One-Step Vital Staining of Presynaptic Terminals and Post-Synaptic Receptors at Neuromuscular Junctions in Mouse Skeletal Muscle

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ABSTRACT

This protocol describes vital staining of neuromuscular junctions in the mouse triangularis sterni muscle in one incubation step, combining presynaptic, motor nerve terminal staining with the styryl dye FM1-43, which labels recycling synaptic vesicles, and TRITC-α-bungarotoxin, which labels acetylcholine receptors in the motor endplate membrane. Curr. Protoc. Mouse Biol. 1:489-496 © 2011 by John Wiley & Sons, Inc.

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BASIC PROTOCOL

The large size and visibility of neuromuscular synapses enabled early light microscopists to define the basic cytological organization of neuromuscular junctions (NMJs). By the early 20th century, silver staining protocols had established a most remarkable feature of NMJs in most vertebrate striated muscle: the mononeuronal innervation of a discrete plaque, occupying 0.1% or less of the total surface area of a muscle fiber. Thus, it was subsequently established, and amply confirmed by all techniques that have superseded silver staining, that in mature animals each motor neuron projects to a discrete muscle, wherein each innervates many muscle fibers (a “motor unit”). However, each muscle fiber is innervated by a collateral branch and terminal derived from the axon of one and only one motor neuron. Moreover, presynaptic and post-synaptic specializations at neuromuscular junctions coincide: motor nerve terminals are intimately associated and align with gutters and folds of motor endplates, which contain a high density of acetylcholine receptors (Costanzo et al., 1999, 2000; Lu et al., 2009). In rodents, the presynaptic and post-synaptic specializations form prenatally (Pun et al., 2002), but the mature pattern of innervation finally emerges during the first three weeks of postnatal development from one of polynuclear innervation (Brown et al., 1976; Walsh and Lichtman, 2003). In addition, the transformation may be driven partly by differences in synaptic strength and activity-dependent competition (Betz et al., 1980, 1990; Fladby and Jansen, 1987; Buffelli et al., 2003; Kasthuri and Lichtman, 2003).

Neuromuscular synapses normally degenerate rapidly (within 18 hr) after peripheral nerve injury, but they are much more slowly removed after nerve injury in the \textit{Wld}⁸ mutant mouse (Gillingwater et al., 2002) and in some forms of motor neuron disease (Schaefer et al., 2005; Murray et al., 2010). Taken together with developmental synaptic remodeling, such observations are consistent with a “compartamental neurodegeneration” hypothesis, according to which axons and synapses are maintained by cellular and molecular mechanisms that are distinct from those that maintain cell bodies (Gillingwater and Ribchester, 2001, 2003). Analysis of neuromuscular synaptic connectivity is therefore
of interest and relevance in the context of development, maintenance, plasticity of motor units, and their vulnerability in different forms of neuromuscular disease.

The “one-step” method for labeling of presynaptic and post-synaptic components of neuromuscular junctions given here is very simple, and it combines vital staining of motor nerve terminals with FM1-43 and staining of acetylcholine receptors at motor endplates with TRITC-conjugated α-bungarotoxin. This method is illustrated using isolated preparations of the triangularis sterni muscle, a muscle lining the pleural side of the thorax that is only one or two fibers thick. The results can readily be observed with conventional fluorescence microscopy, using standard FITC and TRITC filter cubes.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for care and use of laboratory animals.

Materials

- Physiological saline (see recipe)
- Depolarizing saline (see recipe)
- Adult mice (any laboratory strain, e.g., C57B16; any age or gender)
- 1 mg/ml FM1-43 or FM1-43FX (see recipe)
- 500 μg/ml TRITC-α-bungarotoxin stock solution (see recipe)
- 4% paraformaldehyde, optional
- Minutien pins
- Sylgard-lined petri dishes
- Dissecting microscope fitted with lamps or fiber-optic illumination for both transmitted and incident light
- Watchmaker’s forceps (nos. 3 and 5)
- Fine spring scissors
- Iris scissors
- Incubator with rocking platform
- Upright fluorescence microscope or confocal microscope fitted with water-dipping objectives
- Digital camera and driver/image-processing software/PC
- Additional reagents and equipment for euthanizing the animal (Donovan and Brown, 2006)

1. Prepare one liter of fresh “normal” physiological saline solution (i.e., normal saline) following the recipe in the Reagents and Solutions section.
2. Freshly prepare 100 ml of a depolarizing mammalian physiological saline solution following the recipe in the Reagents and Solutions section.
3. Sacrifice a mouse using an authorized, legal method approved by the institution where the research is to be conducted (e.g., see Donovan and Brown, 2006).
   Stunning, immediately followed by cervical dislocation is suitable, swift, and in accordance with approved methods listed under the UK Home Office’s Schedule 1.
4. Using iris scissors, make a circumferential incision through the abdominal skin. Grip the skin rostral to the incision and strip upwards, exposing the thoracic musculature and ribcage. Douse the exposed thorax with normal physiological saline.
5. Using iris scissors, carefully cut through the abdominal body wall following the line of the most caudal ribs. Cut through the ribcage laterally using iris scissors and separate from underlying pleura and connective tissue. Remove the ribcage and pin it to a Sylgard-lined petri dish.
Figure 1  (A) Low-power micrograph taken in a dissecting microscope with both transmitted and incident illumination of triangularis sterni muscle preparation. Note the intact muscle fibers oriented vertically and the intramuscular nerve (arrow) running across it. (B) Examples of intramuscular axons and neuromuscular junctions visualized in a conventional fluorescence microscope. Left panels, upper TRITC-α-bungarotoxin staining; lower, FM1-43; Middle panel, merged channels showing overlay and alignment of motor nerve terminal and motor endplate. (C) Upper panel, FM1-43, middle, TRITC-α-bungarotoxin; lower, merged channels. Yellow fluorescent region indicates the alignment of presynaptic and post-synaptic components of the NMJ.

6. Exchange the bathing fluid with normal physiological saline frequently during the dissection. With the ribcage pinned external face uppermost, carefully cut through the intercostal muscles with watchmaker’s forceps and fine spring scissors, taking care not to damage the underlying triangularis sterni muscle.

The dissection of triangularis sterni is best performed under a dissecting microscope that permits both transmitted and incident illumination simultaneously.

7. Snip the ribs at the sternum using small iris scissors or spring scissors, then reflect and remove the ribs, carefully trimming away adherent tissue and blood vessels. The triangularis sterni muscle should then be exposed as a semi-transparent sheet, only 1 to 3 muscle fibers thick (Fig. 1). Insert fine minutien pins through the lateral intercostal musculature and the sternum to secure the muscle, stretched at about its resting length.

8. Take 10 ml depolarizing saline and add 25 μl of either FM1-43 or FM1-43FX (or AM1-43) from an aqueous 1 mg/ml stock solution. To this solution add 20 μl
from an aqueous 500 μg/ml stock solution of TRITC-α-bungarotoxin. The solution containing both fluorescent dyes will be referred to as staining saline.

Both FM-dyes and labeled bungarotoxin can be divided into aliquots, frozen, and used after thawing. They may be stored in a refrigerator for a few days without loss of activity.

An Alexa-Fluor 555 conjugate of α-bungarotoxin may be used if the preparation is intended for confocal microscopy although TRITC-α-bungarotoxin is suitable for confocal microscopy as well.

9. Drain the dissecting dish of all normal physiological saline and replace it with 5 to 10 ml staining saline containing the fluorescent cocktail. Incubate in this solution for 10 to 30 min on a rocking platform at room temperature. After the incubation, wash three to five times, each time with copious amounts of oxygenated normal saline for 30 to 60 min each time, and for the final wash immerse the dish containing the pinned preparation in 250 ml or more of oxygenated normal medium.

You may need to adjust the time of the incubation depending on the nature of your preparation. Start with 10 min and increase the time, if necessary.

10. Transfer the preparation, still pinned to the Sylgard-lined dish and covered with fresh normal saline to an upright fluorescence compound microscope fitted with water-dipping 10× to 60× objectives.

11. Image and record the glowing pretzel-shape of the primary junctional folds at the motor endplates using the TRITC cube. Obtain images with a digital camera (e.g., a Hamamatsu Orca-12 camera, driven by Improvision/Perkin-Elmer OpenLab software, was used for the images presented in Fig. 1B-C).

The broad emission spectrum of FM-dyes enables terminals to be visualized through standard FITC or TRITC filter cubes. However, better results are obtained using a custom filter cube containing a 435-nm excitation filter and a 515-nm, narrow bandpass (±10 nm) emission filter. A 495-nm dichroic mirror completes the custom cube.

12. If FM1-43fx (or AM1-43) is used instead of FM1-43, then fix the preparation for 30 min in 4% paraformaldehyde after the post-depolarization washes. However, the coloration of the nerve terminals may fade over the following 12 to 24 hr so images are best obtained as soon as possible after dye loading.

13. If the microscope is part of a confocal system, make a z-series at the diffraction limit and either combine these into a maximum-intensity projection or make rotating movies around x, y, or z axes.

In good preparations, it is possible to discern the organization of the secondary junctional folds. ACh receptors are more concentrated at the crests of these folds.

Quantification of end-plate size and fractional occupancy by motor nerve terminals can be accomplished using public-domain Image J software, downloadable from http://rsbweb.nih.gov/ij/.

REAGENTS AND SOLUTIONS
Use deionized, distilled water in all recipes and protocol steps.

Depolarizing saline

Freshly prepare 100 ml of a depolarizing mammalian physiological saline solution, the same composition as the normal physiological saline (see recipe) but with KCl elevated to 50 mM and NaCl reduced by 45 mM to 92 mM. Bubble to equilibration as with normal physiological saline. Do not store.
**FM1-43/FM1-43fx**

1 mg/ml FM1-43/FM1-43FX (Invitrogen) in phosphate-buffered saline (PBS). Divide into 25- to 100-μl aliquots into microcentrifuge tubes and freeze.

_Thawed aliquots can be stored for a few days at 4°C without loss of activity._

**Normal saline**

Freshly prepare one liter of normal physiological saline solution from stocks of NaCl (3 M), KCl (1 M), CaCl₂ (0.2 M), MgCl₂ (0.2 M), NaH₂PO₄ (0.2 M). The following volumes of these, respectively, should be mixed and made up to 1 liter: 40 ml, 5 ml, 10 ml, 5 ml, and 2 ml. To this mix add 2 g NaHCO₃ and 1 g D-glucose. After mixing, bubble through tubing or an aspirator with 95% O₂/5%CO₂ gas mix for at least 10 min before use. Alternatively, instead of mixing NaH₂PO₄ and NaHCO₃, add HEPES buffer to a final concentration of 5 mM, adjust pH to 7.2 to 7.4 and bubble either with air or 100% O₂. Do not store.

**TRITC-α-bungarotoxin**

500 μg/ml TRITC-α-bungarotoxin (Invitrogen) in phosphate-buffered saline (PBS). Divide into 25- to 100-μl aliquots into microcentrifuge tubes and freeze.

_Thawed aliquots can be stored for a few days at 4°C without loss of activity._

**COMMENTARY**

**Background Information**

A potent high-affinity ligand for acetylcholine (ACh) receptors, known as α-bungarotoxin, was originally extracted from the venom of a Taiwanese species of krait. The utilization of this toxin led to detailed molecular and biophysical characterization of these receptors at the mammalian NMJ and elsewhere (Chang and Lee, 1963; Changeux et al., 1970; Mishina et al., 1986). Conjugation of α-bungarotoxin with fluorescent ligands also enables simple, one-step labeling of ACh receptor-rich patches in the membranes of cultured myotubes, or motor endplates in situ (or strictly, the densely packed receptors located in the muscle fiber membranes at the electron dense crests of the junctional folds). Visualizing the distribution of ACh receptors in mammalian skeletal muscles is therefore a very simple procedure (Anderson and Cohen, 1977; Slater, 1982).

Various fluorescent forms of α-bungarotoxin are available: FITC-, TRITC-, or AlexaFluor variants all give good results. Good images can be obtained from any muscle but whole-mounts of thin muscles give the best overviews and images of endplate distribution and fine structure. Excellent preparations of this kind can be prepared using the triangularis sterni and the levator auris longus muscles, but fluorescent α-bungarotoxin also works well with tissue sections or teased preparations of any muscle. Those we have tested and for which we have obtained uniformly good staining results include extensor digitorum longus, soleus, diaphragm, sternomastoid, intercostals, transverses abdominis, flexor digitorum brevis, interosseus, and lumbral muscles. In fact, the lumbral muscles are small and thin enough for excellent data to be obtained from whole-mounts using either conventional fluorescence of confocal microscopy.

Classical silver-staining methods for motor axons and their terminals have the advantage of being high in contrast and durable: well-kept preparations can still be inspected and useful data gained years after the preparation has been made. However, the method is capricious and time consuming; and with the advent of confocal microscopy (useless with silver-stained preparations) and other digital imaging technologies the need for storage of stably stained preparations has diminished somewhat. Other light microscope–based methods for staining and imaging motor nerve terminals have included zinc-iodide/osmium-tetroxide, and methylene blue. However, all these methods were essentially superseded by immunostains of one kind or another, either using a peroxidase-based method to stain NMJ, or using fluorescent secondary antibodies to highlight proteins localized at these synapses (Gillingwater et al., 2002; Ribchester...
Immunofluorescence is particularly well-suited to high-resolution analysis of preparations using confocal microscopy. However, several steps (and days of preparation) are required for making a good immunofluorescent specimen, so methods that are more rapid and reliable offer the potential for a higher throughput.

Lichtman and colleagues showed that fluorescent vital staining of NMJ produces high contrast, effective results in some species, such as snakes (Lichtman et al., 1985). The same group also found that a styryl dye, 4-Di-2-Asp, produces sufficiently high-contrast passive staining of motor nerve terminals, enabling longitudinal study of motor nerve terminals over days, weeks, or months in living mice (Lichtman et al., 1987; Magrassi et al., 1987; Balice-Gordon and Lichtman, 1990). More widespread utilization of vital staining of NMJ was adopted when Betz and colleagues reported the activity-dependent staining and “destaining” of motor nerve terminals in diverse species using the aminostyryl dye “FM1-43” (Betz and Bewick, 1992; Betz et al., 1992). Other related molecules (such as FM2-10 or FM4-64, or RH414 from which FM1-43 was originally synthesized) also stain motor nerve terminals, although the concentrations, loading conditions, and excitation/emission wavelengths differ (Riberch et al., 1994; Barry and Riberch, 1995).

Thin or small muscles give the best results with vital staining of motor nerve terminals using aminostyryl dyes, such as FM1-43 or its fixable analog, FM1-43fx (both from Invitrogen). The amphiphatic molecular structure of styryl dyes like FM1-43 causes them to insinuate into, but not penetrate, exposed membranes. Thus, during exocytosis when the inner membranes of synaptic vesicles become exposed to the extracellular fluid, FM-dye molecules diffuse into the open vesicle through the diffusion pore and become trapped therein, following endocytosis. In this way, styryl dyes stain “recycling” synaptic vesicles. In addition, the fluorescence of membrane-bound FM-dyes is an order of magnitude greater than when the molecules are free in aqueous solution. However, in order to achieve labeling of adequate numbers of vesicles and therefore to produce sufficient fluorescence to observe in the microscope requires intense stimulation, generated either physiologically via long periods of high-frequency nerve stimulation, or by using elevated, depolarizing concentrations of potassium ions. The protocol described here is based on potassium-induced labeling of the terminals.

For activity-independent coloration of axons and motor nerve terminals, nothing presently surpasses the transgenic expression of variants of enhanced green fluorescent proteins (eGFP), under control of a modified thy1 promoter (Caroni, 1997; Feng et al., 2000; Lu et al., 2009). Combinatorial, transgenic expression of GFP variants ultimately led to the generation of “brainbow” mice, with spectacular, aesthetically beautiful, as well as scientifically compelling, unique coloration of neuronal cell bodies, axons, and their terminals (Livet et al., 2007; Ribchester, 2009). While the original “thy1.2-XFP” transgenic lines, especially the “YFP-16” and “YFP-H” lines, give the best results for imaging motor axons and/or their neuromuscular arbors (Keller-Peck et al., 2001; Lu et al., 2009), it is impractical, for many purposes of routine characterization of NMJ structure, to crossbreed one of these YFP lines with another mouse line of interest, then nurture the offspring before appraising the neuromuscular phenotype morphologically (Wong et al., 2009). The utility of these fluorescent proteins combined with minimally invasive live imaging using confocal microendoscopy is described elsewhere in this volume (Ribchester, 2012).

Troubleshooting

**Presynaptic labeling with FM1-43 has not worked**

It is important to appreciate that the staining of nerve terminals depends on an adequate rate of recycling of synaptic vesicles in a living preparation. Thus, the method will not work if the preparation has been damaged or left standing for several hours, or if there are presynaptic neurotoxins present, or if the preparation has been fixed, or if the saline used to bathe the preparation is only a basic phosphate-buffered saline. A physiological saline with normal extracellular Ca concentration (at least 2 mM) is essential.

In order to stimulate vesicle recycling and labeling, depolarization of the living terminals is essential. Check that the staining saline containing FM1-43 has been prepared with depolarizing saline and not normal saline. When exchanging the normal saline with staining saline, ensure that the dish containing the pinned muscle is properly drained. Try a longer incubation (30 to 40 min) period if the problem persists.
Adequate washing is required to rinse passively bound FM1-43 from plasma membranes of the muscle fibers and connective tissue in order to provide adequate contrast. Washing of the preparation must be done with normal saline. If depolarizing saline is used for washing, then the labeled terminals will destain as a result of the maintained exocytosis in the absence of extracellular dye.

**The muscle is saturated with green fluorescence in the muscle fibers**

If substantial numbers of muscle fibers are damaged during the dissection, FM1-3 will enter the muscle fibers and label the extensive sarcoplasmic reticulum and other intracellular membranes, producing incandescent fluorescence that will most likely eclipse the fine detail of motor nerve terminal fluorescence. This internal fluorescence is frequently associated with “contraction clots” in the muscle fibers. This problem can only be avoided or averted by improved dissection technique. In a good preparation, all the muscle fibers adopt the appearance of smooth cylinders along their entire length (Fig. 1A).

**α-Bungarotoxin staining has not worked**

The method is extremely robust because α-bungarotoxin binds to ACh receptors with very high affinity, so the only plausible reasons for failure of the stain are that a bad lot has been purchased (record the lot number of effective batches), or that some mistake has been made in making up or diluting the stock solution. Penetration of the labeled protein may limit staining to superficial muscle fibers in some muscles, but every endplate should become labeled swiftly in triangularis sterni muscle if the protocol here has been followed properly. Another possibility for failure is some pathological feature of the muscle/mouse that reduces the number or density of ACh receptors at endplates.

**Anticipated Results**

Figure 1 shows a typical example of freshly dissected living preparation of triangularis sterni, together with fluorescence micrographs of intramuscular axons and motor nerve terminals stained with FM1-43 and post-synaptic ACh receptors stained with TRITC-α-bungarotoxin. Note that in addition to active, depolarization-induced homogeneous or punctate staining of the motor nerve terminals by FM1-43, ghostly outlines of the intramuscular nerves are also visible. This is mainly passive staining due to the insinuation of the FM1-43 dye molecules into the membranes of the myelin sheaths. Note also the almost perfect alignment between the motor nerve terminal arbor and post-synaptic ACh receptors.

**Time Considerations**

Preparing the solutions takes ~15 min; dissection of triangularis sterni takes, with practice, about 15 to 30 min. Results can therefore readily be obtained within either a morning or an afternoon’s work.

**Literature Cited**


**Pre/Post-Synaptic NMJ Staining in Mouse Skeletal Muscle**

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