Confocal Microscopy for Real-Time Detection of Oral Cavity Neoplasia

Anne L. Clark, Ann M. Gillenwater, Thomas G. Collier, Reza Alizadeh-Naderi, Adel K. El-Naggar, and Rebecca R. Richards-Kortum

Department of Biomedical Engineering, University of Texas at Austin, Austin, Texas 78712 [A. L. C., T. C., R. R-K.], and Departments of Head and Neck Surgery and Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [A. G., R. A-N., A. K. E.]

ABSTRACT

Purpose: The goal of this study was to characterize features of normal and neoplastic oral mucosa using reflectance confocal microscopy.

Experimental Design: Oral cavity biopsies were acquired from 17 patients at the Head and Neck Clinic of The University of Texas M. D. Anderson Cancer Center who were undergoing surgery for squamous cell carcinoma within the oral cavity. Reflectance confocal images were obtained at multiple image plane depths from biopsies within 6 h of excision. After imaging, biopsies were fixed in 10% formalin and submitted for routine histological examination. Reflectance confocal images were compared with histological images from the same sample to determine which tissue features contribute to image contrast and can be potentially imaged using in vivo confocal microscopy.

Results: Confocal images were successfully acquired from 15 biopsy pairs from 17 patients. Depth-related changes in cell diameter and nuclear density were observed at multiple anatomical sites within the oral cavity. In squamous cell carcinomas, densely packed, pleomorphic tumor nuclei could be visualized with distinct differences in nuclear density and morphology distinguishable between confocal images of neoplastic and nonneoplastic oral cavity. Other features of noncancerous and cancerous oral tissue that could be identified in the confocal images included areas of inflammation, fibrosis, muscle fibers, and salivary glands.

Conclusions: Our results support the potential for this tool to play a significant role in the clinical evaluation of oral lesions, real-time identification of tumor margins, and monitoring of response to therapeutic treatment.

INTRODUCTION

Confocal reflectance microscopy is a new technology that can provide detailed images of tissue architecture and cellular morphology of living tissue in near real time. In concept, in vivo confocal imaging resembles histological tissue evaluation, except that three-dimensional subcellular resolution is achieved noninvasively and without stains. In epithelial structure, resolution of 1 μm has been achieved with a 200–400-μm field of view and a penetration depth of up to 500 μm (1–6). Recently, flexible reflectance confocal microendoscopes have been described that can obtain high-resolution confocal images of tissue in vivo in near real time (7–15). Use of this instrument provides the potential to image oral epithelial tissues with subcellular resolution in a clinical setting.

Confocal imaging with reflected light allows for detailed images of cell morphology and tissue architecture using backscattering by various tissue components to provide contrast. In skin (1, 2, 16–18), cytoplasmic melanin provides a strong source of backscattering, enabling detailed morphological images of epithelial cell morphology and tissue architecture throughout the entire epithelial thickness. This technology has been used to image various types of skin pathology, including psoriasis (19), folliculitis (20), and neoplastic skin lesions (21–23). In neoplastic skin lesions, morphological changes in cytoarchitecture and microvasculature were visualized in both basal cell carcinomas and melanomas.

In amelanotic epithelial tissues, cell nuclei provide the primary source of reflected light (6, 24) captured by a reflectance confocal microscope. The backscattering from these nuclei is dramatically enhanced by addition of weak (3–6%) acetic acid (25). Confocal imaging of oral mucosa in the lip and tongue has resolved subcellular detail at depths of 250 and 500 μm, respectively (4). Recent work showed that reflectance confocal imaging of normal and precancerous cervical tissue can characterize nuclear size, nuclear density, and nuclear:cytoplasmic ratio without the need for tissue sectioning or staining. Parameters extracted from confocal images could be used to discriminate high-grade cervical precancers with a sensitivity of 100% and a specificity of 91% in a study of 25 samples (5). These results underscore the potential role of this technology in clinical evaluation of oral lesions and the need for further investigations in oral tissue using multiple anatomical sites and pathological diagnosis.

The goal of this study was to characterize the features of normal and neoplastic oral mucosa using reflectance confocal microscopy. We report results of a pilot study using near real-time reflectance confocal microscopy to image pairs of clinically normal and abnormal biopsies obtained from 17 patients. We find that confocal microscopy can image oral mucosa with...
resolution comparable to histology without the need for tissue fixation, sectioning, or staining. Confocal images provide a detailed view of cell morphology and tissue architecture, demonstrating features of normal epithelium, dysplasia, and squamous carcinoma. Based on these results, we recommend that reflectance confocal microscopy should be explored as a tool to improve early detection of oral cavity neoplasia, to provide real-time determination of mucosal tumor margins, and to determine response to therapy.

MATERIALS AND METHODS

Specimens. Oral cavity biopsies were acquired from 17 patients at the Head and Neck Clinic of The University of Texas M. D. Anderson Cancer Center who were undergoing surgery for SCC\(^2\) within the oral cavity. Informed consent was given by all patients, and the project was reviewed and approved by The University of Texas M. D. Anderson Cancer Center Office of Protocol Research and the Institutional Review Board at the University of Texas at Austin. Biopsies (approximately 3 mm wide by 4 mm long by 2 mm thick) were acquired from one clinically normal-appearing area and one clinically suspicious area and immediately placed in growth medium (DMEM, no phenol red). Reflectance confocal images were obtained at multiple image plane depths from biopsies within 6 h of excision. After imaging, biopsies were fixed in 10% formalin and submitted for routine histological examination by an experienced head and neck pathologist (A. K. E.). Additional sections from each biopsy were stained with MMAC Concentrate Antibody (Zymed Laboratories, Inc.), a broad spectrum monoclonal antibody mixture of clones A1 and A3 that reacts to cytokeratins 10, 14/15, 16, and 19 in the acidic subfamily and all members of the basic subfamily, to assess correlations between confocal image features and the presence of keratin in the specimen.

Confocal System. Reflectance confocal images were obtained from each biopsy using a near real-time, epi-illumination, reflectance confocal microscope (Ref. 26; Fig. 1). Illumination was provided by a continuous wave laser diode operating at 810 nm. A mirror system provided an image frame rate of 7.5 frames/s by scanning illumination light in the sample via a water immersion microscope objective (\(\times 25\), 0.8 NA). Average illumination power was 10–30 mW, focused to a 1-\(\mu m\)-diameter spot on the sample. Light backscattered from the tissue returned to a beam splitter, where it was reflected onto a pinhole lens and then spatially filtered by a 10-\(\mu m\)-diameter pinhole aperture before being detected by an avalanche photodiode. The confocal system operated at a dimensionless pinhole radius of 2.5 to provide maximum optical sectioning for obtaining cellular detail (26). The measured lateral resolution and axial resolution of the system were 0.8 and 2–3 \(\mu m\), respectively. The field of view was adjustable from 300 to 400 \(\mu m\) by changing the system magnification.

Imaging and Image Processing. Before imaging, the biopsies were removed from growth media, rinsed with PBS, and oriented so the image plane of the confocal microscope was parallel to the epithelial surface and would approach the epithelial layer first. A 6% solution of acetic acid was then added to each sample to increase image contrast (25). Frames were acquired at various epithelial depths until either tissue details were no longer resolvable or up to the working distance of the microscope objective (250 \(\mu m\)). To contrast confocal images obtained in this “en face” orientation with the traditional radial orientation, additional confocal images were acquired from one biopsy oriented so that the image plane of the confocal microscope was perpendicular to the epithelial surface and from 200-\(\mu m\)-thick transverse organ cultures prepared from biopsies from one patient.

Each of the confocal image frames presented here was resampled and processed to enhance image quality. Resampling was performed to reduce distortion in the images caused by nonlinearity in the resonant galvonometric scanning system, whereas image quality was improved by increasing brightness and contrast within the images. Brightness was enhanced by adding a selected percentage of full gray scale to each pixel, and contrast was increased by removing another percentage of full gray scale from the image and expanding the remaining midrange gray levels. Brightness and contrast for all confocal images in this paper were increased by 70% and 50%, respectively. Confocal images from the transverse tissues slices were also tiled together to provide large-scale mosaic views of each slice.

Images of stained histological sections were acquired using a color charge-coupled device camera coupled to a bright-field

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\(^2\) The abbreviations used are: SCC, squamous cell carcinoma; MMAC, Monoclonal Mouse Anti-Cytokeratin (Pan).

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**Table 1** Number of clinically normal and abnormal biopsies from each site

<table>
<thead>
<tr>
<th>Location</th>
<th>Normal</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tongue (lateral and ventral surfaces)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Floor of mouth</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Gingiva</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Soft palate</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

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**Fig. 1** Block diagram of the near real-time reflectance confocal microscope used to image oral cavity specimens.
microscope. Reflectance confocal images were compared with histological images from the same sample to determine which tissue features contribute to image contrast and can be potentially imaged using \textit{in vivo} confocal microscopy. The confocal microscope’s small field of view makes it extremely difficult to register exactly where in the biopsy images were acquired, so we identified areas in histological sections that corresponded to features present in our confocal images.

**RESULTS**

Images were successfully obtained from specimens from 15 of 17 biopsy pairs with resolution similar to that provided by bright-field microscopy typically used to examine histological sections; data could not be obtained from 2 patient specimens due to instrument errors, and these were not included in further analyses. Table 1 shows the number of clinically normal and abnormal specimens obtained from each site within the oral cavity. Table 2 lists the histopathological diagnoses for each biopsy from each patient with 15 showing hyperkeratosis or parakeratosis, 6 exhibiting hyperplasia, 1 with dysplasia, 9 moderately differentiated SCCs, 1 well-differentiated SCC, and 3 specimens having no diagnosis due to the lack of epithelium in the histological section.

Fig. 2 shows a comparison of histological (Fig. 2A) and confocal (Fig. 2B) images from the 200-μm-thick transverse organ culture of a normal gingiva biopsy show epithelium and stroma; the basal epithelial nuclei are readily apparent at the epithelial stromal junction (double arrows). Epithelial cell nuclei and fibroblast nuclei (single arrow) are resolved. The histology section (C) and transverse confocal image (D) of a hyperkeratotic tongue biopsy show epithelial cell nuclei from superficial epithelium on the right (double arrows) to the basal layer (single arrow) on the left. Scale bars, 50 μm.

Table 2  Histopathological diagnosis by patient

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Site</th>
<th>Clinically normal biopsy</th>
<th>Clinically abnormal biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tongue (lateral surface)</td>
<td>Hyperkeratosis</td>
<td>Moderately differentiated SCC</td>
</tr>
<tr>
<td>2</td>
<td>Buccal mucosa</td>
<td>Hyperkeratosis</td>
<td>Moderately differentiated SCC</td>
</tr>
<tr>
<td>3</td>
<td>Floor of mouth</td>
<td>Mild hyperkeratosis</td>
<td>Hyperkeratosis, hyperplasia</td>
</tr>
<tr>
<td>4</td>
<td>Tongue (lateral surface)</td>
<td>Mild hyperplasia</td>
<td>Moderately differentiated SCC</td>
</tr>
<tr>
<td>5</td>
<td>Tongue (ventral surface)</td>
<td>Hyperkeratosis</td>
<td>Extreme hyperkeratosis</td>
</tr>
<tr>
<td>6</td>
<td>Tongue (lateral surface)</td>
<td>Mild hyperplasia</td>
<td>Moderately differentiated SCC</td>
</tr>
<tr>
<td>7</td>
<td>Gingiva</td>
<td>None</td>
<td>Well-differentiated SCC</td>
</tr>
<tr>
<td>8</td>
<td>Gingiva</td>
<td>Mild hyperkeratosis</td>
<td>Moderately differentiated SCC</td>
</tr>
<tr>
<td>9</td>
<td>Tongue (lateral surface)</td>
<td>Hyperkeratosis</td>
<td>Moderately differentiated SCC</td>
</tr>
<tr>
<td>10</td>
<td>Tongue (lateral surface)</td>
<td>Hyperkeratosis</td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>Tongue (lateral surface)</td>
<td>Hyperkeratosis, hyperplasia</td>
<td>Moderately differentiated SCC</td>
</tr>
<tr>
<td>12</td>
<td>Floor of mouth</td>
<td>Hyperkeratosis, hyperplasia</td>
<td>Moderately differentiated SCC</td>
</tr>
<tr>
<td>13</td>
<td>Tongue (lateral surface)</td>
<td>Parakeratosis, hyperplasia</td>
<td>Moderately differentiated SCC</td>
</tr>
<tr>
<td>14</td>
<td>Soft palate</td>
<td>Hyperkeratosis</td>
<td>Moderately differentiated SCC</td>
</tr>
<tr>
<td>15</td>
<td>Gingiva</td>
<td>Hyperkeratosis</td>
<td>Mild hyperkeratotic dysplasia</td>
</tr>
</tbody>
</table>

* No epithelium in specimen.
tecture and cell morphology assessed by reflectance confocal microscopy compare well with that assessed by histology.

In clinical applications, reflectance confocal images would be obtained with the image plane parallel to the epithelial surface. Fig. 3 shows reflectance confocal images obtained in this orientation at different depths beneath the surface of the epithelium. Nuclear density, cell size, and cytoplasmic scattering change as the depth of the focal plane increases from (B) 20 to (C) 50 to (D) 150 μm beneath the epithelial surface. Scale bars, 50 μm.

Fig. 3 Transverse histological image (A) and en face confocal images (B–D) obtained at different depths beneath the epithelial surface from hyperkeratotic tissue from the ventral tongue surface. Nuclear density, cell size, and cytoplasmic scattering change as the depth of the focal plane increases from (B) 20 to (C) 50 to (D) 150 μm beneath the epithelial surface. Scale bars, 50 μm.

In contrast, Figs. 4–8 show images that illustrate the features of SCCs with confocal microscopy. Fig. 4 shows histological (Fig. 4, A and B) and confocal images (Fig. 4C) of a moderately differentiated SCC from the lateral surface of the tongue. Extensive variations in cell size, nuclear size, and nuclear morphology are resolved in both the histological section (Fig. 4B) and a confocal image taken 100 μm below the epithelial surface illustrating pleomorphic nuclei (single arrows) and dark areas corresponding to stroma containing inflammation (double arrows). Scale bars, 50 μm.

Fig. 4 Histological and confocal images of a moderately differentiated SCC from the lateral surface of the tongue. A, low-magnification image of a keratin antibody (MMAC)-stained section showing tumor cells interspersed with stroma containing inflammation (×2.5 objective). B, high-magnification image of tumor cells (single arrows) containing pleomorphic nuclei and stroma with inflammation (double arrows). C, confocal image taken 100 μm below the epithelial surface illustrating pleomorphic nuclei (single arrows) and dark areas corresponding to stroma containing inflammation (double arrows). Scale bars, 50 μm.
Epithelial surface (Fig. 4C). Epithelial nuclei (single arrows) appear as bright areas on the confocal image, whereas areas of stroma with inflammation (double arrows) appear dark in the confocal image. Similarly, the histological (Fig. 5, A, B, and D) and confocal images (Fig. 5, C and E) of invasive SCC of the soft palate show regions of tumor cells (single arrows) and interspersed regions of stroma containing inflammatory cells (double arrows). Confocal images obtained 100 μm beneath the epithelial surface show strong backscattering from tumor cell nuclei and dark regions corresponding to areas of inflammation.

Nuclear density as assessed by confocal microscopy in the SCCs of Figs. 4 and 5 is clearly higher than that in the normal tissue displayed in Fig. 3.

Fig. 6 shows histological and confocal images from a moderately differentiated SCC from the buccal mucosa. Increased keratinization in tumor cells are noted in histological (Fig. 6B) and confocal images (Fig. 6C) as higher signal return from cytoplasmic, nonnuclear areas (single arrows). Large, homogeneous keratin pearls (Fig. 6, D and E) were visible in confocal images as areas of high return with a speckled appear-
Fig. 7 Images of a well-differentiated SCC from the gingiva. A, low-magnification image of a keratin antibody (MMAC)-stained section showing tumor cells and keratin pearls interspersed with stroma containing inflammation (×2.5 objective). B, high-magnification histological image of a keratin pearl (double arrows; ×20 objective). C, confocal image taken 50 μm below the surface containing highly reflective keratin (double arrows). D, high-magnification histological image of keratin pearls with highly keratinized tumor cells (single arrow; ×20 objective). E, confocal image taken 50 μm below the surface containing areas of highly reflective keratin with nuclei (single arrow). Scale bars, 50 μm.

Fig. 8 Images of a muscle and fibrosis from a moderately differentiated SCC from the lateral surface of the tongue. A, low-magnification image of a H&E-stained section shows skeletal muscle and extensive fibrosis (×2.5 objective). B, high-magnification histological image of muscle (single arrow; ×20 objective). C, confocal image of muscle (single arrow) taken 100 μm below the surface. D, high-magnification histological image of fibrosis (double arrows) with elongated fibroblast nuclei (single arrow; ×20 objective). E, confocal image of fibrosis (double arrows) taken 50 μm below the surface illustrating reflectance from structural protein fibers and containing elongated fibroblast nuclei (single arrow). Scale bars, 50 μm.

ance (single arrows). These features were also observed in images of a well-differentiated SCC from the gingiva (Fig. 7). The confocal images show both keratin pearls (double arrows; Fig. 7C) and tumor cells (single arrows) surrounded by smaller keratin pearls (Fig. 7E). Confocal image features compare well with corresponding histological images.

In addition to tumor cells and keratin, confocal imaging identified other features in normal and neoplastic oral tissues. Fig. 8 shows images from a moderately differentiated SCC from the lateral surface of the tongue. Fig. 8, B and C, shows histological and confocal images of muscle fibers (single arrows); the muscle fibers do not strongly scatter light and appear dark in the confocal image. Areas of fibrosis in the tumor are clearly discernible in histological (Fig. 8D) and confocal images (Fig. 8E). Confocal images of fibrosis show scattering from individual fibers (double arrows) as well as elongated fibroblast nuclei (single arrows). Fig. 9 shows images from salivary glands (single arrows) in a biopsy specimen from the floor of the mouth. Confocal images of these glands are characterized by bright return from serous demilunes containing serous-secreting cells surrounding darker regions of low return from mucous-secreting units (27).

An important performance measure for confocal imaging in vivo is the maximum depth at which images can be obtained, or "penetration depth." We observed a wide variation in penetration depth throughout this study. We analyzed image stacks from 13 normal samples in which confocal images throughout the epithelium were captured, and we were able to image up to the confocal microscope’s working distance (250 μm) 31% of the time. In 46% of the cases, penetration depth was between 150 and 200 μm, whereas 23% of the time, penetration depth was 100–150 μm. We hypothesize that increased levels of keratin, particularly in the superficial epithelium, can limit the ability of illumination light to penetrate to lower depths due to the high refractive index of keratin compared with cytoplasm \[n_{\text{keratin}} = 1.54 \times 10^{-2} \quad \text{versus} \quad n_{\text{cytoplasm}} = 1.37 \times 10^{-2}\].

DISCUSSION

The confocal images presented here illustrate the ability of reflectance confocal microscopy to image oral mucosa with resolution comparable with histological evaluation without tissue preparation and staining. In normal tissue, depth-related changes in cell diameter and nuclear density were observed at
multiple anatomical sites within the oral cavity. In SCCs, densely packed, pleomorphic tumor nuclei could be visualized with distinct differences in nuclear density and morphology distinguishable between confocal images of neoplastic and non-neoplastic oral cavity. Other features of noncancerous and cancerous oral tissue that could be identified in the confocal images included areas of inflammation, fibrosis, muscle fibers, and salivary glands. Areas of inflammation appear dark in confocal images of the oral cavity.

The images reported here show features in the oral cavity similar to those reported by White et al. (4). In that study, images of the superficial epithelial layers of the lip and anterior tongue were acquired at depths of up to 490 and 250 μm, respectively. Cell nuclei and membranes were clearly resolved in the epithelial layers, correlating well with histology. The use of a low-power objective (×30) allowed the capture of different structures in the lamina propria including collagen fibers and blood vessels. The study presented here provides a more comprehensive survey of the morphological features that can be measured using reflectance confocal microscopy from oral sites such as the floor of the mouth, gingiva, buccal mucosa, soft palate, and lateral surfaces of the tongue and how these features change with the development of SCC.

Confocal microscopy can provide images of many important cellular and architectural features of SCC. Whereas the images presented here were obtained from biopsies measured immediately after excision, we have recently described a fiber optic reflectance confocal microscope that has been used to obtain images of the oral cavity in vivo (7, 8). This flexible confocal microendoscope is small enough that it can be used to examine sites throughout the oral cavity. The tip of the endoscope is placed in contact with the tissue to be imaged. A small drop of saline provides index matching between the tip of the endoscope and the tissue. Weak suction is applied at the distal tip of the microendoscope to pull the tissue up through the image plane of the confocal microscope, to easily obtain images at different depths beneath the surface of the epithelium.

The ability to obtain such images in vivo and at near real time suggests several potential clinical applications for reflectance confocal microscopy such as noninvasive diagnosis of oral lesions and the ability to determine tumor margins in vivo in real time. Visual inspection and palpation remain the standard methods used to assess the extent of mucosal involvement by carcinomas and premalignant lesions. However, molecular and pathological assessments of “normal-appearing” mucosa have revealed molecular and cellular changes in these tissues, illustrating the fallibility of visual detection of dysplasia even by highly trained clinicians (30, 31). To compensate for the limitation of surgeons to exactly determine the margins of carcinoma or dysplasia, it is accepted practice to resect a large cuff (approximately 1–2 cm) of normal-appearing mucosa around the visibly abnormal tissue. This produces better likelihood of complete excision but increased postoperative morbidity due to the greater amount of tissue removed. In tertiary care centers, problems caused by the inability to visually distinguish the margins of carcinoma and dysplasia are ameliorated by the use of frozen section to analyze edges of the resection using light microscopy. However, accurate frozen section analysis is time-consuming, costly, dependent on the experience and skill of the histotechnician and pathologist, and not available in unspecialized medical facilities. Thus, technological advancements such as in vivo confocal imaging that improve the ability of surgeons to accurately identify tumor margins in real time could have substantial benefit for patients. As our results demonstrate, in vivo confocal imaging has the potential to assess features of normal mucosa and SCC and may yield a very attractive alternative method to assess the status of mucosal margins through its capability to visualize cellular morphology and tissue architecture in real time without the need for sectioning and staining. Significant time and cost savings through the use of confocal examination of frozen sections from Mohs micrographic surgery for excision of nonmelanoma skin cancers have already been noted by Rajadhyaksha et al. (32). We acknowledge that a limitation of in vivo confocal microscopy is penetration depth and therefore its inability to assess the deep margin of a large, invasive tumor. In addition, verrucous lesions with extreme hyperkeratosis may not permit adequate light penetration to visualize the epithelial stromal border.

Achieving the clinical potential of in vivo confocal imaging will require further characterization of the cellular and architectural features of oral tissue that are visible with confocal microscopy and assessment of how they match standard histological examination. This includes imaging of more dysplastic lesions and the evaluation of the efficiency of confocal imaging in the clinical assessment of margin involvement and response to treatment. Two primary challenges must be addressed in these studies. The first is to obtain images in vivo and assess their image characteristics in comparison with our previous results and standard histological examination; we have just commenced an in vivo pilot study of fiber optic reflectance confocal microscopy of oral lesions. The second is to explore methods that can increase the penetration depth at which good quality confocal images can be obtained. We are studying methods to increase penetration depth through the use of additional signal filtering.
techniques such as coherence gating to better isolate backscattered light from our focal plane (33) and chemical agents such as glucose and glycercol to improve index matching at the surface (4, 34).

In this study, we have shown the power of reflectance confocal microscopy to visualize, at the subcellular level, features of both normal and neoplastic oral mucosa throughout the oral cavity as well as the composition of SCCs with varying differentiation levels. Our results support the potential for this tool to play a significant role in the clinical evaluation of oral lesions, real-time identification of tumor margins, and monitoring of response to therapeutic treatment.

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