Multiphoton endoscopes for in vivo imaging of unstained tissues

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Multiphoton endoscopes show potential as clinical instruments capable of real-time diagnostics and as replacements for surgical biopsies.

The clinical assessment of tissue health currently involves a physician using a low-magnification white-light laparoscope/endoscope that penetrates into the body to image tissues in situ. Biopsy samples are then extracted, processed into histopathology slides, and analyzed by a pathologist. Although effective, this practice results in delayed diagnosis, high procedural costs, patient discomfort, and inaccuracies because the biopsy samples are located in situ using low-magnification white-light imaging. A different technique—multiphoton microscopy—can acquire images of unprocessed, unstained tissues with resolution and detail comparable to standard histology. Here, we describe our recent advances in imaging unstained tissues using multiphoton microscopy.

The ability to acquire diagnostic-quality images solely from intrinsic tissue fluorophores, such as the enzyme cofactors nicotinamide adenine dinucleotide and flavin adenine dinucleotide, and harmonic generations is a significant advantage for multiphoton imaging. Indeed, although it is customary in the laboratory to use exogenous contrast agents with this technique, there are currently very few of these dyes that are approved for use within a patient owing to concerns of dye toxicity. However, a wide variety of epithelial tissues can be imaged using compact multiphoton endoscopes (MPEs) that enter the body through a natural orifice or small surgical incision. These devices have the potential to be used clinically to assess surgical margins, either as replacements or guides for conventional surgical biopsies.

Several groups have demonstrated small and flexible as well as larger rigid MPEs. The larger rigid devices typically use rodlike gradient-index (GRIN) lenses that are attached to an external microscope. Since only the GRIN lens penetrates the tissue, the scanning mechanism and optics need only be portable and not fully miniaturized. Although previous investigators have used <40mm GRIN lenses, the clinical use of GRIN lenses for diagnosing tissue health requires considerably longer GRIN endoscope systems for human in vivo image acquisition. We recently characterized a number of long (up to 285mm) GRIN lenses. We found that the imaging performance of these systems is adequate for cellular-level imaging. The long GRIN lenses achieve optical resolutions (lateral and axial) that are well suited for resolving cellular details within tissues. Additionally, these long GRIN lenses have suitable fluorescence collection. Furthermore, we incorporated the GRIN lens into a portable hand-held multiphoton microscope and successfully acquired in vivo images of unstained tissues (see Figure 1).

A typical compact and flexible MPE consists of a miniaturized scanning mechanism paired with miniaturized optics. To obtain images from weakly emitting endogenous fluorophores, the scanning mechanism and optics need only be portable and not fully miniaturized. Although previous investigators have used <40mm GRIN lenses, the clinical use of GRIN lenses for diagnosing tissue health requires considerably longer GRIN endoscope systems for human in vivo image acquisition. We recently characterized a number of long (up to 285mm) GRIN lenses. We found that the imaging performance of these systems is adequate for cellular-level imaging. The long GRIN lenses achieve optical resolutions (lateral and axial) that are well suited for resolving cellular details within tissues. Additionally, these long GRIN lenses have suitable fluorescence collection. Furthermore, we incorporated the GRIN lens into a portable hand-held multiphoton microscope and successfully acquired in vivo images of unstained tissues (see Figure 1).
high scan uniformity is essential. We achieve this and fast frame rates using a miniaturized resonant/nonresonant fiber raster scanner. We fabricated the scanner by mounting a double-clad optical fiber (DCF) onto two piezo bimorphs that are aligned such that their bending axes are perpendicular to each other. In this configuration, fast lateral scanning of the laser illumination is achieved by simultaneously driving the DCF cantilever resonantly at 1kHz and nonresonantly at 4Hz in the orthogonal axis. The use of a DCF enables the simultaneous delivery of excitation light along with epicollection of emitted tissue fluorescence.

Using this novel scanner, we developed a compact and flexible MPE capable of acquiring high-resolution ex vivo and in vivo images of unstained tissues. The device has a 40mm rigid distal tip with a 3mm outer diameter—see Figure 2(a) and (b)—that can acquire 110×110μm images with high scan uniformity at a rate of 4.1 frames per second (512×512 pixels per frame). The lateral and axial resolutions for two-photon imaging are 0.8 and 10μm, respectively. This MPE is the first compact and flexible device capable of acquiring multiphoton images of unstained tissues from a live subject: see Figure 2(c).

A clinically useful MPE must achieve imaging parameters such as high-resolution, large field of view (FOV), and fast frame rates while maintaining dimensions suitable for minimally invasive medical procedures. Fast frame rates are necessary to mitigate in vivo motion artifacts caused by respiration, heartbeats, and smooth muscle contractions. A large image FOV is useful so that a clinician can survey a large region of tissue and identify sites of interest, whereas high spatial resolution is necessary to resolve cellular detail within tissues for the medical assessment. We recently demonstrated new techniques that achieve these requirements. We achieved a large image FOV, while maintaining high optical resolution, using a lensed optical fiber (see Figure 3). The use of a lensed fiber reduces the size of an optical fiber’s output beam, thereby allowing for a larger number of independently resolvable points within a large FOV. Furthermore, we demonstrated fast frame rates without sacrificing the signal-to-noise ratio per frame and instantaneous axial sectioning by integrating parallel image acquisition into our original flexible MPE, creating a multifocal multiphoton endoscope (MME, see Figure 4). Translating our MPEs to MMEs is straightforward since this simply involves adding additional excitation DCFs into our miniaturized scanner (i.e., the DCF array raster scanner). We achieved this by bonding multiple DCFs alongside each other to form a rectangular fiber array that is simultaneously driven resonantly along the thin dimension and nonresonantly along the thick dimension. We believe that these features are important for the clinical translation of MPEs.

In summary, imaging unstained tissues with multiphoton excitation is difficult because of the weak emission of endogenous fluorophores. Our devices are able to acquire these images due to efficient delivery of excitation light, good collection properties, and high scan uniformity. In the future, we envision that the compact and flexible as well as large and rigid MPEs will be used as a guide or replacement for surgical biopsies for a wide variety of organs. Owing to the length of the GRIN lenses used in the larger MPEs, these devices would most likely enter the body via small surgical incision. In fact, the small diameters of commercial GRIN lenses means they can ideally be inserted into tissues through needles as small as 22 gauge. Finally, by combining the lensed fibers with the multifocal approach, we have laid a pathway that will enable us to considerably reduce the rigid length of our flexible endoscopes to access a wide range of organs through
Figure 4. Three axially-offset double-clad optical fibers (DCFs) bonded together as part of a miniaturized fiber array scanner for simultaneous axial sectioning. The indicated features (marked with dashed shapes) from an ex vivo mouse lung specimen come into focus initially in the image obtained from the ‘front’ DCF (z = 30 μm) and then come into focus 10 μm deeper into the tissue (z = 40 μm) for the image obtained from the ‘back’ DCF, but are shifted ~50 μm laterally. z: Imaging depth beneath the surface of the lung tissue. Unlabeled scale bars = 10 μm.

natural orifices. The realization of these next-generation MPEs would be a major step toward translating multiphoton imaging into the clinic and constitutes our ongoing work.

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