How-To Guide

Immunofluorescence

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Contents

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 AffiniPure® Rabbit Anti-GFP antibody. Images by Histology Research Core Facility in the Dept. of Cell and Molecular Physiology at University of North Carolina.

Introduction

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Immunofluorescence microscopy is a powerful technique used to visualize cellular dynamics by detecting specific targets 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.

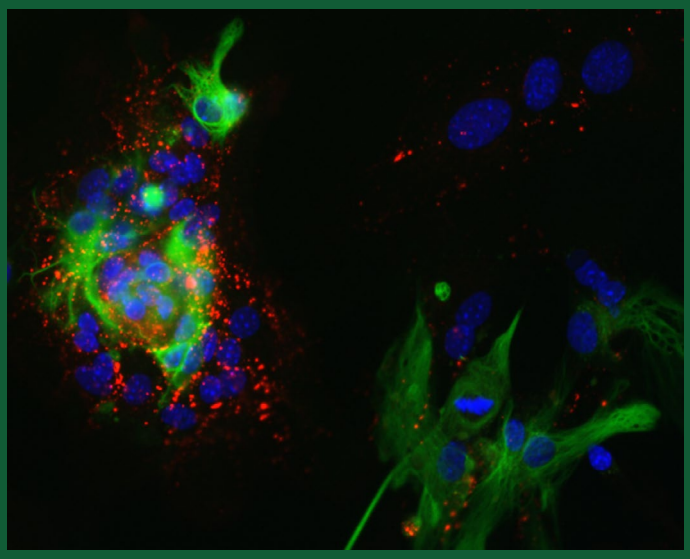

Figure 1. Primary mixed glial culture derived from P0-P3 mouse pups, stained for the astrocytic intermediate filament GFAP (Sigma G3893) and the Gap junction protein Connexin 43. Staining was visualized using Jackson ImmunoResearch secondary antibodies, Alexa Fluor® 488 AffiniPure® F(ab')$_2$ Fragment 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 disrupt 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. For immunofluorescence (IF) on whole animals, ensure that the appropriate fixation and permeabilization protocols are followed to preserve tissue integrity and antigen accessibility.

- **Coverslip and Chamber Selection:** When preparing samples, consider whether to use chambers on a slide surface or cells on a coverslip. The choice between these options can impact the quality of imaging and accessibility of the cells. Coated surfaces, such as poly-L-lysine or collagen, can enhance cell adhesion and stability during the staining process. The selection of coverslips should be based on compatibility with the culturing vessel and the microscope objectives to ensure optimal imaging conditions.

- **Permeabilization Agents:** Use nonionic detergents like Triton X-100 or alcohols/ketones like methanol or acetone to disrupt cellular membranes, allowing antibodies to access intracellular antigens. The goal is not to remove membranes but to create pores that facilitate the entry and exit of staining agents. Optimize the duration and concentration of permeabilizing agents based on the cell type and antigen to achieve the best results.

Step 2: Fixation

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.

Troubleshooting: Optimize fixation methods to prevent protein denaturation or dehydration.

Step 3: Permeabilization

Disrupt membranes to allow antibodies access to intracellular antigens.

Agents: Use nonionic detergents like Triton X-100, or alcohols/ketones like methanol or acetone.

Troubleshooting: Optimize the duration and concentration of permeabilizing agents based on cell type and antigen to achieve the best results without damaging membranes.

Step 4: Blocking

Prevent non-specific binding of antibodies.

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

Troubleshooting: Adjust blocking time and concentration to minimize background fluorescence and non-specific binding.

Step 5: Antibody Incubation

Primary Antibody: Incubate with a primary antibody specific to the protein of interest. These antibodies may be monoclonal or polyclonal, but they should be tailored to the experimental conditions, such as native conditions. Ideally, choose different host species to facilitate labeling of multiple targets.

Secondary Antibody: Choose secondary antibodies that are specific to the host species of the primary antibody. If multiplexing, ideally choose secondary antibodies from the same host species that are cross-adsorbed (Min-x) to prevent cross-reactivity. Check fluorescent conjugates are compatible with the microscope's filters to ensure maximal signal and avoid channel bleed-through.

Troubleshooting: Optimize antibody concentrations to prevent non-specific binding and ensure adequate signal.

Step 6: Washing

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

Troubleshooting: Ensure thorough washing to prevent residual background fluorescence.

Step 7: Mounting

Sandwich the sample between a slide and a coverslip using a mounting medium to preserve fluorescence and prevent photobleaching.

Mounting Techniques: The mounting process varies depending on whether cells are on a slide or a coverslip. If cells are cultured in chambers on a slide, the mounting medium is applied over the cells, followed by placing a clean coverslip on top. This ensures proper coverage and protection of the sample. Conversely, if cells are adhered to a coverslip, the coverslip is inverted onto a slide with the mounting medium, allowing the sample to be sandwiched between the coverslip and the slide.

Alternative Methods: In some cases, particularly when cells are visualized in plates, mounting media may not be necessary. This approach is typically used when immediate visualization is required, and long-term preservation is not a concern.

Troubleshooting: Ensure even mounting conditions across the sample to avoid uneven fluorescence and ensure optimal visualization.

Visualization and Analysis

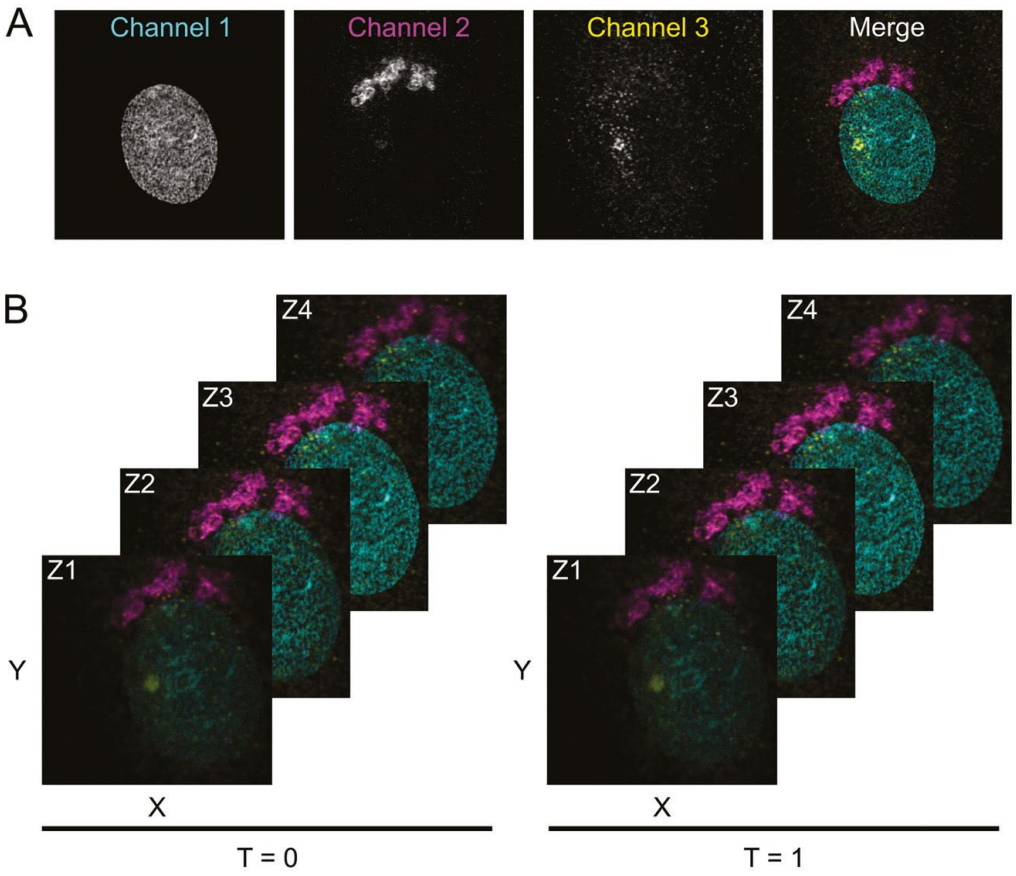

Figure 2. Multiple dimensions of fluorescent microscopy. **(A)** Different molecules of interest can be imaged as different channels in an immunofluorescent microscopy experiment. The nucleus (channel 1), the Golgi apparatus (channel 2), and the centrosome (channel 3) are shown individually in grayscale 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 Flour® 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.

Reference

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

Common Issues and Solutions

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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.

Mounting Conditions: Ensure even mounting conditions across the sample to avoid uneven fluorescence and ensure optimal visualization.

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

Imprint

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