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ORAL
ONCOLOGY

In vivo imaging of oral neoplasia using a miniaturized fiber optic confocal reflectance microscope

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Summary The purpose of this study was to determine whether in vivo images of oral mucosa obtained with a fiber optic confocal reflectance microscope could be used to differentiate normal and neoplastic tissues. We imaged 20 oral sites in eight patients undergoing surgery for squamous cell carcinoma. Normal and abnormal areas within the oral cavity were identified clinically, and real-time videos of each site were obtained in vivo using a fiber optic confocal reflectance microscope. Following imaging, each site was biopsied and submitted for histopathologic examination. We identified distinct features, such as nuclear irregularity and spacing, which can be used to qualitatively differentiate between normal and abnormal tissue. Representative confocal images of normal, pre-neoplastic, and neoplastic oral tissue are presented. Previous work using much larger microscopes has demonstrated the ability of confocal reflectance microscopy to image cellular and tissue architecture in situ. New advances in technology have enabled miniaturization of imaging systems for in vivo use.
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Introduction

Early detection and accurate diagnosis of neoplastic changes could significantly reduce the morbidity and mortality associated with epithelial malignancies. Current techniques rely heavily on the ability of clinicians to recognize sometimes subtle changes in gross morphology and tissue architecture followed by surgical removal of tissue and pathologic assessment. However, premalignant or early malignant lesions can be clinically indistinguishable from more common benign lesions. Diagnostic delays often result because patients and clinicians are reluctant to perform the invasive biopsy currently needed to identify neoplastic changes. A technology that can quickly provide this pathologic information without surgical removal of tissue could improve the management of a number of epithelial malignancies, including cancers of the colon, bladder, skin, esophagus, and oral cavity. Confocal reflectance microscopy currently can provide real-time, non-invasive imaging of intact tissue, and cellular structure in vitro. The purpose of this study is to evaluate whether confocal reflectance microscopy could be used in vivo to aid diagnosis of one representative epithelial malignancy, oral carcinoma. Oral cancer is the fifth most common malignancy worldwide. Roughly 34,000 new cases and over 7500 deaths from this disease occur each year in the United States.[1]

Confocal microscopy (Fig. 1) allows high resolution imaging of intact tissue by selectively imaging light reflected from a specific depth within the tissue and rejecting light originating from tissue layers above and below that plane.[2] Confocal microscopy is a well established biological imaging technique for ex vivo specimens that has recently been applied to human tissue in vivo. In vivo confocal reflectance microscopy can provide images of tissue architecture and cellular morphology with resolution comparable to histology in real time, without tissue removal, sectioning, and staining. This is based on the principal that backscattering of light from cellular and tissue structures provides signal, and image contrast is generated by differences in refractive indexes of cellular components.[3–5] Resolution as low as 1 μm, field of view greater than 400 μm, and penetration depth as great as 500 μm have been achieved in confocal imaging of epithelial tissue.[6–12] Taking advantage of optical fibers,[13,14] miniaturization of optics,[15–17] and improved beam scanning mechanisms,[12,18] confocal microscopes are

becoming more clinically feasible. So far, examples of confocal images have been successful obtained in vivo from the skin,[3,19–21] eye,[22,23] cervix,[24,25] and oral cavity.[7,9,26,27]

Our preliminary studies carried out in vitro demonstrate the capability of confocal reflectance microscopy to identify neoplastic changes, based on morphologic features such as changes in nuclear size and density in oral cavity tissue biopsies.[28] While a few studies have reported in vivo images of the oral cavity of normal volunteers,[9,11] these previous studies of in vivo confocal reflectance imaging of the oral cavity have generally used large, bench-type microscope objectives. For confocal imaging, the objective lens must be in close proximity to the imaging site. Consequently, imaging has been limited to the most accessible areas of the mouth, such as the lip and tongue and is impractical for even pilot clinical trials. We have recently developed a fiber optic confocal reflectance microscope with a miniaturized objective lens, allowing access to mucosal surfaces like the deeper parts of the oral cavity, in a clinical setting.[15] The goals of this study are to obtain confocal reflectance images of normal and neoplastic sites throughout the oral cavity in vivo and to determine if these images can be used to identify neoplastic changes.

Materials and methods

In vivo confocal imaging system

Reflectance confocal images were obtained in vivo from mucosal sites within the oral cavity using a non-invasive, real-time, fiber optic confocal microscope.[15] A schematic diagram of the imaging system is shown in Figure 2 and photographs demonstrating how confocal images are acquired from a subject's tongue lesion with the system are shown in Figure 3. Light at 1064 nm wavelength is directed to the mucosal surface through a 30,000 optical fiber image guide and a complex, water immersion miniature objective lens, with an outer diameter of 7 mm.[29] The miniature objective lens has a magnification of 3.3 and a numerical aperture of 1.0. Raster scanning the laser beam across the fiber bundle allows point scanning in the focal plane in the tissue sample. In this configuration, each fiber acts as a confocal pinhole and reflectance signal is produced by refractive index differences in the tissue. This microscope generates en face reflectance confocal images at 15 frames/s. The measured axial resolu-
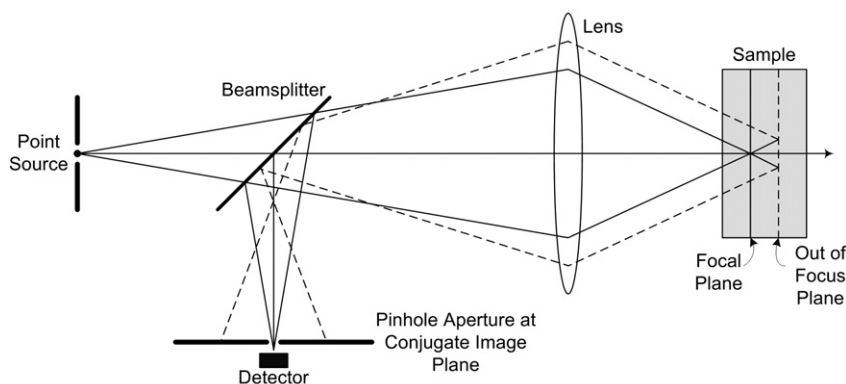

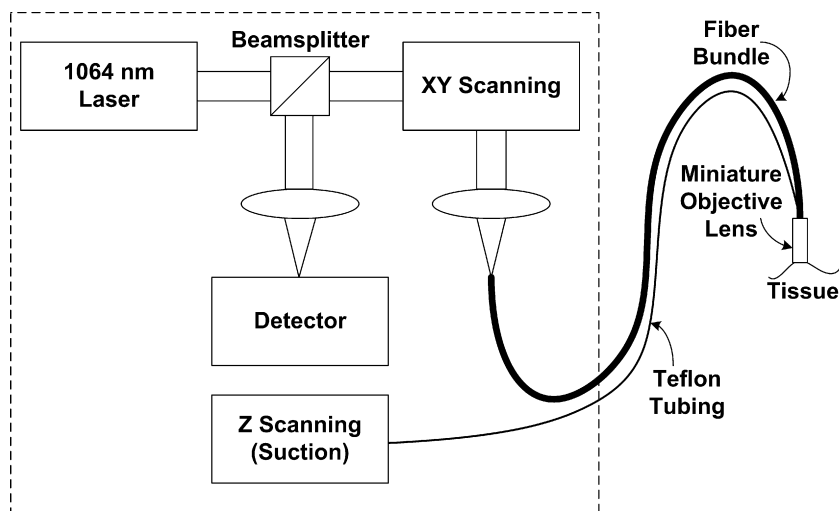**Figure 1** Illustration of optical sectioning by confocal microscopy.

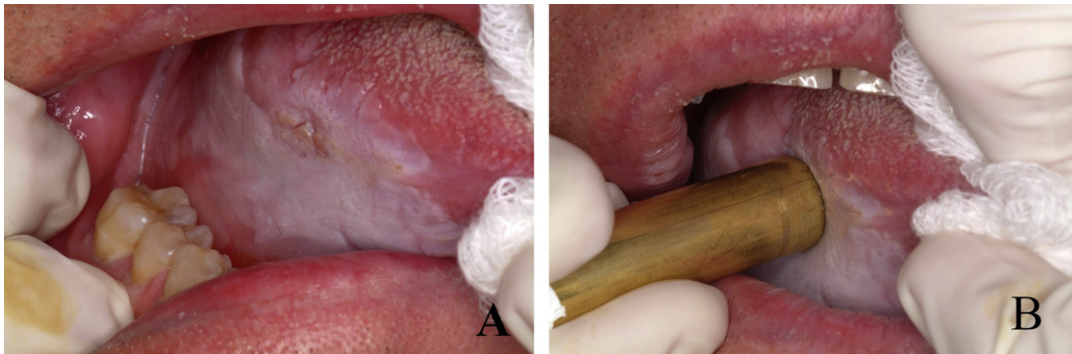**Figure 2**   Schematic of fiber optic confocal microscope.

Figure 3   Photographs of (A) a clinically suspicious imaging site on the ventral tongue and (B) placement of probe on the tissue for confocal reflectance imaging.

tion, or image thickness, is less than 10 μm. The lateral resolution is approximately 2 μm and the imaging field of view is approximately 230×230 μm. The entire epithelial depth is imaged by sequentially moving the tissue through the focal plane of the microscope using hydraulic suction controlled by a syringe pump. Although the imaging depth in the tissue is not quantitatively known, high quality images can be obtained at depths starting at the tissue surface and extending to the basement membrane. Determination of these two positions in the tissue permits visualization of the nuclear density in superficial, intermediate, and basal layers. All of the large optics and electronics are housed on a portable cart that can be wheeled into the operating room or examination room. The miniature objective lens, which is connected to the optical system through the fiber bundle and to the axial scanning system through Teflon tubing, is placed on the tissue for image acquisition. The probe housing which protects the objective lens measures 1 cm in diameter.

In vivo imaging of human oral tissue

All subjects included in this study gave informed consent. The protocol was reviewed and approved by the Institutional Review Boards of The University of Texas M.D. Anderson Cancer Center, The University of Texas at Austin, and Rice University. Eight subjects undergoing surgery for squamous cell carcinoma (SCC) of the oral cavity participated in the study. A head and neck surgeon identified clinically normal and abnormal areas for imaging and applied a sponge soaked in apple cider vinegar, in which acetic acid is the major constituent, to the tissue for approximately 1 min. Low concentration acetic acid is often used for acetowhitening during cervical colposcopy and increases scattering from cell nuclei and improves demarcation of nuclei and cytoplasm.[30,31] The clinician then placed the confocal probe on at least one clinically suspicious tissue site and a contralateral clinically normal-appearing site. Figure 3 shows (A) an example of a suspicious lesion and (B) placement of the fiber optic confocal microscope probe on the tissue. After imaging, biopsies were acquired at each imaged site for histopathologic assessment. Images of histology sections stained with hematoxylin and eosin (H&E) were acquired using a bright field microscope and a color charge-coupled device camera.

Image processing

At each site, confocal video was acquired as the depth of imaging was varied. Individual confocal images from the

superficial and intermediate layers of the epithelium were obtained from video files. In general, viewing of images in video format improves visualization of cellular and tissue structure. Raw fiber optic confocal videos can have image artifacts due to the non-linear scan of one of the two scanning mirrors, non-uniform specular reflection across the fiber bundle, fiber bundle ''pixilation'', and patient or operator motion. Images have been resized in one axis to account for the non-linear optical beam scan,[11] background subtracted, filtered using a median anisotropic diffusion filter to remove the fiber bundle pattern,[32] and contrast enhanced to improve image viewing quality in print form. These image processing techniques could potentially be implemented in real-time during image acquisition.

Results

In vivo reflectance confocal images were successfully obtained from 20 sites in eight patients. Table 1 shows the site location, clinical and confocal appearance, and histopathologic diagnosis of the biopsies taken from each of the 20 imaging sites. Of the 20 sites, eight exhibited hyperkeratosis, five had hyperplasia, four had inflammation, four were diagnosed with dysplasia, and five with invasive SCC. Two clinically normal sites from one subject did not have a pathologic diagnosis because the patient requested that no biopsies be taken from normal-appearing areas. Confocal appearance is classified as ''dispersed nuclei'' for videos with regularly spaced low-density nuclei, ''dense nuclei''

Table 1 Imaging site location, clinical and confocal appearance, and histopathologic diagnosis

| Patient and site	Imaging site	Clinical appearance	Histopathologic diagnosis	Dispersed nuclei	Dense nuclei	Disordered tissue structure
Normal						
6D	Upper lip	Normal	No biopsy taken	×		
6E	Buccal mucosa	Normal	No biopsy taken	Nuclei not visible		
4A	Floor of mouth	Normal	Normal		×	
7A	Ventral tongue	Normal	Normal		×	
8A	Ventral tongue	Normal	Hyperplasia, hyperkeratosis	×		
1B	Ventral tongue	Normal	Hyperkeratosis, mild inflammation		×	
2A	Buccal mucosa	Normal	Hyperkeratosis		×	
3A	Ventral tongue	Normal	Hyperplasia, hyperkeratosis	×		
5A	Ventral tongue	Abnormal	Hyperplasia, hyperkeratosis		×	
6A	Upper lip	Abnormal	Hyperkeratosis, mild inflammation	Nuclei not visible		
6B	Buccal mucosa	Abnormal	Hyperplasia, hyperkeratosis, mild inflammation	×		
7B	Ventral tongue	Abnormal	Hyperplasia, hyperkeratosis		×	
Dysplasia						
5B	Ventral tongue	Normal	Mild dysplasia		×	
4B	Floor of mouth	Abnormal	Moderate dysplasia, severe inflammation		×	
8B	Ventral posterior tongue	Abnormal	Dysplasia	×		
SCC						
1A	Ventral tongue	Abnormal	Moderately differentiated, invasive SCC			×
2B	Retromolar trigone	Abnormal	Moderate − severe dysplasia, invasive focal SCC			×
3B	Anterior floor of mouth	Abnormal	Invasive SCC			×
6C	Lower gum	Abnormal	Invasive SCC			×
8C	Ventral anterior tongue	Abnormal	Invasive SCC			×
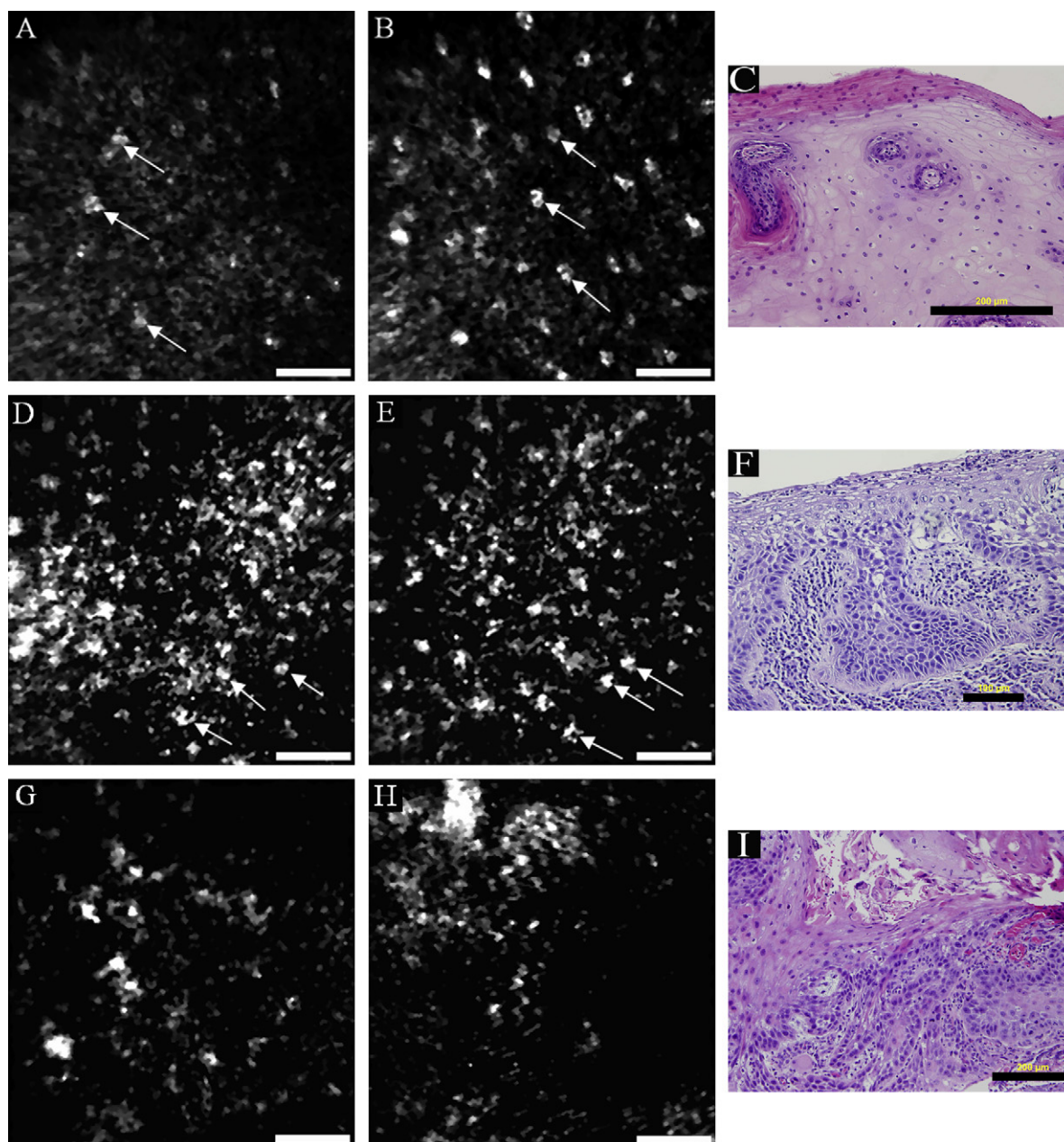
Figure 4 (A, B, D, E, G, and H) In vivo confocal reflectance images and (C, F, and I) photomicrographs of (A–C) normal ventral tongue site #8A, (D–F) floor of mouth epithelium pathologically diagnosed as moderate dysplasia (site #4B), and (G–I) tongue SCC lesion site #1A. Nuclei, which appear white in the confocal images and dark in the histology images, are identified by arrows in the confocal images. Epithelial cell nuclei are clearly visible and orderly spaced in (A) the superficial keratinizing epithelium and (B) the intermediate squamous epithelium of the normal site. (C) The histopathologic diagnosis for this site was hyperplasia and hyperkeratosis. Nuclear crowding disorganization can be seen throughout the epithelium in confocal images of the (D) superficial and (E) intermediate layers and (F) histology section of dysplastic floor of mouth epithelium. (G, H) Confocal images of SCC tissue are characterized by disordered tissue structure. Clusters of nuclei within host stromal and inflammatory cells can be seen in the corresponding histology section (I) of tongue lesion site diagnosed as SCC. Scale bars: (A, B, D, E, G, and H) 50 μm, (F) 100 μm, and (C, I) 200 μm.

for videos with regularly spaced but crowded nuclei, and ''disordered tissue structure'' for videos that show irregular reflective structures and may or may not include nuclei. Confocal videos from two sites showed even, regular tissue structure with few or no visible nuclei, and are therefore not classified into any of the three categories.

Figure 4 shows a comparison of reflectance confocal images acquired in vivo and H&E stained histology sections of normal, dysplastic, and SCC sites. The distribution of nuclei in confocal images (A and B) of normal ventral tongue site #8A compares well with the evenly spaced nuclei in the histology section (C) of a biopsy obtained from the imag-

ing site. (Confocal video included in Supplementary material.) In contrast to the transverse histology section, the confocal image plane is oriented parallel to the epithelial surface. Although the exact depths of the confocal images are unknown, Figure 4A is located in the superficial epithelium and Figure 4B is located in the intermediate epithelium. The white circular features in the confocal images are epithelial nuclei, as indicated with arrows. With low density of nuclei in the superficial layer and regular spacing of nuclei in the intermediate layer, this confocal image shows very typical organization and structure of normal epithelial tissue. The cell morphology and epithelial structure assessed by in vivo confocal microscopy compares well with histology.

Figures 4D and E show similar results for dysplastic tissue; nuclei are visible in the confocal images. The images from the floor of mouth epithelium from clinically abnormal and histologically dysplastic site #4B exhibit characteristic dense nuclei in the in vivo confocal images in both the superficial (Fig. 4D) and intermediate (Fig. 4E) layers of tissue and the histology section (Fig. 4F). (Confocal video included in Supplementary material.) In contrast, confocal images of SCC typically are very disorganized and have irregular structure. Nuclei can occasionally be distinguished in confocal images of SCC; however, it is usually difficult to identify or differentiate various tissue and cell structures. Figures 4G and H show examples of confocal images of tongue lesion site #1A. This site had a histologic diagnosis of SCC (Fig. 4I).

Other tissue structures of interest were visible in some of the confocal images. We were able to image a section of a blood vessel, just beneath the epithelium of normal site #6B. We also detected structures that appeared to be keratin pearls. (Confocal videos of these sites are included in the Supplementary material.)

Discussion

Our results show that in vivo confocal microscopy has significant potential to non-invasively identify changes in tissue structure and cell morphology associated with neoplastic development in the oral mucosa. By qualitatively analyzing the confocal images obtained clinically, one can anticipate the histologic appearance of the surgically removed, fixed, sectioned, and stained tissue. For normal tissue, the nuclei in the confocal image appear regularly organized and spaced. As the tissue transitions to dysplasia, the confocal image shows disrupted and overlapping nuclei throughout the epithelium, as is seen in the histology sections of dysplastic tissue. The confocal images of cancerous oral tissue show markedly disorganized features. If nuclei are visible, they appear haphazardly distributed. As illustrated in Table 1, confocal images of SCC contain abnormal reflective tissue structure that has not been seen in any images of normal tissue. Obviously, these new images, which appear in black and white, with a different orientation and lower resolution, are not comparable to the standard H&E stained pathology images. However, they do not require biopsy, histologic processing, and lateral microscopic examination. Continued advances in imaging technology should enable improvements in the quality and resolution of in vivo confocal microscopy images.[33] These confocal images, which

were acquired non-invasively and in real-time, demonstrate the potential clinical applications for non-invasively identifying changes in tissue structure and cell morphology associated with neoplastic development. Immediate assessment of the mucosal edges of resection for any residual microscopic disease by the surgeon at the time of cancer resection is another potential clinical application.

Confocal reflectance microscopy has previously been demonstrated as a method to image morphologic features of head and neck surgical specimens without fixing, staining, and sectioning of tissue required for histology and without the artifacts associated with histologic processing.[34] As a real-time imaging modality, it has been proposed as an adjunct to frozen-section examination in intraoperative consultation.[34] A study published by White et al. in 1999 comparing in vivo confocal reflectance imaging of normal oral tissue with histology established the foundation for in vivo confocal imaging for disease detection in oral mucosa.[9] The confocal images presented by White et al. correlated well, both qualitatively and quantitatively, with histology. Epithelial cell nuclei and cell borders, circulating blood cells, and extracellular matrix were imaged with confocal microscopy. However, with the large microscope objective, access inside the oral cavity was limited to the lip and tongue mucosa. In this study, we presented in vivo images of various sites of normal, pre-neoplastic, and neoplastic oral tissue, including lip, anterior and posterior ventral tongue, buccal mucosa, floor of mouth, gum, and retrimolar trigone using a miniaturized confocal microscope. Similar to the in vivo confocal images presented previously by White et al., our in vivo confocal images have correlated well to histology obtained from the imaging sites.

While conducting this study, we identified several technological difficulties that need to be resolved to make in vivo confocal imaging practical for diagnosing early neoplastic changes in the clinical setting. With the current fiber optic confocal microscope, we are restricted to imaging specific regions of the oral cavity. We were not able to image gingiva or hard palate sites due to the inability of the suction mechanism to move the rigid tissue through the focal plane. Modifications to move the miniature objective lens relative to the tissue, rather than the tissue relative to the lens, would be mechanically more complicated, but should allow imaging of these sites. Alternatively, a fixed plane imaging system that assessed tissue at one specific depth below the surface would provide cellular resolution of intact tissue but only limited information about all other epithelial layers. Another oral site that is difficult to image is the dorsal tongue. The highly keratinized and irregular surface is extremely reflective and difficult to image with safe laser power levels. Fortunately, tumors of the dorsal tongue are extremely rare, and the areas that have the highest incidence of SCC, the lateral and ventral surfaces, are easily imaged with the fiber optic confocal microscope.

We applied vinegar before imaging the oral mucosa to enhance contrast between nuclei and cytoplasm based on results of previous confocal imaging studies that used acetic acid as a contrast agent in imaging human epithelial tissue.[5,30,31,35] Indeed, while imaging normal volunteers before and after application of vinegar, significant improvement in the ability to visualize nuclei throughout the epithelium after vinegar application was achieved.

However, we have found that the application of vinegar to the oral tissue is the most uncomfortable component of the imaging procedure. To alleviate the discomfort of the taste and smell, we used apple cider vinegar, which yields the same imaging results and was found to be more palatable. In this research protocol, we performed imaging on patients in the operating room under general anesthesia, in order to avoid patient discomfort from the vinegar and subsequent biopsy. However, for this technology to be clinically applicable, it will need to be acceptable to patients in the outpatient clinic setting.

In conclusion, we believe that in vivo fiber optic confocal reflectance microscopy has significant clinical potential to provide a sensitive and specific method for non-invasive detection of precancer and cancer in the oral cavity and this technology should be easily translated to other mucosal sites through the body.

Conflict of Interest Statement

None declared.

Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.oraloncology.2008.02.002.

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