Immunofluorescence on cryosections Entered by Kevin Janes
Janes Lab Protocols 12/12/08

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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*
3. Hydrate slides 3 × 5 min in PBS.
4. 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)*
5. Add primary antibody at the appropriate dilution in blocking solution overnight at room temperature in a humid chamber.
   - *100 μl of primary-antibody solution is sufficient for each coverslip*
   - *Antibody dilutions must be determined empirically, although 1:100 or 1:200 dilutions are common*
   - *Cover the top of each slide with parafilm to reduce evaporation*
6. Wash slides 3 × 5 min in PBS.
7. Add secondary antibody at the appropriate dilution in blocking solution for 1 hr at room temperature in a humid chamber.
   - *100 μl of secondary-antibody solution is sufficient for each coverslip*
   - *We use 1:200 dilution of species-specific, highly-crosslinked Alexa-labeled antibodies from Molecular Probes; these fluorochromes do not need to be protected from ambient light during the staining process*
   - *Cover the top of each slide with parafilm to reduce evaporation*
8. Wash slides 3 × 5 min in PBS.
9. Counterstain slides with 0.5 μg/ml DAPI in PBS (to label nuclei) or 20 μg/ml Alexa-350 wheat-germ agglutinin (to label plasma membranes for image segmentation; Invitrogen #W11263) for 5 min
10. Wash slides 2 × 5 min in PBS
11. 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*
12. Wash slides 5 min in PBS
13. 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).*
    - *Remove any drops of PBS on the slide with a Kimwipe before mounting. These will dilute out the mounting medium and lower its refractive index.*
14. Seal the edges of the coverslip with nail polish and allow to air dry.
15. Remove residual salts with a wet Kimwipe and air dry.
16. 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*