abcam

Immunocytochemistry and immunofluorescence protocol

Procedure for staining of cell cultures using immunofluorescence

Preparing the slide

1. Coat coverslips with polyethylineimine or poly-L-lysine for 1 h at room temperature.
2. Rinse coverslips well with sterile H_2O (three times 1 h each).
3. Allow coverslips to dry completely and sterilize them under UV light for at least 4 h.
4. Grow cells on glass coverslips or prepare cytospin or smear preparation.
5. Rinse briefly in phosphate-buffered saline (PBS).

   For wash buffer we recommend 1x PBS 0.1% Tween 20.

Fixation

The cells may be fixed using one of two methods:

1. Incubating the cells in 100% methanol (chilled at -20°C) at room temperature for 5 min
2. Using 4% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature

The cells should be washed three times with ice-cold PBS.

Antigen retrieval (optional step)

Certain antibodies work best when cells are heated in antigen retrieval buffer. Check the product information for recommendations for each primary antibody being used.

1. Preheat the antigen retrieval buffer (100 mM Tris, 5% [w/v] urea, pH 9.5) to 95°C. This can be done by heating the buffer in a coverglass staining jar which is placed in a water bath at 95°C.
2. Using a small pair of broad-tipped forceps, place the coverslips carefully in the antigen retrieval buffer in the cover glass staining jar, making note of which side of the coverslips the cells are on.
3. Heat the coverslips at 95°C for 10 min.

4. Remove the coverslips from the antigen retrieval buffer and immerse them, with the side containing the cells facing up, in PBS, in the 6-well tissue culture plates.

5. Wash cells in PBS three times for 5 min.

Permeabilization

If the target protein is intracellular, it is very important to permeabilize the cells. Acetone fixed samples do not require permeabilization.

1. Incubate the samples for 10 min with PBS containing either 0.1–0.25% Triton X-100 (or 100 µM digitonin or 0.5% saponin). Triton X-100 is the most popular detergent for improving the penetration of the antibody. However, it is not appropriate for membrane-associated antigens since it destroys membranes.

2. The optimal percentage of Triton X-100 should be determined for each protein of interest.

3. Wash cells in PBS three times for 5 min.

Blocking and immunostaining

1. Incubate cells with 1% BSA, 22.52 mg/mL glycine in PBST (PBS+ 0.1% Tween 20) for 30 min to block unspecific binding of the antibodies (alternative blocking solutions are 1% gelatin or 10% serum from the species the secondary antibody was raised in: see antibody datasheet for recommendations).

2. Incubate cells in the diluted antibody in 1% BSA in PBST in a humidified chamber for 1 h at room temperature or overnight at 4°C.

3. Decant the solution and wash the cells three times in PBS, 5 min each wash.

4. Incubate cells with the secondary antibody in 1% BSA for 1 h at room temperature in the dark.

5. Decant the secondary antibody solution and wash three times with PBS for 5 min each in the dark.

Multicolor immunostaining (optional step)

To examine the co-distribution of two (or more) different antigens in the same sample, use a double immunofluorescence procedure. This can be performed either simultaneously (in a mixture) or sequentially (one antigen after another).

Ensure you have antibodies for different species and their corresponding secondary antibodies. For example, rabbit antibody against antigen A, mouse antibody against antigen B. Alternatively, you can use directly conjugated primary antibodies conjugated to different fluorophores.

Simultaneous incubation

1. Incubate cells with blocking solution for 30 min.
2. Incubate cells with both primary antibodies in 1% BSA in PBST in a humidified chamber for 1 h at room temperature or overnight at 4°C.
3. Decant the solution and wash the cells three times in PBS, 5 min each wash.
4. Incubate cells with both secondary antibodies in 1% BSA for 1 h at room temperature in the dark.
5. Decant the secondary antibody solution and wash three times with PBS for 5 mins each in the dark.

Sequential incubation

1. First blocking step: incubate cells with the first blocking solution (10% serum from the species that the secondary antibody was raised in) for 30 min at room temperature.
2. Incubate cells with the first primary antibody in 1% BSA or 1% serum in PBST in a humidified chamber for 1 h at room temperature or overnight at 4°C, 1% gelatin or 1% BSA.
3. Decant the first primary antibody solution and wash the cells three times in PBS, 5 min each wash.
4. Incubate cells with first secondary antibody in 1% BSA in PBST for 1 h at room temperature in the dark.
5. Decant the first secondary antibody solution and wash three times with PBS for 5 min each in the dark.
6. Second blocking step: incubate cells with the second serum, (10% serum from the species that the secondary antibody was raised in) for 30 min at room temperature in the dark.

7. Incubate cells with the second primary antibody in 1% BSA or 1% serum in PBST in a humidified chamber in the dark for 1 h at room temperature, or overnight at 4°C.

8. Decant the second primary antibody solution and wash the cells three times in PBS, 5 min each wash in the dark.

9. Incubate cells with second secondary antibody in 1% BSA for 1 h at room temperature in the dark.

10. Decant the second secondary antibody solution and wash three times with PBS for 5 min each in the dark.

    If you have to detect more than two antigens, continue following steps 1–5 for the rest of the antibodies.

Counter staining

1. Incubate cells with 0.1–1 µg/mL Hoechst stain or DAPI (DNA stain) for 1 min.

2. Rinse with PBS.

Mounting

1. Mount coverslip with a drop of mounting medium.

2. Seal coverslip with nail polish to prevent drying and movement under microscope.

3. Store in dark at -20°C or +4°C.