Immunofluorescence Assays

Protocol 1: Adherent, 2D Cell Culture Immunofluorescence
Brigitte Arduini, version 3, 2015-Feb-03
Modified from Scott Noggle, 2007-Sep-28

Notes
• For best imaging results, culture cells on no. 1.5 cover glass or optical plastic prior to fixation.
• It is advisable to have a positive control plate for each primary/secondary antibody combination for each experiment.
• It is essential to have a negative (secondary only) control for each antibody for each experiment. These control plates are used to set background levels during microscopy.
• Many antibodies work best with one method of fixation over others, or require distinct permeabilization and staining protocols. It is possible, especially if few antibodies against a particular antigen are available, to optimize these methods to the antibody. However, as multiplex phenotyping is routinely necessary in cell culture experiments, I recommend testing antibodies for use with the same fixation and staining protocol.
• It is possible to use fluorophore-conjugated primary antibodies such as those commonly used in flow cytometry. However, in many cases lack of secondary signal amplification will not provide a high enough signal-to-noise ratio for quality imaging.

Materials
Imaging plates from MatTek (http://glass-bottom-dishes.com/pages/) or ibidi (http://ibidi.com/)
Serum, such as normal donkey serum (Jackson ImmunoResearch, #017-000-121)
Phosphate Buffered Saline (PBS) without Ca²⁺/Mg²⁺ (Life Technologies #14190-144)
Tween-20, Triton-X100
4',6-Diamidino-2-Phenylindole (DAPI, Life Technologies #D1306)
Paraformaldehyde (4%), prepared fresh (see Support Protocol 1)

Procedure
This procedure is for intracellular antigens. For cell surface antigens, omit detergents (Tween, Triton) and wash with PBS only.
1. Wash plates two times in 1X PBS (without Ca/Mg).
2. Fix in 2ml of 4% paraformaldehyde (PFA) for 20 minutes at room temperature.
   **Paraformaldehyde and initial PBS wash should be disposed in hazardous liquid waste containers in a fume hood. Plastics should be disposed in hazardous solid waste containers in the hood.**
3. Wash in 1X PBS (1 X fast, 2 X 15 min) at room temperature. Dishes can be stored at 4°C if sealed with parafilm.
4. Permeablize with 0.1% Triton-X in 1X PBS (PBSTx) for 15 min at room temperature.
5. Block with 3% normal serum in PBSTx for 30 min at room temperature.
   The serum species is dictated by the host of secondary antibody, usually donkey or goat for Alexa-conjugated secondaries.
6. Remove block and add primary antibodies diluted in block solution.
7. Incubate at 4°C overnight.
8. Wash three times for at least 30 min each in PBT (PBS + 0.1% Tween-20) at room temperature.
10. Incubate in the dark at room temperature for 30 min or overnight at 4°C.
11. Wash two times in PBT for 30 min each.
12. Counterstain with DAPI (diluted 1:10,000 in PBT).
   **DAPI solution and initial PBT wash should be disposed in hazardous liquid waste containers in a fume hood. Plastics should be disposed in hazardous solid waste containers in the hood.**
13. Wash two times in PBT for 15 min and leave in PBT. Plates can be stored at 4°C if sealed with parafilm, or imaged immediately.

**Support Protocol 1: Preparation of 8% Paraformaldehyde**

**Notes**
- 8% paraformaldehyde (PFA) is good for approximately two weeks at 4°C.
- Any PFA waste (solid or liquid) must go in a waste container marked as such and is treated as hazardous waste. This includes the weigh boat for weighing out PFA and the first wash of any glassware used in preparation.

**Materials**
- Paraformaldehyde powder (Sigma Aldrich, #30525-89-4)
- Phosphate Buffered Saline (PBS; Life Technologies #14190-144)
- Sodium hydroxide (1N)
- pH strips

**Procedure**
1. For 100 mL measure out 8g paraformaldehyde powder (use caution, PFA is toxic) into a bottle of appropriate size.
2. Add to 80mL PBS and a stir bar; heat and stir vigorously (hot plate temperature can be around 160°C) for at least an hour for complete PFA dissolution.
3. Once solution clarifies, use NaOH to adjust pH to 7.2-7.4 dropwise.
4. Bring the volume up to 100mL with PBS and store in a closed container at 4°C. Leave the stir bar in.
5. To bring to 4% working concentration, dilute 1:1 with PBS.