Whole-mount immunofluoresence of MCF10A 3D structures Entered by Kevin Janes
Janes Lab Protocols 8/24/10

When referring to this protocol, please cite: Wang L, Brugge JS, Janes KA. (2011) Intersection of FOXO and RUNX1 gene-expression programs in single breast epithelial cells during morphogenesis and tumor progression. *Proc Natl Acad Sci*, 108, E803-12.

1. Aspirate the assay medium from the chamber slide, wash gently with 500 μl PBS per chamber, and then fix with 500 μl 2% PFA for 20 min at room temperature.
   - *For all aspiration steps, use a P200 micropipet tip to ensure that the samples are not inadvertently aspirated by the vacuum*
   - *Throughout the assay, use 500 μl washes per chamber on the slide, and add the wash solutions as slowly as possible to avoid detaching the 3D structures*
   - *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*
   - *The lower percentage of PFA in this protocol allows better antibody penetration into the structures*
   - *Alternatively, acini can be fixed in ice-cold 50% methanol-50% acetone for 10 min at –20°C, which can be important for certain antibodies to work successfully*
2. Permeabilize with 200 μl 0.5% Triton X-100 for 10 min at 4°C.
   - *Try to make sure that the permeabilization solution does not touch the top of the chambers, because it will create bubbles in the chambers throughout the rest of the protocol*
3. Wash 3 × 10 min with freshly prepared PBS + 100 mM glycine at room temperature on a rocking platform.
   - *The glycine quenches unreacted formaldehyde groups from the PFA fixative*
4. Block for 1-1.5 hr at room temperature with 200 μl blocking solution: 1× Western Blocking Reagent (Roche #11921673001) diluted in IF buffer.
5. Aspirate the blocking solution and add 100 μl primary antibody diluted in blocking solution. Seal the chamber with Parafilm and incubate at 4°C overnight on a rocking platform.
6. Wash 3 × 20 min with IF buffer at room temperature on a rocking platform.
7. Aspirate the primary antibody solution and add 100 μl secondary antibody diluted in blocking solution. Incubate at room temperature for 45 min on a rocking platform.
   - *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*
8. Wash 3 × 20 min with IF buffer at room temperature on a rocking platform.
9. Counterstain nuclei with 0.5 μM DRAQ5 (Cell Signaling #4084, 1:10,000 dilution) for 15 min at room temperature on a rocking platform.
   - *0.5 μg/ml DAPI in PBS can be used in place of DRAQ5 if the confocal microscope is equipped with a UV or violet laser*
   - *Higher concentrations of DRAQ5 cause fluorescence to increase in the cytoplasm as the probe begins binding RNA*
10. Wash 1 × 5 min with PBS at room temperature on a rocking platform.
11. Aspirate the PBS and gently run a razor blade around the chamber slide to loosen the adhesive binding the chamber separator to the glass slide.
    - *Cut as much of the adhesive as possible, but do not allow the razor blade the nick the matrigel inside the chambers*
12. Use the plastic opener provided with the chamber slides to pry off the separator from the slide.
    - *Using the opener without loosening the adhesive with a razor will often break the glass slide*
13. Carefully aspirate any residual liquid between the chamber samples.
14. Add 4-6 drops of Prolong Gold Antifiade Medium (Invitrogen #P36934) and then gently lay down a 24 × 50 mm No. 1.5 coverslip over the 3D structures.
    - *Do not drop the coverslip on top of the slide; instead, start at one side and lay the coverslip down across the slide*

- *Some bubbles may get trapped between the individual chambers after mounting—do not try to press down on the coverslip to remove these bubbles (this will crush the structures)*
- *After mounting, be careful not to move the slide much, because the coverslip can drift off of the glass slide*

15. Allow the slide to dry overnight, seal the edges with nail polish, and store at 4^{o}C for 1-2 weeks.

Buffer recipes

- **IF buffer** Store at 4°C
  250 mg BSA (final conc: 0.1%)
  125 mg NaN_3 (final conc: 0.05%)
  500 μl Triton X-100 (final conc: 0.2%)
  125 μl Tween-20 (final conc: 0.05%)
  Volume to 250 ml in PBS