How-To Guide

Immunofluorescence

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Introduction

Immunofluorescence microscopy is a powerful technique used to visualize cellular dynamics by detecting specific antigens with fluorescently labeled antibodies. It provides insights into the presence, quantity, and subcellular location of proteins. This technique is applicable to various cell types and requires careful consideration of fixation, permeabilization, and antibody specificity to ensure reliable results.

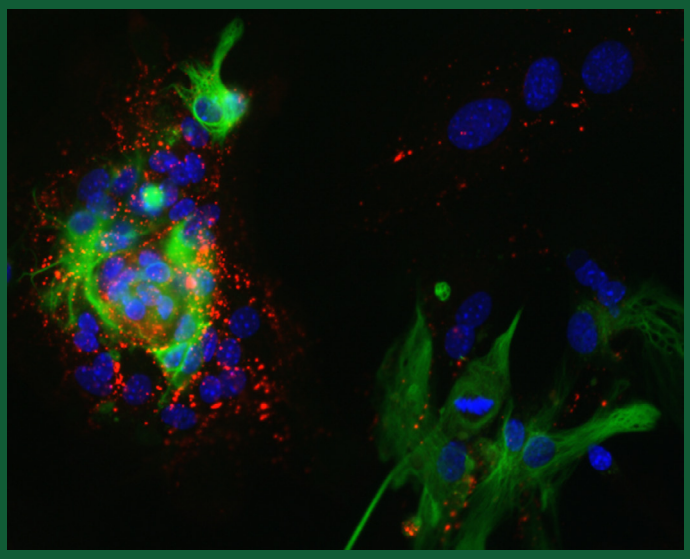

Figure 1. Primary mixed glial culture derived from P0-P3 mouse pups, stained for the astrocytic intermediate filament GFAP (Sigma G3893) and the Gap junction protein Connexin 43. Staining was visualized using Jackson ImmunoResearch secondary antibodies, Alexa Fluor® 488 AffiniPure™ F(ab')$_2$ Fragment Donkey Anti-Mouse IgG (H+L) (715-546-150) and Cy™3 AffiniPure™ Donkey Anti-Rabbit IgG (H+L) (711-165-152). Authors: Carlyn Martina-Mamber, Elly M. Hol.

Essential Materials and Considerations

1. **Samples:** Whole animals, tissues, cultured adherent cells, or suspension cells.
2. **Coverslips:** Choose appropriate sizes and thicknesses to match the culturing vessel and microscope objectives.
3. **Fixatives:** Use aldehydes (glutaraldehyde, paraformaldehyde) for strong crosslinking or alcohols/ketones (methanol, acetone) for rapid fixation.
4. **Permeabilization Agents:** Nonionic detergents (e.g., Triton X-100) or alcohols/ketones to remove membranes and allow antibody access.

Step 1: Sample Preparation

- **Choosing the Sample:** Determine the type of sample, such as whole embryos, tissue sections, adherent cells, or suspension cells. Each type requires specific preparation methods.
- **Coverslip Selection:** Select coverslips of the appropriate size and thickness based on the culturing vessel and the microscope objectives.

Step 2: Fixation

- **Purpose:** Fixation "freezes" cells in time by stopping cellular processes and preserving the position of macromolecules.
- **Methods:**
  - **Aldehyde Fixation:** Use glutaraldehyde or paraformaldehyde for strong crosslinking. Glutaraldehyde provides stronger crosslinking but penetrates cells more slowly.
  - **Alcohol/Ketone Fixation:** Methanol or acetone can precipitate and denature proteins while extracting lipids. This method is rapid but may dehydrate cells.

Step 3: Permeabilization

- **Purpose:** Remove membranes to allow antibodies access to intracellular antigens.
- **Agents:** Use nonionic detergents like Triton X-100, or alcohols/ketones like methanol or acetone. Optimize duration and concentration based on cell type and antigen.

Step 4: Blocking

Purpose: Prevent non-specific binding of antibodies by using a blocking solution, ideally containing the serum of the host species of the labeled antibody.

Step 5: Antibody Incubation

Primary Antibody: Incubate with a primary antibody specific. These may be monoclonal or polyclonal but should be specific to your target in native conditions. Ideally, choose different host species to facilitate labeling of multiple targets.

Secondary Antibody: Choose secondary antibodies that are raised in the same host species which are specific to the species the primary antibodies are raised in. If multiplexing choose antibodies that are cross-adsorbed (Min-X) to prevent cross-reactivity. If using a fluorescent conjugate, check they are compatible with the microscope's filters to ensure maximal signal and avoid crosstalk and bleed-through.

Step 6: Washing

Purpose: Remove unbound antibodies and reduce background fluorescence. Perform multiple washes with a buffer solution after each antibody incubation step.

Step 7: Mounting

Purpose: Mount the sample on a slide with a medium that preserves fluorescence and prevents photobleaching.

Visualization and Analysis

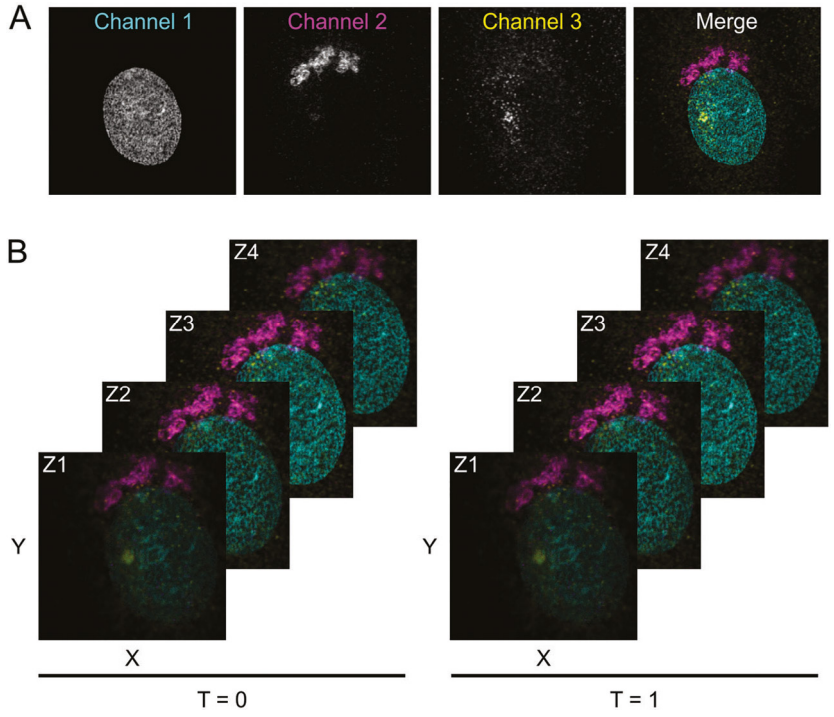

Figure 2. Multiple dimensions of fluorescent microscopy. **(A)** Different molecules of interest can be imaged as different channels in an immunofluorescent microscopy experiment. The nucleus (channel 1), the Golgi apparatus (channel 2), and the centrosome (channel 3) are shown individually in grayscale and as a false-colored merged image. **(B)** Each image is composed of a 2-dimensional array of pixel intensities (the X-Y dimensions). Multiple X-Y dimensions acquired at different focal distances can be stacked together in the Z dimension. When imaging live cells, the X, Y, and Z dimensions can be acquired at different time intervals. The images in this figure are from hTERT-RPE1 cells immunostained with a primary antibody that binds to the Golgi protein Golph2 (Rhodamine Red X was conjugated to the secondary) and a primary antibody that binds to the centrosome protein PCM1 (Alexa 647 was conjugated to the secondary) [1].

Microscopy Techniques: Choose the appropriate microscopy technique based on the resolution required, such as widefield, laser scanning confocal, or super-resolution microscopy.

Optimization: Adjust illumination intensity, camera exposure, and gain settings to acquire non-saturated images. Ensure consistent settings across samples for quantitative analysis.

Troubleshooting

Dim or Uneven Fluorescence: Optimize antibody concentrations, fixation methods, and permeabilization strategies. Check microscope alignment and ensure adequate blocking.

Background Fluorescence: Characterize non-specific binding by using controls, and then establish an appropriate plan to abrogate background, such as adjusting antibody concentrations, increasing blocking time, or switching channels to avoid autofluorescence.

Further Resources

These resources provide a structured approach to conducting immunofluorescence microscopy, ensuring high-quality results through careful preparation, strategic selection of reagents, and effective protocol design. For more expert support, visit Jackson ImmunoResearch.

- A guide to selecting control and blocking reagents
- Blocking: Use of unconjugated Fab fragments to block endogenous immunoglobulins and avoid off-target signal
- Choosing the right affinity-purified secondary antibody for your application
- Cross-adsorbed secondary antibodies and cross-reactivity
- Monovalent Fab fragment affinity-purified antibodies for blocking and double labeling primary antibodies from the same host species
- Multiple labeling for simultaneous detection of several targets

Reference

1. Galati, D.F. and Asai, D.J. (2023). Immunofluorescence Microscopy. *Current Protocols*. https://doi.org/10.1002/cpz1.842.

Cover image

Immunofluorescence microscopy on a transgenic mouse liver constitutively expressing a GFP fusion protein. GFP signal (red channel) was confirmed by immunostaining samples with Alexa Fluor® 647 Rabbit Anti-GFP Images by Histology Research Core Facility in the Dept. of Cell and Molecular Physiology at University of North Carolina.

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