RNA fluorescence in situ hybridization on cryosections Entered by Kevin Janes
Janes Lab Protocols 4/15/11

When referring to this protocol, please cite: Janes KA, Wang CC, Holmberg KJ, Cabral K, Brugge JS. (2010) Identifying single-cell molecular programs by stochastic profiling. *Nat Methods*, 7, 311-7.

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
    - *RNAse 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 $CuSO_4$ in 50 mM NH_4Ac (pH 5.0; acidify with acetic acid) for 10 min
    - *$CuSO_4$ 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 $CuSO_4$ 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 Cu^{2+} ions diffuse out of the tissue*

Buffer recipes

- **0.1 M triethanolamine** Make fresh
  2.64 ml triethanolamine
  ~180 ml H_2O
  pH solution to 8.0 with concentrated HCl
  Volume with H_2O to 200 ml

- **2.9× hybridization buffer** Store at –20°C as 200 µl aliquots
  800 µl 50% dextran sulfate
  200 µl 20 mg/ml yeast tRNA
  400 µl 20× SSC
  1.4 ml total volume
  Add 3 µl probe mix in H_2O 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)