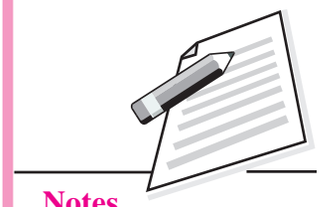


Notes

1

INTRODUCTION TO HISTOPATHOLOGY

1.1 INTRODUCTION

Surgical pathology includes gross and microscopic examination of resected specimens and biopsies by histopathologists for tissue diagnosis. Several steps are followed to get the tissue in a form, by which diagnosis can be made under light microscope.

OBJECTIVES

After reading this lesson, you will be able to:

- list the steps involved in the processing of surgical specimens for histopathologic examination
- explain the after care of the specimens
- explain grossing and gross room
- describe the laboratory hazards and safety measures.

Steps involved in the process are

1. Receipt of specimens from OT
2. Grossing
3. Tissue processing
4. Embedding
5. Section cutting
6. Staining and labelling
7. Dispatch of slides to pathologist for diagnosis

Notes

Once the diagnosis is made, the slides come back to the laboratory. After the reports are sent to the surgeons either as soft copy or hard copy, the laboratory has to perform the following functions -

1. Slides are filed for future reference or teaching/research for at least 10 years.
2. Requisition forms are filed and/or stored in digital form for at least 10 years.
3. Specimens may be divided according to their use
   (a) Well preserved specimens with representative lesion should be kept for
      - teaching
      - research
      - museum
   (b) For future reference (6 months to 1 year)
   (c) Discard – The specimens which are not required or not useful for any of the above purpose should be discarded.

Apart from these essential functions, various other procedures are performed in a surgical pathology laboratory depending upon the requirement, feasibility and availability of instruments and qualified personnel.

- Cryosections
- Histochemical stains
- Immunohistochemistry
- Electron microscopy
- Advanced techniques like in-situ hybridization, immunoflourescence.

1.2 GROSSING

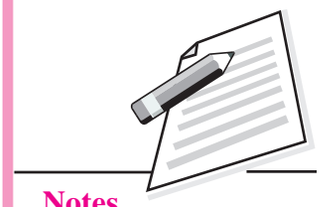
It is the process by which pathology specimens are inspected with bare eye to obtain diagnostic information. Following points should be noted before the tissue is processed for microscopic examination-

A. Identification of the specimen-confirmation of patient and anatomical site from which the specimen has been obtained.

B. Clinical details

C. Gross description – written record of physical appearance of the specimen.

- Only a small portion from the large specimen can be subjected to microscopic examination, hence gross examination should be done by a skilled person.



- Only soft tissue can be cut into small blocks and processed directly.
- Bony specimens need to be decalcified before processing.
- Stones and teeth require special treatment.

Gross room

A. The size and features of surgical pathology gross room depend on the number of specimens, number of staff pathologists and residents and type of institution.

B. The room should be large enough to permit the work to all the pathologists simultaneously. The room should be well illuminated, ventilated and with a exhaust fan to remove the formalin vapors.

Following items should be in a gross room.

(a) A cutting board. The fluid from the board must run directly into the sink.

(b) Shelves for specimen containers.

(c) Ready access to hot and cold water.

(d) Ready access to formalin.

(e) Box of instruments containing forceps of various size, scissors of various types and size, probe, bone cutting saw or electric bone cutter, scalpel handle, disposable blades, long knife and ruler to measure the size of lesion and specimens.

(f) Box with cassettes and labels.

Apart from these items a good gross room should also have -

(a) Large formalin container

(b) Other fixatives

(c) Refrigerator

(d) Photographic facility

(e) Balance for gross specimens

(f) X-ray view box

1.3 LABORATORY HAZARDS AND SAFETY MEASURES

Gross room

1. Formalin vapors are irritant to eyes and throat. Exhaust may be used as outlet for vapors.

2. One should always use mask, apron, eye glasses and gloves to protect oneself from
   - infected material
   - formalin vapors
   - spilt blood or any other fluid
3. Keep the grossing table clean with antiseptic solution.
4. All specimens should be in container with 10% formalin and covered with lid.
5. After grossing specimen should be kept according to accession number.

1.4 HISTOPATHOLOGY LABORATORY

The laboratory should be large enough to accommodate various equipments and personnel to work with ease. The equipments which are kept in this laboratory are -

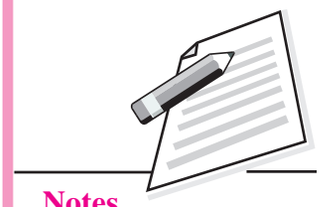
- Tissue processor
- Tissue embedding table
- Microtome
- Tissue warming plate
- Tissue flotation bath
- Slide stainer or glassware for manual staining
- Table to label and dispatch the slides.

The handling of the tissues and description and functioning of various equipments is detailed in the respective lessons.

1.5 LABORATORY HAZARDS AND SAFETY MEASURES

Histopathology laboratory

1. Most of the equipments present in this laboratory are functioning 24x7 days. Electrical connections should be checked before leaving the laboratory every day.
2. Many chemicals are inflammable, hence care should be taken to avoid any fire hazard.
3. Fire extinguisher should always be available.
4. Minimum inflammable substances should be kept in the laboratory. Substances like wax, xylene alcohol, acetone should be stored at a separate place.
5. Some chemicals are carcinogenic or harmful to the skin. Therefore staining and other work should be performed with the gloves on.



INTEXT QUESTIONS 1.1

1. Slides and requisition forms are preserved for future reference for atleast ..... years
2. Specimens may be divided according to their use as ....., ... & .....
3. The process by which specimens are inspected with bare eyes to obtain diagnostic information is .....
4. Formalin vapors may be expelled from the gross room by the use of .....
5. All specimens should be stored in ..... solution

WHAT HAVE YOU LEARNT

- Surgical pathology includes gross and microscopic examination of resected specimens and biopsies for tissue diagnosis
- The steps of process of diagnosis are receipt of specimen, grossing, tissue processing, embedding, section cutting, staining, labeling
- Slides and requisition forms are stored for atleast 10 years for future references
- Specimens may be divided according to their use as well preserved specimen for teaching, research and museum, for future reference from 6 months to 1 year or may be discarded
- Grossing is the process by which the specimens are inspected with bare eyes to obtain diagnostic information
- Gross room should permit the smooth functioning of pathologists, should also be well illuminated and ventilated with exhaust fan
- Gross room should also have cutting board, shelves, formalin, hot and cold water, required instruments
- All the specimens should be stored in 10% formalin container
- Personal protective equipments like gloves, mask, apron, eye glasses should be used for preventive occupational hazards
- Electric equipments should be cared for their functioning.

Notes

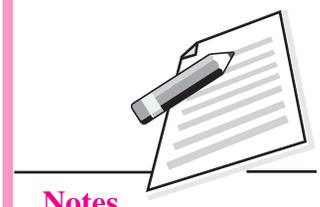
TERMINAL QUESTIONS

1. What is grossing
2. What are the precautions to be taken for maintaining the safety in the laboratory
3. How should the gross room be built

ANSWERS TO INTEXT QUESTIONS

1.1

1. 10 years
2. For museum, future reference and teaching discard
3. Macroscopic examination
4. Exhaust fan
5. 10% Formalin



2

LIGHT MICROSCOPY

2.1 INTRODUCTION

Microscopes are instruments designed to produce magnified visual or photographic images of objects too small to be seen with the naked eye. The microscope must accomplish three tasks: produce a magnified image of the specimen, separate the details in the image, and render the details visible to the human eye or camera. This group of instruments includes not only multiple-lens (compound microscopes) designs with objectives and condensers, but also very simple single lens instruments that are often hand-held, such as a loupe or magnifying glass.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the principle of light microscope
- explain the parts of a light microscope
- learn how to use a microscope.

2.2 LIGHT AND ITS PROPERTIES

Light radiates in all directions, with each ray traveling straight till infinity, unless something interferes its path.

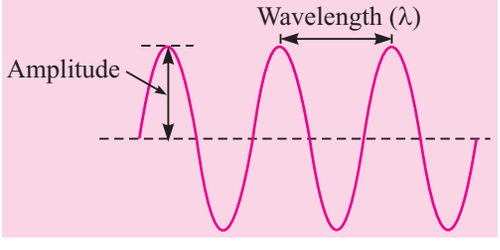


Fig. 2.1: Light represented by a wave showing amplitude and wavelength.

Amplitude refers to the strength of energy or brightness of light. When light passes through any medium, the amplitude decreases depending upon the medium.

Wavelength: The distance between the apex of one wave and the next is the wavelength and measured in nanometers, and determines the color.

Retardation: Media through which light is able to pass, will slow down the speed of light (proportionate to density of medium).

Refraction: If light enters a medium (eg glass) at an angle, a deviation of direction occurs

Image Formation

Focal point: Parallel rays entering a simple lens are brought together to a single point called focal point, where a clear image will be formed.

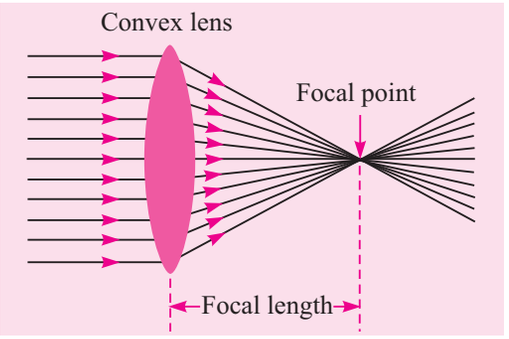


Fig. 2.2: Parallel rays entering a curved lens are brought to a common focus.

Conjugate foci: Object placed at one end of lens will form a clear image on a screen kept at other side of lens. Conjugate foci vary in position. If object is nearer the lens, the image will be formed further away, at a greater magnification and inverted. This "real" image is formed by objective lens of microscope. If the object is placed within focal point of lens, image is formed on same side as object, is enlarged, right way up and cannot be projected on a screen, this is the "virtual image". The eye piece of microscope forms this image.

Image Quality

As white light is composed of all spectral colors, different wavelengths will be refracted to different extent. This lens defect is called **chromatic aberration**. **Spherical aberration** is caused when light rays entering at a periphery are refracted more than those entering the centre of lens. Both these faults can be corrected by using combination of lenses and lens elements.

2.3 COMPONENTS OF A MICROSCOPE

Light source

Light source can be external or inbuilt. Dispersal of heat, collection of greatest amout of light, direction and distance are carefully calculated by the designers of microscope for greatest efficiency.

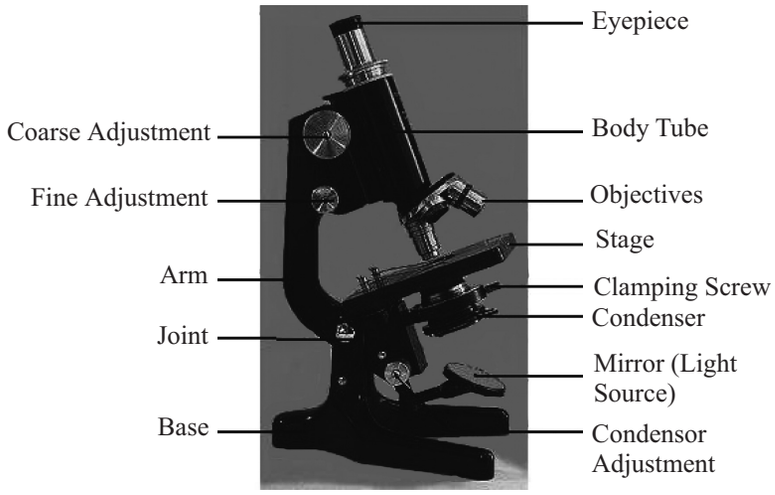


Fig. 2.3: Microscope

Condensers

The purpose of condenser is to concentrate the light into the plane of the object. The more the light at the specimen, better is its resolution. All condensers have aperture diaphragm with which the diameter of the light beam can be controlled.

Object stage

It is a rigid platform with an aperture through which the light can pass. It supports the glass slide. It allows controlled movement in two directions.

Objectives

They are the most important parts of microscope. The main task of objective is to collect the maximum amount of light from the object, unite it and form a high quality magnified real image. Magnifying powers of objectives are from 1:1 to 100:1.

Body tube

Body tube can be monocular, binocular and the combine photo-binocular (also called trinocular). Binocular tubes have provision for inter-pupillary distance adjustment, enabling each observer to adjust for his eyes.

Notes

Eyepiece

The final stage in optical path, the eyepiece's function is to magnify the image formed by the objective within the body tube, and present the eye with a virtual image.

Use of the Microscope

- Illumination should be centered.
- The condenser should be centered and in proper position.
- Objectives should be properly screwed.
- Optical parts should be clean and free from dust.
- Use oil only for oil immersion objective. After use, clean the oil objective with lens tissue. Avoid use of xylene, alcohol or acetone. Eyepieces get dirty by grease from eyelashes, clean them with lens paper.
- When changing slide, always lower the stage before removing the slide or change objective lens to scanner view.
- Make sure the slide is the right way up.

INTEXT QUESTIONS 2.1

1. Parallel rays entering a simple lens are brought together to a single point called
   (a) Focal point (b) Optical point
   (c) Conjugate (d) All of the above
2. Speed of light on entering a medium
   (a) Increases (b) Decreases
   (c) Remains constant (d) Variable
3. Choose the false statement
   (a) Illumination of microscope should be centered
   (b) Objectives should be properly screwed
   (c) Use oil only for oil immersion objective
   (d) When changing slide, always raise the stage
4. Instruments designed to produce magnified visual images of objects are .....
5. Strength of energy or brightness of light is referred as .....
6. Distance between apex of one wave and the next is .....

WHAT HAVE YOU LEARNT

- Microscopes are instruments designed to produce magnified visual or photographic images of objects too small to be seen by naked eye
- Light radiates in all directions, with each ray travelling straight till infinity, unless something interferes its path
- Amplitude refers to the strength of energy or brightness of light
- Wavelength is the distance between the apex of the wave to the next is the wavelength and is measured in nanometers
- Media through which light is able to pass, will slow down the speed of light is described as retardation
- Parallel rays entering a simple lens are brought together to a single point called focal point
- Object placed at one end of lens will form a clear image on a screen kept at other side of lens is described as conjugate lens
- Light source, Condensers, Object stage, Objectives, Body tubes and Eyepieces are the components of microscope

TERMINAL QUESTIONS

1. Define amplitude and wavelength of light with diagram.
2. Define conjugate foci
3. Differentiate between chromatic and spherical aberration
4. Enumerate the different components of light microscope

ANSWERS TO INTEXT QUESTIONS

2.1

1. (a)
2. (b)
3. (d)
4. Microscope
5. Amplitude
6. Wavelength

Notes

3

SPECIAL LIGHT MICROSCOPY

3.1 INTRODUCTION

Microscopes are instruments designed to produce magnified visual or photographic images of objects too small to be seen with the naked eye. The microscope must accomplish three tasks: produce a magnified image of the specimen, separate the details in the image, and render the details visible to the human eye or camera. Compound microscopes are suitable for examination of stained preparations. For some other special conditions we need special microscopes like Dark-ground, phase contrast, polarizing and immuno-fluorescence microscopes.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the principle of Dark-ground, phase contrast, polarizing and immunofluorescence microscopy
- explain the uses of Dark-ground, phase contrast, polarizing and immunofluorescence microscopy.

3.2 DARK GROUND ILLUMINATION

Conditions arise when specimen need to be visualized as unstained or living cells. Since such specimens have refractive indices close to medium in which they are suspended, bright field microscopy is difficult as there is not much contrast.

Principle: Dark ground microscopy prevents direct light from entering the front of the objective, only light which enters is which gets reflected or diffracted by the specimen, thus making them appear bright in a dark background (Fig. 3.1).

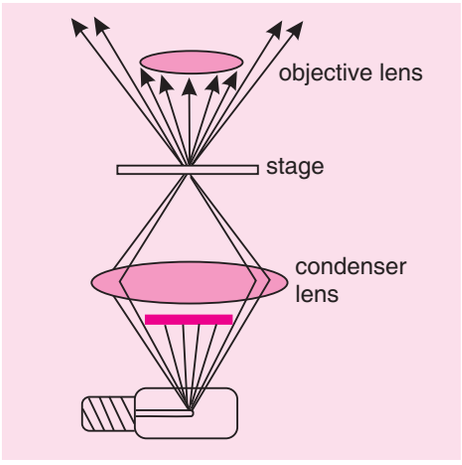


Fig. 3.1: In dark ground illumination, no direct rays enter the objective. Only scattered rays from the edged of structures in the specimen form the image.

Uses of Dark ground microscopy: Useful for spirochaetes, flagellates, cell suspensions, flow cell techniques, parasites, autoradiography, and fluorescence microscopy.

Disadvantage: Resolution is inferior to bright field microscopy. Does not reveal internal details.

3.3 PHASE CONTRAST MICROSCOPY

Unstained and living biological material viewing by bright field and dark ground illumination has problems of reduced illumination and resolution. To overcome these problems, phase contrast microscopes are used.

Principle: It is an optical microscopy illumination technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image. The phase shifts themselves are invisible to the human eye, but become visible when they are shown as brightness changes.

A practical implementation of phase-contrast illumination consists of a phase ring (located in an aperture plane located somewhere behind the front lens element of the objective) and a matching annular ring, which is located in the conjugate primary aperture plane (location of the condenser's aperture).

Two selected light rays, which are emitted from one point inside the lamp's filament, are focused by the field lens exactly inside the opening of the condenser annular ring. Since this location is precisely in the front focal plane of the condenser, the two light rays are then refracted in such way that they exit the condenser as parallel rays. Assuming that the two rays in question are neither refracted nor diffracted in the specimen plane (location of microscope slide), they enter the objective as parallel rays. Since all parallel rays are focused in the

back focal plane of the objective, the back focal plane is a conjugate aperture plane to the condenser's front focal plane (also location of the condenser annulus). To complete the phase setup, a phase plate is positioned inside the back focal plane in annulus.

Uses: It's a quick and efficient way of examining unstained paraffin, resin and frozen sections, studying living cells (cell cultures) and their behavior.

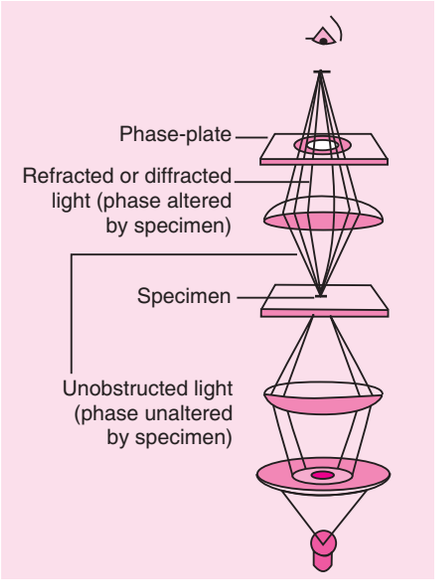


Fig. 3.2: The microscope condenser carries series of annular rings to produce hollow cones of light. Each objective requires a different size of ring, an image of which is formed by the condenser in back focal plane of the objective as a bright ring of light.

3.4 POLARIZED LIGHT MICROSCOPY

Light can be described as an electromagnetic vibration where there are many planes of vibration. Natural light vibrates in many planes or directions, whereas polarized light vibrates in only one plane. It can be produced by passing light through a polarizer. Substances capable of producing polarized light are called birefringent.

Principle: The dedicated polarizing microscope uses two polarizers. One, always referred to as polarizer, is placed beneath the substage condenser. The other is called analyzer and is placed between the objective and the eyepiece. Looking through both polarizers, the light intensity is best when they are both giving light vibrating parallel to each other. However, when the light vibration is at right angles to each other, there is dark background. If a substance capable of birefringence is placed between the two polarisers it gets visualized as brightness against a black background.

Uses: Medicine-Amyloid detection, collagen fibers, urates and other crystals. Metallurgy, Ceramics.

3.5 FLUORESCENCE MICROSCOPY

Principle: Fluorescence is the property of some substances which, when illuminated by light of a certain wavelength, will re-emit the light at a longer wavelength. In fluorescence microscopy, the exciting radiation is usually ultra-violet wavelength or blue region.

A substance which possesses a fluorophore will fluoresce naturally (Primary or autofluorescence) eg Vitamin A, chlorophyll.

Dyes, chemicals and antibodies added to tissues produce secondary fluorescence of structures and are called fluorochromes. When antibodies labeled with fluorochromes are used to detect particular antigens, the technique is called Immunofluorescent technique and is widely used in medicine. Tissue antigens most commonly demonstrable by Immunofluorescence are viruses, protozoa, bacteria, enzymes, hormones, plasma proteins, cells and cell constituents.

Examples of fluorochromes: Fluorescein (apple green emission color), Rhodamine (Orange-red color)

TERMINAL QUESTIONS

1. Define principle and uses of dark ground illumination
2. Define principle and uses of phase contrast microscope
3. Define principle and uses of polarizing light microscopy
4. Define principle and uses of fluorescence microscopy

Notes

4

RECEIVING OF SURGICAL SPECIMENS

4.1 INTRODUCTION

In the laboratory setting, numerous histological specimens are received throughout the day for testing. It is important to maintain a systematic approach to ensure that all samples are accounted for and are being received and tested appropriately. Without it, there is a potential to misplace or lose samples.

OBJECTIVES

After reading this lesson, you will be able to

- explain the process of receiving surgical specimens
- describe preparation of gross room
- receive the samples, label the sample and store.

4.2 RECEIVING OF SURGICAL SPECIMENS

At the time of receiving the specimens, following points should be checked and these points must match between requisition form and label on the sample container

1. Name of the patient
2. Sex and age of patient
3. Registration no, OPD or indoor number
4. Type of sample like appendix or lymph node

After matching the above points carefully, accession number of the Histopathology laboratory should be given on the requisition form and on the sample container like it has been depicted in the form and sample bottle

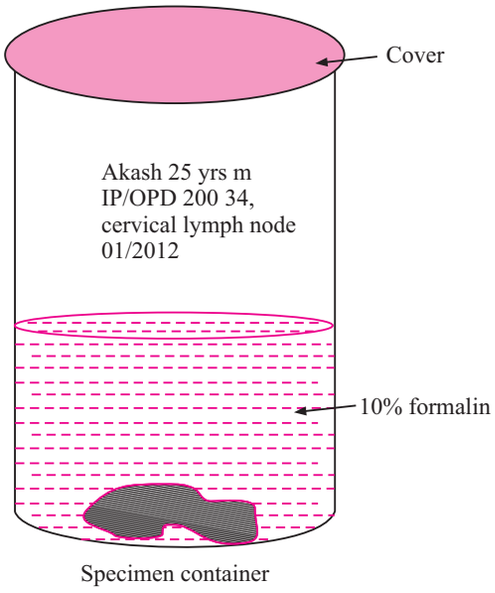


Fig. 4.1: Specimen container

A register should be maintained for record and for future reference

Following points should be noted on the register (sample given)

1. Date
2. Accession number which was given by the pathology department
3. Patients name, age, sex
4. Patients registration number/ OPD/ indoor number
5. Type of sample
6. Number of samples received from one patient
7. Remarks / final diagnosis which may be entered later on

After receiving the samples the consultant should be informed for grossing. If grossing to be done after some time, fixative should be put in all the samples to prevent autolysis of the specimen.

4.3 PREPARATION OF GROSS ROOM

The routine work associated with a surgical pathology specimen includes gross and microscopic examination. Proper preservation of tissues and processing of the tissue are the most important aspects for correct diagnosis.

The size and features of the gross room depends on the number of specimens and type of institution. Gross room should be well illuminated and ventilated. It should have a gross station and racks to keep the specimen in order of accession number.

Notes

Gross station – It should have ventilated hood.

1. Cutting board placed inside the metal box designed in such a fashion that all the fluids should flow directly into the sink
2. Ready access to sink with hot & cold water
3. Formalin – stock and 10% buffered formalin
4. Box of instruments containing
   (a) Scissors
   (b) Forceps
   (c) Malleable probe
   (d) Scalpel handle with disposable blades
   (e) Long knife
   (f) Scale
   (g) Pins for attaching the specimens to corked surface if required
5. Containers with different fixatives
6. Bone cutter
7. Large disposal bin
8. Box with cassettes and labels

These are the essential items. Depending upon the pathology service being rendered to the institution more sophisticated items may be added.

Apart from the above items following items may be of help in keeping the records

1. Photographic facility
2. Refrigerator
3. Balance to weigh the gross specimen
4. X-ray view box
5. Other equipments for tissue bank facility

Sample Copy
Receiving Register

Date 01.08.2012

Acc. No.	Name	Age	Sex	Registration No.	Type of specimen	Remarks / Diagnosis
01/2012	Akash	25 yrs	M	20034	Cervical Lymph Node	
02/2013	Divya	30 Yrs	F	20049	Fallopian tubes	

Histopathology Form (Sample Copy)

			Acc. No – 01/2012
Akash	25 yrs	M	OPD Registraion
Indoor Registration 20034			
Clinical Diagnosis – Tuberculosis – Tuberculosis lymphadenitis			
Clinical Complaints			
Radiological Findings			
Name of Surgeon	Signature	Previous biopsy No. Date of colletion: 1.8.2012	
Type of specimen – Cervical lymph node			
Any special request / remark			

WHAT HAVE YOU LEARNT

- How to receive and hande the surgical specimens. It is paramount to the success of the diagnosis of the specimen. High volumes, multiple steps and multiple human interactions with specimens can create confusion. Therefore, it is important to follow standard procedures which will help in smooth function of the laboratory.

TERMINAL QUESTIONS

1. Write briefly about receiving and labelling of the specimen.
2. How do you prepare a gross room for examination of the specimen.

Notes

5

FIXATION OF TISSUES

5.1 INTRODUCTION

It is a process by which the cells or tissues are fixed in chemical and partly physical state so that they can withstand subsequent treatment with various reagents, with minimal distortion of morphology and no decomposition.

OBJECTIVES

After reading this lesson, you will be able to:

- state the aims of fixation
- explain the principle of fixation
- describe the properties and factors affecting fixation
- explain types of fixation.

5.2 AIMS OF FIXATION

(a) To preserve the tissues as close to their living state as possible

(b) To prevent autolysis and bacterial attack

(c) To prevent tissues from changing their shape and size during processing

(d) To harden the tissues

(e) To allow clear staining of sections subsequently

(f) To improve the optical differentiation of cells & tissues

5.3 PRINCIPLE OF FIXATION

Fixation results in denaturation and coagulation of protein in the tissues. The fixatives have a property of forming cross links between proteins, thereby forming a gel, keeping everything in their in vivo relation to each other.

5.4 PROPERTIES OF FIXATIVES AND FACTORS AFFECTING FIXATION

1. Coagulation and precipitation of proteins in tissues.
2. Penetration rate differs with different fixatives depending on the molecular weight of the fixative
3. pH of fixatives – Satisfactory fixation occurs between pH 6 and 8. Outside this range, alteration in structure of cell may take place.
4. Temperature – Room temperature is alright for fixation. At high temperature there may be distortion of tissues.
5. Volume changes – Cell volume changes because of the membrane permeability and inhibition of respiration.
6. An ideal fixative should be cheap, nontoxic and non-inflammable. The tissues may be kept in the fixative for a long time.

5.5 TYPE OF FIXATION

- Immersion fixation
- Perfusion fixation
- Vapour fixation
- Coating/Spray fixation
- Freeze drying
- Microwave fixation/Stabilization

The most commonly used technique is simple immersion of tissues/smears in an excess of fixative. For all practical purposes immersion fixatives are most useful. These may be divided into routine and special.

5.6 SIMPLE FIXATIVES

1. **Formaldehyde**: Commercially available solution contains 35%-40% gas by weight, called as formalin. Formaldehyde is commonly used as 4% solution, giving 10% formalin for tissue fixation. Formalin is most commonly used fixative. It is cheap, penetrates rapidly and does not over- harden the tissues. The primary action of formalin is to form additive compounds with proteins without precipitation. Formalin brings about fixation by converting the free amine groups to methylene derivatives.

   If formalin is kept standing for a long time, a large amount of formic acid is formed due to oxidation of formaldehyde and this tends to form artefact which is seen as brown pigment in the tissues. To avoid this buffered formalin is used.

Notes

2. **Absolute alcohol** – it may be used as a fixative as it coagulates protein. Due to its dehydrating property it removes water too fast from the tissues and produces shrinkage of cells and distortion of morphology. It penetrates slowly and over-hardens the tissues.
3. **Acetone** – Sometimes it is used for the study of enzymes especially phosphatases and lipases. Disadvantages are the same as of alcohol.
4. **Mercuric chloride** – It is a protein precipitant. However it causes great shrinkage of tissues hence seldom used alone. It gives brown colour to the tissues which needs to be removed by treatment with Iodine during dehydration.
5. **Potassium dichromate** – It has a binding effect on protein similar to that of formalin. Following fixation with Potassium dichromate tissue must be well washed in running water before dehydration.
6. **Osmic acid** – It is used for fixation of fatty tissues and nerves.
7. **Chromic acid** – It precipitates all proteins and preserves carbohydrates. Tissues fixed in chromic acid also require thorough washing with water before dehydration.
8. **Osmium tetraoxide** – It gives excellent preservation of cellular details, hence used for electron-microscopy.
9. **Picric acid** – It precipitates proteins and combines with them to form picrates. Owing to its explosive nature when dry; it must be kept under a layer of water. Tissue fixed in picric acid also require thorough washing with water to remove colour. Tissue can not be kept in picric acid more than 24 hrs.

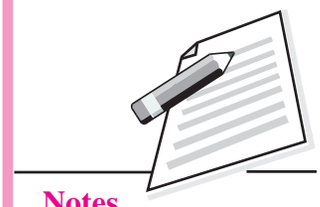
5.7 COMPOUND FIXATIVES

1. **Formal saline** - It is most widely used fixative. Tissue can be left in this for long period without excessive hardening or damage. Tissues fixed for a long time occasionally contain a pigment (formalin pigment). This may be removed in sections before staining by treatment with picric alcohol or 10% alcoholic solution of sodium hydroxide. The formation of this pigment can be prevented by neutralizing or buffering the formal saline.

   Fixation time – 24 hours at room temprature
2. **Formal calcium** – Useful for demonstration of phospholipids.

   Fixation time-24 hours at room temperature
3. **Zenker's fluid** – It contains mercuric chloride, potassium-di-chromate, sodium sulphate and glacial acetic acid.

   Advantages – even penetration, rapid fixation



Disadvantages – After fixation the tissue must be washed in running water to remove excess dichromate. Mercury pigment must be removed with Lugol's iodine.

4. **Zenker's formal (Helly's fluid)** – In stock Zenker's fluid, formalin is added instead of acetic acid.

   Advantages – excellent microanatomical fixative especially for bone marrow, spleen & kidney.

5. **Bouins fluid** – It contains picric acid, glacial acetic acid and 40% formaldehyde.

   Advantages – (a) Rapid and even penetration without any shrinkage. (b) Brilliant staining by trichrome method. It is routinely used for preservation of testicular biopsies.

Points to Remember

1. 10% buffered formalin is the commonest fixative.
2. Tissues may be kept in 10% buffered formalin for long duration.
3. Volume of the fixative should be atleast ten times of the volume of the specimen. The specimen should be completely submerged.
4. Special fixatives are used for preserving particular tissues.
5. Formalin vapours cause throat/ eye irritation hence mask/ eye glasses and gloves should be used.
6. Tissues should be well fixed before dehydration.
7. Penetration of fixatives takes some time. It is necessary that the bigger specimen should be given cuts so that the central part does not remain unfixed.
8. Mercury pigment must be removed with Lugol's iodine.
9. Biopsies cannot be kept for more than 24 hours in bouin's fluid without changing the alcohol.
10. Glutaraldehyde and osmion tetraoxide are used as fixatives for electron microscopy.

Most Commonly used Fixatives in the Laboratory are

10% Formalin

Formaldehyde (40%)	-	10 ml
Distilled water	-	90 ml

Notes

Formal Saline

Formaldehyde (40%)	-	100 ml
Sodium Chloride	-	9 gm
Distilled Water	-	900 ml

10% Buffered Formalin

Formaldehyde (40%)	-	10 ml
Sodium dihydrogen phosphate	-	0.4 gm
Disodium hydrogen phosphate (anhydrous)	-	0.65 gm
Distilled water	-	90 ml

The advantage of this fixative is that it prevents the formation of formalin pigment

Bouin's solution

Saturated picric acid (1.2 gm/ 100 ml)	-	750 ml
Formaldehyde (40%)	-	250 ml
Glacial acetic acid	-	50 ml

Alcoholic formaldehyde

40% formaldehyde	-	100 ml
95% alcohol	-	900 ml

0.5 g calcium acetate may be added to this mixture to ensure neutrality

Alcohol containing fixatives

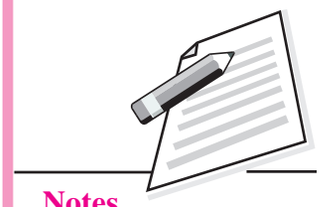
Carnoy's fixatives -

Absolute ethanol	-	60 ml
Chloroform	-	30 ml
Glacial acetic acid	-	10 ml

Mercury salt containing fixatives

Zenker's fluid

Distilled water	-	950 ml
Potassium dichromate	-	25 gm
Mercuric Chloride	-	50 gm
Glacial acetic acid	-	50 gm



B5 fixative

Stock reagent A

Mercuric chloride	-	60 g
Sodium acetate	-	12.5 g
Distilled water	-	1000 ml

Stock Reagent B

10% buffered neutral formalin

Working Solution

Stock reagent A	-	90 ml
Stock reagent B	-	10 ml
Fixation time	-	5-8 hrs

Adequate time should be given for fixation. Formalin fixation should ideally be given for at least 8 hours before processing. (Not the whole specimen but the cut sections).

INTEXT QUESTIONS 5.1

1. Fixation results in ..... & ..... of protein in the tissues.
2. Most commonly used fixation technique is .....
3. ..... is used as fixation for fatty tissues and nerves
4. Most widely used fixative is .....
5. Volume of fixatives shuld be atleast ..... of the volume of the specimen
6. Mercury pigment should be removed with .....
7. ..... prevents the formation of formalin pigment
8. Which is the commonly used fixative for tissues

   (a) Buffered formalin (b) Saline

   (c) Glutaraldehyde (d) Bouin's fluid

9. Which of the following is the best fixative for testicular biopsies?

   (a) Buffered formalin (b) Zenker's solution

   (c) Saline (d) Bouin's fluid

Notes

10. What is used to remove colour from tissues fixed in Zenker's solution?

(a) Alcohol (b) Lugol's iodine

(c) Tap water (d) Acetone

11. Which of the following is used for fixation of tissues for electron microscopy?

(a) Glutaraldehyde (b) Saline

(c) Osmic acid (d) Picric acid

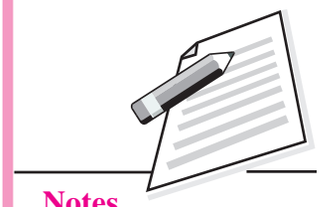
12. What should be the optimum pH of fixative to preserve good morphology?

(a) 5 (b) 6

(c) 7 (d) 8

WHAT HAVE YOU LEARNT

- Fixation of tissues is a process by which the cells of tissue are fixed in chemical and partly physical state so that they can withstand subsequent treatment with various reagants
- Fixation results in denaturation and coagulation of protein in the tissues
- Penetration rate differs with molecular weight of the fixative
- Saturation fixation occurs between pH of 6 & 8 and optimally at 7
- An ideal fixative should be cheap, nontoxic and non inflammable
- Immersion, perfusion, vapour, coating/spray, freeze drying, micro waved fixation are the different types of fixatives used
- The most commonly used technique is simple immersion of tissues/smears in an excess of fixation
- Buffered formalin is the most commonly used fixative and prevents brown pigment formation on tissues
- Following fixation with potassium dichromate tissue must be well washed in running water
- Osmic acid is used for fixation of fatty tissues and nerves
- Osmium tetraoxide and glutaraldehyde are used for electron microscopy
- Formal saline is the most widely used fixative
- Formal Calcium is useful for demonstration of phospholipids
- Bouins fluid is routinely used for preservation of testicular biopsies



- Mercury pigment must be removed with lugol's iodine
- Formalin fixation should ideally be given for atleast 8 hours before processing. Whole specimens should not be fixed without giving cuts.

TERMINAL QUESTIONS

1. What is a fixative?
2. What is the commonest fixative?
3. Write the properties of an ideal fixative.
4. What precautions should be observed when using formalin as fixative?
5. Write names of two special fixatives and their use.

ANSWERS TO INTEXT QUESTIONS

5.1

1. Denaturation and Coagulation
2. Simple immersion
3. Osmic acid
4. Formal Saline
5. Ten times
6. Lugol's Iodine
7. Buffered Formalin
8. (a) Buffered formalin
9. (d) Bouin's fluid
10. (b) Lugol's iodine
11. (a) Glutaraldehyde
12. (c) 7

Notes

6

DECALCIFICATION

6.1 INTRODUCTION

The presence of calcium salts in tissues makes them hard. This causes damage to the knife, difficulty in cutting tissue. Calcium is normally present in bones and teeth. Calcium may also be present in normal tissues in pathological conditions like necrotic tissue in tuberculosis.

OBJECTIVES

After reading this lesson, you will be able to:

- describe decalcification
- explain different methods of decalcification
- describe the chemical and physical tests to estimate the remaining calcium.

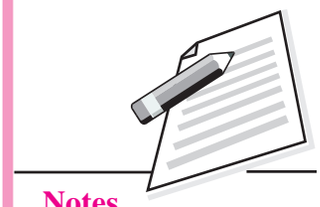
6.2 DECALCIFICATION

Aim – To remove calcium salts from the tissues and make them amenable for sectioning.

Preparation of tissues – The calcified hard tissues should be first cut into small pieces (2 to 6mm) with a thin blade, hacksaw or sharp knife in order to minimize the tearing of the surrounding tissues. This process is followed by fixation in buffered formalin or any other desired fixative. After fixation tissues must be thoroughly washed and excess fixative should be removed before the specimen is subjected to decalcification.

6.3 DIFFERENT METHODS OF DECALCIFICATION

1. Acid decalcification
2. Ion exchange resin



3. Electrical ionization
4. Chelating methods
5. Surface decalcification

Decalcification process should satisfy the following conditions-

- Complete removal of calcium salts
- Minimal distortion of cell morphology
- No interference during staining

Decalcification is a straightforward process but to be successful it requires:

- A careful preliminary assessment of the specimen
- Thorough fixation
- Preparation of slices of reasonable thickness for fixation and processing
- The choice of a suitable decalcifier with adequate volume, changed regularly
- A careful determination of the endpoint
- Thorough processing using a suitable schedule

Methods of Decalcification

The tissue is cut into small pieces of 3 to 5 mm size. This helps in faster decalcification. The tissue is then suspended in decalcifying medium with waxed thread. The covering of wax on thread prevents from the action of acid on thread. The volume of the decalcifying solution should be 50 to 100 times of the volume of tissue. The decalcification should be checked at the regular interval.

Acid Decalcification – This is the most commonly used method. Various acid solutions may be used alone or in combination with a neutralizer. The neutralizer helps in preventing the swelling of the cells.

Following are the usually used decalcifying solutions -

1. Aqueous Nitric Acid-

   Nitric acid - 5 ml

   Distilled water - 100 ml

   If tissue is left for long time in the solution, the tissue may be damaged. Yellow colour of nitric acid should be removed with urea. But this solution gives good nuclear staining and also rapid action.

2. Nitric Acid Formaldehyde

   Nitric acid - 10 ml

   Formaline - 5-10 ml

Notes

Distilled water upto 100 ml

Advantages

- Rapid action
- Good nuclear staining
- Washing with water is not required
- Formalin protects the tissues from maceration

3. Formic Acid Solution

Formic acid	-	5 ml
Distilled water	-	90 ml
Formalin	-	5 ml

In this solution the decalcification is slow. If concentration of formic acid is increased the process is fast but tissue damage is more.

4. Trichloroacitic Acid - This is used for small biopsies. The process of decalcification is slow hence cannot be used for dense bone or big bony pieces.

Formal saline (10%)	-	95 ml
Tricloroacitic acid	-	5 gm

Ion Exchange method – In these ammonium salts of sulfonated polystyrene resin is used. The salt is layered on the bottom of the container and formic acid containing fluid is filled. The decalcifying fluid should not contain mineral acid. X-rays can only determine complete decalcification. The advantages of this method are -

- Faster decalcification
- Well preserved tissue structures
- Longer use of resin

Electrolytic Method – Formic acid or HCl are used as electrolytic medium. The calcium ions move towards the cathode. Rapid decalcification is achieved but heat produced may damage the cytological details.

Chelating Agents – Organic chelating agents absorb metallic ions. EDTA can bind calcium forming a non-ionized soluble complex. It works best for cancerous bone. This is best method for decalcification of bone marrow biopsies as it preserves cytological details best. The glycogen of marrow is preserved.

EDTA Solution

EDTA	-	5.5 gm
Formaline	-	100 ml
Distilled water	-	900 ml

Surface Decalcification – The surface layer of paraffin blocks are inverted in 5% HCl for one hour. About top 30 micron is decalcified. It should be washed thoroughly before cutting.

Factors affecting rate of Decalcification

1. Concentration of decalcifying solution-Increased concentration of the decalcifying agent fastens the reaction.
2. Temperature-The rate of decalcification increases with rise of temperature.
3. Density of bone-Harder bone takes longer time to decalcify.
4. Thickness of the tissue-Small tissue pieces decalcify earlier.
5. Agitation-Agitation increases the rate of decalcification.

6.4 METHODS OF DETERMINING OPTIMUM DECALCIFICATION OR ENDPOINT

- Specimens should **NOT** be crowded together and should **NOT** contact the bottom of container in order to provide complete decalcification.
- Over decalcification can also permanently damage specimen. The following procedure help determine the correct end-point of decalcification.

End-Point of Decalcification:

- X-ray (the most accurate way)
- Chemical testing (accurate)
- Physical testing (less accurate and potentially damage of specimen)

Chemical Test:

The following solutions are needed to chemically test for residual calcium.

5% Ammonium Hydroxide Stock:

Ammonium hydroxide, 28%	5 ml
Distilled water	95 ml

Mix well

5% Ammonium Oxalate Stock:

Ammonium oxalate	5 ml
Distilled water	95 ml

Mix well

Notes

Ammonium Hydroxide/Ammonium Oxalate Working Solution:

Use equal parts of the 5% ammonium hydroxide solution and the 5% ammonium oxalate solution.

Procedure

1. Insert a pipette into the decalcifying solution containing the specimen.
2. Withdraw approximately 5 ml of the hydrochloric acid/formic acid decalcification solution from under the specimen and place it in a test tube.
3. Add approximately 10 ml of the ammonium hydroxide/ammonium oxalate working solution, mix well and let stand overnight.
4. Decalcification is complete when no precipitate is observed on two consecutive days of testing. Repeat this test every two or three days.

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 and pinhole artifacts. 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.

Note: If paraffin embedded bones are not decalcified fully, one can soak the paraffin blocks in the same decalcification solution for a few minutes before cutting. This is usually helpful.

Points to remember

- After completion of decalcification, the specimen should be washed in water
- Over decalcification is more noticeable in staining of nuclei.
- Acid solutions soften bone by removing calcium salts.
- EDTA is used as chelating agent for decalcification.
- To offset the hydrolysis of nucleic acids caused by decalcification, bone marrow is often fixed in Zenker's solution.
- During decalcification, carbon dioxide gas is released.
- Factors affecting decalcification are
  - o Size of specimen,
  - o Concentration of decalcifying solution,

- Time in decalcifying solution
- Amount of decalcifying solution

- For proper decalcification, bone should be cut into 4-5mm thick pieces
- Chelating agents act by binding calcium ions

INTEXT QUESTIONS 6.1

1. Most commonly used method of decalcification is .....
2. In Ion exchange method ..... resin is used
3. ..... or ..... are used as electrolyte medium
4. ..... is used as chelating agents
5. After decalcification, the specimen should be washed in .....

WHAT HAVE YOU LEARNT

- Presence of calcium salts in tissues makes them hard and which causes damages to knife and difficulty in cutting tissues
- Aim of decalcification is to remove calcium salts from the tissues and makes them amenable for sectioning
- The calcified hard tissue should be first cut into small pieces and fixed in buffered formalin
- Acid decalcification, Ion exchange resin, Electrical ionization, Chelating methods and Surface decalcification are different methods of decalcification
- Decalcification must completely remove calcium salts, minimally distort cell morphology and does not interfere during staining
- Acid decalcification is the most commonly used method. Various acids are used in combination of neutralizer. The neutralizer helps in preventing the swelling of the cells
- Ammonium salts of sulfonated polystyrene resin is used
- Formic acid or HCl are used as electrolytic medium
- Organic chelating agents absorb metallic ions and EDTA is used as chelating agent
- Factors like concentration of decalcifying solution, Temperature, Density of bone, thickness of tissue and agitation affects the rate of decalcification

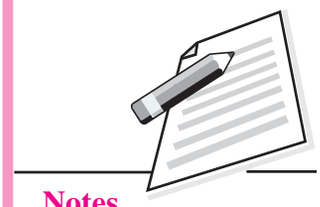
TERMINAL QUESTIONS

1. What is embedding?
2. What is the most common method used for decalcification of bony tissue?
3. Which decalcifying agent is best for bone marrow biopsy?
4. Name the factors affecting rate of decalcification.
5. What are the disadvantages if bony tissue is not decalcified before sectioning?

ANSWERS TO INTEXT QUESTIONS

6.1

1. Acid decalcification
2. Ammonium salts of sulphonated polystyrene resin
3. Formic acid or HCL
4. EDTA
5. Water



7

TISSUE PROCESSING

7.1 INTRODUCTION

The technique of getting fixed tissues into paraffin is called tissue processing. This describes the steps required to take animal and human tissues from fixation to the state where it is completely infiltrated with a suitable wax i.e. paraffin wax and can be embedded and ready for section cutting on microtome.

OBJECTIVES

After reading this lesson, you will be able to:

- define tissue processing
- describe its aim and method of processing.

Aim: To process the fixed tissue into a form in which it can be made into thin microscopic sections.

Processing: The steps in this process are dehydration and clearing.

Dehydration: It is the process of removing water from tissues. It is important because paraffin is not miscible with water. Dehydration is usually complete when less then 3-4% of water remains in the tissues. Time required for this depends on:

1. Permeability of tissues
2. Continuous rotation of fluid to prevent stagnation of fluid around tissues
3. Temperature
4. Vacuum applied

Dehydrants: Ethyl alcohol, Methyl alcohol, Butyl alcohol and Isopropyl alcohol.

The most commonly used dehydrant is ethyl alcohol.

Notes

Alcohol Method: The tissues are passed through a series of progressively more concentrated alcohol baths. Concentration of first alcohol bath depends on the fixative and size and type of the tissue, e.g. delicate tissue needs lower concentration of alcohol and smaller interval between two strengths of alcohol.

Usually 70% alcohol is employed as the first solution and100% as the last solution. After about 40 tissues have passed through the first change of alcohol, it is discarded and all the other changes are brought one step lower. Absolute alcohol at the end is always fresh.

Usually tissues are kept in each solution for 40 to 60 minutes.

Use of copper sulphate in final alcohol: A layer of anhydrous $CuSO_4$ is placed at the bottom of a dehydrating bottle or beaker and is covered with 2-3 filter paper of approximate size to prevent staining of the tissue. Anhydrous $CuSO_4$ removes water from alcohol as it in turn removes it from tissues.

Anhydrous $CuSO_4$ is white in colour while the hydrated form is blue. Therefore, it acts as an indicator for the presence of water.

Advantage of $CuSO_4$ -

1. Rapid dehydration
2. Prolongs life of alcohol
3. Blue colouration of $CuSO_4$ indicates that both alcohol and $CuSO_4$ should be changed.

Acetone - Acetone is clear colourless inflammable fluid which is miscible with water, ethanol. It is used for complete dehydration. Four changes of acetone of half an hour or two changes of one hour are given to achieve complete dehydration of tissues.

Advantages

- Rapid action
- Easily removed by most clearing agents
- Less expensive

Disadvantages

- Highly volatile
- Causes shrinkage and brittleness of tissues
- Dissolves lipid more than ethanol

Clearing – Clearing is a process which leaves the tissues clear and transparent. This term relates to the appearance of the tissues after the dehydrating agent has

been removed. If the refractive index of the clearing agent is similar to the protein of tissue the tissue becomes transparent. The end point of clearing can be noted by the transparent appearance of the tissue. Thus clearing serves two purposes

1. Removes alcohol to make paraffin impregnation complete
2. Acts as solvent for the mounting media which renders the tissues transparent and improves the refractive index, making microscopic examination easier.

Clearing Agents

- Xylene - It is colourless and most commonly used. Two changes of one hour each are given to get the end point. Prolonged treatment hardens the tissues. It is not preferred for brain tissue.

Other Clearing Agents

- Toluene
- Dioxane
- Cedarwood oil
- Cloroform
- Benzene
- Carbol-xylene - clears rapidly, it is kept reserved for material difficult to clear.

7.2 INFILTRATION AND IMPREGNATION

After clearing, tissues are transferred to molten paraffin wax for filtration and impregnation. During this process clearing agent diffuses out and molten wax is infiltrated. The wax which has infiltrated in the tissue gets deposited. This process is called impregnation. Routinely two changes are given in the wax to get proper impregnation. The duration and number of changes required for thorough impregnation of tissue depends on -

1. Size and type of tissues-Longer time is required for thicker tissues. Vacuum reduces the time required for complete impregnation.
2. Clearing agent employed
3. Use of vacuum imbedding

Tissue processing may be performed manually or with the help of automated tissue processor. Routinely 12 containers containing different solutions are used for processing in the following order

Notes

- 10% formalin – container no 1, 2
- 50% alcohol – container no 3
- 90% alcohol – container no 4 & 5
- Absolute Alcohol – container no 6
- Acetone – container no 7 & 8
- Xylene – container no 9 & 10
- Paraffin Wax – container no 11 & 12

INTEXT QUESTIONS 7.1

1. In tissue processing the tissues are impregnated into .....
2. Steps in tissue processing are ..... & .....
3. Process of removing water from tissue is .....
4. Most commonly used dehydrant in tissue processing is .....
5. Usually ..... alcohol is employed as first solution
6. ..... is used as an indicator for the presence of water
7. Clearing is the process which leaves the tissues ..... & .....
8. Clearing serves two purposes namely ..... & .....
9. Most commonly used clearing agent is .....
10. The process by which the infiltrated tissue gets deposited is called .....

7.3 TISSUE PROCESSING (PRACTICAL-1)

Tissue processing can be performed either manually or through automated tissue processor. The device can handle larger number of tissues, process more quickly and produces better quality outcome.

Two types of devices are available

- Tissue transfer or dip dunk
- Fluid transfer or enclosed

Advantages of automated tissue processor - Saves time, decreases human error, effective fluid circulation, Temperature can be adjusted and vacuum/pressure can also be incorporated.

Tissue Transfer Type

The machine consist of a time clock, a circular superstructure that contains basket carrier, a receptacle basket and receptacles (stainless steel or plastic capsules), and a circular deck which holds the reagent beakers and plastic baths. Small blocks of tissue are enclosed in the perforated capsules. These capsules are placed in the basket which in turn is attached to one of the yokes in the superstructure, while it is in the raised position. When the superstructure descends the basket is immersed in the first solution and other reagent beakers are covered preventing evaporation of reagents. To move the basket from one reagent to the next the entire superstructure ascends and descends at scheduled intervals controlled by the time clock. During immersion the basket rotates so the infiltration of fluid into the tissues is optimum. The entire process takes about 16 hours. The machine is started in the evening so that the process is complete in the morning,and embedding is done.

Fig. 7.1: Automated Tissue Processor

Enclosed Type

In this type of tissue processor the tissues remain in one container but reagents get changed at scheduled interval.

Manual

In this process the tissue is changed from one container of reagent to another by hand.

Fig. 7.2: Enclosed Type Tissue Processor

Advantages

- Can be used when the number of tissue blocks is limited
- Non-availability of automated tissue processor

Disadvantages

- Difficult to use when large number of tissue blocks are to be processed
- Proper agitation of reagent not achieved
- More evaporation of reagents
- Process is tedious and requires constant attention

Precautions

1. Labels should be written with graphite pencil, India ink or typed.
2. The fluid used in complete dehydration and clearing tend to become contaminated with fluid carried over from previous vat by the tissue. Every alternate day daily the last solution is the series are replaced by fresh solution of 100% alcohol, acetone and xylene and the previously used once one moved forward while the first one is discarded. Other reagents are changed twice a week or earlier with an average work load. It is far better to change the reagents a day earlier than to have a precious surgical specimen improperly infiltrated.

WHAT HAVE YOU LEARNT

- The technique of getting tissues fixed into paraffin is called tissue processing so that thin microscopic sections can be achieved.

- The main steps in processing are dehydration and clearing
- Dehydration is the process of removing water from tissues and time required depends on permeability of tissues, temperature, vacuum applied and continuous rotation of fluid to prevent stagnation of fluid around tissues.
- Most commonly used dehydrant is ethyl alcohol
- Anhydrous copper sulphate removes water from alcohol as it inturn removes it from tissues and acts as a indicator for the presence of water
- Acetone is used for complete dehydration
- Clearing is the process which leaves the tissues clear and transparent
- Clearing serves two purpose as it removes alcohol to make paraffin impregnation complete and acts as a solvent for mounting media
- Xylene is the most commonly used clearing agent
- Impregnation is the process by which the infiltrated wax gets deposited

TERMINAL QUESTIONS

1. Explain dehydration process of tissue processing
2. Explain clearing process of tissue processing
3. List the advantages of copper sulphate

ANSWERS TO INTEXT QUESTIONS

7.1

1. Paraffin wax
2. Dehydration and clearing
3. Dehydration
4. Ethyl alcohol
5. 70%
6. Anhydrous copper sulphate
7. Clear & Transparent
8. Removes alcohol and acts as a solvent
9. Xylene
10. Impregnation

Notes

8

EMBEDDING

8.1 INTRODUCTION

Embedding is the process in which the tissues or the specimens are enclosed in a mass of the embedding medium using a mould. Since the tissue blocks are very thin in thickness they need a supporting medium in which the tissue blocks are embedded. This supporting medium is called embedding medium. Various embedding substances are paraffin wax, celloidin, synthetic resins, gelatine, etc.

OBJECTIVES

After reading this lesson, you will be able to:

- describe embedding
- explain embedding media
- describe types of moulds
- explain various devices for tissue embedding.

8.2 EMBEDDING

The choice of embedding media depends upon

- Type of microscope
- Type of microtome
- Type of tissue eg. hard tissue like bone or soft tissue like liver biopsy

Paraffin wax with a higher melting point (56 to 62^{o}C) is used for embedding. The molten wax is filtered inside the oven through a course filter paper into another container. This will protect the knife edge.

8.3 OTHER TYPES OF EMBEDDING MEDIA

- **Carbowax**: It is a water soluble wax. Therefore tissues are directly transferred to water soluble wax after fixation and washing.
- **Methacrylate**: It is easily miscible with alcohol and gives a clear and hard block when polymerised Polymerization takes place in the presence of a catalyst. Any trace of water causes uneven polymerization and formation of bubbles in the block around the tissue.
- **Epoxy Resin (Araldite)**: Epoxy polymers of araldite is used in higher resolution work and to see greater details. Epoxy resins are used for electron microscopy. Epoxy polymers of araldite differ from methcrylate in that they are crosslinked causing the cured solid block of araldite to be insoluble in any solvent. Longer filtration is required because the viscosity of resin is greater than methacrylate.

  For electron microscopy araldite is obtained as casting resinCY212, ahardner DDSA and an amine accelerator, DMP (ditrimethylamino methyl phenol) Blocks are suitably cured before sectioning for 48 to 60 hours at 60^{o}C.
- **Agar embedding**: It is mainly used in double embedding. Multiple fragments and friable tissue may be impregnated in one block when sectioning on the cryostat. Another use of agar embedding is for FNAC specimens.
- **Celloidin media**: Celloidin is a purified form of nitrocellulose. It is used for cutting hard tissues.
- **Gelatin**: Its melting point is less than the melting point of agar. Gelatin may be used when frozen sections are required on friable and necrotic tissues.

8.4 TYPES OF MOULDS

A variety of moulds are used for embedding. These may be LEUCKHARD embedding moulds (L mould) paper blocks, plastic moulds. Most of the laboratories use L moulds. L moulds are made up of metal, easy to procure, reusable and may be adjusted to make different size of blocks. One limb of the"L" is longer than the other. The two "Ls" are jointed to form a sides of the rectangular box that act as a cast to make the mould.

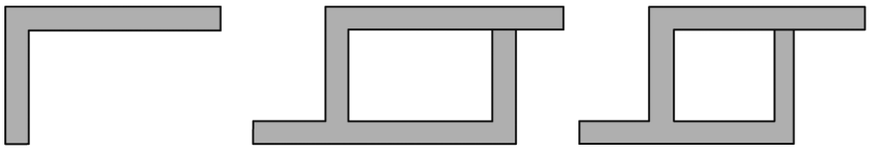


Fig. 8.1: L moulds

Notes

Plastic moulds: Most of the laboratories use plastic embedding rings now. These are relatively inexpensive, convenient and support the block during sectioning and are designed to fit it on the microtome. This eliminates the step of mounting or attaching the block on a holder (metal or wooden holder).

1. **Tissue-Tek System1 or Mark1 system**: In this system plastic embedding rings with stainless steel moulds allow rapid embedding and cutting of tissues. In this system the blocks are stored with the plastic rings; the angle does not change for further requirement of sections.

   The disadvantage of this method is that the space required for storing is more.

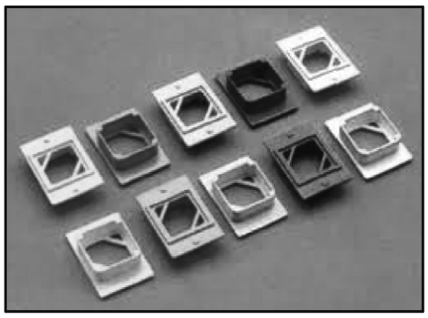


Fig. 8.2: Plastic Embedding Rings

Fig. 8.3: Stainless Steel Moulds

2. **Tissue-Tek system 2or Mark 2 system:** The Mark 2 system has provided a cassette to hold tissue during processing and has a stainless steel lid on the plastic cassette. The cassette has a rough surface on one side of it with a slope where the accession number or the marking is done using a permanent marker.

Fig. 8.4: Plastic Embedding Cassettes

Advantages

- Since the cassette is processed with the tissues and afterwards used for embedding, the writing has to be done once.

- Cassettes are thin so less wax is required.
- The space required for filing the blocks is less.

Disadvantages

- A special clamp has to be used in the microtome for this technique.
- The cassettes are shallow hence thin sections should be taken for processing.

8.5 PARAFFIN WAX ADDITIVES

Various substances can be added to paraffin wax in order to modify its consistency and melting point to improve the efficiency during microscopy.

Additives increase the hardness of blocks. This helps in cutting thinner sections at higher temperature. Stickiness of the medium is increased so better ribbons can be obtained. However if larger quantities of additives are added, undesirable side effects may be seen.

Commonly used additives

- **Ceresin** – It is hard white substance derived from mineral ozokerite. Its melting point is between 61 to70^{o} C. The addition of 0.3-0.5% is sufficient to reduce the crystalline structure of paraffin wax.
- **Bees' wax** - It is yellow substance with melting point of 64^{o} C. This also reduces the crystalline structure of the paraffin wax and improves the ribbon quality.
- **Bayberry wax** - It is a vegetable wax and present in the peal of bayberry. It is extracted from the peal of the fruit. Its melting point is 45° C.

Devices for tissue embedding

Devices designed specifically for tissue embedding are available for laboratories in need of such equipment. These machines vary in size and design depending on the number of samples they are designed to process. Some are designed for specific embedding media, including proprietary compounds intended for specific kinds of histopathology applications. Tissue embedding equipment tends to be expensive. Manufacturers have sales representatives who can provide information and advice when a lab is selecting new or replacement equipment.

Tissue embedding machine

All the blocking steps can be performed with the help of tissue embedding machine. The embedding machine contains the following parts -

- Mould warmer, cassette bath, working surface warmer with a nozzle for pouring the wax, forceps well and cold plate.
- The cold plate is of high efficiency refrigeration system having temperature control ranging from different freezing points to 4 or 5 degree C. It can occupy about 50-60 blocks.
- Large 3-5 litre capacity paraffin reservoir with adjustable temperature of 45-75 degree C.
- Optional vacuum lids, which allows for vacuum infiltration of tissues.
- It has a forceps warmer convenient drain for excess wax.
- The embedding machines are available with many other features.

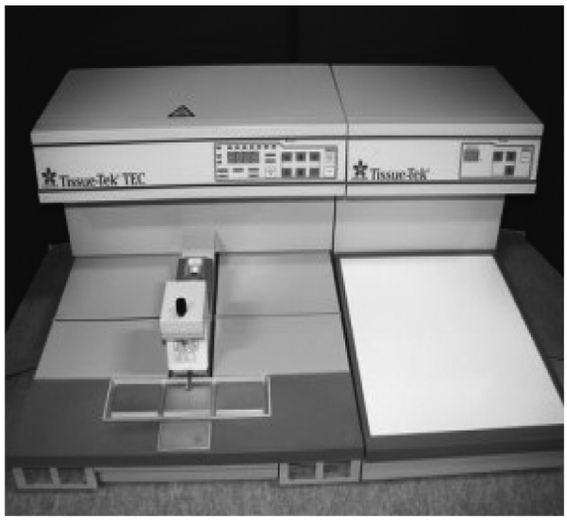


Fig. 8.5

Method of Embedding

1. Open the tissue cassette, check requisition form entry to ensure the correct number of tissue pieces is present.
2. Select the mould; there should be sufficient room for the tissue with allowance for at least a 2 mm surrounding margin of wax.

   Leuckhart mould method-This is the traditional embedding method. The "L moulds are adjusted according to the shape and size of the tissue. Glycerine may be applied to the L pieces and also to the metal or glass plate on which the moulds are placed for embedding. Simple glossed wall or floor tiles may also be used in place of glass plate.
3. Fill the mould with paraffin wax.

4. Using warm forceps select the tissue, taking care that it does not cool in the air; at the same time.
5. Place the tissue in the mould according to the side to be sectioned. This side should be facing down against the mould. A small amount of pressure may be used in order to have more even embedding.
6. Chill the mould on the cold plate, orienting the tissue and firming it into the wax with warmed forceps. This ensures that the correct orientation is maintained and the tissue surface to be sectioned is kept flat.
7. Insert the identifying label or place the labelled embedding ring or cassette base onto the mould
8. Add more paraffin into the mould to fill the cassette and mould.
9. Cool the block on the cold plate.
10. Remove the block from the mould.
11. Cross check block, label and requisition form.

Orientation of different tissue - During embedding the orientation of tissue is important. Correct orientation of tissue in a mould is the most important step in embedding. Incorrect placement of tissues may result in diagnostically important tissue elements being missed or damaged during microtomy.

During embedding it is important to orient the tissue in a way that will provide the best information to the pathologist. At the time of grossing, mark with India ink may be put on the side of the tissue opposite that to be cut. The embedding should be done according to the type of tissue. The requisition form should always be read during embedding for proper orientation.

INTEXT QUESTIONS 8.1

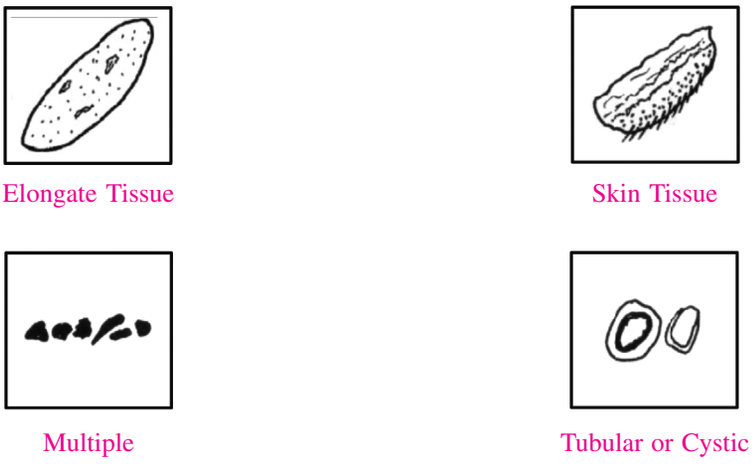
1. ..... is the process by which tissues or specimens are enclosed in a mass of embedding medium
2. The supporting medium is called ..... medium
3. ..... is used for embedding
4. ..... is used for electro microscopy
5. Agar embedding is used for ..... & for .....
6. ..... is used for clotting hard tissues
7. ..... increase the hardness of blocks
8. ..... in a mould is most important step in embedding

Notes

Some general considerations are as follows:

- Elongate tissues are placed diagonally across the block.
- Tubular and walled specimens such as vas deferens, cysts and gastrointestinal tissues are embedded so as to provide transverse sections showing all tissue layers.
- Tissues with an epithelial surface such as skin, are embedded to provide sections in a plane at right angles to the surface (hairy or keratinized epithelia are oriented to face the knife diagonally).
- Multiple tissue pieces are aligned across the long axis of the mould, and not placed at random.

Incorrect placement of tissues may result in diagnostically important tissue elements being missed or damaged during microtomy. In circumstances where precise orientation is essential, tissue should be marked or agar double embedded. Usually tissues are embedded with the surface to be cut facing down in the mould.



Elongate Tissue | Skin Tissue | Multiple | Tubular or Cystic

Fig. 8.6

WHAT HAVE YOU LEARNT

- Embedding is the process in which tissues or specimens are enclosed in a mass of the embedding medium using a mould
- Embedding medium are supporting medium into which the tissue block are embedded

- Various embedding substances such as paraffin wax, celloidin, synthetic resins, gelatine are used depending the type of microscope, type of microtome, type of tissue.
- Epoxy resin is used for electron microscopy
- Agar embedding is used in double embedding and FNAC specimens
- Celloidin media is used for cutting hard tissues
- Gelatin is used when frozen sections are required on friable tissues
- A variety of moulds are used for embedding. There may be L moulds or plastic moulds
- Various substances can be added to paraffin wax in order to modify its consistency and melting point to improve efficiency during microtomy
- Additives increase the hardness of block
- Correct orientation of tissue in a mould is the most important steps in embedding

TERMINAL QUESTIONS

1. Define embedding
2. Explain the types of embedding media
3. Explain the types of moulds

ANSWERS TO INTEXT QUESTIONS

8.1

1. Embedding
2. Embedding
3. Paraffin wax
4. Epoxy resins
5. Double embedding & FNAC
6. Celloidin media
7. Additives
8. Correct orientation of tissue

Notes

9

MICROTOME

9.1 INTRODUCTION

A microtome (from the Greek mikros, meaning "small", and temnein, meaning "to cut") is a tool used to cut extremely thin slices of material, known as sections.

OBJECTIVES

After reading this lesson, you will be able to:

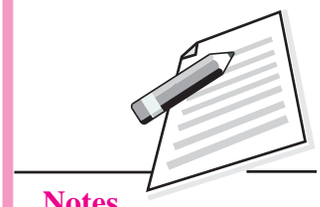
- define Microtome
- describe the application of microtomes
- explain various microtomes
- describe microtome knife and its types.

9.2 MICROTOME

Various types of microtomes are available. Most commonly used microtome for routine histopathology is rotary microtome.

The most common applications of microtomes are:

- **Traditional Histology Technique:** Tissues are hardened by replacing water with paraffin. The tissue is then cut in the microtome at thicknesses varying from 2 to 50 μm. From there the tissue can be mounted on a microscope slide, stained with appropriate aqueous dye(s) after prior removal of the paraffin, and examined using a light microscope.
- **Cryosectioning Technique:** Water-rich tissues are hardened by freezing and cut in the frozen state with a freezing microtome or microtome-cryostat; sections are stained and examined with a light microscope. This technique is much faster than traditional histology (15 minutes vs 16 hours) and is



used in conjunction with medical procedures to achieve a quick diagnosis. Cryosections can also be used in immuno histochemistry as freezing tissue stops degradation of tissue faster than using a fixative and does not alter or mask its chemical composition as much.

- **Electron Microscopy Technique:** After embedding tissues in epoxy resin, a microtome equipped with a glass or gem grade diamond knife is used to cut very thin sections (typically 60 to 100 nanometer). Sections are stained with an aqueous solution of an appropriate heavy metal salt and examined with a transmission electron microscope (TEM). This instrument is often called an ultramicrotome. The ultramicrotome is also used with its glass knife or an industrial grade diamond knife to cut survey sections prior to thin sectioning. These sections are of 0.5 to 1 μm thickness and are mounted on a glass slide and stained to locate areas of interest under a light microscope prior to thin sectioning for the TEM. Thin sectioning for the TEM is often done with a gem quality diamond knife.
- **Botanical Microtomy Technique:** Hard materials like wood, bone and leather require a sledge microtome. These microtomes have heavier blades and cannot cut as thin as a regular microtome.

Rotary Mictrotome - It is most commonly used microtome. This device operates with a staged rotary action such that the actual cutting is part of the rotary motion. In a rotary microtome, the knife is typically fixed in a horizontal position.

A rotary action of the hand wheel actuate the cutting movement. Here the advantage over the rocking type is that it is heavier and there by more stable. Hard tissues can be cut without vibration. Serial sections or ribbons of sections can easily be obtained. The block holder or block (depends upon the type of cassette) is mounted on the steel carriage that moves up and down and is advanced by a micrometer screw.

Auto-cut microtome has built in motor drive with foot and hand control. With suitable accessories the machine can cut thin sections of paraffin wax blocks and 0.5 to 2.0 micrometer thin resin sections.

Advantages

1. The machine is heavy, so it is stable and does not vibrate during cutting.
2. Serial sections can be obtained.
3. Cutting angle and knife angle can be adjusted.
4. It may also be used for cutting celloidin embedded sections with the help of special holder to set the knife.

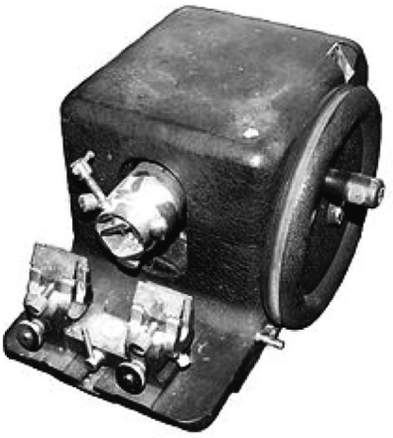


Fig. 9.1: Rotary Microtome

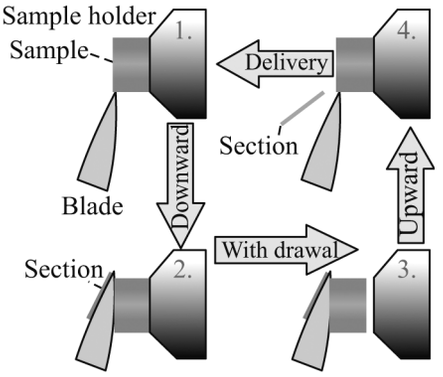


Fig. 9.2: Principle of sample movement for making a cut on a rotary microtome

In the figure to the left, the principle of the cut is explained. Through the motion of the sample holder, the sample is cut by the knife position 1 to position 2), at which point the fresh section remains on the knife. At the highest point of the rotary motion, the sample holder is advanced by the same thickness as the section that is to be made, allowing for the next section to be made.

The flywheel in microtomes can be operated by hand. This has the advantage that a clean cut can be made, as the relatively large mass of the flywheel prevents the sample from being stopped during the sample cut. The flywheel in newer models is often integrated inside the microtome casing. The typical cut thickness for a rotary microtome is between 1 and 60 µm. For hard materials, such as a sample embedded in a synthetic resin, this design of microtome can allow for good "Semi-thin" sections with a thickness of as low as 0.5 µm.

Sledge Microtome is a device where the sample is placed into a fixed holder (shuttle), the sledge placed upon a linear bearing, a design that allows for the microtome to readily cut many coarse sections. Applications for this design of microtome are of the preparation of large samples, such as those embedded in paraffin for biological preparations. Typical cut thickness achievable on a sledge microtome is between is 10 and 60 micron.

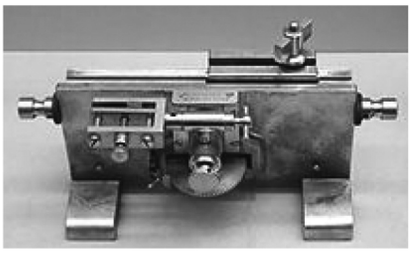


Fig. 9.3: Microtome

Fig. 9.4: A cryomicrotome

Cryomicrotome

For the cutting of frozen samples, many rotary microtomes can be adapted to cut in a liquid nitrogen chamber, in a so-called cryomicrotome setup. The reduced temperature allows for the hardness of the sample to be increased, such as by undergoing a glass transition, which allows for the preparation of semi-thin samples. However the sample temperature and the knife temperature must be controlled in order to optimise the resultant sample thickness

Ultramicrotome

A ribbon of ultrathin sections prepared by room temperature ultramicrotomy, floating on water in the boat of a diamond knife used to cut the sections. The knife blade is the edge at the upper end of the trough of water.

Fig. 9.5: Ultramicrotome

An ultramicrotome is a main tool of ultramicrotomy. It can allow for the preparation of extremely thin sections, with the device functioning in the same manner as a rotational microtome, but with very tight tolerances on the mechanical construction. As a result of the careful mechanical construction, the linear thermal expansion of the mounting is used to provide very fine control of the thickness.

These extremely thin cuts are important for use with transmission electron microscope (TEM) and Serial Block-Face Scanning Electron Microscopy (SBFSEM), and are sometimes also important for light-optical microscopy. The typical thickness of these cuts is between 40 and 100 nm for transmission electron microscopy and often between 30 and 50 nm for SBFSEM. Thicker sections up to 500 nm thick are also taken for specialized TEM applications or for light microscopy survey sections to select an area for the final thin sections. Diamond knives (preferably) and glass knives are used with ultramicrotomes. To collect the sections they are floated on top of a liquid as they are cut and are carefully picked up onto grids suitable for TEM specimen viewing. The thickness of the section can be estimated by the thin-film interference colors of reflected light that are seen as a result of the extremely low sample thickness.

Vibrating microtome

The vibrating microtome operates by cutting using a vibrating blade, allowing the resultant cut to be made with less pressure than would be required for a stationary blade. The vibrating microtome is usually used for difficult biological samples. The cut thickness is usually around 30-500 μm for live tissue and 10-500 μm for fixed tissue.

Saw microtome

The saw microtome is especially for hard materials such as teeth or bones. The microtome of this type has a recessed rotating saw, which slices through the sample. The minimal cut thickness is approximately 30 μm, and can be made for comparatively large samples.

Laser microtome

A conceptual diagram of laser microtome operation

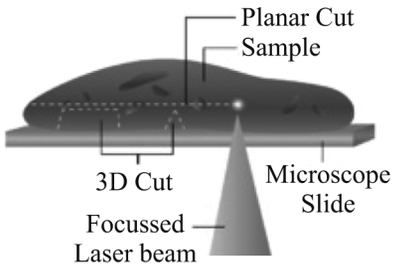


Fig. 9.6: Laser microtome

The laser microtome is an instrument for contact free slicing. Prior preparation of the sample through embedding, freezing or chemical fixation is not required, thereby minimizing the artifacts from preparation methods. Alternately this design of microtome can also be used for very hard materials, such as bones or teeth as well as some ceramics. Dependent upon the properties of the sample material, the thickness achievable is between 10 and 100 μm.

The device operates using a cutting action of an infra-red laser. As the laser emits a radiation in the near infra-red, in this wavelength regime the laser can interact with biological materials. Through sharp focusing of the probe within the sample, a focal point of very high intensity, up to TW/cm^2, can be achieved. Through the non-linear interaction of the optical penetration in the focal region a material separation in a process known as photo-disruption is introduced. By limiting the laser pulse durations to the femtoseconds range, the energy

expended at the target region is precisely controlled, thereby limiting the interaction zone of the cut to under a micrometre. External to this zone the ultra-short beam application time introduces minimal to no thermal damage to the remainder of the sample.

The laser radiation is directed onto a fast scanning mirror based optical system which allows for three dimensional positioning of the beam crossover, whilst allowing for beam traversal to the desired region of interest. The combination of high power with a high raster rate allows the scanner to cut large areas of sample in a short time. In the laser microtome the laser-microdissection of internal areas in tissues, cellular structures, and other types of small features is also possible.

9.3 MICROTOME KNIFE

It is the important instrument used to cut uniform thin serial sections of the tissue. Various types of knives are used with different microtomes. For routine purpose wedge (C type) knife is used. It is plain on both sides. The size varies from 100 mm to 350 mm in length.

Microtome knives are made of good quality of high carbon or steel which is tempered at the tip. Hardness of knife is essential to obtain good tissue sections.

Sharpening of microtome knife - To achieve good sections knife should be very sharp. The knife is put in the knife back to sharpen. Knife can be sharpened manually or by the use of automatic machine.

Honing - This is done to remove nicks and irregularity from the knife edge. Coarse and fine honing is done using different abrasives.

Stropping - The purpose of stropping is to remove the "burr" formed during honing and to polish cutting edge.

Other types of knives are diamond and glass knives. These knives are very expensive and used for ultramicrotomy.

Disposable knife – Nowadays these microtome blades are used. Two types of disposable blades are available.

1. Low profile blade - Usually used for cutting small and soft biopsies like kidney and liver biopsies.
2. High profile blade-Used for any tissue like myometrium, breast tumor or skin.

Notes

Advantages

1. Time is not spent in sharpening, honing or stropping the knife.
2. Resistant to both corrosion and heat.
3. Hardness of blade can be compared with the steel knife.

Disadvantages

1. Relatively expensive
2. Disposable blades are not as rigid as steel knife:

Care of the Microtome Knife

- Store the knife in its box, when not in use.
- The knife should be cleaned with xylene before and after use.
- When knife is being stored for a long time, it should be smeared with grease or good grade of light oil.
- Knife edge should not be touched.
- Knife edge should never be come badly nicked. It is advisable to use separate knife for cutting hard issue like bone.
- The above points are important if re usable knife is being used.

Points to remember

1 For routine histopathology rotary microtome is used.

2 Ultramicrotome is used to cut semi-thin sections or ultrathin sections.

3 Traditional type of knife requires honing and stropping to smoothen the cutting edge.

4 Disposable knives are expensive but do not need honing or stropping.

5 Knife edge is spoiled if properly decalcified tissue is not used.

INTEXT QUESTIONS 9.1

1. ..... is used for cutting extremely thin slices
2. Most commonly used microtome is .....
3. ..... is an instrument for contact free slicing
4. ..... knife is used in routine microtome
5. Nicks and irregularity from the knife edge is removed by ..... technique

6. For polishing the cutting edge ..... is used
7. ..... disposable blade is used for cutting small and soft biopsies
8. ..... disposable blade is used for tissues like myometrium
9. Microtome should be cleaned with .....
10. Knife when stored for long time, should be smeared with ..... or .....

WHAT HAVE YOU LEARNT

- Microtome is a tool used to cut extremely thin slices of material
- Various types of microtome are available most commonly used microtome for routine histopathology is rotary microtome
- Microtome are used in traditional histology, cryosectioning, electron microscopy and botanical microtomy
- Rotary, Sledge, ultra, vibrating, saw, laser microtome are the different types of microtome used
- Microtome knife is the important instrument to cut uniform thin serial sections of the tissue.
- Microtome knife requires Honing and stropping for sharpening the edges
- Currently disposable knifes are commonly used. Low profile blade used for cutting small & soft biopsies & high profile blade is used for any tissue like myometrium
- Knife should be stored in its box and to be cleaned with xylene
- When knife is stored for a long time, it should be smeared with grease or good grade of light oil.

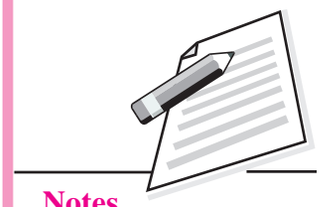
TERMINAL QUESTIONS

1. What is a microtome? Write four types of microtomes and their uses.
2. What are the steps to sharpen the knife?
3. What are the applications of microtome?
4. Name two types of disposable microtome blades and their use.
5. What is the principle of rotary microtome?

ANSWERS TO INTEXT QUESTIONS

9.1

1. Microtome
2. Rotary microtome
3. Laser microtome
4. Wedge c type
5. Honing technique
6. Stropping
7. Low profile
8. High profile
9. Xylene
10. Grease or light oil



10

HEMATOXYLIN AND EOSIN STAINING

10.1 INTRODUCTION

The sections, as they are prepared, are colourless and different components cannot be appreciated. Staining them by different coloured dyes, having affinities of specific components of tissues, makes identification and study of their morphology possible. Hematoxylin and Eosin (H&E) is the most frequently used stain in histology.

OBJECTIVES

After reading this lesson, you will be able to:

- describe Hematoxylin and its preparation
- describe the properties of Hematoxylin
- explain Eosin and its preparation
- describe the method of staining.

10.2 HEMATOXYLIN

It is extracted from the bark of a tree", hematoxylom campechianum". The hematoxylin which we buy is extracted from this bloodwood tree. To obtain the bark of freshly logged tree is chipped off, then boil the chips in water. An orange red solution is obtained, which turns yellow, then black on cooling. The water is evaporated leaving crude hematoxylin. Further purification is done.

Solutions of the dye should be oxidized to retain its staining ability longer. The dye may be oxidized by exposure to the natural light for 3-4 months. Chemical

Notes

oxidation is achieved by using either sodium iodate or mercuric oxide. The chemical oxidation converts the dye almost instantaneously but the product does not have shelf life. Sodium iodate is most commonly used oxidizing agent (0.2 gm oxidizes 1.0 gm hematoxylin).

Hematoxylin is neither a dye nor it has coloring properties. For nuclear staining it is necessary to oxidize the hematoxylin to hematin which is a weak anionic purple dye. Anionic hematin will have no affinity for the nucleic acids of nuclei. Hence a metallic salt or mordant is combined with hematoxylin so that a positive charge to the dye is obtained by virtue of the metal action. Thus the cationic dye –metal complex will bind to the anionic nuclear chromatin. Various mordants are ammonium or potassium alum ferric salt, chrom alum and phosphotungstic acid. The tissue component most frequently demonstrated is nuclear chromatin using an alum mordant in the H&E staining method.

The combination of hematoxylin and mordant is called a hematoxylin lake. The aluminium lake formed with ammonium alum is particularly useful for staining nuclei. Hematoxylin recipes using these mordants are called alum hematoxylin.

10.3 PROPERTIES OF HEMATOXYLIN

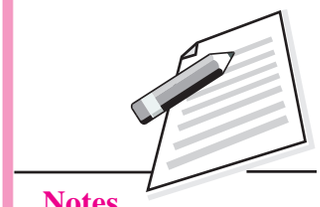
1. Hematoxylin has no staining property
2. Hematin with mordant such as ammonium or potassium alum forms lake which functions as cationic dye and stains anionic tissue components.
3. Hematin in an aqueous solution can be acidic or an alkaline dye depending on pH.
4. Hematin has affinity for several tissues with an appropriate mordant.

Progessive staining - When tissue is left in the stain just long enough to reach the proper end point. The slides have to be examined at different interval to find out when the staining is optimum.

Regressive staining - In this method the tissue is overstained and then destained (differentiate) until the proper endpoint is reached.

Harris hematoxylin is a regressive stain; the overstaining is removed by acid - alcohol. The removal of **this excess dye is called differentiation.**

The hematoxylin alum gives a reddish hue to the tissues because of acidic pH. To convert this colour to the final blue, alkaline pH is required. This process is called “blueing”. It is done either by tap water or by ammonium hydroxide.



Preparation of Harris's hematoxylin

Ingredients :

Hematoxylin	5gm
Absolute alcohol	50ml
Ammonium alum	100gm
Distilled water	1000ml
Mercuric oxide	2.5gm
Glacial acetic acid	40ml

Method - Dissolve the hematoxylin in absolute alcohol and ammonium alum in hot water. Mix the two solutions and heat to boiling. Remove from flame, and add mercuric oxide and cool rapidly. Glacial acetic acid if added gives brisk nuclear staining, but life of the solution is reduced. Hence if acetic acid is to be added, it should be added in working solution.

Preparation of Mayer's hematoxylin

Ingredients :

Hematoxylin	1.0gm
Distilled water	1000ml
Ammonium alum	50gm
Sodium iodate	0.2gm
Citric acid (reduces pH)	1.0gm
Chloral hydrate (preservative)	50gm

Method - Hematoxylin is dissolved in distilled water using gentle heat. Then alum is added and dissolved. Then sodium iodate, citric acid and chloral hydrate are added respectively.

10.4 EOSIN

Eosin is used as the counterstain that stains the cytoplasm rose coloured. The intensity of the eosin is individual choice. The most widely used eosin is "eosin Y". The "Y" stands for yellowish. It is available in either water soluble or alcohol soluble form. Most laboratories use the water soluble form of eosin Y in an alcohol-water solution which is described here.

Eosin Y (water soluble)	1.0gm
Distilled water	80ml

Notes

95% alcohol	320ml
Glacial acetic acid	0.4ml

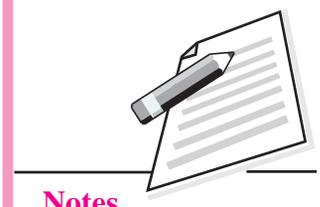
Preparation - Dissolve eosin in water and then add this to 95% alcohol (one part eosin solution with 4 parts alcohol). To the final mixture add a few drops of acetic acid (0.4ml). The acetic acid increases the staining intensity of eosin. When ready to use, the stain should be cloudy; if clear, add a few drops of the acetic acid. The solution should be standardized by staining the control slides.

10.5 METHOD OF STAINING

1. Deparaffinize sections in xylene, 10-20 minutes. Filter Hematoxylin.
2. Rehydrate sections:
   100% alcohol for 1-2 minutes
   95% alcohol for 1-2 minutes
3. Rinse in tap water
4. Rinse in distilled water
5. Stain with Hematoxylin for 3-5 minutes
6. Wash in tap water
7. Differentiate section with 1% HCl in 70% alcohol 1-2 dips and check under microscope. If necessary, return slides to HCl for further differentiation.
8. Wash slides in running tape water for 15 minutes
9. Stain slides in Eosin for 1-4 minutes
10. Dehydration and Differentiation:
    95% alcohol 5-6 dips
    100% alcohol 5-6 dips
11. Clear slides in xylene 2 times
12. Mount slides with mounting media (Permount or DPX)

Note

1. At no stage of staining the section should be dry
2. H&E is a regressive stain in which a tissue is over-stained and then excess dye is removed to obtain desired intensity of stain
3. Filter Hematoxylin each time before staining
4. Change most of alcohol and xylene each time before staining



INTEXT QUESTIONS 10.1

1. Most commonly used stain in histology is .....
2. ..... is the most commonly used oxidising agent
3. Tissue component commonly demonstrated is ..... by hematoxylin.
4. Combination of hematoxylin and mordant is called .....
5. In H & E staining ..... staining technique is followed
6. Process of removing excess dye is called .....
7. Converting red hue to blue colour by use of alkaline pH is called .....
8. ..... is used as counter stain which stains the cytoplasm rose colour

WHAT HAVE YOU LEARNT

- Staining with different coloured dyes makes identification and study of morphology possible
- Haemotoxylin and Eosin is the most commonly used stain in histology
- Sodium iodate is most commonly used oxidising agent
- Nuclear chromatin is usally demonstrated using H & E staining method
- Combination of hematoxylin & mordant is called hematoxylin lake
- Haematoxylin has no staining property, hematin has affinity for several tissues with an appropriate mordant
- Regressive staining is used in H & E staining
- The process of removing excess dye is called differentiation
- Process of converting red colour of tissue using alkaline pH to blue colour is called blueing

TERMINAL QUESTIONS

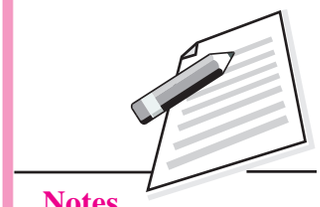
1. Explain the properties of hematoxylin
2. Explain preparation of hematoxylin and Eosin
3. Describe briefly H & E staining

Notes

ANSWERS TO INTEXT QUESTIONS

10.1

1. Hematoxylin & Eosin
2. Sodium iodate
3. Nuclear chromatin
4. Hematoxylin lake
5. Regressive
6. Differentiation
7. Blueing
8. Eosin



11

STAINING METHODS TO DEMONSTRATE SPECIAL/ SPECIFIC TISSUES

11.1 INTRODUCTION

Biological tissue has little inherent contrast in either the light or electron microscope. Staining is employed to give both contrast to the tissue as well as highlighting particular features of interest. Where the underlying mechanistic chemistry of staining is understood, the term histochemistry is used.

OBJECTIVES

After reading this lesson, you will be able to:

- describe various staining methods for demonstrating special tissues.
- demonstrate various staining methods.

11.2 TRICHROME STAIN

A combination of three different dyes is used to identify different cells and tissue elements.

Aim: To identify the collagen and muscle fibers in a histological section.

Reagents

1. Bouin's solution
   - Saturated picric acid 75ml
   - Formaldehyde (37-40%) 25ml
   - Glacial acetic acid 5ml

Notes

- Mix all the reagents well. This solution improves the trichrome stain quality.

2. Weigert's iron hematoxylin stock solution

Stock solution A

- Hematoxylin 1gm
- 95% alcohol 100ml

Stock solution B

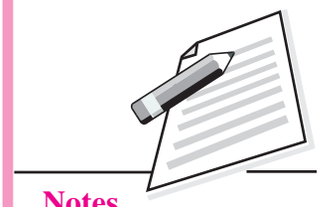
- 29% Ferric chloride in water 4ml
- Distilled water 100ml
- Hydrochloric acid, concentrated 1.0ml

3. Weigert's iron hematoxylin working solution - Mix equal parts of solution A and B (This solution works for three months.)
4. Biebrich scarlet acid fuchsin solution
   - 1% Biebric Scarlet-Acid Fuchsin solution (aqueous solution) 90ml
   - 1% Acid Fuchsin (Aqueous) 10ml
   - 1% Glacial acitic acid 1ml
5. Phosphomolybdic acid-Phosphotungstic Acid Solution
   - 5% Phosphomolybdic Acid 25ml
   - 5%phosphotungstic Acid 25ml
6. Aniline blue solution
   - Aniline blue solution 2.5gm
   - Glacial acitic acid 2ml
   - Distilled water 100ml

Control: skin

Procedure

1. De-paraffinize and rehydrate through graded alcohol.
2. Wash in distilled water.
3. Fix the slides in Bouin's solution for one hour at 56^0C.
4. Rinse in running tap water for 5 to 10 minutes to remove yellow color.
5. Stain in Weigert's Iron Hematoxylin solution for 10 minutes.
6. Rinse in warm tap water for 10 minutes.
7. Wash in distilled water.



8. Put Biebric Scarlet Acid Fuchsin solution for 10 to 15 minutes.
9. Wash in distilled water.
10. Differentiate in Phosphomolybdic-Phosphotungstic Acid solution for 10 to 15 minutes.
11. Put the sections in Aniline blue solution for 5-10 minutes.
12. Rinse in distilled water briefly.
13. Differentiate in acetic acid solution for 2-5 minutes.
14. Wash in distilled water.
15. Dehydrate quickly through 95% alcohol and absolute alcohol. (These steps will wipe off Biebric Scarlet acid Fuchsin staining)
16. Clear in xyline and mount in DPX.

Result

- Glycogen, muscle fibre and keratin — red
- Collagen and bone — blue/green
- Nuclei — brown/black

Note: This stain can be used on frozen sections also.

11.3 VERHEOFF STAIN FOR COLLAGEN

Aim: To identify collagen and elastic tissue in the same section.

Principle: In the presence of ferric salts (oxidizers) elastic fibers stain with hematoxylin, along with the nuclei.

Control: skin

Reagents

1. Verhoeff's solution: Freshly prepared solution gives best result.

   Solution A

   - Hematoxylin — 5gm
   - Absolute alcohol — 100ml
   - Dissolve hematoxylin with the aid of heat, cool and filter.

   Solution B

   - Ferric chloride — 10gm
   - Distilled water — 100ml

Notes

Solution C

- Iodine 2gm
- Potassium iodide 4gm
- Distilled water 100ml
- Add 8ml of solution B into 20ml of solution A and then add 8ml of solution C.

2. 2% Ferric chloride solution
3. 1% aqueous solution of acid fuchsin
4. Saturated aqueous solution of picric acid
5. Van Gieson's stain
   - Acid Fuchsin 1% (aqueous) 5ml
   - Saturated aqueous solution of picric acid 100ml
6. Sodium thiosulphate, 5% (aqueous solution)

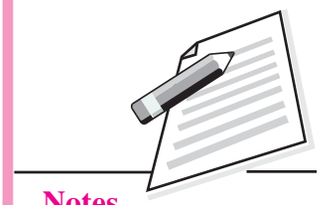
Procedure

1. Deparaffinize and take the section to water.
2. Stain in Verhoeff solution until the section is black.
3. Wash in distilled water.
4. Differentiate in 2% Ferric chloride with agitation for few minutes. Check differentiation by rinsing in distilled water. Under the microscope the elastic fibers and nuclei should stain black and rest of the tissue should be light grey.
5. Put in 5% sodium thiosulphate for 1 minute.
6. Wash in tap water for 5minutes.
7. Counter-stain with Van Gieson's stain for 1-2 minutes.
8. Differentiate in 95% alcohol.
9. Dehydrate in absolute alcohol two times.
10. Clear in xylene and mount in DPX.

Result

- Elastic fibres black
- Nuclei black
- Collagen red
- Other tissues yellow

Note: It is a rapid method but fails to demonstrate fine fibers.



11.4 PRUSSIAN BLUE STAIN

Aim: To demonstrate the presence of iron in tissues.

Principle: The ferric iron in tissue combines with potassium ferro cyanide to form ferric-ferro cyanide. This compound has bright blue color (prussian blue). Prussian blue precipitate is insoluble, hence it can be combined with other staining methods.

Control: Hemosiderin positive tissue

Reagents

1. 2%Hydrochloric acid
   - concentrated hydrochloric acid 2ml
   - Distilled water 98ml
2. 2% potassium ferrocyanide
   - Potassium ferrocyanide 2mg
   - Distilled water 100ml
3. 0.15% Basic fuchsin
   - Basic fuchsin 0.15gm
   - 50% ethyl alcohol 100ml

Procedure

1. Bring section to water.
2. Mix equal volume of 2% potassium ferrocyanide and 2% hydrochloric acid. Pour the solution on the slide and keep it for 20 minutes.
3. Wash thoroughly with water.
4. Counter-stain with basic fuchsin or eosin for 30 seconds.
5. Wash with water, dehydrate, clear in xylene and mount in DPX.

Result

- Ferric iron blue
- Nuclei red
- Other tissues shades of pink

Note: All traces of ferrocyanide should be removed before it is counter-stained, otherwise a dark red fine precipitate will form.

Notes

11.5 MASSON FONTANA SILVER STAINING

Aim: To demonstrate the presence of argentaffin granules.

Principle: Granules in argentaffin cells reduces ammoniacal silver solution to metallic silver. This histo-chemical reaction is due the presence of 5 hydroxy tryptamine(5HT).The 5HT must be converted to tetrahydri-carbolin derivative by formalin fixation before reactions can be demonstrated.

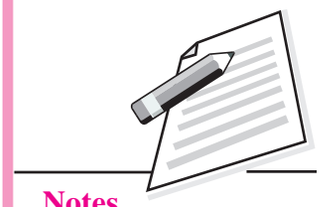
Control: skin or any positive tissue

Reagents

1. Stock 10% silver nitrate solution
   - Silver nitrate A R grade 10gm
   - Distilled water 100ml
2. Fontana masson silver nitrate solution
   - To 50 ml of 10% silver nitrate solution, add one or two drops of ammonium hydroxide. The first drop itself will cause a brown precipitate. Continue to add ammonia solution drop by drop just until the solution is clear. From stock 10% silver nitrate solution, add a little more solution drop by drop dissolving the initial precipitate and stop when a permanent faint turbidity is attained. Let it stand overnight to settle. Before use, decant silver solution, filter and dilute with an equal amount of distilled water. Prepare the fresh solution each time.
3. Gold chloride solution
   - Gold chloride 1gm
   - Distilled water 500ml
   - Keep the solution in refrigerator.
4. Sodiumthiosulfate 5gm
   - Distilled water 100ml

Procedure

1. Bring sections to distilled water
2. Treat with Fontana silver nitrate solution for 1 hour at 56-58IN0C ammoniacal silver solution in a closed jar 15 mins
3. Check microscopically and repeat step 2 if necessary



4. Wash well in distilled water
5. Tone with gold chloride 2 minutes
6. Wash well with distilled water
7. Fix in 2% aq sodium thiosulphate 2 minutes
8. Wash well with distilled water
9. Counter-stain with neutral red stain 1 minutes
10. Rinse in distilled water
11. Rapidly dehydrate well in absolute alcohol, clear and mount

Results

- Melanin — black
- Argentaffin cell granules — black
- Some lipofuscins — black
- Chromaffin — black
- Nuclei — red

Note

- Formalin fixation is essential for argentaffin substances, but not critical for melanin.
- A known positive control section must be used to ensure correct demonstration has been achieved.
- Time of the ammoniacal silver impregnation depends upon the tissue component to be demonstrated. At room temperature, melanin will require 12 hrs, argentaffin 24 hrs. At 60°C melanin blackens within 20 minutes, argentaffin requires approximately 40 minutes. Excessive heat over long periods may cause the silver solution to precipitate, give non-specific background deposits, and cause precipitation of silver on connective tissue fibres.
- Ammoniacal silver solutions can be explosive when allowed to dry. Immediately after use neutralise the silver solution with saturated sodium chloride and discard.

11.6 VON-KOSSA STAIN

Aim: To demonstrate calcium in paraffin sections.

Principle: Tissue sections are treated with silver nitrate solution, the calcium is reduced by the strong light and replaced with silver deposits, visualized as metallic silver.

Notes

Reagents

5% silver nitrate solution

- Silver nitrate 25gm
- Distilled water 500ml

Mix well, pour into acid bottle. Store in refrigerator (stable for one year).

Control: Positive control

Procedure

- Bring sections to water. One section works as negative control.
- Immerse one section in citrate buffer (pH4.5) for 20 minutes to remove calcium if it is present.
- Wash both the slides in distilled water.
- Put 5% silver nitrate solution.
- Expose the slides to bright sun light for 10 to 20 minutes.
- Wash in distilled water several times.
- Treat with 5% sodium thiosulfate for 2 to 3 minutes.
- Wash well in water.
- Counter-stain with neutral red or Van Gieson stain.
- Dehydrate, clear in xylene and mount in DPX.

Result

- Calcium Dark green or black
- Back ground Depends on counter stain.

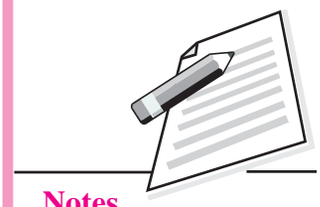
Note: Silver nitrate is tumorogenic and oxidizer. It is strong skin and eye irritant.

11.7 PHOSPHO-TUNGSTIC-ACID-HEMATOXYLIN STAIN

Aim: It is a mix of hematoxylin and phosphotungstic acid. Muscle cross striations, fibrin and glial fibers can be demonstrated by this stain.

Principle: The phosphotungstic acid all of the available hematin to form a blue lake pigment. This lake stains the muscle cross striations, fibrin and nuclei. The rest of the phosphotungstic acid stains red brown, components like collagen.

Control: Skeletal muscle, cardiac muscle, fibrin, cerebral cortex for glial fibers.



Reagents

- Hematoxylin 1gm
- Phosphotungstic acid 20gm
- Distilled water 1000ml

Dissolve the solid ingredients in separate portions of the water, the hematoxylin with aid of gentle heat. When cool mix it. Allow the mixture to ripen or add 177gm of potassium permagnate for immediate use. Properly ripened stain is rich purple in color and opaque.

0.25% potassium permagnate

- Potassium permagnate 0.25gm
- Distilled water 100ml

5% oxalic acid

- Oxalic acid 5gm
- Distilled water 100ml

Procedure

1. Dehydrate and bring section to water.
2. Oxidize in potassium permagnate for 5 to 10 minutes. Discard the solution.
3. Wash in water.
4. Bleach in oxalic acid for 5 minutes or until the sections are colorless.
5. Wash thoroughly. Rinse in distilled water.
6. Put PTAH stain for 12 to 24 hour.
7. Transfer the section into 95% alcohol, followed by absolute alcohol.
8. Dehydrate, clear in xyline and mount in DPX.

Result

- Striated muscle fibers blue
- Astrocytes blue
- Fibrin blue
- Nuclei blue
- Cytoplasm brown red
- Collagen brown pink

Notes

11.8 STAIN FOR RETICULIN FIBRES

Aim: To identify reticulin fibers in sections.

Principle: Reticulin fibers are treated with potassium permagnate to produce sensitized sites for silver deposition. Silver is in a form readily able to precipitate as metallic silver. Formalin, a reducing agent causes deposition of metallic silver at pH 9.0.Excess silver is removed by sodium thiosulphate solution .Treatment with gold chloride produces permanent precipitate.

Control: Normal liver.

Reagents

Acidified potassium permagnate

- 0.5% potassium permagnate 95ml
- 3% sulfuric acid 5ml
- Solution should be made fresh.

Silver nitrate solution

- To 5ml of 10% aqueous silver nitrate, add strong ammonia drop by drop until the precipitate which has formed initially is dissolved. Add 5ml of 3% sodium hydroxide. Again add strong ammonia drop by drop till the precipitate is completely dissolved. Add distilled water to make it 50ml and keep it in a jar.

2% Oxalic acid

- Oxalic acid 2gm
- Distilled water 100ml

4% aqueous iron alum

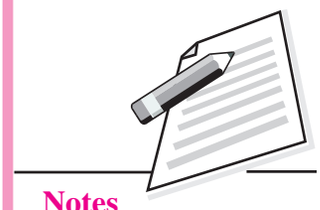
- Ferric ammonium sulphate
- Distilled water 100ml

10% Formalin

- Formaldehyde 10ml
- Distilled water 90ml

0.2% Gold chloride

- Gold chloride 0.2%
- Distilled water 100ml
- Store in refrigerator



2% Sodium thiosulphate

- Sodiun thiosulphate 2gm
- Distilled water 100ml

Neutral red (acidified)

- Neutral red 1gm
- Distilled water 100ml
- Glacial acitic acid 1ml
- Dissolve the dye in distilled water. Add the acid, mix, filter and store.

Procedure

1. Deparaffinize and bring the sections to water.
2. Oxidize in acidified potassium permagnate for 3 minutes.
3. Rinse in distilled water.
4. Decolorize with 2% oxalic acid for 1 minute.
5. Rinse in distilled water.
6. Put iron alum for 10 minutes.
7. Rinse in distilled water.
8. Put ammonical silver solution for 10 seconds.
9. Rinse in distilled water.
10. Immediately reduce with formalin for 2 minutes.
11. Wash in running tap water for 2 minutes.
12. Tone in 0.2% gold chloride for 2 minutes.
13. Rinse in distilled water.
14. Fix in 2% thiosulphate for 2 minutes.
15. Wash in water for 2 minutes.
16. Counter-stain with neutral red for 2 minutes.
17. Dehydrate, clear in xylene and mount in DPX.

Result

- Reticulin fibres black
- Nuclei red

Note

- All glassware should be cleaned thoroughly.

- Silver solution should be made fresh.
- Step 12 should be omitted in liver sections.
- Counter-stain may be omitted in trefine biopsies.

INTEXT QUESTION 11.1

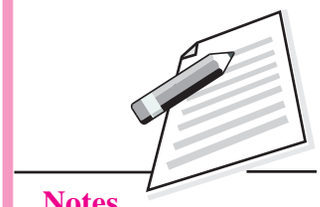
1. ..... stain is used for identifying collagen and muscle fibers in a histological section
2. ..... stain is used to demonstrate the presence of iron in tissues.
3. ..... is the control used in Prussian blue stain.
4. ..... staining is used to demonstrate the presence of argentaffin granules.
5. ..... is used to demonstrate calcium in paraffin sections.

WHAT HAVE YOU LEARNT

- Trichrome stain is used to identify the collagen and muscle fibres in a histological section.
- Trichrome stain uses a combination of three different dyes
- Stain is used as a control in trichrome stain.
- Verheoff stain is used for identifying collagen and elastic tissue.
- Prussian blue stain is used to demonstrate the presence of iron in tissues and hemosiderin positive is used as control.
- Argentaffin granules are demonstrating masson Fontana silver staining.
- Von-kossa stain is used to demonstrate calcium in paraffin sections.
- Muscle cross striations, fibrin and glial fibres can be demonstrated by phospho-tungstic acid hematoxylin stain.

TERMINAL QUESTIONS

1. Explain Prussian blue staining technique.
2. Explain briefly masson Fontana silver staining.



ANSWERS TO INTEXT QUESTIONS

11.1

1. Trichrome
2. Prussian blue
3. Hemosiderin positive tissue
4. Masson Fontana silver
5. Von – kossa stain

Notes

12

METACHROMATIC STAINING

12.1 INTRODUCTION

There are certain basic dyes belonging to aniline group that will differentiate particular tissue components by giving them a different color to that of original dye. The phenomenon is known as metachromasia.

OBJECTIVES

After reading this lesson, you will be able to:

- define metachromasia
- describe the process metachromasia
- know common metachromatic dyes
- describe the factors enhancing metachromasia.

The tissue element reacting in this manner are said to be exhibiting metachromasia.

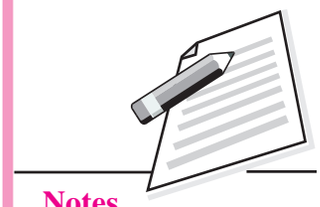
- The generally accepted explanation of this phenomenon is that change in color is due to polymerization.
- Sulfated substances are highly metachromatic e.g. Mast cell granules.
- Mast cells contain Heparin which is highly sulfated.

Some of the common metachromatic dyes are:

- Methylene blue, Methyl violent
- Thionin, Crystal violet
- Toluidine blue

12.2 METACHROMASIA

Metachromasia takes place when certain negatively charged groups on the tissue react with cationic dyes. On polymerization the original colour of the dye changes to another colour (eg mast cell stain pink with toluidine blue).



Metachromatic Staining

Thionin and toluidine blue dyes are commonly used for quick staining of frozen selection using their metachromatic property to stain nucleus and cytoplasm differently.

Metachromasia is enhanced when intermolecular distances are reduced.

Factors which enhance metachromasia are

1. Increasing concentration of dye.
2. Decreasing temperature.
3. pH
4. Water a polar solvent, contributes to the efficiency of van der Waal's forces by which the molecules are held together.

In tissues, where there is a high concentration of anions e.g. in sulphated mucopolysaccharides, the cationic dye molecules may be held in such close proximity to one another that van der Waal's forces can exert their influence and cause the dye to polymerize. Consequently the colour changes from blue to red.

Tissue components often demonstrated by metachromatic stains:

- Amyloid material, Mast cell granules
- Mucin Cartilage

Amyloid Stain -Various stains are used to demonstrate amyloid

12.3 CRYSTAL VIOLET STAIN FOR AMYLOID

Aim: To demonstrate amyloid in tissue sections.

Principle: Amyloid (a glycoprotein) exhibits metachromasia in tissue sections when stained with crystal violet and other cationic dyes.

Control: ositive control.

Reagents

Crystal violet solution

Stock solution

- Crystal violet 14gm
- 95% alcohol 100ml

Working solution

- Stock solution 10ml

- Distilled water 300ml
- Concentrated hydrochloric acid 1ml

Procedure

- Deparaffinize and bring the sections to water.
- Put working crystal violet solution for 1 to 2 minutes and check under microscope.
- Rinse in tap water.
- Mount in water or in water soluble media.
- Put on the coverslip seal the edges with nail polish (Do not let it dry.)

Result

- Amyloid purple violet
- Other tissues blue

12.4 CONGO-RED STAIN FOR AMYLOID

Aim: To demontrate amyloid in tissues.

Principle: Diazo dye attaches itself to amyloid fibrils. The union is affected by H bonds between the OH groups of amyloid and amino side groups of the dye. Congo red dye forms non-polar hydrogen bonds with amyloid. The green birefringence of congo red stained amyloid by polarized light is considered diagnostic of amyloid.

Control: Known positive tissue

Reagents

Congo red solution

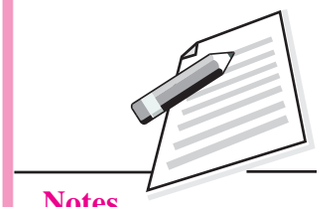
- Congo red 1.0gm
- Distilled water 100ml

Saturated solution of Lithium Carbonate

- Lithium carbonate 1.3gm
- Distilled water 100ml

Procedure

- Bring section to water.



- Pour congo red solution for 20 minutes.
- Pour off the solution and cover the slide with lithium carbonate for 1.5 minutes to differentiate.
- Wash with water.
- Counter-stain with hematoxyline for 5 minutes.
- Differentiate with 1% acid alcohol.
- Wash in running tap water.
- Dehydrate, clear in xylene and mount in DPX.

Result

- Amyloid — bright red which gives apple green birefringence in polarized light.
- Nuclei — blue
- Other structures — unstained to yellow

Notes

1. Sections must be cut at 8 to 10 microns for birefringence
2. Solution must be filtered through glass wool, not paper filters for birefringence to occur
3. Tissue fixed in solutions other than formalin may display false positive birefringence

INTEXT QUESTIONS 12.1

1. ..... & ..... dyes are commonly used for quick staining of frozen section
2. Metachromasia is enhanced when ..... are reduced
3. Tissues demonstrated by metachromatic stain are ....., ... & .....
4. ..... & ..... alcohol is used as fixation in crystal violet stain
5. ..... and ..... are used for demonstrating amyloid in tissues

WHAT HAVE YOU LEARNT

- Basic dye belonging to aniline group differentiates particular tissue components by staining them a different colour to that of original dye by phenomenon known as metachromasia
- Metachromasia occurs due to polymerization
- Methylene blue, methyl violet, thionin, crystal violet and toluidine blue are the metachromatic dyes
- Thionin and toluidine blue dyes are commonly used for quick staining of frozen section
- Metachromasia is enhanced when intermolecular distance are reduced
- Increasing concentration of dye, decreasing temperature, pH, water enhance metachromasia
- Amyloid material, mast cell granules, and mucin cartilages are demonstrated by metachromatic stains
- Crystal violet, cango red stain are used for demonstrating amyloids
- In metachromatic stain amyloid appear pink and the surrounding tissues stain purple
- Crystal violet stain and congo-red stain are used to demonstrate amyloid in tissues.

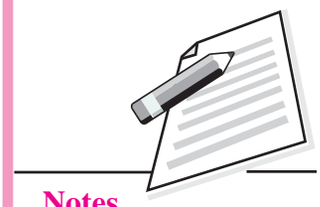
TERMINAL QUESTIONS

1. What are the dyes used in metachromasia?
2. What are the factors which enhance metachromasia?
3. Explain the procedure of metachromasia.
4. Explain briefly stains used for demonstrating amyloid in tissues.

ANSWERS TO INTEXT QUESTIONS

12.1

1. Thionin and toluidine
2. Intermolecular distance
3. Amyloid material, Mast cell granules & Mucin Cartilage
4. Carnoy's and absolute
5. Crystal violet, congo – red stain.



13

LIPID STAIN

13.1 INTRODUCTION

The Oil Red O (ORO) stain can identify neutral lipids and fatty acids in smears and tissues. Fresh smears or cryostat sections of tissue are necessary because fixatives containing alcohols, or routine tissue processing with clearing, will remove lipids. The ORO is a rapid and simple stain.

OBJECTIVES

After reading this lesson, you will be able to:

- explain the principle of lipid stain
- describe various reagents used for lipid stains
- describe the procedure of lipid staining.

13.2 LIPID STAIN

Aim: To demonstrate intracellular lipid in tissue sections.

Principle: The dye being more soluble in the lipid to be demonstrated than in the vehicular solvent. The polyazo group of dyes includes the oil red series, the sudan red series and sudan blacks. All these dyes are interchangeable and may be substituted.

Sudan series - Sudan III

- Sudan IV

- Sudan black

Control - Lipid positive section

Notes

Reagents

1. Oil Red O stock solution -

Oil Red O	0.5gm
Isopropanol	100ml

   Dissolve the dye in isopropanol using gentle heat in water bath.

2. Oil Red O working solution

Stock Oil Red O solution	30ml
Distilled water	20ml

   Dilute the stock solution with distilled water and keep it for 10 minutes, filter and cover it immediately.

3. Glycerine Jelly Mounting medium

Gelatin	10gm
Distilled water	60ml
Glycerol	70ml
Phenol	0.25gm

   Dissolve the gelatin in distilled water using sufficient heat to melt the gelatin, add glycerol and phenol. Mix well and transfer to a small capped bottle and refrigerate.

Procedure

- Fix timer in formalin, wash with running tap water for 5 to 10 minutes.
- Cut frozen section of 8 to 10 micron thickness and air dry.
- Rinse with 60% isopropanol.
- Stain with freshly prepared Oil Red O working solution for 15 minutes.
- Rinse with 60% isopropanol.
- Few dips in Alum hematoxylin to stain nuclei.
- Rinse with distilled water.
- Mount in water or glycerine jelly.

Result

- Lipid red
- Nuclei blue

Note

- Use cryostat sections of 8 to 10 micron thickness or formalin fixed smears.
- working Oil Red O solution should be freshly prepared from stock solution and kept in close container.
- Never take the sections through clearing solvent prior to mounting as this will remove the lipid to be demonstrated.
- Frozen sections should be used to stain neutral triglycerides.
- Lipoproteins may be demonstrated on paraffin sections.
- Alcohol fixation removes most lipids.

INTEXT QUESTIONS 13.1

1. Dyes used in lipid stain is .....
2. ..... is used to stain nuclei
3. Lipid is demonstrated by ..... colour
4. Nuclei is demonstrated by ..... colour
5. Lipoproteins may be demonstrated on ..... section
6. ..... fixation removes lipids

WHAT HAVE YOU LEARNT

- Lipid stain is used to demonstrate intracellular lipid in tissue sections
- Polyazo group of dyes like oil red series, sudan red series and sudan blacks are the dye used for demonstrating lipids in tissue section
- The frozen section should be cut 8 to 10 micron thickness
- Lipids appear as red and nuclei appear blue
- Lipoproteins may be demonstrated on paraffin sections
- Alcohol fixation removes most lipids

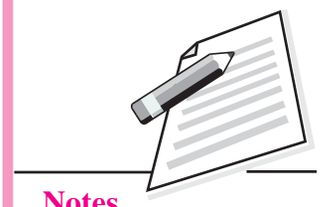
TERMINAL QUESTIONS

1. What is principle of lipid stain?
2. Name three dyes used to demonstrate lipid in tissue sections.
3. What precautions should be observed during lipid staining on tissue sections?
4. What should be the thickness of sections for lipid staining?
5. What is the mounting media used in lipid staining?

ANSWERS TO INTEXT QUESTIONS

13.1

1. Sudan series
2. Alum hematoxylin
3. Red
4. Blue
5. Paraffin
6. Alcohol



Notes

14

STAINING TECHNIQUES FOR DEMONSTRATION AND IDENTIFICATION OF MICROORGANISMS

14.1 INTRODUCTION

Traditional methods for the demonstration of microorganisms in tissue sections can only be based upon staining characteristics and simple morphology because the organisms are fixed and dead. When it is suspected that a disease process may be caused by, or complicated by an infective agent, a sample of fresh tissue is normally provided for microbiological evaluation. The most effective means of isolating and identifying individual species of most organisms is to study their growth patterns and morphology in vitro. The study of these criteria has formed the basis for the identification and classification of microorganisms

OBJECTIVES

After reading this lesson, you will be able to:

- describe Ziehl-Neelsen stain
- demonstrate Fite acid fast stain
- explain periodic acid Schiff, Mucicarmine stain.

IDENTIFICATION OF BACTERIA

Most of the bacteria are demostrated by

Notes

14.2 GRAM STAIN

Some of the bacteria like mycobacterium do not get stained with Gram stain due to their lipid capsule.Ziehl-Neelsen stain is applied to identify mycobacteria which are stained pink to red in colour.

14.2.1 Gram Staining

This reaction may depend on the difference in permeability of cytoplasmic membrane.During staining a dye iodine complex is formed whithin the cell,which is insoluble in water.The complex diffuses freely from gram negative organisms but diffuses less in Gram positive bacteria due to poor membrane permeability.

14.2.2 Preparation of reagents

Crystal violet solution

Crystal violet	1.0gm
Absolute alcohol	20ml
Ammonium oxalate(1%)	80ml

Basic fuchsin

Basic fuchsin	1.0gm
Distilled water	100ml

Gram's iodine

Iodine crystal	1.0gm
Potassium iodide	2.0gm
Distilled water	300ml

14.2.3 Procedure

- Deparaffinize the section and bring it to water
- Put crystal violet for one minute
- Add Gram's iodine for 30 seconds
- Differentiate by dipping the section once or twice in alcohol
- Wash with water and counterstain with safranin for 45 seconds
- Wash with water

- Air dry and mount in DPX.RESULT _Gram positive bacteria stain blue black.
- Gram negative bacteria stain red or pink.
- Some of the bacteria like mycobacterium do not get stained with Gram stain due to their lipid capsule.Ziehl-Neelsen stain is applied to identify mycobacteria which are stained pink to red in colour .

14.3 ZIEHL-NEELSEN STAIN (ACID FAST STAIN)

Aim: To demonstrate Mycobacterium Tuberculosis in paraffin sections.

Principle: When Mycobacterium Tuberculosis are stained with a strong reagent like basic fuchsin in aqueous 5% phenol at high temperature the bacilii resist decolourization by strong acids (20% sulphuric acid). Any decolourized non AFB is counterstained with methylene blue or malachite green.

Control: Mycobacterium tuberculosis positive section

Reagents

1. Ziehl-Neelsen's carbol fuchsin

Basic fuchsin	1gm
Absolute alcohol	10ml
5% phenol (Aqueous)	100ml

Dissolve basic fuchsin in alcohol, and then add 5% phenol.

2. Methylene blue solution

Methylene blue	1gm
Distilled water	100ml

Procedure

- De-wax the sections in xylene and bring to water.
- Flood sections with carbol fuchsin and heat to steaming by intermittent flaming for 10 to 15 minutes or stain in coplin jar at 56°-60°C (oven or water bath) for 30 minutes.
- Wash in water to remove excess of stain.
- Differentiate with 20% sulphuric acid or in 3% hydrochloric acid in 70% alcohol until the tissue is very pale pink colour. Then washed in water (for 5 to 10 minutes).

- Wash in water.
- Counterstain in 1% methylene blue for 10 to 15 seconds.
- Wash in water.
- Dehydrate, clear in xylene and mount in DPX.

Result

Acid-Fast Bacilii	–	red
Nuclei	–	blue
Back ground	–	pale blue

14.4 FITE ACID FAST STAIN - LEPROSY

Aim: To demonstrate mycobacterium leprae (leprosy), in formaline fixed sections.

Principle: This technique combines peanut oil with xylene, minimizing the exposure of the bacteria's cell wall to organic solvent. Thus acid fastness of bacteria is retained.

Control: Leprosy positive tissue.

Reagents

1. Xylene/Peanut Oil Solution:

Xylene	50.0 ml
Peanut Oil	50.0 ml

Mix well. Label with date, solution is stable for 1 year

Caution: The solution is inflammable, irritant

2. Ziehl-Neelsen Carbol-Fuchsin:

As described before

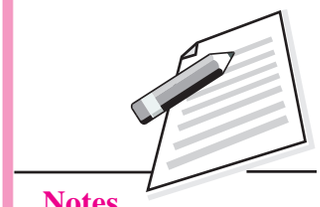
3. 1% Acid Alcohol

4. Methylene Blue:

Methylene blue	1.0 gm
Distilled water	100 ml

Procedure

- De-paraffinize the section for two minutes in xylene and peanut oil mixture



- Drain, wipe excess of oil
- Place the slide in water until ready to stain
- Put Carbol - fuchsin, for 30 minutes, at room temperature
- Wash in tap water
- Dip acid alcohol until pale pink, dip until stain stops running
- Wash in tap water
- Counter-stain in methylene blue solution for 30 seconds
- Wash in tap water
- Blot and air dry
- Dip in xylene and mount with DPX and put coverslip

Results

Acid-fast bacilli – red

Background – blue

Note: Mineral oil may be substituted for peanut oil.

14.5 PERIODIC ACID – SCHIFF (PAS) STAIN

Periodic acid causes oxidation of 1:2 glycol groups in the tissues to di-aldehydes. The di-aldehyde reacts with fuchsin – sulfurous acid solution(Schiff's) to form a magenta colored compound.

Aim: To demonstrate glycogen, epithelial mucin, fungi, ameba and basement membrane.

Control: Liver and intestine

Reagents

Periodic acid 1%

Distilled water 100 m

Schiff's reagent

Basic fuchsin 1 gm

Distilled water 200 ml

1N hydrochloric acid 20 ml

Sodium or Potassium metabitesulfite1gm

Activated charcoal 2gm

Notes

- Dissolve basic fuchsin in boiling distilled water
- Shake for 5 minutes and cool to 50°C
- Filter and add 1N solution.
- Cool further and add sodium or potassium metabisulfite. Keep for 18 hours in dark.
- Add activated charcoal, shake well, filter and store the solution at 40°C.

Procedure

- Bring the sections to water.
- Dip the slide in Periodic acid solution for 5-10 minutes.
- Wash in tap water and rinse in distilled water.
- Put Schiff's reagent on the section for 20 minutes.
- Wash thoroughly in running water.
- Counerstain with Hematoxylin, dehydrate, clear and mount in DPX.

Result

- Glycogen (except non-sulfated acid mucopolysaccharide), basement membrane, fungi, parasites and other positive substances – magenta
- Nucleus – blue or violet

Giemsa stain

Aim: It is used to demonstrate

- Bacteria, Hematologic element, Bone marrow elements, Blood parasites

Reagents

Giemsa stain

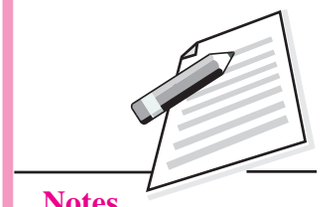
Stock solution:

Giemsa powder	1.0 gm
Methyl alcohol	66 ml
Glycerin	66 ml

Add glycerin to Giemsa powder and put in oven at 60°C for 30 minutes to 2 hours (until the stain dissolves). Then add methyl alcohol.

Working solution:

Stock solution	1.25 ml
Methyl alcohol	1.50 ml
Distilled water	50 ml



Procedure

- Deparaffinize and take the section to water.
- Place the slide in working Giemsa solution overnight.
- Wash with tap water.
- Differentiate with 0.5% aquous acitic acid.
- Dehydrate and mount.

Result

Nuclei	–	blue
Malarial parasite	–	purple
Collagen and other elements	–	pink

Note:

1. Sections stained at room temperature for longer period show better results than sections stained at higher temperature for shorter period.
2. Differentiation with acetic acid removes only blue dye hence gives better red intensity.
3. Giemsa reagent improves with age.

14.6 MUCICARMINE STAIN

Aim: To demontrate mucin glycogen in tissues.

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

Reagents

1. Mucicarmine solution

Carmine alum lake	1.0 gm
Aluminum hydroxide	1.0 gm
50% alcohol	100 ml

- Mix well and add anhydrous Aluminum chloride 0.5 gm
- Boil gently for 2 to 3minutes, cool, filter and refrigerate (may be stored for 6 months)

Notes

2. Metanil yellow solution

Metanil yellow	0.25 gm
Distilled water	100 ml
Glacial acetic acid	0.25 ml

Mix well (may be stored for one year)

3. Harris hematoxylin

Procedure

- Deparaffize and bring the section to water.
- Mayer's hematoxylin for 10 minutes.
- Wash in running tap water for 5 minutes.
- Mucicarmine solution for one hour at room temperature.
- Rinse quickly in distilled water.
- Metanil yellow for 30 seconds to 1minute.(optional)
- Three changes of absolute alcohol.
- Dehydrate, clear and mount in DPX.

Result

Mucin	–	deep rose
Nuclei	–	black
Other tissue elements	–	yellow (if metanil yellow is used)
	–	colorless (if metanil yellow is not used)
Capsule of cryptococci (fungus)	–	deep rose

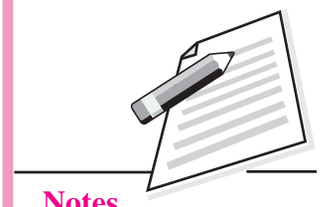
Control small intestine

14.7 GROCOTT-GOMORI'S METHANAMINE SILVER STAIN

Aim: To demonstrate fungi and bacteria particularly to stain carbohydrate. Cell wall of fungi like Pnemocystis Jiroveci is outlined by black stain.

Reagents

- 0.5% aqueous periodic acid



- Methanamine silver stock solution

  3% methanamine 100ml

  5% silver nitrate 5ml

- Add the silver nitrate solution to methanamine solution and mix it properly. A white precipate will form and redissolve on shaking. Filter the stock solution in brown bottle (stable for 6 months at 4°C).

  Methanamine silver working solution-

  Stock solution 50ml

  5% sodium borate 5ml

1. Mix well and filter.
2. Preheat for 10 to 20 minutes at 60°C prior to actual use.
3. 0.2% gold chloride
4. 3% sodium thiosulfate
5. Light green

Procedure

- Deparaffinze slides to distilled water.
- Oxidize in 0.5% periodic acid for 15 minutes at room temperature.
- Rinse three time in distilled water.
- Incubate the slides in methanamine silver working solution for 30 minutes to one hour at 60°C.
- Rinse in hot distilled water and check microscopically.
- Rinse in distilled water at room temperature.
- Tone in gold chloride solution for one minute.
- Rinse in distilled water.
- Treat with sodium thiosulfate solution for 2 minutes.
- Wash in running tap water for 10 minutes.
- Counterstain in nuclear fast red or light green for 5 minutes.
- Dehydrate, clear in xyline and mount in DPX.

Result

Basement membrane black

Fungi cell wall black

Background	pink or green (depends on counterstain nuclear fast red or light green)
Control	kidney or skin

INTEXT QUESTIONS 14.1

1. Mycobacterium Tuberculosis in paraffin section is demonstrated by .....
2. ..... or ..... is used as counter stain
3. Periodic acid Schiff stain is used to demonstrate ....., ... & .....
4. In periodic acid Schiff stain the nucleus appears ..... & glycogen, fungi, parasites appear .....
5. Mucin in tissue is demonstrated by ..... stain
6. ..... stain is used to demonstrate fungi and bacteria
7. In Grocott-Gromori's methanamine silver stain the fungal cell wall and basement membrane appears ..... & background appears .....
8. The control used in Grocott-Gromori's Methanamine silver stain is ..... or .....

WHAT HAVE YOU LEARNT

- Ziel-Neelson stain is used to demonstrate mycobacterium tuberculosis in paraffin sections
- Methylene blue or malachite green is used as counter stain
- Fite acid fast stain is used to demonstrate mycobacterium leprae in fromaline fixed sections and this technique combines peanut with xylene
- Periodic acid schiff stain is used to demonstrate glycogen, epithelial mucin, fungi and amaeba
- Liver and intestine is used as control in periodic acid Schiff stain
- Giema stain is used to demonstrate bacteria and blood parasites

- In Giema stain nuclei appears blue, malarial parasite appears purple and collagen and other elements appear pink
- Mucin in tissue is demonstrated by mucicarmine stain
- Grocott-Gomori's Methanamine silver stain is used to demonstrate fungi and bacteria particularly to stain carbohydrate

TERMINAL QUESTIONS

1. What are the stains which may be used to demonstrate fungi?
2. What is the principle of silver methanamine staining?
3. What is the difference in Acid Fast staining for Mycobacterium Tuberculosis and Leprae?
4. What are the uses of Giemsa stain?
5. What control is used in PAS stain?

ANSWERS TO INTEXT QUESTIONS

14.1

1. Ziel-Neelson stain
2. Methylene blue or Malachite green
3. Glucogen, fungi & ameba basement membrane
4. Blue or violet, Magenta
5. Mucicarmine
6. Grocott-Gomori's Methanamine Silver stain
7. Black, Pink or green
8. Kidney or skin

Notes

15

CRYOSTAT AND FROZEN SECTION

15.1 INTRODUCTION

Sections are prepared quickly for histological examination by freezing the tissue. The section should be thin, and without water crystals. It is an important procedure for quick diagnosis.

OBJECTIVES

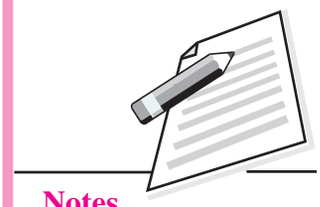
After reading this lesson, you will be able to:

- enlist the indications of frozen section
- explain the disadvantages of frozen section
- describe cryostat.

15.2 PURPOSES OF FROZEN SECTION

Frozen sections are used for following purpose

- Quick diagnosis
- Study the margins of cancer
- Enzyme histochemistry
- Immunohistochemistry
- Detection of lipid
- Some molecular procedures



Disadvantages

- Morphology is distorted
- Cellular details are not well seen,
- Staining is not very good
- Some specials stains cannot be performed.

Handling of specimen

Tissue must reach histopathology laboratory immediately. To avoid tissue being dried it should be kept in saline. The size of the tissue should be small thin, so that good smooth sections can be obtained and freezing is quick. Thickness of the tissue should be about 3mm to 4mm.The tissue can directly be taken to cryostat or can be fixed with 10% formalin or formol –alcohol

Embedding media

Sucrose (20%) or a drop of water may be applied on the chuck. Optimum Cooling temperature (OCT) compounds or 20% sucrose gives good result. Other embedding media are available with cryostat. Completion of freezing is observed by the change of color of tissue which turns glossy white. Freezing should be done fast. This will prevent ice crystal formation. The morphology is better preserved and artifacts are less.

Different freezing substances are used depending upon the availability and feasibility.

Carbon Dioxide gas is most commonly used with freezing microtome. This gives good results. Liquid Nitrogen is another substance used for freezing the tissue. An expertise is required while using liquid nitrogen to get uniform freezing. Aerosol sprays are also used for this purpose.

Cryostat: Cryostat is used in medicine to cut histological sections. They are usually used in a process called frozen section histology. The cryostat is essentially an ultrafine "deli-slicer", called a microtome, placed in a freezer. The cryostat is usually a stationary upright freezer, with an external wheel for rotating the microtome. The temperature can be varied, depending on the tissue being cut - usually from minus 20 to minus 30 degree Celsius. The freezer is either powered by electricity, or by a refrigerant like liquid nitrogen. Small portable cryostats are available and can run off generators or vehicle inverters. To minimize unnecessary warming all necessary mechanical movements of the microtome can be achieved by hand via a wheel mounted outside the chamber. Newer microtomes have electric push button advancement of the tissue. The

precision of the cutting is in micrometres. Tissue are sectioned as thin as 1 micrometre. Usual histology slides are mounted with a thickness of about 7 micrometres.

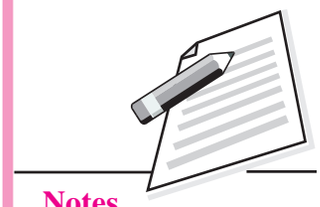
Specimens that are soft at room temperature are mounted on a cutting medium (often made of egg white) on a metal "chuck", and frozen to cutting temperature (for example at -20 degrees C). Once frozen, the specimen on the chuck is mounted on the microtome. The crank is rotated and the specimen advances toward the cutting blade. Once the specimen is cut to a satisfactory quality, it is mounted on a warm (room temperature) clear glass slide, where it will instantaneously melt and adhere. The glass slide and specimen are air dried, and stained. The entire process from mounting to reading the slide takes from 10 to 20 minutes, allowing rapid diagnosis in the operating room, for the surgical excision of cancer. The cryostat section quality is poorer as compared to fixed tissue sections.

INTEXT QUESTIONS 15.1

1. To avoid drying, the tissue should be kept in .....
2. Tissues can be fixed with .....
3. ..... or ..... is used as embedding media
4. ..... gas is most commonly used with freezing microtome

WHAT HAVE YOU LEARNT

- Sections are prepared quickly for histological examination by freezing the tissue
- Frozen section is used for quick diagnosis, studying margins of cancer, enzyme histochemistry, Immunohistochemistry
- Tissues must reach the laboratory immediately and to prevent drying of tissue it should be kept in saline
- The tissue can be fixed with 10% formalin or formal alcohol
- Optimum cooling temperature or 20% sucrose is used as embedding medium
- Ice crystal formation may be prevented by freezing the specimen fast
- Carbon dioxide gas is most commonly used with freezing microtome
- Cryostat is used for cutting histological frozen sections



TERMINAL QUESTIONS

1. What is a cryosection?
2. Write two indications of cryosections.
3. What is a cryostat?
4. What are the substances used in cryostat to cool the device?
5. Write three embedding media used for cryosections.

ANSWERS TO INTEXT QUESTIONS

15.1

1. Saline
2. 10% formalin
3. Optimum cooling temperature or 20% sucrose
4. Carbon dioxide

Notes

16

PROCEDURES FOR DNA, RNA AND MITOCHONDRIA DEMONSTRATION

16.1 INTRODUCTION

Nucleoproteins are combinations of basic proteins and nucleic acids. The two nucleic acids are deoxyribonucleic acid (DNA), which is mainly found in nucleus and ribonucleic acid (RNA) which is located in the cytoplasm of cells, mainly in the ribosomes. Both DNA and RNA molecules consist of alternate sugar and phosphate groups with a nitrogenous base being attached to each sugar group. The sugar in DNA is deoxyribose and in RNA it is ribose. The demonstration of nucleic acid depends upon either the reaction of dyes with the phosphate groups or the production of aldehydes from the sugars.

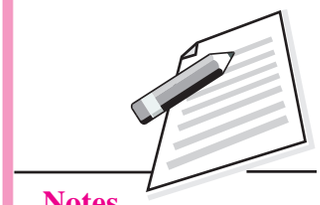
OBJECTIVES

After reading this lesson, you will be able to:

- explain the methods used in demonstrating nucleic acids
- describe the techniques and principles of the methods used.

16.2 DNA

The demonstration of DNA is either by Feulgen technique (which demonstrates the sugar deoxyribose) or the methyl green-pyronin technique (where the phosphates combine with basic dye methyl green at acidic pH). It can also be demonstrated by fluorescent methods using acridine orange, but is considered



less reliable than the above mentioned methods. The definitive and most sensitive technique is in situ hybridization.

16.3 FEULGEN TECHNIQUE

This technique involves mild acid hydrolysis with 1M hydrochloric acid at 60°C to break the purine-deoxyribose bond, the resulting exposed aldehydes are then reacted with Schiff's reagent to stain the DNA red-purple in color.

Feulgen nuclear reaction for DNA

Fixation: Not critical but do not use Bouin's fixative

Solutions

(a) 1 M hydrochloric acid

Hydrochloric acid (conc.)	8.5 ml
Distilled water	91.5 ml

(b) Schiff reagent

(c) Bisulfite solution

10% potassium metabisulfite	5 ml
1M hydrochloric acid	5 ml
Distilled water	90 ml

1. Bring all sections to water.
2. Rinse sections in 1M HCl at room temperature.
3. Place sections in 1M HCl at 60°C
4. Rinse in 1M HCl at room temperature, 1 minute.
5. Transfer sections to Schiff's reagent, 45 minutes.
6. Rinse sections in bisulfate solution, 2 minutes, repeating twice again.
7. Rinse well in distilled water.
8. Counterstain if required in 1% light green, 2 minutes.
9. Wash in water.
10. Dehydrate through alcohols to xylene and mount.

Results

DNA	red-purple
Cytoplasm	green

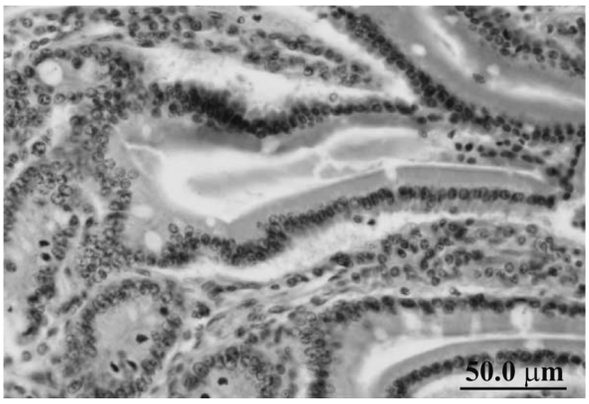


Fig. 16.1: Mouse small intestine stained with Feulgen's reaction and fast green counterstain. DNA is stained a magenta color; the cytoplasm is stained a uniform green .

16.4 RNA

The method of choice for demonstrating RNA is the methyl green-pyronin technique.

Methyl green-pyronin

Methyl green is an impure dye containing methyl violet. When methyl violet has been removed by washing with chloroform, the pure methyl green appears and is specific for DNA. Both dyes are cationinc, when used in combination methyl green binds preferentially and specifically to DNA, and pyronin binds RNA.

Methyl green-pyroninmethod for RNA

Fixation: Carnoy preferred, but formalin acceptable.

Staining Solution: Methyl green pyronin Y

2% methyl green (chloroform washed)	9ml
2% pyronin Y	4 ml
Acetate buffer pH 4.8	23 ml
Glycerol	14 ml

Mix well before use.

Method

1. Take sections down to water.
2. Rinse in acetate buffer pH 4.8.
3. Place in methyl green-pyronin Y solution for 25 min.
4. Rinse in buffer.

5. Blot dry.
6. Rinse in 93% ethanol, then in absolute ethanol.
7. Rinse in xylene and mount.

Results

DNA	green-blue
RNA	red

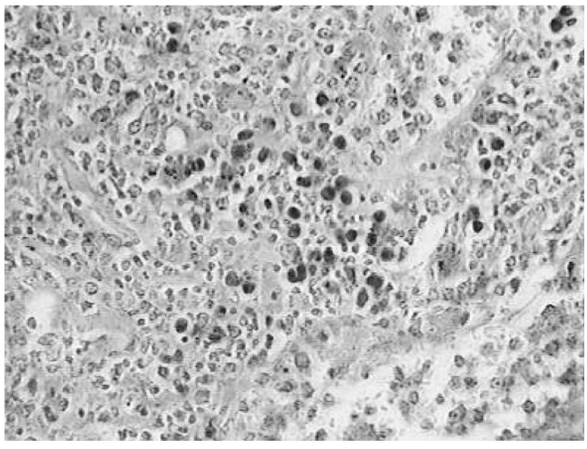


Fig. 16.2: R.N.A. – (notably in plasma cell cytoplasm) – magenta. D.N.A. – green or purplish green.

16.5 MITOCHONDRIA

Mitochondria are the cytoplasmic organelle found in variable numbers in all animal cells. Large number of mitochondria in the cells can change the appearance of cells. Mitochondria are considered the 'power houses' of the cell as many of the energy producing biochemical reactions like oxidative phosphorylation and Krebs cycle activity takes place in mitochondria. Mitochondria can be demonstrated by electron microscopy, enzyme histochemistry and histological methods however electron microscopy is the most satisfactory method. Histopathological methods such as Altman's technique for mitochondria is simple and useful for demonstration of mitochondria.

Altman's technique for mitochondria

Fixation

Champy's fluid is usually recommended, Helly's fluid works equally as well

Aniline-acid fuchsin – saturated solution of acid fuchsin in 5% aniline in distilled water.

Notes

Differentiator 1

Saturated alcohalic picric acid	10 ml
30% alcohol	40 ml

Differentiator 2

Saturated alcohalic picric acid	5 ml
30% alcohol	40 ml

Method

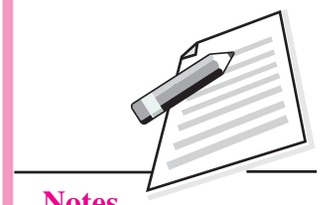
1. Take sections down to water.
2. Flood sections with aniline-acid fuchsin solution.
3. Gently heat the slide until steam rises and leave for 5 min.
4. Rinse in tap water.
5. Differentiate in solution 1 until the excess red stain is removed.
6. Completely differentiate in solution 2, controlling microscopically.
7. Dehydrate rapidly in two changes of absolute alcohal.
8. Clear in xylene and mount in DPX.

Results

Mitochondria	red
RBC and nuclei	red
Background tissue	yellow

INTEXT QUESTIONS 16.1

1. Deoxyribonucleic acid is found in .....
2. Ribonucleic acid is located in ..... of cells
3. Definite and most sensitive technique is .....
4. ..... should not be used as fixation for DNA
5. ..... technique is the method of choice for demonstrating RNA
6. ..... is the preferred fixation for RNA demonstration
7. ..... is used for demonstration of mitochondria
8. In Methyl green pyronin technique the DNA appears ..... and RNA appears .....



WHAT HAVE YOU LEARNT

- Nucleoproteins are combination of basic proteins and nucleic acids
- Two nucleic acids are deoxyribonucleic acid found in nucleus and ribonucleic acid found in cytoplasm of cells
- Demonstration of nucleic acid depends upon either the reaction of dyes with phosphate groups or production of aldehydes from the sugars
- DNA is demonstrated by Feulgen technique and RNA by Methyl green pyronin technique the most definite and sensitive technique is in-situ hybridization
- Carnoy is preferred as fixative in methyl green pyronin technique for RNA demonstration
- Mitochondria is demonstrated by Altman's techniques and is best visualized by electron microscopy

TERMINAL QUESTIONS

1. What are the components of nucleic acids?
2. What are the procedures used to detect DNA and RNA?
3. Describe the method used to detect mitochondria.

ANSWERS TO INTEXT QUESTIONS

16.1

1. Nucleus
2. Cytoplasm
3. In-situ hybridization
4. Bouin's fixation
5. Methyl green pyronin
6. Carnoy
7. Altman's technique
8. Green-blue & Red

Notes

17

SPECIAL PROCESSING

17.1 INTRODUCTION

Some pathological specimens require special handling and need to be processed in a different way to reach the final diagnosis. Examples include eyeball, bones and bone marrow biopsy. The technical person needs to be aware of these special specimens so that appropriate measures can be taken before the grossing procedures are undertaken.

OBJECTIVES

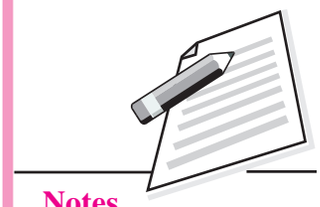
After reading this lesson, you will be able to:

- explain the principle of special processing
- explain the different specimens requiring special processing
- learn how to do special processing procedures.

17.2 BONE

Normal human skeleton has two main types of bones: cortical or compact bone which is hard, solid and very strong and forms shafts of long bones i.e. the femur and tibia etc; and spongy or trabecular/ cancellous bone is found in the marrow cavities and is a mesh of bone strands which is almost ideal weight bearing structure particularly in the femoral head and vertebrae. The three major components of bone are mineral, cells and an organic extra-cellular matrix i.e. collagen fibers. The main bulk of bone is approximately 70% mineral and 30% organic components by weight. Bone cells are relatively few as opposed to marrow cells. The mineral of bone is mainly calcium and phosphate.

Techniques for the demonstration of bone and its components include:



- For decalcified bone: frozen, paraffin, or celloidin sections, transmission electron microscopy.
- For mineralized bone: frozen, plastic, scanning and transmission electron microscopy.

In order to obtain satisfactory paraffin sections of bone, inorganic calcium must be removed from the organic collagen matrix, calcified cartilage and surrounding tissues. This is called decalcification and is carried out by chemical agents, either with acids to form soluble calcium salts or with chelating agents that bind to calcium ions. Any acid, however well buffered, can have damaging effects on tissue staining. The problem increases with acidity of solution and duration of decalcification period. It mostly affects the nuclei which fail to take up hematoxylin and other basic dyes. These effects can be reduced by doing the decalcification end point test, post-decalcification acid removal and adjustment of the stain procedure.

17.3 DECALCIFYING AGENTS

Acids

Acid decalcifiers can be divided into two groups: strong (inorganic) and weak (organic) acids.

Strong Inorganic acids: e.g. nitric and hydrochloric acids may be used as simple aqueous solutions (5-10%). They decalcify rapidly but cause tissue swelling and can seriously damage tissue stainability if used longer than 24-48 hours. Old nitric acid is particularly damaging and should be replaced with fresh stock They also damage tissue antigens for immunohistochemistry and enzymes may be totally lost. They can be used for small needle biopsies to permit rapid diagnosis within 24hrs. They can also be used for large or heavily mineralized bones with decalcification progress being carefully monitored.

Aqueous Nitric acid, 5-10%

Nitric acid	5-10 ml
Distilled water	To make 100ml

Formalin-nitric acid

Formaldehyde (37-40%)	10 ml
Distilled water	80 ml
Nitric acid	10 ml

Weak organic acids: e.g. fromic, acetic and picric acid. Of these three formic acid is the only weak acid which is used in decalcification. Other two are used as components of other fixatives. Formic acid solutions can be aqueous (5-10%),

Notes

buffered or combined with formalin. The formalin-10% formic acid mixture fixes and decalcifies simultaneously and can be used for small biopsies. Formic acid is suitable for most routine surgical specimens, particularly when immunohistochemistry is needed. Decalcification is usually complete in 1-10 days and decalcification progress should be monitered by a decalcification end point test.

Aqueous formic acid

90% stock formic acid	5-10 ml
Distilled water	To make 100 ml

Formic acid-formalin

90% stock formic acid	5-10 ml
Formaldehyde (37-40%)	5 ml
Distilled water	To make 100 ml

Buffered formic acid

20% aqueous sodium citrate	65 ml
90% stock formic acid	35 ml

17.4 CHELATING AGENTS

The chelating agent used for decalcification is ethylene-diaminetetracetic acid (EDTA). Although called an acid, it does not act like acids. EDTA will not bind to calcium below pH 3 and is faster at pH 7-7.4. This is a very slow process that does not damage tissues or their stainability, it also retains good antigen preservation for immunohistochemistry or enzyme staining and electron microscopy. The time required to totally decalcify dense cortical bone may be 6-8 weeks or longer although small bone spicules may be decalcified in less than a week.

Formalin-EDTA

EDTA, disodium salt	5.5 g
Distilled water	90 ml
Formaldehyde (37-40% stock)	10 ml

Aqueous EDTA, pH 7.0-7.4

EDTA, disodium salt	250 g
Distilled water	1750 ml

If solution is cloudy, adjust to pH 7 with sodium hydroxide.

Factors influencing the rate of decalcification

There are several factors influencing the rate of decalcification. The concentration and volume of the active reagent, including the temperature at which the reaction takes place, are important at all times. The more concentrated acids solutions decalcify bone more rapidly but are more harmful to the tissue. The usuall recommended ratio of volume of decalifying fluid to volume of tissue is 20:1 and the fluid should be changed several times during the decalcification

Process. Increased temperature accelerates decalcification, but it also increases the tissue damage and loss of heat sensitive antigen and enzymes. Other factors that contribute include the age of patient, type of bone, size of specimen and solution agitation. Mature cortical bone decalcifies more slowly than immature bone.

Treatment following decalcification

Acids can be removed from tissues or neutralized chemically after decalcification is complete. It can be done by immersing the bone into either saturated lithium carbonate solution or 5-10% aqueous sodium bicarbonate solution for several hours. Many laboratories rinse the specimens with running tap water for a period of time.

Eyeball

The eye should be placed in fixative as soon as practical after removal. Many of the tissues, the retina in particular, are very sensitive to anoxia and the longer you wait to fix the eye, the greater will be the artifacts, making interpretation difficult.

If eyes arrive in formalin and have been fixed for 2 days, wash them in water to remove the formalin (2 changes about 5 minutes each) and place them in enough 50% ethanol to cover the eye. Let the eye equilibrate overnight. Change the alcohol the next day and equilibrate for a second day. The eye should return to a normal volume and should not be indented or shrunken. For sectioning the eye it is best to wait 2 days with the eye in 50% ethanol.

INTEXT QUESTIONS 17.1

1. For sectioning of the eyes, eyes must be placed in ..... for atleast .....
2. Removal of inorganic calcium from the matrix is called .....

Notes

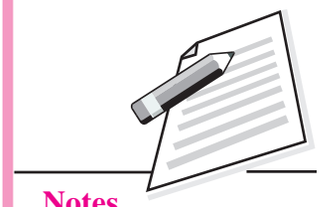
3. Examples of strong inorganic acids are ....., ...
4. Examples of weak organic acids are ....., ... & .....
5. Chelating agent used for decalcification is .....

WHAT HAVE YOU LEARNT

- When eye balls placed in formalin are taken for sectioning, it is best to place the eye ball in 50% ethanol for 2 days before sectioning
- Gross description of the eye should be recorded, appropriate measurements of eye, tumor or pathologic processes also need to be recorded
- For decalcified bone techniques like frozen, paraffin, or celloidin sections, transmission electron microscopy are used
- For mineralized bone frozen, plastic, scanning and transmission electron microscopy techniques are used
- Inorganic calcium must be removed from the matrix by decalcification either by acids or chelating agents
- Acid decalcifiers are strong inorganic acids like nitric acid and hydrochloric acids and weak organic acids like fromic, acetic, picric acids
- Chelating agent used for decalcification is ethylene diaminetetraacetic acid
- Several factors like concentration and volume of active reagent, temperature at which reaction takes place influence the rate of decalcification
- Acids from the tissues be removed from tissues or neutralized chemically after decalcification is complete by either saturated lithium carbonate solution or 5-10% aqueous sodium bicarbonate solution

TERMINAL QUESTIONS

1. Describe decalcification.
2. Enumerate the different methods of decalcification.
3. What are the advantages of EDTA decalcification?
4. What are the factors influencing the rate of decalcification?
5. Describe the special steps required before processing eyeball.



ANSWERS TO INTEXT QUESTIONS

17.1

1. 50% ethanol, 2 days
2. Decalcification
3. Nitric & Hydrochloric acid
4. Fromic, acetic & Picric acid
5. Ethylene-diaminetetraacetic acid (EDTA)

Notes

18

IMMUNOHISTOCHEMISTRY

18.1 INTRODUCTION

The gradual development of immunohistochemical methodologies over the past decades has allowed the identification of specific or highly selective cellular epitopes in formalin-fixed paraffin-embedded tissues with an antibody and appropriate labeling system

OBJECTIVES

After reading this lesson, you will be able to:

- explain the principles of immunohistochemistry
- describe the methods of performing immunohistochemistry.

18.2 IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions, the site of binding can be identified by direct labeling of the antibody or by use of a secondary labeling method.

Antigen-Antibody binding

The amino acid side-chains of the variable domain of an antibody form a cavity which is complementary to a single type of antigen like a lock and key. The precise fit required explains the high degree of specificity seen in antigen-antibody interaction.

Affinity: is the 3 dimensional fit of the antibody to its specific antigen and is a measure of the binding strength between antigen and antibody.

Avidity: is the functional combined strength of an antibody with its antigen. An antibody against more than one epitope of an antigen will bind more strongly to it.

Antibody specificity: is the characteristic of an antibody to bind selectively to a single epitope or an antigen.

Sensitivity: is the relative amount of an antigen that a technique is able to detect.

Primary reagents

Polyclonal antibodies: they are produced by immunizing an animal with a purified specific molecule (immunogen) bearing the antigen of interest. The animal will mount a humoral response to the immunogen and the antibodies so produced can be harvested from animal blood. The serum is polyclonal in nature as it comprises of a mixture of antibodies to different epitopes present on the antigen. Some of these antibodies may cross react with other molecules and produce nonspecific staining.

Monoclonal antibodies: Hybridoma method is used to produce these antibodies and it combines the ability of a plasma cells or transformed B lymphocytes to produce a specific antibody with the in vitro immortality of a neoplastic myeloma cell line. With the technique of cloning, this cell can be grown and multiplied in cell culture to unlimited numbers and can produce large supply of particular antibodies.

Labels: Enzymes are the most widely used labels in IHC, and incubation with a chromogen using a standard histochemical method produces a stable, colored reaction end-product suitable for the light microscope. Horseradish peroxidase is the most widely used enzyme, and in combination with the most favored chromogen, i.e. 3,3'- diaminobenzidine tetrahydrochloride (DAB) it gives a crisp, insoluble, stable, dark brown reaction end-product.

Immunohistochemical Methods

Methods

There are numerous IHC staining techniques that may be used, the selection should be based on parameters such as type of specimen, type of preparation (frozen or paraffin section) and sensitivity required.

Traditional Direct technique: the primary antibody is conjugated directly to the label. The conjugate may be either a fluorochrome or enzyme. The labeled antibody reacts directly with the antigen. The technique is quick and easy to use but provides little signal amplification and is less sensitive, so its used to

Notes

demonstrate immunoglobulins and complements in frozen sections of skin and renal biopsies.

New direct technique (Enhanced polymer one step staining method): available under the commercial name of EPOS. A large number of primary antibodies and peroxidase enzymes are attached to a dextran polymer 'backbone', hence increasing the signal amplification.

Two step indirect technique: A labeled secondary antibody directed against the immunoglobulin of the animal species in which the primary antibody has been raised visualizes an unlabeled primary antibody. It is more sensitive than direct technique.

Antigen retrieval

The demonstration of many antigens can be significantly improved by the pretreatment with the antigen retrieval reagents that break the protein cross-links formed by formalin fixation and thereby uncover hidden antigenic sites. It can be done by enzymatic method and/or heat induced. The most popular enzymes employed today are trypsin and protease. The enzymatic digestion breaks down formalin cross-linking and hence the antigenic sites are uncovered.

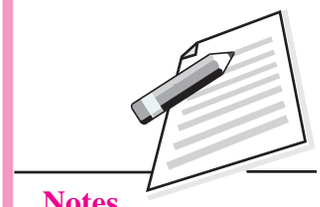
Heat based antigen retrieval methods have brought great improvement in IHC. The theories suggested for the role of heat pretreatment include: heavy salts act as protein precipitant forming insoluble complexes with polypeptides. Another theory is that heat mediated retrieval removes the weaker Schiff bases formed during formalin fixation.

The different methods of heat based antigen retrieval include

1. Microwave antigen retrieval
2. Pressure cooker antigen retrieval
3. Steamer
4. Water bath

Microwave antigen retrieval with a non toxic citrate buffer at pH 6.0 has demonstrated results equivalent to frozen sections. Most domestic microwave ovens are suitable for antigen retrieval . Uneven heating and the production of hot-spots have been reported, but using 400-600 ml of buffer in a suitably sized container can minimize these problems.

Pressure cooker has been suggested as an alternative to microwave oven. Batch variation and production of hot and cold spots in microwave can be overcome. Pressure cooker is said to be more uniform in heating. Also the increased



temperature (120°C) attained under pressure is an advantage in unmasking antigens.

Buffers used for antigen retrieval:

- Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)

  Tri-sodium citrate (dihydrate) 2.94 g

  Distilled water 1000 ml

  Mix to dissolve. Adjust pH to 6.0 with 1N HCl.

  Add 0.5 ml of Tween 20 and mix well. Store at room temperature for 3 months or at 4°C for longer storage.
- 1 mM EDTA, adjusted to pH 8.0

  EDTA 0.37 g

  Distilled water 1000 ml

  Store at room temperature for 3 months.
- Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0)

  Tris 1.21 g

  EDTA 0.37 g

  Distilled water 1000 ml (100 ml to make 10x, 50 ml to make 20x)

  Mix to dissolve. pH is usually at 9.0.

  Add 0.5 ml of Tween 20 and mix well. Store at room temperature for 3 months or at 4°C for longer storage.

IHC staining

All incubations should be carried out in a humidified chamber to avoid drying of the tissue. Drying at any stage will lead to non-specific binding and ultimately high background staining. A shallow, plastic box with a sealed lid and wet tissue paper in the bottom is an adequate chamber, just as long as the slides are kept off the paper and can lay flat so that the reagents don't drain off. A good solution is to cut a plastic serological pipette into lengths to fit your incubation chamber. Glue them in pairs to the bottom of the chamber, with the 2 individual pipette tubes of each pair being placed about 4.0 cm apart. This provides a level and raised surface for the slides to rest on away from the wet tissue paper.

Dilutions of the primary and secondary antibody are listed on the datasheets or are determined by testing a range. Adjust dilutions appropriately from the results obtained. Adhere strictly to all incubation times in the protocol.

For enzymatic methods, horseradish peroxidase (HRP) or alkaline phosphatase (AP) are the most commonly used enzymes. There are a number of chromogens used with these enzymes.

Day 1

1. If using an HRP conjugate for detection, incubate the slides in 0.3% H2O2 in Tris Buffered Saline (TBS) for 15 min
2. Wash the slides 2 x 5 minutes in TBS plus 0.025% Triton X-100 with gentle agitation.
3. Block in 10% normal serum with 1% BSA in TBS for 2 hours at room temperature.
4. Drain slides for a few seconds (do not rinse) and wipe around the sections with tissue paper
5. Apply primary antibody diluted in TBS with 1% BSA
6. Incubate overnight at 4°C

Day 2

1. Rinse 2 x 5min TBS 0.025% Triton with gentle agitation.
2. For enzymatic detection (HRP or AP secondary conjugates): Apply enzyme-conjugated secondary antibody to the slide diluted to the concentration recommended by the manufacturer in TBS with 1% BSA, and incubate for 1 hour at room temperature.
3. Rinse 3 x 5min TBS.
4. Develop with chromogen for 10 min at room temperature
6. Rinse in running tap water for 5 min.
7. Counterstain (if required)
8. Dehydrate, clear and mount with DPX.

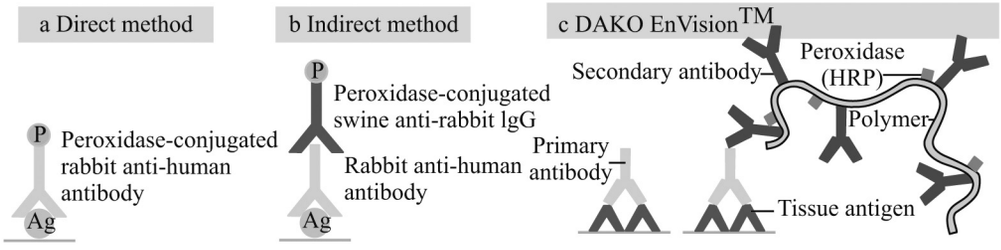
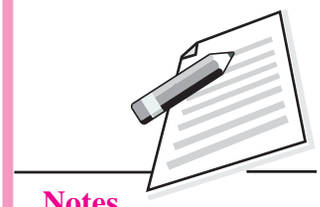


Fig. 18.1: Figure depicting various methods of antigen antibody interaction in IHC



INTEXT QUESTIONS 18.1

1. Technique used for identifying cellular constituents by means of antigen-antibody interaction is .....
2. The site of binding can be identified by ..... of the antibody
3. Three dimensional fit of the antibody to its specific antigen is .....
4. Functional combined strength of an antibody with its antigen is .....
5. Characteristic of an antibody to bind selectively to a single epitope or an antigen is .....
6. Relative amount of an antigen that a technique is able to detect is called .....

WHAT HAVE YOU LEARNT

- Immunohistochemistry is a technique for identifying cellular or tissue constituents by means of antigen antibody interactions
- The site of binding can be identifying labeling of antibody
- Affinity is the three dimensional fit of antibody to its specific antigen
- Avidity is the functional combined strength of an antibody with its antigen
- Antibody specificity is the characteristic of an antibody to bind selectively to a single epitope
- Sensitivity is the relative amount of an antigen that a technique is able to detect
- Primary reagents are polyclonal antibodies, monoclonal antibodies
- Enzymes are sidely used lables in immunochemistry Horse radish peroxidase is the most widely sued enzyme
- Numerous immunohistochemistry staining techniques may be used, and the selection should be based on type of specimen, type of preparation and sensitivity required
- Methods like traditional direct technique, new direct technique or enhanced polymer one step staining method and two step indirect techniques are used
- Methods of antigen retrieval includes microwave, pressure cooker, streamer and water bath antigen retrieval

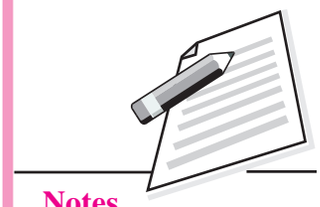
TERMINAL QUESTIONS

1. What is the principle of IHC?
2. What are the various methods of antigen detection in histopathology?
3. Enumerate the various methods of antigen retrieval.

ANSWERS TO INTEXT QUESTIONS

18.1

1. Immunohistochemistry
2. Labeling
3. Affinity
4. Avidity
5. Antibody specificity
6. Sensitivity



19

ELECTRON MICROSCOPY

19.1 INTRODUCTION

Electron Microscopes were developed due to the limitations of Light Microscopes which are limited by the physics of light to a resolution of about 0.2 micrometers. In the early 1930's this theoretical limit had been reached and there was a scientific desire to see the fine details of the interior structures of organic cells (nucleus, mitochondria...etc.). This required 10,000x plus magnification which was just not possible using Light Microscopes. The Transmission Electron Microscope (TEM) was the first type of Electron Microscope to be developed and is patterned exactly on the Light Transmission Microscope except that a focused beam of electrons is used instead of light to "see through" the specimen. The electron microscope was invented in 1931 by Germans Ernst Ruska and Max Knoll. Ernst Ruska later received Nobel Prize for his work in 1986. Conventional transmission electron microscope (TEM) today can achieve a resolution of 0.05nm.

OBJECTIVES

After reading this lesson, you will be able to:

- explain the principle of electron microscopy
- describe the tissue processing for electron microscopy.

19.2 TRANSMISSION ELECTRON MICROSCOPE

The original form of electron microscope, the transmission electron microscope (TEM) is the direct counterpart of conventional light microscope. It uses a high voltage electron beam to create an image. The electron beam is produced by an electron gun, commonly fitted with a tungsten filament cathode as the electron

source. The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam. When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope. The spatial variation in this information (the "image") may be viewed by projecting the magnified electron image onto a fluorescent viewing screen coated with a phosphor or scintillator material such as zinc sulfide. Alternatively, the image can be photographically recorded by exposing a photographic film or plate directly to the electron beam.

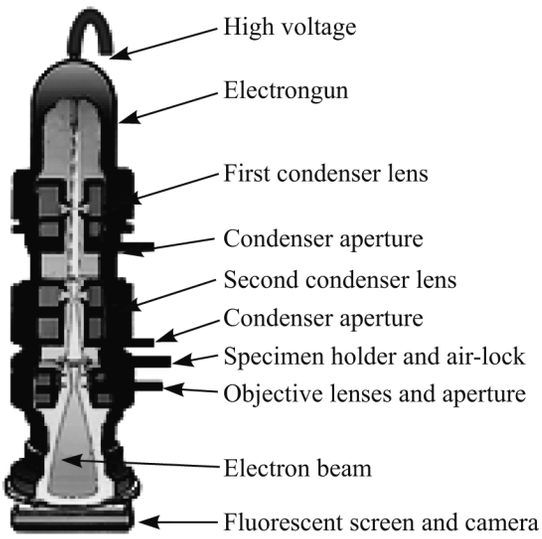


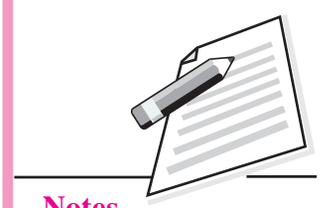
Fig. 19.1: Transmission Electron Microscope

It is the direct counterpart of conventional light microscope. The most obvious differences between TEM and light microscope are the: the 'light' source, the form of the lenses and the manner in which image is formed.

The Electron beam: This is the source of 'light' in an EM, which can be generated by thermionic emission from a tungsten filament using an electron gun. Electrons are produced by passing a heating current through the filament. In some microscopes, the beam can be generated by field emission.

Electromagnetic Lenses: The lenses in EM are electromagnetic coils. To focus an electronic beam onto a given plane, the current passing through the coil is changed. Thus the focal length of these so called lenses can be infinitely variable.

Image formation: In a light microscope, image formation occurs due differential absorption of light rays. In EM, the image is formed partly by differential absorption and partly by scattering.



Scanning Electron Microscope (SEM)

The SEM is an instrument that produces a largely magnified image by using electrons instead of light to form an image. A beam of electrons is produced at the top of the microscope by an electron gun. The electron beam follows a vertical path through the microscope, which is held within a vacuum. The beam travels through electromagnetic fields and lenses, which focus the beam down toward the sample. Once the beam hits the sample, electrons and X-rays are ejected from the sample. Detectors collect these X-rays, backscattered electrons, and secondary electrons and convert them into a signal that is sent to a screen similar to a television screen. This produces the final image.

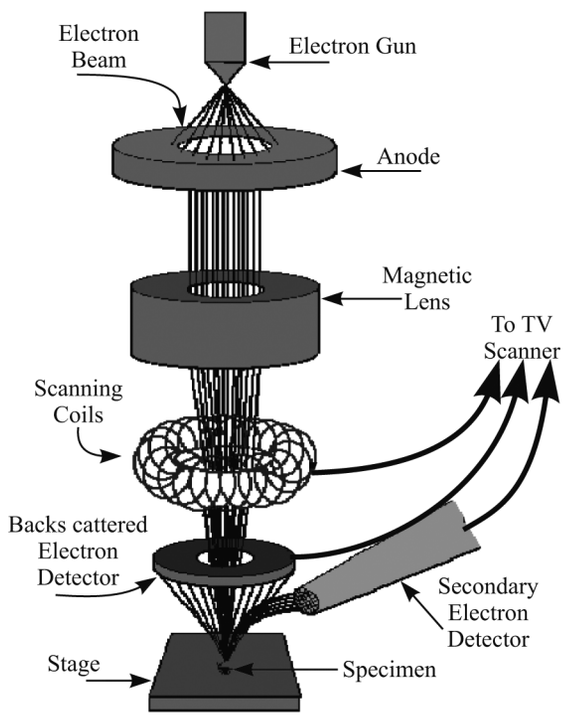


Fig. 19.2: Scanning Electron Microscope

Tissue Processing for EM:

To study the specimen with EM, a series of processing steps are required which are similar but different to light microscopy. The steps involved are fixation, dehydration, embedding, cutting and positive staining with heavy metals.

Specimen Handling & Fixation:

The specimen must be exposed to fixative as soon as possible after interruption of blood supply. Slices of tissues about 1-2 mm thick should be cut and transferred to a container containing fixative. The most popular method of

fixation used is double fixation. It involves primary fixation in an aldehyde followed by secondary (post) fixation in osmium tetroxide. Glutaraldehyde is the most popular aldehyde for fixation of tissues for electron microscopy as it reacts rapidly with proteins and stabilises structures by cross-linking before there is any opportunity for extraction by the buffer . Hence more ground substance of the cytoplasm (glycogen) and of the extracellular matrices is preserved. But, glutaraldehyde alone is not an adequate fixative, since certain cell components especially lipids, are not fixed and may be extracted during dehydration, therefore secondary fixation is required using osmium tetroxide. Depth of penetration of glutaraldehyde is 2 - 3 mm / hour and of osmium tetraoxide is 1mm/hour.

Dehydration

The aim of dehydration is to remove all the free water in the fixed tissue and replace it with a solution miscible with embedding medium. We usually use organic solvents like methanol, ethanol or acetone. It starts with distilled water to 40% ethanol and then through a series of increasing concentrations of ethanol to 100% ethanol.

Protocol for dehydration in ethanol

1. 40% ethanol, 5 min.
2. 70% ethanol, 10 min.
3. 90% ethanol, 10 min
4. 100% ethanol, 3 x 10 min.
5. If ethanol is miscible with the embedding medium then tissue is directly transferred to it. Otherwise, another transitional fluid may be required. Epoxypropane is most commonly used transitional fluid.

Embedding Media

The embedding media for EM are resins, polyester resins and methacrylates. For general electron microscopy epoxy resins have most properties required.

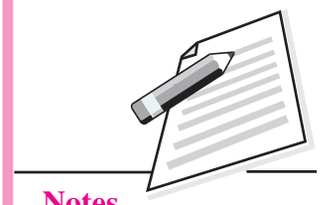
Epoxy resin

Characteristics

(a) Polyaryl ethers of glycerol with terminal epoxy groups.

(b) Transparent yellowish resins which range from viscous liquids to fusible solids.

(c) Require addition of curing agents to convert them to a tough, extremely adhesive and highly inert solid. Polymerization accomplished by the



addition of various bifunctional setting groups which link with the resin to produce a three-dimensional structure.

Embedding

The embedding is carried out in polythene capsules or flat embedding moulds. The capsules are filled with warm bubble-free raisin using a plastic pipette. No air bubbles should be introduced in the medium. Labeling is done at this point. It is best to write the identifier on a piece of paper and embed it along with tissue using marking ink or pencil.

Sectioning

Getting an ultra-thin section is the pre-requisite for EM. It should be approximately 60 nm in thickness. Routine microtomes cannot produce such thin sections, thus special microtome known as ultramicrotome is used which needs special training. Glass knives or diamond knives are used to cut ultra-thin sections on these ultra microtomes.

Applications of Electron Microscopy

1. Kidney biopsy interpretation
2. Skin biopsy interpretation
3. Viral and other infections
4. Inborn errors of metabolism
5. Tumor diagnosis

INTEXT QUESTIONS 19.1

1. Source of light in electron microscopy is .....
2. The lenses in electron microscopy are .....
3. In electron microscopy the image is formed by ..... & .....
4. Surface topography of solid, unsectioned specimens is studied by .....
5. Most popular method of fixation in electron microscopy is .....
6. ..... is used for fixation in electron microscopy
7. ..... is the commonly used transitional fluid during dehydration
8. The thickness of section in electron microscopy should be approximately .....

WHAT HAVE YOU LEARNT

- Transmission electron microscope is the direct counter part of conventional light microscope
- Electron microscope involves the passing of high velocity, homogenous electron beam through a thin enough specimen
- In electron microscopy the source of light is electron beam, lens are electron magnetic coils & image formation occurs due to absorption & scattering
- Scanning electron microscope is used to study the surface topography of solid, unsectioned specimens
- A series of steps namely fixation, dehydration, embedding, cutting & positive staining with heavy metals are used for studying the specimen

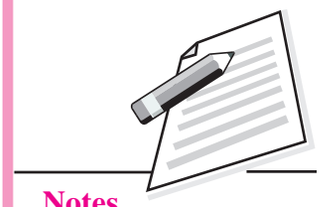
TERMINAL QUESTIONS

1. What is the principle of EM?
2. What are the types of electron microscope?
3. What is the procedure for processing tissue for EM?
4. What are the applications of EM?

ANSWERS TO INTEXT QUESTIONS

19.1

1. Electron beam
2. Electromagnetic coils
3. Absorption & Scattering
4. Scanning electron microscopy
5. Double fixation
6. Gluteraldehyde
7. Epoxypropane
8. 60nm



20

MUSEUM TECHNIQUES

20.1 INTRODUCTION

All teaching hospitals and colleges of Pathology have Museums which serve many functions: permanent exhibition of common specimen for undergraduate and postgraduate teaching purposes, illustrating specimens of rarity, permanent source of histologic material and for gross and microscopic photography.

OBJECTIVES

After reading this lesson, you will be able to:

- explain the methods used in handling museum specimens
- describe the techniques of specimen preservation.

20.2 BASIC MUSEUM TECHNIQUES

Any specimens for museum are handled by following steps:

1. Reception
2. Preparation
3. Fixation
4. Restoration
5. Preservation
6. Presentation

Reception of the Specimen

Any specimen received in the museum should be recorded in a Reception book and given a number followed by year (e.g. 32/2013). This number will stay with specimen even after it is catalogued in its respective place. This number is

Notes

written on tie-on type label in indelible ink and is firmly attached or stitched to the specimen. The reception book should contain all necessary information about the specimen (clinical, gross and microscopic findings).

Preparation of the specimen

An ideal specimen is received fresh in unfixed state. However, it is mostly obtained from pathology laboratory after being examined, thus will already be formalin fixed. If planning to use a specimen for museum, part of it can be kept without disturbing for museum, e.g. in kidney it can be bisected and one half kept aside for museum.

Fixation of the specimen

The objective of fixation is to preserve cells and tissue constituents in as close a life-like state as possible and to allow them to undergo further preparative procedures without change. Fixation arrests autolysis and bacterial decomposition and stabilizes the cellular and tissue constituents. The fixatives used in museums all over the world are based on formalin fixative technique, and are derived from Kaiserling technique and his modifications. Kaiserling recommended that the initial fixation be a neutral formalin (KI) solution and then transferred to a final preserving glycerin solution (KIII) for long term display. Colour preservation is also maintained with these solutions.

Kaiserling's Technique

Fixation of specimen:

The specimen needs to be kept in a large enough container which can accommodate specimen along with 3-4 times volume of fixative. Specimen is stored in the Kaiserling I Solution for 1 month depending on the size of the specimen. The specimen should not rest on bottom or an artificial flat surface will be produced on hardening due to fixation.

Kaiserling I Solution:

Formalin	1L
Potassium acetate	45 g.
Potassium nitrate	25 g.
Distilled water	Make up to 10 litres

Restoration of specimen

It is required to restore the specimens, as they lose their natural color on fixation. The recommended method is the Kaiserling II method. It involves removing the

specimen, washing it in running water and transferring to 95% alcohol for 10 minutes to 1hour depending on the size of specimen. The specimen is then kept and observed for color change for around 1- 1.5 hrs. After this step, specimen is ready for preservation.

Kaiserling II Solution:

Alcohol 95%

*Store specimen in this solution for 10 minutes to 1 hour depending on size of specimen.

Rejuvenator Solution:

Pyridine	100 ml
Sodium hydrosulphite	100 gm
Distilled water	4 litres

*Formalin decreases the natural colour of the specimen. However, rejuvenator solution restores the colour.

Preservation of specimen

The recommended solution for this step is Kaiserling III. This is the final solution in which the specimen will remain for display. It is based on glycerine solution.

Kaiserling III Solution:

Potassium acetate	1416 g.
Glycerine	4 litres
Distilled water	Make up to 10 litres

Thymol crystals added to prevent moulds.

*Leave solution to stand for 2 – 3 days before using to ensure proper mixing of chemicals.

Add 1% pyridine as **stabilizer**. This solution acts as permanent fixative. This solution easily turns yellowish and needs to be replaced to restore colour of the specimen. The specimen will initially float to surface but later sink to bottom.

Presentation of the Specimen

Initially all museum specimens were mounted in cylindrical jars and sealed with sheep bladder walls. Later they were replaced by rectangular glass jars. They

were better than cylindrical ones as the flat surfaces afforded a clear view of specimens without any distortion. They are covered by rectangular glass plates. These jars can be purchased readymade or assembled in museum itself, as per need. Nowadays, Perspex jars are also available, which are lighter than glass jars. However, they cannot be used to store specimens fixed in alcohol or methyl salicylate as they react with plastics.

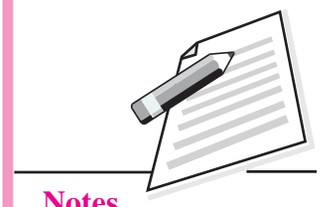
Mounting the Specimens

To support the specimen within its jar, it is attached to the specimen plate or rectangular bent glass rods. It can be done by tying the specimen with nylon threads. Double knots should be made by threads, on the specimen surface.

Fig. 20.1: Museum specimens of cardiovascular system

TERMINAL QUESTIONS

1. Why do we need a museum?
2. What are the steps involved in mounting a specimen?
3. Describe the fixation of specimen.



21

EXFOLIATIVE CYTOLOGY

21.1 INTRODUCTION

Exfoliative cytology, which is a quick and simple procedure, is an important alternative to biopsy in certain situations. In exfoliative cytology, cells shed from body surfaces, such as the inside of the mouth, are collected and examined. This technique is useful only for the examination of surface cells and often requires additional cytological analysis to confirm the results.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the principle of exfoliative cytology
- explain the methods of sample collection for exfoliative cytology.

21.2 EXFOLIATIVE CYTOLOGY

Exfoliative cytology differs from the more precise sampling of known lesions, like needle biopsy. It categorizes collected samples only by analyzing the presence of abnormal or atypical cells, or by showing the presence of malignant cells.

When a woman has a pap smear, she may have a result that show atypical cells. If this is the first exfoliative cytology test that shows atypical cells, then usually, the Pap smear is repeated in six to twelve months. If however, repeated showings of atypical cells are present in exfoliative cytology results, further tests may be undertaken to determine if cancerous cells are present.

Doctors or dentists may also use exfoliative cytology to check for the presence of cancer in the mouth or throat. The test takes a few skin scrapings and can show

Notes

the presence of either malignant or atypical cells. Malignant and atypical cells will probably require a person to undergo a biopsy or closer examination of the area in question to rule out cancer.

Cytologic examination of a serous effusion is of paramount importance because the finding of cancer cells in such a specimen denotes that the patient has cancer that is not only advanced but also almost always incurable. Apart from the finding of cancer cells, cytologic examination of pleural, peritoneal, and pericardial effusions may also reveal information about inflammatory conditions of the serous membranes, parasitic infestations, and infection with bacteria, fungi, or viruses.

21.3 COLLECTION METHOD

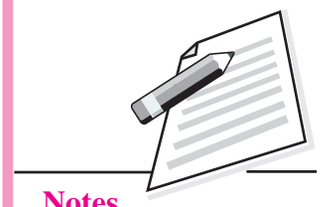
In this method, cells are collected after they have been either spontaneously shed by the body ("spontaneous exfoliation") or manually scraped/brushed off of a surface in the body ("mechanical exfoliation"). An example of spontaneous exfoliation is when cells of the pleural cavity or peritoneal cavity are shed into the pleural or peritoneal fluid. This fluid can be collected via various methods for examination. Examples of mechanical exfoliation include Pap smears, where cells are scraped from the cervix with a cervical spatula, or bronchial brushings, where a bronchoscope is inserted into the trachea and used to evaluate a visible lesion by brushing cells from its surface and subjecting them to cytopathologic analysis.

Spontaneous exfoliation: Peritoneal fluid, pleural fluid, pericardial fluid, urine, cysts, washings (peritoneal, bladder)

The fluid is collected into a clean, dry container, which need not be sterile, and sent to the laboratory as soon as possible. If the fluid cannot be sent immediately, it should be stored in a refrigerator at 4^0C and not allowed to freeze. We do not require anticoagulant or fixative to be added to the fluid. The appearance to the naked eye of a serous effusion sometimes reveals clues about the cause of the effusion and the nature of its cellular contents. Therefore, for every serous effusion received by the laboratory, note should be made of its volume, color, clarity, and any unusual physical features, such as malodor, opalescence, or high viscosity.

Mechanical exfoliation: Cervical pap smear, brushings (Bronchial, gastric, biliary, oral, etc).

Cervical smear is a reliable method for diagnosis of cervical cancer. The smears are usually taken in Gynecology ward or OPD, but sometimes patients are sent



to laboratory for smear purposes. Patients should be advised NOT to douche, use vaginal medications, or have intercourse 24 hours prior to the pap smear preparation. Patients should NOT schedule pap smear exams during menses. These situations may obscure cellular details or remove diagnostic material from the cervix or vagina. The smear is obtained under direct vision after introduction of speculum. A wooden tongue depressor cut with scissors to fit the contour of cervix may be used. Commercially prepared plastic or wooden scrapers are widely available for this purpose. The scraper is rotated under pressure to 360° for 4-5 full rotations. The material is spread on a pre-labelled slide and fixed immediately.

Several types of brushes have also been introduced to overcome the disadvantages of scrapers alone (not being able to reach endocervical canal and transformation zone where the carcinomas originate).

Brushes are also used to scrape cells in respiratory tract, oral mucosa, esophagus, stomach, duodenum, colon and biliary tract. It is preferable to obtain the brush sample before the biopsy because the latter results in bleeding, which both obscures the lesion, and detracts from the quality of a subsequently collected cytologic sample, whereas interpretation of the biopsy is not affected by the reverse order of collection. Collection of a good brush sample usually requires an experienced assistant, because the operator may well be engaged in maneuvering the end of the scope and holding the lesion in focus while the assistant manipulates the brush. Therefore, it is ideal that a Cytology staff member be present for immediate slide preparation of the specimen. They are all taken under vision- direct or through fibreoptic endoscopy. In all these cases a lot of care needs to be taken to make smear immediately from the brush by gently rotating the brush on slides and fixing them immediately. The material should not be crushed. If liquid-based cytology is used, the head of the broom is detached and dropped into the preservative vial.

Once received in laboratory, the usual precautions need to be taken as discussed in specimen receiving, handling and storage.

When only one slide is received, it should be preferable to stain it with Papanicolaou stain. When multiple slides are received- some are air dried (stain with MGG, special stains, etc) and some are wet fixed (stain with Papanicolaou).

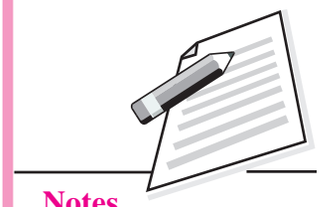
The liquid specimens need to be commented upon the volume, color and turbidity. Specimens need to be centrifuged and cytocentrifuged depending on cellularity. After concentrating they can be used for both air dried smears and wet fixation.

WHAT HAVE YOU LEARNT

- Exfoliative cytology is useful for the examination of surface cells
- Cells are collected either spontaneously shed by body or manually scraped off of a surface in the body
- Brushes have been introduced to overcome the disadvantages of scrapes
- Brushes are used to scrapes cells in respiratory tract, oral mucosa, esophagus, stomach, duodenum, colon and biliary tract
- Care needs to be taken to make smear immediately from the brush by gently rotating the brush on slides and fixing them immediately. The material should not be crushed.
- If liquid-based cytology is used, the head of the broom is detached and dropped into the preservative vial.
- When only one slide is received, it should be preferable to stain it with Papanicolaou stain. When multiple slides are received- some are air dried (stain with MGG, special stains, etc) and some are wet fixed (stain with Papanicolaou).

TERMINAL QUESTIONS

1. Define exfoliative cytology.
2. Give examples of samples on which exfoliative cytology can be performed.
3. Enumerate few precautions to be taken while handling brush specimens.



22

CYTOLOGY : SPECIMEN COLLECTION & STORAGE

22.1 INTRODUCTION

Cytology is the field of diagnostic medicine which deals with study of individual cells and/or tissue fragments spread on glass slides and stained. The final quality of cytodiagnosis depends to a large extent on quality of preparation of the material. It has an advantage of providing a rapid diagnosis. Cytological study can be done on various discharges from body (urine, nipple, sputum, vaginal, sinus, etc), scrapings obtained (buccal mucosa, gastric, respiratory), tap done from fluid collected in body (pleural, peritoneal, pericardial) or aspiration from palpable lumps.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the principle of cytology
- explain the methods of sample collection & receiving
- learn how to store the specimen for examination.

22.2 HEALTH AND SAFETY

There are potential hazards in handling fluid specimens like unfixed sputum, urine and other body fluids. All employees should be aware of all health and safety aspects of laboratory, including fire drills, storage and disposal of chemicals, use of electrical equipment, storage and disposal of biological infectious material.

Notes

Modern approach is to use 'Universal Precautions' to treat all unfixed specimens with care and to handle them in biological safety cabinets. Centrifuges should use sealed buckets.

Main aspects of safety in cytology laboratory

1. Specimen reception- suitable container (disinfectant, autoclave proof); availability of suitable disinfectant (hypochlorite); protocol for leakage/ spillage
2. Specimen preparation- Protective clothes, coats, gloves, eye protection etc; Safety cabinet, Disposal pots with disinfectant, Refrigerator for specimen storage
3. Specimen disposal- Disposal protocol, Autoclave, clinical waste collection
4. Fire hazards and evacuation procedure
5. Storage of chemicals- Inflammables, Poisons, Toxic substances
6. First aid

22.3 SPECIMEN COLLECTION

Most specimens are received in the laboratory either as direct cell spreads (on slides) or as cell suspensions (fluids). Most of Hospitals have FNA (fine needle aspiration) clinic where FNA is done by cytopathologist. Medical technologist is required in the clinic to spread the sample on glass slides and fix the slides appropriately. Some clinics also perform rapid staining to check adequacy of material.

22.4 TRANSPORT AND INFORMATION

Specimens should be sent to the laboratory as early as possible in suitable containers. Lids should be properly secured to prevent any leakage and specimens should be sealed in plastic bags. Glass slides should be kept in suitable slide boxes. All specimens and slides should be properly labeled with patient's name and number. Fixed smears should be submitted in containers that protect against breakage. Slide containers are available in a variety of shapes, volumes and material. Optimal design features include easily opened containers which stay closed during transport, shock resistant material, and enough room to prevent slides from adhering to one another or the container. The slides should be marked clearly with the patient's name, as well as other identifiers if possible. If more than one site is sampled, the slides must be clearly marked as to their

site. Slides in fixative should be submitted in leak proof containers that protect against breakage and are clearly labeled with the patient's name and specimen site(s).

Each fluid specimen should be placed in a clearly labeled container that is leak proof. Enclosure in a transport bag indicating biohazardous contents is careful if a courier system or manual delivery is planned. Paper requisitions that accompany slides in fixative should be placed in an outside pocket to avoid exposure to any leakage of fixative. Needles should never be transported with fluid specimens. Large glass collection containers should be avoided.

Specimen should be accompanied by properly filled requisition form. The requisition must also provide space for the date the sample was collected, the test to be performed, the source of the material, and the name and address or other suitable identifiers of the authorized person requesting the test. The request form should contain essential patient identification data- name, age, sex, ward/OPD, hospital number, any previous sample number. Precise information should be given regarding type of specimen, any fixative used, relevant clinical information and any previous treatment. A written procedure must be in place to handle specimens that are received without adequate information on the request form.

High risk specimens should be clearly marked with biohazard stickers or labels.

The specimen after checking all labels should be given a lab identification number. The laboratory identifier may be generated manually or electronically and may be numeric or alphanumeric and may also be bar coded.

Criteria for the rejection of specimens:

- Unlabeled slides, slides labeled with nonpermanent writing utensils or paper labels and broken slides.
- Mismatched specimens and requisition forms
- Specimen without accompanying requisition form

22.5 STORAGE OF SPECIMENS

Samples should be immediately prepared from the specimen. Record the date of preparation on the specimen container and refrigerate any remaining specimen. Specimen can be stored for one week before disposal. Ideally, samples need to be kept until the specimen is reported by the cytopathologist. The extra sample maybe required for any special tests to aid in the diagnosis.

Notes

INTEXT QUESTIONS 22.1

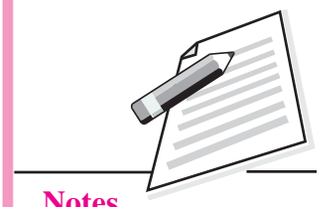
1. The field of diagnostic medicine that deals with individual cells is .....
2. ..... must be used while treating unfixed specimens to prevent transmission of infection.
3. Specimens should be transported to the laboratory as early as possible in ..... containers
4. All patients slides and samples should be labeled with ..... & .....
5. ..... should never be transported with fluid specimens
6. High risk specimens should be clearly marked with .....
7. Specimens can be stored for ..... before disposal
8. Samples should be stored until they are .....

WHAT HAVE YOU LEARNT

- Cytology deals with the study of individual cells or tissue fragments
- Quality of cytodiagnosis depends on quality of preparation of the material
- Universal precautions must be followed while handling specimens
- Specimens should be sent to laboratory as early as possible in suitable container that prevent breakage
- Lids should be properly secured to prevent leakages
- All specimens should be properly labeled with patients name and number
- Needles should never be transported with fluid specimens
- Specimen should be accompanied by filled requisition form
- High risk specimens should be clearly marked with biohazards stickers or labels
- Samples should be stored until it is reported by pathologists

TERMINAL QUESTIONS

1. Define cytology.



2. Give examples of samples on which cytology can be performed.
3. Enumerate few precautions to be taken while handling cytology specimens.
4. Enumerate the points to be kept in mind while transporting and receiving cytology specimens.

ANSWERS TO INTEXT QUESTIONS

22.1

1. Cytology
2. Universal precautions
3. Suitable
4. Patients name and Number
5. Needles
6. Biohazard stickers or labels
7. One week
8. Reported

Notes

23

CYTOLOGY : SPECIMEN PROCESSING & STAINING

23.1 INTRODUCTION

Laboratory sample processing includes steps from the receipt of the specimen in the laboratory to the delivery of a stained slide rea140dy for microscopic examination.

Throughout processing, the identity and integrity of the specimen must be maintained, and the principles of universal precautions followed.

OBJECTIVES

After reading this lesson, you will be able to:

- describe various methods of cytology sample processing
- explain the methods of slide staining
- dispatch labeled slides and forms for cytoscreening.

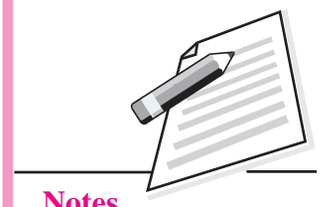
23.2 SPECIMEN PROCESSING

The laboratory should confirm the identity and integrity of the specimen received. Specimens are accepted only when ordered by physicians or other persons authorized by law. Each sample must have a request completed by the authorized provider prior to processing.

1. Specimen Preparation

(a) Smears

The preparation objective of direct smears is a slide with an evenly and thinly applied cellular specimen that is free of mechanical distortion and



free of drying artifact when the slide is fixed in alcohol. Smears fixed in alcohol (wet fixation) are usually stained by the Papanicolaou method; air-dried smears are usually stained with a Romanowsky stain. Smears preserved with spray fixatives should be soaked in 95% alcohol.

(b) Liquid Specimens

Liquid specimens should be processed according to the manner in which they are submitted. Liquid specimens may be received fresh, with heparin, with preservative (alcohol or other fixative), or with physiologic solution or tissue culture medium. Additional processing should be considered for grossly bloody specimens prior to slide preparation. Blood clots should be removed and processed as a cell block.

Specimens of low cellularity and low volume may be cytocentrifuged directly. High volume specimens are usually concentrated prior to preparation. Centrifugation is frequently used with the re-suspended pellet used for direct smears.

2. Specimen staining

The Papanicolaou stain is recommended for the staining of alcohol fixed cytology slides. Romanowsky stains may also be used for wet fixed slides, but are primarily applied to air-dried smears.

(a) Papanicolaou Stain

The Papanicolaou stain uses a standard nuclear stain, hematoxylin, and two cytoplasmic counterstains, OG-6 and EA. The outcome of this method is crisp nuclear detail and transparency of the cytoplasm, which allows the examiner to clearly visualize cellular morphology. Either a progressive or regressive technique may be used for nuclear staining. Several automatic programmable stainers are available.

(b) Romanowsky Stain

A Romanowsky stain is recommended for air-dried smears. Romanowsky stains, mixtures of eosin and methylene blue, are a family of polychrome stains that produce their effect by the production of azure dyes as a result of demethylation of thiazines and the acidic component eosin. Unlike the Papanicolaou stain they are metachromatic. Most Romanowsky stains used in cytology are aqueous stains as opposed to the methyl alcohol based stains of hematology. Many commercial stains are available, and most consist of a methanol-based fixative, and two dyes which result in differentiation of cytoplasmic and nuclear components. Most Romanowsky stains are rapid and are useful in enhancing pleomorphism, and distinguishing extracellular from intracytoplasmic material.

3. Dehydration, Clearing and Coverslipping

(a) Dehydration and Clearing

After staining, the sample is dehydrated by a series of increasing concentrations of alcohol followed by rinsing in clearing solutions. The last clearing solution should be colorless and its refractive index should be close to that of the coverslip, slides and mounting medium. Xylene is the most commonly used clearing agent. Xylene clearing must be performed in a well ventilated area or fume hood to limit exposure to xylene fumes. Slides should remain in the clearing agent until coverslipping is performed.

(b) Coverslipping

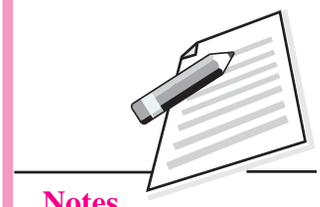
Mounting medium used to bond the slide and the coverslip should be compatible with the clearing agent, transparent, and have a refractive index similar to the glass slide and the stained specimen. Adequate mounting medium should be applied to protect the cellular material from air-drying and shrinkage, and to prevent fading of the cell sample. The cellular material should be covered by a suitably sized coverslip or covering material of appropriate quality.

Different methods used to coverslip include placing the mounting medium on the coverslip, then inverting the coverslip onto the slide surface, or lowering the slide onto a coverslip containing adequate mounting medium. Glass coverslips, coverfilm and automated coverslippers are available. Ideally, the mounting medium should be allowed to dry before the slides are reviewed to reduce movement of cellular material during the slide examination. Chemical waste collected throughout the staining, dehydration, clearing and coverslipping processes must be disposed of or recycled.

The stained and labeled slide(s) should be matched with its requisition or other laboratory document that displays the same information. The information on the slide must correspond to the information on the requisition or laboratory document.

INTEXT QUESTIONS 23.1

1. Smears fixed in alcohol are stained by ..... method
2. Air dried smears are stained with ..... stain
3. Specimens of low cellularity and low volume may be ..... directly
4. ..... is the most commonly used clearing agent



WHAT HAVE YOU LEARNT

- Laboratory sample processing includes steps from the receipt of the specimen in the laboratory to the delivery of a stained slide ready for microscopic examination.
- Throughout processing, the identity and integrity of the specimen must be maintained, and the principles of universal precautions followed.
- The preparation objective of direct smears is a slide with an evenly and thinly applied cellular specimen that is free of mechanical distortion and free of drying artifact when the slide is fixed in alcohol
- Smears fixed in alcohol (wet fixation) are usually stained by the Papanicolaou method
- Air-dried smears are usually stained with a Romanowsky stain. Smears preserved with spray fixatives should be soaked in 95% alcohol.
- Specimens of low cellularity and low volume may be cytocentrifuged directly. High volume specimens are usually concentrated prior to preparation
- The Papanicolaou stain is recommended for the staining of alcohol fixed cytology slides
- After staining, the sample is dehydrated by a series of increasing concentrations of alcohol followed by rinsing in clearing solutions
- Xylene is the most commonly used clearing agent
- Mounting medium used to bond the slide and the coverslip should be compatible with the clearing agent, transparent, and have a refractive index similar to the glass slide and the stained specimen.

TERMINAL QUESTIONS

1. Write briefly about objective of smear making.
2. Write about manners of liquid specimen preparation.
3. What are the main staining methods used in cytology
4. Briefly write about the methods of coverslipping.

ANSWERS TO INTEXT QUESTIONS

23.1

1. Papanicolaou
2. Romanowsky
3. Cytocentrifuged
4. Xylene

Notes

24

CYTOLOGY : DISPOSAL OF HUMAN WASTE

24.1 INTRODUCTION

Hospital waste is "Any waste which is generated in the diagnosis, treatment or immunization of human beings or animals or in research" in a hospital. Hospital Waste Management means the management of waste produced by hospitals using such techniques that will help to check the spread of diseases through

OBJECTIVES

After reading this lesson, you will be able to:

- describe various methods of cytology waste disposal
- practice safe disposal of human and chemical waste.

24.2 DISPOSAL OF HUMAN WASTE

The laboratory should conform to the local practices and guidelines for safe disposal of human and chemical waste generated in the laboratory.

Hospital Waste categories and Disposal

Option	Waste Category	Treatment & Disposal
Category 1	Human anatomical waste	Incineration /deep burial
Category 2	Animal waste	Incineration /deep burial
Category 3	Microbiology & biotechnology waste	Incineration /deep burial

Category 4	Sharps	Incineration / disinfection / chemical treatment /mutilation
Category 5	Medicines and cytotoxic drugs	Incineration / destruction and disposal in secured landfill
Category 6	Solid waste (Blood and Body fluids)	Autoclave/chemical treatment/ burial
Category 7	Solid waste (disposable items)	Autoclave/chemical treatment/ burial
Category 8	Liquid waste (blood & body fluids)	Disinfection by chemicals/ discharge into drains
Category 9	Incineration Ash	Disposal in municipal landfill
Category 10	Chemical waste	Chemical treatment/ secure landfill

24.3 WHO MEDICAL WASTE CATEGORIES

Infectious

Materials containing pathogens if exposed can cause disease.

- Human anatomical waste: waste from surgery and autopsies on patients with infectious diseases;
- Sharps: disposable needles, syringes, saws, blades, broken glasses, nails or any other item that could cause a cut;
- Pathological: tissues, organs, body parts, human flesh, fetuses, blood and body fluids;

Non Infectious (Hazardous)

- Pharmaceuticals: drugs and chemicals that are returned from wards, spilled, outdated, contaminated, or are no longer required;
- Radioactive: solids, liquids and gaseous waste contaminated with radioactive substances used in diagnosis and treatment of diseases like toxic goiter.

Non Infectious (Non Hazardous)

- Domestic waste: from the offices, kitchens, rooms, including bed linen, utensils, paper, etc.

Notes

Care needs to be taken to dispose off the Infectious and non-infectious hazardous waste. The non Infectious (Non Hazardous) waste can be disposed off with regular garbage disposal.

Cytology laboratory generates waste in the form of remnants of fluids (peritoneal, pleural, cysts, etc), sputum, and left over specimen of liquid cytology. The specimens need to be discarded only after chemical decontamination using at least 1% sodium hypochlorite solution; and then discharged into drains/ sewers where it is taken care of by the principle of dilution and dispersal.

Any solid waste needs to be disposed off according to hospital waste management. Before disposal the specimen need to be segregated after proper identification.

Segregation by color coding system

Three categories

- Infectious waste - Red bags
- Domestic waste - Green Bags
- Sharps - Needle cutters / Puncture proof containers

Transportation

- Containers: puncture proof, leak proof,
- Bags: sturdy, properly tied
- Transport trolleys: designated & timely
- Staff protection: provided with protective clothing and other items
- Never put hands in a bag

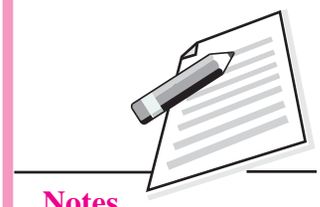
The infectious material in red bags will go for incineration.

The sharps can either go to incinerator or following autoclaving/chemical disinfection can be mutilated. They should never be thrown in regular garbage.

Chemical waste collected throughout the staining, dehydration, clearing and coverslipping processes must be disposed of or recycled according to state and local regulations.

INTEXT QUESTIONS 24.1

1. Specimens before discarding need to be decontaminated with .....
2. Infectious waste is discarded in ..... colour bag
3. Domestic waste is discarded in ..... colour bag
4. Sharps are discarded in ..... container



WHAT HAVE YOU LEARNT

- Hospital waste is "Any waste which is generated in the diagnosis, treatment or immunization of human beings or animals or in research" in a hospital
- Hospital Waste Management means the management of waste produced by hospitals using such techniques that will help to check the spread of diseases through
- WHO categories waste as Infectious, Hazardous Non-infectious and Non-hazardous non-infectious
- The specimens need to be discarded only after chemical decontamination using at least 1% sodium hypochlorite solution; and then discharged into drains/sewers where it is taken care of by the principle of dilution and dispersal
- Any solid waste needs to be disposed off according to hospital waste management. Before disposal the specimen need to be segregated after proper identification.
- Segregation is by color coding system as Infectious waste - Red bags, Domestic waste - Green Bags, Sharps - Needle cutters / Puncture proof containers

TERMINAL QUESTIONS

1. What are the categories of hospital waste?
2. What are the WHO categories of medical waste?
3. What are the main methods of medical waste disposal?
4. What is the color coding for waste disposal?

ANSWERS TO INTEXT QUESTIONS

24.1

1. 1% sodium Hypochlorite
2. Red
3. Green
4. Blue / Puncture resistant

Notes

25

CYTOLOGY : STAINING METHODS

25.1 INTRODUCTION

Consistency and reliability are most important in cytological interpretation. Cytologists rely heavily on the quality and appearance of the stain. The Papanicolaou stain is recommended for the staining of alcohol fixed cytology slides. Romanowsky stains may also be used for wet fixed slides, but are primarily applied to air-dried smears.

Special stains are used as per requirements: Modified Ziehl Neelson (for acid fast bacilli), Gram staining (Bacteria), Mucicarmine (mucins), PAS (for glycogen, fungal wall, lipofuscin, etc), Oil red O (lipids), Perl's Prussian blue (iron), modified Fouchet's test (bilirubin), etc. Recently, immunocytochemistry is also being increasingly used in cytology specimens. These special stains and immunocytochemistry will be discussed along with respective sections in histopathology as the principles and methods remain the same.

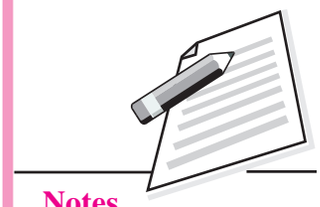
OBJECTIVES

After reading this lesson, you will be able to:

- describe the principle of cytology stains
- explain the methods of staining cytology specimens.

25.2 STAINING IN CYTOLOGY

The universal stain for cytological preparations is the Papanicolaou stain. Harris' hematoxylin is the optimum nuclear stain and the combination of OG6 and EA50 give the subtle range of green, blue and pink hues to the cell cytoplasm.



Papanicolaou stain

Papanicolaou formula

1. Harris' hematoxylin

Hematoxylin	5g
Ethanol	50ml
Potassium alum	100g
Distilled water (50°C)	1000ml
Mercuric oxide	2-5g
Glacial acetic acid	40ml

2. Orange G 6

Orange G (10% aqueous)	50ml
Alcohol	950ml
Phosphotungstic acid	0-15g

3. EA 50

0.04 M light green SF	10ml
0.3M eosin Y	20ml
Phosphotungstic acid	2g
Alcohol	750ml
Methanol	250ml
Glacial acetic acid	20ml

Filter all stains before use.

Original Papanicolaou staining method:

1. 96% ethyl alcohol 15 seconds
2. 70% ethyl alcohol 15 seconds
3. 50% ethyl alcohol 15 seconds
4. Distilled water 15 seconds
5. Harris hematoxylin 6 minutes
6. Distilled water 10 dips
7. Hydrochloric acid 0.5% solution, 1-2 quick dips
8. Distilled water 15 seconds
9. Few dips in 0.1% ammoniated water. The smear turns to blue.

10. 50% ethyl alcohol 15 seconds
11. 70% ethyl alcohol 15 seconds
12. 96% ethyl alcohol 15 seconds
13. OG-6 (orange) 2 minutes
14. 96% ethyl alcohol 10 dips
15. 96% ethyl alcohol 10 dips
16. EA 50 eosin yellowish 3 minutes
17. 96% ethyl alcohol (10 dips)
18. 100% ethyl alcohol (10 dips)
19. Xylene (10 dips)
20. Mount: in DPX using coverslip

Results:

The nuclei should appear blue/black

The cytoplasm (non-keratinising squamous cells) – blue/green

Keratinising cells- pink/orange

Precautions:

1. Use stains only after filtering them
2. Change stains frequently
3. Check staining under microscope for good quality control

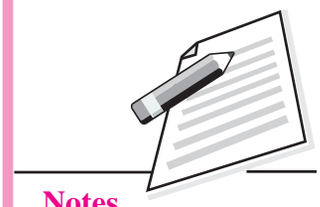
25.3 MAY-GRÜNWALD GIEMSA STAIN

This is one of the common Romanwsky stains used in cytology. It is useful for studying cell morphology in air-dried smears. It is superior to Papanicolaou to study the cytoplasm, granules, vacuoles, basement membrane material etc. For nuclear staining Papanicolaou is superior.

Contents of the staining reagents:

May-Grünwald solution	0.2%
Methanol	99 %
May-Grünwald´s eosin-methylene blue	0.2 %

Contains: Eosin G, Methylene blue



Giemsa solution

Methanol	73 %
Glycerol	26 %
Giemsa´s Azur-Eosin-Methylene blue	0.6 %

Contains: Azur I, Eosin G, Methylene blue

Phosphate buffer

Potassium dihydrogen phosphate/ disodium hydrogen phosphate x $2H_2O$

67.0 mmol/l

Storage

Giemsa solution, May-Grünwald solution: protected from light at 2-25°C.

Unopened reagents may be used until the expiry date on the label.

Phosphate buffer: at 2-8°C. Unopened reagents may be used until the expiry date on the label.

Preparation of working solutions

1. Buffered water: Dilute phosphate buffer with deionised or distilled water 1:20, e.g. 30 ml phosphate buffer + 570 ml deionised or distilled water.
2. Giemsa working solution : Mix 84 ml of Giemsa solution into 516 ml of buffered water.
3. May-Grünwald working solution: Mix 360 ml of May-Grünwald solution into 240 ml of buffered water.

Staining method

1. Fix the air-dried smear specimen in methanol for 10 -20 minutes
2. Stain with May-Grünwald working solution for 5 minutes
3. Stain with Giemsa working solution for 12 minutes
4. Wash with clean buffered water for 2, 5 and 2 minutes
5. Dry the slides in upright position at room temperature
6. Mount the slides with a coverslip using DPX

Any modifications to the staining procedure/working solutions may affect the staining result, and are subject to precise method validation

Sources of errors

Irregular distribution of the blood smear on a glass slide may result in an erroneous cell counts. Alcohols used for wiping the skin may cause hemolysis and artifacts. Do not let the specimens dry at any stage of the staining procedure. Wash properly to avoid dye artifacts. Buffered water is strongly recommended for washing. Staining result is dependent on pH. Alkaline pH increases blue and acidic pH pink or reddish tinge in the stained specimen.

Ziehl-Neelsen stain

Reagents

(1) Carbol Soft Fuchsin

Basic Fuchsin 1 gm

Absolute alcohol 10 ml

Add the basic fuchsin to the alcohol in a 100 ml flask and mix, on a magnetic stirrer for 30 minutes. Add 100ml of 5% aqueous phenol. Mix well. Filter and store in a brown glass bottle.

(2) Acidified Methylene Blue

0.25% methylene blue in 1% acetic alcohol

(3) 0.5% Acid Alcohol

Distiller water 700 ml

Absolute alcohol 300 ml

Hydrochloric acid 5 ml

(6) 5% Sulphuric Acid

Distilled water 475 ml

Sulphuric acid 25 ml

Staining Method

Place fixed slides on the staining rack in serial order, smeared side up. Slides should be separated by a 1 cm gap, and should never touch one another. Cover slides individually with filtered Ziehl's carbol fuchsin working solution. Heat slides from underneath with the flame of a Bunsen burner, an alcohol lamp or an alcohol soaked cotton swab until vapour starts to rise. Staining solution should never be allowed to boil. Do not allow the stain to dry. Keep slides covered with hot, steaming carbolfuchsin for 5 minutes by re-flaming as needed. Rinse slides gently with water to remove excess carbolfuchsin. Drain off excess rinsing water from slides. Sputum smears appear red in colour.

Decolourising: Cover slides with 25% sulfuric acid or acid-alcohol solution and allow to stand for 3 minutes, after which the red colour should have almost completely disappeared. If needed, repeat sequence until the red colour disappears, but do not overdecolourise. Gently wash away the sulfuric acid or acid alcohol and the excess stain with water. Drain off excess rinsing water from slides.

Counterstaining: Cover slides individually with 0.3% methylene blue counterstaining solution and allow to stand for 1 minute. Rinse slides individually with water. Drain water off the slides, which are then allowed to air dry.

A properly stained smear should show a light blue colour due to methylene blue.

Results: Tubercle bacilli, hair shafts, Actinomyces, some fungal elements- red.

Background: pale blue.

INTEXT QUESTIONS 25.1

1. ..... stain is recommended for stains of alcohol fixed cytology
2. ..... stain is used for wet fixed slides
3. ..... stain is used for identification of Glycogen, Fungal Wall
4. ..... stain is used for identification of lipids
5. ..... test is used for identification of Bilirubin
6. The universal stain for cytological preparations is the .....

WHAT HAVE YOU LEARNT

- Consistency and reliability are most important in cytological interpretation. Cytologists rely heavily on the quality and appearance of the stain.
- Special stains such as Modified Ziehl Neelson for acid fast bacilli, Gram staining for Bacteria, Mucicarmine for mucins, PAS for glycogen, fungal wall, lipofuscin, Oil red O for lipids, Perl's Prussian blue for iron, modified Fouchet's test for bilirubin
- The universal stain for cytological preparations is the Papanicolaou stain. Harris' hematoxylin is the optimum nuclear stain
- May-Grünwald Giemsa Stain is one of the common Romanwsky stains used in cytology. It is useful for studying cell morphology in air-dried smears.

- Irregular distribution of the blood smear on a glass slide may result in an erroneous cell counts. Alcohols used for wiping the skin may cause hemolysis and artifacts

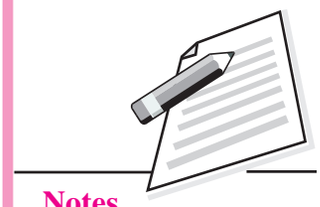
TERMINAL QUESTIONS

1. What are the various stains used commonly in cytology.
2. Write the basic principle of Papanicolaou staining.
3. What cell components are better seen in MGG staining?
4. Name the sources of error in Papanicolaou & MGG staining.
5. Enumerate the substances that get stained red with Ziehl Neelsen staining.

ANSWERS TO INTEXT QUESTIONS

25.1

1. Papanicolaou stain
2. Romanowsky
3. PAS
4. Oil red O
5. Modified Fouchet's
6. Papanicolaou stain



26

CYTOLOGIC SCREENING

26.1 INTRODUCTION

Screening of diseases gained significance in medicine at the end of the nineteenth century, when public health authorities emphasized the importance of screening methods for certain diseases. In 1941, George Papanicolaou demonstrated a test for the early detection of cervical cancer, contributing toward the creation of screening programs. Prevention and early diagnosis are major factors in reducing morbidity and mortality resulting from neoplasia. Screening of diseases involves a test or examination that can detect the existence of a particular disease in a high-risk population, asymptomatic or with minimum symptoms of the disease.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the basics of cytologic screening
- explain the steps involved in cervical cancer screening.

26.2 CYTOLOGICAL SCREENING

Screening of a particular disease requires a precise test, easy to do, at a low cost, and the capability of detecting the presence of a lesion. Cancer of the uterine cervix is an important cause of morbidity and mortality among women worldwide and a leading public health problem. It is the second most common cancer in women, but the most common in developing countries. Because of the phases that precede the lesion in the natural progress of invasive cervical cancer, and because they can be easily discovered and treated, the disease is well suited

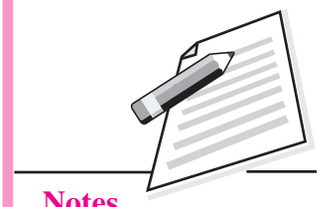
Notes

to screening programs. The Papanicolaou test is an established method for examining the cells collected from the cervix to determine whether they show signs of pre-neoplastic differentiation. Cytologic screening programs have led to a large decline in cervical cancer incidence and mortality in developed countries. However, cervical cancer remains largely uncontrolled in high risk developing countries because of ineffective or no screening.

Cervical cancer can be avoided when there is an early diagnosis of the precursor lesions, without local or systemic compromise. Among the methods available for early detection of cervical cancer, exfoliative cytology, or the Pap test, is recommended worldwide for mass screening, because the efficacy in the detection of premalignant lesions, associated with the social role of the method, permits minimization of costs with curative medicine.

The basic integrated actions include: (1) care with collection, (2) processing of the smears, (3) screening and interpretation of the specimens, (4) follow-up of the patients, and (5) quality control.

1. **Care with collection**: The majority of false-negatives arise from problems with collection of specimens, and for this reason this stage should be systemized and there should be training and recycling of the personnel responsible for taking the samples. The smears must be well identified, slim, uniform, and without contaminants, and contain samples from the transformation zone, where in the majority of cases the cervical cancer develops. There should be a minimum of blood, mucus, or other obscuring material such as lubricating gel. It is also important at this moment to adequately fix the material so as not to compromise subsequent stages.
2. **Processing the specimens**: One of the characteristics of the Pap test is that it consists of various stages. Each stage should be monitored so as to minimize the possibility of error. The condition on arrival of the slides, and the number of slides per case, must be verified. Special care should be taken with the flow of the tests, with adequate numbering and balanced coloration with control of the number of cases colored in each set. The end product of this stage will be fundamental to a good result with the rest.
3. **Screening and interpretation of the specimens**: The screening should be done in as little time as possible, depending on the basic requirements of each program, by trained and qualified personnel. Care should also be taken with excess workloads for cytopathologists and cytotechnicians, and also with refresher courses and recycling. The report on the tests should be systemized and use a unique nomenclature, of which all involved in the preparation and interpretation of the results should be fully aware.



4. **Follow-up of patients**: Mere detection of the lesions will not determine the impact on the natural history of the disease. For this reason the treatment of lesions in a pre-invasive stage is fundamental.

5. **Quality control:** Quality is fundamental in gynecological cytopathology. One of the greatest problems in mass cytology is the false-negative cases. Cytopathology labs must have mechanisms for internal quality control with the objective of avoiding false-negative and false-positive tests. External quality control must be included in the design of the prevention program.

26.3 THE ROLE OF LABORATORY IN SCREENING PROGRAMS

The laboratory can make an important contribution to the structuring and organization of cervical screening programs based on the Papanicolaou test. The lab, when integrated into a screening program, should have among its objectives top quality production, training, and updating of personnel and the guarantee of a secure place of work, where risk factors are under control and the environment is protected.

The system of internal monitoring of laboratory quality includes a set of actions, which should be developed and disseminated in a coordinated way, involving the various stages in the work process, from collecting a sample to issuing the report. The system aims to accompany and evaluate the cyto- and histopathologic diagnostic procedures in the laboratories, thus helping to determine areas where improvements can be planned and implemented, and also evaluate the impact of these actions and the incorporation of new practices.

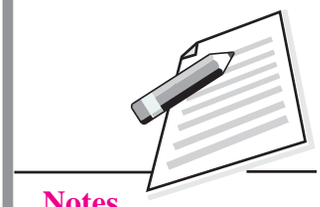
The majority of cervical cancer occurs in developing countries. The success of cervical cancer screening is shown by its ability to reduce the incidence of cervical cancer and the resulting mortality. The integration of procedures is essential for a successful screening program. Recently new technologies for alternative and complementary forms of screening such as liquid-based cytology and automated cytology have been proposed. A combination of methods has been proposed in an attempt to improve the sensibility of the Pap test. Among these, the association of cytology with the molecular test for HPV using hybrid capture has been highlighted. Automated cytology may be used for the purpose of reducing human errors caused by human fatigue, and to detect lesions with a lesser number of abnormal cells in the sample. HPV vaccine will be an additional tool in the strategies to reduce morbidity and mortality from cervical cancer and will be a component of a comprehensive strategy with the long-term goal of eliminating the disease. Cytologic screening can also be performed in selected high-risk populations for lung, esophageal, and bladder cancer.

WHAT HAVE YOU LEARNT

- Screening of diseases gained significance in medicine at the end of the nineteenth century
- George Papanicolaou demonstrated a test for the early detection of cervical cancer, contributing toward the creation of screening programs
- Prevention and early diagnosis are major factors in reducing morbidity and mortality resulting from neoplasia
- Screening of diseases involves a test or examination that can detect the existence of a particular disease in a high-risk population, asymptomatic or with minimum symptoms of the disease
- The Papanicolaou test is an established method for examining the cells collected from the cervix to determine whether they show signs of pre-neoplastic differentiation
- The smears must be well identified, slim, uniform, and without contaminants, and contain samples from the transformation zone, where in the majority of cases the cervical cancer develops
- The screening should be done in as little time as possible
- A combination of methods has been proposed in an attempt to improve the sensibility of the Pap test

TERMINAL QUESTIONS

1. What is meant by screening for a disease?
2. Name few common diseases for which cytology can be used as a tool.
3. What are the actions involved in pap smear screening program?



Notes

27

QUALITY CONTROL IN CYTOLOGY

27.1 INTRODUCTION

Cytopathologists are concerned about and committed to quality assurance and quality control in their laboratories. These practices include, among others, the use of intralaboratory and extradepartmental consultations, case reviews, correlation of cytologic and histopathologic specimens and review of completed diagnostic reports.

OBJECTIVES

After reading this lesson, you will be able to:

- describe Quality control in cytology
- explain various methods of quality control in cytology.

27.2 QUALITY ASSURANCE MEASURES

Cytopathology is a practice of medicine and represents a medical consultation, in both gynecologic and nongynecologic anatomic sites. The basic principles of quality assurance apply to all types of cytologic specimens. The following represents several minimum quality assurance measures.

1. Laboratory Directors

The laboratory should be directed by a legally qualified physician with a specialist qualification in pathology, including special training and expertise in cytopathology. The director or designated medical professional is responsible for proper performance and reporting of all tests done in the cytopathology laboratory. The director or designated cytopathologist should

be physically present in the laboratory to direct the staff, be available for consultations, review all reactive and abnormal gynecologic cytology samples, review fine needle aspiration samples, and review all nongynecologic samples.

2. Cytotechnologists

A suitably qualified person should be recruited for this position.

3. Physical Laboratory Facilities

The laboratory should be clean, well lighted, adequately ventilated, and functionally arranged so as to minimize problems in specimen handling, evaluation, and reporting. The area for specimen preparation and handling should be separate from the area where specimens are evaluated and reported. Formaldehyde and xylene (if in use) should be carefully monitored due to the possible presence of hazardous vapor concentrations.

4. Safety Precautions

Laboratory personnel must be protected against hazards (chemical, electric, fire, infections, or others) by using well-ventilated hoods and biologic safety hoods for handling potentially infectious material. Fire precautions should be posted and tested.

5. Equipment

An adequate number of binocular microscopes of good quality and proper working order must be available. Laboratory instruments and equipment should be under periodic maintenance to monitor and ensure malfunctions do not adversely affect analytical results.

6. Specimen Collection

Cytologic specimens should be accepted and examined only if requested by a licensed medical practitioner and collected in accordance with instructions regarding recommended collection techniques. The cytopathology laboratory should inform the originator of the sample if the specimens are "unsatisfactory" and detail adequacy qualifiers such as presence or absence of a transformation zone component or obscuring factors in "satisfactory samples".

7. Preparation, Fixation, and Staining Procedures

The specimens must be identified with the patient's name and/ or a unique identifier and must be accompanied by a requisition form with the requesting physician's name, address, date of specimen collection, specimen source, and appropriate clinical information about the patient. When the specimen arrives in the laboratory the laboratory staff affix an accession number or bar code label on each slide for further identification. The

laboratory should have written criteria for rejecting specimens. Fixation while the specimen is still wet is recommended for conventional cell samples. The Papanicolaou staining procedure is strongly suggested for most cytologic samples, unless additional staining procedures are warranted. Staining solutions and chemicals used in the cytopathology laboratory should be labeled with the time of preparation, purchase, or both. Staining solutions should be filtered regularly to avoid contamination and should be covered when not in use. Effective measures to prevent cross-contamination between gynecologic and nongynecologic specimens during the staining process must be used.

8. Slide Evaluation Workload

Regulations as to the number of specimens a cytotechnologist may evaluate in a 24-hour period are currently set at 100 slides per an 8-hour day. This regulation may not do justice to the various conditions that influence the quality of the slide evaluation performance. The percentage of atypical cases evaluated versus the percentage of negative cases in varying populations as well as screening of nongynecologic specimens should be considered when workloads are established. This regulation ensures that the number and type of cytologic samples evaluated do not, through fatigue, adversely affect the cytotechnologist's performance.

9. Cytologic Terminology

The vaginal/ectocervical/endocervical cytology sample should be interpreted preferably by using the Bethesda System. The nongynecologic material should be interpreted in medical terms.

10. Laboratory Records, Logs, and Files

Each specimen should be recorded and a sequential accession number assigned together with the name of the patient and the originator of the sample. Test records must be retained for at least 5-10 years. The negative gynecologic cell samples should be retained on file for a minimum of 5 years and negative fine needle aspirates for 10 years or indefinitely if they exhibit abnormal features. The modern cytopathology laboratory should use a computerized file system.

INTEXT QUESTIONS 27.1

1. For conventional cell samples ..... is recommended while the specimen is wet.
2. ..... staining procedure is suggested for most cytologic samples

Notes

3. cytotechnologist may evaluate in a 24-hour period ..... number of slides per an 8-hour day
4. The vaginal cytology sample should be interpreted using ..... System.
5. Test records must be retained for at least ..... years
6. Negative fine needle aspirates should be retained for ..... years

WHAT HAVE YOU LEARNT

- Quality control practices include, the use of intralaboratory and extradepartmental consultations, case reviews, correlation of cytologic and histopathologic specimens and review of completed diagnostic reports.
- The laboratory should be directed by a legally qualified physician with a specialist qualification in pathology, including special training and expertise in cytopathology
- A suitably qualified person should be recruited as cytotechnologists
- The laboratory should be clean, well lighted, adequately ventilated, and functionally arranged so as to minimize problems in specimen handling, evaluation, and reporting
- Laboratory personnel must be protected against hazards
- An adequate number of binocular microscopes of good quality and proper working order must be available
- Cytologic specimens should be accepted and examined only if requested by a licensed medical practitioner and collected in accordance with instructions regarding recommended collection techniques
- The specimen as it arrives in the laboratory should be given an accession number or bar code label on each slide for further identification.
- The laboratory should have written criteria for rejecting specimens.
- Fixation while the specimen is still wet is recommended for conventional cell samples.
- The Papanicolaou staining procedure is strongly suggested for most cytologic samples, unless additional staining procedures are warranted.
- Staining solutions and chemicals used in the cytopathology laboratory should be labeled with the time of preparation, purchase, or both

- Regulations as to the number of specimens a cytotechnologist may evaluate in a 24-hour period are currently set at 100 slides per an 8-hour day
- The vaginal/ectocervical/endocervical cytology sample should be interpreted preferably by using the Bethesda System.
- The nongynecologic material should be interpreted in medical terms.
- Test records must be retained for at least 5-10 years.
- The negative gynecologic cell samples should be retained on file for a minimum of 5 years and negative fine needle aspirates for 10 years

TERMINAL QUESTIONS

1. Write briefly about objective of quality assurance in cytopathology.
2. Enumerate the various measures of quality assurance.
3. How long do you need to maintain the records of cytopathology specimens?

ANSWERS TO INTEXT QUESTIONS

27.1

1. Fixation
2. Papanicolaou
3. 100
4. Bethesda
5. 5-10
6. 10

Notes

28

CYTOMORPHOLOGY

28.1 INTRODUCTION

Light microscopic examination of stained cells in smears is the method of choice of diagnostic cytology. It allows classification of most normal cells as to type and tissue of origin. It also allows the recognition of cell changes caused by disease processes.

OBJECTIVES

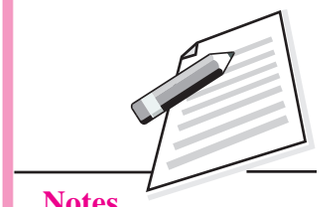
After reading this lesson, you will be able to:

- recognize and classify cells
- identify features cell response to injury
- recognize features of tumors, especially malignancy.

28.2 GENERAL GUIDELINES

The study of cells in smears should take place at several levels:

- A rapid review of the smear with a 10× objective provides information on the makeup of the sample and its cell content. This preliminary review will tell the observer whether the smear is appropriately fixed and stained and will provide initial information on its composition. Smears containing only blood or no cells at all are usually considered inadequate, with some very rare exceptions.
- If the smear contains cells other then blood cells, it should be examined with care. A careful review of the material or screening of smears with a 10× objective is usually required to identify abnormal cells that may be few



in number. Screening is mandatory in cancer detection samples from "well" patients. A microscope stage should be utilized.

- The screening of the smear should lead to the preliminary assessment of the sample and answer the following questions: (1) Does the cell population correspond to the organ of origin? (2) If the answer is positive, the next question pertains to the status of the cell population: (a) is it normal? (b) does it show nonspecific abnormalities of little consequence to the patient? or (c) Does it show abnormalities pertaining to a recognizable disease state that can be identified.

28.3 CELL CLASSIFICATION

In general, the derivation, type of cells, and sometimes their function, are reflected in the cytoplasm, whereas the nucleus offers information on the status of the DNA, which is of particular value in the diagnosis of cancer. Some cells that lack distinct cytoplasmic or nuclear features may be very difficult to classify. For all practical purposes, the cells encountered in cytologic samples are of epithelial and nonepithelial origin. In tissue sections, the cells are often cut "on edge" and are seen in profiles. In cytologic preparations, the cells are whole and are generally flattened on a glass slide, usually affording a much better analysis of the cell components.

Epithelial Cells

An epithelium (plural: epithelia) is a tissue lining the surfaces of organs or forming glands and gland-like structures. Similar epithelia may occur in various organs and organ systems. There are four principal groups of epithelia: (1) squamous epithelia, synonymous with protective function; (2) glandular epithelia with secretory functions; (3) ciliated epithelia; and (4) the mesothelia.

Squamous Epithelium

The squamous epithelium is a multilayered epithelium that lines the surfaces of organs that are in direct contact with the external environment. The growth of the squamous epithelium is in the direction of the surface, that is, the cells move from the basal layer, to parabasal layers, to intermediate layers, to superficial layers. The most superficial cells are cast off. As the cells transit from the basal to the more superficial layers, they are programmed to gradually increase the size of their cytoplasm. The process of cytoplasmic maturation is accompanied by nuclear changes. The nuclei of the basal, parabasal, and intermediate layers of squamous cells appears as spherical, open (vesicular) structures, measuring approximately 8 μm in diameter. As the cells transit from the intermediate to

Notes

superficial layers, their nuclei shrink and become condensed (nuclear pyknosis). In cytology, these cells tend to be flat, polygonal, and sharply demarcated, and they vary in size according to the layer of origin. The smallest cells, measuring about 10 μm in diameter, are the basal cells, which are very rarely seen in normal states. Parabasal cells, derived from the parabasal layers, are somewhat larger, measuring from 10 to 15 μm in diameter. Intermediate cells, derived from the intermediate layers, are still larger, measuring from 15 to 40 μm in diameter. The superficial cells are the largest, measuring from 40 to 60 μm in diameter.

Epithelia with Secretory Function

These epithelia are found mainly in organs with secretory functions and exchanges with the external environment, such as food intake, principally in the digestive tract and associated glands. On cytology, the secretory cells are cuboidal or columnar in shape, averaging from 10 to 20 μm in length and 10 μm in width. Their cytoplasm is transparent because of accumulation of products of secretion, usually mucus. Secretory cells are often polarized, that is, they display one flat surface facing the lumen of the organ.

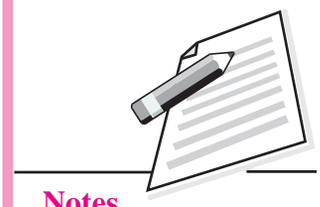
Mesothelia

Organs contained within body cavities, such as the lung, the heart, and the intestine, are all enclosed within protective sacs lined by specialized epithelia of mesodermal origin. These sacs, known as the pericardium for the heart, pleural cavity for the lungs, and peritoneal cavity for the intestine, are lined by an epithelium composed of a single layer of flat cells, known as mesothelial cells. Under normal circumstances, the sacs are filled with only a thin layer of fluid that facilitates the gliding of the two surfaces of mesothelial cells against each other. On cytology, mesothelial cells may form sheets or clusters, in which the adjacent, flattened surfaces of the cells are separated from each other by clear gaps ("windows") filled by microvilli.

Nonepithelial Cells

Endothelial Cells: they line the intima of blood vessels and have many similarities with mesothelial cells but are very rarely observed in diagnostic cytology.

The Immune Cell System: Consist of T & B Lymphocytes, macrophages and plasma cells. Many benign and malignant conditions involving proliferation of these cells can present for cytological evaluation. (Leukemias, lymphomas, multiple myeloma).



INTEXT QUESTIONS 28.1

1. ..... is a tissue lining the surfaces of organs or forming glands and gland-like structures
2. ..... is a multilayered epithelium that lines the surfaces of organs that are in direct contact with the external environment
3. ..... are found mainly in organs of the digestive tract and associated glands
4. The pericardium, pleural cavity and peritoneal cavity, are lined by .....
5. ..... Cells line the intima of blood vessels

WHAT HAVE YOU LEARNT

- An epithelium is a tissue lining the surfaces of organs or forming glands and gland-like structures
- There are four principal groups of epithelia: (1) squamous epithelia (2) glandular epithelia with secretory functions; (3) ciliated epithelia; and (4) the mesothelia
- The squamous epithelium is a multilayered epithelium that lines the surfaces of organs that are in direct contact with the external environment
- The nuclei of the basal, parabasal, and intermediate layers of squamous cells appears as spherical, open structures, measuring approximately 8 μm in diameter
- The smallest cells, measuring about 10 μm in diameter, are the basal cells, which are very rarely seen in normal states
- Parabasal cells, derived from the parabasal layers, measure from 10 to 15 μm in diameter
- Intermediate cells, derived from the intermediate layers, measure 15 to 40 μm in diameter.
- The superficial cells are the largest, measuring from 40 to 60 μm in diameter
- Epithelia are found mainly in organs with secretory functions and exchanges with the external environment, such as food intake, principally in the digestive tract and associated glands

- On cytology, the secretory cells are cuboidal or columnar in shape, averaging from 10 to 20 μm in length and 10 μm in width. Their cytoplasm is transparent because of accumulation of products of secretion, usually mucus.
- The pericardium, pleural and peritoneal cavity, are lined by an epithelium composed of a single layer of flat cells, known as mesothelial cells
- In cytology, mesothelial cells form sheets or clusters, in which the adjacent, flattened surfaces of the cells are separated from each other by clear gaps ("windows") filled by microvilli
- Endothelial Cells line the intima of blood vessels

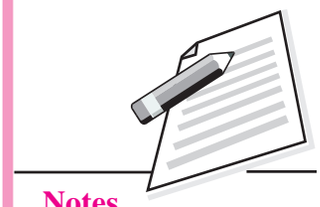
TERMINAL QUESTIONS

1. What are the general guidelines while assessing a cytological smear?
2. Enumerate the different types of cells which can be encountered while examining a cytologic smear.
3. What are the different forms of squamous cells seen in cytology?

ANSWERS TO INTEXT QUESTIONS

28.1

1. Epithelium
2. Squamous epithelium
3. Epithelia
4. Mesothelial cells
5. Endothelial



29

HORMONAL ASSESSMENT

29.1 INTRODUCTION

The established approach to the evaluation of ovarian function and endocrine disorders in the woman is based on serial biochemical analyses of hormones, such as estrogen, progesterone, luteinizing hormones and their metabolites. In women who suffer from menstrual disorders and abnormalities of the ovarian cycle, the biochemical analyses can be effectively supplemented by the old-fashioned endometrial biopsies, or studies of endocervical mucus. In addition, the cervicovaginal smear may sometimes provide useful information and has the advantage of being easy to obtain, rapidly evaluated, and inexpensive. The cytologic approach is particularly valuable if laboratories specializing in endocrine analysis are not readily available. The principle of the cytologic hormonal analysis is simple. The degree of maturation of the squamous epithelium of the female genital tract depends on steroid hormones, mainly estrogen.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the basics of hormonal assessment
- identify the features of squamous cell maturation.

29.2 HORMONAL ASSESSMENT

Naturally occurring estrogen, or the parenteral administration of estrogen or its natural or synthetic substitutes in adequate amounts, produces a rapid and complete maturation of the normal squamous epithelium of the female genital tract with a resulting preponderance of mature superficial squamous cells in smears. The effect takes place regardless of the prior hormonal status, except

Notes

during pregnancy. Conversely, complete atrophy of the squamous epithelium of the vagina and cervix may be equated with complete absence of estrogenic activity. However, there are no reliable data linking intermediate degrees of maturation of the squamous epithelium with the action of a specific hormone or hormones.

Evaluation of the endocrine status of a menstruating woman during the childbearing age belongs among the most difficult tasks in diagnostic cytology. There is considerable variation in the smear patterns from one patient to another, even if matched for age and menstrual history.

Several conditions must be fulfilled before a successful hormonal evaluation of the squamous epithelium may be undertaken.

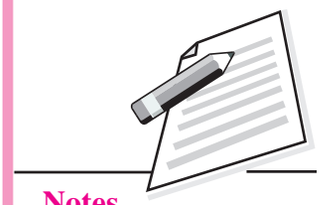
- There must be absence of inflammation or cytolysis.
- There must be no recent medication, either topical or systemic, especially with compounds known to affect the squamous epithelium of the lower genital tract.
- There must be no history of radiotherapy or recent surgery to the vagina or cervix.
- An adequate baseline investigation must have been performed in menstruating women. This should include daily smears during at least one and preferably two complete cycles, or their chronologic equivalent. In nonmenstruating patients, two or three smears may suffice.
- The smears should be obtained from the proximal portion of the lateral wall of the vagina, care being taken to avoid contamination with material from the adjacent cervix.

The Karyopyknotic Index (KI)

The karyopyknotic index expresses the percentile relationship of superficial squamous cells with pyknotic nuclei to all mature squamous cells. Usually, 200 to 400 consecutive cells in three or four different fields on the smear are evaluated. The peak of KI usually coincides with the time of ovulation and was estimated at 50% to 85% of total cells.

The Eosinophilic Index (EI)

The eosinophilic index expresses the percentile relationship of mature squamous cells with eosinophilic cytoplasm to all mature squamous cells, regardless of the status of the nucleus. In a normal menstruating woman, the peak of EI coincides with the peak of KI and may reach 50% to 75% at the time of ovulation.



The Maturation Index (MI)

The maturation index expresses the maturation of the squamous epithelium as a percentile relationship of parabasal cells to intermediate cells to superficial cells. The count should be performed on single cells. For example, in a normal menstruating woman at the time of ovulation, an MI of 0:35:65 would indicate that the smear contained no parabasal cells, 35% of intermediate cells, and 65% of superficial cells.

Other Indices

The folded-cell index represents the relationship of mature superficial or intermediate squamous cells with folded cytoplasm to all mature squamous cells. The crowded-cell index represents the relationship of mature squamous cells lying in clusters of four or more cells to all mature squamous cells.

Alternative Ways of Reporting Hormonal Status

It has been a common practice to base the evaluation of the maturation of the squamous epithelium on an overall visual impression gained during the routine screening of smears. This simplest of methods has not failed in revealing major abnormalities of smear patterns. By comparing the current smear pattern with original baseline smears, a good appreciation of changes in smear pattern may be gained. Small variations in smear pattern have no diagnostic meaning but may strongly influence the indices and thus give a false impression of hormonal "effects." The reporting of smears based on this overall visual impression is always given in reference to age, menstrual history, and possible clinical significance. Some examples follow:

Patient age 35: "Midcycle smear pattern—consistent with functioning ovaries."

Patient age 52: "Absence of maturation of squamous cells consistent with menopause."

Patient age 25: "Absence of maturation of squamous cells—abnormal for age."

Patient age 60: "High level of maturation of squamous cells not consistent with clinical menopause. It is assumed that this patient is not receiving estrogens or other drugs that may account for this smear pattern."

29.3 DETERMINATION OF THE TIME OF OVULATION FROM CERVICOVAGINAL SMEARS

A precise determination of the time of the ovulation is important in artificial insemination and in in-vitro fertilization. The use of the cervicovaginal smears

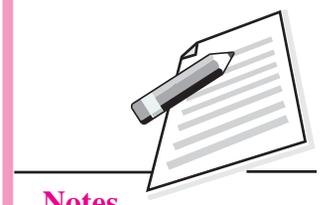
to establish the time of ovulation or the status of the endometrium has been of limited reliability. It is recommended that cytologic methods for estimation of ovulation or status of the endometrium be supplemented by other procedures, such as temperature curves and endometrial biopsies. The examination of endocervical mucus may also be of assistance. Cyclic changes in the physicochemical properties of the cervical mucus have been known for a great many years. Prior to ovulation, the mucus tends to be viscous and when placed on a glass slide, form crystalline, fern-like structures, whereas at the time of and after ovulation, the mucus is more liquid and does not crystallize.

Cytologic evaluation for menstrual abnormalities

1. Cytologic hormonal evaluation may be of assistance in the evaluation of amenorrhea (cessation of menses), in women who have never menstruated (primary amenorrhea) or who stopped menstruating at a young age after a period of normal menses (secondary amenorrhea).
2. Effects of Castration: The effects of castration, either surgical or radiation induced, may be conveniently followed by vaginal smears.
3. Ovarian Tumors: Certain ovarian tumors, particularly the granulosa cell tumors and the thecomas, may produce estrogen-like substances
4. Precocious Puberty in Girls

INTEXT QUESTIONS 29.1

1. The degree of maturation of the squamous epithelium of the female genital tract depends on ..... hormones.
2. ..... index expresses the percentile relationship of superficial squamous cells with pyknotic nuclei to all mature squamous cells
3. ..... index expresses the percentile relationship of mature squamous cells with eosinophilic cytoplasm to all mature squamous cells
4. ..... index expresses the maturation of the squamous epithelium as a percentile relationship of parabasal cells to intermediate cells to superficial cells
5. ..... index represents the relationship of mature superficial or intermediate squamous cells with folded cytoplasm to all mature squamous cells
6. ..... index represents the relationship of mature squamous cells lying in clusters of four or more cells to all mature squamous cells



WHAT HAVE YOU LEARNT

- The evaluation of ovarian function and endocrine disorders in the woman is based on serial biochemical analyses of hormones, such as estrogen, progesterone, luteinizing hormones and their metabolites
- The degree of maturation of the squamous epithelium of the female genital tract depends on steroid hormones, mainly estrogen.
- The smears should be obtained from the proximal portion of the lateral wall of the vagina
- The karyopyknotic index (KI) expresses the percentile relationship of superficial squamous cells with pyknotic nuclei to all mature squamous cells
- The peak of KI usually coincides with the time of ovulation and was estimated at 50% to 85% of total cells.
- The eosinophilic index expresses the percentile relationship of mature squamous cells with eosinophilic cytoplasm to all mature squamous cells, regardless of the status of the nucleus
- In a normal menstruating woman, the peak of EI coincides with the peak of KI and may reach 50% to 75% at the time of ovulation
- The maturation index expresses the maturation of the squamous epithelium as a percentile relationship of parabasal cells to intermediate cells to superficial cells
- The folded-cell index represents the relationship of mature superficial or intermediate squamous cells with folded cytoplasm to all mature squamous cells
- The crowded-cell index represents the relationship of mature squamous cells lying in clusters of four or more cells to all mature squamous cells
- Cytologic hormonal evaluation may be of assistance in the evaluation of amenorrhea

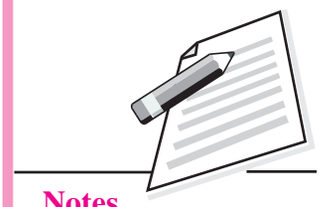
TERMINAL QUESTIONS

1. What are the conditions requiring cytologic hormonal assessment?
2. Enumerate the various methods of hormonal assessment on cytologic smears.
3. Enumerate the precautions required before taking a sample for hormonal assessment of cytologic smears

ANSWERS TO INTEXT QUESTIONS

29.1

1. Estrogen
2. Karyopyknotic index
3. Eosinophilic
4. Maturation
5. Folded-cell
6. Crowded-cell



Notes

30

FINE NEEDLE ASPIRATION CYTOLOGY

30.1 INTRODUCTION

The use of fine-needle aspiration (FNA), a method of aspiration biopsy cytology, continues to grow throughout the World. Improvements in imaging, computed tomography scan (CT), and ultrasound (USG) have fueled the growth of FNA among both radiologists and clinicians. The dominant clinical sites for FNA still remain breast, thyroid, and lymph nodes among superficial tissues.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the techniques of fine needle aspiration cytology
- arrange the clinic for performing FNAC
- assist the pathologist in performing FNAC
- make smears and collect any fluids obtained from FNAC and process appropriately.

30.2 CLINICAL SKILLS REQUIRED

Aspiration biopsy may be indicated whenever there is a palpable tumor mass or a lesion visualized within any organ. For the physician or more specifically for the pathologist performing FNA, some familiarity with general anatomy is essential. For the physician or more specifically for the pathologist performing FNA, some familiarity with general anatomy is essential. For the pathologist performing this biopsy some sharpening of clinical skills, both obtaining a

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focused clinical history and performing a physical examination are required. Clinicians performing aspiration biopsy obviously lack this essential ingredient of experience and knowledge of morphology. Despite the recognized participation and value of cytotechnologists to an aspiration biopsy service, the pathologist must be actively involved in the aspiration biopsy, making both the initial and final evaluation of the smears.

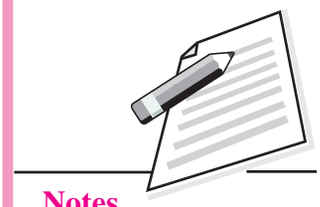
The Thin-needle Aspiration Method

Thin needle generally 22, 23, 25, and 27 gauge, are used for the performance of aspiration biopsy, most often 1.5 in. in length. Special situations may dictate shorter needles and even higher gauge. For example, the very small cutaneous metastasis of breast carcinoma on the chest wall may be sampled more easily with a 27-gauge, 1-in. or even ½-in. needle and with a small, 3.0- to 5.0-mL syringe, approaching the nodule in a plane perpendicular to the skin surface, in the manner of performing a tuberculin skin test. Radiologists most often use the Chiba needle of 21 and 22 gauge for transthoracic and transabdominal aspirations. If one employs only the thin-needle technique, there are virtually no complications, the exceptions being FNA of the thorax (pneumothorax) or some cases of excessive bleeding with transabdominal aspiration biopsy.

Basic Equipment

The basic equipment used for rapid and efficient performance of thin-needle aspiration biopsy are as follows.

1. Cameco Syringe Pistol, Aspir-Gun, or other type aspiration handle;
2. 10 or 20-mL disposable plastic syringe with LuerLok or straight tip, depending on aspiration gun handle size;
3. 22 to 27-gauge, 0.6- to 1.0-mm external diameter disposable needles, 3.8 and 8.8 cm, 15 and 20 cm long, with or without stylus; the needle hub should be clear;
4. Alcohol skin preparation sponges; betadine skin sponges for deeper aspirations, transabdominal, transthoracic, bone (where the cortex is not intact or the periosteum is elevated), or deep soft tissue;
5. Sterile gauze pads;
6. Microscopic glass slides with frosted ends;
7. Small vial of balanced salt solution and/or RPMI tissue culture transport media;
8. Suitable alcohol spray fixatives for immediate fixation of wet smears



9. 10 or 20 mL capped tube with 10% neutral buffered formalin for cell-block
10. Optional vial of local anesthesia, 1-2% lidocaine; topical spray anesthesia for aspirates in children or intraoral aspirates; vials of lidocaine that dentists use for local anesthesia and the dispensing equipment may be useful;
11. Small vial of buffered glutaraldehyde for fixing aspirate for electron microscopy if required or anticipated.

A small plastic tray easily holds all the equipment. Majority of the smears are to be air-dried and later stained with a Romanowsky method, the Diff-Quik stain being preferred. Some smears are usually wet-fixed in 95% ethyl alcohol.

Aspiration Technique

To be successful with an aspiration biopsy, it is important to follow the preliminary steps listed here:

1. Review the history of the patient. Determine the clinical problem and its relevance to the lesion to be biopsied.
2. Determine whether the biopsy is justified.
3. Palpate the mass, attempting to determine its location in relation to surrounding structures. Estimate its depth. Decide on the optimal direction of the needle to accomplish the aspiration biopsy. A mass located deeply in tissue in usually best approached perpendicularly to the skin surface. Small and superficially lying tumors are best approached by penetrating the skin at or very close to a horizontal plane, then feeling for the mass with the needle tip.
4. The patient should be placed in a comfortable position for the aspiration biopsy, but the mass must be easily palpable and immobilized during the biopsy.

   Step 4 is very important for head and neck lesions. The prominence of an enlarged lymph node, or lump, may sometimes depend on whether the patient is supine or erect. The sternocleidomastoid muscle bulk and its close proximity to the cervical lymph nodes require positioning the patient such that the biopsy needle passes through only a minimum of soft tissue and muscle before reaching the target. Avoid aspirating a mass by traversing the sternocleidomastoid muscle. For the aspiration of thyroid lesions, it is usually helpful to place a small pillow under the patient's upper back, extending the neck with the head tilted back.
5. Take time to examine the patient thoroughly. Discuss your preliminary assessment of the patient's lesion. This is an opportunity to describe what

Notes

will take place during the aspiration and what is to be accomplished with it.

6. Obtain informed consent. This consent should indicate that name of the patient who is having the aspiration, the name of the doctor performing the aspiration and a listing of discussed complications.

30.3 PERFORMING THE FNAC

It is essential that the FNAC be performed by a doctor who has knowledge of anatomical structures and pathological lesions expected in the particular region.

Smear Preparation

1. Immediately after completing the aspiration biopsy, the needle should be quickly removed from the syringe; pulled back on the syringe pistol to fill the syringe with air.
2. The needle should be reattached and placed near the center and touching the surface of a plain glass slide.
3. Advancing the plunger of the syringe, will express a small drop of the sample, approximately 2–3 mm in diameter, onto the slide.
4. This procedure should be quickly continued over a series of five to six slides.
5. Invert another plain glass slide over the drop; as it spreads from just the weight of the slide, pull the two slides apart horizontally in a single gentle motion.
6. As an alternative, when the drop spreads in a circular fashion, again from the weight of the slide, pull the two slides apart vertically (compression or pop smears).
7. Repeat the above procedure for all slides; fix some of the slides immediately in 95% ethyl alcohol, or other suitable fixatives, depending on stain preferences, as you make each smear.
8. Allow unfixed smears to air-dry.

Smears are appropriately stained later in the cytology laboratory.

30.4 ORGANIZATION OF THE ASPIRATION BIOPSY SERVICE

Sufficient space to examine patients is required. For the in-hospital clinic it should be located close to the pathology department but should be a separate designated area that is quiet and comfortable for patients and with sufficient

support staff to register patients, provide assistance to the pathologist performing the aspiration, and take care of all clerical matters.

There are a number of considerations in developing and planning a free-standing clinic.

1. **Location**
   a. Convenience
   b. Ground floor
   c. Within an established medical facility
   d. Near offices of physicians referring the majority of the patients
2. **The facility**
   a. Two examining rooms, one somewhat larger than the other
   b. Some counters at waist level for standing
   c. Examining table pointed toward outside windows and away from aspiration instruments and smear preparation area

TERMINAL QUESTIONS

1. What are the equipments required for FNAC?
2. Enumerate the various points to consider while planning an FNAC clinic.
3. Describe the method of smear making in FNA laboratory.
4. What are the usual stains used for staining FNA smears and their fixatives?

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31

MORPHOLOGY OF ORGANS

31.1 INTRODUCTION

Tissue is a cellular organizational level intermediate between cells and a complete organism. A tissue is an ensemble of similar cells from the same origin that together carry out a specific function. Organs are then formed by the functional grouping together of multiple tissues

OBJECTIVES

After reading this lesson, you will be able to:

- Enumerate various types of tissues of the human body
- Describe structure of various tissues of the human body

31.2 TYPES OF TISSUES

Following types of tissues make up all organs of the body:

A. Epithelium

B. Connective tissue-supporting tissue

C. Muscle-striated, smooth and cardiac

D. Nervous tissue

E. Blood-It is found in blood vessles, which are part of connective tissue.

A. Epithelium

Epithelial tissue covers the whole surface of the body. It is made up of cells closely packed and ranged in one or more layers. This tissue is specialized to form the covering or lining of all internal and external body surfaces. Epithelial

tissue that occurs on surfaces on the interior of the body is known as endothelium. Epithelial tissue, is usually separated from the underlying tissue by a thin sheet of connective tissue called as basement membrane. The basement membrane provides structural support for the epithelium and also binds it to neighboring structures.

Types of Epithelial Tissues. It can be divided into two types according to location.

1. Covers the external surface and line all the body cavities and tubes.
2. Secretory; found in glands.

Epithelial tissue can be divided into two groups depending on the number of layers of which it is composes. Epithelial tissue which is only one cell thick is known as simple epithelium. If it is two or more cells thick such as the skin, it is known as stratified epithelium.

Simple epithelium

Simple epithelium can be subdivided according to the shape and function of its cells.

- **Squamous (pavement) epithelium**

  Squamous cells have the appearance of thin, flat plates. The shape of the nucleus usually corresponds to the cell form. Squamous cells, for example, tend to have horizontal flattened, elliptical nuclei because of the thin flattened form of the cell. They form the lining of cavities such as the **mouth, esophagous, anus, uterine cervix** and make up the outer layers of the skin.

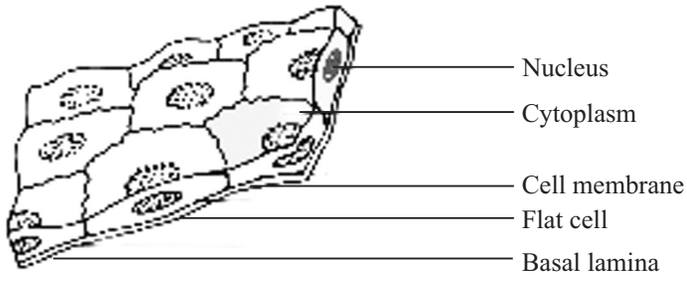

Fig. 31.1: Simple squamous epithelium

- **Simple Cuboidal Epithelium**

  Cuboidal cells are roughly square or cuboidal in shape. Each cell has a spherical nucleus in the centre. Cuboidal epithelium is found in glands and in the lining of the kidney tubules as well as in the ducts of the glands.

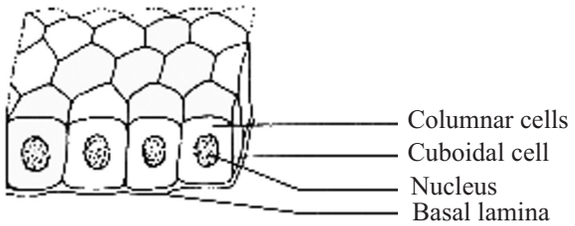

Fig. 31.2: Simple cuboidal epithelium

- **Simple Columnar Epithelium**

Columnar epithelial cells occur in one or more layers. The cells are elongated and column-shaped. The nuclei are elongated and are usually located near the base of the cells. Columnar epithelium forms the lining of the stomach and intestines. Goblet cells (unicellular glands) are found between the columnar epithelial cells of the colon. They secrete mucus, a lubricating substance which keeps the surface smooth.

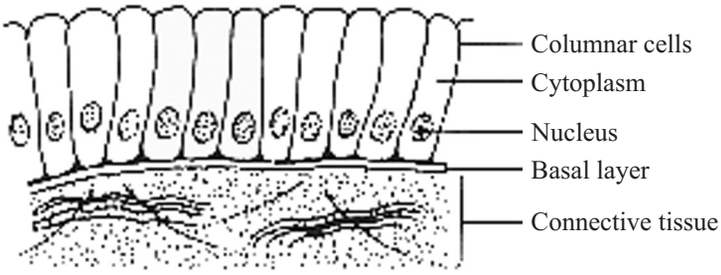

Fig. 31.3: Simple columnar epithelium

- **Ciliated Columnar Epithelium**

These are simple columnar epithelial cells, but in addition, they posses fine hair-like outgrowths, cilia on their free surfaces. These cilia are capable of rapid, rhythmic, wavelike beatings in a certain direction. Ciliated epithelium is usually found in the air passages like the nose. It is also found in the uterus and Fallopian tubes of females. The movement of the cilia propel the ovum to the uterus.

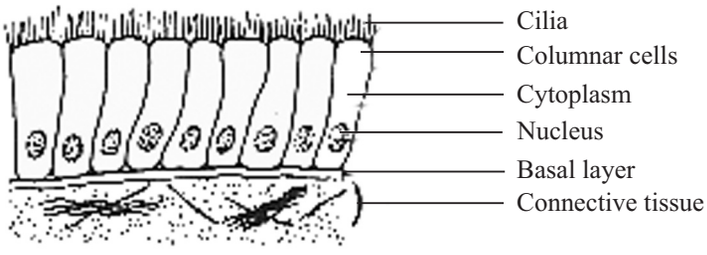

Fig. 31.4: Ciliated columnar epithelium

- **Glandular Epithelium**

Columnar epithelium with goblet cells is called glandular epithelium. Columnar and cuboidal epithelial cells often become specialized as gland cells which are capable of synthesizing and secreting certain substances such as enzymes, hormones, milk, mucus, sweat and saliva.

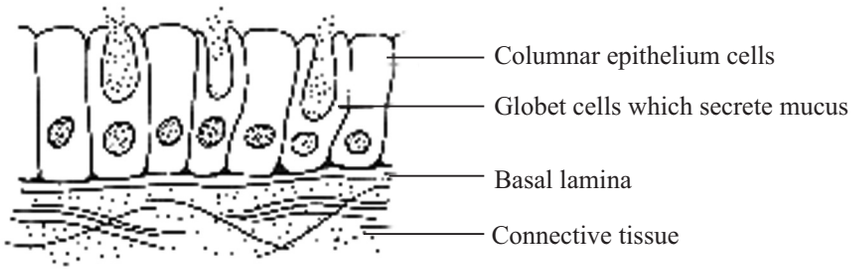

Fig. 31.5: Glandular epithelium

- **Stratified Epithelium.**

Where body linings have to withstand wear and tear, the epithelia are composed of several layers of cells and are then called compound or stratified epithelium. The top cells are flat and scaly and it may or may not be keratinised (i.e. containing a tough, resistant protein called keratin). Human skin is an example of, keratinised, stratified epithelium. The lining of the mouth cavity is nonkeratinising, stratified epithelium.

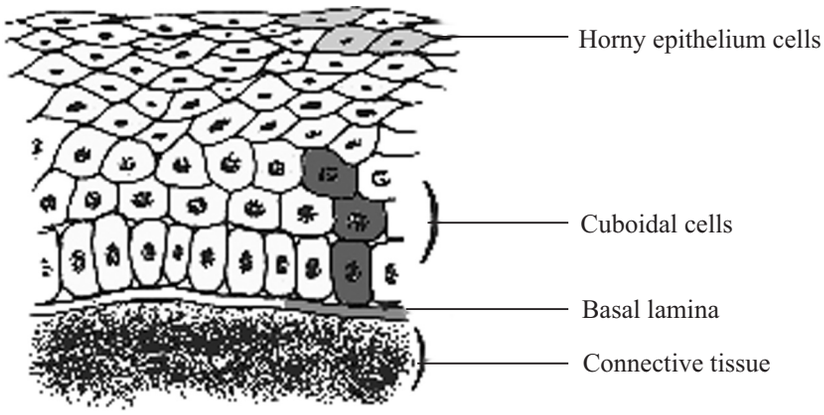

Fig. 31.6: Stratified Epithelium

B. Connective Tissue

This is the most widespread tissue in the human body. Its function is primarily to support, anchor and connect various parts of the body. Although connective tissue exists in a number of forms, all types have three basic structural elements cells, fibres and intercellular substance (ground substance).

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The most common cell type is fibroblast, which produces fibres and other intercellular materials. The two most common types of fibres are: collagen (collagenous) and elastic. Collagen fibres are for strength while elastic fibres provide elasticity to the tissue. Both the cells and the fibres are embedded in the intercellular substance. The consistency of this substance is highly variable from gelatin-like to a much more rigid material.

The proportions of the cells, fibres, and intercellular substance vary, depending on a particular nature and function of the connective tissue. For example, a strong connective tissue needs a greater proportion of the collagen fibres and fewer cells eg. tendons and ligaments.Co nnective tissue composed of mostly cells is loose and soft in consistency like adipose (fat).

Classification of Connective Tissue

I. Connective Tissue Proper – encompasses all organs and body cavities, connecting one part with another and, equally important, separating one group of cells from another.This includes adipose tissue (fat), areolar (loose) tissue, and dense regular tissue.

II. Specialized Connective Tissues — this group includes cartilage, bone, and blood. Cartilage and bone form the skeletal framework of the body.Blood is circulated in the the vessles,made of connective tissues.

Muscles: There are three types of muscles in the body.

Smooth muscle: Muscle tissue that contracts without conscious control, having the form of thin layers or sheets made up of spindle-shaped, unstriated cells with single nuclei.It is present in the walls of the internal organs, such as the stomach, intestine, bladder, and blood vessels.

Cardiac muscle: This type of muscle occurs only in heart. Its cells are joined end to end. The resulting fibers are branched and interconnected in complex networks. Each cell has a single nucleus. At its end, where it touches another cell, there is a specialized intercellular junction called an intercalated disc, which occurs only in cardiac tissue. Cardiac muscles work involuntarily and can continue to function without being stimulated by nerve impulses.

Striated muscle: It is also called voluntary muscle, striped muscle, or skeletal muscle.It is the most common of the three types of muscle in the body. Striated muscles are attached to bones and produce all the movements of body parts in relation to each other. Striated muscle is under voluntary control. Its multinucleated fibers are long and thin and are crossed with a regular pattern of fine red and white lines, giving the muscle its distinctive appearance and its name. These cross striations are better seen with phosphotungstic acid hematoxylin stain.

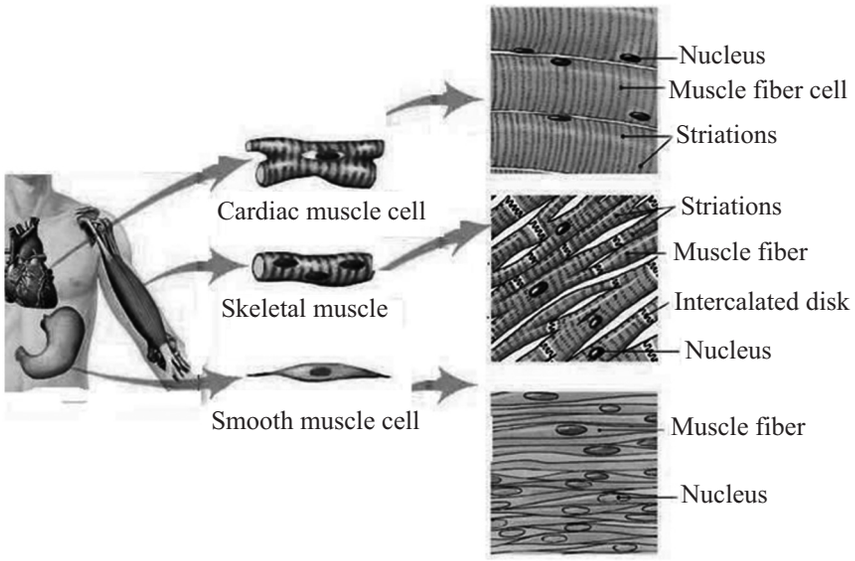


Fig. 31.7: Types of muscle fibre

31.3 BONE

Bone is the basic unit of the human skeletal system and provides the framework for and bears the weight of the body, protects the vital organs, supports mechanical movement, hosts hematopoietic cells**.**

Cartilage and bone are specialized connective tissues and consist of cells and extracellular matrix. The matrix of all connective tissues consists of fibres (collagen, reticular, and elastic) and amorphous ground substance, which contains proteoglycans and hyaluronic acid. The matrix is secreted by some of the cells in connective tissues. In cartilage, it is chondroblasts and chondrocytes that produce the matrix, while in bone, it is osteoblasts and osteocytes.

- **Osteoprogenitor cells**

  They are pluripotent mesenchymal stem cells present in the vicinity of all bony surfaces. On stimulation by growth factor they produce offspring that differentiate into osteoblasts.

- **Osteoblasts**

  They are located on the surface of bones. The cells synthesize, transport and arrange protein of matrix. If osteoblasts become surrounded by newly deposited organic matrix, they transform into osteocytes.

- **Osteocytes**

  Osteocytes communicate with each other and with other cells on the bone surface via an intricate network of cytoplasmic processes that traverse tunnels in the matrix known as canaliculi.

- **Osteoclasts**

These cells are responsible for bone resorption. They are derived from hemopoetic progenitor cells. Mature osteoclasts are multinucleated.

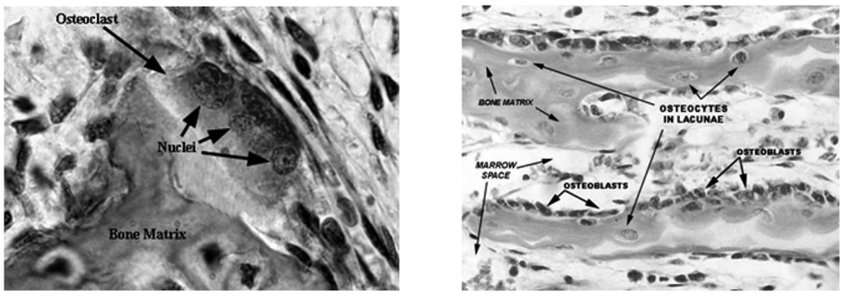


Fig. 31.8: Histology of Bone

31.4 LIVER

The liver parenchyma is divided into thousands of small units called lobules. A lobule is roughly hexagonal in shape, with portal triads at the vertices and a central vein in the centre. In contrast, the hepatic acinus represents a unit that is of more relevance to hepatic function because it is oriented around the afferent vascular system.

The parenchymal cells of the liver are hepatocytes. These polygonal cells are joined to one another in anastomosing plates, with borders that face either the sinusoids or adjacent hepatocytes. Hepatocytes are in contact with blood in sinusoids, which are distensible vascular channels lined with highly fenestrated endothelial cells and populated with phagocytic Kupffer cells. The space between endothelium and hepatocytes is called the Space of Disse which collects lymph for delivery to lymphatic.

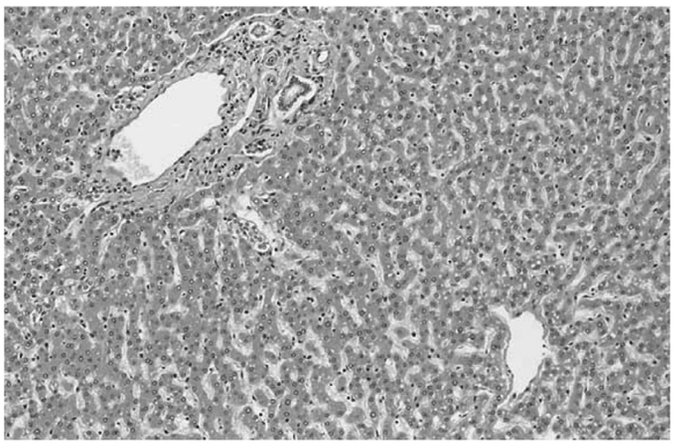


Fig. 31.9

Bile is secreted from the basal surface of hepatocytes, gets collect in channels called canaliculi. These secretions flow toward the periphery of lobules and into bile ductules and interlobular bile ducts, ultimately collecting in the hepatic duct outside the liver. The hepatic duct is continuous with the common bile duct, which delivers bile into the duodenum.

31.5 KIDNEY

Cut surface of kidney shows two parts ;the outer is cortex and inner part is medulla.The two components of renal parenchyma are renal corpuscle and Loop of Henle.

A. Renal Corpuscle

The **renal corpuscle** is the part of the kidney nephron in which blood plasma is filtered. The renal corpuscle of each kidney nephron has two parts - they are the **Glomerulus**, which is a network of small blood vessels called capillaries, and the **Bowman's Capsule**, which is the double-walled epithelial cup within which the glomerulus is contained.

Within the glomerulus are **glomerular capillaries.**The **afferent arterioles** bring blood into the glomerulus and the **efferent arteriole** drain blood out from the glomerulus.

Capsular space is the area between the double-walls of the Bowman's Capsule. The cells that form the outer edges of the glomerulus form close attachments to the cells of the inner surface of the Bowman's Capsule.

B. Renal Tubule

The **renal tubule** is the part of the kidney nephron into which the glomerular filtrate passes after it has reached the Bowman's capsule. The first part of the renal tubule is called the **proximal convoluted tubule.**

The water and solutes that have passed through the proximal convoluted tubule (PCT) enter the **Loop of Henle**, which consists of two portions - first the **descending limb of Henle,** then the **ascending limb of Henle**. The water (and substances dissolved in it) pass from the renal cortex into the renal medulla, then back to the renal cortex through Loop of Henle. When this fluid returns to the renal cortex via the ascending limb of Henle, it passes into the **distal convoluted tubule (DCT).**

The distal convoluted tubules of many individual kidney nephrons converge onto a single **collecting duct.** Many collecting ducts join together to form

several hundred **papillary ducts.** The contents of the papillary ducts drain into the **minor calces** - the channels through which the fluid passes, via the **major calyx**, into the centre of the kidney - called the renal pelvis.

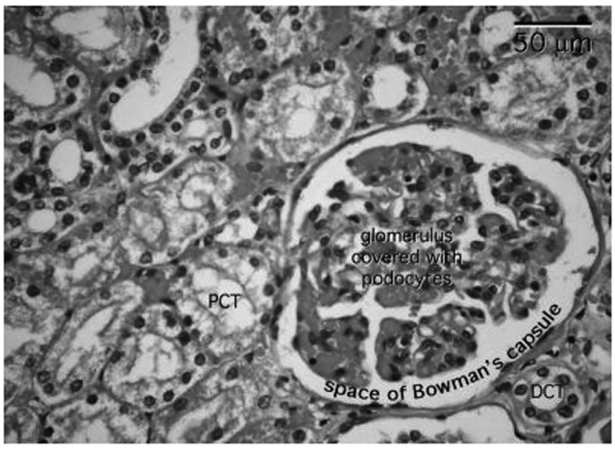


Fig. 31.10

Gastrointestinal tract: It can be divided as

Upper and Lower human gastrointestinal tract

The upper gastrointestinal tract consists of the esophagus, stomach, and duodenum

Lower gastrointestinal tract

The lower gastrointestinal tract includes most of the small intestine,whole large intestine and anus.

- Small Intestine - has three parts:

  Doudenum

  Jejunum

  Ileum.
- Large Intestine: has three parts:
  - Caecum: The Vermiform appendix is attached to the caecum.
  - Colon: Includes the ascending colon, transverse colon, descending colon and sigmoid colon.
  - Rectum and anal canal.

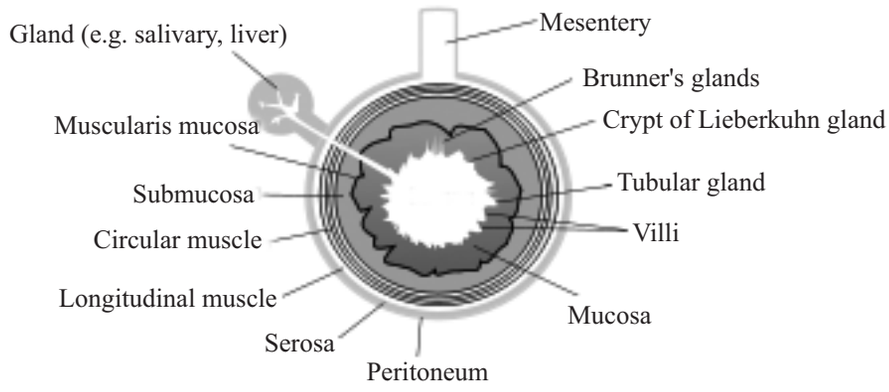


Fig. 31.11: Histology

General structure of the gut wall

The gastrointestinal tract shows four layers on histology with some differences that reflect the specialization in functional anatomy. These layers are in the following order:

- Mucosa
- Submucosa
- Muscular layer)
- Adventitia or serosa

Mucosa

The mucosa is the innermost layer of the gastrointestinal tract.

The mucosa is made up of three layers:

- Epithelium - innermost layer.
- Lamina propria - a layer of connective tissue. Unusually cellular compared to most connective tissue
- Muscularis mucosae - a thin layer of smooth muscle.

In the esophagus, the epithelium is stratified, squamous and non-keratinising, for protective purposes.

In the stomach it is simple columnar, and is organised into gastric pits and glands to deal with secretion.The small intestine epithelium the is organised into plicae circulares and villi, and the enterocytes have microvilli. . In the ileum there are occasionally Peyer's patches in lamina propria.

Notes

The colon has simple columnar epithelium with no villi. There are goblet cells.

The appendix has a mucosa resembling the colon but is heavily infiltrated with lymphocytes.

The ano-rectal junction shows a transition from simple columnar to stratified squamous non-keratinising epithelium for protective purposes.

Submucosa

The submucosa consists of a dense irregular layer of connective tissue with large blood vessels, lymphatics, and nerves branching into the mucosa and muscularis propia. It contains Meissner's plexus, an enteric nervous plexus, situated on the inner surface of the *muscularis* .

Muscularis propia

The muscularis externa consists of an inner circular layer and a longitudinal outer muscular layer.. The layers are not truly longitudinal or circular, rather the layers of muscle are helical with different pitches. The inner circular is helical with a steep pitch and the outer longitudinal is helical with a much shallower pitch.

Between the two muscle layers are the myenteric or Auerbach's plexus. This controls peristalsis. Activity is initiated by the pacemaker cells (interstitial cells of Cajal).

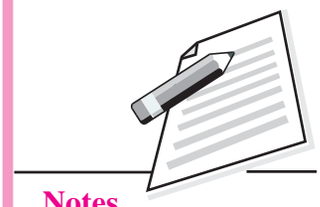
The thickness of muscularis propia varies in each part of the tract. In the colon, for example, the muscularis externa is much thicker because the faeces are large and heavy, and require more force to push along. The outer longitudinal layer of the colon thins out into 3 discontinuous longitudinal bands, known as taeniae coli (bands of the colon). This is one of the 3 features helping to distinguish between the large and small intestine.

The pylorus of the stomach has a thickened portion of the inner circular layer: the pyloric sphincter.

Adventitia/serosa

The outermost layer of the GI tract consists of several layers of connective tissue.

Intraperitoneal parts of the GI tract are covered with serosa. These include most of the stomach, first part of the duodenum, all of the small intestine, caecum and appendix, transverse colon, sigmoid colon and rectum. In these sections of the



gut there is clear boundary between the gut and the surrounding tissue. These parts of the tract have a mesentery.

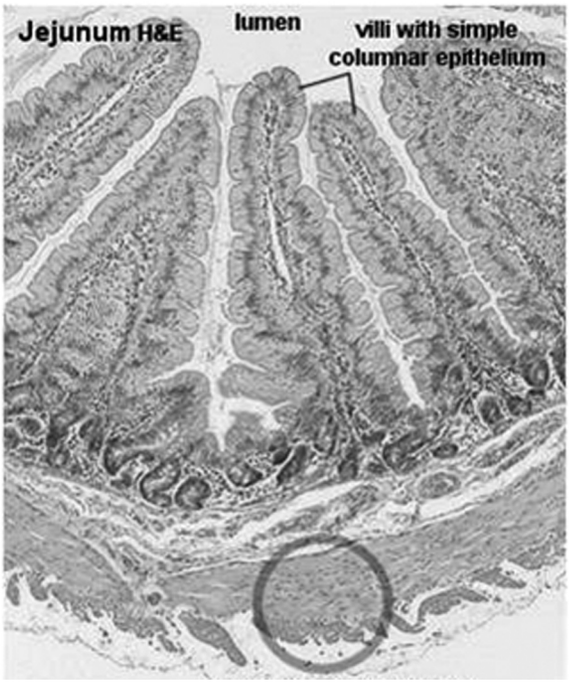


Fig. 31.12: Longitudinal (outside) and circular (inside) layers of smooth muscle

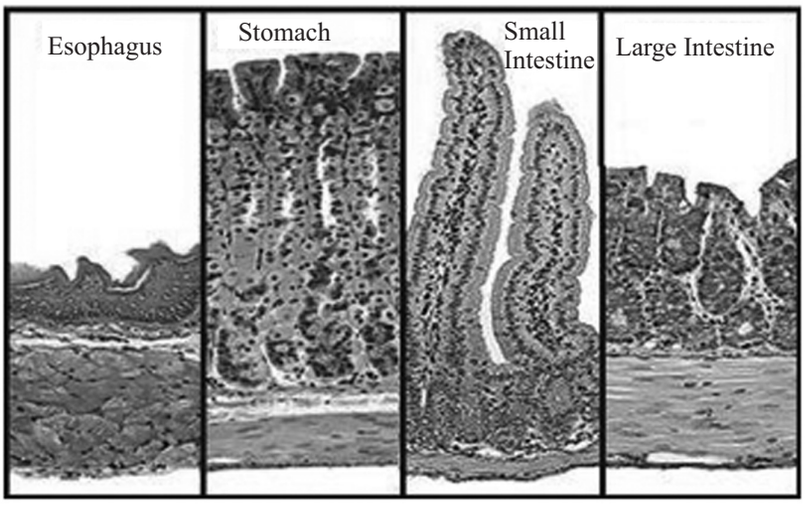


Fig. 31.13