

PERSPECTIVES

Specificity Controls for Immunocytochemical Methods

Richard W. Burry

Department of Neuroscience, College of Medicine and Public Health, The Ohio State University, Columbus, Ohio

SUMMARY Immunocytochemistry is used for antibody localization of proteins in cells and tissues. The specificity of the results depends on two independent criteria: the specificity of the antibody and of the method used. The antibody specificity is best determined by immunoblot and or immunoprecipitation. Absorption of the antibody with a protein does not determine that the antibody would have bound to the same protein in the tissue, and therefore is not a good control for antibody specificity. The specificity of the method is best determined by both a negative control, replacing the primary antibody with serum, and a positive control, using the antibody with cells known to contain the protein. With the increasing use of immunocytochemistry, it is important to be aware of the appropriate controls needed to show specificity of the labeling. (J Histochem Cytochem 48:163-165, 2000)

Immunocytochemistry is a powerful method for identification of proteins in cells and tissues. However, this method is dependent on the specificity of the antibody binding to the epitope of the protein used as an immunogen. Treatment of tissues with chemical fixatives and detergents can change the reactivity of proteins to antibodies because the exposure of epitopes can differ. Recently, Josephsen et al. (1999) discovered that a monoclonal antibody generated to a purified vimentin reacts with a novel epitope on an unrelated protein, but only when the tissue is fixed with glutaraldehyde.

Absorption Controls

Controls for the specificity of an antibody are most important for the correct interpretation of its localization in cells and tissues. One control for specificity is the so-called absorption or preabsorption control, in which the antibody is mixed with the protein or peptide used to generate the antibody, with the objective of eliminating the binding of the antibody to the protein in the tissue. Although the origin of the absorption control is not clear, it was no doubt developed be-

cause of the early use of radioimmunoassays (RIAs) and immunocytochemistry as antibody techniques and the similarity of the absorption control to the RIA procedure. Now, with the use of immunoblots or immunoprecipitation, specificity of the antibody for a specific protein can be determined. As pointed out years ago by Swaab et al. (1977) and more recently by Willingham (1999), the absorption control determines only the specificity of the antibody for the incubating protein or peptide and does not prove the specificity of the antibody for the protein in the tissue. An antibody can bind to any epitope that has the correct conformation, and this potentially includes the protein used for immunization, as well as any protein with a similar epitope. Therefore, the absorption control does not indicate that the protein to which the antibody binds in tissues is exactly the same protein that was used to generate the antibody.

When an absorption control is done, obtaining complete inhibition of antibody binding to tissue is difficult. In theory, incubating the peptide with the antibody at appropriate concentrations will result in occupancy of all binding sites. However, a small but significant dissociation occurs during the incubation, which can lead to dissociation of some of the antibody from the peptide and results in antibody binding to the protein in the tissue. This effect can be overcome by preincubating the tissue with the peptide in addition to preincubating the antibody with the peptide, to reduce the likelihood of antibody binding to tissue

Correspondence to: Richard W. Burry, Dept of Neuroscience, Coll. of Medicine and Public Health, O.S.U., 333 W. Tenth Ave., Columbus, OH 43210.

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proteins. Mark Willingham (personal communication) has found that absorption controls can give complete inhibition of tissue labeling only under conditions in which both the tissue and the antibody were preincubated with the peptide.

The absorption control can actually be positive (i.e., the antibody still produces staining) even though the antibody is specific for the peptide or protein in the tissue. In my experience, binding of the antibody-protein complex may occur via the proteins in tissue sections. For example, adding intermediate filament proteins to an anti-intermediate filament antibody did not eliminate the labeling in tissue sections. Although it is possible that unbound primary antibody could have accounted for some of the labeling, the majority of this labeling occurred because the antibody-protein complex bound to the tissue. It is highly likely that, after antibody binding, other parts of intermediate filament protein were available for interactions with cytoplasmic proteins in the tissue sections. In this case, the protein absorbed by the antibody apparently bound to the tissue through protein-protein interactions of the protein-bound antibody. The strength of this type of binding could be stronger than binding of a single protein alone. When an antibody binds to two separate proteins, one each at an Fab', the thermodynamics of these antibody-bound proteins in solution are slower than those of single proteins, allowing a greater chance of protein-protein binding interaction in the tissue. If both of the antibody-bound proteins bind to proteins in the tissue, the likelihood of either protein dissociating is reduced. The distribution of the antibody-bound proteins in cells is to sites of endogenous protein binding, and therefore labeling will duplicate the distribution of the endogenous protein.

In addition, the results reported by Josephsen et al. (1999) suggest that absorption controls are not always reliable in determining the specificity of the antibody in immunocytochemistry. In this case, the antibody recognized vimentin specifically, as shown by immunoblot analysis. However, glutaraldehyde fixation changed the conformation of an additional protein so that the antibody bound spuriously in tissue sections.

Finally, absorption controls can be difficult to implement because of characteristics of the protein or peptide used as an immunogen. First, the purity of protein used in the absorption is an important consideration. For many proteins, it is not feasible to adequately purify proteins (free of contaminating proteins) to make an absorption control useful. Second, if the protein used as an immunogen is not available, as might be the case for some monoclonal antibodies, absorption controls could not be done. To insist on an absorption control in such cases is inappropriate. Given these problems with absorption controls, it is worthwhile to consider the controls that are necessary

to determine the specificity of antibody labeling by immunocytochemistry.

The specificity of labeling requires two independent sets of criteria: specificity of the antibody and specificity of the method (Petruz et al. 1976). The specificity of the antibody refers to characteristics of an antibody independent of the methods used to employ it, and I will discuss these first.

Specificity of the Antibody

The characterization of antibody specificity requires demonstration that the antibody binds only to the protein that contained the immunogen peptide. Each antibody is produced by methods that determine its specificity. Today, many antibodies, both monoclonal and polyclonal, are generated to synthetic peptides. Many of these antibodies are purified with the immunizing peptide on an affinity column, greatly reducing the possibility that the antibodies will bind to epitopes not found on the original peptide. Immunoblotting with an antibody should show that the antibody recognizes a single protein of the appropriate molecular weight. In cases in which immunoblotting is not possible, immunoprecipitation can be used to bind a protein of the expected molecular weight. Of great importance are the details of the generation, which should be given to the user. The information should include the sequence of amino acids used in the peptide, the species from which the immunogen peptide or protein was obtained, the species of the antibody, and the isotype of immunoglobulin generated. These data are important because many of the antibodies in use recognize an immunogen only in the same or in closely related species. Therefore, the specificity of the antibody is determined by showing specific reaction with the peptide or protein used for immunization with highly sensitive methods other than immunocytochemistry.

Specificity of the Immunocytochemical Method

Controls are essential to ensure that the detection system works and that the primary antibody is responsible for generation of the label. First, to show that the labeling is specifically due to the primary antibody, the primary antibody should be replaced with similarly diluted normal serum from the same species, keeping all the other steps the same, or using a known unreactive antibody of the same isotype. A variation of this control omits the primary antibody, but this procedure is not as rigorous as using normal serum or an unreactive antibody. This latter negative control, which is most likely to detect nonspecific binding due to reactive groups remaining in the tissue or due to poor blocking of a "sticky" section, should be run

with each experiment. Second, sections of known (but different) tissue with cells that contain the protein can be used to confirm the specificity of the antibody. This positive control shows specific labeling in cells containing the protein, whereas other cells are not labeled. For example, an antibody to insulin should specifically label the insulin-producing cells of the islets of Langerhans in the pancreas. An alternative positive control is to use the experimental sections with several different antibodies that are directed against the same protein to show that the same structure is labeled. For example, Josephsen et al. (1999) used several different antibodies prepared to the same protein, vimentin, and showed the same labeling pattern. These antibodies, generated independently to the same protein, must show the same localization within cells and tissues.

Conclusion

Immunocytochemistry is an important method for identification of peptides and proteins in tissues, but the correct interpretation of labeling requires proper controls and thoughtful interpretation of the results of control experiments. In each case, the specificity of the immunocytochemical procedure is validated by negative controls that ensure that the labeling method accurately identifies the antibody bound to the tissue

and by positive controls that show that the antibody is binding to an appropriate structure. The specificity of the antibody is best evaluated by immunoblotting or immunoprecipitation. The absorption control is less important because it cannot determine whether the protein bound in the tissue is the same protein that is used for absorption. Therefore, the recommended guidelines for immunocytochemistry include negative controls and positive controls but do not include absorption controls.

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