**In Situ Protocol for Imaginal Discs and Pupal Wings**

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(\*\*Warning: This page is still under construction. It needs the pages of reagent recipes added).

**AN ANAL RETENTIVE GUIDE FOR DOING IN SITUS ON IMAGINAL DISCS**

A. TERMS FOR MAGGOTS AND METAMORPHOSIS.

The Ashburner book (Drosophila: A Laboratory Handbook) has useful pictures and tables on pgs 182-185, 186-188, and 241-242.

1. Drosophila larvae molt twice before metamorphosis. The stage between molts is an instar, and there are three larval instars. The pointy end of a larva is its head. The most prominent feature of its head are the black mouth hooks which it uses for feeding. About midway through the third instar (3i) a larva undergoes a behavioral change. It stops feeding and wanders up the sides of the container, and it will continue to wander for about 24hr before its stops and pupariates, or prepares to form a pupa. Metamorphosis is carried out by specialized tissues inside the larva that assemble together to form the pupa. This assembly is as bona fide a developmental process as embryogenesis. Much of the larva is discarded during the formation of an entirely different animal.

2. After the larva stops moving its cuticle stiffens, and from a white prepupa stage it will darken ('tan') and harden ('sclerotize') into a brown prepupa stage. A prepupa is the motionless larva during early metamorphosis that has not yet changed into a pupa. Much of the body wall of the pupa is derived from sacs of epithelium called imaginal discs. The imaginal discs evert under the cuticle of the immobilized larva, and rapidly change shape and grow until they assemble the head and thorax of the pupa and its appendages. Most of the abdomen of the pupa is not derived from imaginal discs, but is instead made from scattered islands of cells called histoblasts that spread out and proliferate.

3. The larva cuticle has been transformed by becoming brown and hard, and is now called the puparium. You will note that during pupariation the larva takes on a slightly different shape from a crawling larva, in that it is shortened and has everted a pair of 'horns' at its head. The horns are anterior spiracles. The larva also has a pair of posterior spiracles at the tip of the abdomen. Once the imaginal discs and histoblasts have grown and assembled into a crude fly-like shape, the pupa stage begins with the secretion of the pupa cuticle inside the puparium. Unlike the puparium, the pupa cuticle is very thin, soft, and absolutely transparent.

4. You are probably familiar with insect life cycles where once a pupa is formed inside the cuticle of a larva, a molt then occurs revealing the pupa. The adult is then formed inside the pupal cuticle, and a later molt reveals this adult. Fleas and most flies, however, have a different schedule. The pupa remains inside the puparium (which is the cuticle of the 3i larva), and when the adult cuticle is laid down, the pharate adult remains for a time inside the pupa cuticle, which is inside the former larva cuticle(!). You will see that the area around the head of a puparium is flattened on its dorsal surface (puparia are stuck down on their tummies). This flat area is the operculum. The operculum is encircled by a suture so that it can pop open and allow the adult to hatch out, or to eclose.

5. You can look inside a fly bottle under a dissecting microscope and see this life cycle. This will help you select the right stages for dissection. You can find wandering larvae, some with the anterior spiracles already everted. Immobile white prepupae: some are still able to wander if disturbed, but slightly older ones of this stage cannot wander. These are already stiffened, or sclerotized. The tissues inside the brown prepupae show a two-toned color, with a translucent grey-white anterior one third. These are the imaginal discs that are now assembling together. The tissue inside the abdomen includes opaque white fat. An air bubble in the abdomen marks the relative age of brown prepupae: Those with very small air bubbles (which allow the prepupa to still sink under water) occur in younger stages. As the abdomen fills with air, the pupae can float on water. It is among these stages that a pupa cuticle starts to form. Even after the cuticle is made, the legs and wings of the pupa continue to expand and become more fly-like. Pupae show an outline of a fly inside the puparium. You can see the constrictions between the head and thorax, and between the thorax and abdomen. Pharate adults show bristles and eye color.

B. CONDITION OF STOCKS

1. Either from bottles or vials. Stocks should be uncrowded, so larvae crawling up sides are indeed late 3i looking to pupariate and not looking for food.

2. If from crowded stock, add food/transfer glob of larvae to fresh food. Wait a day or two for them to recover.

3. After adult flies are placed in a bottle it will take about 5 days for 3i larvae and prepupae to appear if raised at 25ï¿½C.

4. To do the trickier procedure of staining late pupa wings, you will want to stage pupae by eye color. These should be white + stock.

C. SELECTING GENOTYPE/SEX OF LARVAE

1. Dominant markers exist for selecting the first (X), second, and third chromosome genotype of larvae and puparia. X chromosome can be marked by y+. One can recognize y+ mouth hooks because they are nearly entirely deep black. y- is dark brown. It is easier to see this against a white background. I find it easier to select and dissect y- larvae by first extracting feeding larvae from the food. I have a later section on how to do this. I recommend learning what y+ and y- looks like with stocks known to be all one or the other. Second chromosome CyO balancer can also be marked with p(y+) in a y- background. Also Black cell (Bc) is a dominant marker that shows brown or black blood cells. Third chromosome is easy: use the TM6B balancer marked with Tb. Older Tb larvae and puparia are much shorter than Tb+ animals.

2. Sex can easily be determined in 3i larvae and younger prepupae. One can see the pair of large male gonad primordia about 2/3 of the way back from the head, on the ventro-lateral sides. They appear as a pair of clear drops imbedded in the fat body in the abdomen. Female gonads are tiny and hard to see.

D. ARE YOU READY TO START?

1. Equipment you will need: stereo dissecting scope, a good light, fine forceps in varying degrees of condition (old forceps for picking up larvae, nice sharp ones for dissection), fine dissecting scissors, small (35X10mm) petri dish bottoms (steal from people making grape juice plates), pipette gun/three valve bulb, 5, 10, 25 ml glass pipettes, 15cm long Pasteur pipettes, razor blades, timer, heating blocks calibrated to give 55ï¿½C and 85ï¿½C, P20, P200, and P1000 pipette persons, 1.5 or 2.0 ml micro centrifuge tubes, and a bucket 'o ice. Additional things for mounting discs: clean microscope slides, large cover slips, cotton swabs, slide warmer.

2. A note on forceps. I use fine dissecting 'jewelers' forceps, but the points do not need to be especially sharp. In fact, under the dissecting scope they look rather blunt. Needle sharp forceps get bent too easily to be practical. What is essential is that they have a perfect 'bite'. The tips can be pressed together exactly at the tips of the forceps, with the jaws well aligned. Also when pressed together the biting surface must not roll back to a position behind the tips, allowing the tips to separate slightly. Take time to carefully file your forceps under the dissecting scope until they are beautiful.

3. Reagents you will need for in situs (refer to media manual): 10X PBS, 1X PBS, 1X PBT (=PBS with 0.1% Tween 20)., 1.3X PBS+ 67mM EGTA, formalin (37% formaldehyde), 100% MeOH, 70% and 100% EtOH, xylenes, -80o frozen aliquots of 10 mg/ml protease K, hybridization buffer, pre absorbed anti-DIG-alkaline phosphatase conjugate, alk phosp staining buffer (freshly prepared before use), Boerringer Mannheim labeling kit tubes 9 and 10, PBT+20mM EDTA, Permount. Except for the anti-DIG, nothing needs to be sterile.

4.Be sure to read the NOTES ON PROTOCOLS SECTION.

E. DISSECTING LARVAE. The idea here is to keep the discs anchored to the carcasses during an arduous series of washes. It is easier to keep track of the larger carcasses than the tiny discs.

1. Be sure you have late 3i larvae crawling up the sides. Have your reagents ready. For example, you will need about 5 ml of in situ fixative. Pipet about 1/2 ml of fix into a 1.5 ml microcentrifuge tube. With old fine forceps, pick larvae off of sides of container and place in small petri dish lid with wet paper (they are happy enough to more or less hang around here while you kill them off). I like to get 15-20 larvae/experiment.

2. Have a small dissecting dish bottom with 4 ml PBS under the microscope. A black background under the dish is essential. Place about a half dozen larvae in the dish (fewer if you are just learning). Also, mount a blue tip with the tip cut off onto the P1000.

3. Cut several (not all at once) of the larvae in half with scissors. Then use forceps to turn them inside out. This is done by gently holding--not squeezing-- the anterior half of a larva in one pair of forceps, and using one jaw from another pair to stuff its head inside its body. This stuffing movement is followed through until the poor thing is turned inside out.

Extra guts must now be picked off (in this order): gut (yellow muscled tube), lateral fat (white, opaque, fluffy fingers of tissue), salivary glands (pair of very large, clear sausages near the head. Avoid trachea (silvery threads), especially the larger trachea, and discs (white, slightly translucent--these will be harder to see until above tissue has been removed). The idea here is to remove excess stuff without loosening the discs. The discs are narrowly anchored to the body, and to a few fine strands of trachea.

4. After a few larvae are dissected gently pick them up one by one and put into the fix. Dissect a few more and put into same tube, etc. until all larvae that were placed into the dish are dissected. This period should take about 5 min. but not more than 10 min. Tip on side so carcasses are spread out and well exposed to fix.. Fix at R.T./30' (I label tube for time to be done fixing), or overnight at 4ï¿½C. Prepare new fixing tube and dissect more until you have dissected enough. While fixing DO NOT ROCK THE TUBES. EVER.

5. After fixing, rinse 4X in MeOH. Sit at R.T. in MeOH while you continue dissecting.

F. DISSECTING PREPUPAE.

1. Look at the sides of your fly bottle under low magnification with the dissecting microscope. Find and pick off puparia of these developmental stages (in chronological order): white, slightly brown, brown without and with an air bubble in the abdomen. Try to avoid puparia that obviously contain pupae. Pupae are recognized by their having a visible profile of a fly head, and elongated legs and wings inside the puparium. Set prepupae aside like the larvae.

2. Dissecting strategies are different, depending on stage.

a. Soft white prepupae: handle as larvae, but cut in half carefully. First, puncture side with scissors, then cut all the way through. Head explodes if you try to do it all at once.

b. Slightly stiffened but still white prepupae: The discs are often stripped off if you turn inside out. Instead, cut in half, then cut anterior half lengthwise (insert scissors blade closely along body wall to not cut off discs). Hold open and pick off guts as before. It is essential to then carefully cut away a wide strip of body wall to keep discs well exposed to reagents. Larva tissue seems to be degenerating at this time. The trachea are much weaker. Do not over clean. c. Brown prepupae: Those with an air bubble may or may not have a pupa cuticle.

Discard those with very large air bubbles--these will have the cuticle and cannot be stained without sectioning. Tear in half with forceps (Pinch up body wall on either side of a segment border and split apart). If crudely formed pupa slips out without tearing in half, then it has a pupa cuticle. If tissue remains in both halves then it is still a prepupa and can be used. young enough (tissue remains in both halves), then continue: Hold down by the anterior spiracle 'horns', and reach inside to pluck out fat, gut. Grab the CNS and carefully pull out. Eye-antennal and many leg discs will come out with it. Peel off the operculum: Pinch up one corner of the operculum and carefully peel away. Peel away the tissue lining underneath it. How one proceeds to dissect these is a matter of personal, idiosyncratic habit. Below I list what I see as stages of brown prepupae, with my weird habits of dissecting them.

APPROXIMATE STAGES OF BROWN PREPUPAE:

From 0 hr white prepupae at 25oC:

1-3hr: Paw stage. Wing discs just under operculum. They are everting and by 3 hr the dorsal and ventral wing surfaces have definitely come together. The wings look like a pair of little paws lying side by side. Pluck out extraneous tissue through the operculum, and nudge the wings out so they are well exposed. Most of the selected prepupae will be around this stage.

4-5hr: Elbow stage. Wings are displaced to the left and right sides of the prepupae, and are bent near the presumptive wing hinge. They are often hard to find. Handle like above, but these wings easily fall out.

6-7hr: Long chubby stage. Elbowed wings have now straightened out. They have more than doubled their length, but the epithelium is still rather columnar. Hence they look chubby. The tissue lining the puparium easily peels away. Pluck out tissue to leave a fairly hollow puparium with the wings still attached inside. This will protect the wings, yet maximize reagent access to these valuable wings.

9-12 hr: Tongue stage: The wings now lie slightly more dorsally. They are very flat and delicate. Easily torn. They readily come out when you are peeling away extraneous body wall. Treat like the long chubbies. A pupa cuticle starts to appear during the tongue stage (certainly by 12hr), and along with it a bubble forms in the abdomen causing the animals to float. Most floaters are too old for whole tissue staining but some are OK. These OK ones will have a relatively smaller bubble.

NOTE: The long chubbies and the tongue stages are in a way easy to do since they tend to clean themselves. Merely pulling the puparia in two often pulls out most the extra tissue.

3. I am sorry to say that only experience, pain, and disappointment after hard work will teach you how to get unimpeded staining of accessible wings without removing so much tissue as to then lose a lot of wings during washing.

4. You will often pull off wings. You can keep some of these by taking an empty half puparium shell and splitting open a short segment of cuticle--either along a segment border, or along the operculum seam. Such a split is a natural clamp--the cuticle is rather springy. Place the tissue inside the puparium, then from the outside reach through the split with forceps, grab the wing by a trachea or near the hinge, tissue, and pull it out through the split in such a way that it is clamped by its wing hinge area with the wing still inside the puparium. Impossible? I do it all the time.

5. You should be processing prepupae along with larvae. Fix as in larvae--no difference here.

6. You can fix larvae and prepupae for 30' at room temperature, or overnight at 4ï¿½C.

G. DISSECTING 18-40 HOUR OLD PUPAE.

This is hard. You should be adept at dissecting before you try this.

1. Pick off puparia that have a visible pupa inside. It looks like a white fly inside there. The head is plainly visible, and the legs and wings can be seen pressed along the ventral side of the body. Look at the pupa stages shown in the Ashburner book if you need to (Pg 183). Reject those that have any visible eye color. These are too old and they will have an adult cuticle. Many that you select will also be too old but you will cull them out as you go. Set aside a large collection--at least 3 dozen--and dissect in small batches.

2. With a small batch in the dissecting dish, extract a pupa from its puparium. First tear off the operculum, and peel off a few segments of anterior puparium cuticle. Reject any with any eye color. Now firmly grip the puparium by the tiny nipple of cuticle at its posterior tip (the posterior spiracles). Grab the pupa by the head (it squishes it), and gently pull until pupa slips out. It sometimes sticks inside, and forms stress creases along length before it pops free. You may have to pull from a dispensable place on the thorax. Damage sometimes occurs, and one should try different methods. I find that it comes out easier if you first peel off much of the puparia cuticle from the abdomen--it sticks the most there. Then pull out the pupa while gripping the posterior-most edge of the puparium.

3. Is the pupa at the right stage? Too-young pupae have puffy looking wings and their cuticle is closely attached to the body epithelium. OK pupae (18-40hrs old) have a visible gap between the epithelium and the clear shiny pupa cuticle. One of the earliest places this gap forms in on the wings (about 18 hrs), followed closely by areas on the head-- especially around the antennae--and the legs. Even relatively very narrow gaps are still quite usable. Too-old pupae will also have these gaps, but the wings have now grown and are folded inside the pupa cuticle--they are too large to fit into the pupa cuticle wing pouch. These will have an adult cuticle. Reject them. The youngest stage to be rejected is where a longitudinal crease forms along the wing parallel to vein 3. Once you get an eye for the pattern of wrinkles of these larger wings, you will be able to see them through the puparium before you bother to extract the pupa. This saves time.

4. Selected pupae have to be eviscerated. Squeezing the pupae forces opaque tissue droplets into the wings, so try to not do this. Cut off the head. Grip the thorax near the chest (a dispensable site), and cut off the tip of the abdomen. Still gripping the chest, insert a Pasteur pipette over the abdomen and gently blow and suck PBS through the pupa. The guts and tissue droplets should be blown out, leaving a translucent husk of a pupa. Pluck out remaining guts. The epithelium of the notum should still be intact, and can be stained for sensilla development. You will have noticed that there are large air- filled trachea, and that air bubbles tend to occur around the legs. The trachea and air can be plucked off, helping the pupa 'torso' to sink. This is optional at this time, but will have be done later.

5. Place them in tube with fixative. In PBS the pupa cuticle sticks to blue tips, so you should use forceps to gently put them one by one straight into a tube with fixative already added. Fix overnight at 4ï¿½C. The fixative will go through the cuticle.

6. A very efficient strategy is to take a small batch of puparia, pull out all pupae. Reject and discard as you go along. All remaining pupae in the dish are selected. Cut off all heads and abdomen tips. Then blow out all torsos. Then fix. This way you arent endlessly picking up and putting down the same damn tools for each pupa.

7. The next day replace the fix with PBT, and sit the tube on ice. Put a few fixed pupae in a dish with PBT. Now you must peel off the raised cuticle from the wings, and this must be done with forceps with a perfect bite. Grip the pupa by the chest and pull off air bubbles (large trachea, etc.). Get the pupa to sink. There are several approaches to peeling off wing cuticle, but they are easier to handle if the wings stay attached to the body. One can pinch up and pull cuticle, starting at the wing 'shoulder', and peeling distalward. One can also start at the distal end and peel up. Or pinch up cuticle on both the dorsal and ventral sides of the wing at the same time, and pull apart. Be sure to remove all the cuticle from both the dorsal and ventral sides of the wing. Use the light to see the shiny cuticle. The trickiest area is along the anterior margin, where the cuticle often sticks to the wing. Peel slowly, carefully, and thoughtfully. Have lots of pupae, since you will tear some wings. Try pulling to a different direction if you get stuck. If you pull off a wing, you can often save it for staining. First, finish peeling off the cuticle while gripping the wing at its base. Then wedge it inside the pupa thoracic cavity. I find that it is often still there after the in situ stain.

8. You should have selected some pupae with youngish wings. The space between the cuticle and wing epithelium is very close, and the wing still looks rather puffy, especially near its base. Another marker for age is the development of the hinge constriction, and the alula which is a posterior flap at the proximal end of the wing. These youngish pupae are harder to peel, but it is important to do some. The best place to start is on the ventral surface of the wing, since the gap is largest here.

9. After your small batch of pupae are peeled, place them in MeOH in a scintillation vial at room temperature. Continue dissections until all are transferred to this MeOH. Many will have formed an air bubble. Pick out these floating torsos and remove the bubble in a dish with MeOH.

H. NOTES ON THE PROTOCOL FOR STAINING IMAGINAL DISCS.

1. For a given session of fixing larvae and puparia, you will need about 5 ml of fixative.

2. Do not under- or over clean the carcasses of their non-disc tissue. Undercleaned carcasses still have wing discs under fat, or pinned against the body wall. Over cleaning shows no fat, very loose wings attached only to the body wall and no longer to the trachea. Kiss these wings good-bye. Ideally, wings remain anchored to the body wall and to the trachea. You will note large trachea trunks. The trachea trunks form an 'H' shape--two trunks along the body, and a cross-trunk just in front of the wings. You can see how these feed into the anterior spiracles. Smaller branches run to the discs. These are your friends.

3. Tricks for cleaning include pulling off the gut and fat rather slowly. Watch out for how these steps tug at the trachea. Stop and adjust your grip if trachea seem to be pulling up too.

4. I fix small batches of carcasses in microcentrifuge tubes. Once in fixative the tubes are tipped on their sides and rocked gently just until the carcasses tumble up and settle out in a spread pattern along the side of the tube. They are then left to lie still during fixation. NEVER continue to rock the tubes after carcasses are spread out. Agitation will break the anchor points of discs.

5. The insitu protocol has an early storage stage in 100% EtOH. During this period the carcasses are in glass scinti vials. The vials are labeled and dated with tape and stored at -20ï¿½C. The label is secured with a small rubber band (tape falls off in the freezer). After storage there is a xylenes wash, and EtOH washes. Early in the EtOH washes the carcasses are pipeted into microcentrifuge tubes again. These use less volume.

6. For a 'wash' or a 'rinse' I add the reagent slowly, with the tube tipped at a steep angle to minimize tumbling of carcasses. I then close the tube and gently spread the carcasses as in during the fixing. Various incubation periods (Protease K, anti-DIG, etc.) are also spent this way. Prehyb and hybridization are done upright.

7. A 'rinse' is just that. One adds a reagent, gently allows the carcasses to swirl once, then removes the reagent for the next step. A 'wash' is 5 min. for steps with alcohols, 10 min. for aqueous. The volumes are 3ml for scinti vial steps, 1 ml for microcentrifuge steps. There are a lot of washes. One could probably cut a few, but I never have. It works.

8. I remove reagents from scinti vials with a vacuum line, but it requires careful use of gravity, friction to the vial sides, and surface tension of liquids, to keep a carcass from being sucked up the line. I always hand pipette out reagents in microcentrifuge tubes, never allowing the Pasteur pipette tip to press onto the carcasses.

9. Stocks of PBS, PBT, and hybridization buffer can get dust in them, and accumulate crud near the bottom. I always use them up rather quickly, and daily check the bottoms of the bottles for crud. I make fresh reagents whenever they seem to be getting grungy. I try to make barely enough hyb buffer so I never have to toss it out.

10. In reading the protocol below, you may wish to flag the "plateaus" as they are noted (steps that can go longer).

11. Many people have a quicker way to dissect. They merely pull off the larva head, which pulls out the brain, eye-antenna discs, and many leg discs. If you pull from a few segments back you also usually get one or both wings. Very little if any cleaning is needed. One disadvantage is that there is little to anchor the wing discs, but since it is done, it may actually work (although I had trouble when I tried it). This problem can be solved by doing the staining protocol in a larger container, with the heads carried in polypropylene tubes with a fine flourtex screen stuck on the tube bottom. This introduces the convenience of doing all solution changes with a vacuum line. The new problems here include the need to use larger reagent volumes, and a constant battle to get the screen tubes to actually drain and re-fill during a solution change. In my hands I found it necessary to move the carcasses into microcentrifuge tubes for the incubation with probe, then back to the screen tubes. It is worthy of consideration, but I think that my old method requires less constant fiddling once past the dissection.

12. Between steps 8 and 9 I often pipette the carcasses back into a dish with PBT, and re- dissect to maximize reagent access. Those elbow, long chubby, and tongue stages will stain better if pulled completely out of the puparia at this time. Also, carcasses often get tangled together, and even sleeved inside each other.

13. For the protease K digests in step 11, assuming you are doing more than one tube: I first remove all PBT from all tubes, add 500 ul P.K. solution to first tube, and spread. I start the timer, wait 1 min., add P.K. to second tube, etc. Each tube is now 1 min. apart, which is enough time to rinse each later on. After each tube is rinsed 2X, they are stored on ice in a 3rd wash until all tubes are rinsed.

I. ISOLATING SELECTED LARVAE FROM FOOD (modified from Ashburner book chapter on fat body purification, pg.299).

To select good numbers of mutant larvae (Say y- mutant larvae in balanced CyO p[y+] stock) it is generally best to just mass extract feeding larvae from the food, isolate the y- mutants, and later harvest the mutants as they wander up from the food and pupariate. So...

1. Rear a large number (6 or more bottles) of CyOp[y+] balanced stock so that feeding larvae are super-abundant and crowded in a liquified layer of eaten food. Prepare to extract them.

2. You will need: a 500 ml beaker, a big flat dish with a diameter about 1/2 inch larger than the beaker (for example a 500ml beaker and a150 mm petri dish), 2 liter 3M NaCl-- enough to overflow the beaker, plus a squirt bottle of the NaCl, a spoon, a vacuum line, and a small fine-screen bottomed cup like the type used to collect embryos.

3. Pour about 300 ml of the NaCl solution into the 500 ml beaker. Dump spoonfulls of the liquified layer of food filled with larvae into this beaker, and stir vigorously with the spoon. Let sit. In 10 minutes or so the food should settle to the bottom, fine food particles first followed by larger food chunks as they becomes infiltrated with NaCl. The larve will stay floating at the top. Most do not drown.

4. Stand the beaker in the wider petri dish. Gently pour NaCl into the beaker until it spills over the top, carrying the larvae down into the dish. Use the squirt bottle with NaCl to hose remaining larvae down into the dish.

5. Hold the screened cup into the dish and vacuum up most of the NaCl through the cup. The screen keeps larvae from being sucked into the line. Rinse 1 or 2 X with H2O to remove food particles. Suck off excess H2O. Larvae are easier to sort if they are floating in a minimal amount of H2O.

6. Sort out the y- larvae under a dissecting scope against a white background. Pick up the larvae with forceps and place on a chunk of food in a small dish (they stay there and eat). When done collecting, put the larvae into a containor with food so they can grow up and be killed later. Clean up your mess.

7. If you can think of ways to improve this protocol I would like to hear about it. For example, it is still hard to suck off H2O. Also one always gets a thousand maggots crawling over the bench after a long day of doing this. This does not bother me personally, but....

**PROTOCOL FOR DISC WHOLE MOUNT IN SITU USING RNA-DIG PROBE**

1. Dissect small batches of larvae and prepupae in PBS. Fix in ufuge tube with 4% formaldehyde in PBS+EGTA buffer/30'. Rinse 4X in MeOH. Sit at R.T. until all are dissected and fixed.

2. Pool in glass scinti vial. Wash 1X in MeOH.

3. Wash 5X in EtOH, and store in about 10 ml EtOH at -20ï¿½ for as long as you like.

4. Wash three times in EtOH.

5. Wash in 50/50 xylenes/EtOH for 60'.

6. Wash 5X in EtOH. After first wash, pipette back into ufuge tubes.

7. Wash 2X in MeOH.

8. Fix/5' in 50/50 methanol/PBT+5% formaldehyde (PBT is PBS+.1% Tween-20). NOTE: between steps 8 and 9 I often return the carcasses to a dish in PBT at this stage and do more dissections on prepupae (not larvae) to maximize wing accessibility.

9. Fix/30' in PBT+5% formaldehyde. Make 2X the amount you will need for this step (see step 13).

10. Wash 5X in PBT.

11. Incubate for 8-10 minutes in PBT+Proteinase K. Stock solutions are 20ul aliquots at 10 mg/ml, made up in ddH2O and stored at -80ï¿½.

a. Diluted stock: 5ul stock in 30ul PBT=diluted stock. Split the rest into 5ul, label, and return to -80).

b. Working solution: per sample add 2ul diluted stock/500ul PBT/sample.

12. Stop the action of the protease by quickly rinsing 2X in PBT; store on ice until all are rinsed. then 2X washes; then 2X rinses in same.

13. Post-fix again in PBT+5% formaldehyde for 30' at R.T./hours at 4ï¿½C (go home for dinner)/overnight at 4ï¿½C.

14. Wash 5X in PBT.

15. Wash in 50/50 PBT/hybridization solution. Start temperature block for 55ï¿½C.

16. Wash 3X in hyb. solution.

17. Pre-hybridize /3hrs in hyb. sol. at 55ï¿½. I usually prehyb overnight.

18. Prepare probe: Have 20-30ml carcasses/tube. Separate carcasses to new tubes if necessary. Pre-dilute probe in hyb buffer:

a. Per tube o'carcasses, add1 ml stock probe to 60ml hyb buffer. Some probes are better with 2-3 ml stock probe. Always wear gloves when handling stock probe vials to protect them from RNAse.

b. Remove as much prehyb buffer from carcasses as possible. Add diluted probe. Knock the tube with your finger so that the carcasses get a bit stirred up; do this periodically as you hybridize for at least 18 hours at 55ï¿½C. 24hrs should be maximum, owing to slow dehydration of the buffer.

19. Remove hybridization solution containing probe (can re-use), rinse 1X in hyb. buffer, then let incubate in hyb. buffer for 1 hr. at 55ï¿½.

20. Change solution every 20' over the next two hours (6X changes). Use more washes if probed longer. Always incubate at the hybridization temp. of 55ï¿½.

21. Do the following washes, each for 15' at room temp.: 750/250 hyb. buff./PBT,

500/500 "

250/750 " volumes in ul.

22. Wash 5X in PBT.

23. Incubate in 500ml PBT+anti-digoxigenin conjugated to alkaline phosphatase. Final antibody dilution from the stock provided in the Boerrhinger-Mannheim kit should be two thousand fold.

a. Before use, antibody should have been pre absorbed against fixed embryos at a one in ten dilution. This is diluted, pre absorbed stock.

b. For working solution dilute diluted stock 200 fold more (2.5ul/500ulPBT).

c. Add 500ul/tube and incubate overnight at 4ï¿½C.

24. Remove antibody and wash 5X in PBT.

25. Rinse once in PBT. Transfer to 24 well plate. Can store O.N. at 4ï¿½C.

26. Rinse once in freshly prepared alkaline phosphatase staining buffer (prepare about 10ml/tube).

27. Wash 3X in staining buffer, while gently swirling on shaker table or on nutator.

28. Prepare staining solution: Per well, pre-mix 500ml staining buffer + 4.5ml NBT (vial 9) and 3.5ml x-phosphate solution (vial 10). Vortex well. Store in dark at R.T. until ready to use.

29. Drain last wash from carcasses, add 500 microliters of staining solution. Wrap the dish in foil and continue swirling in the dark for 20'. Prepare a glass pick from a Pasteur pipette to poke at carcasses later.

30. Look at them under dissecting scope against a white background. Nudge them around with the glass pick to see discs. They may be ready now or they may need several hours of staining in the dark, depending on enzyme activity, transcript abundance, etc.

31. Stop staining reaction with 2X washes of staining buffer, then washes in PBT.

32. To store before mounting, transfer to scinti vials, wash in 70% EtOH, then several washes in 100% EtOH. The stain will slowly diffuse in aqueous soln. Store at -20ï¿½C. Can store indefinitely in EtOH as long as it stays 100%.

GOOD LUCK!!

**TROUBLESHOOTING FOR LOUSY STAINS**

1. I assume all reagents are fresh. Check PH of everything. PH can change, even in buffers.

2. Leaving carcasses in PBS before fixing is bad. None for more than a few minutes.

3. Lousy signal? One solution is to make probe with 5-10X more DNA template than the protocol specifies.

4. High background with or without much signal? Maybe probe is way too concentrated. Try diluting a sample of the probe 1:5 in hyb buffer and use this diluted sample as if full strength. I is surprising but I found this to be a very effective solution even when the signal is very poor, but with high background. My stains with the Dl probe are now done this way with terrific results. Too much protease K is also a common culprit for high background. Fresh batches of p.k. can be especially active. Dilute 1:1 or 1:4 and shorten the digest time. Be sure to aggressively wash out enzyme, and stagger the tubes of carcasses so no tube is left in p.k. overtime.

5. We test new probes and troubleshoot on embryos. These are easier to test different parameters. We keep 50ml tubes of fixed embryos in EtOH in the freezer.

**ANAL RETENTIVE PROTOCOL FOR MOUNTING DISCS**

1. Hydrate carcasses to PBT. Prepare two very clean dissecting dishes with PBT. I scrub the dishes with PBT and a very clean cotton swab. Vacuum up this PBT and add fresh PBT. All PBT here is dispensed through 0.45um filter mounted on an old 50ml syringe. Clean your forceps in alcohol.

2. Put carcasses in dissecting dish with PBT under the microscope, and pick off the discs you want. Avoid touching the discs except at their bases because metal forceps can leave a residue.

3. Transfer discs to a new clean dish with PBT. Swirl discs to the center. Use an eyebrow hair or bent Minutien pin (Fine Science Tools) to sweep clean an area of the dish next to the pile of discs. Look for dust specks sticking to the discs, and one by one nudge the inspected discs into the clean area. It is helpful to look against a black background. Pick off specks with the eyebrow hair/pin. I collect eyebrow hairs with Scotch tape. Return discs to a clean ufuge tube.

4. Wash in filtered 70%, then 5X 5 min. washes in 100%. During these washes pipette them into a very clean (scrubbed) glass scinti vial that has been rinsed with 100% EtOH and now has a 2nd rinse of 100%. A dusty vial negates all the fussy work up to this point. Wash several X in EtOH. Wash1X in 50/50 EtOH and xylenes. Here and elsewhere you may have discs sticking to the glass, especially if significant dehydration occurs while in glass. Dehydrating in ufuge tubes first, and having scinti vials pre-wetted with EtOH before they see the discs minimizes this. If stuck, you can VERY carefully nudge them free with forceps.

5. In hood, wash 5X in xylenes. If they clump, flick them hard until they break apart. If they refuse to de-clump then you did not dehydrate them enough/(were coated with a residue (from polypropylene ufuge tubes?). See end of protocol for what to do for serious clumping. To proceed, set out very clean slides and large (22X40mm) coverslips. You will also need Permount, vacuum line with Pasteur pipette and yellow tip on the pipette, P1000 with cut off blue tip, Pasteur pipettes, cotton swaps, hard white surface (I use the removable center stage of the dissecting scope), aluminum foil. Turn on slide warmer. Turn on vacuum line near slide warmer.

6. My method for mounting is strange, but works well and seems to ensure that most discs come out wing anlage side up. If I dispense the discs onto a slide most of the wing discs come out wing anlage side down. If I do not pre-spread the Permount the discs flow out to the edges when a coverslip is added. My method reduces these problems. You should practice mounting without tissues if you are new to this stuff. Working on clean glass or aluminum foil helps to avoid dust and lint. I cover the linty bench paper in the hood with clean aluminum foil.

7. To prepare slide: Examine slide closely. Pick off dust specks with yellow tipped vacuum line. Add a small drop of Permount onto a slide and quickly spread into very thin layer over an area that matches the size of a coverslip. I use a Pasteur pipette to do this. Set to one side. With experience, you will learn how thin the Permount should be. Set aside in the foil area.

8. Pick up a coverslip, and clean up dust with the vacuum. Set onto white, level background. Pick up discs with blue tip with tip cut off. Let discs settle to bottom of xylenes in blue tip, then dispense onto the coverslip, being careful not to let the discs flow off. 'Herd' discs toward the center, but do not let any overlap. I use very clean forceps to do this. Siphon up excess xylenes with vacuum until a meniscus just surrounds each disc. DO NOT ALLOW THEM TO DRY. Immediately lay down the coated slide--Permount side down-- and flip over the slide with the coverslip now stuck on. Hold down one edge of the slip with a thumb, and use a cotton swab to roll out air bubbles. Remove yellow tip from vacuum line, and vacuum up Permount that oozes out from under the 'slip. Return yellow tip. (See how the vacuum line is used? I pick up excess Permount with the Pasteur pipette, but avoid smearing any of this onto a clean slide by covering this tip with a yellow tip when it is used to pick up dust.)

9. Label frosted area of the slide, using a pencil. It is wise not to label before the coverslip is added, since xylenes can easily wash over the label and carry lots of black graphite to your discs.

10. Dry on slide warmer under weights. To drop on weight first firmly pin 'slip down with two spread fingers, then gently lower the weight onto the slip between the fingers. This keeps the 'slip from spinning as it is crunched down by the weight. A vacuum line by the slide warmer is handy to quickly suck up Permount before it oozes out and gets under the weight.

11. The weight can be removed the next day if they are not stuck onto the slide. If stuck, DO NOT ATTEMPT TO REMOVE. Leave on warmer for about two weeks, then snap off the weight. Clean off excess Permount with a razor blade and a cotton swab soaked in xylenes.

SERIOUS CLUMPING PROBLEMS Could be from discs seeing polypropylene in EtOH and/or xylenes. Keeping all solvents in glass containers, EtOH dehydrated discs in glass scinti vials, are steps that will solve future clumping. For current batch: Gotta re-hydrate -->PBT, hand pick apart. Could then retry, or just mount under 50% glycerol and store in freezer.

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