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Retrograde Tracing of Drosophila Embryonic Motor Neurons Using Lipophilic Fluorescent Dyes

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Department of Cellular Biology
Franklin College of Arts and Sciences
UNIVERSITY OF GEORGIA

August 28, 2019

Editor
JoVE

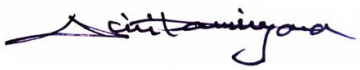
Dear Dr. Jewhurst,

We are submitting to you our manuscript titled "Retrograde tracing of Drosophila embryonic motor neurons using lipophilic fluorescent dyes." In this manuscript, we describe a method for retrograde tracing of the Drosophila embryonic motor neurons using lipophilic fluorescent dyes. By using this technique, we previously unraveled the molecular mechanisms that drive the initiation of motor dendrite connectivity (*Kamiyama et al., Science, 2009; Kamiyama et al., Developmental Cell, 2015*). This technique is highly adaptable for the phenotypic analysis of embryonic motoneurons, enhancing our ability to provide novel insight into the functional architecture of the *Drosophila* nervous system. Given the general use for motor neuron labeling, we expect it would be a great asset of JoVE's Developmental Biology section.

Our suggested reviewers include **Dr. Hiroshi Kohsaka** at the University of Tokyo (kohsaka@neuro.k.u-tokyo.ac.jp) an expert on *Drosophila* embryonic neuromuscular junction development, **Dr. Edward Giniger** at the National Institutes of Health (ginigere@ninds.nih.gov), an expert on *Drosophila* motor neuron development, and **Dr. Thomas Kidd** at the University of Nevada, Reno (tkidd@unr.edu), an expert on *Drosophila* central nervous system development.

We hope this manuscript will receive your favorable consideration.

Sincerely yours,



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TITLE:

Retrograde Tracing of *Drosophila* Embryonic Motor Neurons Using Lipophilic Fluorescent Dyes

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KEYWORDS:

Drosophila, embryo, motor neuron, dendrite, axon, central nervous system, neuromuscular junction, retrograde tracing, lipophilic fluorescent dye

SUMMARY:

We describe a method for retrograde tracing of the *Drosophila* embryonic motor neurons using lipophilic fluorescent dyes.

ABSTRACT:

We describe a technique for retrograde labeling of motor neurons in *Drosophila*. We use an oil-dissolved lipophilic dye and deliver a small droplet to an embryonic fillet preparation by a microinjector. Each motor neuron whose membrane is contacted by the droplet can then be rapidly labeled. Individual motor neurons are continuously labeled, enabling fine structural details to be clearly visualized. Given that lipophilic dyes come in various colors, the technique also provides a means to get adjacent neurons labeled in multicolor. This tracing technique is therefore useful for studying neuronal morphogenesis and synaptic connectivity in the motor neuron system of *Drosophila*.

INTRODUCTION:

The embryonic motor neuron system of *Drosophila* offers a powerful experimental model to analyze the mechanisms underlying the development of the central nervous system (CNS)[1-3]. The motor neuron system is amenable to biochemical, genetic, imaging, and electrophysiological techniques. Using the techniques, genetic manipulations and functional analyses can be carried out at the level of single motor neurons[2,4-6].

During early development of the nervous system, neuroblasts divide and generate a large number of glia and neurons. The spatiotemporal relationship between the delamination and the gene expression profile of neuroblasts has been previously investigated in detail[7-9]. In the case of the motor neuron system, the formation of embryonic neuromuscular junction (NMJ) has been

extensively studied using the aCC (anterior corner cell), RP2 (raw prawn 2), and RP5 motor neurons[2,10]. For instance, when the RP5 motor neuron forms a nascent synaptic junction, the pre-synaptic and post-synaptic filopodia are intermingled[11-13]. Such direct cellular communication is vital to initiate the NMJ formation. Contrary to what we know about the peripheral nerve branches, our knowledge of how motor dendrites initiate synaptic connectivity within the CNS is still primitive.

In this report, we present a technique that allows retrograde labeling of motor neurons in embryos by means of micropipette-mediated delivery of lipophilic dyes. This technique enables us to trace the 38 motor neurons innervating each of the 30 body wall muscles in a hemi-segment at 15 h after egg laying (AEL)[14]. By using this technique, our group has thoroughly investigated numerous gain-of-function/loss-of-function alleles[15-17]. We have recently unraveled the molecular mechanisms that drive initiation of motor dendrite connectivity and demonstrated that a Dscam1-Dock-Pak interaction defines the site of dendrite outgrowth in the aCC motor neuron[17]. In general, this technique is adaptable for the phenotypic analysis of any embryonic motor neurons in wild type or mutant strains, enhancing our ability to provide new insights into the functional design of the *Drosophila* nervous system.

PROTOCOL:

1. Equipment and supplies

1.1. Embryo collection materials for training adult flies to lay eggs

1.1.1. Prepare the filtration apparatus by severing a 50 mL tube and cutting open a hole in the cap to set a mesh filter with pores of 100 µm (**Table of Materials**) in between the tube and the cap.

NOTE: Alternatively, cell strainers with pores of 100 µm (**Table of Materials**) can be used for the filtration step of embryo collection.

1.1.2. Make agar plates with grape agar premix (**Table of Materials**) according to the listed instructions. Briefly, gently stir 1 packet of the powder mix into 500 mL of room temperature (RT, 23 °C) dH_2O and microwave the dissolved mixture to vigorous boil. After cooling down to 70−75 °C, pour the mixture into Petri dishes (60 mm). After the agar is solidified, store plates at 4 °C.

1.1.3. Prepare yeast paste by mixing active dry yeast (**Table of Materials**) and water to a paste consistency, and keep at 4 °C.

1.1.4. Use egg-collection cages (for 60 mm Petri dish, **Table of Materials**) that provide sufficient air flow.

1.2. Preparation of dissection needles and dye injection micropipettes

1.2.1. Prepare dye injection micropipette and dissection needle from the same capillary tubing with an inner diameter of 0.6 mm and an outer diameter of 1.2 mm (**Table of Materials**). Pull the capillary tubing by a micropipette puller at 7% from 170 V maximum output (**Table of Materials**) to create a sharp needle with a taper of ~0.4 cm in length.

1.2.2. For dye injection, adjust the micropipette with a micropipette beveler (**Table of Materials**) by a bubble beveling technique described in instrument's manual.

1.2.2.1. In short, soak the grinder with a wetting agent (**Table of Materials**) to prevent the water from 'dragging' the needle tip. Place the needle on the micropipette clamp at 25−30° and lower the tip onto two-thirds of the radius out from the center of the beveling surface. Grind the needle while a syringe with tubing pushes air into the needle, to ensure that the micropipette will be clear of glass shavings.

1.2.2.2. Mark the micropipette with a fine-tip permanent marker to indicate the position of the opening at the tip after beveling as it is challenging to locate the narrow opening of the micropipette that is formed at an angle.

2. Preparation for embryo collection

2.1. Ensure that the adult flies (20−40 wild-type *Canton-S* or *white* flies), males and females, are maintained in young (<7 days) and healthy conditions for the ideal egg collection.

NOTE: To stimulate egg-laying, flies are trained in their egg collection cage a couple of days prior to egg collection on agar plates streaked with yeast paste at least once every day.

3. Embryo staging

3.1. Allow the flies to lay eggs overnight (or at least 15 h) at RT to collect the embryos at 15 h AEL, i.e., stage 16[18], to view dendritogenesis of the aCC and RP3 motor neurons. In the morning, collect the plate with the eggs.

NOTE: The embryos at 15 h AEL will have a distinct 4-chamber gut[18]. For imaging different stages follow their specific morphological criteria and aging conditions.

3.2. To collect the embryos, dechorionate the eggs laid on the plate with 50% bleach for 5 min.

3.3. Once the chorions have cleared, pour the contents of the plate through the filtration apparatus or cell strainer to separate the embryos. Using a squeeze bottle of water, dilute the bleach left on the plate and gather as many embryos as possible by decanting the mixture into the filter.

3.4. Wash the embryos on the filter 3−4x with more water or until the bleach odor dissipates. Remove the filter from the apparatus and wash the embryos onto another clean plate with water.

Decant the water from the new plate that the embryos are on.

3.5. Prepare a glass slide by covering it with two layers of vinyl tape in the center, forming a rectangle. Cut a rectangular pool out of the tape using a razor blade. Place a thin strip of double-sided tape towards the upper end of the pool, this is where the embryos will be placed as shown in **Figure 1**.

3.6. Using fine forceps, individually select 5–10 embryos at 15 h AEL and place them on the double-sided tape with the dorsal side facing up. Add insect Ringer's saline[19] to the dissection pool to protect the embryos from desiccation (**Figure 1**).

4. Dissection and staining

4.1. Using a glass needle under a dissecting microscope (**Table of Materials**), cut through the midline of a single embryo at its surface from its posterior to its anterior end. Then drag the embryo out from the vitelline membrane from the tape onto the glass (boxed in **Figure 1**). Take care not to damage the interior tissues of the embryo.

4.2. Flip the epithelial tissues from the center and attach the epidermal edge onto the surface of the glass slide (**Figure 1**, inset).

4.3. Using a tube-connected needle with a tip opening of ~300 µm (prepared by breaking the thin tip of a dissection needle), aspirate or blow air to detach and remove the dorsal longitudinal tracheal trunks as well as any remaining guts.

4.4. Use 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) to fix the embryos for 5 min at RT. Wash the embryos 3x with PBS.

4.5. Stain the embryos with 1 µL of anti-horseradish peroxidase antibody conjugated with cyanine 3 dye (anti-HRP Cy3) (**Table of Materials**) in 200 µL of PBS for 1 h. Wash the embryos with PBS 3x after staining.

NOTE: The dye of anti-HRP can be changed based on the lipophilic dyes of choice for injection.

5. Filling of the injection micro-pipette

5.1. Heat lipophilic dyes (5 mg/mL of DiO or DiD, **Table of Materials**) to 60 °C in a 1:10 mixture of ethanol:vegetable oil before use.

5.2. Prepare an oil-dissolved dye slide for the injection micropipette. Place the micropipette into the capillary holder (**Figure 2,** ①). Using the micromanipulator (**Table of Materials**), adjust the micropipette to be over the dye slide. Then, adjust the stage to place the micropipette onto the dye (**Figure 2,** ②).

5.3. To fill up the micropipette, use a microinjector (**Table of Materials**) (**Figure 2,** ③). Collect the dye in the micropipette by setting the P_i (injection pressure) between 200−500 hPa (hectopascal), the T_i (injection time) between 0.1−0.5 s and P_c (compensation pressure) to 0 hPa for 5 min (**Figure 2,** ④).

5.4. Once the dye has been collected, remove the dye slide and place the sample onto the microscope stage. Next, increase the P_c to a range of 20−60 hPa before lowering the micropipette into the sample to prevent contamination of PBS by capillary action.

6. Dye injection into neurons

6.1. Locate the embryo in the center using the microscope with 10x objective lens (**Table of Materials**) and align the micropipette with the embryo.

NOTE: The size of the dye droplet can be adjusted by changing the P_i or the size of the opening of the micropipette tip. The droplet should be 10−20 µm, which is approximately the width of 1 muscle.

6.2. Change the objective lens to a water-immersion 40x lens (**Table of Materials**) and submerge the lens into PBS to see the embryo.

6.2.1. Use fluorescence microscopy to check the neuronal morphology marked by anti-HRP Cy3 and determine the injection site.

6.2.2. During injection, use brightfield microscopy to see the dye droplet. When the embryo is in focus, change the position of the micropipette to make gentle contact with the tip of the axon of interest (e.g., aCC, RP3).

6.2.3. Drop the dye in a right abdominal (A2−A6) hemi-segment at the neuromuscular junction of aCC or RP3 (**Figure 3**) with either DiD or DiO, by using the neurons marked by anti-HRP Cy3. Using the hand control (mouse; **Figure 2**, ⑤) release the dye and remove the micropipette after dropping the dye with the micromanipulator and move onto the next injection site.

NOTE: Unlike other dyes (e.g., Lucifer yellow, calcein) which spread into neighboring cells through gap junctions, lipophilic dyes associate with cell membranes and do not transfer to neighbors. Due to the relatively large size of the dye droplet, however, this technique also results in labeling of the partnering muscles (**Figure 3A**).

6.3. Incubate the sample at RT for 1 h after dye-drop before imaging.

NOTE: The protocol can be paused here before mounting, and the sample can be kept at 4 °C overnight. Lipophilic dyes can also be delivered using iontophoresis, if an intracellular direct-coupled (DC) amplifier is readily available[20].

7. Imaging with a confocal microscope

7.1. Remove the double-sided tape and vinyl tape from the glass slide with the help of forceps.

7.2. Prepare a cover slip (22 x 22 mm square No.1 cover glass) with a small amount of vacuum grease (**Table of Materials**) at the four corners and carefully place on the sample, avoiding air bubbles. Remove any excess PBS using task wipers.

7.3. Push down the coverslip to adjust the working distance between the objective lens and the sample. Completely seal the edges of the cover slip with nail polish.

7.4. Image at 10x and 100x magnification using a confocal microscope.

7.5. Use ImageJ software for processing raw images from the microscope (**Table of Materials**).

NOTE: Observation must begin within 10 min after mounting for the best images. Otherwise, at RT, the dye will spread to sites adjacent to the injection site creating unwanted background for imaging. To slow down the diffusion of dye, the sample can be stored at 4 °C for a couple of hours.

REPRESENTATIVE RESULTS:

A representative image of the aCC and RP3 motor neurons is shown in **Figure 3C** to demonstrate the multicolor labeling of motor neurons at 15 h AEL. Their dendritic morphologies are largely invariant between embryos. The staining pattern obtained with anti-HRP antibody is shown in gray. A small droplet of DiO or DiD was deposited on the NMJ of muscle 1 or 6/7, respectively. **Figure 4** demonstrates the capability to quantitatively measure the phenotype of interest. We counted the total number of dendrite tips in a wild type, compared with a mutant (e.g., *dscam1*$^{-/-}$).

FIGURE LEGENDS:

Figure 1: Setup of the dissection pool. The blue chamber seen on the glass slide is created with vinyl tape keeping the buffers inside. The double-sided tape holds onto the embryos that are properly aligned. Also shown in the bottom left corner is an example of a dissected embryo in saline. The anterior end is on the top in this and all subsequent figures.

Figure 2: Dye injection equipment. Glass pipette labeling in the figure demonstrates the installation site of the glass pipette (①). The epi-fluorescent microscope is equipped with a LED light source and a series of filter sets. The micromanipulator (②) and the microinjection (③) devices are labeled to the right of the microscope. The inset is a close-up of the display of microinjection device (④) with appropriate values of P_i, T_i, and P_c).

Figure 3: Lipophilic dye preparations of retrogradely labeled motor neurons. (**A**) Retrograde-labeled motor neurons and their target muscles. The aCC motor neuron innervating muscle 1 (DiO: excitation/emission, 484 nm/501 nm); the RP3 motor neuron innervating muscles 6/7 (DiD:

excitation/emission, 644 nm/665 nm). Note that muscles 6/7 also receive innervation from another motor neuron (MNISNb/d-Is) in larval stages; however, MNISNb/d-Is does not have an embryonic counterpart[3]. Circles indicate sites of dye applications. (**B**) A schematic diagram of the body wall muscles and peripheral nerve branches in 15 h AEL. The ventral nerve cord (VNC) consists of segmentally repeated and bilaterally symmetrical neuromere with respect to the ventral midline (dotted line). Body wall muscles of each hemi-segment are innervated by 38 motor neurons. The motor neurons project their axons via six major nerve branches (ISN [intersegmental nerve], SNa [segmental nerve a], SNb, SNc, SNd, and TN [transverse nerve]). (**C**) Dendritic branches from the aCC and RP3 motor neurons show extensive overlap. Both neurons are bipolar neurons, meaning that the neurons establish two different populations of dendrites. Each neuron projects one arbor into the ipsilateral neuropil and another into the contralateral neuropil. Arrowheads point to dendritic tips. Fluorescence images were acquired with a 10x objective or a 100x oil immersion objective.

Figure 4: aCC dendritogenesis as revealed with retrograde labeling in hour-15 embryos. (**A**) In wild type, aCC extends its dendrites into both ipsilateral and contralateral neuropils. For simplicity, we only display the ipsilateral dendrites from aCC in this figure. aCC is labeled with DiO, shown in green. (**B**) In *dscam1* mutants (*dscam1*$^{21/21}$ from Dr. Tzumin Lee, Janelia Research Campus), aCC has few ipsilateral dendrites in most cases observed[17]. Arrowheads show dendritic tips.

DISCUSSION:

The use of dye labeling for studying neuronal morphology has several advantages over genetic cell-labeling techniques. The dye labeling technique can minimize the amount of time needed for labeling and imaging the morphologies of motor neurons. The dye labeling process is quite fast as it takes less than 2 h and enables us to define the outline of neuronal projections. As an alternative, one can visualize the aCC motor neuron by choosing a GAL4 line that expresses the yeast GAL4 transcription factor in aCC, and crossing it with a green fluorescent protein (GFP) reporter controlled by the upstream activation sequence (UAS)[21]. A GFP labeling technique as such requires a genetic cross and thus, takes extra few days.

Another advantage of dye labeling is to permit labeling of the plasma membrane at an extremely high density. A sufficient density of lipophilic dyes can be present on every part of the membrane, allowing us to resolve the fine details of a labeled structure. By contrast, the density of GFP molecules is often dependent on the waiting period after the UAS-GAL4 system kicks in. For example, aCC starts to express GFP from 10 h AEL. By 15 h AEL when we observe, the density of GFP molecules is inadequate to cover up the entire membrane. It results in insufficient labeling of fine neuronal projections (D.K., unpublished data).

Although this technique provides several advantages, it is less advantageous when the erroneous projection of motor axons is evident. In the absence of *sidestep*, for example, motor neurons display severe axonal defects such as premature stall, segmental border crossing, and excessive branching[22]. As a consequence, reaching to a certain axon terminal becomes cumbersome. The efficiency of labeling is also age-dependent, being effective in embryos younger than 20 h AEL.

As the extracellular matrix proteins increase with development, the labeling of motor neurons appears to be very intricate.

The technique described here allows us to measure many morphological parameters such as neurite total length and number, and neurite branch pattern and shape[15-17,23,24]. Because lipophilic carbocyanine dyes come in many colors (such as DiO, DiA, DiI, DiD, and DiR), multicolor labeling of adjacent motor neurons is also achievable. As shown in **Figure 4**, dendrites from the aCC and RP3 motor neurons extensively overlap. To further our understanding in motor circuit development, the mechanisms of dendrite-dendrite interaction will be investigated.

Here, we detail the versatile technique that provides an avenue to study neuronal connectivity in the motor circuit. Although the demonstration is restricted to the aCC and RP3 motor neurons in 15 h AEL, this technique can be applied to other motor neurons in different stages of embryonic development. If an axon terminal is accessible with an injection micropipette, this technique could also be applied to labeling of any neuron in the larval and adult stages of flies or even in other organisms.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

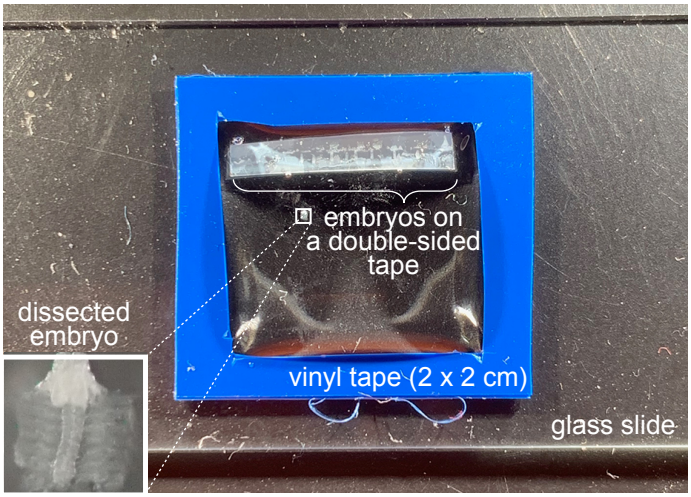


Figure 2

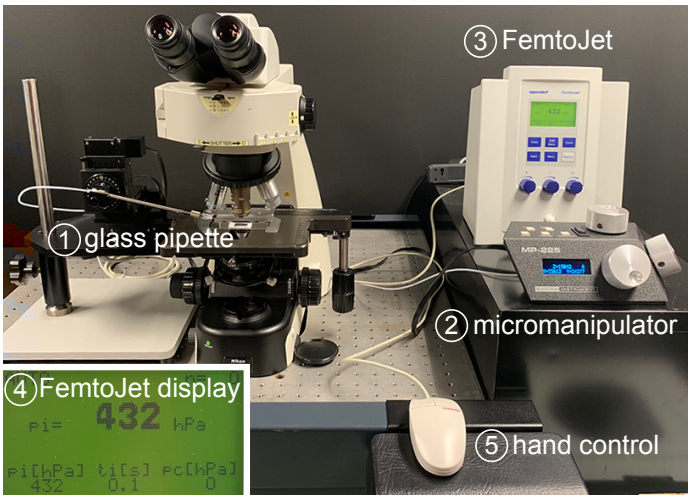


Figure 3

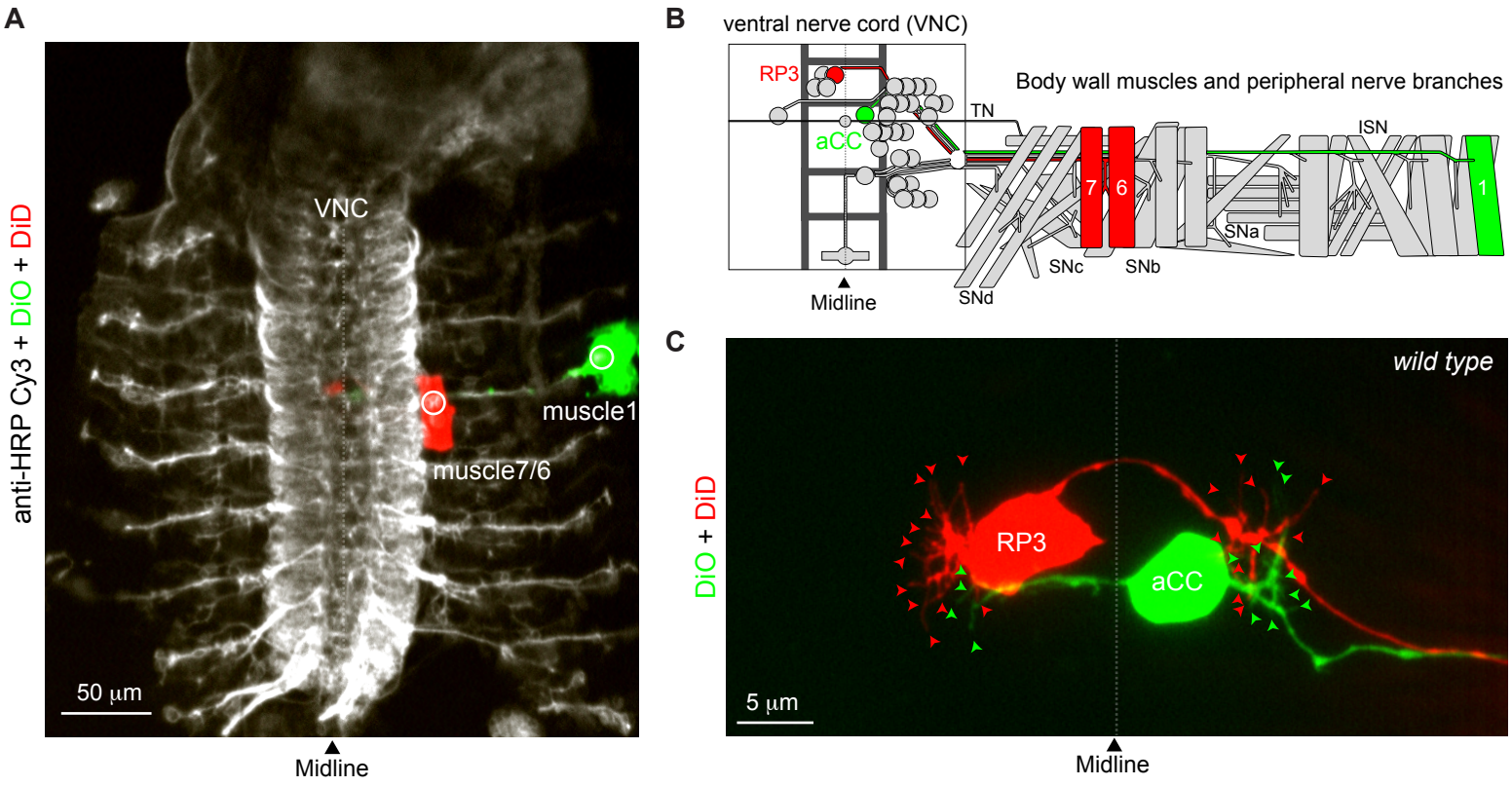
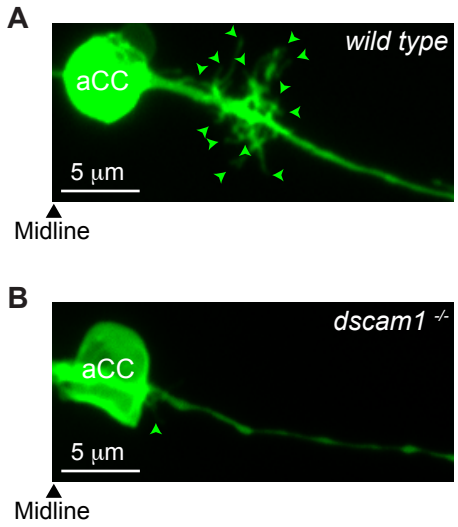


Figure 4



Name of Material/Equipment	Company	Catalog Number	Comments/Description
10x objective lens	Nikon		Plan
40x water-immersion lens	Nikon		NIR Apo
Capillary tubing	Frederick Haer&Co	27-31-1	
Confocal microscope	Andor	N/A	Dragonfly Spinning disk confocal unit
Cover glass	Corning		22x22 mm Square #1
DiD	ThermoFisher	V22886	
DiI	ThermoFisher	V22888	
DiO	ThermoFisher	V22887	
Dissecting microscope	Nikon	N/A	SMZ-U
Double Sided Tape	Scotch	665	
Dow Corning High-Vacuum Grease	Fisher Sci.	14-635-5D	
Dumont #5 Forceps	Fine Science Tools	11252-20	
Egg collection cage	FlyStuff	59-100	
FemtoJet 5247	Eppendorf	discontinued	FemtoJet 4i (Cat No. 5252000021)
ImageJ	NIH		Image processing software
Micromanipulator	Sutter	MP-225	
Micropipette beveler	Sutter	BV-10-B	
Needle puller	Narishige	PC-100	
Nutri-Fly Grape Agar Powder Premix Packets	FlyStuff	47-102	
Nylon Net Filter	Millipore		
Paraformaldehyde 16% Solution, EM grade	Electron Microscopy Sciences	15710	Any EM grades
PBS	Roche	11666789001	Sold on sigmaaldrich, boxed 10x solution
Photo-Flo 200	Kodak	146 4510	Wetting agent
Upright fluorescence microscope	Nikon	N/A	Eclipse Ci with a LED light source
Vinyl Electrical Tape	Scotch	6143	
VWR Cell Strainers	VWR	10199-659	
Yeast	FlyStuff	62-103	Active dry yeast (RED STAR)

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   The e-mail addresses for the co-authors have been provided in lines 9-11 of the manuscript.

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   The commercial names in the manuscript have been removed and substituted with generic terminology when needed. In instances where commercial names were used "(Table of Materials)" was substituted instead. The changes were made in section 1.1.1, the note following on lines 68-69, section 1.1.2, 1.1.3, 1.1.4, 1.2.1, 1.2.2, 1.2.3, 1.2.4, 4.1, 4.4, 4.5, 5.1, 5.2, 5.3, 6.1, 6.2, 7.2, and 7.5.

4. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE."

   The instances of passive voice have been changed to imperative form in the updated manuscript. Parts of these changes were made to as follows: section 1.1.1, 1.1.2, 1.1.3, 1.1.4, 1.2.1, 1.2.3, 2.1, 6.2.1, 6.2.2. Additionally, notes were added after sections 1.1.1, 3.1.1, 6.2.3.1, and 6.3 to provide clarifying information.

5. 1.1.1: What is the mesh size?

   The mesh filter used has 100 µm pores. This point has been addressed in line 65 in the manuscript.

6. 1.2.1: Please specify the settings of the micropipette puller.

   The micropipette puller is set to 7% output with an apparatus of 170V of maximum output. This is addressed in lines 87-88.

7. 4.3: At what temperature are the embryos fixed?

   The embryos are fixed after the tracheal trunks and guts are removed from the tissues at room temperature. This comment is addressed in line 157.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

   Section 1.2.2 is divided into 2 parts 1.2.2 and 1.2.3 resulting in a total of 4 sections under upper section 1.2.
   Section 4.2 is divided into 2 parts 4.2 and 4.3 resulting in a total of 5 sections under mains section 4.
   Section 5.3 is divided into 2 parts resulting in 5.3 and 5.4. The subsequent steps are moved under a new section: "6. Dye Injection into Neurons" to provide clarity between changing steps. Additionally, section 5.5, which is now under section 6, has been divided into 5 distinct steps– 6.2, 6.2.1, 6.2.2, 6.2.3, 6.2.3.1.
   Finally, as a result of creating section 6 for dye injection, imaging step became section 7. Under this, section 7.5 was added to emphasize the software used for image processing.

9. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

The figures have been uploaded individually in .ai format to the Editorial Manager account.

10. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

    The table of materials has been updated to include the objective lenses, cover glass, and the nylon net filter previously addressed only in the manuscript. The ImageJ software was added after the addition of section 7.5 to clarify the software used for image processing. Finally, the table of materials have been alphabetized after these new additions.

11. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

    In-text references have been corrected and references are numbered in order of appearance.

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    The references have been cited in the appropriate format.

Reviewers' comments:

Reviewer #1:

Manuscript summary:
In this study, the authors develop and describe a new technique for retrograde labeling of motor neurons in the model animal drosophila. They use various lipophilic fluorescent dyes dissolved in oil. The delivery of the dyes are via a small droplet injected with a microinjecter at the appropriate locations in the muscles. The technique is potentially useful especially when working with drosophila, but perhaps also in other model organisms. I have no major concerns only minor comments:

Minor comments:

1) First, this technique would be likely be helpful when working in other model organisms. Could this technique be extended to label axons in other animals perhaps even in mammals? I suggest adding some comments about this in the text. If it only works in drosophila, why would it not work in other animals? etc.

Following the suggestion, we have added comments to address this question in the updated manuscript under the DISCUSSION section in lines 328-331:. "…this technique can be applied for other motor neurons in different stages of embryonic development. If an axon terminal is accessible with an injection micropipette, this technique could also be applied to labeling of any neuron in the larval and adult stages of flies or even in other organisms."

2) I presume the dye does no cross gap junctions? Maybe mention this issue somewhere in the text.

The reviewer is correct. Lipophilic dyes normally associate with cell membranes and do not transfer to neighboring cells via gap junctions. We have included this information in the manuscript on page 4 lines 211-214.

3) The text is esoteric. Abbreviations should be spelled out so it is easier to access for non-experts:
HRP, Cy3, aCC, UAS-GAL4 etc.

We have spelled out the abbreviations.

Reviewer #2:

Manuscript Summary:
The protocol from Kamiyama and co-workers describe a method for single cell labeling of Drosophila motoneurons by backfilling from the neuromuscular junction (NMJ). While the method has been around for some time, and its value is well established in various publications, it has only been used in a few labs, in part due to the technical challenges of performing the necessary manipulations. For this reason, it is particularly well-suited to JoVE, where the written protocol will be complemented with a video demonstration. The technique has clear application to a number of biological questions.

Major Concerns:

I have no significant issues with the protocol, which is clearly written and thorough. I have a few minor points.

We thank the reviewer for the encouraging comments.

1. line 87 "the tip is lowered onto two-thirds out" - Did some words get lost here?

We have rewritten the statement: "the tip is lowered onto two-thirds of the radius out from the center of the beveling surface". Please see lines 97-98.

2. line 108 "a distinct 4-gut chamber" - I believe you mean "a distinct 4-chamber gut"

We have corrected this.

3. A scientific question: Backfilling from muscles 6/7 shows clean labeling of the RP3 motoneuron, but don't muscles 6/7 also receive innervation by small boutons from another motoneuron? I don't see anything else labelled in the images that are shown. Are some neurons resistant to labeling, or is there variability in labeling efficiency, or did the authors just select an example with a simple pattern of labeling?

Although another motor neuron (MNISNb/d-Is) is known to innervate muscles 6/7 in larval stages, MNISNb/d-Is does not have an embryonic counterpart (M.D Kim et al., Developmental Biology 336 (2009) 213-221). This is why MNISNb/d-Is is not labelled in our images. These details of MNISNb/d-Is have been added to the FIGURE AND TABLE LEGENDS section. Please see lines 271-273.

4. It would seem that the technique could be modified to apply to labeling of adult motoneuron projections in a fairly straightforward way - is that correct? If so, the authors might wish to say so, or if there is a known problem with this, it would be helpful for them to give an appropriate warning.

Yes, it is correct. This labeling technique can be applied to adult motoneuron projections but the motoneuron terminals must be accessible for injection. To address this comment, we have now included an additional sentence for the DISCUSSION section. Please see lines 329-331.

5. I hope that in the associated video the authors are sure to demonstrate the dissection of the embryos to reveal the CNS. In my own efforts, this has been the most challenging part of the procedure.

We agree with the reviewer that the dissection of embryos is challenging. We will make sure that the procedure is featured in the video.

Reviewer #3:

This manuscript is describing an interesting method for retrograde labelling of neurons. However, it is regrettable that it is a bit difficult to imagine the most important process, application of the lipophilic dyes, at least, just from reading the protocol. It would be better to make an additional description to clarify it for better reproducibility of experiments. For example, should we make injury of a muscle cell or a motor terminal for the dye staining? Or, is it OK just to make a gentle touch of an oil droplet with a dye to the cells? Moreover, is the access OK to be only to muscle and will the dye be transmitted from muscle membrane to the motor terminal? Or, is it needed to make touching of an oil droplet to the terminal? Such detailed description on dye application would make the protocol to be more useful for many researchers. Not only to the neuromuscular system, it may be adopted to central neurons if such detailed instruction is included.

The reviewer brings up an important point about clarity of our explanation for dye injection. Unlike other fluorescent dyes (e.g., Lucifer Yellow, Calcein) which spread into neighboring cells through gap junctions, lipophilic dyes do not transfer to neighbors. Thus, we need to make direct contact with the tip of the axon of interest. Due to the relatively large size of the dye droplet, however, this technique also results in labeling of the partnering muscles (Figure 3A). We have rewritten the statement for section 6.2.2 and added a new NOTE. Please see lines 199-201 for section 6.2.2 and lines 211-214 for the new NOTE on page 4.

Melissa Inal received her Bachelor of Science degree from the University of Georgia (UGA) in Biochemistry and Molecular Biology. She is currently a student in the Master's program at UGA, studying Cellular Biology.

Kota Banzai received his Doctorate degree in Biological Sciences from the Kanagawa University in 2016. He worked as a post-doctoral fellow in the Riken Center for Developmental Biology from 2016 to 2018. Currently he works as a research professional at University of Georgia.

Daichi Kamiyama has had training in cellular biology and neuroscience as a Ph.D. student in the Neuroscience program at the University of Illinois at Urbana-Champaign. During his postdoctoral research at the University of Miami, he pioneered the development of an activation sensor for the Rho family GTPase Cdc42 to visualize the endogenous activation pattern of Cdc42 during neural development in *Drosophila*. He joined the University of California San Francisco as an Assistant Professional Research Scientist to expand his research focus and training into the investigation of the mechanisms of dendritogenesis in *Drosophila* motoneurons using super-resolution microscopy techniques. As an Assistant Professor at the University of Georgia, he is working to illuminate a novel paradigm for cell-cell interactions to establish neural networks in *Drosophila*.

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