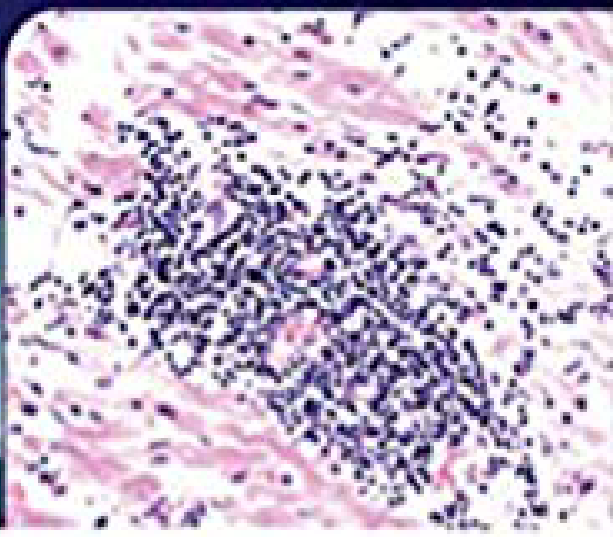
Principles & Interpretation of Laboratory Practices in SURGICAL PATHOLOGY



Shameem Shariff
Amrit Kaur Kaler

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Dedicated to

Our families, who endured with patience our time and efforts,
Our trainees who will advance knowledge further,
Seekers of expertise, whose motivation results in innovations

Preface

The laboratory has been given a central and distinctive role in science education; science educators have suggested that there are rich benefits in learning the accrue from using laboratory activities. Tissues from the body, taken for diagnosis of disease processes, must be processed in the histology laboratory to produce microscopic slides that are viewed under the microscope by pathologists. The techniques for processing the tissues, whether biopsies, larger specimens removed at surgery, or tissues from autopsy, are varied and the persons who do the tissue processing and make the glass microscopic slides are histotechnologists.

While pathologists and cytologists are mainly involved in reporting and interpretation of slides, if the standard criteria for slide preparation are not adhered, this makes the interpretation extremely difficult. As diagnostic personnel, pathologists and cytologists should be aware of all techniques used in the laboratory and have a sound knowledge of the fallacies involved in the preparation. The knowledge of the techniques, which includes processing of specimens, fluids and preparation of these so as to enable them to be transferred to slide material, is all part of the training program for pathologists.

The present book deals exhaustively with methods of processing of tissue including fixation, embedding and cutting, staining techniques and an extensive know-how to combat fallacies in these. The recent techniques with regard to molecular pathology are also dealt with in detail. In addition, certain principles of interpretation are also outlined. In fact, the entire book is user-friendly manual for pathologists, cytologists, cyto- and histotechnologists as well as postgraduate students. It has incorporated our vast experience in cyto- and histopathology.

Shameem Shariff
Amrit Kaur Kaler

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Chapter 1 Fixation

DEFINITION

Fixation is the process by which the constituents of the cells are fixed in a physical and partly chemical state so that they will withstand subsequent treatment with various reagents with minimal loss of significant distortion or decomposition and keep the tissue in as life-like manner as possible.

AIMS

1. Should stop bacterial degeneration, autolysis and putrefaction.
2. Should not distort the cellular constituents by swelling or shrinkage, and maintain as close a resemblance as possible to the natural structure of tissue components.
3. The tissue should withstand the chemicals used at various stages of processing.
4. Allow clear staining of the sections.
5. To increase tissue consistency to permit the cutting of thin slices of tissue at varying microns.
6. To increase optical differentiation of cellular structures.

QUALITIES OF A GOOD FIXATIVE

An ideal fixative is one, which provides:

1. Good tissue penetration.
2. Stabilizes the tissue, preserving the character and distribution of cellular components.
3. Prevents fixation artifacts.
4. Prevents structure deformation, maintaining shape and volume (is isotonic).
5. Preserves cellular constituents.
6. Safe to handle—nontoxic and nonallergenic.

An easy way to remember is '**PRISM', P**enetrate tissue easily, **R**apid in action, **I**sotonic, **S**table and safe to handle, **M**inimal loss or damage to physical and chemical composition of tissue and its components. Effects of fixation on tissues include:

1. Hardening of tissues.
2. Rendering the cells insensitive to hypotonic and hypertonic solutions.
3. Aiding or inhibiting staining.

CLASSIFICATION OF FIXATIVES

Based on Components

Fixatives are classified based on the components present as follows:

1. **Simple fixative:** Contains a single chemical, e.g. formaldehyde (10% formalin), glutaraldehyde, ethyl alcohol, etc.
2. **Compound fixative:** Contains more than one chemical and used as mixtures. Advantage is due to the unequal affinity of each substance for various structural elements:
   a. **Formalin based:** 10% neutral buffered formalin, 10% neutral buffered formol saline and formol calcium.
   b. **Mercurial fixatives:** Zenker's solution, Helly's solution and B5 fixative reagent.
   c. **Dichromate fixatives:** Regaud's solution, Möller's solution and Orth's solution.
   d. **Picric acid fixatives:** Bouin's solution and Gendre's fluid.
   e. **Alcohol-containing fixatives:** Carnoy's and acetic alcohol formalin (AAF).

Based on the Action on Tissues

Fixatives are classified based on the action on tissues or cells as follows:

1. **Fixatives used in histopathology:** They are of two types:
   a. **Microanatomical fixatives:** Preserves the microscopic structure of the tissue, e.g. formol saline, formol calcium, Zenker's fluid, etc.
   b. **Histochemical fixatives:** To demonstrate enzymes, e.g. buffered neutral formalin absolute alcohol.
2. **Fixatives used in cytopathology:** These are used to preserve intracellular structures. They are divided into two types:
   a. **Nuclear fixatives:** Carnoy's fluid, Clarke's fluid and Flemming's fluid.
   b. **Cytoplasmic fixatives:** Champy's fluid and alcohol fixatives.

Based on Their Mode of Action

Fixatives are classified based on their mode of action:

1. Physical methods, e.g. heating, microwaving and freeze drying.
2. Chemical methods, examples are as follows:
   a. Aldehydes (cross-linking): Formaldehyde, glutaraldehyde and acrolein mercurial fixatives.
   b. Oxidizing agents: Osmium tetroxide and chromate-containing fixatives.
   c. Protein denaturation (coagulants/dehydrants): Acetic acid, methyl alcohol and ethyl alcohol.

FIXATION METHODS

Heat Fixation

Heat fixation is the usual mode of preparing bacteriological smears. It generally preserves overall morphology, but not internal structures. After a smear has dried at room temperature, the slide is gripped by tongs and passed through the flame of a Bunsen burner several times, to 'heat kill' and adhere the organism to the slide. This is routinely used with bacteria. Heat denatures the proteolytic enzyme and prevents autolysis. Heat fixation cannot be used in the capsular stain method as heat fixation shrinks or destroys the capsule (glycocalyx), which cannot be seen in stains. It is also used in combination with formal saline.

Heat Fixation on Tissues

The tissue is placed in 20–40 mL of fluid (10% formal saline) and heated below the boiling point over the spirit flame for 1 minute or until the tissue floats on the surface. Then it is cooled immediately and the tissue is taken for processing. This method is generally employed fixation before frozen section.

Microwave Fixation

Microwave fixation is a well-established technique. It provides better fixation than direct heating. In heat fixation, the energy is absorbed from the outer layer and transferred slowly to the rest of the substance, while microwave provides a homogeneous rise in temperature (controlled heating) and all the molecules take up energy simultaneously by a diffusion process. This overcomes the problem of erratic heating by the direct flame.

Principle

Microwave energy interacts with the bipolar molecules resulting in heat denaturation and disulfide bond formation. This process is called microwave stabilization. There is a significant cross-linking of protein molecules with subsequent chemical fixation.

Applications

1. For routine histopathology light microscopy techniques includes staining (standard and special), mucous substance histochemistry, enzyme histochemistry and immunocytochemistry (ICC).
2. For electron microscopy.
3. For rapid fixation of routine surgical specimens and especially for processing urgent cardiac and renal biopsies.
4. For the preparation of botanical and insect material.

Procedure

The tissue is irradiated directly or irradiated after immersing in formalin solution for a period of 4 minutes, followed by irradiation in buffered formalin for another 4 minutes. Alternatively, the tissue is immersed in formalin for 4 hours, followed by microwave irradiation for 1½ minutes in saline, which gives superior results. The optimal temperature required is 45–55°C. Less heating causes poor sectioning quality, while overheating causes vacuolation of the cytoplasm and nuclear pyknosis.

Advantages

1. It reduces the time for fixation from 12 hours to less than 20 minutes.
2. There is little difference in the volume changes in tissues fixed by microwaves compared with conventional formaldehyde-fixed material.
3. Microwave accelerates staining and has no deleterious effect on special staining.
4. Microwave treated tissues (50°C) postfixed in osmium tetroxide gives good results in electron microscopy.
5. It is a prerequisite for immunohistochemical staining methods as it stops the extraction of proteins from tissue.
6. Tissue antigens are better preserved.

Freeze Drying and Fixation

Fresh tissues can be frozen with the following:
- Liquid nitrogen (–190°C)
- Isopentane cooled by liquid nitrogen (–150°C)
- Dry ice [solid carbon dioxide (–70°C)]
- Carbon dioxide gas (–70°C)
- Aerosol sprays (–50°C).

Procedure

The common procedures used in most laboratories are:

1. **Cryostat sections:** In this procedure, tissues are hardened by freezing to temperature as low as –30 to –50°C using a coolant in the cryostat, e.g. Freon 22. Small pieces of tissue sent for rapid diagnosis are transferred onto a chuck of the cryostat in a few drops of optimum cutting temperature (OCT) medium. The metal weight in the cryostat is placed on this for a couple of minutes. This helps in fixation. The chuck is then transferred to the cryotome and serial sections are cut and stained.
2. **Quenching procedure**: Detailed as follows:
   a. The tissue is cut into thin sections (1 mm thick) and placed in a beaker of isopentane suspended and snap frozen in a flask of liquid nitrogen gas at –150°C (isopentane is an extremely volatile and extremely flammable liquid, at room temperature and pressure). This process is known as quenching. This rapid freezing prevents the formation of

ice crystals and preserves the tissue. If only liquid nitrogen is used, it forms vapor bubbles around the tissue, thus producing artifacts around the tissue.

b. The tissue is then transferred to the drying chamber, which is under vacuum and at a higher temperature of –30°C. The ice (tissue water) is removed by sublimation, the water vapor being absorbed by a drying agent such as phosphorus pentoxide. The tissue is impregnated in the embedding medium under reduced pressure as detailed below:
   i. Transfer the dried tissue quickly to a vacuum-embedding medium, i.e. oven containing molten wax.
   ii. On sinking to the bottom of the bath, the tissue will be impregnated with wax and takes approximately 10 minutes for complete impregnation.

Advantages

1. Give better preservation of antigenicity by either ICC or immunohistochemistry (IHC).
2. Not exposed to the organic solvents and therefore minimal chemical alteration of proteins.
3. Minimizes the denaturation of proteins or inactivation of enzymes and particularly useful for enzyme studies in neuropathology as tissues are processed fresh.
4. Little shrinkage of tissue.

Disadvantages

- Lacks precise morphological detail
- Presents a potential biohazard
- Restricted to research laboratory
- Formation of ice crystal artifacts.

Chemical Fixation

Formaldehyde (Aldehydes)

Characteristics

Formaldehyde is commercially available as formalin (40% formaldehyde dissolved in water) and the same is taken as 100% formalin. It is also available as a stable solid form known as paraformaldehyde. Various forms in use are:

1. 10% formalin: This is most commonly used form in laboratories, which contains:
   a Formalin (40% of formaldehyde dissolved in water): 10 mL.
   b Water: 90 mL.

   This in essence is 4% formaldehyde.
2. 10% formol saline:
   a. Distilled water: 90 mL.
   b. Formalin: 10 mL.
   c. Sodium chloride: 0.9 g.

3. Neutral buffered formalin:
   a. Formalin: 100 mL.
   b. Distilled/Tap water: 900 mL.
   c. Sodium phosphate monobasic monohydrate: 4 g.
   d. Sodium phosphate dibasic anhydrous: 6.5 g.

   It is a widely used fixative (pH should be 7.2–7.4). It is tolerant and prevents the formation of formalin pigment. The tissues can be sectioned even after 1 year. It is also used in IHC.
4. Formol calcium acetate:
   a. Tap water: 900 mL.
   b. Formalin: 100 mL.
   c. Calcium acetate: 20 g.

It is good for preserving phospholipids and used for enzyme histochemistry.

Principle

In the first step of fixation, aldehyde groups in formalin form complexes by forming links (methylene bridges) between protein molecules (Fig. 1.1). These methylene bridges subsequently react with several side chains of proteins to form reactive hydroxymethyl side groups. This cross-linkage does not harm the structure of proteins greatly, so that the antigenicity is not lost. However, this cross-linking is reversible by simple washing in water.

Fixation time and temperature

An average tissue requires 10–20 times its volume of formalin/normal buffered formalin for adequate fixation and immersion for 24 hours at room temperature. If the volume of tissues is small a minimum period of 8–12 hours is used. If the temperature is raised to 45°C, fixation time is shortened by 25–40%.

Advantages

- Easily available and cheap
- Good penetration and fixation
- Preservation of fats, myelin, nerve fibers, amyloid and hemosiderin
- Does not cause excessive tissue hardening.

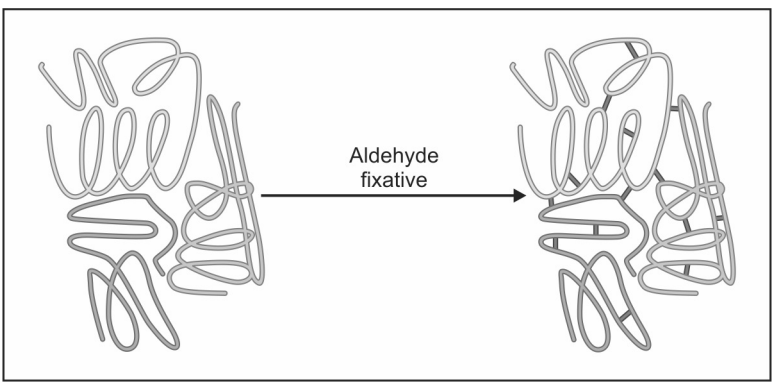


Figure 1.1: Cross-linking of protein molecules by aldehyde groups

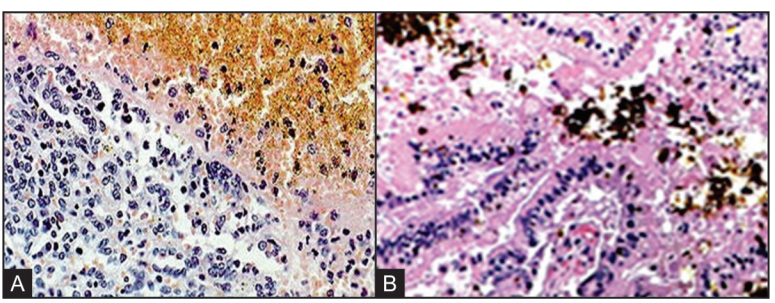
Disadvantages

1. It is slow in action (penetration of 1 mm in 1 hour).
2. On storage it becomes cloudy due to formation of paraformaldehyde, which can be removed by filtration or is inhibited by adding 11–16% methanol in commercial formaldehyde.
3. Traces of formic acid are formed by oxidation, which decreases the quality of nuclear staining and leaches out hemosiderin resulting in formation of brown-black pigment called formalin pigment, also known as acid hematin. This can be prevented by buffering it with a handful of calcium carbonate. Alternatively, when excess pigment gets deposited as shown in Figures 1.2A and B, it should be removed from those sections.
4. Unsuitable for the demonstration of fats and enzymes.
5. It has a denaturing effect on proteins, hence unsuitable for electron microscopy.
6. Irritation to the eyes/ears and causes dermatitis.

Removal of formalin pigment before staining

Formalin removal is done using the following methods:

1. **Schridde's method:** Treat sections for 30 minutes with a mixture of 200 mL of 75% alcohol and 1 mL of 25–28% liquor ammonia. Wash in water.
2. **Verocay's method:** Treat sections for 10 minutes with a mixture of 100 mL of 80% alcohol and 1 mL of aqueous potassium hydroxide followed by thorough washing in water.
3. **Kardasewitsch's method:** Treat sections for 30 minutes to 1 hour with a mixture of 100 mL of 70% ethyl alcohol and 1 mL of 28% ammonia water. Wash in water.
4. **Lillie's method:** Treat sections for 1–5 minutes with a mixture of 50 mL of 75% acetone, 50 mL of 3% hydrogen peroxide and 1 mL of 28% ammonia water; followed by washing in 70% alcohol and then in running water.
5. **Picric acid method:** Treat the sections in saturated solutions of picric acid for 5 minutes to 2 hours.



Figures 1.2A and B: Formaldehyde fixed staining. **A.** Low power view; **B.** Magnified view showing brown-black pigments, i.e. the presence of formalin pigment in the sections. This may hamper interpretation.

Glutaraldehyde

Characteristics
Glutaraldehyde is commercially available as 25 or 50% stock solution. It is best to use as an acid solution (pH is 3–5) and at a temperature of 4°C. It is amber colored due to presence of impurities such as acrolein, glutaric acid and ethanol. It is purified by adding activated charcoal. Glutaraldehyde is used is concentration of 3–6.5% buffered to pH 7.4 with 0.1 M phosphate or cacodylate.

Principle
Glutaraldehyde is a dialdehyde and stabilizes the protein structure by cross-linkages. This cross-linking is irreversible.

Advantages
- Better preservation of cellular and plasma proteins than formaldehyde
- For electron microscopy, as it fixes the tissue rapidly and stabilizes the proteins
- Better ultrastructural preservation than formaldehyde
- Fixes the small tissue fragments and needle biopsies (in 2–4 hours at room temperature)
- Fixation of tissue remains potent for 3 months at 0–4°C
- Less shrinkage of tissue as compared to formalin
- More pleasant smell and less irritating to handle.

Disadvantages
- More expensive
- Slow penetration, hence tissues must be small
- Not suitable for carbohydrates, lipids and immunohistochemical methods
- Secondary fixation with osmium tetroxide may be done for lipids
- It gives a strong periodic acid-Schiff (PAS) reaction, which can be eliminated by immersing the sections in concentrated glacial acetic acid and aniline oil mixture.

Note: Other chemicals used in chemical fixation methods are detailed below.

TYPES OF FIXATIVES

Chemical Fixatives

Osmium Tetroxide

Characteristics
Osmium tetroxide is a fixative not routinely used in the laboratory. It is available as a solid form. It is prepared as a 2% stock solution by adding distilled water at a pH of 7.4.

Principle
Osmium tetroxide cross-links with proteins, reacts with unsaturated lipids and forms monoester as well as diester linkages in the tissues. It rapidly fixes the tissue and stains tissue structure due to formation of hexavalent osmium in proportion to the content of reactive and reducing groups.

Advantages
- Suitable for demonstration of lipids
- Used after postfixation with glutaraldehyde in electron microscopy to retain lipids
- Good preservation of Golgi bodies and mitochondria
- Hardens tissue only slightly.

Disadvantages
- Osmium tetroxide is very expensive
- Vapors are very harmful to the eyes and throat
- Causes swelling of the tissues
- Poor and slow penetration
- Difficulty in counterstaining after its use.

Flemming's fluid
- 1% aqueous chromic acid: 15 mL
- 2% osmium tetroxide: 4 mL
- Glacial acetic acid: 1 mL.

Flemming's fluid is suitable for electron microscopy work and is a good fixative for myelin in peripheral nerves. It is also a nuclear fixative, but is rarely used because of its poor and uneven penetration. Fixation time is 12–24 hours and should be followed by thorough washing, and storing in 80% alcohol.

Mercurials

Mercurials are not commonly used fixatives, but have certain properties good for certain tissues and therefore can be used in such cases. Their main drawback is that several pigments combine with mercury to produce a brownish-black precipitate. These pigments can however be removed by placing sections in iodine solution for 5–10 minutes and then treated with sodium thiosulfate. Mercurial fixatives do not act on lipids.

Zenker's fluid

Compositions of Zenker's fluid are as follows:
- Distilled water: 100 mL
- Mercuric chloride: 5 g
- Potassium dichromate: 2.5 g
- Sodium sulfate: 1 g
- Add 5 mL of glacial acetic acid immediately before use.

Zenker's fixatives are recommended for congested tissues, reticuloendothelial tissues including lymph nodes, spleen, thymus and bone marrow. Zenker's fluid fixes nuclei very well and gives good detail. They are also good for trichrome stains. Duration of fixation is 12 hours, smaller tissues (< 3 mm) are fixed in 2–3 hours. The tissue must be washed overnight to remove the excess dichromate and mercuric chloride pigment must be removed with iodine.

Helly's fluid (Spuler's or Maximow's fluid)

Helly's fluid has the same compositions as that of Zenker's fluid, but differs in 5 mL of formalin is added immediately before use instead of acetic acid.

Zenker formol is slower than Zenker acetate; fixation time is 8–24 hours. It is excellent for bone marrow, spleen, extramedullary hematopoiesis and is recommended for blood-containing organs in general. It preserves cytoplasmic granules and is suitable for Giemsa/Leishman stains.

B5 fixative reagent

1. Stock reagent A:
   a. Mercuric chloride: 12 g.
   b. Sodium acetate: 2.5 g.
   c. Distilled water: 200 mL.
2. Stock reagent B:
   a. 10% buffered neutral formalin.
   b. To prepare the working solution, mix 90 mL stock reagent A with 10 mL stock reagent B.
   c. It is used in bone marrows and on lymph nodes, where lymphomas are suspected. Fixation time is 8–12 hours, also used in IHC.

Picric Acid Fixatives

Many of these fixatives require a saturated aqueous solution of picric acid. Aqueous picric acid (2.1%) will produce a saturated solution and 5% picric acid is a saturated solution in absolute ethyl alcohol.

Bouin's fluid

- Picric acid saturated aqueous solution: 75 mL
- Formalin: 25 mL
- Glacial acetic acid: 5 mL.

This fixative, which keeps well, penetrates rapidly and evenly, and causes little shrinkage. Tissue fixed in it gives brilliant staining with the trichrome methods. It precipitates protein and forms protein picrates (water soluble), which are yellow in color and can be removed through subsequent changes in 50–70% alcohol. Hence, tissues should not be placed in water directly after fixation. The sections are treated in a saturated solution of lithium carbonate in 70% alcohol for a few minutes or alternatively treat the sections in ethyl alcohol followed by 5% sodium thiosulfate, then wash in running tap water.

It is a good fixative for glycogen as well as connective tissue, hence useful for liver and muscle, but Gendre's fluid is better for this purpose. It is also recommended for fixation of testis, gastrointestinal (GI) tract and endocrine tissue. It gives brilliant staining with Masson's trichrome, Giemsa and Mallory stains. It lysis red blood cells (RBCs) and reduces the amount of demonstrable iron. Lipids are altered. It cannot be used for quantitative studies.

Gendre's fluid

- Picric acid saturated solution in 95% alcohol: 80 mL
- Formalin: 15 mL
- Glacial acetic acid: 5 mL.

Recommended for carbohydrate fixation.

Rossman's fluid

- Formalin (neutralized): 10 mL
- Absolute ethyl alcohol saturated with picric acid (approximately 8.5–9%) 90 mL.

It is similar to Gendre's, but without acetic acid; uses are also similar to the above.

Cytological Fixatives

Rapid fixation of smears is necessary to preserve cytological details of cells spread on a glass slide. Fixation means prevention of degeneration of cells and tissue by the autolytic enzymes present in the cells, and preservation of cells as close as possible to the living state. To achieve this, smears are placed in the fixative solutions for specific periods of time before the staining procedure is started. Fixation changes the physical and chemical state of the cells, and determines the subsequent staining reactions that could be carried out on the smears.

Properties of Cytological Fixatives

- Do not excessively shrink or swell cells
- Do not distort or dissolve cellular components
- Inactivate enzymes and preserve nuclear details
- Kill microbes
- Improve optical differentiation and enhance staining
- Maintain properties of the tissues and cell components.

Wet Fixation

The process of submerging of freshly prepared smears immediately in a liquid fixative is called wet fixation. This is the ideal method for fixing all gynecological and non-gynecological smears. Any of the following alcohols can be used. All alcohol fixatives should be discarded or if necessary for reuse it should be filtered (Whatman No. 1 filter paper) after each use.

Ethyl alcohol/Ethanol (95%): The ideal fixative recommended in most of the laboratories for cytological specimen is 95% ethanol. It produces the characteristic effect desired on the nucleus with optimal chromatin detail for cytological preparations. It is a dehydrating fixative. It acts by removing free and bound water thereby changing the structure of proteins so that proteins precipitate, but leave nucleic acids relatively unchanged. It causes cell shrinkage as it replaces water, but only the desired amount of cell contraction.

Absolute ethanol (100%): Produces a similar effect on cells, but is much more expensive.

Ether-alcohol mixture: This fixative was originally recommended by Papanicolaou. It consists of equal parts of ether and 95% ethyl alcohol. It is an excellent fixative, but ether is not used in most of the laboratories because of its safety hazards, odor and hygroscopic properties.

Methanol (100%): This is an acceptable substitute for 95% ethanol. Methanol produces less shrinkage than ethanol, but it is more expensive than ethanol.

Propanol and isopropanol (80%): Propanol and isopropanol cause slightly more cell shrinkage than ether-ethanol or methanol. By using a lower percentage of these alcohols, the shrinkage is balanced by the swelling effect of water on cells. Hence, 80% propanol is a substitute for 95% ethanol.

Denatured alcohol: It is a form of ethanol that has been changed by the addition of additives in order to render it unsuitable for human consumption. One formula in use is 90 parts of 95% ethanol + 5 parts of 100% methanol + 5 parts of 100% isopropanol.

Time of fixation: Minimum 15–20 minutes of smear fixation prior to staining is essential. Prolonged fixation for several days or even few weeks will not affect the morphology of cells. If smears are to be preserved over a long period of time in alcohol, it is better to store them in capped containers in the refrigerator. Alternatively smears may be removed from the fixative after fixation, wrapped in paper and transported or preserved.

Coating Fixative

Coating fixatives are substitutes for wet fixatives. They are aerosols applied by spraying the smear immediately after spreading the cellular contents. They are composed of an alcohol base, which fixes the cells and a wax-like substance, which forms a thin protective coating over the cells, e.g. Carbowax (polyethylene glycol) fixative. Diaphine fixative (hairspray) with a high alcohol content and a minimum of lanolin or oil is also an effective fixative. Most of these agents have a dual action in that they fix the cells and on drying forms a thin protective coating over the smear. These fixatives have practical value in situations where smears have to be mailed to another laboratory for evaluation.

The distance from which the slides are sprayed with an aerosol fixative affects the cytological details and 10–12 inches (25–30 cm) is the optimum distance recommended for aerosol fixation. Aerosol sprays are not recommended for bloody smears, because they cause clumping of erythrocytes. Waxes and oils from hairspray (coating) fixatives can be removed, if it is excess. Smears are kept overnight in 95% alcohol for removal of the coating fixative as these may alter staining reactions if they are in excess.

Acetone Fixation

Acetone fixation should be short (1 hour) at 4°C and used only on small specimens/smears. Acetone produces excessive shrinkage and hardening, and results in microscopic distortion. It is used for IHC, enzyme studies and in the detection of rabies. It facilitates entry of large molecules as in antibodies for IHC studies.

Alcoholic Fixatives/Dehydrant Fixatives

Alcoholic fixatives/dehydrant fixatives can also be used for tissues. Some of the fluids for this purpose are detailed below.

Clarke's fluid

- Absolute alcohol: 75 mL
- Glacial acetic acid: 25 mL.

This solution produces good general histological results for hematoxylin and eosin stains. It preserves nucleic acids, while lipids are extracted. The fixative penetrates rapidly, gives good nuclear fixation and reasonably good preservation of cytoplasmic elements. It is excellent for smears of cell cultures and chromosome analysis.

Carnoy's fixative

- Absolute alcohol: 60 mL
- Chloroform: 30 mL
- Glacial acetic acid: 10 mL.

This is a special purpose fixative for hemorrhagic samples. It penetrates rapidly. The acetic acid in the fixative hemolyzes the RBCs. It is an excellent nuclear fixative as well as preservative for glycogen, but results in considerable shrinkage of cells and tends to produce overstaining with hematoxylin. Overfixing in Carnoy's fluid also results in loss of chromatin material. Carnoy's fixative is also useful for ribonucleic acid (RNA) staining, e.g. methyl green pyronin stain. Carnoy's fixative must be prepared fresh when needed and discarded after each use. It loses its effectiveness on long-standing and chloroform can react with acetic acid to form hydrochloric acid.

Newcomer's fluid

- Isopropanol/Isopropyl alcohol: 60 mL
- Propionic acid: 30 mL
- Petroleum ether: 10 mL
- Acetone: 10 mL
- Dioxane: 10 mL.

This fixative also penetrates rapidly and is excellent for the study of chromosomes for which it is better than Carnoy's.

Dichromate fixatives

Time of fixation is 24 hours (critical). The tissues should be washed after fixation and transferred to 70% ethanol; else pigment precipitation occurs. This may cause excessive shrinkage.

Regaud's fluid or Möller's solution

- Potassium dichromate: 3 g
- Distilled water: 80 mL
- At the time of use add 10% formalin: 20 mL.

Regaud's fluid does not keep well for long time and the solutions should only be mixed immediately before use. It penetrates evenly and rapidly, but has a tendency to overharden the tissues. It may be used as a routine fixative, but is particularly good as a cytoplasmic fixative for mitochondria,

if followed by 4–8 days chromatin in 3% potassium dichromate. Chromaffin tissue is well-demonstrated, but fluids may be improved for this purpose by the addition of 5% acetic acid.

Champy's fluid

- 3% potassium dichromate: 7 mL
- 1% chromic acid: 7 mL
- 2% osmium tetroxide: 4 mL.

The fixative does not keep well for long time and should be freshly prepared each time. It preserves mitochondria and lipids. The tissue must be washed overnight after fixation.

Orth's fluid

- Potassium dichromate: 2.5 g
- Sodium sulfate: 1g
- Distilled water: 90 mL.

At the time of use add 10 mL of formaldehyde.

Postchromatization is the treatment of tissues with 3% potassium dichromate after normal fixation. It may be carried out either before processing when the tissues are left for 6–8 days in dichromate solutions or after processing when sections before staining are immersed in the dichromate solution for 12–24 hours followed in each case by washing well in running water. This technique is employed to mordant tissues for staining, particularly mitochondria and gives, improved preservation and staining of these elements. Phospholipids are also more resistant to extraction.

Dehydrant-cross-linking Fixatives

Compound fixatives with both dehydrant and cross-linking actions include alcohol-formalin mixtures. They produce excellent results in the immunohistochemical identification of specific antigens. In general, most alcohol-based fixatives should be prepared no earlier than 1–2 days before use.

Acetic Alcohol Formalin Fixative

- Formalin: 5 mL
- Glacial acetic acid: 5 mL
- 70% alcohol: 90 mL.

This is the ideal fixative used for cell block preparation of fluid specimens. It is also used for preservation of glycogen.

Alcohol Formalin

- Ethanol 95%: 90 mL
- Formalin: 10 mL.

If desired 0.5 g of calcium acetate may be added for neutrality. The fixatives denature and precipitate due to disruption of hydrophobic bonds that contribute to the tertiary structure of proteins. It is an excellent fixative for glycogen. It penetrates the tissue rapidly, hence used in preparation of tissue for immunofluorescence.

MAILING OF UNSTAINED SMEARS

Coating fixative such as Carbowax fixative and spray-coating fixative can be used primarily to facilitate transport of smears, mailing, etc.

Glycerin Method for Mailing Slides

Smears are first fixed in 95% ethanol for 15 minutes and removed. Two drops of glycerin are placed on smears and covered with a clean glass slide. This may be wrapped in wax paper and mailed to the laboratory in a suitable container.

PREFIXATION OF CYTOLOGICAL MATERIAL

Prefixation of cytological material may preserve some specimens for days without deterioration of cells. The most common solutions used for this purpose are:

1. Ethyl alcohol (50% solution).
2. Saccomanno's fixative (50% alcohol with 2% Carbowax 1540).
3. Mucolex (a commercial mucoliquifying preservative for the collection of mucoid and fluid specimens).

Albuminized slides should be used to prepare smears from prefixed samples. Some of the disadvantages of prefixation are precipitation/coagulation of proteins, hardening of cells in spherical shapes and condensation of chromatin. The coagulation of proteins may interfere with the adherence of cells to glass slides. It also 'rounds up' the cells; causes the cells to gather together into tight clusters making stain absorption and interpretation difficult.

REHYDRATION OF AIR-DRIED SMEARS

Unfixed, air-dried gynecological smears received from peripheral areas can be used for Papanicolaou staining by rehydration method. The simplest rehydration technique is to place air-dried cytological specimens in 50% aqueous solution of glycerin for 3 minutes followed by two rinses in 95% ethyl alcohol and then stained by the routine Papanicolaou method.

FACTORS AFFECTING FIXATION

1. **pH and buffer:** Normal physiological state of pH must be 6–8. More acidic medium of the fixative makes it less reactive. Acidity also favors the formation of formalin-heme pigment that appears as black, polarizable deposits in tissue. Common buffers include phosphates, bicarbonates, cacodylate and veronal.
2. **Duration of fixation and size of specimen:** The thinner the sections (2–3 mm) the better the penetration. There should be a 20:1 ratio of fixative to tissue.
3. **Temperature:** Increasing the temperature, as with all chemical reactions will increase the speed of fixation. Hot formalin will fix tissues faster and this is often the first step on an automated tissue processor.

4. **Osmolality:** Hypertonic solution gives rise to cell shrinkage, while hypotonic solution causes swelling. About 0.9% of sodium chloride works well.
5. **Agitation:** It increases the speed of penetration into the tissues.
6. **Penetration rate:** Penetration of tissues depends upon the diffusibility of each individual fixative. It is expressed as:
   $d = K\sqrt{t}$
   Where,
   d is the depth of penetration;
   K is the coefficient of diffusion (specific for each fixative);
   t is the time.
   For 10% formalin K = 0.78, this means the formalin fixative should not be expected to penetrate more than 1 mm in an hour and it will take approximately 25 hours to penetrate to the center of a 10 mm thick specimen, i.e. 5 mm (= 5^2 hour). The duration of fixation should be the square of distance the fixative penetrates. Formalin and alcohol penetrate the best, and glutaraldehyde the worst.

FIXATION OF INDIVIDUAL TISSUES

1. **Brain:** Adequate preautopsy intra-arterial embalming is done. Formol saline is perfused for minimum of 2 weeks via the middle cerebral arteries. Distortion is prevented by suspending the brain in the fluid by a thread. The brain can then be sliced at 1–2 cm interval after fixation.
2. **Eyes:** Fixed in the lower compartment of the refrigerator (2–8°C) for 48 hours after the optic nerve is removed. To speed up the fixation, one or two windows are made into the globe after 24 hours.
3. **Renal biopsies:** Immunofluorescence biopsies are snap frozen in liquid nitrogen for cryostat sections and then embedded in epoxy resin. Alternatively most laboratories fix the biopsy in an OCT medium in the cryostat itself before sectioning:
   a. For electron microscopy, fixation is done in 2–3% glutaraldehyde.
   b. For paraffin section, neutral buffered formalin.
4. **Gastrointestinal tract specimen:** Flexible fiberoptic endoscopies and small biopsies (2–3 mm) in neutral buffered formalin.
5. **Liver biopsies:** Core biopsies (2 × 10 mm) fixed in neutral buffered formalin or in alcohol fixative.
6. **Lungs:** Infusion with 4% buffered formaldehyde through main bronchi for 1 week, then fixed in neutral buffered formalin.
7. **Lymphoid tissue:** Fixed in neutral buffered formal saline or B5.
8. **Muscle:** Open small biopsies are fixed in neutral buffered formal saline, while deep frozen for histochemistry.
9. **Testis:** Neutral buffered formal saline, Helly's solution and Bouin's fluid gives clear nuclear details, i.e. azoospermia and oligospermia.
10. **Uterus and cervix:** Neutral buffered saline.
11. **Bone:** Neutral buffered saline.

SOFTENING HARD TISSUES

Softening is necessary in tissues such as fingernails, hyperkeratotic skin lesions, fibroids, etc. in which the tissue is processed first by washing the tissue in running water overnight, followed by placing the tissue in 4% aqueous phenol for 1–3 days (Lendrum's method).

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Chapter 2 Decalcification

The process of removing calcium salts from the tissue to make it amenable for sectioning is known as decalcification. Failure to remove calcium from the tissues will result in torn and ragged sections, and damage to the edge of the microtome knife.

TYPES OF BONE AND DECALCIFICATION

Two types of bone are recognized macroscopically in the adult human skeleton:

1. **Cancellous (spongy) bone:** It is found in epiphysis, medullary cavity in diaphysis, vertebra and bone marrow cavities.
2. **Cortical or outer compact bone:** It is solid, hard and forms the shaft of long bones (e.g. femur, tibia, humerus) and external surface of the flat bones (e.g. skull).

The major bulk of the bone is approximately 70% mineral and 30% organic component by weight. The mineralized bone tissue is composed of calcium hydroxyapatite, calcium carbonate in organic collagen matrix and ground substance.

The bone undergoes constant modeling and remodeling, which consists of the deposition and resorption of minerals taking place in equilibrium throughout life, so that the volume and shape of the bone remains constant. In later life, remodeling slows down causing bone porosity and brittleness and in extreme cases osteoporosis.

BONE TECHNIQUES

- Selection of tissue
- Fixation
- Decalcification
- Neutralization of acid
- Thorough washing.

Selection of Tissue

Specimens arriving in the laboratory vary from the size of needle biopsy (a few millimeters long as in diagnostic biopsy of tumor and

hematopoietic disorders) to whole arm or leg amputations (in cases of tumor, chronic osteomyelitis, gangrene, etc.).

Thin slices of bone can be obtained by using a fine–toothed bone saw. Soft and connective tissues, e.g. tendons should be removed before sawing. The first cut is made through midplane and then approximately 4–5 mm thick slabs are cut parallel to the first cut. If bone slabs are too thick, both decalcification and processing is prolonged, while thin bone slabs (< 2 mm) tend to be released from paraffin wax during sectioning. Ideally the thickness for decalcification should be 3–4 mm.

In selection of cases for suspected tumor or infection, sample blocks with the least mineralization should be selected to provide quick diagnosis. These pieces can be fixed, decalcified and processed rapidly to meet urgent requirements. Iliac crest trephine biopsies can be bisected longitudinally, half for decalcification and paraffin sections, and the other half for undecalcified bone methyl methacrylate (MMA) embedding, sectioning for electron microscopy (EM) and light microscopy.

Fixation

Tissues must be fixed adequately before decalcification as acid solutions used in decalcification are injurious to the organic ground substance of the bone. The selected bone is placed in 10% neutral buffered formalin for 24–48 hours. Bone marrow is best fixed in Zenker's fluid.

Decalcification

To obtain satisfactory paraffin or celloidin sections of bone, inorganic calcium must be removed from the organic collagen matrix, calcified cartilage and surrounding tissues. This process is called decalcification. A good decalcifying agent should remove all calcium without damage to the tissues or cells and with no impairment to the subsequent staining or impregnation. The choice of decalcifier will depend upon the urgency of the case, degree of mineralization and the type of staining to be performed. It may be necessary to experiment with different solutions to obtain the best results for a particular material and staining method used.

The selected tissue is suspended by means of waxed thread from a glass rod frame in a jar. The volume of decalcifying fluid should be 100 times that of the tissue. The solution should be checked daily or at regular intervals because calcium ions migrate out of the tissue and cause saturation of the surrounding solution, hence the solution should be changed frequently. Tissues are removed immediately on completion of the process because treatment beyond this point does not improve the cutting qualities, but adversely affects the staining quality.

METHODS OF DECALCIFICATION

Routine decalcification methods include:
- Acid decalcification (strong inorganic acids, weak organic acids)
- Chelating agents
- Ion-exchange resins
- Electric ionization
- Surface decalcification.

Acid Decalcification

Strong Inorganic Acids

The most commonly used is nitric acid (5–10%); 3% hydrochloric acid (HCl), Perenyl's fluid, formalin-nitric acid, etc. are the other solutions used.

Nitric acid (5–10%)
- Nitric acid: 5–10 mL
- Distilled water: To make up to 100 mL.

This solution gives the quickest results and can be used for large and heavily mineralized cortical bone specimens. The length of time for decalcification is from 24 hours to 2–3 days. Fresh solutions of nitric acid are optimal for usage. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are readily hydrolyzed following decalcification. So poor nuclear staining can result if tissues are overdecalcified in acids. Strong acids also tend to damage tissue antigens and enzymes, which may not be detected after the procedure. Decalcification progress should be monitored by a decalcifying endpoint bubble test.

Formalin-nitric acid (10%)
- Nitric acid: 10 mL
- Formalin: 10 mL
- Distilled water: 80 mL.

Formalin is added to nitric acid to protect the tissue from maceration and swelling. It is an excellent preparation for calcified arteries and glands, thyroid and chitinous materials. This is also recommended for urgent biopsies such as needle or small specimen biopsies for decalcification. The only disadvantage is nuclear staining is not as good as in other decalcifying agents such as formic acid or ethylenediaminetetraacetic acid (EDTA).

Perenyl's fluid
- 10% nitric acid: 40 mL
- Absolute ethanol: 30 mL
- 0.5% chromic acid: 30 mL.

This fluid is slow in action, but gives excellent results for small deposits of calcium. In addition to its property as decalcification, it is also used as a softening agent prior to dehydration for dense fibrous tissue hence, hardening does not take place. The cellular details are well preserved and hence, subsequent staining is good. The disadvantage is that it is slow for decalcifying dense bone and the endpoint of decalcification is difficult to detect.

von Ebner's fluid

- Concentrated HCl: 15 mL
- Saturated aqueous NaCl: 175 g
- Distilled water: To make up to 1,000 mL.

Concentrated HCl (0.5%) is added daily until decalcification is complete. It is a rapid decalcifying agent and gives good staining. It is recommended for teeth. Human ribs (5 mm thick) can be decalcified in 36–72 hours.

Weak Organic Acids

The commonly used weak organic acids are 10% formic acid, acetic acid and picric acid. Of these, formic acid is the only weak acid used extensively as a primary decalcifier. The other two cause tissue swelling and are not used alone as decalcifier, but are found as components in Carnoy's and Bouin's fixatives.

Formic acid (5–10%)

- Formic acid: 5–10 mL
- Distilled water: To make up to 100 mL.

It is suitable for most routine surgical specimens. It is gentler on tissues than nitric acid and is safer to handle.

Formalin-formic acid (10%) (Gooding and Stewart's fluid)

- Formic acid: 5–10 mL
- Formalin: 5 mL
- Distilled water: To make up to 100 mL.

It simultaneously fixes and decalcifies the tissue. The time for decalcification is 1–10 days there by taking a longer time to decalcify. Increasing the concentration of formic acid leads to opacity of the solution. This method is also suitable for immunohistochemical staining protocols. It has been proved that decalcification (less than 24 hour) in 5% formic acid can preserve DNA sufficient for fluorescent, in situ hybridization (ISH) or comparative genomic hybridization (CGH).

Buffered formic acid (pH is approximately 2.3)

- 20% aqueous sodium citrate: 65 mL
- 90% stock formic acid: 35 mL.

Buffered formic acid (BFA) counteracts the injurious effects of acids, but increases the time of decalcification.

Citrate-citric acid buffer

- 7.0% citric acid monohydrate: 5 mL
- 1.0% zinc sulfate: 2 mL
- 7.4% ammonium citrate anhydrous: 93 mL.

This solution produces no damage to the tissues and permits excellent staining result, but this method is very slow.

Steps Involved in Treatment of Decalcification with Acids

The following methods may be used for treatment of decalcification with acids:

1. **Neutralization of acid:** Decalcification is done by treating tissues overnight in saturated lithium carbonate solution or 5–10% aqueous sodium bicarbonate solution for several hours.
2. Rinse the tissue in running tap water for 30 minutes for small samples and 1–4 hours for large bones.
3. **Thorough washing:** It is necessary to wash tissues thoroughly before processing to remove the acid or alkali (if neutralization has been done) for optimal staining reactions.
4. Water causes swelling of tissues. So alternatively tissues may be transferred directly to dilute alcohol (70%) with two changes over 12–18 hours. This avoids tissue swelling. Following this, dehydration is continued in the usual way.

Chelating Agents

Chelating agents are organic compounds that have the property of binding certain metals. The EDTA is used as 5–7% solution of its disodium salt (sequestrene or versene) buffered to 7 or 7.4 with phosphate buffer and binds to calcium forming a non-ionizable soluble complex. It is a slow process as calcium is removed in layers from the hydroxyapatite lattice.

Specimens can be decalcified in this solution over several days, depending on the degree of mineralization and size of the specimen. Following fixation in 10% neutral formal saline, tissue is transferred to 50 times its bulk of sequestrene buffered to pH 7.4 with phosphate buffer; for tissues not more than 5 mm thickness, the fluid is changed every 4–5 days. After three such changes, the fluid is changed daily in order to determine the endpoint of decalcification. Temperature and agitation hastens decalcification. Tissue must be removed from the decalcifying fluid as soon as decalcification is complete. This can be tested best by X-ray or by the chemical test for endpoint of decalcification. After decalcification, samples can be washed in water or they are stored overnight in formol saline or neutral buffered saline, routinely processed and embedded in paraffin.

This is also the preferred solution for decalcifying bone material for transmission EM. It also works best on cancerous bone. Several studies have shown that EDTA decalcified bone material preserves DNA better and preferable for ISH analysis. It is also suitable for most immunohistochemical staining protocols.

Ion-exchange Resins

The ion-exchange decalcification (IED) unit incorporates a strong cation ion-exchange resin (ammonium form of sulfonated polystyrene resin) in a weak

acid solution to remove calcium ions from bone, while replacing them with hydrogen ions. The use of resins is limited to those decalcifying fluids not containing mineral acids as in formic acid containing fluids.

The resin is poured on the bottom of the container to a depth of approximately 1 cm and the specimen is allowed to rest on it. The volume of fluid needs to be 20–30 times bulk of the specimen. X-rays are used to determine the endpoint of decalcification. After use, the resin can be used up to three times before discarding. The resin can be regenerated by washing twice in 10N HCl followed by three washes in distilled water.

As IED units are not used with concentrated acid solutions, delicate cellular structures remain intact, cellular detail is not compromised, and a wide variety of routine and special stain procedures can be successfully performed. It also ensures a rapid rate of solubility of the calcium from the tissue and a reduction in the time of decalcification.

Electric Ionization/Electrophoretic Decalcification

Electrophoretic decalcification is based on the attraction of the calcium ions to a negative electrode, i.e. cathode (–), the bone is attached to the anode (+) and a 6 V current is passed through the solution. It utilizes a mixture of formic acid and HCl placed in an electrolyte bath based on a simple electroplating device. X-rays are used to determine the endpoint of decalcification.

The decalcification process is rapid, takes only 2–6 hours and one sample per time can be processed. The process can be hastened by an increase in temperature (40–45°C). The heat generated by this method has a potential for tissue destruction, a total loss of cellular detail and sustainability. Therefore, this is not used as a routine method.

Surface Decalcification

Surface decalcification is the process of decalcifying the surface of blocks in partially decalcified bone or unsuspected mineral deposits on soft tissues found during paraffin sectioning. The exposed tissue surface (up to a thickness of 30 μm) in paraffin block is inverted in 1% HCl or 10% formic acid for 15–20 minutes. The first few sections on subsequent cutting will be calcium free.

ENDPOINT OF DECALCIFICATION

Decalcification requires frequent monitoring as prolonged immersion will result in deterioration of cell as well as tissue morphology and the quality of subsequent staining. It is achieved by the following methods:

1. Specimen radiograph:
   a. Fine detail specimen radiography (the most accurate way).
   b. Microradiography.
2. Chemical testing (accurate).
3. Physical testing (less accurate and potentially damages the tissue).

Specimen Radiograph

Fine Detail Specimen Radiography/X-ray Examination

The detail specimen radiography is the most sensitive test for detection of calcium in the bone or tissues as areas of mineralization and tiny calcifications can be easily identified. The use of fine X-rays as well as high contrast X-ray film provides finer detail and clarity. The 'Faxitron' cabinet X-ray system, used for both bone work and mammography is used with a manual exposure setting of approximately 1 minute. 'Kodak X-ray' film is used for specimen radiography. Fine detail specimen radiography also gives information on:

1. The extent and nature of the lesion.
2. Provides a diagram of the lesion prior to block selection for processing.
3. Confirms the presence of foreign materials, e.g. prosthetic devices, metal or glass fragments.

Microradiography

Microradiographs are high resolution, fine detail contract X-ray of thinner sections of bone for microscopic examination and evaluation of bone mineral density and distribution. The denser, highly mineralized areas appear almost white as fewer X-rays penetrate them. The less denser, non-mineralized areas shows shades of yellowish-gray grading to black pigment against the background of the exposed film. Mineralized bone sections embedded in MMA are preferred. It is less commonly used method of assessing bone calcium. It has applications in bone metabolic diseases and contribution towards fluorescent labeling with tetracycline antibiotic.

Chemical Tests

Chemical tests are simple and a reliable method. The following solutions are needed to chemically test for residual calcium:

1. Ammonium hydroxide stock (5%):
   - Ammonium hydroxide (28%): 5 mL
   - Distilled water: 95 mL.
2. Ammonium oxalate stock (5%):
   - Ammonium oxalate: 5 mL
   - Distilled water: 95 mL.

Working solution: Mixture of 1 part of solution A and 1 part of solution B.

Procedure

1. Insert a pipette into the decalcifying solution containing the specimen.
2. Withdraw approximately 5 mL of the decalcifying solution from the specimen and place it in a test tube. Add a piece of litmus paper.
3. Add 5 mL of the ammonium hydroxide/oxalate working solution, mix well till the litmus paper turns blue (alkaline solution):

a. If a precipitate forms, calcium is present and therefore decalcification needs to be continued (precipitate because of calcium oxalate).
b. Decalcification is complete when no precipitate is observed on two consecutive days of testing.
c. Once decalcification is complete, the cassette containing the decalcified tissue is washed well in running tap water and then processed to a paraffin block as previously described.

Bubble Test

Bubble test can be used as a guide to check the progress of decalcification. On adding a strong acid, gas bubbles form on the bone surface as calcium carbonate combines with acid to form carbon dioxide. If the level of calcium carbonate is reduced, the bubbles will not form. Decalcify the tissue until no bubbles are visible (presence of bubbles can severely disrupt cells).

Physical Tests

The physical tests include bending the specimen or inserting a pin, razor, or scalpel directly into the tissue. The disadvantage of inserting a pin, razor, or scalpel is the introduction of tears as well as pinhole artifacts and may cause false-positive microfractures leading to misdiagnosis. Slightly bending the specimen is safer and less disruptive, but will not conclusively determine if all calcium salts have been removed. After checking for rigidity, wash thoroughly prior to processing.

FACTORS AFFECTING THE RATE OF DECALCIFICATION

The process of decalcification is dependent on the thickness of specimen, compactness or density of bone, strength of solutions and temperature at which the decalcification is carried out:

1. **Age of the patient:** Immature cortical bone decalcifies faster than mature cortical bone.
2. **Concentration of agent:** Increased concentration of acid causes faster decalcification, but is harmful to the tissues.
3. **Temperature:** Increased temperature causes faster decalcification, but has maceration effect on the tissues.
4. **Agitation:** Mechanical agitation hastens the exchange of fluid within the tissue. This can be achieved by low speed rotation, rocking, stirring and bubbling air into the solution.
5. **Thickness of tissue:** A thinner tissue bit takes less time for decalcification.

MORPHOMETRY OF BONE

Morphometry is the method used to quantify the level of cellular activity of osteoblasts and osteoclasts as well as helps in the characterization of mature

trabecular and cortical bone structure. Bone constantly undergoes modeling and remodeling and this may be influenced by an individual's genotype, gender and the specific anatomical region.

Morphometry is performed to determine the underlying cause for osteoporosis and to define alterations in bone morphology associated with metabolic bone diseases, renal osteodystrophy, chronic kidney disease, bone repair, etc. It assesses the relative amounts of bony trabeculae, osteoid, deposition and resorption by a subjective microscopic examination of section. Tetracycline is injected several times prior to biopsy and this helps to determine the amount of active mineralization. This is followed by iliac crest biopsy and MMA embedding as well as subsequent sectioning. Detection of tetracycline lines by fluoroscopy along mineralized bone enables study of the same.

A universal system of nomenclature, confined to trabecular bone has been introduced. They are of two types, structural or kinetic. The first includes trabecular number and separation. The second defines the bone and volume, eroded surface, osteoid surface, mineralized surface, osteoid thickness, wall thickness, mineralization rate, etc. These measurements are integrated with radiological, biomechanical and molecular data, thereby providing a comprehensive set of outcome information that corroborates each other. The only pitfall in the technique of bone morphometry is Paget's disease, where known variations occur from site to site and even within the same bone. Therefore, results at one site are not consistent.

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Chapter 3 Tissue Processing

Tissues must be adequately supported before sectioning for microscopic examination. Once the tissue has been fixed it must be processed into a form in which it can be cut into thin microscopic sections. Therefore, tissues are routinely taken through a series of reagents are infiltrated and embedded in a stable medium, when it becomes hard, provides the necessary support for microtomy. The treatment of tissues through these series of reagents and subsequent embedding in a medium, e.g. paraffin wax (which is similar in density to tissue thus enabling smooth sectioning) is termed as 'tissue processing'. The quality of structural tissue preservation seen in the final stained and mounted section is largely determined by the choice of fixatives, processing fluids and embedding medium. The aim of tissue processing should be minimal morphological cell distortion. When immediate sectioning is required tissues may be sectioned following a range of freezing methods as in frozen section reporting.

TISSUE SAMPLING

Tissue sampling generally follows standard protocols established by each laboratory for various categories of specimens. Tissue blocks for processing should be thin; usually 1–2 mm thick for urgent processing of specimens and 3–5 mm for routine material to be processed overnight. Specimens should not be tightly packed into processing cassettes or containers, but should have sufficient free space to facilitate fluid exchange. Small specimens and tissue fragments, e.g. endometrial biopsies, endoscopic biopsies, etc. are processed, wrapped in lens tissue and placed in fine mesh containers.

PRINCIPLES OF TISSUE PROCESSING

Tissue processing occurs due to diffusion of various substances/fluids into and out of stabilized porous tissues. The diffusion process results from the thermodynamic tendency of processing reagents to equalize concentrations inside and outside the bits of tissue, thus generally conforming to Fick's law. This law states that rate of solution diffusion through tissues is proportional to the concentration gradient (the difference between concentrations of the fluids inside and outside the tissue) as a multiple of temperature-dependent constants for specific substances.

INITIAL PRECAUTIONS BEFORE PROCESSING

Labeling of Tissues

Specimens are generally identified by a numbering system that is not bleached by subsequent fluid and solvent treatment. Examples include:

1. A numbered card label generated by computer-printer (self-adhesive bar code label) or handwritten in soft lead pencil or waterproof ink.
2. Color-coded plastic cassettes (Tissue-Tek system), machine or manually labeled (the label should include a serial number for the specimen and that particular year, e.g. 07/2011 for the seventh specimen received in the year 2011).

Completion of Fixation

Tissues should be fixed before processing is initiated. Poorly fixed tissues are inadequately protected against the physical and chemical rigors of processing. Strategies commonly employed to ensure complete fixation of tissues include:

1. Microwave irradiation of biopsy specimens in normal saline.
2. Continuing fixation on the tissue processor with one or more changes of the routine fixative, often at elevated temperatures of 40–60°C.
3. Secondary fixation of tissues in an alcoholic fixative, which will complete fixation whilst initiating dehydration.

Postfixation Treatment

Fixation with picric acid forms water-soluble picrates, therefore, tissue blocks should be placed directly into 70% ethanol. Tissues fixed in Carnoy's fluid should be placed directly into 100% ethanol, instead of increasing graded alcohols.

STAGES OF TISSUE PROCESSING

- Dehydration
- Clearing
- Impregnation in wax (infiltration)
- Embedding
- Casting or blocking.

Each stage should be of sufficient length to ensure completeness. The tissues are transferred from reagent to reagent in tissue processing cassettes. These are metal or plastic containers with perforations. The cassettes are transported through various reagents in machines called 'tissue processors.' 'Manual processing' may also be done, where cassettes are transported manually from reagent to reagent and is done in small scale laboratories with less number of specimens. Factors affecting the rate of processing are:

1. **Specimen size:** The thicker the specimen, longer is the filtration time.
2. **Agitation:** Effective agitation reduces the overall processing time by 25–50% with improved impregnation of the tissues.

3. **Heat:** Increases the rate of penetration and chilling decreases it.
4. **Viscosity:** The higher the viscosity of the fluid, the slower the rate of penetration.
5. **Vacuum impregnation:** Vacuum considerably reduces the impregnation time.

Dehydration

The first step in processing is dehydration. Water is present in tissues in free and bound (molecular) forms. The water needs to be removed before it is replaced by wax during impregnation and embedding. Wet-fixed tissues (in aqueous solutions) cannot be directly infiltrated with paraffin. The first stage of processing is the removal of aqueous fixatives from the tissues by various dehydrating compounds such as alcohols or a substitute. The dehydrating agents are hydrophilic and attract water molecules from the tissue or affect dehydration by repeated dilution of the aqueous tissue fluids.

Procedure

Dehydration consists of passing the tissue through series of progressively more concentrated alcohol baths. It is best accomplished by the use of graded alcohol, say 70–95% or to 100%. The transfer of tissue directly to a higher grade, i.e. 95% alcohol is risky, since it is liable to cause tissue shrinkage. Tissues are carefully transferred from one container to another at proper intervals (allowing the tissue to drain for few seconds) between each change. The volume of a dehydrating agent in each stage should be at least 10 times the volume of tissue to be dehydrated.

The concentration of the first alcohol depends on the fixative and size and type of the tissue, e.g. delicate tissue such as embryo and brain need lower concentration (50% alcohol to start with) and smaller intervals between two strengths of alcohol. Tissues immersed in alcoholic fixatives such as Carnoy's fluid may be placed directly in 100% alcohol.

Duration of dehydration should be kept to the minimum; consistent with the tissues being processed. Tissue blocks 1 mm thick should receive up to 30 minutes in each alcohol, blocks 4–5 mm thick require up to 90 minutes or longer in each change. Tissues may be held and stored indefinitely in 70% ethanol without harm. Automated programs are also available in tissue processors.

Dehydrating Agents

Alcohols: These are clear, colorless, flammable, hydrophilic liquids, miscible with water and when anhydrous, with most organic solvents. They have dehydrating as well as fixative effect (secondary fixation):

1. **Ethanol:** It is probably the most commonly used dehydrant in histology. It is supplied as 99.85% ethanol (absolute ethanol, 100%) and as special

methylated spirits (99.85% ethanol denatured with 2% methanol). Both are satisfactory for histological purposes. There is some distortion of tissue due to shrinkage produced by immersion in ethanols for a long duration of time. Anhydrous copper sulfate acts as both a dehydrating agent and as an indicator of water content in the last bath of 100% ethanol. Anhydrous copper sulfate is layered (1–2 cm) in the bath and covered with filter paper. If water is present, the anhydrous copper sulfate turns blue.

2. **Isopropyl alcohol (isopropanol):** This is an easily available and cheaper substitute for ethanol. It is slightly slower in action, but a far superior lipid solvent than ethanol. Isopropanol shrinks and hardens tissue less than ethanol and is used to dehydrate hard, dense tissues, which can remain in the solvent for extended periods of time without harm.
3. **Butyl alcohol (butanol):** The dehydrating process is slow, requiring a longer time for immersion. It causes less hardening and shrinkage than ethanol and can be used in combination with ethanol as a dehydrating agent.

Glycol ethers: Unlike the alcohols, these reagents do not act as secondary fixatives:

1. **Dioxane (diethylene dioxide):** 1,4-diethylene dioxide causes less tissue shrinkage and hardening than ethanol. This can also be used as clearing agent because it has a unique property of being miscible with water and paraffin wax. It has a highly toxic vapor as well as high cost, hence should not be recommended for routine use.
2. **Polyethylene glycols (PEG):** These are water-miscible polymers used to dehydrate and embed substances labile to other solvents and heat of the paraffin wax method.

Other dehydrants: Include acetone, phenol, etc.:

1. **Acetone:** It is a fast and effective dehydrant. Though it may cause tissue shrinkage, it may also act as a coagulant secondary fixative. This is clear, highly volatile and easily removed by most clearing agents. Acetone is the best dehydrant for processing fatty specimens due to its fat dissolving properties. This is also a good dehydrating agent for electron microscopy. But it causes tissue shrinkage and causes brittleness, if its action is prolonged. Alcohol and acetone changes are also used in combination. Acetone is not recommended for microwave processing as it causes excessive nuclear shrinkage.
2. **Additives:** About 4% phenol added to 95% alcohol acts as both a dehydrating agent and softening agent for hard tissues such as nail, keratin masses, tendon and dense fibrous tissue.

Note: Tissues, which have been treated with a fixative containing a chromate, must be thoroughly washed in running water prior to treatment with alcohol/dioxane to remove the chromate.

Clearing (Dealcoholization)

Clearing consists of replacing dehydrant with a substance that will be miscible with the embedding medium (paraffin) with which the tissue must be impregnated. The essential requirement of clearing agent is that it should be miscible with both dehydrating agents and impregnating agents. The clearing agents often have the same refractive index as tissues, as a result, when the anhydrous tissue is completely infiltrated with the clearing agent, it becomes translucent. This property is used to ascertain the endpoint and duration of the clearing step. The presence of opaque areas indicates incomplete dehydration. Transition solvents extract certain tissue substances such as lipids, but otherwise do not alter tissue reactivity nor behave as secondary fixatives during processing.

The boiling point of the clearing agent gives an indication of its speed of replacement by paraffin wax. Fluids with low boiling point are easily replaced. Viscosity also influences the speed of penetration.

Criteria for Choosing Suitable Clearing Agents

- Ability to rapidly remove dehydrating agents
- Easily removed by melted paraffin
- It should not produce excessive shrinkage and hardening
- It should not dissolve out aniline dyes used in staining
- Minimal tissue damage
- Least flammable
- Least toxicity
- Cost-effective.

Procedure

After dehydration, the tissue is transferred to a clearing agent. After an appropriate time interval, the tissue is transferred to a second change of clearing agent. The volume of clearing agent is optimally 30–40 times the volume of the specimen. The amount should not be less than 10 times the volume of tissue. The smaller pieces of tissue are cleared in 30 minutes to 1 hour, whereas larger tissues (> 5 mm thick) are cleared in 2–4 hours. The endpoint of clearing can be noted by transparent appearance of the tissue against light. Prolonged exposure to most clearing agents causes the tissue to become brittle and difficult to section.

Clearing Agents

Transition solvents: These are odorless flammable liquids with characteristic petroleum or aromatic odors, miscible with most organic solvents and with paraffin wax:

1. **Xylene:** The most common clearing agent in the laboratory is xylene. It is reasonably cost-effective. It clears rapidly and the tissues are rendered

transparent, facilitating endpoint determination. Long-term immersion of tissue in xylene results in tissue distortions therefore tissues should not be left in it for more than 3 hours.

2. **Toluene:** It is better at preserving tissue structure and causes less hardening of tissues than xylene. It is a suitable clearing agent for automatic tissue processing. It is preferable for processing brain sections. However, toluene is more expensive than xylene, more toxic and a possible carcinogen, so toluene is less commonly used.
3. **Chloroform:** It is an expensive, heavy, highly volatile, slowly penetrating transition solvent. It causes less brittleness than xylene and is often used on dense tissues such as uterus and muscle. The tissues can be cleared overnight without rendering them brittle because of its tolerance. It is also good for nervous tissues, lymph nodes and embryos. But it causes severe health hazard, acts slowly and may lead to sectioning difficulties.

Esters: These are colorless flammable solvents miscible with most organic solvents and with paraffin wax. Methyl salicylate (oil of wintergreen) is safe and effective, clears tissues from 96% ethanol, hardens less and has a pleasant odor. It causes minimal tissue shrinkage and hardening and tissues can remain in it indefinitely without harm. Methyl salicylate, though, is rarely used due to its cost.

Terpenes: These are isoprene polymers found in essential oils. Terpenes clear tissues and celloidin sections from 80 to 95% alcohol, render tissues transparent and have a slow gentle non-hardening action. Most are generally regarded as safe though some have particularly strong odors, which can be overpowering, requiring good laboratory ventilation, e.g. turpentine and oils of bergamot, cedarwood, clove, lemon, *Origanum* and sandalwood.

Histo-Clear (orange oil) has been rigorously purified and stabilized. It offers the best clearing action. It is excellent for preserving fine tissue structure and can often be used in place of xylene. It also enhances the clarity and vibrancy of acidophilic stains and improves the staining of Harris hematoxylin.

Other clearing agents: Includes benzene, butyl acetate, etc.

Impregnating in Wax (Infiltration)

Impregnating is the process in which the clearing agent is replaced by paraffin or its substitute that completely fills all the tissue cavities, giving a firm consistency to the specimen and allowing easier handling and cutting of thin sections without any damage to the tissue or its cellular constituents. This is done at the melting point temperature of wax in use, i.e. 54–64°C, in case of paraffin wax. The volume of the wax should be 25–30 times the volume of the tissue.

Criteria for Ideal Infiltrating Medium

Ideally an infiltrating and embedding medium should be:

- Soluble in processing fluids
- Suitable for sectioning and ribboning
- Capable of flattening after ribbon cutting
- Molten between 30 and 60°C
- Translucent or transparent; colorless at its melting point
- Stable
- Homogeneous
- Nontoxic
- Odorless
- Easy to handle
- Inexpensive.

Types of Wax

Paraffin wax

Paraffin wax is a polycrystalline mixture of solid hydrocarbons produced during the refining of coal and mineral oils. It is colorless or white, somewhat translucent, odorless and has a wide range of melting point, ranging from 56 to 64°C. Tissue-wax adhesion depends upon the crystal morphology of the embedding medium. Small, uniform-sized crystals provide better physical support for specimens through close packing. Crystalline morphology of paraffin wax can be altered by incorporating additives, which result in a less brittle, more homogeneous wax with good cutting characteristics. There is consequently less deformation during thin sectioning. Altering the temperature does not appreciably affect crystal size.

Paraffin wax is routinely used as an impregnating and embedding media. It is cheap, safe, immiscible with water, provides quality sectioning and is easily adaptable to a variety of uses. Paraffin wax forms a matrix that gives hardness and support to the tissues, thus preventing tissue sectioning distortion and provides easy ribbon during microtomy. Tissue blocks can be stored in paraffin wax for a long time without tissue destruction. Low melting point paraffin wax is soft and used for delicate tissues such as fetal and areolar tissues, while higher melting point paraffin wax is hard and used for hard fibrous tissues.

If tissues are processed by hand they will require a total of 4–6 hours in three changes of wax whereas with agitation, 2–4 hours in two baths will suffice. Following impregnation tissues are embedded in a wax block, which enables them to be cut into thin sections (2–8 μ thick) on a microtome. The disadvantage is that paraffin wax requires a long time of immersion to infiltrate tissues such as bones, brain, eyes, etc. Prolonged impregnation causes tissue shrinkage and hardening. All wax should be filtered routinely. If the wax crystallizes due to water content, it may be heated and stirred to remove the water.

Modified paraffin waxes

The properties of paraffin wax are improved for histological purposes by the inclusion of substances added alone or in combination:

- Prolonged heating of paraffin wax at high temperatures or use of micro-crystalline wax improves ribboning
- To increase hardness: Add stearic acid
- To decrease the melting point: Add spermaceti or phenanthrene
- To improve adhesion between specimen and wax (alter crystalline morphology): Add 0.5% ceresin, 0.1–5% beeswax, rubber, wax or phenanthrene.

Examples of modified waxes

1. **Paraplast:** This is a mixture of highly purified paraffin wax containing plastic polymers. It has a greater degree of elasticity and provides excellent tissue infiltration and superior quality sections. It minimizes the tissue compression due to sectioning and gives wrinkle-free serial sections to be cut with ease at 4 mm thick.
2. **Paraplast plus:** This wax of highest purity contains dimethyl sulfoxide (DMSO), which gives faster penetration with a more homogeneous matrix, reduces infiltration times and facilitates thin sectioning. Tissue processing time is therefore reduced.
3. **Ester waxes:** This developed by Steedman, have low melting points (melting point 48°C). These are hard at room temperature and have good adhesive properties. Ester wax is similar to celloidin in that it can be compressed and is therefore less likely to crumble when cutting hard tissues. Like paraffin wax it gives good ribboning, thin sectioning and glass adhesion properties.
4. **Polyester wax:** This developed by Steedman, is a ribboning low melting point wax (38°C), which reduces heat-induced artifacts. It is recommended for heat-labile tissues, to minimize heat-induced hardening in difficult tissues and is an ideal medium for combined light and scanning electron microscopy.
5. **Water-soluble waxes or PEG:** These are water-soluble media used for investigation of heat- and solvent-labile lipids and proteins, and to overcome tissue shrinkage, damage and distortion inherent in the paraffin wax technique. The polyethylene glycols or Carbowax are polymers of varying length. At room temperature, PEG 600 is syrupy liquid, PEG 1000 is soft and slippery, PEG 1500 is hard, and PEG 4000 is hard and brittle. In general, they are less elastic, denser and somewhat harder than paraffin wax. For routine tissues, four changes of Carbowax with agitation (70%, 90% and two times in 100%) at 56°C is used for 30 minutes, 45 minutes and 1 hour each respectively. The specimens are then blocked in fresh Carbowax at 50°C and the blocks are prepared immediately and rapidly cooled in the refrigerator. Crystal slip is a bigger problem than in paraffin and sectioning deformation is mainly nonrecoverable.

Advantages of PEG are:

- It eliminates dehydration and clearing, hence lipids and neutral lipids are not removed and demonstrated in thin sections
- The processing time is reduced
- Technique is good for enzyme histochemistry and immunohistochemistry
- It reduces the shrinkage and distortion.

Disadvantages of PEG are:

- They are difficult to flatten without loss of tissue and adhere poorly to slides
- As Carbowax is soluble in water, sections cannot be floated on water
- Sectioning is difficult because of tendency of the Carbowax to crumble.

Floatation fluids for these composed of:

- Diethylene glycol: 40 parts
- Distilled water: 50 parts
- Strong formaldehyde (40%): 10 parts.

Those waxes with higher (18–22) carbon atoms in their molecules have physical characteristics suitable for tissue embedding.

Vacuum Impregnation

Vacuum impregnation is the impregnation of tissues by a molten medium under reduced pressure. The procedure assists the complete and rapid impregnation of tissues with wax. It reduces the time when tissues are subjected to high temperatures, thus minimizing heat-induced tissue hardening, facilitates complete removal of transition solvents and prolongs the life of wax by reducing solvent contamination. Vacuum infiltration requires a vacuum infiltrator or embedding oven, consisting of wax baths, fluid trap and vacuum gauge, to which a vacuum of up to 760 mm Hg is applied using a water or mechanical pump.

Useful for lung tissue and tissue that contains much air. Also used for splenic tissue, which tend to become hard in routine processing. Care must be taken, while dealing with pieces of lungs, as rapid evacuation of air cause rupture of lung alveoli, which when examined microscopically may be mistaken for emphysema. Continuous supply of electricity is essential, as the apparatus cannot be opened in the middle of the procedure.

AUTOMATED TISSUE PROCESSING METHODS

Tissues are usually more rapidly processed by machine than by manual methods, although the latter can be accelerated by using microwave or ultrasonic stimulation. For routine purposes, tissues are most conveniently automatically processed through dehydration, clearing and infiltration stages by machine. There are two broad principles of automatic tissue processors, tissue-transfer and fluid-transfer types.

Tissue-transfer Processors (Fig. 3.1 and Table 3.1)

Tissue-transfer processors are characterized by the transfer of tissues, contained within a basket, through a series of stationary reagents arranged in line or a circular carousel fashion. The rotary or carousel is the most common model of automatic tissue processor and was invented by Arendt in 1909. It is provided with 9–10 reagent and 2–3 wax positions, with a capacity for 30–110 cassettes depending upon the model. Fluid agitation is achieved by vertical oscillation or a rotary motion of the tissue basket. Processing schedules are card-notched, pin or touch pad programmed.

Tissue-transfer processors allow maximum flexibility in the choice of reagents and schedules. Metal-corrosive fixatives, a wide range of solvents, and relatively viscous nitrocellulose solutions can all be accommodated. These machines have a rapid turnaround time for day/night processing. In more recent models, the tissue basket is enclosed within an integrated fume hood to allow escape of fumes during agitation and transfer cycles thus overcoming the disadvantages of earlier styles. The main disadvantage of this type of processor is that the tissues dry, while being transferred. It is for this reason that, if electricity is interrupted, the tissue processors in routine laboratories are connected to generators.

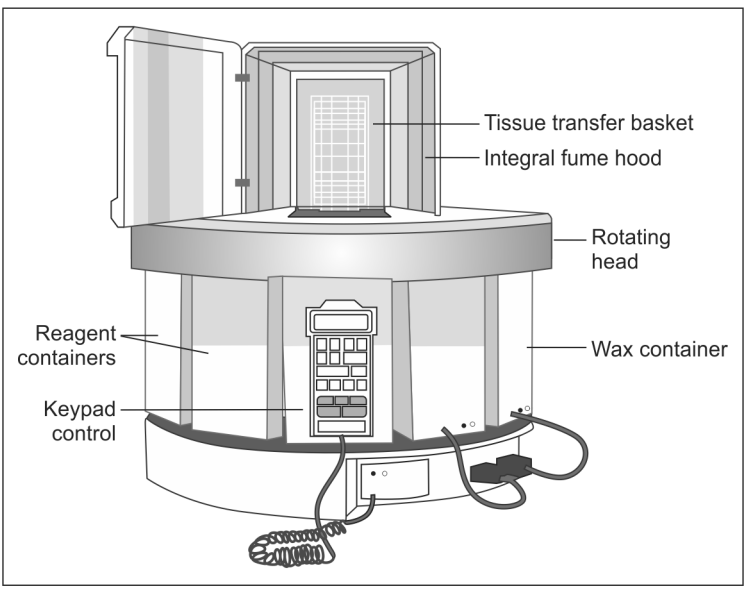

Figure 3.1: A tissue-transfer tissue processor with an integrated fume hood. Tissue cassettes are loaded into the basket on the rotating head, which transfer tissues around the series of reagent containers. Examples of this type of processors are Shandon Citadel, Technicon Ultra and Shandon Duplex.

TABLE 3.1: Example of a processing schedule for a tissue-transfer processor

Step of processing	Duration	
	Daytime	Overnight
Fixative		120 min
Fixative		120 min
70% ethanol		60 min
90% ethanol		60 min
Absolute ethanol	30 min	60 min
Absolute ethanol	30 min	60 min
Absolute ethanol	30 min	60 min
Toluene or substitute	30 min	60 min
Toluene or substitute	30 min	60 min
Paraffin wax	30 min	90 min
Paraffin wax	30 min	90 min
Paraffin wax	30 min	90 min
Paraffin wax (under vacuum)	30 min	30 min
Embed		
Total time	4.5 h	16 h

Note: In day schedule for urgent specimens, tissues 2 mm and fixed in Carnoy's fluid. In overnight schedule for routine processing, tissue blocks 2–3 mm and single load. In weekend processing, tissues are held in fixative or preferably 70% ethanol until Sunday.

Fluid-transfer Processors (Fig. 3.2 and Table 3.2)

In fluid-transfer units, processing fluids are pumped to and from a retort in which the tissue cassettes remain stationary. There are 10–12 reagent stations with temperatures adjustable between 30 and 45°C, three to four paraffin wax stations with variable temperature settings between 48 and 68°C, and vacuum-pressure options for each station. Depending upon the model these machines can process 100–300 cassettes at one time. Agitation is achieved by tidal action. Schedules are microprocessor programmed and controlled. Vacuum pressure cycles coupled with heated reagents allow effective reductions in processing times and improved infiltration of dense tissues.

Fluid-transfer processors overcome the main drawbacks of the tissue-transfer machines. Tissue drying is prevented within the sealed retort and reagent vapors are vented through filters or retained in a closed-loop system. Processors are provided with alert systems and diagnostic programs for troubleshooting and maintenance.

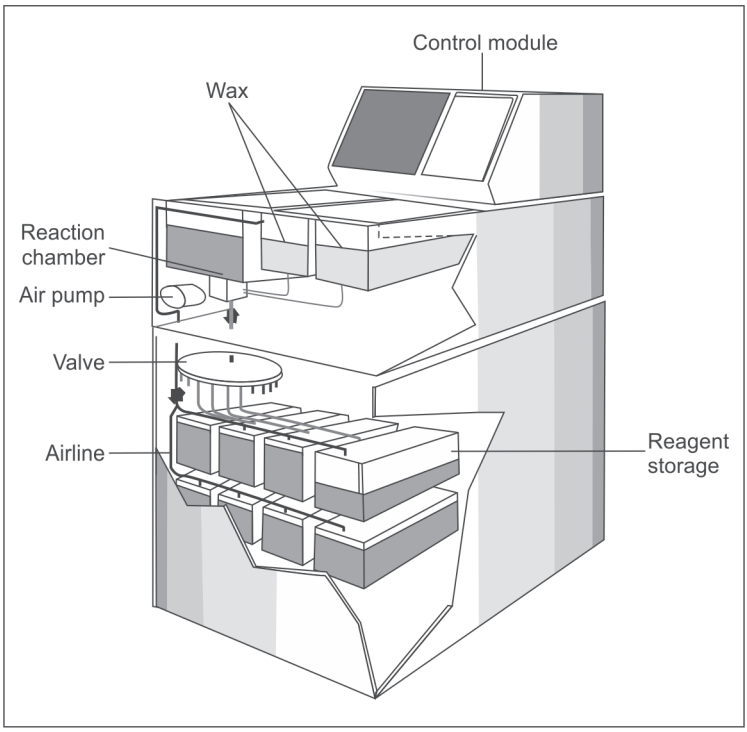

Figure 3.2: A fluid-transfer tissue processor. Tissue cassettes are loaded into the reaction chamber, which processing reagents are pumped 'to and fro' one after another. Contaminated air is not vented externally, but remains within the closed transfer system. Examples of this type are Shandon Hypercenter XP and Pathcenter or Bayers VIP.

TABLE 3.2: Example of a processing schedule for a fluid-transfer processor

Step of processing	Duration					
	Daytime			Overnight		
	Time (min)	*Temperature (°C)*	*P/V**	*Time (h)*	*Temperature (°C)*	*P/V*
Fixative				3.0	35	
Fixative				1.5	35	
70% ethanol	15		ON	1.0	40	
90% ethanol	15	40	ON	1.0	40	
Absolute ethanol	15	40	ON	0.5	45	ON
Absolute ethanol	15	40	ON	0.5	45	
Absolute ethanol	15	40	ON	0.5	45	
Absolute ethanol	15	40	ON	1.5	45	ON
Toluene or substitute	15	40	ON	0.5	50	

Contd...

Contd...

Step of processing	Duration					
	Daytime			Overnight		
	Time (min)	*Temp-erature (°C)*	*P/V**	*Time (h)*	*Temp-erature (°C)*	*P/V*
Toluene or substitute	15	40	ON	1.5	50	ON
Paraffin wax				0.5	60	ON
Paraffin wax	15	60	ON	0.5	60	ON
Paraffin wax	15	60	ON	1.5	60	ON
Paraffin wax	15	60	ON	1.5	60	ON
Embed						
Total time (exclusive of fluid transfer time)	2.75 h			15.5		

Note: Rapid day schedule for endoscopic or needle biopsy. Overnight schedule routine for lightly fixed specimens.
*P/V, pressure vacuum option.

GENERAL CONSIDERATIONS DURING PROCESSING

1. Baskets and metal cassettes should be clean and wax-free type.
2. Tissues should not be packed too tightly in baskets so as to impede fluid exchange.
3. Processors must be free of spilt fluids and wax accumulations to reduce hazards and ensure reliability.
4. Fluid levels must be higher than the specimen containers.
5. Timing and delay mechanisms must be correctly set and checked against the appropriate processing schedule.
6. A processor log should be kept in which the number of specimens processed, processing reagent changes, temperature checks on the wax baths and completion of the routine maintenance schedule are recorded as an integral part of the laboratory quality assurance program.

TISSUE RECOVERY PROCEDURES

Procedures for recovery of tissues that have air dried because of mechanical or electrical failure of the processor or accidentally returned to fixatives are as follows. These tissues will be difficult to section and will always fall short of optimum requirements. However, the next best option is to salvage tissue to provide slides of diagnostic quality particularly where fresh tissue is not available.

Both air-dried tissues as well as tissues accidentally returned into fixative or alcohol following wax infiltration should be transferred to dehydrants

and processed. All contaminant reagents should be discarded. Dried tissues should first be treated overnight in a solution of:

- 70% ethanol: 70 mL
- Glycerol: 30 mL
- Dithionite: 1 g.

TISSUE PROCESSING METHODS

Processing begins with dehydrating solutions and continues to completion.

Manual Tissue Processing (Table 3.3)

Manual tissue processing is usually undertaken for the following reasons:

1. Power failure or breakdown of a tissue processor.
2. A requirement for a non-standard processing schedule as for:
   a. Rapid processing of an urgent specimen.
   b. Delicate material.
   c. Very large or thick tissue blocks.
   d. Hard, dense tissues.
   e. Special diagnostic, teaching or research applications.
   f. Small scale processing requirements.

TABLE 3.3: Manual processing of 1–2 days schedule for well-fixed tissues processed using a magnetic stirrer

Step of processing	Duration	
	1–2 mm tissue thickness	3–4 mm tissue thickness
70% ethanol	20 min	1.5 h
90% ethanol	20 min	1.5 h
Absolute ethanol	20 min	1.5 h
Absolute ethanol	20 min	1.5 h
Absolute ethanol	20 min	1.5 h
Chloroform or substitute	20 min	
Chloroform or substitute	20 min	
Methyl salicylate		Overnight
Paraffin wax	20 min	1.0 h
Paraffin wax	20 min	2.0 h
Paraffin wax		1.0 h
Paraffin wax under vacuum	20 min	0.5 h
Embed		
Total time	3 h 20 min	1.5 day

Resin Embedding

The main advantage of manual processing over automated methods lies in the flexibility of reagent selection, conditions and schedule designed to provide optimum processing for small batches of tissues. Exposure of tissues to the deleterious effects of some reagents can be carefully monitored and regulated through observation and precise timing.

Manual processing can be time-consuming and inconvenient. Care must be exercised so that tissues are left overnight in reagents that will cause minimal harm. A permanent series of solutions in wash bottles simplifies processing small single specimens. Tissues are processed in tubes and agitated on a rotor. Reagents are pipetted or decanted through a fine sieve. Multiple specimens or large blocks are economically processed in large lidded jars of processing fluids. The specimen reagent volume ratio should be at least 1:40 to 50. Agitation is provided by a magnetic stirrer.

Microwave-stimulated Processing (Fig. 3.3 and Table 3.4)

Rapid manual microwave-stimulated paraffin wax processing of small batches of tissues gives excellent results, which are comparable to tissues processed by longer automated non-microwave methods. Processing is undertaken

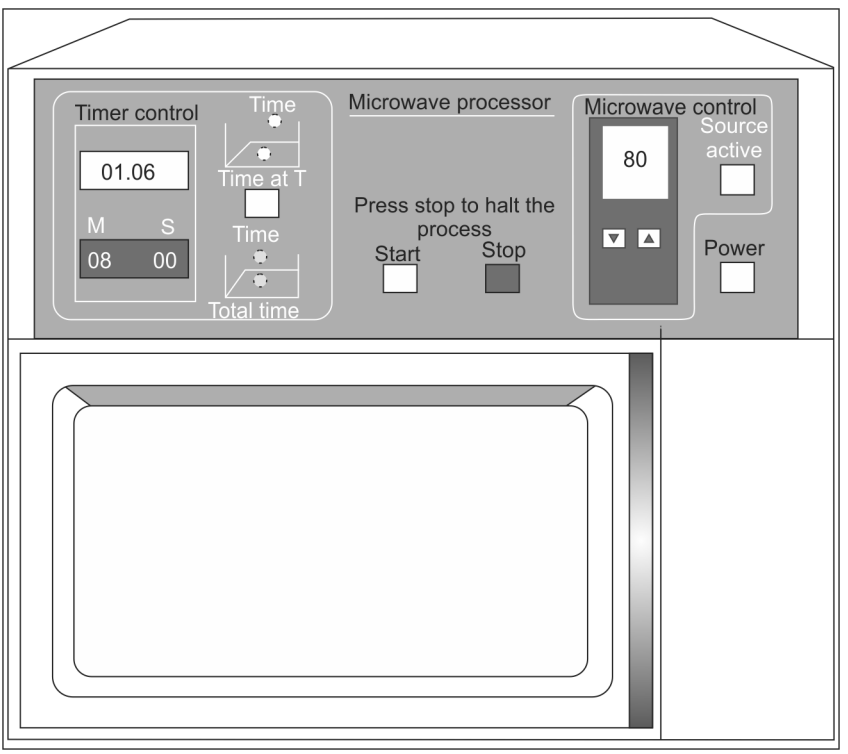

Figure 3.3: A microwave-stimulated tissue processor (T, temperature; M, minute; S, second).

TABLE 3.4: Schedules for microwave-stimulated processing

Step of processing	Temperature (°C)	Tissue block thickness		
		< 1 mm (min)	1–2 mm (min)	2–5 mm (min)
100% ethanol	67	5	15	60
100% isopropanol	74	3	15	45
Paramat wax	67	2	15	30
	82	5	20	60
Embed				
Total time		15	65	195

in a dedicated microwave oven, which is fitted with precise temperature control and timer, and an interlocked fume extraction system to preclude accidental solvent vapor ignition. Agitation is provided by an air-nitrogen system.

Domestic microwave ovens with a temperature probe and timer accurate to seconds are suitable for tissue processing. A turntable or inbuilt radiation disperser facilitates even reagent heating. Toxic and flammable solvent vapors generated during processing cannot always be adequately vented from these ovens and present an ignition hazard if the electrical system is unprotected. Ovens should therefore be used within a fume cupboard to minimize this problem. Calibration of domestic ovens is essential for optimum results and the accuracy of the temperature probe, duration of cycle time and net power levels at various settings must be determined before the oven is used to process tissues. Tissues are placed in conventional plastic cassettes. Transparent glass or solvent-resistant plastic containers of about 200 mL capacity are ideal for processing batches of up to 14 cassettes per container.

Fixation in Microwave Oven

1. For rapid processing, tissues are fixed by microwave irradiation.
2. They can also be fixed in 95% ethanol—PEG 400 from which specimens can be transferred directly to dehydrants.
3. Formaldehyde-fixed tissues must be rinsed in running tap water for 5 minutes before microwave processing and an extra dehydration change incorporated in the schedule.

Hints for Microwave Processing

1. Tissue blocks should be as thin as possible.
2. Length and width are not as important.
3. Process blocks of similar thickness together.
4. Reagent volumes should be at least 50 times that of specimen volume.
5. The temperature probe should be placed centrally in the processing baths.
6. Use a dummy load to check whether heat generation should boil the

reagents on minimum settings; an equal volume of reagent irradiated together with the primary load effectively halves the energy received by the primary load.

7. Preheat the paraffin wax baths in a conventional oven.
8. An increase in the number of cassettes or fluid volumes will require a concomitant increase in power and/or time to achieve the correct processing temperature.

Processing of Tissues for Electron Microscopy (Table 3.5)

The standard protocol for processing of tissues for transmissible electron microscopy (TEM) and electron microscopy involves primary fixation in an aldehyde (usually glutaraldehyde) to stabilize the proteins followed by secondary fixation in osmium tetroxide. For TEM processing, dehydration is performed by passing the specimen through increasing concentration of an organic solvent, e.g. ethanol. Commercially available absolute alcohol contains a small percentage of water, which severely restricts infiltration and polymerization of the resin used for infiltration. Hence, it is necessary to complete dehydration in anhydrous alcohol. Ethanol also requires the use of propylene oxide (1,2-epoxypropane) as a transition solvent to facilitate

TABLE 3.5: Standard processing schedule for solid tissues cut into 1 mm block (at room temperature)

Processing step	Material used/exposure	Duration
Primary fixation	2.5% glutaraldehyde in 0.1 M phosphate buffer	2–24 h
Wash	0.1 M phosphate buffer	2 × 10 min on rotator
Postfixation	1% aqueous osmium tetroxide	60–90 min
Wash	Distilled water	2 × 10 min
En bloc staining (optional)	2% aqueous uranyl acetate	20 min
Dehydration	70% alcohol 90% alcohol 95% alcohol 100% alcohol Anhydrous absolute alcohol	10 min on rotator 10 min on rotator 10 min on rotator 15 min on rotator 2 × 20 min on rotator
Transition solvent (clearing)	1,2-epoxypropane	2 × 15 minute on rotator
Infiltration	50:50 clearant of resin 25:75 clearant of resin Resin only	1 h 1 h 1–24 h (with vacuum to remove bubbles)
Embedding	Fresh resin in embedding capsules	12–24 h at 60–70°C

resin infiltration. Propylene oxide is highly volatile, flammable and forms explosive peroxides; should be stored at room temperature in a flammable solvent facility.

Embedding

The step following dehydration is to infiltrate the tissue sample with liquid resin. In routine TEM, synthetic embedding resins are used that are capable of withstanding the vacuum in the electron microscope column and the heat generated as the electrons pass through the section. This requires gradual introduction of the resin, beginning with 50:50 mix of transition solvent (propylene oxide) and resin followed by 25:75 transition solvent-resin mix, and then finally the resin. An hour in each of the preliminary infiltration steps is usually adequate, although it is preferable to leave the samples in pure resin for 24 hours. Once infiltrated, tissue samples are placed in an appropriate mold, which is filled with resin and allowed to polymerize using heat.

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Chapter 4 Tissue Embedding

Embedding is the process by which processed tissues are surrounded by a support medium such as agar, gelatin or wax, which on solidification will provide sufficient support during sectioning. The choice of the embedding media depends upon the microscope, the type of microtome and also the type of tissue used. Paraffin wax is a suitable embedding media for most tissues from which sections taken of 4–6 μ thickness are satisfactory for most diagnostic purposes. In instances where paraffin wax is unsuitable for embedding (e.g. as in a requirement for better support) and there is a need for very thin sections or preservation of enzymes then plastic may be used in place of paraffin. A modular tissue embedding center is shown in Figure 4.1.

EMBEDDING TISSUES IN PARAFFIN WAX

Tissues are embedded by placing them in a mold filled with molten embedding medium, which is then allowed to solidify. Embedding requirements and procedures are essentially the same for all waxes. The technique for paraffin

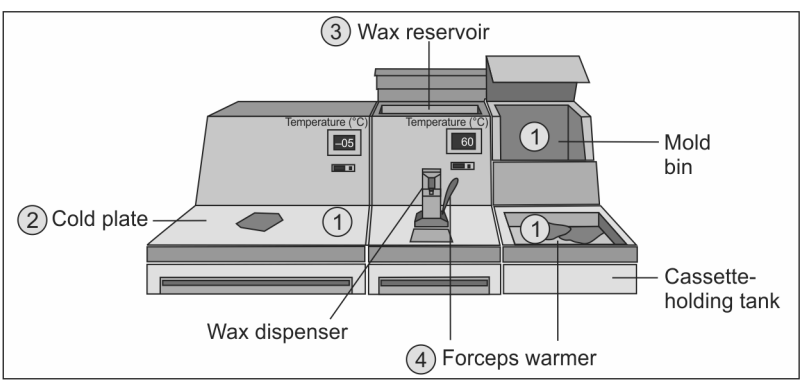


Figure 4.1: Components of a modular tissue embedding center. **1.** Mold warmer, cassette bath, working surface warmer; **2.** A cold plate with a temperature of –5 to +5°C, which occupies 60 blocks; **3.** Large 3–5 L capacity paraffin reservoir with a temperature of 45–60°C; **4.** Forceps warmer, convenient drain for excess wax.

wax is provided here in detail. At the completion of processing, tissues are held in clean molten paraffin wax, which is free of solvent and particulate matter. Requirements for embedding are as follows:

- Supply of clean, filtered paraffin wax held at 2–4°C above its melting point
- Cold plate to rapidly cool the wax
- Supply of molds to embed the tissues.

These elements are conveniently combined in commercially available embedding stations.

TECHNIQUE OF EMBEDDING

1. Open the tissue cassette and check whether correct number of tissue pieces is present.
2. Select the mold. There should be sufficient room for the tissue with at least a 2 mm surrounding margin of wax.
3. Fill the mold with paraffin wax.
4. Place the selected tissue firmly into the wax with a warmed forceps. It is important to ensure that the correct orientation is maintained and the tissue surface to be sectioned is kept flat, facing downwards.
5. Insert the identifying label or place the labeled embedding ring or cassette base onto the mold.
6. Cool the block on the cold plate or carefully submerge it under water when a thin layer has solidified over the wax surface.
7. Remove the block from the mold.
8. Cross check block and label.

DIFFERENT METHODS OF EMBEDDING

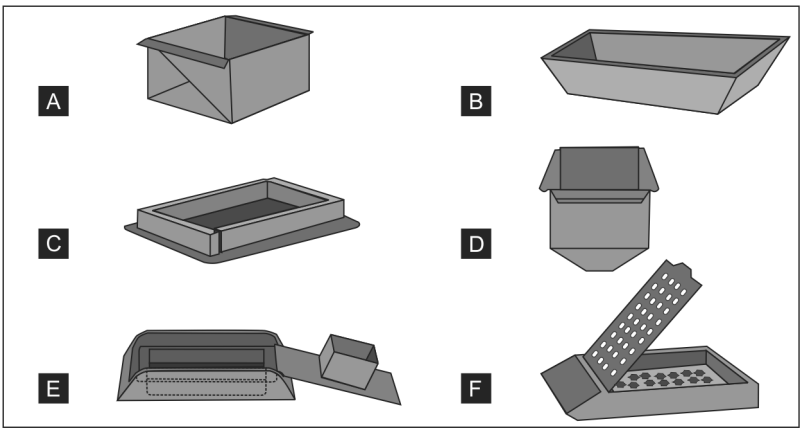
There are four main mold systems and associated embedding protocols presently in use (Figs 4.2A to F).

Leuckart or Dimmock Embedding Irons

Leuckart method is the conventional method of blocking. These consist of two L-shaped pieces of heavy brass or similar metal, a base being formed of copper or brass or glass plate 3 × 2 square inch. Glycerin is applied to the L pieces and also to the metal or glass plate on which molds are arranged before pouring the molten wax. The tissue is then embedded within the molten wax with proper orientation and labeling. After cooling, the molds are removed and wax cakes formed.

Paper Blocks/Boats

Waxed paper blocks are suitable for embedding. The block is convenient to release from the paper mold. Blocked tissue may be stored with an identifying number for a long time.



Figures 4.2A to F: Requirements for embedding: Molds. **A.** Paper boat; **B.** Metal boat; **C.** Dimmock embedding; **D.** Peel-A-Way disposable; **E.** Base used with embedding ring; **F.** Cassette bases.

Peel-A-Way System Using Disposable Plastic Molds

Peel-A-Way systems are present in different sizes. The number tabs are embedded in molten wax, the block number facing against the transparent plastic. The plastic walls are peeled off, once the wax gets solidified. The block requires no trimming and may be placed directly on the microtome.

Tissue-Tek System

Plastic embedding system of this type has replaced all other types of embedding. The plastic molds support the block during sectioning and also eliminate the step of mounting the block to the holder.

Tissue-Tek System I or Mark I System

Tissue-Tek system I comprise of stainless steel base molds in which the tissue block is embedded and a plastic ring, which is laid on the top and filled with wax. The wax is then cooled rapidly in running tap water or in the refrigerator. The base mold is then detached and reused. The plastic ring functions as the block holder and fits directly on to the microtome. The blocks can also be stored with the plastic ring for long periods of time. The drawback is that more space is required for storing of blocks.

Tissue-Tek System II or Mark II System

Tissue-Tek system II is a modification of the above embedding method. The Mark II system uses about one-third wax less than Mark I system and the blocks occupy much less storage space. The drawback is that a special clamp has to be used in the microtome for sectioning and also because the system uses a shallow cassette, thin tissue slices have to be taken for processing.

PARAFFIN OVEN OR INCUBATORS

Ovens should be sufficiently large to accommodate an enamel jug with a funnel inserted for filtering paraffin wax. If not filtered the impurities present may interfere, while cutting the paraffin sections. The ovens used for wax embedding should have a temperature ranging from 50–65°C, about 50 more than the molten wax.

ORIENTATION OF SPECIMENS, WHILE EMBEDDING (Figs 4.3A to D)

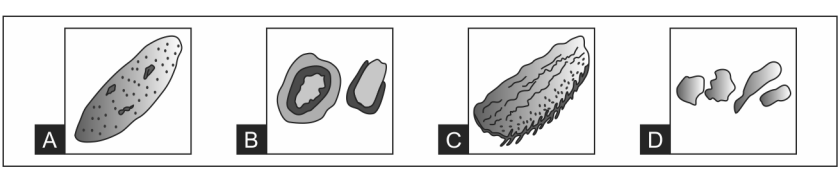
Correct orientation of tissue in a mold is the most important step in embedding. Incorrect placement of tissues may result in diagnostically important tissue elements being missed or damaged during microtomy. Usually tissues are embedded with the surface to be cut facing down in the mold. Some general considerations are as follows:

1. Long tissues are placed diagonally across the block.
2. Tubular and walled specimens such as vas deferens, fallopian tubes, cysts and gastrointestinal tract tissues are embedded so that the knife cuts across the lumen, i.e. it provides transverse sections showing all tissue layers.
3. Skin and other epithelial biopsies such as intestine, bladder, gallbladder, etc. must be positioned so that the plane of the section is across all tissue layers. Hairy or keratinized epithelium is oriented to face the knife last. Cutting the hard keratin layer of the skin at last minimizes compression, scratches and cuts in the subcutaneous layers.
4. Multiple specimens embedded in the same block such as gastric biopsies should be placed side by side with a little space between them.
5. Rectangular pieces should be orientated with their long axis nearly parallel to the knife edge to minimize distortion and wrinkling.
6. Muscle biopsies should be embedded for sectioning both longitudinally and transversely.
7. Inked surfaces and margins: The tissues that have had margins identified with India ink or dye should be so placed that the ink will be visible on the cut section on one aspect.

TISSUE MARKING SUBSTANCES

Criteria for the selection of a suitable tissue marker are:

1. The marking substance must be relatively insoluble in fixative, processing reagents and embedding medium.



Figures 4.3A to D: Tissue orientation in the block. **A.** Elongate tissues; **B.** Tubular or cystic specimens; **C.** Hairy skin, the knife edge cuts the lower block margin last; **D.** Multiple tissue fragments.

2. It must remain on the surface of the specimen and not penetrate tissue.
3. It should not react with histological stains and must be clearly identifiable both macroscopically and microscopically.

Tissue markers are applied to the surface of the specimen using disposable swabs and allowed to dry. Before marking the surface it should be mopped with a swab of cotton to remove surface fluid. The cotton used should be wet, which has been squeezed well to remove excess water, else it leaves cotton fibers on the specimen:

1. India ink provides good black macroscopic and microscopic marking. It is resistant to processing, but takes 15–30 minutes to dry, and may spread beyond the marked area. It is routinely used in the laboratory to mark surgical margins.
2. Silver nitrate (stick) provides a brown-black mark resistant to processing.
3. Alcian blue, 1% aqueous solution, is a rapid and reliable stain for marking resection margins of fixed breast and other biopsies. This method comes handy in excision lumpectomies of the breast where the entire surface needs to be marked.
4. Eosin, erythrosin and rose bengal (1–2% aqueous) are used to stain small translucent specimens. Tissues are stained for 5 minutes, rinsed in water and then processed. They are easily picked up for embedding.

ALTERNATIVE EMBEDDING MEDIA

Alternative embedding media may provide optimum support for tissues in applications for which paraffin waxes are unsuited, for example when:

- Tissue components are heat or reagent labile
- Hard or dense tissues are inadequately supported
- Adhesion between specimen and wax is poor
- Very thick or very thin sections are required
- Sectioning whole organs such as lung or brain.

Aqueous Media

Agar

Agar is not used alone, but as a double embedding media. The high melting point and low gelling temperature of agar make it ideal for double embedding the multiple small tissue fragments. Agar gel does not provide sufficient support for sectioning the tissue when employed alone. Its main use is in acting as a cohesive agent for small friable pieces of tissue before embedding.

Gelatin

Gelatin is used primarily in the production of sections of whole organs in Gough-Wentworth's whole-organ sectioning method. The low melting point of gelatin (35–40°C) makes it unsuitable for double embedding. Gelatin embedding supports large tissue blocks for sectioning. It may be used for frozen sections of friable or partially necrotic tissue or small fragments of

tissue such as uterine curetting and also in phospholipid and enzyme studies for histochemistry with a freezing microtome. This technique has now largely been superseded by other media, the optimum compound temperature (OCT) used for cryotomy.

Plastic (Resin)

Resin was introduced as an embedding media in the preparation of ultra-thin sections required for electron microscopy. The plastic media is a pale liquid and it polymerizes to a solid in blocking. The change in the physical state of an embedding media from liquid to solid is called polymerization and is brought about by joining molecules together to produce a complex macromolecules made of repeating units (poly means many). These agents are readily available, relatively inexpensive, easy to prepare and handle, of low viscosity and allow short infiltration time. Consistently thin sections can be cut and enable a wide range of staining methods to be used. Plastic are classified according to their chemical composition.

Acrylic Media

Butyl Methacrylate/Glycol Methacrylate

Methacrylate was introduced into microtechniques in 1949. The principle use of methacrylate monomers is its ability to polymerize in the presence of a catalyst, such as heat ultraviolet light or artificial catalysts such as benzoyl peroxide (1.5–2.0%), which results in radical formation with subsequent polymerization. Methacrylate is readily miscible in ethanol and gives a clear hard block when polymerized. Methacrylate rapidly infiltrates and dehydrates tissue at room temperature. However, damage to the tissue is common during polymerization because of marked shrinkage of tissue components. Mixes can be devised to produce plastics with a wide range of properties. These resins are more suited for embodiment of hard materials such as decalcified bone and other hard tissues. Glycol methacrylate has been used for high-resolution autoradiography.

Epoxy Resin (Araldite)

An epoxy (epoxide) resin is a substance, which is capable of polymerization to form a three-dimensional structure with cross-linking between molecular chains. The structure once formed is not reversible. Araldite polymerizes in a different way as compared to methacrylate, the cross-linking causes the 'cured' solid block of Araldite to be insoluble in any solvent. Epoxy resin is a carefully balanced mixture of epoxy plastic, catalyst and accelerator; each component has a direct influence on the properties of the cured plastic.

Its advantages are that it maintains the true geometry of the tissue, tends itself to a higher resolution work, shrinkage of Araldite is considerably less and the tissue is not subjected to stress as set up during the polymerization

of methacrylate. Longer infiltration time is required as the resin has a higher viscosity than methacrylate. Araldite embedding solutions consists of:

- Araldite stock solution
- Araldite: 10 g
- Epon: 10 g
- Hardener (anhydride catalyst), dodecenyl succinic anhydride (DDSA): 15 g
- Accelerator, dimethylaminomethyl phenol (DMP).

The disadvantages of Araldite are cutting artifacts, dermatitis in hypersensitive people and its possible carcinogenic effect.

Epon 812: It is a glycerol-based highly reactive epoxy resin with a low viscosity. Hence, infiltrates the tissue faster and makes the sectioning relatively easier. In practice, semi-thin sections 2–3 μ can be cut with ease.

Polyester Resin

Polyester resin has the same cutting consistency as that of methacrylate, but cutting at a later stage on older block becomes difficult, e.g. Vestopal. These days they are seldom used for microscopy and superseded by other epoxides.

Celloidin

Celloidin is the purified form of nitrocellulose obtained by treating cellulose with sulfuric and nitric acid. It is supplied as pulpy, cotton-like material and is referred to as gun cotton. In the past, celloidin was preferred over paraffin as an embedding medium because it provided better support for sectioning hard tissues such as bone, uterus as well as for delicate specimens such as eyes, central nervous system tissues and embryonic tissues. Cellulose does not require heat at any stage of processing and is recommended for infiltrating and embedding tissues that can be damaged by solutions requiring heat. The working strength is 2%, 4% and 8%, the solvent being equal parts of ether and alcohol.

The advantages are that it causes less shrinkage and hardening of the tissues than paraffin because there is no heat involved in the process. The relationship of tissue components is well preserved. It is excellent for embryos and large tissues. The disadvantages are that the procedure is time-consuming, requiring 7–10 days to infiltrate the specimen. Celloidin attracts water, which prevents the solution from solidifying and causes the block to become too soft for sectioning. The block requires storing in 70–80% alcohol so knife and block must be kept moist with 70–80% alcohol. It is also extremely difficult to obtain sections thinner than 10 μ. The preparation of serial sections of celloidin impregnated tissue is normally a tedious procedure since the sections will not adhere to one another in the same manner as paraffin embedding material.

Low Viscosity Nitrocellulose

Low viscosity nitrocellulose (LVN) has a lower molecular weight than celloidin and forms 20% solution with ease. It is insoluble in water, but soluble in most

hydrocarbon solvents. It penetrates tissue rapidly and forms a harder block than celloidin, thus permitting easy sectioning. Sections have a tendency to crack when embedded in pure LVN, hence a small amount of celloidin is added to give elasticity to the block. The average time for impregnation is 6–11 days. Immunohistochemical investigations such as immunophenotyping of lymphoid and non-lymphoid cells are possible on nitrocellulose processed tissues. The disadvantage of LVN is that sections produce cracks during staining, which can be solved by using 95% alcohol as a solvent instead of absolute alcohol. The LVN is an explosive and not handled near fire places. The composition is as follows:

- Celloidin: 4 g
- LVN: 140 g
- Ether: 250 mL
- Absolute ethyl alcohol: 210 mL.

EMBEDDING TECHNIQUE

There are two methods of embedding, dry and wet.

Wet Celloidin Technique

The 'wet' celloidin technique is mainly used for bones and teeth, whole organs and large brain blocks. In this, blocks and sections are stored in 80% alcohol and during cutting both the block and knife have to be kept moist with this alcohol.

Preparation of 'Wet' Celloidin

- Ether-ethyl alcohol 100% equal parts: 24 hours
- Celloidin 4%: 2–3 days
- Celloidin 8%: 2–3 days
- Celloidin 4%: 2–3 days embedding.

Dry Celloidin Technique

The 'dry' celloidin technique is used only for eyes. It obviates the necessity of 80% alcohol for the storage of blocks and during cutting. When the tissue in celloidin is nearly set, it is placed in Gibson's mixture (equal part of chloroform and cedar wood oil). The dry celloidin technique is used to avoid the necessity of having to cut celloidin blocks using 80% alcohol.

Preparation of Dry Celloidin Blocks

- Follow all steps of wet celloidin
- Place tissue in equal parts of cedar wood oil and chloroform for 24 hours
- Place in solution of cedar wood oil three parts and chloroform one part for 24 hours
- Store blocks in cedar wood oil for 24 hours or longer.

This method is not frequently used and the wet celloidin still remains the method of choice.

DOUBLE EMBEDDING AND DOUBLE INFILTRATION METHODS

Double embedding is the process by which tissues are first embedded or fully infiltrated with a supporting medium such as agar, celloidin or nitrocellulose, then infiltrated a second time with wax in which they are also embedded. The main use of this method is for cutting sections of delicate tissue and preparing sections from blocks of tissue of varying consistency, e.g. eyes where retina is easily detached.

Agar-paraffin Wax Double Embedding

Double embedding in agar-paraffin is a reliable and convenient method of handling minute and friable tissue fragments such as curettings and endoscopic biopsies, which can be lost during tissue processing. It also overcomes the difficulty of manipulating small tissue fragments during embedding and facilitates correct orientation and identification of tissues. This method provides additional support to hard tissues and ease of wax microtome.

Procedure of Embedding in Agar and Paraffin

The fixative with the fragments is fitted through Millipore filter using suction and the fragments collected on top of the filter in the filter tube. Molten agar is slowly poured onto the filter, allowed to solidify and then the excess agar is trimmed from around the tissue fragments. The agar block plus the filter paper is now processed and embedded in the paraffin wax. The filter paper provides no difficulty in sectioning the block.

Agar-ester Wax Double Infiltration

Double infiltration of tissues in agar and ester wax aids thin serial sectioning of the tissues at 0.5–1.0 μ. The fine crystalline nature and hardness of ester wax improves tissue-wax adhesion and provides adequate support for thin serial sectioning.

Nitrocellulose-paraffin Wax Double Infiltration

Nitrocellulose-paraffin wax double embedding is mainly used for brain, friable tissues and decalcified bone. It combines the plasticity and support provided by nitrocellulose with convenient handling and microtome of the paraffin technique. Tissues may be infiltrated with a thick nitrocellulose solution, the resulting block hardened in chloroform and then embedded in paraffin wax.

Tissues may be transferred directly from 8% celloidin to two changes of chloroform or benzene and subsequently embedded in paraffin wax, also known as Peterfi's double embedding method. Sections of double-embedded tissues may tend to wrinkle or curl on the water bath. Floating on 95% ethanol softens the LVN and facilitates section flattening.

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Chapter 5

Microtomy

The principle idea of this write-up is to provide a concise presentation of this instrument, its effective use and care, and many rules of thumb acquired through experience to combat difficulties that may be encountered. The data provided in this chapter has been taken from many books as well as from Author's own experience. The secret of microtomy lies mainly in acquiring personal skills in sharpening knives and manipulating the microtome, and this essentially lies hidden in the word 'practice.'

DEFINITION

Microtomy is defined as the means by which tissue can be sectioned and attached to a surface for microscopic examination. Microtomes are defined as precision instruments designed for cutting material into sections, thin enough for examination with a microscope.

HISTORICAL ASPECTS

Microtomes were called 'cutting machines' till 1839 when Chevalier introduced the term 'microtome.' Various instruments were devised to act as microtomes. The earliest known microtome was invented by Cummings (1770), the specimen being held in a cylinder and advanced forward by a fine screw with the knife pivoted across it. Most such instruments were hand models and it was Baker from London, who introduced a table model in addition to the then hand models being produced on a commercial scale. The first table model was introduced in 1840. The mid-19th century foreseen the introduction of a knife holder by Schanze.

PRINCIPLES OF MICROTOMY

Principles Governing Microtome Function

- With a movable knife (block stationary)
- With a stationary knife (block moves).

Some modifications of these principles have resulted in different models as detailed below.

With a Movable Knife (Block Stationary)

Object feed vertical:
- Knife held in hand table and hand models
- Knife moves around a vertical axis at one end with the other end free or supported by a slideway, e.g. freezing microtome
- Knife holder pivots on a parallelogram
- Knife holder moves on a horizontal tract
- Object feeds up an inclined plane.

With a Stationary Knife (Block Moves)

1. Knife vertical:
   - Objects moves along vertical guide (e.g. rotary)
   - Object holder moves around a horizontal axis (e.g. rocking).
2. Knife horizontal:
   - Objects moves horizontally (e.g. base sledge).

Ultramicrotome

- Mechanical advance
- Thermal advance
- Combination of both the above.

Principle on Which the Microtome Works

A pawl is brought into contact with a ratchet wheel, which is connected to a millihead micrometer screw. This action turns the ratchet wheel, which in turn rotates the screw. By these means, the block is moved towards the knife at a predetermined thickness.

CLASSES OF MICROTOMES

- Rotary
- Rocking
- Sliding
- Sledge
- Microtomes for frozen sections:
  - Freezing microtome
  - Cryostat.
- Ultramicrotome.

Rotary Microtome (Figs 5.1A and B)

The rotary microtome was invented by Minot in 1885–1886 in Germany and independently by Pfeiffer in 1886 in the United States. It is the most commonly used microtome in the laboratories. This microtome is so-called because a rotary action of the handwheel actuates the cutting of sections. The block holder is mounted on a steel carriage that moves up and down.

The specimen (block holder) moves vertically down through the cutting surface and returns to the starting position, and is advanced by a micrometer screw on rotation by a handwheel through an angle of 360°.

Principle

The rotary action of the handwheel (360°) advances the specimen and activates the cutting. For details on the parts, refer Figure 5.1A.

Types

- Manual
- Semiautomated: The autocut microtome has a built-in motor drive with foot and hand control; with suitable accessories, the machine can cut thin resin sections of 0.5–2.0 μm thickness
- Fully automated: An example is automated cryostat.

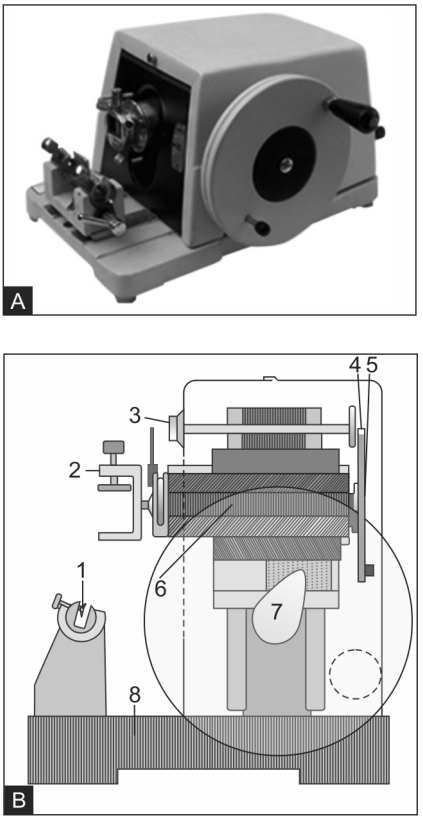
Advantages

- Stable and less of vibration
- Easy adaptation to all types of tissues (hard, fragile and fatty)
- Ability to cut thin 2–3 μm sections
- Cutting angle and knife angle can be adjusted
- Suitable for cutting small tissues embedded in the celluloid and paraffin wax
- Technological advances in the automation has improved the section quality, increased productivity and occupational safety.

Disadvantages

- Complex design
- Initial cost is relatively higher
- Knife is placed in the blade up position and can be dangerous to the operator (to avoid this, knife guard/protector can be used)
- Not suitable for cutting large blocks.

Rotary microtome is very popular all over the world. It is manufactured by several companies including



Figures 5.1A and B: Rotatory microtome. **A.** Photograph; **B.** Diagram (**1.** Knife holder; **2.** Chuck; **3.** Thickness scale; **4.** Ratchet wheel; **5.** Pawl; **6.** Millihead micrometer screw; **7.** Axle with wheel; **8.** Base of microtome).

Leitz (West Germany), AO-Spencer (USA), Reichert-Jung [USA, Austria and Lipshaw (USA)].

Rocking Microtome (Fig. 5.2)

Figure 5.2: Rocking microtome

The rocking microtome was invented in 1881 by Caldwell and Threlfall, and improved on by Charles Darwin in 1885. Now it is manufactured by Cambridge and Baker. The microtome derives its name from the rocking action of the cross arm. The microtome has knife clamps, block holder, microtome adjustment screws, operating handle, feed mechanism, etc.

Principle

In the Cambridge rocking type microtome, knife is fixed and block of tissue moves through an arc, and strikes against the knife by means of a ratchet-operated micrometer thread. The turning of the ratchet wheel rotates the micrometer screw and elevates the lower arm to a more obtuse angle, producing a forward movement. The movement is relayed to the upper arm, thereby moving the blockholder forward. Therefore the sections are cut in a slightly curved plane.

Advantages

- Excellent for serial sectioning (60–90 sections ribbon)
- Very small blocks can be cut
- The instrument is cheap, reliable and easy to maintain.

Disadvantages

- Does not give flat sections because of the rocking movement
- It is light weight, therefore, cutting hard tissues may give vibrations
- Blocks cannot be used with other microtomes.

Sliding Microtome (Fig. 5.3)

Sliding microtome was first developed by Adams in 1798. Two types of models exist, one where the knife is static and the other where the knife moves horizontally against a fixed part. This type of microtome is manufactured principally by Leitz (West Germany) and by Reichert-Jung.

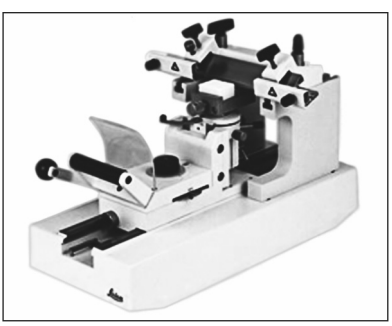


Figure 5.3: Sliding microtome

Advantages

- Used for cutting celloidin-embedded tissues
- Ideal for brain sectioning
- Simple design with no complex moving parts
- Large and serial sections can be cut
- Knife is large and need not be sharpened often as greater cutting edge is available
- Easy to operate and maintain.

Disadvantages

- The sliding knife tends to jump on striking hard tissue
- Difficult to sharpen the long knife.

Sledge Microtome (Fig. 5.4)

Sledge microtome was designed to cut sections of very large blocks. It is similar to a sliding microtome. The block holder is mounted on a steel carriage, which slides backward and forward on guides against a fixed horizontal knife. Usually a wedge-shaped knife is used.

Advantages

- The microtome is heavy, consequently stable and not subjected to vibrations
- Large and hard tissues can be cut
- Used in freezing microtome and also for celloidin-embedded tissues.

Disadvantage

Sledge microtome is much slower to use as compared to other microtomes (unless much practice of the instrument is made).

Freezing Microtome (Fig. 5.5)

The simple type of freezing microtome is a machine, i.e. clamped to the edge of a bench and is connected to a cylinder of carbon dioxide by means of a specially strengthened flexible metal tube. It consists of a radial arm attached to a central pivot. On this arm, two clamps hold a wedge profile microtome knife mounted with a simple block holder, with a cutting edge inclined in a horizontal plane.

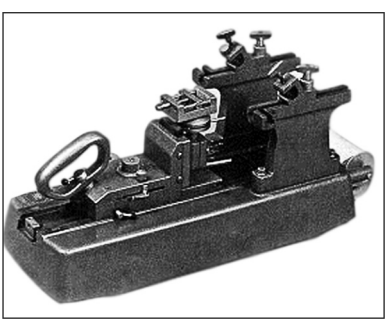


Figure 5.4: Sledge microtome

Principle

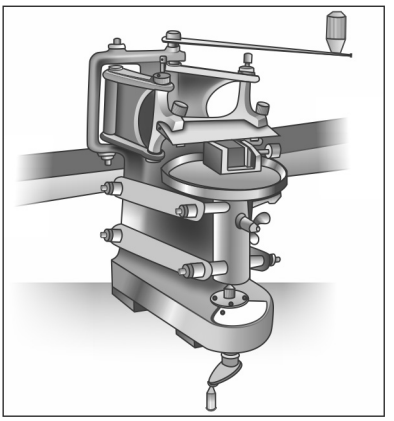


Figure 5.5: Freezing microtome

The object is mounted on a blockholder (chuck) also known as freezing stage, with a centrally advancing screw. The blockholder is perforated and attached to a feed pipe carrying carbon dioxide gas, which can be sprayed on to the tissue for freezing. The knife moves over the block around a horizontal axis when once the tissue hardens.

Thermoelectric cooling device units may be used in place of carbon dioxide gas to freeze the tissue and cool the knife. The cooling produced by the thermoelectric unit depends upon the flow of direct current, which may be regulated by means of power packs. In this stage, temperature can be reduced from ambient to –36°C in 60 seconds, but the optimum cutting temperature for the tissue is usually about –20°C.

Manufacturing company: Leitz, Spencer and MSE.

Advantages

- Freezing microtome is used in the demonstration of fat
- Can be of diagnostic use when affordability of cryostat (which is superior to this) is not possible
- Better demonstration of soluble and diffusible substances.

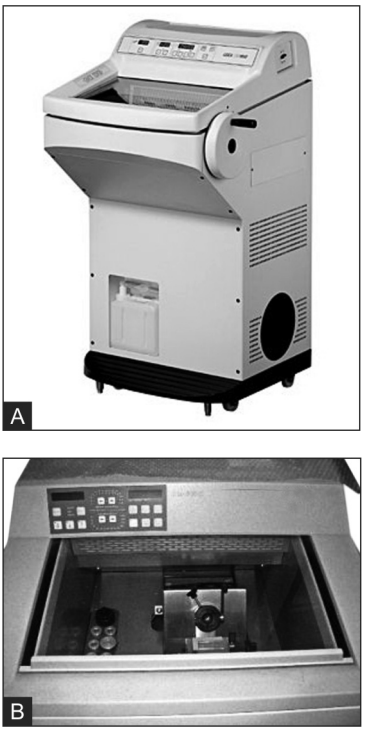
Disadvantages

- The knife and tissue block are exposed to the atmosphere temperature and conditions soon after freezing
- No serial sectioning possible
- Sections less than 8 μ cannot be cut under the best of conditions
- Liquid carbon dioxide must reach the valve in the chuck, therefore cylinders must be held upside down in a special holder or blocks to ensure that the cylinder valve is the lowest point of the cylinder.

Cryostat (Figs 5.6A and B)

The first cryostat ('cryo' meaning cold and 'stat' meaning stationary) was introduced in 1959. Cryostat is a refrigerated cabinet in which a microtome is fixed. The microtome used is usually a rotary type, but may be of sliding type or even rocking type and is rustproof. The microtome is mounted in a stainless steel cabinet (refer Fig. 5.6A) at an angle of 45°. It has an antifogging air-circulating system, a drain for defrosting and a shelf for four to six metal blockholders. The temperature of the cabinet is –5 to –30°C. All microtome

control operations are outside the cabinet. Harris International Microtome is most commonly used. This unit operates on the 'open top-cold box' principle (refer Fig. 5.6B).



Figures 5.6A and B: Cryostat.
A. Photograph; **B.** Microtome in cryostat.

Principle

To create a cold atmosphere around tissue blockholder and microtome by means of a special refrigerator type compressor, capable of taking temperatures below –30 to –50°C. The reason for freezing the tissue is to provide a hardened matrix for sectioning the tissue and at the same time, preserving biochemical or immunological properties of a cell or tissue. The coolant used is usually Freon 22.

Advantages

- Used extensively for rapid diagnosis, fat stains and enzyme histochemistry in neurological applications as well as in fluorescence microscopy
- Both the knife and tissue are maintained at same low temperature
- Capable of slicing sections as thin as 1 μm
- Serial sectioning is possible
- Automatic defrosting and sterilization
- Antifogging air circulatory system.

Disadvantages

1. Constant supervision and maintenance of temperature is required.
2. The whole instrument should be kept in an air-conditioned room to prevent excessive cryostat compressor functioning.
3. Lubricants of special type with a low congealing point have to be used. This prevents the lubricants solidifying at a cooler temperature within the chamber.
4. Freeze artifacts seen as holes in the tissues.
5. If the temperature is too low, the tissue becomes hard and crumbles, and becomes difficult to cut (in such cases, warm the tissue by pressing the thumb over the tissue).
6. Difficulty in sectioning fixed tissue.
7. High cost of the instrument.

Microtomes for Ultrathin Sections

The ultramicrotome was produced by Richards in 1956. It is used for cutting semithin (1 μ or slightly thinner) sections for optical microscopy or ultrathin sections (60–300 μm) almost exclusively for electron microscopy. Tissues are extremely small, rarely more than 0.5 mm in size and the greatest dimension of the block is less than 0.1 cm. Glass or diamond knives are used to cut sections and these are collected in a receptacle made around each knife edge with waterproof sticky tape to contain distilled water. The water acts as a floatation bath on which the sections cut are floated and can be picked up later (Figs 5.7A and B).

Types

Two types of microtomes are in use today:

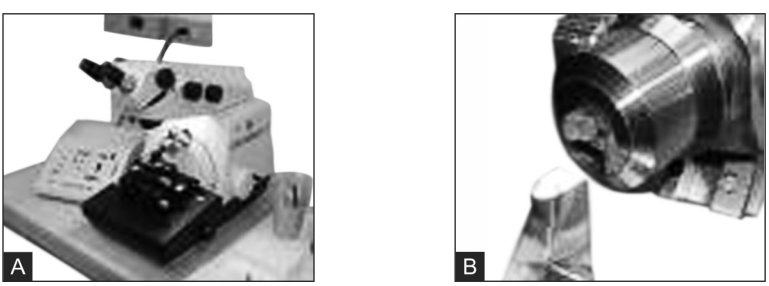
- Mechanical
- Thermal advance
- Also with a combination of the two, i.e. mechanical and thermal advance.

Both types are extremely sensitive to atmospheric temperature variations and both types are used with either glass or diamond knives. In order to achieve even sections, both microtomes are not normally operated by hand, but by an electric motor:

1. **Mechanical type:** Porter-Blum produced by Sorvall, utilizes the principle similar to rotary microtome and movement on a micrometer screw, which advances the specimen for cutting.
2. **Thermal advance type:** Produced by Reichert and LKB, functions on the principle of block advance. This advance is produced as a result of the heat generated between the block and the knife at the time of cutting.
3. **Mechanical and thermal advance type:** Variations and combination of both A and B are available. In general, the mechanical type system is used for semithin sections and the thermal advance system for ultrathin sections.

CARE OF A MICROTOME

Remember that a good microtome is made to last a lifetime. Proper care must therefore always be taken of it. After use, every time microtomes should have



Figures 5.7A and B: Ultramicrotome

all paraffin and other residues on it brushed or wiped away. All moving parts especially the sliding surfaces, the toothed wheel, the axle and the ratchet must be oiled with one or two drops of thin lubricant oil, e.g. '3 in 1' oil. The sliding surface must have light grease applied (of the type used on sewing machines).

Depending on its use, microtome advance on the geared wheel and pawl movement should be checked. One movement of the toothed wheel after a 360° rotation of the axle would indicate a block advance or knife advance (as the case may be) of 1 μ. Similarly, five movements of the toothed wheel would indicate an advance of 5 μ.

MICROTOME KNIVES: TYPES AND PARTS

Microtome knives are meant to make fine sections of various types of materials. It is important to use knives of guaranteed hardness as per the Vicker scale hardness. In the Vicker hardness test, the surface of the knife is indented by a diamond under a standard force of about 5–120 kgf (kilogram-force) for 15 seconds. Vicker hardness test is calculated by dividing the applied force by the surface area of the indentation. This method is very accurate and can be used for all metal knives. The knives are made of different types of material such as:

- Metal knives:
  - Standard steel
  - Razor blades
  - Disposable knives (made of steel).
- Non-metal knives:
  - Glass
  - Diamond.

Metal

Standard Steel Knives

Standard steel knives are made of high-grade steel with a high carbon content (e.g. tantalum) tempered from the tip inwards for one third of width. Actual hardness of the cutting edge may vary between 400 and 900 on the Vickers hardness scale (VHS). The hardness of the knives should be of such a nature as to hold a good sharpening for a long duration. These knives measure usually 120 mm, 180 mm, 210 mm and 260 mm.

Types of knives

Four types of standard steel knives are available (Fig. 5.8):

1. **Biconcave:** It was introduced by Heiffor and used for rocking, rotary or freezing microtome. It has concavity on the both sides. It is less rigid and more prone to vibrations (profile A).
2. **Planoconcave:** It is flat on one side and concave on the other side with varying degree of concavities. The more concave is used for paraffin

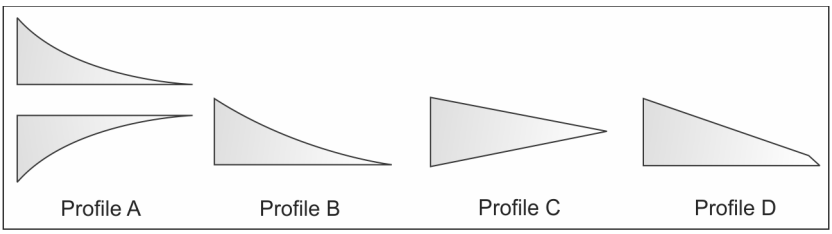


Figure 5.8: Types of metal knives

sections with the sledge, rotary and rocking microtomes, and the less concave ones are used for celloidin (profile B).

3. **Wedge-shaped knife:** It is plain on both sides. Its size varies from 100 to 350 mm in length and varied masses. It is used in routine paraffin sections, frozen sections and all types of microtomes (profile C).
4. **Chisel-shaped knife:** It has a blunt edge, which raises the stability of the knife (profile D) (refer Fig. 5.8).

Razor Blades on Steel Knives

Ordinary shaving razor blades can be used when fixed with a special clamping device to the standard steel knife. This device ensures that the razor blades edge projects slightly over the cutting edge of the standard knife and acts in its stead. The advantage of these blades is that a constant sharp cutting edge is available and the blades when blunt, can always be discarded. No honing needs to be done. The principle disadvantages include fracture of the blade incurred as a result of cutting hard tissue.

Disposable Knives

Nowadays, disposable knives are commonly used. They are made of steel and coated with special polytetrafluoroethylene (PTFE), which allows the ribbons to be easily sectioned.

Types of disposable knives

- Low-profile knives
- High-profile knives.

Stainless steel disposable blade holders are available for high-profile and low-profile disposable blades, which can be fixed on all types of microtomes in place of the conventional microtome razors. Blades are fed into the holder direct from the dispenser cassette and clamped in position by a single twist.

High-profile disposable microtome blade: It is made of heavier gauge steel than most disposable brands, eliminating any vibrating issues when cutting through extremely hard or fibrous specimens. These ultrasharp, high-quality microtome sectioning blades offer striation, distortion and chatter-free sections. The blades are supplied in an ejector dispenser pack, featuring a storage compartment for the safe discard of spent blades. No wiping of the blade is necessary; with all blades providing consistently sharp and clean edges.

Low-profile disposable microtome blades/triple facet microtome blades: These are manufactured from high carbon stainless steel. The blades fit a standard low-profile blade holder and are supplied in an ejector dispenser pack, featuring a storage compartment for the safe discard of spent blades. Teflon- and stainless steel-coated disposable knives are available for use in cryosectioning.

Advantages of disposable knives

- A damaged or dull cutting edge may be replaced by a new, perfect edge within seconds without time consumption and costly process of re-sharpening
- Honing not required
- It can be used as long as it has a keen edge
- Section quality is improved
- Resistant to both corrosion and heat
- Its hardness can be compared to that of standard steel knives
- Easily available and discardable.

Disadvantages of disposable knives

- Relatively expensive
- Not as rigid as other microtome knives, hence, a tendency for some minor vibrations.

Parts of Metal Knife (Figs 5.9A and B)

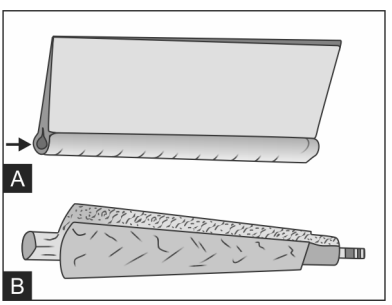
There are four parts of metal knife:

1. The heel of the knife is where the handle can be attached at one end.
2. The toe is the diametrically opposite end.
3. The cutting edge.
4. The back.

Angles of a Metal Knife (Fig. 5.10)

There are several terminologies in use. In author's view, the following are the important ones:

1. **Bevel angle (knife edge angle):** Angle formed between the cutting facets, where they meet at the cutting edge. The angle usually varies between 18° and 30°, the smaller the bevel angle the sharper is the knife. Too small bevel angle results in the elastic distortion of the edge.
2. **Wedge angle:** It is the angle formed when an imaginary extension of the sides of the knife meet at a point, also known as blade angle.



Figures 5.9A and B: Parts of a knife **A.** Knife with knife back [Note: The space for the attachment of the knife handle (arrow). This area is called heel. The other end of the knife is called toe]. **B.** This is the knife handle. This handle should always be attached to the knife first, before the knife back is slid ON or OFF.

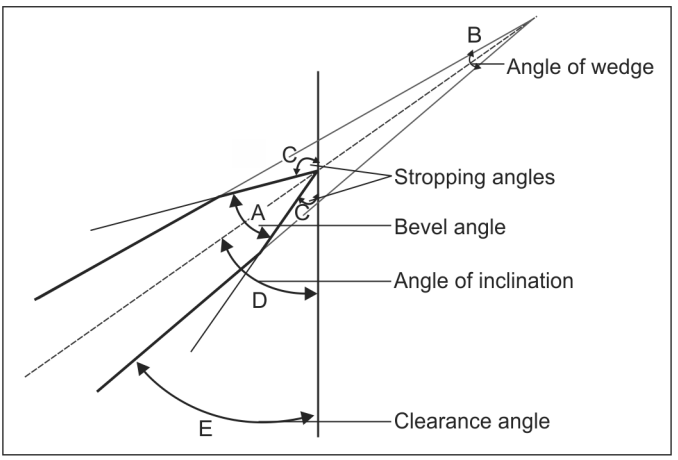


Figure 5.10: Knife and cutting angles. The angles of the metal microtome knife in relation to a paraffin tissue block.

3. **Stropping angle (upper and lower facet angle):** It is the angle between the 'surface of the block and the upper facet and lower facet' respectively of the knife (cutting angle).
4. **Angle of inclination:** Angle formed between the surface of the block and the line bisecting the bevel angle. The optimal angle depends upon the knife geometry and the cutting speed. If the angle is too large, the sample in the block can crumble and the knife can induce periodic thickness variations in the sections (some authors extend this up to the upper facet when it is known as 'cutting angle').
5. **Clearance angle:** Angle between the surface of the block and the lower straight edge of the knife. It may be only 2°–5° angles. It is essential in order to prevent friction between the knife and block. A low clearance angle gives less compression to the tissue block and produces a smooth plastic flow during sectioning.

Non-metal Knives

Non-metal knives are small and used almost exclusively for ultramicrotomy.

Glass Knives

The knives are made from specially made glass strips (e.g. Ralph type) of adequate thickness (0.5–1 cm). These strips are cut at an angle to produce a cutting edge by means of a special instrument called knife maker. The knives are used for both semithin (1 μ) and thin silver gray sections (60–100 μm). Such knives once dull are discarded.

Diamond Knives

Diamond knives are used in electron microscopy for sectioning epoxy resin blocks. They have a cutting edge of 3 mm that has a longer lifespan. Though made of industrial diamonds, these knives cost a considerable amount and cannot be sharpened. Owing to the fact, it tends to fracture if mishandled or if processed hard tissue is sought to be cut by it.

CARE OF A MICROTOME KNIFE

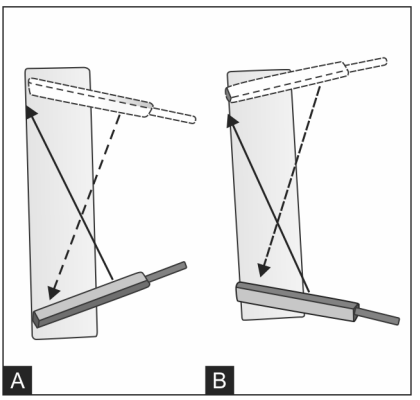
The most important part of the microtome knife is the cutting edge and this edge must always be maintained in good condition in order that ideal sections are obtained. All knives are sharpened at the factory whereby a bevel is obtained with a cutting edge. By usage, these knives develop irregularities and breaks. These irregularities and breaks must necessarily be removed by a process of honing (sharpening) and stropping, whereby a smooth finish is given to the already honed cutting surface. Owing to the brittleness, steel knives should not be dropped or placed on the table with the cutting edge downwards. Both these may result in chipping and even breakage of the knife. The knife should be cleaned in xylene or toluene before or after use.

Sharpening of Metal Knives/Honing (Figs 5.11A and B)

Sharpening of metal knives includes both honing and stropping. By the honing procedure crude nicks are removed from the knife edge and stropping is done after honing to refine the honed edge further. After prolonged use or after cutting very hard tissue, the cutting edge becomes damaged, with a jagged edge. A straight cutting edge and a correct bevel must be restored by grinding the knife on a hone. Honing is the process in which all the nicks and irregularities in the cutting edge of the knife are removed to make the cutting edge straight and sharp.

Hones

Hone stones is done by the use of hand hones or electrically operated hones. In order to hone a hard steel knife, the hone should be even harder and for this purpose either naturally occurring stones or artificially produced material such as plate glass or carborundum may be used. Hones are actually known as sharpening stones and have wide abrasive properties.



Figures 5.11A and B: Sharpening of knives. **A.** Honing (note that movement is from heel to toe), the cutting edge leading; **B.** Stropping.

Naturally occurring hone stones

They are known as oil stones because oil is used as a lubricant. The finer the grain in the stone, the harder is the hone:

- Arkansas stone is a hard pale yellow-white stone and has a polishing effect with medium fineness
- Belgian yellow glass stone, Belgian green glass stone
- Belgian black vein stone—best used for manual sharpening.

Lubricants are used in most sharpening techniques because they act as a coolant, prevent the extreme edge of the knife losing tamper and reduce the tendency of the stone's pores to become blocked with finely divided metal particles. Two types of lubricants are used:

- Aqueous lubricants, e.g. glycerol, soap solution, vegetable oil, liquid detergent (10%)
- Non-aqueous lubricants, e.g. '3 in 1' oil, oils thinned with paraffin oil, Diala oil (liquid paraffin is recommended because it has high viscosity).

Artificially produced hones

1. **Carborundum:** It is an artificial stone having a coarse surface and is useful for badly nicked knives. It is used with an abrasive powder (corundum No. 303, 304, 305), a suspension of which is made in water. The average size of the powder particles for grinding is 20 μm (not > 40 μm) and for polishing is 4 μm (not > 8 μm).
2. **Plate glass:** It is available as 1/4–3/8 inch thick with a length of 14 inches and width of 2 inches. It is also used with abrasive powders, is cheaper, popular, easy to clean and readily available.
3. **Abrasive powders:** Common types of abrasive powders are:
   a. **Diamond:** An ideal abrasive available as paste or aerosol with particle sizes of 0.225–50 μ. Its main advantage is that the particles retain their outline for more periods, so that these can be used for longer periods.
   b. **Silicon carbide/Carborundum:** It is available in 1.00–5 μ size particles, used with light oil for both coarse grinding and polishing of knives.
   c. **Aluminum oxide:** The size of particles is of wide range up to 0.1 μ. Particles are not much hard and they are good for both sharpening and polishing of knives.
   d. **Iron oxide:** The particles are of soft and fine size. It can be used with aqueous and non-aqueous lubricants. It is suitable for polishing the knife edge.
   e. **Ceric oxide:** It has got similar properties as that of iron oxide, but more expensive.
   f. **Chromium oxide:** The particles are softer than alumina and are used on the softer metal plates with aqueous or non-aqueous lubricants.

Methods of Honing (refer Fig. 5.11A)

For both honing and stropping, the knife must have its own knife back. The knife back enables the bevel edge to be sharpened and no knife should be sharpened without the knife back:

1. The knife handle is fitted to the knife if it is sharpened by hand, but in machine sharpening, no knife handle is required.
2. The movement for honing is cutting edge forward in opposition to the honing surface with heel of the knife leading and hone placed on the bench.
3. A small quantity of light oil is applied to the hone and smeared over the surface.
4. The knife is placed at the nearest end of the hone with the cutting edge facing away from the operator.

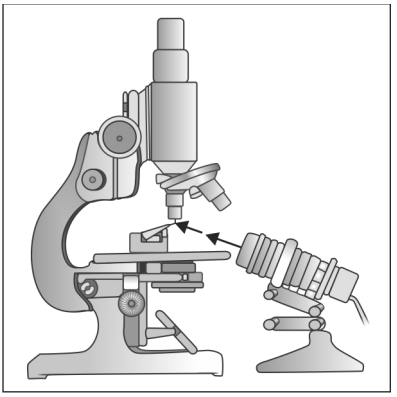


Figure 5.12: von Mohl criteria (examining knife edge using incident light at 100X magnification).

5. The knife is pushed diagonally from heel to toe. The direction of the movement is at such an angle that one entire cutting surface is honed in one upgoing strop, turned over on its back and moved across the hone to its original position (figure-of-eight movement) (refer Fig. 5.11A).
6. In general, 20–30 such strokes are sufficient to hone a knife for an ordinary use.
7. Each knife cutting edge should be examined with a microscope using incident light at a magnification of 100X. A thin white light should be seen (von Mohl criteria) (Fig. 5.12).

Precautions While Honing

- Lubricant must be used
- The blade must be kept vertically flat because if the knife edge is raised slightly during honing, it will cause the edges flat
- After the honing is completed, the knife edge is wiped with a clean soft cloth and moistened with xylene.

Care of a Hone

- The hone is to be kept covered when not in use to prevent dirt and grit from gaining access to the surface
- It is to be wrapped in soft cloth and stored in a shallow wooden box with a lid
- After the use, hone must be washed with warm soap water to remove all metal particles from it, then thoroughly rinsed with water and dried.

Automatic Hones

Automatic hone is designed for safe, quick and convenient onsite use for resharpening of microtome knives. It has a compact and portable structure.

The knife is fitted to a holder, which allows the cutting edge to be in contact with the circular plate made of metal or glass. The knife automatically turns over from edge to edge at suitable intervals.

Semiautomatic hones: They are time saving and easy to manipulate. One big disadvantage of this type of honing is that the manual feeding of the knife across the revolving wheel causes uneven pressure and variation in the rate of honing resulting in an uneven knife edge.

Stropping

Stropping is the final technique for sharpening the knife before cutting sections. It is done to polish the honed cutting edge and remove the fine nicks. Stropping removes the buffs formed during honing. The knife edge cannot be sufficiently sharp to cut good sections directly from honing or sharpening. Stropping must follow honing. Strops are made of horse leather, especially got from horse rump area and are 18 × 3 (or 4) inches mounted on a solid wooden block.

Types of Strops

- Rigid/Fixed strop is preferred as it is easy to manipulate
- Flexible/Hanging with one end fixed.

Methods of Stropping (refer Fig. 5.11B)

1. Since the strop is made of leather, it is cuttable. To strop, a movement is made, which is the reverse to that of honing (i.e. toe to heel), the cutting edge of the knife given no chance to slice into the strop.
2. The knife is placed on the near end of the strop with cutting edge towards the operator.
3. The knife is drawn in a toe to heel direction. Then the knife is turned over and brought back. This action is exactly opposite to that of honing.
4. Usually 20–40 strokes are required and excess stroking may spoil the knife edge.
5. After the stropping is completed, knife edge must be oiled to prevent rusting. Examine the knife edge under microscope.

Care of a Strop

Strops must be wiped clean, as sand/dust can cause nicks in a knife. The strops must be oiled (with thin oil) before use and regularly at intervals of 1 year and dressed with a fine carborundum powder available through the makers of such microtome knife strops.

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Chapter 6 Section Cutting

GENERAL PRINCIPLES OF MICROTOMY

Theory of Sectioning

It is believed that a sharp knife with a narrow edge probably wedges off sections by splitting and tearing rather than shearing. Successful sections require:

1. **Proper material:** Properly prepared material with the supporting medium matching the specimen.
2. **A sharp knife:** Poorly prepared material can sometimes be cut by a good knife, but a well-prepared block may be totally ruined by a poor knife.
3. **A proper microtome:** The choice of microtome should depend on the application. Unless very old, misused, damaged or of poor quality a microtome is rarely the cause of poor sections.
4. **A skilled operator:** Practice is the word in the acquiring of skills. A skilled operator must be able to recognize and correct difficulties as they arise.

PARAFFIN SECTION CUTTING

Trimming of Paraffin Blocks

Once tissues have been blocked, excess paraffin on all aspects of the block of tissues should be removed to leave approximately 3 mm of paraffin around it. Somewhat more paraffin should be available at the back of the block. Once this is done the block is fixed to a metal block object holder or a wooden chuck by means of a hot wooden handled spatula. Both knife and block are then cooled by means of a solid block of ice. In order to arrive at the optimum plane of cutting where adequate sampling of the tissue block is done, coarse trimming is resorted to with the microtome setting at 20–25 μ. A different 'coarse' knife may be used for the purpose or alternatively a different portion of the knife may be used.

Procedure (for Fine Cutting)

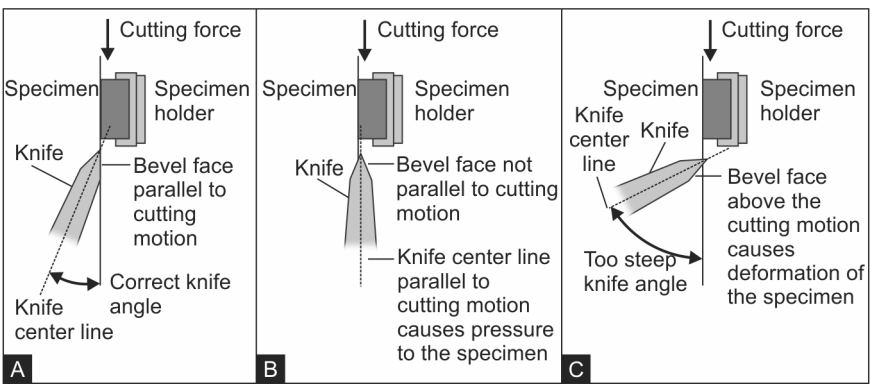
1. The microtome is set for the desired thickness of sections. For highly cellular tissues, e.g. lymph nodes the thickness selector is set at 4 μm to reduce the overlapping of the nuclei; for all the routine tissue 5–6 μm thickness is adequate.
2. The correct position of the properly embedded blocks in the microtome will result in the final preparation of the entire surface, which should be

free of tears, lines, folds or cellular distortion. If the block is not parallel to the knife, readjust the block holder screws (Figs 6.1A to C).

3. Both block and knife should be wiped dry.
4. Cutting is then performed by using regular even strokes. Rapid wheel movement should never be done as often this generates static electricity with sections tending to fly away, crumble, roll or curl.
5. To obtain ribbons, the tissue should be small and block should able to generate sufficient heat and pressure to wield together the edges of the paraffin sections that are cut.
6. Whether single sections are made or ribbons, these sections should be placed on water, whose temperature is 5–8°C below the melting point of the wax used to process and block the tissue. Transport of single sections may be done by means of an artist's brush (Camel No. 2 or 3). Hold on to the free end of the section ribbon, after about six continuous sections have been cut; with a pair of forceps free the attached end and float the ribbon onto the bath. When this is done usually the sections unfold themselves and flatten out.

   At times the section may not unfold in the water bath and this may be achieved by gently inducing it to do so by means of the brush. It may be necessary to add hot water with a dropper pipette to deal with the wrinkles on individual section. A dissecting needle or any other pointed instrument should never be used, since these are likely to produce holes in the section.
7. Ideally spread sections or ribbons may now be transferred to clean new histology glass slides. Many authors believe that a direct transfer may be done without treating the slides with egg albumin. In fact they believe that the tissue protein in the section is sufficient to ensure section adhesion to the glass slides.

   Others feel that egg albumin smearing on slides plays a positive role in tissue adhesion. When egg albumin is used only a minute amount is applied to the slide and spread. If excess amounts are applied, these stain eosinophilic with the hematoxylin and eosin stain, and obscure clear viewing of the tissue section.



Figures 6.1A to C: Procedure for fine cutting: Showing how angles affect cutting of sections

8. Once the desired sections have been cut, remove the block from the blockholder and seal the exposed surface with molten wax. This ensures that tissue will not dry or become hard and brittle, and facilitate re-sectioning of the blocks even after weeks, months and years.
9. The use of adhesives in the water bath promotes the growth of bacteria and fungi. Daily cleaning of the water bath with Clorox, soap and water is recommended to prevent such contamination.

Ancillaries for Paraffin Section Cutting

1. Blockholders.
2. Water bath (floatation method): This is a thermostatically controlled bath with the inside colored black, 10–12 inches in diameter and 3–4 inches deep; the temperature being set at 45°C, i.e. about 8° below the melting point of wax. The black coat on the inner surface makes it is easier to see creases/folds in the sections. Air bubbles, which form at the bottom of the bath during use must be dislodged with the fingers or vibration.
3. Slide warmer (hot plate): Hot stage method is used for drying sections on slides. It consists of a metal top or electric hot plate, which is maintained at a temperature of 45–50°C. The slides on which the sections have been picked up are placed on the hot plate for 30–40 minutes or in a wax oven at 50–60°C for 2 hours. The above procedure helps in section drying, melting of the excess paraffin wax and fixing of tissues to the slides. Drying ovens are available, incorporated with a fan, especially designed for drying tissue sections on slides.
4. Forceps and brush are necessary for handling of sections during cutting and for removal of folds and creases formed in the sections during floating out.
5. Albuminized slides/adhesive mixtures for coating slides: Adhesives are protein solutions used to promote tissue attachment to the glass slides. This is routinely achieved by the application of a smear of glycerin/albumin mixture to the slide before the section is mounted and dried. They are essential for methods entailing significant exposure of sections to strong acids and alkalis (e.g. ammoniacal silver solutions), cryostat sections for immunofluorescence, immunocytochemistry, urgent diagnosis, decalcified tissues of central nervous system (CNS) and those containing blood clot.

Common Preparations

Adhesives act mainly by reducing surface tension and thus producing closer capillary adhesion of the sections to the slides. The only disadvantage is that the albumin used as an adhesive retains some of the stain and gives a dirty background, which may obscure viewing. Some common preparations are:

1. **Mayer's egg albumin-glycerol:**
   - Whites of fresh eggs: 50 mL
   - Glycerol: 50 mL
   - Distilled water: 50 mL.

Mix well and filter through several layers of gauze or coarse filter paper and add a crystal of thymol to prevent the growth of molds.
Stock: Egg albumin flakes (commercially available) 5 g, distilled water 100 mL and one crystal of thymol.
Working solution: Use 50% of stock solution and 50% glycerine [intraperitoneal (IP)].

2. **Gelatin:** About 1% solution of gelatin is superior to egg albumin as an adhesive, but will also give background staining. It can be applied to the slide as a small drop and smeared over the surface or can be added to the water flotation bath (15–30 mL of 1% aqueous gelatin solution).
3. **Chrome alum gelatin solution:**
   - Gelatin: 1.5 g
   - Chromium potassium sulfate: 0.25 g
   - Distilled water: 500 mL.

   Heat the water to 60°C and completely dissolve the gelatin with the aid of a magnetic stirrer. Stir in the chromium potassium sulfate (the solution turns a pale blue). Add a few crystals of thymol as a preservative. For use, 10 mL of solution is added to the floatation bath or slides for use in frozen sections may be dipped in this solution diluted 1:3 in water. It is suitable for decalcified bone sections and frozen sections.
4. **Celloidin:** It is a strong adhesive and is especially effective for decalcified material. Celloidin will take up color if aldehyde fuchsin, Schiff's reagent, mucicarmine or alcian blue are used. Prepare as a 0.5–1% solution in ether/alcohol.
5. **Methyl cellulose:** It is prepared as a 0.5–1% solution in ether/alcohol; can be used in place of celloidin.

Difficulties in Paraffin Sectioning (Table 6.1)

TABLE 6.1: Errors and corrections in paraffin sectioning

Errors	Causes	Corrections
Irregular sections Thick/Thin section (varying thickness)	Insufficient tilt of knife too much or too little clearance angle Clamping screws on the block and knife holder not tight Large blocks	Correct tilt and clearance angle Screw clamp tightly
Scored grooved, smeared and deformed, regular lengthwise scratches and splits in ribbon	Dull knife Defective knife edge with knick dirt or hard material in the tissue itself, e.g. calcium or mercury salt crystals	Sharpen, clean knife, check edge, check block for dirt, decalcify tissue wherever necessary

Contd...

Contd...

Errors	Causes	Corrections
Sections fall out after being mounted on slide	Embedding medium is of inadequate support and consistency compared to the processed tissue	Reblock tissue or if tissue is hard, cool the block
Mushy sections, crumbly sections	Improper fixation Insufficient dehydration Insufficient clearing	Reprocess tissue
Tissue jumps out of the block Fragmented sections	Hard brittle tissue in blocks due to prolonged fixation (Zenker's, Helly's and Bouin's fluid) Prolonged treatment in xylene	Take fresh bits or soak the block surface with an alkaline solution such as 10% ammonium hydroxide This will soften the tissue, prevent cracking and facilitate sectioning
Crooked or uneven ribbons	Edges of block not parallel to knife Block not trimmed parallel Irregular, but sharp knife edge Paraffin of different constituencies in different portions of the block as done in re-embedding One side of the block warmer than the other (e.g. spirit lamp near microtome)	Correct accordingly
Ribbons fail to form	Room too cold Paraffin too hard Tilt too much Section thick Knife too dull	Use softer paraffin with lower melting point; warm knife Tilt knife less Cut thinner sections Sharpen knife, unroll the section with a brush, but do not detach from knife, a ribbon may form
Wrinkled, compressed, crushed and jammed sections	Blunt or dull knife (tilt slight; knife edge coated with paraffin) Cutting too rapidly Room warm Clearance angle too great Micrometer screw set too thin for wax hardness	Rectify accordingly

Contd...

Contd...

Errors	Causes	Corrections
Knife rings and sections scratched	Tilt too great Material too hard Knife too thin	Correct accordingly
Sections lifted from knife	Increased knife tilt Room too warm Knife dull	Correct accordingly
Sections stick to knife	Knife edge dirty Knife tilt too little Dull knife	Correct accordingly
Sections fly and stick to microtome or nearby objects	Static electricity due to dry air	Increase humidity by boiling water in a pan in the room; ground microtome

FROZEN SECTION CUTTING

Many factors operate in the cutting of frozen sections. These include:

- Type of tissue
- Temperature of the cryostat or freezing microtome
- Atmospheric humidity
- The need for tissue fixation prior to frozen section cutting.

The type of tissue may cause problems. This is particularly so with adipose or fibrofatty tissue. The fat released from the cut fat cells results in the knife sliding off the tissue block rather than cutting into it. A decrease in temperature will be required to congeal the fat.

A freezing microtome or even a cryostat to a certain degree is exposed to the atmospheric humidity. During use the atmospheric water crystallizes, these crystals in themselves acting as a knife resulting in the shattering of sections. Common temperatures used in the cryostat are as follows:

1. **Common carcinoma:** About –18 to –20°C at atmosphere humidity of 30%. Sometimes lower temperatures may be needed.
2. **Adipose tissues and sarcomas:** About –22 to –25°C at atmosphere humidity of 30%. These are the rule of thumb values and will vary depending on the other factors described. Atmospheric humidity plays a great role in frozen sectioning. In order to decrease or prevent water crystallization altogether a freezing mixture is used the principle of which is to reduce the freezing point of the tissues by prior impregnation.

Commercially available freezing mixtures are provided by cryostat manufacturers, e.g. optimum cooling temperature (OCT) mixture. Alternatively Hamilton's freezing mixture consisting of solution A and solution B can be readily prepared in the laboratory. The mixture is constituted by mixing equal parts of solution A and B before use, and discarding the

excess after use. The advantage of this over the commercially available one is the cost factor, the latter being much cheaper. Composition of solution A and B are as follows:

1. Solution A:
   - Glucose: 142.5 g
   - Distilled water: 150 mL
   - Formaldehyde 40%: 9 mL.
2. Solution B:
   - Gum acacia: 2.8 g
   - Distilled water: 150 mL
   - Formaldehyde 40%: 5 mL.

A drop of the freezing mixture is placed on the freezing stage, next place the tissue over this, cover it again with the mixture and place the cryostat weight over this for cooling. The question is whether it is advantageous to fix tissues prior to frozen sectioning is a debatable one. Some authors recommend 10% formalin to fix tissues. The formalin being boiled before immersion of tissues into it or after immersion of tissues, the period of boiling being approximately 1 minute.

PROCEDURE FOR ULTRAMICROTOME

Sections cut in an ultramicrotome, which measure 1 μ in thickness are called semithin sections. Such semithin sections are stained by a modified toluidine blue technique in order to scan material available in a tissue block prior to the making of ultrathin sections. Once the user is satisfied as to the amount and adequacy of material in the block, ultrathin sections are made. Tissue meant for ultramicrotomy are finely chopped with each tissue block measuring not more than 0.1 cm (1 mm) in greatest dimension and the blocks are prepared in resin. Processing of these needs a special procedure and is dealt with in the section on processing.

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Chapter 7 Introduction to Staining and Principles of Staining

Staining is the process of applying dyes on paraffin cut sections to study the architectural and cellular pattern of the tissues. Different tissues display varying affinities for most dyes so that they become easily identifiable, thus enabling and establishing presence or absence of disease.

TYPES OF STAINING

1. **Routine staining:** The cells and tissue components combine with the active coloring agent, so that the tissues while being colored (no particular dye is seen) remain relatively clear in morphology; transparent unless deeply stained, e.g. hematoxylin and eosin stain.
2. **Special staining:** This is specific type of staining and involves various principles in the method of staining, e.g. impregnation of silver salts as in reticulin stain, histochemistry as in mucin stains, immunohistochemistry in the demonstration of tissue antigens, etc.
3. **Vital staining:** It is the staining of selective living tissue components, demonstrating cytoplasmic structures by phagocytosis of dye particles, e.g. reticuloendothelium system with trypan blue.
4. **Supravital staining:** It stains living cells immediately after removal from the body. Thin slices of tissue are placed in small staining dishes and enough staining solution is added to cover the tissue, e.g. toluidine blue for reticulocyte count and Heinz bodies; Janus green is taken by mitochondria. Other supravital stains include neutral red, thionine, etc.
5. **Metachromatic staining:** Certain dyes stain and tissues in a color or hue, i.e. quite different from that of the fundamental color of the stain. This is called metachromasia. These are basic dyes of aniline type and belong to the thiazine group; others are triphenylmethane or azo dyes. It is preferable to use frozen sections of fresh or rapidly fixed tissues.

CLASSIFICATION OF STAINS

Based on Applications

1. **Histological stain:** The tissue components are demonstrated in sections by direct contact with the dye producing color to the active tissue component to be stained, e.g. bacterial stains (Gram stain for bacteria), microanatomical stains and other specific tissue stains.

2. **Histochemical:** The various constituents of the tissues are studied through various chemical reactions that will permit microscopic localization of specific tissue substance, e.g. Perls' Prussian blue reaction for hemosiderin and periodic acid-Schiff (PAS) stain for carbohydrates.
3. **Immunohistochemical:** The combination of immunological and histochemical techniques that allow phenotypic markers to be detected and demonstrated under microscope using monoclonal and polyclonal fluorescent or enzyme-labeled antibodies.
4. **Metachromatic stains:** This staining takes place when certain negatively charged groups on the tissue react with cationic dyes. Metachromasia is attributed to stacking of dye cations at sites of high density of anionic groups in the tissue. In simple words, the primary color of the dye is the result of monomeric form in solution and the full metachromatic color is due to formation of dimmers and trimers, e.g. thionine is a blue dye and stains cartilage, mucins and basophilic granules a purple color; methyl violet stains nuclei and cytoplasm in shades of blue, while amyloid (which exhibits metachromasia) stains red; mast cells are stained pink with toluidine blue.
5. **Polychromatic stains:** The dyes, which exhibit two or more shades of coloring reactions to tissue elements, e.g. Giemsa and Leishman's stain.
6. **Orthochromatic stains:** The dyes, which do not change color and the tissues are dyed with the same color of the dye, e.g. α-metachromasia.

Based on Tissue Affinities

1. **Basic stain:** It is an active coloring substance found in a base combined with a colorless inorganic acid radical. Any substance, i.e. stained by the basic dye is considered to be basophilic; it carries acid groups which bind the basic dye through salt linkages, e.g. basic fuchsin is the chloride salt of the base rosaniline; and when using hematoxylin, basophilic structures in the tissue appears blue.
2. **Acid stain:** It is active coloring substance found in an acid component and the basic part being inactive or a substance that is stained by an acid dye is referred to as acidophilic. It carries basic groups, which bind the acid dye, e.g. with eosin, acidophilic structures appear in various shades of pink. Since eosin is a widely used acid dye, acidophilic substances are frequently referred to as eosinophilic; acid fuchsin is the sodium salt of the sulfonic acid derivative of rosaniline.
3. **Neutral stain:** It is formed by mixing the requisite quantities of the aqueous solutions of certain acidic and basic stains, e.g. Romanowsky dyes, Giemsa stain and Leishman's stain for leucocyte differentiation.

Based on Source

1. **Natural dyes:** They are obtained from the plants and animals, e.g. hematoxylin, cochineal, orcein, carmine, saffron and litmus.

2. **Synthetic:** They are derived from coal tar and hydrocarbon benzene, e.g. aniline, coal tar dyes, azo dyes and triphenylmethanes.

METHODS OF STAINING

Direct Staining

Direct staining is the process of giving color to the sections by using aqueous or alcoholic dye solutions, e.g. methylene blue and eosin.

Indirect Staining

The process by which action of the dye is intensified by adding another agent, i.e. a mordant, which serves as link or bridge between tissue and dye.

Mordant: It is a substance (polyvalent metal ion), which forms a coordination complex with the stain or dye and aids in attaching the stain to the tissues during the staining procedure by forming tissue-mordant-dye complex, i.e. insoluble in ordinary aqueous and alcoholic solvents. Mordants used for hematoxylin staining are di or trivalent salts of metals, e.g. potassium alum with Ehrlich's hematoxylin and iron in Weigert's hematoxylin.

Lake: It is a complex between a mordant and certain dyes, which then attaches to the substrate. A dye lake is not strictly a large pool of dye. More accurately, it is an insoluble molecule called lake. The combination of hematoxylin with mordant is known hematoxylin lake and the lakes with different metals have different colors, e.g. aluminum lakes—purple to blue, depending on pH; iron lake—bluish black; copper lake—blue green to purple; tin lakes—red; lead lakes—dark brown, etc. The three methods used for mordanting are:

1. **Premordanting (onchrome):** The substrate is treated with the mordant and then dyed, e.g mercurial or picric acid acts as mordants for phosphotungstic acid and phosphomolybdic acid in connective tissue stains.
2. **Metamordanting (metachrome):** The mordant is added in the dye bath itself, e.g. alum hematoxylin.
3. **Postmordanting (afterchrome):** The dyed material is treated with a mordant. This is not practiced.

Accentuator merely accelerates or hastens the speed of the staining reaction by increasing the staining power and selectivity of the dye. It does not participate in the staining reaction and is not essential to the chemical union of tissue and the dye, e.g. potassium hydroxide in Loeffler's methylene blue, phenol and carbol fuchsin. When accentuators are used for metallic impregnation in the nervous system, they are called accelerators, e.g. certain hypnotic drugs such as barbital and chloral hydrate in methods for the metallic impregnation of nerve fibers.

METACHROMATIC STAINING

Mast cells were first recognized by virtue of their metachromasia in 1877 by Paul Ehrlich (1854–1915, Nobel laureate 1908), who was then a medical

student in Leipzig. The compound responsible for the metachromasia was later identified as heparin.

Types of Metachromasia

1. **Alpha (α):** In this, the color blue remains the same, i.e. monomeric form of the dye, e.g. blue in toluidine blue.
2. **Beta (β):** The color is intermediate due to dimeric and trimeric structure, i.e. the formation of aggregates of two or three molecules or a mixture of α or β type, e.g. violet in toluidine blue.
3. **Gamma (γ):** In this, the color is fully metachromatic and all the surface-bound dye is polymerized, e.g. red in toluidine blue.

Reversal and Induction of Metachromasia

Metachromasia is enhanced by increasing the concentration of dye and by decreasing temperature. Water enhances metachromasia due to its polar nature, contributing to van der Waals forces by which the molecules are held together and metachromasia is lost, if the section is dehydrated in alcohol. Sulfation induces metachromasia, while methylation abolishes it.

THEORIES OF STAINING

The nature of dye-tissue inter-reaction involves either a chemical reaction or physical phenomenon:

1. Physical phenomenon defines the differential solubility and is the basis of staining lipids with alcoholic solutions of such dyes as oil red O, where the dye is more soluble in the fat than the solvent in which it is employed; and adsorption involves the attraction and surface fixation of small molecules by large molecules, e.g. staining of glycogen by iodine.
2. The chemical reaction may be nonspecific; it gives color to the different tissue components and therefore increases the contrast of the cells. Staining of acid nucleoproteins by basic dyes or basic cytoplasmic constituents by acid dyes and dyes staining bacteria are also examples of chemical reactions.

The basic structure of all stains is benzene, which is colorless. Certain chemical compounds when introduced into benzene by substitution of hydrogen will give color to the compound. These groups are called chromophores and the resultant structure is called chromogen, e.g. C=C, N=O, C=S, etc. These compounds absorb light from the visible spectrum and possess color. To convert a chromogen into a dye, a charged group called auxochorme, which gives the compound the power of electrolyte dissociation or the property of forming salts must be added, e.g. hydroxide (OH), amine group (NH_2). Only then, the colored compound stains the tissue and is resistant to simple washing. Therefore a stain has both a chromophore and an auxochorme.

PRINCIPLE OF STAINING METHODS

Two methods are used, progressive and regressive methods. The progressive staining is accomplished by using excessive aluminum salts and thus increasing nuclear selectivity. Staining is continued till the desired intensity is reached. After staining, the slides are washed well in water and counterstained, e.g. Romanowsky dyes stain the nucleus and the cytoplasm in a defined time.

The regressive staining is accomplished by overstaining in a neutral solution and then removing excess stain with acid, ethyl alcohol or some other differentiating agents. The excess stain is removed selectively until the right intensity is obtained. Subsequent neutralization is achieved with a buffer solution such as tap water, lithium, carbonate or ammonia water and counterstained as required, e.g. hematoxylin staining followed by differentiating in acid alcohol to remove the excess dye, e.g. Harris and Mayer's hematoxylins.

TYPES OF DIFFERENTIATORS

1. **Acid differentiators:** They act by combining with metal, thus breaking the union of the tissue or cell components. The acid chosen should form a soluble salt with the metal, so that the latter is dissolved out, e.g. hydrochloric acid and acetic acid.
2. **Oxidizing differentiators:** These substances oxidize the dye to a colorless substance. Components holding least dye will be bleached out, e.g. potassium ferrocyanide, potassium permanganate, chromic acid, picric acid and potassium dichromate.
3. **Mordant differentiators:** These act by binding a dye to the tissue and on the other hand removing the excess dye from its combination with the tissue.

Bluing

Bluing is the process of converting the initially red soluble hemalum to a final blue insoluble form. Tap water alone can blue cells satisfactorily. Scott's tap water substitute (TWS) is an aqueous bluing solution with a pH of 8, which is of intermediate value along the range of pH within that bluing can occur (i.e. 5–11). Scott's TWS is prepared by dissolving in 1 L of water, 2 g sodium bicarbonate and either 10 g anhydrous magnesium sulfate ($MgSO_4$) or 20 g hydrated magnesium sulfate ($MgSO_4.7H_2O$), i.e. Epsom salt. In preparing this solution, add the $MgSO_4$ slowly to water, so that it dissolves rapidly and dissipates the heat produced as it is an exothermic reaction.

Factors Affecting Staining

Staining is a complex process and influenced by both chemical and physical processes and also by the process of fixation:

- Solvent-solvent interaction
- Dye-dye interaction
- Dye-tissue interaction.

HEMATOXYLIN AND EOSIN STAIN

Waldeyer (1863) is credited with introducing the hematoxylin and eosin staining system. This is the most popular stain in the surgical laboratory for routine purposes. A differentiated stain enables a good study of cell morphology and can be preserved for several years.

Hematoxylin is derived from the core or 'heart wood' of the tree *Haematoxylum campechianum*, a tree originally found in the Campeche state of Mexico, but now being actively cultivated in the West Indies. The trees when felled, are stripped of their bark and the external aspect of the trunk called 'sap wood', leaving only the core or 'heart wood'. This 'heart wood' is then cut into 3 cm logs called 'log wood'.

Apparently, hematoxylin had been used in earlier times as a fabric dye of dubious dyeing capabilities by the natives of Mexico after extraction from log wood by hot water and urea. For histological purposes it was found that the active ingredient hematin should be extracted from hematoxylin, which by itself has no staining properties and the stain is then used with a mordant to enhance its color retention.

To extract hematin, oxidation of hematoxylin is done by use of natural methods such as sunlight, etc. called ripening, which may take 6–20 weeks or chemical methods whereby, forced oxidation is resorted to by the addition of oxygen or removal of hydrogen. This chemical method is best utilized with sodium iodate and takes a few weeks to 3–4 months. Other oxidizing agents include mercuric oxide and potassium permanganate. While the chemical methods prove shorter in terms of time and production of quickly and easily utilized hematoxylin, the ripened hematoxylin tends to take a longer time, but it has to have a longer lifespan after having stained the tissues.

Once hematin is extracted, it must be linked to a mordant, which may be either an aluminum salt (alum) or an iron salt. Consequently, the hematoxylins are grouped according to the mordant used:

- **Alum hematoxylin:** Using alum, which is a double salt-like potash alum, e.g. Delafield's, Ehrlich's, Mayer's, Harris and Cole's hematoxylin
- **Iron hematoxylins**: Using ferric chloride and ferric ammonium sulfate, e.g. Weigert's iron hematoxylin and Heidenhain's hematoxylin, respectively.

The alum hematoxylins are commonly used in routine staining with eosin. The major disadvantages are their inability to resist acid solutions as used in the van Gieson stain, their gradual dissipation in staining capacity after many usages and their relative ineffectiveness when used with fixatives, particularly of the acid type. Hence, Weigert's iron is used, which is resistant to picric acid. Ehrlich's hematoxylin is used regressively.

Celestine blue B (Cl 51050) has been successfully used as a mordant for the alum hematoxylins where such hematoxylins are used with acid solutions.

The two iron hematoxylins used today are mentioned above. The salts used for oxidation should be used with great care, as overoxidation is possible with a resultant 'dead' stain. It is the reason that the iron hematoxylins are kept in two separate containers, i.e. stain and mordant, and these mixed only just before use. Heidenhain's hematoxylin is used as a regressive stain.

Formula of Common Useful Hematoxylins

Alum Hematoxylins

Harris alum hematoxylin

- Hematoxylin crystals: 5.0 g
- Absolute ethyl alcohol: 50 mL
- Ammonium or potash alum: 100 g
- Distilled water: 1,000 mL
- Red mercuric oxide: 2.50 g.

Method

- Dissolve hematoxylin in alcohol
- Dissolve alum salts in water by heat
- Remove from heat and mix both solutions
- Bring to boil rapidly
- Remove from heat and add mercuric oxide slowly
- Reheat until dark purple
- Remove and plunge the vessel into cold water until cool
- The stain is ready to use, as soon as possible.

Note: 2–4 mL of glacial acetic acid to every 100 mL stain increases the nuclear stain precision. Always filter before use. Harris alum hematoxylin used as a regressive stain.

Expected results

- Nuclei: Blue
- Background: As counterstain or unstained.

Ehrlich's alum hematoxylin

- Hematoxylin: 2 g
- Absolute ethyl alcohol: 100 mL
- Glycerol: 100 mL
- Distilled water: 1,000 mL
- Glacial acetic acid: 100 mL
- Potash alum: 15 g.

It is an excellent nuclear stain and used as a regressive stain.

Mayer's hematoxylin

- Hematoxylin: 1 g
- Distilled water: 1,000 mL
- Potash or ammonium alum: 15 g
- Sodium iodate: 0.2 g
- Citric acid: 1 g
- Chloral hydrate SLR: 50 g.

This is used as both progressive and regressive stain. It is used as a progressive nuclear counter stain for the demonstration of glycogen.

Harris hematoxylin

Harris hematoxylin is similar to Mayer's hematoxylin, but uses absolute alcohol instead of water as a solvent. The major differences between Harris and Mayer's hematoxylin are detailed in Table 7.1.

TABLE 7.1: Difference between Harris and Mayer's hematoxylin

Mayer's hematoxylin	Harris hematoxylin
Hematoxylin is dissolved in water	Hematoxylin is dissolved in absolute alcohol
Quantity of the dye is less	Quantity of the dye is more
Sodium iodate is used as oxidizer	Mercuric oxide is used as oxidizer
Citric acid used to sharpen the staining	Acetic acid used to sharpen the staining

Gill's hematoxylin

- Distilled water: 730 mL
- Ethylene glycol: 250 mL
- Hematoxylin: 2 g
- Sodium iodate: 20 g
- Aluminum sulfate: 17.6 g
- Glacial acetic acid: 20 mL.

Combine reagents in the order given above, mix and keep for 1 hour at room temperature. The stain can be used immediately.

Examples of other alum hematoxylins

- Delafield's hematoxylin: Naturally ripened alum hematoxylin
- Cole's hematoxylin: Artificially ripened hematoxylin, used in frozen section
- Carazzi hematoxylin: Progressive nuclear stain with a short staining time.

Iron Hematoxylin

Iron salts are used in hematoxylin as both oxidizing agents and as mordants; the most commonly used are ferric chloride and ferric ammonium sulfate. Overoxidation of hematoxylin is a problem with these stains, so it is necessary to prepare and keep separately oxidant/mordant and hematoxylin solutions. Mix them immediately before use. Because of the strong oxidizing property, the mordant/oxidant is also used as a differentiating fluid after hematoxylin staining, as well as a mordanting fluid with the stain.

Iron hematoxylin is used not only as nuclear stain, but also to demonstrate many other structures, including muscle striations, various cellular inclusions and organelles (particularly mitochondria), myelin, chromosomes, keratin, mucosubstances and elastic fibers.

Weigert's iron hematoxylins

The main use of Weigert's hematoxylin is as a nuclear stain in techniques where acidic staining solutions are to be applied to the sections, e.g. van Gieson stain.

But, a more convenient celestine blue alum hematoxylin has largely replaced Weigert's hematoxylin in this role. It remains a useful stain with eosin for central nervous system (CNS) tissues. It is used as a progressive stain.

Solution A: Hematoxylins 1% in ethyl alcohol 95%.

Solution B: Ferric chloride aqueous 29% (4 mL), distilled water 95 mL, concentrated hydrochloric acid 1.0 mL.

Working solution: Mix equal parts of A and B. The mixture should be a violet-black color and must be discarded, if it is brown.

Heidenhain's iron hematoxylin

Heidenhain's iron hematoxylin is used to differentiate many structures based on the degree of differentiation of the stain. After staining, all components are stained black; subsequently the hematoxylin stain is removed from different structures at different rates using the iron solution (solution B); therefore Heidenhain iron hematoxylin is a regressive stain.

Hematoxylin solution

- Hematoxylin: 0.5 g
- Absolute alcohol: 10 mL
- Distilled water: 90 mL.

  The solution must be allowed to ripen for 4–5 weeks.

Iron solution

- Ferric ammonium sulfate (violet crystals): 5 g
- Distilled water: 100 mL.

The following tissue elements may be demonstrated in the order given, depending upon the degree of differentiation, i.e. mitochondria, cross striations of muscle fibers, cytoplasm, nuclear membrane, chromosomes, chromatin, nucleoli and centrioles.

Verhoeff's Hematoxylin

Verhoeff's hematoxylin was developed primarily as a stain for elastic fibers. It also gives a high contrast required for photomicrography, to demonstrate nuclei and myelin, and can be adapted for electron microscopy.

Preparation of stain

Dissolve 1 g of hematoxylin in 22 mL of absolute alcohol in an open dish on a hot plate. Cool, filter and add 8 mL of 10% aqueous solution of ferric chloride and 8 mL of iodine solution (2 g of iodine and 4 g of potassium iodide is dissolved in 100 mL of distilled water). For better results make up fresh solutions just before use.

Ferric chloride solution

- Ferric chloride: 2 g
- Distilled water: 100 mL
- Sodium thiosulfate (hyposulfite) solution 5%: Removes excess of iron
- Sodium thiosulfate: 5g
- Distilled water: 100 mL.

  The counter stain used is van Gieson stain.

Results
- Elastic fibers and nuclei: Black to bluish black
- Cytoplasm and muscle: Yellow
- Collagen: Red.

Tungsten Hematoxylins

Tungsten hematoxylins have the most satisfactory method of preparation; can be ripened naturally or artificially. This process is time consuming, takes months to ripen, but remains stable for several months. Its use is applicable to both CNS material and general tissue structure. Staining is more precise after the sections have been treated with an acid dichromate solution. Good results are obtained with buffered formalin fixative.

Mallory's phosphotungstic acid hematoxylin
- Hematoxylin: 1.0 g
- Phosphotungstic acid: 20.0 g
- Distilled water: 100 mL.

Dissolve the hematoxylin in about 300 mL of water with the aid of gentle heat. Dissolve the phosphotungstic acid in the remainder of the water and when cool combine the two solutions. The stain will ripen in 5–7 weeks, if placed in warm sunshine. Alternatively it may be ripened instantly by the addition of 0.177 g of potassium permanganate or 2 mL of USP (3%) hydrogen peroxide. But with artificial ripening agents (i.e. chemical oxidation) the stain may not reach peak efficiency.

Results
- Nuclei, centrioles, neuroglia, fibrin and cross striations of muscle fibers—blue
- Collagen, reticulin and bone, cartilage, ground substance—yellow to red.

Other Mordants

1. **Molybdenum:** Complexes of molybdenum and hematoxylin are used principally to stain collagen and coarse reticulin.
2. **Lead:** Lead-hematoxylin is used for demonstration of granules in the neuroendocrine system, keratohyalin granules and calcium deposits.
3. **Copper hematoxylin:** It is used to stain fatty acids, acidophils in the pituitary, myelin sheaths and mitochondria.
4. **Chromium alum hematoxylin:** It is used to stain lipoproteins, myelin, phospholipids and cytoplasmic granules in β-cells of the anterior pituitary and pancreatic islets.

Hematoxylin without a mordant (direct staining)
Free hematin stains collagen, elastin and erythrocytes color changes from yellow to orange-brown.

Quality Control of Hematoxylins

The need for consistency of staining is vital to avoid difficult histological interpretation. A preparation may vary from person to person. The age

of the stain, pH difference and degree of usage all these affect the staining properties. New batches of stain must be checked for efficiency against the earlier batches and staining time must be adjusted to give uniformity.

Hematoxylin Quality Control Material

For the daily quality control (QC), the nuclear chromatin of plasma cells, vesicular nuclei of epithelium in the intestine and clear demonstration of keratohyalin granules serve as indicators of hematoxylin staining. The efficacy of hematoxylin can be tested by adding a few drops of hematoxylin to tap water and noting the color change. If it turns blue-purple immediately, it is in working condition. But, if it becomes red to brown, the stain should be discarded. Other nuclear stains include neutral red, safranin O (red) and methylene blue. Counter staining is the application of a different color or stain after the nuclear stain to provide contrast of the structural components to be demonstrated, e.g. cytoplasmic counter staining by eosins.

Eosins

The eosins are the most suitable stain to combine with hematoxylins to demonstrate the cell cytoplasm and general histological architecture of a tissue. Their ability to differentiate between cytoplasm of different types of cells; between different types of connective tissue fibers and matrices, with different shades of pink and red, makes then invaluable as a regular counter stain.

The eosins are acid xanthene or phthalein dyes. The name 'eosin' is derived from the peculiar pale pink color resembling dawn pink. The most frequently used stain among this group is eosin Y. The common members of this group are:

- Eosin Y (eosin yellow water soluble): Cl 45380 BDH Merck
- Eosin R (eosin red spirit or alcohol soluble): Cl 45386 BDH Merck
- Eosin B (eosin blue): Cl 45400 BDH Merck.

Watery eosin solution (used more commonly than eosin R):

- Eosin Y: 10 g
- Distilled water: 1,000 mL
- 0.5 mL acetic acid may be added to shorten staining
- Thymol may be added to prevent fungus formation.

Alcoholic eosin solution:

- Eosin R (spirit soluble): 2g
- Distilled water: 160 mL
- 95% ethyl alcohol: 640 mL.

Dissolve eosin R in distilled water and add 95% ethyl alcohol. If a darker pink shade is required add a drop of acetic acid to each 100 mL of solution.

Other cytoplasmic stains

Although, eosin is the most widely employed cytoplasmic stain, substitutes are available such as phloxine, erythrosin, azophloxin, biebrich scarlet, picric acid, orange G (yellow), light green SF (green), all in concentrations and modes of usage as eosin.

Methods of Staining

Standard: Using Harris hematoxylin and watery eosin Y:

1. Deparaffinize with xylol: 5–10 minutes.
2. Absolute ethyl alcohol: 5 minutes.
   Note: At stage two if tissue is Zenker fixed, treat with Lugol's iodine or 1% alcoholic iodine solution for 10–15 minutes to remove the excess mercury dichromates. Wash in tap water. Treat with 5% sodium thiosulfate for 5 minutes to remove iodine. Wash with tap water.
3. 95% ethyl alcohol: 5 minutes.
4. 80% ethyl alcohol: 5 minutes.
5. 60% ethyl alcohol: 5 minutes.
6. Bring sections to water.
7. Harris hematoxylin: 15 minutes.
8. Rinse with tap water.
9. Differentiate with 1% hydrochloric acid in 70% ethyl alcohol (check with microscope to see background is colorless or pale gray; nuclei should show chromatin).
10. Wash with tap water.
11. 'Blue' with running tap water: 10–20 minutes.
12. Stain with watery eosin: 15 seconds.
13. Depending on the color required, rinse in water: 2 minutes.
14. 80% ethyl alcohol: 2 minutes.
15. 95% ethyl alcohol.
16. Absolute alcohol: 2 changes.
17. Xylene: 2 changes.
18. Mount with Entellan [dibutyl phthalate in xylene (DPX)/Canada balsam].

Results

- Nuclei: Blue to blue-black
- Cytoplasm and other substances: Pink
- Muscle fibers: Deep pink/red
- Red blood cells: Orange
- Fibrin: Deep pink.

Advantages

- Simple procedure
- Cost-effective
- Pleasing colors
- Most histological structures or by products can be located and studied with this stain.

Disadvantages

1. Too much cannot be read into the hematoxylin-eosin stain and with experience ratification with regard to cell structures or products with special stains must be done.
2. Deparaffinization is important and should be adequate. Air bubbles on the section at this stage can cause nonstaining and misinterpretation and should be removed by repeated dipping in the solution.
3. Dehydration after staining is very important:
   a. To increase capability of viewing of the material to be examined (one may not be able to get 'close' to the slide with a high dry or oil immersion lens with improper dehydration and one may think the section is 'thick').
   b. To help preserve the stains in the slide. Remember all stains used are water soluble and any retained water in the final stages of dehydration will lead to 'fading' of the stain after a few weeks or months.

Slides

For normal routine work, 76 × 4 mm slides are available with a variety of thicknesses; those specified as of 1.0–1.2 mm in thickness are preferred, because they do not break easily. Slides of larger size should be used for sectioning of eyes and CNS when such sections do not fit onto the regular size. Special equipment such as slide racks are available for holding slides at any stage of staining.

Slide Numbering

Slide mounted sections are identified during preparation by inscribing the slide with the identification/laboratory accession number or suitable code using a diamond marker or pencil (in case of frosted slides). The use of this diamond pencil leaves small glass splinters on benches, which can easily become embedded in skin or can enter the eye (the safest method is to use a 'frosted end' slide and inscribe using a soft pencil). Automatic slide labeling machines are now available; the increasing use of bar coding will reduce the number of transcription errors.

Care should be exercised when using characters such as A, I, O, T, V and similar, as these appear the same when viewed from either side of the slide; incorrectly identifying the side upon which the section is mounted may lead to poor staining or section damage.

Coverslips

To be compatible with the standard slide, coverslips are 22 mm or 24 mm wide, but vary in length to suit the size of the section. In general, a selection of 22 mm, 30 mm, 50 mm and possibly 60 mm (if preparing cell smears) will accommodate most purposes. Coverslips are manufactured to a specified

thickness to complement the optical specifications of microscope objective lenses (the recommended thickness is indicated on the barrel of the lens and is normally 0.17 mm).

Mounting Media

The final step of preparation of stained section is to cover the slide surface containing tissue with a thin coverslip. This makes the slide permanent and permits microscopic examination, whenever possible. The refractive index of the mounting medium must be approximately equal to that of dried protein, i.e. between 1.53 and 1.54. This is in order to provide the maximum degree of transparency to the stained tissue sections. To be effective, a mountant should possess certain characteristics. These include the following:

- It should be colorless and transparent
- It should have no adverse effect on tissue components
- It should be able to completely permeate and fill tissue interstices
- It should protect the section from physical damage and chemical activity (oxidation and changes in pH)
- It should be resistant to contamination
- It should be completely miscible with the dehydrant or clearing agent
- It should set without crystallizing, cracking or shrinking (or otherwise deform the material being mounted) and not react with leach or induce fading of stains
- Once finally set, the mountant should remain stable.

The mountants can also be classed as adhesives (hydrophobic) or nonadhesive (hydrophilic). In general, adhesives harden through solvent evaporation and thereby fix the accompanying coverslip to the slide. The refractive index of hydrophobic (adhesive) mountants usually approximates that of tissue proteins (fixed) and provides firm adhesion of the coverslip, and hence these mountants are the type most frequently used.

Methods using the hydrophobic mountants generally need the sections to be dehydrated (usually through a series of graded ethanol solutions) and cleared (in a solvent miscible with the mountant) before the mounting medium is applied. Hydrophilic media, although of relatively low refractive index (RI) are essential for procedures in which dehydrant and hydrocarbon type clearant must be avoided. Sections are mountant in hydrophilic media directly from water. These are temporary mounting procedures and slides cannot be preserved on a permanent basis. This will mostly relate to methods for demonstrating lipid, enzyme identification and immunohistochemistry. Disadvantages of hydrophilic mountants are that they may induce stains to leach from the section and many, being nonadhesive, remain soft such that the edge of the coverslip must be sealed to prevent drying out. A sample nail polish may be used for sealing.

Types

Mounting media are of three types:
- Natural resins
- Artificial resins
- Aqueous media.

Natural resins

Canada balsam

Canada balsam is most preferred natural resin. This is an oleoresin obtained from the bark of the fir '*Abies balsamea*'. The dried resin is freely soluble in xylene and other organic solvents. It has a refractive index of 1.523.

Canada balsam is no longer used due to the following disadvantages—it yellows with age, it takes a long time to dry and cationic dyes are poorly preserved; causes the poor preservation of aniline dyes and a stain such as Prussian blue is bleached after mounting.

Formula
- Canada balsam: 55–65 g
- Xylene: 100 mL.

Euparal

Euparal is a mixture of eucalyptol, sandarac, paraldehyde and camsal (camphor and phenyl salicylate). Its relatively low RI, which is usually given as 1.483, makes it useful for mounting unstained sections. Another advantage is that slides may be transferred directly from 95% alcohol eliminating the need for complete dehydration and clearing. Some fading may occur in hematoxylin-stained sections; in this situation the green copper-containing form of euparal is preferred.

Synthetic resins (plastic)

The DPX is one of the most commonly used mountants. It is a colorless, neutral medium in which most standard stains are well preserved. It is prepared by dissolving the common plastic, polystyrene, in a suitable hydrocarbon solvent (usually xylene). It has a refractive index of 1.523.

A major disadvantage of DPX is that it sets quickly and often retracts from the edge of the coverslip. This can be prevented by adding a plasticizer, which is thought to resist this effect by forming a mesh with the polymerized plastic.

Formula
- Polystyrene (distyrene 80): 25 g
- Xylene: 70 mL.

Sections of tissue embedded in plastic compounds (such as epoxy resins) can be successfully mounted in liquid resin of the same type. Sections should be completely dry before applying mountant, which is best set using the same conditions prescribed for tissue blocks. Other examples include, gum chloral mountants, histomount, naphrax, dimethyl hydantoin formaldehyde, etc.

Aqueous media

Aqueous media are employed when dyes and structures are altered or destroyed by dehydration and clearing, e.g. lipid stains and dyes such as crystal violet may lose metachromasia with these steps. Types of aqueous media are as follows:

1. **Glycerine jelly:** It is a useful temporary mountant and has a high RI of 1.47. Ringing the coverslip with a hydrophobic seal will extend the life of mountant sections, although cationic dyes will diffuse into the medium over time. Phosphate buffered glycerol (RI = 1.47) is commonly used to mountant sections for immunofluorescence. Preparation is given below:
   - Gelatin: 10 g
   - Distilled water: 60.0 mL
   - Glycerine: 70 mL
   - Phenol: 1.0 mL.
2. **Apathy's medium:** Sucrose added to gum arabic preparations increases the RI (1.436) and prevents overdrying. The inclusion of potassium acetate will prevent leaching of metachromatic dyes. Dissolve components in warmed distilled water and store in air tight containers. Preparation is given below:
   - Gum arabic crystals: 50 g
   - Cane sugar: 50 g
   - Potassium acetate: 50 g
   - Distilled water: 100 mL
   - Thymol: 100 mg.
3. **Polyvinyl alcohol:** It has been successfully used as a mountant in lipid stains for frozen sections, immunofluorescence microscopy and for immunohistochemistry. A small quantity of this medium can be kept at room temperature for use, but the stock solution can be kept in refrigerator. Discard the solution, if it becomes lumpy. Preparation is given below:
   - Polyvinyl alcohol: 20 g
   - Buffered phosphate saline (pH 7.2–7.4): 80 mL
   - Glycerine: 50 mL.

Method of Mounting Sections

1. Select the appropriately sized coverslip and place on a white paper sheet.
2. Place a drop of mountant in the center of the coverslip.
3. Remove surplus clearent from the back of the slide and around the section, leaving a margin of approximately 3 mm. The section should not be allowed to dry out (unless this is specified in the procedure being undertaken).
4. Invert the slide (section face down) over the coverslip and with one end resting on the paper sheet, gradually lower the other end until the mountant touches the section. Mountant will spread quickly over the section, between slide and coverslip. The slide with the coverslip attached, is then turned upright. Any trapped air is gently squeezed out whilst aligning the coverslip.
5. The mountant is allowed to set. The time required will depend upon the particular agent used, but in some cases warming the slides (37–60°C) will hasten the process. If the result is inadequate, slides are returned to

the solvent (appropriate to the mountant) to have the coverslip removed and the process repeated. In routine practice xylene is used to detach the coverslip.

Mountant sections are often stored for many years and the use of an appropriate mountant is critical to avoid deterioration of the specimen. Slides are usually stored, standing on their short side, in metal or plastic drawers. Labels need to be fixed to these visible ends so the details can be read with the slide in this upright position.

Restaining

Restaining is required to restain slides that have faded appreciably, including those with deteriorated mounting medium. The following steps are undertaken:

1. Coverslips can be removed by soaking in xylene, this may require 1–2 days or alternatively.
2. Slides can be left in xylene at 50–60°C in an oven for up to 15–20 minutes after which the coverslip is gently removed.
3. Dissolve the residual mounting media by placing the slides in staining rack and moving them gently up and down in several changes of xylene.
4. Hydrate through several changes of absolute ethyl alcohol, 95% ethyl alcohol to distilled water.
5. Decolorize slides in 1% acid alcohol.
6. Wash thoroughly to remove the remaining acid alcohol.
7. Restain as indicated with hematoxylin and eosin or special stain.

Repair of Slides

1. If the slide is broken, but the coverslip is intact, attach the broken slide to a new blank slide using plastic cement, which is not soluble in xylene, e.g. Duco cement. Allow to dry overnight.
2. If the tissue is to be removed from the slide, remove the coverslip as described earlier, pour celloidin over the section and allow the excess to run off . Allow the slide to dry thoroughly for several hours. Soak the dry slide in distilled water until the celloidin coating loosens. Slide the razor blade under an end of the celloidin film, then lift off the film of section with a fine forceps. Place the film and slide over the clean slide that has been coated with adhesive. Carefully remove the celloidin coating with multiple changes of acetone. Mount using resinous mounting media.

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Chapter 8 Special Histochemical Stains

HISTOCHEMICAL STAINING

Diagnosis of Lesion

Diagnosis of any lesion at histology rests primarily on the routine hematoxylin and eosin (H&E) stain and a good deal of common sense. The 'eyes cannot see what the mind does not know' is not always true and at times, the unmasking of hidden substances, which can be brought to light with the help of special stains is needed in making a diagnosis.

Definition of Histochemistry

Histochemistry is defined as the identification, localization and quantification in cells and tissues of specific substances, reactive groups and enzyme-catalyzed substances, which are highlighted or colored as a result of the histochemical stains.

Principles

All histochemical stains are based on one of the following principles:

1. Simple ionic interaction between groups of opposite charges.
2. A colored product, e.g. reaction of aldehydes with Schiff's reagent.
3. Detection of an enzyme by providing a substrate, i.e. converted by the enzyme to a colored product.
4. Precipitation of salts of a metal, e.g. precipitation of silver from methenamine silver solution.

Techniques

The various histochemical staining techniques used in histopathology are:

- Routine histochemistry
- Enzyme histochemistry
- Immunohistochemistry.

Routine Histochemistry

Staining of Mucosubstances and Glycogen (Fig. 8.1)

The mucosubstances may occur either in epithelium or in connective tissue and therefore, in either instance, may be called 'epithelial mucosubstances' or 'connective tissue mucosubstances'.

Epithelial mucins

Mucopolysaccharides forms the bulk the substances known as mucins secreted by cells. Mucins are composed of hexosamine units such as glucosamine and galactosamine. These are the predominant epithelial mucins. Other examples are mucoproteins found in thyroid colloid, basement membranes, amyloid and beta (β) cells of the pituitary gland. Mucolipids exist as cerebrosides and gangliosides in the nervous tissue.

Types of connective tissue mucins

Types of connective epithelial tissue mucins are:

- Chondroitin sulfate A found in cartilage
- Chondroitin sulfate B found in skin, heart valves and umbilical cord
- Chondroitin sulfate C found in cartilage, aorta and skin
- Hyaluronosulfate found in cornea
- Keratan sulfate found in cartilage and nucleus pulposus
- Hyaluronic acid found in synovium, skin, aorta, cartilage and bone.

These mucosubstances irrespective of their origin may be either neutral in type being identifiable at pH 5 and above, or acid in type identifiable usually at pH 2.8 and below.

Neutral mucins: Consists of hexosamine and hexose units, and do not have free acid groups, identifiable at pH 5 and above. These are found in lining epithelium of stomach, Brunner's glands of duodenum and in prostatic epithelium. They are periodic acid-Schiff (PAS) positive and Alcian blue (AB) negative.

Acid mucosubstances: These are composed of three different fractions:

1. Carboxylated mucosubstances.
2. Weakly sulfated mucosubstances.
3. Strongly sulfated mucosubstances.

Acid mucins consist of hexosamine and glucuronic acid, iduronic acid or sialic acid, identifiable at pH 2.8 and below. They are AB positive.

Carboxylated mucosubstances [Sialomucosubstances (nonsulfated)]: These sialomucins contain a sialic acid moiety, which is acetylated derivative of neuraminic acid. It reacts with AB at pH of 2.5 and above. These mucosubstances are:

1. Enzyme labile found in goblet cells of peripheral airways of lungs, intestine, mucous cells of submandibular salivary gland. It also contains uronic acid, which is found in connective tissue, synovial fluid of joints and pleural mesotheliomas.
2. Enzyme resistant found in gastric epithelium and mucous gland of major bronchi; non-sulfated sialidase and hyaluronidase resistant is found in gastric pyloric glands.
3. Hyaluronic acid containing mucosubstances (nonsulfated).

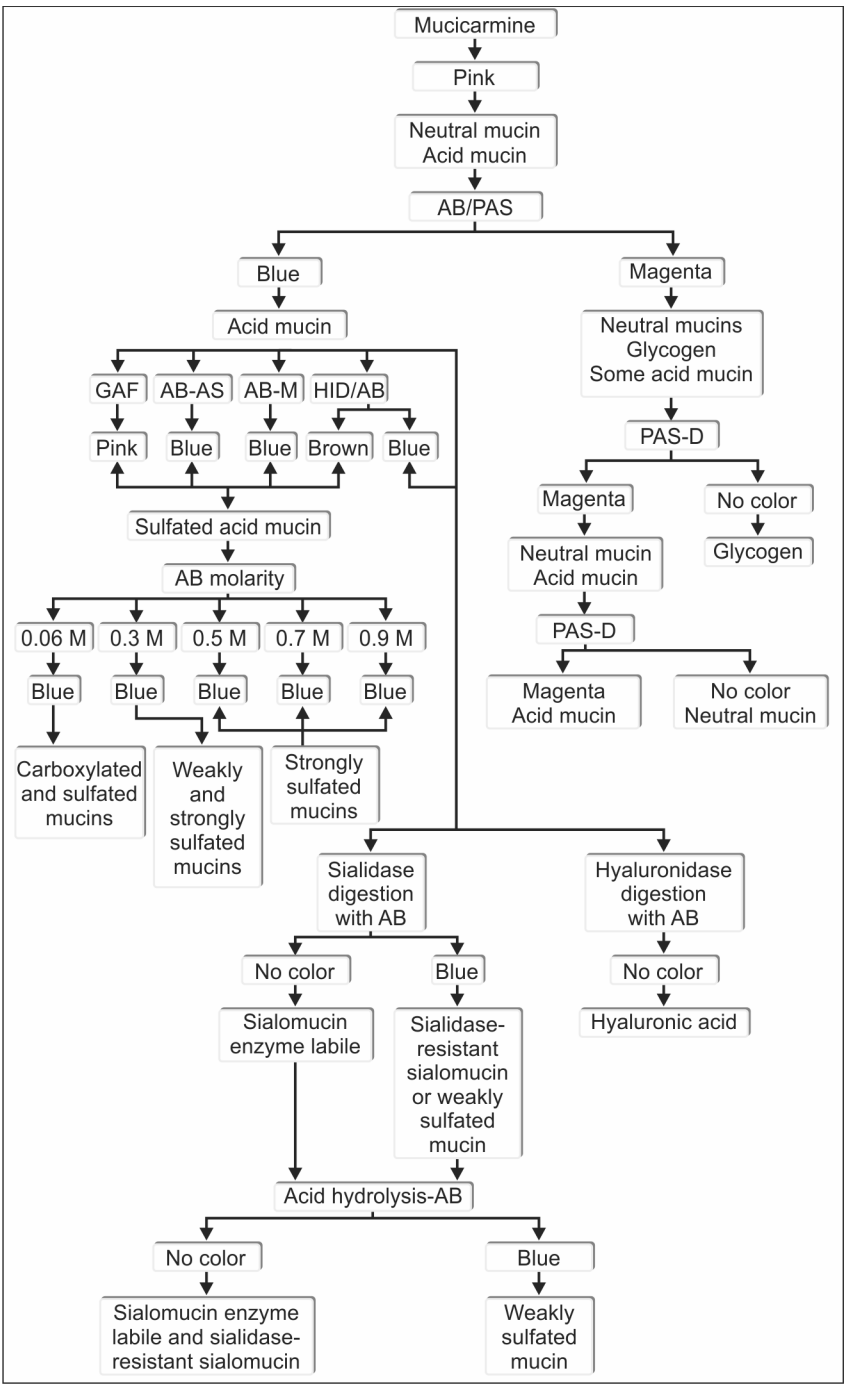

Figure 8.1: Staining for tissue mucins (AB, Alcian blue; GAF, Gomori's aldehyde fuchsin; HID, high iron diamine; M, mucicarmine; PAS-D, periodic acid-Schiff diastase; PAS, periodic acid-Schiff).

Strongly sulfated acid mucins: Comprise of chiefly connective tissue mucosubstances such as chondroitin sulfate, heparan sulfate and keratan sulfate. They are found in bronchial mucus glands (sulfomucins) and minor fraction in intestinal goblet cells, so designated because of their ability to stain with AB at pH levels of 0.5 and below. They are largely PAS negative.

Weakly sulfated acid mucins: These are sialomucins and are usually epithelial in origin, and present in a wide range of cell types and mucous glands, e.g. bronchial submucous glands and in colonic goblet cells. They are PAS positive, which stain with AB at pH 1 and above. Sulfated sialo mucosubstances are also found in prostatic carcinoma and malignant synovioma.

The single universal stain for mucosubstances irrespective of whether these mucosubstances are neutral or acidic is the mucicarmine stain. The PAS tests neutral mucosubstances, chiefly the AB for the acid mucosubstances at pH 2.8. The AB may be used at different molarities too with the addition of varying quantities of magnesium chloride to estimate the amount of carboxylated, weakly sulfated and strongly sulfated acid mucosubstances.

Identification of Different Types of Mucosubstances

Sometimes it becomes necessary in day-to-day practice to identify precisely the type of mucosubstances being secreted by a particular type of epithelium or even a neoplasm. For instance, pleural mesotheliomas are chiefly identified by their capacity to secrete acid mucosubstances, chiefly sulfated and sometimes nonsulfated in types, whereas adenocarcinomas are known to secrete neutral mucosubstances. The types of intestinal metaplasia in the stomach, which is associated with the secretion of sulfomucosubstances predisposes to malignancy. In the cervical epithelium, the presence of sialic acid has been thought to increase the viscosity of mucus. Also normal cervical mucosa secretes chiefly neutral mucins, but cervical carcinoma is associated with secretion of acid mucins. This is also the case in prostate.

MUCICARMINE STAIN

Carmine was first used in 1849, which is extracted from cochineal insect and the active form is carminic acid.

Indication

SouthGate's mucicarmine solution is used for epithelial mucin. The combined AB and PAS technique, however, is more informative to establish presence or absence of tissue mucins.

Procedure

- Carmine (CL 75470) (BDH): 1 g
- Aluminum hydroxide [$Al(OH)_3$]: 1 g

- Aluminum chloride ($AlCl_3$): 0.5 g
- Ethyl alcohol (C_2H_6O): 100 mL.

Dissolve carmine and $Al(OH)_3$ in C_2H_6O, mix by shaking and then add $AlCl_3$, boil for 3 minutes. Cool and make up to 100 mL with the 50% alcohol and store at +4°C.

Principle

Aluminum is believed to form a chelation complex with the carmine by changing the molecule to a positive charge and allowing it to bind with the acid substrates of low density such as mucins:

- **Control:** Small intestine, appendix
- **Fixative:** 10% buffered formalin.

Method

- Bring solutions to water
- Stain nuclei with alum hematoxylin for 10 minutes
- Wash in running water
- Differentiate in 1% acid alcohol for 5 minutes
- Wash well in tap water
- Stain with mucicarmine solution for 20–30 minutes
- Wash in running water
- Bring to mountant and coverslip.

Results

Mucosubstances will be stained pink to red and nuclei will appear blue.

Advantages

- It stains all types of mucosubstances irrespective of their being neutral or acidic in type
- It has a positive prelidection to stain acid mucosubstances
- Stains excess mucin secreted by epithelial cells in intestinal carcinomas, e.g. adenocarcinoma for neutral mucins
- In determining the site of a primary tumor
- Useful in staining encapsulated fungi, e.g. *Cryptococcus.*

Disadvantage

Though rarely seen, tends not to stain or stains lightly neutral mucosubstances.

ALCIAN DYES

Alcian blue is the most popular method for the demonstration of acid mucins. It was introduced by a chemist known Haddock in 1948, who described its composition as being copper phthalocyanine (CuPc) dye. Routinely used is AB 8GX or GS (ICL; merck; sigma; fluka; chroma). Other dyes important are:

- Alcian green 2GX—stains acid mucin as bright green
- Alcian green 3BX—stains acid mucin as blue green
- Alcian yellow—stains acid mucin as yellow.

Method A—Alcian Blue

Indications

In normal tissues, it helps to demonstrate certain cells containing mucin in order to illustrate the normal histological structure, e.g. glandular elements of organs such as prostrate and salivary glands. It stains acidic epithelial mucins such as sialomucins and sulfomucins of large intestine. It stains proteoglycans/hyaluronic acid components of connective tissue and cartilage. Cervical epithelium secretes sialic acid. In diseases it is useful to identify certain type of mucin, so as to assist in the histological diagnosis of certain neoplasms or otherwise:

1. Pleural mesothelioma characterized by its production of hyaluronic acid.
2. Intestinal metaplasia in stomach predisposing to malignancy secretes sulfomucosubstances.
3. Sialomucins and sulfomucins of large intestine are reactive (neutral mucins of gastric mucosa and Brunner's glands are not reactive with AB).
4. It is also useful in identifying mucin-secreting degenerating tumors, e.g. myxomas and neurofibromas.
5. It is helpful in the diagnosis of mucin-secreting tumors such as ovarian tumors.
6. Staining of mucin may be useful in autoimmune and hereditary diseases such as discoid lupus erythematosus (subcutaneous tissue); myxoid degeneration in the dermis in myxedema; in Hurler's disease, hereditary arthro-osteochondrodysplasia, osteogenesis imperfect, etc.

Principle

The dyes are positively charged (cationic dyes) and form electrostatic bonds with certain tissue polyanions (carboxyl or sulfate radicals of acid mucins). The specificity of AB is partly due to its large molecular size, thus staining acidic substrates of low density.

Control

Small intestine, appendix or colon (large intestine contains sulfomucins, which is more strongly acidic as compared to sialomucins of the small intestine):

- Fixative: 10% neutral-buffered formalin (NBF), Bouin's solution
- Technique: 4 μm paraffin sections
- Formula:
  - Alcian blue (8 GX) powder: 1 g
  - 3% acetic acid: 100 mL.

Method

- Bring sections to water
- Stain with AB solution to required depth of staining for 5–10 minutes; wash in tap water
- Counterstain with an alum hematoxylin for 5 minutes or nuclear fast red (Kernechtrot) for a few seconds
- Make up nuclear fast red (Merch; BDH) using 0.1 g in 100 mL of 5% solution of aluminum sulfate with the aid of heat
- Cool, filter and add one crystal of thymol
- Wash in tap water
- Bring to mountant and coverslip.

Result

- Acidic mucosubstances: Light blue to dark midnight blue
- Nuclei: Hematoxylin has a deep blue-purple
- Nuclear fast red: Red.

Advantage

Even prolonged exposure to stain at pH 2.8 will not 'force' other substances to be stained by this stain.

Disadvantages

1. If an alum hematoxylin is used for an excessive period of time, this stain may obscure the blue tinge of the AB.
2. Alcian blue is carcinogenic.

Method B—Alcian Blue with Varying Electrolytic Concentration

Indication

Useful method for separating various acid mucin. Critical electrolyte concentration (CEC) is the point at which the amount of an electrolyte such as magnesium chloride in AB solution is sufficient to prevent staining with AB. This is due to successful competition of electrolyte cations of the salt with dye cations for binding sites on tissue polyanions. Make AB solution as detailed in method A, then add magnesium chloride in the quantities given in Table 8.1 per 100 mL prepared AB solution.

Method

1. Dewax sections and bring sections to water.
2. Stain in various molarities of AB solution overnight at room temperature.

TABLE 8.1: Quantities of magnesium chloride

Molarity (M)	Magnesium chloride (g)	Substances tested
0.06	1.2	Carboxylated and weakly sulfated mucosubstances
0.3	6.10	Weakly and strongly sulfated mucosubstances
0.5	10.15	Strongly sulfated mucosubstances
0.7	14.20	Strongly sulfated mucosubstances
0.9	18.30	Strongly sulfated mucosubstances and keratan sulfate

3. Wash in water and counterstain in the 0.5% aqueous neutral red for 2–3 minutes.
4. Rinse in absolute alcohol.
5. Clear in xylene and mount as desired.

Result

Staining with AB will be retained or lost according to the type of acid mucin present.

PERIODIC ACID-SCHIFF'S REACTION (SCHIFF'S LEUCOFUCHSIN)

Indications

In 1946, McManus first applied the PAS reaction in histology. This technique is closely interlinked to carbohydrate histochemistry and may be used to demonstrate glycogen, neutral mucosubstances, basement membrane, pigments such as lipofuchsin, lipids, e.g. cerebrosides, fungi such as *Candida albicans,* macrophages in Whipple's disease, hyaline proteinaceous renal cysts, intracytoplasmic globules in hepatocytes in α-1 antitrypsin deficiency, trophoblasts in placenta and β-cells in the pituitary. It is also useful in lysosomal storage disorders such as Nimann-Pick disease, Gaucher's disease and in glycogen demonstration in glycogen storage disorders. It is used to differentiate between myeloblasts and lymphoblasts in the diagnosis of acute lymphoblastic leukemia (ALL) (block staining) and acute myeloid leukemia (AML)-M6. The substances positive to PAS are:

1. **Polysaccharides:** Glycogen, cellulose and starch; many leukocytes contain glycogen, capsules of fungi *(Candida albicans, Histoplasma capsulatum, Cryptococcus* and blastomycosis), actinomycosis and bacteria.
2. **Glycoproteins:** Mucins, mucoid secretions of the intestinal tracts, uterine glands, ducts, tracheobronchial tree, hormones [thyroid-stimulating hormone (TSH)], megakaryocytes, etc.
3. **Glycolipids:** Gangliosides, mainly gray matter composed of fatty acids, i.e. cerebrosides and globoid cells of Krabbe's disease.
4. **Noncarbohydrate-containing substances:** Unsaturated lipids, phospholipids and phosphoinositides.

5. **Certain pigments and substances:** Ceroid, lipofuscin, pigment in melanosis coli and Dubin-Johnson pigment (related to lipofuscin).
6. **Plasmogens:** They are acetyl phospholipids, e.g. Russell bodies.
7. **Miscellaneous:** Amyloid, cartilage matrix, colloid, corpora amylacea and ocular lens material.

Principle

The principle is to release the dialdehydes from carbohydrate by oxidation with periodic acid and the subsequent combination of such aldehydes with Schiff's reagent to give a substitute dye, which is red in color (this dye is not basic fuchsin), localized to the site of the aldehyde release.

Oxidation

Periodic acid is the most common substance to be used for oxidation. One of its superior properties is that it will not further oxidize the resulting aldehyde. Thus, standardization is easier. It is used in any strength between 0.5 and 2.5% (preferably 1%). However, four important rules must be observed when periodic acid is used:

1. Oxidation time must be limited to a maximum of 10 minutes.
2. Oxidation should not be carried out at more than 20°C.
3. The periodic acid solution should have a pH of 3–5.
4. Prepared solutions must be kept at 4°C in a refrigerator.

Reagents

Schiff's Reagent

Basic fuchsin (pronounced as fook-sin) when treated with sulfurous acid results in a colorless product called Schiff's reagent. Basic fuchsin is not a pure dye and consists of pararosaniline and magenta II principally. Pararosaniline is usually present in combination with an acetate or chloride and is consequently unstable. For this reason when basic fuchsin is treated with sulfurous acid produces Schiff's reagent. This substance in combination with two aldehyde molecules produces a reddish purple complex. This color is not due to basic fuchsin and may vary in intensity depending on the various aldehydes present in the tissues examined. The two most important rules for preservation of Schiff's reagent are:

1. A deep brown-black bottle to prevent oxidation taking place (if the solution turn pink, discard it).
2. At 4°C temperature.

Sulfrous acid (sulfite) rinse: This step is optional. The purpose of the rinse is to remove excess leukofuchsin, which may become recolorized and give false-positive staining of some structures. The sulfite rinse must be prepared fresh each day.

Procedure for preparing Schiff's reagent

- Schiff's reagent
- Basic fuchsin: 1 g
- Hydrochloric (HCl) acid: 20 mL
- Sodium metabisulfite: 1 g
- Activated charcoal: 2 g
- Distilled water: 200 mL.

Boil the distilled water. Allow to cool to 80°C and then add basic fuchsin. Filter at 50°C and add HCl acid. At 25°C, add sodium metabisulfite, store in dark for 48 hours. Add activated charcoal. Shake for 1 minute and filter; this filtrate should be clear (sodium metabisulphite in presence of HCl releases sulfurous acid with resultant sulfur dioxide, which in turn reacts with basic fucshin to form Schiff's reagent). The activated charcoal absorbs the excess sulfurous acid.

Testing for activity of Schiff's reagent

Pour few drops of Schiff's reagent into 10 mL of concentrated formalin. If the reagent is active, the solution turns reddish pink rapidly. If the blue color develops after some time, the solution is breaking down.

Periodic Acid

The periodic acid 1% solution is prepared by using:

- Periodic acid: 1 g
- Distilled water: 100 mL
- Control: Use skin, aorta or normal liver for positive PAS staining
- Fixative: Standard paraffin section fixed in 10% NBF
- Technique: On paraffin sections 4–5 μm
- Control: Use skin, aorta or normal liver for positive PAS staining
- Fixative: Standard paraffin section fixed in 10% NBF
- Technique: On paraffin sections 4–5 μm.

Procedure

- Bring sections to water
- Rinse in distilled water
- Oxidize with 1% periodic acid for 5 minutes
- Rinse in distilled water
- Use Schiff's reagent for 10 minutes
- Sulfite rinse (optional)
- Wash in tap water for 10 minutes; this intensifies the color reaction
- Stain the nuclei with alum hematoxylin for 5 minutes
- Wash in tap water
- Bring to mountant.

Results

- PAS-positive material: Magenta pink to red
- Nuclei: Blue.

Note: The temperature for oxidants to act should not exceed 25°C as it is likely to oxidize other substances besides aldehydes.

Safety: Basic fuchsin (in Schiff's reagent) is a known carcinogen. It is advisable to wear gloves, goggles, particle mask and laboratory coat, while preparing the solution. Avoid contact and inhalation of HCl, as it is a strong irritant to skin, eyes and respiratory system.

COMBINED ALCIAN BLUE AND PAS REAGENT

Indications

Some substances may require the demonstration of both acidic and neutral mucosubstances at the same time in the very same section. In such instances, an Alcian blue and periodic acid-Schiff (AB-PAS) stain may be used. It is a routine stain for gastrointestinal (GI) biopsies.

Principle

- Control: Intestinal metaplasia in gastric mucosa—known case
- Fixative: 10% formalin
- Technique: On paraffin sections 4–5 μ thick.

Reagents

- Alcian blue (CI 74240) 1 g; 3% acetic acid 100 mL
- 0.5–1% periodic acid (refer PAS technique)
- Schiff's reagent
- Alum hematoxylin.

Procedure

- Bring sections to water
- Stain with AB solution for 5 minutes
- Wash in tap water
- Oxidize with 1% periodic acid for 5 minutes
- Wash in distilled water
- Use Schiff's reagent for 8 minutes
- Wash in tap water for 10 minutes
- Bring to mountant.

If a nuclear stain is required, this may be used after washing with Schiff's reagent. Use alum hematoxylin for 3 minutes or nuclear fast red for a few seconds.

Results

- Acid mucosubstances: Blue
- Neutral polysaccharides: Magenta pink to red
- Mixture of acid and neutral mucosubstances: Purple
- Nuclei: Red (nuclear fast red); blue (alum hematoxylin).

STAINS FOR GLYCOGEN

Glycogen is a polymer of glucose. It is an important storage carbohydrate in man and its presence is frequently tested in cells, e.g. hepatocytes. It is rapidly broken down after death of tissue and therefore rapid fixation after removal at biopsy is necessary to demonstrate it. They are soluble in water and insoluble in alcohol. In the process of fixation and gradual penetration of fixative, aggregates of glycogen are seen in only certain areas of the cell giving a stream-like effect to the section called 'streaming artifact'. This usually cannot be avoided. Formal saline fixation proves adequate as formalin tends to bind glycogen to protein, thus retaining it to a certain extent although much has been written about ethyl alcohol as a fixative for glycogen. The latter usually causes severe artifactual cell shrinkage.

Diastase Method (for Removal of Glycogen)

Principle

Glycogen can be selectively removed by digestion with specific enzymes, e.g. malt diastase, which causes the loss of PAS reactivity of glycogen. The diastase (or amylase) acts on glycogen to depolymerize it into smaller sugar unit (maltose and glucose), that are washed out of the section. Common fixatives have no inhibitory effect on diastase action.

Control

Liver section is taken as the control for this technique.

Method

Bring sections onto two separate slides to water. Treat one (suitably marked) with 0.1% malt diastase in distilled water for 30 minutes. Alternatively, saliva can be used as it contains sufficient diastase to digest glycogen. Wash in running tap water. Stain untreated section (control) and treated section (test) either with PAS reaction or Mucicarmine method.

Result

If glycogen is present, the diastase treated section will not stain with either the PAS reaction or the mucicarmine method. On the other hand, the untreated

section (control) will stain positively. If both do not stain, it would mean glycogen is not present. If both do stain, the positivity of the reaction is due to mucosubstances not digestible by diastase:

- Glycogen: Negative
- Fungus: Magenta
- Nuclei: Blue.

Best Carmine Technique for Glycogen (Best, 1906)

Indication

The technique is highly selective stain for glycogen.

Principle

The staining technique demonstrates glycogen by hydrogen bond formation between hydroxy (OH) groups on the glycogen and hydrogen atoms of the carminic acid. Fibrin, mast cell granules and neutral mucin stain weakly with this method.

Specimen

Standard paraffin section fixed in 10% NBF.

Control

Liver section is taken as the control for this technique.

Reagents

1. **Carmine stock:** Distilled water 60 mL and 2 g carmine (CI 75470), 1 g potassium carbonate and 5 g potassium chloride. Boil gently in a large conical flask for 5 minutes. Cool, filter and add 20 mL concentrated ammonia solution. Allow to ripen at room temperature for 24 hours. Store in a dark container at 4°C and keep for 1–2 months.
2. **Carmine working solution (make fresh each time):** As follows:
   - Stock solution: 15 mL
   - Ammonia concentrated solution: 12.5 mL
   - Methanol: 12.5 mL (staining time to be increased as stock solution ages).
3. **Best's differentiator:**
   - Methanol: 40 mL
   - Ethanol: 80 mL
   - Distilled water: 100 mL.

Procedure

Dewax test with positive control sections and rinse in 100% alcohol, and then in 80% alcohol:

- Duplicate sections may be treated with diastase, if desired
- Stain nuclei of all sections well using one of the iron hematoxylin solutions, e.g. Weigert's iron hematoxylin
- Treat with carmine solution for 10 minutes
- Transfer slides quickly to a Coplin jar of differentiating (Best's) solution
- Wash in alcohol (not water), clear and mount.

Results

- Glycogen: Bright red
- Neutral mucin, mast cells and fibrin: Weak red
- Nuclei: Blue.

STAINS FOR SULFATED ACID MUCINS

Sulfated acid mucosubstances can be identified from non-sulfated acid mucosubstances by the following methods:

- Gomori's aldehyde fuchsin method
- High iron diamine technique
- Aluminum sulfate method
- Alcian blue with varying electrolyte concentrations.

Gomori's Aldehyde Fuchsin Stain

Aldehydes fuchsin stain has a great affinity for sulfated mucosubstances. The traditional method employs an initial oxidation step followed by a variable time in a solution consisting of basic fuchsin, concentrated HCl and paraldehyde. The value of aldehyde fuchsin in identifying mucins is greatly increased when aldehyde fuchsin and AB are combined. This combined technique will give color separation of sulfated and non-sulfated acid mucosubstances. The differentiation depends on the greater affinity of aldehyde fuchsins for sulfated groups than for carboxyl groups as prior staining with aldehyde groups blocks the sulfated mucosubstances and subsequently staining with AB demonstrates the carboxylated acid mucosubstances.

Formula for Gomori's Aldehyde Fuchsin Solution

Dissolve 1 g of basic fuchsin in 100 mL of 60% alcohol. Add 1 mL of concentrated HCl and then 2 mL of paraldehyde (use fresh paraldehyde solution). Allow to 'react' by standing for at least 2 days at room temperatue (solution develops a blue color) and store at 4°C.

Alcian blue solution: 1% AB in 3% glacial acetic acid.

Procedure

- Deparaffinize sections and take down to 70% alcohol
- Stain with aldehyde fuchsin for 20 minutes
- Rinse well in 70% alcohol, then in water

- Stain with AB for 5 minutes
- Wash, dehydrate, clear and mount.

Results

- Strongly sulfated mucins: Deep purple
- Weakly sulfated mucins: Purple
- Non-sulfated acid mucins: Blue.

High Iron Diamine Technique

High iron diamine (HID) is currently the standard method for highly acidic sulfated mucins. A mixture of diamine salt is oxidized to form a black cationic chromogen, which bonds with sulfate ester groups. By counterstaining with AB, a clear color distinction is made between the groups of acidic mucins (sulfated from carboxylated mucins).

Results

- Sulfated mucins: Black-brown color
- Carboxylated mucins: Blue
- Nuclei: Red.

Aluminum Sulfate Method

Aluminum sulfate demonstrates only sulfated mucins and is a confirmatory technique for the presence of this group of acid mucins. It depends on the fact that certain dyes such as thionine, toluidine blue, nuclear fast red and AB when dissolved in an aqueous solution of aluminum sulfate at a certain concentration, will only bind with mucins containing a sulfate group.

Results

- Sulfated mucins: Blue
- Non-sulfated mucins and nuclei: Red
- Mixture of sulfated and non-sulfated acid mucins tends to stain a purple color.

OTHER STAINS TO DETECT PAS-POSITIVE ACID MUCINS

Phenylhydrazine (PAS Technique)

Indication

Generally, acid mucosubstances are AB positive and neutral mucosubstances PAS positive, and the combined AB-PAS reaction detects both these. However, at times a few acid mucosubstances are also PAS positive. In order to identify these, the phenylhydrazine PAS technique is used. This is a reasonably reliable technique and the time of treatment can be safely adhered too.

Principle

Phenylhydrazine condenses preferentially with periodate-treated aldehyde groups of neutral mucosubstances alone, thus blocking any further subsequent reaction with Schiff's reagent. The PAS positivity of the acid mucosubstances is unchanged.

Solutions

- Aqueous periodic acid 1%
- Aqueous phenylhydrazine hydrochloride 5%.

Formula

- About 5% aqueous phenylhydrazine hydrochloride
- Phenylhydrazine hydrochloride: 5 g
- Distilled water: 95 mL.

Procedure

- Deparaffinize sections and take down to distilled water
- Treat all sections (test and controls) with periodic acid solution for 2 minutes
- Wash well in distilled water
- Treat the test and control sections with the phenylhydrazine solution for 1 hour (at room temperature) and the negative control sections with distilled water for the same period of time
- Wash well in distilled water then treat all sections with Schiff's reagent for 8 minutes
- Wash in running tap water for approximately 10 minutes followed by nuclear staining with hematoxylin in the conventional manner
- Dehydrate, clear and mount in either a natural or synthetic resin.

Results

- Neutral mucins: Negative
- Acid mucins: Magenta.

Enzyme Digestion Methods

There are chiefly three types of enzymes, which are in common use for mucosubstances identification work such as sialidase, hyaluronidase and diastase, these when used in conjunction with suitable staining methods provide useful information. The use of diastase in the detection of glycogen has already been outlined. Carboxylated acid mucins are detected by sialidase and hyaluronidase enzyme extraction of the mucins. A potential drawback to the use of certain enzymes is their cost, which prevents their routine use in the laboratory.

Sialidase

Sialidase or neuraminidase extracted from the *Vibrio cholera* group of organisms or *Clostridium welchii* digests sialomucosubstances. The digestion is followed by staining with AB to know whether or not digestion has occurred. Digestion should be followed with the combined AB-PAS techniques; it is seen that the sialidase-labile mucosubstances lose their AB reactivity and give a positive PAS reaction instead, i.e. they change their staining result from blue to magenta. A few sialic acid containing mucosubstances are not digested by sialidase. In such substances, if sialidase digestion is preceded by deacetylation, the sialidase-resistant sialomucosubstances are rendered labile to the enzyme. Alternatively, sulfuric acid (H_2SO_4) hydrolysis may be employed to remove both sialidase–sensitive and sialidase–resistant forms of sialic acid mucosubstances.

Hyaluronidase

The commonly used hyaluronidase is bovine testicular in origin, which digests in addition to hylauronic acid, chondroitin sulfate A and C. The digestion procedure is followed by AB staining.

USE OF METACHROMASIA IN MUCIN STAINS

Both sulfated and carboxylated mucins are metachromatic. Dyes used to demonstrate metachromasia include toluidine blue, thionine, azure A, safranin and Bismarch brown.

Azure A

Azure A technique is for metachromasia staining of mucins. In this technique, even after alcoholic dehydration, the metachromasia is usually well preserved.

Procedure

- Deparaffinize sections in xylene and hydrate through graded ethanols to deionized water
- Azure A solution for 10 minutes (azure A 0.01 g; ethanol 30% 100 mL)
- Rinse in deionized water
- Dehydrate in graded ethanol and clear in xylene
- Counter slip and mount.

Results

- Acid mucins: Purple to red
- Neutral mucins and background: Blue.

CONNECTIVE TISSUE STAINS

The main function of connective tissue is to connect together and provide support to other tissues of the body. It is derived from Latin word meaning

to 'to bind'. Connective tissue usually consists of a cellular portion in a surrounding framework of a non-cellular substance. The cell types of connective tissue include fibroblasts, mast cells, histiocytes, adipose tissue, reticular cells, osteoblasts, osteocytes, chondroblasts, chondrocytes and blood forming cells, etc. The intercellular substance is usually composed of both amorphous (non-sulfated and sulfated mucopolysaccharides) and formed elements (collagen, reticular fibers and elastic fibers).

The collagen fibers are the most common type of intercellular substance and found in abundance in most of the tissues of the body. They can occur individually as in loose areolar tissue, arranged in an open weave pattern or as a large bundles of fibers clumped to form a structure of tensile strength, e.g. tendons viewed under polarized light shows collagen fibers to be birefringent. Connective tissues are divided into the following types:

1. Connective tissue proper includes loose or areolar, dense and reticular tissue.
2. Types of cartilage: Hyaline, elastic and fibrocartilage.
3. Spongy or cancellous bone and dense or cortical tissue.

Types of Collagen

Various types of collagen fibers (Table 8.2) are identified:

1. Type I collagen, the most common form encountered in the human can be found in bone, fibrous connective tissue, ligaments, skin and tendons. The fibrils, are thick, closely packed (75 mm in diameter) and strongly birefringent, but not argyrophilic.

TABLE 8.2: Common types of collagen and their distribution

Types	Fibrillary characteristics	Composition	Distribution
I	Thick and striated	Low in hydroxylysine and glycosylated hydroxylysine	Bone, dentin, dermis, fascia, ligaments, skin and tendons
II	Very thin and fine striations	High in hydroxylysine and glycosylated hydroxylysine	Hyaline and elastic cartilage
III	Loosely packed, thin fibrils and striated	High in hydroxyproline and low in hydroxylysine	Frameworks in organs such as spleen, liver and lymph nodes
IV	Fibers and fibrils are not seen	High in hydroxylysine and glycosylated hydroxylysine	Basement membranes
V	Unknown	High in hydroxylysine and glycosylated hydroxylysine	Blood vessel, endothelium (intercellular) and fetal membranes, and in small quantities with other fibers

2. Type II collagen is present in hyaline and elastic cartilage mainly as very thin, loose fibrils dispersed through the ground substance as a copius meshwork of proteoglycans. Fibrils are birefringent and stain pink to red with sirius red.
3. Type III collagen occurs in conjunction with other types of collagen fibers (e.g. type I) and is a major component of reticulin. It is found as a loose network of thin, striated fibrils surrounded by carbohydrate-rich interfibrillary material. Fibrils are argyrophilic and weakly birefringent.
4. Type IV collagen is a non-fibrillary form, which occurs in basement membranes. It is weakly birefringent and associated with significant amounts of carbohydrate, which is responsible for the strong PAS positivity.
5. Type V collagen, also nonfibrillary is found mainly in blood vessels and fetal membranes although it does occur in small quantities in other tissues. It is found between endothelial cells and other cells involved in the attachment of cells to adjacent structure.

Collagen of both the mature and immature variety stains vary shades of pink with routine hematoxylin and eosin methods. In certain instances, e.g. neoplasms, it would be necessary to define the collagenous nature of a pink substance that is found interlaced with cellular material. It is for this reason that collagen stains are used. These are the van Gieson stain and the Masson's trichrome stain.

Of these stains, perhaps the most insensitive collagen stain is the van Gieson stain, which stains only mature extruded collagen (does not stain reticulin) and cannot differentiate with great ease from such mature extruded collagen and maturing collagen. This need is particularly felt in certain neoplastic spindle cells particularly in fibrous histiocytoma, fibrosarcomas and smooth muscle neoplasms, where collagen is constantly maturing. In such instances a more sensitive stain is required and Masson's trichrome, serves the purpose. The stains also differentiate collagen from smooth muscles as in fibromas from leiomyoma. The Mallory's trichrome stain is also extremely suitable.

Collagen Staining

Aniline (meaning, synthetic) dyes have a strong affinity to adhere collagen fibers in an acid environment. This affinity is taken advantage of by using these dyes in the various collagen stains. It should be remembered that when such an acid requirement is necessary, the nuclear stain, hematoxylin, should be of the 'iron' type, which will not be affected by the acid environment. The common aniline dyes are:

- Aniline blue (spirit solution): Cl 42775
- Aniline blue (water soluble): Cl 42775
- Acid fuchsin: Cl 42685
- Methyl blue: Cl 42780
- Indigo carmine: Cl 73015.

The van Gieson stain combines two or more anionic dyes and relies on differential binding by tissue components. The differentiation is determined by a combination of differences in the relative size of the dye molecules, differences in the physical structure of the tissue, and differences in the amino acid composition of tissue elements.

Principles

The van Gieson's stain is a mixture of picric acid and acid fuchsin. It is the simplest method of differential staining of collagen and other connective tissue such as smooth muscle. When using combined solutions of picric acid and acid fuchsin, the small molecules of picric acid penetrate all of the tissues rapidly, but are only firmly retained in the close textured, red blood cells and muscle. The larger molecules of fuchsin solution displace picric acid molecules from collagen fibers, which have larger pores, and allow the larger molecules to enter.

The acid most commonly used in a stain, e.g. van Gieson is picric acid, which not only provides an acid environment, but also acts as a suitable contrast color for non-collagenous-stained areas and therefore, the combination used in the van Gieson stain is called picrofuchsin stain. The other acids used for collagen staining are the phosphomolybdic acid or phosphotungstic acid, which are the preferred acids for both the Masson's trichrome as also the Mallory's trichrome.

The difference between the van Gieson stain and Masson-Mallory type is that the van Gieson is a relatively simple single unit stain utilizing 'picrofuchsin' as the single unit, staining collagen pink and all other non-collagenous substances yellow. This stain gives sharper differentiation if small amounts of acid are added to stronger fuchsin solutions. The preferred fixative for the van Gieson stain is formalin, whereas for the Masson's trichrome a mercurial fixative (Bouin's fluid or Zenker's fluid) is preferred.

As opposed to the van Gieson stain the Masson's trichrome uses a combination of Biebrich scarlet-acid fuchsin. This combination when used, stains both the collagen and muscle red. With the further use of the counterstain, which may be light green or aniline blue, the collagen stains an intense green (with light green) or a shade of blue (with aniline blue). By implication three dyes are employed (trichrome) one of which is a nuclear stain. The general rule in the trichrome staining is that a smaller dye molecule will penetrate and stain a tissue element, but whenever a larger dye molecule can penetrate the same element, the smaller molecule will be replaced by it. Heat influences the penetration of the larger dye molecules. Erythrocyte protein produces a dense network with small pores between the protein elements and muscle cells will form a more open structure with larger pores. Collagen has the least dense network and is seemingly quite porous. The smaller molecular dyes will penetrate any of the three tissue types. Medium sized dye molecules will penetrate muscle and collagen, but do not react with erythrocytes. The largest dye molecules (aniline and light green) will penetrate only collagen (which is porous), leaving muscle and erythrocytes unstained.

Stains Used

van Gieson Stain

Applications

1. To differentiate between leiomyoma from fibroma and schwannoma; leiomyosarcoma from fibrosarcoma.
2. Peutz-Jeghers polyp to demonstrate the central core smooth muscle.
3. To demonstrate fibrous stroma and blood vessels of endometrial polyp.
4. To show (delineate) the extent of fibrosis in a given tissue.
5. To differentiate amyloid from collagen (specific for collagen).
6. It can be used as counterstain with von Kossa stain (mineralized bone black and osteoid red) and Verhoeff's stain (elastic tissue black and background yellow to red).

Disadvantages

- Immature (young) collagen does not stain with van Gieson
- Control: Artery or skin.

Formula

- Acid fuchsin 1% aqueous solution: 2.5 mL
- Picric acid saturated aqueous solution: 97.5 mL.

Procedure

- Bring sections to water
- Rinse in distilled water
- Stain with Weigert's hematoxylin (freshly prepared) for 10 minutes
- Wash in distilled water
- Stain in van Gieson solution for 1–3 minutes
- Dehydrate in 95% alcohol
- Do not use water
- Clear and mount.

Results

- Collagen: Red
- Muscle and other non-collagenous tissue: Yellow
- Nuclei: Dark blue.

Masson's Trichrome Stain—modified Mallory (Masson, 1929)

The first account of a triple stain was by Gibbs H in 1880 followed by Richardson BW in 1881.

Advantages

1. It is a better differentiator than van Gieson stain for muscle fibers.
2. It is used to differentiate between collagen and smooth muscle in tumors.
3. Liver biopsies to assess early fibrosis and the degree of fibrosis to know the stage and progression of the disease in hepatitis B and C viral infections, alcoholic liver disease, chronic biliary disease and cirrhosis of liver.
4. Routine kidney biopsies.

Formula

- Biebrich scarlet-acid fuchsin solution:
  - 1% Biebrich scarlet aqueous: 90 mL
  - 1%Acid fuchsin aqueous: 10 mL
  - Glacial acetic acid: 1 mL.
- Phosphomolybdic and phosphotungstic acid solution:
  - Phosphomolybdic acid: 5 g
  - Phosphotungstic acid: 5 g
  - Distilled water: 100 mL.
- Light green counterstain solution:
  - Light green: 5 g
  - Glacial acetic acid: 2 mL
  - Distilled water: 250 mL
  - Heat water, dissolve light green. Filter and add the acetic acid.
- Aniline blue counterstain solution:
  - Aniline blue: 2.5 g
  - Acetic acid: 2 mL
  - Distilled water: 100 mL.
- About 1% acetic acid solution:
  - Glacial acetic acid: 1 mL
  - Distilled water: 100 mL.

Procedure

- Bring sections to water
- Rinse in distilled water
- If tissue is fixed in Bouin's or Zenker's solution, proceed to step 4. If not, mordant in Bouin's fixative for 1 hour at 56°C or overnight at room temperature
- Wash in running water until yellow color disappears, later rinse in distilled water
- Expose to iron hematoxylin solution for 10 minutes
- Wash in running tap water for 10 minutes and rinse in distilled water
- Expose to Biebrich scarlet-acid solution for 15 minutes (solution can be reutilized)
- Rinse in distilled water
- Treat with phosphomolybdic and phosphotungstic acid solution for 10–15 minutes
- Aniline blue solution for 5–10 minutes or light green for 5 minutes
- Rinse in distilled water
- Acetic acid solution 1% for 3–5 minutes
- Dehydrate and mount.

Results

- Collagen (mature): Blue or green depends on the counterstain
- Collagen (newly formed): Red
- Muscle (irrespective of skeletal or smooth): Red
- Keratin; red cells: Red
- Nuclei: Blue.

Elastic Tissue Staining

These are strongly eosinophilic and when arranged compactly as in the arterial elastic laminae are easily identified due to their refractivity. They differ from collagen by their insolubility in organic and inorganic solvents (collagen is soluble in 2% acetic acid).

Indications

1. **Malignant hypertension:** To demonstrate hyalinization of blood vessels and reduplication of lamina elastic interna.
2. **Malignant nephrosclerosis:** The arcuate arteries and other renal blood vessels show reduplication.
3. **Arteriovenous malformations:** To demonstrate elastic fibers in the arterial segments.
4. **Aneurysms:** To differentiate true and false aneurysms.
5. **Breast lesions:** Periductal elastosis in infiltrating carcinoma and central elastosis in radial scar.
6. **Skin lesions:** To demonstrate the elastic degeneration of the skin solar elastosis, pseudoxanthoma elasticum, elastofibroma.
7. **Marfan's syndrome:** To demonstrate the depletion and fragmentation of the elastic lamina and vascularity of the media and adventitia.
8. **Giant cell arteritis:** To show the disruption of elastic lamina.

In almost all its applications to the staining of vessels as well as elsewhere, the elastic stain is best combined with the van Gieson stain.

Methods for Demonstration of Elastic Fibers

Several methods are used for the demonstration of elastic fibers. The principles behind the following stains are not well understood:

1. **Verhoeff's method:** The procedure is overstaining with a combination of iodine-ferric chloride ($FeCl_3$) hematoxylin (Verhoeff's iron hematoxylin) followed by $FeCl_3$ differentiation. Elastic fibers have disulfide bridges and these are converted to anionic sulfonic derivatives, which combine with basic dyes. The differentiation is accomplished by busing excess mordant (i.e. 2% ferric chloride) to break the tissue mordant dye complex. The dye is attracted to the larger amount of mordant in the differentiating solution and will be removed from the rest of the tissue. Elastin on the other hand has a strong affinity for the iron-hematoxylin complex formed by the reagents in the stain and will retain the dye longer than the other tissue elements, which are decolorized. The technique works after any fixation, it is the easiest to prepare, quick to perform and is most consistent giving an intense black staining of the coarse elastic fibers, although the thinner fibers are less well demonstrated. The results are permanent and show little fading even after several years.

2. **Weighert's resorcin-fuchsin method:** In this principle of staining involved is that in the presence of Ferric salts, which act as oxidizers, the elastic fibers stain with basic fuchsin to give a brown to purple color. The results obtained with this method are good, but the preparation of the stain is tricky and time consuming, and at times may give variable results or even fail to act. The ferrous salts contained in the $FeCl_3$ may also interfere with the staining.
3. **Gomori's aldehyde-fuchsin stain:** This is to demonstrate elastic fibers, a deep purple color, but because the stain is difficult to prepare and deteriorates rapidly with time, the method has fallen out of favor.
4. **Orcien:** This is a naturally occurring vegetable dye, which is now synthesized and stains elastic fibers in acidic solution. In the resorcin-fuchsin, aldehyde-fuchsin and orcien methods, a hydrogen ion bonding between the stain molecule and the substrate may be responsible for the staining of elastic tissue.

Stains Used

Verhoeff's and van Gieson stain for elastic tissue

Dissolve 1 g of hematoxylin in 22 mL of absolute alcohol on an open dish on a hot plate. Cool, filter and add 8 mL of a 10% aqueous solution of $FeCl_3$ and 8 mL of iodine solution (2 g of iodine with 4 g of potassium iodide dissolved in 100 mL of distilled water). For better results, make up fresh solutions just before use:

1. Ferric chloride solution:
   - Ferric chloride: 2 g
   - Distilled water: 100 mL.
2. Sodium thiosulfate solution:
   - Sodium thiosulfate ($Na_2S_2O_3$): 5 g
   - Distilled water: 100 mL.

Method

- Bring sections from alcohol to distilled water
- Expose to Verhoeff's elastic tissue for 15 minutes
- Wash in distilled water
- Differentiate in 2% $FeCl_3$ only for a few minutes, check under microscope and if differentiated too far, then restain
- Place in 5% $Na_2S_2O_3$ for 1 minute (removes excess iron)
- Wash in tap water for 5 minutes
- Counterstain with van Gieson stain for 1–2 minutes
- Differentiate in 95% alcohol (do not use water)
- Clear and mount.

Results

- Elastic fibers: Blue-black to black color
- Nuclei: Blue to black
- Collagen: Red
- Other tissue elements (cytoplasm and muscle): Yellow.

Reticulin stain

Reticulin is a procollagen. It is finer than collagen, stains black with reticulin stain and is unstained with collagen stain. Collagen fibers on the other hand are coarse, doubly refractile, stain red with a collagen stain, e.g. van Gieson; and yellow, lavender or brown on silver impregnation. Reticulum and collagen are basically similar. Though there may be chemical differences in the amino acid content of collagen and reticulin, most of the observed differences are in the physical arrangements of molecules and the presence of additional bindings or cementing substances in collagen such as a mucopolysaccharides resembling hyaluronic acid.

Principle of staining

The staining procedure for reticulin is an impregnation method. The aldehyde groups of the carbohydrate of reticulin fibers reduce the colorless silver complex to a dark brown oxide of silver, which is precipitated in particulate form on reticulin fibers:

1. The methods of Foot, Bielschowsky-Maresch, Perdrau Da Fano, Wilder, Gorden and Sweet's, Gomori and Lillie, all use silver oxide or hydroxide in ammoniacal solution:
   $AgNO_3 + NaOH = AgOH + NaNO_3$
2. The del Rio Hortega, Foot and Laidlaw variants use ammoniacal solution of silver carbonate:
   $2AgNO_3 + Li_2CO_3 = Ag_2CO_3 + 2LiNO_3$
3. The precipitated silver carbonate is dissolved with ammonia water to given ammonium silver carbonate:
   $Ag_2CO_3 + 4NH_3 = [Ag(NH_3)_2]\ 2CO_3$

So, ammonium silver carbonate (Laidlaw's method) or ammonium silver oxide or hydroxide (other method, e.g. Foot's) are reduced to a dark brown silver oxide by reticulin fibers and subsequently reduced to black metallic silver by formalin.

Reticulin stain—Bielschowsky's method—Foot's modification

Applications

1. In kidney lesions, diabetic glomerulosclerosis display laminated argyrophilia of the Kimmelstiel-Wilson (K-W) lesions and chronic lobular glomerulonephritis, shows a tangle of reticulin and amyloid is colored with a diffuse pale gray with a silver reticulin stain.
2. In liver reticulin is helpful in early cirrhosis and detects creeping in fibrosis.
3. In bone marrow reticulin detects fibrosis in myelofibrosis and metastatic carcinoma shows reticulin-free areas.
4. In diagnosing hemangiopericytoma (vascular tumors), which shows pericytes external to the basement membrane in contrast to hemangioendothelioma, where reticulin surrounds tumor cells to outline vascular pattern in angiosarcoma.
5. In lymph nodes, some early lymphomas are differentiated by production of abundant reticulin with loss of nodal architecture as compared to normal lymph node architecture or follicular hyperplasia.

This application of reticulin stain in lymph nodes is rarely used these days as immunomarkers have replaced the utility value of reticulin in lymph node pathology. Angioimmunoblastic lymphomas and T-cell lymphomas are typically rich in high endothelial venules.

6. In ovarian tumors, granulosa cell tumors show group of cells surrounded by reticulin, while in the comas, individual cells are surrounded by reticulin fibers.
7. Tumors of nervous system arising from mesodermal tissues show abundant reticulin, e.g. gliomas, meningeal tumors and sarcomas. Astrocytomas are characterized by the pattern of angiogenesis.
8. In paraganglioma, the reticulin stain reveals the typical cell nests or organoid (Zell-Ballen) pattern.
9. In endometrial stromal sarcoma, the individual cells are surrounded by reticulin as also an enhanced vascular pattern.
10. A chicken wire pattern of vascularity is characteristic of myxoid liposarcoma and oligodendroglioma.
11. Absence of reticulin fibers may be helpful in the diagnosis of epithelial neoplasia as well as Ewing's sarcoma of bone.

Solution

1. Foot's silver oxide solution: Add 20 drops of 40% sodium hydroxide to 20 mL, 10% silver nitrate. Dissolve the brown precipitate by adding strong (28%) ammonia water drop by drop with constant shaking until only a few granules remain. About 2 mL (theoretically 1.7 cc) is required. Dilute to 80 mL with distilled water; use once. This solution should be freshly made in acid clean glassware. This solution gets reduced by the aldehyde groups of reticulin in tissue to a lower oxide (which is dark brown in color).
2. Potassium permanganate solution:
   - Potassium permanganate: 0.25 g
   - Distilled water: 100 mL.

   Potassium permanganate is used for presilvering for purposes of oxidation. Acidified potassium permanganate is used by Gordon and Sweet (1936). About 4% chromic acid or 0.5% periodic acid may also be used.
3. Oxalic acid solution:
   - Oxalic acid: 5 g
   - Distilled water: 100 mL
   - Oxalic acid is used for bleaching to remove potassium permanganate.
4. Silver nitrate solution:
   - Silver nitrate: 2 g
   - Distilled water: 100 mL.

   This is used as a 'sensitizer' instead of which uranium, $FeCl_3$ or iron alum may also be used. These sensitizers are used as mordants as they also have an oxidizing effect.

5. Formalin solution:
   - Neutral formaldehyde (AR) 37–40%: 5 mL
   - Distilled water: 95 mL
   - Formalin reduces the lower oxide (brown in color) to metallic silver oxide (black). Sodium sulfite or hydroquinone can also accomplish this.
6. Gold chloride solution:
   - Gold chloride solution 1%: 1 mL
   - Distilled water: 99 mL.

   Gold toning is a useful step. In untoned sections, the background is yellowish because of colloidal metallic silver, which is annoying to the eye. Toning removes the silver and replaces it with gold chloride. This is reduced to metallic gold by sodium metabisulfite or thiosulfate giving a pale gray background, which is pleasing to the eye. Toning is essential to differentiate woven bone from lamellar bone as it polarizes well. Toning also enhances counterstaining, if such a counterstain as nuclear fast red is used.
7. Sodium thiosulfate solution (0.5% aqueous): In this stain, sodium thiosulfate has two uses. In the first step, it removes excess of mercury if mercurial fixatives are used. In the last step, however, it removes excess of unreduced silver or gold and prevents further oxidation and does not permit any further reaction. It also reduces gold chloride to metallic gold.

Method

- Bring sections to water
- Treat with 0.5% aqueous $Na_2S_2O_3$ for 5 minutes (skip if mercurial fixatives are not used)
- Wash in distilled water
- Treat with 0.25% potassium permanganate solution for 5 minutes, wash in distilled water
- Treat with 5% oxalic acid for 15 minutes, wash in distilled water
- Treat with silver nitrate solution for 30 minutes, wash in distilled water quickly
- Expose to Foot's silver oxide solution for 20 minutes, quickly wash in distilled water
- Treat with 5% formalin [analytical reagent (AR)] for 2 minutes, wash in distilled water
- Treat with gold chloride solution for 3 minutes
- Fix in 5% $Na_2S_2O_3$ solution for 2 minutes
- Dehydrate, clear and mount.

Gold toning can be totally deleted for routine purposes as gold chloride is extremely expensive.

Results

Reticulin appear dark violet to black. If nuclear stain such as nuclear fast red or hematoxylin is used then nuclei stain is red or blue.

Precautions with reticulin stain

1. The high alkalinity of silver solutions tends to be traumatic to sections and therefore, detaches the imperfectly made or fixed sections from the slides. This may be overcome by:
   - Fixing sections well to albuminized slides
   - Keeping sections prior to staining in the incubator at 60°C for at least 2 hours
   - Celloidinizing the mounted sections by using 1% celluloidin after deparaffinizing, blot the excess stain.

   Note: Take 1 g of celluloidin to which add 40 mL of absolute alcohol and allow to stand for 48 hours in a stoppered bottle. This procedure softens the celluloidin. Add 60 mL of anhydrous ether; this dissolving procedure may take a few days.
2. Sections should be well spread of uniform thickness and from well-processed tissues.
3. Use freshly prepared solution.
4. All salts used in this method and other methods should be of AR quality.
5. Foot's silver oxide solution should also be well filtered using Whatman filter paper to avoid precipitation of silver on the sections.
6. The atmosphere should be dust free as any dust particles will precipitate silver.
7. Always use distilled water throughout the staining procedures.
8. All glasswares, especially that used in the preparation of silver solutions should be washed in 10% nitric acid and then washed in several changes of distilled water.
9. A silvered flask or a silver paper coating of containers for silver solutions may be used to avoid oxidation. These solutions should be colorless and not black or brown at the time of usage.
10. Use alcohol alone for dehydration to get good results.

Explosive Hazard

In the preparation of the commonly used silver impregnation solutions, various chemical reactions occur. With aging or exposure of ammoniacal silver solutions to air or light, shiny black crystals of explosive silver compounds, e.g. 'fulminating silver', silver nitride (Ag_3N) and silver azide (AgN_3) are formed. Violent explosions may occur, while removing a stopper, throwing a solution down a sink or even when holding it up to light. In order to avoid this:

1. All ammoniacal silver solutions should be prepared fresh just before use.
2. Any used solutions should be inactivated by adding excess of sodium chloride solution or dilute HCl.

Other Silver Stains and Applications

Silver stains have varied applications in histopathology, they stain not only reticulin fibers but also neuroendocrine granules, microorganisms as well

as have applications in renal biopsies. This depends on their unique ability to precipitate metallic silver, which is subsequently reduced to give a black color, for example, fungi have polysaccharides in their cell walls, which on oxidation release dialdehyde groups. These aldehyde groups besides being stained by the Schiff's reagent, can also be demonstrated by their ability to reduce methenamine silver nitrate in an alkaline solution. The Grocott's alkaline methenamine silver nitrate solution represents a vehicle, which upon reduction, precipitates nascent silver ions, thus blackening the site. Similarly, spirochetes also have the capacity to bind to silver, which can be reduced by other agents to black silver.

Similarly, the 'argentaffin reaction' is the ability of a silver complex solution to blacken a tissue element without the need of a reducing bath. The term is adjectival and is applied to many methods. The term 'argentaffin reaction' should therefore not be used as a proper name. It is most commonly used to describe the brown-black granularity observed in the carcinoid tumors. The tumors arise from the Kulchitsky cells or neuroendocrine cells. The cells manufacture 5-hydroxytryptamine (5-HT) from tryptophan. When 5-HT is produced in excess, it may lead to peculiar changes in the right heart and lungs. It is also associated with the excretion in the urine of large amounts of the degradation products of 5-HT, i.e. 5-hydroxyindoleacetic acid (5-HIAA). Reserpine reduces the 5-HT content of the enterochromaffin cells.

Probably by virtue of the content of 5-HT or a precursor substance, which may be bound to protein, the enterochromaffin cells give a number of histochemical reactions. They reduce ammoniacal silver solutions by virtue of the phenolic structure of 5-HT or its precursor, i.e. they are argentaffin, e.g. midgut carcinoids. They are also argyrophilic, i.e. they are impregnated by silver solutions when an artificial reducer is employed, e.g. foregut and hindgut carcinoids. The carcinoids arising in the common sites, i.e. appendix and small intestine almost always react and are argentaffin. Those arising in the unusual sites, e.g. rectum, stomach and bronchus more frequently fail to give argentaffin reaction, but are argyrophilic. Examples of silver stains used:

1. Grimelius stain (argyrophil stain) shows positive granules in carcinoid tumors are foregut and hindgut carcinoids (bronchial, stomach, first part of duodenum, esophagus, pancreas, transverse colon last third, descending colon and rectum).
2. Argentaffin stain (refer Masson's-Fontana silver stain)—midgut carcinoids, i.e. second part of duodenum onwards to first two third of transverse colon.
3. Gomori's methenamine silver stain for fungus, e.g. *Pneumocystis jirovecii*.
4. Gridley's silver stain, which stains fungus and bacteria (e.g. C*hlamydia, Klebsiella granulomatis*).
5. The PAS-methenamine silver stains for basement membrane in kidney.
6. Levaditi's stain for spirochetes.
7. Warthin starry sky stain for *Helicobacter pylori (H. pylori)*.

Grimelius/Argyrophil stain

- Fixation: Bouin's or formalin
- Section: Paraffin embedded.

Reagents

1. Silver solution:
   - Acetate buffer pH 5.6: 10 mL
   - Double glass distilled water: 87 mL
   - 1% aqueous silver nitrate (fresh): 3 mL.
2. Reducing solution:
   - Hydroquinone: 1 g
   - Sodium sulfite crystals: 5 g
   - Distilled water (freshly prepared): 100 mL.

Preparation of acetate buffer

Preparation are of two ways:

1. Acetic acid 1.2 mL:
   - Distilled water 100 mL.
2. Sodium acetate 2.7 g:
   - Distilled water 100 mL.

Mix 9 mL of solution type 1 + 91 mL of solution type 2 and adjust pH to 5.6.

Procedure

1. Bring sections to distilled water.
2. Transfer sections to preheated silver solution at 60°C for 3 hours.
3. Drain silver solution from slides thoroughly.
4. Place the sections in freshly prepared reducing solutions at 45°C for 1 minute.
5. Rinse sections in distilled water.
6. Counterstain, if required (light green 0.5% aqueous is recommended).
7. Wash in tap water.
8. Dehydrate to graded alcohols.
9. Clear in xylene and mount in dibutyl phthalate in xylene (DPX).

Results

- Argyrophil islet cells: Positive (brown-black)
- Carcinoid cells: Brown to black
- Other argyrophil cells: Brown to black.

Note:

1. If a weak positive reaction is maintained the result can be improved by double impregnation. After the reducing stage the sections are treated with 5% sodium thiosulfate for 2 minutes, rinsed in distilled water and placed in freshly prepared silver solution at room temperature for 10 minutes. This is followed by fresh reducing solution for 1 minute at 45°C.
2. Counterstains are not normally used.

Masson-Fontana silver staining technique for melanin and argentaffin

Principle

Argentaffin cells exhibit the property of reducing ammoniacal silver solution to metallic silver. Melanin also reduces solutions of ammoniacal silver nitrate to black metallic silver.

Procedure

1. Bring sections to water.
2. Immerse slides in Fontana-silver solutions for 1 hour at 56–58°C in a Coplin jar covered with aluminum foil or overnight at room temperature. The sections appear light brown.
3. Rinse well in distilled water.
4. Tone in gold chloride for 2 minutes.
5. Place in 5% aqueous sodium thiosulfate for 2 minutes.
6. Wash thoroughly in tap water followed by distilled water.
7. Counter stain with aqueous neutral red 0.5%.
8. Rinse in distilled water.
9. Dehydrate, clear and mount in DPX.

Control

Skin is taken as the control for the technique.

Application

1. To show the presence of argentaffin granules or traces of melanin in tumor.
2. To demonstrate 5-HT in certain endocrine cells of the epithelium of the stomach and small intestine, known enterochromaffin cells.

Disadvantage

Ammoniacal silver solutions are potentially explosive, if stored incorrectly (store in a refrigerator and use within 4 weeks). Thoroughly, clean glasswares used for silver solutions as explosive silver compounds might be formed with residues left on glassware.

Fixation

Formalin is best; chromate and mercuric chloride fixatives to be avoided.

Ingredients

Stock 10% silver nitrate solution:

- Silver nitrate AR grade: 10 g
- Distilled water: 100 mL.

Take solution in a glass flask. Use a finely pointed dropping pipette and add concentrated ammonia drop by drop, constantly agitating the flask until the formed precipitate dissolves—end point is faint opalescence. To this add 20 mL of triple distilled water and then filter into a dark bottle.

Result

- Melanin pigment: Black
- Other elements: According to the counterstain used.

SPECIAL STAINS FOR MICROORGANISMS

Microorganisms encountered in routine pathology specimens include bacteria, fungi, protozoa and viruses. Several histochemical stains help in identifying these organisms.

Stain for Bacteria

Gram Stain

The Gram stain is used to stain both bacillary and coccal forms of bacteria.

Principle

Gram stain is based on whether they take up the Gram stain or not. Bacteria that have large amounts of peptidoglycan in their walls retain the methyl violet stain, i.e. they are Gram positive, whereas those who do not retain the color have more lipids and lipopolysaccharides in their cell walls and are termed as Gram negative. It consists of initial staining with crystal/methyl violet. This is followed by Gram's or Lugol's iodine, which acts by allowing the crystal violet to adhere to the walls of Gram-positive bacteria. Decolorization with an acetone and alcohol mixture washes away the methyl violet, which is not adherent to bacterial cell walls. At this stage, Gram-positive bacteria stain blue, while the Gram-negative bacteria are colorless. A carbol fuchsin, a counterstain is then applied, which stains the gram-negative bacteria pink.

Control

Gram-positive and -negative organisms on the tissues harvested from the microbiological plates.

Applications

The most basic application is to differentiate bacteria into Gram-positive and -negative bacteria. When the Gram-positive bacteria die, they become Gram negative.

Advantages

Gram stain enables identification of organisms causing lung abscesses, wounds, septicemic abscesses or meningitis.

Reagents

- 1% crystal violet or methyl violet
- Gram's or Lugol's iodine made up of iodine and potassium iodide.

Method

1. Make a thin smear of the clinical specimen/culture on a clean grease-free glass slide.
2. Air dry and heat, fix the smear.
3. Slide is placed on the staining rack and is flooded with crystal violet for 1 minute.
4. Wash and stain with Gram's iodine for 1 minute.
5. Wash with tap water, decolorize with acetone-alcohol mixture for 2–3 second.
6. Wash with tap water, stain with dilute carbol fuchsin for 30 second.
7. Wash with tap water and air dry.

Results

- Gram-positive organisms: Blue-black
- Gram-negative organisms: Red.

Stains for Mycobacteria Bacilli (Table 8.3)

Ziehl-Neelsen Stain (Carbol Fuchsin Acid and Alcohol Stain)

Mycobacteria cannot be stained by the Gram stain, because it is an aqueous stain that cannot penetrate the lipid-rich waxy mycobacterial cell walls. Mycobacteria have large amounts of a lipid called mycolic acid in their cell walls, which stain by carbol fuchsin as well as resist decolorization by acid alcohol. The latter property is responsible for the commonly used term 'acid-fast bacilli' (AFB). When these organisms die, they lose their fatty capsule and consequently their carbol fuchsin positivity.

Principle

The mycobacterial cell walls are stained by carbol fuchsin. The organisms stain pink with the basic fuchsin, a component of carbol fuchsin. Staining is followed

TABLE 8.3: Comparative grading and magnification correction factor*

Comparative grading		
RNTCP† ZN‡ staining grading (using 100X oil immersion objective and 10X eyepiece)	**Auramine O fluorescent staining grading (using 20X or 25X objective and 10X eyepiece)**	**Reporting/Grading**
> 10 acid-fast bacilli (AFB) per field after examination of 20 fields	> 100 AFB per field after examination of 20 fields	3+
1–10 AFB per field after examination of 50 fields	11–100 AFB per field after examination of 50 fields	2+
10–99 AFB per 100 field	1–10 AFB per field after examination of 100 fields	1+
1–9 AFB per 100 field	1–3 AFB per 100 field	Doubtful positive/repeat
No AFB per 100 fields	No AFB per 100 fields	Negative
Magnification correction factor		
Fluorescent microscopy (FM) objective magnification (power)		**Magnification correction factor**
20		10
25		10
40		5
45		4
63		2

*To obtain the comparative grading divide the observed count of AFB with the FM objective; †RNTCP, Revised National Tuberculosis Control Program; ‡ZN, Ziehl-Neelsen.

by decolorization in acid alcohol; mycobacteria retain the carbol fuchsin in their cell wall (acid-fast) whereas other bacteria do not retain carbol fuchsin. Counterstaining is carried out by methylene blue. Care has to be taken not to overcounterstain, as this may mask the AFB.

Reagents

- Carbol fuchsin:
  - Basic fuchsin: 0.5 g
  - Absolute alcohol: 5 mL
  - 5% aqueous phenol: 100 mL.
- Acid alcohol:
  - Hydrochloric acid: 10 mL
  - 70% alcohol: 1,000 mL.
- Methylene blue (stock) solutions:
  - Methylene blue: 1.4 g
  - 95% alcohol: 100 mL.
- Methylene blue (working) solutions:
  - Methylene blue (stock): 10 mL
  - Tap water: 90 mL.

Method

Deparaffinize and rehydrate through graded alcohols to distilled water. Pour carbol fuchsin solutions for 30 minutes. Staining is aided by the application of heat. Wash well in tap water. Differentiate in acid alcohol until solutions are pale pink (2–5 dips). Wash in tap water for 8 minutes and then dip in distilled water. Counterstain in working methylene blue solutions until sections are pale blue. Rinse in tap water then dip in distilled water; dehydrate, clear and mount.

Results

Mycobacterial, hair shafts, Russell bodies, mast cell granules, fungal organisms and Splendore-Hoeppli bodies around *Actinomyces* red and background pale blue.

Note: Decalcification using strong acids can destroy acid fastness, formic acid to be used.

Modified Fite Method for Mycobacterium leprae and Nocardia

The Fite stain is used for staining of *Mycobacterium leprae (M. leprae),* which has cell walls that are more susceptible to damage in the deparaffinization process.

Principle

The avoidance of solvents (fat-dissolving agents) such as alcohol and xylene helps to conserve this fragile fatty capsule. The Fite procedure thus includes peanut oil in the deparaffinization solvent to protect the bacterial cell wall. The acid used for decolorization in the Fite procedure is also weaker and generally 0.5 or 1% aqueous H_2SO_4 solution is used. Xylene and peanut oil—one part oil for two parts of xylene for deparaffinization.

Reagents

- Carbol fuchsin:
  - Basic fuchsin: 0.5 g
  - Absolute alcohol: 5.0 mL
  - 5% aqueous phenol: 100 mL.

  Mix well and filter before use with filter paper.
- 0.5% H_2SO_4 in 25% alcohol or in water (aqueous H_2SO_4):
  - 25% ethanol: 95 mL
  - Sulfuric acid, concentrated: 5 mL.
- Methylene blue (stock) solutions:
  - Methylene blue: 1.4 g
  - 95% alcohol: 100 mL.
- Methylene blue (working) solutions:
  - Methylene blue (stock): 5 mL
  - Tap water: 45 mL.

Method

Deparaffinize in two changes of xylene and peanut oil, 6 minutes each. Drain slides vertically on paper towel and wash in warm running tap water for 3 minutes (the residual oil preserves the sections and helps accentuate the acid fastness of the bacilli). Pour carbol fuchsin solutions for 25 minutes (solvent may be reused). Wash well in tap water for 3 minutes. Drain excess water from slides on paper towel. Differentiate with 0. 5 or 1% sulfuric acid in 25% alcohol or water, two changes of 1.5 minutes each (do not allow the slides to dry between carbol fuchsin and acid alcohol; sections should be pale pink). Wash in tap water for 5 minutes. Counterstain in working methylene blue solutions, one quick dip (do not overstain the slide sections should be pale blue). Blot sections and dry at 50–55°C oven for 5 minutes. Once dry, one quick dip in xylene. Mount with permanent mountant.

Results

- The AFB including *M. leprae:* Bright red
- Nuclei and other tissue elements: Pale blue.

Auramine-Rhodamine Fluorescence Method

Purpose

To detect the presence of *Mycobacterium tuberculosis (M. tuberculosis)* or other acid-fast organism.

Principle

The exact mechanism of this stain is unknown. Both the dyes used are basic dyes that fluoresce at short wavelengths. Both dyes are used in combination to yield better staining than either dye alone. The mycolic acid in the cell wall of the bacteria has affinity for these fluorochromes. Rhodamine alters the color from the yellow of auramine alone to a reddish or golden yellow.

Fixative

About 10% NBF is preferred. Slides should be scratch free.

Control
A section containing AFB must be used.

Specimen handling
In case of solid or semisolid material, an aqueous suspension of the material is made by taking a small amount of the material and suspending it in a drop of distilled water on a microscope slide. A smear is made, which should not be too thick. In the case of liquid media, a drop is taken directly for smearing. The smear made by either method is air dried and then 'fixed' by passing rapidly through a Bunsen burner flame for two to three times. The smear is allowed to cool before staining.

Reagents
Auramine O and Rhodamine B solution:
- Auramine O: 1.5 g
- Rhodamine B: 0.75 g
- Glycerol: 75 mL
- Phenol crystals: 10 mL (liquefied at 50°C and dissolved by shaking at room temperature in 50 mL distilled water).

Procedure
1. Place the slides on a staining rack, with the smeared side facing up, the slides not touching each other.
2. Flood the slides with freshly filtered auramine-phenol.
3. Let stand for 7-10 minutes and wash well with running water, taking care to control the flow of water, so as to prevent washing away the smear.
4. Decolorize by covering completely with acid-alcohol for 2 minutes, twice. Wash well with running water, as before to wash away the acid alcohol.
5. Counterstain with 0.1% potassium permanganate for 30 seconds.
6. Wash as before with water and slope the slides to air dry.

Precautions
1. Avoid under decolorization with acid-alcohol. Organisms that are truly acid-fast are difficult to overdecolorize since the decolorization procedure with acid alcohol is relatively milder than the 25% sulfuric acid used in Ziehl-Neelsen (ZN) staining procedure.
2. Avoid making thick smears. This will interfere with proper decolorization and counterstain may mask the presence of AFB. Additionally, thick smears have a tendency to flake, resulting in loss of smear material and possible transfer of material to other slides.
3. Strong counterstain may mask the presence of AFB.
4. Smears that have been examined by fluorescent microscopy (FM) may be restained by ZN staining to confirm observations. To restain the same smear for ZN, treat with 5% oxalic acid for 2 minutes, wash and proceed for ZN. However, once smears have been stained by ZN staining, they cannot be used for FM.
5. Fluorescent stained smear are to be read within 24 hours of staining because of fading.

6. Stained smears have a tendency to fade on exposure to light. The slides are to be stored in the slide box to avoid exposure to light. Alternatively, they may be stored wrapped in brown or black paper and kept away from light.

Note: With auramine staining, the bacilli appear as slender bright yellow fluorescent rods, standing out clearly against a dark background:

1. Rule out any artifacts. Grade positive smears into 4° of positivity using the 20X, 25X objective along with 10X eyepiece.
2. Smear needs to be observed in 'linear pattern'. For a trained and experienced laboratory technician (LT), each smear would take approximately minimum of 2 minutes for 100 fields or three horizontal sweeps.
3. In the fluorescent staining, smears are examined at much lower magnifications (typically 250X) than used for ZN-stained smears (1,000X).
4. Each field examined under fluorescence microscopy, therefore, has a larger area than that is seen with bright field microscopy. Thus, a report based on a fluorochrome-stained smear examined at 250X may contain much larger numbers of bacilli than a similar report from the same specimen stained with carbolfuchsin and examined at 1,000X.
5. For the purpose of uniformity for examination and quantitative reporting of results, a method has been suggested (refer WHO Manual on Microscopy Part II) whereby the number of AFB observed under fluorochrome staining could be divided by a 'magnification correction factor' to yield an approximate number that might be observed if the same smear were examined under 1,000X after carbol fuchsin stain. To adjust for altered magnification of fluorescent microscope, when using objectives of 20X or 25X powers, divide the number of organisms seen under FM by the factor of 10.
6. Similarly, if one using a 40X objective the magnification correction factor is 5, and if one using a 45X objective it is 4.

Method

Deparaffinize in two parts xylene and one part peanut oil (two changes each of 3 minute). Drain and blot to opacity. Place slides in Auramine O and Rhodamine B solution in a glass Coplin jar and stain for 10 minutes at 50°. Rinse sections for 2 minutes in tap water. Differentiate sections in two changes of 0.5% acid alcohol for 1½ minutes each (for *M. leprae* use 0.5% aqueous HCl). Rinse sections for 2 minutes in tap water. Differentiate for 2 minutes in 0.5% $KMNO_4$—quenches fluorescence of tissue cells. Wash in tap water for 2 minutes. Blot dry or stand slides on end and thoroughly air dry (for *M. leprae* sections should be mounted in glycerol or liquid paraffin). In case of *M. tuberculosis,* sections may be dehydrated in absolute alcohol (not more than 10 second) cleared quickly in xylene and mounted with a synthetic resin (Harleco fluorescence mountant). Examine sections with a high-dry objective, a undergravel (UG)1 or UG2 exciter filter, and a colorless ultraviolet (UV) barrier filter.

Results
Acid-fast organisms golden-yellow, background black. Slides can be screened on high power (400X) and verified under oil immersion. Staining times vary to suit one's preferences. Switch on the mercury vapor lamp. The bulb takes approximately 10 minutes to reach full intensity. Using the low power objective (magnification 100–150X) first examine a known positive slide to ensure that the microscope is correctly set up.

Silver Stains (Warthin-Starry Stain)

Applications
Silver stains are very sensitive for the staining of bacteria and are reserved for visualizing spirochetes, *Legionella, Bartonella* and *H. pylori. Treponema pallidum* or spirochete causes syphilis. These organisms are infrequently seen in biopsies and a primary chancre is usually diagnosed clinically. The causative organism is seen using dark ground (field) microscopy and is an 8–13 μm corkscrew microorganism, with a kink in the center. Modified Steiner method and Dieterie method or Warthin-Starry stains are used for staining spirochetes. *Leptospira interrogans* is also a spirochete (13 μm) with curled ends and causes Weil's disease. This can also be visualized by the above stains. *Legionella pneumophila* is a Gram-positive organism causing pneumonia. *H. pylori* associated with chronic gastritis is very well visualized by these silver stains. The Gram-negative organism (bacteria), *Afipia felis* and *Bartonella henselae* causing catscratch disease are also demonstrated by the silver stains.

Principle
Spirochetes and other bacteria can bind silver ions from solution, but cannot reduce the bound silver. The slide is first incubated in a silver nitrate solution for half-an-hour and then 'developed' with hydroquinone, which reduces the bound silver to a visible metallic form.

Reagents

Solutions

1. Acetate buffer, pH 3.6:
   - Sodium acetate: 4.1g
   - Acetic acid: 6.25 mL
   - Distilled water: 500 mL.
2. 1% silver acetate nitrate in pH 3.6 acetate buffer.
3. Developer: Dissolve hydroquinone in 10 mL, pH 3.6 buffer and mix 1 mL of this solution, and 15 mL of warmed 5% scotch glue or gelatin; keep at 40°C. Take 3 mL of 2% silver nitrate in pH 3.6 buffer solutions and keep at 55°C. Mix the two solutions immediately before use.
4. Gold chloride 0.2% (optional).

Method
Deparaffinize and rehydrate through graded alcohols in distilled water. Celluloidinize in 0.5% celloidin, drain and harden in distilled water for 1 minute. Impregnate in preheated 55–60°C silver solution for 90–105 minutes.

Prepare and preheat developer in a water bath. Treat with developer (solution) for 3½ minutes at 55°C. Sections should be golden brown at this point. Remove from the developer and rinse in tap water for several minutes at 55–60°C, then in buffer at room temperature. Tone in 0.2% gold chloride; dehydrate, clear and mount.

Results

- Spirochetes: Dark brown to black
- Background: Golden yellow.

Stains for Fungi

Most fungi that infect the subcutaneous and horny layers of skin and hair shafts belong to *Microsporum* and *Trichophyton* groups and appear as yeasts or mycelia forms. These can be readily demonstrated with the common special stains such as Gomori's methenamine silver, Gridley's fungus and PAS, also referred to as 'broad-spectrum' fungal stains. The fungal stains such as AB and Mayer's or Southgate's mucicarmine that readily demonstrate the mucoid capsule of *Cryptococcus neoformans (C. neoformans)* can be termed as 'narrow-spectrum' stains for fungi. This staining reaction differentiates *C. neoformans* from other fungi of similar morphology such as coccidiodes, *Candida* and *Histoplasma.*

When these fungi grow in tissue, they display asexual forms and appear as spherical yeast or spore forms. Some grow as tubular hyphae that may be septate and branched. A mass of interwoven hyphae is called fungal mycelium. Rarely, spore-producing fruiting bodies called sporangia or conidia are produced.

Grocott's Methenamine Silver Stain for Fungi

Principle

Most fungal cell wall is rich in polysaccharides, which can be oxidation to dialdehydes. The Grocott's alkaline methenamine silver nitrate solution represents a vehicle, which upon reduction by the dialdehydes, precipitates nascent silver ions, thus blackening the site.

Advantages

Grocott's methenamine silver (GMS) is preferred for screening, because it gives better contrast and stains even degenerated, and non-viable fungi that are sometimes refractory to H&E. The GMS also stains algae, intracytoplasmic granular inclusions of cytomegalovirus (CMV), *Actinomyces israeli, Nocardia, Mycobacterium* and non-filamentous bacteria with polysaccharide capsules such as *Klebsiella pneumoniae* and *Streptococcus pneumoniae.* Prolonged staining in the silver nitrate solution may be required to adequately demonstrate degenerated fungal elements such as the yeast-like cells of *Histoplasma capsulatum (H. capsulatum).*

Disadvantages

The stain masks the natural color of pigmented fungi, making it impossible to determine whether a fungus is colorless, hyaline or pigmented. Such a

determination is crucial in the histological diagnosis of mycosis. The GMS does not adequately demonstrate the inflammatory response to fungal invasion.

Reagents

- Stock methenamine-silver nitrate solution:
  - Silver nitrate, 5% solution: 5 mL
  - Methenamine, 3% solution: 100 mL.

Add the silver nitrate to the methenamine solution, shaking until the precipitate, which first forms and dissolves. This mixture will keep for 1–2 months at 4°C:

- Working methenamine-silver nitrate solution:
  - Borax 5% solution: 5 mL
  - Distilled water: 25 mL
  - Mix and add methenamine-silver nitrate (stock solution): 25 mL.
- Stock light green solution:
  - Light green, SF: 0.2 g
  - Distilled water: 100 mL
  - Glacial acetic acid: 0.2 mL.
- Working light green solution:
  - Light green (stock solution): 10 mL
  - Distilled water: 50 mL.

Method

Bring sections to distilled water. Oxidize with 5% aqueous chromic acid for 1 hour. Wash in water for a few seconds. Treat sections with 1% sodium metabisulfite 1 minute. Wash in running tap water 5 minutes. Rinse thoroughly in distilled water. Place in preheated working silver solution in a water bath at 56°C for 30–40 minutes until section turns yellowish brown (the incubation time is variable and depends upon the type and duration of fixation; may also be 1–3 hours at 37–45°C). Rinse well in distilled water. Tone sections with 0.1% gold chloride for 4 minutes. Rinse in distilled water. Treat sections with 3% $Na_2S_2O_3$ for 5 minutes (to remove unreacted silver). Wash with running tap water 5 minutes. Counterstain in working 1% light green in 0.1% acetic acid for 15–30 seconds or H&E stain. Rinse excess light green off slide with alcohol; dehydrate, clear and mount.

Results

- Fungi such as *Pneumocystis jirovecii, Histoplasma:* Black
- Inner parts of mycelia and hyphae: Old rose
- *Leishmania* and *Toxoplasma:* Negative
- Mucin: Dark gray
- Red blood cells (RBCs): Yellow
- Background: Pale green.

Gomori's technique is similar to that of Grocott's; in fact the latter is an adaptation of the Gomori's technique with a difference in incubation times.

Gridley's Stain

Advantage: Same as GMS.

Principle
Gridley's stain procedure uses chromic acid to oxidize adjacent glycol groups to aldehydes and the aldehydes then react with Schiff's reagent. Since chromic acid is a stronger oxidizing agent than periodic acid, it further attacks and destroys aldehydes, so fewer reactive groups are left to react with the Schiff's reagent. A less intense reaction is obtained than with the PAS technique, but background staining is also decreased.

Reagents
- About 4% chromic acid solution
- Lillie's cold Schiff's solution
- Sulfite rinsing solution (used to remove excess leukofuchsin, i.e. to remove non-specific background reaction products)
- Gomori's aldehyde-fuchsin solution
- About 0.25% metanil yellow/light green.

Method
Bring the sections to water. Oxidize with 4% chromic acid. Wash in running tap water. Treat sections with Schiff's reagent for 15–20 minutes. Give two sulfite rinses. Wash in water for 15 minutes. Treat the sections with Gomori's aldehyde-fuchsin solution for 15–20 minutes. Rinse in 95% alcohol. Wash in water for 5 minutes. Counterstain with 0.25% metanil yellow/light green.

Results
- Mycelia: Deep blue
- Conidia: Deep rose to purple
- Background: Yellow
- Elastic tissue and mucin: Deep blue.

Rickettsia is also stained. Rickettsial infection causes Q fever, rocky mountain fever or typhus and rarely needs to be demonstrated on tissue sections; can be seen with Giemsa stain.

Staining for Identification of Virus

It is difficult to identify virus under light microscope, but the outlines of virus can be made under electron microscope. Some viruses aggregate within cells to produce viral inclusion bodies that may be both intranuclear and extranuclear, and can be stained with Feulgen reaction. These intranuclear inclusion bodies (e.g. CMV) are acidophilic and impart an owl's eye appearance to the nucleus. The extranuclear inclusion bodies can be basophilic and cytoplasmic, and are PAS positive. Special staining methods are:

1. Feulgen reaction.
2. Modified trichromes using contrasting acid and basic dyes to exploit the differences in charges on the inclusion body and host cell, e.g. Mann's methyl blue-eosin stain for Negri bodies of rabies, phloxine-tartrazine technique for viral inclusions, Shikata's orcien for hepatitis B surface antigen and Macchiavello stain.

Staining for Protozoan Organism

The identification can be made on morphological appearance using H&E and Giemsa stains. The availability of antisera against organism such as *Entamoeba histolytica, Toxoplasma gondii, Leishmania tropica* has made diagnosis much easier in difficult cases.

STAINS FOR PIGMENTS AND MINERALS

Both endogenous and exogenous pigments are seen in routine biopsies.

Common Pigments and Deposits

Exogenous Pigments

- Carbon black (in lungs and associated lymph nodes, especially of city dwellers and coal miners)
- Inks used for tattoos (skin)
- Silica
- Asbestos
- Lead
- Silver.

Fixation artifacts and their removal

- Formalin pigment: Dark brown and granular
- Mercury pigment: Black
- Chromic oxide.

Formalin pigments (artifactual pigments)

Formalin pigments also called acid hematin is formed after several weeks in specimens by the interaction of acidic or alkaline formaldehyde solutions with blood. This dark brown pigment, a product of degradation of hemoglobin, settles out as an insoluble product and can be identified by its presence extracellularly. This artifact is not formed in a NBF solution (refer Chapter 1).

Mercury pigment

Mercury pigment deposits occur in all tissues fixed in liquids containing mercuric chloride including 'B5', Heidenhain's solution and Zenker's fluid. They occur as uniformly distributed brown-black extracellular crystals. The material is removed by brief treatment of sections with alcoholic iodine solution followed by treatment with $Na_2S_2O_3$ to remove the brown iodine stain. It is however advisable not to use iodine solutions prior to staining with Gram method, as they are dissolved out during the procedure.

Reagents: As follows:

- Lugol's iodine 1 g, potassium iodide 2 g and distilled water 100 mL
- 5% aqueous $Na_2S_2O_3$.

Method: Bring sections to water. Place in Lugol's iodine for 15 minutes. Wash in water. Place in thiosulfate for 3 minutes. Wash in water. Stain with H&E or other technique.

Malarial pigment (hemozoin)
Malarial pigments are formed within RBCs that contains the malarial parasite. They can be seen inside the macrophages if infected RBCs are ingested. They can be seen in Kupffer's cells of the liver, sinus lining cells of the lymph node and spleen, and within phagocytic cells in the marrow.

Extraction of malarial pigments: This can be removed with saturated alcoholic picric acid, it requires 12–24 hours for complete treatment.

Reagents: Picric acid (saturated in alcohol) is of 50 mL.

Procedure: Bring sections to water. Place the sections in alcoholic picric acid. Rinse sections in 90% alcohol. Rinse sections in 70% alcohol. Place sections in tap water. Stain with H&E or other routine stain. Alternatively, 10% ammonium hydroxide can be used.

Chromic oxide
These are brownish black granules, which are the result of alcohol treatment following chrome fixation. Such pigment cannot be removed, therefore chrome fixed tissue must be washed in running water for 12–18 hours immediately following fixation. This washing removes excess chromates and then the tissue can be safely dehydrated in alcohol.

Non-hematogenous Endogenous Pigments

- Melanins: Brown to black
- Melanosis coli (lipofuscin-like deposits in colonic mucosa of habitual users of anthraquinone purgatives): Brown
- Lipofuscin (in older people, in cardiac muscle cells, neurons, etc.): Yellow to light brown
- Chromaffin: Pheochromocytoma
- Dubin-Johnson pigment
- Hamazaki-Weisenberg bodies.

Melanins
Melanins are brown to black or yellowish polymeric pigments formed from the amino acid tyrosine by oxidation with tyrosinase [dihydroxyphenylalanine (DOPA) oxidase] in skin cells, hair, eyes (retina, iris and choroid) and in the cell bodies of some neurons, notably in the substantia nigra and locus coeruleus of the brainstem. In skin, melanocytes, which are the branched cells at the junction of the epidermis with the dermis, synthesize the pigment and package it into protein-containing granules called melanosomes. Pathologically, melanin is found in the cells of malignant melanomas and various benign nevus tumors derived from melanocytes.

Demonstration and staining of melanin
Melanin can be indirectly demonstrated by bleaching it with an aqueous solution of either hydrogen peroxide or potassium permanganate allowed to act for 12–24 hours. They are insoluble in organic solvents and soluble in normal sodium hydroxide. Bleaching of sections is also done when cell morphology is obscured by excess melanin pigment. By removing the pigment,

cell nuclear detail is better seen. Histochemical staining of melanin is required when amelanotic melanomas are to be diagnosed or to differentiate minimal pigment from other pigments such as hemosiderin. It is accomplished by exploiting the chemical-reducing properties of this pigment. Two methods are commonly used, i.e. the Masson-Fontana silver method and Schmorl's ferric-ferricyanide reaction. Formalin-induced fluorescence shows a yellow color due to certain aromatic amines such as 5-HT, epinephrine and histamine useful particularly in amelanotic melanomas.

Removal of melanin pigments (bleach)

Principle: Melanin can be bleached by strong oxidizing agents such as potassium permanganate. The H&E stain is done after the bleaching procedure for each patient.

Control: The H&E without bleaching procedure.

Reagents: As follows:

- 0.25% aqueous potassium permanganate
- 5% aqueous oxalic acid.

Method: Deparaffinize and hydrate to distilled water. Oxidize in permanganate solution for 30 minutes. Wash in water and bleach in oxalic acid until white (oxalic acid removes potassium permanganate besides its bleaching action). Proceed as usual to H&E.

Results: Skin melanin is bleached within 30 minutes, ocular melanin takes 2–4 hours.

Schmorl's reaction

Principle: Melanin has the ability to reduce ferricyanide to ferrocyanide, which in the presence of ferric ions forms Prussian blue. This reaction is also seen with lipofuscins, bile and neuroendocrine cell granules.

Control: Melanin pigment in skin biopsy.

Fixation: About 10% NBF.

Reagents: As follows:

- 1% aqueous $FeCl_3$ (freshly prepared)
- 0.4% aqueous potassium ferricyanide (freshly prepared).

Working solution: To 30 mL of 1% $FeCl_3$ add 4 mL of 0.4% aqueous potassium ferricyanide, then add 6 mL of distilled water. Use within 30 minutes.

Method: Deparaffinize and hydrate to distilled water (control and test). Treat with working solution for 10 minutes and wash in running water for several minutes to ensure that all the residual ferricyanide is completely removed from the section. Counterstain with 0.5% aqueous nuclear red for 5 minutes. Dehydrate rapidly in alcohols; clear and mount.

Result: As follows:

- Melanin, argentaffin cells, chromaffin cells, lipofuscins, bile, colloid: Dark blue
- Nuclei: Red.

Enzyme methods

DOPA oxidase (tyrosinase) method for tissue blocks

The most specific method of all, is an enzyme histochemical method called DOPA oxidase. It requires frozen sections for best results, but paraffin sections of well-fixed tissues may also be used. The procedures for both are different.

Principle: The stain works because the DOPA substrate used here is acted upon by the enzyme DOPA oxidase in the melanin-producing cells to produce a brownish black deposit. The enzyme catalyzes the oxidation of tyrosine to DOPA and its final oxidation to melanin pigment.

pH buffer 7.4: Dissolve 42.8 g sodium cacodylate and 9.6 mL HCl in 1 liter of distilled water (sodium cacodylate is a better buffer than phosphate buffer, which may also be used).

Primary fixative: Formalin 10% in the pH 7.4 buffer to which is added 0.44 sucrose (tissue is fixed for 2–3 hour).

Incubating media: DOPA 0.1% in the pH 7.4 buffer.

Method: Fix two pieces of the tissue for test in the primary fixative for 3 hours at 4°C. Rinse in cold (4°C) pH 7.4 buffer for 5 minutes. Incubate one piece of tissue in the buffered DOPA solution for 16–20 minutes at 37°C. The other piece of tissue (negative control) is incubated in the buffer only at 37°C for the same time as the test. Wash both tissues in distilled water for 5 minutes and then fix in a conventional 10% formalin solution for 1–2 days. Paraffin process in the usual way. Cut sections at 10 μm and mount on poly-L-lysine coated slides. Lightly counterstain with Mayer's hemalum for 2 minutes. Wash the blue sections in tap, dehydrate, clear and mount.

Results: As follows:

- Cells with DOPA oxidase: Brown
- Nuclei: Blue.

Formaldehyde-induced fluorescence

Formaldehyde-induced fluorescence can be used to highlight biogenic amines (chromaffin, dopamine, epinephrine, norepinephrine and argentaffin) and melanin in tissues. Formalin fixation imparts a strong yellow autofluorescence to unstained tissues containing these substances.

Method: Frozen sections are fixed in 10% buffered formalin for 5 minutes, dehydrated and placed in xylene; then mounted in the media, i.e. fluorescent free and examined using a fluorescent microscope with BG38, UG1 and a barrier filter. The best results are seen using tissue that has been freeze-dried (snap frozen) and then fixed using paraformaldehyde vapors.

Hematogenous Pigments

- Hemosiderin (in cells that have phagocytized blood; liver in diseases of iron metabolism): Dark yellow to brown
- Bile pigments
- Porphyrins.

Staining for hemosiderin

Hemosiderin is an aggregate of proteins (ferritin)-containing ferric hydroxide. It is an intracellular granular yellow to brown pigment seen in the mitochondria (siderosomes) of histiocytes. Stainable ferric iron is normally found in the bone marrow and in the spleen. Pathologically, it is seen at times in old hemorrhage, intramuscular injections, endometriotic cysts and in hemochromatosis. Commonly ferrous iron is of little interest and may be demonstrated by the Tirmann's method, where 20% potassium ferricyanide and 2% HCl in equal parts are mixed and the sections exposed to this mixture for 1 minute. The results are similar to the Perls' reaction.

Perls' Prussian blue stain

Perls' Prussian blue stain is also called Prussian blue reaction and Turnbull's blue method. This method demonstrates only ferric iron and not ferrous iron.

Historical note: The characteristic blue-green color developed by this reaction is called Prussian blue after the uniform worn by the Prussian army. Prussia was a sovereign state established in the 13th century and become a kingdom in 1701 and is associated with Frederick the Great and Bismarck. At its height, it covered the area of North Germany and parts of Poland. It was formerly dissolved after World War II (1939–1945). Berlin was the capital.

Principle: Ferric iron combines with potassium ferrocyanide to form ferric-ferrocyanide, which gives a bright blue color (precipitate).

Control: Tissue with areas of old hemorrhage.

Advantages: Some of the pathological conditions with hemosiderin deposits are hemorrhages of any kind can be diagnosed hemolytic anemia, some liver diseases, the lungs in congestive heart failure and in the liver, pancreas and skin in hemochromatosis. It is usually seen in enzymes such as peroxidase, cytochrome oxidase and myoglobin.

Reagents: Perls' solution:

- 2% aqueous potassium ferrocyanide: 25 mL
- 2% aqueous HCl: 25 mL.

Method: Bring sections to rinse in distilled water. Transfer to Perls' solution. Rinse in distilled water. Counterstain with 1% nuclear fast red. Rinse in tap water and bring to mountant.

Results: As follows:

- Hemosiderin: Blue
- Nuclei: Red.

Note: As follows:

1. At stage five H&E method may be used instead of nuclear fast red.
2. Perls' stain should be fresh and filtered.
3. Use only AR grade chemicals. All glassware should be cleaned with distilled water to avoid extraneous ferric iron contamination.
4. Use known control slides.
5. Where excessive iron is present, the timing will need to be reduced.

Staining for hemoglobin

Hemoglobin is a basic conjugated protein bound to globin and is the red pigment component, responsible for the transportation of oxygen and carbon dioxide. Heme is composed of protoporphyrin, a substance built up from pyrrole rings and combined with ferrous iron. Two types of demonstration methods are used. The first one demonstrates the enzyme, hemoglobin peroxidase, by benzidine-nitroprusside method, but because of the carcinogenicity of benzidine, these methods are rarely used. The other method is the tinctorial method using amido black technique.

Staining for bile pigments

Staining for bile pigments include both conjugated and unconjugated bilirubin, biliverdin and hematoidin, all of which are chemically distinct and show different physical properties. They are particularly seen in obstructive jaundice patients due to blockage in the normal flow of bile from the liver into the gallbladder. In H&E staining, bile is stained as yellow-brown globules. It needs to be distinguished from lipofuscin, which stains similarly. Lipofuscin is autofluorescent, while bile pigments are not. Hematoidin appears as a bright yellow pigment within the old hemorrhagic areas and in old splenic infarcts, where it contrasts well against the pale gray of the infarcted tissue.

Fouchet's technique

The most commonly used routine method is the modified Fouchet's technique.

Principle: The pigment is converted to the green color of biliverdin and blue cholecyanin by the oxidative action of the $FeCl_3$ in the presence of trichloracetic acid.

Formula: Fouchet's solution:

- 25% aqueous trichloroacetic acid: 36 mL
- 10% aqueous ferric acid: 4 mL freshly prepared before use
- van Gieson: Dissolve 100 mL of 1% acid fuchsin (aqueous) in 100 mL of saturated aqueous picric acid.

Method: Take test and control (distilled water). Treat with the freshly prepared Fouchet's solution for 10 minutes. Wash well in running water for 1 minute. Rinse in distilled water. Counterstain with van Gieson's solution for 2 minutes. Dehydrate, clear and mount in synthetic resin.

Results: As follows:

- Bile pigments: Emerald to blue-green
- Muscle: Yellow
- Collagen: Red.

Staining for porphyrins

The porphyrias are rare pathological conditions that are disorders of the biosynthesis of porphyrins and heme. In erythropoietic protoporphyria, the pigment is seen as focal dark-brown pigment and under polarized light shows a bright red color with a centrally located dark Maltese cross on liver sections, while on frozen section, exhibit brilliant red fluorescence that rapidly fades with exposure to UV light.

Endogenous Elements/Minerals

Calcium

Calcium is present in hydroxyapatite, the insoluble mineral of bones and teeth. Abnormal deposits of calcium phosphate or carbonate can be associated with necrotic tissue in lesions of atherosclerosis, infarction, Gamna-Gandy bodies, malakoplakia of the bladder (Michealis-Gutmann bodies), hyperparathyroidism, nephrocalcinosis, sarcoidosis, tuberculosis and in some tumors. Calcium phosphate crystals can form in the cartilage of joints in a condition known chondrocalcinosis or pseudogout. Calcium salts are monorefringent, but calcium oxalate is birefringent. There are many ways to stain calcium, but only two methods are routinely used in histopathology. They are the von Kossa and alizarin red S technique.

Modified von Kossa's method for calcium

The classic method for the demonstration of calcium and certain other salts in tissues is that of von Kossa (1901).

Principle: It indirectly demonstrates calcium in combination with the anion of phosphate, carbonate and oxalate; it does not stain calcium directly. Silver compounds are formed with phosphates and carbonate anions reduction to metallic silver is achieved by light or hydroquinone.

Control: Calcium containing tissue.

Reagents: As follows:

- 1% aqueous silver nitrate
- 2.5% $Na_2S_2O_3$
- 1% neutral red or van Gieson's picrofuchsin.

Method: Deparaffinize and hydrate to distilled water and rinse well in distilled water. Place in silver nitrate solution and expose to strong light (60 watt bulb at a range of 4–5 inch) for 10–60 minutes. Wash in three changes of distilled water. Treat with $Na_2S_2O_3$ for 5 minutes. Wash well in distilled water. Counterstain with neutral red for 3 minutes; dehydrate, clear and mount.

Results: As follows:

- Mineralized bone: Black
- Osteoid: Red.

This method demonstrates phosphate and carbonate radicals, giving good results with both large and small deposits of calcium. This method is not specific as melanin will also reduce silver to give black deposits. As a general rule, fixation of tissues containing calcium deposits is best using non-acidic fixatives such as NBF, formol alcohol or alcohol.

Alizarin red S method for calcium

Principle: Alizarin red S gives more reliable results with small deposits and is said to be specific for calcium salts at pH 4.2.

Control: Calcium in tissue.

Reagent: As follows:

- 1% aqueous Alizarin red S adjusted to pH 4.2 or 6.3–6.5 with 10% ammonium hydroxide
- 0.05% fast green in 0.2 % acetic acid.

Method: Bring sections to 95% alcohol. Allow the slides to air dry thoroughly. Place sections in a Coplin jar filled with alizarin S solution for 5 minutes. Rinse quickly in distilled water. Counterstain with fast green for 1 minute. Rinse in three changes of distilled water; dehydrate, clear and mount in synthetic resin.

Results: As follows:
- Calcium deposits: Orange-red
- Background: Green.

Copper

Copper is an essential nutrient being a component of cytochromes and many oxidoreductase enzymes. Pathologically, the accumulation of copper is associated with Wilson's disease. This is a recessively inherited metabolic disorder in which a transporter protein in liver cells fails to move copper into the bile and fails to combine copper with ceruloplasmin, the copper-binding protein of blood plasma. Copper associated with albumin and other proteins accumulates in cells of the liver, cornea and corpus striatum of the brain in patients with Wilson's disease. Copper accumulations are seen also in primary biliary cirrhosis and some other liver disorders.

Two reagents are suitable for histochemical demonstration of copper by virtue of reactions employing the oxidative catalyst properties of copper. Dithiooxamide (also known as rubeanic acid) gives a stable dark green polymeric product that can be mounted in a resinous medium.

Modified rhodamine method

Principle: P-dimethylaminobenzylidene rhodanine (DMABR) gives a red product that dissolves in organic solvents and therefore requires an aqueous mounting medium.

Control: Liver with positive material.

Reagents: Rhodamine stock solution:
- 5-p-DMABR: 0.05
- Absolute ethanol: Prepare 25 mL freshly and filter prior to use
- Working solution: Take 5 mL of the stock solution and add to 45 mL of 2% sodium acetate trihydrate
- Borax solution: Disodium tetraborate 0.5 g and distilled water 100 mL.

Method: Take test and control sections to water. Incubate in the rhodamine working solution at 56°C for 3 hours or overnight in 37°C. Rinse in several changes of distilled water for 3 minutes. Stain in acidified Lillie-Mayer or other alum hematoxylin for 10 seconds. Briefly rinse in distilled water and place immediately in borax solution for 15 seconds. Rinse in distilled water. Mount with Apathy's mounting media.

Results: As follows:
- Copper deposits: Red to orange
- Nuclei: Blue
- Bile: Green.

Uric acid and urates

Uric acid is breakdown product of the body's purine (nucleic acid) metabolism, but a small proportion is obtained from the diet. The uric acid is excreted by the kidneys. The uric acid circulating in the blood is in the form of monosodium urate, which is high in patients of gout forming a supersaturated solution. These high levels result in urate depositions, which are water soluble in tissues causing subcutaneous nodular depositions of urate crystals 'tophi', synovitis, arthritis, renal disease and calculi.

Chondrocalcinosis or pseudogout on the other hand is a pyrophosphate arthropathy, which results in calcium pyrophosphate crystals being deposited in joint cartilage of large joints, e.g. knee. With a polarizing microscope, pyrophosphate crystals exhibit positive birefringence and urate crystals exhibit negative birefringence.

Lithium carbonate extraction—hexamine silver technique

Principle: Uric acid and urates are soluble in water; so much of the material will be lost during normal aqueous fixation and subsequent processing. Fixation in 95% alcohol is recommended as it gives a more specific reaction.

Reagent solutions: As follows:

- Grocott's hexamine silver solution
- Saturated aqueous lithium carbonate solution
- 2% aqueous $Na_2S_2O_3$
- 0.2% aqueous light green in 0.2% acetic acid.

Method: Take a test and a control sections to 70% ethyl alcohol. Place the sections in aqueous solution of lithium carbonate for 30 minutes. Rinse all sections in distilled water. Place all sections in hexamine silver solution for 1 hour in distilled water. Wash sections in distilled water. Treat sections with $Na_2S_2O_3$ for 30 seconds. Counterstain with light green solution for 1 minute. Wash in water, dehydrate, clear and mount in synthetic resin.

Results: As follows:

- Extracted sections: Urates are only extracted
- Unextracted sections: Urates and pyrophosphates are blackened
- Background: Green.

Note: Alternatively, 10% ammonium hydroxide can be used.

PRACTICAL APPLICATIONS OF HISTOCHEMICAL TECHNIQUES

Certain stains are employed for optimal evaluation along with morphological evaluation.

Tumor Diagnosis

Special stains can be used for identifying the metastatic tumors from the primary tumors.

Adenocarcinoma

The PAS and mucicarmine are used to identify mucin-producing tumors, e.g. salivary and pancreatic tumors. In carcinomas of the cervix and prostate, the

neoplasms secrete acidic mucins and the normal epithelium secretes neutral mucins. The van Gieson stains bile a bright green and helps to differentiate hepatocellular carcinoma with intracellular pigment from metastatic adenocarcinoma in the liver with no pigment.

Neuroendocrine Tumors

The Grimelius stain identifies argyrophilic and helps to diagnose carcinoids, while argentaffin tumors will be Masson-Fontana positive. The chromaffin reaction is positive in pheochromocytoma, but negative in paraganglioma and chemodectomas.

Mesenchymal Tumors

Glycogen [PAS-diastase (D)] is positive in Ewing's sarcoma, while it is negative in lymphomas. All myxoid stromal tumors show positive staining with AB.

Microorganisms

Gram stain is used for bacterial identification, AFB for tuberculous bacilli, Fite-Faraco stain for lepra bacilli, GMS is used for the staining of fungi, Warthin-Starry for *H. pylori* and Levaditi stains spirochetes.

Biopsies

Lymph Node Biopsy

Reticulin stain helps to differentiate follicular hyperplasia and follicular lymphomas. In lymphomas, the reticulin around the follicle is compressed (also refer topic on reticulin stain). Absence of reticulin fibers is seen in metastatic carcinoma. The PAS highlights the vascular basement membrane in Castleman's disease.

Liver Biopsy

Masson's trichrome is helpful in identifying and grading fibrosis, while orcein is used for hepatitis B surface antigen and copper-associated protein.

Kidney Biopsies

Periodic Schiff-methenamine silver (PASM) is excellent in outlining basement membrane wrinkling, reduplication, tram-track appearance and differentiates sclerosis and hyalinosis.

Gastrointestinal Biopsy

The PAS, AB-PAS and HID are used in the evaluation of gastrointestinal biopsies in intestinal metaplasia and neoplasia.

Bone Marrow

Reticulin is widely used for fibrosis, Perls' stain for hemosiderin and methyl green pyronin for plasma cells and immunoblasts. Reticulin-free areas are noted in metastatic carcinoma.

Metabolic Diseases

Oil red-O is widely used in diagnosing various diseases such as gangliosidosis, e.g. Tay-Sachs disease.

Applications of Pigment Demonstration

1. **Autofluorescence/DOPA reaction:** It is used in diagnosing amelanotic melanomas. An amelanotic melanoma is a tumor with no pigment, but possesses the enzyme tyrosinase.
2. **Lipofuscin:** It is a yellow-brown and reddish-brown pigment found within cells in many parts of the body. The nickname 'wear-and-tear' pigment reflects the accumulation of lipofuscin with advancing age in cells that are either terminally differentiated (e.g. cardiac muscle fibers and neurons) or are infrequently replaced (e.g. adrenal cortex and liver). The pigment is formed from fragments of membranous organelles, which become permanently sequestered in lysosomes. This accumulation of lipofuscin does not appear to interfere with cellular function. Pathologically, lipofuscin is present in the neuronal ceroid lipofuscinoses, a group of several rare inherited disorders within the large category of lysosomal storage diseases. Lipofuscins are demonstrated by PAS stain and Schmorl's ferric-ferricyanide reduction test.
3. **Chromaffin:** This pigment is seen as dark brown granular material in the cells of adrenal medulla in physiological and pathological conditions. Orth's or other dichromate fixatives are recommended. Chromaffin can be demonstrated by Schmorl's reaction, Nile Blue, Masson-Fontan and PAS techniques.
4. **Dubin-Johnson pigment:** It is found in the liver of patients with Dubin-Johnson syndrome and is due to defective canalicular transport of bilirubin. It is characterized by a brownish-black granular and intracellular pigment situated in the centrilobular hepatocytes. The true nature of this pigment is yet to be established. It is related to lipofuscin and demonstrated by the PAS stain.
5. **Pseudomelanosis (melanosis coli pigment):** It is seen in macrophages in the lamina propria of the large intestine and appendix. It is an endogenous lipopigment, whose reactions are those of typical ceroid type lipofuscin.
6. **Hamazaki-Wesenberg bodies:** These are small, yellow-brown spindle-shaped structures found mainly in the sinuses of lymph node, lying freely as cytoplasmic inclusions seen in patients of sarcoidosis.

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Chapter 9 Special Biopsies

KIDNEY BIOPSY

Indications

1. Diagnostic:
   a. Asymptomatic proteinuria 1 g/day.
   b. The recurrent-isolated hematuria and proteinuria when other investigations are not contributory.
   c. Acute nephritis with persisting oliguria.
   d. Nephrotic syndrome (NS), child below 1 year, familial NS and adult with NS.
   e. Acute renal failure without cause.
   f. Systemic disease: Diabetes mellitus complications by renal failure, systemic lupus erythematosus, polyarteritis nodosa and Wegener's granulomatosis.
   g. Kidney transplant: Cellular/humoral rejection, e.g. acute tubular necrosis and glomerulonephritis.
2. Prognostic:
   a. Follow-up of glomerulonephritis.
   b. Assess effect of steroids or immunosuppression in glomerulonephritis or nephritic syndrome.
3. Monitor the progress of disease.
4. Aid in selection of treatment.

Contraindications

1. Absolute:
   - Solitary kidney.
2. Relative:
   - Unconscious patient
   - Severe bleeding diathesis
   - Hypertension
   - Acute renal failure
   - Polycystic kidney
   - Renal artery aneurysm.

Procedure

Percutaneous Needle Renal Biopsy

Site: Posterior lower pole of kidney is chosen.

Needle: Vim-Silverman needle (Fig. 9.1) and Travenol Tru-Cut needle are preferable to take two cores, one for light microscopy, and other for immunofluorescence and electron microscopy. Avoid crushing, use wooden sticks/pipettes instead of needles to handle the liner piece of tissue. If biopsy kept on a Petri dish, cover with gauze moistened with tubuloreticular inclusions (TRIs)/phosphate buffered saline (PBS). When the biopsy is received in the laboratory, check for presence of glomeruli immediately. The core can be examined in a fresh state under 10X objective and glomeruli are recognized as rounded structures in the linear piece of tissue. A linear piece of tissue may also be sectioned at its tip for electron microscopy.

Adequacy of the specimen: Minimum of 5–10 glomeruli. Three cores may be submitted separately for:

1. **Paraffin processing in 10% buffered formalin:** Two or three thin sections are taken for hematoxylin and eosin (H&E) stain, one section each for periodic acid-Schiff (PAS), Jones methenamine silver stain and Masson's trichrome stain.
2. **Cryostat sections for immunofluorescence:** IgG, IgA, IgM, C3, C1q, fibrinogen, κ- and λ-light chains.
3. **Fixed 2–3% glutaraldehyde for electron microscopy:** To detect placement of immune complexes, epithelial cell change and basement membrane morphology.

Staining: Sections 2–3 μm thick:

1. **Hematoxylin and eosin:** General overview, preferred stain for morphology and type of cells. Minimum of 6–7 glomeruli should be present to call it an adequate biopsy. For allograft biopsies 10 glomeruli should be present.
2. **Periodic acid-Schiff:** Glomerular and tubular basement membrane, mesangial matrix, brush border of proximal tubular epithelial cells, nodules of amyloid, Kimmelstiel-Wilson (KW) syndrome and hyalinosis of focal segmental glomerulosclerosis (FSGS).
3. **Periodic acid-silver methenamine (PASM):** Glomerular basement membrane and spikes, splitting, thickening, rounded mesangial regions in nodules of diabetes are silver positive and show layering/lamination of the mesangial substance (sclerotic lesions of FSGS are nonargyrophilic); good for photography.
4. **Masson's trichrome:** Interstitial fibrosis, glomerulosclerosis, arteriosclerosis and protein deposits.

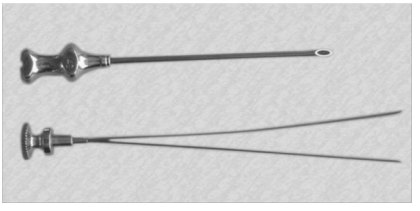


Figure 9.1: Vim-Silverman needle

5. **Congo red:** For amyloid with apple-green birefringence.
6. **Verhoeff's stain:** Fragmentation of elastic fibers in malignant nephrosclerosis.

LIVER BIOPSY

Liver biopsy plays an important role in diagnosing liver lesions along with clinical, biochemical and radiological parameters. Liver biopsy is a safe procedure and though with limitations, remains the choice of investigation for many hepatic disorders, both for primary treatment and monitoring the disease.

Indications

- Liver parenchymal dysfunction
- Cholestatic jaundice
- Portal hypertension
- Hepatomegaly
- Pyrexia of unknown origin (PUO) or metabolic disorders
- Liver abnormalities discovered during laparotomy
- Part of staging procedure in lymphoreticular disease.

Types of Biopsy

- Needle biopsy (Fig. 9.2)
- Wedge biopsy (open biopsy).

Procedure

Biopsy can be done under direct peritoneoscopy control or radiologically guided, especially for focal or uneven lesions. Whenever open wedge biopsy is taken, it is also preferable to take one or multiple needle biopsies simultaneously as subcapsular region in wedge biopsy may show non-specific changes or fibrosis and may not be representative of the liver as a whole.

Fixation: Fix immediately in 10% buffered formalin or alcohol for suspected glycogen storage disorder. Buffered glutaraldehyde for electron microscopy. Fix overnight.

Fixation artifacts: Delay in fixing gives rise to swelling of liver cells in the central part of the specimen; outer layer of liver cells may give unusual staining reactions.

Adequacy: A minimum length of 2–2.5 cm is necessary for needle cores. About four portal tracts should be present in the core for diagnosis of chronic hepatitis and biliary sclerosis.

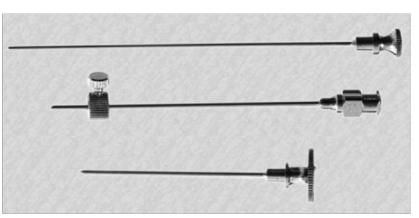


Figure 9.2: Menghini needle

Gross appearance: External surface in wedge biopsy may give information about nodularity or tumor.

Color of biopsy: It gives some idea as follows:

- Pale: Fatty change
- Green: Cholestasis
- Brown: Hemochromatosis
- Brown-black: Dubin-Johnson syndrome
- Slate gray: Malaria.

Sectioning: Step cut sections are preferable. Keep two to three unstained slides for further studies.

Staining: The following are must for a meaningful interpretation of the liver biopsy:

1. Routine H&E: Three sections.
2. Reticulin: For this network, type 3 collagen—black.
3. Masson trichrome: For fibrosis, type 1 collagen—green or blue (Fig. 9.3), more sensitive and preferred as compared to van Gieson stain. Section shows blue fibers, collagen type 1 around hepatocytes in early fibrosis of a fatty liver.
4. The PAS and PAS-diastase:
   - Not resistant—glycogen
   - Resistant—nonglycogen
   - α-1-antitrypsin deficiency globules—red or violet.

Other stains used are:

1. **van Gieson stain:** Fibrosis is pink-red (Masson's trichrome is more sensitive stain for early fibrosis). Bile is stained green.
2. **Prussian blue:** For iron, hemosiderin pigment—blue.
3. **Shikata stain:** For hepatitis B surface antigen (HbsAg), brown; homogeneous intracytoplasmic copper-associated protein as brown-black granules and elastic fibers black.
4. **Rhodanine/Rubeanic acid for copper:** Red and green respectively.
5. **Orcein:** For copper—green.
6. **Fouchet stain:** Bile pigments—emerald green.
7. **Grocott's methenamine silver (GMS):** Acid-fast bacilli (AFB) and Gram's stain for organisms.
8. **Fat stains:** Frozen sections to demonstrate fat.
9. **Immunohistochemistry:** The immunoperoxidase and immunofluorescence are used for HbsAg and hepatitis B core antigen (HbcAg).

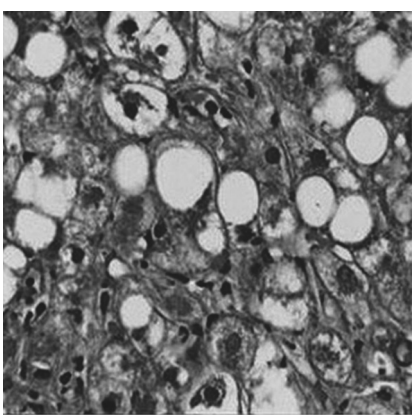


Figure 9.3: Section shows blue fibers, collagen type 1 around hepatocytes in early fibrosis of a fatty liver.

MUSCLE BIOPSY

A muscle biopsy is a surgical procedure to obtain a muscle tissue and is relatively simple and poses little risk to the patient. However, it is a specialized procedure and therefore the tissue obtained must be subjected to proper processing and treatment for optimal information.

Indications

1. Inflammatory muscle disease before beginning treatment.
2. Proximal weakness of uncertain cause, as in floppy baby syndrome.
3. Hereditary myopathies and muscular dystrophies.
4. To exclude treatable disorders, e.g. polymyositis in whom motor neuron disease is suspected.
5. Suspected metabolic myopathies, particularly in patients with muscular cramps, stiffness or tenderness.
6. Autoimmune vasculitis, especially polyarteritis nodosa and even in the absence of muscular symptoms.
7. Other systemic disorders, e.g. sarcoidosis and infestations.
8. To assess the effect of steroid treatment in the management of polymyositis, particularly in relation to the development of steroid myopathy.
9. Occasionally, in carrier detection in female siblings of Duchenne dystrophy.
10. Diagnosis of malignant hyperpyrexia.
11. Research purpose, e.g. exercise physiology, pathological, immunological studies, etc.

Types of Biopsy

- Needle biopsy
- Percutaneous conchotome technique (modified needle biopsy technique)
- Open biopsy technique (can be done under local/general anesthesia depending upon the patient's age and condition).

Procedure

A moderately affected muscle should be selected for biopsy. Generally, quadriceps femoris, deltoid or biceps brachii are preferred for biopsy because no disability is likely to result from the biopsy and normal histological features of these muscles are well-understood. The muscle selected should be actively involved, but not terminally involved by the disease process. The sites of intramuscular injection or electromyographic needling must be avoided as both cause local destruction of fibers.

While selecting the site of incision, the surgeon should avoid the ends of muscle near tendinous attachments. In an open biopsy, the size of the biopsy should ideally be 2 cm in length and 1 cm in diameter. During the

procedure, it is tied with thread at either ends and cut on either side with the help of sharp blade. The piece of muscle is moistened with one drop of normal saline and placed in a Petri dish. It is desirable that the biopsy reaches the laboratory within 4–6 hours. The muscle biopsy is processed in three different ways:

1. Fixed in 10% buffered formalin and submitted for paraffin processing. The paraffin sections are stained by H&E, phosphotungstic acid hematoxylin (PTAH) and Masson's trichrome stain. Other special stains such as PAS for glycogen are employed depending on the necessity.
2. Fresh unfixed muscle is snap frozen in isopentane cooled by liquid nitrogen (–170°C) and sections are cut in cryostat at –18°C to –20°C. These sections are stained by histochemical stains for demonstration of enzyme activity. The enzymes commonly used are nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), succinic dehydrogenase (SDH); adenosine triphosphatase (ATPase) at pH 9.4, 4.6 and 4.3, and phosphorylase.
3. If facilities permit, a small biopsy can be processed for electron microscopic examination after fixation in 2–3% glutaraldehyde.

Muscle fibers of a single motor unit are not congregated into a group, but are randomly distributed among fibers of other motor units. All muscle fibers of a single unit are of the same fiber type. The typical checkerboard or mosaic pattern of normal human muscle is the result of intermingling of type 1, 2A, 2B and occasionally 2C fibers of different motor units.

Characteristics

Light microscopic characteristics to be noted. Size and shape of myofiber, position of nuclei, degenerative changes such as hyalinization, necrosis and myophagocytosis, vacuolar changes such as ring fibers, regenerative activity, increased endomysial and perimysial connective tissue are important diagnostic criteria.

Amongst the histochemical characteristics, one has to note the pattern of fiber type distribution in the fascicles, involvement of particular fiber type in the pathological process, predominance of any one fiber type or presence of grouping of fibers of one histochemical type. Alteration of individual muscle fibers such as presence of mitochondria, cores or vacuoles and target appearances provide important clue to diagnosis.

Principles of Enzyme Histochemistry

1. Histochemical methods rely on the fact that enzymes located in thin (6–8 μm) frozen sections of muscle fibers can be chemically reacted with certain products in order to visualize the activity of the enzyme.
2. First, a substrate (fuel) is provided for the enzyme to be studied. Second, an energy source is provided that allows the enzyme to utilize the substrate. Finally, a reaction product is linked to another product that forms a precipitate, so it can be visualized microscopically.

3. Histochemical methods can be used to classify muscle fibers into fast or slow, oxidative or nonoxidative and glycolytic or nonglycolytic.
4. Histochemical enzyme technique on cryostat sections of muscle biopsies provides a better opportunity for defining fiber types and detecting any alteration in muscle fibers. Muscle biopsies are useful in diagnosing denervating disease through myofiber type atrophy or groupings; certain dystropic fibers are deficient in type 2B fibers, which is also detectable with enzyme histochemistry procedures.

Normal Muscle

Type 1 muscle fibers constitute less than 55% of the fibers in a biopsy and type 2 muscle fibers less than 80%. Predominance of type 1 or type 2 fibers is said to occur, if these figures are exceeded. But, relative predominance of the different fiber types, is to some extent, an inherited characteristic. The pattern of fiber types can be studied by subjecting the sections to:

1. ATPase reaction at pH 9.4, 4.6 and 4.3 based on their pH liability: Type 1 fibers are base labile and acid stable, whereas type 2 fibers possess the reverse property. Types 2B and 2C fibers display activity in solutions containing a wider pH range than the 2A fibers.
2. NADH–TR.
3. Myosin ATPase.

This also differentiate fibers into type 1 and type 2. Histochemical pattern in a normal muscle is illustrated in Table 9.1 and Figures 9.4A to F.

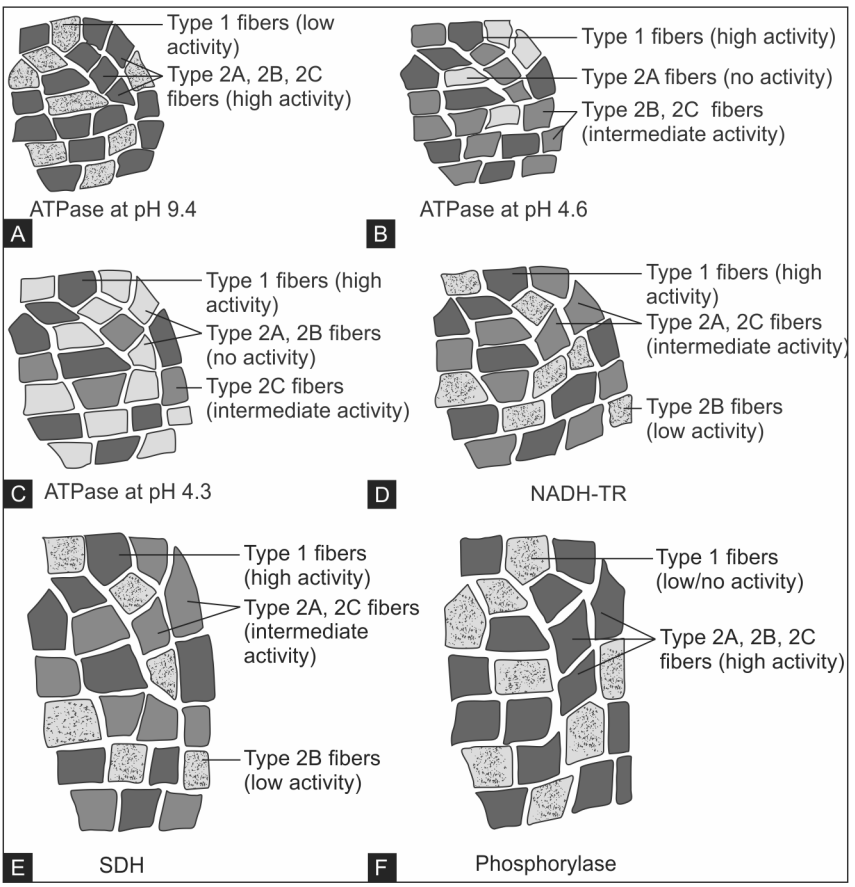
NERVE BIOPSY

Nerve biopsy is an investigation carried out to rule out any pathological condition affecting peripheral nervous system or investigation used for confirmation of tissue. Biopsies to evaluate peripheral neuropathies are employed to establish the diagnosis, to develop a plan of treatment to determine prognosis or to provide information for genetic counseling and for identifying interstitial abnormalities for documenting the extent and chronicity of axonal and Schwann cell involvement in diffuse polyneuropathies.

TABLE 9.1: Histochemical pattern of normal muscle

Muscle fiber type	1	2A	2B	2C
Routine ATPase* (pH 9.4)	+	+++	+++	+++
ATPase (pH 4.6)	+++	0	++	++
ATPase (pH 4.6)	+++	0	0	++
NADH–TR†	+++	++	+	++
SDH‡	+++	++	+	++
Phosphorylase	+/0	+++	+++	+++

Note: 0: No reaction; +: Low activity; ++: Intermediate activity; +++: High activity.
*ATPase, adenosine triphosphate; †NADH–TR, nicotinamide adenine dinucleotide-tetrazolium reductase; ‡SDH, succinate dehydrogenase.



Figures 9.4A to F: Histochemical reaction in human muscle (ATPase, adenosine triphosphatase; NADH-TR, nicotinamide adenine dinucleotide-tetrazolium reductase; SDH, succinate dehydrogenase).

Indications for the Following Clinical Presentations

1. **Acute ascending neuropathy:** Guillain-Barré syndrome, diphtheria neuropathy, toxic polyneuropathy (e.g. organophosphates and arsenic), paraneoplastic neuropathy and vascular neuropathy.
2. **Subacute sensorimotor neuropathy:** Vitamin deficiencies, alcohol neuropathy, toxic neuropathy, paraneoplastic neuropathy, diabetic neuropathy, sarcoidosis and vasculitis.
3. **Chronic peripheral neuropathies:** The chronic inflammatory demyelinating polyradiculoneuropathy, porphyria, toxic neuropathies, vitamin deficiency neuropathies, diabetic neuropathy, uremic neuropathy, diabetic neuropathy, collagen vascular disease, immunocyte-derived amyloidosis, leprosy and hereditary neuropathies.

4. **Hereditary peripheral neuropathies:** The abetalipoproteinemia and Tangier disease, hereditary amyloid neuropathy, Charcot-Marie-Tooth disease, Dejerine-Sottas disease, Refsum disease and adrenoleukodystrophy.
5. **Mononeuropathy or multiple neuropathies:** Primary or metastatic tumors, vasculitis, diabetes mellitus, sarcoid, leprosy, shingles, Lyme disease, acquired immune deficiency syndrome (AIDS), postherpetic neuralgia or traumatic nerve injury.

Major Criteria for Selection of a Nerve for Biopsy

1. It should be purely sensory nerve. It should not have any motor function as it carries the risk of inducing motor deficits.
2. It should have predictable anatomical course and fascicular arrangement.
3. It should be distal nerve.

The most accessible sensory nerve for biopsy is the sural nerve, located behind the ankle. Other nerves that can be biopsied are superficial peroneal nerve, saphenous nerve, superficial radial nerve for polyneuropathies of upper extremities, index branch of radial cutaneous nerve for leprous neuropathy, for motor neuropathy, motor branch of deep peroneal nerve and terminal branch of musculocutaneous nerve to the long head of biceps.

Procedure

After a dose of local anesthesia, the nerve is cut, which results in transient and sudden sharp pain. The major complications are bleeding, infection, scarring, permanent numbness on the outer side of the foot (for sural nerve) and the possibility of developing chronic pain at the site of biopsy. Clinical history and electrophysiological data are helpful both in planning and handling of biopsy, and in interpretation of the findings.

Fixing and transporting nerve biopsies: Specimen is transported in damp gauze and fixed in 10% buffered formalin, and in 4% glutaraldehyde for EM. A small piece of fresh nerve may be snap frozen for biochemical studies, lipid stains and immunofluorescence studies. Care should be taken that nerve is not allowed to bend and do not grasp the nerve with forceps or manipulate the nerve excessively.

Adequacy: About 1–3 cm nerve is usually removed.

Processing of peripheral nerve biopsy: The five most commonly used methods for processing of peripheral nerve biopsy are:

1. **Resin (plastic embedding):** It is the most commonly used procedure. It provides information about the population of both small and large diameter myelinated fibers. It allows assessment of amount of myelin and its thickness. One bit cross section and one bit longitudinal sections are embedded.

2. **Routine H&E staining:** Allows identification of nerve and is the most efficient method to screen focal interstitial lesions such as vasculitis, granulomas and amyloid. It has a limited utility in assessing axonal loss segmental demyelination or myelination.
3. **Frozen section:** For confirmation of nerve, immunofluorescence, to evaluate lipids, metabolic disorders and biochemical analysis.
4. **Teased fiber analysis:** Allows assessment of the pattern of nerve disease along several internodes. It is most sensitive for detecting segmental myelin changes and internodal lengths.
5. **Electron microscopy:** Useful for the study of unmyelinated fiber, cytoplasmic organelles and storage materials. It is helpful in evaluation of autonomic neuropathies.

Examination of a peripheral nerve biopsy: Biopsy examination includes:

1. Assessment of fascicular anatomy and specimen quality.
2. Light microscopy:
   - Epineurium
   - Perineurium
   - Endoneurium.
3. Ultrastructural examination.
4. Immunohistochemical examination.
5. Fiber teasing and morphometry.

BRAIN BIOPSY

Indications

- Tumors of the central nervous system
- Neurodegenerative disorders
- Infections of the central nervous system are viral, fungal, etc.

Types

Types of biopsies taken at craniotomy are:

- Burr hole needle biopsy
- Stereotactic biopsy computed tomography (CT) guided
- Open brain biopsy in the case of tumor or other lesions.

Precautions

Precautions to be taken during the transport of biopsy specimens to the laboratory are:

1. The tissue should be sent in a fresh unfixed state to plan further investigations on the tissue, i.e. for immunocytochemistry and electron microscopic techniques as these are important adjuvants in the diagnosis.
2. The biopsy is placed on a sterile polythene sheet, which is wrapped over the sample by folding it. By doing this we can prevent drying of the

biopsy specimens, which are generally small and sticky. The smaller biopsy specimens can be safely transported in this manner.

Procedure

Brain biopsy may be divided or assorted for the following procedures depending upon the amount of tissue sent and the urgency of the biopsy report required:

1. Formalin fixation (bulk of the tissue) for light microscopy.
2. Freezing in liquid nitrogen for cryostat sections.
3. Glutaraldehyde fixation for electron microscopy.
4. Smear or squash preparation for rapid diagnosis of intracranial tumors.
5. Touch preparation.

Any undefined lesion may be biopsied and inspected by both cytological preparation and frozen sections. Cytology is carried out using the squash or touch technique.

Smear or squash preparation: This technique allows rapid sampling of several areas from a biopsy. All cell types are readily identified by this method. A small piece of tissue about 1 mm is placed at one end of a plain glass slide. A second glass slide is used to gently crush the specimen and is then drawn across the slide to produce a uniform smear. The two slides are held flat together during the smearing, maintaining a gentle and even pressure. Following smearing the slides are immediately fixed in acetic alcohol (95% alcohol and 5% glacial acetic acid).

Staining is routinely done with H&E. Aqueous toluidine blue can also be used. The technique is suitable for small pieces of soft tumors such as gliomas, soft meningiomas and metastatic tumors. Most centers use this as an intraoperative procedure for diagnosis.

Touch preparations: As follows:

1. Touch the surface of the tissue with a clean glass slide.
2. Air dry the smears and stain it with Giemsa stain.
3. The technique is valuable in the case of lymphomas and metastatic carcinomas for rapid diagnosis.

Formalin fixation: Tissue processing as follows:

1. Take measurements in two or three dimensions.
2. Fix the tissue in 10% buffered formalin for 1–2 hours followed by routine paraffin processing.
3. All tissue may be processed. If large quantities are sent then select representative tissue for processing.

Freezing with liquid nitrogen: The tissue is flash frozen in isopentane cooled by liquid nitrogen at -160°C following, which cryostat sections are cut. This is resorted for rapid diagnosis and for biochemical investigations.

Glutaraldehyde fixation for electron microscopy: A small sample of the tissue should be fixed in 2.5% glutaraldehyde for 4 hours, for ultrastructural examination.

ENDOMYOCARDIAL BIOPSY

Endomyocardial biopsy is indicated for early morphological detection, evolution of disease and to follow reversal following treatment.

Indications

1. Rejection in cardiac allograft recipients—diagnosis, grading and follow-up.
2. Anthracycline-induced cardiotoxicity—diagnosis and grading.
3. Acute myocarditis—diagnosis and follow-up.
4. Specific heart muscle disease—diagnosis and follow-up.
5. Idiopathic cardiomyopathy—diagnosis and confirmation.
6. Idiopathic chest pain or arrhythmias—diagnostic aid.
7. Differentiation of restrictive versus constructive heart disease.

Sites in Relation to Indication

- **Left ventricle:** Lesions producing pressure or volume overloading of left ventricle
- **Right ventricle:** Carcinoid and radiation injury
- **Either:** Hemochromatosis.

Types

1. Transthoracic needle biopsy of left ventricle.
2. Open thoracotomy.
3. Catheter needle biopsy—Stanford bioptome (Konno and Sakakibara).

Philip Caves in 1962 introduced more flexible, serial percutaneous transvenous biopsies under local anesthesia.

Complications

- Cardiac perforation
- Right pneumothorax
- Transient paralysis of right laryngeal nerve
- Horner's syndrome
- Refractory atrial arrhythmia
- Postpericardiotomy syndrome.

Procedure

Tissue handling and preservation: Handle the biopsy carefully, pick with needle point and do not use forceps. Immerse the tissue in glutaraldehyde or formalin.

Stains: The most commonly used are:

1. Hematoxylin and eosin.
2. Masson's trichrome: Fibrous tissue, myocytolysis and ischemia.

3. Perl's stain: Hemochromatosis.
4. Congo red: Amyloidosis.
5. Methyl green pyronin: Acute rejection.
6. Verhoeff's stain: Elastin.

Electron microscopy: Fixed in 2% glutaraldehyde and embedded in Epon; screen 5–10 blocks.

Frozen: Snap frozen using liquid nitrogen/isopentane. It is useful for immunohistochemistry (IHC)/immunofluorescence (IF)/in situ hybridization (ISH) studies.

Culture: For suspected myocarditis.

Heart Valves

Specimens received are:

- Entire ring with cusps, chordae with or without papillary muscles
- Piece meal.

In case of the heart valves the gross examination is very important as it reveals more than the microscopy. Hence, photography plays an important role in documenting findings. The aims of gross examination are to:

1. Document severity and etiology.
2. Detect unexpected pathology, e.g. endocarditis.
3. Validate the findings of newer imaging techniques.

Atrioventricular Valves

1. Take photographs to include both the superior and inferior aspects.
2. Description: Fibrosis or calcification, focal or diffuse, involving margins or one surface or both:
   a. Leaflets: Mobile or shortened and stretched or normal.
   b. Vegetations or perforations, if present, single or multiple, large or small.
   c. Commissures fused or not, and to what extent.
   d. Chordae tendinae rupture, shortened, calcified, fused or normal.
   e. If incompetent—it is due to valvular lesion, dilated annulus, ruptured chordae or scarred and shortened papillary muscles.
3. Cut radially through the valve.

Semilunar Valves

1. Take photographs to include the atrial and ventricular valves.
2. Number of cusps:
   a. Whether of equal or unequal size.
   b. Same features as for atrioventricular (AV) valves—calcification, perforation, immobile, stretched or ballooned.
3. Cut radially through the valve, so that the cut goes through the annulus as well as the free margin.

Bits

Several bits in one or more blocks.

BONE MARROW TREPHINE BIOPSY

Bone marrow biopsy allows the study of marrow in its existent architecture, i.e. the relationship of marrow elements to each other; in relation to the stroma, the vasculature and supportive bony trabeculae.

Indications

1. Dry tap on aspiration.
2. Evaluation of marrow suspected for myeloproliferative disorders, lymphoproliferative disorders and hypoplasia/aplasia.
3. Evaluation of marrow in diagnosed cases for staging of lymphoproliferative disorders, round cell tumors of childhood, malignancies with known predilection for marrow involvement (e.g. carcinoma breast and lung).
4. Follow-up in diagnosed cases on treatment for acute leukemia, chronic myeloid leukemia (CML) on certain regimens and myelosuppressive agents.
5. Evaluation of systemic/disseminated disorders, e.g. pyrexia of unknown origin, storage disorders, Paget's disease, osteoporosis and osteogenesis imperfecta.
6. The tumors of bone such as Ewing's sarcoma, chondrosarcoma, osteosarcoma, lymphoma, eosinophilic granuloma and hemangioma.

Contraindications

- Severe coagulopathy
- Hemophilia.

Complications

1. Retroperitoneal hemorrhage following a difficult procedure or in individuals with osteoporosis.
2. Infection at the site.

Procedure

Sites: For trephine biopsy:

1. Posterior iliac crest is universally accepted.
2. Anterior iliac crest is sampled in infants or when posterior iliac crest is not accessible in obese individuals and also in patients with bed sores.
3. Tibial tuberosity in infants below 18 months of age.
4. Under fluoroscopic guidance, the vertebral body and other subcutaneous bones with localized lesion can often yield diagnostically useful specimens.

Routine processing: Bone marrow biopsy core is fixed in Zenker's fluid for 4–6 hours. Regular paraffin embedded sections following decalcification. Special embedding such as plastic embedding in methyl methacrylate will enable study of the remodeling defects on undecalcified bone and also give excellent cytological details of the marrow.

Stains: The most commonly used stains are:

1. **Romanowsky stains:** Giemsa, Wright or Leishman.
2. **Cytochemical stains:** The myeloperoxidase, PAS, Sudan black, acid phosphatase, esterase, reticulin, Perl's, Ziehl-Neelsen stain (decalcification may make interpretation of these difficult).
3. **Immunocytochemical stains:** Monoclonal antibodies for lineage identification.

Electron microscopic studies for immunological markers.

TESTICULAR BIOPSY

Testicular biopsy was introduced by Charny in 1960.

Indications

1. Semen analysis cannot be explained by endocrine evaluation and biopsy required.
2. Absolute azoospermia, oligospermia, teratospermia and atypical cells in ejaculate.
3. Relative in varicocele, cryptorchidism, chronic infection and acute lymphoblastic leukemia.

Note: If FSH is more than three times, it is an indicator of primary hypogonadism, which obviates the use of biopsy.

Types

1. Open surgical incisional biopsy using a window approach.
2. Transcutaneous and fine-needle aspiration (FNA) have been evaluated, but not recommended as there is significant variation in morphology and uneven distribution of lesion and random sampling. However this is practiced in many cytology laboratories in particular for checking the degree of spermatogenesis.
3. Some centers advocate a touch/imprint cytology smear from fresh biopsy tissue; 2–3 mm specimen (in largest diameter) removed without compression.

Procedure

Fixatives: As follows:

- Bouin's picric acid
- Steve's fixative—mercuric chloride.

Note: Formalin is not recommended as it causes excessive shrinkage of tubules and poor preservation of nuclear details.

Stains: Usually stains are:

1. Hematoxylin and eosin.
2. Periodic acid-Schiff: For glycogen and basement membrane.
3. Masson's trichrome and van Gieson's stain; for interstitial fibrosis.

Adequacy: About 100 profiles of seminiferous tubules to be present.

Evaluation: Johnson score for spermatogenesis, nature of basement membrane—thickened or normal, leydig cell hyperplasia, etc.

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Chapter 10 Immunohistochemistry and Immunofluorescence

Although knowledge of histopathology has advanced considerably, the basic principles of diagnosis based upon examination of a tissue section have not changed significantly, since the time of Bennett and Virchow. However, experienced pathologist is the application and interpretation of morphological criteria remains subjective based on the experience of the pathologist. In order to overcome this, search has been made for more sure methods for validating or negating morphological judgments. A variety of 'special stains' were developed to facilitate cell recognition and diagnosis. Most of the stains were based upon chemical reactions of cells and tissue components (histochemistry). In certain circumstances, however, these histochemical stains proved to be of critical value in specific cell identification. The facility for performing a wide variety of truly specific special stains came into effect with the introduction of the immunofluorescence and later immunohistochemistry (IHC) staining procedure.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry is therefore immunohistology, which deals with the study of tissue sections stained with labeled antibodies, an antibody induced by a specific antigenic determinant (the smallest unit of antigenicity). Thus, specific antibodies used as probes can be used to detect the morphological position of antigens within tissue sections or other forms of cell preparations. However neither the antigen nor the antibody or the antigen-antibody complex can be seen with the light microscope unless they are 'colored' or 'tagged' by some method that permits their visualization. A variety of labels or tags have been employed, including fluorescent compounds and active enzymes that can be visualized by virtue of their property of inducing the formation of a colored reaction from a suitable substrate system.

Immunoperoxidase (Immunoenzyme) Methods

Immunoperoxidase methods are based upon attachment of an active enzyme, e.g. horseradish peroxidase (HRP), to a specific antibody. This antibody may be the one detecting an antigen in the tissue (as in the direct method) or one, which combines to other specific antibodies acting as probes to detect antigens in tissues (as in other methods). This enzyme HRP

has also the ability to produce a visible color change in a 'substrate system' thus enabling visualization of the 'antibody-enzyme complex' or indirectly the antigen-antibody reaction.

The 'substrate system' referred to consists of hydrogen peroxide and a chromogen such as 3,3-diaminobenzidine (DAB), tetrahydrochloride or 3-amino-9-ethylcarbazole (AEC). The enzyme HPR reacts with this 'substrate system' to produce a colored molecule and water. Although other enzymes such as alkaline phosphatase and glucose oxidase may also be used, peroxidase is commonly referred because:

1. Its small size does not hinder the binding of antibodies to adjacent sites.
2. It is available in a highly purified form, so that the chance of contamination with other enzymes is minimized.
3. It is very stable and relatively inexpensive.
4. Only small amounts are present in tissue specimens and the endogenous peroxidase activity is easily quenched.
5. Several coloring chromogens are available that can be acted upon by peroxidase to form a colored end product, which will precipitate at the morphological site of the antigen to be localized.

Immunological techniques have one principle in common—to attach a maximum amount of label to the site of localization of the antigen within the tissue with a minimum degree of non-specific (background) binding of the labeled moiety. The following are the important techniques, which have found widespread application.

Direct Method (Fig. 10.1)

Here the specific antibody is chemically linked to peroxidase. This conjugated antibody reacts with the antigen. 'A substrate system' is applied to a colored end product precipitating at the site of antigen-antibody interaction. The most common application of the direct immunoperoxidase method is for the detection of immunoglobulin, complement and immune complex deposits in kidney biopsies from patients with various types of renal disease; in skin biopsies from cases of systemic lupus erythematosus (SLE) and other connective tissue disorders.

Indirect Method (Sandwich Technique) (Fig. 10.2)

An unconjugated antibody reacts with the antigen. A conjugated antibody is then applied, which binds to the first antibody, now acting as antigen.

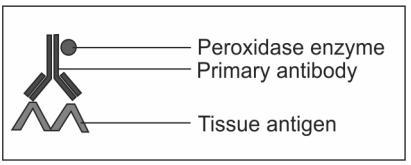


Figure 10.1: Direct method

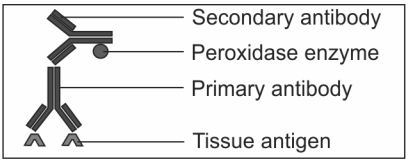


Figure 10.2: Indirect method

A substrate system is added to localize the reaction. The advantage of this technique over the former one is in the fact that a variety of primary antibodies made in the same animal species can be used with one conjugated secondary antibody.

The method gains popularity in the identification of antibodies in the serum of patients with various autoantibodies as well as bacterial and parasitic diseases—thyroid, nuclear, mitochondrial, smooth muscle antigen, treponemal palladium, herpes simplex virus and cytomegalovirus.

Peroxidase-antiperoxidase Method (Fig. 10.3)

Three reagents are utilized. The primary antibody reacts specifically with the antigen, secondary antibody or link antibody binds to the primary antibody and peroxidase-antiperoxidase (PAP) complex. The PAP complex comprises the enzyme peroxidase and an antibody against peroxidase. The peroxidase enzyme is then visualized via a substrate chromogen reaction. The primary antibody and PAP complex are both produced in the same animal species and the link antibody (produced in another species) is, therefore, capable of binding to both.

Absence of the conjugated antibodies in this method provides greater sensitivity than that attributed to the above techniques. This is evident in paraffin processed tissue where strong staining can be observed even though much of the antigen has been destroyed by fixation and processing. The direct and indirect techniques on the other hand are usually performed on cryostat sections. Perhaps, one of the most important applications of PAP methods is in determining the origin of neoplasm by virtue of identifying specific antigens; such cells can produce, e.g. prostate-specific antigen (PSA), kappa and lambda light chains, immunoglobulin G (IgG), IgA, IgM, glial fibrillary acidic protein (GFAP), etc.

Alkaline Phosphatase-antialkaline Phosphatase Method

The principles of the alkaline phosphatase-antialkaline phosphatase (APAAP) method are the same as that of PAP except that the PAP complex is replaced with as APAAP complex.

Major applications are as follows:

- Staining of tissues with high levels of endogenous peroxidase (e.g. bone marrow)
- Double immunostaining in conjunction with peroxidase
- Specific cell types may benefit from the bright red color of alkaline phosphatase.

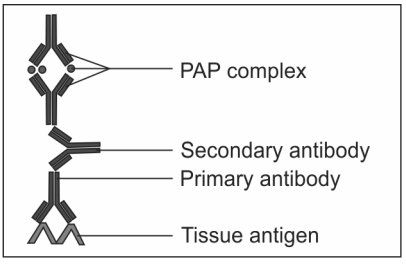


Figure 10.3: Peroxidase-antiperoxidase (PAP) method

Avidin-biotin Method (Fig. 10.4)

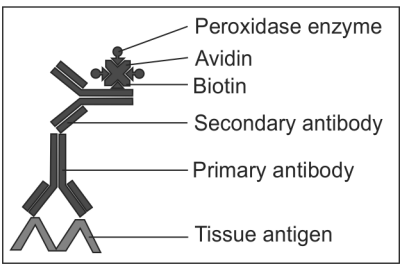


Figure 10.4: Avidin-biotin method

The avidin-biotin method utilizes conjugated antibodies. This method is based on the ability of egg white glycoprotein avidin to non-immunologically bind four molecules of the vitamin biotin. Three reagents are used. The primary antibody is specific for the antigen to be localized. A secondary antibody is capable of binding to the primary antibody and is conjugated to biotin. The third reagent is a complex of peroxidase, conjugated biotin and avidin. The free sites on the avidin molecule allows binding to the biotin on the secondary antibody. Visualization is by using an appropriate chromogen. The strong affinity of avidin for biotin gives this method greater sensitivity and excellent results can be achieved on fixed paraffin embedded specimens. The technique lends itself to the localization of numerous antigens in a variety of specimens—identification of hormones, cell markers in neoplasia, lectin-binding sites, viral proteins and intermediate filaments.

Polymeric Labeling Two Step Method (Fig. 10.5)

Polymeric labeling two step methods utilize a unique technology based on a polymer backbone to which multiple antibodies and enzyme molecules are conjugated. This polymer system contains a dextran backbone to which enzyme molecules horseradish peroxide (HRP) or alkaline phosphatase (AP) are attached along with secondary antibodies with antimouse Ig and antirabbit Ig specificity. This universal reagent can detect any tissue-bound primary antibody of mouse or rabbit origin.

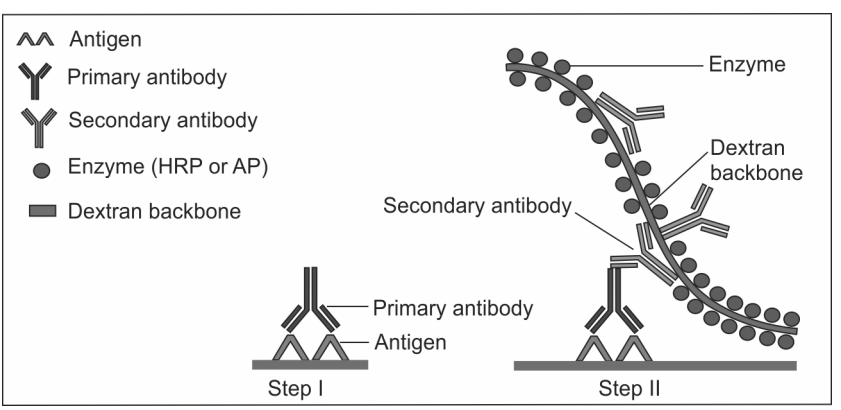


Figure 10.5: Polymeric labeling two step method (AP, alkaline phosphatase; HRP, horseradish peroxide)

Advantages

- Increased sensitivity
- Minimized non-specific background staining due to the absence of the endogenous biotin-associated non-specific staining
- A reduction in the total number of assay steps as compared to conventional techniques.

Interpretation

Deposits of the colored chromogen indicate the presence of antigen and represent specific positive staining.

Applications of Immunohistochemistry

With the introduction of immunochemical techniques into diagnostic laboratory procedure a distinct insight into the pathogenesis of several lesions have been achieved. Formerly these techniques were only utilized on frozen sections. But with the availability of the newly introduced monoclonal antibodies, particularly those with specificity for cell surface antigens, immunohistochemistry can be carried out routinely on paraffin-embedded tissue. The antigens that are better preserved in frozen sections are mainly those located on the cell surface membrane.

Besides frozen sections and paraffin-embedded sections, these procedures could also be carried out on smears. Smears as well as sections of formalin preserved tissues can be treated as discussed here. Fixation methods fall generally into two classes—organic solvents and cross-linking reagents. Organic solvents such as alcohols and acetone remove lipids and dehydrate the cells, while precipitating the proteins on the cellular architecture. Cross-linking reagents (such as paraformaldehyde) form intermolecular bridges, normally through free amino groups, thus creating a network of linked antigens. Cross-linkers preserve cell structure better than organic solvents, but may reduce the antigenicity of some cell components, and require the addition of a permeabilization step, to allow access of the antibody to the specimen. The appropriate fixation method should be chosen according to the relevant application:

1. **Acetone fixation:**
   - Fix cells in –20°C acetone for 5–10 minutes
   - No permeabilization step needed following acetone fixation.
2. **Methanol fixation:**
   - Fix cells in –20°C methanol for 5–10 minutes
   - No permeabilization step needed following methanol fixation.
3. **Ethanol fixation:**
   - Fix cells in cooled 95% ethanol, 5% glacial acetic acid for 5–10 minutes.
4. **Methanol-acetone fixation:**
   - Fix in cooled methanol, 10 minutes at –20°C
   - Remove excess methanol
   - Permeabilize with cooled acetone for 1 minute at –20°C.

5. **Methanol-ethanol mix fixation:**
   - Mixture of methanol and ethanol is 1:1
   - Make the mixture fresh and fix cells at –20°C for 5–10 minutes.
6. **Formalin fixation:**
   - Fix cells in 10% neutral buffered formalin for 5–10 minutes
   - Rinse briefly with phosphate-buffered saline (PBS)
   - Permeabilize with 0.5% triton X-100 for 10 minutes.
7. **Paraformaldehyde-methanol fixation:**
   - Fix in 4% paraformaldehyde for 10–20 minutes
   - Rinse briefly with PBS
   - Permeabilize with cooled methanol for 5–10 minutes at –20°C.

Histogenesis and Typing of Neoplasia (Table 10.1)

In the typing of neoplasia, IHC has gained widespread popularity. A variety of markers are available for use in the market, which include hormones and related proteins, intermediate filament proteins, leukocyte markers, hormone receptors and several other epithelial and non-epithelial antibodies in the identification of surface and intracytoplasmic B-cell immunoglobulins, and T-cell markers in a lymphoid hyperplasia.

TABLE 10.1: Monoclonal antibodies—hematolymphoid

Lineage/Precursors	Antibodies
B lineage	CD19, CD79a, CD20, CD22
T lineage	CD2, CD3, CD5, CD7
Myeloid	CD13, CD33, CD15, CD117, myeloperoxidase (MPO)
Hematopoietic precursors	CD41, CD61
Monocytic	CD14, CD11
Erythroid	CD36, CD7, glycophorin A

Markers for subclassification of acute leukemias (Table 10.2)

TABLE 10.2: Monoclonal antibodies for acute leukemia

Cells	Markers
Pan B cell	CD10, CD19, CD20, CD21, CD22, CD23, CD79a, CD37, CD32
Pan T cell	CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD43, CD57, CD45RO
Monocyte-macrophage system	CD11c, CD13, CD14, CD15, CD33, CD64
Natural killer cell	CD16, CD56

Markers to differentiate lymphomas from other small cell neoplasms (Table 10.3)

TABLE 10.3: Histogenesis

Histogenetic classification	Markers
Epithelial origin	CK*, EMA†, CEA‡, NSE§
Mesenchymal origin	Vimentin, desmin, S-100
Neuroendocrine origin	NSE, chromogranin
Lymphomas	LCA‖, Pan B, Pan T
Melanomas	S-100, vimentin, NSE, HMB-45

*CK, cytokeratin; †EMA, epithelial membrane antigen; ‡CEA, carcinoembryonic antigen; §NSE, neurospecific enolase; ‖LCA, leukocyte common antigen.

Note: Lymphomas has to be differentiated from small cell neoplasms such as carcinomas, rhabdomyosarcomas, etc.

Markers in the subclassification of soft tissue neoplasia (Table 10.4)

TABLE 10.4: Soft tissue neoplasia

Soft tissues	Markers
Myogenic	Spinal muscular atrophy (SMA), desmin, MyoD, myogenin
Nerve sheath	S-100, CD56, CD 57
Vascular	CD 34, CD31, vWF, Factor VIII
Gastrointestinal stromal tumors (GIST)	CD117, CD34, DOG-1
Melanocytic	S-100, HMB-45
Adipocytic	CD34, mdm2, CDK4

Markers in the specific diagnosis of small round cell tumors (Table 10.5)

TABLE 10.5: Small cell neoplasms

Tumors	CK	CD45	S-100	CD99	Desmin	MyoD1/ Myogenin	CD56	WT1
EWS*/PNET†	+/–	–	–	+	–	–	–	–
RMS‡	–	–	–	–	+	+	+	–
DSRCT§	+	–	–	+/–	+	–	+/–	+
WT‖	+/–	–	–	–	+	+/–	+	+
NB¶	–	–	+	–	–	–	+	–
SmCC**	+	–	–	–	–	–	+	–
Melanoma	–	–	+	+/–	–	–	–	–

*EWS, Ewing's sarcoma; †PNET, peripheral neuroectodermal tumor; ‡RMS, rhabdomyosarcoma; §DSRCT, desmoplastic small round cell tumor; ‖WT, Wilms' tumor; ¶NB, neuroblastoma; **SmCC, small cell carcinoma.

Markers in the specific diagnosis of tumors and staining patterns (Table 10.6)

TABLE 10.6: Tumor-specific markers and their staining patterns

Markers	Tumors	Staining patterns
TTF-1*	Lung, thyroid	Nuclear
Thyroglobulin	Thyroid	Cytoplasmic
Villin	Gastrointestinal (epithelia)	Membranous
CDX2	Colorectal	Nuclear
HepPar-1†	Hepatocellular	Cytoplasmic
GCDFP-15‡	Breast	Cytoplasmic
ER/PR§	Breast, ovary, endometrium	Nuclear
Mammaglobin	Breast	Cytoplasmic
PSA‖	Prostate	Cytoplasmic
PAP¶	Prostate	Cytoplasmic
Uroplakin III	Urothelium	Membranous
RCC** marker	Renal	Membranous
Inhibin	Sex cord—stromal, adrenocortical	Cytoplasmic
Melan A	Adrenocortical, melanoma	Cytoplasmic
Calretinin	Mesothelioma, sex cord—stromal, adenocortical	Nuclear/Cytoplasmic
WT-1	Wilms' tumor, ovarian serous, mesothelioma, desmoplastic small round cell	Nuclear/Cytoplasmic/ Membranous
Mesothelin	Mesothelioma	Cytoplasmic/ Membranous
D2-40	Mesothelioma, lymphatic endothelial cell marker	Membranous

*TTF-1, thyroid transcription factor-1; †HepPar-1, hepatocyte paraffin-1; ‡GCDFP-15, gross cystic disease fluid protein-15; §ER/PR, estrogen receptor/progesterone receptor; ‖PSA, prostate-specific antigen; ¶PAP, prostatic acid phosphatase; **RCC, renal cell carcinoma.

Infectious Disease

In the detection of tissue antigens of herpes simplex virus, measles, cytomegalovirus, human papillomavirus, hantavirus and rocky mountain spotted virus.

Immunohistochemistry in Hirschsprung Disease

Demonstrates nerve fiber hypertrophy and ganglion, markers include NSE, S-100, synaptophysin.

Immunohistochemistry in Alzheimer's Disease

Identifies major protein amyloid fibril.

Immunohistochemistry in Barrett's Esophagus

Mucin 5AC (MUC5AC) and MUC3 in superficial columnar epithelium, MUC2 in the goblet cells and MUC6 in the glands.

Immunohistochemistry in Breast Carcinoma

Markers estrogen receptor/progesterone receptor (ER/PR), HER2-neu, basal cell markers (CD5/6) and stem cell markers [CD44(+)/CD24(–/low)/ALDH1(+)].

Modified McCarty's H-scoring System/Histoscore (Estrogen Receptor and Progesterone Receptor) (Figs 10.6A and B)

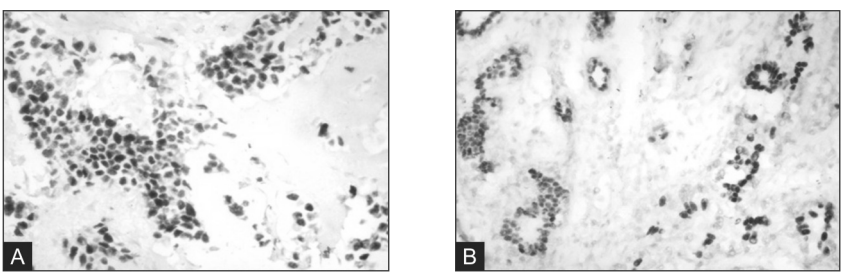
The whole stained section is scanned under low power and an estimation is made on the percentage of tumor cells, which are negative, weakly, moderately or strongly positive. The positive staining is indicated by the presence of brown nuclear stain. Each percentage is multiplied by a number—0, 1, 2 and 3 respectively, reflecting the intensity of their staining. This gives a total score varying from 0 to 300, which is expressed as negative (–ve H-score is 50 or less); weakly positive (+ve H-score is 50–100); moderately positive (++ve H-score is 101–200) or strongly positive (+++ve H-score is 201–300). There is a good correlation between this and other similar semiquantitative scoring systems and the quantitative biochemical assays.

Interpretation by McCarthy's Histoscore

Totally 100 cells have to be counted:

- Percentage of cells with strong positivity × 3 = 20 × 3 = 60
- Percentage of cells with moderate positivity × 2 = 60 × 2 = 120
- Percentage of cells with weak positivity × 1 = 10 × 1 = 10
- Percentage of cells with negative staining = 10 × 0 = 0
- Score = 190/300 (maximum score).

Scores less than 50 are considered negative and such tumors will not show a response to tomaxifen.



Figures 10.6A and B: A. Estrogen receptor positivity in breast carcinoma; **B.** Control showing positivity in the same section, surrounding tissue.

Allred's quick score (Table 10.7): Takes into account two criteria, while scoring—percentage of cells stained and the intensity of staining. Total score is calculated by adding the 2 scores, maximum is 7. Scores above 3 are considered positive.

TABLE 10.7: Allred's quick score

Percentage of positive cells	Score	Intensity of stain	Score
Nil	0	Nil	0
1–25	1	Mild	1
26–50	2	Moderate	2
51–75	3	Intense	3
76–100	4		

HER2/neu stain: The interpretation of the IHC staining is done as follows (Fig. 10.7):

- More than 10% of cells show weak staining of the cell membrane 1+
- More than 10% of cells show moderate staining of the cell membrane 2+
- More than 10% of cells show a continuous strong staining of the cell membrane 3+. It is recommended that all 2+ results need to be confirmed by the FISH technique.

Immunohistochemistry in Renal Transplant Biopsies

In allograft biopsy, CD4 deposition in the peritubular capillaries to diagnose humoral-mediated graft rejection.

Fixation and Processing

Good tissue processing and adequate fixation to ensure preservation of architecture and cell morphology are the cornerstones of IHC. Poor or inadequate fixation, leads to incorrect interpretation of staining patterns, e.g. elution of estrogen receptor from nucleus to cytoplasm, which can be interpreted as negative or nonspecific, since ER has nuclear localization.

Requisites of Fixation in Immunohistochemistry

1. To preserve the position of the antigen (nuclear, cytoplasmic or membranous).
2. To preserve the antigenic protein structure (secondary or tertiary).
3. To provide a target of antibodies that will be used to detect the antigen.

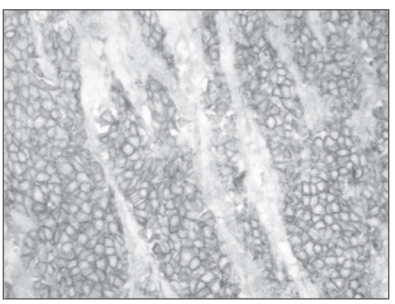


Figure 10.7: HER2/neu positivity in carcinoma breast, this was interpreted as 3+ score

4. To preserve the protein from elution, degradation or other modification.
5. To ensure optimal immunoreactivity with acceptable morphological preservation.

Fixatives: About 10% neutral buffered formalin (NBF), pH 7 (10% NBF).

Precautions: Place the tissue in the fixative (fresh; pH 7.0–7.6) immediately and do not allow it to dry. Fixation time should be fixed between 6 and 48 hours, as overfixing results in antigen denaturation and causes crosslinking, which masks epitopes needed to react with the antibody. Too short a fixation can cause tissue denaturation.

Tissue paraffin embedding: It should be maintained between 56 and 60°C, and if the temperature rises, then it can cause damage to the tissues with severe loss of antigenicity.

Section cutting: The tissue sections should be less than 5 μm in thickness. Avoid getting thick sections as they are more likely to float during antigen retrieval and causes difficulty in interpretation.

Adhesives: Sections that are not flat and that have non-adherent ridges are likely to be digested or torn of the slide during antigen retrieval or during washing. Hence when cut, sections are floated on water and picked up on slides that are coated with adherent material. Commercially available slides (having a positive charge that attracts the negative charge of tissue proteins) are expensive. Slides can also be coated with poly-L-lysine or 3-aminopropyltriethoxysilane—either of which provide a sticky surface for creating flat, adherent sections.

Staining: The following steps need to be taken:

- Antigen retrieval
- Blocking the non-specific binding or endogenous peroxidase or phosphatase activity
- Elimination of background staining
- Primary antibody incubation
- Secondary antibody/polymer incubation
- Chromogen
- Chromogen enhancement
- Counterstaining
- Stringent washing between reagents.

ANTIGEN RETRIEVAL

In formalin-fixed paraffin embedded tissue, most antigens are extensively masked due to cross linking of amino acids. These antigens need to be unmasked for the antibodies to react with epitopes.

Methods for Antigen Retrieval

1. **Enzymatic epitope retrieval:** The sections are exposed to enzymes such as trypsin, protease or proteinase K for 5–15 minutes. This enzyme cleaves the protein crosslinks and allows the epitope to return to its normal configuration enabling more effective antibody binding.

2. **Heat-induced antigen retrieval (HIAR):** Antigen retrieval relies on the application of heat to formalin-fixed paraffin-embedded (FFPE) tissue sections. The slides are immersed in an aqueous medium, referred as 'retrieval solution' and heated. The HIER breaks the formalin-induced cross links and exposes the epitope to the antibody.

Commonly Used Retrieval Buffer Solutions

- Citrate buffer at a pH of 6.0
- Ethylenediaminetetraacetic acid (EDTA) at pH of 8.0
- Trishydroxymethyl aminomethane (Tris-EDTA) at a pH of 9.0.

The degree to which immunoreactivity can be restored is related directly to the duration of incubation and the attained temperature. Optimum length of exposure to heat varies from 10 to 60 minutes. Cooling is usually allowed to take place slowly, requiring another 20–30 minutes.

Various Methods of Heating

- Microwave processing
- Pressure cooker
- Water bath/Steamer.

Combination of HIAR and enzymatic retrieval method is an alternative approach to unmask antigens and is especially used when double or triple labeling for 2–3 antigens, as in colocalization of antibodies.

BLOCKING THE ENDOGENOUS ACTIVITY

Endogenous Peroxidase Activity

The substrate-chromogen reaction used to visualize peroxidase cannot distinguish between the enzyme immunologically localized to the cellular antigen and similar endogenous enzyme activity present in the specimen before staining. The endogenous peroxidase activity is confined only to red- and white-blood cells; if not removed before adding the marking enzyme, false-positive staining will be observed for the specific antigen being looked for. The quickest and the easiest technique to inhibit this endogenous peroxidase is to treat the sections with 3% hydrogen peroxidase before staining. The other alternative is 0.3% hydrogen peroxidase with 0.1% sodium azide in phosphate buffer saline (PBS) or tris phosphate buffer saline (TBS). This is very gentle and leaves the fragile fragments undamaged. The endogenous peroxidase activity should be carried out before the addition of secondary antibody, or else the enzyme label of the secondary antibody will be inactivated by this blocking procedure resulting in false-negative results.

Non-specific Background Staining

Staining of a specimen that is not a result of antigen-antibody binding is termed as non-specific background staining and is most commonly due to highly charged collagen and connective tissue elements of the specimen. These charged sites may bind to the primary antibody and give

a false-positive reaction. This could be avoided by treating the section with an innocuous protein solution such as normal serum of rabbit, which could fill the charged sites, leaving no room for adsorption of the primary antibody.

Blocking the Avidin-biotin or Endogenous Phosphatase Activity

The step is required, if an avidin-biotin detection system is used. In APAAP endogenous alkaline phosphatase activity, the enzyme is usually lost in processing. However, in frozen sections it may be blocked with levamizole at the chromogen blocking step. It is not required with the polymer-based systems.

TYPES OF PRIMARY ANTIBODIES

Polyclonal Antibodies

A single antigen molecule contains several characteristic antigenic determinants or epitopes. When an antigen is injected into animal, the B lymphocyte starts making antibodies against the antigen. One B-cell line is believed to form antibodies against only one antigenic epitope. Since there are many B cells producing antibodies against each epitope, this is called polyclonal (many cells) antibody.

Monoclonal Antibodies

In some instances, it is desirable to have an antibody specific for a single epitope. Since this is produced by a single B-cell line (called clone) it is termed as monoclonal (single cell) antibody, with each antibody molecule produced replicating the other produced by the same B cell. Monoclonal antibodies are obtained in the following ways (Fig. 10.8):

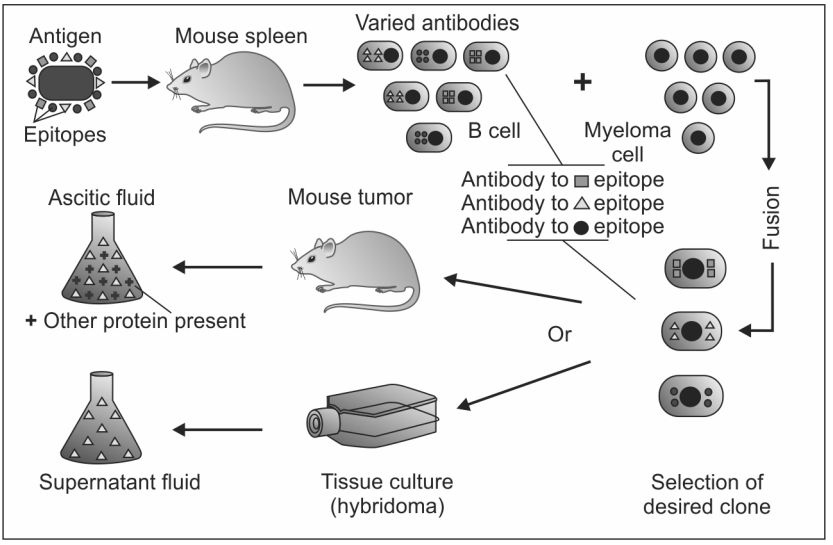


Figure 10.8: Methods for obtaining monoclonal antibodies

1. A mouse is injected with a purified antigen, produces antibodies against it.
2. When large amounts of antibodies are being produced, the mouse is sacrificed and the spleen containing large quantities of B lymphocytes is removed.
3. The B-cell suspension is mixed with myeloma cells in a medium that will cause the cells to fuse. A resulting hybridoma is produced (a hybridoma is the fusion of myeloma cells and B cells of the same species).
4. The hybridomas are tested to determine the clone antibody against the desired epitope. This is the most difficult and time-consuming part of the procedure.
5. The required hybridoma is then grown in tissue culture; the supernatant fluid contains the antibody produced by the hybridoma.

Alternatively, after identifying the cell line, it could also be injected back into a mouse where it will produce a tumor and the subsequent ascitic fluid obtained for antibodies.

Antibody Storage and Maintenance

On receiving the antibodies, care should be taken to note the date of arrival and shelf-life. The antibodies should be stored at –20°C. Once the vial is thawed, it should not be frozen again, as freeze and thaw cycles results in loss of potency of the antibody.

Secondary Antibody/Polymer

The secondary antibody is produced against immunoglobulin of the species from which the primary antibody is derived. Antirabbit secondary antibody is used for rabbit polyclonal primary and antimouse secondary is used for mouse monoclonal antibody, and they are produced in a species different from that in which primary antibody is produced. Secondary antibody is labeled with an enzyme.

Positive and Negative Control

The important step towards quality assurance in IHC is the inclusion of appropriate positive and negative controls with each staining batch.

Positive Control

A positive control is one that is known to contain the antigen under question. It needs to be run with every antibody that is used. It may be an inbuilt control on the same section as the test is being used. For example, smooth muscles of blood vessel acts as control for smooth muscle action.

Negative Control

A negative control is used to confirm the specificity of the method used and to assess the presence of background staining. The tissue here is subjected

identically to all steps as the test section except that the primary antibody step is omitted and replaced with covering the section with wash buffer.

CHROMOGENS USED FOR VISUALIZATION OF REACTIONS

1. **DAB substrate solution (3,3 diaminobenzidine tetrahydrochloride):** This chromogen produces a brown color that is insoluble in alcohol. Slides may be dehydrated with alcohol and coverslipped using conventional mounting media, i.e. dibutyl phthalate in xylene (DPX). It is classified, however, as a possible carcinogen.
2. **AEC substrate solution (3 amino-9-ethylcarbazole):** It forms a red end product that is alcohol soluble. Slides cannot be dehydrated with alcohol and are coverslipped with a water-based medium, e.g. glycerin jelly. The precipitates are therefore temporary unlike with the DAB-stained sections.
3. **Other chromogens:** Naphthol AS-MX phosphate, fast blue-red, new fuchsin and nitroblue tetrazolium.

Methods for Intensifying DAB Reaction Product (Postchromogen Enhancement)

Place the staining dish in a 25°C oven and incubate the slides for 1–5 minutes in 0.5% copper sulfate solution followed by wash under running tap water and counterstaining with hematoxylin (optional).

Apparatus Required for Immunostaining

1. Pyrex/Corning glass dish with a slide rack and cover—for placing slides during staining procedure.
2. Gilson/Eppendorf adjustable digital micropipettes 1:20; 1:100 or 1:200 (any two any be bought at one time).
3. Incubator with accurate regulation at 37–40°C.
4. Plastic cuvettes with stoppers (1 mL) for diluting reagents.
5. Reagents, glasswares, absorbent wipes, etc.

Methods of Doing B-cell Marker on a Section Using a Mouse Primary Antibody

1. Deparaffinize the 4 μ fixed section.
2. Treat with different grades of alcohol and finally bring to PBS, i.e. 100% alcohol, two changes for 3 minutes each; 95% alcohol, two changes for 3 minutes each. PBS buffer 5 minutes.
3. Blocking reagent 3% hydrogen peroxide; incubate for 10 minutes. Rinse well with PBS; incubate for 10 minutes.
4. Blocking reagent—normal serum (rabbit): Dilute 1:5, incubate for 15 minutes; wipe excess.

5. Primary B antibody (mouse monoclonal): Dilute 1:200, incubate overnight. Rinse well with PBS.
6. Secondary antibody (rabbit antimouse): Dilute 1:50, incubate for 30 minutes; rinse well with PBS.
7. Labeling reagent (mouse-Pap complex): Dilute 1:100, incubate for 30 minutes; rinse well with PBS.
8. Acetylethylcarbazole solution: Incubate for 30 minutes, rinse in distilled water.
9. Flood distilled water: Incubate for 2 minutes.
10. Counterstain with hematoxylin.
11. Wash in tap water.
12. Mount, while wet with glycerin jelly.
13. The incubation is carried out at 37°C. All stain should be carried out with a known positive control slide and a negative control slide.

Precautions During Staining

1. Heat-fix paraffin sections (should not exceed for 2–6 hour): Slides may be heat fixed well in advance of staining (alternatively slides may be coated with poly-L-lysine before sections are taken on to them, this ensures better fixation of sections onto the slides through all steps of staining).
2. Never allow sections to dry at any step of staining.
3. All reagents should be stored in the refrigerator. They should be allowed to reach room temperature before use.
4. Fresh dilution of antibodies should be made prior to use.

Fallacies of Staining

Artifacts

Several artifacts are found in specimens, which could produce positive staining and interfere with correct interpretation, e.g. precipitates, tissue artifacts and specific background staining. Precipitates such as unreacted chromogen granules and counterstaining pigments may be found; when these solutions are not filtered; this can be distinguished by the fact that they are not confined within cells.

Troubleshooting

Non-staining of Any of the Slides

- Staining steps not performed in correct order
- Omission of antibody incubation
- Sodium azide present in buffer system
- Improper concentration of hydrogen peroxide
- Specimens not counterstained properly
- Drying out specimens during staining
- Improper fixation and processing of tissues.

Weak Staining of All Slides

- Specimens retained too much liquid after buffer baths
- Use of old substrate solution
- Incubation time is too short
- Improperly diluted antibody solutions.

Excess Background Staining

- Endogenous peroxidase activity not removed
- Non-specific binding to the specimen
- Non-immune serum was hemolyzed
- Improper antibody dilutions
- Improper fixation
- Use of whole serum antibodies
- Excessive application of tissue adhesive
- Improper rinsing of slides
- Overdevelopment of substrate solution
- Increases thickness of specimen.

Crushed, necrotic and hemorrhagic tissue can exhibit non-specific staining. Only the staining pattern of viable cells should be considered for interpretation.

Preparation of Reagents

Phosphate buffered saline pH 7.4 (± 0.2):

- Sodium chloride: 8.0 g
- Sodium phosphate: 1.15 g
- Potassium chloride (KCl): 0.2 g
- Potassium phosphate, monobasic anhydrous (KH_2PO_2): 0.2 g
- Distilled water: 1 L.

3-3, diaminobenzidine substrate solution: Dissolve 6 mg of 3.3 diaminobenzidine tetrahydrochloride in 10 mL of 0.05 M tris buffer pH 7.6. Add 0.1 mL of 3% hydrogen peroxide. The mixture should be filtered if precipitation occurs.

Tris buffer pH 7.6: Dissolve 6.1 g tris (hydroxymethyl aminomethane) base in 5 mL distilled water. Add 37 mL of 1 N hydrochloric acid. Dilute to a total volume of 1 L with distilled water. The pH should be 7.6 ± 0.2 at 25°C.

Acetylethylcarbazole (substrate solution): Dissolve 4 mg of 3 amino-9-ethyl carbazole in 1 mL of N,N-dimethylimidazole. While stirring, add this to 14 mL of 0.1 M acetate buffer of pH 5.2. Add 0.15 mL of 3% hydrogen peroxide. The mixture should be filtered, if precipitation occurs.

Acetate buffer 0.1 M, pH 5.2: Mix 210 mL of 0.1 N acetic acid (5.75 mL glacial acetic acid in 1 L distilled water) an 790 mL of 0.1 M sodium acetate (13.61 g sodium acetate trihydrate in 1 L distilled water).

Checkerboard Titration

Various dilutions for the primary antibody, secondary antibody and PAP complex should be tried to get optimum results before standardization of the technique. A starting dilution for the primary antibody can be usually obtained from the manufacturer. Dilutions are checked out on a graph paper.

Immunohistochemistry is now preferred to the extent that automation in staining has been achieved. The advent of automated immunostainers has contributed to the achievement of slide output, speed, reliable results, standardization of protocols, higher efficiency and superior quality of staining results, consequently increasing the value of these staining results in patient management. It also permits laboratory personnel to perform more complicated procedures. An automated autostainer consists of following components:

1. Slide chamber/carrier, where the slides reside and the incubation and staining steps take place.
2. A reagent delivery system.
3. A computer with a user interface to control the individual staining steps. (refer Chapter 11 'Automation in Histology').

Multiplex Dilution

The multiplex dilution technique and antibody products provide simultaneous testing for morphological distinct markers enabling interpretation of complex problems. Different antibodies can be marked with different chromogens resulting in brown color and red color in the same slide. The innovative AP and HRP micropolymer technology is especially designed for a rapid multiplex stain procedure, i.e. multiple antigens on the same slide. The micropolymer confers a significant increase in staining sensitivity when compared to other conventional dextran polymer detection systems. Detection kits use a combination of AP and HRP conjugated secondary antibodies. The micropolymer multiplex detection kits are optimized for human tissues.

For example, atypical ductal hyperplasia (ADH-5) breast marker cocktail is composed of CK10+/14 + p63 + CK7/18 antibodies. This cocktail can be used in a wide range of applications for breast cancer diagnosis. Invasive versus non-invasive breast lesions can be easily distinguished through the presence or absence of myoepithelium (CK10 and/or p63) (DAB) and glandular staining of breast cancer with CK7/18 (fast red). This five antibody cocktail in one single application can also differentiate hyperplasia of the usual type from atypical hyperplasia and identify microinvasive and basal phenotypes in most breast cancers.

Luminal or cytoplasmic staining may also be observed in CK5/14 and/or CK7/18 staining (bimodal). In certain cases, only CK5/14 luminal staining is observed, representing a basal phenotype classification. Breast cancer with bimodal- and/or basal-like staining are associated with poor prognosis.

IMMUNOFLUORESCENCE

Immunofluorescence is a very sensitive and special method, used for topographical detection of antigens by antibodies, labeled with fluorochromes. Immunofluorescent methods have the potential to define antigen-antibody interactions at the cellular level, e.g. mitochondria, microsomes, as well as identifying small cell surface structures such as receptors, etc. Also these highly specific reactions can be seen in the background of general histological topography of the tissue sections.

Types of Immunofluorescence

- Direct immunofluorescence
- Indirect immunofluorescence
- Microimmunofluorescence.

Direct Immunofluorescence (Figs 10.9 and 10.10)

Direct immunofluorescence was introduced by Coons in 1941. It is used to detect antigen using specific fluorochrome-labeled antibody. The steps involved are fixation of smear/section on the slide, treating with labeled antibody, incubation, washing to remove unbound excess labeled antibody and visualization under a fluorescent microscope. When viewed under fluorescent microscope, the field is dark and areas with bound antibody fluoresce green. This technique has several advantages over the indirect method because of the direct conjugation of the antibody to the fluorophore. This reduces the number of steps in the staining procedure, is therefore faster, and can avoid some issues with antibody cross-reactivity or nonspecificity, which can lead to increased background signal. The disadvantages include lower signal, generally higher cost, less flexibility and difficulties with the labeling

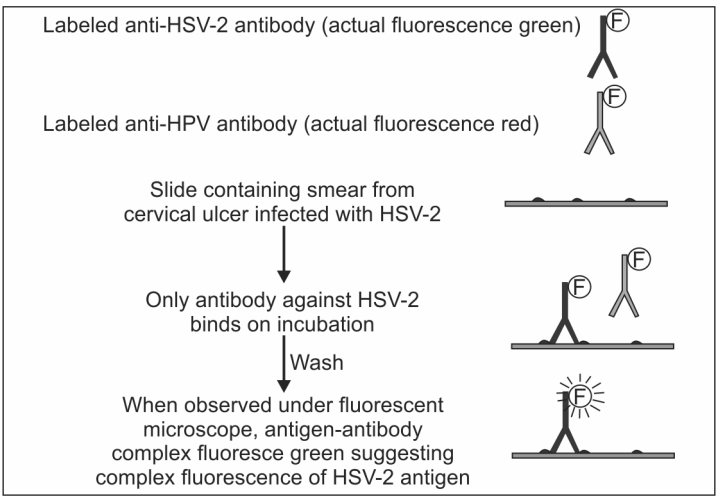


Figure 10.9: Direct immunofluorescence (F, fluorescence; HPV, human papilloma virus; HSV, herpes simplex virus)

procedure when commercially available direct conjugates are unavailable. The best application of direct immunofluorescence is in the evaluation of renal glomerular disease and bullous diseases of skin.

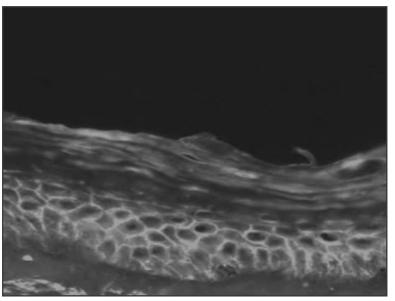


Figure 10.10: Section shows basket-weave positivity of immunoglobulin G (IgG) antibody in pemphigus vulgaris

Methods

1. Wash sections in 0.1 M PBS with three changes over a period of 30 minutes.
2. Drain off the excess PBS and wipe sections with cellulose tissue. Cover the section with diluted conjugate and allow it to react with for at least 30 minutes at room temperature.
3. Drain off the conjugate and wash off three changes of PBS over a period of 30 minutes.
4. Drain off the excess PBS and sections are dried with cellulose tissue. The sections are mounted with PBS/glycerol/DABCO solution.
5. The edges of coverslip are sealed with nail varnish.
6. Read the preparations on a fluorescent microscope and store at 4°C.

Indirect Immunofluorescence (Fig. 10.11)

Indirect immunofluorescence was introduced by Weller and Coons in 1954. Indirect immunofluorescence uses two antibodies; the first (primary antibody) recognizes the target molecule and binds to it, and the second

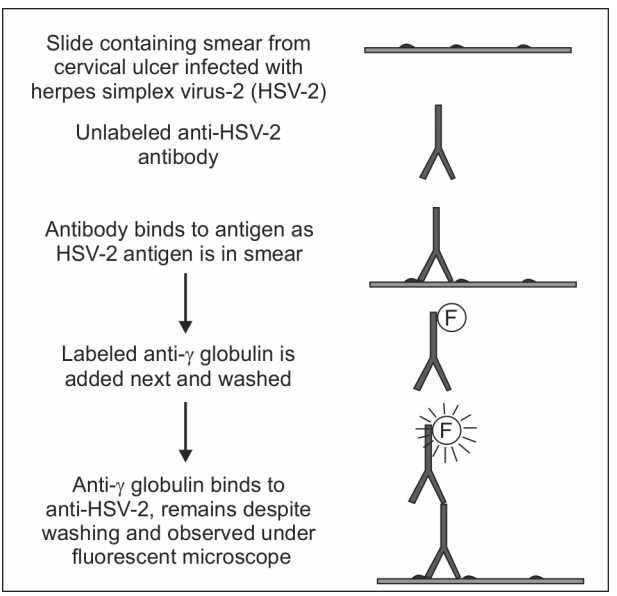


Figure 10.11: Indirect immunofluorescence (F, fluorescence)

(the secondary antibody), which carries fluorophore, recognizes the primary antibody and binds to it. The second antibody is labeled antigammaglobulin antibody. This antibody binds to Fc portion (constant) of first antibody and persists despite washing. The presence of the second antibody is detected by observing under fluorescent microscope.

Different primary antibodies with different constant regions are typically generated by raising the antibody in different species. For example, primary antibodies can be created in a goat that recognize several antigens, and then employ dye-coupled rabbit secondary antibodies that recognize the goat antibody constant (Fc) region ('rabbit antigoat' antibodies). It is often used to detect autoantibodies. For example, in the detection of antinuclear antibodies (ANA) found in the serum of patients with systemic lupus erythematosus (SLE). This protocol is more complex than the direct protocol above and takes more time, but allows more flexibility. It has a greater sensitivity than direct immunofluorescence. There is amplification of the signal in indirect immunofluorescence because more than one secondary antibody can attach to each primary. Commercially produced secondary antibodies are relatively inexpensive, available in array of colors and quality controlled.

Limitations

Photobleaching: It is the photochemical destruction of a fluorochrome due to destruction of reactive oxygen species in a specimen as a byproduct of fluorochrome excitation. Loss of activity can be controlled by reducing the intensity or time span of light exposure, by increasing the concentration of fluorophores and by reducing the availability of singlet oxygen by addition of singlet oxygen scavenger.

Fluorescence overlap: One of the problems that must be dealt when measuring fluorescence of more than one color is the possibility that the emission signals overlap. This should be avoided to prevent false level of more than one color.

Method

1. Prepare the patient's serum dilution in PBS in the ratio of 1:10. Conventionally, 50 μL serum + 450 μL PBS are used. Expose sections to serum dilution of primary antibody for at least 30 minutes at room temperature.
2. Wash over 30 minutes in PBS (three changes).
3. Drain off the excess PBS and wipe sections with cellulose tissue.
4. Cover sections with appropriately diluted antihuman Ig/fluorescein isothiocyanate (FITC) conjugate for 30 minutes.
5. Drain off the excess conjugate and wash with three changes of PBS.
6. Mount coverslip as outlined under 'direct technique'.

Microimmunofluorescence

Microimmunofluorescence is a serological technique employed to detect antibodies in patient serum. It works on the same principle as that of indirect immunofluorescence, but is performed on Teflon slides with many wells dotted with antigens. This technique is used in the serodiagnosis

of Q fever, Mediterranean spotted fever, detection of IgG, IgA and IgM antibodies to chlamydia, toxoplasmosis, epidemic typhus, etc. Applications of immunofluorescence in diagnostic pathology are as follows:

1. Analysis of antigens in fresh, frozen and fixed tissues; subcellular localization of antigens in tissue culture monolayers; observation of bacterial and parasitic specimens.
2. Detection and localization of the presence or absence of specific DNA sequences on chromosomes.
3. Defining the spatiotemporal patterns of gene expression within cells/tissues.

In summary, immunofluorescence is the visualization of antigens within the cells using antibodies as fluorescent probes. The benefits of immunefluorescence are numerous, and the technique has proven to be powerful tool for determining the distribution of known antigens in frozen tissues or in the localization of specific DNA sequences on chromosomes. The method has achieved the status of combining high sensitivity with high resolution in the visualization of antigens and will be a major tool for many years to come that any pathologist studying cells or molecules cannot afford to ignore.

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Chapter 11 Cytopreparatory Techniques

BODY CAVITIES IN DISEASE

The pleural, peritoneal and pericardial cavities are the three body cavities, which have a common embryological origin in the mesenchymal embryonic layer. The body cavities are lined by a single layer of flat cells the mesothelium supported by connective tissue with an appropriate vascular and nervous tissue framework. Structurally all three body cavities are quite similar; layer covering the organs contained within the cavity is referred as visceral layer, whereas that lines outer walls of the respective cavity is called parietal layer. The visceral and parietal layers in continuity with each other form a cavity, which normally is self-contained and not in contact with the outside world.

In the absence of disease, visceral and parietal layers of the mesothelium are separated merely by a thin layer of fluid that facilitates movements of the two membranes against each other. Therefore, these cavities are not true cavities, but virtual spaces. Penetration of gases or fluids into the body fluids will change them into true cavities. The presence of gas constitutes a pneumothorax, a pneumoperitoneum or a pneumopericardium. The presence of fluid constitutes an effusion in which the pleural and pericardial cavities are called pleural and pericardial effusion respectively, and in the peritoneal cavity as ascites. Bloody fluid in body cavities is called hemothorax, hemopericardium and hemoperitoneum respectively.

Effusion of fluid in the pleural, pericardial and peritoneal cavities forms on the basis of plasma ultrafiltration (Table 11.1). Such ultrafiltrates commonly classified as transudates and exudates (Table 11.2). Transudates are effusions caused by factors influencing formation or resorption of fluid, e.g. hypoalbuminemia, increased venous pressure, etc. Exudates are effusions caused by damage to mesothelial lining, as occurs in infections, neoplasms or collagen vascular diseases. Effusions may also form owing to escape of chyle from the thoracic duct.

DIAGNOSTIC CYTOPATHOLOGY

Diagnostic cytopathology is the science of interpretation of cells that are either exfoliated from epithelial surfaces or removed from various tissues. George N Papanicolaou introduced exfoliative cytology as a tool to detect

cancer and precancer in 1928. The Papanicolaou (Pap) smear is now widely accepted method for mass screening in asymptomatic population. This has resulted in a reduction in the incidence of cervical cancer in several countries. Diagnostic exfoliative cytology can be carried out by different methods, which include collection and examination of exfoliated cells such as vaginal

TABLE 11.1: Causes of pleural, pericardial and peritoneal effusions

Site	Exudate	Transudate
Pleural	Neoplasms: • Bronchogenic carcinoma • Metastatic carcinoma • Lymphoma • Mesothelioma (increased hyaluronate and content of effusion fluid) Infections: • Tuberculosis (high percentage of lymphocytes with 1% mesothelial cells) • Bacterial pneumonia • Viral or mycoplasmal pneumonia Trauma (may be associated with and hemorrhagic effusion): • Pleural infarct (may be associated with hemorrhagic effusion) • Rheumatoid disease (low pleural fluid glucose in most cases) • Systemic lupus erythematous (SLE) (cells occasionally present) Pancreatitis (elevated amylase activity in effusion fluid) Ruptured esophagus (elevated amylase activity and low pH in effusion fluid)	Congestive heart failure Hepatic cirrhosis Hypoproteinemia (e.g. nephrotic syndrome) Malignancy due to circulatory obstruction by neoplasm
Peritoneal	Neoplasms: • Hepatocellular carcinoma • Metastatic carcinoma • Lymphoma • Mesothelioma Infections: • Tuberculosis • Primary bacterial peritonitis (may be superimposed on transudate) • Secondary bacterial peritonitis (e.g. appendicitis and intestinal infarct) Trauma Pancreatitis Bile peritonitis (secondary to ruptured gallbladder or needle perforation of bile duct)	Congestive heart failure Hepatic cirrhosis Hypoproteinemia (e.g. nephrotic syndrome) Malignancy due to circulatory obstruction of neoplasm

Contd...

Contd...

Site	Exudate	Transudate
Pericardial	Infections: • Tuberculosis • Bacterial peritonitis • Fungal peritonitis • Viral or mycoplasmal peritonitis Neoplasms: • Metastatic carcinoma • Lymphoma Trauma (may be associated with hemorrhagic effusion) • Myocardial infarct (with hemorrhagic effusion) Secondary to anticoagulant therapy Leakage of aortic aneurysm Metabolic (uremia, myxedema) Rheumatoid disease Systemic LE	
Chylous effusion	Damage or obstruction to thoracic duct, e.g. trauma, lymphoma, carcinoma, tuberculosis and parasitic infestation	

scrapes, sputum, urine, body fluids, etc. Collection of cells by brushing, scraping or abrasive techniques is usually employed to confirm or exclude malignancy. Fiberoptic endoscopes and other procedures can be used for collecting samples directly from the internal organs.

Fine Needle Aspiration Cytology/Biopsy

Fine needle aspiration cytology/biopsy (FNAC/FNAB) has recently been widely accepted as a simple, non-invasive diagnostic procedure, which has

TABLE 11.2: Differences between transudates and exudates

Sl No.	Exudate	Transudate
1.	Result from active accumulation of fluid within the body cavities, apparently associated with damage to the wall of the capillaries	Accumulation is due to filtration of blood serum across the physically intact vascular wall
2.	Specific gravity high (> 1,015)	Specific gravity low (< 1,015)
3.	Protein content > 3 g%	Protein content < 3 g%
4.	Inflammatory cells are increased in number with polymorphs and lymphocytes	Few cells such as mesothelial cells with a few leukocytes and histiocytes
5.	Coagulates on standing due to high-fibrin content	Does not coagulate on standing

largely replaced excision biopsy. This method is applicable to superficial lesions that are easily palpable, e.g. swellings in thyroid, breast, lymph node, etc. Imaging techniques, mainly ultrasonography and computed tomography (CT), offer an opportunity for guided FNAC of deeper structures.

The practice of diagnostic cytology needs proper training of the laboratory personnel, including the cytopathologist, cytotechnologist and cytotechnician. The accuracy of the cytological examination from any body site depends greatly on the quality of collection, preparation, staining and interpretation of the material. Inadequacy in any of these steps will adversely affect the quality of reporting. The introduction of quality assurance and quality control measures with regard to sample preparation techniques, analytical cytology techniques, usage of internationally accepted terminology and advanced technologies including, computerization and automation are important in ensuring high standards in cytology.

COLLECTION/PRESERVATION/EXAMINATION OF BODY FLUIDS

The most important goal of effusion cytology is in the recognition or ruling out of malignancy. Effusions that are commonly sent to a laboratory for diagnostic purposes are pleural fluid, ascitic fluid, pericardial fluid and synovial fluid (SF). Besides these fluids, the other fluids, which may be sent, are cerebrospinal fluid (CSF) and urine. The procedures utilized in the processing of fluids are vital to a proper interpretation and diagnosis.

Essential Requirements

- Freshly tapped specimen
- Immediate processing
- Rapid fixation of slides.

Several parameters influence the following requirements:

- Various methods of specimen collection
- Preservation of specimens after collection
- Method of processing
- Fixatives used on smears
- Staining and mounting.

All laboratories should streamline procedures and aim at good quality control for an ideal report.

Methods of Specimen Collection

1. **Pleural tap:** It is used to collect fluid from the pleural cavity. A wide-bore needle is inserted into the pleural space just above the rib margin with the patient in the sitting posture.
2. **Ascitic tap:** It is the procedure used to obtain fluid from the abdominal cavity. The point of aspiration is usually the right iliac fossa, a little outside the midpoint of a line drawn from the umbilicus to the anterior superior iliac spine with the patient lying supine or slightly propped up.

3. **Urine examination:** Urine may be collected by catheterization, as voided urine and samples of early morning specimens (50–100 mL). To detect or rule out malignancy, urine examination should be done on three consecutive days.
4. **Lumbar puncture (LP):** It is used for collection of CSF.
5. **Synovial tap:** It yields fluid from the joint space. This may be performed on the extensor aspect of the joint where the synovial pouch is superficial and free of nerves and blood vessels.

It must be observed that all paracentesis must be done under aseptic precautions. At least 10 mL of fluid must be available for proper assessment. A maximum quantity of 150–200 mL may be sent for examination at any time. A few tips to remember, do not take fluid for cytological examination from a drainage bottle. This would result in a marked autolytic effect and more over in a bottle; all cells tend to sediment down and may be missed, if fluid is removed from above. For best results immediate processing of fluids is essential.

Preservation of Specimens After Collection

Processing of fluids should be immediate and not later than 2 hours for optimal results. The duration between collection and preparation of the sample before cellular damages can occur, depends on pH, protein content, enzymatic activity and the presence or absence of bacteria. If fresh specimens cannot be brought directly to the laboratory, specimens should be refrigerated. Prefixation in alcohol is not recommended as alcohol coagulates protein and interferes with staining. The following guidelines are useful:

1. **Specimens with high-mucus content** such as sputum, bronchial aspirates and mucocele fluid can be preserved for 12–24 hours if refrigerated. Refrigeration slows down the bacterial growth, which causes cellular damage. Mucus apparently coats the cells, protecting them against rapid degeneration. The cells in specimens diluted with saliva are not well protected and may deteriorate rapidly.
2. **Specimens with high-protein content** such as pleural, peritoneal or pericardial fluids can be preserved for 24–48 hours with refrigeration. The protein-rich fluid in which the cells are bathed acts as a tissue culture medium in preserving cellular morphology.
3. **Specimens with low-mucus or protein content** such as urine or CSF will be preserved for only 1–2 hours even if refrigerated. The fluid medium in which these cells are bathed contains enzymatic agents capable of causing cell destruction. Refrigeration may inhibit bacterial growth, but does not protect the cells.
4. **Specimens with low pH**, such as gastric material, must be collected on ice and processed within minutes of collection to prevent cellular destruction by hydrochloric acid (HCl).

Gross Examination of Fluids

Before fluids can be taken for processing, a gross examination of all fluids yields important information towards diagnosis. The following features may be noted:

1. An excess volume of fluid sent in ½ liter and 1 liter quantifies invariably denotes malignancy.
2. Bloody aspirates also indicate malignancy. These are due to vascular congestion of the subserosal connective tissue infiltrated by the neoplasm.
3. Black color exudates in large amounts indicate metastatic melanoma.
4. Clots of fibrin or blood may sometimes be seen settled at the bottom of the bottle and should be taken for cell block preparations. Groups of cells caught up in this clot may yield important information.
5. Rouleaux effect in a fluid indicates abnormal protein.
6. Pus in the fluid gives it a homogenous milky appearance and would indicate empyema/bursting of an abscess or a pyogenic etiology.
7. Agglutination of red cells may denote presence of cold agglutinins in the fluid.
8. Coagulation in fluid indicates excess of protein and invariably an exudate as also a turbid-yellow color.
9. Watery fluids usually indicate transudates.

METHOD OF PROCESSING

A variety of methods of processing fluids exist and each has its advantages and disadvantages depending upon the type of fluid that one is dealing with.

Centrifugation

The fluid received at the laboratory is stirred briskly to disperse the suspended cells. About 10 mL of this mixed fluid is put in a centrifugation tube and the tube spun at 2,000 rpm for 10 minutes. The sediment is directly smeared and the smears fixed immediately and stained. Any excess sediment may be taken for paraffin processing as a cell block.

Advantage/Disadvantages

Advantage of centrifugation is its simplicity of usage.

Disadvantages are cell loses during pipetting and aerosols during spinning leading to risk of contamination and infection to the user.

Wet Preparation

Besides the permanent stains, a wet preparation of this centrifuged sample may be done for a quick viewing of the sample. The film may be prepared by taking a drop of the top most layer of the sediment and layering it onto a drop of toluidine blue stain on the slide. Coverslip the preparation after mixing the two layers. This may be viewed directly under a microscope.

Advantages of the wet preparation are:

1. To eliminate samples from routine staining after scanning, if found unsuitable.
2. To give an immediate report within 10 minutes on fluids teeming with frank cancer cells.
3. A wet film enables identification of certain constituents, which may not be seen on a permanent preparation—cholesterol crystals, charcot-Leyden crystals, hematoidin crystals, motility in detached ciliary tufts (ciliocytophthoria), etc.
4. Useful in urine cytology.

Samples Analyzed

Proteinaceous fluids

Excess protein hinders cell morphology and therefore cell viewing because of background staining. This could be taken care of by centrifuging the specimen once and discarding the supernatant. To the sediment add normal saline, remix gently by tilting and recentrifuge. This procedure repeated twice gets rid of most of the protein and results in a very clear background against which the cells can be seen.

Alternatively, to any proteinaceous fluid, ethyl alcohol may be added to coagulate the protein. The mixture of fluid and alcohol is then centrifuged and the entire sediment or cell button taken preferably for paraffin processing or even direct smearing.

If a fibrin clot has already formed, the clot may be smashed against the sides of the tube by using an applicator after centrifugation. In case of a large clot, it may be processed as cell block. Place one to two drops of the sediment on the slide and allow it to spread evenly by placing another slide over it. Gently pull slides apart with an easy sliding motion to get alternate thick and thin area.

Serous/Thin fluids

Centrifugation of such fluids yields very little sediment. To concentrate more cells pour off the supernatant after the first centrifugation, add more fluid to the same tube and recentrifuge. This procedure that is repeated twice, collects most of the cells, which may be smeared.

If the cell button is still too small, then add some egg albumin powder or mixture to the sediment and mix well. Addition of ethyl alcohol coagulates the protein or egg albumin flakes, which on centrifugation yield enough sediment for either smears or cell block.

Bloody aspirates

Bloody aspirates show many red blood cells (RBCs) in the background making difficult in viewing. For such fluids, to 50 mL of the sample add either:

1. A few drops of 10% glacial acetic acid.
2. 0.1 N HCl.
3. Carnoy's fluid.
4. Addition of distilled water also causes hemolysis.

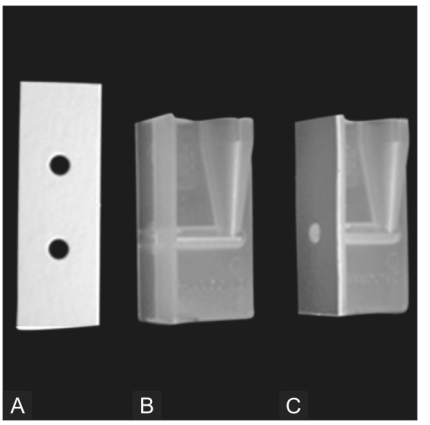
5. In FNAC smears of bloody material, the smear is given one dip in 1% acetic acid before bringing it to water and staining.
6. Newer methods with commercial reagents and now available. These agents not only lyse the RBCs but also fix the other cellular elements. Two such fixatives are CytoRich Red and CytoLyt Solutions. The 1 mL of fixative can be added to 25–50 mL of the sample. After letting the sample sit for few minutes, it is centrifuged, the supernatant is poured off and slides are prepared by the usual method in the laboratory.
7. Another method of dealing with bloody aspirates is that of Yam and Janckila, 1983. The procedure is as following:
   a. Add 5 mL of fluid and 100 μm of heparin. Centrifuge at 1,000–1,500 rpm for 5 minutes. Discard supernatant to make 50% v/v cell suspension.
   b. Mix the sediment.
   c. Load in microhematocrit capillary tubes and seal one end, centrifuge as routine.
   d. Break the microhematocrit tube below buffy coat layer. Attach a microbulb onto the plasma end of the tube.
   e. Express the drops of cells (buffy coat) onto a slide and smear.

Cytocentrifuge or Cytospin (Figs 11.1 and 11.2A to C)

Cytocentrifuge is an instrument used to concentrate small numbers of cells suspended in fluids and is successfully used for the preparation of clear fluids such as urine, CSF and other small–volume specimens. Cytocentrifuge retrieves cells or microorganisms from body fluids directly on the microscopic slides. Ordinary routine centrifuges are not capable of doing this function.

A slide is put into the vessel holder (Cytospin clip) and a suitable filter card is positioned between container and slide. Microtubes with a hole in the bottom, which are placed into the container holes, are used to hold cell suspensions. The well-mixed sample is spun in the

Figure 11.1: Cytocentrifuge or Cytospin



Figures 11.2A to C: Parts of Cytocentrifuge. **A.** Filter card; **B.** Reused cuvette with glued paper residue removed **C.** Cytocentrifuge pre-made cuvettes.

specially designed centrifuge at 2,500 rpm for about 2–5 minutes (speed and time can be set according to preference). Due to capillary forces sample only enters the container, once the centrifugal force is applied. The suspended cells reach the glass surface and stick to it. The microtubes prevent sample loss by stopping a flow on to the filter paper prior to centrifugation.

Depending on the suspension's cell concentration, the sample quantity needs to vary from a few drops to 1 mL. The smaller the sample quantity the better it is absorbed by the filter paper. With larger volumes liquids collect in the bucket's cavity underneath the slide. The result is a monolayer of well-preserved and well-displayed cells within a 6 mm area on the slide. Two such instruments in use are the Shandon Cytospin cytocentrifuge and the Sakura Autosmear. The machine can prepare 12 slides simultaneously.

Advantages of Cytospin method are:

1. The instrument is user friendly on which speed and time are easy to set, and then kept in memory.
2. Up to 12 specimens may run in one spin.
3. A quality monolayer of cells with good morphological presentation is obtained, being especially advantageous in low-yield specimens such as CSF and urine.
4. There is minimal cell loss.
5. Results are reproducible under identical conditions.
6. The sealed head of the tube prevents aerosol of infected samples during spinning and the instrument can be easily sterilized after use.

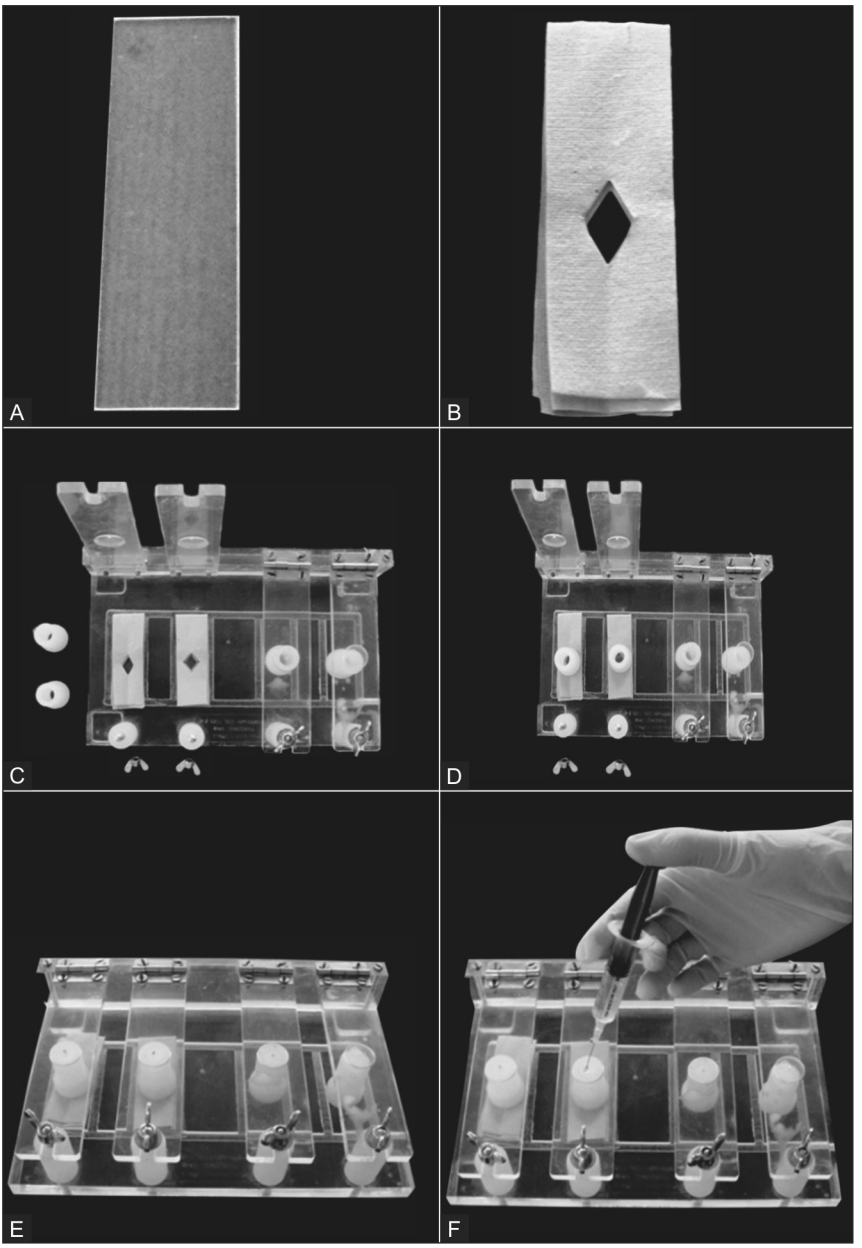
Disadvantages: The only objections to the use of a cytocentrifuge are some distortion of cellular morphology because of air drying artifact and loss of cells because of absorption of fluid into the filter card.

The Shandon Cytospin was the first device to use the principle of carefully controlled centrifugation to separate and deposit a thin layer of cells on slides, while maintaining integrity. It produces better cell capture and good presentation of cell types present in homogenous liquid samples. It provides economical thin layer preparations from any liquid matrix, especially hypocellular fluids such as spinal fluid and urine.

Slow Sedimentation Technique (Figs 11.3A to F)

The principle of this technique rests on the fact that spontaneous sedimentation of cells takes place in a fluid set up in a vertical cylinder at 4°C as the liquid part of it slowly seeps out and is absorbed by a filter paper. The cells settle gently such as snowflakes on the glass slide beneath the tube, undergoing minimal physical change.

The main advantage of this procedure is that very minimal cell distortion occurs as the cells settle by their own weight and force of gravity and excellent cell morphology is obtained. The procedure is ideal for clear fluids and has been used to advantage in urine, CSF and low-protein effusions.



Figures 11.3A to F: Setting up of slow sedimentation apparatus—sequential steps. **A.** Clean glass slide coated with egg albumin; **B.** Slide with filter paper over it; **C.** Slide with filter paper placed on to the stage of apparatus; **D.** Slide with cylinders of the apparatus over it; **E.** Screws tightened and cylinders mounted on to the stage, so that base of cylinder coincides with the hole in the filter paper; **F.** Loading of sample with the help of a disposable syringe in to the cylinder, that is fitted snugly onto the filter paper.

Slow Sedimentation Apparatus

Various apparatus have been devised for this including the sedimentation cylinder with brass rings devised by Blonk and Arentz (1977) and the sedimentation technique of Bots et al (1964), using adjustable weights. An apparatus made of Perspex Sheets and designed in the laboratory by Shariff and Thomas (1985) serves the purpose equally well. Slow sedimentation apparatus is designed by Shariff and Thomas. The apparatus consisted of a series of cylinders with an open base mounted on a stage.

Procedure

1. A clean glass slide coated with a thin layer of egg albumin is placed on to the stage beneath the cylinder; rectangular pieces of filter papers in which a hole has been cut in the center are placed on to the slides.
2. The cylinders are lowered on to the stage such that the center of the hole in the paper coincides with the hole in the base of the cylinder.
3. The screws are tightened to the maximum so that the cylinders sit snugly on the filter papers. The presence of the screws facilitates the rate of liquid seepage.
4. With the help of a disposable syringe of 2 mL, sample is loaded into the cylinder through a hole in the hood. As soon as the fluid is pushed some of it seeps out through the filter paper, but the rest remains in the cylinder.
5. The apparatus is placed aside for 1–2 hours or 6 hours, or kept overnight for cells to sediment onto the slides. Overnight sedimentation is generally used, when fluid is fed into the apparatus in the evenings.
6. After the specified time of sedimentation, screws are loosened and the hoods with the cylinders gently lifted up. The filter papers were gently lifted off from the slides. The sediment corresponded to the circular hole cut in the filter paper.
7. The smears were taken out, gently lowered in a container with 95% alcohol for fixation and stained with hematoxylin and eosin stain (H&E stain)/Papanicolaou stain after 20 minutes of fixation.
8. If the cellularity at the end of 2 hours is inadequate, the slide at the end of 6 hours with more cellularity is used for interpretation. Times of sedimentation can vary depending on the type of fluid (shorter times for protein rich fluids) and the results obtained.

Millipore Filters (Fig. 11.4)

The use of membrane filters for the concentration of cancer cells suspended in fluid was introduced by the late Sam H, Seal of Memorial Sloan Kettering Cancer Center.

Membrane Filters

Figure 11.4: Millipore type membrane filter

Membrane filter is a very simple and useful technique for concentrating the cells that are thinly dispersed in fluids, e.g. CSF, urine, etc. can also be used for fluids with high-protein content, e.g. pleural and ascitic fluids, provided are heparinized as soon as they are removed from the patient. This method is also approved for various 'washing' samples, i.e. gastric and colonic, for isolation of cancer cells. An added quality of this method is an even distribution of cells.

Membrane filters may be used for concentrating cells, e.g. Gelman, Millipore and Nuclepore, filters. The pore diameter is usually around 5 μm. Fluids used in these preparations should be fresh as prefixation coagulates proteins, which may then clog the filters and hardens cells into spherical shapes and prevent flattening of the cells on the membrane surface.

The filters used are cellulose acetate in nature. Filters of different shapes (rectangular or circular) and sizes are available. Most popular is the regular (47 × 19 mm) with a filtering pore diameter of 5 μm and can filter large volumes of fluid without giving a crowded cell picture. This may be mounted whole on a 3 × 2 inch slide, using a cover slip of the same size.

In the monolayer preparation technique, cells are collected on a filter during processing and then transferred to a glass slide before staining. The slide containing the cytospecimen is stained by the Papanicolaou method and examined under a light microscope. This technique provides the added advantage of a clearer background, with the cells of interest concentrated and confined within a demarcated area on the slide.

Filtration Procedure

1. Label Millipore and Gelman filter with indelible ink.
2. Presuspend Millipore and Gelman filter in a Petri dish filled with 95% ethyl alcohol for 10–15 seconds.
3. Moisten the grid of the filter set up with balanced salt solution.
4. Lay the pre-expanded filter on the grid.
5. Place the funnel on top of the filter. Add 15–20 mL of balanced salt solution to the funnel and apply clamp.
6. Add 1–2 drops of the sediment to the solution in the funnel.
7. Start the vacuum up to 100 mm of Hg for Millipore and Gelman filters and up to 20 mm of Hg for Nuclepore filters. As the specimen filters, add balanced salt solutions from a squeeze bottle to rinse the filter well.

The surface of filter should always be covered with fluid and not merely moist or wet looking. Never allow the filter to dry. Add 20–30 mL of 95% ethanol to fix cells in situ.

8. Unclamp the funnel, remove the wet filter. Place the filter, cell-side up in a Petri dish with 95% ethanol for half-an-hour. Specimens are filtered as soon they reach the laboratory.

Cell Block Technique

Cell block material provides valuable diagnostic evidence and can be used on tissue fragments that may not be processed by cytological techniques. It may also be used for processing all residual material (e.g. cell sediments) after completion of smear preparation. This procedure helps in recognition of histological patterns of disease that are not reliably identified in smears or filter preparations. It is of particular use in sputum, bronchial washings and material from gastrointestinal tract and even for FNAC material.

Cell Block Preparation

There are different methods of cell block preparation.

Procedure

Procedures for cell block preparation are:

1. Where tissue fragments are readily obtained, wrap material in a piece of filter paper and place in a tissue capsule after which it may be processed as for regular paraffin processing.
2. Where material is not adequate as in cell sediments after centrifugation, then to the sediment add egg albumin flakes or mixture (previously prepared), some ethyl alcohol and centrifuge. Take the sediment in a filter paper as above. The ethyl alcohol coagulates the protein of the egg albumin, which forms the matrix to hold the cells. Egg albumin mixture preparation from dried albumin is as follows:
   a. Albumin dried: 50 g.
   b. Sodium chloride: 0.5 g.
   c. Distilled water: 100 mL.

   Filter on Buchner's funnel with vacuum. To 50 mL of filtrate add 50 mL of glycerin. Add a crystal of thymol as preservative.
3. Plasma thrombin clot methods are:
   a. Thoroughly mix few drops of an unused sample of blood plasma with the fresh unfixed sediment. If the sample is prefixed with alcohol, the sediment must be washed several times with a balanced salt solution, since alcohol inhibits the clotting action of plasma and thrombin.
   b. Add the same number of drops of thrombin solution as of the pooled plasma and mix well.
   c. This mixture will clot in 1–2 minutes, if the reagents are fresh and not too cold. Place resulting clot in a cassette that has been lined with lens paper to prevent the clot from oozing through the holes.

   d. Put the tissue cassette into a jar of the fixative recommended for cell block. The clot being soft a spatula, instead of forceps, is recommended for its transfer to the embedding mold.
4. Modified technique using glacial acetic acid-alcohol formalin fixative (95% ethyl alcohol 34 mL + Formalin 4 mL + Glacial acetic acid 2 mL):
   a. The cell pellet remaining after preparing smears is mixed with thrice the volume of acetyl alcohol formalin fixative and centrifuged for 10 minutes at 2,000 rpm.
   b. Resuspend the cell button in acetyl alcohol formalin fixative and centrifuge for 10 minutes at 3,000 rpm.
   c. Discard the supernatant. Set aside the centrifuge tube for 4–6 hours. This allows the coagulum to form a gel.
   d. Scrape out the cell button and wrap in lens paper and process in tissue processor.
   e. Fixatives used on smears: It had been discussed in fixatives in detail.
   f. Staining and mounting: As routine.

HYDROCELE FLUID

Hydrocele fluid examination for cytology is a rare procedure in the laboratory diagnostic set up. Although aspiration of fluids accumulating in the hydrocele sac is a common procedure in the wards, a cytological examination is rarely requested. This fluid is usually hypocellular, but occasionally may show several mesothelial cells. When this occur these reactive mesothelial cells may constitute an important source of diagnostic error.

Malignant mesothelioma of the tunica vaginalis is a rare occurrence, but when this occurs the cytology is a very similar to those of mesotheliomas occurring in the pleural cavity. Testicular neoplasm very rarely malignant cells from testicular neoplasm may be encountered in the hydrocele fluid in which case the morphology of the cells is similar to that of the primary neoplasm. Seminomas rarely exfoliate as they are confined to the tunica vaginalis.

SYNOVIAL FLUID

The SF analysis is one of the important diagnostic tests in medicine, yet it is not routinely practiced in most laboratories. Cytology of SF alone is of little value. Therefore, this is one fluid where a careful complete examination including the gross appearance, microscopic examination of wet and stained preparations, chemical examination and if possible, immunological studies is more beneficial for the differentiation of various types of arthritis.

From a practical point of view, a meticulous SF analysis supplemented by a biopsy of a synovial membrane is a useful aid in the evaluation of any rheumatic condition; as well as in its follow-up. The SF is a crystal clear pale yellow or straw-colored viscous fluid that does not clot in its normal state. This fluid has been named 'synovia' by Paracelsus in the 16th century for its apparent resemblance to egg white. The SF differs considerably from serous

cavity fluids, which are plasma ultrafiltrates and also from CSF, which is mostly a secretory product of the choroid complex. The SF is also a plasma ultrafiltrate, but with a hyaluronate-protein complex secreted by the synovial cells.

Procedure of Arthrocentesis

Joint aspiration should be performed under aseptic precautions only by an experienced operator. Almost any joint can be aspirated. With no effusion, a dry tap is a common finding. The usual approach to the joint space is from the extensor surface where the major blood vessels and nerves are sparse and the synovial pouch is most superficial. There are no absolute contraindications to SF aspiration.

Characteristics of Normal Synovial Fluid

The amount of SF in normal joint is quite limited and varies 0.1–3 mL. It is crystal clear and is relative acellular, making it possible to read printed matter through it. The white cell count ranges from 0 to 200/cu mm most of these being lymphocytes and monocytes. The count is remarkably constant in spite of marked peripheral blood leukocytosis. The total protein-content is less than that of serum and averages 1.8 g/dL. The fluid fails to clot even in the presence of blood because of the presence of plasminogen activator. Enzymes such as amylase, protease and lipase are also found in SF.

Examination of Synovial Fluid (Table 11.3)

As much SF as possible should be removed from the joint space and can be collected in tubes for the following purposes:

- Non-sterile tube containing heparin for gross examination, cell counts, mucin test and crystals
- Non-sterile tube with no anticoagulant for cytological examination
- Non-sterile tube with no anticoagulant for serological studies
- Sterile tube containing heparin for bacterial culture.

Gross Examination

1. **Clarity:** Can print be read through it?
2. **Turbidity:** The higher the leukocyte count (due to inflammation), the more turbid is the fluid.
3. **Color:**
   - Yellow: Osteoarthritis
   - Bloody: Traumatic and septic arthritis
   - Xanthochromia: Traumatic and degenerative joint diseases
   - Milky: Gout and pseudogout
   - Greenish: Rheumatoid arthritis.

TABLE 11.3: Synovial fluid analysis in health and disease

Diagnosis	Appearance	Viscosity	Mucin clot	WBC* count % polymorphs	Crystals	RA† cells	Bacteria	Other
Normal	Crystal clear yellow	High	Good	< 200 WBC < 25%	0	0	0	
Noninflammatory:								
• Traumatic arthritis	Bloody or xantho-chromatic (clear yellow)	High	Good	< 2,000 WBC < 25%	0	0	0	Intercellular fat +/-
• Osteoarthritis		High	Good	< 1,000 WBC < 25%	0	0	0	Fragments of cartilage
Inflammatory:								
• Non-infective RA	Turbid, yellow, greenish	Low	Poor	< 15,000–20,000 WBC 75%	Cholesterol crystal	++	0	
• Gout	Turbid, yellow to milky	Low	Poor	10,000–12,000 WBC 60–70%	Urate crystal	0	0	
• Pseudogout	Slightly cloudy/ yellow	Low	Good to poor	10,000–12,000 WBC 25–50%	Calcium pyrophosphate	0	0	
SLE‡	Slightly turbid to yellow	High	Good	5,000 WBC < 10%	0	+/-	0	
Inflammatory:								
• Infective septic arthritis	Turbid, purulent, bloody	Low	Poor	80–20,000 WBCs > 75%	0	0	0	Intracellular fat +/-
• Tuberculous arthritis	Turbid, yellow	Low	Poor	25,000 WBCs 50–60%	0	0	+	

*WBC, white blood cell; †RA, rheumatoid arthritis; ‡SLE, systemic lupus erythematosus.

4. **Viscosity:** This is tested by the drop test. This is performed either by taking a drop of fluid on a slide and pulling out a string from it with the help of a gloved finger or the tip of a syringe. Alternatively drop by drop of the fluid can be ejected with a syringe and the length of the string between two drops noted. An Ostwald viscometer can also be used to compare the viscosity of SF to water:
   - High viscosity (long strings): Normal SF, osteoarthritis, traumatic effusion and systemic lupus erythematosus (SLE)
   - Low viscosity (drops such as water): Rheumatoid arthritis, gout and septic arthritis.
5. **Mucin content (Mucin clot test):** Addition of dilute acetic acid (5%) drop wise to a test tube containing SF forms a clot. Shake after 1 minute to determine friability of the same. The consistency of the clot varies with different diseased conditions:
   - Firm, hard, ropy, non-friable clot (high-mucin content): Seen in normal SF, traumatic SF, osteoarthritis and SLE
   - Loose, friable or flocculent precipitate (low-mucin content): Seen in rheumatoid arthritis, gout and septic arthritis.

Microscopic Examination

Cell counts

The use of ordinary white cell diluting fluid for SF is impractical, since its acidic properties would lead to mucin clot formation. Hence, cell counts need to be performed by using Neubauer counting chamber with saline solution using methylene blue for nuclear staining.

Normal count: Up to 200 cells/cu mm; 25% of these being polymorphs. Leukocytosis occurs in inflammation.

Abnormal count: For various conditions based on abnormal count, refer Table 11.3.

The differential leukocyte count is primarily helpful in determining the degree of inflammation, since a predominant neutrophilic response is seen in highly inflammatory fluids, most notably septic arthritis. A marked SF eosinophilia may be seen:

1. Secondary to an allergic reaction.
2. In parasitic infections, especially filariasis where careful examination of the SF fails to demonstrate microfilaria. Such cases, however, respond to antifilarial therapy.
3. In reaction following the injection of dye for arthrography.
4. In certain types of malignancies.
5. Occasionally in systemic illnesses such as acute rheumatic fever.

Wet mount preparations

Wet mount preparations are needed for the identification of formed elements such as crystals, cartilage fibrils and 'special cells'. The type of crystal identification is not possible during regular staining as the crystals are dissolved due to water. A wet mount comprises of examination of a drop of a

fresh unstained specimen of SF on a slide covered by a coverslip, the edges of which are sealed with either nail polish or correcting fluid. It is examined under an ordinary light microscope at a magnification of 400–1,000 times with a lowered condenser; followed by examination with a polarized microscope. In the absence of a polarized light microscope, the insertion of polarized disks into the ocular and condenser of a standard light microscope is satisfactory for polarized light viewing.

A polarizing microscope has a retardation plate, which is placed between the polarizer (filter that is placed between the substage condensers) and analyzer (filter that is placed between objective and eyepiece); the crystals will then appear yellow or blue depending on their direction in relation to the direction of the slow ray of the retardation plate.

Urate crystals are termed negatively birefringent, since their color is yellow when their long axis is parallel to that of the slow ray of the retardation plate whereas, calcium pyrophosphate crystals are positively birefringent and blue when aligned in the same direction. The SF for this purpose should not be collected with double oxalate as the calcium in the SF combines with the oxalate and may cause confusion with urate and calcium pyrophosphate crystals (Fig. 11.5).

Six types of crystals: It can be identified in various types of arthritis, which are as follows:

1. **Urate crystals:** These are birefringent crystals and appear as short rods with rounded ends. Rarely, they are needle-like. They are pathognomonic of gouty arthritis.
2. **Calcium pyrophosphate dihydrate crystals:** These are rhomboid forms with sharp corners. They appear in chondrocalcinosis or pseudogout.
3. **Apatite crystals:** These are sometimes considered as a cause of synovitis and appear as shiny crystals.

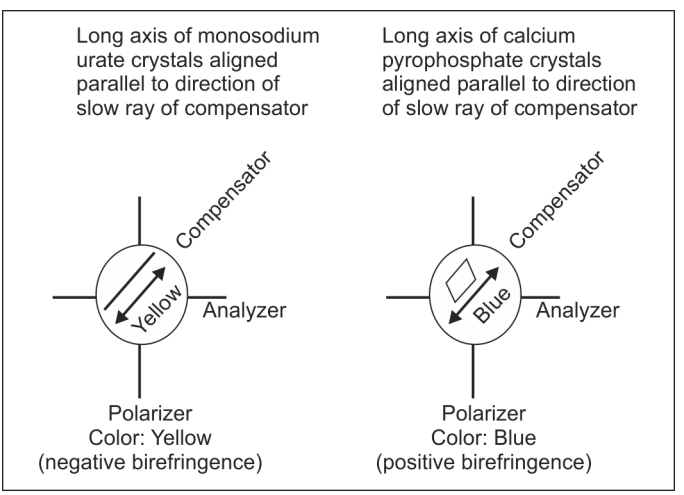


Figure 11.5: Polarizing microscopy for examination of crystals in synovial fluid

4. **Cholesterol crystals:** These are large, plate-like, flat with sharp corners and are mosaics of color.
5. **Talcum crystals:** Present as Maltese cross appearance and are small. Such crystals may produce arthritis when they are introduced during joint surgery.
6. **Steroid crystals:** These are pleomorphic and sometimes needle-like. They are positively or negatively birefringent.

Fragments of cartilage: It may appear birefringent, needle-like and are seen in osteoarthritis or traumatic arthritis.

The report should note whether the crystals especially urate and calcium pyrophosphate are lying free in the SF or have been injected by polymorphs. Intracellular location of crystals suggests that these are responsible for acute arthritis and if found extracellularly in the fluid, it is unlikely that they are responsible for an acute attack.

Examination of a Stained Smear

The principle types of cells present in the SF are as follows:

1. Polymorphs having morphology identical to those of blood, it may contain fat droplets, microorganisms and sometimes crystals.
2. Polymorphs in rheumatoid arthritis shows number of changes, which are suggestive of the disease. They are as follows:
   a. Hypersegmented nuclei and a marked vacuolated cytoplasm.
   b. Intracytoplasmic globules of DNA identified by Feulgen stain representing ingested nuclear debris from degenerative leukocytes.

Rheumatoid arthritis cells or rhagocytes or inclusion body cells: In an unstained wet preparation with an ordinary microscope, the SF show intracytoplasmic inclusions in polymorphs ranging from 0.5 to 2 μm in size. These are proteinaceous masses composed of immunoglobulins (IgG, IgM) and complement components. Immunofluorescent stains have shown the inclusions to the result of neutrophils taking up immune complexes from the fluid.

Lymphocytes: Identical to small lymphocytes of the blood.

Monocytes: Similar to that of blood, but may contain fat droplets and sometimes crystals.

Presence of synovial cells has no particular diagnostic significance. However, with Sudan Black staining, type A synoviocytes are stained positive and type B synoviocytes are stained negative.

Chemical Examination

An adjuvant chemical examination of SF is of great help in diagnosis. Glucose in normal SF is identical to or slightly less than plasma glucose. Since equilibration between blood and SF glucose is slow, samples of blood and SF should be obtained to note the difference between the two.

In non-inflammatory arthritis, the blood SF glucose difference is about 10 mg/dL. In inflammatory arthritis (e.g. septic, rheumatoid or tuberculosis), the glucose difference is about 25 mg/dL. Protein in SF is about 2 g/dL.

The concentration of it in SF also depends on plasma levels. It is markedly increased in rheumatoid arthritis:

- Uric acid concentration in SF does not play a role even in gouty arthritis
- Lactate and pH values are non-specific indices of inflammation
- Enzyme measurements appear to have little clinical value.

Immunological Studies

Include tests for:

- Rheumatoid factor
- Antinuclear antibodies
- Complement measurements
- LE cell phenomenon.

These tests, however, should not be considered as part of routine examination of SF.

Miscellaneous Points

1. In questionable cases, one may identify a given sample of fluid as SF, if the clot test is positive with 2% acetic acid and metachromasia is observed with toluidine blue.
2. A malignant effusion is encountered, when the synovium is infiltrated by carcinoma, sarcoma or leukemia.
3. Large vacuolated macrophages have been observed in the SF from cases with Whipple's disease and abnormal histiocytes in cases of histiocytic disorders.
4. Amyloid may be present within SF polymorphs in rare cases of amyloid arthropathy associated with multiple myeloma.
5. Intracellular fat droplets may be demonstrable with fat stains in case where there is a breach in the articular surface after trauma and in 'aseptic arthritis'.
6. To summarize, a careful complete examination of the SF may be considered for all practical purposes, a liquid biopsy from the site of inflammation.

CEREBROSPINAL FLUID

The CSF and other fluids of small volume have considerable bearing on diagnostic accuracy, larger the sample, the result is better. If several samples are obtained the second or third should be used for cytology. The addition of an equal amount of ethyl alcohol to the CSF is recommended, if a delay in processing is anticipated.

Main Indications for CSF Cytology

- The diagnosis of intracranial or spinal tumors, which are suspected to seed the CSF

- To confirm or rule out CSF involvement in lymphoma or leukemia and follow-up on treatment
- To determine the nature of cerebral infection.

Preparation of Material

Considering the low volume and cellularity, CSF specimen should be processed by cytocentrifugation, by using a Cytospin or by the slow sedimentation technique. The latter technique gives particularly good results.

Staining

All stains, alcohol and xylene should be filtered beforehand. This is done in order to avoid any possible 'floaters' from other specimens or the staining reagents. The error because of a false positive should be avoided at any cost.

A routine Papanicolaou staining technique is used except when special stains such as Gomori's methenamine silver (GMS) and periodic acid-Schiff (PAS) are used to demonstrate microorganisms. Air-dried smears may be stained with hematological stains such as Leishman or May-Grünwald Giemsa (MGG) when leukocyte morphology needs to be clearly seen.

Normal Cytology

The CSF is mostly acellular as the blood–brain barrier (BBB) prevents the entry of all cells except a few mononuclear cells, which are mostly lymphocytes and occasional monocytes. Ependymal and meningeal cells are very rarely encountered.

Changes in Benign Cell Population

1. Non-specific transformations of lymphocytes to immunoblasts and monocytes to macrophages occur in viral and tuberculosis meningitis.
2. Myelograms result in monocyte transformation and engulfment of yellow radiopaque material by macrophages.
3. Neutrophilic polymorphonuclear leukocytes, which never cross the BBB always indicate an acute inflammatory process.
4. Eosinophils occur as a consequence of parasitic infection.
5. The RBCs usually do not cross the BBB, are most commonly found in traumatic tap, a surgical intervention or in trauma. Hemosiderin indicates a past hemorrhagic event. An iron stain differentiates iron from melanin.

Other Cells

Plasma cells indicate either a chronic inflammatory event or multiple myeloma. Squamous cells or anucleate squames, bone marrow cells and cartilage cells originate from the skin or vertebral bone, which may be inadvertently entered during the procedure.

Malignant Tumors

Primary

With the exception of medulloblastoma and related tumors, the CSF is rarely studied for the diagnosis of primary brain tumors. This family of tumors is characterized by tumor cells, which resemble primitive neurons, often arranged in rosettes around a central lumen-like area filled with neurofilaments. These highly malignant tumors, which occur in children are capable of metastasis and are the only tumors of the CNS, which are consistently shed into the CSF.

In CSF, the tumor cells are usually numerous and readily identified as malignant. The cells remain fairly monotonous and have hyperchromatic nuclei with visible nucleoli. The scanty cytoplasm is sometimes elongated on well-fixed preparations. The cells may occur in clusters, singly or formed rosettes. These cells cannot be distinguished from neuroblastoma and can mimic a lymphoma.

Metastatic

The recognition of metastatic tumors particularly lymphomas/leukemias and solid tumors is the most important task in most cytology laboratories. This has assumed more importance recently because:

1. In the setting of acquired immunodeficiency syndrome (AIDS) the development of cerebral or meningeal lymphoma is a feared and common complication.
2. Malignant lymphoma with central nervous system (CNS) involvement is also seen in organ transplant recipients with associated immunosuppression.

A small increase in the number of lymphoid cells under appropriate clinical circumstances calls for careful cytological evaluation. In acute leukemia and chronic leukemia in blast crisis the blast cells are identified in Pap stain because of their large size (two to four times that of normal lymphocyte) and the presence of nucleoli and nuclear protrusions (the latter being found also in malignant lymphoma). Hematological stains (MGG or Leishman) may be used on dried CSF smears to observe nucleoli more clearly. The CSF involvement in chronic myelogenous leukemia is very uncommon.

One of the goals of CSF cytology is to monitor the effects of therapy particularly in children, in whom this may be an occult site of involvement when the peripheral blood and bone marrow show a good response to therapy.

Major Malignant Types

Malignant lymphoma: Cerebromeningeal involvement in non-Hodgkin's lymphoma is sufficiently frequent to warrant examination of CSF. Cells generally lie singly with nuclei showing irregular contours and protrusions in the form of small tongues. Large-cell lymphomas have in addition prominent

large and irregular nucleoli. False-positive diagnosis could be made in cases of viral and fungal meningitis. Sequential samples of CSF should be examined to monitor treatment.

Plasmacytoma: A diagnosis of plasma cell myeloma should be considered, when plasma cells form the sole population of cells in CSF sediment. Isolated plasma cells may, however, be observed in Hodgkin's disease and in chronic inflammatory processes.

Epithelial tumors: The most common identified metastatic carcinomas in CSF are of mammary and bronchogenic origin.

Bronchogenic (small cell carcinoma): A good response of the primary tumor and local metastasis to intensive chemotherapy regime still fails to prevent cerebral and meningeal involvement. Cytological detection of tumor cells in CSF at an early stage has led to regimes of aggressive treatment. Small cell carcinomas are often shed singly or in clusters. Sometimes the cells are arranged in short chains with nuclear molding resembling a string of vertebrae. Adenocarcinomas and epidermoid carcinomas shed large tumor cells, often in clusters.

Mammary carcinoma: Ductal types are readily recognizable and may show cytoplasmic protrusions. Cells have large nuclei, prominent nucleoli and sometimes exhibit mitotic activity.

Inflammatory disease: As the identification of leukocyte cell types is important in inflammatory diseases, air-dried smears stained with hematological stains are very useful.

Acute bacterial meningitis: It may be caused by various organisms; CSF in this condition shows a predominance of neutrophilic polymorphonuclear leukocytes.

Viral meningitis: The CSF cytology is characterized by a population of activated lymphocytes. Polymorphonuclear leukocytes and macrophages are absent.

Tuberculosis meningitis: In the early stages, the CSF is rich in a panorama of cell, which includes transformed lymphocytes, plasma cells, activated macrophages and polymorphonuclear leukocytes. With therapy, the polymorphonuclear leukocytes decreases lymphocytes persist. Multinucleated giant cells have also been seen.

Fungal meningitis: The most common fungus causing meningitis in the immunocompromised or debilitated is *Cryptococcus neoformans*. These are round yeast organisms measuring 4–10 μm with thick mucoid capsules, which stain readily with mucicarmine, PAS or India ink preparations. Other fungi such as *Candida albicans (C. albicans)*, *Aspergillus* and *Mucor* have also been observed in the immunocompromised.

URINE

Urine microscopy consists of the observing of formed elements such as the epithelial cells, RBCs, leukocytes and study of casts and other elements such as crystals and amorphous material. What is realized is that epithelial cells

and circulating blood cells are normally found in small numbers in urine and that the numbers in which these cells are found vary considerably from laboratory to laboratory depending on the methodology. The cells found in normal urine come from either the desquamation of lining the urinary tract epithelium or from the circulating blood. Cells of the circulating blood include leukocytes and RBCs. Casts on the other hand are formed in renal tubules and ducts.

Indications

A urine examination may be done in:

1. The investigation of a suspected case of urinary tract infection.
2. The investigation of symptomatic patients in urinary tract cancers.
3. The investigation of asymptomatic patients in high-risk groups, e.g. aniline dye workers and other industrial workers.
4. Follow-up of patients in known cancers of urinary tract.
5. Low-grade papillary tumors and flat dysplasias can be detected using urine cytology.

Advantages

The main advantage of this procedure is the non-invasive nature of the investigation, thus enabling it to be repeated any number of times with ease. An early morning sample provides the maximum number of exfoliated cells, however better morphological details are obtained on a random sample.

Preparation

The urine specimen should be examined, while fresh as cells and casts begin to lyse within 1–3 hours. Refrigeration at 2–8°C helps to prevent the lysis of pathological entities. Laboratory specimen preparation methods include cytocentrifugation, Cytospin method, slow sedimentation technique, membrane filter and monolayer preparations. Cytocentrifugation is the most basic method, where the urine is centrifuged to concentrate the cells, as it normally contains few cells. Centrifuge the urine for 10 minutes (1,500 rpm) and place one or two drops of sediment on a glass slide, spread the material and fix immediately.

Method of Examination

For the examination of urinary sediment, unstained and stained preparations are used.

Examination of an Unstained Preparation

In order to obtain sediment of a fresh sample (passed 1–3 hour earlier) an unstained preparations should be used. Cell lysis will thus be avoided. The sample should be mixed, poured into a 10 mL graduated centrifuge tube and

centrifuged at 1,500 rpm for 10 minutes. The supernatant is decanted and the sediment resuspended in 0.5 mL of urine. A drop of this placed on a slide and cover slipped as an unstained preparation. Identification of leukocytes (neutrophils, eosinophils and lymphocytes), histiocytes, renal epithelial cells, viral inclusion cells, neoplastic cells and cellular casts may be difficult in unstained preparations; as such examination of these on urine sediment may be performed by:

1. **Bright field microscopy:** Is examination of urine sediment under subdued light using a bright field and condenser to delineate the more translucent elements of the urine such as hyaline casts, crystals and mucus threads.
2. **Phase contrast microscopy:** In this microscopy the use of an annular diaphragm in the condenser and an annular phase plate in the objective results in the separation of diffracted and non-diffracted light rays. The diffracted rays are retarded by the specimen in contrast to those that pass through the surrounding media. The resulting interference of these rays provides a darker image contrast, which helps in revealing even small details within unstained cells. This is used for rapid screening of the urine, where contrast between epithelial and non-epithelial cells can be well-made out. Atypical cells can also be picked up by a trained observer and the specimen later subjected to permanent staining. Phase contrast microcopy is also used for the detection of more translucent formed elements of the urinary sediments and is preferable to bright field microscopy.

   By these methods the red cells appear as refractile gray biconcave disks, while the white cells appear granular with lobulated nuclei. Usually epithelial cells of the tubules and urinary tract cannot be distinguished from blood leukocytes. In order to make this differentiation clear, the nucleus can be brought more vividly into view by allowing a small drop of glacial acetic acid to run under the cover glass. Epithelial cells have a single round nucleus and leukocytes show segmented nuclei. The name 'glitter cells' is given to neutrophils with many cytoplasmic particles, which are characteristic of chronic pyelonephritis. When stained with gentian violet these appear pale blue and contain refractile granules that exhibit 'Brownian movement'. Casts are best seen at the edge of the coverslip.
3. **Polarizing filters:** These are best used to distinguish crystals and fibers from cellular and protein cast material. With the addition of a retardation plate crystals may be further identified as being positively or negatively birefringent. The details of the polarizing microscope have already been given in the section on SF.

Examination of Stained Smears of Urine

The permanent stains used on smears prepared on urine sediment are Papanicolaou stain, H&E stain, etc. The urinary sediment may also be viewed as a stained wet preparation. The stain commonly used for this is made up as

solution I and solution II, and mixed just before use as 3 parts of solution I and 97 parts of solution II. These are mixed and filtrated. The mixtures should be discarded every 3 months. Keep solution I and II indefinitely at room temperature, but stain precipitation occurs in highly alkaline solution:

- Solution I:
  - Crystal violet: 3 g
  - 95% ethyl alcohol: 20 mL
  - Ammonium oxalate: 0.8 g
  - Distilled water: 80 mL.
- Solution II:
  - Safranin O: 1 g
  - 95% ethyl alcohol: 40 mL
  - Distilled water: 400 mL.

Add one or two drops of crystal violet and safranin stain to 1 mL of concentrated urine sediment. Mix with pipette and place a drop of this suspension on a slide and coverslip. Methylene blue and toluidine blue may also be used as simple quick supravital stains. The clinical significance of cells, bacteria, fat, casts and crystals in the urinary sediment is given in Tables 11.4 to 11.6. The urinary findings of the sediment in glomerulonephritis and pyelonephritis are given in Table 11.7.

Cytology

Normal Urine

1. **Cells:** Vary greatly in size.
2. **Superficial cells:** They are also called 'umbrella cells' and usually seen as flat or polygonal cells with a convex smooth border, which corresponds to the lumen of the bladder. They show a lace-like cytoplasm by a Romanowsky stain and may contain more than one nucleus.
3. **Deeper cell:** When fresh have a granular nucleus and single round nucleolus.
4. **Background:** Clear, free of leukocytes with an occasional erythrocyte.
5. **In women:** Admixture of squamous cells because of vaginal contamination and the cells derived from vaginal type of epithelium from the trigone of the bladder.
6. **Catheterized specimen:** Rich in squamous cells.

Cytology of Urine in Non-Specific Chronic Inflammation

1. The RBCs.
2. Increased epithelial cells and degenerated cells.
3. Atypical cells are variable sized nuclei with irregular margins. However the nuclear chromatin lacks the features of malignancy.
4. 'Decoy cells' or 'comet cells' with tail-like cytoplasmic processes and homogenous degenerated nuclei simulating carcinoma cells.
5. Leukocytes, bacteria and debris suggesting inflammation.

TABLE 11.4: Clinical significance of cells, bacteria and fat in urinary sediment

Cells	Appearance	Origin	Normal values	Significance
Red blood cells	Refractile gray biconcave disks	Anywhere in genitourinary tract	An occasional RBC is seen in normal urine Addis count: 0–500,000/12 hour	Disorders of genitourinary (GU) tract, e.g. acute nephritis, cystitis, pyelonephritis and calculi; tumors of GU tract Systemic diseases such as hemorrhagic diseases, systemic lupus erythematosus (SLE) Drugs: Anticoagulant therapy Large doses of aspirin
White blood cells	Granular cells with lobulated nuclei; the granules occasionally show Brownian movement; also refer 'glitter cells'	As above	3–5/high power field Addis count: 0–1,000,000/12 hour	Infection of urinary tract Non-infectious inflammatory disease, e.g. because of drugs
Renal epithelial cells	Granular cells with oval nuclei	Kidney	A few cells can occur in normal urine Addis count: 750–330,000/hour	Recovery from acute tubular necrosis Tubular damage because of other causes, e.g. secondary to parenchymal disease, large doses of analgesics
Bacteria	Solid gray rods or chains of cocci	Anywhere in the GU tract	Sterile	Infection of the GU tract
Fat bodies	Shiny globules	Degeneration of tubular cells	Not seen	Nephrotic syndrome Diabetic glomerulosclerosis

TABLE 11.5: Casts and its significance [normal range: 0–5,000/12 hours (Addis count)]

Type of cast	Appearance	Composition	Origin	Formation	Clinical significance
Hyaline cast	Clear cylinder	Mucoprotein	Distal tubules	Urinary pH acid Increase in protein concentration and coagulability	Occasionally found in normal urine, febrile illness after strenuous exercise, congestive cardiac failure (CCF)
Red cell cast	Clear cylinder filled with red blood cells (RBCs)	Mucoprotein + RBCs	As above	Renal parenchymal disease (for RBCs in urine)	Acute glomerulonephritis; subacute bacterial endocarditis with embolization Renal vein thrombosis and allergic vasculitis
White cell cast	Clear cylinder filled with white blood cells (WBCs)	Mucoprotein + WBCs	As above	Renal infection (as for pus cells in urine)	Pyelonephritis, systemic lupus erythematosus Interstitial nephritis because of analgesics
Epithelial cell cast	Clear cylinder filled with epithelial cells	Mucoprotein + epithelial cells	As above	Sloughing of tubular epithelium	Tubular necrosis, tubular damage because of parenchymal disease or analgesic overdosage
Granular casts	Cylinder with coarse or fine granules	Possible degeneration of cellular casts (refer white and epithelial cell cast)	Distal tubules	Parenchymal disease	Chronic renal disease, diuretic phase of acute renal failure, removal of obstruction
Waxy casts	Smooth, refractile	Possible degeneration granular cast clusters	As above	Chronic parenchymal disease	Same as above
Fatty casts	Cylinders with oval bodies	Protein with cholesterol esters	As above	Tubular fatty change	Nephrotic syndrome, diabetic glomerulosclerosis
Broad casts	Similar to narrow casts		Collecting tubules		End stage

TABLE 11.6: Crystals in normal and abnormal urine

Type of crystal	Structure	Urine pH	Seen in
In normal urine			
Calcium oxalate	Small, colorless octahedrons, resembles envelope, insoluble in acetic acid	Acid/Neutral	Chronic renal disease, ethylene glycol or methoxyflurane toxicity and oxaluria
Uric acid and urates	Four sided, flat, yellow or reddish brown; other shapes rhomboid plates, oval forms, wedges, rosettes, irregular plates; they polarize and show interference colors	Acid	Indicate nucleoprotein turn over chemotherapy of leukemia/lymphoma, stones in ureters, gout (urate nephropathy)
Phosphate	Variation in size; colorless, 3–6 sided prisms with oblique ends referred to as coffin lids; flat forms and fine sheaves (calcium phosphate)	Alkaline	Have little clinical significance Often seen in infected urine of alkaline pH
Dicalcium phosphate	Colorless prisms arranged in stars and rosettes 'stellar phosphates'	Alkaline, acid or neutral	Have little clinical significance May be seen in bladder stones
Ammonium biurate	Opaque yellow crystals usually seen as spheres with fine or coarse spicules; they dissolve on addition of acetic acid	Alkaline	Decomposing urine
In abnormal urine			
Cystine	Colorless, refractile, hexagonal plates, sometimes twinned; soluble in water, resembles uric acid crystals, but dissolves in dilute HCl, whereas uric acid does not	Acid	Cytsinuria
Tyrosine	Fine silky needles arranged in sheaves or clumps; soluble in dilute, HCl, but not in alcohol	Acid	Tyrosine and leucine occur together in severe liver damage
Leucine	Yellow oily appearing spheres with radial and concentric striations Soluble in acids and alkalis; unlike fat globules, leucine is not soluble in either and may be differentiated from fat	Acid	Tyrosine and leucine occur together in severe liver damage
Sulfonamide	Various forms are seen depending on the form of drug used; sheaves of wheat with central binding arrow heads, petals, needles, etc.	Acid	Drug therapy
Ampicillin	Long fine colorless crystals, coarse sheaves on refrigeration	Acid	Drug therapy

TABLE 11.7: Urinary findings

Urinalysis observations	Acute glomerulonephritis	Chronic glomerulonephritis	Acute pyelonephritis	Chronic pyelonephritis
Volume	Oliguria	Increased	Oliguria may be present	Increased
Color	Brown or smoky	Normal	Microscopic hematuria +/-	Normal
Specific gravity	High	Low: Fixed 1,010	High	Low
Protein	Present, usually less than 5 g/24 hour	Present	Present, less than 2 g/24 hour	Present
Sediment	Red blood cells are present in large numbers	Red blood cells +/-	Red blood cells + Leukocytes are present in large numbers	WBC +/- Epithelial casts +/-
Casts	Red cell casts: Present	Granular casts and waxy casts: Present in large numbers	Leukocyte casts: Present	Leukocyte casts + RBCs and RBC casts are infrequent Glitter cells present

Specific Inflammations

1. **Fungal inflammations:** The most common fungus observed in the urinary sediment is *C. albicans*. In the urine, organisms are observed commonly as fungal spores, but pseudohyphae may occasionally be observed.
2. **Viral infections (cytomegalovirus):** Epithelial cells are markedly enlarged and carry within their nuclei very large conspicuous basophilic inclusions surrounded by clear halos:
   - Multinucleation
   - Seen commonly in AIDS patients and renal transplant recipients.
3. **Herpes simplex virus:** It may occasionally be detected in urine sediments with multinucleated epithelial cells, 'ground glass' appearances of the nuclei and occasionally eosinophilic intranuclear inclusions.
4. **Polyoma virus (rare infection):** The virus forms single dense basophilic homogeneous intranuclear inclusions that fill the enlarged nucleus completely.
5. **Decoy cells:** This is identified in inflammatory urine and may reflect an infection with polyomavirus.
6. **Parasitic infections (schistosomiasis):** Numerous squamous cells, ova rarely seen.
7. **Trichomonas vaginalis:** Organism may rarely be seen in urine of females.

In Malakoplakia

Malakoplakia are cells with single or multiple Michaelis-Gutmann bodies. These may vary in size and may be calcified and resemble small psammoma bodies. Others may take a uniform H&E stain. Such cells appear in the urine more commonly after a biopsy as malakoplakia is separated from the lumen by normal bladder epithelium.

In Carcinoma

Early changes
Early changes are variation of nuclear size, nuclear enlargement and mild hyperchromasia. Such cells appear intermittently and for a close follow-up. Such patients may show a flat or papillary lesion of low grade malignancy. Occasionally the urine sample may be negative.

Stages of positive cytology
Carcinoma may be associated with papillae or non-papillary clinically obvious bladder cancer or with carcinoma in situ (sessile). It becomes important to particularly detect the latter condition as one fourth of the patients with carcinoma in situ of the bladder are completely asymptomatic. About 75–90% of bladder carcinomas can be categorized as being transitional in origin. The cytology would therefore show in differentiated tumors, cells similar to transitional epithelial cells with little evidence of malignancy may give rise to false-negative results. However, the presence of numerous cells in a freshly voided random sample is unusual and should raise the suspicion of a low-grade lesion. In frank carcinomas and undifferentiated tumors:

- Cells appear in groups and vary in size and shape
- Nuclei in groups; display striking abnormalities of chromatin pattern with clumping and marginal condensation of chromatin.

The cellular differences between low-grade and high-grade urothelial neoplasms are given in Table 11.8.

TABLE 11.8: Cellular difference on low- and high-grade urothelial neoplasm

Cells	Low grade	High grade
Arrangement	Papillary and loose clusters	Isolated and loose clusters
Size	Increased, uniform	Increased, pleomorphic
Number	Often numerous, resemble urothelium	Variable
Cytoplasm	Homogenous	Variable
Nucleocytoplasmic ratio	Increased	Increased
Nuclei		
Position	Eccentric	Eccentric
Size	Enlarged	Variable
Morphology	Variable in aggregates	Variable
Borders	Irregular, notches, creases	Irregular
Chromatin	Fine, even	Coarse, uneven
Nucleoli	Small, absent	Variable

The features of malignant cells arising from a transitional cell carcinoma of the ureter or the renal pelvis are essentially similar to those of malignant cells from a transitional cell carcinoma of the bladder.

Squamous cell carcinoma: It is identified by the exfoliation of fairly mature cell recognizable as being squamous in origin with elongated, spindled and fusiform configurations.

Primary adenocarcinoma: It is rare; cells are similar to that occurring elsewhere in the body. Cells tend to cluster and have a poorly stained lucent vacuolated cytoplasm. The chromatin tends to aggregate along the smooth nuclear borders resulting is so called 'clear areas.'

Reasons for false-positive diagnosis of malignancy

1. **Radiation therapy**:
   a. Nuclei are enlarged, show pyknosis and karyorrhexis.
   b. Multinucleation.
   c. Vacuolated cytoplasm.
   d. Enlarged nuclear-cytoplasmic ratio may also be observed.
2. **Drug-induced changes**: Certain alkylating agents such as thiotepa, Endoxan, busulfan and cyclophosphamide administered for the treatment of cancer, exercise is a marked effect on the epithelium of the urinary bladder:
   a. Cell enlargement.
   b. Hyperchromatic nucleus; chromatin is coarse, but uniform in distribution.
   c. Multiple nucleoli.
   d. Nuclear pyknosis and karyorrhexis.
   e. Changes may mimic carcinoma closely.
3. **Urinary calculi:** Abnormal cytological changes with alteration in shape and size of epithelial cells with nuclear hyperchromasia, prominent nucleoli and thickened nuclear membranes. Several 'umbrella cells' may be seen because of frequent stone abuse of epithelium. Many of these are multinucleated.
4. **Papillary aggregates:** These occur in specimens obtained as a result of instrumentation when von Brunn's nests get avulsed and appear as papillary processes. A false-positive diagnosis occurs in mistaking these as a reliable sign of low-grade neoplasia.

Reason for false-negative diagnosis of malignancy

1. **Low-grade neoplasms:** In the absence of a tissue pattern, which includes a delicate fibrovascular stalk, many papillary transitional cell tumors may be compose of cells, which differ so little from normal that they can hardly be considered as neoplasms.
2. **Prolonged exposure of cells to urine:** This has detrimental effect on interpretation. Exfoliated cells enter a hostile environment of high acidity and low osmolality, which may obscure essential diagnostic features.

CYTOLOGY OF THE GASTROINTESTINAL TRACT

Cytology of gastrointestinal tract is a technique, not universally applied in diagnosis of the lesions of the gut. It is usually carried out in institutions,

where gastroenterologists and cytologists work in close association. The dawn of this dates back to as early as 1910, when Giovanni Marini illustrated by drawing benign and malignant cells that he encountered in gastric washings. The inability to distinguish a high-grade dysplasia from carcinoma in situ and invasive carcinoma by experienced cytologists have resulted in a reluctance to accept this procedure for definitive diagnosis; however, the combined use of cytology and biopsy during endoscopic procedure have improved greatly the scope of cytology as an adjuvant to biopsy in arriving at a diagnosis. Cytology specimens have an advantage over biopsy specimens as they sample a wider surface area of the mucosa.

Indications for Doing Gastric Cytology

1. In the diagnosis and detection of early cancers of oral cavity, esophagus, stomach and intestine.
2. In diffuse superficial spreading cancers of the stomach where no mucosal abnormality is seen grossly, then brushings yield malignant cells.
3. Inflammatory conditions, e.g. fungal and viral esophagitis.
4. In lymphomas.
5. Bile cytology in obstructive biliary stenosis to detect malignancy.
6. The FNAC of gastrointestinal lesions plays a vital role in diagnosis and helps to differentiate intra-abdominal visceral from retroperitoneal lesions.

Methods and Techniques in Gastric Cytology

It may be emphasized that it is one branch of cytology, where success in cytology is 80% preparations and 20% interpretation.

Lavage or Washing of the Organ Under Suspicion

Washing of the organ under suspicion is one of the ways of obtaining exfoliated cells for study. Generally a gastric lavage is performed. Blind gastric washings may be performed on patients using 250 mL or 300 mL of buffered saline solution introduced through a plastic or rubber tube with several holes at the bottom and passed through the patient's nose or mouth. After passage of the buffered saline, the abdomen is massaged and the patient made to flex or extend the body to induce exfoliation of cells. The washings are then aspirated and smears made from the sediment after centrifugation. This technique is however superseded by endoscopically directed brush cytology. The procedure may also be used in large bowel cytology through a colonoscope/sigmoidoscope.

Balloon Cytology

Balloon cytology is used to study esophageal samples. In this method, an introducing tube is swallowed to just beyond the cricopharynx (15 cm from teeth). The introducing tube is stiffened by an internal style, which is then

removed to pass a special catheter with a deflated balloon, through the cardioesophageal junction (40 cm from teeth). The balloon is inflated with about 5 mL of air and then passed up and down the esophagus at the desired level, three times causing slight patient discomfort. After deflation the balloon and catheter are withdrawn. The collapsed balloon is taken and the cells are transferred from it onto glass slides, immediately fixed in 95% alcohol and stained. The morphology is excellent and interpretation relatively easy.

Endoscopically Directed Brush Cytology

After endoscopy, a brush sample may be taken and is combined with a biopsy. It is preferable to obtain a brush sample before the biopsy because the latter results in bleeding, which obscures the lesion and affects the quality of a subsequently collected cytological sample. Several ranges of brushes are available and enclosed within a transparent Teflon sheath. Multiple samples can be collected at a time. When taking a smear the brush is either plunged firmly and briskly into the mucosa 5–6 times so that the lamina propria is penetrated or just rolled firmly on the surface to obtain an adequate sample. The brush sample when obtained is pulled just back within the Teflon sheath and the whole sheath withdrawn. Endoscopically directed jet wash may also be used and is known to yield good results.

Fine Needle Aspiration Technique

The FNAC is usually performed either directly on palpable masses or under guidance. It can be used on all abdominal masses including upper GI tract, lower GI tract and mesenteric lesions. The standard technique using a syringe holder and a 10 cm^3 syringe is used. The aspiration is performed under negative pressure and is greatly aided by ultrasound or CT guidance. A direct or indirect smear technique is used for cellular and blood/fluid mixed aspirates respectively.

CYTOLOGY OF RESPIRATORY TRACT

Respiratory tract malignancies can be detected mainly by sputum cytology or by examination of material collected from bronchoscopy.

Sputum Cytology

Sputum specimens can be obtained from the patient either spontaneously or by aerosol–induced method. Morning specimen resulting from overnight accumulation of secretion yields best results. Induced method is indicated when the patient cannot produce spontaneous deep cough sputum. It includes inhalation for 20 minutes of a solution composed of 15% NaCl and 20% propylene glycol, heated at 37°C. Inhalation of this solution stimulates mucus production. Sputum should be examined for 3–5 consecutive days to ensure maximum diagnostic accuracy in suspected malignancies.

Preparation and Method

Sputum specimens are sent to the laboratory in a fresh state or in a prefixed state in 70% ethyl alcohol, or Saccomanno's fixative or in CytoLyt solution:

1. **Fresh unfixed specimen:** The specimen must be carefully inspected by pouring the specimen into a Petri dish and examining it against a dark background. The sample is examined grossly for tissue fragments and other suspicious areas. Smears are randomly sampled by picking up small fragments and spreading the specimen evenly between two glass slides until a thin uniform layer is formed. The slides are fixed immediately, in 95% alcohol and stained by the Papanicolaou stain.
2. **Prefixed sputum:** Sputum is expectorated into a wide mouthed jar half filled with 70% ethyl alcohol. Fragments are picked up and smeared on albumin or poly-L-lysine coated slides.
3. **Saccomanno technique:** Sputum is collected in a solution composed of 50% ethyl alcohol and 2% polyethylene glycol. This is subsequently broken-up in a food blender, centrifuged and the smears are prepared from the cell button.

Advantages

- Concentration of the cells is satisfactory
- Preparation of teaching slides.

Disadvantages

- Cell to cell relation is disrupted because of blending
- Tissue fragments with fungal elements, actinomycotic and botryomycotic colonies are disrupted
- Difficult to classify the type of malignant cells
- Increased risk of aerosol infection (especially tuberculosis).

ThinPrep

- Sputum is collected in a special preservative (CytoLyt solution)
- Multiple samples from one patient are collected in the same container.

Advantages

- Eliminates RBC's and mucus
- One slide is prepared for examination
- Reduces the fatigue factor
- Can be mailed to distant laboratories.

Disadvantages

- The only disadvantage is high cost.

Examination of Materials Collected from Bronchoscopy

Indications

Bronchoscopy is usually done:
- To evaluate lung lesions of unknown etiology
- Origin of unexplained positive sputum cytology
- To obtain microbiology material
- In diffuse and focal lung lesions
- Investigate unexplained paralysis of vocal cord, superior vena cava (SVC) syndrome and chylothorax.

Sample Collection

Fiberoptic bronchoscope permits the visualization of the lower respiratory tract and a variety of samples can be obtained.

Bronchial aspirate and washing

1. Bronchial aspirates are obtained by introducing the bronchoscope in the lower respiratory tract and aspirating the secretions by a suction apparatus.
2. Bronchial washings from the visualized areas are collected by instilling 3–5 mL of a balanced salt solution through the bronchoscope and reaspirating the same. Cytopreparation such as direct smear, centrifugation with smear preparation from cell buttons, centrifugation with embedding of the buttons as cell blocks (paraffin embedding and histological sectioning).

Satisfactory bronchial aspirates and washings include the presence of ciliated cells, squamous cells, goblet cells and alveolar macrophages.

Advantages

It is easier to evaluate the slide because of the lesser number of cells as compared to sputum.

Disadvantages
- The procedure is unpleasant for the patient
- It has to be performed by a highly skilled physician.

Bronchial brushings

Cell samples are obtained with small brushes from the surface of suspected tumor site under the visual central fiberoptic bronchoscope or by employing imaging guidance technique. Procedure generally performed prior to biopsy.

Method I: Brush is firmly rolled onto a series of glass slides, which are then fixed in 95% alcohol.

Method II: Agitating the brush in 5 mL of isotonic saline and centrifuging the fluid in the laboratory.

Advantages
- Samples a wider surface
- Allows the investigation of primary, secondary and tertiary bronchi.

Complications/Disadvantages

- Bronchospasm
- Laryngospasm
- Pneumothorax.

Bronchoalveolar lavage

Technique and processing

1. Performed via transnasal route under local anesthesia by 409 mm fiberoptic bronchoscope.
2. Wedged into a subsegmental brochus at the level of 4th or 5th branching.
3. About 0.9% sterile saline is instilled by a syringe and reaspirated in 20–50 mL aliquots. Repeated 5 times at each site.
4. Performed at site of maximum radiological opacity or middle lobe in case of diffuse disease.

Invalid procedure if:

1. Purulent secretions are present.
2. Volume recovered is less than 40% of volume infused. Used when the disease is in the alveoli:
   a. Opportunistic infections in immunocompromised patients.
   b. Interstitial lung diseases.
   c. Idiopathic pulmonary fibrosis.
   d. Hypersensitivity pneumonitis.
   e. Extrinsic allergic alveolitis.
   f. Asbestosis-related interstitial disease.
   g. Silicosis and berylliosis.

Fine needle aspirations

Transbronchial needle aspiration (TBNA) was first described by the Argentinian surgeon Eduardo Schieppati in 1949. A thin flexible needle is inserted through the bronchial wall into the suspected lesion via bronchoscope and the cellular material is aspirated and processed as for percutaneous biopsies. Specific modification of fine needle aspiration done under flexible bronchoscopic, fluoroscopic and CT guidance have been described in the late 1970s and 1980s. The TBNA is greatly dependent on skill of the operator and availability of cytopathology support for performing rapid onsite evaluation (ROSE) of the cytological sample.

Indications of TBNA

- External bronchial compression
- Upper lobe lesions
- Lung neoplasm, which has not breached the mucosa
- Hilar lesions, e.g. mediastinal tumors, Hodgkin lymphoma, sarcoidosis and other granulomatous diseases
- Intraluminal lesions, which are necrotic with submucosal (carcinoids, bronchial gland tumor) extensions
- In the staging intrathoracic malignancies.

Transthoracic fine needle aspiration cytology

A fine needle (with external diameter 0.6 mm, length 10–20 cm) is passed through the chest wall into the pulmonary and mediastinal mass, visualized

by fluoroscopy, CT or US guidance. The patient is asked to hold breath during the procedure. Core needle biopsies can also be performed and touch imprints or roll-preps of the core biopsies can also be used. The yield of transthoracic fine needle aspirations cytology (TTNA) is lower for central than for peripheral lesions.

Indications of TTNA
- Lung cancer
- Suspected infectious process
- Mediastinal lymphadenopathy.

Contraindications
- Debilitated uncooperative patients who have uncontrolled cough
- Vascular lesions and pulmonary hypertension
- Echinococcosis (contamination of adjacent lung tissue/hypersensitivity reactions).

Complications
- Pneumothorax
- Air embolism
- Hemothorax
- Hemoptysis.

Endoscopic ultrasound-guided fine needle aspiration

Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) was first described in early 1990s to obtain cytological material in pancreatic masses, but has become a popular method to diagnose a variety of intra-abdominal and intrathoracic masses. The EUS-FNA is preferred as lymph stations 2L, 4L, 7, 8 and 9 are only accessible through a transesophageal approach.

Indications
- It is cost effective in the initial mediastinal staging of non-small cell lung cancer (NSCLC) patients
- It is useful in biopsy of mediastinal masses
- It can be used even in patients with previous non-diagnostic conventional TBNA.

Complications
- Bleeding
- Infection.

Endobronchial ultrasound-guided needle aspiration

Endobronchial ultrasound (EBUS) provides real time imaging of mediastinal structures adjacent to major airways. Radial and linear EBUS are the two available modalities. Radial EBUS provides a 360° view of the airway wall and surrounding structures. It is used to determine whether a mediastinal tumor invades the airway and the surrounding structures (such as esophagus cancer invading the membranous wall of the airway), to help elucidate in a more precise way the extent of resection required in lung cancer.

INTRAOPERATIVE CYTOLOGY

The utility of cytological examination of smears and imprints has been long established in intraoperative examination, but remains underutilized because

of the traditional belief that frozen section is the best technique to diagnose lesions in such settings. Intraoperative cytology (IC) adds new dimensions to morphological interpretation and is essential in many instances to arrive at correct diagnosis.

Applications

The IC is used as an adjunct to frozen section and can be applied to any tissue. It is most helpful in the field of neuro-oncopathology, infections, lymphomas as well as epithelial tumors.

Methods for Acquiring Cytological Material from Specimens

Imprints

Imprints were developed by Dudgeon and Patrick in 1927 and serve as a rapid diagnostic procedure equal to frozen section with an accuracy rate of 95.28%. The entire procedure is completed within few minutes and can be performed whenever required before frozen section cutting and as a complementary procedure to it.

Imprint cytology gives excellent cytological clarity. Imprints are preferred for lymph node, breast biopsies and conjunctiva; very useful in the diagnosis of non-Hodgkin's lymphoma and their classification as cells are much better preserved and their differentiation easier. Imprints should always be interpreted in light of gross findings. A direct imprint is prepared by pressing a glass slide gently on to the freshly cut surface of the surgical specimen, avoiding a gliding movement, which will distort the shape of the cells. The imprint slide is immediately fixed in 95% ethyl alcohol for 5–6 seconds and then stained (rapid H&E stain). Toluidine blue can also be used for staining purpose.

Squash smear technique

Squash smear technique is a reliable rapid method for intraoperative diagnosis and is commonly applied in neurological diagnosis. This technique was introduced in 1923 and has a diagnostic accuracy of 83–94%.

Advantages

- Rapid intraoperative diagnosis of space occupying lesions of the nervous system can be made
- High degree of diagnostic accuracy
- Small amounts of tissue (1–2 mm) is required
- Better cellular detail and higher quality preparation as compared to frozen section
- Neurological toxoplasmosis and other viral infections can be diagnosed.

Limitation

Hard and fibrotic tissue can cause difficulty in obtaining crush preparations.

Technique

The smear is prepared by taking a small piece of tissue with a scalpel blade (less than 0.5 mm in diameter) and placing it on a slide, and subsequently

the specimen is smeared with another slide using gentle uniform pressure, holding the second slide above in approximation and at right angles to the first (similar to the routine FNAC procedure). It is important not to smear too large in a specimen, which may yield a slide too thick for optimal cytological detail. Recognition of the fine fibrillary processes that are often associated with glial tumors is dependent on thin smearing. The smears are immediately fixed in alcohol and stained with H&E stain.

Scrape: The cut surface of the freshly cut specimen is scraped with a sharp scalpel or the end of a glass slide, from the most appropriate site. A semifluid drop thus obtained is spread over a glass slide in the same manner as in FNAC. Fixation is done in 95% alcohol or in alcoholic acetic acid mixture (95% alcohol + 5% glacial acetic acid) for 2–3 minutes. Staining is done as usual on wet-fixed smears using rapid H&E and Pap stain, air-dried smears are subjected to Leishman or MGG. The slides can be examined immediately and reported as intraoperative procedure.

Advantages of IC Over Frozen Section

1. Rapidity of preparation with the same accuracy rate in interpretation as frozen section.
2. The simple and inexperienced method, not requiring any special instruments.
3. Excellent preservation of cellular details devoid of freezing artifacts.
4. Preservation of the remaining tissue in cases of very small samples avoiding freezing artifacts.
5. Possibility of identifying focal (macroscopically undetectable) neoplastic lesions in large tissue fragments.
6. Possibility of identifying adipose tissue or calcified tissues, unsuitable for frozen section.
7. Avoidable use of cryostat, when dealing with infective samples.
8. Ratification and promotion of cytology learning as a result of comparison with histological sections.

Limitations of IC Over Frozen Section

- Does not distinguish in situ from infiltrating carcinoma
- Depth of infiltration or margins of resection of tumors cannot be done
- In celomic neoplasms, does not distinguish between borderline and frankly malignant invasive tumors.

CONJUNCTIVAL IMPRESSION CYTOLOGY

As conjunctiva is a surface tissue, the various conjunctival disorders would seemingly lend themselves to appropriate simple examination techniques. Recently, conjunctival impression cytology (CIC) has emerged as a non-invasive outpatient diagnostic tool for the diagnosis of conjunctival disorders and their appropriate follow-up. It is of immense help in identifying

subclinical vitamin A deficiency cases in a population, where the prevalence rate of this deficiency is low and in patients with various ocular surface disorders and to diagnose early ocular surface changes before they become symptomatic. It is also useful in contact lens users and dry eyes. The CIC can also be studied in similar fashion with patients with epithelial cell storage diseases such as mucopolysaccharidosis, inactive trachoma, patients with open angle glaucoma who are on antiglaucoma therapy.

Preparation

The CIC is based purely on squamous epithelial and goblet cell abnormalities of the exfoliated cells from the conjunctival epithelium obtained by impression on a Millipore membrane filter paper of 0.025–0.22 μ. The paper strips, which are gas sterilized using ethylene oxide, are applied on the conjunctiva with the dull surface down. They have been marked at various anatomical regions such as bulbar, fornical and palpebral conjuctiva to obtain surface cells.

Procedure of Conjunctival Impression Cytology

Collection of the most superficial layers of the ocular surface is done by applying different collecting devices (usually filter papers), so that cells adherent to that surface and are subsequently removed from the tissue, and further processed for a diversity of techniques. It represents therefore a non- or minimally-invasive biopsy usually the conjunctiva. Impression cytology was first introduced in 1977 when it was noticed that absorbent filters (e.g. Millipore membrane filter VSWP 0.025 μm) would not only remove mucus secretions from the conjunctival surface but also sheets of epithelial cells, which included goblet cells, as well.

Once the specimen is obtained, the cells are fixed and then stained for analysis. In general terms, CIC involves the following. A piece of filter paper is applied to the conjunctival surface for approximately 2–5 seconds. Some authors recommend instilling anesthetic, but the procedure can be carried out without it. The filter paper is removed from the conjunctiva in a peeling motion to ensure maximal collection of surface cells. The cells are fixed by various means. The cells that are adherent to the filter paper are stained to enhance the visibility of the goblet cells with counter staining of the epithelial cells to increase the contrast of the goblet cells. The specimen is examined under a light microscope and various analyzes of the visible cells are conducted. Tissue fixation is a prerequisite to all cell and tissue staining procedures. Fixation is necessary for a number of reasons to inhibit autolysis and bacterial contamination, prevent solubility of those components that are of interest and provide conditions that will enhance the effects of various biological dyes. Fixatives are commonly used in CIC such as formaldehyde (formalin), glutaraldehyde, ethanol and methanol. Some authors make use of a spray fixative, but do not give specific names. The paper strips are gas sterilized.

Simple Grading System for the Changes on Cytology

The changes on cytology are graded as follows:

1. **Grade 0:** Epithelial cells are small and round with eosinophilic cytoplasm. Nuclei are large and basophilic with a nuclear-cytoplasmic ratio of 1:2. Goblet cells are abundant, plump and oval, and have intensely PAS-positive cytoplasm.
2. **Grade I:** Epithelial cells are slightly larger and are more polygonal, and have eosinophilic stained cytoplasm. Nuclei are smaller with nuclear-cytoplasmic ratio of 1:3. Goblet cells are decreased in number, but still are plump and oval, and have intensely PAS-positive cytoplasm.
3. **Grade II:** Epithelial cells are large and polygonal, occasionally multinucleated with variably staining cytoplasm. Nuclei are small with a nuclear-cytoplasmic ratio of 1:4 or 1:5. Goblet cells are markedly decreased in number, are smaller, less intensely PAS positive and have faintly-defined cellular borders.
4. **Grade III:** Epithelial cells are large and polygonal with basophilic staining cytoplasm. Nuclei are small, pyknotic and in many cells are completely absent. Nuclear/Cytoplasmic ratio is 1:6 or more. Goblet cells are completely absent.

FEMALE GENITAL TRACT

Cervical cancer is a leading cause of cancer deaths in women of many economically underdeveloped countries. Incidence and death rates are particularly high in Latin America, Africa, India and Eastern Europe. Squamous cell carcinoma and its intraepithelial precursor follow a pattern typical of sexually transmitted disease.

Cervical Intraepithelial Neoplasia

Cervical cancer screening relies on the fact that carcinoma of the cervix is preceded by intraepithelial changes known as cervical intraepithelial neoplasia (CIN) several years before it can be clinically manifest. As these changes rarely produce any specific obvious clinical alteration, its firm diagnosis can only be established by microscopic examination of tissues or cells derived from it. The detection of these precursor lesions by exfoliative cytology and therefore their prompt treatment is the basis of the screening programs. In the screening and reporting of cervical smears, a variety of classifications are used throughout the world. Criteria for the diagnosis vary somewhat between pathologists, the important characteristics of the lesion being—cellular immaturity, cellular disorganization, nuclear abnormalities and increased mitotic activity. The degree of neoplasia is determined from the extent of nuclear atypia, immature cell proliferation and mitotic activity. If this is present only in the lower one third of the epithelial thickness, the lesion is usually designated as CIN I. Involvement of the lower and middle

third is known as CIN II and full thickness involvement is CIN III. Foci of CIN I, II and III may coexist in the same lesion. The term cervical intraepithelial neoplasia as proposed by Richart in the year 1967 refers only to a lesion that may progress to invasive carcinoma. Changes of CIN are sometimes referred to as dysplasia, which a term first proposed by Papanicolaou in 1945 and later reintroduced by Reagan in 1953 as atypical hyperplasia. Because the term dysplasia also means abnormal maturation without mitotic activity the preferred term is cervical intraepithelial neoplasia.

Specimen Collection

The cytological specimens collected from the female genital tract (FGT) include cervical smears, vaginal smears and aspiration from the posterior fornix of vagina (vaginal pool smear) as well as endometrial smears.

Vaginal Pool Smear

The technique allows collection of cells under direct vision from the posterior fornix pool using an unlubricated speculum. It is a collection of exfoliation of cells from the vaginal wall, cervix, cervical canal, endometrium, using a Pasteur pipette. When a speculum is not employed the pipette is gently introduced in to the vagina until resistance is encountered.

Advantages

The advantages are that the smears can be obtained in the presence of an intact hymen. Vaginal smears are used for hormonal studies. The vaginal smear is efficient in the detection and diagnosis of endometrial cancers (but fails to detect nearly 50% of all precancerous lesions). Yet, it complements the cervical smear and offers several major advantages, particularly in women who past the age of 40 years. Cells from endometrium, the fallopian tube, the ovary and occasionally from other distant sites are found in the vaginal pool smear and usually not in the direct smear of the uterine cervix.

Endometrial Aspiration Smear

After preliminary visualization and cleaning of the cervix, a sterile endometrial cannula is introduced into the endometrial cavity without preliminary dilatation of the endocervical canal. A 20 mL syringe attached to this provides the negative pressure necessary for aspiration of material. The material is aspirated and subsequently expressed on a slide, smeared and fixed in the usual cytological fixative. Alternatively, the material is placed in a fixative, spun and embedded in paraffin and examined as microbiopsies.

Advantages

The advantages are that the method provides a reliable means for the diagnosis of early endometrial carcinoma and information about the status of the endocervical canal.

Cervical/Pap Smear (Figs 11.6 and 11.7)

Georgios Nicholas Papanicolaou (Fig. 11.8) was a Greek pioneer in cytology. Inventor of the 'Pap smear', which was the genesis for cervical screening for dysplasia and intraepithelial neoplasia. He published a paper on the diagnostic value of vaginal smears in the cancers of uterus and the 'Atlas of Exfoliative Cytology' way back in 1941. His name will always remain immortal in the world of cervical cytology.

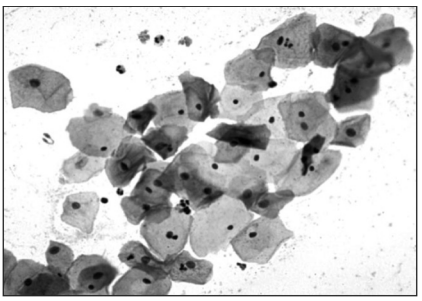


Figure 11.6: Normal Pap smear

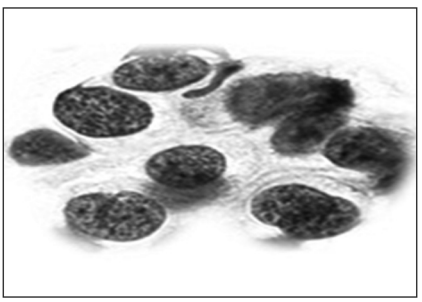


Figure 11.7: Abnormal Pap smear

Advantages of Pap smear

- Can be carried out as an outpatient procedure
- It is painless and simple
- Does not cause bleeding
- Does not need anesthesia
- Can detect cancer and precancer
- Can identify non-specific and specific inflammations.

Patient Preparation

Proper patient preparation is the beginning of good cervical cytology. The patient should be instructed before coming for smear collection that the patient should not douche the vagina for at least a day before the examination. No intravaginal drugs or preparations should be used for at least 1 week before the examination and the patient should abstain from coitus for 1–2 days before the examination.

Precautions

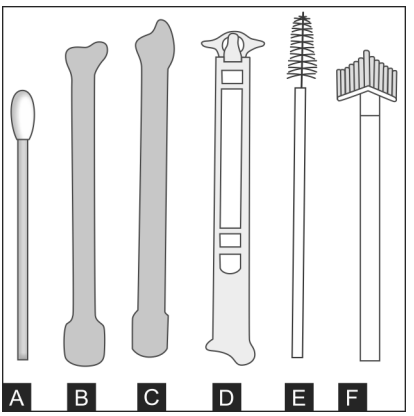
1. A smear should not be taken during menstrual bleeding, to avoid contamination with blood, endometrial component, debris and histiocytes.
2. Lubricants should not be used, while examining, as it can obscure cells during smear examination.
3. The cervix must first be wiped with a large moistened cotton swab in order to remove any mucus from the endocervical canal.

Figure 11.8: George Nicholas Papanicolaou

Different methods used in preparation of cervical smears (Figs 11.9A to F)

1. **Cotton swab (Fig. 11.9A):** It is simple and handy to use, however, the use of a cotton swab for collection of cervical smear is to be discouraged, in view of the drying artifacts and loss of cells, which are caused by this method.
2. **Spatulas:** For any device to sample the target area successfully, it must be able to recover as many representative cells as possible and effectively transfer them to the glass slide with maximum ease and rapidity, and without undue mechanical distortion (crushing, air drying, clumping, mixing with blood, etc.).

A variety of wooden and plastic spatulas are available. The Ayre spatula (Figs 11.9B and C) was developed in 1947 and it has been widely used. It is especially designed with a bifid end, one end of the scraper slightly longer than the other, so that the spatula fits the external os. The scraper is rotated 360° (Fig. 11.10), the longer end used as a pivot within the external os. Smears obtained with the Ayre's spatula (Fig. 11.11) result in adequate yield and are easy to screen.



Figures 11.9A to F: Requisites for smear collection. **A.** Cotton swab; **B.** Ayre spatula; **C.** Modified Ayre spatula; **D.** Multispatula; **E.** Cytobrush; **F.** Cervex-Brush.

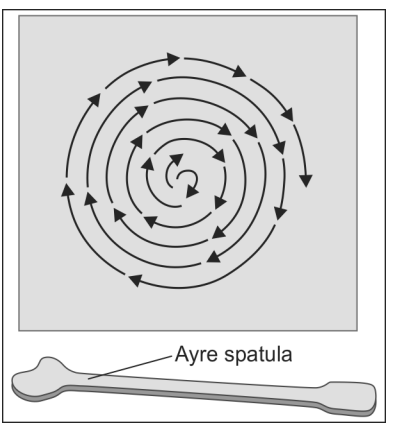


Figure 11.10: Rotation of Ayre spatula

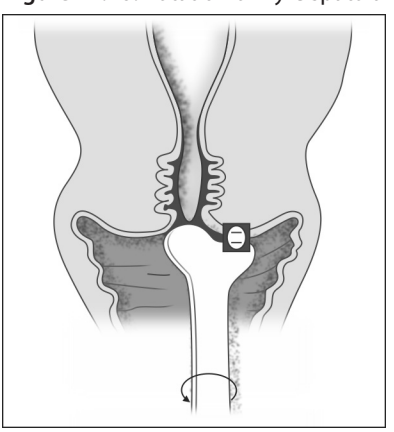


Figure 11.11: Collection of cervical smear by Ayre spatula

Preparation of Smear

After smear collection, the cellular sample is evenly smeared on to the center of the non-frosted glass slide, by rotating both sides of the scrape end of the spatula in multiple clockwise swirls in contact with the slide and fixing it immediately. Excessively thin or thick smears

can result in false-negative reports. The smear should be visually inspected after fixation. If it does not appear satisfactory, repeat it during the same examination and submit both slides for cytological examination. Most gynecologist smear one slide with endocervical material and a second slide with ectocervical material.

Wooden Spatula

The pointed Aylesbury spatula, a wooden spatula with an extended tip is designed to sample cells from both endocervix and the transformation zone (TZ) of the cervix. The disadvantage is that this method is traumatic to the patient and edge of the scraper may not fit the external os and fails to remove some of the valuable material from the squamocolumnar junction. Wooden spatulas are the least expensive sampling devices and have a long history of successful sampling, but have been criticized for their 'trapping effect' of diagnostic cells, which could lead to false-negative results. Modifications generally include variably sized hooks and extension of tips, which easily reach into the endocervical canal, e.g. modified Ayre spatula.

Multispatula

Multispatula consists of two parts—a body and a long central portion, which slides with the body (Fig. 11.9D). At the cervical end, the body has two wing-like protuberances, which surround the central portion. The sliding into the endocervical canal is controlled by a button and allows an endocervical penetration of 8 mm, when fully extended. The main advantage of multispatula is to increase the endocervical cell content of the smear in both nonpregnant and pregnant patients. A concave end (front end) fits the cervix and the convex end curves into the vaginal wall and vaginal pool scrapings.

Cytobrush (Bottle Brush)

A cervical brush for the sampling of endocervical cells was introduced in 1946. The Cytobrush (Fig. 11.9E) is superior to the Ayre spatula in obtaining adequate smears. It consists of a twisted metal wire core with perpendicular plastic bristles attached to the end of a plastic handle. The pliable bristles allow insertion of the narrow tip into a small cervical os. The instrument is highly effective in obtaining endocervical material and in collecting large numbers of cells. Optimal transfer of cells is accomplished by rolling the brush across the slide. The only problem with the Cytobrush is the trauma that occurs to the cervix as a result of the stiff bristles on the instrument. The resulting bleeding can obscure the transformation zone, as well as creating

a bloody, thick Pap smear, which is hard to screen. Patients occasionally complain of cramping pain, when the Cytobrush is used.

Cervex-Brush

The Cervex-Brush (Fig. 11.9F) is a new sampling device developed in Netherlands. It combines features of both the spatula and Cytobrush, and allows placement of an adequate amount of very well-preserved cytological material from both the ecto and endocervix on a slide, with far less trauma to the patient, resulting in better preserved cellular monolayers having less blood. The plastic bristles extend parallel to the plastic handle and are attached to a base in a 'V' shape manner. The bristles are softer and thicker than those of the Cytobrush, and taper toward the end. The longer strands, when carefully positioned into the ectocervical os, can extend into the canal, while the shorter strands will spread over the portio and lateral ectocervix.

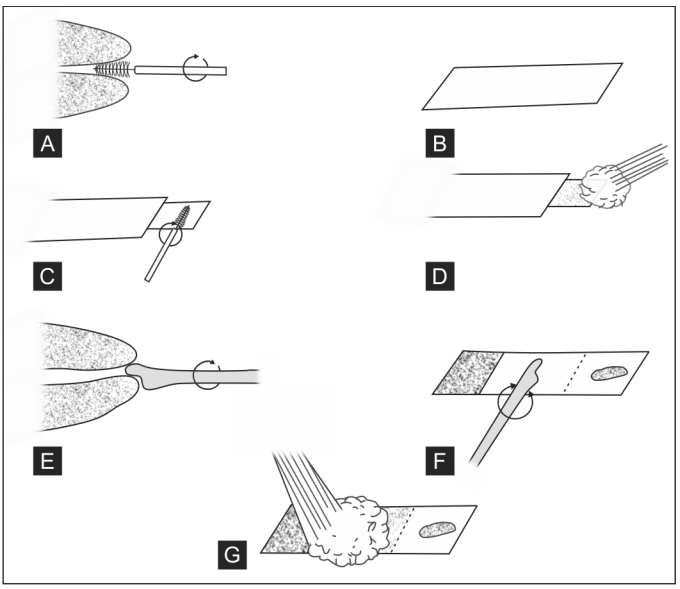
The brush is applied to the cervix with the central bristles inserted into the canal and rotated. It is then smeared longitudinally across the glass slide in a single motion, turned to an angle of 180° rotation and again swept across the slide. If performed correctly, the endocervical sample will be located in the center of the slide and ectocervix component will be concentrated towards the edges. It can be quickly accomplished with minimal air drying and used in those patients with cervical stenosis, and in menopausal women with transformation zones not clinically visible. This instrument, however, is the most expensive of all the available sampling devices.

Combined Spatula and Cytobrush Technique

Combined spatula and Cytobrush technique is a very effective sampling combination for both endocervix and ectocervix. Smears are taken with the brush initially, followed by the spatula (Figs 11.12A to G). Smears are prepared after both collections using the entire length of the slide; rolling the brush on one side and then smearing with the spatula in a monolayer, on the unused side of the slide. In those with a prolapsed uterus, the cervix is first soaked with normal saline and the scrape is collected with a Cytobrush.

Triple Smear or the Vaginal-cervical-endocervical Technique

Triple smear or the vaginal-cervical-endocervical (VCE) technique provides the best results. The material is collected from three sources (vagina, portio vaginalis and endocervix) on a single slide. This offers the advantage of rapid screening material. This method requires considerable dexterity on the part of the clinician to obtain material rapidly in order to avoid drying of the smears.



Figures 11.12A to G: Schematic representation of the combined spatula-Cytobrush method

Liquid-based Cytology

The idea of liquid based cytology (LBC) was developed in 1970. It is an inexpensive method for preparation of monolayer slides from liquid-based gynecologic cytology samples. Two systems of LBC are available, ThinPrep and SurePath.

ThinPrep

ThinPrep processor is appropriate for workloads of up to 20,000 samples per annum. The cells are collected in the ThinPrep vial of the preservative solution using a broom–type device or endocervical brush/spatula as a collection device. The vial is processed as both semiautomated the T2000 processor or fully automated the T3000. The TP2000 is a bench top model, which is loaded manually with individual pots and consumables TP3000 is fully automated, and allows for up to 80 samples per batch.

In either case, the method remains the same. The fluid in the vial is agitated and made to suck through a Micropore filter. The RBCs, mucus cells and neutrophils pass through the filter. While epithelial cells block the pores of the filter, leads to a pressure differential across the filter, which is detected by the machine and used to determine when sufficient cells have stuck on the filter. The filter is removed and then dabbed onto an electrically charged slide, causing the cells to transfer onto the glass slide and stained as in the conventional smear. The TP3000 is heavily automated and converts vials into slides ready for staining without any intervention.

SurePath

The PrepStain processor (Fig. 11.13) is semiautomated, but has a higher capacity than the ThinPrep T3000. This system works on the principle of a density gradient. Vials are mixed to resuspend cells. An aliquot is placed into the centrifuge vial and treated through a density gradient centrifugation process, which removes the unwanted cells and produces a concentrated pellet of cells. This is then resuspended and the aliquot is transferred to a settling chamber mounted on a microscopic slide, cells sediment to form a thin layer. Staining is an integrated part of the process.

The TP5000 is a fully automated bench top instrument, but is more flexible and can also process non-gynecological samples and make multiple preparations from a single vial. It allows for processing in batches of 20, with an option to add extra pods, which will enable up to 160 samples to be loaded.

All staff working in SurePath preparation must undergo the manufacturers training and be certified as competent before they can run the process. The British Society for Clinical Cytology (BSCC) supports these recommendations and where the scientific head participates in examining cervical samples, it is recommended and emphasized that they must:

- Examine a minimum of 750/3,000 cases per annum depending on the specific role undertaken by them and participate in the relevant regional external quality assessment (EQA) scheme
- Undertake 3 days of update training every 3 years
- Undertake 4 days of in-house training per annum
- Undertake relevant formal documented in house training, if returning to cervical cytology after a period or absence of more than 3 months. If the absence exceeds 6 months, then external training may be required.

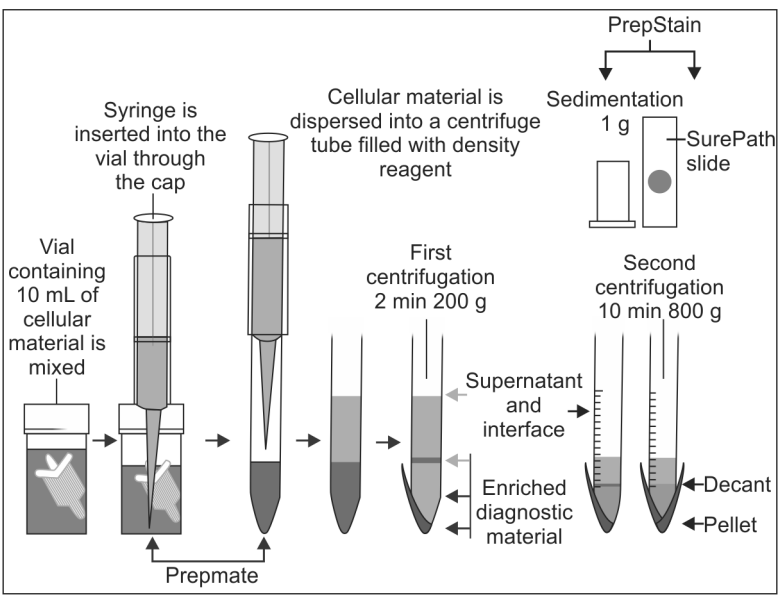


Figure 11.13: PrepStain technique

Reporting of Cervical Smear (Bethesda System)

1. In December 1988, the National Cancer Institute (NCI), Bethesda, Maryland, sponsored the workshop to address the Papanicolaou system, and to develop a uniform, descriptive and diagnostic terminology.
2. In 1991, The Bethesda System (TBS) came into existence at Bethesda, Maryland.
3. Revised The Bethesda System has been published in 2001.

The following is the format of reporting a cervical smear based on TBS, 2001.

Specimen Type

Indicate conventional smear (Pap smear) vs liquid based vs other.

Specimen Adequacy

Satisfactory for evaluation (with the presence or absence of endocervical/transformation zone component). Noted and any other quality indicators, e.g. partially obscuring blood, inflammation, etc.:

- Unsatisfactory for evaluation (specify reason)
- Specimen rejected/not processed (specify reason)
- Specimen processed and examined, but unsatisfactory for evaluation of epithelial abnormality because of.... (specify reason).

General Categorization (Optional)

- Negative for intraepithelial lesion or malignancy
- Epithelial cell abnormality: Refer 'Interpretation/Result' (specify 'squamous' or 'glandular' as appropriate)
- Other: Refer 'Interpretation/Result' (e.g. endometrial cells in a woman more than 40 year of age).

Automated review: If case examined by automated device, specify device and result.

Ancillary testing: Provide a brief description of the test methods and report the result, so that it is easily understood by the clinician (TBS 2001 recommends that the results of HPV-DNA testing can be added, if appropriate and preferably reported with the cytology result).

Interpretation/Result

1. **Negative for intraepithelial lesion or malignancy** (when there is no cellular evidence of neoplasia, state this in the 'General Categorization' above and/or in the 'Interpretation/Result' section of the report; whether or not there are organisms or other non-neoplastic findings may be mentioned).

2. **Organisms:**
   a. *Trichomonas vaginalis*.
   b. Fungal organisms morphologically consistent with *Candida* species.
   c. Shift in flora suggestive of bacterial vaginosis.
   d. Bacteria morphologically consistent with *Actinomyces* species.
   e. Cellular changes consistent with herpes simplex virus.
3. **Other non-neoplastic findings** (optional to report; list below not inclusive):
   a. Reactive cellular changes associated with inflammation (includes typical repair).
   b. Radiation.
   c. Intrauterine contraceptive device (IUD).
   d. Glandular cells status posthysterectomy.
   e. Atrophy.
4. **Other:** Endometrial cells (in a woman more than 40-year of age) (specify if negative for squamous intraepithelial lesion).

Epithelial cell abnormalities

Squamous cell

- Atypical squamous cells (ASC) of undetermined significance (ASC-US)
- The ASC cannot exclude high-grade squamous intraepithelial lesion (HSIL)—ASC-H
- Low-grade squamous intraepithelial lesion (LSIL) encompassing HPV/mild dysplasia/CIN
- The HSIL encompassing moderate and severe dysplasia, CIS/CIN 2 and CIN 3
- The HSIL with features suspicious for invasion (if invasion is suspected)
- Squamous cell carcinoma.

Glandular cell

Endocervical, endometrial, extrauterine, not otherwise specified (NOS):

- Atypical:
  - Endocervical cells (NOS or specify in comments)
  - Endometrial cells (NOS or specify in comments)
  - Glandular cells (NOS or specify in comments).
- Atypical:
  - Endocervical cells and favor neoplastic
  - Glandular cells and favor neoplastic
  - Endocervical adenocarcinoma in situ
  - Adenocarcinoma.

Other

Specify other malignant neoplasms, if any.

Educational notes and suggestions (optional)

Suggestions should be concise and consistent with clinical follow-up guidelines published by professional organization.

FINE NEEDLE ASPIRATION CYTOLOGY

The FNAC is the study of cellular samples obtained through a fine needle under negative pressure. The technique is relatively painless and inexpensive. When performed by well-trained pathologists/surgeons/clinicians and reported by experienced cytologists, it can provide unequivocal diagnosis in most of the situations.

Indications and Advantages

It is useful in lesions that are easily palpable, such as subcutaneous soft tissue tumors, lesions of the thyroid, lymph nodes, salivary glands and breast. Guided aspiration by imaging techniques such as CT or ultrasonography allows FNA of lesions of internal/deep seated organs, i.e. the lung, mediastinum, abdominal and retroperitoneal lesions, etc. The low-risk of complications allows it to be performed as an outpatient procedure. It is highly suitable in debilitated patients, in multiple lesions and is easily repeatable.

Prerequisites

The three prerequisites for a meaningful diagnosis on FNAC are:

1. Proper techniques are procedure, preparation of smears, fixation and staining.
2. Careful microscopic evaluation of smears.
3. Correlation of morphology with clinical picture.

Technical Requirements

Attention to technique is necessary to optimize the yield of the sample, making its interpretation easier and more reliable. Expertise regarding the technique comes from constant practice.

Location

The FNAC can be performed as an outpatient procedure or at the patient's bedside.

Equipments/Instruments

Needles: Standard disposable 22–24 gauge 1–1½ inch needles are used for FNAC. The length and caliber of the needle should fit the size, depth, location of the target. For small subcutaneous lesions, a 1½ inch, 22 or 23 gauge needle is ideal, while for deep-seated lesion, longer and larger needles are required. Finer needles (23 gauge) are also recommended in children and in vascular organs, i.e. thyroid. LP needles with sharp ends and Sheeba needles are some of the other instruments, which are routinely used in deep seated and guided lesions.

Syringes: Standard disposable plastic syringes of 10–20 mL are used. The 5 cm^3 syringes can also be used, but without the syringe holder as this is not designed to hold small syringes.

Syringe holder: The use of a Cameco syringe holder leaves one hand (left hand) free to immobilize and hold the lesion, while the right hand may be used for aspiration.

Other supplies: Test tubes, pencil for marking, alcohol swabs for skin, watch glass, saline, adhesive dressing, gloves, etc. are needed.

Procedure

Common Steps to be Followed for Performing the Aspiration

1. Request for an FNAC should include relevant history and clinical details, radiological findings, provisional diagnosis, etc. Site of FNA must be clearly stated.
2. The procedure must be clearly explained to the patient; consent and co-operation ensured. Patient anxiety needs to be allayed to the best of one's ability. Ignoring this simple, but crucial step can result in failure.
3. The lesion to be aspirated is palpated and its suitability for aspiration assessed. The appropriate needle is selected accordingly.
4. Before starting the procedure, check that all the required equipment and instruments are available.
5. All universal precautions for sterility should be followed during the procedure.

Positioning the Patient

The FNA is usually carried out with the patient lying supine on an examination couch unless the lesion demands other position.

Immobilization of the Lesion

The skin is cleansed firmly twice with spirit swab and the lesion is fixed between the thumb and index finger of the left hand.

Penetrating the Lesion

Fixing the lesion with left hand, the syringe with the syringe holder is held by the dominant hand and introduced through the skin into the lesion, carefully and swiftly. The angle and depth of entry varies with the type of lesion. For small lesions, aspiration of the central portion is indicated. For a larger lesion a single entry multidirectional technique is used.

Creation of a Vacuum and Obtaining the Material

Once in the lesion, a negative pressure is applied by withdrawing the piston to the fullest extent, while maintaining the suction. The needle is moved vigorously

back and forth in one direction before changing the direction, ensuring that the needle is inside the mass/lesion throughout; the whole procedure taking only 4–8 seconds. The to and fro movements as well as changing the direction of the needle, while it is still inside the lesion are the two crucial steps in procuring an adequate representative sample. The direction is changed as the needle comes up and goes down in a new direction not when it is well within the lesion, in order to avoid tissue tear. Movement of the needle is adjusted according to the type of lesion. A sclerotic lesion will require more force than a soft tumor.

Release of Vacuum and Withdrawal of the Needle

When material is seen in the hub of the needle (as a result of the cutting motion of the needle), the procedure is discontinued. Before withdrawing the needle, suction is released and needle pulled straight out. The piston is just allowed to slowly fall back by itself (never push). Failure to release negative pressure within the lesion will cause the aspirated material to enter the syringe, which is difficult to recover. The purpose of syringe is not to collect material, but to provide suction facilitating entry of cells into the needle hub and then to expel them from the needle, while making smears.

Immediately after withdrawing, detach the needle, draw air into the syringe, reattach the needle and express the material in the needle onto a slide. The needle tip is brought into light contact with the slide and the aspirate is carefully expressed onto the slide. Spraying into the air, which can form aerosols that are potentially infectious, should be avoided. An ideal aspirate is of creamy consistency with numerous cells suspended in a small amount of tissue fluid without admixture with blood. Such aspirates are smeared immediately using another slide or cover slip immersed into the fixative.

When fluid is aspirated, its color, consistency and amount should be recorded. The fluid can be taken in a bottle for centrifugation and preparation of smears. Material for culture can also be obtained. In cystic lesions, especially of breast and salivary gland, a large cyst may obscure a small malignant tumor. Hence cysts should be completely aspirated and residual lump if any, should be reaspirated and labeled separately.

In sclerotic/fibrotic lesions of the breast, little or no material will be obtained and a tissue biopsy may be more useful. Vascular organs such as the thyroid must be sampled rapidly with minimal movement of the needle. Observations, while doing the aspiration regarding site, size, and consistency (solid/cystic/soft/sclerotic/vascular) must be correlated, while interpreting the smears.

In guided FNA, the needle is guided by imaging into the mass, the procedure and smear preparation is similar to the routine method. Special anatomical areas require specially designed instruments in order to reach them as in prostatic aspirations where the needle requires guide for insertion (Fig. 11.14).

Smear Making and Staining

There are two fundamental methods of making smears obtained by FNA-direct and -indirect. Direct smear is for a cellular aspirate. The smear is

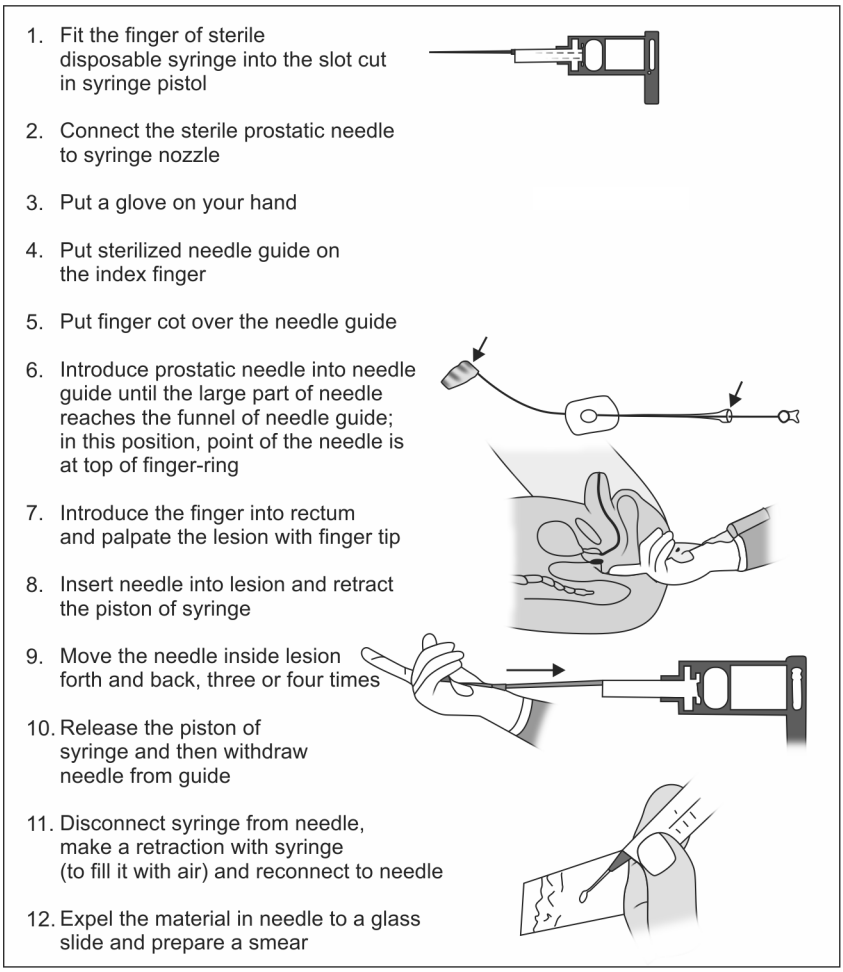


Figure 11.14: Prostate biopsy with Cameco instruments

made using another slide, which is placed perpendicular to the one with the aspirate so that the top part of the second slide is flat on the cellular material. Using gentle uniform pressure the second slide is pulled across resulting in uniform thickness of the smear. The indirect method is for bloody aspirates and deals with tilting the slide with the expressed material so as to drain out the blood and then making smears as in the direct method.

Smears are prepared and fixed according to the requirements of the stain to be used—Pap stain, MGG or H&E is recommended as per the requirements of the reporting cytologist:

1. **Air-dried:** In this method, smears are intentionally air-dried and stained by MGG hematological stains such as MGG, Diff Quik, Giemsa, etc. Cytoplasmic details are well preserved in this method. Colloid, mucin, endocrine cytoplasmic granules, etc. are better brought out in air-dried preparations. Paravacuolar granules in the thyroid cells

are well brought out. It is also ideal for morphology in hematological malignancies such as lymphoma or leukemia.

2. **Diff-Quick stain:** This is rapid stain and is used in practice to check adequacy of material, while an FNA procedure is being performed in particular a guided FNA. This will enable repeating the procedure immediately, while the patient is still available in case cellularity is not enough. It is therefore recommended to carry a microscope and tray with Diff-Quick stain to the site of a guided FNA and in all other cases where it would be difficult to repeat an aspiration at a later date.
3. **Alcohol fixation:** Rapid fixation in alcohol (wet fixation) is essential followed by Pap or H&E stains. Smears are dipped in absolute alcohol as soon as they are made. The Pap stain brings out nuclear details clearly, allowing better identification of malignant cells. Nuclear details with crisp chromatin granularity is preserved in Pap stain, whereas, nuclear chromatin transparency is less in Giemsa and the Pap stain is therefore called transparent stain. Thus the limitation of one method can be counterbalanced by the benefits in the other method.

Modified Cell Block Technique for FNAC Material

Method

If any tissue fragments are present, pick them up with an applicator stick or forceps and wrap them in a small piece of filter paper. Place this in a tissue cassette with a label; fix in 10% neutral buffered formalin and then process the material as usual. The procedure is used for sediments also. In case of paucity of the later, the sediment can be embedded in the agar gel.

Procedure

1. About 4 g of agar in 100 mL of water is liquefied.
2. Transfer sediments into the well of the agar. A vial lid will serve the purpose as a mold and a few drops of the agar put into it with sediment with in. This is allowed to solidify.
3. Processed as a normal histopathological specimen.

Cell block for immunocytochemistry (ICC) in FNAC: Some of the FNAC material is immediately fixed in 4% formaldehyde for 3–4 hours. The sample may be centrifuged and the cell button processed as a cell block for immunohistochemistry (IHC).

Advantages

- It produces histological sections of very good quality
- It gives excellent results for all IHC markers
- Accuracy is 97%, sensitivity is 95% and specificity is 100%
- Processing of multiple sections of the same material for routine staining, special staining and immunocytochemical procedure can be done
- Less cellular dispersion
- Storing of cell block for prospective studies.

Disadvantages

- Risk of losing material during preparation
- Time consuming
- Loss of cytological detail with respect to smears.

Applications

1. It is useful in diagnosis as several immunomarkers, can be used on scanty material.
2. Diagnosis of infectious antigens such as herpes, hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg), measles, cytomegalovirus (CMV), human papillomavirus (HPV), chlamydial antigen in urethral and prostatic aspirates.
3. The IHC/ICC markers are used as prognostic indicators, e.g. breast carcinoma.
4. To demonstrate glial and neuronal cells in the amniotic fluid of anencephalic pregnancies.

Sensitivity, Specificity and Predictive Value of a Positive Value Result in FNAC

Many times it becomes necessary to ratify results obtained at cytology with histopathology, which is generally used by all cytologists as gold standard. Various indices are calculated using histopathology as gold standard can be.

Sensitivity

Sensitivity means positive in disease. As per Frable (1984), it can be calculated as follows:

$$\text{Sensitivity} = \text{Positive in disease} = \frac{\text{True positive} \times 100}{\text{True positive} + \text{False negative}}$$

In a nutshell it means if 100 FNAC smears are compared to the histological paraffin sections; what percentage of these smears gives the correct diagnosis? In different centers it varies in between 85 and 100%, i.e. in approximately 85–100% of cases, a correct diagnosis is given at cytology.

Specificity

Specificity means negative in disease (Frable, 1984).

$$\text{Sensitivity} = \text{Negative in disease} = \frac{\text{True negative} \times 100}{\text{True negative} + \text{False positive}}$$

If 100 FNAC smears with a negative diagnosis are compared to the corresponding paraffin sections; what percentage of these smears could definitely be told that there was no disease at cytology? Varies at center from 95 to 100%.

Predictive Value of the Positive Result

Predictive value means when histology is taken into consideration, what percentage of cases are predicted at cytology.

$$\text{Predictive value of a positive result} = \frac{\text{True positive} \times 100}{\text{True positive} + \text{False positive}}$$

For example, in 200 histologically proven breast lesions it was seen that at FNAC 165 cases were reported as being carcinomas (true positive at FNAC); 27 cases were reported as being negative at FNAC.

STAINS SPECIFIC IN CYTOLOGY

Papanicolaou Staining Method

Papanicolaou staining method (Table 11.9) is the routine staining procedure used in any cytopathology laboratory. This technique is named after George N Papanicolaou, the father of exfoliative cytology and is devised for the optimal visualization of cells exfoliated from epithelial surfaces of the body. It is a polychrome staining reaction designed to display many variations of cellular

TABLE 11.9: Papanicolaou staining procedure

Step	Chemical/Stain	Duration
1	90% ethanol (fixation)	15 min
2	80% ethanol	2 min
3	50% ethanol	2 min
4	Distilled water	5 dips
5	Distilled water	5 dips
6	Hematoxylin stain	2 min
7	0.05% HCl solution	10 seconds, if necessary regressive stain
8	Running tap water (bluing)	10 min
9	50% ethanol	2 min
10	80% ethanol	2 min
11	80% ethanol	2 min
12	95% ethanol	2 min
13	OG-6 stain	2 min
14	95% ethanol	2 min
15	95% ethanol	2 min
16	95% ethanol	2 min
17	EA-36 stain	2 min
18	95% ethanol	2 min
19	95% ethanol	2 min
20	95% ethanol	2 min

Contd...

Contd...

Step	Chemical/Stain	Duration
21	95% ethanol	2 min
22	Absolute ethanol	2 min
23	Absolute ethanol	2 min
24	Absolute ethanol + xylene (1:1)	2 min
25	Xylene	5 min
26	Xylene	5 min
27	Xylene	Till clear
28	Mount in dibutyl phthalate in xylene (DPX)	

morphology showing the degree of cellular maturity and metabolic activity. The use of Papanicolaou stain results in well-stained nuclear chromatin and differential cytoplasmic counterstaining.

Eosin Alcohol Solution

Eosin alcohol (EA) is a polychromatic stain and composed of two components. Eosin stains the mature squamous cells, erythrocytes, nucleoli and red cells in blue-green; while light-green stains for the metabolically active cells, i.e. parabasal cells, intermediate cells, histiocytes, columnar cells and malignant cells. Many variables of EA are available and vary from concentration of light green, eosin and Bismarck brown. They have similar formulas, but solvents are different. Solvent is probably ethanol, but some may use methanol. The EA-36 and commercial preparation EA-50 have a similar formula. Each contains twice the amount of light green used in EA-65. The increased amount of light green in EA-50 and EA-36 tends to stain the background of thick non-gynecological smears too intensely and for such smears EA-65 is preferred.

EA-50 (or EA-36)

- Light-green SF (yellowish) 0.1% solution in 95% ethyl alcohol
- Bismarck brown 0.5 % solution in 95% ethyl alcohol
- Eosin (yellowish) 0.5 % solution in 95% ethyl alcohol
- Phosphotungstic acid lithium carbonate [saturated aqueous solution, (about 2 g saturates)] 10 drops.

EA-65

The formula is similar to the formula for EA-50, but the amount of light-green SF (yellowish) is halved. For this reason it is preferred to EA-36 for staining non-gynecological smears.

Orange G (OG-6)

- Orange G stock solution (0.5% ethyl alcohol): 100 mL
- Phosphotungstic acid: 0.015 g.

The OG is a monochromatic stain, 'O' stands for orange and 'G' stands for 'gelb', a German word for yellow. The dye has a relatively small molecule,

which enables the stain to rapidly penetrate the cytoplasm. It stains keratin brilliant orange.

Principles of Staining Procedure

Fixation: The cytology smears are fixed as soon as made in 95% ethyl alcohol or in other substitutes for a minimum of 15 minutes.

Nuclear staining: It is done by using the hematoxylin stain. Harris hematoxylin or its modified form is used in Papanicolaou staining in the regressive method, in which the smears are deliberately overstained with hematoxylin and the excess stain removed by using a differentiating solution such as acid alcohol (0.05% HCl in 70% ethyl alcohol) or 0.05% aqueous solution of HCl alone.

As hematoxylin is used in an acid pH, a pink color, which is not stable develops. In order to make it stable, the compound is brought to alkaline pH (bluing) by treating with a weak alkaline solution. Running tap water, which is slightly alkaline (pH 8) is used as a bluing solution. Ammonium hydroxide solution (15 mL of ammonium hydroxide 28–30% weight/volume to 985 mL of 70% ethanol) can also be used.

Cytoplasmic staining: Cytoplasmic stains are OG-6 and EA-36. Both are synthetic stains.

Dehydration: Rinse the smears in absolute alcohol for two or three changes for the removal of water. Smears left in rinses for long will lose too much stain. Alternative to 100% ethanol are 100% isopropanol and 100% denatured alcohol.

Clearing: Cells are not transparent, while the smear is in the staining or alcohol solutions. During clearing, alcohol is replaced with xylene, which is also miscible in mounting medium. Xylene has a refractive index as that of glass and mounting medium and it prevents cellular distortion.

Mounting: The mounting media must be miscible with the clearing agent to prevent fading of the stains. Practice is essential to achieve well-mounted slides, free of air bubbles and artifacts. Too much mounting medium interferes with microscopic detail, making the cell film appear hazy or milky when examined under the high-power objective.

Precautions

1. Immediate fixation of smears is essential.
2. Smears should never be allowed to dry before placing the coverslip.
3. Hematoxylin is filtered every day before use.
4. All solutions and other stains are filtered daily after use, to keep them free of sediment.
5. Avoid contamination during placing of the coverslip, with the dropper used to dispense the mounting medium.
6. Place the coverslip on the microslide slowly without trapping airbubbles.

Rapid Papanicolaou Staining

The purpose is to save staining time and money by combining OG and EA, and reducing the number of rinses. This procedure needs to be done only for emergency situations and not for routine use. Rapid Pap stains are useful at times when a cytology decision is demanding instantaneously as at a surgical operative procedure or in mass cytology screening camps. Many commercial stains composed of rapid Pap-nuclear stains, rapid Pap-cytoplasmic stains as well as dehydrants are available.

Hematoxylin and Eosin Staining Method

Some laboratories use routine H&E stain for non-gynecological smears. The benefits of using Papanicolaou stains are clear definition of nuclear details and differential counter staining giving nuclear and cytoplasmic transparency. The H&E stain does not satisfy these criteria and hence is unacceptable for cervical smears and cancer screening. It can be used for staining FNAC material and is preferred by some authors as comparison to histological sections is closer.

May-Grünwald-Giemsa Staining Method

Many laboratories use MGG (Romanowsky-type stain) staining method for cytological diagnosis of non–gynecological specimens. The combination of all these stains increases the efficiency of microscopic interpretations. MGG stain is performed in air–dried aspirates or fluids. Stock solutions of May-Grünwald Reagent and Giemsa Stain are available commercially.

Staining Procedure

- May-Grünwald solution: 5 min
- Running water: 1 min
- Geimsa solution: 15 min
- Running water:1–2 min
- Air-dry.

Artifacts because of faulty techniques

1. Delay in processing may lead to degenerating smear picture with loss of cell morphology and plenty of bacteria in the smear background.
2. Delay in fixation may lead to air-drying artifacts, pale stained nuclei, lack of differential cytoplasmic staining, cytoplasmic and nuclear eosinophilia.
3. Contamination from other smears and cell from effusion smears to other slides should be avoided. All the alcohol and xylene solutions should be filtered every day using Whatman No.1 filter paper. The fixative should be filtered after each use.

CYTOLOGY AND INDEXING

The last half of the 20th century has witnessed the growing importance of cytology as an 'important diagnostic tool' not only in the West but also in centers all over India. As such there has arisen a basic need for documentation of cytology results in order to enable retrieval of such results whenever necessary. This topic deals with a cytology indexing system now in use based on the 'systematized nomenclature of pathology' (SNOP) developed by the committee on Nomenclature and Classification of Disease of the College of American Pathologists. The system can be used in an abbreviation form or otherwise to suit any requirement.

Cytology has played a secondary role to histopathology in the last few decades, but it has become increasingly obvious especially with the resurge of the use of the FNAC that cytology has come into its own. This is particularly so because of the ease with which a diagnosis may be arrived at, in most instances, with least patient morbidity. It has now become imperative, for the various reasons stated below, that a cross-indexing system should be introduced for this discipline as has been done in various areas in pathology.

Chief Reasons for Indexing

- To study the pattern of diseases at the geographical and socioeconomic levels
- To provide statistics with regard to a particular disease process
- To make follow-up of individual patients possible
- To provide a feed-back to the cytologist on the accuracy of a cytological diagnosis.

These requirements are definite need for both institutional cytology units as also to the private cytologist, who require to maintain records on patients.

SYSTEMATIZED NOMENCLATURE OF PATHOLOGY

SNOP Indexing System (Box 11.1)

Various indexing systems have also been used to advantage in histopathology, one of the later systems being SNOP. The nomenclature is based on four areas:

- **Topography:** Where the portion of the body affected plays a part in the diagnosis, the code letter being 'T', e.g. Breast, liver or abdomen
- **Morphology:** Where structural changes are essential to the diagnosis, the code letter being 'M', e.g. adenocarcinoma, papilloma
- **Etiology:** Where the etiological agent is known, the code letter being 'E', e.g. herpes simplex virus infection
- **Function:** Where body functional manifestations are essential to the diagnosis, the morphology playing only an ancillary role, e.g. thyrotoxicosis.

BOX 11.1: Cytology indexing (SNOP system)

T-Y4: Abdomen including retroperitoneal M0001: No pathological diagnosis M4000: Inflammation M8013: Carcinoma 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-Y5: Abdominal visceral—general M-NOS 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-Y4: Ascites M0001: No pathological diagnosis M3880: NOS M4100: Acute inflammation M4014: Lymphocytic inflammation M8013: Carcinoma, NOS M8016: Carcinoma, metastatic M9051: Mesotheliomas, NOS 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-1100: Bone M0001: No pathological diagnosis F9492: Tuberculosis M4000: Osteomyelitis M9251: Giant cell tumor M9223: Chondrosarcoma M9260: Aneurysmal bone cyst M9733: Myeloma, plasma cell M9593: Malignant lymphoma M9183: Osteogenic sarcoma M8016: Metastatic carcinoma 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-0400: Breast M0001: No pathological diagnosis M7816: Lactation alteration M4000: Mastitis F9492: Tuberculosis M7631: Mammary dysplasia M7631: Cystic disease, chronic cystic mastitis M9010: Fibroadenoma M9020: Giant fibroadenoma M8513: Medullary carcinoma

Contd...

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M8023: Anaplastic carcinoma M8016: Metastatic carcinoma 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-X1: Cerebrospinal fluid M0001: No pathological diagnosis M4000: Inflammation, NOS M9383: Glioma, NOS M8013: Carcinoma, NOS 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-83: Cervix uteri M0001: No pathological diagnosis M4000: Cervicitis M7500: Metaplasia M7600: Dysplasia M8012: Carcinoma in situ M8072: Squamous cell carcinoma M7100: Atrophy E3141: Herpes simplex virus 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-5600: Liver M0001: No pathological diagnosis M4174: Abscess E4420: Ameba E4725: *Echinococcus* M4850: Cirrhosis M8173: Liver cell carcinoma M8023: Anaplastic cell carcinoma M8016: Metastatic carcinoma 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-28: Lung M0001: No pathological diagnosis M8013: Carcinoma, NOS M8016: Carcinoma, metastatic 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification

Contd...

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T-08: Lymph nodes M0001: No pathological diagnosis M4000: Lymphadenitis including reactive changes F9492: Tuberculosis M9633: Hodgkin's lymphoma M9823: Lymphocytic leukemia M8016: Metastatic carcinoma 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-87: Ovary M8013: Carcinoma, NOS 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-3X: Pericardial fluid M4000: Inflammation M0001: No pathological diagnosis M9051: Mesothelioma, NOS 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-29: Pleura M0001: No pathological diagnosis M4150: Pleuritis M9051: Mesothelioma, NOS 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-2Y: Pleural fluid M0001: No pathological diagnosis M4000: Inflammation M8013: Carcinoma NOS M9051: Mesothelioma, NOS 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-7700: Prostate M0001: No pathological diagnosis M8013: Carcinoma NOS M7300: Benign hyperplasia 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification

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T-55: Salivary gland M4000: Sialadenitis M8940: Pleomorphic adenoma M8433: Mucoepidermoid carcinoma M8013: Carcinoma, NOS M8560: Papillary cystadenoma lymphomatosum 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-01-02: Skin M8013: Carcinoma M8073: Squamous cell carcinoma F9391: Leprosy 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-1900: Soft tissue M0001: No pathological diagnosis M4000: Inflammation F9492: Tuberculosis M9300 to 9500: Miscellaneous specific infectious diseases M8850: Lipoma/Fibrolipoma M8810: Fibroma M8853: Fibroliposarcoma M8873: Leiomyosarcoma 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-1Y: Synovial fluid M4000: NOS M9043: Synovial sarcoma M9040: Synovial benign 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification
T-78: Testis M-NOS 6971: Cytological alteration, suspicious 6972: Cytological alteration, positive 6973: Cytological alteration, deferred classification

Contd...

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T-9600: Thyroid
M0001: No pathological diagnosis
M4000: Thyroiditis
M7661: Thyroiditis—Hashimoto's
F4621: Thyrotoxicosis
M7233: Goiter—colloid
M7234: Goiter—adenomatous
M8001: Neoplasm
M8330: Follicular adenoma
M8140: Adenoma
M8333: Follicular carcinoma
M8053: Papillary carcinoma
M8513: Medullary carcinoma
M8023: Undifferentiated carcinoma
6971: Cytological alteration, suspicious
6972: Cytological alteration, positive
6973: Cytological alteration, deferred classification
T-8100: Vagina
M0001: No pathological diagnosis
M4000: Vaginitis
M4464: *Trichomonas vaginalis*
M7821: Proliferative
M7822: Secretory
F0101: Pregnancy NOS
M7100: NOS
6971: Cytological alteration, suspicious
6972: Cytological alteration, positive
6973: Cytological alteration, deferred classification

NOS is 'not otherwise specified'

Topography with Other Areas

1. In the utilization of this nomenclature, topography is always used in conjunction with any of the other three areas.
2. To promote an expanded possibility for utilization of each of these areas, a four–digit number system is allocated to each one of these areas. The first digit on the left hand side indicates the broad category under which the diagnosis made is to be indexed. The subsequent numbers towards the right signify the finer ramification of this broad category. Example, in the area of topography:
   a. T-9100 is vagina.
   b. T-8200 is uterus.
   c. T-9210 is body of uterus.
   d. T-8300 is cervix.
3. In this sequence the number '8' signifies the female genital tract in general and the numbers 1, 2 and 3 that follows immediately after the number '8' signify the various portions of the female genital tract in ascending order. The number 8210 indicated a further ramification of

8200, wherein the body of the uterus is specifically required for indexing purposes. Thus suppose a patient has had a fibroadenoma of the breast diagnosed, the indexing card would read as:

a. T-0400 is breast.
b. M9010 is fibroadenoma.

4. In certain instance, the term topography may be used to not only indicate anatomical, organ in the strict sense but also an area too. In such instances a two digit code is used. It was realized when this two-digit was introduced for topography, that expansions on this coding system would be required and so the letters 'X' and 'Y' have been replaced after the letter 'T'. For example:
   a. T-Y4 is abdomen and includes retroperitoneum.
   b. T-Y5 is abdominal viscera in general.
5. Whereas this system is complete for utilization for histopathology indexing, in cytology where diagnosis depends on cell populations and cell morphology, the cytologist is prone to use terms such as, 'definite, consistent with' (where only about 80% of standard criteria for a diagnosis are satisfied), 'suspicious' (where only 40–50% of the recognized criteria are satisfied) and when a diagnosis cannot be made the notation 'no diagnosis possible'. These terms are subjective to each cytologist and cannot therefore be indexed. The SNOP system can be modified to take care of such exigencies, the equivalents of which are shown below:
   a. Suppose a patient has been cytologically diagnosed as fibroadenoma of the breast and the cytologist has seen all criteria to satisfy such a diagnosis the index card to be used will hold the following numbers:
      - T-0400 is breast
      - M9010 is fibroadenoma
      - 6972 is cytological alteration, positive.
   b. Should only 40–50% of the criteria for a diagnosis of this lesion be satisfied, another card would be used with the following notations:
      - T-0400 is breast
      - M9010 is fibroadenoma
      - 6971 is cytological alteration, suspicious.
6. The indexing system that has been suggested for use is essentially meant for the cytopathologist and the cytopathology unit, for the purposes already stated. It has however been found by the authors that the requesting clinician receiving a cytology report is never able to assess as to how definite the cytopathologist is, with regard to a particular diagnosis. This is particularly so when terms such as 'consistent with' or 'suspicious of' are used. In order to indicate to the clinician the surety of diagnosis a grading system on the report being sent to the clinician is used. This system runs from 5 (definite diagnosis) to 0 (no diagnosis) (Table 11.10). For example:
   a. On the outgoing report the statement made as impression would read in the case of a fibroadenoma breast aspirate where all criteria have also been satisfied as:
      - 'Aspirate showing cytological features of a fibroadenoma of the breast (5)'

TABLE 11.10: Systematized nomenclature of pathology (SNOP) system for cytologists

Terms used by cytologists	SNOP	Grading
Definite	6972 Cytological alteration, positive	5
Consistent		4
Suspicious	6971 Cytological alteration, suspicious	3 2
No diagnosis	6972 Cytological alteration, classification deferred	1 0

b. Where only a suspicion of the lesion exists the impression would read as:
   - 'Aspirate showing cytological features suspicion of a fibroadenoma (3)'.

It should be noted that for each lesion pertaining to each organ and each type of cytological change there should be one card (Table 11.11). The indexing system for the breast as per the lesions outlined in the appendix would be 12 lesions with 3 possibilities each (12 × 3) amounting to 36 cards for the breast alone.

It should be also noted that the intrinsic to this indexing system is the maintenance of a report copy and relevant requisition form on file. Once any cytological number is located on the index card, the name, age, sex and other details can automatically be derived by tracing the report copy and relevant requisition form.

TABLE 11.11: Sample card (organ/change specific)

Organ code No. T-0400	Diagnosis Code No. M9010	Cytology Code No. 6972
Breast	Fibroadenoma	Cytological alteration, positive
1990		
C–1240		
Entry of cytology numbers		

ORGANIZATION OF CYTOPATHOLOGY LABORATORY

A basic cytopathology laboratory requires minimal infrastructure and investment and is an essential part of a diagnostic center. Exfoliative cytology/brush cytology and fine needle aspiration cytology have become important procedures. Ultrasound and CT-guided diagnostic FNACs have added to these diagnostic modalities. Various guidelines for functioning of these laboratories have been formulated by various government and private organizations—the National Accreditation Board for Testing and Calibrating Laboratories (NABL), Indian Academy of Cytology (IAC), etc.

Aims of Any Cytology Laboratory

- Screening of cancers
- Diagnosis of disease
- Monitoring of a disease
- A profitable organization.

Status

The laboratory can be a division of the department of pathology, obstetrics and gynecology, family planning clinic, private laboratory or an independent center involved in training, service and research.

Workload

A minimum of 500 cases per year are mandatory:

- Small laboratory: 1–50 cases/day (cytology and histopathology)
- Medium laboratory: 51–500 cases/day
- Large laboratory: > 500 cases
- Super-spacey laboratory: With ICC, flow cytometry and polymerase chain reaction (PCR) facilities.

Staff

1. At least 1 part time cytopathologist/pathologist and 1 cytotechnologist.
2. The chief of the laboratory should be a cytopathologist/pathologist.
3. Cytotechnician should have a diploma in medical laboratory technology from a recognized institution and should have undergone 6 months training course from an accredited laboratory.
4. **Receptionist:** Computer skills, good communication skills and pleasing personality.
5. **Office boy:** Paying bills and delivering reports.
6. **Cleaners:** As required.
7. **Consultants:** Computer expert, service engineer, tax consultant, electrician, plumber, printer and binder.
8. **Suppliers:** For equipment and reagents.

Setting up a Cytopathology Laboratory

Location

The placement of a cytology laboratory should be planned out in a manner to facilitate smooth processing and reporting of samples and finally, dispatch of reports. It should be easily accessible to patients and to the staff, away from the noise and preferably on the ground floor.

Size

Minimal area of the laboratory should preferably be 20 × 12 ft with scope for further expansion.

Design of the Laboratory

The main objective should be maximum utility with minimum wastage of space and energy. The laboratory must be well designed and conveniently located to enable the professional and support personnel to perform their duties effectively. It must contain four definitely separated areas:

1. **Reception area:** With an adequate waiting area with comfortable seating for patients and attendants.
2. **Specimen collection room:** For fluids and other cytology samples.
3. **Separate room/Cubicle for special procedures:** The FNAC, taking pap smears, etc. They should have a couch/examination table, desk and chair, work area with relevant instruments, etc.
4. **Working area/Technical room:** The technician should have an isolated corner for processing and staining with a sink and 24-hour water facility, which should be well ventilated and should have an exhaust fan. The work bench with 2.5 ft width and 3 ft height from the floor may be at two adjacent sides of the laboratory to fit one corner at the side where there is enough ventilation. The bottom of the work bench can be made as cupboards with one or two racks for storage of materials. A reagent shelf may be fitted over the workbench. Laboratory sink must be fitted at one end of the work bench. Power plugs with a capacity of 15 and 5 A must be made available at the other end of the work bench.
5. **Pathologists reporting room:** Should have sufficient space and comfortable seating arrangement to permit easy performance of microscopic examination. Screening areas should be well lighted and ventilated.

Laboratory Equipments

The laboratory should be well equipped to suit the requirements, but one should always keep the budget in mind. After consulting friends and experts, a thorough study of the manufacturer's reputation, service, record and the equipment essential for the laboratory should be ordered. Equipment bought should have supportive infrastructure such as electric power requirements, grounding, plug points, spares to last for 5 years, etc. All equipment should be of a good quality and useful for long-term service although at times it is not practical.

Essential equipments are microscope (binocular) minimum two, FNAC syringe holder, diamond pencil, centrifuge (4 tubes capacity), cytospin/slow sedimentation apparatus, autoclave, hot air oven, electric heater, refrigerator, microtome, stainless steel electrical sterilizer, bivalve vaginal speculum sims, a kidney tray, metal tray, punch holding forceps (stainless steel), forceps (stainless steel), gynecological couch (with adjustable step and focusing lamp), slide tray, filing cabinet (10,000 capacity), rubber sheet (for gynec couch), diamond glass marking pencil; if finance allows ThinPrep machine.

DPX Mounting Media Chemicals

Stains: Glaxo: BDH (British Drug House).

Chemicals: Hematoxylin powder, Giemsa, Leishman, xylene extra pure, DPX mountant, ethyl alcohol and isopropyl alcohol.

Consumables number

- Ayre spatula (wooden)
- Disposable tongue depressor
- Disposable syringes 10 mL and 20 mL
- Disposable needle 22 G
- Gloves (disposable)
- Koplin jar
- Glass staining jar with lid
- Slide carrier (20 slides capacity stainless steel)
- Slide trough
- Microslides
- Filter paper Whatman No. 1
- Blotting paper
- Alcohol can be purchased in bulk; substitutes are acetone, butyl alcohol, isopropyl alcohol; also cotton gauze and blotting paper
- Dettol/phenolic disinfectant 5 liter can be purchased
- Sodium hypochlorite/bleaching powder
- Disposable syringes and needles
- Gloves, face masks and aprons
- Marking pens
- Stationery items: Register, printing papers, pencils, pens and labels
- Glass slides: 75 × 25 mm, ideally 0.65 mm thick, but at least < 1.1 mm. Blue star is the best available, 50 slides/box. Surface should be parallel edges, should be smooth and polished. Cover slip for cytology, the ideal size 22 × 50 for wet mounts. Wax concealing should be 59–60 cm^3.

Glass wares number

1. Glass ware of good quality (Borosil and Corning):
   - Measuring conical flasks: 250 mL and 500 mL
   - Round bottom flask: 1,000 mL
   - Measuring cylinder: 100 mL and 500 mL
   - Centrifuge tube, test tubes (should have a lip for easy pouring)
   - Rapid heating and cooling is possible with such glassware. They should be clear, transparent with no air bubbles.
2. Pipettes (10 mL): The tips are important, should be regular and must not be broken.
3. Analytic balance for weighing chemicals.
4. Distilled water unit.
5. Non-technical equipment:
   - Computer
   - Generator

- Telephone
- Furniture
- Fire extinguisher.

Inventory, Laboratory and Purchase

1. Recurring and non-recurring expenditure: Maintenance of an inventory book, there should be stock card for each item.
2. Inventory to be taken every 6 months.
3. Laboratory supplies to be ordered every 3 months. Requirement should be assessed from the previous consumption. Buffer stock to be maintained. Breakages to be taken into account. Indent to be approved (Box 11.2), supplies to be checked before issue of payment.

BOX 11.2: Request and report forms of laboratory inventory

Request forms

Name of patient: _____
Name of referring physician or facility: _____
Dates of sample collection, receipt and issue of report: _____
Clinical diagnosis: _____

Reports

Name of patient: _____
Laboratory number: _____
Name of referring physician or facility: _____
Name and address of the laboratory: _____
Dates of sample collection, receipt and issue of report: _____
Adequacy of sample: _____
Cytological diagnosis: _____
Signature of cytotechnologist and cytopathologist: _____

Maintenance of the Laboratory

Laboratory cleanliness, plumbing, electric, equipment service contract, microscope, daily cleaning, stains should be filtered and quality of stains to be maintained.

Storage

Stores must be well ventilated and must have a powerful exhaust fan. The chemicals and volatile substances used in the specimen processing area must be stored in a cool metal cabinet. Glassware properly stored in shelves, flasks plugged with cotton wool. Pipettes in drawers that have sections. Chemical and reagents in strict alphabetic order. Record keeping is very important to enable proper data retrieval for various purposes. A system for proper maintenance of registers or use of computer for maintaining data is advisable. Smears must be preserved for 5 years, FNAC for a period of 10 years, in yearwise accession number after reporting. This is important as a quality control measure and also for follow-up.

Safety

Precautions to minimize the possibility of hazards from these sources should be followed. Pipetting to be done using bulbs. No mouth pipetting. Protective clothing to be worn during cytopreparation. All material to be treated as potentially infectious. They can be rendered them noninfectious by fixation. Use of disposables. All articles used in cytopreparation to be sterilized before reusing.

Archives

Clerical and record keeping system should be located near the screening area for rapid retrieval of data. Health and fire regulations of the state and local authorities must be observed for the personal safety of laboratory personnel.

Disinfection of Work Areas

A solution of sodium hypochlorite is used as disinfectant. Handwashing after handling materials and chemicals; keep hands away from nose, eyes and mouth. Never smoke or eat in the laboratory area. Clean up spills promptly.

First Aid

- Maintain a first aid for treating minor burns, chemical burns and eye wash equipments
- Fire extinguisher, blankets, fire alarms, etc. should be available.

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Chapter 12 Quality Control in Cytopathology

TERMINOLOGIES

Quality control: It is defined as a system for verifying and maintaining a desired level of proficiency in any cytology procedure that takes place in a laboratory. Quality control governs the functioning of a laboratory from the start of specimen collection and processing of specimens through interpretive analysis to postanalytic assessment.

Quality assurance: It is defined by the College of American Pathologists as a systematic monitoring of quality control results and quality practice parameters to assure that all systems are functioning in an efficient manner to promote clinical patient care and match the health needs of the society.

Donabedian model: The most outstanding theoretician of quality assurance in the field of health care is Avedis Donabedian, who in 1988, proposed the partition of clinical practice into three elements—structure, process and outcome (Table 12.1).

Continuous quality improvement: The term 'quality assurance' has been replaced by the term 'continuous quality improvement' (CQI). It includes

TABLE 12.1: Donabedian model

Elements	Clinical practice
Structure	Resources (staff number and qualification, equipment) Organization (availability of a mission and vision statements, availability of job descriptions, clarity of hierarchical relationships, policies to develop and update procedures, and to monitor their implementation)
Process	Workload and productivity (e.g. number of slides processed per year by the laboratory and examined per day by individual cytologist) Quality of data collection and reproducibility (i.e. reliability, accuracy and completeness of data) of final reporting and recording Implementation of internal and external quality control
Outcome	Diagnostic accuracy and reliability Timeliness of diagnosis (i.e. time from intake to delivery or turnaround time) Client satisfaction

traditional assurance, but has a wider scope. It involves not only taking corrective action if the laboratory falls below an agreed standard but also higher and new standards once the original targets have been achieved, so that the quality of the service can be enhanced even further (Table 12.2).

Quality control is maintained by guidelines laid down for laboratory practices by professional bodies. The process of creating guidelines represents a consensus for 'best practices' within a specialty and endorsement by national professional organizations. The following account of standard laboratory procedures is extracted from guidelines laid down by the American Society of Cytopathology, the European Union Guidelines and British Society of Clinical Pathology coupled with standard practicing cytology procedures utilized in Indian accredited laboratories. It has been written with the intention of helping a cytopathologist to maintain levels of quality services in his/her laboratory.

MAINTAINING QUALITY CONTROL

Each laboratory must perform and maintain records of routine quality control relating to specimen receipt, preparation and staining, maintenance of copies of reports, microscope and instrument maintenance as well as instrument calibration records, as these activities are a requirement by laboratory

TABLE 12.2: Continuous quality improvement (CQI) program

Managerial requisites	Functions
Management commitment	Without it, quality activities are doomed to remain fragmented and intermittent Coping with problems and attending to them for future; once corrected it will result in performance improvement
Delineation of clear responsibility and assignment of adequate resources to quality activities	It may be advisable to appoint a quality coordinator or facilitator and a quality committee with the participation of all staff categories, under the chairmanship of the laboratory director or his/her closest collaborator Identify the basic requirements needed to proceed with the implementation of CQI Discuss the obstacles preventing the successful implementation of CQI Describe the major components of a quality improvement plan and how they relate to each other Describe how strategic planning and CQI can be integrated, and why this is important
Training and education	For all staff in basic principles of CQI
Human resource management policy	It should include rewards for the participation in quality activities as well as in professional training
Periodic review of the quality system	Particular attention should be given to the implementation of corrective measures and the assessment of their effects

accreditation agencies. A brief account of the methods for maintaining this can be dealt under the following headings.

Specimen Handling

Non-gynecological Cytology

All cytology specimens sent to the laboratory should be accompanied with a request form. The requisition should include:

1. Full patient name.
2. Medical record number.
3. Date of birth and age.
4. Date and time of specimen collection.
5. Source or site of specimen.
6. Type of specimen and examination requested.
7. Clinical history including pertinent physical findings, radiographic findings, previous histological and cytological abnormalities.
8. Ordering clinical's full name with telephone number for contact.
9. Signature of the clinician/authorized person requesting the examination.

All material from infectious sources [acquired immunodeficiency syndrome (AIDS) patient, hepatitis B and C patients] should have a 'biohazard label' tagged on the container indicating that the contents should be handled with care.

Fluids: Proper collection of specimens is essential for good results. Clinical personnel should be trained well in procedural techniques and in the appropriate collection and submission of samples. The laboratory should provide instructions for proper collection of specimens and procedures should be available at the locations where specimens are collected. There are various methods by which fluids can be processed in the laboratories such as centrifugation, cytospin technique, Millipore filters, slow sedimentation cytology, liquid-based cytology and cell block technique. All laboratories should standardize these methods for optimal results and the laboratory supervisor should routinely check both methodology and staining performed. Processing of fluids should be immediate and not later than 2 hours for optimal results.

Gynecological Cytology

The Papanicolaou smear test is used routinely in all laboratories to detect cervical cancers. The diagnostic accuracy and reliability in reporting cervical smears minimize the risk of errors and provides high-quality service. The following measures should be followed:

1. **Reporting personnel and workload:** Medical regulations requires that individual examining a gynecological cytology specimen should be a qualified cytopathologist or pathologist in a certified laboratory. In primary screening of cervical smears, it requires to examine and evaluate every cell in the smear to detect relatively low number of abnormal

cells, less than 50 cells, scattered among large number of normal cells (300,000–500,000):

a. These individuals may examine up to a maximum of 100 slides per 24 hours (average 12.5 slides per hour) and in not less than 8 hours.
b. It is advised to screen smears for not more than 2 hours without break.
c. It is advised that screeners should screen not more than 6 hours/day.

This is the case for primary screening and each laboratory should establish individual workload limits for each cytotechnologist, and the limits reviewed 6 monthly, using laboratory-defined performance standards. All specimens must be reported using descriptive nomenclature easily understood by clinicians.

2. **Review of abnormal gynecological cases:** A cervical cytology specimen initially evaluated by a cytotechnologist as reactive, atypical, premalignant or malignant must be referred to a pathologist for final interpretation and report:
   a. Discordance between cytotechnologist's and pathologist's report is a reason for continuing education.
   b. Peer review may be taken for difficult cases and an outside consultation for cases with a discrepancy and significant clinical implications. The latter constitute part of quality assurance program and documentation of all reviews is essential.
   c. Clinicopathological discussions should be conducted in all the problematic cases.
3. **Rescreening of negative cases:** Most regulatory bodies governing cytology quality programs, specify that at least 10% of samples interpreted as negative by each cytotechnologist be rescreened by a pathologist or a qualified supervisor prior to reporting:
   a. Specimens from women considered to be at increased risk for cervical cancer must be included in the review process. High-risk patients are those with multiple sexual activity and those having infection with high-risk strains of human papillomavirus (HPV), i.e. 16, 18, 31, 35, etc.
   b. Rapid rescreening is a popular approach, which involves a supervisory staff who performs a partial review under a low-power objective of all the cervical smears reported as negative. Rapid review is a better method of quality control than random rescreening in terms of quality control, in that rapid review identifies more false-negative reports than 10% random rescreening in the same amount of time.

Stain Quality Control

Maintenance of good staining requires that the stains are filtered and replaced on a regular schedule determined by either the number of slides stained or the time elapsed, since stains were last replaced. Measures include:

1. Written standard operating procedures (SOPs) to ensure that staining protocols are adhered to the same, all reagents (including fixatives) and stains are clearly labeled and stored under appropriate conditions.

2. A daily record is kept for the need to top up fixatives and stains, and the replacement of same as the stains are replaced more frequently in hot weather or if there is a large throughput of smears.
3. Daily check of the quality of staining, i.e. the intensity of nuclear staining, contrast between eosinophilic and cyanophilic staining of cytoplasm, definition of nuclear chromatin, quality of dehydration of slide and clarity of mountant, etc. should be done by the technical supervisor.
4. A random selection of smears should be checked at yearly intervals to determine the extent of fading the stain and inadequate dehydration. Well-stained slides should maintain their color for at least 3 years.
5. Slide files should be checked random at 6 months intervals to ensure that slides can be readily retrieved, if necessary.

Standard Terminology of Reporting

Terminologies should be assorted to and the terms used should be easily understood by the clinicians. Although no minimal data sets currently exist for reporting exfoliative cytology, synoptic reports and templates are used in many departments. These should be agreed upon by the reporting team and may be used for the sake of improving consistency and turnaround time of reporting. The Bethesda system of reporting (2001) cervical smears is now well accepted in most centers. It should include all the relevant portions of the report.

Cytology-histology Correlation and Clinical Follow-up

All premalignant and malignant gynecological cytology reports should be correlated with subsequent histopathology, if available. Causes of any discrepancy should be determined. Cytohistological correlation can be a helpful educational tool used to refine methods of evaluation for both cytology and biopsy specimens. The correlation process should be documented in the laboratory quality assurance program. Negative biopsy specimens in the context of recognized squamous intraepithelial lesion (SIL) or cancer by cytology may be the result of a surgical sampling discrepancy. Comments regarding such cytological discordance in the surgical pathology report may be helpful in directing further patient management. The laboratory must have a clearly defined policy regarding the methods used for cytohistological correlation. If histological material is not available, the laboratory may attempt to obtain follow-up material or information on patients by sending letters.

Retrospective Reviews

All negative cervical cytology obtained within last 5 years must be reviewed, when any further abnormality on the patient is detected. If reviews affect current patient care then an amended report must be issued. Where the review does not result in issuance of a corrective report, then these interpretive discrepancies need not be documented. It should be kept in mind that knowledge of clinical outcome, context of slide examination and

hindsight all plague retrospective reviews. Every reasonable effort should be made to minimize bias, when reviewing cases or slides for laboratories or individual performance evaluation. There are a number of methods to attempt this including:

- Review by multiple individuals
- Review without knowledge of clinical outcome
- Review of the index case embedded in a slide sequence containing a range of normal and abnormal cases.

Measures of Screening Performance

Laboratory Performance

Cervical cytology is a highly successful screening test, but limited by both false-positive and false-negative results. False positives are likely to occur at some level because of difficult, subjective interpretive character of the cytological evaluation and due to pressures to minimize false-negative results. These results may be a consequence of:

1. Patient (inadequate) sampling by the clinician.
2. Laboratory screening or interpretation where abnormal cells are present on the slide, but are not identified by screening or misinterpretated during screening.

False-positive proportion should be aimed at keeping below 5–8% in any laboratory. The false-negative proportion calculated for a laboratory represents an estimate of the staff's average screening sensitivity. In most laboratories, it approaches 20% and an attempt should be made to limit it to this figure or even less.

Individual Performance

In a laboratory, individual performance also be assessed although specific methods of evaluation have not been laid down in literature. The most frequently used measures include random rescreening, targeted rescreening of specific patient groups, seeding abnormal cases into screening and rescreening pools, and retrospective rescreening of negative cervical cytology specimens from patients with a current high-grade abnormality. Since, accuracy of rescreening has a major impact on the rates of false negatives, it should be strictly implicated and monitored.

Proficiency Testing

Testing proficiency has been mandated for individuals examining gynecological and non-gynecological specimens. A number of state and national programs are available towards this and can be applied too. Liquid-based cervical cytology specimens should be included in the proficiency testing programs for laboratories that use this methodology. Laboratories using automated screening devices must follow the manufacturer's directions

that have been approved by the food and drug administration (FDA). Ongoing education is a requirement for proficiency in cytology. This requirement can be fulfilled by participating in proficiency testing, intradepartmental slide review sessions, attending workshops and symposia, teaching cytotechnology students, pathology students and fellow, and independent study.

Archiving and Retention of Data

Each laboratory is responsible for a good archiving of request forms, samples and reports. Procedures should comply with standard regulatory norms.

Slide Storage and Retrieval (Nongynecological)

1. Cytology laboratories must retain all non-gynecological slide preparations regardless of diagnosis for a minimum period of 5 years from the date of microscopic examinations.
2. Fine-needle aspiration glass slides must be retained for a minimum of 10 years.
3. Slides may be stored on site in the laboratory or on institutional premises or even off site, but must be retrieved within a reasonable amount of time for histocytological correlation.
4. When breakage is discovered there should be appropriate documentation of the incident and repair of the slide, if possible.

Storage of Documents

1. Test requisitions must be retained for 2 years from the date of receipt.
2. Test reports must be retained for 10 years from the date of report.
3. Logs and accession records for cytology specimens must be retained for 2 years from the date of receipt.
4. Quality control records for cytology specimens must be retained for 2 years from the date they were created and generated.

Gynecological Specimens

1. All gynecological slides must be stored for a minimum period of 10 years in good condition.
2. The request forms must be stored for a minimum period for 3 years.
3. All reports must be stored for a minimum period of 10 years.

Quality Assurance

1. Accreditation of laboratories should be carried out. The accreditation boards in India are Indian Academy of Cytology (IAC), National Accreditation Board of Laboratories (NABL) and ISO certification.
2. A laboratory should take part in external quality assurance programs and have internal quality control programs on a regular basis. These can be in the form of supervision, slide discussions, interdepartmental discussions, patient follow-up, etc.

3. A cytopathologist is responsible for the management of the cytopathology laboratory. Final responsibility for all activities performed in the laboratory is with the registered cytopathologist managing the laboratory.
4. The position of each employee in the pathology laboratory should be recorded in an organization flow chart.
5. A reasonable maximum workload, in terms of number of slides per day to be screened, should be established within the laboratory. This should take into account that cytotechnicians also have a task in quality control procedures, etc.
6. The laboratory in charge is responsible for selecting referral laboratories. The referring laboratories must report all test results from the referral laboratory without alterations. All opinions given should be recorded and conveyed to the treating clinician.

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Chapter 13

Quality Control in Histopathology

QUALITY CONTROL/QUALITY ASSURANCE

Quality control is the process through which quality performance is measured and compared with a specified standard and the difference is acted upon. In the laboratory, quality control is a day-to-day monitoring of reproducibility or precision, designed to detect any serious errors in any day's analytic procedures. Precision and reproducibility in histopathology means that same diagnostic label is applied to the same lesion and consequences that arise from the label are the same in the minds of all concerned with patient care.

In both histopathology and cytopathology, final report is the professional's opinion of an individual and is based on that individual's experience and judgment. Histopathology, thus differs from other pathology disciples because both technical and diagnostic performance is subjective as compared to clinical chemistry and hematology, which lend themselves to a statistical approach for control.

Quality assurance is a management system designed to achieve an acceptable level of quality of service with a minimum of expenditure. Quality assurance is usually a retrospective performance check on an individual or a laboratory, primarily by detecting constant deviations from the norm. It is based on process approach, whereas quality control is product-based approach. Both are complementary and necessary to produce a good histopathology standard.

DIAGNOSTIC STANDARDS

Diagnostic standards in pathology laboratories are maintained and improved by:

- Internal quality control
- External quality assessment (EQA) programs
- Audit
- Laboratory accreditation
- Continued medical education
- Clinicopathological case review meetings.

These processes are interrelated. For example, feedback from EQA provides opportunities for continuing medical education and participation in relevant EQA schemes enable compliance with accreditation standard.

Internal Quality Control

Internal quality control is carried out in most departments. It consists of continuous checking of routinely stained sections for quality and adequacy of staining, thereby reducing cutting artifacts. Inclusion of control slides with every batch should be a routine subjected to special techniques. Internal quality evaluation has three main benefits:

1. It is a valuable educational exercise for both consultants and trainee pathologists.
2. It promotes uniformity of nomenclature and diagnostic criteria.
3. It is a valuable guide to the suitability of individuals for the responsibility of surgical reporting.

External Quality Assessment

External quality assessment is an essential part of an overall laboratory quality assessment program. Many external quality assessment schemes have developed in the last few decades for which participation is voluntary. Diagnostic histopathology EQA schemes involve the circulation of test material to participants, usually consisting of histological sections with appropriate clinical information. Diagnosis and comments are returned to the person who organizes the scheme and reports relating to individual and institutional performance are returned to the participants.

The educational aim in histopathology EQA is twofold. First, viewing the material circulated with adequate clinical information enhances knowledge. Performance assessment to individuals can be assessed. The circulating slides must be typical of routine practice and not the rarities, which are more suited to slide seminars. The diagnosis should be discussed by the organizers at an open meeting of participants. However, the communication of results between organizer and participants must be confidential. Schemes must have a defined minimum rate of participants. Second, feedback must be measurable (scorable). The provision of a personal score to each participant is the most important way in which EQA schemes differ from slide clubs. Educational value is derived not only from the content but also from personal feedback, which allows individual participants to identify and correct problems in their own performance.

The use of quantitative feedback inevitably means that some pathologists will perform better in EQA schemes than others. This is also a mechanism for defining and investigating substandard performance in the pathologist.

Diagnostic histopathology EQA schemes exist on a local as well as national basis. Local histopathology EQA schemes cater to the needs of general histopathologist. These should cover any material in which a general histopathologist should be capable of reporting specialist. Better organized national schemes have been set up relating to the specific organ systems, e.g. gynecological pathology, renal pathology, neuropathology, etc.

Schemes for technique comprise slices of formalin-fixed tissue measuring approximately 1 × 1 × 0.5 cm and made from a spectrum of tissues ranging

from skin, adipose tissue, thyroid, fibrotic/hyalinized tissue, bone, mailed to each of the participating laboratories who in turn process the tissue, make stained sections and return slides to the nodal laboratory for pre-analytical assessment. The stained sections are scored by an independent expert and can be categorized as:

- Score 1: Unsatisfactory
- Score 2: Poor
- Score 3: Average
- Score 4: Good
- Score 5: Excellent.

A total of 15 marks are allotted under the following headings:

- Processing: 5 marks
- Sectioning: 5 marks
- Staining: 5 marks.

Laboratories that score less than 3 in any of the areas are advised to take immediate corrective actions.

The EQA is carried out under clinical pathology accreditation (CPA) and National Accreditation Board Laboratories (NABL). Other schemes are those of the Indian College of Pathologists/Indian Association of Pathologist and Microbiologist/NABL/Quality Control.

Audit

The audit was introduced by Carne in 1991. The derivative of audit is from the Latin word meaning 'hear' and a simple definition of medical audit is hearing from peers. It is also defined as an assessment of efficiency of the laboratories and allows histopathologists to analyze their own work. It is a systematic activity, with peer view of medical care, comparing actual practice with explicit standards of practice. The primary objective of medical audit and quality assurance is to improve the outcome of medical care to community health care.

Audit in histopathology refers to evaluation of the quality in histopathology report. The outcome of the reporting process is a diagnostic decision made by a histopathologist. The final report is the professional opinion of an individual and is based on that individual's experience and judgment.

Audit conducted at the departmental level is a necessary component of laboratory management and examines many aspects of the reporting process. In most local histopathology audit schemes, overall staining quality, assessment of laboratory speed and turnaround time are measured. Audit also assesses the workload both for the laboratory and for individual pathologists. Audit also plays a role in defining diagnostic criteria in histopathology. The technique of analyzing and evaluating patterns of diagnosis is derived from matching the histological impression with other factors such as age, sex, anatomical site, clinical history and incidence ratio.

A pathology service must audit its operations as part of the quality system in order to determine compliance of the service with current regulatory and accreditation requirements. Laboratories must be continuously enrolled,

participate and perform to an acceptable standard in external proficiency testing programs that cover all test methods performed in the laboratory where such programs are available. The laboratory must be able to track the specimen and procedures performed at all times. Procedures are required for:

1. The auditing of the organization's facilities, records and documents, methods, instruments, calibrations, staff training and competency, and quality control at appropriate intervals according to a predetermined schedule.
2. Reviewing the quality system.
3. Assessing user satisfaction and complaint handling.
4. Check tests, retrospective assessments, analysis of EQA schemes, proficiency testing, performance of autopsies, etc.

Laboratory Accreditation

Accreditation is the assessment or evaluation by an external agency of an individual or organization against defined criteria. It is the mechanism by which an agency or an organization, service or program of study meets specific predetermined standards.

It is also a professional as well as national recognition reserved for those who intend to provide high-quality health care. Accreditation is similar to licensing or registration, but is voluntary and given out by a recognized external agency, i.e. CPA or United Kingdom Accreditation Service (UKAS). A laboratory must fulfill certain criteria and be open for examination to qualify for a certificate of accreditation. The procedure is meant to assure the public and the health organizations that its practice is professionally competent. The problem with accreditation is that whilst it shows the standards set are being met at present, it cannot ensure that the service will meet these in future; neither is it organized to improve quality continuously.

Continuous Medical Education and Clinicopathological Case Reviews

Continuous medical education and clinicopathological case reviews are done to maintain satisfactory levels of performance by histopathologists, to impart further knowledge and advice to the clinicians, and take the feedback and vice versa.

PHASES OF QUALITY CONTROL IN HISTOPATHOLOGY

Quality control in histopathology is applied in three phases:

1. Preanalytical phase.
2. Analytical phase.
3. Postanalytical phase.

These steps provide a performance check for the duties, responsibilities and activities of the laboratory personnel. They monitor the breakdown of workload, turnaround time, costing of tests, the effect of staff changes, efficiency of the department, etc. These steps are discussed in detail.

Preanalytical Phase

Preanalytical phase includes the various steps from the submission of specimen to the issue of stained slides. Services provided must be in response to a documented request identifying the patient, requesting practitioner, the tests requested and appropriate clinical information. The laboratory must ensure accurate identification of the patient, labeling of samples and integrity of the specimen appropriate to the proposed testing, e.g. immunohistochemistry required for specific biopsies should have them fixed in neutral buffered formalin.

A unique accession number that is traceable to the specimen is of prime importance. Bar code technology can be of profound importance to minimize errors in sample accession. Recording of relevant information regarding the specimen is required, e.g. date and time of collection, anatomical site of the specimen, person performing the collection, specimen characteristics that may provide relevant information to the interpretation of result. Retention of the original request for testing or examination is required, in a readily accessible form in compliance with legislative and accreditation requirements and agencies, e.g. NABL, etc.

Planned changing of chemicals used for processing should be done based on the number of tissues processed. The laboratory personnel should record the number of tissues passed through every day and compulsorily change the chemicals once the predetermined limit is reached.

Access to protocols or standard operating procedures (SOP) for specimen collection, processing and storage must be easily available to the laboratory staff for reference. Documentation outlining the procedures authorized for use are required to be available in the appropriate work area. Periodic documentation review should be conducted as part of the audit process. Only appropriate standard validation records and recognized procedures should be available as part of the quality system, all test procedures should be documented in sufficient detail to enable the test to be performed properly. The equipment records include the overall history of each item of equipment, its date of acquisition, commencement of operation within the laboratory, initial acceptance and validation testing, maintenance service and repair, cleaning and decontamination, and ongoing conformity testing.

Analytical Phase

The analytical phase is related to actually carrying out the test. The assessment of analytical aspects in histopathology depends upon the subjectivity of the reports. Various methods of internal and external audit have been explained before. The review of selected cases (intradepartmental consultation), comparison with other reports (frozen/histopathology; cytopathology/histopathology), re-reporting of random cases by the same or different person to check the precision and accuracy. Sections obtained from one common

tissue block are stained with hematoxylin and eosin, and distributed to all EQA participating laboratories along with relevant clinical history and other information, which improves the analytical phase.

Postanalytical Phase

Reports commensurate with good medical laboratory practice and patient care must be provided to the requesting practitioner. Reports may be written or electronic, provided if they have been appropriately authorized/validated. Computers can perform simple and complex tasks under a rule-based system and can therefore evaluate the data by assessing their completeness and quality. Computers can also validate laboratory accession numbers, patient identification data and coded diagnosis. Accurate data capture can be achieved by a good retrieval system working on a clean database. A telephone or verbal report is always to be followed by an authorized written or electronic report, which include reasons for reporting (such as urgent request, critical values, recipient, staff member, date and time of call). Interpretive comments and appropriate additional information may be added to reports at the discretion of the person responsible for the issued report. Retention of patient data, specimens, test procedures and equipment records is required to be in compliance with good medical laboratory practice and statutory, and accreditation requirements. Appropriate storage to ensure their preservation and to facilitate retrieval is necessary.

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Chapter 14 Telepathology and Telecytology

As per European Union Committee, telemedicine is defined as a group of activities, which include investigation, monitoring and management of patient data by means of systems, which allow quick access to expert advice and patient information, no matter where the patient and the relevant information. Telemedicine is used in various branches such as radiology, endoscopy, cardiology, dermatology, pathology, etc. Telemedicine is more important in developing countries, since it allows extension of medical facilities in remote and rural areas.

TELEPATHOLOGY

Telepathology means practice of pathology at a distance, viewing histological, cytological or macro images transmitted along telecommunication pathways on a video monitor rather than directly through a microscope. Telepathology allows pathologists, surgeons and radiologists to communicate with each other over diagnostic dilemmas and overcoming the barrier of distance. It brings expert services closer to the patient, eliminating the unnecessary shifting to referral hospitals. It also provides a higher quality of education. This acquisition of images may be useful for diagnosis, consultation or continuing medical education.

Components

Components of telepathology network (Fig. 14.1) are:

- A conventional microscope
- A method for image capture, i.e. a camera mounted on a microscope
- Telecommunication link between the sending and receiving sites
- A work station at the receiving site with a high quality video monitor.

Types

Telepathology can be of two types namely 'static telepathology' and 'dynamic telepathology'.

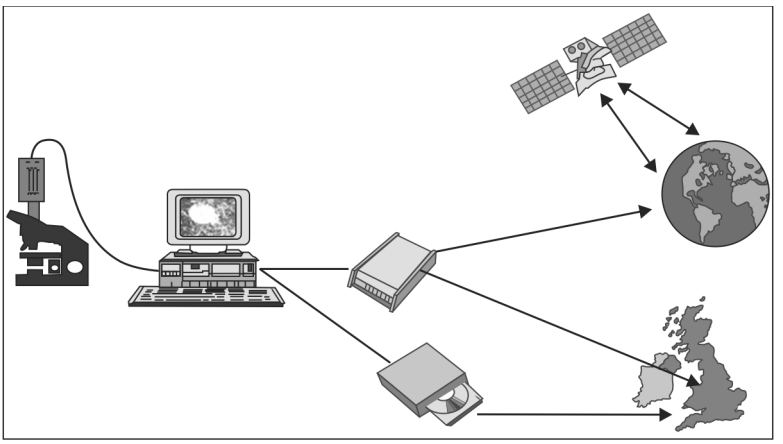

Figure 14.1: Telepathology network

Static Telepathology

Static telepathology is otherwise 'store and forward' method. It is cheap and simple, and needs only a standard telephone and internet connection. A limited number of images 'varying from 1 to 40' are captured and stored in the hard drive or compact disk-read only memory (CD-ROM) for later transmission.

Advantages: Small transfer size and no special software needed.

Disadvantages: Selection by nonexpert, may miss important areas, no facility for recipient control, number of images are limited, sampling errors due to inability to view the entire slide. Bias, i.e. selection of images that support one's own diagnosis, dependent upon video image quality, interpretation is based on the skill and experience of the pathologist in viewing static images. To the pathologist who is trained to see the slide in continuity, this may appear a suboptimal approach.

Dynamic Pathology

Dynamic pathology permits the recipient pathologist to control movement of the slide by means of a joystick and view 'real time' images on a high-resolution monitor. The sections are viewed in entirely, thus eliminating problems caused by inadequate/inappropriate field selection. It has a motorized microscope with remote control software.

Synonyms: Robotic interactive telepathology and real time telepathology.

Advantages: Selection of field of interest by expert, very fast and high resolution. Studies from different centers has shown that there is a high rate of concurrence even for frozen section biopsies and the time taken for diagnosis is little more.

Disadvantage: Complex technology.

Both static and dynamic telepathology can be combined to advantage and are not mutually exclusive (Table 14.1).

Utilities of Telepathology

Validated Applications

- Intraoperative frozen section
- Surgical pathology consultations
- Expert-to-expert consultations
- Quality assurance
- Distance education.

Provisional Applications

- Autopsy pathology
- Cytogenetics
- Cytometry imaging
- Cytopathology
- Fine-needle aspirates
- Hematopathology
- Immunohistochemistry.

Future Applications

- Point of care pathology services
- Ultra-rapid turnaround time surgical pathology particularly for difficult cases
- Proficiency testing.

TABLE 14.1: Difference between static and dynamic telepathology

Features	Static	Dynamic
Image system	Still	Live
Motorized microscope	No	Yes, bidirectional
Robotic remote control	No	Yes
Images per case	5 or more	Unlimited
Specimen sampling	Limited	Comprehensive
Image selection	Referring physician/pathologist	Telepathologist
Transmission time per image	45 second	1/15 second
Average time per diagnosis	15 minute	3–10 minute
Video conferencing	No	Yes
Network capacity	28.8 kbps	1.54 Mbps

Technical Aspects of Telepathology

Image Acquisition

Digitalized images are in the form of bitmaps, which are represented by a two-dimensional array of dots. These images could be in true colors derived from a pellet of 16.8 million combination of red, green and blue or in 256 shades of gray. Each true color picture element or pixel is composed of 24 bits of color information.

A video camera mounted over the microscope captures the desired fields. The camera of 2.1 megapixel or a higher resolution should be chosen. The number of pixels is a representation of the image resolution achievable by the camera. These cameras capture the 1,024 × 768 resolution images that are recommended for telecytological diagnosis. The camera must have screw threads on the inside of lens, which makes mounting of the camera to the microscope much easier (camera port). The image can be focused and viewed on the camera screen, and stored in memory space. A special processing program such as Adobe Photoshop will ensure high-quality imaging.

Standard personal computers designed for photorealistic videogames and multimedia, with a memory of minimum 256 MB random access memory (RAM) helps to assist in processing the imaging data. As image files can be large up to 5 MB before compression disk space in hard drive must exceed 20 GB. Programs allow to crop images, rotate images as well as to correct minor flaws such as underexposure, poor contrast and slight focus deficiencies.

With the help of a solid-state charge-coupled device (CCD) sensor, the image is converted to an electronic form. Using a three-chip video camera, each handling a single color, gives the best image color and sensitivity. The most important step is to select appropriate images that make sense of the pathological process. The lack of focusing ability and the issue of image manipulation (contrast, brightness and color) may all be important impediments to a successful outcome.

Image Compression

Standard color images occupy much storage space and need to be reduced in order to facilitate transmission. This is achieved with the help of compression algorithms, which are of two types:

1. **Lossy type:** It discards unnecessary data during compression.
2. **Lossless type:** It retains data at the expense of image size.

Some of the commonly used formats are:

1. **Joint photography experts group (JPEG):** This is a loose technique used for still image compression. Excessive compression may lead to false color and blockiness.
2. **Portable network graphics (PNG):** This is a lossless format approved by World Wide Web (WWW).

3. **Fractals:** This system uses a finite set of mathematical equations to describe an image and is ideally suited for real-time compression, but is time-consuming.
4. **Storage:** Hard drive is the most suitable for short-time storage. Other options include CD-ROM, scanners and extra hard disks.
5. **Computer networks:** A local area network (LAN) is a high-speed system, which links computers within a small geographical area and allows users to share resources. A wide area network (WAN) is operated by interchange carriers such as telephone companies. The internet can also be used for online discussion of cases with experts.

Telepathology Consultation Centers

There are a number of telepathology consultation centers around the world using static telepathology methods to help colleges across the globe. The centers of renown are:

1. Armed Forces Institute of Pathology started in 1993. Reports are given within 24 hours and faxed to the referring pathologists.
2. The Union for International Cancer Control (UICC), Telepathology Consultation Center at Berlin, as a panel consisting of 62 experts across the globe. Its services are free and reports are received by email or fax within 3 working days.

The idea of telepathology has also gained popularity in India. Baruah MK, from Dibrugarh, India, has been instrumental in bringing the telepathology concept to India. He is the telepathology expert in the World Health Organization (WHO) sponsored project on teleoncology in India. John Marshall Johnson from Bengaluru is the pioneer in the use and promotion of the internet in the field of pathology in India. He is the founder and moderator of the popular Pathoindia website—www.pathoindia.com, through which telepathology in India got its platform.

TELECYTOLOGY

Telecytology, a component of the broader field of telepathology, is the practice of cytology at a distance, using telecommunication networks (e.g. internet) to transfer digital images from one site to another. The emergence of fast and high-resolution digital imaging technology and computers with high-processing capacity has made telecytology possible.

Indications

1. Both gynecological and non-gynecological cases are amenable to telecytology for primary interpretation and secondary opinion consultations.
2. The lesion-best suited for telecytology are fine-needle aspiration cytology (FNAC) images of any site.

Drawbacks

Tissues or neoplasms that do not readily exfoliate or excessively bloody aspirates prove even more difficult to diagnose via telecytology as by traditional glass slides. The quality of the microscope has a profound effect on the quality of the images.

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Chapter 15 Molecular Diagnostic Pathology

IN SITU HYBRIDIZATION

In situ hybridization (ISH) technique was originally developed by Pardue and Gall (1969) and independently by John et al (1969). In 1970, ISH was applied to histological sections by Buongiorno Nardelli. In 1981 and 1986 radioactive probe labeling, e.g. biotin detected by avidin coupled to a fluorochrome, was developed. Pinkel and coworkers were the first ones to apply the fluorescence in situ hybridization (FISH) technique for the study of human chromosomes. Since 1986 and up to 1992, multicolor FISH experiments have been routinely performed for cytogenetic analysis.

In situ hybridization is defined as the morphological localization of genetic sequences. It involves application of a labeled probe to the tissue section and in detecting the label as a means of identifying where hybridization between probe and tissue has occurred allowing the deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) target molecules to be localized to specific cells in sectioned, but otherwise intact tissues. The objective is to determine the presence or absence of specific DNA or RNA sequence and to localize those sequences to particular cellular or chromosomal sites. The genetic target in molecular genetic analysis of tissues is nucleic acid either DNA or RNA.

The DNA mutations, which may be expressed as a structural alteration of RNA transcribed from the mutant genes, act as diagnostic markers in various pathological conditions. These DNA sequences are labeled with fluorescent dyes and applied to a metaphase spread or interphase nuclei of the sample. When this hybridization reaction is used to analyze the nucleic acid content of a sample, the process is called hybridization assay. The basic steps required to be followed during all hybridization techniques are:

1. Generation of a nucleic acid probe, labeled by a fluorescent dye to enable subsequent detection.
2. Preparation of chromosomal spreads or fixation of tissues (on the sample to be tested).
3. Pretreatment of tissues to increase accessibility of target nucleic acid.
4. Hybridization of labeled probe to chromosomes or tissue samples.
5. Washing under conditions that remove the non-hybridized probe.
6. Detection of the labeled probe, revealing the location of the target cellular nucleic acid on the sample.

Probe

Probe refers to a stretch of nucleotides that is used to detect a specific region of DNA or RNA, as a function complementary to the target sequence. Different types of nucleic acid probes can be prepared for use in ISH:

1. Long single-stranded DNA probes can be prepared by primer extension on single-stranded templates or by asymmetric polymerase chain reaction (PCR), with one of the nucleotides labeled.
2. Oligonucleotides (short probes), typically 20–40 bases in length, can be synthesized and allow specific probes to be readily designed from published sequences.
3. Single-stranded RNA probes can be synthesized by the use of an RNA polymerase to transcribe sequences downstream of the appropriate polymerase initiation site.
4. Radiolabeled probes, e.g. phosphorus 32, sulfur 35, tritium have high sensitivity, quantifiability and reproducibility of results. The disadvantages are safety issues relating to radioisotopes, reduced stability and lack of resolution.
5. Non-radioactive labels, e.g. biotinylated probes, enzyme-conjugated probes, hapten-conjugated probes and fluorescent probes. These overcome many of the problems seen in radioactive labels, but they reduce hybridization efficiency and lower the sensitivity.

Note: Longer probes can give stronger signals because it is possible to incorporate more labeled nucleotides into them. However, probes that are too long, give weaker signals because they penetrate less efficiently into the cross-linked tissue.

Preparation of Tissue

A wide variety of tissues can be used for ISH. These include fixed tissues, frozen tissue, cells from touch preparations, exfoliated, aspirated or cultured cells.

Fixation

About 4% of paraformaldehyde, 4% of formaldehyde or 1% of glutaraldehyde are most commonly used. These cross-linking fixatives give a greater accessibility and retention of cellular RNA.

Embedding and Sectioning of Tissue

Paraffin wax is the most popular embedding medium since sections up to 1 μm can be cut. Tissues can also be prepared on cryostat or embedded in plastic, which allows thinner sections to be cut.

Coating of Slides

Slides are coated with poly-L-lysine or aminopropyltriethoxysilane so that cells or tissues adhere on to the slides and are retained for the entire procedure.

Pretreatment of Tissue

Prior to hybridization, the tissues are subjected to a series of pretreatment that increases the efficiency of hybridization:

1. Whole tissues or sections are usually treated with organic solvents (ethanol or methanol) to permeabilize the cells by removing lipid membranes.
2. In order to increase the accessibility of the target RNA, the tissue is treated with protease (proteinase K), which partially digest cellular proteins.
3. Non-specific binding of probe to positively charged amino groups can be prevented by acetylation of these residues with acetic anhydride.
4. For whole mount hybridization, tissues are prehybridized by incubation in hybridization solution lacking a probe in order to block non-specific binding.
5. For double-stranded DNA targets, a heat denaturation step is carried out.

Hybridization and Washing

Following the pretreatments, hybridization is carried out under optimal conditions for annealing of the probe to target nucleic acid in the tissue. For DNA probes and targets, this requires initial separation of the double-stranded nucleic acid molecules into their single strands called denaturation. This can be achieved either by chemical means or by heating the double-stranded DNA to above its melting temperature, either in an oven or using a microwave. For DNA detection using DNA probes, both probe and target molecules must be denatured. This can be achieved in two ways either separately or by codenaturation. Denaturation is not necessary for RNA detection. Once the probe and target molecules have been rendered single stranded, all that is required for annealing to take place is for probe and target molecules to be brought together. For this, the incubation temperature is to be reduced to below the melting temperature of the required hybrids. Washing of the hybridized sections is carried out to remove excess probe that has bound to any sequences related to, but distinct from the integral target or nonspecifically to other cellular components.

Visualization of Signal

The final step of ISH is detection of the labeled probe in the tissue. The method for this depends upon the type of label that has been incorporated into probe:

1. **Radioactive probes:** For sulfur-labeled probes, a low-resolution signal can be obtained by placing the slide adjacent to an X-ray film with overnight exposure. A much greater resolution is obtained by dipping the slide in liquid nuclear track emulsion, which is then dried, exposed at 40°C and developed.

2. **Hapten-related probes:** The location of this can be visualized by fluorescence microscopy, directly if the probe is fluorescently labeled or indirectly with a conjugated antibody.

Applications

Detection of Infectious Agents

Gene probes are available for the identification of the genetic material of numerous bacteria, mycobacteria and viruses:

1. Specific localization of the organism with a specific cell type or histologic lesion, thus providing essential pathological link between the presence of an organism's genetic material and the presence of disease.
2. Valuable in viral infections, in which probes have the ability to provide insight into the molecular mechanisms of viral-induced cell injury and to determine the viruses in developmental anomalies, chronic degenerative diseases and oncogenesis.
3. The ISH has advantage over immunohistochemistry (IHC) that it can detect latent infections as well as active infections.
4. The ISH has potential to elucidate the mechanism of spread of viruses in tissues and to define their life cycles.

Cytogenetic

The ISH is the method of choice for localization of a gene at a specific locus. Nucleic acid probes are commercially available for large portions of every chromosome as well as specific gene arrangements associated with certain forms of neoplasia:

1. It is the most powerful tool for chromosomal mapping of single and multiple genes in the haploid genome, for delineation and classification of many genetic diseases.
2. Prenatal and postnatal detection of several genetic diseases.
3. Used in genetic counseling.

The FISH is the major growth field in cytogenetics and is predicted to have wide applicability in diagnostics, particularly when applied to interphase nuclei and perhaps when combined with flow cytometry.

Neoplasia

The ISH has emerged as a remarkably powerful tool for the assessment of gene expression in tumor cells at the cellular level. In contrast to IHC, which is dependent on protein content of cells, hybridization analysis offer the advantage of identifying cells on the basis of their contents of specific messenger RNA (mRNA) encoding the products of interest:

1. The ISH provides critical approach for the analysis of heterogenicity in tumors that typically contain cells at different phases of neoplastic progression, at multiple levels of differentiation and at different levels of functional activity.

2. Proven to be effective for the analysis of oncogene overexpression in a variety of different tumor types, e.g. lymphoma.
3. The demonstration of mRNA of encoding cytoskeletal proteins including intermediate filaments, actin and tubulin.
4. Considerable application for tumor typing when IHC techniques have given negative or equivocal results with either because of very low levels of intermediate filament synthesis and storage or antigen masking.

Neuroendocrinology

The ISH has proved to be of upgrade value in experimental and clinical neuroendocrinology:

1. Messenger RNAs encoding a large series of regulatory peptides have been demonstrated in both the central and peripheral neuroendocrine systems. In some instances, positive hybridization signals for certain regulatory peptide mRNAs such as somatostatin have been observed in the presence of negative immunohistochemical reactions for the peptide.
2. The demonstration of a particular mRNA by ISH is a reflection of the rate of gene transcription and mRNA turnover. These methods have proven to be of particular value for the analysis of certain neuroendocrine tumors.
3. In instances of ectopic hormone production, e.g. ISH can resolve the question of whether cells are simple concentrating their product from circulation or whether they are actively transcribing the specific mRNA for peptide synthesis.

FLUORESCENCE IN SITU HYBRIDIZATION

The FISH combines molecular biology, histology and cytogenetic techniques to analyze DNA in tissues and chromosomes. 'Fluorescent' means emitting light that comes from a reaction within the emitter. 'In situ' refers to the fact that this technique is done with the chromosomes, cells or tissue in place (in situ) on a microscopic slide. The technique does not require dividing cells and so can be performed on interphase nuclei, eliminating the need for cell culture and reducing turnaround time. It is used for the determination of the number of copies and location of a specific DNA sequence within human cells. The FISH can be performed on a wide range of specimen types, including fixed tissues and allows accurate chromosome identification from poor quality preparations.

Principle

The procedure allows on the ability of DNA probes to specifically hybridize to a DNA target sequence in tissue, cells or isolated chromosomes by making a probe complementary to the known sequence. These probes are labeled with a fluorescent marker, e.g. fluorescein.

The DNA inserts of human are in the order of 100,000–200,000 base pairs, which defines the limit of resolution of FISH for identifying chromosomal changes. Thus this ability of FISH to circumvent the need for dividing cells is invaluable.

Procedure

The procedure of FISH is as follows:

1. The chromosomes are put on a microscopic slide and the DNA is denatured.
2. The denatured probe is added on to the microscope slide and then letting the probe to hybridize to its complementary site.
3. Wash off the excess probe and look at the hybridization site in a fluorescent microscope. The probe will show as one or more fluorescent signals in a microscope, depending on how many sites it can hybridize to.

Types of Probes

1. **Chromosome sequence probes (chromosome painting):** The whole chromosome fluorescences.
2. **Repeat sequence probes:** They are isolated from telomerase/centromere regions and used in chromosome enumeration.
3. **Unique sequence probe:** Isolate from a clone disease-causing gene, used to identify the presence or absence of that gene.

Clinical Applications in Cytogenetics

1. By using a particular probe, chromosomal material of unknown origin can be identified:
   a. Identification of marker chromosomes.
   b. Identification of chromosomal variants or polymorphisms.
2. The FISH allows rapid screening of a large number of metaphases or interphases for a particular chromosome or other target sequence:
   a. Rapid screening for chromosomal mosaicism.
   b. Rapid screening for chromosomal aneuploidy (e.g. Down syndrome) in prenatal samples.
   c. Monitoring residual disease status in patients with leukemia.
   d. Monitoring the sex-matched bone marrow engraftment after transplantation.
3. For cancer diagnosis and prognosis:
   a. **Gains and losses:** The identification of abnormal chromosome number in cancer, e.g. trisomy 8 in many hematological tumors, trisomy 12 in chronic lymphocytic leukemia (CLL), gain of chromosome 3, 7, 17. Gene deletions—inactivation of tumor suppressor gene, such as p53 and RB, is commonly observed genetic event in cancer. The FISH deletional analysis is particularly useful for cytological specimens, e.g. 9p21 deletion can help to identify

transitional cell carcinoma in urine cytology and deletion of 1p, 3p or 22q is potentially useful for detection of malignant mesothelioma.

b. **Chromosome rearrangements:** Identification of chromosomal translocation, e.g. the detection of t(9;22) (q34;22q11) is more than 90–95% of chronic myelogenous leukemia (CML) and in small subset of ALL and result in fusion of the break point region (BCR) to the Abelson murine leukemia (*ABL*) gene. Use of two differently-labeled probes (dual color FISH) for both BCR and ABL loci will show the presence of fusion signal, which strongly suggest presence of BCR-ABL and is diagnostic of CML. In small round cell tumor, t(11;22) (q24;q12) identifies Ewing's sarcoma/primitive neuroectodermal tumor (PNET). In spindle cell tumors, t(X;18) (p11;q11) is characteristic of synovial sarcoma; t(11;14) (q13;q32) is diagnostic of mantle cell lymphoma; t(14;18) (q32;q21) identifies follicular center cell lymphoma.
c. **Gene amplification:** It is an oncogene mechanism observed in most cancers, e.g. detection of multiple gene copies of HER2/neu in breast adenocarcinoma, c-MYC in carcinomas and leukemias, and n-MYC in neuroblastomas.

TISSUE MICROARRAY

Tissue microarray, a recent innovation in the field of pathology was first reported 25 years ago by Battifora. The format for this tissue microarray was first conceived by Wan and colleagues in 1987. The microarray contains many small representative tissue samples from hundreds of different cases assembled on a single histological slide, therefore allows analysis of multiple specimens at the same time. It designed as molecular biology technique for researchers that allows for assessment of expression of disease-related genes or gene products simultaneously on hundreds of tissue samples. This method enables pathologists to perform large scale analysis using FISH, IHC or RNA/ISH at substantially faster and at markedly lower costs compared with conventional approaches. Tissue microarray technology should not be confused with DNA microarrays, where each tiny spot represents a unique cloned cDNA or oligonucleotide. Tissue microarrays differs from DNA microarrays as in the former, the spots are larger and contain small histological sections from unique tissue or tumors.

Tissue Microarray Technique (Fig. 15.1)

Tissue microarray is a technique for organizing minute amounts of biological samples on a solid support. These are composite paraffin blocks constructed by extracting cylindrical tissue core 'biopsies' from different paraffin donor blocks and re-embedding these into a single recipient (microarray) block at defined array coordinates.

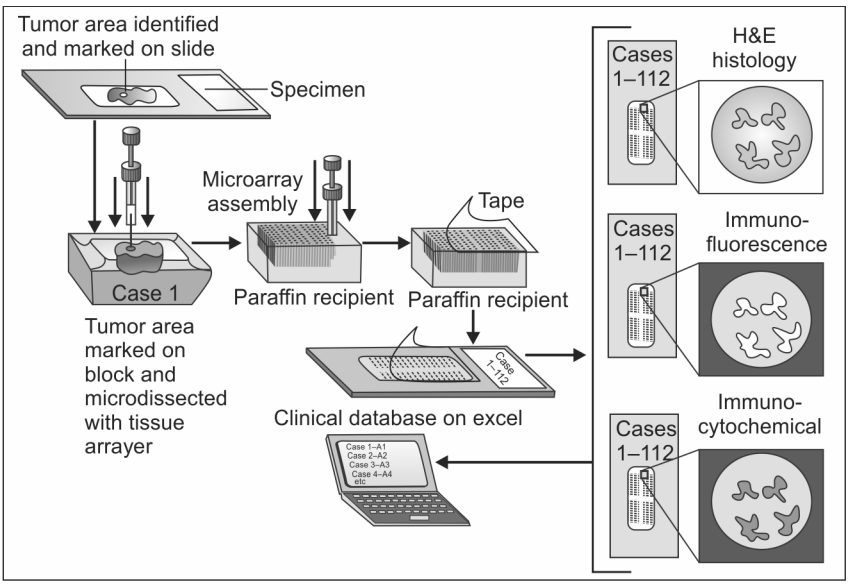

Figure 15.1: Tissue microarray technique (H&E, hematoxylin and eosin)

Method

At first, the donor blocks are retrieved and sectioned to produce standard microscopic slides that are stained with hematoxylin and eosin. An experienced pathologist examines the slides to mark the area of interest, commonly an area of cancer, following which the samples can be arrayed. A special tissue microarray instrument (e.g. Beecher instruments, Wisconsin, USA) is used to acquire a tissue core from the donor block. The extracted core is then placed in an empty paraffin block—the recipient block. The currently available Beecher instruments arraying device is designed to produce sample circular spots that are 0.6 mm in diameter at a spacing of 0.7–0.8 mm. The surface area of each sample is 0.282 mm^2 or in a pathologist's term, about the size of 2–3 mm high-power fields. The sampling process can then be repeated many times from different donor blocks until hundreds or even thousands of cores are placed into one recipient block. The number of spots on a single slide is variable depending on the array design; the current comfortable maximum is about 600 spots per standard glass microscopic slide with a 0.6 mm needle core.

Advantages and Applications of Tissue Microarray

1. **Amplification of a scarce resource:** A standard histological section is about 3–5 mm thick, sections can be cut 50–100 times depending on the skill of technician. On an average, each archived block yields material for a maximum of 50–100 assays. If the same block is processed for optimum microarray construction, it would routinely be needle

biopsied 200–300 times or more depending upon the size of tumor in the original block. Thus instead of 50–100 conventional sections or samples for analysis from one tissue biopsy, the microarray technique could produce material for 500,000 assays (assuming 250 biopsies per section times 200) (sections 2.5 μm thick), per 5 mm array block represented as 0.6 mm disks of tissue. Thus the technique amplifies (up to 10,000 fold) the limited resource.

2. **Simultaneous analysis of very large numbers of specimens:** Tissue microarrays provide high-data acquisition and methodology. For instance, if a tissue microarray block contains 1,000 cores and is cut 200 times, this allows for 200,000 individual assays.
3. **Experimental uniformity:** With each technology, each tissue sample is treated in an identical manner and microarrays are amenable to a wide range of techniques, including histochemical stains, immunological stains with either chromogenic or fluorescent visualization, ISH (including mRNA, ISH and FISH) and even microdissection techniques.
4. **Decreased assay volume, time and cost:** As only a small amount of reagent is required to analyze an entire cohort, less laboratory personnel are required to perform the experiments. This method has proven to be very efficient, of shorter duration and cost-effective, especially with expensive reagents.
5. **Does not destroy original block for diagnosis and thus conserves valuable tissue:** There are occasions where the original block must be returned to the patient or donating to institution. In these cases, the block may be cored a few times without destroying the original tissue block.

Disadvantages

The most common criticism of tissue microarray is that the small cores sampled may not be representative of the whole tumor, particularly in heterogeneous cancers such as germ cell tumors and Hodgkin's lymphoma.

FLOW CYTOMETRY

The first multiparameter flow cytometry was invented by Kamentsky in the year 1965 and the first fluorescence-based flow cytometer was invented by Wolfgang Göhde from the University of Münster, Germany in 1968. It is interesting to note that the original name of flow cytometry was pulse cytophotometry. In 1978, at a conference in Florida, its name was changed to flow cytometry. It is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity and relative fluorescence intensity. These characteristics are determined using an optical to electronic coupling system that records how the cell or particle scatters incident light and emit fluorescence. It is a powerful tool for the investigation of normal and

neoplastic hematopoietic cells. Its ability to measure multiple parameters on individual cells at high speed is ideal for the study of lymphomas and leukemias. Flow cytometry immunophenotyping using highly specific monoclonal antibodies (moAbs) to cell surface antigens and intracellular components has greatly improved the knowledge of origin, and differentiation of acute and chronic leukemias as well as non-Hodgkin lymphomas. A flow cytometer is made up of three main systems—fluids, optics and electronics.

The fluidic system transports particles in a stream to the laser beam for interrogation. The optical system consists of the laser to illuminate the particles in a simple stream and optical filters to direct the resulting light signals to the appropriate detectors. The electronic signals convert the detected light signals into electronic signals that can be processed by the computer (Fig. 15.2). For some instruments equipped with a sorting feature, the electronic system is also capable of initiating sorting decisions to charge and deflect particles.

In flow cytometry, particles are carried to the laser intercept in a fluid stream. Any suspended particle or cell from 0.2 to 150 μm in size is suitable for analysis. Cells from solid particles are disaggregated (as in lymph nodes) before analysis. The portion of fluid stream, where particles are located is called sample core. When particles pass through laser intercept, they scatter laser light. If any fluorescent particles present on these, it fluoresces. The scattered and fluorescent light is collected by appropriate positioned lenses. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. Both forward scatter (FSC) and side scatter (SSC) are unique for every particle, and a combination of the two may be used to differentiate different cell types in a heterogeneous sample. The detectors produce electronic signals proportional to the optical signals striking them. Measurement of light scattered by the cell in a forward

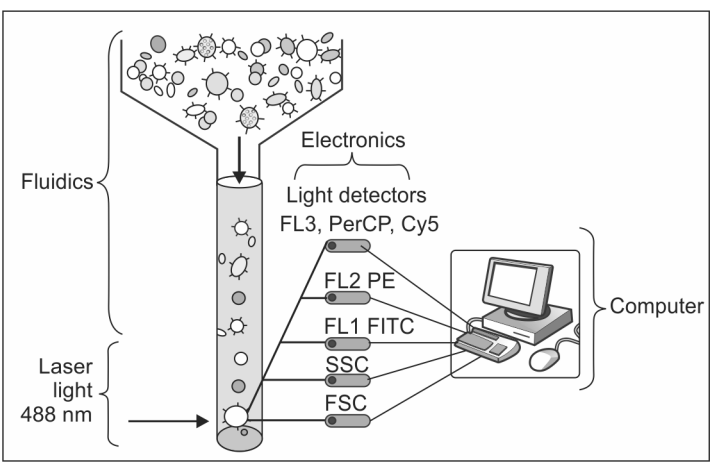

Figure 15.2: Scattered and emitted light signals are converted to electronic pulses that can be processed by the computer (FITC, fluorescein isothiocyanate; FL, fluorochrome; FSC, forward scatter; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SSC, side scatter).

direction is proportional to cell size, whereas light scatter at 90° is related to cell granules. By using these two physical parameters it is possible to discriminate lymphocytes, monocytes and granulocytes.

List mode data are collected on each particle or event. The characteristics or parameter of each event are based on its light scattering and fluorescent properties. The purpose of a fluorescent probe, such as a fluorochrome-conjugated antibody, is to directly target an epitope of interest and to allow its biological and biochemical properties to be measured more easily by the flow cytometer.

Fluorescent probes are useful in a wide range of applications including—identifying and quantifying distinct populations of cells, cell surface receptors, or intracellular organelles, cell sorting, immunophenotyping, calcium influx experiments, determining nucleic acid content, measuring enzyme activity, and for apoptosis studies. By changing the excitation light and using more than one fluorochrome, it is possible to analyze several parameters of the sample at any one time. The data are collected and stored in the computer. This data can be analyzed to provide information about subpopulations within sample.

Although flow cytometry technology offers the advantage of rapid cellular analysis with better objectivity, morphological correlation is lost. It is critical to review the smear or cytospin preparation to determine the composition of cell suspension submitted for flow cytometry for correlation.

POLYMERASE CHAIN REACTION

The PCR was first developed in 1987 by Kary Mullis (who won a noble prize) and his associates. It is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology because it is quick, inexpensive and simple. This technique amplifies specific DNA fragments from minute quantities of source DNA material, even when that source is of relatively poor quality. The PCR serves to copy DNA. It uses repeated cycles, each consisting of three steps (Fig. 15.3):

1. The reaction solution containing DNA molecules (to be copied), polymerase (which copy the DNA), primers (which serves as starting DNA) and nucleotides (which are attached to the primers) is heated to 95°C. This causes the two complementary strands to separate, a process known as denaturation or melting.
2. Lowering the temperature to 55°C causes the primers to bind to the DNA, process known as hybridization or annealing. The resulting bonds are stable only if the primer and DNA segment are complementary, i.e. if the base pairs or DNA segments match. The polymerases then begin to attach additional complementary nucleotides at these sites, thus strengthening the bonding between primers and DNA.
3. Extension: The temperature is again increased, this time to 72°C. This is the ideal working temperature for the polymerases used, which add further nucleotides to the developing DNA strand. At the same time, any loose bonds that have formed between the primers and DNA segments

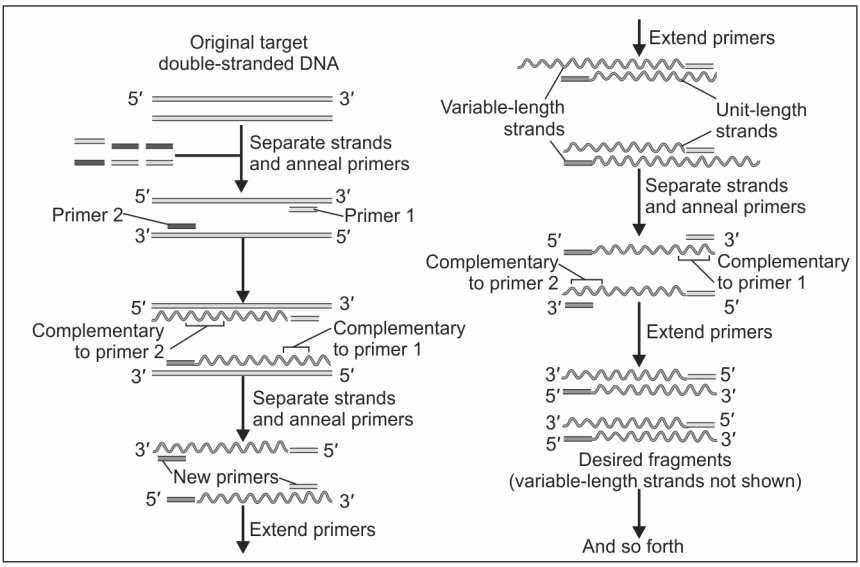

Figure 15.3: Amplification of target sequence

that are not fully complementary are broken. Each time these steps are repeated, the number of copied DNA doubles. After 20 copies a million copies are cloned from a single segment of double-stranded DNA.

DNA polymerase known as Taq polymerase is named after the hot spring bacterium *Thermus aquaticus* from which it was originally isolated. The enzyme can withstand high temperature needed for DNA strand separation and can be felt in the reaction tube.

The reaction products are separated by gel electrophoresis. Depending on the quantity produced and the size of the amplified fragment, the reaction products can be visualized directly by staining with ethidium bromide or a silver staining protocol, or by means of radioisotopes and autoradiography.

Applications

1. Amplifications of small amount of DNA for further analysis by DNA fingerprinting.
2. Mapping the human (and other species) genome.
3. The isolation of a particular gene of interest from a tissue sample.
4. Generation of probes: Large amount of probes can be synthesized by this technique.
5. Production of DNA for sequencing: Target DNA is cloned using appropriate primers and then its sequence determined.
6. Analysis of mutation: Deletion and insertion in a gene can be determined by differences in size of amplified product.

7. Diagnosis of monogenic diseases (single gene disorders): For prenatal diagnosis, PCR is used to amplify DNA from fetal cells obtained from amniotic fluid. The PCR has also proved to be useful in carrier testing.
8. Detection of microorganism: Especially of organisms that take long time to culture.
9. Crucial forensic evidence may be present in small quantities, e.g. one human hair, body fluid stain (blood, saliva, semen). The PCR can generate sufficient DNA from a single cell.

Limitations of PCR

1. The PCR is extremely sensitive technique, but is prone to contamination from extraneous DNA, leading to false-positive results. Another potential problem is cross contamination between samples.
2. Reagents and equipment are costly, hence cannot be afforded by small laboratories.

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Chapter 16 Laboratory Waste Management

INTRODUCTION

Management of biomedical wastes is a critical area and of great concern primarily because of three important considerations:

1. Biomedical waste could be highly infectious.
2. Unscientific disposal of waste can generate more number of sick patients through exposure to infectious agents.
3. Produces toxic substances on earth in the form of dioxins and furans.

IMPACT OF IMPROPER WASTE MANAGEMENT

The impact of adverse effects on the environment can be categorized into air, water and soil pollution. Air pollution is predominantly due to harmful emissions from formalin fumes and suspended particulate matter. Water pollution is the result of chemical and liquid wastes being put in the sewers and drainage systems. Soil pollution results from improper disinfecting and dumping of waste in landfills and incinerating the same. The ash is heavy in toxic substances or metals.

PRINCIPLES AND OBJECTIVES OF WASTE MANAGEMENT

Basic Principles of Waste Management

1. Minimize waste by use of 3R (reduce, reuse and recycle).
2. Biosafety measures to protect the healthcare workers and personnel in charge of final disposal of waste residues.
3. Proper collection, handling, storage and disposal of basic medical waste.
4. Optimum use of sterilization/disinfection.
5. Development of policies and programs.

Objectives of Waste Management

Objectives of waste management should be:

- To reduce the hazardous nature of the waste
- To prevent misuse/abuse of the waste
- To reduce the volume of the waste
- To ensure occupational safety.

HEALTHCARE WASTE GENERATION

From Tertiary Care Hospital

In a tertiary care hospital, the waste may be composed of:

- 80%: General healthcare waste, may be dealt with normal domestic and urban waste management system
- 15%: Pathological and infectious waste
- 1%: Sharps waste
- 3%: Chemical and pharmacological waste
- Less than 1%: Special waste—radioactive or cytotoxic waste, pressurized containers, broken thermometers, used batteries.

From Pathology Laboratory

The pathology laboratory waste generated during diagnostic procedures is composed of:

- Liquid (urine and cytology samples) and solid (body tissues, organs) wastes
- Soiled swabs, gloves, stain remnants
- Soiled linen
- Sharps (needles, broken slides and coverslips).

CATEGORIES OF WASTE

The hospital wastes are commonly categorized depending on their physical or chemical composition and their effects in health and environment. As per the 'Biomedical Waste Rule, 1998' there are 10 categories of waste (Table 16.1) [Government of India, Gazette Notification SD(E) of 20-07-1998].

HOSPITAL WASTE DISPOSAL

The Government of India has prescribed strict procedures and guidelines for each step in waste management leading to waste disposal in hospitals. The following are the brief outline of these steps.

TABLE 16.1: Categories of biomedical waste—treatment and disposal

Category number	Waste category	Treatment and disposal
1	Human anatomical waste (human tissues, organs, body parts)	Incineration*/Deep burial
2	Animal waste (animal tissues, organs, body parts, carcass, bleeding parts, fluid, blood and experimental animals used in research, waste generated by veterinary hospital colleges, discharge from hospitals, animal houses)	Incineration*/Deep burial

Contd...

Contd...

Category number	Waste category	Treatment and disposal
3	Microbiology and biotechnology wastes (from laboratory cultures, stocks or specimens of microorganisms, live or attenuated vaccines, human or animal cell culture in research and industrial laboratories, wastes from production of biological toxins, dishes and devices used for transfer of cultures)	Local autoclaving/ Microwaving/ Incineration*
4	Waste sharps (needles, syringes, scalpels, blades, glass, etc. that may cause puncture and cuts; this includes both used and unused sharps)	Disinfection-chemical treatment†/ autoclaving, microwaving and mutilation/shredding
5	Discarded medicines and cytotoxic drugs (wastes comprising of outdated, contaminated and discarded medicines)	Incineration‡/ Destruction and drug disposal in secure landfills
6	Solid waste (items contaminated with blood, and body fluids including cotton dressing, soiled plaster casts, linen, beddings, other material contaminated with blood)	Incineration*/ Autoclaving and microwaving
7	Solid waste generated from disposable items other than the waste sharps such as tubings, catheters, intravenous sets, etc.	Disinfection— chemical treatment†/ autoclaving, microwaving and mutilation/shredding‡
8	Liquid waste (generated from laboratory and washing, cleaning, housekeeping and disinfecting activities)	Disinfection—chemical treatment† and discharge into drains
9	Incineration ash (from incarnation of any biomedical waste)	Disposal in municipal landfill
10	Chemical waste (chemicals used in production of biologicals, in disinfection, as insecticides, etc.)	Chemical treatment† and discharge into drains for liquids and secured landfill for solids

*There will be no chemical pretreatment before incineration. Chlorinated plastics shall not be incinerated.

†Chemical treatment is using at least 1% hypochlorite solution or any other equivalent chemical reagent. It must ensure that chemical treatment assures disinfection.

††Mutilation/Shredding must be as such so as to prevent unauthorized reuse.

Segregation

In this different categories of waste are sorted and placed in different containers or bags (Table 16.2). Segregation helps in reducing total treatment cost and chances of infecting healthcare workers. General waste does not become infectious.

Transportation

Internal and external transportation is an integral part of the hospital waste management system. Within the hospital, plastic bag containing waste may be tied well and transported by a handcart to a storage point. Equipment used for transport should be frequently cleaned using disinfectants. Designated vehicles used for external transport of wastes should be roadworthy and should carry the wastes in covered containers.

Storage

Daily laboratory wastes from different facilities of the laboratory awaiting final disposal are stored in a store room meant for the purpose. The store room should be away from the service areas and should be dry and well secured to prevent rodent nuisance.

Labeling

In medical waste disposal, it is essential to utilize proper labeling system. Waste container to be sent to off-site treatment and disposal should be clearly marked immediately after packaging. The label should indicate the type of waste, time, date and treatment method.

TABLE 16.2: Color coding and type of container for disposal of biomedical wastes

Color coding	Type of container	Waste category	Treatment options as per schedule I
Yellow	Plastic bag	Category 1, 2, 3 and 6	Incineration/Deep burial
Red	Disinfected container/ Plastic bag	Category 3, 6 and 7	Autoclaving/Microwaving/ Chemical treatment
Blue/White translucent	Plastic bag/Puncture-proof container	Category 4 and 7	Autoclaving/Microwaving/ Chemical treatment and destruction/shredding
Black	Plastic bag	Category 5, 9 and 10 (any solid)	Disposal in secured landfill

Notes: Categories 8 and 10 (liquid) do not require containers/bags. Category 3 if disinfected locally need not be put in containers/bags. Waste collection bags for waste types needing incineration shall not be made of chlorinated plastics.

Record Keeping

Effective hazardous hospital waste management requires accurate record keeping of all aspects of the program to document expenditures and evaluate minimization efforts.

Treatment

The treatment modifies waste before final disposal. The objectives are:

1. Documentation of the waste to render it noninfectious by steam sterilization or autoclaving or chemical disinfection.
2. Reduction of bulk volume by incineration.
3. To give the waste an esthetic look, e.g. for body parts.
4. To destroy reusable-infected materials such as needles and blades.

Methods of Disinfection

1. **Chemical disinfection:** Liquid waste from laboratories may be decontaminated chemically. Bleaching powder, sodium hypochlorite, chloramines, isopropyl alcohol, povidone iodine, glutaraldehyde, alcohols or quaternary ammonium compounds may be used. Factors such as concentration and stability of chemicals, surface contact time, etc. determines effectiveness of a chemical disinfectant.
2. **Autoclaves:** It is a low heat process with a holding time of 20–30 minutes at a temperature of 121°C. In this method, the waste is put inside an autoclave and the required temperature and pressure is maintained for a fixed duration of time during which all microorganisms are destroyed.
3. **Microwave:** It is a low-heat thermal process in which decontamination occurs through electromagnetic waves passing through the waste material. The waves penetrate into the material for a period of about 25 minutes.
4. **Incineration:** It is the process by which combustible materials are burned, at high temperature under controlled conditions, by use of oil, electricity or conventional fuel. This oxidation process reduces organic and combustible waste to inorganic matter and results in a very significant decrease of waste volume as well as weight. This process is usually selected for wastes that cannot be recycled, reused or disposed in a landfill site. Incineration hospital waste categories include organic waste from surgery, autopsy and delivery, blood and body fluids from dialysis and transfusion, microbial and pathological waste, etc. Heavy metals such as mercury, cadmium and chloride-based plastic (e.g. polyvinyl chloride) cannot be incinerated at low temperature (800°C) as at these temperatures they produce hazardous particulate matter and gases such as hydrogen, chloride, dioxins and furans.

Final Disposal of Hospital Wastes

Depending upon the quantity of general, non-hazardous solid wastes generated in a hospital, the following disposal options are available.

For Small Quantity of Wastes

Landfilling can be done by trench, ramp or area method. The depth should be at least 2 meters as per Biomedical Waste Rules, 1998. The pit is filled with waste to 50 cm of surface and rest filled with soil. The place is chosen far away from habitation and usable ground water.

For Large Quantities of Wastes (in Commercial Scale)

1. Composting through thermophilic aerobic bacteria.
2. Pelletization technology where wastes get converted into pellets by treating with heat, humidity and pressure.
3. Gasification technology in which aerobic digestion is followed by production of biogas.

CONCLUSION

The handling of biomedical waste with appropriate technology is mandatory (by law). However, the type of technology, that is suitable for any given institution or country would largely depend on the finances available. Hence in developing countries those technologies that are cost-effective should be adopted rather than going for very high technology for which infrastructural facilities may not be available.

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Chapter 17 Principles in the Functioning of Microscopes

HISTORICAL ASPECTS

A microscope is the most common instrument used in the laboratory. In Greek 'mikros' means 'small' and 'skopein' is 'to see'. The term was coined in 1625 by Giovanni Faber, 48 years before Leeuwenhoek developed a functional microscope in 1673.

The first simple microscopes were glass globes filled with water and these were described in the 1st century AD by Pliny. Lenses were used as spectacles since the 13th century. In 1610, Galileo improved a microscope with a concave mirror. 50 years after Galileo, Robert Hooke and the Curator of the Royal Society prepared lenses of considerable magnification by globules formed by fusing threads of spun glass. He described the structure of nettle stings, bee stings, flies, feet lice and spiders are laid the earliest foundation of the cell theory of biological structures. Hooke was unaware of the importance of what he saw. Marcello Malpighi used a magnifying lens and described the structure of lung, capillaries and breathing tubes of insects and the pores of the leaves. The indefatigable Dutchman Jan Swammerdam described the compound eyes of the bees and the red cells of the frogs. All these pioneers used single lenses. Antonie van Leeuwenhoek (1632–1723), a Dutch dry goods clerk, was the first to describe microorganisms in the tartar of his own teeth as 'animalcules'. All these pioneers used single lenses of short focal length and these had their limitations. The enormous potential of the microscope was realized with the invention of the compound microscope. This discovery of using three lenses together was accidental when the children of two Dutch spectacle makers Johan and Zacharias Jansen, while playing viewed the church spires through two lenses—a convex and a concave. The church spires appeared very near and their shouts of joy attracted the attention of their elders. The Jansens made the first telescope and presented it to prince Maurice of Nassau. Galileo copied the discovery and discovered the four moons of Jupiter. Several technical and optical difficulties had to be overcome before the compound microscope came into its own.

LENSES

The lens is the basic component of a microscope and its aberrations or faults place most of the limitations on a microscope. Lens refers to a piece of glass or other transparent material, usually circular, which has its faces ground and

polished in such a manner that rays of light passing through it either converge or diverge. Lenses, which converge light rays and form images are called positive lenses; those that cause rays to diverge and which do not form real images are called negative lenses (Fig. 17.1).

The principle focus in a lens is that point at which a lens forms a sharp image. In addition to the principle focus, positive lenses also have conjugate foci, i.e. they form sharp images on a screen at a distance, which is dependent upon the distance of the object from the lens. Bringing the object closer to a lens causes the image to be more distant and therefore more magnified. Therefore:

$$\text{Magnification} = \frac{\text{Screen distance from the lens}}{\text{Object distance from the lens}}$$

For example: $\frac{160}{4} = 40$ mm

As in the case of a high-dry objective lens of a microscope, in this case, however, moving the object really close to the lens causes the image to disappear (to form at infinity) and finally reappear as a virtual image on the same side as the object. It is the virtual image on that we see when looking through the microscope.

Aberrations

Spherical Aberrations

Spherical aberration occurs in a convex lens. The cause of this defect is the curvature of the lens. Rays passing through periphery of the lens are bent more than rays passing through the central parts of the lens. The rays bent more will therefore come to focus nearer the lens than those that are bent less. Hence, a point source of light will not be focused sharply and the image is hazy (Fig. 17.2). The spherical aberration can be corrected to a large extent, by a combination of convex and concave lenses.

Chromatic Aberrations

An ideal lens should not split the incident light into true primary colors—VIBGYOR, but in reality this is not so. White light is composed of colors of

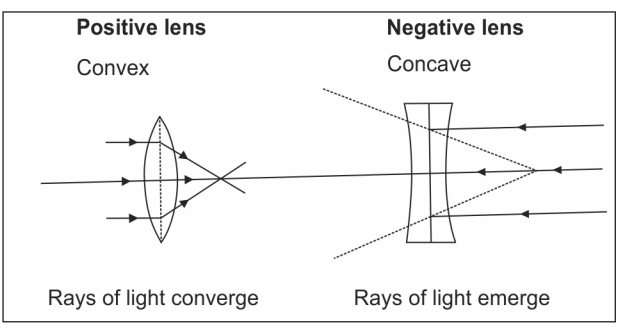

Figure 17.1: Negative and positive lens

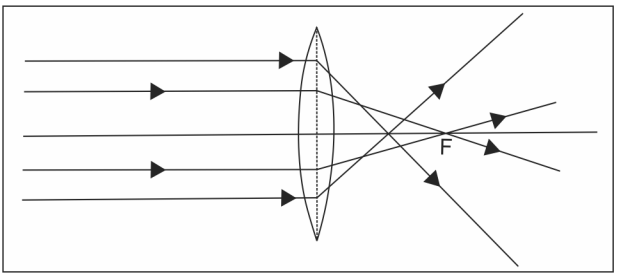

Figure 17.2: Spherical aberration

the spectrum, each of which has a different wavelength. Each spectral color is refracted differently when it passes through the lens system. Colors with shorter wavelengths are bent to a greater extent than colors with longer wavelengths. Thus red is bent the least, while blue is bent the most (Fig. 17.3). Thus, blue comes to focus nearer the lens, while red color comes to focus further away. The image formed has a red fringe surrounding a blue fringe or vice versa. A lens, which is corrected for two wavelengths of light in the green-blue region is called 'achromatic lens.' The incorporation of fluorite allows correction of three wavelengths of light and such lenses are known as 'fluorite lenses.' If they are corrected for all wavelengths, they are called 'apochromatic lenses.'

COMPOUND MICROSCOPE (FIG. 17.4)

The compound microscope is a combination of lenses—the objective lens system (lens closer to the object) and ocular lens system (lens closer to the eye) are used in combination to form the image to retina, which is virtual image disappearing below the plane of the object.

Objective Lenses

Objective lenses in wide use today are the achromatic, which fulfill all requirements for ordinary work. The objective screws into the bottom of the body tube by means of a standard thread. This makes all objectives interchangeable. However, this practice of interchanging them should be avoided because many manufacturers use the objective and ocular interdependently to correct some aberrations.

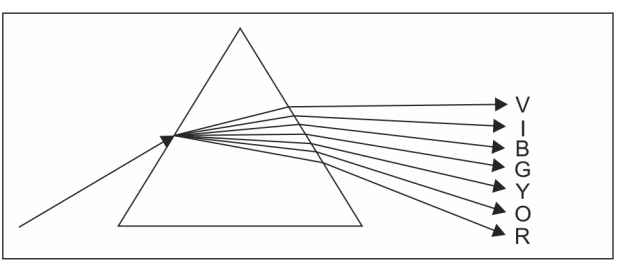

Figure 17.3: Chromatic aberration [splitting of white light to seven colors by a prism (lens system)]

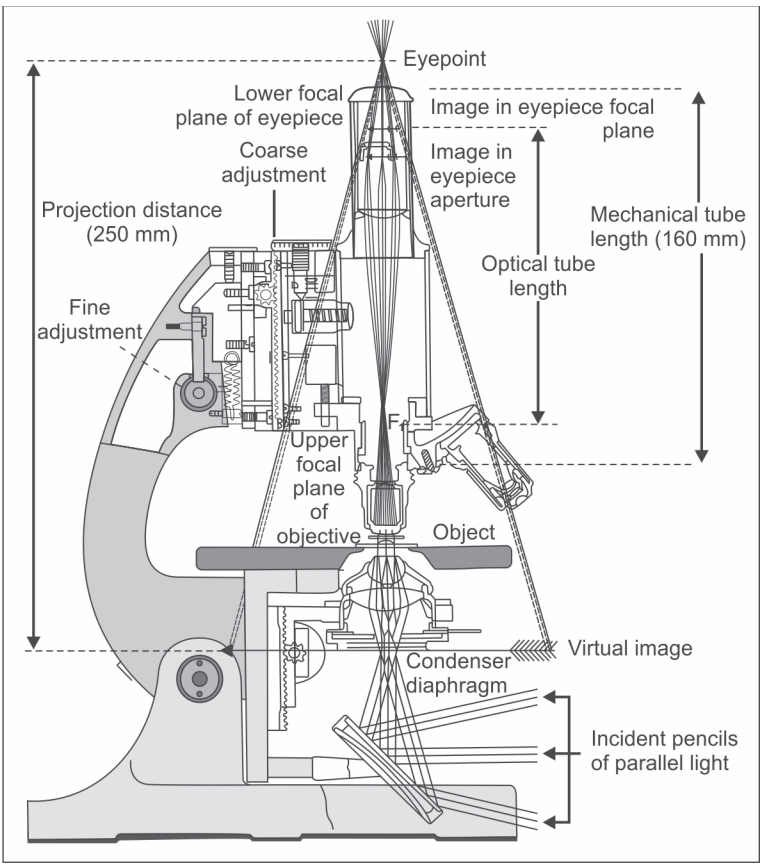

Figure 17.4: Compound microscope

Objectives usually are marked with their magnifying power (2.5X, 10X, 40X and 100X), but this only applies when they are used at a fixed tube length (distance from the top of the eyepiece slot to the nosepiece where the objective screws in), which is normally 160 mm. Dividing the tube length by the focal length will give the magnifying power of the lens, e.g. the high-dry lens has a focal length of 4 mm, which divides 160 mm to give 40X, which is its magnification. Information, which may be marked on an objective are its magnification, numerical aperture, mechanical tube length at which it should be fixed and the cover glass thickness (Table 17.1).

Numerical Aperture

The quality of an image is dependent upon the amount of light admitted by a lens and this is directly dependent upon the aperture of the lens. The mathematical formula for the numeric aperture (NA) is 'N sin μ,'

TABLE 17.1: Markings on objective lens

Markings	Description
40/0.65	40: Magnification 0.65: Numerical aperture
160/0.17	160: Mechanical tube length 0.17: Cover glass thickness

where N = refractive index (RI) of the material between the object and the lens (e.g. air = 1.0 and the immersion oil = 1.51) and the sin is the sign of the half angle of the cone of light entering the object (Fig. 17.5).

The NA of high-dry objective should theoretically be 1 and of oil immersion with an RI of 1.51, should be 1.51. However, in practice these values are usually 0.65 and 0.95, respectively.

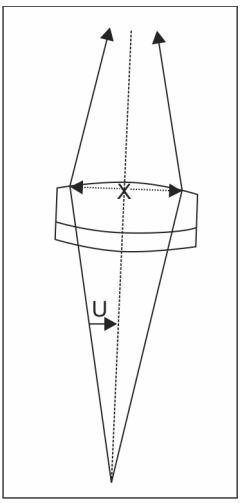

Figure 17.5: Aperture of the lens
(X, angular aperture; U, numerical aperture)

Resolution

Resolution is the ability to differentiate two points as separate and this resolving power is related to NA of the objective and wavelength of the light used. The shorter the wavelength and greater the NA, the more will be the resolution. In bright field microscopy, blue filter is used as blue light has a shorter wavelength than others in the visible spectrum (among VIBGYOR—violet and indigo lights, which are annoying are excluded).

Oculars

The main function of the eyepiece is to magnify further the primary image produced by the objective. There are two basic types of oculars:

- Huygenian or negative ocular
- Ramsden or positive ocular.

The huygenian type of ocular was designed originally by Huygens C for use with the telescope. It consists of two simple lenses (planoconvex), the lower of which (the field lens) collects the image, focuses it just slightly above the plane of the fixed diaphragm (which is between the two lenses). This image from the field lens is magnified by the top lens, to produce the virtual image seen by the eye. Since, the objective (image from field lens) is focused just above the fixed diaphragm, it follows that an optically plane glass disk, carrying an engraved scale, e.g. a micrometer eyepiece or grid, or even a pointer will be seen (in focus) superimposed upon the object image (Fig. 17.6).

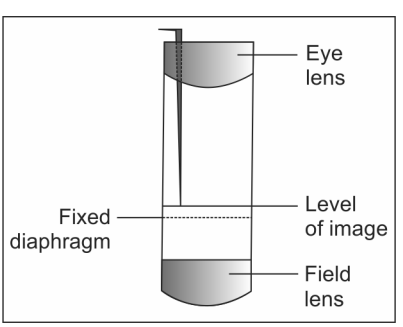

Figure 17.6: Huygenian or negative ocular

Ramsden oculars: These are composed of double or triplet component lenses. The plane side of the bottom lens is towards the object (reverse of huygenian). The virtual image from the objective is formed by entire lens system. The other oculars are 'compensating eyepiece' and 'wide field eyepiece,' which are designed to give a more flat field. The magnification of the oculars is usually indicated on them, e.g. 5X, 10X, etc.

Condensers

The condenser functions to direct the beam of the light of designed numeric aperture and field sizes on to the specimen. The numerical aperture of the condenser must be always equal to the NA of the objective. The iris diaphragm is used to vary the numeric aperture and not to cut down light. There are several types of condensers. The most common are the Abbe, the variable focus and the achromatic (Fig. 17.7). The Abbe condenser is a 1.3 NA condenser utilizing only two lenses and the simplest of the three. It does not, however, provide correction for spherical or chromatic aberration. The variable focus condenser is a two lens condenser with a 1.3 NA maximum, in which upper lens element is fixed and the lower one focusable. This enables the low power objective field to be filled without removing the top element. The achromatic condenser is a 1.4 NA condenser, which is corrected for both chromatic and spherical aberrations, and is used usually for color photomicrography.

To obtain numeric apertures over 0.95 in the condenser system for critical work, it is necessary to bring the condenser and the specimen slide into contact with an oil drop. It is very important that the condenser be centered accurately in the optical axis of the instrument and most instruments have centering screws or adjustment knobs for this.

Use of Mirrors to Direct Light

Plane mirrors are used:

1. To reflect light from infinity (daylight).
2. When substage is being used.

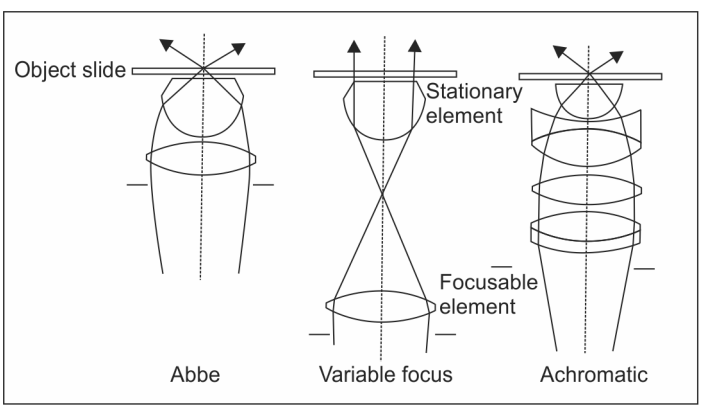

Figure 17.7: Types of condensers

Concave mirrors are used:

1. To direct light from an artificial source.
2. When substage lens is not available.

Filters

Usually blue filters are supplied with microscopes. These cut off yellow light, which is abundant in light sources made up of tungsten filaments.

Magnification in a Microscope

Greatest magnification that can be obtained is about 1,000 times the NA; higher the NA, the more complex (and expensive) lens system becomes. With a high dry having a NA of 0.65, it should therefore be 0.65 × 1,000 = 650 times. This is never achieved in practice because beyond a limit the magnification becomes empty since no more details are seen. The magnification of a microscope may be calculated as follows:

$$\text{Magnification} = \frac{\text{Tube length} \times \text{NA of eyepiece}}{\text{Focal length of objective}}$$

$$\text{For example: } \frac{160 \times 10}{4} = 400 \text{ mm}$$

Magnification is usually increased by using a higher power objective because the resolving power is also increased. Changing the ocular lenses has limited use because the resolving power is not increased leading to empty magnification.

Illumination

Two types of illumination are used in microscopy:

1. Nelson or critical system (Fig. 17.8), where the light source is imaged directly on the specimen.
2. In Köhler illumination (Fig. 17.9), the latter system is more commonly used and requires a field diaphragm, control where the source is imaged in the aperture of the system. It is also the system of choice for photomicrography.

Use of Microscope

Guidelines

1. Both eyes should be kept open.
2. Focus upwards.
3. Fine adjustments only for exact focusing; not to be turned more than one revolution.
4. Fine adjustments to be done with hands, not eyes.

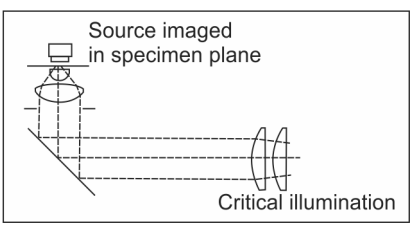

Figure 17.8: Nelson or critical illumination

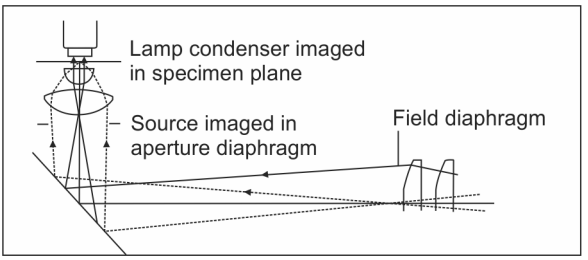

Figure 17.9: Köhler illumination

5. Work with the low-power objective as far as possible.
6. Objects should be centered for clarity.
7. External illumination should be subdued.
8. Use vernier scale to mark objects or use object markers on slide.
9. A slight disturbance also causes harm to the microscope. Even a slight resistance should never be forced.
10. Should be kept scrupulously clean and dust free. Use xylol sparingly. Lens to be cleaned by lens paper. Do not allow oil to dry on objectives.

Cleaning of the Microscope

Daily maintenance

1. Dust it daily. Polish the outer surface of lens with lens paper.
2. Polish the top lens of ocular with lens paper to remove finger marks.
3. Set the microscope for correct illumination.
4. Rotate the eyepiece. If any dust is seen to rotate with the eyepiece, then clean the lens of ocular.
5. Clean the substage condenser lens and mirror. If dust is allowed to remain on the lens, some of the particles being chemically corrosive, may damage the lens.

Weekly maintenance

1. The sides of coarse adjustment, mechanical stage and substage condenser should be wiped with a cloth dampened with xylene to remove the dust. Oil supplied by the manufacturer should be applied and the sides replaced. The latest models do not require this.
2. The lens system should be checked and cleaned.
3. Clean the oculars.
4. Clean the interocular adjustment once a month. Take care, not to disturb the prism arrangement.

Various Types of Microscopy

Dark-field Microscopy

Dark-field microscopy is achieved by blocking out the central rays of light and directing the peripheral rays against the microscope and objective from the side. Only those rays that strike the object and are reflected upward pass into the objective lens; the object thus appears on a black ground. This

type of illumination is most frequently used in the detection of *Treponema pallidum*. Dark-field condensers depend upon the use of high-numeric aperture with a hollow cone of light.

Bright-field Microscopy

Bright-field microscopy is examination under subdued light using a bright-field objective and condenser to delineate more translucent elements in a given specimen.

Phase Contrast Microscopy

Unstained and living biological specimens have little contrast with their surrounding medium, even though small differences of RI exist in their structures. To see them clearly, it involves either closing down the iris diaphragm of the condenser, which reduces its NA producing diffraction effects that destroys the resolving power of the objective or by using dark-field illumination, which enhances the contrast by reversal, but often fails to reveal internal detail. Phase contrast microscope overcomes the problems by controlled illumination using the full aperture of the condenser and improving resolution. The higher the RI of a structure, the darker it will appear against a light background, i.e. with more contrast (Fig. 17.10).

In phase contrast microscopy, the use of an annular diaphragm in the condenser and an annular phase plate in the objective result in the separation of diffracted and non-diffracted light. The diffracted rays are retarded by the specimen in contrast to those that pass through the surrounding media due to a variation in the refractive indices. The resulting interference of these rays provides a darker image, which helps in revealing small details in unstained cells.

To achieve the phase contrast, the microscope requires modified objectives and condenser, and relies on the specimen retarding light by

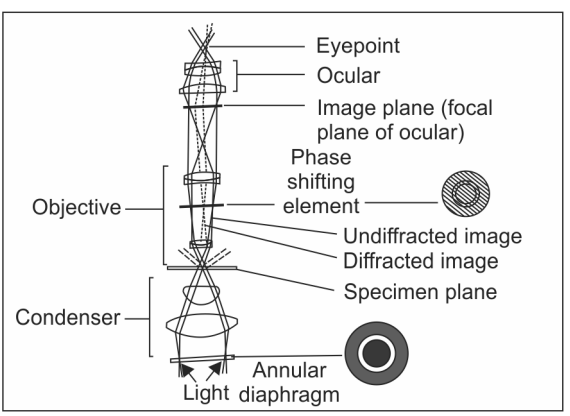

Figure 17.10: Basic principles of phase contrast illumination. The annular diaphragm controls the illumination and the phase shifting element creates a one quarter wavelength retardation of the image.

between one-eighth and one-fourth wavelength. An intense light source is required to be set up for Köhler's illumination. The microscope condenser usually carries a series of opaque glass, with a clear narrow ring, to produce a controlled hollow cone of light. Each objective requires a different size of annulus, an image of which is formed by the condenser in the back focal plane (BFP) of the objective as a bright ring of light. The objective is modified by a phase plate, which is placed at its BFP. A positive phase plate consists of a clear glass disk with a circular trough etched on it, to half the depth of the disk. The light passing through the trough, which has a phase difference of one-fourth wavelength compared to the rest of the plate. The trough also contains a neutral density light-absorbing material to reduce the brightness of the direct rays, which would otherwise obscure the contrast obtained.

When the hollow cone of direct light from the annulus enters the specimen, some will pass unaltered, while some rays will be retarded (or diffracted) by approximately one-fourth wavelength. The direct light will mostly pass through the trough in phase plate, while the diffracted rays pass through thicker clear glass and are further retarded. The total retardation of the diffracted rays is now half wavelength and interference will occur when they are recombined with the direct light. Thus the image of contrast is achieved revealing even small details within unstained cells.

Applications

Rapid screening of urine, a quick way of examining unstained paraffin, resin and unstained sections.

FLUORESCENCE MICROSCOPE

Immunofluorescence is an antigen-antibody reaction, where the antibodies are tagged (labeled) with a fluorescent dye and the antigen-antibody complex is visualized using ultraviolet (UV) (fluorescent) microscope. Fluorochromes are dyes that absorb UV rays and emit visible light. This is called fluorescence. Commonly used fluorochromes are acridine orange, rhodamine, lissamine and calcofluor white. This label or dye has the property of absorbing radiation in the form of UV light. This absorbed radiation causes the molecule to attain an excited state leading to electron redistribution and emission of radiation at a different wavelength. The emitted light is invariably of a longer wavelength and width in the visible spectrum.

Principle (Fig. 17.11)

Protein or protein-containing compounds can be tagged by means of a chemical combination between such material and a fluorescent dye or fluorochrome. Such dyes have advantages in that they neither affect the biological property of the protein nor the protein-containing compound, but can also be detected with UV light at much lower concentrations than ordinary dyes.

The UV light has an extremely short wavelength of between 0.4 μm, just beyond the violet of the visible spectrum to about 4.0 mm on the border of the X-ray region. Only certain portions of the UV spectrum are, however, useful for fluorescence microscopy and are from around 300 to 550 nm.

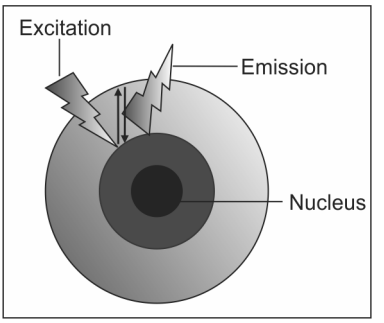

Figure 17.11: Principle of immunofluorescence (the actual color appears for excitation spectrum is 'blue' and emission spectrum is 'green')

The actual utilization of fluorescence microscopy therefore entails the emission of UV light with a wavelength of between 300 and 500 nm from specific exciter filters that permit transmission of only this specific range wavelength of UV light on to a fluorochrome-tagged specimen. The fluorochrome dye absorbs only a single wavelength of UV light (between 300 and 500 nm) and continues to absorb this wavelength of UV up to its maximum called absorption maximum.

Once total absorption is reached, the fluorochrome begins to fluorescence. The fluorescence emitted by the dye (attached to the tagged material) is stopped by what is termed as a suppression filter (placed above the tagged object), which suppresses all other light except the visible fluorescence emission of the fluorochrome that is viewed as apple green or orange (in normal practice) against an almost totally dark background. Since the mechanism of fluorescence was first explained by a British scientist, Sir George Stokes, in 1852, the shift in wavelength from short to long during fluorescence is called 'stokes shift' (Fig. 17.12).

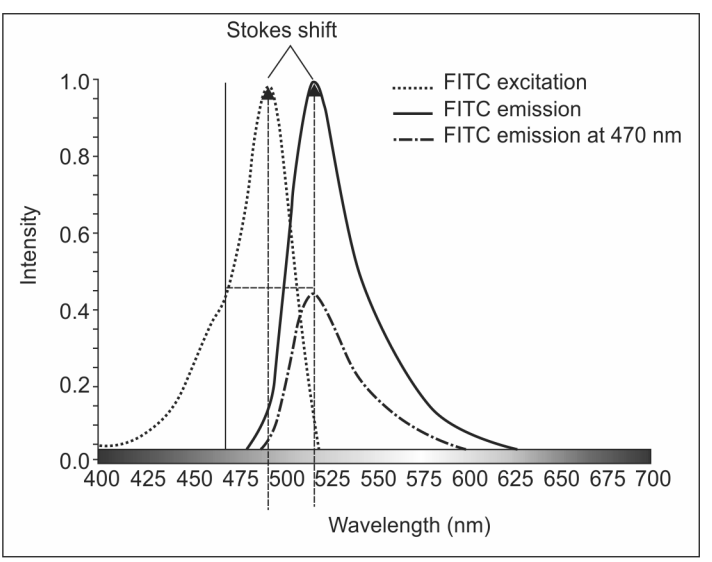

Figure 17.12: Stokes shift (FITC, fluorescein isothiocyanate)

Types of Fluorescence

There are two types of fluorescence:

1. Primary fluorescence.
2. Secondary fluorescence.

Primary fluorescence is defined as an inherent property of the substance and also called autofluorescence, e.g. chlorophyll, oils, calcium particles, etc.

Secondary fluorescence is defined as when non-fluorescent substances are made to fluorescence after staining with a fluorescent dye, e.g. acridine orange, auramine, calcein, phosphine, etc. Certain tissues of an organism (bone, teeth) store fluorescent compounds such as the antibiotic tetracycline and can therefore show fluorescence.

Immunofluorescence is a very sensitive and special method, used for topographical detection of antigens by antibodies, labeled with fluorochromes. Immunofluorescence methods have the potential to define antigen-antibody interactions at the cellular level, e.g. mitochondria, microsomes, as well as identifying small cell surface structures such as receptors, etc. Also these highly specific reactions can be seen in the background of general histological topography of the tissue sections.

Essential Components for Fluorescent Microscopy

The essential components for fluorescent microscopy include:

- Fluorochrome dye
- UV light source
- Fluorescence microscope
- Exciter filter system
- Suppressor filter system
- Other ancillary equipment.

Fluorochrome Dyes

- Fluorescein isothiocyanate (FITC):
  - Absorption maximum: 495 nm
  - Color on fluorescence: Brilliant apple green.
- Lissamine rhodamine B:
  - Absorption maximum: 575 nm
  - Color on fluorescence: Orange.
- 1-dimethylaminonaphthalene-5-sulfonic acid (DANS):
  - Absorption maximum: 310–370 nm
  - Color on fluorescence: Green.
- Tetramethylrhodamine (TRITC):
  - Absorption maximum range: 510–545 nm
  - Color on fluorescence: Orange.

It is important to know that the absorption maximum for these common fluorochromes, so that the correct exciter filters may be used.

Ultraviolet Light Source

1. **High-pressure mercury vapor lamp 50–1,000 watts (common usage 200 watts):** Utilizes electrodes housed in a quartz (containing mercury and argon) and requires a power pack and ballast to provide the arcing voltage, housed in a special lamp housing, with a long warm up and cooling time. The lamp has a limited time and blackens with time and has a potential for explosion.
2. **Iodine:** Quartz lamp (tungsten-halogen) 100 watts (Toshiba, Japan).
3. **Others:** Xenon-mercury arc lamp; cadmium lamp. Emission spectrum 365, 405, 445 and 546.

Advantage

A wide range of UV light for various fluorochromes of different absorption spectrum are made use of with a single mercury bulb.

Disadvantages

1. Excessive heat.
2. Likelihood of spontaneous bursting.
3. Bulb when installed must be free of grease to prevent differential bulb glass heating, consequent glass herniation and explosion.
4. Maximum emission is usually reached after 30 minutes of putting ON.
5. Bulblife span is usually about 200 hours, after which if light is emitted, the UV range may not be satisfactory.
6. Bulblife span is also governed by the number of putting—OFF and ON. The more the putting OFF, the shorter the life.

Fluorescent Microscope (Fig. 17.13)

The basic task of the fluorescence microscope is to let excitation light radiate the specimen and then sort out the much weaker emitted light from the image. First, the microscope has a filter that only allows the specific wavelength of light that matches the fluorescent material. The radiation collides with the atoms in the specimen and its electrons are excited to a higher energy level. When they relax to a lower level, they emit light. To become detectable (visible to the human eye) the fluorescence emitted from the sample is separated from the much brighter excitation light by a second filter called barrier filter. This works because the emitted light is of lower energy and has a longer wavelength than the light that is used for illumination. The dichroic mirror is a key element of the fluorescence microscope. It is used to separate excitation and emission light paths.

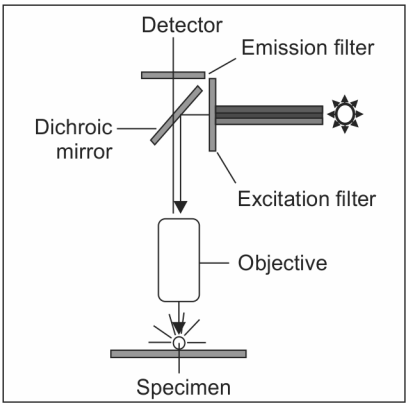

Figure 17.13: Fluorescent microscope

Exciter Filter System (UV Interference Filters)

The general peculiarities of this filter system are to transmit UV light and cut-off visible illumination, permit transmission of UV light frequencies of specific ranges up to 600 nm and prevent heat transmission. They are made of heat-resistant suitably colored glass made by Corning, USA; Kodak, USA; Chance Pilkington, UK; Schott and Genossen, Germany; and are available under designations such as UG 1, BG 38, OG 530 and at certain thickness of 1 mm, 1.5 mm, 2 mm and 4 mm. Additional heat stopping filters are also used to save these UV filters from breakage. The manufactures of microscopes can supply the required filter range.

Suppression Filter System

Suppression filter system suppresses all background fluorescence and transmit only the particular UV absorption maximum of a particular fluorochrome. They are placed always between the fluorescing object and the ocular, but also tend to scratch easily producing uneven viewing through the microscope.

Types available: Suitable for common fluorochromes 410, 470, 500, 530, 570 (the numbers indicate the wavelength of UV light, permitted to pass through) and should match the absorption maxima of the fluorochrome dye.

Mountants

For the fluorescent microscope should not have fluorescent qualities and these can be glycerin jelly, glycerol or solution of 0.5% of gelatin.

Material

Fresh tissue can be transported in a transport medium such as Michel's medium. Fresh tissues may be cut with frozen section, freeze drying (with liquid nitrogen at –40°C). Smears may also be used. Sections are placed on slides coated with gelatin adhesive or polylysine at a dilution of 1:10.

Applications

1. This technique can be used to detect viral, parasitic, tumor antigens from patient specimens or monolayer of cells.
2. Direct immunofluorescence:
   a. Cutaneous/Mucosal biopsies for direct immunofluorescence are stained with fluorescence-labeled antibodies/immunoglobulins (IgG, IgA, IgM), complement (C3) and collagen IV, e.g. bullous pemphigoid, pemphigus vulgaris, dermatitis herpetiformis, vasculitis and lupus.
   b. Kidney: Evaluation with antibodies against IgG, IgM, IgA, C3, C1q, fibrinogen, albumin, kappa and lambda are performed by direct immunofluorescence. Transplant kidney biopsies are stained for C4d to assess the presence of humoral injection.

3. Indirect immunofluorescence: Blood without anticoagulant is required. The serum is separated and serial dilutions (1:20–1:280) of serum is inoculated onto the tissue substrate along with fluorescence-labeled IgG, e.g. systemic lupus erythematosus (SLE), bullous pemphigoid, pemphigus vulgaris and dermatitis herpetiformis.

Disadvantages of Fluorescence Microscopy

1. Incapability of detecting minute quantities of an antigen, antibody or antigen-antibody complex.
2. Lack of stability over prolonged UV exposure resulting in fading of fluorescence called quenching.
3. Difficulty in recording results, as UV emissions from fluorochromes are low with consequent long film exposure times.
4. Most fluorochromes preparations are unstable and must be used fresh.

ELECTRON MICROSCOPE

Electron microscope is a high resolution microscopy. The resolving power, which is the limit where two closely spaced points can be distinguished as two distinct entities, is much higher in an electron microscope as compared with that achieved by a light microscope. Since the time the first transmission electron microscope was produced (Ruska, 1934) with the advanced technology, production of electron microscopes has undergone a rapid development to achieve a high resolution of 2.5 Å (Angstroms) (10,000 Å = 1 μ). The transmission electron microscope (TEM) uses a high-voltage electron beam to create an image. The electron beam is produced by a tungsten filament cathode. The electron beam passes through the specimen. Those electrons, which do not heat the obstacle (i.e. atoms of heavy metals used in specimen staining) do not deviate from their course, while those that form an electron image are picked up and focused by the objective lens and magnified by a combination of various lenses. A vacuum is created for the passage of electrons. The electron image is visualized on a fluorescent screen and appropriate areas are photographed by an in-built camera. The resolving power of the microscope depends on factors such as wavelength, accelerating voltage and scattering power. The shorter the wavelength, the better is the resolution. Different kinds of electron microscopes exist, e.g. TEM, high-voltage electron microscope (HV-EM), scanning electron microscope (SEM) and scanning transmission electron microscope (STEM).

Image of objects formed in electron microscopy also uses the principle of light interference and diffraction, Fresnel images and wavelengths.

Comparisons Between Light and Electron Microscopes

The electron microscope can be explained just similar to a projection microscope with ground glass screen. There are three basic lens systems:

- Condenser lens
- Objective lens
- Projection lens as in the light microscope.

But the difference is the presence of an additional intermediate lens to change the focal length by which magnification of the electron system can be controlled. The optics in both microscopes are more or less same with basic differences being:

1. The source of illumination is electron gun instead of light rays.
2. In the electron microscope, the column has vacuum to enable the flow of electrons.
3. Magnification can be increased or decreased by changing the lens current or accelerating voltage as compared to light microscope where the magnification of a particular lens system remains unaltered.
4. The image is formed on a fluorescent screen, which cannot be visualized otherwise.

Scanning Electron Microscope

The SEM uses a focused beam of high-energy electrons similar to a TEM to generate a variety of signals at the surface of solid specimens. The SEM is mainly a surface microscope. Its resolution (100–200 Å) is about 10 times more than that of the light microscope and nearly 50 times less than that of the TEM. The magnification is usually between some 100 and 1,000 folds, and seldom reaches 10,000 or more.

The signals give information about the sample including external morphology (texture), chemical composition and crystalline structure. In most applications, data are collected over a selected area of the surface of the sample and a two-dimensional (2D) image is generated that displays spatial variations in these properties. Areas ranging from approximately 1 cm to 5 μm in width can be imaged in a scanning mode using conventional SEM techniques. The SEM is also capable of performing analyzes of selected point locations on the sample; this approach is especially useful in qualitatively or semiquantitatively determining not only morphology but also chemical compositions, crystalline structure and crystal orientations.

Scanning and Transmission Electron Microscope (Basic Principle)

In SEM, the backscatter of electrons on the surface of the object are detected, whereas in TEM the forward scattering of the electrons is used to construct the image.

CONFOCAL MICROSCOPY

A confocal microscopy creates sharp images of a specimen that would otherwise appear blurred when viewed with a conventional microscope. This is achieved by excluding most of the light from the specimen that

is not from the microscope's focal plane. The image has less haze and better contrast that of conventional microscope and represent a thin cross section of the specimen. Thus, apart from allowing better observation of fine details it is possible to build three-dimensional (3D) reconstructions of a volume of the specimen by assembling a series of thin slices taken along the vertical axis.

Confocal microscopy was pioneered by Marvin Minsky in 1955. Minsky's invention would perform a point-to-point image construction by focusing a point of light sequentially across a specimen and then collecting some of the returning rays. By illuminating a single point at a time, confocal microscope avoided most of the unwanted scattered light that obscures an image when entire specimen is illuminated at the same time. Additionally, the light returning from the specimen would pass through the second pinhole aperture that would reject rays that are not directly from the focal point. The remaining desirable light rays would then be collected by a photomultiplier and the image gradually reconstructed using a long persistence screen. To build the image, specimen can be scanned by moving the stage rather than the light rays. This was to avoid the challenge of trying to maintain sensitive alignment of moving optics. Using a 60 Hz solenoid to move the platform vertically and a lower frequency solenoid to move it horizontally, a frame rate of approximately one image every 10 seconds can be obtained.

Modern Confocal Microscopy

Modern confocal microscopy has kept the elements of Minsky's design, the pinhole apertures and point by point illumination of the specimen. Advances in optics and electronics have been incorporated into current designs and provide improvements in speed, image quality and storage of the generated images.

The majority of confocal microscopes image either by reflecting light off the specimen or by stimulating fluorescence from dyes (fluorochromes) applied to the specimen. The focus of this entry will be on fluorescent confocal microscopy as it is the mode, which is most commonly used in biological applications. These microscopes are often used for:

1. Imaging structural components of small specimens, e.g. cells.
2. Conducting viability studies on cell populations (are they alive or dead?).
3. Imaging the genetic material within a cell [deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)].
4. Viewing specific cells within a larger population with techniques, e.g. FISH.

Basic Step of Confocal Microscope

Light from the laser is scanned through the specimen by the scanning mirrors. Optical sectioning occurs as the light passes through the pinhole as it passes through the detector (Fig. 17.14).

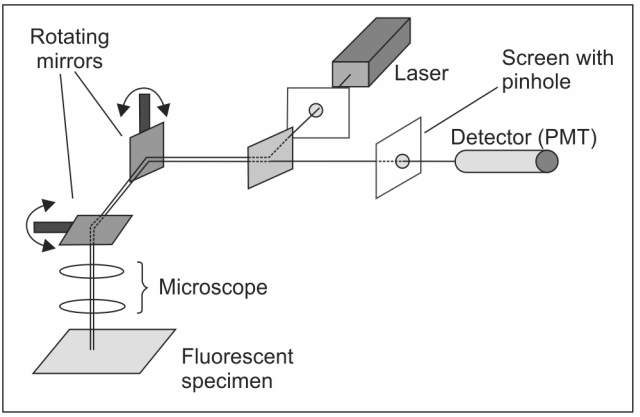

Figure 17.14: Basic step of confocal microscope (PMT, photomultiplier tubes)

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