Immunofluorescence staining

This page provides protocols for direct and indirect immunofluorescence as well as extra information on immunofluorescence.

Direct Immunofluorescence
Staining cells with antibodies directly linked to fluorochromes is known as direct immunofluorescence (DIF). DIF lends itself to multicolour experiments where a cell suspension is simultaneously stained with two, three or four antibodies, each tagged with a different fluorescent dye. When designing multicolour experiments it is important to use dyes that are compatible with each other. If in doubt speak to Simon. The inclusion of Sodium Azide prevents "capping" and so enables shorter incubations at room temperature to be used. If you are in any doubt about which fluorochromes can be used on our instrument (FACS Calibur) or any other aspect of your experiment design then please ask.

Controls
All multicolour experiments require compensation controls where the same cells are stained with each of the fluorochromes separately. The correct negative control for allimmuno fluorescent experiments are cells treated in the same way but incubated with isotype matched control antibodies with no known specificity tagged with the same fluorochromes as the test antibodies. Sometimes when dealing with suspensions where there will be negative and positive cells such as peripheral T-Cells, it is permissible to consider the unstained cells in the sample the negatives and exclude the negative control.

Equipment and reagents

- Tissue culture medium with 10mM SodiumAzide (TCM-N3)
- Wash Buffer: DPBS with 10mM Sodium Azide(DPBS-N3)
- Fixative :1 % paraformaldehyde in PBS (PFA), see notes 1
- Bench centrifuge
- 12X75 mm plastic test tubes
- Antibodies

Method

1. Harvest cells in usual way. Do not use trypsin to detach adherent cells. Rinse cells with cation free PBS, then use EDTA in PBS (see notes 2). Wash cells in TCM-N3 by centrifuging in 50 ml conical tubes for 10 minutes at 2000 rpm. Aspirate supernatant and resuspend in 1 ml (TCM-N3). Count your cells using a hemocytometer. The ideal number of cells is about 5x105 per tube and less than 5x104 is too few. Resuspend in nXml of TCM-N3 where n is the number of tubes you will have.
2. Pipette 1 ml of the cell suspension into each of your 12x75 mm tubes (having labelled them first). Centrifuge for 5 minutes at 1500 rpm. Aspirate all but about 10 µl of the supernatant. Resuspend cells by vortexing or flicking the tube.
3. Dispense your antibodies into microcentrifuge tubes, with multicolour experiments all the antibodies for one tube can be pre-mixed before addition to cells. Centrifuging briefly in a microcentrifuge removes aggregates and reduces background staining. Add the appropriate amount of antibody to each tube (read manufacturers suggestions) and incubate for 15 minutes at room temperature.
4. Resuspend cells by flicking the tube or vortexing gently and add 1 ml of DPBS-N3. Centrifuge tubes at 1500 rpm for 5 minutes. Aspirate supernatant, resuspend cells by flicking or vortexing. Add 0.5 ml of 1 % formaldehyde in PBS by adding slowly while vortexing gently. Samples should be left for at least one hour in the formaldehyde solution before running on cytometer if they are virus infected or possibly virus infected.

Notes 1 Paraformaldehyde is the solid form polymerized formaldehyde. Formaldehyde solution should be made fresh and not kept for more than a week. The most convenient form is to buy vials of 20 % formaldehyde from Tousimis cat1008A. Old formaldehyde can spoil your data by increasing non specific background fluorescence. If you have to make it up from the powdered paraformaldehyde, mix the powder with PBS and leave in a water bath for a day or two. Heating on a hotplate is not recommended as the fumes are dangerous.

Notes 2 A method of removing adherent cells from flasks:

1. Make up PBS with 0.53 mM EDTA. It is important that there are no divalent cations, for example Ca2+ or Mg2+ in the PBS.
2. Wash the monolayer free of tissue culture medium with PBS-EDTA. Decant off fluid and add more PBS-EDTA. Incubate for 15 minutes at 35 °C. Rocking the flask may help. For really stubborn cells, you may need to give the flask a few hard raps to detach them.

Indirect immunofluorescence
Staining cells with antibodies that are not directly conjugated to fluorochromes and then using a second labelled

reagent to bind to your primary antibody is known as indirect immunofluorescence (IIF). This method was pretty standard before so many antibodies became available directly conjugated to fluorochromes. Mostly with unlabelled antibodies the second step reagent will be another antibody with a specificity for the primary antibody allotype or sometimes isotype. Typically antibodies against human antigens and made in mice so are mouse immunoglobulins usually mouse IgG1. The typical second step reagent would be goat anti-mouse IgG conjugated to FITC. However, care should be taken to ensure your second step reagent will recognize the primary antibody. For instance, if your primary antibody is mouse IgG2a andyour second step reagent is anti-mouse IgG1, then it will not work. Also some primary antibodies are made in hamster or rat, beware. Some primary antibodies are sold conjugated to biotin. In this case, the second step reagent will be a fluorochrome labelled streptavidin or avidin. The affinity of streptavidin forbiotin is awesome, so short incubations can be used. Multicolour experiments are much more diffecult with indirect immunofluorescence, but are possible. The following protocol assumes it is a single colour experiment. If you are in any doubt about which fluorochromes can be used on our instrument (FACS Calibur) or any other aspect of your experiment design, then please ask.

Controls
The correct negative control for all immunofluorescent experiments, are cells treated in the same way but incubated with isotype matched control antibodies with no known specificity, tagged with the same fluorochromes as the test antibodies. Sometimes, when dealing with suspensions where there will be negative and positive cells, such as peripheral T-cells, it is permissible to consider the unstained cells in the sample as the negatives and exclude the negative control.

Equipment and reagents

- Tissue culture medium with 10 mM SodiumAzide (TCM-N3)
- Wash Buffer: DPBS with 10 mM Sodium Azide (DPBS-N3)
- Fixative 1% paraformaldehyde in PBS (PFA), see notes 1
- Bench centrifuge
- 12X75 mm plastic test tubes
- Antibodies

Method

1. Harvest cells in usual way. Do not use trypsin to detach adherent cells. Rinse cells with cation free PBS, then use EDTA in PBS (see notes 2). Wash cells in TCM-N3 by centrifuging in 50 ml conical tubes for 10 minutes at 2000 rpm. Aspirate supernatant and resuspend in 1 ml (TCM-N3). Count your cells using a hemocytometer. The ideal number of cells is about 5x105 per tube and less than 5x104 is too few. Resuspend in nXml of TCM-N3, where n is the number of tubes you will have.
2. Pipette 1 ml of the cell suspension into each of your 12x75 mm tubes, having labelled them first. Centrifuge for 5 minutes at1500 rpm. Aspirate all but about 10 µl of the supernatant. Resuspend cells by vortexing or flicking the tube.
3. Dispense your antibodies into microcentrifuge tubes. Centrifuging briefly in a microcentrifuge removes aggregates and reduces background staining. Add the appropriate amount of antibody to each tube (read manufacturers suggestions) and incubate for 15 minutes at room temperature.
4. Resuspend cells by flicking the tube or vortexing gently and add 1ml of DPBS-N3,centrifuge tubes at 1500 rpm for 5 minutes. Aspirate supernatant.
5. Repeat step 4, leaving about 10 µl of fluid in the tube. Add the second step reagent, incubate for 15 minutes at room temperature. Incubations can probably be reduced with streptavidin.
6. Resuspend cells by flicking the tube or vortexing gently and add 1ml of DPBS-N3,centrifuge tubes at 1500 rpm for 5 minutes. Aspirate supernatant, resuspendcells by flicking or vortexing. Add 0.5 ml of 1 % formaldehyde in PBS by adding slowly while vortexing gently. Samples should be left for at least one hour in the formaldehyde solution before running on cytometer if they are virus infected or possibly virus infected.

Immunofluorescence extras

Blocking Fc receptors during immunofluorescence staining

1. Some cell types have a lot of Fc receptors that cross react with mouse Igs and produce a lot of non specific staining. Monocytes, macrophages and B-cells in particular can cause problems. Saturating these receptors with human immunoglobulin before staining with antibodies usually greatly improves results.
2. Prepare cells in the usual way, spin them down and aspirate supernatant.
3. Resuspend cell pellet in 50 µl 10 % heat inactivated human serum in PBS. Incubate for 15 minutes at room temperature.
4. Without washing, add your antibody and incubate for your usual time.
5. When washing cells use the same 2 % human serum PBS. If you are going to fix the cells the last wash should be just PBS, straight up!

Dual immunofluorescence staining when one antibody is directly conjugated and the other is not

1. Prepare cells in the usual way, spin them down and aspirate supernatant.

2. Incubate cells first with the unconjugated antibody. Wash, then incubate with the second step reagent, usually goat antimouse-fitc or PE, and wash again twice.
3. Incubate cells with 20µl 10% mouse serum (heatinactivated) in PBS for 10 minutes at room temperature.
4. Add without washing your directly conjugated antibody, incubate as usual, wash and fix.

Immunofluorescence staining of lymphocytes using whole blood method

1. Draw blood into tubes containing EDTA. Immediately before staining the cells, invert the tube half a dozen times to suspend cells.
2. Dispense 100 µl of blood into Falcon 2054 tubes. Add the recommended amount of antibody, usually 20 µl with BD antibodies. If you are not looking for very rare cells, 50 µl blood and 10 µl AB can be used. Incubate for 10 minutes at room temperature in the dark. When staining with more than one antibody and if the antibodies are directly conjugated, they can all be added at the same time.
3. Add 1 ml of 1X FACS lyse, 0.5 ml if you halved the volumes. Incubate 10 minutes at room temperature. Centrifuge at 500 rpm for 5 minutes.
4. Resuspend pellet by flicking the tube, add 1 ml DPBS with 10 mM sodium azide. Centrifuge at 1500 rpm for 5 minutes.
5. Repeat step 4.
6. Resuspend pellet by flicking the tube. Add 0.5 ml 1% paraformaldehyde in DPBS slowly while gently vortexing the cells.