Section *In Situ* Hybridization (SISH) of Embryonic Kidneys with Digoxigenin-Labeled Probes

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This in situ protocol is suitable for routine use with moderate to strong riboprobes on frozen embryonic and newborn tissues.

Tissue Preparation

The following steps have been successfully used on embryonic and newborn tissues for SISH.

- Dissect "trunks" of embryos from just below the forelimbs to the tail. The tail may be removed for PCR genotyping.
- Fix in 4%PFA/PBS at 4°C on a rotator for 24 hrs.
- Rinse in PBS for 5 min, 3x. The final PBS wash can be extended for 4h-O/N for convenience.
- Treat kidneys in 30% sucrose/PBS at 4°C O/N on a rotator.
- Remove sucrose solution and add fresh 30% sucrose/PBS for 1hr. Add an equal volume of OCT and mix well/rotate for 30min at room temperature.
- Swirl kidney pairs/embryonic trunks 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.
- Store frozen kidney blocks at -80oC.
- Section kidneys at 20µm thickness. Be sure to use "precleaned superfrost plus" slides (e.g. VWR 48311-703) or similar charged slides. Air-dry sections for at least 30min. Store at -80°C up to 1 month.

Note: We tested fixation times of 3, 6, 9, 12, 15, 18, 21 and 24 hours. No difference was seen, therefore 12-15h (overnight) was chosen. We also tested 10, 16, 25, and 50um-thick sections were and found 20um sections produced balance between sensitivity and resolution.

Note: We find reduced sensitivity with prolonged storage of the sectioned material. Limit the storage of slides to one month and use air sealed containers to avoid reduced sensitivity. Cryopreservation of the uncut blocks preserves sensitivity indefinitely if properly sealed to avoid moisture loss. This is best achieved with zip-lock freezer bags containing the blocks.

Hybridization

Preparation Steps:

- Remove slides from -80°C freezer to room temperature and allow to dry for 1 hour.
- Prepare Coplin jars. Use two sets, a PRE-HYB and POST-HYB set so that RNase never touches the PRE-HYB set. Rinse thoroughly!
  1. Fix sections in 4% PFA/PBS for 10 min. *Do not discard, save for step 5.*
  2. Wash in PBS for 3 min, 3x.
  3. Incubate with 10µg/ml of Proteinase-K in PBS for 10 min.

4. Wash for 3 minutes in PBS, 3x.
5. Fix in 4% PFA / PBS for 5 min.
6. Wash in PBS for 3 min, 3x.
7. Acetylation: To make 30ml for a single Coplin jar, combine the following in a conical tube:
    a. 29.5ml H2O
    b. 400ul Triethanolamine
    c. 52.5ul 37% HCl
    d. 112.5ul Acetic Anhydride
    e. Close tightly and shake vigorously to mix. Be ready to add to the slides immediately after mixing.
    f. Discard the PBS wash from slides and add the acetylation solution. Place on shaker for 10 min.
    g. !!Acetylation reagents must be disposed of in the designated chemical waste jar when done!!
8. Wash in PBS for 5 min, 3x.
9. Wash with 0.85% NaCl for 3min.
10. Rinse with 70%EtOH/0.85%NaCl for 5min.
11. Rinse with 95% EtOH for 5min.
12. Air dry for 10min.
    i. 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°C for 5 min.
    i. Arrange slides horizontally in a humidified chamber (humidified with 50% formamide/5X SSC), remove excess solution, and apply ~250µl of hybridization buffer with probe to each slide, and apply Parafilm. Incubate at 68oC overnight.

Note: Proteinase K concentration of 0, 5, 10, 15, and 20ug/ml were tested. 10ug/ml ProtK produced the strongest signal with good tissue morphology.

Note: 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.

Post-Hybridization Washes

Preparation Steps:

- A. Heat oven and Coplin jars to 68°C.
- 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 37°C for 10min.
    a. 800ml total
    b. 8ml 1M Tris pH 7.5
    c. 80ml 5M NaCl
    d. 1.6ml EDTA
    e. Fill with H_2O

3. RNase A (2ug/ml) in TNE at 37°C for 15min.
4. TNE at 37°C for 10min.
5. 2xSSC at 65°C for 20min.
6. 0.2xSSC at 65°C for 20min x2.
7. MBST wash at RT for 5min x3.
8. Encircle sections on slide with a PAP pen.
9. Encircle sections on slide with a PAP pen and arrange slides horizontally in a humidified chamber (use MBST to humidify chamber). Apply ~250µl of 20% HISS + 2% BR/MBST to each slide. Incubate at RT for 1-2 hrs.
10. While slides are incubating, prepare the antibody solution (for anti-Dig-AP, it is not necessary to pre-absorb, just dilute Ab at 1:4000 in 1%HISS+2%BR/MBST):
11. Keep slides arranged horizontally in a humidified chamber (humidified with water), remove excess solution, and apply ~150µl of antibody solution to each slide. 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.

Development, Fixation, and Preparation for Data Collection

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.
    a. 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.
    b. 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).

<u>Mounting</u>

1. Confirm that the slides have had enough time to develop.
2. Rinse slides in PBS for 5 min, 3x.
3. Fix slides in 4% PFA/PBS + 0.2% gluteraldehyde at RT for 30min or overnight at 4°C.
4. Rinse slides in PBS for 5 min, 3x.
5. Rinse slides briefly in 70% ethyl alcohol.
6. Let slides dry.
7. Mount cover slips with Glycergel (which has been heated to 60°C per manufacturer's instructions).
    a. NOTE: We have also used Cytoseal 60 with success. The solvent in this sealant does not appear to affect the BM purple precipitate signal and is easier to use than Glycergel.

Photography

- 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”. The two letter type code is as follow: SX, section; IM, immunostaining; WM, whole-mount; GM, gross morphology.
- Adjust white balance 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.
- Record filename, gene name, sex, zoom, and BM Purple development time.

Authors/Contributors to prior versions of this protocol.
This protocol is a revision of protocols by Jason Bielagus and Arindam Majumdar based on protocols from M. Wijgerde, Jill and Andy McMahon. Post-hybridization washes are modified from Cliff Tabin’s laboratory protocol. M. Wijerde’s protocol was based on protocols from the Jessell Lab. Jill and Andy 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).

Jing Yu in Andy McMahon’s laboratory developed the optimization and other adjustments that produced the kidney optimized form for high throughput SISH (~40-60 slides) that is published on the GUDMAP.org website (2007).

Todd Valerius has further modified this protocol for small scale (~9-18 slides) suitable for routine SISH using his preferred equipment.

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 MgCl2
0.1ml Tween-20
Add H2O 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.
Sheep serum (Sigma S2263) should be heat inactivated to kill endogenous alkaline phosphatase activity by heating serum at 70°C for 30 minutes, then stored at -20°C in aliquots. Be sure to open cap slightly and swirl serum, occasionally, during inactivation. We have found certain lots coagulate and opening the cap helps reduce this behavior.

Embryo powder (if used)

We do not use embryo powder in our experiments on kidney, but the procedure is left here in case needed.

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 10min. Remove supernatant.
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°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°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 150oC 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°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:
Hyb Buffer 2
50% formamide
5X SSC
5X Denhardt's
250mg/ml Baker's yeast RNA (Sigma R6750)
500ng/ml herring sperm DNA
Decrease the hybridization temperature by 5-10°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.

What is the difference between a "wash" and a "rinse" in this protocol? A wash indicates agitation on a rotator. A rinse is done at the bench without added agitation. The act of pouring in the solution mixes well enough for the purpose of the rinse steps.

I prefer to use glass Coplin jars in this protocol for several reasons.
Each holds 9 slides and working with 9-18 slides is comfortable.
The glass holds temperature well enough for the hot washes.
Changing solutions is very simple by dumping the wash off while holding the slides with a gloved finger.
The volumes are reasonable – 30mls for each wash. This colume is easily mixed in 50ml conicals also.