Multicolor Immunofluorescence Imaging

In order to study localization of cellular proteins with high specificity, it is frequently necessary to multiplex antibody probes conjugated to fluorescent markers. Today’s technology provides the researcher with a variety of fluorescent probes that can be combined in studies of cellular function.

High-resolution, multi-probe imaging places a number of demands on imaging technologies; advanced CCD and cMOS imaging solutions from QImaging are engineered to produce impressive results in this context.

General Concepts of Immunolabeling

Protocols for immunolabeling vary widely due to experimental goals. Interactions that contribute to antibody binding include hydrogen bonds, ionic bonds, hydrophobic interactions and van der Waals forces. The strength of each of these interactions is weak compared to a covalent bond. Consequently, a large number of noncovalent interactions are required to stabilize antibody binding to an antigen. Each of these noncovalent interactions operates over a very small distance (1 Å or less), therefore a strong antibody-antigen interaction depends on a very close fit between the antigen and antibody. This fit requires a high degree of complement between antigen and antibody. This requirement is the basis of the specificity that characterizes antigen-antibody interactions.

Methods used to prepare samples for microscopic visualization may confound immunolabeling; some antigenic epitopes (the small structural element recognized by an antibody) are destroyed by temperature extremes or the organic solvents commonly used to process histological samples. Different fixatives may destroy epitopes or make them less accessible. The choice of blocking agent may impact the level of non-specific background staining. For success in immunolabeling it is crucial to carefully optimize labeling conditions; in order to do this it is important to understand some basic concepts regarding antibody-antigen interactions. It is also important for an investigator to be aware of appropriate control experiments which will aid in troubleshooting poor labeling results.

Antibody Selection

Antibodies are glycoproteins and can themselves function as potent antigens to induce an immune response. For example, antibodies from a rabbit may be used to immunize a goat to produce anti-rabbit antibodies. These goat anti-rabbit antibodies may be used as a reagent to detect the binding of mouse antibodies In Vitro. Such a detection scheme is known as indirect labeling and an advantage of this approach is that the signal resulting from the binding of the primary antibody can be amplified (Figure 2). A disadvantage of indirect labeling is that non-specific staining is often more prevalent. Secondary labeling reagents may be specific for the species (xenotype) from which the primary antibody is derived, the class (isotype), the

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subclass (allotype) and the light chain of the primary antibody.

Highly specific secondary antibody reagents may reduce nonspecific staining significantly (as compared to secondary reagents that are specific for the xenotype and isotype of the primary antibody alone). For a well characterized monoclonal antibody, the species, isotype, allotype and light chain component will be known. For instance, a mouse derived monoclonal antibody might be designated as IgG2bk, reflecting the antibody isotype, allotype and light chain type. A well matched secondary probe would be specific not just for murine IgG antibodies, but for IgG2bk in particular.

Control Strategies

The data from immunolabeling is greatly strengthened with the judicious use of positive and negative controls. Positive controls are valuable tools to confirm the reactivity of the antibody to antigen under a particular protocol. If the antigen of interest is available in purified form, it can be adsorbed onto a nitrocellulose membrane. This membrane may then be blocked using a blocking reagent. The resultant “spot blot” is done using typical Western Blotting techniques and confirms the functional antibody-antigen interaction.

Alternatively, cells or tissues that are well documented to possess the antigen of interest should be used under identical labeling conditions to the experimental system.

In order to explore the possibility that a monoclonal antibody is cross-reacting with an irrelevant antigen in the experimental system, a monoclonal antibody to an alternative epitope on the antigen of interest may be used. Using monoclonal antibodies to different epitopes on the same antigen will strengthen the evidence that the antigen being detected is, in fact, the antigen of interest.

To assess the degree to which non-specific binding of the labeling antibody plays in the signal distribution, an isotype control may be used. In the most basic form of this test, the negative control specimen is exposed to an antibody of the same isotype as the experimental antibody probe, but the control antibody is specific for an irrelevant antigen. Suppliers of monoclonal antibodies often offer well characterized isotype control antibodies. Ideally, the isotype control antibody will be of the same species, and have the same heavy and light chain antigens as the probe antibody.

In a ligand-blocking control, the antibody probe is allowed to react with an excess of purified antigen. This pre-blocked antibody is then incubated with the intended target. The antibody-blocking control is the inverse of the ligand-blocking control. In the antibody blocking control, the cells or tissues of interest is pre-incubated with probe antibody that has not been conjugated to a signaling molecule. The blocked cell or tissue is then probed with the conjugated antibody probe.

Indirect labeling studies require controls for the nonspecific binding of the secondary antibody as well as the primary antibody; in many cases simply omitting the primary antibody from the labeling protocol will give an indication of non-specific binding of secondary probes. This can be taken one step further by using an isotype control for the secondary antibody and omitting the primary antibody.

Imaging Technology

Although relationships between labels on a multiple labeled specimen may be intuitively evaluated through the microscope eyepiece using our innate color vision, color cameras are not considered the best choice for specimens displaying multi-color fluorescence. There are several reasons for this; one of the most compelling is that color cameras possess only a small fraction of the light gathering efficiency of monochrome cameras.
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Because the conditions of fluorescence imaging typically involve low light conditions and chromophores that fade with exposure to the illumination source, it is advantageous to make efficient use of the light coming back from the specimen. For this reason, the QImaging Retiga R™ Series CCD cameras present a user friendly and high-performance solution that support the very specific requirements of immunofluorescence imaging.

Visualization of the individual colors can be achieved with a monochrome camera using a specialized interference filter for each fluorochrome emission color in the microscope. The images for each color are typically acquired in a serial fashion, one after the other, and then assembled into a single image using software. One such software, QImaging Ocular™ allows a user to acquire each channel as many times as desired to get the perfect image of each fluorophore before finalizing the multicolor overlay image. Such a streamlined image acquisition workflow is a great benefit to the scientist performing repeated multicolor imaging experiments.

The QImaging Retiga R Series cameras use a CCD chip that is highly optimized for light gathering ability. The cameras’ microlenses and low noise performance enable the ability to maintain high resolution and provide the flexibility needed for fast readout and updating the image information for fluorescence microscopy.

Additionally, monochrome camera technology has an inherently linear response, which ensures the relative contributions of individual fluorochromes are measured reliably. This is not advisable with color cameras because they are designed to emphasize green wavelengths over blue or red. This is an intentional bias designed into color cameras to better replicate the human eye response. However, it is a detriment to quantitative analysis.

The low light levels that are typical of fluorescent imaging may require longer exposure times. For this reason, the best imaging sensors for immunofluorescence are typically cooled to decrease the contributions from camera dark noise at longer exposures. Cooling to an absolute temperature mitigates fluctuations of the room temperature on camera performance. The Retiga R Series cameras are peltier-cooled to a regulated -12°C, providing the most reliable low-noise performance under conditions that require longer exposure times.

With any modern experiment, there is a myriad of experimental and technological parameters to consider for multicolor immunofluorescence imaging. There is no substitute for good chemistry (probe creation) solid biology (labeling of samples) and expert application of physics and engineering (the camera). Of course, software is how interaction with images occurs and enables the ability to create publication-quality data, so that should not be overlooked. Taken in this context, multicolor immunofluorescence is an advanced technique that labs are employing for a multitude of research applications.

Careful attention to all of these details ensures productive data and cover-worthy images.

Learn more about the high performance cameras from QImaging: http://www.qimaging.com/products/cameras/scientific

References