Confocal theta line-scanning microscope for imaging human tissues

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A confocal reflectance theta line-scanning microscope demonstrates imaging of nuclear and cellular morphology in human skin and oral mucosa in vivo. The illumination and detection are through a divided objective lens pupil, resulting in a theta-microscope configuration. A line is directly scanned in the pupil and descanned onto a linear detector array such that the theta line scanner consists of only seven main optical components. The experimentally measured lateral resolution is 1.0 μm and optical section thickness is 1.7 μm under nominal conditions at 830 nm wavelength. Through full-thickness human epidermis (i.e., in the dermis) the measured lateral resolution is 1.7 μm and the optical section thickness is 9.2 μm. The lateral resolution, sectioning, and image quality in epidermal (epithelial) tissue is comparable to that of point scanning confocal microscopy. © 2007 Optical Society of America

OCIS codes: 180.1790, 180.5810, 180.6900.

1. Introduction

Point-scanning reflectance confocal microscopes have proved to be successful for noninvasive imaging of nuclear and cellular detail in thin optical sections within living human skin, oral mucosa, and cervical tissue.1–6 Point scanners are being increasingly used in basic, translational, and clinical research. However, point-scanning confocal technology is complex and expensive. Widespread use of confocal microscopes, especially in clinical and surgical settings, will require scanners that are simple, robust, easy to duplicate and use on humans with repeatable performance in either laboratory or commercial manufacturing environments, easy to configure into hand-held instruments, and low cost. Line scanning offers an alternative that may meet many of these requirements because it is relatively less complex than point scanning.

Two configurations of confocal line-scanning microscopes have been previously developed. One is the standard full-pupil configuration with coaxial illumination and detection.7–13 Two recent commercial versions include the LSM-5 from Carl Zeiss (Germany) and LiveScan from Nikon (Japan). The other is a divided-pupil configuration, in which the illumination and detection are through symmetrically opposite portions of a divided objective lens pupil.14,15 With a divided pupil, the illumination and detection line spread functions intersect at a half-angle θ. Consequently, the divided-pupil line scanner resembles the theta-microscope configuration,16,17 albeit with a single objective lens. The use of a single objective lens in the divided-pupil configuration significantly simplifies the alignment and assembly, compared with the use of two separate lenses in the original theta microscope.18–20

Most of the previous confocal line-scanning microscopes are based on rescanning the detected descanned beam onto a two-dimensional detector, through intermediate optics and relay telescopes in a standard confocal arrangement. Such line-scanning configurations tend to be complex. The use of newly available linear detector arrays eliminates the need for rescanning and offers a relatively simpler configuration. A full-pupil line-scanning confocal microscope using a linear charge-coupled device detector array was recently demonstrated.21 Optical sectioning and images of reflectance test targets were shown.
Compared with the full-pupil coaxial configuration, the divided-pupil provides slightly reduced optical sectioning and resolution, but the theta configuration results in higher contrast, especially within scattering tissues, due to better rejection of multiply scattered light. Thus, the divided-pupil theta configuration offers user control of both sectioning and contrast, which will be useful in highly scattering tissue such as skin.

The design of a simple divided-pupil confocal theta line-scanning microscope with optical sectioning and imaging in human epidermis that is comparable to that of current point scanners was recently reported. Scanning is directly in the objective lens pupil, and detection is with a linear complementary metal oxide semiconductor (CMOS) array, such that the theta line scanner consists of only seven main optical components.

Here we report on a new analytical model of the design, experimental line spread function (sectioning and lateral resolution) measurements, and images of human epidermis and oral mucosa in vivo. Of particular clinical relevance is the imaging performance of confocal scanners deep within highly scattering and aberrating tissues such as human skin (which has not yet been investigated, to the best of our knowledge). Experimental measurements of line spread functions are shown for nominal instrumental conditions as well as for deep dermal (through full-thickness epidermis) conditions. The results show the influence of scattering and aberration on confocal line-scanning performance in actual skin.

2. Instrumentation

The confocal reflectance theta line-scanning microscope is designed for specifications that are similar to current point-scanning technology to meet the requirements for clinical imaging. The performance specifications are summarized in Table 1. Figure 1 shows the optical design of the microscope. The design combines Koester’s divided pupil configuration and the Steltzer and Lindek and the Webb and Rogomentich theta microscope configurations.

The three main components of the microscope are the illumination path, the detection path, and the scanning electronics.

A. Illumination Optics

We use an 830 nm laser diode (56ICS115/HS Melles Griot, Carlsbad, California) light source that provides an optimum combination of optical sectioning and depth penetration. The Gaussian beam is expanded by a beam expander (EX 16, Micro Laser Systems, Garden Grove, California) consisting of two convex lenses ($L_1, L_2$), to a collimated beam diameter of 13 mm. The beam fills a cylindrical lens ($CL_1$) of focal length 200 mm. The plano–cylindrical discrete lens is positioned close to the pupil and only in the illumination portion of the objective lens ($L_3$).

The objective lens is custom made (Lucid, Rochester, New York) with a focal length of 17.5 mm and a pupil diameter of 28 mm. The objective lens was designed to meet the needs of clinical and pathology applications: low magnification of 10× to provide a large field of view of 1 mm, high NA of 0.8, water immersion, and corrected for 1 mm thick cover glass window. We placed a divider strip of width 14 mm in the center of the objective lens pupil (see Figs. 1 and 4) to separate the illumination path and the detection path. Thus, the circular objective lens pupil is divided into D-shaped illumination and detection pupils.

The combination of the cylindrical lens and objective lens results in two orthogonal lines in the object space as illustrated in Fig. 2. The primary line, parallel to the y axis (orthogonal to the page), is formed at the focus of the objective lens and is used for imaging. The secondary line (not used for imaging) is parallel to the x axis, and is focused 1.6 mm closer to the objective lens because the cylindrical lens is placed approximately 6.5 mm in front of the back focal plane of the objective lens. Since the incident beam is collimated, the length of the primary line is determined as $L_p = d(f_{obj} - f_{net})/f_{net}$, where $d$ is the diameter of the beam entering the cylindrical lens, $f_{obj}$ is the focal length of the objective lens, and $f_{net}$ is the net focal length of the cylindrical lens and objective lens. Hence, the primary line is of length 1.13 mm. The design results in the line being confocal in the x direction and nonconfocal along the length of the line (y direction).

The primary illumination line is scanned in the x direction by the first facet of a prismatic mirror ($M_2$)}
that is driven by an oscillating nonresonant galvanometer (MiniSAX, GSI Lumonics, Billerica, Massachusetts). The prismatic scanner is physically close to but not exactly in the pupil of the objective lens. Scanning in (or close to) the objective lens pupil eliminates the need for a relay telescope between the scanner and the objective lens. This reduces the complexity and size of the microscope. Since we do not scan exactly in the pupil plane of the objective lens, the illumination beam slightly walks across the pupil. However, there is no vignetting at the edges of the field of view because the 11 mm diameter beam (with a scan of ±0.9 mm) sufficiently overfills the D-shaped illumination portion along the x direction (7 mm) of the objective lens pupil. The scan angle of ±1.6° produces a total scanned XY plane of ±0.5 mm (~1 mm × 1 mm) within the tissue. The x dimension of the objective and cylindrical lens pupil is overfilled to keep the confocal direction resolution close to the diffraction limit, while still providing maximum light transmission through the system.

The measured light throughput of the illumination path is ~25%. The low light throughput in the present setup is due to the lack of near-infrared antireflection coatings in the beam expander and cylindrical lens and also overfilling of the cylindrical lens and objective lens pupil (x direction). The current optics is not optimized for maximum light transmission at 830 nm, but this may be easily accomplished in the next generation instrument.

B. Detection Optics

The diametrically opposite portion of the objective lens pupil is used to collect light that is backscattered from the object plane. The backscattered light is descaned by the second facet of the prismatic mirror (M3). A prismatic galvanometric scanner has the advantages that any fluctuations in scan rate are synchronized in both illumination and detection paths. Moreover, the scanner is a small, compact, and simple optical component. The collimated light is focused by a biconvex lens \((L_d)\), and passes through a slit \(S_1\) that is placed conjugate to the object plane. A CMOS line detector (LIS 1024, Photon Vision Systems, Cortland, New York) is used for detection. (Note: Photon Vision Systems is now Panavision.) The detector consists of a linear array of 1024 pixels, each pixel being 7.5 μm (length—y direction) × 125 μm (width—x direction). Slits of 5–100 μm widths were used in front of the detector for resolution measurements and imaging experiments. Compared with the standard theta microscope configuration that uses two separate objective lenses,17–20 the use of a single objective lens with a divided pupil allows for easy alignment and control of the angle (half-angle \(\theta\)) between the intersection of the illumination and detection paths. Since the focal length of the detector lens \((L_d)\) is 100 mm, the effective magnification of the detection side is \(f_d/f_{obj} = 5.7\). Therefore 1 μm in the object plane scales to 5.7 μm on the detector array.

C. Scanning Electronics

The timing electronics for the line scanning and detection are on a detector board that contains the CMOS linear detector. The board electronics provide a pixel clock of 5.71 MHz, a line clock of 5.55 kHz, and a frame rate of 4.86 frames/s. The pixel-, line-, and frame-clock drive a PCI-frame grabber (Digital Meteor II, Matrox, Inc.) that converts the analog detector signal into an 1143 × 1024 pixel, 8 bit image. The video timing and control signals are shown in Fig. 3. The linear detector board performs all the timing needed to interface the CMOS line detector to the frame grabber.

An oscillator-driven field programmable gate array (FPGA programmable logic) generates the pixel clock (PCLK) that drives the linear detector to produce pixels. The analog-to-digital converter (ADC) is on the sensor board with the ADC close to the detector to minimize noise. The analog amplifier provides a gain of ~5 and has a bandwidth of approximately 10 MHz. The detector board produces horizontal synchronization pulses (H-SYNCH, one per line) that trigger the

![Diagram](https://via.placeholder.com/150)

Fig. 3. Video timing and control electronics for the linear CMOS detector, galvanometric scanner, and frame grabber.

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line scans and clock the line counter (see Fig. 3). As
the lines are counted down, the output of the line
counter drives a DAC to produce a sawtooth ramp
(V-RAMP), which then drives the galvanometric
scanner. Counter reset pulses (V-RAMP RESET) at
the end of each countdown or sawtooth cycle are pro-
duced to reset the counter and to generate the frame
synchronization pulses (V-SYNCH, one per frame).
Images are nominally of 1143 lines of which 1024 are
active and 119 are retraces at the start and end of
scan; each line contains 1029 pixels of which the last
four are absolute black. The complete displayed
frames are 1142 lines by 1024 pixels. Four integra-
tion times are available in the range of 200–50
\( \text{s/line} \), corresponding to 5–20
frames/s. All the images with the current line scan-
er were taken at 4.86 frames/s, which corresponds
to an integration time of 200 \( \text{s/line} \) (i.e., 200 \( \text{\mu s/pixel} \)).

3. Resolution and Optical Sectioning

A. Analytical Model
Since our design does not contain circular pupil con-
ditions, an analytical model was developed to aid in
the design of the microscope. Figure 4 illustrates a
representative model to predict the section thickness
\( \Delta z \) and lateral resolution \( \Delta x \) of the confocal theta
line-scanning microscope. The illumination and de-
tection line spread functions (LSFs) intersect at half-
angle \( \theta \) at the focus of the objective lens. D-shaped
pupils are awkward to model because the NA varies
from minimum to maximum. Moreover, the Gaussian
beam overfills the illumination pupil in the \( x \) direction,
but the exponential tails fill the pupil in the \( y \) direction [see Fig. 4(B)]. To account for these effects,
we consider equivalent pupils with circular shapes.
On the basis of equal areas, the D-shaped pupils are
modeled as circular pupils of diameter
\[
d = 2 \left( \frac{r^2}{\pi} \cos^{-1} \left( \frac{u}{r} \right) - \frac{u}{r} \left( 1 - \frac{u^2}{r^2} \right) \right)^{1/2},
\]
where \( u \) is the half-width of the divider strip and \( r \) is
the radius of the objective lens pupil. For a Gaussian
beam, the diffraction-limited lateral resolution, in
terms of the full width at half-maximum (FWHM)
of the illumination LSF, is
\[
\Delta x_{\text{ill}} = 0.76 f_{\text{obj}} \lambda / d,
\]
where \( \lambda \) is the wavelength in the immersion medium (\( \lambda = \lambda_0 / n \), \( \lambda_0 = 830 \text{ nm in vacuum and } n = 1.33 \text{ for water} \)).
For the confocal theta microscope, the lateral re-
solution \( \Delta x_\theta \) and section thickness \( \Delta z_\theta \) have been
determined to be\(^{14,16–18} \) \( \Delta x_\theta = \Delta x_{\text{ill}} / \cos \theta \) and \( \Delta z_\theta = \Delta x_{\text{ill}} / \tan \theta \).

Thus, in terms of the divider strip half-width \( u \) and
the objective lens pupil radius \( r \), the FWHM
optical section thickness and lateral resolution are
determined to be
\[
\Delta x_\theta = \frac{0.76 f_{\text{obj}} \lambda}{d} \left[ \frac{r^2}{4} \left( 1 + \frac{u^2}{r^2} \right) + f_{\text{obj}} \right]^{1/2},
\]
\[
\Delta z_\theta = \frac{0.76 f_{\text{obj}} \lambda}{d \cos \theta}.
\]

Fig. 4. (Color online) Schematic of the objective lens pupil, divided into an illumination pupil and a detection pupil (4A). The illumination
and detection paths intersect at a half-angle \( \theta \). (B) The actual D-shaped pupils are modeled as circular pupils of equivalent area. The
intersection of the illumination and detection LSFs at the focus is shown as an expanded view in (B).
The ratio \( u/r \) determines both the illumination and the detection pupil sizes and half-angle \( \theta \) at which the illumination and detection LSFs intersect. For \( u/r = 0-0.5 \), Eq. (2) predicts the diffraction-limited section thickness \( \Delta z_s \) to be 1.1–1.3 \( \mu \)m and Eq. (3) predicts the lateral resolution \( \Delta x_s \) to be 0.5–0.8 \( \mu \)m. We designed our objective lens with a radius \( r \) of 14 mm and placed a divider strip of half-width \( u \) of 7 mm. Under this condition of \( u/r = 0.5 \), the predicted diffraction-limited section thickness is 1.3 \( \mu \)m and lateral resolution is 0.8 \( \mu \)m, which are sufficient for imaging human skin and oral mucosa.23

**B. Experimental Results**

Experimental measurements of confocal theta LSFs were made to determine optical section thickness and lateral resolution of the system. The LSFs were measured under two conditions, diffraction-limited (i.e., nominal instrument) conditions as well as under scattering and aberrating conditions of actual human skin (i.e., deep within human epidermis). The nominal instrumental optical section thickness was measured by the standard method of axially translating a \( \lambda/20 \) flat mirror surface through focus and measuring the detected power versus axial position \( z \). Freshly excised specimens of human epidermis were harvested and placed on the mirror to measure LSFs under actual tissue-induced scattering and aberrating conditions.

Figure 6 is an experimental plot of an axial theta LSF under nominal instrument conditions and through full-thickness (approximately 75 \( \mu \)m thick) human epidermis. The figure shows the influence of tissue-induced scattering and aberrations on the axial theta LSF for a slit width of 5 \( \mu \)m (A) and 50 \( \mu \)m (B) placed in front of the detector. (Note: 5 \( \mu \)m corresponds to one lateral resolution element in the detector plane.) Thirty measurements were made with each slit. The FWHM of the axial theta LSF plot represents the section thickness. For a slit width of 5 \( \mu \)m, the nominal instrumental section thickness is 1.7 ± 0.1 \( \mu \)m, and through full-thickness epidermis is 9.2 ± 1.7 \( \mu \)m. This measured nominal section thickness compares well to the analytically predicted value of 1.3 \( \mu \)m. Correspondingly, for a 50 \( \mu \)m slit, the nominal section thickness is 4.8 ± 0.3 \( \mu \)m and through full-thickness epidermis is 12.2 ± 3.2 \( \mu \)m. The scattering and aberration due to the epidermis reduces the sectioning ability of the microscope in the dermis.

Figure 7 shows the optical section thickness for slit widths of 5–100 \( \mu \)m, which corresponds to 1–20 times the diffraction-limited lateral resolution. The measurements indicate that slits of width 25 or 50 \( \mu \)m provide adequate axial sectioning (to visualize nuclei and cells in the epidermis) as well as adequate detected signal-to-noise ratio and signal-to-background ratio (contrast) as observed in the images.
Lateral resolution in the confocal direction (perpendicular to the primary line—x dimension) and nonconfocal direction (y dimension) was measured by the standard method of imaging a chrome-on-glass edge (Ronchi Ruling, F38-566, Edmund Scientific, Barrington, New Jersey). An experimental plot of the theta lateral LSF under nominal instrument conditions and through full-thickness human epidermis, for a 25 μm slit in front of the detector is shown in Fig. 8(A). Previously, it had been determined that, when a Gaussian spot scans an edge, the lateral resolution in terms of the e−2 radius is obtained as 0.94 times the 10%–90% points.20 With a slit of width 25 μm in front of the detector, the nominal lateral resolution is approximately 0.9 μm, and through full-thickness epidermis, approximately 1.7 μm.

Figure 8(B) shows the lateral resolution along the confocal direction (x direction) for slit widths of 25, 50, and 100 μm, which corresponds to 5–20 times the diffraction-limited lateral resolution. The measured nominal lateral resolution with the smallest slit width of 5 μm is 0.9 μm, which compares well with the analytical prediction of 0.8 μm. In the nonconfocal direction, the lateral resolution was measured as 2.5, 3.1, and 3.1 μm for slit widths of 25, 50, and 100 μm, respectively.

4. Imaging of Human Skin and Oral Mucosa In Vivo
With the system described above, we demonstrated imaging in human skin and oral mucosa, both ex vivo and in vivo with high resolution and high contrast. The image acquisition details and example images are presented.

A. Objective Lens-to-Tissue Interface
Excised tissue specimens (ex vivo) were mounted in a fixture that consisted of an aluminum plate with a rectangular 15 mm × 10 mm × 1 mm cover glass window. The window was taped to the plate for quick removal and cleaning. The plate is translated relative to the objective lens with a motorized nanostepper stage (Nanomotion II, Melles Griot, California). The immersion medium of water-based hair gel (Suave Naturals) was used between the cover glass and the tissue specimen. Another drop of gel was then placed on the outer surface of the cover glass to index match the objective lens to the cover glass.

For in vivo imaging, the aluminum plate is mounted onto a ring that threads into the objective lens housing. Volunteers press the skin or oral mucosa against the 1 mm cover glass window, and focusing into the tissue is achieved by rotating the ring relative to the tissue.

B. Image Acquisition
Based on the sectioning and resolution measurements and the sensitivity of the current CMOS detector, imaging was performed with 25, 50, and 100 μm slit widths. Although slit widths of 5–10 μm provide diffraction-limited sectioning, resolution and
very high contrast, slit widths of 25–100 μm were necessary in practice to detect more signal when imaging deeper. The images that are shown here were obtained with the 10×, 0.8 NA water immersion objective lens and a 50 μm slit in front of the detector. Individual images and sequential z stacks were captured using a frame grabber (Meteor II, Matrox Inc.) and image capture and processing software (MATLAB version 6.5, MathWorks, Natick, Massachusetts).

Lateral (en face) images were obtained at various depths (z) within human tissue both ex vivo and in vivo. The z depth in ex vivo tissue was controlled by the motorized nanopositioner with a resolution of 100 nm through a general purpose instrument bus (GPIB) card (National Instruments, PCI-GPIB). The rate and range of motion for the nanopositioner is controlled with MATLAB software. To obtain serial z-depth images, the nanopositioner was advanced 1 μm between each image capture. Depending on the maximum depth, 100 to 300 images were captured for each series.

In vivo z-depth control was obtained by rotating the threaded fixture ring. The imaging rate of 5 frames/s provided real-time observation of tissue morphology including blood flow through capillary loops in the dermis of human skin as well as within the papillae layer at the epithelial junction of the oral mucosa. The images were cropped and enhanced for brightness and contrast, with image processing software (IPLab Spectrum, v7.5, Scanalytics, Fairfax, Virginia). The current in vivo tissue fixture ring design provided sufficient stability for capturing individual images. However, tissue motion was too severe to capture controlled z-stack images in the same field.

C. Images

In vivo images taken with the confocal theta line scanner illustrate the optical sectioning and dynamic

Fig. 9. Confocal theta line-scanning images of human skin in vivo, showing (A) stratum corneum; (B) granular layer, illustrating the dark nuclei in the cells (arrows); (C) the spinous layer, showing the smaller dark nuclei (arrows); (D) the basal cells at the dermal-epidermal junction, where the nuclei (arrows) are visible around the dermal papillae. The dermal papillae appear as ring-shaped clusters of basal cells. Scale bar 100 μm.
changes within biological tissue. Nuclei and cells are visible in all the layers of the epidermis (in skin) and epithelium (oral mucosa), demonstrating sectioning and lateral resolution that corroborates the LSF measurements. In the top layers of the skin, the stratum corneum, granular cells, and spinous cells are seen, as shown in Figs. 9(A), 9(B), and 9(C), respectively. In these layers the cellular cytoplasm appears bright and grainy, with nuclei (arrows) appearing as dark ovals within the cells. Deeper in the epidermis [Fig. 9(D)], at the dermal and epidermal junction, ring-shaped clusters of basal cells on dermal papillae are easily seen. Basal cells are smaller with dark nuclei (arrows). Basal cells appear brighter due to the pigment melanin.\(^3\) These images appear similar to those of human skin in vivo obtained with confocal point scanners.\(^2,3,20\)

Confocal reflectance theta line-scanning images of human oral mucosa in vivo are shown in Fig. 10. Superficial epithelial cells at a depth of 25 μm are shown in Fig. 10(A). The nuclei appear bright (arrows), probably due to condensed chromatin in the highly mitotic cells. At a depth of 100 μm, the nuclei are smaller with higher density as shown in Fig. 10(B). Below the papillae at the epithelial junction [Fig. 10(C)], blood flow (arrows) is readily visualized in real time at a depth of 200 μm. At increasing depths [Fig. 10(D)], connective tissue is seen in the superficial lamina propria. These images, too, appear similar to those of human oral mucosa in vivo, obtained with confocal point scanners.\(^4,5\)

5. Conclusions

The confocal theta line-scanning microscope provides optical sectioning and resolution that compares well with that of current point scanning within the weakly scattering and aberrating conditions of human epidermal (skin) or epithelial tissues (oral mucosa). Nuclear, cellular, and tissue morphologic detail is resolved in the human epidermis and in the epithe-
lum and connective tissue of oral mucosa in vivo. However, within strongly scattering tissue such as deeper dermis in skin, the contrast in theta line-scanned images degrades more rapidly than that in point scanned images. The contrast degrades mainly due to the line being nonconfocal in one dimension (parallel to itself) that results in pixel cross talk in the linear detector array.

The design is simple in terms of both optics and electronics, such that a low-cost hand-held theta line scanner may find clinical applications that require imaging of superficial and/or weakly scattering tissues. Example applications include mapping of lateral cancer margins to guide microsurgery either in fresh excisions (surgical pathology-at-the-bedside) or intraoperatively (directly on the patient).

The authors thank Bill Fox and Scott Grodevant at Lucid Inc. for their help with the instrument electronics, and James Zavislan at the University of Rochester and Charles Koester at Columbia University for their optics expertise. This work is supported in parts by NIH/NCI (Awards 1R43CA93106 and 2R44CA93106) and the NSF Partnerships in Education and Research Program (Award EEC-012931).

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