BASIC SCIENCE REVIEW

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FLUORESCENCE SPECTROSCOPY: A TECHNIQUE WITH POTENTIAL TO IMPROVE THE EARLY DETECTION OF AERODIGESTIVE TRACT NEOPLASIA

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Abstract: *Background.* Any innovation which facilitates the early detection of neoplastic changes in upper aerodigestive tract mucosa has potential to greatly improve survival and quality of life in persons prone to develop malignancies in this area. One technology that has shown great promise during initial investigations is fluorescence spectroscopy. Fluorescence spectroscopy evaluates the physical and chemical properties of tissue by analyzing the intensity and character of light emitted in the form of fluorescence. This technology has been investigated for the noninvasive detection of malignancy in various sites including the gastrointestinal tract, lung, breast, and cervix.

Methods. This article reviews the recent work investigating the capabilities of fluorescence spectroscopy to discriminate between normal and neoplastic mucosa in the oral cavity. Also discussed are potential applications for the detection and diagnosis of premalignant and malignant lesions of the upper aerodigestive tract, and some of the obstacles to overcome to make this technology feasible. © 1998 John Wiley & Sons, Inc. *Head Neck* **20:** 556–562, 1998.

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Despite improvements in chemotherapy, radiotherapy, and surgical techniques for the treatment of malignancies of the upper aerodigestive tract, survival rates for patients with these malignancies have not improved substantially over the past three decades. Patients with head and neck squamous cell carcinomas (HNSCC) are usually seen initially with advanced-stage disease. Treatment for these patients, compared with that for patients with smaller malignancies, is more difficult, more expensive, more disfiguring, and less successful. Early detection of neoplastic changes may have the greatest potential for improving patient quality of life and survival rates.

The diagnosis of early malignancies of the upper aerodigestive tract is usually made by visual identification and recognition. Thus, the detection of HNSCC relies heavily on the clinical experience of the examiner at recognizing suspicious lesions during physical examination. However, dis-

tinguishing premalignant and early malignant lesions from the more common benign inflammatory conditions can be extremely difficult even for experienced practitioners. The development of a noninvasive and accurate technique for the screening and diagnosis of oral lesions in real time would have great potential for improving the early detection of neoplastic changes in the aerodigestive tract mucosa.

Physicians have relied on the interactions of light within tissue to aid in the diagnosis of disease for over a hundred years. Common examples include the use of a Woods lamp to detect tinea capitis or the use of histologic stains of organ specimens to provide color differentiation of tissue structures. Thus, the concept that alterations in tissue architecture and cellular composition are reflected in variations in the optical properties of human tissue is not new to the medical field. However, recent technological advances have now markedly increased the potential applications of light-tissue interactions for the recognition of disease.

Fluorescence spectroscopy is a technique for evaluating the physical and chemical properties of a substance by analyzing the intensity and character of light emitted in the form of fluorescence. Although this technique has been used extensively for over a hundred years in other scientific fields, fluorescence spectroscopy has only relatively recently been investigated for use in the medical field. Technological advances now allow subtle alterations in tissue architecture and biochemical composition induced by processes such as dysplasia and inflammation to be detected in real time noninvasively using fluorescence spectroscopy. Although much research remains to be done, it appears that this technique has great potential to bridge the gap between clinical examination and invasive biopsy and thus facilitate the early detection and diagnosis of neoplastic changes. This article will review some of the recent work investigating the technique of optical spectroscopy and its potential for the detection and diagnosis of neoplasia of the upper aerodigestive tract.

OPTICAL TISSUE INTERACTIONS

Energy in the form of a light photon can activate certain molecules within a cell. The subsequent radiative relaxation of the molecule is accompanied by the release of a reemission photon in a process termed "fluorescence." This is illustrated schematically in Figure 1. The wavelength of the photon causing the activation is termed the "excitation wavelength"; the wavelength of the emitted photon is the emission wavelength. Fluorescence spectroscopy is the detection and analysis of this reemitted optical signal. Two types of fluorescence spectra can be produced. An emission spectrum is made when the excitation wavelength is fixed and the fluorescence is measured over a range of emission wavelengths. In an excitation spectrum, the emission wavelength is fixed and the fluorescence is measured over a range of excitation wavelengths. An excitation–emission matrix (EEM) is composed of fluorescence intensities as a function of excitation and emission wavelengths.

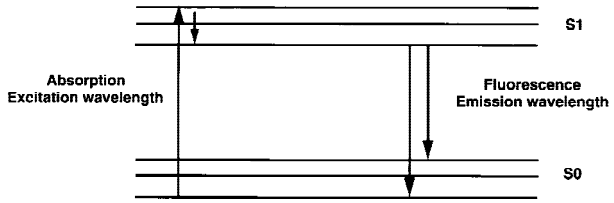

FIGURE 1. Jablonski diagram depicting the energy level transitions that occur during the process of fluorescence.

Molecules that can fluoresce are called "fluorophores." Many of the endogenous fluorophores in tissue have been identified and are summarized in Table 1. Nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) play important roles in the energy metabolism of cells. The reduced form of NADH and the oxidized form FADH2 are fluorescent at wavelengths near 500 nm. The aromatic amino acids—

Table 1. Endogenous tissue fluorophores.*

Fluorophores	Excitation maxima (nm)	Emission maxima (nm)
NADH	290,340	440,450
FAD	450	515
Collagen cross-links	325	400
Elastin cross-links	325	400
Collagen powder	280,265,330,450	310,385,390,530
Elastin powder	350,410,450	420,500,520
Tryptophan	280	350
Tyrosine	Not determined	300
Phenylalanine	Not determined	280
Pyridoxine	332	400
Lipofuscin	340-395	430-460,540
Eosinophils	370,500	440,550

**Adapted from: Richards-Kortum R, Sevick-Muraca E. Quantitative optical spectroscopy for tissue diagnosis. Ann Rev Phys Chem 1996;47:555–606.*
Abbreviations: NADH, nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide.

tryptophan, tyrosine, and phenylalanine—contribute to the fluorescent signals emitted by biologic tissue. The structural proteins collagen and elastin also fluoresce, with emission maximum in the blue region around 400 nm. Porphyrins, which are present at low levels in normal tissue and are produced by certain bacterial species, fluoresce in the red spectral region (>600 nm).

The spectroscopic systems currently being evaluated for use in obtaining the fluorescence spectra from oral mucosa consist of a source of monochromatic light (either laser or lamp with appropriate filters to obtain specific wavelengths), an optical fiber probe, and a spectrometer and detector to record fluorescence intensities at specific wavelengths. One example of a fluorescence spectroscopy system is depicted schematically in Figure 2. Most probes described in the literature require direct contact with the mucosal surface. In this regard, Dhingra et al[1] have noted that the optimal pressure at the mucosal surface is 300 g/cm$_2$.

DIAGNOSTIC APPLICATIONS

Alterations in the concentrations or forms of endogenous tissue fluorophores in pathologic states such as inflammation or neoplasia should be detectable as differences in the overall fluorescent signature of the tissue. For example, fluorescence in the red spectral region (>600 nm), which was noted in tumors as early as 1924[2] and was originally attributed to bacteria, is now thought to be secondary to endogenous porphyrins. More recently, investigators who were focusing on the ability of fluorescence tumor markers, such as hematoporphyrin derivative (HPD), to facilitate diagnosis and treatment of early tumors, discovered a native tissue fluorescence that varied between normal and malignant areas.[3] Other researchers subsequently confirmed these findings.[4,5]

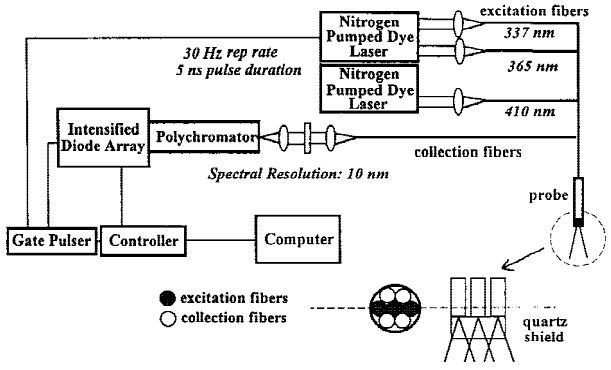

FIGURE 2. Schematic diagram illustrating one example of a fluorescence spectroscopy system designed for evaluation of tissue in vivo.

One of the first clinical applications of autofluorescence spectroscopy investigated was the detection of dental decay.[6] Other groups later adapted this technology to the detection of atherosclerosis.[7] In 1987, Yang et al[8] investigated the fluorescence spectra of various types of tissues. Using an excitation wavelength of 365 nm, this group noted a difference in the autofluorescence spectra between normal and malignant tissue specimens. They also performed one of the first clinical trials to evaluate in vivo fluorescence spectroscopy for the detection of malignancy. Their results demonstrated good diagnostic potential for this technique.[8] Since that time, studies of the application of fluorescence spectroscopy to the diagnosis of neoplastic changes have been conducted in a variety of sites including the gastrointestinal tract,[9–11] lung,[12] breast,[13] and cervix.[14] Fewer studies have been conducted on the fluorescent properties of upper aerodigestive tract mucosa.

SPECTROSCOPIC EVALUATION OF ORAL MUCOSAL LESIONS

In Vitro Studies. Investigations into the fluorescent properties of oral mucosa were performed initially on human tissue specimens in vitro. In vitro testing has the advantage of allowing analysis of fluorescence spectra over a complete range of excitation wavelengths. In this way, complete EEMs can be obtained, and the wavelengths with the greatest discrepancy between normal and abnormal tissues can then be determined. A typical EEM obtained from an oral cavity squamous cell carcinoma is shown in Figure 3. Ingrams et al[15] analyzed the fluorescence properties of oral cavity biopsies over excitation wavelengths from 250 to 500 nm. As a result, they documented differences in the fluorescence spectra between normal oral mucosa and malignant lesions in several wavelengths, especially around 370 and 410 nm.[15]

In Vivo Studies. Several small clinical trials have been performed to evaluate the potential of fluorescence spectroscopy to discriminate between benign, dysplastic, malignant, and inflammatory tissue in the oral cavity. One study analyzed two emission spectra (at 300 and 340 nm excitation) and two excitation spectra from oral lesions in 31 patients. Ratios of the peak fluorescence intensi-

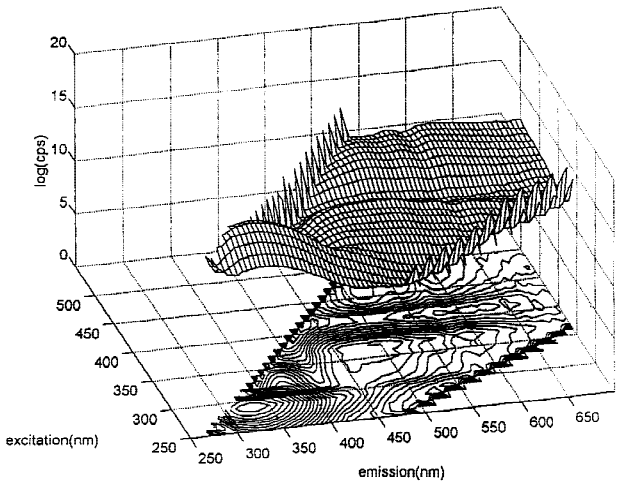

FIGURE 3. Excitation-emission spectra (EEM) obtained in vitro from a surgical specimen of oral tongue squamous cell carcinoma.

ties at two wavelengths were used to compare normal with abnormal tissue. A correct diagnosis of abnormality was achieved in 24 of 31 cases using two such algorithms.[16] Dhingra et al[1] evaluated autofluorescence spectra in 19 lesions from 13 patients using excitation wavelengths of 370 and 410 nm. Consistent differences were found between the spectra obtained from abnormal lesions and those from a corresponding normal control.

Another study evaluated 33 sites within the oral cavity from 15 patients using excitation wavelengths of 337, 365, and 410 nm. Although there was considerable patient-to-patient and site-to-site variability, consistent differences were found between the fluorescence spectra obtained from abnormal tissue and normal controls at all three wavelengths tested. Furthermore, diagnostic algorithms were developed that were able to discriminate between abnormal and normal tissue, with a sensitivity and specificity of 88% and 100%, respectively.[17]

Although the spectroscopic systems and wavelengths used by different groups varied slightly, the alterations in fluorescence spectra of malignant lesions compared with normal showed some similarities among the different studies. First, there was a decrease in the fluorescence intensity of dysplastic and neoplastic mucosa when compared with normal mucosa. Second, the spectral line shape between abnromal and normal tissues had consisted differences, particularly an increased fluorescence in the red region of the abnormal specimen.

Fluorescence Intensity. A typical fluorescence spectra obtained from a patient with an oral cavity carcinoma is shown in Figure 4a. The greatest intensity occurred in the blue region between wavelengths 455 and 490 nm (this is often referred to as a blue peak). When the malignant specimen is compared with the normal specimen from the same patient, the peak intensity is much less in the abnormal tissue. This decrease in intensities in the blue region is not specific to oral tumors. Similar results have been obtained in studies with tumors of the cervix,[18] bronchus,[12] and colon tissue.[11] Yet, despite numerous investigations in various organ systems, the etiology for the decreased fluorescence intensity in the blue region of malignant tissue versus normal tissue is still unclear. Natural fluorophores whose emission maxima occur in this wavelength region include collagen, elastin, the aromatic amino acids, pyridoxine, NADH, and FAD. Any alterations in tissue architecture that inhibit the ability of excitation photons to reach the natural fluorophores or of the fluorescence emission photon to escape from the tissue and be detected by the spectroscopic system would effect the fluorescent signature. Additionally, changes in the concentration or form of natural fluorophores such as collagen would alter the emitted fluorescence. The pyridine nucleotides and the flavins have different fluorescence capabilities depending on whether they are in the reduced or oxidized state. There-

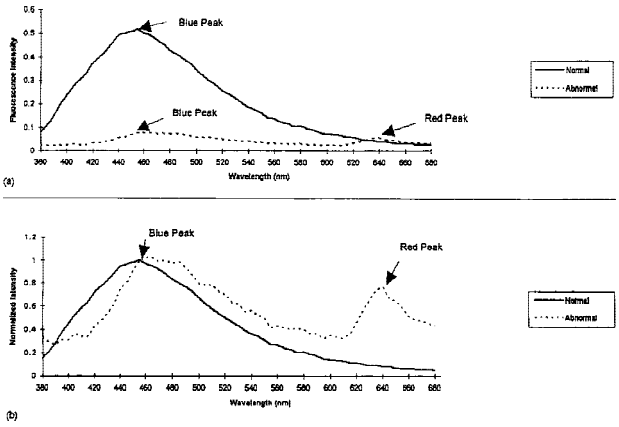

FIGURE 4. (a) Emission spectrum obtained from a patient with squamous cell carcinoma of the gingiva at a 365-nm excitation wavelength. Note that the intensity of the carcinoma sample is less than that of the normal gingiva in this patient. Fluorescence intensity is presented in calibrated units. **(b)** When the spectrum of the cancer specimen is normalized to the spectrum of the corresponding normal specimen, differences in the spectral line shape become apparent. Note the increased intensity of the cancer spectrum in the red region.

fore, changes in the tissue oxidative state due to the neoplastic process might account for changes in the overall fluorescence spectra. A considerable amount of research will need to be performed to better understand the etiology of the altered fluorescence intensity in the blue region during malignant progression.

Fluorescence Spectral Line Shape. When the peak intensities of the normal and malignant spectra are normalized, differences in the spectral line shape become evident. An example is shown in Figure 4b. The spectral line-shape changes predominantly manifest as a shift of the peak intensity of abnormal tissues to a longer wavelength and an increase in the fluorescence of abnormal tissues in the red region (>600 nm). The increased red fluorescence of oral cavity tumors compared with the surrounding normal mucosa was one of the first observations made regarding fluorescent characteristics of abnormal tissues.[2,19] The red fluorescence of tumors has been attributed to porphyrin,[20] and in a more recent work, Ghadially[21] indicated that it is due to protoporphyrin. Porphyrins are intermediate products generated during the synthesis of the metaloporphyrins such as heme, and though the concentration of porphyrins in tissue is normally low, abnormalities in the synthesis pathway of heme can result in the accumulation of porphyrin. In addition, bacteria such as *Bacteroides, Propionibacterum, Clostridium, Actinomyces,* and *Pseudomonas* can produce porphyrins.[23,24] The absorption maxima for porphyrins are 380–420 nm and 480–650 nm, and the fluorescence emission maxima are in the 600–700-nm range.[8] Whether or not the increased red fluorescence noted in oral cavity tumors is related to intrinsic abnormalities in porphyrin metabolism within cancer cells or abnormal bacterial growth and porphyrin production has not been determined.

Fluorescence Properties of Normal Mucosa. To better characterize the variations in fluorescence spectra between neoplastic and normal oral mucosa, it is important to understand the normal variability of the fluorescence spectra obtained from oral mucosa from different sites within the oral cavity and from different individuals. Some preliminary investigations have been performed to help determine this normal distribution. Kolli et al[16] showed significant variability between individuals in the fluorescence spectra obtained from the buccal mucosa and the dorsal tongue. This variability in fluorescent signals among different individuals was confirmed in later studies[1,17] and is illustrated in Figure 5. Thus, in developing algorithms to differentiate between neoplastic and normal tissue, it is important to compare each person's suspicious lesion with a corresponding normal area. In this regard, Dhingra et al[1] showed that, although there was no difference in peak fluorescence intensity in one anatomic site versus in the contralateral corresponding anatomic site in one individual over time, there was significant variation among the different anatomic sites within the oral cavity.

DIAGNOSTIC HURDLES AND LIMITATIONS

The results obtained in the various investigations performed to date demonstrate the exciting potential for the application of this new technology to the diagnosis of neoplasia in the oral cavity and upper aerodigestive tract. However, before this technique can be brought to the general public, several major obstacles must be overcome. First, larger clinical trials need to be performed to confirm the preliminary results obtained in the initial clinical trials. Second, the optimal excitation and emission wavelengths needed to differentiate between normal and abnormal tissue at each oral cavity location must be ascertained. It is important to note that, to date, the only investigations using complete EEMs (ie, analysis at all wavelengths within a given range) have been performed in vitro. Because there are substantial differences between the results obtained in vitro and

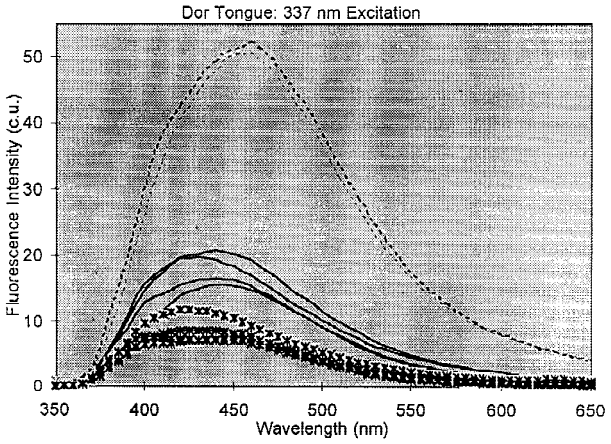

FIGURE 5. Spectra obtained at a 337-nm excitation wavelength from several sites on the oral tongue in three different individuals. The spectra from each subject is depicted by a different line form. Note that the intersubject variability is much greater than the intrasubject variability.

in vivo, clinical investigations using complete EEMs are needed to assess the optimal excitation and emission wavelengths.

In addition, further investigation is required to determine the ability of fluorescence spectroscopy to differentiate between invasive carcinoma, dysplasia, and inflammation. The optimal analytic algorithm for differentiating between these populations needs to be determined and tested. Because of the marked variation of native autofluorescence between different individuals, it is necessary at this time to base all diagnostic algorithms on a comparison between the abnormal tissue and a contralateral normal tissue in each patient.

There are some aspects of this technique that may limit its diagnostic usefulness to certain situations. First, all of the probes described in the literature so far require direct contact with the mucosal surface to obtain a spectroscopic reading. This will make application to lesions in the oropharynx, nasopharynx, and larynx more difficult without general anesthesia. Second, the monochromatic light signal penetrates only about 500 μm deep into the tissue. Thus, this technique is capable of analyzing only the most superficial portions of oral lesions. This will limit the application of this technique for detection of submucosal disease.

FUTURE PERSPECTIVES

Fluorescence spectroscopy has great potential to facilitate the noninvasive identification and detection of neoplasia at an early stage. Perhaps its greatest application would be for the screening of oral cavity neoplasias in unskilled hands. Once the proper algorithms have been established and the instrumentation standardized, this technique could be made available to dental clinics and primary-care facilities to be used by dental hygienists, public-health nurses, and primary-care physicians and physician assistants to screen for early neoplastic lesions in the oral cavity. In a tertiary-care setting, fluorescence spectroscopy would also be extremely useful for the identification of subsequent primary lesions in patients who have already undergone treatment for an upper aerodigestive tract tumor and as intermediate end-point markers for therapy trials. The development of this technology, however, will rely on the combined efforts and expertise of head and neck cancer specialists, biomedical engineers, pathologists, and patients.

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