30.1 Introduction

Light can react with tissue in different ways and its response can yield information about the physiology and pathology of tissue. The light used to probe tissue does so in a nonintrusive manner; in contrast to therapeutic applications, very low levels of light are typically used. The use of fiber optics allows light to probe tissue in a minimally invasive manner. Because tissue response is virtually instantaneous, the results are obtained in real time, and data processing techniques and computers allow for automated detection of disease. These factors have resulted in a variety of applications that employ light and optical modalities for tissue diagnosis.

When a photon is incident on a molecule, it may be transmitted, absorbed, or scattered. The various techniques that are based on these different light–tissue interactions include:

- Absorption spectroscopy
- Reflectance spectroscopy
- Fluorescence spectroscopy
- Raman spectroscopy

Each of these four techniques has been studied for the purpose of tissue diagnosis with varying degrees of success. Of these techniques, fluorescence spectroscopy is perhaps the most researched one for tissue diagnosis.
Considerable evidence indicates that fluorescence spectroscopy of exogenous (see, for example, Reference 1) and endogenous chromophores can be used to identify neoplastic transformations in the breast,2 lung,3 bronchus,4 oral cavity,5 cervix6 and gastrointestinal (GI) tract.7,8 Despite the success of this technique, results indicate that fluorescence spectra of precancerous tissues of the cervix and colon and benign abnormalities such as inflammation and metaplasia are similar in many patients.6–8 This suggests that the use of fluorescence diagnosis in a screening setting, where the incidence of precancer is expected to be low, may result in an unacceptably high false-positive rate. Many groups consider vibrational spectroscopy to be the solution to these problems because its molecular specificity enhances the specificity of spectroscopic diagnostics. The focus of this chapter is the application of Raman spectroscopy in biomedicine, especially as it pertains to disease detection.

Raman spectroscopy has been used for many years to probe the biochemistry of various biological molecules.9,10 In recent years, there has been interest in using this technique in diagnostics.11–14 Most of the diagnostic applications are still in the early stages of development. They range from the detection of (pre)malignant lesions in various organ sites to quantitative detection of blood analytes such as glucose, from measurement of skin hydration to the study of aging. The focus of all of these applications is on quantitative, in vivo, nonintrusive, automated detection in a real-time manner.

This chapter describes the concept, instrumentation, and application of Raman spectroscopy as it applies to biomedicine. We begin with a description of the basic concepts underlying Raman spectroscopy and Raman signatures of materials. We then review the past, present, and future directions of instrumentation that facilitates the use of this technique for detection of disease. Finally, we examine sample applications of Raman spectroscopy for disease diagnosis, with reference to work at the molecular and microscopic levels. Although many of the reported articles are based on in vitro work, this chapter focuses on the potential of the application of Raman spectroscopy to clinical diagnosis.

### 30.2 Principles of Raman Spectroscopy

Raman spectroscopy is based on the Raman effect that results from energy exchange between incident photons and the scattering molecules. When the energy of the incident photon is unaltered after collision with a molecule, the scattered photon has the same frequency as the incident photon. This is Rayleigh or elastic scattering.15,16 During the collision, when energy is transferred from the molecule to the photon or vice versa, the scattered photon has less or more energy than that of the incident photon. This is inelastic or Raman scattering (Figure 30.1).

Quantum mechanically, Raman scattering is a result of an energy transition of the scattering molecule to a virtual excited state and its return to a higher (or lower) vibrational state with the emission of an altered incident photon (Figure 30.1). Because energy can be transferred from the photon to the molecule or from the molecule to the photon, the scattered photon can have less or more energy compared to the incident photon. When the scattered photon has less energy than the incident photons, the process is referred to as Raman Stokes scattering. When the scattered photon has more energy than the incident photons, the process is referred to as Raman anti-Stokes scattering.

A Raman spectrum is a plot of scattered intensity as a function of the frequency shift (which is proportional to the energy difference) between the incident and scattered photons (Figure 30.2). The frequency shift is characteristic of the molecule with which the photon collided, and the resulting spectrum is characterized by a series of Raman bands that correspond to the various vibrational modes of that molecule. The locations of the Raman bands are typically presented in terms of relative wavenumbers (or Raman shift) defined as shifts in wavenumbers (inverse of wavelength in cm$^{-1}$) from the incident frequency, which is set to zero. This is because the location of Raman bands for a given molecule in relative wavenumbers remains constant, regardless of the incident frequency (within the physical constraints of the light–tissue interaction).

Raman signals are usually weak and require powerful sources and sensitive detectors. Typically, Raman peaks are spectrally narrow (a few wavenumbers) and, in many cases, can be associated with the vibration of a particular chemical bond (or normal mode dominated by the vibration of a single functional group)
in a molecule. Consider a complex molecule such as glucose (Figure 30.2). Each of the stretching and bending modes of vibration of this molecule has a characteristic frequency. When a photon is incident on this molecule, each mode of vibration results in a characteristic shift in frequency that is indicated in the Raman spectrum. Because each type of bond has characteristic modes of vibration, each type of molecule has its own spectral “fingerprint.” Thus, in a complex mixture of molecules such as in tissue, the presence of the unique bands of glucose can be traced; this results in the quantitative evaluation of the sample's chemical composition, which can then be related to the tissue pathology for diagnosis.

Raman spectroscopy probes characteristics of materials other than fluorescence. The energy transitions of molecules are solely between the vibrational levels. When a photon is incident on a molecule, it may be transmitted, absorbed, or scattered. Fluorescence results from the emission of absorbed energy; Raman scattering results from perturbations of the molecule that induce vibrational or rotational
transitions. Only a limited number of biological molecules such as flavins, porphyrins, and structural proteins (collagen and elastin) contribute to tissue fluorescence, most with overlapping, broadband emission. In contrast, most biological molecules are Raman active with fingerprint spectral characteristics. Indications are that vibrational spectroscopy may overcome some of the limitations of fluorescence diagnosis of precancers and cancers.

Several different modalities of Raman scattering have been used to analyze the structure of various biological molecules. These techniques include near-infrared (NIR) dispersive Raman, Fourier transform Raman (FT-Raman), surface-enhanced Raman (SERS), and ultraviolet resonance Raman (UVRR) spectroscopy. In NIR dispersive Raman spectroscopy, NIR radiation typically in the range of 780 to 1100 nm is used for excitation. The advantage of this technique is that minimal fluorescence is produced, thus making detection of the weak Raman signal easier. In FT-Raman spectroscopy, the Fourier transform of the signal is detected and then inversely transformed to give the actual Raman signature. This technique yields improved signal-to-noise ratio (SNR) of hard-to-detect events, but requires high collection times. Recent developments in spectroscopic instrumentation have resulted in reduced application of FT-Raman techniques in tissue diagnosis. Thus, while some early results from FT-Raman studies are referenced, the focus of this chapter is on dispersive Raman spectroscopy.

SERS is used to investigate the vibrational properties of single adsorbed molecules. It was discovered that the rather weak Raman effect can be greatly strengthened (by a factor of up to 14 orders of magnitude) if single molecules are attached to nanometer-sized metal structures. Metal surfaces must be of high reflectivity and of a suitable roughness. In this way, many groups have detected single molecules attached to colloidal silver particles adhered to a glass slide or even in an aqueous solution. The advantages of this method are that it is fast, it can supply some structural information about the molecules, and it does not bleach the molecules. Single-molecule detection is of great practical interest in chemistry, biology, medicine, and pollution monitoring; examples include DNA sequencing and the tracing of interesting molecules such as those used in bioterrorism. Again, because the focus of this chapter is on clinical in vivo diagnosis, SERS will not be discussed here.

When the excitation frequency approaches or enters the region of electronic absorption of a molecule, the resonance Raman spectrum of that molecule is obtained. By choosing appropriate excitation frequencies, a sample is selectively excited at the maximum absorption frequency of a characteristic molecule to detect that feature above all else. Resonance excitation increases the scattering intensity by several orders of magnitude. Typical absorption frequencies of biological molecules such as proteins and nucleic acids are in the ultraviolet portion of the spectrum, where the Raman and fluorescence signatures may be spectrally resolved. However, the high intensities may cause photolysis of the sample and destroy it over time. Besides, this signal may be attenuated by simultaneous intensified absorption and fluorescence emission. In addition to these factors, the mutagenicity of UV radiation makes this technique inviable for clinical in vivo use. Therefore, UVRR studies are excluded from this chapter.

Many groups have recognized the potential of Raman spectroscopy in the study and diagnosis of disease because of its successful application in biology. Early attempts to measure Raman spectra of cells and tissues were hindered by two factors: (1) the highly fluorescent nature of these samples and (2) instrument limitations, which necessitated long integration times and high-power densities to achieve spectra with good S/N ratios. Improvements in instrumentation in the last decade, particularly in the near-infrared region of the spectrum where fluorescence is reduced, have resulted in a dramatic increase in biomedical applications of Raman spectroscopy. Recent reviews of this field illustrate the diversity of potential applications, ranging from monitoring of cataract formation in vivo to the precise molecular diagnosis of atherosclerotic lesions in coronary arteries. Reports have begun to appear validating the potential of Raman spectroscopy to diagnose disease in human tissues in vivo.

### 30.3 Instrumentation Considerations

Despite the wealth of information provided by Raman spectroscopy about the structure of biological molecules, early attempts to measure Raman spectra of tissues were limited by two factors: (1) the highly
fluorescent nature of these samples and (2) instrument limitations, which necessitated long integration times and high-power densities to achieve spectra with good S/N ratios. The initial Raman spectra of tissue were measured with visible laser excitation, using primarily the argon laser lines (see, for example, Yu and East\textsuperscript{28} and Clarke et al.\textsuperscript{29}). With the development of interferometers, FT-Raman spectroscopy was used to measure tissue Raman spectra, typically using 1064 nm (Nd:YAG) for excitation with germanium detectors.\textsuperscript{10}

The development of diode lasers and cooled silicon CCD cameras sensitive in the near-IR has resulted in a wide range of applications beyond the laboratory. Diode lasers can provide excitation in the region of 750 to 850 nm, which allows the use of silicon detectors (sensitive only to 1100 nm). There are two advantages of this technique: (1) fluorescence emission is reduced and (2) spectra with acceptable SNRs ratios can be achieved with relatively short integration times on the order of a few seconds. Thus, Raman spectroscopy is now commonly used in environmental monitoring and manufacturing. Advances in the Raman instrument by the commercial sector that supplies this industry have positively impacted the application of Raman spectroscopy in biomedicine, making clinical use viable.

### 30.3.1 Basic Instrumentation

The basic instrument capable of measuring Raman spectra is similar to any spectroscopic system. It consists of a light source, which is typically a laser, light delivery and collection, and a detection system. Figure 30.3 shows a typical clinical Raman system used today.

The weak nature of the Raman phenomenon makes the details of this basic system challenging. These three basic components interact in terms of overall system performance, but as a first approximation they can be viewed as individual components contributing to the system. Significant technological advances in each of these areas have resulted in Raman instruments superior to similar systems from the previous decade. We consider each of the three components of this basic system individually; however each is dependent on the intended use of the system. For example, a system capable of confocality requires a single-mode laser and appropriate optics. A clinical fiber-based Raman system requires a multimode diode laser with stable frequency output and rugged design that facilitates portability.

![Figure 30.3](image)

**FIGURE 30.3** Schematic of a typical near-infrared dispersive Raman spectrometer used for tissue diagnosis today. This system is shown with an Enviva Raman probe.
30.3.1.1 Light Sources

Traditional Raman systems used the argon ion (Ar⁺) laser for visible excitation, the Nd:YAG laser for FT-Raman applications and the Titanium:Sapphire (Ti:Sapph) laser for NIR excitation due to their high output powers, single spatial and longitudinal mode operation, and Gaussian beam profile that allows for near-diffraction-limited optical performance. However, the size and electronic and cooling requirements of these lasers make them impractical in a portable clinical system. Some current Raman instruments, especially those with confocal capabilities, still use the Ti:Sapph laser. However, advances in diode laser technology have completely changed the fingerprint of a typical Raman system.

Diode lasers utilize electro-optical components (diodes), which emit light as a function of applied current and operating temperature. The laser diodes are small (<1 mm³) and require highly accurate controlling electronics to obtain stable output. Laser diodes require highly stabilized temperature controllers to minimize thermoelastic effects on the laser cavity length (and thus output frequency), and highly stabilized current sources to minimize output power fluctuations. However, even with highly accurate controlling electronics, laser diodes are still susceptible to output variations, so users of these lasers must be aware of their limitations. In addition to the frequency and power considerations described, laser diodes are also characterized by their elliptical beam output (due to the rectangular shape of the output facet) and astigmatism (due to unequal beam divergence from each dimension of the rectangular facet). These issues also need to be accounted for when using a diode laser. Most commercial diode lasers, however, are available with a pig-tail option where a fiber is coupled directly to the laser diode, thus minimizing losses due to astigmatism and elliptical nature of the beam.

A diode laser with tunability in its wavelength is available; such a system tends to be more susceptible to changes in the laser cavity length, resulting in so-called "mode-hops" in which the primary resonant mode of the laser jumps from one frequency to another. Because Raman scattering is very sensitive to the frequency of the incident photon, a source stable in frequency with high line widths is essential to obtain reliable spectra. Diode lasers that are optimized for highly stable frequency output and powers of the order of 300 mW specifically designed for Raman spectroscopy are now commercially available (such as that offered by Process Instruments Inc., Salt Lake City, UT).

Most published reports of confocal Raman microspectroscopy utilize bulky Ar⁺ and Ti:Sapph lasers for excitation sources. However, the size of these lasers, and their electronic and cooling requirement, make use in a portable clinical system impractical. Thus, in order to develop a truly portable confocal Raman microscope, an alternative excitation source is needed. By extending the length of the resonant cavity of a laser diode, the distance between the diode's longitudinal modes can be extended, thereby minimizing the effect of small thermoelastic changes on the output frequency. Such a source is called external cavity diode laser (ECDL). Compared to a bare laser diode, the ECDL nearly eliminates mode-hops, minimizes spectral bandwidth of the output light, allows substantial wave length tunability, and markedly decreases frequency dependence on temperature stabilization. A commercial ECDL single-mode system, previously available at a high price, is no longer on the market. An ECDL excitation source can be built in-house using off-the-shelf optics, hardware, and electronics at a price (~$6,000) far less than the cost of commercially available Ar⁺ and Ti:Sapph laser systems (= $20,000) typically used in confocal Raman systems.

30.3.1.2 Detectors and Spectrometers

A typical dispersive Raman detection system consists of a short focal length imaging spectrograph attached to a cooled CCD camera. Clinical implementation of the Raman system requires spectral acquisition on the order of a few seconds. This fast acquisition in turn needs a fast spectrograph and a highly sensitive detector, especially given the weak nature of the Raman signal. A typical CCD camera used in spectroscopy consists of a rectangular chip wherein the horizontal axis corresponds to the wavelength axis. The vertical axis is used to stack multiple fibers for increased throughput and is subsequently binned for improved SNR. Technological advances have led to CCD chips with quantum efficiencies on the order of 90% in the near-infrared. While various types of chips are commercially available for different applications, a back-illuminated, deep depletion CCD is highly recommended for NIR
Raman spectroscopy. These chips are known to be susceptible to the so-called “etaloning effect” wherein the thin silicon chip acts as an etalon, resulting in the introduction of sharp peaks in the sample signal that are hard to resolve from the narrow Raman signal. However, commercial cameras are now available (such as that offered by Roper Scientific Inc., Princeton, NJ) that incorporate technology that effectively eliminates this etaloning effect.

Most laboratory-grade systems currently utilize a liquid nitrogen–cooled CCD that allows the detector to be cooled to at least −80°C, resulting in extremely low dark noise as well as read-out noise. While this level of noise is important in a laboratory grade system where integration times and other system parameters are still being evaluated, a clinical system with a liquid nitrogen–cooled detector is cumbersome and impractical. Most recently, thermoelectrically cooled detectors are now available with multistage Peltier cooling systems that allow the camera to be cooled down to −80°C, thus solving this dilemma. It should be noted that shot noise due to tissue fluorescence continues to be a limiting factor.

Silicon-based detectors are relatively inexpensive and have high quantum efficiencies in the NIR, making them ideal detectors down to about 1100 nm. For longer wavelengths, other types of detectors such as indium gallium arsenide (InGaAs) and germanium (Ge) detectors need to be used. While the longer wavelengths do result in lower fluorescence interference, the quantum efficiency and noise exhibited by these detectors are less than silicon detectors. Research needs to be conducted in this area to verify that Raman spectra with adequate S/N can be acquired from tissue using 1064 nm excitation. New technological advances have yielded low-cost InGaAs and Ge detectors with compact Nd:YAG lasers resulting in extremely compact 1064 nm Raman systems (TRI, Inc., Laramie, WY).

A Raman-sensitive detection system requires an appropriate imaging spectrograph that couples to the light delivery device (such as a fiber probe) on one end and the CCD of choice at the other end. In order to resolve details of the Raman bands, the Raman system should have a spectral resolution of at least 8 cm⁻¹. Commercial spectrographs optimized for Raman use are now available that provide f-number matching with standard fiber optics and high throughput for rapid acquisition using holographic gratings. These spectrographs (such as that offered by Kaiser Optical Systems, Inc., Ann Arbor, MI) are compact by design and rugged for portable use. Additional components of the detection system include laser line rejection filters that remove any laser light as well as the elastically scattered light from the detected signal. Holographic notch filters can block the laser wavelength with an optical density of six with steep edges and provide 90% transmission elsewhere with a relatively flat curve.

Most Raman systems used in medical applications today are state-of-the-art, high-cost, high-performance Raman systems. This system at its best consists of a Kaiser imaging spectrograph, a Roper Scientific liquid nitrogen–cooled CCD detector, a 300 mW Process Instruments diode laser, and a custom fiber probe. Typical integration times are on the order of 5 to 10 sec for this type of system.

30.3.1.3 Light Delivery and Collection (Fiber Optics)

Raman scattering is a weak phenomenon, but most materials are Raman active, which allows for molecular-specific study of samples. On the other hand, because most molecules are Raman active, the materials used in the Raman system themselves interfere with the detection of sample signal. The development and availability of diode lasers, imaging spectrographs, and cooled CCD cameras has made it possible to build compact NIR Raman systems that acquire spectra with short integration times. However, the limiting factor remains the signal generated in the delivery systems (luminescence and Raman) used for remote sensing. Light is typically delivered using optical fibers made of silica in a remote sensing spectroscopic system. However, silica has a strong Raman signal, which overrides sample signal. The signal generated is proportional to the fiber length and limits the detection capability of the technique.

Figure 30.4 shows the Raman spectrum of fused silica fibers used to design a probe. Raman signal was observed to be generated from the core as well as the cladding and buffer of the fiber. This signal can have magnitudes equal to and sometimes greater than those of the sample under study and thus demands careful consideration. Fiber signal is generated in the delivery fiber by the excitation light. In addition, background signal is generated in the collection fibers by the elastically scattered excitation light returning to the collection fibers. Mathematical techniques cannot be used to remove this unwanted fiber signal.
A feasible probe design must prevent unwanted signal generated in the delivery fiber from illuminating the sample as well as prevent elastically scattered excitation light from entering the collection fibers and generating unwanted signal.

Several different designs have been proposed for potential clinical acquisition of Raman spectra using fiber optic probes. Early on, Myrick and Angel developed different dual fiber probes that could be used under different conditions with maximum collection efficiency but minimum fiber interference. Most fiber designs since have been based on similar concepts with modifications. In general, Raman probe designs utilize a bandpass filter placed after the excitation fiber lens, thus allowing only the transmission of the excitation light from the delivery fiber, and a longpass or notch filter placed in front of the collection fibers that blocks the transmission of the Fresnel reflected excitation light and also prevents the elastically scattered light from entering the collection fibers. These filters are placed at the tip of the probe for maximum effectiveness and they must be sized on the order of a few millimeters or smaller. There is a demand in biomedicine for high-quality optical coatings and micro-optical components to simplify the design of much-needed compact fiber optic probes for Raman spectroscopy.

Multiple fiber bundles have been utilized for fluorescence measurements of tissue by several groups. Such a bundle typically consists of a central excitation fiber surrounded by many collection fibers linearly aligned in front of the spectrometer. McCreery et al. used this design and tested it on different samples. Although spectra with good SNR ratio could be obtained from transparent samples, fiber background was still a serious problem in samples with high elastic scattering such as tissue. Feld et al. have adapted an old design (used in solar energy collection) for improved signal collection to allow spectral acquisition in a few seconds. A hollow compound parabolic concentrator (CPC) was used at the distal tip of the probe to yield signals with seven times greater collection than a fiber probe without the CPC. Fiber background was reduced by using a dichroic mirror and separate excitation and collection fiber geometries. Excitation light was reflected by the mirror (placed at 45° with respect to the sample normal) through the CPC onto the sample. Raman spectra were collected by the CPC, transmitted through the mirror into a collection fiber bundle (with about 100 fibers) to a detector. This probe design was used to acquire Raman spectra for transcutaneous blood glucose measurements.

In vivo applications thus far have been mostly confined to exposed tissue areas where fiber background could be circumvented using a macroscopic arrangement — e.g., noncontact probe (DLT, Laramie, WY) used for breast tissues by McCreery et al. and CPC probe for transcutaneous measurements of blood analytes by Feld et al. However, other applications such as in the colon, cervix, and oral cavity, require...
a more compact configuration and probe design. Thus, while many reports have been published assessing the feasibility of Raman spectroscopy for disease detection, few reports have applied this technique in vivo. The main obstacle continues to be fiber probe design.

One of the first designs of a compact fiber probe used clinically in the cervix was reported by the author. As stated previously, a feasible probe design must prevent unwanted signal generated in the delivery fiber from illuminating the sample, as well as prevent elastically scattered excitation light from entering the collection fibers and generating unwanted signal. Experimental results show that significant proportions of silica signal are generated in the excitation and collection fibers, indicating the need for filters in the excitation and collection legs of the probe. Figure 30.5 shows a transverse section of the design that was implemented as a Raman probe for the cervix. Rather than placing the excitation leg at an angle, a mirror surface is placed in the excitation path to deflect the beam onto the sample. This probe was designed using the smallest available physical dimensions of the bandpass and the holographic notch filters at that time. An angularly polished gold wire was glued in place such that the deflected excitation and normal collection spots overlap. One of the collection fibers was used to provide an aiming beam during placement of the probe on the sample. A quartz shield was used at the tip of the common end of the probe, forming a barrier between the probe optics and the sample. Quartz was selected as the material of choice because its fluorescence and Raman signal were known and any additional background signal from the probe could be identified. The inner surfaces of the metal tubings used to house the probe optics were anodized to reduce the incidence of multiple reflections of light.

The probe was tested in a pilot clinical study approved by the Institutional Review Board (IRB). NIR Raman spectra of the cervix were successfully measured in vivo, as shown in Figure 30.6, using integration times in the order of 90 sec. Since this initial report was produced, several designs for a Raman probe have been reported. One design in widespread use is the beam-steered Enviva Raman probe designed by Visionex Inc. (now Cirrex Corp., Atlanta, GA), which incorporates in-line filtering of the laser light and the scattered light at the tip of the probe. The beam steering allows improved overlap of the excitation and collection volumes and thus improves signal collection efficiency (see Figure 30.3).

Another unique feature of this probe was its dimension in the order of 1 to 3 mm, which was not seen in other probes available commercially. However, the company no longer manufactures these probes and
thus a need for such slickly designed Raman probes still exists. Attempts have been made by other companies such as InPhotonics Inc. to fill the gap. In a survey of currently available probes for biomedical Raman use, it was found that all commercially available probes were bulky (up to several millimeters in diameter) and expensive (~$3500 or more). Proponents of Raman spectroscopy for tissue diagnosis must rely on custom-designed fiber probes built using commercial vendors.

Other new designs have been recently reported. Most recently, the Feld group has proposed a newer design for breast cancer detection, based on micro-optics and optical coatings. Results of spectra acquired with this probe have not yet been reported in vivo. The recent developments in the field of microfabrication (MEMs) open a new avenue for compact probe designs using reflective optics. Others (including the author) continue to develop new ideas for feasible probe designs that should be tested and reported in the near future.

30.4 Effect of Sample Conditions

The design and construction of a Raman system is clearly a critical part of the process. An often forgotten aspect in the use of a new technique deals with the application itself. In developing and applying a new technique, the technology must first be tested in a model system before it can be applied in vivo. Subsequently, when it is ready for in vivo use, several factors unique to clinical use must also be considered. These issues are addressed below.

30.4.1 Tissue Model Selection

In the field of spectroscopy, there are two philosophies on the approach to use in testing the feasibility of a given technique. Some researchers prefer to use an animal model that resembles human tissues in function and structure to simulate the behavior of human tissues, while others prefer in vitro human tissue studies before tackling in vivo studies. Each of these approaches needs to be carefully considered in the context of the human in vivo model.

Although an animal model allows in vivo testing, Raman spectra of animal tissues can in some cases differ from those of human tissues, resulting in conclusions not valid for humans. One example of this is the use of mouse eye lenses for the study of lens aging. In early studies, Yu suggested that the intensity of Raman bands at 2580 cm$^{-1}$ due to sulphhydryl groups (–SH) and 508 cm$^{-1}$ due to disulfide groups (–S–S) can be used to calculate their relative concentrations. These can then be related to aging and

FIGURE 30.6 In vivo NIR Raman spectra of cervical tissue measured using the probe shown in Figure 30.5.
cataract formation. In an extensive study of mouse lenses, a fall in –SH concentration and a correspond-
ing increase in –S–S concentration was observed along the visual axis of mouse lens nucleus from age
1 to 6 months. A tandem increase in protein development with a decrease in sulfhydryl was concluded
to accompany the normal aging process. However, on repeating these studies on guinea pig and human
lenses, a different phenomenon was observed. Although guinea pig lenses also showed a decrease in
sulfhydryl intensity, no corresponding increase in disulfide band was observed. Human eye lenses
behaved similarly to guinea pig lenses. This study clearly indicates the pitfalls in improper selection of
an animal model and that independent verification of the model is needed to ensure that results will be
applicable to human conditions.

A primary concern in devising a clinical diagnostic system based on Raman spectroscopy is whether
the spectra of excised tissue resemble those of tissue spectra acquired in vivo and whether the information
obtained from these in vitro studies can be applied to a clinical setting. It is well known that the spectra
acquired from in vitro tissues (especially those that have undergone the freeze–thaw process) differ from
those acquired in vivo. Spectra from intact human stratum corneum were compared to those from
excised human stratum corneum; significant differences in the Raman spectral features were observed.
Increased intensity of the C–C stretching vibrational bands at 1030 and 1130 cm–1 was observed in vivo.
An additional spectral band at 3230 cm–1 of unknown origin is observed only in vivo. Thus, it is important
to be aware of the potential differences that may occur when moving from in vitro studies to in vivo
conditions. Most researchers who follow this approach use the in vitro studies as proof of concept before
moving directly into in vivo studies.

### 30.4.2 In Vivo Considerations

In vivo clinical application of the technology is the goal of all researchers in this field. However, various
issues specific to working in vivo must be considered in the planning of these clinical studies. Logistic
issues such as IRB approval and consent forms have become challenging processes. When building a
clinical system, some issues need to be considered. Because the probe must be sterilized or disinfected,
depending on the application, it must be designed to withstand the physical and chemical conditions of
these processes. The power consumption of the Raman system must not overload hospital and clinic
systems. Such requirements can be verified by the biomedical engineering department of the hospital.
Acquisition times must be on the order of a few seconds for the sake of both the patient and clinician
participation. The system needs to be self-contained and nonobtrusive. Some of these issues may appear
trivial, but they all contribute to smooth conduct of clinical studies.

Any technique that uses lasers must comply with certain safety standards to avoid potential hazards.
Safety standards for laser exposure of skin and eye are established by the American National Standards
Institute (ANSI). The power densities used for successful Raman spectral acquisitions can be quite high
and thus warrant consideration as safety hazards. The maximum permissible exposures as set by ANSI
are wavelength dependent and can be calculated for a given exposure time following the directions
specified in the ANSI laser safety manual. Sources of variability that need to be evaluated experimentally include ambient effects, physician variation, pressure effects, and procedural interference. Regardless of the short duration of the integration
times used for Raman acquisition, ambient effects can be significant for spectral integrity. Although room
lights may be turned off and windows may be light-tight in a laboratory setting, these are more critical
issues in a clinical setting and require careful planning. Variability from physician to physician in terms
of single-site placement, as well as site-to-site placement for a given pathology, must be evaluated. The
effect of varying pressures applied to the probe during acquisition should also be considered. Another
critical parameter is the measurement-site to biopsy-site variability. Because histology continues to be
the gold standard against which we must compare this technology, it is critical that the biopsy be obtained
from the precise site of spectral measurement using such techniques as marking inks, etc. placed appro-
priately. It is essential to account for sources of variability when acquiring Raman spectra from human
patients in vivo.

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30.5 Processing

Once a Raman spectrum is acquired using a feasible Raman system, it must be processed for different reasons, including calibration, noise smoothing, and fluorescence elimination and binning. Other processing methods may be applied depending on the requirements of the analysis tools and the variability in the data.

30.5.1 Calibration

Because different researchers have different approaches in the development of a given application for Raman spectroscopy, a standard must be set to allow transferability of data across systems and methods. Instrument and data calibration are key components of data processing immediately following data acquisition. Some calibration must occur at the time of acquisition and other calibration may be implemented later. Most biomedical Raman systems currently in use are based on dispersive CCD systems where the spectra are recorded as a function of pixel numbers along the horizontal axis. Thus, the spectrum of a known calibrated source such as a neon lamp is typically taken to calibrate the horizontal axis. Raman spectrum of a strong Raman scatterer such as naphthalene, a strong Raman scatterer and fluorophore such as rhodamine 6G, and a weak Raman scatterer such as methylene blue are used as intensity standards to account for day-to-day variations in the spectral intensity. A spectrum of the laser source is taken to verify the location (and thus the wavelength) of the laser line. This spectrum is also used with the neon spectrum to convert the horizontal axis in terms of relative wavenumbers.

Finally, there exists a wavelength-dependent efficiency of all the various components of the detection system such as the grating, the filters, optics, quantum efficiency of the CCD chip, etc.; therefore, the wavelength dependency of the system response must be calibrated. This is typically done using an NIST-calibrated source such as a tungsten lamp to generate correction factors for the instrument variation. As various researchers acquire calibrated Raman spectra, a library of tissue Raman spectra is created that can be used to study cross links in the chemical compositions and correlation in tissue pathologies as well as to strengthen and standardize the use of Raman spectroscopy for tissue diagnosis.

30.5.2 Fluorescence Elimination

In recent years we have witnessed an explosion in the use of Raman spectroscopy for biological purposes such as tissue diagnosis, blood analyte detection, and cellular examination. The greatest benefits of this technique are its high sensitivity to subtle molecular (biochemical) change and its capacity for noninvasive application. Because biological applications of Raman spectroscopy involve turbid, chemically complex, and widely varying target sites, much of the challenge in using Raman spectroscopy for biological purposes is not only the acquisition of viable Raman signatures but also the suppression of inherent noise sources present in the target media. Perhaps the greatest contributor of “noise” to biological Raman spectra is the intrinsic fluorescence of many organic molecules in biological materials. This fluorescence is often several orders of magnitude more intense than the weak chemical transitions probed by Raman spectroscopy and, if left untreated, can dominate the Raman spectra and make analysis of tissue biochemistry as probed by the technique impractical. Therefore, in order to extract Raman signal from the raw spectra acquired, it is necessary to process the spectrum to remove this fluorescence.

A number of techniques, implemented in both hardware and software, have been proposed for fluorescence subtraction from raw Raman signals. Hardware methods such as wavelength shifting and time-gating have been shown to effectively minimize fluorescence interference in Raman spectra, but require modification of the spectroscopic system to achieve their results. Mathematical methods implemented in software require no such system modifications, and have thus become the norm for fluorescence reduction. These methods include first- and second-order differentiation, frequency-domain filtering, wavelet transformation and manual polynomial fitting. Although each of these methods has been shown to be useful in certain situations, these methods are not without limitations.
Differentiation can be implemented in various ways. One way is to measure the spectra at two slightly shifted excitation wavelengths and take their difference. The fluorescence remains unchanged at both excitation wavelengths whereas the Raman peaks are shifted. The difference of the two spectra is comparable to the first derivative of the Raman spectrum; integrating the difference spectrum yields the original Raman signal. A similar result can be obtained by measuring the spectrum at a single excitation wavelength and taking the first derivative of the spectrum. The Raman spectrum can be obtained by integrating the noise-smoothed derivative spectrum following baseline correction. The derivative method is entirely unbiased and very efficient in fluorescence subtraction, yet it severely distorts Raman line shapes and relies on complex mathematical fitting algorithms to reproduce a traditional spectral form.

Frequency filtering can be achieved by using the fast Fourier transform (FFT). In this technique, the measured spectrum is Fourier transformed to the frequency domain by taking the FFT of the signal. The FFT signal can then be multiplied with a linear digital filter to eliminate the fluorescence. The inverse FFT then yields the Raman spectrum free of fluorescence. This method can cause artifacts to be generated in the processed spectra if the frequency elements of the Raman and noise features are not well separated. Wavelet transformation, a more recently utilized method, is highly dependent on the decomposition method used and the shape of the fluorescence background.

A simple, accurate, and more elegant method to subtract fluorescence is to fit the measured spectrum containing both Raman and fluorescence information to a polynomial of high enough order to describe the fluorescence line shape but not the higher-frequency Raman line shape (Figure 30.7). A fourth- or fifth-degree polynomial has been found to be optimal. Polynomial curve-fitting has a distinct advantage over other fluorescence reduction techniques because of its ability to retain the spectral contours and intensities of the input Raman spectra. However, simply fitting a polynomial curve to the raw Raman spectrum in a least-squares manner will not efficiently reproduce the fluorescence background because the fit is based on minimizing differences between the fit and the measured, which includes the fluorescence background and the Raman peaks. Subsequent subtraction of this fit polynomial results in a spectrum that varies about the zero baseline.

This technique traditionally relies on user-selected spectral locations on which to base the fit from regions that do not include the more intense Raman bands. Unfortunately, this intervention has several drawbacks. It is time consuming because the user must process each spectrum individually to identify...
non-Raman-active spectral regions to be used in the fit. In addition, identification of non-Raman-active frequencies is not always trivial because biological Raman spectra sometimes contain several adjacent peaks or peaks that are not immediately obvious. The end effect is a method that is highly subjective and prone to variability. In order to address the limitations of existing methods of fluorescence subtraction, various methods to automate polynomial curve-fitting have been developed that retain the benefits of manual curve fitting, without the need for user intervention. One implementation of this automation is the modified-mean method.

In one implementation of this automation, the basis is a simple sliding-window mean filter, in which the center pixel intensity in the window is set equal to the mean of all the intensities within the window. The modification involved in this method is that any filtered pixels with an intensity value higher than the original are automatically reassigned to the original intensity; therefore, the smoothing only eliminates high-frequency Raman peaks while retaining the underlying shape of the baseline fluorescence. This smoothing is repeated over the entire spectrum until a specified coefficient of determination ($R^2$) of the processed spectrum with an $n$th order polynomial (typically fifth order) is obtained. The smoothed spectrum is then subtracted from the raw spectrum to yield the Raman bands on a near-null baseline. This algorithm can be implemented easily in MATLAB, thus automating the entire procedure.

Figure 30.8 shows the Raman spectra of rhodamine 6G, as well as the processed Raman spectrum using derivative, FFT, and automated polynomial fluorescence subtraction techniques for direct comparison of the methods. The figure shows the effectiveness of the automated polynomial method for removing the slow varying fluorescence baseline while retaining the Raman features of rhodamine, especially in comparison to the other methods.

Each of the different techniques has advantages and disadvantages and the method should be selected based on the specific application and measurement technique used. Mosier-Boss et al. tested the use of the shifted excitation, first derivative, and FFT techniques for fluorescence subtraction; a preference for using the FFT technique was indicated due to its ability to filter out random noise from the spectrum. In an analysis of the different techniques, the use of a polynomial fit was found to be the simplest technique from both experimental and computational points of view.
30.5.3 Other Preprocessing Methods

Because Raman scattering is such a weak phenomenon, the SNRs of most measured Raman spectra are such that noise-smoothing filters must be included in the processing procedure. Various types of filters that have been effectively used include the Gaussian filter, whose full-width-half-maximum is typically set equal to the spectral resolution of the system, and the Savitsky-Golay filter of various orders. When using any method of noise smoothing, care should be taken to retain the integrity of the spectral line shape, especially in the case of multiple peaks.

Other preprocessing methods include binning of the spectral dataset for computational ease because of a large number of variables. Depending on the variability in the acquired data and the needs of the analysis methods used, other methods include normalization to intensity standards, normalization to its own maximum intensity, mean-scaling the spectra acquired from a given patient, etc.

30.6 Analysis

One advantage of spectroscopic diagnosis is automation, which allows objective and real-time diagnosis of pathologies. Differences in spectral features can be incorporated into diagnostic algorithms; several techniques have been identified and applied to enhance the differentiation and classification of tissues for potential automated clinical diagnosis. Because Raman spectroscopy is a molecular-specific technique, the contribution of the various participating chromophores can also be extracted from the measured spectra. These can, in turn, be used in diagnostic algorithms and in the understanding of the spectral signature as it pertains to the disease process. In addition, concentration of specific components such as glucose can be obtained for diagnostic use.

30.6.1 Automated Diagnosis

Early studies analyzed biological Raman spectra for differences in intensity, shape, and location of the various Raman bands between normal and non-normal materials such as tissues. Based on consistent differences observed among the various tissue categories, diagnostic algorithms are developed using empirical methods. These algorithms may be based on changes in intensity or ratios of intensities or number and location of peaks. For example, it has been observed that the intensity ratio of the CH₂ bending vibrational mode at 1450 cm⁻¹ to the amide I vibrational mode at 1655 cm⁻¹ varies with disease in several applications, including breast, gynecologic, and precancers. These empirical algorithms indicate the specific changes that occur in the spectra acquired and provide information about the biochemical processes that result in these spectral differences.

This empirical analysis, however, has two important limitations. First, clinically useful diagnostic information is typically contained in more than just the few wavenumbers surrounding peaks or valleys observed in tissue; a method of analysis and classification that includes all the available spectral information can potentially improve the accuracy of detection. Second, empirical algorithms are optimized for the spectra within the study. Hence, the estimates of algorithm performances will be biased toward that tissue population. An unbiased estimate of the performance of the algorithms is required for an accurate evaluation of the performance of Raman spectroscopy for tissue diagnosis. To address these limitations, multivariate statistical techniques have become the practice to develop and evaluate algorithms that differentiate between normal and non-normal tissues.

In recent years, the potential for using multivariate techniques for spectroscopic data analysis in disease detection has been exploited with great success. Discrimination techniques such as linear regression, as well as classification techniques such as neural networks, have been used. Data compression tools such as principal component analysis (PCA) are used to account for variability in the data. Subsequently, methods such as hierarchical cluster analysis (HCA), linear discriminant analysis (LDA) and others have been used to yield classification algorithms for disease differentiation. Partial least squares, a regression-based technique, and hybrid linear analysis have been used to extract
accurate concentrations of analytes such as glucose using NIR Raman spectra for transcutaneous blood analysis.59

The consensus of the scientific community is that, while any of these methods may form the basis of performance estimates, the true measure of success of Raman spectroscopy for tissue diagnosis can be assessed by extensive unbiased (and independent) validation alone. This may best be accomplished by random distribution of the subject population into two equal datasets: one for calibration or testing and the other for validation. When this is not feasible, one can then rely (in a limited manner) on the leave-one-out or leave-five-out methods of cross validation.

### 30.6.2 Component Extraction

Analyzing Raman spectra for the purpose of disease classification may be sufficient when the goal of the study is to achieve tissue diagnosis alone; however, most researchers want to know if and why they can diagnose disease. In the case of Raman spectroscopy, the wealth of information provided in these spectra, especially with respect to biochemical composition, allows us to study the question of “why” in detail that can subsequently be used for the purpose of detection as well.

While most researchers study the biochemical basis of the measured Raman spectra from biological tissues, some groups have been particularly active in this area. For example, Puppels et al. have utilized skin Raman spectra to obtain quantitative information about skin hydration;13 Feld et al. have developed comprehensive models for biochemical component extraction that have subsequently been used for disease classification.60 Each of these methods is based on the acquisition of Raman spectra from individually identified chromophores as morphological tissue components or as extracted biochemical constituents.

Pixelated Raman microspectroscopy is typically used (with or without confocality) to measure Raman spectra from individual morphologic tissue components using tissue sections. A Raman spectrometer is coupled to a microscope and is scanned across the tissue section to obtain Raman images that can then be correlated with serial hematoxylin- and eosin-stained sections to identify relevant morphologic components and their Raman signature. Alternatively, tissue chromophores can be extracted from biochemical assays and Raman spectra can be acquired from each of these extracted chromophores.60 By using mathematical models such as those developed by the Feld group, contributions of the various extracted or morphologic components to intact tissue spectra can then be extracted.60

### 30.7 Clinical Application of Raman Spectroscopy

*In vivo* studies are necessary to fully evaluate the potential of Raman spectroscopy for clinical diagnosis. Several groups have initiated this process with varying degrees of success.26,27,62 This progression has been made possible by the development of sensitive instrumentation, use of fiber optics, and development of automated algorithms. However, despite these significant developments, the majority of reported studies on the application of Raman spectroscopy for tissue diagnosis continue to be from *in vitro* studies. Only a limited number of reports exist on the application of Raman spectroscopy *in vivo* in humans in a clinical setting; most of them are pilot clinical studies. Nevertheless, these critical studies create the roadmap for the future of Raman spectroscopy in biomedicine.

Several biological molecules such as nucleic acids, proteins, and lipids have distinctive Raman features that yield structural and environmental information. These molecules have been studied in solutions and in their natural microscopic environments.20 The molecular and cellular changes that occur with disease result in distinct Raman spectra that can be used for diagnosis. The transitional changes in precancerous tissues as well as in benign abnormalities such as inflammation can also yield characteristic Raman features that allow their differentiation.

Several groups have indicated the potential of vibrational spectroscopy for disease diagnosis in various organ sites. These groups have shown that features of the vibrational spectrum can be related to molecular and structural changes associated with disease. Raman spectroscopy has been studied extensively for tissue diagnosis in four main organ sites: breast,60 esophagus (and the GI tract),63 cervix (and other
27. Other organ sites include the brain, the eye, and biological fluids. Some examples of the application of Raman spectroscopy for disease diagnosis are detailed in the following sections.

30.7.1 Breast

Until recently, perhaps the most widespread application of Raman spectroscopy in cancer research has been for breast cancer detection. Breast cancer is the most common cancer in women, accounting for 18% of all cancer deaths in women. The breast consists of mammary glands arranged in lobes separated by fibrous connective tissue and a considerable amount of fatty tissue. Fibrocystic changes are benign proliferative processes that vary from the innocuous to those associated with increased risk of carcinomas. These benign changes take three forms: (1) cyst formation and fibrosis, (2) hyperplasia, and (3) adenosis. In most afflicted breasts, all forms occur simultaneously in fibroadipose stroma.

Breast tumors may arise from the ductal and lobular epithelium or connective tissue. These tumors vary from benign adenomas and malignant adenocarcinomas to benign fibromas and malignant fibrosarcomas. Infiltrating ductal carcinoma (IDC), the most frequent invasive form of breast cancer, has been studied using Raman spectroscopy. IDCs typically show an increase in dense fibrous stromal tissue and malignant proliferation of ductal epithelium. Although routine screening using mammography can aid in early detection of malignancy, lesions identified with this method must be biopsied and evaluated histopathologically to determine the presence of malignancy. In recent years, spectroscopic techniques have been used for breast cancer diagnosis.

Several groups have studied the potential of Raman spectroscopy for pathologies of the breast, from detection of breast cancers to study of capsules from breast implants. Using an FT-Raman system, Alfano et al. obtained the first Raman spectra from excised normal human breast tissues and benign and malignant breast tumors and discussed the feasibility of using FT-Raman spectroscopy for differentiating normal and malignant breast tissues. The vibrational spectra of benign breast tissues (which include normal tissues) showed four characteristic bands at 1078, 1300, 1445, and 1651 cm−1. The spectra of benign tumors showed bands at 1240, 1445, and 1659 cm−1, and malignant tumors displayed only two Raman bands at 1445 and 1651 cm−1. The intensity ratio of 1445 to 1651 cm−1 was found to be larger in benign tissues compared to benign tumors and the same ratio was found to be even lower in malignant tumors.

A subsequent study by McCreery et al. assessed the feasibility of using NIR Raman spectroscopy for breast cancer detection. Chromophore contributions differed as excitation was shifted from the visible (yielding carotenoid and lipid bands) to the NIR (yielding only lipid bands) in normal tissues. NIR Raman spectroscopy yielded signals with lower fluorescence interference and higher SNR. NIR Raman spectra were measured from excised normal human breast tissues, tissues with fibrocystic change, and infiltrating ductal carcinoma (IDC) at 784 nm excitation (Figure 30.9). The ratio of the areas under the peaks at 1654 cm−1 and 1439 cm−1 were found to increase in malignant breast tissues as compared to normal breast tissues. This increase is consistent with the changes reported by Alfano et al. The intensity of the 1654 cm−1 C = C stretching band varies with the degree of fatty acid unsaturation; the CH2 scissoring band at 1439 cm−1 depends on the lipid-to-protein ratio. The spectra from IDC tissues showed an overall decrease in intensity with respect to normal tissue.
Several differences were also observed when IDC tissues were compared with benign abnormal tissue. In benign tissue, the intensities of the bands at 1656 cm\(^{-1}\) and 1259 cm\(^{-1}\) were smaller than the band at 1449 cm\(^{-1}\) and this band is further shifted to 1446 cm\(^{-1}\). The region of 850 to 950 cm\(^{-1}\) showed only two bands in benign tissue as compared to four in IDC samples. The peaks observed in normal tissues were primarily attributed to oleic acid methyl ester, a lipid, and the peaks observed in IDC and benign tissues were primarily attributed to collagen I. This is consistent with histopathology where IDC and benign tissues show an increase in interstitial tissues microscopically. These spectral differences were found to be significant; subsequently, this group reported the development of a clinical probe and system for in vivo testing. Preliminary testing of the probe was reported on in vitro samples but no other reports have since been published.

Similar results were also obtained by Manoharan et al. using a comparable system. The Raman spectra acquired from this study were analyzed using multivariate statistical techniques and results yielded no false negatives. This group has subsequently reported the development of a clinical Raman system and in vivo testing will be reported in the future.

### 30.7.2 Cervix

The various gynecologic tissues differ both structurally and functionally from one another and they have been studied using Raman spectroscopy. Because of its well-characterized disease process and its significance, the cervix has been studied extensively in the development of spectroscopic detection. The cervix is the most inferior portion of the uterus, which typically measures 2.5 to 3.0 cm in diameter in

![Figure 30.9](https://example.com/figure30.9)

**FIGURE 30.9** Raman spectra of (A) normal, (B) malignant and (C) benign human breast tissue at 784-nm excitation. (Modified from Frank, C.J., McCreery, R.L., and Redd, D.C., Anal. Chem., 67, 777, 1995.)
the human adult female. The cervix is covered by two types of epithelia. The multilayered squamous epithelium covers most of the ectocervix and is separated from the stroma by the basal layer; the columnar epithelium consists of a single layer of columnar cells and covers the surface of the endocervical canal. The interface of the two epithelia is called the squamo–columnar junction. Over time, the columnar epithelium is replaced by squamous epithelium, which causes the squamo–columnar junction to move toward the os. This transitional epithelium is termed squamous metaplasia.70

Virtually all squamous cervical neoplasias (new growth) begin at the functional squamo–columnar junction and the extent and limit of their precursors coincide with the distribution of the transformation zone.70 Cervical intraepithelial neoplasia (CIN) refers to the precancerous stages of cervical carcinoma and is often also referred to as cervical dysplasia. Here we generally refer to them as “precancers.” Other pathologies that affect the cervix include cervicitis or inflammation, which is usually the response of tissue to injury,70 and the human papilloma viral (HPV) infection. Similarities observed in the morphological changes of the epithelial cells between those induced by HPV and precancer have led to the suggestion that certain strains of HPV may be involved in the incipient stages of cervical precancer and other strains may aid in the progression of the disease.71 Thus, HPV is typically placed in the same category as mild precancers and is clinically treated as such. Endocervical cancers are typically adenocarcinomas, arising within the endocervix as opposed to cervical epithelial lesions, which arise in the squamo–columnar junction.70

Cervical cancer is the second most common malignancy found in women worldwide. It was estimated that in 2001, 4100 deaths occurred in the United States from this disease and 13,000 new cases of invasive cervical cancer were diagnosed.65 Although early detection of cervical precancer has played a central role in reducing the mortality associated with this disease over the last 50 years, the incidence of preinvasive squamous carcinoma of the cervix has risen dramatically, especially among women under the age of 50. The primary screening tool for cervical precancer is the Papanicolaou (Pap) smear, where scrapings from the walls of the ecto- and endocervix, which contain a variable number of cells, are examined and diagnosed.70 Although the widespread application of the Pap smear as a screening tool has greatly decreased the incidence of cervical cancer,72 sampling and reading errors lead to high false-positive and -negative rates. Treatment ultimately relies on directed biopsies and subsequent pathological findings.

Alfano et al. were the first to report on the feasibility of using FT-Raman spectroscopy for detecting cancers from various gynecologic tissues.57 Characteristic Raman features of normal tissues and malignant tumors from the cervix, uterus, endometrium, and ovary were described. Three significant peaks were noted to differ in the Raman spectra of normal and benign cervix compared to cancerous lesions. In cancerous tissues, the intensity of the amide I stretching vibration band at 1657 cm–1 is less than the intensity of the C–H bending vibrational band at 1445 cm–1. The amide III band at 1262 cm–1 is broadened in cancerous lesions. An additional unidentified peak at 934 cm–1 is observed only in normal and benign cervical samples. A possible diagnostic algorithm could be based on the relative intensities of the two peaks where I1657 > I1445 in normal and benign tissues and I1657 < I1445 in cancerous samples. Alfano et al. attributed these peaks primarily to collagen and elastin.

The author has reported in vitro as well as in vivo studies on the application of Raman spectroscopy for cervical precancer detection.27,58 In her early work, Raman spectra of cervical tissues were measured to characterize the spectral signatures of the different cervical tissue types and to assess the feasibility of using Raman spectroscopy for cervical precancer diagnosis.58 Primary tissue Raman peaks were observed at 626, 818, 978, 1070, 1246, 1330, 1454, and 1656 cm–1 (±10 cm–1), present in all samples. The peaks at 626, 818, and 1070 were attributed as primarily due to silica from the optics of the system. Both empirical and multivariate techniques were used to explore the diagnostic capability of NIR Raman spectra from cervical tissues. A multivariate discrimination algorithm developed using the entire Raman spectrum could differentiate cervical precancers from nonprecancers with a sensitivity and specificity of 91 and 90%, respectively.58 A discrimination algorithm developed using intensities at just 8 Raman bands gave an unbiased estimate of the sensitivity and specificity as 82 and 92%, respectively.58 Thus, the success of the in vitro Raman studies for precancerous cervical tissue recognition warranted the development of a clinical system.

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In a pilot clinical study approved by the IRB, NIR Raman spectra of the cervix were successfully measured \textit{in vivo} from 24 sites (11 normal, 4 inflammation, 4 metaplasia, 1 low-grade, and 4 high-grade lesions) in 13 patients.\textsuperscript{27} Raman spectra were measured from normal and abnormal areas of the cervix using the fiber probe described in Figure 30.5 and a Raman system similar to the one shown in Figure 30.3. Figure 30.10 shows typical Raman spectra of the different types of cervical tissues acquired \textit{in vivo}.\textsuperscript{27}

Cervical tissue Raman spectra show peaks in the vicinity of 1070, 1180, 1210, 1245, 1270, 1330, 1400, 1454, 1580, and 1656 cm\(^{-1}\). The ratio of intensities at 1454 to 1656 cm\(^{-1}\) is greater for precancerous tissues than for all other tissue types, while the ratio of intensities at 1330 to 1454 cm\(^{-1}\) is lower for samples with cervical precancer than for all other tissue types. A simple algorithm based on these two intensity ratios separates high-grade lesions from all others with a sensitivity of 100\% and a specificity of 95\%.\textsuperscript{27} Preliminary results thus indicate that it is possible to measure Raman spectra \textit{in vivo} and extract potentially diagnostically useful information.

\textit{In vivo} Raman spectra, in general, appeared similar to \textit{in vitro} Raman spectra previously obtained from cervical biopsies. Due to the small number of patients included, the number of samples within a particular category was small; only one of the investigated samples was histologically identified to be low grade. Thus, while promising results were obtained in discriminating high-grade lesions from all other tissue types, significant research still remains to be done to characterize the spectra of low-grade lesions and other tissue pathologies of the cervix before Raman spectroscopy can be validated as a viable method for the diagnosis and potential screening of cervical precancers.\textsuperscript{73}

\section*{30.7.3 Skin}

The skin is the largest organ of the body and has many different functions. It is divided into two main regions: the epidermis and the dermis. The epidermis primarily consists of keratinocytes and varies in thickness throughout the body. Melanocytes, the pigment-producing cells of the skin, are found throughout the epidermis.\textsuperscript{66} The dermis consists mostly of fibroblasts, which are responsible for secreting the collagen and elastin that give support and elasticity to the skin. There are two major groups of skin cancers: malignant melanoma and nonmelanoma skin cancers. Cancers that develop from melanocytes are called melanoma. Nonmelanoma skin cancers are the most common cancers of the skin. Two of the most common nonmelanoma types are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC).
About 75% of all skin cancers are BCCs, which have a high likelihood of recurrence after treatment, either at the same site or elsewhere. SCCs account for about 20% of all skin cancers; these carcinomas are more likely to invade tissues beneath the skin and distant parts of the body than are BCCs.

Although most people believe they are not at risk for skin cancer, cancers of the skin (including melanoma and nonmelanoma skin cancers) are the most common of all cancers and account for about half of all cases in the United States. The American Cancer Society estimated approximately 9800 skin cancer deaths for 2002, 7800 from melanoma and 2000 from other skin cancers. Melanomas account for about 4% of skin cancer cases but cause about 77% of skin cancer deaths. The number of new cases of melanoma found in the United States is on the rise. The American Cancer Society predicted 53,600 new cases of melanoma in the United States in 2002. For most skin cancer patients (including melanoma and nonmelanoma skin cancer), early diagnosis and thorough treatment (i.e., complete resection) are critical for a favorable prognosis.

Current diagnostic methods for skin cancers rely on physical examination of the lesion in conjunction with skin biopsy. Suspicious areas are selected upon visual inspection by the clinician, after which those lesions are partially or wholly biopsied for complete histological evaluation. The biopsy is then sectioned and stained for pathological investigation and diagnosis. This protocol for skin lesion diagnosis is accepted as the gold standard, but it is subjective, invasive, and time consuming; hence, there is considerable interest in developing a noninvasive diagnostic tool that can accurately detect skin lesions noninvasively in real-time, especially in the early stages.

Previous studies on skin cancer detection using optical spectroscopy have been primarily limited to fluorescence and diffuse reflectance spectroscopy and have produced limited success. The interference of skin pigment and external agents such as creams and soaps has been the major limitation to the success of these techniques. Despite the relative ease of studying the skin, the first published reports of skin Raman spectra appeared only in the early 1990s. These early studies, however, focused on characterizing skin components, and included research on skin hydration, skin aging, and the effect of UV radiation.

Williams et al. utilized FT-Raman spectroscopy to examine a number of skin features and correlate the Raman spectra with the biochemical agents responsible. Caspers et al. used confocal Raman microspectroscopy to ascertain the in vivo Raman signal characteristics emanating from each layer of normal human skin. They showed that each layer of the skin contained Raman features that could be highly correlated with the protein and chemical content of each respective layer. A recent study has also shown that it is possible to extract carotenoid Raman spectra in vivo from various skin tissues. Gniadecka et al. utilized FT-Raman spectroscopy to successfully differentiate BCC from normal, healthy skin in vitro in 16 patients. Although promising results were reported, FT-Raman spectroscopy is not a feasible technique for clinical diagnosis.

A recent study exploring the use of confocal Raman microspectroscopy for skin cancer detection was also reported. Results from two pilot studies performed on a novel bench top confocal Raman spectrometer were encouraging. In the first study, Raman spectra were acquired in vitro from normal and various types of malignant skin tissues obtained from human patients undergoing excision. Figure 30.11 shows the Raman spectra of normal skin, BCC, SCC, and melanoma tissues at a depth where optimal differences between the various tissue types were observed. Key differences in the spectra are observed at several Raman bands including those seen at 860, 940, 1120, 1220 to 1340, and 1550 cm⁻¹. Another study assessed the capability of the confocal Raman system to acquire Raman spectra in vivo and the variability in the Raman spectra from various types of normal skin. Raman spectra were acquired from the dorsal area of the hand from volunteers of various ethnicity and skin color (white, African-American, east Asian, and south Asian) at various depths of tissue. Subsequent statistical analysis of the Raman spectra at various depths revealed significant variations of Raman signal in the upper strata of the skin, presumably due to differences in melanin content. However, Raman spectra from locations deeper in the skin (~60 μm) showed much more similarity visually and statistically. These results indicate that confocal Raman spectroscopy can limit the measurement volume to specified depths of the skin,
which may circumvent the problems in skin color differences traditionally associated with optical measurement.

### 30.7.4 Other Applications

The preceding examples show the various stages of development in the implementation of Raman spectroscopy for disease detection in vivo. Additional applications of this versatile technique not specifically reported here include the detection of various pathologies in the brain\(^1\) and the GI tract, especially the esophagus,\(^6\) ovary,\(^5\) and colon.\(^6\) Raman spectroscopy has also been used to determine various analytes such as those in the blood. For example, studies have been reported on the determination of glucose concentration in diabetes patients.\(^5\) A recent study reported the transdermal measurement of blood glucose using tissue modulation for signal extraction.\(^8\) Researchers are using Raman spectroscopy in clinical settings and results appear promising. Raman spectroscopy is poised to move to the next level of implementation.

### 30.8 Future Perspectives

Recent technological developments have made clinical implementation of Raman spectroscopy feasible. Advances in diode laser technology and CCD detector technology have contributed tremendously to this process. However, the need still exists for compact Raman probes and integrated systems that would make this technique even more versatile. Currently, several integrated systems are available for the application of Raman spectroscopy in environmental and manufacturing processes. A similar advancement is required in the field of biomedicine. The introduction of micro-optics and microfabrication can further aid in the development of compact small-diameter probes.

This chapter presents a review of concepts, instrumentation, and sample applications of nonresonance Raman spectroscopy for disease detection in vivo. The success of the technique has led to the development of feasible clinical systems that can measure Raman signals from tissue with short collection times. Many of these systems have already undergone preliminary testing and several others are currently in the process of being tested. These studies clearly indicate that clinical application of Raman spectroscopy is imminent and may be expected to change the face of disease detection in the near future. While more extensive

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FIGURE 30.11 NIR Raman spectra of normal and cancerous skin lesions acquired *in vitro* using a confocal Raman microspectrometer.
studies are still needed to form a true assessment of the capability of Raman spectroscopy for disease detection, preliminary results are extremely encouraging. What makes this technique so invaluable is not only its use for disease classification, but also the possibility of evaluating the acquired spectra for biochemical composition. This technique is a viable clinical tool and an important research tool for furthering both the technology of patient care and the understanding of the disease process.

References


