Nanosurgery in live cells using ultrashort laser pulses

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ABSTRACT

We selectively disrupted the cytoskeletal network of fixed and live bovine capillary endothelial cell using ultrashort laser pulses. We image the microtubules in the cytoskeleton of the cultured cells using green fluorescent protein. The cells are placed on a custom-built inverted fluorescence microscope setup, using a 1.4 NA oil-immersion objective to both image the cell and focus the laser radiation into the cell samples. The laser delivers 100-fs laser pulses centered at 800 nm at a repetition rate of 1 kHz; the typical energy delivered at the sample is 1–5nJ. The fluorescent image of the cell is captured with a CCD-camera at one frame per second.

To determine the spatial discrimination of the laser cutting we ablated microtubules and actin fibers in fixed cells. At pulse energies below 2 nJ we obtain an ablation size of 200 nm. This low pulse energy and high spatial discrimination enable the application of this technique to live cells. We severed a single microtubule inside the live cells without affecting the cell's viability. The targeted microtubule snaps and depolymerizes after the cutting. This nanosurgery technique will further the understanding and modeling of stress and compression in the cytoskeletal network of live cells.

Keywords: Ultrashort laser pulses, cell surgery, biology, ablation, photodisruption, microtubule, actin

INTRODUCTION

Ultrashort laser pulses are versatile tools for micromachining and precise ablation. However, their first application in biology was primarily in imaging.[1,2,3] Nowadays, they are increasingly used to micromanipulate and ablate nanoscale structures in living cells and other biological materials.[4,5,6] When femtosecond laser pulses are confined to a very small focal volume by focusing with high numerical aperture objectives (NA>1), multiphoton absorption can be induced at the laser focus, even in normally transparent materials. This process generates very high concentrations of free electrons in the focal volume, resulting in ablation of material.[7] As the wavelength of an ultrashort laser is typically in the near infrared, for example centered around 800 nm in our case, high penetration depth into tissues is possible. The amount of laser radiation which is aborbed in regions otuside the focus is negligible. The nonlinear nature of the optical absorption makes it possible to treat any transparent sample, regardless of its linear absorption coefficient as the multiphoton absorption is limited to the focus region. With a pulse duration of about 100 fs, only a few nanojoules of energy are necessary to achieve ablation.[4,6] As possibly harmful effects like cavitation and heat deposition scale with the amount of applied laser energies, ultrashort laser applications offers a way to manipulise live cells with very low side effects, the low energy minimizes collateral damage in the vicinity of the laser focus and reduces the likelihood that the cell will be injured or killed .

In several studies, especially with glasses and different metals, the dependence of material ablation on pulse energy has been studied,[8] but no comprehensive studies of the this relationship have been undertaken in biological samples.The

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ablation of isolated human chromosomes has been studied using atomic force microscopy (AFM) and fluorescence microscopy,[9] but neither technique is a good measure of the removal of material from the bulk of the sample. AFM examines only the surface of the sample and fluorescence microscopy cannot discriminate between photobleaching and material ablation. Because irradiation can alter the staining properties of the sample, restaining[6] also does not allow differentiating between photo-induced chemical changes and removal of material. A recent transmission electron microscopy (TEM) study confirms the cutting of microtubules using picosecond laser pulses at 532 nm at pulse energies of only a few nanojoules, but noticed a considerable amount of photobleaching effects.[10] However, the boundaries of photobleaching and material ablation have not been established quantitavely, especially for low-energy femtosecond pulses.

To address this issue, we studied the effects of femtosecond laser irradiation in fluorescently-labeled structures in the cytoskeleton and nuclei of fixed cells using a combination of fluorescence microscopy and whole mount TEM. We used fluorescence microscopy to evaluate the extent of apparent photodamage, and TEM to determine the actual degree of material removal. Once we found the optimum set of parameters to achieve highest cutting resolution at lowest energies, we applied this technique to live cells.

EXPERIMENTAL METHODS

The laser radiation is generated in a custom-built chirped-pulse amplified titanium-sapphire laser system. A passively mode-locked oscillator delivers 100-fs pulses at a repetition rate of 80 MHz and a central wavelength of 790 nm. These pulses are regeneratively amplified to energies of up to 1 mJ. As these energies are far too high for subcellular ablation, we reduce the pulse energy to the nanojoule range with an attenuator. An adjustable neutral density

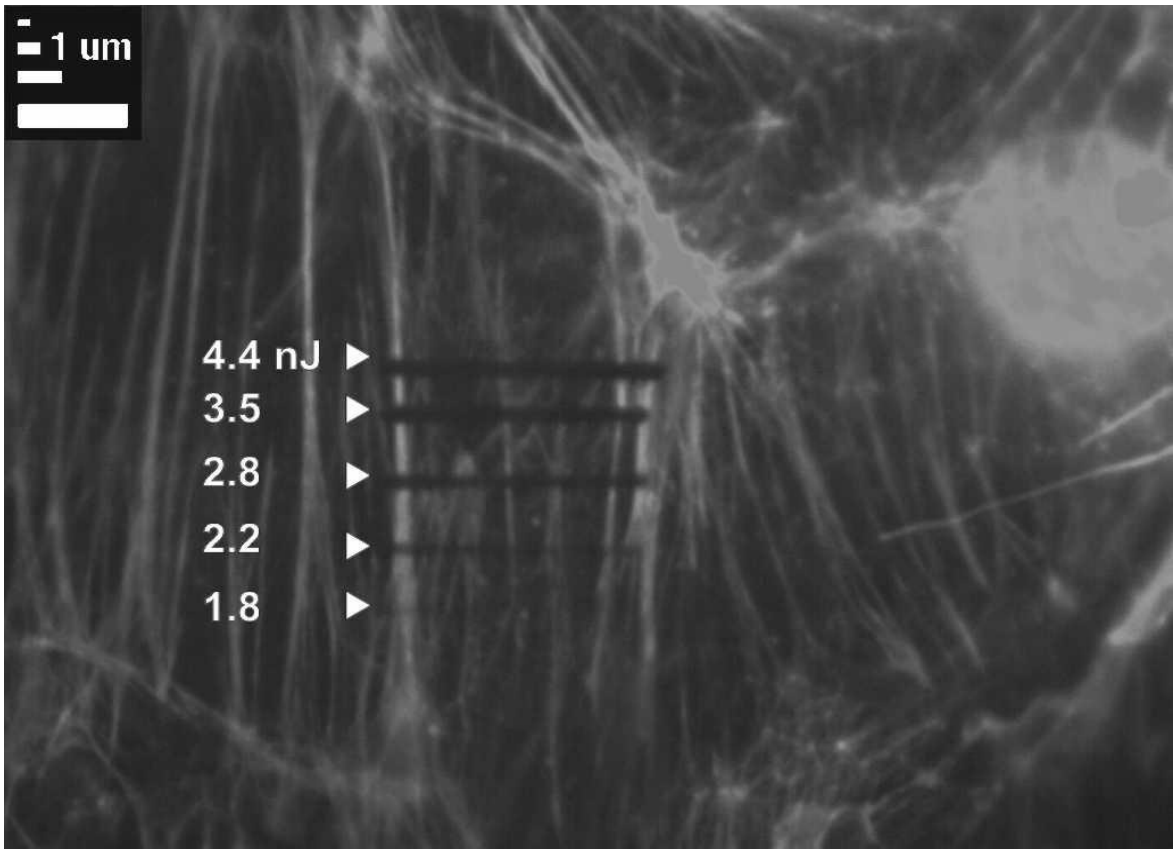

Fig. 1 Cuts through fluorescently-labeled actin fibers in a fixed endothelial cell obtained by irradiation with femtosecond laser pulses of energies between 1.8 nJ and 4.4 nJ[16].

filter wheel is used to regulate the energy at the sample. The laser light is focused into the sample with a 1.4-NA oil-immersion objective. The sample is placed on a piezo-controlled microscope stage that permits sample positioning with 10-nm precision along all three axes. A UV lamp illuminates the sample and the emitted fluorescence is collected through the objective using a standard filter cube and recorded with a CCD camera.

Bovine capillary endothelial cells (passage 1015) were maintained at 37 °C in 10% CO_2 on tissue culture dishes in a complete medium composed of low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal calf serum (FCS) (Hyclone), 10 mM HEPES (JRH-Biosciences), and glutamine (0.292 mg/ml)/penicillin (100 U/ml)/streptomycin (100 g/ml) as previously described.[11] For experiments, cells were then trypsinized (Trypsin EDTA, Gibco), harvested, and seeded either onto glass-bottomed 35 mm dishes (MatTek) or onto carbon- and formvar-coated Embra TEM finder grids (Electron Microscopy Sciences) in complete medium. After allowing the cells to attach and spread for 12–24 hours, the cells were fixed in 4% formaldehyde (Electron Microscopy Sciences) in phosphate buffered saline (PBS) for 40 minutes, permeabilized in 0.1% Triton X-100 in PBS for 5 minutes, blocked in 1% bovine serum albumin in PBS for 1 hour, and stained for either actin (Alexa Fluor 488 phalloidin, Molecular Probes) or nuclear DNA (Hoechst 33348, Molecular Probes), all at room temperature. Following laser treatment, cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4 °C for one hour and then washed and stored in this buffer at 4 °C until processing. Prior to TEM imaging with a Philips CM-10 microscope, cells were fixed in 1.5% osmium tetroxide in 0.1-M cacodylate buffer, pH 7.4, at 4 °C for 30 minutes, washed in the same buffer, dehydrated in graded ethanol solutions, critical point dried, and carbon coated.

For live cell experiments, endothelial cells were transfected for 48 h with an adenoviral vector encoding green fluorescent protein (GFP)-tagged tubulin[12], trypsinized (Trypsin-EDTA, Gibco), harvested, and seeded onto glass-bottomed 35 mm dishes (MatTek) in complete medium. Prior to imaging, cells were transferred into a nonfluorescent, CO2-independent medium based on Hank's balanced salt solution (pH 7.3) as described[13] and in the presence of 10% calf serum.

RESULTS

Figure 1 shows the fluorescence from the actin network of an endothelial cell after it has been irradiated along five parallel lines with various pulse energies. The sample was translated at a speed of approximately 0.7 μm/s

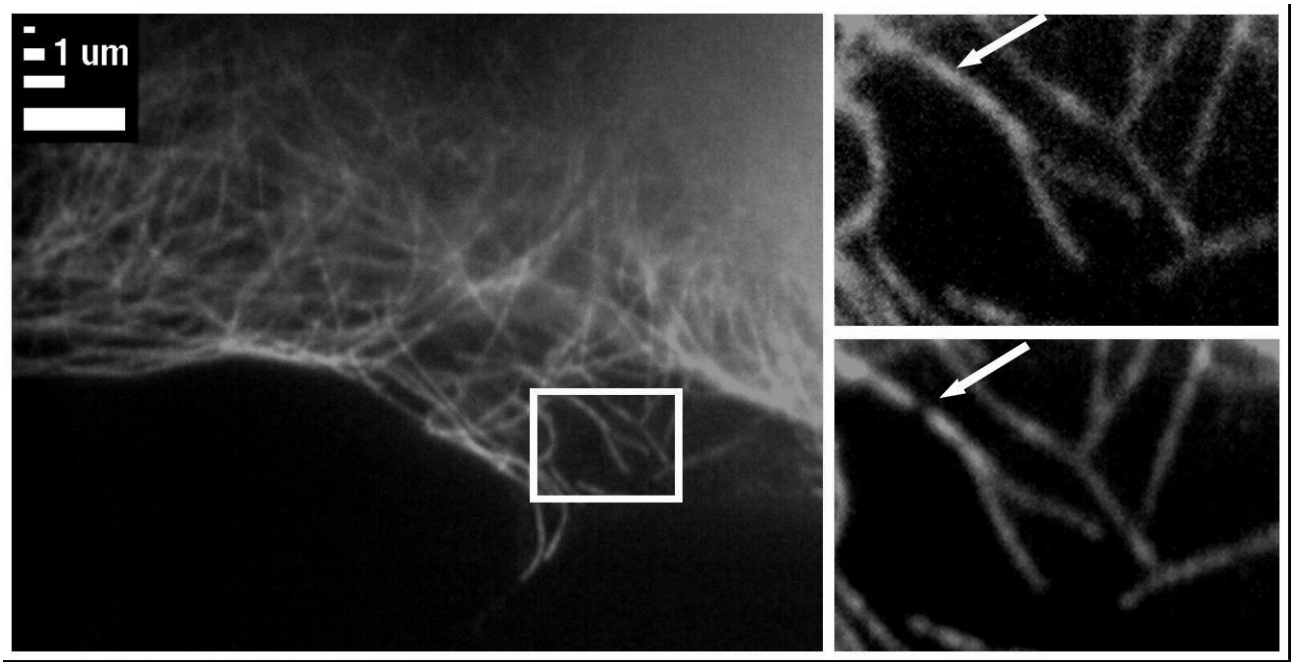

Fig. 2 Laser cuts in the microtubule-network in side a fixed BCE-cell: The magnification shows a microtubule before (upper image) and after the laser application (lower image). The microtubule is cut with an energy of 1.4 nJ.

corresponding to roughly 15,000 pulses per line. The image shows that the fluorescence intensity following irradiation depends strongly on pulse energy. At 1.8 nJ the effect of irradiation is barely visible in the fluorescence image. Increasing the pulse energy to 2.2 nJ produces a clear decrease in fluorescence with a width of 240 nm. At higher energy the width of this decrease in fluorescence scales with pulse energy, from 360 nm at 2.8 nJ, to 500 nm at 3.5 nJ and 600 nm at 4.4 nJ.

In Figure 2 a fluorescence microscopy picture of a fixed cell is shown, which was stained for microtubules. One of the microtubules was severed by several laser pulses of 1.1 nJ energy, see magnification on the right of Figure 2. However, it is not clear from these images, whether the microtubule is cut or just photobleached by the laser radiation. Figure 3 shows that loss of fluorescence does not always correspond to removal of material. It shows both fluorescence and TEM images of the nucleus of a fixed endothelial cell after irradiation at three different energies. While a slight loss of fluorescence can be observed for a pulse energy of 1.45 nJ, the TEM image shows no material removal. The loss of fluorescence must thus be due to photobleaching. At higher energies we see clear "cuts" in both images.

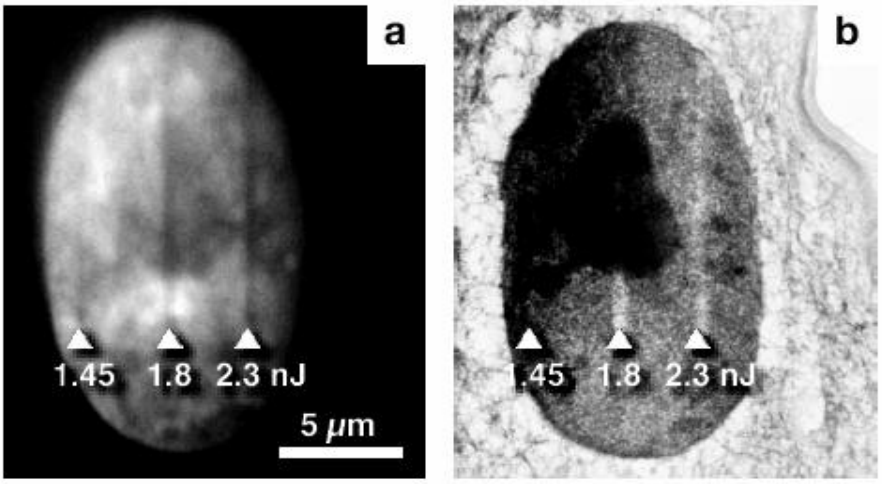

Fig. 3 Cuts in the nucleus of a fixed endothelial cell at various laser energies, imaged by (*a*) fluorescence microscopy and (*b*) electron microscopy[16].

The data allow us to define three regimes of irradiation: no interaction (no damage visible in either image), photobleaching without material loss (only the fluorescence image shows a change), and ablation of material (both images show cuts). For pulse energies above 10–15 nJ, a much larger part of the cell is ablated (not shown). This phenomenon is most likely due to cavitation, which has been observed during laser irradiation of water, soft materials, and biological tissues,[14,15] Part of the energy delivered to the sample cannot be dissipated through thermal diffusion, producing rapid, local increase in material temperature, leading to an explosive expansion of the material and, thus, damage far from the laser focus.

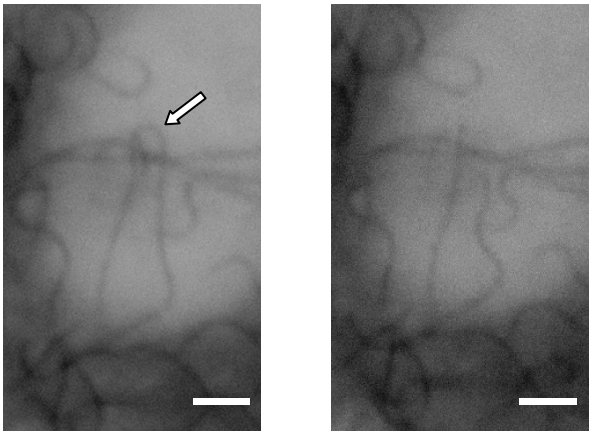

Fig. 4 Laser cuts (*arrow*) inside a living BCE-cell: The GFP-tagged microtubule (MT) before cutting (*left*) and after cutting (*right*). The MT snaps back and starts to depolimerize. The scale bar is 2 μm.

While the thresholds vary from sample to sample, the energy threshold of ablation is at most 20% higher than the photobleaching threshold. In other words, at energies exceeding 1.2 times the threshold for which fluorescence disappears, one can be assured of ablation. The TEM and fluorescence microscopy measurements reveal that the ablation width depends strongly on pulse energy, with pulse energies between 1.2 and 1.7 nJ producing material loss as small as 200 nm. Above 1.7 nJ, the

ablation width increases with energy; around 3 nJ the ablation width is approximately one micrometer. If such laser energies are applied to live cells, micromanipulation at resolution below a micrometer within the cells is possible. Thus, we tried to cut single microtubules in a live cell. Microtubules consist of protein subunits of tubulin, which form a stiff hollow tube with typical diameters of about 25-30 nm and length of up to several 10 μm and are part of the cytoskeleton. If such a structure is cut by a laser, it depolimerizes rapidly due to its dynamic instability. This can be seen by the fluorescence microcscopy images in Figure 4. The laser was aimed at a curved microtubule in the middle of the image (*see arrow*). After cutting, both ends of the microtubule snapped back and started to depolimerize, as shown in the right of Figure 4, taken approximately 16 seconds after the laser cut. Both ends of the microtubule are already several micrometer apart, prooving that the microtubule is clearly cut.

CONCLUSION

The use of ultrashort laser pulses for ablation of cells and subcellular structures with simultaneous fluorescence imaging is gaining increasing use in cell and developmental biology. Here, we have established thresholds for fluorescence photobleaching and material ablation, using a combination of fluorescence and electron microscopy. The size of the ablated region depends strongly on the laser pulse energy. In particular, there is a range of energies, at this focusing condition, which is sufficient to photobleach fluorescent structures but not to ablate them. The optimal range for material ablation is 20% higher than that for photobleaching. The energy thresholds shown here will prove to be a useful to guide and interpret the femtosecond laser material ablation and real time fluorescence imaging to investigate cell structure and function at increasingly short length scales. The feasibility of using a femtosecond laser for minimally disruptive subcellular ablation also opens up the integration of the laser simultaneously as an illumination source and an ablation instrument in a multiphoton microscope. As proven by live cell application, this technique has a spatial selectivity smaller than 0.5 μm and can be applied to the study of real time dynamic effects in live cells without compromising cell viability.

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