Immunofluorescent staining and imaging of the pupal and adult *Drosophila* visual system

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This immunofluorescence protocol can be used to assay cell morphology, cell positioning and subcellular localization of proteins in the fly eye at stages of development from early pupation to adult. The protocol includes the following procedures: collecting and developmentally staging *Drosophila* pupae, dissecting fly eyes at defined stages of development, immunostaining of retina and preparing visual system samples (i.e., retina and optic lobe) for confocal microscopy. It is supplemented with images of key dissection steps, guidelines for troubleshooting and examples of data obtained using these methods. Overall, this protocol takes up to 9 d to complete. The amount of hands-on time required on each day varies, ranging from 30 min to several hours depending on the number of stages and/or genotypes one wishes to study.

INTRODUCTION

Drosophila eye organogenesis requires the precise and intertwined regulation of cell proliferation, specification, apoptosis, shape and polarity. *Drosophila* genetics enable the experimenter to easily remove (loss-of-function approach)[1] or ectopically express (gain-of-function approach)[2] a given gene. This includes the newly emerging genome-wide *in vivo* RNAi gene silencing strategy[3] that has been furthered in part by the NIG-FLY group at the National Institute of Genetics in Japan[4] and the *Drosophila* RNAi group at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences[5]. As the eye is not essential for the survival of the animal, one can uncover information that would normally be inaccessible due to lethality in an *in vivo* system. For these reasons, the developing eye represents a good model system in which to study organogenesis.

The compound eye consists of approximately 800 light-gathering units called ommatidia[6]. Each ommatidium contains 20 cells, including eight photoreceptor neurons labeled R1–R8 that bear an apical light-gathering organelle called the rhabdomere[7]. After early specification in the larval imaginal disc, the eye develops during the pupal life of the animal. It is possible to stage the pupa with reference to defined key events in the morphogenesis of the photoreceptors. Early in pupal development, a set of accessory cells known as the cone cells make contact with one another and close over the ommatidial photoreceptor cluster engaging in new cell–cell junctions[7]. A direct consequence of this process is a 90° rotation of the apical portion of the photoreceptor membrane, now aligned with the proximodistal axis of the developing retina. During subsequent pupal life, the retinal cells undergo extensive morphogenesis, with a tenfold increase in their length along the proximodistal axis of the retina. Concurrent with this increase in cell length is the elaboration of the rhabdomere[7,8]. The formation of a functional compound eye depends on two key processes. First, optimum cell stacking within the ommatidial unit relies on specifying the correct cell number and positioning within the retinal cell lattice[6]. Second, specifying proper photoreceptor morphogenesis is a highly regulated aspect of eye development that relies heavily on the proper establishment and maintenance of apical–basal polarity.

Powerful genetic tools such as the FLP/FRT system[1] and refinements such as the MARCM system[9] allow for the study of given factors at the single cell level within mosaic tissues that contain both wild-type and mutant cells. Further genetic tools have been developed to specifically study gene function in the eye[10,11]. The ability to study gene function *in vivo,* combined with the fact that proper photoreceptor morphogenesis depends on the coordinated function of a number of key events, makes these cells ideal for studying a wide variety of important aspects of cell biology[12–17].

This protocol can be used to examine cell morphology, cell positioning and discrete protein localization by immunofluorescence on retinal preparations taken from early-, mid- and late-stage *Drosophila* pupae. We have included detailed descriptions of the dissection methods used at three discrete stages of photoreceptor development which correspond to the onset of rhabdomere morphogenesis with the induction of microvilli within the apical membrane of the cell (37% pupal life), the opening of the ommatidium at mid-pupal life (55% pupal life) and finally the imaging of the mature cells (95% pupal life).

The success of this protocol depends largely on obtaining cleanly dissected retinal samples that have not been excessively torn or distorted. First-time users may find it helpful to practice with wild-type samples before proceeding to dissect precious samples, and might choose to sacrifice one of the two eyes, thereby focusing on dissecting one eye per head. Although it is desirable to preserve as much of the retina as possible, because the compound eye consists of approximately 800 repeated units, satisfactory data can often be obtained with less than a whole retina. This is certainly true when processing samples in which the whole eye has been made mutant for a gene of interest or when RNAi has been induced in the whole eye. A little extra care should be taken when dissecting eyes in which mosaic clones have been induced, as having more tissue will obviously increase the number of clones that can be analyzed. In some cases, especially when dissecting eyes with severe morphological defects, the eye may be too fragile for even the most experienced person to successfully dissect (see **? TROUBLESHOOTING**).

This protocol can also be used to dissect adult eyes. This may be necessary if fly stocks used to build flies of a desired genotype do

not carry markers that can be scored during pupation. However, some antibodies that give satisfactory results on pupal retinas do not work well on adult retinas. When adult flies are collected, it is recommended that they be dissected within 2 d of eclosion to avoid confusing a phenotype associated with eye development with a possible subsequent retinal degeneration phenotype.

In certain cases, the primary antibody used for immunofluorescence is sensitive to the fixation conditions used. Alternate fixation methods are provided for this purpose. As previously noted, in some cases a primary antibody that works well on pupal retinas will not give satisfactory immunofluorescence results on adult retinas. Provided that the fly stocks used to build flies of interest carry balancers that can be scored during pupation, retinas at 95% pupal development can be used instead of adult retinas. At 95% pupation the morphology of the retina differs little from that of the adult retina, and it is easier to dissect the eye.

Finally, the method described here assumes that the reader is familiar with the basic principles of rearing flies in the lab and is able to set up a mating cross to produce flies of the desired genotype. For users who are new to working with flies, several excellent print reference materials are available that describe the basic principles of working with flies[18].

MATERIALS

REAGENTS

- PBS (see REAGENT SETUP)
- 1× PBS + 0.3% Triton X-100 (Sigma cat. no. T8787) ! CAUTION Triton X-100 is an irritant. Wear gloves.
- Formaldehyde (Sigma F8775) fixative (see REAGENT SETUP) ! CAUTION Formaldehyde is a fixative and suspected carcinogen. Wear gloves and use in a well-ventilated area.
- Primary antibody solution (see REAGENT SETUP)
- Secondary antibody solution (see REAGENT SETUP)
- Fluorescently labeled phalloidin; rhodamine-conjugated phalloidin (Sigma cat. no. P1951) is suitable for most applications.
- Alexa-conjugated phalloidins are also available (e.g., Invitrogen cat. nos. A12379 and A12381). These are more expensive, but are ideal if your choice of available secondary antibodies precludes you from using rhodamine for phalloidin staining.
- Antifade mounting medium (Vectashield Mounting Medium (H-1000) or Vectashield Mounting Medium plus DAPI (H-1200))

EQUIPMENT

- Dissecting tweezers; Dumont #5 (Fine Science Tools 11252-30) or Dumont #55 (Fine Science Tools 11255-20) (see EQUIPMENT SETUP)
- Dissecting pins (see EQUIPMENT SETUP)
- Sylgard dissecting dish (see EQUIPMENT SETUP)
- Nine-depression glass spot plates (Corning Life Science 7220-85)
- Cover slips (22 × 22 mm no. 1.5 and 22 × 46 mm no. 1.5)
- Dissecting microscope
- Paintbrushes. These can be purchased from art supply stores
- 6 cm plastic petri dish

REAGENT SETUP

Standard 1× PBS at pH 7.4 is suitable for this protocol. Store at 4 °C and discard if a precipitate forms.

4% formaldehyde is the fixative commonly used, prepared in 1× PBS. ! CAUTION Formaldehyde is toxic. Wear gloves and use in a well-ventilated area.

Alternative fixative Retinas can be fixed in either PLP fixative[16] (10 mM sodium periodate, 75 mM lysine, 2% paraformaldehyde in 1× PBS) supplemented with 0.05% saponin or microtubule-stabilizing buffer[19] (4% paraformaldehyde in 5 mM EGTA, 2 mM $MgCl_2$, 80 mM K-PIPES [pH 6.5]). Fix samples for 20 min at room temperature (22 °C). If the retinas are excessively fragile they can be fixed overnight at 4 °C in glutaraldehyde fixative (2% formaldehyde, 2% glutaraldehyde (Agar Scientific R1020), 0.1 M sodium cacodylate). However this fixative may affect the antigenicity of some epitopes and compromise antibody staining.

Blocking solution 10% Normal goat serum in 1× PBS supplemented with 0.3% Triton X-100.

Primary and secondary antibody solutions are prepared in 1× PBS + 0.3% Triton at the dilution appropriate for the antibody of choice.

EQUIPMENT SETUP

Dissecting tools (see Fig. 1) (i) Care must be taken to keep dissecting tools sharp for optimum results. Tweezers can be periodically re-sharpened using Surgical Black Arkansas sharpening stones, which are widely available online. (ii) Prepare two dissecting pins by inserting a single regular minutien pin (Fine Science Tools, cat. no. 26002-15) into a pin holder (Fine Science Tools, cat. no. 26016-12) and screwing tight. You will need to leave one pin straight. Using tweezers, bend the end of the second pin to form a shallow hook (**Fig. 1c**). (iii) Sylgard dissecting dishes are prepared by partially filling a glass Petri dish or plastic base with Sylgard (**Fig. 1a**). Instructions for preparing the Sylgard mixture are provided with the kit (Sylgard 184 Silicone Elastomer Kit (Dow Corning)).

PROCEDURE

Collecting and staging *Drosophila* pupae (Days 1–5) ● TIMING Approximately 1 week, with 30 min of hands-on time per day

1| Approximately 5 d after setting up a cross at 25 °C, late third instar larvae will begin to wander up the sides of the vial and enter pupation.

2| Place a moistened piece of tissue in the bottom of an empty 6 cm plastic Petri dish. Mark grids on the top of the plate so that pupae collected at the same time can be grouped together and labeled.

3| Select pupae as soon after they enter pupation as practical by gently nudging them from the side of the vial

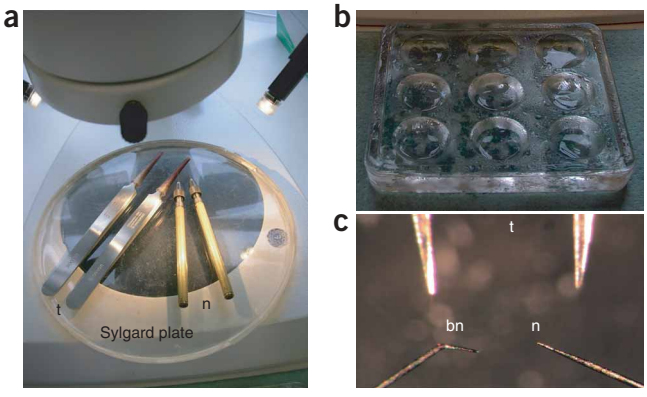

Figure 1 | Dissecting station. (**a**) Binocular microscope equipped with directional optic fibers. Lights should be positioned to achieve an indirect illumination of the sample to avoid light reflection. Sylgard dissecting plate with two tweezers (t) and two dissecting pins (n). (**b**) Multi-well glass plate used during the immunostaining protocol. (**c**) Close-up view of the dissecting tools, showing the tweezers (t), a straight dissecting pin (n) and a bent dissecting pin (bn).

with the back end of a paintbrush. Pupae can be distinguished from slow moving third instar larvae as their anterior spiracles are everted. Pupae should be white or very light yellow. Older pupae are dark yellow or brown and should not be used.

TABLE 1 | Timing of pupal development at 20 and 25 °C (in hours).

Incubation temperature (°C)	37%	55%	75%	Eclosion
20	64	95	129	172
25	37	55	75	100

4| Using the brush end, transfer each pupa to the top inside lid of the Petri dish. The pupa should stick to the top of the lid of its own accord. If not, brush a very small drop of water onto the lid. Hydrostatic forces should hold the pupa in place.

5| Label the top of the plate with the date and time at which the pupae were collected. It is helpful to mark the position of each pupa with a line so that if a pupa falls from the top of the lid it can be identified and returned to its group. Close the Petri dish and store right side up in an incubator.

6| Collect pupae at 3 h intervals during the day.

7| Incubate staged pupae at the desired temperature until you are ready to dissect retinal samples. The time it takes to reach eclosion depends on the temperature at which the flies are stored. **Table 1** lists the time it takes to proceed from early pupae to eclosion at 20 and 25 °C.

Dissection of *Drosophila* retina (Day 7) ● TIMING Approximately 1–2 h per genotype and developmental stage

8| On the appropriate day, dissect pupae of the desired stage (see **Table 1**). Start with between 6 and 8 pupae per genotype at the desired developmental stage(s) to ensure that there will be enough usable material at the end of the protocol. The method used to dissect the retina differs according to the stage of development. Methods for dissecting retinas from pupae at early (option A, **Fig. 2a–i**), mid (option B, **Fig. 2j–n**) and late stages (option C, **Fig. 2o–q**) of pupal development are described below.

(A) Option 1: 30–40% pupal retina dissection (Day 7) ● TIMING Approximately 1–2 h per genotype

(i) Pipette 200 µl of fixative solution into two wells of a glass multi-well plate (**Fig. 1b**) and place on ice. Pipette approximately 500 µl fixative solution onto the Sylgard dissecting dish (**Fig. 1a**).

(ii) Using one dissecting pin, gently hold the pupa at the posterior end (**Fig. 2a,b**). The pupa should not be submerged in fixative at this stage. Take care not to damage the animal inside the pupal case.

(iii) With tweezers, create a window exposing the ventral side of the head by carefully removing the anterior part of the case by gently snipping away the ventral portion of the anterior pupal case (**Fig. 2b,c**).

(iv) Remove circular portions of the pupal case from the anterior side of the pupa, taking care not to damage the head and eyes (**Fig. 2c–e,d′,e′**).

(v) Place the pupa in the fixative solution. Create a split in the head at the level of the mouth parts (**Fig. 2f,f′**) by inserting the tweezers through the membrane and opening them. Gently open up the hole so that the entire mouth is exposed. Alternatively, an incision can be made at the top of the head using a dissecting pin or bent forceps.

(vi) Insert a P200 pipette tip into the opening at the mouth or top of the head. Gently suck up the contents of the pupal case, making sure to take the entire head (**Fig. 2g,g′**).

(vii) Expel the contents into a glass well containing fixative solution.

(viii) Locate the two retinas (**Fig. 2h**). Using a snipped pipette tip transfer them to fresh fixative in another well. Leave the optic lobes attached to avoid the tissue folding on itself. Prepare 6–8 retinas per genotype in this manner.

(ix) Incubate the retinas for 20 min at room temperature with gentle shaking. Pipette the fixative from the glass well and replace with 1× PBS + 0.3% Triton X-100.

(x) Proceed to immunostaining.

(B) Option 2: 55–75% pupal retina dissection (Day 7) ● TIMING Approximately 1–2 h per genotype

(i) Pipette 200 µl of fixative solution into one well of a glass multi-well plate and place on ice. Pipette approximately 500 µl fixative solution onto the Sylgard dissecting dish.

(ii) Proceed with Steps (ii)–(iv) of dissection protocol outlined in Option A.

(iii) Using dissecting tweezers, remove the head from the thorax (**Fig. 2j**). Heads may tend to float on the outside of the fixative droplet due to air trapped under the antennae.

(iv) Insert one tweezer into the occipital foramen and gently hold the head face down against the dissecting dish (**Fig. 2k**). With another tweezer, grab the proboscis and pull it away from the head. If it does not come away with the rest of the proboscis, remove the labrum (**Fig. 2l**).

(v) Using the tweezers, bisect the head by tearing both retinas apart using small alternating motions (**Fig. 2m**). It is helpful to leave a small amount of head capsule at the eye margins for later dissection steps.

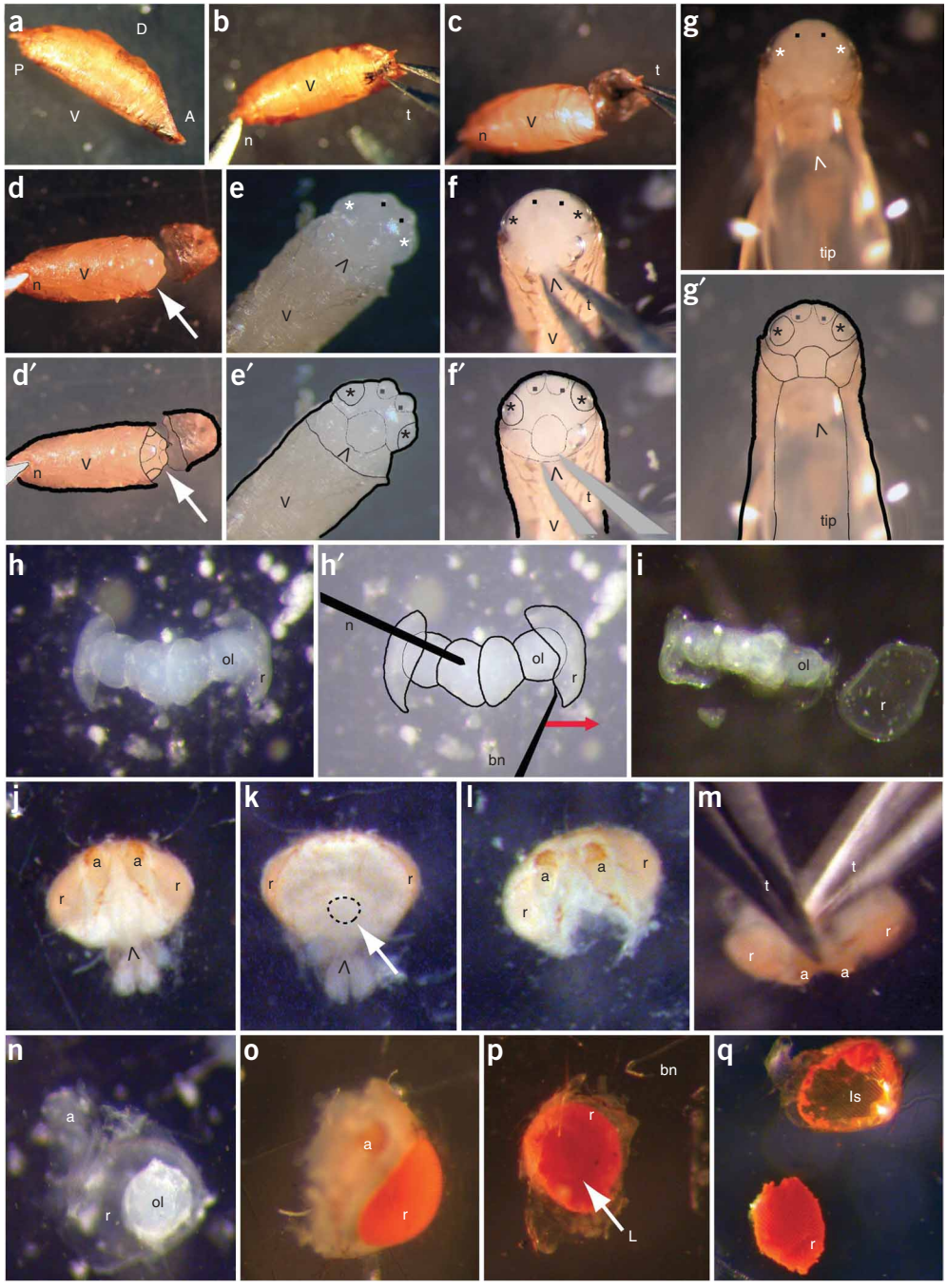

Figure 2 | Pupal visual system dissection. (**a**) 37% pupal development animal. The dorsal (D), ventral (V), posterior (P) and anterior (A) poles are labeled. (**b–e**) Successive dissection steps used to gain access to the animal head. (V) indicates the ventral side of the pupae. (^) points to the labrum, (*) mark the developing eyes and (squares) mark the developing antennae. In (**b–d**), (n) marks the needle while in (**b** and **c**) a (t) marks the tweezer. In (**d**), the white arrow points to the head of the animal. In (**f**) the head has been exposed and tweezers (t) are used to open a hole in the mouth of the animal. In (**g**), the pupal animal is held in fixative and the whole visual system is pipetted (tip) out from the opened head. In (**d′–g′**) key anatomical features have been traced onto the original image to aid visualization. In (**h**) the whole visual system is extracted from a 37% pupal development animal in fixative; (r) marks the retina and (ol) the optic lobe. In (**h′**) the visual system preparation has been traced for clarity. A straight dissecting pin (n) and a bent pin (bn) are drawn onto the image to show where they should be positioned in order to detach the retina from the optic lobe. In removing the retina, the straight pin is used to hold the preparation in place and the bent pin is moved in the direction indicated by the red arrow. (**i**) A 37% pupal retina (r) detached from its optic lobe (ol). (**j–n**) Successive steps of the dissection of a 55–75% pupal retina. In (**j–n**) the retinas are labeled (r), the antennae are labeled (a), and (^) marks the mouth parts where appropriate. (**j**) Anterior view of a mid-stage pupal head. (**k**) Posterior view of a mid-stage pupal head. A white arrow marks the position of the occipital foramen (also circled with a black dashed line). In (**l**), the mouth parts have been removed. In (**m**) tweezers (t) are used to bisect the head with local, alternating motions. (**n**) A dissected 55% pupal development retina (r) detached from its optic lobe (ol). The developing retina is indicated as (r) and the antenna is labeled (a). (**o–q**) Successive steps of the dissection of a 95% pupal development retina. In (**o**), one head was bisected and one of the retinas (r) is floating in fixative. In (**p**) the same retina has been cleaned using a bent dissecting pin (bn). In this preparation the lamina (L) is visible. In (**q**), the retinal cells (r) have been separated from the lens (ls).

(vi) Remove excess brain material and debris using the dissecting pins to obtain a clean visual system preparation (**Fig. 2n**).

(vii) Pipette the sample using a P200 with a snipped pipette tip and place it in fixative in a glass well on ice. Using dissecting pins, gently brush away any bubbles from the surface of the retina to ensure that it stays submerged in the fixative. Prepare between 6 and 8 retinas per genotype in this manner.

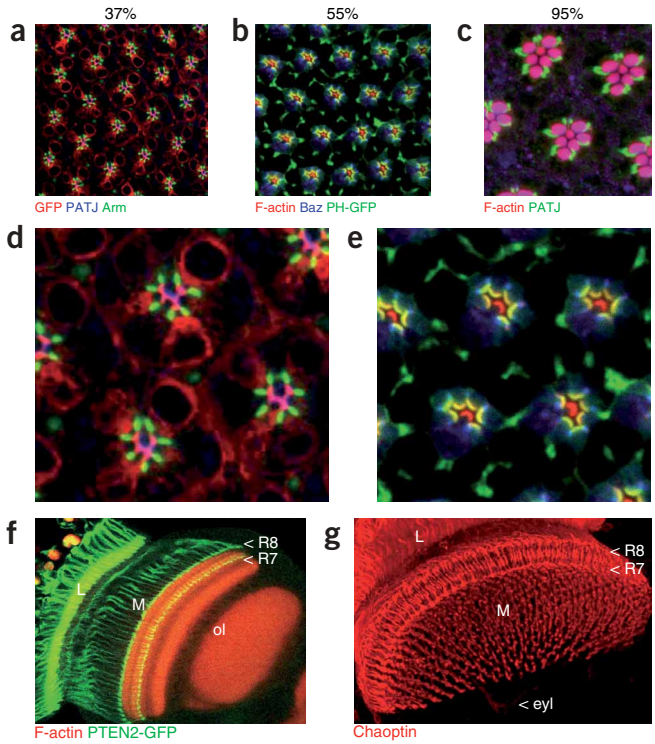

Figure 3 | Immuno-imaging of whole mount retinas and optic lobes (**a**) 37% pupal development flat retina stained for a ubiquitous GFP (red), the apical marker PATJ (blue) and the *zonula adherens* marker armadillo (Arm, green). (**b**) 55% pupal retina stained for F-actin using rhodamine-phalloidin (red), *bazooka* (the *Drosophila* homologue of Par3) (Baz, blue) to label the developing *zonula adherens* and PH-GFP (green), which is a marker of the phosphoinositol triphosphate PInst(3,4,5)P3[17]. (**c**) 95% pupal development retina stained for F-actin (purple) present in the outer and inner photoreceptors (forming the characteristic trapezoid shape), and PATJ (green) to label the apical stalk membrane. Panels (**d**) and (**e**) are close-up views of panels (**a**) and (**b**) respectively. (**f**) 55% pupal optic lobe corresponding to the retina shown in (**b**) stained for the lipid phosphatase/tumor suppressor PTEN using a PTEN2:GFP transgene and phalloidin staining F-actin in red. The optic lobe is indicated by (ol) while the lamina and medulla parts of the optic lobe are marked (L) and (M), respectively. The axonal terminations of the R7 and R8 inner photoreceptors are indicated by a white (<). (**g**) Whole mount 95% pupal development optic lobe stained for the chaoptic epitope (24B10, red). The lamina and medulla are labeled as in (**f**), while the eyelet axon is indicated by a white (<) and labeled eyl. Image manipulation was fully compliant with guidelines for proper digital image handling outlined in Rossner and Yamada[20].

(viii) Pipette the fixative from the glass well and replace with fresh fixative solution. Incubate at room temperature with gentle shaking for 20 min.

(ix) Pipette the fixative from the glass well and replace with 1× PBS (with 0.3% Triton X-100).

(x) Peel away the optic lobe and lamina to allow for optimal antibody penetration. Hold the eye with the lens down with a straight dissecting pin at the eye margins (head capsule).

(xi) Insert the hooked pin between the floor of the retina and the lamina. Gently prod between the layers until the lamina begins to detach.

(xii) Once about one-third of the lamina has detached, draw the hook sideways along the retinal floor to remove the lamina entirely. The optic lobes and lamina can be reserved for immunostaining if desired. Prepare 4–6 retinas per genotype in this manner

(xiii) Proceed to immunostaining.

(C) Option 3: 75–95% pupal retina dissection (Day 7) ● TIMING Approximately 1–2 h per genotype

(i) Pipette 200 µl of fixative solution into two wells of a glass multiwell plate and place on ice. Pipette approximately 500 µl fixative solution on to the sylgard dissecting dish.

(ii) Proceed with Steps (ii)–(iv) of the dissection protocol outlined in option A.

(iii) Proceed with Steps (xiii)–(xv) of the dissection protocol outlined in option B in order to bisect the head (**Fig. 2j–m**).

(iv) Remove all the excess material that is not retinal tissue. This includes the surrounding head capsule, central brain, optic lobe and excess connective tissue. The head capsule should be removed using gentle and local tweezer motions similar to those shown in **Figure 2m**. The lamina should not be removed (**Fig. 2p**). The optic lobes can be reserved for immunostaining if desired.

(v) Remove the lens to allow for optimal antibody penetration and optimal imaging. With the lens side down, hold down the retina by pinning the ventral excess head capsule with a straight dissecting pin.

(vi) Gently insert the point of the hooked dissecting pin between the lens and the top of the retina. Without pulling the lens away, try to separate the two layers along approximately one-quarter of the eye.

(vii) Insert the hooked dissecting pin into the opening between the lens and the retina. Gently drag the hook sideways along the lens, applying a slight downward pressure.

(viii) Once the lens is separated from half of the retina, use a scooping motion to separate the remaining tissue. It is not essential to remove the whole retina perfectly. If at least a third of the retina is intact, there should be enough tissue to proceed. Prepare 4–6 retinas per genotype in this manner (**Fig. 2q**).

(ix) Proceed to immunostaining.

Immunostaining of retinal preparations (Days 7 and 8) ● TIMING Day 7, 30 min (10 min manipulation and 20 min incubation), Day 8, 2.5–4.5 h (30 min manipulation and 2–4 h incubation)

9| Day 7: Replace 1× PBS + 0.3% Triton with 200 µl blocking solution. Incubate with shaking for 20 min at room temperature.

10| Remove blocking solution and add primary antibody solution. Incubate with shaking overnight at 4 °C.
■ **PAUSE POINT** In most cases an overnight incubation with primary antibody at 4 °C is suitable. In our experience this incubation could be extended to 2.5 days at 4 °C if needed. However, it should be noted that this can increase the background and should be avoided if the primary antibody is known to give a high background signal. If extending the incubation to 2.5 d, ensure that the sample is well covered. A shorter incubation of one hour at 22 °C can be used 30–40% pupal retina samples. This shorter incubation is not recommended for retinas beyond 55% pupal development as one hour is insufficient for the antibody to penetrate these thicker retinas.

11| Day 8: Remove primary antibody solution and wash 3 × 5 min in PBS + 0.3% Triton. Add secondary antibody solution and incubate with shaking at room temperature for 2–4 h.

12| Wash twice briefly with 1× PBS + 0.3% Triton. Wash a third time and incubate with shaking at 4 °C overnight.
■ **PAUSE POINT** This final wash step can be extended to 2.5 days, at 4 °C if needed. Ensure that the sample is well covered.

Preparation of retinal samples for confocal microscopy (Day 9) ● **TIMING: Approximately 20 min per preparation**
13| Using a snipped pipette tip, pipette the retina onto the center of a clean glass slide.

14| Prepare retinas for mounting. The method used depends on the stage of development (option A is used for early stage pupal retinas while option B is used for mid and late stage pupal retinas).
(A) Option 1: 30–40% pupal retina
(i) If dissecting 30–40% pupal retinas, use the dissecting pins to remove the optic lobe, including the lamina (see **Fig. 2h,i**), to obtain the corresponding retina free of any material (**Fig. 2i**). Use a straight dissecting pin to hold the preparation in place and insert a bent dissecting pin between the retina and optic lobe. Gently pull the retina away from the optic lobe using the bent dissecting pin.
(ii) Mount the corresponding retinas, flat with a 22 × 46 mm no. 1.5 coverglass that will be sealed at its four corners using nail varnish.
(B) Option 2: 55–75% pupal retina and 75–95% pupal retina
(i) For mid- and late-stage retinas and optic lobe preparations, pipette the sample using a P200 with a snipped pipette tip onto a clean glass slide. Remove excess PBS and cover with a drop of mounting medium.
(ii) Place two pieces of bridging material (coverglass 22 × 22 mm no. 1.5) on either side of the preparation to form a gap about 5–8 mm wide.
(iii) Arrange the lensless retina with the apical side facing upward, using the dissecting pins.
(iv) Spread the mounting media using a hooked dissecting pin into the space defined by the two 22 × 22 mm flanking coverglasses forming the bridge, so that the entire well is covered. As you spread the mounting media the retina should move slightly, but not freely. If it does move freely, carefully remove excess mounting media with a clean tissue. If there is not enough media to cover the slide, carefully add a small amount.
(v) Carefully cover with a rectangular coverglass. Seal at the corners with nail varnish.

15| Store slides at 4 °C. Ideally preparations should be imaged within 48 h.

● **TIMING**
Steps 1–7, collection and staging pupae: Approximately 1 week, with 30 min of hands-on time per day
Step 8, dissecting: Approximately 1–2 h per genotype
Steps 9–10, antibody staining: Approximately 30 min
Steps 11–12, antibody staining: Approximately 2.5–4.5 h
Steps 13–15, preparation of samples for microscopy: Approximately 20 min per genotype
In this protocol, Day 1 is defined as the day on which the user begins to collect pupae. We have defined Day 7 assuming that the user intends to dissect retina at all stages of development described in this protocol at the same time following incubation at 20 °C. This timing will obviously vary depending on the exact stage(s) or development that the user wishes to prepare and the incubation temperature at which the pupae are stored (See **Table 1**).

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Problem	Possible reason	Solution
Weak or no signal (Step 15)	Antigenicity of the epitope is sensitive to the fixation condition used	Try an alternative fixation method
	Epitope is not accessible to the antibody	Use an antibody against another part of the protein. Use a transgenic line expressing a tagged protein with the tag fused to a different position
	Solutions are no longer at the correct pH	Check the pH of 1× PBS and 1× PBS + 0.3% Triton
	Antibody is not effective on adult retinal preparations	Provided the pupae can be genotyped, dissect retina at 95% pupal development
	Low expression of the protein of interest	Use a transgenic line to overexpress the protein of interest with a fusion tag
High background (Step 15)	Insufficient washing after incubation with secondary antibody	Increase the number of wash steps
Retinas are fragile and very difficult to dissect (Step 8)	Defect in eye development severely compromises retinal tissue integrity	Use a weaker allele of the gene of interest to make whole mutant eyes
		Leave brain tissue attached to the retina and fix overnight at 4 °C in glutaraldehyde fix. Continue with the dissection the next day. Note that the glutaraldehyde fix may affect the antigenicity of some epitopes and therefore compromise immunostaining
		Analyze mosaic mutant retinas rather than whole mutant eyes
		Use a different RNAi insertion line that gives a milder phenotype
		Use a weaker Gal4 driver to express RNAi in the eye
		Use a heat shock Gal4 driver to express RNAi for a shorter period of time during development
30–40% pupal retinas do not detach from the rest of the head (Step 8, Option A (viii))	Pupae are older than expected	Ensure that only white pupae are selected for staging and when collecting pupae discard any pupae that are obviously too old, to avoid confusion
		Check that the temperature of the incubator is not higher than what is set
		There is some inherent variability in staging pupae. The times listed in **Table 1** are a general guide, but the actual stage may vary by 2 h or more in either direction

ANTICIPATED RESULTS

The fly retina offers an opportunity to study the morphogenesis of a complex polarized cell *in vivo* using genetics. The protocols described here allow one to study a given gene's function at the single cell level, using multi-channel staining in whole retina preparations. The different cell types present in the adult fly eye originate from common multi-potent precursor cells (because there is no cell lineage in the fly eye). This allows for the possibility to generate mosaic eyes for a given mutation, with ommatidia consisting of wild-type and mutant cells. The widespread use of fluorescent proteins or various other markers such as β-galactosidase allows one to easily visualize the corresponding mutant and wild-type cells. Because the different membrane domains of the photoreceptor (apical organelle, *zonula adherens,* baso-lateral membrane and axon) are well defined, confocal analysis of the corresponding preparation allows the experimenter to assay the fine distribution of a given gene product.

This is illustrated in **Figure 3** with examples of wild-type retina stainings at different stages of development. Optic lobes, which in this protocol are dissected along with the retina, can also be examined in confocal microscopy. This point is illustrated in **Figure 3f,g**. Confocal analysis of whole mount retina is particularly powerful when combined with classical electron microscopy. These two approaches should be used in parallel to achieve optimal characterization of a given mutant phenotype.

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