Femtosecond Laser Nano-Surgery for Nerve Regeneration Studies

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***Abstract*- Ultrashort laser pulses are increasingly being used to ablate subcellular structures inside living cells and multi-cellular organisms. We have recently demonstrated the feasibility of nano-axotomy in the nematode *Caenorhabditis elegans* (*C. elegans*) and observed nerve regeneration of the severed axons. Neural regeneration process can be impaired by the amount of collateral damage. Such damage is due to expansion of the thermoelastic stress-induced cavitation bubbles and the shock-waves expanding beyond the focal volume of the laser beam. This study presents a systematic characterization of the extent of damage in *C. elegans* created by 100-fs pulses at 1-kHz repetition rate with energies varying from 2-nJ to 20-nJ per pulse and the total number of pulses ranging from 1 to 1000. The minimum energy required for ablation greatly depends on the total number of pulses used. While a single shot of 21-nJ of energy can severe an axon, the threshold drops to as low as 3.8-nJ for 1000 pulses.**

I. Introduction

Microsurgery using nanosecond (ns) laser ablation has been used for the study of the biological systems since early 80's [1]. However, ablation with ns pulses is largely a thermal process depositing significant heat into the targeted tissue. The excess of heat causes the nearby cells to heat to the point where the surrounding tissue suffers collateral damage. This unwelcome effect greatly limits surgical precision and challenges the applicability of ns-laser microsurgery techniques in sensitive regions, such as the nervous system.

In contrast, when lasers operate in the femtosecond (fs) regime, the ablation process requires substantially lower pulse energies. The large peak intensities of fs-laser pulses rapidly ionize the tissue inside the focal volume with only a few nano-Joules of pulse energy while causing very limited thermal damage to the surrounding tissue. Using fs-laser pulses of only 10nJ of energy, we have succeeded to cut individual axons in the nematode *C. Elegans* and studied nerve regeneration of the severed axons [2]. The high precision of fs-laser nanosurgery retained the tissue surrounding the severed axon undamaged and provided the first demonstration of the long-hypothesized neural regeneration in *C. elegans*.

To better understand the fundamentals of fs-laser nano-surgery for nerve regeneration, we have characterized the extent of damage as a function of laser energy and number of pulses and further explored nerve regeneration in *C. Elegans*.

II. Nerve Regeneration

The nematode *C. elegans* is a versatile and most widely used model organism for experimental studies in neurobiology. It consists of only 302 neurons with well-defined functionality and wiring diagram that are similar in every adult worm. Its simplicity and the availability of genetic tools, make *C. elegans* the most powerful model organism for in-vivo studies of nerve regeneration and degeneration. The application of fs-laser axotomy techniques in these animals will help in the rapid identification of genes and molecules that affect nerve regeneration and degeneration.

We have performed nanosurgery on the axons of both touch and motor neurons of *C. elagans*. The worms were first anesthetized using phenoxy-propanol and the surgery was performed by tightly focusing (using NA=1.4) 400 laser pulses (100-fs) with energy of 10-nJ per pulse.

Figure 1 shows an example of fluorescence images of a GFP-labeled axon (touch neuron) before and after axotomy. Right after the surgery, the proximal and distal ends of the worm were about 2-µm apart as observed by the disappearance of the GFP signal in Fig. 1b. After 12 hours, the proximal end regrew and connected with the distal end (Fig. 1c). Dye-filling of axotomized neurons (phasmid neurons) confirmed that the observed axon gaps were not due to photobleaching, but due to physical disconnection of the axons.

To evaluate functional recovery associated with nerve regeneration, we also tested the behavior of the operated worms. Through studies of backward motion, related to motor-neuron function, we showed that the worms recovered their functionality after their motor neurons were severed [2]. A movie demonstrating the recovery of worms can be found at http://www.me.utexas.edu/ben-yakar/files/movies/regeneration.avi.

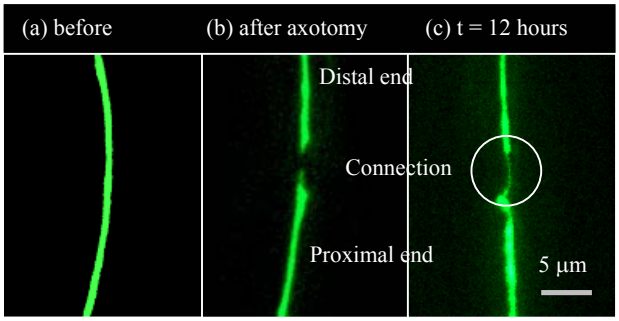

Fig. 1. Fluorescence images of axons before (a) and right after the axotomy and regrowth of the severed axon after 12 hours (c).

III. Extent of Damage

The assessment of damage and resulting response of the tissue after laser operation is important for understanding tissue permissivity factors on nerve regeneration. Topologically, the motor neuron processes traverse muscle cell membranes that are less than 100-nm away. To assess the extent of the peripheral photo-damage (including both ablation and photobleacing), we used a strain where both nerve processes and muscle cell membranes that are in close proximity to the axons were labeled by GFP. We severed the GFP labeled axons with varying pulse energies and measured ensuing surrounding damage by loss of GFP signal [3].

Figure 2 shows fluorescence images of axons and their surrounding muscles after axotomy with different pulse energies and number of pulses. The observed damaged (dark) region consists of a central region where the tissue is actually removed (ablated/evaporated) and a peripheral region where GFP is photobleached.

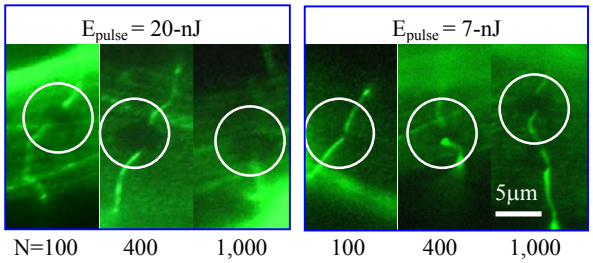

Fig. 2. Fluorescence images of the extent of damage induced by fs-laser ablation for two different pulse energies and different number of pulses.

Figure 3 summarizes the measured size of the total extent of photo-damage. For each number of pulses, the damage increases rapidly with pulse energy. Above 30-nJ, the large extent of damage raptures the worm. For each energy level, there is a minimum number of pulses that initiates ablation. For example, at 2-nJ, there is no ablation even for 100,000 pulses (100 seconds of laser exposure) while at 4-nJ, ablation is observed by using as few as 1,000 pulses. Once ablation is initiated with the minimum number of pulses, the amount of ablation does not increase much by a longer exposure to the laser.

A statistic evaluation of the error for the ablation size was done on based on the ablation of 10 different axons of several worms. When using more than 100 pulses, the size of the extent of damage varied slightly (0.3-0.4-μm) from experiment to experiment, while when using less than 75 pulses, this variation became much larger (about 1-μm) due to scattering and absorption of the cuticle and accumulated bacteria that vary from worm to worm.

We also measured the minimum energy required to observe ablation for different pulse trains. In the single-shot mode, the threshold energy is 21-nJ. At 25 pulses, the threshold decreases to 8-nJ, going down as the number of pulses increases: 7.2-nJ at 50, 6.3-nJ at 75, 5.6-nJ at 100, 5-nJ at 200, 3.8-nJ at 1,000 pulses to finally reach 2.6-nJ at 10,000 pulses and beyond. These observations indicate the necessity to use sufficiently low pulse energies to reduce the extent of photo-damage to the surroundings in laser surgery of tissue.

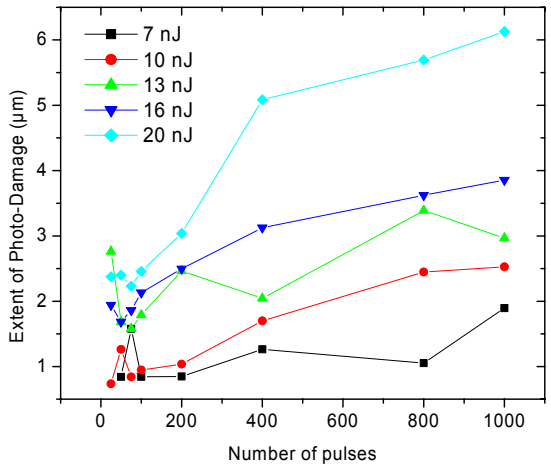

Fig. 3. Extent of photo-damage induced by fs-laser ablation of axons in *C. elegans* as a function of pulse energy and total number of pulses.

IV. Prospects

The characteristics of fs-laser ablation make this technique an attractive alternative to conventional lasers for microsurgery applications. For example, fs-laser ablation is well suited for removal of small cancerous and precancerous lesions in the epithelium, which is where 85% of cancers first originate. By detecting and removing cancerous cells in this early stage, the cancer can be eliminated before it ever poses a risk to the patient.

To this end, we are currently developing a small endoscope-on-a-chip capable of detection and treatment of oral cancers. By taking advantage of the pathological changes in endogenous fluorophores for cell differentiation, lower-energy fs-pulses can detect cancer cells via two-photon microscopy and then remove the cells with higher-energy pulses.

To further aid this process, small metallic nanoparticles can be conjugated to the cancerous cells, allowing for deeper imaging and targeted ablation by using the near-field enhancement effect of plasmonic scattering. Although our current work focuses on the diagnosis and treatment of oral cancers, this technology is well suited for many diseases in sensitive areas where precise confinement of damage is a top priority, such as in the brain or vocal cords.

Acknowledgement

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