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

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. Plate cells at the desired density on 22 × 22 mm No. 1.5 coverslips.
   - *No. 1.5 coverslips provide the proper working distance for microscope objectives*
2. Aspirate the culture medium and wash cells with an appropriate volume of PBS.
3. Fix coverslips in freshly prepared 3.7% PFA and incubate for 15 min at room temperature.
   - *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*
4. Wash coverslips 3 × 5 min in PBS.
5. 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% Triton X-100.
   - *Humid chambers can be constructed with an inverted pipette-tip box containing a paper towel saturated with water*
   - *Coverslips should be handled with fine forecеps and lifted off the bottom of the 6-well plate with a curled hypodermic needle while the PBS is still in the well*
   - *5% BSA can be substituted for Western Blocking Reagent, but nonspecific intracellular staining may increase*
   - *90–100 μl of blocking solution is sufficient for each coverslip*
   - *Place each coverslip face-down on a piece of Parafilm in the humid chamber*
6. Add primary antibody at the appropriate dilution in blocking solution overnight at room temperature.
   - *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*
   - *Place each coverslip face-down on a piece of Parafilm*
7. Wash coverslips 3 × 5 min in PBS.
   - *Washes can be done in a clean 6-well plate*
8. Add secondary antibody at the appropriate dilution in blocking solution for 1 hr at room temperature.
   - *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*
   - *Place each coverslip face-down on a piece of Parafilm in the humid chamber*
9. Wash coverslips 3 × 5 min in PBS.
10. Counterstain coverslips with 0.5 μg/ml DAPI in PBS for 5 min.
11. Wash coverslips 2 × 5 min in PBS.
12. Mount coverslips with 10 μl 0.5% N-propyl gallate in 90% glycerol + 1× phosphate buffer (pH 8.0).
    - *Be sure to get as much of the residual PBS off as possible so that the mounting medium spreads uniformly after mounting; touch the edge of the coverslip on a paper towel and then aspirate any residual drops of PBS*
    - *Warm the mounting medium to room temperature so that it is easier to pipet when mounting*
    - *Place the mounting medium directly on the coverslip (face up) and then try to wick the coverslip gently onto an inverted SuperFrost Plus slide*
13. Seal the edges of the coverslip with nail polish and allow to air dry.
14. Remove residual salts with a wet Kimwipe and air dry.
15. Store slides at 4°C until imaging.