Time-Resolved Fluorescence Spectroscopy as a Diagnostic Technique of Oral Carcinoma

Validation in the Hamster Buccal Pouch Model

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Objective: To investigate the benefit of using time-resolved, laser-induced fluorescence spectroscopy for diagnosing malignant and premalignant lesions of the oral cavity.

Design: The carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) was applied to 1 cheek pouch of 19 hamsters. The contralateral pouch and the cheek pouches of 3 hamsters without DMBA exposure served as controls.

Setting: University of California, Davis.

Participants: Twenty-two golden/Syrian hamsters.

Intervention: A nitrogen pulse laser was used to induce tissue autofluorescence between the wavelengths of 360 and 650 nm.

Main Outcome Measures: Spectral intensities and time-domain measurements were obtained and compared with the histopathologic findings at each corresponding site.

Results: Spectral intensities and lifetime values at 3 spectral bands (SBs; SB1 = 380±10 nm; SB2 = 460±10 nm, and SB3 = 635±10 nm) allowed for discrimination among healthy epithelium, dysplasia, carcinoma in situ, and invasive carcinoma. The lifetime values at SB2 were the most important when distinguishing the lesions using only time-resolved parameters. An algorithm combining spectral fluorescence parameters derived from both spectral and time-domain parameters (peak intensities, average fluorescence lifetimes, and the Laguerre coefficient [zero-order]) for healthy epithelium, dysplasia, carcinoma in situ, and invasive carcinoma provided the best diagnostic discrimination, with 100%, 100%, 69.2%, and 76.5% sensitivity and 100%, 92.2%, 97.1%, and 96.2% specificity, respectively.

Conclusions: The addition of time-resolved fluorescence-derived parameters significantly improves the capability of fluorescence spectroscopy–based diagnostics in the hamster buccal pouch. This technique provides a potential noninvasive diagnostic instrument for head and neck cancer.


Despite the significant medical advances of the past several years, the survival rate of patients with head and neck cancer has not improved significantly. In fact, within the oral cavity, survival outcomes may be decreasing. Most of these oral and pharyngeal tumors present themselves at an advanced stage despite many of them being easily visible through either direct visualization or simple awake endoscopy.

Patients with oral cavity cancer most often present with a painful lesion in the mouth. A diagnosis of head and neck cancer is obtained after this lesion is visualized, biopsied, and then inspected under the microscope. Definitive treatment is then performed with surgery, radiation, and chemotherapy in different combinations, dependent on tumor location and patient preference. The goals of the treatment include complete eradication of the tumor with maximum preservation of function and aesthetics. Despite advances in reconstruction, treatment causes significant morbidity and impairment of many critical functions, such as speech, swallowing, and taste, as well as facial appearance. Earlier detection allows smaller surgical procedures and less aggressive nonsurgical treatment. Subsequently, patients will experience fewer treatment adverse effects and have an improved quality of life and survival. Developing a straightforward, noninvasive diagnostic technique to allow for more accurate and earlier diagnosis would significantly benefit patients.

Fluorescence spectroscopy has shown promise as a noninvasive technique to aid
in the diagnosis of head and neck cancer.3-6 This technique relies on the molecular contrast offered by either endogenous fluorophores present in all tissues or targeted fluorescent exogenous probes. Typically, a light source in the wavelength range of near UV to visible light is used to excite these fluorophores. The fluorescence is then recorded as an emission spectrum. Patterns of peak intensities and spectral line shapes can then be compared for differences among distinct tissues.

Fluorescence spectroscopy is generally divided into steady-state (spectrally resolved or intensity measurements) and time-resolved (time-domain and frequency-domain) techniques. Although steady-state approaches have been extensively tested as a diagnostic tool for head and neck tumors,3,7-9 the potential of time-resolved techniques to improve the diagnostic ability of these tumors has been scarcely investigated. Time-resolved measurements resolve fluorescence intensity decay in terms of lifetimes, thus providing additional information with regard to the underlying fluorescence dynamics. The use of time-resolved (lifetime) fluorescence to study biological systems offers several distinct advantages. For example, biomolecules with overlapping fluorescence emission spectra but different fluorescence decay times can be discriminated. These measurements are more robust to changes in fluorescence excitation-collection geometry, presence of endogenous absorbers (eg, hemoglobin), photobleaching, and changes in fluorophore concentration, light scattering, and excitation intensity, which thus makes them more suitable for clinical investigations. In addition, a complete fluorescence emission spectrum (steady state) can be obtained by recording the time-resolved fluorescence emission at a number of wavelengths across the emission spectrum.

The hamster buccal pouch carcinogenesis model provides a means to evaluate the ability of fluorescence spectroscopy to distinguish healthy, premalignant, and malignant epithelial cells. In this model, a known carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA), is applied to the buccal pouch of immunocompetent animals 3 times a week.10-13 Repeated exposure to the carcinogen leads to consistent temporal development of precancerous lesions followed by cancerous growths. Although many animal models involve injection or implantation of tumor cells into the animals, the hamster cheek pouch model allows study of the sequence of events from healthy epithelium to carcinoma in an immunocompetent animal. After visible tumors are generated, the surrounding epithelium may be healthy or dysplastic. This allows sampling using fluorescence spectroscopy of a wide variety of histopathologic specimens. The objectives of this study were to evaluate the ability of time-resolved, laser-induced fluorescence spectroscopy (TR-LIFS) to serve as a noninvasive diagnostic technique for squamous cell carcinoma in the hamster cheek pouch model and to determine whether time-resolved, fluorescence-derived parameters can add diagnostic value to fluorescence spectroscopy intensity measurements in distinguishing normal tissues from premalignant and malignant epithelium.

ANIMAL CARE

Twenty-two male, golden/Syrian hamsters, 5 to 6 weeks of age, were obtained from Charles River Laboratories (Wilmington, Massachusetts). The experiment was approved by the Institution for Animal Care and Use Committee at the University of California, Davis, to ensure humane treatment of the hamsters, and strict adherence to the protocol was observed. The animals were housed 2 to 3 per cage under controlled conditions with a 12-hour light and dark cycle and given water and standard laboratory chow ad libitum. The study was conducted from February 27, 2007, through August 9, 2007.

INTERVENTION

All animals were anesthetized at the time of the procedure with ketamine hydrochloride and xylazine hydrochloride. While the hamsters were anesthetized, the hamster cheek pouch was everted and examined for lesions. Once the desired buccal tissue was exposed, the tissue was spectroscopically investigated with a prototype TR-LIFS apparatus (Figure 1). This apparatus is similar to a previously used system described in detail in other studies.11,13 In brief, tissue autofluorescence was induced with a pulsed nitrogen laser (337 nm, 700-picosecond pulse width). The collected fluorescence was dispersed by an imaging spectrometer/monochromator (Medel MicroHR, 13.8, 600-g/mm grating; HORIBA Jobin Yvon Inc, Edison, New Jersey) and then detected with a gated multichannel plate photomultiplier tube. The laser triggering, wavelength scanning, and data acquisition, storage, and processing were controlled by means of a computer workstation and custom analytical software written in LabVIEW (National Instruments Corporation, Austin, Texas) and MATLAB (The Mathworks Inc, Natick, Massachusetts).

The fiber-optic probe was placed perpendicular to the surface of the cheek pouch regions presumed to be healthy tissue and regions suggestive of carcinoma. The probe was held in position with a specially designed, metallic, 3-dimensional micromanipulator, and each sample investigated was 1 mm in diameter. The fluorescence emission of each sample was scanned in the 360-nm to 650-nm range at 5-nm intervals, with a time resolution of 100-picosecond and at a scanning speed of 0.8 second per wavelength. At each wavelength, 16 fluorescence pulses were collected and averaged by the oscilloscope. The total acquisition time was approximately 45 seconds across the scanned emission spectrum. After each measurement sequence, the monochromator was tuned to a wavelength slightly below the excitation laser line. The laser pulses reflected by the sample were measured and used to represent the temporal profile of the laser pulse. This profile was later used as input to the deconvolution algorithm for the estimation of fluorescence lifetimes.

The data were recorded from regions visually identified as either healthy or suggestive of carcinoma. The suggestive lesions ranged from one to several millimeters in diameter. A total
of 96 TR-LIFS measurements were collected from 37 biopsied locations. Between 1 and 4 measurements were taken at each biopsy site, and all spectroscopic measurements from each biopsy specimen were found to be similar. The time-integrated fluorescence (spectral emission) was computed as in previous studies.16

In the context of TR-LIFS, the intrinsic fluorescence impulse response functions, \( h(n) \), describe the real dynamics of the fluorescence decay. The impulse response functions were recovered by numerical deconvolution of the measured input laser pulse from the measured fluorescence response transients. The Laguerre expansion technique was used for deconvolution. This analytical approach for characterization of fluorescence decay was recently developed by our research group and described in detail elsewhere.17

HISTOLOGIC ANALYSIS

Once the spectroscopy data had been collected from the desired areas of the cheek pouch, incisional punch biopsies were performed at these locations. By means of the same pathologic classification scheme described in a previous publication by Meier et al,13 a pathologist (B.P.) masked to the study scored standard hematoxylin-eosin–stained slides of these biopsy specimens on a scale 1 to 7 (1, normal; 2, papilloma; 3, mild; 4, moderate; 5, severe dysplasia; 6, carcinoma in situ [CIS]; or 7, invasive carcinoma; the original magnification of all specimens was between \( \times 10 \) and \( \times 20 \)).

STATISTICAL ANALYSIS

Significant spectral intensity peaks were noted at 3 spectral bands (SBs): SB1, 380±10 nm; SB2, 460±10 nm (SB460); and SB3, 635±10 nm. At these SBs, ratios of the normalized peak intensity were compared among the varying histologic classifications by means of 2-way analysis of variance and significance determined at \( P < .05 \). The lifetime values and Laguerre expansion coefficients at SB1, SB2, and SB3 were also analyzed by means of 2-way analysis of variance. As described in previous studies,15,16 a stepwise linear discriminant analysis was used to determine the combination of predictor variables that accounts for most of the differences in the average profiles of the 4 tissue groups (normal epithelium, dysplasia, CIS, and carcinoma) and to generate a classification algorithm for samples classification. The classification accuracy was determined for 5 groups of predictor variables: (1) spectral features only, (2) temporal features only, (3) both spectral and temporal features, (4) spectral and temporal features with the Laguerre coefficients (zero, first, second, and third order), and (5) spectral and temporal features with the Laguerre coefficients (zero order only). A leave-1-out method was used to create the test and training set. The classification accuracy was determined by computation of the sensitivity and specificity. Linear discriminant analysis was performed with the statistical software package SPSS (SPSS Inc, Chicago, Illinois).

RESULTS

HISTOLOGIC ANALYSIS

Histologic examination confirmed healthy tissue in 18 of the 19 specimens presumed to be healthy, with 1 specimen that revealed hyperplasia. In the 18 biopsy specimens taken of lesions suggestive of cancer, 3 were found to have dysplasia, 9 were diagnosed as CIS, and 6 were confirmed to have invasive carcinoma. Of the 96 sites that underwent spectroscopic analysis, 44 were histologically classified as healthy mucosa, 3 as hyperplasia, 5 as moderate dysplasia, 1 as severe dysplasia, 26 as CIS, and 17 as carcinoma. No specimens were categorized as papilloma or mild dysplasia. Because of the small sample of hyperkeratotic and dysplastic lesions, those with hyperkerato-
keratosis were included in the healthy group and all grades of dysplasia were pooled, which left 4 histopathologic groups (healthy epithelium, dysplasia, CIS, and carcinoma). The cross-sectional depth of the tissue differed among the groups. Epithelium depth varied from 30 to 100 µm in normal specimens, 120 to 200 µm in dysplastic specimens, 100-1100 µm in CIS specimens, and 100 to 1500 µm in carcinoma specimens.

**FLUORESCENCE SPECTROSCOPY**

Both steady-state spectra and time-resolved emission features were found to be useful in distinguishing healthy tissue, dysplasia, CIS, and carcinoma. Typical fluorescence impulse response functions for each type of lesion are depicted in Figure 2. The fluorescence decay dynamic was different for healthy epithelium cells, dysplasia, CIS, and carcinoma along the emission spectrum.

**Figure 2.** Representative of fluorescence impulse response functions. A, Healthy epithelium cells; B, dysplasia; C, carcinoma in situ; and D, carcinoma. AU indicates arbitrary unit; nm, nanometers; and ns, nanoseconds.

**Figure 3A** shows normalized integrated fluorescence emission spectra of the healthy cheek pouch mucosa, dysplasia, CIS, and carcinoma. Well-defined peaks are noted at 380, 460, and 635 nm. These peaks are known to correspond to collagen (peak, 380-390 nm), nicotinamide adenine dinucleotide (NADH) (460 nm), and porphyrin (633 nm) fluorescence emission, respectively. The normal mucosa presents a main peak at 380 nm with a smaller peak at 460 nm. However, as carcinogenesis ensues, the peak significantly diminishes at 380 nm and the 460-nm peak becomes more prominent. Carcinoma displays a sharp peak at 635 nm, which is less pronounced in CIS and dysplasia. Figure 3B depicts the intensity ratio at 2 SBs, I_{460}/I_{635} and I_{460}/I_{660}, for each tissue type. This ratio demonstrates that porphyrin fluorescence intensity increases with disease progression (healthy: I_{460}/I_{635} = 0.065 [0.00]; dysplasia: 0.16 [0.01]; CIS: 0.39 [0.08]; and carcinoma: 0.66 [0.13]), and the intensity values at these SBs allow for the discrimination of healthy from diseased tissue and the staging of disease.

The time-resolved emission features of carcinoma were found to be distinct from those of the healthy mucosa, dysplasia, and CIS. The τ values (lifetime) for each tissue type are depicted in Figure 3C. The τ values at the 3 SBs associated with the fluorescence peak emissions support further that the fluorescence of normal and diseased tissue investigated in this study corresponds also to collagen, NADH, and porphyrins. Reported τ values for collagen, NADH, and porphyrins are 0.4 to 2.4, 0.4, and 9 to 18 nanoseconds, respectively. Significant differences were seen among each tissue type in the fluorescence lifetimes at 460 nm (Figure 3D). The mean (SE) lifetimes were 1.44 (0.01)
Figure 3. Spectral intensities and time-domain measurements of the spectral bands. A, Fluorescence intensity spectra values of healthy cheek mucosa (NOR), dysplasia (DYS), carcinoma in situ (CIS), and carcinoma (CA). B, Ratio of fluorescence intensities at 635-nm and 460-nm spectral bands (\(\text{ISB}_{635}/\text{ISB}_{460}\)) for each tissue type. C, Lifetime values for each tissue type. D, Average lifetime values at the 460-nm spectral band (\(\tau_{460}\)) for each tissue type. E, Laguerre expansion coefficient, zero order (LEC-0) for each tissue type. F, LEC-0 at the 460-nm spectral band (\(\text{LEC}_{0}\)) for each tissue type. AU indicates arbitrary unit. Results are presented as mean (SE) of the data from each independent measurement.
nanoseconds for healthy mucosa, 1.25 (0.02) nanoseconds for dysplasia, 1.28 (0.01) nanoseconds for CIS, and 1.34 (0.02) nanoseconds for carcinoma. Varying lifetimes between healthy tissue and carcinoma were also noted at 380 and 635 nm. However, among all tissue types, the lifetime differences at these wavelengths did not reach statistical significance. Laguerre coefficients (zero order) of normal cheek mucosa, dysplasia, CIS, and carcinoma are portrayed in Figure 3E. The values were noted to be significant at 460 nm (Figure 3F). Laguerre coefficients (first, second, and third order) were also determined (data not shown).

Stepwise linear discriminant analysis was then used to determine which combination of predictor variables provided the most clinically useful information. The classification accuracy (sensitivity and specificity values) is summarized in the Table. On the basis of data derived from the steady-state spectroscopy (peak intensity ratios) only, the sensitivity and specificity for discrimination of healthy from diseased tissue are high at 97.9% and 100%, respectively. However, the classification accuracy for the staging of the disease remains poor (eg, sensitivity of 23% for CIS and 47% for carcinoma). When only fluorescence lifetime data were used, the sensitivity for the CIS (39%) and carcinoma (71%) groups improved. Classification on the basis of a combination of spectral-domain and time-domain parameters (peak intensity ratios, lifetimes, and Laguerre coefficients zero order) resulted in significant improvement of both sensitivity (eg, 69% for CIS and 77% for carcinoma) and specificity for all tissue groups.

### Table. Sensitivity, Specificity, and Accuracy of Diagnostic Algorithm

<table>
<thead>
<tr>
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<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Overall cross-validation classification performance, %</th>
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<tr>
<td></td>
<td>Spectral Ratio Only</td>
<td>Average Lifetime Only</td>
<td>Spectral Ratio, Average Lifetime, and Laguerre Coefficient (Zero Order)</td>
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<td>Healthy tissue</td>
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<tr>
<td>Carcinoma</td>
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<td>Healthy tissue</td>
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<td>76.0</td>
<td>79.2</td>
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Abbreviations: ISB(380), intensity at spectral band 380 nm; $\tau_{380(380)}$, lifetime at spectral band 380 nm; LO$_{380(380)}$, zero order Laguerre expansion coefficient at spectral band 380 nm; nm, nanometers.

In this study, both spectral-domain and time-domain fluorescence features were helpful in distinguishing healthy from neoplastic tissue. Use of these combined features, along with the Laguerre coefficients derived from the numerical deconvolution of the fluorescence impulse function, significantly improved the diagnostic potential of laser-induced fluorescence spectroscopy in the hamster model. Fluorescence spectroscopy in the hamster cheek pouch model has been studied previously. However, this was the first study, to our knowledge, that used the hamster pouch as a model for evaluation of the potential of the time-resolved fluorescence technique to discriminate among distinct pathologic stages in oral carcinoma. Previous studies focused solely on spectrally resolved measurements. Balasubramanian et al evaluated ex vivo, minced cheek pouch tissue treated for 16 weeks with DMBA and compared it with control tissue. They found a significant fluorescent peak at 630 nm in the tissue treated with DMBA. Another ex vivo study showed significant differences in fluorescence at both 380 and 460 nm. Dhingra et al performed in vivo measurements and noted a difference in fluorescence between normal and neoplastic lesions at a peak centered between 630 and 640 nm. Additional in vivo studies were able to distinguish healthy epithelium from precancerous and cancerous tissue. However, these groups were unable to successfully delineate the various stages of carcinogenesis. Wang et al used partial least-squares and logistic regression and had better success in distinguishing the stages of carcinogenesis. In our study, the addition of time-resolved data and the Laguerre coefficients to the spectral intensities significantly improved our ability to discriminate precancerous and cancerous lesions.

Fluorescence spectroscopy as a diagnostic instrument has been evaluated in several anatomical sites, including the colon, cervix, esophagus, and upper aerodigestive tract. Gillenwater et al have extensive research using fluorescence spectroscopy in evaluation of the oral cavity. Early results from their group showed excellent discrimination between healthy and diseased tissue but revealed the limitations of spectrally resolved data in discrimination of precancerous from cancerous lesions. Recently, Mallia et al found promising results in distin-
guishing carcinoma from dysplasia and hyperplasia by comparing spectral intensity ratios at 500 and 685 nm. Müller et al10 were able to distinguish cancerous and dysplastic lesions from healthy epithelium with 96% sensitivity and 96% specificity and discriminate cancerous from dysplastic tissue with 64% sensitivity and 90% specificity by means of trimodal spectroscopy. Trimodal spectroscopy combines intrinsic fluorescence, diffuse reflectance, and light-scattering spectroscopy. Lastly, by means of time-resolved fluorescence spectroscopy at 633 nm, Chen et al11 used time-resolved parameters and could diagnose dysplasia with 93% accuracy and hyperplasia with 75% accuracy.

The discriminant analysis used in this study can distinguish healthy from diseased epithelial cells with 100% sensitivity and specificity. However, lower classification accuracy was encountered when distinguishing among the various stages of carcinogenesis. A few limitations in this study may account for these challenges. Only a small number of preneoplastic lesions were evaluated. To take multiple measurements on several health-appearing locations of a DMBA-treated pouch would increase the number of dysplastic specimens and possibly improve the ability to distinguish carcinoma from dysplasia. The diagnostic algorithm also revealed some overlap between carcinoma and CIS. This overlap could be attributed to the way the specimens were processed and read by the histopathologist. Possibly, some of the histologic slides evaluated did not include the actual portion of tumor that contained invasion of the basement membrane, and the histologist unknowingly erred on the side of underscoring the lesions. Future studies could address these limitations. Nevertheless, the use of the time-resolved parameters in the classification model improved the overall classification accuracy from approximately 69% (spectral parameters only) to approximately 87% (spectral and temporal characteristics combined).

The addition of the time-resolved data increased the specificity of the fluorescence measurements, which allowed for greater than 92% specificity across all histopathologic groups. Also, the lifetime fluorescence measurements contribute to a better understanding of the tissue morphologic features and molecular makeup at each stage of carcinogenesis. During carcinogenesis, changes in metabolic activity (NADH concentration and protein binding), stroma (collagen cross-linking), and neovascularization (porphyrin) can be detected with variations in tissue autofluorescence.

These findings highlight the importance of time-resolved fluorescence data in improvement of the diagnostic potential of laser-induced fluorescence spectroscopy. Studies are currently under way to assess the validity of this algorithm in patients with head and neck cancer. Further development of this technique could lead to a noninvasive diagnostic technique for head and neck squamous cell carcinoma and assist surgeons intraoperatively when evaluating surgical margins.

Submitted for Publication: March 3, 2009; final revision received June 8, 2009; accepted September 13, 2009.

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Author Contributions: Dr Farwell had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Farwell, Meier, Enepekides, and Marcu. Acquisition of data: Farwell, Park, Sun, Coffman, and Tinling. Analysis and interpretation of data: Farwell, Meier, Park, Sun, Poirier, and Phipps. Drafting of the manuscript: Farwell, Meier, Park, and Coffman. Critical revision of the manuscript for important intellectual content: Farwell, Meier, Park, Sun, Poirier, Phipps, Tinling, Enepekides, and Marcu. Statistical analysis: Meier, Park, and Sun. Obtained funding: Farwell. Administrative, technical, and material support: Farwell, Meier, Sun, Coffman, Poirier, and Tinling. Study supervision: Farwell, Enepekides, and Marcu.

Financial Disclosure: None reported.

Funding/Support: This work was supported in part by National Institutes of Health grants R01-HL67377 and U1.I RR024146 and by the NSF Center for Biophotonics Science and Technology and the Cancer Center at University of California, Davis.

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