J. Biophoton. **2**, No. 10, 557–572 (2009) / **DOI** 10.1002/jbio.200910053

Journal of BIOPHOTONICS

REVIEW ARTICLE

Surgical applications of femtosecond lasers

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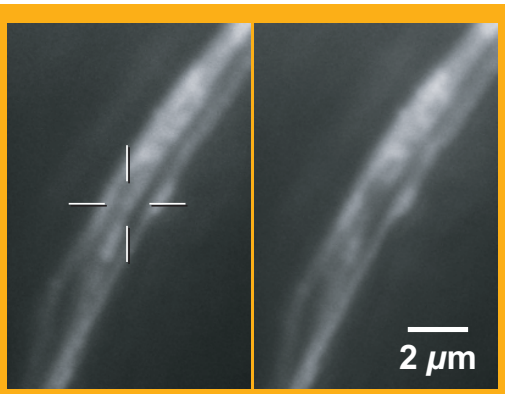
Received 20 December 2008, revised 19 June 2009, accepted 27 June 2009
Published online 21 July 2009

Key words: subcellular laser ablation, nanosurgery, femtosecond lasers

PACS: 06.60.Jn, 42.66.–p, 87.16.–b, 87.19.L–

Femtosecond laser ablation permits non-invasive surgeries in the bulk of a sample with submicrometer resolution. We briefly review the history of optical surgery techniques and the experimental background of femtosecond laser ablation. Next, we present several clinical applications, including dental surgery and eye surgery. We then summarize research applications, encompassing cell and tissue studies, research on *C. elegans*, and studies in zebrafish. We conclude by discussing future trends of femtosecond laser systems and some possible application directions.



C. elegans dendritic bundle and femtosecond laser ablation of middle dendrite (reproduced from [123]).

1. Introduction

In the search for new methods of creating finer dissections with minimal collateral damage, femtosecond laser ablation is emerging as an exquisite tool to perform non-invasive, submicrometer-sized surgeries in the sample bulk. In addition, advances in laser technology are making femtosecond laser systems more accessible in terms of cost and maintenance. As a result, the number of research laboratories and clinical settings using femtosecond laser ablation has increased significantly in recent years, leading to a rapid growth in the number of publications in the field.

Femtosecond laser ablation has been applied to a wide range of research in materials science and the life sciences. Some of the first researchers transitioned from micromachining transparent materials to ablating living cells and tissues. Others interested in laser tweezer manipulation or multiphoton microscopy discovered they could permanently disrupt their targets by increasing the laser power. Physicists concerned with a theoretical understanding of laser ablation proceeded to model the effects of shorter pulses as they became readily available. Biologists and clinicians interested in a more precise tool for their applications have also joined the field. Because

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the femtosecond laser ablation field is so diverse and interdisciplinary, the literature contains many synonymous terms for surgery by femtosecond lasers, depending on the application. A sampling includes *laser microbeams*, *femtosecond laser surgery*, *subcellular laser ablation*, *nanosurgery*, *nanoscissors*, *nanoprocessing*, *nanoscalpels*, *nanodissection*, *nanoneurosurgery*, and *nanoaxotomy*. For simplicity, we will use the general term *femtosecond laser ablation* throughout. Also, pulsed lasers have numerous applications, including laser tweezers [1], optical coherence tomography [2], stimulation of neurons [3], catapulting [4], and even artwork restoration [5]. Many of these techniques are worthy of a review in their own right, but in this review we shall focus on the history, experimental background, applications, and future trends of high-precision surgeries by femtosecond laser ablation.

2. Historical background

Surgery by electromagnetic radiation was first demonstrated in 1912 using an ultraviolet (UV) microbeam [6]. The introduction of the laser in 1960 [7] made high collimated intensities readily available in the laboratory, and surgical applications rapidly followed [8, 9]. A number of conventional (i.e., nonfemtosecond) lasers have been used to perform dissections on a variety of biological media, including the retina [10], cellular organelles such as mitochrondria [11, 12], the algae *Spirogyra* [13], skin cells [14], mouse and rat melanoma [15], teeth [16], cell membranes for micropuncture [17], chromosomes [18], and chloroplasts [19].

While the transparency of many biological media allows for direct optical observation of phenomena, it can also prevent absorption of laser light, which is necessary for surgery. There are three methods of overcoming this obstacle: first, the target of interest can be labeled with a chromophore or dye that absorbs at the laser wavelength. The laser energy is deposited as destructive thermal energy [20] or the label breaks down, releasing free radicals which destroy nearby chemical bonds [21]. While this technique has a high resolution, labeling a single structure with an exogenous chemical can be difficult, and labels can be toxic to the sample. Second, one can use a laser supplying a short enough wavelength so that linear absorption occurs. An example is UV "microscissors", which have found wide use in whole-cell ablations. One drawback is that linear absorption leads to deposition of laser energy and varying amounts of damage all throughout the beam path, not only in the targeted volume [22]. Third, absorption at the laser wavelength can occur by moving to short laser pulses and stimulating a nonlinear absorption. The remainder of this review paper focuses on applications of this last method, which can yield higher resolution within the sample bulk without relying on labels. Reviews of the theoretical and experimental background of pulsed laser ablation are available in the literature and range from the presentation of a simplified model (e.g., Section 2 of [23]) to in-depth examinations of the ablation mechanisms [24–26].

Nonlinear absorption occurs when the incident optical intensity is sufficient to stimulate the simultaneous absorption of multiple photons. To minimize the pulse energy and collateral damage, this is typically done using pulsed lasers with pulse durations of nanoseconds or less. Nanosecond and picosecond laser ablation has become a mainstay in many biology laboratories and industrial applications. Improvements in generating and amplifying femtosecond lasers in the early 1980s made ultrashort laser pulses available in the laboratory, reducing the pulse energy needed for surgery and improving resolution [27, 28]. The first studies employing femtosecond laser pulses for surgery were published in 1987–91 and examined laser interaction with retinal tissue [29, 30] and skin [31].

3. Experimental background

A number of papers have reported on the efficacy and precision of femtosecond laser ablation, and others have detailed the post-surgery viability of the cells, tissues, and organisms. Ablation was confirmed to depend strongly on pulse duration [32], numerical aperture (NA), and pulse energy [33]. Ablated areas were probed by scanning electron microscopy (SEM) [34], atomic force microscopy (AFM) [35, 36], and fluorescence microscopy to ascertain the completion and resolution of surgery. Studies of femtosecond laser ablation in a new medium typically establish the fundamental surgery characteristics, such as surgical resolution and ablation threshold (usually for a fixed pulse duration and NA).

In living samples, post-surgery examination of viability is essential. A few of the first studies using femtosecond lasers observed the decreased cloning efficiency of cells restrained by optical traps [37–39] or imaged with two-photon microscopes [40]. Some cells become giant cells with multiple nuclei [41], and others undergo apoptosis because of laser-generated reactive oxygen species [42]. However, proteins involved in recognizing and repairing DNA are also present after surgery to mitigate damage [43]. Cellular viabilities and survival rates after femtosecond laser ablation are generally high, and many studies present data from controls to confirm confinement of surgical effects.

There are a number of practical considerations when implementing femtosecond laser ablation for surgery. First, if the target structures are in the sample bulk, the sample must be transparent at the laser wavelength and the target volume must be within the working distance of the microscope objective. Any significant linear absorption results in absorption and collateral damage throughout the laser beam. Second, a major disadvantage of femtosecond laser systems is cost. Oscillators, which generate femtosecond pulses, are pumped by another laser, adding to cost. Amplified systems often used for surgery are approximately double the cost of a commercial oscillator system. Fortunately, some newer high-power oscillators and fiber lasers are powerful enough to perform surgery without amplification, reducing cost. Third, the investment of time, energy, and resources into femtosecond laser systems can be considerable. Despite laser companies claims of turn-key performance, the specific reliability of each laser system can only be truly known through actual experience in the laboratory. Some systems require weekly maintenance and adjustment while others require it only yearly. Fourth, laboratories with a femtosecond laser must invest time in safety training, both initially upon system installation and incrementally for each new laser operator. There are a number of primary hazards (e.g., eye safety and material flammability) and secondary hazards (e.g., particulate and X-ray emissions) associated with femtosecond laser pulses [44, 45].

4. Clinical applications

A limited number of targets in the human body are accessible by femtosecond laser ablation because of tissue transparency and shallow penetration depth requirements (see previous section). Most of the clinical applications of femtosecond laser ablation are either dental or ocular. Targets on the teeth are accessible because they are superficial, but the transparency of the eye permits access to its internal targets. While preliminary research is carried out in fields such as dermatology (both skin [46] and fingernail [47]) and otology (inner and middle ear [48]), this research has not yet generated a critical mass of interest, perhaps because other simpler laser systems or treatment methods exist.

4.1 Dental surgery

Initial conventional laser ablation studies on teeth using ruby or CO_2 lasers noted significant thermal deposition leading to collateral tooth damage [16, 49]. Other lasers, such as Er : YAG, reduce thermal effects considerably but cannot compete with the speed or quality of mechanical drills [50, 51]. Many systems also produce micro-scale cracks due to the large pulse energy associated with long pulse durations [52]. Since the mid-1990s a growing number of studies have demonstrated that femtosecond laser ablation represents the first viable alternative to mechanical dental surgery [25]. Table 1 summarizes the content of peer-reviewed journal publications using femtosecond laser ablation for dentistry. Femtosecond lasers have successfully ablated dentin, enamel, calculus (tartar), and hydroxyapatite, which is the primary mineral component of dentin and enamel. Most studies have focused on determining the ablation thresholds, measuring the ablation rates, observing the morphology of ablated regions by SEM, and measuring the temperature increase during surgery. In brief, femtosecond ablation rates are about an order of magnitude faster than picosecond laser ablation rates and are comparable to mechanical drills. The femtosecond laser "drill" is much quieter and causes less pain than traditional drills, or no pain at all. Scanning electron microscopy and other tests confirm the surgeries are crack-free and have a resolution below 10 μm (see Fig. 1a, b), which is ideal for fillings and far more precise than surgeries by other lasers. Finally, the temperature rise of the tooth bulk is below 5 °C when the ablated tooth is air-cooled.

A few studies have shown that femtosecond laser ablation can select between carious (cavity-damaged) and healthy tissue. As shown in Fig. 1c, carious dentin ablates at a faster rate and lower pulse

Table 1 Publications on dental applications of femtosecond laser ablation.

	Dentin	Enamel	Calculus	Hydroxyapatite
Ablation threshold	[146–148]	[58, 146, 147, 149]	[150]	
Ablation rate	[34, 147]	[34, 146, 147, 149, 151]		
Morphology	[34, 147, 152, 153]	[34, 147, 149, 151, 153–157]		
Temperature	[34]	[149, 155, 158]		
Selectivity (carious *vs.* healthy)	[156]		[150]	
Spectral analysis		[146, 154, 155]		[157, 159]

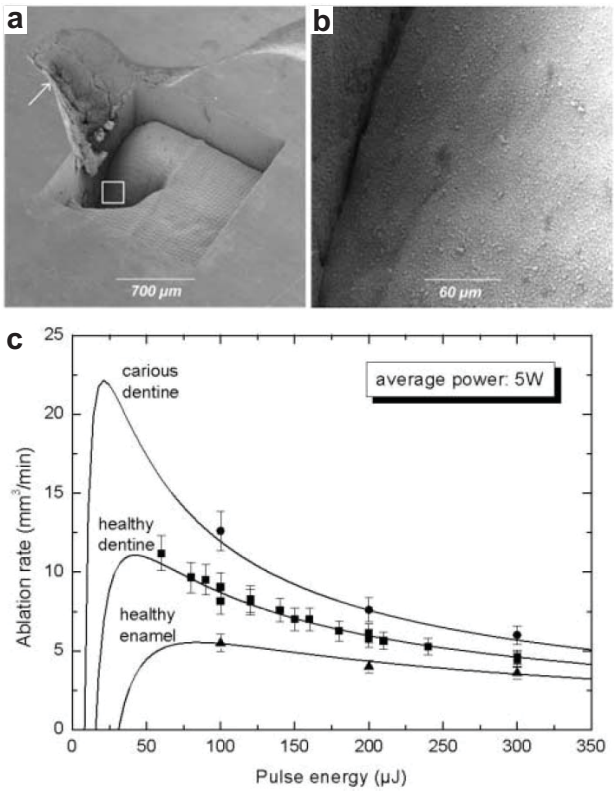


Figure 1 (**a**) Scanning electron micrograph of a carious lesion (white arrow) in dentin after femtosecond laser ablation. Note the precision of laser processing. (**b**) Enlargement of rectangular section in (a) shows caries-free surface. (**c**) Ablation rates of carious dentin, healthy dentin, and healthy enamel. Rates are extrapolated at a constant average power of 5 W. Reproduced from Fig. 2 and 4 in Ref. [53] with kind permission of Springer Science+Business Media.

energy than healthy dentin. Furthermore, spectral analysis of the laser-induced plasma shows that carious dentin has less intense calcium transitions than healthy dentin, due to carious demineralization. This difference may allow for real-time surgical feedback of the actual material being ablated [53].

Eye safety issues and technical difficulties of integrating a femtosecond laser into a dental hand piece represent two challenges to clinical acceptance, but the primary challenge is cost. There is reason to believe the cost of femtosecond laser systems will continue to decline in the future, which should permit widespread clinical application in dentistry. Femtosecond laser ablation may also find use in non-surgical applications, such as machining of hydroxyapatite or ceramics for fabricating dental restorations [53, 54]. Barriers to using femtosecond lasers (e.g., target positioning, laser safety) are mitigated in some of these applications because they are industrial rather than clinical.

4.2 Eye surgery

Ocular applications of femtosecond laser ablation are an extremely heavily-researched field and account for more scientific papers than all other femtosecond laser surgery fields combined. In addition, eye surgery by femtosecond lasers is cost-effective and more precise than conventional methods. Initial papers in the late 1980s detailed the effects of femtosecond laser ablation in retina tissue [29] and found that the excisions made are ultrastructurally superior to those made with nanosecond lasers [30]. For many years, however, research focused on picosecond Nd : YAG and YLF lasers due to the technical challenges of working with femtosecond laser systems. Interest in femtosecond laser ablation for ocular applications surged in the mid-1990s, leading to a number of biophysical studies of laser-tissue interactions in animal and cadaver samples [55–63], theoretical models of ablation and tissue removal [58, 64], and clinical trials [65, 66]. Femtosecond laser ablation is currently applied to refractive surgery and is also under consideration for use in keratoplasty and in correcting presbyopia.

Refractive surgery: Laser *in situ* keratomileusis (LASIK) [67] involves the removal of tissue in the bulk of the cornea (stroma) to correct for nearsightedness, farsightedness, or astigmatism. Suction immobilizes the eyeball, and a microkeratome blade partially separates the epithelium, leaving a hinge of uncut tissue at one end. The epithelium flap is pulled back, exposing the stroma, which is ablated by excimer laser. The flap is then folded back, and it bonds to the new surface as it heals.

Current clinical applications of femtosecond LASIK replace the microkeratome with a femtosecond laser. While ablation by excimer laser is already extremely precise, there is ongoing research to replace it as well [61, 62]. As shown in Fig. 2a, the femtosecond laser traces an outline of the flap and cuts out a volume to be removed (lenticule) within the bulk of the cornea. The flap is opened, the lenticule is extracted, and the flap is repositioned (see Fig. 2b). Femtosecond-LASIK holds a number of advantages over conventional LASIK. First, the accuracy of the flap thickness is significantly greater due to the absence of forces from direct contact by the microkeratome. Second, femtosecond laser cuts are smoother than microkeratome cuts. Third, the interior of the cornea is exposed for less time and in direct contact with fewer instruments, improving sterility [68].

Keratoplasty: If disease or injury damages the cornea, it can be replaced by grafting in new corneal tissue. Using femtosecond laser ablation rather than

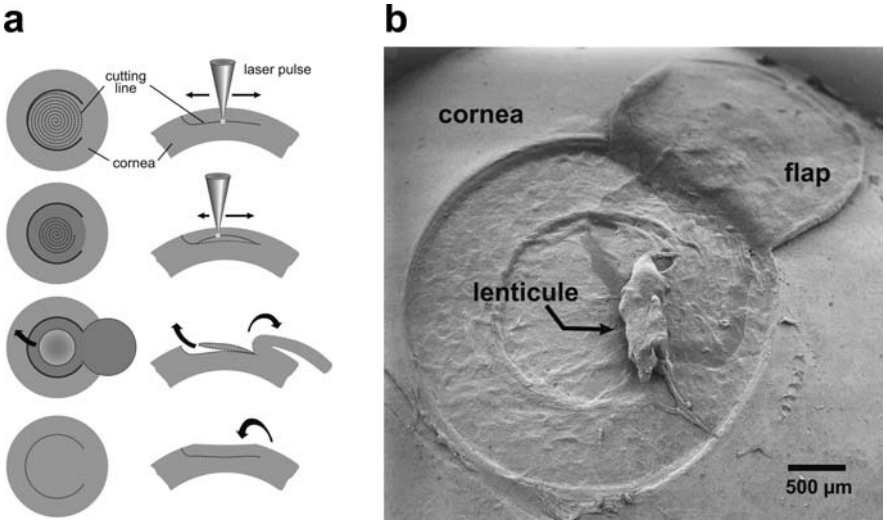


Figure 2 Femtosecond LASIK surgery of the eye. (**a**) Femtosecond laser ablates through eye interior to create lenticule. After its removal, the flap is repositioned. (**b**) Scanning electron micrograph of cornea with open flap. Reproduced from Fig. 6 and 7 in Ref. [62] with kind permission of Springer Science+Business Media.

mechanical cutting methods improves accuracy and smoothness of cuts. In addition, femtosecond lasers are capable of cutting arbitrary geometries on the transplanted cornea and the recipient eye. Various shapes such as a "top hat" or zigzag pattern increase resistance to wound leakage after keratoplasty [69, 70]. Furthermore, greater selectivity is possible in removing the damaged tissue, allowing for more of the original cornea to remain intact.

Presbyopia: With age, the eye gradually loses the ability to focus at close distances; the loss of lens flexibility is generally accepted to be the cause [71, 72]. Presbyopia is currently one of the most-heavily researched topics in ophthalmology, perhaps because it is the most common refractive error and has no permanent treatment options [73]. There are a number of different treatments under development, including scleral implants, corneal surgeries, pharmacological interventions to soften the lens, artificial intraocular lens implants, and laser surgery of the lens [74]. Lenticular surgery aims to restore flexibility by breaking the hardened-tissue structures and creating gliding planes of tissue [75]. Previous surgical attempts with nanosecond or picosecond lasers left behind residual gas bubbles, but femtosecond laser ablation of the lens yields lesions with bubbles that resolve with time [76]. Researchers are also pursuing a theoretical understanding of laser-tissue interactions to assist in the execution of surgeries [73].

5. Research applications

The research applications of femtosecond laser ablation fall into three general biological fields: cell and tissue studies, *C. elegans*, and zebrafish. There is ongoing work in other animals, such as *Drosophila* [77, 78], sea urchin, starfish, and squid [79], but the scope of applications in most of these animals is fundamentally limited by tissue opacity and target depth. It is also worth noting that green fluorescent protein [80] and various dyes have greatly facilitated the targeting and post-surgical observation of biological structures [81] but are not required for femtosecond laser ablation.

While research applications include some of the most precise and innovative uses of femtosecond laser ablation, the technique has yet to find widespread acceptance by the general biological community. Several factors might contribute: first, a significant initial cost in time and money is associated with purchasing and setting up a femtosecond laser. Second, the efficacy of the technique needs to be demonstrated in a wider range of applications. If these impeding factors can be mitigated, femtosecond lasers could find broader biological application.

5.1 Cell and tissue studies

Work in cell and tissues dominate biological femtosecond laser ablation research. Cell studies include selective disruption of individual chromosomes and organelles, ablative severing of cytoskeletal fibers, and membrane puncture for transfection. Tissue studies have focused on neural and vascular tissues in various model animals.

Chromosomes and organelles: The first application of femtosecond laser ablation at subcellular resolution was the nanometer-sized dissection of chromosomes, published in 1999 [82]. A similar study published simultaneously also showed DNA damage after femtosecond irradiation, but the mechanism was enzymatic rather than ablative [83]. As shown in Fig. 3, further

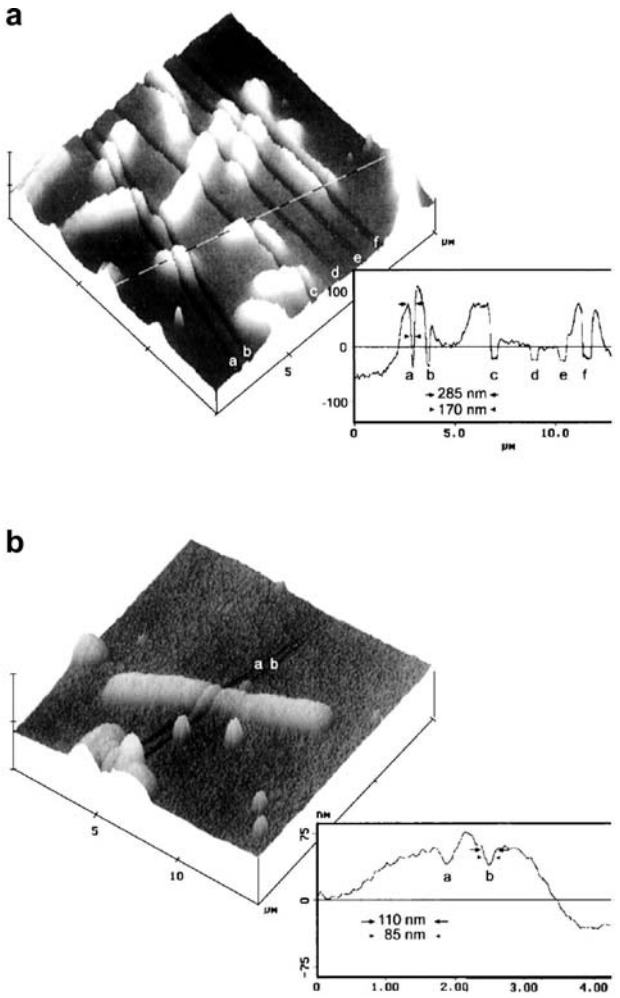


Figure 3 Topographical images and depth profiles of human chromosomes ablated by femtosecond lasers. (**a**) Complete dissections with widths below 200 nm. Depth profile of zero corresponds to bottom of chromosomes; negative values reveal cuts in underlying matrix. (**b**) Partial cuts with widths below 100 nm. Reproduced from Fig. 1 and 2 in Ref. [35] with kind permission of Optical Society of America.

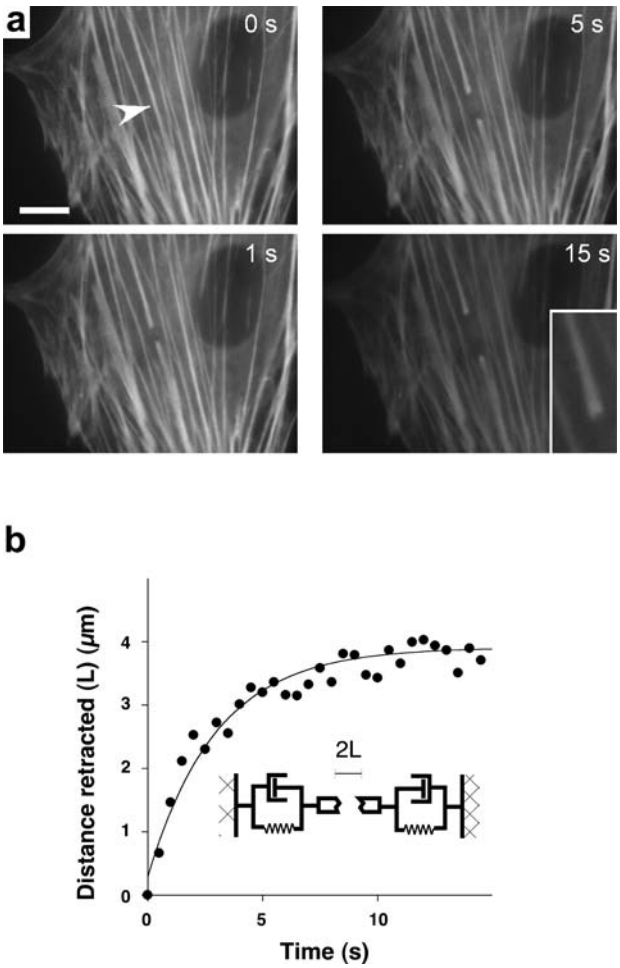


Figure 4 Dynamics and kinetics of stress fiber retraction. (**a**) Fluorescent image of severed actin retraction from point of surgery (arrow). (**b**) Time course of retraction. Line corresponds to predicted retraction of viscous and elastic elements in parallel. Scale bar is 2 μm. Reproduced from Fig. 1a and 3a in Ref. [92] with kind permission of Biophysical Society.

ablative work produced chromosomal cuts as small as 85 nm, measured by AFM [35]. Recent work includes using metal nanoparticles to preferentially absorb light and damage particular locations on the chromosome [84] and a demonstration of an integrated 1-GHz repetition rate system for multiphoton tomography and surgery with staining [85]. Femtosecond laser ablation has also selectively disrupted individual plastids in plant tissue [86] and mitochondria in HeLa [87] and capillary endothelial [36] cells. In summary, subcellular femtosecond laser ablation of chromosomes and organelles results in experimentally-straightforward dissections of unprecedented precision but have yet to move beyond the proof-of-principle phase to become common tools in cell biology [88–91].

Cytoskeletal fibers: The cell actively controls its shape through diverse networks of cytoskeletal stress fibers, which transmit forces from the extracellular environment and within the cell. An understanding of the molecular and biophysical mechanisms behind these dynamics provides insight into cell motility, differentiation, and apoptosis [92]. Femtosecond laser ablation can sever single fibers without disrupting the rest of the cell, allowing *in situ* perturbation of the cytoskeleton. Three proof-of-principle experiments in 2005 determined the optimal laser parameters and submicrometer surgical precision of femtosecond laser ablation of fluorescently-labelled cytoskeletal fibers [33, 36, 93]. Post-surgery dynamics demonstrate that actin is under tension and microtubules are under spring-like compression [33]. Another study observing the retraction of the ends of

severed actin found that stress fibers behave as simple viscoelastic cables (see Fig. 4), and the retraction was quantified under various pharmacological inhibitors. The extent of post-surgery cell deformation scales with the flexibility of the culturing substrate [92], and the mechanical forces released by the surgery propagate through the entire cell within about 100 μs [94]. Femtosecond laser ablation was also used to show that the unbinding rate constant of the focal adhesion protein *zyxin* increases as cellular traction forces decrease [95]. While one of the original studies described the depolymerization of microtubules after severing [33], a later study showed that microtubules near the nucleus depolymerize at a faster rate than ones at the cell periphery [96]. After severing the dynamic F-actin network by femtosecond laser pulses, both actin and E-cadherin, a spot adherent junction (SAJ) protein, redistribute away from the ablation point, indicating that the actin network restrains the mobility of SAJs by a tether mechanism [97]. A number of reviews cover femtosecond laser ablation of the cytoskeleton, usually in combination with other cell biology techniques for characterizing cell mechanics [91, 98–101].

Transfection, poration, permeabilization: Biologists often seek to introduce exogeneous material into cells or embryos for purposes such as transfection, staining, or biopreservation [102]. Conventional methods include direct injection by microneedles [103], electroporation [104], and gene delivery by viral vectors [105]; however, these techniques are plagued by complications such as toxicity, decreased viability due to cell damage, or lack of specificity in targeting. In 1984, a study showed that conventional laser pulses could perforate cell membranes transiently and transfect cells with greater efficiency [106]. Later, femtosecond laser pulses of significantly lower energy were employed to open smaller pores for the delivery of foreign DNA, drugs, or stains into plant or animal cells [107, 108]. A number of papers (e.g., [109, 110]) have since characterized the increased cell viability (about 80%), superior transfection efficiency (about 60%), and cellular volume exchanged during femtosecond optoporation. Efforts to facilitate the application of this technique are proceeding along several fronts, including nondiffracting light beams to simplify targeting of the cell membrane [111] and optical fiber-mediated transfection to simplify delivery of pulses from fiber laser systems [112]. As the technology becomes more accessible, more studies will demonstrate the efficacy of femtosecond optoporation in more applications, enabling mainstream use.

Neural and vascular tissue studies: The ablation and imaging capabilities of femtosecond lasers have been applied to study brain histology and vascular disruption in mouse and rat models. An *in vitro* proof-of principle experiment in 1998 determined the ablation speed, morphology, and threshold for surgery of bovine brain tissue [113]. Another experiment used femtosecond lasers for both surgery and imaging in mouse. Two-photon imaging is typically limited to a depth of hundreds of micrometers, but deeper tissue becomes optically accessible by ablating previously imaged tissue sections. Using an amplified femtosecond laser system, the entire neocortical column was serially visualized with micrometer resolution [114].

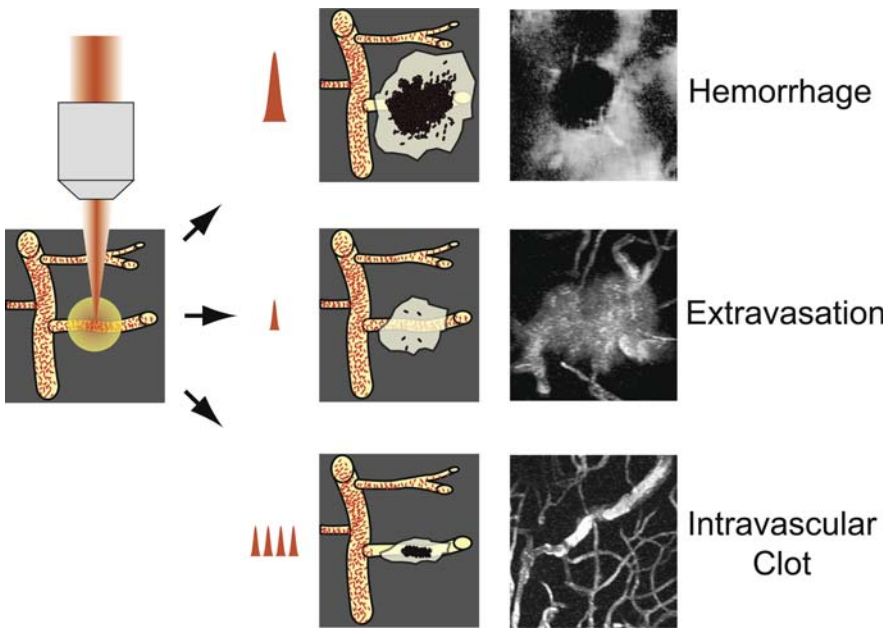


Figure 5 (online color at: www.biophotonics-journal.org) Femtosecond laser ablation of blood vessels leads to three distinct vascular lesions. High energies yield hemorrhage (top), low energies leave vessels largely intact, with transient leaks (middle), and multiple low energy pulses lead to intravascular clot (bottom). Scale bar is 50 μm. Reproduced from Fig. 1d in Ref. [115] with kind permission of Macmillan Publishers Ltd: Nature Methods.

Ablating blood vessels with laser pulses of varying energies leads to three different forms of damage, which could be useful stroke models (see Fig. 5) [115]. Laser pulses of high pulse energy produce vessel rupture and hemorrhage. Pulses of lower pulse energy induce extravasation of plasma and red blood cells from the vessels but continued flow within the vessels. Multiple low energy ablations yield intravascular clot formation and impaired blood flow.

5.2 *C. elegans*

The nematode worm *Caenorhabditis elegans* is an extremely useful model organism for studying biological phenomena [116]. A short lifespan, simple and inexpensive cultivation, and essentially identical individuals composed of only 959 cells make the worm straightforward to study. Widely-available genetic and molecular tools permit inactivation of proteins, removal of cells, and many other disruptions that illuminate the mechanisms of biological processes. Broad spectrum transparency and a small 100 μm diameter permit white light imaging, fluorescence microscopy, and precise laser surgery on any cell in the worm [117]. Conventional laser ablation with microsecond, nanosecond, and UV lasers has long been used to ablate whole cell bodies to probe cellular contributions to a variety of phenomena, such as development [118] and lifespan [119]. The submicrometer precision of femtosecond laser ablation enables the selective dissection of significantly more structures. For instance, a neuronal fiber connecting sensory cilia or distal synapses to a cell body is often bundled with the fibers of other neurons. Only a scalpel with submicrometer precision in the bulk can dissect this fiber without collaterally damaging neighbors (see Fig. 6). While the number of possible targets for ablation in the worm is enormous, the application of femtosecond laser ablation has so far been limited to studying two topics: neuronal regeneration and the neuronal origins of behavior. A significant broadening of applications is likely contingent on the technology becoming more accessible.

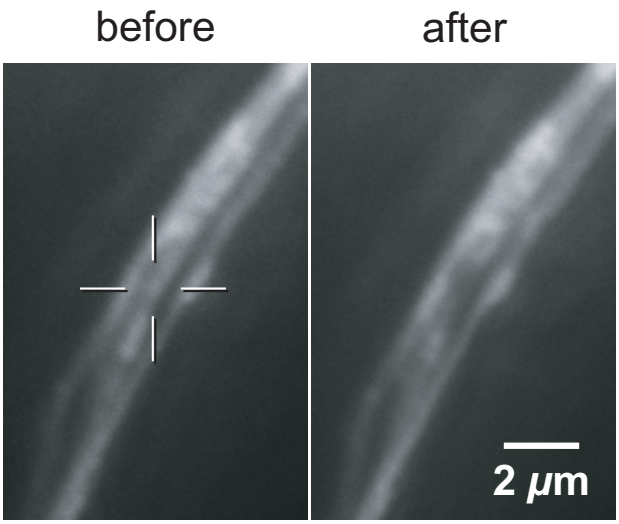


Figure 6 *C. elegans* dendritic bundle before and after femtosecond laser ablation of middle dendrite. Neighboring dendrites less than 500 nm away are not damaged. Reproduced from Fig. 1b in Ref. [123].

Neuronal regeneration: In 2004, the first published study applying femtosecond laser ablation to *C. elegans* described post-surgery functional regeneration of motor axons [120, 121]. While a number of other papers have confirmed varying degrees of axonal regrowth after axotomy, it remains the only publication in *C. elegans* presenting evidence for functional regeneration. Axonal regrowth significantly improves as pulse energy is decreased but is less dependent on the number of pulses [122]. This is expected for surgeries in the kilohertz regime as the size of the damage region depends on the pulse energy but not the number of pulses. Regrowth is also influenced by neuronal type [123], age of the worm, ephrin signaling, and location of the cut [124]. An in-depth, time-lapse examination of post-surgery axon regrowth in a variety of mutants showed significant deviations from initial axon development. The molecular requirements for development and regrowth are different, and axon growth during development is very precise and stereotyped, while regrowth may involve multiple unsuccessful axons and imperfect pruning [125].

Neuronal origins of behavior: Worm behavior is completely specified by the structure and function of the nervous system, which comprises only 302 neurons in stereotyped positions and morphologies, with a largely invariant neuronal connectivity [126]. Behavioral deficits in post-surgery worms can illuminate the contribution of the specific neuronal component that was eliminated by lesion. Femtosecond laser ablation has been used to study *C. elegans* thermotaxis, mechanosensation and locomotion, electrotaxis, olfaction, and egglaying.

Post-surgery assays localized the AFD neuron thermosensation to its dendritic tips and showed that the AFD is involved in gating but not executing the mechanism for cryophilic movement (crawling down thermal gradients) [123]. Further experiments observing the post-surgery calcium dynamics of the AFD showed that the memory of the cultivation temperature is encoded at the dendritic tips [127]. Finally, an analysis of post-surgery behavior showed that an olfactory neuron modulates cryo-

philic movement above the cultivation temperature and that suppression of its neuronal activity is necessary for isothermal tracking at the cultivation temperature [128].

An increase in the undulatory frequency of the worms swimming gait after surgery of mechanosensory neurons suggests that they might play a role in altering the temporal dynamics of the locomotory circuit [129]. Femtosecond laser ablation was used to clarify precisely which neurons are involved in electrotaxis, from sensing electric fields to making reorientation decisions and to executing them [130]. The response of mock and post-surgery swimming worms to temporal variations of odor in their environment is consistent with aggregation phenotypes obtained in previous crawling assays [131]. Femtosecond laser ablation has also been used to demonstrate the autonomous generation of activity in the egglaying neuron by isolating its cell body from all synaptic inputs [132].

5.3 Zebrafish

Originally native to freshwater lakes in India, the zebrafish has become both a common household pet and a powerful vertebrate model organism for biological research [133]. Embryos develop outside the mother, so the early development is rapid and the larvae are precociously capable of behaviors necessary for survival. Like *C. elegans*, young larval zebrafish are also transparent, allowing non-invasive optical imaging of their cells. Zebrafish studies focus on development, genetics, the nervous system, and regeneration.

The application of femtosecond laser ablation to zebrafish research is still in its early stages; however, the power of the zebrafish as a laser-accessible vertebrate model suggests a more widespread application in the future. Proof of principle experiments for ablation in zebrafish were published in 2003 and 2007 [79, 134]. A couple of papers specify the optimal laser parameters for femtosecond laser optoporation of embryonic cell membranes and the resulting short and long-term viabilities, which are generally above 90% [135, 136]. In another study, two-photon calcium imaging identified specific spinal projection neurons whose activity correlate with stimuli evoking turns. Subsequent femtosecond laser ablation of a small subset of those neurons eliminates turning toward the ablated side, confirming the subset as a necessary component of the turning circuitry [137]. A recent study also imaged microscopic flow in developing embryos by using femtosecond laser ablation to generate fluorescent debris [138].

6. Future trends and summary

6.1 Femtosecond laser systems

The state of the art in femtosecond pulse generation has mostly remained constant over the last 15 years. Pulse durations from femtosecond lasers have decreased only marginally, and control over the pulses has improved only slightly. What has improved significantly is the accessibility of the lasers. Three of the primary factors in accessibility are cost, complexity, and stability of the laser systems. As previously mentioned, cost is perhaps the limiting factor in the development of clinical applications. It is possible that as the systems become simpler, a broader use will help lower price.

One development that has reduced complexity and cost is the relatively recent emergence of higher-power oscillators. Previously, the maximum pulse energy of oscillators was approximately 10 nJ, so amplified systems were necessary to produce single-shot regime surgeries. Newer oscillators can produce up to 500 nJ pulse energy, making amplifiers unnecessary for most ablations in either regime. Eliminating the amplifier in a laser system increases stability and reduces the complexity and cost of the setup by about a factor of two.

Another ongoing technological advance is the proliferation of femtosecond fiber lasers with sufficient pulse energy to ablate. Fiber lasers create and amplify pulses inside an optical fiber rather than in free space, significantly reducing sensitivity to environmental changes. The output of the fiber laser can also be connected directly to the input port of a microscope, making alignment for surgery trivial. Although most fiber lasers currently operate at 1 μm, 800 nm fiber lasers with ablation capability may be forthcoming.

In principle, a completely solid-state semiconductor device emitting femtosecond pulses at 800 nm would be an ideal laser system. Unlike current femtosecond laser systems, a semiconductor laser requires no alignment by the user. Such a device could simply be attached onto a microscope for use, and it would be almost completely insensitive to vibration, temperature, and humidity. It would allow virtually anyone to perform femtosecond laser ablation with no more difficulty than using common imaging instruments, such as a laser scanning microscope.

6.2 Applications

As stated in the previous section, a number of recent innovations have made femtosecond laser systems significantly more simple, stable, and affordable.

Although femtosecond laser technology will undoubtedly continue to improve, current systems are sufficiently accessible for non-experts to acquire and operate. Thus, current systems are poised to become prevalent in both medical and research communities. In the remainder of this section, we discuss two powerful imaging tools which might further enhance femtosecond laser ablation and the possible future contributions of femtosecond laser ablation to clinical and research applications.

Femtosecond laser ablation is often used in combination with imaging techniques and tools, including epifluorescent or two-photon imaging, various staining agents, and fluorescent proteins. Significant improvements in imaging are proceeding along two fronts that could increase the precision and efficiency of femtosecond laser ablation. First, subdiffraction-resolution imaging permits discrimination of nanometer-sized objects and can be used to improve pre-surgery targeting and post-surgery observation. Many "superresolution" techniques exist, but two are generating much interest because of their simplicity and adaptability to existing microscopy setups. Photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) iteratively activate sparse subsets of fluorescent proteins and localize their position [139–141]. Stimulated emission depletion (STED) increases resolution by deactivating fluorophores in a donut-shaped region at the focal volume periphery [142, 143]. These techniques currently require substantial amounts of time for image reconstruction and laser scanning, respectively. If the speed of sub-diffraction-resolution imaging can be improved, it will be possible to use femtosecond laser ablation in conjunction with an imaging technique with commensurate submicrometer spatial resolution, allowing unprecedented surgery precision. Second, quantum dots might provide an alternative to conventional staining or fluorescent proteins [144]. The advantages of quantum dots include increased brightness, resistance to photobleaching, greater control over their fluorescence spectrum, and targeting specificity after surface functionalization. Moreover, quantum dots can also target tumors and deliver drugs [145]. If significant issues of cytotoxicity can be resolved, quantum dots might become a robust and flexible imaging partner to femtosecond laser ablation.

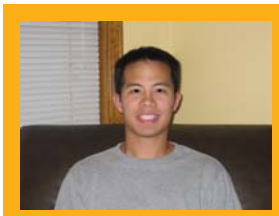
Extrapolating current trends, femtosecond laser ablation stands to make significant, but distinctly different, contributions in clinical and research applications. Clinicians (and research for clinical use) are mostly focused on optimizing existing applications of femtosecond laser ablation, while researchers are generally pursuing new applications. This distinction may stem from fundamental differences between medicine and basic science: medicine usually employs more established methods for treatment, but basic science is devoted to discovery of new methods and phenomena. It may also result from having relatively fewer accessible targets in the human body compared with cell culture or translucent, small model animals. Thus, the future contribution of femtosecond laser ablation to clinical fields might primarily be measured by the precision of surgery or the number of procedures performed. An increasing affordability of femtosecond lasers and a significant reduction in pain during surgery will encourage use in dentistry and possibly lead to a commercial device. The considerable volume of research into eye surgery will likely yield ocular applications beyond femtosecond-LASIK, which is already commercially available. The development of more clinical applications is largely dependent on gaining access to deeper targets in the human body (e.g., by transmitting pulses by endoscope). Femtosecond laser ablation is likely to contribute to the three research fields (mentioned in Section 5) dissimilarly as well. Due to size and amenability, studies performed on cell and tissue culture will probably continue to encompass the most precise work, both in terms of surgical resolution and resulting data. In relation, applications in *C. elegans* are likely to be more diverse as an entire organism is accessible to the laser. Applications in the vertebrate zebrafish are limited by the short window of developmental time when young larvae are accessible but could also produce a diverse set of findings more relevant to human physiology. The cells of other model animals, such as *Drosophila*, might also be accessible for surgery at early developmental stages. In summary, femtosecond laser ablation will contribute to multiple clinical and research fields in the future as femtosecond laser systems continue to develop.

6.3 Summary

Femtosecond laser ablation can perform submicrometer-resolution surgeries in bulk without collateral damage, and surgical applications of femtosecond laser ablation are increasing in number and diversity. Clinical applications of femtosecond laser ablation currently include several types of dental and eye surgeries. At present, research applications fall into three fields: cell and tissue studies, *C. elegans*, and zebrafish. Recent innovations in laser design have made femtosecond lasers significantly more accessible and will facilitate widespread use in the clinical and research communities.

Acknowledgements Several people contributed to the creation of this paper. S.H.C. wrote the first draft of the manuscript; both authors subsequently took part in the revision process and approved the final copy of the manu-

script. A. Heisterkamp, E. C. Chung, P. Tayalia, E. D. Diebold, T. Shih, K. Vora, and M. Sher provided feedback on the manuscript throughout its development. S.H.C. is supported by the National Science Foundation under contracts DMR-0213805 and PHY-0555583.



Samuel Chung is a pioneer of femtosecond laser ablation, which can perform submicrometer-resolution surgeries within the bulk of living animals and cells. For his doctoral research, he applied the technique to probe the neuronal origins of behavior in the worm *C. elegans*. In 2009, he obtained a Ph.D. degree in Applied Physics from the School of Engineering and Applied Sciences at Harvard University, and he is currently pursuing postdoctoral research with Christopher Gabel at Boston University.

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