Video Article

Constructing a Low-budget Laser Axotomy System to Study Axon Regeneration in *C. elegans*

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Abstract

Laser axotomy followed by time-lapse microscopy is a sensitive assay for axon regeneration phenotypes in *C. elegans*[1]. The main difficulty of this assay is the perceived cost ($25-100K) and technical expertise required for implementing a laser ablation system[2,3]. However, solid-state pulse lasers of modest costs (<$10K) can provide robust performance for laser ablation in transparent preparations where target axons are "close" to the tissue surface. Construction and alignment of a system can be accomplished in a day. The optical path provided by light from the focused condenser to the ablation laser provides a convenient alignment guide. An intermediate module with all optics removed can be dedicated to the ablation laser and assures that no optical elements need be moved during a laser ablation session. A dichroic in the intermediate module allows simultaneous imaging and laser ablation. Centering the laser beam to the outgoing beam from the focused microscope condenser lens guides the initial alignment of the system. A variety of lenses are used to condition and expand the laser beam to fill the back aperture of the chosen objective lens. Final alignment and testing is performed with a front surface mirrored glass slide target. Laser power is adjusted to give a minimum size ablation spot (<1um). The ablation spot is centered with fine adjustments of the last kinematically mounted mirror to cross hairs fixed in the imaging window. Laser power for axotomy will be approximately 10X higher than needed for the minimum ablation spot on the target slide (this may vary with the target you use). Worms can be immobilized for laser axotomy and time-lapse imaging by mounting on agarose pads (or in microfluidic chambers[4]). Agarose pads are easily made with 10% agarose in balanced saline melted in a microwave. A drop of molten agarose is placed on a glass slide and flattened with another glass slide into a pad approximately 200 um thick (a single layer of time tape on adjacent slides is used as a spacer). A "Sharpie" cap is used to cut out a uniformed diameter circular pad of 13mm. Anesthetic (1ul Muscimol 20mM) and Microspheres (Chris Fang-Yen personal communication) (1ul 2.65% Polystyrene 0.1 um in water) are added to the center of the pad followed by 3-5 worms oriented so they are lying on their left sides. A glass coverslip is applied and then Vaseline is used to seal the coverslip and prevent evaporation of the sample.

Video Link

The video component of this article can be found at https://www.jove.com/video/3331/

Protocol

1. Construction of a laser ablation system

Wear laser safety goggles and use good laser safety practices during the initial alignment. Never look through the oculars when the laser is on.

1. Arrange the components on the breadboard as shown in Fig. 1 (see also example[2]). Bolt down the laser (with a riser plate if needed), periscope post, and the elevated rail supports.
2. Position your microscope to align with the rail axis. Align with the transmitted light beam from the microscope condenser lens. Align the rail height (use a level) and position with an adjustable aperture or a lens affixed to the rail. The aperture or lens must be aligned at **both ends** of the rail to the condenser beam. Lab jacks can help with the rail height adjustment.
3. Align the Glan-Thompson polarizer and half-wave plate to the laser beam. You do not need the laser on to do this alignment. The beam just needs to go through both without hitting their edges. Rotate the polarizer to provide a convenient orientation and position for the beam dump (fig.1). You will rotate the half wave plate to adjust laser power. Secure the components to the breadboard.
4. Turn on the laser, set the pulse frequency to 100, continuous mode, and reduce power to a minimum using the half-wave plate. Use a Post-It note or lens paper to visualize the laser beam.
5. Adjust and fix the kinematically mounted mirrors in sequence from the laser to bring the beam up to the mirror that is mounted on the end of the elevated rail. The laser beam should be aligned roughly to the center of the mirrors. The mirrors should be oriented roughly 45 degrees to the laser beam axis (fig.1).

6. Coarsely adjust the kinematically mounted mirror on top of the periscope and on the end of rail to align the laser beam to the center of the transmitted light beam from the microscope. Both the laser beam and the transmitted light beam from the microscope can be simultaneously visualized by inserting paper in the beam path. Make sure any ND filters and apertures in the intermediate module are out or fully open.
7. Finely align the laser beam to the center of the transmitted light beam with the fine adjustments on the top periscope and rail mirrors. Hold the paper close to the rail mirror and align the laser beam to the center of the transmitted light beam with the top periscope mirror. Hold the paper near the microscope port and align the laser beam to the center of the transmitted light beam with the rail mirror adjustments (fig.2).
8. Position a Post-It note in the beam path between the aligned condenser lens and the open objective turret. You should see the transmitted light beam with the smaller laser beam near the center. Use the fine adjustments on the rail mirror to center the laser beam. Fix the microscope in place with clamps.

Steps 1.9-1.12 are optional, but it is easy and useful to assess alignment and laser ablation before adding the beam expanding lenses.

9. Turn off the laser and engage the mechanical safety shutter. Rotate the 50% dichroic out of the beam path. Even though no direct laser light will pass to the oculars, the reflected light can be quite intense. Mount the target slide for the fine alignment and image with the 60X oil objective. Focus on scratches on the slide. Put in the ND4 and ND8 filter in intermediate module.
10. Image the target slide with your CLSM, Spinning disk, or CCD camera system. Rotate the 50% dichroic into the laser path. **From this point, you should never look through oculars while the ablation laser is on**. Turn on the ablation laser and set the mode to triggered, frequency to 100, and pulse number to 10. Make sure the power is still set to minimum with the half-wave plate and then open the mechanical safety shutter. You can simultaneously image and use the ablation laser.
11. While imaging the target slide, trigger the ablation laser. Check the target slide for an ablation spot 1-10 um in diameter. Sequentially remove ND filters until an ablation spot is seen. Adjust the ablation spot to the center of the image with the rail mirror. You will want to add back a ND filter to have room to finely increase laser power with the half-wave plate to give a <1 um ablation spot. Image at 0.2 um/pixel or less and finely adjust the ablation spot to the image center (position 256,256 with 512X512 image).
12. Check the depth of focus and axis of the laser beam. Focus up and down 1-2 um and check the ablation position and ablation spot size. If the laser beam is axially aligned the ablation spot will not move. The unconditioned laser beam will distribute power over several um (about 3-5 um from top to bottom of focus).

2. Add lenses to expand the laser beam to fill the objective back aperture and adjust convergence to control focus

1. This system uses dual Galilean beam expanders to expand the laser beam to fill the back aperture of the objective (10 mm). Mount the 4 lenses on to carriers and attach them to the rail (L1f1+L2f2 and L3f1'+L4f2'). Adjust the positions so that all lenses are at the same optical axis and exactly orthogonal to the laser beam (fig. 2).
2. Turn on the laser and adjust it to minimum power and continuous mode. Roughly adjust the beam alignment through each lens using paper to see the laser beam and transmitted light beam from microscope condenser. Turn off and shutter the laser.
3. Remove the clamps from one side of the microscope and slide the microscope out of the laser beam path. Turn on the laser and remove safety shutter.
4. The fine alignment of the lenses and the laser beam can be performed by viewing the expanded laser beam on a nearby wall. The beam should expand and contract symmetrically when the lenses are moved. Adjust the last two kinematically mounted mirrors to align the beam (fig.3).
5. The aligned and expanded beam profile should be circular and of uniform brightness (fig.3). The size and uniformity of the beam can be viewed with a Post-It note at the estimated position of the objective back aperture.
6. The beam should be adjusted to zero convergence for infinity optical systems. Convergence can be estimated by noting how the beam size changes as you move a Post-It note farther from lenses. Turn off the laser and engage the mechanical shutter.
7. Slide the microscope back into the laser beam path using the fixed clamps to define the correct alignment. Replace the clamps on the free side of microscope, but do not tighten down.
8. Turn on the laser and remove the safety shutter. Rotate the objective turret to an open position. Place a Post-It note on the stage. You should see the expanded and uniform laser beam centered on the transmitted light beam from the condenser (fig.4). You may need to center it by loosening the microscope clamps and carefully nudging the microscope.
9. Engage the safety shutter, and set the laser to trigger mode. Rotate the 60X objective in place and image the surface scratches on the target slide.
10. Remove the safety shutter, trigger the ablation laser and adjust the laser power for the minimum ablation spot. The ablation spot should be circular and within 5-10 um of the image center.
11. Center the ablation spot with fine adjustments of kinematically mounted rail mirror. You will need to iteratively adjust the rail mounted and top periscope mirror to both center the ablation spot and maintain a uniform beam profile.
12. Evaluate the Z focus of the laser by moving the focus systematically up and down in 1 um steps and triggering the ablation laser. The expanded, aligned, and convergence adjusted beam should ablate maximally at the image focus and weakly or not at all 1 um above or below focus (fig 5).
13. Adjust the Z focus of the expanded beam by moving lens L3 of the Galilean telescope (fig.2).

3. Laser axotomy and time-lapse microscopy of axon regeneration

1. Microwave 10% Agarose (RPI Molecular Biology Grade EEO 0.1 A20090) in balanced saline till fully melted. Set on a hot plate to keep it melted during use for mounting.
2. A drop of molten agarose is placed on a glass slide and flattened with another glass slide into a pad approximately 200 um thick (a single layer of time tape on adjacent slides is used as a spacer).
3. A "Sharpie" marker cap is used to cut out a uniformed diameter circular pad of 13mm.

4. Anesthetic (1ul Muscimol 20mM) and Microspheres (Chris Fang-Yen, personal communication) (1ul 2.65% Polystyrene 0.1 um in water) are added to the center of the pad followed by 3-5 worms oriented so they are lying on their left sides. A glass coverslip is applied and then Vaseline is used to seal the coverslip and prevent evaporation of the sample (fig.6).

5. The ablation laser is aligned to crosshairs, as described above, at the beginning of each session. Engage the laser safety shutter.

6. Remove the laser alignment target and image a mounted adult worm with a suitable fluorescent marker in the target neurons. Image axons at a similar magnification (0.2 um/pixel or higher magnification) and position under the crosshairs (fig.7).

7. Open the laser safety shutter and trigger the ablation laser while imaging. Evaluate the axon after triggering the laser and slowly increase laser power until the axon is cut. You will need about 10X more laser power to cut axons than to form an ablation spot on the alignment target slide (about 1uJ/ ns pulse). We typically cut with 100 pulses at 2.5 kHz and power set to about 0.27mW (average power measured at specimen with Coherent FieldMaxII power meter and laser set to continuous at 2500 Hz). The ablation laser power setting will be consistent for cutting axons in future experiments (fig.7).

8. A successfully cut axon will show a break of about 0.5-1 um without a loss of brightness in the two cut ends (fig.7). A large loss of brightness or a large gap (2-10 um) often signifies extensive damage beyond the axon by a cavitation bubble. The proximal and distal axons will separate and form retraction bulbs over the next 30 minutes (fig. 8 and Movie).

9. Equilibrate your mounted worms with your microscope stage for 30 minutes before starting your time lapse recording. This will minimize drift due to temperature differences and agarose contraction.

10.) Time-lapse imaging parameters are set up using the imaging software associated with your system. We typically image 10-20 Z steps (1um/ step) at 0.1-0.2 um/pixel using gain, pinhole, scan rate, and imaging laser power settings that minimize exposure while giving the desired spatial resolution. Sampling intervals are typically 1-5 minutes over 15 hours depending on the desired temporal resolution (fig. 8 and Movie).

11. Time-lapse image data are converted into movies using NIS Elements or ImageJ.

4. Representative Results:

Laser axotomy using this system is a reliable and routine. The results shown in figure 7 are typical. Time-lapse imaging of axon regeneration is very robust using this protocol. We routinely cut and image up to 5 axons in 5 different worms on a single slide using a motorized stage. The only limitation is the time it takes to collect the images of each axon, e.g., if it takes 20 seconds to collect a stack for an axon then at most you can sample 9 axons (9 stacks) if you are sampling every 180 seconds. The example shown in Figures 8 and 9 is a good representative result. About 10% of experiments give results of this quality. The remaining experiments provide good data on the regeneration phenotype, but are esthetically unappealing because of small jittering movements of the worm that generally begin after 5-8 hours of immobilization.
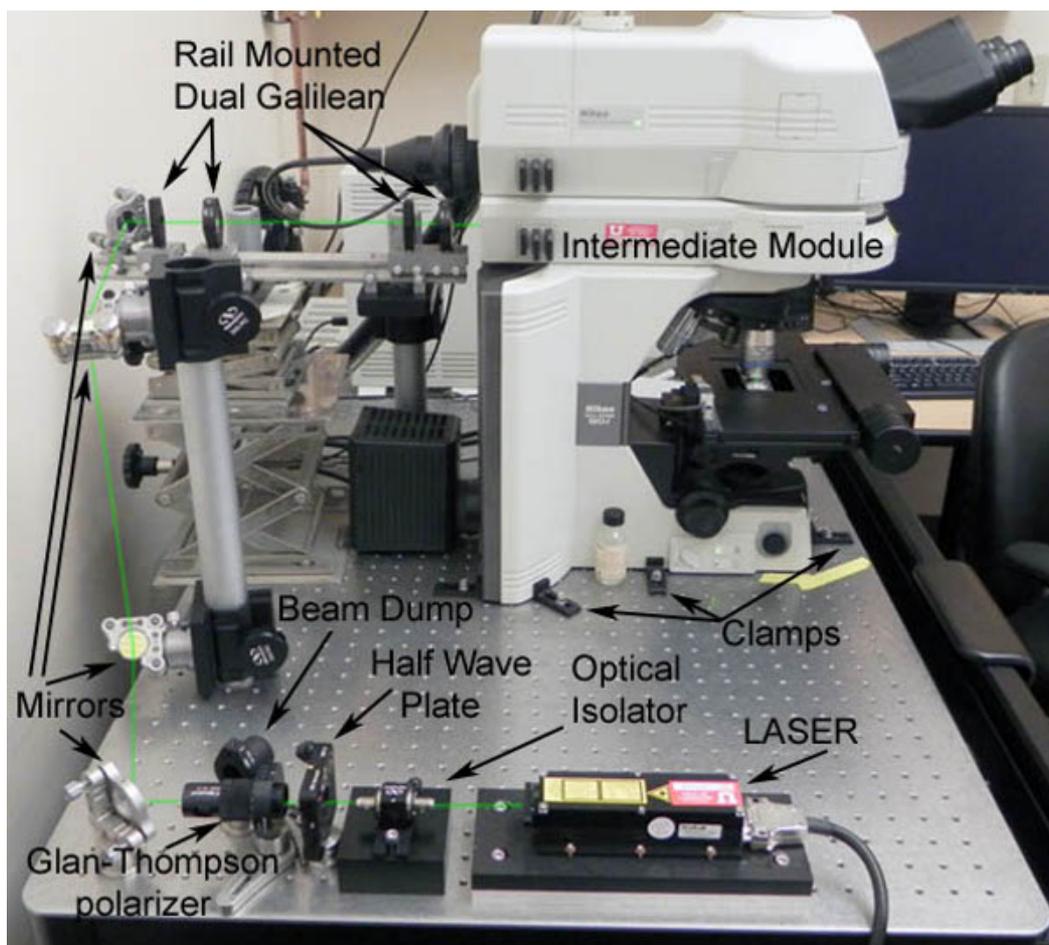

Figure 1 Laser Ablation System. The 532 nm Nd:Yag laser is mounted on a riser plate to bring it to the height of the other components and bolted to the breadboard. (Optical isolator is optional and probably not needed). The Glan-Thompson polarizer and half-wave plate are used

to finely control the laser power. They are in finely calibrated rotation mounts. Take care setting the beam dump. The corner mirror directs the laser beam to the lower periscope mirror. The upper periscope mirror directs the beam to the rail mirror. The rail is mounted on two rod supported platforms. Dual Galilean lenses are attached to sliding rail mounts. Notice the added intermediate module dedicated to the ablation laser. The system could be made more compact by removing the optical isolator and the corner mirror, and repositioning the laser, Glan-Thompson polarizer and half-wave plate to the back edge of the breadboard.
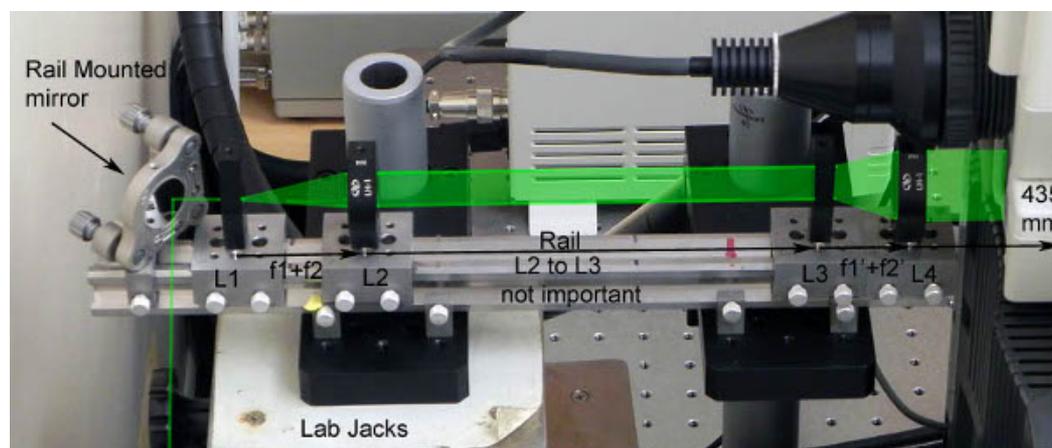

Figure 2 Dual Galilean Conditioning lenses are used to expand the 300 um TEMoo laser beam to a zero convergence uniform 10 mm size. A lab jack is positioned under one of the platforms holding the rail. This helps in adjusting the rail height during the alignment steps. Crudely adjust the rail height by mounting the L1 lens to the rail and using the condenser beam as an alignment guide. Adjust the rail so that the condenser beam aligns to the center of the lens at **both** ends of the rail. This will ensure the rail is aligned parallel to the optical axis of the microscope. You may need to first adjust the microscope to match the optical axis of the rail. Use clamps to fix the position of the microscope (see fig. 1). Remove the rail-mounted lens. Now align the laser beam to the center of the condenser beam at **both** ends of the rail. A paper in the beam path is a convenient way to see both beams simultaneously. Use the top periscope mirror to align the laser beam to the condenser beam close to the rail-mounted mirror. Use the rail mounted mirror to align the laser beam to the condenser beam at the far end of the rail (closest to microscope). Now mount all four lenses to the rail as shown in the figure. The length of the rail, focal length of the lenses, and the lens arrangements will vary depending on your microscope and physical set up. Critical parameters are the laser beam diameter and the size of the back aperture of the chosen objective. Infinity optical systems will focus a perfectly parallel laser beam (zero convergence). Distance between L1 and L2 should be sum of the lenses focal lengths, f1+f2, and distance between L3 and L4 should correspondingly be f1'+f2'. The distance between the dual Galilean pairs is not critical. Position of L3 can be finely adjusted to control beam convergence; <1mm adjustments will be needed to adjust the focus of the laser to the image focus. Be sure to remove all lenses, filters, apertures, etc. from the intermediate module that might be in the beam path (or be aware of what they do and adjust your system accordingly).
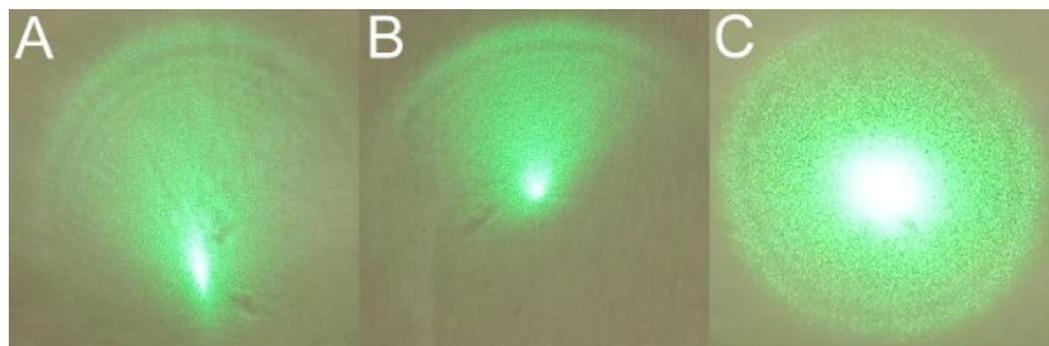

Figure 3 Alignment of the laser beam through the dual Galilean lenses. Remove the microscope clamps from one side of the microscope so the microscope can be moved out of beam path, but the remaining clamps will allow the microscope to be precisely repositioned. **Think laser safety**. Make sure the laser power is adjusted to a minimum with the Glan-Thompson polarizer. Turn on the laser and view the laser beam pattern on the wall. You may find a paper taped to the wall helps to view the laser spot. Systematically adjust the positions of the lenses to get a feel for what they do to the beam size and appearance. You should see something that looks like panel A, where an intense spot is eccentrically located in a dimmer profile. Use the rail-mounted mirror to adjust the intense spot to the center as shown in panel B. Now use the top periscope mounted mirror to give a uniform peripheral profile. If everything is aligned correctly you should now find that moving the lenses causes the beam to expand and contract symmetrically. Move the lenses back to the starting positions as shown in figure 2. Now intercept the beam at the distance of the objective back aperture. It should be circular, at least large enough to fill your back aperture, and of a uniform brightness. It is OK if it is larger, as long as you have sufficient laser power for your ablations. Evaluate convergence by comparing beam diameter at the distance of the objective back aperture and the wall. Beam diameter should be identical for a zero convergence beam. Scale bar is 10mm.
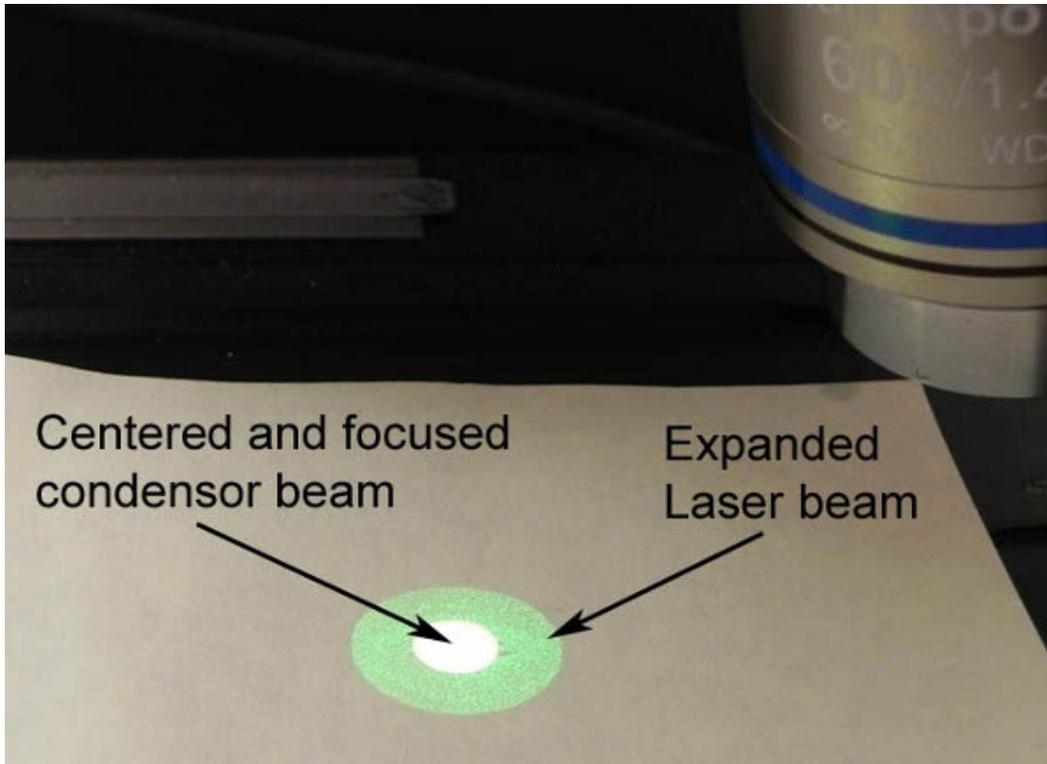
Figure 4 Alignment of the expanded laser beam to the condenser beam. Shutter the laser and move the microscope back into the path of the laser beam using the clamps as alignment stops. Turn on, align, and focus the condenser beam using an objective lens. **Tape paper over the oculars to prevent anyone from viewing the intense reflected laser light**. Rotate the objective turret to an open position. Put a piece of paper on the stage to occlude the condenser beam. Turn the laser on to continuous mode and remove the mechanical safety shutter. You should see some laser light coming though the microscope. Loosen the microscope clamps and gently nudge the microscope to align the laser beam with the condenser beam. The laser beam should be circular and of uniform brightness. It is sometimes useful to adjust the rail-mounted lenses. You should be able to expand and contract the laser beam uniformly if the alignment is correct. You can center the contracted laser beam to the minimum centered condenser beam with the rail-mounted mirror and then expand it to the desired size. Once you adjust the position of the contracted beam with the rail mirror, you will need to readjust the top mounted periscope mirror to maintain a uniform brightness of the expanded beam. If you cannot get a circular centered laser beam then you will need to go back through the earlier alignment steps.
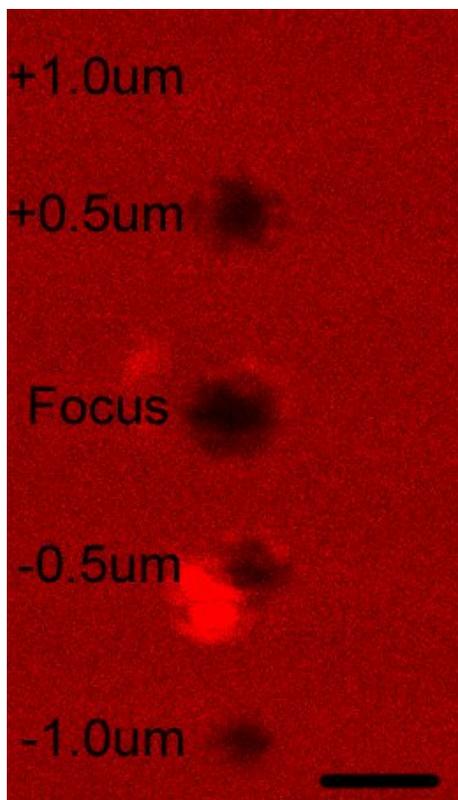
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Figure 5 Center the ablation spot and adjust the laser beam convergence for optimum focus. Turn off and shutter the laser. Remove the paper and mount the mirrored target slide (you can also use permanent marker ink on a coverslip as a target[2]). Image scratches on the mirrored surface with the 60X 1.4na oil lens) Now image the surface with a confocal or CCD. **Do not look through the oculars while the laser is on**. Turn on and unshutter the laser. Set the laser to trigger mode, lowest power, and 100 Hz. Trigger about 10 pulses. You should see an ablation spot of 1-5 um in diameter within 10 um of the center of view. If you do not see an ablation spot you can increase the laser power in 5-10% steps. Once you identify an ablation spot, center it to the field of view. Put the cross hairs at 256, 256 coordinates if viewing 512X512 image. If you adjust the laser spot to the center then it will not change position with zooming. Use the rail mounted mirror to move the ablation spot to the center crosshairs. Reevaluate the uniformity of the laser beam by looking at the beam with the objective removed as described above in Figure 4. Adjust the beam uniformity with the top periscope mounted mirror. Repeat the evaluation of the ablation spot position. This is an iterative process that should be repeated until the ablation spot is centered and the expanded beam is uniform. If done correctly the ablation spot will be circular as seen in the ablation spot at Focus in the figure. Next evaluate the focus of the laser beam in the Z axis. Trigger the laser to produce an ablation spot of about 1 um in the center. Now move the slide about 5 um laterally and focus 1 um below the surface of the target slide. Trigger the laser using the same laser settings as you used for the first ablation spot. Repeat after focusing 1 um above the surface of the target slide. If the laser beam is focused to the image focus then you should see a pattern similar to that shown in this figure. The largest ablation spot should correspond to the image focus and the ablation spots above and below focus should get smaller symmetrically. Adjust lens L3 to align focal planes. Moving lens L3 away from the microscope will make the beam more convergent and the ablation spot will move closer to the objective. Small <1mm adjustments will be needed near focus. You are now ready to cut axons. Scale bar is 1 um.
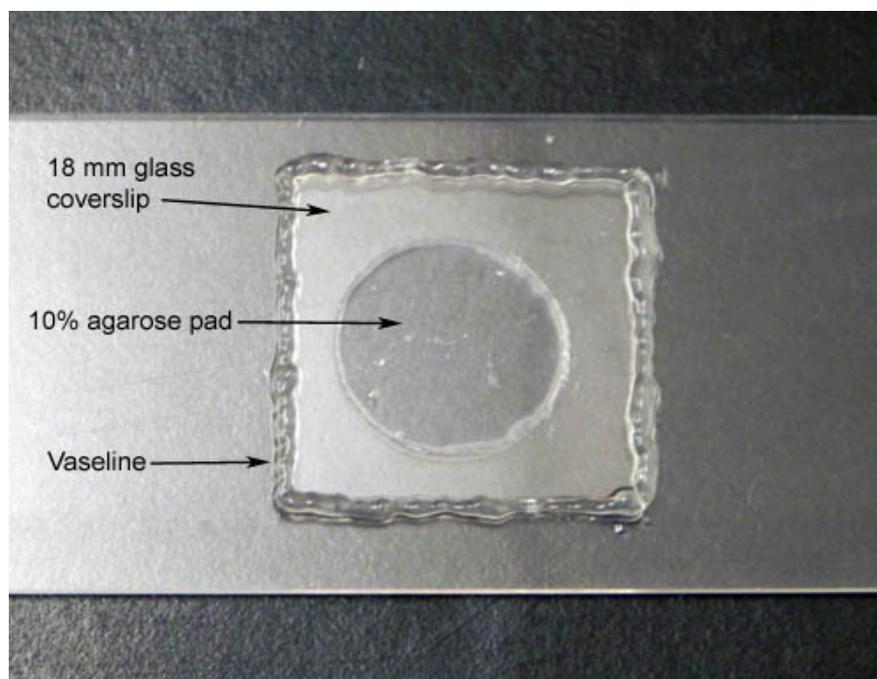
Figure 6 Mounting worms for laser axotomy and time-lapse imaging. Agarose 10% in saline is heated till fully melted. A small drop is placed on a glass microscope slide and then flattened with another slide to form a flat pad. The thickness of the pad is set by tape on two adjacent slides. The pad is allowed to set for about 1 minute and then the slides are separated. A "Sharpie" top is used as a cookie cutter to form a uniform sized pad of about 13mm. 1 ul of 10mM Muscimol and 1 ul of microspheres are added to the center of the pad (Fang-Yen personal communication). Worms are added to the 2 ul drop and oriented before putting on the glass coverslip. Vaseline is then applied from a syringe (20-25 ga. needle) to the edge of the coverslip to seal it.
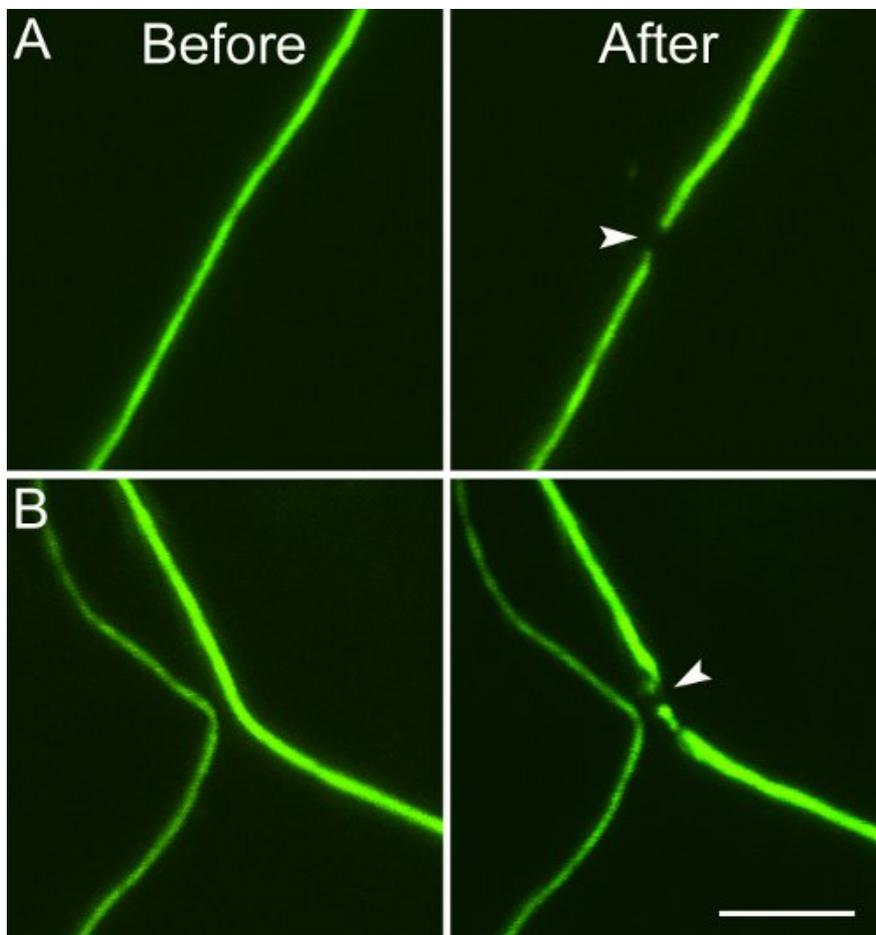
Figure 7 Typical results of laser axotomy in C. *elegans*. In A you can see the intact axon shortly before and after laser axotomy. The gap is normally about 0.5-1um. B shows laser axotomy of one axon without damaging a nearby axon about 1 um away. Laser was set to about 10% power (0.3 mW average at 2500 Hz), and 100 pulses. Scale bar is 5 um.
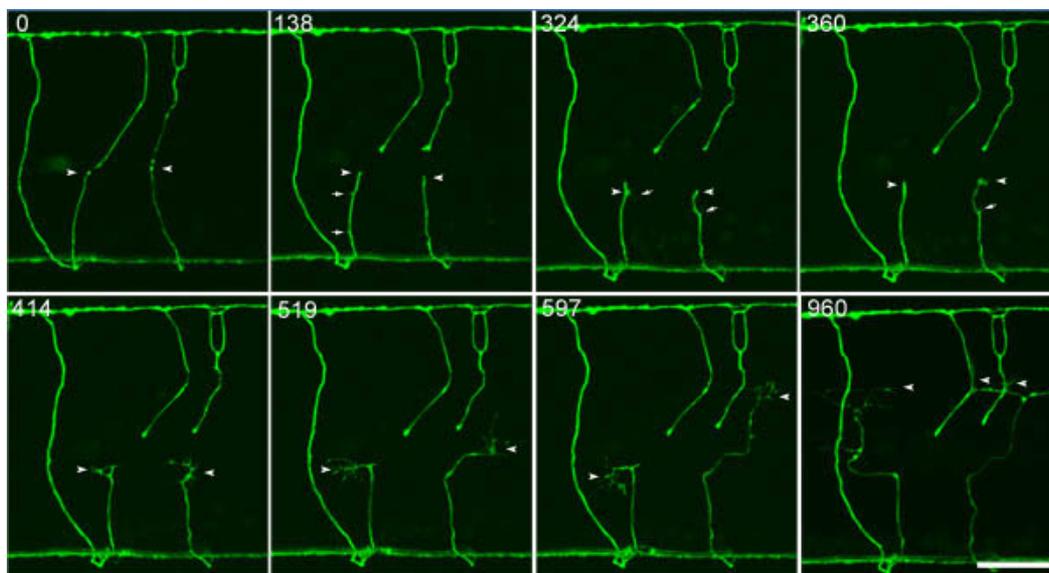

Figure 8. Typical wild type L4 regeneration. This is a time series showing ablation and time-lapse recording of axon regeneration. Shortly after laser axotomy at 0 minutes you can see the retraction bulb. By 138 minutes the retraction bulb has pulled back to its farthest extent and now begun to remodel. Sporadic membrane protrusions (mircospikes or filopodia) can be seen along the axon shaft (arrows 138 and 324). The right stump extends a well-formed compact growth cone at 360 minutes. At 414 minutes both stumps have extended growth cones. The initial embryonic-like growth cones become dystrophic as they extend toward the dorsal nerve cord (414, 519 and 597 minutes). The dystrophic growth

cones stall short of the dorsal nerve cord (960 minutes). Arrowheads point out proximal stump and growth cones. Arrows point out membrane protrusions along the proximal axon shaft. Scale bar 20um.

Movie 1. Movie of wild type axon regeneration. This movie goes along with the time points shown in Figure 8. Worms were mounted as described in the protocol and axons were imaged every three minutes for about 10 hours. Z stacks (20 X 1um) were taken at each time point and merged into single images by a maximum projection algorithm (NIS Elements). Click here to watch the movie.

Discussion

There are several good discussions of laser microsurgery with different laser systems[3,5-11]. Femtosecond IR lasers are the "gold standard" for subcellular laser ablation[12] and convenient if associated with an imaging facility, but they are often too costly for individual users. If you require a femtosecond IR laser for imaging your sample because of depth of imaging then you will probably need one for laser axotomy. Transparent tissues with target axons within 30-50 um of the surface are probably feasible with blue and green pulse lasers in the 20 uJ/pulse range targeted through a high na immersion lens. There will be more collateral damage with the nano and pico second lasers compared with the femtosecond laser, especially as the depth of the target axon increases. *C. elegans* is transparent and all axons are within 20-30 um of the surface. We routinely cut motor axons that are within 5 um of the surface. We have also easily cut axons within the nerve ring that are about 20 um from the cuticle surface. We found the limit for cutting axons to be about 30-50 um through the diameter of an adult worm. It is likely that the laser ablation system described here would work well with many different preparations that fit the criteria of transparency and depth of target axons. Still, it is a bit surprising, given the theoretical advantages of the femtosecond IR lasers, that nano and picosecond 355 nm and 532 nm lasers perform so well for laser axotomy in *C. elegans*[6,13]. We see no differences in axon regeneration in response to laser axotomy with nanosecond 440 nm, nanosecond 532 nm, and femtosecond IR lasers.

Solid state 355 nm UV lasers are about the same cost as the 532 nm diode pumped passively Q-Switched solid state laser, but require either higher powers or optics that efficiently pass these shorter wavelengths. Most optics are designed to perform well with visible 400-700 nm light. 355 nm lasers would offer some advantages[6] such as reduced plasma threshold, smaller spot size, and a long pass dichroic would efficiently direct 100% of the ablation laser to the target and allow simultaneous imaging of GFP without loss of sample signal. Blue 440 nm lasers would retain the advantages of working with a visible light laser (good performance with standard optics and safety). Unfortunately, the cost of a DPP Q-switched solid state 440 nm laser is 3 times the cost of a 355nm or 532 nm laser of similar power at this time. If we were designing a new system for *C. elegans* , where target depth is minimal, we would opt for a UV transparent lens (cost about $3,000 for a 40X 1.3na oil lens with 50-70% transmittance at 355nm) and a 355 nm laser producing 5-10 uJ/pulse at 1-20 kHz.

Axon ablation is thought to be due to plasma formation. Shorter pulses will produce plasma thresholds at lower power and the plasma volumes will be smaller[6]. The goal is to produce the smallest and shortest-lived plasma by adjusting the pulse energy to the lowest power. Larger long-lived plasmas will generate cavitation bubbles that damage surrounding cells. We have generally found that ablation using about 50-100 pulses at the highest frequency (i.e. 2.5kHz) gives the best results. This suggests that damage can be gradually integrated over a series of pulses to better control the extent of damage. The laser ablation system described here is very forgiving if the beam is aligned and expanded accurately. We start cutting axons in adult worms at 10% laser power (average of 0.3 mW measured at 2500 Hz and estimated 1 uJ/pulse) and can cut with limited localized damage through 14% power. You can observe larger areas of damage from 15-39% laser power, but only above 40% power (about 1 mW average measured at 2500 Hz) do the worms explode due to large cavitation bubbles and damage to the worm's cuticle.

The Steinmeyer et al. 2010[2] paper is an excellent resource for constructing a laser ablation system. Leon Avery also has a nice practical description of a laser ablation system based on an inexpensive N2 gas 337 nm laser and he provides some great practical advice (elegans.swmed.edu/Worm_labs/Avery/). When designing your own system you should first talk with your microscope representative to determine how to target the ablation laser to the microscope objective. Your microscope should be mounted on a vibration isolation table (Newport, TMI, Thor). A breadboard top is optimal, but a solid steel top can still be used with magnetic positioners. A dedicated intermediate module on an upright scope is nice because all optical elements can be left in place. However, it may be less expensive and more convenient to use a camera port (inverted) or a "combiner" attached to an epi-fluorescent port. You need to know the size of the back aperture and the transmittance (at the ablation laser wavelength) of the objective lens you intend to use. Once you decide on a laser (e.g., Crylas, TEEM, Crystal, or CRC) you should contact Thor or Newport for help with selecting the correct optical elements, hardware and safety equipment. We decided on a dual Galilean beam expander to save space, but a simpler Keplerian expander is an option (f2/f1=expansion factor). A custom beam expander will be the least expensive, but Newport, Thor, and several of the laser manufacturers offer excellent quality commercial beam expanders. Some laser manufacturers also offer manual and electronically controlled attenuators that may be cost effective and space saving, but not as versatile as the Glan-Thompson polarizer and half wave plate. You will also need to decide how to control the laser. You can design a LabView based controller, use a commercial TTL generator, or software provided by the laser company. Discuss the options with the specific laser manufacturer.

We have provided a list of the hardware we have used only as a general guide. The system described here cost about $15,000 when added to our existing imaging system. Your local Physics department will often have someone willing to provide a practical introduction to safely working with free beam lasers.

Alternative methods for immobilization and time lapse imaging of *C. elegans* have been recently described.[14]

Troubleshooting

The most difficult and critical step is alignment of the centered expanded beam to the optical axis of the microscope. Once you have the beam centered and expanded through the Galilean lenses you should work hard to get it aligned to within 5 um of the microscope's optical axis BEFORE using the steering mirrors for the final alignment to the center. Excessive use of the steering mirrors to align the beam to the microscope will move it off axis through the beam expander. **USING EXTREME CAUTION** you can attenuate the laser beam with the appropriate ND filter and directly view the laser beam profile on a reflective target (front surface mirror). You can gently nudge the microscope to precisely center the beam in the 60X objective field of view (if it can't be centered in the up/down range you need to adjust the rail height). The beam profile can be used to precisely align the microscope optical axis to give a centered beam that is circular and "flares" symmetrically. An

asymmetric flare is an indication that the microscope axis is not perfectly aligned (or the rail is not level). Finally, you should adjust L3 and see an even and symmetrical expansion and contraction of the beam. Focus the microscope precisely on the target surface and then adjust L3 to focus the laser beam to the smallest spot. You should now find that the laser beam is within 5 um of the center when imaged via LSCM or CCD system and the ablation spot is within 1um of Z focus.

If you find you are "blowing up" worms or consistently generating large cavitation bubbles to cut axons your problem is most likely the fine alignment of the ablation laser. Make sure the minimum (<500nm) ablation spot is localized to the Z focus (adjust convergence with L3 lens) and to the XY fiduciary spot (adjust with last kinematically mounted mirror).

Targeting axons closer to the surface will require less laser power. Similarly, smaller animals (L1 and L2) will require less laser power for axotomy compared to targeting the same axons in adults.

If your wild type axons do not regenerate or the axons bleach you should try reducing imaging laser power or decreasing the sampling rate. If your worms die or become sickly try reducing the percent Agarose in 1% steps. Make sure you are not moving the coverslip after mounting worms as this will often cause them to die when using high percentage agarose and microspheres.

If your worms burst or die during a time-lapse session it is due to: 1. Percentage agarose is too high. 2. Damage to cuticle by ablation laser. 3. Movement of coverslip after mounting. If damage to the cuticle is at fault it will only affect mounted worms that have been targeted with the ablation laser.

If your worm moves too much during a time-lapse session try increasing the percentage agarose in 0.5% steps. Healthy axons in healthy worms are always "active" and display a consistent level of fluorescence. If you see a sudden decrease in fluorescence or activity the worm is dying or dead. Multiple beaded or fragmented axons are a sure sign of a dying or dead worm.

If you have trouble keeping your axons in focus during the entire time lapse session there may be several different problems. First check your stage drift by imaging an inert sample on a glass slide over 10 hrs. A few microns drift may be due to thermal instability, but greater than 5 um is probably due to mechanical issues with your microscope. Problems with mounting are more common. Let your mounted worms equilibrate with your microscope stage for 30 minutes before starting your time lapse. Check that your Vaseline seal did not develop leaks during the course of your time lapse session.

Disclosures

No conflicts of interest declared.

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