

Femtosecond Laser Pulses in Biology: From Microscopy to Ablation and Micromanipulation

by Marco Arrigoni, Coherent Inc.

Until recently, biological applications for ultrafast laser pulses were confined to sample interrogation, but now the lasers also are being used for sample manipulation.

Ultrafast laser technology continues to mature, and today's products are more reliable, are easier to use, and cover a wider power and wavelength range than ever. This, in turn, has stimulated new applications for these lasers. The use of ultrafast lasers is evolving from purely imaging purposes to applications that use amplified pulses to remove layers of tissue for deep imaging, to selectively cut and/or destroy cells and to ablate single organelles. These functions

promise increased understanding in cellular and systems physiology.

The short (100 fs) pulse duration of ultrafast mode-locked lasers and amplifiers results in very high peak powers, with commercial laser systems ranging from kilowatts to the terawatt regime. When this high peak power is focused into a transparent material, it causes a number of phenomena related to the interaction of multiple photons with the material. Among these effects are multiphoton excitation and absorption. With appropriate control of the focusing optics and the laser power, they occur only at the focused beam waist, where the laser fluence is highest.

In multiphoton microscopy, nanojoule pulse energies excite fluorescence from exogenous or native fluorophores. As the laser's focus is scanned through the sample, the microscope captures an image of the fluorescence intensity. Compared with single-photon confocal microscopy using blue-green laser light, multiphoton microscopy using near-IR laser pulses offers advantages such as deeper tissue penetration, minimal or no damage to the sample, low scatter and high spatial resolution.

Higher pulse energies can be used to drive the multiphoton absorption process more intensely, removing material by directly breaking molecular bonds. This process is called multiphoton ablation. The limited heat generation and short pulse duration enable very precise control of the volume of tissue ablated and the peripheral thermal damage.

Multiphoton ablation

Two research groups at the forefront of using this effect are led by professors David Kleinfeld at the University of California, San Diego, and Eric Mazur of Harvard University in Cambridge, Mass. Many of their experiments involve a combination of multiphoton ablation and multiphoton microscopy. They have used ablation to selectively destroy tissue over a wide size range — from individual organelles to multicellular layers.

The emergence of these techniques highlights the need for ultrafast laser amplifiers that are user-friendly and that

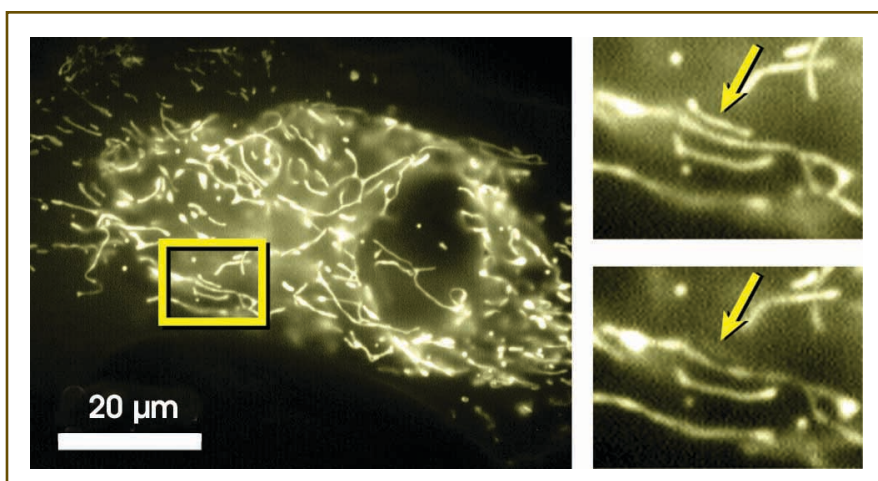


Figure 1. Femtosecond laser ablation can remove a single mitochondrion with no damage to nearby structures, such as other mitochondria.

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provide high throughput. The required high throughput and the size of the features being manipulated drive the need for laser pulses in the range of 1 to 10 μJ and high repetition rates, an operating envelope that matches the domain of continuous-wave-pumped femtosecond amplifiers such as the RegA 9000, produced by Coherent Inc. of Santa Clara, Calif.

Subcellular ablation

Cell biologists already have a number of chemical labeling and disabling tools that are species-selective but not site-selective, Mazur explained. However, his group realized that the ultrafast laser could provide a highly specific site-selective tool and began some interesting collaborations with cell biologists.

Mazur's team is using 800-nm, 100-fs pulses in the 2- to 8-nJ range to ablate structures at the subcellular level. The laser is focused in the sample to a diffraction-limited 500-nm spot diameter, using a typical pulse repetition frequency of 1 kHz, to avoid any peripheral thermal effects.

One of the scientists' first experiments demonstrated the spatially selective capabilities of ultrafast ablation. They tagged mitochondria in a mouse capillary endothelial cell using fluorescein dye and then eliminated a single mitochondrion without visibly damaging another only 500 nm away (Figure 1). They call this technique nanosurgery.

The group has collaborated with Dr. Donald E. Ingber, a professor at Childrens Hospital Boston, Harvard Medical School, to apply nanosurgery to cytoskeleton studies. Ingber, who is interested in the mechanism of signal transmission along the cytoskeleton, uses nanosurgery to cut specific fibers of the cytoskeleton as part of the research. When a fiber is cut, the recoil of the ends reveals whether the fiber is under tension or stress. If it is under tension, the cut ends move apart; if under

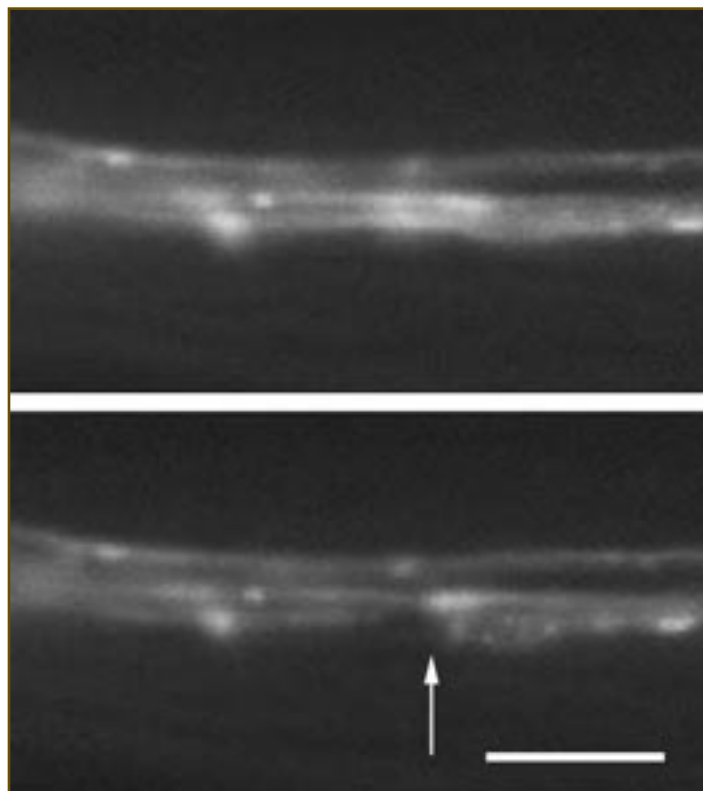


Figure 2. Two *C. elegans* axons on either side of one that a laser ablated continued to operate normally more than 30 minutes after the ablation. The scale bar is 5 μm .

stress, the ends push past each other to overlap. In a related study, his group has used laser nanosurgery to cut individual microtubules associated with mitosis.

In collaboration with Howard C. Berg of the Harvard biology department, the Mazur team is using nanosurgery to cut into *E. coli* to provide direct biochemical access to the flagellar motor. When the laser punctures the cell membrane, the contents spill out under huge pressure, and the loose ends of the membrane tend to reseal after surgery because the hydrophobic parts prefer to come together rather than be exposed to water. The group is looking to use chemical means to hold the cell membrane open after surgery.

Cutting single cells

Moving up slightly in size scale, the Mazur team has begun working with biophysicists led by professor Aravi Samuel of the Harvard physics department using ultrafast ablation in studies of the *Caenorhabditis elegans* worm. This worm

measures 1 mm \times 80 μm , with a high neuron ratio: Of the organism's 1300 cells, 302 are neurons. A major attraction of this organism is that the collective structure of the neurons is fairly well-known and varies little from individual to individual.

In the past, researchers have studied mutated worms that are missing a few neurons, to see how their absence affects functions such as locomotion and response to thermal stimuli. But ultrafast ablation allows the Harvard group to arbitrarily target and cut individual axons without killing the nerve cell or disrupting the surrounding tissue (Figure 2).

Mazur said, "Formerly, this type of research was completely limited to the available mutations. But now we can select single axons for cutting. Moreover, we can successively cut multiple axons, monitoring the worm's response after each cut."

The researchers use several Ti:sapphire laser systems, all pumped by Verdi lasers. Besides using commercial laser oscillators, such as the Mira laser, they have built their own oscillators and amplifiers for use in this work. For example, one device is a novel long-cavity Ti:sapphire with a slower pulse repetition frequency. This provides high-energy pulses without amplification (the pulse energy of a laser scales as the average power divided by the pulse repetition frequency).

The group is now setting up a combined instrument to perform both nanosurgery and multiphoton microscopy. For simplicity (and cost), this microscope is equipped with a single Ti:sapphire laser oscillator, used primarily for imaging. When nanosurgery is required, the oscillator output is momentarily diverted into a high-repetition-rate amplifier, whose output is also aligned with the microscope axis.

Kleinfeld and co-workers are using ultrafast laser ablation to perform tasks on

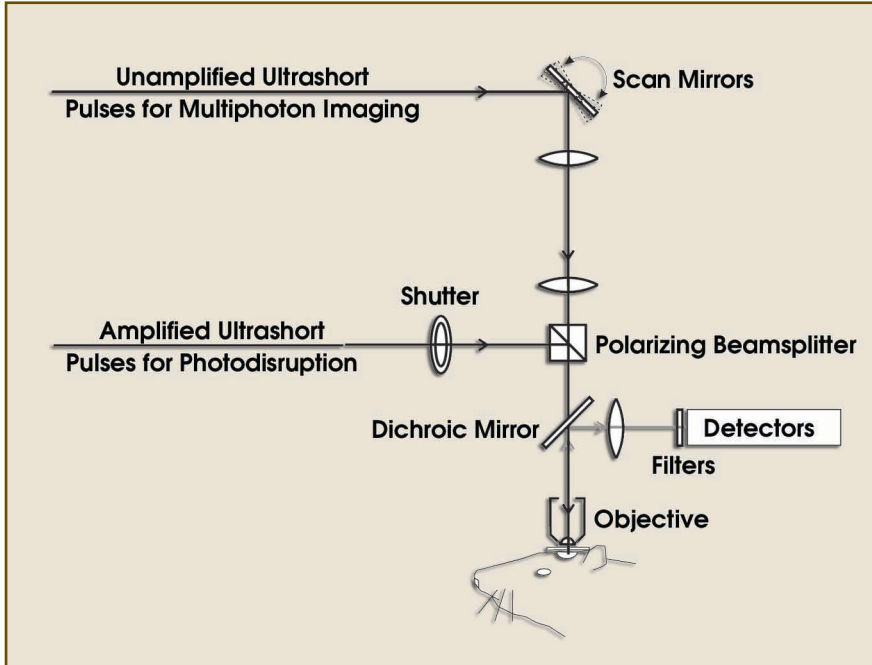


Figure 3. A two-photon laser-scanning microscope modified for delivery of amplified ultrashort pulses for microvascular photodisruption produces high-resolution images of vessel disruption, in real time, and quantifies the speed of blood cells in individual vessels.

a slightly larger scale. One project, with Dr. Patrick D. Lyden, a professor at the University of California, San Diego, School of Medicine, involves the disruption of deep-lying microscopic blood vessels in the rodent brain (neocortex).¹⁻⁴

Penetrates beyond surface vessels

Neurovascular clotting causes fatalities and ischemic strokes in humans, but small, diffuse neurovascular disruptions also have been linked recently with forms of dementia. The researchers have shown that ultrafast laser ablation can target and induce precise local vascular disruption in the neocortex with no significant damage to surrounding material. This provides a means to assess the neurological impact of vascular disruptions. Kleinfeld noted that other researchers have previously used lasers to disrupt surface blood vessels, but a critical advantage of ultrafast ablation is its ability to target vessels throughout the upper cortex, at depths of up to 500 μm .

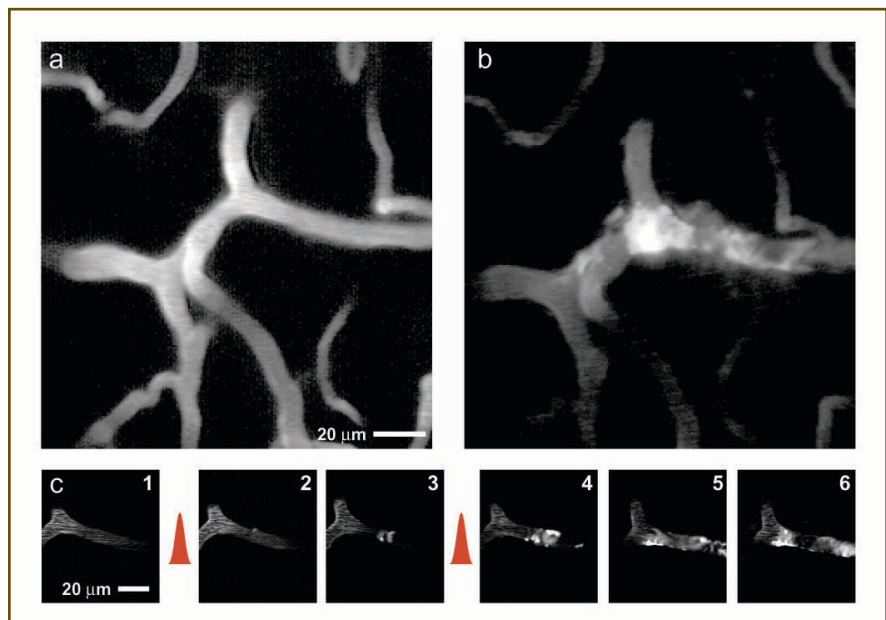
In the experimental setup, a glass window replaces part of the rat's skull, and

the blood plasma is labeled with a fluorescent dye (Figure 3). The anesthetized rat is positioned under a multiphoton microscope that was modified to allow delivery of amplified ultrafast pulses from a homemade amplifier, pumped by a Corona laser, with a repetition rate of 1 to 2 kHz and pulse energies up to 10 μJ or more at the focus. A Mira Ti:sapphire oscillator provides the imaging and seed pulses for the amplifier. The microscope obtains high-resolution images of the vessel disruption, in real time, and quantifies the speed of blood cell flow in individual vessels.

The threshold pulse energy for observable vessel disruption was found to vary from vessel to vessel, even within the same animal, from 0.1 to 5 μJ per pulse. By varying the laser pulse energy and the number of pulses (2 to 10), the researchers found that they could produce one of three types of vascular injury:

- At low laser power, the first observable effect is blood plasma extravasation. In this case, fluorescein-labeled plasma leaks from the vessel, but blood flow does not change.
- After further pulses, the vessel undergoes an intravascular clot, a thrombosis that completely blocks the target vessel. In the targeted vessel lumen, the coalescence of red blood cells, and possibly platelets, appears as dark areas in the two-photon laser scanning microscopy image (Figure 4). After its formation, the clot in this figure remained unchanged during observation (two hours).
- When the laser pulse energy increases to a factor of 10 greater than a vessel's observed disruption threshold, applying multiple pulses (up to 10) causes a hemorrhage, a larger disruption of the targeted

Figure 4. An intravascular clot appears in a time series of two-photon laser-scanning microscope images. The pulse icon indicates when irradiation occurs, by 10 pulses at 0.3 μJ per pulse, in this case. The clot appears as a dark area.



vessel. Both plasma and blood cells leak out of the vessel.

Kleinfeld said that this study shows that multiphoton laser ablation can be used to target vessels deep in the cortex, enabling subsequent study of the physiological effects of disruption at the cellular level. Moreover, he said that observing localized extravasation may provide insight on a nonischemic route to neuronal death. (Some blood plasma proteins are known to be toxic to neurons.)

All-optical histology

On an even bigger scale, Kleinfeld's group has combined multiphoton ablation and microscopy to obtain three-dimensional images from within thick samples using a technique referred to as all-optical histology. A previous article in *Biophotonics International* (September 2003, page 56) discussed the method, which was devised in collaboration with professor Jeff Squire of Colorado School of Mines in Golden, but a brief mention serves to provide a complete picture of the capabilities of combining ablation with microscopy.

Multiphoton microscopy can produce 3-D images of live cells, with greater depth of view than other optical techniques. However, thick multicellular samples often require reconstruction from sequential image layers; i.e., section-based histology, in which tissue is physically sliced before imaging. But this requires frozen samples and significant manual labor, and it suffers from problems of misalignment and distortion in reassembling labeled structures from the individual sections.

All-optical histology uses virtually the same basic optical setup as vascular disruption. The multiphoton microscope obtains an image block 100 to 200 μm thick. Amplified pulses then carefully ablate this layer, with no damage to underlying tissue. The next layer is imaged, followed by more ablation, and the image blocks are then combined. Because the sample is never disturbed or removed from the microscope, reconstruction of underlying 3-D structures is straightforward, with no alignment problems. Moreover, Kleinfeld noted, the technique is well-suited to commercial automation. \square

Meet the author

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References

1. D. Kleinfeld et al (1998 and 1999). Fluctuations and stimulus-induced changes in blood flow observed in individual capillaries in layers 2 through 4 of rat neocortex. *PNAS*, 95:15741-15746; corr. 96:8307c.
2. P.S. Tsai et al (2003). All-optical histology using ultrashort laser pulses. *NEURON*, Vol. 39, pp. 27-41.
3. B. Friedman et al (2004). Heterogeneous changes in blood flow in response to single vessel blockages and MCA occlusion in rat parietal cortex as revealed by in vivo two-photon laser imaging. *STROKE*, Vol. 35, p. 81.
4. N. Nishimura (2004). Targeted disruption of deep-lying neocortical microvessels in rat using ultrashort laser pulses. In Proc. SPIE, *Commercial and Biomedical Applications of Ultrafast Lasers IV*. J. Neev, A. Ostendorf and C.B. Schaffer, eds.; SPIE, Bellingham, Wash.