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Use of Antibodies in Immunocytochemistry

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1. Introduction

Immunostaining can be used to pinpont the subcellular localization of a protein antigen, to follow its changing position as cells respond to stumuli, or to compare its position to other proteins in the same cell. Using these methods one can follow an antigen's distribution during development, mark the location of a particular cell type in a multicellular in vivo setting, or determine the presence of an antigen in a diseased tissue. The protocoles normally require multiple steps over several days as well as extensive knowledge of architecture of the tissues been studied. These procedures demand methods to preserve the structure of tissues, which unfortunately are often damaging to antigens.

 The important propeties of the antibodies are; they specifically bind to a particular protein or molecule, that are called antigens. This binding property keeps the antibody binding unaltered in the physiological conditions. Also, any other noval antibodies could be made to other interested molecules. These are the crucial properties of the antibodies that are used in immunocytochemistry. An antigen is defined as a substance that can be bound by an antibody molecule through its antigen-binding sites, also called epitopes or antigenic determinants. Treatment of tissues with chemical fixatives and detergents can change the reactivity of proteins to antibodies because the exposure of epitopes to these chemicals can change the chemical and physical nature of the molecules in the epitope region. Many substances can, and are known to be antigenic, for example: proteins; nucleic acids: DNA, RNA; carbohydrates or sugar groups; lipids; small chemical groups; peptides (10-15 amino acids long). Thus antibodies can bind almost any repertoire of antigens, including chemicals and things B-cells have never encountered before.

Development, characterization and manufacturing of antibodies has made tremendous progress in using the antibodies in many research, diagnostic, and therapeutic areas. However, it has been emphasized that antibodies both monoclonal and polyclonal remain the primary site in the vast majority of research and dianostic applications (Leduc and Connolly., 1955). New types of antibodies, recombinant and synthetic, have been developed and validated. Recombinated antibodies can be produced in transgenic mice (He et al., 2002), also using bacteriophage (Hoogenboom and Chames., 2000) high quantities of high-affinity antibodies can be produced. Synthetic antibodies (diabodies, triabodies, tetrabodies)

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are generated using chemical or biological cross-linking to produce di-, tri-, and tetrameric multivalent conjugates exhibiting enhanced specifity and functional activity (Tomlinson and Holliger., 2000). There are some investigations on antibodies that have been made to replace amino acids by other biological molecules to ctreate chemical diversity and produce nucleic acid-based molecules forming specific binding to target antigens (Proske et al., 2005).

Antibodies are the responsible proteins that hold the key step in immunocytochemistry. Antibodies have been used as research reagents for many years. Technology has improved and created new techniques and enhanced their value in immunocytochemistry. Antibody is tagged with a visible label. The visual marker which may be a fluorescent dye, colloidal metal, hapten, nanocrystal, radioactive marker or the more commonly in the light microscopic field, an enzyme. Experimental samples ranging from frozen sections, cell culture/suspension, to whole tissue samples have been used. Ideally, maximal signal strength along with minimal background is required to give optimal antigen demonstration.

There are multiple ways of performing immunological stains on tissues, some of which include the direct method of staining, where the antibody is bound directly onto the antigen on a cell with a fluorescent or colored dye bound directly to the antibody. Another method includes the indirect method where the antigen is reacted with a primary antibody which binds directly. This is followed by a secondary antibody which binds to the primary antibody. Next, a tertiary reagent is applied, which binds to the secondary antibody, with an enzymatic end. When the quarternary reagent is applied, the enzymatic end of the tertiary reagent converts the substrate into a chromogen (DAB; Diaminobenzadine or others), which stains the cell, usually a brown color.

In the immunocytochemical application, since there are many different staining methods, the investigator should know how to optimize the staining method when the sataining fails. Fore example; there may be other methods that although using the same primary antibody concentrations, the degree of the staining intensity may be increased due to the method.

Recommendations of the optimal dilutions of the primary antibody should be aquired. When the sataining does not work the investigators should have the basic nowledge to deal with the sepisific problem. In, particular, tissue fixation and tissue processing can have inportant effect on the antigenicity of the protein by changing the conformation of the epitope, therefore, creating non-specific background. Specificity of the antibodies needs to be tested in control experiments to avoid non-specific staining due to nonspecific binding to tissue components or binding to other proteins that share similar epitopes.

2. Antibody molecules

The antibodies, or immunoglobulins (Igs) are a group of glycoproteins present in the serum and tissue fluids. They are produced when the host's lymphoid cells come into contact with immunogenic foreign molecules called antigens, and they bind specifically to the antigen which induced their formation.

Each antibody molecule consists of four polypeptides– two heavy chains and two light chains joined to form a "Y" shaped molecule. Antibodies are further classified into multiple classes or isotypes. Five distinct classes of immunoglobulin molecule are recognized in most higher mamals, namely IgG, IgA, IgM, IgD and IgE (Table 1). These differ from each other in

size, charge, amino acid composition and carbohydrate content. In addition to the differences between classes the immunoglobulins within each class are also very heterogeneous.

IgG;	Is the major immunoglobulin in normal human serum accounting for 70-75% of the total immunoglobulin pool. It is distributed evenly between the intra- and extravascular pools, is the major antibody of secondary immune responses and the exclusive anti-toxin class.
IgA;	Represents 15-20% of the human serum immunoglobulin pool. In man more than 80% of IgA occurs as the basic four chain monomer but in most mammals the IgA in serum is mainly plymeric and occurs mostly as a dimer. It is the predominant immunoglobulin in sero-mucous secretions such as saliva, tracheobronchial secretions, colostrum, milk, and genito-urinary secretions.
IgM;	Accounts for about 10% of the immunoglobulin pool. The molecule has a pentameric structure.This protein is largely confined to the intravascular pool and is the predominant "early" antibody frequently directed against antigenically complex infectious organisms.
IgD;	Accounts for less than 1% of the total plasma immunoglubulin but it is known to be present in large quantities on the membrane of many circulating B lymphocytes. It is involved in initial immune response
IgE;	Though a trace serum protein, is found on the surface membrane of basophils and mast cells in all individuals. This class may play a role in active immunity to parasites and commonly associated with immediate hypersensitivity reactions.

Table 1. Ig classes

Essentially each immunoglobulin molecule is bifunctional; one region of the molecule is concerned with binding to antigen while a different region mediates binding of the immunoglobulin to host tissues. IgG isotype is the immunoglobulin that is used in immunocytochemistry because it is generated in high quatities and its binding property is more consistent. Knowledge of the IgG structure (Fig. 1), is important in order to understand the mechanisms of the antibody and antigen reactions. The basic structure of IgG and all other immunoglobulin molecules is a unit consisting of two identical light polypeptide chains (variable region) and two identical heavy polypeptide chains (constant region) linked together by disulphide bonds.

The class and subclass of an immunoglobulin molecule is determined by its heavy chain type. Thus the four IgG subclasses (IgG1, IgG2, IgG3 and IgG4) have heavy chains called γ1, γ2, γ3, and γ4 which differ only slightly although all are recognizable γ heavy chains. The differences between the various subclasses within an immunoglobulin class are less than the differences between the different classes; thus IgG1 is more closely related to IgG2, 3, or 4 than to IgA, IgM, IgD or IgE. The amino acid sequence in the tips of the "Y" varies greatly among different antibodies. This variable region, composed of 110-130 amino acids, give the
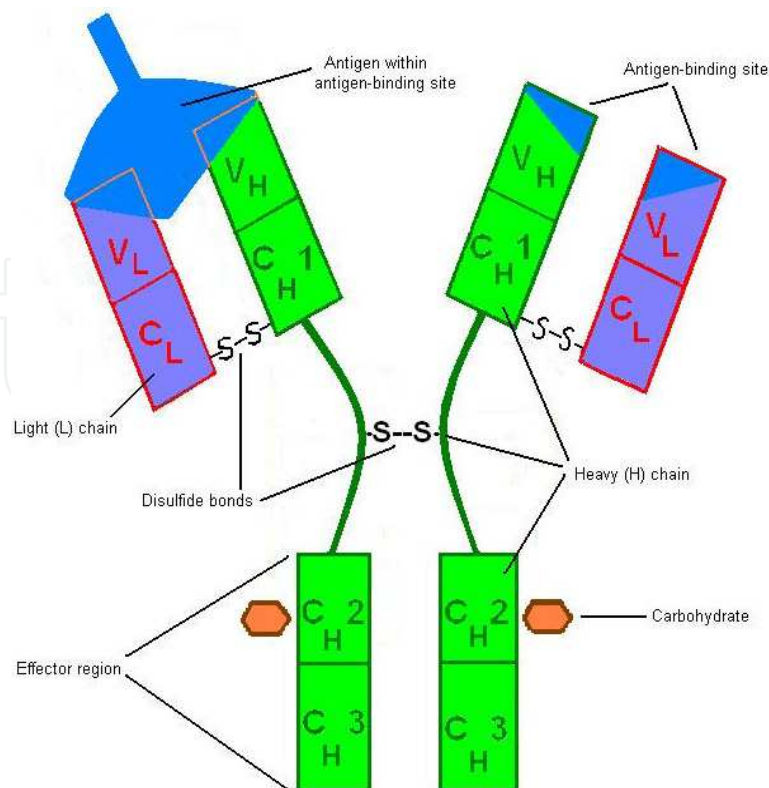
Fig. 1. The antibody. An IgG antibody has a single constant region (C) and the variable region. The constant region containing light (C_L) and heavy chain (C_H) with the fragment crystallizable (Fc) region (effector region). The variable region contains the the antigen binding site. The small protein, only in the variable region, is known as the light chain; the large protein that is part of the constant and variable region is the heavy chain. The IgG can be digested by the protease enzyme, papain, into an Fc end (constant end) and a Fab end (variable end).

antibody its specificity for binding antigen. The variable region includes the ends of the light and heavy chains. When the primary amino acid structure of a large number of light and heavy polypeptide chains is examined it is found that the variability between their V domains is not distributed evenly throughout the length of these regions. Some short polypeptide segments are termed hypervariable regions. It is now generally accepted that such hypervariable regions are involved directly in the formation of the antigen binding site. Hypervariable regions are sometimes referred to as Complementarity Determining Regions (CDR) and the intervening peptide segments as Framework Regions (FR). In both light and heavy chain three hypervariable regions exist – HV 1, 2 and 3. Four FR regions which have more stable amino acids sequences separate the HV regions. Treating the antibody with a protease can cleave this region, producing fragment antigen binding (Fab) that include the variable ends of an antibody. The constant region contains species specific sequences and the Fc portion that binds an Fc receptor, which is found on circulating white cells, macrophages, and natural killer cells. The Fc portion also has species-specific sites that are unique to the animal species in which the antibody was generated. Thus, generation of an antibody against IgG from rabbit will result in antibodies that bind the constant region from rabbit IgG only and not, for example, from mouse IgG.

Antibodies or IgG molecules are generated to other IgG molecules by injecting purified IgG molecules from one species into another species. In the case of mouse IgG injected into rabbit, it will produce rabbit anti-mouse IgG antibodies. Antibodies made against an IgG will only bind to the constant region or Fab region of the IgG. The variable end of the antibody contains the unique epitope-binding regions that give each antibody its specificity (Fig. 1). This variable region is the fraction antigen binding (Fab) portion. The unique configuration of the Fab specifically binds the epitope. When an antigen is injected into a rabbit, the resulting antibodies against the antigen have Fab portions that are unique to the antigen, but the rest of the IgG is similar to other IgG molecules. Each IgG antibody has two Fab ends, which can bind to two identical epitopes at the same time. The advantage of this bivalent epitope binding is that it can amplify the epitope detection.

An antigen is a protein, peptide, or molecule used to cause an immune response in an animal. The animal responds by making antibodies to individual epitopes or antigenic determinant region located on the antigen. An individual antigen has multiple epitopes that can generate antibodies. An epitope can be an amino acid sequence on a denatured peptide or a several sequences on the surface of a folded protein. Animals frequently generate multiple antibodies to the same epitope. Also, an epitope on one protein might also exist on a different, unrelated protein because it has the same sequence or the same surface configuration.

3. Producing antibodies

When the macrophage engulf the bacteria, proteins (antigens) from the bacteria are broken down into short peptide chains and those peptides are then presented on the macrophage membrane attached to special molecules referred to as Major Histocompatibility Complex Class II (MHC II). Bacterial peptides are similarly processed and displayed on MHC II molecules on the surface of B lymphocytes. When a T lymphocyte determines the same peptide on the macrophage and on the B cell, the T cell stimulates the B cells. The stimulated B cell undergoes repeated cell divisions, enlargement and differentiation to form a clone of antibody producing plasma cells. Therefore, by recognizing a specific antigen, clonal expansion and differentiation of B cells to the plasma cells are acquired and all these cells start to produce the same specific antibody to only a single epitope. Epitopes are regions on an antigen that an antibody can bind to, and are also known as antigenic determinants. Epitopes can be: conformational; in which the antibody recognizes the secondary structure of the molecule, linear; in which the antibody binds to the determinant in both the denatured protein and the native protein; neoantigenic, which is an epitope which is not present in the native protein but becomes an epitope after the protein is cleaved by a protease.

3.1 Polyclonal antibodies

Polyclonal antibodies are multiple clones of antibodies produced to different epitopes of the antigen. In Fig. 2, the serum from an immunized rabbit contains antibodies from many clones of B-cells. In the rabbit serum, the different clones of antibodies have multivalent property, consisting of antibodies that bind to several regions (epitopes) of the antigen molecule, providing a strong dectecting capacity. This feature is important in that it gives high levels of staining for a single antigen. However, shared epitopes on different proteins

can label multiple proteins which can cause false evaluation, and it is referred to as cross-reactivity.

Polyclonal antibodies depend on a living animal thus if the animal dye no more antibody of that specific type can be produced. In addition, when a new animal is immunized with the same antigen, the exact epitopes generating antibodies will be different. An immunocytochemical study using a polyclonal antibody to detect IGF-I protein is demonstrated in Figure 3.
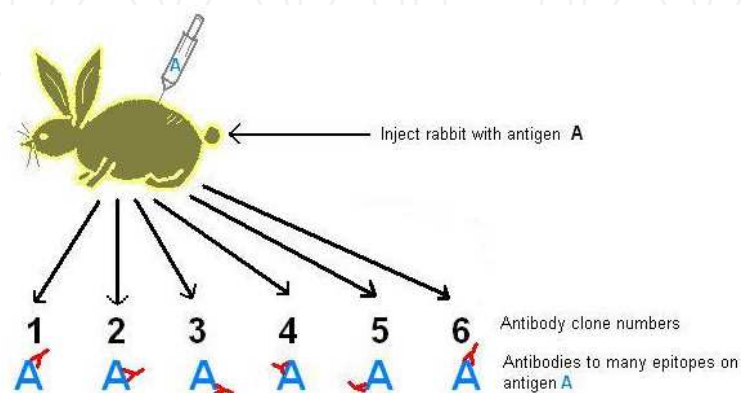

Fig. 2. Schematic representation of the production of polyclonal antibodies. Polyclonal antibody preparations are usually a mixture of different specific antibodies known as "antibody clones"which all recognize the same antigen (A). The specificity difference means the antibodies bind to different epitopes on the antigen with different strength. The blood serum that is obtained from the rabbit that contains polyclonal antibodies is known as "antiserum".
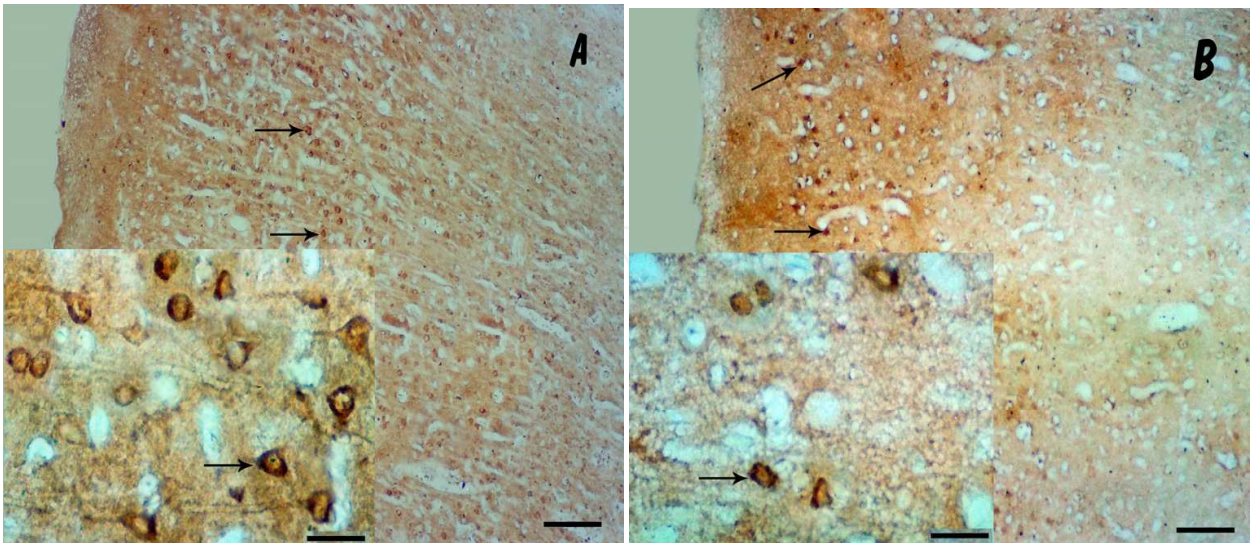

Fig. 3. Photomicrographs of IGF-I immunoreactivity in the cortex. In the present immunostaining antihuman IGF-I polyclonal antibody is used to detect the IGF-I protein in various areas in the cortex. In the present study the siginificant decrease in density of IGF-I immunoreactive neurons in ethanol treated (B) compared to the control (A) rats. At a higher magnification neuronal processes are evident (inset). (Dalçik et al., 2009).

3.2 Monoclonal antibodies

Monoclonal antibodies, originally from one mouse, contain a single antibody from one clone of B-cells to a single epitope on the antigen. This procedure was first described by Cesar Milstein, (Milstein et al., 1979; Milstein C., 1980). The antibodies are produced in mice (Fig. 4), and when antibodies are produced, the spleen of the immunized mouse is removed. Then the spleen cells are dissociated. These spleen cells so-called the B-cells are fused with mouse myeloma cells in culture. The fusion process allows the hybrid cells to continue to proliferate. Each cell produces only one type of antibody. The procedure consists of screening the culture fluid from various clones for antibody via RIA, ELISA, or immunocytochemistry (Ritter M.A., 1986). One mouse spleen can give many different antibodies to different epitopes on the same antigen molecule. Antibodies spesific to fragments of the molecules could be produced in this way and, the culture can be stored until further production is required. Rabbit and mice monoclonal antibodies are available than other animals because good rabbit and mice myeloma cell lines are available.
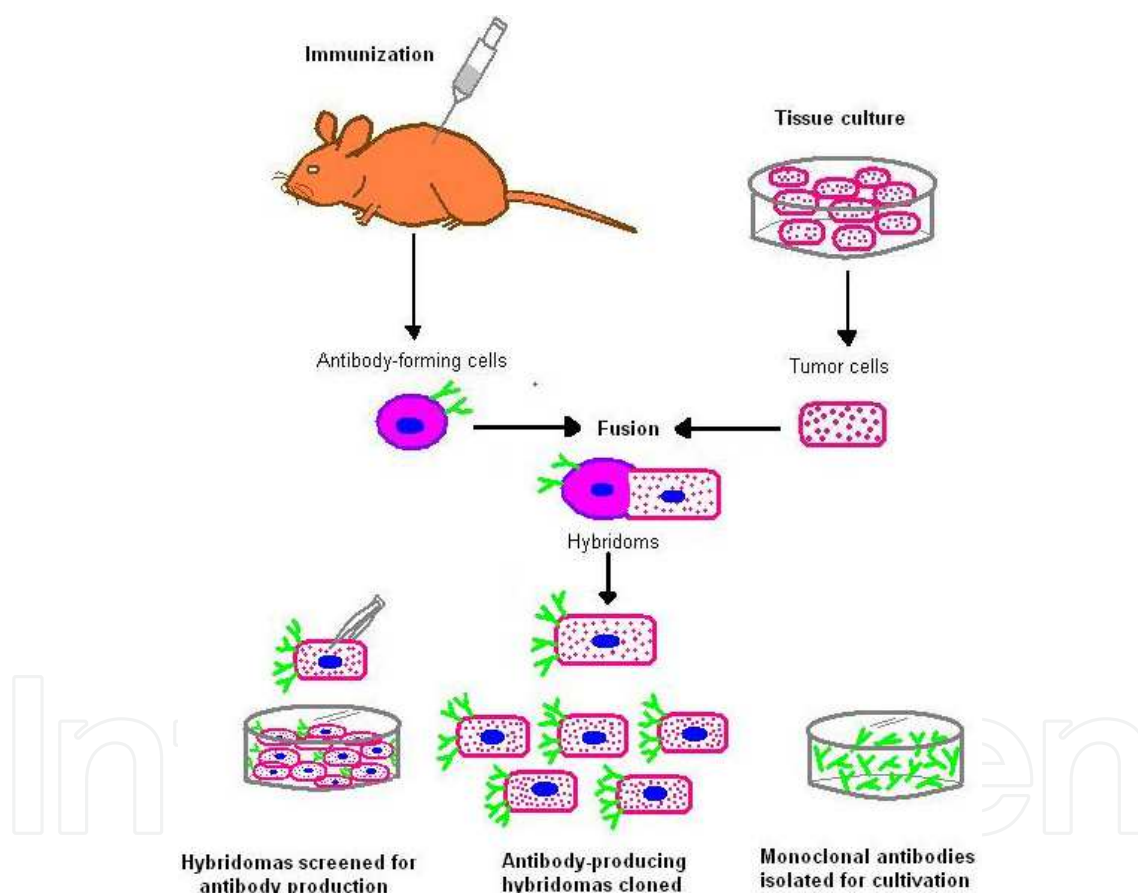

Fig. 4. Monoclonal Antibody production is the process by which large quantities of antibodies are produced. A mouse is immunized by injection of an antigen to stimulate the production of antibodies targeted against that particular antigen. The antibody forming cells are isolated from the mouse's spleen. Monoclonal antibodies are produced by fusing single antibody-forming cells to tumor cells grown in culture. The resulting cell is called a hybridoma. Each hybridoma produces relatively large quantities of identical antibody molecules. By allowing the hybridoma to multiply in culture, it is possible to produce a population of cells, each of which produces identical antibody molecules. These antibodies are called "monoclonal antibodies" because they are produced by the identical cloned antibody producing cell.

The advantage of monoclonal antibodies compared to polyclonal antibodies is their absolute specificity for a single epitope on the antigen molecule, and different clones of antibodies can be generated to different epitopes on the same antigen. Therefore, since the cross-reactivity is very much decreased the immunostaining can be clean without any artifacts on the slide. However, if the monoclonal antibody happens to bind to an antigenic sequence that is shared by other substance then in this case cross-reactivity will occur in the usage of monoclonal antibodies. Since the monoclonal antibodies bound to only one site on the molecule its binding results in weaker staining. In addition, since the specific sequence of the epitope may be altered with the fixation this may also cause no staining compared to the polyclonal antibodies because in polyclonal antiserum there are several regions (epitopes) in the antigen that the antibody can bind, if one region gets altered the others can be available. Therefore, in many cases frozen materials are been used rather than fixed paraffin sections. If using paraffin sections it may be wise to use many different antibodies spesific to different regions of the same antigen molecule to get better results.

Although much work is required to generate monoclonal antibodies, with the aid of cultured hybridoma cells it can be generated indistinctly. However, polyclonal preparations are generally easier and less expensive to generate than monoclonal antibodies, and they can withstand greater variations in temperature and pH. An immunocytochemical study using a monoclonal antibody to detect IGF-I protein is depicted in Figure 5.
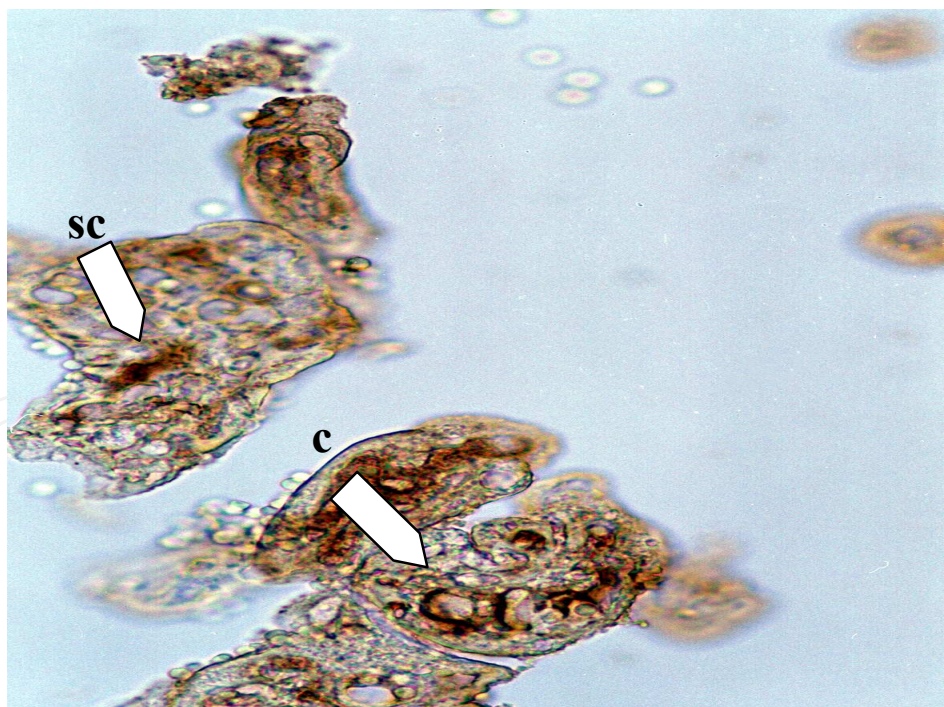

Fig. 5. Photomicrograph showing IGF-I immunoreactivity from the placenta of the intrauterine growth retarded placental villus. In the present immunostaining anti-human IGF-I monoclonal antibody is used to detect the IGF-I protein in various areas in the placental villi. IGF-I immunoreactivity is observed in the stromal cells (sc) and capillary endothelial cells (Dalçik et al., 2001).

4. Selection of the antibody

In the immunocytochemical applications the desired antibody used, is referred to as the "primary antibody". There is also a "secondary antibody" to detect and bind to the primary antibody. The secondary antibodies usually have tag to make the primary-secondary antibody complex visible under the microscope. In order to detect a protein for example IGF-I protein in human tissue with anti-human rabbit polyclonal or anti-human mouse monoclonal antibody, it is appropriate to use an anti-rabbit secondary or anti-mouse secondary antibodies. This is followed by using either a horseradish peroxidase (HRP)-DAB (Fig. 4,5) or alkaline phosphatase-Vector Red reagents to produce a color in order to detect the localization of the desired antigen. Other colorimetric detection systems are also commercially available.

Blocking reagents are used during the immunostaining procedure to avoid the problem of detecting endogenous immunoglobulins that are detected by the secondary antibody, in order to reduce the background staining. On the other hand, in the immunofluorescence technique the secondary antibodies are chemically conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC). There are two types of immunofluorescence staining methods; direct immunofluorescence staining and indirect immunofluorescence staining. In the direct immunofluorescence staining, the primary antibody is labelled with fluorescence dye and in the indirect immunofluorescence staining (Fig. 6) the secondary antibody is labelled with the fluorochrome (FITC or Texas Red). Immunofluorescence staining can be performed on cells that are fixed on slides and also tissue sections and these can be examined under a fluorescence microscope.
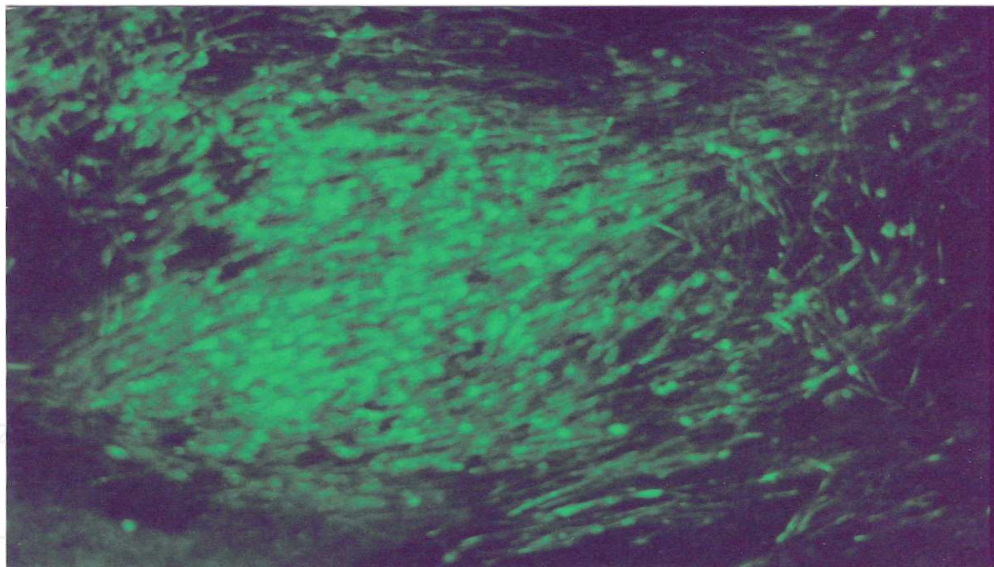

Fig. 6. Photomicrograph showing nestin immunoreactivity from a 9 day old embryonic stem cells. In the present study mouse anti-nestin monoclonal antibody is used to detect the nestin protein which are the primary marker for the neuronal differentiation, and anti-mouse IgM FITC is used for secondary antibody (Tas et al., 2007).

5. Handling of the antibody

Commonly the antibodies are commercially available. There are many companies that manufacture and sell antibodies. For example, antibodies may be sold in the form of hybridoma tissue culture supernatant, ascites fluid or crude serum.

It is important to get a good and efficeint antibody. Before obtaining the primary antibody, relevant work that has been done by other investigators should be examined thoroughly. In addition, recommendations from collegues that have used the antibody in their previous study is a good way to obtain a reliable antibody. After purchasing the antibody, one should read the product information and instructions in the datasheets provided by the supplier. The company supplies the antiserum in a liquid or powder form. Antibodies in powder form are liyophilized and transfered at room temperature. Antibodies in liquid form are transfered in ice packages.

Antibodies should be stored as indicated by the vendor or supplier. When receiving the powder or liquid form of the antibody to prepare the stock solution it should be diluted and saved as small aliquots in microcentrifuge tubes at -20 ^{0}C or -70 ^{0}C. The aliquots should not be less than 10μl. When using the antiserum the antibody should be thawed, the left over antibodies should not be freezed again, repeated freezing and thawing is not recommended since these processes destroy the stability of the antibody. Damage can be reduced by adding glycerol to the solution. Extreme pH, and high salt environments can also damage the structure of the antibody. However, in some cases the antibodies are supplied as "ready to use" form. In this case, without diluting the antiserum it is applied directly on the tissue sections.

When using the antibody it is note worthy to keep in mind that the antiserum can be contaminated by bacteria. The manufacturer supplies the antibody with a preserver substance such as sodium azide (NaN_3). If there is a bacteria contamination it will be wise to discard the antibody and not use it, because the antiserum will create significant amount of background staining.

A good antibody should have high affinity (binding strength) for its antigen. Therefore, they are not washed off the sections during the immunocytochemical staining. In addition, the concentration of the antibody is important to get clean appeareance of the tissue. A high dilution means decreased population of antibodies is present in the used antiserum and that unwanted antibodies are removed. Therefore, unwanted reactions are discarded. A low dilution means; increased population of antibodies is present in the used antiserum. The optimum dilution is required to get a better binding and staining, also to get low background staining (maksimum signal to noise ratio). High dilutions of the antibody are advantageous because they allow the total use of the available antibodies. If monoclonal antibodies are used in the immunocytochemical procedure, since the primary antibody is specific to the antigen, it is likely that unwanted reactions are absent, and therefore, the dilution in many cases becomes less important. Polyclonal antibodies come in as whole serum or as purified antibodies and an antibody concentration is given as μg/ml. The initial dilution of the monoclonal antibodies obtained via the hybridoma technology should be between 1:5-1:100. The secondary antibody used in the immunocytochemistry should be used at the optimum dilution such as; 1:200. The dilution propotion differ according to the type of tissue used and according to the tissue sections either paraffin or frozen section. The dilution is usually made commonly by phosphate buffered saline (PBS) solution. In order to obtain the working dilution (for example; 1:1000) the stock antibody is removed from the refrigerator, first 1:100 diluted solution is prepared than in the second step the solution is further diluted 10 times and 1:1000 working solution is prepared. The incubation period of the antibody with the tissue section in the antiserum can provide important information for the binding charecteristics of the antibody. Frequently, the incubation period of the antibody

with the sections at 37 ^{0}C is between 30 min. to 2 hour, at room temperature is 1-6 hour, at +4 ^{0}C is 6-24 hour.

The methods where the antibody is used other than immunocytochemsitry (ICC) are; immunfluorescence (IF), western blot (WB), immunoblot (IB), immunoprecipitation (IP) and ELISA.

6. Control application of the antibody

It is important to make control studies in the immunocytochemistry procedures to make sure that the staining is specific. When the antibody targets a certain protein, the antibody should be unique to that particular protein. Before performing the study, other related protein family in the cell that may have homologous peptide sequence should be evaluated by identifing their aminoasid sequence. Because these proteins may cross react with the antibody and therefore, nonspecific binding and background staining may occur. The antibody specificity is best determined by immunoblot and or immunoprecipitation. The specificity of the method is best determined by preabsorption/preincubation control (Swaab et al., 1977; Willingham., 1999), negative control, replacing the primary antibody with serum, and a positive control, using the antibody with cells known to contain the protein. In the preabsorption control, the antibody is mixed with the desired antigen (protein or peptide) before it is delivered to the sections. The goal of this process is to eliminate the binding of the antibody to the target protein. Therefore, when the antibody-protein complex at appropriate concentrations is applied to the tissue section, and if there is no staining then we can conclude that the antibody used is specific to that particular protein. We should also remember that the antibody can bind to any epitope of a protein that has the right conformation. Therefore, the competitive blocking experiment may produce a false data because, many peptides may bind nonspecifically to formalin-fixed tissues and can increase the immunostaining. Another test for antibody sensitivity and specificity is performing immunocytochemistry on negative control.

7. Conclusion

Immunocytochemistry is a technique that is used for staining cells or tissues using antibodies against target antigens or proteins. It is common to use unlabelled first antibody also referred to as the primary antibody, and then use the second antibody also referred to as secondary antibody directed against the first (anti-IgG). This secondary antibody is conjugated either with enzymes for colorimetric reactions, or fluorochromes, or gold particles (for EM) to visulaize the location of the antigen.

In order to get best of the immunocytochemical technique the antibody used should have specificity, ability to reach and bind to the antigen. On the other hand, in the target antigen or protein epitope alterations can occur, and therefore weak staining will be visualized. The antibodies should be carefully selected. Search for specific antibodies and related protocols from the literture is necessary. Since monoclonal antibodies recognize single epitope they have greater specificty. However, polyclonal antibodies recognize multiple epitopes, and have increased risk of binding non-specific molecules with similar epitope. Thus, significant staining variations could occur according to different batches. Once receiving the antibody before using it in the immunocytochemical procedure, it should be tested at higher and lower concentrations in order to define the best dilution to study.

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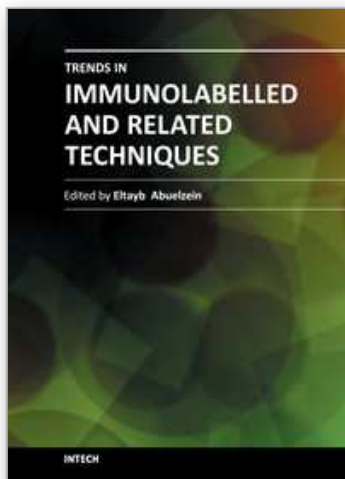
Trends in Immunolabelled and Related Techniques

Edited by Dr. Eltayb Abuelzein

ISBN 978-953-51-0570-1

Hard cover, 360 pages

Publisher InTech

Published online 27, April, 2012

Published in print edition April, 2012

The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Hakkı Dalçık and Cannur Dalçık (2012). Use of Antibodies in Immunocytochemistry, Trends in Immunolabelled and Related Techniques, Dr. Eltayb Abuelzein (Ed.), ISBN: 978-953-51-0570-1, InTech, Available from: http://www.intechopen.com/books/trends-in-immunolabelled-and-related-techniques/use-of-antibodies-in-immunocytochemistry

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