Slit-scanning confocal microendoscope for high-resolution in vivo imaging

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We discuss the design and construction of a novel imaging system in which a fiber-optic imaging bundle and miniature optical and mechanical components are used to allow confocal fluorescence microscopy in remote locations. The instrumentation has been developed specifically for cellular examination of tissue for optical biopsy. Miniaturization of various components makes the device usable in a clinical setting. The numerical aperture of the beam in the tissue is 0.5, and the field of view is 430 μm. The measured lateral resolution of the system is 3.0 μm. The axial point and the axial planar response functions of the confocal system were measured with a FWHM of 10 and 25 μm, respectively. In vitro and in vivo images obtained with cell cultures, human tissue specimens, and animal models indicate that the performance of the device is adequate for microscopic evaluation of cells. © 1999 Optical Society of America

1. Introduction

Confocal microscopy is currently used in many biological imaging applications. Recently, there have been efforts to develop instrumentation capable of performing confocal imaging in vivo. Benchtop systems have been constructed for noninvasive investigation of skin with reflectance confocal microscopy. Other applications have included investigation of the ocular fundus and corneal tissue. For in vivo investigations of tissue inside the body, confocal imaging systems utilizing fiber-optic imaging bundles have been developed by various groups. One system for in vivo confocal imaging has been developed based on micromachined optical components that allow incorporation of the scanning mechanism inside the endoscopic head.

In this paper we present the design and demonstration of a confocal optical microendoscope. This instrument is a fluorescence imaging system that is minimally invasive and designed to provide high-resolution images of tissues to aid physicians in the diagnosis of pathology in situ. The instrument is designed to visualize the morphology of cells both on the surface and at selected depths below the surface. This capability would allow an examiner to determine if an abnormality is localized on the surface or is more aggressive (i.e., invading below the surface).

The basic system concept was initially described by Gmitro and Aziz and is based on the use of a fiber-optic imaging bundle. The system described here has been reconfigured as a slit-scanning confocal microscope with a miniature objective lens and mechanical positioning assembly at the distal end of the fiber-optic imaging bundle. The device will allow for cellular-level imaging of tissues in regions that are accessible by endoscopes such as the lung, bladder, cervix, uterus, colon, and peritoneum.

2. Confocal Microscopy

The confocal microscope was initially conceived by Minsky. In most confocal imaging systems a point source illuminates a point on the sample and the light emitted from this point is imaged through a pinhole aperture onto a detector. Light coming from out-of-focus points is largely rejected because it does not produce a point focus at the pinhole aperture. By scanning the sample in both lateral dimensions, a two-dimensional image of a thin slice within the sample can be constructed. Alternatively, the source

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and detector can be scanned in tandem to achieve the same effect. Imaging a thin slice within a thick sample by a confocal microscope is known as optical sectioning. Because of its optical sectioning capability, confocal microscopy is useful for improving contrast when imaging thick biological specimens.

The confocal system allows three-dimensional imaging. If the position of the sample or the focal plane is shifted longitudinally along the optical axis, a different plane in the thick sample comes into focus. Multiple planes can be imaged in a thick sample, and a three-dimensional image of the object can be obtained.

A slit aperture represents an alternative geometry to the pinhole apertures used in standard two-dimensional scanning systems. In the slit-scan system, multiple detectors are required along the length of the slit, but scanning is necessary only in the direction perpendicular to the slit. A slit aperture has been employed in the system discussed here because scanning in only one dimension increases the speed of image acquisition. Since the device is intended for in vivo imaging, fast acquisition is important to minimize artifacts due to motion. However, with slit scanning, the axial resolution (i.e., the effective thickness of the image slice) is degraded relative to a point-scan system with the same numerical aperture. Based on geometric arguments, the integrated intensity falls as $1/z^2$ for a pinhole-scanning system versus $1/z$ for a slit-scanning system, where $z$ is the distance from the nominal focal plane. Calculations based on diffraction theory are necessary to quantify more accurately the axial resolution performance of a slit-scan system at a particular numerical aperture. Results of such calculations for the system discussed here are presented in Section 4.

3. System Design

Figure 1 shows the general system layout of the confocal microendoscope for fluorescence imaging. It consists of three major subassemblies: (1) the illumination arm, (2) the detection arm, and (3) the fiber-optic catheter.

A. Illumination Arm

The illumination arm subassembly is an anamorphic optical system designed to convert a spherical wave front from a laser beam into a cylindrical wave front that forms a line image at the proximal face of the fiber-optic imaging bundle. In this system an argon-ion laser operating at 488 nm is used as the light source. The point source formed by the pinhole spatial filter is collimated by a positive focal-length lens and refocused by a second positive focal-length lens. A negative focal-length cylindrical lens collimates the beam in the meridional plane only. In the sagittal plane the cylindrical lens has no power, so the beam is unaffected by this component and continues converging to a focus. This focusing behavior combined with the collimation in the meridional plane yields a line image at the location indicated in the diagram. The outer relay pair consists of a positive spherical lens and a Zeiss $10\times$ Plan-Neofluar microscope objective that images this line of light onto the proximal face of the fiber bundle with a demagnification of 7.5.

The length of the line illumination should match the diameter of the bundle, and the linewidth should match the diameter of the individual fibers in the bundle. However, the laser-beam intensity distribution at the proximal face of the fiber bundle is Gaussian. Therefore the $1/e^2$ width of the intensity distribution is matched to the width of an individual fiber element, and the FWHM of the intensity distribution along the length of the line image is matched to the diameter of the fiber bundle. This provides reasonable uniformity in illumination intensity from the center to the edge of the field of view.

The design of the illumination system incorporates the ability to change the length of the line formed on the proximal end of the fiber so that the illumination uniformity can be traded for light throughput efficiently. By adjusting the location of the negative cylindrical lens, the length of the line image can be changed. One undesirable effect of moving the cylindrical lens from its nominal design position is that the beam emerging from this lens is no longer collimated in the meridional plane and hence not collimated as it enters the fiber. The beam is either converging or diverging as it enters the fiber; however, the angles are small compared with the numerical aperture of the fiber bundle ($NA = 0.35$), so coupling efficiency into the fiber is not adversely affected.

The scanning apparatus is a flat mirror mounted on a galvanometer. The scan angle is $\pm 0.63$ deg and is determined by the diameter of the fiber-optic imaging bundle and the focal length of the Zeiss microscope objective. It is essential that the scanning mirror be located at a pupil plane of the illumination arm optical system to ensure that variation of energy throughput with scan angle is minimized. The aperture stop of the Zeiss microscope objective is the aperture stop of the illumination arm. Therefore
the scan mirror location is made conjugate to the entrance pupil of the microscope objective by using the inner relay pair of lenses.

The scan mirror performs three functions simultaneously in this system. First, it scans the illumination across the proximal face of the fiber bundle. Second, it effectively scans the fixed detection slit across the proximal face of the fiber bundle in synchronization with the illumination. Finally, it sweeps the collected light exiting the detection slit across the face of the CCD, constructing a two-dimensional image of the sample. This design of a single-scan mirror is preferable to using multiple mirrors because no synchronization is required.

B. Detection Arm

The proximal face of the fiber bundle is the object for this optical system. The light coming from the bundle is collimated by the microscope objective. This beam passes through the afocal inner relay and is reflected by the scan mirror and the dichroic beam splitter toward another lens. This lens converges an image of the bundle with a magnification of 8.75. A slit aperture is inserted at the location of this intermediate image to effect the confocal operation of the system. The aperture width, set at 20 μm, matches the diameter of the image of a fiber element. Increasing the slit width increases the throughput of the system but reduces the axial resolution (the optical sectioning performance). Decreasing the slit width improves axial resolution at the expense of throughput. This improvement is minimal, however, since the diameter of the fiber element sets the limit on the axial resolution of the system. By matching the slit width to the fiber element diameter, a balance between throughput and resolution is achieved.

A combination of a collimating lens and a zoom lens images the detection slit onto the CCD. As the scan mirror sweeps the illumination line and detection slit in tandem across the tissue, it simultaneously sweeps the image position on the CCD, building up a two-dimensional image. A long-pass filter is inserted in the collimated space between the collimating lens and the zoom lens to reject reflected illumination light and to pass the fluorescence emission. The zoom lens has a focal-length range of 75–300 mm, allowing variable magnification of the fiber face image on the CCD. A scientific-grade, low-noise, cooled CCD camera from Princeton Instruments (CCD512-EFT) is used for recording the images. This camera is a 512 × 512 frame transfer system with a 12-bit digitizer operating at 1 MHz. The maximum frame rate is 4 frames/s.

C. Fiber-Optic Catheter

The fiber-optic catheter consists of a fiber-optic imaging bundle, a miniature objective lens, and a focusing mechanism. The central component of the system, which dictates many of the parameters of the illumination and detection subassemblies, is the fiber-optic imaging bundle. A Sumitomo IGN08/30 fiber bundle is used in this system. This bundle consists of 30,000 2-μm-diameter elements with a center-to-center spacing of 3 μm. The diameter of the active area is 720 μm. This particular fiber bundle was chosen because it provides an appropriate balance between resolution, energy throughput, and flexibility. The fiber-optic imaging bundle serves to relay images between the proximal and the distal ends of the bundle while maintaining the spatial integrity of the system. The incorporation of this component in the system allows for confocal imaging of a sample located in a remote location. There is significant Fresnel reflection of the illuminating light at the glass–air interfaces at both the proximal and the distal ends of the fiber. Even with the spectral filter a portion of this reflected light gets through the detection arm to the CCD camera and reduces the contrast of the fluorescence signal. By placing glass coverslips on each end of the fiber, the glass–air interfaces are moved away from the fiber faces. In a conventional fluorescence microscope, moving the glass–air interface makes no difference since there is no rejection of out-of-focus light. However, in the confocal configuration, light coming from these planes is out of focus and rejected by the aperture. The outer (glass–air) surface of both coverslips is antireflection coated to minimize further the reflected illumination light.

Figure 2 shows the miniature objective lens system designed and fabricated for this application. The system is an F/1, achromatic, water-immersion lens with a nominal magnification of 1.67 from tissue (object) to fiber (image). It was designed to provide a minimum contrast of 0.5 out to 287 line pairs/mm in object space (170 line pairs/mm in image space). The lens is doubly telecentric to alleviate changes in magnification with focus and to maintain uniform fiber coupling across the field. The lens consists of 10 optical elements. Elements 2 and 11 in Fig. 2 are 150-mm-thick coverslips that are glued on the outer housing of the lens assembly and the fiber face, respectively. Lens elements 3 and 10 are field-flattening components placed near the object and image planes to correct for field curvature. Elements 4 and 9 are catalog aspheres. The doublets (elements 5, 6 and 7, 8) primarily help to correct for chromatic aberrations. The nominal object plane is indicated as surface 1, and the image plane (fiber surface) is 12. This lens has a maximum diameter of 5 mm and a total length of 3 cm. The peak-to-valley optical path difference is λ/4. Figure 3 shows the modulation transfer function (MTF) of this lens de-
Figure 3. Polychromatic MTF plot of miniature objective lens.

Figure 4. Schematic of focus and control subassemblies of miniature positioning mechanism (patent pending).

Figure 5. Objective lens and subassembly of focusing mechanism.

sign, indicating that it achieves nearly diffraction-limited performance over the full field of 430 μm at the object plane.

To alter the depth of the focal plane below the tissue surface, the position of the distal end of the fiber must move relative to the lens. To achieve a depth range of 100 μm in the tissue, the fiber must move ∼300 μm relative to the lens. This range of motion is beyond what can be achieved with piezoelectric devices. A miniature hydraulic system was designed and fabricated for positioning the distal face of the fiber bundle relative to the miniature objective lens. This device has two subassemblies: a focus subassembly and a control subassembly as shown in Fig. 4. The focus subassembly consists of an outer housing, a moving piston, and a reservoir to hold the hydraulic fluid (baby oil). The fiber-optic imaging bundle is inserted through a channel in the center of the piston and is secured to the piston with epoxy. As fluid is injected into the reservoir of the focus subassembly, the piston and the distal face of the fiber move toward the miniature objective lens, and the effective focal plane in the tissue moves farther below the surface. When fluid is removed from the reservoir, the piston retracts and the focal plane moves closer to the tissue surface. A spring between the lens and piston provides a restoring force to move the piston back as fluid is withdrawn.

The hydraulic fluid is added to and removed from the focus reservoir by way of the control subassembly that is located remotely and connected to the focus subassembly by a long section of flexible tubing. The control subassembly is another piston–reservoir system. When the piston is pushed forward, fluid from the reservoir is sent through the tubing into the focus subassembly reservoir. When the piston is pulled back, the fluid is returned to the control subassembly reservoir. The control subassembly is a dual-adjustment system allowing for both coarse and fine adjustment of the position of the distal face of the fiber. When coarse position adjustment is required, a threaded screw is turned, displacing a relatively large amount of fluid. When finer motions are desired, the micrometer-based adjustment is used to obtain axial adjustment with a precision of 2–3 μm. The fabricated focus subassembly of the positioning mechanism and the miniature objective lens are shown in Fig. 5. These two components fit together inside a cylindrical housing. This rigid end of the microendoscope is 7.9 mm in diameter and 60 mm long.

4. System Characterization

In this section the performance of the individual components and subassemblies is presented followed by a description of the performance of the system as a whole.

A. Illumination Arm

The illumination arm subassembly was characterized by measuring the line-spread function (LSF) at the proximal face of the fiber bundle. This measurement was based on a magnified image of the two-dimensional intensity distribution captured with a CCD. An average, one-dimensional distribution was calculated by projecting the two-dimensional distribution in the direction of the line. The width of the illumination line, measured at the minimum of
the central lobe, is \( \sim 2 \mu m \), which closely matches the core diameter of the fiber elements. The central lobe of the measured LSF is slightly wider than the ideal LSF based on the diffraction-limited performance of the Zeiss 10× Plan-Neofluar objective. This behavior is expected, considering the contribution of aberrations from other lenses in the illumination arm, especially the cylindrical lens. The throughput of the illumination arm from the laser output to the tissue plane is \( \sim 5\% \). The primary locations of energy loss are at the spatial filter (\( \sim 74\% \) throughput), the dichroic beam splitter (\( \sim 85\% \) throughput), and the fiber bundle that transmits only 50% of the incident energy. However, the laser has sufficient output to accommodate these losses and deliver adequate illumination to the tissue (>5 mW at 488 nm if necessary).

B. Detection Arm
The detection arm was characterized by comparing the ideal image of the detection slit aperture at the location of the proximal face of the fiber bundle with the measured image. The ideal image was determined by convolving the detection slit aperture with the diffraction-limited point-spread function (PSF) of the Zeiss microscope objective. The width of the detection slit aperture is 20 \( \mu m \). At the proximal fiber face the width of the diffraction-limited image of the detection slit is 3.6 \( \mu m \), taking into account the magnification between the slit aperture and the proximal face of the fiber bundle. The measured FWHM of the image of the slit is 5 \( \mu m \). This is larger than the FWHM of the ideal image, which is expected, considering the aberrations in the imaging system. The throughput of the detection arm from the tissue plane to the CCD detector is approximately 15% with primary losses due to the fiber bundle and the dichroic beam splitter.

C. Fiber-Optic Catheter and Objective
The fiber-optic imaging bundle is used to transfer an image from inside the body. Its performance, especially in terms of spatial resolution, is critical to the overall performance of the system. The bundle is technically a shift-variant system. Therefore its operation cannot be modeled mathematically as a convolution of the input with a PSF. However, pixelated components such as CCD’s and fiber bundles are often modeled approximately as shift-invariant systems with an average PSF or LSF.\(^{12}\)

Figure 6 shows a plot of the average LSF at the proximal and the distal faces of the fiber-optic imaging bundle. These average one-dimensional input, and output distributions were formed by taking the projection of the measured two-dimensional energy distributions along the direction of the line illumination. The output distribution is quite different from the input distribution. First, the central lobe of the output distribution is wider. This is due to the behavior of light spreading inside the fibers. Second, there are significant tails in the output distribution. Because of irregular fiber packing, the illumination line does not necessarily fall on a straight line of fiber elements. In some cases the illumination falls between fiber elements, leading to coupling of energy into multiple fibers and into the cladding structure. These effects widen the central lobe and produce the tails seen in the output distribution.

The average LSF distributions at the proximal and the distal faces of the fiber (Fig. 6) were used to extract the effective, incoherent fiber PSF.\(^{12}\) This PSF is plotted in Fig. 7. The central lobe is wider than the width of a single element, and there are secondary lobes, which are a manifestation of energy coupled into the cladding structure and/or cross talk between fiber elements.

The MTF of the objective lens was measured to assess its performance. The specifications called for a contrast of at least 0.5 at a maximum spatial frequency of 287 line pairs/mm at the tissue plane (170 line pairs/mm at the fiber plane). This spatial frequency represents the Nyquist cutoff frequency of the fiber bundle. The initial test of lateral resolution involved imaging a U.S. Air Force bar target with the objective and measuring the contrast,

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C = \frac{I_{\text{max}} - I_{\text{min}}}{I_{\text{max}} + I_{\text{min}}},
\]

of each group/element. The objective has a magnification of 1.67, and additional optics were necessary to magnify the image formed by this lens. The con-
contrast measurements of the entire optical system were made with and without the objective. By dividing the contrast measurements made with the objective by the contrast measurements made without the objective, the contrast of the objective lens alone was calculated. Figures 8(a) and 8(b) show the magnified images of the bar target without and with, respectively, the objective lens. Note that the image of the bar target obtained with the objective lens in the system has a slight background haze. This haze is probably a result of scattering caused by the diamond-turned aspheric lenses used in the system.

The contrast at each spatial frequency was calculated with the images in Fig. 8 and is plotted in Fig. 9. Theoretically, the curve should be monotonically decreasing; however, there is some variation in the curve due to measurement noise and slight variations in illumination across the field. Still the measurements indicate that the lateral resolution of the objective lens is excellent. The data are plotted out to 228 line pairs/mm because that is the highest spatial frequency on the U.S. Air Force bar target. Extrapolation of these data indicates that the contrast exceeds the design specification at 287 line pairs/mm.

The performance of the focusing mechanism was evaluated with a microscope mounted on a translation stage. Longitudinal displacements of the distal fiber face could be measured very accurately by focusing the microscope on the fiber face. The proximal face of a fiber bundle was illuminated with white light. The position of the distal end of the fiber face was adjusted by using the fine adjustment on the focusing mechanism. A 15-mm adjustment of the fine-focus micrometer corresponded to a 330-μm motion of the fiber face. The fiber face returned to the original position when the fine-focus micrometer was returned 15 mm to its starting position. Qualitative assessments of performance, including image drift and hysteresis of the focusing mechanism, were performed. There was no noticeable drift of the image plane over short periods of time (~5 min). However, over long periods of time (i.e., 24 h), the fiber did move slightly as the spring decompressed by forcing hydraulic fluid past the O-rings of the piston. A small amount of hysteresis was noticeable in the device. Although an accurate quantitative evaluation of the focusing mechanism was not performed, this device worked well enough to control focus adequately in the image experiments described below.

D. System Performance

The confocal system was assembled, and the performance of the system as a whole was evaluated for fluorescence imaging. Careful measurements of the lateral and the axial resolution of the system were made and compared with expected performance. The expected performance of the system was modeled with GLAD software (Applied Optics Research, Rochester, N.Y.). The inputs to this model included the measured illumination arm and detection arm distributions, the measured fiber PSF, the operating numerical aperture of the objective lens, and the Zernike polynomials of the nominal lens design that describe the aberrations of the miniature objective lens.

A fluorescent edge (step function) was imaged to
determine a system edge response function. Since the pixel structure of the fiber is observed in the image, the two-dimensional image was projected along the direction of the edge to obtain an average edge response function. The derivative of this average edge response function was then taken to obtain the system LSF. The measured average edge response function and system LSF are shown in Figs. 10a and 10b, respectively. The LSF curve exhibits some oscillation on the right-hand side. This is due to the pixel structure of the fiber bundle, which is not completely eliminated by the projection operation.

The FWHM of the LSF provides one measure of the lateral resolution. The FWHM of the measured LSF is 3.0 μm, which is somewhat larger than the value predicted by the model (1.7 μm). This discrepancy may be due to the performance of the imaging optics between the detection slit aperture and the CCD not being included in the computer simulation. In addition, the actual aberrations of the objective lens are not included in the model. Only the aberrations predicted by the lens design code were included in the model. Finally, there are inherent errors in the technique used to measure the lateral resolution. It was difficult to obtain a high-resolution fluorescent edge, which leads to some blurring in the measured edge response function. Thus measurement of the lateral resolution represents an upper bound on the actual lateral resolution of the system.

Two different measures of axial resolution were considered, the axial point response function and the axial planar response function. The axial point response describes how well two fluorescent points at different locations along the optical axis are resolved. The axial planar response function describes how well two fluorescent planar objects are resolved. Each measure is useful in different circumstances. For example, a tissue sample in which the cells are sparsely distributed can be considered as a collection of point objects. In this case the system performance would be more accurately described by the axial point response and the axial resolution would be defined as the FWHM of this function. If the tissue sample had a high density of cells, it would be more appropriately modeled by the axial planar response. In this situation the axial resolution would be given by the FWHM of the axial planar response function.

Measurements of the axial point response and the axial planar response functions were made with fluorescent microspheres. A 5-μm fluorescent bead with emission at 520 nm was placed at the appropriate focal plane, and images of the bead were recorded at defocus intervals of 2 μm. These images were used to determine the variation in on-axis intensity versus defocus to generate the axial point response. These images were also used to characterize the variation in integrated intensity as a function of defocus to generate the axial planar response. The integrated intensity at each position was calculated by summing the values over a region encompassing the fluorescent bead. The background/noise level was calculated by summing the values over an identically sized region not containing fluorescent beads. The background value was subtracted from the total integrated intensity to yield a background-corrected integrated intensity value. Figures 11 and 12 show the measured axial point and planar response functions compared with predictions of the GLAD models.

In both cases the measured and the predicted results are in close agreement. The slight discrepancy between the measured and the simulated response is attributed to two shortcomings of the model. First, the modeling does not incorporate the actual aberrations of the objective lens. Since the aberrations of
the fabricated lens are most likely worse than the aberrations of the designed lens, the measured performance is expected to be slightly worse than the predicted performance. Second, the assumption of a planar bead object (delta function in $z$) for the model is also not exact. The fluorescent bead is a sphere, and as such it has a finite depth of approximately 2–3 μm. The finite depth of the bead will have a broadening effect on the axial response functions. This broadening effect would lead to a measured performance that is slightly worse than the predicted performance.

5. Imaging Results

To demonstrate how the system would perform when imaging microscopic objects with sizes of the order of a cell, a sample with 15-μm fluorescent microspheres (Molecular Probes, Eugene, Ore.) was prepared. These microspheres were excited with the 488-nm line of a Lexel argon-ion laser. The emission wavelength of the microspheres was 605 nm, allowing excellent separation of the emission from the excitation. Figure 13(a) shows an image of the microspheres obtained with a frame rate of 2 frames/s. A similar experiment was performed with 6-μm microspheres (Polysciences Corporation, Phoenix, Ariz.) with the same excitation wavelength but an emission wavelength of 520 nm. This image is shown in Fig. 13(b).

The confocal microendoscope was used to image live cells grown in culture. PC3 human prostate cells (American Type Cell Culture, Va.) were grown on a microscope coverslip in a solution of Delbecco’s
Modified Eagle Media (DMEM)/F12 with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were incubated at 37°C and passed every three to four days. For the imaging experiments the cells were stained with SYTO-16 (Molecular Probes, Eugene, Oregon). SYTO-16 is a nucleic acid stain with a peak emission at 518 nm. This dye has a number of properties that make it attractive for in vivo imaging.\textsuperscript{15} SYTO-16 can penetrate almost all cell membranes including mammalian cells and bacteria. It has very low fluorescence (quantum yields of less than 0.01) when it is not bound to nucleic acids. When the dye is bound to nucleic acids the quantum yield is greater than 0.4 (DNA binding). Finally, SYTO-16 bound to DNA has twice the quantum efficiency of SYTO-16 bound to RNA. This difference in quantum efficiency not only allows visualization of the nucleus but also produces a visible contrast between the nucleus and cytoplasm, making assessment of the nuclear:cytoplasmic ratio possible.

While photobleaching is observed, this dye is fairly robust, requiring long illumination times or high-power densities to quench the fluorescence. With illumination levels of \textasciitilde 400 W/cm\textsuperscript{2}, photobleaching was not observed until after continuous illumination for several minutes.

SYTO-16 is provided in dimethyl sulfoxide, which aids in penetration of the dye through cell membranes. The 1-mM concentration of SYTO-16 was further diluted by mixing 2 \( \mu l \) of solution with 98 \( \mu l \) of phosphate buffered saline, yielding a final concentration of 20 \( \mu M \). The entire 100-\( \mu l \) mixture was added to the cell culture and allowed to sit for 15 min. After this time period the coverslip containing the cells was rinsed with phosphate buffered saline and imaged with the fiber-based confocal system. Figure 14 shows an image of this cell culture acquired with the confocal system. The cell nuclei are nicely

Fig. 15. Images of normal human prostate tissue taken at various depths: (a) tissue surface; (b) 15 \( \mu m \) below tissue surface; (c) 30 \( \mu m \) below tissue surface; (d) 45 \( \mu m \) below tissue surface.
resolved, and variations in nuclear size and shape are easily visualized.

In vitro images of human prostate tissue were obtained to demonstrate the system performance with a real tissue sample. Samples of human prostate tissue were obtained from tissue extracted during prostate surgery. Core biopsies taken from the extracted prostate were 2–3 mm in diameter and 20–30 mm in length. These samples were placed in a balanced salt solution. The core samples were sectioned on a special microtome at 4 °C producing sections 100–250 μm thick. These sections were then grown in a rotating organ culture system in keratinocyte media with growth factors for 48 h. Following the incubation period, the sample was stained with SYTO-16 and imaged with the confocal microendoscope system. Figure 15 shows a series of four images that highlight different zones of a hemispherical-shaped distribution of cells. This structure is a duct in the prostate gland, and as the

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Fig. 16. Images of abnormal human prostate tissue: (a) indications of hyperplasia; (b) region of aggressive cancer.

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Fig. 17. In vivo images of a mouse: (a) top surface of peritoneal lining; (b) underlying peritoneal and smooth muscle cells.
tissue sample is scanned in depth, the back side of the duct comes into view and the sidewalls of the duct start to disappear. These images were taken at depth increments of 15 µm with a frame rate of 2 frames/s.

The confocal microendoscope system was used to search for areas on the tissue sample that appeared abnormal. Images of abnormal tissue are shown in Fig. 16. Figure 16(a) shows a region exhibiting hyperplasia. Hyperplasia is a condition in which the cells have started to multiply faster than normal, leading to a higher cell density. In the normal prostate gland, high cell density is usually present in regions lining the ducts. In Fig. 16(a) a higher cell density is noticeable away from the duct lining. In Fig. 16(b) the cell density of the tissue is extremely high and there is no particular organization to the cells. This is indicative of a cancerous state. In these two figures we highlight the capability of the system to distinguish between normal and abnormal regions of human prostate tissue by allowing visualization of cell density and organization.

The confocal microendoscope was also used to obtain in vivo images of the peritoneum of a mouse. A mouse was anesthetized, and the abdominal area was exposed to allow for access with the confocal microendoscope. A region of the peritoneum was stained with SYTO-16, and, after a few minutes, the scope was placed in contact with the peritoneal lining of the abdominal cavity to acquire images at a rate of 4 frames/s. In Fig. 17(a) we show an image of the peritoneum. The bright, punctate structures in the image are the nuclei of the cells in the peritoneum. The elongated structures are the underlying muscle fibers of the abdomen. In Fig. 17(b) an image of the tissue is provided at an approximate depth of 20 µm below the top surface. In this image the muscle fibers in the underlying abdominal tissue are seen more clearly. The small, bright structures in this image are the nuclei of the muscle cells. Since this specimen did not have cancer, these images are representative of normal tissue.

6. Discussion

The results with the confocal microendoscope are encouraging. The imaging performance of the system appears to be adequate for visualizing cells and subcellular organelles. The use of the microendoscope in contact with the tissue specimen combined with a frame rate of 4 frames/s appears to be sufficient to avoid image blurring due to patient motion. Although high-quality images were obtained in vivo with SYTO-16, it remains to be shown whether this dye is applicable to in vivo human imaging. Certainly there are many fluorescent stains that may be useful in the context of optical biopsy. The toxicity, specificity, and sensitivity of these dyes need to be carefully studied before a system such as this is ready for clinical application.

The depth imaging properties of this confocal system have not been fully investigated in the context of in vivo imaging. The results in Fig. 15 demonstrate the focusing and the depth discrimination of the confocal microendoscope but are showing cells only on the tissue surface. Presumably, only the surface layer of cells is stained by topical application of the dye. The two images in Fig. 17 show cells at different depths (peritoneum and underlying smooth muscle) but these layers are separated by less than 20 µm and the peritoneum is relatively transparent. Even if cells below the surface layer are stained, the ability to image deep in the tissue will be limited. Light at 488 nm penetrates only 100–200 µm below the surface before scattering reduces the primary beam intensity to negligible levels. However, this depth may still provide meaningful results in vivo by allowing visualization of tissue surface topology, which can make more careful analysis of surface inhomogeneities possible. Longer-wavelength illumination with an appropriate dye will be required for imaging deeper inside the tissue.

This effort has moved the confocal microendoscope instrumentation closer to clinical use. The distal end of the confocal microendoscope is similar in size to many endoscopes used in clinical practice. However, it is desirable to make the rigid end of this flexible endoscope even smaller to allow imaging in more confined spaces. The design of a smaller objective and focusing mechanism is the subject of ongoing work in our laboratory.

7. Conclusions

The primary objective of this research was development of a fiber-based confocal microendoscope for imaging pathology in situ. A major emphasis was development of miniature optical and mechanical components of the fiber-optic catheter having the performance necessary for microscopic evaluation of tissue. The characterization of the fabricated and assembled components shows that the system performance meets design specifications. The lateral resolution of the system is approximately 3 µm. The axial resolution specified in terms of both an axial point response (10 µm) and an axial planar response (25 µm) shows good agreement between measured and predicted results. The imaging experiments conducted with the cell culture model demonstrate that cellular and nuclear morphology can be visualized with the system. In vitro and in vivo results demonstrate that the system allows for visualization of cell morphology, density, and organization when imaging real tissue samples.

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