Digoxigenin-Labeled *In Situ* Hybridization for P1 Mouse Kidney Sections

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This protocol is a revision of protocols by Jason Bielagus based on protocols from Dr. M Wijgerde and Drs. J and A McMahon. Post-hybridization washes are modified from Cliff Tabin laboratory protocol. M Wijerde's protocol was based on protocols from the Jessell Lab. J and A McMahon's protocol was based on protocols from D Wilkinson, P Ingham, R Conlon, B Rosen, and R Harland, which are described in detail in In Situ *Hybridization: A Practical Approach*, edited by D Wilkinson, IRL Press, Oxford University (1992).

I. Tissue Preparation

1. Isolate mouse kidneys.
2. Fix in 4% PFA / PBS at 4^{o}C on a rotator for 24 hrs.
3. Rinse in PBS for 5 min, 3x. The last PBS wash 4h-O/N.
4. Treat kidneys in 30% sucrose / PBS at 4^{o}C on a rotator overnight.
5. Remove sucrose solution and add fresh 30% sucrose / PBS for 1hr. Add equal volume of OCT, 4C rotate 30min.
6. Swirl kidney pairs in 5 dishes of OCT, incubate for about 5' each. Freeze kidneys in OCT by placing kidney pairs in plastic molds, pouring in OCT, arranging the kidneys, and floating the mold on a bath of ethyl alcohol and dry ice.
7. Store frozen kidney blocks at -80^{o}C.
8. Section kidneys into 20μm sections. Be sure to use "precleaned superfrost plus" slides (VWR 48311-703). Air dry sections for at least 30min. Store at -80^{o}C.

Note:
1. Duration of 4%PFA fixation of 3, 6, 9, 12, 15, 18, 21 and 24h were compared. No difference was seen, therefore 12-15h (overnight) was chosen.
2. 10, 16, 25, and 50um-thick sections were tested. The 10um section produced better resolution of single layers of cells, and was therefore chosen.

II. Probe Transcription

See "Plasmid purification, probe synthesis and manual cloning" Protocol.

Note: probe length of 0.5kb, 1kb, and 1.5kb was tested. The signal intensity was not affected, so 1kb probe is chosen.

III. *In Situ* Hybridization – Hybridization

Part A

Preparation Steps:
A. Remove slides from -80 freezer to room temperature and allow to dry for **1 hour.**
B. Wash glass trays and slide holder with Rnase Zap. Rinse thoroughly!
C. Make 4% PFA by combining PFA powder and PBS (no need to regard volume change!). Place in 65C oven and shake from time to time in order for the PFA powder to dissolve. The mixture is ready when it appears transparent. This will usually take at least **30 minutes**. Finally, filter sterilize the 4% PFA and place on ice.

1. **Fix:** Fix sections in 4% PFA / PBS for **10 min**. !!Do not discard, save for step 5!!

2. Rinse in PBS for **3 min, 3x**.

3. **Proteinase K:** Treat with 10µg/ml of Proteinase K in PBS for **10 min**.

4. Rinse for **3 minutes** in PBS, **3x**.

5. **Fix:** Fix in 4% PFA / PBS for **5 min**.

6. Rinse in PBS for **3 min, 3x**.

7. **Acetylation:** In a glass histology jar on a stirrer, combine

	H_2O	200.00ml
	Triethanolamine	2.66ml
	37% HCl	0.35ml
Begin stirring and add:	Acetic Anhydride	0.75ml

Note: It is helpful to have the slides on hand when the acetic anhydride is added, to allow a quick change of reagents.
Place rack of slides in jar, while stirring, for **10 min**.

!!Acetylation reagents must be disposed of in the designated chemical waste jar!!

8. Rinse in PBS for **5 min, 3x**.

9 Rinse with 0.85% NaCl for **3min**.

10 Wash with 70%EtOH/0.85%NaCl for **5min**.

11 Wash with 95% EtOH for **5min.**

12 Air dry for **10min** on Wattman Paper.
Note: This time can be used to dilute probes.

13. **Hybridization:** Dilute probes with prehybridization buffer such that the probe concentration is 500ng/ml. Heat the diluted probe to 80^{o}C for 5 min.

Arrange slides horizontally in a humidified chamber (humidified with 50% formamide/5X SSC), remove excess solution, apply ~250µl of hybridization buffer with probe to each slide, and apply parafilm. Incubate at 68^{o}C overnight.

Note:
1. Proteinase K concentration of 0, 5, 10, 15, and 20ug/ml were tested. 15ug/ml ProtK produced the strongest signal with good tissue morphology.
2. 1.3xSSC hybridization buffer (Henrique lab) and 5xSSC hybridization buffer were compared with Tcn2 and Ihh probes. The signal intensity is similar with both hybridization buffers, but the background is weaker with 1.3xSSC hybridization buffer.

III. *In Situ* Hybridization – Antibody

Part B

Preparation Steps:

A. Heat oven to 68C.

B. Make necessary reagents (listed in steps below) and heat to designated temperatures. Allow about **1 hour** for preparation time!

Immerse slides in 5X SSC at 68°C to allow parafilm to separate (**5 min**).

1. Wash slides in 1X SSC/50% formamide at 65°C for **30 min**.

2. TNE (10mM Tris pH7.5, 500mM NaCl, 1mM EDTA) at **37C for 10min**.
   800 ml total
   8 ml 1M Tris pH 7.5
   80 ml 5M NaCl
   1.6 ml EDTA
   Fill with H2O

3. RNase A (2ug/ml) in TNE at **37C for 15min**.

4. TNE at **37C** for **10min**.

5. 2xSSC at **65C** for **20min**.

6. 0.2xSSC at **65C** for **20min x2**.

7. MBST wash at RT for **5min x3**.

8. Make 20% HISS + 2% Blocking/MBST. Arrange slides horizontally in a humidified chamber (slide mailer humidified with water), remove excess solution, apply ~250µl of 20% HISS + 2% BR / MBST to each slide, and apply parafilm. Incubate at RT for 1-2 hrs.

9. While slides are incubating, prepare the antibody solution (for anti-Dig-AP, it is not necessary to preabsorb, just dilute Ab at 1:4000 in 1%HISS+2%BR/MBST):

10. Keep slides arranged horizontally in a humidified chamber (humidified with water), remove excess solution, apply ~150µl of antibody solution to each slide, and apply parafilm. Incubate at 4°C overnight.

Note:
Anti-Dig AP of 1:1000, 1:2000, 1:4000, and 1:8000 dilutions were tested. The signal intensity was not affected with the different dilutions. So 1:4000 is chosen for all analysis.

III. *In Situ* Hybridization – BM Purple AP Substrate

Part B

If parafilm was used, immerse slides in MBST to separate it from the slides.

1. Rinse slides in MBST on a rotator at RT for **5 min, 3x**.

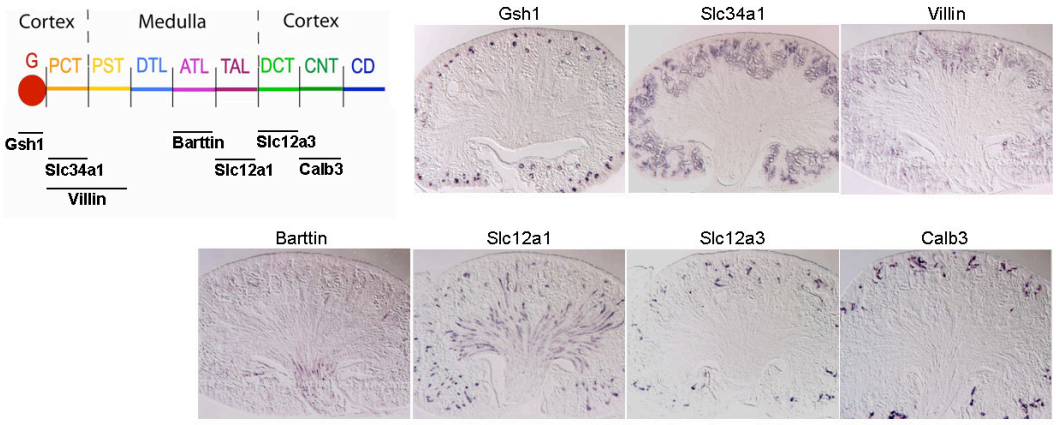
2. Rinse slides in NTMT, pH9.5 for **10 min**.

3. Spin BM purple at 2000rpm for 5min (try to avoid using BM Purple from the bottom of the bottle). Add BM Purple to slides in humidified chamber. Incubate at RT in dark.

   For low-signal probes (e.g. *Shh*), a longer development time than overnight may be required. Maximum development time is 7days before background comes up.
   The slide mailers hold up to 15mls but less is needed to cover the sections. Each slide mailer holds 5 slides (when develop in slide mailers).

Control Probes and Markers

SISH References



IV. Archiving and Photography

Mount Slides

Confirm that the slides have had enough time to develop.

1. Rinse slides in PBS for **5 min, 3x**. PBT pH 4.5 can be used instead of PBS. TE pH 8.0 can be used in place of the first PBS rinse.

2. Fix slides in 0.2% gluteraldehyde + 4% PFA / PBS at **RT for 30min** or **overnight at 4C**.

3. Rinse slides in PBS for **5 min, 3x**.

   Optional – Rinse slides briefly in 70% ethyl alcohol.

4. Let slides dry.

5. Mount cover slips with Glycergel (which has been heated to 60^{o}C).

Photography

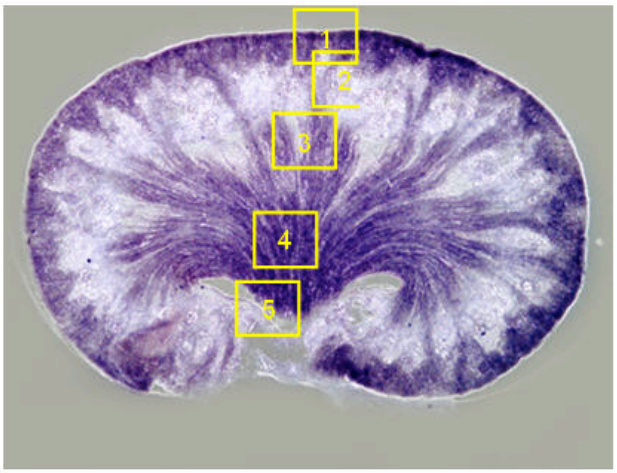
1. We photograph samples with a Nikon DXM1200 digital camera attached to a Nikon Edge scope.

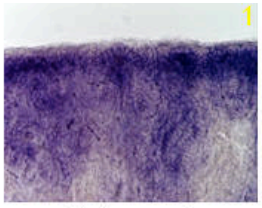
2. 6 photos per gene: 1 global view at 100X and 5 regional (outter cortex, juxtamedullary cortex, outter medulla, inner medulla, renal pelvis), each at 4000X.

   White balance should be adjuted so that the color of the digital image matches the color of the real image. The center of the bladder generally provides a good white reference point. Non-expressing regions can also provide a white reference.

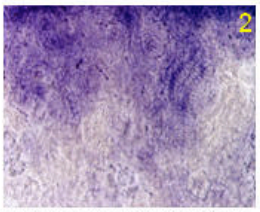
3. Naming protocol is "YYMMDDi[type][initial]_###". For example, the eighth photograph of a whole mount *in situ* hybridization sample taken by John Harvard on May 15, 2001 is called "010515iWMjh_008."

   Also, as each sample is photographed, a separate Excel document is maintained to record filename, gene name, MTF, zoom, and BM Purple development time

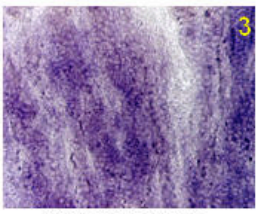




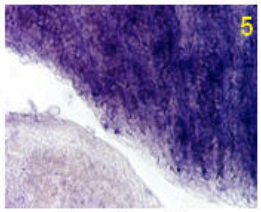
Outer cortex



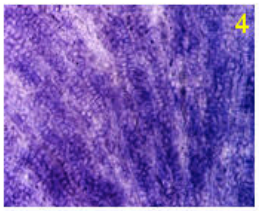
Juxtamedullary cortex



Outer medulla



Renal pelvis



Inner medulla

V. Solutions and Materials

Hybridization Buffer (5xSSC)	50% formamide (Fisher BP 227 100) 5X SSC pH 4.5 (use citric acid to adjust pH) 50µg/ml yeast tRNA (Gibco 15401-011) 1% SDS 50µg/ml Heparin (Sigma H8514)
Hybridization buffer (1.3xSSC)	50% formamide 1.3x SSC pH4.5 5mM EDTA (pH8.0) 50ug/ml yeast tRNA 0.2%Tween-20 0.5% CHAPS 100ug/ml Heparin
Proteinase K	(Roche 161 519). Dilute with water. Store at –70°C.

SSC Adjust pH to 4.5 with citric acid (~1.35g/100ml)

10xMBST stock (no Tween-20)
1M Maleic acid (Sigma M0375)
1.5M NaCl
pH with NaOH to pH7.5 (start with 70g/l)

MBST Dilute from 10xMBST and add Tween-20 to 0.1%.

NTMT
2ml 5M NaCl
5ml 2M Tris, pH9.5
5ml 1M $MgCl_2$
0.1ml Tween-20
Add H_2O to 100ml
2mM Levimasole

BR 2% Blocking Reagent (Boehringer Mannheim 1096 176) / MBST. Solution must be heated and agitated for BR to go into solution.

HISS Heat Inactivated Sheep Serum

Embryo powder

1. Isolate E12.5-14.5 mouse embryos.
2. Homogenize embryos in a minimal amount of PBS.
3. Add a volume of acetone 4x greater than the volume of the homogenized embryos in PBS. Mix and incubate for 30 min.
4. Spin at 10,000rpm for 10 min. Remove supernant.
5. Repeat steps 3 and 4.
6. Scrape pellet into a paper towel. Let the pellet dry, covered in a paper towel, at RT overnight.
7. Grind the pellet with a mortar and pestle.
8. Store dry, as powder, in a dessicator, at 4^{o}C.

Anti-Digoxigenin-AP Fab Fragments (Roche 1093 274)

BM Purple AP Substrate, precipitating (Roche 1 442 074)

Notes

For a humidified chamber, use either a plastic box with pipettes glued to the bottom (upon which the slides will rest, above the solution in the bottom), or a 100-slide slide box. To properly humidify the chamber, the solution in the bottom need not be too deep, but it needs to entirely cover the bottom of the box.

Be sure to keep the sections from drying out. Do try to remove as much of the previous solution from a slide before applying a new solution, but take care not to let the tissue sections on the slide become dry. Drying out the sections increases background.

Some solutions must be heated and agitated to go into solution (e.g. PFA, BR). Some ways to heat and agitate solutions are as follows:

- Combine solute and solvent in a beaker on a stirrer/hotplate, cover solution, and set stirrer and hotplate to low (2 or less). If PFA is done this way, the stirrer/hotplate and solution should be under a hood.
- Combine solute and solvent in a sealed tube and place in a rotator in a hybridization oven set to 65^{o}C.
- Combine solute and solvent in a sealed tube and place in a rotator in the warm room.

NT (NTT without the Tween-20) can be used in place of NTT in steps III.3.4 and III.5.2.

If there is a need to decrease background signals, try decreasing the antibody concentration up to half (i.e. 1:8000 in place of 1:4000), use 1.3XSSC hybridization buffer, or include a RNase step post hybridization.

Decant BM Purple before using. Try not to use BM Purple from the bottom of the bottle. A yellow-white precipitate forms at the bottom of the bottle, which, if used, leaves white and blue speckles on the material.

If “precleaned superfrost plus” slides are unavailable, regular slides can be used if pretreated:

1. Dip slides successively in 10% HCl / 70% EtOH, distilled water, then 95% EtOH.
2. Dry in 150^{o}C oven for 5 min.
3. Dip in 2% TESTA / acetone for 20 sec, then wash in acetone 2x, and in water 3x.
4. Dry at 42^{o}C overnight.

TESTA -- 3-Aminopropyltriethoxy-silane (Sigma A-3648)

If there is difficulty in visualizing a low-signal probe (e.g. *Shh*), try the following:

- Alternative hybridization buffer:

  Hybridization Buffer (2)
  50% formamide
  5X SSC
  5X Denhardt's
  250mg/ml Baker's yeast RNA (Sigma R6750)
  500ng/ml herring sperm DNA
  For Denhardt's, see *Molecular Cloning*.

- Decrease the hybridization temperature by 5-10^{o}C. If the hybridization temperature is reduced, then likewise reduce the temperature of the subsequent washes.

- Increase the probe concentration.

- Increase the probe length.

- Use RNase after hybridization and before the antibody.

- Decrease the antibody concentration by up to a half (i.e. 1:8000 in place of 1:4000).

- Increase the development time of BM Purple.