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Imaging *Drosophila* Pupal Wing Morphogenesis

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Summary

Drosophila pupal (P) wing development entails a series of dynamic developmental events, such as epithelial and glial morphogenesis, that are of outstanding interest to cell biologists. Here, we first describe how to prepare P and prepupal (PP) wings for immunofluorescence microscopy. This protocol has been optimized to visualize wing epithelial architecture, such as polarized cortical domains of planar cell polarity proteins. We then provide a protocol to prepare pupae for whole mount live imaging of P wings. This procedure has allowed us to live-image glial cell migration and proliferation along wing sensory nerves.

Key Words: *Drosophila*; glial cell migration; immunofluorescence microscopy; live imaging; planar cell polarity; pupal wing.

1. Introduction

During pupal (P) metamorphosis a series of dramatic tissue rearrangements mediate the transformation of imaginal discs into adult fly structures. In the course of the first day of P development wing imaginal discs evaginate, increase their surface area by flattening, secrete and ecdyse a cuticle layer, undergo cell divisions, acquire a regular epithelial packing geometry, and grow hairs ***(1–4)***. In addition to these events that bring about epithelial morphogenesis, the wing gives rise to a sensory nervous system that is made up of peripheral neurons and their associated glial cells ***(5,6)***. During early P development glial cells proliferate and migrate extensively to completely cover the wing nerves ***(7,8)***. The timeline in **Fig. 1.** illustrates this sequence of events and the stages at which they occur. A detailed description of the wing morphology during these stages can be found in **refs. *4,9***. This information will aid the understanding of the issues raised in this chapter and allow you to develop a staging protocol for your specific application and your lab conditions.

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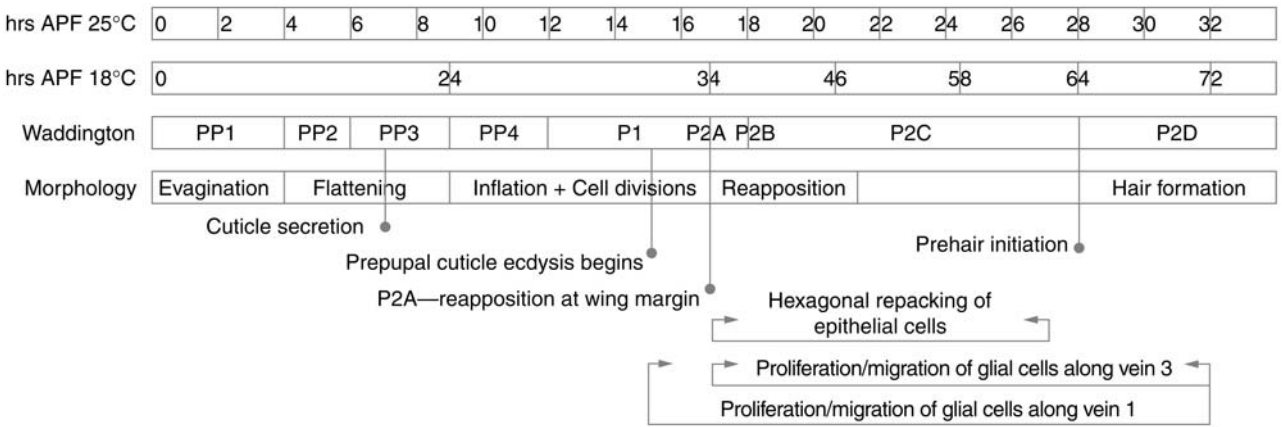

Fig. 1. Early P wing development. In this timeline we delineate morphological landmarks of P wing development, the associated Waddington's stages, and the times at which they occur at 18°C or 25°C temperatures. We use Waddington's staging terminology throughout this chapter. Development within the P case is divided into PP and P stages that are separated by a molt. During evagination, in the first PP stage (PP1), the dorsal and ventral halves of the wing pouch begin to adhere along their basal surfaces and the wing epithelium folds along the future wing margin to assume an approximately semicircular shape. In PP2, the tissue elongates and narrows until it resembles a thick cylinder three times as long as it is wide (at about 5 h APF at 25°C). During PP3 (6–9 h APF at 25°C), the wing cells flatten and the wing dramatically increases its surface area. Cuticle deposition begins at this time, and forms a chitinous sheath during PP4, when the wing becomes inflated and the dorsal and ventral surfaces move apart. This leaves the wing looking like a balloon. During inflation, a wave of cell divisions runs through the wing. Ecdysis of the cuticle—the shedding of the chitinous cuticle from the apical side of the wing epithelium—starts late in the first P stage (P1). Waddington subdivides the second P stage into substages **A–D**. In P2A, dorsal and ventral wing surfaces begin to reappose beginning at the wing margin, and the adhesion of the two basal surfaces spreads from the distal, anterior, and posterior ends of the wing during P2B and early P2C. During P2A–P2C the wing epithelium acquires a hexagonal cell packing geometry. P2D starts with the initiation of prehairs. Eventually hairs will cover the whole wing. Proliferation and migration of glial cells along vein 3 initiates in the beginning of P1 (by 15 h APF at 25°C). Migration, accompanied by proliferation of glial cells along vein 1 begins at approximately late P1 (by 17 h APF at 25°C). Glial cells cover the sensory nerves in vein 1 and 3 by mid P2D (32 h APF at 25°C). Abbreviations: APF, after puparium formation; Hrs APF 18°C, hours APF at 18°C; Hrs APF 25°C, hours APF at 25°C.

The dramatic events that occur during P wing development have posed interesting challenges to the curiosity of cell biologists. Some of the questions that have been addressed, so far, include integrin-mediated adhesion between epithelial cell sheets ***(10,11)***, vein cell fate specification by Notch signaling ***(12,13)***, establishment of wing hair planar cell polarity ***(3,14)***, and endocytic trafficking of E-cadherin during junctional remodeling ***(1)***.

The method section will first outline some general comments on staging P development (**Subheading 3.1.**). Then we will describe how prepupal (PP) (**Subheadings 3.2.** and **3.4.**) and P wings (**Subheadings 3.3.** and **3.4.**) are prepared for immunofluorescence microscopy. This protocol has been optimized in our lab to visualize polarized cortical domains of planar cell polarity proteins during PP and P stages ***(1)***. You may have to adapt some details to your specific application. In **Subheading 3.5.**, we will describe how a pupae is prepared to carry out live imaging on the P wing. This protocol has been successfully used to study the highly dynamic development of peripheral glial cells and their interactions with the sensory neurons ***(8)***, but may be used for other applications.

We will not discuss the process of imaging the samples at the microscope in this chapter as microscopy devices differ between labs. We successfully used laser scanning confocal microscopes built by Leica or Zeiss for immunofluorescence and live imaging.

2. Materials

*2.1. Immunofluorescence Microscopy (***see** *Subheadings 3.2.–3.4.)*

1. Fixatives.
   a. Fix 1: 8% paraformaldehyde (PFA) in phosphate-buffered saline (PBS)—prepare fresh or from 20% PFA stock stored at −20°C.
   b. Fix 2: 8% PFA, 200 m*M* sodium cacodylate, 100 m*M* sucrose, 40 m*M* potassium acetate, 10 m*M* EGTA (*see* **Note 1**).
2. PBS.
3. Washing solution PBT: PBT/PBT 0.01% TritonX-100/PBS (*see* **Note 2**).
4. Blocking solution PBTN: PBTN/PBTN 0.01% TritonX-100/PBS + 5% normal goat serum.
5. ProLong gold antifade mounting medium (Invitrogen, Karlsruhe, Germany) (*see* **Note 3**).
6. Microwell minitrays with lids (*see* **Note 4**)—60 wells, low profile, 10 µL well volume (Nalge Nunc, Wiesbaden, Germany).
7. Dumont forceps 55—tip measures: 0.05×0.02 mm^2, Fine Science Tools (*see* **Note 5**).
8. Round Petri dish lids (for dissection).
9. Watchmaker's glass dishes.

*2.2. Live Imaging (***see** *Subheading 3.5.)*

1. Glass bottom dishes—specialized Petri dishes for imaging (*see* **Note 6**).
2. 10S oil (Voltalef, PROLABO, Paris, France): store at room temperature in a dark vial.

3. A pair of spring scissors with small straight blades (8 cm long with 3 mm effective cutting edge, Fine Science Tools, Heidelberg, Germany).
4. Dissection forceps.
5. Tape (*see* **Note 7**).
6. Standard cover slips (*see* **Note 8**).
7. A stereo microscope with at least ×40 magnification.

3. Immunofluorescence Microscopy of PP and P Wings

3.1. Staging and Collecting Pupae

Even at the same temperature, the distinct developmental events outlined in **Fig. 1** can progress at different rates depending on the fly strain and on the specific lab conditions. Therefore, the careful establishment of a staging timeline under your lab conditions according to morphological landmarks (*see* **Fig. 1**) is recommended.

For staging, pick white prepupa (*see* **Note 9**) with a wet brush (*see* **Note 10**) off the wall of the fly culture flask and transfer to the wall of an Eppendorf tube. Note down the time and strain. Recover the pupa or prepupa at the desired stage by picking them off the Eppendorf tube wall with a wet brush.

*3.2. Dissecting and Fixing PP Wings (***see** *Note 11)*

1. Place a prepupa in 50 µL of PBS on the top of a Petri dish lid.
2. Gently make an incision using two forceps in the middle of the prepupa and separate the prepupa in two halves. Displace the posterior half.
3. Take the remaining gut and fat body out of the anterior half. Be careful not to pull on the white tissues (remaining larval epidermis) and the evaginating imaginal discs located in the head region.
4. Using one pair of forceps, pin down the prepupa in between the anterior spiracles. The ventral side of the prepupa needs to face up.
5. Slide the other pair of forceps (closed) along the ventral inside of the P case into the most anterior head region. The prepupa wings lie more dorsally on the lateral sides of the P case. Therefore, if you stay ventrally, you should not damage them when sliding the forceps past them into the most anterior head region.
6. Grab one of the two trachea near their attachment site to the anterior spiracle and slowly pull it back out posteriorly. Repeat with the wing on the other side. Each wing is attached to one tracheal branch and should therefore, ideally, be pulled out with it. By pulling on the trachea you will avoid having to touch the fragile wings directly (*see* **Note 12**).
7. Free the wings of surrounding tissue. Remove most of the debris from the drop and add 50 µL of fixative 1. Gently mix the liquid by sucking and expelling small volumes with the pipet. Leave the wings to fix in this droplet for 20 min (*see* **Note 13**).
8. Pick up the wings in a small drop between the two arms of the forceps and transfer them to a watchmaker's glass dish with a large volume of PBT. Wash them once with PBT using a P1000 pipet.
9. For staining and mounting follow the protocol in **Subheading 3.4.**

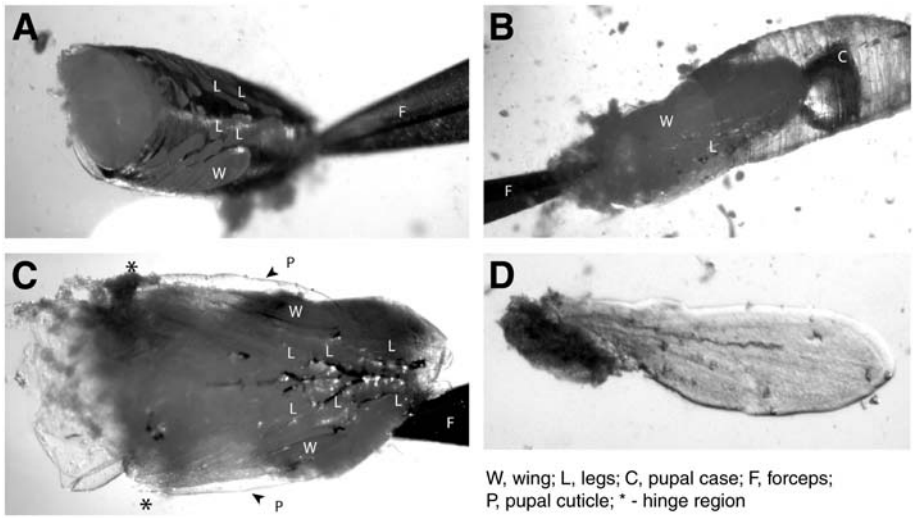

Fig. 2. Dissection for immunofluorescence microscopy. **(A)** The puparium operculum has been removed to expose the pupa's head. The pupa is fixed in position by pinning down the posterior spiracles with forceps. A small incision in the head cuticle has been made. You can see the outlines of wings and legs through the P case. **(B)** The pupa is pulled out of the P case using forceps by tugging on the thoracic P cuticle and fixing the P case at the posterior spiracles (*see* A). Image was taken viewing the lateral side of the pupa; the region where the wing and legs are located is labeled. **(C)** You can see the wing epithelium inside the translucent cuticle sac. Hold pupa in position, dorsal side down, with one pair of forceps. With the other pair of forceps make a little hole in the P cuticle by gently tugging on it near the wing hinge region. **(D)** Dissected wing (stage late P2C) with some excess hinge tissue. In all images anterior is to the left.

3.3. Dissecting and Fixing P Wings (see *Note 14*)

During the dissection it is important to allow for sufficient fixation in between the individual dissection steps. Therefore you can dissect 4 pupae in parallel, carrying out each dissection step on all 4 pupae before going on to the next dissection step. Each dissection step is represented by one numbered bullet point below.

1. Place four small droplets of fixative 2 onto the lid of a Petri dish and place one pupa into each one of them.
2. With one pair of forceps gently grab the posterior spiracles to hold the pupa and pin the forceps down onto the dish in order to fix the position of the pupa within the droplet. Fix the pupa into position like this during the subsequent steps (*see* **Note 15**). Take off the puparium operculum with the other forceps. Peel it off and remove enough P case to expose the head of the pupa. Make a little incision into the P head by gently poking the forceps into the head cuticle (*see* **Fig. 2A**).
3. Increase the size of the incision by sticking the closed forceps into the cuticle hole and gently releasing them to allow the forceps to open a little.

4. Move your forceps into the pupa through the head opening and remove some of the thoracic tissue inside the pupa. Repeat this step twice (*see* **Note 16**).
5. Carefully grab the ventral thoracic cuticle near the head incision and remove the P body from the case by gently tugging on the pupa (*see* **Fig. 2B** and **Note 17**).
6. Transfer the pupa to a fresh droplet of fixative solution.
7. Pin down the pupa at the posterior abdomen with one pair of forceps. With the other make a little hole into the translucent cuticle at the hinge region of the wing to allow the fixative to reach the wing epithelium (*see* **Fig. 2C** and **Note 18**).
8. Again, pin down the pupa at the posterior abdomen. Open the hole in the hinge cuticle a little bit more, so that the wing can fit through. Move your forceps into the hole and grab the hinge of the wing epithelium. Carefully pull the wing out of the cuticle sac (*see* **Fig. 2D**).
9. Free the wing of excess hinge tissue and debris. Pick up the wings in a small drop between the two arms of the forceps and transfer them to a watchmaker's glass dish with a large volume of PBT. Wash them once with PBT using a P1000 pipet.

3.4. Staining PP and P Wings

1. Always track your wings under the microscope. Exchange PBT in the watchmaker's glass dish with PBTN by using a P1000 pipet. Block wings for 20 min at room temperature.
2. Work all following steps with a P20-pipet; working volume is about 10–15 µL. Suck up the wings in a small volume into the yellow tip. Transfer the wings to microwell tray wells (*see* **Note 19**).
3. Make sure that for each of the following steps the wings are submerged in liquid; therefore, always leave a little bit of liquid in the wells when you exchange solutions. Take off the PBTN and then add the primary antibody diluted in PBTN to each well.
4. Incubate overnight on a gentle rocking platform at 4°C.
5. Take off the primary antibody/PBTN and wash with PBT (3X quickly followed by 3X for 10–15 min).
6. Block with PBTN for 20 min at room temperature.
7. Take off the PBTN and add the secondary antibody diluted in PBTN to each well.
8. Incubate for 3 h at room temperature on a gentle rocking platform.
9. Take off the secondary antibody/PBTN and wash with PBT (3X quickly and 3X for 10–15 min).
10. Wash once with PBS.
11. Take off PBS and add a few microliters of mounting medium into the wells. Mix the wings into the mounting medium by gently sucking them up and down in a small volume into the yellow tip (*see* **Note 20**).
12. Suck up the wings in a small volume into the yellow tip and transfer them into 12 µL of mounting medium placed on a microscope slide.
13. Spread out the drop at the edges and make sure that the wings lie flat. Slide an 18×18 mm^2 cover slip on them. Keep the slide at room temperature in the dark for 24 h to allow the mounting medium to solidify.

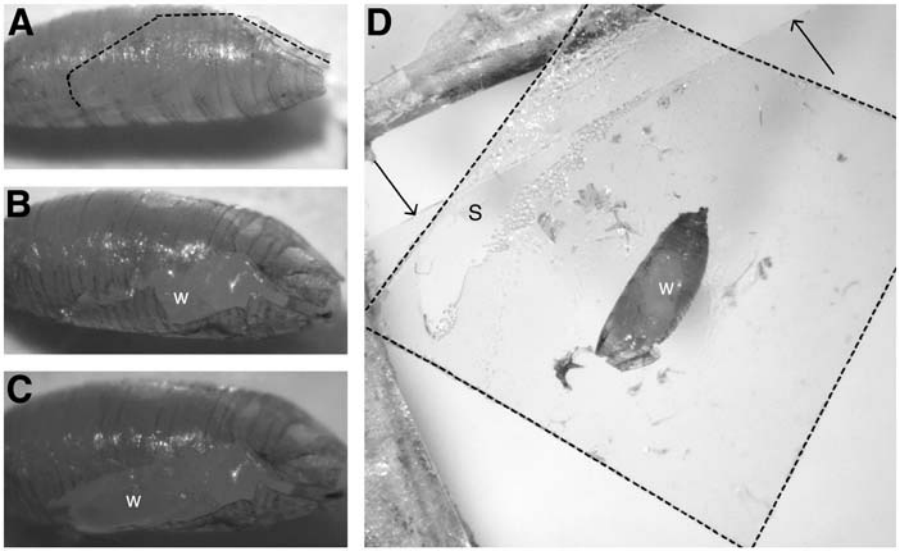

Fig. 3. Dissection for live imaging. **(A)** Once you have cut the puparium operculum, scale the P case surrounding the wing along the dashed line. **(B)** After scaling along one margin of the wing, now cut the P case on the ventral side of the animal close to the legs to release the wing. **(C)** The entire puparium case lying over the wing has been removed including additional puparium case above and below the wing (the equivalent of half a P leg width). **(D)** Tape the animal in a glass bottom dish with the wing facing the cover slip. S and dashed lines denote the region where the tape sticks to the cover slip. Arrows in (d) point to the cover slip edge. Image in (D) has been taken from below the glass bottom dish. In (A–C), anterior is to the right. In (D), anterior is south west. W indicates the P wing.

3.5. Live Imaging of Drosophila *P Wings (see Note 21)*

1. Recover the pupa to be dissected from the Eppendorf tube and place it on a facial tissue to dry it (*see* **Note 22**).
2. Cut a 5 cm long piece of tape.
3. Appose the 5 cm piece of tape onto the dried pupa.
4. From now on put the taped pupa under the objective of the dissection stereomicroscope. Place two fingers on the tape, one on the left and one on the right of the animal, to hold the tape and to reorient the pupa during dissection.
5. At this stage the pupa does not yet strongly adhere to the tape, so you can easily reposition it. Using your forceps, orient the pupa as indicated in **Fig. 3A**—the left lateral side of the pupa should be in contact with the tape. Then gently press with the closed forceps onto the puparium operculum and onto the posterior spiracle to increase the adhesion of the pupa to the tape (*see* **Note 23**).

6. Using a pair of fine scissors cut the puparium operculum just above the P head (*see* **Fig. 3A**). Slide one of the scissor blades in between the puparium case and the pupa, without injuring the pupa. The sharp side of the blade should face dorsally.
7. Use the sharp side of the blade to break and "scale" the P case surrounding the wing (*see* **Fig. 3B**). Do so by repeatedly tilting the blade up without closing the scissors. Try to follow the hollow folds surrounding the wing: start from the dorsal side of the wing hinge region and scale the puparium until you reach the distal tip of the wing. Scale the puparium case in this region as well. Reposition your scissors to the wing hinge region.
8. Place one of the two blades underneath the puparium case (which is now movable). Slide the blade ventrally (toward the legs) and progressively cut the puparium case along the ventrally lying wing margin until you reach the distal tip of the wing.
9. Remove additional P case surrounding the wing to expose the equivalent of half the width of a P leg below and above the wing (*see* **Fig. 3C** and **Note 24**).
10. Cover the exposed P wing with a small amount of 10S oil. To do so, dip a 20 μL yellow tip into a small drop of oil that has been placed in the middle of a cover slip. Remove the excess oil by rolling the yellow pipet tip over the oil-free area of the cover slip. Then, carefully touch the wing with the oily tip (*see* **Notes 25** and **26**).
11. Cut the tape (using normal scissors) at a 1 mm distance from the head and from the posterior spiracle of the animal and at a 3 mm distance from the dorsal and ventral sides of the pupa. Take this rectangle of tape using forceps and tape it in the glass bottom dish so that the wing of the animal faces the cover slip (as shown in **Fig. 3D**).
12. The pupa is now ready to be imaged (*see* **Notes 27** and **28**).

4. Notes

1. Especially to sensitively visualize polarized cortical PCP domains, it is important for the wing tissue to be fixed quickly. This improves the quality of staining and makes the dissection easier. Therefore, 8% PFA and the addition of Cacodylate is strongly recommended; they greatly speed up fixation.
2. In principle, only very low levels of detergent are required for immunolabeling of the P wing epithelium. Some antibodies may require higher levels of detergent. However, low detergent concentrations aid the preservation of membranous structures for imaging, such as small endocytic vesicles.
3. Other solidifying mounting media should work as well. The wings are extremely fragile and get easily destroyed or squished in nonsolid mounts.
4. Staining is carried out in miniwell-trays as they allow for easy tracking of wings under the dissection microscope throughout the protocol. Furthermore, only a minimal volume of diluted antibody solution (10 μL) is required.
5. Tungsten needles or sharp injection needles may be additional helpful tools for dissection.
6. Numerous glass bottom dishes are commercially available but make sure that the glass bottom dish you plan to buy is of appropriate size for your imaging devices. If you cannot find an appropriate glass bottom dish, you can easily make your own glass bottom dish by piercing a hole in the bottom of a plastic dish and sticking a cover slip below using double-sided tape.

7. Use the everyday tape that you find in general stores but take care that the tape you choose sticks well and that it is not difficult to cut with scissors.
8. The cover slips can be of any size but pay attention that they are dust free.
9. White prepupae are easy to recognize: they are immobile like pupae, they exhibit the characteristic shape of pupae but are white like larvae. Be careful to always pick white prepupae at the same stage. Consistency is crucial for good morphological concurrence because dramatic morphogenetic rearrangements may occur within 30 min.
10. Using a wet brush, instead of forceps, prevents damage to the pupae.
11. PP wings within the first hour after puparium formation can be dissected like larval imaginal discs. PP wings between 1 and 7 h after puparium formation (APF) at 25°C are dissected as described in **Subheading 3.2.**
12. If the wings do not remain attached to the trachea and stay inside the P case then you should try to carefully pull out the white tissues (evaginating imaginal discs, including wings, and larval epidermis) from the P case. At PP stage PP3, the PP body has already acquired a compact morphology, where the thoracic imaginal tissues have converged and fused. At this stage, you will be able to pull the entire anterior half of the pupa out of the P case in one step. The wings will lie at the side of the pupae and can be easily cut off from the rest of the pupae body for fixation.
13. The wings become extremely flimsy and fragile between 3 and 7 h APF at 25°C. Take care to never lift the wings out of the dissection droplet. They easily break and tangle up if they are removed from the droplet before fixation. Therefore, the fixative is added to the drop to a final concentration of 4% PFA—this stabilizes the tissue before transferring the wings for subsequent steps. If you feel that there is too much debris in the dissection droplet, then prefix the wings for 3 min (this is enough to stabilize the tissue) and then transfer them to a fresh droplet of fixative for the remainder of the incubation period.
14. In general, P wings from stage P2A–P2D (17–32 h APF at 25°C) can be prepared for immunofluorescence microscopy. PP cuticle ecdysis initiates in late P1. Thus, during P2A you will be able to peel the cuticle off the wing epithelium, thereby granting access of the antibody to the entire wing surface. However, as both wing blades have just started to appose at their basal sides the wing tissue is extremely fragile in P2A and P2B. P2C and P2D wing tissue is much more firm, and thus easier to dissect.
15. Alternatively, you can remove the posterior spiracles and stick one arm of your forceps into the ensuing hole in the P case and then close the forceps around the wall of the case. If you now also pin down the forceps onto the plastic dish at the same time you will be able to fix the pupa into a stable position.
16. Ideally, you should remove the remains of the larval salivary glands and gut (big green blob). Removing some of the thoracic tissue prevents it from being squeezed into the wing sac during subsequent steps. This often destroys the wings.
17. This may not work immediately because often the P cuticle sticks tightly to the P case. If this happens, then just gently tug on the pupa to allow the fixative to gain some access into the space between case and pupae. Give the fixative some time to

work by continuing with the other three pupae. Once you arrive back at the pupa it should be much easier to remove it from the case.

18. Especially in stage P2A the cuticle is still closely apposed to the wing epithelium. Once the fixative reaches the wing epithelium through the small hinge incision it will cause the tissue to dissociate from the cuticle. Therefore, it helps to repeat this step twice; in the second round you should try to increase the size of the hole at the hinge by tugging on the cuticle.
19. To prevent the wings from sticking to the tray plastic you can block the wells for 5 min with PBTN before transferring the wings into them. Collect 3–4 wings per well; too many stick to each other and are more difficult to track during the subsequent steps.
20. Avoid creating bubbles; the wings stick to them, thus it will be more difficult to mount the wings.
21. Wings can be live imaged starting from 13 to 14 h after puparium formation and until adulthood.
22. Wet pupae will not adhere to the tape properly and will be very difficult to dissect. To dry a pupa you can either let it for a couple of minutes on the facial tissue or if you cannot wait, gently roll them on the facial tissue using a dry brush.
23. The pupa now strongly adheres to the tape and you should not try to move the animal anymore or you may kill it.
24. Take much care to remove sharp pieces of the puparium case above and below the wing because they may damage the wing later on.
25. The small quantity of oil you have placed on the wing must not flow inside the puparium case; otherwise, this means that you have used too much oil. If you use too much oil the animal will die.
26. If you have damaged the wing with your scissors at any time during the dissection, or if you have forgotten to remove sharp fragments of cuticle you will see black spots (probably necrotic cells) appear on the wing (<3 h after dissection). You need to improve your dissection skills until you do not see these black spots anymore.
27. After the live imaging, the glass bottom dish can be cleaned with water and reused up to three times. The cover slip becomes more and more opaque after each use.
28. If you want to recover adults from the imaged pupae, place the pupae in a chamber with wet paper after imaging in order to prevent them from drying out. It is also important to cut as much tape as possible around the animal; otherwise, the fly will survive but get stuck on the tape soon after its eclosion.

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