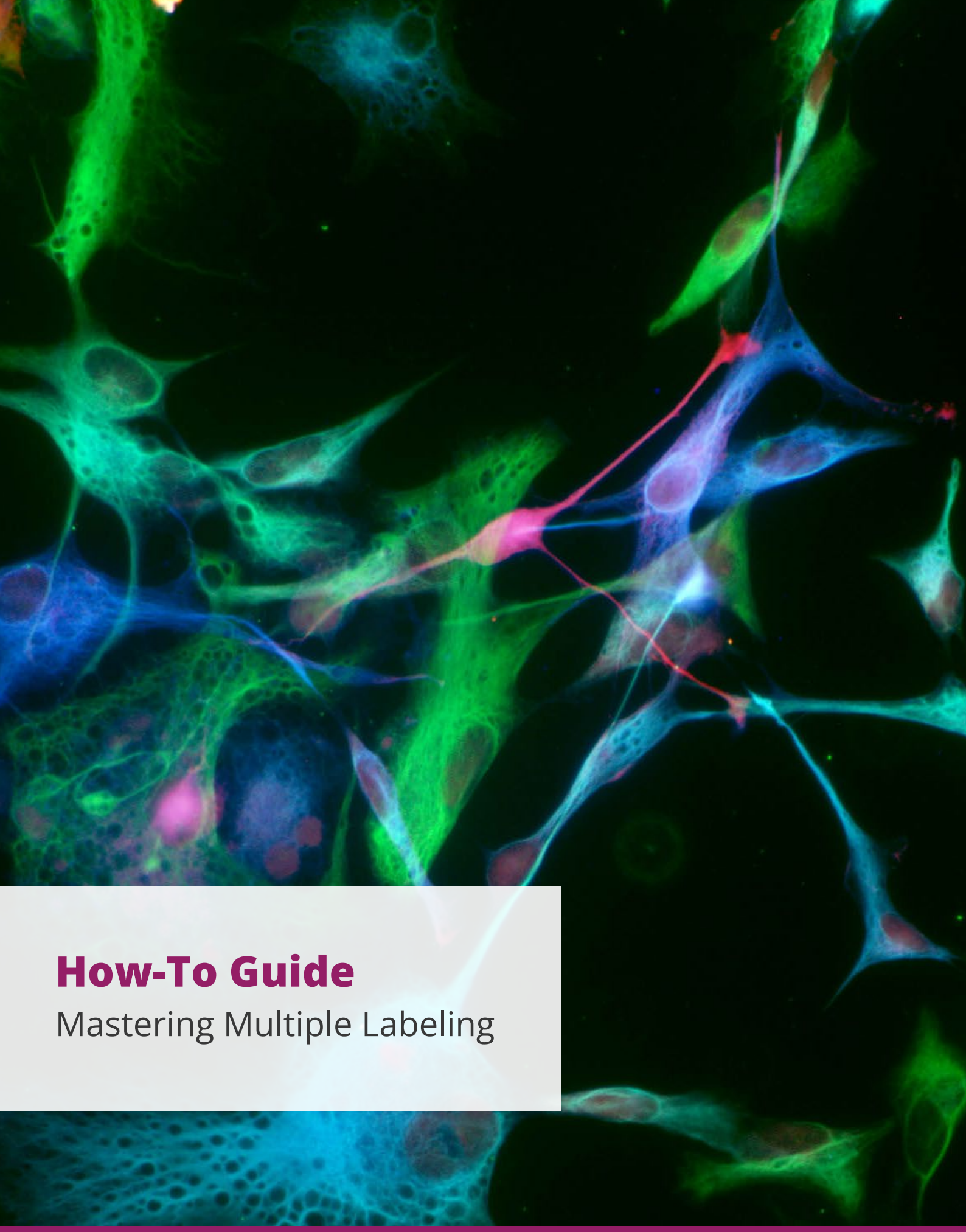

How-To Guide

Mastering Multiple Labeling

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Contents

Cover image

Human brain progenitor cells, immunocytochemically stained for GFAP (blue AMCA 715-155-150), Doublecortin (red Cy2 703-225-155), and Nestin (green RRX 711-295-152). Dr. Philip Schwartz and Boback Ziaeian, Children's Hospital of Orange County: Neuroscience Research.

Introduction to Multiple Labeling

Multiple labeling in immunoassays enables the simultaneous detection of several target proteins 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.

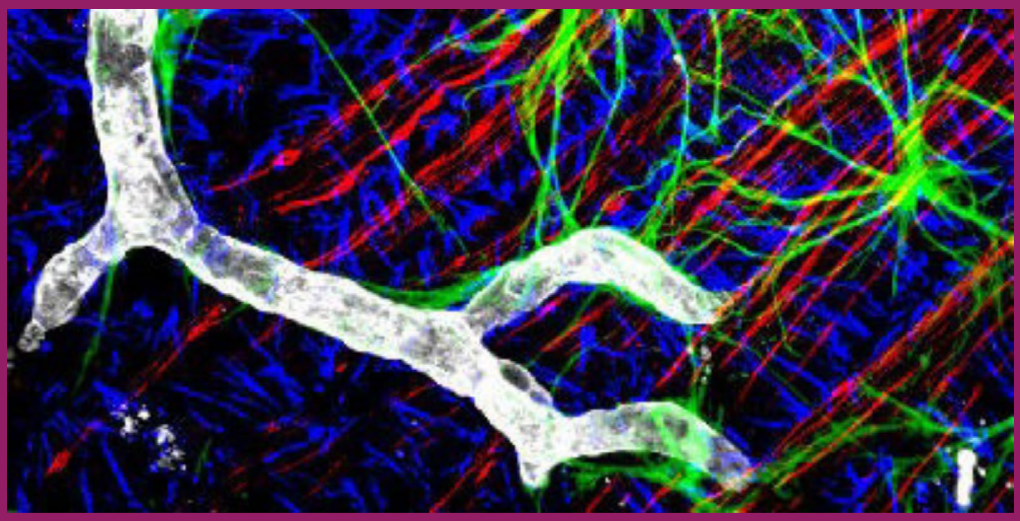


Figure 1. Mouse GFAP (green), NF (red), Collagen IV (grey), Vimentin (blue) z1.
Image courtesy of Gabe Luna, Neuroscience Research Institute, UC Santa Barbara.

Designing Your Experiment

Select Primary Antibodies

Choose primary antibodies that detect your protein/s of interest. Preferably, validate their specificity in-house with purified protein. When detecting two or more targets, ideally choose primary antibodies raised in different host species. This prevents cross-reactivity, where secondary antibodies may detect unintended primary antibodies, leading to unclear 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. 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, sectioning, and epitope retrieval.

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.

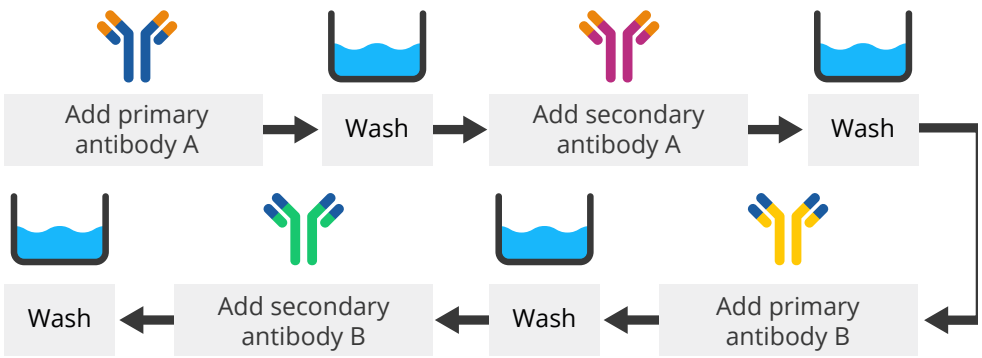
Step 3: Antibody Pair Application

Establish optimal incubation times for each antibody pair to achieve effective penetration. Titrating antibody concentrations is crucial for efficient usage and minimizing background noise.

Sequentially add each pair of primary and secondary antibodies per target protein (e.g., Goat Anti-Target 1 with Donkey Anti-Goat, Rabbit Anti-Target 2 with Donkey Anti-Rabbit, Rat Anti-Target 3 with Donkey Anti-Rat). 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 and Imaging

Optimize exposure times, filters, and image processing parameters for each fluorophore channel. Adjust microscope settings to maximize signal-to-noise ratio while avoiding saturation. Consider using sequential imaging for each channel to prevent bleed-through. Process images consistently to allow for accurate comparison and quantification of results.

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.

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.

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

- **Secondary-Only Control:** Incubate the sample with only secondary antibodies to determine the presence of any background signal. This control step is crucial for verifying that the secondary antibodies do not cross-react with the sample.

  When comparing a secondary-only control to a sample stained with both primary and secondary antibodies, you should not observe a matching staining pattern. If the pattern matches or a signal is present, optimize your blocking protocol and ensure that the secondary antibodies are cross-adsorbed against the sample species to eliminate the signal.

- **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.

- **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.

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

Blocking and Control Reagents

ChromPure® Purified Proteins: Use ChromPure® proteins as a negative control.

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. VHH fragments (nanobodies) also lack an Fc domain. This makes them particularly useful for avoiding binding to Fc receptors, which are present on certain immune cells.

By using $F(ab')_2$ fragments or nanobodies, 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. Additionally, simply titrating the secondary antibody to a lower concentration can help reduce background signal. Titrate your antibodies to ensure concentrations for optimal signal to noise ratio.
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 the target protein with another technique, such as Western blotting. 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.

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