

Video Article

# In vivo Laser Axotomy in *C. elegans*

Alexandra B. Byrne<sup>\*1</sup>, Tyson J. Edwards<sup>\*1</sup>, Marc Hammarlund<sup>1</sup>

<sup>1</sup>Department of Genetics, Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine

\*These authors contributed equally

Correspondence to: Marc Hammarlund at [marc.hammarlund@yale.edu](mailto:marc.hammarlund@yale.edu)

URL: <http://www.jove.com/video/2707>

DOI: [doi:10.3791/2707](https://doi.org/10.3791/2707)

Keywords: Neuroscience, Issue 51, *C. elegans*, axotomy, regeneration, GABA neurons, pulsed laser, in vivo

Date Published: 5/19/2011

Citation: Byrne, A.B., Edwards, T.J., Hammarlund, M. *In vivo* Laser Axotomy in *C. elegans*. *J. Vis. Exp.* (51), e2707, doi:10.3791/2707 (2011).

## Abstract

Neurons communicate with other cells via axons and dendrites, slender membrane extensions that contain pre- or post-synaptic specializations. If a neuron is damaged by injury or disease, it may regenerate. Cell-intrinsic and extrinsic factors influence the ability of a neuron to regenerate and restore function. Recently, the nematode *C. elegans* has emerged as an excellent model organism to identify genes and signaling pathways that influence the regeneration of neurons<sup>1-6</sup>. The main way to initiate neuronal regeneration in *C. elegans* is laser-mediated cutting, or axotomy. During axotomy, a fluorescently-labeled neuronal process is severed using high-energy pulses. Initially, neuronal regeneration in *C. elegans* was examined using an amplified femtosecond laser<sup>5</sup>. However, subsequent regeneration studies have shown that a conventional pulsed laser can be used to accurately sever neurons *in vivo* and elicit a similar regenerative response<sup>1,3,7</sup>.

We present a protocol for performing *in vivo* laser axotomy in the worm using a MicroPoint pulsed laser, a turnkey system that is readily available and that has been widely used for targeted cell ablation. We describe aligning the laser, mounting the worms, cutting specific neurons, and assessing subsequent regeneration. The system provides the ability to cut large numbers of neurons in multiple worms during one experiment. Thus, laser axotomy as described herein is an efficient system for initiating and analyzing the process of regeneration.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/2707/>

## Protocol

### 1. Assembling the system

The specific components of our system are outlined below. When properly assembled, the user should be able to focus the microscope with one hand, move the stage with the other (either with the mouse or the joystick), activate the laser with the foot pedal, and have a clear view of the on-screen image.

1. MicroPoint laser mounted to the epi-fluorescence port of a compound microscope. We use a Nikon 80i, but any research-grade upright or inverted scope should work. The MicroPoint system will include a custom dichroic, which should match the fluorophore that labels the cells you wish to cut. Cutting cells labeled with alternative fluorophores requires additional dichroics. Additionally, a pulse generator and foot pedal come with the MicroPoint system.
2. 100x, 1.4 NA oil objective for performing surgery and assessing regeneration, plus additional objectives as necessary for locating the sample on the slide. We use a Nikon Plan Apo VC for surgery, and find that a 4x is useful for coarse focusing and finding the sample.
3. Motorized stage with joystick. A stage with extremely high accuracy and repeatability is unnecessary, since the stage is only used for targeting. We use a Prior OptiScan II (see discussion).
4. Camera. The camera needs to provide a frame rate sufficient to focus and target the labeled neurite with the 100x objective, while visualizing the image on the computer monitor. We prefer attenuating the illumination light during the axotomy procedure to avoid potential confounding effects from photo-bleaching or damage (see 1.7, below). Thus, the camera needs to be sensitive enough to accommodate this reduced light level. We use a Hamamatsu Orca 05G, and find that imaging at 16.3 Hz is sufficient. Equivalent cameras could be substituted.
5. Computer, monitor, and imaging software that can drive the camera. Additionally, we use the imaging software to manipulate the motorized stage with a mouse (see discussion).
6. Air table. This requirement may vary depending on location, but since surgery is performed with a 100x objective, we find vibration isolation to be essential.
7. Light supply with light guide and shutter mechanism for illuminating the fluorophore. The light guide will attach to the MicroPoint. The shutter mechanism is useful to reduce the illumination intensity independently of the laser. We use a Prior Lumen 200 or a Leica EL6000, and attenuate up to 80% of total light intensity for axotomy.

## 2. Laser setup

A detailed protocol to initially align and focus the laser is provided with the MicroPoint laser system. We assume that procedure has been followed successfully upon initial setup. This includes aligning the laser with the crosshairs in the eyepiece. Here we provide a protocol for regular maintenance of the laser's focus and intensity.

As noted in the MicroPoint manual, the pulsed nitrogen laser is capable of severely damaging the human eye. Care should be taken to never look directly into the laser beam. Once installed, the barrier filter should safely block radiation through the eyepieces.

Cutting neurons in an organism the size of a grain of sand can be challenging. Since the area of damage done by the laser is very small, it is essential to know the three-dimensional location of the laser's focus in order to target the sample precisely. The laser focus is found using the mirrored slide. First, we check that the z location of the laser focus matches the focus of the imaging path. Next, the x-y location of the damage focus is marked with crosshairs in the imaging software.

### Focusing in the z-plane

1. Push in the neutral density filter ND16 (Figure 1a). We use neutral density filters in the epi-illumination path to attenuate the laser while focusing. For even finer focusing, push in both the ND16 and ND4 fluorescent filters.
2. Set the MicroPoint laser to 1 pulse with the switch turned to 'Select' (Figure 1b), and then move the filter wheel to the appropriate long-pass barrier filter (Figure 1c).
3. Place the mirrored slide on the stage and focus on pinholes within it with the 4X objective using transmitted light.
4. Add a drop of immersion oil to the mirrored slide and refocus on pinholes with the 100X objective. Open the camera shutter with the optical path switching lever (Figure 1d) and the laser shutter (Figure 1e). If necessary, reduce transmitted light intensity so the edges of the pinholes are crisp: this will aid in focusing exactly.
5. Press the foot pedal. This should produce a small hole in the mirror if the laser is focused properly.
6. Focus the microscope slightly above the plane of the mirrored slide and shoot the laser again. This should make an even smaller hole than the first shot, or leave no mark at all. Repeat by focusing below the mirrored slide.
7. If a hole is not produced in 2.5, or if a larger hole is produced when focusing above or below the surface of the mirror, refocus the laser with the focus ring on the ablation head. Verify that the hole is still properly aligned with the crosshairs in the eyepiece. With consistent use, the z-plane focus rarely needs to be adjusted.

### Focusing in the x-y plane

8. Follow steps 2.1 through 2.3 above and press the foot pedal to make a small hole in the mirror slide.
9. Initiate 'Live' capture mode from the Elements 'Acquire' menu. In the 'Measure' toolbar select 'ROI editor', then select the 'double cross' tool and drag the crosshairs to align them with the center of the new hole. Click 'Save ROI' and then 'Exit editor' and resume Live capture mode.
10. Activate the 'mouse XY' setting in Elements to manipulate the stage with the mouse.
11. Pull the neutral density filter(s) back to the original position(s), set the laser pulses to 10, and remove the mirrored slide.

### Adjusting the power of the laser

12. The power of the laser should be adjusted to the minimum power necessary to cut the neuron of your choice. Adjust the power by moving the attenuator plate (Figure 1f) while cutting living worms (described below). If the laser power is too high, it will cause peripheral damage or blast a hole in the worm. If the laser power is too low, it will not sever the neuron. Once the system has been set up, the attenuator position rarely needs to be changed. If the laser becomes weak, and the attenuator plate needs to be moved to continue cutting, it could be a sign that the dye in the dye cell needs to be replaced (see discussion).

## 3. Immobilize the worms

1. Prepare a solution of 3% molten agarose in M9 (22mM KH<sub>2</sub>PO<sub>4</sub>, 42mM Na<sub>2</sub>HPO<sub>4</sub>, 85mM NaCl, 1mM MgSO<sub>4</sub>). Dispense 5 mL of molten agarose into a 15 mL falcon tube. Fill another tube with water. Place tubes in a modular heating element set to 55°C. Fill the heating element with water to create mini water baths for each tube. Note: agarose can be prepared in large quantities in advance and re-melted for subsequent experiments.
2. Place two layers of labeling tape on each of two slides and a clean slide between these slides. Dispense a drop of agarose using a plastic bulb pipette to the middle of the clean slide and place another slide perpendicular to the first so that the resulting agarose pad is the thickness of two pieces of tape (Figure 2). Remove one of the two slides from the pad after 30 seconds. Rinse and store the plastic pipette in the water-filled falcon tube for use with subsequent slides.
3. Next, place 3-5  $\mu$ L of 0.10  $\mu$ m or 0.05  $\mu$ m polystyrene beads in the center of the pad. Add 5-10 worms expressing fluorescent protein in the neurons of interest. If the neurons of interest are asymmetrically distributed on the right or left side of the worm, use a platinum pick to flip worms so that the correct side is facing up. Worms must be placed on slides within 10 minutes of preparing the agarose pads.
4. Carefully place a cover slip on the worms. After placing the cover slip, do not move it relative to the agarose pad as this will disrupt the cuticle and destroy the worms. Place the prepared slide on the microscope stage.

## 4. Cut neurons

1. Direct light into the eyepieces with the optical path switching lever. Focus on the worm to be cut with the 4X objective, place a drop of oil on the cover slip, and switch to the 100X objective. Return the optical path to the camera.
2. Use the mouse to move the neuron of your choice under the center of the crosshairs. Press the foot pedal to fire the laser and sever the neuronal process. If needed, two sets of pulses can be used to sever the neuron. At first, it is helpful to track precisely which neurons have

been severed in individual worms in order to assess subsequent regeneration. When performed correctly, laser axotomy produces a small break in the neuron without causing significant damage to the surrounding skin tissue or other neurons (see Figure 3). In certain cases, a small scar may form around the site of injury.

3. Recover the worms by removing the cover slip in a direct upward movement, being careful that the worms remain on the agarose pad. Do NOT slide the coverslip off. Then, cut the pad around the worms with needle-nose forceps and place the section of agarose on a freshly seeded NGM plate containing OP50<sup>8</sup>. Place 10  $\mu$ L of sterile M9 on the pad to free the worms from the beads and remove agarose.

## 5. Score neurons for regeneration

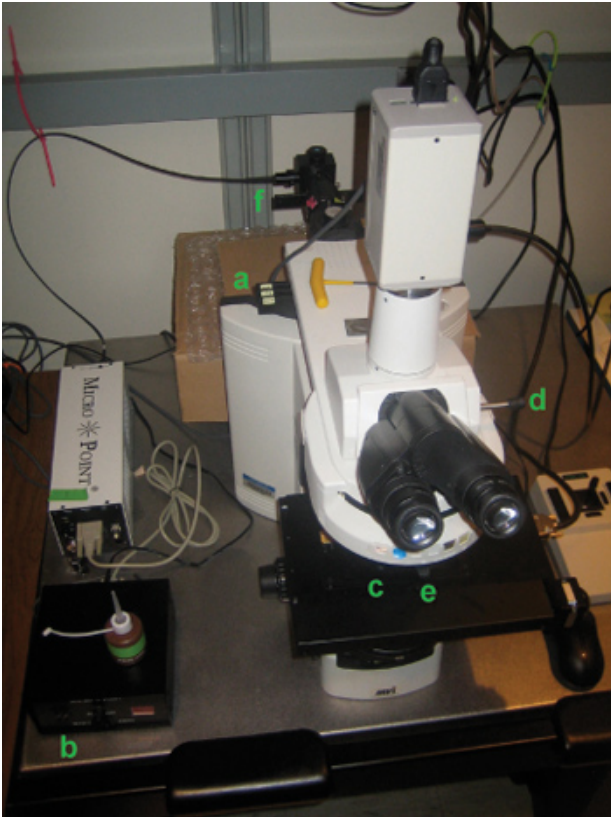
1. Prepare agarose pad as outlined in step 2.2.
2. Place worms in 3-5  $\mu$ L of polystyrene beads. Apply a coverslip. Slides can be prepared sequentially, or alternatively all the worms can be prepared in advance and the slides kept in 10 cm culture dishes, each containing a damp Kimwipe to keep the agarose moist.
3. Use the 100X objective to visualize the severed neurons. In many neurons, a neuronal stump distal to the cut site will remain. We use the presence of this remnant as an indication that the neuron was indeed severed (Figure 4). The presence of this stump makes it possible to distinguish a neuron that has been cut and regenerated from one that was not cut. Note: both the distal and proximal stumps initially retract after being cut.
4. Assess regeneration. A variety of parameters can be determined. One simple assay is whether the injured neuron has formed a growth cone (Figure 4a) and regenerated, or not (Figure 4b). Alternatively, neurite length can be traced and compared.

## 6. Representative results

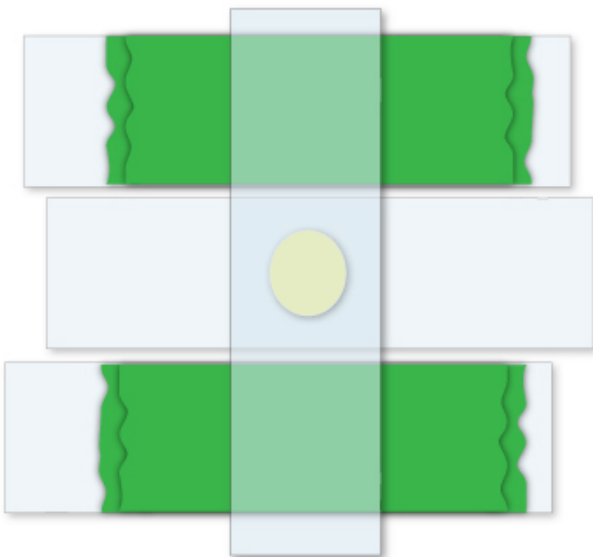
As an example, we describe characterization of regeneration of the  $\gamma$ -Aminobutyric acid (GABA) motor neurons. These neurons, which reside in the ventral nerve cord and extend processes circumferentially to the dorsal nerve cord, are essential for proper locomotion<sup>9</sup>. GABA neurons can be visualized by expressing a genetically encoded fluorophore, such as green fluorescent protein (GFP), under the control of the *unc-47* or *unc-25* promoters (strains EG1285 and CZ1200, respectively, available from the *C. elegans* Genetic Center).

Mount L4-stage worms as described, flip the worms so the GABA neurons on their right sides are facing up, and place the cover slip. Cut 1-3 of the posterior commissures in each worm, midway between the dorsal and ventral cords. Avoid cutting commissures that cross or fasciculate with other commissures, as these are difficult to score later. Recover worms as described.

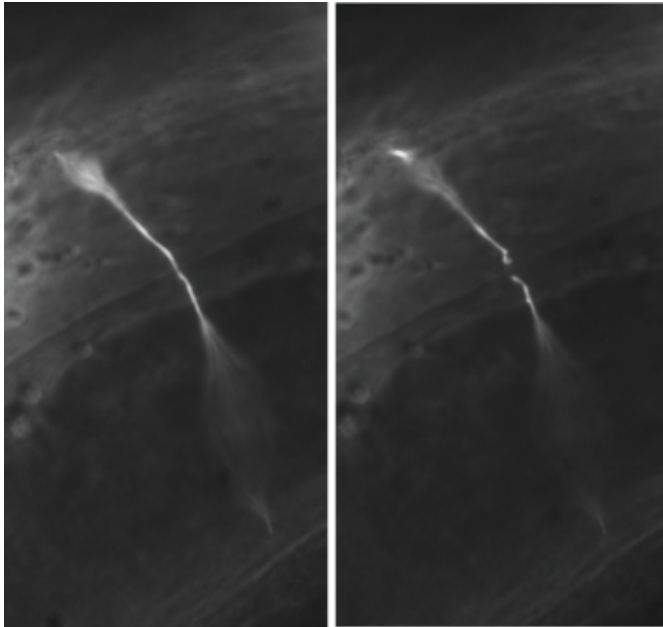
18 to 24 hours after a successful axotomy, the worms will have recovered from surgery and exhibit normal locomotory and egg-laying behaviors. Discard worms that are dead or sick. Remount the remainder as described, and assess regeneration. We usually aim to assess at least 30 cut neurons per experimental condition. In *ox/s12* animals, typically 60-70% of severed L4 GABA neurons will have initiated a growth cone structure, evidenced by a broadening of membrane at the tip and the extension of several neurite branches, and migrated from the cut site (Figure 4a). In many cases, the growth cones will have migrated to reconnect with the dorsal nerve cord, an adjacent neuronal commissure, or the distal stump. The remaining 30% of neurons will have either initiated no growth, forming a stump proximal to the site of axotomy, or will have extended only small filopodia (Figure 4b).



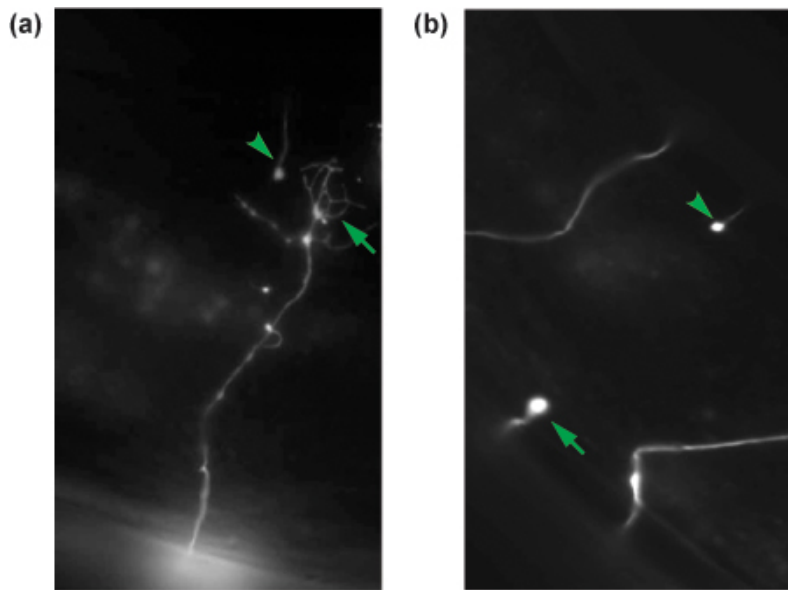
**Figure 1. The UV pulsed laser axotomy system.** Specific components are: (a) ND filters (b) pulse selector (c) filter wheel lever (d) optical path switching lever (e) laser shutter and (f) attenuator plate.



**Figure 2. Preparing an agarose pad.** To prepare an agarose pad of the desired thickness, two layers of tape (green) are placed on two slides. A slide is placed between the first two, and a drop of agarose is then added to the clean slide. Finally, a fourth slide is placed perpendicularly to the first three, resulting in a pad that is the thickness of two pieces of tape.



**Figure 3. Representative GABA neuron axotomies.** In a typical experiment, GABA neuron commissures are severed at the middle of the lateral aspect of the worm. A severed commissure is shown immediately before (left panel) and after (right panel) axotomy. All images were taken with a 100X oil objective.



**Figure 4. Representative results of GABA neuron axotomies.** 24 hr post-axotomy severed axons are scored for regeneration. A regenerating axon with a growth cone (arrow) is shown in (a) while a non-regenerating axon is seen as a proximal stump (arrow) in (b). The distal stump of each cut axon is shown in each panel (arrowhead) and is an indication that the axon has been severed.

## Discussion

A variety of laser systems have been used to cut neurites in *C. elegans*, and several studies have examined their performance in detail<sup>3,7,10,11</sup>. The MicroPoint laser used in our protocol is a turnkey system that is easy to set up and maintain, and is available at a low cost to researchers compared to a Ti-Sapphire laser system. Compared to a Ti-Sapphire system, however, the MicroPoint laser is expected to cause damage to a larger area, which may be disadvantageous for some applications. If a Ti-Sapphire system is desired, an excellent protocol on building such a system is available<sup>12</sup>.

The current protocol can be performed on a variety of neurons in *C. elegans*; however, we note that differences in regenerative abilities between distinct types of neurons are expected<sup>13</sup>. Additionally, different transgenic backgrounds can affect regenerative success. Although the percentage

of regenerating GABA neurons is fairly consistent between different transgenic marker strains, we have noted an overall slight increase in regeneration in *juls76*<sup>14</sup> vs. *oxIs12*<sup>15</sup> worms. Differences between markers of the touch neurons have also been described<sup>2</sup>.

We prefer to immobilize the worms with microbeads rather than anesthetics as the beads result in faster and more consistent immobilization<sup>16</sup>. This is also advantageous as we are able to observe regeneration free of any possible confounding effects due to anesthesia<sup>17</sup>. An alternative anesthetic-free method for immobilization is the use of microfluidic devices. The use of microfluidics for axotomy has been extensively described<sup>17-22</sup>.

We find that with consistent use, the laser functions best when the Coumarin 440 in the dye cell is changed once each week following the procedure outlined in the MicroPoint manual. If necessary, the attenuation slider can be used to increase power, but this may be an indication of old dye or laser misalignment. Also, the dye cell has a limited lifetime and will eventually need to be rebuilt or replaced (See Troubleshooting).

It can be difficult to maneuver the target neurite under the crosshairs that mark the laser focus, particularly if the animal is incompletely paralyzed. We find that a manual stage is not optimal for this purpose, although it is certainly usable. A joystick-controlled motorized stage is more precise, and we find that using software that supports image dragging with the mouse to move the motorized stage is best. Nikon Elements provides this feature and is described in this protocol, but the free Micromanager package, as well as other imaging software, may have similar functionality. A different way of fine targeting is to move the laser focus, rather than the animal. A galvanometer beam-steering mechanism is available as an add-on to the MicroPoint laser, if this approach is preferred.

Besides its application to the study of neuronal regeneration, the laser can be used to ablate other cell types, such as skin, muscle, or specialized cells, or to disrupt specific neuronal synapses<sup>23-27</sup>. Moreover, pathways that regulate the degeneration of neurons that accompanies injury or disease could be investigated with this system. As such, the use of pulsed lasers will continue to shed light on both the genetic factors and the cell biological changes that facilitate neuronal regeneration and other relevant processes.

### Troubleshooting:

Described here are some common problems and their associated solutions.

1. **The laser is not cutting properly.** This is likely because either the laser is out of focus (see section 2), or the dye in the dye cell needs to be replaced (see MicroPoint manual). Alternatively, the dye cell may need to be replaced or rebuilt.
2. **The worms are moving on agarose pad.** This is generally a sign that the agarose pad is too moist. We find that pads need to be left for approximately 30 seconds before worms are placed on them to circumvent this problem. You can also try using the smaller sized (0.05  $\mu\text{m}$ ) microbeads.
3. **The recovery of worms is difficult and inefficient.** This can be caused by incorrect coverslip removal or by a dry agarose pad. If the pad is too dry, you may notice that the axons develop a beaded appearance. In some cases, this is because the worms were not placed on the pad soon enough after it was made. Alternatively, an old stock of agarose solution may have a higher than 3% concentration after repeated melting.

### Disclosures

No conflicts of interest declared.

### Acknowledgements

Work in the Hammarlund lab is funded by: NIH grants R01 NS066082-01 and T32GM007223, the Beckman Foundation, and the Ellison Medical Foundation.

### References

1. Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E.M., & Bastiani, M. Axon regeneration requires a conserved MAP kinase pathway. *Science* 323 (5915), 802-806 (2009).
2. Ghosh-Roy, A., Wu, Z., Goncharov, A., Jin, Y., & Chisholm, A.D. Calcium and Cyclic AMP Promote Axonal Regeneration in *Caenorhabditis elegans* and Require DLK-1 Kinase. *Journal of Neuroscience* 30 (9), 3175-3183 (2010).
3. Wu, Z. *et al.* *Caenorhabditis elegans* neuronal regeneration is influenced by life stage, ephrin signaling, and synaptic branching. *Proceedings of the National Academy of Sciences* 104 (38), 15132-15137 (2007).
4. Yan, D., Wu, Z., Chisholm, A.D., & Jin, Y. The DLK-1 Kinase Promotes mRNA Stability and Local Translation in *C. elegans* Synapses and Axon Regeneration. *Cell* 138 (5), 1005-1018 (2009).
5. Yanik, M.F., *et al.* Neurosurgery: Functional regeneration after laser axotomy. *Nature* 432 (7019), 822-822 (2004)
6. Gabel, C.V., Antoine, F., Chuang, C., Samuel, A.D.T., & Chang, C. Distinct cellular and molecular mechanisms mediate initial axon development and adult-stage axon regeneration in *C. elegans*. *Development* 135 (6), 1129-1136 (2008).
7. Rao, G.N., Kulkarni, S.S., Koushika, S.P., & Rau, K.R. *In vivo* nanosecond laser axotomy: cavitation dynamics and vesicle transport. *Optics Express* 16 (13), 9884-9894 (2008).
8. Wood, W.B. The Nematode *Caenorhabditis Elegans*. (Cold Spring Harbor Laboratory Press, 1988).
9. McLntire, S.L., Jorgensen, E., Kaplan, J., & Horvitz, H.R. The GABAergic nervous system of *Caenorhabditis elegans*. *Nature* 364 (6435), 337-341 (1993).
10. Bourgeois, F. & Ben-Yakar, A. Femtosecond laser nanoaxotomy properties and their effect on axonal recovery in *C. elegans*. *Optics Express* 15 (14), 8521-8531 (2007).



11. Chung, S. & Mazur, E. Femtosecond laser ablation of neurons in *C. elegans* for behavioral studies. *Applied Physics A: Materials Science & Processing* 96 (2), 335-341 (2009).
12. Steinmeyer, J.D. *et al.* Construction of a femtosecond laser microsurgery system. *Nature Protocols* 5 (3), 395-407 (2010).
13. Wang, Z. & Jin, Y. Genetic dissection of axon regeneration. *Current Opinion in Neurobiology In Press*, Corrected Proof (2010).
14. Huang, X., Cheng, H.-J., Tessier-Lavigne, M., & Jin, Y. MAX-1, a Novel PH/MyTH4/FERM Domain Cytoplasmic Protein Implicated in Netrin-Mediated Axon Repulsion. *Neuron* 34 (4), 563-576 (2002).
15. McIntire, S.L., Reimer, R.J., Schuske, K., Edwards, R.H., & Jorgensen, E.M. Identification and characterization of the vesicular GABA transporter. *Nature* 389 (6653), 870-876 (1997).
16. Fang-Yen, C., Gabel, C.V., Samuel, A.D.T., Bargmann, C.I., & Avery, L. Laser microsurgery in *C. elegans* in *Methods in Cell Biology: Caenorhabditis elegans: Modern Biological Analysis of an Organism* (Elsevier Academic Press, to be published).
17. Guo, S.X. *et al.* Femtosecond laser nanoaxotomy lab-on-a-chip for *in vivo* nerve regeneration studies. *Nature Methods* 5 (6), 531-533 (2008).
18. Zeng, F., Rohde, C.B., & Yanik, M.F. Sub-cellular precision on-chip small-animal immobilization, multi-photon imaging and femtosecond-laser manipulation. *Lab on a Chip* 8 (5), 653-656 (2008).
19. Rohde, C.B., Zeng, F., Gonzalez-Rubio, R., Angel, M., & Yanik, M.F. Microfluidic system for on-chip high-throughput whole-animal sorting and screening at subcellular resolution. *Proceedings of the National Academy of Sciences* 104 (35), 13891-13895 (2007).
20. Hulme, S.E., Shevkoplyas, S.S., Apfeld, J., Fontana, W., & Whitesides, G.M. A microfabricated array of clamps for immobilizing and imaging *C. elegans*. *Lab on a Chip* 7 (11), 1515-1523 (2007).
21. Ben-Yakar, A., Chronis, N., & Lu, H. Microfluidics for the analysis of behavior, nerve regeneration, and neural cell biology in *C. elegans*. *Current Opinion in Neurobiology* 19 (5), 561-567 (2009).
22. Samara, C. *et al.* Large-scale *in vivo* femtosecond laser neurosurgery screen reveals small-molecule enhancer of regeneration. *Proceedings of the National Academy of Sciences* (2010).
23. Nelson, F.K. & Riddle, D.L. Functional study of the *Caenorhabditis elegans* secretory-excretory system using laser microsurgery. *Journal of Experimental Zoology* 231 (1), 45-56 (1984).
24. Sulston, J.E., & White, J.G. Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. *Developmental Biology* 78 (2), 577-597 (1980).
25. Kimble, J. Alterations in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans*. *Developmental Biology* 87 (2), 286-300 (1981).
26. Allen, P.B., *et al.* Single-synapse ablation and long-term imaging in live *C. elegans*. *Journal of Neuroscience Methods* 173 (1), 20-26 (2008).
27. Pujol, N., *et al.* Distinct Innate Immune Responses to Infection and Wounding in the *C. elegans* Epidermis. *Current biology : CB* 18 (7), 481-489 (2008).