Tissue Preparation

- Fix dissected implantation sites in 4% PFA/1x PBS O/N at 4°C
- Rinse in 1x PBS three times for 5 min
- Incubate tissue in 10% sucrose/1x PBS overnight
- Incubate tissue in 25-30% sucrose/1x PBS overnight
- Embed tissue in OCT (let sit in OCT for at least 1 hour at 4°C before freezing or leave O/N in OCT at 4°C before freezing)
- Place tissue in mold and freeze in ethanol bath with dry ice – put into -70°C
- Cut 7-10 µm sections at -20°C and transfer to Superfrost (+) slides… store at -70°C

Prehybridization Steps and Hybridization

- Remove sections from -70°C freezer and warm to RT wrapped in paper towels (about 30-40 min). Then open box and remove slides.
- Rinse slides in 1x PBS for 5 min
- Fix in 4% PFA/1x PBS for 10-15 min
- 1x PBS 5min
- 1x PBS 5 min
- Proteinase K treatment ~10 min (in Pro K buffer) – Must determine optimal time and concentration for each tissue/stage used… I have used…

  Placenta – E10.5-E18.5 use 10 minutes with 600 µl ProK
  Placenta – E7.5-E9.5 use 5 minutes with 300 µl ProK

- 1x PBS 5 min
- 4% PFA/1x PBS for 5 min
- 1x PBS 5 min
- Acetylation: Add 500 µl Acetic Anhydride to 200 ml Acetylation Buffer 5 min
  Add another 500 µl Acetic Anhydride, mix with pipette 5 min
- 1x PBS 5 min

- Heat probe diluted in Hybridization buffer to 70°C for 10 min
  (Dilute 2 µl of a 20 µl DIG probe synthesis in 18 µl of hyb buffer [can be stored at -80 °C this way]. THEN add 1 µl of DILUTED probe per 200µl hyb buffer. If doing double ISH, add 1 µl of each probe at this point.)
- Add 200 µl per section (cover with a sigmacote coated coverglass if desired - dip coverglass into sigmacote, drain and set aside to dry prior to this step – use dried coverglass). If not using coverglass – circle sections with PAP pen so hyb solution does not run out over whole slide (I prefer this method as I can do several probes on one slide). If doing whole slide with coversglass I use 300-350 µl of hyb.

Day 1 – RNase Free

- Hybridize overnight at 65°C in a sealed humidified box (paper towels or Whatman filter paper soaked in 1X salts/50% formamide)

Post Hybridization - Washes

- Remove coverslips by immersion in wash solution in 50 ml Falcon Tube
- Transfer slides to rack and start washes in post-hyb washing solution at 65°C

- Wash 2X in post-hyb wash solution 30 min at 65°C
- Wash 2X in 1x MABT for 30 min at RT
- Wash 1X in 1x RNA wash for 10 min at 37°C
- Wash 1X in 1x RNA wash with RNAse A added (400 µl of 10 mg/ml RNAse A per 200 ml) for 30 min at 37°C
- Wash 1X in 1xRNA wash for 5 min at 37°C
- Wash 1X in 1x MABT at RT for 5 min

Blocking and Antibody

- Block in 1x MABT + 2% blocking reagent + 20% heat inactivated sheep serum (or goat serum) for >1 hour
  [5ml = 1ml block reagent + 1ml serum + 1ml 5x MABT + 2ml H_2O + 50 µl tween-20]
- Dilute anti-DIG antibody 1:2500 in blocking solution (or anti-Flourosceine antibody depending on what your probe is labeled with)
- Add 200 µl diluted antibody to each section and coverslip as before (coverglass without sigmacoat), unless used PAP pen initially, then no need for coverglass
- Incubate overnight in humidified chamber (paper towels soaked with 1x PBS) at 4°C

Post Antibody Washes

- Wash 4 times in 1x MABT for 15 min at RT
- Wash in NTMT for 10 min at RT
- Wash in NTMT + Levamisole (100 mg/200 ml) for 10 min at RT

- Transfer slides to a box and add 200 µl staining solution per section and coverslip (Unless used PAP pen initially – may need to touch up PAP pen at this point)
- Cover box with foil or cardboard box and incubate at RT until precipitate forms, hours to days depending on probe (purple)
- Stop reaction in 1x PBS
- If doing single ISH skip to Counterstain and Mounting

Day 2/3 - Not RNase Free

*To Do Doule ISH

Always do NBT/BCIP (purple) colour first… then detect with second AP substrate (INT/BCIP or Fast Red). Also, develop the least abundant gene first using NBT/BCIP as it is the most sensitive. Extended colour development with INT/BCIP can result in clumpy crystals to precipitate out of the solution – very ugly.

- Heat slides in 1x MABT for 30 minutes at 65°C
- Wash 2X in 1x MABT 10 minutes at RT
- Incubate in 0.1M Glycine-HCl pH 2.2 for 30 minutes
- Wash in 1x MABT 10 minutes at RT
- Block in 1x MABT + 2% blocking reagent + 20% heat inactivated sheep serum (or goat serum) for >1 hour
  [5ml = 1ml block reagent + 1ml serum + 1ml 5x MABT + 2ml H_2O + 50 µl tween-20]
- Dilute anti-DIG antibody 1:2500 in blocking solution (or anti-Flourescein antibody depending on what your probe you are detecting)
- Add 200 µl diluted antibody to each section and coverslip as before (without sigmacoat), unless used PAP pen initially, then no need for coverglass
- Incubate in humidified chamber (with 1x PBS) at 4°C O/N

Post Antibody Washes

- Wash 4 times in 1x MABT for 15 min at RT
- Wash in NTMT for 10 min at RT
- Wash in NTMT + Levamisole (100 µg/200 ml) for 10 min at RT

- Transfer slides to a box and add 200 µl staining solution per section and coverslip (Unless used PAP pen initially – may need to touch up PAP pen at this point)
- Cover box with foil or cardboard box and incubate RT until precipitate forms, hours to days depending on probe (brown or red depending on substrate you are using)
- Stop reaction in 1x PBS

*To Do Immunohistochemistry following an ISH

To do an immuno following the ISH (single ISH) continue from here after stopping the NBT/BCIP colour reaction in 1xPBS.

- Wash 3X 5 min in 1x PBS
- Wash 1X 5 min in 1x PBS/1% BSA
- Block 1 hour at RT in 1x PBS/1% BSA/5% serum

  (Use serum from animal planned secondary Ab is raised in)

- Incubate with primary Ab diluted in block O/N at 4°C

- Wash 3X 5 min in 1x PBS
- Wash 1X 5 min in 1xPBS/1% BSA
- Incubate with secondary diluted in 1x PBS/1% BSA/5% serum for 1 hr at RT
- Wash 3X 5 min in 1x PBS

- Add DAB substrate (see Dako DAB protocol) for up to 1-10 min
- Rinse slides gently in distilled water

- Counterstain and mount as described below for single ISH technique as the DAB substrate can withstand the alcohol and xylene dehydaration

Counterstain and Mounting (For NBT/BCIP)

- Immerse slides in nuclear fast red (sigma – undiluted) for 30 seconds -1 minut (2 min if older solution)
- Rinse slides in tap water 1-2 min
- Dehydrate slides through alcohol to xylene:

    - 30% EtOH 1min
    - 50% EtOH 1min
    - 70% EtOH 1min
    - 95% EtOH 1min
    - 100% EtOH 1min
    - 100% EtOH 1min
    - Xylene 2 min
    - Xylene 2 min

Add mounting medium and coverslip slides – air dry in fumehood

Counterstain and Mounting for Double ISH (For INT/BCIP)

INT/BCIP precipitate is SOLUBLE in alcohol and xylene…

Therefore…

- Counterstain in Nuclear Fast Red for 30 seconds – 1 minutes
- Rinse slides in tap water 1-2 minutes
- add 2 drops Crystal Mount Aqueous Mounting Medium (Sigma) to sections an let dry according to manufacturers instructions (RT O/N)
- After dry can add Xylene based mounting medium on top, it should form a barrier, and coverslip as usual. DO NOT take through alcohol and xylene first though.

- Option – can mount under 50% glycerol/1x PBS temporarily and you can take pictures. However, the INT/BCIP brown ppt will eventually dissolve under thes conditions.

SOLUTIONS

Proteinase K Buffer

10 ml 1M Tris pH 8
2.5 ml 0.2M EDTA pH 8
187.5 ml DEPC water
600 µl Proteinase K (10 mg/ml) – added just before use

Proteinase K Stock

100 mg Proteinase K (Roche)

Dissolve in 5 ml 100 mM Tris pH 8.0 (Made in DEPC water)
6 mM $CaCl_2$ (DEPC)
Add 5 ml 100% glycerol (sterile)
Aliquot and store -20°C

Acetylation Buffer

3.72 g Triethanolamine
200 ml DEPC water
500 µl Acetic Anhydride – added just before use

10X Salts

NaCl	114 g
Tris HCL	14.04 g
Trise Base	1.34 g
$NaH_2PO_4 \cdot 2H_2O$	7.8 g
Na_2HPO_4	7.1 g
0.5M EDTA pH 8	100 ml

Make up to 1000 ml with DEPC water

Yeast RNA

Dissolve in DEPC water at 10 mg/ml and freeze in 1 ml aliquots

50X Denhardt's

Bovine Serum Albumin (BSA)	1 g	1%(w/v)
Ficoll™	1 g	1%(w/v)
Polyvinylpyrrolidone (PVP)	1 g	1%(w/v)

Make up to 100 ml with DEPC water

Hybridization Buffer

	1 ml	10ml	50 ml	Conc
10X Salt	100 µl	1 ml	5 ml	1X
Deionized Formamide	500 µl	5 ml	25 ml	50%
50% Dextran Sulfate	200 µl	2 ml	10 ml	10%
Yeast tRNA	100 µl	1 ml	5 ml	1 mg/ml
50x Denhardt's	20 µl	200 µl	1 ml	1X
DEPC water	80 µl	800 µl	4 ml	

5x MABT

NaCl 21.91 g
Maleic Acid 29.02 g
NaOH 18 g
450 ml nanopure water
Adjust pH to 7.5
Adjust to 500 ml
Filter

Add 0.1% Tween on day of use

Post-Hyb Wash Solution

1x SSC, 50% Formamide, 0.1% Tween-20

500 ml = 250ml formamide + 25 ml 20x SSC + 5 ml 10% Tween-20

RNAse A Stock

500 mg RNase A (Sigma)

Dissolve in 45 ml 0.01M sodium acetate pH 5.2
Heat to 100°C for 15 min.
Cool to RT and add 5 ml 1M Tris pH 8.0
Aliquot and freeze at -20°C

RNA Wash Buffer (10X) 500 ml

5M NaCl 400 ml
1M Tris pH 7.5 50 ml
0.5M EDTA 50 ml

Blocking Reagent

Roche Blocking Reagent is made up in 1x MAB as 10% stock (10g/100ml). Heat to dissolve, autoclave, aliquot and freeze -20°C

NTMT

	200 ml
5M NaCl	4 ml
1M MgCl	10 ml
1M Tris pH 9.5	20 ml
10% Tween-20	2 ml
Levamisole	100 mg

Staining Solution (per 1 ml in NTMT with Levamisole)

NBT	6.7 µl (50 mg/ml)
BCIP	5.25 µl (50 mg/ml)

OR

INT/BCIP	35 µl in 5 ml NTMT with levamisole