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REVIEW ARTICLE

IMMUNOHISTOCHEMISTRY – A TECHNICAL REVIEW

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ABSTRACT

In routine histopathology, Haematoxylin and Eosin (H&E) stain is the commonly used standard procedure for the diagnosis of benign and malignant conditions. Though this conventional procedure gives a clue to the diagnosis of the lesion, it might be challenging in determining the exact origin, type, behaviour and progression of a given neoplastic tissue. Detection of cell markers of the neoplastic tissue will be helpful in identifying the exact nature of the lesion. For this, a technique called immunohistochemistry is used. Using the principle of antibodies binding specifically to antigens in biological tissues, this technique detects and visualises the individual or multiple antigens of biopsy tissue sections. This technique has an increasing role in modern pathology and has developed into a powerful research tool in the armamentarium of every biomedical research field. It has three major implications in our histopathology – diagnostic, prognostic and detecting the predictive markers for the specific cancer therapy. This review emphasises briefly on the steps involved in staining process.

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INTRODUCTION

Diagnosis of many diseases is fundamentally based on the microscopic examination of cells and tissues. With time, the practice of pathology is expanding its frontiers with the advent of newer diagnostic and prognostic techniques that aid in analysis of various tissue components in a precise and efficient manner and yield promising results. (Jordan *et al*., 2002) Immunohistochemistry is one such technique. It has become a powerful tool in the armamentarium of the pathologist. It has provided insight into tumor histopathogenesis and has contributed to the accurate determination of patient prognosis. (Jordan *et al*., 2002) It is basically a technique for identifying tissue constituents (antigens) by means of antigen antibody interactions. The antigens/target proteins in the tissue are recognized by the antibodies that are highly specific to those antigens. In the recent days many antibodies specific to the antigenic components have been developed and the method is being widely utilized for attaining diagnosis and also to study various aspects of the disease processes. (Jordan *et al*., 2002) Predictable tumour expression of many of the same antigens as their cells of origin or normal tissue counterparts validates the principle of tumour classification by Immunohistochemistry. (Jordan *et al*., 2002) Immunohistochemical staining process is technique sensitive and it involves a series of steps each of which is very important and need to be performed carefully.

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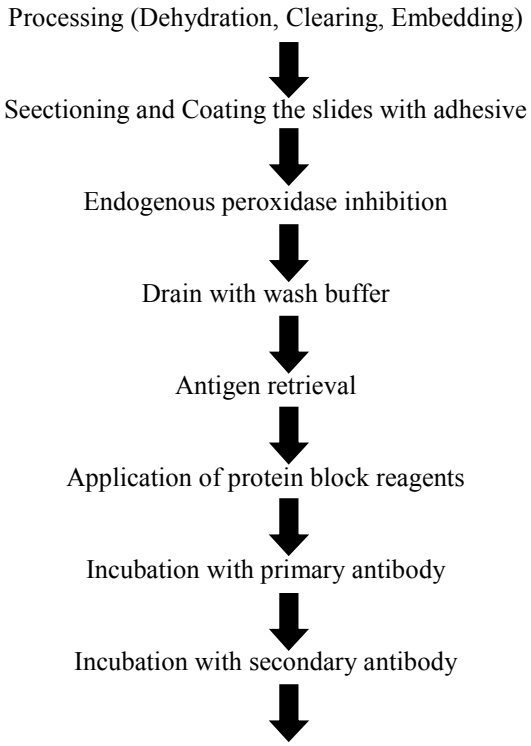
This review article will discuss briefly on the steps involved in the staining process in order.

Tissue fixation



Incubation with Streptavidin-HRP

Application of Chromogen

Nuclear staining with haematoxylin

Steps in technique

Tissue fixation

It is important to establish proper fixation and processing procedure which will allow for good morphology and henceforth maximize the ability of the pathologist to identify the antigens that aid in diagnosis. Fixation in this technique should preserve the position of the antigen, whether nuclear, cytoplasmic or membrane bound and preserve as much as antigenic secondary and tertiary structure to provide target for antibodies. (Shi *et al*., 2001) The fixative of choice is 10% neutral buffered formalin at a pH of 7.0 -7.6. The time duration for fixation ranges from 24 – 48 hrs and is preferably done at room temperature. In case of over fixation, cross-linking can mask the epitopes that are needed to react with antibody. (Shi *et al*., 2001)

Tissue processing

The aim of the tissue processing is to embed the tissue in a solid medium that is firm enough to support the tissue and give it sufficient rigidity to enable thin sections to be cut and yet soft enough not to damage the knife or tissue. (D'Amico *et al*., 2008) The grossed tissue specimen is subjected to dehydration through graded concentrations of alcohol (70%, 80%, 90%) for 45min each followed by 3 changes of absolute alcohol for 1hr each. Propanol or ethanol is the most commonly used dehydrating agents. Tissue is then cleared through 2 changes of xylene for 1hr each. It is then impregnated with 3 changes of paraffin wax for 1hr each and then it is embedded in the same material (paraffin wax).

Sectioning and coating the slides with adhesive

Tissue sections of 5µm thickness are made using microtome and the sections are made to float on warm water bath. Sections are then taken over the slides coated with APES or VECTABOND adhesives which provide sticky surface for flat adherent sections. Slides are heated to 60^{o}C to soften the wax for 30mins. Slides are then deparaffinized in 2 changes of xylene, 5min each and then rehydrated in decreasing concentrations of alcohol, initially in 2 changes of absolute alcohol for 5 min each followed by 95%, 70% and 50% alcohols for 5 min each.

Endogenous peroxidase inhibition

This block is an inhibitor that prevents an enzyme originating within a cell or tissue from causing a reaction with another substrate. Peroxidase and alkaline phosphatase are the commonly generated enzymes in the cell. Peroxidase inhibition is achieved by immersing the sections in 3% hydrogen peroxide solution in methanol at room temperature for 10min.

Wash buffers

They are used for removing excess or unwanted reagents or complexes formed during each step. Commonly employed wash buffers for use are Tris buffered saline (TBS) /phosphate buffered saline (PBS).

Antigen retrieval

It is the process by which antigenic epitopes that are made unavailable because of fixation induced protein cross-linking are rendered accessible to antibodies for binding. Antigen retrieval involves exposing the tissue sections to heat or proteolytic enzyme digestion before commencing immunochemical staining. (Jordan *et al*., 2002) The success of retrieval depends on the duration of fixation, the antigen being demonstrated, and the type and conditions of the antigen-retrieval process itself. Tissues undergoing antigen retrieval should be on tissue-adhesive coated slides to help adherence to the glass.The harsh conditions of heat induced epitope (antigen) retrieval (HIER) often cause tissues to lift off the slides.Slides coated with APES (aminopropyltriethoxysilane) and poly-L-lysine appears to be the most popular. (Bancroft and Gamble, 2002) Numerous methods of antigen retrieval exists and the method of choice depends on the antigen and antibody under study and is usually determined by trial and error study (Jordan *et al*., 2002).

The following are the various antigen retrieval methods:

I. Water Bath Methods
II. Pressure Cooker Heating
III. Autoclave Heating
IV. Microwave Oven Heating
V. Proteolytic Pre-treatment
VI. Combined Proteolytic Pre-treatment and HIER
VII. Combined De-paraffinization and Target Retrieval

The mechanisms of antigen retrieval are not well established and many theories have been proposed which include breaking of the cross links, renaturation, formation of holes and gaps, extraction of diffusible proteins, precipitation, stabilization and rehydration of protein epitopes. (C.F.A Culling 1974)

Heat-induced epitope (antigen) retrieval (HIER)

HIER involves heating tissue sections in various buffer solutions and can be performed using a variety of means such as a microwave, pressure cooker, vegetable steamer or autoclave. Each method has its advantages and disadvantages, but has the common end point of antigen retrieval.

Buffer solutions commonly used include 0.1 M citrate buffer (pH 6), 0.1 M EDTA (pH 8), 0.5 M Tris base buffer (pH 10) and 0.05 M glycine/HCl buffer

Proteolytic (enzymatic) antigen retrieval

Proteolytic antigen retrieval can be carried out using a variety of enzyme solutions such as trypsin, pepsin, pronase, and proteinase K. This method has largely been set aside following the arrival of HIER, since a much wider range of antigens can be demonstrated. Enzymatic antigen-retrieval solutions can also be difficult to work with due to the enzymes being sensitive to factors such as temperature and pH.

Once antigen retrieval is done slides are allowed to cool for 20-30mins. Followed by rinsing in PBS, 2 changes for 5min each

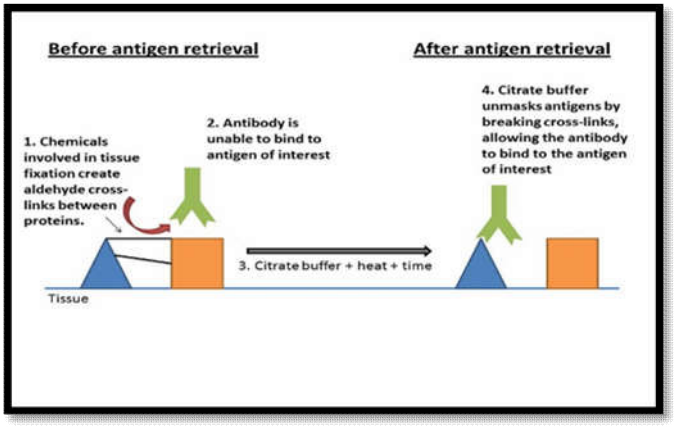


Fig.1. Structure of an antibody

Protein blocks

Protein blocks refer to the process where in different reagents are used to reduce the chances of nonspecific reactions of antibody with components other than antigens thereby reducing the non immune-specific reactions. They also eliminate background staining. Blocking buffer *(10% fetal bovine serum in PBS)* is added onto sections and incubated in humidified chamber at room temp for 1hour. Excess blocking buffer is drained off from the slide.

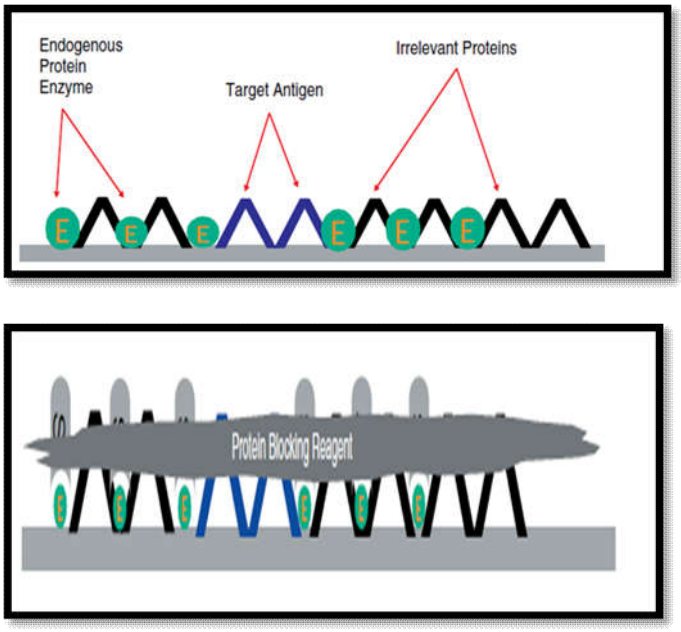
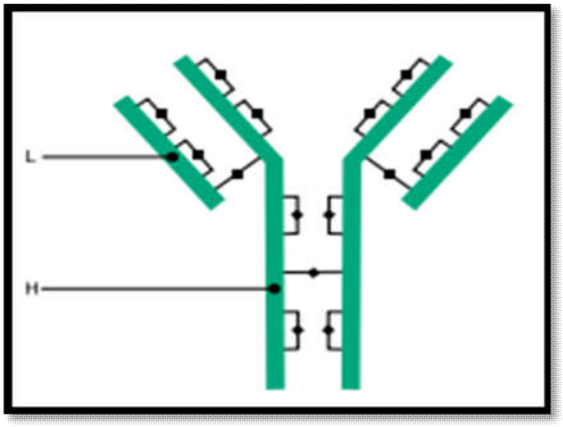


Fig.2. Protein Block

Antibodies

They constitute an important reagent to all immunohistochemical techniques. They belong to a group of proteins called immunoglobulins that are present in the blood of immunized animals. Immunoglobulins are comprised of five major classes namely IgG, IgA, IgM, IgD, IgE in decreasing orders of their concentrations of which IgG and IgM are the most frequently used in Immunohistochemistry. IgG is the most abundant antibody in a newly immunized individual wheras IgM is the first detectable humoral antibody. (Shi *et al*., 2001)



L – LIGHT CHAIN
H – HEAVY CHAIN

Fig.3. Structure of an antibody

Monoclonal antibodies

They are derived from a single clone of plasma cells and hence are immunochemically identical. They react with a specific epitope on the antigen against which they are raised. Mice are frequently used for the production of monoclonal antibodies. Advantages of monoclonal antibodies over their polyclonal counterparts include high homogeneity, absence of non-specific antibodies, ease of characterization and minimal batch-to-batch variability. (Shi *et al*., 2001)

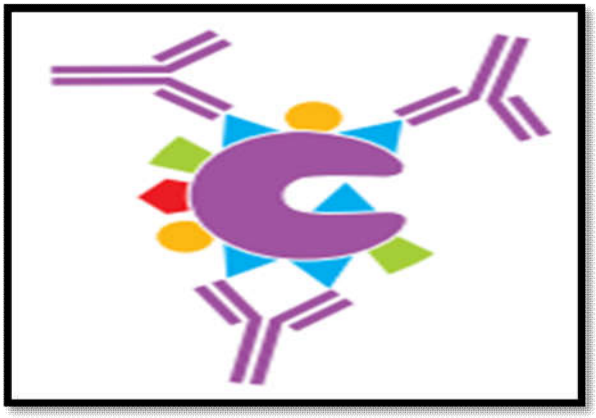


Fig.4. Monoclonal Antibody

Polyclonal antibody

They are derived from different cell types and hence are immunologically dissimilar. They react with the epitopes on the antigen against which they are raised. Animals that are frequently used for their production are rabbits followed by goat, pig, sheep, horse, guinea pig and others. Frequent use of rabbits is attributed primarily to their ease of maintenance and secondly the human antibodies to rabbit proteins are rare when compared to those from other animals like goat. Also the rabbit antibodies precipitate human proteins over a wider range of either antigen or antibody excess. New Zealand white rabbit is the most frequently used animal for the production of polyclonal antibodies. (Shi *et al*., 2001)

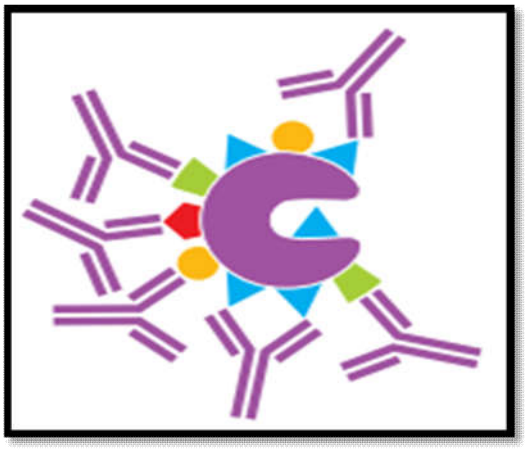


Fig.5. Polyclonal Antibody

Antibody titre

Optimum antibody titre may be defined as the highest dilution of an antiserum that results in maximum specific staining with the least amount of background staining under specific test conditions.For polyclonal antisera, titres vary from 1:100 to 1:2000 and for monoclonal antisera, they vary from 1:10 to 1:1000 in cell culture supernatants and upto 1:1,00,000 in ascites fluid. (Immunohistochemical staining methods 4th edition)

Incubation with primary antibody

Primary antibodies areraised against the antigen of interest and are typically unconjugated/unlabelled. They are diluted with Antibody Dilution Buffer at proper working concentration according to manufacturer's guidance.

100-400 µl of diluted primary antibody is added to each section and incubated at 37 ºC for 1hour or 4ºC for 12-24 hours in closed incubation chamber. Slides are then washed in PBS, 2changes, 5mins each.

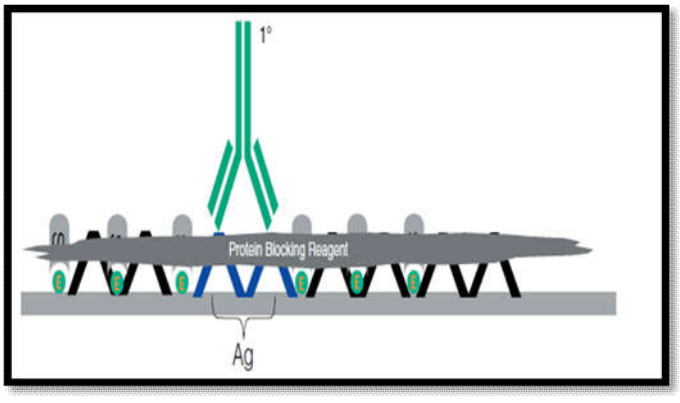


Fig.6. Incubation with Primary Antibody

Incubation with secondary antibody

Raised against immunoglobulin of the primary antibody species, usually conjugated to linker molecule. It is biotinylated. Avidin and streptavidin possess 4 binding sites for biotin (vitamin). In ABC method secondary antibodies are conjugated to biotin and function as links between primary antibody and avidin peroxidase complex.

Each section is immersed in 100-400 µl diluted biotinylated secondary antibody for 30min at 37 ºC. Slides are then washed in PBS, 2changes, 5mins each

Streptavidin – HRP incubation

Enzyme-substrate reactions convert colorless chromogens into colored end products. Horseradish peroxidase and calf intestine alkaline phosphatase are common.HRP is used because of convenience and clarity. Streptavidin – HRP antibody is diluted with Antibody Dilution Buffer at proper working concentration according to manufacturer's guidance. 100-400 µl of diluted Streptavidin–HRP is added to each section and incubated at 37□ for 30min in an incubation chamber. Slides are then washed in PBS, 2changes, 5 mins each. (Immunohistochemical staining methods 4th edition)

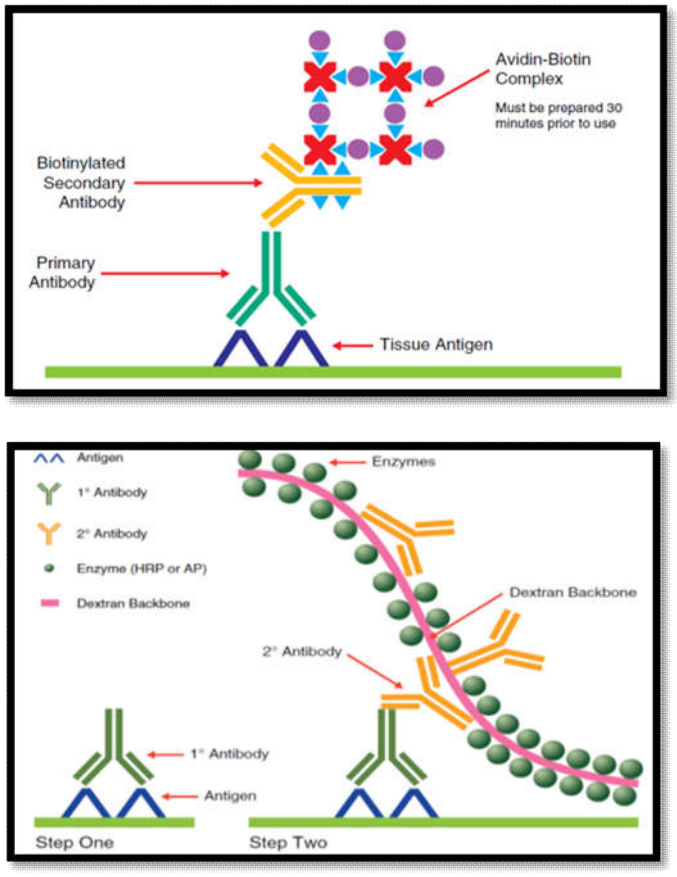


Fig.7. Streptavidin-HRP Incubation: Avidin Biotin Complex Method

Chromogen

DAB/AECare the commonly used chromogens. They produces a brown end product that is highly soluble in alcohol and other solvents. Enzymatic activation of the chromogen results in visible reaction product at the antigen site. 100-400 µl of freshly prepared *DAB (diaminobenzidine*) reagent is added to each section to reveal the color of antibody staining. Colour is allowed to develop. Sections are washed in PBS for 3changes, 2mins each to stop staining.

Hematoxylin stain

Sections are counterstained in haematoxylin reagent according to manufacturer's instructions. Slides are rinsed in running tap water for about 15min. Dehydrated through 4 changes of

alcohol (95%, 95%, 100%, 100%), for 5min each. Clearing is done in 3 changes of xylene and then coverslip is mounted. Then the stained slide is viewed under the proper magnification of a commonly used light microscope and is interpreted.

Conclusion

Immunohistochemical studies have conventionally focused on specific cell markers for detection of a particular tumour type that aids in the diagnosis as well as guide for the specific cancer therapy. Due to its reliability, sensitivity and specificity, it has become an integral technique with a broad application not only in surgical and molecular fields but also in routine clinical practice.

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