Supplementary Information

"Axon regeneration in C. elegans after femtosecond laser axotomy"

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Methods:

The worms were maintained on standard NGM agar plates at 20^{o}C. Axotomy is performed on L4 stage worms. For axotomy and following fluorescence imaging of axons, the worms were anesthetized for short periods (15 minutes) on agarose pads with 5 μl phenoxypropanol per 1ml agarose. Sham operated worms were also subject to the same procedure, but the laser beam was focused 5-10 μm distance away from the axons instead of focusing on them.

The femtosecond laser axotomy technique is quite rapid. It takes less than a minute to find, position, focus and cut an axon. Including the time to prepare slides, anesthetize worms, cut 15 D-type axons, and recover axotomized worms, the entire process is completed within 10 minutes per worm. We found that with 40nJ pulse energy, the damage spot size is optimal so that we can focus the laser on the target easily and perform axotomy rapidly. We can cut axons with 100% success rate.

Behavioral Studies:

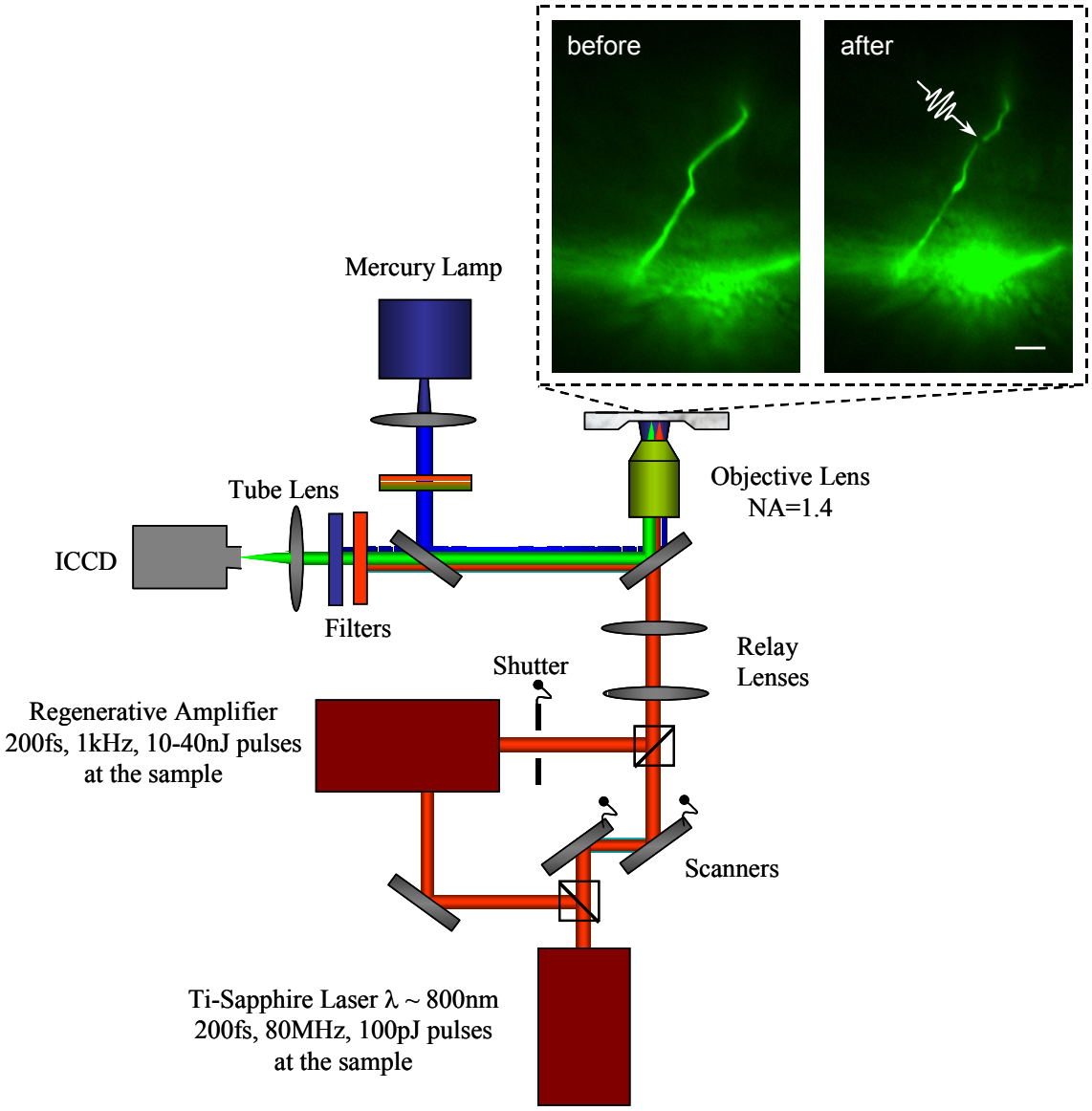
We scored behavioral recordings of axotomized and sham operated *juIs76* animals for backward movements. By using criteria such as, number of backward waves upon each touch to the head, presence or absence of spontaneous backward movement, deviation from wild type sinusoidal movement, each movie was given a general score. All the movies were scored blindly for both experimental conditions and recording time. The scorings at multiple times were always consistent. Worms were recorded at (or around) 3, 12, 24, 36, 48, 54, and 60 hours after axotomy. 6 worms were sham operated and showed wild type behavior. 17 worms were axotomized and scored as follows:

Score 1: No backward movement, animal shows shrinker behavior.

Score 2: Animal can move back up to two waves. It uses segmental muscle contractions instead of sinusoidal ones.

Score 3: Animal can move backwards more than two waves, but uses segmental moves instead of sinusoidal ones.

Score 4: Animal can move backward four or more waves by using sinusoidal muscle contractions - WT behavior.

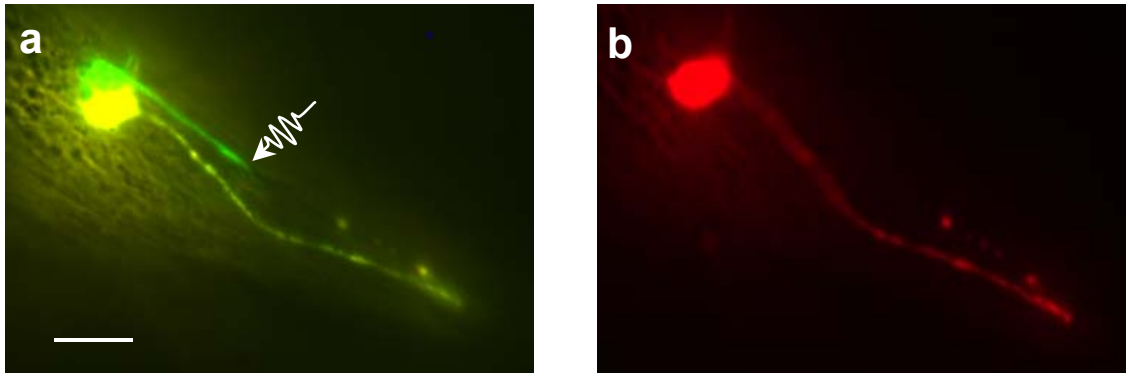

before
after
Mercury Lamp
Tube Lens
ICCD
Filters
Objective Lens
NA=1.4
Relay
Lenses
Shutter
Regenerative Amplifier
200fs, 1kHz, 10-40nJ pulses
at the sample
Scanners
Ti-Sapphire Laser λ ~ 800nm
200fs, 80MHz, 100pJ pulses
at the sample

Supplementary Figure 1: Experimental set-up for femtosecond laser axotomy. A tunable Ti-Sapphire mode-locked laser (Coherent, "Mira 900") produces 200 fs short pulses with a repetition rate of 76 MHz and 5 nJ energy. For axotomy, a regenerative amplifier (Positive Light, "Spitfire") seeded by the Ti-Sapphire laser generates 1 mJ energy, 200 fs short pulses at 1 kHz repetition rate. The laser energy on the specimen can be precisely varied using two attenuators. Each attenuator involves an electronically controlled rotating half-wave plate that rotates the polarization of the laser beam and a cube beam splitter in which the intensity of the transmitted light depends on its polarization. A fast mechanical shutter is used to select the desired number of pulses for the ablation experiments. The laser beam is tightly focused inside the sample through an oil-immersion high numerical aperture objective lens (Zeiss 64×, NA=1.4). The fluorescence imaging system consists of a mercury lamp, and an FITC filter set. The excitation filter and the emission filter transmit wavelength ranges of 460-500 nm and 510-560 nm, respectively. The dichroic mirror reflects wavelengths smaller than 510 nm. A cold mirror is used to transmit the laser beam but reflect the fluorescence emission towards either an intensified camera (Roper Scientific, "PI-Max") for two-photon imaging or an interline transfer CCD camera (Princeton Instruments, "Micromax") for epifluorescence imaging. The resulting emission from the focal spot is collected by the tube lens and detected by the CCD camera (6.5 μm pixel size and 2×2 binning). A filter blocks the scattered laser light. A camera exposure time of 300 to 600 ms is used. Simultaneously with laser axotomy, the system can also perform two-photon imaging with a pair of piezo-driven mirrors that scan the laser beam. A relay-lens system with a pair of lenses (focal lengths of 75 mm & 160 mm) are used to image the center of the scanning mirrors to the back aperture of the objective lens to prevent laser beam displacement during scanning. This relay lens system also enlarges the laser beam to fill the back aperture of the objective lens and to achieve diffraction limited focusing. **The inset** on the right upper corner shows an example of femtosecond laser axotomy with 10 nJ pulse energy (200 fs duration, 400 pulses at 1 kHz rate). Fluorescence images of a D-neuron axon before and 10 minutes after axotomy are shown. The rightmost image shows a photo-disrupted region with 1.2 μm length. This indicates that a volume of a few femtoliters is ablated at the focal point. Assuming an axon diameter of 0.3 μm, we estimate that a volume of the axon less than 0.1 femtoliter (1.2 μm × π (0.15 μm)^2) is ablated. Scale bar of the inset is 5 μm. The ventral part of the worm is towards the bottom of the image.

Dye-filling Studies to Assess Axotomy, Specificity and Damage Extent:

In order to test whether the observed axon gap after axotomy is simply due to photobleaching or not, we have stained phasmid neurons with DiO-green before cutting the dendritic processes that connects the cell body to the sensory ending. After cutting the dendrites of neurons, we have stained with DiI-red. While the axotomized neurons showed no red fluorescence (Supplementary Figure 2), their distal ends and the unoperated neurons were filled with DiI-red. Thus, although outside-connected distal stumps can take up the dye, they cannot pass it to the neurons due to physical disconnection.

In DiO/DiI staining experiments, we can specifically cut one of the dendrites among two nearby dendrites (only few microns apart) while leaving the other dendrite completely intact (Suppl. Fig. 2). This experiment shows that not only the damage extent is less than a few microns, but also we can selectively cut nerve processes.



Supplementary Figure 2. Axotomy of PHAR phasmid neurons. Prior to axotomy, two nearby phasmids have uptaken DiO-green dye. (a) After axotomy of one of the two nearby dendrites, they are visualized with FITC (green) filter. The other dendrite is not axotomized. (b) Worms are incubated in DiI-red dye. The phasmid whose dendrite was cut is not visible with red optical filter indicating that its dendrite is physically disconnected and the phasmid cannot update DiI-red dye. The other phasmid that was not axotomized fluoresces red. The scale bar is 5μm. The arrow is indicating the axotomized part of the dendrite. The tail of the worm is towards the right bottom corner of the image.

GFP fluorescence photobleaching and recovery studies:

We have also performed controlled photobleaching experiments with our setup by exposing cell bodies to laser pulses with 1-2 nJ energy at 1.0 kHz repetition rate. We have completely photobleached both touch and motor neurons, and observed that fluorescence signal could recover from complete photobleaching within 3 hours. The recovery duration is consistent with previously reported photobleaching studies using Argon Ion laser based confocal microscopy [11], which show that completely photobleached neurons are able to synthesize and recover GFP fluorescence within 3 hours. Furthermore, immediately after we photobleach all motor neurons, the worms can still move backwards exactly like the wild type. In the regime of the pulse energies (10-40 nJ) we used in axotomy, the neurons never recover GFP fluorescence even after 6 hours, indicating that a permanent damage occurs instead of simple photobleaching. The worms also cannot move backwards.

Furthermore, during photobleaching, the entire neuron loses its fluorescence intensity instead of the local laser spot that is being photobleached. This indicates that GFP molecules rapidly diffuse in and out of the photobleached spot, and that only a localized region cannot stay as photobleached. This is also another indication that the local gap observed after axotomy is not simply photobleached.

Aberrated Growth Cases:

For the 10-40 nJ energies we use for axotomy, we observe cases where aberrant growth or no-regrowth occurs, which is also suggestive of successful axotomy. If our observations were simply photobleaching, no aberrant growth should have been observable. In fact, operated axons mostly show a "de tour" extension.

Movie:

Behavioral response of an axotomized worm. The movie includes two parts: Part I shows an axotomized worm 3 hours after the axotomy. The worm can move forward like wild type. When we touch to its head, it attempts to move backwards, but it cannot move, instead its body segments shrink. Part II shows the same worm 24 hours after the axotomy. It is now able to move backwards with sinusoidal body motions when we touch to its head.

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