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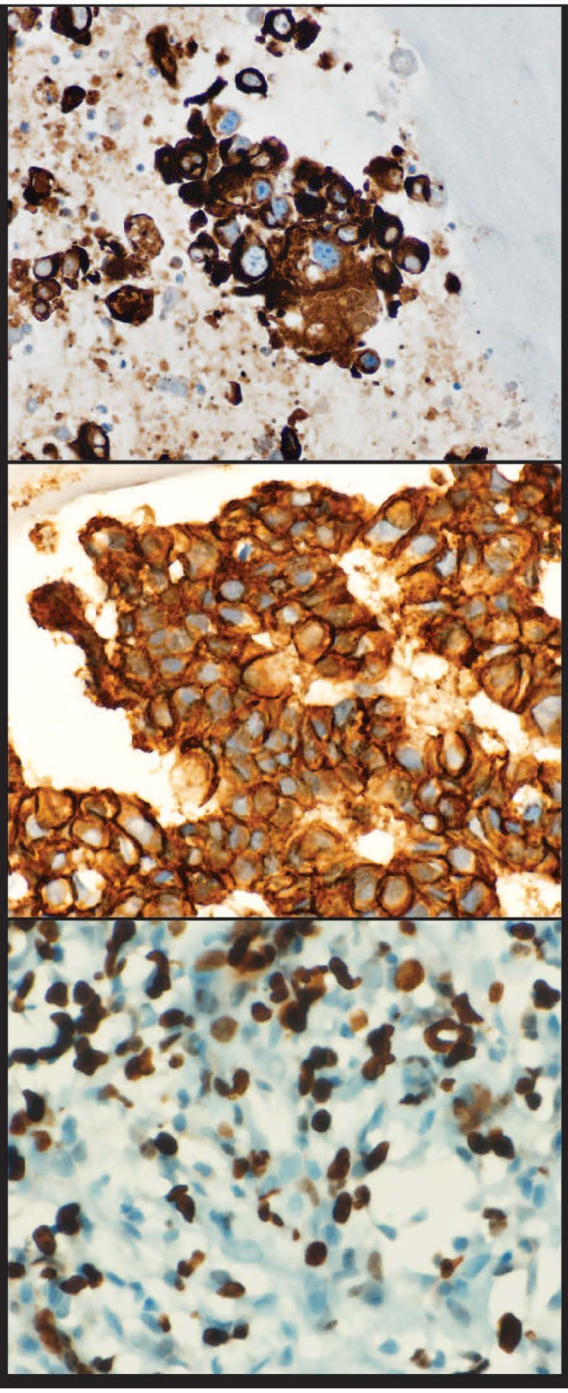

Pranab Dey

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CHAPTER 2

Visualization of an Antigen-antibody Reaction

INTRODUCTION

Antigen (Ag) and antibody (Ab) reaction cannot be visualized by light microscope. The detection system is needed by visualizing this reaction. The enzymes or fluorescent probes are used to demonstrate the Ag-Ab reaction. In the case of enzyme labelling a suitable substrate is used to develop an insoluble chromogenic product.

The various types of visualization system are described here (**Box 1**).

DIRECT METHOD

In the direct staining method, the primary Ab is tagged with the enzyme (**Fig. 1**). This Ab combines directly with the epitope of the Ag. Subsequently, with the help of chromogenic substrate, the Ag-Ab reaction is visualized.

Advantages

- Rapid technique
- Relatively simple

Disadvantages

- The Ab should be pure and highly specific otherwise erroneous staining my happen.
- The sensitivity of the direct staining technique is much lower than the indirect method.
- The various Ab should be labeled separately.
- A higher concentration of primary Ab is needed, and therefore, it is costly.

INDIRECT METHOD

Here the conjugated (enzyme/fluorochrome) secondary Ab is used against the unlabeled primary Ab. The Ag-Ab reaction

BOX 1 **Different technique of visualization of antigen-antibody reaction.**

Direct technique:

- The primary Ab is tagged with the enzymes
- Rarely used

Indirect method:

- The enzyme-conjugated secondary Ab is used against the unlabeled primary antibody

Peroxidase-antiperoxidase (PAP) method:

- The PAP reagent is a stable complex molecule that contains horseradish peroxidase (HRP) Ag and Ab against it. The bridging secondary Ab is used that binds with both the primary and PAP complex

Avidin and biotin method:

- The secondary Ab is chemically attached with biotin that reacts with the primary Ab
- The avidin conjugated to HRP binds with the biotinylated secondary Ab

Avidin and biotin complex (ABC) technique:

- Primary Ab, biotinylated secondary Ab, and ABC are used
- The biotinylated secondary Ab attaches with avidin of ABC

Biotin–streptavidin method:

- Primary Ab, biotinylated secondary Ab, and enzyme-conjugated streptavidin enzyme conjugated streptavidin
- Streptavidin binds with biotin

Polymer-based labelling method:

- A polymer is used as backbone with embedded multiple enzymes and secondary Abs

Tyramine signal amplification:

- The biotinylated tyramine is activated by HRP in the presence of hydrogen peroxide
- The activated biotinylated tyramide forms covalent bond with tyramine in the tissue and deposited in the vicinity of HRP
- The biotinylated tyramide is further demonstrated by HRP-conjugated streptavidin-biotin technique with 3,3'-diaminobenzidine (DAB) as substrate

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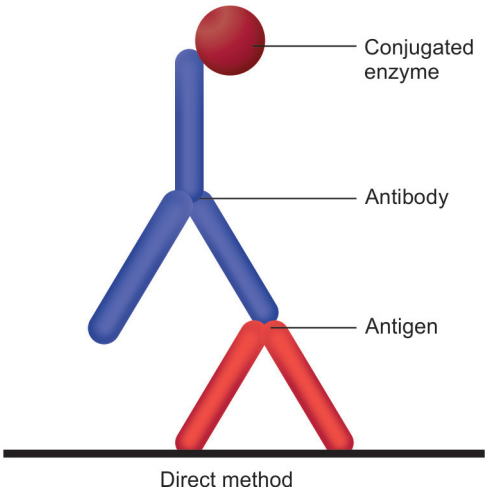

FIG. 1: Schematic diagram showing the direct technique of immunostaining.

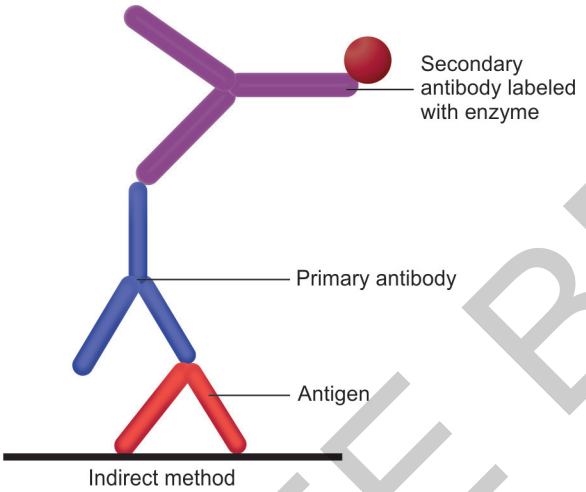

FIG. 2: Enzyme labeled secondary antibody against the primary antibody.

is demonstrated by a suitable substrate forming a colored product (**Fig. 2**).

Advantages

- The primary Ab is used in higher dilution, and so the cost is less.
- Easy to conjugate a single type of secondary Ab.
- The secondary Ab can be produced easily with high specificity against the primary Ab.
- Easy to have negative control by omitting the primary Ab.

PEROXIDASE-ANTIPEROXIDASE METHOD

The peroxidase-antiperoxidase (PAP) technique is introduced by Sternberger.[1] The PAP reagent is a stable complex molecule that contains horseradish peroxidase (HRP) Ag and Ab against it. The bridging secondary Ab is used that binds with both the primary and PAP complex (**Fig. 3**). The primary Ab and PAP complex must be of the same species, or they may have

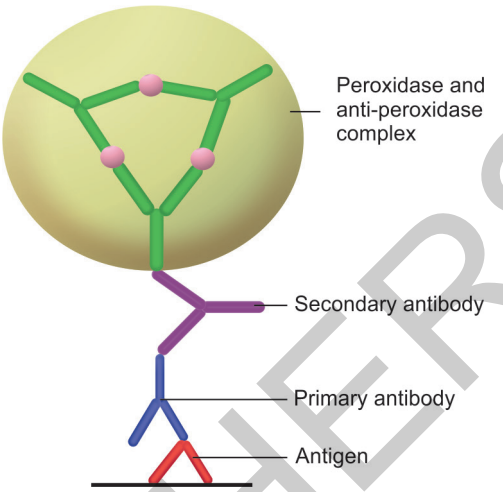

FIG. 3: Schematic diagram of peroxidase-antiperoxidase.

an almost similar antigenic determinant site. The secondary Ab is developed from another species and has specificity against both the primary Ab and Ab present within the PAP complex.

Advantages

Peroxidase-antiperoxidase method is a very sensitive technique.

AVIDIN AND BIOTIN METHOD

Avidin and biotin have high affinity with each other, and this is exploited in this technique. The secondary Ab is chemically attached with biotin that reacts with the primary Ab. Now the avidin conjugated to HRP is used that binds with the biotinylated secondary Ab (**Fig. 4**). The reaction is visualized by adding the substrate.

Advantages

- Relatively rapid technique
- Good sensitivity

Disadvantages

- The normal tissue may contain endogenous biotin and may produce false-positive reaction by binding with HRP-conjugated avidin. This reaction can be prevented by using a suitable blocking agent.
- The binding affinity between avidin-biotin may differ from batch to batch and thereby, may affect the sensitivity and reproducibility of the technique.

AVIDIN AND BIOTIN COMPLEX TECHNIQUE

The avidin and biotin complex (ABC) method is the modification of the previously described avidin-biotin technique.[2]

The essential ingredients of this technique are: (1) Primary Ab, (2) Biotinylated secondary Ab, and (3) ABC.

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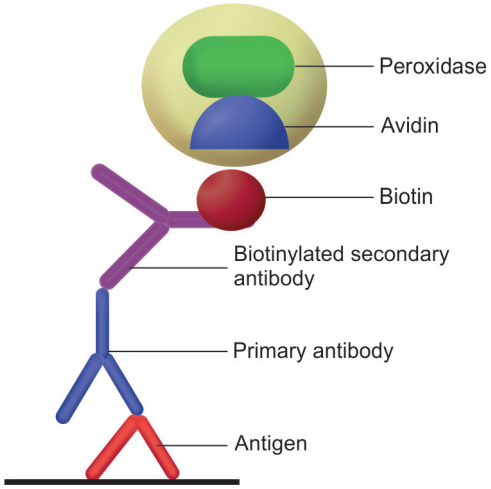

FIG. 4: Schematic diagram showing avidin-biotin technique.

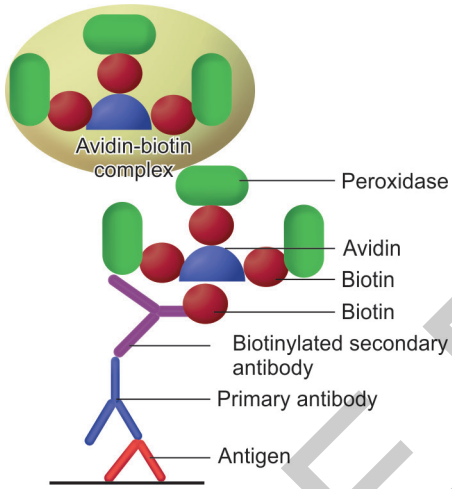

FIG. 5: Avidin-biotin complex technique.

The preformed ABC contains avidin and biotin-labeled peroxidase. This large lattice-like molecule of ABC has a strong affinity with biotin molecule. The secondary Ab is also tagged with biotin that attaches with avidin of ABC as the ABC molecule contains multiple peroxidase enzymes, so the signal intensity is high (**Fig. 5**).

Advantages

- Sensitivity is many-fold higher than simple avidin-biotin technique.
- High dilution of primary Ab can be used.

Disadvantages

Endogenous biotin in tissue may also react with ABC.

BIOTIN–STREPTAVIDIN METHOD

In the biotin–streptavidin (B–S) technique instead of avidin, we use enzyme-conjugated streptavidin that has a very high affinity with biotin (**Fig. 6**). The B-S technique is better than the avidin-biotin method due to its higher sensitivity.

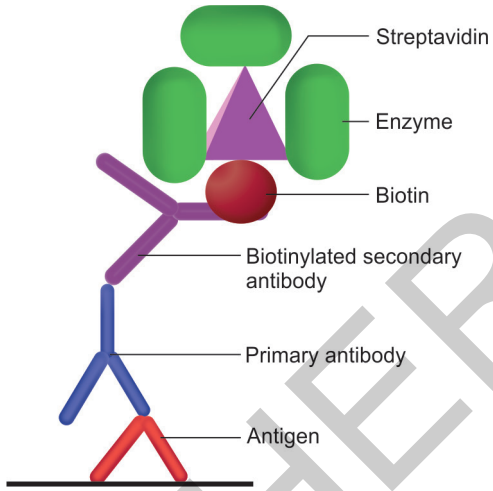

FIG. 6: The schematic diagram showing the streptavidin and biotin technique.

Advantages

- Streptavidin does not contain any carbohydrate, so there is no cross-reaction with a lectin-like molecule that is present in liver, kidney, etc.
- The streptavidin-enzyme complex is more stable, and therefore, the diluted form can be stored a long time and immediately usable.
- No nonspecific staining develops in this technique.

ALKALINE PHOSPHATASE–ANTIALKALINE PHOSPHATASE METHOD[3]

The alkaline phosphatase–antialkaline phosphatase (APAAP) technique is not used nowadays. Here, similar to PAP technique, we apply a complex of APAAP along with a secondary Ab that binds both the complex and primary Ab. The primary Ab and the Ab in the APAAP complex should be from the same species, and secondary Ab should be from some other species.

Advantages

- The APAAP technique is useful in the tissue sections that are rich in endogenous peroxidase.
- Dual labelling with PAP technique.
- The APAAP complex is relatively stable.

POLYMER-BASED LABELING METHOD

Polymer-based labeling method is a simple and very sensitive technique. Here a polymer is used as a backbone with multiple embedded enzymes and secondary Abs.[4] The polymer can be made of dextran, dendrimers or polypeptides. Recently micropolymer of very small size is used to prevent steric hindrance. This system is extremely sensitive because each polymer contains a large number of secondary Abs and

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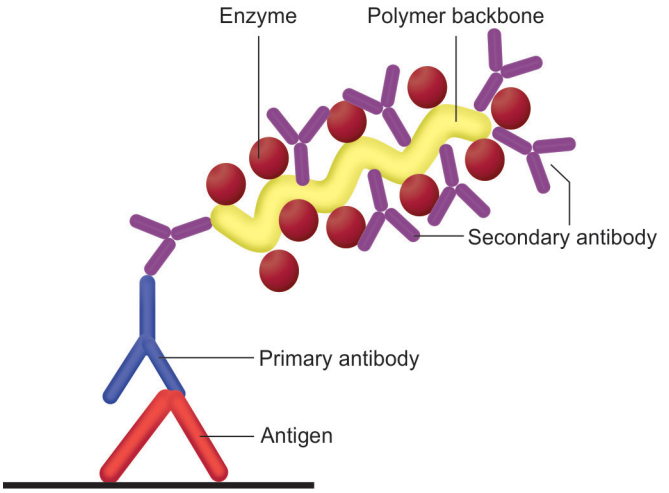
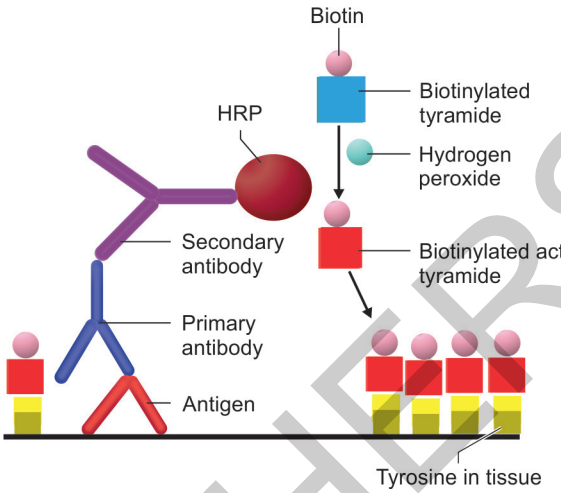


FIG. 7: Polymer-based labelling method.



(HRP: horseradish peroxidase)

FIG. 8: Tyramine signal amplification method.

enzymes (**Fig. 7**). As no biotinylated Ab is used so there is no need to use any blocking agent to prevent cross-reaction with tissue biotin.

Advantages

- Extremely sensitive technique as a large number of enzymes are embedded in polymer.
- Rapid, simple two steps method.
- No cross staining as no biotin or avidin is used.

TYRAMINE SIGNAL AMPLIFICATION

Tyramine signal amplification (TSA) method is also called a catalyzed reporter deposition technique. It is based on the deposition of the activated biotinylated tyramine.[5] The biotinylated tyramine is activated by the oxidizing agent HRP in the presence of hydrogen peroxide. Subsequently, this activated-biotinylated tyramide forms a covalent bond with electron rich tyramine in the tissue and deposited in the vicinity of HRP (**Fig. 8**). The biotinylated tyramide is further demonstrated by HRP-conjugated streptavidin-biotin technique with 3,3'-diaminobenzidine (DAB) as a substrate.

Steps of TSA:

- At first, complete the step up to the secondary Ab with HRP.
- Now add biotinylated tyramine.
- After proper wash add HRP attached streptavidin.
- Develop color by adding DAB substrate.

MULTIPLE IMMUNOCYTOCHEMISTRY IN A SINGLE SECTION

Multiple immunocytochemistry in a single tissue section helps to detect the various Ag in the tissue localized in the different cell or the same cell in the different subcellular compartment. It provides crucial information about the distribution of the Ags and the character of the neighboring cells.

The prerequisite before the designing of the multiple staining:

- *The target section*: Formalin-fixed paraffin-embedded or cytology smear
- *Reagents available*: Primary Ab of the same species or different species and also the secondary Ab against the primary Ab from the same or different species.
- *Sequential or simultaneous staining*: The staining of all the Ags at the same time happens in the simultaneous staining, and it requires less time whether the staining of the Ag in a stepwise manner is sequential and needs more time.
- Nature of the target antigen.

Selection of primary Ab: The selection of the primary Ab from the different host species is the simplest technique.

Selection of secondary Ab: The secondary Abs are usually raised against the primary Ab of the host species. However, these different secondary Abs are developed in the same species. The normal serum of the host species of the secondary Ab is used as blocking agent to prevent the nonspecific immunostaining.

Selection of the enzymes and substrates: The enzyme-substrate combination should be designed in such a way to have red-blue combination than red-brown combination because red-blue combination of the final color is easy to distinguish. To develop blue color chromogenic product alkaline phosphatase and fast blue BB is the most popular combination, whereas, for the red color development, HRP and AEC (3-amino-9-ethylcarbazole) is the desirable combination.

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Steps:

- After Ag retrieval, please wash the sections
- Apply primary Abs
- Wash
- Endogenous biotin blocking
- Wash
- Apply secondary Ab
- Peroxidase blocking by hydrogen peroxide
- Wash
- Incubate with New fuchsin chromogen
- Wash
- DAB
- Wash
- Mount and see

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This book highlights the basic technique and diagnostic applications of immunocytochemistry in cytology. The author has provided the recent information in this area which may be very helpful for the pathology students, technical staffs, and pathology practitioners. There are a large number of tables, boxes, and microphotographs in this book to facilitate the interpretation of immunocytochemistry in various tumors. This is a concise and informative book in this subject.

Pranab Dey is a renowned cytologist in India. He is working in Cytology and Gynecologic Pathology for more than 30 years. Presently, he is a Professor in the Department of Cytology and Gynecologic Pathology in the Postgraduate Institute of Medical Education and Research, Chandigarh, India. Dr Dey is the author of nine more books in Cytology and Gynecologic Pathology.

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