventional morphological analyses, and an IR spectroscopic image of the distribution of chemical components is shown in Figure 12 (see color insert). A comprehensive segmentation of prostatic tissue into its constituent cell types was undertaken using a 10-class model. All cell types could be identified to a high degree of accuracy (area under the receiver operating characteristic curve ranged from 0.89 to 0.995). In particular, epithelial cells could be readily distinguished from other tissue. Because 95% of prostate cancers arise in this cell type, their optical isolation is important in the examination of prostate tissue for spectral markers of malignancy.

Preliminary studies (103) reinforce the concept that the analysis of localized spectral changes is an attractive means for characterizing spectral changes for disease diagnosis and for monitoring its progression. Because the spectral changes involved are subtle, large numbers of samples must be examined employing FTIR imaging techniques, which provide superior data quality. In comparing normal tissue sections to malignant sections derived from the same patients, a small cohort was employed to demonstrate that malignancy could be accurately detected for all patients (103). Another study (123) compared the ratio of peak areas at 1030 and 1080 cm\(^{-1}\), corresponding to the glycogen and phosphate vibrations, respectively, and suggested that this metric could serve as a potential marker for the differentiation of benign from malignant tissue. The study also demonstrated that the results from a linear discriminant algorithm were promising for segmenting tissue and separating spectra arising from different pathologic grades. The elucidation of tissue histology and the evaluation of such biomarkers is an on-going process for many tissue types. The low throughput of IR microspectroscopic measurements based on single-element detection has been addressed with the advent of FTIR spectroscopic imaging methods, but the translation of these preliminary studies to clinical protocols requires statistical measures that can be best obtained using high throughput sampling in conjunction with FTIR imaging. A route to this achievement is discussed in the next section.

**Clinical Pathology**

Although we have enumerated various illustrative reports in the literature regarding the utility of IR spectroscopic imaging, there are excellent reviews on the applications of solely single-element detection IR microscopy to biomedical problems. In particular, IR microscopy has been extensively employed in preliminary tissue studies and cancer detection (124). It is recognized now that these early studies cannot be translated to clinical practice and the lack of reliable clinical applications is due to three technological, rather than fundamental, factors: a lack of high quality data, spatial nonspecificity of the recorded signal, and the complexities
arising in the interpretation of the spectral data, which are further confounded by variations in patient populations. Considerable progress has been made in resolving these problems (103), and an effective route for applying IR spectroscopic imaging to the histopathologic analysis of clinically related tissue samples has been demonstrated. The fundamental advancement that has permitted high throughput spectroscopic imaging is clearly the development of high fidelity imaging systems. Conventional IR spectroscopic studies of tissues examine 1 to 100 spectra derived from 1 to 100 samples. These studies render questionable the statistical significance of observed biomarkers when applied later to large populations. Unfortunately, there was no alternative to this strategy and, until a few years ago, investigators were limited to making progress with a paucity of data. Hence, a number of controversial issues had arisen that questioned the validity of applying IR spectroscopy to tissue analyses. The first issue was one of sample control. A variety of approaches were suggested to appropriately include effects arising from sample preparation. For example, the use of cryo-sectioned samples (125), observing cells in a controlled growth state (126), and the necessity of extensive control samples (127) became relevant. Discussions in the literature have illuminated the many factors (128) precluding the definitive vibrational spectroscopic analyses of cells and tissues; unfortunately, useful spectroscopic protocols that can function in the presence of these factors have not been articulated. An alternative (101) rationale addresses directly wide population sampling and numerical analyses through the use of tissue microarrays (Figure 13, see color insert). Under this paradigm, all possible states of preparation and growth could be incorporated into a robust mathematical procedure.

The utility in imaging tissue microarrays allows hundreds of unstained samples, processed using standard histopathology procedures (103), to be spectroscopically visualized. This approach allows the sampling of all cell types and tissue pathologies, permits the recording of data from growth states in their natural abundance, samples a wide range of preparative effects and processing conditions, and, finally, allows extensive statistical validation of protocols that have been developed. The tissue microarray format will also lead to extensive intrapatient and interpatient validations. In our study (103), samples were obtained from at least 30 different hospitals, including data from commercial array vendors. For example, we currently have a total data set of approximately 870 samples and more than 9.5 million spectra to establish, for the first time, that variations in tissue populations can be accounted for through robust mathematical modeling and that normally prepared pathologic samples can be directly employed for IR spectroscopic analyses. Thus, spectral imaging-based protocols remain compatible with archival and current tissue processing protocols, greatly facilitating further applications of vibrational spectroscopic imaging in pathology.

Single Cells

The high spatial resolution of IR imaging, which has allowed the examination of small morphological features in tissues, may also be employed to image single,
biological cells (129). An example of single-cell imaging and the associated spectral data that are collected are shown in Figure 14 (see color insert) for African green monkey kidney cells. In the future, the spatial resolution limits and spectral SNRs that can ultimately be obtained will allow more complete chemical characterizations of single cell assemblies. Until recently, the spectral region below \( \sim 1500 \text{ cm}^{-1} \) has been particularly difficult to analyze owing to a lack of high quality spectral data (130–132). Even until a year ago, this has not been an easy task to accomplish. For example, in one study employing a globar source, more than 1500 spectra were averaged to achieve high SNR spectra at moderate spatial (15 \( \mu \text{m} \)) and spectral (8 \( \text{cm}^{-1} \)) resolutions, resulting in extended acquisition times for the mapping of single cells. The same study reported FTIR imaging spectra acquired in 2 min with an average SNR of tens of averaged pixels to be \( \sim 70 \). We average 100 scans with an FPA of a smaller pixel size than employed in that study and report an approximate rms absorbance noise level of \( \sim 5 \times 10^{-4} \text{ a.u.} \), whereas the noise level for a separate raster-scanned system was \( \sim 2 \times 10^{-4} \text{ a.u.} \), providing a single pixel SNR of \( \sim 100 \) for the Amide I peak. We adjusted our data acquisition parameters to provide approximately the same noise levels to facilitate spectral comparisons and generalize statistical results based on noise levels. These values do not present the upper limits of performance and can be improved by invoking the usual rapid scan spectrometry trade-offs.

As expected, rapid scan data acquisition can permit high levels of performance that have not been previously attained. Averaging 100 consecutively acquired data sets to allow the highest SNR requires that the coaddition be performed after all data are recorded. Attempts to improve the SNR of acquired data by time averaging when employing large FPAs, however, involve one additional complication. If data are corrected and coadded between scans, then several interferogram scans would necessarily be omitted after the collection of every data, allowing instrumental drift to likely mitigate the advantages of time averaging. Data collection without real-time coaddition, however, results in very large data sets, which present limits on data storage. Hence, current computer and detector technology may present real limits to FTIR imaging experiments. Deciding between the two options above is not trivial and may not be possible for all cases, as instrumental drift is difficult to quantify and impossible to predict. In practice, we acquire interferograms rapidly and to compensate we have increased computer data storage. Experiments conducted in this manner have an additional advantage if multivariate methods are employed subsequently to eliminate noise (44), as successive interferograms can be subtracted to yield an estimate of the pixel variance. Although the decrease in noise by averaging 100 consecutively acquired interferograms is clear, the significance of the noise level for disease detection is unclear. Markers of pathologic changes have been examined in ensembles of breast tissue cells using TR-FTIR imaging and pattern recognition techniques (133). Spectral differences that could potentially offer an automated method for discriminating between diseased and normal cells were noted. Multivariate data processing and an artificial neural network model were used to provide differentiation despite relatively poor spectral
quality and significant pixel-to-pixel variability. The approach used was based on the assumption that the diseased state was reflected in the reproducibly measured IR spectra. The assumption is clearly affected adversely by noise and variations. To build a robust classifier, the authors noted that the acquisition of large numbers of representative training spectral data were essential and that better data analysis techniques were required. Though the development of FTIR spectroscopic imaging provides the ability to collect large volumes of data, studies such as the one discussed above will now clearly benefit from the introduction of high throughput sampling platforms that use robust mathematical approaches. This example underscores the need to develop integrated platforms of imaging, data acquisition, and analysis if the promise of FTIR spectroscopic imaging is to be fully realized.

Pharmaceuticals

**FUNDAMENTAL STUDIES ON POLYMER-LIQUID SYSTEMS**  The dissolution of polymer films by liquids has been a subject of much investigation in polymer science and is an important parameter in the formulation of drug delivery systems. FTIR imaging presents a method to determine rapidly both spatial and spectral content of a polymer-solvent interphase and is, thus, well suited to the study of the kinetics of polymer dissolution. By monitoring the spatial distribution of concentration as a function of time from an initially known state, the diffusion of polymer and solvents as well as the dissolution rate can be determined; this experiment was among the first reported applications of FTIR imaging to real-time phenomena. The dissolution of a PMMA film was monitored and the polymer-solvent diffusion coefficient calculated (134). In another study, it was shown that faster dissolution processes could also be monitored in real time using a combination of smaller collection times and coaddition processes. The dissolution rate of the polymer film could then be obtained using its concentration profile as a function of time. The nature of the diffusion profiles provides clues to the nature of the diffusion process. For example, a Case II behavior was found for MIBK diffusing into poly(\(\alpha\)-methyl styrene) (PAMS) (135).

Not only can dynamic phenomena in time be measured readily, but changes in liquid-polymer systems due to reaction processes can also be determined. A polymer-liquid system consisting of phase-separated mixtures of uncured poly-(butadiene) and diallyl phthalate were studied to characterize morphology differences before and after the curing process (136). Optical microscopy of these systems is particularly challenging as the resultant phases have similar refractive indices. However, good image contrast was achieved by FTIR imaging because of the inherent chemical differences manifest in their IR spectra. The morphological changes were characterized over a period of time with the post-cured sample exhibiting homogeneity at the resolution level of the instrument. These model studies may be useful in determining both the formation of microstructures and the static morphology of polymer-based drug delivery systems. One such application is the study of the encapsulation of biologically active proteins in sustained release
devices, as, for example, microparticles composed of poly(lactic-co-glycolic acid) (PLGA) (137). As the capsule of biologic containing polymer is prepared, it is subjected to several manufacturing processes that may denature the biological molecule. In such cases, it is advantageous to have a method available to perform a spectroscopic analysis on the final product without the need to extract the biologic. In one such example, PLGA containing egg-lysozyme was analyzed using FTIR imaging (138). Protein was present inside the microspheres and distributed evenly at the scale of examination. Little protein was found on the surface; this result was confirmed by subsequent ATR and photoacoustic spectroscopic measurements. These results led to a suggestion for an alternative mechanism of release (139, 140).

PHARMACEUTICAL TABLETS The distribution of different components in pharmaceutical tablets affects not only their structural properties but also their dissolution and drug release profiles. FTIR spectroscopic imaging of laboratory and commercial tablets (52) demonstrated the existence of domains of a model drug (caffeine) for concentrations exceeding 3.5% and allowed the differentiation of two additional components (the drug and starch). Although information concerning the overall distribution of each component in the tablet obtained by compaction of the mixture on the diamond crystal employed for imaging, the in situ manufacturing of the tablet required further studies on the dissolution behavior of tablets. Using micro-ATR imaging, the study also demonstrated the feasibility of observing clearly various domains at a detection limit of $\sim 0.5\%$. A combination of micro- and macro-ATR-IR spectroscopic imaging methods proved useful in detailing structural information useful in optimizing both the blending and compaction processes in pharmaceutical tablet manufacture.

CONCLUDING REMARKS FTIR imaging, employing an interferometer and focal plane array detection system, considerably enhances the ability to obtain, noninvasively, chemical measurements by combining the advantages of optical microscopy with those of IR spectroscopy. The instrumentation, although sophisticated, is now commercially available, providing a reliable platform both for research studies involving dynamic and static systems and for routine analyses. FTIR imaging instruments will soon decrease in cost as technical developments continue to enhance the applicability of the technique. An important contemporary application for which the speed of detection is particularly suited lies in the biomedical arena, specifically, in considering high throughput analyses of large numbers of millimeter-sized tissue samples exhibiting subtle histopathologic changes. At the same time, we foresee vibrational spectroscopic imaging applications to new problems in polymer physical chemistry because of the capability of imaging dynamic processes of composites, for example, on the millisecond and minute coads using TR and kinetics approaches. Along with increasingly efficient methods of data collection, the
past several years have seen an increase in the number and sophistication of data analysis tools and strategies. The pace in further development of numerical analysis procedures should accelerate, resulting in superior spatially derived images and in the ability to perform unsupervised data analyses. Unsupervised multivariate analysis is particularly suited to the large volumes of data that typically arise in process control procedures, an area in which IR spectroscopic imaging has, thus far, not been applied. In the biomedical sciences, we are now able to acquire data at rates that should prove clinically useful; the development of automated spectroscopic image analyses has the potential to provide new tools to aid, particularly, the pathologist in screening for diseased tissue. Clearly, we are only at the initial stages in the design of effective strategies for new and novel applications of this versatile, interdisciplinary vibrational spectroscopic imaging technique.

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CHAPTER NOTE

1. There is a fundamental issue in reading out a large number of pixels that prevents true equivalence to be achieved. If FPA-detector readout could be done at the same speed as that of single-element infrared detectors, for example, by massive parallel readout or by employing a small array detector, the data acquisition in both modes should require the same time period.

2. Specific detectivity, $D^*$, increases approximately one order of magnitude as the modulation frequency is increased from none to 1 KHz. Further increase in modulation frequency up to 1 MHz provides little benefit.

3. A commercial instrument has recently been introduced by SensIR technologies, CT based on this principle.

4. The capability to perform IR microscopic and fluorescence measurements on the same instrument is available in a configuration from Thermo Inc.

5. Spotlight FT-IR Imaging system by Perkin-Elmer Company.

6. Continuum XL by Thermo Nicolet Inc.

7. In addition to various optical arrangements providing different spatial resolution and sample sizes, a number of different ATR crystals—including germanium, diamond, and zinc selenide—have been employed for ATR measurements.
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Figure 7  (a) Infrared (IR) color scale images of a normal bone human iliac crest biopsy. The spatial distribution hydroxyapatite generated from the integrated intensities of the phosphate $v_1$, $v_3$ contour across the $64 \times 64$ array of the HgCdTe (MCT) elements is an indication of the mineral present in the sample. The elliptical structure corresponds to a single osteon. The color coding used to generate the images is indicated on the scale to the right of the Figure. (b) IR color scale image of the spatial distribution of protein generated from the integrated intensities of the Amide I mode contour across the $64 \times 64$ array of MCT elements. Axes and spatial resolution are as in Figure 4a. (Images courtesy of Prof. Richard Mendelsohn, Rutgers University.)
Figure 9 (a) Hematoxylin and Eosin (H&E) stained esophageal tissue section and the (b) glycojen (~1080 cm\(^{-1}\)) and protein (Amide I) specific modes’ absorbance distribution in a corresponding section of unstained tissue. Unstained vacuoles of glycogen storage indicate a gradient in cell maturation in the H&E stained image and appear as regions of high intensity in the absorbance image (b, left) and complementary Amide I distribution (b, right). (c) The spectrum of a pixel from the glycogen-rich region (bottom) was compared to a spectrum of glycogen embedded in a KBr disk. (d) Cell maturation and storage are likely confounding variables in attempts to distinguish benign from malignant cells using spectra alone. The variability in absorbance owing to cell maturation is demonstrated in spectra from cells containing high (black) and low (green) glycogen, which masks differences between the benign and malignant cell spectra (red).
Figure 10  Absorbance images at 1032 cm$^{-1}$, which are indicative of glycogen concentration in esophageal tissue, show contrast in both stained (left) and unstained (right) images. The gradient in absorbance, decreasing from the lumen (right) to the interior of the tissue (left), in both images, as indicated by the lower absorbance profile.
Figure 11  Optical microscopy image of a skin section. Stratum corneum, epidermis, and dermis in skin can be readily identified from spectra obtained from layers ~10 µm thick. Differences in spectra can be used for image segmentation using the methodologies discussed in the numerical analysis section.
Figure 12  Optical microscopy images obtained after staining and absorbance images from unstained prostate tissue using infrared (IR) spectroscopic characteristics of various cell types show good correspondence. (A) Hematoxylin and Eosin (H&E) stained biopsy section of human prostate tissue showing epithelial cells defining the lumen (top). The relative protein (middle) and phosphodiester (bottom) concentration determined, without the use of external reagents, from IR spectral absorbance at 1545 and 1080 cm\(^{-1}\), respectively, from a corresponding, unstained section demonstrate noninvasive, nondestructive tissue segmentation similar to that achieved by stains. Absorbance intensities are indicated by the color bar at the bottom. (B) Characteristic IR absorbance spectra of ten histologic classes comprising prostate tissue are (from bottom to top) normal epithelium, fibrous stroma, mixed stroma, muscle, nerve, lymphocytes, stone, ganglion, endothelium, and blood. The bar indicates an absorbance of 0.2 a.u. (Reproduced from D.C. Fernandez et al. Nat. Biotech., Submitted.)
Figure 13  Tissue microarrays, consisting of a large number of small-sized samples arranged in a regular grid format, provide a high throughput platform for using infrared (IR) spectroscopic assessment of samples from numerous patient cohorts. Stained tissue (top) can be compared to the “digitally stained” image of a sequential unstained array obtained by a fast computation of IR spectral data. (Reproduced from D.C. Fernandez et al., Nat. Biotech., Submitted.)

Figure 14  (a) An image based upon the intensity of the asymmetric CH$_2$ stretching vibrational mode at 2919 cm$^{-1}$ demonstrates a typical distribution of cells on a CaF$_2$ substrate. Cells with a central high intensity nuclear region have a tendency to cluster together. (b) Single pixel spectrum (top), average water vapor spectrum (middle), and corrected spectrum (bottom) from the image. (c) and (d) show sections of characteristic spectra from nuclear material and cytoplasmic material rich pixels.