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ORIGINAL ARTICLE

Synchronous Luminescence Spectroscopy as a Tool in the Discrimination and Characterization of Oral Cancer Tissue

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Abstract
High incidence of oral cancer is primarily due to ongoing tobacco epidemic. In this work, synchronous luminescence spectroscopy (SLS) has been used to characterize and discriminate oral cancer tissue. Spectral deconvolution method is employed to compute the fluorescence intensity, peak wavelength, and full width half maxima for different endogenous fluorophores. The fluorescence measurements were made on 21 normal and 88 oral squamous cell carcinoma biopsy tissues. Besides, variations in relative concentration of collagen, NADH, and FAD, peak shifts and broadening of peaks are observed for tryptophan, NADH, and FAD, in oral cancer tissues indicating both biochemical and micro environmental changes at cellular level. Linear discriminant analysis showed that oral cancer tissue is discriminated with a sensitivity and specificity of 100% and 95.2% respectively.

Keywords Synchronous luminescence spectroscopy · Tissue · Oral cancer · Spectral deconvolution method

Introduction

Global cancer statistics estimated that there will be 354,864 new cases and 177,384 deaths due to oral cavity cancer in 2018 worldwide and it is the leading cause of cancer death among men in India. The major risk factors for oral cancer are due to smoking, alcohol use, smokeless tobacco product like betel quid, and HPV infection. The high incidence of oral cancer is primarily attributed to ongoing tobacco epidemic. Invasive oral squamous cell carcinoma is preceded by premalignant lesions of white or red patches known as leucoplakia and erythroplakia. As cancer develop, formation of non-healing ulcer is observed. Late stage symptoms include bleeding, loosening of teeth, development of neck mass, difficulty in swallowing, difficulty in articulation of speech, and painful swallowing. Unfortunately, many patients present for diagnosis and treatment until they have stage III or stage IV disease which accounts for low survival rate of oral cancer [1, 2]. In spite of the fact that oral cavity is readily available for examination, unavailability of non-invasive and rapid diagnostic method is the main reason for high mortality. Effective non-invasive spectroscopic based diagnostic methods may reduce the incidence and increase survival rate due to oral cancer.

Recently, molecular diagnosis has gained attraction not only to understand the molecular mechanism of transformation from normal to cancer but also in discriminating the cancer from normal. Among various optical spectroscopic methods, native fluorescence spectroscopy is widely used in oncology for the reason that it is simple, non-invasive and highly sensitive to metabolic changes in cells. This is because various cancer related abnormalities may change conformation and/or distribution of key fluorophores at cellular and tissue level. For example, coenzymes flavoproteins and nicotinamide nucleotides are fluorescent which acts as biomarkers for monitoring the oxidation – reduction state in cells. Tryptophan is an amino acid, fundamental building block of protein and the alteration of this amino acid level may reflect conformational state function of the protein. Structural protein collagen is primarily present in extracellular matrix of tissue and its fluorescence emission may elucidate changes in structural integrity of cancer tissues. Fluorescence emission of the endogenous fluorophores of the tissue is also sensitive to their microenvironment [3, 4]. Many have reported on the diagnosis of oral cancer by using different complimentary techniques of fluorescence spectroscopy viz. steady state fluorescence, excitation emission matrix, and

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synchronous luminescence spectroscopy, and fluorescence lifetime spectroscopy [5–10]. Among these, synchronous luminescence spectroscopy also referred as SLS has been emerged as a tool in cancer diagnosis due to its unique advantage of reduced overlap of the broad-band spectra, capability of estimating multiple fluorophore components from one single spectrum and thus facilitating low photo bleaching of biomolecules [11–13]. However, only limited reports are available on the use of SLS in cancer diagnosis.

Majumder et al. have investigated breast cancer tissues and achieved 100% sensitivity and specificity [14]. Yang et al. [15] have used this technique to diagnose breast and prostate cancer using non negative least square fitting method. Subsequently, Ebenezar et al. applied this technique for diagnosing both prostate and cervical cancers using ratiometric method [16, 17]. Further, Borisovo et al. [18] have used this technique for diagnosis of basal cell carcinoma and Genevo et al. [19] for stage wise discrimination of colorectal cancer. Rajesekaran et al. reported on the use of SLS in characterizing urine of cervical and oral cancer patients [20]. However, only limited reports are available on the use of SLS for oral cancer diagnosis.

The present study is aimed for in vivo characterization of oral tissue using SLS. The spectral deconvolution algorithm is employed to extract key parameters of different endogenous fluorophores. Deconvolution algorithm improves both diagnostic potential and clinical interpretation of spectroscopic results in discriminating oral cancer. The extracted parameters of tissues were used as input variable for linear discriminant analysis.

Materials and Methods

Tissue Sample

Tissue biopsies of 21 normal and 88 oral cancer tissues were obtained from the Aringar Anna cancer hospital and research centre (Kancheepuram, India) with previous consent of the patient and ethical clearance from the Hospital. The samples were placed in normal saline to avoid dehydration and the freshly excised tissues were all measured within 5 h of excision. The biopsy tissues were transported in an ice box at 4 °C and thawed to room temperature before measurement.

Data Acquisition

The commercially available fluorescence spectrometer Fluoromax-2 manufactured by Horiba Jobin Yvon was used to measure the SL spectra from the biological tissue biopsies at room temperature. The excitation source, 150 W Ozone free Xenon arc lamp was used and the fluorescence emission from the sample were collected by an emission monochromator connected to a photomultiplier tube (R928P; Hamamatzu, Shizuoka-Ken, Japan). The gratings on the excitation and emission monochromators had a groove density of 1200 grooves/mm^{-1} and were blazed at 330 nm and 500 nm, respectively. Excitation and emission slit width were fixed as 5 nm. The acquisition interval and the integration time were set as 1 nm and 0.1 s respectively.

The offset wavelength ($\Delta\lambda$) for SL spectra was selected such that maximum number of fluorophores are obtained in a single scan with good signal to noise ratio. Among all the $\Delta\lambda$ values, $\Delta\lambda = 20$ nm showed five identifiable key fluorophores with good signal to noise ratio and was set as an optimal offset value for the entire experiment. A similar offset wavelength ($\Delta\lambda = 20$ nm) has been used by researchers in the discrimination for normal and cancer cells and tissues (14,16,17,20). However, use of higher offset wavelength was also preferred by researchers [15] as it yield higher sensitivity but at the cost of spectral resolution.

Data Processing

Normalization

Each SL spectrum was normalized by dividing the fluorescence intensity at each emission wavelength by the peak emission intensity of the spectrum. Normalizing an SL spectrum removes absolute intensity information, and the main advantage of utilizing normalized spectrum is that it is independent of source and detector profile.

Deconvolution Method

The normalized SL spectrum of tissue was mathematically described in Eq. (1). Here an assumption was made that the SL spectrum is due to fluorescence emission of multiple endogenous fluorophores in tissue and the fluorescence emission can be described as Gaussian function.

$$Intensity_{modeled} = \sum a\, e^{-\frac{(x-b)^2}{2\,c^2}} \tag{1}$$

Where a, b, and c are positive constants.

The number of fluorophores and the corresponding parameters a, b, and c are computed according to quasi Newton's algorithm. An approximate initial value for the free parameters were fixed. The SL spectrum is estimated using Eq. (1) for the wavelength range from 250 to 750 nm. Root mean square variance (error) between measured and modeled SL spectra was estimated using Eq. (2). Values of free parameters were updated according to quasi Newton's algorithm (fmin subroutine in Matlab) until the error reaches a minimum value.

$$\text{Error} = \sqrt{\sum_{\lambda=250}^{750}\left[I_{obs}(\lambda) - I_{mod}(\lambda)\right]^2} \tag{2}$$

Where I_{obs}, I_{mod} are measured and modeled SL spectra respectively.

Area under the curve (Intensity), full width half maxima (FWHM), and peak wavelength (λ_{max}) were calculated using the relation Eqs. (3), (4), and (5) respectively (https://ned.ipac.caltech.edu/level5/Leo/Stats2_3.html).

$$AUC = a\ c\ \sqrt{2\pi} \approx\ 2.50662\ a\ c \tag{3}$$

$$FWHM = 2\sqrt{2\ln 2}\ c \approx 2.35482\ c \tag{4}$$

$$\lambda_{max} = b \tag{5}$$

Statistical Analysis

To quantify the results and estimate the diagnostic potentiality of the present technique, 15 input variables (Intensity, FWHM, λ_{max}) were introduced. Mean and standard deviation values of all the variables were calculated and a two-tailed t test was performed to determine the level of significance (*P* value) with which each variable discriminates cancer tissues from normal. In this study, both receiver operator characteristic analysis and stepwise multiple linear discriminant analysis was performed to find the sensitivity and specificity of the present technique to discriminate the cancer from normal tissues.

Results

SL spectra of 21 normal tissues and 88 oral cancer tissues were measured in the wavelength region of 250 nm to 750 nm with 20 nm wavelength offset. To analyze the spectral differences between normal and cancer, the normalized mean SL spectra of normal and oral cancer tissues and its corresponding difference spectrum were computed and they are shown in Fig. 1.

From the Fig. 1 (a and b), it is observed that normalized mean SL spectra of normal and oral cancer tissues have many emission peaks centered at 300, 350, 440, 530, and 630 nm. These emission peaks may be attributed to tryptophan, collagen, NADH, FAD, and porphyrin, respectively [4]. From the difference spectrum between normal and cancer tissues, considerable variations in the spectral signatures between normal and cancer tissues is observed. A decrease in peak intensity of tryptophan, collagen, and NADH in oral cancer tissue with respect to its normal counterpart are observed. FAD peak at 530 nm is found to be increased in cancer tissues with that of normal tissues. Apart from these variations in the spectral intensity, changes in the FWHM and peak shifts are also observed.

To evaluate the spectral shift and FWHM variations between normal and cancer tissues, the normalized SL spectra were processed using spectral deconvolution method. A representative of measured and modeled SL spectra and the corresponding extracted fluorescence emission spectra due to endogenous fluorophores for a normal and cancer tissues are shown in Fig. 2 (a and b). The modeled SL spectrum generated by spectral deconvolution method, is fitted well with the measured SL spectrum with root mean square error of less than ±1%. This confirms the validity of fitting by Gaussian function. The integrated area of the curves (Intenstiy), line width (FWHM), and peak wavelength position (λ_{max}) were estimated using Eqs.3 to 5 respectively for different endogenous fluorophores.

Discrimination by Student T-Test Analysis

Two tailed student t-test analysis was performed across 15 variables extracted using spectral deconvolution method. It was found that, out of 15 variables, only seven variables turned out to be significant and they are listed in Table 1 along with their *p*-values.

From the Table 1, it is observed that peak position wavelength of tryptophan for cancer tissues is red shifted in comparison with that of its normal counterpart ($p < 10^{-2}$). NADH molecule exhibit a lower line width in cancer tissues compared to normal ($p = 0.05$). In contrast, spectral broadening is observed for FAD molecule in cancer tissues with respect to normal tissues ($p = 0.04$). Intensity of collagen shows remarkable decrease in cancer tissues than that of normal tissues ($P < 10^{-3}$). NADH molecule exhibit a decrease in cancer tissues with respect to normal tissues ($p = 0.02$), while FAD molecule exhibit an increase in cancer tissues ($p = 0.02$).

Discrimination by Receiver Operator Characteristic Curve

Figure 3 shows the ROC graph for tryptophan λ_{max} and collagen intensity. The cut off values for each variable and their respective sensitivity and specificity are given in Table 2. From Table 2, it is observed that the Tryptophan λ_{max} provides 87.5% sensitivity and 85.7% specificity for discriminating normal and cancer. Collagen intensity provides 76.2% sensitivity and 85.2% specificity.

Discrimination by Stepwise Multiple Linear Discriminant Analysis

The stepwise multiple linear discriminant analysis performed across the whole set of 88 cancer tissues and 21 normal tissues by use of both SL spectra and variables extracted using deconvolution algorithm. Linear discriminant analysis provides 97.7% sensitivity and 76.2% specificity when SL spectra was used as input variables. However, use of deconvolution method improved both sensitivity and specificity compared to conventional SLS method. Linear

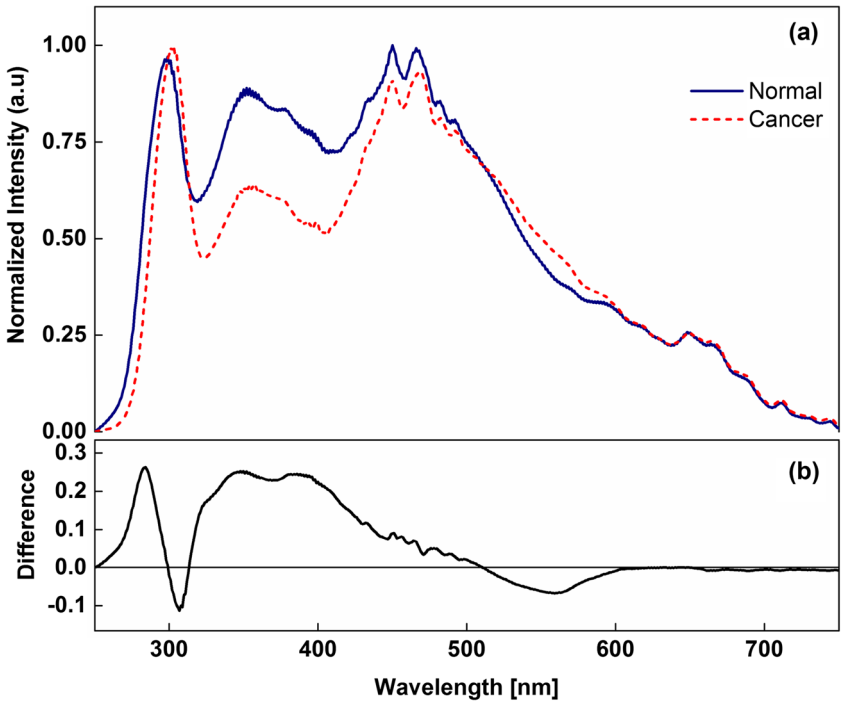

Fig. 1 (**a**) Normalized mean SL spectra of normal and oral cancer tissues and (**b**) difference spectrum

discriminant analysis provides 100% sensitivity and 95.2% specificity when deconvolution method is used. Table 3 comparers the results of discriminant analysis for both these methods. The scatter plot of discriminant function (deconvolution method) for normal and cancer tissues was plotted in Fig. 4.

Discussion

Native fluorescence spectroscopy has been emerged as one of the non-invasive tools to monitor the metabolic and pathological conditions of the cells and tissues [3, 4]. During neoplastic progression, tissues of oral cavity show considerable variations in spectral signature with respect to their normal counterpart. These variations may be attributed to altered photophysical characteristics of native fluorophores present in cells and tissues. Based on these, native fluorescence spectroscopy has been successfully applied in oral cancer diagnosis with varying percentage of sensitivity and specificity (80–100%) [5–10].

Among various complimentary techniques of fluorescence spectroscopy, SLS has the unique advantage of providing diagnostic information from a single spectrum and improving spectral resolution of overlapping fluorescence emission from different fluorophores [11–13]. In this context, researchers showed interest in characterizing various normal and cancer tissues using SLS towards disease diagnosis viz. breast, prostate, cervical, lung, skin, and colorectal [14–20]. As, only limited studies were reported on SLS for oral cancer diagnosis, the present study was conducted to investigate the SL

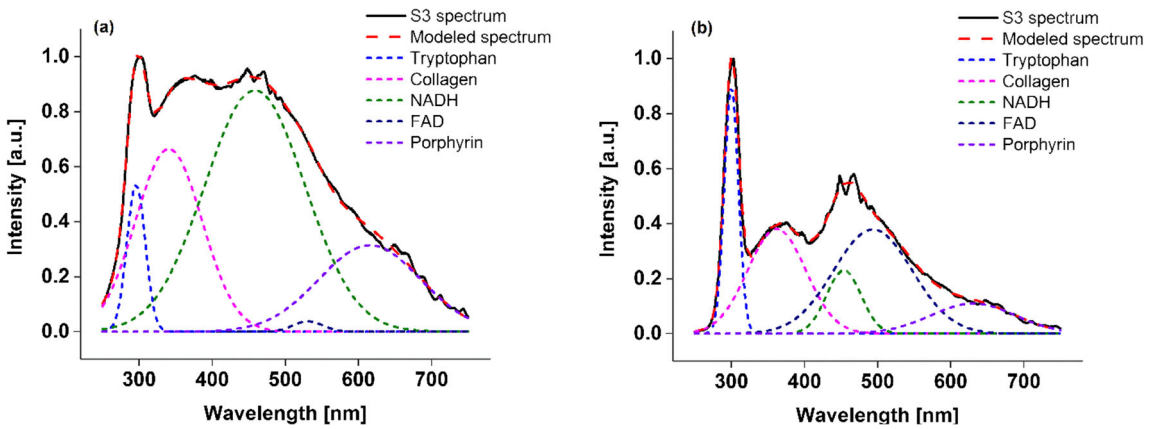

Fig. 2 Schematic representation of measured and modeled stokes shift spectra using spectral deconvolution method by assuming Gaussian as the fitting function (**a**) for normal tissue (**b**) for cancer tissue

Table 1 The average, standard deviation, and *p* value of all the statistically significant variables for normal and cancer tissues

Variable	Normal	Cancer	*P* values
Tryptophan (λ_{max})	295.31 ± 3.36	298.97 ± 4.94	0.00
NADH (FWHM)	120.65 ± 37.92	101.54 ± 40.68	0.05
FAD (FWHM)	56.55 ± 31.30	74.13 ± 35.76	0.04
Collagen (Intensity)	54.25 ± 18.91	38.16 ± 12.91	0.00
NADH (Intensity)	96.09 ± 50.41	72.37 ± 39.56	0.02
FAD (Intensity)	18.43 ± 26.26	36.20 ± 33.49	0.03

spectra of normal and malignant oral tissues and to probe the physicochemical changes of native fluorophores during the transformation of normal into cancer cell.

Figure 1 (a and b) shows the normalized mean SL spectra of normal and cancer tissues and their corresponding difference spectrum. It is observed that the spectral signatures of normal and oral cancer tissues show significant variations at wavelength of emissions lesser than 600 nm. The spectra exhibit prominent maximum at 300 nm, 350 nm, 440 nm which may be attributed to tryptophan, collagen, NADH respectively and a small hump around 520 nm may be due to FAD [4]. The emission above 570 nm is mainly due to porphyrin [4]. From Fig. 1b, it is observed that SL intensity of tryptophan, collagen, and NADH increases in oral cancer tissues while SL intensity of FAD decreases.

In the case of SL signature of amino acid, tryptophan, there is a 4 nm difference in the emission maxima between normal and cancer tissues, i.e. cancer tissues exhibiting a 4 nm red shift with that of normal. This shift may be attributed due to possible changes associated with the microenvironment (temperature, pH, viscosity and polarity), and molecular conformation that occur due to the transformation of normal tissues into cancer [2, 4]. Similar spectral shifts were reported on the SL spectra of normal and malignant tissues [16, 17]. The

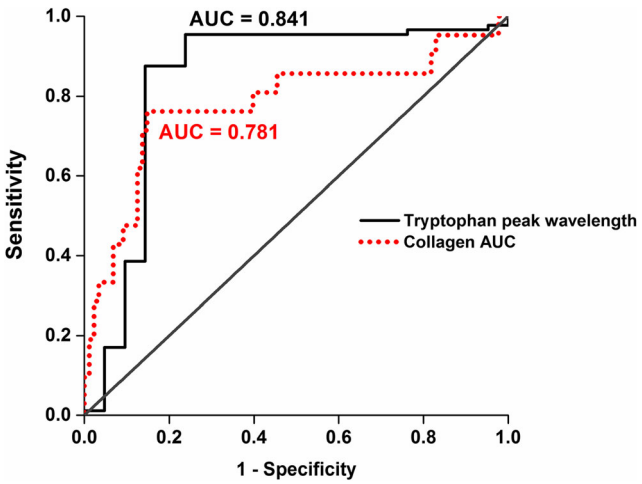

Fig. 3 ROC curves for the variables extracted using spectral deconvolution method

Table 2 Cutoff value, AUC, sensitivity and specificity of the extracted variables

Variable	Cutoff value	AUC	Sensitivity	Specificity
Tryptophan (λ_{max})	296.91	0.841	87.5%	85.7%
Collagen (Intensity)	48.92	0.781	76.2%	85.2%

considerable difference in peak position wavelength of tryptophan between normal and cancer tissues could also be due to difference in the distribution and/or conformational changes [21]. Earlier studies have reported that conformational changes and the polarity of microenvironment alter the photophysical characteristics of tryptophan [22, 23]. Hence, tryptophan peak position wavelength shifts to a longer wavelength indicates that there may be tryptophan residues undergoing conformational changes such as partial unfolding of key proteins and/or microenvironment changes such as steric hindrance around tryptophan molecule by surrounding apolar and polar residues that occur during the transformation of normal into cancer tissues [24, 25].

It is also evident from the spectral profiles that a significant decrease in collagen fluorescence in cancer tissues is observed and it may be due to breakdown of collagen crosslinks in the stroma [26, 27]. Destruction of extracellular matrices by cancer cell due to proliferation and increased metabolism was noted in the work of Peter A. Jones [27]. The loss of collagen autofluorescence intensity in cancer tissue may also be attributed due to increased epithelial scattering and thickness [28]. A similar observation of reduced collagen fluorescence in malignant condition was noted in other types of cancer [14–17].

Further, it is observed that NADH and FAD molecule exhibit significant variations in both fluorescence emission intensity and line width. The decreased NADH intensity and increased FAD intensity in cancer cells is observed and the reason for this may be attributed due to change in oxidation – reduction state of the cell. Generally, the metabolic coenzymes FAD and NADH are the primary electron acceptor and donor, respectively, in oxidation phosphorylation process. Variation in redox ratio may be due to a shift from oxidative phosphorylation to glycolysis in cancer cells [29, 30]. The redox ratio is also sensitive to cellular metabolism and vascular oxygen supply [29, 30]. Glassman et al. reported that metastatic breast cancer cell line HTB22 has lower NADH and higher FAD than a non-metastatic cell line HTB126 [31]. Pradhan et al. reported that non-metastatic melanoma, sarcoma, and lung

Table 3 Classification results of discriminant analysis

Input variables	Sensitivity	Specificity
SL normalized spectra	97.7%	76.2%
Variables extracted using deconvolution method	100%	95.2%

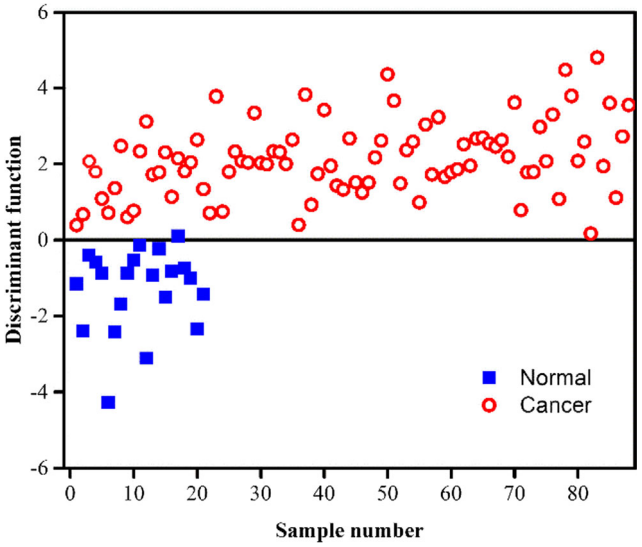

Fig. 4 Scatter plot of discriminant function for normal and oral cancer tissues

cancer cell line have a lower redox ratio; however more metastatic cancer cells have found to exhibit higher redox ratio (NADH/FAD) [32]. Similar observations of increased redox ratio in metastatic cancer cells were reported by Glassman et al. in gynaecological tract, Ostrander J H et al. in breast cancer cell lines, and Sivabalan et al. in oral submucous fibrosis [7, 33, 34]. The change in line width of NADH and FAD may be due to various possible reasons such pH, solvation and oxidation state, and oxygenation concentration [17]. Earlier report by skala et al. suggest decrease of protein bound NADH and increase of protein bound FAD in high-grade epithelial precancer compared with normal tissues [35]. Thus change in line width of NADH and FAD may also indicate conformational changes and/or protein free/bound conditions of these molecules during the onset of cancer [35].

To quantify the spectral variation, spectral deconvolution method is applied to decompose the SL spectra of normal and cancer tissues into different Gaussian peak components. Figure 2 a and b, shows five Gaussian peak components which are centered at 300 nm, 350 nm, 440 nm, 530 nm, and 630 nm. These peaks are primarily attributed due to the emissions from tryptophan, collagen, NADH, FAD, and porphyrin, respectively [3]. Further, the spectral peak position, line width, and the integrated intensity of all five fluorophores were estimated using Eqs. 3–5 respectively and they were subjected to two-tailed student t test analysis. Among the 15 variables extracted, only six of them turned out to be statistically significant which are listed in Table 1 along with their *p*-values.

By considering the difference in the extracted variables between normal and cancer tissues, stepwise linear discriminant analysis and ROC analysis were carried out. Six variables extracted using the deconvolution algorithm and listed in Table 1 have been chosen as input variables for linear discriminant analysis. Out of 88 cancer tissues all are correctly classified yielding a sensitivity of 100% and out of 21 normal tissues, only one normal tissue is misclassified as cancer tissues yielding a specificity of 95.2%. Figure 3 shows the ROC curve generated for the diagnostically significant variable tryptophan peak position wavelength and collagen intensity. Accuracy of the ROC analysis is measured by the area under the ROC curve (AUC). The AUC calculated from the ROC for tryptophan peak position and collagen intensity is 0.84 and 0.78 respectively. The tryptophan peak position discriminates oral cancer tissues and normal with a sensitivity and specificity of 87.5% and 85.7% respectively. Collagen integrated intensity yields sensitivity and specificity of 76.2% and 85.2% respectively. From both the analysis, it is clear that six variables calculated using deconvolution method discriminates the oral cancer tissues from that of normal.

Conclusion

Synchronous luminescence spectroscopy (SLS) characterization and discrimination of normal and oral cancer tissues were carried out. SL spectra of normal and oral cancer tissues exhibit various fluorophores presence such as tryptophan, collagen, NADH, FAD, and porphyrin with marked differences in spectral signature between them. Spectral deconvolution method was employed to extract integrated area, peak position, and line width for different endogenous fluorophores. Among them, tryptophan peak position, and collagen intensity are found to be statistically significant with p value of 0.002 and 0.000 respectively in discriminating oral cancer tissues from normal. Further to evaluate the efficacy of SLS technique linear discriminant analysis was carried out and it yielded a sensitivity and specificity of 100% and 95.2% respectively in discriminating oral cancer tissues from normal. SLS technique along with deconvolution method is found to demonstrate excellent efficacy. Hence, this technique can be considered for monitoring metabolic changes in oral cancer tissues.

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Compliance with Ethical Standards

Ethical Clearance Ethical Clearance has been obtained from Health and Family Welfare Department, Government of Tamil Nadu, India. (Ref. no. 47846/ E2 /2012–1), to collect samples from Government Arignar Anna Cancer Hospital, Kanchipuram, Tamilnadu, INDIA.

Conflict of Interest The authors declare that there is no conflict of interest regarding the publication of this article.

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