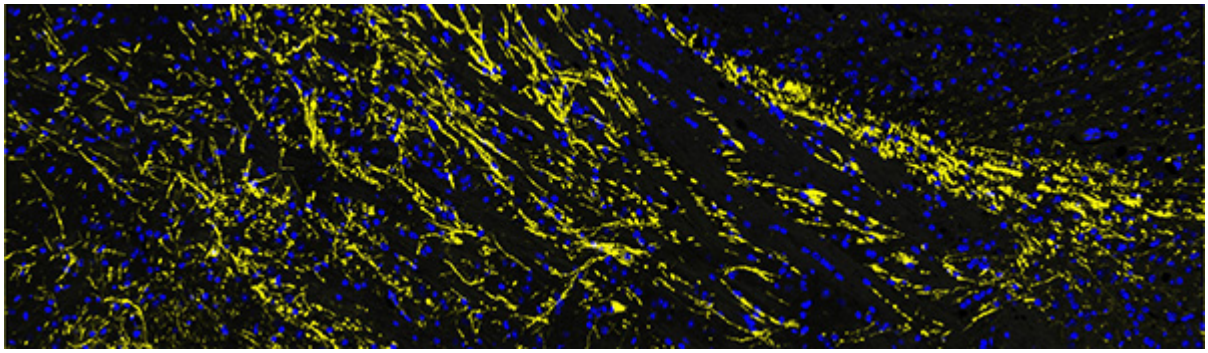
A major advantage of fluorescent detection is that it allows for multiplexing. Depending on the immunoassay technique being performed, as many as 20 or more different analytes can be measured in the same sample. However, multiplex immunofluorescence presents unique challenges for experimental design. Not only must researchers optimize each of the protocol steps common to all immunoassay experiments, but they must also consider fundamental fluorophore properties to ensure that target analytes are accurately identified.



Immunoassays that use fluorescent detection

While fluorescent detection was once reserved mainly for flow cytometry, immunohistochemistry (IHC), and immunocytochemistry (ICC), technological advances have allowed almost any immunoassay to produce a fluorescent readout. Today, researchers can perform fluorescent western blotting, fluorescence-linked immunosorbent assays (FLISAs), and a broad range of bead-based fluorescent immunoassays, as well as more recently established techniques such as spectral flow cytometry and various advanced microscopy applications.

Principles of fluorescence

Fluorescence occurs when specialized molecules known as fluorophores absorb light within a certain range of wavelengths (the absorption spectrum) before emitting it at another range of wavelengths (the emission spectrum). Because some of the absorbed energy is lost as heat during this process, the emitted light has a longer wavelength than the light used for excitation and is, therefore, a different color. During a typical fluorescence-based immunoassay, the excitation-emission cycle repeats thousands of times for each fluorophore. Due to this, and the fact that multiple fluorophore molecules are present on each antibody being used for analyte detection, the fluorescent signal is amplified to allow for measurement with an appropriate reader.

Key fluorophore properties

The peaks of the excitation and emission spectra, known respectively as the absorption and emission maxima, are key fluorophore properties that must be factored into experimental design. Specifically, they must be paired with the instrument’s light sources and detectors. Other fluorophore properties to consider include brightness, which is a property of both the extinction coefficient (ε) and the quantum yield (Φ), and the Stokes shift, which is the difference in wavelength between the absorption and emission maxima. In addition, the fluorophore’s photostability is important, especially for live-cell imaging studies.

Direct vs. indirect detection

When performing fluorescence-based immunoassays, researchers must decide between direct and indirect detection. Direct detection uses fluorophore-labeled primary antibodies to identify the target of interest, shortening the experimental workflow by eliminating the need for a secondary antibody incubation step and its associated washes. Indirect detection instead combines unlabeled primary antibodies with fluorophore-labeled secondary antibodies, offering the advantages of signal amplification and access to a wider range of reporter molecules.

Tips for performing multiplex immunofluorescence experiments

Whichever type of assay you intend to use for multiplex immunofluorescence, the same best practice recommendations apply. Here are our five top tips for performing multiplex immunofluorescence experiments.

Be rigorous with blocking

Blocking is essential to prevent unwanted signals due to non-specific antibody binding. Common blocking buffers include bovine serum albumin (BSA), non-fat milk, and gelatin, although normal serum (5% v/v) from the same species as the secondary antibody host is recommended for assays based on indirect detection.

We offer a broad range of blocking agents, including BSA free of IgG, which can otherwise serve as an antigen for cross-reacting secondary antibodies, and normal serums from almost 20 different species.

If indirect detection involves a primary antibody that shares the same host species as the sample, it is often advised to block endogenous immunoglobulins with an excess (20-40 µg/ml) of unconjugated Fab antibody immediately after routine blocking. This will prevent secondary antibodies from binding to sample IgGs, which can especially present issues for ICC and IHC applications.

Our FabuLight™ antibodies are Fab fragment secondary antibodies specific to the Fc region of IgG or IgM primary antibodies; they are available unconjugated for blocking endogenous IgGs, as well as conjugated with nine different fluorophores to enable the detection of primary antibodies without a secondary antibody incubation step.

We do not recommend that the blocking agent be included in antibody diluent buffers.

Optimize single staining before sequential staining

Before performing a multiplex immunofluorescence experiment, it is important to individually optimize the staining conditions for each primary antibody or primary/secondary antibody pair. This means titrating each of the antibody reagents to identify conditions with low background and a strong positive signal.

For assays based on indirect detection, it is also recommended to confirm the specificity of each secondary antibody for its intended primary antibody during optimization. This is easily achieved by attempting to label primary antibodies with the ‘wrong’ secondary antibodies. Additionally, running ‘secondary antibody only’ controls is advised to identify any sources of non-specific secondary antibody binding.

Choose the right secondary antibodies

While a lot of attention is given to primary antibody selection, choosing the right secondary antibodies is equally critical. When performing multiplexed experiments, it is recommended to select secondary antibodies that share the same host, if possible, to prevent them from binding to one other. It is also advised to choose secondary antibodies that have been cross-adsorbed against the other species that might be encountered in the experiment to ensure they will not recognize other primary antibodies or tissue being used in the same experiment.

To provide an example, if primary antibodies from goat, rat, and mouse hosts will be used for indirect detection of three different analytes, a sensible approach would be to choose three secondary antibodies from a donkey host, one of which is cross-adsorbed against rat and mouse, one against goat and mouse, and one against goat and rat.

We offer an extensive selection of cross-adsorbed secondary antibodies, for which we confirm that species cross-reactivity has been minimized by ELISA and immunoelectrophoresis (IEP) testing.

Assign fluorophores carefully

Besides ensuring that the excitation and emission maxima of each fluorophore are compatible with the instrument being used, researchers must think carefully when assigning fluorophores to different targets. Best practice recommendations include matching brighter fluorophores to less abundant analytes or lower affinity primary antibodies to increase the likelihood of target detection, and spreading fluorophores across as many lasers and detectors as possible to minimize spillover.

If the instrument has only a handful of lasers, the number of targets being analyzed can be increased using fluorophores with similar excitation maxima but different Stokes shifts, or by introducing tandem dyes into staining panels. The excitation and emission maxima, and Stokes shifts, of different fluorophores, are easily visualized using a Spectra Viewer.

Speak with us!

Jackson ImmunoResearch specializes in producing secondary antibodies for life science applications and is always happy to provide guidance for product selection. Whether you're looking for a particular fluorophore conjugate, need help with panel design, or are thinking about purchasing fluorophore-conjugated antibodies in bulk to support a long-term study, contact us today to discuss how we can advance your project.

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