Femtosecond laser disruption of subcellular organelles in a living cell

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Subcellular organelles in living cells were inactivated by tightly focusing femtosecond laser pulses inside the cells. Photodisruption of a mitochondrion in living cells was experimentally confirmed by stacking three-dimensional confocal images and by restaining of organelles. The viability of the cells after femtosecond laser irradiation was ascertained by impermeability of propidium iodide as well as by the presence of cytoplasmic streaming.

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1. Introduction

Studying intact organelles and their function in a living cell is essential to understand cell dynamics [1]. It is difficult to directly reveal the characteristics of organelles *in vivo* by conventional molecular and biochemical techniques; optical methods, however, can manipulate or modify the behavior of intact organelles in living cells. A focused continuous-wave or long-pulse ultraviolet (UV) and visible laser beam has been used for microsurgery of organelles. Dissection and inactivation of subcellular organelles in plant [2] and animal cells [3-6] were demonstrated with submicron spatial resolution by laser-induced plasma formation [6]. However, continuous-wave or long-pulse lasers in UV and visible regions have some disadvantages, namely, low light penetration depth, collateral damage outside the focal volume, and the possibility of photodamage to living cells due to absorption. In addition, UV radiation is also known to be responsible for oxidative stress leading to apoptosis.

Femtosecond (fs) lasers operating in the near infrared (NIR) region have attractive advantages compared with conventional UV lasers, such as efficient two-photon ionization, no out-of-focus absorption, and the absence of significant transfer of heat or mechanical energy to surrounding structures due to nonlinear optical effects around the focal volume. A two-photon microscope using NIR fs laser pulses was developed to observe subcellular structures [7]. The high penetration depth of the NIR region provides the possibility of optical sectioning in deep tissue. Due to the lack of out-of-focus photodamage and photobleaching,

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two-photon NIR microscopy appears to be a safe tool having no adverse effects on cellular metabolism, reproduction, and viability [8]. However, above a certain threshold energy, photodamage occurs [9-12]. König *et al.* found two types of photodamage when non-labeled cells were irradiated by scanning of NIR fs laser pulses operated at a high-repetition-rate (~80 MHz) over the entire area of each cell [13]. One is a slow process probably based on two-photon excitation of endogenous absorbers, for example, by the coenzymes NAD(P)H and flavins as well as by porphyrins; excitation of these endogenous photosensitizers can result in the formation of destructive reactive oxygen species (ROS). It was reported that mitochondria and the Golgi apparatus were the major targets of NIR laser radiation [14] and the generation of ROS induced apoptosis-like death [15]. The second damage process is of immediate effect and requires high intensities; it is based on multiphoton ionization, optical breakdown phenomena, and intracellular plasma formation. Laser-exposed cells either fail to divide, become giant cells, or die through cell fragmentation. In addition, the irradiated cells manifest membrane-barrier dysfunction, drastic alterations in the morphology of the nuclear envelope, and DNA fragmentation. It should be noted that the photodamage described above was induced when a number of laser pulses were launched into nonlabeled cells by scanning of fs laser pulses over the entire area of each cell.

One of the salient features of focusing fs laser pulses is the limited interaction region in three-dimensional space. When fs laser pulses are tightly focused, the intensity in the focal volume can become high enough to cause nonlinear absorption, through multiphoton, tunnel and avalanche ionization process, resulting in optical breakdown and the formation of a high-density plasma. This hot plasma results in a permanently damaged region in the cell with sub-micron size. König *et al.* proposed a novel nanosurgery tool with NIR fs lasers to perform dissection of chromosomes [16] and DNA transfection [17]. NIR laser-based nanoprocessing of cellular structures without compromising the viability of cells has potential applications in cell biology because one can control functional interactions between organelles and inter-cell communication only at targeted locations. A single organelle was completely knocked out without disturbing surface layers and affecting the adjacent organelles or the viability of both plant cells [18] and animal cells [13]. Recently Mazur *et al.* investigated the threshold energy for plasma formation in water and thermal diffusion in terms of the numerical aperture (NA) of the focusing objectives [19,20]. They also demonstrated knocking out of individual mitochondria in a living cell without disturbing the rest of the cell on a scale of a few hundred nanometers using tightly focused NIR fs laser pulses at a low repetition rate (1 kHz) [19,20]. NIR laser-based nanoprocessing of cellular structures without compromising the viability of cells has potential applications in cell biology because one can control functional interactions between organelles and inter-cell communication only at targeted locations. After focusing fs laser pulses, fluorescence from the target organelles disappears; however, the question remains whether it is due to photodisruption, bleaching of the fluorescence, or just movement of organelles. In the case of photodisruption, the targeted organelles are completely disrupted and become dysfunctional permanently. To the best of our knowledge, there is no direct evidence for photodisruption of organelles in living cells by fs laser irradiation. In this paper, we demonstrate the experimental confirmation of photodisruption of subcellular organelles in a living cell by tightly focusing fs laser pulses inside the cell. Photodisruption of organelles in living cells was confirmed by restaining of organelles after irradiation with fs laser pulses. We also acquired three-dimensional confocal image stacks to determine if organelles were moved out of the focus by fs laser irradiation. Impermeability of propidium iodide (PI) and the presence of cytoplasmic streaming suggested that the cells are alive after fs laser irradiation.

2. Femtosecond laser photodisruption

2.1 Experimental setup

Figure 1 shows a schematic diagram of the setup used for femtosecond laser photodisruption of subcellular organelles in living cells. To visualize organelles in a living cell, a confocal laser scanning microscope was used. The laser scanning microscope was adapted from an

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Olympus FV300 scanning unit combined with an Olympus IX71 inverted microscope. A collimated laser beam of a He-Ne laser (wavelength 543 nm, Melles Griot, 05-LGR-171) or an Ar-ion laser (wavelength 488 nm, Melles Griot, IMA101-010-BOS) was reflected by DM1 and DM2 in the FV300 and expanded by a pupil transform lens and a microscope tube lens so that it matched the back aperture of a high NA objective lens (OB), which focused the laser beam, in the microscope IX71. The objective lens was a water-immersion objective (Olympus Corporation, UplanApo60×W/IR, NA 1.2) whose chromatic aberration was optimized for the visible and NIR region for short-term observation, or an oil-immersion objective (Olympus Corporation, PlanApo60×O2, NA 1.4) for long-term observation. Scanning of the focused laser beams in the *xy* plane was accomplished by a pair of high-speed galvanometer mirrors (Cambridge, 6210) inside the FV300. The back-propagated one-photon fluorescence was collected using the same objective and epi-detected with photomultiplier tubes (PMT1 and PMT2) (Hamamatsu Photonics, R928P) to record the fluorescence intensities point by point to form two-dimensional confocal cross-sectional images at a certain depth inside the cell. Before PMT1 and PMT2, bandpass filters BP1 (transmission wavelength; 510 nm - 640 nm) and BP2 (transmission wavelength; above 585 nm for MitoTracker Red, 560 nm - 600 nm for PI) were placed. The depth scanning was achieved by moving the objective with a stepping motor to obtain three-dimensional confocal images.

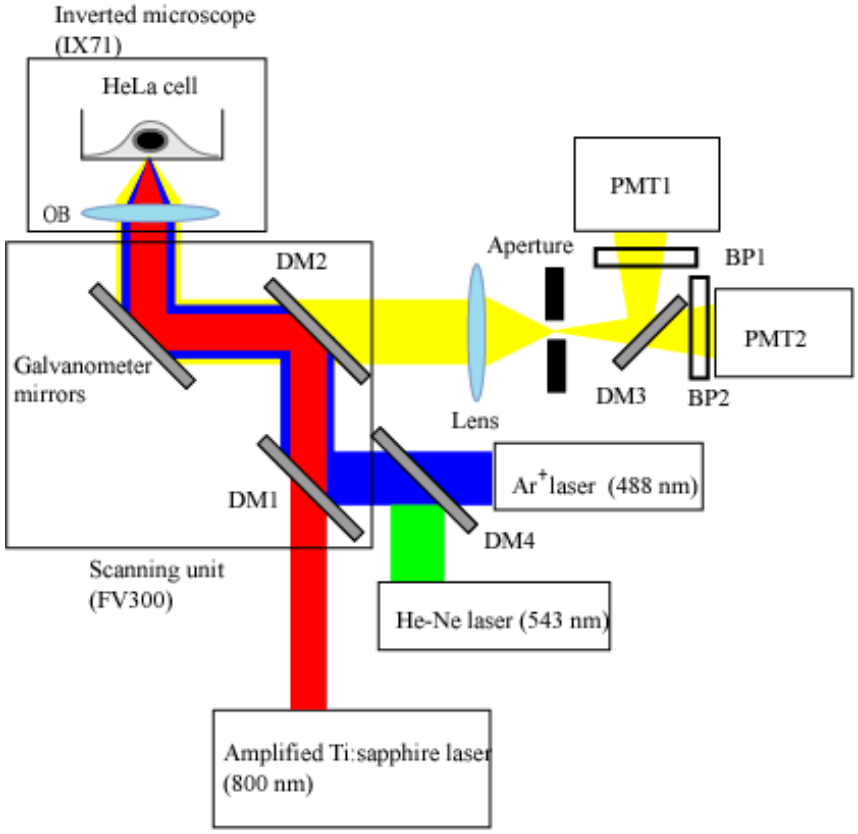

Fig. 1. Schematic of the experimental setup. OB, DM, BP, and PMT denote objective lens, dichroic mirror, bandpass filter, and photomulutiplier tube, respectively.

Irradiation with femtosecond laser pulses was performed using a regeneratively amplified Ti:sapphire laser system which produced 150-fs, 800-nm, 1-kHz pulses (Spectra Physics, Spitfire). The beam diameter was approximately 5 mm. After the beam was reduced to 2 mm in diameter with a beam expander, it was directed into the FV300 scanning system through DM1 and DM2 to fill the back aperture of the OB. The laser pulses were focused at the

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desired location of an organelle inside the cell with a spot size close to the diffraction limit using OB. The working distance of the objective lens (150 μm) allowed subcellular photodisruption. The pulse energy was reduced by inserting neutral density filters. The number of pulses supplied was selected by an electro-magnetic shutter (Sigma Koki, Σ-65L).

2.2 Experimental protocols

Human carcinoma cell line HeLa cells were cultured and harvested as previously reported [21]. Target organelles in the experiments were mitochondria in the HeLa cells. Among organelles, mitochondria in particular are essential for cell viability because they function as the powerhouse for production of energy in cells. Mitochondria of the HeLa cells were stained with 2.5 ng/ml MitoTracker Red (chloramethyl-X-rosamine, Molecular Probes), which is a mitochondria-selective dye, for 30 minutes at room temperature. To determine the effects on cell viability after fs-laser irradiation, 1 μg/ml PI (Sigma) was added to the medium 30 minutes after the irradiation. Fusion constructs containing a gene encoded an enhanced yellow fluorescent protein (EYFP) and the mitochondrial targeting sequence from human cytochrome C oxidase (pEYFP-Mito; Clonetech) were transfected into the HeLa cells using the calcium phosphate method [22].

2.3 Methods

First, one-photon fluorescence images of HeLa cells were obtained by excitation with cw He-Ne laser or Ar^+ laser. The recording speed per frame with 512×512-scanned points was less than 1.7 seconds. Next, we set the focal point of the objective lens to a target organelle inside the HeLa cells. By opening the shutter, fs laser pulses were focused into the target. Finally, after fs laser irradiation, one-photon fluorescence images of the HeLa cells were obtained by excitation with the cw He-Ne laser or Ar^+ laser. The time duration necessary to obtain two confocal images, that is, before and after fs laser irradiation, was 40 seconds.

3. Experiments

3.1 Laser damage to living cells

The viability of the cells after fs laser radiation was ascertained by using PI. PI is impermeant to the membranes of living cells, whereas it only penetrate dead or apoptotic cells with compromised membranes. Mitochondria of the HeLa cells were stained with MitoTracker Red. Before fs laser irradiation, one-photon fluorescence images of individual cells were observed by excitation with the He-Ne laser, as shown in Fig. 2(a). The fluorescence appears red under green illumination with a He-Ne laser at a wavelength of 543 nm. The fs laser pulses were focused at the same depth inside cells α and β indicated by the arrows in Fig. 2(a) through the 1.2-NA objective at energies of 7 nJ/pulse and 3 nJ/pulse, respectively. The exposure time was one-quarter of a second and the number of laser pulses was two hundred fifty. Just after irradiation of fs laser pulses, PI was added to the culture. The fluorescence images were observed 30 minutes after addition of the PI. Figure 2(b) shows the confocal image of the cells after fs laser irradiation and the addition of PI. Figure 2(b) shows that, at the energy of 7 nJ/pulse, additional red fluorescence derived from PI was observed in the nuclei and the cytoplasm of the cell α because the plasma membrane was distorted or destroyed and then PI penetrated the cell. Above the energy of 5 nJ/pulse, the morphological changes in mitochondria were observed and cytoplasmic streaming in the cells was not observed. However, the nuclei of the cell β irradiated at the energy of 3 nJ/pulse were not stained with PI from the surrounding medium. The experiments show that the cells remain viable after irradiation of laser pulses below the energy between 2 nJ/pulse and 4 nJ/pulse even when 250 laser pulses were focused at different fifty cells through the 1.2-NA water-immersion objective. The energy was dependent on cell activity. Furthermore, we did not observe any apoptotic phenotypes in the cell β 24 hours after irradiation. Fluorescence images and

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transmission images after fs laser irradiation revealed virtually no adverse effects on the pattern of cytoplasmic streaming in the cells.

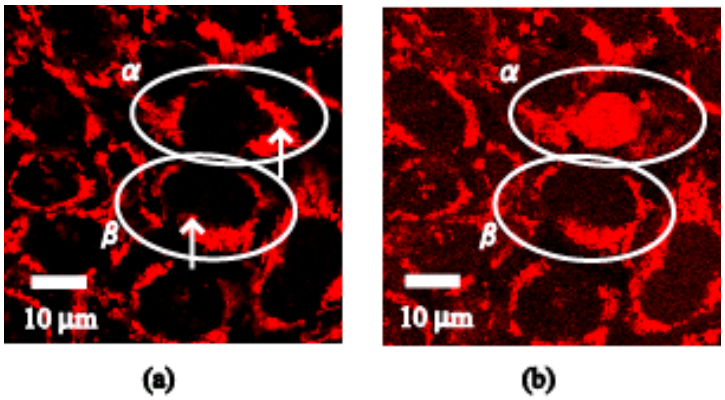

Fig. 2. Confocal images of the cells (a) before and (b) after laser irradiation. Red fluorescence shows mitochondria of HeLa cells stained with MitoTracker Red. The white circles and arrows indicate individual HeLa cells and target mitochondria, respectively. Additional red fluorescence in Fig. 2b is derived from propidium iodide (PI). The laser pulses were focused inside cells α and β at energies of 7 nJ/pulse and 3 nJ /pulse, respectively.

3.2 Photodisruption of mitochondria in living cells

We show the disruption of individual mitochondria in living HeLa cells. Mitochondria in the HeLa cells were stained with MitoTracker Red. Figures 3(a) and (b) show the confocal images of HeLa cells obtained before and after fs laser irradiation. Figures 3(c) and (d) show magnified views of the square areas indicated in Figs. 3(a) and (b), respectively. Femtosecond laser pulses at an energy of 3 nJ/pulse were focused with the water-immersion objective at a mitochondrion indicated by the arrow in Fig. 3(a), or at the center of the circle in Fig. 3(c). Figure 3 shows that fluorescence from a single mitochondrion within a size of 1 μm disappeared. Neighboring mitochondria were not photodisrupted or photobleached even though they were only a few micrometers away. The positional difference of the mitochondria outside the focal region between the before and after images was attributed to cytoplasmic streaming, which indicated the viability of the cells.

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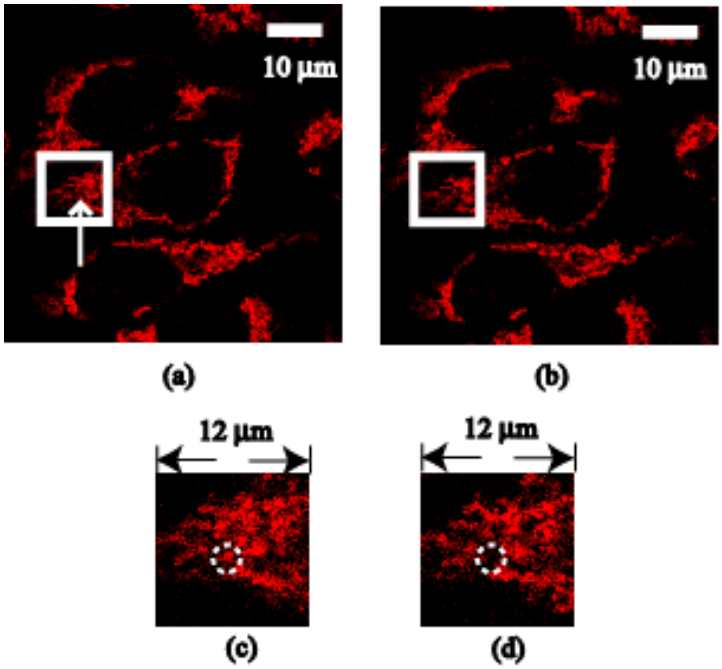

Fig. 3 Confocal images of HeLa cell before and after fs-laser irradiation. (a) and (b) confocal images of the HeLa cell before and after femtosecond laser irradiation. Red fluorescence shows mitochondria of HeLa cells stained with MitoTracker Red. A target mitochondrion is indicated by a white arrow. (c) and (d) magnified views of square areas indicated in (a) and (b), respectively. The center of the dotted circles show target mitochondria.

If the mitochondria move away from the focal volume, the movement should be observable in three-dimensional confocal images. Figures 4 (a) and (b) show stacked three-dimensional confocal images at different depths before and after fs laser irradiation. Twenty-one images were obtained by translation of the objective lens along the optical axis by 6 μm in steps of 300 nm. Figures 4(c) and (d) show magnified views of the square areas indicated in Figs. 4(a) and (b), respectively. The figure shows that the mitochondria did not move in the depth direction. Any fluorescence recovery was not observed after fs laser irradiation.

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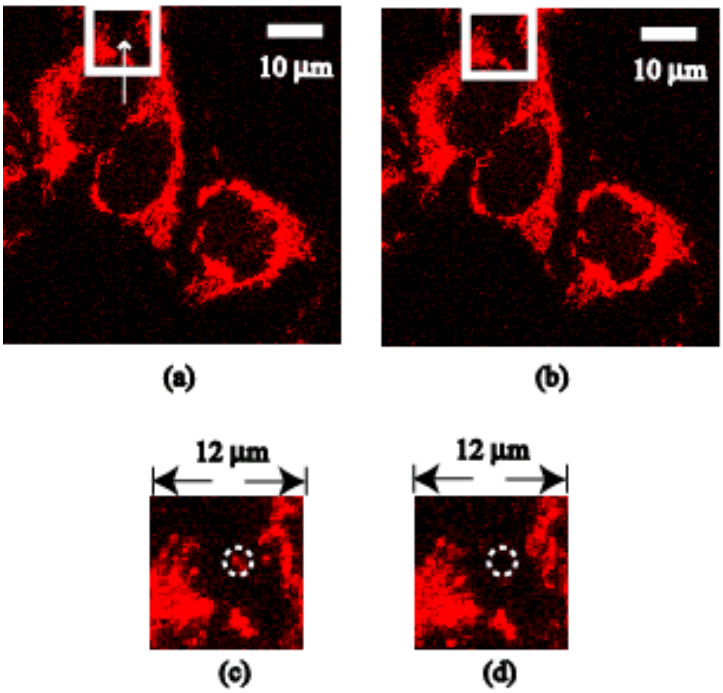

Fig. 4. (a) and (b) stacked three-dimensional confocal images at different depths before and after femtosecond laser irradiation. Red fluorescence shows mitochondria of HeLa cells stained with MitoTracker Red. A target mitochondrion is indicated by a white arrow. Twenty-one images were obtained by translation of the objective lens by 6 µm in steps of 300 nm. (c) and (d) magnified views of square areas indicated in (a) and (b), respectively. The center of the dotted circles show target mitochondria.

3.3 Results after restaining

We performed an experiment on restaining of the cells, to investigate whether the absence of fluorescence intensity was due to disruption or due to bleaching of the fluorescence. The HeLa cells were incubated in DMEM without phenol red at 37 ^{o}C and 100% air humidity in a microscope incubator (Tokken, TK-INCO$_2$). In this experiment, the cells were transfected with an EYFP fused with a mitochodria-targeted sequence of cytochrome C oxidase. One-photon fluorescence images of cells were observed by excitation with the Ar^+ laser at a wavelength of 488 nm, as shown in Fig. 5(a). Figure 5(c) shows a magnified view of the square area indicated in Fig. 5(a). Femtosecond laser pulses at an energy of 3 nJ/pulse were focused with the oil-immersion objective (NA, 1.4) used for long-term experiments. The energy for disruption was between 2 nJ/pulse and 4 nJ/pulse, which was dependent cell activity, with the 1.4-NA objective from the experiments on different fifty cells and was almost the same as that with the 1.2-NA water-immersion objective. Figure 5(b) shows the confocal image obtained after fs laser irradiation and Fig. 5(d) shows a magnified view of the square area indicated in Fig. 5(b). No fluorescence from the surrounding mitochondria in a region a few microns wide from an irradiated mitochondrion was observed. We restained the mitochondria with MitoTracker Red after fs laser irradiation. If the mitochondria were not disrupted, the remaining mitochondria would be detected with MitoTracker Red within the irradiation area. MitoTracker Red was added to the medium immediately after fs laser irradiation. Because mitochondria were normally stained 30 minutes after addition of MitoTracker Red, confocal images obtained 35 minutes after addition of MitoTracker Red are shown in Fig. 5(e) and (f). Figure 5(e) shows the confocal image obtained by excitation with

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the Ar^+ laser, whereas Fig. 5(f) shows the confocal image obtained by excitation with the He-Ne laser. The figures show that the mitochondria in the laser-irradiated region were not restained after addition of MitoTracker Red. In contrast, after the YFP fluorescence in mitochondria was photobleached using the single photon source (Ar^+ laser with the wavelength of 488 nm), the mitochondria were restained with MitoTracker Red (data not shown). However, the YFP fluorescence in mitochondria focused by fs laser pulses was not restained with MitoTracker Red. The results clearly indicated that the mitochondria were disrupted by the fs laser irradiation.

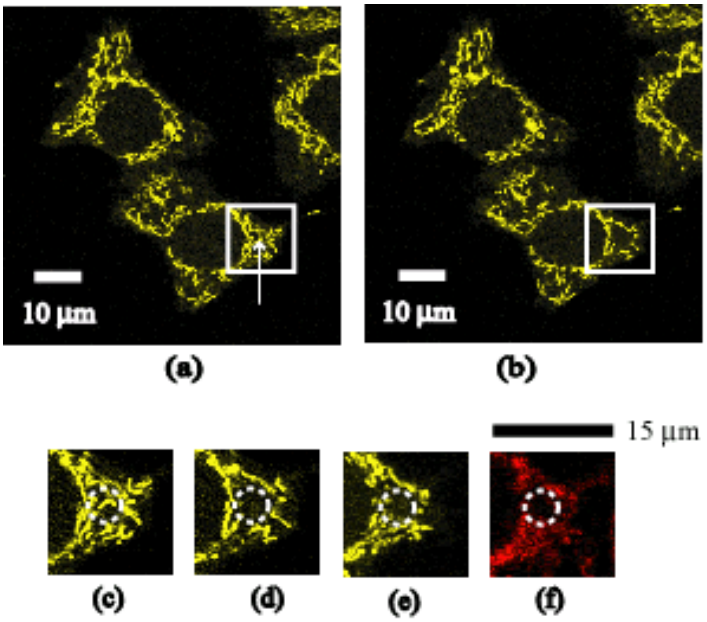

Fig. 5. (a) Confocal images of laser-irradiated cells before and after restaining. Yellow fluorescence shows mitochondria visualized by EYFP. Target mitochondrion is indicated by a white arrow. (c) magnified view of square area indicated in (a) before femtosecond laser irradiation. (b) confocal image obtained after femtosecond laser irradiation. (d) magnified view of square area in (b). (e) and (f) confocal images obtained after restaining by MitoTracker Red. (e) confocal image obtained by excitation with the Ar^+ laser. (f) confocal image obtained by excitation with the He-Ne laser. Dotted circles show target mitochondria.

We investigated the behavior of other organelles when a single mitochondrion was disrupted. In this experiment, mitochondria in the HeLa cells were stained with MitoTracker Red and microtubules were visualized by EYFP with α-tubulin. The fluorescence intensities from microtubule did not vary before and after fs laser irradiation although a single mitochondrion was disrupted. The experiment shows that morphology of the cell remains same before and after photodisruption of a mitochondrion. In addition, fluorescence images and transmission images after fs laser irradiation revealed that the irradiated cell was alive by the presence of cytoplasmic streaming in the cells.

4. Discussion

Subcellular organelles in living cells were disrupted by tightly focusing NIR fs laser pulses, however, the physical mechanisms of the photodisruption have not been elucidated. A possible mechanism is explained similar to laser-induced plasma formation in fs laser ablation [23-25]. When intense fs laser pulses are tightly focused at the targeted organelles inside a cell, the intensity in the focal volume can become high enough to cause nonlinear absorption, through multiphoton, tunneling and avalanche ionization processes. The high intensity of the

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fs laser pulse promotes electrons from the valence band to the conduction band via multiphoton ionization, and these free electrons act as the seed for avalanche ionization, which exponentially increases the free carrier density. The free electrons absorb energy from the electromagnetic field of the laser pulse, leading to laser-induced optical breakdown, and the generation of high-density plasma [23,24]. Recently it has been demonstrated that multiphoton ionization does not play a significant role in optical damage and that the dominant optical damage mechanism is tunneling ionization and tunneling-seeded avalanche ionization in fs laser ablation. The high peak intensities achieved by fs laser pulses allow the pulse to generate its own seed electrons by the tunneling mechanism [25]. The optical field of the fs laser pulse leads to tunneling by electrons from the valance band to the continuum to form an electron plasma. The density of the plasma increases due to additional tunneling and avalanche ionization. In both cases, the increase in electron density leads to absorption of the fs laser pulse and the high-density plasma expands with hypersonic velocity that drives a shockwave [26-29]. The shockwave plays a role in photodisruption of organelles [19,20]. There is still room for investigation as to whether multiphoton ionization or tunneling ionization plays an important role for the first stage of the increasing electron density. In our experiments, the targeted organelle is labeled by exogenous fluorescent dye or protein for visualization. Two-photon absorption by fluorescent labels contributes to plasma formation as a seed for avalanche ionization because of the higher two-photon absorption cross-section at the wavelength of 800 nm, compared to those of endogenous absorbers in nonlabeled cells or in water. The disrupted region in organelles was smaller than a few microns and the threshold energy was 3 nJ/pulse for 1.4-NA and 1.2-NA objectives. The threshold energy was the same order as that previously reported [19,20].

Finally we discuss the application of fs laser photodisruption. Currently lower-intensity NIR fs laser pulses are used to cause photobleaching of fluorescence from subcellular organelles. Fluorescence recovery after photobleaching (FRAP) is the recovery of fluorescence in a defined region of a sample after a bleaching event [30]. FRAP results from the movement of unbleached fluorophores from the surrounding areas into the bleached area. Fluorescence loss in photobleaching (FLIP) is the decrease or disappearance of fluorescence in a defined region adjacent to a repetitively bleached region [31]. Both FRAP and FLIP can be used to define the dynamics of two-dimensional or three-dimensional mobility of fluorescence molecules in membranes or the cytoplasm of living cells. However, unlike FRAP and FLIP, fs laser photodisruption does not cause photobleaching but disruption – removal of organelles. Focusing fs laser pulses can destroy targeted organelles with order of micrometers to sub-micrometer and bring their dysfunction. For instance, when fs laser pulses were focused to some mitochondria, we can analyze whether the removal of some mitochondria give influence to the activity of respiration. In the case of disruption or dissection of cytoskeletons including microtubules and actin filaments, the detail mechanisms of cell division might be obtained. Such analyses by the disruption of organelles using fs laser pulses will give us novel information on function and dynamics of organelles in living cells.

5. Conclusion

Photodisruption of organelles in living cells with focused fs laser pulses was experimentally confirmed by restaining organelles after irradiation by focused fs laser pulses, and by stacking three-dimensional confocal images. The viability of the cells after fs laser irradiation was verified by impermeability of PI into the cells as well as by the presence of cytoplasmic streaming. This fs laser disruption technique has possible applications in the removal of specific organelles in living cells and may provide information on function and dynamics of organelles in living cells and lead to the elucidation of functional interactions between organelles and inter-cell communication.

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