

Introduction

Immunofluorescence is the labeling of antigens with fluorescent dyes. This technique is often used to visualize subcellular distribution of biomolecules of interest. Immunofluorescent-stained tissue sections or cells are studied using a fluorescence microscope, confocal microscopy or flow cytometry.

The first step is cell or tissue preparation. To allow easy handling of microscope procedures the cells or tissue are attached to a solid support. The second step is to fix and, if necessary, permeabilize the cells, to ensure free access of the antibody to its antigen. The third step of staining involves incubation with antibody. Unbound antibody is removed by washing, and the bound antibody is detected either directly (if the primary antibody itself was labeled) or indirectly, using a fluorochrome-labeled secondary reagent. Finally, the staining is evaluated using fluorescence microscopy.

**Materials/
reagents**

- **Cultured cells grown on coverslips, coupes, or cells in suspension**
- **Primary antibody**
- **Fluorochrome-labeled secondary antibody**
- **Antibody controls**
- **Phosphate Buffered Saline (PBS)**
- **Aqueous mounting medium**
- **95%, 100% ethanol**
- **Propidium iodide (PI) or DAPI**
 - A: Methanol (-20 °C)
 - Acetone (-20 °C)
 - B: 3-4% Paraformaldehyde in PBS
 - Methanol (-20 °C)
- **3-4% Paraformaldehyde in PBS:**
 - 1:1 solution of 8% paraformaldehyde stock solution and PBS
 - Prepare on the day of use
- **Wash buffer:**
 - 0.05% Tween 20 in PBS
- **Antibody buffer:**
 - 2% Bovine Serum Albumine (BSA) in PBS
- **Blocking buffer:**
 - 10% serum from host species of secondary antibody diluted in PBS or 2% BSA diluted in PBS.

Procedure**Cell preparation**

Cell preparation can be achieved by several methods: adherent cells may be grown on microscope slides, coverslips, coupes, or an optically suitable plastic support. Suspension cells can be centrifuged onto glass slides (cytospins), bound to solid support using chemical linkers, or in some cases handled in suspension. The procedure of staining cells handled in suspension is described in the protocol [flow cytometry \(FC\)](#). For immunofluorescence on tissues the protocols for [immunohistochemistry](#) can be used. The preparation of cell lines, frozen sections and cytospins are described below:

Cell lines:

1. Grow cultured cells on support overnight at 37°C.
2. Inspect under inverted light microscope to verify the desired appearance.
3. Wash briefly with PBS, remove excess solution.

Frozen Sections:

1. Use liquid nitrogen, embedded in Optimal Cutting Temperature (OCT) compound in cryomolds to snap the frozen fresh tissues.
2. Store frozen blocks at -80°C.
3. Cut 4-8 µm thick cryostat sections and mount on superfrost plus slides or gelatin coated slides.
4. Store slides at -80°C until needed.
5. Before staining, warm slides at room temperature for 30 minutes.

Cytospins:

1. Prepare a cell suspension of not more than 0.5×10^6 cells/ml of protein-containing medium.
2. Pre-label the slides.
3. The glass slide and card is inserted/extracted from the cytospin cuvette .
4. Place empty cuvettes.
5. Load up to 200 µl of this suspension in each cuvette.
Spin at 800 rpm for 3 min.
6. Extract the slide, paper and cuvette without disarranging.
7. Carefully detach the cuvette and the paper without damaging the fresh cytospin. It is very important to hold firmly together glass slide and cuvette when extracting from metal holder.
8. Mark the area around the cytocentrifuged cells with dry point or permanent marker.
9. Proceed with either immediate fixation or air-dry at room temperature. Proceed with air dried sample

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within two days.

Fixation

Wide ranges of fixatives are commonly used, and the correct choice of method will depend on the nature of the antigen being examined and on the properties of the antibody used. Fixation methods fall generally into two classes: organic solvents and cross-linking reagents. Organic solvents such as alcohols and acetone remove lipids and dehydrate the cells, while precipitating the proteins on the cellular architecture. Cross-linking reagents (such as paraformaldehyde) form intermolecular bridges, normally through free amino groups, thus creating a network of linked antigens. Cross-linkers preserve cell structure better than organic solvents, but may reduce the antigenicity of some cell components, and require the addition of a permeabilization step, to allow access of the antibody to the specimen. Fixation with both methods may denature protein antigens, and for this reason, antibodies prepared against denatured proteins may be more useful for cell staining. Organic and cross-linking fixation methods (A and B) are described. One of the appropriate fixation method should be chosen according to the relevant application.

A: Methanol-Acetone Fixation

1. Fix in cooled methanol for 10 minutes at -20°C .
2. Remove excess methanol.
3. Permeabilize with cooled acetone for 1 minute at -20°C .

B: Paraformaldehyde-Methanol Fixation

1. Fix in **3-4% paraformaldehyde** for 10-20 minutes at room temperature.
2. Rinse briefly with PBS.
3. Permeabilize with cooled methanol for 5-10 minutes at -20°C .

Staining

Please read entire procedure before staining sections. Perform all incubations in a humidified chamber and do not allow sections to dry out. Isotype and system controls should also be run and must be matched to the isotype of each primary antibody to be tested.

1. Rinse sections twice in wash buffer for 2 minutes.
2. Incubate sections with **blocking buffer**.
3. Block for 30 minutes in blocking buffer to block non-specific binding of immunoglobulin.
4. Dilute primary antibody in **antibody buffer** to appropriate dilution.
5. Incubate the samples in antibody buffer with primary antibody for 60 minutes at room temperature or overnight at 4°C (it is recommended to use a humidified chamber).
6. Wash three times (at least 5 minutes each) with wash buffer.

Secondary antibody is applied only in indirect assays.

7. Dilute labeled secondary antibody to appropriate dilution in antibody buffer. Incubate the samples in antibody buffer with secondary antibody for 30 minutes at room temperature.

Protect the slides from light, because of the fluorescence, starting from step 7 to the end by covering slides with aluminum foil or black box.

8. Wash three times (at least 5 minutes each) with wash buffer.
9. Remove excess wash buffer.
10. Counterstain the cells with PI or DAPI if desired.
11. Wash in wash buffer.
12. Dehydrate through 95% ethanol for 2 minutes and twice through 100% ethanol for 3 minutes.

Evaluation

1. Mount coverslip with anti-fade mounting medium (e.g. Vectashield) and invert onto glass slides.
2. Inspect under a fluorescence microscope.
3. Record the results. It is recommended to photograph the labeled cells.

Safety

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Notes

- When making buffer(s) fresh before you start, there is no need to add sodium azide to the buffer.
- Protect fluorescent conjugates and labeled slides from the light. Incubate samples in the dark and cover whenever possible.
- It is advisable to run the appropriate negative controls. Negative controls establish background fluorescence and non-specific staining of the primary and secondary antibodies. The negative control reagent should be isotype-matched, not specific for cells of the species being studied and of the same concentration as the test antibody. The degree of autofluorescence or negative control reagent fluorescence will vary with the type of cells under study and the sensitivity of the instrument used. For fluorescent analysis of cells with Fc receptors, the use of isotype-matched negative controls is mandatory.

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Technical assistance

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