Miniature in vivo MEMS-based line-scanned dual-axis confocal microscope for point-of-care pathology

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Abstract: There is a need for miniature optical-sectioning microscopes to enable in vivo interrogation of tissues as a real-time and noninvasive alternative to gold-standard histopathology. Such devices could have a transformative impact for the early detection of cancer as well as for guiding tumor-resection procedures. Miniature confocal microscopes have been developed by various researchers and corporations to enable optical sectioning of highly scattering tissues, all of which have necessitated various trade-offs in size, speed, depth selectivity, field of view, resolution, image contrast, and sensitivity. In this study, a miniature line-scanned (LS) dual-axis confocal (DAC) microscope, with a 12-mm diameter distal tip, has been developed for clinical point-of-care pathology. The dual-axis architecture has demonstrated an advantage over the conventional single-axis confocal configuration for reducing background noise from out-of-focus and multiply scattered light. The use of line scanning enables fast frame rates (16 frames/sec is demonstrated here, but faster rates are possible), which mitigates motion artifacts of a hand-held device during clinical use. We have developed a method to actively align the illumination and collection beams in a DAC microscope through the use of a pair of rotatable alignment mirrors. Incorporation of a custom objective lens, with a small form factor for in vivo clinical use, enables our device to achieve an optical-sectioning thickness and lateral resolution of 2.0 and 1.1 microns respectively. Validation measurements with reflective targets, as well as in vivo and ex vivo images of tissues, demonstrate the clinical potential of this high-speed optical-sectioning microscopy device.

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References and links
1. K. Sokolov, K.-B. Sung, T. Collier, A. Clark, D. Arifler, A. Lacy, M. Descour, and R. Richards-Kortum, “Endoscopic microscopy,” Dis. Markers 18(5-6), 269–291 (2002).
2. J. T. C. Liu, N. O. Loeveke, M. J. Mandella, R. M. Levenson, J. M. Crawford, and C. H. Contag, “Point-of-care pathology with miniature microscopes,” Anal. Cell Pathol. (Amst.) 34(3), 81–98 (2011).
3. J. M. Jabbour, M. A. Saldua, J. N. Bixler, and K. C. Maitland, “Confocal endomicroscopy: instrumentation and medical applications,” Ann. Biomed. Eng. 40(2), 378–397 (2012).
4. C. Macaulay, P. Lane, and R. Richards-Kortum, “In vivo pathology: microendoscopy as a new endoscopic imaging modality,” Gastrointest. Endosc. Clin. N. Am. 14(3), 595–620 (2004).
5. K. B. Sung, C. Liang, M. Descour, T. Collier, M. Follen, A. Malpica, and R. Richards-Kortum, “Near real time in vivo fibre optic confocal microscopy: sub-cellular structure resolved,” J. Microsc. 207(2), 137–145 (2002).

6. J. J. Shin, M. C. Pierce, D. Lee, H. R. Ra, O. Solgaard, and R. Richards-Kortum, “Fiber-optic confocal microscope using a MEMS scanner and miniature objective lens,” Opt. Express 15(15), 9113–9122 (2007).

7. Y. S. Sabharwal, A. R. Rouse, L. Donaldson, M. F. Hopkins, and A. F. Gmitro, “Slit-scanning confocal microendoscope for high-resolution in vivo imaging,” Appl. Opt. 38(34), 7133–7144 (1999).

8. A. L. Polglase, W. J. McLaren, S. A. Skinner, R. Kesslich, M. F. Neurath, and P. M. Delaney, “A fluorescence confocal endomicroscope for in vivo microscopy of the upper- and the lower-GI tract,” Gastrointest. Endosc. 62(5), 686–695 (2005).

9. W. Piyawattanametha, H. Ra, Z. Qiu, S. Friedland, J. T. C. Liu, K. Loewke, G. S. Kino, O. Solgaard, T. D. Wang, M. J. Mandella, and C. H. Contag, “In vivo near-infrared dual-axis confocal microendoscopy in the human lower gastrointestinal tract,” J. Biomed. Opt. 17(2), 021102 (2012).

10. M. C. Pierce, Y. Guan, M. K. Quinn, Z. Xiang, W.-H. Zhang, Y.-L. Qiao, P. Castle, and R. Richards-Kortum, “A pilot study of low-cost, high-resolution microendoscopy as a tool for identifying women with cervical precancer,” Cancer Prev. Res. (Phila.) 5(11), 1273–1279 (2012).

11. T. Ota, H. Fukuyama, Y. Ishihara, H. Tanaka, and T. Takamatsu, “In situ fluorescence imaging of organs through compact scanning head for confocal laser microscopy,” J. Biomed. Opt. 10(2), 024010 (2005).

12. H. Makhlouf, A. F. Gmitro, A. A. Tanbakuchi, J. A. Udovich, and A. R. Rouse, “Multispectral confocal microendoscope for in vivo and in situ imaging,” J. Biomed. Opt. 13(4), 044016 (2008).

13. K. C. Maitland, A. M. Gillenwater, M. D. Williams, A. K. El-Naggar, M. R. Descour, and R. R. Richards-Kortum, “In vivo imaging of oral neoplasia using a miniaturized fiber optic confocal reflectance microscope,” Oral Oncol. 44(11), 1059–1066 (2008).

14. S. Y. Leigh and J. T. C. Liu, “Multi-color miniature dual-axis confocal microscope for point-of-care pathology,” Opt. Lett. 37(12), 2430–2432 (2012).

15. P. Kim, M. Puoiris'haag, S. Friedland, C. P. Lin, and S. H. Yun, “In vivo confocal and multiphoton microendoscopy,” J. Biomed. Opt. 13(1), 015015 (2008).

16. K. Carlson, M. Chidley, K.-B. Sung, M. Descour, A. Gillenwater, M. Follen, and R. Richards-Kortum, “In vivo fiber-optic confocal reflectance microscopy with an injection-molded plastic miniature objective lens,” Appl. Opt. 44(10), 1792–1797 (2005).

17. C. L. Arrasmith, D. L. Dickensheets, and A. Mahadevan-Jansen, “MEMS-based handheld confocal microscope for in-vivo skin imaging,” Opt. Express 18(4), 3805–3819 (2010).

18. M. Rajadhyaksha, R. R. Anderson, and R. H. Webb, “Video-rate confocal scanning laser microscope for imaging human tissues in vivo,” Appl. Opt. 38(10), 2105–2115 (1999).

19. J. Knittel, L. Schnieder, G. Buss, B. Messerschmidt, and T. Possner, “Endoscopy-compatible confocal microscope using a gradient index-lens system,” Opt. Commun. 188(5-6), 267–273 (2001).

20. E. S. Flores, M. Cordova, K. Kose, W. Phillips, A. Rossi, K. Nehal, and M. Rajadhyaksha, “Intraoperative imaging during Mohs surgery with reflectance confocal microscopy: initial clinical experience,” J. Biomed. Opt. 20(6), 061103 (2015).

21. J. T. C. Liu, M. J. Mandella, N. O. Loewke, H. Haeberle, H. Ra, W. Piyawattanametha, O. Solgaard, G. S. Kino, and C. H. Contag, “Micromirror-scanned dual-axis confocal microscope utilizing a gradient-index relay lens for image guidance during brain surgery,” J. Biomed. Opt. 15(2), 026029 (2010).

22. J. T. C. Liu, M. J. Mandella, H. Ra, L. K. Wong, O. Solgaard, G. S. Kino, W. Piyawattanametha, C. H. Contag, and T. D. Wang, “Miniature near-infrared dual-axes confocal microscope utilizing a two-dimensional microelectromechanical systems scanner,” Opt. Lett. 32(3), 256–258 (2007).

23. P. J. Dwyer, C. A. DiMarzio, J. M. Zavislan, W. J. Fox, and M. Rajadhyaksha, “Confocal reflectance theta line scanning confocal reflectance microscopy for imaging human skin in vivo,” Opt. Lett. 31(7), 942–944 (2006).

24. T. D. Wang, S. Friedland, P. Sabhaie, R. Soetikno, P.-L. Hsiung, J. T. C. Liu, J. M. Crawford, and C. H. Contag, “Functional imaging of colonic mucosa with a fibered confocal microscope for real-time in vivo pathology,” Clin. Gastroenterol. Hepatol. 5(11), 1300–1305 (2007).

25. F. Jean, G. Bourg-Heckly, and B. Viellerobe, “Fibered confocal spectroscopy and multicolor imaging system for in vivo fluorescence analysis,” Opt. Express 15(7), 4008–4017 (2007).

26. V. Becker, T. Vercauteren, C. H. von Weyhern, C. Prinz, R. M. Schmid, and A. Meining, “High-resolution miniprobe-based confocal microscopy in combination with video mosaicing (with video),” Gastrointest. Endosc. 66(5), 1001–1007 (2007).

27. J. B. Pawley, “Handbook of biological confocal microscopy,” 985 (2006).

28. K. E. Loewke, D. B. Camarillo, W. Piyawattanametha, M. J. Mandella, C. H. Contag, S. Thrun, and J. K. Salisbury, "In vivo micro-image mosaicing," IEEE Trans. Biomed. Eng. 58(1), 159–171 (2011).

29. B. Rosa, M. S. Erdem, T. Vercauteren, B. Herman, J. Szewczyk, and G. Morel, “Building large mosaics of confocal edomicroscopic images using visual servoing,” IEEE Trans. Biomed. Eng. 60(4), 1041–1049 (2013).

30. K. Kose, M. Cordova, M. DuFly, E. S. Flores, D. H. Brooks, and M. Rajadhyaksha, “Video-mosaicing of reflectance confocal images for examination of extended areas of skin in vivo,” Br. J. Dermatol. 171(5), 1239–1241 (2014).

31. Y. Chen, D. Wang, and J. T. C. Liu, “Assessing the tissue-imaging performance of confocal microscope architectures via Monte Carlo simulations,” Opt. Lett. 37(21), 4495–4497 (2012).
1. Motivation and Background

Over the past century, the most reliable method of diagnosing diseases has been the microscopic visualization of glandular, cellular, and subcellular features from thinly sectioned tissues mounted on glass slides. This practice of histopathology is universally accepted by the medical community as a gold-standard diagnostic method. Unfortunately, the art and science of preparing histology slides from freshly excised or biopsied tissues is labor intensive and time consuming. The most standard histology method involves chemically fixing tissues, embedding them in paraffin wax, sectioning them, adhering them onto glass slides, staining them, and finally visualizing the thin (~5-μm thick) tissue sections with a microscope. For certain clinical scenarios, frozen-section pathology is also performed, in which tissues are rapidly frozen and sectioned with a cryotome prior to staining and microscopic visualization. While frozen sections can be prepared in as short as 20 min, this time frame is still an obstacle for intraoperative or point-of-care use. Moreover, histopathology requires the invasive removal of tissues from a patient, which introduces risk of iatrogenic injury and also hinders its use for the diagnostic screening of sensitive sites or large areas of tissue. In light of these concerns, there has been a growing consensus of the need for miniature in vivo microscopes to enable noninvasive point-of-care pathology.

Various prototypes and commercial devices have been developed for clinical microscopy applications, many of which have been based on the technology of confocal microscopy, which can utilize relatively low-power and inexpensive laser sources (as opposed to multiphoton microscopy techniques) [5–26]. Confocal microscopy utilizes spatial filtering to reject out-of-focus and multiply scattered background light, thereby enabling the “optical sectioning” of intact fresh tissues [27]. Previous handheld and endoscopic confocal microscopy prototypes have largely utilized a point-scanned configuration in which an image is generated one pixel at a time by scanning a focal volume in two dimensions within the sample. The complexity and physical limits of a two-dimensional scanning mechanism often results in limited frame rates for a point-scanned clinical system (typically <10 frames/sec), which results in motion artifacts during handheld or endoscopic use, and also hampers image-based mosaicking algorithms that require successive image frames to contain common image features [9, 20, 26, 28–30]. Therefore, in the device described in this manuscript, a line-scanned configuration is utilized in which an image is generated by scanning a focused line along one dimension within a tissue sample (instead of two dimensions for a point-scanned device). Using the line-scanning technique, we demonstrate the ability to perform fluorescence optical-sectioning microscopy in fresh tissues at 16 frames/sec. Faster frame
rates may be possible in the future, as the microelectromechanical system (MEMS) scanner utilized in our device has a mechanical resonance frequency of >100 Hz.

Unlike conventional single-axis confocal (SAC) microscopes, which have become standard equipment in life-science and clinical laboratories, the miniature microscope developed in this study utilizes a dual-axis confocal (DAC) architecture. Compared to a SAC microscope, a DAC microscope with comparable axial resolution (optical-section thickness) is capable of improved rejection of out-of-focus and multiply scattered background light, which enables high-contrast microscopy within highly scattering tissues [31–33]. The DAC architecture utilizes off-axis low-numerical aperture (NA) illumination and collection beams that intersect at their foci, which also defines the focal volume of the microscope [31–33]. As mentioned in the previous paragraph, the DAC microscope device described in this report utilizes line scanning (LS) to enable high-speed microscopy while only requiring beam scanning along one dimension. Unlike a point-scanned confocal microscope, in which an illumination beam is focused to a tight spot within tissue and a pinhole is used for rejection (spatial filtering) of background light, a line-scanned confocal microscope utilizes a slit to reject background light from an illumination beam that is focused to a thin line within the tissue [34]. Line-scanned confocal microscopes thus sacrifice one dimension of confocality and typically exhibit reduced contrast (signal-to-background ratio, SBR) and tissue-imaging depth compared to point-scanned systems [31, 34]. Nevertheless, simulations and experiments have demonstrated that a LS-DAC microscope is capable of achieving adequate contrast (SBR) when imaging near tissue surfaces (approximately 100- to 200-μm deep) [31, 34, 35]. In addition, while the low-NA collection of light would be expected to limit the collection efficiency and signal-to-noise ratio (SNR) of DAC microscopes, the relatively isotropic resolution of the DAC microscope enables the collection of light from a larger focal volume compared to a SAC microscope with an equivalent optical-sectioning thickness. Coupled with the increased pixel dwell times afforded by line scanning, the LS-DAC architecture is capable of sensitive optical-sectioning microscopy of fresh tissues (ex vivo and in vivo) at high frame rates (>16 frames/sec), as exhibited here as well as in a previous study with a large tabletop LS-DAC prototype [34, 35, 39].

2. Miniature microscope design

2.1 Microscope modules

The miniature LS-DAC microscope developed in this study consists of three major modules (Fig. 1): (1) a main body housing the optics that are unique for the illumination beam (blue) and collection beam (green), the MEMS scanning mirror and alignment mirrors; (2) a relay objective lens with a lens cap that provides 3x magnification from the focal plane of the microscope within tissue to the back focal plane that is scanned by the main-body optics; and (3) a detector array module.

Compared to a previous tabletop LS-DAC system [39], the only major differences for this miniature system are the incorporation of a miniature MEMS scanner (Mirrorcle Technologies Inc.), rather than a large galvanometric scanner, as well as the design and utilization of a custom 3x relay objective (Fig. 1). The 3x objective lens not only boosts the crossing angle ($\theta$) and NA of the dual-axis beams ($\alpha$), but is also designed to provide effective index matching of these high-NA beams into fresh tissue specimens with minimal aberrations. As a result, the spatial resolution of the miniature system surpasses that of the previous tabletop LS-DAC system (see sections 2.5 and 3.2 for details).
L_2 \alpha = 0.20 \text{ rad} \quad \theta = 25.3 \text{ deg}

660 \text{ nm} \quad \text{Laser source}

Index-matching gel \quad \text{Coverslip}

Fig. 1. Optical circuit of the miniature LS-DAC microscope. The illumination beam path is colored blue whereas the collection beam path is colored green. The main body houses the MEMS scanning mirror, the mirrors (M1 and M2) used to align the dual-axis beams such that they intersect at the back focal plane of the objective lens, and the primary optics (L1, L2, L3 & L4) that focus the dual-axis beams at the back focal plane. The custom objective lens relays the beams from the back focal plane (at the left side of the objective) to the front focal plane in tissue (right side) with 3x de-magnification. The focusing angle of the beams in tissue, \alpha, and crossing angle, \theta, enable high-contrast optical sectioning with a resolution of 1 – 2 \mu m in the lateral and axial dimensions, respectively (see section 2.5).

2.2 Illumination focusing module

A singlemode optical fiber (SM670) is used to couple laser radiation at a wavelength of 660 nm into the illumination path of the main body (Gaussian beams are assumed throughout this work). An illumination fiber module, assembled by GRINTECH GmbH in Jena, Germany, consists of a series of three doublet achromat lenses packaged within a stainless steel cylindrical tube with an inner diameter of 3.0 mm and an outer diameter of 3.2 mm as shown in Fig. 2(a). Lenses L1 and L2 are spherical achromats that were purchased from Edmund Optics in Barrington, NJ (catalog numbers 45262 and 45090, respectively) and then reduced in diameter (3.0-mm diameter) by BMV Optical in Ottawa, Ontario, Canada. Lens C is a custom cylindrical achromat lens, fabricated by BMV Optical, that is based on the lens prescription of a spherical achromat from Edmund Optics (catalog number 45420). Without the cylindrical lens, C, the focusing module would generate a focused spot. The addition of the cylindrical lens converts the illumination fiber module into an anamorphic focusing system, which causes one axis of the illumination beam to focus first, thereby generating a focal line as desired for LS-DAC microscopy. This optical design is similar to our previous large-scale tabletop LS-DAC prototypes [34, 35]. The focal lengths of lenses L1 and L2 are 9 mm and 12 mm, respectively. This causes the NA of the illumination beam to be reduced from the 0.12 1/e^2 NA emitted from the singlemode fiber to a focusing NA of 0.09 at the back focal plane of the 3x objective. The focal length of lens C was selected to provide a focal line with a full-width at half maximum (FWHM) length of 1.2 mm, which corresponds to a 0.4-mm long focal line within tissue (after the 3x objective).

2.3 Main body design

As shown in Figs. 2(c) and 2(d), the main body features two cylindrical channels, one for the illumination fiber module (described in the previous section), and one for the collection-side optics. The collection-side channel holds a pair of doublet achromat lenses, L3 and L4, purchased from Edmund Optics (catalog numbers 63692 and 45345, respectively) and then reduced in diameter (3.2-mm diameter) by BMV Optical. The focal lengths of L3 and L4 are 12 mm and 60 mm, respectively. Therefore the lenses serve to magnify the image of the focal line onto the linear detector array. As described later, this 5x magnification, coupled with the 3x magnification of the objective lens, provides sufficient magnification (15x) to allow the
detector array (with 6.5-μm pixel spacing) to fully sample the focal line (lateral resolution of ~1 μm in tissue) according to the Nyquist sampling criterion. Both the illumination and collection beams are designed to focus and intersect at the back focal plane of the objective lens at a half crossing angle, $\theta$, of 11 deg. The alignment of the beams is achieved by rotating and translating (axially) a pair of 45-deg mirrors that fit within the cylindrical channels in the main body (Fig. 2(b)–2(d)). The alignment mirrors are adjusted to deflect the illumination and collection beam paths at 11 deg with respect to the $x$ axis and are then permanently fixed in place. The MEMS mirror is positioned above the beams to re-direct them in the axial ($z$) direction, with a half crossing angle of 11 deg with respect to the $z$ axis.

Fig. 2. (a) Line-focusing illumination fiber module. (b) Illumination and collection optics within the main body. (c) Main body with detector. (d) Main body scan head. (e) Microscope package (in progress). (f) Handheld device (in progress).

2.4 MEMS scanning mirror

The MEMS scanning mirror utilized in our device was purchased from Mirrorcle Technologies in Richmond, CA. The MEMS mirror surface has a circular profile, with a diameter of 3.8 mm, and is actuated in two dimensions (tip / tilt) with electrostatic comb drives. In the LS-DAC device, only one dimension of scanning is necessary to create a two-dimensional image (in a line-by-line fashion). However, the extra dimension of scanning may be useful for correcting certain image distortions that may be present in our current LS-DAC system. The resonant frequency of the MEMS mirror is in the ~200 Hz range. This would be too slow for a point-scanned system but is more than adequate for a line-scanned system in which a maximum frame rate of 200 frames/sec would theoretically be possible if photon counts are sufficiently high, as may be the case for reflectance microscopy. The square MEMS scanner chip measures 4.3 by 4.3 mm, and is glued and wirebonded by the manufacturer (Mirrorcle Tech. Richmond, CA) within a rectangular surface-mount LCC18 package that measures 8.89 by 7.24 mm. The LCC18 package is soldered onto a PCB chip (measuring 10.16 by 8.64 mm) that was custom designed by our group and fabricated by Advanced Circuits Inc. The PCB chip provides a mounting surface for the MEMS package,
and also routes the electrical connections from the LCC18 MEMS package to a row of conductive vias located on the backside of the PCB. Note that a total of 8 electrical connections, plus a ground connection, are required to fully actuate the two-dimensional MEMS mirror. The 8 high-voltage MEMS connections are supplied by wires that are connected to the backside of the PCB through a two-part miniature 9-pin connector (“Nano-miniature” line) from Omnetics Connector Corp., Minneapolis, MN (part numbers A79000-001 and A79001-001, respectively) as shown in Fig. 2(d).

2.5 Objective lens and optical ray tracing

The purpose of introducing the objective lens into the miniature system is to increase the focusing NA of the illumination and collection beams located at the back focal plane of the objective, as well as to increase the crossing angle of the beams, which improves the resolution of the miniature system [21, 33]. As mentioned previously, the 1/e² NA of the Gaussian illumination beam at the back focal plane of the objective (in air, where \( n_1 = 1 \)) is equal to \( n_1 \sin \alpha_1 = 0.09 \). The objective lens magnifies the NA at the back focal plane by a factor of 3 and the sample is assumed to have an index of approximately \( n_2 = 1.34 \). Therefore,

\[
\alpha'_1 = \sin^{-1} \left( 3 \cdot \frac{n_1}{n} \sin \alpha_1 \right) = 0.202
\]

Likewise, since \( \theta_1 = 11 \) deg, \( \theta_2 \) at the front focal plane in tissue is 25.3 deg.

According to diffraction-theory calculations [21, 33], in which the illumination and collection beams are assumed to be symmetric and identical (approximately true for our device), these parameters should result in the following theoretical spatial resolutions (in tissue with an index \( n = 1.34 \)):

\[
\Delta x = \frac{0.446 \lambda}{n(\pi / 2 \cdot \alpha)} = 0.77 \mu m; \quad \Delta y = \frac{0.446 \lambda}{n(\pi / 2 \cdot \alpha)} = 0.70 \mu m; \quad \Delta z = \frac{0.446 \lambda}{n(\pi / 2 \cdot \alpha) \sin \theta} = 1.64 \mu m
\]  

A ray-trace analysis of the miniature system was performed in ZEMAX and is shown in Fig. 3. The spot diagram from the illumination path (Fig. 3(b)) shows that the focal line within tissue does not exhibit significant aberrations, with a root mean square (RMS) spread of < 1 \( \mu \)m across the line. As shown in Eq. (2), this spread is within the diffraction-limited FWHM width of the microscope. When the MEMS mirror is tilted by 4.5 deg, the line is translated by 200 \( \mu \)m in the \( x \) direction. Again, the spot diagram shows an RMS spread of < 1 \( \mu \)m across the line, which is excellent for the edge of the field of view. Note that due to the geometry of the beam-scanning method, the focal line tilts slightly as it scans in the \( x \) direction. However, this artifact is easily removed in software during image reconstruction and display. For the collection path, we analyzed the optical performance by setting up three ideal point sources at various locations along the focal line in tissue and carried out ray-trace simulations from these ideal point sources to the detector plane. The spot diagrams at the detector plane, shown in Fig. 3(d), demonstrate that the RMS spread is significantly less than the diffraction-limited FWHM spot size of ~15 \( \mu \)m that is expected at the detector. This corresponds to a 1-\( \mu \)m spot size within the tissue, due to the 15x magnification provided by the 3x objective plus the 5x collection optics (section 2.3). The custom objective was designed and fabricated in collaboration with Photon Gear Inc. (Ontario, NY).

2.6 Detector array

A miniature linear-detector array module, shown in Fig. 2(c), is currently being developed to fully package our microscope as a handheld device for clinical use. For preliminary assessment of the optical design and to evaluate the performance of the microscope, a sCMOS camera (Hamamatsu Orca Flash 4.0) with a two-dimensional (2D) detector was used to mimic the one-dimensional (1D) linear detector that will be incorporated in the final design. A thin
rectangular region of interest within the sCMOS array serves as a digital slit, as described previously for a large-scale tabletop LS-DAC microscope system [34, 35, 39]. In short, the sCMOS camera allows rapid acquisition of a 2048 × 8 pixel region at the center of the camera. Since the pixel spacing is 6.5 μm, we bin the center 3 rows of pixels to create a digital slit of thickness 6.5 × 3 = 19.5 μm. This approximately matches the diffraction-limited FWHM spot size of ~15 μm that is expected at the detector (see section 2.5). Exposure times of 125 μs (a line-acquisition rate of 8 kHz) are utilized to collect 500 lines per frame at an imaging rate of 16 frames/sec. In summary, our device images a field of view (FOV) of 300 × 300 μm with a sampling density of 500 × 600 pixels (x by y).

3. Results

3.1 Machined and fabricated components

As shown in Fig. 4(a), an illumination fiber module forms a subassembly with the main body (section 2.2). The main body with optical components is shown in Fig. 4(b). The illumination fiber module (Fig. 4(a)) is inserted into a cylindrical illumination channel and is secured with set screws (Fig. 4(b)). The collection lenses, L3 and L4, as well as the alignment mirrors, M1 and M2, are secured within their respective channels using UV-curing glue.
Figure 4(c) shows the scan head of the main body (CAD model shown in Fig. 2(d)). The clamps support the MEMS chip at a 45-deg angle and at the correct axial position to ensure proper alignment of the device. The main body and the objective lens (fabricated by Photon Gear Inc., Ontario, NY) are shown in Fig. 4(d). In the eventual hand-held device, an outer tube will surround and hold these components together (Figs. 2(e) and 2(f)).

3.2 Reflectance-based characterization of device performance

The axial response of the miniature system to a flat reflective surface was measured. Figures 5(a) and 5(b) are plots of the axial response on a linear and log scale, respectively. The FWHM optical-sectioning thickness at the center of the field of view (FOV) is measured to be ~2.0 μm. Figure 5(b) shows that the background signal, in the absence of a mirror, is below 0.1% of the maximum intensity from a mirror at the focus. The presence of diffraction sidelobes is due, in part, to slight clipping of the Gaussian beams by apertures, such as the 3.0-mm diameter illumination lenses. The edge response of the microscope to a chrome knife edge on glass is plotted in Fig. 5(c), showing a 10% to 90% transition width in the x direction ($X_{10-90}$) of 1.1 μm. The corresponding transition width in the y direction is similar, as expected based on Eq. (2), and is measured to be $Y_{10-90} = 1.0$ μm. The image of a reflective 1951 USAF resolution test chart, shown in Fig. 5(d), shows the ability of the microscope to resolve features at the micron scale. Note that there is vignetting at the edges of the FOV due to slight field curvature introduced by the scanning MEMS mirror. However, this field curvature (< 10-μm deviation in focal depth over the entire FOV) is not a significant issue when imaging thick three-dimensional tissues. As expected, the resolution is slightly degraded at the edges of the FOV (+/− 150 μm from the center). The worst FWHM resolution values measured at
the edges of the FOV were approximately 3.1 μm in the axial dimension (section thickness) and approximately 1.3 μm in the lateral dimensions.

3.3 Tissue images

To demonstrate the ability of our device to acquire fluorescence images, we imaged fresh mouse tissues and blood flow within the capillaries of a mouse ear. Fresh mouse tissues were stained in 1% methylene blue for 10 - 30 min, and then rinsed in PBS to remove excess methylene blue from the tissue surface. Methylene blue is known to preferentially label cell nuclei, as is apparent from the kidney images shown in Figs. 6(c) and 6(e). Corresponding H&E pathology images, from the same approximate tissue regions imaged by the miniature LS-DAC microscope device, are also shown in Fig. 6.

To demonstrate the ability to visualize dynamic processes at 16 frames/sec, we imaged red blood cells trafficking within the capillaries of a mouse ear (see Visualization 1). The blood plasma was labeled via retro-orbital injection of a high-molecular-weight fluorescent dextran (Cy5.5-dextran, M.W. = 500 kDa, 2.5 mg per injection in a 150-μL volume, Nanocs Inc., DX500-S5-1). The intravenous injection of the fluorescent dextran causes the red blood cells to appear as dark shadows against the bright fluorescence from the plasma within the capillaries (see Visualization 1). Figure 7 shows a maximum-intensity projection (in the axial or depth direction) of the vasculature over a range of depths from 120 to 170 μm. The colormap in Fig. 7 encodes for depth, with shallower vessels colored blue and deeper vessels colored red. Visualization 2 shows en face image sections of fluorescently stained mouse tongue over a range of depths from 25 to 75 μm. Visualization 3 shows en face image sections of fluorescent vasculature in a mouse ear over a range of depths from 75 to 125 μm.
Fig. 6. (a) Mouse tongue image at a depth of ~50 μm. (b) Histologic section (H&E staining) of corresponding tissue. (c) Mouse kidney image at a depth of ~100 μm. (d) Histologic section (H&E staining) of corresponding tissue. (e) Mouse kidney image at a depth of ~50 μm. (f) Histologic section (H&E staining) of corresponding tissue. (g) Mouse colon image at a depth of ~70 μm. (h) Histologic section (H&E staining) of corresponding tissue. The scale bar represents 50 μm.
Fig. 7. (a) Depth projection of vasculature in a mouse ear, imaged between 120- and 170-μm deep. The trafficking of blood cells can be observed within the vessels at an in vivo imaging speed of 16 frames/sec (see Visualization 1). The scale bar represents 50 μm.

4. Discussion

Previous miniature point-scanned dual-axis confocal (PS-DAC) microscopes have had modest axial resolutions (optical sectioning) in the 5 – 10 μm range, limited frame rates of up to 4 frames/sec, and have relied on sophisticated MEMS scanners designed to operate at frequencies in the kHz range [14, 21]. The slow frame rates of such devices made them susceptible to motion-induced image artifacts, and the fast 2D MEMS scanners were challenging to develop and optimize for use in a reliable clinical device. Therefore, in this study, we developed a miniature line-scanned dual-axis confocal (LS-DAC) microscope that utilizes a robust commercial MEMS mirror to achieve a high frame rate of >16 frames/sec (and potentially much higher). While line-scanned confocal microscopes sacrifice confocality in one dimension and are therefore less efficient at rejecting out-of-focus and multiply scattered background light compared to their point-scanned counterparts, this study demonstrates that a miniature LS-DAC microscope is capable of high-speed imaging within fluorescently labeled fresh tissues (ex vivo and in vivo) with excellent sensitivity and resolution.

A major goal of this study was to demonstrate the ability to miniaturize a LS-DAC microscope without sacrificing performance compared to a table-top LS-DAC prototype that was recently developed for high-speed optical sectioning [33, 39]. The miniature system described here exhibits a lateral and axial resolution of 1 – 2 μm, which is slightly superior to that of our previous tabletop systems. However, as shown in the axial response plot in Fig. 5(b), the miniature system exhibits diffraction noise that slightly deteriorates the contrast (SBR) of the microscope in comparison to the tabletop LS-DAC devices. We believe that these diffraction sidelobes are a result of the beam clipping that is difficult to avoid due to the necessity for miniature optical components in our design – a necessary trade-off. However, at the shallow imaging depths (~100 – 200 μm) at which the LS-DAC technology is designed to image best [34, 35], this slight loss of contrast is not a major factor and our results demonstrate the ability to image fluorescently labeled tissues with high contrast and resolution. Furthermore, we demonstrate the ability image fluorescent vasculature with sufficient sensitivity and contrast to visualize the trafficking of blood cells at 16 frames/sec.

In future work, we will fully package the main body optics, 3x objective lens, and linear detector array into a handheld package for clinical use, including for oral-cancer detection and for guiding brain-tumor resection procedures. We are also developing a sterilizable lens cap to provide an effective interface between the distal tip of the microscope and the patients’
tissues, as well as to enable passive or active axial translation of the focal plane by clinical end users for imaging at various depths.

Intraoperative and point-of-care diagnostic use of our device could be facilitated by implementing real-time mosaicing algorithms, as evidenced by recent studies that explored the use of such algorithms for microscopy and scanning fiber endoscopes [20, 26, 28, 36]. Recent advances in surgical robotics and telesurgery also motivate the integration of the LS-DAC system with robotic devices [36, 37]. Additional studies have demonstrated the feasibility of co-registering images from wide-field-of-view (FOV) devices (e.g. MRI and CT) with limited-FOV devices (e.g. miniature microscopes) to facilitate device localization in traditional and robot-assisted surgeries [38]. This work represents a first step towards realizing real-time in vivo pathology for a variety of diagnostic and therapeutic applications and will pave the way for several first-in-human feasibility studies in the near future.

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