Modified 7/27/08

Tissue Preparation (Abdomen)

It is very important that the tissue used for hybridization is treated gently. Don't bend or stretch samples more than needed.

1.) Heat pupae for 40 seconds @ 100° C in 100 µl PBS. This relaxes the muscles, helping to maintain the morphology during fixation. Don't heat longer than 40- 60 seconds, as the fat bodies and other tissues start to melt: it will be harder to clean out the abdomen. Place tube immediately on ice.
2.) If working with eclosed flies, remove PBS, and bring up in 100% ethanol to remove hydrophobic waxes…this makes the dissection step easier. Let the flies sit in ethanol (agitate a couple of times) for ~1 minute.
3.) Remove the ethanol and bring up in cold PBS. Place the tube on ice until you dissect (I like to dissect and fix before ~30 minutes).
4.) Dissect in a glass dish of cold PBS. Make a cut down the ventral midline. Clean the specimen: Gently wash away fat bodies by blowing with a P-200. Remove reproductive organs, trachea, etc using forceps and scissors.
5.) Add to a tube of PBT + 4% paraformaldehyde fix on ice
6.) When a batch of pupae has been dissected, fix at room temperature 30 minutes
7.) Wash 2X in MeOH
8.) Wash 2X in EtOH
9.) Store in EtOH at -20C indefinitely

in situ day 1

Sometimes abdomens collect bubbles, and float to the top of the tube. The bubbles can be teased out with forceps in a staining dish.

Wash = 5 min for organic solution, 10 min for aqueous solution.

1.) take carcasses from the –20C freezer (all should be in 100% EtOH

2.) wash 3X in 100% EtOH ○○○

3.) incubate in 50:50 xylenes:EtOH for 30 min in hood ○

4.) wash 5X in 100% EtOH ○○○○○

5.) wash 2X in MeOH ○○

6.) Wash 3X in PBT ○○○

7.) fix 30 min in PBT + 4% paraformaldehyde ○

8.) wash 5X in PBT ○○○○○

9.) incubate 8-10 in PBT + Proteinase K ○
Final [] 1:25,000 – 10mg/ml Stock -> 1:100 -> 6 µl in 1.5 ml PBT

10.) Rinse 2X in PBT; store all on ice until all have been rinsed, then wash 2X for 10 min in PBT ○○○○

11.) Post-fix 30 min in PBT + 4% paraformaldehyde ○

12.) Wash 5X in PBT ○○○○○

13.) Wash in 50:50 PBT/Hyb for 10 min ○

14.) Wash 3X in Hyb at Room temp ○○○

15.) Prehybridize for 1 hr in Hyb solution @ 65C ○

16.) While hybridizing, prepare probes; dilute probe in Hyb solution ○

17.) Setup 6-10 carcasses (the more the merrier) in each tube for each probe. remove all old hyb with p200 pipetperson ○

18.) Add probe to each tube. Flick tubes to mix. Incubate 18 – 24 hours @ 65C. mix a couple times during incubation ○

in situ day 2

1.) Rinse once in Hyb solution pre-warmed to 65 C. ○

2.) Incubate in Hyb solution (pre-warmed) 1 hr at 65 C. ○

3.) Incubate 3X for 30 min in Hyb solution (pre-warmed) at 65 C. ○○○

4.) Wash 2X for 15 min in 50/50 PBT/Hyb solution. ○○

5.) Wash 5X for 10 min each in PBT. ○○○○○

6.) Remove all solution, add 0.3 mL antibody. Incubate overnight at 4 C. ○
Antibody: Roche anti-DIG AP Fab fragments. 1:6000 in PBT

in situ day 3

1.) Wash 5X for 10 min in PBT. ○○○○○

2.) Wash 3X for 10 min in Staining Buffer. ○○○

3.) Remove carcasses from tubes into glass viewing dishes. ○

4.) Remove liquid, add 0.4 mL Staining Solution. ○

5.) Stain in the dark, watching the pattern periodically develop (20 min to 4+ hrs, depending on probe). ○

6.) To stop staining, wash 2X for 10 min in Staining Buffer. ○○

7.) Wash 2X in PBT. ○○

8.) Add 0.5 mL Glycerol Mountant. ○

9.) Incubate overnight at 4 C. ○

10.) For Abomens, I mount samples on slides that are supported by 2-3 layers of double sticky tape. Cut a hole in the stack of tape, and place the tape on a new slide. After adding mount to the center and placing samples, add a cover slip. This method keeps the abdomen from getting crushed by the coverslip, and allows the sample to be preserved indefinitely.

PBT

1X PBS
0.1% Triton-X

PBT + 4% Paraformaldehyde

4 mL 10X PBS
10 mL 16% paraformaldehyde (pre-mixed ampules from E.M.S.)
400 µL 10% Triton-X

PBT + Proteinase K (for 15 mL)

1.5 mL 10X PBS
6 µL 10 mg/mL stock Proteinase K
150 µL 10% Triton-X

Glycerol Mountant

0.1 M Tris Hcl pH 8.0
80% glycerol

Hybridization (hyb) Solution	**STOCK**	**Amt/200mL**
50% Formamide		100mL
5X SSC	20X	50mL
pH to 5.5 (for 65C Hyb)		
Filter Sterilize		
100 ug/mL SSp DNA	10mg/mL	2mL
100 ug/mL Heparin		.02g
0.1% Tween		200µl

Staining Buffer	**STOCK**	**Amt/50mL**
100 mM NaCl	5M	1mL
50 mM $MgCl_2$	1M	2.5mL
100 mM Tris HCl pH 9.5	2M	2.5mL
0.1% Tween-20		50µl

Staining Solution (per 1.5 mL)

1.5 mL Staining Buffer
10.2 mL NBT (promega)
5.25 mL BCIP (promega)

Probes

I find that raw PCR products make the best template. If I have a pGem or Topo clone, I determine the directionality, and amplify my template using vector-specific primers, so as to include the right promoter (T7, T3, SP6). If you get a single, robust band, the raw product will work great for *in vitro* transcription to make a dig labeled probe. I use the 10X Dig mix from Roche, and the polymerases from promega. When the in vitro transcription is finished, I ethanol precipitate (using linear acrylamide as a carrier), and resuspend the probe in hyb solution.