Confocal microscopy offers a powerful tool for imaging in biological tissue, and it can be used in vivo if the lenses and scanning mechanisms can be made sufficiently small. In the conventional, single-axis configuration, the same lens facilitates illumination and image collection, and the optics cannot be reduced to millimeter scale without sacrificing resolution, field of view (FOV), or working distance. The scanning mechanism is located proximal to the objective, requiring multiple optical elements to correct aberrations and achieve high performance. Furthermore, the use of standard objectives in vivo is rather cumbersome.

By combining a dual-axes confocal architecture with biaxial, post-objective scanning, we have been able to collect fluorescence (non-coherent) images in horizontal cross sections parallel to tissue surface. This design uses two low-numerical-aperture (NA) lenses to achieve high axial resolution and long working distance. A scanning mirror located distal to the lenses rotates along orthogonal axes to produce arc-surface images over a large FOV. With fiberoptic coupling, this microscope can be scaled down to millimeter dimensions via micro-electro-mechanical systems (MEMS) technology. Moreover, low-NA lenses are easy and inexpensive to fabricate.

Design and Innovation

Dual-axes architecture uses separate low-NA objectives for the illumination and collection (see figure 1). The NA or half-cone angle of each lens is determined by the angles $\alpha_i$ and $\alpha_c$. The point spread function of either objective (purple ovals) has a long axial dimension and a narrow transverse dimension, so that the length of the overlapping region (black oval) represents the overall axial resolution of the system. The length of this overlap depends on the transverse dimension primarily, rather than...
the axial dimension of the beams at the focus. The illumination beam is incident to the tissue at angle $\theta$, and the fluorescence, which originates from the overlapping focal volume at the intersection of the two beams, is collected off-axis. As a result, light scattered along the illumination path (grey region) outside of the focal volume is unlikely to arrive at the collection objective with the angle necessary for detection, since the required combination of scattering events occurs with low probability. On the other hand, a standard confocal microscope objective has a single axis and requires a much higher NA to achieve an equivalent axial resolution. As a result, the collected light emerges from within the same, large-angle cone (dashed lines) as that traversed by the illumination, which results in increased noise from scattering.

We use a novel scanning method whereby the illumination and collection beams pass through their respective objectives on-axis, independent of the angle of the scan mirror. A single-plane, biaxial mirror located distal to the objectives steers the beam (see figure 2). With the mirror in this location, we define the scanning distance as the displacement between the mirror's surface and the focal volume. Note that this parameter differs from the working distance as defined in figure 1. In the biaxial mode, the scanning mirror rotates about the x- and y-axes and scans the illumination and collection beams synchronously, such that the intersection of the two axes remains oriented at a constant angle $2\theta$. The focal volume remains overlapped, and without changing shape, moves over an arc surface within the tissue.

We can consider this optical system equivalent to the outer annular ring of a high-NA objective that is illuminated with two parallel collimated beams. Low-NA objectives focus the separate beams. A single-plane mirror that is distal to the objectives steers the beams together and preserves the dimensions of the overlapping focal volume, as long as no aberrations are introduced. This is a good approximation when the maximum angle of deflection during scanning is small compared to $\theta$. Note that it would be difficult to use post-objective scanning with a conventional, single-axis microscope because of the short working distance.

We constructed a bench-top prototype with an inverted configuration. The axes of illumination and collection are oriented at $\theta = 30^\circ$ to the
midline. A frequency-doubled semiconductor laser (Sapphire; Coherent; Santa Clara, CA) delivers illumination at \( \lambda = 488 \) nm into a single-mode polarization, which maintains optical fiber SMF 1 with a mode-field diameter of 2.9 \( \mu \)m, NA = 0.11. We achieve biaxial scanning by driving the scan mirror (GSI Lumonics; Billerica, MA) with a 50-Hz, sinusoidal waveform and 0.1-Hz, linear-ramp waveform along the fast and slow axes, respectively. This yields a raster scan with an FOV of about 500 \( \mu \)m after clipping the non-linear portions at the edges of the sinusoidal scan.

**Performance**

For a fluorescence image collected from a mouse cerebellum at a depth of \( z = 30 \) \( \mu \)m, we collected horizontal scans at increasing axial depths of up to about 100 \( \mu \)m, beyond which the system could detect no signal (see figure 3). Larger round structures (arrows) with high fluorescence intensity, about 20 \( \mu \)m in diameter, are consistent with Purkinje cell bodies and aligned side by side in a row.

From fluorescence, the signal-to-noise ratio (SNR) for the Purkinje cells, molecular layer, and internal-granule layer is 13.1 \( \pm \) 0.5, 4.4 \( \pm \) 0.3, and 4.3 \( \pm \) 0.4, respectively. The contrast ratio between the Purkinje cells and the molecular layer and internal-granule layer is 3.0 and 3.1, respectively.

We also collected fluorescence images from Drosophila melanogaster embryos that expressed the engrailed gene labeled with green fluorescent protein, a marker of developmental regulation. The cylindrical embryos are about 200 \( \mu \)m in diameter by about 500 \( \mu \)m in length. We created a series of transverse images, which we collected at increasing axial depths in 10 \( \mu \)m increments through the thickness of the embryo, and created 3-D reconstructed images using Amira volume modeling software. Fluorescence images collected with a conventional, single-axis confocal microscope provided a basis for comparison.

A fluorescence image collected at a depth of \( z = 100 \) \( \mu \)m shows 14 stripes of engrailed, a marker of embryonic development (see figure 4). Single lines of cells mark the posterior parts of the segments. The SNR of the stripes is 15 \( \pm \) 3, and the contrast ratio between the stripes and the embryo protoplasm is 115. A 2-D projection of a 3-D reconstructed image from 18 horizontal sections shows the middle stripes are more intense and the spacing is somewhat irregular. In the single-axis confocal image, the stripes of engrailed have similar intensity and are equally spaced. These differences, which occur because the image is collected over an arc surface rather than a plane, can be corrected with appropriate image-processing software.

**Putting It in Perspective**

Using this system, sub-cellular structures such as nuclei and cell membranes can be distinguished from cells in a monolayer and within bulk tissue specimens over axial depths of several hundred microns. The collection of light off-axis selects against light scattered along the illumination beam, thus reducing the image noise.

Combined with fiberoptic coupling, the dual-axes instrument could be reduced in size to millimeter dimensions and used for in vivo collection of fluorescence images in transgenic models. With post-objective scanning, scaling down the dual-axes architecture will change the working distance and FOV, but not the image resolution. A miniature confocal microscope that uses the dual-axes approach can be used for in vivo molecular and cellular imaging as a handheld device or implanted instrument.

The instrument’s long working distance provides greater...
will use custom optics for diffraction-limited performance in future systems.

For this technique to be used in vivo in anesthetized animals, we would like an image collection time of less than 2 s to minimize motion-induced artifacts. The fluorescence images shown required 90-s collection intervals, but we could reduce this time significantly by optimizing the system design. To achieve the same SNR as that in the images shown, we could increase the fluorescence-detection efficiency by using a photomultiplier tube with a multi-alkali, rather than

ewase of use for imaging from the surface of the skin or from surgically exposed fields. For longitudinal studies, this instrument could be inserted through an implanted cannula that is cemented to some outer surface of a transgenic mouse. The microscope could be mechanically translated deeper into the tissue to image remote structures. While the imaging performance of the dual-axes instrument does not equal that of the single-axis instrument, these images, along with the system’s suitability for miniaturization, demonstrate the potential for in vivo biological investigations.

The biaxial scanning mechanism and the simple objective lenses are the key features. Scanning the illumination and collection beams behind the objectives, rather than in front of them, eliminates the need for additional optical elements to correct off-axis aberrations. The approach allows us to use low-NA lenses that are economical, simple to fabricate, and easy to miniaturize. For diffraction-limited performance, we expect wavefront aberrations of better than 0.25λ at all points in the image. However, we do not achieve this value here because we used readily available optics to demonstrate proof of principle. We

Figure 3 Fluorescence image (left) from mouse cerebellar cortex shows Purkinje (P) cell bodies (arrows) separating the granule (G) and molecular (M) layers; FOV 500 µm, scale bar 50 µm. The corresponding histology section (right) shows the granule layer, with dense cellularity; the intervening Purkinje layer, with several aligned neuronal cell bodies (arrowheads); and the molecular layer (lower diagonal half), with relatively few cells.

Figure 4 Fluorescence images of Drosophila embryo expressing stripes of engrailed were collected at a depth of z = 100 µm with dual axes system (left); scale bar 50 µm. A 2-D projection of the 3-D reconstructed image (middle) shows 18 irregular horizontal sections. The single-axis, confocal image (right) shows stripes of engrailed with similar intensity and equal spacing.
bi-alkali, photocathode, which would increase the detector quantum efficiency by a factor of about two. If we use objectives and optical fibers with a total collection NA of better than 0.20, rather than NA = 0.11, we could gain a factor of about four. We could also generate more fluorescence photons by increasing the laser power by a factor of about two or by decreasing the overfill of the illumination aperture, gaining a factor of about four. Alone, these changes can increase the fluorescence signal by a factor of about 60. In addition, we can accept images with a lower SNR, and develop novel reporters such as quantum dots that promise significantly greater fluorescence quantum yield. We will implement these changes in a future MEMS prototype.

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References

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