Fiber-Optic Confocal Reflectance Microscope With Miniature Objective for *In Vivo* Imaging of Human Tissues

Kung-Bin Sung, Chen Liang, Michael Descour, Tom Collier, Michele Follen, and Rebecca Richards-Kortum*

***Abstract*—We have built a fiber-optic confocal reflectance microscope capable of imaging human tissues in near real time. Miniaturization of the objective lens and the mechanical components for positioning and axially scanning the objective enables the device to be used in inner organs of the human body. The lateral resolution is 2 micrometers and axial resolution is 10 micrometers. Confocal images of fixed tissue biopsies and the human lip *in vivo* have been obtained at 15 frames/s without any fluorescent stains. Both cell morphology and tissue architecture can be appreciated from images obtained with this microscope.**

***Index Terms*—Confocal microscopy, fiber-optic, *in vivo*, laser, reflectance, scanning.**

I. Introduction

OPTICAL technologies are being increasingly used to perform real time assessment of tissue pathology *in vivo*. One particularly promising technology is confocal microscopy, which samples small volumes of tissue, producing images with microscopic resolution at depths up to several hundred micrometers within tissue. This optical sectioning capability of confocal microscopy enables cellular structures to be imaged without taking biopsies from the human body. Miniaturized objective optics are required to enable confocal imaging of internal organs for *in situ* detection of pathology.

Nonfiber-optic reflectance confocal imaging systems that achieve *in vivo* imaging have been reported for accessible tissues such as the skin [1]–[3], and the lip and tongue [1]. Epithelial cell morphology and tissue architecture are well resolved by these systems. Confocal imaging in epithelial tissues other than the skin has been limited because it is difficult to bring the tissue of interest in contact with the microscope objective. Several groups have attempted to develop flexible endoscopes to record fluorescence confocal images *in vivo* based on single mode optical fibers [4]–[8]. Two groups have developed systems with potential for *in vivo* fluorescence confocal imaging using fiber-optic bundles and miniaturized objectives; they have reported fluorescence confocal images of biological samples including human prostate tissue, mouse peritoneum [4] and rat colon tissue [5]. Fluorescent stains have been used to yield sufficient signal in these studies. For *in vivo* imaging these dyes must be nontoxic to the tissue and able to penetrate to deeper layers within tissue. Confocal systems that image reflected light have no need for dyes and can achieve deeper penetration than fluorescence systems by utilizing longer wavelength sources. Two such reflected light confocal systems have been developed by raster scanning the proximal end of a fiber bundle [6] and using a digital micromirror device to selectively illuminate fibers within a fiber bundle [7]. Another approach is to use a single-mode optical fiber and a miniaturized scanning head including micromachined scanning mirrors and objective lens [8]. Images of biological tissues have not been reported by these groups.

Recently, we developed a fiber-based reflected light confocal microscope and imaged epithelial cells and tissues *in vitro* and *in vivo* without any fluorescent stains [9], [10]. A commercial microscope objective was used to image the specimens. Here, we present a modified system that has a miniature objective lens for easier access to tissues and an axial scanning mechanism to hold the objective stably against the tissue and provide controllable axial scanning. The miniaturized dimensions of the end piece attached to the distal fiber end enable use of the system on internal organs such as the cervix and inner oral cavity.

II. Materials and Methods

A. Fiber-Optic Confocal Reflectance Microscope (FCRM)

Fig. 1 illustrates the schematics of the FCRM. The details of system design and performance are reported elsewhere [9]. The commercial objective and relay optics used in the previous system are replaced by a miniature objective that is 22 mm long and 7 mm in diameter including the housing. The key component of the FCRM is the fiber bundle (IGN-15/30, Sumitomo) that is located between the custom-designed lens system L1 and the miniature objective. This image guide has 30 000 fibers, an overall outer diameter of 2.5 mm, and a nominal numerical aperture (NA) of 0.3. The fibers inside this bundle have an average core diameter of 4 μm and average center-to-center spacing of 7 μm. Lens system L1 focuses the illumination beam onto the proximal end face of the fiber bundle such that only one fiber is illuminated at one time. At the distal end of the bundle, the

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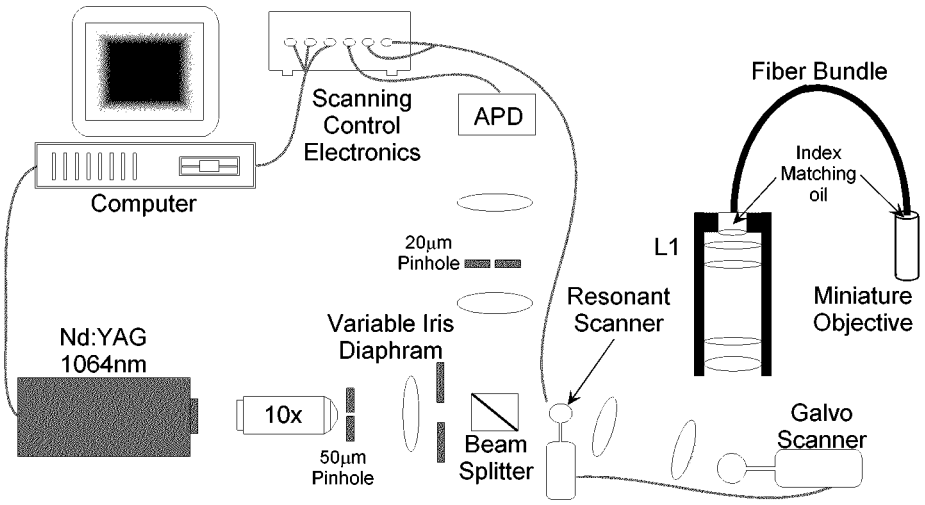

Fig. 1. System diagram of the FCRM.

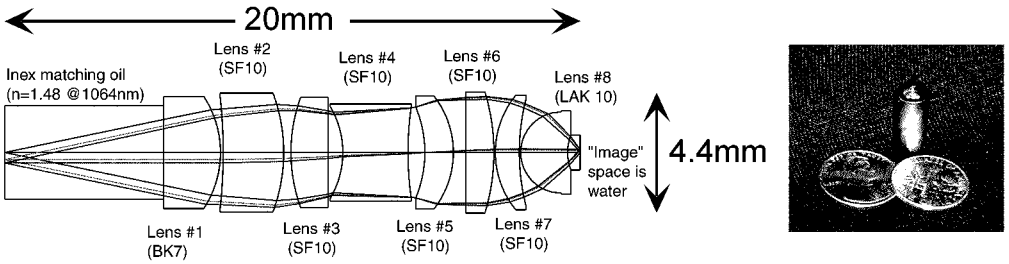

Fig. 2. The optical design and photograph of the miniature objective lens. The overall outer diameter is 7 mm and length is 22 mm including the housing.

illumination fiber is imaged to the sample by the miniature objective. The illumination fiber also serves as the detection pinhole in a conventional confocal microscope [11]. Backscattered light from the sample goes back through the fiber bundle and is deflected toward an avalanche photodiode (APD) by the beam splitter. A secondary pinhole, located in front of the APD, is adjusted to a position that is conjugate to the illumination/detection fiber. Backscattered light from locations other than the illumination/detection fiber will be mostly rejected by the pinhole. Therefore, the condition for confocal microscopy is achieved. The specular reflection resulting from the surfaces of the fiber bundle is reduced by using index-matching oil at both ends of the fiber bundle.

Fig. 2 shows the optical layout and a photograph of the miniature objective. The detailed design approach and testing results of this miniature objective are described separately [12]. Diffraction-limited performance has been achieved. The level of Fresnel reflection is reduced to less than 0.5%/surface by use of antireflection coatings on the elements of the miniature objective. The optical efficiency of the objective is measured to be 82%. The object plane (tissue) of the miniature objective is immersed in water and the image plane (fiber bundle) is immersed in oil. The NA at the image plane is designed to be 0.3, matching the NA of the fiber bundle. The NA at the object plane needs to be as large as possible in order to have high magnification from tissue to fiber. In this case the NA is 1.0 and the magnification is 3.33. Given that the spacing between adjacent fibers is 7 μm, the separation between illumination spots in the sample is 2.1 μm. The lateral resolution of the system is limited by the spatial sampling property of the fiber bundle. The axial resolution of the FCRM is calculated to be 3.1 μm following the method of Gu *et al.* [13].

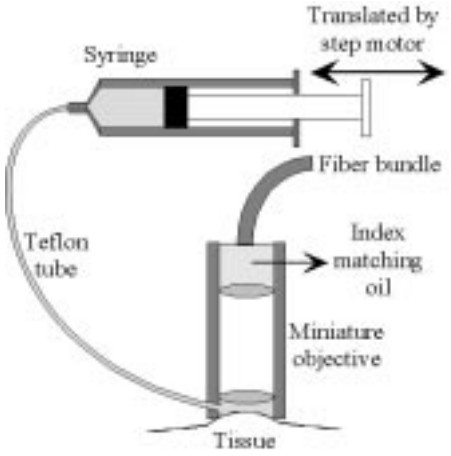

Fig. 3. Schematic of the axial scanning mechanism. The lens elements in the miniature objective are not drawn to scale here.

In order to obtain confocal images of tissues the illumination spot in the sample needs to be scanned in the transverse directions. Two galvanometric scanning mirrors that produce a raster scan at 15 frames/s achieve *en face* scanning of a thin slice within the sample. Axial scanning of the focal plane within the sample is achieved by a hydraulic system (Fig. 3) that moves the tissue in the field of view (FOV) along the optical axis. The space between tissue and lens #8 of the miniature objec-

tive is filled with water and connected to a syringe through Teflon tubing. The tubing and the syringe are also filled with water. The syringe can be translated by moving a stepper motor (Compumotor with ZETA6104 indexer drive, Parker Automation) connected to the syringe. After the objective is in contact with tissue the motor moves outward and sucks water from the tissue chamber. Negative hydraulic pressure draws the tissue upward and helps hold the objective against the tissue stably. The holder of the miniature objective is designed such that the image plane of the objective is recessed 1 mm from the end. Therefore, the surface of the tissue can be imaged when the objective is held tightly against the tissue, reducing motion artifacts from positioning of the objective. Deeper layers of the tissue can be imaged by moving the motor outward.

B. Samples Imaged

The system performance was tested by imaging standard samples including a glass Ronchi grating and polystyrene microspheres. Cervical biopsy specimens were imaged in order to test optical sectioning of the system. Biopsies were excised from patients undergoing colposcopic examination at the M.D. Anderson Cancer Center in Houston, Texas. Informed consent was obtained from each patient, and the study was reviewed and approved by the Internal Review Boards at the University of Texas M.D. Anderson Cancer Center and the University of Texas at Austin. These biopsies were immersed in 6% acetic acid solution and then fixed with 10% formaldehyde. During image acquisition the biopsy specimens were moved using translation stages. *In vivo* images of the lower lip of one of the authors (KBS) were recorded while the axial scanning system was used to change the imaging depth in the tissue. The maximum optical power that entered the tissue was less than 40 mW, which is comparable to that used by another *in vivo* study [1]. 6% acetic acid was added to the lip to enhance image contrast [14]. Background subtraction was applied to images of microspheres and biological specimens to reduce residual specular reflection from the fiber end faces. The contrast and brightness of the resultant images were adjusted for better presentation.

III. Results

A Ronchi grating on a glass slide was imaged to calibrate the scale and measure the lateral resolution of the system. A high degree of speckle was observed in images of highly reflective samples such as a mirror and the glass grating. To reduce the effect of speckle a short video file was recorded for1 s and the stack of images from the video was averaged to produce the resultant image shown in Fig. 4(a). The grating has 1000 line pairs/in and each of the bright and dark bars has a width of 12.7 μm. The dimension of the FOV is calculated as 180 μm$\times$ 170 μm accordingly. The grating image appears to be wavy because the image is stretched horizontally at the left and right edges by the sinusoidal line-scan pattern of the resonant scanner. The brightness is not uniform over the FOV, which is mostly attributed to field curvature of the miniature objective.

The lateral resolution of the system was obtained by measuring edge response from the image of the grating. Fig. 4(b) shows a line profile across one of the edges in the image. Since the image is pixilated by the fiber structure, the line profile is averaged over a line width of 200 pixels or equivalently 80 μm. The distance between 10% and 90% of the average edge response is 1.6 μm, which is slightly smaller than the calculated distance between adjacent fiber illumination spots in the tissue.

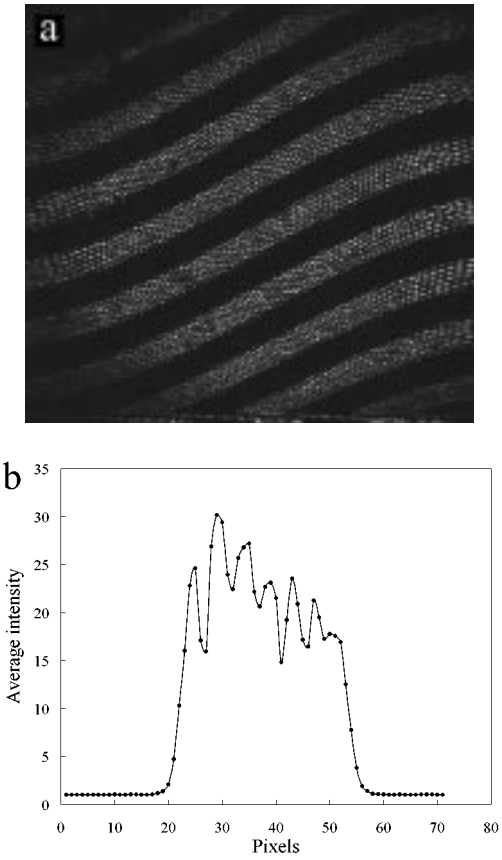

Fig. 4. (a) Confocal image of a Ronchi grating with 1000 line pairs/in, which corresponds to a period of 25.4 μm. The FOV is 180 μm$\times$ 170 μm. (b) Profile plotted along a line across the edge. The values are averaged over a line width of 200 pixels or equivalently 80 μm. The distance between 10% and 90% of intensity in the edge response is 1.6 μm.

The axial response of the confocal microscope was measured by moving the Ronchi grating through the focus of the miniature objective and recording a series of videos at different axial positions. An average image was calculated from each of the videos. A bright region in the images was selected and average intensities over this region were calculated on each of the average images. The average intensity versus axial position is shown in Fig. 5 and the full-width at half-maximum (FWHM) is 10 μm. The spatial resolution measured from planar samples are sufficient to image epithelial cell nuclei, which have an average diameter of 5–10 μm.

In order to assess system performance on microscopic objects, 4.3-μm polystyrene microspheres were immersed in water and imaged by the FCRM (Fig. 6). Individual microspheres as well as clumps were visualized in the image. The image of the microspheres demonstrates the ability of the FCRM to image objects of similar size to epithelial cell nuclei.

Fig. 7 shows an image of a cervical biopsy taken from abnormal epithelial tissue. The image plane was located at ap-

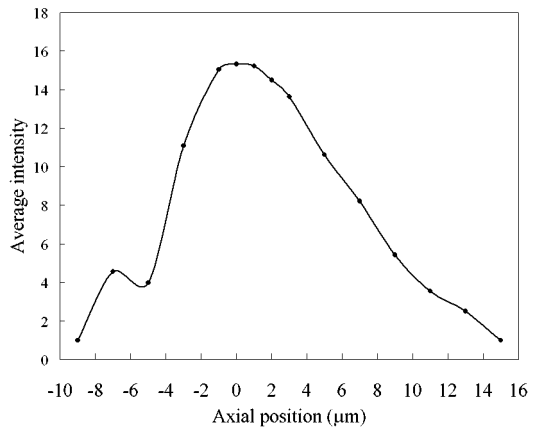

Fig. 5. Axial response of the confocal microscope was measured by moving the Rochi grating through focus of the objective and recording a series of images at different axial positions. An average intensity was calculated over the same bright region on the grating for each image. The average intensity versus axial position is plotted and the FWHM is 10 μm.

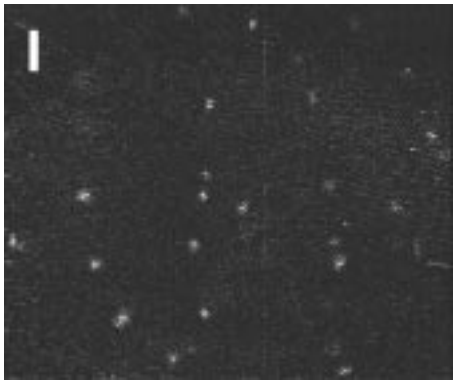

Fig. 6. Image of 4.3-μm polystyrene microspheres in water. Scale bar is 20 μm.

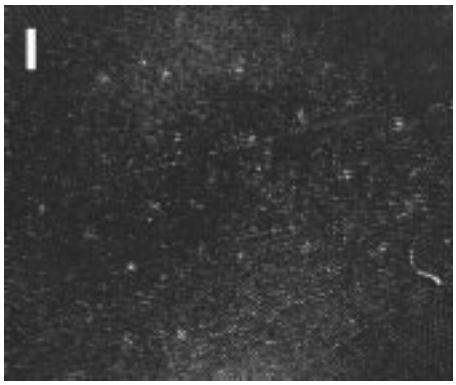

Fig. 7. Image of an abnormal biopsy taken from the cervix. The image plane is approximately at 100 μm below the surface. Scale bar is 20 μm.

proximately 100 μm below tissue surface. An *in vivo* image of the lip is shown in Fig. 8. The exact depth of the image is unknown but approximately 30 μm below the surface. Cell nuclei are clearly visualized in these images. Both video files and still images were obtained from these biological specimens. Still images, even with background subtraction and contrast/brightness adjustment, do not appear to be as good as video images. Small

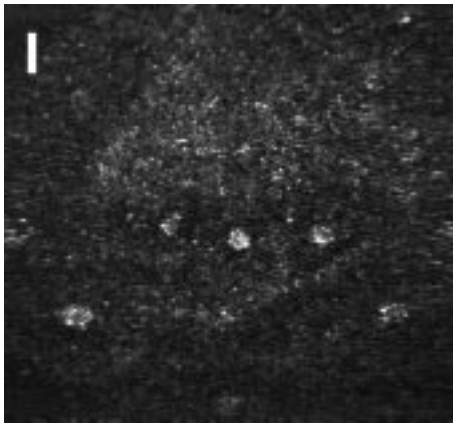

Fig. 8. *In vivo* image of human lip. The exact depth of the image is unknown but approximately 30 μm below the surface. Scale bar is 20 μm.

movements of the object across the FOV greatly facilitate perception and recognition of small or dim features over a constant background in the videos. Persistence of vision provides a time-averaging effect on the image sequence so the effects of noise and pixilation are greatly reduced.

IV. Discussion

The images show that the system is able to image cell morphology and tissue architecture in epithelial tissues *in vivo*. The system performance obtained by using the miniature objective is comparable to that using a commercial objective [9]. Videos acquired and displayed at 15 frame/s provide better visualization than still images, which partly compensates for the pixilated appearance due to the fiber bundle. Although images of fixed biopsies show good contrast at 100 μm below tissue surface, the maximum depth of imaging has not yet been explored *in vivo*.

The suction device (Fig. 3) is designed to achieve axial scanning of the focal plane within tissue. When soft tissue such as the oral mucosa is imaged, the image plane can be stably maintained and controlled with cellular resolution. However, large movements of the objective relative to the tissue must be minimized; otherwise motion artifacts overwhelm positioning stability provided by suction. This can be achieved while holding the probe steadily with one hand. Air bubbles trapped in the space between tissue and the objective can also impede the effectiveness of suction. However, trapped air bubbles, if any, can be easily removed before placement of the objective against tissue.

V. Conclusion

We have built a fiber-based confocal microscope that has a miniature objective lens and a positioning and axial scanning mechanism. The system shows sufficient resolution and sensitivity to image biological samples with cellular and subcellular resolution at half video rate. No fluorescent stain is needed since reflected light from the samples is detected. We believe that the FCRM will be highly useful for the recognition and monitoring of pathology in epithelial tissues *in vivo* and the system will be used in clinical trials on the cervix and oral cavity in the near future.

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