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INTRACELLULAR MANIPULATION BY FEMTOSECOND LASERS: REVIEW

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Multiphoton absorption of femtosecond laser pulses focused through an objective with high numerical aperture (NA) can be used to image and manipulate cellular and intracellular objects. This review highlights recent advances in intracellular manipulation, including nanosurgery and labeling in living cells with femtosecond lasers.

Keywords: Femtosecond laser; nanosurgery; ablation; multiphoton microscopy; photoconvertible fluorescent protein.

1. Introduction

Lasers have been used for imaging as well as for manipulating cellular structures in living cells non-invasively. Since the advent of femtosecond lasers, multiphoton microscopy has become an important tool for the observation of sub-cellular structures.[1] Focusing of intense femtosecond laser pulses allows manipulation of cellular and intracellular structures to be carried out.[2] Femtosecond laser manipulation techniques offer attractive advantages, including high-resolution in 3-dimension, reduced photon-induced damage, and deep penetration into thick samples because they are based on multiphoton process. In this review, we report on state-of-the-art technology of intracellular manipulation with femtosecond lasers by highlighting selective labeling and surgery for intracellular structures.

2. Femtosecond Laser Nanosurgery

An intense focused continuous wave (CW) or long-pulse laser beam in ultraviolet (UV) and visible region has been used for selective targeting of cellular and intracellular structures.[3–7] Surgery in plant

and animal cells was demonstrated with sub-micron spatial resolution by laser-induced plasma formation. Ultraviolet lasers have some disadvantages, namely, their low-light penetration depth, collateral damage outside the focal volume, the risk of photodamage to living cells due to absorption, and the induction of oxidative stress leading to apoptosis. The focused long-pulse laser beam causes thermal damage and denaturation of the protein molecules around the laser focus and collateral damage due to heating and shockwave propagation. The main disadvantage of using UV and visible lasers is that the viability of the cells after laser surgery is relatively low.

2.1. *Chromosome dissection using femtosecond nanosurgery*

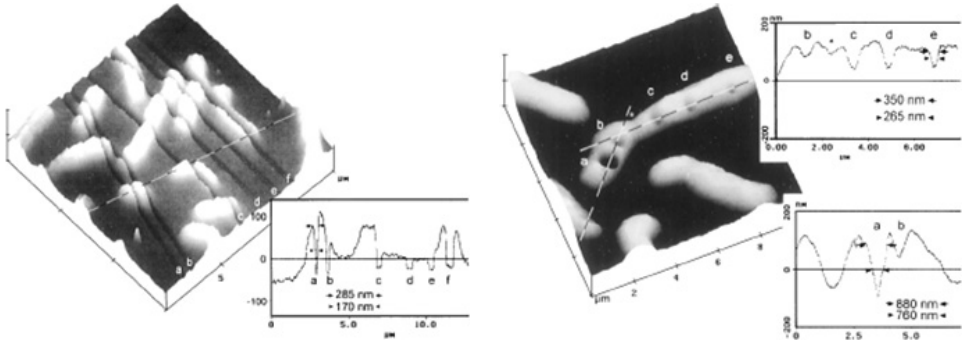
Femtosecond lasers can also be employed as highly precise nanosurgical tools for tissues, cells, and intracellular structures when using higher intensities. König *et al.*[8] first proposed nanosurgery with femtosecond lasers in 1999. König *et al.*[9] demonstrated the dissection of human chromosomes by tightly focusing high repetition rate (80 MHz) femtosecond laser pulses. Measurements with the atomic force microscope reveal chromosome dissection with a cut size below 300 nm. In addition, the removal of chromosome material with a precision of 110 nm was obtained (Fig. 1). The cells remained alive and completed cell division after laser surgery. The limited heat generation enables precise control of the modification of chromosomes, avoiding peripheral thermal damage. The femtosecond laser can thus provide spatially selective manipulation of a targeted structure.

2.2. *Femtosecond laser transfection*

Femtosecond laser pulses have also been used for efficient targeted transfection by transient opening of the cellular membrane and the subsequent diffusion of the foreign DNA into the cytoplasm. König *et al.*[10] demonstrated single-cell gene transfection of Chinese hamster ovary cells by creating a 100-nm sized puncture in the cell membrane that stayed open long enough to allow for enhanced green fluorescent protein (EGFP)-tagged protein to infuse in the cell and later be expressed. The transfection effficiency was investigated using Chinese hamster ovary cells.[11] Femtosecond laser transfection is applied to canina mammary cells MTH53a[12] and stem cells.[13] Kohli *et al.* reported that exogenous material has been delivered into developing zebrafish embryos by femtosecond laser pulses.[14–16]

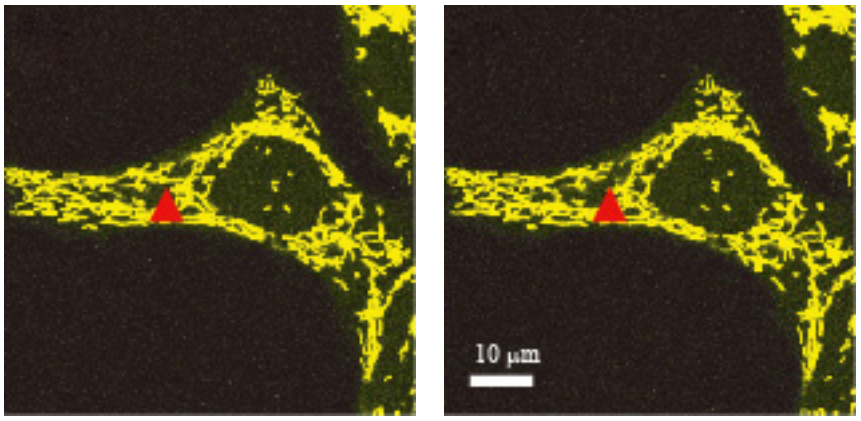
2.3. *Femtosecond nanosurgery of organelles*

Femtosecond laser pulses can be employed for nanosurgery of targeted organelles within a living cell with high spatial resolution.[8] A single organelle (cytoskeleton, mitochondrion etc.) was completely disrupted or dissected without disturbing surface layers and affecting the adjacent organelles or the viability of both plant[17] and animal cells.[2] Several researchers have adopted femtosecond laser oscillators for nanosurgery of organelles, ablation in *Drosophila* embryos to induce modulation of specific movements, and nanosurgery of structures within yeast mitotic spindles.[18–22] Figures 2(a) and (b) show the examples of nanosurgery of a targeted mitochondrion in a HeLa cell before and



(a) (b)

Fig. 1. (a) Dissection of human chromosomes with 800 nm femtosecond laser pulses and (b) nano-ablation of Giemsa-stained chromosomes. Reprint with permission from Ref. 9.



(a) (b)

Fig. 2. (a) Ablation of a single mitochondrion in a living HeLa cell. Target mitochondrion (marked by arrow) (a) before and (b) after laser surgery using high repetition femtosecond laser pulses. The red arrowhead indicates a targeted mitochondrion. Reprint with permission from Ref. 20.

after irradiation with femtosecond laser pulses with a wavelength of 800 nm and a repetition rate of 76 MHz at an energy of 0.39 nJ/pulse. The vitality of the cells after nanosurgery has been ascertained by the exclusion of propidium iodide from the cell as well as by the presence of cytoplasmic streaming.[17,18] In addition, the cell undergoes normal division.[2,17,20] It has been shown that disruption and bleaching are distinguishable using fluorescence recovery and the restaining method.[20]

Amplified femtosecond laser systems with a low repetition rate can also be used for nanosurgery. Mazur *et al.*[23] demonstrated the knocking-out of a single mitochondrion in a living cell without disturbing the rest of the cell on a scale of a few hundred nanometers using a few nJ femtosecond laser pulses at a repetition rate of 1 kHz. Mazur *et al.*[24,25] also demonstrated dissection of individual actin filament in endothelial cells and investigated the tension in actin stress fibers in living endothelial cells (Fig. 3).

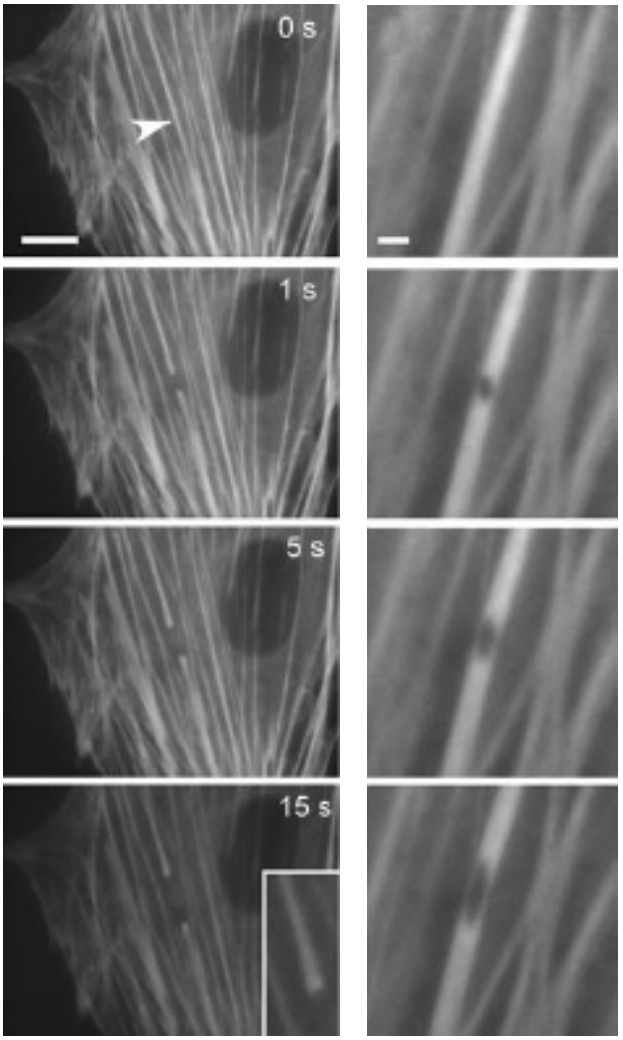
Heisterkamp *et al.*[26] confirmed the difference between bleaching of the fluorophore and disruption in fixed cells after focusing femtosecond laser pulses by transmission electron microscopy. Watanabe *et al.*[27,28] restained the mitochondria with MitoTracker Red after femtosecond laser nanosurgery of a single mitochondrion labeled with enhanced yellow fluorescent protein (EYFP). The mitochondria in the laser-irradiated region were not restained after the addition of MitoTracker Red. The results indicated that the mitochondria were disrupted by the femtosecond laser irradiation. Femtosecond-laser-based nanosurgery of intracellular structures without compromising the viability of cells has potential applications in cell biology.

2.4. *Disconnection of nematode axons in Caenorhabditis elegans*

Another application of femtosecond laser nanosurgery is to dissect neurons within living tissues or animals. Femtosecond laser nanosurgery enables us to directly observe the function of individual neurons of *C. elegans* by severing neuronal fibers (Fig. 4). Yanik and Ben-Yakar severed the axons that control the crawling motion of *C. elegans*.[29] After the laser nanosurgery, the backward crawl of the nematode was greatly hindered. Femtosecond laser nanosurgery can control neural regrowth and investigate the important biochemical and genetic pathways responsible for neuronal regeneration. Most experiments in femtosecond laser axotomy are performed using low-repetition rate femtosecond lasers.[29–31]

2.5. *Mechanisms for nanosurgery*

Femtosecond laser surgery has been demonstrated by the use of both low-repetition-rate (1 kHz–250 kHz) amplified laser systems and high-repetition-rate oscillators ($\sim$80 MHz). Nanosurgery at high-repetition rate is performed in the low-density plasma regime at pulse energies below the optical breakdown threshold. It is mediated by free-electron-induced chemical decomposition (bond breaking) in conjunction with multiphoton-induced chemistry, and hardly related to heating



(a) (b)

Fig. 3. Dissection of stress fibers in living cells by focusing femtosecond laser pulses. (a) Severing and retraction of a single stress fiber bundle in an endothelial cell expressing EYFP-actin. Scalar bar, 10 μm. (b) Strain relaxation of a single stress fiber bundle after a 300-nm hole was ablated in the fiber. The white arrowhead indicates a laser ablation point. Scale bar, 2 μm. Reprint with permission from Ref. 25.

or thermoelastic stresses.[32] When the energy is raised, accumulative heating occurs. The energy was lower than that achieved by the amplified laser, and therefore, many pulses are applied. By contrast, nanosurgery at low-repetition rate is performed using more than 10-fold larger pulse energies and relies on thermoelastically induced formation of minute transient cavities with lifetimes <100 ns.[32] For example, Watanabe *et al.* investigated intracellular nanosurgery of a single mitochondrion by femtosecond lasers with both low- and high-repetition rates. They performed nanosurgery of a mitochondrion using 250 pulses at an energy of 3 nJ/ pulse from a 1-kHz Ti: sapphire amplifier.[27,28] With a Ti: sapphire oscillator at a repetition rate of 76 MHz, intracellular nanosurgery of a mitochondrion was performed using 2.4×10^6 pulses at an energy of 0.39 nJ/ pulse.[20] The energy was lower than that achieved by the amplified laser, and therefore, many pulses were necessary at a high-repetition rate.

3. Femtosecond Laser Photoconversion and Photoactivation

In combination with fluorescent proteins, the applications of manipulation of sub-cellular structures using multiphoton excitation include photoconversion or photoactivation. The intracellular structures are not directly ablated and the fluorescent properties are altered at a focal volume. Photoactivation and photoconversion enables selective conversion or activation of fluorescence signals after optical irradiation.[33–35] Most photoactivation and photoconversion processes via one-photon excitation require light in the UV region, which causes several problems. In order to overcome these problems, there have been some reports describing the use of femtosecond laser pulses to attempt two-photon excitation of photoactivatable fluorescent proteins. Problems such as low contrast and low spatial resolution were encountered.[36–39] Here, we show spatially selective labeling of a single mitochondrion by using two-photon conversion of Kaede, and tracking the dynamics of mitochondria.[40,41]

3.1. *Labeling of an organelle by femtosecond laser pulses*

Watanabe *et al.*[40] showed spatially selective labeling of a single mitochondrion by using two-photon conversion of Kaede, and tracking the dynamics of mitochondria. Tobacco BY-2 cells whose mitochondria were labeled with photoconvertible fluorescent proteins, Kaede, was used. In order to alter fluorescence spectrum, femtosecond laser pulses with a wavelength of 750 nm and a repetition rate of 76 MHz were focused at the targeted mitochondrion indicated by the arrow through an oil-immersion objective lens (NA 1.4). After photoconversion of the mitochondrion, time-lapse images of the green and red fluorescence using a one-photon fluorescence microscope were obtained (Fig. 5). A volume of the mitochondrion of approximately 1 femtoliter ($= 1.0\,\mu\text{m} \times \pi \times (0.5\,\mu\text{m})^2$) was photoconverted

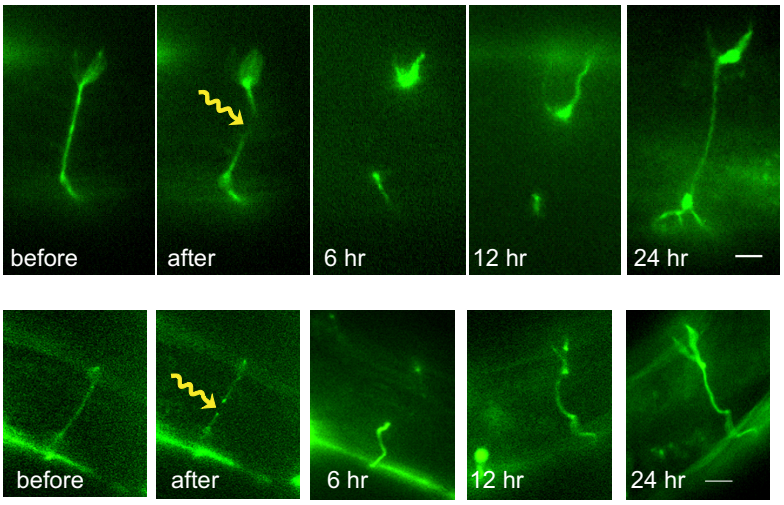


Fig. 4. Femtosecond laser axotomy in *Caenorhabditis elegans* worms using femtosecond laser pulses. Fluorescence images of axons labeled with green fluorescent protein before, after. The yellow arrows indicate laser ablation points. Scale bar, 5 μm. Courtesy of Yanik, M. F.

around the focal point. Two-photon photoconversion by the femtosecond laser irradiation enables us to perform site-specific labeling of a single organelle in 3-dimensional space.

Labeling technique can be used to monitor the dynamic behavior of an organelle. By observing time-lapse photoconverted red fluorescence of Kaede after photoconversion allowed tracking of the movement of the target mitochondrion. The technique has potential application in direct tracking of selective cellular and intracellular structures.

4. Outlook and Conclusions

We have reviewed recent advances in the manipulation of intracellular organelles using femtosecond

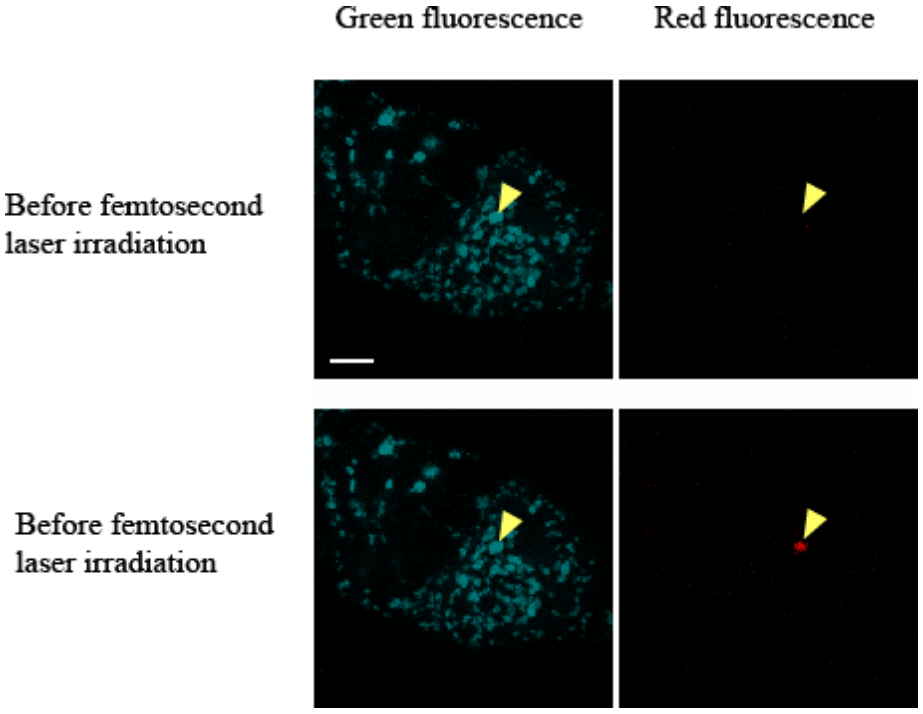


Fig. 5. Selective photoconversion of mitochondria with Kaede in a fixed BY-2 cell. A single mitochondrion was photoconverted from green to red by 750-nm femtosecond laser pulses. A target mitochondrion is indicated by yellow arrowheads. Scale bar: 10 μm. Reprint with permission from Ref. 40.

laser pulses. The manipulation techniques encompass site-specific photoconversion and nanosurgery of intracellular organelles. Femtosecond lasers can manipulate targeted cells and intracellular objects without using genetic methods or chemical agents used in conventional molecular and biochemical techniques.

The application of sub-20 fs has been used for nanosurgery of living cells.[13,16,42,43] König *et al.*[13] have demonstrated the efficient optical transfection of stem cells by sub-20 fs laser microscopy by dispersive-mirror compressors. The management of pulse duration and pulse shape will be an important topic for precise nanosurgery.

Femtosecond lasers can be used to stimulate chemical reactions[44] in a target cell without using chemical agents. In addition, chromophore-assisted laser inactivation (CALI) using multiphoton excitation confirmed to be an effective chromophore for inactivation of a protein's function without nonspecific photodamage in living mammalian cells.[45]

The next-generation femtosecond nanosurgery is plasmonic nanosurgery, which performs nanosurgery with higher resolution. Local field enhancement in the near-field of metal nanoparticles irradiated with laser pulses is a promising technique.[46–48] Femtosecond laser nanosurgery becomes a versatile tool for regeneration study in combination of microfluidic chips.[49,50] Yanik demonstrated a method for non-invasive and high-throughput on-chip immobilization of physiologically active C. elegans with a femtosecond laser oscillator.[51]

Femtosecond lasers can also be used to modify the structure of biological tissues. There have been reports on the applications of femtosecond laser nanosurgery to ocular refractive surgery[52–54] and vessels in living animals.[55] Femtosecond laser manipulation can be employed as a highly precise manipulating and nanosurgical tool for tissues, cells, and intracellular compartments without collateral damage, and will be a versatile and feasible tool in cell biology and clinical applications.

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