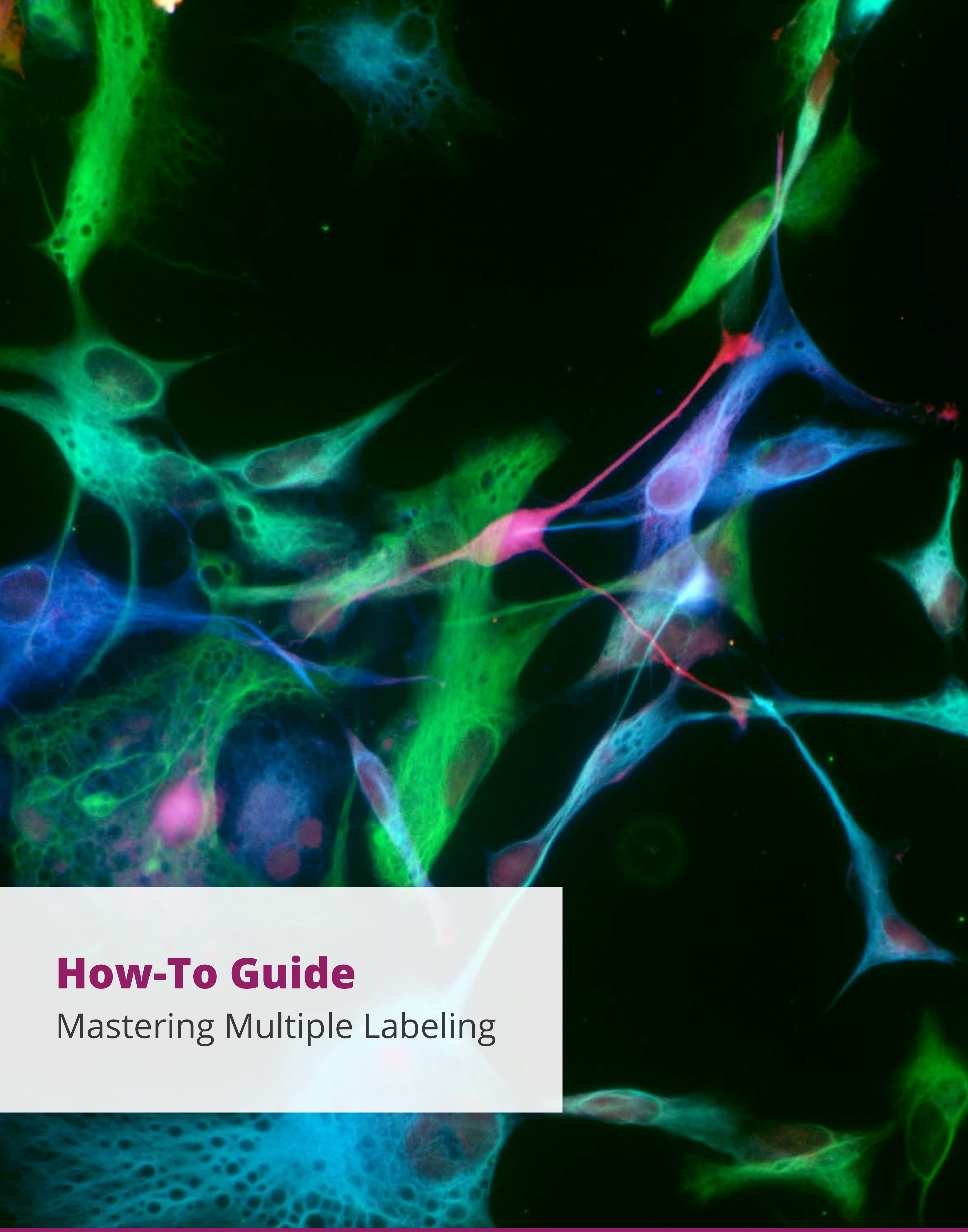


How-To Guide

Mastering Multiple Labeling

WILEY

CURRENT PROTOCOLS®
The Fine Art of Experimentation
A Wiley Brand

Jackson ImmunoResearch Laboratories Inc.

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 to Multiple Labeling

Multiple labeling in immunoassays enables the simultaneous detection of several antigens within a single sample. This technique is invaluable for studying complex biological systems, allowing researchers to visualize the spatial relationships and interactions between different targets. By using distinct labels, such as fluorophores or chromogens, multiple labeling provides a comprehensive overview of cellular and tissue architecture in a single experiment.

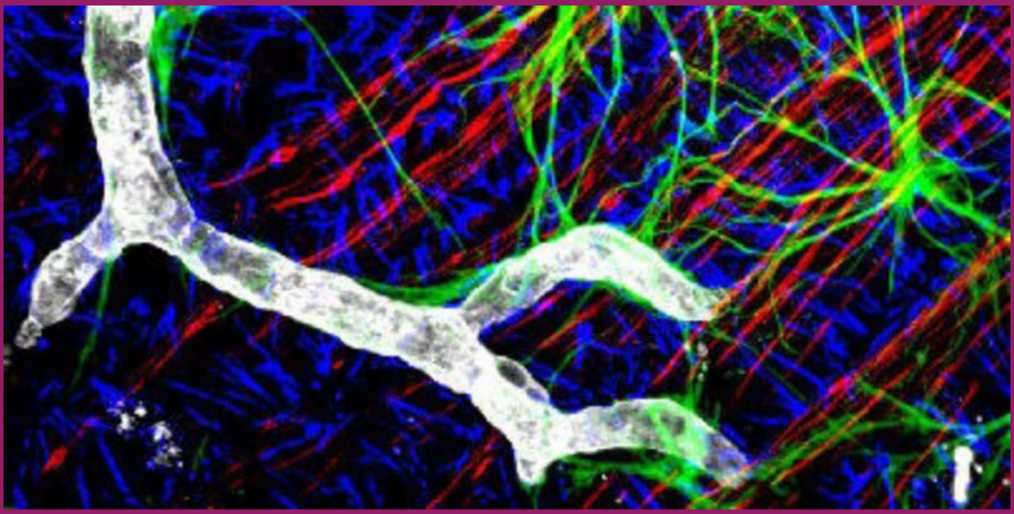


Figure 1. Mouse GFAP (green), NF (red), Collagen IV (grey), Vimentin (blue) z1.
Image courtesy of Gabe Luna, Neuroscience Research Institute, UC Santa Barbara.

Designing Your Experiment

Select Primary Antibodies

Choose primary antibodies that detect your protein/s of interest, ideally validate their specificity internally with purified protein. When detecting two or more targets, ideally, choose primary antibodies raised in different host species to prevent detection of each other by subsequent secondary antibodies (cross-reactivity) that would prevent clear detection of the target proteins. If it is unavoidable to use primaries from the same host species, implement a Fab fragment blocking protocol tailored to your antibody selection. When the primary antibody host matches the sample species, a species-on-species (SOS) blocking protocol is required to minimize background and off-target labeling.

Select Secondary Antibodies

Ideally, select secondary antibodies that are all raised in the same host species for simplicity and to reduce the risk of cross-reactivity. When designing a multiple labelling experiment, choose secondaries that are cross-adsorbed (min-x) against the other primary antibody host species and the sample species, as this ensures the secondary only detects its intended primary antibody.

Use Appropriate Fluorescent Probes

Opt for fluorophores or chromogens with well-separated spectra to avoid signal overlap. Carefully consider the detection system to ensure each target can be distinctly visualized, which is crucial for accurate interpretation of results.

Ready for Immunostaining

Multiple Labeling Setup

Step 1: Sample preparation

Ensure sample integrity for accurate labeling. Proper sample preparation is essential for maintaining tissue integrity and antigenicity. Depending on the sample type, this may involve fixation, permeabilization, and sectioning.

Step 2: Blocking

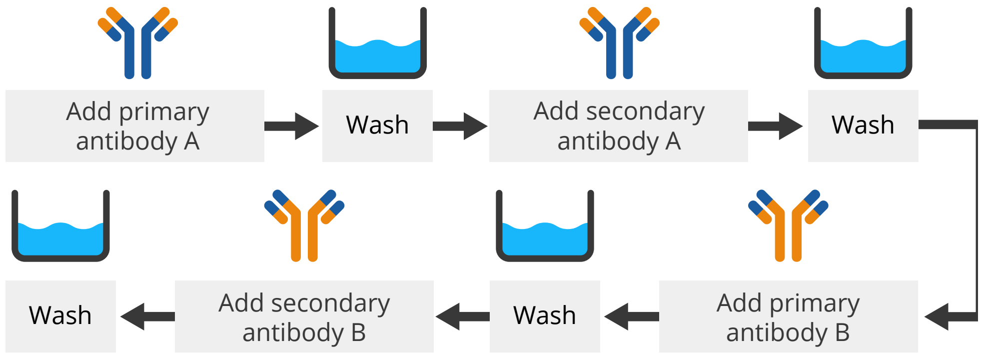
Apply 5% normal serum from the same species as the labeled antibody to block non-specific binding sites. This step is critical for reducing background and ensuring that antibodies bind only to their intended targets.

Step 3: Antibody Pair Application

Sequentially add each pair of primary and secondary antibodies per target protein (e.g., Goat Anti-A with Donkey Anti-Goat, Rabbit Anti-B with Donkey Anti-Rabbit, Rat Anti-C with Donkey Anti-Rat). Establish optimal incubation times for each antibody pair to achieve effective penetration. Titrating antibody concentrations is crucial for efficient usage and minimizing background noise. Optimize each antibody pair for the specific antigen, considering factors like sample thickness and penetration. You may choose a nanobody or F(ab')2 fragment to reduce complex size, improve penetration, and minimize linkage error to increase resolution.

Step 4: Washing

Perform thorough washes after each antibody application using an appropriate buffer. This removes unbound antibodies and reduces background, ensuring that only specific binding is detected.



For thicker samples, consider extending washing incubations to ensure thorough removal of unbound components.

Step 5: Visualization

Select detection probes (fluorophores or chromogens) that provide strong, non-overlapping signals. Place the brightest fluorophore on the weakest-expressing antigen to maximize visibility. Proper visualization allows for clear differentiation of each target antigen in the final image.

Optimization

Blocking: Use 5% normal serum from the host species of the labeled antibody as a standard to reduce background from non-specific binding. If using primary antibodies from the same species as the sample, an SOS blocking protocol using Fab fragments to block endogenous Ig is essential. In a situation when two or more primary antibodies of the same species are unavoidable, implement a Fab fragment blocking protocol suited to your antibody selection to prevent mislabelling. Normal serum or Fab fragments are effective tools for minimizing background and enhancing specificity.

Visualization: Choose fluorophores with well-separated emission spectra to ensure each target is easily distinguishable. This prevents spectral bleed-through and allows for accurate quantification and localization of each antigen. Applications such as FluoroFinder's spectral view can help you select compatible fluors.

Application of Controls

Optimize Conditions for Each Antibody Pair: Test and adjust incubation times, concentrations, and buffer conditions for each antibody pair to achieve optimal specificity and signal strength. This ensures reliable detection of all targets.

Use Isotype Negative Controls: Include isotype-matched negative controls to confirm the specificity of primary antibodies. This helps distinguish the true signal from a nonspecific background caused by a nonspecific primary antibody.

Example of Expected Observations:

When using a mouse IgG1 primary antibody to target a specific antigen, you should also apply a mouse IgG1 isotype control to a separate sample or section of the same sample. The isotype control should not bind to any specific target in your sample.

What to Expect:

With Primary Antibody: You should observe specific staining or signal in areas where the target antigen is present. This indicates that the primary antibody is binding specifically to the target antigen.

With Isotype Control: Ideally, you should see minimal to no staining or signal. This lack of signal confirms that any observed staining with the primary antibody is due to specific antigen-antibody interactions and not nonspecific binding.

If the isotype control shows significant staining, it suggests that the primary antibody's signal might include nonspecific binding, and further optimization of the blocking or washing steps may be necessary.

Test Secondary Antibodies with 'Irrelevant' Primaries: Incubate secondary antibodies with primary antibodies that are not intended to target any antigens in your sample. This control step is crucial for verifying that the secondary antibodies do not cross-react with other primary antibodies or components in the sample.

Clarification:

Use a primary antibody that is not relevant to the target antigens in your experiment. For example, if your experiment uses a rabbit primary antibody to target a specific protein, you can use a mouse primary antibody that targets a different, irrelevant protein as the control.

The goal is to ensure that the secondary antibody does not produce any signal when paired with an irrelevant primary antibody, confirming the specificity of the detection system.

This approach helps ensure that any signal observed in your experiment is due to specific interactions between the primary and secondary antibodies and not due to cross-reactivity with other proteins or antibodies.

- **Secondary-Only Control:** Incubate the sample with only secondary antibodies to establish what the background signal from tissue is. This background level can be compared to the signal achieved with antibody pairs to establish if the primary is specific. You can use this to establish if blocking protocols are sufficient, as the signal should be abrogated.

- **Tissue Only Control:** Visualise the sample in the absence of reagents to establish any autofluorescence in the channels you plan to visualise.

- **Positive Control:** If possible, test primary antibody specificity on a sample known to express the target protein/protein of interest. In the absence of this, it may be useful to check how a primary antibody performs in a technique such as western blotting on purified protein; however, this may not reflect the specificity of the primary antibody under native conditions.

- **Test Fluorophore:** It’s a great idea to see if your fluorophore can be excited under the experimental conditions. Set up your microscope as desired, and place a drop of the labeled antibody onto a slide. Check if you can successfully excite and detect the fluorophore with your setup.

Blocking and Control Reagents

- **ChromPure™ Purified Proteins:** Use ChromPure™ proteins as negative .

- **Normal Serums:** Normal serum from the same species as the labeled antibody can effectively block non-specific binding sites in tissue samples, but for specifically avoiding Fc receptor binding, using F(ab')2 fragment antibodies without an Fc region is recommended.

Additional info:

Normal serum contains a variety of proteins that can occupy potential binding sites, thus reducing background noise. However, it is not specifically designed to block Fc receptors.

F(ab')2 fragments are antibodies that have had their Fc (fragment crystallizable) region removed. This makes them particularly useful for avoiding binding to Fc receptors, which are present on certain immune cells. By using F(ab')2 fragments, researchers can minimize Fc receptor-mediated non-specific binding, leading to clearer and more specific results in experiments.

- **Monovalent Fab Fragments:** Fab fragments are monovalent antibody fragments that can be used to block endogenous immunoglobulins when using primary antibodies from the same species as the sample, or they can be employed in blocking protocols that enable the use of primary antibodies from the same host species.

Troubleshooting Common Issues

Problem	Solution
Background Signal	Visualise tissue without reagents to establish autofluorescence. Use a secondary-only control to determine the source of the background signal. If no signal appears, the primary antibody may be non-specific—select a different primary. If background persists, confirm that secondary antibodies are cross-adsorbed against the tissue species. Apply appropriate blocking reagents, such as normal serum from the host species of the labeled antibody. Use Fab fragments to block endogenous immunoglobulins.
No Signal	Always include positive controls to confirm the specificity of your primary antibody. If no signal is detected, check antibody storage, concentration, and incubation conditions, verify antigen presence in the sample, and/or establish if the primary antibody can detect POI with another technique, such as Western blotting. Spot labeled antibody on a slide to check that the fluor is compatible with the equipment laser/filter setup. Use an app like FluoroFinder to check fluor compatibility with equipment/sources, etc.
Background from Endogenous Ig Recognition	Select secondary antibodies that are cross-adsorbed against the tissue species to avoid recognition of endogenous immunoglobulins. If using primary antibodies of the same species as the tissue, block endogenous Ig with Fab fragments to reduce background.

Further Resources

These resources provide comprehensive guidance for successful immunofluorescence microscopy:

- How-To Guide: Immunofluorescence
- 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

For more expert support and detailed protocols, visit Jackson ImmunoResearch.

Imprint

Email: info@wiley-vch.de

Editor: Róisín Murtagh