Fiber optic confocal reflectance microscopy: a new real-time technique to view nuclear morphology in cervical squamous epithelium

in vivo

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Abstract: We present a fiber optic confocal reflectance microscope (FCRM) which can be used to image epithelial tissue with sub-cellular resolution in vivo. Confocal images of normal and abnormal appearing cervical tissue were obtained in vivo from eighteen patients undergoing colposcopic examination of the cervix; biopsy specimens were taken from imaged sites. The measured lateral and axial resolutions of the system were 1.6 µm and 3 µm, respectively. Morphologic features, including nuclear size and nuclear-to-cytoplasmic ratio, were extracted from confocal images obtained at various depths beneath the epithelial surface. Image features extracted from confocal images compared well with features extracted from confocal images obtained in vitro and from previous histopathologic studies. This study shows that fiber optic confocal reflectance microscopy can be used to visualize the morphology of cervical epithelium in vivo.

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OCIS codes: (180.1790) Confocal microscopy; (170.3880) Medical and biological imaging; (110.2350) Fiber optics imaging; (170.4440) ObGyn

References and links


1. Introduction

Confocal microscopy is an indispensable tool in cell biology; the optical sectioning ability of confocal imaging enables the study of molecular and morphologic changes in thick biologic specimens with sub-cellular resolution. Typically, because of the need for large microscope objectives, toxic fluorescent dyes for image contrast, and relatively long image acquisition times, confocal microscopy has not been used to examine living tissue. Recent developments in photonic and electronic technology provide the ability to adapt confocal microscopes to non-invasively assess tissue abnormalities in real time. In vitro studies with biopsy specimens suggest that confocal imaging can provide useful diagnostic information about the presence of precancerous lesions; confocal images of normal and dysplastic cervical biopsy specimens obtained with a confocal reflectance microscope showed a strong correlation between nuclear morphologic features extracted from confocal images and histopathologic diagnosis [1]. This in vitro work suggests that the ability of confocal microscopy to visualize tissue morphology without biopsy provides a potential tool to improve the detection of precancerous tissue in vivo.

Until recently, in vivo confocal images of reflected light in humans have only been obtained non-invasively from cornea [2], the skin [3-4], the lip and tongue [3,5] because these organ sites are more easily accessible with conventional microscopes. The ability to obtain confocal images of normal and diseased tissue in vivo is limited by the ability to bring the tissue of interest in contact with the microscope objective. Flexible confocal microscopes incorporating either a solitary optical fiber [6,7] or a fiber optic imaging bundle [8] are needed to facilitate in vivo imaging of less accessible organ sites. In vivo confocal imaging of fluorescent light has been demonstrated with fiber optic confocal microscopy; however the application of fluorescent dyes is required to achieve sufficient signal level and image contrast [9,10]. In confocal reflectance microscopy, differences in refractive indices of the cell nucleus and cytoplasm provide a source of image contrast [11]. These differences can be enhanced using simple, non-specific contrast agents such as acetic acid [12] which are routinely used in clinical examination. Designing fiber optic confocal microscopes to image reflected light is
more challenging because of the difficulty of rejecting specular reflection from the proximal and distal surfaces of the fiber optic.

We recently reported the design of a fiber optic confocal reflectance microscope (FCRM) and showed it can image ex vivo cervical biopsy specimens with sub-cellular resolution [13,14]. Here we report the results of the first pilot clinical trial using this FCRM to image cervical epithelium in vivo in a group of 18 women. The goal of this study is to determine whether confocal images obtained with the FCRM could be used to identify morphologic features associated with nuclei throughout the epithelium. One of the main hallmarks of cervical precancer is an increase in nuclear size and an increase in the ratio of the size of the nucleus to the size of the cell; thus the ability to image nuclear morphology in vivo represents the first step toward developing a non-invasive diagnostic tool for in vivo detection of epithelial precancers and cancers.

2. Materials and methods

2.1 Confocal imaging system

A schematic diagram of the FCRM is shown in Fig. 1. A fiber optic bundle consisting of 30,000 fibers is located between the scanning mirrors and the objective lens. The illumination laser beam is coupled into only one fiber at a time on the proximal end of the fiber bundle. On the distal end of the fiber bundle each fiber serves both as a point light source and a detection pinhole. This illuminated fiber is imaged onto the tissue by a miniature objective lens; backscattered light from the tissue is imaged by the objective lens and relayed through the fibers to a beam splitter and photodetector. Backscattered light from out-of-focus regions in the tissue is distributed over multiple fibers and mostly rejected by a pinhole aperture in front of the detector. En face confocal images are produced by raster scanning the illumination spot across the proximal face of the fiber bundle. Index matching oil is utilized to reduce the specular reflection from both fiber end faces. A more detailed description of the fiber optic confocal microscope has been previously reported [14].

Three modifications were made to the system to perform the in vivo study presented here. First, a polarizing beam splitter was used to further reduce the unwanted background due to specular reflection. Light from the laser source is linearly polarized and the beam splitter is oriented such that most of the incident light is transmitted by the beam splitter. Specular reflection from the proximal end of the fiber bundle is also transmitted by the beam splitter because the polarization orientation of specular reflection is not changed. The incident light is depolarized as it travels through the fibers to the tissue. Therefore, about 50% of the
backscattered light from the distal end of the fiber bundle or the tissue is reflected by the beam splitter toward the detector. In comparison to the previous system, the total throughput is decreased but the overall image quality is enhanced because the background radiation resulting due to specular reflection from the proximal fiber end is reduced. Secondly, polishing the distal end of the fiber bundle at 7° further reduced the background resulting from the residual mismatch of refractive indices at the distal fiber end.

![Fig. 2. Photograph of the imaging probe, showing the fiber optic bundle (F), Teflon tube (T) and the brass tube (B).](image)

Finally, the distal end of the system was assembled into a probe as shown in Fig. 2. The miniature objective was attached to the fiber bundle through a custom-made connector. In order to obtain confocal images from living tissue the tissue needs to be held in stable contact with the end of the probe. In addition, to obtain images from different depths within the tissue, a provision for axial scanning is required. To achieve both goals, we used a simple hydraulic system that moves the tissue in the field of view along the optical axis. The holder of the miniature objective was designed such that the object plane of the objective is recessed 1 mm into the holder. The space between the miniature objective and the tissue is filled with water and connected to a syringe through Teflon tubing. After the objective is put against the tissue, water is sucked out the tissue chamber, thus creating a negative hydraulic pressure that draws the tissue upward and holds the objective against the tissue stably [14]. The distal end of the fiber bundle, objective lens and Teflon tubes were enclosed in a brass tube and sealed at the end for easier handling and cleaning. The dimensions of the imaging probe were 25 cm long and 1.0 cm in diameter, which was adequate for use to image the uterine cervix.

Prior to clinical use, the FCRM was used to image a reflective Ronchi grating to measure the size of the field of view and the lateral and axial resolution of the imaging system.

2.2 In vivo imaging of human uterine cervix

Cervical Intraepithelial Neoplasia (CIN) is a precursor to cervical cancer. The stage of CIN correlates with the proportion of the total epithelial thickness that shows morphologic abnormalities, including increased nuclear size, increased nuclear-cytoplasmic ratio, nuclear hyperchromasia, and pleomorphism. Eighteen patients participated in this study at the colposcopy clinic of the University of Texas M.D. Anderson Cancer Center. The patients were referred to this clinic for suspected CIN on the basis of an abnormal cervical cytology or for removal of the cervical tissue using the loop electrical excision procedure (LEEP) due to a previous diagnosis of cervical precancer. Patients who were aged at least 18 years and not pregnant were eligible for this study. Written informed consent was obtained from each of the participating patients. The study was reviewed and approved by the Surveillance Committee at the University of Texas M.D. Anderson Cancer Center and the Institutional Review Board at the University of Texas at Austin. Immediately after standard colposcopic examination wherein 6% acetic acid was applied to the cervix, the imaging probe was placed against a colposcopically abnormal location and a colposcopically normal location on the cervix by the clinician who performed the colposcopy exam. The suction device was used to move the
tissue toward the objective lens. Confocal images were acquired using a frame grabber and displayed on a computer monitor at 15 frames per second. Images displayed on the monitor were also saved into video files in AVI format for later processing. Following video acquisition, biopsies were taken from the imaged sites and submitted for routine histological examination using hemotoxylin and eosin (H&E) stain. The stained sections were examined by an experienced gynecologic pathologist (AM).

2.3 Image processing

Individual images with clearly seen nuclei were selected from the video files; these images were background-subtracted and resized using the procedures described previously [13]. In order to avoid redundancy in the data, the images from different tissue depths were chosen carefully so that no nuclei appeared in more than one image. The cell nuclei were identified and segmented manually to allow analysis of nuclear morphologic features. The number of pixels in each nucleus was counted and nuclear area was calculated using the calibration factors obtained from the grating image. Nuclear-cytoplasmic ratio (N/C) was calculated by dividing the total nuclear area in an image by area of the active field of view. Areas without any visible signal or blocked by air bubbles were excluded from the active field of view.

3. Results

The image field of view was 160-250 μm, depending on the magnitude of scanning. The measured lateral resolution was 1.6 μm. Figure 3 shows normalized intensity of the Ronchi grating versus distance of the grating from the focal plane. Thirty images were captured with a 1 μm increment in axial position of the grating. The gray-scale values of pixels were averaged over a 16 x 36 μm area in the center of the field of view for each image. The full width at half magnitude of the axial response is 3 μm, which agrees well with the predicted value of 3.1 μm previously reported [14].

![Fig. 3. The optical sectioning capability of the system is illustrated by imaging a planar reflective grating at different positions along the optical axis. Average intensity over a 16x36 μm area from thirty images of the grating is normalized and plotted. The full width at half the maximum magnitude is 3 μm.](image)

In this in vivo clinical study, we successfully obtained confocal images of cell nuclei from 15 cervical sites on 9 patients. Thirteen of the 15 sites with visible nuclei were diagnosed
histologically as normal; one site was diagnosed as CIN 1 and one was diagnosed as CIN 3. The major problem preventing visualization of nuclei was malfunction of the suction device, which was due to air bubbles trapped between tissue and the miniature objective and/or inadequately sealed between tissue surface and the probe tip; during image acquisition on one of the 18 participants, the suction device failed because the Teflon tube was clogged with blood and/or tissue debris.

Figure 4 shows a confocal video of normal cervical epithelium in vivo. The depth of the image plane in the tissue increases, starting at the superficial epithelium and reaching the basement membrane. The field of view is 250 x 240 µm. Cell nuclei can be visualized in real time throughout the whole thickness of epithelium. The borders of cells are also visible in the images of the superficial and intermediate layers. The nuclei are more densely packed in the deeper layers close to the end of the video. Beyond the basal layer, the image contrast is insufficient to resolve cell nuclei in the stroma or tissue structure. A total of 16 or 17 layers of cells can be seen throughout the entire epithelium in the video. The maximum depth imaged is estimated to be 190 µm assuming an average thickness of 8 µm for the upper eight cell layers and an average thickness of 15 µm for the deeper layers [15].

Figure 5 shows three representative confocal images of cervical squamous epithelium and photographs of H&E sections from the corresponding biopsy specimens. The confocal images were obtained from superficial layers of the epithelium. Figures 5(a) and (c) show confocal images of epithelial tissue diagnosed as normal from two different patients. Nuclear size and number of nuclei per unit area are similar in these confocal images. The corresponding H&E sections are shown in Figs. 5(b) and 5(d) respectively. Figure 5(e) shows a confocal image with the benign findings of hyperkeratosis and atrophy taken from another patient. Figure 5(f) shows the corresponding H&E section. The confocal image in Fig. 5(e) shows a higher level of background and a slightly larger nuclear size. The higher level of backscattering could be due to higher level of keratin and/or the densely packed nuclei near the basement membrane.
Fig. 5. (a) and (c) Confocal images of normal cervical epithelium taken from two patients. (e) Confocal image of hyperkeratotic and atrophic cervical epithelium from another patient. Photographs in (b), (d), and (f) show the corresponding H&E sections of biopsy specimens taken from the imaged tissue sites. The scale bars are 20 µm in the confocal images and 50 µm in the H&E sections.
Confocal images were also obtained from two samples with cervical precancer. Figure 6(a) shows the confocal image obtained from the superficial epithelium of the sample with CIN 1. The corresponding H&E section in Fig. 6(b) shows undifferentiated cells with enlarged nuclei in the lower one-third of the epithelium, which is characteristic of CIN 1 lesions. Confocal images were successfully obtained only from the superficial epithelium in the sample due to insufficient suction provided by the syringe pump. Since tissue diagnosed as CIN 1 is typically normal throughout the upper two-thirds of the whole epithelial thickness, the confocal images from this patient are not able to provide information to differentiate CIN 1 lesions from normal epithelium. Figure 6(c) shows the confocal image obtained from the superficial epithelium of the sample with CIN 3; Figure 6(d) shows the corresponding H&E section. Nuclear features are difficult to obtain from this sample due to the relatively low image quality and the limited number of images with visible nuclei.

A total of 103 images were selected from 15 confocal videos of normal cervical tissue and one tissue site with CIN 1. 1263 nuclei were hand segmented from the selected images and used to characterize nuclear morphologic features of normal cervical epithelium in vivo. Images from the CIN 1 site were included in the analysis because only the superficial layers of epithelium were imaged with the FCRM. The average nuclear area and nuclear-cytoplasmic ratio (N/C) from these 15 video segments are plotted as open triangles in Fig. 7.
Each point in the graph represents measurements from one confocal video. The nuclear area was averaged over all of the segmented nuclei from each video. The N/C was averaged over selected images from each video.

![Graph showing scatter plot of measured average nuclear area and nuclear-cytoplasmic ratio (N/C) from this in vivo study (open triangles) and a previous in vitro study reported by Collier et al. (black diamonds) [1]](image)

**Fig. 7.** Scatter plot of measured average nuclear area and nuclear-cytoplasmic ratio (N/C) from this *in vivo* study (open triangles) and a previous *in vitro* study reported by Collier et al. (black diamonds) [1]

The results obtained from this *in vivo* study were compared to results of a previous study to image nuclear area and N/C from normal cervical biopsies and samples with CIN 1 *in vitro*. Collier et al. measured nuclear area and N/C of *ex vivo* cervical biopsy specimens with a non-fiber optic confocal microscope [1]; morphologic features extracted from Collier’s study are plotted as black diamonds in Fig. 7. Collier obtained one reflectance confocal image at 50 µm below the tissue surface from each of 29 biopsy specimens diagnosed as normal or CIN 1. Each point in the graph represents measurements from one confocal image. On average, the results of the previous *ex vivo* study show a slightly larger average nuclear area and higher N/C values than the current *in vivo* study. The difference in measured nuclear area is partly due to underestimation of nuclear size with the FCRM system because of the pixelation in the images caused by the fiber optic bundle. The larger difference in measured N/C values suggests shrinkage of tissue volume during the process of biopsy.

We compared results measured from confocal microscopy obtained *in vivo* in this study and *in vitro* by Collier to morphologic features measured from normal and precancerous cervical tissue by Walker [15]. Walker et al. measured the nuclear diameter and N/C of normal cervical tissue, CIN 1, 2, and 3 in histology sections. A 20% reduction in the linear dimensions of tissue has been estimated due to the processes of removing and fixing tissue for histological analysis [16]. The data shown here are taken from measurements of the upper half of the epithelial thickness. Figure 8(a) compares the average nuclear diameter and Fig. 8(b) compares the N/C measured in these three studies. The average nuclear diameter and N/C obtained from the *in vivo* confocal microscopy study compare well with the values reported from normal and CIN 1 specimens in the previous studies. As tissue progresses from normal to CIN 3, the average nuclear diameters increase slightly. The N/C increases dramatically between specimens diagnosed as CIN 2-3 and those diagnosed as normal or CIN 1, as assessed both by reflectance confocal microscopy and histology.
4. Conclusions and discussion

Images obtained in vivo with the FCRM show that reflectance confocal microscopy can be used to image the morphology of cervical squamous epithelium. In particular, image features such as nuclear size and nuclear-cytoplasmic ratio correspond well with those assessed by histopathology. These features are important in the diagnosis of precancerous epithelial lesions, thus we suggest that reflectance confocal microscopy can potentially improve early detection of precancers.

We imaged cell nuclei in 13 normal cervical sites and only one CIN 3 site in the current in vivo study due to the relatively low specificity of colposcopy. The imaging conditions were not optimized during imaging of the CIN 3 site. In a previous ex vivo study with a non-fiber optic confocal reflectance microscope, images taken from four cervical biopsy specimens with CIN 3 showed both increased nuclear size and density as well as a higher degree of scattering than images of normal cervical biopsy [17]. This is consistent with results from a three-dimensional finite-difference time-domain model of normal and dysplastic cervical cells [18]. The increase in backscattering from dysplastic cells is due to increased refractive index heterogeneities within the nuclei of dysplastic cells, which could provide additional information in discrimination between normal and precancerous tissue.
To our knowledge, we present the first noninvasive imaging system to provide reflectance confocal images of cervical epithelium with sub-cellular resolution. The capability of the FCRM to visualize individual cells and cellular structures such as nuclei is a significant improvement to diagnostic imaging techniques. Cell morphology and tissue architecture can be appreciated throughout the entire epithelium as in histopathologic examination. Although the resolution of the FCRM is slightly inferior to that of the conventional microscopes used in histology, nuclear morphologic features such as nuclear area and nuclear-cytoplasmic ratio can be extracted from the confocal images and used to differentiate between dysplastic and normal tissue [1]. The FCRM system provides high-resolution, noninvasive imaging in real time, which can potentially allow the physicians to survey large area of tissue without taking excessive biopsy specimens. This could improve the accuracy and efficiency of detecting intraepithelial neoplasia in the cervix and other organ sites such as oral cavity and colon. Studies with larger number of subjects are needed to assess the sensitivity and specificity of using FCRM to detect precancerous lesions. Other potential applications of the FCRM include real time margin detection of tumors during surgery and determination of response to chemotherapy or chemoprevention.

Acknowledgments

Financial support from the National Institutes of Health is gratefully acknowledged (grant number R02-CA82880). The authors would like to thank Tom Collier for providing data obtained with the non-fiber confocal system and Brette Luck for the help in image processing.