Oral cancer detection using diffuse reflectance spectral ratio R540/R575 of oxygenated hemoglobin bands

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Abstract. A low-cost, fast, and noninvasive method for early diagnosis of malignant lesions of oral mucosa based on diffuse reflectance spectral signatures is presented. In this technique, output of a tungsten halogen lamp is guided to the tissue through the central fiber of a reflection probe whose surrounding six fibers collects tissue reflectance. *Ex vivo* diffuse reflectance spectra in the 400 to 600-nm region is measured from surgically removed oral cavity lesions using a miniature fiber optic spectrometer connected to a computer. Reflectance spectral intensity is higher in malignant tissues and shows dips at 542 and 577 nm owing to absorption from oxygenated hemoglobin (HbO_2). Measurements carried out, within an hour of surgical excision, on malignant lesion and adjoining uninvolved mucosa show that these absorption features are more prominent in neoplastic tissues owing to increased microvasculature and blood content. It is observed that reflectance intensity ratio of hemoglobin bands, R540/R575, from malignant sites are always lower than that from normal sites and vary according to the histological grade of malignancy. The diffuse reflectance intensity ratio R540/R575 of the hemoglobin bands appears to be a useful tool to discriminate between malignant lesions and normal mucosa of the oral cavity in a clinical setting. *© 2006 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.2165184]

Keywords: diffuse reflectance spectroscopy; oral cancer diagnosis; hemoglobin absorption peaks; reflectance spectral ratio R540/R575.

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1 Introduction

Early detection of neoplastic changes in the oral cavity and initiation of treatment is important to improve the quality of life of patients and their survival rates. Eighty-five percent of malignancies of the oral cavity are squamous cell carcinomas[1] (SCCs). Despite tremendous advancements made in ablative and reconstructive techniques for treating SCC of the oral cavity, survival rates over the past 30 yr are not very significant. Patients usually present themselves for checkup when the cancer of their oral cavity is already in an advanced stage. The 5-yr overall survival rates remain around 50% and are even less in an advanced stage of the disease.[2] The treatment modalities of the oral cancer in an advanced stage is expensive and often disfiguring and debilitating. In addition, there is the additional risk of metastasis, which again requires timely detection and follow up treatment.

Discrete lesions of the oral cavity such as oral leukoplakia (white plaques) and erythroplakia (reddish, velvety mucosal lesions) have the potential for malignant transformation. Many of the currently available screening and detection techniques for oral cancer do not provide adequate sensitivity and specificity for detecting precancerous tissues. Although the most common screening method of staining with agents such as toludine blue and Lugol's iodine has a sensitivity of around 90%, its specificity is lower and requires experienced clinicians for diagnosis. Sometimes, the whole tissue lining of the oral cavity of patients is at risk or has premalignant changes and it becomes difficult to determine biopsy location for diagnosis. Thus, more sensitive, noninvasive, cost-effective, and real-time screening and diagnostic methods are essential to detect cancer in its early stages.

The optical properties of tissues are important and informative, and the spectroscopic aspects are preeminent in the field of lesion localization and determination. Therefore, diagnostic techniques based on optical spectroscopy are fast, noninvasive, and quantitative and utilize the biochemical and morphological properties of tissues. This technique can also be used to elucidate key tissue features, such as the cellular metabolic rate, vascularity, intravascular oxygenation, and alterations in tissue morphology, which helps in precisely locating the neoplastic mucosal tissues of the oral cavity.

A number of new quantitative optical techniques, including diffuse reflectance spectroscopy, fluorescence spectroscopy, and Raman spectroscopy, have been investigated for detecting epithelial neoplasms.[3–8] Many of the instruments developed and tested, both *in vitro* and *in vivo*, show promise to increase both sensitivity and specificity. Among them, diffuse reflec-

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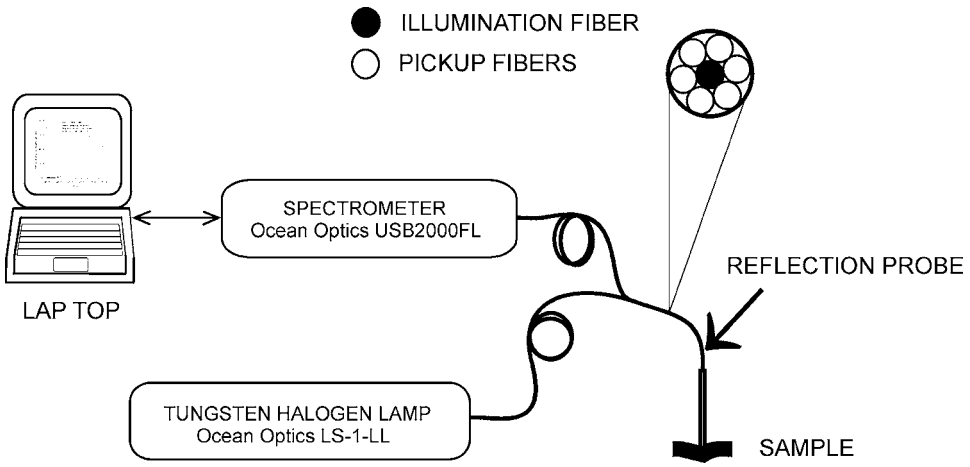

Fig. 1 Experimental setup for diffuse reflectance spectral measurements from intact tissues.

tance spectroscopy is one of the simplest and most cost effective methods for understanding biological tissue characteristics. This technique involves detection and analysis of a portion of the incident light that undergoes multiple elastic scattering owing to inhomogeneities in the refractive index of the tissue. Recent results have shown that tissue back scattering is altered as the size of the nucleus increases and the nuclear texture becomes coarser.[9] Tissue absorption in the UV and visible region is dominated by hemoglobin, with the oxygenated and deoxygenated forms having different absorption spectral features.[10] The potential for using diffuse reflectance spectroscopic techniques is greater because elastic interactions are much stronger than inelastic interactions.

By illuminating the tissue with continuous-wave white light from an optical fiber, and collecting the emanating light at various distances using several other fibers, the optical properties of the tissue (absorption and scattering coefficients) can be calculated.[4,11,12] Many researchers have explored the use of reflectance to correct the tissue fluorescence spectral data and for understanding the effect of changes in oxygenation and tissue perfusion.[13,14] Mourant et al.[6] used reflectance between 330 and 370 nm, affected by nuclear-to-cytoplasmic ratio, to distinguish malignant from nonmalignant sites in the bladder and found a sensitivity of 100% and a specificity of 97%. Ge et al.[15] used an algorithm that utilized intensities at the absorption peaks of oxy- and deoxyhemoglobin to distinguish neoplastic from nonneoplastic sites in colonic tissue and reported a sensitivity of 86% and specificity of 84%. Reflectance spectroscopy was used to detect Barrett's oesophagus with an agreement of 71% in comparison to the diagnosis by four different pathologists.[16] Zonios et al.[7] used diffuse reflectance data from human adenomatous colon polyps to fit an analytical model and found that differences can be attributed to hemoglobin concentration and effective scatterer size. Elastic light scattering spectroscopy with polarized illumination/detection, which reduces the contribution from hemoglobin absorption, was used by Sokolov et al.[17] to characterize *in vitro* cervical biopsies and *in vivo* oral cavity mucosa. Utzinger et al.[18] have investigated the slope of reflectance spectral features between 530 and 585 nm at different source-detector separations for *in vivo* characterization of ovarian cancer and found that reflectance spectral slopes between 510 and 530 nm, strongly affected by oxy- and deoxyhemoglobin absorption, provided useful discriminatory information. These bands, seen in both normal and malignant tissues, have been utilized to extract intrinsic tissue fluorescence, which is free from artifacts induced by tissue scattering and absorption.[19]

The diffuse reflectance spectrum shows characteristic hemoglobin absorption valleys at 430, 542, and 577 nm on excitation with white light and it is reported that hemoglobin content is more pronounced in malignant and premalignant lesions owing to increased microvasculature.[7] In this study, we explore the potential of using the diffuse reflectance spectral ratio (R540/R575) of the oxygenated hemoglobin absorption bands at 540 and 575 nm for *ex vivo* detection of oral cavity SCC. Surgically excised tissues from various parts of the oral cavity have been examined and the results presented show the potential of diffuse reflectance spectral ratio R540/R575 for *in vivo* quantitative analysis of mucosal tissues.

2 Materials and Methods

2.1 *Study Material*

Oral tissue samples were brought for examination to the laboratory situated within a short distance (3 km) from the Regional Cancer Centre (RCC), Trivandrum, immediately following surgical excision, without fixing or immersion in any solvent. The tissues were transported in clean and dry petri dishes covered by cellophane, in a light-tight bag. The lesions removed from patients had varying histological grades of SCC and belonged to different sites in the oral cavity. A tissue sample removed during surgery from one of the margin areas (1 cm away from the lesion) that has the appearance of a normal tissue was used as control. The size of the lesions were of the order of 1 to 2 cm in length, 1 to 1.5 cm in breadth, and 0.5 to 1 cm in thickness, enabling point monitoring from at least 10 different sites on each sample, considering the heterogenity of oral cavity lesions. For comparison with normal healthy mucosal tissues, *in vivo* reflectance spectra from the oral cavity of three consenting volunteers were used.

2.2 *Instrument*

The portable spectroscopic system for measurement of tissue reflectance is shown in Fig. 1. The system consists of a compact tungsten halogen lamp (Ocean Optics, USA, model: LS-1-LL) whose output is sent through the central fiber (400 μm diameter) of a 3-m long reflection probe that has six surrounding fibers (400 μm diameter, each) for collection of diffuse reflectance from the lesion. The reflection probe tip is terminated in a stainless steel ferrule (15 cm long and 6 mm in diameter) for easy access to interior areas and to facilitate sterilization, before and after use. The distance from the fiber tip to a lesion was optimized to obtain maximum overlap between the excitation and collection areas. A spacer was used to maintain this distance during measurements. The optical fiber probe tip is kept in close contact with the tissue surface to avoid ambient light from entering the detection system and to avoid potential light loss through the specimen edges. The light emerging from the collection fiber is delivered to a miniature fiber-optic spectrometer (Ocean Optics, USA, model: USB 2000FL) driven via the universal serial bus (USB) port of a laptop computer. This spectrometer is equipped with a 2048-element linear silicon CCD array that gives a resolution of 8 nm in conjunction with the 400-μm-diam. optical fiber light guide. The diffuse reflectance spectrum is recorded in the 400 to 600-nm region using the OOI software supplied with the spectrometer.

2.3 *Data Acquisition*

Before measurements the probe was cleaned with distilled water, sterilized, and dried using sterile gauze. The tissue samples were cleaned in running water and crusts, if any, present in the border of the lesion were removed and the samples were dried before initiation of diffuse reflectance measurements. Histological examination was carried out at RCC on a portion of the surgically excised tissue sample. Within an hour of excision, the diffuse reflectance spectra were recorded, with an integration time of 100 ms and boxcar width of 10 nm, from 10 random sites on the lesion by point monitoring. The reflectance intensity ratio (R540/R575) of the oxygenated hemoglobin absorption bands at 540 and 575 nm was determined from the recorded spectra of normal and malignant lesions. To account for the broad nature of the peaks and sample-to-sample variation in peak position owing to absorption differences between lesions, the average reflectance intensity over a ±5-nm interval at the absorption peak was used to determine the ratio. The results are then analyzed to determine the extent of variation in the R540/R575 ratio for different types of lesions and correlated with histopathological findings.

3 Results

The diffuse reflectance spectra depend on tissue morphology, constitution, and surface features. Figure 2 shows the mean of 10 measurements of the *ex vivo* (immediately after surgery) diffuse reflectance spectra of the excised lesion from the buccal mucosa of a patient, pathologically confirmed as moderate to well-differentiated SCC, and of an adjoining uninvolved area, considered as normal. Average *in vivo* tissue reflectance spectrum from the buccal mucosa of three healthy volunteers (10 measurements each) and the diffuse reflectance spectrum

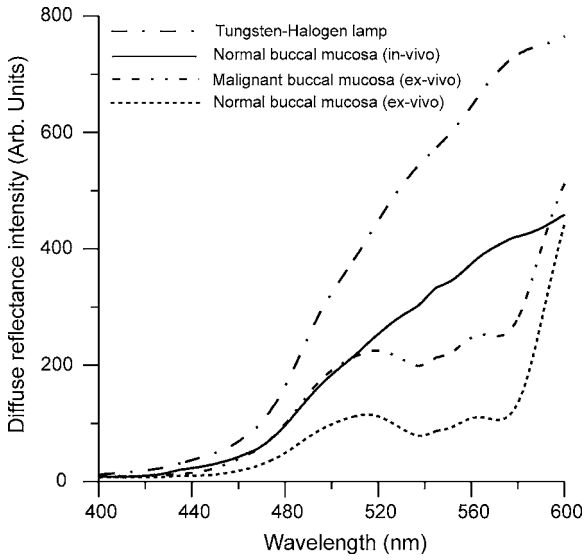

Fig. 2 Mean reflectance spectra of malignant and normal buccal mucosal tissues superimposed on the diffusely scattered light from a tungsten-halogen lamp. Mean *in vivo* reflectance spectra from the normal buccal mucosa of three healthy volunteers is also shown for comparison.

of the tungsten halogen lamp, with a ground glass plate as the scatterer, are also shown in Fig. 2 for comparison. In all our measurements, the reflectance spectral intensity from malignant lesion was always greater than that of normal mucosa. Notable differences were observed in the short-wavelength region where the broad hemoglobin (Hb) absorption valley around 420 nm is prominent. Secondary dips in reflectance spectra at 542 and 577 nm owing to absorption from oxygenated hemoglobin (HbO_2) bands were evident in both malignant lesions and adjoining uninvolved areas. As compared to normal tissues, absorption at the 542- and 577-nm bands were more prominent in malignant lesions, signifying increased Hb presence and microvascular volume. However, the hemoglobin bands are not seen in the *in vivo* diffuse reflectance spectra of healthy volunteers.

Mean R540/R575 ratios, with their standard deviations, are shown in Table 1, for normal and malignant tissue samples from various sites of the oral cavity of the patients studied. Irrespective of tissue location, the mean R540/R575 ratio was always found to be lower for malignant lesions as compared to the adjoining uninvolved normal areas. Significant changes were also noticed in the diffuse reflectance ratio according to the stage of malignancy. In the case of moderate to poorly defined SCC of the tongue, which is the highest grade of SCC studied, the percentage decrease in the diffuse reflectance ratio from 0.94 to 0.82 was maximum (12.77%). The patient with buccal mucosa, pathologically diagnosed as moderate to well-defined SCC, reported minimum variation (8.54%) from 0.82 to 0.75. Thus, the R540/R575 ratio derived from the absorption due to oxygenated hemoglobin bands appears to be a useful parameter in the grading of oral cavity SCC.

The temporal profile of *ex vivo* diffuse reflectance spectra is shown in Fig. 3. The overall *ex vivo* diffuse reflectance spectral intensity and absorption band intensities at 540 and 575 nm in both normal and malignant tissues were found to

Table 1 The mean reflectance spectral intensity ratio R540/R575 of surgically excised oral lesions from patients with different stages of oral cavity squamous cell carcinoma (SCC), within 1 h of tissue removal.

Age (yr)/Sex	Site	Tissue Type	*N*	Mean R540/R575	Percentage of decrease
48/M	Tongue	Normal	10	0.94±0.07	12.77
		Mpdscc	10	0.82±0.02	
40/M	Tongue	Normal	10	0.81±0.04	11.11
		SCC	5	0.72±0.01	
69/M	Lower alveolus	Normal	6	0.79±0.03	10.13
		Mdscc	8	0.71±0.02	
49/F	Tongue	Normal	6	0.81±0.02	9.88
		Mdscc	6	0.73±0.02	
42/M	Buccal mucosa	Normal	10	0.82±0.01	8.54
		Mwdscc	10	0.75±0.01	

Tissue nomenclature: Mpdscc, moderate to poorly differentiated SCC; Mwdscc, moderate to well-differentiated SCC; Mdscc, moderately differentiated SCC. *N* denotes the number of measurements.

decrease gradually during the 3-h measurement period owing to tissue degradation. During this period, the percentage variation in R540/R575 ratio between the normal and malignant lesions also decreased from 12.5 to 1.5% (Fig. 4), thereby demonstrating the necessity to carry out *ex vivo* diffuse reflectance spectra measurements within the shortest possible time for maximum tissue discrimination. This also points to the significance of using oxygenated hemoglobin band ratios (R540/R570) as indicators of malignancy.

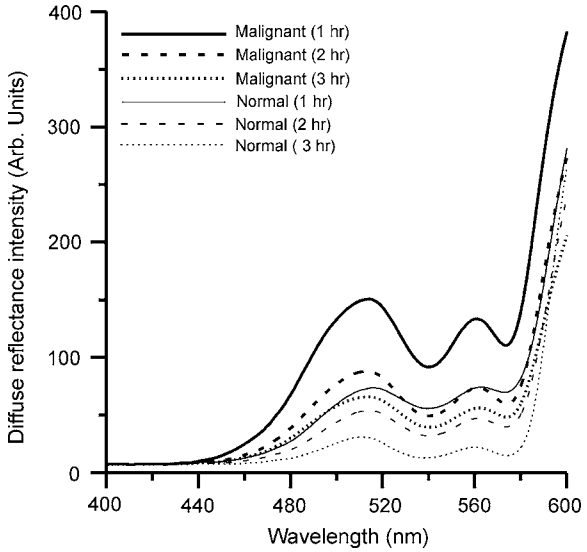

Fig. 3 Mean diffuse reflectance spectra of malignant and adjoining normal lesion from the tongue diagnosed as moderate to poorly differentiated SCC. These spectra were recorded after 1, 2, and 3 h of surgical excision.

4 Discussion

Tumor or cancerous tissues are known to exhibit increased microvasculature and hence, increased blood content.[20] They often have abnormally low hemoglobin (Hb) oxygenation owing to the disturbed metabolism.[21] Zonios et al.[7] observed in the case of adenomatous colon polyps an increase in Hb concentration, a larger average effective scatter size, and a smaller average scatter density, with no significant change in Hb oxygenation. Using morphometry and vascular casting, they observed that precancerous tissues are characterized by increased microvascular volume. Our results are in agreement with these observations as the malignant tissues exhibit increased absorption at the oxygenated hemoglobin bands and overall enhancement in spectral intensities with concomitant decrease in the R540/R575 reflectance ratio.

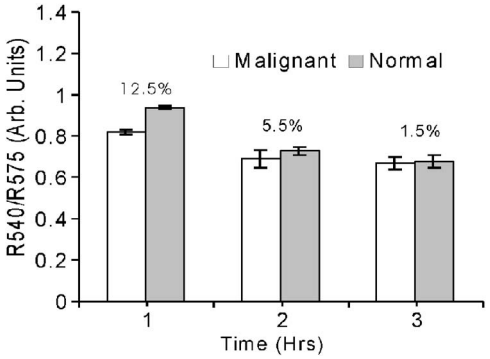

Fig. 4 Mean R540/R575 ratio of surgically excised malignant lesion of the tongue and its adjoning normal lesion. The percentage decrease in ratio with respect to the normal lesion and standard deviation for each ratio are shown over the histogram bar.

Palmer et al.[5] observed that the reflectance intensities at 415, 540, and 570 nm remained relatively constant over a 90-min time period. However, in the case of malignant tissues, the decrease of *ex vivo* reflectance intensity at 540 and 575 nm in 120 min, was 46.4 and 46.3%, respectively. They also reported that the diffuse reflectance of both *ex vivo* and *in vivo* tissues are similar at 500 nm and decreases at longer wavelengths due to tissue deoxygenation and changes in tissue scattering. Our measurements (Fig. 2) also show similarity in reflectance intensity profile between *in vivo* normal tissues with *ex vivo* malignant tissues up to 500 nm and around 600 nm. However, the temporal variations in reflectance intensity (Fig. 3) are prominent in the 500 to 580-nm region where the oxygenated Hb bands are absorbing, whereas at around 600 nm the changes are minimal between normal and malignant tissues. This increase in tissue absorbance is due to increase in hemoglobin absorption and hemoglobin deoxygenation brought about by cell lysing that increases the amount of blood content in tissues.[5]

Studies carried out on surgically excised tissue samples show that decrease in the diffuse reflectance intensity ratio R540/R575 of HbO_2 bands at 542 and 577 nm can be used to discriminate between malignant lesions and normal tissues and in determining the grade of malignancy. Since the normal tissue samples used for comparison were from areas adjoining the tumor site, there is every possibility that some parts of these tissues could also be malignant. Note that histology was not performed on the same tissue samples, but on another portion of the tissue removed during surgery. To check the validity of results, pathological analysis was conducted in a tissue sample from upper alveolus, fixed immediately after surgery, and also on a lesion fixed in the laboratory after optical sampling. In both of these cases, biopsy reports showed the lesions as moderately differentiated SCC. However, tissues from uninvolved areas considered as normal, also showed up as squamous epithelial lining with focal mild dysplasia. Hence, under *in vivo* conditions where one has the opportunity to sample healthy and suspicious tissues from the oral cavity of the same patient, the changes in the diffuse reflectance ratio between normal tissues and malignant lesions could be much more pronounced.

In comparison, the *in vivo* diffuse reflectance spectra from the buccal mucosa of healthy volunteers showed the absence of the oxygenated hemoglobin bands. The mean reflectance spectra shown in Fig. 2 relate to sampling from both sides of the buccal cavity of the three volunteers who had no visible symptoms of any oral cavity disease or inflammatory condition. Since the surgical procedures are usually not carried out in the presence of overt inflammatory conditions, the changes seen in diffuse reflectance spectral could be attributed to the vascularity associated with increased blood content in malignant lesions. Further, the percentage of decrease in the reflectance ratio R540/R575 due to increase in vascularity of the lesions was found to depend on the grade of malignancy (Table 1).

The precancerous condition is usually characterized by cellular and microvascular proliferation and, hence, increased volume occupied by cells in tissue. Since visible light penetration is limited to the top one-half millimeter of the mucosal layer, where precancerous changes occur, the diffuse reflectance ratio of oxygenated Hb bands could help in early detection of changes to the mucosal layer of the oral cavity and cervix. Since the *ex vivo* and *in vivo* tissues have similar diffuse reflectance spectral features between 400 to 600 nm, the *ex vivo* measurements, within a reasonable time frame, have the advantage of reproducing clinically relevant *in vivo* conditions.

On the basis of the results obtained in this study, the diffuse reflectance ratio technique appears to have potential for the *in vivo* detection of oral and cervical cancer and superficial tumors of internal organs with the help of endoscopes. The optical fiber probe could further aid in demarcation of malignant lesions during surgical interventions, thereby limiting tissue removal to the barest minimum. Moreover, the diffuse reflectance instrument due to its compactness, low cost, and portability proves to be an advantageous and suitable alternative for *in vivo* cancer screening through community extension centers.

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