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LarvaSPA, a Method for Mounting Drosophila Larva for Long-Term Time-Lapse Imaging

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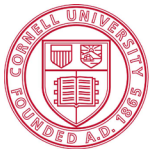

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Aaron Berard, Ph.D.
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Dear Aaron,

Thank you for giving us the opportunity to revise our manuscript. I am happy to submit the revised manuscript with the same title "LarvaSPA: a method for mounting *Drosophila* larva for long-term time-lapse imaging" for publication at JoVE.

In the revised manuscript, we addressed all concerns raised by the reviewers by adding new figure panels and revising the manuscript. We believe that the paper has been significantly improved and hope that the editor and reviewers will find our revised paper now suitable for publication.

Thank you for the consideration.

With best wishes,

Chun Han, Ph.D.

TITLE:

LarvaSPA, a Method for Mounting *Drosophila* Larva for Long-Term Time-Lapse Imaging

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SUMMARY:

This protocol describes a method for mounting *Drosophila* larvae to achieve longer than 10 h of uninterrupted time-lapse imaging in intact live animals. This method can be used to image many biological processes close to the larval body wall.

ABSTRACT:

Live imaging is a valuable approach for investigating cell biology questions. The *Drosophila* larva is particularly suited for in vivo live imaging because the larval body wall and most internal organs are transparent. However, continuous live imaging of intact *Drosophila* larvae for longer than 30 min is challenging because it is difficult to noninvasively immobilize larvae for a long time. Here we present a larval mounting method called LarvaSPA that allows for continuous imaging of live *Drosophila* larvae with high temporal and spatial resolution for longer than 10 h. This method involves partially attaching larvae to a coverslip using a UV-reactive glue and additionally restraining larval movement using a polydimethylsiloxane (PDMS) block. This method is compatible with larvae at developmental stages from second instar to wandering third instar. We demonstrate applications of this method in studying the dynamic processes of *Drosophila* somatosensory neurons, including dendrite growth and injury-induced dendrite degeneration. This method can also be applied to study many other cellular processes that happen near the larval body wall.

KEYWORDS:

long-term time-lapse imaging, live imaging, in vivo imaging, LarvaSPA, confocal microscopy, *Drosophila* larva, body wall, dendritic arborization, da neurons, neurodegeneration, neurodevelopment, cell biology

INTRODUCTION:

Time-lapse live imaging is a powerful method for studying dynamic cellular processes. The spatial and temporal information provided by time-lapse movies can reveal important details for answering cell biology questions. The *Drosophila* larva has been a popular in vivo model for investigations using live imaging because its transparent body wall allows for noninvasive imaging

of internal structures[1,2]. In addition, numerous genetic tools are available in *Drosophila* to fluorescently label anatomical structures and macromolecules[3]. However, long-term time-lapse imaging of *Drosophila* larvae is challenging. Unlike stationary early embryos or pupae, *Drosophila* larvae move constantly, necessitating immobilization for live imaging. Effective ways of immobilizing live *Drosophila* larvae include mounting in halocarbon oil with chloroform[4], anesthetizing using isoflurane or Dichlorvos solution[5], and compressing between the coverslip and the microscope slide[6]. Although some of these methods have been used for microscopy, none of them is effective for long-term live imaging. Other methods were developed for imaging body wall neurons in crawling larvae using conventional confocal microscopy or light-sheet microscopy[7–9]. However, these methods are not ideal for monitoring cellular dynamics due to the movement of the larvae.

New methods have been developed to achieve long-term time-lapse imaging of *Drosophila* larvae. Using a polydimethylsiloxane (PDMS) "larva chip", *Drosophila* larvae can be effectively immobilized through vacuum-generated suction in a specialized microchamber without anesthetization. However, this method does not offer high temporal resolution for cell biology studies and it has strict limitations on animal size[10]. Another method using an anesthetization device achieved live imaging of *Drosophila* larvae at multiple time points and has been applied to study neuromuscular junctions[11–16]. However, this method also does not allow for continuous imaging for longer than 30 min and requires using desflurane repeatedly, which can inhibit neural activity and affect the biological process studied[17,18]. Recently, a new method that combines microfluidic device and cryoanesthesia has been used to immobilize larvae of various sizes for short periods of time (minutes)[19]. However, this method requires specialized devices such as a cooling system and longer periods of immobilization require repeated cooling of the larvae.

Here we present a versatile method of immobilizing *Drosophila* larvae that is compatible with uninterrupted time-lapse imaging for longer than 10 h. This method, which we call "Larva Stabilization by Partial Attachment" (LarvaSPA), involves adhering the larval cuticle to a coverslip for imaging in a custom-built imaging chamber. This protocol describes how to make the imaging chamber and how to mount larvae at a variety of developmental stages. In the LarvaSPA method, the desired body segments are affixed to the coverslip using a UV-reactive glue. A PDMS cuboid additionally applies pressure to the larvae, preventing escape. The air and moisture in the imaging chamber ensure the survival of the partially immobilized larvae during imaging. Advantages of LarvaSPA over other techniques include the following: (1) It is the first method that allows for continuous live imaging of intact *Drosophila* larvae for hours with high temporal and spatial resolution; (2) The method has fewer limitations on larval size; (3) The imaging chamber and PDMS cuboids can be manufactured at a minimal cost and are reusable.

In addition to describing the larval mounting method, we provide several examples of its application for studying dendrite development and dendrite degeneration of *Drosophila* dendritic arborization (da) neurons.

PROTOCOL:

1. Making the imaging chamber

1.1. The metal frame can be constructed from an aluminum block in a typical machine shop. The specifications of the frame are illustrated in **Figure 1A**.

1.2. To construct the imaging chamber, seal the bottom of the metal frame using a long coverslip (22 mm x 50 mm) and UV glue (**Figure 1A**). Cure the UV glue using a hand-held UV lamp.

2. Making PDMS cuboids

2.1. Prepare the mold for PDMS cuboids.

2.1.1. Attach layers of packaging tape to the inner surface of a rectangular (80 mm x 55 mm) Petri dish or round cell culture plate. Use one layer (0.063 mm thick) for second instar larvae, 2 layers (0.126 mm thick) for early third instar larvae, or three layers (0.189 mm thick) for late third instar larvae (**Figure 1B**).

2.1.2. Cut the tape into strips of specific width with a razor blade: 1.5 mm for the 1-layer tape, and 2 mm for 2-layer or 3-layer tape. The width and thickness of the strip will determine the size of the larvae that the final PDMS cuboid can hold. Leave at least a 5 mm space between the two strips. Remove the tape layers covering the space (**Figure 1B**).

2.1.3. Remove dust from the inner surface of the plate using sticky tape. The mold is ready for use.

2.2. Prepare the PDMS mix.

2.2.1. Mix 7 g of PDMS base and 0.7 g of curing agent (10:1 ratio) thoroughly in a small container.

2.2.2. Place the container in a vacuum desiccator for at least 15 min to remove air from the mixture.

2.2.3. Slowly pour about 5.5 g of PDMS mixture onto the mold to reach a 1–2 mm thickness (**Figure 1B**).

2.2.4. Place the PDMS mixture in the vacuum desiccator again for at least 15 min to remove remaining air bubbles from the mixture. Break the last few bubbles with a pipette tip.

2.2.5. Cure the PDMS on a flat surface in a heat incubator at 65 °C for 2 h.

2.2.6. Use a razor blade to loosen the cured PDMS along the edge of the mold and detach it from the mold. Store the PDMS between two pieces of large sticky tape at room temperature.

2.2.7. For early and late third instar larvae, cut the PDMS into 8 mm x 2 mm x 1 mm cuboids

(along the dotted lines in **Figure 1B**) by positioning the groove created by the tape strip (step 2.1.2) at the center of the long side of the cuboid (**Figure 1B,C**). For second instar larvae, cut the cuboid to 8 mm x 1 mm x 1 mm.

3. Mounting larvae for long-term time-lapse imaging

3.1. Prepare the top coverslip for mounting.

3.1.1. Choose six PDMS cuboids with grooves matching the sizes of the larvae. Follow the recommended groove and size of PDMS based on steps 2.1.1, 2.1.2, and 2.2.7.

3.1.2. Remove dust from the surface of the PDMS with sticky tape.

3.1.3. Attach four pieces of double-sided tape (12 mm x 5 mm) on a long coverslip (22 mm x 50 mm) for fixing PDMS cuboids later. The spaces between the two pieces of double-sided tape should be the same as the width of the PDMS groove.

3.1.4. Apply a small drop (~1.2 µL) of UV glue into the groove of each PDMS cuboid and add six small drops of UV glue into the space between the double-sided tape on the coverslip.

3.2. Prepare the larvae for mounting.

3.2.1. Using a pair of forceps, clean the larvae in water to remove food from the body surface.

3.2.2. Place clean larvae on a small piece of moistened tissue paper in a small (35 mm) Petri dish without a lid. Place the small Petri dish into a large (60 mm) Petri dish containing a piece of dry tissue paper. In a chemical hood, apply 8–12 drops (160–240 µL) of isoflurane onto the dry tissue paper using a plastic transferring pipette and close the lid of the large Petri dish.

3.2.3. Wait 2–3 min while monitoring the larvae. Take out the larvae from the large Petri dish once their mouth hooks stop moving.

3.3. Mount the animals.

3.3.1. To image structures on the dorsal side of the animal, place the immobilized larvae onto the UV glue between the double-sided tape on the coverslip with the dorsal cuticle facing the coverslip.

3.3.2. Cover each larva with a PDMS block and fit the trunk of the larva into the groove of the PDMS. Leave the head and the tail of the larva outside the PDMS groove. Avoid blocking the spiracles of the larva by the glue.

3.3.3. Press down on the ends of the PDMS block onto the double-sided tape without applying force on the groove. Gently pull on the tail of the larva to flatten the cuticle under the PDMS.

3.3.4. Cure the UV glue for 4 min using a hand-held UV lamp at the high setting (at about 0.07 mW/mm^2).

CAUTION: Protect eyes with safety glasses while using the UV lamp.

3.3.5. Flip the coverslip upside down and repeat step 3.3.4.

3.3.6. Moisten a small piece of lens paper (15 mm x 30 mm) with 20 µL–30 µL of water. Place the moistened lens paper at the bottom of the imaging chamber (**Figure 1A**,**D**).

3.3.7. Place the coverslip on the chamber so that the larvae are facing the inside of the chamber. Adhere both ends of the coverslip to the metal surface using UV glue (**Figure 1A**,**D**). The dorsal side of the larvae is ready for imaging under confocal microscope (**Figure 1E**).

4. Imaging

4.1. Image larvae with an appropriate microscope. All results shown in this protocol (**Figure 2, Figure 3**, **Video S2**, **Video S3**, and **Video S4**) were acquired using a confocal system with a 40x (1.30 NA) oil objective.

5. Recovery of imaging chamber and PDMS cuboids

5.1. After imaging, remove the oil on the top coverslip using a lens paper. Detach the top coverslip from the metal frame by cutting into the space between the coverslip and the metal frame with a razor blade. The imaging chamber is ready for reuse.

5.2. Detach the PDMS cuboids from the top coverslip with forceps. Roll the PDMS cuboids on sticky tape to remove glue residue and dust. The PDMS cuboids are ready for reuse.

RESULTS:

The larva imaging chamber is constructed by gluing a custom-made metal frame and two coverslips together. The design of the metal frame is specified in **Figure 1A**. *Drosophila* larvae inside the chamber are adhered to the top coverslip with the aid of UV glue and PDMS cuboids. The groove on the PDMS cuboid and the double-sided tape the cuboid is attached to create the space to hold the larvae (**Figure 1B**,**C**). The PDMS also applies gentle pressure to flatten the larval body wall and physically restrict larval movement. Lastly, a small piece of wet lens paper is placed at the bottom of the chamber to provide moisture. This setup can immobilize *Drosophila* larvae for longer than 10 h for continuous imaging. Most of the animals are alive after 10 h and can be recovered to grow into the pupal stage. The imaging chamber can accommodate up to nine larvae at once. **Figure 1D** shows six late third instar larvae mounted in the chamber. The trunks of the larvae are fixed while their heads and tails are free to move (**Figure 1E** and **Video S1**). This method has been successfully used to image second instar to wandering third instar larvae with continuous high-resolution imaging for up to 15 h. The chamber is designed for imaging using

upright microscopes, but the setup also works for inverted microscopes by simply flipping the chamber.

Here we demonstrate the application of LarvaSPA in studying neuronal dendrite dynamics and dendrite degeneration using class IV da (C4 da) neurons as a model (**Figure 2**, **Figure 3**, **Videos S2**–**S4**). C4 da neurons are somatosensory nociceptors located on the larval body wall, whose dendrites innervate the larval epidermis[1,20–22]. C4 da dendrites exhibit highly dynamic growth behaviors throughout the larval development, resulting in complete coverage of the body surface or space-filling[23]. C4 da neurons have also been successfully used to study dendrite degeneration and regeneration after physical injury[24–27].

For imaging dendrite dynamics, larvae ranging from 48 h after egg laying (AEL) at second instar to 120 h AEL at wandering third instar were mounted in the imaging chamber for time-lapse imaging using point-scanning confocal microscopy. Time-lapse movies were taken with a 3 min interval to capture growth behaviors of C4 da dendrites labeled by *ppk-CD4-tdTom* or *UAS-CD4-tdTom* driven by *ppk-Gal4*[6]. Throughout the imaging period (up to 12 h), the high order dendrite branches of C4 da neurons exhibited complex growth behaviors, including extension, retraction, branch formation, and branch elimination (**Figure 2A**–**2F**), indicating the health of the neurons. Our movies also captured homotypic dendro-dendrite repulsions in which dendrite tips retracted after contacting other dendrites (**Figure 2F**). Overall, these results demonstrate that time-lapse imaging using LarvaSPA is effective for studying neurodevelopment.

To image dendrite degeneration, we used a MaiTai laser to sever primary dendrites near C4 da neuronal cell bodies. The larvae were recovered and mounted in the chamber for imaging starting from 1.5 h after injury (AI) (**Figure 3**, **Video S4**). The movies recorded key events during dendrite degeneration, including dendrite swelling, dendrite fragmentation, and clearance of dendrite debris. In the same experiment, the larval fat body was also engineered to secrete Annexin V-GFP (AV-GFP), a sensor that labels externalized phosphatidylserine (PS) on the cell surface[27]. We observed specific labeling of the degenerating dendrites by AV-GFP (**Figure 3**), suggesting that PS was exposed on the surface of degenerating dendrites to serve as an eat-me signal for subsequent clearance by phagocytosis[27].

FIGURE LEGENDS:

Figure 1: The imaging chamber for LarvaSPA mounting. (**A**) Diagrams of the imaging chamber with detailed specifications, showing both the top view and the side view. The light blue shading in the top view indicates the moistened lens paper. The chamber is sealed by a top coverslip and a bottom coverslip. The position of a mounted larva is illustrated. (**B**) Diagrams of the PDMS mold (the top view) and the mold after filling with the PDMS mixture (side view). Strips in grey indicate two 1-layer, two 2-layer, and two 3-layer tape strips, respectively. The yellow shading in the side view indicates the PDMS mixture in the mold. The dotted lines indicate where to cut the cured PDMS. The side view of the diagram is not drawn to scale. (**C**) Photographs showing a top view and a side view of a PDMS cuboid. Scale bar = 1 mm. (**D**) Photographs showing an imaging chamber before mounting, and an imaging chamber with six immobilized late third instar *Drosophila* larvae mounted dorsal side up. Scale bar = 10 mm. (**E**) A closer view of a larva in (**D**).

Scale bar = 1 mm.

Figure 2: Time-lapse imaging of dendrite dynamics. (**A**-**F**) Selected frames from time-lapse movies of class IV da neuron dendrites at specific time points after the start of imaging. Images of the larvae were taken 48 h AEL (**A** and **F**), 72 h AEL (**B**), 96 h AEL (**C**), and 120 h AEL (**D**). The neurons are labeled by *ppk>CD4-tdTom* (**A**, **D**, **E**, and **F**) or *ppk >CD4-tdTom* (**B** and **C**). Blue arrows indicate dendrite retractions compared to the previous time point. Yellow arrows indicate dendrite extensions compared to the previous time point. (**E**) Dendrite morphology at the end of 12 h of imaging. (**F**) Consecutive frames with 3 min intervals in a movie illustrating how a dendrite branch in a second instar larva extended (yellow arrows) and subsequently retracted (blue arrows) after it made contact with another dendrite.

Figure 3: Time-lapse imaging of dendrite degeneration and exposure of an eat-me signal. Selected frames from a time-lapse movie of degenerating dendrites of a class IV da neuron after laser injury. Dendrites were labeled by *ppk>CD4-tdTom*. The eat-me signal PS on degenerating dendrites was detected by Annexin-GFP (AV-GFP), which is secreted by the fat body. Yellow arrows point to the branches showing AV-GFP labeling. Blue arrows point to dendrites undergoing fragmentation.

Video S1: A third instar larva is immobilized in the notch of a PDMS cuboid.

Video S2: Dendrites extend and retract dynamically in a second instar larva.

Video S3: Dendrites extend and retract dynamically in a wandering third instar larva.

Video S4: Dendrites degenerate and expose phosphatidylserine after laser injury in a third instar larva.

DISCUSSION:

Here we describe LarvaSPA, a versatile method of mounting live *Drosophila* larvae for long-term time-lapse imaging. This method does not require recovering or remounting larvae, enabling uninterrupted imaging. It is therefore ideal for tracking biological processes that take hours to complete, such as dendrite degeneration and regeneration. This method can be also used for imaging intracellular calcium dynamics and subcellular events such as microtubule growth. As the larval body wall is stable during the imaging, the spatial and temporal resolution can be adjusted to fit the imaging application at hand (e.g., to track fast subcellular vesicle movement or to monitor slow global changes of neuronal branch patterns). In addition, this method is compatible with larvae at various developmental stages and does not require special equipment. Thus, LarvaSPA can potentially be used by many *Drosophila* labs to address diverse questions.

Factors affecting the success of the method, including the nature of the PDMS cuboid and the developmental stage of the larvae

The size of the PDMS cuboid and the depth of the groove need to match the size of the larvae. A PDMS cuboid too wide for the larvae can cover the head and the tail and cause hypoxia. However,

a narrow PDMS cuboid may not be effective in preventing the larvae from moving. A too deep groove similarly would not generate enough pressure to restrain the movement of the larvae. A too shallow groove would not hold enough glue to adhere the larvae to the coverslip. Based on our experience, the following dimensions of the PDMS and the groove are recommended for various stages of larvae: 8 mm x 1 mm x 1 mm PDMS cuboids with 1.5 mm x 1 mm x 0.063 mm grooves for second instar larvae (~48 h AEL); 8 mm x 2 mm x 1 mm PDMS cuboids with 2 mm x 2 mm x 0.126 mm grooves for early third instar larvae (~72 h AEL); 8 mm x 2 mm x 1 mm PDMS cuboids with 2 mm x 2 mm x 0.189 mm grooves for late third instar larvae (~96 h AEL or older).

Wandering third instar larvae (at or older than 120 h AEL) move less compared to younger ones and require less moisture to survive once mounted in the chamber. They also have a thicker cuticle and can endure more physical stress. Therefore, experiments using wandering third instar larvae have the highest success rate. In our hands, more than 80% of wandering third instar larvae survived and remained immobile 12 h after mounting. To prevent larvae from pupariating during imaging, we recommend mounting the larvae between 96 h to 120 h AEL. Younger third instar larvae have a greater chance of escape or death during imaging. Typically, at least 2 out of 6 young third instar larvae mounted in the same chamber would survive and remain immobile 10 h after mounting. Our experience with second instar larvae is limited and the survival rate is hard to estimate. Nevertheless, we have successfully imaged second instar larvae for 7 h using this method.

Potential concerns of long-term imaging

Although LarvaSPA has been very useful for imaging dendrite growth dynamics and degeneration, there are a few potential caveats to consider. First, in our current method, the larvae are deprived of food for the whole duration of imaging, which may lead to starvation-induced responses in many tissues. We have observed formation of granular structures in epidermal cells around 10 h after mounting, which may result from autophagy. Therefore, the results obtained in the first few hours of imaging should be more physiologically relevant. Results over longer time scales require more cautious interpretation. To minimize this concern, our procedure could potentially be adapted to allow for larval feeding. Second, our method may interfere with the rapid growth of younger larvae due to the physical constraint and the lack of nutrient intake. However, this concern may not apply to older animals. For example, wandering third instar larvae can turn into pupae even after 12 h of continuous imaging, making this method ideal for investigations of early metamorphosis. Third, imaging of deeper structures such as the fat body and the gut presents some challenges. A main reason is that deeper tissues often move during imaging and therefore require motion correction in postprocessing. Additionally, mismatches of refractive indices in the light path can cause spherical aberration when imaging deeper tissues. While oil objectives are appropriate for imaging da neurons because their dendrites are within 15 μm from the body surface, water immersion objectives could be better choices to correct refractive-index mismatches for imaging deeper inside the larvae. Fourth, because C4 da neurons can be activated by UV light[28], using UV light during the larval mounting can potentially alter C4 da neuron physiology. Although the light intensity we recommend is far below the levels that robustly activate C4da neurons[28], results related to C4da neuronal activity need to be cautiously interpreted. The UV glue has been used for imaging a wide variety of biological samples[29–31] and

does not cause obvious toxicity to the larvae in our hands. Lastly, one should consider the proper microscope setup for in vivo live imaging. For example, resonance scanners and spinning disc confocal microscopes are better options for long-term live imaging because they cause less phototoxicity and photobleaching; multi-photon microscopy is more effective for imaging deeper internal structures in the larvae; long-term imaging could be prone to sample or focus drifting, which could potentially be corrected by post-imaging processing.

The most common causes of failure for LarvaSPA and the recommended solutions to address them

1. Larvae move too much or escape: Two common reasons could contribute to this problem. The first is that the PDMS groove may be too shallow or too deep for the larvae. Trying another PDMS cuboid with a different groove depth may solve the problem. The second reason is that there is too much moisture in the chamber, weakening the UV glue. Reducing the volume of water added to the lens paper in the chamber may help immobilize the larvae. In addition, try to image only the segments covered by the PDMS cuboid, because the head and the tail are free to move.

2. Larvae die during imaging: If a larva dies soon after imaging, it is likely that it was exposed to too much anesthetic. Properly anesthetized larvae should wake up after mounting and show motions including mouth hook extension and retraction and dorsal vessel contraction. To solve this problem, less isoflurane could be used to anesthetize larvae. Transfer a larva out of the anesthesia Petri dish immediately after the mouth hook stops moving. If the larva dies about one hour after imaging, it is usually because of dehydration. Remember to place a piece of moistened lens paper in the imaging chamber before sealing. Another common reason of lethality is that the UV glue blocks the spiracles. Try to limit UV glue to the middle segments of the larva and avoid covering the head and the tail. An overly wide PDMS cuboid can also easily cause the UV glue to block the spiracles.

3. Folds on the body wall interfere with imaging: To avoid generating folds during mounting, it is important to fully immobilize the larvae during the anesthesia step. When a larva is paralyzed, it is easier to straighten and stretch the body. For animals older than 96 h AEL, gently dragging the tails before curing the UV glue can effectively reduce fold formation. Dragging the tails of young larvae is not recommended because their body walls are fragile.

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DISCLOSURES:

The authors declare no competing interests.

REFERENCE:

1. Grueber, W. B., Jan, L. Y., Jan, Y. N. Tiling of the Drosophila epidermis by multidendritic sensory neurons. *Development.* **129** (12), 2867–2878 (2002).
2. Schmid, A. et al. Activity-dependent site-specific changes of glutamate receptor composition in vivo. *Nature Neuroscience.* **11** (6), 659–666 (2008).
3. Venken, K. J., Simpson, J. H., Bellen, H. J. Genetic manipulation of genes and cells in the nervous system of the fruit fly. *Neuron.* **72** (2), 202–230 (2011).
4. Daniele, J. R., Baqri, R. M., Kunes, S. Analysis of axonal trafficking via a novel live-imaging technique reveals distinct hedgehog transport kinetics. *Biology Open.* **6** (5), 714–721 (2017).
5. Csordas, G. et al. In vivo immunostaining of hemocyte compartments in Drosophila for live imaging. *PLoS One.* **9** (6), e98191 (2014).
6. Han, C., Jan, L. Y., Jan, Y. N. Enhancer-driven membrane markers for analysis of nonautonomous mechanisms reveal neuron-glia interactions in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America.* **108** (23), 9673–9678 (2011).
7. He, L. et al. Direction Selectivity in Drosophila Proprioceptors Requires the Mechanosensory Channel Tmc. *Current Biology.* **29** (6), 945–956 (2019).
8. Heckscher, E. S., Lockery, S. R., Doe, C. Q. Characterization of Drosophila larval crawling at the level of organism, segment, and somatic body wall musculature. *The Journal of Neuroscience.* **32** (36), 12460–12471 (2012).
9. Vaadia, R. D. et al. Characterization of Proprioceptive System Dynamics in Behaving Drosophila Larvae Using High-Speed Volumetric Microscopy. *Current Biology.* **29** (6), 935–944 (2019).
10. Mishra, B. et al. Using microfluidics chips for live imaging and study of injury responses in Drosophila larvae. *Journal of Visualized Experiments.* **84**, e50998 (2014).
11. Andlauer, T. F., Sigrist, S. J. In vivo imaging of the Drosophila larval neuromuscular junction. *Cold Spring Harbor Protocols.* **2012** (4), 481–489 (2012).
12. Andlauer, T. F., Sigrist, S. J. Building an imaging chamber for in vivo imaging of Drosophila larvae. *Cold Spring Harbor Protocols.* **2012** (4), 476–480 (2012).
13. Andlauer, T. F., Sigrist, S. J. Quantitative analysis of Drosophila larval neuromuscular junction morphology. *Cold Spring Harbor Protocols.* **2012** (4), 490–493 (2012).
14. Andlauer, T. F., Sigrist, S. J. In vivo imaging of Drosophila larval neuromuscular junctions to study synapse assembly. *Cold Spring Harbor Protocols.* **2012** (4), 407–413 (2012).
15. Zhang, Y. et al. In vivo imaging of intact Drosophila larvae at sub-cellular resolution. *Journal of Visualized Experiments.* **43**, e2249 (2010).
16. Fuger, P., Behrends, L. B., Mertel, S., Sigrist, S. J., Rasse, T. M. Live imaging of synapse development and measuring protein dynamics using two-color fluorescence recovery after photo-bleaching at Drosophila synapses. *Nature Protocols.* **2** (12), 3285–3298 (2007).
17. Sandstrom, D. J. Isoflurane depresses glutamate release by reducing neuronal excitability at the Drosophila neuromuscular junction. *The Journal of Physiology.* **558** (Pt 2), 489–502 (2004).
18. Sandstrom, D. J. Isoflurane reduces excitability of Drosophila larval motoneurons by activating a hyperpolarizing leak conductance. *Anesthesiology.* **108** (3), 434–446 (2008).

19. Chaudhury, A. R. et al. On chip cryo-anesthesia of Drosophila larvae for high resolution in vivo imaging applications. *Lab on a Chip.* **17** (13), 2303–2322 (2017).
20. Han, C. et al. Integrins regulate repulsion-mediated dendritic patterning of drosophila sensory neurons by restricting dendrites in a 2D space. *Neuron.* **73** (1), 64–78 (2012).
21. Kim, M. E., Shrestha, B. R., Blazeski, R., Mason, C. A., Grueber, W. B. Integrins establish dendrite-substrate relationships that promote dendritic self-avoidance and patterning in drosophila sensory neurons. *Neuron.* **73** (1), 79–91 (2012).
22. Grueber, W. B. et al. Projections of Drosophila multidendritic neurons in the central nervous system: links with peripheral dendrite morphology. *Development.* **134** (1), 55–64 (2007).
23. Poe, A. R. et al. Dendritic space-filling requires a neuronal type-specific extracellular permissive signal in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America.* **114** (38), E8062–E8071 (2017).
24. Han, C. et al. Epidermal cells are the primary phagocytes in the fragmentation and clearance of degenerating dendrites in Drosophila. *Neuron.* **81** (3), 544–560 (2014).
25. Song, Y. et al. Regeneration of Drosophila sensory neuron axons and dendrites is regulated by the Akt pathway involving Pten and microRNA bantam. *Genes, Development.* **26** (14), 1612–1625 (2012).
26. Stone, M. C., Albertson, R. M., Chen, L., Rolls, M. M. Dendrite injury triggers DLK-independent regeneration. *Cell Reports.* **6** (2), 247–253 (2014).
27. Sapar, M. L. et al. Phosphatidylserine Externalization Results from and Causes Neurite Degeneration in Drosophila. *Cell Reports.* **24** (9), 2273–2286 (2018).
28. Xiang, Y. et al. Light-avoidance-mediating photoreceptors tile the Drosophila larval body wall. *Nature.* **468** (7326), 921–926 (2010).
29. Desjardins, M. et al. Awake Mouse Imaging: From Two-Photon Microscopy to Blood Oxygen Level-Dependent Functional Magnetic Resonance Imaging. *Biological Psychiatry: Cognitive Neuroscience and Neuroimaging.* **4** (6), 533–542 (2019).
30. Huang, C. et al. Long-term optical brain imaging in live adult fruit flies. *Nature Communications.* **9** (1), 872 (2018).
31. Low, R. J., Gu, Y., Tank, D. W. Cellular resolution optical access to brain regions in fissures: imaging medial prefrontal cortex and grid cells in entorhinal cortex. *Proceedings of the National Academy of Sciences of the United States of America.* **111** (52), 18739–18744 (2014).

Figure 1

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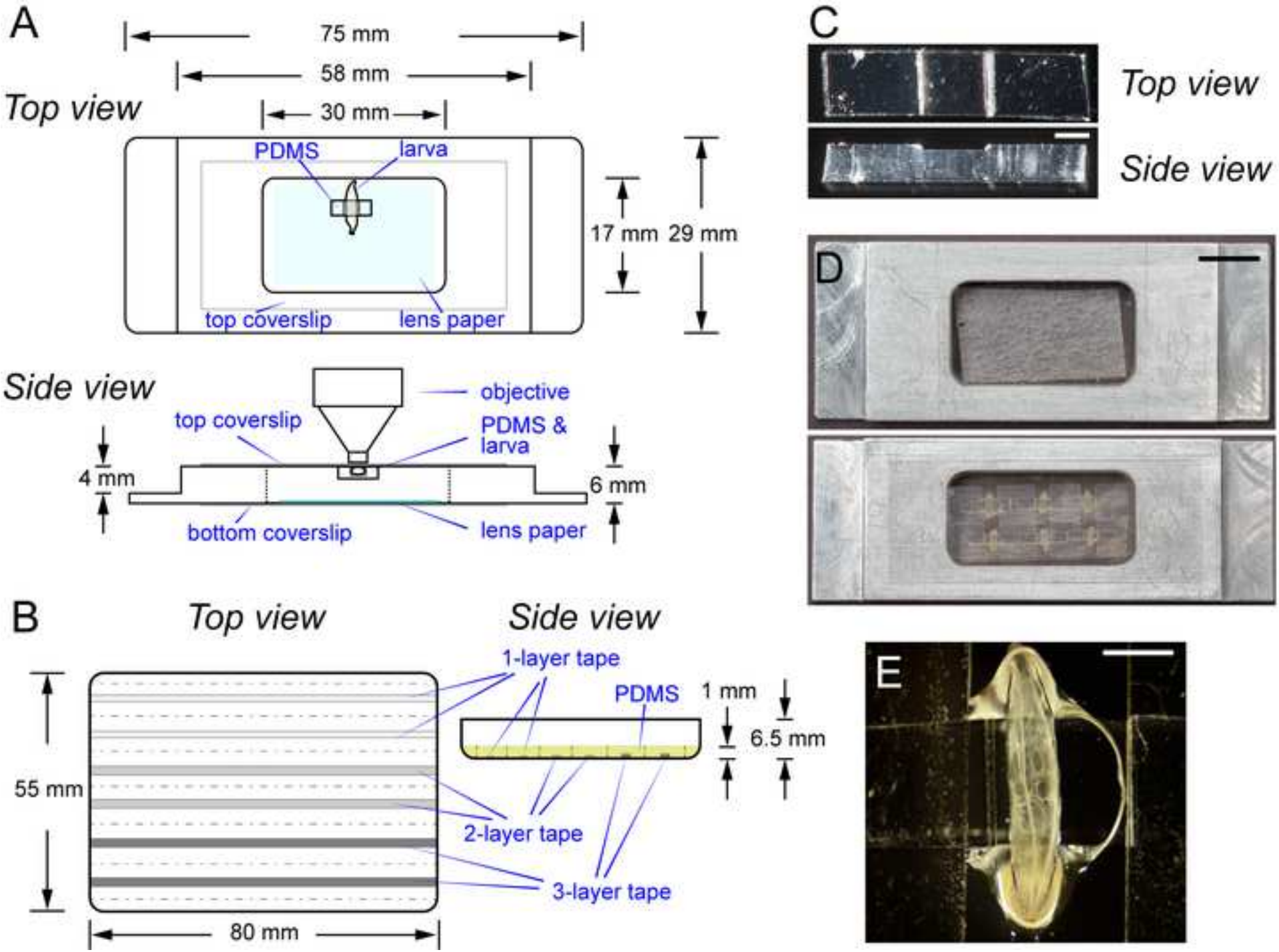

Figure 2

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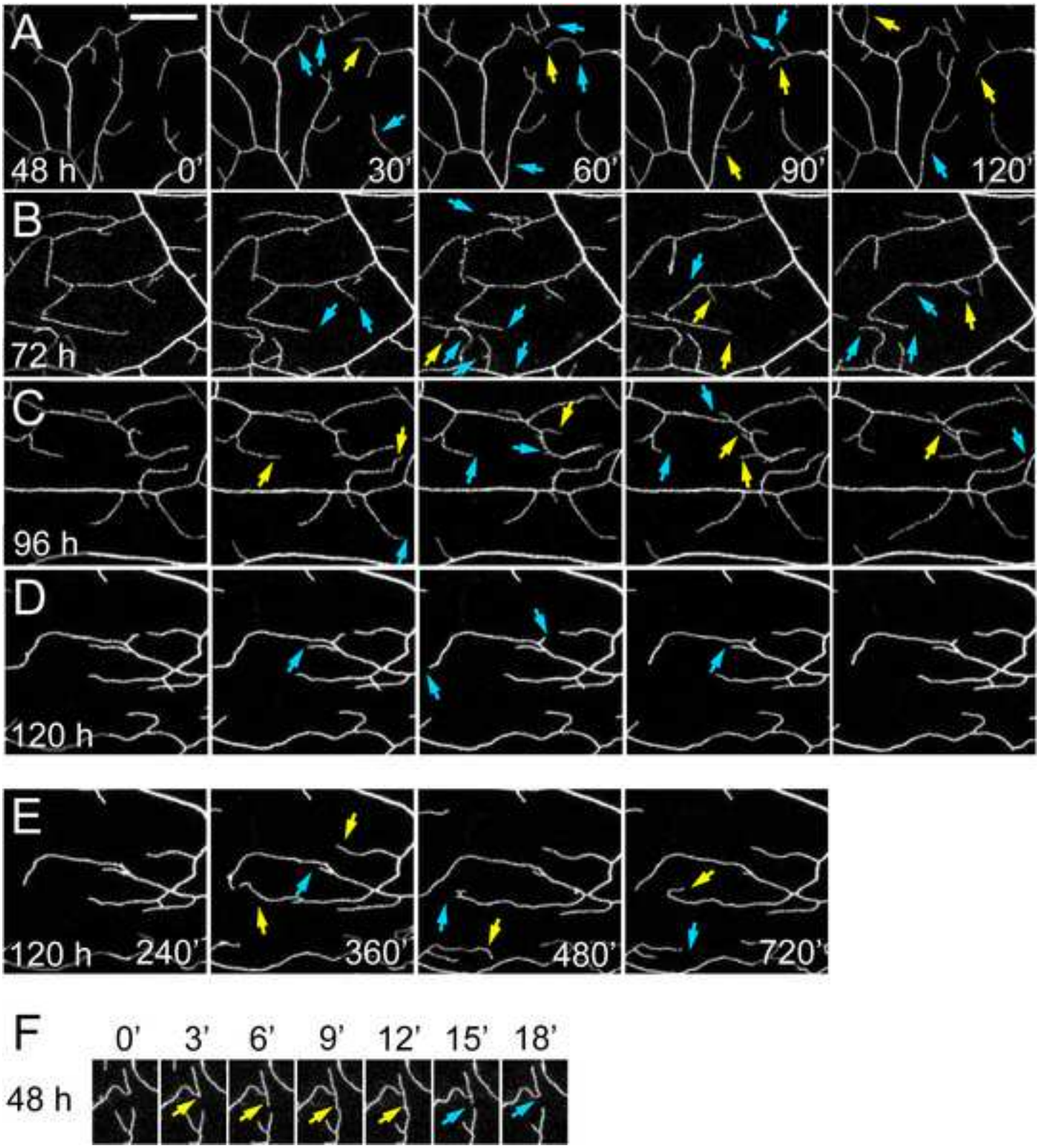

Figure 3

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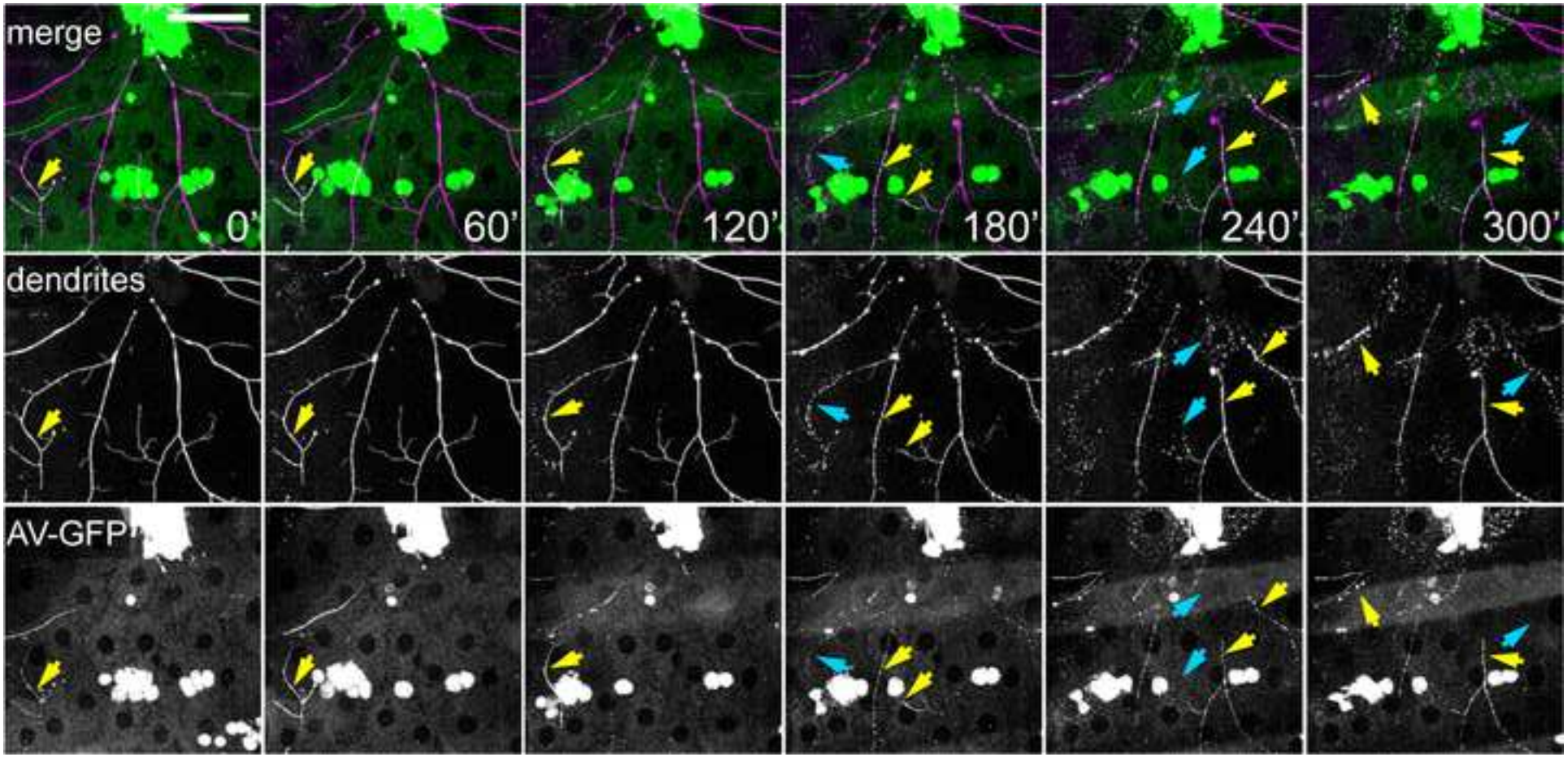

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Video S4

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
6061 Aluminum bars	McMaster-Carr	9246K421	
3M double-sided tape	Ted Pella, Inc.	16093	
3M Scotch Packaging tape	3M		1.88"W x 22.2 Yards
DUMONT #3 Forceps	Fisher Scientific	50-241-34	
Glass coverslip	Azer Scientific	1152250	
Isoflurane	Midwest Veterinary Supply	193.33161.3	
Leica Confocal Microscope	Leica	SP8 equipped with a resonant scanner	
Lens paper	Berkshire	LN90.0406.24	
Petri dishes (medium)	VWR	25373-085	
Petri dishes (small)	VWR	10799-192	
Razor blade	Ted Pella, Inc.	121-20	
Rectangular petri dish	VWR	25384-322	
SYLGARD 184 kit (PBMS kit)	Electron Microscopy Sciences	24236-10	
Transferring pipette	Thermo Fisher Scientific	1371126	
UV glue	Norland products	#6106, NOA 61	Refractive Index 1.56
UV lamp (Workstar 2003)	Maxxeon	MXN02003	
Vacuum desiccator	Electron Microscopy Sciences	71232	
Wipes	Kimberly-Clark	Kimwipes	

We greatly appreciate the reviewers' constructive suggestions. We have addressed the issues raised by the reviewers by adding new figure panels and revising the manuscript. We believe that the paper has been significantly improved and hope that the editor and reviewers will find our revised paper now suitable for publication. The following are our point-by-point responses to reviewers' comments.

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have thoroughly proofread the manuscript before submission.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Response: We corrected this.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts (without braces) after the appropriate statements.

Response: We corrected this.

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For example: 3M, Kimwipe, Leica

Response: We corrected this.

Protocol:

1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: We corrected this.

Figures:

1. Figure 2: Please include a space between all numbers and their corresponding units, and use 'h' instead of 'hr'.

Response: We corrected this.

References:

1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
Response: We applied the JoVE style downloaded from Endnote website.

2. Please do not abbreviate journal titles.
Response: We applied the JoVE style downloaded from Endnote website.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
Response: We corrected this.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The authors demonstrate an efficient method to immobilize a second or third instar Drosophila larva to image cells close to the body wall. They focus on structural changes of neuronal dendrites to introduce their method. There are more advanced techniques existing, eg SCAPE, but for the experiments suggested in the manuscript, this method is sufficient, less complicated and allows a higher throughput because several larvae can be mounted in parallel. Additional advantages of the method are long continuous imaging (>10h) without using anesthetics as well manufacturing of a reusable imaging chamber at low cost. The protocol is described clear and they highlight problems of the method and suggest solutions.

Major Concerns:
none

Minor Concerns:

- Title: include, that this method is for cells in the body wall
Response: Although the specific imaging examples shown in our manuscript were all done on the larval body wall, our mounting method is in fact compatible with imaging deeper into the body. For example, we have successfully used this method for imaging muscles, the fat body, and the salivary gland. However, we agree with the reviewer that imaging deeper tissues presents more challenges. In the revised discussion section, we discussed sources of the challenge (sample movement and spherical aberration) and suggested solutions for these challenges.

- Elaborate if other applications are possible (e.g. functional imaging, imaging sub-cellular mechanisms)
Response: Thanks for the suggestion. In the revised discussion, we now included the possibility of doing calcium imaging and imaging sub-cellular events.

- *Citations: [4-7] revisit the statements in the manuscript and compare with the cited paper*
Response: We have revised the reference to better match the methods and cited papers.

- *Consistency using imaging chamber, metal chamber or metal frame would improve reading quality*
Response: We replaced "metal chamber" with "metal frame" to keep the consistency. We kept "imaging chamber" to refers to "metal frame" with the bottom sealed by a coverslip.

- *Step 1.1.2 (line 98) and step 2.3.3 (line 127) are referred to, but do not exist in manuscript*
Response: We corrected this.

- *Using the UV glue brought up concern about its toxicity and matching the refraction index during imaging, especially if the study would focus on cells deeper in the tissue or focus on intracellular mechanisms. It would be helpful if the imaging through the glue (even it is just a thin layer) could be discussed in more detail.*
Response: The refractive index of cured glue matches those of the coverslip and the immersion oil and therefore should be appropriate for oil objectives. We added the refractive index of the glue in the material table. In the revised manuscript, we discussed how refractive-index mismatches may affect imaging of shallow and deep tissues. The UV glue has been widely used in imaging, and we have not observed cytotoxicity in our experiments. We added this information in the manuscript.

- *Introducing feeding, as mentioned in the manuscript, would be a big advantage for the long imaging time. If the authors feel comfortable discussing this point more in detail, the usefulness of the method would be even bigger, but adding this information is not absolutely necessary for the quality of the method.*
Response: We agree that introducing feeding could potentially be very useful. However, we have not explored this idea enough to give comments on its feasibility.

- *Adding a schematic how to prepare the PMDS molt would be helpful*
Response: As suggested, we have added a schematic to illustrate how to prepare the PDMS cuboid in Figure 1B.

- *The authors mentioned that the PMDS block and imaging chamber can be reused. It would be helpful to add advice about cleaning without damaging the devices.*
Response: We described details of the recovery of the imaging chamber and PDMS blocks in a newly added step 5 in the protocol.

- *Figure 3 (bottom) the AV-GFP labeling (yellow arrows) are not clearly identifiable*
Response: We tried to adjust the brightness and contrast of the green channel to make AV-GFP signals more identifiable in Figure 3.

Reviewer #2:

Manuscript Summary:

This manuscript provides a detailed protocol for imaging immobilized Drosophila larvae for up to 10 hours. Currently, methods for imaging intact larvae are limited to ~30 mins, and there is no suitable method for longer-term live imaging in this system. The method discussed in this manuscript provides compelling demonstration of its effectiveness in achieving long term imaging by showing successful time lapse imaging of second/third instar Drosophila larvae for up to 10 hours.

Overall, the protocol detailed in this manuscript is straightforward and easy to follow. The authors provide thorough explanations and cautionary points about factors that can impact the success of this method, as well as tips for troubleshooting. However, there are some areas that should be addressed to improve the clarity and reproducibility of the LarvaSPA method.

Major Concerns:

1. As the authors point out, UV light can activate the Class IV da neurons imaged in proof of concept experiments. The LarvaSPA method requires curing the UV glue for 4 mins using UV light. Therefore, the authors should caution for potential experimental confounds of the UV exposure during such experiments as injury and other activity-dependent processes.
Response: The 365nm UV light intensity we used was about 0.07 mW/mm^2. According to Xiang et al. (2010), 0.07 mW/mm^2 of 340 nm UV light causes a slight increase of firing frequency of Class IV da neurons, while 0.07 mW/mm^2 of 380 nm UV light does not change the firing frequency (doi:10.1038/nature09576, Figure 2h and Figure S6). Therefore, the wavelength and the intensity of UV light we used would unlikely activate the Class IV da neurons. However, we have added discussion about the potential concern of using UV light to call for a stronger caution.

2. Important, the authors suggest the imaging results obtained in the first few hours are the most physiologically relevant. Although the authors propose ways to circumvent this limitation by feeding larvae, a stronger caution to the reader should be made about results over longer time scales perhaps being influenced by declining health and secondary factors.
Response: We have reiterated this concern in the discussion.

3. The part of the manuscript detailing PDMS cuboids and larva mounting can be improved to enable the reader to more fully understand the entire process. The authors may consider the following to improve this area:

A. Schematic: It would be helpful to draw schematic graphs of 1) a top view of strips cut out on the rectangular petri dishes; and 2) a side view of the PDMS liquid covering the 3M strips on the bottom of petri dishes.

Response: Thanks for the suggestion. In Figure 1, we added a schematic of the rectangular petri dish as suggested, including a top view and a side view.

B. Extra labeling to figures: It would improve and add clarity to the descriptions by labelling the size and distance of the cuboids and grooves shown in Figure 1B and C. In addition, adding time stamps in video S2, S3 and S4 would help the reader to understand the time scale and dynamics of the dendritic development shown.
Response: A scale bar has been added in images of the PDMS cuboid and the imaging chamber. A time stamp has been added to the Movie S2, S3, and S4.

4. Fig 2 shows serial imaging of dendritic extension and retraction. Out of curiosity, is it possible to characterize these structural scaling strategies by comparing de novo vs. recuring branching and comparing branching of higher and lower dendritic orders based on current imaging data? Perhaps the authors can comment on this in the manuscript.
Response: This is a very interesting possibility. Quantification of the branching events turns out to be a significant endeavor. We are still in the process of developing effective algorithms to analyze dendrite branching dynamics. We hope to be able to report the analysis in the future.

5. Finally, in the Discussion, the authors suggest using older third instar larva to maintain higher survival rate during long term imaging. However, old 3rd instar larva might start to molt during the imaging session. Can the authors comment on the optimal timing and limits of imaging third instar larvae to ensure molting is avoided? Does the molting process have any impact on dendritic mobility in the DA neuron?
Response: When wandering larvae molt under microscope, changes of the dendrite pattern are easy to tell from the movies. During molting, dendrites become stretched along the dorsal-ventral axis and compressed along the anterior-posterior axis because segments become narrower. Meanwhile, cell bodies sink deeper inside the larva. Any part of the movie that is not desired can be trimmed easily. To avoid imaging pupae, one can mount larvae that are 96 h to 120 h after egg laying so that the animals will remain larvae at the end of the imaging session.

Minor Concerns:

1. Abstract: "immobilizing" should be "immobilize"
Response: We corrected this.

2. Line 42: 'due' - 'due to'
Response: We corrected this.

3. Page 8: "wondering" should be "wandering"
Response: We corrected this.

4. Figure 1: The appearance of the imaging chamber, particularly panel B, can be improved.
Response: We have improved the quality of images for PDMS cuboids and imaging chamber.

5. Final minor point - adding a bit more detail about how other labs can establish this approach in their own labs, including more information about the chamber, would be useful.
Response: We have tried to cover all the details that we think are important. Hopefully, a video recording of the key steps will help other labs to implement this method.

Reviewer #3:

Manuscript Summary:
This protocol provides a highly useful method to perform log-term timelapse imaging of Drosophila larvae. Under normal conditions, larvae can only be imaged for short time periods due to hypoxic conditions. The authors devised a clever and simple solution for larval long-term imaging of up to 10h. In comparison to other methods using microfluidic chambers, the described approach stands out by its ease of use and efficiency. This is a major advance and highly useful for this field. The protocol describes in detail how to build the imaging chamber in a step-wise manner and gives illustrative examples of results. I strongly recommend the publication of this protocol.

Major Concerns:
none

Minor Concerns:

1. It is not quite clear to me what the dimensions of the aluminum block are, as Fig. 1 shows a schematic of the block with PDMS and lens paper attached. I guess the aluminum block needs a rectanglular hole and has a different thickness on the edges. I would be nice to get the exact specifications for it The cover slip glued to the metal holder is not indicated. Maybe images of the individual components before assembly would help as well.
Response: We added images of imaging chamber before and after assembly in Figure 1D. Also, we included the top and bottom coverslip in the schematic graph of imaging chamber in Figure 1A. Hopefully these will help improve the clarity of the setup.

2. In 2.2.3, mention that the PDMS mixture has to cover the double sided tape strips to make a chamber of the correct depth.
Response: We added a schematic graph illustrating how to make PDMS cuboids in Figure 1B. We hope it will help.

3. Fig 1B. A scale bar would be helpful to assess the PDMS chamber dimensions
Response: A scale bar has been added to the images of PDMS cuboids and imaging chamber.

4. In 3.2.2., isofluorane is used, which is highly volatile and usually need a vaporizer to be applied. Could cold anesthesia be an alternative if isofluorane is not available?
Response: We have not extensively tested other anesthesia methods. Cold anesthesia might work if the larvae are quickly immobilized in the cured UV glue before they crawl away.

5. In 3.3.4, it mentions that one should repeat step 2.3.3, but there is no such step.
Response: We corrected this.

6. In 3.3.6 it is not quite clear to me if this is the cover slip to which the larvae are glued. Is the lens paper covered by a 2nd over slip? If so, this should be more clearly described. The discussion mentions a 2nd cover slip which I did not notice in the protocol.
Response: The coverslip mentioned in 3.3.6 is the one to which the larvae are glued. The other coverslip attached to the bottom of the chamber was described in step 1.2. We have now specified the top and bottom coverslip in our drawing in Figure 1A.

Reviewer #4:

Manuscript Summary:
This protocol describes an inventive and useful method for long-term imaging of Drosophila larvae, with a specific application to larval dendrite development and regeneration. The method is straightforward, relies mostly on commonly-kept materials and fills a need not provided by other described methods developed for similar goals. Data are provided that demonstrate the utility of the method. The description of methods and materials is appropriately detailed and also straightforward to follow. I think this will become a standard method in the field of dendrite development and will permit many questions to be addressed in a way that was not possible before. Altogether this is an excellent contribution.

Major Concerns:
none

Minor Concerns:

It looks like there is a mistaken "go to" on step 3.3.4. There is no 2.3.3 in the protocol.
Response: We corrected this.

The sized of cuboids for the different stages is helpful and I'd recommend having them in main protocol rather than the discussion.
Response: The recommended sizes of PDMS cuboid are now described in step 2.1.1, 2.1.2 and 2.2.7.

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Author(s):	Hui Ji and Chun Han

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