Immunohistochemistry: A Review of Practical Procedure

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Immunohistochemistry (IHC) is a simple, yet a highly essential, research and diagnostic tool in the field of molecular biology and medical science.

It is a procedure of antigen detection in a tissue by using the principle of antigen antibody reaction. This is the technique by which different tumor markers are detected in tumor samples. The method of staining tissue sections and detecting antigen in the tissue is called immunohistochemistry. Similar process to detect antigen in a cell is called immunocytochemistry.

Grossly there are 3 steps in IHC namely slide preparation, slide staining and slide interpretation. More precisely the whole procedure can be divided into different sections as follows- deparaffinization and rehydration, activation of antigen, endogenous peroxidase blocking, primary antibody incubation, secondary antibody incubation, antibody detection, counterstaining, and slide mounting. It takes about 6 hours to complete the immunostaining and can be divided into two parts and done in two consecutive days.

The purpose of this article is to review the basic principle and simplify the procedure of IHC. The target group is the young medical practitioners who have every scope of carrying out different research works.

Key words: IHC, Immunostaining, Procedure, Technology

IHC is an important research tool in medical science. It is a technique of detecting a protein, an antigen, in the tissue by detecting antigen antibody reaction with the help of markers. Among various markers, fluorescent dyes and enzymes are the most widely used ones. [3] The method of staining tissue sections using this technique is called immunostaining. The similar process to detect antigen in cells is called immunocytochemistry.

It is routinely used for research, prognostic and diagnostic purpose.[4, 5, 9, 16] However, it is not yet commonly practiced in developing countries like Nepal due to various reasons. This article is targeted to the young medical practitioners like doctors, nurses, medical technologists and research workers.

The main goal of this article is to review the procedure of immunostaining and has been written on our own experience basis following a standard protocol. [15]

Procedure

IHC was first developed in the middle of 19th century.[3] Since then, the steps of this technique have been reviewed and improved many times.[1, 2, 3] IHC can usually detect one antigen at a time. However, the new techniques and technologies are being developed so that two or more antigens can be detected simultaneously. Moreover, further advancement in this technology like development of immunofluorescent study, development of new reagents and their application have led to the development of advanced diagnostic capability. [13]

Grossly IHC consists of 3 steps

- slide preparation,
- slide staining (immunostaining) and
- slide interpretation.

Detail explanation of each and every step of IHC is out of scope of this small review paper. Therefore, slide staining procedure is the main focus of this article.

The slide staining (immunostaining) is very simple and less time consuming. The whole procedure can be further sub divided into following steps for simplicity. [6-12]

Steps

1. Deparaffinization and rehydration
2. Activation of antigen
3. Endogenous peroxidase blocking
4. Primary antibody incubation
5. Secondary antibody incubation
6. Antibody detection
7. Counter staining
8. Slide mounting

The whole procedure can be divided into 2 parts for our practical convenience which can be performed in 2 consecutive days. Each part takes about 3 hours. First part includes deparaffinization of slides, antigen activation, endogenous peroxidase blocking and primary antibody incubation. Second part includes rest of the procedures.

Phosphate Buffer Saline Solution (PBS; 0.01M, pH-7.4 to 7.6) is used throughout the procedure to rinse and wash slides after every step. Tris buffer solution is an alternative to PBS.

The tissue specimen that can be used for immunostaining is either formalin fixed paraffin embedded blocks or fixed frozen sections which is collected in liquid nitrogen during surgery and stored in refrigerator in -80° C. [2] However, the former one is not only the ultimate standard for IHC but also an easier way as far as facilities in laboratories in our country is concerned. [17-21]

Deparaffinizaton and Rehydration

Deparaffinization is important to remove the excess paraffin from the tissue. Because of paraffin fixation there is crosslink formation that covers the active sites of antigen preventing it to react with antibody. Xylene solution removes the excess paraffin. It is necessary to maintain the hydration of the tissue through out the procedure. Dryness will destroy the tissue section. Dip the slide holder with slides into xylene, 3 times, 5 minutes in each, and then into alcohol 100%, 95 %, 85% and 75% successively 2-3 minutes in each. Wash the slides in water by dipping the slide holder in a tub full of water for 3 minutes.

Activation of antigen

Even after removal of excess paraffin from the tissue section, the antigens are still masked by cross link formation by paraffin and formalin. So the antigens can be unmasked by various methods of antigen activation. Heat Induced Epitope Retrieval (HEIR) and Proteolytic Induced Epitope Retrieval (PIER) are the common methods. Usually one of these is sufficient to activate the antigens but using both of the methods is an alternative if either of them is not strong enough to unmask the antigen. HEIR is more commonly used and is described here. HEIR is a method of antigen activation by heat treatment using Citrate Buffer Solution (CBS, pH 6.0). The solution is heated in a microwave oven for 8 minutes till it gets boiled. Put the slide holder in the solution and reheat it for about 20 minutes in the boiling state. Once it is boiled take it out and let it cool down to room temperature or 37°C which will take about 45-60 minutes.

Endogenous peroxidase blocking/ enzyme digestion or blocking

Enzyme blocking is an important step in IHC. Sometimes endogenous enzymatic activities are found in the tissue and its reaction with the antibody causes non specific background staining. One of them is endogenous peroxydase. This endogenous peroxidase activity can be eliminated by the pretreatment of the tissue section with hydrogen peroxide (H2O2) prior to primary antibody incubation. Enzyme blocking solution is prepared by mixing 10 ml of 30% H2O2 with 90 ml of 100% methanol. After antigen activation the slide holder is dipped into this solution for 30 min. Other rarer enzymes are endogenous alkaline phosphatase, endogenous biotin etc.

Washing with PBS solution

After this step, the tissue sections need to be washed with PBS solution thrice 5 minutes each. In fact, this washing is essential after every step.

Antibody incubation

Antibody incubation is done to facilitate the detection of antigen through antigen antibody reaction. [1, 14] On the basis of antibody incubation, IHC can be grouped into 2 types, direct and indirect. Direct method is "one step staining method" which involves only one antibody incubation. The incubated antibody is the labeled one and thus antigen antibody reaction is detected with the help of enzyme or fluorescent dye which is fixed in the antibody. Indirect method is "two steps staining method" which involves primary and secondary antibody incubation. Primary antibody is unlabeled and it reacts with antigen. Secondary antibody is labeled and it reacts with primary antibody. Secondary antibody is detected with the help of enzyme or fluorescence, depending on which it is labeled with. Indirect method is more sensitive and thus is more widely used. ABC (Avidin Biotin Complex) method is more commonly used among various indirect methods. The improved technique of ABC method is SAB (Strept Avidin Biotin) method. In this method, there are three steps or layers. First layer is unlabeled primary antibody. Second layer is biotinylated secondary antibody. Biotin is a low molecular weight vitamin which can be conjugated to a variety of biological molecules like antibodies. The third layer is streptavidin, a large glycoprotein with high affinity

for biotin, labeled with enzyme, commonly peroxidase which can be visualized by the application of appropriate substrate. The process of antibody incubation is illustrated in the figure. SAB kit, manufactured by Nichirei Co. Tokyo, Japan is one of the commonly used kits for this technique which consists of 3 different agents namely normal serum (blue in color), secondary antibody (yellow in color) and enzyme peroxidase (red in color).SAB kit blue is the serum of the animal in which secondary antibody was raised. It is used to limit any nonspecific protein binding to the specimen. It is used in Avidin-Biotin system to prevent nonspecific binding of avidin or biotinylated products to tissue proteins. It ensures all endogenous biotin, biotin receptors, or avidin binding sites present in tissues are blocked prior to the addition of the labeled avidin reagent

Once the slides are washed with PBS solution after endogenous peroxidase blocking, preparations for antibody incubation should be done. The necessary items needed for that are SAB kit (blue, yellow and red), moisturizing box and kimwipe paper. Tissue paper can be used instead of kimwipe paper if not available. The slides are wiped and made dry with kimwipe paper and are placed on the moisturizing box one by one. Biotin blocking should be done first before primary antibody incubation by adding 2-3 drops of blue SAB kit (normal serum) over the tissue section. Once SAB kit blue is added to all the slides, the moisturizing box is closed with its cover and left as it is for 30 minutes in room temperature.

While waiting for the SAB kit blue incubation for 30 min, the primary antibody (PAb) of appropriate dilution should be prepared by adding PBS solution. The usual dilution of primary antibody ranges from 1:100 to 1:1000 which varies depending on the antibody.

After SAB kit blue incubation for 30 minutes, primary antibody can be added to the slides directly without washing with PBS solution. In fact this is the only step where we don't need to wash the slides. Once PAb (about 300 MicroL) is added to the every slide, moisturizing box is closed with its cover and it is put inside the refrigerator (4° C) over night. This is the end of the first part and the next step can be started on the next day. Alternatively PAb incubation can also be done for 1 hour in room temperature and the next step can be continued on the same day after that.

Secondary antibody incubation

The remaining process of IHC starts from here on the next day. The moisturizing box is taken out of the refrigerator. Washing of the slides is done by dipping the slide holder in the PBS solution thrice 5 minutes each. Secondary antibody (SAB Kit Yellow) is then applied to all the slides and moisturizing box is closed and left in room temperature for 30 minutes.

Antibody detection

Once antibody incubation is done, it is ready for its detection according to the principle of IHC. This is a very important step, as without this the antigen cannot be detected and the whole procedure will be in vein. In this step the streptavidin enzyme conjugate (streptavidin +peroxidase) is applied on the tissue section. The streptavidin of this third layer will conjugate with the biotin of secondary antibody. The closed moisturizing box is left in room temperature for 30 minutes and then the slides are washed with PBS solution as before.

Since the end result of antigen antibody reaction in the slide is not visible either to naked eye or to microscope, the further process is needed to make it visible. The substrate for the enzyme peroxidase is prepared by mixing 10 mg of Diaminobenzidine (DAB) in 100 ml of PBS solution. It should be noted at this point that DAB has a neurotoxic effect. So it should not be handled with naked hand. Once it is well mixed, the slide holder is dipped in the DAB solution. The time for this step is not fixed and it depends on the antigen to be detected. Usually it ranges from 2-10 minutes. When the color of the tissue section in the slide is changed to brownish, this is the approximate time required for DAB treatment. The slide holder is taken out and dipped in the PBS solution. The slide, then, is observed under the microscope one by one. If the cells in the tissue section are well visualized with brownish nuclei inside them, that means DAB treatment is adequate. If not yet so clear, continue DAB treatment for some more time. However this expertise will develop with practice.

Counterstain

Mayers Hematoxillin solution is used as a nuclear counter stain in IHC and also in HE staining which gives nucleus blue color. First it gives purple or reddish purple stain to the tissue. But finally after exposure to alkaline solution like lithium it gives blue color to the tissue especially nucleus. Put the slide in Mayer hematoxillin solution for about 1 min. and then wash in water by dipping it in the tub full of water for 3-5 minutes. Dip in alkaline solution like lithium 2 -3 times. Dip in water for few minutes and look under microscope, nucleus is seen as a blue spot. By this IHC, immunostaining is complete.

Slide mounting

Slide mountaining is done to preserve the already stained slides for longer period. For this purpose the slides need to be dehydrated first as follows. Dip the slides into alcohol 75%, 85%, 95% and 100% 2-3 min each then into xylene 3 times 5 min each. Wipe the slide as usual, put a drop of glue over the tissue section and then cover with the cover slip of appropriate size

Slide interpretation

Slide interpretation is the final and a very vital step in any staining procedure. Interpretation is done by examining the slide under the appropriate magnification of light microscope. Whether the cell is positive or negative for the protein in question depends on whether the protein is present in the cell, either nucleus or cytoplasm or both, or

not. The positive cell, i.e. cell which contains the protein in question looks brown in color due to treatment with DAB solution. But the cell which is negative i.e. doesn't contain the protein, is blue in color due to counterstaining with hematoxillin solution. If the protein is localized in the nucleus, it looks brown and the cytoplasm looks blue. If the protein is localized in the cytoplasm, the nucleus looks blue and the cytoplasm is brown. If the protein is present in both the nucleus and cytoplasm, both of them look brown. But if the cell is negative, both nucleus and cytoplasm look blue.

Conclusion

IHC is one of the important tools in daily clinical practice as well as in the field of medical research. Young medical practitioners and researchers should possess the knowledge of this procedure which can broaden the horizon of medical practice. In the lack of this technology, we are still lagging far behind especially in the field of oncology.

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