Chick *In Situ* Hybridization

0. You want antisense mRNA (and sense, as a control, unless you already know what pattern to expect).

1. Get your gene in a vector with transcriptional promoters (T3, T7, or SP6). Make template: linearize it by cutting with an enzyme which cuts near the 5' end. Make sure the enzyme doesn't cut within the gene! Also avoid any enzyme that leaves a 3' overhang (including Pvu I, Pst I, and Kpn I). After digestion, phenol extract and precipitate the DNA - it must be clean before you transcribe it.
2. Make probe: do the transcription reaction with enzyme which goes from the 3' direction (= antisense). Resuspend the probe in 1 ml of hybe solution and leave them in the -80 °C. You can reuse them a few times.
3. For a single-probe run, do like the *in situ* directions that follow. For 3-color fluorescent detection, see 3-color fluorescent in situs. For a 2-probe run, you need to:
   a) make 2 probes - the first one being dioxygenin (DIG)-, the second being flourescein-labelled, UTP. Precipitate the fluorescein probe twice (do **not** put it on dry ice - only -20 °C, after you get a pellet the first time, resuspend in TES, add lithium, ethanol, glycogen, and precipitate again) and wash it a few times with 70% EtOH.
   b) put in both probes, and hybridize them together. Save the strongest probe for last. Go through the procedure, and detect with anti-DIG phos, etc.
   c) Use DEPC water throughout - even in TBST etc
   d) On day 4, after the color reaction is complete (and try to make color reaction as brief as possible since it degrades probe:mRNA hybrids), do the following.
   e) wash it twice in PBST, 30 min. in 4% para (no gluteraldehyde!), wash in TBST
   f) 30 min. at 65 °C (in TBST)
   g) start with the blocking (2.5 hr) step of day 2, using the anti-flourescein Alk-Phos conjugated antibody
   h) Detect the second probe with either "magenta-Phos" instead of x-phos and no NBT (made up as in standard protocol), or 350 λ magenta phos instead

of x-phos + 450 λ Tetrazolium red (made up as if it were NBT in 70% dimethyl formamide) in 150 ml NTMT

i) The detection takes longer (at least overnight at room-temp) and you may have to replace the substrate since it poops out.

The procedure: (4 days; do everything in max. volume of scintillation vials).

Preparation: (need PBS, PBT, MetOH series in PBT)

1) Dissect embryos into PBS as needed, and fix in 4% paraformaldehyde overnight or a couple of nights for late embryos, an hour or two for early ones. You can apparently also fix in 4% formaldehyde in 1X PBS.
2) Wash in PBT, 2 times (once, for early embryos) for 10 min. at 4 °C with gentle rocking.
3) Wash for 10 min. with rocking at room temp. in 25%, 50%, 75% MetOH:PBT series.
4) Wash 2 times in 100% MetOH, 5 min. rocking, room temp.; these can be stored for a couple of months at -20 °C.

Day 1: (need PBT, MetOH series, RIPA buffer, probe, hybe solution)

1. Rehydrate samples in 75%, 50%, 25% MetOH:PBT series. Wash 2 times in PBT for 5 min. at room temp.
2. Do two 45 min. washes in RIPA buffer (for embryos younger than st. 9), or one 45 min. wash in RIPA buffer followed by 1 μg/ml Proteinase K in PBT for 15 min. at room temp., followed by 2 mg/ml glycine in PBT for 10 min. at room temp. (make fresh and filter) (for any < st. 21 embryos). For older embryos, can bleach with 6% hydrogen peroxide in PBT for 1 hour, rocking at room temp. (1 ml 30% hydrogen peroxide + 4 ml PBT) first.
3. Postfix with 4% para + 0.2% gluteraldehyde in PBT for 20 min. at room temp. (= 800 λ of 25% glut in 100 ml Para in PBT). Warm up the hybe oven to 68-70 °C (look at the analog thermometer inside, to be sure of the right temp.).
4. Wash twice with PBT for 5 min. at room temp.

5. Replace PBT with prehybe solution, and swirl gently. Be careful as embryos will float to the top at first. Prehybe at 70 °C for at least1 hour. You can store them at -20 °C in prehybe, before or after heating.
6. Remove most of the prehybe, leaving about 1/3 of an inch of liquid from the bottom (make sure embryos won't dry out when the scint vials tips in rocker, but minimal volume other than that). Add about 500 λ of the probe reaction (after it's resuspended in 1 ml of prehybe) to each scint vial.
7. Hybe overnight at 70 °C with very slow shaking or rotation.

Day 2: (need solution I, solution III, TBST) - antibody incubation

If you have problems with background, use RNAse, or a higher wash temp. on this day).

1. Pre-warm solution I to 70 °C. Wash 3 times in solution I for 30 min. each (early embryos can go twice for 45 min.), at 70 °C, rocking.
2. If you want to use RNAse, do it here (I never use this with chick embryos):

   Wash with pre-warmed solution I and solution II mix (1:1) for 10 minutes at 70 °C.

   Wash with solution II twice for 5 minutes at room temp.

   Wash with 100 μg/ml RNAse in solution II twice for 30 minutes at 37 °C.

   Wash with solution II for 5 minutes at room temp.
3. Wash 3 times with solution III for 30 min. at 65 °C (early embryos can go twice for 45 min.) (to increase stringency, make this 70 or more °C).
4. Wash with fresh TBST 3 times for 5 min., at room temp., rocking (2-layer embryos can go twice for 10 min.).
5. Add 10% sheep serum (heat-inactivated is best, but use it straight if you absolutely have to) in TBST for 2.5 hours at room temp., rocking (2-layer embryos can go 1 hour).
6. Meanwhile, make up the AB mix: into a 50 ml falcon tube (or glass bottle), for each vial of embryos you'll have, add: 4 ml of 1% sheep serum in TBST, 10 λ sheep serum, 2 λ anti-dig antibody. Swirl to mix well.
7. Remove blocking solution from embryos, add 4 ml AB mix. Rock (vertically)

gently overnight at 4 °C.

Day 3: (need TBST) - wash off excess antibody:

1. Wash 3 times for 5 min. in TBST, rocking at room temp. (2-layer embryos can go twice for 10 min.)
2. Wash 4 times 1-1.5 hours in TBST, rocking at room temp. (2-layer embryos can go for three one-hour washes).
3. Wash overnight (up to 3 nights) in TBST rocking at 4°C (2-layer embryos don't really need this, and if you are impatient, you can do the detection after another 1-hour wash).

Day 4: (need NTMT, pH 5.5 PBT) - detection:

1. Wash 2 times in NTMT for 10 min. rocking at room temp.
2. Make reaction mix:

   74 mg NBT in 1 ml 70% dimethyl formamide (doesn't have to be de-ionized)

   50 mg BCIP (= x-fos) in 1 ml 100% dimethyl formamide

   reaction mix = 675 λ NBT/DMF + 525 λ BCIP/DMF + 200 ml NTMT
3. Remove NTMT, add max. volume of reaction mix to each vial.
4. Cover tubes with aluminum foil and place on rocker at room temp. Check, and then monitor about once per hour (not too often, since light will bring up the background) for progress. Some may need to go overnight.
5. When complete, wash twice in pH 5.5 PBT for 10 min. at room temp. rocking
6. Postfix with 4% Para + 0.1% glut for 1 hour at room temp.
7. Wash, and photograph.
8. Transfer into 100% MetOH and store at -20 °C.
9. To clear (for older embryos): put it into 1:2 benzyl alcohol : benzyl benzoate. Clears immediately. Be careful - it's very nasty stuff (and melts plastic).

Solutions

PBST: DEPC PBS + 0.1% Tween-20 (we have clean 10X PBS in boxes).

Hybe solution (kept as 1 L in -20 °C):

	100 ml
50% de-ionized formamide	50 ml formamide
5X SSC pH 4.5	25 ml 20 X SSC (pH 4.5)
50 µg/ml yeast RNA (Sigma R-6625!!)	5 mg Yeast RNA
50 µg/ml Heparin	5 mg Heparin
1% SDS	10 ml 10% SDS

Solution I: (you need 3 x 25 ml x # of vials)

	20 ml =
50% de-ionized formamide	10 ml formamide
5X SSC, pH 4.5	4 ml 20X SSC (pH 4.5)
1% SDS	2 ml 10% SDS
	4 ml good water

Solution II: (you only need it if using RNAse):

	40 ml =
	4 ml 5 M NaCl
	0.4 ml 1M Tris pH 7.5
	40 λ Tween 20
	35.6 ml good water

Solution III: (you need 2 x 25 ml x # of vials)

	20 ml =
50% formamide	10 ml formamide

2X SSC, pH 4.5	2 ml 20X SSC (pH 4.5)
	8 ml H_2O
	10 λ NP-40

20X SSC (pH 4.5):

1 L = 175.3 g NaCl + 88.2 g sodium citrate.

Make up in 900 ml of DEPC water, add enough citric acid (monohydrate) to make the pH = 4.5, and then add more DEPC water to 1 L.

RNAse A stock (prepare away from *in situ* area!!!):

dilute power 10 mg/ml in 10 mM Tris HCl, pH 7.5

15 mM NaCl

Heat to 100 °C for 15 min., cool slowly, aliquot and store at -20 °C.

Sheep serum:

Inactivate by heating to 70 °C for 30 min., store in aliquots at -20 °C.

10X TBS: (store at RT)	1 Liter =
	80 g NaCl
	2 g KCl
	250 ml 1 M Tris HCl, pH 7.5
	750 ml good water

TBST:

1X TBS + 0.1% Tween 20, add 0.05 g Levamisole for every 100 ml

NTMT:

	100 ml =
100 mM NaCl	2 ml 5 M NaCl
100 mM Tris HCl pH 9.5	10 ml 1 M Tris HCl

50 mM $MgCl_2$	5 ml 1 M $MgCl_2$
0.1% Tween 20	0.1 ml Tween 20
2 mM Levimasole	0.05 g Levimasole
	88 ml good water

RIPA buffer: (make 2 L and store at room temp.)

30 ml 5M NaCl

10 ml Nonidet P-40

10 ml 10% SDS

2 ml 5M EDTA

5 g deoxycholic acid (Na salt)

50 ml 1M Tris pH=8

add DEPC water to 1 L, filter.

Yeast mRNA: has to be Sigma R-6625 or Roche 109223 for wholemounts, or Invitrogen 15401-011 for sections.