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Using Femtosecond Laser Subcellular Surgery as a Tool to Study Cell Biology

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Using femtosecond laser subcellular surgery as a tool to study cell biology
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Abstract

Research on cellular function and regulation would be greatly advanced by new instrumentation using methods to alter cellular processes with spatial discrimination on the nanometer-scale. We present a novel technique for targeting submicrometer-sized organelles or other biologically important regions in living cells using femtosecond laser pulses. By tightly focusing these pulses beneath the cell membrane, we can vaporize cellular material inside the cell through nonlinear optical processes. This technique enables non-invasive manipulation of the physical structure of a cell with sub-micrometer resolution. We propose to study the role mitochondria play in cell proliferation and apoptosis. Our technique provides a unique tool for the study of cell biology.

Introduction/Background

Complex cellular processes and function — metabolic synthesis and transportation, cell division, differentiation, death, signal transduction, and cellular communication — take place in or are controlled by defined and spatially distinct subcellular domains. To study these behaviors one could structurally modify or remove these functional domains within single living cells with spatial discrimination on the nanometer scale. Conventional dissection tools such as microneedles, however, are invasive, have spatial resolution no better than one micrometer and usually disturb the physical relationships between structures. Other optical microdissection devices use UV radiation, which is known to introduce many biological changes, including DNA mutations. Therefore, in order to study cellular processes, it is necessary to develop a precise subcellular "surgery" technique that provides submicrometer resolution, minimal alteration to cellular environment, and that can be applied to living biological samples. We have demonstrated previously a novel nanosurgery technique using near-infrared femtosecond laser pulses to disrupt or excise subcellular structures inside living cells (Shen et al., 2001; Shen et al., 2005). This subcellular surgery technique provides a direct, non-contact method for manipulating or removing individual intracellular organelles or chromosome sections in live cells. The technique opens the door to precise studies of organelle function and cellular processes by allowing observation of cell behaviors after the perturbation or removal of an individual organelle or subcellular nanodomain.

Biological tissues such as cell cultures normally do not absorb light in the near infrared range. However, when a short laser pulse (< 10 ns) is tightly focused into tissue, the laser intensity in the focal volume can become high enough to induce nonlinear absorption of laser energy by the tissue through multiphoton, tunneling, and avalanche ionization (Bloembergen, 1974; Lenzner et al., 1998; Sacchi, 1991; Soileau et al., 1989; Sparks et al., 1981; Stuart et al., 1996). Because the absorption is nonlinear, it exhibits a sharp threshold behavior, and the laser energy

is only absorbed at the focus where the laser intensity is above the threshold. By placing the focus beneath the surface, tissue within the bulk of the sample can be disrupted without affecting the surface itself (Juhasz et al., 2000; Juhasz et al., 1999; Niemz et al., 1993; Nishimura et al., 1998). Studies of the interaction of tightly focused femtosecond laser with water have revealed that this nonlinear energy absorption produces an excited plasma in the focal volume. The plasma expands supersonically into the surrounding tissue (the region of this supersonic expansion is referred to as the shock zone), and launches a pressure wave as the plasma expansion slows to acoustic velocity (Glezer et al., 1997; Juhasz et al., 1996; Vogel et al., 1996; Zysset et al., 1989). Ultimately, vaporized material at the focus forms a cavitation bubble, which expands outward and then collapses under external pressure (Juhasz et al., 1996; Vogel et al., 1996).

Laser-induced plasma formation vaporizes tissue in the focal volume, providing the surgical effect (referred to as photodisruption). However, the supersonic plasma expansion, the expansion of the cavitation bubble, as well as thermal diffusion all cause unwanted collateral damage to the surrounding tissue, limiting surgical precision (Juhasz et al., 1999; Noack et al., 1998; Vogel et al., 1999; Zysset et al., 1989). The size of the region affected by these mechanical (plasma and cavitation bubble expansion) and thermal effects increases as the amount of laser energy that is deposited into the sample increases. To minimize collateral damage, the amount of energy deposited into the sample must be minimized while still maintaining a sufficiently high laser intensity to produce photodisruption through plasma formation. The shorter the pulse duration, the higher the intensity is for a given pulse energy. Also, the larger the numerical aperture (NA) of the focusing lens, the tighter the focusing is and the lower the energy required to reach the intensity threshold for vaporization. Therefore, the threshold intensity is reached with the least amount of energy for femtosecond pulses under 1.4 NA focusing.

Figure 1 shows a schematic diagram of the cell surgery setup. A femtosecond Ti:sapphire laser system delivers 800 nm wavelength 100-fs pulses with a pulse energy of 2–5 nJ. A shutter in the beam path controls the number of pulses that irradiate the sample. The sample is mounted on a temperature-controlled stage, placed on top of a computer-controlled *x*-*y* translation stage. We focus the collimated femtosecond laser beam into the sample using a 1.4 NA oil immersion microscope objective, which is 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, we use the same objective to obtain an epi-fluorescence microscope image of the sample cell. The epi-fluorescence microscope allows us to observe the photodisruption *in situ* and to monitor the resulting cell response in real-time.

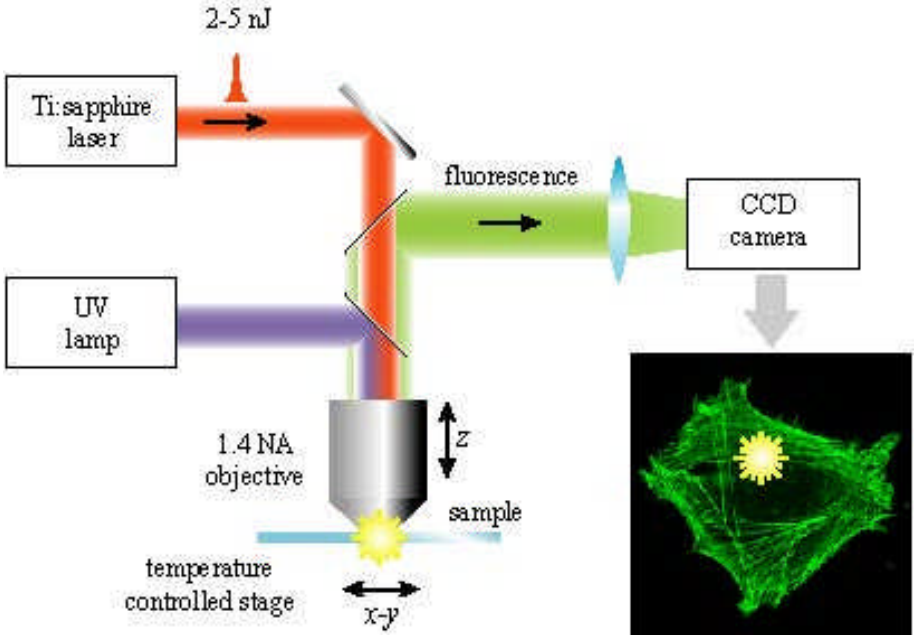

Figure 1. Schematic diagram of the experimental setup.

Preliminary subcellular surgery study performed on fixed 3T3 fibroblast cells to cleave actin fibers showed that femtosecond laser produced localized photodisruption with a resolution of ~300 nm. Cellular materials outside the disrupted region were not affected (see Figure 2).

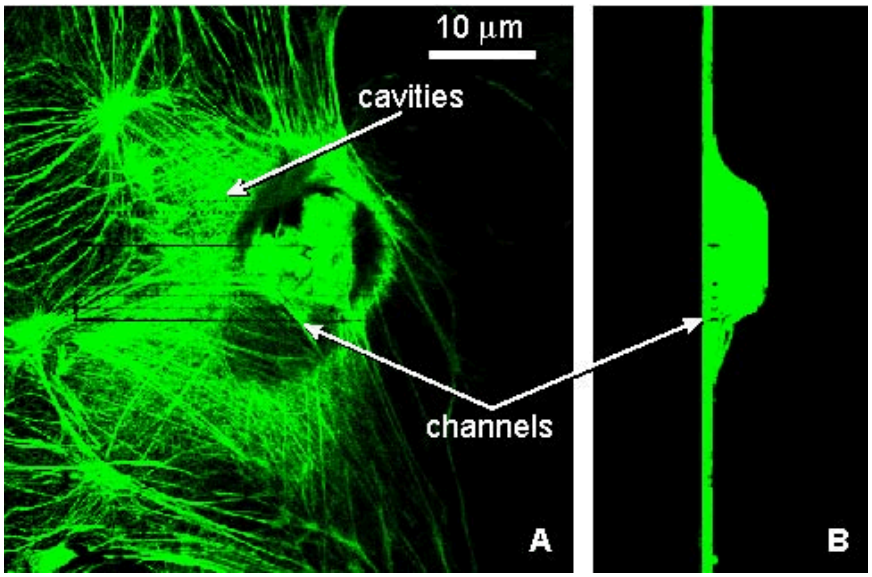

Figure 2. Laser scanning fluorescence confocal microscope images of a 3T3 fibroblast cell with Alexa Fluor stained actin fibers photodisrupted using femtosecond laser pulses. (A) Top view of a mid plane section through the cell in which channels and cavities were produced. (B) Reconstructed orthogonal side view image of the same cell where the spatially defined ablated regions corrspond to the channels seen in (A).

Research Activities

Instrumentation

The experimental setup was constructed for the project. It consists of a femtosecond laser system, a Nikon Eclipse TE2000 inverted microscopy, and a CoolSnap HQ CCD camera from Roper Scientific for cell imaging (Figure 1). We reconfigured the standard Mira 900 femtosecond laser oscillator from Coherent, Inc. to include a cavity dumper system. The modified laser has an extended cavity compared to its original configuration, which leads to a reduction in the maximum pulse repetition rate the system generates. Increased cavity length also means that the energy output per pulse increases (up to ten times higher than a standard Ti:sapphire oscillator system). We now have the ability to test femtosecond laser disruption in a cell with a much larger energy range. The cavity dumper system also allows us to accurately control both the number of laser pulses that reach a cell sample and the separation in time between consecutive pulses in order to achieve the most desirable surgical effect.

Cell sample preparation

To study mitochondria functions in cell proliferation and apoptosis, we used both the BJ1 Infinity^{TM} human fibroblast cells, HeLa, a human epithelial cells, and bovine endothelial cells. BJ1 fibroblast is a telomerase-immortalized cell line eliminates losses in cell viability due to natural cell aging. We successfully transfected the cell line with plasmid DNA that encodes a fusion of enhanced yellow fluorescent protein (EYFP) and the mitochondrial targeting sequence of the cytochrome c oxidase.

Laser cell surgery

We investigated how cells respond to femtosecond laser surgery. Cellular structures, such as a single mitochondrion, were disrupted with femtosecond laser pulses at different energies and under different focusing conditions. Cellular behaviors and functions were then monitored over generations to study long-term effect of laser surgery on cells.

Mitochondria play an important part in eukaryotic cell physiology. They are the sites of aerobic respiration, and generally are the major energy production center. Mitochondria are self-replicating organelles, and eukaryotes are known to constantly recycle worn out mitochondria. However, does there exist a critical number of functional mitochondria, a threshold for a cell to remain viable and mitochondria to continue their function?

Laser assisted delivery of nanoparticle sensors into live cells

The ability to determine the chemical characteristics, such as the pH value, of a microenvironment inside a live cell with high sensitivity and spatial resolution is important for the understanding of many basic biological processes. Studies by Laurence *et al.* of using SERS particles as local chemical sensors have shown great promises achieving this goal. These nanoparticles may also help better assess the effect of femtosecond laser surgery in live cells. One of the challenges of using the SERS nanoparticle sensor is to deliver them into live cells reliably. We tested the feasibility of delivering the nanoparticles through live cell membrane by transiently creating a pore with the femtosecond laser surgery technique. The study involved determining the proper laser parameters, with which a transmembrane pore can be created without permanently damaging the cell.

Results/Technical Outcome

Effects of focused near-IR laser irradiation on living cells

We first looked at the physical effect of ultrashort laser pulses on the structures of biological samples. Femtosecond pulses at 2 nJ were focused on the surface of live fibroblast cells which were fixed immediately after the experiment for atomic force microscopy analysis. The left panel in Figure 3 shows the optical image of the channels inscribe on the surface of the fibroblast cells, and the panel on the right is the AFM image of a small region of the sample.

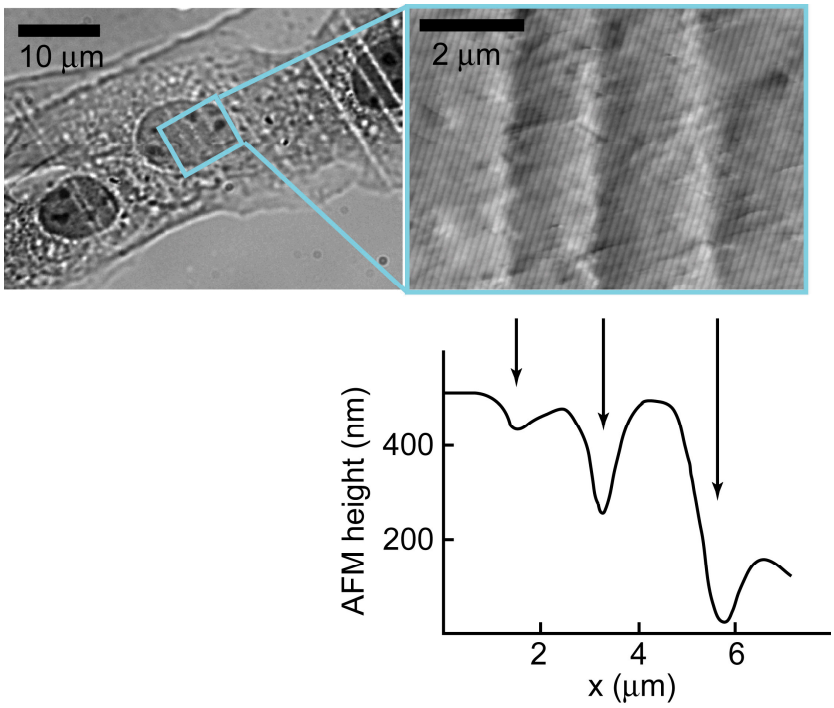

Figure 3. Light (left panel) and AFM (right panel) microscopy images of femtosecond laser irradiated cells.

Using regular BJ1 and BCE cells (without EYFP fusion protein), we monitored mitochondria dynamics and function over time by detecting their membrane potential variations using JC1 (a membrane potential sensitive dye) after laser disruption of a single mitochondrion. Cells without laser irradiation were used as control. At low laser pulse energy, mitochondria in target cells remain active during our observation period (Figure 4 a&b). But with increasing pulse energy, we start to see a gradual decrease in mitochondrial membrane potential.

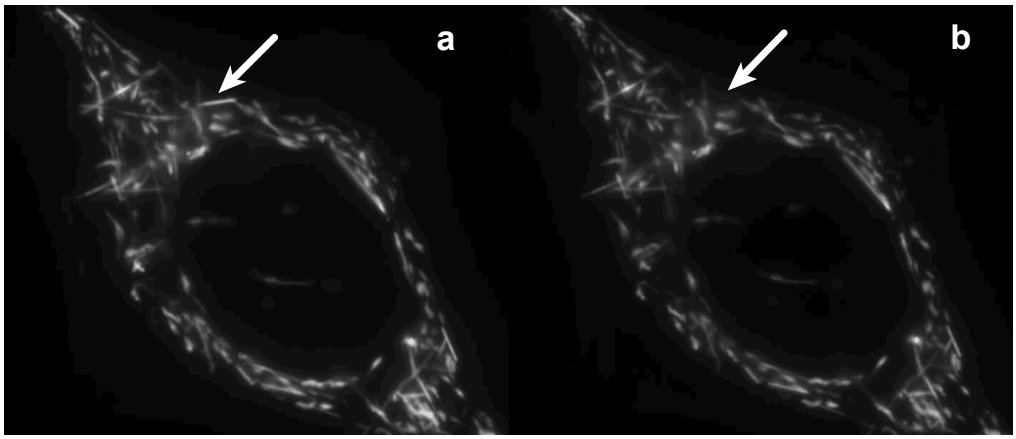

Figure 4. a&b: Fluorescence microscopy images of bovine capillary endothelial cell stained with JC1 (**a**) before, and (**b**) after laser irradiation. Mitochondria in the cell appear polarized and active. The arrows indicate the position of the target single mitochondria.

We also tested this nanosurgery technique on living cells. Individual fluorescent protein labeled mitochondria were disrupted using 100-fs, 2-nJ laser pulses (see Figure 5). Neighboring mitochondria less than 1 micrometer away continued to function after laser photodisruption took place. Also cell membrane integrity remained intact. Cell functions such as division was monitored after laser disruption of single mitochondria. Figure 5 shows that cells continue to divide even though localized structural modification has taken place within the cell.

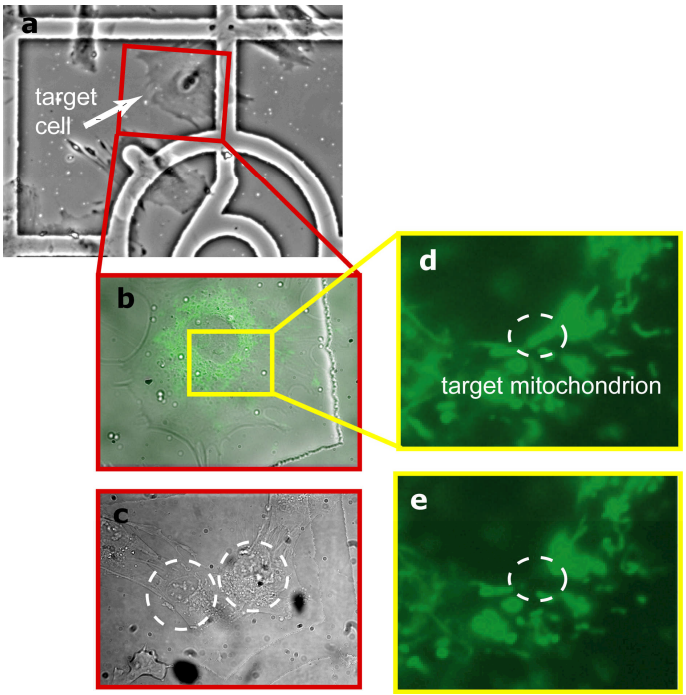

Figure 5. Cell viability test after laser disruption of a single mitochondrion. (a&b) Phase contrast image of the target cell; (d&e) Fluorescent image of the cell before and after disruption of a single mitochondrion; (c) Cell division after ~24 hours.

Mitochondria function and cell apoptosis

As shown in figure 6 below, we targeted ~30 mitochondria in a single live cell (~10% of the total mitochondria population) using 2-nJ femtosecond pulses. Although there was significant photobleaching over the time of the experiment, the overall shape of the cell remained intact suggesting there was no immediate change in cell viability during the couple of hours of observation. When we increased the laser pulse energy, we can induce with control the apoptosis of a single cell within a cell colony.

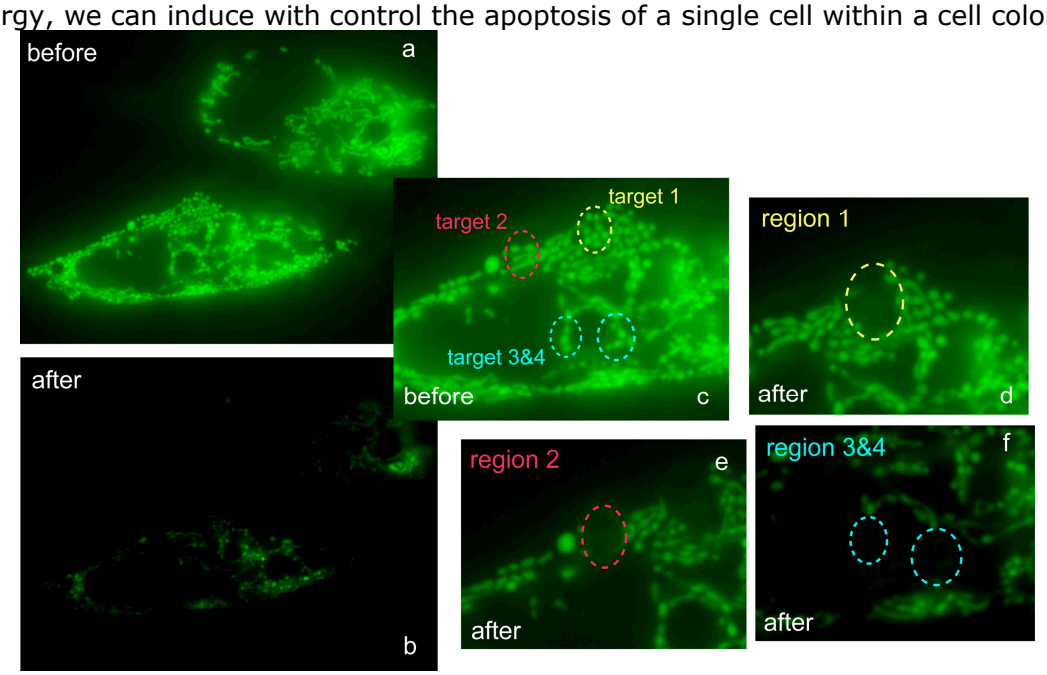

Figure 6. Fluorescent microscopy images of live fibroblast cells with fluorescent protein labeled mitochondria. (a,c) Before laser irradiation; (b, d, e, and f) After laser irradiation.

Laser micro-injection

It has been demonstrated that molecules can be injected into cells by creating a transient membrane pore using focused femtosecond laser. We tested the possibility of local delivery of nanoparticles into a cell. We have observed the intake of nanoparticles by the target cell upon local laser disruption at the cell membrane. However, the delivery efficiency varies a lot, and depends on a number of parameters, such as laser pulse energy and the particle density where the transmembrance pore is created.

Exit Plan

The focus of this research was to develop and test a novel single-cell technique to study responses and signal transduction in cellular systems. We demonstrated new LLNL capability utilizing laboratory expertise in both laser and life sciences. The resulting data supports DOE missions in understanding cellular mechanisms. Furthermore, the understanding is important in helping to determine complete mechanisms for host response to stimulation, advance health biology studies, and serve to identify new targets for therapeutic intervention and biowarfare countermeasures. The result from this study has been used to successfully compete for research funding on pathogen biology.

Summary
This project provides proof-of-concept and preliminary data on the application of using femtosecond laser disruption for structural manipulation of organelles within living cells. Unique instrumentation combining ultrafast laser and high resolution imaging was developed to achieve the high precision surgery with live cells. We have demonstrated the subcellular localization and non-invasive properties of this laser surgery technique. Using this tool, we looked at the mitochondria function in cells.

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