Confocal microscopy: Imaging cervical precancerous lesions

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Abstract

Objectives. We explore the clinical potential of reflectance and fluorescence confocal microscopy to image the morphologic and biochemical changes associated with precancer, in order to aid in the detection and diagnosis of cervical dysplasia.

Methods. Cervical epithelial tissue samples imaged ex vivo or in vivo were obtained from M. D. Anderson Cancer Center and Lyndon B. Johnson Hospital in Houston, Texas. Confocal reflectance microscopy was used to image ex vivo cervical biopsies and in vivo cervical tissue. Confocal fluorescence microscopy was used to image ex vivo cervical tissue slices.

Results. We present reflectance and fluorescence confocal images of cervical tissue demonstrating the ability to differentiate between normal and abnormal cervical tissue.

Conclusions. We believe that there is significant clinical potential for confocal microscopy to provide a sensitive and specific method for cervical precancer detection.

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Introduction

Cancer of the cervix is the second most common cancer in women worldwide, following cancer of the breast. Cervical cancer might well be completely prevented by vaccines that prevent the viral infection and detection and removal of precancerous lesions that occur on the cervix. The Papanicolaou (Pap) smear screening and timely treatment (when necessary) have significantly reduced the incidence of cervical cancer in the developed world. However, in the developing world, where approximately 83% of new cases occur [1], women have limited access to the necessary supplies and trained personnel needed to screen for this disease. Additionally, women in developing countries are much less likely to return to receive their Pap smear results or return for follow-up or treatment. Typically, a Pap smear can take 2 to 3 weeks for processing and analysis. Immediate results at the patients’ first visit may increase the number of appropriate treatments performed and reduce the mortality associated with this disease.

Precancerous lesions are distinguished by morphological and biochemical changes in the epithelium, which are currently characterized by tissue biopsy, physical sectioning of the biopsy, and microscopic imaging of the tissue sections. Ideally, in vivo imaging could allow real-time identification of these changes. With in vivo reflectance confocal microscopy, we can visualize the increase in nuclear-to-cytoplasmic ratio and nuclear density associated with the progression of cervical intraepithelial neoplasia (CIN) without tissue removal. With fluorescence confocal microscopy, we can detect the changes in cellular metabolism that accompany dysplastic progression, which is characterized by the metabolic indicators NADH and FAD.
Confocal microscopy is an optical imaging technique that has the potential to provide a more sensitive and specific method to detect cervical precancerous lesions in real-time. The “optical sectioning” principle of confocal microscopy, as illustrated in Fig. 1, allows high-resolution structural imaging at the cellular level at varying depths in the cervical epithelium, non-invasively. This schematic demonstrates light pathways in reflectance confocal imaging of tissue. The point source illumination (solid line) is focused by a lens to a point within the sample. Since tissue is highly scattering, some of the illumination light is reflected from all points illuminated within the sample. The light rays reflected from the focal region of the lens (solid line) are refocused by the lens and partially reflected by the beam splitter to a point at the conjugate image plane. A pinhole aperture placed at the conjugate image plane allows light from the focal region to pass to the detector; whereas, light from other depths in the tissue (dashed line) is out of focus and significantly rejected. Similar rejection occurs for light coming from depths less than the focal region. Optical sectioning is accomplished for lateral points as well since light returning from these points is imaged lateral to the pinhole. Thus, the confocal system is able to isolate light returning from a finite volume, without the need for physical sectioning. Scanning the focal spot in the axial and radial dimensions forms a map, or image, of the reflectance values from the focal region of each point in the sample.

Materials and methods

Cervical tissue specimens

All research protocols received approval from the Internal Review Boards of the University of Texas at Austin, The University of Texas M. D. Anderson Cancer Center, The University of Texas Health Science Center at Houston, and the Lyndon Baines Johnson Hospital and Harris County Hospital District. All patients who had biopsies removed signed an informed consent and entered a registered protocol. All cervical tissue specimens, both normal and dysplastic, were obtained at M. D. Anderson Cancer Center or Lyndon B. Johnson Hospital in Houston, Texas. Sites were chosen based on the colposcopic appearance by a trained clinician. Confocal reflectance imaging was performed on ex vivo intact cervical biopsies and in vivo cervical tissue. Ex vivo confocal fluorescence imaging was also performed on 200 μm thick, transverse, fresh tissue slices taken from biopsies. The diagnoses of the specimens used for both ex vivo and in vivo imaging were determined by fixing and slicing of the tissue, staining with hematoxylin and eosin (H & E), and examination by a pathologist.

Confocal microscopy

Images of cervical biopsy specimens were obtained using the epi-illumination confocal reflectance microscope previously described by Smithpeter et al. [2] and Collier et al. [3]. Briefly, this confocal microscope uses 810 nm continuous wave laser illumination. The frame rate is 7.5 frames per second, and the measured lateral and axial resolution are 0.8 and 2–3 μm, respectively. Images from this confocal microscope have been resized to compensate for a non-linear scanning mirror, and contrast and brightness have been enhanced.

In vivo confocal images of cervical epithelium were acquired with a fiber-bundle confocal reflectance microscope previously described by Kung-Bin et al. [4]. A glass miniature microscope objective [5] and a fiber bundle enable real-time imaging of the cervix in vivo. A 1064 nm laser beam is scanned across the fiber bundle at a rate generating 15 frames per second. The measured lateral and axial resolution are 2 and 3 μm, respectively. Additionally, confocal images of cervical biopsies have been obtained using the same fiber optic confocal microscope with a new inexpensive injection-molded plastic miniature objective lens [6,7]. Fiber confocal images have undergone image processing to remove the pattern of the fiber bundle from the images. Images have been background subtracted, resized to compensate for a non-linear scanning mirror, and median filtered using an anisotropic median diffusion filter [8]. Finally, brightness and contrast have been enhanced to improve viewing.

Contrast in reflectance microscopy is provided by spatial variation in refractive index in the tissue [9]. Additionally, the application of 6% acetic acid solution increases contrast,
improving visualization of nuclei in confocal reflectance images [10]. All confocal reflectance images of cervical tissue presented here were obtained after application of 6% acetic acid.

Fluorescence confocal images of cervical epithelium and stroma were acquired with an inverted Leica confocal laser scanning fluorescence microscope and a 40× oil-immersion objective. Fluorescence was excited at 351–364 nm and detected with a 405 nm band pass filter [11].

Results and discussion

Confocal reflectance microscopy—ex vivo results

In a pilot study of ex vivo cervical biopsies from 25 patients [3], we found that confocal reflectance microscopy can detect the presence of dysplasia with a sensitivity of 100% and a specificity of 91%, based solely on the nuclear-to-cytoplasmic ratio in the images. In comparison, the sensitivity and specificity of the colposcopic appearance compared with histopathologic diagnoses of these specimens were 91% and 62%, respectively. This demonstrates the significant clinical potential of confocal microscopy to detect cervical dysplasia. Since the algorithm was calculated from a pilot study, it may be over-trained, producing artificially high sensitivities and specificities. A pilot study of this device in patients is ongoing.

Fig. 2 illustrates representative confocal reflectance microscopy images of cervical epithelium from normal (a) and dysplastic (b) cervical epithelial tissue. White round objects identify nuclei in the confocal images. These images of cervical biopsies clearly demonstrate the increase in nuclear-to-cytoplasmic ratio in the epithelium associated with dysplasia.

Fiber optic confocal reflectance microscopy—in vivo results

In a pilot clinical study of in vivo imaging of the cervix using a fiber optic confocal reflectance microscope, we have obtained exceptional images in real-time with sub-cellular resolution of normal and precancerous cervical epithelium [12]. Preliminary results show that the nuclear-to-cytoplasmic ratio of cells in these images is comparable to other studies. Fig. 3 demonstrates images obtained in vivo of both normal (a, b) and abnormal (c, d) cervical sites from a single patient. At histologic examination, the abnormal site was diagnosed as CIN II/III. Again, white circles identify nuclei. The two images of normal epithelium demonstrate an increase in the nuclear-to-cytoplasmic ratio from the differ-

Fig. 2. Ex vivo reflectance confocal images from a normal/abnormal cervical biopsy pair. Increased nuclear density can be seen in the confocal image of the abnormal sample (b), in comparison to the normal sample (a). The focal planes of both images are near the surface of the epithelium. The field of view is approximately 515 μm × 660 μm.

Fig. 3. In vivo fiber optic confocal reflectance images extracted from videos of normal (a, b) and abnormal (c, d) cervical epithelium from patient 34. Images obtained near the surface of the tissue (a, c) and close to the basement membrane (b, d) demonstrate the relative change in nuclear density at different depths in the epithelium for normal and dysplastic cervical tissue. Field of view for all images is approximately 200 μm × 200 μm.
entiated superficial epithelium (Fig. 3(a)) to the dense basal epithelium (Fig. 3(b)). In contrast, the images of dysplastic epithelium show little change from the upper layer (Fig. 3(c)) to the basal layer (Fig. 3(d)) of the epithelium. These images demonstrate the potential to use confocal microscopy to image tissue in vivo, providing clinicians with structural information in real-time.

A new injection-molded plastic miniature objective lens has been used to obtain images of cervical epithelium with this fiber optic confocal microscope. This inexpensive alternative to the glass objective lens allows wide distribution of this imaging technology. A confocal image of the basal layer of cells in a normal cervical biopsy (Fig. 4) demonstrates resolution, penetration depth, and image quality similar to the glass miniature objective lens (Fig. 3).

Confocal fluorescence microscopy—tissue slices

Fluorescence confocal images from two patients showed two types of fluorescence patterns that can be distinguished in cervical epithelium [11]. Cytoplasmic fluorescence, attributed to mitochondrial NADH or FAD, is seen in the cytoplasm of basal cells and sometimes around the nuclei of intermediate and superficial cells. Peripheral fluorescence is also seen around the periphery of cells in the superficial and intermediate region and is currently thought to be caused by cytokeratins. Representative images of normal cervical tissue are shown in Figs. 5(a) and (d). Here, cytoplasmic fluorescence is usually limited to the basal and parabasal regions of the epithelium, leaving a large part of the total epithelial thickness (intermediate and superficial regions) occupied by peripheral fluorescence. Fluorescence from the cervical stroma has a very strong intensity and originates from stromal fibers. Previous research indicates that collagen and elastin are the major components of the fibrous stromal matrix [13], and collagen and elastin crosslinks are the major fluorophores in the stroma [14,15].

With cancer progression, cytoplasmic fluorescence, originating from mitochondrial NADH or FAD becomes more dominant then peripheral fluorescence and spreads through the total thickness of the epithelium. In LGSIL, cytoplasmic fluorescence occupies about 1/3 of the total epithelial thickness (Fig. 5(b)), whereas in HGSIL, cytoplasmic fluorescence occupies at least two thirds of the
distance between the basement membrane and the surface of the epithelium (Fig. 5(c)). This is in agreement with the previously established model of dysplastic developments in the cervix, where abnormal cells originating from the basal region invade the rest of epithelium. In addition, stroma adjacent to dysplastic epithelium (Figs. 5(e) and (f)) was observed to be less dense and to have a lower fluorescence than stroma next to normal epithelium (Fig. 5(d)). A decrease in stromal fluorescence was hypothesized to be caused by decomposition of the collagen fibers, accompanied by a reduction in the concentration of collagen crosslinks [11].

Conclusion

We have presented images exhibiting the clinical potential of confocal microscopy to aid in the detection and diagnosis of cervical dysplasia. Confocal reflectance and fluorescence microscopy may be able to identify the morphological and biochemical changes associated with the progression of precancer both non-invasively and real-time. With advancement of effective, cost efficient imaging technologies, this imaging modality may be able to provide a more sensitive and specific detection tool for the less resource-rich developing world.

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References