Optical pathology of oral tissue: A Raman spectroscopy diagnostic method

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Raman and fluorescence spectroscopy methods are being considered as techniques which could be complementary or even alternative to biopsy, and pathology and clinical assays in many medical applications. The present paper discusses the results of Raman spectral studies on oral tissues for optical pathology. It is shown that Raman spectra of oral tissues can be classified into spectra of normal and malignant sets and a model based on such a classification can be used to analyse oral tissue for detection of oral malignancy. Sensitivity and specificity calculated from 90 test spectra are better than 85 and 90 per cent respectively.

AT present many disease conditions, and almost all cases of cancer are diagnosed by subjecting a biopsied tissue sample to pathological examination. Vigorous research is going on at present[1–3] in finding whether optical spectroscopy methods like Raman, FTIR and fluorescence can be employed for such diagnostic applications, especially in areas like early detection of cancer. In our laboratory, we have studied Raman spectra of normal as well as different stages of malignant oral tissues and have developed a Raman spectroscopy technique for optical pathology of oral tissue. To the best of our knowledge, that is the first time such a detailed analysis of sufficiently large number of samples has been carried out to prove the efficacy of the use of Raman spectroscopy for optical pathology in oral cancer. The Raman spectra have also led to identification of the biochemical changes in the two types of tissues. The results are presented and discussed in this paper.

Oral tissue samples obtained by biopsy (25) or surgical resection (24) were supplied by the College of Dental Surgery and Sai Baba Cancer Hospital, Manipal, India. Of these, 37 were squamous cell carcinoma and rest were normal, obtained from the same group of patients. More than 140 spectra were recorded using different sites on these samples. Raman spectra of the samples, kept moist with saline, were recorded within half an hour of tissue removal, though trial runs have shown that the spectra remain unchanged even for several hours after biopsy, if kept in saline. For each sample, spectra were recorded from several points, separated by few hundred microns to few millimetres. All standard chemicals were obtained from Sigma Chemical Co. and used as such.

Spectra were recorded on a Spex Triax 320 spectrometer equipped with a Spectrum One liquid N_2 cooled, CCD detector, and 600 g/mm (900 nm blazed) grating. Excitation was with a 785 nm diode laser (SDL 8530) with laser power of 100–150 mW. A holographic filter (Kaiser Optics) was used to remove the background emission from the laser and a notch filter (Kaiser Optics) was used in the collimated scattered beam to reduce the laser background.

Figure 1 shows typical spectra of normal and malignant buccal mucosa samples. Even a visual examination of the spectra is sufficient to discriminate between the normal and malignant specimens. In general, the spectrum of normal oral tissue is closer to the lipid spectrum, while the spectrum of malignant samples resembles the spectrum of proteins. Figure 2 shows the Raman spectra across a sample which had been diagnosed pathologically as malignant at the centre, the edges being normal. It can be seen that the spectral characteristics change from malignant to normal as we scan across the sample. It is thus clear that for many medical and surgical applications like screening of general population, decision of surgical boundaries, follow up for regression or recurrence and progress during therapy, Raman spectra can be successfully used as a routine probe. With a fibre optic probe, spectra can be obtained *in situ* in a few minutes and this facilitates all the applications mentioned above. A biopsy followed by pathological examination is not so convenient in all the above cases, because of the need to remove and examine the tissue, which takes considerable time.

In the present study we have collected more than 140 spectra and these have been analysed to derive statistically significant quantitative information about the spec-

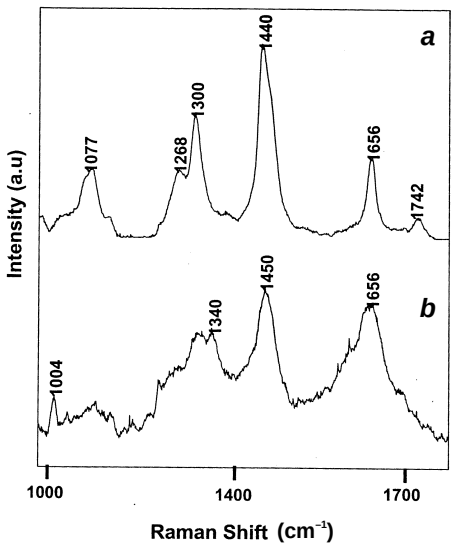

Figure 1. Raman spectra of buccal mucosa: ***a***, Normal; ***b***, malignant.

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tral components. This was done by carrying out a principal component analysis (PCA) of the various sets of spectra, and the derived spectral and statistical parameters[4–6] have given quantitative information on the samples.

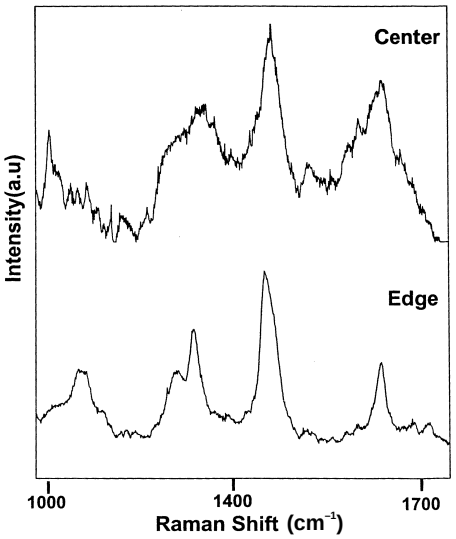

Figure 2. Raman spectra recorded across a surgically resected sample.

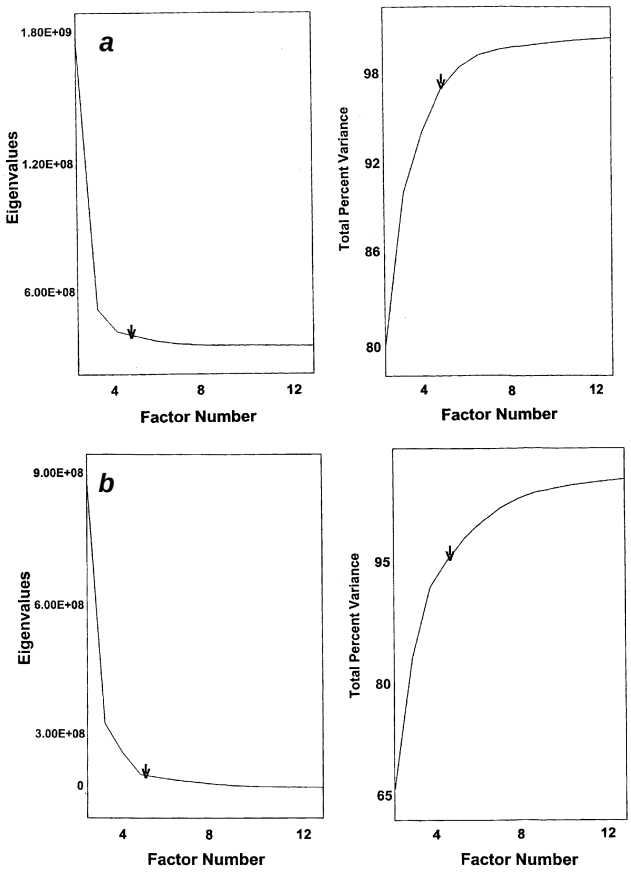

Figure 3. PCA eigenvalues and total per cent variance (see text) for a model set of (***a***), 25 normal tissue samples and (***b***), 25 malignant tissue samples. Arrows indicate the number of PCA factors required to fit the spectra well.

In PCA, spectra from a sufficiently large number of samples, which belong to a given class (for example normal oral tissue) are subjected to an eigenvalue–eigenvector analysis. The entire spectral data from all standard samples are first used in calculating a set of eigenvectors and scores. The eigenvectors can be considered as the spectral equivalents of the principal components (or factors) of the samples, while the scores correspond to the contribution of each principal component to a given sample. Each sample spectrum can be regenerated by multiplying the eigenvectors with the scores for that sample and adding the products for all scores. The spectra of a given set of samples may have contribution only from a limited number of factors and many of the eigenvalues may be close to zero with no practical contribution to the spectra. The number of significant factors can be decided by several techniques[5,7]. Figure 3 *a* shows the eigenvalues for the factors and total per cent variance (i.e. total percentage contribution to the spectra with increasing number of factors) for 25 spectra of normal oral tissue, while Figure 3 *b* shows the same for a set of malignant tissue spectra. It is seen that the eigenvalues decrease very rapidly and are almost zero after 6–7 factors, and about 99% of the total spectral contribution come from these factors. Figure 4 shows the 'spectral' contribution of the first, second, eighth and ninth factors for normal tissue. It can be seen that the spectral loading (contribution) of factors eight and nine simple compensates only for the noise in the spectra, and need not be included in any assessment. This can be further confirmed by using an appropriate number of factors from the model set and regenerating the spectrum of any sample. The difference between the observed and regenerated spectrum, expressed as residual errors squared sum can be used as a measure of the desired number of factors, as well as the accuracy of the model. Figure 5 shows such a regenerated

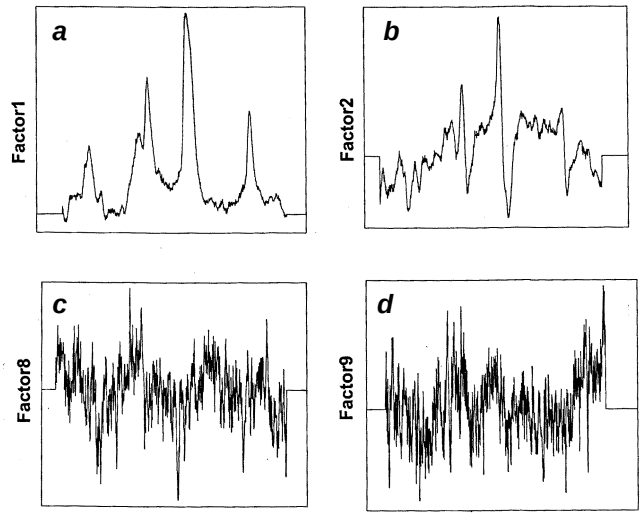

Figure 4. Spectral loading of PCA factors for model set of normal tissue samples. ***a***, Factor 1; ***b***, factor 2; ***c***, factor 8; and ***d***, factor 9.

spectrum for one sample with the corresponding residual spectrum, using only the first five factors of the PCA. The fit is seen to be excellent.

The advantage of PCA is that once a set of spectra have been analysed and classified as belonging to a particular class, any new spectrum can be compared with this model to decide whether it will fit into that class. A new tissue sample spectrum can thus be objectively classified as normal or malignant (or anywhere between) by comparing it with the standard models for normal and malignant spectra obtained from pathologically classified normal and malignant samples. The statistical significance of such a decision can be quantified by various parameters. We have employed the Mahalanobis distance[7] (*M*-distance) and the residual errors squared sum (spectral residual) as the criteria. The *M*-distance for a sample is a function of the scores for that sample when compared to a model set and will thus clearly show whether that sample belongs to the model set, with well-defined statistical probability. Figure 6 shows a plot of the *M*-distance against residual errors squared sum for a new set of about 90 samples, compared to a model set of malignant spectra. It is seen that all samples diagnosed as malignant by pathological examination in the new set fall in the lower left-hand corner of the plot. If we take an *M*-distance of 1 as acceptance, then almost all the malignant samples fall within 3 times this value, while all samples classified as normal lie far outside. The sensitivity and specificity of the technique is thus quite good. A closer examination of Figure 6 shows a very small number of samples outside the acceptable range of either normal or malignant species. This might have happened because these samples belong to borderline cases, and only long-term follow up can classify them correctly. We are doing this at present. The sensitivity and specificity based on 90 test spectra are better than 85 and 90 per cent, respectively.

In addition to being a diagnostic tool, Raman spectroscopy can give considerable information on the biochemical molecular composition of the samples. Table 1 shows the assignments of the Raman bands from general considerations of infrared and Raman spectral studies[8,9]. These assignments show that in general the spectra of normal tissue samples resemble those of lipids (C=C, C=O, C–C), while the malignant tissue samples resemble protein spectra (amide I, amide II, phenylalanine).

There may be several reasons for the observed spectral differences between normal and malignant tissue samples. We have to note that the spectra were recorded using 785 nm excitation. Radiation of this wavelength can penetrate deep into the tissue, so that several layers of the tissue (e.g. epidermis, dermis, and subcutaneous tissue for skin) are exposed to the laser. Similarly, the longer wavelength Raman scattered light also can exit out even from deep inside (several hundred microns) the tissue. Since the composition of major cellular materials is not very different from normal to malignant cells, the observed spectral differences cannot arise only from such small compositional differences and other reasons may exist for the observed differences.

In all our experiments the laser beam is usually focused onto the surface of the sample. The fraction of the radiation which penetrates into the tissue is highly scattered, since the scattering coefficient for tissue is very large compared to the absorption coefficient[10] at our excitation wavelength. As a result, any radiation which penetrates into the tissue, as well as any scattering from inside the tissue is lost to the observation system, having been scattered far away from the collection optics. In tissue systems, it is thus the surface species which contribute significantly to the observed Raman spectrum. It is well known that cells which are malignant or have lost growth control by some means have associated with their external membrane, a broad variety of receptor-type molecules, which are mostly glycoproteins[11]. Also normal tissue con-

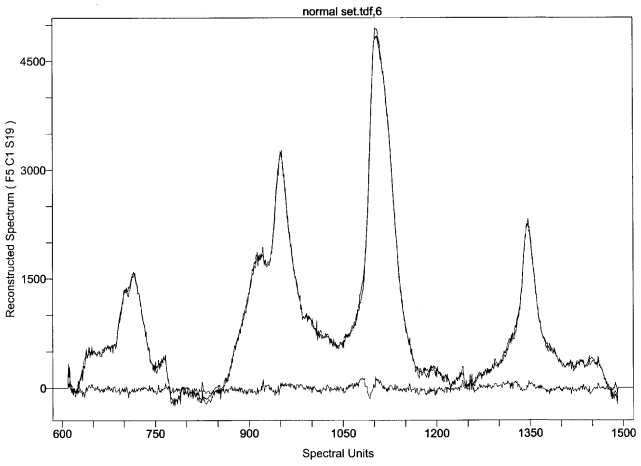

Figure 5. Observed and regenerated spectra using 5 factors, with the residual shown below. The spectrum appearing slightly more noisy is the observed one.

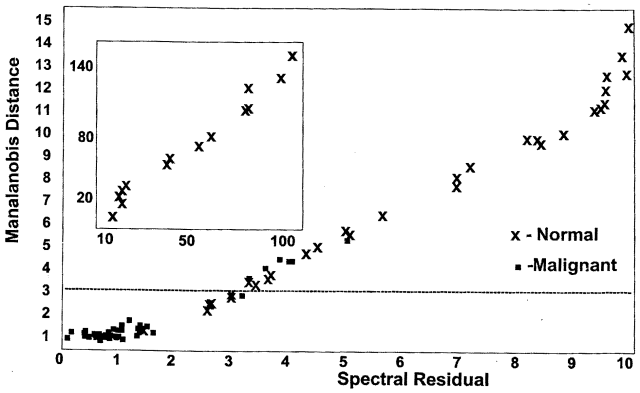

Figure 6. Classification of 88 spectra (44 malignant, 44 normal) compared to the model set of malignant spectra. A Mahalanobis distance up to 3 can be considered as malignant. (*Inset*) Normal samples which lie far out on the plot.

Table 1. Assignment of Raman bands in oral tissue

Raman shift (cm^{-1})			
Normal tissue	Squamous cell carcinoma	Assignment	Change from normal to malignant
3008 VW	–	=C–H stretch (lipid)	Disappears
–	2998 VW	CH stretch (protein)	Appears new
–	2979 sh, W	„	„
2956 sh, W	–	Asym. CH_3 (lipid)	Disappears
2936 sh, ms	–	*a*-CH_2 (lipid)	„
–	2928 vs	CH (protein)	Weak in normal
2894 vs, b	2896 s, sh	CH (lipid, protein)	–
2859 vs, sp	–	CH_2 sym (lipid)	Very weak in malignant
1744 m, sp	–	C=O (lipid)	Not observed in malignant
1656 s, sp	1656 s, b	C=C (lipid); amide I (protein)	Broader and stronger in malignant
–	1609 W	Aromatic amino acid (protein)	
–	1445 s	CH_2 deformation (protein)	Appears in malignant
1440 vs, sp	–	CH_2 deformation (lipid)	Disappears in malignant
–	1339 s	CH bend (protein)	Appears in malignant
–	1332 m	CH_2 bend (protein)	Not observed in normal
1330 vs, sp	–	CH_2 twist (lipid)	Very weak in malignant
–	1273 m	Amide III (protein)	Not observed in normal
1268 m, sp	–	=CH in plane (lipid)	Overlapped by amide III in malignant
–	1210 VW, sh	Aromatic amino	Not present in normal
–	1172 VW	acid (protein)	„
–	1129 VW, sp	C–N (protein)	
1120 W, sh	–	Skeletal	Not observed
1080 s	–	C–C	in malignant
1071 W, sh	–	(lipids)	
–	1004 m, sp	Phenylalanine (protein)	Not observed in normal
–	946 w, sp	C–C (protein)	„

VW, Very weak; W, Weak; sh, Shoulder; s, Strong; b, Broad; m, Medium, sp, Sharp.

sists of well-differentiated and architecturally highly ordered group of cells which present a regular assembly of bilayer membranes to the laser beam. On the other hand, malignant tissues consist of anaplastic cells with no recognizable pattern of orientation. Moreover, the stroma made up of connective tissue is crucial to the growth of neoplasm. Many cancer cells induce a dense, abundant, fibrous stroma (desmoplasia), which will enhance the spectrum of the corresponding fibrous component, namely collagen-like protein. These two factors may contribute to the differences in the Raman spectra of normal and malignant oral tissues. We are now carrying out biochemical analyses, as well as scattering model calculations to understand more about the spectral difference between normal and malignant tissue samples.

Several studies indicate the potential of laser-induced fluorescence (LIF) to distinguish between normal and malignant tissues[12–15]. But it is not easy to extract biochemical information from the broad fluorescence bands, whereas Raman spectra, a seen from Table 1, easily give such information which makes it more suitable for diagnostic purposes. With present-day advances in instrumentation, Raman spectroscopy is comparable in sensitivity to fluorescence[16] and hence it can be used routinely for diagnostic applications.

Raman spectroscopy can be routinely used for optical pathology of oral tissue to diagnose malignancy. The technique is fast, can be applied *in situ* and combined with data processing algorithms, can be made highly objective.

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