Immunohistochemistry: Basics and Methods

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Springer

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Cover Illustration: Double immunolabeling of cytokeratins in a duct wall of the human mammary gland, see Chap. 8, Fig. 3 (photo by Igor B. Buchwalow, Gerhard-Domagk-Institut für Pathologie, Münster, Germany)

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Preface

Combining two different scientific disciplines – morphology and immunochemistry – immunohistochemistry has developed as an important instrument in research and clinical pathology. A basic understanding of underlying principles and potential problems is unavoidable if you want to be successful in your use of immunohistochemistry, as well as in getting your papers published and your research grants funded.

While many excellent texts and monographs exist which cover various aspects of immunohistochemistry, the lack of a concise comprehensive guide to using these methods was a major motivation for writing this book. Our intention was to create an easy-to-read and focused resource based on state-of-the-art information for a broad audience ranging from students and technical assistants to experienced researchers. This handbook has a concise format, with protocols and instructions for methods immediately following the short introductory theoretical material in each chapter. Being conscious of the growing role of Internet as an information source, we have found it reasonable in many cases to substitute citing books and journal publications with corresponding Internet websites. Where possible, commercial sources of reagents, kits, and equipment are listed throughout the text instead of in a separate index. Though each chapter is small and introductory, this handbook itself is self-sufficient and provides a comprehensive look at the principles of immunohistochemistry. For readers wanting further depth of knowledge, each chapter is backed up by a short list of carefully selected original articles.

During the last decade, pioneering efforts of histochemists have led to an immense improvement in the reagents and protocols. The researcher is urged always to determine the reason for every method and step before doing it. This handbook is intended to help readers to avoid troubles in the choice of an adequate method, which happens when using standard textbooks. For this handbook, we carefully selected established methods and easy-to-adopt protocols, paying attention to modern developments in immunohistochemistry, such as antigen retrieval, signal amplification, the use of epitope tags in immunohistochemistry, multiple immunolabeling or diagnostic immunohistochemistry. Each of the methods described in this handbook

was proved by the authors; many of these methods are routinely used in daily practice in their institute. All the practical methods advocated are clearly described, with accompanying tables, and the results obtainable are illustrated with colour micrographs.

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Chapter 1
Antibodies for Immunohistochemistry

The first use of the term *Antikörper* (the German word for *antibody*) occurred in a text by Paul Ehrlich (Fig. 1.1) in the conclusion of his article "Experimental Studies on Immunity," published in October 1891. Paul Ehrlich was born in 1854 in Strehlen (the German Province of Silesia, now in Poland). As a schoolboy and student of medicine he was interested in staining microscopic tissue substances. In his dissertation at the University of Leipzig, he picked up the topic again ("Contributions to the Theory and Practice of Histological Staining," Beiträge zur Theorie und Praxis der histologischen Färbung) (http://en.wikipedia.org/wiki/Paul_Ehrlich). In 1903, Paul Ehrlich published the ever first comprehensive textbook describing histological and histochemical staining techniques ("Encyclopedia of Microscopical Technique," Enzyklopädie der Mikroskopischen Technik). His first immunological studies were begun in 1890 when he was an assistant at the Institute for Infectious Diseases under Robert Koch. In 1897, Paul Ehrlich proposed his theory for antibody and antigen interaction, when he hypothesized that receptors on the surface of cells could bind specifically to toxins — in a "lock-and-key interaction" — and that this binding reaction was the trigger for the production of antibodies. He shared the 1908 Nobel Prize with Mechnikoff for their studies on immunity (http://en.wikipedia.org/wiki/Antibody).

Albert H. Coons (Fig. 1.2) was the first who attached a fluorescent dye (fluorescein isocyanate) to an antibody and used this antibody to localize its respective antigen in a tissue section. The concept of putting a visible label on an antibody molecule appeared both bold and original. His initial results were described in two brief papers in the early 1940s (Coons et al. 1941, 1942), but the research was halted while he joined the army and spent the next 4 years in the South Pacific. His later studies (Coons and Kaplan 1950) contributed immensely to the use of the fluorescent antibody method in a wide variety of experimental settings. In our time, the use of antibodies to detect and localize individual or multiple antigens in situ has developed into a powerful research tool in almost every field of biomedical research (http://books.nap.edu/html/biomems/acoons.pdf).

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Fig. 1.1 Paul Ehrlich

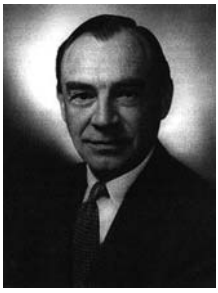

Fig. 1.2 Albert H. Coons (Courtesy of the Harvard Medical School Countway Library)

1.1 Structure of Antibodies

An antibody or immunoglobulin (Ig) is a glycoprotein used by the immune system to identify and neutralize substances foreign to the body, such as bacteria and viruses and other infectious agents, known as antigens. Some immunologists will argue that the word immunoglobulin covers more than just antibodies but we will not complicate matters by going in to the details here. In mammals, there are five classes of antibodies: IgG, IgA, IgM, IgE and IgD. For immunoassays, two Ig classes are of importance — IgG and IgM.

IgM eliminates pathogens in the early stages of B cell mediated immunity before there is sufficient IgG. The IgG molecule has two separate functions; to bind to the pathogen that elicited the response, and to recruit other cells and molecules to destroy the antigen. Generated during the secondary immune response and produced by B lymphocytes, IgG molecules provide the majority of antibody-based immunity against invading pathogens. IgG is one of the most abundant proteins in serum of adult animals and constitutes approx. 75% of serum immunoglobulins. Therefore, it is primarily used in the experiments for production of antibodies. IgG antibodies are further divided into four subclasses (also referred to as isotypes, IgG1, IgG2, IgG3 and IgG4).

The classical "Y" shape of the IgG molecule (MW ~150 kD) is composed of four polypeptide chains — two identical light chains (each has a molecular weight of ~25 kD), and two identical heavy chains (each has a molecular weight of ~50 kD)

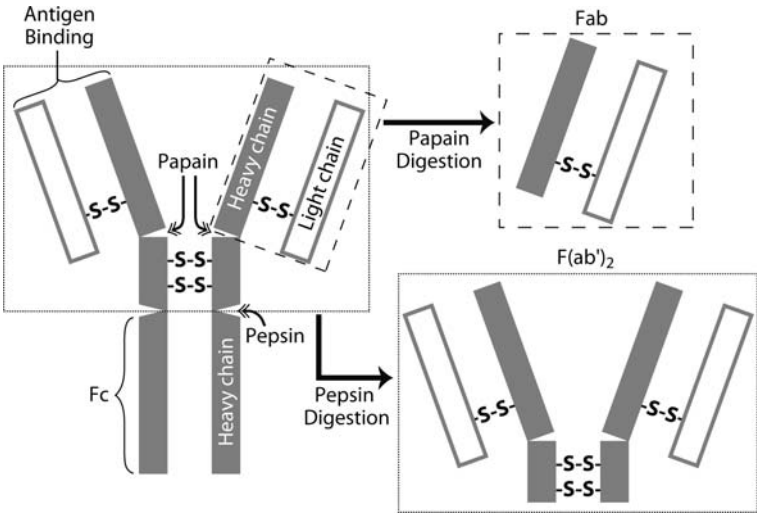

Fig. 1.3 Schematic representation of an antibody molecule. Adapted from http://probes.invitrogen.com/handbook/boxes/0439.html

— which are connected by disulfide bonds (see Fig. 1.3). Each end of the forked portion of the "Y" on the antibody is called the *Fab* (*Fragment, antigen binding*) region. Antigen specific Fab "arms" are responsible for antigen binding. A Fab fragment is comprised of one light chain and the segment of heavy chain on the N-terminal side. The light chain and heavy chain segments are linked by interchain disulfide bonds. The *Fc* "tail" (*Fragment, crystallizable*) is a region of an antibody composed of two heavy chains on the C-terminal side. Fc has many effector functions (e.g., binding complement, binding to cell receptors on macrophages and monocytes, etc.) and serves to distinguish one class of antibody from another (Harlow and Lane 1999).

Fc and Fab fragments can be generated through digestion by enzymes. An antibody digested by papain yields three fragments, two Fab fragments and one Fc fragment. Pepsin attacks the antibody molecule on the heavy chain below disulfide bonds connection, cleaving the antibody into a $F(ab')_2$ fragment and a Fc fragment. Along with the whole antibody molecules, Fab, $F(ab')_2$ and Fc fragments are useful tools in immunoassays. Bivalent $F(ab')_2$ fragments can be used as secondary antibodies to avoid the nonspecific binding to Fc receptors in tissue sections, especially on the cell surface. Unconjugated monovalent Fab fragments can be used to convert the primary antibody into a different species. Labeled monovalent Fab fragments are used for haptenylation of primary antibodies in double or multiple immunostaining when primary antibodies belong to the same species (see Sect. 8.2). The Fc fragment serves as a useful "handle" for manipulating the antibody during most immunochemical procedures. Antibodies are usually labeled in the Fc region for immunohistochemical labeling.

Since IgG is the most abundant immunoglobulin in serum (http://probes.invitrogen.com/handbook/boxes/0439.html), it is primarily used in the production

of antibodies for immunoassays. IgM accounts for approximately 10% of the immunoglobulin pool, and is also used in the production of antibodies, but to a lesser extent than IgG. The IgM molecule contains five or occasionally six "Y"-shaped subunits covalently linked together with disulfide bonds. The individual heavy chains have a molecular weight of approximately 65,000 and the whole molecule has a molecular weight of 970,000 (Harlow and Lane 1999).

1.2 Polyclonal Antibodies

Polyclonal antibodies are produced by immunization of suitable animals, usually mammals. An antigen is injected into the animal, IgG specific for this antigen are produced by B lymphocytes as immune response, and these immunoglobulins are purified from the animal's serum. Thereby, specific IgG concentrations of approximately 1–10 mg/ml serum can be obtained (see Table 4.1). Antibodies produced by this method are derived from different types of immune cells, and hence are called polyclonal. Animals usually used for polyclonal antibody production include goats, horses, guinea pigs, hamsters, mice, rats, sheep and chickens. Rabbit and mice are the most commonly used laboratory animals for this purpose. Mice are, however, mostly used for production of monoclonal antibodies (see Sect. 1.3). Larger mammals, such as goats or horses, are used when large quantities of antibodies are required. Many investigators favor chickens because chickens transfer high quantities of IgY (IgG) into the egg yolk, and harvesting antibodies from eggs eliminates the need for the invasive bleeding procedure. One week's eggs can contain ten times more antibodies than the volume of rabbit blood obtained from one weekly bleeding (http://en.wikipedia.org/wiki/Polyclonal_antibodies).

1.3 Mouse Monoclonal Antibodies

Whereas polyclonal antibodies are multiple antibodies produced by different types of immune cells that recognize the same antigen, monoclonal antibodies are derived from a single cell line (also referred to as clone). In monoclonal antibody technology, tumor cells that can replicate endlessly are fused with mammalian cells that produce an antibody. The result of this cell fusion is called a "hybridoma," which will continually produce antibodies. Monoclonal antibodies are identical because they were produced by immune cells that all are clones of a single parent cell. Production of monoclonal antibodies requires immunizing an animal, usually a mouse, obtaining immune cells from its spleen, and fusing the cells with a cancer cell (such as cells from a myeloma) to make them immortal, which means that they will grow and divide indefinitely (see Fig. 1.4). In fermentation chambers, antibodies can be produced on a larger scale. Nowadays, bioengineering permits production of antibodies also in plants.

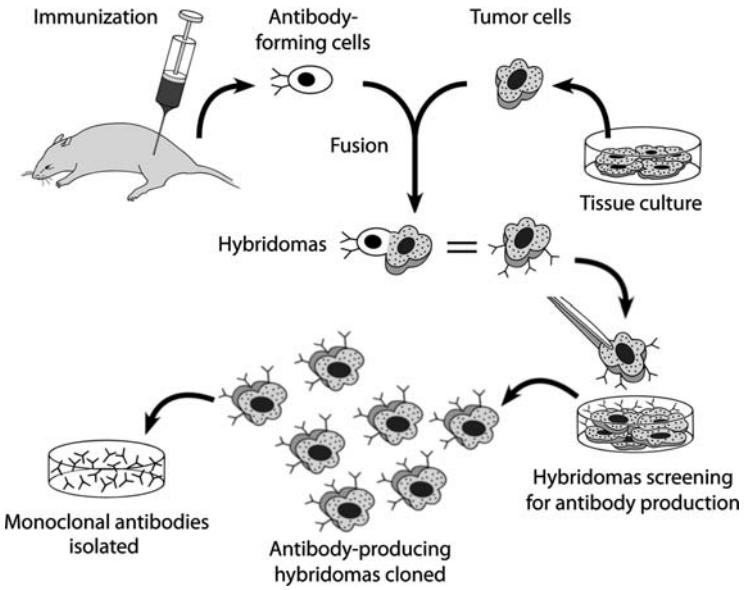

Fig. 1.4 Monoclonal antibody production. Adapted from http://www.accessexcellence.org/RC/VL/GG/monoclonal.html

Monoclonal antibodies can be produced not only in a cell culture but also in live animals. When injected into mice (in the peritoneal cavity, the gut), the hybridoma cells produce tumors containing an antibody-rich fluid called ascites fluid. Production in cell culture is usually preferred, as the ascites technique may be very painful to the animal and if replacement techniques exist, may be considered unethical. The process of producing monoclonal antibodies described above was invented by Georges Köhler, César Milstein, and Niels Kaj Jerne in 1975; they shared the Nobel Prize in Physiology or Medicine in 1984 for the discovery (http://en.wikipedia.org/wiki/Antibody).

1.4 Rabbit Monoclonal Antibodies

Monoclonal antibodies have traditionally been produced in a mouse and to a lesser extent in a rat. Cloning techniques produce highly specific antibodies. However, polyclonal antibodies produced by rabbits are known to have a higher affinity than antibodies raised in mice, which means — to put it simply — a better antigen recognition. Additionally, the rabbit immuno-response recognizes antigens (epitopes) that are not immunogenic in mice. It is often for this reason that rabbit polyclonal antibodies are developed and used in many research and even some diagnostic applications. In order to combine benefits of better antigen recognition

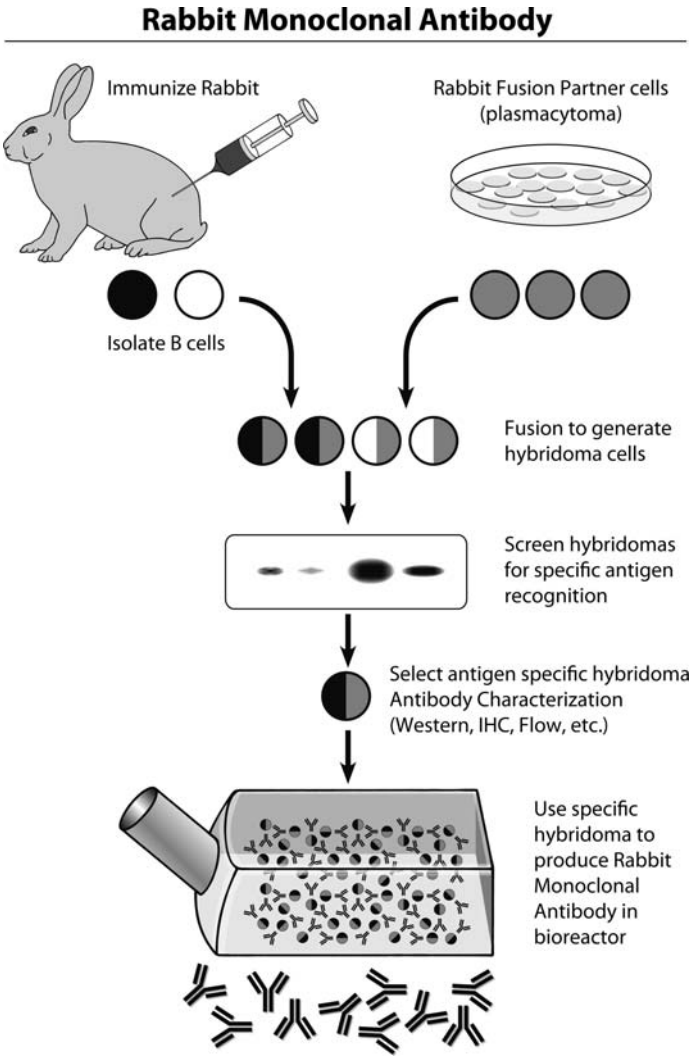

Fig. 1.5 The general outline that Epitomics uses for producing a rabbit monoclonal antibody

by polyclonal antibodies with the specificity and consistency of a monoclonal antibody, Epitomics (http://www.epitomics.com/) developed a proprietary unique method for making monoclonal antibodies from rabbits (Fig. 1.5). Rabbit monoclonal antibodies are now available also from Lab Vision Corporation (http://www.labvision.com/rabmab/Rabmab.cfm) and Bethyl Laboratories (http://www.bethyl.com/default.aspx).

The basic principle for making the rabbit monoclonal antibody is the same as for mouse monoclonals. Rabbit fusion partner cells can fuse to rabbit B-cells to create the rabbit hybridoma cells. Hybridomas are then screened to select for clones with

specific and sensitive antigen recognition, and the antibodies are characterized using a variety of methods. The resulting rabbit monoclonal antibody has ten times the affinity of the mouse antibody, thus resulting in a more specific and much more sensitive antibody.

1.5 Protein A and Protein G in Immunohistochemistry

Protein A and protein G are bacterial proteins that bind with high affinity to the Fc portion of various classes and subclasses of immunoglobulins from a variety of species. Protein A is a constituent of the *Staphylococcus aureus* cell wall. Protein A was introduced in immunocytochemistry for the localization of different antigens at the light and electron microscope levels in the 1970s (Roth et al. 1978). Later, another protein isolated from the cell wall of group G streptococcal strains, known as protein G, was found to display similar IgG-binding properties. Like protein A, protein G has a high affinity for IgGs from various mammalian species (Table 1.1). Protein G was, however, found to interact with a broader range of polyclonal and monoclonal antibodies from various mammalian species with higher avidity (Bendayan and Garzon 1988). Protein A/G is a genetically engineered 50,460 Da protein that combines IgG binding domains of both protein A and protein G. Protein A/G contains four Fc binding domains from protein A and two from protein G. It is a gene fusion product secreted from a non-pathogenic form of *Bacillus*. Protein A/G binds to all subclasses of mouse IgG but does not bind mouse IgM. Mouse monoclonal antibodies commonly have a stronger affinity to the chimeric protein A/G than to either protein A or protein G.

The use of protein A and protein G in immunohistochemistry is based on the same principle as that using secondary anti-IgG antibodies in the indirect two-step approach. In the first step, an antigen–IgG complex is formed, which is then revealed in the second step by incubation with labeled protein A or protein G.

Table 1.1 Binding profiles of protein A, protein G and protein A/G. Adapted from http://www.2spi.com/catalog/chem/gold_conjugate.html)

Primary IgG	Protein A	Protein G	Protein A/G
Chicken	+/−	+	+/−
Goat	+/−	++	+++
Guinea pig	++	++	++
Mouse IgG_1[a]	+/–	++	+/−
Mouse (other subclasses)	++	++	++
Rabbit	+++	+++	+++
Rat	+/−	+	+++
Sheep	+/−	++	+++

[a]IgG_1 binds best to protein A at a pH of 8–9. + Moderate binding, +++ Strong binding, – Weak or no binding