Video Article

Immunohistological Labeling of Microtubules in Sensory Neuron Dendrites, Tracheae, and Muscles in the *Drosophila* Larva Body Wall

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Abstract

To understand how differences in complex cell shapes are achieved, it is important to accurately follow microtubule organization. The *Drosophila* larval body wall contains several cell types that are models to study cell and tissue morphogenesis. For example tracheae are used to examine tube morphogenesis[1], and the dendritic arborization (DA) sensory neurons of the *Drosophila* larva have become a primary system for the elucidation of general and neuron-class-specific mechanisms of dendritic differentiation[2-5] and degeneration[6].

The shape of dendrite branches can vary significantly between neuron classes, and even among different branches of a single neuron[7,8]. Genetic studies in DA neurons suggest that differential cytoskeletal organization can underlie morphological differences in dendritic branch shape[4,9-11]. We provide a robust immunological labeling method to assay *in vivo* microtubule organization in DA sensory neuron dendrite arbor (Figures 1, 2, Movie 1). This protocol illustrates the dissection and immunostaining of first instar larva, a stage when active sensory neuron dendrite outgrowth and branching organization is occurring [12,13].

In addition to staining sensory neurons, this method achieves robust labeling of microtubule organization in muscles (Movies 2, 3), trachea (Figure 3, Movie 3), and other body wall tissues. It is valuable for investigators wishing to analyze microtubule organization *in situ* in the body wall when investigating mechanisms that control tissue and cell shape.

Video Link

The video component of this article can be found at https://www.jove.com/video/3662/

Protocol

1. Preparation of reagents

Notes before beginning: Dissection and immunohistochemical staining are carried out in a magnetic chamber and the larva is pinned down using specially shaped insect pins. Detailed instructions on the construction of a magnetic chamber, and preparation of these pins can be found in related references [14,15]. In brief, a 1x1cm square hole is cut into a magnetic sheet and a coverslip affixed to the rear of the sheet to make a small chamber. The sides of the chamber are sealed with epoxy glue; after this glue has set the chamber is washed several times with 70% ethanol before use. Dissection insect pins are prepared by bending to the required shape and then glued onto a metal tab[14,15]. Alternatively to a metal tab, we have used an inverted flat-head steel drawing pin with a handle made from a cut-off yellow tip. Use of this magnetic chamber arrangement allows close control over pin positioning and tissue stretching during dissection.

To drive reporter gene expression in different subsets of DA neurons investigators may use several different Gal4 lines (summarized by Shimono and colleagues[16]). Many of these lines are available from public stock centers. In this representative protocol, we carry out immunostaining of a line in which two contrasting classes of DA neuron are co-labeled: the most simple- class I and most complex- class IV *(P10-Gal4[17,18], UAS-mCD8::Kusabira-Orange (KO))*.

1. Prepare Ca^{++}-free HL3.1 saline
    1. In mM: 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 5 HEPES, 115 sucrose, and 5 trehalose; pH 7.2[19]. Filter-sterilize and store at 4°C.
    Note: Ca^{++}-free solution prevents muscle contraction during dissection.

2. Prepare 2x PHEM buffer
    1. In mM: 130 PIPES, 60 HEPES, 20 EGTA, 4 $MgSO_4$; pH 7.0. Filter-sterilize and store at 4°C.
    Note: These materials will not dissolve until the pH approaches 7.0.

3. Prepare the fixative freshly immediately before the fixation.
    1. In order to prepare a 25ml solution, first mix 2g paraformaldehyde, 100µl 1M NaOH and 10ml water in a 50ml Falcon tube.
    2. Shake fixative in a 55°C water bath with shaker until the solution is clear. (Total volume will be about 11.5 ml.)
    3. Cool fixative on ice.
    4. Add 12.5ml 2x PHEM buffer.
    5. Adjust pH to 7.0 with 1M HCl.
    6. Fill the solution with water until the volume is 25ml.
    7. Filter the solution using Whatman paper.

2. Larval dissection

Note before beginning: Microtubule networks, and particularly those in sensory dendrites, will breakdown rapidly after the initiation of dissection. Achieving fast dissection in less than five minutes followed by immediate fixation are key factors in the success of this protocol.

1. Wash the larva in water and quickly move them into this drop using a loop of hair.
2. Orient the larva dorsal side up and the ventral side on the glass. Note: This orientation is to examine the ventral cluster neurons. To examine dorsal cluster neurons, invert the orientation.
3. Use the center insect-pin to pin the anterior end near the mouth hooks. Place the pin close to the end for best results.
4. Place a drop of HL3.1 saline in the dissection chamber.
5. Cut the very posterior tip of the larva off with microscissors. Note: This step opens an aperture at the posterior end of the larva that will allow access for the microscissors (step 2.7).
6. Grab with the forceps the region of gut that is now poking out of the aperture at the posterior end of the larva. Gently pull out the whole gut.
7. Place the tip of one blade of the microscissors through the aperture and cut along the ventral midline towards the anterior.
8. Using the corner pins, pin the now free corners, posterior first, then anterior. Simultaneously gently stretch open the larval fillet.

3. Fixation, blocking, staining, and mounting of larval fillets

Notes before beginning: All fixation and staining steps are carried out in the dissection chamber. During this process, be careful not to knock the insect pins holding the larva as this may lead to tissue damage. To prevent the experiment from drying out, do all staining steps in a small Tupperware container surrounded by moistened tissues.

1. Aspirate the Ca^{++}-free HL3.1 buffer using a yellow tip. Immediately add the fixative using another pipetteman.
2. Gently pipette up and down to mix the fixative with the remaining traces of Ca^{++}-free HL3.1 buffer in the chamber. Immediately aspirate it and then add fresh fixative into the chamber.
3. Incubate at room temperature for 20 minutes.
4. Wash 6x 10mins in PBST (0.1% Triton X-100 in PBS).
5. Block with 5% goat serum in PBST for 20 mins at RT.
6. Replace the blocking solution with primary antibodies diluted in 5% goat serum in PBST. The primary antibodies used are mouse anti-α-Tubulin (DM1A) and rat anti-CD8 (5H10) both diluted 1/1000.
   Note: The investigator may wish to substitute mouse anti-α-Tubulin (DM1A) with mouse anti-Futsch (22C10)[20,21] diluted 1/1000 in some circumstances (see discussion).
7. Incubate overnight (for at least 16h) at 4°C.
8. Wash 6x 10mins in PBST.
9. Add secondary antibody solution diluted in 5% goat serum in PBST. The secondary antibodies used are Alexa Fluor 488 goat anti-mouse IgG and Cy3 donkey anti-rat IgG. Keep the sample covered to prevent fluorophore photo-bleaching[22].
10. Incubate either at RT for two hours, or overnight at 4°C followed by one hour at room temperature.
11. Wash 6x 10mins in PBST.
12. Place the larval fillet on the slide cuticle-side down, mount in 80% glycerol, and seal the sides of the coverslip with nail polish for a 'quick' mount.
    Note: For improved tissue clearing, and a permanent stable sample, mount in DPX as previously described[23].

4. Representative Results:

Fluorescent staining was examined under a confocal microscope. In Figures 1-2, different branches within a dendrite arbor have different cytoskeletal organization. Figure 1 shows a region of the arbor of a class IV DA neuron at the 1st instar larval stage. The whole arbor is marked with mCD8::KO and detected using an anti-CD8 antibody and fluorescent secondary (Cy3). Tubulin is detected using anti α-Tubulin antibody and fluorescent secondary (Alexa Fluor 488). The main branches are positive for Tubulin, some thin side branches are Tubulin-negative. Movie 1 is a set of serial sections through a similar staining of a class I DA neuron. Figure 2 shows a region of the arbor of a class IV DA neuron stained with antibodies against Futsch and CD8 at the 1st instar larval stage. The main branches are Futsch-positive, some thin side branches are Futsch-negative. Figure 3. Tracheae in the larval body wall show a complex microtubule organization. Movies 2 and 3 show serial sections of staining through body wall muscles and trachea.

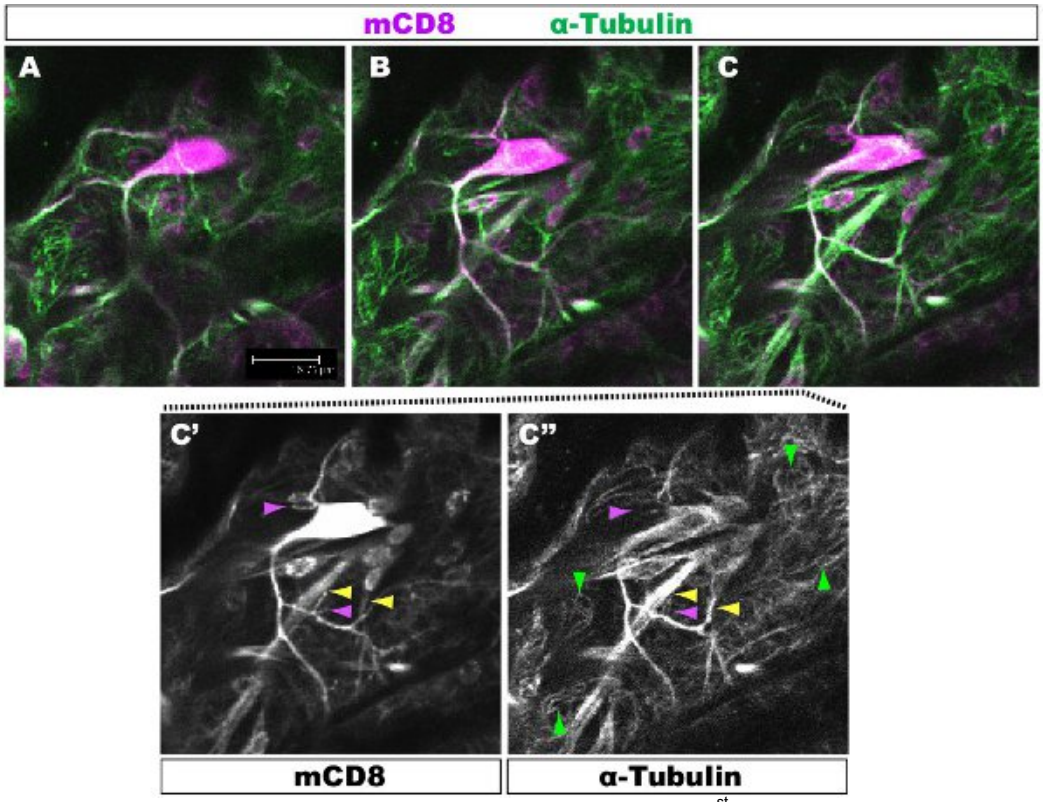

Figure 1. Fig. 1 shows a region of the arbor of a class IV DA neuron at the 1st instar larval stage. The whole arbor is marked with mCD8::KO and detected using anti-CD8 antibody and fluorescent secondary (Cy3). Tubulin is detected using anti-&-Tubulin antibody and fluorescent secondary (Alexa 488). Panels A-C sequential confocal z-sections (0.5µm), C'-C", single antibody staining from panel C. Example of branches with (yellow arrowhead) or without (purple arrowhead) microtubules are highlighted. Red arrowheads highlight microtubules in the underlying epithelial cells.

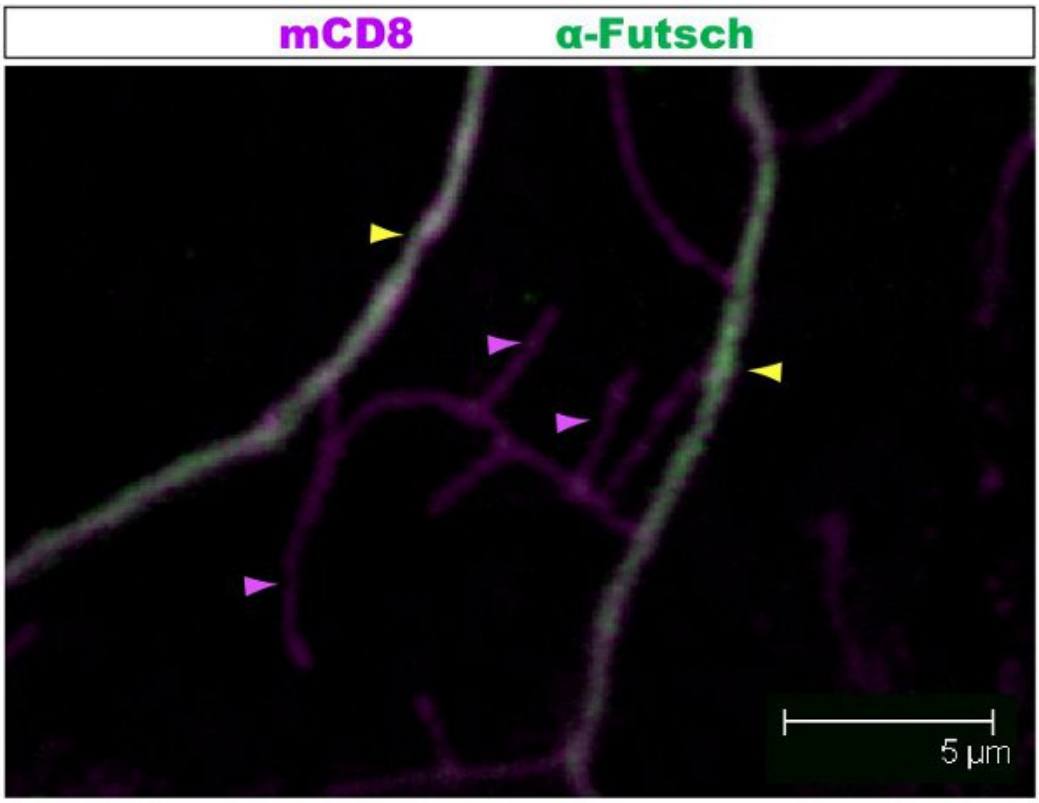

Figure 2. Fig. 2 shows a similar region of the arbor of a class IV da neuron stained with antibodies against Futsch and CD8 at the 1st instar larval stage. The whole arbor is marked with mCD8::KO and detected using anti-CD8 antibody and fluorescent secondary (Cy3). Futsch is detected

using anti-Futsch antibody and fluorescent secondary (Alexa 488). The main branches are Futsch-positive, some thin side branches are Futsch-negative. Example of branches with (yellow arrowhead) or without (purple arrowhead) Futsch are highlighted.

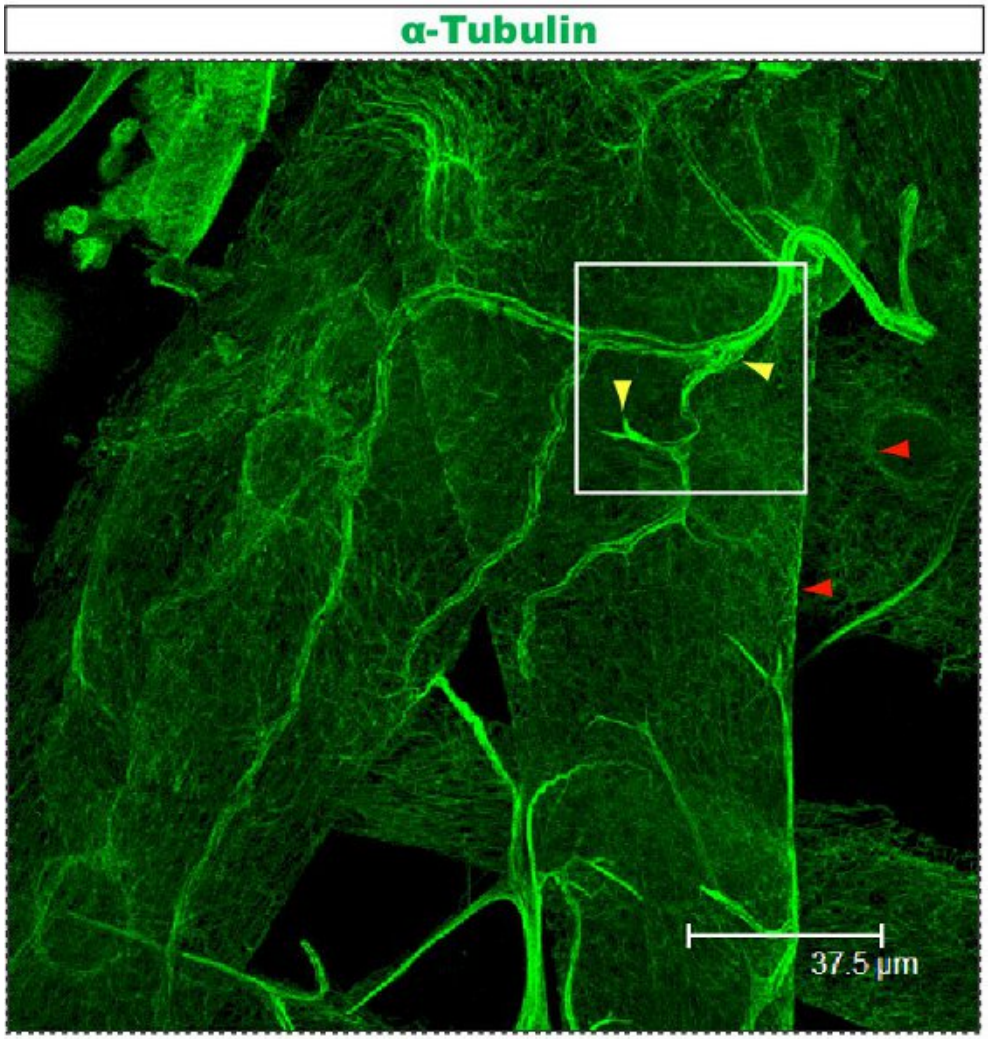

Figure 3. Trachea stained using the anti-Tubulin protocol described above. This larva is third instar and dissected as previously described[23,24]. Movie 3 shows enlarged serial sections from the field marked by a square.

Movie 1. Serial sections tracing Tubulin staining throughout the dendritic arbor of a class I neuron. The whole arbor is marked with mCD8::KO (Magenta) and detected using anti-CD8 antibody and fluorescent secondary (Cy3). Tubulin (Green) is detected using an anti-α-Tubulin antibody and fluorescent secondary (Alexa Fluor 488). Scale: one side of the video image corresponds to 46.88µm in the section. Click here to view the Movie.

Movie 2. Serial sections tracing Tubulin staining in body wall muscles of a third instar larva. Tubulin is detected using anti-α-Tubulin antibody and fluorescent secondary (Alexa Fluor 488). Scale: one side of the video image corresponds to 46.88µm in the section. Click here to view the Movie.

Movie 3. Serial sections tracing Tubulin staining in a body wall trachea of a third instar larva, marked with a square in Figure.3. Tubulin is detected using anti-α-Tubulin antibody and fluorescent secondary (Alexa Fluor 488). Scale: one side of the video image corresponds to 46.88µm in the section. Click here to view the Movie.

Discussion

To understand how complex cell shapes are achieved it is important to be able to accurately assay microtubule organization. Here we describe a robust immunohistological labeling method to assay microtubule organization of dendritic arborization neuron sensory dendrites. In addition to staining sensory neurons, this method achieves robust immunohistological staining of trachea, muscles and other body wall tissues.

We use this protocol to examine microtubule organization in the developing sensory dendrites of DA neurons. These dendrites are very sensitive to injury or stress[25], and break down rapidly after the beginning of dissection[23]. Dissection should be carried out in five minutes or less. Microtubules in the trachea and other body wall tissues are more stable. This protocol is adapted from those used prepare larval fillets for analysis of the neuromuscular junction[14,15], and is optimized for quick fixation after a rapid dissection.

When analyzing the dendrites of DA neurons, robust anti-Tubulin staining in overlying muscle, and underlying epithelial cells[26] can lead to a complicated staining pattern (Figure 1, Movie1). Hence careful tracing of dendritic staining through several serial sections (Movie1) may be required for a full analysis of microtubule organization in the DA neuron dendrite arbor. Monoclonal antibodies to the specific *Drosophila* homologue of MAP1B, Futsch[20,21], can also be used to label the microtubule cytoskeleton in the *Drosophila* peripheral nervous system[5,9,20]. Futsch is expressed only in neurons and hence it produces a less complicated staining pattern than α-Tubulin (Figure 2). GFP-tagged-endogenous-Tau has also been used as a marker of the DA neuron dendritic microtubules[27]. Use of these markers is effective, but it should be noted that in the axon-termini of the neuromuscular junction Futsch specifically labels bundled microtubules[28]. The exact spatial relationships between Futsch, Tau, and microtubules in DA dendrites are yet to be fully characterized. In this protocol we have addressed endogenous microtubule organization. It is also possible to label microtubules using overexpression of GFP tagged Tau or Tubulin. Care should be taken against inducing unanticipated phenotypes when overexpression approaches are used[29].

In our hands, immunohistochemical staining of microtubules in DA neurons and other body wall tissues is best achieved when the secondary antibodies used to detect the anti-Tubulin primary antibody is coupled to an easily-visualized fluorophore such as Alexa Fluor 488. Hence when using transgenic tools to label DA neurons in concert with anti-tubulin labeling e.g. via the Gal4/UAS system, LexA system, or in MARCM[23] and MARCM derivatives, we prefer marker proteins without spectral overlap with this secondary, such as mCD8::Cherry and mCD8::KO.

Disclosures

We have nothing to disclose.

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