

Wholemout in situ hybridization (WISH)

<Preparation of DIG-labeled RNA probes>

Mix the reagents in the following order at room temperature

Sterile DDW (0.2 um filtered)	up to 20 ul
10x transcription buffer	2 ul (Roche, 1 465 384, unit 1 ml)
0.1 M DTT	2 ul (promega, P117B, unit 250ul)
DIG RNA labeling mix	2 ul (Roche, 1 277 073, unit 40ul)
Linearized plasmid	1 ug
(Protector) RNase inhibitor	1 ul (Roche, 3 335 399, 40 U/ul)□
RNA polymerase (T3/T7/SP6)	1 ul (Roche, T3-1 031 171, T7-881 767, SP6-810 274, all 20 U/ul)
→ 37 C 2 hrs.	
DnaseI (RNase free)	2 ul (Roche, 776 785, 10 U/ul)
→ 37 C 15 min	

Purify probes using mini quick spin column (Roche). Yield is usually 4-5 ug. Dilute to 0.01 ug/ul using hybridization buffer, and store at -80 C.

* Linearized plasmid : cut about 20 ug, and purify with QIAquick PCR purification kit (cat# 28104, unit 50 columns, QIAGEN) using 30 ul of elution buffer -> ~ 0.5 ug/ul

<Preparation of the embryos>

1. Dissect embryos in PBS. Can stay on ice for several hours. For E8.5, puncture amnion. For E9.5 and older, puncture heart, forebrain, and hindbrain. For E8.5, process the embryos in 1.5 ml tube to minimize damage. For E9.5 and older, process them in 2 ml screw cap tube.
2. Fix in 4% paraformaldehyde in PBS at 4 C o/n with gentle rocking.
3. Wash in PBT (PBS + 0.1% Tween 20) 10 min x2 at 4 C, then 25%, 50%, 75% methanol in PBT, 5 min each at room temp, then 100% methanol 10 min x2 at room temp. Can store embryos in MeOH at -20 C at least 6 months.

<In situ hybridization>

Day1:

1. Rehydrate embryos 75%, 50%, 25%, MeOH in PBT, then rinse in PBT 2x. All 5 min each at room temp. Transfer the embryos into 4 ml glass vial (not for E8.5, up to ~ 6 E10.5 embryos/vial)
 2. Bleach with 6% hydrogen peroxide in PBT -> 1 hr at room temp on nutator (3 ml of 30% + 12 ml PBT). Wash with PBT 5 min x3 at RT.
30% Hydrogen peroxide : Mallinckrodt, cat# 5240, unit 500 ml
 3. Treat with 10 ug/ml Proteinase K in PBT (Proteinase K, Roche, 3 115 836, 25 mg).
- Timing is important-should be adusted based on the tissue of interest.

- E7.0 for ? 2 min
- E7.5 for ? 3 min
- E8.5 for 4 min
- E9.5 for 15 min

E10.5 for 23 min

E11.5 and E12.5 (hemisected) - 30 ug/ml, 25 min.

Let the glass vial sit on the table on its side, and role gently every ~3 min. Do not shake. Embryos very fragile after PtK digestion before the second fixation.

4. Wash with fresh 2 mg/ml glycine in PBT (filtered). Glycine: Mallinckodt, cat# 7728, unit 1 kg). Wash with PBT 5 min x2 at RT.

5. Refix with 0.2 % glutaraldehyde/4% paraformaldehyde in PBT -> 20 min at RT.
(80 ul of 25% -> 10 ml para/PBT)

Wash with PBT 5 min x2 at RT.

6. Transfer embryos to 2 ml conical bottom O-ring screw cap tubes. Add 1 ml hybridization buffer (see SISH protocol) -> 1 hr at 70 C, rotating in Hybaid oven. (can store in hybridization buffer before or after heating)

7. Dilute probes to 500 ng/ml in hybridization buffer. Remove prehybridization sol. from the embryos, add 0.5-1 ml probe, mix, make sure the volume covers the embryos -> 70 C, o/n in Hybaid oven. Do not need to rotate.

Day2:

1. Take out probes and store for recycle (can use once or twice more). Add SolI to tube and pour embryos into 4 ml glass vials. Rinse embryos with SolI 1x.

Wash with SolI for 30 min. x2 at 70 C. Prewarm solI.

Solution I : 30 ml formamide
12 ml 20X SSC pH4.5
6 ml 10% SDS
12 ml DDW
60 ml

2. Wash with 1:1 SolI:SolII -> 10 min at 70 C. Pre-warm mix.

Solution II: 12 ml 5M NaCL
1.2 ml 1 M Tris pH 8.0
0.48 ml 25% Tween-20
106.3 ml DDW
120 ml

3. Wash with SolIII, 5 min x3, at RT.

4. Wash 1x with 100 ug/ml RNase A in Sol II -> 37 C, 1 hr.
RNase A: Sigma R 4875, 100 mg

5. Wash with Sol II then Sol III, 5 min at RT.

Solution III: 30 ml formamide
6 ml 20X SSC pH 4.5
1.2 ml 10% SDS (optional)
24 ml DDW
60 ml

6. Wash with Sol III, 30 min x2 at 65 C (prewarm Sol III). Make up MBST during this incubation.

7. Wash with MBST, 5 min x3 at RT.

MBST:	100 mM Maleic acid pH 7.5	1 M	100 ml
	150 mM NaCl	5 M	30 ml
	0.1% tween 20		1 ml
	DDW		<u>869 ml</u>
			1 L

8A. Pre-block embryos with 10% HISS in MBST plus 2% Blocking reagent (see SISH protocol), 1 ml per 4 ml vial -> 3 – 4 hrs at RT, vial sitting upright on rotating platform.

8B. Weigh out 3 mg embryo powder into 1.5 ml eppendorf tube. Add 0.5 ml MBST plus 2% Blocking reagent -> 70 C for 30 min, rotating in Hybaid oven. Vortex for 10 min at RT, cool on ice, add 5 ul sheep serum, 1 ul anti-DIG AP (see SISH protocol), shake at 4 C gently for 1 hr on nutator. Spin in microcentrifuge for 10 min at 4 C. Dilute supernatant to 4 ml with 1% sheep serum in MBST plus 2% Blocking reagent. (spin thoroughly, take only supe (take only ~350 ul))

Embryo powder:

- 1) homogenize E12.5 – E14.5 mouse embryos in a minimum volume of ice-cold PBS.
- 2) Add 4 volumes of ice-cold acetone, mix and incubate on ice for 30 min.
- 3) Centrifuge at 10000 g for 10 min, remove the supe, and wash the pellet with ice-cold acetone and spin again.
- 4) Spread the pellet out, let dry o/n at RT, covered.
- 5) Grind it into a fine powder using mortar and pestle. Store dessicated at 4 C.

It is important to use embryo powder prepared from the species that you are studying. e.g., for the analysis of chick embryos, prepare chick embryo powder by this method.

9. Remove blocking solution from the embryos, transfer the embryos to 2 ml O-ring screw cap tube, and add 1 ml of antibody solution. Incubate on rocker at 4 C o/n.

Day3,4:

1. Transfer embryos to 4 ml glass vial. Wash with MBST for 5 min x3 at RT.
2. Wash with MBST 1 hr x8 at RT -> leave o/n in MBST at 4 C.
3. Repeat 2. for day 4. Embryos can be left up to 3 days washing at 4 C without apparent loss of signal. The larger the embryos, the longer the washes should be.

Day5:

1. Wash with NTMTL 5 min x3 at RT

NTMTL:	2 ml of 5 M NaCl
	5 ml of 2 M Tris pH 9.5

5 ml of 1 M MgCl₂
0.4 ml of 25% Tween 20
88 ml DDW
0.1 ml of 2 M Levamisole
100 ml

2. Transfer embryos to 2 ml O-ring screw cap tube, replace NTMTL with 1 ml BM purple, wrap in foil and incubate at RT on nutator 2 hr – 2 days. Monitor signal every couple of hours.
 3. When reaction is complete, wash in PBT, pH 4.5 (pH with phosphoric acid) 5 min x3 at RT, keeping in dark.
 4. Fix in 4% paraformaldehyde + 0.1% glutaraldehyde, for 1 hr – o/n.
 5. Glycerol series 50%, 75%, 85% in PBT (or PBS). Store in 85% glycerol for at least a week before taking pictures.
- Alternatively, wash with PBT 2x and then dehydrate in MeOH, 30%, 50%, 30%, 100%^{x2}, 10 min each at RT. Put into benzyl alcohol:benzyl benzoate (1:1) to clear completely. Note: BABB melts plastic. Embryos are fragile in BABB, so return to MeOH for storage.