Video Article

Using Linear Agarose Channels to Study *Drosophila* Larval Crawling Behavior

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Abstract

Drosophila larval crawling is emerging as a powerful model to study neural control of sensorimotor behavior. However, larval crawling behavior on flat open surfaces is complex, including: pausing, turning, and meandering. This complexity in the repertoire of movement hinders detailed analysis of the events occurring during a single crawl stride cycle. To overcome this obstacle, linear agarose channels were made that constrain larval behavior to straight, sustained, rhythmic crawling. In principle, because agarose channels and the *Drosophila* larval body are both optically clear, the movement of larval structures labeled by genetically-encoded fluorescent probes can be monitored in intact, freely-moving larvae. In the past, larvae were placed in linear channels and crawling at the level of whole organism, segment, and muscle were analyzed¹. In the future, larvae crawling in channels can be used for calcium imaging to monitor neuronal activity. Moreover, these methods can be used with larvae of any genotype and with any researcher-designed channel. Thus the protocol presented below is widely applicable for studies using the *Drosophila* larva as a model to understand motor control.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54892/>

Introduction

The overall goal of this method is to study *Drosophila* larval crawling in detail. Experiments on locomotion have played an important role in developing and testing theories on motor control². Traditionally locomotion has been studied in aquatic animals (e.g., leech, lamprey, tadpole)³. The repetitive nature of locomotion in these animals has allowed for the study of rhythmogenesis, for analysis of the biophysical events driving locomotion, and for monitoring the neural firing patterns that accompany locomotion.

The use of *Drosophila* larvae for studies of locomotion presents a unique combination of advantages over other model systems: facile genetics, well-characterized development, a body that is optically clear at first and second instars, and an ongoing transmission electron microscopic reconstruction of the entire nervous system⁴⁻⁶. However, *Drosophila* larval locomotion on flat open surfaces is somewhat complex including pauses, turns, and meandering crawls⁷. This publication presents a method to use linear agarose channels to guide *Drosophila* larval locomotor behavior such that larvae perform sustained, straight, rhythmic crawling behavior.

Studying *Drosophila* larval behavior in agarose channels, instead of behavior on flat open surfaces, has several advantages. First, it allows researchers to specifically select crawling behavior from the many movements that are part of the larval behavioral repertoire. Second, by adjusting the width of the channel versus the larval body size, crawling speed can be adjusted. Third, channels allow for the larva to be viewed from dorsal, ventral, or lateral side depending on how the larva is loaded and oriented within the channel. This versatility in larval orientation allows for any structure of interest to be continually visible during crawling. Fourth, channels are amenable for use with a wide variety of microscopes and objectives. For example, linear channels can be used for low-resolution imaging on bright-field stereoscopes and/or for high-resolution imaging on spinning-disc confocal microscopes¹. Fifth, this method can be used in combination with optogenetic/thermogenetic neuronal manipulations in any genetic background. Finally, because both the larval body (at first and second instars) and agarose channels are optically clear, channels can be used when studying the dynamic movements, or changes in fluorescent intensity of larval structures labeled by genetically-encoded fluorescent probes.

The method described is appropriate for detailed kinematic studies of first and second instar *Drosophila* larval behavior. This publication analyzes the dynamic changes in fluorescent intensity of the CNS during forward larval crawling to demonstrate the use of channels and as a precursor to neuronal calcium imaging.

Protocol

1. Preparation of Larvae

- One week before recording behavior, set up a cross (minimum of 25 virgins and 5 males). Maintain all crosses and progeny at 25 °C.
NOTE: The temperature of culture conditions can be altered, but the time line described below would need to be adjusted to account for changes in developmental speed.
- 5 days before recording, first thing in the morning, place the cross into a collection cage with an agar/juice cap and a dab (0.5–1 ml) of yeast paste in the center of the agar/juice cap.
 - To make a collection cage, poke holes into a 6 oz. square polyethylene *Drosophila* bottle.
 - To make agar/juice caps, mix 18 g of agar with 600 ml water in a conical flask. In a separate flask mix 20 g sucrose with 200 ml apple juice. Microwave until solids are dissolved. Combine and stir, allowing mixture to cool to 60 °C. Add 20 ml 10% methyl-p-hydroxybenzoate in 95% ethanol, and stir. Add ~7 ml to the bottom half of a 35 x 10 mm round petri dish. Allow agar/juice to solidify for 1 hr at RT. Store at 4 °C.
 - To make yeast paste, add equal parts by volume dry yeast and water. Store at 4 °C.
- Check the health of larval collections.**
 - In the morning, 4 and 3 days before recording, remove the cap and place a fresh cap on the cage. Change the caps at the same time each day to get a 24 hr collection of eggs and newly hatched larva.
 - After each cap is removed, examine it to see how many eggs the cross is laying. Expect between 500–2,000 eggs. Adjust the number of adults, if needed.
NOTE: A few eggs should have hatched into larvae, and the newly-hatched larvae will be found away from the yeast paste.
 - Age each cap for 24 hr. Re-examine the cap to determine whether larvae are healthy.
NOTE: Most eggs should have hatched into larvae, and the larvae should have crawled into the yeast paste. Signs of poor health include a large number of unhatched eggs, dead larval bodies away from the yeast paste, and larvae with significant dark patches in the abdomen, indicating an immune reaction. If larvae are unhealthy, change the cage, make fresh caps/yeast paste, and check the genotype.
 - Discard the old caps.
- Collect larvae for behavioral recordings.**
 - At both 2 and 1 day before recording, remove the cap and replace with a fresh cap.
 - Label each removed cap and keep at 25 °C. This will produce a staged series of larvae for behavioral recordings. Larvae from newly-hatched (0–4 hr posthatch) through second instar (24–48 hr posthatch) can be used in the channels. Save caps for recordings on consecutive days.
 - Discard all caps after 48 hr.
 - If needed, repeat steps 1.4.1–1.4.3 for up to 5 additional d. After that fewer fertilized eggs are produced.

2. Preparation of Channels

- Prepare the linear channel PDMS (Polydimethylsiloxane, a silicone elastomer) casting mold (**Figure 1A**). PDMS casting molds are available from the Heckscher lab upon request.
 - Clean the casting mold with isopropyl alcohol. Air dry, dab dry with laboratory wipes, or use canned air.
 - Place the casting mold into a 10 cm Petri dish lid or other container.
- Prepare and pour agarose solution.**
 - Make a 3% agarose solution in water (3 g in 100 ml) by microwaving until fully dissolved.
 - Cool to 55 °C, allowing bubbles to rise to surface.
 - Pour the agarose mixture into the petri dish containing the casting mold so that the mold is just covered. Let agarose set until solidified.
- Remove the agarose channels from the casting mold. Trim the edges of the agarose disc with a razor blade.
 - Put the channels in a 10 cm Petri dish immersed in water. They can be kept at 4 °C for 7 days.

3. Loading a Larva into a Channel to Record Behavior

NOTE: If storing channels at 4 °C, allow channels to come to RT before using for behavioral recording.

- Use a clean razor blade to cut a single channel from those prepared in Step 2 (**Figure 2A**).
- Use a pair of forceps to place the channel grooved-side-up on a glass coverslip (**Figure 2B**). The size and thickness of the coverslip depends on the specific scope used for imaging.
- Use fine forceps to select a larva from the agar/juice cap prepared in Step 1 and place into the cut channel (**Figure 2C**).
 - Do not squeeze the larva. Pick it up gently with the tip of the forceps tine. It is also possible to manipulate larva with a paintbrush with a fine point.
NOTE: This is important as larvae are very sensitive to touch. If they are not handled with care, nociceptive responses might dominate their behavior during the first few seconds of the experiment.
 - Wash the larvae by briefly submerging them in water.

3. After placing the larva onto the cut channel, use the tip of the forceps tine to gently tease the larva into the channel groove.
NOTE: For most applications the width of the larva and the width of the channel should match. The average width of larvae at different stages of development follows: 0 hr posthatch - 140 μm , 24 hr posthatch - 180 μm , 48 hr posthatch - 320 μm , 72 hr posthatch - 500 μm , 96 hr posthatch - 750 μm ⁸. Channels come in the following widths: 100, 150, 200, 250, and 300 μm , and all channels are 150 μm deep.
4. Use the tip of a forceps tine or paintbrush to adjust the position of the larva within the channel. It can be oriented in any direction: ventral up, ventral down, ventral to one side, depending on what structure is to be imaged.
4. Using forceps, gently flip the channel over such that the larva is now on the cover glass. Place one or two drops of water at the ends of the channel so that it fills with water (**Figure 2D**).
 1. Gently lift the sides of the channel to remove any air bubbles.
 2. Adjust the orientation of the larva. To do so, gently nudge the channel but not the cover glass to roll the larva. If this does not change the orientation, remove the cover glass and adjust larval position. The larva is ready to be imaged.
 3. Image the channel/coverslip on an inverted microscope. If imaging on an upright microscope, simply invert the channel/coverslip.

4. Measure Feature of Interest in Behavioral Recording

1. For a structure of interest (e.g., tail, CNS), measure a feature of interest (e.g., fluorescence intensity, location) over time.
NOTE: Structures such as the head and tail can be manually annotated. For example, the head can be identified as containing dark H-shaped mouth hooks, and the tail can be identified as containing posterior tracheal spiracles. This paper focuses on the nerve cord. Use the neuronal GAL4 line *elav-GAL4* to drive a *UAS-myr-GFP* transgene (*elav>GFP*)^{9,10} to fluorescently label the central nervous system (CNS). The CNS contains two anterior brain lobes attached to the nerve cord, which extends to the posterior (**Figure 3**).
2. Measure the pixel intensity of the nerve cord ($f_{(NC)}$) at each time point (t). Below describes a manual annotation approach, but this step could be automated.
 1. In Fiji (or similar software) in the 'Analyze' menu use the "Set Measurements ..." dialogue box to report the mean gray value and slice position.
 2. Manually draw a box in the center of the nerve cord in the first frame of the movie (**Figure 3**). This step could also be automated with image segmentation and registration algorithms.
 3. Use the "Measure" command in the 'Analyze' menu to report the average pixel intensity in the boxed region of interest. This generates a "Results" window.
 4. In the next frame, manually reposition the box without resizing by using the arrow keys. Use the "Measure" command again. The updated results will be shown in the "Results" window. Repeat this step for each frame of interest.
 5. Save the "Results" using the 'Save As...' command from the File menu. The results will be exported as an .xls file.

5. Analyze the Measurements

1. **Normalize each $f_{(NC)}$ value to a value between 0 and 1 ($z_{(t)}$).**
 1. In spreadsheet (or other program), open the Results.xls file. This file will have two columns representing frame number and average fluorescence intensity.
 2. Make a new column and use the formula $z_{(t)} = f_{(NC[t])} - \min(f_{(NC)}) / \max(f_{(NC)}) - \min(f_{(NC)})$ to generate a normalized fluorescence value corresponding to each time point.
2. **Determine the stride period for each stride in the recording.**
NOTE: A stride cycle is the unit of repetitive whole body motion for forward (and reverse) crawling. By convention a forward stride starts (and ends) with the forward movement of the tail, head, and other internal organs such as the gut or CNS¹.
 1. Manually inspect the behavioral recording and record the initiation times (i) of each stride ($i_{(Stride\ Cycle[n])}$).
NOTE: In the example below, forward movement of the CNS as the initiation of a stride cycle was used (**Figure 3**).
 2. For each stride calculate the period: $\Delta t_{(Stride\ cycle)} = i_{(Stride\ Cycle\ [n+1])} - i_{(Stride\ Cycle\ [n])}$.
3. **Calculate percentage of stride cycle elapsed for each time point of the recording.**
 1. Make an additional column in the spreadsheet file representing time elapsed since the start of a stride cycle (c). Set time since stride initiation to zero at the initiation of each stride ($c_{(Stride\ Cycle[n])} = 0$). Adjust times after initiation accordingly.
 2. Make an additional column in the spreadsheet file representing percent stride cycle elapsed (% stride cycle). Divide adjusted times by stride period and multiply by 100 to convert to percentage of stride cycle elapsed: $\% \text{ stride cycle} = (c_{(Stride\ Cycle[n])}) / (\Delta t_{(Stride\ cycle)}) * 100$.

6. Generate Polar Coordinate Plots to Represent Dynamics of Structures of Interest over the Crawl Cycle

1. In MATLAB, use the 'Import Data' dialogue in the Home tab to import the updated Results.xls.
2. Make a new array "Theta" containing percent stride cycle values (% stride cycle). ("Theta = Var1" where Var1 represents % stride cycle).
3. Make a new array "Rho" contain normalized fluorescence values ($z_{(t)}$). ("Rho = Var2" where Var2 represents $z_{(t)}$).
4. Use the 'polarplot' command to make polar coordinate plots with the percent stride cycle as the angle, and the fluorescence as the radius ("polarplot(Theta, Rho)").

Representative Results

This article describes a method for guiding *Drosophila* larval behavior using agarose channels and for measuring the dynamics of larval structures over a crawl cycle. Larvae in linear channels perform sustained bouts of rhythmic crawling (**Figure 3**). Because both larvae and channels are optically clear, channels can be used with larvae expressing fluorescent probes expressed in any structure of interest. We recorded larvae expressing GFP in all neurons (*elav-Gal4/+; UAS-myr-GFP/+*) and monitored the dynamic changes in fluorescence intensity in the nerve cord over the crawl cycle. We show that the CNS moves forward at nearly the same time as the larval head and tail (**Figure 4A-B**). As a wave of muscle contraction passes along the body axis, the CNS moves in and out of the plane of focus causing the fluorescence of the nerve cord to change (**Figure 4**). To quantify changes in nerve cord fluorescence intensity for several strides in several animals we represented the data on a polar coordinate plot (**Figure 4C**). Plotting the data on polar coordinate plots shows that the dynamics of the nerve cord fluorescence over the stride cycle follows a reproducible pattern.

Figure 1: Design of Linear Channels to Guide *Drosophila* Larval Crawling Behavior. (A) The design of the microfluidic device used to make linear agarose channels is shown. The widths of channels in this device vary from 100-300 μm by increments of 50 μm . The depth is 150 μm . (B) A *Drosophila* larva is loaded into an agarose channel. A dorsal view is shown with anterior (head) to the right. Scale bar = 200 μm . [Please click here to view a larger version of this figure.](#)

A. Cut channel.

B. Place channel groove side up on cover glass.

C. Add larva.

D. Invert channel onto cover glass and water.

Figure 2: Diagram Illustrating How to Load a Larva into a Channel. See protocol section 3 for details [Please click here to view a larger version of this figure.](#)

Figure 3: A Fluorescently-labeled *Drosophila* Larva Performs Sustained, Rhythmic, Linear Crawling when Placed into an Agarose Channel. At left, a schematic of a larva expressing GFP in all neurons (*elav>GFP*) is shown. A box shows the region where fluorescence intensity of the nerve cord (distinguished by its elongated morphology) can be measured. At right is an example of a larva crawling through a channel at one second intervals. A dorsal view is shown with anterior up. Arrows indicate the initiation of a stride. Scale bar = 200 μ m. [Please click here to view a larger version of this figure.](#)

Figure 4: Dynamics of Fluorescence Changes in Nerve Cord Over the Crawl Cycle are Presented on Polar Coordinate Plots. (A) A diagram of a single stride. Percentages refer to percent of stride cycle completed. By convention forward movement of the tail, head, and internal organs such as the CNS marks the initiation of a stride (or 0% of stride cycle). Note that the CNS (white) moves forward and backward, as well as up and down. A side view is shown with anterior to the right. (B) A kymograph shows the movement of the head, tail, and CNS. Note that the fluorescence intensity of the nerve cord during the stride cycle is dynamic. (C) A polar coordinate plot shows the dynamic changes in normalized fluorescence intensity of the nerve cord over the stride cycle. Each dot represents a normalized fluorescence intensity of a single larva at a single time point ($n = 3$ larvae, 3 strides each). [Please click here to view a larger version of this figure.](#)

Discussion

A microfluidic device was built to make linear agarose channels that can accommodate *Drosophila* larvae (Figure 1). When *Drosophila* larvae are placed in these linear agarose channels their behavioral repertoire is limited to crawling, which allows for detailed observation of the dynamics of larval structures over the crawl cycle.

A successful recording occurs when a larva perform a series of rhythmic strides (Figure 3). If this does not occur, check for obstacles like an air bubble in the channel, and check the health of the larva. Another important element of a successful recording is that the larva is oriented optimally to visualize structures of interest. If the larva is not oriented correctly, or if the larva crawls out of the channel, simply remove the coverslip and remount the larva. In our experience, ~20% of larvae initially mounted yield excellent behavioral recordings without adjustment.

In the past, measurements were taken of the position of larval structures such as the mouth hook, gut, and abdominal segments during crawling behavior. To visualize the movement of these structures over the crawling stride cycle, polar coordinate plots were generated. In this paper, the fluorescence intensity of the nerve cord was measured and polar coordinate plots used to visualize the dynamics of fluorescence over the crawling stride cycle (Figure 4). There are several advantages to representing the data on polar coordinate plots: it eliminates crawl speed as a variable, it can summarize data from many animals and many strides, and it allows visualization of both overall trends and variation in data¹¹. Notably, it is possible to measure the dynamics of any fluorescently-labeled structure of interest. In principle, this analysis is applicable to tracking any type of dynamic changes that occurs over a crawl cycle.

There is a wide array of applications for the methods described in this paper. In the past, linear agarose channels have been used to record larval behavior at the whole organism, segment and individual muscle levels¹. These data showed that larvae use a "visceral-pistoning" mechanism for both forward and reverse crawling, and they allowed the neuromuscular mechanism driving both forward and reverse crawling to be determined¹. In the future, researchers can use channels to study crawling in different genetic backgrounds. In addition, it should be possible to use channels to analyze the activity of larval neurons using calcium imaging during crawling. This should lead to the understanding of which neurons fire in phase with particular movements of the crawl cycle. Finally, there is no reason that channels must follow the linear design presented in this paper; using channels with different dimensions will no doubt help answer a variety of question about *Drosophila* larval locomotion and motor control as a whole.

Disclosures

The authors have nothing to disclose.

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