Multiphoton Microscopy of Endogenous Fluorescence Differențiates Normal, Precancerous, and Cancerous Squamous Epithelial Tissues

Melissa C. Skala,1 Jayne M. Squirrell,3 Kristin M. Vrotsos,1 Jens C. Eickhoff,2 Annette Gendron-Fitzpatrick,4 Kevin W. Eliceiri,3 and Nirmala Ramanujam1

Departments of 1Biomedical Engineering and 3Biostatistics and Medical Informatics, 2Laboratory for Optical and Computational Instrumentation, and 4Research Animal Resources Center, University of Wisconsin, Madison, Wisconsin

Abstract
This study characterizes the morphologic features and the endogenous fluorescence in the stratified squamous epithelia of the 7,12-dimethylbenz(a)anthracene-treated hamster cheek pouch model of carcinogenesis using multiphoton laser scanning microscopy (MPLSM). MPLSM allows high-resolution, three-dimensional image data to be collected deeper within thick tissue samples with reduced phototoxicity compared with single-photon imaging. Three-dimensional image stacks of normal (n = 13), precancerous (dysplasia, n = 12; carcinoma in situ, n = 9) and cancerous tissue (nonpapillary squamous cell carcinoma (SCC), n = 10, and papillary SCC, n = 7) sites in the hamster cheek pouch were collected in viable, unsectioned tissue biopsies at a two-photon excitation wavelength of 780 nm. Five features were quantified from the MPLSM images. These included nuclear density versus depth, keratin layer thickness, epithelial thickness, and the fluorescence per voxel in the keratin and epithelial layers. Statistically significant differences in all five features were found between normal and both precancerous and cancerous tissues. The only exception to this was a lack of statistically significant differences in the keratin fluorescence between normal tissues and papillary SCCs. Statistically significant differences were also observed in the epithelial thickness of dysplasia and carcinoma in situ, and in the keratin layer thickness of dysplasia and SCCs (both nonpapillary and papillary). This work clearly shows that three-dimensional images from MPLSM of endogenous tissue fluorescence can effectively distinguish between normal, precancerous, and cancerous epithelial tissues. This study provides the groundwork for further exploration into the application of multiphoton fluorescence endoscopy in a clinical setting. (Cancer Res 2005; 65(4): 1180-6)

Introduction
Over half of all human cancers occur in stratified squamous epithelia. Approximately 1 million cases of nonmelanoma cancers of the stratified squamous epithelia are identified each year (1). Tissues with stratified squamous epithelia include the cervix, skin, and oral cavity. Such tissues consist of a surface layer, the epithelium (several cell layers thick), a basement membrane, and an underlying stroma (containing structural proteins and blood vessels). Neoplastic cells originate near the basement membrane and can move progressively upward through the epithelium (2). These cells can eventually occupy the full thickness of the epithelium and ultimately break through the basement membrane and invade the stroma. Currently, the diagnosis of squamous epithelial cancers is carried out through visual inspection, followed by biopsy. In patients at high risk for malignancy, the entire epithelium may potentially be diseased. Therefore, it is difficult to identify the best location to biopsy based on visual inspection alone. Techniques that can diagnose epithelial precancers and cancers more accurately than visual inspection alone are needed to guide tissue biopsy.

Multiphoton laser scanning microscopy (MPLSM) is a potentially attractive technique for the diagnosis of epithelial precancers and cancers. This technology can noninvasively generate high-resolution, three-dimensional fluorescence images deep within tissue while maintaining tissue viability (3–6). This technique enables the visualization of cellular and subcellular structures with exceptional resolution. Visualization of these structures is important because it is well known that the development of precancers and cancers is accompanied by changes in cellular and subcellular morphology (7). MPLSM can also exploit the intrinsic fluorescence contrast of molecules already present in tissue, thus obviating the need for exogenous contrast agents. It has been previously shown that the endogenous fluorescence of certain molecules, such as reduced nicotinamide adenine dinucleotide (NADH) within tissue is altered with precancer (8–10) and that these sources of intrinsic fluorescence contrast can be exploited for the early detection of epithelial precancers and cancers.

MPLSM has been used to image the endogenous fluorescence in tissues in several feasibility studies (6, 11–13). These collective studies show that MPLSM can image the endogenous fluorescence deep within thick tissues and that qualitative morphologic differences can be observed between malignant and nonmalignant tissues. In a more recent study (6), MPLSM was used to image the endogenous fluorescence within the stroma of the normal, precancerous, and cancerous hamster cheek pouch model in vivo. Images were obtained from a total of five sites per animal in a total of 70 animals. The diagnosis of tissues was based on a blinded observer evaluation of the morphologic features resolved with MPLSM (collagen matrix and fibers, cellular infiltrates, and blood vessels). The blinded observer evaluation agreed with the gold standard, histopathology for 88.6% of the samples.

The study reported in this article characterizes the morphologic features and endogenous fluorescence in the stratified squamous epithelia of the 7,12-dimethylbenz(a)anthracene (DMBA)-treated hamster cheek pouch model of carcinogenesis using MPLSM. Three-dimensional image stacks of viable tissue biopsies were

Requests for reprints: Nirmala Ramanujam, University of Wisconsin, Madison, 2144 Engineering Centers Building, 1550 Engineering Drive, Madison, WI 53706. Phone: 608-265-8267; Fax: 608-263-9239; E-mail: nimmi@engr.wisc.edu. ©2005 American Association for Cancer Research.
buffered formalin and submitted for histopathology. The samples were kept moist and nourished at a physiologic temperature. All imaging was completed within 7 hours after biopsy. This tissue culture medium served to approximate an in vivo environment by keeping the tissue moist and nourished at a physiologic level.

Materials and Methods
Sample Preparation
Fifteen male Golden Syrian hamsters (152 ± 14 g) were examined in this study. Animal care and procedures were in accordance with the guidelines in the U.S. Department of Health and Human Services and NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Wisconsin. The DMBA-treated hamster cheek pouch model was selected for this study because it has been shown to mimic the dysplasia-carcinoma sequence in the human oral cavity (7, 19, 20) and different stages of epithelial precancer because it has been shown to mimic the dysplasia-carcinoma sequence in vivo (CIS) and cancerous tissues (nonpapillary and papillary squamous cell carcinoma (SCC)). The only exception to this was that the lack of statistically significant differences in the keratin fluorescence between normal tissues and papillary SCCs. Statistically significant differences were also observed in the epithelial thickness of dysplasia and CIS, and in the keratin thickness of dysplasia and SCCs (both nonpapillary and papillary).

Imaging Instrumentation and Protocol
The optical workstation at the Laboratory for Optical and Computational Instrumentation at the University of Wisconsin was used for MPLSM imaging (21). The two-photon excitation source was a titanium sapphire laser (Spectra Physics (Mountain View, CA) Tsunami pumped by 5W Millennia), and a photomultiplier tube (Thorn-EMI-9924B) was used as the detector. The excitation light was delivered to and the emitted light was collected from the sample through an inverted Nikon Eclipse microscope (40× oil-immersion lens, numerical aperture of 1.3, working distance of 0.2 mm) coupled to a Bio-Rad (Hercules, CA) confocal scan head (21). The lateral and axial resolution of the microscope was 0.35 and 1.25 μm, respectively. The diameter of the focused spot size on the sample was calculated to be 0.80 μm. The average and peak powers of the titanium sapphire laser were monitored by tapping a fraction of the beam with a microscope slide into both linear and nonlinear detectors. Infrared-sensitive silicon diodes (Hamamatsu, Hamamatsu City, Japan, S2386-4K) were used to monitor the average power at the two-photon excitation wavelength and UV/visible-sensitive GaAsP diffusion diodes (Hamamatsu, G1116) measured two-photon-induced electron/hole pairs for two-photon excitation level comparison (21, 22). The average power, peak power, and the focused fluorescence incident on the sample were approximately 15 mW, 300 GW/cm², and 3.7 MW/cm², respectively. The image z stacks were generated using a 2 μm step size. The scan area at each image plane was 283 × 283 μm (512 × 512 pixels). The dwell time per pixel was 11.5 μs and the scan time per image plane was approximately 3 seconds.

A two-photon excitation wavelength of 780 nm was used for MPLSM imaging of the control and DMBA-treated hamster cheek pouch biopsies. A biopsy from the control cheek was imaged first, then biopsies from the DMBA-treated cheek were imaged, and finally a second biopsy from the control cheek was imaged. Each biopsy was placed (epithelium down) on a coverslip (which was part of a chamber that contained medium) and the tissue was not compressed at any point before or during the imaging. Within each biopsy, three z stacks were imaged. Each of the three z stacks was obtained from a different tissue region (arbitrarily chosen), as verified by the image position on an x-y stage motor. The first image of each z stack was taken above the epithelial tissue surface and then the remaining images of each z stack were recorded until the signal was completely attenuated and/or the working distance of the objective was reached.

Histopathology
Biopsies were placed in 10% buffered formalin, embedded in paraffin, cut as 5-μm transverse sections (containing the epithelial and stromal layers) and stained with H&E. Each H&E-stained tissue section was evaluated by a board-certified veterinary pathologist (AGF) and diagnosed based on established histologic criteria (23). Tissues were assigned to one of the following categories: normal, hyperplasia (increased thickness of epithelium), early dysplasia, moderate dysplasia, severe dysplasia, papillary hyperplasia/papilloma, carcinoma in situ (CIS), or nonpapillary/papillary squamous cell carcinoma (SCC). Dysplasia was characterized as precancerous change associated with atypical cell proliferation (24), CIS was a focal preinvasive carcinoma, and SCC was an invasive carcinoma of the surface epidermis (24). Papillary SCC was defined as a lesion that protruded from the mucosal surface, as identified by visual inspection, before pathologic diagnosis as SCC. The histologic diagnosis was determined from evaluation of multiple sections cut from each biopsy sample. If any of these tissue biopsies were found to have more than one disease state (e.g., severe dysplasia and SCC), then the most severe diagnosis was assigned to that biopsy. MPLSM images were obtained from a microscopic (283 × 283 μm) field of view of each tissue biopsy. Thus, it was difficult to precisely mark the image location for subsequent correlation with histopathology. The approach that was taken in this study design was to obtain MPLSM images from three nonadjacent sites on each biopsy sample and to then assign the histologic diagnosis for that biopsy to all three MPLSM images. Each MPLSM image was sorted by its histologic diagnosis before performing qualitative and quantitative analyses of the images. To more closely correlate MPLSM images to histologic diagnoses, the pathologist (AGF) and the investigator (MCS) evaluated MPLSM images for each biopsy simultaneously with histologic sections of the same biopsy. It was found that most images illustrated the histologic diagnosis assigned to them.

Quantification of Morphologic and Fluorescence Features
Thickness of the Keratin and Epithelial Layers. The thickness of the keratin layer was determined from the total number of image planes (at 2-μm separations) between the surface of the tissue and the bottom of the keratin layer (at which the epithelial cells were first visually identified and/or the diffuse keratin fluorescence was no longer present). The thickness of the epithelial layer was determined from the total number of image planes between the bottom of the keratin layer and the epithelial/stromal interface (at which collagen was first identified and/or the cellular fluorescence was no longer present). In a subset of the image stacks obtained from
precancerous and cancerous tissues, the epithelial/stromal interface could not be imaged due to the attenuation of the fluorescence signal or because the structures were beyond the working distance of the objective. In these samples, the maximum depth of the epithelial layer was defined as the image plane at which the average intensity (averaged over all pixels) was two fifths of the maximum average intensity of the image planes within the epithelium. The value of two fifths was selected empirically in order to include all the image planes in which morphologic features could be visually resolved.

**Nuclear Density Ratio.** The number of nuclei was counted in two image planes of each image stack using the ImageJ (NIH, available from http://rsb.info.nih.gov/ij) "Threshold" and "Analyze Particles" functions. The accuracy of this technique was verified by comparing it to the number of nuclei counted manually from six image planes from two normal biopsies. In the normal samples, the number of nuclei was counted in the first plane in the epithelium ($d_{0n}$) and in the last plane in the epithelium ($d_{1n}$). In the diseased samples, the number of nuclei was counted at the first plane in the epithelium ($d_{0d}$) and at a depth, $d_{1d} = d_{0d} + (d_{1n} - d_{0n})$, where $d_{1n}$ and $d_{0n}$ were obtained from a subsample image of a normal biopsy from the same animal, imaged at the beginning of the day. Thus, the depth separating $d_{0n}$ and $d_{1n}$ was the same as the depth separating $d_{0d}$ and $d_{1d}$. The nuclear density was calculated by dividing the total number of nuclei by the area of the image plane. The change in nuclear density with depth, which is referred to here as the nuclear density ratio was defined as the number of nuclei at depth $d_{1n}$ divided by the number of nuclei at depth $d_{0n}$ for the normal samples and as the number of nuclei at depth $d_{1d}$ divided by the number of nuclei at depth $d_{0d}$ for the diseased samples.

**Fluorescence Intensities of the Keratin and Epithelial Layers.** Fluorescence intensities originating from the surface keratin layer and from the epithelium were quantified using ImageJ. First, the fluorescence intensity from each voxel in the three-dimensional image stacks were normalized to a reference value related to the two-photon excitation peak power at the sample (measured using the nonlinear detector). Next, the average fluorescence per voxel was calculated by dividing the total fluorescence intensity integrated over the volume of interest by the total number of voxels in that volume, for the keratin and epithelial layers. The average stromal fluorescence was not calculated because collagen was not visually identified in most precancerous and cancerous tissues (due to the increased thickness of their epithelium compared with that in normal tissue).

**Statistical Analysis of Morphologic and Fluorescence Features**

Variance component analysis (25) was used to examine variability within the morphologic and fluorescence intensity features of subsample images (subsamples are the three areas imaged within a tissue biopsy sample). If significant subsample variability was found in these features, then the features obtained from the subsamples were analyzed separately. Otherwise, the features obtained from the subsamples were averaged for further analysis. The subsamples were averaged for all fluorescence intensity variables except for the fluorescence per voxel in the keratin layer for nonpapillary and papillary SCCs. Subsamples were not averaged for any of the morphologic variables. Statistical comparisons between normal tissues and each precancer and cancer tissue category, as well as comparisons between dysplasia versus CIS/nonpapillary SCC/papillary SCC, CIS versus nonpapillary SCC/papillary SCC, and nonpapillary SCC versus papillary SCC were done using nonparametric Wilcoxon rank sum tests (unpaired; ref. 26). For each significance test, a general two-sided significance level of 5% was applied. Significance levels were adjusted for multiple comparisons by Bonferroni correction. Statistical analyses were done using SAS software version 8.1 (SAS Institute Inc., Cary, NC).

**Results**

**Histopathology.** Of the 51 tissue biopsies imaged, 13 were diagnosed as normal, 12 were diagnosed with dysplasia (1 mild dysplasia, 7 moderate dysplasias and 4 severe dysplasias), 9 were diagnosed with CIS, and 17 were diagnosed with SCC, 7 of which were papillary SCC and 10 of which were nonpapillary SCC. Dysplastic samples were grouped into one category for further analysis due to the small number of samples in each category. Note that three images were collected per tissue biopsy, resulting in a total of 153 images from all 51 tissue biopsies.

**Viability Results.** To ensure that the tissue biopsies were viable during the imaging protocol, the viability of six normal tissue biopsies was verified after these biopsies had been subject to the full imaging protocol. The viability was verified with ethidium homodimer-1 (Molecular Probes, Eugene, OR). Ethidium homodimer-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (excitation and emission maxima of 495 and 635 nm, respectively). Ethidium homodimer-1 is excluded by the intact plasma membrane of live cells. Tissue biopsies were stained with ethidium homodimer-1 (40-minute incubation at a concentration of 700 μmol/L) at the end of the 7-hour imaging protocol, and then imaged immediately after incubation at 900 nm excitation. Figure 1 shows MPLSM images of tissue biopsies labeled with ethidium homodimer-1, following either (a) our experimental imaging protocol or (b) a 30-minute incubation in methanol to cause cell death. Both samples were imaged approximately 7 hours after biopsy under identical experimental conditions. Sample (a) was subject to the full imaging protocol before labeling. The lack of fluorescence observed from the ethidium homodimer-1 in tissue biopsy (a) verifies that the cell membranes are not damaged (27). However, the fluorescence observed from tissue biopsy (b), which was soaked in methanol before being stained with ethidium homodimer-1 verifies that the cells membranes are damaged. Thus, this staining procedure provided a way to assess whether the cell membranes in the hamster cheek pouch tissue biopsies were damaged during the 7-hour imaging protocol.

**Qualitative Differences between Normal and Neoplastic Epithelial Tissues Are Resolved with Multiphoton Laser Scanning Microscopy.** The focus of the first part of the study was to qualitatively determine whether MPLSM could resolve morphologic differences as well as differences in the fluorescence of normal, precancerous, and cancerous tissues. Figure 2 shows representative MPLSM images at a two-photon excitation wavelength of 780 nm of tissues diagnosed as normal (Fig. 2A), moderate dysplasia (Fig. 2B), CIS (Fig. 2C), nonpapillary SCC (Fig. 2D), and papillary SCC (Fig. 2E). The images in Fig. 2 were corrected for the two-photon excitation peak power at the sample; thus, the fluorescence intensities in Fig. 2A-E can be compared.

Morphologically, all normal samples were similar. In the representative normal sample shown in Fig. 2A, the surface keratin layer is characterized by bright, diffuse fluorescence. The underlying epithelium (starting at 10-μm depth) has several layers of cells containing dark nuclei surrounded by bright cytoplasmic fluorescence. The nuclear density (and the nuclear-to-cytoplasmic ratio) increases with depth in the epithelium. The stroma can be identified by the fluorescence/second-harmonic generation from fibrillar collagen below the basal cells (40-μm depth).

Unlike in normal tissue (Fig. 2A), the nuclear density in precancerous tissues (dysplasia, Fig. 2B, CIS (Fig. 2C) and cancerous tissues (nonpapillary SCC, Fig. 2D; papillary SCC,
Fig. 2E) varies minimally with depth. In addition, the precancerous and cancerous samples generally show increased keratin and epithelial layer thickness relative to normal tissues. These morphologic changes are consistent with those identified with the gold standard, histopathology (7). Keratin layer fluorescence is weak in precancerous tissues and nonuniform in cancerous tissues compared with that in normal tissues. Epithelial cellular fluorescence becomes progressively weaker and more perinuclear in precancerous and cancerous tissues when compared with normal tissues. White et al. found “perinuclear aggregates of mitochondria” with neoplastic progression using sectioned tissues from the hamster cheek pouch viewed under an electron microscope (7). This suggests that the perinuclear fluorescence seen in these images is likely due to NADH from perinuclear mitochondria.

**Morphologic and Fluorescence Intensity Differences between Normal and Neoplastic Epithelial Tissues Can Be Quantified Using Multiphoton Laser Scanning Microscopy.** Three of the morphologic features that were qualitatively found to differentiate normal and neoplastic tissues were quantified to determine whether these morphologic features were statistically different between normal and neoplastic tissues. The three morphologic variables that were quantified from the MPLSM images were changes in nuclear density with depth, keratin layer thickness, and epithelial layer thickness. The nuclear density ratio was used to describe the change in nuclear density with depth. A nuclear density ratio equal to one indicates no change in nuclear density with depth, a nuclear density ratio greater than one indicates a decrease in nuclear density with depth, and a nuclear density ratio less than one indicates an increase in nuclear density with depth. The average fluorescence per voxel of the keratin and epithelial layers was also quantified from the MPLSM images. For simplicity, these two variables will be referred to as keratin fluorescence and epithelial fluorescence, respectively.

Table 1 shows the average and SD of each of the morphologic and fluorescence intensity variables for normal, dysplastic, CIS, and SCC samples. The average nuclear density ratio for the normal samples is less than unity, indicating that their nuclear density increases with depth. The average nuclear density ratio of the dysplastic, CIS, nonpapillary SCC and papillary SCC samples are close to unity indicating that their nuclear density varies minimally with depth. The average and SD of the keratin and epithelial layer thicknesses in the normal samples is $16 \pm 4$ and $23 \pm 5 \mu m$, respectively, indicating that depth measurements of the keratin/epithelial layer interface and the epithelial/stromal layer interface were fairly consistent between normal samples. The average keratin thickness tends to increase as epithelial tissues progress from a normal to precancerous to cancerous state. The average epithelial thickness increases as tissue progresses from a normal to dysplastic to CIS state. The thickness of the epithelial layer in the SCCs is comparable to that in dysplastic tissues. The average keratin fluorescence is dramatically decreased in dysplasia and CIS, but not as low in the nonpapillary and papillary SCCs when compared with normal tissue. The average epithelial fluorescence is decreased in all the precancerous and cancerous tissues compared with that in normal tissue.

Table 1 presents the morphologic and fluorescence intensity variables which showed statistically significant differences (at least $P < 0.05$) between the normal, precancerous, and cancerous tissue categories based on unpaired Wilcoxon rank sum tests (26). All three morphologic variables show statistically significant differences between normal tissues and all precancerous and cancerous tissues. The fluorescence intensity variables also display statistically significant differences between normal tissues and all precancerous and cancerous tissues. However, no statistically significant differences are observed in the keratin fluorescence of normal tissues and papillary SCCs. Finally, statistically significant differences are observed in the epithelial thickness of dysplasia and CIS, and in the keratin thickness of dysplasia and both types of SCCs.

### Table 1. Quantitative variables for differentiating normal, precancerous, and cancerous epithelial tissues with MPLSM

<table>
<thead>
<tr>
<th>Morphologic variables</th>
<th>Keratin layer thickness ($\mu m$)</th>
<th>Epithelial thickness ($\mu m$)</th>
<th>Fluorescence intensity variables</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Keratin layer fluorescence (c.u.)</td>
</tr>
<tr>
<td>Normal</td>
<td>$0.51 \pm 0.13$ ($n = 39$)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>$16 \pm 4$ ($n = 39$)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>$23 \pm 5$ ($n = 39$)&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Dysplasia</td>
<td>$1.12 \pm 1.32$ ($n = 35$)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$22 \pm 9$ ($n = 35$)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$33 \pm 11$ ($n = 35$)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CIS</td>
<td>$0.95 \pm 0.48$ ($n = 25$)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$30 \pm 16$ ($n = 27$)&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>$51 \pm 23$ ($n = 25$)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nonpapillary SCC</td>
<td>$0.99 \pm 0.59$ ($n = 22$)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$38 \pm 19$ ($n = 28$)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>$35 \pm 14$ ($n = 22$)&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Papillary SCC</td>
<td>$0.82 \pm 0.12$ ($n = 7$)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$41 \pm 15$ ($n = 19$)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>$37 \pm 12$ ($n = 7$)&lt;sup&gt;bc&lt;/sup&gt;</td>
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**Note:** The average and SD of each of the morphologic and fluorescence intensity variables for normal, dysplastic, CIS, and SCC samples. The total number of images from which these parameters were extracted is indicated in parentheses and includes the three subsample images for each biopsy. Note that a subset of the subsample images had to be discarded due to sample and/or imaging artifacts. The actual epithelial thickness of the precancerous and cancerous samples were greater than those listed in the table because the epithelial/stromal interface could not be identified at or before the maximum imaging depth was reached in all of these samples. Within a column, values with different superscript letters are statistically different (at least $P < 0.05$) and values with the same superscript letter are statistically similar ($P > 0.05$). For example, in the keratin layer thickness column, dysplasia, CIS, nonpapillary SCC, and papillary SCC are statistically different from normal, and dysplasia is statistically different from nonpapillary SCC and papillary SCC. The number of data points included in the Wilcoxon tests differ from that reported in the table when subsample images were averaged before the statistical comparison (based on variance component analysis). In cases when subsamples were averaged, the number of data points for normal, dysplasia, CIS, nonpapillary SCC, and papillary SCC are $n = 13, 12, 9, 10$, and 5, respectively (two biopsies diagnosed with papillary SCC had no identifiable epithelium).

Abbreviation: c.u., calibrated units.
To assess whether the differences in fluorescence intensity described above could be ascribed to deterioration of the tissue sample over the course of the imaging period and/or the sample-handling protocol, statistical comparisons were made of the keratin and epithelial fluorescence between normal biopsies (from the same animal) imaged at the beginning and end of the imaging session using a Wilcoxon signed rank test (paired analysis; ref. 26). The fluorescence intensities of these samples were not different from one another ($P > 0.25$), indicating that the intensities measured from the normal tissue samples, on a given day, remained relatively constant over the course of the experiment (eliminating the possibility of sample deterioration) and between tissues obtained from the same animal (eliminating the possibility of variations in the sample handling protocol).

**Discussion**

This work shows that three-dimensional images from MPLSM of endogenous tissue fluorescence can effectively distinguish between normal, precancerous, and cancerous epithelial tissues. The high-resolution capability of MPLSM enabled the identification of three morphologic features (nuclear density ratio, keratin, and epithelial thickness), which showed statistically significant differences between normal and both precancerous and cancerous tissues. Moreover, statistically significant differences were observed in the keratin thickness of dysplasia and both types of SCCs and in the epithelial thickness of dysplasia and CIS. The lack of statistically significant differences in the epithelial thickness of dysplasia and SCCs may be because the imaging depth was limited by signal attenuation in the SCCs, thus underestimating the epithelial thickness in these samples. The statistically significant morphologic changes that accompany precancer and cancer development in the MPSLM images (Table 1) are similar to those identified with standard histopathology techniques (7, 23, 28, 29). However, unlike histopathologic techniques, MPLSM has the key advantage of not requiring physical sectioning of the tissue.

The complementary fluorescence intensity measurements achieved with MPLSM also show statistically significant differences between normal and neoplastic tissues. The quantitative analysis of fluorescence intensity differences was achieved by segregating the fluorescence contribution from individual layers within the epithelial tissue into an average fluorescence per voxel for the keratin and epithelial layers. The qualitative differences observed from the images were quantified in this manner to simply allow for a statistical analysis of these parameters. The fluorescence intensity variables displayed statistically significant differences between normal tissues and tissue with dysplasia, CIS, and nonpapillary SCC. There are also statistically significant differences in epithelial fluorescence of normal tissues and papillary SCCs. However, no statistically significant differences are observed in the keratin fluorescence of normal tissues and papillary SCCs. The lack of statistical differences in the keratin fluorescence between normal tissues and papillary SCCs may be explained by the differences in the keratin production in dysplasia, CIS, and SCC tissues as discussed below.

The average keratin fluorescence (Table 1) in dysplastic and CIS samples is less than that in normal tissues and also less than that in the SCC samples (papillary and nonpapillary). Dysplasia and CIS lesions most often consisted of less differentiated cells; differentiation in the squamous cells is toward keratinization (24). Thus, it is not surprising that there is a decrease in keratin fluorescence in tissues diagnosed with dysplasia or CIS compared with normal tissues. Tissues diagnosed with SCC, however, show exuberant desquamation of keratin over the uncontrolled growth of the squamous tissue (24). Thus, the SCC samples would be expected to show increased keratin fluorescence compared with tissues with dysplasia or CIS.

The primary endogenous fluorophore in the epithelium at a two-photon excitation wavelength of 780 nm is most likely NADH (16). Previous fluorescence microscopy studies of transverse slices of human cervical tissues carried out at an equivalent single-photon excitation wavelength support this statement (30). Specifically, the fluorescence of fresh cervical epithelial tissue slices was imaged before and after treatment with potassium cyanide. The perturbation resulted in an increased fluorescence from the cells within the epithelium. It is well known that the addition of potassium cyanide (which inhibits electron transport) will increase the fluorescence from NADH (31). Thus, the increase in the fluorescence of the cervical epithelial cells was attributed to NADH. In addition, the results of our study show a decrease in epithelial fluorescence with precancer and cancer, which suggests a decrease in the concentration of NADH. This conclusion is consistent with the results from a previous publication, which used a biochemical assay to show that the concentration of NADH decreases with oral cancer in the human oral cavity (32).

In the MPLSM experiments, the measured fluorescence per voxel is not only a function of the endogenous fluorescence but is also affected by the absorption and scattering in the tissue (33). Thus, differences in the fluorescence intensity for the subsurface epithelial layer between normal and neoplastic tissues may not necessarily be due to the endogenous fluorescence differences in that layer, but indirectly due to the effects of absorption/scattering of overlying layers. Tissue absorption is primarily due to hemoglobin and tissue scattering is primarily due to cellular organelles and extracellular structural proteins (16). The absorption and scattering properties of tissue can change with epithelial precancer and cancer, and thus modulate the intensity of the measured fluorescence from the epithelial layer. Thus, caution should be used in interpreting the differences observed in the epithelial fluorescence between normal and precancerous/cancerous tissues in this study.

The maximum imaging depth of the MPLSM is a function of several variables including the working distance of the objective, the absorption and scattering properties of the sample, the
excitation wavelength used, the incident laser power, and the detector efficiency. In our study, the goal was to evaluate the superficial epithelial layer in these tissues (because this is where neoplasia originates); thus, we used a high-numerical-aperture objective (which provides better resolution) at the expense of limited penetration depth (not being able to evaluate the stroma in the diseased samples). However, longer working distance objectives, water-immersion objectives, and higher incident power levels can be used to image deeper in these tissues, thus allowing for the evaluation of the basement membrane and the stroma in future studies.

In closing, the high resolution of MPLSM provides three important pieces of information. First, it provides information on the microscopic morphologic changes that occur in precancerous and cancerous tissue such as changes in nuclear density with depth, keratin, and epithelial thickness. Second, it not only provides insight into the changes in fluorescence intensity with precancer/cancer development but also where these intensity changes occur on a cellular level. All of this information can be used to assess whether a tissue is normal, precancer, or cancer. Moreover, MPLSM does not require tissue biopsy, sectioning, or staining and in the future could be applied in vivo in a clinical setting with multiphoton fluorescence endoscopy (34–39).

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