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RAMAN SPECTROSCOPY FOR CANCER DETECTION: A REVIEW

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Abstract

This article reviews recent developments in the attempt to develop diagnostic techniques for cancers and precancers based on Raman spectroscopy. The article summarizes some facts about cancer biology as it pertains to Raman diagnosis. It reviews certain instrumentation considerations and finally surveys the application of Raman spectroscopy for diagnosis on intact tissues. The article concludes with our perspective on the current status of Raman spectroscopy for clinical applications and its future.

Keywords: Raman spectroscopy, cancer, precancer, tissue, diagnosis

Introduction

There is much evidence to indicate that fluorescence spectroscopy of both exogenous and endogenous chromophores can be used to identify neoplastic transformations in cells and precancer and cancer in various organ sites (see for example [1,2]). Despite the success of this technique, the 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 [1,2]. 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. To enhance the specificity of spectroscopic diagnosis, vibrational spectroscopy has been considered.

Raman spectroscopy has been used for many years to probe into the biochemistry of various biological molecules [3]. In recent years, there has been interest in using this technique in diagnostics [3]. Raman spectroscopy probes different characteristics of materials than fluorescence [4]. 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. Raman scattering arises from perturbations of the molecule that induces 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; because of this, vibrational spectroscopy may overcome some of the limitations of fluorescence diagnosis of precancers and cancers.

Principles of Raman Spectroscopy

Classically speaking, 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 [4]. When energy is transferred either from the molecule to the photon or vice versa, the scattered photon has less or more than the energy of the incident photon. This is inelastic or Raman scattering. A Raman spectrum is a plot of scattered intensity as a function of the energy difference between the incident and scattered photons. The loss (or gain) in photon energies corresponds to the difference in the final and initial vibrational energy levels of molecules participating in the interaction. The resultant spectra are characterized by shifts in wavenumbers (inverse of wavelength in cm^{-1}) from the incident frequency. 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.

Several different modalities of Raman scattering have been used to analyze the structure of various biological materials [3]. Some of these techniques include near infrared (NIR), fourier transform infrared (FT-IR) and ultraviolet (UV) resonance Raman spectroscopy [4]. In NIR Raman spectroscopy, NIR radiation typically in the range of 800-1100 nm is used for excitation. The advantage of this technique is that minimal fluorescence is produced making detection of the weak Raman signal easier. In FT-IR Raman spectroscopy, the Fourier transform of the signal is detected which is then inversely transformed to give the actual Raman signature. This technique yields improved signal to noise ratio of hard to detect events but requires high collection times (~30 minutes) [4]. Here, we review the development and use of Raman spectroscopy to diagnose and follow the progression of precancerous and cancerous lesions.

Review of Cancer Biology

The term neoplasia, which literally means new growth, is used clinically to describe pathologic tissue masses which grow independent of and faster than normal tissues [5]. A neoplasm (tumor) may be classified as benign or malignant, based on its potential to harm the host body. Malignant tumors are also called cancers. The nomenclature of a neoplasm reflects its microscopic origin as well as the potential to harm the host. For example, cancers arising from glandular epithelial tissue are called adenocarcinomas, where the suffix carcinoma indicates the epithelial origin and the prefix adeno reflects the glandular origin. On the other hand, cancers arising from the mesenchymal tissue are called sarcomas.

It is generally believed that most cancers have a monoclonal (single cell) origin [5]. This single cell can give rise to a focus of neoplastic cells; in the case of epithelial tissues, if the neoplastic cells are confined to the epithelium, the lesion is regarded as a precancer. These pre-cursor lesions can progress by invading the basement membrane to become full cancers; however, because the incidence of precancers is so much higher than that of cancer, it is widely believed that many pre-cursor lesions naturally regress. Neoplastic cells are characterized by increased nuclear material, an increased nuclear to cytoplasmic ratio, increased mitotic activity, abnormal chromatin distribution, and decreased differentiation. There is a progressive loss of cell maturation, and proliferation of these undifferentiated cells results in increased metabolic activity. Histologically, neoplasms are characterized by cellular crowding and disorganization. The increased metabolic activity induces rapid angiogenesis and results in the formation of leaky vessels.

These general features of neoplastic cells result in specific changes in nucleic acid, protein, lipid and carbohydrate quantities and/or conformations [5]. For example, neoplastic cells are known to produce more lactate than normal cells. The DNA protein interaction is also disturbed in malignant transformations resulting in repeated duplication and amplification of DNA sequences. The morphologic and biochemical changes that occur with neoplasia are numerous and in many cases, depend on the specific type and location of the cancer. It should be noted however, that some of these markers may also be present in certain benign abnormalities such as cirrhosis, hepatitis etc.

In summary, there are multiple molecular markers, located in the membrane, the cytoplasm, the nucleus and in the extra-cellular space which may be indicative of neoplasia. Marker molecules include proteins, lipids, and nucleic acids; changes in either the marker itself or its interaction with another molecule may be expected.

Instrumentation Considerations

Based on the success of Raman spectroscopy in biology, many groups have recognized its potential in the study and diagnosis of disease. Although Raman spectra have been successfully measured from intact tissues; collection and interpretation of tissue Raman spectra has proven to be more challenging because of the strong fluorescence of the tissue chromophore and the complex mixture of biological molecules. Because high S/N Raman spectra, free of fluorescence, are so difficult to obtain, we first briefly review the instrumentation used to measure tissue Raman spectra and then the tools that have been developed to separate Raman signal from the background fluorescence.

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 signal to noise ratios. Improvements in instrumentation in the last decade, particularly in the near infrared region of the spectrum, where fluorescence is reduced, have engendered a dramatic increase in biomedical applications of Raman spectroscopy. The initial Raman spectra of tissue were measured with visible laser excitation, using primarily the argon laser lines. With the development of interferometers, Fourier transform Raman spectroscopy was used to measure tissue Raman spectra, typically using 1064 nm (Nd:YAG) for excitation with germanium detectors. FT-IR Raman spectroscopy uses pre-resonance excitation to reduce the fluorescence contribution and thus allows the measurement of Raman spectra even from highly fluorescent samples with little or no fluorescence and yields spectra more detailed when compared to that obtained using visible excitation [3]. This technique yielded acceptable S/N with moderately high power densities but collection times on the order of 30 minutes were required to obtain spectra of highly fluorescent, scattering tissues such as human artery [3].

The development of diode lasers and cooled silicon CCD cameras sensitive in the near-IR, has enabled the measurement of tissue Raman spectra with NIR excitation. Diode lasers can provide excitation in the region of 750-850 nm which allows the use of silicon detectors (sensitive only to 1100 nm). The advantage of this technique is that the fluorescence emission is reduced and spectra with acceptable S/N ratios can be achieved with relatively short integration times (< 1 min) [3].

Fluorescence Elimination

Even with NIR and IR excitation, some residual fluorescence is detected and accurate subtraction of this signal to yield the vibrational spectrum, continues to present a significant challenge since the fluorescence and Raman signals lie in the same spectral range. In biological samples, the fluorescence background is due to the intrinsic tissue autofluorescence which is difficult to eliminate without altering sample composition.

Several experimental as well as mathematical tools have been proposed to separate the contribution of fluorescence to yield the intrinsic Raman scattering [3]. A simple approach to prevent fluorescence interference could be the addition of a fluorescence quencher; however this requires a quencher that is itself not Raman active. A more feasible technique involves using a suitable detector gate to temporally differentiate between Raman (with short lifetimes) and fluorescence (with longer lifetimes) signals. The wavelength of the excitation light can also be modulated at low frequencies to reject fluorescence. Since fluorescence is independent of shifts in excitation wavelength, modulation yields a time-invariant fluorescence signal and a modulated Raman signal that is the derivative of the Raman signal (with respect to wavelength) free of fluorescence. This concept can be simply applied by measuring the spectra at two slightly shifted excitation wavelengths and taking their difference. A similar result can be obtained by measuring the spectrum at any excitation wavelength and taking the first derivative of the spectrum. With any of these methods, the Raman spectrum can be obtained by integrating the noise-smoothed derivative spectrum following baseline correction. Another mathematical tool that can be used is the fast Fourier transform (FFT). In this technique, the measured spectrum is noise-smoothed and then 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. Another simple and accurate method to subtract fluorescence is to fit the spectrum containing both Raman and fluorescence information to a polynomial of high enough order to describe the fluorescence lineshape but not the higher frequency Raman lineshape. A 5th degree polynomial was found to be optimal. The best fit polynomial was then subtracted from the spectrum to yield the Raman signal alone [3].

Each of the different techniques have advantages and disadvantages and the method used should be selected based on the specific application and measurement technique used. In an analysis of the different techniques by our group, the use of a polynomial fit was found to be the simplest technique from experimental as well as computational points of view.

Cancers & Precancers in Tissues

Several biological molecules such as nucleic acids, proteins and lipids have distinctive Raman features that yield structural and environmental information. Hence, the molecular and cellular changes that occur with cancer may result in distinct Raman spectra from normal and cancerous tissues. The transitional changes in precancerous tissues as well as in benign abnormalities such as inflammation could also yield characteristic Raman features that allow their differentiation. For example, one of the more prominent changes that occur with cancer and precancer is increased cellular nucleic acid content; the extensive DNA studies indicate that it may be possible to sample this change using Raman spectroscopy [3,5]. Several groups have shown that features of the vibrational spectrum can be related to molecular and structural changes associated with neoplastic transformation. Raman spectroscopy has been applied towards *in vitro* detection of cancers of epithelial and mesenchymal origin such as breast, brain, colon, bladder and gynecologic tissues.

Brain and Other Sarcomas

The brain is one of the most complex organs in the human body making it a very difficult target of study. Different types of tumors arising in the brain have differing characteristics and constituents which result in different Raman signatures. These signatures are separate from normal brain signature which can be used for disease identification [5].

Several groups have been responsible for the Raman study of human brain and some of its tumors (see for example [6]). Mizuno *et al* have extensively studied the FT-IR Raman spectra of different kinds of brain tumors relative to that of normal human brain tissue excised from human patients. In general, the spectra of normal white and gray matter of the brain show primary peaks due to lipids and proteins [6]. The spectra of white matter show a greater contribution from lipids, cholesterol and proteins as compared to the spectra of gray matter which show a greater contribution from water as is consistent with normal brain structure [3].

The spectra of different tumors were studied: glioma II and III (origin - astrocytes), neurinoma (origin - schwann cells) and neurocytoma (origin - arachnoid choroid plexus) [5]. FT Raman spectra of grade II gliomas were similar to that of gray matter. However, grade III gliomas showed several spectral differences relative to normal brain tissue at 856, 1130 and 1245 cm^{-1} [Fig 1(b)]. Neurinomas show spectral characteristics similar to gray matter [Fig 1(a)] except for bands at 956, 1006, 1157 and 1524 cm^{-1}, attributed to carotenoids that are absent in normal tissues

[Fig. 1(c)]. Neurocytomas display a sharp band at 960 cm^{-1}, (Fig. 1(d)). Thus, each tumor type shows specific bands that can be used as potential diagnostic features.

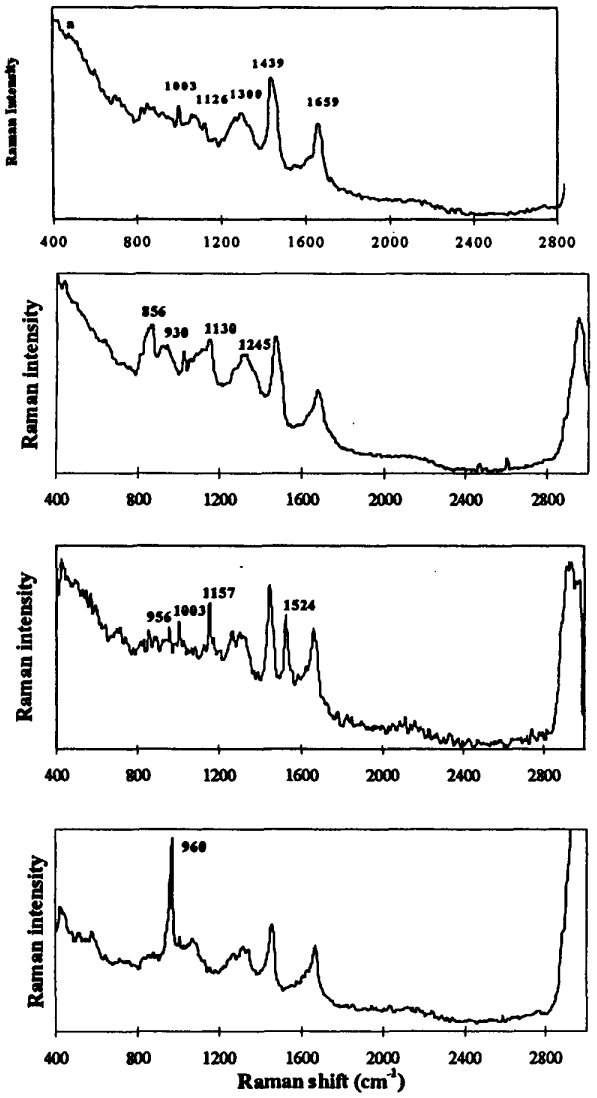

Figure 1: FT Raman spectra at 1064 nm excitation (200-300 mW, 1000-3000 scans) of (a) normal gray matter, (b) glioma grade III, (c) of acoustic neurinoma and (c) central neurocytoma. (Modified from [6,7]).

Feld *et al* studied liposarcomas, a cancer of adipose tissues occurring in the extremities [7]. NIR Raman spectra were obtained from liposarcomas and normal adipose tissues. The spectrum of normal tissues show lipid bands due to C-H, C-C, C=C and C-O vibrations. In addition to lipid bands, the spectrum of liposarcoma show carotenoid bands at 1528 and 1156 cm^{-1}. The intensity ratio of the CH_2 bending mode at 1442 cm^{-1} to the C=C stretching band at 1667 cm^{-1} is observed to decrease with the grade of malignancy when compared to normal tissue. Thus, these features can potentially be used as diagnostic parameters to identify malignant tumors and determine their grades.

Breast Cancer

Perhaps the most extensive work on the use of Raman spectroscopy for cancer detection has been for breast cancers. This is the most common type of cancer among women, accounting for 18% of all cancer deaths among women[3]. 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 attempted for breast cancer diagnosis [3]. Although fluorescence spectroscopy has shown some promise as a diagnostic tool, Raman spectroscopy may provide more definitive characteristics that allow differentiation of benign and malignant tumors [8].

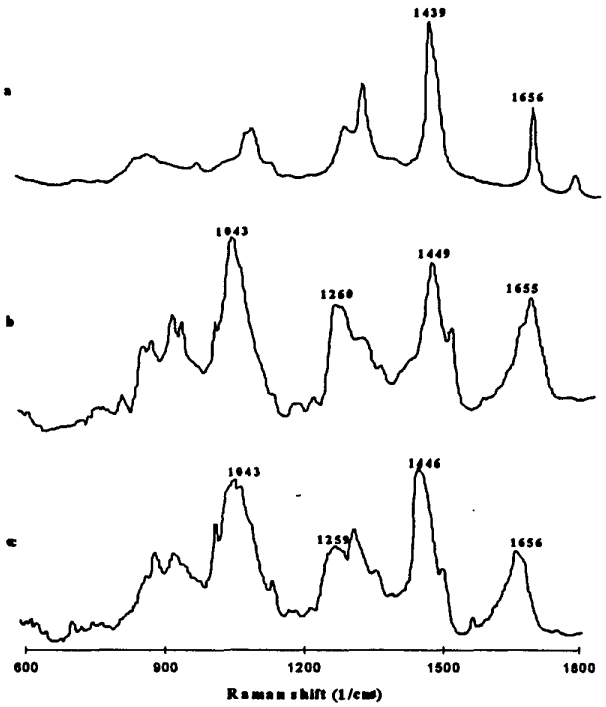

Figure 2: Raman spectra at 784 nm excitation of (a) normal breast (100 mW, 300 sec), (b) malignant (100 mW, 300 sec) and (c) benign tissue (200 mW, 300 sec) (Modified from [9]).

Several groups have explored the potential of Raman spectroscopy for breast cancer detection. Raman

spectroscopy using visible excitation was used to study excised human breast tissues, and spectra characteristic of normal, benign and malignant tissues were obtained [3]. In a subsequent study, the feasibility of using NIR Raman spectroscopy for breast cancer detection was assessed. NIR Raman spectra were measured from excised normal, benign and malignant human breast tissues at 784 nm excitation by McCreery *et al* [9]. Figure 2 shows Raman spectra from normal, malignant and benign breast tissues. The ratio of the areas under the peaks at 1654 cm^{-1} and 1439 cm^{-1} were compared for normal and malignant breast tissues. The spectra from malignant tissue showed an overall decrease in intensity with respect to normal tissue [Fig. 2(b)]. 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} [Fig. 2(c)]. The region of 850-950 cm^{-1} showed only two bands in benign tissue as compared to four in malignant samples. Similar results were also obtained by Feld *et al* using a comparable system [7].

Colon and Bladder Cancer

Preliminary work was conducted by Feld *et al* using NIR Raman spectroscopy to identify colon cancer; small differences were found in the Raman spectra of normal samples and adenocarcinoma [7]. The difference spectrum formed by subtracting the spectrum of normal from malignant tissues show the variations in the vibrational bands with cancer. Peaks at 1662, 1576, 1458 and 1340 cm^{-1} corresponding to nucleic acid modes were found to be more intense in carcinoma samples indicating an increased nuclear content with carcinoma. In addition, several peaks corresponding to lipids were found to be more intense in normal samples.

Bladder cancers are most commonly of epithelial origin. NIR Raman spectroscopy was also used to study bladder cancers [7]. The Raman spectra were dominated by contributions from protein bands; however, spectral differences similar to those observed in the colon were also displayed. Increased intensity of the nuclear bands were accompanied by decreased lipid bands in bladder cancer samples relative to normal samples.

Cancer of Gynecologic Tissues

Alfano *et al* were the first to report on the feasibility of using FT-IR Raman spectroscopy for detecting cancers from various gynecologic tissues [8]. Characteristic features of normal tissues and malignant tumors from the cervix, uterus, endometrium and ovary were described. The gynecologic tissues studied differ structurally as well as functionally [5]. However similar spectral characteristics were observed. Three significant peaks at 1262, 1445 and 1657 cm^{-1} were consistently observed in all gynecologic tissues studied. The intensity ratio of 1657 cm^{-1} to 1445 cm^{-1} could be used for differentiation in all gynecologic tissues. In uterine and cervical samples, the intensity ratio was greater in normals than malignant samples. The same ratio was greater than one in normal and cancerous endometrial samples. In ovarian tissues, the ratio was less than 1 in normal ovary and greater than 1 in malignant samples. The bands were shifted to 1651 and 1453 cm^{-1} in malignant ovarian tissues. Although Raman spectra were obtained from normal, "benign" and malignant cervical tissues, it should be noted that Alfano *et al* did not attempt to differentiate between normal tissues and precancerous lesions [8].

In a study performed by our group, NIR Raman spectra were measured from normal and precancerous human cervical tissues *in vitro* [10] Detection algorithms based on peak intensities, ratios of peak intensities as well as multivariate statistical techniques were investigated. Intensities at 1070 and 1656 cm^{-1} can differentiate precancers from other tissues with an average sensitivity and specificity of 88% ± 4 and 92% ± 4 in a paired analysis, by normalizing the peak of an unknown sample to that of a known normal from the same patient. The ratio of unnormalized intensities at 1656 to 1330 cm^{-1} can separate precancers from all other tissues with a sensitivity and specificity of 82% and 80%, and the ratio of unnormalized intensities at 1656 to 1454 cm^{-1} can separate high grade and low grade precancers with a sensitivity and specificity of 100% without the need for spectra from a known normal sample. Using multivariate methods, intensities at 8 frequencies can be used to differentiate precancers from all other tissues with a sensitivity and specificity of 82% and 96% in an unbiased test. All Raman algorithms can correctly separate benign abnormalities such as inflammation and metaplasia from precancers.

The abundance of diagnostic features in the tissue spectra clearly indicate the potential of this technique for clinical application. In comparing the fingerprint features of the Raman spectra from different tissues and their cancers, several similarities and differences were observed. The ratio of intensities at 1655 cm^{-1} to 1450 cm^{-1} has been consistently

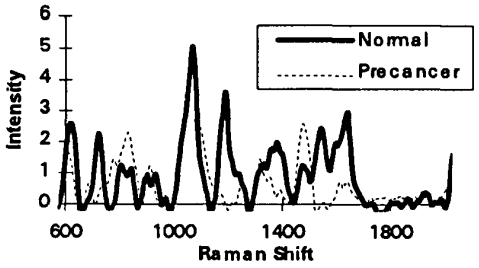

Figure 3: NIR Raman spectra of human cervical tissue from a typical patient at 789 nm excitation (25 mW, 15 min)

used to differentiate normal and cancerous tissues at different sites including the brain, breast and gynecologic tissues. In several tissues, the second amide band at 1260 cm^{-1} contributes towards differentiation as well. In addition, cancers show significant nucleic acid contribution compared to normal tissues and can be used as a diagnostic tool [7]. On the other hand, fatty tissues such as breast and endometrium show identical peaks assigned to lipids [8].

Clinical Use of Raman Spectroscopy

In order to fully evaluate the potential of Raman spectroscopy for clinical detection of precancers and cancers, *in vivo* studies are required. Several groups have initiated this process with varying degrees of success. This progression has been made possible by the development of sensitive instrumentation, use of fiber optics and development of automated algorithms.

Automated Diagnosis

One of the potential advantages of spectroscopic diagnosis is automation, which allows objective and real-time diagnosis of pathologies. Differences in spectral features can be incorporated in diagnostic algorithms; several techniques have been identified and applied to enhance the differentiation and classification of tissues for potential automated, clinical diagnosis. The simplest algorithms are based on empirically identified diagnostic features. These differences may be variations in intensity, intensity ratios and number and location of peaks. For example, the intensity ratio of 1450 cm^{-1} to 1655 cm^{-1} has been observed to vary with disease in several applications including breast cancers and gynecologic cancers and precancers [8,9].

Often, several spectral features contribute to the differences observed in normal and abnormal tissues and more elaborate techniques such as multivariate statistical methods may improve differentiation. Partial least squares, a regression based technique, was used to extract accurate concentrations of dissolved glucose and bicarbonate using NIR Raman spectra for transcutaneous blood analysis [11]. Our group has developed a multivariate algorithm where principle component analysis (PCA) is used for data reduction and Fisher discriminant analysis (FDA) is used for the classification [10]. Several other techniques have also been attempted. These include building a library of known spectra which was used to classify the origin of tissue based on its Raman spectra [3]. Such techniques allow automated diagnosis that enhance the potential of real-time clinical detection of disease.

Fiber Optic Remote Sensing

With the development and availability of diode lasers, imaging spectrographs and cooled CCD cameras, it is now possible to build compact NIR Raman systems that acquire spectra with short integration times [3,7]. Fiber optic probes can be designed to deliver and collect signal efficiently thus allowing remote access. However, a significant problem with this idea has been that of signal generated by the fibers themselves (luminescence and Raman) [3]. This signal can have magnitudes equal to and sometimes greater than that of the sample under study and thus needs careful consideration [3]. Fiber signal is generated in the delivery fiber by the excitation light. In addition, background signal is also generated in the collection fibers by the elastically scattered excitation light returning into the collection fiber(s). 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. Angel *et al* developed different dual fiber probes which could be used under different conditions with maximum collection efficiency but minimum fiber interference [3]. One of these probes which can potentially be used *in vivo* consists of separate single fibers for delivery and collection placed at a small angle relative to each other. A bandpass filter was placed after the excitation fiber lens and a long pass filter was placed between the sample and collection fiber lens. Thus the bandpass filter allows only the transmission of the excitation light from the delivery fiber, and the longpass filter blocks the transmission of the Fresnel reflected excitation light as well as the elastically scattered light from entering the collection fibers.

Several other fiber designs have been attempted, including a 6 x 1 fiber bundle accessible through a biopsy needle and an integrated 2 x 2 inch non-contact probe (DLT, Laramie, WY), and tested it on breast tissues *in vitro* by McCreery *et al* [3] and a hollow compound parabolic concentrator (CPC) with a dichroic mirror and separate excitation and collection fiber geometries for transcutaneous blood glucose measurements by Berger *et al* [11].

Other successful *in vivo* measurements have been in the eye, nail and skin [3]. *In vivo* applications thus far have been confined to exposed tissue areas where fiber background could be circumvented using a macroscopic arrangement; e.g. DLT probe for breast tissues by McCreery *et al* [9]. However, other applications such as in the colon, cervix and oral cavity, require a more compact configuration

and probe design. Although several groups have concentrated their efforts in this direction, no reports of success were found. Our group has recently developed a compact Raman probe (1.5 cm diameter) which has been successfully tested clinically on the human cervix. The probe has separate excitation and collection legs with bandpass and notch filters that reduce fiber signal [12].

Perspectives on the Future

The success of non-resonance Raman spectroscopy for precancer and cancer detection has led to the development of feasible clinical systems that can measure Raman signals from tissue with short collection times [3,8]. For example, McCreery *et al* state the use of two potentially clinical fiber-optic probes; one for non-contact measurements and one accessible through the biopsy channel [9]. Although measurements were not performed *in vivo*, the probes were tested on model breast tissues. Feld *et al* have developed a clinical Raman system using a uniquely designed probe incorporating a CPC and spectra were measured from coronary arteries *in vitro* [3]. These studies clearly indicate that clinical application of Raman spectroscopy is imminent and may be expected to change the face of cancer detection in the near future.

The complexity of tissue structure and environment make the interpretation of tissue Raman spectra difficult. To achieve the maximum benefit from Raman based diagnostic systems, an understanding of the molecular, microscopic and macroscopic origin of observed tissue Raman signals is required. *In vitro* results have demonstrated contributions from proteins, lipids and nucleic acids which are altered under neoplastic transformations. However, extracting more detailed information will require more detailed chemical and microscopic studies to confirm the molecular basis of tissue signal and the development of models to relate the macroscopic signal to its microscopic origins. This process has been initiated with the analysis of Raman spectra from arterial tissue by Feld *et al* to obtain relative contributions from participating chromophores [3]. We believe that applying similar tools to analyze the Raman spectra of neoplastic tissues will yield results that will maximize the clinical potential of this technique.

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