

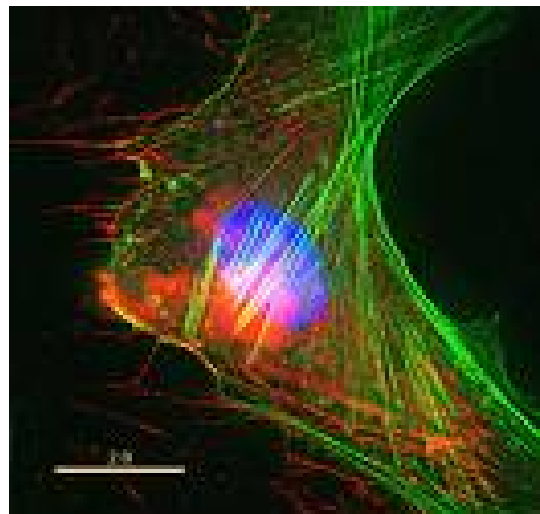
QImaging® Camera Application Notes Multicolor Immunofluorescence Imaging

In order to image localization of intracellular 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 immunofluorescent 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 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 non-covalent interactions are required to stabilize antibody binding to an antigen. Each of these non-covalent interactions operates over a very small distance (1 Å or less) and so 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.



The methods used to prepare a sample 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 important control experiments which will aid in troubleshooting poor labeling results.

Polyclonal and Monoclonal Antibodies

Most antigens are complex and contain many different antigenic determinants, or epitopes, and so the immune system generally responds by producing antibodies to several of them (Figure 1). Each B-cell produces a monoclonal antibody, the collective output of these monoclonal B-cells is considered to be a polyclonal antibody response to an immunizing antigen. The polyclonal antibody produced *in vivo* is beneficial to the organism, however polyclonal antisera has disadvantages for *in vitro* applications such as immunolabeling. Such experiments require precise control of antibody quantity, properties and specificity, particularly where multiple label strategies are concerned. The method for *in vitro* production of monoclonal antibody was devised by Kohler and Milstein (1975) and their work was later recognized with a Nobel Prize in 1984. The basic approach involves fusing of an antibody producing B-cell with a myeloma cell. Monoclonal antibodies are generally well characterized, an important consideration for optimizing immunolabeling studies.

Antibody Selection

Antibodies are glycoproteins, and they can function as potent antigens to induce an antibody 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 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.

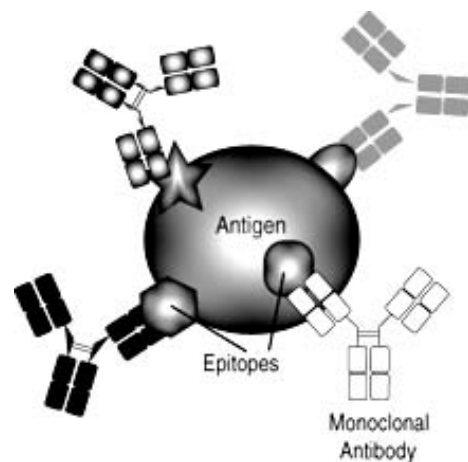


Figure 1. Monoclonal antibodies binding to different epitopes

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. 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.

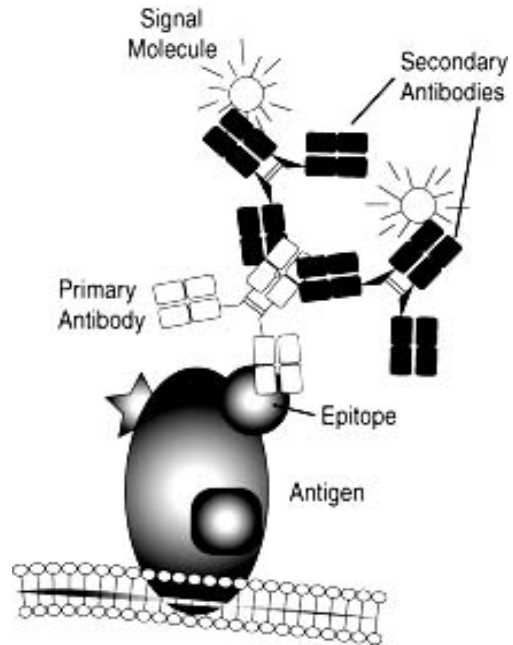


Figure 2. Signal amplification through indirect labeling

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 are most intuitively evaluated through the microscope eyepieces using our innate color vision, color cameras are not generally considered the best choice for multi-color fluorescent specimens. 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. 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. The QImaging® SRV has been conceived as user friendly a high-performance solution to immunofluorescence imaging requirements.

Segmentation of the individual colors with a monochrome camera is achieved at very high efficiency using specialized interference filters 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 overlaid into a color image using software. This strategy permits acquisition of images equal in resolution to color cameras that have much lower light-gathering ability due to their typically much smaller pixels and the light absorbing color Bayer mask. The QImaging® SRV utilizes a microlensed CCD chip highly optimized for light gathering ability and low noise performance while maintaining high resolution and the flexibility for fast readout and update of image information for fluorescence microscopy.

Another major consideration is that monochrome camera technology is considered to have an inherently linear response and so the relative contributions of individual fluorochromes can be 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 so that they better replicate the human eye response, it is, however, a detriment to quantitative analysis.

The low light levels typical of fluorescent imaging may require longer exposure times. For this reason, the best imaging sensors for immunofluorescence imaging are typically cooled to decrease the relevance of camera dark noise at longer exposures. Cooling to an absolute temperature mitigates fluctuations of the room temperature on camera performance. The QImaging® SRV is peltier-cooled to a regulated -30 degrees to provide the most reliable low-noise performance under conditions requiring longer exposures.

QImaging® monochrome cameras have the option of being seamlessly equipped with an innovative liquid crystal color filter that can be moved in and out of the

light path. This option permits easy acquisition of brightfield RGB color images at high resolution under brightfield conditions as well as high efficiency quantitative monochrome imaging for fluorescence. Such flexibility adds to the value of QImaging® monochrome cameras and is like having two specialized acquisition strategies in a single technology package.

To learn more about the high-performance CCD cameras from QImaging, please visit:

<http://www.qimaging.com/products/cameras/scientific/index.php>

References

Kohler, G. and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 256:495.