CHAPTER 14

Cellular–Molecular Diagnostic Techniques in Clinical Practice

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LEARNING OBJECTIVES

FROZEN/CRYOSTAT MICROTOMY TECHNIQUES
- Frozen section technique
- Cryostat section technique
- Cutting of tissue section
  - Ultramicrotomy
  - Laser microtomy

ELECTRON MICROSCOPY AND POLARIZING LIGHT MICROSCOPY
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- Polarizing light microscopy

TISSUE FIXATION, DECALCIFICATION, HANDLING OF SURGICAL SPECIMENS AND HISTOCHEMICAL STAINS
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- Tissue decalcification
- Handling of surgical specimens
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FROZEN/CRYOSTAT MICROTOMY TECHNIQUES

FROZEN SECTION TECHNIQUE

Frozen section technique is used for rapid diagnosis, study of margins of malignant neoplasm, enzyme histochemistry, demonstration of lipid and molecular studies. Drawbacks of frozen section technique include distortion of morphology of tissue and cellular cytomorphologic details and poor staining. Tissue sample must reach the histopathology laboratory immediately. It should be kept in normal saline to prevent drying of sample. The tissue can directly be taken to the cryostat or can be fixed with 10% formalin or formalin-alcohol.

- Frozen sections are stained normally, because this is faster for one or a few individual sections. Toluidine blue stain generally used as a progressive stain to demonstrate adipose tissue in which the section is left in contact with the stain until the desired tint is achieved.

Table 14.1 Examples of fresh frozen section evaluation

Anatomic Sites	Indications	Comments
Skin	▪ Squamous cell carcinoma (eyelid, nose or ear) ▪ Basal cell carcinoma (eyelid, nose or ear) for evaluation of margins	Not indicated for melanoma skin
Breast	Breast carcinoma	Evaluation of margins is sometimes required
Lymph nodes	Metastases of breast carcinoma in lymph nodes (sentinel node)	Sensitivity (60%)
Thyroid	Papillary carcinoma of thyroid (imprint smear cytology for nuclear groove)	Not suitable for follicular neoplasms
Female genital system	Intraoperative ovarian neoplasm (most important)	Not suitable for cervical carcinoma
Pancreas	Intraoperative pancreatic carcinoma to look for margins	Accuracy 98%

- The Oil red O (ORO) stain can identify neutral lipids and fatty acids in touch smears and tissues.
- Fresh touch smears or cryostat sections of tissue are necessary because fixatives containing alcohols, or routine tissue processing with clearing will remove lipids.
- The Oil red O stain is used to identify lipid-laden macrophages in bronchoalveolar lavage (BAL) cytologic samples to diagnose fat embolic syndrome (FES).
- Other fat stains are Sudan black, Sudan III and Sudan IV. Examples of fresh frozen section evaluation are given in Table 14.1.

CRYOSTAT SECTION TECHNIQUE

Cryostat is a device by which temperature can be maintained at a low level. In histopathology, cryostat consists of a microtome within its chamber for cutting sections of frozen tissue. The cryostat is essentially an ultrafine 'deli-slicer', called a microtome, placed in a freezer. It is usually stationary upright freezer, with an external wheel for rotating the microtome.

- The temperature within cryostat can be varied, depending on the tissue being cut—usually – 20° to –30°C. Low temperature may be maintained within cryostat by using various refrigeration methods, most commonly using cryogenic fluid bath such as liquid helium or nitrogen.
- Although cryosections are physically less stable than paraffin-embedded tissue sections. Cryosections are generally superior for the preservation of antigenicity and therefore the detection of antigens by immunohistochemistry. The preparation of cryosections does not involve the dehydration process. Rapid freezing of tissue reduces the formation of ice crystals, thus minimizes morphologic damage.

CUTTING OF TISSUE SECTION

ULTRAMICROTOMY

Ultramicrotomy is a technique for cutting tissue specimens into extremely thin transparent sections. The ultrathin sections can be studied at different magnifications in a transmission electron microscope (TEM).

- Ultrathin sections must be very thin because the 50–125 kV electrons of the standard electron microscope cannot pass through biological material much thicker than 150 nm. For best resolutions, tissue sections must be 30–60 nm in thickness.
- Prior to the cutting ultrathin sections, the biological materials are usually placed in a more rigid fixative used in the process known as embedding. This is achieved by the inflow of a liquid substance around the sample, such as paraffin or epoxy.

LASER MICROTOMY

Recent development in the microtomy is the use of a laser microtome, which has a capacity to cut in few seconds. No sample preparation is required. The thickness of tissue sections varies 10–100 nm. Using a vibrating knife improves the results when dealing with soft materials. This is the only technique yielding directly 30 nm sections of constant thickness. Laser microtomy technique cannot be applied to very heterogenous, very hard, very soft and very fragile samples.

ELECTRON MICROSCOPY AND POLARIZING LIGHT MICROSCOPY

ELECTRON MICROSCOPY

Electron microscope (EM) was invented by Ernst Ruska in 1933, for which he was awarded the Nobel Prize in Physics in 1986.

- Electron microscopes are scientific instruments that use a beam of energetic electrons to examine objects on a very fine scale. It was developed due to the limitations of resolution power of light microscope that is limited by physics of light.
- Electron microscopes are basically of two types: (a) transmission electron microscopy (TEM); (b) scanning electron microscopy (SEM).
- Principle of light microscopy, transmission electron microscopy and scanning electron microscopy is shown in Fig. 14.1A to C. Technical processes used for electron microscopy is given in Table 14.2.

Table 14.2 Technical processes used for electron microscopy

Process	Methodology
Fixation of tissue	2% glutaraldehyde
Processing of tissue	▪ Application of tetraoxide in post-fixed tissue ▪ Dehydration done in a graded ethanols ▪ Infiltration of tissue with EM epoxy resin
Embedding	Tissue embedded in mold
Sectioning of tissue	▪ Heating of resin infiltrated tissue hardened into blocks ▪ Sections obtained with microtome ▪ Sections but on a specimen grid
Electron microscopy	▪ Section image studied under electron microscope ▪ Digital image obtained and analyzed

TRANSMISSION ELECTRON MICROSCOPY

In transmission electron microscopy (TEM) source is a beam of high velocity electrons accelerated under vacuum, focused by condenser lens (electromagnetic bending of electron beam) onto specimen, which causes loss and scattering of electrons by individual parts of specimen. Emergent electron beam is focused by objective lens. Final image is formed on a fluorescent screen for viewing.

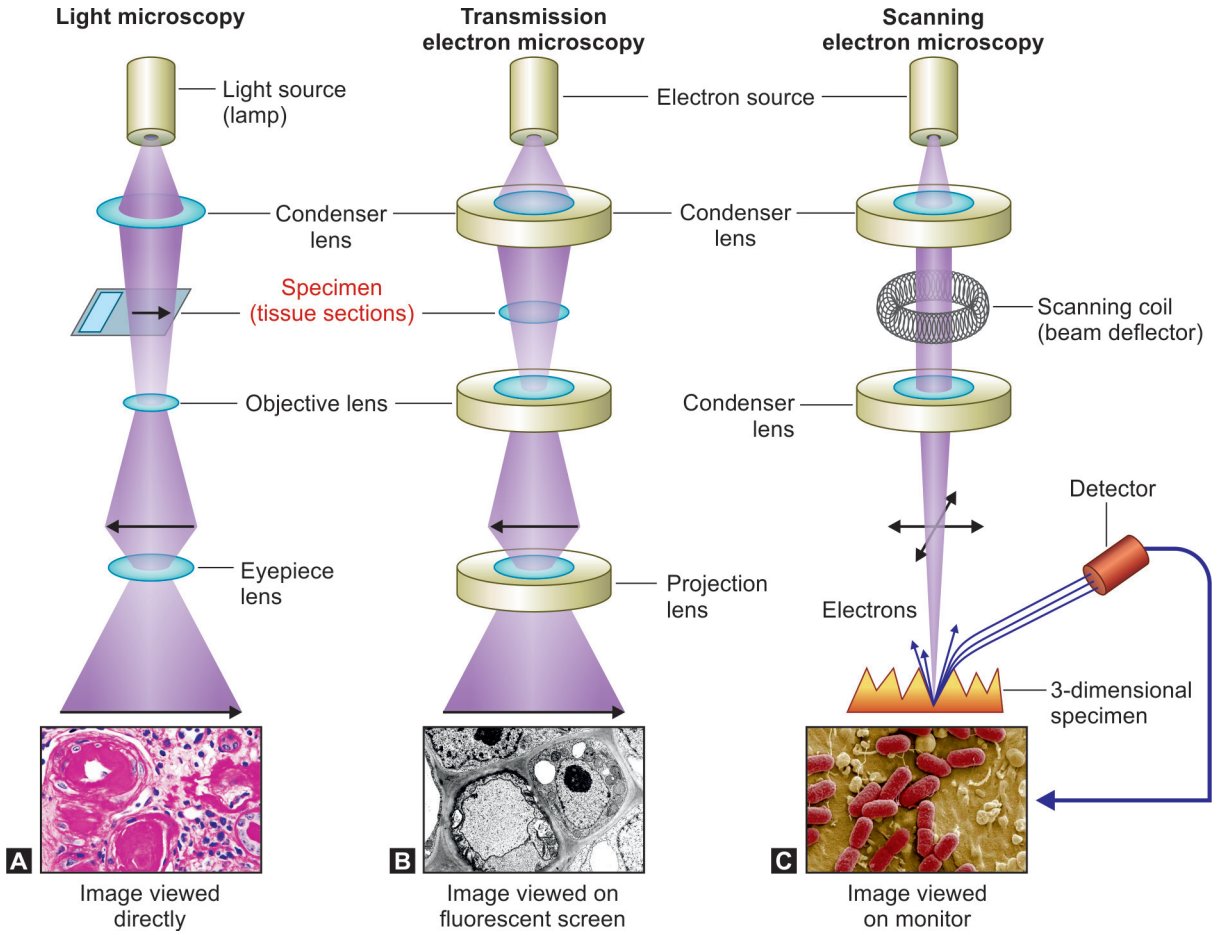

Fig. 14.1A to C: Principle of light microscopy, transmission electron microscopy and scanning electron microscopy.

- Standard transmission electron microscope has a resolving power to about 15–30 angstroms.
- Magnified images are typically formed from 1000X to 50,000X. By using fine grain photographic film, it is possible for further enlargements of images from 150,000X to 1,000,000X.

SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) differs from standard transmission electron microscopy in that the electrons are bounced off the surface of an object with SEM, not passed through it. The major advantages of SEM are the great depth of field (more of the specimen can be in focus at once) and simple specimen preparation.

Principle of Scanning Electron Microscopy

The basic principle of scanning electron microscopy is that a beam of electrons is generated by a suitable source, typically a tungsten filament or a field emission gun.

- The electron beam is accelerated through a high voltage and passes through a system of apertures and electromagnetic lenses to produce a thin beam of electrons. Then electron beam scans the surface of the specimen.
- Electrons are emitted from the specimen by the action of the scanning electron beam and collected by a suitably positioned detector.
- Comparison between light microscope and electron microscope is given in Table 14.3. Comparison between transmission electron microscopy and scanning electron microscopy is given in Table 14.4.

Applications of Scanning Electron Microscopy in Surgical Pathology

Scanning electron microscopy is a useful diagnostic technique to supplement morphologic, immunohistochemical, cytogenetic and molecular analysis of tissues, although immunoperoxidase techniques have largely replaced EM for tumor diagnosis in surgical pathology.

- Scanning electron microscopy is used to diagnose alcoholic liver disease, genetic disorders, neoplasms, glomerulonephritis and identification of microorganisms.
- Diagnostic utility of electron microscopy in various disorders is given in Table 14.5.

POLARIZING LIGHT MICROSCOPY

Some materials such as crystals or fibers have the property of birefringence which is the ability to pass light in a particular plane. Such materials (crystals or fibers) are called anisotropic substances because of this property.

- Normally, most materials are isotropic substances because any light that passes through them will be scattered in all directions.

Table 14.3 Comparison between light microscope and electron microscope

Light Microscope	Electron Microscope
Small and portable	Very large and operated in a special room
Resolution is 200 nm	Resolution is 0.4 nm (in SEM) or 0.5 Å (in TEM)
Magnification is 1000X	Magnification is 2 million (in SEM) or 50 million (in TEM)
It is unaffected by magnetic fields	It is affected by magnetic fields
Preparation of material is quick and simple	Preparation of material is lengthy and requires expertise
Preparation rarely distorts the material	Preparation may distort the material
Cheap to purchase and operate	Expensive to purchase and operate
Natural color of object is observed	Images are in black and white

Table 14.4 Comparison between transmission electron microscopy and scanning electron microscopy

Transmission Electron Microscopy (TEM)	Scanning Electron Microscopy (SEM)
TEM is based on transmitted electrons	SEM is based on scattered electrons
Electrons are directly pointed towards the sample in TEM	The scattered electrons in SEM produce the image of sample after the microscope collects and counts the scattered electrons
TEM shows the sample as a whole	SEM shows the sample bit by bit
TEM has up to 50 million magnification	SEM has up to 2 million magnification
TEM provides a two-dimensional image	SEM provides a three-dimensional image
The resolution in TEM is 0.5 Å (1 Å = 10^{-10} m)	The resolution in SEM is 0.4 nm (1 nm = 10^{-9} m)

Table 14.5 Diagnostic utility of electron microscopy in various disorders

Subcellular Features in Cell or Tissue	Diagnostic uses in Clinical Practice
Genetic disorders	
▪ Cilia in epithelial cells	▪ Primary ciliary dyskinesis
▪ Lysosomes in neurons	▪ Demonstration of lipids and mucopolysaccharides in tissues
▪ Absence of peroxisomes in liver and kidneys	▪ Zellweger syndrome and neonatal adrenoleukodystrophy
Acquired non-neoplastic disorders	
▪ Increased number of peroxisomes in hepatocytes	▪ Alcoholic liver disease, chronic venous congestion, oral contraceptives
▪ Siderosomes in mitochondria	▪ Sideroblastic anemia
Neoplasms	
▪ Intercellular junctions in epithelial neoplasms and lymphoma	▪ Distinction between carcinomas and lymphomas (intercellular junctions present in carcinomas and absent in lymphomas)
▪ Intracellular or intercellular in glandular epithelium	▪ Diagnosis of adenocarcinoma
▪ Microvillous core rootlets in glandular epithelium of intestines	▪ Diagnosis of metastatic deposits from gastrointestinal carcinoma
▪ Cytoplasmic tonofibrils in squamous epithelium	▪ Diagnosis of squamous differentiation of epithelial neoplasms
▪ Premelanosomes and melanosomes in melanotic cells	▪ Diagnosis of melanoma
▪ Neurosecretory granules in neuroendocrine and neuroectodermal cells	▪ Diagnosis of neuroendocrine and neuroectodermal neoplasms
▪ Birbeck granules in Langerhans' cells	▪ Diagnosis of Langerhans' cell histiocytosis
Miscellaneous disorders	
▪ Viruses and parasites in solid tissues, fecal specimens and body fluids	▪ Identification of infectious agents in tissues
▪ Electron dense deposits in glomeruli	▪ Identification and classification of glomerulonephritis
▪ Electron dense deposits present adjacent to vascular smooth muscle	▪ Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) syndrome

- When viewed under polarized light, however, anisotropic materials will be brightly visible in one plane (birefringent), but will be dark in a plane turned 90°. Principle of polarizing microscope is shown in Fig. 14.2A and B.

POSITIVE/NEGATIVE BIREFRINGENCE

The birefringence is categorized into positive or negative based on the property of birefringence in which rays of light travelling through the anisotropic material

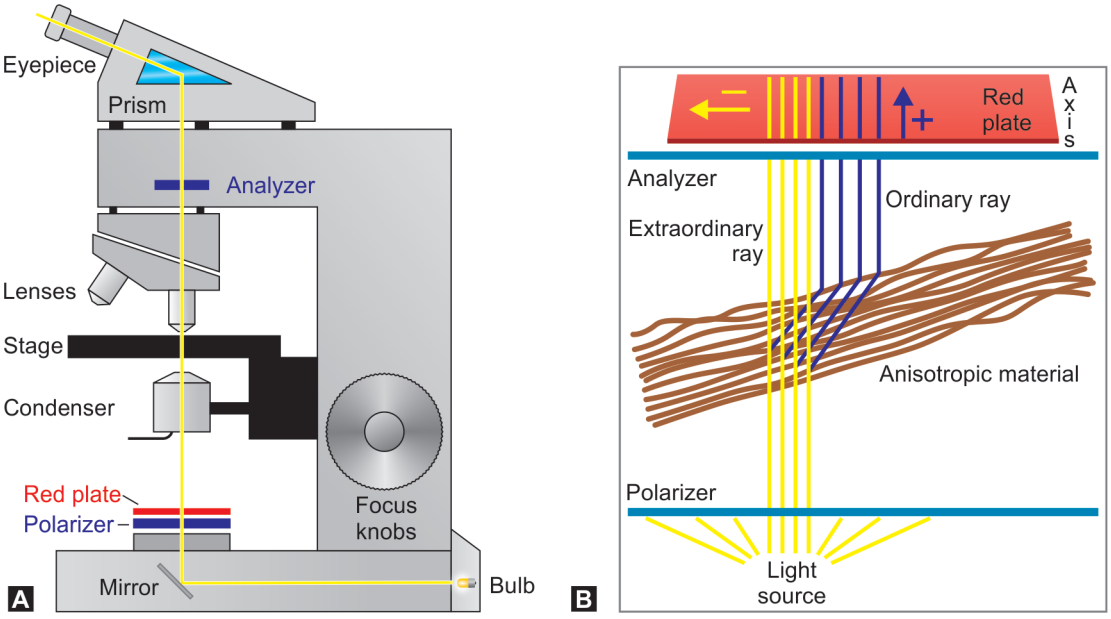

Fig. 14.2A and B: Principle of polarizing microscope.

in perpendicular planes (at right angles) will travel at different velocities through the material.

BIREFRINGENT MATERIAL REFRACTIVE INDEX

Birefringent material has two refractive indices: high for fast rays and small for slow rays. Fast rays travel in parallel with the crystalline structure of the birefringent material than the extraordinary's low rays traverses the material. Negative birefringence occurs when the ordinary ray becomes the slow ray when it is reflected and travels across the crystalline structures.

POLARIZER AND ANALYZER FILTERS IN POLARIZING MICROSCOPE

There are two polarizing filters made of glass or plastic material in polarizing microscope: polarizer and analyzer. These filters pass light in only one plane.

APPLICATIONS OF POLARIZING MICROSCOPY

Exogenous crystalline material, such as talc crystals, is demonstrated by polarizing microscopy found in subcutaneous injection site, lungs, and organs of the mononuclear phagocyte system of persons engaging in injection drug use. Applications of polarizing microscopy in clinical practice are given in Table 14.6.

Table 14.6 Applications of polarizing microscopy in clinical practice

Disorders	Characteristics
Sodium urate crystals gout	Negative birefringence
Calcium pyrophosphate crystals	Positive birefringence
Amyloid deposits in various organs	Apple green birefringence
Silicosis in organs	Insignificant finding in lungs and lymph nodes
Formalin-heme pigment (artefact of poor fixation)	Bright white birefringence
Collagen: Collagen fibrils	Dull yellow-white

TISSUE FIXATION, DECALCIFICATION, HANDLING OF SURGICAL SPECIMENS AND HISTOCHEMICAL STAINS

TISSUE FIXATIVES

Tissue fixatives preserve tissues in their natural state and fixes all components by denaturing or precipitating proteins. The ideal tissue fixative would prevent autolysis and bacterial decomposition. Microorganisms are also fixed and killed during tissue fixation. Most tissue fixatives act by denaturing or precipitating proteins which then form a sponge or meshwork tending to hold the other cell constituents. Formaldehyde is tissue fixative of choice.

CLASSIFICATION OF TISSUE FIXATIVES

Tissue fixatives are classified according to mechanism of action: aldehydes (formaldehyde and glutaraldehyde), mercurials, alcohols, oxidizing agents and picrates (Table 14.7), routine 10% formalin saline tissue fixative (Table 14.8), routine 10% buffered formalin tissue

Table 14.7 Tissue fixatives used in histopathology

Categories of Tissue Fixatives	Examples of Tissue Fixatives	Advantages of Tissue Fixatives	Disadvantages of Tissue Fixatives
Aldehyde fixatives	▪ Neutral formalin (10%) ▪ Buffered formalin (10%) ▪ Buffered glutaraldehyde (%)	▪ Immunoperoxidase techniques ▪ Immunoperoxidase techniques ▪ Prevents formalin-heme pigment ▪ Electron microscopy	▪ Formalin-heme pigment ▪ Nil ▪ Not good for immunoperoxidase techniques ▪ Nil
Mercurial fixatives	Zenker's fixative	Hematopoietic and reticuloendothelial tissues, i.e. lymph nodes, spleen, thymus, and bone marrow	Marked shrinkage and hardness of tissues. It reduces the amount of demonstrable glycogen
Alcohol fixatives	Carnoy's fluid	▪ Suitable for small biopsies giving nuclear details ▪ Preserves glycogen	Causes lysis of RBCs, dissolution of lipid and myelin; and shrinkage of tissue

Contd...

Table 14.7 Tissue fixatives used in histopathology (*Contd...*)

Categories of Tissue Fixatives	Examples of Tissue Fixatives	Advantages of Tissue Fixatives	Disadvantages of Tissue Fixatives
Chromate fixatives	Helly's fluid	Used to demonstrate chromaffin tissue (adrenal medulla), mitochondria and preservation of phospholipids	Glycogen preservation poor
Picrate fixatives	Bouin's fluid fixative (40% formalin, picric acid and glacial acetic acid)	▪ Testicular, gastrointestinal tract, and endocrine tissue biopsies ▪ Gives yellow color to the tissue and gives good nuclear details	▪ Makes tissue hard and brittle ▪ Lysis of RBCs, reduce amount of demonstrable ferric iron

Table 14.8 Routine 10% formalin saline tissue fixative

Constituents	Composition
Formalin	100 ml
Water	900 ml
Sodium chloride	8.5 g

Disadvantage: Routine 10% formalin saline fixative forms formalin-heme pigment giving black discoloration to tissues.

fixative (Table 14.9), composition of Zenker's tissue fixative (mercurial fixative) (Table 14.10), Schaudinn's sublimated alcohol tissue fixative (mercurial tissue fixative) (Table 14.11), composition of Carnoy's fluid (alcohol tissue fixative) (Table 14.12), Orth's fluid tissue fixative (chromate tissue fixative) (Table 14.13) and Regaud's (Moller's) fluid (chromate tissue fixative) (Table 14.14).

Table 14.9 Routine 10% buffered formalin tissue fixative

Constituents	Composition
Formalin	100 ml
Water	900 ml
NaH_2PO_4	3.5 g
Na_2HPO_4	6.5 g

Advantage: 10% buffered formalin prevents formation of formalin-heme pigment.

Table 14.10 Composition of Zenker's tissue fixative (mercurial tissue fixative)

Constituents	Composition
$HgCl_2$	50 g
Potassium dichromate	25 g
Sodium sulfate	10 g
Distilled water	1000 ml

Mercurial fixatives are used to fix lymph nodes, spleen, thymus, and bone marrow.

Table 14.11 Schaudinn's sublimated alcohol tissue fixative (mercurial tissue fixative)

Constituents	Quantity
$HgCl_2$	3 g
Distilled water	50 ml

Schaudinn's sublimated alcohol fixative is most useful fixative for making smears of loose cells on a slide.

Table 14.12 Composition of Carnoy's fluid (alcohol tissue fixative)

Constituents	Quantity
Absolute ethyl alcohol	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

Table 14.13 Orth's fluid tissue fixative (chromate tissue fixative)

Constituents	Composition
Potassium dichromate (2.5%)	100 ml
Formalin solution	10 ml

Formalin solution is added just before using as fixative.
Chromate fixatives are used to demonstrate chromaffin tissue (adrenal medulla), mitochondria, Golgi apparatus and mitotic figures.

Table 14.14 Regaud's (Moller's) fluid (chromate tissue fixative)

Constituents	Composition
Potassium dichromate (3%)	80 ml
Formalin	20 ml

Formalin solution is added just before using it as fixative.
Chromate fixatives are used to demonstrate chromaffin tissue (adrenal medulla), mitochondria, Golgi apparatus and mitotic figures.

Table 14.15 Factors affecting fixation of tissue

Parameters	Characteristics
Concentration of fixative	▪ Formalin 10% used for tissue fixation ▪ Glutaraldehyde 0.25–4% used for electron microscopy
Volume of fixative	10–20 times of tissue sample
Agitation of specimen	Enhances tissue fixation
Buffers used to prevent formalin-heme pigment	▪ Neutral buffered formalin prevents excessive acidity and formation of formalin-heme pigment ▪ Common buffers (phosphate, bicarbonate, cacodylate and veronal)
Penetration of tissue fixative	▪ Formalin and alcohol fixatives (best penetration) ▪ Glutaraldehyde (worst penetration) ▪ Mercurial fixatives somewhat in between ▪ Sectioning of specimen 2–3 cm enhances fixation
Temperature	Increasing temperature of formalin enhances the speed of tissue fixation
Time interval between fixation and removal of tissue	Tissue should be fixed faster and removed within 6–8 hours. Longer tissue fixation leads to nuclear shrinkage and loss of organelles

Formalin used to fix tissue forms formalin-heme pigment.

FACTORS AFFECTING TISSUE FIXATION

There are a number of factors that will affect the fixation process: volume, buffering, penetration, temperature, concentration and time interval. Fixation is best carried out close to neutral pH, in the range of 6–8. Commercial formalin is buffered with phosphate at a pH of 7. Factors affecting fixation of tissue are given in Table 14.15.

TISSUE DECALCIFICATION

Bone and other tissues containing calcified areas and calcium deposits are extremely firm and which will not section properly with paraffin embedding owing to the difference in densities between calcium and paraffin. Hence, calcium must be removed prior to embedding to allow tissue sectioning. Mineral acids (nitric acid and hydrochloric acid), organic acids (acetic acid and formic acid), EDTA, and electrolysis are used to decalcify such tissues.

STRONG ACIDS

Strong acids, i.e. nitric acid and hydrochloric acid are used to decalcify dense cortical tissue at a rapid rate; but also damage cellular details. These are not recommended for delicate tissues such as bone marrow.

ORGANIC ACIDS

Organic acids such as acetic acid and 10% formic acid are better suited to bone marrow, because these act more slowly on dense cortical bone. Formic acid in 10% concentration is the best all-around decalcifier.

ETHYLENEDIAMINETETRA-ACETIC ACID

Ethylenediaminetetra-acetic (EDTA) acid can remove calcium slowly but is expensive in large amounts. Electrolysis removes calcium slowly, hence not suited for routine purpose.

COMMERCIAL DECALCIFYING SOLUTIONS

Commercial decalcifying solutions containing formic acid with formalin is available to decalcify tissues.

HANDLING OF SURGICAL SPECIMENS

Under normal circumstances, the specimens are received in 10% neutral buffered formalin. Tissue fixation process prevents autolysis and decomposition of specimen. Gross processing of surgical specimens comprises description of size, shape, color and overall appearance. The tissues placed in cassettes are designated number. When a malignancy is suspected, then the specimen is often covered with ink in order to mark the margins of the specimen.

AUTOMATIC TISSUE PROCESSOR

This multistep process is done by an automatic machine which has multiple jars containing different chemicals, i.e. formalin, ascending series of alcohols, xylene and paraffin through which tissue is passed on a preset time scale. Automatic tissue processor with digitally programmed schedule is given in Table 14.16.

PARAFFIN EMBEDDING OF PROCESSED TISSUE

Properly fixed and processed tissue in the tissue processor are embedded in the molds containing paraffin with melting point 54–62°C to prepare them for cutting in microtome. During paraffin embedding, the tissue is oriented with the surface placed down in the mold becoming the face of tissue block.

MICROTOMY

Tissue embedded in paraffin can be sectioned at 4–8 microns in thickness with microtome having thick metallic knife.

- Tissue ribbon is then floated on warm water bath at a temperature 6–8°C below the melting point of paraffin, which aids in mounting tissue sections on a glass slide properly labelled for light microscopy.

Table 14.16 Automatic tissue processor (histokinette) with digitally programmed schedule

Jar Numbers	Chemical Used	Time Variable Depending on Convenience and Fixation of Tissues	Step
1.	70% alcohol	½ hour	Dehydration of tissue is done to replace fixative and water by a dehydrating fluid
2.	95% alcohol	½ hour	
3.	100% alcohol	1 hour	
4.	100% alcohol	1 hour	
5.	100% alcohol	1 hour	
6.	100% alcohol	1 hour	
7.	100% alcohol	1 hour	
8.	100% alcohol + xylene	1 hour	Clearing is performed to replace dehydrating fluid with a medium totally miscible with embedding medium (molten paraffin wax)
9.	Xylene	1 hour	
10.	Xylene	2 hours	
11.	Molten paraffin wax	2 hours	A vacuum can be applied inside the tissue processor to assist penetration of the embedding paraffin agent. This process replaces water content of tissue and clearing agent by paraffin wax used in molten state
12.	Molten paraffin wax	2 hours	

- Thin tissue sections for electron microscopy are best obtained with a diamond knife which is very expensive. After embedding tissues in epoxy resin, a microtome equipped with a glass or diamond knife is used to cut very thin sections (typically 60–100 nm). Thin tissue sections are stained and examined with a transmission electron microscope.

HEMATOXYLIN AND EOSIN STAINING

The staining process makes use of a variety of dyes that have been chosen for their ability to stain various cellular components of tissue. Hematoxylin and eosin (H&E) stain is routinely done.

- Hematoxylin had been derived from the logwood tree in the past. Eosin at pH 4.6–5.0 has high affinity for positively charged tissue protein group.
- However, special histochemical stains are employed in specific situations according to the diagnostic need.
- Commercially ripened synthetic hematoxylin is commonly used due to less availability of logwood tree. Hematoxylin is the oxidized/ripened product of the logwood tree known as hematein.
- Hematoxylin, a basic dye has an affinity for the nucleic acids of the cell. Eosin, an acidic dye has affinity for cytoplasmic components of the cell. Problem with eosin may occur due to overstaining of decalcified tissues.
- Routine hematoxylin and eosin staining technique in surgical pathology is given in Table 14.17. Applications of hematoxylin stains for different purpose in surgical pathology are given in Table 14.18.

Table 14.17 Routine hematoxylin and eosin staining technique in surgical pathology

Steps	Purpose	Procedure
Step 1	Deparaffinization of tissue section	Deparaffinize the section with xylene (three changes)
Step 2	Rehydration of tissue section in graded alcohol	Rehydration is done by putting section in graded alcohol and then water for 1–2 minutes (2 changes)
Step 3	Nuclear staining by hematoxylin	Put hematoxylin on section and keep for 5–15 minutes
Step 4	Differentiation of tissue section	Differentiation of section is done by putting in 1% acid alcohol (2 dips and then wash section in tap water
Step 5	Bluing of tissue section in alkaline solution (ammonia water)	Wash in running tap water for 5 minutes until section becomes blue
Step 6	Counterstaining of tissue section by eosin	1% eosin Y for 2–3 minutes
Step 7	Dehydration of tissue section	Put section in graded alcohol (70%, 95%, 100%) for 3–5 minutes in each graded alcohol or until excess of eosin is removed
Step 8	Clearing of stained section	Clearing of section in xylene two charges of two minutes each
Step 9	Mounting of stained section	Mount section in DPX (mixture of distyrene, a plasticizer and xylene)

Table 14.18 Applications of hematoxylin stains for different purpose in surgical pathology

Hematoxylin Types	Purpose
Harris hematoxylin	▪ Routine stain on histologic sections and cytology smears (nuclei blue, cytoplasm pink) ▪ Stain acidophilic in nature and lacks alcohol
Mayer's hematoxylin	▪ Carbohydrates ▪ Stain contains alcohol
Baker's acid-hematein	Phospholipid
Tungsten hematoxylin	▪ Fibrin ▪ Cross striations of skeletal muscle
Verhoeff's iron hematoxylin	Strong affinity for connective tissue fibers
Iron hematoxylin	▪ *Entamoeba histolytica* ▪ *Wuchereria bancrofti*
Gill's hematoxylin	▪ Conventional stain used on histologic sections, cytology smears and immunohistochemistry ▪ Nuclear chromatin
Heidenhain's iron hematoxylin	▪ Photomicrography ▪ Skeletal muscle striations ▪ Mitotic structures blue black
Ehrlich's hematoxylin	▪ Counterstaining in immunohistochemistry and cytochemistry ▪ Nuclear stain

Mordant to Tissues

Hematoxylin will not directly stain tissues, but needs a mordant or link to the tissues, i.e. metal cation iron, aluminum, or tungsten. Depending on the mordant used, there is variety of hematoxylins, which vary in intensity.

Progressive Hematoxylin Staining

Hematoxylin and eosin staining can be either progressive or regressive. With a progressive staining the slide is dipped in the hematoxylin until the desired intensity of staining is achieved such as with a frozen section. This is simple for a single slide, but lends itself poorly to batch of slides processing.

Regressive Hematoxylin Staining

With a regressive hematoxylin and eosin staining, the slides are left in the solution for a set period of time and then taken back through a solution such as acid alcohol that removes part of the stain. This method works best for large batches of slides to be stained and is more predictable on a day-to-day basis.

HISTOCHEMICAL STAINS

Commonly used stains in histopathology are given in Table 14.19. Carbohydrate classification for the purpose of histochemical staining is given in Table 14.20. Mucin types and their origin for the purpose of histochemical staining are given in Table 14.21.

Table 14.19 Commonly used stains in histopathology

Special Stains	Uses	Result
Hematoxylin and eosin stain	Routine stain	Nucleus blue, cytoplasm pink, bone blue, mucoid and cartilaginous area pale blue, RBC and eosinophil granules orange red
Stains for carbohydrates		
Periodic acid–Schiff's stain	▪ Glycogen ▪ Fungi ▪ Neutral mucin ▪ Amyloidosis ▪ Basement membrane ▪ Glycoproteins ▪ Glycolipids	▪ Red color ▪ Red color ▪ Red color ▪ Red color ▪ Red color ▪ Red color ▪ Red color
Periodic acid–Schiff with diastase stain	Neutral mucin	
Alcian blue stain at pH 2.5	▪ All acidic mucopolysaccharides ▪ Sulphated mucin	▪ Blue color ▪ Blue color
Mucicarmine stain	▪ Acidic epithelial mucin ▪ Cryptococcus capsule	▪ Blue color ▪ Blue color

Contd...

Table 14.19 Commonly used stains in histopathology (*Contd...*)

Special Stains	Uses	Result
Colloidal iron stain pH 1	Prussian blue reaction (Perls reaction) to demonstrate hemosiderin	Blue color
Congo red	Amyloid	▪ Pink color under light microscope ▪ Green birefringence for under polarizing microscope
Methyl violet (metachromatic stain)	Amyloid	Rose pink color
von Gieson stain	Amyloid	Khaki color
Crystal violet (metachromatic stain)	Amyloid	Pink color
Thioflavin T and thioflavin S	Amyloid	Yellow to yellow green color under immunofluorescent microscopy
Stains for microorganisms		
Bacteria		
Ziehl-Neelsen (ZN) stain (20% H_2SO_4)	*Mycobacterium tuberculosis*	Red bacilli
Auramine-rhodamine	*Mycobacterium tuberculosis*	Yellow fluorescence in auramine-rhodamine stain
Fite acid-fast stain (5% H_2SO_4)	*Mycobacterium leprae*	Red bacilli
Papanicolaou's stain, May-Grünwald Giemsa stain and Gomori methenamine silver stain	Actinomycosis	Clustered bacilli with sunray appearance
May-Grünwald Giemsa stain, Gram stain, Modified acid-fast stain	Nocardia	Thin slender bacilli
May-Grünwald Giemsa stain, hematoxylin and eosin stain, Gram stain, Steiner's stain	*Helicobacter pylori*	Flying bird-like bacilli in row
Warthin-Starry stain	*Treponema pallidum*	Black color against yellow to pale brown background
Fungi		
Papanicolaou's stain, May-Grünwald Giemsa stain, Gomori methenamine stain and periodic acid–Schiff (PAS) stain	*Candida albicans*	Thin slender-like pseudohyphae and spores
Papanicolaou's stain, periodic acid–Schiff (PAS), hematoxylin and eosin stain, Gram stain	Aspergillus	Slender bacilli with acute angle branching
Papanicolaou's stain, hematoxylin and eosin stain, Gomori methenamine silver stain	Mucormycosis	Broad wide-angle branching, nonseptate
Mucicarmine stain, Masson-Fontana silver stain	*Cryptococcus neoformans*	Small 3–5 µm round structure
Gomori methenamine silver stain, periodic acid–Schiff (PAS) stain	*Histoplasma capsulatum*	Small 2–3 µm round structure
Gomori methenamine silver stain	*Pneumocystis jiroveci*	Organism appears as ping pong balls or folded spheres
Parasites		
Periodic acid–Schiff (PAS) stain, hematoxylin and eosin stain, iron hematoxylin and eosin stain	*Entamoeba histolytica*	Round-shaped small organism
Hematoxylin and eosin stain	*Giardia lamblia*	Pear-shaped small organisms
Hematoxylin and eosin stain, Gomori methenamine silver stain, periodic acid–Schiff (PAS) stain	*Echinococcus granulosus*	Hooklets demonstrated on fine needle aspiration cytology

Contd...

Table 14.19 Commonly used stains in histopathology (*Contd...*)

Special Stains	Uses	Result
Stains for connective tissue		
Reticulin stain (used to highlight outlines of parenchyma of organs and highlighting growth pattern of neoplasia)	Reticulin fibers	Black against clear background
Jones methenamine silver stain	Similar to reticulin stain	
Masson's trichrome stain (used in chronic active hepatitis with hepatocytes collapse, cerebral abscess and scleroderma with fibrosis of gastric submucosa)	▪ Elastic fibers ▪ Collagen fibers ▪ Background	▪ Black color ▪ Red color ▪ Yellow color
Verhoeff-van Gieson (VVG) stain (used to outline arteries)	Elastic fibers	Black color
PTAH (phosphotungstic acid hematoxylin)	Cross striations in muscle fibers	Deep purple color
Pentachrome stain	▪ Elastic fibers ▪ Collagen fibers ▪ Fibrin ▪ Muscle ▪ Mucin	▪ Black color ▪ Red color ▪ Red color ▪ Red color ▪ Red color
Oil red O	Fat stain	Red color
Stains for nervous system		
Bielschowsky's stain	Nerve endings, neuron fibrils, tangles and plaques	Black color
Luxol fast blue	Myelin	Blue color
Pigments and minerals		
Perls Prussian blue reaction	Iron	Blue color
Hall's bile	Bile pigments	Emerald green color
von Kossa stain	Calcium	Black color
Alzarin red S (pH 4.8)	Calcium	Red color
Rhodamine stain	Copper	Red to orange color
Gomori's methenamine silver stain	Urate crystals	Black color
Masson-Fontana stain (used in melanoma)	Melanin	Black color
Churukian-Schenk stain	Argyrophil granules	Black color

PAS reactive tissue components are glycogen, starch, mucin, α_1-antitrypsin, fungal capsule, pancreatic zymogen granules, thyroid, colloid, corpora amylacea and plasma cell of Russell bodies.

Table 14.20 Carbohydrate classification for the purpose of histochemical staining

Parameters	Simple Carbohydrates	Glycoconjugates
Composition	Composed of pure carbohydrate	Composed of carbohydrates and other molecules such as protein and lipid
Examples	▪ Monosaccharides (glucose, mannose, galactose) ▪ Oligosaccharides (sucrose, maltose) ▪ Polysaccharides (glycogen, starch)	▪ Proteoglycans ▪ Mucins ▪ Other glycoproteins

Table 14.21 Mucin types and their origin for the purpose of histochemical staining

Parameters	Neutral Mucins	Acidic Simple, Nonsulfated Epithelial Mucins (Sialomucins)	Acidic Complex, Sulfated Epithelial Mucins (Sulfomucins)	Acidic Simple, Mesenchymal Mucins Contain Hyaluronic Acid	Acidic Complex, Connective Tissue Mucins
Location of mucin	▪ Surface epithelia of gastric mucosa ▪ Brunner's glands and prostatic epithelia	▪ Bronchial submucous glands ▪ Salivary glands ▪ Goblet cells	Bronchial mucous glands	Sarcomas	Tissue stroma, cartilage, and bone (chondroitin sulfate or keratan sulfate)
PAS stain	Positive	Positive	Positive	Positive	Negative
Alcian blue at pH	Negative	Positive (pH 2.5)	Positive (pH 1)	Negative	Selectively positive at pH 0.5
Colloidal iron	Negative	Positive	Positive	Negative	Negative
Mucicarmine	Negative	Positive	Positive	Negative	Negative
Metachromatic stain	Negative	Positive	Positive	Negative	Negative

PAS stain is used to demonstrate mucin and glycogen. Mucicarmine stain is specific for epithelial mucins. Acidic sulfomucins resist hyaluronidase digestion.

IMMUNOHISTOCHEMISTRY TECHNIQUE

IMMUNOHISTOCHEMISTRY TECHNIQUE: OVERVIEW

Immunohistochemistry (IHC) technique is the most powerful and widely used ancillary methods in surgical pathology to detect antigens in tissue sections by use of labeled antibody through antigen–antibody reactions. These are visualized by using fluorescent dye, enzyme, radioactive element or colloidal gold.

- Immunofluorescence microscopy is still used in the evaluation of medical renal biopsies. Currently, antigens are detected in formalin fixed paraffin-embedded tissue sections by immunoperoxidase technique.
- Common indications for immunohistochemistry technique are characterization of neoplasms, detection of infectious organisms and evaluation of prognostic or predictive factors.

TISSUE FIXATION

Tissue should be fixed in neutral pH formalin for 12–48 hours at room temperature. It is worth mentioning that formalin induces crosslinks that may mask some epitopes resulting in loss of immunoreactivity. Decalcification of bone samples also reduces immunoreactivity.

TISSUE SECTION USED FOR IMMUNOSTAINING

Unstained tissue sections are cut from paraffin block and placed on charged slides coated with poly-L-lysine or gelatin or albumin. Immunostaining should be performed immediately because unstained sections exposed to air for prolonged time may lose antigen immunoreactivity.

Frozen Section

If the unfixed tissue is frozen, the tissue sections obtained may need to be used for immunoassays. Tissue antigens in frozen sections are not altered. Frozen tissue sections may fall off the slides.

Formalin-fixed Paraffin-embedded Section

If the tissue is formalin-fixed paraffin-embedded, deparaffinize tissue sections to remove the infiltrated paraffin wax by using organic solvents. The tissue section then needs to be rehydrated by sequential immersion in graded alcohols (100%, 70%, 50% and then PBS, i.e. phosphate buffered saline). The deparaffinized tissue section may need to be treated to expose buried antigenic epitopes with either proteases or by heating in low pH citrate buffer or high pH EDTA buffer (antigen retrieval).

CLASSIFICATION OF ANTIBODIES

Antibodies can also be classified as primary or secondary antibodies used in immunohistochemistry technique.

PRIMARY ANTIBODIES

Primary antibodies are immunoglobulin molecules that bind to the target antigen in the tissue sections. Antibodies used for specific detection can be

Table 14.22 Comparison between polyclonal antibody and monoclonal antibody

Parameters	Polyclonal Antibody	Monoclonal Antibody
Synthesis	Mixture of synthesis of different antibodies to a single antigen	Synthesis by single clone commonly raised in mice
Specificity	Multiple epitopes	Single epitope

monoclonal derived via the hybridoma technique or polyclonal obtained from an antiserum (Table 14.22). Primary antibodies are unconjugated antibodies. In general, monoclonal antibodies are considered to exhibit greater specificity than polyclonal antibodies.

SECONDARY ANTIBODIES

Secondary antibodies are raised against primary antibodies, which recognize immunoglobulin of a particular species. These secondary antibodies are not visible with standard microscopy, hence labeled with fluorochrome (fluorescein, rhodamine), enzymes (horseradish peroxidase, alkaline phosphatase). Labeling secondary antibody is done in such a manner that it does not interfere with their binding specificity. For electron microscopy study, scattering compounds such as ferritin and colloidal gold are used for labeling.

BACKGROUND STAINING

Background staining occurs due to binding of nonspecific antibody or endogenous enzymes to the chromogenic substrate. Therefore, working dilution of primary antibody must be done, that yields contrast between specific staining and background staining. Endogenous enzymes present in red blood cells, neutrophils, eosinophils, monocytes/macrophages and neoplastic cells yield background staining. Endogenous peroxidase activity can be blocked by incubation with hydrogen peroxide.

DETECTION SYSTEMS OF IMMUNOHISTOCHEMISTRY TECHNIQUE

The principle of immunohistochemistry technique is to localize antigens in tissue sections by the use of labeled antibodies as specific reagents through antigen–antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold.

DIRECT CONJUGATE-LABELED ANTIBODY METHOD FOR IMMUNOSTAINING

Direct conjugate-labeled antibody method is the application of labeled nonspecific antibody directly to tissue section obtained from skin or renal biopsies. It is one step immunohistochemical staining technique, that utilizes only one antibody.

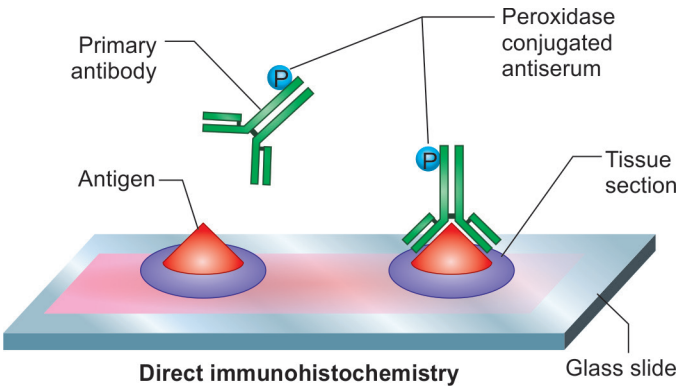

Fig. 14.3: Direct method. Enzyme-labeled primary antibody reacts with tissue antigen.

- The immunomarker then demonstrates the cellular location of the antigen in the tissue section. The label used can be an enzyme, biotin, fluorochrome or colloidal gold.
- There are several procedures that are being used to enhance the direct conjugate-labeled antibody method. The most common one is the biotin label to conjugate the antibody, and then the biotin will bind to the receptor of either the labeled avidin or streptavidin.
- With the second layer of labeling, the staining becomes simplified, which gives quick results, however, labeling intensity is low. Drawback of this procedure is requirement of large amount of antibody for labeling. Secondly, there is lack of signal amplification (Fig. 14.3).

INDIRECT OR SANDWICH METHOD FOR IMMUNOSTAINING

Indirect or sandwich method includes two major procedures: (a) indirect labeled procedure, and (b) indirect unlabeled antibody procedure.

- Indirect or sandwich medhod for immunostaining is more sensitive due to signal amplification through secondary reactions with different antigens. In addition, this method is also economical. Since one labeled second layer of antibody can be used with many first layer antibody to different antigens.
- Indirect labeled procedure has two layers of antibodies. The primary unlabeled antibody is specific

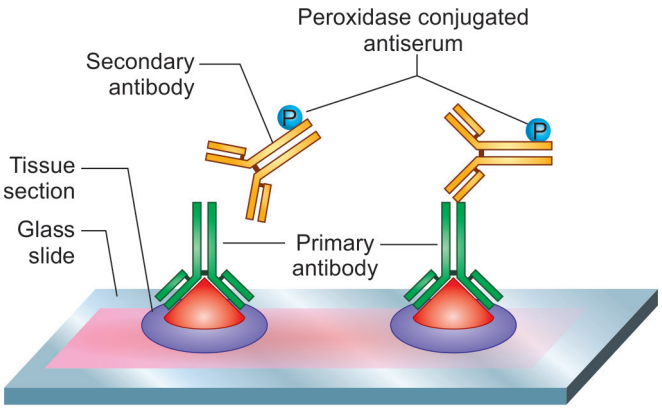

Fig. 14.4: Two-step indirect method of immunostaining. Enzyme-labeled secondary antibody reacts with primary antibody bound to tissue antigen, if the primary antibody is prepared in rabbit or mouse, the secondary antibody must be directed against rabbit or mouse immunoglobulins, respectively.

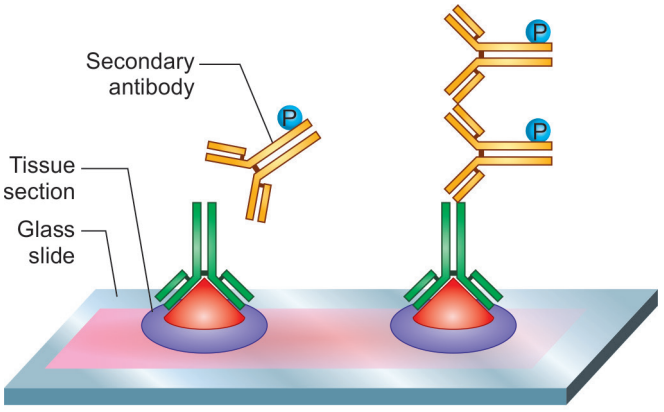

Fig. 14.5: Peroxidase–antiperoxidase (PAP) indirect method of immunostaining. In this three-step indirect method, enzyme-labeled tertiary antibody reacts with enzyme-labeled secondary antibody. Three-step indirect method provides a simple way to further increase the staining intensities compared to the two previous techniques.

for a particular tissue antigen. The secondary labeled antibody with fluorescent dye or peroxidase enzyme is an anti-immunoglobulin antibody. The marker then identifies the sites of the tissue antigen.

- The unlabeled antibody method has three layers of antibodies. The primary antibody is specific for a particular antigen. The secondary unlabeled antibody is an anti-immunoglobulin that reacts to both the primary antibody and to the tertiary antibody. The tertiary antibody is an anti-enzyme antibody, which reacts to a particular enzyme is demonstrated by its reaction with the substrate. Two-step indirect method of immunostaining is shown in Fig. 14.4.

Peroxidase–Antiperoxidase (PAP) Indirect Method

Unlabeled antibody technique is also known as peroxidase–antiperoxidase (PAP) indirect method, which uses an unlabeled primary antibody, an unlabeled bridge antibody and an antiperoxidase antibody and peroxidase molecule.

- Unlabeled bridge antibody directed against both primary antibody and the antiperoxidase antibody links the primary antibody to tissue antigen via signal generated by peroxidase. The sensitivity is about 100–1000 times higher since peroxidase molecule is not chemically conjugated with anti-IgG.
- Although peroxidase molecule is immunologically bound, yet it does not lose its enzymatic activity. It also permits much higher dilution of primary antibody resulting in elimination of many unwanted antibodies and reducing nonspecific background staining. Peroxidase–antiperoxidase (PAP) indirect method of immunostaining is shown in Fig. 14.5.

Pathology Pearls: Immunoperoxidase Staining

Tissue antigen markers that can be demonstrated by immunoperoxidase staining of formalin fixed paraffin sections as mentioned below:

- CD45 is a common hematopoietic lineage marker.
- S-100 is a neurological and neural crest cell marker. It is a calcium-binding protein soluble in 100% saturated ammonium sulfate.
- Synaptophysin is a neuroendocrine cell marker.
- Desmin is a skeletal muscle tissue marker.
- HMB-45 is human melanoma, black protein is common marker for melanoma and other tumors with melanocytic differentiation. It stains immature melanosomes.

Avidin–Biotin Complex (ABC) Indirect Method

Abiotinylated secondary antibody is used to recognize primary antibody. Avidin complexed with biotinylated peroxidase is then bound to secondary antibody. The peroxidase is then developed by DAB (3,3′-diaminobenzidine) used as chromogen in immunostaining or other substrate to produce different calorimetric end products. This reaction delivers several peroxidase molecules to the primary antibody sites and boosts its sensitivity. Disadvantage of this avidin–biotin complex method is background staining (Fig. 14.6).

Labeled Streptavidin–Biotin (LSAB) Indirect Method

Streptavidin derived from *Streptococcus avidin* is recent substitute of avidin. A biotinylated secondary antibody is used to recognize primary antibody. Streptavidin complexed with biotinylated peroxidase is then bound to secondary antibody. This labeled streptavidin–biotin technique is similar to standard avidin–biotin complex method. This reaction delivers several peroxidase molecules to the primary antibody