Adherent Cells:

1. Plate cells at low density on 15mm round coverslips cleaned according to attached protocol and sterilized by autoclaving. Allow cells to settle at least 15 minutes, up to overnight.

2. Rinse coverslips 3x by dipping in consecutive beakers containing 1x PBS and blot off excess liquid. Place cell-side up on warming device (if desired) and add 120µl of 2% PFA solution. * Incubate for 30 minutes in a humid chamber.

   Alternate fixation method:
   Place coverslip in 100% Methanol at -20°C for 5 minutes. No subsequent permeabilization is required.

   Note: Cells may be stained with rhodamine-phalloidin by simultaneously fixing, permeabilizing, and staining using PFA/Saponin or Tx-100 and a 1:100 dilution of Rh-Ph for only 10-15 minutes.

3. Again, rinse coverslips 3x by dipping in consecutive beakers containing 1x PBS.

4. Permeabilize PFA-fixed samples by addition of 120µl either 0.02% Saponin or 0.1% Triton X-100 in 1x PBS (both detergents may be stored as a 1% solution at 4°C) for 10 minutes in a humid chamber.

5. Rinse as in #3.

6. Add primary antibody. Primary antibody is diluted in 1x PBS plus 0.1% BSA (NOT immunoglobulin-free) beginning with a 1-2µg/ml dilution. Sequential dilutions may be used to maximize results. Do not dilute more than 30 minutes prior to use. Add 120µl onto the fixed coverslips and incubate either at 37°C or at room temperature for 30-60 minutes. Add both primary antibodies in double-labeling experiments.

7. Rinse as in #3.

8. Add secondary antibody (or antibodies in a double-labeling experiment). Dilute in 1x PBS plus 0.1% BSA at concentrations recommended by the manufacturer. Incubate 30-60 minutes in a humid chamber. If desired labeling nuclei, Hoechst dye (#33342, purchased from Polyscience and diluted to 1mg/ml in DMSO) is added at a concentration of 2µg/ml during this incubation. Always check the labeling by secondary antibodies alone (preferably using a control sample to which immunoglobulin identical, in species and isotype, to the primary antibody has been added – BD or Santa Cruz).
9. Rinse as in #3 and blot excess liquid. Invert over a drop (15-20µl) of Vectashield (from Vector Labs) placed on a clean glass slide.

10. Remove excess liquid from edge, and seal with 1:1 (v:v) beeswax (Fisher)/Vaseline.

11. Examine. For best results, take photographs immediately. Slides can be stored at -20°C.

* Fixative – 2% Paraformaldehyde in PBS

Use at room temperature.

CAREFUL, PFA IS TOXIC – USE IN HOOD!

If using commercially prepared 16% PFA:
• Dilute to 2% with PBS and water. Adjust pH to 7.4.

If using solid PFA:
• 1g PFA + 25ml H₂O. Gently heat under a hood. Let it steam, but not boil.
• Add 1N NaOH (~ 2 drops) to clear. Let cool.
• Add 2x PBS up to 25ml.
• pH 7.4 – Be sure to adjust pH to 7.4 before using. Refrigerate. Last for 2 weeks.