Title: RNA in situ hybridization (from Dr. Eric Domyan)
Rationale and background: To visualize gene expression by binding a tagged probe to target mRNA
Protocol:

DAY 0 – Dissect tissue, fix in 4% paraformaldehyde (4% PFA) at 4°C overnight. Dehydrate through methanol gradient into 100% MeOH at 4 °C, store at -20°C.

DAY 1 – All incubation steps to be performed with gentile agitation on an orbital shaker, unless otherwise indicated.

1. Prepare supplies and preheat solutions.
- **A.** Transfer prehyb stock solution into sealed conical tube and preheat to 60.5°C.

2. Rehydrade fixed tissue (In 6-well baskets or nunc tubes. Use 5mL/wash for baskets and 2mL/wash for nunc tubes.)
- **A.** Wash tissue 1 x 15 min each in 75%MeOH/25% PBSTw, 50%MeOH/50%PBSTw, 25% MeOH/75% PBSTw.
- **B.** Wash tissue 2 x 15 min in PBSTw.

3. Incubate tissues for 30 min at 25°C in 2mL/well of 6% H_2O_2.
4. Wash tissues 4 x 5 min at 25°C with 2mL/well PBSTw.
5. Incubate tissues 12 min at 25°C in 2mL/well proteinase K solution.
6. Wash tissues 1 x 5 min at 25°C with 2mL/well PBSTw.
7. Incubate tissues 20 min at 25°C in 2mL/well post-fix solution.
8. Wash tissues 2 x 5 min at 25°C with 2mL/well PBSTw.
9. Transfer tissues into separate 2mL Nunc tubes, 1 tube per probe.
10. Prehybridization step: Incubate for at least 1 hr at 60.5°C in 1.5 to 2mL prehyb buffer.
11. Hybridization step: Add 0.65mg probe/well and incubate sections overnight in hybridization chamber at 60.5°C in probe+prehyb buffer.
12. Prepare and preheat solutions for Day 2: Add SDS to solution 1 (to a final concentration of 1%) and preheat overnight at 60.5°C.

DAY 2 - All incubation steps to be performed with gentile agitation on an orbital shaker, unless otherwise indicated.

1. Transfer tissue into baskets of a 6 well plate. Remove hyb buffer from each well and wash tissues 3 X 30 min at 60.5°C with 5mL/well Solution 1.
2. Prepare and preheat solutions.
- **A.** Prepare 50/50% mix of Solution1/Solution 2 and preheat to 60.5°C.
- **B.** Add RNase to Solution 2 and preheat to 37°C.
- **C.** Preheat one wash volume of Solution 3 to 25°C and two wash volumes to 60.5°C.

3. Wash tissues 1 X 10 min at 60.5°C with 5mL/well of 50/50% mixture of Solution 1/ Solution 2.
4. Wash tissues 4 X 10 min at 25°C with 5mL/well Solution 2.
5. Incubate tissues 15 min at 37°C in 5mL/well RNase solution.
6. Wash tissues 1 X 10 min at 25°C with 5mL/well Solution 2.
7. Wash tissues 1 X 10 min at 25°C with 5mL/well Solution 3.
8. Wash tissues 2 X 1 hr at 60.5°C with 5mL/well Solution 3.
9. Preheat Tissue Blocking (TB) buffer to 25°C.
10. Wash tissues 3 X 10 min at 25°C with 2ml/well TBSTw.
11. Transfer tissues into new 2mL Nunc tubes.
11. Blocking step
- **A.** Incubate tissues at least 2 hr at 25°C in 2mL/well TB buffer.

12. Antibody step
- **A.** Dilute anti-DIG AP antibody 1:2000 in TB buffer.

- **B.** Add 2 mL of TB containing the antibody to each Nunc tube.
- **C.** Incubate tissues overnight at 4°C in 2mL TB buffer + antibody.

Day 3 - All incubation steps to be performed with gentile agitation on an orbital shaker, unless otherwise indicated.

1. Remove antibody solution and save (can be reused).
- **A.** Add sodium azide to a final concentration of 0.2mM to prevent microbial growth.
- **B.** Store antibody solution at 4°C and reuse up to two additional times.

2. Transfer tissues into baskets of 6 well plate.

3. Wash tissues 8 X 30 min at 25°C with 5mL/well TBSTw containing 2 mM levamisole.

4. Remove tissues from baskets, use forceps to separate sections & remove visible debris, transfer tissues into clean Nunc tubes.

5. Wash tissues 1 X 10 min at 25°C with 1.5mL NTMT containing 2 mM levamisole.

6. Detection step
- **A.** Protect tissues from light and incubate at 25°C in 1mL/tube of a 60/40% mixture of NTMT containing 2mM levamisole/BM Purple.
- **B.** Color development time ranges from several hours to several days. Change NTMT/BM Purple solution as needed (substrate will precipitate over time).
- Once color is fully developed, wash tissues 2 X 5 min at 25°C with 1mL/tube NTMT containing 2mM levamisole.

7. Bleaching step
- **A.** Post-fix tissues overnight at 4°C in 1mL/tube 4% PFA.
- **B.** Remove PFA and incubate tissues for 30 min at 25°C in 1mL/tube PBSTw containing 3% H2O2.
- **C.** Wash tissues 1 X 10 min at 25°C in 1mL/tube PBSTw.
- **D.** Store tissues at 4°C in 1mL/tube 4% PFA.

Solutions

Day 1

PBSTw: 1X PBS containing 0.1% Tween-20 and 0.2mM sodium azide. Pass through 0.2µm filter to remove insoluble material/contaminants.

6% H_2O_2: 1mL 30% H_2O_2 per 4mL PBS

Proteinase K: 0.25µL 20mg/mL proteinase K per 1mL PBSTw

Post-fix: 8µL 25% glutaraldehyde per 1mL 4% PFA

Prehybridization solution

stock solution	**final conc**	**for 1000mL**
100% formamide	50%	500mL
20X SSC	5X	250mL
Blocking reagent	1%	10g
10mg/mL yeast tRNA	10µg/mL	1mL
10mg/mL heparin	10µg/mL	1mL
dH_2O to vol.	--	to 1000mL

Aliquot 50mL volumes into conical tubes & store at –20°C.

Solution 1

stock solution	**final conc**	**for 500mL**

100% formamide	50%	250mL
20X SSC	5X	125mL
dH_2O	--	75mL
10% SDS	1%	50mL

Mix formamide, SSC & dH_2O as shown above & store at –20°C.
DO NOT add SDS to freezer stock solution because SDS will precipitate in the cold.
Prior to using Solution 1 for ISH, add 1mL of 10% SDS per 9mL Solution 1 stock.

Day 2

Solution 2

stock solution	**final conc**	**for 500mL**
1M Tris-HCl, pH 7.5	10mM	5mL
5M NaCl	0.5M	50mL
100% Tween-20	0.1%	0.5mL
0.2M sodium azide	0.2mM	0.5mL
dH_2O	--	444mL

Pass through 0.2µm filter to remove insolubles/contaminants, store at 25°C.
RNase: 5µL RNase (50µg/mL) per 1mL Solution 2

Solution 3

stock solution	**final conc**	**for 500mL**
20X SSC	2X	50mL
100% formamide	50%	250mL
dH_2O	--	200mL

Store at –20°C

Tissue blocking (TB) buffer

stock solution	**final conc**	**for 500mL**
10X TBS	1X	50mL
100% sheep serum	10%	50mL
10% blocking reagent	1%	50mL
BSA	1%	0.5g
dH_2O to vol.	--	to 500mL
100% Tween-20	0.1%	0.5mL

Mix TBS, serum, blocking reagent, BSA & dH_2O as shown above.
Filter through #2 Whatman filters.
Add Tween-20.
Aliquot 6mL volumes into conical tubes & store at –20°C.

Sheep serum (must be heat-inactivated before use).
To heat inactivate:

- thaw new bottle of serum
- incubate at 70°C for 30 min
- aliquot & store at –20°C

10% Blocking reagent

stock solution	final conc	for 100mL
maleic acid	100mM	1.2g
5M NaCl	150mM	3mL
dH_2O to vol.	--	to 100mL
Blocking reagent	10%	10g

Mix maleic acid, NaCl & dH_2O according to above, pH to 7.5 (note: strong buffer so difficult to pH, try using solid NaOH pellets to raise pH initially).
Add blocking reagent, microwave briefly to aid solubility (avoid boiling over, solution will be cloudy & viscous so watch carefully to ensure blocking reagent is completely dissolved in solution).
Aliquot 10 mL volumes into conical tubes & store at –20°C.

Day 3

2M Levamisole: Dissolve 4.82g levamisole in ~7mL double-distilled H_2O (total volume should equal 10mL), aliquot 200µL volumes & store stocks at –20°C.
TBSTw + levamisole: 1X TBSTw containing 2mM levamisole.
NTMT + levamisole (inhibits endogenous alkaline phosphatases):

stock solution	**final conc**	**for 500mL**
1M Tris-HCl, pH 9.5	100mM	50 mL
5M NaCl	100mM	10mL
1M $MgCl_2$	50mM	25mL
dH_2O	--	415mL
100% Tween-20	0.1%	0.5 mL
2M Levamisole	2mM	0.5mL

Mix Tris, NaCl, $MgCl_2$ & dH_2O as shown above.
Pass through 0.2µm filter to remove insoluble material/contaminants. Store at 25°C.
DO NOT add Tween or levamisole to stock solution.
Prior to using NTMT for ISH, add 1µL of 100% Tween-20 and 1µL of 2M levamisole per 1mL NTMT stock.
3% H_2O_2: 1mL 30% H_2O_2 per 9mL PBSTw.

Abbreviations

BSA = bovine serum albumin
DIG = digoxigenin
H_2O_2 = hydrogen peroxide
PFA = paraformaldehyde
PBS = phosphate-buffered saline
PBSTw = 1X PBS + 0.1% Tween-20

SDS = sodium dodecyl sulfate (aka lauryl sulfate)
SSC = saline sodium citrate
TBS = Tris-buffered saline
TBSTw = 1X TBS + 0.1% Tween-20

Reagents and Supplies

Anti-DIG antibody, Fab fragments, cat # 11214667001, Roche
Blocking reagent, cat # 11096176001, Roche
BM Purple AP substrate, precipitating, cat # 11442074001, Roche
BSA, cat # BP1600-100, Fisher
Formamide, cat # F5786-1L, Sigma
Glutaraldehyde, 25% solution in H_2O, cat # G6257-100ML, Sigma
Heparin, sodium salt, cat # H3393, Sigma
Hydrogen peroxide, 30% solution in H_2O, cat # BP2633-500, Fisher
Levamisole, cat # L9756, Sigma
Magnesium chloride, cat # M33-500, Fisher
Maleic acid, cat # M0375-500G, Sigma
Paraformaldehyde, cat # 101176-014, VWR
PBS, w/out Ca & Mg, MP Biomedicals powdered media, cat # ICN1760420, Fisher
Proteinase K solution, 20mg/mL, biotechnology grade, cat # E195-5ML, Amresco
RNase, cat # R6513, Sigma
SDS, cat # S529-500, Fisher
Sheep serum, cat # S2263-500mL, Sigma
Sodium azide, granular, cat # S227I-100, Fisher
Sodium chloride, cat # BP358-212, Fisher
SSC, 20X solution, cat # S24022-4000.0, Research Products International
Tris-HCl, cat # BP153-1, Fisher
Tween-20, cat # BP337-100, Fisher
Yeast tRNA, cat # 109495, Roche
Polyester mesh, 33 micron, 12" x 24", cat # CMY-0033-D, Small Parts Inc.

Title: Bacterial Toxicity Assay (from Dr. Eric Domyan)
Rationale and background: Test the toxicity (to *E. coli*) of IPTG-induced expression of different plasmid inserts.
Protocol:

1. For each construct you want to test, add 15 mL LB + 15uL 100mg/mL carbenicillin to a 15mL culture tube.
2. Pick a single colony from a plate with a micropipette tip; swirl tip in your tube of LB+ carb.
3. Incubate tube of LB at 37°C with shaking at 300rpm for 1 hr.
4. Split the culture into 6 x 2 mL aliquots in 15 mL culture tubes.
5. To 3 of the tubes, add 45uL 100mg/mL IPTG.
6. Incubate tubes O/N with shaking at 37°C.
7. The next day, determine the OD600 with a spectrophotometer.

   LB recipe: pH=7.0 (1L of ddH2O)
   10g NaCl
   10g Bacto Tryptone
   5g Yeast Extract

   1. Fill container with 750L of ddH2O