Journal of Visualized Experiments

Dissection, immunohistochemistry and mounting of larval and adult Drosophila brains for optic lobe visualization

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61273R1
Full Title:	Dissection, immunohistochemistry and mounting of larval and adult Drosophila brains for optic lobe visualization
Section/Category:	JoVE Developmental Biology
Keywords:	Drosophila; optic lobe; Brain; medulla; lamina; lobula; IPC; OPC; neuroblasts
Corresponding Author:	Ted Erclik University of Toronto - Mississauga Mississauga, Ontario CANADA
Corresponding Author's Institution:	University of Toronto - Mississauga
Corresponding Author E-Mail:	ted.erclik@utoronto.ca
Order of Authors:	Urfa Arain
	Ishrat Maliha Islam
	Priscilla Valentino
	Ted Erclik
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Mississauga, Ontario, Canada

Cover Letter

Ted Erclik
Assistant Professor

Department of Biology
University of Toronto Mississauga

3359 Mississauga Road
Mississauga, Ontario L5L-1C6 CANADA
Phone: 905-569-5755; Fax: 905-828-3792
Email: ted.erclik@utoronto.ca
http://www.utm.utoronto.ca/biology/

March 28, 2020

Dear Dr. Phillip Steindel,

Please find here the submission of our revised manuscript entitled “Dissection, immunohistochemistry and mounting of larval and adult *Drosophila* brains for optic lobe visualization” for consideration by the *Journal of Visualized Experiments (JoVE).*

This resubmission includes revised versions of the Manuscript (with all edits marked as track changes), Figures and Table of Materials. We have also uploaded a detailed rebuttal letter that addresses each of the editorial and reviewer comments. We thank the reviewers and editors for these comments, as we feel that the revised version of the paper is significantly improved.

Sincerely,

Ted Erclik

TITLE:

Dissection, Immunohistochemistry and Mounting of Larval and Adult *Drosophila* Brains for Optic Lobe Visualization

AUTHORS AND AFFILIATIONS:

Urfa Arain[1], Priscilla Valentino[1], Ishrat Maliha Islam[1], Ted Erclik[1]

[1]Departments of Biology and Cell & Systems Biology, University of Toronto – Mississauga, Mississauga, Canada

Email Addresses of Co-Authors:

urfa.arain@mail.utoronto.ca
priscilla.valentino@mail.utoronto.ca
ishrat.islam@mail.utoronto.ca

Corresponding Author:

Ted Erclik (ted.erclik@utoronto.ca)

KEYWORDS:

Drosophila, optic lobe, brain, medulla, lamina, lobula, IPC, OPC, neuroblasts, neurons

SUMMARY:

This protocol describes three steps to prepare larval and adult *Drosophila* optic lobes for imaging: 1) brain dissections, 2) immunohistochemistry and 3) mounting. Emphasis is placed on step 3, as distinct mounting orientations are required to visualize specific optic lobe structures.

ABSTRACT:

The *Drosophila* optic lobe, comprised of four neuropils: the lamina, medulla, lobula and lobula plate, is an excellent model system for exploring the developmental mechanisms that generate neural diversity and drive circuit assembly. Given its complex three-dimensional organization, analysis of the optic lobe requires that one understand how its adult neuropils and larval progenitors are positioned relative to each other and the central brain. Here, we describe a protocol for the dissection, immunostaining and mounting of larval and adult brains for optic lobe imaging. Special emphasis is placed on the relationship between mounting orientation and the spatial organization of the optic lobe. We describe three mounting strategies in the larva (anterior, posterior and lateral) and three in the adult (anterior, posterior and horizontal), each of which provide an ideal imaging angle for a distinct optic lobe structure.

INTRODUCTION:

The *Drosophila* visual system, comprised of the compound eye and underlying optic lobe, has been an excellent model for the study of neural circuit development and function. In recent years, the optic lobe in particular has emerged as a powerful system in which to study neurodevelopmental processes such as neurogenesis and circuit wiring[1–8]. It is made up of four neuropils: the lamina, medulla, lobula and lobula plate (the latter two comprise the lobula

complex)[1–6]. Photoreceptors from the eye, target neurons of the lamina and medulla which process visual inputs and relay them to the neuropils of the lobula complex[1–6]. Projection neurons in the lobula complex subsequently send visual information to the higher order processing centers in the central brain[1,5,9]. The complex organization of the optic lobe, necessitated by a need to maintain retinotopy and to process different types of visual stimuli, makes it an attractive system for studying how sophisticated neural circuits are assembled. Notably, the medulla shares striking similarities in both its organization and development with the neuroretina, which has long been a model for vertebrate neural circuit development[3,8].

Optic lobe development begins during embryogenesis, with the specification of ~35 ectodermal cells that form the optic placode[2,4–8]. After larval hatching, the optic placode is subdivided into two distinct primordia: 1) the outer proliferation center (OPC), which generates the neurons of the lamina and outer medulla and 2) the inner proliferation center (IPC), which generates neurons of the inner medulla and lobula complex[4–6,10]. In the late second-instar larva, the neuroepithelial cells of the OPC and IPC begin to transform into neuroblasts that subsequently generate neurons via intermediate ganglion mother cells[4,5,11,12]. Optic lobe neuroblasts are patterned by spatially and temporally-restricted transcription factors, which act together to generate neural diversity in their progeny[11–14]. In the pupa, the circuits of the optic lobe neuropils are assembled via the coordination of several processes, including programmed cell death[11,15], neuronal migration[12,16], axonal/dendritic targeting[10,17], synapse formation[18,19] and neuropil rotations[10,17].

Here, we describe the methodology by which larval and adult brains are dissected, immunostained and mounted for imaging the optic lobe. Given its complex three-dimensional organization, analysis of the optic lobe requires that one understand how its adult neuropils and larval progenitors are positioned relative to each other and the central brain. Thus, we put special emphasis on how the orientation of mounting relates to the spatial organization of the optic lobe structures. We describe three mounting strategies for larval brains (anterior, posterior and lateral) and three for adult brains (anterior, posterior and horizontal), each of which provide an optimal angle for imaging a specific optic lobe progenitor population or neuropil.

PROTOCOL:

1. Preparing larval brains for confocal imaging

1.1. Dissections

NOTE: Before starting the dissection, prepare the fix (4% formaldehyde in phosphate buffered saline (PBS)) and PBT (0.1-0.3% Triton in PBS) solutions. The fix solution should be placed on ice during the dissection. Although paraformaldehyde (PFA) fixative is used in this protocol, alternative fixing strategies (using PLP[20] or PEM[21]) have been described for specific epitopes. For larval dissections, two pairs of forceps (Dumont #5 or #55s) are needed. See the **Table of Materials** for more details.

1.1.1. Start by filling each well of the dissecting dish with 400 µL of 1x PBS. Use a pair of forceps to gently pick wandering third-instar larvae crawling on the inside of the vial. Place larvae in the first well of the PBS-filled dish.

1.1.2. Take one larva and transfer it to the middle well. Using the non-dominant hand, hold the body of the larva down at the base of the well.

1.1.3. With the dominant hand, gently grasp the larval mouth hook and pull away from the body. The larval brain should be attached to the mouth hook and other accessory tissue and will come off as the tissue is pulled away.

1.1.4. Remove accessory imaginal discs and transfer the larval brain to the third well of the dissection dish. The imaginal discs are epithelial tissues surrounding the larval brain, which generate adult structures such as the antennae, eyes, legs and wings[20]. If left on the tissue, the imaginal discs may obstruct proper immunostaining of the larval brain.

1.1.4.1. To remove the imaginal discs, use a pair of forceps to firmly grip the brain via the ventral nerve cord. Using another pair of forceps, gently pluck away the discs.

1.1.4.2. Remove the eye disc carefully, since it wraps over the surface of the brain lobes. To remove the eye disc, use a pair of forceps to hold the brain against the base of the dish. Instead of holding the brain via the ventral nerve cord, use the forceps to lightly grasp the brain lobe being careful to not squeeze the lobe. Using another pair of forceps, gently pull away the eye disc.

NOTE: The eye imaginal disc will eventually give rise to photoreceptors of the retina. For those interested in studying neural connectivity between the retina and optic lobe, the eye disc can be left on the brain. However, for those solely interested in optic lobe structures, it is recommended to remove the eye disc as it may hinder image quality due to its location on the surface of the brain.

1.1.5. Repeat steps 1.1.2–1.1.4 until a sufficient number of brains have been collected (or 30 min of dissection time have elapsed). Dissections should not go longer than 30 min to ensure the integrity of the tissue is not compromised.

1.1.6. Once the dissection period is over, use a P200 pipette to carefully remove PBS from the third well. Ensure that a small volume of liquid remains in the well to keep the brains immersed.

NOTE: It is crucial to keep the brains immersed in liquid at all points of the protocol. If the tissue dries out, the quality of the stain may be compromised by unwanted autofluorescence in the confocal image.

1.1.7. Add 500 µL of the cold fix solution into the third well. Use a pair of forceps to stir the liquid gently, allowing the brains to swirl in the dish. Cover the dish with a glass slide and place it on ice for 30 min to allow for tissue fixation.

1.1.8. Remove the fixation solution and wash the brains with 400 µL PBT, 5 times.

NOTE: Triton is a detergent in PBT that keeps the brains from sticking to each other or the dish, and prepares the tissue for optimal antibody penetration. Make sure to discard the fix solution safely. The protocol can be paused here by covering the dish with a glass slide after adding the final 500 µL of PBT. Wrap the dish and slide with laboratory film, and store the brains at 4 °C. It is recommended to not pause this step for longer than a week. For researchers new to the optic lobe, it may be easier to leave accessory tissue on the brains prior to and during fixation. After the tissue has been fixed and washed, the brains can be carefully cleaned prior to adding primary antibody solution. This will allow researchers to maximize the number of brains obtained in a given dissection period. Additionally, to preserve the integrity of the tissue before it is fixed, researchers can place the dissected brains in a well on ice during the dissection period.

1.2. Immunohistochemistry

NOTE: There are several primary antibodies available from the Developmental Studies Hybridoma Bank (DSHB) that can be used to label specific optic lobe structures or cell types. DE-Cadherin labels the IPC and OPC neuroepithelia, Bruchpilot (Brp) marks the developing neuropil and Dachshund labels lamina and lobula neurons (as well as a small subset of medulla neurons). Additionally, Elav can be used to label neurons, Prospero to mark ganglion mother cells and Repo to identify glia.

1.2.1. Prepare the primary antibody solution by adding antibodies to PBT upto a total volume of 100 µL. A blocking agent (10% normal goat serum) can be used to prevent non-specific binding between the primary antibody and tissue[20,22,23]. The antibodies used in this protocol specifically label brain tissue without the need for blocking agents.

1.2.2. Remove the final post-fixation wash and add the primary antibody solution. Ensure that there is only a small amount of PBT in the well prior to adding the antibody solution. After adding the solution, gently stir the brains with forceps 5–10 times. Cover with a glass slide, seal with laboratory film and incubate at 4 °C overnight.

1.2.2.1. Alternatively, if a refrigerated orbital shaker is available, place the dish on the shaker to optimize the overnight incubation.

NOTE: The primary antibody incubation and all subsequent steps can alternatively be performed in a 1.5 mL microcentrifuge tube. The volume of primary and secondary incubations can remain 100 µL, but the wash steps should be performed with 800 µL or more of PBT.

1.2.3. After incubation, wash out the primary antibodies with PBT as in step 1.1.8 of the previous section.

NOTE: The primary antibody solution should be saved and stored at 4 °C as it can be reused for subsequent experiments. Many primary antibodies can be reused up to four times.

1.2.4. Add 400 µL of PBT to the brains for a final wash. Cover the dish with a slide, seal with laboratory film and place it on an orbital shaker at low speed (100 rpm) and room temperature (RT) for ~4 h.

NOTE: The protocol can be paused here. Brains can be left in wash for 2–3 days at 4 °C.

1.2.5. Prepare the secondary antibody solution in a total volume of 100 µL.

NOTE: In this protocol, all secondary antibodies are used at a dilution of 1:500, without blocking agents.

1.2.6. Remove the PBT wash from the well and add the secondary antibody solution. Mix the tissue in the solution using a pair of forceps. Cover the dish with a slide and laboratory film. Place the dish on an orbital shaker at RT for a minimum incubation period of 2 h. Since secondary antibodies contain light-sensitive fluorophores, cover the dish with aluminum foil.

NOTE: The protocol can be paused here. Brains can be left in secondary antibody solution overnight at 4 °C.

1.2.7. Wash out the secondary antibodies as described in step 1.2.3. Add 400 µL of PBT to the brains for a final wash. Cover the dish with a slide, seal with laboratory film and foil. Place the brains on a shaker at RT for 4 h.

NOTE: The protocol can be paused here. Brains can be left in wash for 2–3 days at 4 °C.

1.3. Mounting

1.3.1. Remove final PBT wash and replace with two drops of fluorescence-safe mounting media. Place a drop of the mounting media onto the center of a slide.

1.3.2. Transfer brains from the well to the drop on the slide using forceps. To avoid damaging the optic lobes, the brains can be held by the ventral nerve cord while transferring to the slide.

1.3.3. Mount the brains as per the mounting strategies that follow (**Figure 1A**).

1.3.4.1. Anterior side up

NOTE: For those interested in visualizing the anterior medulla, lamina or lobula plug, this mounting orientation is ideal. The best way to distinguish the anterior versus posterior mounts is by examining the position of the ventral nerve cord relative to the brain lobes.

1.3.4.1.1. Use the anterior orientation, to observe the ventral nerve cord projecting out over the lobes (**Figure 1B**).

1.3.4.2. Posterior side up

NOTE: This orientation is recommended for those interested in visualizing the posterior tips of the OPC (pOPC) or IPC [10,11].

1.3.4.2.1. Use the posterior orientation, to view the ventral nerve cord projecting out from under the brain lobes (**Figure 1C**).

1.3.4.3. Lateral view

NOTE: A lateral mounting orientation is used to visualize the crescent of lamina, medulla or lobula plug neurons in both the dorsal-ventral and anterior-posterior axes in a single plane (**Figure 1G**).

1.3.4.3.1. For a lateral mount, split the brain lobes from each other to fall flat on their side, with the lamina and lobula plug facing up.

1.3.4.3.2. Using two pairs of sharp forceps, split the ventral nerve cord in half, starting from where the cord attaches to the lobes. Note that the two lobes are already separate from one another, with the ventral nerve cord holding them intact. Thus, split the ventral nerve cord down from the cleavage point between the lobes, to separate them. Then flip each brain lobe and attached ventral nerve cord on their sides with the lateral surfaces facing up.

NOTE: For lateral view mount, it is recommended to use a tungsten needle with a fine tip to orient the brain lobe on its side. To clearly visualize the orientation of the lobes and the direction of the ventral nerve cord when mounting, the microscope lighting can be adjusted. If using a gooseneck LED light source, the goosenecks should be positioned parallel to the surface of the slide for clear visibility. Also, the lighting intensity can be increased or decreased to enhance contrast between the brain structures and provide clarity while mounting.

1.3.4. Place a small drop of PBS on either side of the mounting media containing the brains. Place one cover slip onto each drop, and a final coverslip over the brains. The right and left edges of the top coverslip should rest on the two other coverslips (**Figure 1A**).

NOTE: Prior to placing a coverslip over the brains, a bridge must be built. Larval brains are approximately 200 µm thick, and thus, to keep the integrity of the tissue, a bridge is created that increases the distance between the cover slip and the surface of the slide.

1.3.5. Seal the edges of the bridge with nail polish to secure the mounted brains.

1.3.6. Image the brains using confocal microscopy.

2. Preparing adult brains for confocal imaging

2.1. Dissections

NOTE: Adult brain dissections are more challenging than larval brains and require careful handling. It is strongly recommended to use at least one pair of ultra-fine forceps (Dumont #55) for this part of the protocol.

2.1.1. Anesthetize flies with CO_2, using either a needle or flypad, and place them on a laboratory wipe or paper towel over ice (to keep them anesthetized).

2.1.2. Add 400 µL of PBS to all three wells of the glass dish. Use a pair of forceps to gently grab one adult fly by the wings and place it into the first well of the dish.

2.1.3. Use a pair of forceps held in the non-dominant hand to hold the thorax of the fly against the base of the well. With the dominant hand, gently pull off the head from the rest of the body.

2.1.4. Transfer the head into the second well. Often, the head will float in the PBS and can be difficult to handle. Use a pair of forceps to hold it down against the base of the well by the proboscis.

2.1.5. Peel away a region of the cuticle between the eyes using both forceps. Since the brain sits right below the cuticle, be as gentle as possible to prevent damaging the underlying tissue. Continue to peel cuticle away one piece at a time, until the brain is exposed. Remove any accessory tissue (i.e., retina, trachea, air sacs) that may remain attached to the brain.

NOTE: Removal of the retina has been described in previous protocols[22,24], but the lamina can also be separated from the rest of the optic lobe. This is useful for those wanting to study the medulla or lobula complex, since the lamina sits at the surface and may obstruct visualization of these other optic lobe neuropils when imaging. To remove the lamina, use a pair of sharp forceps to gently pull at the indentation between the lamina and medulla neuropils. The lamina will slowly begin to peel away from the medulla. Repeat this motion until the entire lamina is removed. During this process it is important to maintain a firm grip of the brain. Use another pair of forceps to hold the brain against the base of the dish by positioning it so that the top and bottom of the central brain fit perfectly between the tips of the forceps. A firm grip around the central brain will prevent unwanted damage to the optic lobe.

2.1.6. Transfer the clean brain to the third well. Repeat steps 2.1.2–2.1.6 until a sufficient number of brains are obtained or a maximum dissection period of 30 min has elapsed.

2.1.7. Remove the PBS in the third well and add 500 µL of fix solution. Ensure that the brains do not dry out here or during any wash steps as this will lead to background fluorescence in the confocal images. Cover the dish with a glass slide and allow the brains to incubate in fix for 20 min at RT.

2.1.8. After fixation, wash the brains with 400 µL of PBT, 5 times.

NOTE: The protocol can be paused here. Brains can be covered with a glass slide and laboratory film and left in wash for 2–3 days at 4 °C. For researchers new to the system, it may be easier to leave accessory tissue on the brains before and during fixation. This will allow researchers to maximize the number of brain samples obtained in a given dissection period. After the tissue has been fixed and washed, the brains can be carefully cleaned before adding primary antibody solution. Adult brains with accessory tissue tend to float, and therefore risk drying out. As a result, it is recommended to use a pair of forceps to carefully pool all of the brains into the center of the dish upon adding fix solution, and to clean the brains immediately after fixation.

2.2. Immunohistochemistry

2.2.1. Perform immunohistochemistry as described for larval brains in section 1.2.

2.2.2. Useful primary antibodies from the DSHB include those that are mentioned in the protocol of larval immunohistochemistry in section 1.2. See the **Table of Materials** for a complete list of antibodies and corresponding dilutions.

NOTE: Extra care must be taken during the wash steps for adult brains, since they may float to the surface of the wash solution and risk drying out on the sides of the well when the solution is removed (which will lead to background fluorescence). A good practice is to hold the pipette in one hand to add or draw away liquid, and a pair of forceps in the other to keep the brains submerged.

2.3. Mounting

2.3.1. Prior to the mounting step, perform a final wash with PBS (instead of PBT). PBS causes the brains to become slightly sticky, which allows them to remain in the desired orientation during mounting.

2.3.2. Remove the final wash and replace with three drops of fluorescence-safe mounting media. Place a drop of the mounting media onto the center of a microscope slide.

2.3.3. Transfer brains from the well to the drop on the slide using forceps. To avoid damaging the optic lobes and prevent the tissue from drying out, carefully retrieve a brain via capillary action. Slowly close a pair of forceps around a brain until a small volume of liquid containing the brain is drawn up between the tips.

NOTE: When transferring the brain to the slide, be careful to not close the forceps as this will damage the brain. Alternatively, a P200 pipette can be used to transfer the brains. Cut off the end of the tip to widen the opening and pre-rinse with PBT to prevent the brains from sticking to the inside of the tip.

2.3.4. Mount the brains as per the following strategies (**Figure 2A**).

2.3.4.1. Anterior side up

NOTE: To visualize lamina and medulla neurons, orient the brain with the anterior side up (**Figure 2B**). This can be achieved by examining the anatomy and curvature of the central antennal lobes.

2.3.4.1.1. With a pair of forceps, gently turn the brain on its side to examine both the anterior and posterior sides. Observe that on the anterior side, the curvature of the brain is pronounced and the antennal lobes protrude slightly outward from the center.

2.3.4.2. Posterior side up

2.3.4.2.1. Position the brains with the posterior (flat) side facing up and the antennal lobes down (**Figure 2C**).

NOTE: This will bring the lobula and lobula plate closer to the imaging surface and is ideal for those interested in visualizing lobula complex neurons.

2.3.4.3. Horizontal view

NOTE: To visualize all optic lobe neuropils simultaneously, use a horizontal mounting strategy (**Figure 2G**). In this orientation, neuronal cell bodies, axonal trajectories and dendritic arborizations can be visualized in the same plane.

2.3.4.3.1. Initially, orient the brain with the anterior side up. With a tungsten needle, gently tilt the brain 90° upwards so that it sits on its dorsal side with its antennal lobes facing outwards. Check that both the anterior and posterior sides of the brain are visible in this orientation.

2.3.5. Instead of a cover slip bridge, use clay to elevate the cover slip from the slide. The use of clay allows for re-mounting of the brains after imaging (described in the discussion section).

2.3.5.1. Place a small piece of clay on each corner of a cover slip. Ensure that each piece of clay is relatively the same thickness. The pieces of clay should be between 0.5–1 mm in thickness. Gently place the coverslip over the brains and apply slight pressure to the corners. The coverslip should immediately catch the liquid and form a seal. The slide is now ready for imaging.

NOTE: For long-term storage of the slide, it is recommended to seal the coverslip with nail polish and store at 4 °C away from light.

REPRESENTATIVE RESULTS:

Confocal images of larval and adult optic lobes mounted in the orientations described in the protocol are presented in **Figure 1** and **Figure 2**.

Figure 1 shows schematics and representative confocal slices of larval brains positioned in the anterior, posterior and lateral orientations. In the anterior mounting orientation, the OPC epithelium (DE-Cadherin), medulla neuroblasts (*deadpan*>βgal) and lamina neurons (Dachshund) appear at the surface as bands of cells that wrap around the brain (**Figures 1B**,**D**). The OPC is spatially patterned along its dorsal/ventral axis by the differential expression of transcription factors[6,11,12]. Vsx1 labels the central OPC (cOPC), Optix labels the main OPC (mOPC) and Rx is expressed in the posterior OPC (pOPC), which represents the tips of the crescent[11,12,25]. The anterior mount is ideal for the visualization of the cOPC, as this region of the crescent lies at the surface in this orientation (**Figure 1D**). Neurons of the lamina are also visible on the lateral side of the lobe in this orientation. Deeper into the brain along the z-axis, additional regions of the OPC, as well as other structures, become visible (**Figure 1E**,**F**). At a middle point in the z-stack, the mOPC epithelium, along with its respective neuroblasts and neurons, are visible (**Figure 1E**). Additionally, the proximal region of the IPC (p-IPC) and the lobula plug, which give rise to neurons of the lobula and lobula plate[6,13,26], are visible in these intermediate slices. The deepest z-slices depict the other side of the brain, where the pOPC tips are located (**Figure 1F**). The superficial tip of the IPC (s-IPC) is also present in these deepest slices[13].

The structures and cells located at the bottom of an anteriorly-mounted brain correspond to those that would appear on the surface of a posteriorly-mounted brain. Due to their proximity to the imaging objective, the optic lobe structures closest to the surface of the brain resolve better in confocal images compared to those located at the bottom. At the surface, there is minimal light scattering between the tissue and the objective. In deeper parts of the tissue, more light scattering leads to a weaker fluorescence signal. Thus, a posterior mounting strategy permits optimal imaging conditions to visualize the tips of the OPC or ventral-IPC (**Figure 1F**), whereas an anterior mounting strategy is better-suited for visualizing cells of the lamina or cOPC (**Figure 1D**). If the region of interest is the lobula plug or mOPC and its progeny, either mounting orientation is suitable, as these structures are located towards the middle of the brain (**Figure 1E**).

A laterally-mounted larval brain lobe (**Figure 1G**) can be used to visualize the medulla, lamina or lobula plug neuronal crescents in one focal plane. Different structures can be visualized at different depths along the z-axis. At the surface, the lamina crescent (Eya) is visible with the lobula plug crescent located between its arms (**Figure 1H**). The medulla neuronal crescent (Bsh, Eya and Svp) appears at a slightly deeper z-position (**Figure 1I**). In a single z-slice one can visualize the entirety of the neuronal crescent along both the dorsal-ventral and anterior-posterior axes. Thus, this orientation is suitable for a researcher interested in determining where their gene of interest is expressed with respect to the spatial axes of the OPC.

Figure 2 shows schematics and representative confocal slices of adult brains positioned in the anterior, posterior and horizontal orientations. The lamina has been removed in these images to better show the underlying medulla and lobula complex. The medulla, lobula and lobula plate are each comprised of a cortex, which contains neuronal cell bodies, and a neuropil, which is made up of axonal and dendritic arborizations. In the anterior orientation (**Figure 2B**), the medulla cortex and neuropil are located at the surface, whereas in the posterior orientation (**Figure 2C**), the lobula and lobula plate are the first structures imaged. **Figure 2D–F** display representative images of an anteriorly-mounted adult optic lobe at three Z-positions. Since the medulla is located at the surface of the anterior mount (**Figure 2D**), the medulla cortex (Vsx1) is immediately visible. Cell bodies in the cortex project their arborizations into the neuropil (labeled by Bruchpilot), which can be visualized at an intermediate z-position (**Figure 2E**). The lobula also appears at this level, located perpendicular to the medulla. At the deepest z-position, the lobula plate is visible (**Figure 2F**). Thus, researchers interested in studying the neurons of the lobula complex should use a posterior-mount orientation, whereas those interested in the lamina and medulla should mount their brains in an anterior orientation.

A horizontally-mounted optic lobe is achieved when the brain is flipped 90° on to its side from an initial anterior position (**Figure 2G**). In this view, all of the neuropils and cortices of the optic lobe are visible within a single plane (**Figure 2H**,**I**). This mounting orientation is recommended for the visualization of retinotopic projections and the projections of neurons that target multiple optic lobe neuropils.

FIGURE AND TABLE LEGENDS:

Figure 1. Larval brain mounting orientations. (**A**) Cartoon schematic showing late 3rd instar larval brains mounted on a slide in three orientations. Orientations can be distinguished from one another based on the position of the ventral nerve cord (VNC) relative to the brain lobes. (**B**) In the anterior mount, the VNC comes over the top of the brain lobes. In this orientation, the medulla neuroblasts (NBs), anterior OPC neuroepithelium (NE) and neurons of the lamina are visible. (**C**) In the posterior mount, the VNC protrudes from beneath the brain lobes. This orientation permits the visualization of both posterior (tip) medulla NBs and the ventral IPC NE. (**D–F**) Confocal images of an anteriorly-mounted 3rd instar larval brain stained for the OPC and IPC NE marker DE-Cadherin (blue), lamina marker Dac (red) and NB marker *dpn>lacZ* (β-gal) (green). D-F are Z-slices from the top (D), middle (E) and bottom (F) of the same confocal stack. (**G**) Schematic depicting a late 3rd instar larval brain mounted in a lateral orientation. (**H**, **I**) Confocal images of a laterally-mounted 3rd instar larval optic lobe stained for the lamina marker Eya (blue), and lamina and medulla neuronal markers Bsh (red) and Svp (green). In this orientation, the lamina (H) and medulla (I) neuronal crescents can be visualized along the entire dorsal-ventral and anterior-posterior axes in a single plane. H and I are Z-slices from top (H) and middle (I) of the same confocal stack. Scale bars = 50 um.

Figure 2. Adult brain mounting orientations. (**A**) Cartoon schematic showing adult brains mounted on a slide in three orientations. Orientations can be distinguished based on the location of the antennal lobes. (**B**) In the anterior orientation, the antennal lobes are facing up and the

medulla neuropil is located at the surface. (**C**) In the posterior orientation, the brain is mounted with the antennal lobes facing down and the lobula and lobula plate neuropils located at the surface. (**D–F**) Confocal Z-slices of an anteriorly-mounted adult brain labeled with the medulla neuronal marker Vsx1 (magenta) and the neuropil marker Brp (red). D-F are Z-slices from the top (D), middle (E) or bottom (F) of the same confocal stack. (**G**) Schematic of an adult brain in the horizontal orientation, which can be achieved by mounting the brain on its side. (**H**) Confocal image of a horizontally-mounted brain stained with Vsx1 (magenta) and Brp (red). In this mounting orientation all three optic lobe neuropils and cortices are visible in one Z-slice. This orientation is ideal for visualizing the morphologies of optic lobe neurons across the neuropils within a single plane. (**I**) Confocal image of a horizontally-mounted brain labeling a medulla neuron (Dm4) with GFP (green) and the neuropils with Brp (red). Dm4 neurons send arborizations to layers 3 and 4 of the medulla neuropil. Scale bars = 50 μm.

DISCUSSION:

In this protocol, we describe a method to immunostain larval and adult *Drosophila* brains and mount them in several orientations. While methods to stain larval and adult brains have been previously described[22–24,27,28], mounting strategies for the optimal visualization of specific optic lobe structures have received less attention[28]. It is anticipated that the protocol described here will provide researchers with a greater understanding of the relationship between mounting orientation and the optic lobe structures visualized.

In addition to the orientations described in this protocol, alternative angles of adult and larval optic lobe visualization can be achieved by separating the optic lobe from the central brain. The optic lobes can be split from the central brain using insect scissors, forceps or a tungsten needle. In the adult, this can be a useful strategy in cases where the curvature of the central brain inhibits flat mounting of the lobes, resulting in uneven angles during imaging. It should be noted that an isolated lobe will be more challenging to mount without the reference points provided by the central brain (i.e., antennal lobes, brain curvature, etc.) that are used to determine mounting orientation. This limitation can be overcome by analyzing optic lobes under a fluorescence GFP microscope (if the brain is stained for the appropriate fluorescent marker) to ensure the desired orientation is achieved before adding the coverslip. Similarly, in the larva, the removal of a brain lobe from the attached contralateral lobe and ventral nerve cord, allows the brain to be mounted in any orientation. A GFP-microscope can be used to determine the mounting angle with respect to the optic lobe structures of interest.

Brains can also be imaged in multiple orientations by removing the coverslip after imaging and remounting the brain. For remounting, the original bridge should be made with clay and nail polish should not be applied. To re-orient brains, a pair of forceps can be inserted underneath the coverslip to break the seal. Once the coverslip is lifted, most of the brains should remain in the mounting media. The brains can then be remounted and a new coverslip can be placed on top of the brains. This technique has previously been used to image a single brain in multiple orientations to build a high-resolution three-dimensional image of a medulla neuron's morphology[17]. While remounting is often done with adult brains, the technique can also be applied to larval brains, which would also require building the bridge with clay. It is important to

handle larval brain samples carefully when remounting because their fragility makes them more likely to tear when the coverslip is removed.

The above-mentioned protocols can also be applied to pupal brain tissue[10,22,24]. Since pupal brains undergo rapid morphogenic changes during development, the mounting orientations for early pupa (0–30 h APF) resemble those of larval brains, whereas mid-late stage pupa (>50 h APF) are closer to adult brain mounting orientations. Pupal brains are more fragile than larval and adult brains, and therefore require extra care when being manipulated.

Finally, in addition to fixed and stained tissue, an understanding of brain mounting orientations is important for live imaging applications. Larval and adult brains can be cultured and imaged under live conditions to follow cell divisions and changes in neuronal morphology and activity over time[24,27,29]. Here, the mounting orientation used is critical, as the weaker endogenous fluorescence demands that cell types of interest are located as close as possible to the surface of the brain for optimal signal detection during imaging.

ACKNOWLEDGMENTS:

We would like to thank Claude Desplan for sharing with us an aliquot of the Bsh antibody. The DE-Cadherin, Dachshund, Eyes Absent, Seven-up and Bruchpilot monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. This work was supported by an NSERC Discovery Grant awarded to T.E.. U.A. is supported by an NSERC Alexander Graham Bell Canada Graduate Scholarship. P.V. is supported by an Ontario Graduate Scholarship.

DISCLOSURES:

The authors declare that they have no competing financial interests.

REFERENCES:

1. Fischbach, K.-F., Dittrich, A. P. M. The optic lobe of *Drosophila melanogaster*. I. A Golgi analysis of wild-type structure *Cell Tissue Research.* **258**, 441M75 (1989).
2. Hofbauer, A., Campos-Ortega, J. A. Proliferation pattern and early differentiation of the optic lobes in Drosophila melanogaster. *Roux's Archives of Developmental Biology.* **198**, 264–274 (1990).
3. Sanes, J. R., Zipursky, S. L. Design Principles of Insect and Vertebrate Visual Systems. *Neuron.* **66**, 15–36 (2010).
4. Nériec, N., Desplan, C. From the Eye to the Brain. Development of the Drosophila Visual System. in *Current Topics in Developmental Biology*. **116**, 247–271 (Academic Press Inc., 2016).
5. Apitz, H., Salecker, I. A Challenge of Numbers and Diversity: Neurogenesis in the *Drosophila* Optic Lobe. *Journal of Neurogenetics.* **28**, 233–249 (2014).
6. Contreras, E. G., Sierralta, J., Oliva, C. Novel strategies for the generation of neuronal diversity: Lessons from the fly visual system. *Frontiers in Molecular Neuroscience*. **12**, 140 (2019).

7. Erclik, T., Hartenstein, V., Lipshitz, H. D., McInnes, R. R. Conserved Role of the Vsx Genes Supports a Monophyletic Origin for Bilaterian Visual Systems. *Current Biology* **18**, 1278–1287 (2008).
8. Erclik, T., Hartenstein, V., McInnes, R. R., Lipshitz, H. D. Eye evolution at high resolution: The neuron as a unit of homology. *Developmental Biology.* **332**, 70–79 (2009).
9. Wu, M. et al. Visual projection neurons in the Drosophila lobula link feature detection to distinct behavioral programs. *eLife* **5**, (2016).
10. Ngo, K. T., Andrade, I., Hartenstein, V. Spatio-temporal pattern of neuronal differentiation in the Drosophila visual system: A user's guide to the dynamic morphology of the developing optic lobe. *Developmental Biology.* **428**, 1–24 (2017).
11. Li, X. et al. Temporal patterning of Drosophila medulla neuroblasts controls neural fates. *Nature* **498**, 456–462 (2013).
12. Erclik, T. et al. Integration of temporal and spatial patterning generates neural diversity. *Nature* **541**, 365–370 (2017).
13. Apitz, H., Salecker, I. A region-specific neurogenesis mode requires migratory progenitors in the Drosophila visual system. *Nature Neuroscience.* **18**, 46–55 (2015).
14. Suzuki, T., Kaido, M., Takayama, R., Sato, M. A temporal mechanism that produces neuronal diversity in the Drosophila visual center. *Developmental Biology.* **380**, 12–24 (2013).
15. Hara, Y., Sudo, T., Togane, Y., Akagawa, H., Tsujimura, H. Cell death in neural precursor cells and neurons before neurite formation prevents the emergence of abnormal neural structures in the Drosophila optic lobe. *Developmental Biology.* **436**, 28–41 (2018).
16. Morante, J., Erclik, T., Desplan, C. Cell migration in Drosophila optic lobe neurons is controlled by eyeless/Pax6. *Development.* **138**, 687–693 (2011).
17. Millard, S. S., Pecot, M. Y. Strategies for assembling columns and layers in the Drosophila visual system. *Neural Development.* **13**, 1–17 (2018).
18. Takemura, S. Y., Lu, Z., Meinertzhagen, I. A. Synaptic circuits of the Drosophila optic lobe: The input terminals to the medulla. *Journal of Comparative Neurology.* **509**, 493–513 (2008).
19. Akin, O., Bajar, B. T., Keles, M. F., Frye, M. A., Zipursky, S. L. Cell-type-Specific Patterned Stimulus-Independent Neuronal Activity in the Drosophila Visual System during Synapse Formation. *Neuron.* **101**, 894-904.e5 (2019).
20. Spratford, C. M., Kumar, J. P. Dissection and immunostaining of imaginal discs from drosophila melanogaster. *Journal of Visualized Experiments.* (91), e51792 (2014).
21. Dokucu, M. E., Zipursky, S. L., Cagan, R. L. Atonal, rough and the resoluton of proneural clusters in the developing Drosophila retina. *Development (Cambridge, England).* **122**, 4139–47 (1996).
22. Hsiao, H. Y. et al. Dissection and immunohistochemistry of larval, pupal and adult Drosophila retinas. *Journal of visualized experiments.* e4347 (2012).
23. Kelly, S. M., Elchert, A., Kahl, M. Dissection and immunofluorescent staining of mushroom body and photoreceptor neurons in adult Drosophila melanogaster brains. *Journal of Visualized Experiments.* **2017**, e56174 (2017).
24. Ryan Williamson, W., Robin Hiesinger, P. Preparation of developing and adult Drosophila brains and retinae for live imaging. *Journal of Visualized Experiments.* e1936 (2010).
25. Bertet, C. et al. Temporal patterning of neuroblasts controls notch-mediated cell survival through regulation of hid or reaper. *Cell.* **158**, 1173–1186 (2014).

26. Pinto-Teixeira, F. et al. Development of Concurrent Retinotopic Maps in the Fly Motion Detection Circuit. *Cell.* **173**, 485-498.e11 (2018).
27. Plevock, D. A., Rusan, K. M. Live Imaging of Drosophila Larval Neuroblasts. *Journal of Visualized Experiments.* e51756 (2014).
28. Ting, C. Y. et al. Analyzing dendritic morphology in columns and layers. *Journal of Visualized Experiments.* **2017**, e55410 (2017).
29. Özel, M. N., Langen, M., Hassan, B. A., Hiesinger, P. R. Filopodial dynamics and growth cone stabilization in Drosophila visual circuit development. *eLife.* **4**, (2015).

Figure 1

Your PDF file "Arain_JoVE_Figure_1.pdf" cannot be opened and processed.
Please see the common list of problems, and suggested resolutions below.

Reason:

Other Common Problems When Creating a PDF from a PDF file

You will need to convert your PDF file to another format or fix the current PDF file, then re-submit it.

Figure 2

Your PDF file "Arain_JoVE_Figure_2.pdf" cannot be opened and processed.
Please see the common list of problems, and suggested resolutions below.

Reason:

Other Common Problems When Creating a PDF from a PDF file

You will need to convert your PDF file to another format or fix the current PDF file, then re-submit it.

Table of Materials

Click here to access/download;Table of Materials;Arain_JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number
10x PBS	Bioshop	PBS405
37% formaldehyde	Bioshop	FOR201
Alexa Fluor 488 (goat) secondary	Invitrogen	A-11055
Alexa Fluor 555 (mouse) secondary	Invitrogen	A-31570
secondary	Invitrogen	A-21450
Alexa Fluor 647 (rat) secondary	Invitrogen	A-21247
Cover slips	VWR	48366-067
Dissecting forceps - #5	Dumont	11251-10
Dissecting forceps - #55	Dumont	11295-51
Dissection Dish	Corning	722085
Dry wipes	Kimbery Clark	34155
Goat anti-Bgal primary antibody	Biogenesis	
antibody	Gift from Claude Desplan	
antibody	Erclik et al. 2008	
Laboratory film	Parfilm	PM-996
Microcentrifuge tubes	Sarstedt	72.706.600
Microscope slides	VWR	CA4823-180
Mouse anti-dac primary antibody	Developmental Studies Hybridoma Bank (DSHB)	mabdac2-3
Mouse anti-eya primary antibody	DSHB	eya10H6
Mouse anti-nc82 primary antibody	DSHB	nc82
Mouse anti-svp primary antibody	DSHB	Seven-up 2D3
Polymer Clay	Any type of clay can be used	
Rabbit anti-GFP	Invitrogen	A-11122

antibody	DSHB	DCAD2
Slowfade mounting medium	Invitrogen	S36967
Triton-x-100	Bioshop	TRX506

Comments/Description
use at 1:501
use at 1:500
use at 1:503
use at 1:502
use at 1:1000
use at 1:500
use at 1:1000
use at 1:20
use at 1:20
use at 1:50
use at 1:100
use at 1:1000

use at 1:20

Vectashield mounting medium (cat# H-1000) can also be used

Editorial comments:
General:

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

   The manuscript has been proofread.

2. Please include all authors' emails in the manuscript itself.

   This comment has been addressed.

3. Please reduce the length of the Summary to 10-50 words.

   This comment has been addressed.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
   For example: Parafilm, Eppendorf, Kimwipe.

   This comment has been addressed.

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

   The relevant text has been highlighted in yellow.

2. 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.

   This comment has been addressed.

Figures:

1. Please provide scale bars for all images taken with a microscope.

Scale bars have been added.

References:

1. Please do not abbreviate journal titles.

   This comment has been addressed.

Table of Materials:

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

   This comment has been addressed

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript describes a protocol for the dissection immunohystochemistry and mounting of larval and adult Drosophila brains. Special emphasis is given to the orientation while mounting the brains for subsequent imaging of optic lobe neuropiles. The protocol is described in sufficient detail for the reader to replicate the procedures. The illustrations and images are of good quality.

Minor Concerns:

1. The authors should clarify that the description of the brain orientation refers to the body axis of the larvae or adult fly and does not refer to the neuraxis. To illustrate the orientations more clearly the authors could add an illustration like in Contreras et al., 2019. Figure 1A, or 1B.

   The descriptions of the brain orientations used here are consistent with those used in other optic lobe papers. Therefore, we feel that an attempt to clarify which axis the orientations are relative to may be confusing for the reader.

2. Could the authors explain in more detail what they mean by flusher mount (line 430)

   This comment has been addressed.

3. The authors could mention previous immunohystochemistry protocols for Drosophila brains with focus on the optic lobe: Weng, Komori and Lee., 2012, Perruchoud and Egger., 2014, Powell and Salecker., 2019

   This comment has been addressed.

Reviewer #2:

Manuscript Summary:
The authors of this study describe techniques for dissection of Drosophila larval and adult brains, immunofluorescent staining of these tissues, and subsequent mounting of the stained tissue on slides. The authors focus on imaging of the optic lobe in particular since it has been used in multiple studies to better understand the processes of neurogenesis and neuronal circuit formation (neurite outgrowth, pathfinding, synaptogenesis, etc.). The methods described in this manuscript complement previously published articles in JoVE and other journals by describing in more detail the ways in which the brains can be placed onto slides for optimal confocal microscopy imaging. The authors provide some background data on the overall organization of the fly optic lobe, highlighting several specific regions as well as antibodies that can be used to highlight cells within these regions. Expected results are included with the manuscript. Although the content of this article overlaps with previously published technique-focused manuscripts about Drosophila brain dissections and immunofluorescent microscopy, the addition of a section focused on the orientation of the brain when placed onto slides is (as far as I'm aware) a new addition. Some suggestions for changes/alterations to the manuscript are given below.

Major Concerns:

1. One of the main concerns that seems worth raising is the argument the authors make about the importance of positioning the brain correctly on the slide for confocal microscopy imaging. Since the fly brain is relatively transparent and ~150-200µm thick, it is fairly straight forward to image through the entire brain in either of the described "anterior" or "posterior" mounted positions. This is especially true when the brain has been incubated in anti-fade mounting medium. The authors suggest in the text (lines 349-351) that "cells located at the bottom of an anteriorly-mounted brain correspond to those that would appear on the surface of a posteriorly-mounted brain." If both sides of the brain can be observed in both mounting orientations, is there a reason why one would be preferred? Perhaps a more thorough discussion of the benefits of one mounting orientation over the other would help to clarify this point. I also realize that the "representative data" is just representative, but if the authors are focusing on a particular brain region, such as the "tips of OPC or ventral-IPC" (line 352) that require a posterior mounting strategy, could data be provided from both the posterior and anterior mounts to show the benefit of one vs. the other?

   This is an excellent point. Towards the end of the second paragraph in the Representative Results section we have added a few sentences that explain why we think orientation matters, even if the entire brain can be imaged in one orientation. Our justification is that the confocal signal gets significantly weaker as one images deeper into the brain.

2. One point that the authors could discuss more thoroughly is the removal of imaginal eye/antennal discs from the larval brains. On line 93, the authors briefly describe removing "accessory tissues" such as imaginal discs. However, given the focus of this article on imaging the optic lobes of the brain and the fact that the imaginal discs are attached to the optic lobes, a more complete/thorough description of how the imaginal discs are removed without damaging brain tissue would be helpful to novice

researchers who are just learning this technique. Including the distinction between brains containing eye discs and those without could potentially broaden the readership of the article since the authors could then include a brief discussion of experiments that could be performed if eye discs were left attached, such as investigation of connection patterns between retinal neurons and the optic lobe using anti-chaoptin antibodies (available from the DSHB as 24B10).

We have added a note to this part of the dissection protocol that addresses these points.

3. On line 233, the authors discuss removing the retina from the adult fly brain. Previously published JoVE protocols have been written to address this particular part of the procedure so it might help to reference these other protocols or describe this step in more detail. Since the main focus of the current paper under review here is on correctly imaging the optic lobes, more detail should be given in the methodology for removing optic lobes in a way that does not significantly damage the optic lobes. Given that the retina is so closely associated with the lamina of the optic lobe, retina removal could potentially be problematic for researchers first learning this protocol. Although this topic is partially addressed starting on line 311, this portion of the protocol could be placed earlier (near line 233) to help clarify how the retina can be removed.

   We have added two JoVE references for retinal dissection protocols to this section.

Minor Concerns:

1. On line 78 of the protocol, the authors describe making the fix solution. While 4% paraformaldehyde is a very typical fixative, it is not the only fixative that is typically used. The authors should discuss alternative fixing strategies that are also occasionally used for imaging specific epitopes. Two examples are:
   a. PLP is 2% paraformaldehyde, 0.01 M NaIO4 , 0.075 M lysine, 0.037 M NaPO4, pH7.2.
      See: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4542997/

   b. PEM is 0.1 M PIPES pH7.0, 2 mM MgSO4 , 1 mM EGTA (final conc) and is conveniently made up as a 2X stock to be diluted with 8% formaldehyde (ie final 4% para).
      See the above citation and https://www.ncbi.nlm.nih.gov/pubmed/9012533

   Thank-you for this information. These fix alternatives and the references you provided have been added to the protocol.

2. Doing all washes and fixing steps in 0.3% PBT may be slightly too much detergent for some antibodies. This could be a discussion point or a point that might require optimization. In addition, fixing the tissue in PBS containing 0.1% or even 0.05% Triton-X (instead of 1x PBS) might help prevent the tissue from adhering to the sides of the dish or to help the tissue stay submerged in the fixative solution.

Thank you for your comment. In order to maximize the integrity of the tissue, we refrain from adding detergent to our fixative.

3. On line 127, the authors discuss creating the primary antibody solution in general terms but should provide more specifics. For example, is a blocking reagent used (normal goat serum, BSA, etc. and if so how much?)? The addition of a table for some of the suggested antibodies might be useful. The table might include suggested starting dilutions and references (if available) from which those dilutions were obtained.

   This is a great suggestion. We have added a note on blocking alternatives in the immunohistochemistry section and have added the suggested antibody information to the Table of Materials.

4. On line 132, the authors refer to stirring the brains gently with forceps after the primary antibody solution has been added. More detail could be given here. Are the brains stirred 5 times or 10 times? Or are they gently stirred for 15 seconds? More detail would be useful here.

   This comment has been addressed.

5. For the NOTE on line 135, more detail seems to be necessary. For example, how are the brains transferred to an Eppendorf tube? What size Eppendorf is used? How much volume is used? Is the tissue incubated on a rocker or stationary?

   This comment has been addressed.

6. On line 140, is there an estimate of how many times a primary antibody solution can be reused? At least in the authors' experience, how many times do they typically reuse primary antibody solutions?

   This comment has been addressed.

7. One line 143, the authors suggest using a shaker. Could more detail be provided here? For example is this a "nutator" type shaker, a rocker, or an orbital shaking platform? If possible, could the speed of shaking be given (assuming this can be changed)? This would also apply to line 153.

   We have added more detail when describing the shaker.

8. On line 148, for the secondary antibody dilutions - the same comments apply as those for the primary. Are blocking solutions/reagents used? What are the suggested dilutions of the secondary antibodies being used?

   This information has been added in the Table of Materials.

9. On line 153, the authors suggest that the minimum time for incubation with the secondary antibody is ~2 hours. Is there a maximum time suggested by the authors? A more complete range of times could be added here

   This comment has been addressed.

10. On line 170, when the authors discuss picking up the brain, could they describe the area of the brain they grasp with the forceps in order to transfer the brains to slides?

    This comment has been addressed.

11. On line 260, is the space under the cover slip "bridge" empty or filled with mounting media? Leaving the area open to air could cause issues/artifacts when imaging on the confocal microscope. If the drop of mounting media is large enough when the brains are oriented in it then that might suffice.

    Thank you for your comment. Usually, we ensure that the drop of mounting media is large enough for all of the tissue to be fully submerged under the bridge. Thus, open air between the central coverslip and flanking coverslips does not cause any issues, especially if the bridge is sealed with nail polish.

12. When mounting the dissected and stained brains (both larval and adult) onto the slides, the authors should describe in more detail how they perform this task. Do they use forceps to move the brains into a specific orientation or some other tool? In particular different lighting conditions can help immensely when using dissecting stereomicroscopes. If gooseneck LED light sources are available, moving these goosenecks so they are parallel with the benchtop provide extra contrast for the brains and help to visualize them in the field of view.

    Thank you for this comment. It is very helpful and a paragraph has been added to the larval mounting section that addresses these points.

13. While the benefits of the anterior vs. posterior mount orientations could use some clarification and/or additional data (see the point above at the beginning of this review), the horizontally-mounted brains demonstrate a clearly different brain orientation that can be used to image different sets of neurons. However, between the larval and adult brain descriptions this mounting orientation is described differently ("lateral" for the larval brains and "horizontal" for the adult brains). Was this intentional? Perhaps the authors should keep these labels the same for consistency or more clearly define the differences between "lateral" and "horizontal."

    Thank you for your comment. Both “lateral” and “horizontal” mounts enable a cross-sectional view of the tissue, but we have found that, in the literature, lateral is used in the larva and horizontal in the adult. Moreover, in the larval lateral orientation, the brain is mounted on the mediolateral axis, enabling researchers to visualize the dorsoventral axis within one plane. In the adult horizontal orientation, the brain is mounted on the dorsoventral axis, such that the mediolateral axis is visible within the same plane.

14. For the text associated with Figure 1, the "top," "middle," and "bottom" labels should be referenced more clearly. There is a reference to the "middle point in the Z-stack" on line 344, but I do not see a clear reference to the top and bottom of the Z-stack as referenced in Figure 1.

    This comment has been addressed.

15. In Figure 2, it would be useful to have the Dorsal/Ventra surfaces labeled as in Figure 1. This would be especially helpful for the diagram in G. Labeling the antennal lobe is useful but having Anterior/Posterior sides of the brain labeled would also be useful.

    This comment has been addressed.

Reviewer #3:

Manuscript Summary:
In this manuscript, Arain et al. aim to develop a protocol for visualization of the optic lobe structure in larval and adult Drosophila brains. Drosophila is an important model organism and has been widely applied to study brain development and neurodegeneration. The anatomy of the Drosophila optic lobe has been well characterized (Fischbach, K-F., and A. P. M. Dittrich. "The optic lobe of Drosophila melanogaster. I. A Golgi analysis of wild-type structure." Cell and tissue research 258.3 (1989): 441-475.). This timely protocol will help researchers choose the optimal mounting orientation for optic lobe structure visualization. The protocol is well-written with all procedures easy to follow. I have the following suggestions

Major Concerns:

1. In the introduction section, I would suggest the authors cite Fischbach's paper so that the readers can refer to this paper and have a comprehensive review of the anatomy of the Drosophila optic lobe.

   This is an excellent suggestion and the citation has been added.

2. I would suggest the authors provide the concentrations of primary and secondary antibody solutions applied in the illustrations.

   The concentrations have been added in the Table of Materials.

3. In the Table of Materials, the primary antibody information for Bsh, Svp, Eya, Vsx1, and DE-Cad need to be provided.

   This comment has been addressed.

4. Line 135-136, does the primary antibody incubation need gentle shaking at 4 degree?

   It can be beneficial, but is not necessary, to have the brains shaking. This has been clarified in the protocol.

5. In Figure 1 and Figure 2, scale bars of the images need to be provided.

   Scale bars have been added.

6. The authors may provide representative images of the posterior mount in Figure 1 and Figure 2, and compare the visualization of structures to the anterior and horizontal/lateral mount.

   Thank you for this comment. The optic lobe structures shown in the deepest parts of an anterior mount (Figure 1F and Figure 2F) would be first visible across a posterior mount. Due to this reason, we have included just one mounting orientation. We have referenced this in the text (line 482-496).

Minor Concerns:

1. it is better to indicate the time required to wash the brains with PBT every time after fixation and antibody incubation.

   Thank you for your comment. We wash the brains sequentially five times, with no duration between washes. Only the final washes prior to a subsequent step (i.e. fixation to primary, primary to secondary) are conducted over a duration of time, which is stated in the protocol.

2. The blocking step before antibody incubation was not indicated.

   This comment has been addressed in Step1 of the larval immunohistochemistry section.

3. It would be constructive to include the tip of pre-wet the pipette tip with PBT when transfer brains from PBS solution to avoid the adherence of dissected brains to the inner wall of the tip.

   Thank you for this comment. We have added this advice to Step 4 of the adult mounting section.

Reviewer #4:

Manuscript Summary:
This is a useful protocol that describes with clarity and in detail the main steps needed to image Drosophila optic lobes at the larval and at the adult stages. In contrast to previous publications that focused on the dissection and immunostaining of the Drosophila optic lobe, this paper highlights and discusses different mounting strategies, which will help those researchers who are interested in imaging different regions/neurons during larval development and in adult flies.

Major Concerns:
I have no major concerns.

Minor Concerns:

1. Lines 32 and 68: The mounting strategies that the authors describe here are also critical for imaging under optimal conditions distinct regions of the optic lobe and the neurons/subcellular compartments they contain, i.e. in order to minimize light scattering and thus not only to provide an ideal imaging angle. For instance, with both posterior and anterior mountings of the adult optic lobe, one could manage to image with a very similar angle the dendrite of a particular neuron in medulla layer 3. However, with the anterior mounting, this dendrite will be closer to the objective and will be imaged under better conditions with less light scattering compared to posterior mounting. This is critical when the researcher aims to image dendrites/axons to analyze in detail their arbors/shapes. I strongly recommend that the authors emphasize this point when mentioning the advantages of the different mounting strategies along the manuscript (lines: 352, ...), similarly to what they do in lines 454-457.

   This is an excellent point. Towards the end of the second paragraph in the Representative Results section we have added a few sentences addressing this issue.

2. Introduction: The authors describe developmental processes that take place during larval and pupal stages, and in the last paragraph of the introduction, the authors describe their methodology to image larval and adult brains. The authors might want to highlight why it is important to image the adult brain in the context they presented. In addition, it would be very useful, and it would also make the manuscript very complete, to include protocols for different mounting strategies of optic lobes during pupal development. Many interesting steps of circuit formation occur during pupal development, as the authors state in lines 57-59.

   We have added a paragraph at the end of the Discussion on applying our protocol to the pupal optic lobe.

3. Line 43: Reference 8 is not related to what the sentence states. The authors should cite other studies, for instance: "Wu et al., 2016 Elife", "Fischbach and Dittrich, 1989 Cell Tissue Res", etc.

This comment has been addressed.

4. Lines 43-45: The authors could describe the organization of the optic lobe in more detail, i.e. highlighting the columnar structure of all neuropils and the layered structure of all neuropils except the lamina. They could further link this to the functional aspects that they already mentioned: retinotopy and processing of different types of stimuli.

   This comment has been addressed.

5. Lines 51-53: From a recent review (Contreras et al., 2019), I understood that the OPC generates neurons of the outer medulla and the lamina, while the IPC generates neurons of the inner medulla and the lobula complex. Could the authors clarify this discrepancy with what they wrote, please?

   This comment has been addressed.

6. Line 53: The authors should cite "Apitz and Salecker, 2015 Nat Neurosc" when they mention that the IPC generates neurons of the lobula plate.

   This comment has been addressed.

7. Lines 56-57: The authors could also cite other studies showing that temporally-restricted TFs generate neuronal diversity in the Drosophila optic lobe, for instance: "Apitz and Salecker, 2015 Nat Neurosc" and "Suzuki et al., 2013 Dev Bio".

   This comment has been addressed.

8. Line 83: Please, specify the kind of forceps used for each step (#5 or #55 type?)

   This comment has been addressed.

9. Line 94: Alternatively, the authors could keep the dissected brains in a well plate on ice to preserve them better.

   This comment has been addressed.

10. Line 120: Other immunohistochemistry protocols recommend using blocking buffer for a blocking step before adding the 1ary antibodies solution, as well as to have 5% normal goat serum in the antibody solution. Please, clarify this difference with previous studies. Also, the authors could specify somewhere (may be last table) the dilution factor for antibodies used in this protocol.

    This comment has been addressed.

11. Line 153: It could be mentioned that, as an alternative, brains with 2ary antibodies solution can be incubated overnight at 4º C (covered with aluminum foil).

    This comment has been addressed.

12. Lines 170 and 279: In order to avoid damage, brains could be transferred by using a P200 pipette.

    This suggestion has been added.

13. Line 176: "Lobula plug" was not introduced earlier in the manuscript. I would recommend that the term is briefly explained, or to use another term more in accordance to the most recent papers in this field.

    We agree with this point and have revised the sentence to define the plug as mentioned.

14. Line 183: The authors might consider using a nomenclature for the different IPC regions that corresponds to the one used in the most recent papers in this field ("Filipe-Pinto et al., 2018", "Apitz and Salecker, 2015", "Schilling et al., 2019").

    We agree with this point and have labelled the correct IPC structures in Figure 1, with reference in the text.

15. Lines 202 and 291: Please, specify the thickness of the coverslips used for making the bridge and to cover the brains. This is important to achieve good imaging conditions, as well as to avoid too much pressure on the brains (if the bridge is too low), which might affect their integrity and alter the structures to image. The authors might consider highlighting this.

    This comment has been addressed.

16. Line 289: Please, specify the minimum thickness of clay used for making the bridge. This is important to achieve good imaging conditions, as well as to avoid too much pressure on the brains (if the bridge is too low), which might affect their integrity and alter the structures to image. The authors might consider highlighting this.

    This comment has been addressed.

17. Line 307: This could be done with forceps that are not so sharp in order to avoid damage of the brains.

    This comment has been addressed.

18. Line 346: The term "lobula IPC" is a bit confusing, and the "lobula plug" needs to be defined somewhere.

    This comment has been addressed.

19. Lines 380-383: The authors could also mention that the horizontal mounting is especially suited for the examination of the layered structure of the neuropils, as well as to analyse which layers are innervated by different neuronal types. This mounting is also very good for the examination of retinotopic projection patterns. I think this change would make reader more aware of the advantages of the different mounting strategies.

    This comment has been addressed.

20. Line 392: I would write something like "in this orientation,… neurons of the lamina are closer to the surface or to the miscrope's objective".

    This comment has been addressed.

21. Line 393: I would write something like "This orientation permits optimal imaging conditions for…"

    This comment has been addressed.

22. Line 518: There is a duplication with this reference (line 510).

23. Figure 1 D-F: I think that for non-experts, it might be difficult to match some of the labels with the corresponding regions/structures. This could be improved by using arrows.

    Arrows have been added.

24. Figure 1: Please, define somewhere in the text the meaning of cortex and neuropil. This might be helpful for non-expert readers.

    This comment has been addressed.

25. Figure 2H: I strongly recommend that the authors replace this by a panel in which a specific neuronal type is also labeled (with enhancer-Gal4, UAS-memGFP for instance) to show that the horizontal mounting is especially good to examine the neuropils innervated by this neuronal type, as well as its retinotopic projection patterns and the different neuropil layers it innervates. Different layers in the neuropils could be also labelled by using published antibodies (like anti-Connectin). I think this would substantially improve the Figure and and make the reader more aware of the advantages of this mounting strategy.

    Thank you for this excellent comment. We have added an additional panel (Figure 2I) labelling a medulla neuron (Dm4) with GFP and co-stained with Brp. Dm4 dendritic arborizations in medulla layers M3 and M5 are nicely shown in this orientation.