

## **Special Topic: Advanced Basics of Immunostaining and Antigen Retrieval.**

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The ability to immunologically link microscopic tags to specific proteins has produced major advances in all forms of microscopy. Initially researchers needed to make and label their own antibodies. This required a good working knowledge of immunology. Today labeled antibodies are readily available from a variety of vendors. Although this has greatly facilitated the expansion of immunolabeling techniques, it has also led to their use by researchers with little or no background in immunology. While this is not a problem for simple, straightforward situations, the quality of data produced in more specialized experiments can suffer. Being an expert in immunology is not necessary for the average user of immunohistochemistry techniques, but some basic understanding of what antibodies are, how they are formed, and how best to use them as a cellular probe can greatly enhance the results of “non-routine” staining procedures. In this regard it is worth reviewing some basic immunology principles for the naive user and sharing some insights with the more experienced user. This is the general goal of this session. Specifically, we will discuss what antibodies are, we will review the variables in a “standard” immunostaining protocol (fixation, washing, choice of primary and secondary antibodies)

Immunoglobulins, particularly immunoglobulin G (IgG), are the basic tool of immunohisto/cytochemistry. An antibody is an immunoglobulin molecule. It is produced by cells of the immune system in response to a foreign challenge (antigen). A good antibody binds to its antigen specifically and with high affinity. This binding is exploited to tether a microscopically visible marker to the antigen. In this way, the molecule can be specifically localized within cells or tissue. To produce superior localization without artefact, a good antibody is required. A little time spent obtaining and testing antibodies to insure they are useful in immunostaining will save the investigator a lot of time and trouble later.

There are a variety of paradigms for immunostaining. The most basic procedure is to label the antibody directly with a microscopic marker. This simplifies the staining procedures but interactions between the label and the immunoglobulin often lessens the usefulness of the antibody as a histochemical reagent. The most common method is the two-step or indirect method. In this method the antibody is reacted with the sample and allowed to bind to the antigen. Then the preparation is incubated with a second antibody. This antibody contains the label and has been carefully selected because its properties are not adversely affected by the tag and because of its specificity for binding to the first antibody. For instance, to localize human acid phosphatase enzyme an IgG antibody can be raised in a rabbit that recognizes human acid phosphatase. After tethering the anti-acid phosphatase to the acid phosphatase molecules in the cell, the anti-acid phosphatase can be localized by tethering a labeled goat IgG that specifically binds all rabbit IgGs but does not bind human IgG. These general labeled secondary antibodies are readily available commercially. Although this two-step method takes a little more time, it saves the researcher the time of making multiple primary antibodies, labeling each antibody, and testing each for specificity after labeling in order to find one that is useful. Variations on this theme include exploiting the ease of biotinylating antibodies and the strong affinity of biotin for avidin as a means of localizing an

antigen. Since each avidin will bind four biotins, modifications of the technique can be used to amplify the labeling of an antigen that occurs in low abundance.

Among the more mystical of variables in deciding on a specific immunohistochemistry technique are those classified as “antigen retrieval” procedures. The goal is to unmask “hidden” epitopes and thus increase immunostaining or allow immunostaining with antibodies that were previously not useful for *in situ* staining. Antigen retrieval procedures have been most useful with paraffin embedded sections but are now receiving some notoriety for their success with whole mount preparations and with specimens for electron microscopy. Basically, the idea is to open up sites that were hidden during processing or are occult in the native protein. The method by which these procedures work is not known but it is thought that most function by either denaturing the protein and/or removing the crosslinking effect of fixative within an active site. The procedures can be divided into two basic techniques. The first employ chemical means such as detergents or proteases and the second physical means such as heat and/or pressure. We will review some of these methods, the various devices on the market to facilitate antigen retrieval, and the factors that most effect the outcome of an antigen retrieval procedure.

Several critical factors go into the decision of the exact protocol to be used in an immunolocalization. These include how cell integrity is to be maintained, how the antigen is going to be made accessible to the antibody, what type of tag will be used, and how the localization data will be acquired and analyzed. For instance, are the cells to be fixed and, if so, what fixative should be employed? Do the cells need to be permeabilized to allow the antibody access to the antigen or is the antigen readily accessible? Do you need to visualize multiple antigens at the same time? Do you need the resolution of an electron microscope or is light microscopy sufficient? All of these are considerations in choosing a methodology. Hopefully, this session will provide you the tools to design better protocols for your studies.