In vivo manipulation of biological systems with femtosecond laser pulses

Nozomi Nishimura[a,b], Chris B. Schaffer[b], David Kleinfeld[a]
[a]Dept. of Physics, University of California at San Diego, La Jolla, CA USA, 92093;
[b]Dept. of Biomedical Engineering, Cornell University, Ithaca, NY USA, 14853

ABSTRACT

Femtosecond laser pulses have the unique ability to deposit energy into a microscopic volume in the bulk of a material that is transparent to the laser wavelength without affecting the surface of the material. Here we review the use of this capability to disrupt specifically targeted structures in live cells and animals with the goal of elucidating function and modeling disease states. Particular attention will be paid to recent work that uses femtosecond laser disruption to injure cerebral blood vessels that lie below the brain surface in a live, anesthetized rat. By varying the degree of injury, the vessel can be made to leak blood plasma, to rupture, or to clot. This technique thus provides a versatile model of cerebrovascular disorders such as small-scale stroke.

Keywords: femtosecond, ultrafast, biology, *in vivo*, ablation

1. INTRODUCTION

Femtosecond lasers have been well established as a tool for subsurface machining of transparent materials[1] and ultra-precise machining of solid state materials[2]. Recently, femtosecond ablation has been adapted as a biological tool to manipulate structures and chemistry on a microscopic scale. Femtosecond laser ablation can produce ionization that is highly spatially confined in three dimensions. This can be used to disrupt organelles inside individual cells with subcellular precision, multiple cells, or parts of cells in whole animals. This allows unprecedented study of biological dynamics and reactions. These capabilities allow the function of a particular structure to be studied in an *in vivo* context, and can also be used to reproduce disease states of relevance to human health in animal models.

2. MECHANISMS OF FEMTOSECOND ABLATION

At ordinary intensities of light, photons that have insufficient energy to ionize a material pass through the material without interaction; for example, visible light passes through transparent materials such as water and glass. However, at light intensities around 10^{13} W/cm^2 that are achievable by femtosecond lasers, the density of photons is sufficient that the chance of multiple photons interacting with the same molecule nearly simultaneously becomes high. The cooperative action of multiple photons can result in ionization of an ordinarily transparent material via several conceptually different processes (Fig. 1a). In multiphoton ionization, an unexcited electron simultaneously absorbs the energy of several photons, so that the combined energy of these photons is sufficient to boost the electron to an excited state or may free the electron to form an electron-ion plasma. Because overcoming the energy gaps in transparent materials require multiple photons, the probability of ionization depends on intensity to a power equal to the number of photons. This process requires that the photon density is high enough that the probability of absorbing multiple photons at one time is non-negligible. Another mechanism for cooperative action of photons is called avalanche ionization. In this process a previously ionized electron can absorb energy from the laser photons. Collisions between this electron and an un-ionized electron can result in the ionization of the second electron. This process results in an exponentially increasing number of ionized electrons. Avalanche ionization also requires high laser intensities because it requires that an ionized electron absorb the energy of multiple photons so that its excess energy exceeds the band-gap energy before colliding with another electron.

Ionization leads to an electron-ion plasma in which the energy from the laser pulse is stored in the separation of the positively charged ions and negative electrons and their kinetic energy. After about 10 ps to 1 ns, the electrons and ions in the plasma recombine. The energy from the recombination contributes to highly non-equilibrium conditions, which can overcome the tensile force of the material around the focus . With sufficient energy, this leads to a microexplosion and shockwave (Fig. 1b)[3, 4]. The recombined material, including molecular hydrogen and oxygen[5], remains as a hot gas bubble. This cavitation bubble expands and contracts with complex dynamics as it equilibrates to the surrounding conditions.

All the steps in the process of femtosecond laser ablation can produce effects in biological materials. For example, the material ionized at the laser focus may be altered substantially from its original chemical composition. Ionization can also lead to excited molecules that participate in chemical reactions. Both the formation of reactive oxygen species from the ionization of water and the direct breaking of chemical bonds in cellular structures can lead to biological effects[6]. The microexplosion, shockwave, and cavitation bubble can add mechanical disruption to chemical effects[7, 8].
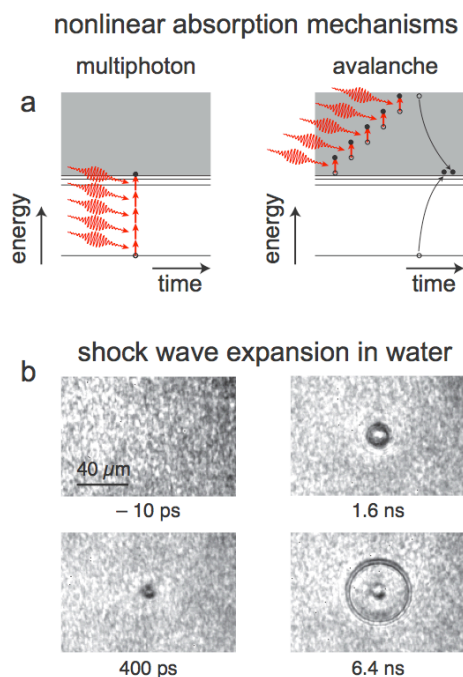

Fig. 1 Mechanisms of femtosecond laser ablation. (a) Schematic diagrams of multiphoton ionization and avalanche ionization. (b) Images of ionization at focus, shock wave and cavitation bubble in water after irradiation by a femtosecond laser pulse (100 fs, 1 μJ, 800 nm, 0.65 NA). Data adapted from Schaffer et al., 2002[3].

One of the most useful properties of femtosecond laser ablation is the limited size of the affected volume. Chemical alteration by ionization, mechanical and thermal effects of the shock wave and cavitation bubble can all lead to permanent alterations in the target material, but these effects can be limited to microscopic volumes. Two factors play a role in minimizing the disrupted volume. First, tight focusing ensures that nonlinear absorption occurs only in a three-dimensionally localized volume. With sufficiently tight focusing[6] the necessary intensity for any significant amount of multiphoton ionization is only achieved at the focus. It has been demonstrated that under carefully controlled conditions, only the central portion of the laser focal volume will ionize a significant number of electrons, so that volume of ionized material is actually smaller than the diffraction limited spot size of the laser[9]. Second, with femtosecond-duration laser pulses, sufficient intensity to drive nonlinear optical absorption can be achieved with low pulse energy. The total energy deposited determines the magnitude of effects of the shockwave and cavitation bubble. The energy required to achieve ionization can be very small, so that the size of the affected material can be microscopic – very close to the diffraction-limited focusing volume. For example, ionization of water in the focal volume of a 0.65-NA objective requires only 0.1 μJ using 100 fs pulses at 800 nm[3].

Properties such as the size of the laser-processed volume can be controlled by varying the laser pulse energy, repetition rate, and number. After a single laser pulse, the sizes and energies of the cavitation bubble and shock wave depend on the total amount of energy deposited in the material. As laser power is increased well above the threshold energies for ionization, energy is deposited in an increasingly large volume, even as the focusing is held constant, leading to a larger volume of ionization. The fraction of the energy in the incident laser pulses that is absorbed increases from less than 20% at the threshold energy to nearly all the energy in the laser pulse at higher energies[10]. In addition, more energy in the laser pulse leads to an increase in the number of electrons, and consequently, larger shock waves and cavitation bubbles that can lead to macroscopic cuts and cavities.

Irradiation of the target material by repetitive pulses can lead to cumulative effects that can be tailored to the application. Despite the low total energies required, femtosecond laser ablation can lead to significant thermal and chemical effects under certain conditions. These cumulative effects may often occur at energies below the threshold for photodisruption. If the time between the laser pulses is small relative to the time heat takes to diffuse out of the vicinity of the focal volume, heat can accumulate near the focal region[1]. Although the energy of each laser pulse is small, at the repetition rates of a laser such as titanium:sapphire oscillators typically used for two-photon microscopy a significant amount of heat can build up at the focus if the laser irradiates the same spot for an extended period of time. In solid state materials, such as glass, this effect is used to produce refractive index changes via the melting and cooling of material outside the laser focus[11]. While the chemical changes caused by a single femtosecond laser pulse can be negligible for cells, continuous irradiation over time can lead to a significant number of excited molecules that can participate in potentially useful or harmful chemical reactions[6].

Femtosecond laser ablation has been demonstrated to be useful for processing extracted and fixed tissues. The lack of collateral and thermal damage makes femtosecond laser ablation an ideal tool for heat sensitive tissues such as bone and brain[12-15]. In vitro experiments show that the precision of femtosecond laser ablation may also be useful for treatment of eye conditions such as glaucoma by making channels through the sclera or trabecular meshwork in the eye[16]. Another advantage of femtosecond ablation is that the threshold energy for many materials is very similar[10]. This trait is useful in the ablation of heterogeneous samples in which material properties may vary significantly. For example, femtosecond laser ablation was used as part of a 3-D histology system used to image fixed mouse embryos[17]. This required simultaneous ablation of bone and soft tissue without adjusting the laser parameters. Recent work has expanded use of femtosecond lasers from the processing of solid state materials and extracted tissue, to the manipulation of living tissue. This has important applications in medical technologies and provides an important research tool to investigate the function of biological structures in dynamic systems and disease models.

3. APPLICATIONS

Here, we highlight some outstanding applications of femtosecond lasers in living systems from a number of research groups that exploit the unique properties of nonlinear interactions of light with matter.

3.1 Eye surgery with femtosecond lasers

One of the earliest and most commercially successful applications of femtosecond lasers is in laser eye surgery. To correct near- or farsightedness in a person, portions of the cornea are cut and reshaped so that the cornea will then correctly focus. In the earliest versions of this procedure, radial keratectomy, mechanical blades were used to reshape the cornea with radial cuts. A great improvement came from the used of lasers. In photorefractive keratectomy, UV laser light is used to photoablate and reshape the cornea, but also ablates the protective endothelial layers on the corneal surface. In a modification of this procedure, laser-assisted *in situ* keratomileusis (LASIK), the corneal tissue is first exposed by a cutting a flap from the surface layer with a mechanical blade. Then, UV light is used to photoablate the corneal tissue, and the flap is replaced. The preservation of the surface endothelial layer helps speed recovery from the surgery. While UV laser ablation via linear absorption was a great improvement over mechanical cutting of the corneal tissue, new techniques using femtosecond laser ablation offer further improvements in the surgical outcome. Much of the complications in the surgical procedure come not from the reshaping of the cornea,

but from damage to the surface layer of the cornea, the epithelial cells, when removed with a mechanical blade. Juhasz *et al.* developed an optical technique to cut this flap by taking advantage of the nonlinearity of ablation by femtosecond laser[18]. The femtosecond laser is focused beneath the surface of the epithelial layer, and via nonlinear absorption mechanisms ionizes just enough tissue to cut under the corneal surface. After scanning the laser focus in a spiral pattern, the surgeon is able to lift a flap of the surface layer. The laser-based cutting of the flap is far more precise and less error-prone than with a blade. This procedure is now performed at many clinics and offers significant reduction in side effects[19].

3.2 Disrupting development in fly embryos

Femtosecond lasers are also a powerful tool in research and allow manipulation inside living organisms. The differentiation of embryos into various types of tissues is governed by factors called morphogens. Many morphogens are chemicals that induce the development of different cell types. Recently, research on drosophila embryos revealed that mechanical forces can act as morphogens and affect the development of cells[20, 21]. Femtosecond lasers were used in living embryos to disrupt the cells thought to be responsible for generating some of the mechanical forces that triggered subsequent development. The success of the experiment depended on preserving the viability of the embryos and also the ability to disrupt only a selected subset of cells, without compromising the surrounding membrane, the vitelline membrane. In recent work, Supatto *et al.*[21] ablated dorsal cells in vital drosophila embryos and measured changes in the development of the embryo. The embryos were imaged via third harmonic generation of intrinsic features or by two-photon fluorescence of transgenically inserted yellow-fluorescent protein. First, Supatto *et al.* carefully studied the laser parameters that lead to the appropriate type of disruption. Their femtosecond laser has a high pulse-repetition rate, 76 MHz, so that when the focus of the laser was moved slowly or held still, the time between each pulse was insufficient for the energy from one pulse to diffuse away before the arrival of the next pulse. At low energies and small number of pulses, only a change in the endogenous fluorescence was observed. With increased number of pulses, at the same energy, increasingly large bubbles were produced (Fig. 2a-c). The size of bubbles could also be increased by changing the energy of pulses while holding the number constant.

The suitability of the ablation mechanism for manipulating embryonic development was demonstrated by the ablation of ~100 x 40 x 20μm in the dorsal region of the embryo. Parameters were adjusted so that the size of the bubbles guaranteed the complete disruption of the target cell, 5-6μm in diameter. Different ablation locations resulted in different deficits in the development of structures over the next hour after ablation. The results suggested that the ablation resulted in a mechanical perturbation of an area which participates in the morphogenic movement, eliminating the signals that cause cells to differentiate and form specific structures. Further, the lack of cell differentiation and movement in ablated embryos lead to a deficit in the expression of *twist*, a gene whose expression is thought to be modulated by mechanical deformation of cells. Femtosecond laser ablation enabled these researchers to add precise mechanical manipulations inside intact embryos to elucidate the role of movement and deformations in gene expression.

3.3 Neural ablation in *C. elegans*

The use of femtosecond lasers as a precise, subsurface scalpel has also been extended to the worm *C. elegans*, in which changes in behavior in whole active animals have been observed after laser ablation. Yanik *et al.* used 10-40 nJ pulses to cut single axons in neurons that are responsible for coordinating muscles that cause the worm to move backwards after a touch on the nose. Femtosecond lasers are ideal tools for this research because the subsurface ablation capability enabled work in whole animals, allowing the observation of behavioral changes. In addition, the microscopically-sized affected volume allowed this research team to cut a single axon at a time. In animals in which multiple axons were cut lost the ability to go backwards after being touched on the nose. Remarkably, the laser ablation was mild enough that over half the cut neurons showed signs of regrowth within 24 hours. The majority of animals with cut neurons recovered some ability to move backwards after 24 hours, indicating that at least some of the axons had recovered functionality. Other techniques for eliminating neurons in *C. elegans* can disrupt whole cells. However, after elimination of the whole neuron, rather than just the axon, the worm does not recover the

ability to move backwards even after 48 hours. The femtosecond laser ablation helped elucidate difference between losing a portion of a cell, rather than the entire cell.

Femtosecond laser ablation has also been used to elucidate the role of specific neurons involved in sensory systems in *C. elegans* (Fig. 2d) [22]. It had been previously known that a group of neurons were involved in the regulating behaviors related to temperature. *C. elegans* is known to be sensitive both to absolute temperature and also to changes in temperature. In this work from Chung *et al.*, individual dendrites in the neural network responsible for temperature sensing were cut by femtosecond laser, and the reaction of the worms to different temperatures and temperature gradients was measured. From these dissections, the authors were able to determine that a particular neuron is involved in generating a response to absolute temperature, but is not involved regulating behavior in response to relative changes in temperature. Both of these experiments are remarkable in that they relate the physical "wiring" of a neural network all the way to behavior.

3.4 Subcellular nanosurgery of cell cytoskeletons

Femtosecond laser ablation was also used to investigate the mechanical properties of single cytoskeletal filaments in living cells. Using 1-kHz, 200-fs pulses, a single microtubule fiber was cut and was observed to recoil and then further depolymerize[23]. Using a similar technique, Kumar *et al*. cut single actin stress fibers in living cells and observed the retraction dynamics and changes in the cell shape (Fig. 2e)[24]. In order to confirm that the actin fibers were truly severed, a portion of the fluorescently labeled actin fiber was photobleached before cutting. When the actin fiber was cut at a location far away from the photobleached area, the photobleached area also recoiled, indicating that the entire fiber contracted, rather than just the tips. By chemically blocking molecular motors, it was found that in addition to the passive elastic properties of the fibers, the cell actively exerts force on these fibers with molecular motors[24].

3.5 Subcellular nanosurgery of mitochondria

Mitochondria are extremely important as the energy factories of cells. Abnormalities of or changes in number of the mitochondria can adversely affect the health of an organism. Femtosecond lasers have been demonstrated as a method to selectively remove mitochondria inside cells in culture[25, 26]. The cells remain viable and can even divide successfully after ablation. In these experiments the mitochondria was fluorescently labeled. Confirmation of the elimination of the targeted mitochondria, rather than just photobleaching of the fluorescent dye was confirmed by a lack of labeling with mitochondria-specific dyes after ablation. Additional work by Shen *et al.* suggests that mitochondria are separate entities rather than continuous across the cell[27].
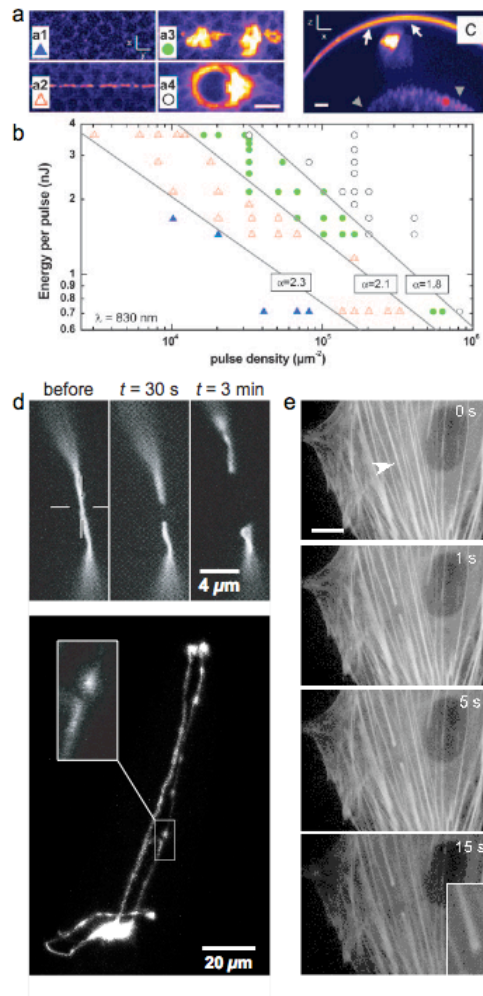
Fig. 2. Use of femtosecond lasers to manipulate living biological systems. (a-c) Femtosecond laser pulses were used in fly embryos to study the effect of removal of cells on changing the development. Supatto *et al.* were able to change the type of disruption (a1-a3) by adjusting the laser energy and number of pulses (b). (c) A fly embryo after irradiation. The region of irradiation shows strong autofluoescence. Reproduced from Supatto *et al.*, 2005[21]. (d) Cutting of individual dendrites in *C. elecgans* worms. The top images show a dendrite contracting after a cut at t = 0. The bottom shows confocal images of the a worm with a cut dendrite. Reproduced from Chung *et al.*, 2006[22]. (e) A fluorescently-labeled actin filament retracts after cutting by femtosecond laser ablation. (Arrow head indicates the position of the laser spot; bar = 10 μm). Reproduced from Kumar *et al.*, 2006[24].

3.6 Photochemistry and membrane poration

When focused into a sample for long periods of time, the titanium:sapphire oscillators typically used for imaging with two-photon microscopy can generate phototoxicity and chemical changes through multiphoton processes that may be useful in experiments on living cells. The photochemical effects generally only become pronounced with relatively long periods of illumination. At energies that do not initiate the full photodisruption sequence, biological function can be influenced by chemical changes, temperature increases and the production of reactive molecules. These changes are still confined to small volumes at the laser focus and maintain subcellular precision.

Tirlapur *et al.* demonstrated the use of low energy femtosecond laser pulses for cutting chromosomes[28]. Such a feat had been demonstrated by using linear optical effects[29], but because femtosecond lasers accomplish the task at non-UV wavelengths, they may be less damaging to cells. Femtosecond laser irradiation at high repetition rate can be used to make microscopic lesions in embryos, dendrites, squid axons, and oocytes to disrupt the normal function of these cells[30]. For example, cell division in one cell of many cells in an embryo was halted by femtosecond irradiation of the mitotic pole[30]. The three-dimensional spatial resolution of femtosecond lasers may prove to be a crucial technology for such optical techniques such as chromophore-assisted laser inactivation (CALI) of specific proteins[31]. CALI makes use of reactive oxygen species created by the absorption of a dye coupled to a protein of interest to inactivate just the target protein. Femtosecond laser pulses and CALI combine the spatially specificity of nonlinear optics and the molecular specificity of biochemical labeling. The membrane of cells can also be made permeable to large molecules by femtosecond laser irradiation. This has been put to use as a means to transfer foreign genes into cultured cells as demonstrated in 2002 by the expression of GFP plasmids[32]. This femtosecond laser version of optoporation for gene transfer has a much greater success rate and cell survival rate compared to other traditional methods.

3.7 Exciting neurons in brain slice

Hirase *et al.* took advantage of the ability of nonlinear processes that occur in the depth of a scattering sample and demonstrated the activation of neurons in mouse brain slice experiments[33]. Neurons in brain slice were irradiated with 80-MHz laser pulses from an oscillator while the intracellular voltage was recorded by an electrode. Irradiation of the plasma membrane led to depolarization which in turn could lead to the firing of action potentials. Varying the laser power and the length of irradiation time generated two types of depolarization. One, with low energies but long irradiation times (many seconds), lead to a slow effect that was reduced with the addition of anti-oxidants. This suggested that a multiphoton photochemical reaction lead to depolarization. At higher energies, still attainable with the oscillator, shorter ~ 30ms bouts of irradiation lead to faster voltage dynamics. In these cases, some leakage of dye from the cell was observed, suggesting that the plasma membrane was porated. This is reminiscent of earlier work with laser-based photostimulation of dye-stained neurons by linear optical processes[34], but incorporates the improved spatial localization of nonlinear optical processes.

3.8 Calcium signaling in cell culture

Femtosecond laser pulses were used by Smith *et al.* to manipulate the release of internal calcium stores in cultured cells[35]. Irradiation of cultured HeLa cells in the cytosol and on the plasma membrane led to a rise in intracellular calcium concentration. An application of an extracellular calcium chelator reduced the calcium response only in the case of plasma membrane irradiation, indicating that the calcium from irradiation of the cytosol came from inside rather than outside the cell. In addition, the calcium in neighboring cells also rose after irradiation, indicating that there is an intercellular activation mechanism.

3.9 Microstroke in rodents

Femtosecond lasers were used to mimic a human disease in rodents. Recent clinical work has established the detrimental effects of small strokes on the cognitive health of older people[36]. The source of these small strokes is thought to be blockages or leaks in the small vessels of the brain. Surgically-induced strokes in animals models have elucidated many mechanisms in the sequence of events that progress from blood flow changes to neural death. Much of this work is done in animal models of large stroke, in which blood flow is compromised in major arteries. Until recently, there were no methods for producing a clot in a targeted microvessel, and therefore no controllable animal models of small strokes. Femtosecond lasers were used to ablate individual, subsurface blood vessels in rat to produce occlusions and hemorrhages[37]. Two-photon excited fluorescence (2PEF) microscopy of intravenously injected fluorescein dye was used to image the structure of the cortical vasculature through a glass-covered window in the skull. The velocity of red blood cells was measured by 2PEF before and after the induction of microvessel lesions.

For ablation, femtosecond laser pulses from a multipass titanium:sapphire amplifier with energies ranging from 0.1nJ to 1μJ were focused into the lumen of a subsurface cortical vessel. The photodisruption caused a localized injury to target vessel, which caused some blood to leak into the surrounding tissue. Proteins in the blood are toxic to neural tissue and such leakage may mimic leakages in the brains of elderly humans. In some cases, often with additional irradiation, the injury was sufficient to trigger the intrinsic clotting cascade and occlude the target vessel. Blood flow dropped dramatically to ~10% of baseline after the occlusion in the vessels immediately downstream from the target vessel. This level of blood flow decrement is dangerous to the health of neurons, suggesting that occlusions of small blood vessel could be the cause of small strokes observed clinically. Recent clinical studies have found small hemorrhages in the brains of peoples with age-related and other types of dementia[38]. Using femtosecond laser ablation, similar-looking hemorrhages can be induced in the rat. To generate such lesions, femtosecond laser pulses several times higher than the energy of pulses used to make leakages and occlusions were focused into a single vessel. Single, high-power pulses resulted in a mass of red blood cells pushing into the brain tissue.
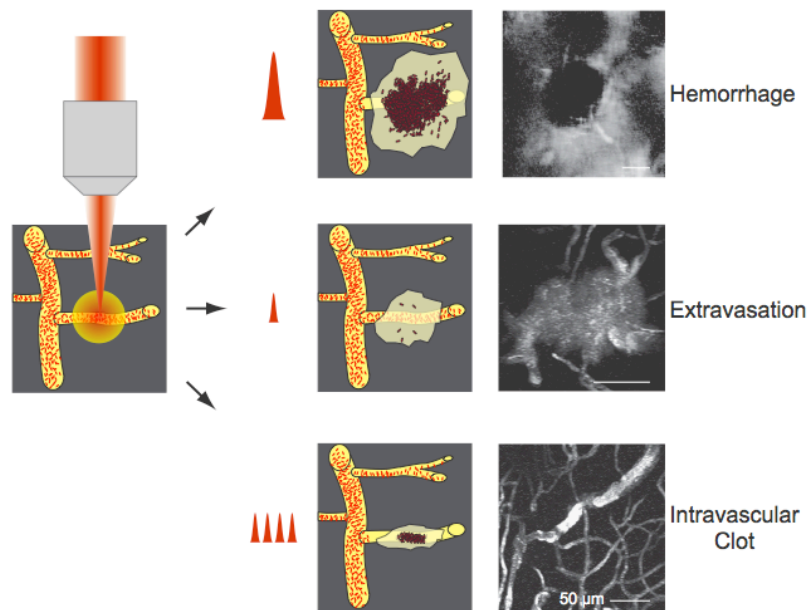

Fig. 3. Lesions in microvasculature in rat cerebral cortex. Femtosecond laser pulses are focused into the lumen of a sub-surface blood vessel. Irradiation with high energy pulses generates a hemorrhage (top). Irradiation with lower energy pulses can lead to extravasation or leakage of blood vessel contents (middle). Additional irradiation can lead to an occlusion of the target vessel (bottom). Adapted from Nishimura *et al.,* 2006[37].

This animal model of small vessel disease was used to evaluate the effect of decreasing blood viscosity as a means to increase blood flow after blockages. Red blood cell speed was measured before and after occlusion of a target vessel. Physiological saline, approximately half of the volume of the rat's own blood, was slowly injected intravenously. After the injection, red blood cell velocity was remeasured. While the red blood speed did not recover to baseline, there was an increase in velocity relative to the measurement immediately after the clot that may help mitigate the pathological effects of the occlusion[37].

4. CONCLUSION

The modification and ablation of cells and tissue with femtosecond laser light is becoming an important tool in biology and in medicine. The same properties of femtosecond lasers that are advantageous in

micromachining solid state materials are also applicable to biological applications. The ease, precision and the three-dimensional localization of femtosecond laser ablation within living organisms open new opportunities in areas that range from studies of subcellular functionality to medical research on human diseases.

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