Published in final edited form as:
Nat Protoc. 2010 March ; 5(3): 395–407. doi:10.1038/nprot.2010.4.

Construction of a femtosecond laser microsurgery system

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Abstract

Femtosecond laser microsurgery is a powerful method for studying cellular function, neural circuits, neuronal injury and neuronal regeneration because of its capability to selectively ablate sub-micron targets *in vitro* and *in vivo* with minimal damage to the surrounding tissue. Here, we present a step-by-step protocol for constructing a femtosecond laser microsurgery setup for use with a widely available compound fluorescence microscope. The protocol begins with the assembly and alignment of beam-conditioning optics at the output of a femtosecond laser. Then a dichroic mount is assembled and installed to direct the laser beam into the objective lens of a standard inverted microscope. Finally, the laser is focused on the image plane of the microscope to allow simultaneous surgery and fluorescence imaging. We illustrate the use of this setup by presenting axotomy in *Caenorhabditis elegans* as an example. This protocol can be completed in 2 d.

INTRODUCTION

The non-linear absorption that results from focusing a train of near-infrared (NIR) femtosecond laser pulses through a transparent specimen enables the specific ablation of submicron-scale features with minimal collateral damage[1]. This technique has been used to study the function of microtubules[2], mitochondria[3] and other organelles[4] in cultured cells, tissues and whole organisms, as well as for optotransfection[5] and laser microdissection[6,7]. In addition, femtosecond laser microsurgery has been used to investigate the effects of neural injury and other biological processes in model organisms such as *Caenorhabditis elegans*[8–16], *Drosophila melanogaster*[17] and rodents[18]. We previously used this technique to make the first observation of regeneration in *Caenorhabditis elegans* by cutting neurites of motor neurons *in vivo* using 100 pulses of NIR light at a repetition rate of 1 kHz with low

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AUTHOR CONTRIBUTIONS C.L.G. and C.B.R. developed the laser axotomy techniques described in this protocol. M.A.S. and J.D.S. developed the beam expander structure. M.A., C.B.R. and C.L.G. developed the other elements of the system. M.A.S. developed the laser alignment technique. J.D.S., C.L.G. and C.P.-M. wrote the manuscript, and M.A. and M.F.Y. commented on the manuscript at all stages.

energies (10–40 nJ per pulse) and ultra-short pulse durations (200 fs)[8]. Full neurite regrowth and recovery of locomotive response upon touch stimulus was observable within 24 h. We had also previously demonstrated the first microfluidic system[19] to capture and mechanically immobilize whole organisms (*C. elegans*) for the purpose of sub-cellular resolution femtosecond laser microsurgery[20,21], two-photon imaging[20] and high-throughput screening[19,22,23]. UV lasers[24] and pulled glass pipettes[25] have also been used for microsurgery in cultured cells and tissues; however, the inaccuracy of these techniques and the damage done to surrounding tissue limits their use *in vivo*. Although femtosecond laser systems are complex pieces of equipment, their costs have come down significantly in recent years as the technology has matured.

In this protocol, we detail a procedure to add a femtosecond laser microsurgery capability to a standard fluorescence microscope using commercially available components. The femtosecond laser microsurgery system (Fig. 1 and corresponding component descriptions in Table 1) is designed to take up minimal space while allowing access to facilitate easy and reproducible alignment. The design provides flexibility in the choice of objectives, filter cubes, laser wavelengths and laser–pulse repetition rates while requiring no modification of the fluorescence microscope. The protocol begins with the installation of major components including the optical table, the microscope and the laser. Next, beam-conditioning optics (optical isolator, electro–optic modulator (EOM), Glan–Thompson polarizer and half-wave plate) are assembled and mounted. The construction and installation of a dichroic mounting adapter, an assembly that brings the laser beam in line with the optical axis of the objective, is then described. The protocol then details the installation of a periscope to raise the beam from the table to the microscope input port and a laser beam expander to resize the beam to fill the back aperture of the objective. The protocol concludes with step-by-step instructions for axotomy in *C. elegans*, which is included as an example application of the system. Adapting the system for other microsurgical applications, such as those mentioned above, involves modifications only to the standard sample preparation steps[2–7] and use of different objective lenses[1]. For example, although an air objective lens with a numerical aperture (NA) of 0.75 is sufficient for axonal ablation in *C. elegans*, for axotomy in zebrafish larvae, researchers have used water-immersion objective lenses with 0.8 or higher NAs[26]. For ablation of cytoskeletal filaments of cells *in vivo*, oil-immersion objective lenses with higher NAs of 1.4 are used[7]. In addition, different laser–pulse repetition rates have been used in the literature[1,7,11], where the lower pulse repetition rates yield reduced heat accumulation in specimen[1], and therefore less collateral damage. The high speed EOM and the high pulse-repetition rate laser used in this protocol provide the ability to choose any pulse repetition rate ranging from sub-KHz to 80 MHz, thus providing sufficient flexibility.

The system described in this protocol does not include a two-photon imaging capability. However, this capability can be easily added by replacing the top periscope mirror with scanning mirrors and by adding a photomultiplier tube to one of the camera ports of the microscope[27]. To adapt the laser system for simultaneous use by multiple microscopes simply requires the addition of a beam splitter, EOM, periscope, beam expander and dichroic mounting adapter for each additional microscope. If necessary, a regenerative

amplifier or a higher-power laser can be used to ensure that sufficient power is delivered to each setup.

MATERIALS

REAGENTS

- Experimental animals. Although transgenic strains of cells/animals expressing fluorescent reporter proteins are not required for successful surgery, they do ease in the identification and targeting procedures. (*C. elegans*; the *zdIs5* strain can be used for GFP-expressing mechanosensory neurons.)

  ! CAUTION All animal experiments must comply with the relevant institutional and national animal care guidelines.

- Cover glass (0.175 mm × 25 mm × 50 mm)
- Microscope cover slides (1 mm × 25 mm × 50 mm)
- Agarose gel 1.5%
- Sodium azide 10 mM

EQUIPMENT

General equipment

- Femtosecond laser (Spectra Physics Mai Tai HP Ti:Sapphire, tunable-wavelength Class IV laser). More cost-effective femtosecond lasers with a fixed wavelength can also be used including: Polarynox femtosecond fiber lasers, Del Mar Photonics Ti:Sapphire custom made kit, model TISSA100 and HighQLaser FemtoTrain, model IC-1045-3000. Minimum recommended pulse energy is ~20 nJ (i.e., average power 1.6 W for a laser with 80 MHz pulse repetition rate)
- Laser warning sign and safety equipment which meet requirements set by institutional as well as local and national safety standards. Laser goggles with minimal safety rating of OD7 + are recommended (Diode/Nd:YAG 42F goggle, Kentek, cat. no. KGG-42F)
- Optical table with sufficient surface area (1) (3.0 m × 1.5 m) and set of four air-damped legs (Newport, cat. nos. RS-4000-510-12 and I-2000-413.5, respectively)
- Inverted fluorescence microscope system (1) (Nikon Eclipse TI or similar model, Nikon) including a 70-mm stage-up kit to provide sufficient clearance for the rear-entry of the laser beam and a stage plate capable of holding a standard glass slide
- Image acquisition software (1) (NIS-Elements 2.0) and compatible computer.
- High-resolution CCD camera (1) (Photometrics Coolsnap HQ2)
- RMS-threaded IR and VIS alignment disk (400–640 nm and 800–1700 nm) (1) (Thorlabs, cat. no. RMSIR)
- RMS 45 to CFI 60 objective adapter (1) (Nikon, cat. no. MXA20750)

- IR card (1) (Newport, model no. F-IRC4)
- Infrared viewer (1) (Newport, cat. no. IRV1-2000)
- Optical power meter and detector (1) (Newport, part nos. 1918-C and 818P-010-12, respectively)
- Ø1/2″ Post (2″) (1) (Thorlabs, cat. no. TR2)
- Ø1/2″ Post holder (2″) (1) (Thorlabs, cat. no. PH2-ST)
- Standard base (1) (Thorlabs, cat. no. BA2)
- Function generator with single-pulse generation capability (1) (BK Precision, model no. 4030) ▲ **CRITICAL STEP** If another model or device is used, ensure that the function generator, or any source in general driving the high-voltage (HV) amplifier, is capable of driving the input load of the high voltage amplifier. For example, if the input impedance of the HV amplifier is 50 Ω and the maximum input voltage is 1 V, the function generator must be able to supply at least 20 mA to have access to the full input range.
- Oscilloscope (Tektronix, TDS2024)
- 3/8″ corded drill driver (1) (RIDGID, model# R70002)
- Drill set (Ryobi, model# AR2074)
- 1/4″-20 cap and set screws and #8–32 cap and screws (ThorLabs, cat. nos. HW-KIT2 and HW-KIT3, respectively)

Laser setup

- Optical isolator (1) (Conoptics, cat. no. 712TGG)
- Half-wave plate for 600–1050 nm high power applications (1) (Thorlabs, cat. no. AHWP05M-950)
- High-precision rotation mount for Ø1″ Optics (1) (Thorlabs, cat. no. PRM1)
- Glan–Thompson polarizer with 600–1050 nm anti-reflection coating (1) (Thorlabs, cat. no. GL10-B)
- Polarizing prism mount (1) (Thorlabs, cat. no. SM1PM10)
- Kinematic mount for thin Ø1″ Optics (1) (Thorlabs, cat. no. KM100T)
- EOM (1) and requisite HV amplifier (Conoptics, cat. no. 350-160 EOM with amplifier) **! CAUTION** The cables connecting EOM to the HV amplifier carry high voltage. Only the appropriately rated cables from the EOM manufacturer should be used. (Optional, see Steps 10–12) A high-speed mechanical shutter capable of providing a 2.2 ms pulse can be substituted for the EOM; however, the EOM enables the added benefit of two-photon excitation targeting (see Step 93).
- Optical isolator mount (1) (Conoptics, model M102, modified to hold the isolator)
- EOM mount-labjack (1) (Conoptics, model M102) (optional, see Steps 10–12)

- Safety shutter (1) (Electro-Optical Products SH-10 Interlock safety shutter with silver coated mirror, for NIR and DSH-10-110 V controller)
- Beam block (includes TR3 post) (7) (Thorlabs, cat. no. LB1)
- 2″ High universal post holder (for use with beam blocks) (7) (Thorlabs, cat. no. UPH2)
- Ø1/2″ post (2″) (3) (Thorlabs, cat. no. TR2)
- Ø1/2″ post holder (2″) (3) (Thorlabs, cat. no. PH2-ST)
- Standard base (3) (Thorlabs, cat. no. BA2)
- 50-Ω BNC cables of sufficient length (2) to connect the function generator to the oscilloscope and to the HV amplifier (or high-speed shutter controller if that option is used). T-joint BNC-cable connector (http://Cablesnmore.com, cat. nos. N23713 and X15305, respectively)

Dichroic mounting adapter

- IR dichroic mirror (1) (Chroma, cat. no. 670dcspxr)
- Nikon adapter plate (‘dust cover’) (70 mm stage-up kit, Nikon)
- Ø1″ (1″) pedestal pillar post (1) (Thorlabs, cat. no RS1P)
- Compact kinematic mount (1) (Thorlabs, cat. no KMS)
- BA1 Standard base (1) (Thorlabs, cat. no. BA1)
- Dichroic cube holder (1) (large aluminum filter cube, Nikon)
- High-performance epoxy (1) (Loctite Fixmaster, Loctite, part no. L08FA12920)
- #8–32 screw × 1/4′ with low-profile head (1) (McMaster–Carr, cat. no. 91770A190)

Periscope and beam expander

- 40 mm focal length, Ø1″, NIR achromatic doublet lens (1) (Thorlabs, cat. no. AC254-040) (dependent on back aperture of objective lens; see Step 43)
- 200 mm focal length, Ø2″, NIR achromatic doublet lens (1) (Thorlabs, cat. no. AC508-200) (dependent on back aperture of objective lens; see Step 43)
- Single-axis position stage with micrometer (1) (Newport, cat. no. 460P-X)
- Ø1.5″ post clamp adapter plate (1) (Thorlabs, cat. no. C1520)
- 14″ tall Ø1.5″ mounting post (3) (Thorlabs, cat. no. P14)
- Ø1.5″ post mounting clamp (4) (Thorlabs, cat. no. C1501)
- Right-angle kinematic cage mount (2) (Thorlabs, cat. no. KCB1)
- Ø1″ gold mirror (3) (Thorlabs, cat. no. PF10-03-M01)
- 12″ optical rail (1) (Thorlabs, cat. no. RLA1200)

- Ø2″ lens mount (1) (Thorlabs, cat. no. LMR2)
- Ø1″ optics, translating lens mount (1) (Thorlabs, cat. no. LM1XY)
- Ø1″ iris (4) (Thorlabs, cat. no. ID25)
- Rail carrier (4) (Thorlabs, cat. no. RC1)
- Ø1/2″ post (2″) (7) (Thorlabs, cat. no. TR2)
- Ø1/2″ post holder (2″) (6) (Thorlabs, cat. no. PH2-ST)
- Standard base (5) (Thorlabs, cat. no. BA2)
- Ultra-stable kinematic Ø1″ mirror mount (1) (Thorlabs, cat. no KS1)

Microscope optics

- High-NA objective lens for laser surgery (1). NA greater than 0.7 recommended. Examples: Mag: 20×, NA: 0.75, air lens (Nikon, cat. no. MRD00200). For work in *C. elegans* and *Danio rerio* axotomy, a 20×, NA: 0.75 objective provides a suitable balance between field of view and NA for rapid and reliable axotomy. If a different objective is used, the focal lengths of lenses L1 and L2 may need to be altered as discussed in Step 43 of the protocol below.
- Fluorescence filter cube for use with the fluorescent reporter protein expressed by the organism (1) (For GFP, Nikon HQ:F, Nikon)
- Optical notch filter at the laser wavelength (1) (Thorlabs, cat. no. FES0700)
- Standard square cage plate, SM1 threaded inner bore (1) (Thorlabs, cat. no. CP02)
- Epi and bright-field shutters (1) (Sutter, cat. no. LB-SC and IQ25-SA)

PROCEDURE

Laser safety section

! CAUTION Before initiating the protocol, ensure that the appropriate ‘Laser in Use’ safety signs are installed at the proper locations. In addition, the laser warning system as well as the laser system itself must comply with the relevant institutional, local and national laser safety guidelines. It is important to observe good laser safety practice, including not wearing reflective items such as jewelry and wristwatches when working near the laser. Do not look through the microscope eyepieces when the laser is on and when either the internal laser shutter or the safety shutter is open. Use beam blocks to safely terminate the laser beam while aligning. The laser should be shuttered while inserting components into the beam path. When installing a component into the beam path, use the IR card and/or IR viewer to visualize the location of the beam. Close either the internal laser shutter or the safety shutter (depending on the component location) and then place the component in the path. Following this, open the shutter and view the location of the beam in relation to the component being aligned. Close the necessary shutter and make more adjustments. If necessary, repeat this on/view–off/adjust cycle multiple times to obtain satisfactory alignment.

▲ **CRITICAL STEP** These steps are time independent and may be stopped and started when necessary; however, it is imperative that the laser is either properly shuttered or turned off when not in use.

Setting up the optical table, laser, power meter, microscope and optical isolator ● TIMING 6 h

1| Install the optical table (this should be done by the manufacturer).

2| Install the femtosecond laser system such that the beam is aligned down the center of the optical table (this should be done by the manufacturer).

3| Install the microscope body and supporting equipment at least 1.5 m from the output of the laser to allow sufficient room for the beam-conditioning optics (installation of the microscope should be done by the manufacturer).

4| Attach the optical power detector to the power meter. Mount the detector on one of the Ø1/2″ posts and insert the post into a Ø1/2″ post holder mounted on a standard base (BA2) for easy insertion and positioning of the detector in the beam path. Turn on the attenuator in front of the optical power detector to avoid damage (see manufacturer's manual for details). The optical power meter should be brought to zero to calibrate it for ambient radiation.

! CAUTION It is critical both for user and equipment safety that the power meter is accurately calibrated.

5| Turn on the laser, leaving the internal shutter closed and allow it to warm up. Set the laser wavelength to 800 nm.

6| Set the output power of the laser to a low yet stable level (~150 mW) and ensure that the laser is not pulsing. This state of the laser is used for aligning optical components in steps below.

! CAUTION Do not allow the output power of the laser to exceed 200 mW.

7| Install the optical isolator (Component B in Fig. 1) with its mounting hardware in front of the laser to eliminate back reflections (follow the instructions of the manufacturer for alignment). The optical isolator prevents reflection back into the femtosecond laser, which can result in instabilities in the output power and disrupt mode-locking.

! CAUTION The optical isolator contains a powerful magnet that can attract metallic tools such as screwdrivers during installation, thus causing damage. It is also imperative that sufficient beam block assemblies, composed of one beam block with post (LB1) and one 2″ high universal post holder, are placed around the isolator to absorb any beams reflected from the crystal surfaces of the isolator as shown in Figure 1 by the dashed orange lines. The placement and number of beam blocks is dependent to the alignment of the optical isolator.

Installing the equipment to control the laser power ● TIMING 2 h

8| Install the half-wave plate (Component C in Fig. 1) using the high precision rotation mount, one Ø1/2″ post, one Ø1/2″ post holder and a standard base (BA2) at the output of the optical isolator. Rotate the half-wave plate mount to the 0° position.

9| Install the Glan–Thompson polarizer (Component D in Fig. 1) using the polarizing prism mount, the kinematic mount for thin Ø1″ optics, one Ø1/2″ post, one Ø1/2″ post holder and a standard base (BA2). Place a beam block assembly to absorb the rejected beam.

10| Install the EOM (Component E in Fig. 1) (or high-speed shutter; see equipment list), and place a beam block assembly to absorb the rejected beam. Follow the installation instructions of the manufacturer.

! CAUTION When aligning the EOM, 200 mW or less average power must be used to avoid damage.

11| Install the HV amplifier (if using EOM), but do not turn it on. Connect the HV amplifier to the EOM using the included HV-rated cables.

! CAUTION The HV-rated cables look similar to standard low-voltage-rated BNC cables. Using improperly rated cables increases the risk of electrocution and may damage equipment.

12| Use a T-joint connector and two 50-Ω BNC cables to connect the output of the function generator both to the input port of the HV amplifier (or high-speed shutter controller) and also to the input of the oscilloscope. To visualize single electrical pulses on the oscilloscope screen, set the oscilloscope to trigger and hold on the rising edge at 0.5 V. Consult the oscilloscope manufacturer's manual for detailed operation instructions.

13| Set the function generator to produce a square pulse with a 2.2 ms duration and 1.0 v amplitude (or the voltage required by the high-speed shutter control). Consult the function generator manufacturer's manual for detailed operation instructions.

14| Mount the safety shutter using one Ø1/2″post, one Ø1/2″ post holder and a standard base (BA2) as well as a beam block assembly to serve as a rejected beam dump (Components F in Fig. 1). Using the IR card and IR viewer, adjust the position of the safety shutter and the beam block such that the beam is not clipped when the safety shutter is open and the beam is reflected into the center of the beam block when the safety shutter is closed.

15| Close both the internal laser shutter and the safety shutter.

16| Turn the laser to its maximum emission power and ensure that it is pulsing. For the Mai Tai HP laser the expected maximum emission power is approximately 3.0 watts at 800 nm.

? TROUBLESHOOTING

17| Place the optical power detector at the output of the EOM. Turn on the HV amplifier. Open the internal laser shutter and adjust the position of the detector to maximize the power displayed on the meter.

18| Adjust the bias voltage of the HV amplifier until the measured power reaches its minimum.

19| Turn off the HV supply.

20| Rotate the half-wave plate (Component C in Fig. 1) until the power measured is appropriate for surgery: for axotomy in *C. elegans*, 1.2 W is sufficient when using a 20× objective lens with NA = 0.75 and a 2.2 ms exposure time. For higher NA objectves or longer exposure times, lower power levels are sufficient[1].

21| Turn on the HV amplifier and record the value on the power meter. This is the minimum amount of transmitted laser power. The ratio of the power set in Step 20 to this value gives the contrast ratio of the laser power at the sample. A ratio of at least 50 is desirable.

? TROUBLESHOOTING

22| Close the internal laser shutter.

23| Remove the power detector from the beam path.

Assembly of the dichroic mounting adapter ● TIMING 1 h and a 24-h pause

24| The dichroic mounting adapter, shown in detail in Figure 2, is composed of the dichroic mirror, the Nikon adapter plate from the 70 mm stage-up kit for a Nikon Eclipse TI, one Ø1″ (1″) pedestal pillar post, one compact kinematic mount, one standard base (BA1), one dichroic cube holder. The dichroic mounting adapter directs the laser beam into the objective lens without interfering with the normal optical paths of the microscope.

25| Mount the dichroic mirror in the dichroic cube holder.

▲ **CRITICAL STEP** Ensure that the coated side of the dichroic mirror is facing the laser.

26| Glue the dichroic cube holder to the standard base (BA1) using two-part epoxy following the diagram in Figure 2.

27| Glue the compact kinematic mount to the standard base using two-part epoxy following the diagram in Figure 2.

▲ **CRITICAL STEP** To ensure that the components are glued properly, place the assembly upside-down on a flat surface overnight while the epoxy cures.

28| Attach the pedestal pillar post to the compact kinematic mount using one #8–32 × 1/4″ set screw.

29| Hold the dichroic cube holder so that the dichroic mirror is centered above the large hole in the Nikon adapter plate. Use a marker to draw a circle on the Nikon adapter plate around the bottom of the pedestal pillar post.

30| Using a hand-held power drill, make a 0.25″ hole in the Nikon adapter plate at the center of the marked circle as shown in Figure 2.

31| Use a low-profile #8–32 × 1/4″ screw to attach the pedestal post to the dust cover through the hole drilled in Step 30.

32| Attach the completed dichroic mounting adapter (Component N in Fig. 1) to the top of the microscope's fluorescent filter turret (Component P in Fig. 1) using the screws and hardware included in the 70-mm stage-up kit.

Assembly of the periscope ● TIMING 1 h

33| Construct a periscope (Component I in Fig. 1) using one Ø1.5″. Mounting post, one BA2 standard base, two right-angle kinematic cage mounts, two Ø1″gold mirrors and two Ø1.5″post mounting clamps. Place the periscope assembly on the optical table as shown in Figure 1 so that it will be able to direct the beam into the laser entry port of the microscope.

34| Install a Ø1″gold mirror in the ultra-stable kinematic Ø1″mirror mount.

35| Repeat Step 7 to lower the laser output power.

36| Turn off the HV amplifier.

37| Mount the ultra-stable kinematic Ø1″ mirror mount using a one Ø1/2″ post, one Ø1/2″ post holder and a standard base (BA2) (Component G in Fig. 1) on the optical table. Open the internal laser shutter and the safety shutter. Coarsely align the kinematic mirror to center the laser on the periscope's lower mirror. Close the safety shutter and secure the kinematic mirror to the optical table.

38| Place a Ø1″ iris at the input of the periscope (Component H in Fig. 1). This iris will ease the process of realignment when needed.

39| Coarsely align the periscope to direct the laser horizontally into the back port of the microscope.

40| Adjust the angle of the lower periscope mirror to center the laser on the upper periscope mirror.

41| It may be necessary to repeat Steps 37–40 several times iteratively to obtain a satisfactory alignment.

42| Close both the safety shutter and the internal laser shutter.

Assembly of the laser beam expander ● TIMING 1 h

43| Determine the focal lengths of lenses L1 and L2 needed to fill the back aperture of the objective used for axotomy. In this protocol, the laser beam diameter of 3 mm must be expanded to fill the 15-mm-in-diameter back aperture of the objective lens, and therefore the beam must be expanded fivefold. The amount

of expansion is determined by the ratio of the focal length of lens L2 to that of lens L1, which in this protocol is 200 mm/40 mm = 5. The distance between the lenses is the sum of their focal lengths f_1 and f_2 as shown in Figure 3, which in this protocol is 40 mm + 200 mm = 240 mm.

44| Construct the beam expander following the diagram in Figure 4 using two Ø1.5″ mounting posts, two standard bases (BA2), two Ø1.5″ post mounting clamps, one 12″ optical rail, three rail carriers, one single-axis position stage with micrometer, one Ø1″ translating lens mount, one Ø2″ lens mount and four Ø1/2″ post holders.

45| Place the beam expander on the optical table next to the microscope as shown in Figure 1. Positioning of the beam expander optics close to the microscope in this manner provides two benefits: first, it allows for a single person to (re)align the setup, thus facilitating quick troubleshooting. Second, it reduces the amount of real estate on the optical table consumed by the optics and supporting hardware.

46| Use a bubble level to ensure the 12″ optical rail is level. The rail carriers slide onto the optical rail and are handtightened with the included screws. The single-axis position stage with micrometer is mounted using cap screws.

47| Space the outer Ø1/2″ post holders by a distance equal to $f_1 + f_2$. The distance between these two Ø1/2″ post holders is finely adjusted below in the protocol.

48| Lower the laser power as in Step 6.

49| Completely close the iris closest to the objective. Close the remaining two irises leaving an aperture of approximately 5 mm.

50| With the aid of the IR viewer and IR card, adjust both the height and the angle of the upper periscope mirror until the laser beam passes through the two open irises.

51| Open all three irises.

52| Center the beam on the dichroic mirror by adjusting only the height of the upper periscope mirror and the position of the periscope assembly on the optical table. If the periscope assembly is displaced, repeat Steps 37–40 to recenter the beam on the lower periscope mirror.

53| Close all three irises leaving an aperture of approximately 5 mm. Adjust the height of the 12″ optical rail until the laser passes through all three irises. Use a bubble level to ensure the 12″ optical rail is level.

? TROUBLESHOOTING

54| Close both the safety shutter and the internal laser shutter.

Coarse alignment of the laser ● TIMING 30 min

55| Construct the IR alignment tool by drilling a 1/8″ diameter hole through the center of the RMS-threaded IR alignment disk and then insert the modified IR alignment disk into an RMS 45 to CFI 60 objective adapter.

! CAUTION The alignment disk is built from brittle plastic so drilling must be done with care.

56| Thread the completed IR alignment tool into the microscope nosepiece.

57| Place a cover glass on the stage plate to act as a reflecting surface for laser alignment.

58| Lower the laser power for alignment as in Step 6.

59| Tilt the condenser arm away from the stage to facilitate viewing of the IR alignment tool.

60| Open the internal laser shutter and the safety shutter.

61| Using the IR viewer, observe the top of the IR alignment tool as shown in Figure 5.

! CAUTION Do not hold the IR viewer directly in the beam path as this could result in damage to the IR viewer.

62| If the laser is not passing through the center hole then a glowing dot will appear on the surface of the IR alignment tool as shown in Figure 5a. Use the compact kinematic mount of the dichroic mounting adapter to center the laser on the IR alignment tool.

? TROUBLESHOOTING

63| If the laser beam is not normal to the cover glass two spots will appear on the surface of the IR alignment tool as shown in Figure 5b: a glowing ring around the center hole (the ‘transmitted beam spot’) and a spot off to the side (the ‘reflected beam spot’).

64| Note the location of the reflected beam spot.

65| Adjust the angle of the upper periscope mirror to move the transmitted beam spot approximately half the distance towards the initial location of the reflected beam spot (from the previous step). In doing this, the reflected beam spot will itself move and may disappear, as the transmitted beam is no longer passing through to the center hole.

? TROUBLESHOOTING

66| Adjust the angle of the dichroic mirror using the compact kinematic mount of the dichroic mounting adapter to bring the transmitted beam spot back to the center hole. The reflected beam spot should move back towards the center hole at approximately twice the rate as the transmitted beam spot, and they will meet in the middle as shown in Figure 5c.

? TROUBLESHOOTING

67| Close the safety shutter. Insert L2 making sure that the curved surface of the lens faces away from the objective.

68| Open the safety shutter. Using the IR viewer, observe the IR alignment tool and center the transmitted beam spot by adjusting the position and pitch of L2.

? TROUBLESHOOTING

69| Close the safety shutter. Insert L1 making sure that the curved surface of the lens faces L2.

70| Open the safety shutter. Using the IR viewer, observe the IR alignment tool and center the transmitted beam spot by adjusting the position and pitch of L1 using both the Ø1/2″ post and the Ø1″ translating lens mount. Note that the transmitted beam spot will be larger and diffuse after inserting L1 as shown in Figure 5d. Ensure that the beam is not clipped (i.e., the transmitted beam spot should be a full, symmetric and circular disk on the IR alignment tool).

? TROUBLESHOOTING

71| Close the safety shutter and internal laser shutter.

Fine alignment of the laser and image focal planes ● TIMING 1 h

72| Insert the objective to be used for surgery. Make sure that no filter cube is in the active slot of the epifluorescence filter turret. Turn on the HV amplifier and adjust the half-wave plate as discussed in Steps 17–21.

73| Close the internal laser shutter and the safety shutter.

74| Draw lines on a clean cover glass using a non-water-based permanent marker (e.g., Sharpie). Place the cover glass on the stage plate.

▲ **CRITICAL STEP** Use only a single pass with the marker, as a thick coating will not allow the laser to produce thin cuts.

75| Turn on the camera and image acquisition software.

76| Open the internal laser shutter and the safety shutter.

77| Locate the image of the laser in the preview window of the image acquisition software. Center the beam in the preview window by adjusting the angle of the dichroic mirror using the compact kinematic mount of the dichroic mounting adapter.

? TROUBLESHOOTING

78| Mark the position of the laser on the computer monitor with a piece of tape.

▲ **CRITICAL STEP** Do not move the image acquisition software preview window after marking the beam location.

79| Close the internal laser shutter and the safety shutter.

80| Insert the laser notch filter into a 30 mm-standard square cage plate.

81| Place the laser notch filter between the dichroic mirror and the microscope epifluorescence filter turret.

82| Tilt the condenser arm to its original upright position. Turn on the bright field source, adjust the exposure of the camera and focus on the edge of a marker line. Move the stage to position the edge of a marker line under the piece of tape on the computer monitor.

83| Open the safety shutter. Depending on the locations of L1 and L2 set in Steps 67–70 the region of marker underneath the tape will be ablated to varying degrees of sharpness, symmetry and size. While staying focused on the edge of the marker line, adjust the position of L1 along the optical rail using the micrometer to adjust the focus of the laser beam to increase the sharpness of the ablation. In addition, adjust L1 using its translating mount axes to further improve the shape of the ablation.

? TROUBLESHOOTING

84| Characterize the laser alignment by using the single-pulse button on the function generator to generate spots and compare the result to the image in Figure 6. When the system is properly aligned, the cutting patterns shown in Figure 6 should be ~3 μm wide and the firing patterns should be ~9 μm across. Note that these values have been found to be suitable for axotomy in *C. elegans* using a 20×, NA: 0.75 objective.

? TROUBLESHOOTING

85| Close the safety shutter and the internal laser shutter.

Example application: *in vivo* laser axotomy on *C. elegans* ● TIMING 25 min

86| Prepare the microscope for fluorescence imaging and remove the laser notch filter (1 min).

● **TIMING***:* These steps are strongly time dependent and must be completed within 2 h once the anesthetic has been administered to ensure minimal toxic effects (*C. elegans*). The rapid development cycle of the animals may influence the biological process under study, so care should be taken to ensure that the animal is in the proper developmental stage.

87| Turn on the HV amplifier and adjust the half-wave plate as discussed in Steps 17–21 (3 min).

88| Open the internal laser shutter and the safety shutter (2 min).

89| Locate the image of the laser in the preview window of the image acquisition software and adjust the piece of tape on the monitor if necessary (2 min).

? TROUBLESHOOTING

90| Close the safety shutter and return the laser notch filter to its position above the microscope filter turret (2 min).

91| Immobilize an animal on a cover glass using standard procedures[28]. Place the cover glass on the stage plate. Ensure that the animal is as close to the cover glass as possible (5 min).

92| Using fluorescence imaging, locate the animal and move the stage to bring the axon or cell to be ablated directly under the piece of tape. Bring the target axon or cell into focus (< 30 s).

93| Open the safety shutter and adjust the focus to achieve two-photon fluorescence of the target axon, indicative of proper focusing. Adjust stage position and focus if necessary (< 30 s).

94| Press the single-pulse button on the function generator to ablate the target axon or cell. Immediately close the safety shutter to avoid damaging the organism/cell (< 10 s).

? TROUBLESHOOTING

95| Recover the animal using standard procedures[28] (5 min).

96| At the end of the experiment, close the safety shutter and internal laser shutter. Turn off the laser and the fluorescence and bright field sources (3 min).

● TIMING

Steps 1–7, Setting up the optical table, laser, power meter, microscope and optical isolator: 6 h

Steps 8–23, Installing the equipment to control the laser power: 2 h

Steps 24–32, Assembly of the dichroic mounting adapter: 1 h + 24 h pause

Steps 33–42, Assembly of the periscope: 1 h

Steps 43–54, Assembly of the laser beam expander: 1 h

Steps 55–71, Coarse alignment of the laser: 30 min

Steps 72–85, Fine alignment of the laser and image focal planes: 1 h

Steps 86–96, *In-vivo* laser axotomy on *C. elegans*: 25 min

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

ANTICIPATED RESULTS

Characterization of the femtosecond laser surgery system

The setup described in this protocol produces a circular laser spot with a full-width at half-maximum (FWHM) of 1.7 μm at the focal plane (see Fig. 7). An objective with a higher NA can be used to generate a smaller laser spot size at the expense of the working distance and/or the field of view. Using the components described in this protocol, the total power loss between the output of the EOM and the sample is 47.5%.

Axotomy of *C. elegans* mechanosensory neurons

With a 20× (NA = 0.75) objective, we use a 1.20 w (measured at the output of the EOM, which corresponds to 0.63 watts at the sample), 2.2 ms-long laser pulse train to carry out axotomy on *C. elegans zdIs5* strain (GFP-labeled mechanosensory neurons) (see Fig. 8). Although low-magnification objective lenses have smaller NAs, they allow simultaneous visualization of the cell body and large portions of the axons, enabling surgery of the axons at precisely measured distances from the soma.

Lower laser power and shorter pulse trains can be used; however, the reproducibility of surgery decreases at lower powers in part because of increased sensitivity to laser focus1. In addition, it becomes difficult to distinguish photodamage from photobleaching. Photobleaching is often followed by spontaneous recovery of fluorescence in the axon at the site of injury within a few minutes, whereas photodamage leads to permanent non-fluorescent regions. The threshold power levels for photobleaching versus photodamage can be characterized using dye-filling techniques[8,10]. Conversely, higher laser powers and/or longer pulse trains often result in extensive scarring, causing widespread injury to the animal[1,10,29].

Upon ablation, we often briefly observe fluorescent protein leaking from the axon terminals and diffusing into the surrounding area. The brightness of the axon terminals decreases initially, and recovers within several minutes, leaving a non-fluorescent region at the site of surgery. Within several minutes both the proximal and distal axon terminals retract by several microns (arrow 2 in Fig. 8b). Within a few hours, the proximal axon terminal starts regrowing (arrow 3 in Fig. 8b)[8]. The regrowing proximal axon terminal may also fuse with the distal axon terminal[10].

Acknowledgments

We thank the following funding sources: NIH Director’s New Innovator Award Program (1-DP2-OD002989–01), Packard Award in Science and Engineering, Sloan Award in Neuroscience, Lincoln Laboratory Advanced Concepts Committee, NSF Career Award, NSF Graduate Research Fellowship, ‘La Caixa’ Fellowship, NIH Biotechnology Training Grant and the NDSEG Fellowship.

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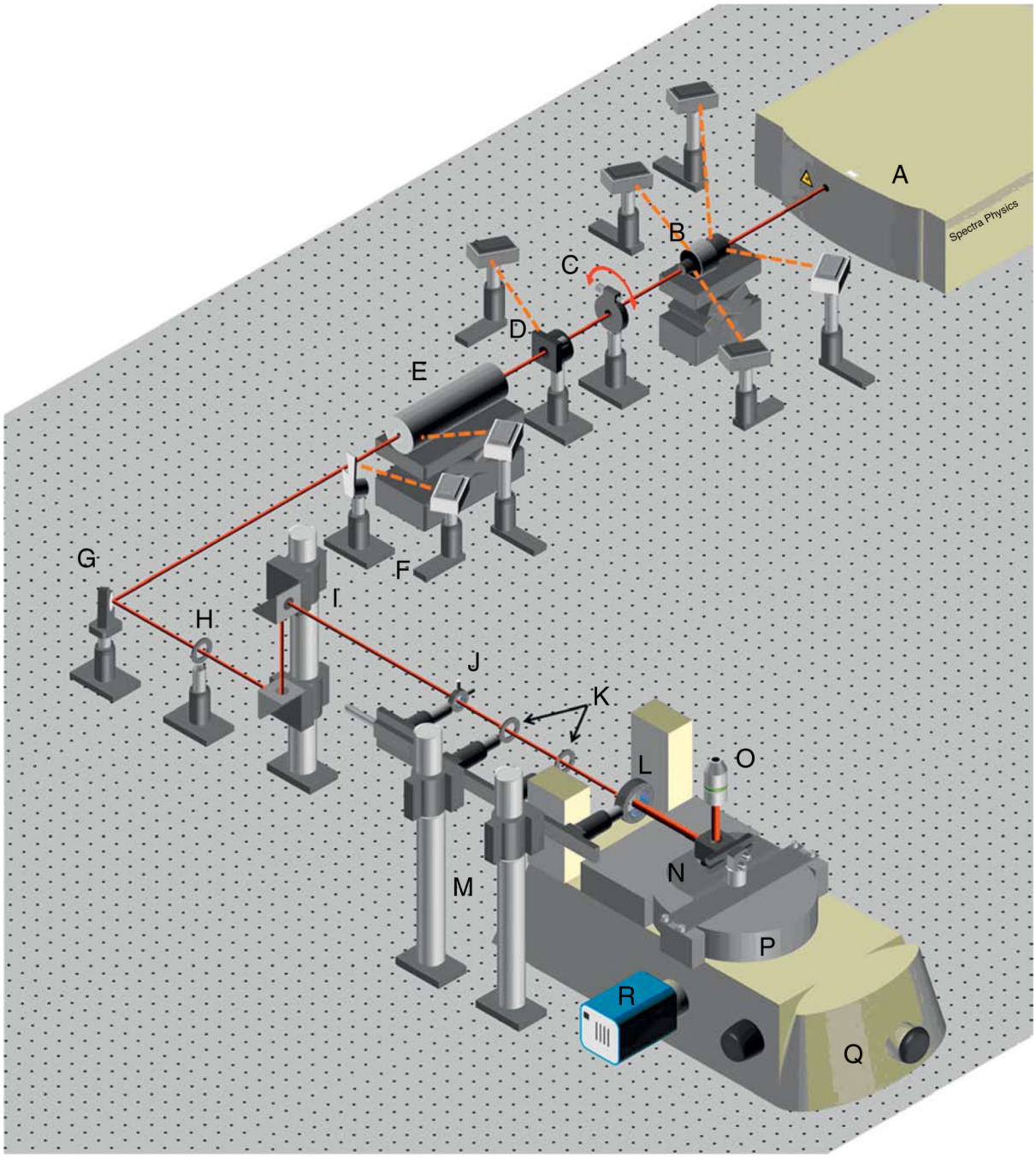

Figure 1.
Optical system layout. See Table 1 for component list.

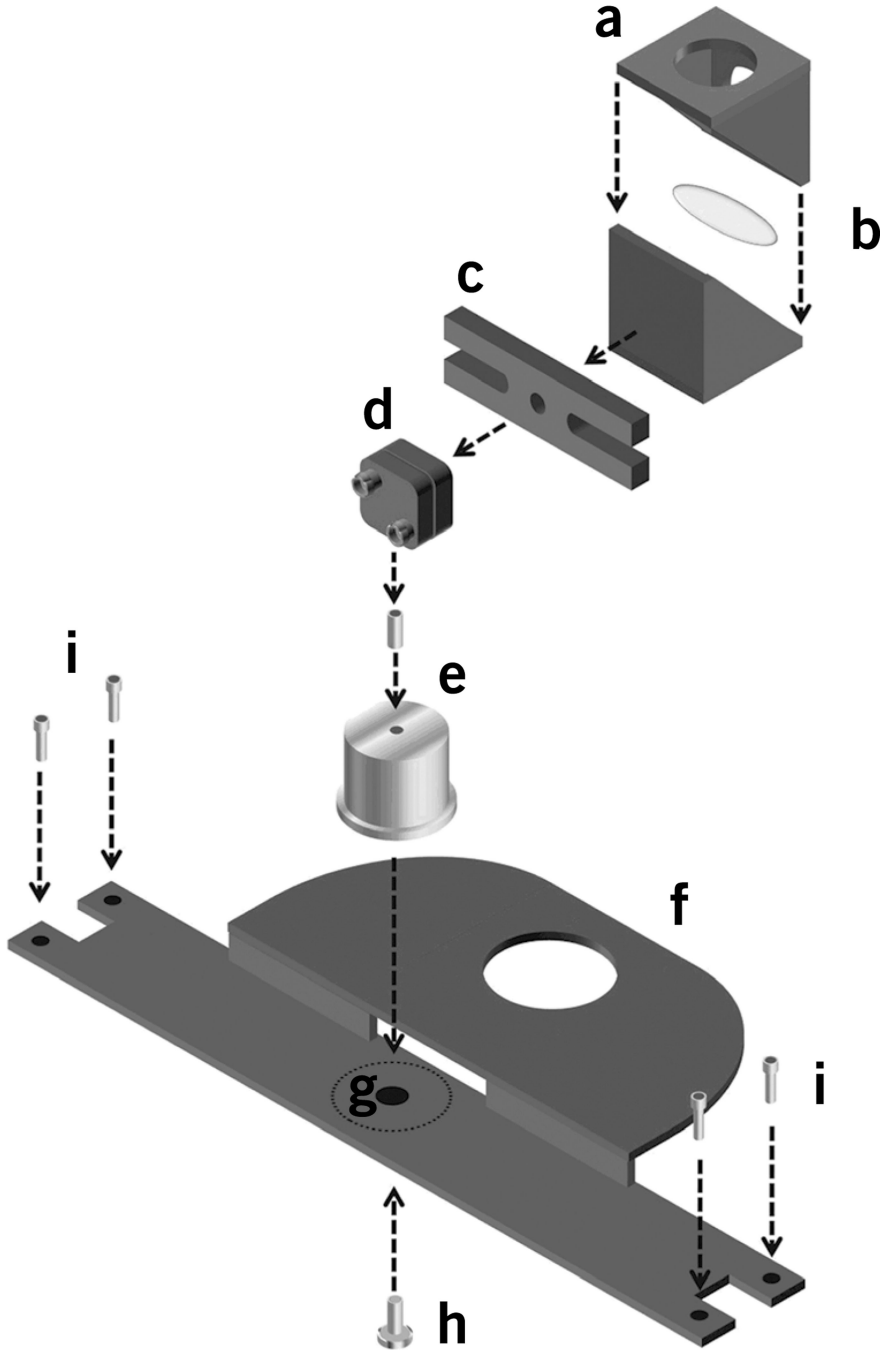

Figure 2.

An exploded view of the dichroic mounting adapter. (**a-b**) (**a**) The dichroic mounting adapter is composed of a metal filter cube, which contains an infrared (IR) dichroic mirror (**b**). (**c**) The filter cube is attached to a BA1 standard base using glue. (**d**) The opposite face of the BA1 standard base attaches to a two-axis compact kinematic mount also using glue. (**e**) The compact kinematic mount is attached to a Ø1″ (1″) pedestal pillar post with a #8–32 × 1/4″ set screw. (**f**) The assembly comprising components **a–e** mounts to a Nikon adapter plate from a 70 mm stage-up kit so that the dichroic mirror sits in the beam path. (**g-h**) (**g**)

This is accomplished by drilling a hole and using a #8–32 × 1/4″ screw (**h**) to position the dichroic mirror over the opening in the adapter plate. (*i*) The entire assembly is affixed to the microscope using screws included in the 70 mm stage-up kit. Figure 1 shows the location of the dichroic mounting adapter on the microscope.

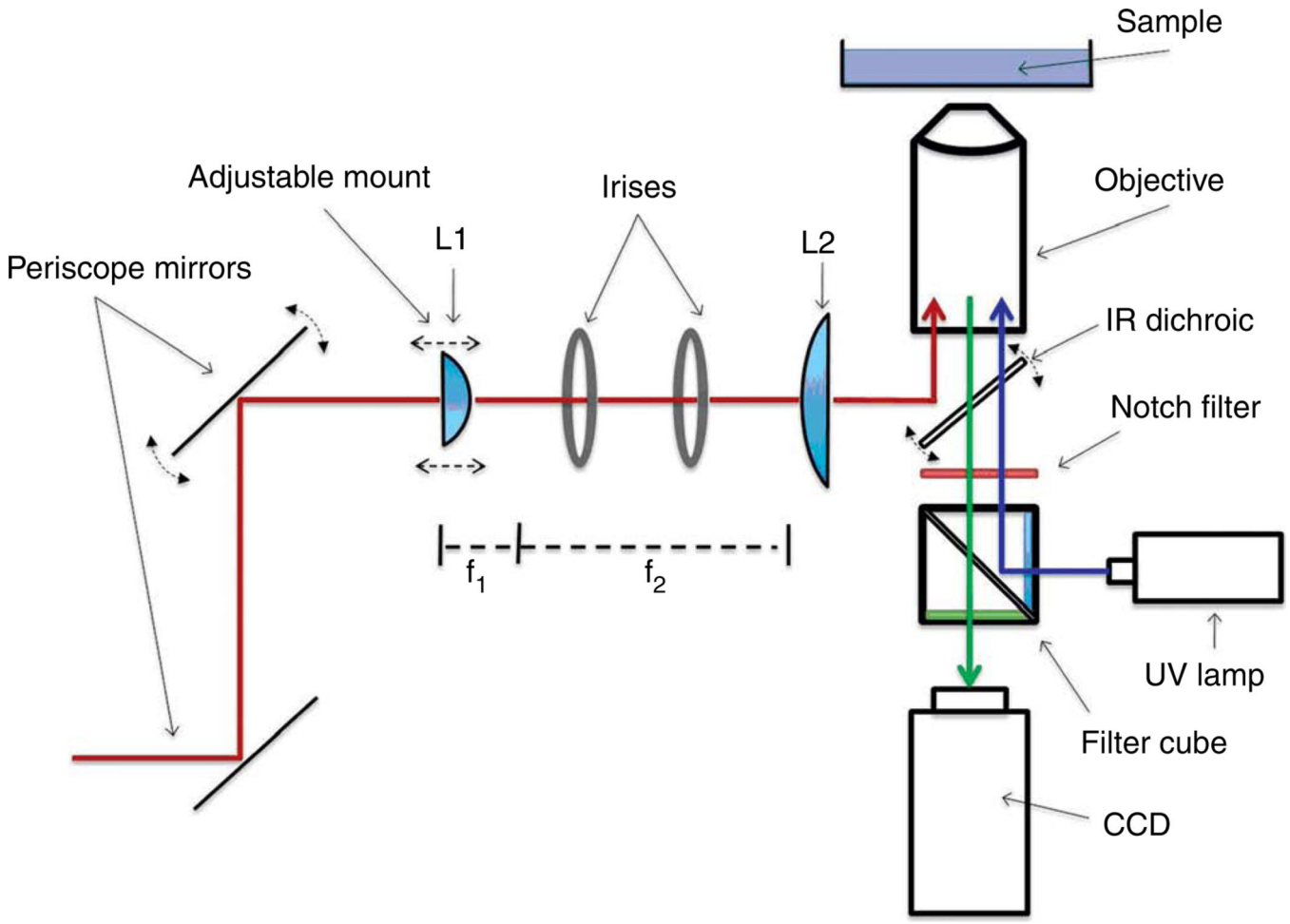

Figure 3.

Optical path for simultaneous epifluorescence imaging and laser axotomy. The femtosecond laser, indicated by the red line, passes through beam conditioning optics before being directed up by the near infrared (NIR) dichroic mirror into the back aperture of the objective lens. The epifluorescence excitation, indicated by the blue line, is simultaneously directed into the back aperture of the objective lens by the filter cube. The fluorescence emission, indicated by the green line, passes through multiple filters and is captured by the camera.

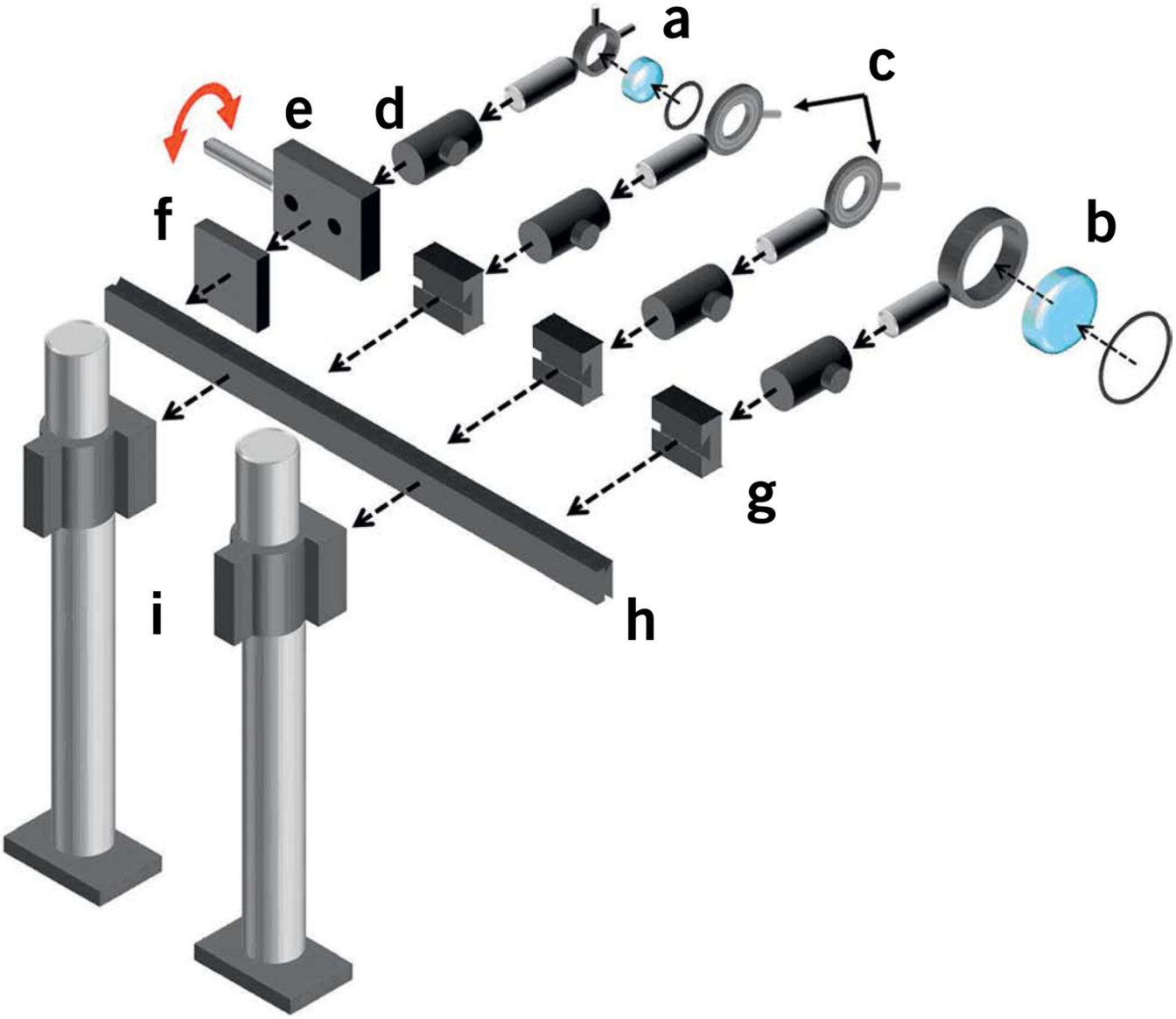

Figure 4.

An exploded view of the beam expander. Lenses L1 and L2 (**a** and **b**, respectively) sit in their mounts that are attached to Ø1/2″ posts. Two Ø1″ irises (**c**) are also attached to Ø1/2″ posts. All four posts sit securely in Ø1/2″ post holders (**d**), one of which is attached to a single-axis stage (**e**) with rotatable micrometer and Ø1.5″ post clamp adapter plate (**f**), whereas the remaining three are attached to rail carriers (**g**). These four assemblies firmly attach to the 12″optical rail (**h**) which is mounted to the two Ø1.5″ posts by two Ø1.5″ post mounting clamps (**i**). The entire assembly is mounted using BA2 standard bases.

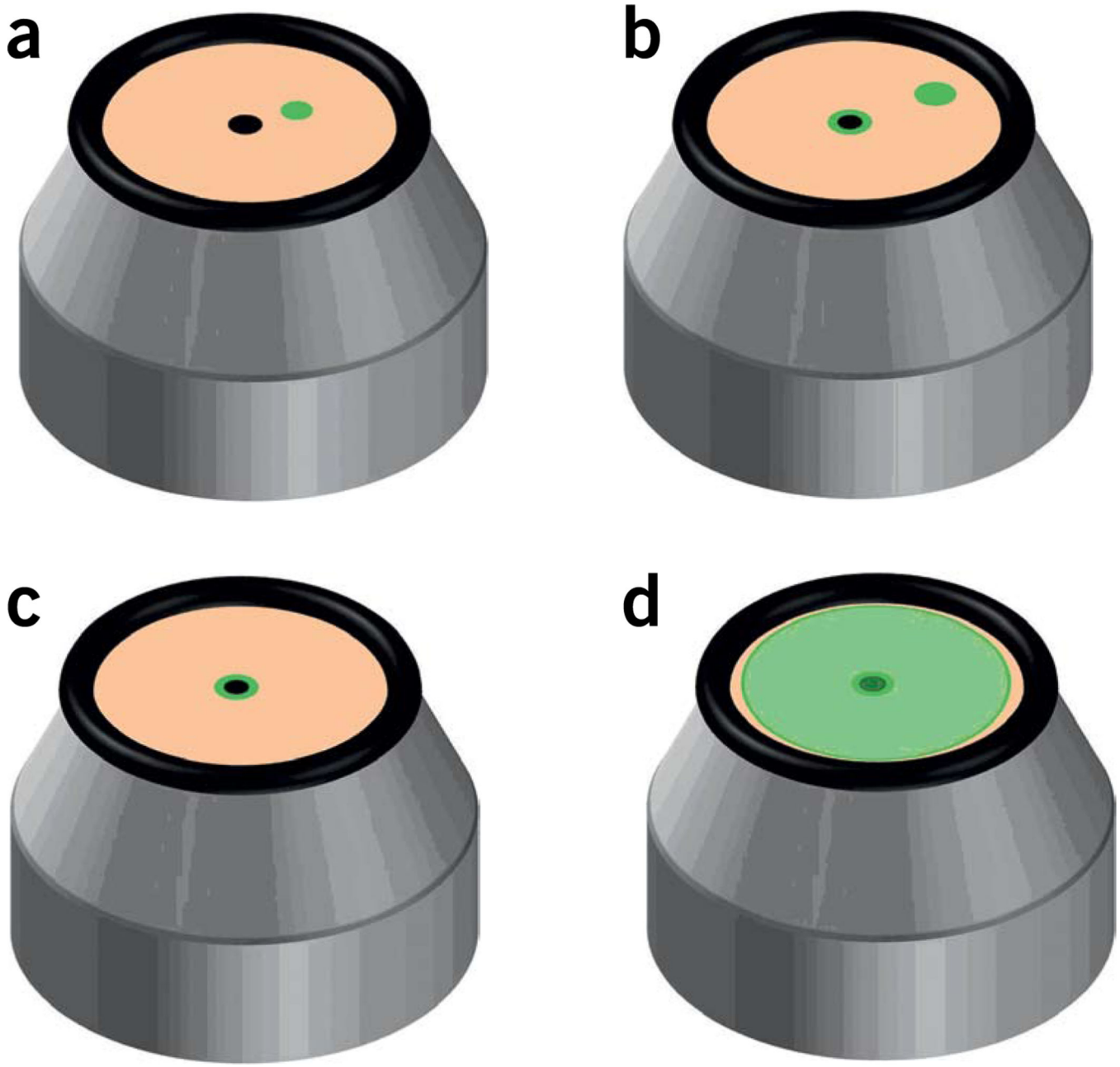

Figure 5.

Use of the infrared (IR) alignment tool. The IR alignment tool is composed of an RMS IR-aligning disk and an RMS 45 to CFI 60 objective adapter. (**a**) Without lenses L1 and L2, the transmitted beam is directed to the center of the dichroic mirror, thus resulting in a glowing spot on the field of the disk. (**b**) Adjusting the dichroic mirror causes the transmitted beam to pass through the center hole and an additional spot caused by the reflection of the beam from the cover glass appears on the field of the disk. The reflected spot is caused by the non-normal incidence of the transmitted beam on the cover glass. Moving the transmitted spot half-way towards the initial location of the reflected spot by adjusting the upper periscope mirror and then moving the transmitted spot back to the center hole by adjusting the angle of

the dichroic mirror, achieves normal incidence of the beam on the cover glass. (**c**) Normal incidence is indicated by both reflected and transmitted beams passing through the center. (**d**) Inserting both lenses L1 and L2 into the beam path (Steps 68–70), results in a large, symmetric circular illumination on the IR alignment tool.

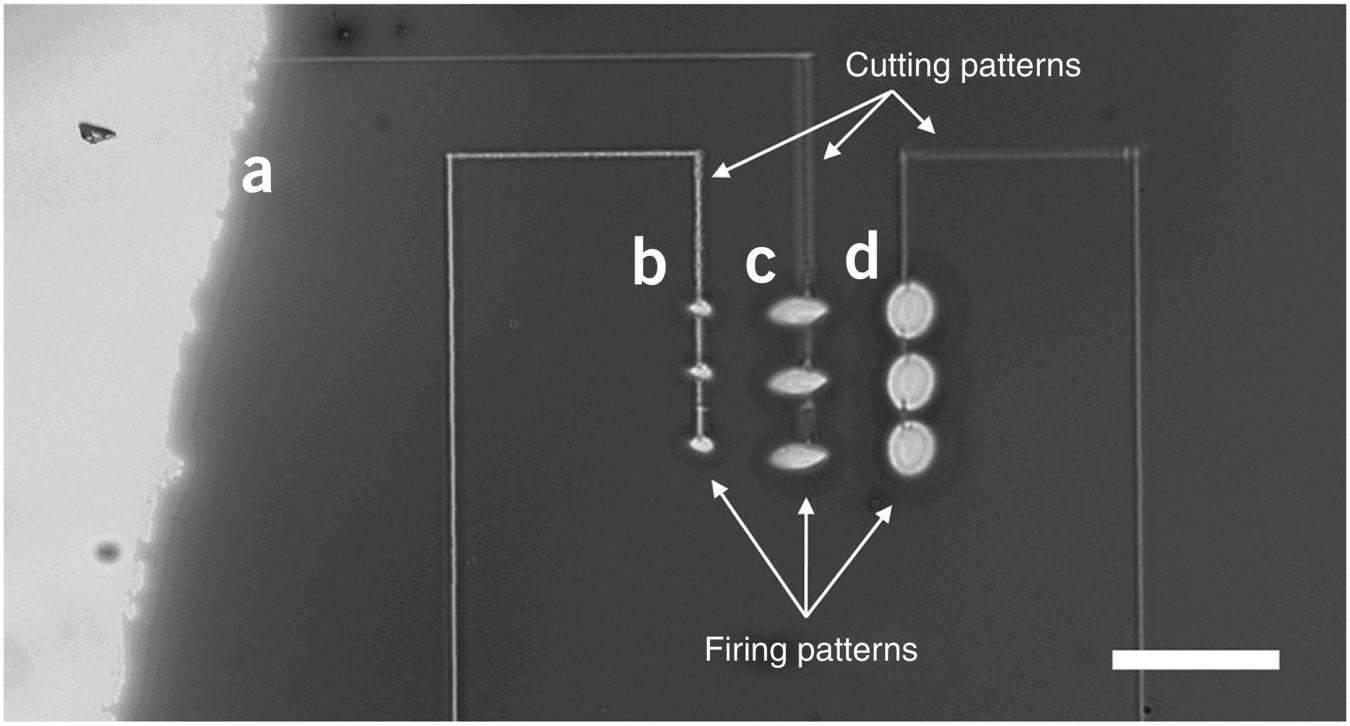

Figure 6.

Ablated patterns in permanent marker on cover glass under different alignment conditions. (**a,b**) When the image plane is focused on the boundary of the marked and unmarked glass surfaces, and when the system is properly aligned, the resulting cutting pattern is narrow and symmetric (**a**), whereas the firing pattern is relatively small and also symmetric (**b**). If the beam is clipped and/or lenses L1 and L2 are misaligned, the cutting pattern is wider in one direction than the orthogonal direction (**c**). In addition, the firing pattern becomes asymmetric. If the laser is out of focus, the cutting pattern is blurred and the firing pattern is larger (**d**). Scale bar is 50 μm.

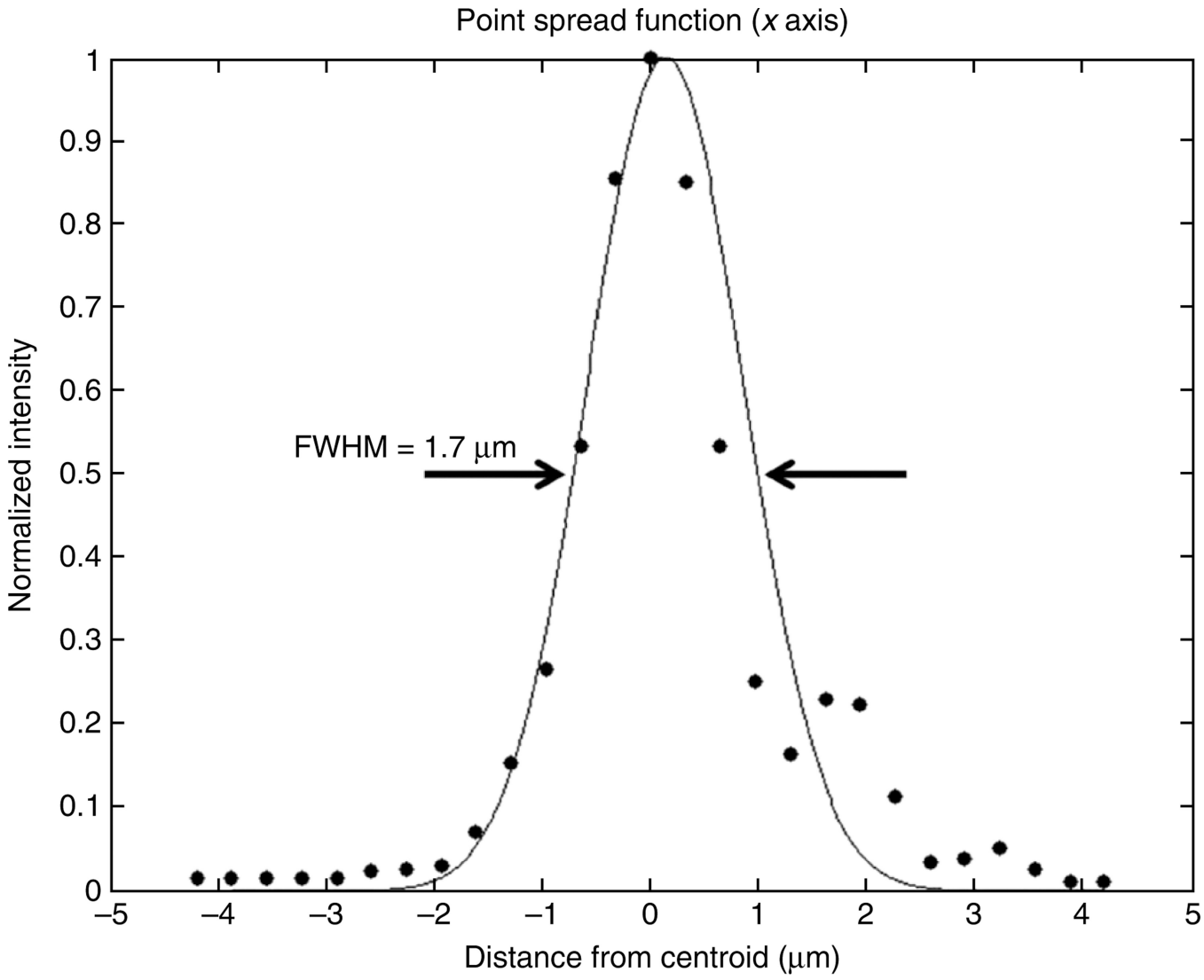

Figure 7.

Point spread function of the laser at the focal plane. The system described in this protocol generates a circular laser spot at the sample with a full width at half-maximum of 1.7 μm.

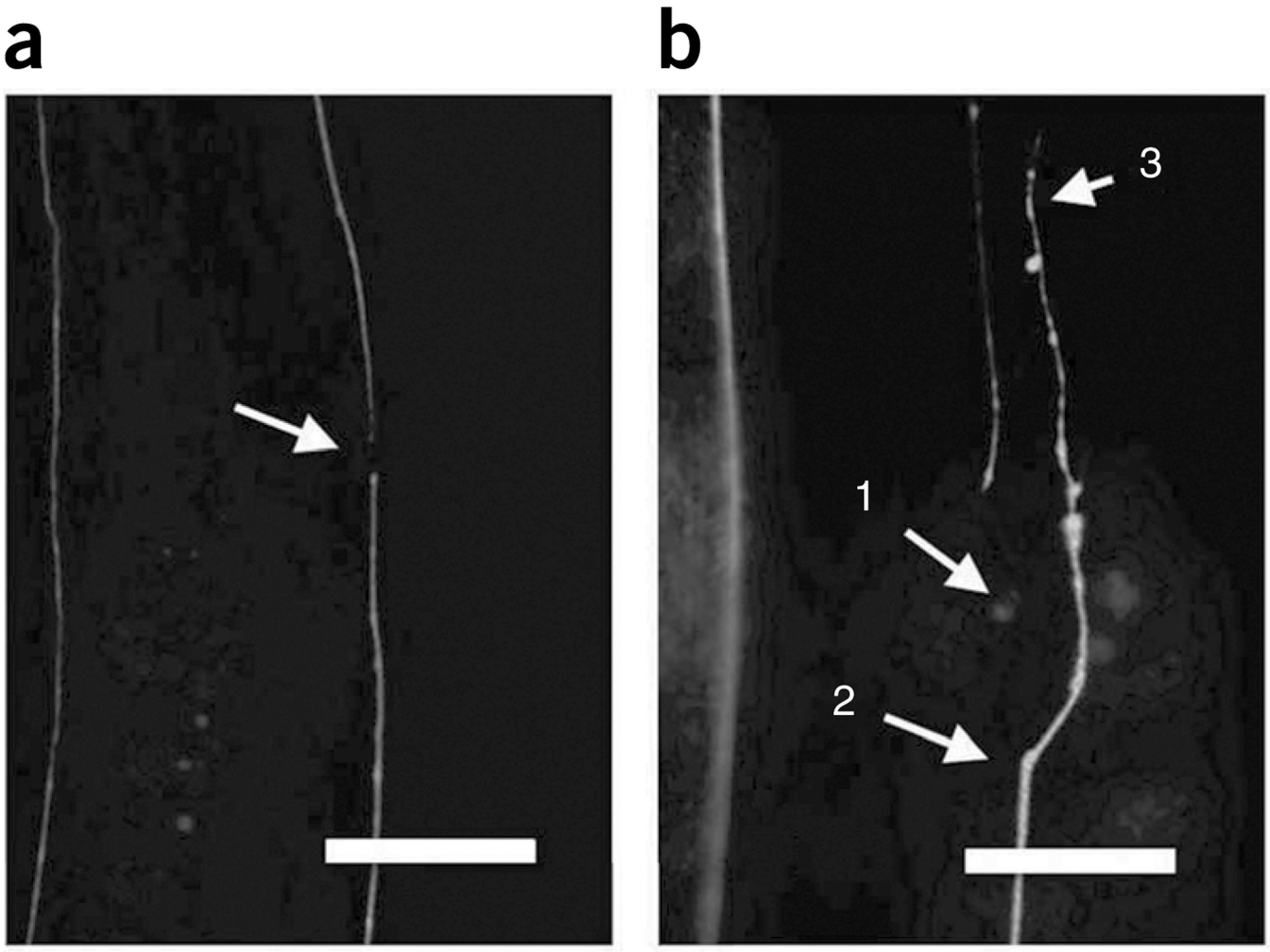

Figure 8.

Femtosecond laser microsurgery. (**a**) A highly localized region (arrow) of a *Caenorhabditis elegans* mechanosensory neuron is ablated using the system described in this protocol. (**b**) Following surgery at point 1, the ablated process first retracts to point 2 and then regenerates to point 3. Scale bar represents 10 μm.

Table 1

Component list for the optical system layout of Figure 1.

Component	Description
A	Femtosecond laser
B	Optical isolator
C	Half-wave plate
D	Glan–Thompson polarizer
E	Electro–optic modulator (optional)
F	Safety shutter and beam block
G	Kinematically mounted mirror
H	Ø1″ Iris
I	Periscope
J	Ø1″ Lens L1 on z-adjustable stage
K	Two Ø1″ irises
L	Ø2″ Lens L2
M	Beam expander
N	Dichroic mounting adapter
O	20× 0.75 Numerical aperture (NA) objective
P	Epifluorescence filter turret
Q	Lower half of microscope body
R	Camera

Table 2

Troubleshooting table.

Step	Problem	Possible reason	Solution
16	Laser is not pulsing	Optical isolator is not functioning properly, allowing back reflections into the laser	Check optical isolator manual and ensure the component is aligned correctly
		Laser power is set too low	Check the power output of the laser
21	Power ratio at electro–optic modulator (EOM) is insufficient	EOM is misaligned or damaged	Realign and/or consult manufacturer
		Bias voltage for high-voltage (HV) amplifier is incorrect	Repeat Step 18
53	The laser beam will not pass through all three irises	The 12″ optical rail is not parallel to the beam path	Carefully rotate the entire beam expander structure to bring the 12″ optical rail parallel to the beam path
62	Cannot find laser spot on the infrared (IR) alignment tool	Dichroic mirror is severely misaligned	Adjust the rotation of the Ø1″ pedestal post holding dichroic and/or compact kinematic mount on the adapter plate and realign
65, 66	Transmitted beam spot disappears as it is moved	The beam is being clipped	Adjust the position and/or rotation of the Ø1″ pedestal pillar post; if insufficient, realign the periscope, beam expander and dichroic mounting adapter
68	The beam spot cannot be properly centered using only L2	The laser beam is not passing through the center of L2	Adjust the beam expander hardware, in particular the height of the 12″ optical rail from the table
70	Expanded beam shape is asym-metric or irregular in shape	Clipping of the beam and/or poor orientation of the lenses	Check alignment using IR viewer and IR card and correct the misaligned components
77	Cannot find laser in preview window	Beam may not be falling on the CCD of the camera	Rotate the camera in its mount if the sensitive area is particularly small
		Filter cube may be in the active slot of the epifluorescence filter turret and may be blocking the laser beam	Remove the filter cube
83	Cannot obtain sharp cuts on the permanent marker	Imaging and laser plane of focus are mismatched	Find both planes of focus; if the laser plane of focus is below the imaging plane, move L1 closer to L2 with the micrometer; if laser focus is above, move lenses apart
		Laser is not pulsing	Change the settings of the laser to pulsing mode or go to troubleshooting of Step 16
84	Firing pattern is asymmetric or large	Clipping of laser beam	Trace the beam path using the IR card to determine the point of clipping and correct the misalignment
		Misalignment of lenses	Realign the lenses
		Laser is not focused on the image plane	Adjust the position of L1 along the 12″ optical rail using the micrometer
		Power level is too high	Verify the high-power level using Steps 17–21
		Laser is not centered on or overfilling the back aperture of the objective lens	Redo Steps 63–71 to correctly align the incoming beam on the back aperture of the objective
	The position of cuts shifts when focusing	Non-normal incidence of laser beam onto the back aperture of the objective	Redo Steps 62–66 to correctly orient the incoming beam
89	Laser spot has moved significantly since last usage	The alignment of components have changed	Check alignment using IR viewer and IR card and correct the misaligned components
94	Failure to successfully ablate the target	Laser is not focused on the image plane	Repeat Steps 72–85
		Energy of the laser is too low	Check maximum power of the beam using power detector at the output of the EOM; see Step 17
		Target is too deep in the tissue for efficient cutting; excessive scattering	Reorient the sample

Step	Problem	Possible reason	Solution
		Numerical aperture (NA) of the objective is too low	Use objectives with higher NA
		Laser is not pulsing	Change the settings of the laser to pulsing mode or go to TROUBLESHOOTING of Step 16
		Significant laser pulse dispersion	Analyze the entire beam path for portions which may be causing significant dispersion and adjust/replace components with ones suited for high-speed near infrared (NIR) lasers
	Specimen is damaged at low power, even when not firing	Minimum transmitted power is too high	Check/correct EOM bias voltage and the rotational angle of the half-wave plate