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TITLE:

Dissection and Immunohistochemistry of the *Drosophila* Adult Leg to Detect Changes at the Neuromuscular Junction for an Identified Motor Neuron

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KEYWORDS:

Neurodegeneration, ALS, *Drosophila* leg motor neuron, *Drosophila* adult neuromuscular junction

SUMMARY:

We describe a dissection technique that preserves the architecture of the neuromuscular junction and enables a detailed immunocytochemical study of motor neurons in the adult *Drosophila* leg.

ABSTRACT:

Drosophila melanogaster represents a genetically tractable model to study neuronal structure and function, and subsequent changes in disease states. The well characterized larval neuromuscular junction is often used for such studies. However, rapid larval development followed by muscle histolysis and nervous system remodeling during metamorphosis makes this model problematic for the study of slow age-dependent degenerative changes like those occurring in amyotrophic lateral sclerosis. Alternatively, adult flies live for 90 days and the adult leg can be used to study motor neuron changes over the course of adult lifespan using *in vivo* fluorescent imaging through the cuticle. Here, we describe a leg dissection technique coupled with immunocytochemistry, which allows for the study of molecular changes at the neuromuscular junction of identified adult leg motor neurons. These techniques can be coupled with a myriad of antibodies labeling both pre- and post-synaptic structures. Together these procedures allow a more complete characterization of slow age-dependent changes in adult flies and can be applied across multiple motor neuron disease models.

INTRODUCTION:

Motor neuron (MN) diseases encompass a group of heterogenous conditions that include progressive degeneration leading to muscle wasting and paralysis as a primary clinical

phenotype[1]. Although rare with a global prevalence of 4.5 per 100,000, this prevalence is expected to increase with an aging population[2]. Amyotrophic lateral sclerosis (ALS) is the most common MN disease (MND) and is typically fatal within a short time of diagnosis with no existing disease-modifying treatments available[3]. MNDs share in common a protracted presymptomatic phase with early molecular biomarker changes and functional imaging changes seen in patients[4]. Early presymptomatic cellular pathology is also observed in non-human disease models[5-8]. The study of early changes at the neuromuscular junction is important for understanding MN disease pathogenesis and may aid in developing early diagnostics and potential therapeutics.

A wealth of genetic and molecular tools exists in *Drosophila* to dissect the structure and function of the neuromuscular junction (NMJ, see[9] for a review of the well-characterized larval NMJ). These tools combined with a short lifespan make *Drosophila* an excellent model to study neurodegenerative changes at the NMJ. Specifically, MNs innervating adult muscles are present throughout the ~90-day adult lifespan and are subject to normal aging processes[10-13]. The adult MNs therefore provide an opportunity to study slow degenerative changes in contrast to larval NMJs which exist for only a short ~1 week time-period prior to metamorphosis[14,15].

Here, we describe a dissection procedure that allows us to conduct immunocytochemical analysis of MNs in the adult leg. Each adult leg is innervated by ~50 MNs, which synapse onto the associated leg muscular to drive locomotion. The leg anatomy, mechanical physiology, and neurobiology has been well described[16-18]. Axon arbors of leg MNs have previously been characterized by imaging through cuticle in back-filled or genetically labeled cell populations using the bipartite Gal4/UAS system and imaging methods have been published previously[19]. The dissection methods presented here preserves axon branching morphology and allows us to exploit a diverse range of antibodies to label different molecular components of the NMJ. Our previous work has focused on projections of a defined MN in the metathoracic (3rd) leg, which innervates the tibia levator muscle (tilm) and shows consistent arborization patterns and bouton numbers. Initially we studied age-dependent changes in *Drosophila superoxide dismutase 1* (*dsod1*) mutants and found alterations consistent with dismantling of the NMJ[20]. These dissection methods offer the opportunity to better characterize slow degenerative changes at the NMJ for other ALS models, basic studies of aging and other MN associated diseases.

PROTOCOL:

The procedures for preparing working solutions used in conjunction with this protocol are described in **Table 1**.

[Place Table 1 here].

1. Initial fixation of tissues

1.1. Select approximately 10 flies for each genotype and age. Anesthetize on a fly pad under carbon dioxide (**Figure 1A, B**).

NOTE: Begin with more flies than necessary to ensure a large enough sample size after dissection.

1.2. Using a paintbrush, transfer flies to cold methanol in a glass well or dish for approximately 30 seconds to 1 minute (**Figure 1C**). The methanol solubilizes the cuticular hydrocarbons and flies can now be submerged rather than float in aqueous solutions.

1.3. With forceps, carefully transfer flies to PBS. Rinse 3x in ice-cold PBS to remove excess methanol and keep flies in PBS on ice until dissection and fixation. At this point, dissect flies and fix within the shortest time possible (<30 minutes).

1.4. To isolate legs, transfer flies to a silicone elastomer dissection dish filled with cold PBS, remove the metathoracic legs at the coxa using two pairs of #5 Dumont forceps or cut the legs with Vanna scissors (**Figure 1D**). Transfer the legs to a well in a plastic 24 well plate filled with 1 mL of PBS and keep the plate on ice until all legs are removed and transferred to wells.

NOTE: Each well can hold at least 20 legs.

1.5. Replace the PBS solution with 1 mL of FA solution and rotate on a nutator for 30 minutes (**Figure 1E**). The nutator setting should be set at a medium speed (17 rpm). Ensure the legs are completely submerged in FA solution during this time for adequate fixation.

1.6. To remove FA solution, wash in 1 mL of PBS 3x quickly followed by an additional 3 washes for 5 minutes each in 1 mL of PBS. Hold tissues in 1 mL of PBS on ice prior to, and during the dissection steps described below.

2. Removing the Leg Cuticle and Antibody Staining

2.1. Removing the leg cuticle

2.1.1. The dissecting forceps are critical for success. Introduce slight parallel bends in both prongs at the end of #5 super fine forceps to provide a bevel that allows the cuticle to be grabbed superficially rather than be poked, which can ruin the tissue (**Figure 1F, G**).

NOTE: Prongs bent in parallel should still make contact with each other throughout the length of the prong when closed (**Figure 2**).

[Place Figure 2 here]

2.1.2. Transfer legs to a silicone elastomer dish in PBS for dissection. Orient a leg so that the anterior side is facing up (see **Figure 3** for leg anatomy and orientation information). Using one pair of forceps, hold the tibia segment against the silicone elastomer dish. Using the other forceps held bevel side down, grab a piece of cuticle on the distal end of the femur and pull in the proximal direction toward the trochanter.

2.1.3. Keep methodically removing cuticle until the naked muscle is visible throughout the proximal end of the femur (**Figure 1F, G, H**).

NOTE: Only make superficial contact with the legs using the beveled side of the forceps to avoid pulling muscle.

2.1.4. Once all legs are dissected, replace PBS with FA to post-fix legs for 30 minutes with shaking on a nutator at medium speed (**Figure 1I**). Wash samples in 1 mL of PBS for 3 times quick and then 3 times for 5 minutes each in PBT (**Figure 1J**).

NOTE: If staining with monoclonal antibody NC82 (anti-bruchpilot) to label active zones, post-fix for 20 minutes as this antigen is sensitive to longer fixations.

2.2. Antibody staining

2.2.1. To block tissues for antibody staining, replace 1 mL of PBT with blocking solution consisting of 1 mL of 5% NGS diluted in PBT. Incubate dissected legs for 4 hours at room-temperature or overnight at 4 °C while rocking on a nutator at medium speed (17 rpm). During all incubations, cover wells with sealing tape in addition to the plastic cover (**Figure 1J**).

NOTE: 24 well plates are used for immunocytochemistry rather than 1.5 mL or 2 mL microcentrifuge because previous attempts to use microcentrifuge tubes resulted in broken legs and damaged tissue.

2.2.2. Remove blocking solution and add 300 mL of primary antibodies diluted in fresh blocking solution. The small volume of antibody used should be sufficient to cover the tissues. Re-seal wells with laboratory sealing tape and plastic lid and incubate overnight at 4 °C with shaking on a nutator at medium speed (**Figure 1J**). The working antibody reagent information and concentrations used in for these studies are described in **Table 2**.

2.2.3. Wash primary antibodies in 1 mL of PBT for 3 times briefly and then 3 times for 15 minutes each (**Figure 1J**).

NOTE: Diluted primary antibodies can be saved and reused if stored at 4 °C for up to 2 weeks.

2.2.4. Block tissues again in 1 mL of 5% NGS for at least 2 hours at room temperature or overnight at 4 C (**Figure 1J**).

2.2.5. Remove the 5% NGS blocking solution and add 300 µL of the appropriate diluted fluorescent conjugated secondary antibodies. Additionally, add 1:2000 dilution of fluorescence-conjugated phalloidin to label muscle (**Figure 1J**).

2.2.6. For secondary antibody incubations, seal wells with laboratory sealing tape and lid. Also, wrap plates in aluminum foil to protect fluorophores from light. Incubate for 6-8 hours at room-

temperature or overnight at 4 °C.

2.2.7. Wash secondary antibodies and phalloidin as described in step 2.2.3 above. Cover plates with aluminum foil in between washes to protect fluorophores from light.

3. Mounting Legs

3.1. Transfer legs to a slide using forceps and orient anterior side up. Cover the legs with mounting media (**Figure 1K, L**).

NOTE: The dissected legs can be aspirated with a P1000 pipette tip if the bottom bore is widened by cutting with a razor blade.

3.2. Add clay spacers to a 22x22 mm^2 coverslip (#1.5 thickness) by scraping the coverslip corners across a small ball of modeling clay. Each corner should have a small amount of clay 1-2 mm thick (**Figure 1K**).

3.3. To cover the slide, add the coverslip with the clay spacers facing towards the slide and carefully push on the corners until the coverslip just touches the surface of the femur.

3.4. To prevent evaporation, seal the edges of the coverslip with nail polish and let dry in a dark place (about 10 minutes) before storing at 4 °C until imaging.

4. Imaging

4.1. Image by confocal microscope (**Figure 1M**). Include a transmitted light channel to better assess the quality of the dissection and samples with visibly disrupted muscle fibers in the area of interest should be discarded.

4.1.1. Begin imaging z-stacks with 20x magnification with 2x zoom and a total image depth of ~ 40 mm corresponding to the thickness of the femur. For fluorescent signal detection, capture images 1024 x 1024 pixels with a dwell setting 8-10 µs/pixel. The signal intensity should be in the linear range which is achieved by adjusting the high voltage gain settings. Once the gain settings are set for a series of samples within an experiment, they should not be altered so that signal intensities can be compared between samples.

4.2. For imaging synaptic boutons and other subcellular structure, capture confocal images at ≥60x magnification. The detector settings should be in the linear range, while the pixel density, dwell settings and z-depth should be similar for images captured at lower magnification (step 4.1).

REPRESENTATIVE RESULTS:

Figure 4 shows a representative example of a metathoracic leg stained with anti-hrp, anti-dlg, and phalloidin. For dissections that remove cuticle from the proximal portion of the femur,

stereotyped arbors will be apparent near the tendon which is detected easily by autofluorescence. Note that antibody penetration into the leg occurs for a short distance beyond the region in which cuticle has been removed (**Figure 4A**). These regions can be imaged effectively when strong fluorescence signal is present. Imaging at low magnification (20x with a 2x zoom) allows an easy determination of 1) how much cuticle is removed, and 2) whether damage occurred during dissection. Increased magnification (60x) shows stereotyped projections onto the tilm (**Figure 4B**). Our work has focused on one MN, likely derived from the I- MN lineage which innervate the tilm in the proximal femur (box, **Figure 4B** and **Figure 4C**). Increasing magnification further (100x with 2x zoom) allows for effective visualization of synaptic boutons (**Figure 4D**).

To study morphological changes at the adult NMJ over time, we have previously used dsod1 mutants as a model of ALS. Bouton swelling occurs in aged *dsod1*^{H71Y} mutants relative to *dsod1*^{null} and *dsod1*$^{+}$ (**Figure 5A, B**). At the larval NMJ, monoclonal antibody NC82 is often used to label active zones and this these structures can be easily visualized at the adult NMJ (**Figure 5C**). Weakly-positive HRP axon branches are abundant in *dsod1*^{H71Y} mutants and these branches often show weak and diffuse BRP localization (arrows).

Important for neurodegeneration, commercially available antibodies can detect ubiquitinated proteins often found in aggregates, and we have detected ubiquitinated aggregates within terminal axons of MNs in aged mutant *dsod1* flies as well as within muscle (**Figure 6A**). Also, antibodies labeling mitochondria can also detect morphological changes with age (**Figure 6B**).

For some preparations, muscle damage during the dissection makes the sample unusable. At first, such damage can be a common occurrence, but improvement occurs with practice. Examples of good dissections are show **Figure 7A, C** while poor dissections are shown in **Figure 7B, D**. Poor dissections cause disorganization of muscle fibers as detected by phase contrast microscopy (**Figure 7B**) or missing muscle fibers visualized by fluorescent-conjugated phalloidin (**Figure 7D**, arrow). The combination of using phalloidin to label muscle, and transmitted light images can help to detect such muscle damage which may not be apparent when viewing under a dissecting microscope.

FIGURE AND TABLE LEGENDS:

Figure 1. Workflow summary for dissecting legs. See protocol for detailed steps. (**A,B**) Flies are selected and anesthetized. (**C**) Flies are transferred to methanol and washed with PBS. (**D**) The metathoracic legs are removed at the base of the coxa while visualized with a dissecting microscope (~30x magnification); scale bar = 500 mm. (**E**) The legs are then fixed in 3.7% formaldehyde/PBS (FA) solution for 30 minutes within wells of 24-well plates and then FA is removed by washes with PBS. (**F, G, H**) Legs are transferred to silicone elastomer dissecting trays and a piece of cuticle is removed from the proximal femur using beveled forceps while visualized under a dissecting microscope at 80x; scale bar = 50 mm. (**I**) Legs are post-dissection fixed in FA and washed in PBS and then PBT (PBS+ 0.1% non-ionic surfactant). (**J**) Legs are subjected to immunocytochemical staining. (**K,L**) Legs are transferred to a glass slide, cleared in mounting media, and covered with a coverslip containing clay spacers; scale bars = 2 mm and 500 mm. (**M**)

Legs are imaged by confocal microscopy.

Figure 2. Modified forceps used for dissecting adult legs. (**A**) The ends of the forceps are bent and then flattened at the bottom (arrow) creating a bevel by filing on a sharpening stone. (**B**) In contrast, the prongs of unmodified forceps are not bent. Scale bar = 1 mm

Figure 3. Anatomy of the *Drosophila* leg. (**A**) View of the anterior side of a metathoracic leg, characterized by the presence of bristles in contrast to the prominent naked cuticle present on the posterior side. The segmented leg is comprised of the coxa (Cx), trochanter (Tr), femur (Fe), tibia (Ti), 5 tarsal segments (Ta1-5), and claw (Cl) in order from proximal (pr) to distal (di). Dorsal (d) and ventral (v) sides of the femur are also indicated. Scale bar =100 mm. (**B**) Diagram of the femur containing the tibia levator (tilm), tibia depressor (tidm), long tendon muscle 2 (ltm2) and tibia reductor (tirm) muscles and axon projections in the proximal femur innervating the tilm. These stereotyped axonal projections are presumed to be derived from I-lineage neuroblasts[16], and the second arborization is the easiest to access by dissection (boxed). Scale bar = 100 mm.

Figure 4. Dissected metathoracic femur revealed stereotyped MN architecture innervating the tilm. Immunocytochemistry was used to mark neurons (HRP), discs-large (DLG) and muscle (phalloidin). Z-stacks were captured by confocal microscopy imaging through the entire femur and shown as a maximum series projection. (**A**) A transmitted light channel was also included to illustrate no detectable muscle damage occurred during dissection. Image was captured at 20x magnification, scale bar =50 mm. (**B**) Identified arbors associated with the tilm (boxed area, center), scale bar =20 mm; and (**C**) at 60x magnification, scale bar = 20 mm. (**D**) DLG surrounding boutons was apparent in wild type animals when imaged at 100x magnification with 2x zoom; scale bar = 2 mm

Figure 5. Example of age-dependent changes in bouton morphology which can be detected using the leg dissection technique. Bouton swelling is seen in aged *dsod1^{H71Y}* mutants. (**A**) Representative images of HRP stained boutons from young (newly eclosed, day 0 adults) and old (day 10) flies, scale bar = 1 mm. (**B**) Bouton sizes were quantified from respective genotypes using the measure function within ImageJ. ***p<0.0001 (2-way ANOVA with post-hoc Tukey test). (**C**) Active zones within synaptic boutons labeled with monoclonal antibody NC82 (anti-bruchpilot); scale bar = 1 mm. Figure modified from[20]

Figure 6. Common markers associated with neurodegenerative disease used in *Drosophila* leg preparations. (**A**) Subcellular polyubiquitinated aggregates are detected in terminal axons and muscle of aged *dsod1^{H71Y}* flies. Immunocytochemistry using anti-polyubiquitin (FK2) detects puncta (arrows) in aged *dsod1$^{H71Y/H71Y}$*, scale bar = 20 mm (left) and 5 mm (right). (**B**) Swollen mitochondria can be detected using anti-ATP5A, scale bar = 1 mm. Figure modified from[20]

Figure 7. Examples of good and poor dissections. (**A**) Dissection which did not disrupt the underlying muscle architecture. (**B**) An example of a poor dissection which showed frayed muscle fibers (arrow) and could not be used for analysis. Both samples were imaged by phase-contrast microscopy. Scale bar = 50 mm. (**C**) Example of a good dissection imaged by confocal microscopy

with HRP (green) and phalloidin (blue). (**D**) A poor dissection missing dorsal muscle fibers of the TILM missing (arrow). Scale bar = 50 mm

Table 1. Solutions for performing immunocytochemistry of the adult *Drosophila* leg.

Table 2. Antibodies and dilutions. The commercial suppliers of the antibodies used in these studies are listed in the Materials List.

DISCUSSION:

The *Drosophila* adult leg is an ideal model to study neurodegeneration given relative simplicity with well-characterized MNs mapped from neuroblast lineages and stereotyped arborization patterns. Several reports have previously used leg MNs for the study of neurodegenerative disease[21,22]. These studies utilized GFP-expressing lines combined with mosaic analysis with a repressible cell marker (MARCM) to image through the cuticle and documented a series of morphological changes. Imaging adult NMJs by immunocytochemistry with resected cuticle enables further characterization with the ability to track complex molecular changes using a toolbox of antibodies available.

The immunocytochemistry portion of this protocol is relatively standard and can be implemented independent of genotype (see[23] for an excellent description of general antibody staining methods for use with *Drosophila*). Furthermore, parameters such as fluorescence intensity, axon branch length, and bouton numbers and size can be determined using a variety of available ImageJ macros once images are captured and detailed methods for quantitative analysis have been published (for example, see[24-26]). Thus, the dissection technique is the main innovation described here. Prior to dissection, flies are submerged in an alcohol to strip cuticular hydrocarbons. Both ethanol and methanol are commonly used for this purpose; however, we have only used methanol. Critical to dissection success are several factors: First, using modified forceps with a bevel allows for very superficial contact with the cuticle. Second, using a dissecting microscope capable of 60-100x total magnification so that the surface of the cuticle is clearly visible. For microscopes with lower maximum magnification, 2x objectives are available for most common brands and should be sufficient when combined with existing lenses. Third, the initial fixation step makes the cuticle brittle and easier to pull away without damaging muscle underneath. Over-fixation at this step makes the entire leg too stiff for effective dissection. Therefore, the initial fixation should be limited to 30 minutes. The formaldehyde fixative will not penetrate enough to effectively crosslink the underlying tissue during this short period and thus a second fixation step is necessary. Prior to the second fixation, tissues should be kept on ice to prevent degradation and changes in morphology. Fourth, we have found dissecting samples while cold is also important, likely for similar reasons in that cuticle is brittle and a small piece can be more easily removed.

With practice, we find ~50% of dissections will be usable within no discernable tissue damage. Although this percentage may seem low relative to some other tissues, the dissection procedure is rapid, and many legs can be processed in 30- 60 minutes. Therefore, even if success rates are low initially, it is feasible to obtain 4-5 good samples for each experimental group. However, a

limitation may be the number of flies available at any given time if genotypes and/or age result in substantial lethality.

Another limitation is that we have not been able to dissect other areas of the leg beyond the proximal region of the femur due to size. Thus, we can study identified MN arbors innervating the TILM reliably and it is possible to dissect cuticle above the tibia depressor muscle with small changes to the way the leg is oriented when dissecting. However, accessing other regions of the leg have proved more difficult without disrupting axonal architecture during dissection.

Here, we present dissection methods to detect changes at the adult NMJ for defined MNs innervating the tilm using immunocytochemistry. The leg is useful as a simple system, innervated by only ~50 MNs and containing 14 muscles with well-described anatomy. The dissected leg preparation can be used across genotypes and a suite of antibodies are available for NMJ visualization without the need for building genotypically complex stocks of reporter gene constructs in mutant backgrounds. This approach will enable a more detailed characterization of changes at the NMJ for MN diseases and other age-related conditions.

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DISCLOSURES:

The authors declare no conflicts of interest.

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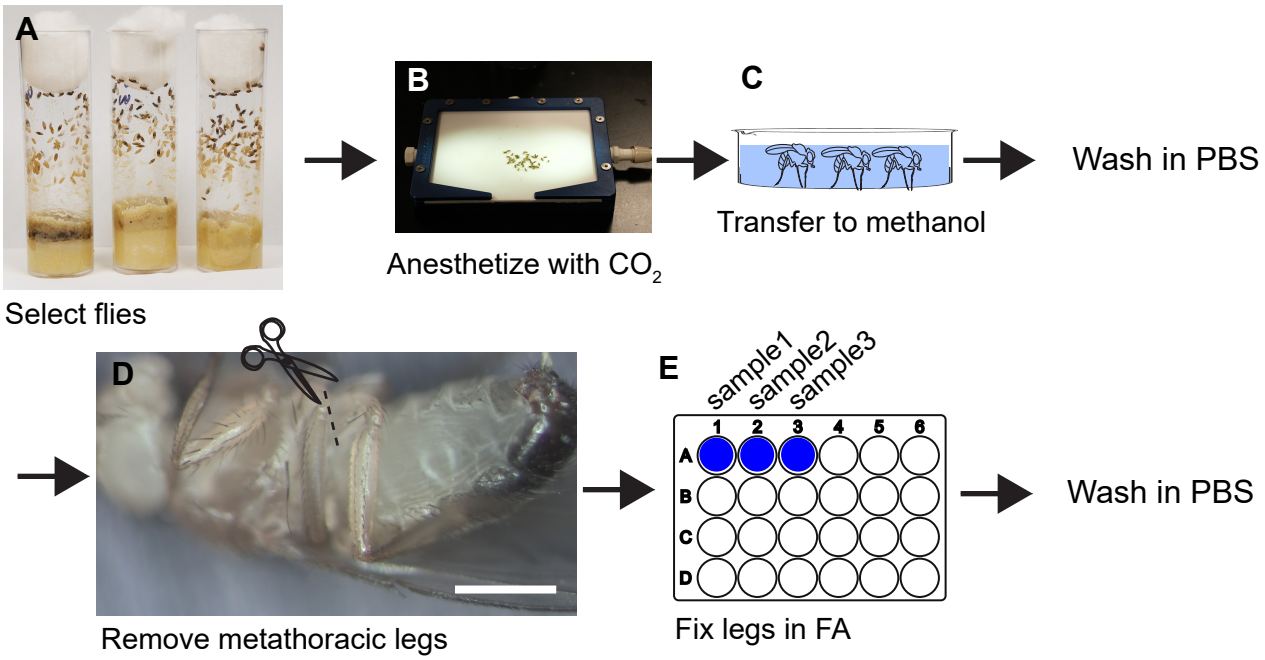
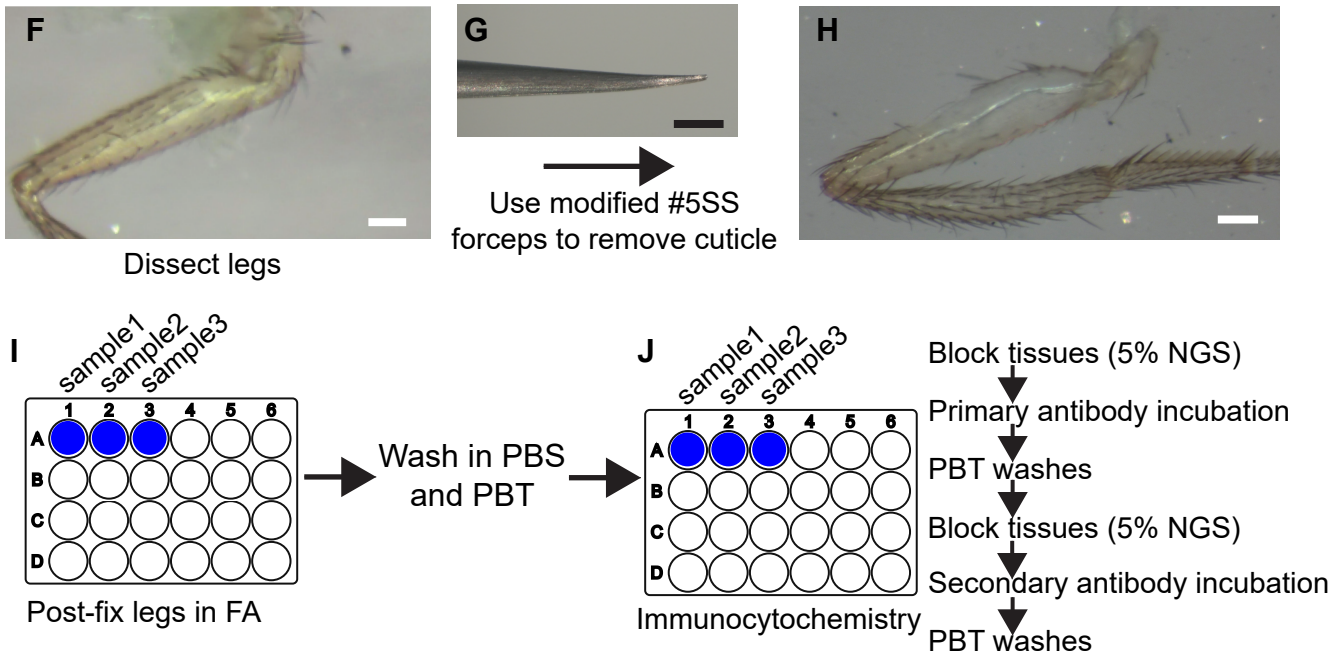
1. Initial Fixation of Tissues



2. Removing Leg Cuticle and Antibody Staining



3. Mounting Legs

4. Imaging

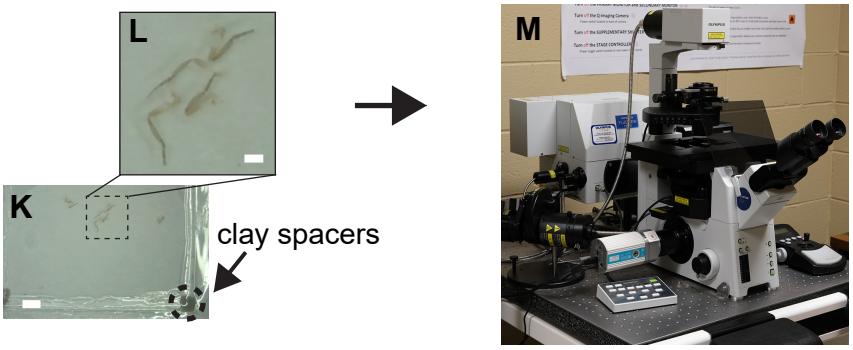
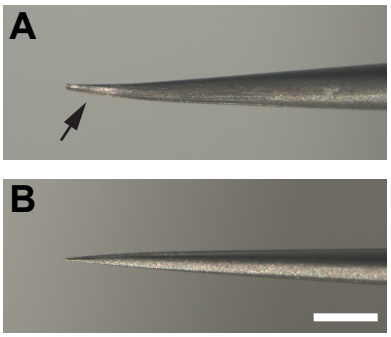


Figure 2- Forceps



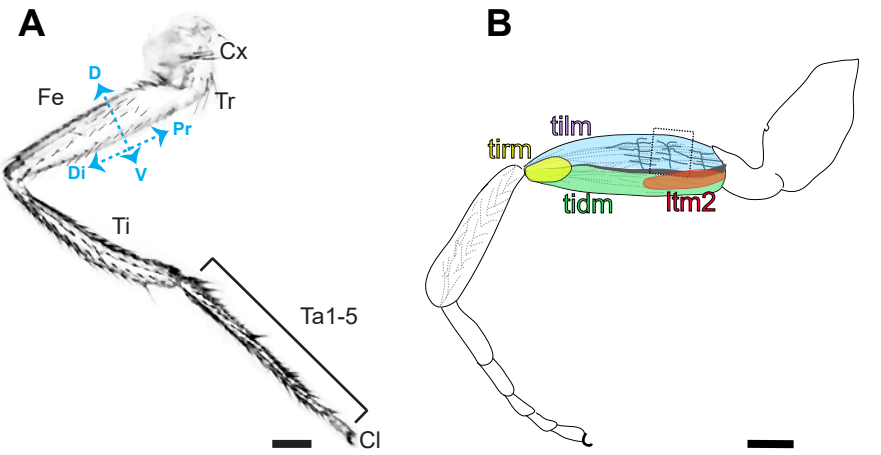
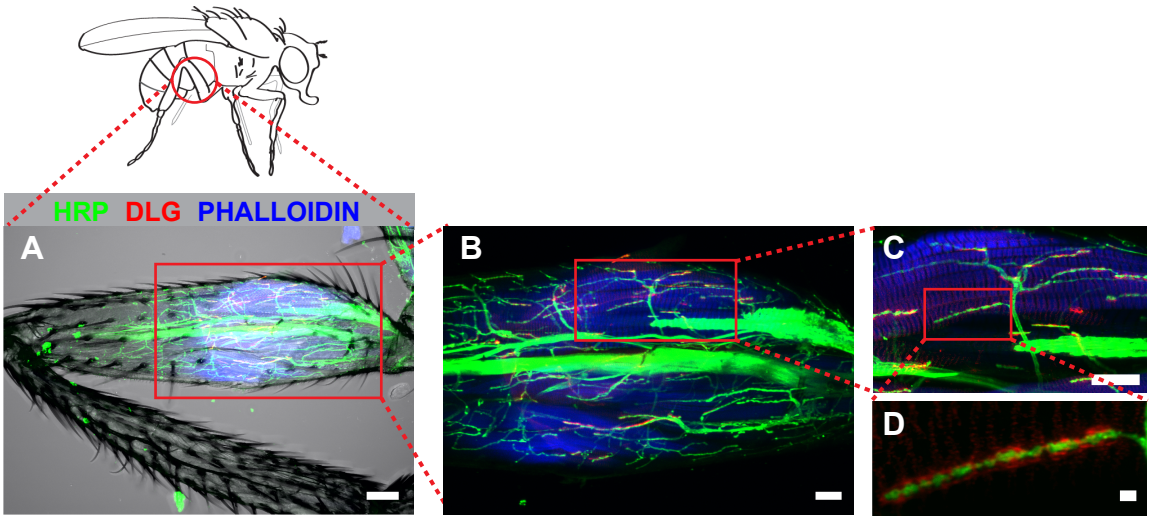
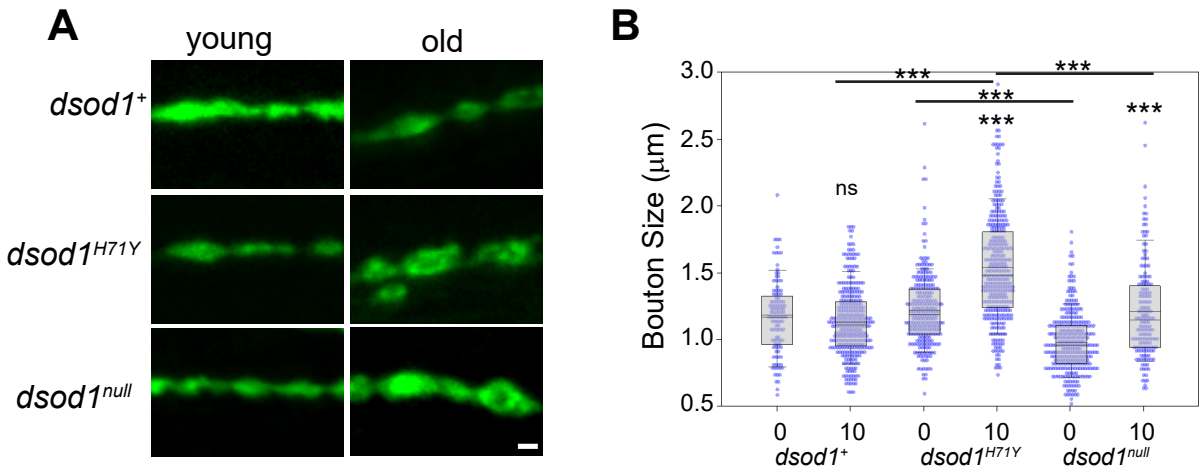


Figure 4- Example

Click here to access/download;Figure;Figure4__FINAL.pdf





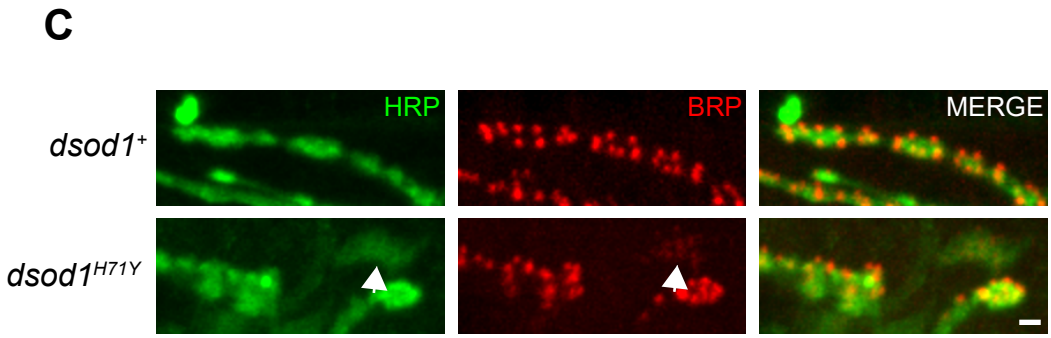


Figure 6- Neurodegeneration Model

Click here to access/download;Figure;Figure6__FINAL.pdf

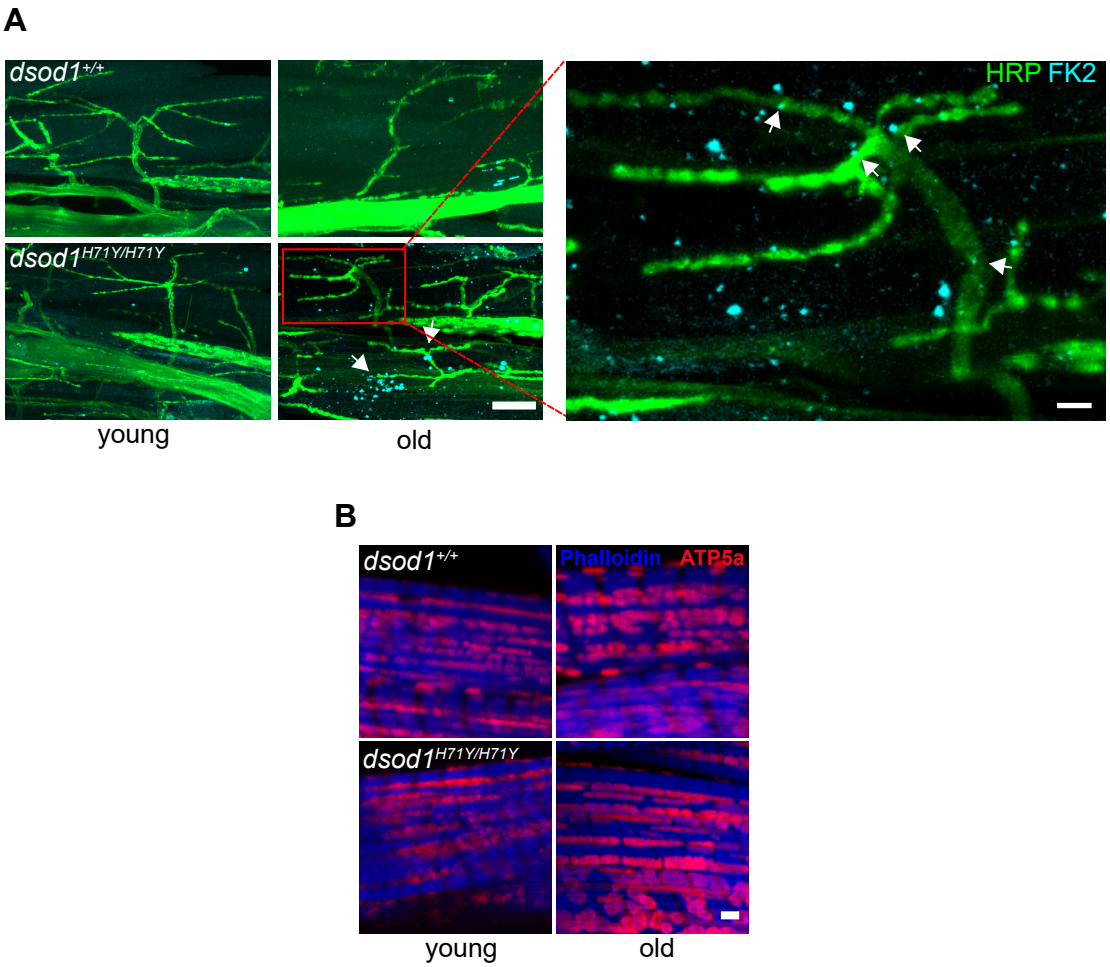


Figure 7- Good vs bad dissection

Click here to access/download;Figure;Figure7_FINAL.pdf

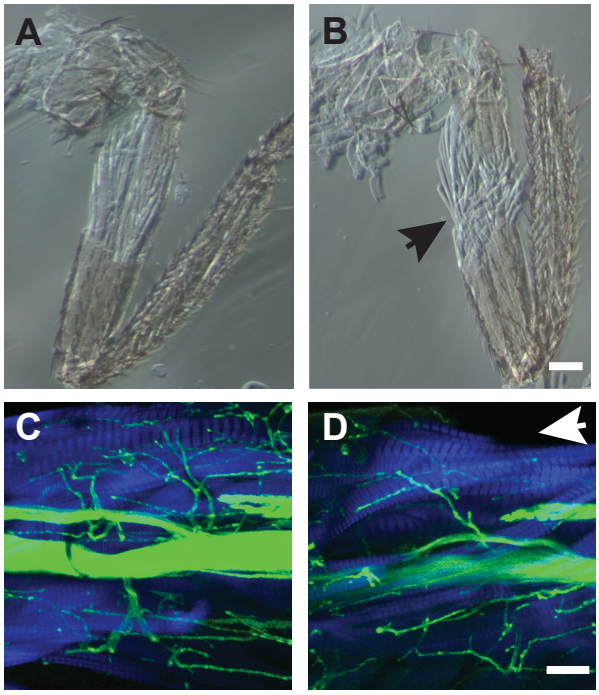


Table 1

Click here to access/download;Table;Table1_ReagentPreparation.xlsx

Reagent	Preparation
PBS	Make a working stock of 1x PBS from a 10x PBS stock solution by diluting in distilled water. The pH of the working PBS stock should be 7.2- 7.4
PBT	1x PBS solution with 0.1% non-ionic surfactant.
FA	3.7% formaldehyde solution made from a 37% formaldehyde stock and diluted in 1x PBS. CAUTION: Formaldehyde supplied as 37% stock solution is a potential carcinogen and should be diluted in a fume hood.
5% NGS	5% normal goat serum diluted in PBT. The serum used should match the species of the secondary antibody to be used.

Storage

4 °C for 1+ months until bacterial contamination is visible.

4 °C for 1+ months until bacterial contamination is visible.
Room temperature. Make fresh each day of dissection

4 °C for several weeks until bacterial contamination is visible

Table 2 - Antibodies

Click here to access/download;Table;Table2_AntibodyDilutions.xlsx

Antibody/Stain	**Dilution***
Anti-ATP5A	1:500
Anti-bruchpilot (nc82)	1:20
Anti-cysteine string protein (ab49)	1:50
Anti-discs large (4F3)	1:200
Anti-hrp	1:550
Anti-polyubiquitin (FK2)	1:1000
Anti-repo (8D12)	1:5
Goat anti-mouse secondary antibody	1:800
Phalloidin	1:2000
	*Dilute in 5% NGS

Table of Materials

Click here to access/download

Table of Materials

MaterialsList_v2.xlsx

Geoff Stilwell, PhD
Rhode Island College
Fogarty Life Sciences, Room 236
600 Mount Pleasant Ave.
Providence, RI 02908

Aug. 20, 2021

Vineeta Bajaj, MS, PhD
Manager of Review
JoVE

Dear Dr. Bajaj,

Please find our enclosed revised manuscript entitled “Dissection and immunohistochemistry of the *Drosophila* adult leg to detect changes at the neuromuscular junction for an identified motor neuron”. We thank reviewers for their many constructive comments and have incorporated these suggestions throughout the manuscript. Our responses and associated changes to the document are listed individually (highlighted in yellow).

We have also included a document with webpage links which clarifies our copywrite holder status of previously published material.

If you have any questions or concerns regarding this revision, please feel free to contact me. We believe we have been responsive to the reviewer’s comments; however, we would be happy to make additional changes to the text or video if needed.

Best regards,

Geoff Stilwell

Manuscript Revisions

Introduction:

- Introduction: Introducing the motor neuron diseases, including the statistics, indicating the number of individuals affected by these diseases worldwide every year will increase the impact of the study.

  Added the statement: “Although rare with a global prevalence of 4.5 per 100,000, this prevalence is expected to increase with an aging population[2]. “

- Introduction section, line: "Motor neuron (MN) diseases encompass a group of heterogenous conditions which include progressive degeneration leading to muscle wasting and paralysis as a primary clinical phenotype". Please provide the references.

  Reference added to support this statement

- Introduction section, line: "Conversely, the adult MNs which are formed mainly during pupal development are present throughout the ~90-day adult life". Please provide references.

  Specifically, MNs innervating adult muscles are present throughout the ~90-day adult life-span and are subject to normal aging processes[10-13].

Protocol:

- Protocol section, line: "Replace the PBS solution with 1 mL fixative (FA) solution and rotate on a nutator for 30 minutes. Ensure the legs are completely submerged in solution during this time". Which specific fixative was used paraformaldehyde or glutaraldehyde and at what working concentration?

  Thanks to reviewer for catching our unclear use of abbreviations. We have added a table in which we define solutions specifically (Table 1). FA refers to 3.7% formaldehyde from 37% stock diluted in PBS.

- Protocol section, line: "Once all legs are dissected, replace PBS with FA to post-fix legs for 30 minutes with shaking on a nutator. Wash samples in 1 mL PBS for 3 times quick and then 3 times for 5 minutes each in PBT". Please be specific and provide details about PBT. It's full form, stock and working solution concentration, how was it made to be used in the study, dilution, name of company etc.

  Table 1 clarifies our abbreviation PBT.

- Line "Remove 5% NGS blocking solution and add 300 µL of diluted primary antibodies in fresh 5% NGS". Please indicate the dilution of all primary antibodies used in the study". company of secondary antibodies, phalloidin used in the study".

  Table 2 was added to manuscript which lists all antibodies and dilutions we have used previously

- Line "Using forceps, orient legs so that the dissected anterior side is facing up. Carefully remove the excess PBS and add 50 µL mounting media". Which mounting media was used? any specific company?

  Slowfade diamond, listed in Materials List.

- The manuscript lacks detailed explanation on how the images were analyzed and quantification was performed. Any particular software that was been used? how was the analysis conducted?

  Once images are captured, there are many software platforms available for analysis. Most notable are the multitude of macro plug-ins within ImageJ, many of which have been described in JOVE methods themselves. The main goal of our manuscript and video is provide methods on dissection and staining. A detailed description of image analysis is lengthy and therefore beyond the scope of our methods. However, we have referenced other published sources including JOVE which provide image analysis methods.

- Section 5 (solutions) can be nicely represented in the form of table indicating how the stock and working solutions were made and used

  A solutions table has been added.

- Imaging section: needs to be elaborated. Please indicate the details of confocal microscope, transmitted light channels used in the study. Were all the imaging parameters kept consistent to avoid any variation during quantification? Was automated quantification software used? Can the results be replicated?

  Yes, imaging parameters were kept consistent for each experiment performed. To clarify imaging, we added microscope information to the materials list and added image capture information in Step 4.1. Although beyond the scope of this article to detail automated quantitative analysis of images, we have referenced other published articles which focus on such methods.

- The dilution of NC82, anti-ATP5A needs to be specified.

  Table 2 was added which lists antibodies used and dilutions.

- Line "Re-seal wells with parafilm and plastic lid and incubate overnight at 4 C with shaking on a nutator or platform shaker". At what speed?

Clarified in documents: medium speed (17 rpm). Nutator also added to the materials list

- Figures: Figure 1 legend needs to be elaborated. The orientation of the images, magnification needs to be indicated in figure legends. All figures needs to be properly labelled (A,B,C..), numbered (1,2,3..) and referred in the text. Please provide scale bars wherever applicable in adult fly images as well. Confocal images needs to specified with antibodies used to stain the tissue with.

  Done

- I have one question about the protocole. In section 1.2. The author used methanol. Why not ethanol which is less toxic? Do they see a difference between both ethanol and methanol?

  We have not tried ethanol and have always used methanol for removing wax from the cuticle. While there is likely little difference between these two short-chain alcohols, methanol does appear to penetrate faster into at least some tissues and is used as a stabilizer for formaldehyde.

- 4, line 94: How much is the volume of "PBS with FA solution"?

  Clarified amount (1 mL)

- Line "Re-seal wells with parafilm and plastic lid and incubate overnight at 4 C with shaking on a nutator or platform shaker". At what speed?

  Specified medium speed (17 rpm) in text.

Additional Edits:

- Please correct this sentence: Each of the three sets of adult legs contain(s)
  Done

- 'Conversely, the adult MNs which are formed mainly during pupal development are present throughout the ~90-day adult life'. The sentence is a bit incorrect. Adult leg MNs are mostly produced during the larval stages and they morphologically differentiate during metamorphosis.

  Clarified this point in the text

- 'Each of the three sets of adult legs contain ~50 MNs'. The leg do not contain MNs , they contain the axons. Maybe this sentence is a bit more correct: Each of the three sets of adult legs contain are innervated by ~50 MNs'

  Done

- Were electrophysiology studies conducted and results were confirmed? Replications of the experiments needs to be specified.

  We believe electrophysiological studies would be a powerful compliment to these methods but have not been conducted yet.

- Please provide full form of acronyms (such as TILM, HRP, GFP, NMJ, MNDs etc) the first time they are mentioned in the manuscript. It help readers understand the subject better.

  Done

- Authors affiliations needs to be indicated properly. Individual contributions of all the authors needs to be specified at the end.

  It seems JoVE articles do not typically contain this section. However, I have added it to the revised manuscript.

- JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Sylgard, parafilm, etc.

  Done. We only use the trademarked name in the comment section of the Table of Materials and Reagents, similar to that in other JoVE articles we have seen.

1, line 65: Why only "males"? Are there any reasons for this description?

Removed wording about males. While there is a very slight difference in arboration pattern between males and females, it is very small.

2, line 75: How many legs can be put in a well? One leg in a well, or 10-20 legs in a well?

We have used 20 legs at most per well. We have clarified this point in the document.

3, lines 85-92: This dissection procedure should be carefully described.

We have clarified this as best as possible in written form and hope the video demonstration adds to the understanding of the dissection method. Furthermore, we have added Figure 3 which describes leg anatomy and orientation.

5, lines 102-103: What do the authors intend to say? Should the microcentrifuge tubes not be used? Describe what you want to say clearly.

The purpose of adding this note was to clarify why we use 24 well plates rather than commonly used 1.5 mL or 2 mL tubes. We have modified this statement as follows:

Note: 24 well plates are used for immunocytochemistry rather than 1.5 mL or 2 mL microcentrifuge because previous attempts to use microcentrifuge tubes resulted in broken legs and damaged tissue.

6, Figure 3: NMJ on TILM seems to be useful. Label which is TILM in Figure 3 and describe how to find the TILM. In addition, are there any other adult NMJs useful for the analysis of disease-related mutation?

We have added an additional figure describing leg anatomy. To address the other comment, we believe an important study would be one which compares changes between MNs with different morphology and/or physiology. However, this study has not been done in the context of a disease model to the best of our knowledge.

2. In figure 1 step 2, the graphic implies that the flies are not submerged in the methanol, which I believe to be an error.

We altered this image to convey complete submersion in methanol.

3. For the actual dissection procedure (Steps 2.1-2.2) I think it may be helpful to include a diagram of the femur indicating 1)orientation/direction (e.g. which side is anterior vs. posterior, proximal vs. distal, etc), 2)where to place dissection pins, 3)any relevant landmarks to help orient the reader, and 4)approximate start and end points for the actual dissection. This is likely to be the most technically challenging portion of the entire protocol so a detailed diagram for reference would be extremely useful.

Added figure describing anatomy and orientation.

4. Regarding the modified forceps in Figure 2/step 2.2 of the protocol, are the tips bent so that they are pointed toward each other (like a claw) or in parallel (to form more of a spade shape)?

In parallel. We have modified the protocol wording as follows:

2.1.1. The dissecting forceps are critical for success. Introduce slight parallel bends in both prongs at the end of #5 super fine forceps to provide a bevel which allows the cuticle to be grabbed superficially rather than be poked which can ruin the tissue (Figure 1F, G).

NOTE: Prongs bent in parallel should still make contact with each other throughout the length of the prong when closed.

6. For mounting, in Step 3.4 the authors mention using cover slips with clay spacers. Are these commercially available or home-made? If the latter, a brief description of how to make and use them (or a reference) would be helpful. It would also be useful to know the approximate thickness of this spacer, should the reader wish to use other commercially available options.

Clarified this point in the protocol. The step now reads:

3.2. Add clay spacers to a 22x22 mm^2 coverslip (#1.5 thickness) by scraping the coverslip corners across a small ball of modeling clay. Each corner should have a small amount of clay 1-2 mm thick (Figure 1K).

7. A table of commonly used antibodies (including at the very least the ones used in the representative results section) including appropriate dilutions and the structures they mark should be included.

Added as Table 2

8. The title references "an identified motor neuron" but the text does not indicate the specific neuron to which this refers. A diagram of the stereotyped motor neuron architecture in the dissection region, with the specific neuron(s) analyzed in the representative results would be helpful here.

Added this information in Figure 3.

9. Other changes made

- Checked for grammar and spelling
- Italicized Drosophila throughout
- Reworked Figure 1 with additional details and to better match the protocol.
- Added details to most figure captions
- Provided keywords

Video Edits:

Introduction:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

We have reshot and recorded many sections to harmonize the video/audio with the written manuscript. Also, all figures (except the outline, Figure 1) are shown in the video.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

See above comment

3. Format:

• Please consider capitalizing first letter of all important words in Title Card.

Done

• Title Card in the end seems to be missed, please consider adding Title Card in the end of the video.

We apologize for an oversight and did not add this into the revised video file. If possible, we would be happy to add a closing title card and upload this revision after our submission deadline.

• Conclusion Card seems to be missed, please consider adding conclusion card after the Results section.

Done

4. Please ensure that the protocol section titles are same in the text and in the video.

Done

5. Video Comments:

• All "dip to black or fading" transition can be shorten, as sometimes it starts fading in/out while the narrator is speaking.

Done

• 0:16 Text background can be changed to more darker shade to improve the visiblity of text for the viewers.

Done

• Throughout the video there are some parts which can be removed or trimmed as, leaving in footage that has no accompanying instruction can slow down the pacing and cause 'drag' with the audience. Mentioning some ranges for reference:

- 2:00 - 2:04
- 2:35 - 2:50
- 4:00 - 4:16
- 4:26 - 4.31
- 7:04 - 7:09
- 6:58 The transition seems jarring and distracting for viewers, please consider putting cross dissolve/dip to black/fadde transition between the two footages to avoid it.

Done

• 8:42 - 11:53 Consider highlighting the parts as narrator speaks about them, it will be more understandable for viewers.

Reshot these screens indicating referenced areas

• 9:14 - 9:15 Can be removed as it seems jarring and distracting for viewers.

Done

• 10:37 The picture must be clear enough for the audience to understand the content. Consider replacing this image with a high quality picture.

We believe this image conveys the appropriate information and is reprinted from a previous peer-reviewed publication.

• 8:42 - 9:14 & 10:37 - 11:53 Images can be scaled to fit in video frame size and it will also be good for viewers visibility.

We enlarged images for video

6. Audio Comments:

• Audio seems high and low in some parts of the video. Throughout the video, audio levels should peak between -12 to -6 dB. Time range where audio level needs to be reduced (and keep it between -12 to -6 dB):

• 0:15 - 1:07

• 1:08 - 1:23 (This can be fixed by reducing only 2 dB)

• 4:32 - 4:52

• 7:44 - 8:35

• 11:54 - 12:06 (This can be fixed by reducing only 2 dB)

We have changed audio levels throughout to standardize as much as possible and to be within specified parameters. There are still places where the levels are different but we hope the difference is now within an acceptable range.

Except for the above-mentioned time stamp whole audio level needs to increase so that it peaks between -12 to -6 dB.

Done

• Audio starts at 1:58 which can be started at 1:56 for better use of the narration.

Done

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Additionally, I am including an email exchange with the Permissions Editor at Bio Open who clarified the Bio Open policy in writing on the following attached page.

From: permissions <permissions@biologists.com>
Sent: Tuesday, August 10, 2021 6:36 AM
To: Stilwell, Geoff E. <gstilwell@ric.edu>
Cc: permissions <permissions@biologists.com>
Subject: RE: reproduce figures for JoVE?

Dear Geoff,

Thank you for your permission enquiry.

Since authors retain copyright of their articles, they are free to reproduce material from their own work in other publications, without the need to obtain permission from us separately. As a matter of courtesy we do recommend that you inform your co-authors of your intention.

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With best wishes,
Alice

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Sent: 09 August 2021 4:05 PM
To: permissions <permissions@biologists.com>
Subject: reproduce figures for JoVE?

Dear Biol Open,

I was solicited to submit a Journal of Visualized Experiments (JoVE) article and video. For this submission, we are detailing methods for a novel dissecting technique we used in our published Biol

Open paper (ref. pasted below). As part of the JoVE submission, we wish to use some of the images we published in Biol Open and we are requesting permission to do so. JoVE specifically states it is possible to reproduce figures for the purposes of demonstrating the effectiveness of a technique or procedure and we would like to use images from Biol Open for this reason.

Please let me know if this request is possible and thank you for your consideration.

Best,

Geoff

MS ID#: BIOLOPEN/2019/049692
MS TITLE: Age-dependent degeneration of an identified adult leg motor neuron in a Drosophila SOD1 model of ALS
AUTHORS: Anthony Agudelo, Victoria St. Amand, Lindsey Grissom, Danielle Lafond, Toni Achilli, Asli Sahin, Robert Reenan, and Geoff Stilwell

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