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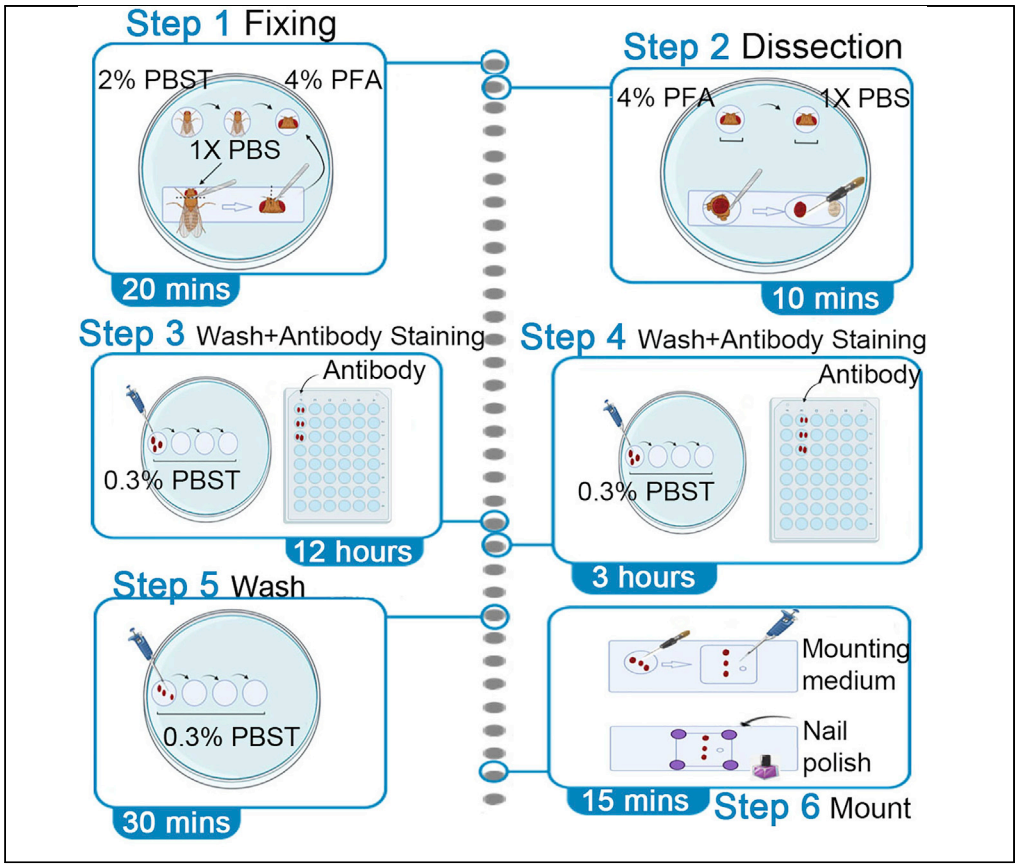
Protocol

Whole-mount immunofluorescent labeling of the adult fly retina



The adult *Drosophila* compound eye is an ideal *in vivo* model for studying biological questions. However, light microscopy of this tissue requires cumbersome embedding and sectioning. Here, we document detailed whole-mount procedures for immunolabeling the adult retina, enabling high-quality studies of fluorescent-tagged targets with straightforward preparations. We describe the steps for visualizing the nuclear lamina, membrane-associated protein, and actin-rich rhabdomere, but this robust protocol can apply to other cellular structures and target proteins.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Reproducible protocol to study fluorescent-marked targets in adult *Drosophila* retina

Utilizes straightforward immunolabeling and mounting techniques

Can be performed in pupal eyes with modified tissue isolation procedures

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Whole-mount immunofluorescent labeling of the adult fly retina

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SUMMARY

The adult *Drosophila* compound eye is an ideal *in vivo* model for studying biological questions. However, light microscopy of this tissue requires cumbersome embedding and sectioning. Here, we document detailed whole-mount procedures for immunolabeling the adult retina, enabling high-quality studies of fluorescent-tagged targets with straightforward preparations. We describe the steps for visualizing the nuclear lamina, membrane-associated protein, and actin-rich rhabdomere, but this robust protocol can apply to other cellular structures and target proteins.

For complete details on the use and execution of this protocol, please refer to Chang et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for using antibodies and dyes to label adult *Drosophila* compound eyes. However, we have also used this protocol in pupal eyes with modified tissue isolation procedures.

Prepare buffers

Timing: 0.5–1 h

1. Prepare 2% (v/v) Triton X-100 in PBS (2% PBST). Dilute the detergent Triton X-100 stock in 1× PBS.
2. Prepare 0.3% (v/v) Triton X-100 in PBS (0.3% PBST). Dilute the detergent Triton X-100 stock in 1× PBS.

Note: All 1× Phosphate Buffered Saline (PBS) buffer with pH 7.4 at 25°C.

Prepare fixing solution (4% paraformaldehyde)

Timing: 30 min

3. For 5 mL of 4% paraformaldehyde (PFA), add 5 mL of 1× PBS to a 20 mL disposable scintillation vial on a stir plate in a fume hood.

⚠ CRITICAL: Paraformaldehyde is a toxic substance, prepare the 4% PFA solution in the fume hood while wearing gloves.

4. Add 0.2 g of paraformaldehyde powder to the PBS solution.
5. Heat while stirring to approximately 200°C. Once boiled, put it aside to cool down.
6. Once the bubbles are settled, re-boil again then set aside to cool down once more.
7. Wait until it is room temperature, then the solution can be stored at 2°C–8°C for up to 3–4 days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Phalloidin–Tetramethylrhodamine B isothiocyanate (1:20)	Sigma-Aldrich	Product No. P1951
Mouse anti-Lamin Dm0 (1:20)	Developmental Studies Hybridoma Bank, DSHB	DSHB Cat# ADL67.10
Rabbit anti-Lamin Dm0 (1:20)	Dr. Paul A. Fisher at Stonybrook University, USA	
Alexa Fluor® 647 AffiniPure Goat Anti-Mouse IgG (H + L) (1:100)	Jackson ImmunoResearch Laboratories	Code Number: 115-605-146
Mouse monoclonal 4F3 anti-discs large (DLG) (1:100)	Developmental Studies Hybridoma Bank, DSHB	DSHB Cat# 4F3 anti-discs large
Alexa Fluor® 488 AffiniPure Goat Anti-Mouse IgG (H+L) (1:100)	Jackson ImmunoResearch Laboratories	Code Number: 115-545-003
Alexa Fluor® 647 AffiniPure Goat Anti-Rabbit IgG (H+L) (1:100)	Jackson ImmunoResearch Laboratories	Code Number: 111-605-003
Chemicals, Peptides, and Recombinant Proteins		
10× PBS, pH 7.4 (Phosphate Buffered Saline buffer)	BioMate™	Product No. BR110-1L
Paraformaldehyde	Sigma-Aldrich	Product No. P6148
TRITON® X-100	US Biological	CAS: 9002-93-1
Nail Polish	N/A	N/A
Antifade mounting media	Vectashield	REF: H-1000
Experimental models: Organisms/strains		
Adult *D. melanogaster*: UAS-*LacZ*	Bloomington Drosophila Stock Center	BDSC# 1777
Other		
60-well clear Terasaki plate with lid	Electron Microscopy Sciences	Product No. 70438-15
Microscope cover glass 18 × 18 mm #1.5	Deckglaser	LOT:40434 017
Microscope cover glass 18 × 18 mm #1 (for shattered glass)	Deckglaser	LOT:37695 017
DGS® Microscope Slide	Bioman	DG75001-07105
Tungsten needles	A-M systems	LOT:545636
Tweezers	Dumont	0207-5TI-PS

⚠ CRITICAL: Paraformaldehyde is a toxic substance, exposure to the skin, eye, and respiratory tract may cause irritation and severe damage. Work in a fume hood and wear gloves when handling.

Note: Commercially available aqueous formaldehyde solutions can be used as an alternative to paraformaldehyde powder.

STEP-BY-STEP METHOD DETAILS

Fixation of fly head

Timing: 20 min

Fixing the fly head with 4% paraformaldehyde will aid in easier dissection of the eye.

1. Incapacitate flies using CO_2 then place the flies in a drop of 2% PBST. Wait until anesthetized then transfer to 1× PBS.
2. Place two layers of double-sided tape on a Sylgard dissection plate.
   a. Use tweezers to move each individual fly onto the tape by its wings.
3. Remove the fly head by cutting between the head and thorax with a scalpel.
   a. Make a small incision at either the posterior or anterior end of the head, but do not bisect the head at this time so the sample handling for the next two steps will be easier.
4. Fix the head tissue by placing each individual head into a droplet of 4% PFA at room temperature for 10–12 min.
5. After fixation, remove the heads from 4% PFA and place in a droplet of 1× PBS.

Note: Work with 3–4 heads (6–8 eyes) at a time, this is the minimal amount for quantification.

Note: White-eyed flies may be more difficult to work with due to the similar color to the brain. Work with caution.

Dissection of the eye

Timing: 1–2 h

Dissecting the eye for better antibody staining and confocal microscopy observation.

6. Transfer one head from 1× PBS and place on tape. Cut the head along the incision made earlier, separating the two eye tissues.
   a. Place one eye back in 1× PBS and remove unwanted tissues on the other eye (such as gena, vibrissae, and brain tissue behind the eye) (Figures 1A and 1B).
7. With the compound eye facing downward, use the tungsten needle to flick the cornea off the eye.
   a. Gently place the needle on edge of cornea and flick it off of the eye tissue (Figure 1C, Methods video S1).
8. Use a pipette to place the finished eye in 1× PBS for later and continue on the remaining eye.

Note: Place all heads in 1× PBS and dissect one head at a time to prevent the heads from drying out.

Antibody staining

Timing: 2 days

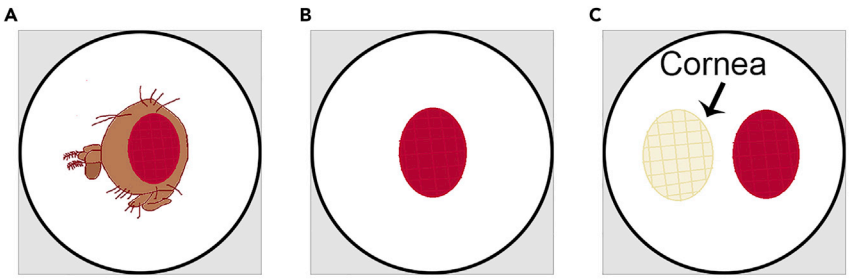

Figure 1. Dissection of the eye
(A) Overhead view of fly eye.
(B) Overhead view of fly eye after removing the unwanted tissues.
(C) The removed cornea placed next to the eye tissue.

Primary and secondary antibody staining of eye to observe target protein in eye.

9. For primary antibody staining, wash the eyes in 0.3% PBST at 23°C–25°C 3 times for 10 min each.
   a. To wash, use a dropper to place 3 drops of 0.3% PBST adjacently on a Sylgard dissection plate. Use a pipette to transfer the eyes to the first drop of 0.3% PBST, slightly pipetting the eyes in the drop. Transfer to the next drop after 10 min and repeat.
   b. After washing, place the desired antibodies and eyes into each individual well on the Terasaki plate.
   c. Place on shaker and shake 6–12 h in 4°C.
10. For secondary antibody staining, take the eyes out of the wells and wash them again at 23°C–25°C in 0.3% PBST 3 times for 10 min each.
   a. Place the desired antibodies and eyes into each individual well on the Terasaki plate.
   b. Place on shaker and shake for 2 h in room temperature at 25 rpm.
   c. After 2 h, remove the eyes from the Terasaki plate and wash in 0.3% PBST at 23°C–25°C 3 times for 10 min each for the final time.

Note: The 0.3% PBST drops should be new for each wash.

Mounting

Timing: 0.5–1 h

Mounting the eyes in mounting medium for observation under the confocal microscope.

11. Use a pipette to transfer the eyes into a drop of antifade mounting medium on the dissection plate.
   a. Pipette both the eyes and the mounting medium onto the edge of the microscopic slide.
12. Use the tungsten needle to flip the compound eye upward (the curve of the eye facing up).
13. Use a tweezer to place a small piece of randomly shattered cover glass (#1) near the eyes to prevent the cover glass (#1.5) from directly touching the eyes.
   a. With the tungsten needle, carefully place the cover glass on top of the shattered glass and eyes (Figure 2).

Note: Place the cover glass at an angle to prevent direct pressure on fly eyes.

14. Use a pipette to fill up the remaining space between the microscopic slide and cover glass with mounting medium. Avoid air bubbles.
15. Cover the four corners of the cover glass with nail polish and let dry.

Note: Line up the eyes in the middle of the slide for easier observation.

Note: Our lab uses the LSM 510 two-photon confocal microscope with ZEN software and 40× water immersion objective to scan the *Drosophila* eye at wavelengths 488, 543, and 647. Different magnifications, objectives, and wavelengths may be modified to the desired outcomes.

EXPECTED OUTCOMES

The typical whole-mount immunofluorescent labeling results of the adult fly retina are shown in Figures 3 and 4. The bicyclic peptide phalloidin is used to stain actin filaments enriched in the specialized photon-sensing domain known as the rhabdomere. Each unit eye comprises eight photoreceptor cells (R1 to R8). The rhabdomeres of R1 to R6 cells organize into a trapezoid shape extending through the retinal layer, whereas R7's rhabdomere is located above R8's counterpart

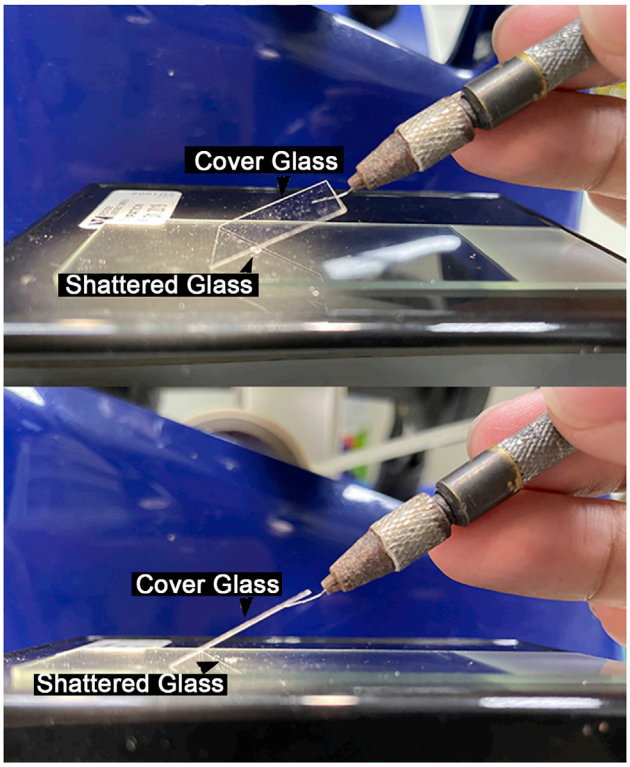

Figure 2. Placement of the cover glass
Angled placement of cover glass over fly eyes and shattered glass. Shown at two angles.

in the center (Tomlinson and Ready, 1987). Therefore, an optical cross-section of a normal adult retina shall reveal a repetitive trapezoidal ornament with seven phalloidin-labeled ovals. This protocol is feasible for labeling different target proteins, organelles, and structures if the appropriate antibodies are applied. Figure 3 shows immunolabeling of anti-Lamin, which marks lamina underneath the inner nuclear membrane that conveniently outlines the nucleus. Figure 4 shows immunolabeling of anti-Lamin and anti-DLG (discs large) co-stained with phalloidin.

Similar to common immunolabeling of sectioned samples, pilot trials for deciding the best conditions of antibody/dye dilutions, blocking reagents and required durations, and detergent concentrations are highly recommended. The trials should include genetic materials to validate the procedures better if they are available.

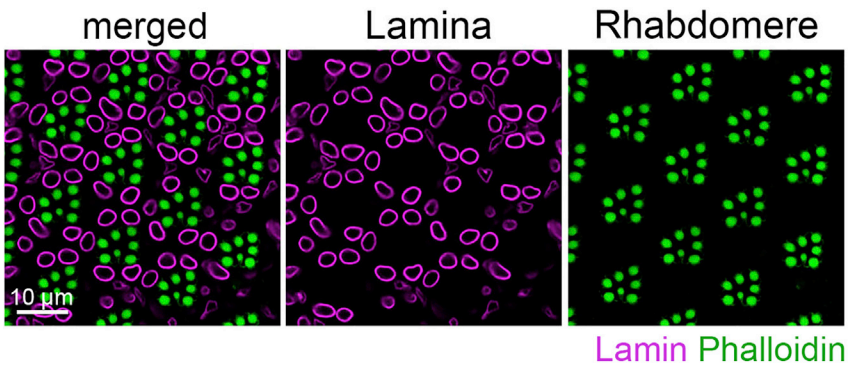

Figure 3. Confocal images of an adult retina stained with phalloidin (green) and anti-Lamin (magenta) antibody

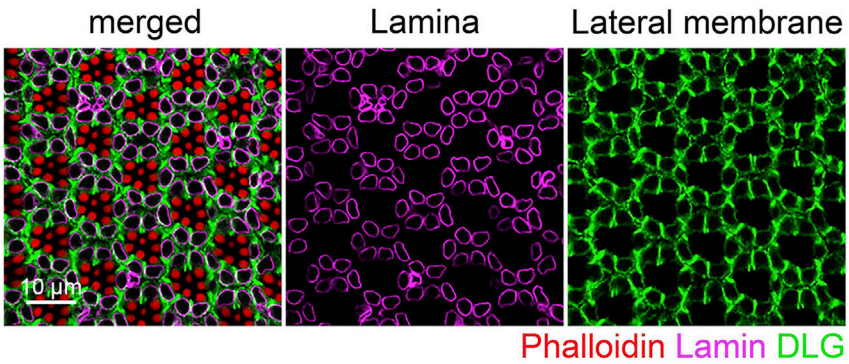

Figure 4. Confocal images of an adult retina stained with phalloidin (red), anti-Lamin (magenta), and anti-DLG (green) antibodies

LIMITATIONS

This technique labels fixed macromolecules; thus is not suitable for live imaging.

TROUBLESHOOTING

Problem 1

The following reasons may result in a weak staining signal (steps 1–3):

- The antigen-antibody affinity is low.
- Inadequate fixation and/or washing/blocking procedures may affect the antigen-antibody interaction and physical structure of the sample itself.
- The dilution and reaction time of the antibody is not appropriate.
- The cornea is not removed completely.
- The laser condition or microscopic setting is not well-suited.

Potential solution

- Test the fidelity of the selected antibody with suitable genetic controls included in the test.
- Verify the best immunostaining condition of the antibody, including blocking/washing condition and reaction time.
- Ensure the aforementioned sample preparation steps are followed.
- Check the microscopy settings with a standard slide from the manufacturer or a recently viewed sample with the matched fluorescent dyes.

Problem 2

The following mishandlings may cause artificial tissue damage (steps 1 and 2):

- Incomplete fixation of the sample.
- Pressure is applied directly onto the retina.
- Removal of the cornea damaged the underneath retina.

Potential solution

- Ensure the fixative used in sample preparation is freshly prepared within 5 days.
- Avoid tissue removal before fixation and never compress the eye surface during dissection.
- Check the tungsten needle to ensure it is straight and flexible before the experiment. A bent rod may affect the separation of the cornea and damage the retina.

Problem 3

The following reasons may lead to high background (steps 1 and 3):

- Excessive fixation.
- Autofluorescence of the eye pigment.
- Inadequate antibody/dye dilution.
- Inappropriate microscopic setting.

Potential solution

- When fixing adult eyes with 4% Paraformaldehyde, the fixation should not exceed 1 h. Determine the best fixation time for your selected antibody/chemical dye in pilot trials.
- Extending the washing time with occasional pipetting, or increasing the detergent concentration could reduce the eye pigments.
- Conduct pilot tests to seek the best antibody dilutions.
- Adjust the microscope to the best signal-to-noise ratio.

Problem 4

Fly retinas are prone to stick to surfaces, including pipette tips (steps 3 and 4). This may cause eye loss or tissue damage during transfer between washes and antibody staining, resulting in insufficient eyes for quantitative analysis.

Potential solution

- Increase the pipette's volume and pipette to wash the eyes down.
- Cut open the tip and use another pipette to retrieve the eye.

Problem 5

When filling up the space between the microscopic slide and cover glass with mounting medium, the eyes may drift away from the original position, causing a displacement (steps 4). This may cause confusion or misjudgment in reading the results if different phenotypes are involved.

Potential solution

- Place the shattered glass close to the eyes.
- Pipette the mounting medium in slowly.
- Make sure the eyes are placed upward (the curve of the eye facing up).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, [Tzu-Kang Sang] (tksang@life.nthu.edu.tw).

Materials availability

This protocol did not generate new unique reagents.

Data and code availability

This protocol did not generate new datasets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101430.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.C. and T.S.; methodology, H.C. and T.S.; formal analysis, M.H., T.H., B.Y., and T.C.; investigation, T.S. and H.C.; validation, M.H., T.H., B.Y., T.C., and T.S.; resources, T.S.; supervision, T.S.; funding acquisition, T.S.; writing – original draft, M.H., T.H., B.Y., and T.C.; writing – review & editing, T.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Chang, Y.C., Peng, Y.X., Yu, B.H., Chang, H.C., Liang, P.S., Huang, T.Y., Shih, C.J., Chu, L.A., and Sang, T.K. (2021). VCP maintains nuclear size by regulating the DNA damage-associated MDC1-p53-autophagy axis in Drosophila. Nat. Commun. *12*, 4258. https://doi.org/10.1038/s41467-021-24556-0.

Tomlinson, A., and Ready, D.F. (1987). Neuronal differentiation in the Drosophila ommatidium. Dev. Biol. *120*, 366–376. https://doi.org/10.1016/0012-1606(87)90239-9.