

Chapter 2

Antibodies

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Introduction

An antibody (Ab) is the key reagent of immunocytochemistry. To use antibodies effectively, consider their structure, function, and generation. Such basic knowledge about antibodies is essential to succeed in identifying suitable experimental design, finding antibodies, and trouble-shooting problems.

- Immunocytochemistry takes advantage of three properties of antibodies:
1. Antibodies uniquely bind to a protein or other molecule.
 2. Antibody binding to molecules is essentially permanent at physiological conditions.
 3. New antibodies can be made tailored to new interesting molecules.

Antibody Molecules

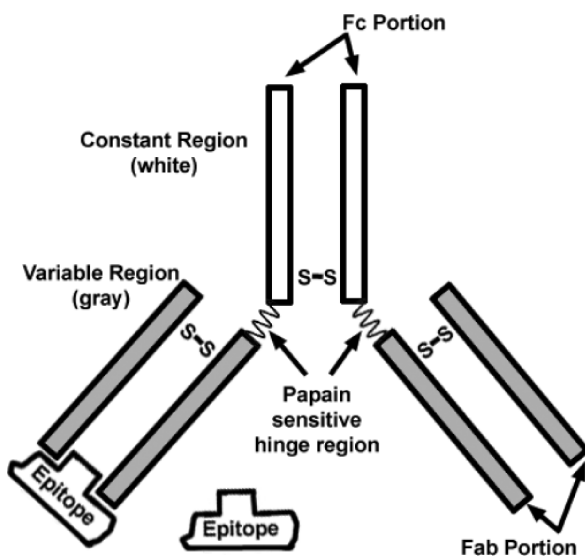
An immune response generates antibodies or proteins called *immunoglobulins (Igs)*. Antibodies are further classified into multiple *isotypes or classes* (Table 2.1). In immunocytochemistry, the *IgG* isotype is preferred because its generation and binding is more consistent. *IgM* antibodies can be used if no other isotype is available. The *IgG* molecules can be broken down into four *subclasses*, *IgG1*, *IgG2*, *IgG3*, and *IgG4*. In immunocytochemistry experiments, these subclasses do not matter for most species of animals, but they are important for antibodies generated in mouse monoclonal antibodies (*IgG1*, *IgG2a*, *IgG2b*, and *IgG3*), as we will see in later chapters.

Table 2.1 Ig isotypes

Antibody isotype	Action
IgA	Gut, respiratory, urinary response
IgD	Initial immune system response
IgE	Response to allergens – histamine release
IgG	Immune response to invading pathogens
IgM	Early immune response for pathogens

In using antibodies, knowledge of the *IgG* structure is important (Fig. 2.1). *IgG* has a *constant region* and a *variable region*. The *constant region* contains species-specific sequences and the *Fc portion* that binds an *Fc receptor* (Fig. 2.1, clear end), which is found on circulating white cells, macrophages, and natural killer cells. The *Fc* portion also has species-specific sites that are unique to the animal species

Fig. 2.1 The antibody. An *IgG* antibody has a single constant region (white) with the *Fc* portion and the species-specific antigens. The variable region (gray) contains the *Fab* portion that binds the epitope portion of the antigen. The small protein, only in the variable region, is known as the light chain; the large protein that is part of the constant and variable region is the heavy chain. The *IgG* can be digested by the protease enzyme, papain, into an *Fc* end (constant end) and a *Fab* end (variable end)



in which the antibody was generated. Thus, generation of an antibody against IgG from rabbit will result in antibodies that bind the constant region from rabbit IgG only and not, for example, from mouse IgG.

Immunocytochemistry uses antibodies against IgGs. Antibodies or IgG molecules are generated to other IgG molecules by injecting purified IgG molecules from one species into another species. In the case of mouse IgG injected into rabbit, it will produce rabbit anti-mouse IgG antibodies. Antibodies made against an IgG will only bind to the constant region or Fab region of the IgG.

The variable end of the antibody contains the unique epitope-binding regions that give each antibody its specificity (Fig. 2.1, gray end). This *variable region* is the fraction antigen binding (*Fab*) portion. The unique configuration of the Fab specifically binds the epitope. When an antigen is injected into a rabbit, the resulting antibodies against the antigen have Fab portions that are unique to the antigen, but the rest of the IgG is similar to other IgG molecules.

Each IgG antibody has two Fab ends, which can bind to two identical epitopes at the same time. The advantage of this *bivalent epitope binding* is that it can amplify the epitope detection. The orientation of the two epitopes is not restricted as there are hinge regions (Fig. 2.1) in the IgG molecule that connect the Fab portion to the Fc portion of the IgG. The hinge region allows movement and rotation of each individual Fab, thus facilitating binding to adjacent identical epitopes.

Heavy chains or long protein (Fig. 2.1, light and dark bars connected by a papain-sensitive hinge) and light chains or short protein (Fig. 2.1, short dark bar) IgG molecules are made of two proteins that are held together by disulfide bonds of the amino acid cysteine (Fig. 2.1; S–S between bars).

The enzyme, papain, can digest the hinge regions of IgG and can generate two identical Fab portions and one Fc portion. The individual Fab portion can be used for immunocytochemistry, where single epitope-binding region is needed without species-specific binding.

An *antigen* is a protein, peptide, or molecule used to cause an immune response in an animal. The animal responds by making antibodies to individual *epitopes located on the antigen*. An individual *antigen has multiple epitopes* that can generate antibodies. In Fig. 2.2, the “&” represents an antigen and the light gray areas on the edge represent individual epitopes. An *epitope can be an amino acid sequence on a*

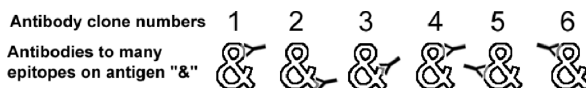


Fig. 2.2 Antibody generation. Antigens are the molecules injected into animals that generate antibodies (“&” is an antigen). Epitopes are small parts of antigens that generate a specific antibody (short gray lines on “&” are epitopes). Here, six antibodies (small Ys) are generated to epitopes on the antigen “&.” Each different antibody is from a clone of B-cells (with numbers); each B-cell produces antibodies to only one epitope; some clones can produce antibodies to the same epitope as other clones (clones No. 1 and No. 4)

denatured peptide or a several sequences on the surface of a folded protein. Animals frequently generate multiple antibodies to the same epitope (Fig. 2.2, clones 1 and 4). Also, an epitope on one protein might also exist on a different, unrelated protein because it has the same sequence or the same surface configuration.

Making Antibodies

An animal injected with an antigen will generate multiple antibodies to many epitopes. Antibodies are produced by B-cells and a *single clone of B-cells produces antibodies to only a single epitope.* Once a B-cell begins producing a single type of antibody, it will divide and give rise to many B-cells, all producing that single antibody to just one epitope; this is called a *B-cell clone*. Sometimes there are multiple clones of B-cells that produce antibodies to a single epitope (Fig. 2.3, clones 1 and 4). Parts of injected proteins and molecules make better antigens than others. As a result, some proteins do not generate many antibodies. An example is G-coupled receptors, a class of membrane receptors, that do not generate antibodies well.

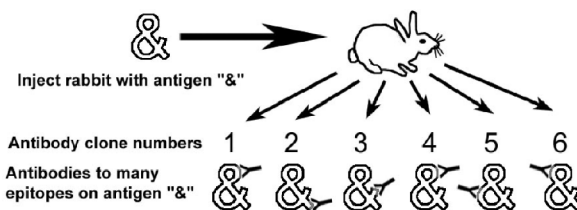


Fig. 2.3 Polyclonal antibodies. An animal injected with an antigen will generate B-cell clones that can produce antibodies to multiple epitopes. The serum from the animal has different antibodies to these multiple clones, thus the name, polyclonal

Polyclonal antibodies contain multiple clones of antibodies produced to different epitopes on the antigen. In Fig. 2.3, the serum from an immunized rabbit contains antibodies from six clones of B-cells. In serum from the rabbit, the six different clones of antibodies will increase the labeling of the antigen because there are multiple epitopes on the antigen. Polyclonal antibodies are in the form of serum from animals and are made in different species of large animals (rabbit, donkey, goat, sheep, and chicken). Chicken polyclonal antibodies are purified from unfertilized egg yolks, with the advantage that eggs are easy to collect and large amounts of an antibody can be isolated from a single chick.

Advantages of Polyclonal Antibody

- Multiple clones give high levels of labeling for a single antigen because they contain many antibodies to different epitopes on the same protein.

Disadvantages of Polyclonal Antibody

- Shared epitopes on different proteins can label multiple proteins that are not the antigen protein.
- Obtaining the antibody depends on a living animal and the ultimate death of the rabbit means no more antibody.
- When a new rabbit is immunized with the same antigen, the exact epitopes generating antibodies will be different and a different number of clones are generated.

Monoclonal antibodies, originally from one mouse, contain a single antibody from one clone of B-cells to a single epitope on the antigen. This procedure was first described by Georges Kohler and Cesar Milstein, for which they received the Nobel Prize in 1984. Monoclonal antibodies are made by immunizing a mouse, and when antibodies are produced, the spleen of immunized mouse is removed (Fig. 2.4). The spleen cells are dissociated including the B-cells producing antibodies (Fig. 2.4, different gray levels). Because B-cells will not divide in culture, they must be fused with a continuously dividing cell line that produces antibodies. Such a cell line is the mouse myeloma cell line.

The spleen cells are fused with mouse myeloma cells to become a continuous hybridoma cell line. A continuous hybridoma cell line with multiple B-cell clones produces many different antibody clones indicated by the different gray levels of

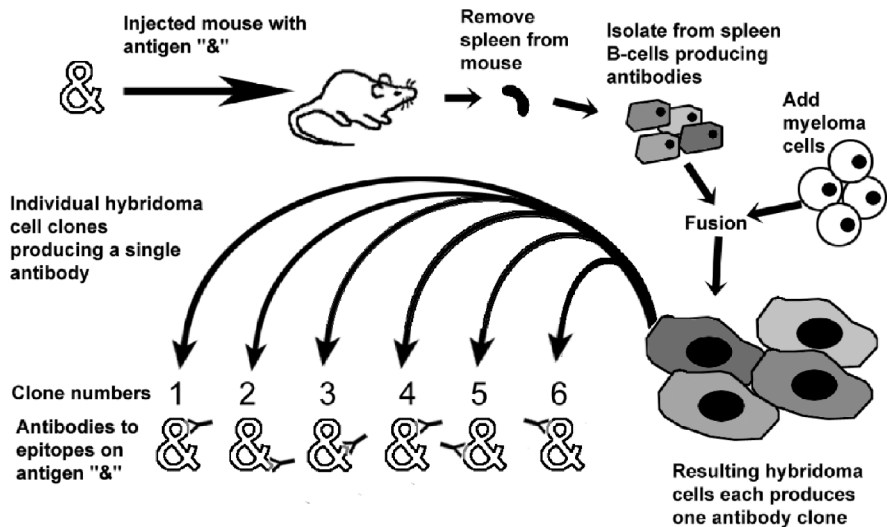


Fig. 2.4 Monoclonal antibodies. After injecting the antigen and generating several clones of antibodies, the spleen containing B-cells is removed. Hybridoma cells are made by fusing spleen B-cells with a myeloma cell culture line. To isolate the individual hybridoma cells producing one clone of antibody, the mixed hybridoma culture is highly diluted and plated in 96-well plates with one cell or less per well

the cells in Fig. 2.4. Next, the population of *hybridoma cells producing many antibodies is cloned* in 96-well plates and each single B-cell clone of cultured cells produces one antibody. Individual clones producing a separate antibody are named by location in the 96-well plate (e.g., 5B12 plate 5, row B, column 12). One mouse spleen can give many different antibodies to different epitopes on the same antigen. Monoclonal antibodies are raised in either tissue culture media, called *supernatant*, or generated from hybridoma cells injected into the peritoneal cavity (abdominal cavity), called *ascites fluid*. Until recently, all monoclonal antibodies were generated exclusively from mice because of the limitations with generating good myeloma cell lines for other species of animals. Rabbit monoclonal antibodies are now available because a good rabbit myeloma cell line is now available. Rabbit monoclonal antibodies have high sensitivity and excellent response to antigens from mouse tissue.

As a result of the popularity of rabbit monoclonal antibodies, confusion exists when using the term monoclonal. Previously, monoclonal antibodies were always from mouse and so detection systems were always based on binding to mouse monoclonal antibodies. Now with the popularity of rabbit monoclonal antibodies, it is not possible to use the term monoclonal to identify the species of the antibody.

Advantages of Monoclonal Antibodies

- Single clone monoclonal antibodies bind to a single epitope, which is selected for high specificity for the antigen.
- Different clones of antibodies can be generated to different epitopes on a single antigen.
- Single clone can be generated to a posttranscriptionally altered protein (e.g., phosphorylated amino acid).
- Clones to an epitope shared with multiple proteins (gene products) can be rejected.
- The same antibody can be generated indefinitely from cultured hybridoma cells in a process that creates a stable reagent.
- The identical clone sold by different companies will be the same antibody.

Disadvantages of Monoclonal Antibodies

- Much work is required to generate a successful monoclonal antibody, especially in the cloning and selection process.
- Low levels of labeling occur because the monoclonal antibody binds an infrequent epitope on a protein or binds with low affinity.
- Monoclonal antibodies are mostly from mice because of a strong myeloma cell line.

Talking About Antibodies

Terminology is important in describing the source and specificity of antibodies used in immunocytochemistry. *The species used to generate antibodies are used to differentiate antibodies.* An antibody generated in rabbit to the protein tubulin would be a “rabbit anti-tubulin antibody.” With both mouse and rabbit being used to make monoclonal antibodies, the species of the animal generating the monoclonal antibody must be stated, and not simply “monoclonal” to mean antibodies produced in mouse. To identify an antibody, use the species of animal where the antibody was generated and not the term monoclonal.

Concentrations of IgG in

serum is 1–10 mg/ml;
ascites is 1–2 mg/ml; and
supernatant is 0.4–1 mg/ml

Antibodies can come in a variety of forms and purities. Polyclonal antibodies can come as *whole serum* or as purified antibodies with an IgG concentration of 1 mg/ml. Monoclonal antibodies come as isolated tissue culture media from hybridoma cells called *supernatant*. The antibody from supernatants is between 50 and 100 µg/ml, which means that the working antibody dilution for immunocytochemistry will be lower than whole serum. In addition, monoclonal antibodies can be *ascites fluid* giving antibodies that are highly concentrated of 1 mg/ml. Today, generation of ascites may be restricted by federal regulations for care of research animals.

To increase the purity or to concentrate an antibody solution, it may be purified. Purification is done with a range of techniques applied to whole serum, supernatant, or ascites fluid. At the first level, the *purified Ig* will be separated from other serum proteins and will select all IgGs including the IgG of interest and other IgG molecules. These purification steps can be done by using ammonium sulfate to precipitate the Ig molecules or it can be done by binding antibodies to a Protein A and/or Protein G columns. Proteins A and G are produced by the bacteria, *Staphylococcus aureus*, and bind to different species and subclasses of antibodies by the Fc receptor. After the antibodies have attached, they are washed out by changing the buffer.

The next level of purification is *affinity purification*, where the antigen is available and can be bound to a column, the serum or supernatant is passed over the column binding to the antigen. The antibodies are washed off with low salt and detergent-containing buffers. The third level of purification is used if the antigen is not available. A band from a gel containing the protein of interest can be cut out and used to purify the antibody. Affinity-purified antibodies are, in theory, the best because they have bound to the antigen. However, some of the strongest binding antibodies cannot be eluted from the affinity columns and recovered, so there is controversy about the value of affinity purification.

All antibody solutions should be clear and free of particles or other precipitated material essential to eliminating background labeling in immunocytochemistry. IgG purification removes any particulate material from the whole serum, supernatant, or ascites fluid that could cause background.

Finding and Getting Antibodies

Selecting an antibody can be a daunting task. Most commonly, antibodies will be purchased from a vendor. There are hundreds of vendors selling antibodies. Finding good antibodies is best done by looking in journal articles or by getting a recommendation from someone who is using a specific antibody. Regardless, to successfully use an antibody requires information about that antibody. What follows is a list of items that should be available from vendor in the *product information* for all antibodies.

Catalogue information – The catalogue number and the price.

Description or background – The name of the antigen, its molecular weight, alternative names, and something about antigen's function.

Antibody type or host – The name of the species used to generate the antibody, the isotype of the antibody, and the clone name/number, if the antibody is monoclonal. If the antibody is a Fab fragment, it should be stated.

Source of antigen – The nature of the injected antigen (protein, peptide from a specific sequence) and the species of the antigen. Sometimes antibodies to specific parts of molecules are needed (e.g., the extracellular domain, a specific sequence of amino acids or a posttranslational modification).

Packaging, product, or purification – The amount of the liquid product, the concentration of antibody in the product (1 mg/ml is ideal), additives (e.g., sodium azide, glycerol), the source (e.g., whole serum, supernatant, ascites), and purification, if any.

Specificity – A description of how the producer determined that the antibody binds only the listed antigen. Most of the time this is a western blot (with a blot shown), but it can be immunocytochemistry (with an image shown). Sometimes data are included about binding to other related proteins or to posttranslationally modified (e.g., phosphorylated) proteins. Some vendors who use a peptide for making an antibody will also sell the peptide for an absorption control. More information about specificity is discussed in the Chapter 9, Controls. Frequently, there are no data given for the specificity.

Uses or application – The methods where the antibody has been tried are generally any of the following: immunohistochemistry (IHC), immunocytochemistry (ICC), or immunofluorescence (IF); western blot (WB) or immunoblot (IB); and immunoprecipitation (IP). This information should

also include recommended dilutions for the listed applications. When purchasing of antibodies for immunocytochemistry, focus only on those tested with immunocytochemistry.

Species reactivity – The species where the antibody binds the antigen. If the antibody does not react to the species of your tissue or has not been tested against the species you are using, do not use it. Some vendors will send a sample to test for a new species. Alternatively, the species that the antibody does not react with might also be listed. This is important because not all antibodies will bind equally to antigens from different species.

Protocols – The recommended protocol that can be useful to understand any unique fixation, detergent, blocking, or incubation conditions. Many vendors will list references for papers that use their antibody rather than list a protocol.

Some antibodies generated in research labs are available from individuals. Antibodies obtained from noncommercial sources will not have the level of documentation expected from commercial sources; however, sometimes it is available. The advantage of antibodies from individual scientists is that if the antibody does not work, the person giving you the antibody will usually help you to get the antibody to work. A disadvantage is that the antibody obtained once may not be available when you need more to complete a study.

Choice of Primary (1°) Antibodies

In immunocytochemistry, the antibody that binds to the antigen is called the *primary antibody* (1° antibody). In searching for 1° antibodies, the first source of information is the published literature. The best search will show several antibodies to the antigen presenting a choice of antibody possibilities. If there are several antibodies available, the factors that determine your choice should be, in the following order: recommended in literature, product literature recommendation for immunocytochemistry, high specificity for the antigen of interest including same species as your tissue, species where the antibody was generated (important when used for multiple antibody experiments), and price.

Note that publication of research using results with an antibody requires that the author includes in the manuscript the source of the antibody and how its specificity was determined. List the company, the product name, product number, lot number, the supplier-specificity results, and any specificity experiments that were performed. Antibodies from individuals generally require negotiation with the individual because the individual might require that he/she be designated a co-author on any paper using his/her antibody. Alternatively, be sure that you acknowledge the individual as the source of the antibody in any manuscript.

Antibodies Handling and Storing

Antibodies are variously supplied in specific forms and shipped frozen, on ice or at room temperature depending on the antibody. Antibodies should be stored as indicated by the vendor or supplier. Antibodies are reasonably stable but can be damaged by repeated freezing and thawing, extreme pH, and high salt environments.

Recommended Storage Freezer, -20°C

- Repeated freezing and thawing will denature antibodies. Damage is reduced by diluting in 30% glycerol.
- Aliquot antibodies so they will be thawed once.
- Passive or nonfrost-free freezers at -20°C or freezers at -70°C . Not recommended are frost-free freezers that have circulating air which will dehydrate frozen antibody in months.

Recommended Storage Refrigerator, 4°C

- Prevents damage due to freeze–thaw.
- Nonfrost-free refrigerators. Frost-free refrigerators will dehydrate and concentrate and eventually dry the antibody solutions.
- Add 0.02% sodium azide to inhibit growth of bacteria (many companies do this). Note: sodium azide will inhibit and enzyme used for immunocytochemistry, horseradish peroxidase (HRP).

Storage of antibodies is controversial. Hint: After years of antibody use, we recommend storing antibodies in small aliquots in microfuge tubes in a -70°C freezer. These aliquots of about 10 μl contain enough liquid for one or two uses. These antibodies will last for many years at this temperature and will not dry because there is no circulating cold air. The downside is that this process requires a lot of space and a great record system to keep track of where individual antibodies are located.



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