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Objective Detection of Oral Carcinoma with Multispectral Fluorescence Lifetime Imaging *In Vivo*

Bilal H. Malik^{1,2,*†}, Joohyung Lee^{3,†}, Shuna Cheng¹, Rodrigo Cuenca¹, Joey M. Jabbour¹, Yi-Shing Lisa Cheng⁴, John Wright⁴, Beena Ahmed⁵, Kristen C. Maitland¹, and Javier A. Jo¹

¹Department of Biomedical Engineering, Texas A&M University, College Station, TX 77843 USA

²QT Ultrasound Labs, Novato, CA 94949 USA

³Department of Electrical and Computer Engineering, Texas A&M University, College Station, TX 77843 USA

⁴Department of Diagnostic Sciences, Texas A&M Health Science Center – Baylor College of Dentistry, Dallas, TX 75246 USA

⁵Department of Electrical and Computer Engineering, Texas A&M University at Qatar, Doha, Qatar

Abstract

Successful early detection and demarcation of oral carcinoma can greatly impact the associated morbidity and mortality rates. Current methods for detection of oral cancer include comprehensive visual examination of the oral cavity, typically followed by tissue biopsy. A noninvasive means to guide the clinician in making a more objective and informed decision towards tissue biopsy can potentially improve the diagnostic yield of this process. To this end, we investigate the potential of fluorescence lifetime imaging (FLIM) for objective detection of oral carcinoma in the hamster cheek pouch model of oral carcinogenesis *in vivo*. We report that systematically selected FLIM features can differentiate between low-risk (normal, benign and low-grade dysplasia) and high-risk (high-grade dysplasia and cancer) oral lesions with sensitivity and specificity of 87.26 % and 93.96 %, respectively. We also show the ability of FLIM to generate ‘disease’ maps of the tissue which can be used to evaluate relative risk of neoplasia. The results demonstrate the potential of multispectral FLIM with objective image analysis as a noninvasive tool to guide comprehensive oral examination.

Introduction

Autofluorescence imaging has been widely used for rapid and non-invasive evaluation of potentially malignant oral lesions, and a number of such devices are now commercially available (1). While these devices have shown early optimism in their ability to detect potentially malignant lesions in selected clinical settings, most available devices to date have

*Corresponding author: bilal.malik@qtultrasound.com (Bilal H. Malik).

†These authors contributed equally to this work.

not been able to provide definitive evidence to suggest any improvement in sensitivity and specificity over conventional oral screening methods (2-6). Most of such autofluorescence imaging devices rely on changes and variations in the steady-state fluorescence signal, and, therefore, are limited in their diagnostic power due to intensity artifacts such as overlapping emission spectra of endogenous fluorophores, variation in excitation intensity and probe-to-tissue distance. Alternatively, fluorescence lifetime imaging (FLIM) is less sensitive to such artifacts: it can resolve signal from fluorophores with overlapping spectra and is less sensitive to variation in excitation intensity and photobleaching (7). In addition, FLIM of endogenous markers also provides a relative measurement of biochemical makeup and changes in the biological tissues, which are one of earliest hallmarks of precancer and cancer progression (8-11).

It is well studied and understood that the metabolic demand of cells is increased in the premalignant and malignant stages (12). This change in metabolism manifests as shifts in the electron transport chain and the citric acid cycle, in turn modulating the levels of cofactors such as the reduced form of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD). Both NADH and FAD are considered as the primary contributors to epithelial autofluorescence. In this manner, the changes in epithelial cell and tissue autofluorescence are correlated with malignant transformation (1, 13, 14). In addition to NADH and FAD, the collagen cross-links within the stromal region underlying the epithelium are also a major source of fluorescence (1, 15, 16). The path to premalignancy and malignancy is usually associated with a decrease in stromal fluorescence due to both degradation of collagen and thickening of the epithelium, making it a useful biomarker towards detection of precancerous lesions (17). However, the emission spectrum of collagen has a substantial overlap with that of NADH and, to some extent, FAD, and can therefore significantly confound the epithelial fluorescence. Time-resolved measurement of emitted light, as mentioned above, can differentiate between these sources of autofluorescence on the basis of their fluorescence lifetimes, thereby providing a contrast mechanism typically unavailable through steady-state measurements (7).

Despite the aforementioned advantages of FLIM, its application to imaging of oral cancer *in vivo* has been somewhat limited and its ability to distinguish potentially malignant lesions has not yet been fully established. More importantly, most of the embodiments had a relatively smaller field of view (FOV) making it unfeasible to examine large areas of tissue typically required for practical clinical application of this technique (18-20). Sun et al. have demonstrated application of FLIM *in vivo* by developing a compact and clinically compatible imaging system (18, 21). They presented the design of a FLIM module for intraoperative disease diagnosis and validated it in a hamster model of oral carcinogenesis *in vivo*, showing the ability of FLIM to determine normal and tumor regions on a tissue (18). More recently, they demonstrated the application of wide-field FLIM endoscopic probe for intraoperative image-guided cancer demarcation, and noted that tissue differentiation based on time-resolved information was more significant in comparison to that of fluorescence intensity (21). In both instances, FLIM imaging was performed with a 4 mm FOV but the acquisition times were relatively long (~ 120 seconds for a single wavelength emission band). Other embodiments of FLIM include imaging systems based on multi-photon microscopy (MPM) (11, 20), as well as using FLIM in conjunction with other

complementary imaging modalities, such as ultrasound backscatter microscopy, confocal microscopy, optical coherence tomography and photoacoustic tomography (19, 22-24). Our group has reported multispectral FLIM imaging at FOV as large as 16×16 mm with acquisition time of less than 20 seconds (400×400 pixels), and as fast as less than 1 second across three wavelength emission bands while maintaining practical FOVs (6.5 – 13 mm) in both benchtop and endoscopic implementations (22, 25).

Here, we report objective detection of potentially malignant lesions in the hamster cheek pouch model of oral carcinogenesis *in vivo* using FLIM at practical spatial resolution and acquisition speed, and demonstrate the ability of this technique to distinguish benign and low-grade precancerous lesions from high-grade precancer and carcinoma, while maintaining adequate sensitivity and specificity.

Materials and Methods

Tissue preparation and imaging

The experiment was performed using 12 Syrian (Golden) hamsters (male, aged 5-6 weeks, Harlan Labs). The animal use protocol was reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC), and a veterinarian managed the clinical aspects of this study. We utilized the hamster cheek pouch model of oral carcinogenesis administered using 7, 12-dimethylbenzene[a]anthracene (DMBA) as the chemical carcinogen. This model is one of the most well characterized animal models of oral chemical carcinogenesis and resembles closely the development of human oral cancers (26). The right cheek pouch of all the animals was painted 3 times a week with 0.5% solution of DMBA (Sigma-Aldrich) dissolved in mineral oil (Sigma-Aldrich). The left cheek pouch was treated with mineral oil only and served as control. The treatment was performed for 8-12 weeks, generating oral lesions at different stages of carcinogenesis. At the time of imaging, the hamsters were anesthetized using 10% solution of urethane (Sigma-Aldrich) in de-ionized water. Once anesthetized, the hamster cheek pouch was pulled and braced using a custom- machined mount to maximize exposure of cheek pouch tissue. Throughout the imaging procedure, the anesthetized hamster was kept warm with a heating pad. Finally, the hamster was euthanized with a solution of pentobarbital. The cheek pouches were harvested, formalin fixed and processed for H&E histology.

Instrumentation and data processing

The hamster cheek pouches were imaged using a recently reported multispectral FLIM system (27). Briefly, a frequency-tripled Q-switched Nd:YAG laser (355 nm, 10 kHz repetition rate, 1 ns pulse width, Advanced Optical Technology) was used for excitation. A 50 μ m core diameter multimode fiber was using to deliver light from the laser to a pair of close-coupled galvanometer mirrors which allowed two-dimensional raster scanning. A UV-to-NIR corrected triplet with focal length of 45 mm and effective numerical aperture (NA) of 0.06 was used to focus light onto the cheek pouch tissue. The fluorescence emission light was separated from excitation using a dichroic mirror (Chroma Technology Corporation). The emission was further separated into three spectral bands corresponding to the peak emission wavelengths of collagen (390 ± 20 nm, Channel 1), NADH (450 ± 22.5 nm,

Channel 2) and FAD (>500 nm, Channel 3). The three decay profiles corresponding to each pixel were time multiplexed using three multimode fibers of 1, 13 and 25 meters, providing a time delay of approximately 60 ns between two consecutive spectral bands before being detected by a single micro-channel plate photomultiplier tube (Hamamatsu Corporation) followed by a high-speed digitizer (National Instruments). Digitization was performed at 6.25 GS/s resulting in temporal sampling at 160 ps. Note that the temporal resolution is determined by either the detector or the digitizer. In our system, the detector had a response time of 25 ps, and hence the temporal resolution of 320 ps was determined by the digitizer. The lateral resolution of the imaging system was characterized using a 1951 USAF target (Edmund Optics) and was observed to be 62.5 μm . The field of view set to be 16 $\times$ 16 mm sampled at 400 $\times$ 400 pixels per image. Image acquisition took $\sim$ 20 seconds. A single time series of images was acquired per cheek pouch. Note that high sensitivity detectors, such as PMTs, often result in impulse noise resulting in salt-and-pepper like appearance of the acquired image. Image smoothing with an average filter can reduce some of the resultant intensity variations while preserving most of the edges. Therefore, the acquired time series was smoothed using a spatial average filter of 3 $\times$ 3 kernel, which reduced the noise within the images. This consequently increased the signal-to-noise ratio (SNR) of the time-resolved fluorescence signal per pixel without reducing the temporal resolution.

Laguerre FLIM Deconvolution

The intrinsic fluorescence decays at each pixel were estimated using previously reported Laguerre FLIM deconvolution algorithm (28). Typically, the series of time-gated fluorescence decay $y(n)$ is given by the convolution of impulse response function (IRF) $h(n)$ with the instrument response $x(n)$:

$$y(n)=T \cdot \sum_{m=0}^{K-1} h(m)x(n-m), n=0, \dots, N-1 \quad (1)$$

where T is the sampling interval, N is the number of measured samples, and K determines the time length of the IRF(29). The Laguerre deconvolution technique uses a set of discrete Laguerre functions (DLF) as an orthonormal basis to represent IRF:

$$h(n)=T \cdot \sum_{j=0}^{L-1} c_j^\alpha b_j^\alpha(n) \quad (2)$$

where c_j^α are the Laguerre Coefficients (LC), which are to be estimated from the input-output data, b_j^α denotes the j th order orthonormal DLF, and L is the number DLFs used to model IRF (29). The Laguerre parameter α determines the rate of asymptotic decline of the DLFs. The larger the α , the longer α DLF is spread over time. For a given pixel, the measured fluorescence decay $y(n)$ can thus be written as:

$$y(n) = \sum_{j=0}^{L-1} c_j^\alpha v_j^\alpha(n) \quad (3)$$

where,

$$v_j^\alpha(n) = \sum_{i=0}^{K-1} b_j^\alpha(m) x(n-m) \quad (4)$$

The decay profiles obtained by Laguerre analysis are then integrated to generate absolute fluorescence intensity maps corresponding to each wavelength band (I_1 , I_2 and I_3). The normalized intensities are calculated by $I_{1n} = I_1/(I_1 + I_2 + I_3)$, $I_{2n} = I_2/(I_1 + I_2 + I_3)$ and $I_{3n} = I_3/(I_1 + I_2 + I_3)$. Both the average fluorescence lifetime (t_1 , t_2 and t_3) and 1/e lifetime (τ_1 , τ_2 and τ_3) are calculated using their general mathematical definition (7). In addition, the first four orders of normalized Laguerre coefficients (LC1_i, LC2_i, LC3_i and LC4_i; i=1, 2, 3) for each of the channels are also used to generate two dimensional maps, wherein, each order corresponds to a component of the fluorescence decay dynamics as described above.

Histopathology analysis

Each cheek pouch was sectioned at five to seven different locations depending on the number of suspicious lesions within the tissue. The sections were processed and evaluated by oral pathologists (JMW and YLC). Based on the histopathological evaluation, the regions across tissue sections were classified as: normal, cellular atypia, hyperplasia, hyperkeratosis, low-grade dysplasia, high-grade dysplasia, and carcinoma. Sections from only seven out twelve treated hamsters presented with dysplasia and carcinoma, and were selected for further evaluation. Regions of interest from these sections were grouped as “normal”, “benign” including (cellular atypia, hyperplasia and hyperkeratosis), “low-grade dysplasia” and “high-grade precancer/cancer” (including high-grade dysplasia and carcinoma). Note that only the DMBA treated cheek pouches were used in the analysis below and the same cheek pouches were used to identify ‘normal’ tissue regions of interest (ROIs).

Statistical analysis

In order to build (training and cross-validation) a classifier, 65 ROIs were identified across the seven cheek pouch tissues. The number of ROIs per cheek pouch tissue varied from 7 to 13 depending on the variety of lesions presented within a single tissue. While ideally each pixel within the image would be labeled to one of the groups, this requirement is very difficult to meet since perfect co-registration between histopathology sections and FLIM maps is virtually impossible. This problem was somewhat avoided by treating an 11×11 pixel ($440 \times 440 \mu\text{m}$) block as one ROI and classified to belong to a single histopathological class. The size of ROIs was similar to the resolution of histology sectioning that could be performed with confidence on such large and heterogeneous tissue sections of varying

topologies. Further, distances on both the images and the respective tissue sections were measured and correlated with respect to the four edges.

The ability of the 21 FLIM features (I_{in} , t_i , τ_i , $LC1_i$, $LC2_i$, $LC3_i$, and $LC4_i$; $i=1, 2, 3$) to distinguish between the four histopathological classes was analyzed as follows: A Kruskal-Wallis test was first performed on each FLIM feature having all four tissue types as groups. When the null hypothesis was rejected (at a $P < 0.01$), then multiple comparisons between pairs of group was performed using the Mann-Whitney Rank-Sum test, and the Holm correction was applied to control the probability of false positive error accumulated in a sequence of test on the same dataset. FLIM features which showed insignificant differences were excluded from further analysis. The capacity of the remaining pertinent features was successively evaluated by Sequential Floating Forward Selection (SFFS) method. The reduced feature set then served as feature vector in K-Nearest-Neighbor (K-NN) algorithm for statistical classification. This algorithm identifies the K nearest training data points (measured in terms of Euclidean distance) from each test data point subject to classification. The data point is assigned to the majority class among K nearest training points. A leave-one-animal-out cross-validation strategy was adopted to evaluate the performance of the classification. This consisted of using all the ROIs except those from one animal to train the classifier. The predictive ability of this model was tested using the ROIs from the animal that was excluded from building the model. The procedure was repeated $n = 7$ times for all the hamsters, and the outcomes from all the iterations were pooled together to calculate the sensitivity, specificity and accuracy of the model. Finally, a 'disease map' was generated by classifying each pixel of an image to one of the histopathological groups.

Results

Histopathology analysis

Each of the 65 ROIs showing approximately uniform histopathological characteristics were classified as normal ($n=19$), benign ($n=17$), low-grade dysplasia ($n=12$) or high-grade dysplasia/carcinoma ($n=17$). The resulting database was used for feature selection, classification design and cross-validation for performance estimation. The remaining areas of the images were used to further validate the classifier and generate disease maps.

FLIM imaging

Figure 1 shows maps of the 21 FLIM features from a representative cheek pouch tissue diagnosed with high-grade dysplasia or cancer. A complete set of maps for each tissue consists of the normalized fluorescence intensity, average and $1/e$ fluorescence lifetimes, and first four orders of normalized Laguerre coefficients for each of the three emission channels. As seen in the normalized intensity maps, the channel 1 signal is usually strongest due to the contribution of collagen, the dominant fluorophore in the oral mucosa. While the peak emission wavelength of collagen is closer to 390 nm, there is still some signal leakage into the NADH and FAD channels due to the long red tail of collagen fluorescence emission (30). Therefore, the lifetime values in all three channels resemble those of collagen (lifetime: 5-6 ns). Nevertheless, the contribution of NADH in the second channel results in a decrease

of lifetime values. The maps of Laguerre coefficients provide an additional set of parameters that are correlated with the decay dynamics of the fluorophores.

Regions 1 and 2 marked in the channel 1 normalized intensity map were histopathologically interpreted as a hyperplastic lesion and a tumor, respectively. Comparing the two regions, significant thickening of the epithelial layer was noted in comparison to the benign lesion. This resulted in reduction of both excitation light reaching the lamina propria and the corresponding fluorescence emission reaching the top surface for detection, resulting in a decrease of collagen contribution to the fluorescence signal. The corresponding region in channel 3 intensity map shows a relative increase in the signal in comparison to the benign region, which can be attributed to increased FAD contribution due to higher metabolic demand of the tumor. Note that since the sum of all normalized intensities is equal to 1, reduction in total fluorescence contribution in one channel can result in an apparent increase in another channel or channels.

Statistical analysis and classification

The results from the statistical analysis are summarized in Figure 2. The Wilcoxon test results (only fluorescence intensity and lifetime results are shown here) suggested that only high-grade dysplasia/carcinoma (HG-dysplasia/carcinoma) could be distinguished consistently from other histopathological groups. At this point, the remaining classes (normal, benign and low-grade dysplasia) were pooled into one group namely “No or Low-Grade Dysplasia” (N/LG-dysplasia).

Once the two classes were established, the Wilcoxon test was again performed to identify the parameters which could distinguish between N/LG-dysplasia and HG-dysplasia/carcinoma. The features which were able to differentiate with $p < 0.01$ were retained for further evaluation. Fifteen of the features passed this criterion: I_{1n} , I_{3n} , t_1 , t_2 , τ_1 , τ_2 , τ_3 , $LC1_2$, $LC1_3$, $LC2_1$, $LC2_2$, $LC3_2$, $LC3_3$, $LC4_1$, and $LC4_2$. This feature set was subjected to SFFS in combination with K-NN analysis. Based on a given criterion, the SFFS method can iteratively select the smallest subset of features which can still provide sufficient accuracy in classification. For the purpose of K-NN analysis, we tested multiple values of K (= 3, 5 and 7). Even values of K were not included since those can result in a tie. As K decreases, the performance can shift towards higher sensitivity due to over-fitting of data. For instance, K=1 estimates the probability based on a single sample, and hence can be sensitive to bias due to noise and outliers in the data. For this reason, K=1 was also excluded from the analysis. On the other hand, higher K values tend to lead to higher specificity at the expense of sensitivity. When optimizing for lowest error rate, classifiers with different K values showed similar error rates, and specificity in all instances was higher than 90%; however, sensitivity varied from ~73% to ~87% for different combination of K-values and number of FLIM features. Highest sensitivity was achieved with K=3 with two FLIM features. Specifically, the feature subset providing the least error rate in differentiation of N/LG-dysplasia and HG-dysplasia/carcinoma included normalized fluorescence intensity in the FAD channel (I_{3n}) and 1/e lifetime in the NADH channel (τ_2), identified in first and second iterations of the SFFS method. The 3-NN analysis after the second iteration provided a correct prediction rate (=100 – error rate) of 92.2 % with corresponding sensitivity and

specificity of 87.26 % and 93.96%. Additional iterations did not provide any significant improvement in the predictive values and hence only two features were selected for further validation.

Figure 3 shows the receiver operating characteristic (ROC) curve corresponding to 3-NN data analysis. Points on the ROC curve were determined by varying the threshold. When using k-Nearest Neighbor method, adjusting the classification decision rule can vary the threshold. When assigning the classification for each test datum, which represents the ROI that needs to be classified by the classifier, each test datum will have three nearest training data points-training ROIs. These training ROIs can have four different classification distributions: when three nearest neighbors, that would belong to either N/LG-dysplasia (N/LG) or HG-dysplasia/carcinoma (HG/C), consist of (i) 0 'HG/C' and 3 'N/LG', 2) 1 'HG/C' and 2 'N/LG', 3) 2 'HG/C' and 1 'N/LG', and (iv) 3 'HG/C' and 0 'N/LG'. Rule (i) would result in the highest specificity (specificity=1, sensitivity=0) whereas rule (v) would show the highest sensitivity (sensitivity=1, specificity=0). The area under this ROC curve was calculated to be 0.918.

Disease maps

The 3-NN classifier developed above was then used to classify the full cheek pouch tissues. Note that this model utilizes two features: normalized fluorescence intensity in the FAD channel (I_{3n}) and 1/e lifetime in the NADH channel (τ_2). An example of this classification is shown in Figure 4 along with the actual photograph of the tissue. The white and black colored areas across the map correspond to regions classified as N/LG-Dysplasia and HG-Dysplasia/Carcinoma, respectively. The square boxes mark the different ROIs of this particular animal used in building the classifier. The histology section corresponding to the vertical dashed line in 4b is shown in Figure 4c. Note that ROIs marked by two black arrows on Figure 4b correspond to N/LG-dysplasia category as identified by the initial histopathological classification. A section of the tissue between these two ROIs (marked by a red arrow) was categorized as HG-dysplasia/carcinoma by the classifier. Subsequent location specific histopathological analysis of that region, shown in Figure 4c and 4d, confirmed it as high-grade dysplasia. More importantly, the location of this lesion was not as evident by visual inspection (as can be observed in the corresponding photograph), in comparison to areas classified as HG-dysplasia/carcinoma, demonstrating the ability of the FLIM features to distinguish between the two different classes. Note that the classifier used not only the 11 ROIs from the specific hamster cheek pouch (shown in Figure 4) but the tissue was classified based on all 65 ROIs from images from seven hamster cheek pouches using leave-one-animal-out validation.

Discussion

The primary sources of fluorescence in the oral tissue are collagen in the connective tissue, and NADH and FAD in the epithelial layer, and the change in relative contributions from these biomarkers is expected to correlate with progression of precancer and cancer. The multispectral FLIM system, which can simultaneously collect fluorescence signal in three spectral bands corresponding to the peak emission wavelengths of the aforementioned

endogenous markers, enables discrimination of benign and low-grade precancer from high-grade precancer and cancer. As reported in this study, while multispectral FLIM can generate a plethora of features related to biochemical contrast across the tissue, carefully selected fewer parameters can still provide a meaningful and objective method for detection of diseased regions of the tissue. In addition, fewer numbers of features are also computationally less expensive to process, and provide a mechanistic basis for rapid classification of large areas of tissue.

The assessment and grading of dysplasia is based on histopathologically observed architectural disturbances along with cytological atypia (31). Although epithelium is often thickened during dysplasia, high-grade dysplasia is occasionally accompanied by significant epithelial atrophy (32). We noticed this behavior in tissue from one of the hamsters wherein the thickness of the epithelium was similar to that observed in histopathologically normal tissue layers. Interestingly, the ROIs associated with the corresponding tissue section and histopathologically interpreted as high-grade dysplasia were consistently classified as false negatives. Typically, the relatively thicker epithelial layer in high-grade dysplasia reduced the amount of light both reaching the lamina propria and emitted by that region, thereby reducing the contribution of collagen fluorescence to the overall signal, a behavior consistent across most of ROIs with HG dysplasia/carcinoma. This indicates a limitation of our system (and classifier) towards detection of high-risk lesions – it is currently unable to detect severe dysplastic regions if the epithelium is atrophic. When the corresponding twelve ROIs were removed from the statistical analysis and classification, the correct prediction rate improved to 98.9 % with associated sensitivity and specificity of 97.7 % and 99.2 %, respectively, demonstrating enhancement in the classifier's performance.

As observed in the Results section, while the FLIM system allows measurement of fluorescence lifetimes in three distinct wavelength bands, the actual lifetime values in all three channels resemble those of collagen due to its emission across wide range of wavelengths. Moreover, a superficial keratinized layer is usually present in hamster cheek pouch tissue, and often present in human oral tissue from specific anatomic sites such as palate and gingiva, as well as in clinically apparent leukoplakia (1). This layer is composed of keratinized cells that typically have a fluorescence signature very similar to that of collagen in the underlying lamina propria (33, 34). Our current FLIM system lacks the ability to differentiate and isolate the source (layer) of fluorescence within the oral tissue due to which the signal from both collagen and keratin can confound the signal from NADH and, to some extent, FAD. To address this concern, we are working towards depth sensitive approaches to FLIM imaging which can potentially isolate the fluorescence signal collection to a specific depth in tissue and thereby discriminate the signals originating in the topmost keratin, the epithelium and the stroma. Such an arrangement may allow better characterization of fluorescence signal from NADH and FAD, and may also lead to demarcating more subtle differences between normal, benign and low-grade dysplastic lesions.

It is worth noting that while application of FLIM for detection of oral carcinoma has been reported to be able to detect oral cancer progression, such instances have been limited to case-by-case basis, i.e. only a few lesions representative of the disease have been used to

show quantitative differences (18, 22-24). Our group has previously demonstrated the ability of FLIM to differentiate between normal and disease regions of the oral mucosa *in vivo*, however such a comparison was strictly qualitative and no attempt was made towards tissue classification (13). In addition, any attempt at classification of such lesions has been mainly limited to show differences in normal and tumor regions only (21). While such differentiation can still be useful to the clinician, the ability to differentiate lesions before malignant transformation would have greater diagnostic value, since the differences at that level are clinically more subtle in nature and relatively more difficult to identify with visual examination. Our classification mechanism based on supervised learning results in slightly different grouping and can discriminate between the two groups: (1) normal, benign and low-grade dysplasia, and (2) high-grade dysplasia and cancer. The cross-validation method using leave-one-subject-out shows that variation across different individuals can be accounted for using such analysis. Nevertheless, we acknowledge that this study was performed in an animal model of oral carcinogenesis, and actual potential of this method should be tested in oral precancer/cancer of the human oral cavity. We would also like to point out that the ROIs considered to be 'normal' in our analysis were acquired from the tissues that were treated with DMBA. While these ROIs were histopathologically interpreted as normal tissue, the phenomenon of 'field cancerization' can potentially result in precancerous changes wherein epithelium would still have no unusual microscopic features (as identified by routine H&E histopathology) (35). Such early markers of field cancerization e.g. change in number and volume of nucleolar organizer regions and genetic variations, could affect the FLIM features that are sensitive to the fluorophore microenvironment. Consequently, this can potentially be a reason that our classifier was unable discriminate normal tissue from benign and early dysplastic lesions.

Conclusion

Our results demonstrate that multispectral FLIM has significant potential to noninvasively identify biochemical changes within the tissue *in vivo*, and should be further explored as a tool towards detection of oral precancer. Once calibrated, the FLIM parameters can generate a biochemical 'disease' map of the tissue which can be instantly recognized and used by the clinician in making a more informed and objective decision in designating an area of tissue for biopsy. In order to realize the clinical potential of FLIM, we have recently initiated an *in vivo* pilot study to examine lesions in the human oral cavity using a multispectral FLIM endoscope prototype, wherein we will investigate the efficacy of our classification technique in differentiating different types of oral lesions in patients.

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Figure 1.

Fluorescence lifetime imaging (FLIM) images of a representative hamster cheek pouch tissue. From top to bottom, the seven rows correspond to seven different sets of FLIM features across the three spectral channels. The histology sections correspond to Regions 1 (hyperplastic) and 2 (tumor with high-grade hyperplasia and carcinoma) in the Channel 1 intensity image.

Figure 2.

Results of the statistical analysis on the multispectral fluorescence lifetime imaging (FLIM) features (a) normalized intensity, (b) average fluorescence lifetime, and (c) 1/e fluorescence lifetime. The horizontal brackets with asterisks above the bars indicate significant difference among pairs ($p < 0.01$).

Figure 3. Receiver operating characteristic (ROC) curve corresponding to K-NN data analysis with $K=3$. The area under the curve was calculated to be 0.918.

Figure 4.

Sample biochemical 'disease' map of a hamster cheek pouch tissue sample showing different regions of histopathology. (a) Photo of the cheek pouch tissue sample, and (b) the corresponding binary classification map. The white and black colored areas across the map correspond to N/LG-Dysplasia and HG-Dysplasia/Carcinoma, respectively. The colored boxes across (b) represent the regions of interest (ROIs) from this tissue, where blue and red boxes correspond to 'N/LG-Dysplasia' and 'HG-Dysplasia/Carcinoma', respectively. Note that the two ROIs in (b) marked by black arrows were identified as low-grade dysplasia for training of the classifier. The area marked by red arrow in (b) corresponds to the region marked in (a) and was identified as HG-Dysplasia/Carcinoma by the classifier. The corresponding histopathological (c & d) evaluation confirmed this area as high-grade dysplasia. Scale bars: 2 mm in (a) and (b), 1 mm in (c), and 50 μ m in (d).