Alice, Michelle and Audrey's adult internal structure *in situ* hybridization protocol (5/22/03)

Based on Audrey's embryo *in situ* hybridization with modifications from Alice and Michelle.
Audrey's note: RNA probes seem more sensitive but are less stabile and harder to make.

Make probe using favorite method. This probe should incorporate digoxigenin (or comparable nucleotide analog with changes in detection protocol where appropriate) and it should be digested to produce ~75-200 bp (Michelle and Alice use 200bp) fragments in some manner such as this method:

MA/AF probe: This takes about 2 days

To make antisense and sense probes, cDNAs were linearized using restriction enzymes with sites near the 5' or the 3' end, respectively. The restriction enzymes were separated from the linearized template using a Qiaquick column, the eluted DNA was lyophilized and then was resuspended in DEPC-treated water (about 10ul).

To make DIG-labeled probes for in situ hybridization analyses, transcription reactions were performed using linearized cDNAs, Roche 10X DIG RNA Labeling Mix, Ambion RNA polymerase enzymes and buffers following the Ambion Maxi-script product protocol (including the DNase and EDTA steps). The reactions are 20 ul total volume. Before hydrolysis step, add 30 ul DEPC water and remove 3ul to assay on a gel. We found that to check the RNA reactions, adding 3 ul 2X RNA loading buffer (ambion kit has this, or look in Maniatias) to RNA, and running the RNA on a "regular" 1% agarose gel was fine and that it was not necessary to run a formaldehyde gel. Remember that if you use a DNA ladder on this gel, it is not appropriate as a size marker as it is double-stranded and runs differently than RNA. Can estimate size of probe based on DNA template size.

The RNA probes were then hydrolyzed to ~200 base pair fragments according to Boeheringer Mannheim protocol:

add 30ul 200mM Na_2CO_3 and 20ul 200mM $NaHCO_3$ to 50 ul of probe reaction.

time in minutes at 60°C= Lo-Lf/ (K)(Lo) (Lf)
Lo= length original
Lf= length final
K= rate constant .11 kb/minute

immediately add
5ul 10% acetic acid
11 ul 3M sodium acetate ph 6 (Rnase Free)
1 ul 10mg/ml tRNA stock (R-7125 Sigma)
1.2 ul 1M $MgCl_2$
300ul cold ethanol (2.5 volumes)

overnight at –20°C, spin at least 20 minutes at room temp. remove ethanol, do not overdry or pellet will be very difficult to resuspend. We resuspend in hybe buffer (see below) and store at –20°C.

Probes are quantitated after hydrolysis step using a spot assay compared to a control DIG labeled RNA that is purchased from Roche. We followed the manufactures instructions and found assaying a 1:10, 1:100, and 1:1000 dilution of probe gave an appropriate range for this assay. For the spot assay a kit of buffers is recommended by the manufacturer (Roche) which we bought. Contains wash buffer, block, development solution etc.

Dissect and **Fix adult tissue**

Day one

1. Collect flies under anesthesia.
2. Dissect in spot dish in PBS (or other buffer such as Grace's medium).

    For internal genitalia: use a small amount of buffer in dish, 2 pair of forceps. Roll fly onto back with forceps. Pinch with both pairs of forceps (oriented anterior and posterior) on the ventral abdomen just anterior to genitalia -- rip open a small hole about the size of the external genitalia. Continue holding on with posterior forceps and hold down fly/abdomen with anterior pair. Pull gently to remove internal genitalia and connecting gonads. Move remaining carcass aside. With higher magnification, the last few tergites and sternites can be removed as can the trachea, gonads (if desired) and as much of the gut as appropriate. Be careful when removing gut in male; the ejaculatory duct wraps around the rectum.

    For Dissecting CNS tissue make sure and remove all the membrane that surrounds the tissue as the NBT/BCIP reaction has a tendency to stick non-specifically. Also, this procedure is performed in a spot dish and not eppendorf tubes, as you will lose your CNS tissue.

3. Transfer dissected tissue to eppendorf tube with fix and incubate with occasional movement O/N at 4°C:

(Michelle and Alice: 100 ul 10X PBS
200 ul 20% paraformaldehdye (purchase EM grade parafomaldehdye that is a premade 20% solution; Electron Microscopy Sciences phone is 215-646-1566, Cat# 15713)
700 ul depc water)

Standard paraformaldehyde fix:

0.8 ml [0.1M Hepes, pH 6.9/2 mM MgSO4/1mM EGTA]
0.2 ml 20% paraformaldehyde/PBS

PBS can be substitued for the buffer; other fixes may work as well. We haven't tried this one which is good for multiple tissue layers in embryos:

DMSO fix (from Rob Ray):

0.4 ml DMSO

0.2 ml 20% paraformaldehyde (A. Vincent says it'll work better if its freshly made)
0.3 ml H2O
0.1 ml 10X PBS

***in situ* protocol** (from C. Rauskolb)
Day 2
1. Wash tissue 3x5' in PBS/0.1% Tween-20 (a.k.a., PBT, PT, PTw... I'll call it PT; other detergents may work, too).

2*. Incubate in PT + 50ug/ml Proteinase K for 4'15" (RNA probe) or 4' for DNA probe. *May not be necessary for single tissue layers (A. Vincent) and it does worsen morphology. Alice compared a prd probe with and without the ProK and found the in situs cleaner and clearer with the ProK. If you omit this step, go directly to step 7. (2.5 ul of 10mg/ml solution in 500 ul of PT + embryos)

MA and AF do proteinase K step for CNS tissue and internal genitalia (proteinase K Gibco-BRL 25530-049)

3. Rinse 2X in 2mg/ml glycine/PT (make fresh).
4. Rinse 2X 5' in PT.
5. Refix in 4% PP for 25' (RNA probe) or 20' (DNA probe).
6. Rinse 5X 5' in PT.
7. Rinse 10' in PT:Hyb wash (1:1).

Hyb wash: **50% formamide (molecular biology grade or high grade)**

5X SSC

100ug/ml salmon sperm DNA (we purchased phenol extracted ss DNA from sigma, not RNAse free, but worked fine since in formamide solution; D-7656 Sigma)

50ug/ml heparin (H-3393 Sigma. make in 4X SSC/DEPC treated, final concentration 50mg/ml and store at 4°C)

0.1% Tween-20

according to one protocol, hybe wash can be stored long term at 4°C if ss sperm DNA has not been added. ss sperm can be added fresh to small aliquots of the hybe wash and is what we routinely do. We do not add ss DNA to hybe wash solutions on day three as these are wash steps.

10. Rinse 10' in Hyb wash.

(note-this is a good time to separate tissue into tubes for different probe conditions. This takes time and so it is best done before the samples are equilibrated at the higher temperature).

11. Prehybridize 1+hr in Hyb wash:
    45°C for DNA probe
    55°C for RNA probe
12. Hybridize O/N with probe at same temperature.
    DNA probe will require **Boiling** to separate the strands:
    Boil 10ul for 10'
    Quick chill on dry ice/EtOH
    Add 40ul cooled hyb solution.
    Mix and pulse down in centrifuge.
    Add to tissue after removing as much of the prehyb wash as possible.

use 0.5 ul RNA probe or 10 ul DNA probe in 50ul Hyb wash

(Alice and Michelle)
RNA probes are diluted into hybe solution (~2.5 ng/ ul; probes are quantitated using membrane spot assay compared to a known control) heated at 80°C for 5-10 minutes, and then chilled on ice. Probes are added to sample and then incubated overnight as described above.

May wish to parafilm tops to maintain volume; A. Vincent suggests immersing the tubes below the surface of the waterbath to prevent condensation on the lid and altering the concentration of solution in the reaction mixture -- easily done with a little wet paper towel.

Day 3

13. Remove probe solution (save DNA probe solution to reuse 2 more times).
    Wash at hybridization temperature:
        2-3X for total 2 hrs in Hyb wash
        20' in PT:Hyb wash (1:4)
        20' in " (2:3)
    Wash at RT:
        20' in " (3:2)
        20' in " (4:1)
        2X 20' in PT
14. Incubate with anti-digoxygenin Ab (Fab fragment) conjugated to AP for 2 hr at RT or O/N at 4°C; use 2.5ul of 1:10 dil anti-DIG in 500ul PT.

Purchase antibody from Roche (Sheep anti-Dig 1-093-247 150 U and 200 ul)

15. Rinse 4X 20' in PT.
16. Rinse 3X 5' in AP staining buffer:
    100mM NaCl
    100mM Tris, pH 9.5

50mM Mg Cl2
0.1% Tween-20

17. Stain with BCIP/NBT in AP staining buffer while wrapped in foil.
for 1 ml: 3.5ul BCIP, 6.75ul NBT (Promega)

USE: 3.5 ul/ml of a 50 mg/ml BCIP and 4.5 ul/ml of 75 mg/ml NBT (MA and AF use Gibco-Brl 18280-016)

Check periodically to determine the extent of staining.
If staining is slow, increase temperature to 37°C.
If staining is strong, change buffer and BCIP/NBT when substrate used.
If staining is too strong/quick, try slowing reaction on ice or by reducing substrate.

(note: staining comes up really fast and so it is best to perform the reaction one probe at a time)

When CNS tissue is equilibrated in development solution it has a tendency to stick to glass and ceramic. Do not pull it off as it will tear the tissue. Once the tissue is reequilabrated in PT it does not stick to the surface.

18. When done staining, rinse in PT 2X.

19. Pass tissue through an ethanol series, e.g., 30%, 50%, 70%, 95%, 100% X2 5' each (can skip a little but not too much). Rinse for 1 hour in 95-100% EtOH to perform the color change. Tissue can be stored for a short period in 50-70% EtOH (we found that a couple days was fine). Mount in preferred medium.

70-90% Glycerol
rehydrate to PT (70%, 50%, 30%, PT 2Xs, DAPI, PT 2Xs- 5 minutes each step, DAPI optional)
add 50% glycerol and let mix and settle
remove most of mix
add a bit more glycerol
take up tissue in pipetman tip and mount in slide
Store at 4°C

(note: for blue Alk phos reaction, Alice and Michelle preferred glycerol mounting. We stained with DAPI after the ethanol color change reaction, by incubating in 1X dapi/PT solution-see above)

Permount
dehydrate
rinse 2X in Xylenes
place on slide
let xylenes almost evaporate
add permount and cover

Araldite (hazardous -- neurotoxin; bake all trash until solid)
dehydrate
rinse 2X in acetone
sit several hours or O/N in acetone:araldite mixture
let tissue settle
transfer to slide and orient if possible
incubate O/N in 60° oven or slide warmer
add araldite and coverslip (beware of dripping)
return to oven until solid