Diagnostic Fluorescence Spectroscopy of Oral Mucosa

[1,3] Krishnendu Roy, [1]Ian D. Bottrill, [1]Duncan R. Ingrams, [1]Michail M. Pankratov, [1]Elie E. Rebeiz,
[1] Peak Woo, [2]Sadru Kabani, [1]Stanley M. Shapshay,
[4]Ramasamy Manoharan, [4]Irving Itzkan, [4]Michael S. Feld

[1] Otolaryngology Research Center for Advanced Endoscopic Applications
Department of Otolaryngology-Head and Neck Surgery,
New England Medical Center, Tufts University School of Medicine, Boston, MA 02111,
[2]Department of Oral Pathology, Tufts University School of Dental Medicine, Boston, MA 02111,
[3]Department of Biomedical Engineering, Boston University, Boston, MA 02215,
[4]George R. Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology, Cambridge, MA 02139

ABSTRACT

Autofluorescence characteristics of normal, dysplastic, and malignant squamous tissues from the oral cavity were measured with a spectrofluorometer in the excitation range of 250-500 nm and emission range of 350-750 nm. Fluorescence excitation-emission matrices (EEM) were obtained from samples collected from patients in the clinic and in the operating room. The same samples were submitted for histopathological examination following spectroscopic measurements. The contour plots obtained from the EEMs of the samples showed consistent differences between normal and abnormal tissues. All the abnormal samples showed enhanced red region (> 600 nm) fluorescence with a prominent peak at 635 nm, when excited by 410 nm light. A ratio contour plot (abnormal/normal) enhanced spectral differences in the red region. A fiber-optic based spectrofluorometer for EEM measurements is being developed for further investigations

Keywords: Fluorescence, Spectroscopy, Dysplasia, Oral Mucosa

1. INTRODUCTION

There are more than eighteen thousand new cases of intraoral cancer per year in the USA[1] and these are mainly seen in patients who have a history of tobacco use and alcohol consumption. The oral cavity and the upper aero-digestive tract is lined in most parts by non-keratinizing squamous epithelium. Under the influence of a variety of oncogenic factors, this epithelium undergoes a spectrum of changes (dysplasia) that may end in the growth of squamous cell carcinoma. These changes can occur in an isolated area or more commonly be associated with multifocal sites (temporal and spatial). In the latter case this is termed field cancerization. The chances of surviving oral cavity carcinoma are markedly increased by early detection and treatment, but early lesions can be very difficult to identify by conventional techniques. Current management of suspicious lesions includes excisional biopsy, perhaps under general anesthesia, followed by repeated observations in clinic and further biopsies of any area of concern. The need for a non-invasive technique for easy, fast and accurate assessment of such lesions is evident.

A decade ago it was shown that fluorescence spectroscopic techniques can be used in medicine for diagnostic purposes.[2] Since then several researchers have extensively studied the feasibility of such techniques to diagnose malignant and premalignant tissues in different parts of the human anatomy. Alfano et al.[3] reported studies in gastrointestinal and colon tissues as well as human breast tissues[4] and lung cancers.[5] Cothren et al.[6] and Shomacker et al.[7] reported prospective studies in colon cancer and adenomatous polyps with a high degree of diagnostic sensitivity and specificity. Richards-Kortum et al.[8-10] have performed extensive studies in atherosclerotic plaques and cervical lesions. Several other groups including Kapadia et al,[11] Feld et al,[12] have reported the effectiveness of such technique in diagnosing malignant and

premalignant lesions. All these groups use different methods (in terms of wavelengths, diagnostic algorithms etc.) for differentiating between normal and diseased tissues.

Only a few preliminary studies have been reported involving fluorescence spectroscopy of squamous epithelium from the upper aero-digestive tract (Kolli et al[13] and Koenig et al[14]). The field of spectroscopic diagnosis of oral lesions is still essentially unexplored. We are presently studying the autofluorescence characteristics of normal, dysplastic and malignant squamous tissues from the oral cavity *in vitro*. Our ultimate goal is to develop a portable *in vivo* diagnostic system for clinical use to detect malignant and dysplastic lesions in the upper aerodigestive tract. This will bypass the need of multiple invasive biopsy and will provide physicians with a fast and effective tool to easily assess the oncological characteristics of oral lesions. Furthermore a quantitative and qualitative analysis of the fluorescence spectra can provide deep insight into the biochemical changes associated with the gradual development of squamous cell carcinoma.

Other methods for non-invasive measurements of tissue condition include, elastic scattering of light[15] and Raman Spectroscopy. Light scattering is based on the fact that changes in tissue architecture due to disease will alter the elastic scattering properties of light incident on the sample. These changes in back-scattering can be correlated to the extent of dysplasia. Studies involving Raman spectroscopy to diagnose cancer are also in progress but although Raman spectroscopy is very specific it requires much more sophistication and expertise than fluorescence measurements. Fluorescence spectroscopic techniques provides quantitative information about the biochemical composition of the tissue specimen and can provide a easy, fast and accurate non-invasive tool, if proven effective. This paper describes *in vitro* autofluorescence measurements and data analysis of tissue specimens collected from the oral mucosa of patients in the clinic and the operating room. Comparison of spectral signals between histologically normal and diseased tissues are made. Design and development of a fiber optic based spectrofluorometer, suitable for more effective measurements, is underway.

2. METHODS AND INSTRUMENTATION

2.1 Specimen handling procedure:

Samples were collected from patients in the Department of Otolaryngology-Head and Neck Surgery and the Department of Oral Surgery, New England Medical Center, Boston, MA. These were biopsied in the clinic from patients with suspicious lesions and in the operating room from patients undergoing surgical removal of lesions. Informed consent was obtained from each patient and this study was reviewed and approved by the Human Investigation Review Committee of the New England Medical Center and Massachusetts Institute of Technology. Biopsies were taken from sites that appeared abnormal to the physician and from sites which appeared to be normal. In each patient, tissue specimens approximately 5mm x 5mm were removed and then flash frozen in Isopentane cooled in liquid nitrogen. The frozen specimens were stored at -80°C. They were then transported in a dry ice container to MIT's spectroscopy laboratory where *in vitro* spectroscopic measurements were performed using a fluorometer. After measurements the specimens were fixed in 10% formalin and submitted for histopathological analysis.

2.2 Instrumentation:

A fluorescence spectroscopic bench system (Spex Fluorolog) was used to obtain the excitation-emission scan of each specimen. This system consists of a continuous white light source (Xe lamp), two monochromators (Spex 6810), a sample chamber, a photomultiplier tube detector cooled with a peltier cooling system and a personal computer with Spex DM3HOST data acquisition, control and analysis software. The output of the light source is broadband white light which enters the excitation monochromator through an adjustable entrance slit. The light is dispersed by the grating and individual monochromatic beams come out through the exit slit into the sample chamber. The tissue specimen is placed inside a quartz cuvette inside the chamber. The excitation light shines on the specimen and the fluorescence signal is collected from the front face of the sample. The collection slit is connected to the emission monochromator which disperses the fluorescence signal into individual wavelengths which are then detected by the photomultiplier tube. There is a second

photomultiplier tube connected to the emission monochromator to monitor the intensity of the emitted fluorescence in order to take into account the wavelength to wavelength variability of intensity of the excitation light. All these operations of wavelength selection and detection are controlled through the software and it directly plots the spectrum (intensity vs. emission wavelengths) of the specimen for each individual excitation wavelength. A block diagram of the measurement system is shown in Figure1.

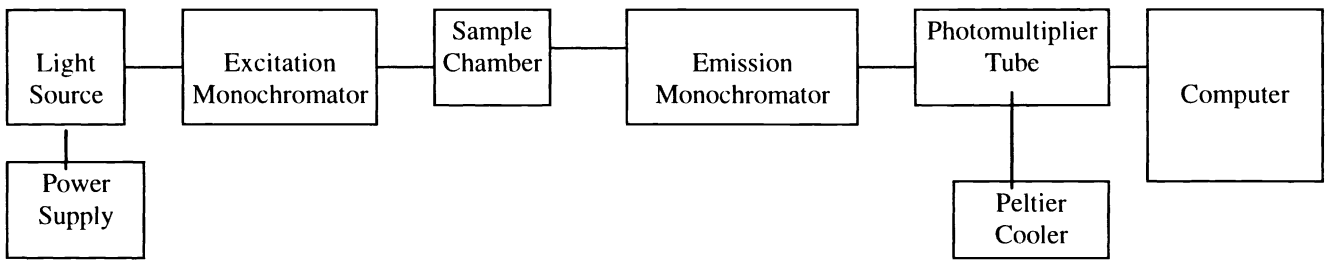

Figure 1: Block diagram of instrument for in vitro measurement of tissue fluorescence

2.3 Measurement protocol:

Our primary goal is to find the best excitation wavelength(s) which will give us maximum diagnostic differences between normal and diseased tissues. In order to obtain the excitation-emission characteristics of the specimens, emission spectra are measured at different excitation wavelengths so that an excitation-emission matrix (EEM) can be calculated. Each frozen sample is allowed to thaw for few minutes before measurement. It is then placed inside the cuvette in such a way that the excitation light illuminates the mucosal surface. For each sample the excitation wavelength is varied from 250 to 500 nm in 10 nm steps and fluorescence emission spectra are collected in the range of 350 to 750 nm using this basic rule: lm = (lx + 10 nm) to (2*lx -10 nm) where lm = emission wavelengths (nm), lx = excitation wavelength (nm). For excitations from 250 nm to 340 nm, emission are collected only from 340 nm onwards since the correction factor file starts from 340 nm. Each datafile is corrected with a correction factor file generated by measuring the fluorescence of standard white light. After measurements the samples are fixed in 10% formalin and submitted for histopathological analysis. The pathology results are then correlated with the spectroscopic signals to find a diagnostic difference between normal and diseased tissue samples.

2.4 Data Processing:

The spectroscopic measurement of each sample generated datafiles for each excitation wavelength and contained the intensities of fluorescence for different emission wavelengths. These files were then processed to generate a single excitation-emission matrix (EEM) for each sample. The columns of these matrices are intensities at different emission wavelengths for a particular excitation wavelength. If we look at a single row it gives us the intensities at different excitation wavelengths for a particular emission wavelength (excitation spectra). After obtaining the EEM, a contour plot is obtained using the GRAPHTOOL software. This gives equal intensity lines for different excitation and emission wavelengths. A MATLAB program was written to plot the ratio contour (abnormal vs normal). Such ratios were calculated for normal and abnormal samples obtained from the same patient. Individual spectra were also plotted to study

the difference in fluorescence signals at different wavelengths. These data were then correlated with the histopathological classifications of each tissue.

3. RESULTS

Fluorescence spectra from 20 normal and diseased tissue samples were measured in vitro using the spectrofluorometer system described above. Among these, 19 samples were from the oral cavity and one was from the larynx. A classification of the collected specimens showing their location, clinical impression and pathological diagnosis are tabulated below. The last column describes results of spectroscopic measurements and shows whether the sample shoed increased red fluorescence or not.

Table: Location and Types of Samples used for *in-vitro* study of Squamous Dysplasia of Oral Mucosa (In the last column, '-' means low red region fluorescence and '+' means increased red region fluorescence with a prominent peak at 635 nm when excited by 410 nm light).

Number of Sample	Location of Lesion	Clinical Impression	Pathological Diagnosis	Spectroscopic Findings
1	Right Floor of Mouth	Normal	Normal	-
2	Right Floor of Mouth	Carcinoma	Normal	-
3	Gingiva	Dysplasia	Normal	-
4	Right Buccal Mucosa	Normal	Normal	-
5	Base of Tongue	Carcinoma	Carcinoma	+
6	Floor of Mouth	Normal	Normal	-
7	Right Buccal Mucosa	Dysplasia	Carcinoma (Verrucous)	+
8	Left Gingiva	Dysplasia	Dysplasia (Mild)	+
9	Right Buccal Mucosa	Normal	Normal	-
10	Left Buccal Mucosa	Dysplasia	Carcinoma	+
11	Left Buccal Mucosa	Dysplasia	Carcinoma	+
12	Floor of Mouth	Dysplasia	Dysplasia (Mild)	+
13	Left Buccal Mucosa	Normal	Dysplasia (Moderate)	+
14	Lateral Border Tongue	Carcinoma	Ulceration, Inflammation	+
15	Left Buccal Mucosa	Normal	Normal	-
16	Tongue	Carcinoma	Carcinoma	+
17	Left Buccal Mucosa	Dysplasia	Dysplasia (Mild-Moderate)	+
18	Left Buccal Mucosa	Normal	Normal (Hyperkeratosis)	-
19	Left Lateral Tongue	Dysplasia	Dysplasia (Mild-Moderate	+
20	Ventral Tongue	Normal	Normal (Hyperkeratosis)	-

EEMs were constructed and contour plots were obtained from each specimen. All the samples shows strong characteristic peaks around 330 nm (the datafile can only collect the tail of this peak), around 480 nm and around 515 nm. All the pathologically abnormal (dysplasia, carcinoma-in-situ and carcinoma) samples collected from the oral cavity showed an additional fluorescence peak at 635 nm when excited by 410 nm light. There was also increased red fluorescence in the 690-700 nm region at this same excitation wavelength. Ratio contours were computed for samples from the same subject and they showed prominent peaks at 635 nm and 690 nm at 410 nm excitation. Typical normal and abnormal contours are shown in Figure 2. A typical ratio contour is plotted in Figure 3.

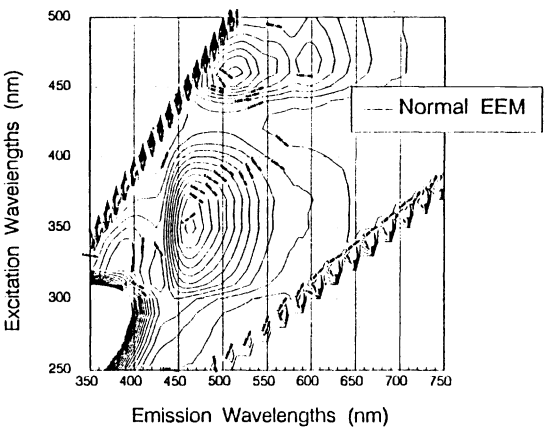

Figure 2: Typical EEM contour plot of Normal Oral Mucosa

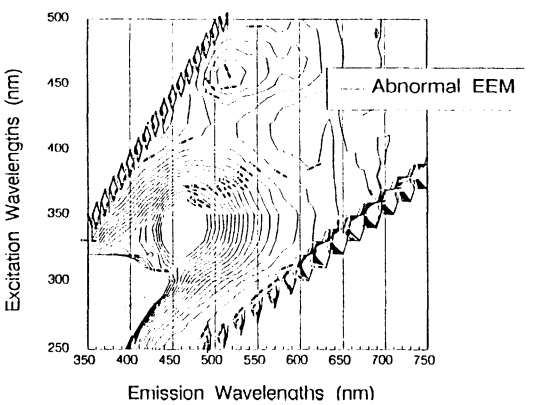

Figure 3. Typical EEM contour plot of abnormal (dysplastic) oral mucosa

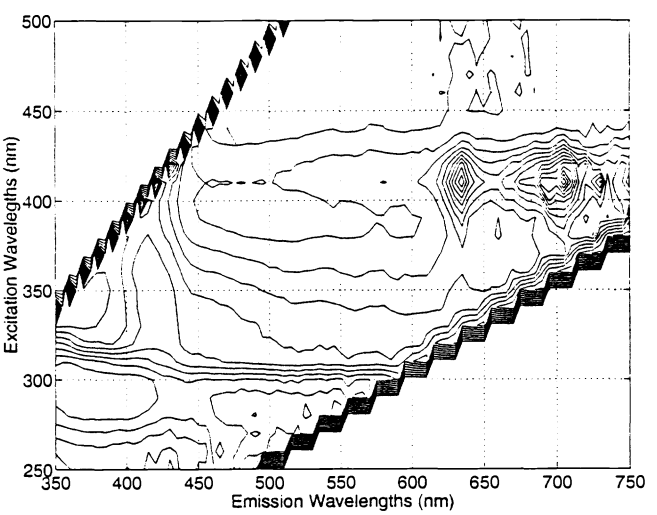

Figure 4. Typical ratio contour (abnormal vs. normal specimens)

Individual spectra at different excitation wavelengths were also plotted for analysis. The most significant differences were obtained when an excitation wavelength of 410 nm was used. Almost all the abnormal specimens showed a prominent emission peak at 635 nm with increased red fluorescence. None of the normal samples showed this peak nor did they show high red fluorescence. Typical 410 nm emission spectra are plotted in figure 5 to show the differences between normal and abnormal specimens. Figure 6 shows the same pair of spectra where the data have been normalized to their highest value to enhance the differences in the red region.

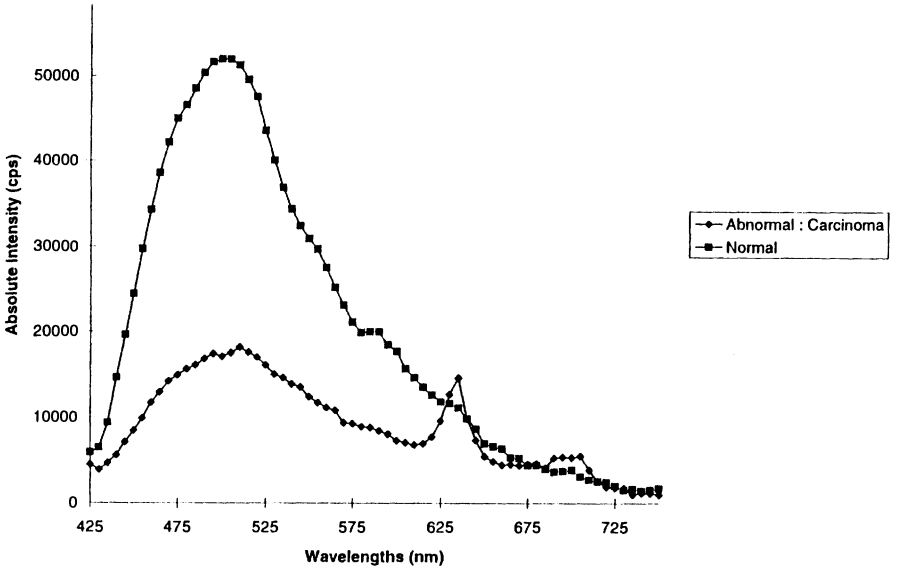

Figure 5. Typical 410 nm Emission Spectrum of normal and abnormal oral mucosa

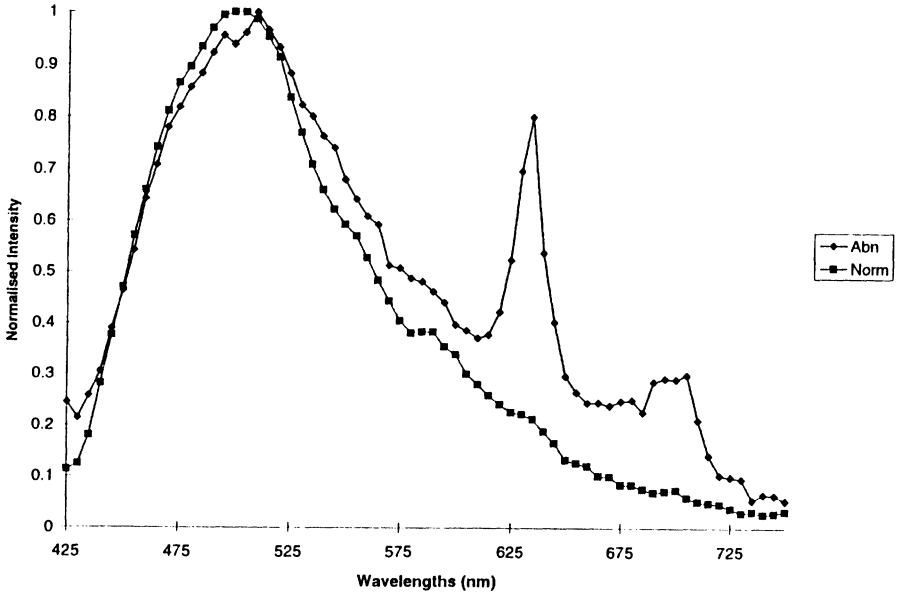

Figure 6. Typical 410 nm Emission Spectrum (Normalized) of normal and abnormal oral mucosa

4. DISCUSSION

This preliminary *in vitro* study of the autofluorescence characteristics of normal, pre-malignant and malignant tissues from different parts of the oral mucosa shows interesting and very promising results. The consistent differences in the contour plots, especially in the red region fluorescence indicates that such technique has definite potential to be used as diagnostic tool to differentiate tissue condition. The 410 nm excitation wavelength seems to be the most promising wavelength to be used for this purposes, but detailed *in vivo* studies under more controlled conditions must be done before anything can be definitively said.

It is to be noted that the intensity peaks observed in the normal and abnormal samples are consistent with the peaks from other tissues reported by different researchers. The common fluorophores present in tissues, like collagen, elastin, NADH, flavin etc. are likely to cause these peaks. The cause of increased red fluorescence is still unknown. A detailed biochemical study needs to be performed to exactly assign the spectral signatures to individual chromophores. Some researchers[14] attribute the red region peak and the increased red fluorescence to porphyrins produced by certain bacteria.

The spectrometric measurements obtained from the fluorometer by placing the sample in the cuvette are found to have intensity variations from day to day. There were wide intensity variations among normal samples also. Few of the samples showed very low fluorescence intensity. These differences can be attributed to sample size, thickness etc. Also it was found that the position of the sample in the cuvette may change during the measurement. Several samples were quite small which may have been responsible for the lower intensity. In lieu of these factors comparisons have been made on the basis of line shapes only. But measurements performed by other researchers have shown that the intensity informations are also useful to develop effective diagnostic tool. So, before we can decide on any sort of diagnostic algorithm we need to perform more detailed measurements and an extensive *in vivo* study comparing both line shapes and fluorescence intensities at different wavelengths.

We are in the process of building up a portable fiber optic system to ensure constant probe geometry and more accurate measurements. This system will be based on a nitrogen pumped dye laser that will generate different excitation wavelengths. The laser light will then be delivered through a fiber probe to the tissue and the fluorescence signal will be collected by a fiber bundle. A 1024 diode array detector will be used with a optical multichannel analyzer to obtain the fluorescence spectra. This system will ensure accurate in vitro measurements as well as will be used to perform in vivo measurements in the next phase of the study. Our new fiber optic system will incorporate a simultaneous reflectance measurement of the sample to obtain the intrinsic fluorescence. We will explore the utility of the method of Wu et al.[16] to extract the intrinsic fluorescence of a sample by measuring the usual (extrinsic) fluorescence signal and the white light reflectance signal of the sample, with the same probe geometry and then taking the ratio of the two.

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