Pupal RNA in situ hybridization.

Suggested timetables and stopping points:

This protocol assumes that you already have a labeled probe. If you do not, see the DIG labeling protocol. There are two stopping points. One is compulsory: the actual hybridization has to go overnight. The second stopping point is optional: fixed pupae can be stored for days or weeks in ethanol at –20C before hybridization.

The whole protocol can therefore be completed in either two or three days. You can either dissect and fix the pupae and set up hybridization overnight on Day 1, than finish the staining on Day 2. Or, you can dissect, fix, and dehydrate the pupae on Day 1, re-hydrate them and set up hybridization overnight on Day 2, and finish the staining on Day 3. It is better to follow the three-day schedule until you acquire enough experience with pupal dissections and can do it quickly.

Solutions.

Assemble all solutions before you start! Remember that DEPC treatment has to go overnight.

PBS
140 mM NaCl
7 mM Na_2HPO_4
3 mM KH_2PO_4
Prepare a 10x stock, which is good for months, and dilute it as needed.

DEPBT
Treat PBS with 0.1% DEPC overnight with a magnetic mixer, autoclave, and add 0.1% filter-sterilized Tween 20. You will need to make fresh DEPBT every couple of months.

Hybridization Buffer
5X SSC or SSPE
50 µg/ml heparin
0.1% Tween 20
(100 µg/ml **denatured** Salmon Sperm DNA)
50% formamide (deionized).
Formamide needs to be deionized before use by incubating it with an ion-exchange resin overnight.
To make 20x SSC, dissolve 175.3g of NaCl and 88.2g of sodium citrate in 800ml of distilled H2O, adjust the pH to 7.0 with a few drops of 1M HCl, adjust the volume to 1L with additional distilled H2O, and sterilize by autoclaving.
Make the stock solution without ssDNA, and add ssDNA to small aliquots as needed. To denature ssDNA, boil it in a water bath (or heat it at 99C in a PCR machine, which is easier) for 10 minutes, then cool **very quickly** on ice. If the hybridization buffer is ever cooled, the ssDNA should be considered to have been re-natured, and you need to add fresh one.

Staining Buffer
100 mM NaCl
50 mM $MgCl_2$
100 mM Tris pH 9.5
0.1% Tween 20
1 mg/ml levamisole
Make the buffer without levamisole, and a 10x levamisole stock (store at –20C). Add levamisole immediately before use.

PMG
0.1 M PIPES
2 mM EGTA
1 mM $MgSO_4$
adjust pH to 6.9 by NaOH addition (PIPES will not dissolve until pH is increased).

Preabsorbed anti-DIG, AP-conjugated antibody.
The antibody should be prepared ahead of time by diluting 1:200 in DEPBT and preabsorbing for 2 hours against 100 µl packed volume of fixed tissue (e.g. fixed inverted larvae. Preabsorbed antibody appears to be stable for several weeks at 4 C, but needs to be spun down hard before use. Dilute the preabsorbed antibody an additional 10 fold for the incubation.

Staining solution
4.5 µl NBT and 3.5 µl x-phosphate (aka BCIP) per 1 ml of staining buffer

You will also need:
16% EM-grade ultrapure formaldehyde (from Polysciences)
Morphological, non-fluorescing dye (e.g. methylene blue or toluidine blue).
10µg/ml Proteinase K in DEPBT. Prepare a single batch of Proteinase K solution, aliquot it, and store it at –20C. Label each tube with the date or a batch ID – you will need to know each batch intimately.
2 mg/ml glycine in DEPBT.
Capillary tips.
Vacuum aspirator.
Hybridization oven.
Nutator or rocking platform.
Boiling water bath or PCR machine to denature the probe and ssDNA.

Pupal dissection and fixation.

Prepare the following materials:
A bucket of ice
1x PBS buffer + 0.2% Tween-20 or NP-40
16% EM-grade ultrapure formaldehyde
Methylene blue or toluidine blue solution.
Extra-sticky packing tape.
Plastic slabs.
Depression glasses.

Two pairs of extra-fine dissecting forceps.
Dull forceps.
Microsurgical scissors.
Highest quality shaving razor blades.
Fly cultures at the **right stage**.

Dissection forceps and microsurgical scissors are fine instruments that are easily damaged. Avoid mechanical damage by keeping them in their protective sheaths when not in use, and clean them often and carefully to avoid rust. Ask Artyom how to care for these instruments.

1. **Several days before you plan to dissect**, prepare the fly cultures by sticking a Kimwipe into a bottle where mature third-instar larvae are about to pupate in large numbers. Depending on the age of the pupae you want, this needs to be done 1-4 days in advance. It helps to keep plenty of vigorous bottle cultures, staggered by 3-4 days, on hand.

2. Pull the Kimwipe out of the bottle and place it on a paper towel under the dissecting microscope. Select the pupae at the right developmental stage (staging pupae is a separate issue). Only use dull (i.e. cheap) forceps for this crude work. Fine dissecting forceps are to be treasured and protected. Attach a piece of wide extra-sticky packing tape to a plastic slab, sticky side up. Line the pupae up in a neat row, top to bottom, on the sticky tape. Having a neat vertical row of pupae makes it much easier to cut them in the next step! If you need the dorsal and/or ventral halves of the body wall, line up the pupae on their sides. If you need the lateral halves, put the pupae dorsal side up. After you have lined up, press on them **very** gently with your finger to make them stick to the tape.

3. Cut the pupae in half (longitudinally) with a razor blade. <u>This is a very critical step</u>! "Industrial" razor blades are **not** sharp enough. Use a real blade (made for shaving), and get the best one you can afford. If the blades are sold oiled, clean them well with 95% ethanol before use. Since RNA degrades rapidly, cut only 10-15 pupae at a time (at most) before placing them on ice. Razors dull quickly; each corner is good for only 15-20 pupae. Cut the pupae **very gently** with a back-and-forth, sawing motion. Apply as little downward pressure as possible. After a good cut, the pupa should still look almost intact. If its contents spills out, you must have pressed too hard, and this pupa is now completely ruined – do not use it.

4. Remove the pupal halves from the pupal cases by grabbing them with sharp forceps by their heads, and put them in an Eppendorf tube containing 1 ml of DEPBT. RNA and proteins are degraded fast. Do not cut more than 10-15 pupae at a time before placing them in buffer. Keep the tube in a bucket of ice.

5. Once you have accumulated enough pupae (20-30, or 40-50 if you can do this fast), close the tube and rock it **gently** by hand for 30-60 seconds to remove most of the gut and fat body. <u>This is another highly critical step</u>. Use **very smooth** motions. If you rock the tube too hard, you will destroy the epidermis, which is only loosely attached to the pupal membrane. If you do not rock it hard enough, you will fail to wash out the fat body,

which will harden during fixation and become impossible to remove. You will need to develop a good feel for this step. Monitor your progress by looking at the tube against a bright light. The body walls should become somewhat translucent, but not completely transparent.

6. Let the pupal body walls settle to the bottom of the tube. This takes time, and the pupae are almost transparent at this point. Be careful not to lose them!

7. Remove most of the buffer with a vacuum aspirator. Be very careful not to suck in any of the pupae! Remove the remaining buffer with a pipette with a long capillary tip. Add 750 mcl of DEPBT, 250 mcl of 16% formaldehyde (to a final concentration of 4%), and a drop of methylene blue. Fix for 20 minutes on a Nutator, preferably at 4C.

8. Remove the buffer as above, and rinse the pupae in 1 ml of DEPBT. Dump them out into a **clean** depression glass. Under the microscope, identify the best-looking pupae (this will be 30-50% of them if you are lucky), and finish dissecting them with the extra-sharp forceps. The pupae will now be blue and easy to see. Remove the trachea, flight muscles, the dorsal heart, and as much of the fat body as you can. Be careful not to damage the epidermis, which is **extremely fragile**. Place the pupae in a tube with 1 ml of DEPBT on ice. Do this quickly – this step is not entirely RNase-free!

9. Add 16% formaldehyde to a final concentration of 4%, and fix again for 20 minutes on a Nutator. Remove the fixative and wash in DEPBT **3 times for 10 minutes each**.

At this step, you have two choices:

10A. Continue with the hybridization procedure.
10B. Dehydrate and store the pupae for future use.
To dehydrate, wash once for 10' with 70% ethanol / 30% DEPBT, wash once for 10' with 100% ethanol, replace the 100% ethanol, and store at –20C.
The ethanol will wash out methylene blue, and the pupal body walls will be almost completely transparent and **very hard to see**. They also settle down to the bottom of the tube very slowly, especially in 70% ethanol. Be very careful not to lose the pupae!!! Use capillary tips, and wait for the pupae to settle. Take your time.

Hybridization – Day 1.

0. If necessary, rehydrate the pupae by washing them once for 10' in 70% ethanol / 30% DEPBT, and 3 times for 10' each in DEPBT. If you did not dehydrate the pupae, proceed immediately to step 1.

1. Digest the pupae ~2-3 minutes in 10µg/ml Proteinase K. **This is a very critical step!** If the treatment time is too short, you'll get high background. If it is too long, morphology will deteriorate beyond recognition. Remember that the epidermis is very fragile – you do not want to destroy it. 2-3 minutes work OK as starting conditions, but in practice you will need to calibrate each batch of Proteinase K solution and adjust digestion times based on your experience. Prepare a single batch of Proteinase K solution, aliquot it, and store it at –20C. Calibration will need to be repeated for every new batch that you make.

2. Stop the reaction by washing the pupae in 2 mg/ml glycine in DEPBT for 2'.
3. Rinse once in DEPBT, then wash 3 times for 5' each in DEPBT.
4. Post-fix the pupae for 30' in 4% formaldehyde (EM grade) in DEPBT. This step kills the Proteinase K and restores tissue integrity (a little…).
5. Rinse twice in DEPBT, then wash 3 times for 5' each in DEPBT and rinse once more in DEPBT.
6. Wash 10' in 50% DEPBT / 50% hybridization solution (without probe or ssDNA).
7. Wash 10' in hybridization solution (without probe or ssDNA) at room temperature.
8. Pre-hybridize for 1-3 hours in hybridization solution with ssDNA, but still without the probe, at 55 C*
9. In the meantime, add 1-3 mcl of probe to 50 mcl of hybridization solution containing denatured ssDNA. Heat this cocktail to 70C for 5 min and then put **very quickly** on ice. The probe is now denatured – **do not allow it to renature!** Keep it on ice until ready to use.
10. Remove the pre-hybridization solution, and add 500 µl of fresh hybridization solution with **denatured** salmon sperm DNA and the freshly **denatured** probe cocktail. Hybridize overnight (10 hours or more) at 55 C* with rocking

*55C is a good starting point, but hybridization temperature may need to be adjusted experimentally for each probe. In general, use the lowest temperature you can get away with for the pupal epidermis, and rock the pupae as gently as possible. It is a very fragile tissue, and harsh hybridization conditions destroy it. To mix the hybridization reaction, rotate the tube around the **long axis**. Do not invert the tube.

Washing and detection –Day 2.

(This is a very long day – you won't be home for some time. There are no stopping points here!).

1. Remove the hybridization solution and rinse 1X in 1 ml of hybridization solution without ssDNA. (all subsequent washes are in 1 ml and without ssDNA).
2. Wash 5X 30 min each in hyb at 55C with very gentle mixing (see above).
3. Wash 20 min in 8/2 hyb/DEPBT at room temp (all subsequent steps at room temp with gentle rocking)
4. Wash 20 min in 6/4 hyb/DEPBT
5. Wash 20 min in 4/6 hyb/DEPBT
6. Wash 20 min in 2/8 hyb/DEPBT
7. Wash 20 min in DEPBT
8. Post-fix for 30 minutes in 4% EM-grade formaldehyde.
9. Rinse twice in DEPBT, and wash 3x for 10 min each in DEPBT.
10. Incubate for 1.5-2 hours at room temp with gentle rocking in 300-500 µl DEPBT with **preabsorbed** Alkaline Phosphatase anti-DIG antibody at 1:2000 final dilution.
    The antibody should be prepared ahead of time by diluting 1:200 in DEPBT and preabsorbing for 2 hours against 100 µl packed volume of fixed tissue (e.g. fixed inverted larvae. Preabsorbed antibody appears to be stable for several weeks at 4 C, but needs to be spun down hard before use. Dilute the preabsorbed antibody an additional 10 fold for the incubation.
11. Rinse once in DEPBT, and wash 4X 20 min each in DEPBT (1 ml)

12. Rinse in staining buffer. The staining buffer does not have any detergent in it (detergents inhibit the AP reaction), so the pupae will start sticking to the tube walls at this point).
13. Wash 2X 5 min each in staining buffer
14. Place the sample in a depression glass and add 1 ml staining solution (staining buffer + NBT and BCIP). Incubate **in the dark** for 20 minutes - 2 hours, monitoring periodically under dissecting scope until the staining pattern is clear. Developing times may vary widely across genes. For best results, replace the staining solution with fresh one every 30 minutes or so.

Once the staining is done to your satisfaction, pick the best pupae with forceps and place them in a tube with DEPBT.

15. Rinse once in DEPBT, and wash 5x 5 min in DEPBT.
16. Wash once for 5 minutes in 70% ethanol / 30% DEPBT.
17. Rinse in 100% ethanol, and wash twice for 10 minutes each in 100% ethanol.

At this point, you have two choices.
18A. Store the pupae in fresh 100% ethanol at –20C for future mounting.
18B. Rehydrate and mount immediately (it's getting pretty late by now).

Mounting.

1. Wash once for 5' in 70% ethanol / 30% DEPBT.
2. Wash twice for 5' each in DEPBT.
3. Dump the pupae out in a depression glass. Take the pupae one at a time, and put them on a regular glass slide containing a drop of 80% glycerol / 20% DEPBT. Use the microsurgical scissors to cut out the best-looking piece of epidermis. This piece should have little curvature to avoid creasing when you mount it. The sides and the first and last abdominal segments will need to be cut off with the scissors.

The microsurgical scissors are very easily damaged. Do not touch any hard objects with the tips or edges. Prevent rust by keeping them as clean as possible and dehydrating them in 100% propanol. Ask Artyom how to care for the scissors!

4. Remove the unneeded pieces of the pupa from the slide, add a little glycerol/DEPBT if needed, and mount the selected piece of epidermis under a cover slip.

5. Photograph the pupa within a few hours of mounting. AP-stained specimens accumulate background very fast. The mounted slides can be stored at 4C in the dark overnight, but for best results take you pictures immediately after mounting.