



Neuromuscular Junction Protocol: Dissection, Staining and Fluorescent Imaging

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1 Objective: Why stain for NMJ? Why focus on TVA muscle?

The objective of this SOP is to provide a step-by-step protocol for the staining, imaging and morphological assessment of neuromuscular junctions (NMJs). This protocol is adapted to the mouse model of spinal muscular atrophy (SMA) but can be used to stain the TVA muscle in any mouse model. Within the NMJ, common characteristics of SMA include; the presence of immature endplates that lack a pretzel-like morphology, a decrease in the innervation/occupancy of endplates, as well as the inappropriate accumulation of neurofilament protein at the terminal synapse. These features can be assessed through the staining and imaging of NMJs, as outlined in this SOP and can be applied to mice of any genotype. While this SOP can also be applied to mice of any age, NMJs can be more clearly visualized in mice below age P30.

The transverse abdominal muscle is a proximal muscle that is known to show denervation and neurofilament protein accumulation in the Delta7, 2B- and SMN^{-/-};SMN2 mouse models of SMA. Its thin structure makes for easy staining and clear imaging of the neuromuscular junction.

Woschitz, V., Mei, I., Hedlund, E. et al. Mouse models of SMA show divergent patterns of neuronal vulnerability and resilience. *Skeletal Muscle* 12, 22 (2022). <https://doi.org/10.1186/s13395-022-00305-9>.

2 Scope and Applicability

This SOP is intended for researchers and students who are interested in examining NMJ pathology in a mouse model of SMA but may be adapted to image NMJs in any mouse model. This protocol may be of particular relevance when measuring the impact of novel therapeutics on the neuromuscular system. This SOP has been successful in staining the transverse abdominal and the internal/external oblique muscles.

3 Materials and equipment required

- Microdissection tools: scissors, forceps and angled spring scissors with 10mm cutting edge.
- Sylgard lined petri dish (made according to manufacturing instructions) with dissection pins
- Dissecting microscope
- 24 well plate
- Glass slides and cover slips
- Fluorescent microscope

4 Step-by-Step Procedure

4.1 Tissue Collection and Preparation

Dissection of the Transverse Abdominal Muscle

A video of the procedure can be found here: Murray, Lyndsay et al. "Dissection of the transversus abdominis muscle for whole-mount neuromuscular junction analysis." *Journal of visualized experiments : JoVE*, 83 e51162. 11 Jan. 2014, doi:10.3791/51162

1 Dissection of abdominal muscles

2 Fixing and Isolation of TVA muscle

3 Staining of TVA muscle

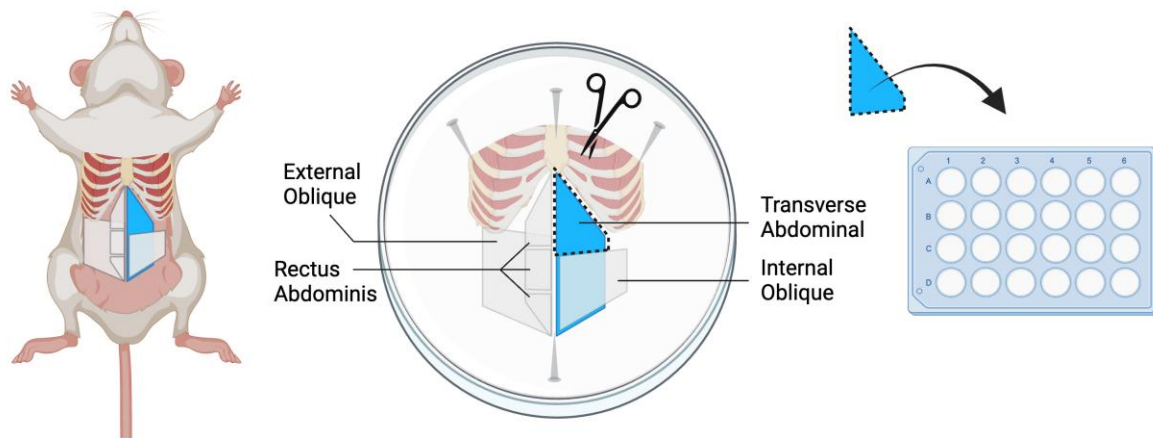


Image created using BioRender

1. Mice should be culled using the appropriate methodology dictated by the investigator's location. Microdissection tools (Scissors and forceps), a sylgard lined-petri dish and inset pins are required for this dissection.
2. Collection of the bottom half of the ribcage and abdominal muscles should be performed rapidly and in a gross fashion. Ensure that the diaphragm is removed from the ribcage. Work gently with the tissue and avoid pulling on the muscle as this can cause breakage between the neurofilament and endplates.
3. Gently pin the tissue onto a sylgard lined-petri dish and immerse in cold 1X PBS solution. Remove any excess fat from the tissue. If multiple dissections are being performed, the dish should be placed on ice.
4. Fix the muscle tissue in 4% paraformaldehyde for 12 minutes on a rocking plate at room temperature.
5. Wash muscle tissue in 1X PBS for 10 minutes, repeat for a total of 3 washes.

6. Using a dissection microscope, carefully dissect and remove the outer external oblique, rectus abdominal and internal oblique muscles to uncover the transverse abdominal muscle. Carefully cut and collect the proximal half of the transverse abdominal muscle and place it into a 24-well plate containing cold 1X PBS solution.

Stopping point: At this point muscle tissue immersed in 1X PBS can be placed at 4°C overnight if a stopping point is required.

4.2 NMJ Staining

Reagent List

Reagent	Company	Catalogue number
α -bungarotoxin	Invitrogen	T1175
Triton-X	Sigma/milliore	X100-100
Bovine Serum Albumin	Sigma/millipore	A4503-100g
NF 2H3 antibody	Developmental Studies Hybridoma Bank	2H3-s
SV2 antibody	Developmental Studies Hybridoma Bank	SV2-s
Goat-anti-mouse IgG 488	Invitrogen	A11001
Dako Fluorescence Mounting Medium	Agilent	S3023

Wrap the 24-well plate in aluminum foil to prevent light exposure. Steps 1-3 and 5-8 are completed at room temperature. Solutions should be prepared fresh for each batch of staining.

1. Incubate the muscle tissue in α -bungarotoxin (1:1000) in 1X PBS, for 30 minutes on a rocking plate.
2. Remove the liquid, incubate in 2% triton-X for 30 minutes on a rocking plate.
3. Remove the liquid, incubate in blocking reagent for 40 minutes.
 Blocking reagent:
 - 4% Bovine Serum Albumin
 - 1% Triton-X (in 1X PBS)
 - Make up in 1X PBS
4. Remove liquid, incubate in blocking reagent with antibodies overnight at 4°C.
 Blocking reagent with antibodies:
 - NF 2H3 (1:100)
 - SV2 (1:250)
5. The following day, remove the liquid and gently wash the samples with 1X PBS for 10 minutes, repeat for a total of 3 washes.
6. Remove the liquid, incubate in 1X PBS containing secondary antibody for 2 hours.

1X PBS containing secondary antibody:

- Goat-anti-mouse IgG 488 (1:250)
7. Remove the liquid, gently wash the samples with 1X PBS for 10 minutes, repeat for a total of 3 washes.
 8. Mount the samples onto glass slides using Dako mounting media (Agilent, S3023), place a cover slip over the tissue, avoid creating air bubbles. Press gently on the cover slip to remove air bubbles if they occur.
 9. Allow the slides to rest face up overnight at 4°C.
 10. The following day, a thin layer of nail polish can be applied to the sides of the cover slip to help seal it to the glass slide.
 11. Image tissue using a standard fluorescent microscope, performing a z-stack can increase the clarity of the neuromuscular junctions. It is best to image within 1 week of staining.

4.3 Imaging

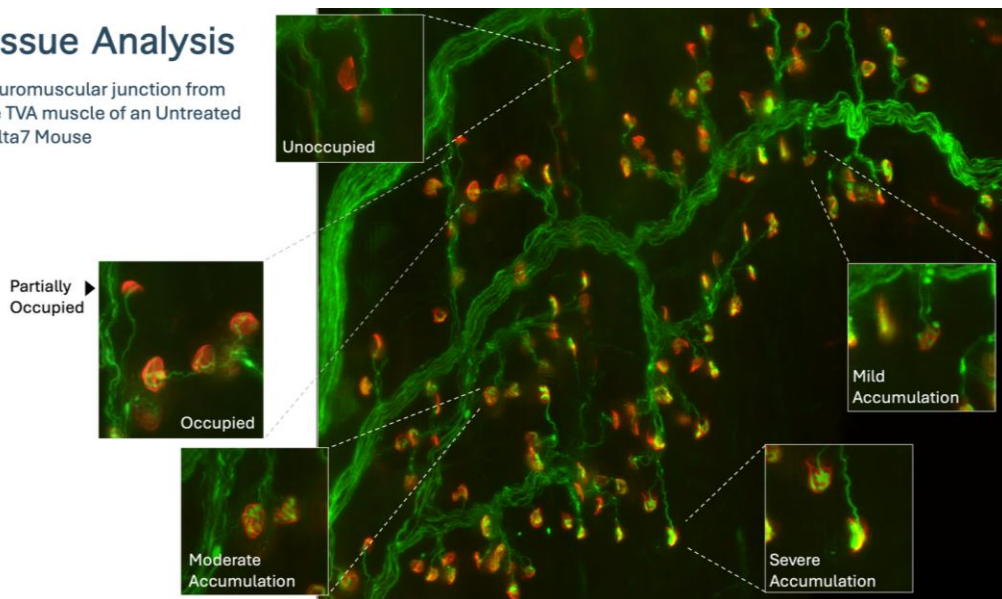
Using a fluorescent microscope, images can be taken at 20X magnification for assessment of multiple NMJs or at 40x magnification for individual endplate analysis.

4.4 Analysis

Images can be quantified with ImageJ. Using the counting tool the number of occupied, partially occupied and unoccupied endplates can be counted, as well as the number of terminals displaying neurofilament accumulation. Other quantification tools such as NMJ Morph can also be applied to these images.

Tissue Analysis

Neuromuscular junction from the TVA muscle of an Untreated Delta7 Mouse



5 Safety Precautions

Use normal laboratory pre-cautions.

6 References

Sutton, Emma R et al. "Liver SMN restoration rescues the $Smn^{2B/-}$ mouse model of spinal muscular atrophy." *EBioMedicine* vol. 110 (2024): 105444. doi:10.1016/j.ebiom.2024.105444

Reilly, Aoife et al. "Long term peripheral AAV9-SMN gene therapy promotes survival in a mouse model of spinal muscular atrophy." *Human molecular genetics* vol. 33,6 (2024): 510-519. doi:10.1093/hmg/ddad202

Reilly, Aoife et al. "Central and peripheral delivered AAV9-SMN are both efficient but target different pathomechanisms in a mouse model of spinal muscular atrophy." *Gene therapy* vol. 29,9 (2022): 544-554. doi:10.1038/s41434-022-00338-1

Murray, Lyndsay et al. "Dissection of the transversus abdominis muscle for whole-mount neuromuscular junction analysis." *Journal of visualized experiments : JoVE* ,83 e51162. 11 Jan. 2014, doi:10.3791/51162

7 Examples & Tips

- Adipose tissue can affect the quality of the staining, during the dissection stage remove as much fat from the muscle as possible. For this reason, it is easiest to use mice aged P30 and below for this protocol.
- When making the blocking reagent, allow the BSA to dissolve slowly into the 1X PBS before adding triton-x.
- Failing to adequately wash tissues and/or decreasing the incubation period in step 8, can result in increased background fluorescence.