Optical redox ratio differentiates early tissue transformations in DMBA-induced hamster oral carcinogenesis based on autofluorescence spectroscopy coupled with multivariate analysis

To cite this article: R Sethupathi et al 2016 Laser Phys. 26 116202

View the article online for updates and enhancements.

Related content
- Raman spectroscopy detects biomolecular changes associated with nanoencapsulated hesperetin treatment in experimental oral carcinogenesis
  K Gurushankar, M Gohulkumar, Piyush Kumar et al.
- Fluorescence spectroscopy to discriminate neoplastic human brain lesions: a study using the spectral intensity ratio and multivariate linear discriminant analysis
  Shalju S Nazeer, Ariya Saraswathy, Arun Kumar Gupta et al.
- Evaluation of the chemopreventive response of naringenin-loaded nanoparticles in experimental oral carcinogenesis using laser-induced autofluorescence spectroscopy
  N K Sulfikkarali and N Krishnakumar

Recent citations
  Jarlath McKenna
  Jarlath McKenna
Optical redox ratio differentiates early tissue transformations in DMBA-induced hamster oral carcinogenesis based on autofluorescence spectroscopy coupled with multivariate analysis

R Sethupathi¹, K Gurushankar² and N Krishnakumar²

¹ Research & Development Centre, Bharathiar University, Coimbatore, Tamilnadu 641 046, India
² Department of Physics, Annamalai University, Annamalainagar, Tamilnadu 608 002, India

E-mail: nskumarphyamu@gmail.com

Received 29 April 2016
Accepted for publication 23 July 2016
Published 14 October 2016

Abstract
Fluorescence intensity measurements have the potential to facilitate the diagnoses of many pathological conditions. The changes in fluorescence intensity may be influenced by factors such as tissue architectures, endogenous fluorophores, cellular metabolism and light penetration depth in tissue. Two of the most diagnostically important endogenous fluorophores are reduced nicotinamide dinucleotide (NADH) and flavin adenine dinucleotide (FAD), which can be used to monitor dramatic metabolic changes in cells and tissues. The goal of this study is to investigate changes in the endogenous fluorophore emission and to quantify metabolic changes in the redox state of various tissue transformation conditions with respect to control tissues in dimethyl benz[a] anthracene (DMBA)-induced hamster oral carcinogenesis for measuring emission spectrum at 320 nm excitation. In the present study, collagen, NADH and FAD emission of well-differentiated squamous cell carcinoma (WDSCC) showed decreased intensity at ~385 nm, ~450 nm and ~520 nm compared to hyperplasia, dysplasia and control tissues. Furthermore, a significant decrease in the optical redox ratio is observed in WDSCC tissues, which indicates an increased metabolic activity compared to the control tissues. Moreover, the principal component linear discriminant analysis (PC-LDA) algorithm together with the leave-one-out cross-validation (LOOCV) method yield an overall diagnostic sensitivity of 77.7% and a specificity of 88.8% in the classification of control, hyperplasia, dysplasia and WDSCC tissues, respectively. The results from this study demonstrated that fluorescence-based tissue analysis combined with PC-LDA has tremendous potential for the effective discrimination of control from neoplastic tissues; furthermore it also detects early neoplastic changes prior to definite morphologic alteration.

Keywords: endogenous fluorophores, oral carcinogenesis, redox ratio, multivariate analysis

(Some figures may appear in colour only in the online journal)
cancer detection and therapy, the mortality rate of oral cancer remains high and the five year survival rate is among the lowest of the major cancers. The overall prognosis for these patients is poor with a five year survival rate of ~50% that has not changed over the last three decades [4]. Descriptive histopathology is currently considered the gold standard for diagnosis in oral cavity and oropharynx cases; it examines the morphology, degree of differentiation and number of mitosis present in the sample. However the accuracy of this technique is highly dependent on the appropriate sampling of the lesion and also a correct pathological interpretation. Moreover, this technique is time-consuming and has a limited statistical confidence level due to inherent operator variability. Therefore, a technique for non-invasively detecting cancer or helping the clinician choose the appropriate site for biopsy can save patients from multiple biopsies and allow a broader range of diagnoses, which can aid the early detection of oral cancer [5]. Bio-optical methods could overcome these limitations by relying on a common principle: when illuminating the tissue with light, the optical spectrum derived from the tissue contains information about the molecular/chemical composition of the tissue and/or its surface character [6]. The main notable changes within a light spectrum after interaction with tissue are the result of either the absorption or scattering of light, or are otherwise due to fluorescence and Raman scattering [7]. As techniques have advanced, interest has moved to assessing the ability of bio-optical methods to diagnose cancers at much earlier stages, placing a higher demand on spectroscopic data quality.

Auto-fluorescence (AF) spectroscopy is one of such optical spectroscopic techniques which has the potential to monitor biochemical, metabolic and morphological changes in various tissue types/conditions and is a minimally-invasive or non-invasive technique [8–10]. Fluorescence spectroscopy is a very sensitive tool. Its applicability to detect cancer depends on many factors related to the lesions investigated; such factors include their pigmentation, morphology, localization and stage of growth. This technique relies on the expression of either endogenous naturally occurring fluorophores, such as tryptophan, collagen, elastin, nicotinamide dinucleotide (NADH), flavin adenine dinucleotide (FAD) and others. The fluorescence signatures of these substances—along with their local distributions—have the ability to report on the tissue disease state. Endogenous fluorophores under excitation with light at a suitable wavelength give rise to an AF emission, the properties of which depend on the nature, amount, physico-chemical state, intratissue distribution and microenvironment of these biomolecules, in a close relationship with morphological and metabolic conditions of the biological substrate [11]. Two of the most diagnostically-important endogenous fluorophores are reduced NADH and FAD, which can be used to monitor dramatic metabolic changes in cells and tissues. NADH and FAD are important indicators of cellular metabolism. Hence, large-scale cell proliferation or tumor growth can be identified by significant changes in NADH and FAD fluorescence [12]. NADH and FAD are two of the principal electron donors and acceptors in cellular metabolism, respectively. NADH and FAD AF and the associated redox ratio, ([FAD]/[NADH] + [FAD]), have been exploited in a number of in vitro and in vivo studies for detecting changes in metabolic activity that are typically associated with neoplastic transformation [13, 14]. This advantage is important because it eliminates possible artifacts in metabolic measurements that can be introduced by tissue excision, processing or staining [15]. The AF technique shows a promising potential to provide real-time information of endogenous fluorophores that are associated with morphological structure and metabolic processes. The principle of AF spectroscopy is based on the fact that different diseased tissues contain different morphohistological characteristics and intrinsic fluorophores that give rise to different fluorescence emission spectra when the tissues are excited at a suitable wavelength [16]. Many research groups have reported that excitation around 320 nm is the ideal wavelength to study the variation of collagen and NADH and FAD [17, 18]. Thus, systematic investigations of oral tissue constituent fluorescence in progressively-complex biological systems that range from cells to tissue cultures to living animal models of oral cancer may provide an effective approach towards understanding the differences in the endogenous fluorescence of malignant and non-malignant oral tissues.

Although no animal model is perfectly applicable to every kind of human cancer, it is generally agreed that the use of a suitable preclinical animal model is helpful in attempting to elucidate the molecular pathogenesis of squamous cell carcinoma of the oral cavity. The Syrian golden hamster buccal pouch (HBP) carcinogenesis model closely mimics events in the development of precancerous lesions and epidermoid carcinomas of the oral cavity in humans using topically and chronically-applied 7,12-dimethyl benz[a]anthracene (DMBA) [19]. DMBA is a polycyclic aromatic hydrocarbon (PAH) known to cause mammary tumors in rats [20]. PAHs are common organic environmental pollutants derived from the incomplete combustion of fossil fuels and which are also present in tobacco smoke and various foods [21]. Based on the emission spectrum of the fluorescer in areas of disease or changes in AF spectra, control and neoplastic tissues can be distinguished. Hence, the present study is designed to investigate changes in the endogenous fluorophore emission and quantify the metabolic changes in the redox state of various tissue transformation conditions with respect to control tissues in a hamster oral carcinogenesis model for measuring emission spectrum at 320 nm excitation. Further, the principal component and linear discriminate analysis (PC-LDA) multivariate statistical techniques are employed to develop effective diagnostic algorithms for differentiations between control, hyperplasia, dysplasia and well-differentiated squamous cell carcinoma (WDSCC) in HBP tissues. In addition, the receiver operating characteristic (ROC) curve was also employed to assess and compare the accuracy of both diagnostic algorithms.

2. Materials and methods

Sections of various hamster buccal mucosa from 24 samples obtained from a previous experiment [10, 22] were submitted to
histological and spectroscopic analysis to determine the composition, the morphology and hence the metabolic changes. One section of each cheek pouch was immediately frozen in liquid nitrogen and stored at $-80^\circ$C for further fluorescence spectroscopic analysis. A second portion of the pouch was fixed in 10% neutral buffered formalin for histopathological studies.

2.1. Histological preparation and analysis
The specimens were embedded in paraffin. Sections, 4–5 $\mu$m in thickness, were cut on a rotary microtome and stained with haematoxylin and eosin to observe general pathology features and morphology. The histopathology results confirmed that six were diagnosed as control, six were diagnosed as hyperplasia, six were diagnosed as dysplasia (one mild dysplasia, two moderate dysplasias and three severe dysplasias) and six were WDSCC. Typical histopathological results in various oral transformations conditions such as control, hyperplasia, dysplasia and WDSCC tissues are shown in figure 1. For the final evaluation, only specimens for which the pathologist agreed on the diagnosis were used. The spectroscopic data was further classified according to the histological diagnosis.

2.2. Fluorescence instrumentation
AF spectra of HBP tissue samples were measured using a Fluorolog-III spectrofluorometer (Jobin Yvon Inc., USA). The excitation light was obtained from a Xenon lamp (450 W) by passing through a monochromator and was focused on to a $2 \times 6$ mm spot on the surface at an angle of $45^\circ$ to the sample. The emission from the sample was collected at a $22.5^\circ$ angle with respect to the excitation beam. The emission light of the selected wavelength range was then passed into the photomultiplier tube for analysis. A bifurcated Y-type fiberoptic probe coupled to the sample compartment facilitated in vivo measurements. This multimode fiberoptic probe with an outer diameter of 1 cm consists of illumination fibers and collection fibers with a numerical aperture of 0.22. The excitation light was guided to illuminate samples by one arm of a Y-type fiber bundle, and the emission fluorescence was collected by another arm of the fiber bundle and sent to the photomultiplier detector. The excitation wavelength of 320 nm was selected using Datamax™ software (Datamax, Round Rock, Texas, USA) and the in-built double-grating monochromator. Emission spectra in the range of 350–550 nm in 1 nm increments were recorded for a 320 nm excitation wavelength. In the present study, a total of 48 fluorescence spectra (12 from each group) were successfully recorded at different anatomical locations randomly in various tissue transformations conditions such as control, hyperplasia, dysplasia and WDSCC tissues.

2.3. Data processing
To compensate for variations, the spectra were normalized using the average fluorescence emission intensities at 450 nm from the control, hyperplasia, dysplasia and WDSCC tissues. Normalizing a fluorescence spectrum removes absolute intensity information; algorithms developed from normalized fluorescence spectra rely on differences in spectral line shapes for diagnosis. An analysis of variance (ANOVA) was performed using the Statistical Package for the Social Sciences (SPSS 17.0) software to assess for significant differences in the
fluorescence emission spectra between the control, hyperplasia, dysplasia and WDSCC tissues.

2.4. Redox ratio

To accurately determine the redox ratio at a 320 nm excitation, the detected NADH and FAD AF intensity was considered the measurement of cellular metabolism [15]. The reduction and oxidation ratio was computed using the relation [17].

\[
\text{Redox ratio} = \frac{\text{FAD intensity}}{\text{FAD intensity} + \text{NADH intensity}}
\]

where, FAD intensity and NADH intensity are the emission intensity at ~520 nm and ~450 nm, respectively.

2.5. Curve-fitting

Curve or peak fitting is mostly used for resolving the overlapped peaks and finding more precise peak parameters e.g. position, height, width and area. The advantage of this process lies in the fact that the data is de-noised to a great extent and overlapping is largely minimized. In the present study, spectral curve fittings were performed from the averaged AF spectra from the control, hyperplasia, dysplasia and WDSCC tissue groups using Origin 8.0 software with Gaussian spectral functions. Curve fitting is performed by step-wise iterative adjustments towards a minimum root-mean-square error of the different parameters, determining the shape, peak position, intensity and area of the constituent bands in the spectra.

2.6. Multivariate analysis

To reduce the high dimension of the spectral space (fluorescence emission spectrum ranging from 350 to 550 nm with a set of 200 intensity variables), principal component analysis (PCA) was employed to generate a few (PCs) that account for the majority of the whole variance in the original spectra while retaining the most diagnostically-significant information regarding the differentiation of tissues. In the present study, PCA on the normalized spectra of each category was performed and the data were reduced into seven PCs using SPSS version 17.0 for Windows. These PCs were used in a supervised classification model, LDA, for automated group discrimination and data classification.

LDA optimizes data classification and separation by maximizing the between-group separation variance while minimizing the within-group variance. It is also closely related to PCA; they both look for linear combinations of variables which best explain the data. The performance of the PC-LDA diagnostic algorithm was validated using the leave-one-out cross-validation (LOOCV) method. SPSS software was used to perform PCA based on fluorescence intensities as variables. The PCs obtained from PCA were analyzed as variables in LDA using SPSS software.

2.7. ROC analysis

The classification performance, based on the estimated class probabilities, was evaluated using ROC analysis [23], using pairwise comparisons of all tissues. In addition, the area under the ROC curve summarizes the performance of the test; the higher the discriminatory ability of the test, the more the area under the curve approaches 1.

3. Results

The typical averaged AF spectrum of the control and various oral transformations conditions such as hyperplasia, dysplasia and WDSCC tissues at the 320 nm excitation wavelength is shown in figure 2. It can be observed that all the groups show two maxima—one at ~385 nm and the other at ~450 nm. The ~385 nm emission band is generally attributed to collagen,
while the difference in the longer wavelength band associated with NADH emission, which has its peak at ~460 nm. The valley peak was also observed at ~520 nm for control tissues, known as the characteristic FAD emission peak. The fluorescence intensity at the 380 nm emission wavelength decreased in the order of control tissue to hyperplasia, dysplasia and then to WDSCC tissues. As for 460 nm, the fluorescence intensity of the control tissues was higher than those of hyperplasia, dysplasia and WDSCC tissues. The decreased collagen fluorescence at 320 nm excitation, which may result from degradation of the extracellular matrix by matrix metalloproteinase, has been noted in numerous spectroscopic studies [24–27].

The rise in NADH, a constituent of the electron transport chain (ETC), is attributed to the increased energy requirements by a dysplastic cell due to the rapidity of the divisions [28]. Moreover, cancer cells undergo aerobic glycolysis which results in elevated NADH:NAD⁺ ratios, where NAD⁺ is the non-fluorescent oxidized form of NADH. This may be one of the reasons for the observed lower intensity of the fluorescence maxima of NADH in neoplastic tissues.

Following data processing, a method of normalization was adopted to remove the absolute intensity information from the spectra that might be affected by many unavoidable experimental factors. In the case of fluorescence, the spectrum from each group of a sample was normalized with respect to the 450 nm emission. Figure 3 shows the normalized average spectra of the control, hyperplasia, dysplasia and WDSCC tissues at the 320 nm excitation wavelength. The normalized peak intensity at ~450 nm was not considered for further analysis. A direct relationship was observed between the changes of the fluorescence spectra and the tumor transformation. In the current study, control tissues exhibited a valley at ~520 nm; this is attributed to the emission of FAD. It has been corroborated in previous studies which stated that the blue fluorescence peak at 460 nm and the yellow fluorescence peak at 530 nm are contributions of cellular NADH and FAD, which are the enzymes carrying information on cellular metabolism [13, 27].

3.1. Area under a specific AF peak

Figure 4 demonstrates the results of curve fitting for the fluorescence spectra of control and various oral tissue transformations such as hyperplasia, dysplasia and WDSCC tissues. The areas for peaks 385 nm, 450 nm and 510 nm at 320 nm excitation for the control, hyperplasia, dysplasia and WDSCC tissues...
tissues are also given in figure 5. It shows that the amounts of collagen, NADH and FAD were decreased in the neoplastic oral tissues. However, the overall mean peak area is more in the case of control tissues. It clearly indicates that the distribution and conformation of these endogenous fluorophores may be varied with respect to tumor progression.

3.2. Redox ratio

Metabolic states of tissues can provide important parameters for the diagnosis, prognosis and treatment of various diseases. Thus, \([\text{FAD}/(\text{NADH} + \text{FAD})]\), a ratio of reduced and oxidized electron carriers, can be used as a sensitive metabolic index of the energy metabolism status of the tissues [10, 17]. The box plot relating the variations in the redox ratio of different groups is shown in figure 6. A drastic decrease in the optical redox ratio is observed for WDSCC, dysplasia and hyperplasia compared to control tissues, with the decrease being highly significant in the case of WDSCC tissues. A decrease in the redox ratio is thought to indicate an increase in the metabolic rate that it is typically observed in tumor tissues.

3.3. Multivariate analysis

Figure 7 shows the discriminant analysis of a scatter plot based on the discriminant scores, showing the classification of each spectrum for the control, hyperplasia, dysplasia and WDSCC groups. It is possible to visualize discrimination between groups by plotting the individual scores for the two discriminant functions. The results of the classification efficiency analysis are shown in table 1.

The separating lines for the scatter plots, as shown in figure 8, were calculated using the LDA model for the
7

diagnostically-significant pairs of groups (PCs): control versus hyperplasia, hyperplasia versus dysplasia and dysplasia versus WDSCC, respectively. Sensitivity is defined as the ratio of true positive (TP) cases to the sum of TP and false negative (FN) cases: \[ \frac{TP}{TP + FN} \]. Specificity is defined as the ratio of true negative (TN) cases (normal) to the sum of TN and false positive (FP) cases: \[ \frac{TN}{TN + FP} \]. The dividing line produces a diagnostic sensitivity of 83.3%, 75.0% and 75.0%, a specificity of 91.6%, 83.3% and 91.6%, and an accuracy of 87.5%, 79.1% and 86.9%, for discriminating control versus hyperplasia, hyperplasia versus dysplasia and dysplasia versus WDSCC tissues. These results are summarized in table 2.

Furthermore, the two most diagnostically-significant PCs (PC1 and PC2) were identified for diagnostic classification; direction comparisons between the control, hyperplasia, dysplasia and WDSCC groups are presented in figure 9. The different tissue groups are clustered into four separated groups based on the significant PCs and the corresponding separating lines in figure 9.

3.4. ROC analysis

The ROC curve is a sensitive method of reflecting the relationship of sensitivity and specificity. It is obtained by calculating the sensitivity and specificity of different diagnostic thresholds. Figure 10 displays ROC curves illustrating the TP rate against the FP rate to further evaluate the performance of the PC-LDA. The resulting AUC from this ideal scenario approaches the maximum value of 1, meaning that it is a perfect test (i.e. perfect sensitivity and specificity). The ROC gave an area under the curve of 1.00, 0.92 and 0.96, for control versus hyperplasia, hyperplasia versus dysplasia and dysplasia versus WDSCC tissues.

4. Discussion

As a pathological condition develops in tissue, changes in biochemical and physicochemical properties at the cellular and tissue level occur and alterations in the fluorescence emission
spectrum can be observed. The HBP model is ideal for studying the whole spectrum of oral carcinogenesis because of the great similarity between the lesions caused and those which occur in humans as a result of exposure to chemical carcinogens [29]. Several studies have demonstrated that the analysis of the light/tissue interaction can result in the discrimination of changes in tissue structure and metabolic activity such as breakdown of the collagen matrix and change in

**Figure 8.** Pairwise discriminant function scatter plot of different group pairs: (a) control versus hyperplasia, (b) hyperplasia versus dysplasia and (c) dysplasia versus WDSCC tissues.
the concentration of NADH, FAD, tryptophan, tyrosine and porphyrin [30]. Identification of these fluorophores present in the different tissue types and estimation of their concentration can help us understand the observed differences in the AF spectra. As a result of these alterations that occur during tumor transformation, the fluorescence signatures of collagen, NADH and FAD are different in control, hyperplasia, dysplasia and WDSCC tissues. In recent years, research has discovered that 320 nm is the ideal wavelength to study the variation of collagen, NADH and FAD, which are the main fluorophores involved in the pathogenesis of oral cancer. These fluorophore molecules may appear in different amounts in tumor evolution and structure and these changes in the metabolic status of the tissue are expected, therefore, resulting in a change in its concentration.

Peak intensity patterns and spectral line shapes using these fluorophores can be compared to distinguish control from neoplastic tissues. The significant intensity differences were observed in the AF spectra of control, hyperplasia, dysplasia and WDSCC tissues, and these differences could be attributed to the differences in fluorophores’ expression. In the current study, the normalized fluorescence spectra of control, hyperplasia, dysplasia and WDSCC tissues show peaks at ~385 nm, ~450 nm and ~520 nm; this is thought to arise from collagen, NADH and FAD. Collagen is one of the main contributors to oral mucosa fluorescence, and the decreased emission is related to the breakdown of the fiber links, resulting from tumor cells’ invasion. The fluorescence intensity of the control tissue was higher than that of neoplastic tissues. This might be due to the incrassation of the mucosa resulting from neoplastic transformation, so that the lower the fluorescence quantum yield of collagen mainly distributing in the submucosa, the lower the total fluorescence intensity at 385 nm consequently observed in neoplastic tissues. Further, neoplastic progression is associated with the regulation of matrix-degrading proteases essential in invasion and metastasis. Müller et al [31] have shown that oral cavity carcinoma is accompanied by an increase in epithelial thickness, which reduces the depth of penetration of the excitation light that reaches stromal collagen, thus reducing its contribution to the total signal. Further, it has been noticed that a clear redshift at ~450 nm has been observed for WDSCC compared to hyperplasia, dysplasia and control tissues. Previous spectrally-resolved studies have shown reduced overall intensity and a wavelength shift to longer wavelengths in malignant lesions of the head and neck [32, 33]. Shaiju et al [34] reported a similar progressive reduction in fluorescence intensity and wavelength shift in tumor brain tissues compared to normal tissue, with an excitation wavelength of 320 nm. These findings were consistent with the results from this study. The changes in the fluorescence intensity of neoplastic tissues may be influenced by factors such as tissue architectures, endogenous fluorophores and light penetration depth in tissue. Furthermore, it is also observed in the normalized spectrum, that the valley around at ~520 nm is prominent in the case of control tissues when compared with hyperplasia, dysplasia and WDSCC tissues. This observation is consistent with Ramanujam [35] who found that the flavin concentration was reduced in tumors, suggesting a ‘deficient’ aerobic oxidation system. The changes in NADH and FAD fluorescence probably arise due to the fact that DNA damage leads to the destruction of cellular organelles, namely mitochondria, which might in turn alter NADH and FAD.

Table 2. Diagnostic accuracies obtained by PC-LDA for the discrimination of control, hyperplasia, dysplasia and WDSCC tissue groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>MCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control versus hyperplasia</td>
<td>83.3</td>
<td>91.6</td>
<td>87.5</td>
<td>90.9</td>
<td>84.6</td>
<td>0.92</td>
</tr>
<tr>
<td>Hyperplasia versus dysplasia</td>
<td>75.0</td>
<td>83.3</td>
<td>79.1</td>
<td>81.8</td>
<td>76.9</td>
<td>0.83</td>
</tr>
<tr>
<td>Dysplasia versus WDSCC</td>
<td>75.0</td>
<td>91.6</td>
<td>86.9</td>
<td>90.0</td>
<td>78.5</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Figure 9. PC score plot of the first two PCs for the spectral dataset acquired from four different groups (i.e. control, hyperplasia, dysplasia and WDSCC tissues).

Figure 10. The ROC curves of discriminant scores of control versus hyperplasia (AUC = 1.00), hyperplasia versus dysplasia (AUC = 0.92) and dysplasia versus WDSCC (AUC = 0.96) tissues.
metabolic states. Mitochondrial metabolism is reflected in the redox state of the NAD system, which is tightly coupled with the flavin redox state in flavoproteins. The matrix of the mitochondrion contains a mixture of enzymes that catalyze the citric acid cycle (also called the Krebs cycle). The citric acid cycle produces the electron donors NADH (reduced nicotinamide adenine dinucleotide) and FADH$_2$ (reduced FAD) that feed into the ETC. Changes in the mitochondrial permeability affect the ETC and subsequently alter the balance between the reduced and oxidized state of molecules. Furthermore, metabolic endpoints show particular promise because shifts in cellular metabolism often occur sooner than changes in tumor size. Cellular metabolism is particularly sensitive to upstream molecular interventions, and therefore may be a powerful biomarker of early neoplastic changes prior to definite morphologic alteration. The redox ratio provides a dynamic measure of cellular metabolism [36, 37]. The fluorescence contributed by two fluorophores (NADH and FAD) has been explored to estimate optical redox ratios to reflect the variation in the metabolic rate of tissues [38]. The redox ratio, defined as ([FAD]/([NADH] + [FAD])), provides a quantitative metric to compare relative metabolic rates of measured samples. In the present study, it was found that the redox ratio of WDSCC tissues is significantly lower compared to that of control tissues. On the contrary, a ratio value of redox was significantly higher in hyperplasia and dysplasia compared to WDSCC tissues (figure 6). Changes in the optical redox ratio across different pathological tissues are consistent with proliferation rates. The redox ratios in tumors are generally lower than those in control tissues, which indicate a higher metabolic rate in tumors. This observation agrees with published microscopic studies in breast cancer cell lines [36, 39]. The underlying mechanism is that neoplastic cells have an increased metabolic demand due to rapid cell division compared with normal tissues [17].

A multivariate analysis approach, PCA coupled with LDA, was hence used for identifying significant spectral differences between different pathological tissues and to build a classification model for automatically discriminating different groups. To visualize the extent of group separation, we included a sufficient number of the PCs into the LDA and generated a scatter plot based on the estimated first and second discriminant functions (figure 7). Since the scores obtained by LDA can either be negative or positive, the scatter plot for each pair is given separately. The cut-off value in the scatter plot, which is the weighted mean of the paired values, is used to classify different lesions. Noticeable separation between the control and the neoplastic data can be observed in scores for the control, hyperplasia, dysplasia and WDSCC groups (figure 8). To further evaluate the performance of the PC-LDA, the ROC curve was generated from the scatter plot in figure 10 by varying the threshold level. The ROC gave an area under the curve of 1.00, 0.92 and 0.96, for control versus hyperplasia, hyperplasia versus dysplasia and dysplasia versus WDSCC tissues. These results demonstrate the potential of diagnostic fluorescence spectroscopy in an objective to distinguish dysplasia, hyperplasia and WDSCC from control oral mucosa. Furthermore, the results from this animal model can guide the development of techniques to identify early treatment responses in cancer.

5. Conclusions

In the present study, the AF technique demonstrated its ability to discriminate between the control from hyperplasia, dysplasia and WDSCC tissue groups based on endogenous fluorophores, mainly collagen and NADH and FAD, which presented the metabolic activity of tissues. The results show that there is a reduced contribution from the emission of collagen, NADH and FAD in WDSCC tissues as compared with the control tissues. Significant differences in the redox ratios of the control and different stages of the pathological tissues were observed, along with notably different box plots. From the study, it was concluded that the measure of fluorescence emission spectra of tissue along with the redox ratio in different tissue types not only provides information on the distribution and concentration of fluorophores, but also about metabolic rates at a cellular level. The results further suggest that fluorescence spectra in conjunction with a simple multivariate statistical analysis can be used as a potential tool for the discrimination of early neoplastic changes with improved classification accuracy.

Acknowledgments

The authors thank Dr Ramapurath S Jayasree from the Bio-photons & Imaging Laboratory, SCTIMST, Trivandrum, India for the opportunity to use the AF system. The authors are also grateful to Dr R Nirmal Madhavan from the Department of Oral & Maxillofacial Pathology, Annamalai University, Annamalainagar, India for his help in histopathological evaluation.

References

component analysis and k-means nearest neighbor analysis


[29] Vairaktaris E et al 2008 H-ras and c-fos exhibit similar expression patterns during most stages of oral oncocogenesis *In Vivo* **22** 621–8


[33] Badizadegan K et al 2004 Spectroscopic diagnosis and imaging of invisible pre-cancer *Faraday Discuss.* **126** 265–79


