Confocal Microendoscopy of Neuromuscular Synapses in Living Mice

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ABSTRACT

Here we describe a step-by-step method for vital imaging of neuromuscular junctions (NMJ) and axons using fiber-optic confocal microendoscopy (CME). A commercially available system, the Cellvizio Lab, can be applied to transgenic mouse lines expressing yellow fluorescent protein in all or pseudorandom sub-subsets of motor neurons. Microscopic imaging in vivo is achieved by means of a flexible optical fiber probe that excites and collects the emitted light from fluorescently labeled structures. The hand-held probe is introduced through small skin incisions to visualize nerves and neuromuscular junctions from superficial muscles. Interpolation software then reconstructs the images in real time. The images are of sufficient quality to permit screening of axonal and neuromuscular synaptic integrity and other aspects of their phenotype in live animals.

INTRODUCTION

Confocal microendoscopy (CME) is a versatile, optical-fiber-based technique for live imaging of neural and other tissues. The method we describe here utilizes a Cellvizio Lab instrument. This enables minimally invasive observation of fluorescent internal structures, because it employs imaging probes that are between 300 μm and 4.2 mm in diameter and that provide better than 5 μm of lateral resolution. This means that the imaging probe tip can be inserted and manipulated, either by hand or using a micromanipulator, through quite small skin incisions in an anesthetized mouse, which can then be allowed to recover. The procedure can then be repeated in subsequent imaging sessions, thus achieving longitudinal study by repeated visualization of the same structures on successive days, weeks, or months. The protocol we describe here is simple and has been used effectively to study the morphology of axons and neuromuscular junctions (NMJ) undergoing degeneration or regeneration after nerve injury, and in transgenic mouse models of neuromuscular disease. We have also used the procedure for forward-genetics, in a phenotype-driven screen of ethylnitrosourea (ENU)-mutated lines for axonal and synaptic neuroprotection (Wong et al., 2009).

A vital fluorescent marker or reporter is required to illuminate the morphology of axons and their endings in living/anesthetized mice. It is feasible in principle to use nontoxic dyes, like fluorescein or cresyl violet, for visualization of living tissue. However, these fluorescent dyes do not selectively label cells in nerve or muscle; therefore, their nonspecific binding produces background staining that outshines the fluorescence of axons and their endings. In addition, although flexible and providing high-speed image acquisition in real time (>12 fps), the Cellvizio has a relatively low spatial resolution and is not as sensitive to fluorescent light as most conventional confocal microscopes. However, for the time being, images with sufficient resolution and contrast to evaluate the integrity of axon and presynaptic structures in mice can be achieved using the transgenic...
reporter lines thy1.2-YFPH and thy1.2-YFP16 (see Commentary) that strongly express fluorescent protein selectively in neurons.

In order to study the effects of specific mutations on axonal and NMJ integrity, genetic crosses must be set up to either of the YFP reporter lines, or an equivalent one. Crossing and genotyping strategies to generate a cohort of mutant mice that also carry YFP-labeled motor neurons will depend on the nature of the mutation and its associated phenotype. Once the reporter lines have been generated in the desired genetic background, in vivo visualization of axons and presynaptic structures requires the following steps: preparation of the mice for a minimally invasive protocol, a brief surgical procedure, collection of movies from fluorescent structures, and recovery of the mice from anesthesia.

Preparation and surgical procedures vary according to the intended objective of the observations. For axonal regeneration experiments, the sciatic nerve may be exposed in anesthetized mice and then either cut or crushed. At later time points, an incision through the skin at a distal point along the path of the nerve (the tibial nerve is convenient and accessible) can then be carried out and images collected of the regenerating axons. Recurrent observations within days or weeks can be performed with careful monitoring of the wounded area. For direct observations of innervation patterns, a small incision in the skin is carried out under anesthesia in the region most likely to provide access to superficial neuromuscular junctions (e.g., over muscles adjacent to the tibial nerve or the gastrocnemius muscle). Once the muscle is partially exposed, the hand-held optical-fiber probe can be introduced through the skin wound. Real-time imaging of the subcutaneous area is used to guide the probe towards the fluorescent axons and presynaptic terminals of the NMJs, which are observed on the monitor of an Apple Mac computer using the ImageCell software bundled with the Cellvizio system. Movie clips or still frames from these fluorescent structures can then be recorded in standard formats.

**IMPORTANT NOTE:** In the UK, animal surgery conducted for the purposes of justified research experimentation requires local ethical approval by the research institution; training and testing in practical and legal constraints; and authorization through successful application for Project and Personal Licenses administered by the Home Office. It is illegal to conduct the regulated procedures described below without having such licensed procedures authorized. Such authorization is normally given for research only: it is also illegal in the UK to demonstrate, teach, or practice the techniques described on living animals where such demonstration, teaching, or practice is the only objective. Most countries and research institutions have similar requirements for ethical approval and regulation before such procedures may be carried out for research purposes. In the United States, before beginning any work with mice, proper training and protocol approval must be obtained from the Institutional Animal Care and Use Committee (IACUC) or equivalent and conform to government regulations.

**SURGICAL PROCEDURE: SCIATIC NERVE LESION**

To evaluate axon regeneration or reinnervation phenotypes by CME, an appropriate nerve must be injured and allowed to regenerate. Sciatic nerve transection or crushing is a widely used nerve injury paradigm. Animals receiving unilateral or even bilateral section of the sciatic nerve learn to adapt to their immediate motor deficits within a few hours. This evidently occurs by the animals recruiting their hip and thigh musculature to carry out movements or to support their bodies, compensating for the disuse of the distal muscles supplied by the sciatic nerve or its derivative branches. After about a day, animals with unilateral sciatic nerve injury will move about their cages with apparent ease and no special provisions for accessing their food or water supply are normally needed.
Axons regenerate to target muscles in mice within 2 to 8 weeks of sciatic nerve injury (Brown and Ironon, 1978; Gillingwater et al., 2002). Axonal and neuromuscular synaptic morphology may be carried out before or after regeneration at different time points following nerve injury within the same animal (Rich and Lichtman, 1989; Balice-Gordon and Lichtman, 1990; Wong et al., 2009). For CME following sciatic nerve cut or crush, this is accomplished by making a small incision in the skin distal to the injury (the tibial nerve is very accessible) and/or over the lower limb muscles, e.g., flexor digitorum longus, tibialis anterior, or gastrocnemius. The fluorescent nerves and neuromuscular synapses can then be visualized using the Cellvizio confocal microendoscope (Basic Protocol 2).

**Materials**

- Thy1.2-YFP16 or thy1.2-YFPH transgenic mice (Jackson Labs)
- Vetergesic (buprenorphine 4.2 μg/ml)
- 3% to 5% isoflurane/O2
- Antiseptic
- Fur clippers
- Iris scissors and fine spring scissors (e.g., Fine Science Tools)
- Watchmakers forceps (e.g., Fine Science Tools)
- Wound clips or silk suture with integral suture needle (e.g., Ethicon, 7-0)
- Heating lamp

1. Familiarize yourself with the required nerve and muscle anatomy by dissection of mouse cadavers before attempting any surgery on anesthetized live animals. A suitable guide adequate for mouse neuroanatomy is “The Anatomy of the Rat” (Greene, 1963). Check that the necessary authorization for carrying out the procedures on living animals described below has been obtained from local ethical committee(s) and other statutory regulatory agencies.

2. Prepare the mice for surgery with a subcutaneous injection of 100 μl of Vetergesic (buprenorphine 4.2 μg/ml), following anesthesia by inhalation of 3% to 5% isoflurane/O2 (0.5 to 1 liter/min). Ensure an adequate depth of anesthesia is maintained throughout the following steps by frequently checking for absence of eye-blink, ear-twitch, and other reflex responses to nociceptive stimuli, such as toe-pinching.

3. Using fur clippers, trim the fur on the lateral thigh and swab the skin with antiseptic. Expose the sciatic nerve unilaterally through a skin incision of ∼3- to 8-mm made with scissors at mid-thigh level. Bluntly dissect and divide the inter-muscular fascia parallel to the femur with forceps and scissors, exposing the sciatic nerve.

4. Either section the exposed nerve with scissors, optionally removing a 1-mm piece for other analysis, or crush it firmly between the jaws of watchmaker’s forceps for 30 sec. Check the continuity of the perineurial sheath of the sciatic nerve after nerve crush

5. Close the skin wounds with either wound clips or careful stitching with 7-0 silk suture. With skill, and if the initial skin incision is small, between one to three sutures should be sufficient to close the wound.

6. Discontinue the supply of inhalation anesthetic.

7. Monitor the recovery of the mouse under a heating lamp, and, at the first signs of recovery (restoration of reflexes and voluntary movement), return the animal to its cage. Monitor the condition of the animal and its wound at least once per day thereafter and report any adverse effects of the surgery on health or behavior, other than hind limb paralysis, to the veterinary surgeon appointed by the institution.
CONFOCAL MICROENDOSCOPY (CME) OF AXONS AND PRESYNAPTIC TERMINALS

The CME technique we describe here utilizes a commercial instrument, manufactured by Mauna Kea Technologies (MKT) and currently marketed as the “Cellvizio Lab” by VisualSonics. The principle of operation of this microendoscope is that monochromatic light (wavelength 488 nm) from a diode laser is scanned across a bundle of optical fibers. The tips of these are used to illuminate structures labeled with a fluorescent dye of appropriate excitation wavelength. The working distance of the standard “S” probes we use is zero (that is, the image plane is less than 100-μm below the interface between the probe and the tissue) and the horizontal (X-Y) resolution is ∼5 μm (see http://www.maunakeatech.com/pre-clinical-research/705/proflex-microprobes). The emitted fluorescence is collected and transmitted along the same optical fibers—hence the imaging is “confocal.” The Cellvizio can be fitted with optical-fiber probes of different diameters. We have routinely used the standard 1.5-mm “S” probe supplied by the manufacturer and found it to be of robust construction and suitable for repeated, heavy, or frequent use. The application of Cellvizio systems to CME has been described in several publications focused on imaging different tissues (see http://www.maunakeatech.com/pre-clinical-research/437/bibliography), but we confine ourselves here to the application for imaging peripheral nerves and neuromuscular junctions (Pelled et al., 2006; Vincent et al., 2006; Wong et al., 2009).

Materials

- Thy1.2-YFP16 or thy1.2-YFPH transgenic mice (Jackson Labs)
- Vetergesic (buprenorphine 4.2 μg/ml)
- 3% to 5% isoflurane/O2
- Antiseptic
- Cellvizio Lab (Mauna Kea Technologies)
- Proflex S-1500 probe (Mauna Kea Technologies)
- Fur clippers
- Iris scissors and fine spring scissors (e.g., Fine Science Tools)
- Adhesive tape
- Dissection board or homeothermic blanket
- Watchmakers forceps (e.g., Fine Science Tools)
- ImageCell software
- Micromanipulator, optional
- Wound clips or silk suture with integral suture needle (e.g., Ethicon, 7-0)
- Heating lamp

1. Follow the manufacturer’s instructions for attaching the chosen Proflex probe to the Cellvizio instrument and continue with the onscreen procedure for its calibration, using the dilute solution of Alexa 488, hydrogen peroxide bleaching solution, water, and suspension of fluorescent beads provided in the manufacturer’s calibration kit. The Proflex S-1500 probe has a tip diameter of 1500 μm and a robust protective stainless steel shield around the tip (Fig. 1A). Smaller probes can be used, but they are more fragile and there is no particular advantage for visualization of mouse peripheral nerve or muscle.

2. Familiarize yourself with the required nerve and muscle anatomy by dissection of mouse cadavers before attempting any surgery on anesthetized live animals. A suitable guide adequate for mouse neuroanatomy is “The Anatomy of the Rat” (Greene, 1963). Check that the correct authorization for carrying out the procedures on living animals described below has been obtained from local ethical committee(s) and other statutory regulatory agencies.
Figure 1  (A) Medial aspect of a mouse hind limb (cadaver), in which the tibial nerve (arrow) has been exposed through a small wound. The stainless-steel tip of a Proflex S-1500 probe is positioned in the wound over the exposed nerve. (B) CME image of intact axons in the tibial nerve of an anesthetized thy1.2-YFP transgenic mouse. Only about 5% of the axons express YFP in this line and seven of them are visible in this image. (C) CME image of intact axons in the tibial nerve of an anesthetized thy1.2-YFP16 transgenic mouse. All the motor axons are labeled by YFP expression in this line. (D-F) CME images of a group of NMJs in axotomized flexor digitorum longus muscle of a WldS mutant mouse, obtained in imaging sessions conducted on successive days 3 (D), 4 (E), and 5 (F) after sciatic nerve section, following Basic Protocols 1 and 2. There is a delayed, progressive degeneration of motor axon terminals in this mutant strain following such nerve injury (arrows). Reprinted from Wong et al. (2009) with permission.
3. Prepare the mice for surgery with a subcutaneous injection of 100 μl of Vetergesic (buprenorphine 4.2 μg/ml) following anesthesia by inhalation of 3% to 5% isoflurane/O2 (0.5 to 1 liter/min). Ensure that an adequate depth of anesthesia is maintained throughout the following steps by frequently checking for absence of eye-blink, ear-twitch, and other reflex responses to nociceptive stimuli, such as toe-pinching.

4. Carefully trim the fur using scissors or clippers from the medial side of the lower hind limb. Secure the mouse with adhesive tape to a firm surface, such as a dissection board or homeothermic blanket, and expose the medial surface of the limb. Make a small incision, about 2 to 5 mm in length, and identify the tibial nerve lying beneath the connective tissue fascia and adjacent to the posterior tibial blood vessels. Expose the nerve by teasing away the connective tissue with small spring scissors and watchmakers forceps, taking care not to damage either the nerve or the blood vessels.

5. Activate the Cellvizio laser from the ImageCell software interface. The orientation and position of the probe tip can be manipulated by hand while monitoring the images acquired in real time on the computer monitor.

A micromanipulator can be used to position the tip of the probe onto the surface of the tibial nerve instead, but we have found that panning the probe tip along the nerve with a steady hand offers more flexibility in positioning. Once a region of interest has been found, fluorescent images may be captured at >12 frames/sec and saved by activating an on-screen switch or depressing the footswitch on the device included with the hardware (Fig. 1B,C)

6. Displace the tip of the probe either posteriorly to the flexor digitorum longus or anteriorly to flexor hallucis longus, or other muscles lying beside and deep with respect to the exposed surface of the tibial nerve. In YFP16 mice it is not difficult, with fine adjustments to the positioning of the probe tip, to reveal fluorescent sprays of motor nerve terminals (Fig. 1D-F). This is more problematical in the YFPH line, due to the pseudorandom nature of the expression of YFP between motor neurons in this line. However, in fortunate cases, sprays of motor nerve terminals belonging to the same motor unit can be observed.

7. Close the skin wounds with either wound clips or careful stitching with 7-0 silk suture. With skill, and if the initial skin incision is small, between one to three sutures should be sufficient to close the wound.

8. Discontinue the supply of inhalation anesthetic.

9. Monitor the recovery of the mouse under a heating lamp and at the first signs of recovery (restoration of reflexes and voluntary movement) return the animal to its cage. Monitor the condition of the animal and its wound at least once per day thereafter and report any adverse effects of the surgery on the animals’ health or behavior to the veterinary surgeon appointed by the institution.

10. For longitudinal studies, the above steps may be repeated hours, days, weeks, or months later on the same animal, revisualizing the same NMJs and thus facilitating description of the development or maturation of phenotypes or appraisal of the effects of treatments or mutations designed to mitigate the effects of nerve trauma or disease (Fig. 1D-F).
Background Information

Several methods have been described for vital imaging of NMJs, the most notable being the methods developed by J.W. Lichtman and his colleagues for repeated visualization of NMJs in mouse sternomastoid muscle (Lichtman et al., 1987; Balice-Gordon and Lichtman, 1990; Walsh and Lichtman, 2003). Initially, these authors used a membrane-permeant styryl dye (4-di-2-Asp) as a vital stain for motor nerve terminals, visualizing the fluorescence in anesthetized and ventilated animals using a standard microscope and SIT or intensified CCD cameras. This staining method was superseded by two motor neuron reporter lines that have become widely used since they were first described and are now commercially available from Jackson Laboratories: the thy1.2-YFP16 and the thy1.2-YFPH transgenics (Feng et al., 2000). The thy1.2-YFP16 line expresses yellow fluorescent protein (YFP) in all motor neurons. YFP fluoresces when excited with the 488-nm line of an argon or diode laser. In the thy1.2-YFPH line, only about 5% of motor neurons are rendered fluorescent, but those that are labeled are visible in their entirety, including the full length of the axon, its collateral branches, and their terminals (Feng et al., 2000; Keller-Peck et al., 2001; Kasthuri and Lichtman, 2003; Beirowski et al., 2004; Wong et al., 2009). More recently, two-photon microscopy combined with use of gradient-index (GRIN) lenses has been used to obtain high-resolution images of skeletal muscle in vivo, utilizing its autofluorescence (Llewellyn et al., 2008; Barretto et al., 2009). The CME method described here is complementary to these approaches.

CME offers flexibility, speed of image acquisition, and minimal invasiveness, but at the cost of lower spatial resolution and light sensitivity. A further shortcoming is that the Cellvizio systems currently available are monochromatic: that is, only one factory-set excitation wavelength can be used to generate fluorescent images. Finally, the equipment is expensive and the only commercial manufacturer of the type of CME equipment we have described here is Mauna Kea Technologies. However, despite these shortcomings the method has proved straightforward and successful for judging the integrity of axons and neuromuscular junctions, for instance after neural or neuromuscular trauma, in models of disease, and in a mutagenesis screen for synaptic protection phenotypes (Pelled et al., 2006; Vincent et al., 2006; Wong et al., 2009). Significant improvements, perhaps increasing the utility of the CME method and opening up more applications, would result first from design or discovery of better methods for acute, selective, high-contrast fluorescent staining of axons and their terminals that does not require genetic engineering; and second, from the ability to visualize two or more fluorochromes simultaneously.

Troubleshooting

There is excessive bleeding at the sites of either nerve injury (Basic Protocol 1) or imaging (Basic Protocol 2). Be careful with sharp dissecting instruments in the vicinity of blood vessels. Twist a small strip of Kleenex-type tissue to make a wick and use this to soak up bleeding until clotting has occurred.

The wound sutures have disappeared overnight leaving a gaping wound. Sometimes animals groom or bite the site of the injury resulting in removal of the sutures. Consult the appointed institutional veterinary surgeon for professional advice. If indicated, the animal should be re-anesthetized and the wound re-sutured.

The dynamic range of the images appears low. Check the calibration procedure has been followed correctly in accordance with the manufacturer’s instructions and the on-screen guidance. We have found that the concentration of Alexa 488 in the manufacturer’s calibration kit is highly variable. Choose a tube that gives a strong signal. Using the Autolevel on-screen button optimizes the range of gray levels displayed on screen without altering the raw data.

The on-screen image is non-uniform, excessively grainy, or shows static dark spots. The Proflex probe tip is dirty. Clean it using a cotton bud soaked in hydrogen peroxide or water and recalibrate. If the problem persists, soak the tip overnight by immersion in a tube containing a detergent, such as Triton X-100. Always clean the probe tip at the end of each imaging session, disconnect it from the Cellvizio, and store the probe carefully. Plug the exposed imaging port to prevent dust entering the instrument between imaging sessions.
Anticipated Results
Figure 1 shows typical images of axons and neuromuscular junctions obtained using the Cellvizio microendoscope, in mice of either the transgenic thy1.2-YFP line (Fig. 1B) or thy1.2-YFP16 line (Fig. 1C-F).

Time Considerations
In order to detect presynaptic morphology with the Cellvizio confocal, reporter thy1.2-YFP16 or thy1.2-YFP mice must be crossed with mice bearing known or suspected phenotypes at axonal or presynaptic levels. This can take considerable time depending on the breeding fitness of the strains involved. In particular, generating a viable colony of mice homozygous for the mutation and the reporter, an ideal situation given that no further genotyping would be needed, can be a very lengthy process. We found the use of in vitro fertilization using the sperm from the thy1.2-YFP:16 strain to fertilize eggs of superovulated females carrying the mutation a useful method to speed up this process. Large progenies of double heterozygous mice can be obtained in this way and intercrossed to generate double homozygous.

Once a routine procedure has been established, the time for carrying out a sciatic nerve lesion, from anesthesia to recovery, is typically about 10 to 15 min per mouse. For imaging sessions, from induction of anesthesia, CME imaging, appraisal of axonal and neuromuscular phenotype, to wound-suture and recovery of the mouse, can also be as short as 10 to 15 min per mouse. The method therefore has considerably more potential for throughput than other assays of axonal or neuromuscular synaptic morphology, or other aspects of neuromuscular phenotype.

Literature Cited