Fly/Arthropod Lab

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INTRODUCTION

In this module you will learn about the development of the model arthropod, *Drosophila melanogaster*, and how our study of *Drosophila* development contributes to our understanding of arthropod evolution and diversity. We will start by looking at some pre-stained embryos that will help to visualize the events of segmentation, body regionalization, and neural development. You will also have the opportunity to try your own hand at antibody staining of embryos and larvae, with an emphasis on examining the regionalization of the body plan through the action of Hox genes, and neural patterning during larval development. We will also have on hand several fly stocks containing various GFP and RFP constructs that can be used for live imaging. Finally, we will visualize the process of neuronal remodeling during metamorphosis and of eye morphogenesis using fly lines that express GFP in body wall sensory neurons and in photoreceptor neurons. If we are able to find other insects, or crustaceans, you can also use several of the broadly crossreactive antibodies to compare development between arthropod species.

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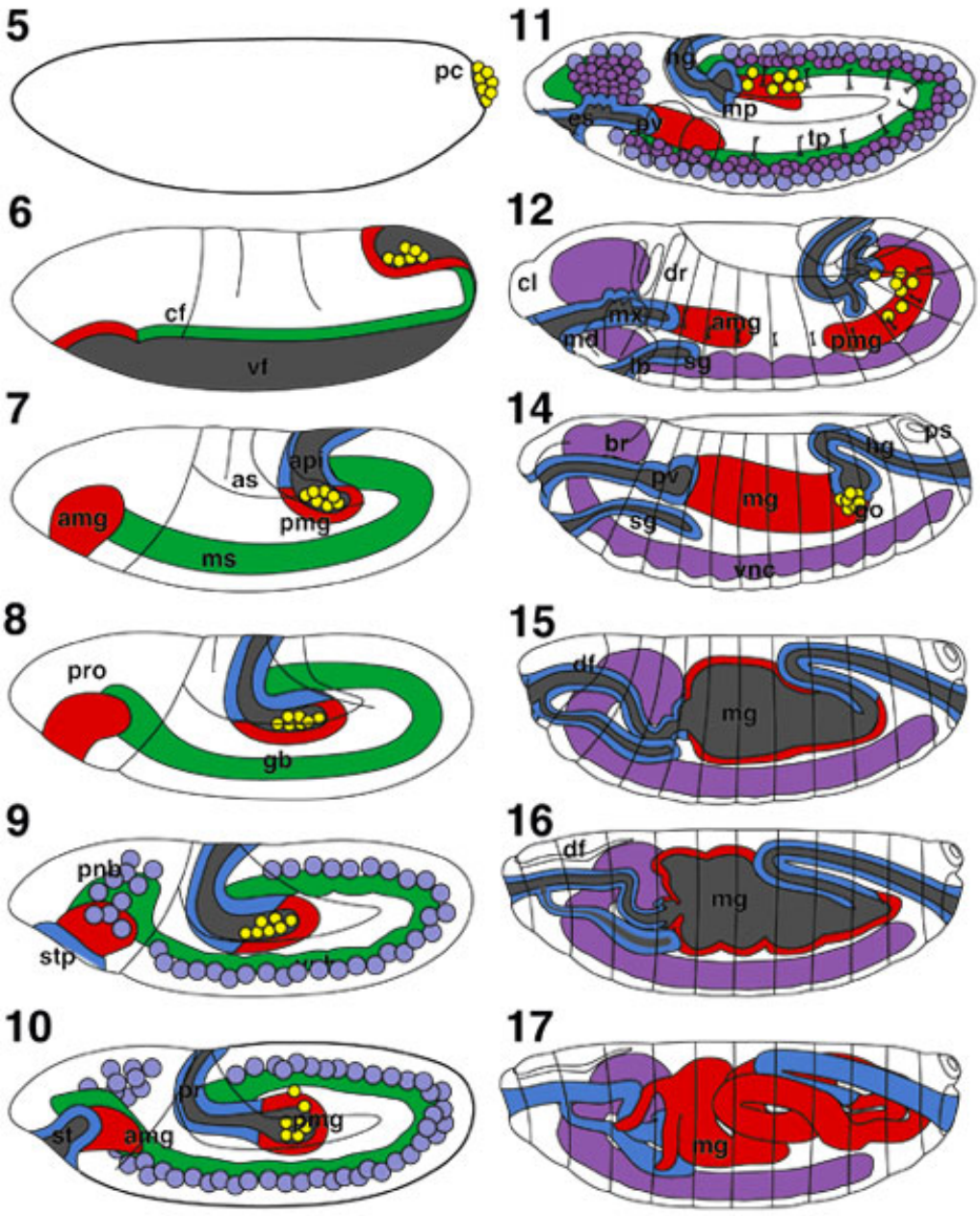
DROSOPHILA **DEVELOPMENT & STAGING**

Embryogenesis

Drosophila embryos complete embryogenesis in 22 to 24 hours at 25°C, and take approximately twice as long at 18°C. Gastrulation and segmentation are completed within the first several hours, and the last half of embryogenesis is mainly dedicated to organogenesis. Because development is so rapid, it can easily be observed under the compound microscope. The detailed study by Volker Hartenstein and José Campos-Ortega (see refs) remains the definitive description of embryogenesis, and this handout includes some of the staging tables and images from that study. Schematic diagrams from the fly atlas are also helpful in staging embryos.



Atlas of *Drosophila* Development Volker Hartenstein
All embryos are in lateral view (anterior to the left).
(amg) anterior midgut rudiment;
(br) brain;
(cf) cephalic furrow;
(cl) clypeolabrum;
(df) dorsal fold;
(dr) dorsal ridge;
(es) esophagus;
(gb) germ band;
(go) gonads;
(hg) hindgut;
(lb) labial bud;
(md) mandibular bud;
(mg) midgut;
(mg) Malpighian tubules;
(mx) maxillary bud;
(pc) pole cells;
(pmg) posterior midgut rudiment;
(pnb) procephalic neuroblasts;
(pro) procephalon;
(ps) posterior spiracle;
(po) proventriculus;
(sg) salivary gland;
(stp) stomodeal plate;
(st) stomodeum;
(tp) tracheal pits;
(vf) ventral furrow; (vnb) ventral neuroblasts; (vnc) ventral nerve

Here is a schematic diagram that you can use to identify fly embryo developmental stages:

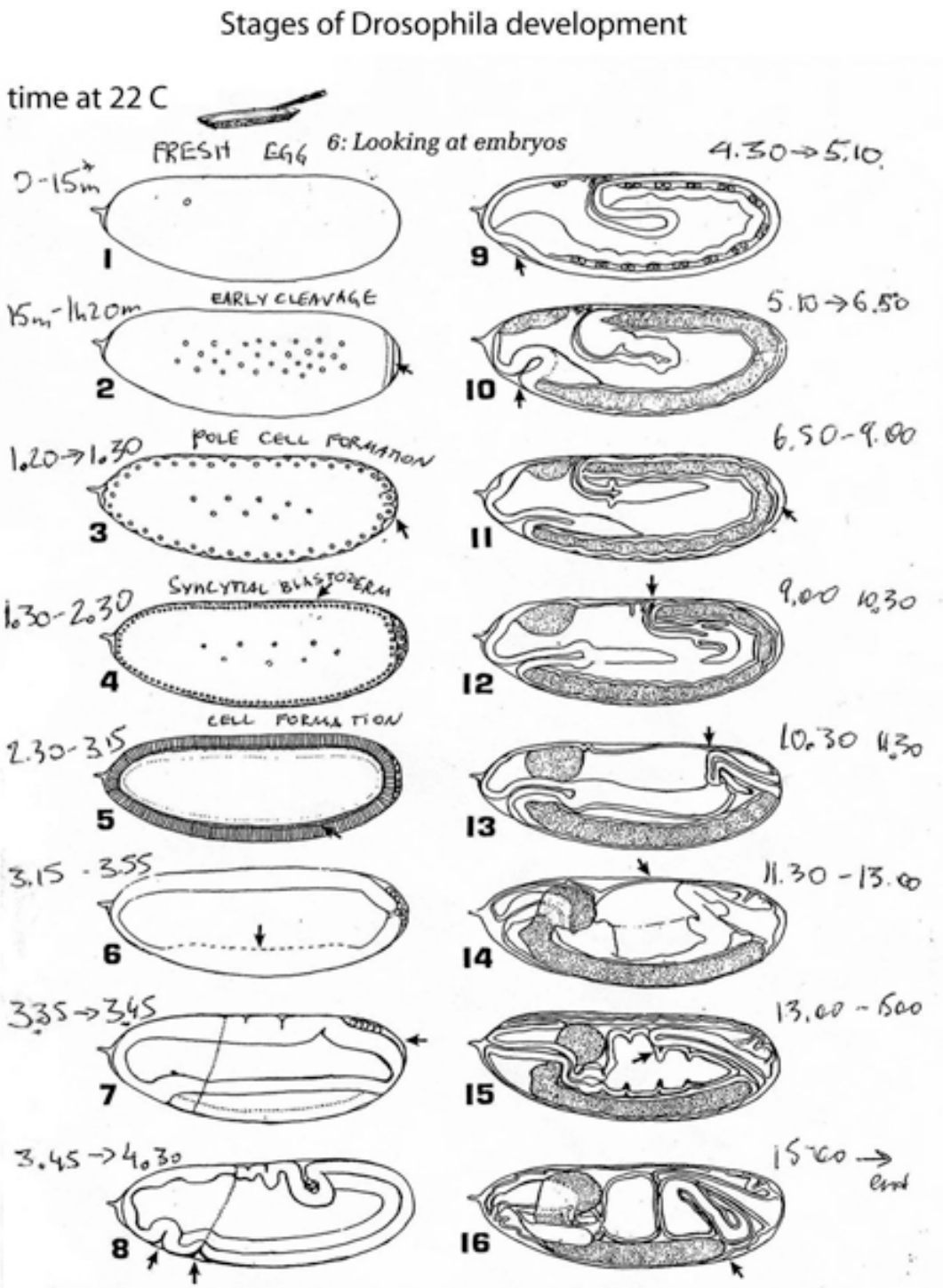
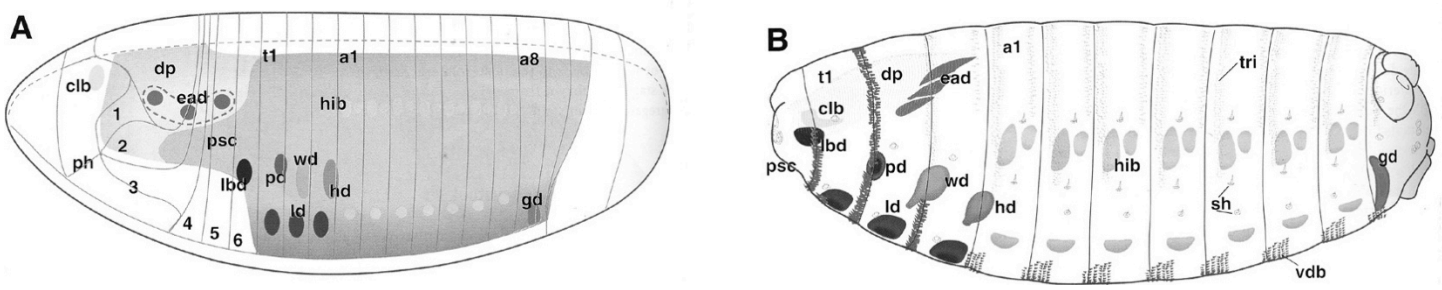


Figure 9. Schematic diagrams of 16 stages of *Drosophial* embryogenesis. The small *arrows* in each diagram indicate those morphological features which are most diagnostic of that particular stage (see text). In stages 9–16, the developing nervous system has been stippled. Stages 14 and 16 are depicted as slightly parasagital optical sections to emphasize the three-dimensional locations of the various organ systems.

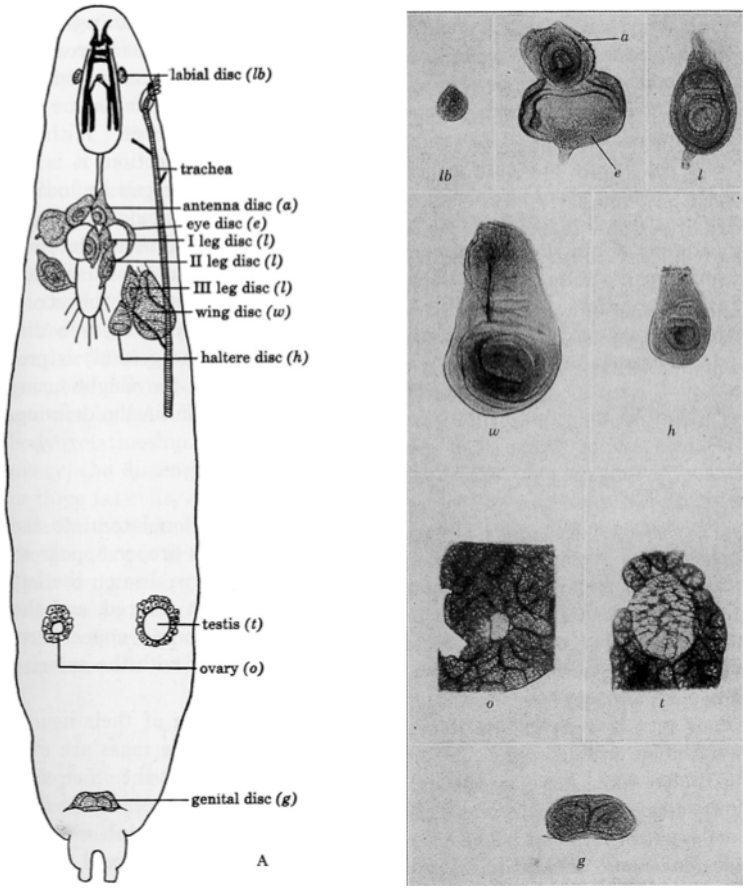
Larval Development and Morphogenesis

After hatching from the egg, the first instar larva begins to feed and grow immediately. The first and second larval instars (L1, L2) last approximately 24 hours each, and the third larval instar (L3) lasts about three days. Groups of cells specified during embryonic development proliferate during these three larval stages, and form clumps of cells called **imaginal discs** in the lumen of the larva. These discs are the primordia of virtually all of the cuticular and ectodermally derived tissues in the adult.



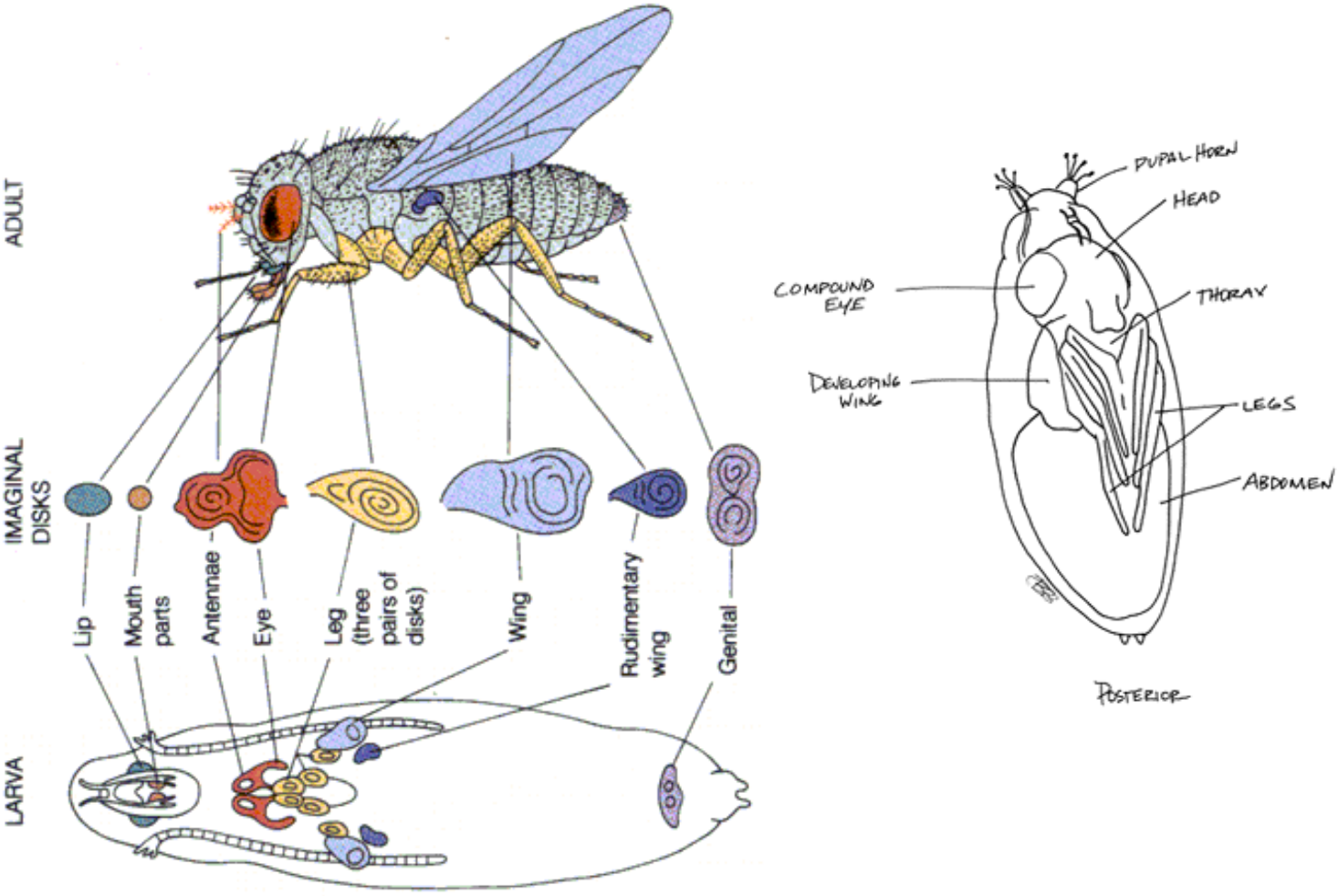
Left: Fate map of imaginal disc primordia in a blastoderm stage embryo. Imaginal disc anlagen are represented as ovals. Right: Position of the imaginal disc primordia at the end of embryogenesis. Anterior is to the left. (ead) eye-antennal disc (wd) wing discs (ld) leg disc (hd) haltere disc (gd) gential disc

The imaginal discs of the eyes/antennae, head structures, legs, halteres, wings and genitalia are easily identified in L3, but are slightly more difficult to isolate from L2 and L1. Hox genes, among others, play important roles in specifying imaginal disc identity, such that specific cuticular structures develop on different body segments. The patterning of these discs during embryonic and larval development is the basis of pattern formation in the adult fly.



Left: Position of the imaginal discs in an L3 larva. Right: enlarged view of dissected imaginal discs and gonads of both sexes of an L3 larva. Anterior is up. Discs are shown in roughly the order that they appear in the larvae, anterior to posterior.

After the end of the L3 stage (approx. 5 days after egg laying (AEL)), the larva leaves the food and begins to crawl in search of a place to pupate. The anterior and posterior spiracles are everted, the puparium (pupal case) is formed, and the process of metamorphosis begins. Most of the larval tissue is destroyed by histolysis, but the imaginal discs continue to grow and undergo the morphogenetic movements that will form the adult fly. Dissecting pupae can be difficult since they are basically bags of mush with the imaginal discs floating around in them, but with practice it can be done.



Left: Colored coded scheme showing the imaginal discs with their respective adult structures.
Right: Ventral view of a pharate adult within the pupal case prior to eclosion.

For more information on Drosophila embryo, larval, and adult anatomy, see

http://flybase.org/static_pages/imagebrowser/imagebrowser10.html

DROSOPHILA PROTOCOLS

General Fixation and Antibody Staining (Drosophila embryos)

Fixation:

1. Transfer embryos from agar collection plates into a nylon mesh basket using water and a small paintbrush.
2. Place the egg baskets in a small glass beaker partially filled with 50% bleach solution. Gently swirl the basket or use a Pasteur pipette to rinse the embryos. Dechorionation should take about 3 min; however, the potency of bleach varies so monitor the process under a dissection scope and stop it once the chorion has dissolved away.
3. Immediately wash thoroughly with room temperature water.
4. Transfer the embryo to a 20 ml glass scintillation vial containing 10 ml of heptane and 10 ml of 3.2% paraformadehyde in PBS (or PEM buffer). You can do this by shaking the mesh directly into the heptane phase and can use heptane to wash down any embryos stuck to the side of the basket.
5. Mix gently for 15-20 minutes.
6. Remove the aqueous phase (lower phase). Add more heptane if needed to maintain a volume of at least 8 mls of heptane. Try to remove all of the aqueous phase.
7. Add 10 ml of methanol and shake vigorously for 15-30 seconds. Devitellinized embryos will fall to the bottom (methanol phase).
8. Pipette out the embryos from the bottom and transfer them to a new tube.
9. Wash embryos several times with methanol to remove traces of heptane.
10. Embryos can be stored in methanol at -20°C for several years.

Rehydration and Staining:

1. Rehydrate embryos from methanol with 3 X 5 minute PT washes. Only rehydrate what you need for the day, leaving rest in methanol for future use. As a rule of thumb, 15 μl of settled fly embryos in MeOH will be about 20 μl when rehydrated, and this 20 μl volume is what you want per 1.5 ml eppendorf microcentrifuge tube.
2. Incubate 10-30 min in 300 μl of PT+N (PT + 5% Normal Goat Serum). The normal goat serum (NGS) will help to block nonspecific antibody binding sites. Gently mix by spinning the tubes. Avoid shaking or flicking the tubes as the embryos will splash up onto the walls of the tube and dry out resulting in either unstained or non-specifically stained embryos.
3. Add the appropriate amount of primary antibody to achieve the desired final concentration (see antibody table).
4. Gently mix the embryos and antibody solution and incubate overnight at 4°C (continued mixing is not necessary during the overnight incubation)
5. Wash 3 X 1 min with PT. Before these washes are started, it is possible to recover the diluted primary antibody, and this used antibody can often be re-used several more times. Store this diluted antibody at 4°C.
6. Wash 3 X 30 min with PT.
7. Incubate 10-30 min in 300 μl of PT+N as in step 2 above.
8. Add appropriate secondary antibody to the proper final concentration (1:500 - 1:1000 for most of the Alexa conjugated secondaries).
9. Mix the embryos and secondary antibody solution gently and incubate for 2 hrs at room temperature.

10. Wash 3 X 1 min with PT.
11. Wash 3 X 30 min with PT.
12. If you used a fluorescently tagged secondary antibody, add 200 μl 50% glycerol with DAPI (either 0.1 or 1.0 μg/ml DAPI) for 15-20 minutes, and then replace with 300 μl 70% glycerol (no DAPI). AlexaFlour conjugates are very fade resistant even without the addition of an anti-fade compound.

Mounting:
Transfer approximately 20-100 embryos in 20-50 μl of 70% glycerol to a microscope slide. Place two #1 thickness coverslips (18X18 mm) to each side of the drop of embryos. Place 5-10 μl drop of 70% glycerol onto a third coverslip (1.0 or 1.5 thickness; 18X18 mm square) and (drop side down), lower this coverslip on top of the embryos. This should be done so that the top coverslip is resting on the edge of the lower two coverslips. This keep the top coverslip from crushing the embryos. Very small drops of nail polish can be used to hold down all the coverslips. Observe using your microscope. For confocal analysis, it is useful to mount single embryos and role them into the proper orientation.

Rapid Antibody Staining Protocol (*Drosophila* embryos)

While this protocol produces antibody stains in just a few hours, it **only works well on very robust antibodies**. Use this only for antibodies listed with a "60 min" concentration on the antibody list.

1. Rehydrate 3X 1 min followed by 1X 10 min with PT.

2. Incubate 10 min in 200 μl PT+N (PT + 5% NGS).

3. Add primary antibody to the appropriate final concentration. ("60 min" concentration)

4. Mix and incubate in the primary antibody at room temperature for 60 min.

5. Wash 3X 1 min with PT.

6. Wash 3X 10 min with PT.

7. Add secondary antibody (in 200 μl of PT+ 5% NGS). It is not necessary to pre-block with PT+NGS. Use fluorescently labeled secondaries at a dilution of 1:500 when using this rapid protocol (as opposed to 1:500 - 1:1000 with the "normal" protocol). Incubate for 60 min at room temperature.

8. Wash 3X 1 min with PT.

9. Wash 3X 10 min with PT.

10. Add 200 μl 50% glycerol with 1 μg/ml DAPI and incubate 15 min.

11. Remove 50% glycerol and add 70% glycerol (no DAPI). Embryo will be ready to view in about 15 minutes. Mount as described in the standard protocol above.

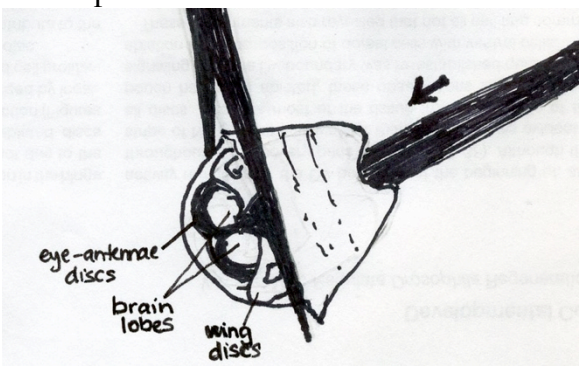
Dissection of Post-embryonic Tissues (Imaginal Discs and CNS of *Drosophila*)

Dissecting imaginal discs:

On a Slygard coated dish filled with 1X PBS, dissect imaginal discs by one of the following methods:

Method A:

- Tear larvae in half (cut across the "waist") with forceps
- Turn the anterior half of the larva "inside-out" by pushing in at the head/mouth-hooks with the tip of one pair of forceps while drawing the cuticle back over this pair of forceps with a second pair.



- Gently remove the fat body, salivary glands and gut, leaving the brain and imaginal discs intact. These are at the anterior end of the larva near the mouth hooks. (Tip: leave the main lateral trachea in place as this will almost always guarantee recovery of the wing discs)
- The wing discs are pinned to the sides of the larva by the two prominent lateral trachea. They are the largest imaginal discs.

Method B:

- Grab the mouth hooks with one pair forceps
- Grab in the middle of the larval body with 2nd pair forceps
- Pull mouth hooks (and connected imaginal discs) out of body while holding the body in place with your 2nd pair of forceps.
- Wing discs will be in a bunch with other discs and brain. Clean away any extra tissue such as fat body or salivary gland. The wing disc is largest and has a prominent pocket.

Ideally your discs will spend no more than 15-20 min in PBS before you begin your fixation. The importance of this will vary depending on the antibody you are looking at. You can either do the initial dissection (inverting carcasses or pulling mouth hooks), then add fix and clean off the extraneous tissue while fixing or you can complete your dissections in PBS and transfer just the carcass + discs or mouth hooks + discs into an eppendorf for fixation. How you do this will depend largely on personal preference and dissection speed/skill.

Fixation and Staining:

1. Fix wing discs (either attached to the inverted carcass for A or to the mouth hooks and other discs for B) in 3.2% praformaldehyde in 1X PBS for 15-20 minutes.
2. Wash at least 3X 10 min in PT to remove residual fix.
3. Block for 30 min in PT+N (PT + 5% Normal goat serum).

4. Add the appropriate amount of primary antibody to achieve the desired final concentration, mix gently and incubate overnight at 4°C.
5. Rinse once in PT, then wash 3X 20 min in PT.
6. Block for 10 - 30 min in PT+N.
7. Add the appropriate secondary antibody to the proper final concentration, mix gently and incubate with for 2hrs at room temperature or overnight at 4°C.
8. Rinse once in PT, then wash 3X 20 min in PT.
9. Add 200 μl 50% glycerol with 1.0 - 0.1 μg/ml DAPI and incubate 15 min.
10. Remove 50% glycerol and add 70% glycerol (no DAPI). Disks will be ready to view in about 15-30 minutes.

Mounting:

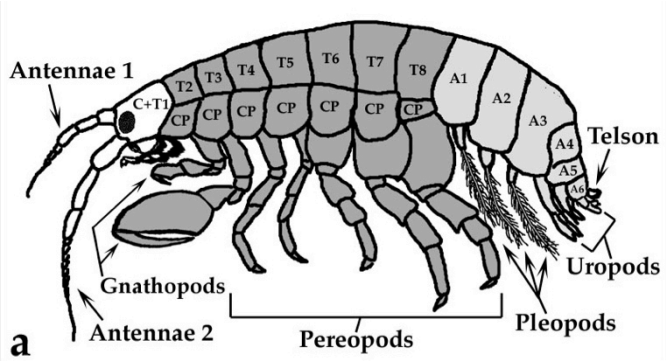
Transfer 1-5 disks in 20-50 μl of 70% glycerol to a microscope slide. Place two #1 thickness coverslips (18X18 mm) to each side of the disks. Place 5-10 μl drop of 70% glycerol onto a third coverslip (1.0 or 1.5 thickness; 18X18 mm square) and (drop side down), lower this coverslip on top of the disks. This should be done so that the top coverslip is resting on the edge of the lower two coverslips. This keeps the top coverslip from crushing the disks. Very small drops of nail polish can be used to hold down all the coverslips. Observe using your microscope.

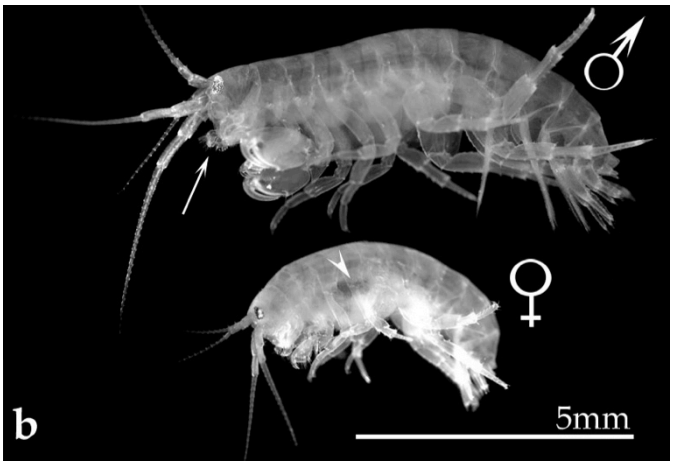
PARHYALE DEVELOPMENT & STAGING

(Modified from Browne et al (2005) Genesis 42:124-49 and Gerberding et al, (2002) Development 129:5789-5801.)

Introduction

Studying the relationship between development and evolution and its role in the generation of biological diversity has been reinvigorated by new techniques in genetics and molecular biology. However, exploiting these techniques to examine the evolution of development requires that a great deal of detail be known regarding the embryonic development of multiple species studied in a phylogenetic context. Crustaceans are an enormously successful group of arthropods and extant species demonstrate a wide diversity of morphologies and life histories. One of the most speciose orders within the Crustacea is the Amphipoda. The embryonic development of a new crustacean model system, the amphipod *Parhyale hawaiensis*, is described in a series of discrete stages easily identified by examination of living animals and the use of commonly available molecular markers on fixed specimens. Embryogenesis is completed in approximately 250hrs at 26°C and has been divided into 30 stages. This staging data will facilitate comparative analyses of embryonic development among crustaceans in particular, as well as between different arthropod groups. In addition several aspects of *Parhyale* embryonic development make this species particularly suitable for a broad range of experimental manipulations.

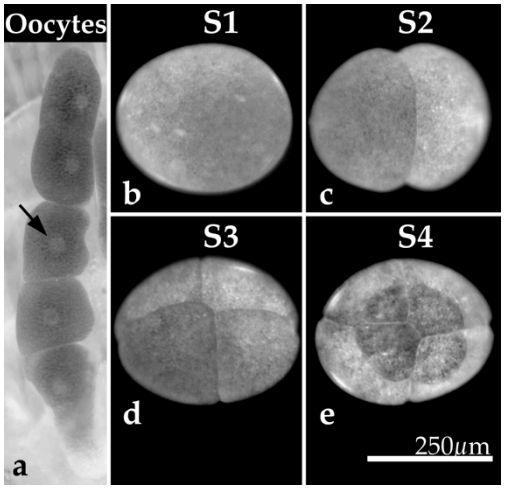


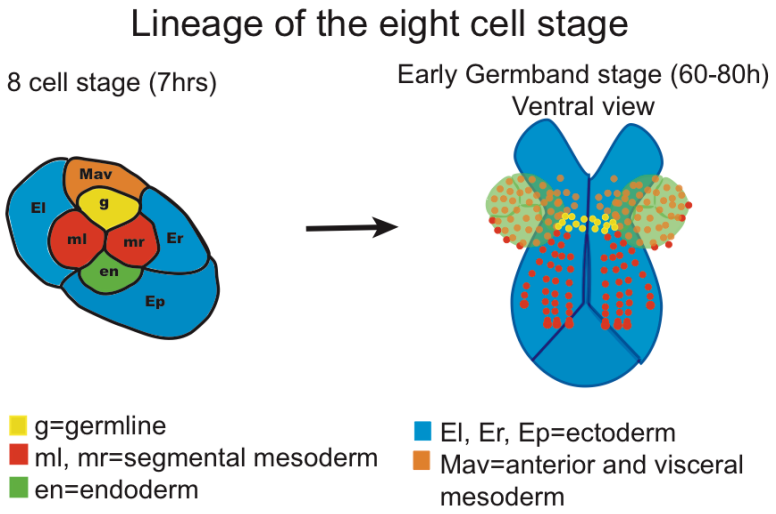


Reference Guide to *Parhyale* Development

1. **S1-4** Oocyte to eight cell stage, lineage of eight cell stage, 0-9hrs of development

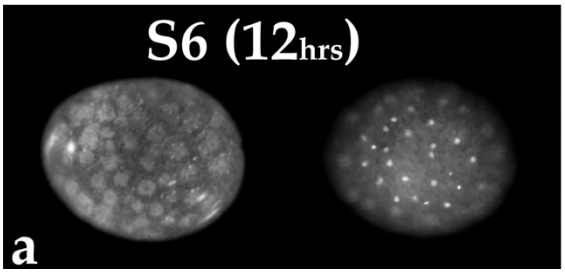
Early cleavages are total or holoblastic, resulting at the eight cell stage in an embryo with 4 micromeres and 4 macromeres. The lineages of these early blastomeres are restricted early in development such that the mesoderm is derived from only 3 blastomeres: ml (mesoderm left side), mr (mesoderm right side) and Mav (anterior and visceral mesoderm), while the ectoderm is derived from the El (left), Er (right) and Ep (posterior and midline) blastomeres.





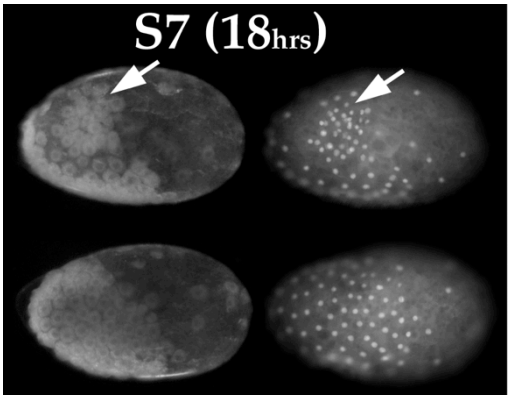
2. **S6** Soccerball stage

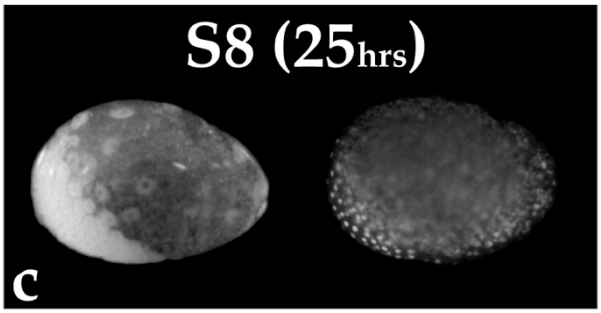
Cells are approximately the same size at this point; the divisions are asynchronous and the yolk is shunted internally to center of embryo.



3. **S7-S8** Rosette stage - Gastrulation

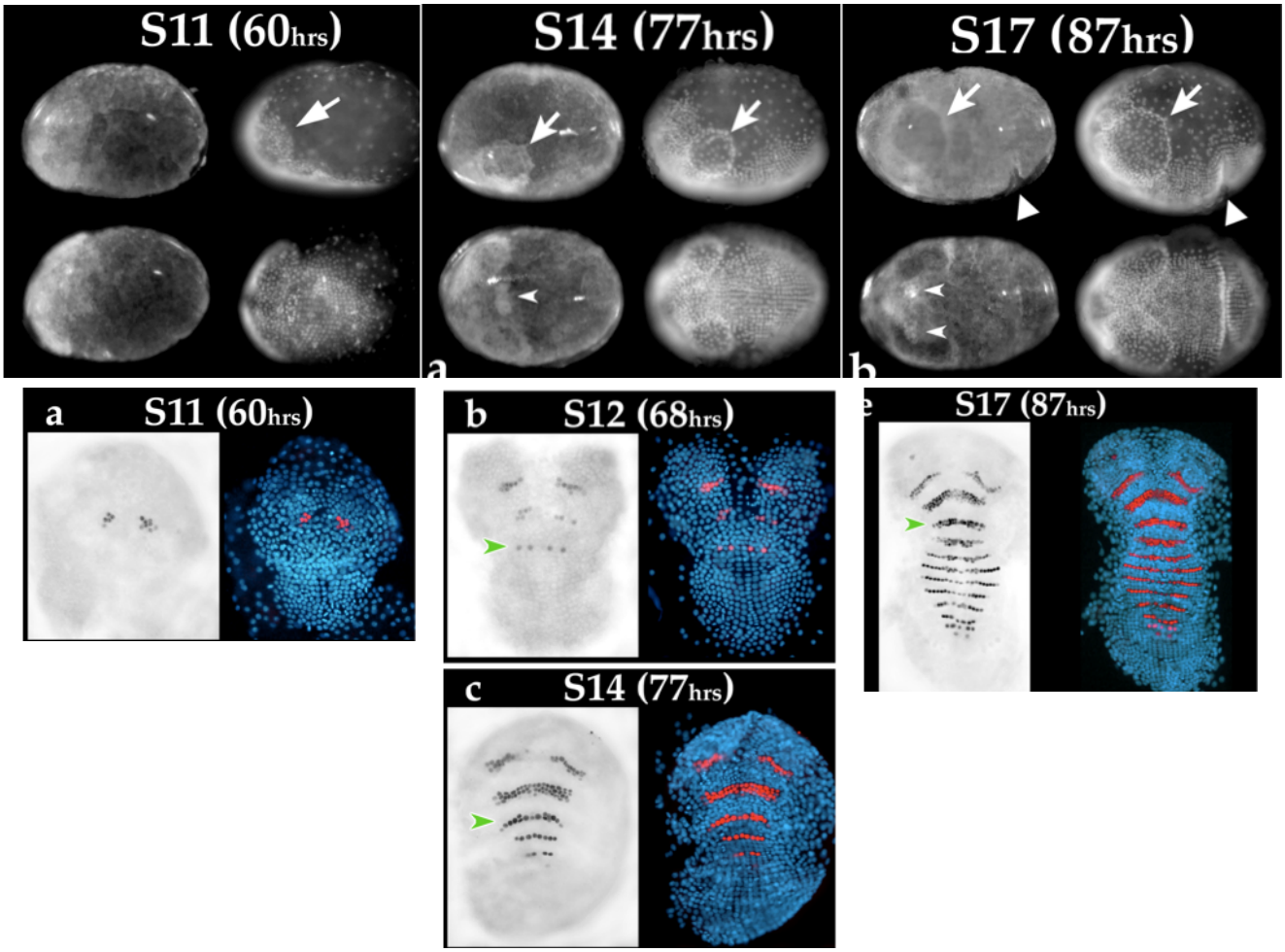
The rosette, which is made up of Mav and g progeny, marks the future anterior side. The ectoderm will migrate ventrally, and then over the rosette and mesoderm progeny. The rosette is no longer visible by S8. After this the germdisc continues to condense on the anterior ventral side.





4. **S9-17** Germband formation and elongation

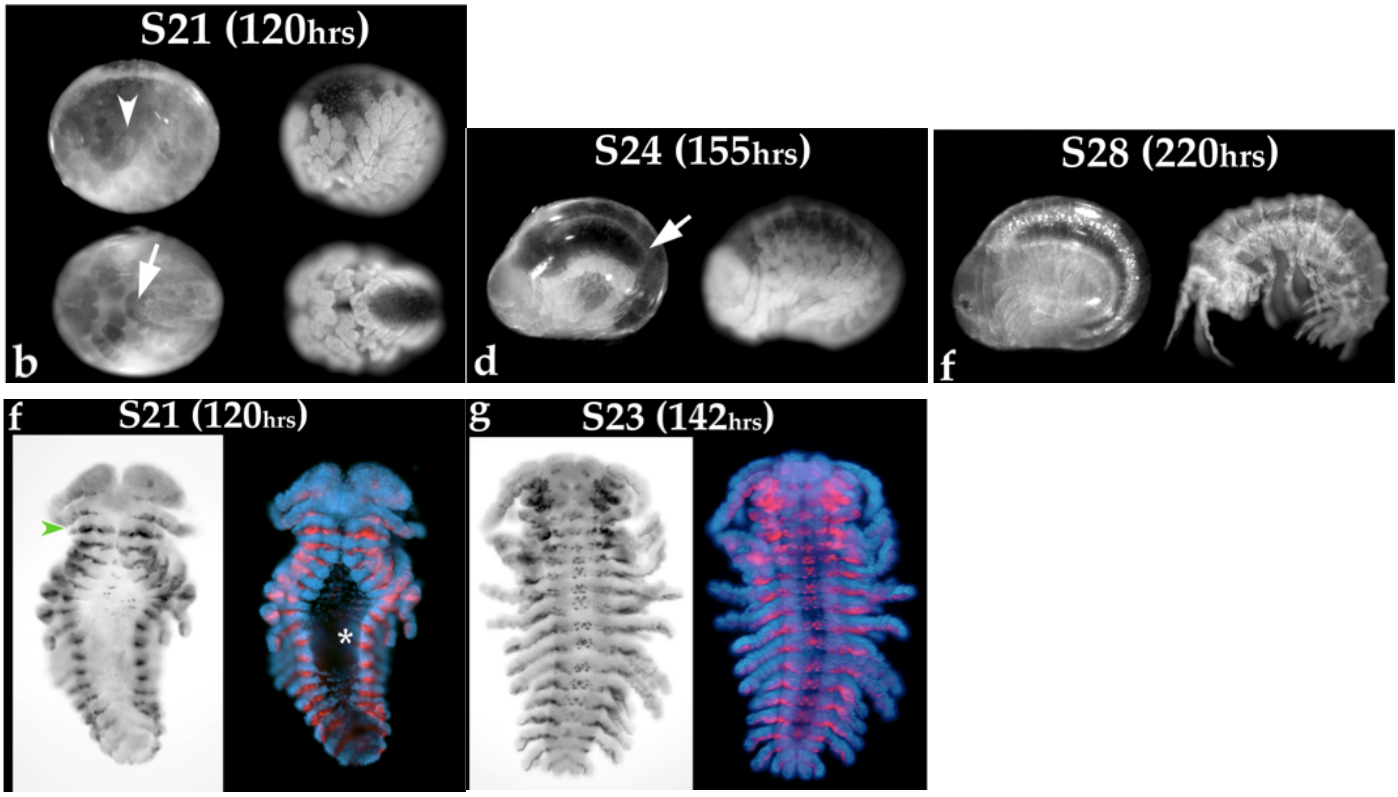
Ectodermal and mesoblast rows are organizing along the ventral surface in transverse rows. The midgut anlagen is visible as an aggregate of cells on either side of the head lobes that becomes more organized as an ovoid anlagen (triangles). By S17, the caudal furrow is visible at the posterior (arrowhead) and the germcell cluster has split into bilateral clusters (arrowheads). Limb buds are developing on the anterior region of the animal. On the right is a series of dissected embryos stained with the segment polarity gene, Engrailed and counterstained with a nuclear dye, DAPI, showing the progression of segmentation along the A/P axis.



Left panel: live image and matching DAPI (nuclear) images, Right panel: ventral view of embryos stained with Engrailed Brightfield and DAPI images shown

5. **S18-30** Appendage formation, organogenesis and neurogenesis to hatching

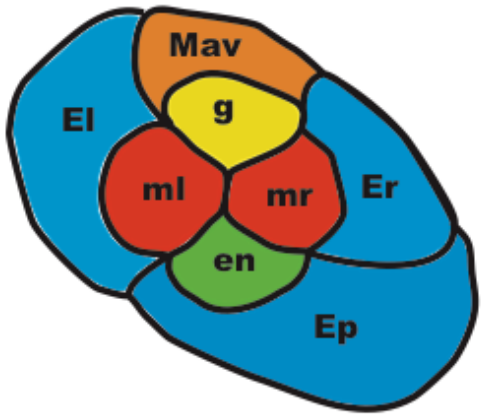
At these stages, the posterior regions (telson), gut and limbs become well developed. The germcells migrate to a lateral position between the ectoderm and midgut at S21 (arrowhead) and then by S28 have migrated dorsal medially as the embryo undergoes dorsal closure. The hindgut proctodeum (arrow at S21) is visible at the posterior terminus and digestive cecum begins to extend posteriorly (arrow at S24). Eye fields and a beating heart also begin to form by S28, followed by cuticle thickening and muscular twitching before hatching at S30 (250hrs)



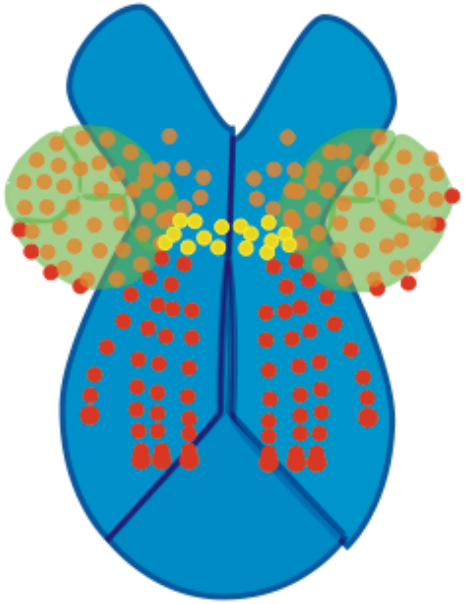
Left panel: live image and matching DAPI (nuclear) images, Right panel: ventral view of animals stained with Engrailed, Brightfield and DAPI images shown.

Lineage of the eight cell stage

8 cell stage (7hrs)



Early Germband stage (60-80h)
Ventral view



- g=germline
- ml, mr=segmental mesoderm
- en=endoderm

- El, Er, Ep=ectoderm
- Mav=anterior and visceral mesoderm

***PARHYALE* PROTOCOLS**

Parhyale embryo extraction

Being **patient** is the most important part of embryo dissections. A small number of embryos that have been dissected and fixed well may be more valuable than numerous embryos in pieces.

Extracting embryos from females using clove oil:

Gravid *Parhyale* females brood their embryos in a ventral pouch (See Section VIII.1, white arrow in lower panel (b) of first figure). To extract embryos without sacrificing the mother, you can put your female *Parhyale* to sleep using clove bud oil in seawater. **It is especially important to put your females to sleep if they are transgenic, you want to be able to use these females again!**

1. Putting amphipods to sleep:
    a. Add 10uL of clove bud oil to 50mL of filtered seawater in a falcon tube. Shake vigorously.
    b. Collect gravid *Parhyale* in a Petri dish or a medicine cup.
    c. Remove as much water as possible.
    d. Add your clove oil / seawater mixture (cover the *Parhyale*).
    e. Wait for them to completely stop moving – 5 to 10 minutes should do the trick.
    f. Caution: Leaving amphipod in the clove oil too long (hours) will kill them.
2. Embryo extraction:
    a. After the *Parhyale* are asleep, transfer them to a sylgard plate using forceps or a plastic transfer pipette (with tip cut off so amphipod will fit through opening).
    b. With the forceps at a steep angle, corral the waist of the animal, i.e. between the forward- and rearward-facing walking legs. Hook the glass probe between the walking legs and rotate the animal ventral side up. Grasp the waist of the animal until the legs splay just a bit. They're slippery, so it will take some practice.
    c. Use the small blunt end of the probe [to make probe see additional protocol] or forceps to sweep out the embryos by starting at the posterior end and moving the probe through the brood pouch.
    d. ** Be careful not to damage the embryos - the younger animals are very soft and are squished easily! Also, do not damage the females, especially the transgenic ones!**
    e. Transfer embryos to a new petri dish or tube. Wash with seawater to remove clove oil.
    f. To wake mother amphipods up, remove them from the clove oil and place them in a dish or cup of clean seawater until they recover. Return clove oil mixture to a Falcon tube – this can be reused.
    g. Put the adult amphipods back into their tank. Note: Do not place sleeping amphipods back into their tank – they will be eaten by the others in the tank!

Any embryos removed from their mothers should be stored in filtered seawater and placed in a humidity chamber (a.k.a. pipette tip box lined with wet paper towels) on a bench top, or a 26 degree incubator.

Parhyale embryo dissection and fixation

You will need:

Forceps (optional)	3 well glass dish or Plastic Petri dishes (one with Sylgard)
Dissecting needles	Medicine cups (optional)
3.7% formadehyde in filtered seawater	PT to rinse fixed embryos
Eppendorf tubes	Glass Pasteur pipettes and/or plastic transfer pipettes

Helpful setup tips:

- In the 3-well glass dish, fill the first well with filtered seawater, this will be your "corral" of embryos waiting to be fixed; fill the middle well halfway with fix, this is where you'll fix and dissect simultaneously; and fill the last well with filtered seawater, this will be where you rinse the fix off of your dissected embryos.
- **Embryos tend to stick to glass pipettes and even the sides of the wells in the glass dishes**. An easy way to prevent sticking is to use some yolk/material from the first group of embryos you dissect to swirl around the bottom of the dish, also use to coat the insides of your transfer pipette prior to using with fixed intact embryos. Only pull embryos up into the narrow neck of the pipette – avoid the expanded upper region of the transfer pipettes, embryos tend to get trapped there.

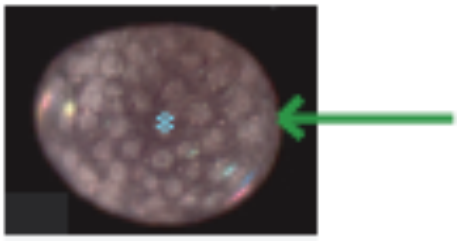
1. Place a few embryos (start with 2-3 and increase with experience) in a dish containing fixative.
2. Holding each embryo in place with your forceps or one of your dissecting needles (forceps are optional), poke a shallow hole in the eggshell with a dissecting needle. A shallow hole will avoid damaging embryonic tissues, and try to poke a hole in the yolk away from most of the embryo if possible (see figure next page). This is tricky for very early embryos because their cells are evenly distributed around the yolk (the cells of older embryos condense to one side). Start your timer (or note the time on the clock) after you have poked a hole or made some kind of tear in each embryo in fix.
3. Allow fix to enter the embryo for a couple of minutes – this assists in the dissection – however do not wait too long because the embryo is also fixing to the outer membrane(s).
4. Carefully peel away the outer egg shell membrane starting at the hole you made with your dissecting needle. Sometimes it helps to make a slight tear in the shell at the poked hole because it will produce a flap or loose end that you can hold with a needle or forceps.
5. While holding a piece of free membrane with one needle, carefully peel the membrane away from the embryo with the other needle. Sometimes it may be easier to hold a piece of membrane and try to roll the embryo away from the membrane with another needle. Either way, it is important to remove the membrane from the tissue as gently as possible. Be careful of appendages sticking to the membrane – it is very easy to dismember the embryos.
6. If you are lucky, the inner membrane (germband stages and older) will come off with the egg shell membrane! If not, repeat steps 4 & 5 for inner membrane.
7. Try to get the embryo mostly out, and exposed to fix relatively quickly – the time it takes you to remove the membrane will influence the amount of time you actually fix the embryo. Try to remove the membranes within 5-10 minutes and then allow the embryos to continue to fix for 10 more minutes (total time in fix should be around 15-20 minutes).

8. Once fixed, carefully pipette embryos into a dish/tube containing PT to rinse embryos before beginning the staining protocol.

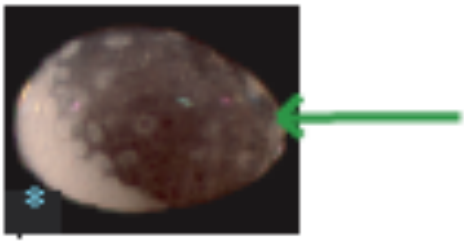
General things to keep in mind while dissecting: Be careful. Don't make any sudden or jerking movements, this will tear the embryo. Be patient – large pieces of embryos will also give you some data. You do not have to remove every bit of yolk from the embryo when antibody staining as long as enough yolk has been removed to expose the tissue sufficiently, your staining will work fine.

Helpful hints for dissecting different age *Parhyale* embryos:

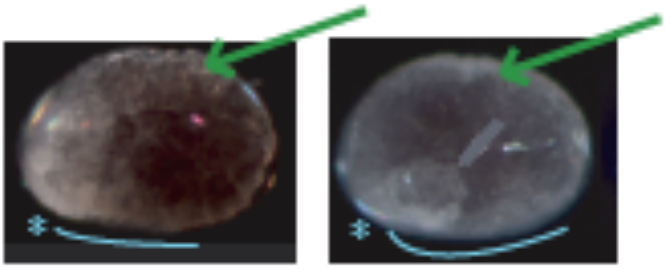
0-18hrs: These embryos are very yolky and it is difficult to maintain overall shape while dissecting away the single membrane surrounding them. Poke a very shallow hole to begin. You may want to initially fix for a few minutes while you dissect and start the real time of fixing once you have totally removed the membrane. For example, you may want to spend 5 minutes in seawater plus formaldehyde (9:1) to poke a hole and remove the membrane followed by another 15 minutes in fresh/another fixative (9PEM:1PBS(10x):1Formaldehyde) once the membrane has been removed.



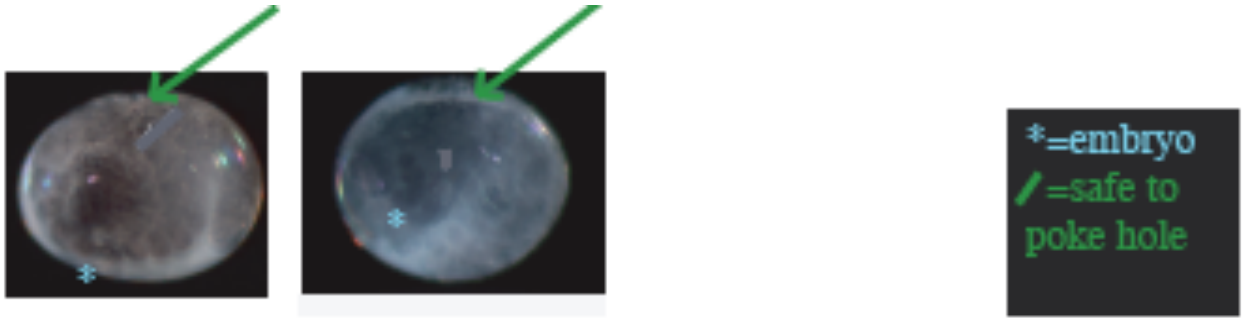
1-2 day: These embryos are relatively easy to dissect because they are surrounded by a single membrane and since most cells have condensed to one side of the embryo, you can pretty much hack off the yolky side of the embryo without losing too many cells.



60hrs - 3days: This is another tricky stage because the embryo is not easy to identify and the yolk has developed two membranes – one of which is easy to remove (outermost) and another inner membrane that is more difficult to remove. You still want to poke a hole in the embryo to begin your dissection. One good way to locate the embryonic tissue is to roll the embryo on its side in the well with fix. As it rolls around, you will notice an arc of whitish or more opaque region with respect to the purplish yolk and totally clear space sometimes seen between the membrane and the yolk. Pierce the embryo on the opposite side from the opaque region. The only other hint at this stage is to dissect fewer embryos at a time – therefore you can remove both membranes quickly before the inner one sticks to the embryo like plastic wrap. The longer the embryo sits in fix, the more the inner membrane becomes fixed to the embryo. If you cannot remove all the membrane, remove all the yolk on the opposite side – this exposure is generally enough for antibody staining to work, however a through removal of the inner membrane is best for good *in situ* results.



4+ days: Once the embryo has grown appendages, the trick is to remove the membrane without breaking off appendages. It is easier to dissect older, leggy embryos if you poke a hole dorsally just posterior of the head. This will give you more room to begin removing membranes. After poking a hole it can be helpful to let them sit for 1-2 minutes undisturbed in fix.



Parhyale Staining

For antibody staining, follow *Drosophila* antibody staining protocol beginning at the step after rehydration of fly embryos in PT.

Phalloidin Staining (for cell outlines at early stages and muscle staining at later stages)
Note: Embryos must not have been exposed to methanol!

1. Fix in 3.7% formaldehyde 20-30minutes at RT
2. Wash 2X with PT 5-10 minutes*
3. Wash 10 minutes with 70% cold acetone
4. Wash 10 minutes with100% cold acetone
5. Wash 2X with PT 5-10 minutes
   (if your hatchlings are more than a few days old, sonicate a few seconds)
6. Incubate 1:500 phalloidin overnight at 4 °C (a couple of hours at RT will also work)
7. Rinse with PT 3X
8. Stain with DAPI/50% glycerol, if desired
9. Store in 70%glycerol

*If you want to stain with phalloidin and an antibody, perform the antibody stain first.

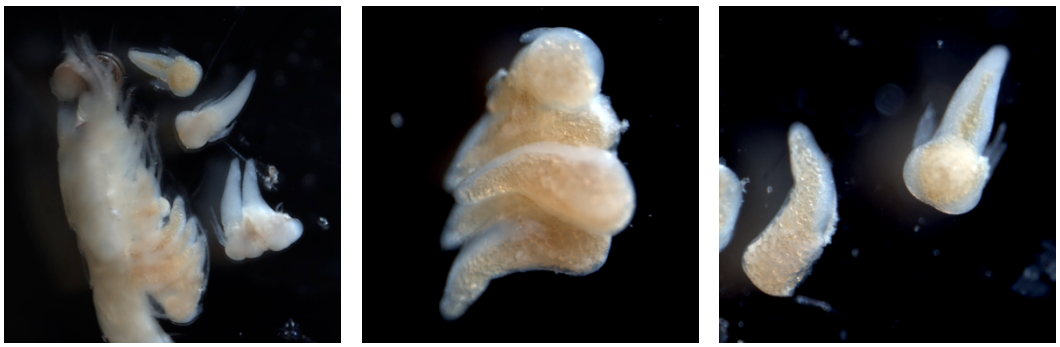
MYSID STAGING & PROTOCOLS

Mysids develop within the brood pouch of the female in four days, corresponding to Stages 1-4. Whole females with embryos are fixed with 3.7% PFA, stored in methanol, and when needed, can be rehydrated in PT. Each female carries three to six embryos.

Stage 1: Yellow ball. Corresponds to the germband stage. Square cells are organized into perfect rows and columns. The eggs are easily removed by grasping the female with forceps and brushing the brood pouch with another pair of forceps. The embryos must then be must be dissected out of the vitelline membrane with forceps or tungsten needles. Do not sonicate this stage. Embryos hatch out of the vitelline membrane following Stage 1, so all subsequent stages do not need to be dissected.

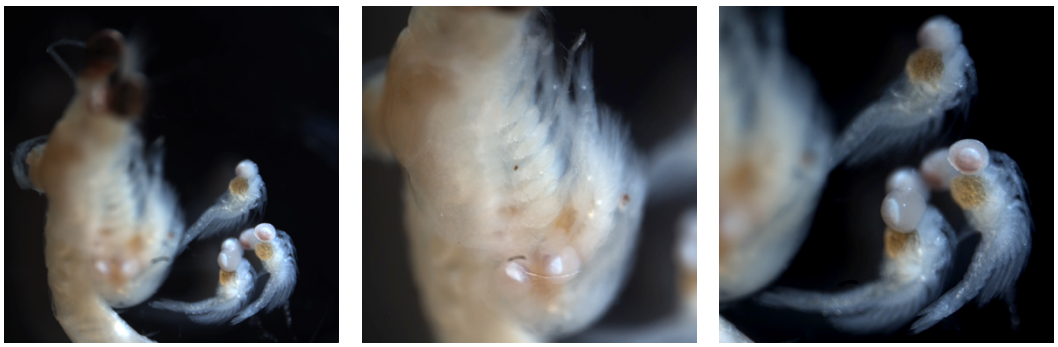
(left) A female with Stage 1 embryos next to four Stage 1 embryos. (middle) Cluster of stage 1 embryos. (right) Stage 1 embryo with membrane removed.

Stage 2: Tadpole. The embryo has legs, but they are held tight against the body, so they are difficult to see. Stage 2 embryos stick together and must be gently pulled apart. This stage may not need sonication, but can be gently sonicated to open up the cuticle for weaker stains.



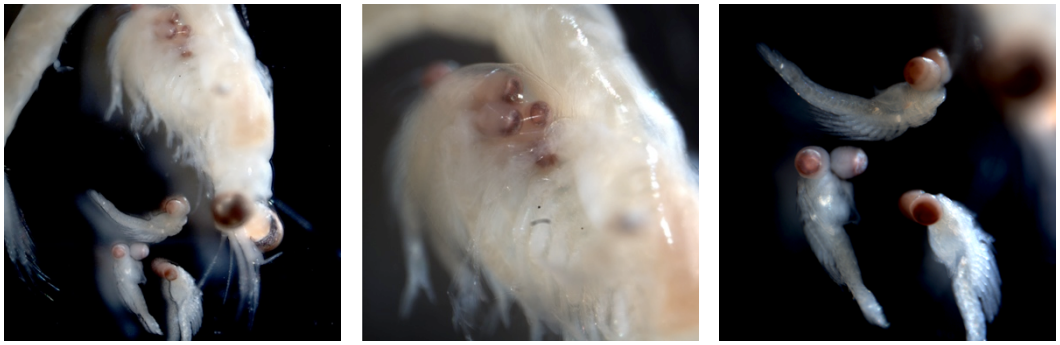
(left) A female with Stage 2 embryos next to four Stage 2 embryos. (middle) Cluster of stage 2 embryos. (right) Separated stage 2 embryos.

Stage 3: White eyes. The embryo's legs are now visible, and it has white eyes. This stage must be sonicated.



(left) Female with Stage 3 embryos next to three Stage 3 embryos. (middle). Brood pouch with Stage 3 embryos. (right) Isolated Stage 3 embryos.

Stage 4: Red eyes. The embryo has red eyes and more detailed features. This stage must be sonicated.



(left) Female with Stage 4 embryos next to three Stage 4 embryos. (middle). Brood pouch with Stage 4 embryos. (right) Isolated Stage 4 embryos.

Sonication: Place embryos to be sonicated in 1.5 ml eppendorf tubes in 1 ml PT. Stage 2: sonicate 40-60 seconds. Stage 3 and 4: Sonicate 15 minutes. All sonication in Branson 1510 water bath sonicator located in cold room; put eppendorfs into floaties to sonicate. After sonication, rinse with PT and begin antibody staining as you would Drosophila (block, add primary, etc.).

DROSOPHILA TRANSGENIC LINES

Wild type Development – Fluorescent Imaging in Living Embryos

- Gap 43-Venus Cell surface marker for following gastrulation.. For more information, see Mavrakis *et al*. (2009). Plasma membrane polarity and compartmentalization are established before cellularization in the fly embryo. Dev Cell. **16**: 93-104.
- ***btl*-GAL4 + UAS-actin-GFP**. *btl* (*breathless*) is expressed in developing trachea.
- ***nanos*-GFP** *nanos* (*nos*) easily marks the pole plasm and thus the pole cells; it can be used to follow migration of germ cells to the gonads.
- **Histone 2A-EGFP**. Labels nuclei with GFP
- **Histone2A-RFP; G147 *jupiter*- GFP**. *jupiter* is a microtubule binding protein. Nuclei are labeled with RFP and cell membranes are labeled with GFP.

Metamorphosis of the nervous system

- ***ppk*-gfp** The *pickpocket* (*ppk*) gene is expressed in body wall sensory neurons. For more information, see Williams, D.W. and Truman, J.W. (2005). Cellular mechanisms of dendrite pruning in *Drosophila*: insights from in vivo time-lapse of remodeling dendritic arborizing sensory neurons. Development **132**(16): 3631-42.
- **GMR-GAL4 + UAS-GFP** The GMR enhancer (*glass* multiple reporter) is expressed in photoreceptors as they differentiate in the eye/antennal disc, project into the brain and cause the proliferation and differentiation of the optic lobe neurons. For more information, see Huang, Z. and S. Kunes (1996). *hedgehog*, transmitted along retinal axons, triggers neurogenesis in the developing visual centers of the *Drosophila* brain. Cell **86**(3): 411-422.

SOLUTIONS

10X PBS:

18.6 mM	NaH_2PO_4 (2.56 g $NaH_2PO_4 \cdot H_2O$ per 1000 ml dH_2O)
84.1 mM	Na_2HPO_4 (11.94 g Na_2HPO_4 per 1000 ml dH_2O)
1750.0 mM	NaCl (102.2 g NaCl per 1000 ml dH_2O)

Adjust pH to 7.4 with NaOH or HCl. Prepare 1X PBS by diluting 1:10 with dH_20. Both 1X and 10X PBS can be kept indefinitely at room temp.

PT:

1X	PBS
0.1%	Triton X-100

Mix 100 ml 10X PBS, 899 ml dH_2O, and 1 ml Triton X-100. Store at 4°C or at room temp.

PT + N (PT + 5% Normal Goat Serum):

1X	PBS
0.1%	Triton X-100
5.0%	Normal Goat Serum (NGS; Gibco-BRL Cat. No. 200-6210AG)

Heat inactivate the serum at 56°C for 30 min. Filter through a 0.22 μm filter while still warm. Aliquot into sterile tubes. Store aliquots at –20°C. Once thawed, aliquots are stable for several months at 4°C. To prepare the PT+NGS solution, mix 4.75 ml PT with 0.25 ml Normal Goat Serum and store at 4°C. Solution will usually last at least several weeks. Discard if bacterial growth is detected (solution will turn cloudy).

Formaldehyde Fixative:

3.2% paraformaldehyde in PBS
Mix 1 ml 32% paraformaldehyde with 9 ml 1X PBS

Glycerol solutions:

Use ultrapure glycerol (Boehringer Mannheim, Cat. No. 100 647). Prepare 50% and 70% glycerol solutions by mixing the appropriate volume of glycerol with 1X PBS. Use pH paper to make certain that the pH of the glycerol solutions is around 7.4. Glycerol solutions can be stored at room temperature. Glycerol solutions with DAPI (1.0 to 0.1 μg/ml) should be stored in dark at 4°C.

PRIMARY ANTIBODY LIST - Quintay 2014

Antibody Name	Antibody type	Species specificity	Recognizes	Description	Suggested Dilution (in parentheses dilution for Rapid)	
A/P AXIS						
Hb (1G10)	Mouse IgG	Drosophila	Hunchback	Gap pattern blastoderm, CNS (neuroblasts plus first born GMCs) and other tissues later.	1:10	1G10
Rb α Eve	Rabbit IgG	Drosophila	Even-skipped	Pair-rule pattern at blastoderm, subset of CNS neurons and subset of dorsal muscle and heart later.	1:150 (1:100) *	10900; diluted 1:100
2B8	Mouse IgG	Drosophila, Tribolium, most insects, some crustaceans (mysids, crayfish). Not in Parhyale.	Even-skipped	In Drosophila and Tribolium, pair-rule pattern early, subset of CNS neurons and subset of dorsal muscle and heart later.	1:60 (1:40 60 min)	
DP312	Mouse IgG	All bilateria	Pax3/7 family (in Drosophila - Prd, Gsb, and Gsb-n). Also reacts with Rx	In Drosophila, pair-rule pattern early (prd), then segmental pattern (gsb), then CNS pattern (gsb-neuro). Rx reactivity gives brain staining. Same patterns seen in Tribolium and Grasshopper. In Parhyale, just segment polarity and CNS patterns.	1:50 (1:40 60 min)	

Antibody Name	Antibody type	Species specificity	Recognizes	Description	Suggested Dilution (in parentheses dilution for Rapid)	
4D9	Mouse IgG	Almost all insects (not lepidoptera), most crustaceans (including mysids, but not Artemia and Triops), many vertebrates including zebrafish and chickens (not mice, weak in Xenopus)	Engrailed/invected	For arthropods, segment polarity pattern (posterior compartment). Posterior compartment of Drosophila imaginal disks. Vertebrate patterns include midbrain/hindbrain boundary and subset of muscles (in zebrafish)	1:40	
4F11	Mouse IgG	Almost all insects (including lepidoptera), some crustaceans (including Artemia and Triops), no vertebrates. Superior to 4D9 for Drosophila imaginal disc staining, but not as good for embryos	Engrailed/invected	For arthropods, segment polarity pattern (posterior compartment). Posterior compartment of Drosophila and butterfly imaginal disks.	1:30	
2A1	Rat IgG1	Drosophila	Cubitus interuptus	Segment polarity pattern (anterior compartment of embryo and disks)	1:20	2A1-s 12/6/12-48ug/mlIg
4D4	Mouse IgG1	Drosophila	Wingless	Segment polarity pattern in embryos. D/V stripe in wing disk.	1:30 (1:20)	4D4-s
Ptc	Mouse IgG2a	Drosophila	Patched (apa 1)	Segment polarity pattern. Expressed in either side of engrailed stripe	1:20	Drosophila Ptc (apa 1)-s 8/18/11-45ug/ml Ig
HOX (homeotic)						
Rb α Ubx	Rabbit IgG	Drosophila, other species not tested	Ubx	T2/T3 and posterior	1:50 (1:25) *	11278; diluted 1:100
FP3.3	Mouse IgG	Drosophila specific	Ubx	T2/T3 and posterior	1:20 (1:10 60 min)	
FP6.87	Mouse IgG	All arthropods	Ubx and abd-A	T2/T3 and posterior	1:20	

Antibody Name	Antibody type	Species specificity	Recognizes	Description	Suggested Dilution (in parentheses dilution for Rapid)	
α Abd-B (1A2E9)	Mouse IgG1	Drosophila	Abdominal-B	Posterior abdomen	1:50 (1:25)	1A2E9
α Antp (8C11)	Mouse IgG	Many insect species including Drosophila, Tribolium, and grasshoppers	Antennapedia	Ectoderm - Thorax; CNS - thorax and posterior	1 : 40 (1:20 60 min)	8C11
α Scr (6H4.1)	Mouse IgG	Drosophila and Parhyale	Sex combs reduced	Mx/La/T1	1:20	6H4.1
IMAGINAL DISKS						
4D9	Mouse IgG	Almost all insects (not lepidoptera), most crustaceans (including mysids, but not Artemia and Triops), many vertebrates including zebrafish and chickens (not mice, weak in Xenopus)	Engrailed/invected	Posterior compartment of the wing and leg discs	1:40	
4F11	Mouse IgG	Almost all insects (including lepidoptera), some crustaceans (including Artemia and Triops), no vertebrates. Superior to 4D9 for Drosophila imaginal disc staining, but not as good for embryos	Engrailed/invected	Posterior compartment of the wing and leg discs	1:30	
2A1	Rat IgG1	Drosophila	Cubitus interuptus	Anterior compartment of the wing disc	1:15	
4D4	Mouse IgG1	Drosophila	Wingless	A theta around the wing pouch.	1:30	
Ptc	Mouse IgG2a	Drosophila	Patched	A stripe along the A/P boundary in the anterior.	1:40	

Antibody Name	Antibody type	Species specificity	Recognizes	Description	Suggested Dilution (in parentheses dilution for Rapid)	
NERVOUS SYSTEM						
BP102	Mouse IgG	Drosophila	Unknown antigen	Stains all CNS axons (not PNS)	1:200 (1:50 60 min)	
22C10	Mouse IgG	Drosophila	22C10	Stains cytoplasm and cell membranes of axons of all PNS neurons plus a subset of CNS neurons	1:40 (1:20 60 min)	
G α HRP 488	Goat; directly conjugated to Alexa 488; No 2°Ab needed	All arthropods, probably most Ecdysozoa	Carbohydrate epitope present on many neural proteins	All neurons (CNS and PNS)	1:200 (1:100 60 min)	
G α HRP 647	Goat; directly conjugated to Alexa 647; No 2°Ab needed	All arthropods, probably most Ecdysozoa	Carbohydrate epitope present on many neural proteins	All neurons (CNS and PNS)	1:200 (1:50 60 min)	
1D4	Mouse IgG	Drosophila	Fasciclin II	Subset of CNS axons (including motoneurons)	1:30	
9F8	Mouse IgG	Drosophila	Elav	Stains nuclei of all neurons (elav gene product encodes neural-specific splicing factor)	1:50 (1:30 60 min)	
Rt α Elav	Rat IgG	Drosophila	Elav	Stains nuclei of all neurons (elav gene product encodes neural-specific splicing factor)	1:50 (1:30 60 min)	7E8
8D12	Mouse IgG	Drosophila	Repo	Glial cells (nuclear)	1 : 50 (1:30)	
MR1A	Mouse IgG1	Drosophila	Prospero	Ganglion mother cells and neurons	1:20	

Antibody Name	Antibody type	Species specificity	Recognizes	Description	Suggested Dilution (in parentheses dilution for Rapid)	
MISC.						
Rat α Tropo	Rat mAb	All insects, probably all arthropods	Tropomyosin	Muscle	1:50 (1:30 60 min)	
2A12	Mouse Ig**M**	Drosophila	Trachae (antigen unknown)	Trachae	1:10	
Rb α pHist	Rabbit IgG	All bilaterians	Phospho-Histone H3 (Ser 10)	Cells in mitosis	1:20 *	diluted 1:100;Rabbit α phospho-Histone H3 (Ser10) Mitosis Marker; Rb monoclonal MC463
anti GFP	Mouse IgG1	All forms of GFP	Green fluorescent protein	Where GFP is expressed	1:20	predilute as needed

SECONDARY ANTIBODIES & OTHER REAGENTS

USE AT dilutions of 1:1000 (overnight) to 1:500 (Rapid protocol)

Use Alexa 488 and 555 as your first choice;
Alexa 647 should be used only when you need a third label

The anti-IgM antibodies listed here are not meant for multi-labeling experiments. They will cross-react to anti-IgG antibodies

ANTI-MOUSE SECONDARY ANTIBODIES

goat anti-mouse IgG (Alexa 488 conjugated)

goat anti-mouse IgG (Alexa 555 conjugated)

goat anti-mouse IgM (Alexa 488 conjugated) Use only with IgM primary

goat anti-mouse IgG (Alk Phos conjugated)

ANTI-RAT SECONDARY ANTIBODIES

goat anti-rat IgG (Alexa 488 conjugated)

goat anti-rat IgG (Alexa 555 conjugated)

goat anti-rat IgG (Alexa 647 conjugated)

goat anti-rat IgG (Alk Phos conjugated)

ANTI-RABBIT SECONDARY ANTIBODIES

goat anti-rabbit IgG (Alexa 488 conjugated)

goat anti-rabbit IgG (Alexa 555 conjugated)

goat anti-rabbit IgG (Alk Phos conjugated)

ADDITIONAL REAGENTS

PHALLOIDIN (at –20°C) Use 1:500

Phalloidin RITC conjugated

NUCLEAR DNA STAIN

DAPI (1 mg/ml in dH_2O)

Normal Goat Serum; NGS (for blocking) Use at 5%

4 ml aliquots at 4°C (Boveri)

Glycerol (diluted with 1X PBS)

50% no DAPI - 10 ml aliqouts at 4°C (Boveri)

50% DAPI 1.0 µg/ml - 4 ml aliqouts at 4°C (Boveri)

70% no DAPI - 10 ml aliqouts at 4°C (Boveri)