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Ultrafast laser nanosurgery in microfluidics for genome-wide screenings

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The use of ultrafast laser pulses in surgery has allowed for unprecedented precision with minimal collateral damage to surrounding tissues. For these reasons, ultrafast laser nanosurgery, as an injury model, has gained tremendous momentum in experimental biology ranging from *in vitro* manipulations of subcellular structures to *in vivo* studies in whole living organisms. For example, femtosecond laser nanosurgery on such model organism as the nematode *Caenorhabditis elegans* has opened new opportunities for *in vivo* nerve regeneration studies. Meanwhile, the development of novel microfluidic devices has brought the control in experimental environment to the level required for precise nanosurgery in various animal models. Merging microfluidics and laser nanosurgery has recently improved the specificities and increased the speed of laser surgeries enabling fast genome-wide screenings that can more readily decode the genetic map of various biological processes.

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Introduction

Nanosurgery using ultrafast laser pulses permits precise ablation of cellular and subcellular structures with minimal collateral damage and without compromising the cellular viability. Its nanoscale precision and minimal invasiveness allow studying the cellular organization from interactions of subcellular organelles to biological pathways. Laser nanosurgery is versatile enough to resect subcellular structures in cultured cells and in small living multicellular organisms for the purpose of dissecting the biological role of organelles and assessing the kinetics of regeneration. When linked to advances in microfluidics, this approach can be used to reveal subcellular and molecular function at the nanoscale.

In the same way that recent advances in microfabrication techniques have impacted the electronics industry, microfluidics promises a great impact in fields ranging from analytical chemistry to biology and medicine. Microfluidic devices can increase throughput and decrease cost by densely integrating complex assays and analytical measurements in a chip format. Among potential advantages of microfluidic devices, the use of parallel sample processing and/or automation make them ideally suited for high-throughput screening applications. The use of high-throughput microfluidic devices has recently been used to refine a number of biological assays and procedures such as DNA microarrays, enzymatic reactors, cell or larvae sorting, and stimuli testing [1–3].

The merging of the fields of nanosurgery and microfluidics has enabled large-scale screening with nanoscale manipulation, and has led to the ability to perform drug and gene screens on complex biological phenomena such as nerve regeneration. These technological advances can be used to rapidly reveal the biochemical basis of multifaceted processes.

Ultrafast laser nanosurgery

The advantage of using ultrashort (picosecond and femtosecond) laser pulses in tissue ablation is their ability to evaporate an extremely small volume of tissue with minimal heating or damaging the surrounding cells. It is the nonlinear nature of the ultrafast laser–tissue interaction that leads to the three-dimensional submicron confinement of the laser absorption below the surface. The high peak intensities of ultrashort laser pulses provide a high flux of photons that can be absorbed nonlinearly by the electrons. The ultrafast duration of the absorption process leads to rapid and efficient plasma generation where the beam is focused. Therefore, pulse energies as low as a few nano-Joules are sufficient for ablation of subcellular structures when the beam is focused to submicron spot size.

The damage mechanism of ultrafast laser nanosurgery depends on the pulse intensity (energy per area per time), total number of pulses, and repetition rate of the laser and has only a slight dependence on the wavelength. The absorption of ultrashort laser pulses produces free electrons through multiphoton ionization, electron tunneling, and cascade ionization. Depending on the density of these free electrons, several different damage mechanisms can lead to the dissection effect of tissue [4]. A summary of these damage mechanisms is provided in

Table 1

Damage mechanisms in ultrafast laser nanosurgery

Damage mechanism	Photochemical damage	Thermoelastic stress confinement	Plasma-mediated ablation
Intensity threshold	0.26×10^{12} W/cm^2	5.1×10^{12} W/cm^2	6.54×10^{12} W/cm^2
Electron density at threshold	2.1×10^{13} cm^{-3} One free electron in the focal volume	0.24×10^{21} cm^{-3} Induced thermal stress overcomes the tensile strength of water	1.0×10^{21} cm^{-3} Critical electron density for optical breakdown
Description	Free electrons participate in chemical reactions to form destructive reactive oxygen species and lead to breaking of chemical bonds	Since thermalization of the plasma occurs faster than the acoustic relaxation time, confinement of thermal stresses leads to formation of nanoscale transient bubbles	The damage is created by the high pressure and high temperature plasma and the accompanying shock wave and cavitation bubble
Pulse repetition rate	>1 MHz A large number of pulses are required. For practical reasons high repetition rate lasers are preferable	<1 MHz Bubble lifetime is 100–500 ns. We need avoid heat accumulation and long lasting bubble formation	

The intensity thresholds and the free electron densities were estimated for 100 fs, 800 nm laser pulses focused with a 1.3 NA lens [4].

Table 1. For a comprehensive discussion of the fundamental physics of ultrafast laser nanosurgery, we refer the reader to a review by Vogel *et al.* [4].

For the past 15 years, ablation by ultrashort laser pulses has earned a considerable interest among scientists as a precise surgical tool for biological studies. Over the past five years, its application in living cells has proven invaluable for the removal of organelles, such as mitochondria and lysosomes, and for the cutting of subcellular structures, such as actin filaments, microtubules, or mitotic spindles. Recently, a study on cellular stress fibers has given invaluable information on the shape stability of cells [5]. Not only the viscoelastic properties of stress fibers have been proven, but it was also shown that the stress fibers participated in the extracellular matrix compliance and the overall cellular shape. For example, the disruption of a single stress fiber of a cell anchored on compliant extracellular matrix compromises the entire cellular force balance and contributes to a large cell elongation (more than 5% of the cell size) [5]. A complementary study investigated the location dependence of microtubule depolymerization rates [6]. Such depolymerization can be as much as four times faster around the cell nucleus than in the periphery of the cell, confirming the interconnection of stress fibers with extracellular matrix components, which can affect the dynamics of the cellular skeleton. The comprehension of stress fiber and microtubule mechanics may lead to a better understanding of the healing of wounds or the malignant transformation of tumors.

Ultrafast laser ablation has also been used for the transfection of membrane impermeable substances into cells through cellular membrane poration [7], with efficiencies as high as 80% when cell viability is taken into account [8]. Transfection has also been demonstrated by using Bessel laser beams instead of the conventional Gaussian beams [9]. Bessel beams are radiative electromagnetic fields whose amplitude is described by a Bessel function. Unlike Gaussian beams, they are not diffracting. They are obtained by focusing Gaussian beams through an axicon. With these Bessel beams, transfection was achieved over axial distances 20 times greater than those of Gaussian beams: at 20% transfection efficiency, the focal spot may be as far as 90 μm on either side of the membrane when using Bessel beams, whereas it had to be within 4.5 μm of the membrane with Gaussian beams [9]. However, confinement of the focal volume is critical to achieve ablations with submicron precision and minimal collateral damage within the tissue.

In vivo application of ultrafast laser surgery has recently provided new unique injury models by manipulating structures *in vivo* inside whole living organisms. It has been successfully applied to severe axons [10] and dendrites of neurons [11], blood vessels [12], and membranes of embryos (chorion) [13], whose dimensions may vary from microns to as small as a couple of hundred nanometers. The biological response to such nanosurgeries may be best assessed when performed in a whole organism *in vivo*. In such a study in 2004, we showed nerve regeneration in the live nematode *Caenorhabditis elegans* after injuring the axons (axotomy) of the motor neurons with femtosecond laser pulses [10]. Since then, axotomy by femtosecond laser pulses has been used to investigate nerve regeneration in the worm *C. elegans*. Studies showed that nerve regrowth and guidance depend on the type of neuron and on the age of the worm whether axotomy was performed during development or at adult stage [14], and that the molecular and cellular mechanisms involved in nerve growth and guidance are distinct during and after development of the worm [15]. Ultrafast laser nanosurgery has been used on other model organisms as well. In 2007, DNA was delivered into dechorionated embryos of zebrafish (*Danio rerio*), through pores created at the

blastomere–yolk interface by femtosecond laser pulses [13]. Chorionated embryos that underwent the same procedure survived, proving that the laser beam travelling through the chorion did not damage it [13,16••]. On larger animals, mice, *in vivo* nanosurgery on dendrites of cortical neurons revealed the morphological changes of dendritic spines [11]. This observation of neuronal morphofunctionality opens the path to studying neuronal circuitry and its response to injury.

As one can imagine, immobilization of samples and localization of targets are paramount before nanosurgery that requires submicron accuracy. These two prerequisites can become problematic to implement. The conventional method to immobilize animals relies on the use of anesthetics. However, we showed that in the case of the *C. elegans*, anesthetics did have an effect on nerve regeneration and thus could influence the outcome of nerve regeneration or degeneration studies [17••]. The ideal method of immobilization would be a mechanical trap. Microfluidic devices are the solution.

Microfluidic devices for laser nanosurgery

Since the revolution that soft lithography brought to microfabrication [18], scientists have long exploited microfluidic systems, and their applications in biology [1–3], starting with cells and continuing to more complex biological systems like eggs, embryos, and larvae. Following the same trend, the microfluidic devices have also evolved to highly integrative systems that can monitor whole cell cultures [19] or profile the gene expression of human embryonic stem cells [20].

Several unique properties of microfluidics make them ideal for their application in biology: first, the availability of simple and cheap microfabrication techniques; second, the use of transparent materials such as glass or PDMS, allowing transmission of light for optical imaging and manipulation; third, small dimensions on the order of 10–100's of microns, providing precise and fast manipulation of biological systems and reducing the use of chemicals to small amounts; fourth, the scalability to handle a large sample population either in parallel or in series for high throughout; and fifth, the possibility to interface with the currently available robotic handling technologies that use microtiter plates, thus enabling the screening of a large collection of chemical compounds in a rapid, automatic, and parallel fashion.

When considering the use of various light manipulation techniques, such as laser nanosurgeries, optical tweezers, and light stimulations, microfluidic devices can provide the ideal laboratory environment on-a-chip, especially, unique experimental conditions necessary for studying small multicellular model organisms [21]. Several microfluidic devices have recently been developed for handling model organisms such as the fruit fly *Drosophila melanogaster* [22] and the nematode *C. elegans*.

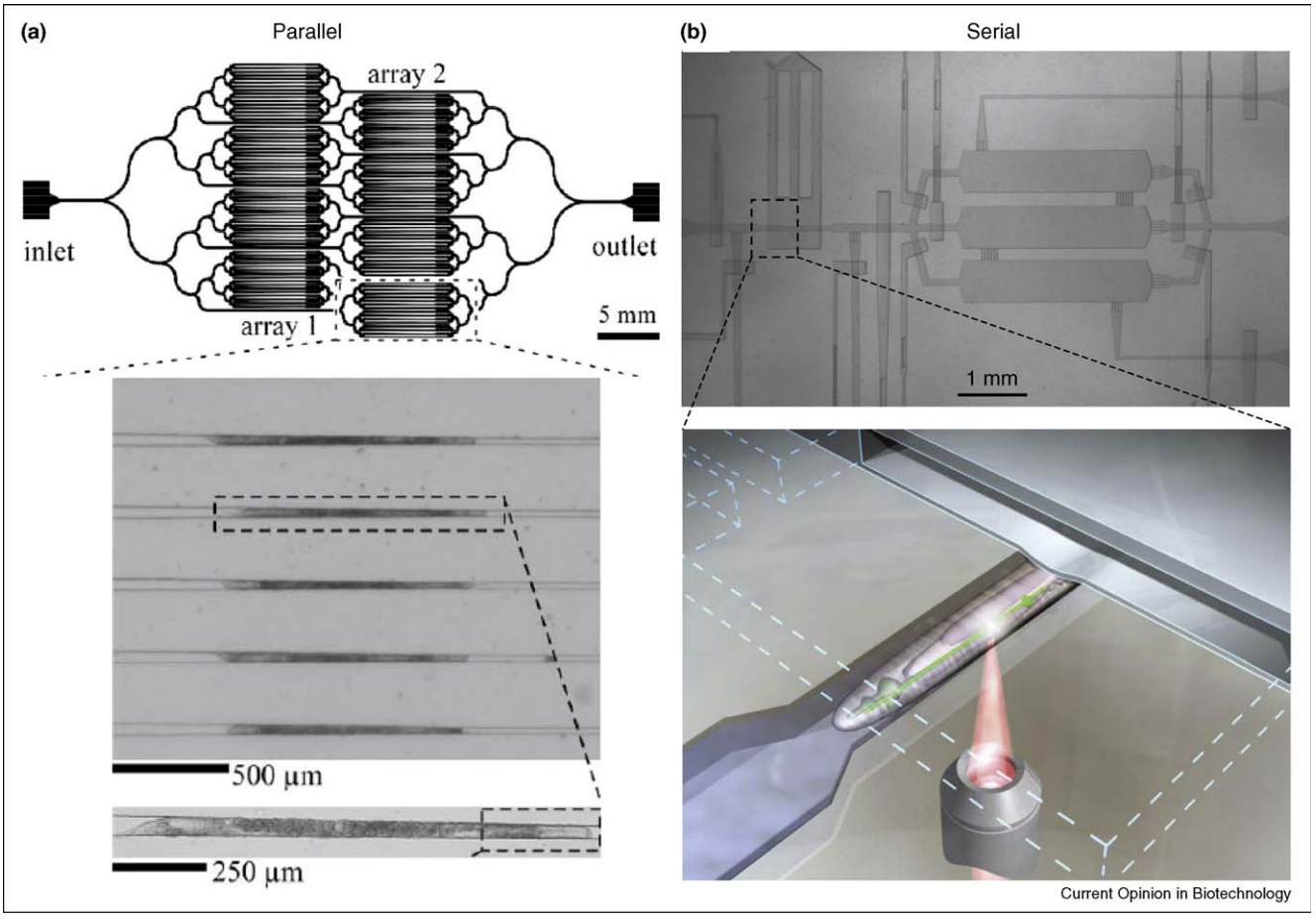
The first microfluidic devices to have been employed with *C. elegans* were designed to control environmental conditions to cultivate the worms [23] or to study the chemical-dependent, mechanical-dependent, and environmental-dependent behavior of worms [24,25]. Later, with the development of various worm-trapping methods [26,27], it became feasible to image the neuronal activity to these chemical and mechanical stimuli [28,29] and to capture and sort worms for high-throughput screening [30••]. Most recently, novel-trapping methods provided the ability to immobilize worms with very high accuracy to perform precise laser nanosurgeries [11,17••].

Two different trapping approaches have been explored for performing laser nanosurgery. The first approach (Figure 1a) utilizes single-layer geometry where worms are squeezed in tapering down channels in a microfluidic maze structure [31•]. This approach allows for rapid immobilization of a large number of worms in parallel. It has been shown that the worms can be recovered after being trapped for a short period of time without any effect on viability. Such approach was later used to keep the worms immobilized for the ablation of single synapses and for the duration of the follow-up observations up to five hours [32•].

The second approach (Figure 1b) adopts a two-layer configuration for serial trapping of worms [17••]. The bottom layer against the glass slide houses the worms in liquid and the upper layer contains the pressurized air that controls the mechanical trap. In between these two layers, a thin PDMS membrane exits in the trapping area that is deflected downward, pressing the animal against the glass when pressure is applied in the upper microchannel. Using this approach, we could completely immobilize worms and perform the first *in vivo* nanoaxotomy and subsequent time-lapse imaging of regrowing axons, in the absence of anesthetics. The precision and accuracy of this mechanical trap was similar to those achieved on agar pads with anesthetics. An additional study adopted the two-layer design described above and showed that their original immobilization technique based on suction [33] was improved greatly, finally providing the precision necessary to perform nanosurgery [34]. We also discovered that axonal regeneration on-a-chip occurs faster than previously obtained for both touch and motor neurons showing how microfluidic devices can be effective in avoiding the unwanted side effects of anesthetics [17••].

These two microfluidic trapping approaches possess many advantages over the conventional immobilization techniques that were formerly used in the studies of *C. elegans*, such as anesthesia on agar pads or glue, including:

Figure 1



Examples of parallel and serial integration of laser nanosurgery in microfluidic devices trapping whole living model organisms, *C. elegans*, for *in vivo* neuronal studies. **(a)** The parallel approach uses a single-layer chip where the worms are trapped in parallel in the device consisting of 128 tapered channels. The worms are pushed by constant pressure until they are trapped along the tapering channels [31•]. **(b)** The serial approach utilizes a double layer design where the worms are pressed down by a flexible membrane activated by pressurization of the upper layer. The worms are sequentially operated in the trap and sent to either any external storage device or the recovery chambers as shown in the picture [17••].

first, no chemical other than the liquid growth medium will interfere with the physiological processes of the worms; second, the worms do not need a recovery period after surgery, permitting immediate behavioral study of the postaxotomy functionality; and third, the sample population is well contained and experiment conditions are easily reproducible since the trap for surgery and the environment for recovery are on the same chip.

Both approaches can easily be adapted to immobilize other model organisms and to incorporate other manipulation techniques, such as optical trapping [35], and light stimulation [36,37]. The two-layer approach can also provide an additional benefit of serial immobilization where model organisms can be trapped and manipulated sequentially, widening the possibilities of high-throughput biological investigations. For example, the incorporation of full automation and the interfacing of the microfluidic device to multi-well plates [33,38] will greatly facilitate genome-wide reverse screening using RNA-interference.

Conclusion

Ultrafast laser nanosurgery undergoes a tremendous research momentum because of the need of understanding biological phenomena using small model organisms. The submicron precision of the ultrafast laser pulses does indeed guarantee minimally invasive surgeries. Combining laser nanosurgery with microfluidic devices permits the user to control both the environment of the sample and its immobilization — the latter being particularly imperative because of the accuracy requirement of nanosurgery, and it allows rapid high-throughput screening at low costs. The two aforementioned approaches integrating these two technologies are devoted to studying nerve regeneration and degeneration in the nematode *C. elegans*.

But applications of nanosurgery microchips are endless when one considers how far both technologies have progressed and how diverse their uses in biology and medicine are. All it takes is integration.

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