Novitch lab
David Geffen School of Medicine at UCLA

Immunostaining Cultured Cells

1. Remove media and wash cells 3 times with PBS. Be aware that some cells may not be very adherent to the plate and will come off during the washes. To minimize this, carefully add the PBS to the side of the dish or well. Quick washes are sufficient here. One note for this step and all others is to be sure to keep the cells hydrated at all times. If you leave the cells too dry, the salt in the PBS, which is used as the base for most of the solutions will crystallize out, and cause very bad background staining problems in the final steps. The best way to avoid this is to work with a small number of wells or plates at one time, and to leave the lid on the plates whenever possible.
2. Fix the cells in 4% paraformaldehyde made up in 0.1 M PB or in 1X PBS. Freshly made paraformaldehyde is always preferable, but one can usually get away with stuff that is a few days old. Use about 1 ml per well for a 12-well dish, more for other sizes. Let this sit on the cells for 10 minutes at room temperature.
3. Remove the paraformaldehyde and wash cells one time with PBS (quick wash).
4. Add 1 ml of 0.25% Triton X-100 in 1X PBS to each well to permeabilize the cell membranes. Let this sit on the cells for 10 minutes.
5. Quick wash the cells with PBS-T.
6. Add primary antibody(ies) diluted in blocking solution. Use 0.5 ml per well of a 12-well dish (try to use just enough to sufficiently cover the cells, as you don't want to unnecessarily waste antibody, but don't let the cells dry out). Multiple primary antibodies can be mixed together here, as long as they are made in different species (i.e. rabbit, mouse, guinea pig, goat, etc). The dilution factor for each primary antibody must be empirically determined. Leave the primary antibody solution on the cells for at least 1 hour at room temperature (occasionally, you can get away with 30 minutes if your antigen is abundant and the antibody is good). You can also leave it on overnight at 4°C, but make sure that the cells don't dry out. You can minimize the drying out problems by placing the dishes in a humidified chamber.
7. Remove the primary antibody solution. Then wash the cells 3 times with PBS-T, leaving each wash on for about 5 minutes.
8. Add secondary antibody(ies) diluted in blocking solution. You can add multiple secondary antibodies here, as long as they are proven to be species-specific for each of the primary antibodies that you are using (i.e. you want to have an anti-rabbit IgG antibody that does not cross-react with mouse IgG, etc.). Most of the secondary antibodies that are commonly used are conjugated to fluorescent molecules though some may be conjugated to enzymes. More details on secondary antibodies will be listed below. I often add a DNA dye such as DAPI or Hoechst 33258 to the secondary antibody solution (final concentration of 0.5 μg/ml) to allow visualization of all of the nuclei with the fluorescent microscope. Leave the secondary antibody solution on for at least 30 minutes. You should also keep the sample in the dark (in a drawer or covered in foil) from this point onward, as room light may cause some fading of the fluorescent molecules attached to the antibodies.
9. Wash cells 3 times with PBS-T, leaving each wash on for about 5 minutes.
10. Leave the cells with an ample covering of either PBS-T or PBS and examine cells under the microscope. If you are planning on leaving the cells sitting for a while, consider adding sodium azide to the PBS or PBS-T to a final concentration of 0.05% to prevent microbial growth. Also be careful to not let the dishes evaporate as salt crystals will form.

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Solutions:

4% Paraformaldehyde
For 50 ml:

In a small beaker, heat 25 ml of water in a microwave. You want the water to be warm, but not boiling. Add a few drops of 10 N NaOH and then 2 g of paraformaldehyde. Add a stir bar and mix in a fume hood. It will take several minutes for the paraformaldehyde to go into solution. Once clear, add 12.5 ml of 0.4 M PB solution (or 2.5 ml of 20X PBS), and check the pH using pH paper to make sure that the pH is around 7. Add NaOH or HCl to obtain the proper pH. Bring the final volume up to 50 ml with water and filter through a 0.2 μm membrane. Store at 4°C, and use within a few days.

20X PBS
for 1L:

Mix 160 g NaCl
4.0 g KCl
43.2 g Na_2HPO_4-$7H_2O$ (or 23 g Na_2HPO_4 anhydrous)
4 g KH_2PO_4
ddH_2O to 1 L

pH to 7.4 with NaOH or HCl. Filter through a 0.2 μm membrane.

0.25% Triton X-100 in PBS
For 100 ml:

Mix 1.25 ml 20% Triton X-100
5 ml 20X PBS
ddH_2O to 100 ml

PBS-T (PBS with 0.1% Triton X-100)
Add Triton X-100 to a final concentration of 0.1% in 1X PBS

Antibody blocking solution
For 250 ml:

Mix 2.5 ml horse serum (will be a final concentration of 1%)
1.25 ml 20% Triton X-100
12.5 ml 20X PBS
2.5 ml 5% sodium azide (final concentration of 0.05%)
ddH_2O to 250 ml

Sterile filter through a 0.2 μm membrane. Store at 4°C.

Note: sodium azide is poisonous, so avoid getting it on your skin. Also, sodium azide will inhibit the activity of Horseradish Peroxidases (HRP), so this blocking solution should NOT be used for HRP-conjugated secondary antibodies.

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Secondary antibodies:

The secondary antibodies that we routinely use are as follows:

Alexa^{488}- this emits a green color under the fluorescent microscope. It is more photostable than FITC, and generally preferable to FITC. All of our Alexa^{488}antibodies come from Invitrogen and should be used at a 1:1500 dilution.

CY3- this emits a red color under the fluorescent microscope. Strong CY3 fluorescence will also carry over into the green visualization channel on the fluorescent microscope. For this reason, it is preferable to use CY3-conjugated antibodies to detect the least abundant or hardest to detect antigen. All of our CY3 antibodies come from Jackson Immunoresearch and should be used at a 1:1000 dilution.

FITC (Fluoroscein)- this emits a green color under the fluorescent microscope. One drawback to FITC is that it tends to fade more rapidly when examined on the fluorescent microscope. All of our FITC antibodies come from Jackson Immunoresearch and should be used at a 1:500 dilution.

CY5- this emits a color in the far-red range that is not excited by the lamp used in the fluorescent microscope. It can only be used with a confocal microscope or flow cytometer, which use an Argon laser to excite the fluorochromes. Most of the time, the CY5 channel is pseudo-colored as blue by the computer programs used to run the confocal or flow cytometer. All of our CY5 antibodies come from Jackson Immunoresearch and should be used at a 1:700 dilution.

DAPI or Hoechst 33258- this are dyes that will stain the DNA in the nuclei of cells. It can be detected with the fluorescent microscope equipped with the proper filter set. It emits a blue color. Unfortunately, the Argon laser used in both the confocal microscope and the flow cytometer does not excite this dye. Thus, it cannot be visualized with these instruments. Nevertheless, I still routinely add it to all of my antibody stains, as you need to focus on your sample using the fluorescent microscope first, and this stain is very helpful for this step. Either dye should be added to a final concentration of 0.5 μg/ml.

Goat vs. donkey secondary antibodies:

We have secondary antibodies that have been made in both goats and donkeys. The biggest advantage to the donkey antibodies is that you can use these to detect primary antibodies made in goats. A second advantage is that the donkey antibodies are highly purified such that they show remarkable species selectivity, guaranteeing that you don't have any cross-reactivity problems. A disadvantage is that they are much more expensive than goat secondary antibodies. When doing a simple anti-mouse IgG and anti-rabbit IgG stain, the goat secondary antibodies are perfectly fine.