
1. Allow a five-slide mailer of cryosections to equilibrate to room temperature for 15 min
   - Frost on the cryosections can damage morphology

2. Open mailer and identify the corners of each section with a lab marker
   - Once the slides have been hydrated, it will be difficult to tell where the sections are on the slide
   - Once the slides have been hydrated, do not let them dry out again! This will irrecoverably increase the autofluorescence of the tissue.

3. Pretreat sections with 0.2 N HCl for 10 min
   - Acid treatment dramatically increases signal by denaturing RNP’s and freeing transcripts for hybridization
   - Fixing in 3.7% PFA + 5% acetic acid can also increase signal, but the background is higher
   - Proteinase K pretreatments cause unpredictable loss of cells from the slides

4. Wash slides in PBS for 1 × 5 min

5. Postfix slides in freshly prepared 3.7% PFA and incubate for 15 min
   - Stock solutions of 37% PFA should be prepared according to Wang et al. Meth. Enzymol. 85:514 (1982) and stored in single-use aliquots at −20°C
   - Fix slides in a fume hood and dispose of PFA as hazardous waste

6. Wash slides in PBS for 2 × 10 min
   - The same PBS jar can be used for both washes

7. Equilibrate slides in freshly prepared 0.1 M triethanolamine (pH 8.0) for 10 min

8. Acetylate slides with 0.25% acetic anhydride in freshly prepared 0.1 M triethanolamine (pH 8.0) for 5 min
   - Acetic anhydride has a half-life of ~1 min in aqueous solutions
   - Add 100 µl of acetic anhydride to 40 mL of triethanolamine, shake vigorously, and add slides immediately (process multiple jars separately)

9. Wash slides in 2× SSC for 10 min

10. Dehydrate slides in 70% EtOH for 2 min, 95% EtOH for 2 min, 100 EtOH for 2 min, and then air dry for at least 5 min
   - EtOH dehydration increases the specificity of hybridization by drawing the hybridization mix better into the section

11. Add 10 µl hybridization solution to each section (20 µl per slide), cover with parafilm, seal with rubber cement, and incubate in a humid chamber for 14–16 hr at 42°C
   - Prehybridization causes an unacceptable loss of cells from the slides (in my hands)
   - Small squares of parafilm keep the hybridization solution from evaporating but are gentler to remove than glass coverslips
   - Lower hybridization temperatures (37°C) increase background, whereas higher temperatures (50°C, 55°C) decrease signal
   - Shorter or longer incubation times have not been tested
   - Water-saturated chambers appear to be fine if the samples are sealed in rubber cement

12. Soak slides in 2× SSC for ~5 min at 37°C

13. Remove rubber cement and parafilm from each section and wash slides in 2× SSC/50% formamide for 30 min at 55°C
   - RNAses treatments of any kind dramatically decrease signal intensity
   - Wash slides in a fume hood and dispose of formamide as hazardous waste
   - Higher temperatures (60°C) dramatically decrease signal intensity

14. Wash slides in 0.1× SSC for 30 min at 55°C
   - Higher temperatures (60°C) dramatically decrease signal intensity

15. Equilibrate slides in PBS for 10 min

16. If using Alexa-labeled riboprobes alone, proceed directly to Step 19
17. Block slides in a humid chamber for 1 hr at room temperature in blocking solution: 1× Western Blocking Reagent (Roche #11921673001) diluted in PBS + 0.3% Tween-20
   - Humid chambers can be constructed with an inverted pipette-tip box containing a paper towel saturated with water.
   - 5% BSA can be substituted for Western Blocking Reagent, but nonspecific intracellular staining may increase.
   - 100 µl of blocking solution is sufficient for each slide.
   - Cover the top of each slide with parafilm to reduce evaporation.
   - Substituting Triton for Tween increases background staining of the Matrigel (permeabilization is not required for thin sections).

18. Add anti-digoxin (Jackson ImmunoResearch #200-002-156) at 1:500 dilution or anti-dinitrophenyl (Invitrogen #A6430) at 1:1000 dilution in blocking solution and incubate 1 hr at room temperature:
   - 100 µl of primary-antibody solution is sufficient for each slide.
   - Cy3-conjugated anti-digoxin can be used for direct detection, but signal will be amplified if secondary anti-mouse antibodies are used.
   - Longer incubations with Cy3 conjugate may improve sensitivity but will be incompatible with other mouse primary antibodies.
   - Cover the top of each slide with parafilm to reduce evaporation.

19. Wash slides 3 × 5 min in PBS.

20. Add Alexa-conjugated anti-mouse and anti-rabbit at 1:200 dilution in blocking solution and incubate 1 hr at room temperature.

21. Wash slides 3 × 5 min in PBS.

22. Counterstain slides with 0.5 µg/ml DAPI in PBS (to label nuclei) plus 2 µg/ml DyLight 750 wheat-germ agglutinin (to label plasma membranes for image segmentation) for 5 min:
   - We have custom labeled wheat-germ agglutinin (MP Biomedicals #790164) with the Cy7 analog, DyLight 750 (Pierce #46423) for dual staining with DAPI.
   - Alternatively, 20 µg/ml Alexa-350 wheat-germ agglutinin (Invitrogen #W11263) can be used without DAPI as a substitute for DyLight 750 wheat-germ agglutinin.

23. Wash slides 2 × 5 min in PBS.

24. Incubate sections with 10 mM CuSO₄ in 50 mM NH₄Ac (pH 5.0; acidify with acetic acid) for 10 min:
   - CuSO₄ treatment dramatically reduces autofluorescence of sections, while only slightly diminishing the fluorescence of Cy- and Alexa-family dyes (Schnell et al. J. Histochem. Cytochem. 47:719 (1999)).
   - Higher concentrations of CuSO₄ reduced signal, whereas lower concentrations are less effective at quenching autofluorescence.

25. Wash slides 5 min in PBS.

26. Mount with an antifade mounting medium and seal with nail polish:
   - We use 0.5% N-propyl gallate in 90% glycerol + 1× phosphate buffer (pH 8.0).
   - To reduce spherical aberration, use a minimal amount of mounting medium between the coverslip and slide. We use 10 µl per 24 × 50 mm coverslip (allow to spread slowly across the entire surface of the coverslip).

27. Seal the edges of the coverslip with nail polish and allow to air dry.

28. Remove residual salts with a wet Kimwipe and air dry.

29. Store slides at 4°C until imaging.
   - Autofluorescence will be suppressed in mounted sections for a few days, but it will increase thereafter as Cu²⁺ ions diffuse out of the tissue.
Buffer recipes

- **0.1 M triethanolamine** Make fresh
  2.64 ml triethanolamine
  ~180 ml H₂O
  pH solution to 8.0 with concentrated HCl
  Volume with H₂O to 200 ml

- **2.9x hybridization buffer** Store at –20°C as 200 µl aliquots
  800 µl 50% dextran sulfate (Chemicon #3730-100ML)
  200 µl 20 mg/ml yeast tRNA (Invitrogen #15401-029)
  400 µl 20× SSC (Promega #V4261)
  1.4 ml total volume
  Add 3 µl probe mix in H₂O to 10 µl formamide, denature at 65°C for 30 sec and hold at 42°C. Then, add 7 µl 2.9× hybridization buffer at 42°C, mix well, and add to slide (10 µl per section)