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Basics of Immunohistochemical procedure

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Abstract
The field of diagnostics has emerged with some varied new techniques in recent past enabling pathologists to reach to a more conclusive diagnosis. One such technique is immunohistochemistry which in recent past has gained a lot of importance. Although histopathology has long been considered to be gold standard in diagnosis but immunohistochemistry has further facilitated pathologists to decipher the cell lineage as well. Hereby we discuss immunohistochemistry especially the procedure and principle of each step of procedure.

Keywords: Immunohistochemistry, fixation, antigen retrieval techniques.

Introduction
Oral pathology basically encompasses the diagnostic means to reach to a conclusive diagnosis of a disease **[1]**. Albeit histopathology has been considered a gold standard technique in the field of diagnosis yet on various occasions the histopathological diagnosis has been proven as dubious [2]. In recent years diagnostic field has experienced upsurge in molecular techniques owing to it sensitivity and specificity amongst which immunochemistry comes as a feasible technique and ensuring better cell preservations than other techniques [3]. Immunohistochemistry has served in confirmation of various infectious diseases by identification of bacterial DNA or RNA while applied in cytological specimens as sputum, fluid obtained from fine needle aspiration cytology for same. Another spectrum of IHC is in understanding gene specific functions in biological processes as development and apoptosis [2].

History
The principle of immunohistochemistry has known to exist since 1930s. However it was in 1941 when Professor Albert H Cons and colleagues demonstrated a breakthrough technique of visualizing the tissue constituents using antibody labeled with fluorescent dye. In 1966 Nakane and Pierce reported the use of secondary antibody which was conjugated with peroxidase conjugate to an antibody and visualized it with an appropriate chromogen as diaminobenzidine (DAB). In 1970 Sternberger described the peroxidase anti peroxidase method (PAP method). In 1971 Engvall and Perlman reported use of alkaline phosphatase labeling. The following discoveries of alkaline phosphatase-antialkaline phosphatase (APAAP) method by Cordell *et al* in 1984 significantly expanded the application of immunohistochemistry technique. Heggness and Ash in 1977 proposed use of avidin biotin for immunofluorescence. This technique was further used by Guesden *et al* in 1979 [3]**.** In 1981 Huang *et al* discovered antigen retrieval methods while Hsu *et al* introduced the system of secondary antibody detection, which enabled application of technique in pathology diagnostic routines [3] Vyberg and Nielson in 1998 reported about Dextran polymer technology which when used as indirect technique offered comparable sensitivity to the traditional avidin biotin technique and this could avoid labelling of endogenous biotin **[3]**. Immunohistochemistry has contributed tremendously to the field of routine surgical pathology, hemapathology, cytopathology, oncologic pathology and neuropathology [4].

Immunohistochemistry Procedure
Broadly IHC consists of 3 steps

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

Immunohistochemistry is a very special technique for in situ localisation of tissue constituent by means of specific antigen antibody interaction where the antibody has been tagged with a visible label through electron dense, fluorescent or enzyme markers [5].

Immunohistochemical techniques can be divided into following steps

1. Fixation step
2. Processing tissue for Embedding
3. Antigen retrieval techniques
4. Counterstaining following immunochemical staining
5. Mounting of slide

Fixation

The main cause of variation in the reproducibility of immunohistochemical staining is the tissue fixation. Usually neutral buffered formalin (10%) is used. To avoid overfixation it should be carried out for about less than 24-48 hours. Paraformaldehyde has been proven an excellent cross linking agent although not good for many membrane proteins while recently microwave fixation has come up as better alternative causing minimal loss of protein and maintaining ultrastructure and specificity of labelling [9]. Microwave alone or in combination with other fixatives has yielded excellent preservation of cell structure, maintaining antigenicity and not affecting staining quality [6, 7].

To summarize following types of fixatives can be used,

1. **Aldehydes**: formaldehyde (paraformaldehyde, formalin); glutaraldehyde; acrolein; glyoxal; formaldehyde mixtures containing mercuric chloride, acetic acid, zinc, and periodate lysine
2. **Protein-denaturing agents** (precipitants): acetic acid; methanol; ethanol; industrial methylated spirits
3. **Oxidizing agents**: osmium tetroxide; potassium permanganate; potassium dichromate
4. **Other cross-linking agents**: carbodiimides
5. **Physical**: heat; microwaves
6. **Miscellaneous/unknown**: nonaldehyde-containing fixatives, acetone, picric acid [8].

Processing Tissues for Paraffin Wax Embedding

Paraffin wax acts as an embedding medium that supports the tissue during microtomy. As fixatives employed for tissues destined for paraffin embedding are aqueous in nature and paraffin wax is hydrophobic, there is a need for an intermediate phase that will be miscible with both. Histological-grade paraffin wax typically melts between 50 and 60 °C, but exposing tissues to this temperature can have deleterious effects on the staining of some antigens, for example vimentin. Tissue sections are also exposed to such temperatures during slide drying, in order to increase their adherence to the slide. The duration and intensity of heating tissues during embedding or slide drying should therefore be kept to a minimum and certainly no longer than overnight [8].

Table 1: Advantages and Disadvantages of Some Embedding Resins [9].

Embedding Resins	Advantages	Disadvantages
Acrylic resins	1. Water compatible 2. Background labelling is reduced 3. Low temperature is required hence stabilises antigen	1. Weak adherence between acrylic resins and tissue. 2. Tissue shrinkage
Epoxy resins	1. Best morphological tissue preservation and reliable immunostaining. 2. uniform polymerisation	1. Occasionally decrease immunolabelling intensity 2. Highly hygroscope

Antigen Retrieval Techniques

Formaldehyde fixation leads to a major artifact that is masking of tissue antigens due to crosslinking of protein amino acid residues involving hydroxymethylene bridges. Hence there are techniques devised for antigen retrieval by unmasking the antigen epitope that have been altered by fixation, processing and storage or resin interaction [9].

Table 2: Antigen Retrieval Methods

Methods	Possible mechanisms
Enzymatic digestion	
Trypsin	Etching effect due to protein cleavage
Pronase	
Pepsin	
Acid treatments	Solubilisation of amyloid proteins
Formic acid	
Alkaline hydrolysis	Saponification
Detergent	Solubilisation
Urea solution	Protein structural unfolding
Refixation with Zn- solution	Protein precipitation
Freeze and thawing	Increase in membrane penetration
Freeze and drying	
Heating	
Simple heating	
High temperature heating	Protein structureal unfolding
Microwave	
Autoclave	
Mechanical forces	Unfolding and aggregation
Ultrasound treatment	
Pressure	

The purpose of carrying out of these steps in immunohistochemical studies is to create the conditions for specific signal growth and to reduce the background. Labelling density is believed to be doubled after antigen retrieval procedure for most fixatives, largest reported with use of 4% paraformaldehyde and 2% formaldehyde [9].

The two most common methods employed for antigen retrieval comprise of

1. Antigen retrieval with enzymes-Protease induced eptitope retrieval was first introduced by Huang *et al*. Other enzymes used for this purpose are trypsin, proteinase K, pronase, ficin and pepsin. The disadvantage of this method being low number of antigen available for staining while possible alteration of tissue and epitope was suspected which led to advent of newer technique [10].
2. Heat induced epitope retrieval (HIER) – It was introduced by Shi *et al* and worked on principle that the chemical reaction between protein and formalin could be reversed atleast in some part by high temperature or strong alkaline hydrolysis. Several solutions have been employed in HIER. Sometimes multiple antigen retrieval methods are applied [10].
3. Miscellaneous - Pretreatment with concentrated formic acid improves the signal in some IHC tests. Another AR method is incubation of slides in strong alkaline solution, urea, acid solutions, borohydride, and a solution of sucrose [10].

Washing Steps

In the procedure of immunohistochemistry there can be antigen antibody precipitates which can cause an undesired background and hence interfering with the proper results. Therefore it is necessary to perform some steps of washing with PBS and distilled water thus removing traces of non-reacted antibody [9].

Labelling Methods with Conjugated Antibodies

The need to precisely reveal the scarce macromolecules, such as collagen type IV in basement membrane, led to elaboration of a wide spectrum of immunohistochemical amplification procedures. This antibody tagging is possible due to the fact that on heavy chain there is are free amine groups allowing the chemical attachments of another molecule [9]. The various techniques in labelling of antibodies are:

1. Direct method which consist of reaction of primary antibody with a label including fluorochromes, enzymes, colloidal gold and biotin [10]. Some of these are discussed further as :

Labelling With Enzyme Tagged Antibody: Most commonly enzymes coupled with antibodies are peroxidase from horseradish and alkaline phosphatase. Perioxidase has great ability to enter rapidly into tissue because of its small size where it interacts with hydrogen peroxidase in presence of diaminobenzidine (chromogen) to form insoluble brown product which can be viewed under light microscope. While under electron microscope it shows electron dense products reacting along with osmium tetraoxide. Another used chromogen is 3-amino-9-ethylcarbazole (AEC) and 4-chloride-1-napthol and tetramethylbenzidine (TMB) [9].

Labelling With Fluorochrome Tagged Antibody: Fluorochrome represents that class of marker which is used in immunohistochemistry of extracellular matrix molecules which is able to detect components with high sensitivity and selectivity. Most commonly used fluorochromes are fluoresceine, rhodamine, texas red, phycoerythrin. Whereas fluorochrome coupled antibodies are conjugates of fluoresceine, rhodamine, of isothiocyanate type. Fluoresceine is the most often used fluorochrome having benefit of strong fluorescence which differs from tissue autofluorescence and is most sensitive to pH of 8-9. Two major shortcomings of this method are background labelling and photobleaching of tissue [9].

Labelling With Colloidal Gold Tagged Antibody: Colloidal gold is a non-discolouring, electron dense marker which has ability to bind to several molecules. It is used in light microscopy following autometallography and at polarised light, the wide range of colloidal gold size allows simultaneous location of several antigen by electron microscopy. The variety of colloidal gold sizes range from 1 to 40 nm. Particles less than 1 nm must be silver enhanced to be visualised by transmission electron microscopy. However colloidal gold may also cause non-specific background labelling and it does not show staining in presence of low amounts of antigen [9].

2. Indirect methods on other hand include various techniques as [10].

- Avidin biotin complex method
- Labelled streptavidin biotin method
- Peroxidase antiperoxidase method
- Polymeric labelling two step method
- Tyramine amplification method
- Immune rolling circle amplification method (Antigen retrieval)

All avidin-biotin methods majorly rely on the strong affinity of avidin or streptavidin for the vitamin biotin. Streptavidin (from Streptomyces avidinii) and avidin (from chicken egg) both possess four binding sites for biotin. The biotin molecule is conjugated easily to antibodies and enzymes [13]. One major setback in this method is presence of endogenous biotin which causes significant background labelling which led to advent of polymer based immunohistochemical methods. These methods utilize a unique technology based on a polymer backbone to which multiple antibodies and enzyme molecules are conjugated. EPOS (Enhanced Polymer One Step) system based on dextran polymer and Envision system have also been recognized recently [9, 11].

Counterstaining following immunohistochemical staining

Few immunochemists appear to give much consideration to nuclear counterstains employed to visualize nuclear detail after immunochemical staining has been performed. However, it is important to get an appropriate contrast between the immunochemical stain and the nuclear counterstain. Counterstains can be classed into two groups: tinctorial which comprise of Hematoxylin, Toulidine blue, Methylene blue, Light green and Fast red stains. While fluorescent stains comprise of DAPI, Hoechst and Propidium iodide. Whichever nuclear counterstain is employed, it is important to use one of a different color to that of the label being used in order to avoid obvious confusion**.** Amongst all the tinctorial nuclear counterstains Hematoxylin is by far the most popular. Alum hematoxylin used are Harris's Hematoxylin, Mayer's Hematoxylin and Carazzi's Hematoxylin. Other tinctorial stains are not very popular in immunochemical application in comparison with hematoxylin. Manufacturer's instruction for staining should be followed [8].

Slide mounting

Slide mounting is done to preserve the already stained slides for longer period. It should not react with, leach or fade stains and reaction product from immunohistochemical procedures. The choice of mounting medium following immunochemical staining is mainly dictated by the label (and, in the case of enzymatic labels, the chromogen) used to visualize the antigen [5, 8].

Limitations of IHC

Some of the basic limitations are experience of pathologist as to selection of appropriate reagents and controls, availability of antibodies and antigen loss [12]. Apart from this some of the tumours have no specific antibodies available for their diagnosis, hence requiring multimodal approach [13]. Most often antibodies yield a false positive results thereby requiring testing of its specificity [14]. Finally the field of diagnostics has always dealt with interobserver and intraobserver bias which also exists in immunohistochemical analysis [15].

Conclusion

Immunohistochemistry is an upcoming sector of molecular diagnostics with advent and proposals of newer methods and modifications in existing protocols therefore the need of hour is to train pathologists in the methodology and interpretation of immunohistochemistry which will serve in a great way in diagnosis of diseases previously considered an enigma.

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