Video Article

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

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## 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*.

## Video Link

The video component of this article can be found at https://www.jove.com/video/60716/

## 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)[1,2,3]. 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[2,4,5,6].

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[7,8,9]. 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[11,12,13]. 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[15,16,17]. 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. Materials for collecting embryos and training adults to lay eggs
    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.
    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.
    3. Prepare yeast paste by mixing active dry yeast (**Table of Materials**) and water to a paste consistency, and keep at 4 °C.

 

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

2. Preparation of dissection needles and dye injection micropipettes
    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.
    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. 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.
        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

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

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.
2. To collect the embryos, dechorionate the eggs laid on the plate with 50% bleach for 5 min.
3. Once the chorions have cleared, pour the contents of the plate through the filtration apparatus or cell strainer to isolate 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.
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.
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**.
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

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.
2. Flip the epithelial tissues from the center and attach the epidermal edge onto the surface of the glass slide (**Figure 1**, inset).
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. Use 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) to fix the embryos for 5 min at RT. Wash the embryos 3x with PBS.
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

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.
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).
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).
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

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.
2. Change the objective lens to a water-immersion 40x lens (**Table of Materials**) and submerge the lens into PBS to see the embryo.
   1. Use fluorescence microscopy to check the neuronal morphology marked by anti-HRP Cy3 and determine the injection site.
   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).
   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**).
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

1. Remove the double-sided tape and vinyl tape from the glass slide with the help of forceps.
2. Prepare a cover slip (22 x 22 $mm^2$ 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.
3. Push down the cover slip to adjust the working distance between the objective lens and the sample. Completely seal the edges of the cover slip with nail polish.
4. Image at 10x and 100x magnification using a confocal microscope.
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*$^{-/-}$).

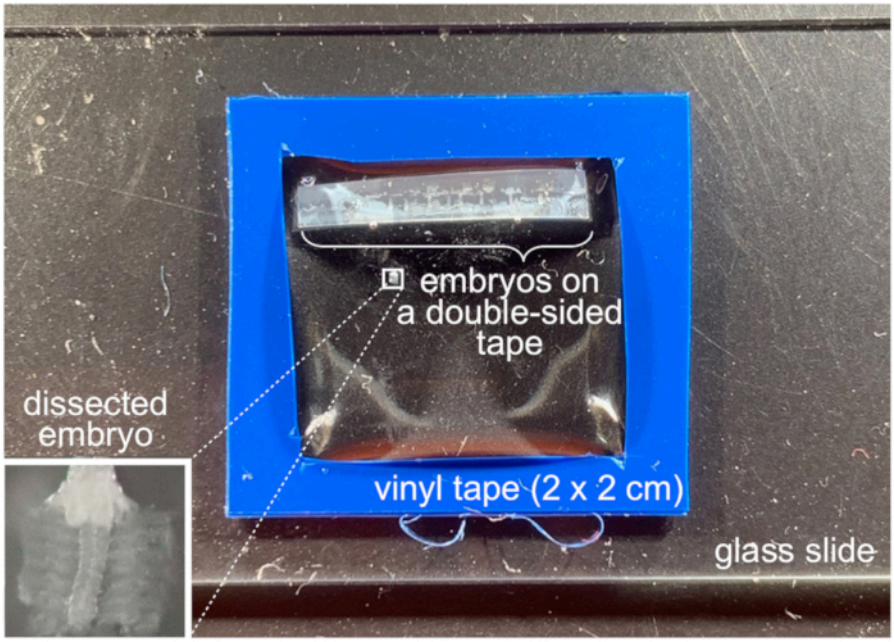


**Figure 1: Setup of the dissection pool.** The blue chamber seen on the glass slide is created with vinyl tape keeping the buffers inside. The double-sided tape holds onto the embryos that are properly aligned. Also shown in the bottom left corner is an example of a dissected embryo in saline. The anterior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

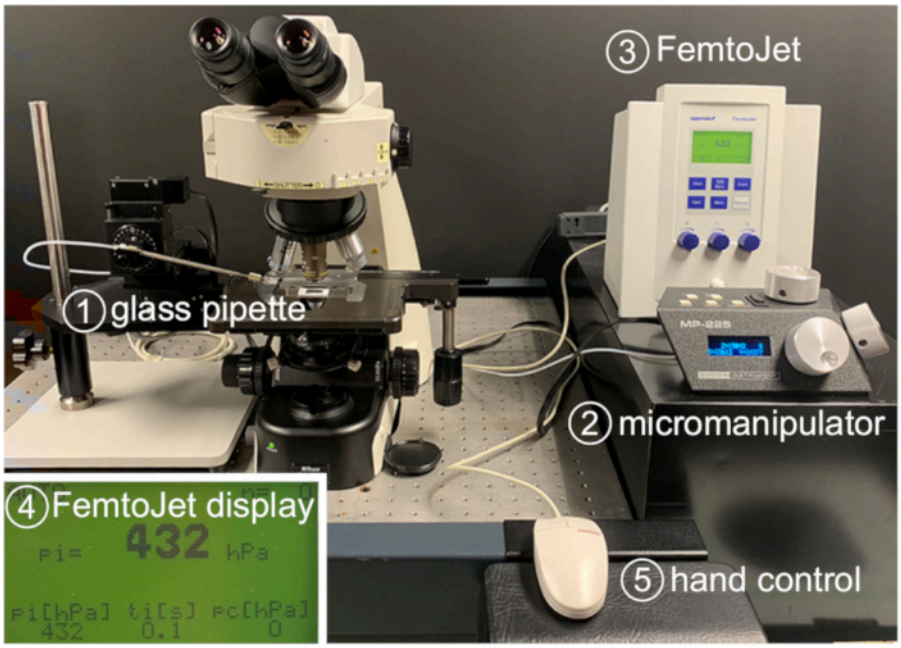


**Figure 2: Dye injection equipment.** Glass pipette labeling in the figure demonstrates the installation site of the glass pipette (1). The epi-fluorescent microscope is equipped with a LED light source and a series of filter sets. The micromanipulator (2) and the microinjection (3) devices are labeled 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 $P_c$). Please click here to view a larger version of this figure.

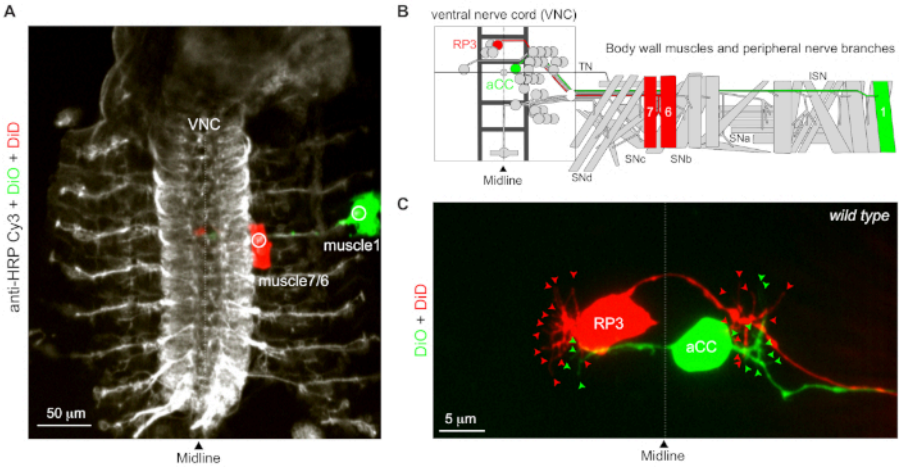


**Figure 3: Lipophilic dye preparations of retrogradely labeled motor neurons.** (**A**) Retrograde-labeled motor neurons and their target muscles. The aCC motor neuron innervating muscle 1 (DiO: excitation/emission, 484 nm/501 nm); the RP3 motor neuron innervating muscles 6/7 (DiD: excitation/emission,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

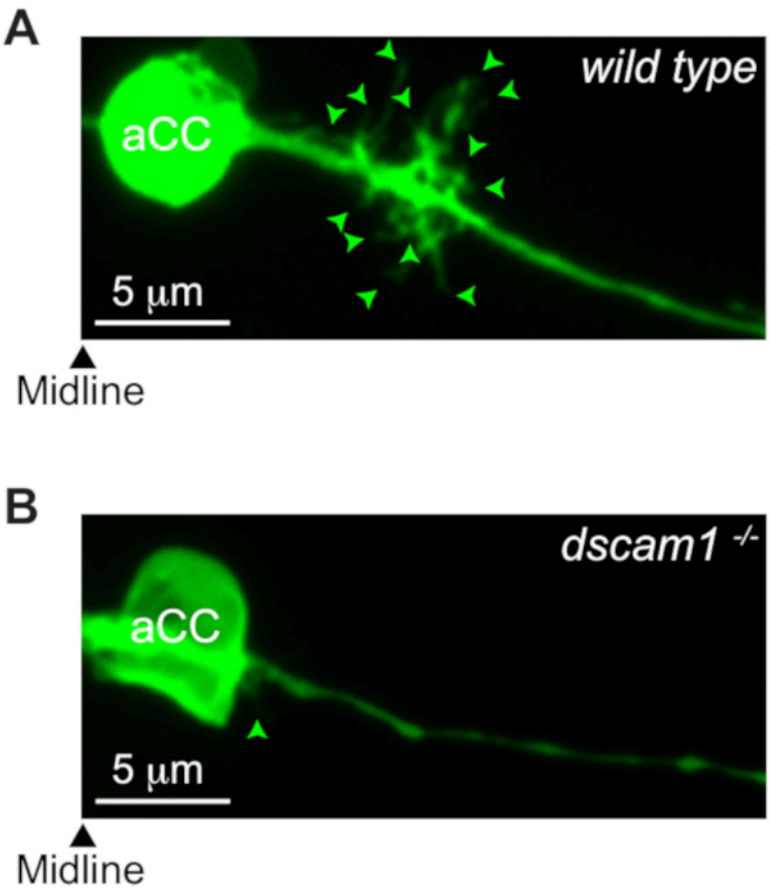


**Figure 4: aCC dendritogenesis as revealed with retrograde labeling in hour-15 embryos.** (**A**) In wild type, aCC extends its dendrites into both ipsilateral and contralateral neuropils. For simplicity, we only display the ipsilateral dendrites from aCC in this figure. aCC is labeled with DiO, shown 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. Please click here to view a larger version of this figure.

## 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[15,16,17,23,24]. 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.

## Disclosures

The authors have nothing to disclose.

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