Techniques in Life Science and Biomedicine for the Non-Expert
Series Editor: Alexander E. Kalyuzhny

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Immuno-histochemistry

Essential Elements and Beyond

Springer

Techniques in Life Science and Biomedicine for the Non-Expert

Series editor

Alexander E. Kalyuzhny, University of Minnesota, Minneapolis, MN, USA

The goal of this series is to provide concise but thorough introductory guides to various scientific techniques, aimed at both the non-expert researcher and novice scientist. Each book will highlight the advantages and limitations of the technique being covered, identify the experiments to which the technique is best suited, and include numerous figures to help better illustrate and explain the technique to the reader. Currently, there is an abundance of books and journals offering various scientific techniques to experts, but these resources, written in technical scientific jargon, can be difficult for the non-expert, whether an experienced scientist from a different discipline or a new researcher, to understand and follow. These techniques, however, may in fact be quite useful to the non-expert due to the interdisciplinary nature of numerous disciplines, and the lack of sufficient comprehensible guides to such techniques can and does slow down research and lead to employing inadequate techniques, resulting in inaccurate data. This series sets out to fill the gap in this much needed scientific resource.

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Alexander E. Kalyuzhny

Immunohistochemistry

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Alexander E. Kalyuzhny
University of Minnesota
Minneapolis, MN
USA

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To Dora and Henry

Preface

This volume champions a new book series, of which I am honored to be the editor and to have the opportunity to be the first contributor. As with any endeavor, it is both exciting and frightening to be the first. What if the format of the book is not an optimal match for the presented subject? What if the illustrations are not clear and are difficult to digest? What if it is too long, and after reading the first few pages the reader feels bored and the book finds its place on a dusty shelf among outdated manuals and last century's catalogs? Fortunately, we managed to convert all of these "what if" worries into a concise writing plan that serves as a road map to help us reach our goal: showing the elements of immunohistochemistry and explaining how they come together to make this technique work.

I wish to acknowledge Martin Wessendorf, who made the biggest impact on my career development by introducing me to the exciting world of immunohistochemistry more than twenty years ago.

Martin remains my mentor and good friend, and this book could not have been written without his encouragement and support.

Finally, my special and sincere thanks go to Jennifer Kalyuzhny for her outstanding editorial assistance.

Contents

Introduction

Immunohistochemistry (IHC) has arisen at the junction of three independent disciplines: immunology, histology, and chemistry. Although IHC may not appear as sophisticated as its parental disciplines, it is quite complicated in its own right. Being a good immunohistochemist is like being an artist who plays harp and drums while dancing at the same time: this is not just about excelling in each of the arts, but rather about blending them together to create a totally new form of performance. The deceiving simplicity of IHC can play tricks on researchers whose overconfidence eventually turns into frustration. Seasoned IHC experts sometimes joke that IHC is not a science so much as an art, and that performing a new IHC experiment is like creating a new masterpiece. Jokes aside, IHC is one of the most frequently used techniques in life science research as well as in diagnostics and forensic investigations. IHC allows for precise tissue localization of stem cells, dying neurons, and metastasizing cancer cells, as well as subtle intracellular changes in protein activation and trafficking within the cell. If it were possible to expand IHC technique for use in ordinary life, it would be the perfect tool for finding the needle in a haystack. Over the years, IHC evolved into a state-of-the-art technique and keeps steadily moving up the sophistication and complexity ladder, making its learning challenging for both novices and experts trained in other fields. Currently there is no shortage in printed textbooks, specialized scientific journals, and online resources covering advanced IHC techniques aimed at experts who have already been using IHC for quite some time. Such publications are of great help to experienced researchers who are interested in polishing their skills and looking for solutions to their cutting-edge experiments. However, there are almost no concise introductory guides for IHC novices or experienced researchers and professionals from other fields who have to deal with unfamiliar IHC technique. Thus, the ultimate goals of this book are to fill this gap and introduce readers to principles of IHC technique and overview reagents and instruments needed to run IHC protocols. This book elaborates on IHC workflow, selection of primary and secondary antibodies, chromogenic and fluorescence detection chemistry, simultaneous detection of multiple tissue targets, IHC controls, and acquisition of digital IHC images and their presentation. In addition, this book will cover the experimental setup, emphasizing components of critical importance.

By trying to unravel the "quantum mechanics" of IHC, we hope the book will serve not only as an introduction to this fascinating technique but will also arm the readers with necessary skills to perform simple IHC staining protocols on their own. There is absolutely no intent to pretend this book is a substitute for advanced IHC guides, and we will provide references to such resources for readers who wish to expand their existing knowledge. To make IHC learning more accessible, this book includes numerous illustrations that can be easily understood by readers with different professional backgrounds and education, as well as numerous quizzes to enable readers to check their learning success.

Chapter 1
Short Historic Overview

Immunohistochemistry, or IHC, is a technique that utilizes antibodies—products of the immune system—to analyze histological tissues under the microscope. IHC evolved from histochemistry, a technique that utilizes chemical and biochemical techniques for probe histological tissues. It appears that the founder of histochemistry was George Gomori (1904–1957), a Hungarian-born scientist who immigrated to the United States in 1938 and co-founded the Histochemical Society in 1950. In the introduction to his classical textbook *Microscopic Histochemistry: Principles and Practice*, published by the University of Chicago Press in 1952, he defined histochemistry as "…a borderline field between histology and analytical chemistry or biochemistry. Its subject matter is the identification and localization of chemical substances in the tissues on a cytological scale." He emphasized that the term histochemistry refers to nondestructive analysis of histological tissues using only methods "…in which the identifying chemical reaction is observed directly through the microscope, in tissues of which the architecture is not grossly altered." In his remarkable textbook *Histochemistry: Theoretical and Applied*, A.G. Everson Pearse further defined histochemistry as "the identification, localization and quantification, in cells and tissues and by chemical or physical tests, of specific substances, reactive groups, and enzyme-catalyzed activities." The evolution from histochemistry into immunohistochemistry occurred due to the efforts of Dr. Albert Hewett Coons, who suggested using antibodies conjugated to fluorescent dyes for visualization of antigens in tissue sections, which he described as "putting tail lights on antibodies." In 1941, he published his pioneering paper describing the coupling of the fluorescent dye anthracene isocyanate to anti-pneumococcus serum. In addition to agglutinating the cocci by the antibodies in the serum, the cocci turned brightly fluorescent and could be easily visualized under the microscope. Dr. Coons's concept that antibodies can be labeled to visualize tissue antigens gave birth to IHC. Other researchers have since found that in addition to fluorescent probes, antibodies can be also labeled with many other visualization-enabling tags

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including biotin, enzymes, and colloidal gold and silver, and short oligonucleotides. Since Dr. Coons's discovery, hundreds and thousands of scientific IHC papers have been published and IHC has become an indispensable technique for research and diagnostic applications. It is absolutely impossible to imagine modern-day life sciences without IHC, which still remains a very dynamic developing discipline.

Chapter 2
Primary Antibodies

To a certain extent, IHC can be compared to GPS navigation: to find the place of interest, we must enter its address—which is a unique identifier—and the electronic navigator will bring us there. In IHC, the unique identifier is the structure of the molecule we want to detect, while primary antibodies serve as the chemical rather than electronic GPS navigator. The most critical reagent in IHC protocol is a primary antibody. What is the antibody and why is it primary? As we learned before, the major task of IHC is to detect and visualize proteins of interest in thin sections of animal and human tissues. In order to be detected, the protein in the tissue has to be recognized by another molecule that binds to tissue protein. Recognition by the detecting reagent should be very accurate and selective, so that thousands of other irrelevant proteins composing the tissue remain undetected. Unfortunately, there are no efficient man-made reagents allowing for selective detection of a specific protein among thousands of others. But the good news is that Mother Nature has already designed such a tool and has let us borrow it for our research needs. The tool is called the antibody (Fig. 2.1).

Antibodies are made by the immune system to defend against numerous foreign substances, antigens, which enter the living organism. This process is called adaptive immunity. Antibodies are produced by B lymphocytes (B cells) during a complex cascade of cellular interactions between different types of immune system cells and belong to the class of molecules called immunoglobulins, or Ig. Based on their chemical structure, Ig molecules belong to G, M, A, D, and E types depicted as IgG, IgM, IgA, IgD, and IgE respectively. Most primary antibodies used in IHC belong to IgG type, which, in turn, is represented by four classes, or isotypes: IgG1, IgG2, IgG3, and IgG4.

Antibodies are very selective in recognizing their antigens, which makes antibodies a perfect tool for IHC. Since antigens induce production of antibodies under natural conditions it is possible to imitate the same process by synthesizing antigens resembling the structure of a naturally occurring protein and injecting antigens into host animals like sheep, goats, rabbits, etc.

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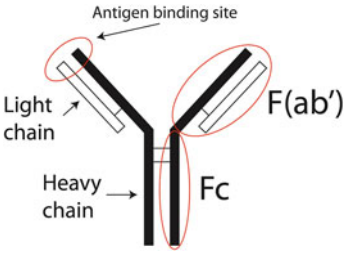

Fig. 2.1 Simplified schematic overview of antibody molecule. Interactions of antibodies with their antigens occurs only at their antigen binding sites

For example, if we want to study the distribution of cells producing insulin in tissue sections of a pancreas, we either use a full size insulin molecule or just its shorter fragment unique to the insulin molecule as the antigens. Antigens used for injection into host animals to stimulate the production of antibodies are also called immunogens. Antibodies made by B cells are then secreted into the bloodstream and can be found in blood serum, which immunohistochemists refer to as immune serum. Immune serum can be used for IHC staining either without any further processing or after being subjected to antibody purification. Immune serum can be used for IHC as is, but the problem is that immune serum is a cocktail of antibodies: in addition to antibodies against the injected immunogen, it also contains antibodies to other unknown antigens to which the animal was exposed during its life. Such contaminating antibodies may bind to irrelevant tissue proteins and produce unwanted background staining, which can obscure a specific staining generated by the antibodies of interest. To overcome such a problem, specific antibodies should be isolated from the immune serum. One of the very efficient ways to purify specific antibodies is to run immune serum through a column (called the purification column) filled with beads of a special resin coated with immunogen: only specific antibodies will bind to the immunogen and become retained in the resin matrix, while contaminating antibodies will pass through and get discarded. Retained specific antibodies attached to the resin beads can be isolated by flushing the column with a very acidic elution buffer, and the collected eluate will only contain specific antibodies. This process is called antibody affinity purification, and purified antibodies are referred to as affinity-purified antibodies.

When affinity-purified antibodies are added to tissue sections they bind to tissue protein that resembles the immunogen. Binding of antibodies to its specific tissue antigen is the primary step in tissue antigen detection, and therefore such antibodies are designated primary antibodies. Primary antibodies can be generated by immunizing animals of different host species, including small laboratory rodents (such as mice, rats, rabbits, or guinea pigs) as well as large farm animals (such as goats, sheep, or llamas). In addition, the antibodies can be also made in chicken. What are the reasons for choosing different host species? Are antibodies raised in rabbits better than those raised in sheep? There are several reasons why antibodies are made in different host species. First of all, it is a matter of convenience: handling small laboratory rodents is much easier than injecting immunogens into large farm animals and collecting their blood. Another reason is the cost: handling large farm

animals requires assistance from a trained veterinarian and therefore increases the cost of antibody production. Because of this, low cost mice, rats, rabbits, and guinea pigs are favorite species in academic laboratories, whereas more expensive sheep, goats, and llamas are mostly utilized by commercial organizations that make money by selling their antibodies to customers: much more immune serum can be produced by a goat compared to a guinea pig, which results in a much higher yield of antibodies for sale. However, due to recent advances in antibody production technologies, small animal sizes are no longer an issue as researchers have learned how to generate an almost endless supply of antibodies from mice, rats, and rabbits. As mentioned before, antibodies are produced by B cells of the immune system and there are slight differences in antibodies made by different B cells. These differences include the specificity of antibodies. It has been found that antibodies secreted from myriad B cells have different specificities toward the antigen: antibody specificity is defined as its capacity to bind with only a certain part of the antigen but not to others or to irrelevant proteins. Specificity also determines whether antibodies bind exclusively to their target (high specificity) or cross-react with proteins bearing resemblance to the specific target. The higher the potency of the antibody to cross-react with irrelevant proteins, the lower its specificity.

Another important feature is the affinity of the antibody. Affinity is defined as the strength of the antibody binding to its antigen and is determined by the chemical structure of the antigen and the binding site on the antibody. Antibodies may have a high or low affinity, and the higher the affinity the better the detection of the antigen in tissue sections, which is always required in IHC.

Immunologists have discovered that B cells exist as clones and a single B cell clone divides to produce a large number of descendant B cells, which produce antibodies with the same specificity. However, the immune response to the injected immunogen activates multiple B cell clones, which produce antibodies with high and low specificities. Antibodies combined from different B cell clones into a common pool are called polyclonal antibodies. But what if we want to produce antibodies with the highest specificity to the antigen? Is there a way to isolate the corresponding B cells from a single clone, culture them in a dish (called in vitro—outside the body of a host animal), and collect the antibodies secreted by these cells into a culture media where they live? The answer to all these question is "yes," and antibodies produced by a single B cell clone are called monoclonal antibodies. Unfortunately, B cells cultured in vitro have a very short life that is not long enough for the production of antibodies in quantities required for IHC staining. This problem was solved in 1975 by Georges Köhler and César Milstein in Cambridge, UK, when they found a way to immortalize B cells by fusing them with cancer myeloma cells. Such immortalized B cells can be cultured long enough to produce large quantities of monoclonal antibodies. Köhler and Milstein received the Nobel Prize in Physiology or Medicine in 1984 for their discovery.

Which antibodies are better for IHC, polyclonal or monoclonal? Each type has its pros and cons (Fig. 2.2). Cost-wise, making polyclonal antibodies is less

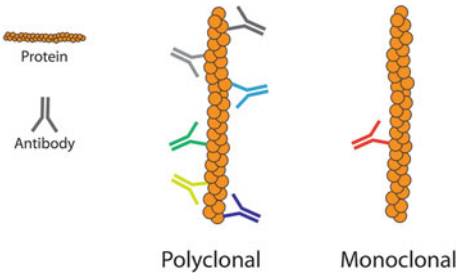

Fig. 2.2 Polyclonal antibodies can be considered as a mixture of monoclonal antibodies which, due to variations in their antigen specificity, bind to different regions of the same protein molecule. Monoclonal are the antibodies of the same specificity that bind to the same region of a protein molecule

expensive. Theoretical considerations suggest that due to the presence of antibodies with different specificities that bind to different regions of tissue antigen, IHC staining using polyclonal antibodies should be much stronger compared to monoclonal antibodies. Unfortunately, in practice this does not always hold true and researchers have reported it going both ways. The commercial drawback in making polyclonal antibodies is that their supply is limited and depends on the quantity of serum blood collected from the immunized animals (not much can be collected from guinea pigs and rabbits), whereas monoclonal antibodies can be supplied in unlimited quantities. The major advantage of monoclonal antibodies is the consistency in their specificity between different production batches. That is why monoclonal antibodies are widely used for IHC diagnostics when high accuracy in detecting the pathological tissue changes is of critical importance. For IHC research it appears that monoclonal antibodies tend to produce staining with less of a background compared to their polyclonal siblings. It appears that mice and rats are the most frequently used animals for the production of monoclonal antibodies, but recently new technologies have emerged for making rabbit monoclonal antibodies. Buying monoclonal antibodies for IHC research costs more than buying polyclonal ones, and therefore due to budget constraints some academic laboratories prefer choosing polyclonal antibodies at the expense of the quality of tissue staining.

Currently, many small and large companies manufacture antibodies for sale and reading the labels on the antibody vials may be challenging. First of all, the species of antibodies must be abbreviated due to small printing area of the label: goat—Gt, sheep—Sh, rabbit—Rb, guinea pig—Gp, mouse—Ms, rat—Rt. Antibodies are usually made to stain tissues of a particular species like human, rat, mouse, etc., which are shortened to just the first letter: human—h, mouse—m, rat—r. The typical label on the vial may look like this (except that lines are not numbered, which has been done for sake of explaining their meaning in point-by-point manner below):

(**1**) anti-hmrActivin
(**2**) Purified rat monoclonal
(**3**) Clone: 123765
(**4**) Catalog Number: M1534
(**5**) Lot Number: B12
(**6**) 0.05 mg
(**7**) For Research Use Only
(**8**) Best Antibodies, Inc.

What does this label say?

Line 1—Antibodies were made to cross-react with Activin molecules in human, mouse, and rat tissues. In other words, such antibodies are suitable for IHC staining of human, mouse, and rat tissue sections.

Line 2—Antibodies are monoclonal and were raised in rats.

Line 3—Clone number designated by the manufacturer. There may be different clones of the antibody against Activin molecule that work in different applications: one clone works for IHC, another for Flow Cytometry, and a third for Western Blotting.

Line 4—Catalog number assigned by the manufacturer, which helps to find the antibody on the vendor's web site.

Line 5—Production lot number assigned by the manufacturer, usually to a group of vials with the same antibodies, that were produced on the same date from the same bulk material. Sometimes different lots of the same antibody do not produce the same IHC staining due to so-called lot-to-lot variations. Customers can request that they be sent antibodies from the best-performing lot for IHC.

Line 6—The quantity of antibody in a vial. If antibodies are supplied in a dry (lyophilized) format the quantity is given in milligrams, (mg) or in micrograms (can be written either as μg or ug). When antibodies are supplied in liquid form their volume is given in milliliters (mL) or in microliters (can be written either as μL or uL). Microgram and microliter are 1/1000 parts of a milligram and a milliliter respectively. When antibodies are sold as a liquid, the label will also indicate their concentration, for example, 1, 0.5 mg/mL, etc. Knowing the original concentration of primary antibody is very important in order to make correct working dilutions.

Line 7—Intended use of the antibody. When antibodies are made for research rather than diagnostic purposes the manufacturer must state this on the label. Unlike antibodies for research, IHC diagnostic antibodies must be subjected to much more rigorous quality testing according to the rules set forth by the United States Food and Drug Administration (FDA).

Line 8—The name of the antibody vendor. No matter how much information is put on the label, it is always a good idea to visit the antibody vendor's web site and carefully read the actual antibody datasheet, which, in addition to the antibody details, may also provide examples of IHC images as well as the tissue staining protocol and troubleshooting recommendations.

Antibodies are supplied by commercial vendors in a concentrated form and need to be further diluted, defined as preparing antibody working dilutions. Typical

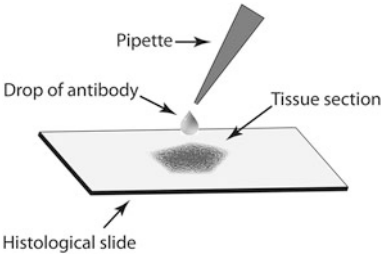

Fig. 2.3 Application of primary antibody solution to tissue section on the histological slide

working dilution of antibodies in IHC ranges from 1 to 20 micrograms per 1 mL of solution. To make a working dilution of immune serum in which the concentration of specific antibodies is not known, it is usually diluted at 1:100–1:2000. One important thing to remember: the higher the concentration, the higher the risk of getting nonspecific background staining.

Antibodies can be supplied either as liquid or dry (lyophilized) concentrated stock reagents, which should be further diluted according to the manufacturer's instructions to make working dilution that can be applied to tissue sections (Fig. 2.3). Liquid antibody concentrates usually need to be stored between 4 and 8 °C and are usually stable for up to a year. If vial with antibodies cannot be used within its shelf life term due to low volume of IHC experiments, it is recommended to make aliquots. For example, a single 0.5 mL stock solution can be aliquoted into 50 vials containing 10 uL. Aliquots should be stored frozen and used to prepare a working solution immediately before use. Original stock solution vials can be also stored frozen, but have to be taken out from the freezer and thawed to make working solutions and then transferred back to the freezer. Repetitive freezing and thawing of antibodies is the worst enemy, which destroys their staining capacity. Unopened vials of dry, lyophilized, antibodies can be stored for years at −20 °C and lower, but after reconstitution with the diluents, they have to be treated like liquid stock solutions. To extend the shelf life of antibody stock solutions, some vendors recommend adding glycerol and storing them in freezers at −20 to −80 °C as glycerol prevents the solution from freezing and therefore eliminates the risks of freeze–thaw cycles.

2.1 Summary

The key reagent in IHC technique is primary antibodies. Primary antibodies are made by B cells in response to natural pathogens, but can be also produced for IHC needs artificially by immunizing host animals with antigens of a known chemical structure. Primary antibodies can be applied as immune serum, affinity purified polyclonal and monoclonal antibodies. Polyclonal antibodies may produce unwanted background staining in addition to specific staining, whereas monoclonal antibodies produce cleaner and more consistent IHC staining.

2.2 Quiz (True or False)

1. Antibodies are called “primary” because they are made by B cells: **T F**
2. Primary antibodies are not an essential reagent for IHC and there are many other reagents that can be used in place of primary antibodies: **T F**
3. Immune serum is the source of primary antibodies: **T F**
4. Antigens used for immunization are not the same as immunogens: **T F**
5. Polyclonal antibodies are a pool of antibodies with identical specificities: **T F**
6. The major advantage of monoclonal antibodies is consistency in their specificity between different production batches: **T F**
7. Monoclonal antibodies are preferred over polyclonal in IHC diagnostics: **T F**

Chapter 3
Choosing Commercial Suppliers of Primary Antibodies

As IHC dogma puts it, the higher the quality of primary antibodies, the more compelling and credible the scientific data. There are two main sources of primary antibodies, either generated in-house or purchased from commercial suppliers. It appears that by choosing the in-house route, researchers are in full control of the antibody production process and can generate an endless supply of antibodies free of charge that can be shared with other labs. The main drawback in making antibodies by researchers on their own is the timing: it can take months and therefore delay experimental work that is needed for generating data for publications and writing grant application by set deadlines. Another problem is that making a good antibody is not an easy task: researchers can control immunization of the host animals, but they cannot control its immune system. It is not the researcher, but the immune system of the host animal that "runs the show." Thus, in order to raise IHC-reliable antibodies, the immunization cycle may need to be repeated several times, which is quite time-consuming and costly. Making antibodies for IHC in someone's own lab was a widespread practice until commercial suppliers specializing exclusively in antibody production entered the game. Making antibodies for sale takes a lot of effort and requires immunizing a large number of animals of different host species to improve the success rate. Companies use complicated protocols and employ robust quality control protocols not only of developed antibodies, but also of reagents and instruments utilized for antibody production. If a company releases low quality antibodies, it risks its reputation and turning the customers away from its products. That is why good companies spend a lot of money on developing and validating antibodies before adding them to their catalogs. There is one fundamental question: what is a good IHC antibody? The most important attribute of the antibody is its specificity, which means that the antibody should detect only the protein of interest and should not cross-react with irrelevant tissue targets. Unfortunately, IHC staining on its own cannot prove or disprove antibody specificity and requires other tests. One of the most popular tests is a so-called western blotting technique, which allows for the analysis of antibody specificity based on the molecular weight of the protein to which it binds. If we

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want to make sure that the IHC staining in liver tissue is specific, we subject liver samples to western blotting and if the latter reveals that the antibody detects proteins of wrong molecular weights, such antibody is considered nonspecific, regardless of specific-looking IHC staining. A good IHC antibody does not only mean getting a strong staining signal, but also being absolutely sure that IHC staining makes histological and physiological sense.

Unfortunately, the antibody specificity issue is not black and white, but rather grey. What if the antibody strongly detects a specific protein but weakly stains nonspecific ones? What is the extent of acceptable nonspecific staining? There is no written mandatory guide on specificity of antibodies for IHC, which gives a large degree of freedom in data interpretation and acceptance to individual investigators. The good news is that diligent commercial suppliers address this issue and for the most part release antibodies of high specificity.

How about choosing a reliable commercial supplier of antibodies? There are two major profiles of antibody sellers: bonafide antibody producers and distributors who do not develop antibodies but source them in from manufacturers. Some manufactures are also distributing antibodies developed by other vendors. Companies that make their own antibodies are in full control of their production, including antigen design, antigen synthesis, immunization, antibody purification, testing, and quality control. But is it safe to purchase antibodies from a distributor? It depends on the status of such a distributor. If a distributor has a technical support team and offers troubleshooting support and either offers money back or a product credit if the antibody fails in the customer's hands, then there should not be any problem dealing with such a vendor. It is always a good idea, before placing the order, to contact the company by phone or e-mail to make sure they are providing qualified IHC support, because ordering the antibody for IHC is less an event than a process. If you are not satisfied with your IHC staining, in many cases the company can help by either offering a different lot or an antibody raised in a different species, or by suggesting alternative detection reagents and modifications of IHC protocol. The conclusion is: spend some time on preordering research to make a well-calculated decision on which vendor to choose.

There are contact research labs that offer antibody validation services serving as an intermediary between antibody supplier and the end user. Such antibody validation services may look attractive, but what if the antibody tested and recommended by it does not work in the customer's hands? On the other hand, what if the validation service calls nonspecific the antibody that has been characterized and approved by the manufacturer? Whose opinion should the customer take into consideration? These situations can be quite confusing to both the customers and antibody vendors. It appears that it would be very helpful to set forth antibody IHC guidelines requiring commercial suppliers to adhere to certain antibody validation and their commercial release rules. For example, such rules would require commercial suppliers to meet certain criteria confirming antibody specificity. What would really help the customers is having a vendor ranking system with more stars given to the most reliable antibody suppliers. Having such a system in place would

definitely save customers time and money, and hopefully one day we will see these ranking system up and running.

Vendors supply primary antibodies either as liquid (neat) or as dry lyophilized stock concentrates. Primary antibodies can be supplied as a sterile solution in special buffers that also contain other chemicals to stabilize the molecule of primary antibodies (e.g., protein albumin and sugar trehalose) and anti-microbial compounds/preservatives (e.g., sodium azide, Pro-Clean 400). Stocks of primary antibodies are highly concentrated and should not be used "right out of the box" for IHC because tissues will become overstained and not analyzable. Stock antibodies have to be further diluted per the vendor's recommendations to make working solutions/dilutions suitable for IHC staining. Stock antibodies are usually supplied with preservatives to prevent bacterial contamination that degrades primary antibodies. Neat antibodies can be used right away for making working solutions, but lyophilized ones have to be first liquefied (or reconstituted, in IHC terminology) with special antibody diluents, which are made of buffers with different chemical additives. Liquid primary antibody stocks can be stored for a short period of time at 4 °C, but it is strongly recommended to aliquot into smaller volumes so that one or two vials contain enough material for a single IHC experiment. If primary antibodies are stored frozen in their original vials, they have to be thawed each time for making working solutions for IHC experiments. However, repetitive (as few as 3–5 cycles) freezing and thawing can completely destroy the antigen-binding capacity of antibodies, making them unusable because they do not produce any tissue staining. The volume of aliquots is calculated based on the estimated working dilution: the higher the working dilution, the less amount of antibody stock solution is aliquoted. For example, if the working dilution is known to be 100 fold, then aliquots may be 20 uL (one aliquot is enough for making 2000 uL (100 × 20 uL) working solution. If the working dilution is 400 fold, then aliquots may be 5 uL, which is also enough to make 2000 uL of working solution (400 × 5 uL) from a single aliquot. Aliquoted primary antibodies should be stored at −20 °C and lower and thawed either on ice or in the fridge at 4–8 °C when preparing working solutions and starting IHC staining. Leftovers of working solutions can be stored for few hours in the fridge and, if not used, they are usually discarded.

3.1 Summary

Instead of making antibodies on their own, which is costly and time-consuming, researchers can purchase antibodies from commercial suppliers. Primary antibodies for IHC can be bought either from companies that manufacture their own antibodies or from companies that distribute antibodies made by manufactures. Before ordering antibodies, it is advisable to check the vendor's background to make sure that qualified technical support will be provided when needed. In addition, it is recommended to deal with suppliers that either guarantee money back or offer product credits for antibodies failed in IHC. Specificity of antibodies is the most

important feature. IHC staining per se cannot establish whether an antibody is specific or not and western blot testing is required to find this out. Currently there are no mandatory guidelines for antibody vendors with regard to manufacturing and validating antibodies for IHC research. Primary antibodies may lose their staining power if stored improperly. Because the stock solution is highly concentrated, it is recommended to aliquot primary antibodies received from the vendor into smaller vials and store frozen so that each vial has enough antibodies for a single IHC experiment. Repetitive freezing and thawing of primary antibodies significantly impairs their activity, which results in weak and even lack of tissue staining.

3.2 Quiz (True or False)

1. Specificity of antibodies is not their important attribute: **T F**
2. Antibodies should be only purchased from the vendors who manufacture them: **T F**
3. Making antibodies by researchers on their own is costly and time-consuming: **T F**
4. IHC staining alone is sufficient to determine specificity of the antibody: **T F**
5. Western blotting is done to find out whether antibodies bind proteins of correct molecular weight: **T F**
6. When checking specificity of IHC staining on liver tissue, western blotting should be done using tissues other than liver (such as kidney, brain, skin, etc.): **T F**
7. Antibodies can be either specific or nonspecific with nothing in between: **T F**
8. The industry must follow mandatory rules for developing and validating antibodies for IHC research: **T F**
9. Primary antibodies should not be stored at room temperature: **T F**
10. Frequent freeze-thaw cycles do not have a negative effect on the performance of primary antibodies: **T F**

Chapter 4
Detection and Visualization of Antigens

As we know now, the first step in detecting tissue antigens requires incubation of tissue sections with the primary antibodies. However, this does not allow for the detection and visualization of the antigen-antibody complex because primary antibodies are colorless. There are two major approaches researchers can use to turn the colorless antibody-antigen complex into a colored, easily detectable signal. The first approach, direct detection, is to modify the primary antibody by attaching either a color label or a color-generating tag to it, turning the antibody into a so-called direct conjugate of the primary antibody. This is exactly what Dr. Coons did in his pioneering IHC experiments: attaching fluorescent tags allowed him to detect stained cells under the microscope. In the second approach, indirect detection, instead of making direct conjugates of primary antibodies, researchers use so-called secondary antibodies that recognize and bind to the primary antibody, which, in turn, is bound to tissue antigen. Secondary antibodies have to be conjugated to color labels or color-generating tags allowing their detection. Both direct and indirect detection techniques can be combined with additional signal amplification techniques. Usually, color labels for direct and indirect detection techniques include fluorescent dyes, whereas color-generating tags represent enzymes that convert other colorless chemical solutions into precipitated colored buildup (Fig. 4.1).

Although both approaches have their pros and cons and are commonly used in IHC protocols. In general, the decision of whether to use direct or indirect detection techniques is made based on the abundance of tissue antigens and their levels: for strong antigens, direct detection may be sufficient, whereas for weak antigens indirect detection may be a better option. One of the major drawbacks in using direct detection methods is that conjugation of primary antibodies to color labels may significantly impair specificity of primary antibodies and reduce their binding efficiency with tissue antigens. Unfortunately it is impossible to predict which antibody is at a higher risk of losing its sensitivity until tested experimentally in side-by-side comparisons with indirect detection technique. Advocates of direct detection point out that this is the simplest way for simultaneous detection of

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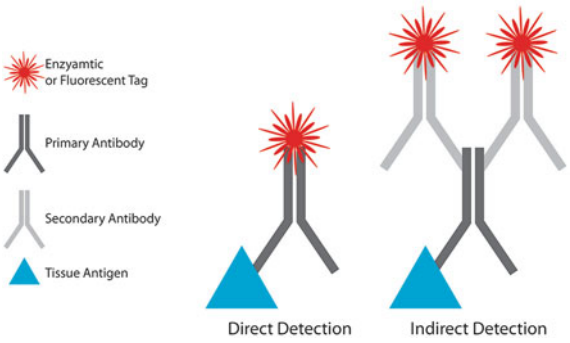

Fig. 4.1 Direct versus indirect IHC detection. Note that indirect detection provides higher sensitivity because two labeled secondary antibodies can bind to one primary antibody

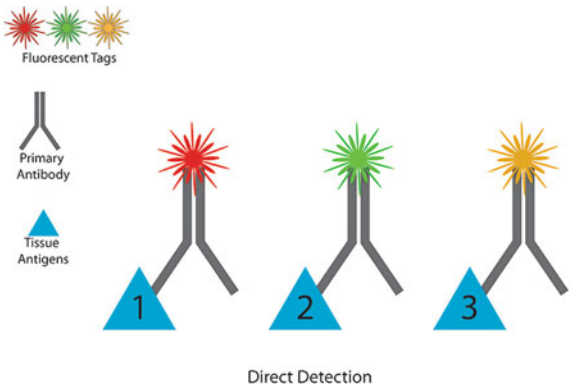

Fig. 4.2 Direct IHC detection using primary antibodies conjugated to fluorescent tags of different colors that bind to 3 different tissue antigens

several tissue targets using a mixture of primary antibodies conjugated to labels of different colors (Fig. 4.2).

Although indirect detection can be also used for multiplex IHC, there is a risk of nonspecific binding of secondary antibodies to tissue sections resulting in background staining that can obscure the specific signal.

4.1 Secondary Antibodies

Practically, indirect detection is used far more often in IHC than the direct technique. The main reason is the higher sensitivity of indirect detection, which allows for generating a stronger tissue staining. The simplest explanation is that two molecules of secondary antibodies can simultaneously bind to one molecule of the primary antibody. In other words, indirect detection employs two times more color labels (or color-generating tags) than direct detection.

Secondary antibodies are sold by a large number of commercial suppliers and the same recommendations apply for deciding which vendor to purchase from as those for choosing the source of primary antibodies. The first step in identifying

which secondary antibody to use is dictated by the host species of primary antibodies used in the IHC experiment. For example, if primary antibodies are raised in rabbits, goats, or sheep, secondary antibodies have to be anti-rabbit, anti-goat, or anti-sheep respectively. Using secondary antibodies that mismatch their primary antibody counterparts will not produce any specific signal. For example, anti-rabbit, rather than anti-goat, secondary antibodies should be used if the primary antibodies are raised in rabbits.

Secondary antibodies are produced using the same approach that is used to make primary antibodies except that host animals are immunized not with a specific protein or its fragment but with antibodies from a different species. To raise anti-rabbit secondary antibodies we can immunize donkey (or any other species) with rabbit immunoglobulins. Any host species can be used for making secondary antibodies other than the same species the secondary antibodies are raised against. For example, rabbits cannot be used to produce anti-rabbit antibodies because the immune system of a healthy animal does not develop antibodies against its own proteins. Secondary antibodies can be made against the entire IgG molecule, IgG molecules from different classes (e.g. anti-IgG1), and against fragments of IgG molecules.

Vendors sell secondary antibodies in two major formulations: the whole IgG molecule and F(ab) fragments (monovalent F(ab) as well as divalent $F(ab)^2$ fragments). The less expensive and most widely used secondary antibodies are whole IgG molecules. Whole IgG secondary antibodies bind to all classes (IgG1, IgG2, IgG3, and IgG4), which makes them versatile detection reagents. If whole IgG secondary antibodies are made only against the IgG1 class of molecules, such antibodies will not be able to bind to primary antibodies of IgG2, IgG3, and IgG4 classes. The more expensive F(ab) fragments of secondary antibodies are recommended for IHC if whole IgG antibodies are producing excessive background staining. Besides the low background, another advantage of using F(ab) fragments is that they are smaller than the whole IgG antibodies and therefore can better penetrate into the tissue, which is beneficial for the detection of antigens localized deep inside the cell, such as in cell nuclei.

The working dilution of secondary antibodies is usually less compared to the recommended dilution of primary antibodies and can range between 1 and 10 μg per milliliter of antibody diluent. Some vendors do not disclose the concentration of their secondary antibodies and only say the recommended dilution is 100 fold, which means that 1 part of the stock antibody solution should be mixed with 99 parts antibody diluent. Vendors may or may not give recommendations about the incubation time with secondary antibodies, but it usually ranges from 30 min to 2 h for thin tissue sections (2–15 microns) mounted onto histological glass slides, and 3–4 h for thick tissue sections (50–250 microns) that are not mounted onto the slides but are stained as free-floating tissue sections.

There are some disadvantages of using secondary antibodies. First of all, the IHC protocol will become longer, requiring more tissue incubation and washing steps. The second disadvantage is more serious: secondary antibodies may bind to tissue sections nonspecifically and produce nonspecific background staining, which

may obscure specific staining. To solve the latter problem, researchers have to use secondary antibodies that have been affinity purified and further processed to have minimal interaction with irrelevant host IgGs: for example, in staining mouse, rat, or human tissue sections using primary rabbit antibodies, secondary antibodies should have minimal interaction with mouse, rat, and human IgGs, respectively, but strongly bind only to rabbit IgGs. Such secondary antibodies are marked by commercial vendors as cross-absorbed against IgGs from other species. The more species secondary antibodies are cross-absorbed against, the more expensive they are. For example, the data sheet that comes with a secondary antibody may say "anti-rabbit IgG1 antibodies," which means that this secondary antibody binds to the IgG1 class of primary antibodies, but should not interact with other classes of immunoglobulins including IgG2a, IgG2b, and IgG3.

Although IgG is the most common type of antibodies, there are other types designated IgM, IgA, IgD, and IgE that are structurally different from IgG. It is beyond the scope of this book to elaborate on the structure and function of other types of antibodies. For the purposes of our discussion, we just want the reader to keep in mind that in addition to primary antibodies belonging to IgG type, secondary antibodies may also interact with IgM, IgA, IgD, and IgE types of primary antibodies. Many vendors cross-absorb secondary antibodies not only against IgG classes (e.g., IgG2a, IgG2b, and IgG3), but also against other types of antibodies (e.g., IgM, IgA, IgD, and IgE). For example, the anti-IgG1 antibody may not only be specific for the IgG1 isotype but may also have minimal cross-reactivity with IgM, IgA, IgD, and IgE.

For example, a secondary antibody data sheet may say: "F(ab) Donkey anti-Rabbit IgG1 (rat, mouse, guinea pig, goat, chicken cross-absorbed); (IgM, IgA, IgD, and IgE cross-absorbed) secondary antibody, conjugated to FITC." This description states the following:

1. Secondary antibodies were raised in a donkey host animal and are F(ab) fragments;
2. Will bind to rabbit IgG1;
3. Will not cross-react with primary antibodies raised in rat, mouse, guinea pig, goat, or chicken;
4. Will not cross-react with IgG2a, IgG2b, or IgG3 antibodies; and
5. Secondary antibodies are conjugated to fluorescent dye FITC.

4.2 Summary

Due to its high sensitivity, indirect detection is used far more often in IHC than the direct technique. Secondary antibodies are sold by commercial vendors who provide recommendations on working dilutions and storage conditions. The critical step in choosing secondary antibodies is their complementarity or match with primary antibodies: anti-rabbit secondaries for rabbit-made primaries, anti-mouse

secondaries for mouse-made primaries, etc. Secondary antibodies can be made either with the whole immunoglobulin molecule or its fragments and can be pre-absorbed against different IgG classes. In addition, secondary antibodies by themselves can be either the whole IgG molecule or its monovalent F(ab) and bivalent $F(ab)^2$ fragments. Working dilution of secondary antibodies for IHC ranges from 1 to 10 μg per milliliter of the diluent and incubation time varies from 30 min to 4 h depending on the thickness of the tissue section.

4.3 Quiz (True or False)

1. Secondary antibodies are used for indirect detection technique: **T F**
2. Indirect detection technique is more sensitive than direct detection: **T F**
3. When using primary antibodies raised in rabbits it does not matter which secondary antibodies to use, anti-rabbit, anti-mouse, anti-goat, etc.: **T F**
4. Secondary antibodies are always made against the entire IgG molecule but not against its fragments: **T F**
5. Secondary antibodies can be stored at room temperature for long periods of time without a negative impact on the quality of antibodies: **T F**
6. Incubation with secondary antibodies should be always done for 30 min: **T F**

4.4 How to Choose Secondary Antibodies for IHC?

For the most part, the choice of secondary antibodies is determined by the nature of the primary antibodies. It is of critical importance to use secondary antibodies that match the host species in which the primary antibodies were raised. For example, if the primary antibodies are raised in rabbits, the secondary antibodies should be anti-rabbit: using anti-goat or anti-mouse secondary antibodies will not produce specific IHC staining. This rule applies to both immune serum and affinity-purified primary antibodies (Fig. 4.3).

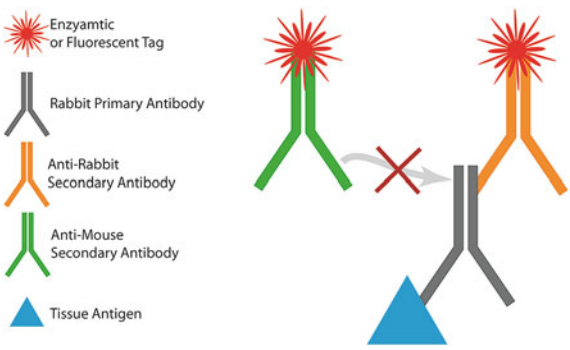

Fig. 4.3 For the detection of antigens in indirect technique secondary antibodies should match the species of primary antibodies: using anti-mouse secondary antibodies with primary antibodies raised in rabbit will give no staining

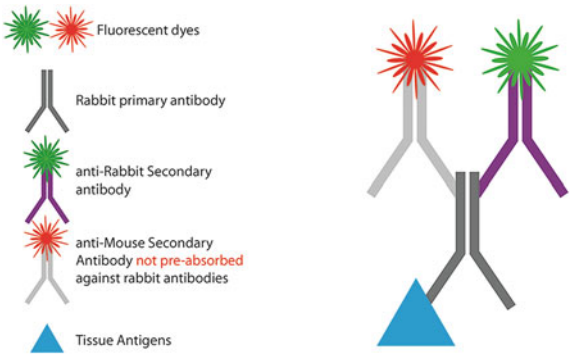

Fig. 4.4 Binding of secondary antibodies that were not preabsorbed with irrelevant primary antibodies will result in nonspecific tissue staining

How important is it in IHC to use antibodies that are cross-absorbed against different species? It all depends on the experimental design. For example, when only a single host (e.g., raised in rabbit) primary antibody is used for the detection of a single tissue target, it does not matter if secondary antibodies were cross-absorbed against IgG molecules from other (e.g., rat, mouse, goat, etc.) host species. However, if primary antibodies from more than one host animal are being used for the detection of two or more different tissue targets, it is of critical importance to choose secondary antibodies that were cross-absorbed against different species to avoid cross-reactivity between irrelevant primary and secondary antibodies (Fig. 4.4).

Another important factor to consider is which IgG class of primary antibodies is used: for example, if mouse monoclonal antibodies belong to IgG2a class, it is necessary to use secondary antibodies that bind to IGg2a but are not cross-absorbed against this immunoglobulin so they interact with classes other than IgG2a (e.g., IgG1, IgG2b, or IgG3), which would result in no staining.

When either immune serum or affinity-purified primary antibodies are being used, it is recommended to use secondary antibodies that interact with the all IgG classes/isotypes because immune serum and affinity-purified antibodies are the mixtures of different IgG classes. In other words, using secondary antibodies cross-absorbed against select IgG classes will most likely result in weak tissue staining.

Sometimes, using secondary antibodies that do not cross react with unwanted classes and types of antibodies is done to minimize background tissue staining. Secondary antibodies can be supplied either as liquid or dry lyophilized concentrated stocks, which should be further diluted according to the manufacturer's instructions to make a working dilution which can be applied to tissue sections. Liquid antibody concentrates usually need to be stored between 4 and 8 °C and are usually stable for up to a year. If a vial with secondary antibodies cannot be used within its shelf life term due to low volume of IHC experiments, it is recommended to make aliquots. For example, a single 0.5 mL stock solution can be aliquoted into 50 vials containing 10 μL. Aliquots should be stored frozen and used to prepare a working solution immediately before use. Original stock solution vials can be also

stored frozen, but have to be taken out from the freezer and thawed to make working solutions and after that transferred back to the freezer. Repetitive freezing and thawing of secondary antibodies can destroy their staining capacity. Unopen vials of dry, lyophilized, antibodies can be stored for years at −20 °C and lower but after reconstitution with the diluents, they have to be treated like liquid stock solutions. To extend the shelf life of antibody stock solutions, some vendors recommend adding glycerol and storing them in freezers at −20 to −80 °C: glycerol prevents solution from freezing and therefore eliminates the risks of freeze–thaw cycles. Secondary antibodies with added glycerol can be stored in their original vials making small size aliquots unnecessary.

Secondary antibodies are colorless molecules and cannot be used for visualization of tissue targets. To become visible, they have to be combined with reporter molecules. There are two major types of reporter molecules: (1) fluorescent dyes and (2) special proteins called enzymes, which convert colorless chemical compounds (called substrates) into colored precipitating deposits. Fluorescent dyes produce very high-contrast images resembling the picture of the bright stars against the dark sky. To visualize fluorescence staining, it is necessary to use fluorescence microscopes, which are equipped with special illumination to be able to see fluorescent tags. The images produced with enzyme-substrate detection chemistry look like reversed fluorescent images with a dark signal against a light background and have to be examined using a bright-field microscope. Although bright-field images have a lower contrast compared to fluorescent ones, enzymatic detection is considered to be more sensitive than the use of fluorescent dyes because the conversion of the substrate by its enzymes works in a "snowball" fashion: the longer the chemical reaction, the more colored precipitate is formed and the stronger the tissue staining becomes.

4.5 Summary

Secondary antibodies should match primary antibodies species specificity-wise. Both IgG class polyspecific and cross-absorbed secondary antibodies can be used for IHC. Vendors sell secondary antibodies in dry (lyophilized) and neat (liquid) forms. To avoid repetitive freezing and thawing of the same vial with secondary antibodies, which impairs their quality, it is recommended to make small volume aliquots for one-time use in IHC experiments.

4.6 Quiz (True or False)

1. It is always required in IHC to use cross-absorbed secondary antibodies: **T F**
2. It would not be right to use secondary antibodies preabsorbed against the same IgG class as the class of primary antibodies: **T F**

3. Adding glycerol to stock solution of secondary antibodies prevents them from freezing when stored at subzero temperatures: **T F**
4. In order to be visible and detectable, secondary antibodies should be conjugated to different reporter molecules: **T F**
5. Enzymatic detection is less sensitive than the use of fluorescence: **T F**

4.7 Fluorescent Probes

As we know now, antibodies are colorless molecules. They are therefore useless for the detection of tissue targets in their original, unmodified form and must be combined with reporter molecules that can "light" antibodies up. One such group of reporter molecules includes fluorescent probes. Fluorescence is a part of a luminescence phenomenon that is described as light emission by a molecule previously irradiated by light. As history goes, the term "fluorescence" was coined in 1852 by physicist George Gabriel Stokes at the University of Cambridge after he discovered that the mineral fluorite can emit visible light after being exposed to invisible ultraviolet illumination. The fluorescence phenomenon has since been explained based on the behavior of electrons orbiting around the atoms (Fig. 4.5).

Currently there are a lot of different fluorescent compounds, or fluorophores, sold by numerous vendors for IHC applications. Different vendors not only offer

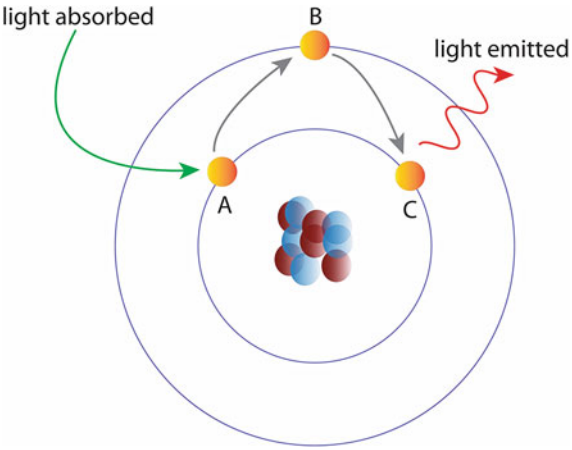

Fig. 4.5 After being hit with light, the electron (**a**) absorbs a photon and becomes excited (acquires additional energy) and jumps to a higher orbit (**b**). However, this is an unstable state and the electron eventually returns to its original orbit (**c**). During this "home run," the electron loses its energy and emits photon, which is recorded as a visible light. Cycling through the excitation/emission loop, fluorescent dyes produce bright light visible under the microscope

fluorophores with comparable characteristics based on price competition, but also introduce to the market fluorophores with significantly improved characteristics. Which fluorophore properties are of critical importance? It appears that the most important properties are: (1) brightness; (2) fading; (3) stock shift; and (4) resistance to organic solvents.

Brightness. Brightness is defined by how much light is emitted by the fluorophore. The brighter the fluorophore is, the more sensitive its detection. Brightness is a function of the fluorescence quantum yield of the fluorophore molecule, which entirely depends on its molecular structure. A fluorophore becomes visible and detectable when it emits a photon, or lite. But photon emission can only happen if a fluorophore is first hit, or excited, by another photon coming from an external source, such as a laser. The number of photons emitted by a fluorophore is less than the number of photons that hit it, and the fluorescence quantum yield is defined as the ratio of the number of photons emitted to the number of photons absorbed by the fluorescent dye. This ratio is usually less than 0.05.

Fading (*Photobleaching*). Fading of the fluorophore is the loss of its ability to produce fluorescence during its excitation and emission cycles (Fig. 4.6). The sooner the fluorophore fades, the dimmer its light and the less time the researcher has for examining the tissue staining under the microscope before the fluorescent staining disappears. Different fluorophores have different fading dynamics that are determined by their capacity to cycle through numerous excitation/emission states, which, in turn, depends on their molecular structures: the more cycles a dye can withstand, the longer its fluorescence. Some dyes retain fluorescence for a long period of time (strong fluorophores), whereas others photobleach very quickly (weak fluorophores). To prevent fading, special anti-fade reagents are often used in the form of mounting media.

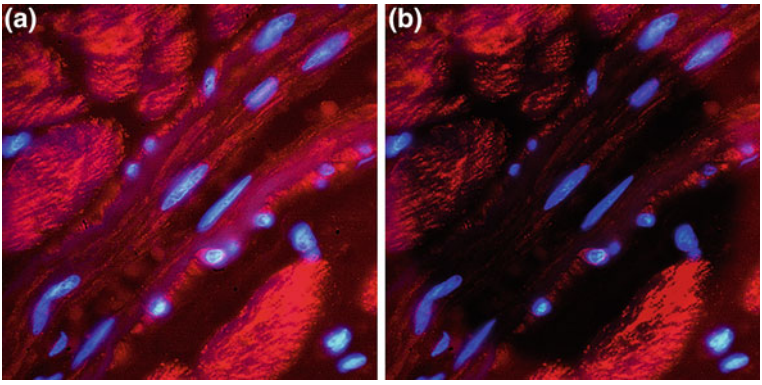

Fig. 4.6 Example of photobleaching. Labeling on freshly stained heart tissue appears uniformly bright across the image (**a**). After examining this tissue under the fluorescence microscope, the area most exposed to light shows signs of profound fading (**b**)

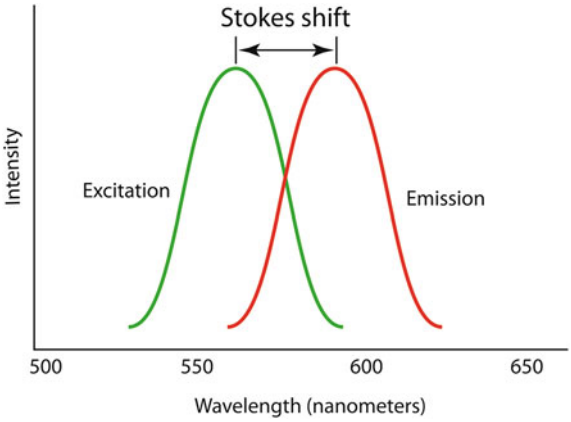

Fig. 4.7 Example of a Stokes shift profile of a fluorescent dye that has a maximum excitation around 560 nm and emits the light of the wavelength around 590 nm, which corresponds to a red spectrum of light

Stokes shift. This phenomenon is named after George G. Stokes and represents the difference between the peaks of maximum absorption and the emission spectra of a particular fluorophore. Different fluorophores are characterized by unique Stokes shift signatures, allowing the researcher to determine which excitation/emission filter combination should be used for maximum brightness of a particular fluorophore. Another important aspect of the Stokes shift is that it can be a measure of the sensitivity of a particular fluorophore: the farther apart the maximum excitation and emission peaks are, the stronger and higher contrast the fluorescent signal is. Alternatively, the closer these peaks are, the higher the background and lower the contrast of staining. Besides information about the wavelength of excitation, Stokes shift diagrams provide very important information about the wavelength of emission, which determines the color of the staining (Fig. 4.7).

Resistance to organic solvents. In some cases, after finishing the IHC staining, tissue sections need to be cleared in special organic solvents like xylenes and alcohol in order to be mounted under coverslips into permanent mounting media. Some fluorophores, like the widely used FITC, can completely lose their fluorescence after exposure to xylenes and alcohol. Therefore, when choosing a fluorophore it is necessary to check whether its photostability can be affected by tissue-clearing chemicals.

There are a large number of fluorescent dyes belonging to different families, including but not limited to: Fluorescein (FITC), Rhodamine (TRITC, Rhodamine Red X), Cy® dyes (Cy2, Cy3, Cy5, CY7, etc.), Alexa Fluor® (350, 405, 488, 546, 610, etc.), DyLight® Fluor (DyLight 350, DyLight 488, DyLight 550, etc.) and Oyster® dyes (Oyster-488, Oyster-550, Oyster-645, Oyster-800, etc.).

A standalone group of fluorescent dyes includes Qdot® (Quantum Dots) dyes, which are tiny (just a few nanometers in diameter) nanocrystals of semiconductor material. Chemically, Qdots®, unlike other fluorescent dyes, are not small single-molecule dyes but rather larger multimolecular particles. The major advantage of Qdots® is their extraordinary brightness and anti-fade stability.

4.8 Enzyme Conjugates and Their Chromogenic Substrates

Another way of detecting tissue antigens in tissue sections is to use enzymes and their chromogenic substrates: via chemical reaction, enzymes convert colorless chromogens into tiny colored and insoluble particles that precipitate over tissue antigens so they can be observed and recorded under the microscope. Enzymatic detection has high sensitivity, which is important for the detection of low abundant, or weak, antigens. Using chemical conjugation procedure, enzymes can be directly attached, or conjugated, to primary antibodies, secondary antibodies, and other molecules like avidin and streptavidin.

Enzymatic detection chemistry is sensitive to acidity and the temperature of the reacting mixture: even minor changes may significantly affect the enzymatic activity, which, in turn, results in low staining intensity. Enzymes are also sensitive to chemicals present in solutions. For example, the preservative sodium azide can completely inactivate the enzyme horseradish peroxidase. The four most commonly used enzymes in IHC are: horseradish peroxidase; alkaline phosphatase; glucose oxidase; and beta-D-galactosidase.

Due to its properties, horseradish peroxidase, or HRP—purified from the root of the horseradish plant *Amoracia rusticana*—is the most frequently used enzyme: it has a small size that allows good intracellular penetration and a fast conversion rate of chromogenic substrates into a colored precipitate. HRP is easy to conjugate to antibodies and other molecules, which adds to its popularity. Chromogenic substrates for HRP that are often used in IHC include: DAB (3,3′-diamino-benzidine), which produces a brown color; AEC (3-amino-9-ethyl-carbazole), which produces a red color; CN (4-chloro-1-naphthol), which produces a blue color; and p-Phenylenediamine dihydrochloride (Hanker—Yates reagent), which also produces a blue color.

Another enzyme that is also popular among researchers is alkaline phosphatase, or AP. One form of this enzyme is widely distributed in different tissues, whereas the other is found only in the intestine. The enzyme used for IHC labeling is isolated from calf intestines. The advantage of using AP is that its substrate-converting reaction is linear, which means that incubation with the chromogenic substrate may go longer. This will boost the sensitivity of antigen detection without the risk of over-staining of the tissue sections. Another advantage of using AP conjugates is to avoid nonspecific staining in tissues with high levels of endogenous peroxidase, which limits the application of HRP conjugates. There are different AP chromogenic substrates, but those most frequently used are BCIP/NBT (Bromo-chloro-indolyl-phosphate plus Nitro blue tetrazolium), and Fast Red TR (4-chloro-2-methyl-benzenediazonium salt). BCIP/NBT produces strong blue precipitate and Fast Red TR produces red, both of which retain their color for a long period of time.

Glucose oxidase (or GOD, from the fungus *Aspergillus niger*) and beta-D-galactosidase (or (beta-Gal, from the bacterium *Escherichia coli*) are two other

enzymes used in IHC in place of HRP if tissues are known to have high endogenous peroxidase levels that may produce nonspecific staining when using HRP substrates. The advantage of using GOD is that there is no activity of this enzyme in mammalian tissues and therefore there is no risk of getting nonspecific staining. Different GOD substrates can be used for IHC, including TNBT (tetranitroblue tetrazolium), NBT (nitroblue tetrazolium), and INT (iodophenyl-nitro-phenyl tetrazolium), which produce brown-black, blue-black, and red precipitates respectively. Unlike GOD, the choice of substrates for beta-Gal that produces an insoluble end product is limited, and X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galatopyranoside), forming blue precipitate, is the one most frequently used.

4.9 Advantages and Disadvantages of Fluorescence and Enzymatic Chromogenic Detection

Advantages and disadvantages of both detection techniques are summarized in the table below.

Features	Fluorescence	Enzymatic chromogenic
Time needed to complete IHC staining	The entire procedure is short: one step if using primary antibodies conjugated to fluorophore, and two steps if using secondary antibody conjugates	The protocol is longer. Beyond incubation with primary antibodies, additional steps are required to block endogenous enzymatic activity (e.g., peroxidase activities in blood cells), prepare chromogenic substrate solution, and add it to tissue section for color development. If using avidin-biotin detection chemistry (discussed below), it may also be necessary to block endogenous avidin and biotin to avoid nonspecific staining of tissue sections
Multiplexing	It is easy to simultaneously detect multiple overlapping (i.e., located in the same part of the tissue section and/or intracellular compartment: cell membrane, cell nuclei, Golgi vesicles, endosomes, etc.) and nonoverlapping profiles. Due to the construction of the fluorescence microscope, each color in multi-color staining	Multi-color is possible only for nonoverlapping tissue antigens. For example, on brain tissue sections, one color can be used for the detection of astrocytes and another for neurons. Multi-color is also possible if intracellular targets are confined to different compartments and organelles (e.g., nuclei and plasma membrane). If antigens overlap

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Features	Fluorescence	Enzymatic chromogenic
	depicting different antigens is detected via individual color channels. Individual colors can then be digitally combined together if a multi-color image is needed for presentation. On digital multi-color images, individual colors can be turned on and off so they don't obscure each other	(e.g., both are expressed in cell nuclei), a resulting color mix would make it difficult or even impossible to determine the exact localization of each individual antigen. Multi-color detection protocols are usually cumbersome and lengthy
Fine details resolution	Fluorescence has excellent resolution of finest details. Stained samples can be examined using a laser-scanning confocal microscope, or LSCM (see its description in the section below). By minimizing out-of-focus details, LSCM enables obtainment of images with very sharp details. In addition, LCSM allows collection of a stack (called a z-stack) of a large number of images from the same tissue section at different focal planes. Images from a z-stack can be combined together to reconstruct a 3D view of the antigens distributed within the tissue	Contrast, sharpness and resolution are lower compared to fluorescence detection. Stained tissues cannot be observed and analyzed using LSCM
Detection of co-localized antigens	The ability to toggle between individual and merged colors that overlap makes fluorescence an ideal tool for co-localization studies	Using this method, it is impossible to accurately extract individual colors from the color mix depicting overlapped targets. This method is not suitable for analysis of co-localized antigens
Color stability	Fluorescent dyes can fade during examination under the microscope and during storage	Colors are more stable and won't fade during examination of stained tissue sections under the microscope. Stained tissue sections retain color for years
Tissue type limitations	Fluorescence is not recommended for tissues with endogenous autofluorescence (e.g. human or primate brains), as autofluorescent signals can be confused with specific staining	There are no tissue type limitations because endogenous autofluorescence does not affect chromogenic signals
Availability of reagents	Reagents are available from many commercial sources	Reagents are available from many commercial sources

4.10 Summary

For tissue antigens to be visible, reporter molecules that recognize and bind to tissue antigens have to be attached to antibodies. Two major types of labels are widely used in IHC: (a) fluoresecent dyes and (b) enzymes. Both fluorophore- and enzyme-antibody conjugates can be purchased from a large number of commercial vendors. Fluorescent probes can be selected based on their excitation and emission spectra, fluorescent quantum yield, and resistance to fading (photobleaching). Unlike fluorescent dyes, which can be visualized on their own, enzymes are colorless and need to be mixed with their respective chromogenic substrates to produce colored precipitate. The most frequently used enzymes in IHC are Horseradish Peroxidase (HRP) and Alkaline Phosphatase (AP). Color development using HRP is faster compared to AP, which may result in tissue over-staining when using HRP conjugates. Fluorescence detection protocol is short and can be used for the detection of multiple overlapping and nonoverlapping tissue targets. Chromogenic protocol is longer and can only be used for the detection of nonoverlapping tissue targets. Fluorescent labels produce high-contrast images with excellent resolution of fine details compared to enzymatic chromogenic detection. However, due to fading, fluorescent dyes lose their brightness faster than chromogenic labels. If tissues show endogenous autofluorescence, use of chromogenic rather than fluorescence detection technique is recommended.

4.11 Quiz (True or False)

1. Two major labels are used in IHC: fluorescent dyes and enzymes: **T F**
2. The higher the fluorescence quantum yield, the weaker the fluorescent signal: **T F**
3. Fading (or photobleaching) of the fluorophores reduces their brightness: **T F**
4. Fluorescent probes can only be used for the detection of nonoverlapping antigens: **T F**
5. HRP and AP are the most frequently used enzyme labels for chromogenic detection in IHC: **T F**
6. AP has a faster reaction rate than HRP, which may lead to over-stained tissue sections when using AP conjugates: **T F**
7. Chromogenic detection is recommended for the detection of multiple overlapping antigens: **T F**
8. Chromogenic labels do not lose their color as fast as fluorescent dyes: **T F**
9. When tissues show endogenous autofluorescence, the use of chromogenic rather than fluorescence detection is recommended: **T F**

Chapter 5
Ancillary Reagents for IHC

Although primary and secondary antibodies are essential for IHC procedure, they cannot be used alone and require ancillary reagents: buffer solutions, antibody diluents, and mounting media. Without them, IHC staining cannot be successfully started and completed. Let us go over each of them and discuss their role in IHC procedure.

5.1 IHC Buffers

An important prerequisite for antibody binding to its tissue antigen is the proper acidity/alkalinity of the reacting mixture. In chemistry, acidity and alkalinity is expressed numerically on a so-called pH (pronounced *pi:'eɪtʃ*) scale ranging from 0 to 14. Solutions with a pH less than 7 are acidic, and the lower the number the stronger the acid (for example, lemon juice has a pH of 2.0 and less acidic tomatoes have a pH of 4.4). Solutions with a pH higher than 7 are alkaline, and the higher the number the stronger the solution (for example, household bleach has a pH of 12.2). From our ordinary life experience, we know that acidic and alkaline solutions can be dangerous because solutions of a high or low pH are not generally compatible with normal human physiology (with the exception of gastric acid secreted in the stomach, which has a pH around 2.5). Solutions at a pH of 7.0 are considered neither acidic nor alkaline, and therefore pH 7 is referred to as neutral (which is the average pH of the human body). Antibodies are products of the immune system, and that is why the optimal pH for antibodies to bind to their antigens is around the neutral pH of 7.0. To maintain the optimal pH for antibodies to bind to tissue antigens, scientists use buffer solutions (called buffers for short), which, due to their chemical composition, prevent drastic shifts of pH in either direction. The main types of buffers used in IHC are listed below.

Wash Buffer. This buffer is used to wash tissue sections between incubations with different reagents. For example, after incubation with primary antibodies, tissues

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have to be washed before starting incubation with secondary antibodies. This is done to wash off excess primary antibodies not bound to tissue antigens. When primary antibodies are added to tissue sections, they bind to their antigen (specific binding) as well as to a large number of irrelevant tissue targets (nonspecific binding). Specific binding is much stronger than nonspecific binding, which is why antibodies bound to non-specific targets can be dislodged with wash buffer while primary antibodies tightly bound to specific targets will not be removed. If the washing step is not done and secondary antibodies are added right away, they will bind to primary antibodies that are bound to both specific and nonspecific targets, producing a strong background staining that masks a specific signal.

Wash buffers commonly used in IHC include phosphate buffered saline (PBS) at a pH of 7.2–7.6, which is created by dissolving such chemicals as NaCl, KCl, Na_2HPO_4 and KH_2PO_4 in distilled water, and Tris buffered saline (TBS) at a pH of 7.4–7.6, created by mixing the organic compound Tris (Tris (hydroxymethyl) aminomethane) with NaCl in distilled water. These buffers may be used as is or in some cases with the added detergent Tween 20 to facilitate better penetration of the wash into the tissue.

Both PBS and TBS can be purchased from commercial sources as 10x concentrated solutions, which can be diluted with distilled water to 1x ready-to-use buffers, as well as in dry powder form, which can be dissolved in water immediately before use.

Block buffer. Specific binding of antibodies to epitopes on tissue antigens occurs via hydrophobic interactions, ionic interactions, and hydrogen bonding. However, the same mechanisms also underlie the nonspecific binding of primary antibodies to irrelevant tissue proteins that results in nonspecific background staining, which is a common problem in IHC experiments. It can be challenging to eliminate or reduce nonspecific interactions while leaving specific ones unaffected. For example, primary and secondary antibodies may interact with serum proteins and stick to tissue sections as a result of ionic interactions. A high nonspecific background can mask specific staining and interfere with the detection of antigens of interest and hinder analysis of their spatial distribution.

Thus, the main goal in IHC is to label only tissue antigens of interest while leaving irrelevant ones undetected. Unfortunately, due to the inherent nature of histological tissues they are not an ideal material for IHC studies: various endogenous substances that are present in the tissue can obscure specific staining of the targets of interest, and minimizing the influence of such endogenous conditions is of ultimate importance in IHC. This goal is accomplished using specially formulated reagents under the collective name "block buffers." Because primary antibodies can bind to both specific and nonspecific tissue targets, and it is necessary to block nonspecific tissue targets so they do not bind primary antibodies and produce background staining. This is done using block buffers, which contain normal animal serum (e.g., goat, donkey, swine, horse, etc.) in PBS. Incubation with block buffers should be done before starting the incubation with primary antibodies. A protein called serum albumin, which is abundant in animal serum, binds to nonspecific targets and makes them inaccessible for primary antibodies.

Using normal animal serum helps to minimize nonspecific binding of secondary antibodies as well, which can react non-specifically with tissue sections and cause a strong background staining (Figs. 5.1 and 5.2). This problem can be solved by incubating tissues with an excess amount of antibodies from the same animal host species in which the secondary antibodies were raised. For example, if specific secondary antibodies were raised in donkey and they were found to bind non-specifically to tissues, the solution would be to pre-incubate this tissue with unlabeled antibodies from the same species (donkey). Bound nonspecific antibodies will mask the sites to which specific secondary antibodies can bind. The source of such nonspecific blocking antibodies can also be normal serum because, in addition to serum albumin, normal animal serum has a variety of antibodies developed in response to the animal's exposure to different immunogenic substances during its life. Thus, pre-treatment of tissues with normal animal serum can minimize non-specific staining with both primary and secondary antibodies.

In addition to using normal animal serum, a pure solution of a protein called bovine serum albumin (BSA) can be also used as a block buffer in IHC. Some block buffers also combine normal animal serum and BSA.

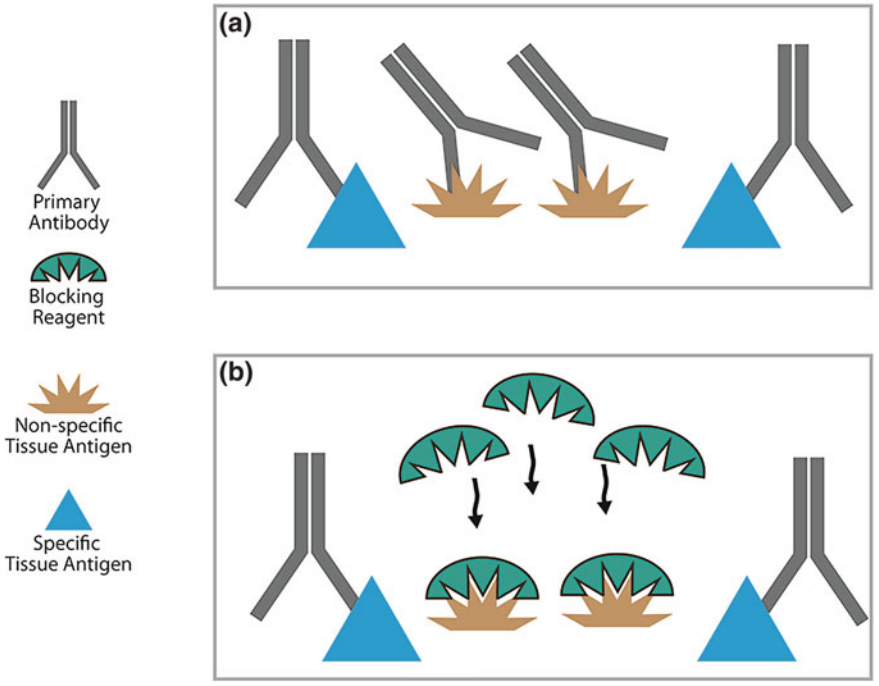

Fig. 5.1 Schematic illustration of blocking nonspecific tissue staining. In addition to specific tissue antigens, primary antibodies can also bind to nonspecific antigens resulting in background staining (**a**). Blocking reagents react with nonspecific targets to mask them from primary antibodies, which then bind only to specific tissue antigens (**b**). Blocking results in the elimination or reduction of nonspecific staining

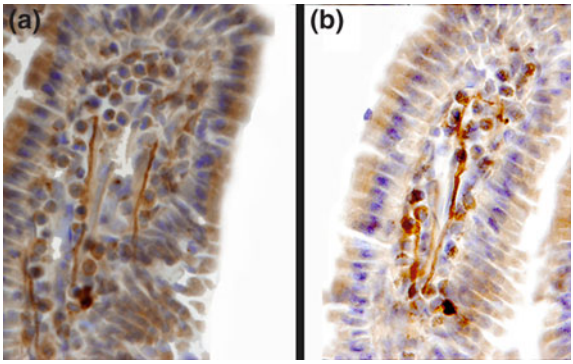

Fig. 5.2 Example of IHC staining without using blocking reagents (**a**) and with using them to block nonspecific staining (**b**). Use of blocking reagents allows for much cleaner and crisper labeling of specific tissue antigens

Besides nonspecific binding of primary and secondary antibodies to tissues there are other sources of nonspecific signal: endogenous enzymes and biotin. Endogenous enzymes pose threat when using enzymatic chromogenic detection protocols: if endogenous enzymes are not blocked they can convert chromogenic substrate solutions into colored precipitates which is similar to effects produced by exogenously added enzyme conjugates. In the case of using horseradish peroxidase (HRP), it is necessary to block tissue endogenous peroxidases, which is done using hydrogen peroxide (H_2O_2) solution, and if using alkaline phosphatase (AP) conjugates, tissues need to be treated with a solution of organic compound levamisole to block endogenous AP. Unfortunately, levamisole does not block endogenous AP in intestinal tissue and blocking can only be done using acetic acid.

When using enzymatic chromogenic detection, which utilizes Avidin-Biotin bridging reagents (avidin-biotin detection chemistry is discussed below), it is necessary to quench endogenous biotin. To block endogenous biotin, tissues are pre-incubated with Avidin followed by incubation with Biotin to block the remaining Biotin binding sites on the Avidin molecule. Unfortunately, endogenous Biotin cannot be completely blocked, which is why Biotin-free detection is gaining popularity as an alternative IHC technique.

Permeabilization buffer. To facilitate penetration of primary and secondary antibodies inside cells and cellular nuclei, tissues can be treated with PBS supplemented with chemicals resembling household soap called detergents. Detergents work by making holes in the plasma membrane that surrounds the cell. Cell membranes represent a continuous bilayer of lipid molecules with proteins randomly inserted into them. Detergents form structures that imitate this lipid bilayer into which proteins from the natural cell membrane translocate. This process gives rise to the formation of large "pores" in the cell membrane through which antibodies can enter the cell much more easily. Nonionic detergents are used in IHC, which only change the interactions between lipids but have no major effect on the structure of proteins, which retain their antibody-recognition properties. Most commonly, nonionic detergents used in IHC include Tween 20, Triton X-100, Saponin, Nonidet P40, and BRIJR.

Antibody dilution buffer. As its name goes, this buffer is used to make working dilutions of primary and secondary antibodies from their concentrated stock solutions. It is possible to use plain PBS as the antibody diluent, but usually PBS is supplemented with normal serum and BSA to block nonspecific binding of antibodies as well as a detergent like Triton X-100 to improve tissue permeability. Antibody diluent may also include antimicrobial chemicals like sodium azide (NaN_3) and ProClin, but these chemicals have to be handled with extreme care as both can be fatal if swallowed. When handling these chemicals, it is extremely important to wear gloves, goggles, and a lab coat.

Antigen retrieval buffers. These buffers are recommended to improve the intensity of specific staining in tissue sections. Before tissues can be taken for IHC studies, they usually undergo fixation with special chemicals like formaline and formaldehyde to preserve tissue morphology and antigens of interests. Fixation results in cross-linking proteins resembling a very dense net formed by so-called methylene bridges. The longer the duration of fixation, the denser this net becomes. This molecular net may become so dense that it shields protein antigens from antibodies, which results in either weak or no tissue staining. Thus, before adding antibodies to the tissue, it is necessary to reduce the density of threads in the net by incubating tissues with antigen retrieval buffers, which are also referred to as "antigen unmasking" or "epitope retrieval" buffers (Fig. 5.3). There are two major methods of antigen retrieval: (a) using enzymes, and (b) using buffers of low or high pH at a high (95–98 °C) temperature. High-temperature buffers are used for a so-called Heat-Induced Epitope Retrieval (HIER) procedure. For enzymatic retrieval, tissues can be treated for 5–15 min at 37 °C with either Trypsin, Pepsin, or Proteinase K enzymes. The drawback of enzymatic retrieval is that tissues can be physically damaged by the enzyme, making them unusable for further IHC staining. Enzymatic retrieval requires optimization of enzyme concentration and treatment

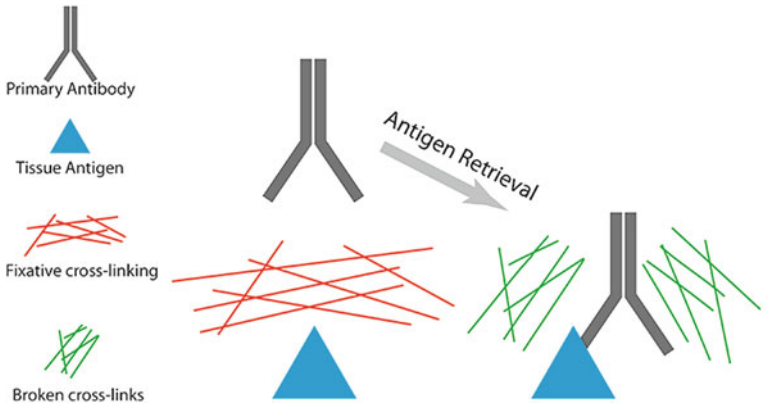

Fig. 5.3 Antigen unmasking using heat-induced epitope retrieval (HIER). Fixative cross-links interfere with the primary antibody binding to its tissue antigen. Subjecting a tissue section to HIER breaks the fixative cross-links, making the antigen accessible to the primary antibody

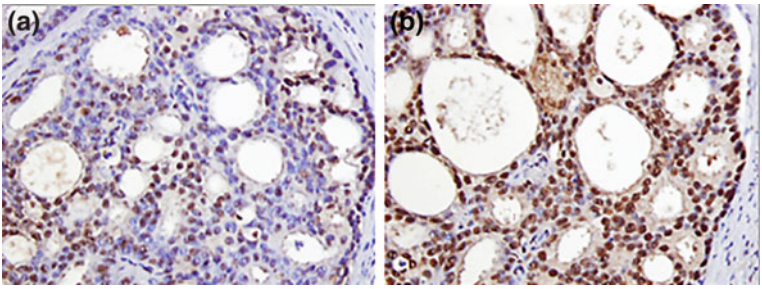

Fig. 5.4 Effects of heat-induced epitope retrieval (HIER) on the intensity of IHC staining. Without HIER (**a**), antigen labeling is much weaker compared with HIER-treated tissue (**b**). Tissue sections were stained using HRP-DAB detection (*brown color*) and counterstained with hematoxylin (*blue color*)

time for different tissue antigens, which may be a tedious procedure. Unlike enzymatic retrieval, HIER is more user-friendly, has a less damaging effect on tissues, and produces consistent results from one experiment to the next (Fig. 5.4). There are several HIER buffers that are frequently used in IHC, including Tris-EDTA at either a pH of 8.0 or 9.0, sodium citrate buffer at a pH of 6, and a solution of just EDTA at a pH of 8. The extent of antigen retrieval depends on the buffer used, and different tissue antigens may require different treatment with different buffers for optimal IHC staining. Usually testing of different HIER solutions is recommended to find the best for one for the antigen of interest. Treatment of tissues with HIER buffers can be done using either an expensive, specially designed chamber with a control panel to set the temperature and monitor the pressure, or with a conventional pressure cooker and/or vegetable steamer commonly used in the kitchen. Usually HIER treatment is done for 5–20 min and the optimal time interval needs to be determined by the investigator.

5.2 Mounting Media

Mounting media is an essential reagent in IHC. After IHC staining is completed, stained tissue sections need to be protected from damage during their examination under the microscope and during storage. Tissue sections are covered with very thin pieces of glass called coverslips that, in addition to providing protection, also serve as an observation window. Before coverslips are placed on the tissue section they must be wet with few drops of mounting media, which serves as a glue for coverslips (Fig. 5.5).

Mounting media protects tissue sections from drying and improves their clarity, which is important for microscopy observation. There are two kinds of mounting media used after chromogenic detection: nonaqueous or organic and aqueous.

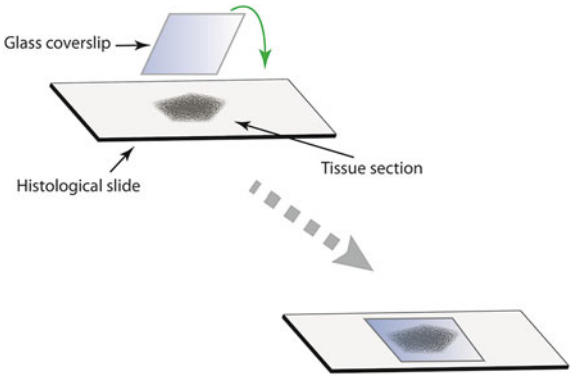

Fig. 5.5 Schematic overview of covering stained tissue sections with glass coverslips

Organic mounting media is used with precipitated dyes that are not soluble in organic solvents, like DAB, whereas aqueous media is used to mount tissues stained with dyes that can be dissolved in organic solvents and lose their color, like AEC. If tissue sections are mounted under coverslips after IHC staining using nonaqueous xylene-based mounting media, they need to be dehydrated using increasing (30–100 %) concentrations of ethanol followed by bathing them in xylene, which is miscible with mounting media: xylene quickly evaporates around the edges of the coverslip and air-exposed mounting media dries, turning into a strong permanent adhesive gluing the coverslip to the slide. When IHC is done using fluorescence detection, a special mounting media is used that contains chemicals to prevent the fading of fluorescent dyes. Mounting media is inexpensive and can be purchased from different commercial sources.

5.3 Summary

Besides antibodies, IHC requires the use of ancillary reagents including buffers, antibody diluents, and mounting media. Buffers are solutions that prevent the pH of the reacting mixture from fluctuating in either the acidic or the alkaline direction. The main buffers used in IHC include wash buffer, block buffer, antibody dilution buffer, and antigen-retrieval buffer. Wash buffer is used to rinse tissues between incubations with antibodies and detection reagents. Permeabilization buffer is recommended for improving penetration of reagents inside the cells for better staining of intracellular targets. Antigen retrieval buffer is used to unmask tissue antigens in chemically fixed tissues. To prevent stained tissue sections from damage and to minimize fading of fluorescence dyes, aqueous or nonaqueous mounting media has to be used in combination with glass coverslips, which create a physical barrier that protects tissue sections.

5.4 Quiz (True or False)

1. Buffer solutions are essential reagents for IHC procedure: **T F**
2. Buffers work by preventing fluctuations of pH toward acidic and alkaline ranges: **T F**
3. Wash buffer is used to permeabilize cells to improve staining of intracellular targets: **T F**
4. Permeabilization buffer is used wash tissue sections between incubations with antibodies and detection reagents: **T F**
5. Antibody diluent is used to make working dilutions of primary but not secondary antibodies: **T F**
6. Block buffer should be used to block nonspecific staining: **T F**
7. Antigen-retrieval buffer is used to prevent the fading of fluorescent dyes: **T F**
8. To prevent stained tissue sections from physical damage, they can be allowed to dry and then just covered with coverslips: **T F**

Chapter 6
Preparing Tissues for IHC Staining

Fresh histological tissues cannot be used "as is" and must undergo special processing to turn them into a suitable experimental material: tissues need to be first chemically fixed and then cut into thin sections using special histological instruments. Fixation and tissue sectioning are critical to the success of IHC staining. If they are not done correctly, staining quality can be badly compromised regardless of the use of high-quality primary and secondary antibodies, buffers, and detection reagents. It is important to note that compromised tissue staining cannot be corrected: such tissues cannot be re-stained, and staining problems cannot be corrected on digital images. That is why it is of critical importance to master the skills necessary for preparing tissue samples acceptable for IHC staining.

6.1 Tissue Fixation

Fixation is the process of treating freshly dissected tissues with chemicals to: (1) preserve their histological integrity/morphology; (2) preserve tissue antigens; and (3) make cells composing the tissue permeable to antibodies and detection reagents. Preserving the morphology of cells composing the tissue means that cells in the tissue retain their natural original shapes and sizes. Morphology preservation is not only important for accurate identification of cells under the microscope, but also for the prevention of cellular deformations that may cause translocation of antigens from the sites of their natural localization to irrelevant cellular and extracellular compartments (e.g., from cell membrane to cytoplasm). Preservation of tissue antigens is needed so they retain their molecular structure for primary antibodies to bind to them: if tissues are under-fixed, protein antigens may be partially destroyed due to proteolytic degradation caused by enzymes localized in intracellular organelles called lysosomes. Such degraded proteins have a reduced capacity for recognition by their antibodies, which results in either weak or no tissue staining. Fixation inhibits lysosomal enzymes and prevents proteolytic

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degradation. Alternatively, if tissues are over-fixed, protein antigens may become masked by the excessive number of fixative molecules, causing both weak non-specific staining and high non-specific background that can obscure specific staining. It is extremely difficult to work out optimal fixation conditions, and researchers generally prefer having mild over-fixation, because over-fixation can be corrected whereas under-fixation cannot. Over-fixed tissues can usually be recovered using antigen-retrieval reagents before starting IHC staining, while under-fixation artifacts can only be seen after IHC staining is completed. Based on their chemical reaction with protein antigens, fixatives can be divided into two groups: (1) cross-linking and (2) precipitating. Cross-linking is a chemical reaction when one molecule of the fixative links two molecules of antigens and stabilizes them. Precipitating fixatives do not cross-link protein antigens, but rather cause their precipitation or the transformation of solubilized proteins (those with a high content of water inside the cells) into insoluble solid aggregates.

6.1.1 Cross-Linking Fixatives

The most common types of fixatives used in IHC include formaldehyde and formalin solutions. Formaldehyde fixative (chemical formula CH_2O) cross-links not only proteins, but also proteins with nucleic acids by forming methylene bridges ($–CH_2–$) (Fig. 6.1). Formaldehyde can link amino (NH_2) groups among themselves as well as with peptide linkage groups (CONH) of different amino acids, which compose proteins. In addition, it can link NH_2 and NH groups. Usually, good fixation can be accomplished using a 2–4 % formaldehyde solution. Another type of fixative is neutral buffered formalin (NBF) solution, which is often referred to as simply formalin. NBF is a formaldehyde fixative that contains about 10–15 % methanol to prevent inactivation of formaldehyde via its polymerization during long-time storage: it is the presence of methanol that makes the difference between formalin and formaldehyde solutions. Authors often define formalin fixative in scientific literature as 10 % FBS, which actually does not mean that this solution is 10 % formaldehyde solution with added methanol. Concentrated stock solution of formalin contains 37 % formaldehyde (i.e., 37 g of formaldehyde per every 100 mLs of liquid), and it must be diluted ten times to make a working solution (defined as 10 % solution). But dilution of a 37 % formalin solution ten times will result in making 3.7 % working solution. It may sound confusing, but 10 % NBF is actually a 3.7 % formaldehyde solution. Formalin is the most common fixative used in human histopathology, while formaldehyde fixative is used in experimental biology to fix tissues of small laboratory animals like mice and rats. Fixation turns histological tissues from very soft, mushy substances into a sturdy material that is much easier to handle without any risk of causing inadvertent damage. Due to the presence of methanol, FBS is more stable for a much longer time than pure formaldehyde fixative, which should be made fresh right before a tissue fixation procedure.

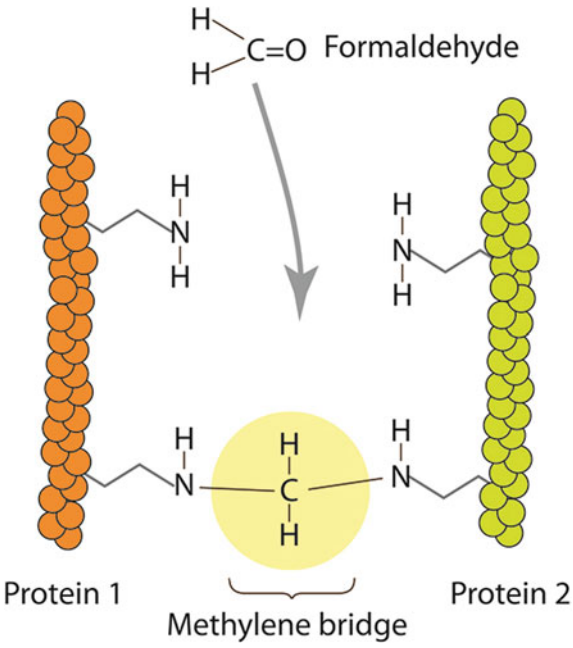

Fig. 6.1 Chemical principle of tissue fixation with formaldehyde. Fixation is based on cross-linking of proteins via the formation of methylene bridges

In addition to formaldehyde-based fixatives, tissues can be also fixed with glutaraldehyde $CH_2(CH_2CHO)_2$ reagent, which also cross-links protein molecules. Glutaraldehyde has a long hydrocarbon chain flexibly connecting two aldehyde groups that cross-link proteins. Glutaraldehyde was first introduced to fix tissues for electron microscopy, for which it is currently in frequent use. It can be used on its own or mixed with formaldehyde. There are some disadvantages of using glutaraldehyde: (1) it introduces free aldehyde groups into fixed tissue, which can react with primary and secondary antibodies and result in high background staining; and (2) due to its strong autofluorescence over a wide spectral range, it is incompatible with fluorescent detection chemistry.

6.1.2 Precipitating Fixatives

The action of precipitating fixatives is based on changing the solubility of proteins that compose histological tissues. Solubility of proteins depends on their electrical charge and the extent of their hydration, which is determined by the amount of water molecules layered around them: proteins will precipitate if they lose their water shell and electrical charge.

Fixatives that preserve protein antigens by precipitating them include alcohols and acetone, which resemble water molecules and therefore can compete with the latter by displacing them. The most common alcohols used by researchers include

methanol (CH_3OH) and ethanol (C_2H_5OH), which replace water molecules bound to proteins and reduce proteins' dielectric constant, thereby forcing them to precipitate. Unlike formalin and formaldehyde, alcohols are not good reagents to preserve intricate tissue morphology and are usually used to fix either specimens with simple morphology (e.g., lymphocytes) or tissues preserved by physical means (e.g., snap frozen tissues). Acetone $(CH_3)_2CO$ works similarly to the actions of alcohols causing precipitation of proteins, and is usually used to post-fix cells and frozen tissue sections that have already been fixed with alcohols. Some precipitating reagents are not used on their own, but as additives to cross-linking fixatives. For example, picric acid ($C_6H_3N_3O_7$) is a very popular reagent that is added to formaldehyde-based fixatives and acts by neutralizing the positive charge of the proteins, thereby causing their precipitation.

Both cross-linking and precipitating fixatives only interact with proteins rather than with lipids or carbohydrates, which undergo physical rather than chemical fixation by being trapped and immobilized between cross-linked and precipitated proteins.

There are two major approaches to treating tissues with fixatives: (1) perfusion fixation and (2) immersion fixation.

6.1.3 Perfusion Fixation

Perfusion fixation is the technique of using an animal's own blood vessels (vasculature) as a natural delivery route for the fixative to penetrate many body organs, and it is done through the animal's heart as it is the hub of major incoming and outgoing blood vessels piercing every organ in the body. Whole body perfusion fixation is usually done on small laboratory animals like rats and mice.

6.1.4 Immersion Fixation

Another fixation approach is just submerging freshly dissected tissue into the fixative. This type of fixation is done on tissues that cannot be fixed by perfusion fixation, including tissues from large animals (e.g., horses, pigs, or dogs) and humans. However, to improve detectability of some protein antigens, tissues that have already been fixed by perfusion can be additionally fixed by immersing them into either the same or a different type of the fixative: this is commonly referred to as post-fixation. Although formaldehyde is a very small molecule that can easily penetrate the tissue, the fixation efficiency depends on the size of the tissue that is immersed into the fixative: the larger the size, the more difficult it becomes for the fixative to reach areas deep inside the tissue. It appears that the optimal size for tissue to be fixed by immersion fixation is not larger than $10 \times 10 \times 3$ mm. Fixation time is also important to ensuring complete fixation, and it usually takes 24–48 h at room temperature to

achieve good fixation of a 10 × 10 × 3 mm piece of tissue. To prevent degradation of tissue antigens via proteolysis, tissues should be immersed in the fixative as soon as possible after dissection from the body and the volume of the fixative solution should exceed that of the tissue to be fixed by 30–100 times. Unfortunately, there is no universal "one-size-fits-all" recipe for tissue fixation because based on their inherent histological nature, different types of tissues require working out optimal fixing conditions such as composition of the fixative, duration of fixation, temperature, and pH of the fixative. For example, a certain fixative may be an excellent choice for preserving tissue morphology but fails to preserve the antigen of interest and vice versa. It does not appear practical to spend time on developing an ideal fixative that excels at preserving both tissue morphology and antigens. It usually makes more sense to find the fixative with maximal antigen-preserving capability and simply adequate preservation of tissue morphology.

6.2 Cutting Tissue Sections

After fixation, tissues need to be cut into thin sections to perform IHC staining. Staining thin sections rather than entire thick piece of fixed tissue is done for several reasons: (a) antibodies and other staining reagents can penetrate thin sections better, and (b) thin sections are optically transparent, which is important for their examination under the microscope. Usually, larger pieces of fixed tissues are cut into sections 5–15 microns thick using a special instrument called a microtome. However, fixed chunks of tissue cannot be taken out of the fixative solution and cut as-is because they are still very soft and the cutting knife will jam them rather than cut. This would be analogous to trying to cut thin slices from a piece of butter that was sitting on a kitchen table for a couple hours at room temperature. To turn tissues into a rigid, easy-to-cut material, tissues can either be frozen or embedded into paraffin similar to that used in making candles. Both animal and human tissues can be cut when frozen or paraffin-embedded, but the majority of IHC experiments on tissues from small laboratory animals like mice and rats is done on frozen sections, while human tissues are generally paraffin-embedded for IHC diagnostics. Advantages and disadvantages of the use of frozen and paraffin-embedded tissues are given in the table below.

Features	Frozen	Paraffin-embedded
Time needed to prepare tissues for cutting	Short. Does not require special instrumentation. No need to dehydrate the tissue	Long. Requires a special, expensive paraffin-embedding station. Because paraffin does not mix with water, the tissue block must first be dehydrated
How easy to make	Easy	Difficult if done manually and easy if using special automated systems

(continued)

(continued)

Features	Frozen	Paraffin-embedded
Preservation of antigens	Very good	Good in general, but some antigens may suffer when tissues are exposed to high temperatures (~60 °C) while embedding into paraffin
Durability of tissue sections	Low. May get damaged during staining and washing IHC steps. May be completely destroyed if using Heat-Induced Epitope Retrieval (HIER) procedure, which requires exposing tissue sections to temperatures of 95–98 °C	High. Tissue sections usually do not lose their integrity when subjected to HIER treatment and can withstand staining and washing conditions
Storage conditions and shelf life of tissue blocks and tissue sections cut from them	Tissue blocks are usually cut right away after being frozen or can be stored in a freezer at −20 to −80 °C for no more than 1–2 weeks before cutting tissue sections. Prolonged storage at low temperatures causes drying of frozen tissue blocks, making them unusable for cutting tissue sections Tissue sections can be stored for no longer than 6–12 months in the freezer. If stored longer, significant degradation of antigens may occur	Tissue blocks and tissue sections cut from them can be stored either at room temperature or in the refrigerator for years with no significant damage to tissue antigens. However, for human IHC diagnostic, pathologists recommend staining freshly cut tissue sections to ensure the highest expression levels of tissue antigens, which is of critical importance for correct diagnosis
Compatibility with fluorescent and chromogenic IHC protocols	Compatible with both	Compatible with both
Treatments required to condition tissue sections for IHC staining	No treatments necessary. Tissue sections can be used right out of the storage box	Tissue sections cannot be used as-is and have to be processed through a clearing procedure to remove paraffin from tissues and to rehydrate them. Clearing step may take 30–40 min

6.2.1 Frozen Tissue Sections

What could be simpler than freezing chunk of tissues? Just put them into the freezer and wait a couple hours until they turn solid. However, freezing tissues for IHC can actually be a tricky process. The critical issue when freezing tissues is to prevent transformation of the water inside cells into ice: ice has a lower density than water, so ice crystals fill a larger volume than water. As a result, ice will expand and causing damage to intracellular organelles and even the cell membrane, grossly affecting cell morphology and translocation of antigens within the cells. Therefore, tissue freezing must be done in such a way as to prevent the formation of ice crystals. One way to accomplish this is to use sucrose ($C_{12}H_{22}O_{11}$) as a cryoprotectant: after finishing perfusion or immersion fixation, tissue is placed for a few days into a 10–20 % sucrose solution. Sucrose displaces water, minimizing the risk of intracellular ice formation. Prior to freezing, tissues are embedded into an Optimal Cutting Temperature (OCT) compound, which helps cut tissue sections adhere to histological slides. Freezing can be done either, by exposing tissue blocks to gaseous CO_2, or by using slurs of dry ice and methanol.

After tissue is frozen, it is ready for cutting sections using a microtome mounted inside a cryostat, a vertical freezer with a top-mounted sliding glass lid and the temperature set between −10 and −20 °C (Figs. 6.2 and 6.3).

Tissue sections are cut into sections 5–15 μm thick and placed onto 1 mm thick 75 × 25 mm histological glass slides (these are the standard dimensions of histological slides and do not change from one vendor to another). Adherence of a cut

Fig. 6.2 Example of a cryostat manufactured by the company Leica, which is used to cut frozen tissue sections. The temperature inside the chamber can be set using controls on the front panel of the instrument. The rotary handle on the right side of the cryostat is connected to the microtome inside: by rotating the handle clockwise, the tissue block on the microtome moves against the blade, which cuts a tissue section from the frozen tissue block

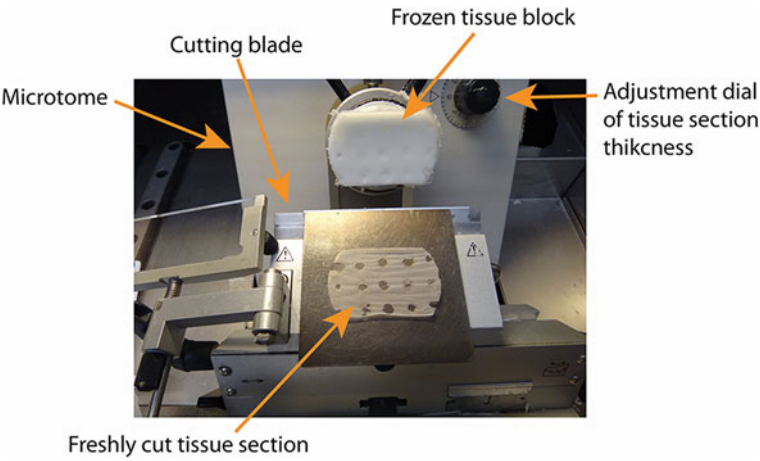

Fig. 6.3 View of the inner chamber of a Leica cryostat with a microtome holding a frozen tissue block. A tissue section is cut when the rotary handle moves the tissue block downward so that it goes against a steady cutting blade. When the tissue block is moved upward, the microtome also advances it forward so that another tissue section can be cut as it travels downward. The distance of the forward advancement of the tissue block is set in microns using the adjustment dial: the higher the number, the longer the advancing step and the thicker the tissue section will be cut

Fig. 6.4 Typical storage box containing histological slides with tissue sections. Boxes come in different sizes to store 25, 50, or as shown in the picture, 100 slides

tissue section to the slide occurs by heating its opposite side either with the warm metal roll bar or simply by touching it with the finger: the frozen tissue section and the OCT in which it is embedded melts down, making the tissue section firmly "glued" to the slide.

This operation is called "thaw-mounting" of frozen tissue sections.

Tissue sections mounted onto slides have to be placed on a slide warmer at 37 °C for 20–30 min to completely dry. After drying, slides with tissue sections can either be used right away for IHC staining or placed into a special slide box for storage (Fig. 6.4). Usually, a desiccant like silica gel in small packets or capsules is placed inside the box to minimize moisture condensation on tissue sections and prevent deterioration of tissue antigens.

Boxes with slides are stored in a freezer (−20 to −80 °C) and before starting IHC staining they are brought to the lab and should be kept unopened for 20–30 min to adjust to room temperature: if the box is opened right after being taken from the freezer, water condensate will accumulate on tissue sections damaging them.

6.2.2 Paraffin-Embedded Tissue Sections

Paraffin-embedding of tissues is usually done using a special instrument. It is not recommended to do it manually, because this is a long (∼12 h) procedure requiring the use of such volatile organic chemicals as alcohol and xylene while precisely maintaining the temperature at 65–70 °C to keep paraffin wax in melted, liquid condition. Paraffin has a melting point at about 56–60 °C, but because paraffin is quite viscous at this temperature the use of higher temperatures is recommended to keep paraffin in liquid condition. After fixation, tissues cannot be embedded right away into paraffin because they contain water, which is not mixable with paraffin wax. Therefore, the water must be replaced with chemicals miscible with paraffin. This is a multistep process that is started with tissue dehydration using increasing (30–100 %) concentrations of ethanol (which mixes well with water), followed by replacing 100 % ethanol with xylene (which mixes well with ethanol). Then, xylene can be replaced with paraffin, which infiltrates the tissue all the way through. After that, chunks of tissues infiltrated with paraffin are embedded into paraffin blocks attached to special plastic cassettes that can be attached to the microtome (Fig. 6.5).

Cutting of paraffin-embedded tissues is similar to cutting frozen tissue, but mounting the cut tissues on a slide is quite different. Freshly cut paraffin-embedded sections are first put into a water bath with warm water (37 °C), and because paraffin is lighter than water, sections float to the surface and are stretched by surface tension, making them perfectly flat for adhesion to the slides. Floating tissue sections are mounted onto histological slides by immersing slides at an angle into the water underneath the tissue sections and lifting them up so that the tissue sections stick to the slide. After that, slides with adhering tissue sections are placed on a hot plate (37–40 °C) for about an hour. Drying increases the adhesion of tissue

Fig. 6.5 Image of the typical paraffin-embedded tissue block

sections to the slides, which is important for preventing the detachment of tissue sections that would be lost during the IHC procedure (Fig. 6.6).

Unlike frozen tissue sections, paraffin sections cannot be used as-is for IHC staining and must be processed to make them suitable for staining. This processing is done in the reverse of the paraffin-embedding procedure: tissue sections need to be cleared of paraffin and rehydrated so that primary antibodies can penetrate the tissue section and interact with their targets. This is done by bathing slides holding tissue sections in xylene, followed by rehydrating the tissues using decreasing (100, 95, 70 and 50 %) concentrations of ethanol, and finally transferring them into either distilled water or a buffer like PBS.

For Paraffin-Embedded Tissue Sections:

(a) Clear slides in xylene: 3 times for 5 min each.
(b) Immerse slides into 100 % alcohol: 3 times for 5 min each.
(c) Immerse slides into 95 % alcohol: 2 times for 5 min each.

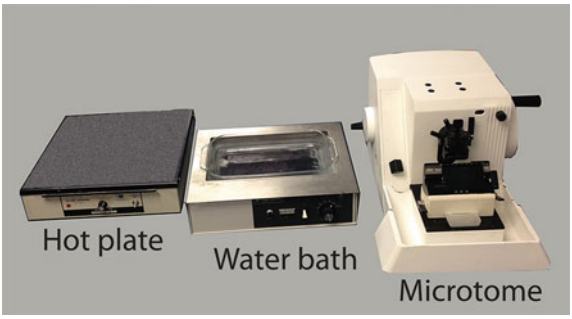

Fig. 6.6 Commonly used setup for cutting paraffin-embedded tissue sections. After tissue sections are cut on the microtome, they are transferred to the water bath. Floating tissue sections in the water bath are mounted onto histological slides and placed on the hot plate for drying. The microtome for cutting paraffin-embedded tissue sections is operated similarly to that in the cryostat for cutting frozen tissue sections

(d) Immerse slides into 70 % alcohol: 5 min.
(e) Immerse slides into 50 % alcohol: 5 min.
(f) Transfer slides into distilled water.
(g) Transfer slides into buffer.

After these steps are complete, slides are ready for IHC staining.

6.3 Summary

Freshly dissected tissues cannot be used for IHC and need to be fixed with chemical fixatives to preserve tissue morphology and tissue antigens. Fixation turns tissues into a rigid material that can be easily cut into 5–15 micron sections. Cross-linking and precipitating fixatives are the major fixing reagents of tissues for IHC. The most commonly used cross-linking fixative solutions include formaldehyde, formalin, and glutaraldehyde, and the most commonly used precipitating fixatives are alcohol and acetone. Tissue fixation can be accomplished either by perfusion or immersion fixation. After fixation, tissues can be either frozen in OCT or embedded into paraffin wax. The shelf life of frozen uncut tissues is typically 1–2 weeks at −20 to −80 °C, whereas paraffin-embedded tissues can be stored for years at room temperature. Paraffin-embedded tissue sections are cut at room temperature, whereas frozen sections are cut at −10 to −20 °C using a cryostat. Frozen tissue sections can be used for IHC staining as-is, whereas paraffin-embedded ones need to be cleared in xylene, rehydrated by treating them with decreasing concentrations of alcohol, and transferred into either distilled water or a buffer solution.

6.4 Quiz (True or False)

1. Freshly dissected tissues need to fixed before using them for IHC: **T F**
2. Precipitating fixatives cross-link tissue proteins: **T F**
3. The commonly used cross-linking fixatives include formaldehyde and acetone: **T F**
4. Tissue fixation is done to preserve tissue morphology and tissue antigens: **T F**
5. Embedding tissues into paraffin is much simpler and shorter than freezing tissues: **T F**
6. Frozen tissue sections are cut using cryostat at sub-zero temperatures: **T F**
7. Paraffin-embedded tissue sections are cut at 60 °C: **T F**
8. Paraffin sections are usually more sturdy than frozen ones: **T F**
9. Before starting IHC staining, paraffin tissue sections need to be cleared of paraffin wax and rehydrated: **T F**

Chapter 7
Staining Protocols

Due to the inherent nature of different types of histological tissues composing the body as well as the complexity of tissue antigens, there are no universal "one-size-fits-all" staining protocols in IHC: the approach that works for a particular type of tissue and "to-be detected" antigens may be completely ineffective for other types of tissue and antigens. That is why the most critical step in IHC is the optimization of a staining protocol allowing for the best detection of tissue antigens. Usually, the first step in designing an IHC experiment is determining which antigen detection strategy should be used: fluorescence or chromogenic. As soon as this is done, basic IHC protocol is taken as the foundation and tweaked to suit the experimental needs. To save reagents, slides with tissue sections are stained in a horizontal position with tissue sections facing up: unlike incubating slides vertically in a jar requiring 50 mLs or more of IHC reagents, staining them horizontally requires only 50–200 microliters per slide.

Generally, a workflow of IHC staining looks like this:

a. *Hydrophobic tissue protection*: Using a special pen (called a PapPen), a hydrophobic barrier line is drawn around the tissue section to prevent leakage of reagents from the slide during incubation;
b. *Blocking*: Tissue sections are treated with reagents to block endogenous sources of nonspecific staining such as (i) enzymes, (ii) endogenous peroxidase, (iii) free aldehyde groups, (iv) immunoglobulins, and other irrelevant molecules that can mimic specific staining;
c. *Permeabilization (this step is not mandatory and only used as needed)*: Tissue sections are incubated with permeabilization buffer to facilitate penetration of antibodies and other staining reagents into the tissue;
d. *Incubation with primary (often depicted as 1°Ab) antibody*: This incubation is done for 1–24 h either at room temperature or in a cold room at 6–8 °C depending on the affinity of antibodies and abundance of tissue target;

A.E. Kalyuzhny, *Immunohistochemistry*, Techniques in Life Science and Biomedicine for the Non-Expert, DOI 10.1007/978-3-319-30893-7_7

e. *Rinsing with wash buffer*: This step is usually done as 3–5 short, 5–15 min repetitive cycles using fresh wash buffer and, for example, can be depicted in the scientific literature as "3 × 15 min washes";
f. *Incubation with secondary (may be depicted as 2°Ab) antibody*: This is usually done for 1–2 h at room temperature;
g. *Rinsing with wash buffer*: This is a repeat of step "e"; and
h. *Incubations with detection reagents, mingled with rinsing with wash buffer*: This is done when applicable (for example, in chromogenic detection).

Let us take a look at basic IHC protocols and discuss their advantages and limitations.

7.1 Fluorescence Protocols

Fluorescence (also frequently referred to as immunofluorescence) detection is a widely used IHC technique that can be either direct or indirect: direct indicates that primary antibodies are labeled with fluorescent probes and implies that there is no need to use fluorescent secondary antibodies, while indirect means that primary antibodies are unlabeled and secondary antibodies conjugated to fluorescent dyes have to be used for detection. Which technique is better, direct or indirect? There is no simple answer to this question as each technique has its advantages and limitations, which are discussed below.

7.1.1 Direct Fluorescence Detection

Direct detection was developed by Dr. Coons and is considered as the ancestor of all other fluorescence protocols used in IHC. It is the fastest and shortest IHC protocol, requiring incubation of tissue sections with only a primary antibody conjugated to the fluorophore of choice.

The advantage of direct fluorescence detection is that tissue staining can be obtained quickly.

Unfortunately, this technique sometimes does not work because after the conjugation of fluorescent dye to the primary antibody, its antigen-recognition capacity may be either impaired or lost, which results in weak or no IHC staining. That is why direct detection is better suited for the detection of strong, highly expressed tissue antigens. Direct detection is the technique of choice when, due to the host species of the primary antibodies and the histological nature of tissue, use of secondary detection antibodies may cause strong nonspecific staining. For example, a researcher has to use primary mouse monoclonal antibodies for staining mouse

lymph nodes because anti-mouse secondary antibodies will not only interact with mouse primary antibodies bound to the tissue target, but also to endogenous immunoglobulins abundantly present in the lymph node. This would result in strong nonspecific staining, and therefore the better option would be to employ a direct detection technique using mouse primary antibodies conjugated to a fluorophore.

7.1.2 Indirect Fluorescent Detection

Indirect detection is more sensitive than direct, which is why it is the more popular technique. The higher sensitivity of indirect detection is the result of the possibility of two secondary antibodies labeled with fluorophores interacting with a single molecule of primary antibody bound to its tissue target.

Another advantage of indirect detection is the ability to choose secondary antibodies with fluorophores of different colors, Stokes shifts, quantum yield, and fade resistance. For example, if the tissue has a strong endogenous red autofluorescence, the researcher can use secondary antibodies conjugated to a fluorophore emitting green light. The major disadvantage of indirect detection is the risk of nonspecific staining due to the interaction of secondary antibodies with irrelevant tissue targets: if nonspecific staining is observed, tissue sections have to be treated with blocking solutions, thereby adding time and expense to the IHC experiment. Usually it is assumed that secondary antibodies have a tendency to bind nonspecifically, and therefore incubation with blocking buffers is generally done before applying primary antibodies.

7.1.3 Multi-Color Fluorescence Detection

Fluorescence detection allows for simultaneous detection of multiple overlapping and nonoverlapping tissue targets. Multicolor detection can be done using either direct or indirect detection, or a combination of both techniques (Fig. 7.1).

Usually in doing multicolor fluorescence IHC researchers use primary antibodies raised in different host species to avoid cross-reactivity of secondary antibodies with irrelevant primary antibodies.

Unfortunately, quite often the selection of the hosts of primary antibodies is limited and the researcher has only primary antibodies raised in the same host species. One approach to avoid cross-reactivity of secondary antibodies with primary antibodies is to modify the primary antibodies by conjugating them to Biotin and Digoxigenin followed by detection using Streptavidin and anti-Digoxigenin antibodies conjugated to fluorescent dyes, respectively. There are other approaches

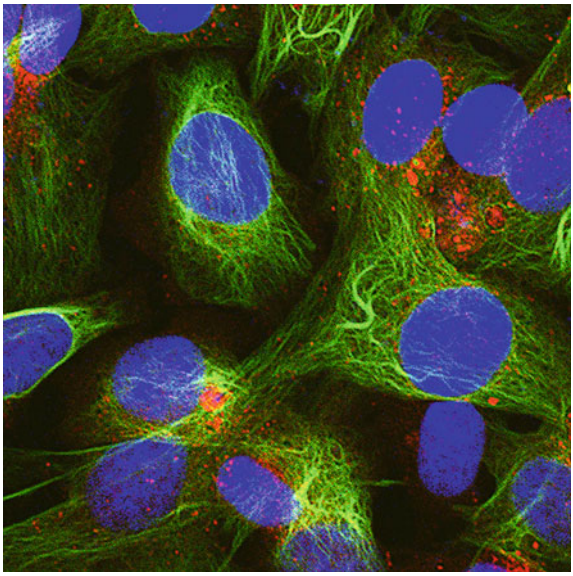

Fig. 7.1 Example of 3-color fluorescence IHC image. Nuclei were labeled *blue*, the cytoskeletal network *green*, and the cytoplasmic proteins *red*

for using primary antibodies raised in the same host species for multicolor fluorescence as well, but they are quite complicated and involve advanced IHC techniques detailed in the references listed at the end of this book.

7.1.4 Fluorescent Counterstaining

Counterstaining is the staining of tissue sections with dyes that allow one to see the entire "landscape" of the tissue section and serve as a reference for the main color used for the detection of tissue targets. Such dyes can stain cell nuclei, the cell membrane, or the entire cell. The key to tissue counterstaining is choosing a counterstain color that is distinctly different from the main, antigen-detecting color. Most often, researchers counterstain cell nuclei using these dyes:

a. DAPI, which binds to nuclear DNA and emits strong blue light;
b. Hoechst blue stain, which binds to nuclear DNA and emits strong blue light; and
c. Propidium iodide, which binds to nuclear DNA and emits strong red light.

Counterstaining of the intracellular cytoskeletal network can be done using Phalloidin conjugated to fluorescent dyes. Phalloidin is a toxin that tightly binds to Actin filaments in a cell's cytoplasm, which then become clearly visible under the microscope (Fig. 7.2).

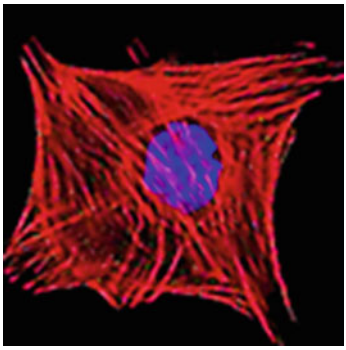

Fig. 7.2 Cell counterstained with Phalloidin conjugated to red fluorophore (labeling is in cytoskeletal filaments) and with DAPI (labeling is in the cell nucleus—*blue* color)

7.2 Chromogenic Protocols

The majority of chromogenic IHC protocols are based on the use of Avidin and Biotin molecules because detection sensitivity of a simple antigen-antibody reaction in many cases is quite low. It has been found that Avidin, which is egg white glycoprotein, has a strong binding capacity to Biotin (also known as vitamin B7, which plays an important role in maintaining healthy skin and hair). Avidin-Biotin binding serves to bridge antigen-bound antibodies with detection reagents, allowing amplification of the staining signal. Unfortunately, due to being heavily glycosylated, Avidin has a tendency to bind to tissues nonspecifically and replaced by Streptavidin (frequently abbreviated as SA) from bacterium *Streptomyces avidinii*, which is not glycosylated and therefore does not bind to tissues nonspecifically. Besides strong interactions, each molecule of Avidin and its sibling SA can bind four molecules of Biotin, which is the chemical nature of signal amplification due to the formation of large multimolecular enzyme-containing complexes. The most frequently used Biotin-based techniques include labeled SA-Biotin (LSAB) and Avidin-Biotin Complex (ABC) detection. There are also non-Biotin-based detection techniques utilizing primary antibodies either conjugated directly to enzymatic labels or to a long polymer containing multiple copies of enzymatic labels.

7.2.1 LSAB Detection

This detection technique utilizes secondary antibodies conjugated to Biotin that link primary antigen-bound antibodies to SA conjugated to an enzyme. The first step in LSAB detection is the incubation of tissue sections with primary antibodies followed by incubation with biotinylated secondary antibodies. After that, SA conjugated to the enzyme of choice (e.g., AP, HRP, etc.) is added to tissue sections followed by adding appropriate enzyme substrate. The enzyme converts substrate into colored particles precipitating at the sites of antigen localization, which can

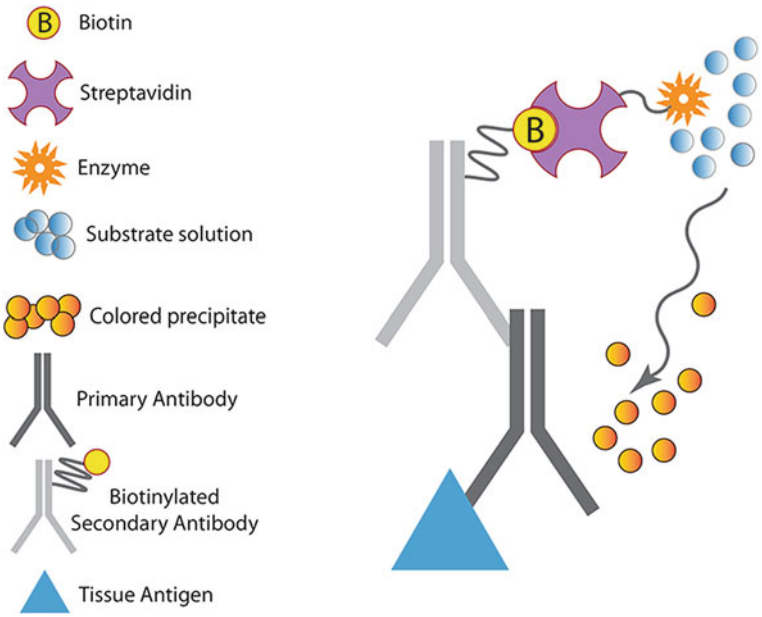

Fig. 7.3 Detection using Labeled Streptavidin Avidin Biotin (LSAB) chemistry

then be observed under the microscope. LSAB technique can be shortened using biotinylated primary antibodies, eliminating the need for incubation with biotinylated secondary antibodies (Fig. 7.3).

7.2.2 ABC Detection

The initial steps—incubation with primary and biotinylated secondary antibodies—in ABC detection are the same as in LSAB, but the next steps and reagents are quite different. Avidin and biotinylated enzymes are first mixed and incubated together for about 30 min at room temperature and then added to tissue sections. During this incubation, Avidin interacts with the biotinylated enzymes, forming large complexes densely packed with enzyme molecules—far exceeding the concentration found in the LSAB detection technique—that boost the sensitivity of antigen detection. The critical drawback of ABC technique is that the large size of these formed complexes impairs their ability to penetrate inside the cells, which reduces detection sensitivity. Like LSAB, ABC protocol can be shortened using biotinylated primary antibodies (Fig. 7.4).

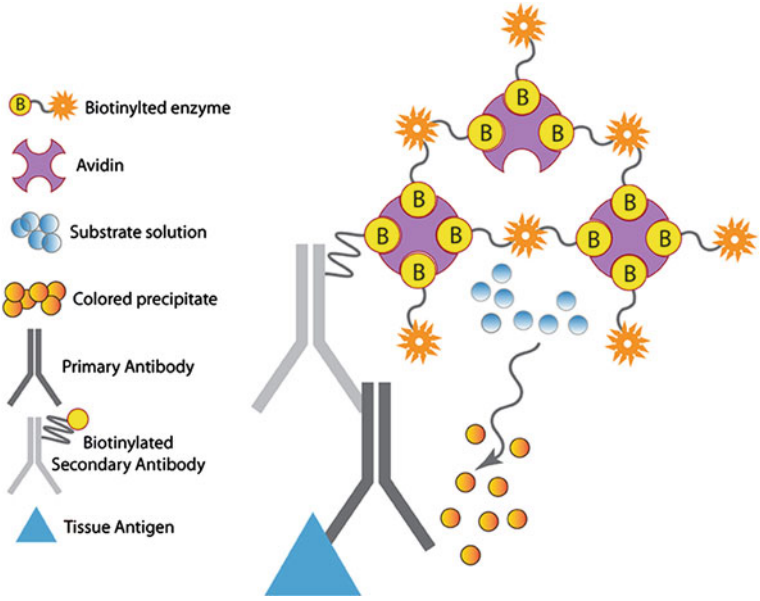

Fig. 7.4 Detection using Avidin-Biotin Complex (ABC) formation

7.2.3 Non-Biotin-Based Detection

Non-Biotin detection techniques have gained popularity because they are devoid of such limitations of Avidin-Biotin detection as nonspecific background staining due to the endogenous biotin that is abundant in different types of animal tissues, including kidney, brain, and placenta.

Non-Biotin protocols are shorter and simpler because they do not require the blocking of endogenous biotin or incubation of tissue sections with biotinylated secondary antibodies and either Avidin or SA enzyme conjugates. Detection can be done either using primary/secondary antibodies conjugated directly to enzymes like AP or HRP, or by employing polymer-enzyme chemistry, which offers much stronger sensitivity compared to direct enzyme conjugates of antibodies and LSAB and ABC detection (Fig. 7.5).

7.2.4 Counterstaining in Chromogenic Detection

In chromogenic IHC, tissue counterstaining serves the same purpose as it does in fluorescence detection: to visualize the entire layout of the tissue section and label organelles of the same type. Usually counterstaining is done to label cell nuclei that should not be of the same color as the main color depicting antigens of interest. For example, if the main color is red (AEC chromogen) or brown (DAB chromogen),

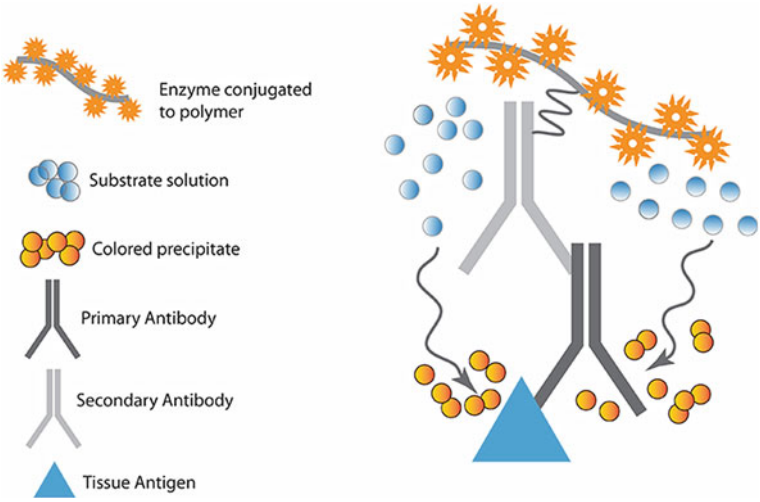

Fig. 7.5 Detection using enzyme-conjugated polymer linked to secondary antibody. A polymer packed with a large number of enzyme molecules is attached to secondary antibodies. The high density of the enzyme results in fast conversion of the substrate into a dense colored precipitate marking the sites of antigen localization. The polymer complex is small, which allows good intracellular penetration and increases the detection sensitivity

nuclei may be stained using Hematoxylin, which produces a blue color, or Methyl Green, which produces a green color. If the main color is blue (BCIP/NBT chromogen), then nuclei may be counterstained red using Nuclear Fast Red dye. In cases when tissue antigen is localized in cell nuclei, the duration of their counterstaining may be either shortened to make them barely visible or even skipped to avoid masking the main IHC color.

7.3 IHC Controls

Running controls side-by-side with stained tissue sections is a critical part of IHC experiments. The ultimate purpose of IHC controls is to confirm the specificity and relevance of detected tissue antigens. Usually, it is recommended to perform (i) tissue antigen, (ii) endogenous tissue condition, and (iii) reagents specificity controls.

7.3.1 Tissue Antigen Controls

There are two types of tissue antigen controls: positive and negative. Positive tissue control is running IHC staining on tissues that are known to express the antigen of

interest, whereas negative tissue control is staining tissues known to lack the antigen. Positive controls are a must when investigating the expression of a known antigen in a new type of tissue. For example, when studying the localization of antigen "X" in the brain, which is known to be expressed in the liver, the latter will serve as a positive control. But if antigen "X" is known not to be expressed in the kidney, then kidney tissue sections can be used as a negative control. Seeing staining in a positive control and a lack of staining in a negative control indicates that the primary antibodies are specific and therefore suitable for IHC experiments. However, if negative control tissue becomes stained, this can indicate that either primary antibodies are nonspecific or further optimization of staining protocol is required.

7.3.2 Endogenous Tissue Condition Controls

Endogenous tissue condition controls need to be done to ensure that there are no endogenous substances in the tissue that can either obscure or mimic specific staining. Usually these controls are essential for fluorescence but not for chromogenic enzymatic detection, because it is important to make sure that after IHC staining is complete, fluorescent staining observed under the microscope depicts targets of interest rather than artefacts. Nonspecific staining may be due to endogenous tissue autofluorescence, nonspecific binding of secondary antibodies, or cross-reactivity of primary antibodies with irrelevant tissue targets.

Special IHC controls need to be employed to rule out the interference of nonspecific staining with specific staining. For example, endogenous tissue autofluorescence can be easily ascertained by analyzing unstained tissue sections under the fluorescence microscope. If a tissue's autofluorescence has a green color, the use of fluorescent labels other than green is recommended. It is always advisable to quench endogenous autofluorescence using treatment strategies tailored to its nature. Autofluorescence may be inherent to a particular type of tissue or it can be induced by tissue processing. Tissues may naturally autofluoresce due to the presence of organic compounds called porphyrins (which are abundant, for example, in red blood cells) and flavins (a component of vitamin B_2 or Riboflavin involved in energy production and the oxidation of fatty acids). Porphyrins and flavins are not stable in organic solvents and therefore do not represent a serious problem when doing IHC on paraffin-embedded tissue sections processed using xylene and alcohol. The presence of Porphyrins and Flavins is more of an issue when staining frozen tissue sections, which do not require organic solvents for their processing. Another compound with a strong autofluorescence is Lipofuscin, nicknamed an "aging pigment," which is a product of degraded cellular material accumulated in cytoplasmic organelles lysosomes. The biggest problem with Lipofuscin is that it can be excited by the light within a wide range (360–647 nm), emitting orange, green, yellow, and red light that can resemble fluorescent signals emitted by the most frequently used fluorophores conjugated to primary and

secondary antibodies. One efficient anti-Lipofuscin remedy is the use of Sudan Black B, a histological dye that binds to lipids in lysosomes to mask them: after finishing IHC staining, tissue sections are incubated in a solution of Sudan Black B, which blocks Lipofuscin fluorescence but does not affect specific fluorescent signals emitted by specific probes. Unfortunately, in some tissues treated with Sudan Black B the level of endogenous autofluorescence remains strong, and therefore the only option left would be to switch to chromogenic detection.

In addition to natural occurrence, autofluorescence can be also induced by tissue processing. For example, fixation with formaldehyde and glutaraldehyde fixatives, in addition to cross-linking proteins, leads to formation of free aldehyde groups that can cause autofluorescence. In order to block free aldehyde groups, tissue sections can be treated with sodium borohydrate, which converts CHO aldehyde groups to OH hydroxyl groups. Another way of blocking free aldehyde groups is to treat tissues sections with reagents containing NH_2 amino groups such as the amino acids glycine and lysine: the chemical reaction between NH_2 amino and CHO aldehyde groups leads to the reduction of the number of free aldehyde groups.

To check whether staining is produced by the nonspecific binding of fluorescent secondary antibodies to tissue sections, instead of incubating tissue sections with primary antibodies it is recommended to incubate just with antibody diluent lacking primary antibodies followed by incubation with secondary antibodies. If it turns out that secondary antibodies bind to tissue sections nonspecifically, then it is recommended to optimize their working dilution and also prolong the incubation of tissue sections with blocking buffers. If none of these solutions help, then it is advisable to switch vendors of secondary antibodies.

Although it is tempting to run endogenous peroxidase and enzyme controls in advance of a chromogenic detection IHC experiment, they may be skipped because it is assumed that tissues have such problems and therefore it is more practical to simply employ peroxidase and enzyme blocking steps.

7.3.3 Reagents Controls

In order to make sure that primary antibodies do not cross-react with irrelevant tissue targets, it is recommended to employ what is called an absorption control. Primary antibodies are mixed with the immunogen that was used to immunize host animals. It is expected that the binding of immunogen to antibodies will neutralize, or absorb them and adding such absorbed primary antibodies to tissue should not generate any specific staining. If staining is produced by immunogen-absorbed primary antibodies, then such staining is most likely nonspecific. If optimization of the working dilution of primary antibodies does not help, then it may be recommended to use a different antibody (made in-house or by a different commercial vendor) against the same tissue target.

When using primary monoclonal antibodies, it is recommended to run an isotype control by incubating tissue sections with nonimmune antibodies of the same

isotype as the primary antibodies (e.g., IgG1, IgG2b, or IgG2a). If there is staining in the isotype control, then it may be necessary to find antibodies of a different isotype that do not bind nonspecifically to tissue sections.

7.4 Selection and Optimizaion of Primary Antibodies

When planning IHC experiments, one the most frequently questions asked is that of which primary antibodies to use: polyclonal or monoclonal? As we already know, monoclonal antibodies are made by a single B-cell clone and therefore represent a homogeneous population of antibodies that have the same affinity and specificity toward a single epitope of the tissue antigen. Such consistency is particularly useful when the purpose of an IHC experiment is the detection of a single member of a large protein family when its other members have similar amino acid sequences. However, due to tissue fixation, the conformation of the target protein may change in such a way that its antibody-interacting epitope may become inaccessible to the antibody, which results in a lack of tissue staining. Unlike monoclonal, polyclonal antibodies are the mixture of antibodies produced by different B-cell clones that can bind to multiple different epitopes: it is unlikely that all epitopes become inaccessible during the changes in target protein conformation. In other words, "back-up" epitopes always remain that are recognized by polyclonal antibodies. Because of this, polyclonal primary antibodies are thought to be a better choice for IHC experiments.

Primary antibodies are the most critical IHC reagents, and their optimization should be the first step in developing IHC protocol. Optimization usually means determining the appropriate concentration of primary antibodies, their incubation time with tissue sections, and the incubation temperature. To find the optimal concentration, tissue sections are usually incubated with three-fold serial working dilutions of primary antibodies starting with the highest concentration. For example, the highest concentration of polyclonal antibodies in IHC does not exceed 10–20 μg/mL and for monoclonal antibodies, it does not exceed 20–30 μg/mL. For immune serum, the highest antibody concentration is usually obtained when it is diluted 1:100. Serial working dilutions of polyclonal antibodies may be, for example, as follows: 18 , 6, 2, and 0.7 μg/mL, and for immune serum – 1:100, 1:300, 1:900, 1:2,700. When doing IHC with a new antibody for the first time, it is recommended to do a pilot study that tests a broad range of antibody concentrations. The purpose of this task is to determine the concentration that produces the strongest specific and most minimal nonspecific, or background, staining. As a common starting point in determining incubation time and temperature, it is recommended first to run overnight incubations at a low (4–8 °C) rather than room temperature. If the specific signal is strong and there is no background staining, then incubation time may be shortened to 2–3 h and done at room temperature. When comparing tissue sections stained with different antibody concentrations, it is critical to use a consistent time and temperature for the incubation step.

Antibody optimization is tissue dependent, which means that the optimal conditions established for one type of tissue (e.g., brain) cannot be blindly applied when staining another type of tissue (e.g., liver): antibodies will need to be re-optimized. Re-optimization may also be required when staining tissue of the same type that is sectioned at different thicknesses. For example, in staining thicker, 15 um tissue sections, the concentration of primary antibodies needs to be higher than with Sects. 5 um thick in order to allow the primary antibodies to better penetrate and interact with tissue targets localized deeper in the tissue section. The incubation time for thicker sections may need to be longer than for thinner sections. Primary antibodies raised against the same tissue antigens in the same host species (e.g., rabbit, mouse, goat, etc.) may be supplied by different vendors or by the same vendor but made in different host species (for example, rabbit antihuman Insulin versus mouse antihuman Insulin). Although it is tempting to assume that such antibodies are the same based on their antigen specificity, they may, and in many instances do, have different affinities to the same target: high-affinity antibodies require a shorter incubation time and working dilutions of a lower concentration, whereas low-affinity antibodies require higher concentrations and a longer incubation time. In other words, antibodies against the same targets originating from different sources (e.g., suppliers or/and host species) need to be treated as completely different antibodies requiring optimization.

7.5 Summary

Commonly, basic IHC protocol is used as a foundation for designing a specific IHC protocol to suit experimental needs. Incubation of tissue sections with antibodies and staining reagents is alternated with buffer washes to avoid cross-contamination of IHC solution. Direct fluorescence IHC detection is less sensitive than indirect detection, but the latter protocol is longer and more expensive. Direct fluorescence detection can be combined with indirect when doing multicolor IHC detection. Most chromogenic IHC protocols utilize Avidin-Biotin detection chemistry such as LSAB and ABC techniques. Non-biotin, HRP polymer detection chemistry is gaining popularity due to its high sensitivity and the lack of interference from tissue endogenous biotin. In addition to the main IHC staining, tissues are also counterstained to allow researchers better orientation when analyzing stained tissue sections under the microscope. IHC controls are an essential part of staining protocols, which must be done to rule out artefacts that can mimic specific staining signals. Primary antibodies are critical IHC reagents, which must be optimized to obtain a strong specific signal with as little background staining as possible. Primary polyclonal antibodies are preferred over monoclonal for IHC staining.

7.6 Quiz (True or False)

1. There are no universal "one-size-fits-all" staining IHC protocols: **T F**
2. To save staining reagents, slides with tissue sections are incubated vertically in jars: **T F**
3. Direct and indirect fluorescence detection can be combined in a single IHC protocol: **T F**
4. The majority of chromogenic IHC protocols are based on Avidin-Biotin detection chemistry: **T F**
5. LSAB is the abbreviation of "Low Sensitivity Avidin Binding": **T F**
6. Biotin-free HRP polymer detection chemistry does not have the advantage over Avidin-Biotin detection: **T F**
7. Tissue counterstaining is done to enhance nonspecific staining: **T F**
8. IHC controls are essential to every IHC experiment: **T F**
9. Background staining can occur as a result of endogenous tissue conditions or it can be introduced by tissue processing: **T F**
10. Primary antibody optimization is only required when doing fluorescence detection IHC: **T F**
11. Primary polyclonal antibodies are generally preferred over monoclonal for IHC: **T F**

Chapter 8
Microscopy Examination and Collection of Digital Images and Their Presentation

After finishing an IHC experiment, stained tissue sections need to be examined under the microscope and images are commonly captured using the camera attached to the microscope. Due to enormous progress in digital photography, capturing IHC images is no different than taking pictures of friends using dedicated "point-and-shoot" cameras or cell phones. Although taking digital IHC images appears to be simple and easy, it is important to bear in mind that digital cameras are inanimate and cannot choose which part of a stained image to show and what magnification should be used for the best illustration of antigen distribution. These choices can be determined only by the researcher. This, in turn, requires the researcher to know how to operate a microscope and follow certain guidelines in choosing the best tissue layout and orientation to be shown in captured IHC images.

8.1 Operating a Bright-Field Microscope and Collecting IHC Images

If primary antibodies are the critical IHC reagents, a microscope is the most important and most frequently used IHC instrument. It generally takes some time for novices to learn how to operate even a simple laboratory microscope. The main reason for using a microscope is the ability to analyze stained tissues under high magnification: conventional bright-field microscopes provide up to 1000X magnification, which is sufficient for distinguishing individual cells ranging in size from 10 to 50 μ. Below is an overview of a typical bright-field laboratory microscope equipped with a digital camera connected to a computer (Fig. 8.1).

Microscopes commonly come with 10X eyepieces and a set of objective lenses of low (2X, 4X, and 10X) and high (20X, 40X, 60X, and 100X) magnification. Objective lenses are very expensive, and therefore it is not unusual to see microscopes with only the 2–3 lenses of different powers needed most by the researcher

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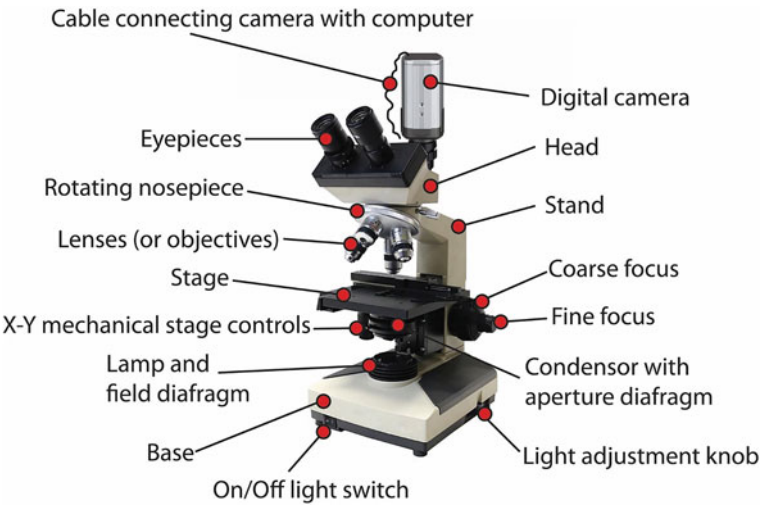

Fig. 8.1 Typical bright-field laboratory microscope and its components

who does not want to spend money on lenses solely for the sake of having the entire set. To calculate the total magnification, it is necessary to multiply the magnification of the eyepieces by that of the objective lens. For example, when using a 40X lens, the total magnification is 10X (eyepieces) × 40X (lens) = 400X. Examination of stained tissues usually starts by focusing using low-power objective lenses, such as 4X or 10X, and then rotating the nosepiece to set a higher magnification objective. Objectives are parfocal, which means that when they are changed from low to high magnification the examined sample remains largely in focus, requiring only subtle adjustments with the fine focus knob. When rotated, coarse and fine focusing knobs move the stage holding the slide up and down. Although microscopes are built by different manufacturers, they have at least one constructive feature in common: one 360° rotation of the fine focus knob moves the stage 0.2 mm up or down depending on the clockwise or counter-clockwise rotation. Objective lenses are perfectly centered: if the region of the sample is positioned in the center of the field of view for one lens, it will remain in the center when switching to another lens by rotating the nosepiece.

Objective lenses are the most critical components of a microscope: they collect and focus the light that passes through the stained tissue section and project the image to either eyepieces or a digital camera, allowing visualization and recording of the finest localization and morphological details of labeled antigens. Objective lenses are mounted into a rotating nosepiece, which allows quick changes in magnification by simply turning it left or right.

Objective lenses have numbers engraved on their metal casings that provide important technical information to the user.

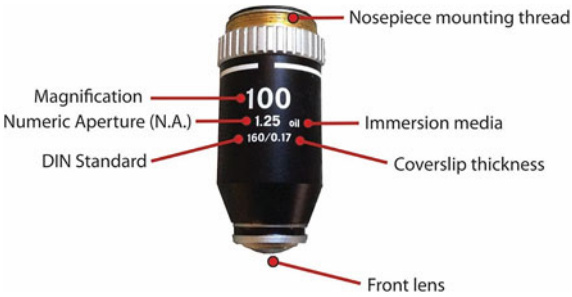

Fig. 8.2 Objective lens with engravings on its casing

For example, the objective lens shown in Fig. 8.2 has 100X magnification and should be used with a special immersion oil as an intermediary medium between its front lens and the coverslip mounted on top of a stained tissue section. This lens is corrected for coverslips that have a thickness up to 0.17 mm DIN, which is the abbreviation of "Deutsche Industrie Norm," and states that the distance from the objective lens flange to the eyepiece flange is 160 mm. There is also a Numerical Aperture (N.A.) number, which is calculated using a mathematical formula based on the relationship between the angular aperture of the lens and the refraction index of the immersion medium that goes between the front lens and the surface of the stained tissue. Importantly, N.A. is a measure of the resolving power of the objective lens, which is defined in microscopy as the ability to distinguish between two points located in close proximity to each other: the higher the resolution, the shorter the distance between two points that can be distinguished as standalone profiles. There is a direct correlation between N.A. and resolution: the higher the N.A value, the higher the resolution of the lens. For example, N.A. values for low-magnification 4X and 10X lenses are typically around 0.1 and 0.25, respectively, whereas for high-magnification 60X and 100X lenses they are around 0.7 and higher. Based on mathematical calculations, N.A. cannot be higher than 1 for so-called dry lenses, which do not use immersion media between the front of the objective lens and the coverslip placed on top of the stained tissue section. Using immersion media, it becomes possible to increase N.A. values above 1: with immersion oil, N.A. can be as high as 1.5, and with distilled water, it can be as high as 1.3. Oil and water-immersion objective lenses provide higher N.A. values because oil and water have refractive indexes higher than 1. Immersion objective lenses can be used without immersion media, but this will reduce the sharpness of the images (Fig. 8.3).

It should be remembered that lenses designed for oil immersion should not be used with water and vice versa because using inappropriate media may damage the lens. There are two types of immersion oil, A and B, which have different viscosities but a similar refractory index. Usually a small drop of oil is added on top of the coverslip and the lens is slowly moved downward using the focusing knobs until the front lens of the objective touches the oil. The refractive index of oil (1.5) is very close to that of glass (1.52), and therefore oil serves as a link creating optical continuity between the glass coverslip and the glass front lens. Such a linkage

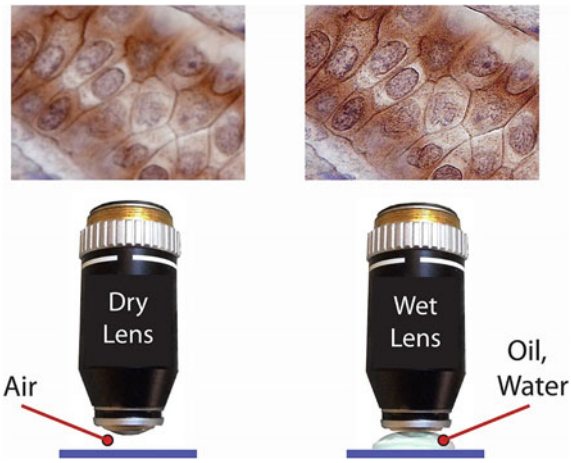

Fig. 8.3 Effect of immersion media on the sharpness of the image. Use of immersion media allows increased sharpness and resolution of IHC images

provides an effective way for both straight as well as oblique light rays to pass through a stained tissue section, entering the lens and then reaching the eyepieces or/and digital camera. In other words, visual information is not lost due to refraction, which refers to a change in the direction of a ray of light when it passes obliquely from one transmission medium into another. To ensure maximum performance of the lens, it is important to use a condenser on the microscope with an N.A. that is either the same as or higher than the lens number. For example, the resolution of a 100X and 1.25 N.A. lens will be impaired if matched with a 0.65 rather than 1.5 N.A. condenser, whereas the resolution of a 60X lens with 0.7 N.A. will be at its highest when using a 1.5 N.A. condenser.

The choice of high- or low-magnification lenses is always dictated by the level of detail required for recording. If subcellular localization of the antigen is under investigation, then high-magnification 60X and 100X lenses are better choices than 4X and 10X lenses. But if an IHC experiment is dealing with analyzing the distribution of labeled cells regardless of intracellular antigen localization, then use of 4X and 10X lenses will suffice. A researcher can use the Rayleigh formula (named after English physicist Lord Rayleigh, who was awarded the Nobel Prize for discovering Argon) for calculating the resolution of a lens to get an idea which lens best suits the researcher's needs.

$$R = 0.61 \times \frac{\lambda}{\text{N.A.}} \tag{8.1}$$

Equation 8.1. Rayleigh formula allowing calculation of the approximate resolution of the lens.

R = resolution in microns; λ = wavelength of light; N.A. = Numerical Aperture of the objective lens.

For example, if N.A. is 1.25 and examination is done under visible light with a λ of 0.55 μm, then the resolution is 0.61 × 0.55/1.25 = 0.27 μ. In other words, using this lens it would be possible to distinguish between two spots if they are not closer to each other than 0.27 μ, which is probably the best resolution the bright-field microscope can produce.

Another important characteristic of a microscopy objective is its working distance, which is the distance between the front lens and the surface of the coverslip when the stained tissue section is in focus. The working distance is engraved on the lens barrel of some objectives, but on others it may not be indicated at all. This may not be a big problem because it is known that usually the higher the magnification, the shorter the working distance. In turn, the shorter the working distance is, the smaller the field of view of the sample. Some high-magnification lenses have working distances less than 0.2 mm, and when using such lenses it is necessary to be cautious so as not to "overdo" focusing, which can cause the objective to push against the slide and crack it as well as scratching the front lens, compromising its optical quality (Fig. 8.4).

To make microscopy work comfortable, it is recommended to adjust eyepieces so they are set at correct interpupillary distance: the two-part images seen with left and right eyes must coincide to become a single image. This adjustment can be done by looking into the eyepieces and pushing them closer together or further apart until one sees a single image with both eyes. To accommodate researchers who wear glasses, modern microscopes are equipped with eyepieces designed in such a way that removal of eyeglasses is not required. After finishing interpupillary adjustment, it is necessary to adjust the focus of the eyepieces. While looking into the eyepieces, coarse and fine focusing knobs are rotated so that the image seen with the right eye is the sharpest. Then the focus needs to be adjusted for the left eye as follows: with the right eye closed and left eye looking into its eyepiece, the

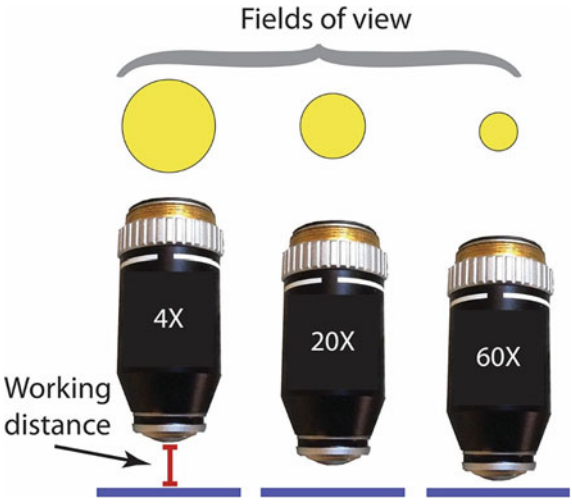

Fig. 8.4 Schematic representation of the relationship between the magnification of the objective lens and its working distance and the field of view. The higher the magnification the shorter the working distance and the smaller the field of view

diopter adjusting ring on the left eyepiece tube is turned until the image becomes the sharpest. Although it is tempting to use coarse and fine focus knobs, they should not be used at all when adjusting the focus for the left eye. Only the diopter ring on the left eyepiece tube should be used for such an adjustment.

A histological slide with a stained tissue section is placed on the stage, which has a central opening to allow the light from the illuminator to pass through the tissue section (Fig. 8.5). Usually the slide is mounted into a slide holder attached to the stage, which prevents the slide from unwanted movements during observation and image collection. Positioning of the stage can be precisely controlled by two mechanical X-Y controllers on the left part of the stage. Usually, while looking into the eyepieces, the left hand is used to move the stage while the right-hand adjusts the focus with the coarse and fine focus wheels on the right side of the microscope stand. The intensity of light can be regulated by either a dimmer dial or by a rheostat slider on the base of the microscope: the light level should be always comfortable rather than blinding to the eyes. In addition, the intensity of light can be adjusted using a field diaphragm built into a light illuminator protruding from the stand underneath the stage. Although the light can also be regulated by adjusting the diaphragm of the condenser (located between the stage and the light illuminator), this is not advisable because the condenser's main function is to adjust the contrast: by closing its diaphragm, the beam of light gets narrower, which increases the contrast of stained tissue sections.

Recording images of stained tissue sections is quite easy and is commonly done using a digital camera attached to the microscope. Vendors usually sell microscope cameras with designated image-capturing software that can be easily installed by the end user. This software allows the user to set the required image resolution, choose between manual or automatic camera exposures, and correct colors on the images. The user can save images in different formats, including such popular ones as TIFF, BMP, and JPEG, which can then be postprocessed and analyzed using different image-processing software packages like Adobe Photoshop (which is quite expensive) or ImageJ (which is free from the National Institute of Health).

8.2 Operating Fluorescence Microscope and Collecting IHC Images

In general, a fluorescence microscope is the same as a bright-field laboratory microscope but with few additional parts: a fluorescence illuminator and a set of excitation and emission filters combined in cube-shaped metal housings. The fluorescence illuminator is located at the back of the microscope and includes high intensity mercury or xenon lamps to excite fluorescent molecules in tissue sections. Basic principles of fluorescence microscope operation and image capturing using a digital camera connected to it are the same as for the bright-field microscope.

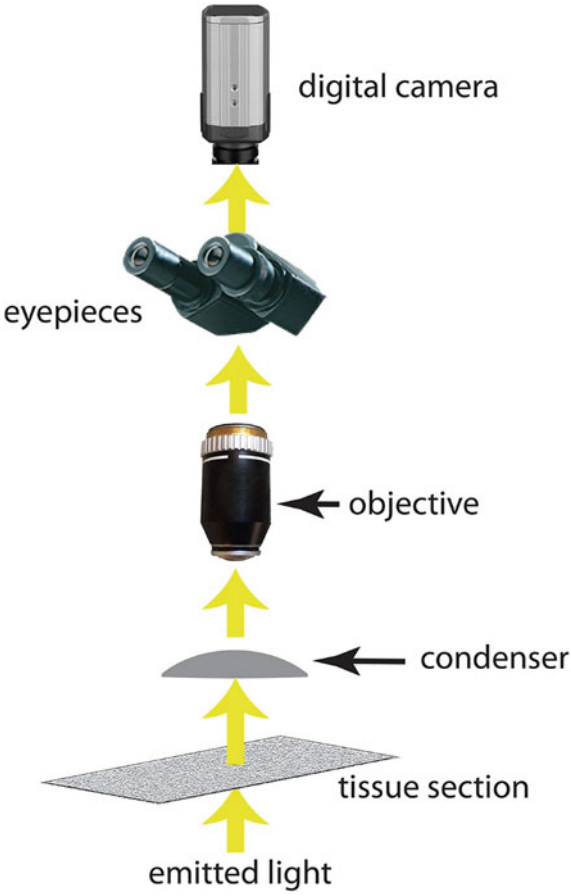

Fig. 8.5 Schematic overview of bright-field illumination in the upright microscope (parts are not shown to scale). Light emitted by either a halogen lamp or LED source passes through the stained tissue section, condenser, and objective lens into the eyepieces and/or a digital camera

Unlike a bright-field microscope, in which the signal is produced by the light transmitted through the tissue section, the signal in a fluorescence microscope (Fig. 8.6) is generated by the light reflected by the tissue section. Such a way of generating the signal is called epi-fluorescence, and the microscope is referred to as an epi-fluorescent microscope.

Modern epi-fluorescent microscopes can accommodate up to six cubes with excitation and emission filters matching different fluorescent dyes.

In addition to mercury and xenon lamps, microscopes can be also equipped with laser light sources such as argon-ion, argon-krypton, and more recently, solid-state lasers. Laser light sources are an essential component of laser-scanning confocal microscopes (CLSMs), which are widely used to produce very sharp images by suppressing out-of-focus image details. Due to their high power, lasers can produce illumination of very high intensity, allowing CLSMs to "look through" thick tissue sections and collect images at different tissue depths for three-dimensional reconstructions of antigen distribution.

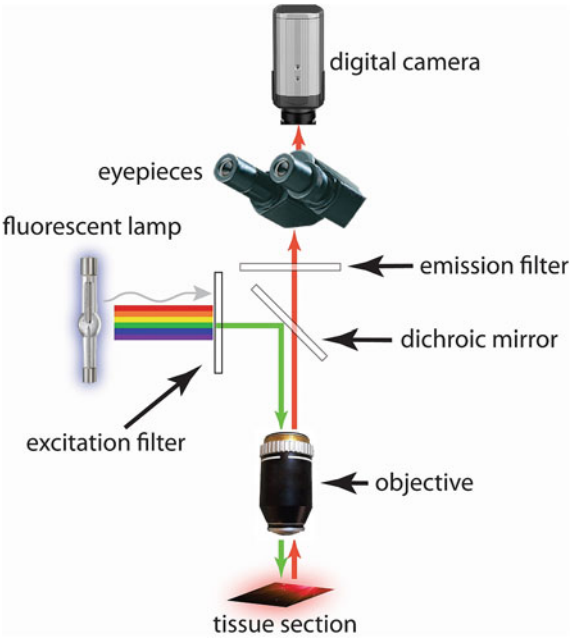

Fig. 8.6 Schematic overview of fluorescence illumination (parts are not shown to scale). Light that radiates from a fluorescent lamp (mercury or xenon) is filtered by the excitation filter to a wavelength (green, for example) that can excite a fluorescent dye used in IHC staining. This filtered light is then reflected by the dichroic mirror and passes through the objective lens onto a stained tissue section. Fluorescent dye used for tissue staining becomes excited and emits a light of a different, longer wavelength (e.g., red), which passes back through the same objective lens into the eyepieces and/or a digital camera

8.3 Presenting Immunohistochemistry Data

The main piece of IHC data is the images of stained tissue sections collected on the microscope, and therefore the saying "a picture is worth a thousand words" applies perfectly to the presentation of IHC data. What could be easier than showing an image? Just add saved IHC images to PowerPoint slides and you are all set for scientific presentation! Unfortunately, the process is not that simple and requires following some rules. First of all, it is necessary to decide what magnification to use for shown images: if the goal is to show an overview emphasizing the quantity of labeled cells, then it makes more sense to show a low-magnification image. However, if the goal is to illustrate the intracellular distribution of an antigen, then high-magnification images should be presented. In other words, the image must provide details that can be easily recognized by the reader. In many cases low- and high-magnification images of the same stained tissue are shown side-by-side with the area corresponding to the high-magnification image outlined on a low-magnification image with circular or rectangular shapes to guide the reader (Fig. 8.7).

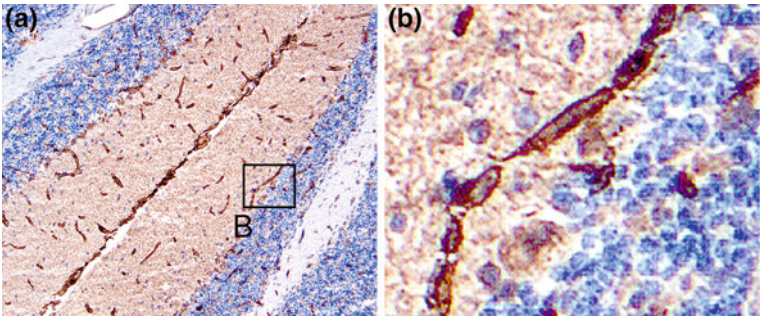

Fig. 8.7 Example of presentation of IHC images. Image **a** is a low-magnification overview of a stained tissue section taken with a 4X objective lens of the cerebellum in a rat brain showing labeling of blood vessels using the HRP-DAB (*brown color*) detection technique. Image **b** is a high-magnification image taken with a 40X lens of the region outlined by the black rectangular box on the low-magnification image **a**. Image **a** illustrates the density of blood vessels, whereas image **b** allows the visualization of fine histological details of these blood vessels, which would hardly be possible using only the low-magnification of image **a**. Tissue sections were counterstained with hematoxylin (*blue color*)

Another important technique for IHC illustrations is showing side-by-side images of specific staining and staining in the control. This is done so the reader can make an unbiased judgment of tissue labeling specificity. Controls can include, for example, (1) absorption control, (2) IgG control, or (3) no-primary antibody control (Fig. 8.8).

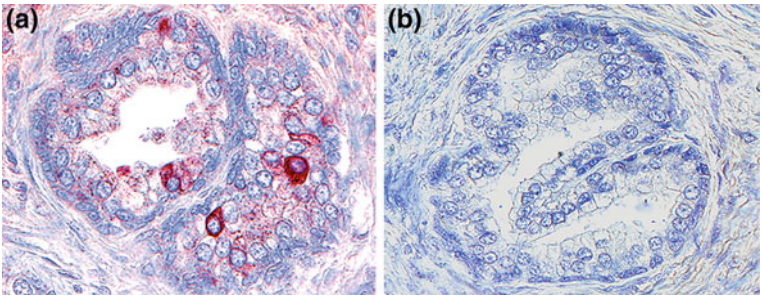

Fig. 8.8 Panel of side-by-side images depicting specific staining (**a**) and staining in the control (**b**). Image **a** shows cancer cells in a human prostate tissue section identified by IHC labeling for one of the cancer biomarkers using the HRP-AEC detection technique (*red color*). Image **b** shows staining in a no-primary antibody control. The lack of *red color* on image **b** indicates that specific staining on image **a** was due to the interaction of primary antibodies with the antigen of interest rather than by nonspecific binding of secondary antibodies and detection reagents to the tissue section. The *blue color* on images **a** and **b** resulted from counterstaining of tissue sections with hematoxylin

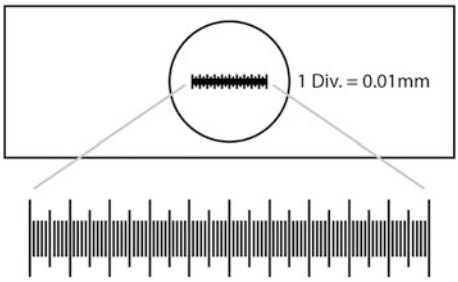

Fig. 8.9 A simple stage micrometer represents a transparent glass slide with a scale resembling a ruler printed on it. The distance between adjacent vertical lines (e.g., divisions) is printed next to the scale. Stage micrometer slides have the same dimensions as histological slides used for mounting tissue sections

When showing IHC images, it is also important to provide a reference to the final magnification of shown images, which is done by including a scale bar of a known size in the image. If there is a panel of several images, the scale bar can be added only to one of them if the images are shown at the same magnification. However, if images are shown at different final magnifications, scale bars have to be added to each of the images. How does one calculate the dimensions of a scale bar to be added to IHC image? This can be done by capturing the image of a scale on a so-called stage micrometer using the same objective lens on the microscope that was used to capture the image of stained tissue section (Fig. 8.9). Then, the IHC image and the image of stage micrometer can be digitally overlaid and a scale bar of an optimal length and thickness can be drawn using image-generating software like Adobe Photoshop or Adobe Illustrator. Nowadays, many image-capturing software applications provide the option of adding a scale bar on captured images automatically without the need to use stage micrometers (Fig. 8.10).

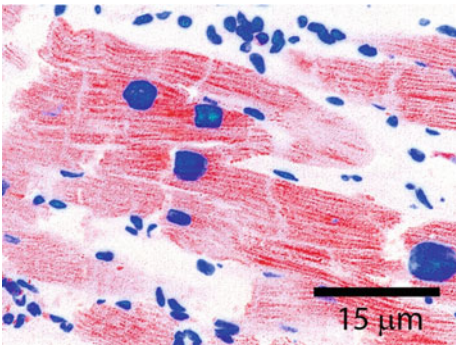

Fig. 8.10 Example of a scale bar added to an IHC image that shows labeling for cytoskeletal proteins in a tissue section of a human heart using HRP-AEC detection (*red color*) and counterstaining of cell nuclei with hematoxylin (*blue color*). A scale bar and its dimension in microns are added to the lower right-hand corner of the image so they can be easily referred to while not obscuring labeling of the antigen

Finally, IHC images must be accompanied by legends that start with a short descriptive title, then elaborate on the histological nature of antigen detection and point out important image details that may be overlooked by the reader due to their subtle appearance. Unless a single IHC image is shown, multiple images comprising a single panel are usually labeled alphabetically and these panels are then numbered and referred to in the text of a scientific paper as "figures".

8.4 Summary

Laboratory microscopes are the main tools for analyzing stained tissue sections after completing IHC staining. Operation of microscopes is not difficult, but it does require practice and adherence to certain rules. Microscopes come with objective lenses of different magnifications, which can be either "dry" or "immersion" as indicated by engraving on their barrels. Using water or oil as immersion media allows for the increase of Numerical Aperture (abbreviated N.A.) above 1, which, in turn, increases the resolution of the lenses. This resolution can be calculated using the Rayleigh formula. The higher the magnification of the objective is, the smaller the field of view and the shorter the working distance. In bright-field microscopy, light passes through the tissue sample (transmitted light illumination), whereas in fluorescence microscopy, light is reflected by the tissue sample (incident light illumination). To make microscopy work comfortable, it is recommended to adjust the eyepieces so they are focused for each eye and set at the correct interpupillary distance. Although bright-field and fluorescence microscopes are generally similar in operation, the user of a fluorescence microscope has to set the correct cube with excitation and emission filters matching the fluorescent dye used in IHC staining. Illumination on bright-field microscopes can utilize halogen bulbs and LED devices, whereas in fluorescence microscopy, light sources include mercury or xenon lamps as well as lasers. Images of IHC staining must be presented at the appropriate magnification, side-by-side with control images, and must include a scale bar.

8.5 Quiz (True or False)

1. Microscopes are the main instrument used to analyze stained tissue sections: **T F**
2. Microscopes usually come with a set of objectives of different magnification: **T F**
3. When starting examination of stained tissues under the microscope, high-magnification objectives should be used first followed by low-magnification ones: **T F**

4. The higher the Numerical Aperture (N.A.) of the objective, the higher its resolution: **T F**
5. Microscopes with bright-field illumination can be used to examine fluorescent samples: **T F**
6. In fluorescence microscopy, excitation and emission filter sets should match the fluorescent dyes used for IHC staining: **T F**
7. The Rayleigh formula is used to calculate the working distance of an objective: **T F**
8. Oil- and water-immersion objectives are recommended for use with oil and water-immersion media, respectively: **T F**
9. For presentation, IHC images must be shown only at the highest magnification possible: **T F**
10. Control images have to be shown side-by-side with IHC images of specific staining: **T F**
11. Scale bars need to be added only to images of fluorescent staining: **T F**

Chapter 9
Concluding Remarks

If our book has been read through to this point, then we consider our mission successfully accomplished. We hope that this short book was both informative and entertaining in covering the basics of immunohistochemistry technique. In some sections of this book, we could not avoid using high-level terminology because there was no way to accurately explain the subject matter using only layman's terms. Our ultimate goal was to introduce the reader to IHC technique focusing exclusively on its essential bits and pieces. This is by no means an immunohistochemistry encyclopedia, but rather a stepping stone for novices to learn and understand this technique as well as a refreshing reference for those who practice it only occasionally.

Writing this book was an exciting and pleasant experience, as nothing could be more satisfying for an educator and a researcher than sharing his knowledge and experimental tricks with his peers.

A.E. Kalyuzhny, *Immunohistochemistry*, Techniques in Life Science and Biomedicine for the Non-Expert, DOI 10.1007/978-3-319-30893-7_9

Quiz Answer Keys

Chapter 2 Quiz (True or False)

1. **F**
2. **F**
3. **T**
4. **F**
5. **F**
6. **T**
7. **T**

Chapter 3 Quiz (True or False)

1. **F**
2. **F**
3. **T**
4. **F**
5. **T**
6. **F**
7. **F**
8. **F**
9. **T**
10. **F**

Section 4.1 Quiz (True or False)

1. **T**
2. **F**
3. **F**
4. **F**
5. **F**
6. **F**

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Section 4.2 Quiz (True or False)

1. **F**
2. **T**
3. **T**
4. **T**
5. **F**

Sections 4.3–4.5 Quiz (True or False)

1. **T**
2. **F**
3. **T**
4. **F**
5. **T**
6. **F**
7. **F**
8. **T**
9. **T**

Chapter 5 Quiz (True or False)

1. **T**
2. **T**
3. **F**
4. **F**
5. **F**
6. **T**
7. **F**
8. **F**

Chapter 6 Quiz (True or False)

1. **T**
2. **F**
3. **F**
4. **T**
5. **F**
6. **T**
7. **F**
8. **T**
9. **T**

Chapter 7 Quiz (True or False)

1. **T**
2. **F**
3. **T**
4. **T**
5. **F**
6. **F**
7. **F**
8. **T**
9. **T**
10. **F**
11. **F**

Chapter 8 Quiz (True or False)

1. **T**
2. **T**
3. **F**
4. **T**
5. **F**
6. **T**
7. **F**
8. **T**
9. **F**
10. **T**
11. **F**

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