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## Editorial

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# An Open Letter to Our Readers on the Use of Antibodies

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The use of immunohistochemistry has become ubiquitous in neuroscience. A large majority of papers now published in *The Journal of Comparative Neurology* use immunohistochemistry, and some papers may employ a battery of ten or more antibodies to examine issues of colocalization or cell typing. This pattern has resulted in a flood of new information. . . . but also a flood of misinformation.

The *Journal* has repeatedly, over the last few years, received distressed communications from authors, who have had to withdraw papers because an antibody against a novel marker was found to stain tissue in knockout animals, who lack that target protein. In many cases these papers contained careful characterization of the antibodies and immunocytochemical controls. This issue has sensitized the Editors to the problem of antibody specificity, and we soon realized that many of the papers we were publishing had very limited characterization or controls for antibodies that were used. Subsequently the Editors noticed that a number of commercially available antisera, particularly against G-protein coupled receptors, gave staining patterns that did not match mRNA distributions, and that these antibodies still stained tissue from animals in which the receptor had been knocked out. We felt that the integrity of scientific communication was being threatened by the proliferation of poorly characterized antibodies that produce artifactual staining patterns, and therefore came up with a minimal set of rules for identification, characterization, and controls for immunohistochemistry that we are attempting to apply to all manuscripts that we publish (Saper and Sawchenko, 2003).

The result has been a substantial degree of confusion about what information is necessary for a paper in JCN (or any other journal, for that matter), to provide a reasonable level of assurance that an antibody is actually recognizing what it is supposed to be staining. In the interest of demystifying this procedure further, we present the following brief description of the three basic elements that are necessary in describing an antibody for use in neuroscience:

**1.) Complete information on the antibody.** It is important to recognize that antibodies are not simple reagents that always identify the same thing. Thus, to describe an antibody as completely as possible, so that other scientists can replicate your work, you need to provide information of two kinds:

—Identification: What was the source of the antibody? If another lab has donated the antibody, give the antiserum code number, and if possible, the bleed. If it was obtained from commercial sources, give the catalog, and if possible the lot number.

—Preparation of the antibody: What was the antibody actually raised against? Give the precise structure of the immunizing antigen, not just vague information about the part of the molecule that was used. What species was the antiserum raised in? Was it a polyclonal or monoclonal preparation? Please note that some antibody manufacturers deliberately try to obscure the information about the structure of the antigen. We have received complaints from authors that several manufacturers have claimed that sequence information on the antigen was “proprietary,” and they would not provide it. Because work using these antibodies is inherently not repeatable, such papers are not acceptable for publication. Our advice is:

**NEVER BUY ANTIBODIES FOR WHICH THE MANUFACTURER WILL NOT DISCLOSE THE STRUCTURE OF THE IMMUNIZING ANTIGEN. THESE REAGENTS ARE NOT FIT FOR SCIENTIFIC WORK, AND THE WORK YOU DO WITH THEM WILL NOT BE PUBLISHABLE.**

Examples of antibody descriptions that would be acceptable:

“This rabbit antiserum (Company XYZ #30248) was prepared against a synthetic peptide representing amino acids 121-142 from tyrosine hydroxylase.”

“This mouse monoclonal antibody, kindly donated by Dr. John Smith, University of Alabama, was raised against human placental choline acetyltransferase.”

**2.) How has the specificity of the antibody been characterized?** If the antiserum is against a large protein, it is important to know what it stains on a gel from the tissue and species you are using. Ideally, an antibody should stain a single band (or several bands if the antigen has several known molecular configurations) of appropriate molecular weight. This information is often included by the manufacturer in the technical information, and can be cited, as can previous studies that provided this information. However, it is not sufficient just to state “this antiserum was previously characterized (Jones et al.,

2002).” You need to provide a reasonably critical reviewer with information on what characterization was done, and what it showed.

Examples of antibody characterization that would be acceptable:

“The antiserum stains a single band of 55 kD molecular weight on Western blot (manufacturer’s technical information).”

“This antiserum stains the 150kD but not the 130kD or 110kD forms of the molecule on Western blot (Fig. 1).”

**3.) What controls are necessary for immunostaining?** We are constantly surprised by the oddly trusting nature of many of our colleagues, who seem to believe (or want to believe) that an antibody will stain what the manufacturer claims. In fact, in many cases nothing could be further from the truth. It is always important to provide the highest level of control you can to assure that the antibody is staining what you want it to stain.

The gold standard in this regard is: does the antibody stain the tissue of interest from which the molecule of interest has been removed? This standard is simple to achieve for antibodies against tracers, or bromodeoxyuridine, or green fluorescent protein, which are not normally present in tissue. A similar level of assurance can be obtained when a knockout mouse (or other animal) is available (i.e., if the antibody stains tissue in a wild-type animal and not the animals from which the gene has been deleted). But this is only applicable if you are looking at tissue from an animal for which a knockout exists.

Most of us, most of the time, must make due with lesser degrees of certainty. A useful control, which is especially important for antibodies raised against synthetic peptides, is to preadsorb the antiserum against the peptide. If the staining disappears, it is at least likely that the staining component of the antiserum is indeed raised against that antigen. This does not protect you from other tissue proteins that may cross-react, however, and we have had a number of papers eventually retracted when an antiserum that passed the preadsorption test also were shown to stain tissue from knockout animals. This strategy cannot be used for monoclonal antibodies, which always will be adsorbed out by their antigen, even if they are staining something entirely different in the tissue.

Lacking the antigen (e.g., if it is a large protein), there are several alternative strategies for establishing staining specificity. If the pattern of staining of the antigen is well known (e.g., antibodies against glial fibrillary acidic protein or tyrosine hydroxylase), it is reasonable to show that tissue of the type and species you are studying, when

stained with your antibody, produces a pattern that is identical to that previously reported. Alternatively, it can sometimes be demonstrated that one antibody (e.g., a monoclonal antiserum against a large protein) produces a pattern of staining that is identical to another antiserum (e.g., a polyclonal serum against a synthetic peptide) that is better characterized (and does pass the adsorption test). Antisera against different parts of the same molecule which produce the same staining pattern, or can be shown to be colocalized in double label studies, provide an important strategy for establishing specificity. Similarly, comparison with the pattern of mRNA expression by using *in situ* hybridization histochemistry can demonstrate that the immunostaining for the protein is genuine.

Note that omission controls (staining without the primary antibody) are **NOT** controls for specificity of the primary antibody at all. They control only for the specificity of the secondary antiserum.

Examples of acceptable controls for immunostaining (in roughly descending order of rigor):

“No staining was seen when the antibody was used to stain tissue from an orexin knockout mouse.”

“All staining was abolished when 1 ml of the diluted primary antibody was preincubated with 50  $\mu$ g of the immunizing peptide.”

“Staining with this antiserum was colocalized with *in situ* hybridization for the mRNA for the same protein (Fig. 3).”

“This antiserum against the N-terminal 13 amino acids of the protein gave the same staining pattern as another antiserum against the C-terminal cyanogen bromide fragment of the protein (Smith et al., 2001).”

“Staining of sections through the pons produced a pattern of tyrosine hydroxylase immunoreactivity that was identical with previous descriptions (Smith et al., 1992).”

“The GFAP antiserum only stained cells with the classic morphology and distribution of fibrillary astrocytes (Fig. 2; see Smith and Jones, 1998).”

The full information for any antibody usually requires only two or three sentences. Often this information can be contained within a table. However, it is critical that for each antibody used, the authors must supply sufficient information to assure that the result is replicable and likely to be correct. Blind faith that the antibody will stain whatever the manufacturer claims is not consistent with good science.

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