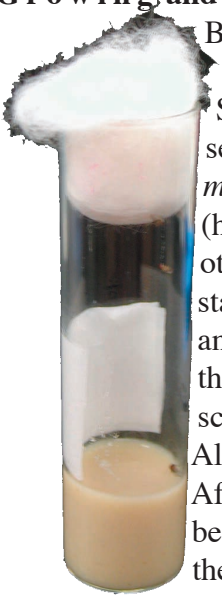
Immunostaining on Drosophila pupal abdominal epidermis

Nicolas Gompel, December 2003

Growing and harvesting pupae



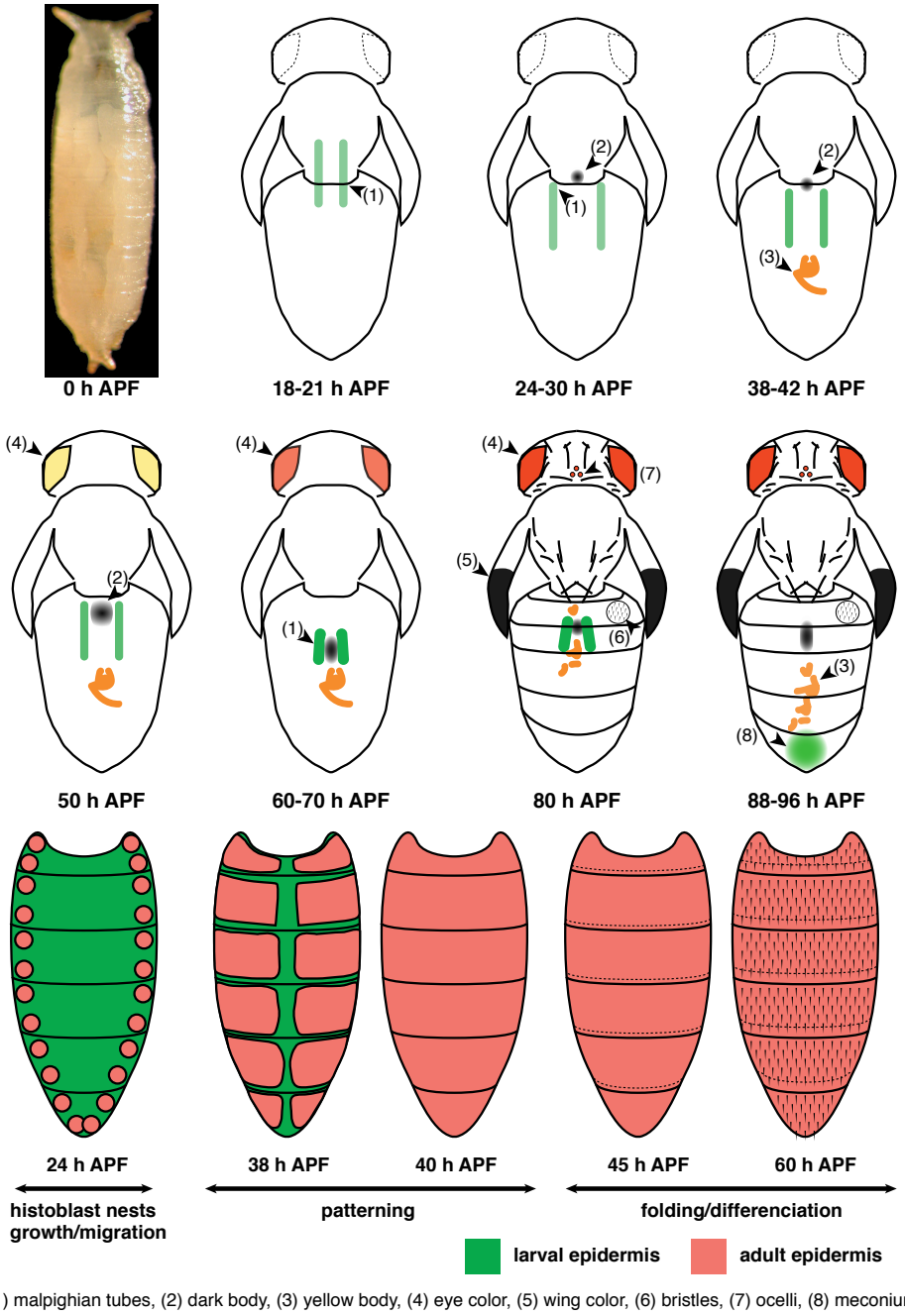
Because bigger pupae are easier to dissect, it is convenient to allow larvae to grow fat in non-crowded conditions. Standard cornmeal or Wheeler-Clayton (Yoon, 1985 ; see recipe hereafter) medium is appropriate for *D. melanogaster*. Set 30-50 mature adults per glass vial (height: 10 cm ; diameter: 2.5 cm) and transfer them every other day. When many pupae have reached the desired stage, spread water on the vial wall, wait about 2 minutes and harvest them from the vial wall with a spatula. Clean them gently in a mesh basket with water using a squeeze bottle and sort them under the dissecting scope.

Alternative method: After adult flies have been removed from the vial, use a spatula or a glass wand to place a folded square of wet Acclaim or Kleenex facial tissue on the wall of the vial, about 1 mm above the food. Clean the tools, hands and work bench with 70% ethanol before manipulation to prevent mold contamination. The square of tissue must be kept moist. Mature third instar larvae will crawl out of the food and pupate into the paper. The moisture will keep the pupae healthy and allow easy harvesting and staging of many clean pupae.

Staging pupae

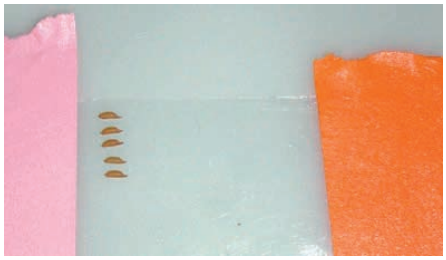
The most accurate staging of pupae requires histological sections (Madhavan and Madhavan, 1980). However, reliable estimation is possible for some stages, based on external features that become apparent during pupal life. Staging can be refined *a posteriori* when specimens are examined under a compound microscope.

-a priori staging based on external criteria involves white shell *vs* yellowish shell, presence and position of green, black and yellow



bodies (see schematic), eye color (not for transformants!), presence and coloration of bristles (the characters shown on top panels of the staging figure can be seen through the puparium when it's wet) (Ashburner, 1989).

-a posteriori staging is based on regression of larval cells, segment folding, bristle development and presence of the adult cuticle (Madhavan and Madhavan, 1980).



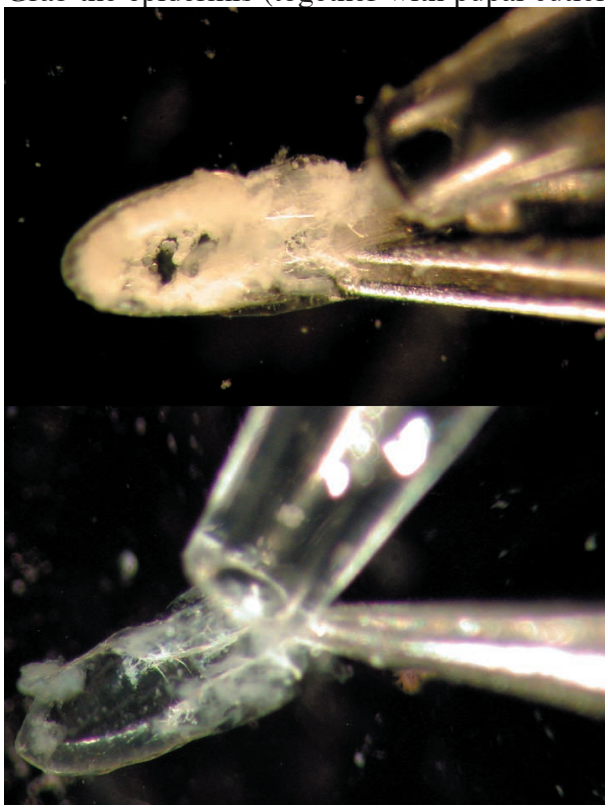
Dissecting pupae

Pupae can be dissected either dorso-ventrally or sagitally. Use a dissecting plastic board (~3x6 in) coated with a piece of strong tape (Scotch brand, ref. 3750), glue side facing up, and held to the board by pieces of tape. It is critical that the Scotch tape is not loose. Line-up selected pupae on the tape, on their side (for dorso-ventral dissection) or their back (for sagital dissection). Let them sit until their shell is dry and firmly attached to the tape. (Alternatively, let them dry and move them to the tape, but it's much more difficult to re-position them this way because they'll stick firmly to the tape).

Multi-well glass plates are required for the next steps. Fill a 9-well plate with cleaning solution (1x PBS). Pupae are processed for dissection and cleaning under a dissecting microscope. Each pupae should be cut with a double side razor blade (e.g. Wilkinson brand) as shown. Each side of the blade is generally sharp enough to cut 10-15 pupae.

Cleaning the epidermis

Grab the epidermis (together with pupal cuticle for pupae younger than 50 h APF) by the thorax with a forceps, and transfer it from the pupal case to a well filled with PBS. Plunge it quickly into the liquid to avoid spreading of the epidermis onto the surface of the liquid (cuticle is very hydrophobic). This and the following steps should be monitored under a dissecting scope. While holding the dissected tissue by the thorax, use a P20 pipette set on 5 μl to blow PBS into the abdominal cavity until the fat body and internal organs are gone. Be gentle enough to preserve the epidermis and avoid shearing.



Transfer the cleaned epidermis to a new well (PBS), either by grabbing it by the thorax, or by transporting the entire carcass in a droplet at the tip of the forceps.

Fixation

Transfer the epidermis to a fix solution (25 μl PFA/475 μl fix buffer) in a new well for 10'. Do not shake. Wash in PBT 2 x 5' (transfer the epidermis to a new well each time).

Preabsorb antibody

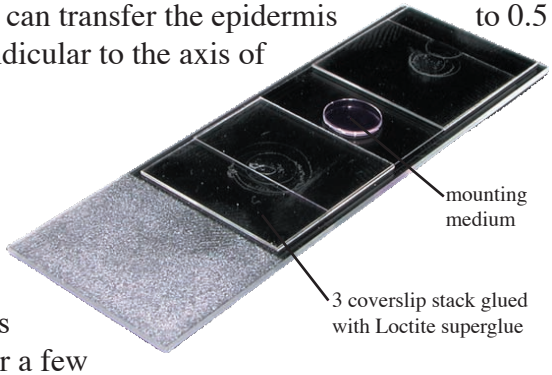
- dilute 50 μl of larval powder in 1 ml PBS. Vortex, spin, discard PBS resuspend in 1 ml PBTB.

- preabsorb primary (and secondary, optional) in this PBTB/larval powder mix, overnight @ 4˚C. Spin, harvest supernatant, filter with Centrex 0.45 μm.

Staining

- Block 1 hour in PBTB (possibly store in PBTB @ 4˚C with azide for a few days).
- Incubate epidermis in a glass well with primary antibody overnight @ 4˚C or 2-3 hours at room temperature. In either case, cover the well with a microscope slide to limit evaporation.
- Wash 3x fast, then 6x20'@ 4˚C in PBTB.
- Incubate epidermis in a glass well with secondary antibody (fluorochrome conjugated) diluted in PBTB for 4 hours @ 4˚C or 2-3 hours at room temperature. Keep in the dark. Cover the well with a microscope slide to limit evaporation.
- Wash 3x fast, then 6x20'@ 4˚C in PBT.
- Equilibrate samples in Vectashield overnight and store @ 4˚C until ready to mount and analyze.

If you are concerned about mixing during antibody incubation, you can transfer the epidermis to 0.5 or 1.5 ml eppendorf tubes and roll them gently (parallel, not perpendicular to the axis of rotation).



Mounting

Transfer carcasses to a new well containing a drop of Vectashield mounting medium (or 100% glycerol). Equilibrate for 20' at room temperature. Mount in a drop of Vectashield medium or 100% glycerol between a slide and a coverslip. Use 3 or 4 coverslip stacks as spacers. Process for confocal imaging, or store flat in the dark for a few days.

Recipes

Fix buffer

Fix Buffer	Stock	50 ml	Stock	50 ml
0.1 M PIPES pH 6.9	0.5 M	10 ml	1 M	5 ml
1 mM EGTA pH 6.9	0.5 M	0.1 ml	0.5 M	0.1 ml
1% Titon X-100	100%	0.5 ml	100%	0.5 ml
2 mM $MgSO_4$	1 M	0.1 ml	10 mM	10 ml

Make 1 ml of 1.85% formaldehyde fix solution just prior to fixation (950 μl fix buffer+ 50 μl 37% formaldehyde).

PBT

PBS pH 7.3, 0.03% Triton X-100

PBTB

PBS, 0.1% Triton X-100, 0.1% proteose peptone, 0.5% casein enzymatic hydrolysate. Autoclave to eliminate any enzymatic activity

Larval powder

- Grow wild-type Drosophila in a bottle under crowded conditions.
- When third instar larvae are crawling on the bottle wall discard adult flies and fill the bottle with 3 M NaCl. The larvae will float and the food will sink.
- Collect larvae from the surface with a pipet or a spoon, wash them several times with the NaCl solution, then with PBS.

- Homogeneize larvae in the smallest amount of PBS with a tissue tearor.
- Fix for 2 hours at room temperature in 8% paraformaldehyde/1x PBS.
- Wash in PBS, spin, discard supernatant 3 times.
- Wash in methanol, spin, discard supernatant 3 times. Store @ -20˚C in methanol.

Tucson Stock Center Wheeler-Clayton mix (makes approx. 80 glass vials)

Top-layer ingredients	Amount
Cornmeal	20.1 g
Special K	6.75 g
Wheat Germ	6.75 g
Product 19	3.78 g
Brewer's yeast	13.5 g
Hi-protein baby cereal	10.3 g
Green banana	30 g)
Tap water	675 ml
95% EtOH	4.3 ml
Propionic acid	4.3 ml
Agar (powder)	3 g

Bottom-layer ingredients	Amount
Instant Drosophila medium (Carolina biologicals)	27 g
Boiled tap water	120 ml

Directions for cooking on stove top

1. Mix the instant Drosophila medium with the boiled tap water in a small Ziploc baggie. Allow closed Ziploc to sit and cool to a touchable temperature. Cut a corner out of the baggie and squeeze a small dab (about 1 ml) into the bottom of each vial.
2. Take out the dry cereal mix (containing all of the cereals, blended to a powder – dry mixes are prepared ahead of time and stored a -20˚C in a Ziploc bag for up to 6 months in advance).
3. In a pot, bring 300 ml of water to a rapid boil. Add the agar and *stir constantly*. Cook at a boil until agar is clear. This process takes 5-10 minutes. Be careful not to burn the agar.
4. Blend the bananas, the dry mix, and 175 ml in a blender.
5. Once the agar solution is clear, add in the wet mixture, plus 200 ml of water. (Use this water to wash all of the cereal mix out of the blender and into the pot of agar).
6. Lower the temperature slightly to prevent scalding.
7. Stir constantly; allow the mixture to simmer for 10 minutes.
8. Carefully add 95% EtOH, then the propionic acid; mix thoroughly.
9. Continue simmering for 5 more minutes.
10. Remove from heat and pour into containers.

References

Ashburner, M. (1989). *Drosophila. A laboratory manual*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Madhavan, M. M. and Madhavan, K. (1980). Morphogenesis of the epidermis of adult abdomen of Drosophila. *J Embryol Exp Morphol* 60, 1-31.

Yoon, J. S. (1985). Drosophilidae II: Drosophila species other than *D. melanogaster.* In *Handbook of insect rearing*, vol. II (ed. R. F. Moore and P. Singh), pp. 85-91. Amsterdam ; New York: Elsevier.

***

(Please send feed-back comments to improve this protocol to ngompel@wisc.edu)