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Looking Deeper

New technique allows for deep brain imaging

By Jennifer Patterson Lorenzetti

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The brain is an organ that jealously guards its mysteries. Not only is it a complex structure, its function is also incompletely understood. For example, while researchers know that the nerve cells in the hippocampus region are critical to learning and memory, little is understood about how these critical functions that define humanity actually take place. Further, when something goes awry – like Alzheimer's or Parkinson's disease – it is a challenge to understand exactly how the brain has been affected.

Researchers would like to obtain images at the cellular level inside living animals and human beings in order to better understand how cellular behavior affects the organism as a whole, including normal functions like learning or abnormal situations like disease. Until now, however, imaging a complex organ like the brain has been a difficult undertaking.

Part of the challenge lies in the constraints of imaging. Traditionally, it has been difficult to image the brain at the cellular level using conventional techniques. Electron microscopy cannot be used on living tissue; optical microscopy cannot penetrate deep enough into tissue because light scatters as it travels through tissue near the surface. Therefore, traditional microscopic techniques have only been useful for imaging near the surface of the living brain, while understanding the baffling neurological functions and disorders demands a deeper look.

Now, researchers at the James H. Clark Center for Biomedical Engineering and Sciences at Stanford University in California have developed a minimally invasive optical imaging technique that can capture images from deep inside the brain by using a device that fits in the palm of the hand. The device combines two-photon microendoscopy and the use of optical probes to deliver detailed images in a small, convenient package. The results of the team's work appear in a recent issue of *Optics Letters*.

The Science Behind the Images

Fluorescence microscopy has long been used to image tissue. In conventional, one-photon fluorescence imaging, a dye is injected into the tissue, and then the tissue is subjected to a bright light. The light collides with atoms in the dye, and their electrons are excited to a higher level. As they return to their original level, they "fluoresce" – or emit photons – which are packets of light. The fluorescing areas can be observed by the microscope, where they stand out against a darker background, thus creating a picture of the tissue under investigation.

The fluorescing areas can occur naturally, such as with tissues containing chlorophyll, or they can be differentiated by use of fluorescing chemicals, such as dyes. The dyes – or naturally occurring fluorescing chemicals – are sensitive to a specific wavelength of light, meaning that anatomical structures which contain the fluorescing chemical will react to the light, while surrounding tissues will react less or not at all. This allows certain structures to show up clearly in imaging; for example, injecting a dye that is picked up by blood plasma will allow cerebral blood vessels to show up

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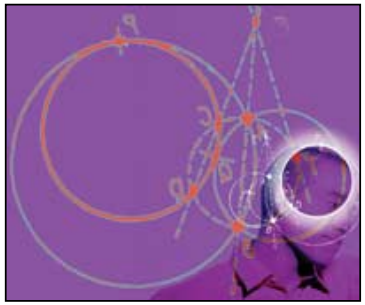

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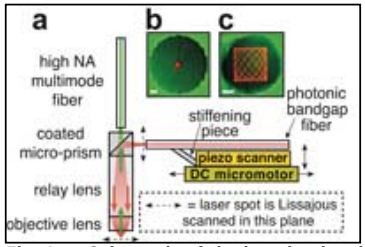
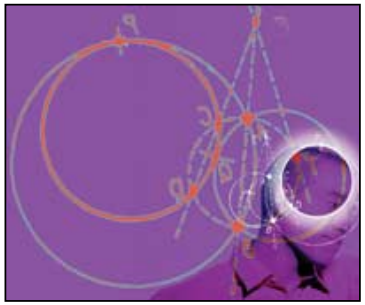


Fig. 1. a, Schematic of the imaging head. Arrows extending from the micromotor and scanner indicate directions of movement. b, Photographic overlay of near-field intensity distribution of infrared light in the lowest-order mode (red pseudocolor) exiting the bandgap fiber (green pseudocolor). The scale bar is 10 m. The mode appears off center from the 6.8- m-diameter air core due to a small shift between image acquisitions. c, Endoscope probe (green pseudocolor) and a real image of the Lissajous scanning pattern (red pseudocolor) that arises from a reflection at the junction of the GRIN objective and relay lenses. The Lissajous driving frequencies were 538 and 698 Hz. Only a portion of the pattern appears due to an image acquisition time of 0.067 s. The scale bar is 200 m. (Benjamin A. Flusberg)



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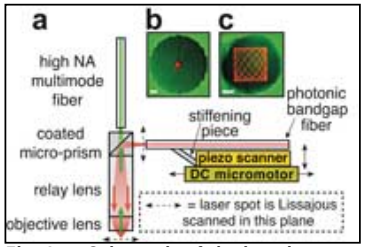

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against surrounding brain tissue.

However, one-photon imaging is not practical for imaging deep tissue. Deep tissue will cause the photons to scatter, ricocheting as they return to the detector, and creating a background haze in the resulting image. The haze is a cloud through which it is difficult to accurately perceive the image.

Two-photon fluorescence imaging, on the other hand, reduces the photon scattering and resulting background haze by subjecting the tissue to two streams of photons, each with half of the energy required to excite the fluorescing material. The combined energy of the two photons will cause the sample to fluoresce, but molecules outside the area of interest – or deeper in the tissue – will be less likely to absorb a pair of photons simultaneously and fluoresce in response. This reduces the background haze and creates a clearer image.

"One of the many virtues of two-photon fluorescence microscopy is that it enables imaging deeper into highly scattering biological tissue than one-photon fluorescence microscopy," says Benjamin Flusberg, graduate student in the Stanford lab and lead author of the *Optics Letters* paper. "However, by "deeper,' I mean up to [about] 500 microns deep through tissue, as opposed to [about] 50 to 100 microns deep. Therefore, two-photon fluorescence microscopy, which has been around since the early '90s, is still limited to structures that are fairly close to the tissue surface," he says. Therefore, traditional two-photon microscopy, while an improvement over the one-photon method, is still not a solution to imaging deep within structures like the brain.

But what if the two-photon technique could be delivered more deeply into a tissue structure? This is what Mark Schnitzer, PhD, assistant professor with a joint appointment in the departments of biological sciences and of applied physics at Stanford and principal investigator on this project, has done. "Professor Schnitzer and colleagues previously developed the technique of performing two-photon imaging through microendoscope optical probes," says Flusberg. "These probes, which are up to 1 mm in diameter, can be inserted into biological tissue in a minimally invasive manner. We regularly insert them many millimeters deep into tissue."

Therefore, the optical probes deliver the two-photon technique deeper into tissue, making it theoretically possible to view microscopic activity deep in the human brain. "When performing two-photon imaging through these probes, one can image tissue structures that are up to [about] 300 microns deep from the tip of the microendoscope probe," says Flusberg. "Thus, because the probe can be inserted many millimeters or more into biological tissue, this technique of using microendoscope optical probes enables one to image tissue structures that are many millimeters below the tissue surface."

Deep Imaging Made Easy

With the science in place, Schnitzer's team could turn its attention to making the technique portable and easy to use. "This technique of combining two-photon images and microendoscope probes has, until our current *Optics Letters* paper, been incorporated into bulky and immobile optical equipment, such as a tabletop microscope," Flusberg says. Using some of the latest advances in micromotors, lensing and fiber optics, the team has designed a small, palm-sized device that does the work of the bulky equipment, thus making the technique much easier to use. The two-photon microendoscope is an inch-and-a-half long and weighs in at 4 grams.

"Our main innovation in this work is to combine these two techniques, two-photon imaging and microendoscope probes, with a flexible optical fiber in order to build a fully functional, portable device that fits in the palm of one's hand. The device is fully functional in that it contains a scanning mechanism, an optical alignment mechanism and a focusing mechanism, and it is capable of obtaining "in vivo' to photon images from deep tissue locations. The flexible optical fiber, which is a special kind of fiber called a

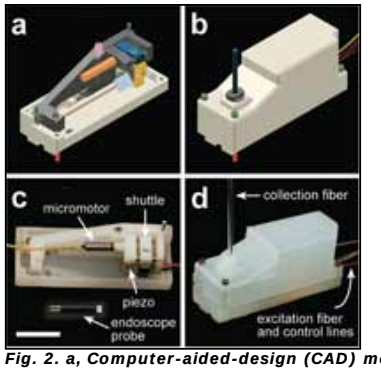


Fig. 2. a, Computer-aided-design (CAD) model of device components mounted on the baseplate. White, baseplate (nylon); red, endoscope probe and bandgap fiber; light blue, piezo actuator; gold, piezo clip (titanium); gray, wedge mechanism for coarse focusing (acetal); pink, spring for wedge mechanism; orange, micromotor; blue, micromotor shuttle (acetal) for finer focusing; green, screws and rails (stainless steel). b, CAD model of the imaging head. White, baseplate and casing (nylon); blue, fluorescence collection fiber; green, screws (stainless steel). The bandgap fiber (pink), actuator leads (red and purple), and motor control lines (yellow) exit through a slot in the casing. c, Photograph of components assembled as in panel a. Scale bar is 1 cm. d, Photograph of the imaging head.

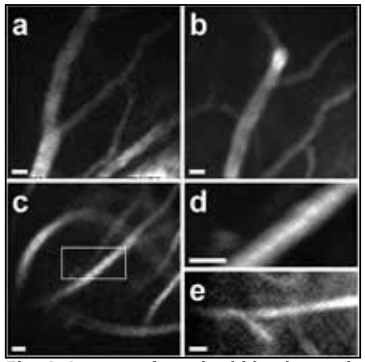
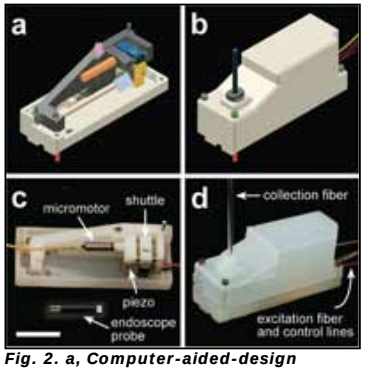


Fig. 4. Images of cerebral blood vessels labeled with fluorescein in anesthetized mice. The frame rate was 2 Hz. a, b, Vessels near the neocortical surface. Excitation power was 15 mW at the specimen and the endoscope had a working distance (WD) of 250 m in air. Lissajous driving frequencies were 537 and 699 Hz. c–e, Hippocampal vessels imaged through a 152 m cover glass at the tip of a guide tube that was implanted just dorsal to hippocampus. The WD was 160 m in air. Driving frequencies were 538 and 698 Hz. c, Vessels 20 m below the dorsal hippocampal surface imaged with 30 mW power. d, Magnified image of the boxed region in c, obtained by decreasing the Lissajous driving voltages. e, Capillaries 80 m below the hippocampal surface imaged with 80 mW power. Images a–c are 128 128 pixels. Images d and e are 128 62 and 96 46 pixels, respectively. a and c display single image frames, while b, d, and e show averages over six frames. All scale bars are 10 m.



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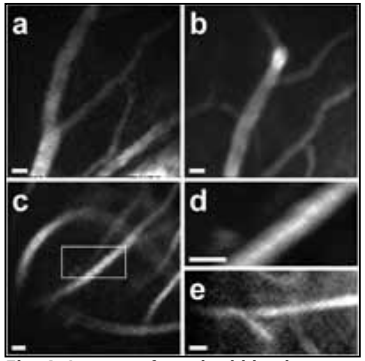

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"photonic bandgap fiber,' gives the device its portability," says Flusberg.

Standard optical fibers control light by their construction of concentric layers of glass around a solid core, which contains light by total internal reflection. However, the photonic bandgap fiber makes use of silica glass fashioned in a precise pattern. The pattern repeats itself at regular intervals, creating a photonic bandgap in which certain frequencies of light are blocked from exiting the fiber. The spacing of the pattern determines which frequencies will be blocked. Rather than using the concentric layers of glass used by typical optical fibers, the photonic bandgap fiber is built by stacking hollow glass rods. The construction blocks certain frequencies, thus guiding the light.

Photonic bandgap fibers can also help reduce distortion of the signal. In conventional fiber optic cables, the intensity of light required for two-photon microscopy will interact with the surrounding glass, causing distortion. With the photonic bandgap fibers, the pulses of light travel through an air-filled core running through the length of the fiber, never interacting with the glass at all. Therefore, distortion is reduced.

"We're bringing two-photon imaging to endoscopy, and we're putting it all into a miniaturized package. This is a portable handheld device with the power of two-photon imaging – the full functionality of a microscope that fits in the palm of your hand," says Schnitzer.

The *Optics Letters* paper details the team's tests of the handheld device to image the blood vessels in the hippocampus of live mice. The mice were anesthetized and injected with a fluorescing dye, and then the optical probes – which were long enough to reach any portion of the mice's lima bean-sized brains – were inserted. The fluorescing dye labeled the blood plasma in the mice's cerebral blood vessels so that it would react to the light delivered to the area. Through this technique, the team was able to obtain images of cerebral blood vessels located more than 1 mm below the surface.

Schnitzer's next goal is to design a device that can be used on unanesthetized mice. To achieve this goal, Schnitzer and his team are working with Nikolas Blevins, MD, assistant professor of otolaryngology, and Lawrence Recht, MD, professor of neurology and neurological sciences, both at Stanford. Blevins' work centers on the inner ear, while Recht is using microendoscopic probes to image brain tumors in mice.

This early success indicates that the handheld device may be highly marketable, but it is still has long way to go before it becomes commonplace in the imaging suite. For one thing, it is a long way from imaging the lima bean-sized brain of a mouse to imaging the brain of a living human being, and, although Schnitzer believes the technology will eventually find applications in imaging human patients, the team does not anticipate testing on humans anytime soon. "We believe such technology will have clinical applications, but we do not have specific human patient trials planned," says Flusberg.

With human trials yet to be planned, it stands to reason that the team cannot predict a date by which this technology could hit the market. "We believe that this kind of technology will have an important impact, but we are currently at a very early stage, years away from commercialization," says Flusberg. However, some anticipated applications the team is looking into include biomedical research and clinical imaging.

Flusberg is excited about the possibilities of convenient, portable two-photon microscopy using optical probes. "Even conventional two-photon fluorescence microscopy is limited to imaging tissue structures within [about] 500 microns of the tissue surface. Most biological tissue, including the brain, is much thicker than 500 microns, and therefore there are many brain and other tissue structures that remain out of reach to conventional two-photon microscopy. Many of these deep brain structures play a role in certain types of learning, memory and disease, and much can be learned

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from imaging them in vivo," he explains. "Our technique of using microendoscope optical probes enables us to perform in vivo imaging of deep tissue structures, such as the mouse hippocampus, with micron-scale resolution. Other techniques would not be able to perform this type of imaging," he says.

— Jennifer Patterson Lorenzetti is a freelance technology writer in Centerville, Ohio, and owner of Hilltop Communications. Questions and comments can be directed to editorial@rt-image.com.

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