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Visualizing synaptic degeneration in adult Drosophila in association with neurodegeneration

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TITLE:
Visualizing Synaptic Degeneration in Adult *Drosophila* in Association with Neurodegeneration

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SHORT ABSTRACT:
The goal of this procedure is to dissect the dorsal longitudinal muscle (DLM) tissue to assess the structural integrity of DLM neuromuscular junctions (NMJs) in neurodegenerative disease models using *Drosophila melanogaster*.

LONG ABSTRACT:
Drosophila serves as a useful model for assessing synaptic structure and function associated with neurodegenerative diseases. While much work has focused on neuromuscular junctions (NMJs) in *Drosophila* larvae, assessing synaptic integrity in adult *Drosophila* has received much less attention. Here we provide a straightforward method for dissection of the dorsal longitudinal muscles (DLMs), which are required for flight ability. In addition to flight as a behavioral readout, this dissection allows for the both DLM synapses and muscle tissue to be amenable to structural analysis using fluorescently labeled antibodies for synaptic markers or proteins of interest. This protocol allows for the evaluation of the structural integrity of synapses in adult *Drosophila* during aging to model the progressive, age-dependent nature of most neurodegenerative diseases.

INTRODUCTION:
Synaptic dysfunction is among the earliest known hallmarks of most major neurodegenerative diseases[1-6]. However, very little is known regarding how these structural and functional impairments relate to later stages of disease progression. *Drosophila* has proven to be a useful model system for understanding synapse growth and development using larval NMJs[7-9]. However, the third larval instar stage only lasts a few days, limiting their utility in studying progressive, age-dependent neurodegeneration. An alternative to assessing larval NMJs is to examine synaptic structures in adult *Drosophila*, such as the synapses formed on the Dorsal Longitudinal Muscles (DLMs) that are required for flight[10-16]. These tripartite synapses are

structurally organized in a similar manner to mammalian synapses[17], providing a unique advantage for assessing models of neurodegenerative diseases.

Here we describe a straightforward method for analyzing the structural integrity of adult NMJs in a *Drosophila* model of neurodegeneration. Previous DLM dissection methods and studies have emphasized the importance of preserving muscle tissue for a variety of applications[18-23]. Our protocol provides a comprehensive method to preserve both neuronal and muscle tissue to investigate neurodegenerative diseases. Another major component of studying these diseases is the ability to understand neuronal loss in an age dependent manner. Previous work provides a critical and in-depth understanding of how the DLM NMJs are formed during metamorphosis into early adulthood[11,12,14-16,24]. Our protocol establishes a method to build upon this work to investigate DLM NMJs in an age-dependent manner in aging and neurodegenerative diseases.

PROTOCOL:

1. Generation of transgenic flies

1.1. To generate transgenic flies for this experiment, collect OK371-Gal4[25] virgin female flies and males of UAS-TDP-43^{M337V} [26] (**Figure 1A**) by anesthetizing flies with CO_2 on a pad to sort.

1.2. Sort anesthetized flies into vials with standard Drosophila media for the cross. Place labeled vials at 25 °C for the next generation to emerge.

NOTE: Clear the adults from the vials before the progeny emerge to ensure the proper genotype.

1.3. Once progeny emerge, collect the transgenic flies into vials and sort by sex to begin aging for experimental conditions.

1.4. Once flies are collected, transfer flies to fresh food every 2 days until the flies are 21 days old.

2. Dissection prep

2.1. To prepare for dissections, obtain room temperature phosphate buffered saline (1x PBS), a 10 cm dissection dish coated with a silicone elastomer, straight edge dissecting scissors, one set of blunt dissection forceps, a P200 pipette and pipette tips, 2.0 mL microcentrifuge tubes, standard office scissors, 70% ethanol, a 6 cm Petri dish, and 32% formaldehyde diluted to 4% with 1x PBS.

2.2. Label tubes for each genotype or condition and add 900 µL of 1x PBS (room temp) and 150 µL of 32% formaldehyde to each tube. Obtain a 200 µL pipette tip and cut off about 1/5th, or approximately 10 mm of the tip in order to transport thorax samples. Wear gloves and safety glasses when preparing the 4% formaldehyde fixative.

2.3. Anesthetize 6–10 flies per group directly from the vial with CO_2 and submerge flies into a 6 cm Petri dish with 70% ethanol. Press flies down into the ethanol using a paint brush to ensure that specimens are fully submerged. This will remove the layer of oil on the outer cuticle.

3. Thorax isolation and fixation

3.1. Before dissecting each specimen, add approximately 7–10 mL of 1x PBS to the dissection dish coated with silicone elastomer. This volume should ensure that the tissue samples are completely submerged.

3.2. Transfer one fly to the dissection dish from the 70% ethanol using blunt forceps and grasping either the wings or the legs.

3.3. Focus the sample in the dissection dish under a dissecting microscope. Next submerge the sample in 1x PBS, and carefully remove the wings using blunt Dumont #5 fine forceps.

3.4. Using Vannas straight edge spring dissection scissors, remove the legs by creating a small incision in the ventral side of the cuticle. In step 3.8, this incision will allow the formaldehyde to penetrate the tissue.

3.5. Take the scissors in one hand and hold the forceps in the other to position the fly ventral side up. While holding the specimen in place with the blunt forceps, remove the head and abdomen with the dissection scissors.

3.6. Transfer the isolated thorax using the modified pipette tip into the labeled tube, from step 3.2.

NOTE: Set the pipette to 40 µL to avoid adding extra 1x PBS to the fixative.

3.7. Repeat steps 3.2–3.6 above for each specimen.

3.8. Fix samples for 30 min at room temperature.

3.9. Remove the fix using a Pasteur pipette and discard it in the proper waste container under the fume hood. Rinse samples three times with 1.5 mL of 1x PBS each using a Pasteur pipette. Complete a fourth rinse using only 750 µL and leave tissues in 1x PBS.

NOTE: At this point, tissue samples can remain at 4 °C for up to 3 days before proceeding to the next steps.

4. Flash freezing and thorax bisection

4.1. Before beginning the bisections, fill a Dewar flask with liquid nitrogen wearing proper cryo-protective gloves and safety glasses. Obtain a blade breaker, feather blades, one pair of fine forceps, ice, ice-cold 1x PBS, and cryogenic tweezers.

4.2. Prepare an ice bucket to keep 1x PBS ice-cold.

4.3. Use the blade breaker to grab the feather blade at an angle, and bend the blade in order to break off a small piece. The blade breaker can then lock the blade in position for use as a small scalpel.

NOTE: One blade should last for all groups. Switch if blade breaks or becomes dull.

4.4. Add a clean pipette tip to the P200 and remove 1/5th of the tip to transport samples.

4.5. Prepare a new microcentrifuge tube for each group and add 200 µL of 1x PBS to each tube. This second tube will be used to collect the final DLM preps.

4.6. Remove all 1x PBS from tubes using a Pasteur pipette.

4.7. Wearing proper protective equipment, submerge the tube into the liquid nitrogen flask for 10 s with the cryogenic tweezers.

NOTE: The tubes should be closed tightly to keep the tube from exploding.

4.8. Remove the tube from the liquid nitrogen and add approximately 300 µL of ice-cold 1x PBS to the samples with a Pasteur pipette. Keep the samples on ice.

4.9. Add ice-cold 1x PBS to the 10 cm dissection dish coated with silicone elastomer and dispense the first thorax with the modified 200 µL pipette.

4.10. Place the thorax ventral side up. In one hand use a dull pair of forceps to position the thorax and in the other use a fine pair of forceps to remove some of the thoracic ganglion to expose the midline of the thorax.

4.11. Use the midline of the thorax as a guide to make a shallow cut through 1/3rd of the thorax with the blade.

4.12. Remove the blade from the thorax and position the thorax at a 45° angle with the blunt forceps. Reinsert the blade and cut straight down the midline of the thorax. This will result in two hemithoraces.

4.13. Take one hemithorax at a time and remove the excess tissue under DLM muscle fiber F (**Figure 1B**), the most ventral fiber. Use the blade to carefully make one or two cuts to remove the excess tissue without damaging the DLMs.

4.14. Once isolated, transfer the hemithorax to the correct tube with 1x PBS.

4.15. Repeat steps 4.6–4.14 until 10 dissected hemithoraces per group are made.

5. Structural staining

5.1. After bisecting the thorax samples, place the tissue in blocking buffer (1x PBS with 0.1% normal goat serum, and 0.2% Triton X-100 at pH 7.4) to permeabilize the tissue and prevent non-specific staining. Use a Pasteur pipette to remove excess 1x PBS and add 1.5 mL of blocking buffer to each tube. Block tissues for at least 1 h at 4 °C.

5.2. Prepare the samples for structural staining using a fluorescently conjugated antibody, horseradish peroxidase 488 (anti-HRP-488) at a dilution of 1:200 and Phalloidin-647 at a dilution of 1:1000 in blocking buffer to stain motor neurons and muscle tissue, respectively. Make enough stain to have 150 µL per tube. Store the stain at 4 °C covered in foil or in a dark box until ready for staining.

5.4. After blocking, remove the excess blocking buffer with a glass Pasteur pipette.

5.5. Before dispensing the structural stain, vortex the stain. Add 150 µL of the stain to each tube. Place the samples in a dark box on the rotator at room temperature for 2 h.

5.6. Remove the stain and wash the tissues four times in 1.5 mL of room temp 1x PBS with 0.3% Triton X-100 for 5 min on the rotator in a dark box. The samples are now ready to mount to a slide.

6. Mounting tissue

6.1. After washing samples in PBST, prepare a microscope slide to mount tissue for staining. Prepare additional supplies including glass cover slips, a P200 pipette, 200 µL pipette tips, scissors, clear reinforcements, straight edge forceps, anti-fade fluorescent mounting media, nail polish, and a dark box to cover the slides.

6.2. Label the slide to identify the samples and clean the slide with kimwipes to ensure there are no smudges.

6.3. To ensure the hemithorax samples are not damaged by the cover slip, build a “bridge” using reinforcement labels. Take one reinforcement label, cut it in half, and place each half approximately 15 mm apart. This distance must be smaller than the width of the cover slip. Repeat this step four times to complete a “bridge” that is 5 labels high.

6.4. Take the P200 pipette and modify a tip by cutting off 1/5th of the tip to transfer the samples to the slide. Samples should be transferred onto the slide in the center of the bridge.

6.5. Take the edge of a lab wipe and remove any excess PBST. Using forceps, arrange the DLMs such that all samples are facing muscle side up and cuticle side down.

6.6. Using a standard P200 pipette tip, apply 70 µL of mounting media to the slide, avoiding air bubbles. Dispense the media in a circular pattern inside the reinforcements starting from the outside into the center.

6.7. Place a cover slip over the reinforcements.

6.8. Use nail polish to coat the outside edges around the perimeter of the coverslip. Apply generously to form a complete seal of the tissue.

6.9. Place the slide on a flat surface in the dark, allowing at least 10 min to dry and prevent photo-bleaching or loss of fluorescence. Slides may now be used for imaging immediately, or otherwise stored in a slide folder at -20 °C for later viewing.

7. Alternative: Staining with primary antibodies

NOTE: This section is **optional** and should be used directly between sections 4 and 5 if desired.

7.1. To stain tissue with primary antibodies, submerge tissue in blocking buffer for at least 1 h.

7.2. Prepare primary antibody with proper dilution in blocking buffer. At minimum, prepare enough antibody mixture to have 150 µL per group. Note that the samples are kept still. Store at 4 °C until ready for use.

7.3. Remove excess blocking buffer with a Pasteur pipette. Briefly vortex the primary antibody and add 150 µL of antibody mixture to each group and place samples at 4 °C overnight.

7.4. On the next day, remove primary antibody and wash tissue 4 times with PBST for 5 min each on a rotator.

7.5. Prepare secondary stain in blocking buffer. Add the secondary stain to the sample and then keep it a room temperature for 2 h in a dark box on the rotator.

NOTE: The secondary staining can also include HRP and phalloidin.

7.6. After the 2 h incubation, to remove the secondary stain wash tissue 4 times for 5 min with PBST and proceed to mounting.

REPRESENTATIVE RESULTS:

The generation of transgenic flies expressing human Tar-Binding Protein of 43 kDa mutant (TDP-43^{M337V}) is represented by the schematic (**Figure 1A**). This demonstrates the application of the

binary Gal4/UAS system in *Drosophila*[27]. The illustration depicts a hemithorax with six muscle fibers, A–F going from the most dorsal fiber A to the most ventral F (**Figure 1B**)[11,12]. To assess synaptic integrity, NMJs were stained with HRP and Phalloidin (**Figure 1C–E**). Motor neurons in TDP-43^{M337V} mutants (**Figure 1F**) have little to no HRP staining by Day 21, while WT (Oregon-R) remains intact (**Figure 1C**). There are no visible differences in muscle staining (**Figure 1D,G**). The changes in gross morphology observed in TDP-43^{M337V} mutants demonstrates how synaptic integrity can be implicated in a neurodegenerative disease model of amyotrophic lateral sclerosis (ALS) using the adult DLM model. In addition to structural staining, staining the DLM NMJs can also provide an assessment of synaptic integrity with presynaptic (**Figure 2A–R**) and post synaptic (**Figure 2S–X**) markers. Together, these results illustrate how this dissection protocol could be applied to studying DLM tissue in neurodegenerative diseases.

One key aspect of this dissection is the application of liquid nitrogen to flash freeze the tissue to make the bisection easier. The utility of the liquid nitrogen is demonstrated in WT flies with liquid nitrogen where muscle tissue has no damage or nicked fibers (**Figure 3A–C**). Without liquid nitrogen, the tissue can be more difficult to dissect. For example, following this protocol and skipping the liquid nitrogen flash freezing step allows the tissue to be more susceptible to damage from the dissection tools such as damaged neurons (**Figure 3D**) or damaged muscle fibers (**Figure 3E**). The application of liquid nitrogen helps to prevent tissue damage that could occur when working with DLM tissue regardless of the genotype of the specimen (**Figure 3C and 3F**).

FIGURE AND TABLE LEGENDS:

Figure 1: Progressive denervation of DLM synapses in a *Drosophila* model of ALS. (**A**) The generation of ALS transgenic flies expressing a human mutant form of Tar-Binding Protein of 43 kDa (TDP-43) are shown in the schematic. (**B**) The illustration depicts the shape and orientation of a hemithorax in an adult *Drosophila*. Using the protocol, we can observe the progressive loss of synaptic integrity of DLM NMJ synapses through structural staining of motor neurons with HRP (green) and muscle tissue with Phalloidin (magenta). Our model depicts the loss of synaptic integrity in an adult model of ALS through the generation of adult flies expressing a mutant from of human TDP-43^{M337V} in motor neurons (**Figure 1F–H**) in comparison to WT (**Figure 1C–E**) flies in muscle fiber C. Arrows highlight examples of a WT synapse (**Figure 1C**) and an example of loss of synaptic integrity. Scale bar =20 μm at 63x magnification.

Figure 2: Assessing synaptic integrity using presynaptic markers at adult NMJs. Synaptic integrity can also be assessed using presynaptic and postsynaptic markers in WT flies that are 14 days old in muscle fiber C. The presynaptic markers Synapsin (**B**), Syntaxin (**H**), and Bruchpilot (BRP) (**N**) are co-stained with HRP (**A**, **G**, **M**). The staining depicts the localization of these markers to the presynaptic terminals (**C**, **I**, **O**). At higher magnification, the images illustrate the localization of Synapsin (**E**), Syntaxin (**K**), and BRP (**Q**) with HRP (**D**, **J**, and **P**) in more detail (**Figure F**, **L**, and **R**). We also show a postsynaptic marker Glutamate Receptor III (GluRIII) (**T**) co-stained with HRP (**S**). The co-staining demonstrates the utility of these markers (**U**). At higher magnification the representative images exemplify the localization (**X**) of GluRIII (**W**) and HRP (**V**) to the postsynaptic muscle tissue and the presynaptic terminals, respectively. Scale bar for panels

A–C, G-I, M–O, S–U represent 20 µm at 63x magnification. Scale bar for panels D–F, 2J-2L, 2P-2R, and 2V-2X represent 10 µm at 63x magnification.

Figure 3: Utility of liquid nitrogen for DLM dissections. To demonstrate the utility of liquid nitrogen for the DLM dissections, we show a comparison of day 21 WT flies with and without liquid nitrogen from muscle fiber C. With liquid nitrogen, Phalloidin (**B**) remains intact and does not compromise the HRP staining (**A**, **C**). Without liquid nitrogen, muscle tissue becomes stringy and difficult to bisect (**E**) and HRP staining (**D**, **F**) becomes compromised due to technical error. White arrows show an area of no muscle damage in with liquid nitrogen (**B**) and damaged muscle tissue (**E**). Scale bar = 20 µm at 63x magnification.

DISCUSSION:

Using the methods described in this protocol, we provide a straightforward approach for dissection of the DLM tissue and demonstrate how this can be applied to assess synaptic integrity through structural staining and synaptic markers in adult *Drosophila.* One critical step in the protocol that makes the DLM tissue easier to dissect is the flash freezing with liquid nitrogen. Without this step, the tissue is less firm and more difficult to cut precisely as observed in **Figure 3**. This protocol builds upon previous dissection methods to allow the preservation of both motor neurons and muscle tissue[18-23]. One limitation of this protocol is that when making the cut down the midline for the bisection, it can be difficult to get two clean preps per thorax. One way to ensure at least one hemithorax per fly, you can purposely cut off to one side of the thorax to get one clean prep. With this modification, one may also need to remove additional excess tissue from the cut to clean up the sample with the blade breaker. For those new to this technique, with continued practice, accuracy of the bisection will increase.

The method described here allows researchers to easily assess structural integrity of adult DLM NMJs at any time throughout their lifespan. A major advantage of this protocol is the ability to access synaptic integrity in neurodegenerative disease models by using synaptic markers. We demonstrate that this application can help visualize changes in gross morphology with structural staining (**Figure 1C–H**). Additionally, synaptic integrity can be assessed with staining of presynaptic markers including but not limited to Synapsin[28] (**Figure 2A–F**), Syntaxin[29] (**Figure 2G–L**) and BRP[30] (**Figure 2M–R**). The postsynaptic muscle tissue can also be assessed using the Glutamate Receptor III subunit antibody[31] (**Figure 2S–X**), demonstrating the utility of this protocol.

Researchers can also utilize this dissection method to complement functional data to comprehensively examine the structural integrity of synapses associated with a wide variety of diseases. These synapses also allow for functional analysis through electrophysiological recordings[32-34] and the flight assay[10]. This protocol can also provide ease of access to the tissue for many applications and assays. Future studies, for example, could use this protocol to quantify synaptic changes through quantification of the density and number of synapses[15,16]. While the protocol described here specifically examines synaptic integrity of motor neurons, complementary protocols for assessing muscle cell loss can also be performed with this dissection using TUNEL staining[35]. To examine neuronal loss, dissection of the thoracic ganglion[36]

could also be used with TUNEL staining. We expect that the dissection described here will have more applications to future studies assessing age-related pathologies as well as neurodegenerative diseases.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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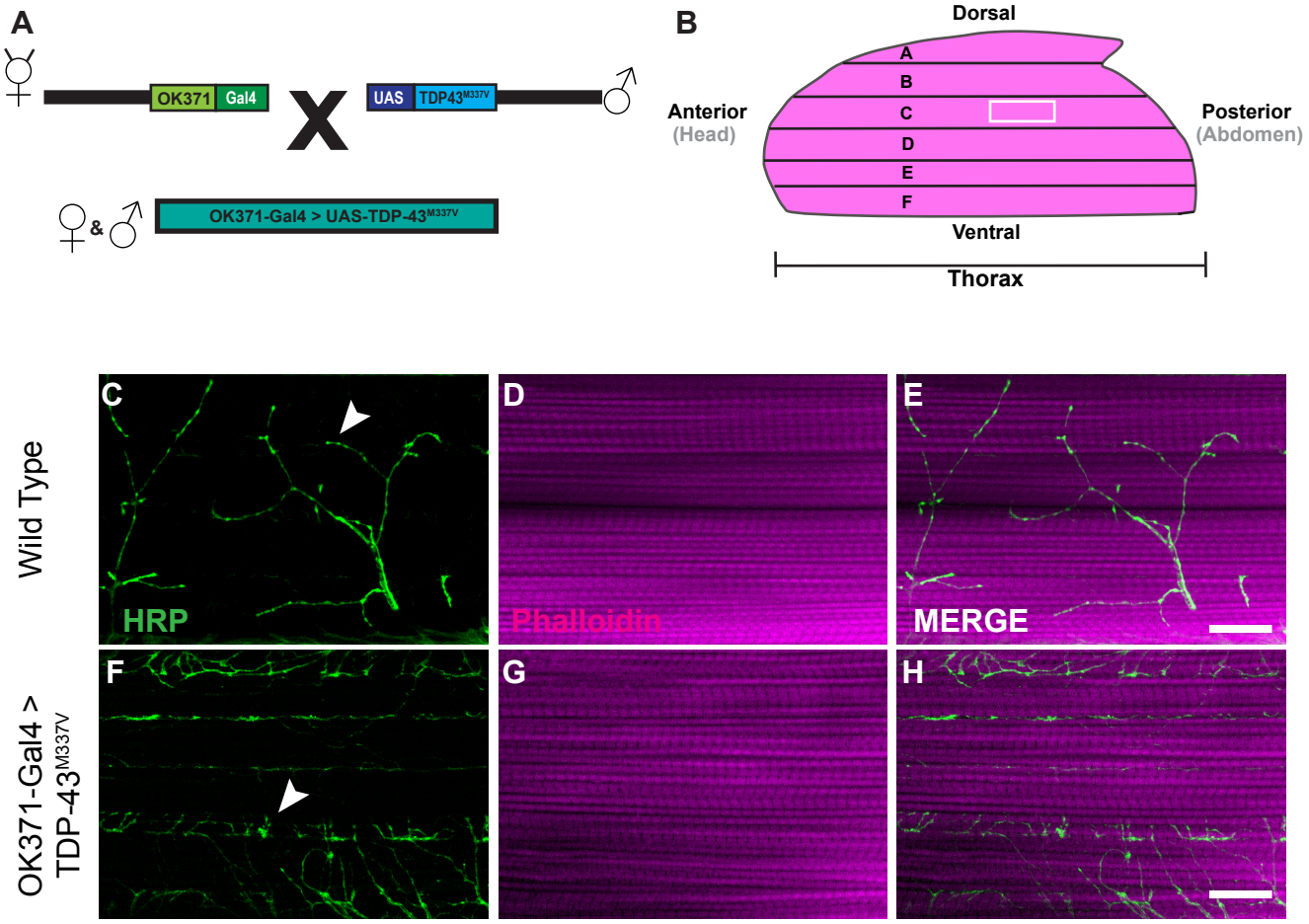

Figure 2

Click here to access/download;Figure;Jove Figure 2 Synaptic Markers with Insets 3-29 JMS (1).ai

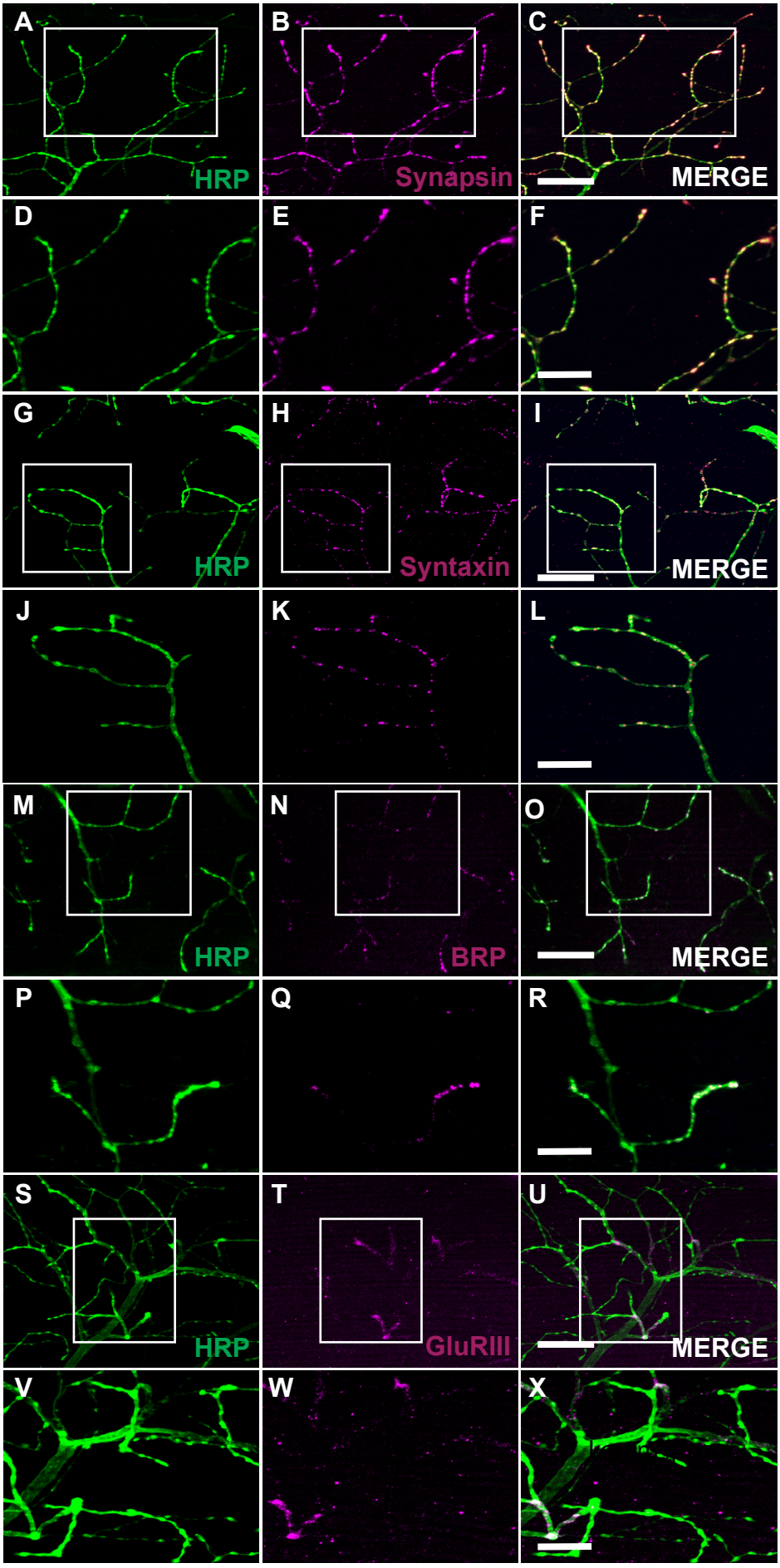

Figure 3

Click here to access/download;Figure;Jove Figure 3 Dissection w& without liquid nitrogen (1).ai

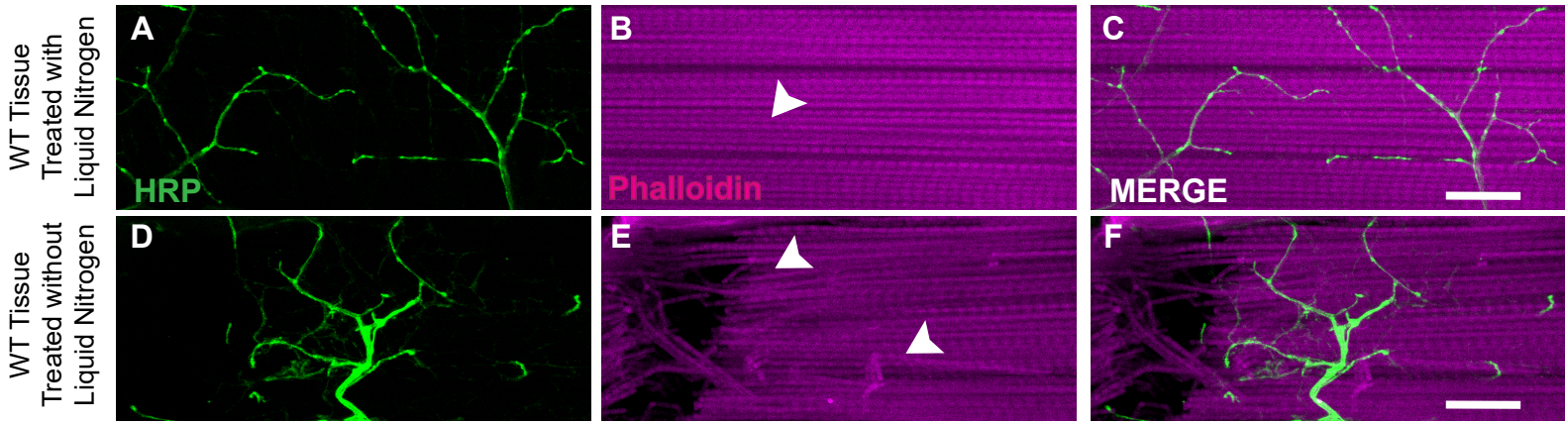

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
32% Formaldehyde	Electron Microscopy Sciences	15714	Tissue preservation
Alexa Fluor 568 goat anti mouse	Fisher Scientific	A11031	Labels primary antibodies. Used at 1:200 concentration.
Alexa Fluor 568 goat anti rabbit	Fisher Scientific	A11036	Labels primary antibodies. Used at 1:200 concentration.
anti- Bruchpilot (BRP) antibody	Developmental Studies Hybridoma Bank	NC82	Stains the active zones in presynaptic neurons. Used at 1:25 concentration.
anti-GluRIII antibody	Gift from Aaron DiAntonio	N/A	Labels glutamate receptor subunits. Used at 1:1000 concentration.
anti-Synapsin antibody	Developmental Studies Hybridoma Bank	3C11	Labels the synaptic protein synapsin. Used at 1:50 concentration.
anti-Syntaxin antibody	Developmental Studies Hybridoma Bank	8C3	labels the synaptic protein syntaxin. Used at 1:10 concentration.
BenchRocker	Genesee Scientific	31-302	Rotating samples during staining
Blade Breaker	Fine Science Tools	10053-09	Used for holding feather blade

cover slips	Fisher Scientific	12548A	For mounting tissue
cryogenic gloves	VWR	97008-198	protect hands from liquid nitrogen
cryogenic tweezers	VWR	82027-432	Hold 2.0 mL tube in liquid nitrogen
dewar flask-1900 mL	Thomas Scientific	5028M54	Hold liquid nitrogen
Feather Blades	Electron Microscopy Sciences	72002-01	Scalpel Blades
Fine Forecps x 2	Fine Science Tools	11252-20	One fine pair for Clearing midline of thorax. The other pair can be dulled using a sharpening stone.
FITC-conjugated anti HRP	Jackson Laboratories	123-545-021	Stains Motor Neurons. Used at 1:100 concentration
freezer box (Black)	Fisher Scientific	14100F	Protects samples from light
glass pasteur pipettes	VWR	14637-010	Used to transfer samples
glass slides	Fisher Scientific	12550143	For mounting tissue

mounting media (vectashield) anti-fade	VWR	101098-042	Mounting media retains fluorescent signaling
nail polish	Electron Microscopy Sciences	72180	Seals microscope slides
normal goat serum	Fisher Scientific	PCN5000	Prevents non-specific binding of antibodies
paint brush	Genesee Scientific	59-204	Transferring flies
PBS	Fisher Scientific	10-010-023	Saline solution for dissecting and staining
Phalloidin 647	Abcam	AB176759	Stains F-Actin in muscle Tissue. Used at 1:1000 concentration
plastic petri dish (100 mm)	VWR	25373-100	Dissection dish
reinforcement labels	W.B. Mason	AVE05722	Provides support for glass coverslip over the mounted tissue
sharpening block	Grainger	1RDF5	Keeping fine forceps sharp and also dulling separate pair
slide folder	VWR	10126-326	Sample storage

standard office scissors	W.B. Mason	ACM40618	Cutting reinforcement labels
Sylgard 184	Electron Microscopy Sciences	24236-10	Coating for dissection dish
Triton-X-100	Electron Microscopy Sciences	22140	Helps to permeabilize tissue
Vannas Disssection Sissors	Fine Science Tools	1500-00	Ued for removing fly legs and making an incision on thorax

We would like to thank the editors and reviewers for providing helpful comments regarding our initial submission. These comments have resulted in several additions and changes to our revised manuscript, and we feel that they enhance the clarity and quality of the protocol. Please find below our point-by-point response to each of these comments, and where these updates can now be fine in the revised manuscript.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have checked the manuscript for spelling and grammatical accuracy.

• Please include a minimum of 6 keywords/phrases.

We have increased the number of keywords to 6. These can be found on Page 1.

• **Introduction:** Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies ; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

The introduction has now been expanded to include specific advantages over pre-existing methods, and information to help determine if readers will find this protocol helpful for their research.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

1) Line 68: What type of forceps?

We now list the specific type of forceps used. Details can also be found in the updated materials list.

2) Line 70: what kind of scissors?

We now list the specific type of forceps used. Details can also be found in the updated materials list.

3) Line 74: Under a dissecting scope?

We have specified that this action takes place under a microscope.

4) 78: What tool is used for transfer?

We clarify that a pipettor is used for this action.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. Please add a one-line space after each protocol step.

We adjusted the format accordingly.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next. Currently the highlighting is disjoint.

2) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

We have highlighted approximately 2.5 pages of protocol text.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We adjusted the discussion accordingly.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Sylgard.

We changed the specific product name "Sylgard" to "silicone elastomer". The specific item that we used is now mentioned in the materials list.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be

copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

We confirm that all figures are original and not published elsewhere.

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

This is a very useful technique to study age-dependent neurodegeneration, especial in the motor system, such as ALS. It could also be useful to study the pace of Glutamatergic synaptic loss in other fly models focusing on dementia. The description of the protocol is quite thorough.

Minor Concerns:

In the sample experimental data sections: to show the synaptic integrity, it will be more appropriate to use both pre- and post-synaptic markers, instead of using only presynaptic markers as the authors did. Antibodies against DLG or any other Glutamate receptors can be

used for such purposes. Labeling both sides of the synapses provide more insight into the nature of synaptic loss.

We agree with the reviewer that labeling both sides of the synapse provides greater insight into the nature of synaptic loss in this protocol. Our updated Figure 2 now includes the presynaptic markers Bruchpilot, Synapsin, and Syntaxin, along with the postsynaptic marker Glutamate Receptor subunit III (GluRIII). Discs Large (DLG), unfortunately, does not stain at these synapses because the DLM synapses lack a Subsynaptic Reticulum.

Reviewer #2:

Manuscript Summary:

The manuscript by Sidisky and Babcock (JoVE61363) describes a method for visualizing synaptic degeneration in adult Drosophila. The method is a great addition to the Drosophila toolbox, and the representative results are a great illustration of the methods utility. In general, the method is described in sufficient detail for a novice to carry it out. However, there are numerous places were additional information would be helpful. There are a lot of suggested changes below, but they are all minor and should be easy to address.

Major Concerns:

None

Minor Concerns:

1. The materials list is incomplete. It is missing the following items: mounting media, slide folder, glass cover slips, straight edge dissection scissors, razor blades, glass Pasteur pipettes, sylgard dish, blunt forceps, straight edged forceps, reinforcement labels, paint brush, rotator, normal goat serum, Triton, anti-fade mounting media, nail polish, dark box, PBS, PBST, and 32% formaldehyde.

Thank you for pointing this out. We have updated our materials list to include these items.

2. Lines 18-19, most people will not know what DLM and NMJ stand for. If the word limit allows, write out the acronyms.

We have written out Dorsal Longitudinal Muscles and Neuromuscular Junction prior to listing the acronyms for clarification.

3. Line 37, I assume that the word "transient" is meant to convey the fact that the larval stage of development lasts a short time, but it could be interpreted by the reader as meaning that the larval structures, not the larvae themselves, last a short time. Clarifying this point would be helpful.

We agree with the potential ambiguity of our previous statement. We now clarify that “transient” refers to the fact that the third larval instar only lasts a few days, making this developmental stage difficult to assess progressive, age-dependent phenotypes.

4. Line 41, change structural to structurally.

This change was made.

5. Lines 48-50, what is the difference between "straight edge dissecting scissors" (line 48) and "dissection scissors" (lines 49 and 50)?

We now clarify that the scissor mentioned in lines 49 and 50 refer to standard office scissors and not dissection scissors. These items have also been updated in our materials list.

6. Line 50, is the formaldehyde diluted from 100% to 32% or is it purchased at 32%?

We have clarified that the formaldehyde is purchased at 32%. The specific item has also been updated in our materials list.

7. Line 54, it may be more helpful to say how many millimeters to cut off the pipette tip rather than saying to cut off 1/5th of the tip.

For clarity, we have added the phrase “approximately 10 mm” this step. We are also highlighting this step so that it will be shown directly in the protocol.

8. Line 55, I assume that "fixative" is being used as jargon for 32% formaldehyde. If so, this should be clarified. In the same vein, also clarify the term "fix" that is used as a verb in lines such as in lines 83 and 85.

We have now clarified that 32% Formaldehyde is the “fix”or “fixative” that is used upon its first use.

9. Lines 62-63, it would be more helpful to specify the volume of PBS, rather than say "enough volume".

We have included the phrase “approximately 7-10 mls” to describe the volume of PBS. The exact amount of PBS may vary slightly depending on the precise amount of sylgard added to the dish originally.

10. Line 71, to clarify this instruction, change it to "In step 8, this incision…".

We have included a statement mentioning what this incision will be used for in order to provide further clarification.

11. Line 78, to clarify this instruction, change it to "…the labeled tube, from step 2".

This change has been made

12. Line 94, it may be helpful to describe the blade breaker. Many readers will not be familiar with this tool.

We have added a brief description of how the blade breaker works. We have also highlighted this step in the text so that this new description will be part of the filmed protocol.

13. Line 108, you may want to add a note indicating that the tubes should closed tightly before submerging them in liquid nitrogen. If liquid nitrogen leaks into the tubes, they will explode.

A note mentioning that tubes should be closed tightly has been added to this step.

14. Line 117, what are dull forceps? Are they just forceps whose tips are worn down by heavy use or are they purposely dulled in some way?

Yes, these forceps are purposely dulled using a sharpening stone. We have updated our materials list to include these items.

15. Line 122, should "scalpel" be "blade"?

We have changed the word to “blade”.

16. Line 129, it would be helpful to provide a detailed description of what DLM muscle fiber F looks like.

The current text describes muscle fiber F as being the ventral-most muscle in this group. We have also updated Figure 1B to identify the location of this muscle. This portion of the text will also be filmed to show the viewer this location under the microscope.

17. Line 135, I assume that "preps" is jargon for a dissected hemithorax. This should be clarified.

We have clarified that “preps” refers to the dissected tissue samples.

18. Line 139, specify the type of Triton, such as Triton X-100.

Yes, we are using Triton X-100. We have clarified this in the text, and also updated our materials list to include this specific item.

19. Line 152, splitting the sentence into two sentences would improve clarity. Change "…vortex the stain and add…" to "…vortex the stain. Add…".

Thank you for this recommendation. We agree that splitting this into two sentences makes this step easier to understand.

20. Line 156, indicate how much Triton is in PBST.

We have specified that PBST includes 0.3% Triton X-100.

21. Line 175, be more specific about what you mean by "some".

We have changed “some” into the phrase “any excess PBST”. We will also make sure that this section is filmed for clarity.

22. Line 188, should -20oF be -20oC?

Indeed. Thank you for pointing this out.

23. For the figure legends, it would be helpful to the reader to include a more detailed description of the data. For example, describe what the parts of the neurons in Figures 1A and D and the muscle fibers in Figures 1B and C.

We have added a description to specify synaptic structures and axon terminals, and this is now included in the updated figure legends.

24. In the materials list, fix the spelling of forceps.

This spelling has been corrected.

Reviewer #3:

Manuscript Summary:

The manuscript by Sidisky and Babcock presents a protocol for analysing adult neuromuscular junctions from DLM flight muscles. Their main contribution is a protocol for dissecting intact

DLMs. However, the protocol that they describe is quite complex compared to the simple, dorso-ventral medial sagittal cut of the fly thorax that quickly yields two hemi-thoraces that can be directly fixed and processed.

Major Concerns:

It is not clear that the increase in resources, specially time and effort, associated with the proposed protocol represents a reasonable advantage in comparison to the simple bisection. This needs a more convincing justification. Furthermore, the extra freezing step might alter other synaptic antigens that are usually analysed when studying synaptic integrity (Dlg, GluRs, Futsch, Fasciclins, cac, csp, synaptotagmin, synapsin, between others), glial integrity or general NMJ morphology. The figures do not address these issues. A clear comparison of NMJ structure and immunoreactivity between both simple and complex (proposed) protocols would be necessary. These should clearly justify the advantages of the proposed procedures.

A major advantage of this protocol is maintaining the structural integrity of the dissected tissue. Because the DLMs are fibrillar muscles, they are prone to tear off and form fibrils upon cutting. This makes it very difficult to reliably assess NMJ structures near the surface of the muscles. This often results in arbitrarily choosing sections of the tissue without as much damage. To clarify this issue, we have included a sample image of the dissected tissue using the simple bisection compared to our protocol described here. These images can be seen in Figure 3.

We also include a number of sample images showing presynaptic and postsynaptic markers using our protocol showing the immunoreactivity of these markers. These images can be seen in Figure 2.

Minor Concerns:

*The title is quite misleading and ambitious. There is no mention to quantitative methods for measuring synaptic degeneration or visual examples

Our title specifically describes a visual assessment of synaptic degeneration, and we also include an example of this using a Drosophila model of ALS. Quantitative methods for assessing NMJ structure have been previously described, and we have added a statement mentioning these references in the discussion.

(Figure 1 shows axonal degeneration in a model of ALS; axonal and synaptic degeneration are distinct pathological processes). In addition, the title seems to imply that synaptic degeneration and neurodegeneration (neuronal death/loss) would be simultaneously analysed, which is not the case.

We clarify that our current protocol does not directly monitor cell death in neurons. In our discussion, we mention that neuronal cell death can be analyzed separately using TUNEL or caspase staining, but those are distinct protocols from what we describe here.

Finally, "degeneration" involves age-dependent phenotypic progression, but procedures for managing flies to control age (and other variables) are not described.

We mention in part 1 of the protocol that flies can be aged by transferring them to fresh food every 2 days..

*In line 47, step 1 of the protocol: why 2 types of dissecting scissors?

Thank you for pointing this out. Please see our response to this comment from Reviewer 2.

*Line 106: "Remove all PBS from with washed samples using a Pasteur pipette", should say "remove form tubes"?

This change has been made.

*Figures 1 and 2: what is the age of the flies?

This information has been added to the figure legends.

*There may be more recent references describing updated DLM electrophysiology protocols.

We have included additional references for assessing DLM function using electrophysiology.

Reviewer #4:

Manuscript Summary:

This protocol article presents a detailed technique for examining synaptic structure and integrity of the neuromuscular junction in adult Drosophila melanogaster. The technique is innovative and could be used to assess synaptic morphology in age-dependent manner and in various Drosophila models of neurodegenerative diseases.

The protocol is well written, however, I have few minor recommendations, which, if addressed, to my opinion, will improve the quality of the manuscript.

Major Concerns:

n/a

Minor Concerns:

Abstract: the authors could include that synapses and muscles of the dissected DLM preparation can be labeled with fluorescently labeled antibodies and/or fluorescent probes. These steps are part of the protocol and acan be included in the abstract.

We agree that adding such a statement in the abstract describes the utility of this protocol to the reader early on. This statement can now be found in the abstract.

Line 41: Do the authors mean structurally?

Yes, this change has been made.

Protocol

Prior the first step of the protocol, to my opinion, the authors should include a step/part describing how they obtain the flies of the desired genotype (e.g. OK371-Gal4>UAS-TDP43M337V). Because results for these flies are presented in the Representative results section, to maximize reproducibility of the method, a step describing the cross can be helpful.

We have added a description of the genetic cross used to generate the genotypes used in our protocol. This can be found in Figure 1A.

Line 47 and remaining of the manuscript: The authors should state what concentration of PBS they are using (e.g. PBS1X). In many cases concentrated stock solutions of PBS can be purchased (e.g. 10X), and it will be helpful for researchers to have this information.

We have clarified that the PBS used in this protocol is 1XPBS.

Line 57: Are the flies anesthetized on a fly pad or in the vial and directly taken from the vial? Please, specify.

We have clarified that flies are anesthetized in the vial directly.

Line 65: Is the fly transferred from the EtOH 70% dish to the dissection dish? Please, specify.

Yes, we have clarified that the flies are transferred from the ethanol to the dissection dish.

Line 145-146: Please, specify that this is an antibody (anti-HRP). Also, please specify the dilutions used for both the anti-HRP antibody and the Phalloidin stain. I would also recommend providing the full name of the Phalloidin stain here and in the reagents table (e.g. Phalloidin-Alexa 647).

We have clarified in the text that the HRP is an antibody used to stain neuronal membranes. We have also included the full name of the specific product used in our Materials List.

Line 152: is there a centrifugation step after vortexing the structural stain? if yes, please specify.

No there is not a centrifugation step.

Line 155: Do the authors mean Phosphate Buffered Saline with Triton 0.3%? If yes, I recommend moving 0.3% after Triton.

We have adjusted the wording to clarify that the PBS includes 0.3% Triton X-100.

Line 160: The authors can add "Prepare" before additional. (“prepare additional materials that include…)

This change has been made.

Line 197: is there a centrifugation step (quick spin down) after vortexing? If yes, please, specify. Also, please specify if the samples are kept still or shaking.

No there is not a centrifugation step. We have also clarified that at this stage the samples are kept still.

Line 203: Please, specify whether samples are still or shaking.

We have clarified that at this stage the samples are gently shaking on a rotator. We have also added the rotator to our updated Materials List.

Line 210: The authors could be a little more specific and provide the exact genotype of the TDP-43 mutants, which matches the genotype in Figure 1.

We have added a description of the precise genotypes used to Figure 1A.

Line 216: Please state which pre-synaptic markers can be used.

We have updated Figure 2 to include Brp, Synapsin, Syntaxin, and GluRIII.

Figures: The authors can possibly include a diagram showing the Drosophila thorax and then circle the region of the thorax that is being examined and for which representative pictures are shown. This will help to better visualize the anatomical region examined.

Thank you for this suggestion. Figure 1B now includes a diagram of the thorax illustrating both the overall location of the DLMs as well as a box showing the location over which the representative images are taken.

Table: Please add anti-Bruchpilot.

This change has been made.