WHOLE MOUNT IN SITU HYBRIDIZATION

Dissect embryos in cold PBS, wash in cold PBS in a 10 or 50 ml tube, and fixate o/n in cold 4% PFA/PBS at 4°C, while gently rocking. Young embryos or dissected tissues should be fixated shorter. Wash 2x in cold PBS. Dehydrate by a wash in cold 70% EtOH, rocking gently at 4°C for at least 1 hr, and subsequently in EtOH abs. Embryos/tissues can be stored at 4°C/ -20°C (up to several years) in EtOH .

Transfer embryos to pointed-bottom 2 ml tubes, fill to ~2 ml. All subsequent steps are carried out by replacing the top-most ~1.8 ml in this tube (don't let tissue fall dry!). Incubate while gently rocking, and at T_{room} unless denoted otherwise.

Bleach embryos 1 hr in freshly prepared 4:1 mix of **EtOH abs** and **30% H_20_2**. Wash 3x5' in PBT.

Digest the embryos for **maximum 5'** with 15 μg/ml **proteinase K in PBT**. Duration of treatment is OK for lungs or whole embryos, but may vary with age of embryo, type of tissue or probe used.

Block proteinase K with **a short rinse** and then 2 washes in freshly prepared 2 mg **glycine /ml PBT**. Wash 2x5' or longer in PBT.

Refixate embryos 20' in freshly prepared **0.2% glutaraldehyde/ 4% PFA/PBS**. Wash 2x5' in **PBT**.

It is possible to conserve samples in hybridization buffer without SDS at –20 °C (SDS precipitates at this temperature)

Replace fixative by **prewarmed (68°C) hybridization buffer**, rock gently until embryos sink (indicating that formamide has well penetrated the embryos). Replace once with prewarmed hybridization buffer and prehybridize at 68°C for 30' to 2 hrs.

Replace with 0.5 ml prewarmed hybridization buffer, add **probe** to 1 μg/ml, hybridize o/n at 68°C in incubator or waterbath.

Wash embryos 2x30' in **prewarmed**, freshly prepared solution **I** at 68°C.

Wash embryos 10' in a **prewarmed** 1:1 mix of solutions **I and II** at 68°C.

Wash embryos 3x5' in solution **II** at $\mathbf{T_{room}}$.

Incubate 2x30' in 100 μg/ml **RNase A in solution II** at 37°C (e.g. in shaking water bath).

Wash 2x30' in **prewarmed**, freshly prepared solution **III** at **65°C**.

Wash 3x5' in **TBST/ 2mM levamisole**.

Block embryos for at least 1 hr in 10% h.i. FCS/ 2% blocking reagent/ TBST/ 2 mM levamisole.

Meanwhile, heat inactivate a pinch of embryo/acetone-powder in 500 μl TBST/ 2 mM levamisole for 30' at 68°C.

Spin down 20 seconds and resuspend pellet in 1% h.i. FCS/ 2% blocking reagent/ TBST/ 2 mM levamisole. Preabsorb **anti-digoxigenin-Ab** diluted 1:1000 in this solution for at least 1 hr at 4°C. Spin down to use only the supernatant.

Incubate embryos o/n at 4°C in the preabsorbed antibody sup. solution diluted 1:1 in the 10% h.i. FCS/ 2% blocking reagent/ TBST/ 2 mM levamisole that is already in the tube with embryos.

Wash embryos 3x quickly, then 5 of 6x 1 hr , then o/n in **TBST/ 2 mM levamisole** at + 4 °C

Wash 2x20' in freshly prepared NTMT (+ 2 mM levamisole). Warm BM Purple to 37°C in the dark.

Transfer embryos to 1 ml culture wells containing sufficient **prewarmed BM Purple** to cover the embryos. Incubate 30' to o/n in the dark at 37°C to develop the purple substrate. Monitor hourly.

Stop precipitation reaction by washing 3x5' in **PBS**.

Post-fixate the embryos in freshly prepared **4% PFA** and store until further use in PFA at 4°C. Embryos can then be cleared or dehydrated for embedding and sectioning.

Reage nts and solutions

PFA	1 kg, Prolabo 28794.295	4°C
30% H2O2	0.5 l , Merck 1.07209	4°C
Proteinase K, 14 mg/ml	5 ml, TrisHCl pH 7.5, Roche 1373196	4°C
Glycine	500g, Life technologies, 15527-013	2% =20 mg/ml PBT –20°C [6ml]
Glutaraldehyde, 25%	Electron Microscopy Sciences 10200	–20°C
Levamisole, hydrochloride	5g, Sigma L9758	2M, =500 mg/ml in TBST (1000x) at –20°C [6ml]
FCS, heat inactivated (h.i.)	Gibco-BRL ?	–20°C
Blocking Reagent	50g, Roche, 1096176	10% in PBS at –20°C
BM Purple Alkaline Phosphate substrate	100 ml, Roche, 1442074	4°C
Rnase A	100mg, Roche, 109169	*10 mg/ml in {10mM Tris pH7,5/15mM NaCl} (100x) at –20°C [1ml]
Deionized Formamide	Quantum appligene FORMD002	4°C
Heparin	100 000 U, Sigma, H9399	50mg/ml in H_2Odd –20°C [0,2ml]
t-RNA from brewer's yeast	100mg, Roche, 109517	40mg/ml in H_2Odd –20°C [0,5ml]
Anti-dig AP Fab fragments	150U/200 µl, Roche 1093274	4°C

[x ml] : Volume pratique des aliquots

*Rnase A : prepare at 10mg/ml en {10mM Tris pH7,5/15mM NaCl} - heat 15 min / 100 °C – when solution is at T_{room}, aliquote and conserve at –20°C

10x PBS, 1 l	80 g NaCl 2 g KCl 14.4 g Na2HPO4 2.4 g KH2PO4 800 ml H20, adjust to pH 7.2 H20 to 1000 ml	Dilute 10x for use as PBS
10x PBST, 1 l	10 ml Tween-20 to 1 liter 10x PBS	Dilute 10x for use as PBST (0.1% T)
10x TBS, 1 l	80 g NaCl 2 g KCl 30 g Tris 800 ml H20, adjust to pH 7.6 with HCl	Dilute 10x for use as TBS
10x TBST, 1 l	H20 to 1000 ml 10 ml Tween-20 to 1 liter 10x TBS	Dilute 10x for use as TBST (0.1% T)
20x SSC, 1 l	175.3 g NaCl 88.2 g NaCitrate H20 to 1000 ml	
(Pre)Hybridization buffer, 10 ml	5 ml deionized formamide 2.5 ml 20x SSC 12.5 µl 40 mg/ml tRNA 500 µl 20% SDS 10 µl 50 mg/ml heparin 2 ml H20 (1978,5 µl exactly)	50% deionized formamide 5x SSC 0.05 mg/ml tRNA 1% SDS 0.05 mg/ml heparin

Solution I, 25 ml	12.5 ml deionized formamide 6.25 ml 20x SSC 1.25 ml 20% SDS 5 ml H20	50% deionized formamide 5x SSC 1% SDS
Solution II, 50 ml	5 ml 5M NaCl 500 µl 1M TrisHCl pH 7.5 500 µl Tween-20 44 ml H20	0.5M NaCl 10mM TrisHCl pH 7.5 0.1% Tween-20
Solution III, 20 ml	10 ml deionized formamide 2 ml 20x SSC 8 ml H20	50% deionized formamide 2x SSC
NTMT, 50 ml	1 ml 5M NaCl 5 ml 1M TrisHCl pH 9.5 2.5 ml 1M MgCl2 500 µl 10% Tween-20 41 ml H20	100mM NaCl 100mM TrisHCl pH 9.5 50mM MgCl2 0.1% Tween-20
Blocking Reagent : --Maleic acid buffer -- Blocking stock solution		 100mM maleic acid 150mM NaCl Adjust to pH7,5 with NaOH Dilute to 10% (w/v) in maleic acid buffer Heat to dissolve ; 1ml aliquots ; at –20°C

Préparation of mouse embryo acetone powder (Harlow and Lane, 1988)

1- Homogenize 11,5-13,5 dpc embryos in a minimum of Ca/Mg free PBS (1ml/g of tissue) on ice using a tissuemizer.

2- Add 4 volumes of cold acetone and mix vigorously. Keep on ice for 30 min with occasional vigorous mixing. Collect the precipitate by centrifugation at 10000g for 10 min. Remove and discard the supernatant.

3- Resuspend the pellet with cold acetone and mix vigorously. Allow to sit on ice for 10 min. Spin at 10000g for 10 min. Transfer the pellet to a clean piece of filter paper, spread the precipitate and allow to air dry at T_{room}. As it dries, continue to spread and disperse the pellet. After the powder is dry, transfer to an airtight container and store at –20 °C.

We are not using embryo powder anymore: we found that the antibodies were very clean