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Neuromuscular junction imaging on whole muscles and muscle sections in CMD animal models

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1. OBJECTIVE

This document describes a method for staining, imaging and assessing changes at the neuromuscular junction (NMJ), the skeletal muscle and motor neuron synapse, in a mouse model for congenital muscle dystrophies (CMD) in different muscles and stages of disease progression.

2. SCOPE AND APPLICABILITY

The NMJ is essential for the normal functioning and survival of skeletal muscles and motor neurons. In neuromuscular diseases, the NMJ undergoes morphological alterations [1-4] that strongly correlate with disease progression [5, 6]. Thus, structural alterations that occur at the NMJ could serve as the basis for testing therapeutics to slow or stop the initiation and progression of CMD. In this SOP, we describe procedures for visualizing and analyzing rodent NMJs.

3. CAUTIONS

To capture the native structure of the NMJ, it is imperative that muscles are properly fixed. Lack of proper fixation will cause muscle fibers to shrink, resulting in changes to the microanatomy of the NMJ including fragmentation of the postsynaptic apparatus, loss of acetylcholine receptors and localization of synaptic vesicles away from the nerve terminal. Insufficient fixation could also result in the partial or full detachment of nerve terminals from muscle fibers. On the other hand, excessive fixation can prevent antibodies from penetrating the tissue, resulting in lack or unreliable detection of presynaptic changes. Such changes are often visualized using antibodies against proteins localized throughout the axon or exclusively at the nerve terminal. Transgenic animals that express fluorescence proteins selectively in neurons, and therefore allow for the visualization of innervating axons [7], could be used to overcome these issues. However, caution must be taken during fixation and in interpreting results obtained using such animals. Lack of proper fixation can result in the disappearance or abnormal aggregation of fluorescence proteins, giving the impression that axonal dystrophy and muscle denervation has occurred. More importantly, it has been reported that expression of fluorescence proteins could cause or accelerate axonal degeneration [8]. Hence, it is important to compare NMJ changes in such transgenic animals with those in control animals in the absence and presence of mutations that cause muscular dystrophy.

4. MATERIALS

4a. Materials needed to sacrifice animals and prepare muscles for staining:

- 1 x PBS
- 4 % PFA
- Two 30mL syringes and two 26cc needles are needed for manual perfusion.
- Instead perfusion can be performed using a variable-flow peristaltic pump (Fisher Scientific; Cat # 13-876-3).
- 500cm dish filled with Sylgard (Sylgard 184 Silicone elastomer kit).
- Dissection tools (Fine Science Tools: Student Fine Scissor, Cat# 91460-11; Student Vannas Spring Scissors, Cat # 91500-09; Dumont #5SF Forcep, Cat # 11252-00).
- Dissecting microscope with good illumination.
- 0.5mL tubes
- Embedding material (Tissue-Tek: Cryomold, Cat # 4565; OCT freezing compound, Cat # 4513).
- Sucrose
- Cryostat (Leica CM1850)
- Charged slides (Lab Scientific, Cat# 7796; See Appendix for protocol for coating slides with gelatin).

4b. Materials needed to stain and image the NMJ:

- 1 x PBS
- Blocking and antibody diluting solution (3% BSA + 5 Goat/Sheep/Lamb Serum + 0.5% or 0.1% Triton-X in 1 x PBS)
- Pap Pen (Ted Pella, cat # 2209)
- Nail Polish
- α -bungarotoxin (α -BTX) conjugated with fluorophore of interest (Invitrogen)
- Primary antibodies (Neurofilament, smi-312, Covance; Synaptotagmin-2, Zebrafish International Resource Center)
- Secondary antibodies (Invitrogen: Alexa-488 anti-mouse IgG1 and Alexa-647 anti-mouse IgG2A).
- Rocking platform.
- Magnets and plate (Eclipse Magnetics: Alnico button magnets, Cat # E823)
- Anti-fade mounting medium (Vector Lab: Vectashield, Cat # H-1000)
- Fluorescence microscope (Epifluorescence and confocal microscopes).

5. METHODS

We describe two approaches for examining the structure of the NMJ. In one, intact (whole-mount) muscles are used to image NMJs. This method can be used to stain most neonatal

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muscles and a subset of adult muscles, mostly thin or those that can be easily separated into distinguishable sub-compartments. To image NMJs in thick adult muscles, we detail a protocol for preparing and staining muscle sections. Whenever possible, however, we recommend that whole-mounts are used to examine structural changes. In contrast to sectioned muscles, the entire structure of every NMJ can be imaged in whole-mounted muscles. Because the muscle remains intact, it is also possible to correlate structural changes at the NMJ with those in the muscle fiber.

5a. Sacrificing and fixing animals.

Neonatal stages: animals are sacrificed via decapitation. The abdominal cavity should then be completely exposed and limb, neck and head skin should be cut and separated to expose muscles. The animals are then immersed in cold 4% PFA for at least 2 hours at 4C.

Adolescent and adult mice: Immediately after euthanization and before the heart stops beating, animals are first perfused with 1 x PBS and then with 4 % PFA. Although the perfusion volume will depend on the weight of the animal, it is important that animals are thoroughly perfused with 4 % PFA, especially because we do not recommend postfixing of adult skeletal muscles to stain for axonal and presynaptic markers.

5b. Preparation of muscles for staining.

Neonatal stages: The large majority of muscles can be isolated and stained intact until postnatal day 9.

Adolescent and adult mice: In these animals, there are numerous muscles that can be analyzed intact and others that must be cryosectioned.

Examples and preparation of muscles for whole-mount analysis:

- 1) Extensor Digitorum Longus (EDL): This muscle can be separated into four pieces that remain perfectly intact. The tendons that attach to the distal and middle toe phalanges are grabbed and pulled apart resulting in the division of the EDL into four separate muscles. These muscles are thin enough to allow sufficient penetration of antibodies. They can also be flattened enough to allow NMJs in any muscle fiber to be imaged using a 40X oil immersion objective.
- 2) Soleus muscle: This muscle cannot be subdivided into unique components. However, it can be teased apart using forceps to allow for proper staining with most antibodies, proper mounting and imaging of all NMJs. It is important to remove the superficial fascia to allow antibodies to properly penetrate.
- 3) Gracilis: This muscle can be whole-mounted without the need to separate it into smaller parts. It is important to remove the superficial fascia to allow antibodies to properly penetrate.
- 4) Diaphragm: Only the left or right hemidiaphragm is used per experiment. It is important to remove the superficial fascia to allow antibodies to properly penetrate.

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- 5) Triangularis Sterni: Only the left or right triangularis sterni is used per experiment. This is a thin muscle with high-depth of penetration for most antibodies used to stain the presynapse, easy to mount and to image every NMJ.
- 6) Levator Auris Longus (LAL): Very thin muscle with high-depth of penetration for most antibodies used to stain the presynapse, easy to mount and to image every NMJ. It is also subdivided into regions that vary in their susceptibility to muscle and motor neuron diseases [3]. It is important to remove the superficial fascia to allow antibodies to properly penetrate.

After isolation, muscles are immersed in blocking buffer containing 0.5% Triton-X 100 for at least 1 hr at room temperature or at 4 C overnight. Primary antibodies are then added and the muscles are then incubated at 4 C overnight on a rotating platform. The muscles are then washed three times with 1 x PBS at room temperature for 15 minutes each time. This is followed by incubation with secondary antibodies and fluorescently labeled α -BTX for at least 2 hours at room temperature or overnight at 4C on a rotating platform. The muscles are again washed 3 times with 1 x PBS, placed on a slide and bathed with antifade mounting medium and covered with a cover slip. In order to image every NMJ, muscles are flattened using magnets placed on the slide and the coverslip and applying pressure, prior to sealing with nail polish.

Examples and preparation of sectioned muscles:

- 1) Tibialis Anterior (TA)
- 2) Medial and lateral gastrocnemius
- 3) Sternomastoid

For cryosectioning, muscles are incubated in 30% sucrose in 1 x PBS overnight. The muscles are then longitudinally positioned on a mold, covered with OCT and placed at -80C for at least 15 minutes. Using a cryostat, 40-50 μ m longitudinal sections are captured on charged slides. Muscles are washed two times with 1xPBS to remove OCT, blocked for at least 30 minutes and then incubated in primary antibodies for 3-4 hours at room temperature or overnight in a humidified chamber. The next day, muscles are washed three times with 1 x PBS for 5 minutes each time, incubated with secondary antibodies in blocking buffer containing 0.1% Triton-X 100 and fluorescence α -BTX. The muscles are finally washed three times with 1 x PBS, mounted using vectashield and imaged.

5c. Image Acquisition.

To assess the effect of CMD causing mutations, the entire NMJ, with the pre- and post-synaptic sites stained with different fluorophores, should be imaged. Such images are best acquired using confocal microscopes, but epifluorescence scopes capable of acquiring images at different depth of the tissue can also be used. Irrespective of microscope used, maximum intensity projections should be generated using images representing the different depth of the tissue to better visualize the architecture of the NMJ. Whenever possible,

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image acquisition parameters should remain constant when acquiring images. Importantly, NMJs residing in the same region of control and CMD muscles should be compared in order to more accurately determine deleterious structural changes caused by the progression of CMD. This is especially important in muscles such as the Diaphragm, which contains a mixture of all muscle fiber types.

6. EVALUATION AND INTERPRETATION OF RESULTS

Maximum intensity projections of confocal stacks, of at least 20 NMJs per muscle and from a minimum of three control and three CMD animals, are necessary to fully determine whether any of the following NMJ features are compromised: 1) Fragmentation: has the NMJ formed or maintained a pretzel like structure? Most NMJs in young adult and healthy mice contain fewer than five AChR islands and are characterized by primary and secondary folds that yield a highway like structure. An increase in the number of AChRs islands and the appearance of irregularly shaped AChR clusters is often a feature of deleterious alterations in most muscles (Figure 1). 2) Faint AChR clusters: has the NMJ lost AChRs? Using fluorescence α -BTX, the relative density of AChRs can be compared between adjacent muscle fibers. Hence, a reduction in the fluorescence intensity of α -BTX would suggest that a loss of AChRs has occurred. 3) Innervation: are the presynaptic and postsynaptic sites mostly or completely apposed? A hallmark of a stable and normal functioning muscle fiber is the near perfect apposition between the nerve terminal (presynapse) and cluster of AChRs (postsynapse). Denervation occurs progressively and strongly correlates with the health status of motor neurons and muscle fibers. Hence, muscles are often found partially denervated and eventually completely denervated as muscles wither away. 4) Sprouting: is the nerve regenerating or searching for a healthier partner? As muscles deteriorate, nerves often form additional branches that extend beyond the NMJ. 5) Axonal swelling: Is the axon degenerating? Large and anomalous bulging of the axon proximal to the postsynapse often correlates with muscle denervation and nerve degeneration. 6) Axonal atrophy: is the motor neuron receiving sufficient trophic support? Lack of proper trophic support can cause axonal thinning, often 2 μ m smaller than the diameter of axons from motor neurons receiving sufficient trophic support. 7) Multiple innervation: are multiple axons competing for the same muscle fiber? Deleterious changes in muscles also cause multiple axons to compete for the same muscle fibers, a hallmark of axons attempting to find a healthy muscle partner with which to form and maintain a normal functioning NMJ.

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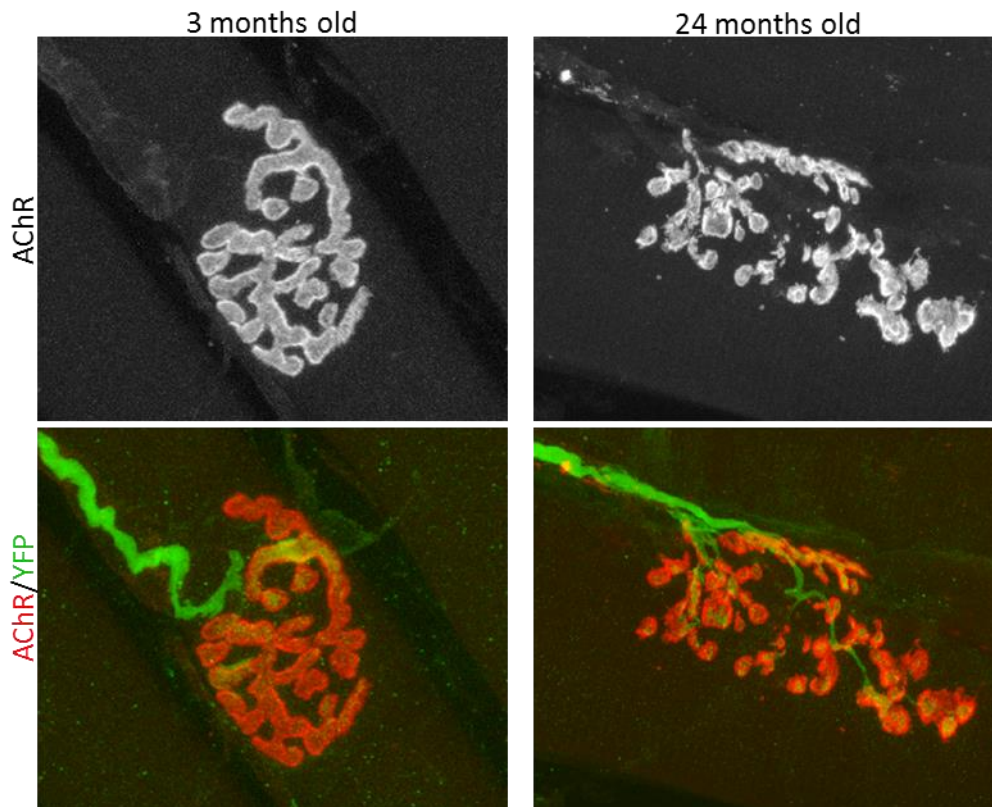


Figure 1: Neuromuscular junctions fall apart in atrophying muscle fibers. Acetylcholine receptors (AChR - Red) aggregate in defined patterns that delineate the architecture of the postsynaptic apparatus at NMJs. In atrophying old muscle fibers (right images), the postsynaptic apparatus breaks into small clusters, compared to NMJs in young mice (left images).

7. REFERENCES

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8. APPENDIX

Coating gelatin slides

1. Prepare fresh 0.5% gelatin, 0.05% chromium potassium sulfate coating solution.

Gelatin (300 bloom)	2.5 g
Chromium Potassium Sulfate	0.25 g
ddH ₂ O	to 500 mL

- a) Add gelatin to ~300 mL of water. Heat to dissolve (not >60°C)
 - b) Cool to room temperature (IMPORTANT)
 - c) Add Chromium Potassium Sulfate (water must be cool)
 - d) Add water to 500 mL
2. Clean slides:
 - a) Dip slides in 70% EtOH overnight.
 - b) Rinse slides in MilliQ water and change the water several times (for 20 minutes).
 3. Dip clean slides in coating solution. Let slides dry overnight in a dry place with no dust.
 4. Store slides in slide boxes at 4°C, or they can be stored at -20°C forever.