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Multi-color miniature dual-axis confocal microscope for point-of-care pathology

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Abstract

We present a miniature micro-electro-mechanical systems (MEMS)-based dual-axis confocal microscope capable of spatially co-registered fluorescence imaging at multiple wavelengths. This device has a 10-mm diameter scan head with a 2-mm diameter tip for convenient use during surgery to guide tumor resection. The microscope has an adjustable focal depth from 20 – 200 microns and is capable of imaging with an axial resolution of 9 microns and in-plane resolution of 4 microns over a field of view of 450×450 microns. Simultaneous two-color imaging of individual optical sections is achieved by using a pair of grating-prism assemblies (GRISMs) to compensate for chromatic dispersion in the 2-mm diameter gradient-index (GRIN) relay lens at the distal tip of the device. Experimental measurements of the axial response of the microscope as well as two-color images of a reflective bar target and fresh mouse brain tissues demonstrate the performance of our device and its potential for multi-color *in vivo* optical-sectioning microscopy.

In recent years, miniature optical-sectioning microscopes have played a critical role in the rapidly developing field of *in vivo* microendoscopy and point-of-care pathology for both clinical applications and biological investigations. Microscope designs have utilized various approaches towards achieving optical sectioning such as multiphoton excitation [1–4], single-axis confocal microscopy [5,6], dual-axis confocal (DAC) microscopy [7] and structured illumination [8]. Designs have also differed in their scanning mechanisms, including proximally-scanned coherent fiber bundles [5,6], distally-scanned fiber tips [3] and MEMS scanners [4,7].

DAC microscopy achieves subcellular resolution with several advantages over conventional single-axis confocal microscopy. The latter generates its optical sectioning capabilities with pinhole illumination and detection through a high-numerical-aperture (NA) objective lens, where the short working distance of the high-NA objective necessitates pre-objective beam scanning. This, in turn, results in off-axis aberrations that must be mitigated through the use of multiple corrective lenses. DAC microscopy achieves optical sectioning by using two low-NA off-axis beams for illumination and collection that intersect and focus at a single point in space. The use of low-NA focusing lengthens the microscope's working distance, allowing for post-objective beam scanning over a large field of view with minimal aberration. Furthermore, we have shown through diffraction theory and Monte-Carlo scattering simulations that the DAC architecture results in superior rejection of out-of-focus and multiply scattered light, which is important for preserving contrast during deep-tissue imaging [9].

Multi-color microscopy is important in that it offers the ability to observe labeled components in context with each other, such as nuclei and cytoplasm in the case of

pathological determinations, or multiple cell types for investigating correlated behavior [10]. In addition, multi-color imaging can be used for ratiometric quantification [11] or correction of image intensities [12] to improve diagnostic accuracy. Thus, we present here a method for dispersion compensation based on grating-prism assemblies (GRISMs) that counteracts chromatic aberrations introduced by the gradient-index (GRIN) relay lens at the distal end of our miniature DAC microscope. This solution was modeled and optimized via ray-trace simulations and validated experimentally.

The device presented in this Letter is a modified version of the first miniature DAC microscope [7] to combine MEMS scanning technology with a 2-mm diameter GRIN relay lens for surgical use. The basic optical circuit of our device (Fig. 1) consists of two parallel collimated beams (illumination and collection beams) that are reflected off a parabolic mirror such that they focus to, and intersect, at a single point that defines the focal volume of the microscope. The focusing half-angle of each beam and the intersection half-angle between the beams are 0.075 radians ($1/e^2$ intensity) and 22.2 degrees, respectively. A 2D scanning MEMS mirror [13] deflects these beams forward towards a GRIN triplet relay lens that is positioned with one face at the intersection (foci) of the illumination and collection beams [7]. The GRIN lens then relays the focal plane (1-pitch relay) towards the specimen to be imaged. In terms of the optical circuit from source to detector, laser illumination is provided by two diode-pumped solid-state lasers (Coherent Laser Inc.) at 488 nm and 561 nm, which are combined into a single-mode optical fiber before being coupled to a fibered achromatic doublet collimator (2.99-mm focal length, GRINTECH GmbH). The fibered collimator generates a collimated illumination beam with a $1/e^2$ diameter of 0.66 mm that travels through a GRISM before being focused by the aforementioned parabolic mirror (focal length = 6.0 mm). Reflectance or fluorescence signal generated at the focus within the sample at the distal tip of the GRIN relay lens is collected by a path that is axially symmetric to the illumination path of the optical circuit. Fiber-coupled fluorescence signals at two wavelengths are demultiplexed by a dichroic mirror (FF555-Di02, Semrock) and filtered (LP02-488RU, SP01-561RS and LP02-561RU, Semrock) before being detected by photomultiplier tubes (H7422-40, Hamamatsu).

In order to design the color-correcting GRISM elements appropriately, ray-trace simulations were performed with ZEMAX software. In the absence of the color-correcting GRISM elements, all of the optical components prior to the GRIN relay lens are achromatic, such that illumination and collection beams of all wavelengths intersect and focus at the same point on the proximal side of the GRIN lens. However, the chromatic behavior of the GRIN lens causes the imaging depth (axial position of the intersecting foci) to be wavelength dependent at the distal end of the GRIN lens (Fig. 2c).

To correct for this chromatic aberration, we utilize a low-dispersion grating with an effective line density of 40.64 line pairs/mm to introduce chromatic dispersion (Fig. 2b) that compensates for the GRIN lens' dispersion effects. This grating causes the collinear collimated beams at 488 nm and 561 nm to be dispersed (first-order diffraction) such that they travel at an angle of 0.17 deg with respect to each other ($\theta_{561} - \theta_{488}$). Since the first-order diffracted beams at 488 nm and 561 nm are directed at an angle of $\theta_{488} = 1.14$ deg and $\theta_{561} = 1.31$ deg with respect to the original collimated beam (rotated about the x-axis), it is necessary to use a 2.3-deg prism (N-BK7 glass) to re-steer both beams towards their original axial orientations. This allows the first-order diffracted beams to be focused well by the parabolic reflector while steering any residual zero-order (dashed line in Fig. 2b) and higher-order diffracted beams away from an axial orientation, thus preventing them from introducing significant background signals.

Ray-trace simulations demonstrate how a well-designed GRISM allows the illumination and collection beam paths at 488-nm and 561-nm to intersect at the same point at the distal end of the GRIN relay lens (Figure 2d). A slight tradeoff is required in that the 488-nm and 561-nm beams are focused slightly before and after their common intersection point, respectively. This is due to the fact that the collimated beams at the two wavelengths cannot both be oriented exactly in the axial direction after being dispersed by the GRISMs. However, since the DAC architecture utilizes low-NA beams, the beams still intersect well within one Rayleigh range (~220 microns) of their foci, which prevents degradation in spatial resolution.

Experimental validation involved the construction of the GRISMs, which consisted of volume phase holographic gratings from Wasatch Photonics glued to prisms from Tower Optical. The GRISMs were mounted in custom holders (Creative Metal, San Jose, CA) and inserted within the 10-mm diameter housing of our miniature GRIN-relayed DAC microscope. The low-dispersion gratings are constructed by placing two high-dispersion gratings back-to-back in opposite directions, such that the effective dispersion is the difference between the two higher dispersions. This design enables light to be diverted with high efficiency (>90%) into the first diffractive order at the required angles. It should be noted that the low-dispersion gratings exhibit peak efficiency at roughly 525 nm and that our microscope, with GRISM compensation, is designed to be achromatic over a wide range of wavelengths within approximately ± 100 nm of 525 nm. Experimental validation was performed with reflectance and fluorescence imaging at two discrete illumination wavelengths: 488 and 561 nm.

The device's axial response to a reflective mirror surface was measured both with and without dispersion compensation to confirm co-localization of imaging planes at 488-nm and 561-nm with the GRISM elements in place (Fig. 3a, 3b). The measured full width at half-maximum (FWHM) axial resolution of the device in air was measured to be 9 microns and 10 microns for 488-nm and 561-nm illumination, respectively. Additional validation of image plane co-localization was obtained first in reflectance mode (Fig. 3c, 3d) and then fluorescence mode (Fig. 3e, 3f). Images were obtained by driving each axis of the biaxial MEMS mirror at its respective resonance frequency to obtain a maximum field of view of 450 $\times$ 450 microns. Pixel intensities captured along the Lissajous trajectory were mapped with a custom-written LabVIEW (National Instruments) framegrabber program [7].

To demonstrate the ability of our device to acquire dual-channel fluorescence images, we imaged fresh mouse brain tissues. Animals were anesthetized before being injected retro-orbitally with tetramethylrhodamine-dextran ($\lambda_{ex/em} = 555/580$ nm, 70kDa, Invitrogen) and fluorescein-dextran ($\lambda_{ex/em} = 494/521$ nm, 70 kDa, Invitrogen) as blood-pooling agents. Following decapitation, the brain was removed and imaged immediately with our miniature multi-color DAC microscope where incident intensities were 1 mW and 1.6 mW for 488-nm and 561-nm light, respectively. Vasculature containing fluorescently labeled dextrans were readily visualized with spatial co-registration between channels (Fig. 3e, 3f), thus demonstrating the ability of our approach for multi-spectral optical-sectioning microscopy.

We have presented a design and a demonstration of a miniature MEMS-scanned DAC microscope that is capable of spatially co-registered imaging at multiple wavelengths. This design utilizes a custom GRISM to compensate for the chromatic aberrations that are introduced by a 2-mm diameter GRIN relay lens at the distal tip of the device. Future plans are to utilize this device for quantitative ratiometric imaging of molecularly targeted fluorescent contrast agents. These technologies have the potential to delineate tumor margins in real time, with accuracy approaching that of frozen-section pathology, for guiding brain tumor resection.

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References

1. Llewellyn ME, Barretto RPJ, Delp SL, Schnitzer MJ. *Nature*. 2008; 454:784–788. [PubMed: 18600262]
2. Grewe BF, Langer D, Kasper H, Kampa BM, Helmchen F. *Nat. Methods*. 2010; 7:399–405. [PubMed: 20400966]
3. Wu Y, Leng Y, Xi J, Li X. *Opt. Express*. 2009; 17:7907–7915. [PubMed: 19434122]
4. Hoy CL, Durr NJ, Chen P, Piyawattanametha W, Ra H, Solgaard O, Ben-Yakar A. *Opt. Express*. 2008; 16:9996–10005. [PubMed: 18575570]
5. Tanbakuchi AA, Rouse AR, Udovich JA, Hatch KD, Gmitro AF. *J. Biomed Opt.* 2009; 14:044030. [PubMed: 19725741]
6. Maitland KC, Gillenwater AM, Williams MD, El-Naggar AK, Descour MR, Richards-Kortum RR. *Oral Oncology*. 2008; 44:1059–1066. [PubMed: 18396445]
7. Liu JTC, Mandella MJ, Loewke NO, Haeberle H, Ra H, Piyawattanametha W, Solgaard O, Kino GS, Contag CH. *J. Biomed Opt.* 2010; 15:026029. [PubMed: 20459274]
8. Lim D, Chu KK, Mertz J. *Opt. Letters*. 2008; 33:1819–1821.
9. Liu JTC, Mandella MJ, Crawford JM, Contag CH, Wang TD, Kino GS. *J. Biomed Opt.* 2008; 13:034020. [PubMed: 18601565]
10. Henrickson SE, Mempel TR, Mazo IB, Liu B, Artyomov MN, Zheng H, Peixoto A, Flynn MP, Senman B, Junt T. *Nat. Immunology*. 2008; 9:282–291. [PubMed: 18204450]
11. Liu JTC, Helms MW, Mandella MJ, Crawford JM, Kino GS, Contag CH. *Biophys. J.* 2009; 96:2405–2414. [PubMed: 19289065]
12. Themelis G, Yoo JS, Soh KS, Schulz R, Ntziachristos V. *J. Biomed Opt.* 2009; 14:064012. [PubMed: 20059250]
13. Ra H, Piyawattanametha W, Taguchi Y, Lee D, Mandella MJ, Solgaard O. *J. Microelectromech. Sys.* 2007; 16:969–976.

Figure 1.
Optical circuit of multi-color DAC microscope.

Figure 2.

Ray-trace simulations (a) GRISMs compensate for chromatic aberration in the GRIN relay lens at the distal end of the device. (b) The grating portion of the GRISM introduces chromatic dispersion into the first diffractive order. Dotted arrows indicate zero-order (undiffracted) light. (c,d) Ray-trace simulations show that a GRISM-free design results in a wavelength-dependent focal volume position (axial offset of 80 microns between 488-nm and 561-nm imaging planes), which is corrected by the GRISMs.

Figure 3.

Experimental results of GRISM-based color correction. (a,b) Axial scan of a reflective target shows that an uncorrected device causes light at 488 nm and 561 nm to focus 70 μm apart, which is corrected to within 3–4 μm by the GRISMs. (c,d) Reflectance image of group 7 of the USAF bar target at a single axial position (only the center is in focus due to inherent field curvature, which is not of concern in 3D tissues). (e,f) Fluorescence image sections of mouse brain vasculature (depth = 55 μm) containing both fluorescein-dextran and tetramethylrhodamine-dextran excited at 488 nm and 561 nm, respectively. Scale bars = 50 μm .