Video Article

Removal of *Drosophila* Muscle Tissue from Larval Fillets for Immunofluorescence Analysis of Sensory Neurons and Epidermal Cells

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URL: https://www.jove.com/video/54670
DOI: doi:10.3791/54670

Keywords: Neuroscience, Issue 117, *Drosophila*, larva, fillet, dissection, muscle, dendritic arborization neuron, epidermis

Date Published: 11/2/2016

Citation: Tenenbaum, C.M., Gavis, E.R. Removal of *Drosophila* Muscle Tissue from Larval Fillets for Immunofluorescence Analysis of Sensory Neurons and Epidermal Cells. *J. Vis. Exp.* (117), e54670, doi:10.3791/54670 (2016).

Abstract

Drosophila larval dendritic arborization (da) neurons are a popular model for investigating mechanisms of neuronal morphogenesis. Da neurons develop in communication with the epidermal cells they innervate and thus their analysis benefits from *in situ* visualization of both neuronally and epidermally expressed proteins by immunofluorescence. Traditional methods of preparing larval fillets for immunofluorescence experiments leave intact the muscle tissue that covers most of the body wall, presenting several challenges to imaging neuronal and epidermal proteins. Here we describe a method for removing muscle tissue from *Drosophila* larval fillets. This protocol enables imaging of proteins that are otherwise obscured by muscle tissue, improves signal to noise ratio, and facilitates the use of super-resolution microscopy to study da neuron development.

Video Link

The video component of this article can be found at https://www.jove.com/video/54670/

Introduction

Drosophila larval dendritic arborization (da) neurons provide a valuable model for studying neuronal development due to their amenability to genetic manipulation and the ease with which they can be imaged. These sensory neurons have been instrumental in the identification of numerous pathways that control dendrite morphogenesis[1-3].

Four classes of da neurons (class I - IV) innervate the larval epidermis. These neurons lie between the basement membrane and the epidermis, with their dendrites forming largely two-dimensional arrays[4,5]. Of the four classes, class IV da neurons have the most highly branched arbors and, like sensory neurons of other animals, the elaboration of these arbors requires intrinsic factors as well as cues from neighboring tissues, particularly the epidermis, for their development[6-9].

Studies to determine how such neuronal and extra-neuronal factors control dendrite morphogenesis benefit from the ability to detect protein expression *in situ* by immunofluorescence. The outer cuticle of the larva is impenetrable to antibodies, but this impediment is easily overcome by the preparation of larval fillets through well-established dissection methods[10,11]. However, the body wall muscle tissue that lies just interior to the basement membrane presents several challenges towards visualization of da neurons and epidermal cells. First, the muscle tissue, which lines most of the body wall, greatly obscures fluorescent signals emanating from neuronal or epidermal tissue. This substantially reduces the signal to noise ratio in the sample. Second, many relevant proteins may be expressed in the muscle tissue as well as in the neurons or epidermis. This muscle-derived fluorescence signal is likely to further obscure detection of a fluorescence signal from the neuron or epidermis. Finally, advances in microscopy technologies now permit imaging of samples at sub-diffraction resolution and may be especially helpful in discerning the localization of proteins that are expressed in neurons and surrounding epidermal cells[12,13]. However, imaging via super-resolution microscopy benefits from a strong signal to noise ratio and close proximity of the sample to the coverslip. In addition to reducing the signal to noise ratio, the larval body wall muscle distances da neurons from the coverslip, thereby limiting the improved image resolution that can be achieved with super-resolution microscopy methods. Besides challenges for immunofluorescence analysis, muscle tissue presents a barrier to electrophysiological recording from sensory neurons in the larval body wall. Its removal therefore benefits neurophysiological manipulation of sensory neurons[14].

Here a method for manual removal of *Drosophila* larval muscle tissue is described. We demonstrate that our protocol permits immunofluorescence imaging of proteins that are otherwise obscured by muscle tissue, improves the signal to noise ratio for visualization of class IV da neurons, and enables the use of super-resolution microscopy to better discriminate spatial relationships of proteins and cellular structures in da neurons and the epidermis.

Protocol

Note: The procedure for muscle removal (**Figure 1**) is a modification of previously described methods for preparing larval fillets. The steps that precede and follow muscle removal are outlined briefly and the reader is referred to previous work [10, 11] for more detailed descriptions.

1. Dissect Larva in Cold Saline

1. Prepare a working dilution of cold HL3.1 saline[15] or cold Ca^{2+}-free HL3.1 saline[11] (**Table 1**). Place the larva in a silicone elastomer dish with just enough cold saline to cover the bottom of the dish.
   NOTE: See Discussion regarding the choice of saline.

1x HL3.1 saline (pH 7.2)
5 mM HEPES
70 mM NaCl
5 mM KCl
1.5 mM $CaCl_2$ (omit for Ca^{2+}-free saline)
4 mM $MgCl_2$
10 mM $NaHCO_3$
5 mM trehalose
115 mM sucrose
Filter sterilize and store at 4 °C
Note: Composition mimics insect hemolymph

Table 1. Composition of Cold Saline.

2. Position the larva with the ventral side up. The ventral surface of the larva can be identified by the abdominal dentical belts and the dorsal side by the primary larval tracheal tubes running from anterior to posterior. Stretch the larva in the anterior-posterior direction and pin the head and tail to a silicone elastomer dissecting dish using insect pins. Use fine dissecting scissors to cut along the ventral midline, beginning at one end and progressing toward the other.
   NOTE: This orientation preserves the commonly studied dorsal cluster of da neurons.
3. After the larva is cut open, pin the four corners to the dissecting dish as though opening a book. Use forceps to grab and remove internal organs including the CNS, gut, and trachea. Adjust insect pins so that the fillet is taut but not maximally stretched.
   NOTE: Muscles are anchored to the body wall at segmental boundaries. Although muscles cover most of the body wall, they are absent in a narrow region near the dorsal midline.

2. Muscle Removal

1. Locate the dorsal midline of the larva, where muscle tissue is absent. Position a single forceps prong such that it can be inserted underneath the muscle tissue in the flattest possible orientation.
2. Starting at the dorsal midline, near the anterior boundary of the segment, carefully slide the forceps prong between the muscle and epidermis, taking care to minimize contact between the forceps and epidermis.
3. Pull the forceps upwards to break the attachment of the muscle to the body wall at one anchor point. Repeat this process for the remaining hemi-segment(s) of interest.
   NOTE: This protocol optimizes preservation of the posterior of each da neuron dendrite field. To preserve the anterior part of the dendrite field, it is best to insert the forceps prong at the posterior end of each segment.
4. Re-adjust insect pins such that the larval fillet is maximally stretched in all directions.
5. Fix the fillet while it is still pinned to the dissecting dish using cold, freshly prepared 4% formaldehyde in PBS for 25 min.
6. Rinse 5 times in PBS.
7. Use forceps to carefully pull away muscle tissue from the remaining anchor points, taking care to minimize contact with the epidermis.
8. Unpin and remove the fillet from the dissecting dish to a 1.5 ml microcentrifuge tube. Perform all subsequent washing, blocking, and immunofluorescence staining steps, as previously described[11].

3. Mount the Larval Fillet

1. First remove the head and tail using fine dissecting scissors in order to make the sample as flat as possible. Mount the fillet on a coverslip in antifade mountant with the inner surface of the fillet facing the coverslip.
2. Place a microscope slide on the coverslip and press gently to disperse the mounting medium. Flip the microscope slide over and seal the coverslip on the slide using nail polish. Slides can be stored at -20 °C for at least one month.

Representative Results

We demonstrate the utility of the muscle removal procedure for improving signal to noise ratio in immunofluorescence experiments to co-visualize the septate junction proteins Coracle (Cora) and Discs-large (Dlg) together with class IV da neurons labeled with the membrane marker CD4-tdTomato.

Cora has been previously used to identify tracts where da neuron dendrites are enclosed by epidermal cells and is one of many identified epidermal factors that have been studied in association with the morphogenesis and function of da neuron dendrites[4,6-9,16]. Immunostaining with anti-DsRed to detect the neurons (red) and anti-Cora antibody (green) was performed on larval fillets with muscle removed (muscle-off) and with muscle intact (muscle-on). For each condition, the posterior dendrite field of the class IV da neuron was imaged using a laser scanning confocal microscope. Images were obtained using identical imaging parameters for both conditions. We additionally imaged muscle-on fillets using increased laser power to compensate for interference from the muscle tissue.

In muscle-off samples, Cora could be clearly detected at epidermal cell boundaries, in intermittent tracts along class IV da neuron dendrites, and outlining the class IV da neuron soma (**Figure 2C**). In contrast, its localization to these domains was largely obscured in muscle-on samples, even when laser power was increased (**Figure 2A-2B**). In muscle-on samples, Cora was primarily visualized in muscle, trachea, and at the neuromuscular junction (NMJ), which become separated from the body wall as a result of the muscle removal procedure. The fluorescence signal emanating from these tissues obscured most of the Cora that localizes to epidermal cell boundaries and to dendrite tracts, except for in the narrow strip of the body wall near the dorsal midline, where muscle is absent. Cora that outlines the class IV da neuron soma was not visualized in muscle-on samples (**Figure 2A-2B**). Visualization of epidermally expressed proteins that also have high muscle expression, such as Dlg, is particularly problematic. In muscle-on samples, the distribution of Dlg in the epidermis was almost completely obscured by fluorescence from the muscle and NMJ (**Figure 2D**). After muscle removal, Dlg is readily detected at epidermal cell boundaries, in intermittent tracts along da neuron dendrites, and outlining the class IV da neuron soma (**Figure 2E**). Thus, muscle removal enables visualization of epidermally and neuronally expressed proteins otherwise obscured by muscle and associated tissues.

In addition, it was observed that the fluorescence intensity of the class IV da neuron marker appeared reduced in muscle-on fillets as compared to muscle-off fillets when imaging parameters were held constant between the two sample preparations (**Figure 2A, 2C**). With increased laser power, the apparent fluorescence intensity of the class IV da neuron marker could be matched to muscle-off samples but background noise was increased (**Figure 2B, 2C**). Therefore, muscle removal improves the signal to noise ratio in our samples.

Lastly, we tested whether the improvements gained by removing muscle tissue could be applied to imaging of class IV da neurons at sub-diffraction resolution. To do this, 3D structured illumination microscopy (SIM) imaging was performed, which achieves approximately 120 nm lateral resolution[13]. As with confocal imaging, we compared muscle-on and muscle-off fillets immunostained for Cora and class IV da neurons, first under identical imaging conditions and then after optimizing laser power, exposure time, and image processing for muscle-on samples. Similarly to confocal microscopy, a substantially reduced signal to noise ratio in muscle-on was observed as compared to muscle-off fillets (**Figure 3A-3C**). Additionally, image reconstruction appeared sharper in muscle-off samples. Compared to muscle-on samples, fewer apparent artifacts were observed and Cora dendrite tracts were more readily resolved (**Figure 3B, 3C**). Dendrite membranes could be measured at approximately 114 nm in width in muscle-off fillets but appeared to be 272 nm wide in muscle-on fillets (**Figure 3D, 3E**). Thus, our protocol enables the application of super-resolution microscopy to visualization of class IV da neurons.

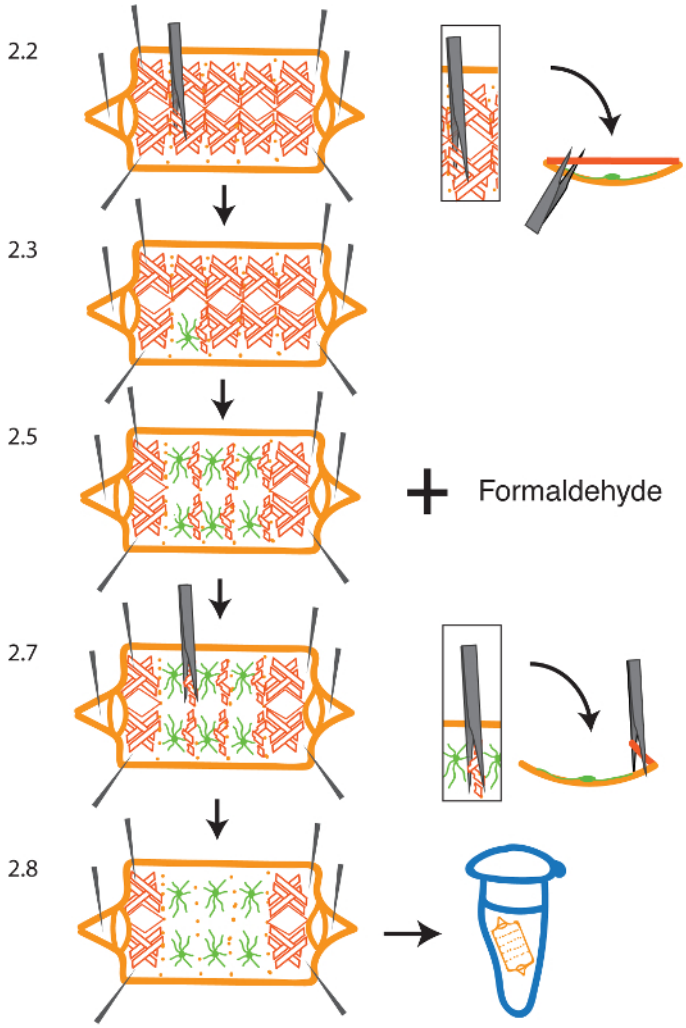

Figure 1. Overview of Muscle Removal Procedure. A larva is dissected and pinned to a silicone elastomer dissecting dish. To remove muscle tissue, one forceps prong is inserted between the muscle and epidermal cell layer, starting from the dorsal midline near a segment boundary (2.2). The forceps is pulled upwards to break the attachment between the muscle and body wall (2.3). This is repeated for segments of interest and then the larva is fixed in formaldehyde (2.5). After fixation, forceps are used to separate the remaining muscle tissue from the body wall (2.7 - 2.8) The larval cuticle and epidermis are depicted in orange, class IV da neurons in green, and muscles in red. Insets in (2.2) and (2.7) represent cross-sectional views of a single larval hemi-segment. Please click here to view a larger version of this figure.

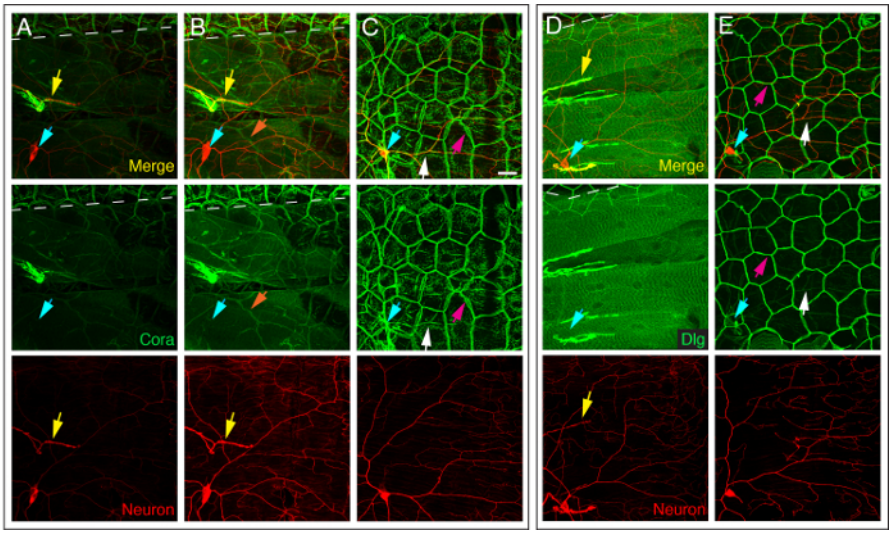

Figure 2. Muscle Removal Improves Confocal Imaging of da Neurons and the Epidermis. Immunofluorescence of Cora (green; **A-C**) or Dlg (green; **D-E**) was detected with muscle tissue (**A, B, D**) and without muscle tissue (**C, E**). Class IV da neurons were labeled using the *GAL4*^{477} driver to express *UAS-CD4:tdTomato* and detected with an anti-dsRed antibody (red). Muscle-on and muscle-off samples labeled for Cora were first imaged with a confocal microscope under identical conditions (**A, C**) and then with increased laser power to compensate for muscle interference (**B**). Muscle-on and muscle-off samples labeled for Dlg were imaged under individually optimized conditions. All images are confocal Z-projections. The middle (green) and bottom (red) panels show individual channels; the top panels are the merged channels. The boundary of the muscle tissue (dashed lines), the class IV da neuron soma (cyan arrows), Cora and Dlg dendrite tracts (white arrows), epidermal cell boundaries (magenta arrows), NMJ (yellow arrows), and trachea (orange arrows) are indicated. Scale bar = 20 µm. Please click here to view a larger version of this figure.

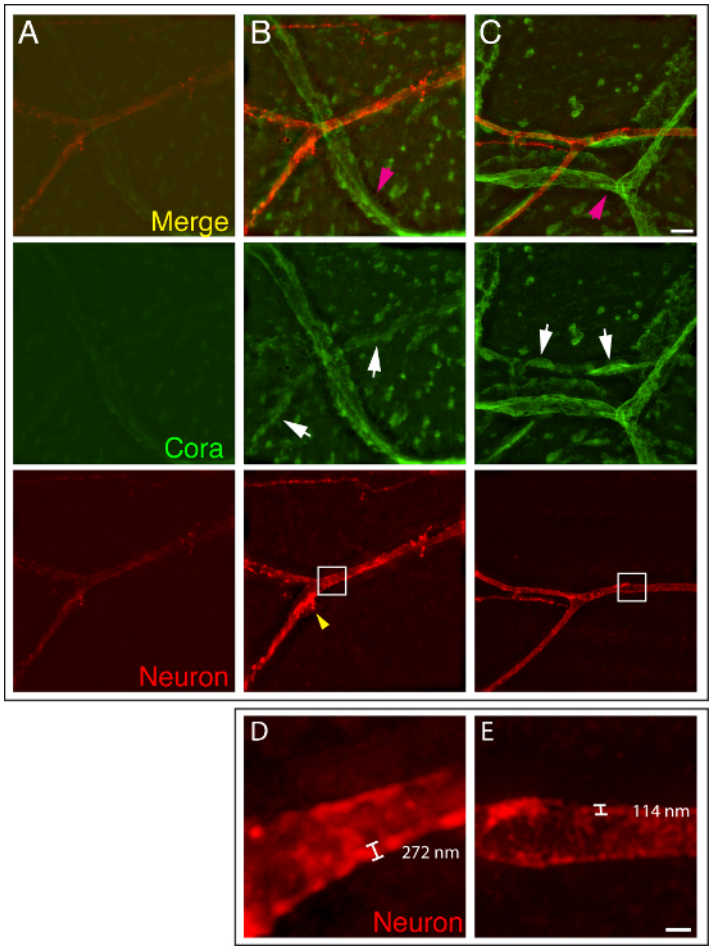

Figure 3. Muscle Removal Enables Super-resolution of Imaging of da Neurons and Epidermal Cells. Immunofluorescence detection of Cora (green) in class IV da neurons with muscle intact (**A, B**) and after muscle removal (**C**). Class IV da neurons were labeled as in **Figure 2**. Muscle-on and muscle-off samples were imaged with a structured illumination microscope first under identical conditions (**A, C**); the muscle-on sample in (**A**) was then re-imaged with increased laser power and exposure times to compensate for muscle interference (**B**). All images are Z-projections. The middle (green) and bottom (red) panels of A-C show individual channels. The top panel of A-C is a merge. Epidermal cell boundaries (magenta arrows), Cora dendrite tracts (white arrows), and an image reconstruction artifact (yellow arrowhead) are indicated. Insets in the bottom panels of B and C correspond to D and E, respectively. The apparent width of the dendrite membrane is shown in muscle-on (**D**) and muscle-off (**E**) samples. Scale bars = 3 µm (**A-C**) and 0.5 µm (**D, E**). Please click here to view a larger version of this figure.

Discussion

Here a protocol is described for manual removal of muscle tissue from *Drosophila* larval fillets. This protocol modifies previously described larval dissection techniques[10,11]. After the larva is dissected in a silicone elastomer dish, the dorsal midline is located. A single forceps prong, in its flattest possible orientation, is carefully inserted between the muscle tissue and the epidermis, near the dorsal midline. The forceps are gently pulled upwards to separate muscle tissue from one anchor point in each larval segment of interest. The larval fillet is then fixed in cold formaldehyde after which forceps are used again to pull away muscle from the remaining anchor points.

While it is tempting to perform the entirety of the muscle removal procedure post-fixation, we find that stretched muscle, once fixed, remains flattened and closely apposed to the epidermis even after detachment from an anchor point, rendering it difficult to manipulate without damaging the underlying tissue. Additionally, in our experience, fixed muscle tissue is brittle and tears easily, preventing its complete removal from the body wall. In contrast, when muscle is detached from one anchor point prior to fixation, it will contract towards the remaining anchor point during fixation, leaving tissue stubs that can be more easily grabbed by forceps and pulled from the larval body wall.

In order to best preserve samples, considerable care must be taken to minimize contact between the forceps and the epidermis during the muscle removal procedure. As a result, our protocol may add several minutes to previously described dissection techniques[10,11]. To avoid tissue degradation, the number of larvae that are dissected prior to fixation should be limited such that elapsed time is no more than 25 minutes. Additionally, we recommend testing multiple pairs of forceps — forceps of the same model may vary considerably in shape and sharpness — and adjusting the extent to which fillets are stretched prior to muscle removal in order to improve tissue preservation and dissection speed.

Lastly, we recommend experimenting with the use of Ca^{2+}-free HL3.1 saline versus standard HL3.1 saline during larval dissection. In the absence of calcium, larval muscle contractions are greatly reduced. Thus, larval fillets may be easier to manipulate when dissected in Ca^{2+}-free HL3.1 saline. However, we find that muscle contraction creates space between the muscle and epidermis and this can facilitate the insertion of

forceps between these tissues while minimizing risk of damage to the epidermis. As a result, standard HL3.1 may be preferable for preserving the integrity of the epidermis and da neurons. We obtain good results with either saline and the choice is left to the user.

While our protocol increases the duration and complexity of previously described dissection techniques[10,11], it offers several improvements that are useful for imaging larval sensory neurons and epidermal cells. First, it permits imaging of neuronally and epidermally expressed proteins that are otherwise obscured by muscle tissue. Second, it improves the fluorescence signal to noise ratio. Lastly, it enables use of super-resolution microscopy for superior discrimination of protein spatial relationships in da neurons and the epidermis. The improved imaging quality that we observe following muscle removal is likely the result of reduced photon absorption and scattering as well as reduced distance between the sample and the coverslip. Although not demonstrated here, muscle removal likely also improves antibody access to the epidermis and da neurons, improving the quality of immunofluorescence. Greater antibody accessibility may be particularly useful for immunofluorescence protocols performed in the absence of a tissue permeabilizing agent[4].

The improvements gained by use of this protocol are likely to benefit studies of the neuronal and epidermal factors that regulate dendrite morphogenesis in sensory neurons. Furthermore, muscle removal has previously facilitated electrophysiological study of sensory neurons in the larval body wall and a modified version of this protocol may be useful for future studies that employ neurophysiological manipulation of larval sensory neurons. Lastly, a modified version of this protocol may be useful towards other applications such as the isolation of single da neurons for gene expression profiling.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank Gary Laevsky for helpful discussions on microscopy. This work was funded by NIH grants R01GM061107 and R01GM067758 to E.R.G.

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