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Ablation of cytoskeletal filaments and mitochondria in live cells using a femtosecond laser nanoscissor

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Abstract: Analysis of cell regulation requires methods for perturbing molecular processes within living cells with spatial discrimination on the nanometer-scale. We present a technique for ablating molecular structures in living cells using low-repetition rate, low-energy femtosecond laser pulses. By tightly focusing these pulses beneath the cell membrane, we ablate cellular material inside the cell through nonlinear processes. We selectively removed sub-micrometer regions of the cytoskeleton and individual mitochondria without altering neighboring structures or compromising cell viability. This nanoscissor technique enables non-invasive manipulation of the structural machinery of living cells with several-hundred-nanometer resolution. Using this approach, we unequivocally demonstrate that mitochondria are structurally independent functional units, and do not form a continuous network as suggested by some past studies.

keyword: Nanoscissor, nanosurgery, femtosecond laser, photodisruption, cytoskeleton.

1 Introduction

Complex cell behaviors that are manifested at the micrometer scale, such as growth, differentiation, motility, and apoptosis, are made possible through the integrated action of nanometer-sized supramolecular complexes that provide specialized chemical and mechanical functions within spatially-distinct subcellular domains. To better define the relation between cell structure and function, it is important to be able to structurally modify or ablate specific structures or functional domains within single living cells with spatial discrimination on the nanometer scale. Techniques currently used for microdissection of cells include microneedles [Maenner *et al.* (1996), Weimer *et al.* (1999), Harsch *et al.* (2001)], Atomic Force Microscopy (AFM) tips [Hansma *et al.* (1992), Bushell *et al.* (1995)], "microscissors" using focused UV light [Berns *et al.* (1981), Greulich and Weber (1992)], chromophore-assisted laser inactivation (CALI) [Jay and Sakurai (1999)], nanosecond lasers [Brenner *et al.* (1980), Khodjakov *et al.* (1997), Khodjakov *et al.* (2000)], and picosecond lasers [Botvinick *et al.* (2004)]. Microneedles are invasive and usually cause disturbance to the physical relationships between cellular structures. The spatial resolution is on the order of tens of micrometers and the procedure is often labor intensive. AFM tips work only on the sample surface and are also invasive. Commercially available UV microscissors use a near-ultraviolet (337 nm) nanosecond laser pulse to dissect tissue with a resolution of about 1 μm. However, because most cell components have strong linear absorption in the near-ultraviolet, any components in the light path outside the focal plane also absorb the radiation, making it difficult to ablate subcellular organelles in live cells without compromising the cell membrane. CALI involves the irradiation with visible light of specific proteins bound to visible light-absorbing dyes generates free radicals that selectively denature the proteins; however, these inactive proteins are not physically removed from these regions. The effects of CALI are also short-term and recruitment of new functional proteins to the disrupted site occurs within hours. The effect of focused nanosecond green lasers for microsurgery varies greatly for different types of cellular structures and denaturation of the protein molecules in and around the laser focus has been reported [Brenner et al. (1980), Khodjakov et al. (1997),

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Khodjakov et al. (2000)]. In order to study nanoscale biological processes in their physiological context, there is therefore a need for a precise subcellular surgery technique that provides nanometer-scale resolution, results in minimal alteration to cell structure, and can be applied to live biological samples.

Recent advances in laser technology have made it possible to explore precise ablation of biological materials using lasers of varying pulse durations, from continuous wave (CW) to tens of femtoseconds. Femtosecond lasers show great promise because they can be used with any type of biological tissue, and they exhibit the highest precision and best control in three dimensions [Fujimoto *et al.* (1985), Krueger *et al.* (1996), Nishimura *et al.* (1998), Vogel *et al.* (1998), Juhasz *et al.* (2000), Botvinick et al. (2004)]. High repetition-rate trains of femtosecond laser pulses have been used to cleave isolated chromosomes [Konig *et al.* (1999), Konig *et al.* (2001)] and to rupture the surface membrane of living cells to enhance DNA transfection efficiency [Tirlapur and Konig (2002)].

Here, we describe a nanosurgery technique using near-infrared low-energy, low repetition-rate femtosecond laser pulses that can be applied to ablate subcellular structures inside live cells with 300-nm resolution and without affecting neighboring structures or compromising cell viability. Using this technique, we demonstrate cleavage of actin fiber bundles in fixed cells and ablation of individual mitochondria in live cells without altering the structural integrity of neighboring organelles. The technique provides a direct, non-invasive method to probe cytoplasmic and nuclear structure-function relations by allowing observation of cell behavior after the removal of an individual organelle or subcellular nanodomain.

2 Materials and methods

2.1 Femtosecond laser cell surgery setup

To develop a nanosurgical tool for probing cell structure, we built a femtosecond Ti:sapphire laser system that delivers 100-fs pulses at 800 nm with a pulse energy of 2–5 nJ at a 1-kHz repetition rate at the samples (Fig. 1). A mechanical shutter in the beam path controlled the number of pulses that irradiate the sample. The sample was mounted on a temperature-controlled stage, placed on top of a computer-controlled x-y translation stage. The collimated femtosecond laser beam was focused into the sample using a 1.4 NA oil-immersion microscope objective, which was mounted on a separate stage allowing the laser focus to be moved in the z-direction. In order to monitor the sample and position the subcellular target at the focus of the femtosecond laser beam, the same objective was used to obtain an epi-fluorescence microscope image of the sample cell, which allows us to observe the ablation *in situ* and monitor the resulting cell response in real-time.

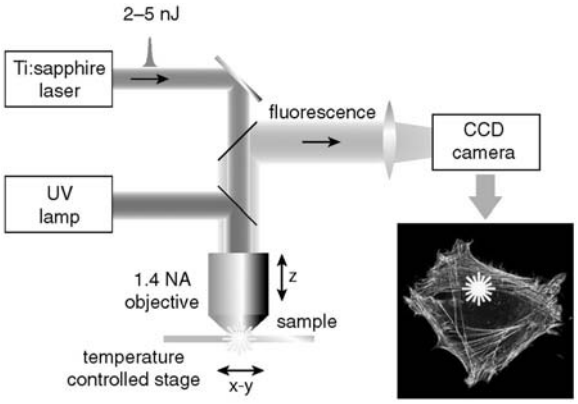

Figure 1 : Schematic diagram of the experimental setup for nanosurgery. Laser pulses of 100-fs duration at 800 nm are focused through a 1.4 Numerical Aperture (NA) microscope objective onto a small region (asterisk) within a fluorescently-labeled cell (stained with Alexa Fluor 488 in this inset view). The sample is mounted on a temperature-controlled microscope stage and simultaneously imaged under epi-fluorescence using the same 1.4 NA objective.

2.2 Cell sample preparation

Mouse 3T3 fibroblasts and bovine adrenal capillary endothelial cells were cultured, harvested, and processed for fluorescence microscopy as previously reported [Park et al. (2001), Wang et al. (2001)]. Before ablation, the fibroblasts were washed in phosphate buffered saline (PBS) with calcium, fixed in 4% paraformaldehyde in PBS, and stained with Alexa Flour phalloidin 488 (Molecular Probes). Capillary endothelial cells were placed in CO_2-independent media (Gibco) with 10 μg/ml high-density lipoprotein, 5 μg/ml transferrin, and 5 ng/ml basic fibroblast growth factor, and 10 mM HEPES buffer while on the microscope stage. To determine effects on cell viability, 10 mM ethidium bromide (Molecu-

lar Probes) was added to the medium 30 minutes before the femtosecond ablation experiment. Fusion constructs containing a gene for Enhanced Yellow Fluorescent Protein (EYFP) and the mitochondrial targeting sequence from human cytochrome C oxidase (pEYFP-Mito; Clonetech) were transfected into the endothelial cells using the Effectene (Qiagen) technique, as previously described [Wang et al. (2001)].

2.3 Microscopy analysis

Both wide field fluorescence microscopy and laser scanning confocal microscopy (Carl Zeiss, Inc.) were used to image cell samples. Fluorescence filter sets (Chroma Technology Corp.) were selected to optimize each fluorophore used to label the subcellular structures. To characterize femtosecond laser disruption in single cells, we also performed atomic force microscopy studies on fixed cell samples in solution using tapping mode (Asylum Research).

3 Results and discussion

To demonstrate the capabilities of the nanosurgical technique, the femtosecond laser was first used to ablate the actin cytoskeleton within fixed 3T3 fibroblasts that were stained with the green fluorescent F-actin-binding dye, Alexa Fluor 488-phalloidin. Nanometer scale channels were created within the cytoskeleton by translating the cell at 5 μm/s during irradiation with a 1-kHz pulse train in a direction perpendicular to the laser beam (Fig. 2A). The diameter of these linear channels decreases as the pulse energy is lowered from 3 to 1.5 nJ, with the smallest diameter being close to the optical resolution of the microscope. One can also create small spherical cavities deep in the cytoplasm, as demonstrated in horizontal and vertical cross-sections obtained with confocal microscopy (Fig. 2B), by applying twenty 100-fs pulses (2.3 to 3 nJ/ pulse) at the same location.

To confirm that the disappearance of fluorescence after irradiation is due to ablation of the actin fibers, rather than bleaching of the fluorophore [Periasamy (2001)], we restained the same samples after laser irradiation with phalloidin labeled with a red fluorophore (Alexa Fluor 546). The channels remain dark after restaining (Fig. 2C, D) and thus the appearance of these channels cannot simply be due to photobleaching of the dye. At a pulse energy of 1.5 nJ material ablation is still observed,

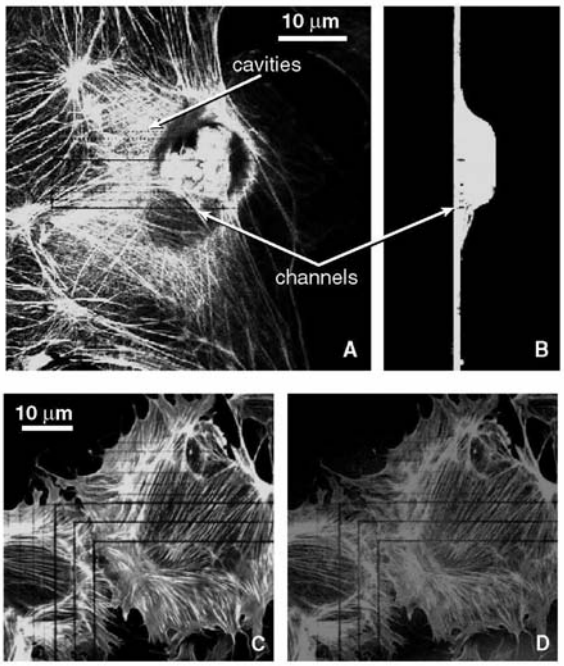

Figure 2 : Laser confocal microscopic images of a fixed 3T3 fibroblast stained for F-actin with a green fluorescent dye. (**A**) Top view of a mid-plane horizontal section through the cell showing channels and cavities produced by femtosecond laser ablation. The top and bottom channel were obtained at a pulse energy of 3 nJ; those in between at pulse energies ranging from 1.5 to 2.3 nJ. (**B**) Reconstructed orthogonal image of the same cell. (**C**) A cell that was prestained for F-actin with a green fluorescent dye immediately after ablation with 2-nJ (wide channels) and 1.5-nJ (narrow channels) laser pulses. (**D**) The same irradiated cell after restaining for F-actin with a red fluorescent dye.

but at 1 nJ photobleaching dominates — that is, channels visible with the green stain are no longer visible after restaining with the red dye. This establishes 1.5 nJ as the energy threshold for ablation of the actin cytoskeleton for 100-fs laser pulses at 800 nm focused with a 1.4-NA objective. This threshold value is consistent with the energy threshold for plasma generation in water for 100-fs laser pulses focused with a 1.3-NA microscope objective [Vogel *et al.* (1996), Noack *et al.* (1998), Vogel et al. (1998), Vogel and Noack (2001), Vogel *et al.* (2002)].

To confirm the ablation of material at these pulse energies, we performed atomic force microscopy of channels incised directly on the cell surface using 2-nJ pulses.

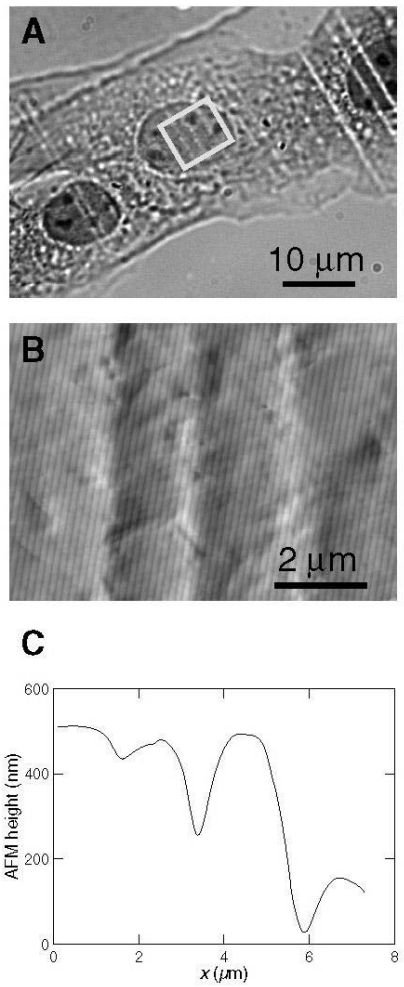

Figure 3 : Femtosecond laser ablation on the surface of fibroblast cells at a laser pulse energy of 2 nJ and a scanning rate of 10 μm/s and a repetition rate of 2.7 kHz. (**A**) Phase contrast microscopy image of three fixed fibroblast cells showing femtosecond laser ablation channels. (**B**) Atomic force microscopy image of the rectangular outlined area shown in Figure 3A showing three channels ablated on the surface of a cell. **(C)** Depth profile across the three cuts shown in part B. The left cut was obtained in a single pass, the other two in four passes.

These images confirm that cellular matter was removed from grooves that were incised into the cell membrane and that longer exposures remove more material (Fig. 3).

Having demonstrated the feasibility of using the femtosecond laser system as a nanosurgical tool in fixed cells, we then applied this method to analyze how mitochondria are organized at the nanometer scale in living cells because past studies have produced conflicting reports. Some studies suggest that these energy-generating organelles form a physically continuous network similar to the endoplasmic reticulum [Rizzuto *et al.* (1998), De Giorgi *et al.* (2000)], while others suggest that mitochondria are organized as structurally independent functional units [Park et al. (2001), Collins *et al.* (2002)]that are linked by cytoskeletal filaments [Lin *et al.* (1990), Wang et al. (2001)]. We studied the connectivity of mitochondria by selectively damaging a single mitochondrion and examining the effect on its neighbors. If each mitochondrion is physically isolated from the others, then damage to one should not compromise the structural integrity of its neighbors.

Capillary endothelial cells were transfected with Enhanced Yellow Fluorescent Protein (EYFP) fused to cytochrome C oxidase to visualize mitochondria [Huang *et al.* (2000), Wang et al. (2001)] (Fig. 4A), and then these living cells were exposed to 100-fs laser pulses through a 1.4-NA objective. Figures 4B and C show the ablation of a single mitochondrion, about 5 μm in length and separated by less than 1 μm from multiple neighboring mitochondria. After irradiating a fixed spot on the organelle with a few hundred 2-nJ laser pulses at a 1-kHz repetition rate, the entire mitochondrion disappears from the image, whereas neighboring mitochondria are not affected by the irradiation even though they are only a few hundred nanometers away (Fig. 4C). This result provides clear evidence that mitochondria do not form a structurally continuous network, as previously suggested [Rizzuto et al. (1998), De Giorgi et al. (2000)]. These results also clearly demonstrate that the laser irradiation produces material removal. If the laser pulses were photobleaching the EYFP in the target mitochondrion, the fluorescence signal would disappear only at the 0.5-μm diameter spot where the laser is focused. Instead, we observe immediate disappearance of the fluorescence throughout the entire mitochondrion indicating that local ablation of the target mitochondrion causes a major structural change in the organelle resulting in the destruction of that single organelle, but not its neighbors.

To verify that the nanoscissor produces these effects on this organelle without rupturing the cell's surface membrane, we carried out similar studies with cells that were incubated with the DNA-binding dye, ethidium bromide. Because this dye cannot penetrate across the cell membrane, little nuclear fluorescence is observed in living

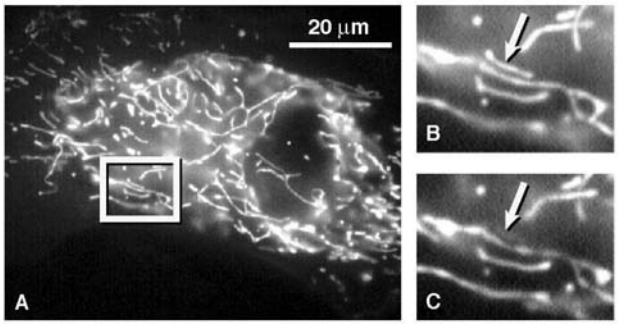

Figure 4 : Ablation of a single mitochondrion in a living cell. (**A**) Fluorescence microscopic image showing multiple mitochondria in yellow before femtosecond laser irradiation. Target mitochondrion (marked by arrow) (**B**) before and (**C**) after laser ablation with 2-nJ pulses.

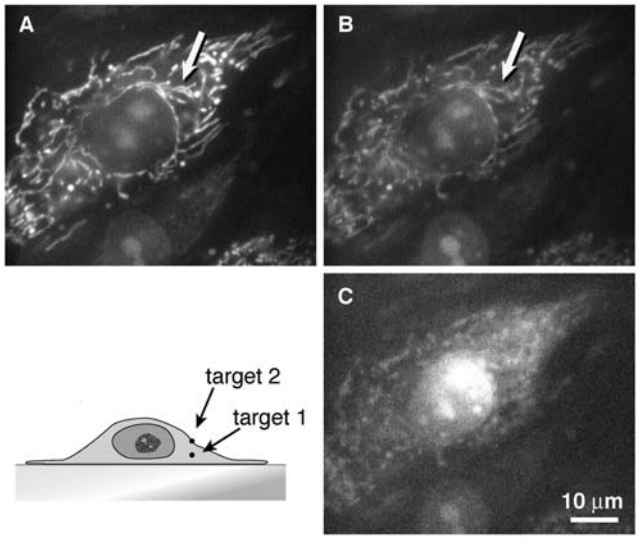

Figure 5 : Ablation of a mitochondrion in a live cell without compromising cell viability. Fluorescence microscopic images of a live cell containing EYFP-labeled mitochondria in a cultured medium containing ethidium bromide (4 μM) (**A**) before femtosecond laser irradiation, (**B**) after ablation of a single mitochondrion within its cytoplasm (target 1 in diagram at bottom left), and (**C**) after irradiation of the apical cell membrane (target 2). Note the large increase in nuclear ethidium bromide staining that only occurs after irradiation of the apical membrane.

cells prior to laser exposure (Fig. 5A). Ablation of a single mitochondrion (marked by the arrow head) using several hundred 2-nJ pulses does not produce any significant increase in nuclear fluorescence (Fig. 5B). In contrast, when we focus the objective at the top of the cell and irradiate the apical cell membrane with similar pulse energies, the intensity of fluorescent staining in the nucleus immediately increases by a factor of ten as the ethidium bromide rapidly diffuses into the cell through the incision in the surface membrane (Fig. 5C). In conjunction with the absence of any morphological change in the cell or other mitochondria over a period of one hour following ablation, the results indicate that the nanosurgical technique presented in this paper can be used to selectively ablate internal cell structures without producing generalized injury or cell necrosis.

Laser radiation affects materials in a number of ways: through heating, bond breaking, and ablation. While ablation is limited to the focal volume, which can be made of submicrometer size, heating and indirect mechanical stresses caused by the laser excitation result in unwanted side effects on a much larger scale. Because living cells are restricted to a narrow range of temperatures, even a little heating can cause cell necrosis, limiting the total amount of energy that can be deposited in the sample.

The degree of heating in a cell is proportional to the amount of laser energy being absorbed by the cellular material. The larger the numerical aperture (NA) of the objective, the smaller the volume illuminated at the focal point, and the lower the energy required to reach the intensity threshold for ablation. Studies on laser-induced breakdown in water have demonstrated that the threshold energy for breakdown drops from microjoules for nanosecond pulses to only a few nanojoules for femtosecond pulses at high NA [Vogel et al. (1996), Noack et al. (1998), Vogel et al. (1998), Vogel et al. (2002)]. As a result, the amount of heating and the volume of the thermally affected region are minimized for femtosecond laser pulses.

Heating also becomes more pronounced when a cell is irradiated with trains of pulses at repetition rates above 1 MHz, when the separation between pulses is shorter than the heat diffusion time. Irradiation with a 25-MHz train of femtosecond laser pulses of just 5 nJ heats glass to the melting point over a region that is 50 times larger than the focal volume [Schaffer *et al.* (2003)]. Furthermore, as multiphoton microscopy using high repetition rate femtosecond lasers becomes more widely applied in biological studies, researchers are starting to investigate thermal effects on tissue by low energy but high repetition femtosecond laser pulses. Irradiation of only 80

μs by focused 150-fs, 760-nm, 80-MHz laser pulses at a mean power of 3 mW is enough to cause impaired cell division in half the irradiated cells [Konig et al. (1999)]. Cutting chromosomes using high repetition rate femtosecond lasers requires a mean power of 30–40 mW and an exposure time between 40 and 500 μs. [Konig et al. (1999), Konig et al. (2001)] Although the cells in these past studies with MHz laser pulses do not show clear loss of short-term viability, the potential for functional damage due to excessive thermal stress is high, based on knowledge of the effect of such pulse trains on a solid such as glass. To prevent unnecessary damage in cells other than the targeted structure for laser surgery, it is therefore essential to minimize the thermal load to the sample by lowering the repetition rate of the pulses to well below 1 MHz, as we did in the present study.

Laser-induced breakdown at the focus causes mechanical effects on the material surrounding the focal volume. Within a few hundred picoseconds after excitation, the plasma expands into the surrounding tissue and launches a pressure wave as the laser-induced plasma expansion slows to acoustic velocity [Fujimoto et al. (1985), Zysset *et al.* (1989), Juhasz *et al.* (1996), Vogel et al. (1996), Glezer *et al.* (1997), Noack et al. (1998), Vogel *et al.* (1999), Venugopalan *et al.* (2002)]. In addition, the ablated material at the focus forms a cavitation bubble that expands outward and then collapses under external pressure [Vogel *et al.* (1986), Juhasz et al. (1996), Vogel et al. (1996), Vogel et al. (1999), Vogel and Noack (2001), Vogel et al. (2002)]. The size of the region affected by these mechanical effects, called the 'shock zone', increases with the energy deposited by the laser pulse during ablation. As the duration of the laser pulses increases, the minimum pulse energy required to achieve ablation also increases, leading to larger shock zone and hence greater collateral damage. For 1-mJ pulses of nanosecond duration, the size of the shock zone is about half a millimeter [Fujimoto et al. (1985)]. For shorter pulse durations (and consequently lower pulses energies), the shock zone is much smaller: at 40 ps and 8 μJ it is on the order of 100 μm [Fujimoto et al. (1985)] and at 100 fs and 1 μJ it is only 11 μm [Glezer et al. (1997)]. The exact thermal and mechanical distribution of absorbed laser energy (shock wave and cavitation bubble) depends on the laser pulse duration, energy, and focusing conditions. Compared to picosecond and longer pulses, mechanical effects are minimized for femtosecond laser pulses near the ablation threshold and therefore most of the absorbed laser energy is used for ablation of cellular material. For example, for a 2-nJ laser pulse, the estimated cavitation bubble is less than 50 nm assuming a worst-case scenario that all the pulse energy is absorbed [Vogel et al. (1999)]. The small size of the cavitation bubble ensures that femtosecond laser induced material ablation is the dominant effect in cell surgery.

Collateral thermal and mechanical effects within biological materials are further minimized by using tightly-focused subpicosecond laser pulses at low repetition rate as these pulses afford higher ablation precision than picosecond and longer pulses. Such pulses offer two additional advantages. First, because the absorption is nonlinear [Bloembergen (1974), Sacchi (1991), Du *et al.* (1994), Stuart *et al.* (1996), Lenzner *et al.* (1998), Schaffer *et al.* (2002)], the use of subpicosecond pulses does not require the sample to have any linear absorption at the laser wavelength and any region within the medium can be targeted regardless of the wavelength of the laser. Secondly, by placing the focus beneath the surface of a cell that is transparent at the laser wavelength (*e.g.*living cells), material within the bulk of the sample can be ablated, forming sub-surface cavities, without affecting the surface itself and any material around the focal volume [Niemz *et al.* (1993), Krueger et al. (1996), Kurtz *et al.* (1997), Krueger *et al.* (1998), Nishimura et al. (1998), Juhasz *et al.* (1999), Juhasz et al. (2000), Shen *et al.* (2001), Watanabe *et al.* (2004)], as confirmed experimentally in the present study.

In conclusion, the nanosurgical method presented here, using low-energy, low repetition-rate femtosecond laser pulses allows one to ablate subcellular domains within live single cells with high spatial selectivity. Structures outside the submicrometer focal region remain intact, and cell viability is preserved. The pulse energy required for ablation is only a couple of nanojoules and requires only a femtosecond laser oscillator [Rundquist *et al.* (1997), Backus *et al.* (1998)]. Such oscillators typically have a pulse repetition rate of about 80 MHz and so only a few pulses out of the pulse train are needed for ablation, leaving the remainder available for simultaneous high-resolution multiphoton microscopy [Denk *et al.* (1990), Periasamy (2001)]. The technique will be useful in studies of cell dynamics, chemotaxis, cell polarity, spatially regulated signaling, drug screening and other phenomena involving intracellular compartments, sub-

cellular heterogeneity, and structure-function relations on the nanometer scale.

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