Non-Invasive *in vivo* Screening of Oral Malignancy Using Laser-Induced Fluorescence Based System

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About 4000 spectra from oral cavity sites have been recorded *in vivo* from 380 volunteers, under normal (n=133), potentially malignant (n=155), and malignant (n=92) conditions, by excitation with 325 nm CW He-Cd laser. The analyses of the spectra show that the overall spectra arise from about 7–8 bands from different molecular species. In healthy condition (from subjects with no abnormal oral conditions, including tobacco use), buccal mucosa, lip underside, and tongue bottom give spectra very similar to each other, while tongue top, tongue lateral, and palate give spectra different from these as well as from each other. Under potentially malignant and malignant conditions all sites give spectra which are noticeably different for different sites and under different conditions. It is therefore advisable to use separate reference data sets of normal, potentially malignant, or malignant conditions for different sites for optical diagnostic applications.

Key words: laser-induced fluorescence; screening oral malignancy; oral cancer.

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Russian

Неинвазивный скрининг злокачественных новообразований полости рта *in vivo* с применением метода лазерно-индуцированной флюоресценции

В ходе исследования у 380 добровольцев зарегистрировано *in vivo* около 4000 спектров флюоресценции с участков ротовой полости в нормальном (n=133), потенциально злокачественном (n=155) и злокачественном (n=92) состоянии при возбуждении непрерывным волоконным He-Cd лазерой с длиной волны 325 нм. Анализ показал, что суммарные спектры из 7–8 полос возникают в различных видах молекул. В здоровом состоянии (у лиц без патологий полости рта, в том числе связанных с потреблением табачных изделий) слизистые оболочки щек, нижней губы и нижней поверхности языка имеют очень похожие спектры, в то время как спинка языка, его боковая поверхность и небо дают отличные от них и друг от друга спектры. При потенциально злокачественных и злокачественных заболеваниях спектры заметно отличаются на различных участках при различных состояниях. В связи с этим при оптической диагностике целесообразно использовать отдельные эталонные наборы данных о нормальных, потенциально злокачественных или злокачественных состояниях для различных участков.

Ключевые слова: лазерно-индуцированная флюоресценция; скрининг новообразований полости рта; рак полости рта.

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Introduction

Considerable work has been carried out by many groups during the last several years to develop the technique of tissue fluorescence spectroscopy as a suitable method for early diagnosis of oral cancer [1–20]. Almost all these studies were done on buccal mucosa, though other sites in the oral cavity, like tongue, lips, and upper and lower palates, also are often found to be sites of malignancy. It is well known that these different sites have tissue structure differing from one another [21], and as such, their spectra, though superficially similar, exhibit noticeable differences. This is more so in oral diseases which may precede potentially malignant and malignant conditions. Proper interpretation of the observed fluorescence spectra of these different sites is thus necessary for, not only to develop suitable optical methods for diagnosis, but also to understand the complex biological processes that take place during induction, progression, and regression of oral malignancy, and follow up in therapy. In earlier studies on fluorescence spectra of different sites in the oral cavity mostly spectra above 450 nm have only been recorded, and excitation wavelengths have also been limited to 337 nm and above [6, 9, 16, 20].

Of the various biochemical species present in tissues, the amino acids such as tryptophan, tyrosine, and phenylalanine, do not absorb (so do not give any fluorescence) excitation above 300 nm. Pyridoxine, lipo-pigments, collagen, elastin, nicotinamide adenine dinucleotide (NADH), porphyrins, and flavins have strong absorption bands in the 300–500 nm range, giving rise to fluorescence in the 350–700 nm range [2]. Though all these species have fairly broad absorption bands (40–50 nm half width FWHM), which overlap in several cases, excitation at a given wavelength may not give fluorescence from all species which absorb at that wavelength. For example, excitation around 275 nm, gives mainly protein spectrum, though NADH and flavins also have strong absorption at that wavelength. A change in excitation wavelength of 20–30 nm can produce considerable changes in the fluorescence spectrum. The problem is more acute when pulsed lasers are used for excitation, and spectra are recorded after some delay from the laser pulse, since a few nanoseconds jitter in the laser or intensified charge-coupled device (ICCD) trigger and gate, can give spectra which may vary from time to time, due to fast decay of the fluorescence of some of the species [17]. Some workers have used excitation wavelengths around 400 nm and have shown that fluorescence in the 500–700 nm region, from porphyrins, is capable of discrimination between normal and malignant conditions [6, 20]. However, this difference presumably from increased angiogenesis in malignancy, may lead to misleading results, since conditions like inflammation of tissue, wound healing, and bacterial invasion produce similar situations. Only flavins will absorb above 450 nm to give fluorescence in the 500–600 nm region. Unfortunately, normal, and malignant conditions show very similar fluorescence in this case, any changes observed being in absolute intensity only, and diagnostic accuracy from such spectra may vary from user to user, unless all instruments are accurately calibrated for absolute intensity measurements. In general, radiation in the wavelength region of 300–350 nm may be more suitable for laser-induced fluorescence (LIF) diagnosis of oral cavity malignancy [17].

Excitation wavelengths in the range 300–450 nm, have been used in the studies by different groups [15–20]. Fluorescence spectra from different sites of the oral cavity, in the range 455–867 nm, using excitation in the 350–450 nm region, have also been studied for normal, healthy subjects [14]. In many of these studies, only a limited number of subjects/samples were used, mostly *in vitro*, and combined data from all the sites (or selected sites only) were used for evaluating the suitability of fluorescence spectrum for discrimination of normal, potentially malignant, and malignant conditions. Not much effort was made to understand the basic spectral differences or the desirability or otherwise of using combined data from different sites, for diagnosis. We have studied the fluorescence spectra *in vivo* from different sites of the oral cavity in normal, potentially malignant, and malignant conditions using a fairly large number of subjects in each case. In our earlier studies we have explored the effect of using different excitation wavelengths 275–425 nm [17], for the present study, excitation by 325 nm from a He-Cd laser only was used for two reasons; one, the use of a CW laser needs no ICCD and any effects of time delays between laser and detector in pulsed laser-ICCD trigger combination is avoided and two, excitation around 310–330 nm causes fluorescence from many components present in tissue compared to excitation at higher wavelengths [2]. The spectra from the different sites were analyzed by curve fitting, difference spectral calculations, and simulation to get a better understanding of changes in tissue spectra as the system proceeds from normal to malignant state through different potentially malignant conditions like leukoplakia, erythroplakia, and sub-mucosal fibrosis. The results of our studies are presented and discussed in this paper.

Materials and Methods

Laser-induced fluorescence instrumentation. A schematic layout of our portable laser fluorescence set up used in the *in vivo* studies is shown in Figure 1. The system consists of a CW He-Cd laser (5 mW) (IK5351R and KR1801C; Kimmon, Japan), an Acton spectrograph (ARC-SP150i; Acton, USA) with CCD (DU920N-BU; Andor, Ireland), and a fiber optic probe fabricated in our laboratory. The probe consists of 7 optical fibers (NA=0.22, core diameter 200 μm) (Ocean Optics, USA) with the center fiber used for excitation and the surrounding 6 fibers for fluorescence collection. The

fiber tip is enclosed in a removable stainless steel cap with a quartz window (1 mm thick) which is replaced after use on each subject. The spectrograph was calibrated with a Hg-Ar lamp (HG-1; Ocean Optics, USA). The entire system has been shown to be very stable, the mean variation in wavelength over several months being around 1 nm. The system was thoroughly tested and optimized for *in vivo* measurements [22].

Samples and spectra acquisition. LIF set up was kept in two locations for the oral cancer screening, i.e. first location was Shirdi Sai Baba Cancer Hospital and Research Center, affiliated to Kasturba Hospital, Manipal, Karnataka, India and the other was Manipal College of Dental Sciences, Manipal and Mangalore, Karnataka, India. We could screen total 380 volunteers (i.e. 133 normal, 155 potentially malignant, and 92 malignant cases) who visited these centres. Among 380 volunteers, all normal and except few potentially malignant cases diagnosed clinically by well experienced clinicians of Oral Medicine and Radiology Department of Manipal College of Dental Sciences, Mangalore, India. Potentially malignant and all malignant cases were confirmed by taking post LIF screening biopsy and histopathology report. Ethical clearance was obtained from MU Ethics Committee.

A total of 3994 fluorescence spectra were recorded with LIF system (50 accumulations with integration time of 0.02 s) *in vivo* from 380 volunteers with 3 categories (133 normal, 155 potentially malignant, and 92 malignant). The protocol for taking spectra was as follows: calibrate the spectrograph and take a background spectrum, with the laser on and the probe tip against a dark background to record the background, which is subtracted from all spectra. Wash the mouth with ordinary water 3 times; rinse with saline 2 times; rinse again with HPLC grade distilled water; record at least two *in vivo* fluorescence spectra each, from the lesion site and corresponding unaffected site. The probe was always placed in contact and as close to normal as possible with reference to the tissue surface, with minimum applied pressure. The sample details are given in Table 1. The spectral data distribution is summarized in Figure 2.

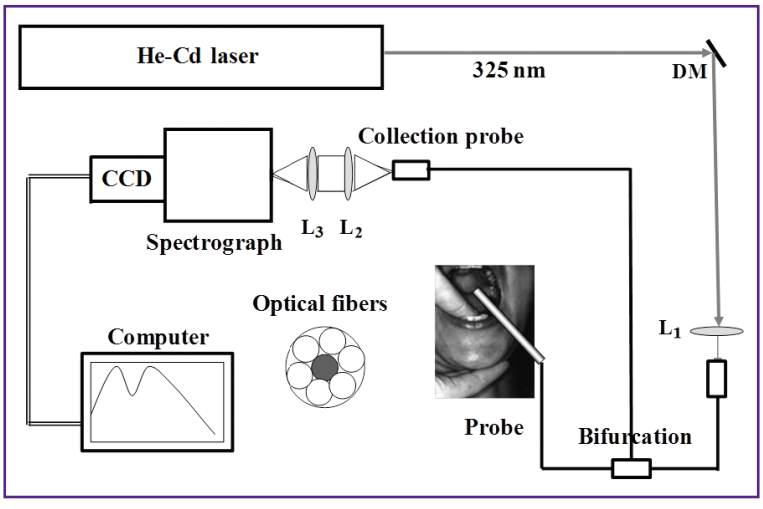

Figure 1. Schematic layout of portable laser-induced fluorescence set up
DM: dichroic mirror; L_1, L_2, L_3: lenses

Table 1

Data distribution among different conditions and anatomical sites

Class	Normal		Potentially malignant										Malignant			
			Leukoplakia		Speckled leukoplakia		OSMF		Lichen planus							
	No. of subjects	No. of spectra	No. of subjects	No. of spectra	No. of subjects	No. of spectra	No. of subjects	No. of spectra	No. of subjects	No. of spectra	No. of subjects	No. of spectra	No. of subjects	No. of spectra	No. of subjects	No. of spectra
Buccal mucosa	113	453	46	137	13	31	59	227	19	55	91	284	45	115	85	293
Tongue lateral	113	226	3	5	2	2	43	84	5	15	104	203	30	72	81	145
Lip	113	180	10	16	3	4	47	49	3	7	92	92	13	25	80	83
Tongue tip	113	113	1	1	0	0	35	35	0	0	116	116	9	10	82	83
Tongue top	113	118	2	4	0	0	35	35	3	4	115	120	10	15	79	79
Tongue bottom	113	113	2	2	0	0	44	45	0	0	107	107	15	27	71	72
Palate	64	64	3	5	0	0	1	2	0	0	0	1	7	20	0	0

N o t e. Total number of subjects — 380 (133 normal, 155 potentially malignant, and 92 malignant). Total number of spectra recorded — 3994 (≈10 spectra from each subject). OSMF — oral submucous fibrosis.

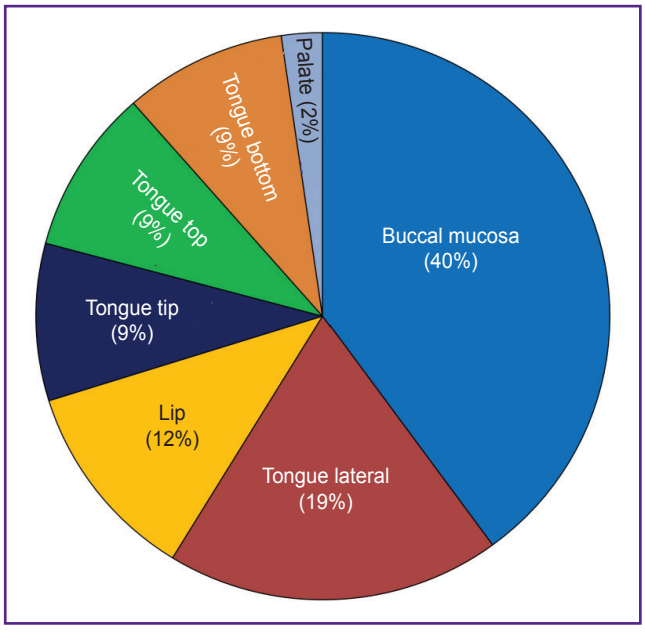

Figure 2. Spectral data distribution from 380 subjects

Data processing and data analysis. Details of our data processing and data analysis (GRAMS/AI, Thermo Fisher Scientific, USA) are given elsewhere [23–31]. In our analyses all spectra were subjected to pre-processing which involve background subtraction, smoothing, baseline correction, and normalization. The baseline correction reduces shift in signal level due to changes in efficiency of the system like grating efficiency and CCD sensitivity. Normalization is done with reference to the peak of the strong fluorescence band around 460 nm. Though information on absolute intensities is lost in this normalization, it has two advantages; one, it gives immediate direct information on relative intensity changes; two, it enables to a good extend transfer of data between institutions using different, but comparable, instrumentation without need for precise intensity calibration.

Though clinical samples like tissue contain many bio-molecules only a few are present in relatively large amounts. Under normal conditions they will all be present within specific concentration ranges in all samples of a given type, for example, buccal mucosa. Further, at the shorter wavelengths used for fluorescence spectroscopy, the radiation penetrates only a few hundred microns, and the resultant fluorescence, when measured at the illumination point, will be mostly from this top layer [32]. It is thus possible to represent the total profile as a sum of fluorescence profiles of combinations of 2–3 species, each combination varying only slightly from sample to sample. The consequence of this is that if we take the mean of several profiles of a given class, buccal mucosa from normal subjects, each individual member of the class will differ from the mean only by small amounts.

Results and Discussion

Oral cavity sites — normal conditions. As mentioned earlier, though there are noticeable differences between normal and malignant conditions in absolute intensities of the fluorescence spectra, it may not be convenient to use them for diagnostic applications. Instrumental components (spectrograph, detector, probes) and experimental conditions may vary from user to user and for transferability of absolute intensities between users accurate calibration will be required. On the other hand, relative intensities can be made transferable provided they are normalized to a common specification. We have, therefore normalized all spectra to the peak intensity of the band in the 450–460 nm region. This band was chosen because, it remained more or less unchanged in shape, and, since intensity variations will remain minimum at the peak, any small errors in wavelength calibration will cause only minimum error in normalization. Choice of a fixed wavelength for normalization may result some times in use of points showing rapid intensity variations, which can cause considerable error, since any small difference in wavelength calibration can change intensity considerably from run to run.

The mean spectra of the different sites from clinically normal subjects — no oral diseases, no tobacco habits — recorded *in vivo* are shown in Figure 3. De Veld et al. [14] have measured the auto-fluorescence spectra from 13 sites in the oral cavity for healthy oral mucosa. At 405 nm excitation, with spectra recorded in the 455–867 nm range, they have shown that dorsal side of tongue (tongue top), and vermillion border of lip only were noticeably different from 11 other sites which more or less resembled each other quite well. They had concluded that the remaining 11 locations can be combined in a reference data base for future lesion diagnostics. The differences seen by them were mainly of two types; one, appearance of proto-porphyrin type bands for tongue top, and two, differences in intensities for the 490 nm band when normalized by area of fluorescence spectrum. They have not apparently studied spectra below 450 nm.

An examination of Figure 3 shows that in the normal tissue, at least three quite strong fluorescence peaks are observed by excitation with 325 nm radiation. Though there are broad similarities in the spectra from different sites, there are also large differences between some of the sites, for example, between tongue tip/tongue top, palate, and the rest. Lower lip and tongue bottom are similar to buccal mucosa, and were considered to be of the same type as buccal mucosa [14]. Excitation with 325 nm gave bands at 390, 430, and 455 nm, none of which were observed by 405 nm excitation. The spectra also show possible weak intensity coming from the 500 nm band [14].

The differences are brought out more clearly in Figure 4 where the difference spectra (mean normal buccal mucosa — mean normal other site) are shown. For normal condition, this indicates more fluorescence

compared to buccal mucosa in the collagen region for all sites except lip and tongue bottom. Tongue top and bottom mainly differ by the presence of numerous, slender, filiform papillae with cornified tips which are present only at the top, bottom having a very smooth mucosa. Even for tongue lateral site, which is considered similar to buccal mucosa there are noticeable differences in all regions which are evident from the difference spectra.

The difference spectra clearly show that the spectra of the different sites differ at many places across the entire wavelength region, as indicated by the several small peaks, shoulders, and dips (eg. 378, 390, 400, 430, 455, 478, 500, 530 etc.) seen clearly in the difference spectra (See Figure 4). This indicates that the overall spectrum cannot be considered as a superposition of just 2 components [11] (collagen and NADH), but is a combination of several different closely similar overlapping bands, since, otherwise, the difference spectra would have appeared with only a few symmetric, peaks and dips and the relative intensities in different sections in the spectra would have been maintained in the difference spectra also. But as seen from Figure 4 (buccal mucosa — tongue top), the 450–500 nm range is negative, while the 530–600 nm is positive. If this entire range is to be attributed to only NADH [11], the difference spectrum in the whole range would have been either wholly positive or wholly negative. It should be noted at this point, that, even the dips around 540 and 577 nm that are supposed to be due to hemoglobin absorption [14], may not be really so, since they are not observed for the others, and have also been observed for cell pellets [11] which are unlikely to have any hemoglobin.

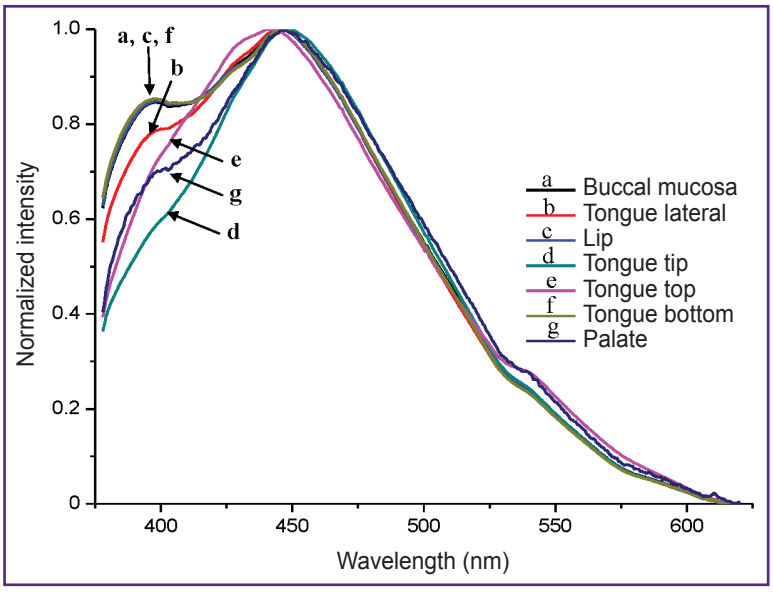

Figure 3. Mean spectra of oral cavity sites — normal

From Figure 4 it is clear that the major difference between other sites and buccal mucosa, lip underside, and tongue bottom, is a substantial decrease in relative fluorescence in the collagen region. Unfortunately, this is the same characteristic which discriminates malignant buccal mucosa from normal. It is thus obvious from even primary considerations that the other sites cannot be clubbed with these three, without incurring possible wrong diagnosis in detection of malignancy. In principal

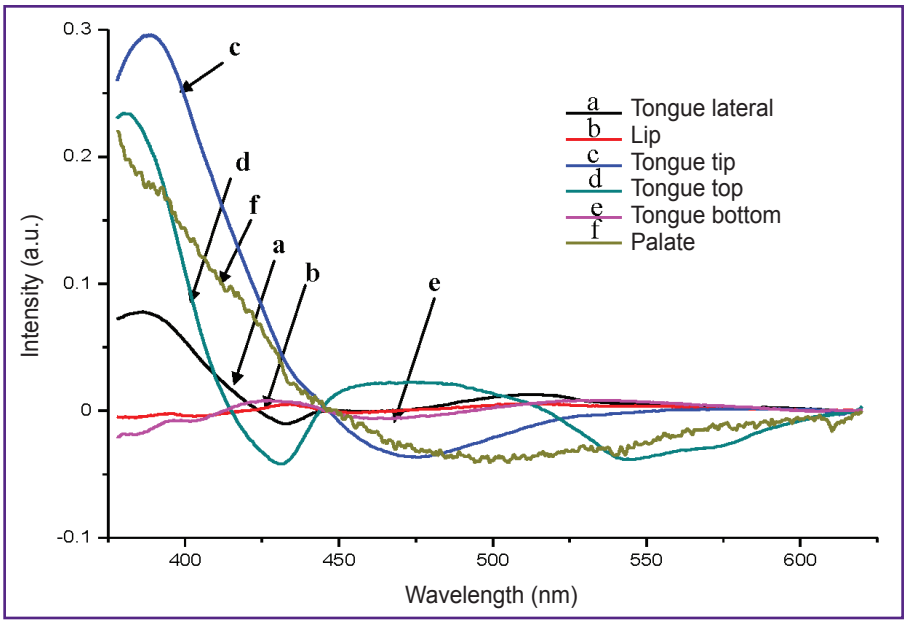

Figure 4. Difference spectra of normal oral cavity sites (mean buccal mucosa — mean of other site)

component analysis (PCA) (or any other statistical pattern analysis), parameters like scores of factors, characterizing fluorescence spectra of each class are first derived to discriminate between different classes.

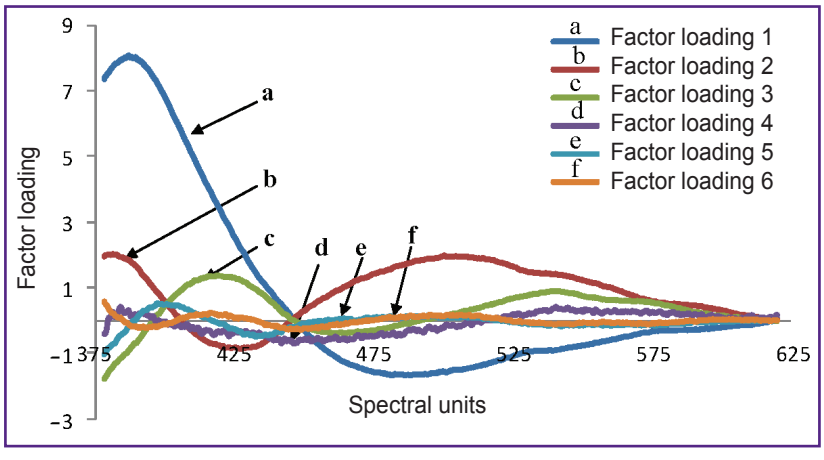

Figure 5. Factor loadings for normal oral cavity sites

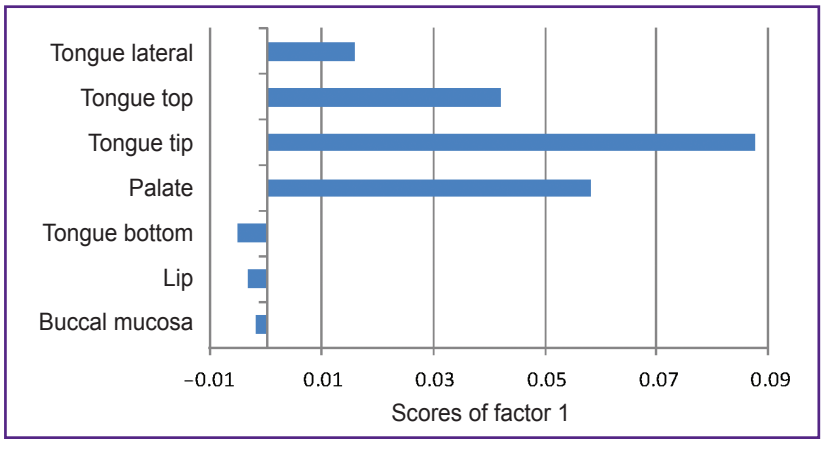

Figure 6. Distribution of scores of factor 1 vs normal oral cavity sites

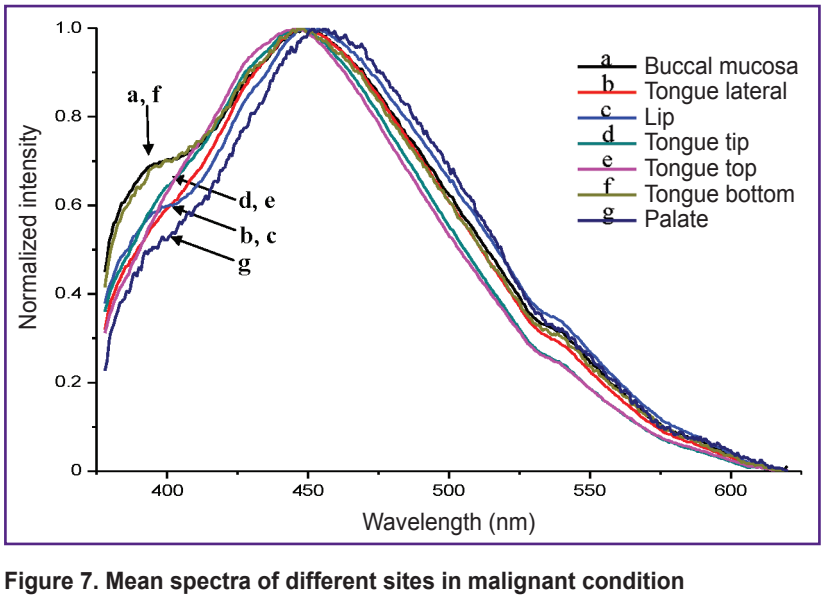

Figure 7. Mean spectra of different sites in malignant condition

Physiological samples come from subjects with widely varying life styles, food habits, social and economic status, age (pre- and post-menopause), substance abuse (tobacco, alcohol), etc. As a result, any diagnostic test will give not single values for the decision-making parameters but values spanning a distribution (eg. fasting glucose for normal subjects 60 to 110 mg/dl). This effect will be more in disease, depending upon stage of the disease at the time of the test. Pattern analysis methods like PCA, will thus give a range of values for the parameters determining the spectra, even for a single class of samples, and clubbing together different types will increase the probability of wrong classification.

To evaluate how far one is justified in combining the data of all sites to form a "Reference Database", we did a PCA of the combined data. Using 12 factors to fit the data, it was found that the first 6 factors contributed to 99.7% of the variance of the samples from the "mean". Figure 5 shows the relative contributions of these 6 factors. It was found that factor 1 contributes 84%, factor 2 another 10%, and factor 3 about 4%, the rest contributing less than 1% each. It is interesting to note that the number of significant factors also indicate that 3 major and 3 minor independent spectral combinations are required to express the spectrum fairly well. Since, as mentioned earlier, each factor can correspond to a combination of more than one molecular species, it is clear that more than 5–6 molecular components are required to reproduce the spectral emission for the different sites. It can be seen that the major contribution (factor 1) arises out of differences in the collagen region, and to some extent the NADH–flavin regions as expected from the spectral distribution. If we now look at the distribution of the scores of factor 1 for different anatomical sites of oral cavity shown in Figure 6 suggests that while buccal mucosa, lip underside, and tongue bottom may be clubbed together for normal tissues, the palate, tongue tip, and tongue top form classes separate from buccal mucosa. This has important implication for early, objective diagnosis. Because of resultant broad distribution in the decision making parameters — in this case score of factor 1 — through combining samples which may differ slightly, the distribution

of the parameter will be broadened, making it difficult to decide the cut off/ threshold value, necessary to include a test sample in a given class, in this case normal tissue. We will see later that similar considerations will apply for potentially malignant and malignant conditions also. In view of this, though lip (underside) and tongue bottom can be considered to form a combined reference data base with buccal mucosa, all the other sites (tongue tip, tongue top, tongue lateral, and palate) have to be treated separate, not only from buccal mucosa, but also from each other for more dependable classification.

Oral cavity sites — malignant conditions. Figure 7 shows the mean fluorescence spectra of different oral cavity sites under clinically malignant conditions. As before, if we look at the difference spectra (mean malignant buccal mucosa site — mean malignant other sites) we can get a better idea of the implications in clubbing together spectra from different sites. It is seen (Figure 8) that though buccal mucosa, tongue bottom, and lip underside gave very similar spectra in normal conditions, all sites, including lip underside (except tongue bottom), show noticeable differences from buccal mucosa, in malignancy. In view of this even for lip underside it is necessary to have separate reference data sets of normal and malignant conditions for diagnosis. Similarly, tongue tip, tongue lateral, tongue top, and palate also differ from each other in the 400–600 nm region, indicating that individual reference data sets are necessary for these sites for optical diagnostic applications.

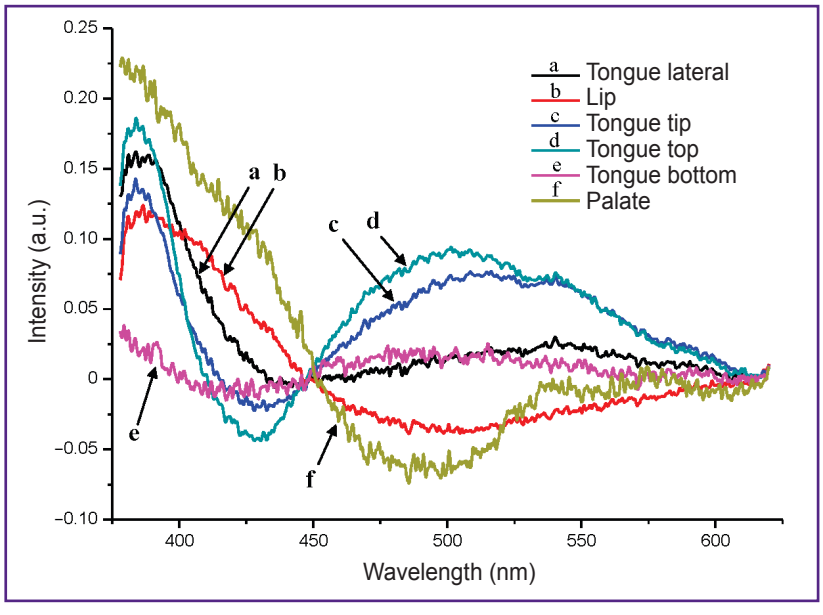

Figure 8. Difference spectra of malignant sites (mean malignant buccal mucosa — mean malignant other site)

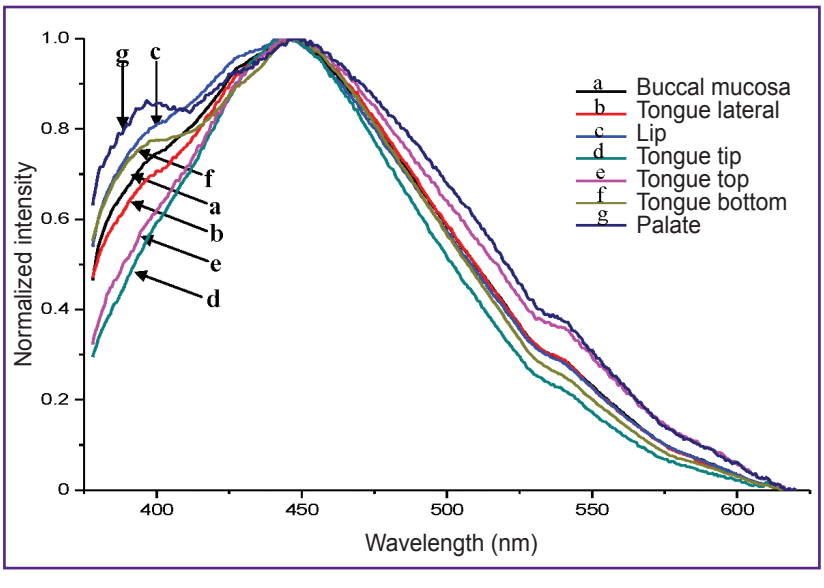

Figure 9. Mean spectra of oral sites in leukoplakia

Oral cavity sites — potentially malignant conditions. From the spectra of different oral cavity sites in clinically normal and malignant conditions, we have seen that there are 5–6 different molecular species contributing to the fluorescence spectra in the 375–600 nm region. Also, except, may be for buccal mucosa and tongue bottom, it is advisable to have different sets of reference data bases for each site. These conclusions are further strengthened by study of potentially malignant conditions on the different sites.

Oral malignancies are often preceded by potentially malignant conditions like leukoplakia, erythroplakia, and oral submucous fibrosis (OSMF). It is reported that about 25% of leukoplakia cases go over into squamous cell carcinoma [32]. As seen from Table 1 we had a reasonably large number of subjects for leukoplakia of buccal mucosa and lip, and for all sites (except palate) for OSMF. Figure 9 and Figure 10 show the mean spectra from different sites for leukoplakia and OSMF, respectively. Once again, it is seen that under same potentially malignant conditions different oral cavity sites give noticeably different spectra, which span a range of values in several regions. Clubbing together some (or all) of these together, will widen the distribution of diagnostic parameters, leading to larger uncertainties in the statistical reliability of diagnostic evaluation.

Fluorescent species in oral cavity. Müller et al. [11] have attributed spectra obtained by 337 nm excitation

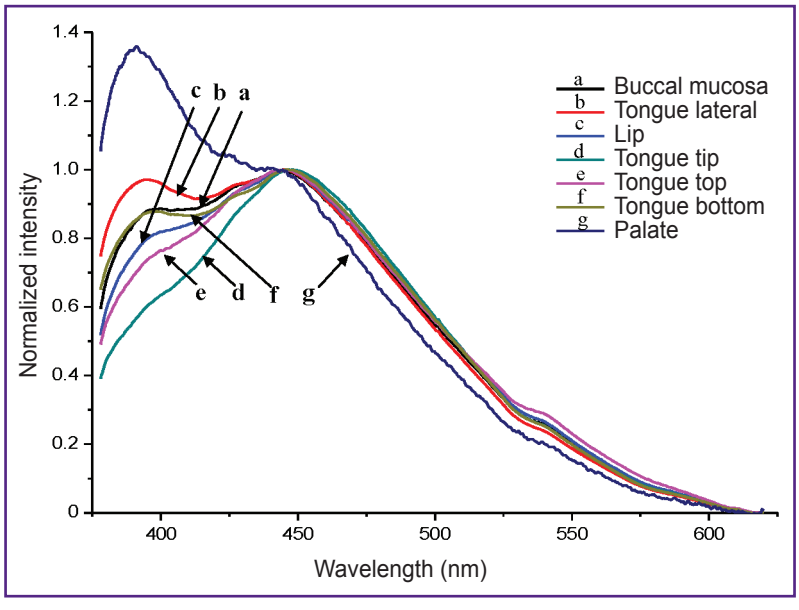

Figure 10. Mean spectra of oral sites in oral submucous fibrosis

to only two species, collagen and NADH, and fitted the observed fluorescence with fluorescence of these two species, modified with hemoglobin absorption. However, the presence of three clearly separate fluorescence peaks observed in tongue bottom, lower lip, tongue lateral, and buccal mucosa indicate that at least three different fluorescent species are present in these tissues. It is well known that bound NADH, gives a spectrum at shorter wavelengths compared to free NADH and this may account for the peak around 430 nm [33]. Fluorescence spectra of endometrial curettage and other tissue specimens have also been analyzed by curve fitting with combinations of several bio-molecular species [34, 35].

Excitation in the 320–340 nm range produces an extended fluorescence spectrum spanning 350–650 nm (or more) range from oral cavity sites. The fluorescence has been attributed to superposition of fluorescence bands from several species, each giving rise to fairly broad (several tens of nanometers) fluorescence peaks. Under these conditions curve fitting techniques can give good fit of the observed spectrum, with a suitable combination of even two individual bands. It is therefore not very correct to use simple curve fitting methods for assignment of the observed spectrum to different fluorescent species. More important, using the bands of the pure components for fitting the actual spectrum can be only a poor approximation, since intermolecular interactions and environmental factors in the tissue can considerably alter the spectra of the pure components in the tissue. It is therefore necessary to have a good idea of the number of possible components before attempting any curve fit.

An idea of the number of components can be had from the difference spectra. As we have discussed earlier, in a difference spectrum, any change in concentration of a given species can give a difference band, positive or negative, depending on whether there is an increase or decrease in concentration of that species. Also, whenever two or more bands overlap in such a way that the overlapping produces a spectrum which looks like a single

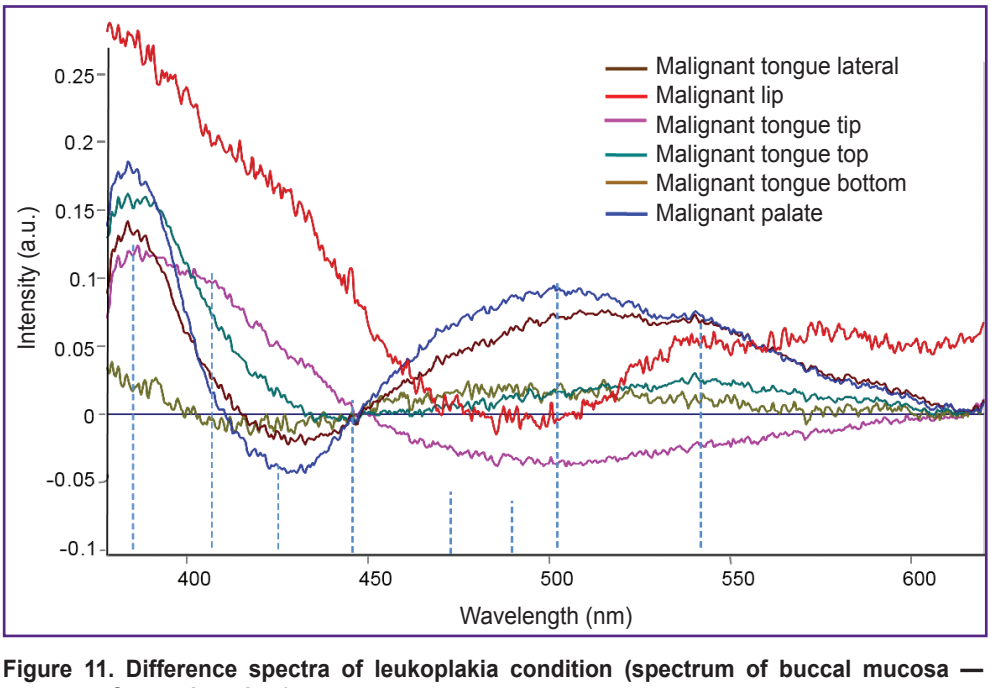

Figure 11. Difference spectra of leukoplakia condition (spectrum of buccal mucosa — spectrum from other sites)

band for one site, in a difference spectrum they will get resolved into the individual components, because it is highly unlikely that concentrations of the two species producing the single-looking overlapping band, change in such a way that the appearance of the single looking band is unchanged for both sites.

As was seen from our difference spectra in Figures 4 and 8 these spectra do give a better idea of the number of independent bands. This is more clearly seen in Figure 11 which shows the difference spectra (spectrum of buccal mucosa — spectrum from other sites) for leukoplakia of the different sites. It is seen that there are at least 7–8 fluorescence bands in the spectra. It is also seen that the 500–600 nm region, in some cases gives a single, broad difference band while in other cases it shows two bands, one positive, and the other negative (see Figure 4) clearly showing that there are at least 3 bands in this region.

A careful examination of all our difference spectra, for different tissue conditions of the different oral cavity sites, gives an idea of the possible bands. It is to be noted that some of these peaks show slight changes under different conditions. Even if we assume these changes are due to different conditions of the tissue we still have at least 8 separate molecular species giving rise to the fluorescence. Table 2 gives the peak positions and probable assignments for these bands. Curve fitting of the spectra with the values of peak positions and half widths shown in Figure 12 gave very good fit for all sites.

Normal, potentially malignant, and malignant conditions of oral cavity sites — screening and diagnosis. We have seen

Table 2

Peak positions and assignments of laser-induced fluorescence spectrum of buccal mucosa (Figure 12)

Peak No.	Peak max. (nm)	Peak max. (cm^{-1})	Area	Assignment
1	391.36	25,552.04	2012.91	Collagen
2	425.14	23,521.70	101.19	Pyridoxine
3	445.50	22,446.70	36.56	Elastin
4	453.65	22,043.32	3421.28	Nicotinamide adenine dinucleotide
5	510.98	19,570.33	485.82	Carotene (given wavelength — 525 nm) [34, 35]
6	552.42	18,102.06	118.53	Flavin adenine dinucleotide
7	586.65	17,045.99	29.35	New

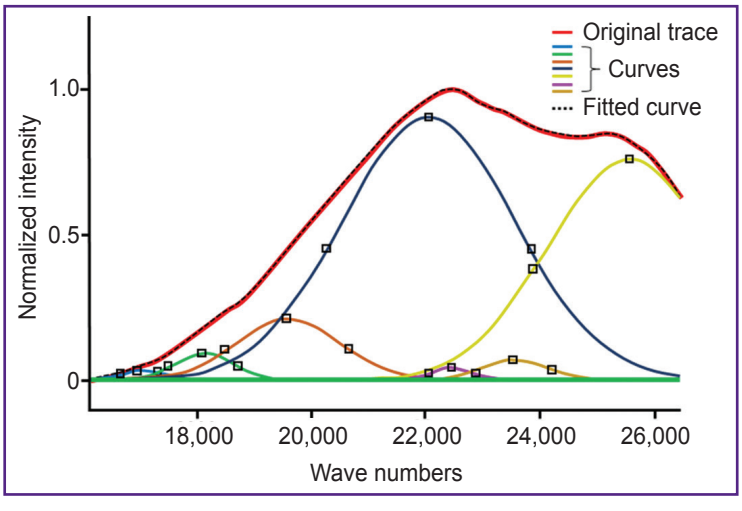

Figure 12. Curve fitting of laser-induced fluorescence spectrum of buccal mucosa showing the peak positions and assignments. The curves in seven colors stand for seven fluorophores

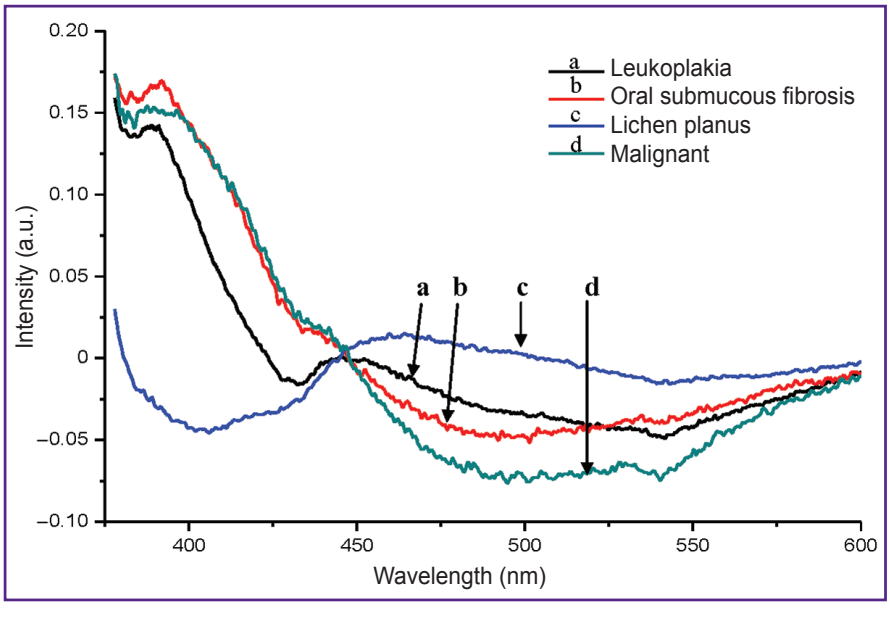

Figure 13. Difference spectra of normal buccal mucosa (normal buccal mucosa — buccal mucosa with different clinical conditions)

that the different sites of the oral cavity give noticeably different fluorescence spectra on excitation with 325 nm, not only in the normal but also under potentially malignant and malignant conditions, making the use of a common reference data set not very advisable. Since the clinician knows which site he is examining, more important for diagnostic purposes are possible differences in different clinical conditions for the same site. As a typical example of such differences, in Figure 13 we show the mean spectra of buccal mucosa under different disease conditions, as difference spectra from normal buccal mucosa.

From Figure 13 it is clear that the different disease conditions of buccal mucosa give sufficiently different fluorescence spectra, which enable correct identification of the clinical condition making them quite suitable for diagnostic purposes. Similar results (Figure 14) were obtained for the other sites also.

It is seen from Table 1 that, except for buccal mucosa, only fewer subjects are available for potentially malignant and malignant conditions of the other sites. In view of this it is helpful to check whether combined reference data sets can be used for diagnostic applications. It was observed that the spectral changes at different sites, for different clinical conditions, vary over a wide range and combining them to form a single reference data set is not advisable because of the possible wide dispersion in decision making parameters with such a combined set.

Optical biopsy: screening in oral malignancy. The identification of potentially malignant/malignant conditions is usually done at present by biopsy and histopathology. But the effects of field cancerization leading to multi-centric lesions, inter-observer and intra-observer variations in reading the degree of epithelial dysplasia, and the possibility that a biopsy may not be representative of the whole lesion, lead to the high likelihood of a biopsy not being reliable for diagnosis [36]. It is thus essential that potentially malignant conditions in oral cavity should be followed by regular periodic observations. Obviously, biopsy is not suitable for this purpose. Optical biopsy by spectroscopic methods, which are non-invasive, highly objective, and operator-independent is ideal for this purpose. The usefulness of the fluorescence optical technique can be appreciated by a look at the spectra of oral cavity sites of susceptible population, mainly tobacco users. The fact that tissue alterations have already been taking place in such cases is observed by changes in fluorescence spectra of tissue samples, diagnosed as normal by histopathology, from subjects under malignant and potentially malignant conditions. To confirm this, we determined the scores for the mean spectra of samples judged to be normal visually by the clinician, for all the sites, with various clinical conditions, adding them as test samples to the normal buccal mucosa set. The results are shown in Figure 14. It is seen that tissue sections adjacent to lesion sites and considered to be normal by clinician, give factor 1 values closely similar to malignant spectra for buccal mucosa, lip, tongue bottom and the other sites. If such conditions are observed during routine screening of susceptible groups (tobacco users) through LIF, remedial action can be taken (advise the patients to quit tobacco smoking, administer the required therapy) early enough, without any need for biopsy and histopathology. Optical biopsy by LIF is quite suitable for this purpose. Though clinical samples like tissue contain many biomolecules only a few are present in relatively

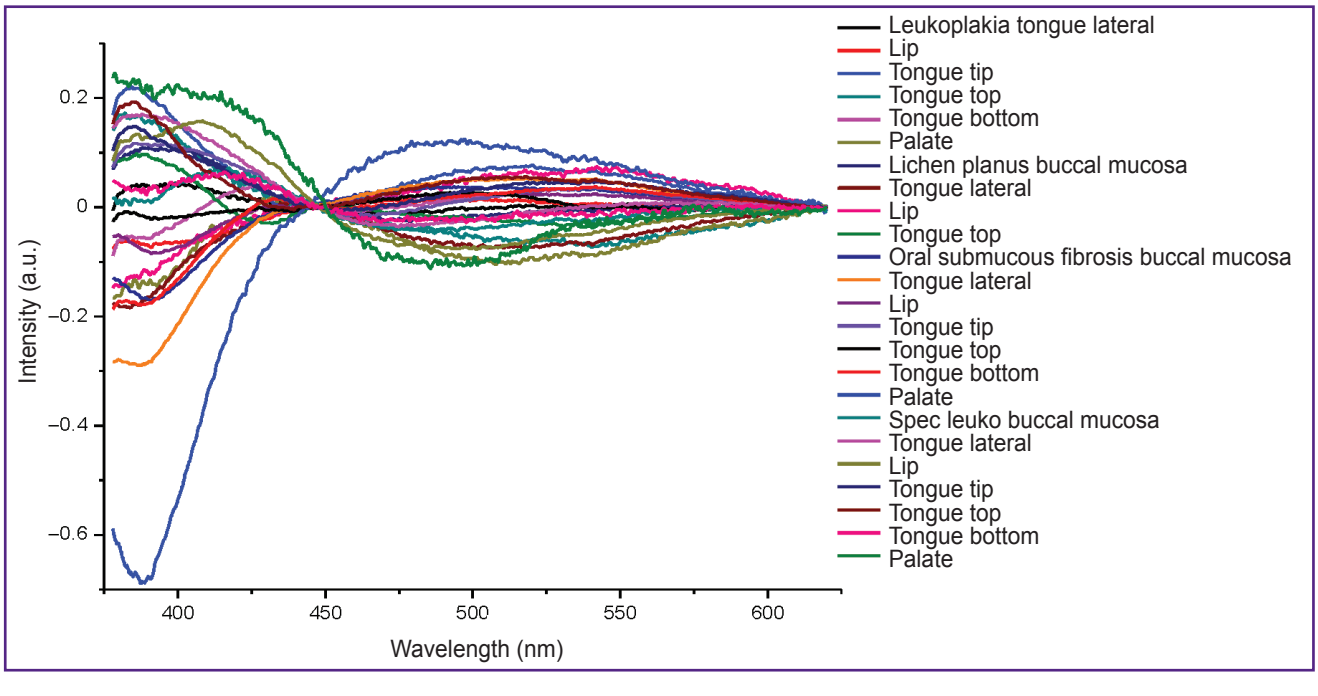

Figure 14. Scores of factor 1 for normal sites from the malignant and potentially malignant conditions

large amounts. Under normal conditions they will all be present within specific concentration ranges in all samples of a given type, for example, buccal mucosa. Further, at the shorter wavelengths used for fluorescence spectroscopy, the radiation penetrates only a few hundred microns, and the resultant fluorescence, when measured at the illumination point, will be mostly from this top layer [31]. It is thus possible to represent the total profile as a sum of fluorescence profiles of combinations of 2–3 species, each combination varying only slightly from sample to sample. The consequence of this is that if we take the mean of several profiles of a given class, buccal mucosa from normal subjects, each individual member of the class will differ from the mean only by small amounts.

Conclusions

In healthy conditions (from subjects with no abnormal oral conditions, including tobacco use), buccal mucosa, lip underside, and tongue bottom give spectra very similar to each other, while tongue top, tongue lateral, and palate give spectra differing from these as well as from each other. The curve fitting analysis has shown that the variations in the spectra may be due to the presence of 7–8 fluorophores with varying concentrations. Under potentially malignant and malignant conditions all sites give spectra which are noticeably different for different sites in different conditions. It is the usual practice that common spectra are being used for comparison of normal oral tissue with respect to disease conditions of oral cavity. It is therefore not advisable to use common reference data sets of normal, potentially malignant, or malignant conditions of different sites for diagnostic applications. By matching test samples to standard calibration sets for each anatomical site, for different conditions, accurate diagnosis can be achieved for potentially malignant and malignant states, with high sensitivity and specificity. The fluorescence spectroscopy technique can thus be routinely employed for regular screening of susceptible population groups, for diagnostic applications to discriminate potentially malignant and malignant conditions, and for follow up in therapy, without the need for repeated biopsy.

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