

Tabin Lab**Single, double, or triple section *in situ* hybridization**

Adapted from Lassar lab protocol

I- Tissue Preparation and Sectioning*Paraffin Sections*

- Dissect embryo in PBS
- Fix 1hr-o/n in 4% PFA/PBS 4°C
- Wash in PBS
- Dehydrate to 100% EtOH

25% EtOH/PBS	10'
50% EtOH/PBS	10'
75% EtOH/dH ₂ O	10'
100% EtOH	10'
100% EtOH	10'

- Tissue can be stored in 100% EtOH at -20°C for several months
- Clear tissue in xylene
2 x 5'-30' (or longer depending on tissue)
- 50/50 xylene:paraffin at 60°C 15'
- 100% paraffin 3 x 1hr 60°C (or longer--can go overnight)
- Vacuum 1+ hours (can go overnight)
- Embed
- Store blocks at 4°C until needed
- Section and collect on TESPA treated slides
- Dry sections O/N 37°C and store at 4°C until needed

Frozen Sections

- Dissect embryo in PBS
- Fix O/N in 4% PFA/PBS
- Transfer embryo to 5% sucrose/PBS, rock at RT until embryo sinks
- Replace with 30% sucrose/PBS, rock O/N 4°C
- Add equal volume of OCT mounting medium and rock until solution is homogenous
- Embed embryo in OCT on dry ice and store blocks at -80°C
- Section and collect on Superfrost Plus slides (VWR or Fisher)
- Air dry sections 20'-3hrs and store sections at -80°C

II- Probe Preparation

- For probe template use either linearized template or PCR template
- Combine:

10.5μl	dH ₂ O-DEPC
2μl	10X Transcription Buffer (Roche)
2μl	10X Nucleotide Labeling Mix, DIG, FITC, or Biotin (Roche)
4μl	Template (~1μg)
0.5μl	RNase Inhibitor (Roche)
1μl	RNA Polymerase (Roche)

Incubate 37°C 2hrs

Run 1μl to check synthesis reaction

Add 1μl DNase (RNase free, Roche) 37°C 15'

Add 100μl TE-DEPC, pH8
10μl 4M LiCl-DEPC
400μl 100% EtOH

Incubate at -20°C 60' - O/N

Spin 10' 14,000rpm at 4°C

Wash 70% EtOH

Spin 2' 14,000rpm at 4°C

Airdry 5-10'

Resuspend in 100μl dH₂O-DEPC

For Biotin-, FITC-labeled probes, or DIG-labeled probes with high background, precipitate a second time.

III- Preparation of Slides for Hybridization

Use DEPC treated solutions for all steps prior to hybridization.

Paraffin Sections

- Bake slides on hot plate at 60°C 1hr

- Allow slides to come to RT
- Dewax in xylene 2 x 5'
- Rehydrate to PBS

100% EtOH	5'
100% EtOH	5'
75% EtOH/dH ₂ O	5'
50% EtOH/PBS	5'
25% EtOH/PBS	5'
PBS	5'
PBS	5'

Frozen Sections

- Thaw slides and air dry for 15-20' at RT

IV- Hybridization

- 4% PFA/PBS 10'
- PBT Rinse
- PBT 5'
- PBT 5'
- PK (Roche) in PBS (generally 1 μ g/ml for 10', but concentration and length of incubation can be varied).
- PBT 5'
- PBT 5'
- 4% PFA/PBS 5' (Re-use PFA from first post-fix)
- PBT Rinse
- PBT 5'
- PBT 5'
- Acetylation 10'
Add 625 μ l acetic anhydride to 250ml 0.1M TEA (triethanolamine) and shake well—use immediately.
- PBT Rinse
- PBT 5'
- PBT 5'
- Rinse H₂O
- Air dry slides ~10'-30'
- Combine 100 μ l prewarmed hybe solution and 1 μ l of probe, add to slides, and coverslip (coverslips cut from polypropylene bags).

For double or triple in situs, add 1ml of each probe to 100ml hybe soln.

- Place slides in humidified slide box (use Whatman paper or paper towels soaked in 5XSSC/50% formamide)
- Incubate O/N at 65°C

V- Post-Hybridization Washes

Pre-warm wash solutions

- Remove cover slips and rinse in 5X SSC
- 1X SSC/50% formamide 30' at 65°C
- TNE 10' at 37°C
- TNE + RNase A (20µg/ml, Roche) 30' at 37°C
- TNE 10' at 37°C (Re-use TNE from first wash)
- 2X SSC 20' at 65°C
- 0.2X SSC 20' at 65°C
- 0.2X SSC 20' at 65°C

VI- Antibody Incubation and Detection

- MABT 5' at RT
- MABT 5' at RT
- Block in 20% HISS/MABT 1+ hrs (For mouse probes include 2% Boheringer Blocking reagent—heat to 55°C to dissolve).
- α-DIG-AP (1:2500, Roche) in 5% HISS/MABT—1ml/slide and incubate O/N at 4°C in humidified chamber.
- MABT Rinse
- MABT 5'
- MABT 5'
- MABT 5'
- NTM pH9.5 10'
- NTM pH9.5 + NBT + BCIP
Using stock solutions of NBT and BCIP: to 1ml NTM, add 5µl NBT and 5µl BCIP
- Develop 1hr-3 days in the dark, change solution as necessary
- Rinse NTM pH9.5
- PBS 5'
- PBS 5'
- 4% PFA/PBS 30'

Fix for 10' if doing double or triple detection.

- PBS 5'
- PBS 5'

- ***For single detection:*** rinse slides in dH₂O and mount in gelvatol (heat gelvatol to 65°C before using).
- ***For double detection:*** go on to section VII.

VII- Detection of Second Probe

For double in situ hybridization, detect with NBT/BCIP first, followed by either INT/BCIP or Fast Red. For triple in situ hybridization, detect with NBT/BCIP first, INT/BCIP second, and Fast Red third.

- MABT 5'
- MABT 5'
- Block in 20% HISS/MABT 1+ hrs (For mouse probes include 2% Boheringer Blocking reagent—heat to 55°C to dissolve).
- α-FITC-AP (1:2500, Roche) in 5% HISS/MABT—1ml/slide and incubate O/N at 4°C in humidified chamber.
- MABT Rinse
- MABT 5'
- MABT 5'
- MABT 5'

Detection using INT

- NTM pH 9.5 10'
- NTM pH9.5 + NBT + BCIP
Using stock solutions of INT and BCIP: to 1ml NTMT, add 3.5μl INT and 7μl BCIP
- Develop 1hr-3 days in the dark, change solution as necessary
- Rinse NTM pH9.5
- PBS 5'
- PBS 5'
- 4% PFA/PBS 20'

Fix for 10' if doing triple detection

- PBS 5'
- PBS 5'
- ***For double detection:*** rinse slides in dH₂O and mount in gelvatol (heat gelvatol to 65°C before using).
- ***For triple detection:*** go on to section VIII.

Detection using Fast Red

- NTM pH 8.0 10'
- Dissolve Sigma Fast Red TR/Naphtol AS-MX ready made tablets in buffer provided.

- Filter through 0.2-0.8 μ m syringe filter
- Dilute 1:10 in NTM pH8.0 and add to slides
- Develop 1hr-3 days, change solution as necessary
- Rinse NTM pH8.0
- PBS 5'
- PBS 5'
- 4% PFA/PBS 20'

Fix for 10' if doing triple detection

- PBS 5'
- PBS 5'
- ***For double detection:*** rinse slides in dH₂O and mount in gelvatol (heat gelvatol to 65°C before using).
- ***For triple detection:*** go on to section VIII.

VIII- Detection of Third Probe

- MABT 5'
- MABT 5'
- Block in 20% HISS/MABT 1+ hrs (For mouse probes include 2% Boheringer Blocking reagent—heat to 55°C to dissolve).
- α -Biotin-AP (1:2500, Roche) in 5% HISS/MABT—1ml/slide and incubate O/N at 4°C in humidified chamber.
- MABT Rinse
- MABT 5'
- MABT 5'
- MABT 5'

Detection using Fast Red

- NTM pH 8.0 10'
- Dissolve Sigma Fast Red TR/Naphtol AS-MX ready made tablets (Sigma) in buffer provided.
- Filter through 0.2-0.8 μ m syringe filter
- Dilute 1:10 in NTM pH8.0 and add to slides
- Develop 1hr-3 days, change solution as necessary
- Rinse NTM pH8.0
- PBS 5'
- PBS 5'
- 4% PFA/PBS 10'
- PBS 5'
- PBS 5'
- Rinse slides in dH₂O and mount in gelvatol (heat gelvatol to 65°C before using).

Antibody Staining Following Probe Detection

- PBS 5'
- PBS 5'
- 4% PFA/PBS 10'
- PBS 5'
- PBS 5'
- ***If detecting antibody with DAB***—bleach 0.3% H₂O₂/PBS 30'
- PBS 5'
- PBS 5'
- 1° antibody in 5% HINGS or HISS/PBS—1 ml/slide and incubate O/N at 4°C in humidified chamber (to conserve antibody, use 200µl/slide and coverslip).
- PBS Rinse
- PBS 5'
- PBS 5'
- PBS 5'
- Block in 20% HINGS or HISS/PBS 1+ hrs
- 2° antibody in 5% HINGS or HISS/PBS—1ml/slide and incubate O/N at 4°C in humidified chamber. ***Fluorescent, HRP, or AP-conjugated 2° antibody can be used.***
- PBS Rinse
- PBS 5'
- PBS 5'
- PBS 5'
- Fluorescent 2° antibody—Rinse slides in dH₂O and mount in gelvatol (heat gelvatol to 65°C before using).
- HRP-conjugated 2° antibody—detect with DAB Substrate Kit (Vector Labs).
- AP-conjugated 2° antibody—detect with NBT/BCIP, INT/BCIP, or Fast Red (as described above), depending on color reactions used for probe detections. ***As with in situ hybridizations, detect with NBT/BCIP first, INT/BCIP second, and Fast Red third.***
- PBS 5'
- PBS 5'
- PBS 5'
- 4% PFA/PBS 10'
- PBS 5'
- PBS 5'
- Rinse slides in dH₂O and mount in gelvatol (heat gelvatol to 65°C before using).

IX- Solutions

10X PBS (1 liter)	80g NaCl 2g KCl 14.4 g Na ₂ PO ₄ 2.4 g KH ₂ PO ₄ adjust to pH 7.4 with HCl and bring volume to 1 liter with water
PBT	1X PBS + 0.1% Tween-20
Hybridization Solution	10mM Tris pH7.5 600mM NaCl 1mM EDTA 0.25% SDS 10% Dextran Sulfate (American Bioanalytical 50% solution) 1X Denhardt's 200µg/ml yeast tRNA (Gibco) 50% formamide Store at -20°C
SSC	20X stock pH 7.0
10X TEA	1M stock pH8.0
TNE	10mM Tris pH7.5 500mM NaCl 1mM EDTA
MABT (1X)	100mM Maleic Acid 150mM NaCl bring to pH7.5 with NaOH 0.1% Tween-20
NTM pH 9.5	100mM NaCl 100mM Tris pH 9.5 50mM MgCl ₂

NTM pH 8	100mM NaCl 100mM Tris pH 8 50mM MgCl ₂
NBT stock	50mg/ml in 70% DMF (store at -20°C)
BCIP stock	25mg/ml in water (store at -20°C)
INT stock	50mg/ml in DMSO (store at -20°C)
HISS	Sheep Serum, heat inactivated at 56°C for 30' and store at -20°C.
HINGS	Goat Serum, heat inactivated at 56°C for 30' and store at -20°C.