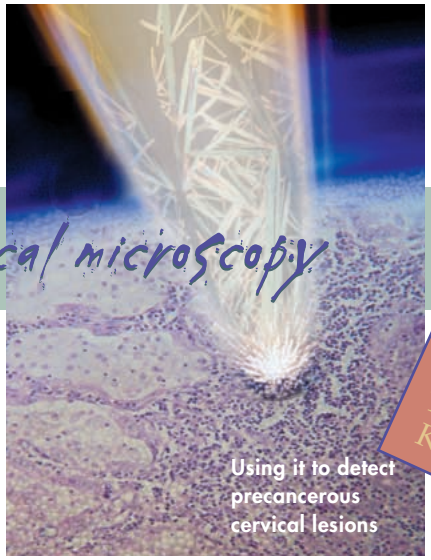
Confocal microscopy



Using it to detect precancerous cervical lesions

Brette Luck, Kristen Carlson, Tom Collier and Kung-Bin Sung

Optical technologies, such as reflectance and fluorescence microscopy, may help detect and diagnose cancers that originate in the epithelium. The epithelium is the layer of tissue that is exposed to the environment and lines the body's cavities. Cancers that originate in the epithelium include cervical, oral, colon, lung, stomach, bladder and skin cancers.

Since epithelial tissue protects the body from the environment, it has a high turnover of cells and, consequently, a greater chance of cell mutation. The basement membrane separates epithelial tissue from the next layer of tissue within the organ. The goal is to detect and remove lesions of cancerous cells within the epithelium before they penetrate through the basement membrane. This action prevents the precancer from establishing tumors in the neighboring tissue and metastasizing to other organs. In other words, removing precancerous lesions prevents the formation and progression of invasive cancer.

The Optical Spectroscopy Lab at the University of Texas at Austin is developing and applying fluorescent, confocal and polarized reflectance microscopes as well as contrast agents to enhance reflectance and fluorescence for detecting and diagnosing epithelial precancers. The goal is to perform diagnosis *in vivo*, i.e. in the body. The lab currently has an *in vivo*, fiber optic reflectance confocal microscope (FOCM) in clinical trials at the University of Texas M.D. Anderson Cancer Center in Houston.

Precancerous cervical lesions

Cancer of the cervix, the uterine cap, is the second most common cancer among women worldwide. (Breast cancer is number one.) As with other epithelial-originating cancers, cervical cancer can be entirely prevented by detecting and removing precancerous lesions.

The current screening method for cervical precancer is the Pap smear. This cytological technique consists of scraping cellular samples off the surface of the cervix. The samples are examined for their nuclear size and nuclear to cytoplasmic ratio.

When a Pap smear is abnormal, the next steps for diagnosis are a colposcopic examination of the cervix and a biopsy of the abnormal looking tissue. A colposcope is a microscope designed to examine cervices for precancerous lesions. Gynecologists identify abnormal areas of the cervix by cellular arrangement and aceto-whitening.

The biopsy taken from a potentially precancerous site is sliced, stained, and, like the cells acquired through the Pap smear, examined for cells with larger nuclei and a larger nuclear to cytoplasmic ratio then cells in normal tissue. Figure 1a shows a stained slice from a normal cervical tissue biopsy. Figure 1b shows a stained slice from a precancerous cervical tissue biopsy. For this stain, the nuclei are blue and the cell cytoplasm is pink. Note how the nuclei in the precancerous tissue are larger and more densely packed than the nuclei in normal tissue. Pathologists use nuclear size and density information to diagnose the presence or degree of cervical precancer. If a patient has cervical precancer, a gynecologist will remove the lesion, effectively preventing the development of cervical cancer.

Figure 1 also demonstrates how precancerous lesions begin at the basement membrane and spread upward. Once the precancerous lesion reaches the top of the epithelium, it most likely will invade the stroma through the basement membrane and become cancer. This is why early detection is important. Cervical cancer can spread quickly because of its close proximity to other organs.

Confocal microscopy

In vivo confocal imaging can provide similar information to histological analysis of biopsies just described. However, that 3D sub-cellular resolution is achieved without removing tissue, and contrast is provided without stains. The optical sectioning principle of confocal imaging is illustrated in Fig. 2. The illumination light (solid line) passes through a beam splitter and 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. Ordinarily, this reflection would degrade image resolution. However, the light rays reflected from the focal region of the lens (the solid line) are refocused by the lens and partially reflected by the beam splitter to a point at the conjugate image plane.

If a small pinhole aperture is centered on the focused beam in the conjugate image plane, most of the light returning from the focal region in the tissue is passed to the detector. Light reflected from depths greater than the focus region (the dashed line) diverges and spreads out when it reaches the pinhole so the aperture significantly reduces its intensity and increases resolution. Similar rejection occurs for light coming from depths less than the focal region. 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 an image of the reflectance values from the focal region of each point in the sample.

Moving tissue towards the lens allows confocal microscopes to resolve nuclei at deeper depths in the tissue. Figure 1 C-J shows confocal images of both normal and precancerous tissue at various depths. In confocal images, nuclei appear white. As with the histology slides (Fig. 1 A, B), Fig. 1 C-J illustrates the increase in nuclear size and density found in precancerous cervical lesions. Unlike histology, which physically sections the tissue perpendicular to the surface, confocal microscopes optically section the tissue parallel to the surface.

Figure 1 C-J also shows that the depth of confocal imaging is limited by the penetration of light into the tissue. Decreased resolution in tissue as a function of light penetration is analogous to decreased visibility on a hazy day. On hazy days, small particles of

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moisture in the air scatter light, preventing resolution at far distances. Since each layer of tissue scatters light, light will only penetrate a few hundred microns into the tissue. The light penetration of the Optical Spectroscopy Lab's bench-top confocal microscope in normal tissue is 250 µm, which is nearly the depth of the entire epithelium layer (200-300 µm depending on the patient).

Sensitivity and specificity

Sensitivity and specificity are statistics used to compare similar medical tests' usefulness. Sensitivity is the percentage of actually diseased samples that the test identified as diseased. Specificity is the percentage of actually healthy samples that the test identified as healthy. The two statistics give complementary information that reflects a test's overall accuracy. For example, a test that diagnoses every sample as diseased would have a high sensitivity but a low specificity. Likewise, a test that diagnoses every sample as healthy would have a high specificity but a low sensitivity. A good test is one that is high in both areas, having the diseased tissue always diagnosed as diseased and the healthy tissue as healthy.

Colposcopy images the surface of the epithelium, while confocal microscopy images throughout the epithelium. Recalling that precancerous cervical lesions originate at the base of the epithelium (see Fig. 1), one would expect confocal microscopy to have higher sensitivity and specificity. Studies conducted by the Optical Spectroscopy Lab have shown that in discriminating between normal tissue and high grade precancers, confocal imaging has a sensitivity of 100% and a specificity of 91%.

These findings are appreciably better than colposcopy, which has a sensitivity of 85% and specificity of 69% for detection of high-grade precancerous lesions. Since processing biopsies is expensive, fewer false positive biopsies significantly decreases the overall screening costs for precancerous cervical lesions, not to mention the stress on the patient.

Contrast agents

Areas containing cervical precancer will turn opaque white when exposed to acetic acid as shown in Fig. 3. Gynecologists use this aceto-whitening effect to identify abnormal areas during colposcopic examination. Using instrumentation developed by the Optical Spectroscopy Lab, studies have shown

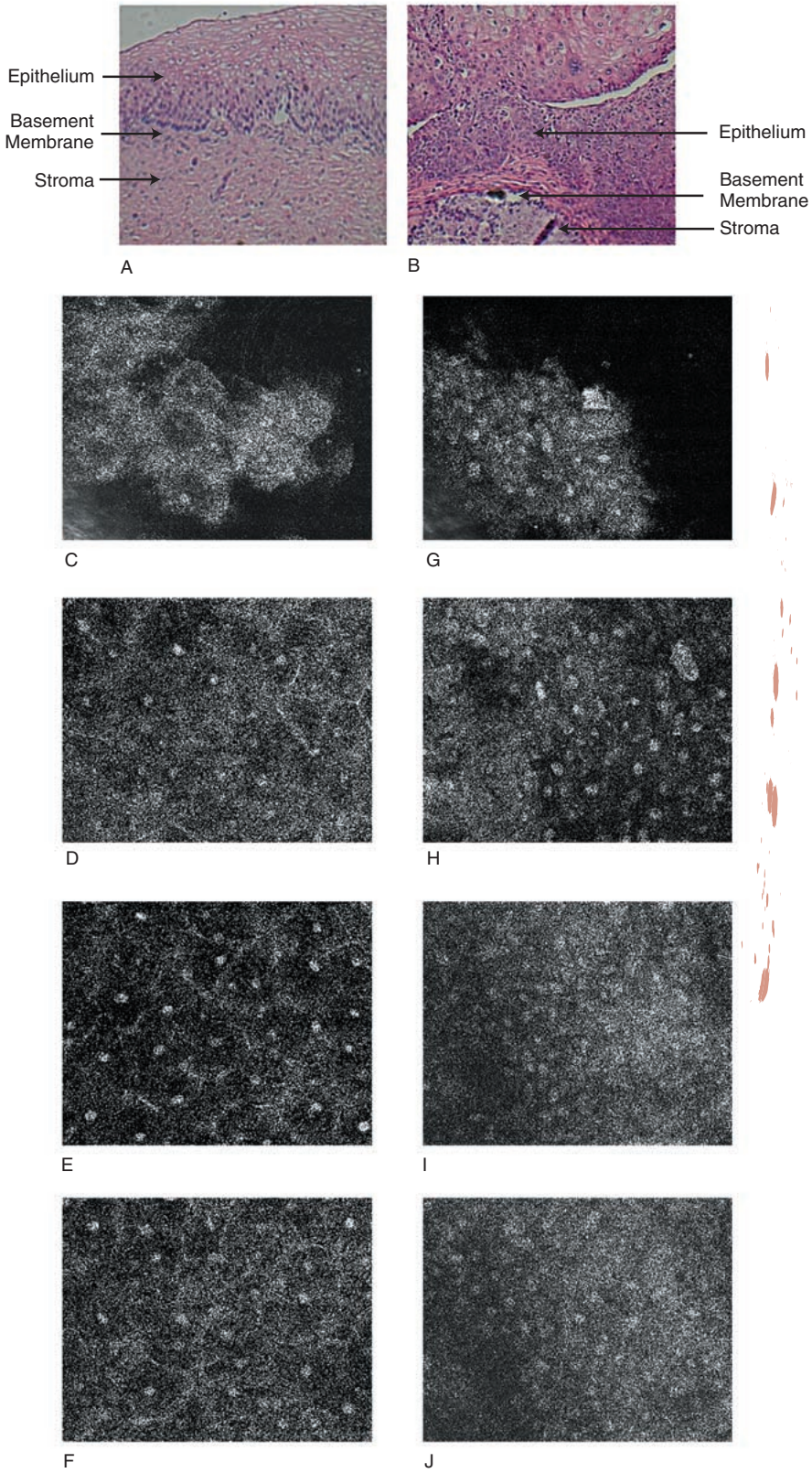

Fig. 1 Images of normal and abnormal cervical tissue biopsies. Stained slice of (A) normal and (B) abnormal cervical tissue biopsy slice. The nuclei are purple and the cell walls are pink. Confocal image of the normal cervical tissue biopsy at (C) 00µm (D) 50µm (E) 100µm and (F) 150µm. Confocal image of the abnormal cervical tissue biopsy at (G) 00µm (H) 50µm (I) 100µm and (J) 125µm. The nuclei are white. Figure 1 was originally published in: T. Collier, A. Lacy, R. Richards-Kortum, A. Malpica, M. Follen, "Near Real-Time Confocal Microscopy of Amelanotic Tissue: Detection of Dysplasia in *Ex Vivo* Cervical Tissue," *Academic Radiology*, vol. 9, no. 5, pp. 504-12, May 2002.

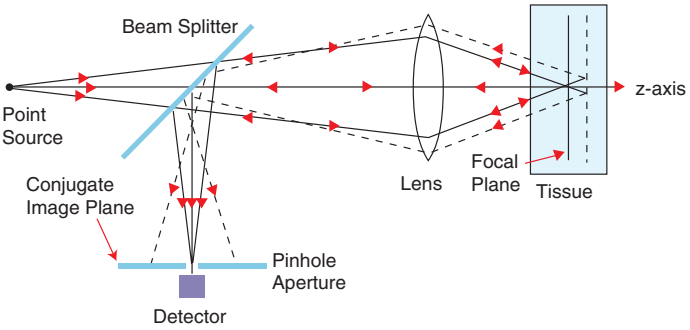

Fig. 2 Schematic of confocal microscopy for a single point. The pinhole aperture rejects out of focus light, thus optically sectioning the tissue. Figure 2 is adapted from T. Wilson, *Confocal Microscopy*. Suffolk: St Edmundsbury Press Ltd, 1990.

that acetic acid increases the ability of cervical epithelial nuclei to scatter light. Since cancerous lesions contain more nuclei, those tissue areas will scatter more light. Thus, they will appear white during colposcopic examination, as shown in Fig. 3.

The increase in nuclear scattering after applying the acetic acid also improves the contrast in confocal images. Figure 4 shows four images of cervical tissue biopsies taken by the non-fiber confocal system. The left images were taken without applying acetic acid, and the right images were taken a few minutes after applying acetic acid. As the images show, the nuclei appear much brighter in both the normal and precancer biopsies after applying the acetic acid. Although these images are from biopsies, the same effect occurs in vivo Note the acetic acid does not hurt the patient when done *in vivo*.

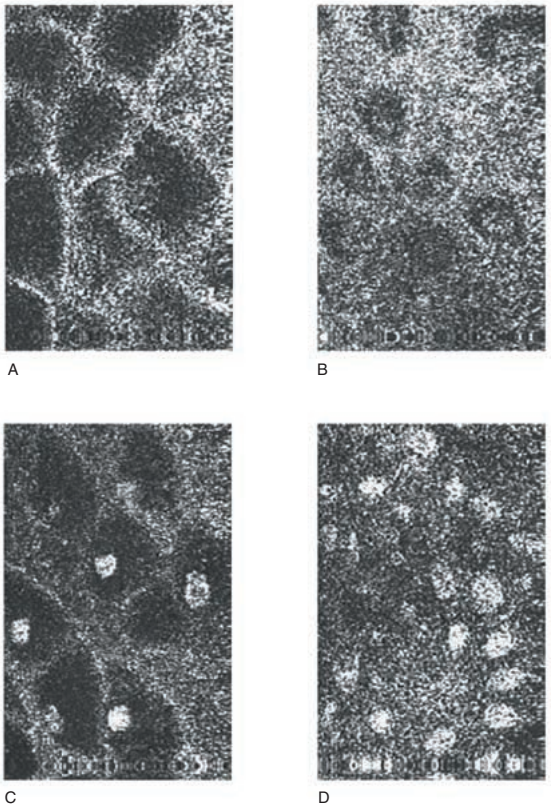

Fig. 4 Images form normal and abnormal cervical biopsies before and after application of acetic acid. The biopsies had been frozen and thawed before imaging. Images of normal cervical biopsy before (A) and after (B) application of 6% acetic acid. Note that the field of view is different for the images. Images of colposcopically abnormal cervical biopsy before (C) and after (D) addition of acetic acid. Note that the field of view is different for the images.

by the Optical Spectroscopy Lab (Fig. 5) uses a coherent fiber bundle image guide with 30,000 fibers and an outer diameter of 2.5 mm. Two scanning mirrors raster scan a focused laser beam (1064 nm wavelength) across the proximal end, or system side, of the fiber bundle illuminating one fiber at a time. The fiber bundle is scanned at a rate that provides 15 images per second. At the distal end, or tissue side, of the fiber bundle, the tissue is illuminated by the same fiber. In both the single fiber and fiber bundle configurations, the fiber core acts as the pinhole aperture (from Fig. 2) in the confocal system.

A specialized miniature microscope objective focuses the light onto the tissue, collects the light reflected from inside the tissue, and focuses the light back onto the fiber. The reflected light collected from the tissue returns through the same fiber that illuminated

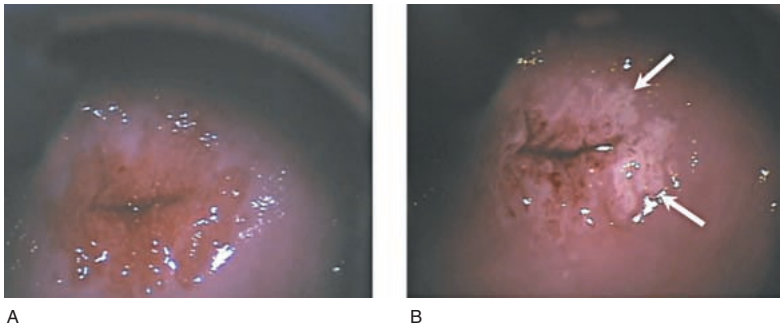

Fig. 3 A cervix (A) before and (B) after application of acetic acid. The opaque white areas in (B) may be precancerous lesions

Fiber-optic, *in vivo* confocal microscope

To use this valuable tool *in vivo* on cervical tissue, the confocal microscope has to be modified into a flexible endoscope. This adaptation is accomplished with 1) an optical fiber that separates the objective (lens) from the main part of the microscope, 2) miniaturizing the objective lens to a comfortable size for the patient, and 3) developing a way to move the tissue with respect to the objective lens.

A confocal microendoscope can be made using 1) a single optical fiber that scans across a region of interest, or 2) a stationary optical fiber bundle that builds up an area's image through raster scanning. The reflectance fiber-optic confocal microscope (FOCM) developed that spot. In collaboration with the University of Arizona, the complex objective lens in the FOCM was designed to have a wide acceptance angle at the tissue to collect as much reflected light as possible and a narrow focus at the fiber side to specifically match the fiber.

The miniature objective lens is only 22 mm long and 7 mm in diameter. The field of view for the system is approximately 250 μm x 250 μm, large enough to see a sufficient region of cells and small enough to be able to characterize individual cells. Because the resolution is limited by the center-to-center spacing between fibers (7 μm)—and is improved by the magnification of the objective lens (3.3x)—the lateral resolution can be calculated as 7 μm/3.3 = 2.1 μm. Since the diffraction-limited spot size of the fibers on the tissue (1.1 μm) is smaller than the spot separation, the pattern of the fiber bundle is superimposed onto the image of the tissue (Fig. 6). The

measured axial resolution is 3 µm.

In bench-top confocal microscopes, the stage holding the sample is usually moved axially to image deeper into the tissue. Moving tissue in vivo relative to an endoscope tip is much more complicated. The FOCM incorporates a hydraulic system for axial scanning.

The focal plane of the objective lens, located 450 µm from the surface of the objective lens, is offset from the tip of the endoscope casing. This setup forms a cavity that the tissue can be drawn into. To reduce the reflections from the lens/air and air/tissue surfaces, the well is filled with water before applying the probe tip to the tissue of interest. After application, a syringe pump, connected through a Teflon tube, extracts water out of the well that draws the tissue up into the well, through the focal plane of the microscope.

Atlas of cervical epithelium pathology

The *in vivo* as well as *ex vivo* cervical tissue imaged using the confocal microscopes were also examined by pathologists. The images and pathological findings were archived together to create an atlas of cervical epithelium pathology. This database is being used to develop algorithms to automatically recognize precancerous cervical lesions. Additionally, it is a resource for studying the development of cervical epithelial precancer.

Conclusions

The curable precursors to cervical cancer are cervical epithelial lesions that have larger and more densely spaced nuclei. A fiber-optic confocal microscope (FOCM) has been developed at the Optical Spectroscopy Lab to help detect and diagnose these lesions *in vivo*. With the aid of acetic acid as a contrast agent, the FOCM shows nuclear size and density information throughout the epithelium, presenting the same information as histology but without removing, staining and slicing cervical epithelial tissue. (There are also spatial resolution requirements for showing cell nuclei.) The Optical Spectroscopy Lab continues to develop confocal microscopic instrumentation, new contrast agents, and image processing techniques to improve early detection of precancerous cervical lesions.

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Read more about it

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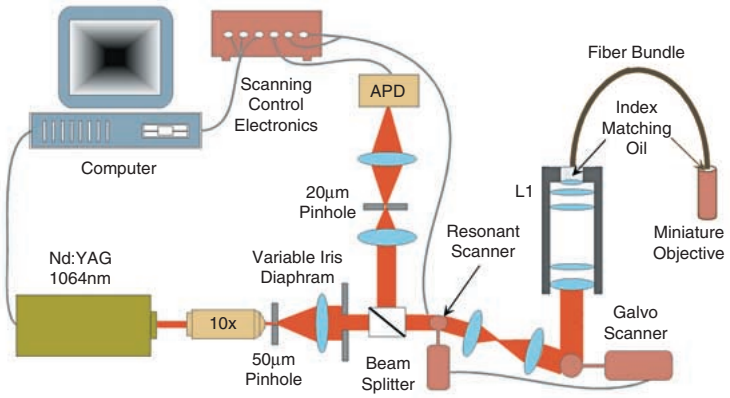

Fig. 5 Fiber Optic Confocal Microscope system diagram. Figure 5 was originally published in: K. Sung, C. Liang, M. Descour, T. Collier, M. Follen, R. Richards-Kortum, "Fiber-Optic Confocal Reflectance Microscope with Miniature Objective for In Vivo Imaging of Human Tissues," *IEEE Transactions on Biomedical Engineering*, vol. 49, no. 10, pp. 1168-72, Oct. 2002.

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Brette Luck received her BS in Electrical and Computer Engineering from The Ohio State University in June 2002 and began graduate work at the University of Texas at Austin's ECE department in August 2002 as a NSF Integrative Graduate Education and Research Training (IGERT) research fellow. Her advisors are Dr. Rebecca Richards-Kortum and Dr. Alan Bovik. She is interested in medical image processing for diagnosis and is currently working on an image-processing algorithm to automatically identify nuclei in fiber-optic confocal videos of cervical epithelium.

Kristen Carlson received both her BS and MS in Electrical Engineering from California Polytechnic State University, San Luis Obispo, in 2002. She is currently a NSF IGERT research fellow in the Biomedical Engineering Department at the University of Texas at Austin. Kristen is developing the next generation fiber-optic confocal microendoscope in Dr. Rebecca Richards-Kortum's Optical Spectroscopy Laboratory.

Tom Collier received his Bachelor of Architecture in 1987 from the University of Texas at Austin. He worked as a registered architect until reentering the University of Texas where he received his Masters of Electrical Engineering in 2000. He is currently pursuing a PhD in Electrical Engineering at the University of Texas at Austin. His thesis research explores the use of confocal microscopy as a tool for diagnosing precancers.

Kung-Bin Sung was born in Taipei, Taiwan. He received his BS degree in Electrical Engineering from the National Taiwan University, Taiwan, in 1996. He entered graduate school at the University of Texas at Austin in 1998 and received the MS degree in Biomedical Engineering in 1999 and the PhD. degree in Biomedical Engineering in 2002. He is currently working at Intel.

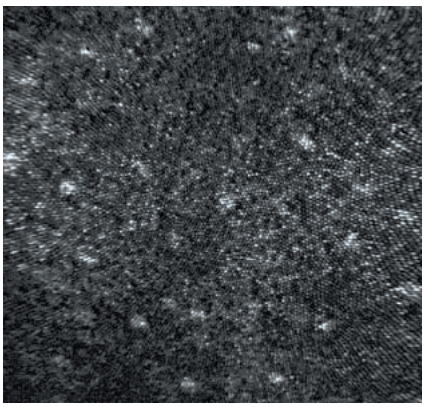

Fig. 6 A FOCM image of cervical epithelium. Notice that the fiber pattern is superimposed on the image.