ADVANCED MICROTECHNIQUE

Microtechnique in Medical

The microtechnique is divided to two main ways:

-1- Sectioning method
-2- Non sectioning method

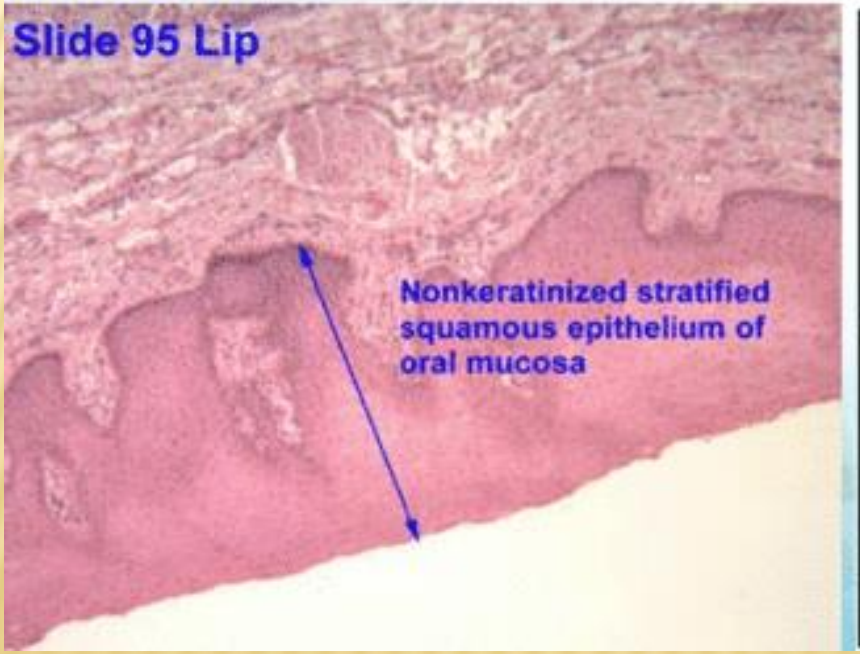
- The sectioning method is the way we deal with the solid sample (tissue) which needs to be sectioned by the microtome to obtain very thin sections which can be studied under light microscope.
- The non sectioning method is the way to deal with the human body fluid, (like: pleural effusion, ascetic fluid, C.S.F,,,, et) and these specimens don't need to be sectioned.

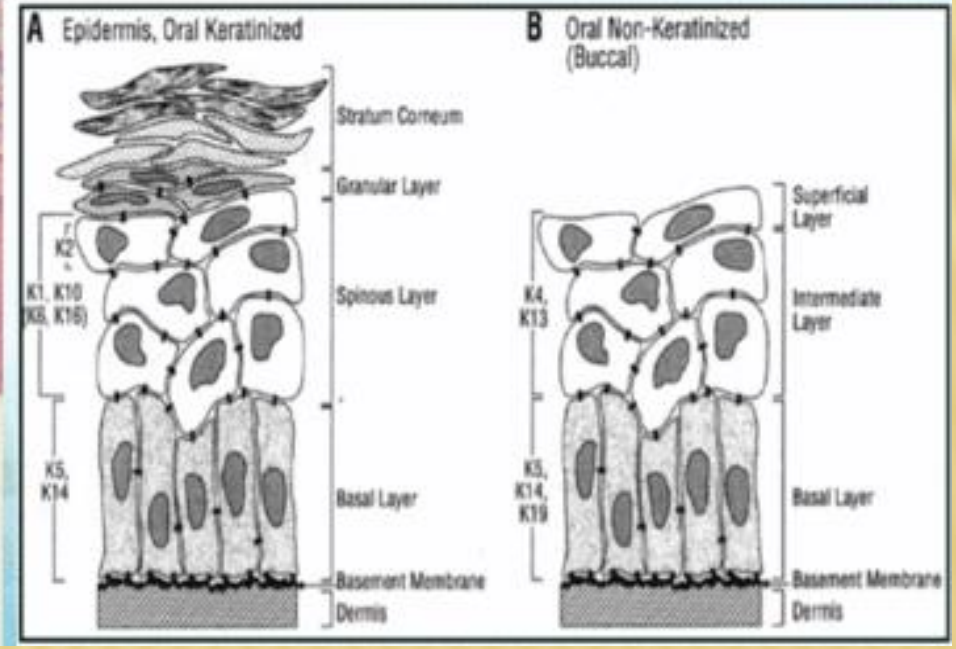
BUCCAL SMEAR

The cells which line the inside of your cheeks form a mucous membrane and are classified as a stratified squamous epithelium tissue. These flat, scale-like Buccal cells (pronounced "buckle") resist friction and are shed constantly as the tissue is renewed. By gently scraping the inside of your cheek, these cells can be harvested, and when smeared and stained, may be used to illustrate a number of important biological phenomena including cell and tissue structure, oral bacterial flora and morphology, etc.

BUCCAL SMEAR

This tissue is non-keratinized and therefore the surface cells are living and still possess their nuclei, in contrast with shed epidermal cells.





EQUIPMENT

Soap and water

Microscope slides

Dropper bottle of dH2O

Dropper bottle of 0.3% methylene blue

Toothpicks or swap (optional)

Bunsen burner or alcohol lamp,

Paper towel (“non-linty”) or bibulous paper

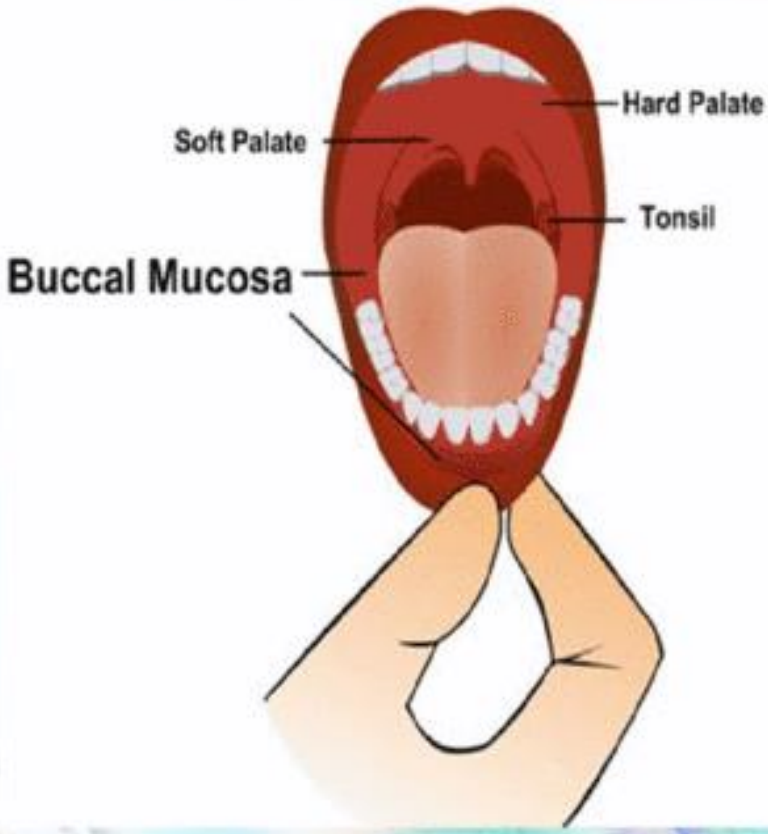
SLIDE PREPARATION

1. Clean a microscope slide well with soap & water, dry with a "non-linty" paper towel.

Cleanse very thoroughly under the. 2 nail of your index finger. (This step is not necessary if you use swap or

(toothpick

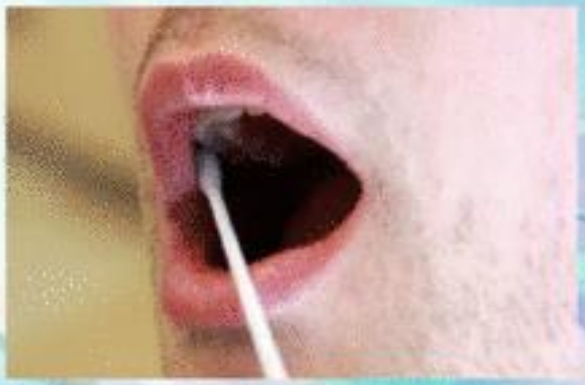
3. Place a *tiny* drop of dH20 in the center of the very clean slide.



HARVEST THE CELLS, PREPARE THE SMEAR

4. *GENTLY* scrap the inside of your cheek to pick up some of the shed stratified squamous cells. Do NOT scrape chunks. A toothpick may be used if you have no fingernails. *Gentle* scraping is the watchword, there should be *no* discomfort...

5. Express the material from under your nail by pressing with your thumb, and press into the drop of water, mix and spread the material around to the size of a dime.



FIX AND STAIN THE SMEAR

FIX THE SMEAR:

6. Pass the slide briefly through the flame several times to warm and fix the smear. Do *NOT* heat the slide above a temp which is comfortable. You are "gluing" the smear to the slide. (you can also use 96% Methanol or Ethanol for fixation)

STAIN THE SMEAR:

7. Place a drop of 0.3% methylene blue on the specimen. Let sit for 1 minute.

. Rinse off the excess stain with tap water. 8

9. Blot dry with an non-linty paper towel or bibulous paper. *Do not rub*.

10. Flame again briefly to dry slide by warming.

:EXAMINE UNDER THE MICROSCOPE

11. Examine first with the 4x objective, scanning the entire field to find a well-distributed region with individual cells (no big clumps). Then view with the 10x and 40x objectives. Illustrate the 400x view noting 1) the nucleus, 2) nucleolus, 3) cell boundary and 4) the variety of bacteria colonizing the surface of the cells.

For Microbiology only: you will be instructed on oil immersion use, then view and illustrate bacterial morphologies with the 100x oil immersion objective (1000x view).

BUCCAL SMEAR PREPARATION

In babies

- rubbing the inside of cheek with gloved finger > spread immediately

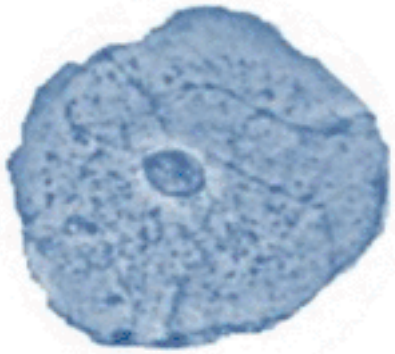
In any patient except babies

- Rinse the mouth with water
- The inside of mouth scraped with Ayres spatula
- The resultant > slightly turbid fluid spread on 5 slides

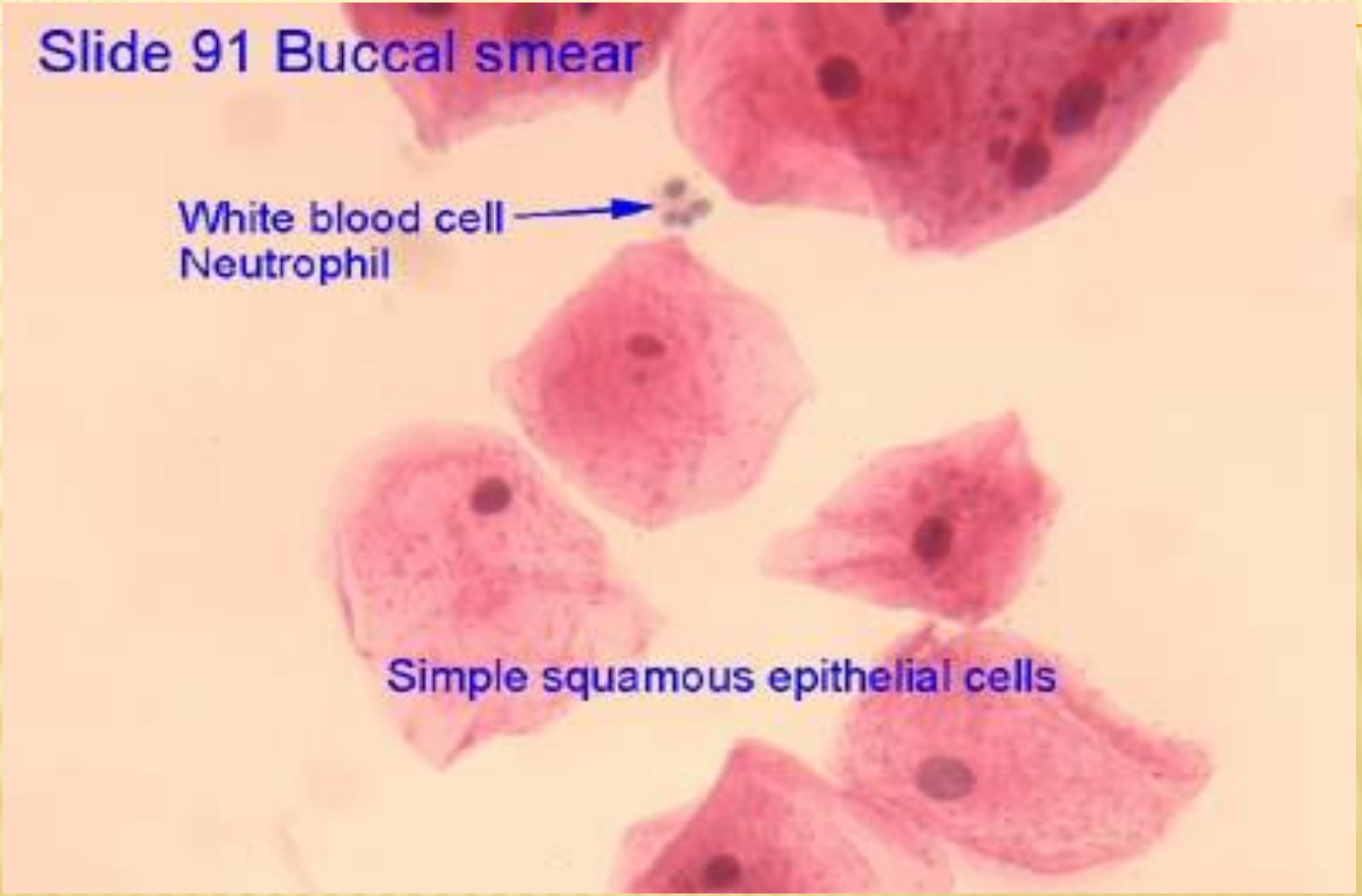
BUCCAL SMEAR

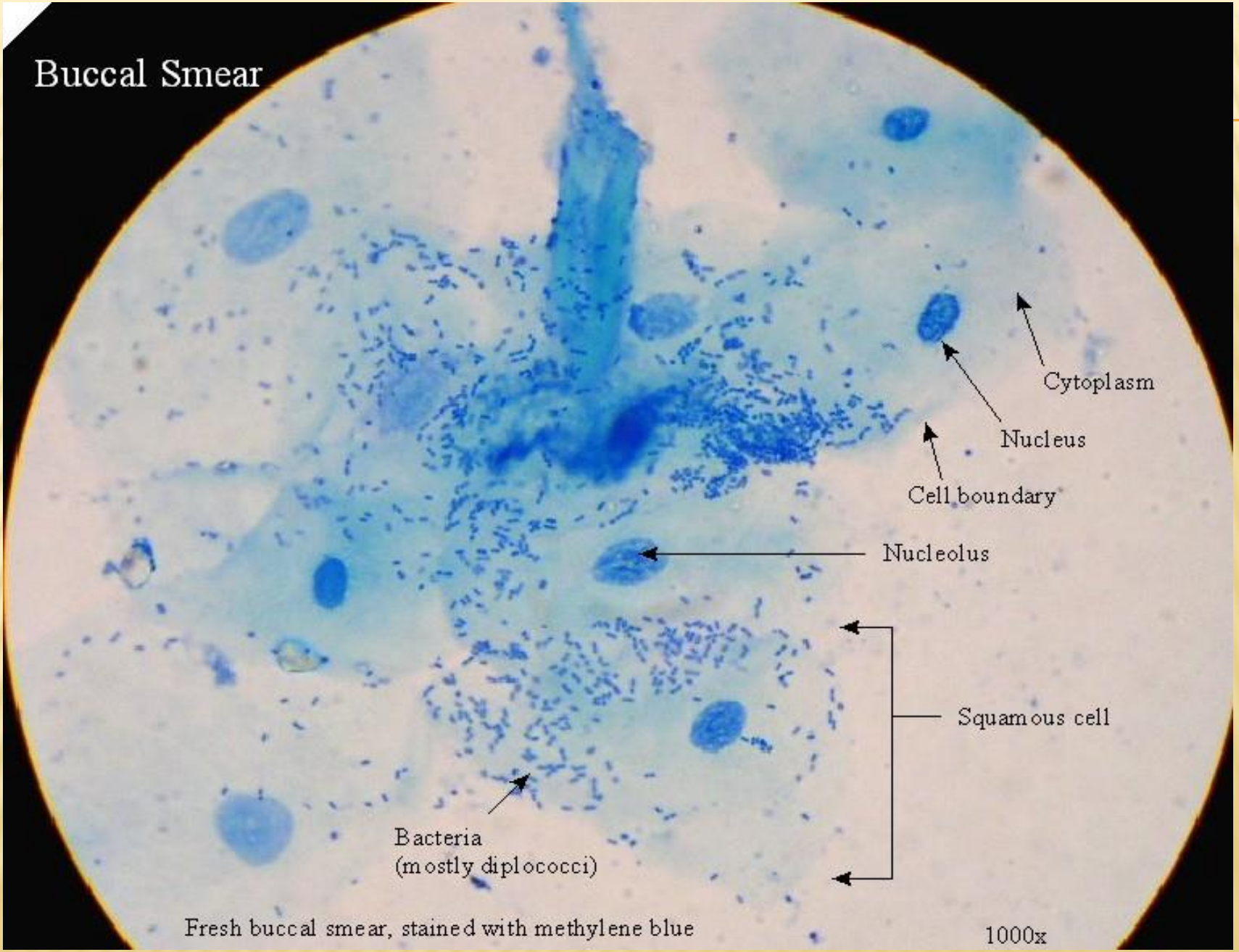
squamous epithelium

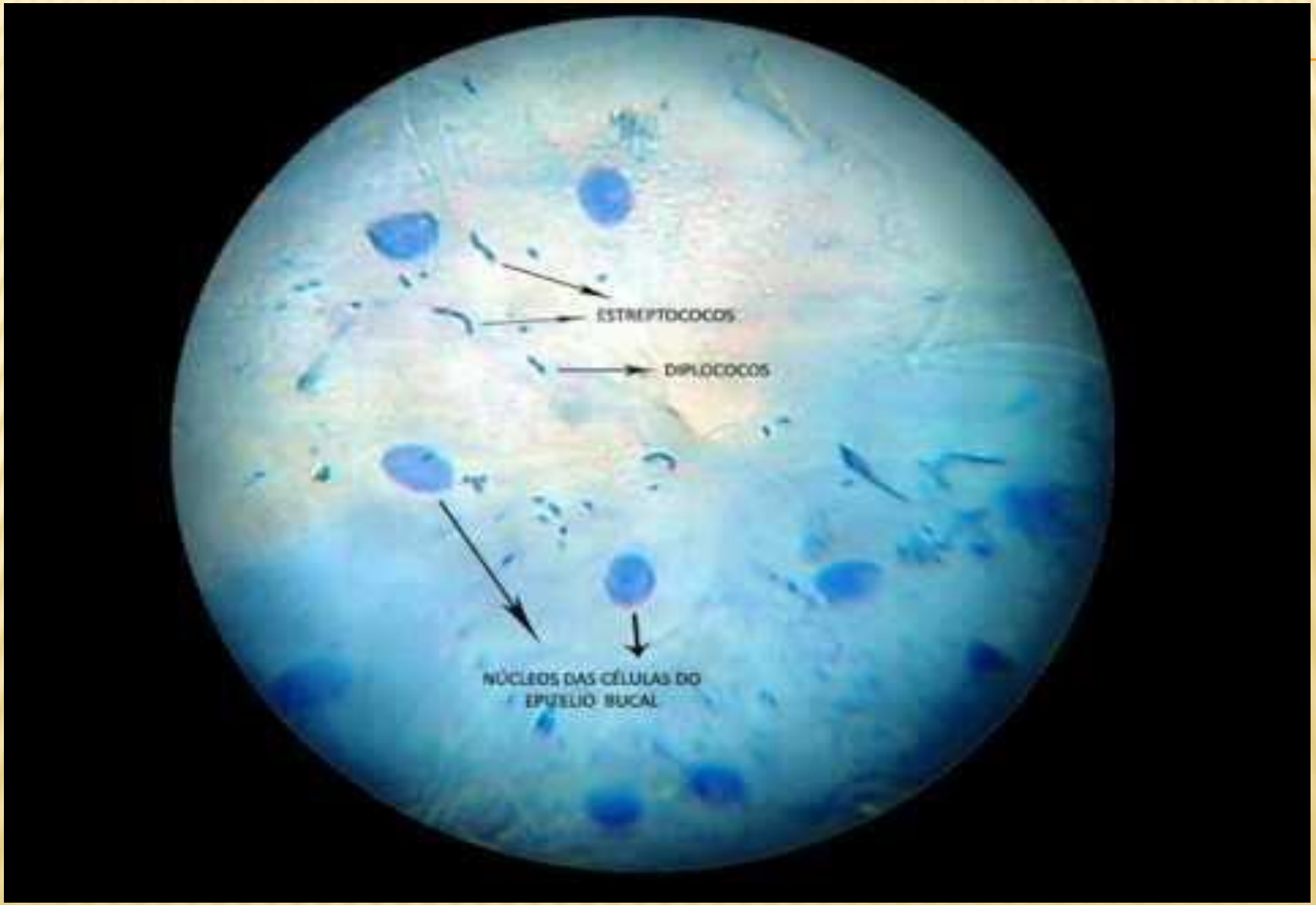
Observe your preparation under the low power of a microscope.

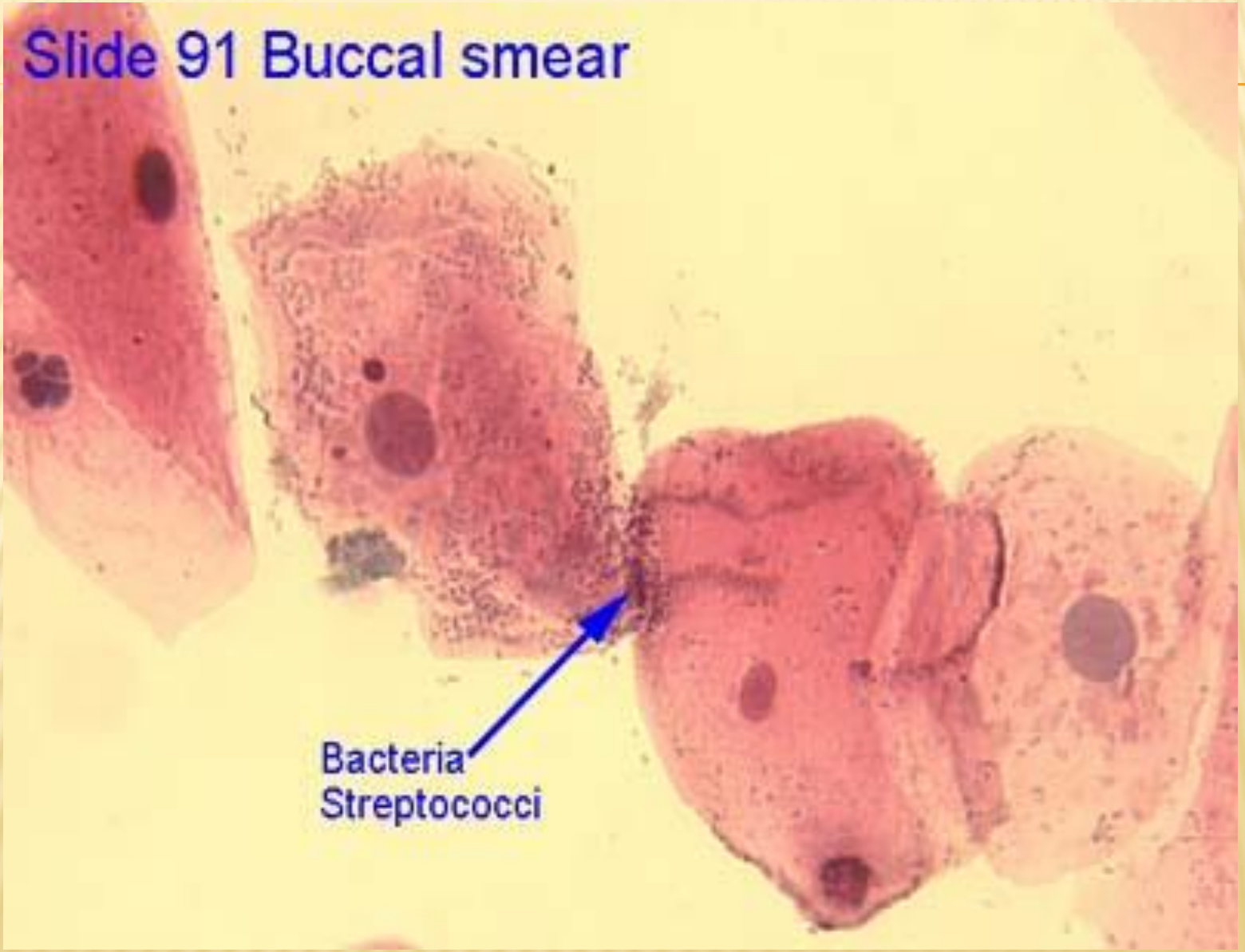


squamous epithelium
a single magnified cell

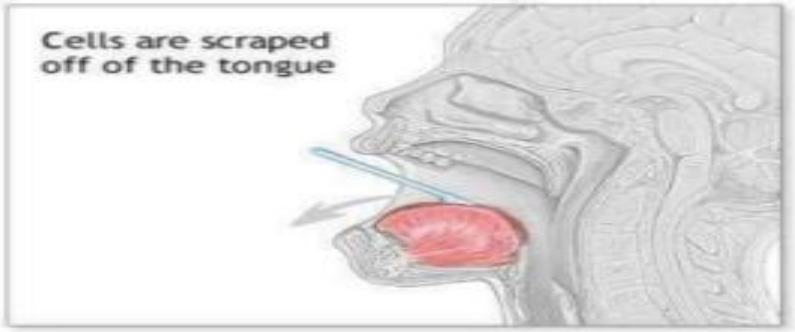

Slide 91 Buccal smear
White blood cell
Neutrophil
Simple squamous epithelial cells


Buccal Smear
Cytoplasm
Nucleus
Cell boundary
Nucleolus
Squamous cell
Bacteria
(mostly diplococci)
Fresh buccal smear, stained with methylene blue
1000x


ESTREPTOCOCOS
DIPLOCOCOS
NÚCLEOS DAS CÉLULAS DO
EPITÉLIO BUCAL


Slide 91 Buccal smear
Bacteria
Streptococci

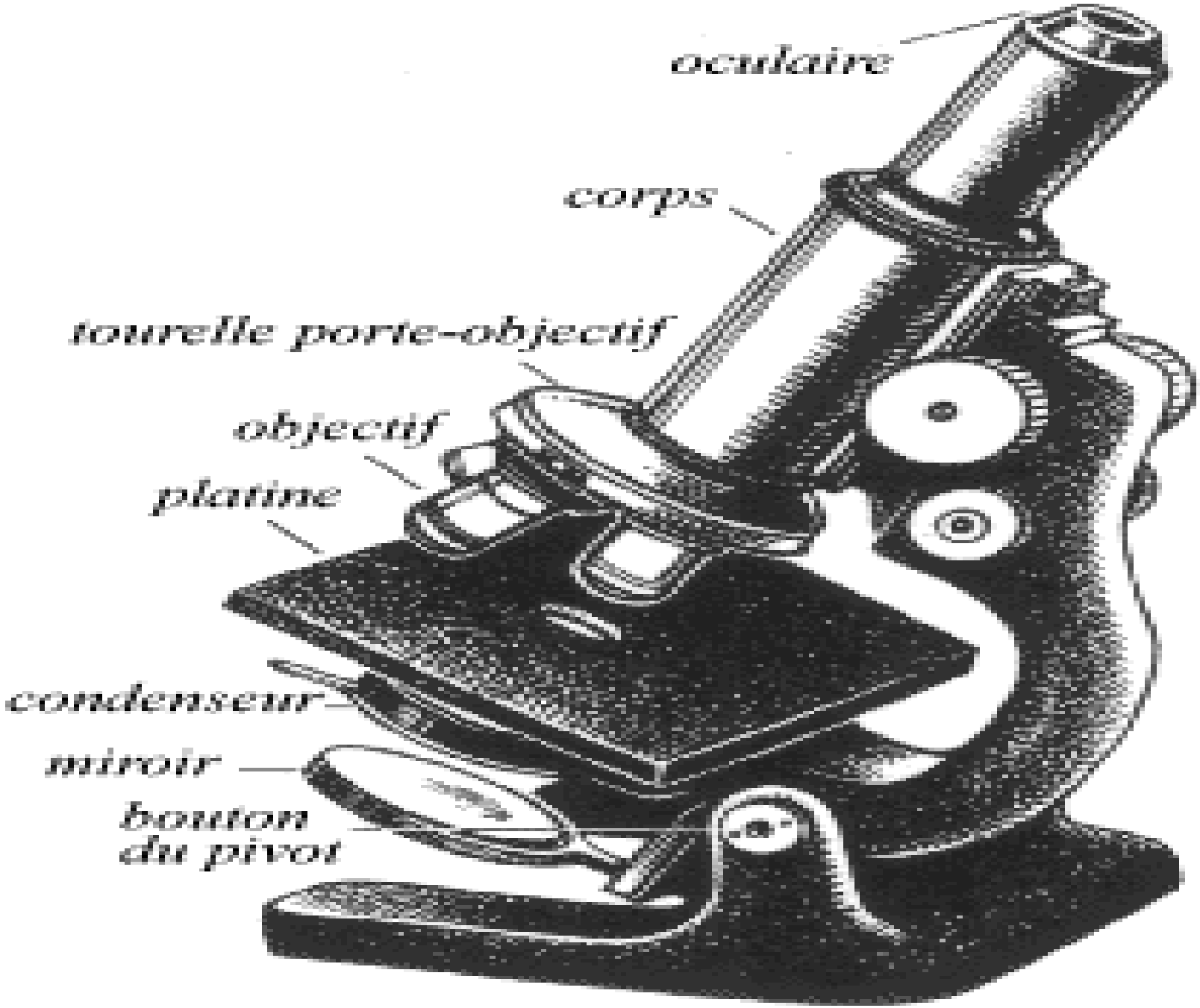
Scrapping of Cheek cells



A buccal smear is a test where cells are taken from the tongue. Cells are collected by scraping the tongue with a spatula. The cells are then placed on a slide and the sample is taken to the laboratory for evaluation. The cells are evaluated for the presence of Barr bodies (a mass seen in a normal female sex chromosome). The buccal smear test can confirm whether the patient is a male or female.

The cells which line the inside of your cheeks are classified as a stratified squamous epitheliumtissue and are the surface of a mucous membrane. These flat, scale-like buccal cells (pronounced "buckle") are shed constantly as the tissue is renewed. By gently scraping the inside of your cheek, these cells can be harvested, and when smeared and stained, may be used to illustrate a number of important biological phenomena including cell and tissue structure, oral bacterial flora and morphology, etc. This tissue is non-keratinized and therefore the surface cells are living and still possess their nuclei, in contrast with shed epidermal cells.

(Microtechnique) Technology laboratory preparations

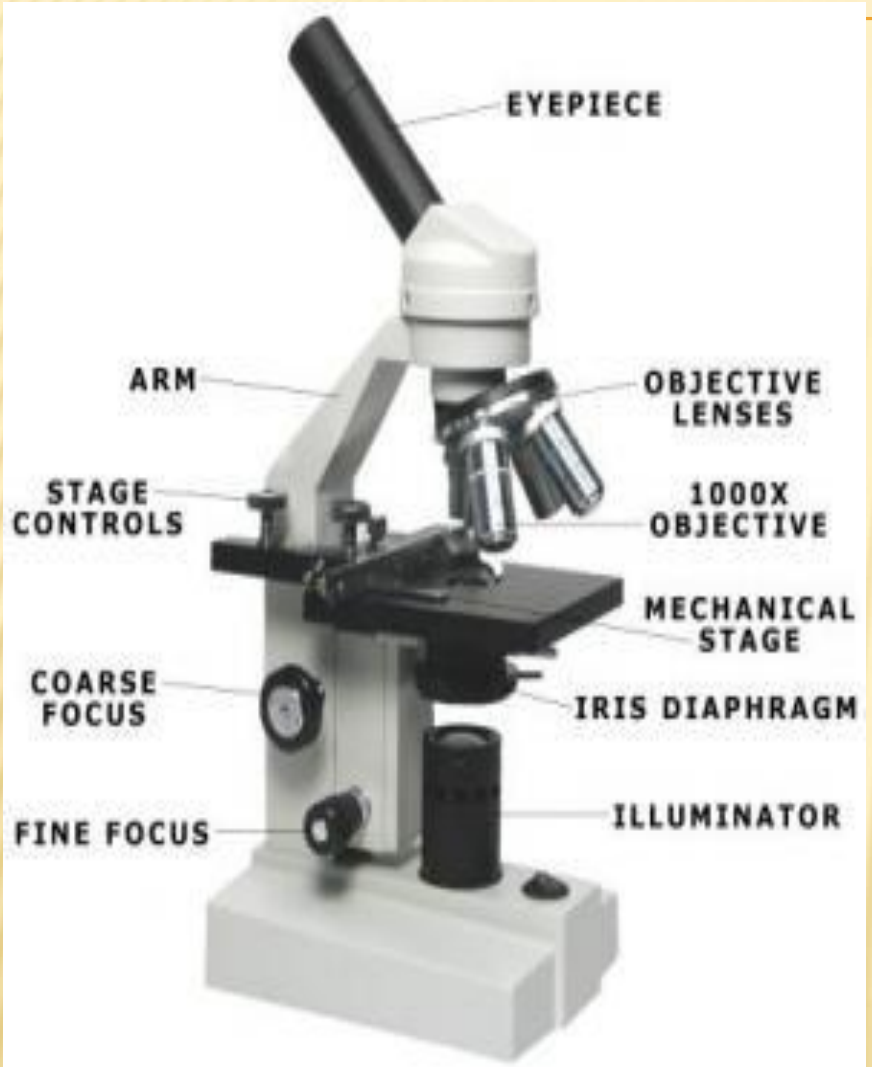

oculaire
corps
tourelle porte-objectif
objectif
platine
condenseur
miroir
bouton
du pivot

TYPES OF MICROSCOPES

- Compound Microscope
- Dissection Microscope
- Scanning Electron Microscope (SEM)
- Transmission Electron Microscope (TEM)
- Phase Contrast Microscope

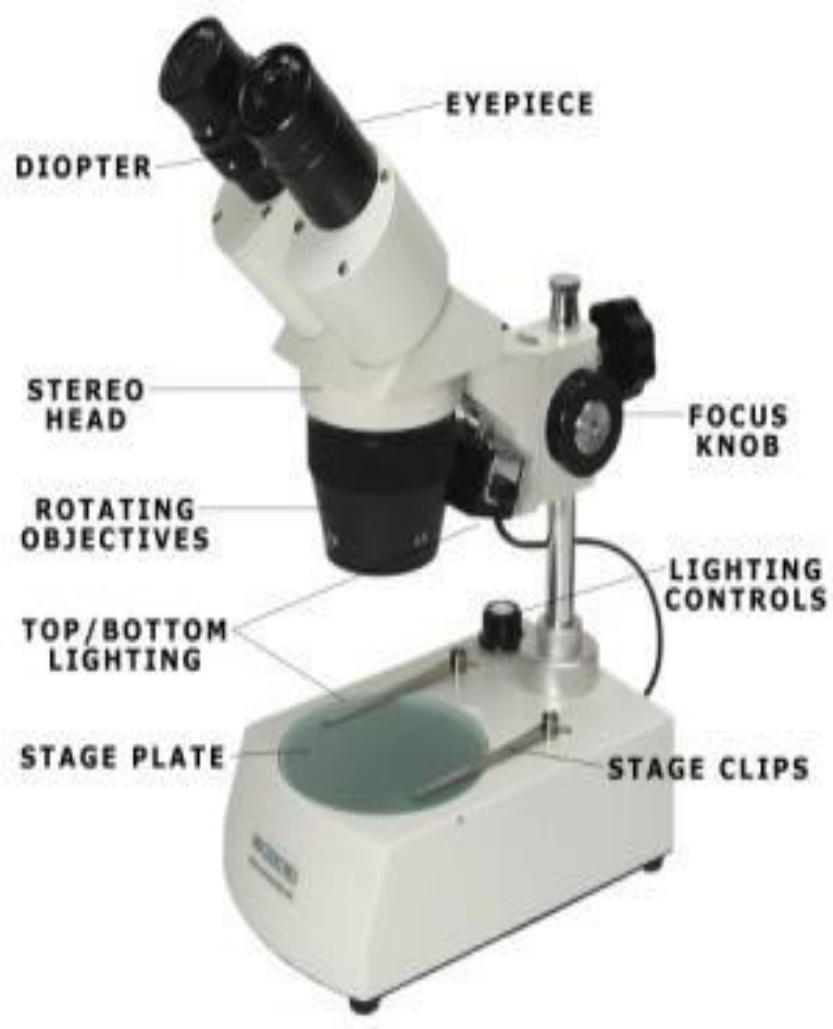
COMPOUND MICROSCOPE

- Compound microscopes are light illuminated. The image seen with this type of microscope is two dimensional. This microscope is the most commonly used. You can view individual cells, even living ones. It has high magnification. However, it has a low resolution.


EYEPIECE
ARM
OBJECTIVE LENSES
STAGE CONTROLS
1000X OBJECTIVE
MECHANICAL STAGE
COARSE FOCUS
IRIS DIAPHRAGM
FINE FOCUS
ILLUMINATOR

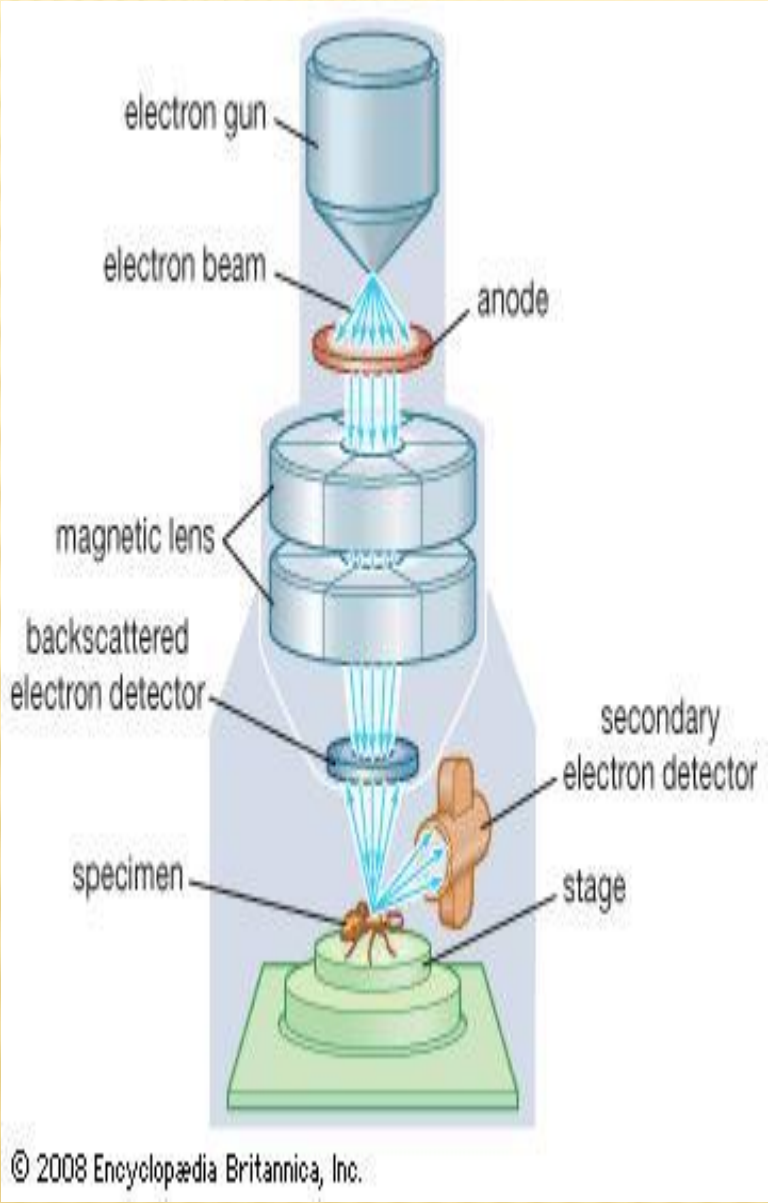
DISSECTION MICROSCOPE

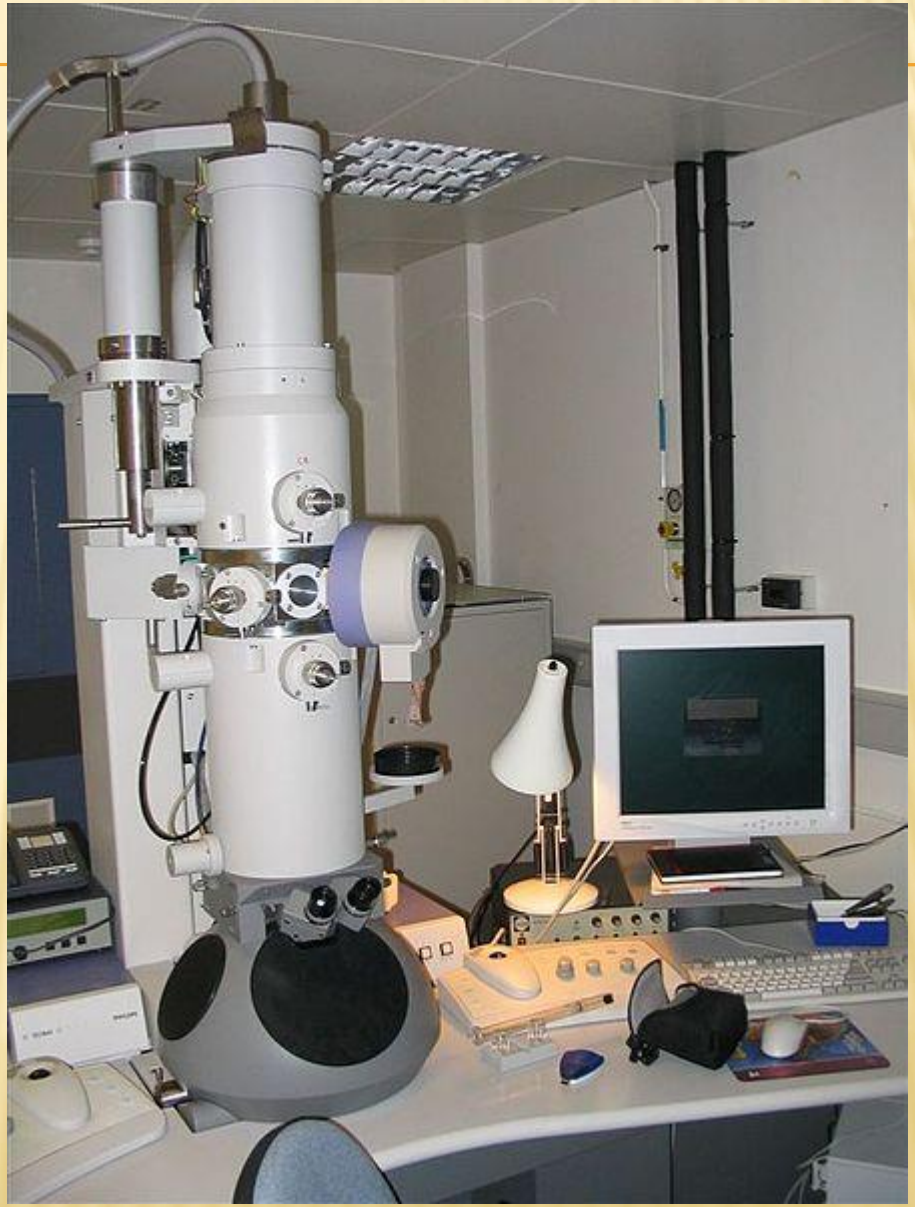
- A dissection microscope is light illuminated. The image that appears is three dimensional. It is used for dissection to get a better look at the larger specimen. You cannot see individual cells because it has a low magnification.
- (also called stereo microscope)


EYEPIECE
DIOPTER
STEREO HEAD
FOCUS KNOB
ROTATING OBJECTIVES
LIGHTING CONTROLS
TOP/BOTTOM LIGHTING
STAGE PLATE
STAGE CLIPS

SCANNING ELECTRON MICROSCOPE- SEM

- SEM use electron illumination. The image is seen in- 3D. It has high magnification and high resolution. The specimen is coated in gold and the electrons bounce off to give you and exterior view of the specimen. The pictures are in black and white.


electron gun
electron beam
anode
magnetic lens
backscattered
electron detector
secondary
electron detector
specimen
stage
© 2008 Encyclopædia Britannica, Inc.

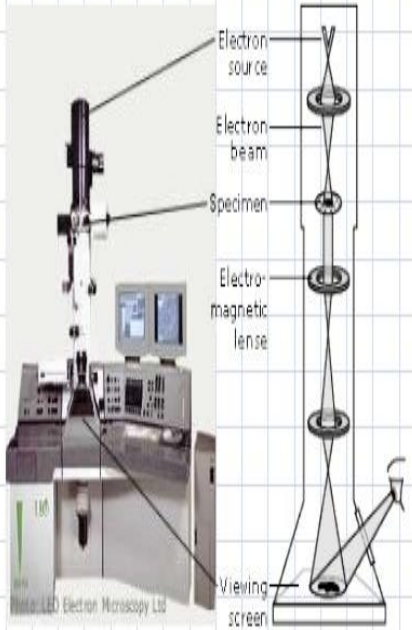


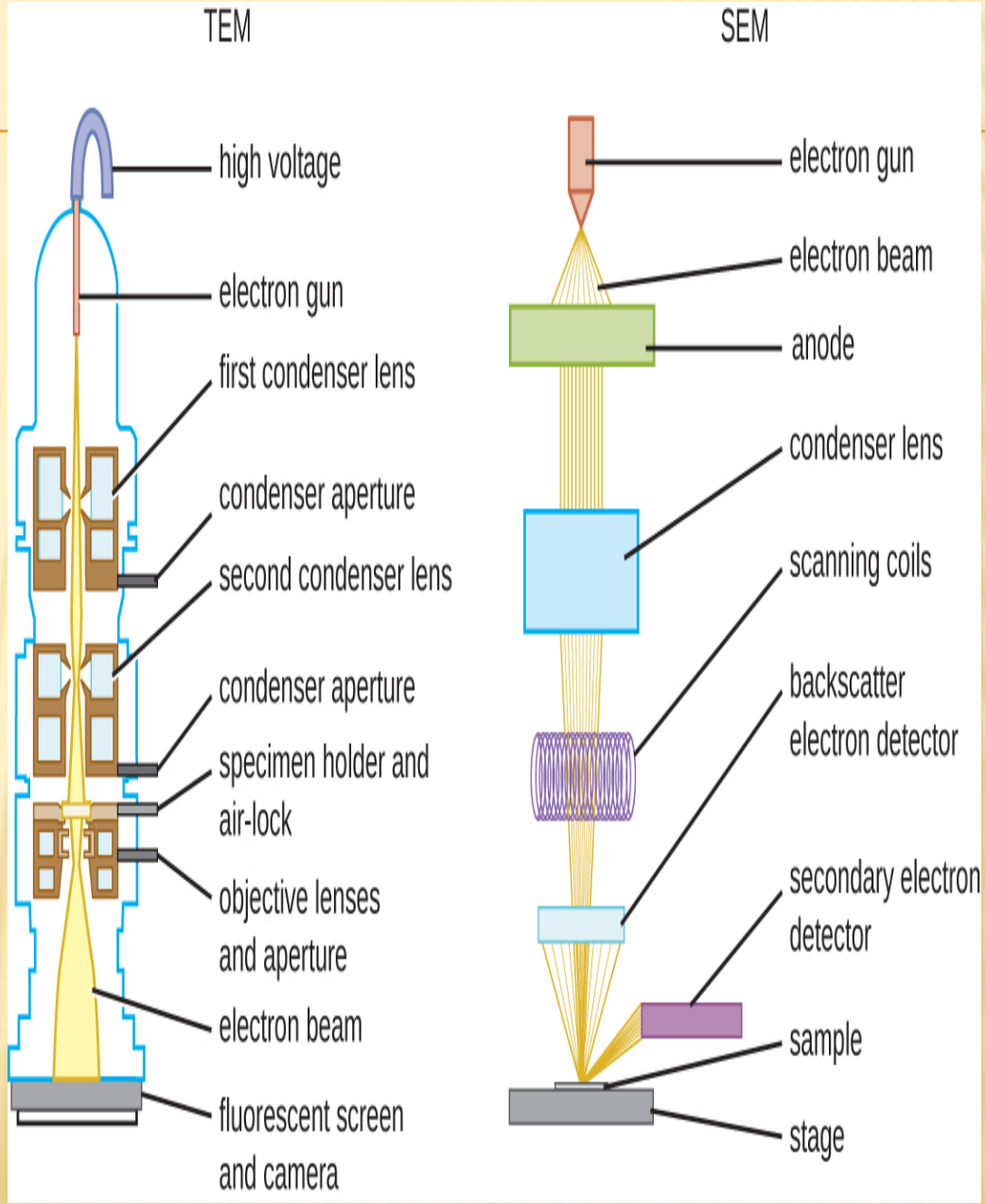
TRANSMISSION ELECTRON MICROSCOPE- TEM

- TEM is electron illuminated. This gives a 2D view. Thin slices of specimen are obtained. The electron beams pass through this. It has high magnification and high resolution.

Transmission Electron Microscopy

TEM is a unique tool in characterization of materials crystal structure and microstructure simultaneously by diffraction and imaging techniques.





Phase-contrast microscopy

Phase-contrast microscopy is an optical-microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image. Phase shifts themselves are invisible, but become visible when shown as brightness variations.

Phase-contrast microscopy is particularly important in biology.

MICROSCOPE CARE

- Always carry with 2 hands
- Never touch the lenses with your fingers.
- Only use lens paper for cleaning
- Do not force knobs
- Keep objects clear of desk and cords
- When you are finished with your "scope", rotate the nosepiece so that it's on the low power objective, roll the stage down to lowest level, rubber band the cord, then replace the dust cover.

Microscopic preparation methods

The most important scientific instruments relevant to microscopic preparations:-

Microscopes
Cutting devices (Microtomes)
Refrigeration
Heating devices
Centrifuges

Incubator

Oven

However, fresh material is needed for the following purpose:

1. Frozen section
2. Immunocytochemistry
3. Cytological and histological examination
4. Microbiological sampling before histopathology
5. Chromosome analysis
6. Research purpose
7. Museum display

HISTOLOGY : It is the branch of science which deals with the gross & microscopic study of normal tissue .

HISTOPATHOLOGY : It is the branch of science which deals with the gross & microscopic study of tissue affected by disease.

Tissue for study can be obtained from: Biopsies – Autopsies.

HISTOTECHNIQUES: The techniques for processing the tissues, whether biopsies, larger specimen removed at surgery, or tissues from autopsy so as to enable the pathologist to study them under the microscope.

THE MOST COMMON WAYS TO PREPARE MICROSCOPIC SAMPLES

How to get the sample from the animal:-

Slaughter

Paralysis

Beating on the back of the head

Anesthesia

SLAUGHTER

Operations easier and fastest on vertebrate animals

Conditions:

Constipation good animal

Use a knife or a very sharp blade

The process of slaughtering cut veins and arteries of the neck with the trachea and pharynx

PARALYSIS

Paralyse animal full and separation of the spinal cord about nervous system or brain
This method is:
Inserts a needle anatomy acute among the first vertebera of the cervical spine and the skull until you reach the spinal cord
Move the needle left and right in order to ensure total separation of the spinal cord for the nervous system
This applies to: frogs, deer.

ANESTHESIA

Must be carried out by qualified
Type of drug depends on the nature of the research and the type of animal
Prefers not to be used in cellular studies
Used in the case study of how members function and histological studies

TYPES OF ANESTHESIA

Non-lethal narcotic material: animal anesthesia for a specific period of time so that recovery occurs
Article deadly narcotic (euthanasia): exposing the animal to the strong narcotic substance eventually lead to his death.

Types of narcotic substances:-

Chloroform

Ethyl alcohol

Menthol

Ether vapor

METHODS OF FIXATION THE SAMPLE

The aim of the fixation is:

1-Nature conservation cell and tissue structures to the greatest possible on the original nature

2-Reduction of the changes that can happen after death Not no Sticky perfect fits all studies but study identifies the type of fixative

3-Must reduce the size of the sample to be fixation (2-4 mm) only

4-Fixative amount greater than 20 times of the size sample.

What happens to a piece of tissue from a living organism isolated not received special attention?

Lose a lot of water content by air and shrinks changed
Shrink or swell depending on the nature of the osmotic pressure of the solution
Exposed to the risk of bacteria
lysis automatically because they contain enzymes analyst

MENTION THE IMPORTANT OF FIXATIVES?

Installer: Maintain

solution is characterized by its ability to

- Prevents swelling or shrinkage
- Kills bacteria
- Inhibits the action of enzymes self-analysis
- Strengthens the tissues so that they can be buried and chipping
- Increases the speed of interaction with histological dyes

CONDITIONS SHOULD BE AVAILABLE IN THE FIXATIVE

Be fast permeability through parts of the sample
That converts soluble protein to insoluble materials
That have the ability to prevent bacterial decomposition and cellular self
Not cause any deformation cells

DEFINITION OF MICRO TECHNIQUE:

Micro technique :is the preparation and staining of tissue sections from surgical or autopsy material for microscopic study.

Cytology : is the study of individual cell microscopically.

Histology: is the study of specialized cell aggregates from definite structures with specific function.

SOURCES OF TISSUE

Tissue is taken from human or laboratory animals. Small specimen is taken 0.1 – 10 mm from selective area of tissue that contain significant changes that can be observed macroscopically (by naked eye examination) .

The specimen of tissue has specific term according to its source:

Biopsy: is the tissue taken from living animal or human being.

Autopsy or (necropsy) **: is the tissue taken from dead animal or human being.**

HISTOLOGICAL TECHNIQUE

Histological technique deals with the preparation of tissue for microscopic examination. The aim of good histological technique to preserve microscopic anatomy of tissue and make them hard, so that very thin section (4 to 5 micron)(**1000 micron = 1 millimeter**) can be made. After staining, the section should represent the anatomy of the tissue as close to as possible to their structure in life. This is achieved by passing the total as selected part of the tissue through a series of process.

These processes are:

1. Fixation
2. Dehydration
3. Clearing
4. Embedding
5. Cutting
6. Staining

FIXATION PROCESS

Before start of this process we making prefixation(Washing) with saline solution.

Fixation is usually the first stage in a multistep process to prepare a sample of biological material for microscopy or other analysis. Therefore, the choice of fixative and fixation protocol may depend on the additional processing steps and final analyses that are planned. For example, immunohistochemistry uses antibodies that bind to a specific protein target. Prolonged fixation can chemically mask these targets and prevent antibody binding. In these cases, a 'quick fix' method using cold formalin for around 24 hours is typically used.

Fixation: is the process by which the constituents of cells and tissue are fixed in a physical and partly also in a chemical state so that they will withstand subsequent treatment with various reagents with minimum loss of rchitecture. This is achieved by exposing the tissue to chemical compounds, call fixatives.

fixation is a chemical process by which biological tissues are preserved from decay, either through **autolysis** or **putrefaction**. Fixation terminates any ongoing biochemical reactions, and may also increase the mechanical strength or stability of the treated tissues.

Fixation preserves a sample of biological material (tissue or cells) as close to its natural state as possible in the process of preparing tissue for examination

AIM OF FIXATION:

1- Confers chemical stability on the tissue
2- Hardens the tissue (helps further handling)
3- prevent enzyme autolysis
4- Prevent bacterial putrefaction
5- May enhance later staining techniques
6- protect the cells from distortion and shrinkage when they are subjected to alcohols and hot paraffin.

Mechanism of action of fixatives:

Most fixatives act by denaturing or precipitating proteins which then form a sponge or meshwork, tending to hold the other constituents.

Good fixative is most important factors in the production of satisfactory results in

histopathology. Following factors are important:

- Fresh tissue
- Proper penetration of tissue by fixatives
- Correct choice of fixatives

No fixative will penetrate a piece of tissue thicker than 1 cm. For dealing with specimen thicker

than this, following methods are recommended:

1. Solid organ: Cut slices as necessary as but not thicker than 5 mm.
2. Hollow organ: Either open or fill with fixative or pack lightly with wool soaked in fixative.
3. Large specimen, which require dissection: Inject fixative along the vessels or bronchi as in case of lung so that it reaches all parts of the organs.

PROPERTIES OF AN IDEAL FIXATIVE

1. Prevents autolysis and bacterial decomposition.
2. Preserves tissue in their natural state and fix all components.
3. Make the cellular components insoluble to reagent used in tissue processing.
4. Preserves tissue volume.
5. Avoid excessive hardness of tissue.
6. Allows enhanced staining of tissue.
7. Should be non-toxic and non-allergic for user.
8. Should not be very expensive.
9. penetrate rapidly and kill the tissue to prevent post mortem changes.
10. Harden the tissues
    a. As the fixative comes into contact with the structural components of cells and tissues, especially the proteins, they become insoluble and precipitate out in place. This hardens the tissue.
    b. The hardening of the tissue protects against shrinkage and distortion during dehydration , embedding and sectioning .
11. Allow cell parts to become clear visible by means of dyes and improved refractive indices .

Temperature:

The fixation can be carried out at room temperature. Tissue should not be frozen once it has been placed in the fixative solution, for a peculiar ice crystals distortion will result.

Speed of fixation:

The speed of fixation of most fixative is almost 1 mm/hour. Therefore, a fixation time of several hours is needed for most specimens.

Amount of fixative fluid:

This should be approximately 10-20 times the volume of the specimen. Fixative should surround the specimen on all sides.

FACTORS AFFECTING FIXATION

1. Size and thickness of piece of tissue.
2. Tissue covered by large amount of mucous fix slowly.
3. The same applies to tissue covered by blood or organ containing very large amount of blood.
4. Fatty and lipomatous tissue fix slowly.
5. Fixation is accelerated by agitation.
6. Fixation is accelerated by maintaining temperature around 60oc.
7. pH Should be kept in the physiological range, between pH 4-9. The pH for the ultrastructure preservation should be buffered between 7.2 to 7.4
8. Osmolarity Hypertonic solutions give rise to cell shrinkage.

Hypotonic solutions result in cell swelling and poor fixation.

9. Temperature: Increasing the temperature, as with all chemical reactions, will increase the speed of fixation,. Hot formalin will fix tissues faster, and this is often the first step on an automated tissue processor. However, care is required to avoid cooking the specimen.

10. Concentration of fixative: should be adjusted down to the lowest level possible, because you will expend less money for the fixative. Formalin is best at 10%; glutaraldehyde is generally made up at 0.25% to 4%. Too high a concentration may adversely affect the tissues and produce artifacts.

11.Volume of the Fixative

At least 15-20 times greater than tissue volume

12.Duration: As a general rule 1hr per 1mm

13.time interval from of removal of tissues to fixation: The faster you can get the tissue and fix it, the better. Artifacts will be introduced by drying, so if tissue is left out, please keep it moist with saline. The longer you wait, the more cellular organelles will be lost and the more nuclear shrinkage and artifactual clumping will occur.

14. the rate of penetration of the fixing fluid

CLASSIFICATION OF FIXATIVES

A. Tissue fixatives

a. Buffered formalin

b. Buffered gluteraldehyde

c. Zenker's formal saline

d. Bowen's fluid

B. Cytological fixatives

a. Ethanol

b. Methanol

c. Ether

C. Histochemical fixatives

a. Formal saline

b. Cold acetone

c. Absolute alcohol

METHODS OF FIXATION

There are generally four types of fixation process:

1. **Heat fixation:** After a smear has dried at room temperature, the slide is gripped by tongs or a clothespin and passed through the flame of a Bunsen burner several times to heat-kill and adhere the organism to the slide.

2. **Perfusion:** Fixation via blood flow. The fixative is injected into the heart with the injection volume matching cardiac output. The fixative spreads through the entire body, and the tissue doesn't die until it is fixed. This has the advantage of preserving perfect morphology, but the disadvantages that the subject dies and the cost is high (because of the volume of fixative needed for larger organisms)

3. **Immersion:** The sample of tissue is immersed in fixative of volume at a minimum of 20 times greater than the volume of the tissue to be fixed. The fixative must diffuse through the tissue to fix, so tissue size and density, as well as type of fixative must be considered. Using a larger sample means it takes longer for the fixative to reach the deeper tissue.

4. **Chemical Fixation**

In this process, structures are preserved in a state (both chemically and structurally) as close to living tissue as possible. This requires a chemical fixative that can **stabilize** the proteins, nucleic acids and mucosubstances of the tissue by making them **insoluble**.

TYPES OF CHEMICAL FIXATIVES

By far the most commonly used fixative in histology is formaldehyde. It is usually used as

10% Neutral Buffered Formalin (NBF), that is aprox. 3.7% formaldehyde in phosphate buffered saline. Because formaldehyde is a gas at room temperature, formalin=formaldehyde gas dissolved in water (~37% w/v)-is used when making the former fixative.

Routine Formalin:

Formalin is sold as 40% w/w solution of formaldehyde gas in water. It is used as 10% solution in water or normal saline. It does not precipitate protein but combine with NH2 group to form an insoluble gel, preserve particularly all elements including fats. It keeps phospholipids insoluble in fat solvents. Tissue can remain in it for prolonged periods without distortion. It is compatible

with most special stain. It is the cheapest and most popular fixative.

Another popular **aldehyde** for fixation is glutaraldehyde. It operates in a similar way to formaldehyde by causing deformation of the alpha-helix structures in proteins. However glutaraldehyde is a larger molecule, and so its rate of diffusion across membranes is slower than formaldehyde. Consequently glutaraldehyde fixation on thicker tissue samples may be hampered, but this problem can be overcome by reducing the size of the tissue sample. One of the advantages of glutaraldehyde fixation is that it may offer a more rigid or tightly linked fixed product—its greater length and two aldehyde groups allow it to 'bridge' and link more distant pairs of protein molecules. It causes rapid and irreversible changes, fixes quickly, is well suited for electron microscopy, fixes well at 4°C, and gives best overall cytoplasmic and nuclear detail. However it is not ideal for immunohistochemistry staining.

Some fixation protocols call for a combination of formaldehyde and glutaraldehyde so that their respective strengths complement one another.

glutaraldehyde advantages and disadvantages

Formalin advantages and disadvantages

2. ACOHOLS:

The most common precipitating fixatives are ethanol and methanol. They are commonly used to fix frozen sections and smears. Acetone is also used and has been shown to produce better histological preservation than frozen sections when employed in the Acetone Methylbenzoate Xylene (AMEX) .technique

The protein denaturants - methanol, ethanol and acetone - are rarely used alone for fixing blocks unless studying nucleic acids.

Ethanol, are used primarily for cytology smears. Ethanol (95%) is fast and cheap. Since smears are only a cell or so thick, there is no great problem from shrinkage, and since smears are not sectioned, there is no problem from induced brittleness

For fixing frozen sections, you can use just about anything--though methanol and ethanol are the best.

a. It shrinks, distorts and hardens tissue

b. Is generally used only when combined with other fixatives

Acetic acid is a denaturant that is sometimes used in combination with the other precipitating fixatives. The alcohols, by themselves, are known to cause considerable shrinkage and hardening of tissue during fixation while acetic acid alone is associated with tissue swelling; combining the two may result in better preservation of tissue morphology.

3. OXIDISING AGENTS

The oxidizing fixatives can react with various side chains of proteins and other biomolecules, allowing formation of crosslinks that stabilize tissue structure. However they cause extensive denaturation despite preserving fine cell structure and are used mainly as secondary fixatives.

Osmium tetroxide is often used as a secondary fixative when samples are prepared for electron microscopy. (It is not used for light microscopy as it penetrates thick sections of tissue very poorly.)

Potassium dichromate, chromic acid, and potassium permanganate all find use in certain specific histological preparations.

4.MERCURIALS

Mercurials such as B-5 and Zenker's have an unknown mechanism that increases staining brightness and give excellent nuclear detail. Despite being fast, mercurials penetrate poorly and produce tissue shrinkage. Their best application is for fixation of hematopoietic and reticuloendothelial tissues. Also note that since they contain mercury care must be taken with disposal.

Mercuric chloride fixatives:

1.Helly's fluid - fixation time 12-24 hours.

2.Zenker's fluid - fixation time 4-24 hours.

Advantages and disadvantages of mercuric chloride fixatives (Zenker's & Helly's fluids)

5.PICRATES

Picrates penetrate tissue well to react with histones and basic proteins to form crystalline picrates with amino acids and precipitate all proteins. It is a good fixative for connective tissue, preserves glycogen well, and extracts lipids to give superior results to formaldehyde in immunostaining of biogenic and polypeptide hormones However, it causes a loss of basophilia unless the specimen is thoroughly washed following fixation

picric acid fixatives:-

1. Bouin's fluid - fixation time 6 hours.
2. Gender's fluid

Advantages and disadvantages of picric acid fixatives (Bouin's & Gender's fluids)

6.HOPE FIXATIVE

hepes-glutamic acid buffer-mediated organic solvent protection effect (hope) gives formalin-like morphology, excellent preservation of protein antigens for immunohistochemistry and enzyme histochemistry, good rna and dna yields and absence of crosslinking proteins.

7.FROZEN SECTIONS

small pieces of tissue (5x5x3mm) are placed in a cryoprotective embedding medium - oct, tbs or cryogel - then snap frozen in isopentane cooled by liquid nitrogen. tissue is then sectioned in a freezing microtome or cryostat. sections are then fixed in one of the following fixatives: absolute acetone for 10-15 minutes, 95% ethanol for 10-15 minutes or absolute acetone 10minutes followed by 95% ethanol 10 minutes

frozen sections are prepared using a cryostat.
a cryostat is a cooled chamber, or cabinet that houses an instrument to section. frozen samples; a rotary microtome and knife (or blade) holder, and a means to freeze samples.

Advantages and Disadvantages of Frozen Sections ???????

8-CARNOY'S FLUID – FIXATIVE (TIME 1-3 HOURS)

Advantages and Disadvantages OF Carnoy's fluid

POST FIXATION:- WASHING

Washing tissue after fixation:-

1-After the tissue is fixed for the proper length of time, excess fixative must be washed out to prevent over-fixation. Washing also removes substances in the fixative which might interfere with the subsequent processing.

2- Since most fixatives are aqueous solutions, the washing is usually carried out for a specific period of time in tap water or isotonic saline solution.

GENERAL RULES FOR WASHING

Aqueous fixatives

1-Aqueous solutions containing any of the following :-

Potassium dichromate , and formalin ; wash in water.

2. Aqueous solutions containing picric acid or mercuric chloride such as Zenker's fluid ; wash in 70% alcohol.

3. Boun's fluid : wash in 70% alcohol.

B. Alcoholic fixatives; these are always to be washed with alcohol.

Washing in water

This is the most efficient and least troublesome method for objects which are of sufficient size and firmness to withstand such treatment.

Washing in water

1. Washing is generally continued until all or nearly all of the uncombined fixative has been removed .
2. Tissue should not remain in water longer than is necessary, because they become softened or even partially disintegrated by prolong soaking.
3. The approximate time required for thorough washing in running water is 3 -6 hrs. for small and permeable about slices of tissue objects , and 6 – 12 hrs. up to 5 mm thick and 18 – 24 hrs. for larger or less permeable objects.
4. After fixation with formalin , a few minutes in water will most purposes , or the alcohol used for suffice for dehydration can be counted on to remove the fixative.

Washing in alcohol

1. Following alcoholic fixatives, the general rules is to transfer material to alcohol of about the same percentage contained in the fixing fluid or the next lower percentage, change this several times before replacing it with stronger or weaker alcohol.

2. Following Carnoy's acetic acid-alcohol or the chloroform (Carnoy II), modification containing transfer to 95% alcohol, change this for two to three times at intervals of one to several hrs.

3. For the most formalin acetic acid – alcohol mixture , transfer material to 70% alcohol.

Washing of mercuric chloride

(Zenker's and Helly's fluids, wash material in running water for 12 to 24 hrs, then place it successively in 30 , 50 , and 70% alcohols).

Iodine solutions for mercury deposits removal.

Washing for aqueous picric acid solution

Picric acid washed out with alcohol not weaker than 50% and preferably in 70% alcohol.

Tissue Processing

Tissue processing is a long procedure and required 24 hours. Tissue processing can be done by manually or mechanically.

Manual Tissue Processing:

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

Mechanical Tissue Processing:

Automatic tissue processors are available. In this processor, there are different jars containing reagents. These are arranged in a sequence. The tissue is moved from one jar to another by a mechanical device. Timings are controlled by a timer which can be adjusted in respect to hours and minutes. Temperature is maintained around 60°C.

The processing, whether manually or mechanically, involves the same steps.

Dehydration

Tissues are dehydrated by using increasing strength of alcohol; e.g. 50%, 70%, 90% and 100%. The duration for which tissues are kept in each strength of alcohol depends upon

1.the size of tissue,

2. fixative used

3. type of tissue

to be dehydrated slowly starting in 30% ethyl alcohol directly whereas most tissue specimens may be put into 70% alcohol. Delicate tissue will get high degree of shrinkage by two great concentration of alcohol.

The volume of alcohol should be 50-100 times that of tissue

1.Tissues contain much "free" water which does not mix well with the paraffin used later in the procedure. Therefore, water in the tissues must be removed by submerging the tissue in alcohol, a process known as alcohol dehydration.

2.If this is done too rapidly, the large out flow of water can damage the morphology of the cells and tissues.

3.In this step, tissue is placed into a series of gradually increasing concentrations of alcohol, usually ethyl alcohol (30, 50, 70 ,80, 95, and 100%), for specific periods of time.

Dehydrating agents

1.Alcohol

Advantages of alcohol:-

They are fast acting

Non toxic

Reliable

Disadvantages?????

2.Dioxane (diethylene dioxide)

Dioxane is mixable with water, ethyl alcohol and melted paraffin. It is inflammable as ethyl alcohol and its vapours are toxic, and should be used in a well ventilated room.

Advantages of Dioxane:

Tissue can be left in this reagent for long time without affecting the consistency of the tissue or its staining properties

It is readily miscible with water ,alcohol , xylene and paraffin

Produce less shrinkage than alcohol

It both dehydrate and clear

It can be used as final dehydrator only in place of absolute alcohol

Disadvantages are?????

3.Acetone

Time recommended for dehydration of tissue is 20 minutes in 4 fresh changes of acetone.

Advantages of Acetone:

It is very rapid in action

Less expensive than some of other dehydrants

Disadvantages are?????

Clearing
(Removal of Alcohol)

During dehydration water in tissue has been replaced by alcohol. The next step alcohol should be replaced by paraffin wax. As paraffin wax is not alcohol soluble, we replace alcohol with a substance in which wax is soluble. This step is call clearing.

The following reagents of clearing are:-

- Xylene
- Chloroform
- Benzene
- Carbon tetrachloride
- Toluene

Impregnation with Wax
(infiltration)

This is allowed to occur at melting point temperature of paraffin wax, which is 54-60oC. Volume of wax should be about 25-30 times the volume of tissues. The duration of impregnation depends on size and types of tissues and the clearing agents employed. Longer periods are required for larger pieces and also for harder tissue like bones and skin as compared to liver kidney, spleen,lung etc. Xylene is easiest way to remove. Total duration of 4 hours is sufficient for routine impregnation.

Infiltration: is to impregnate the tissue with supporting medium to facilitate its cutting by microtome knife.

Types of Wax employed for Impregnation:-

1. Paraffin wax
2. Water soluble wax
3. Other material, like colloidin, gelatin, paraplast etc.

Procedures of infiltration:-

1. after tissue specimens have been completely dehydrated and cleared , they are immersed in melted paraffin for 2 – 4 hours .
2. two or more changes of paraffin are required to eliminate traces of the solvents which prevent the paraffin from hardening properly.
3. the liquid paraffin infiltrate the tissue , and when cold , it will solidified and provide the supporting necessary for cutting thin sections.
4. rapid chilling of the melted paraffin is recommended on the principle that slow cooling of a liquid which normally crystallize on cooling will proved a fine crystalline structure capable of fitting closely to the individual cells , thus providing adequate support.

Embedding (Blocking)

Impregnated tissues are placed in a mould with their labels and then fresh melted wax is poured in it and allowed to settle and solidify. Once the block has cooled sufficiently to form a surface skin it should be immersed in cold water to cool it rapidly.

After the block has completely cooled it is cut into individual blocks and each is trimmed. Labels are made to adhere on the surface of the block by melting the wax with a metal strips sufficiently wormed


TISSUE EMBEDDING CENTER


ARAPLAST

Embedding:

a. is to make a block of embedding media with the tissue inside it.

b. Or is the enclosing of the tissue or specimen in a solid mass of the embedding medium .

1-The surface of the section to be cut should be placed parallel to the bottom of the container in which it is cast .

2-To orient the tissue for proper embedding , you must notch with a scalpel or mark with India ink the side of the tissue opposite that to be cut.

Embedding media

Embedding media: are all materials used by histological technique to infiltrate , support and enclose specimens which are to be cut into thin sections by a device or instrument called microtome.

Characters of embedding media

1.must be capable of being converted readily from liquid to solid form.

2. in the liquid form the embedding media must penetrate easily into the interstices of the tissue , and then is converted into solid.

The conversion into solid may be brought about by:

a-Crystallization (paraffin)

b-Evaporation of solvent (celloidin)

c-Polymerization (plastics)

Paraffin Wax

- The paraffin used in histology laboratory is a refined , white , filtered paraffin , to which has been added :
- 1. beeswax and Paraffin wax 2. Paraplast 3. Paraplast plus 4. Gelatin 5. Celloidin , to facilitate riboning:
- Paraffin is sold at room temperature . Heat renders paraffin fluid , so that it can permeate the tissue.
- Hardness of paraffin used for infiltration is matched to the hardness of the tissue.
- Cakes of paraffin are placed in clean metal or enamel pitchers and melted down in a paraffin oven regulated at a temperature just above the melting point of paraffin.
- The melted paraffin is filtered within the oven by coarse filtered paper and is then ready to use (to reduce injury to the knife edge when sectioning the tissue.

BLOCKING

Types of Moulds:

Leuckhart's Moulds: L- shaped brass pieces which is placed in opposing positions & can be manipulated to increase or decrease the size of the block to be prepared.

B. Glass or Metal petri dishes.

C. Watch glass

D. Paper boats.

Orientation of specimen

Orientation of specimen

1- Select proper size mold to allow segments of specimen to be embedded all flat to the bottom of the container and still have a margin of few millimeter around all edges. The mold must also be deep enough to allow paraffin to be added to about twice the thickness of the specimen.

2- Specimen without layers: all pieces of tissue should be embedded firmly to the bottom of the container so that the cut section will present a valid representation of the tissue submitted.

3- Stratified tissue like cyst wall, gallbladder wall, and skin section must be carefully oriented on the edge so that the side of the tissue to be sectioned is positioned vertically to the bottom of the mold.

4- Light and loose texture materials such as cell block and scanty endometrial curetting are best stirred up toward the center of the mold with wormed forceps while the paraffin is still worm .

5- It is best not to embed a very small soft specimen in the same mold with a large dense block.

Trimming

The appropriate size mold should be used whenever possible. A margin of paraffin in excess of a few millimeters around the tissue is unnecessary and may cause compression , as the sections are cut.

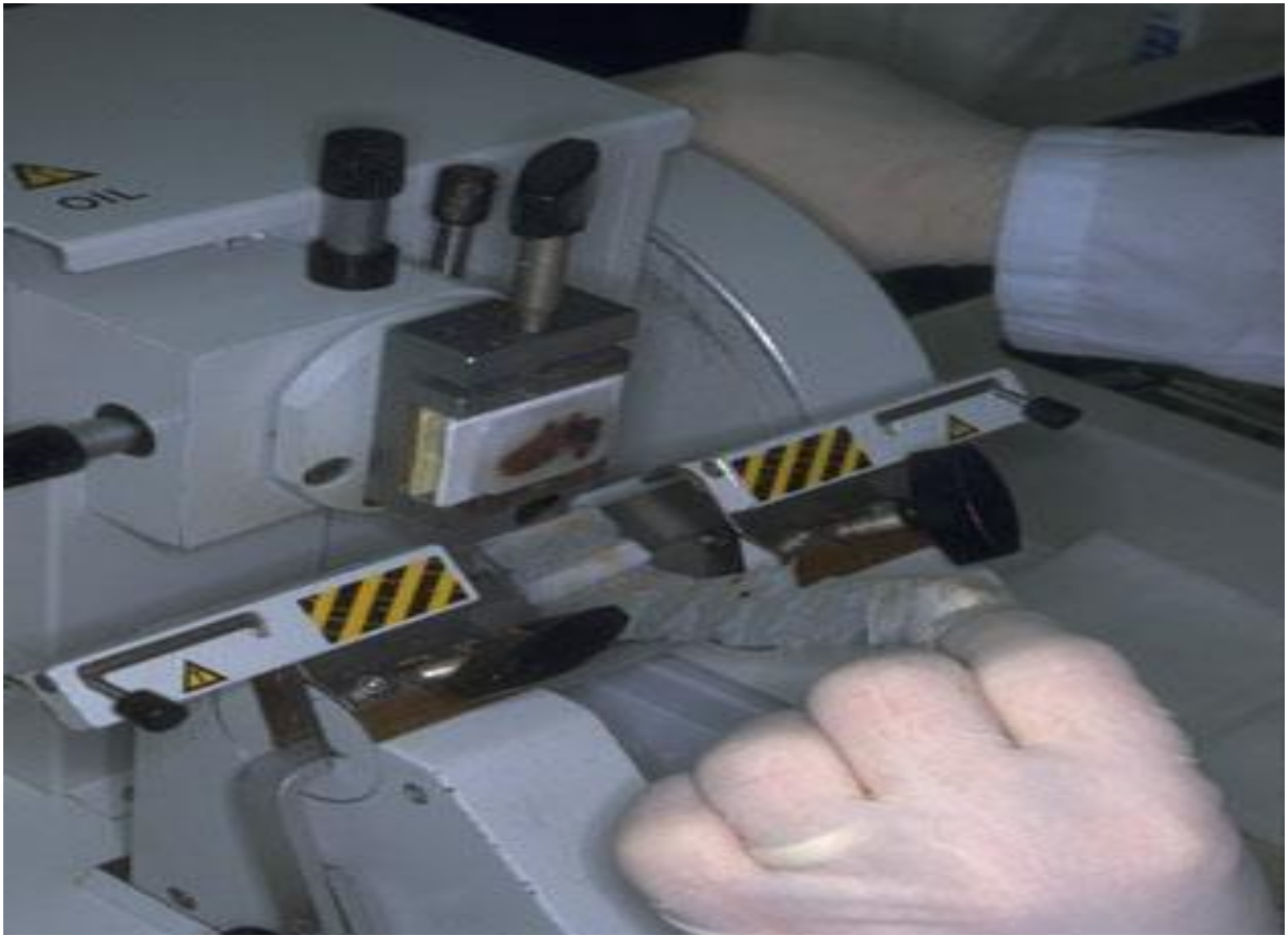
If paraffin surround the tissue in excess of two millimeters around the object, it may be trimmed after it is mounted on the microtome with razor blade.

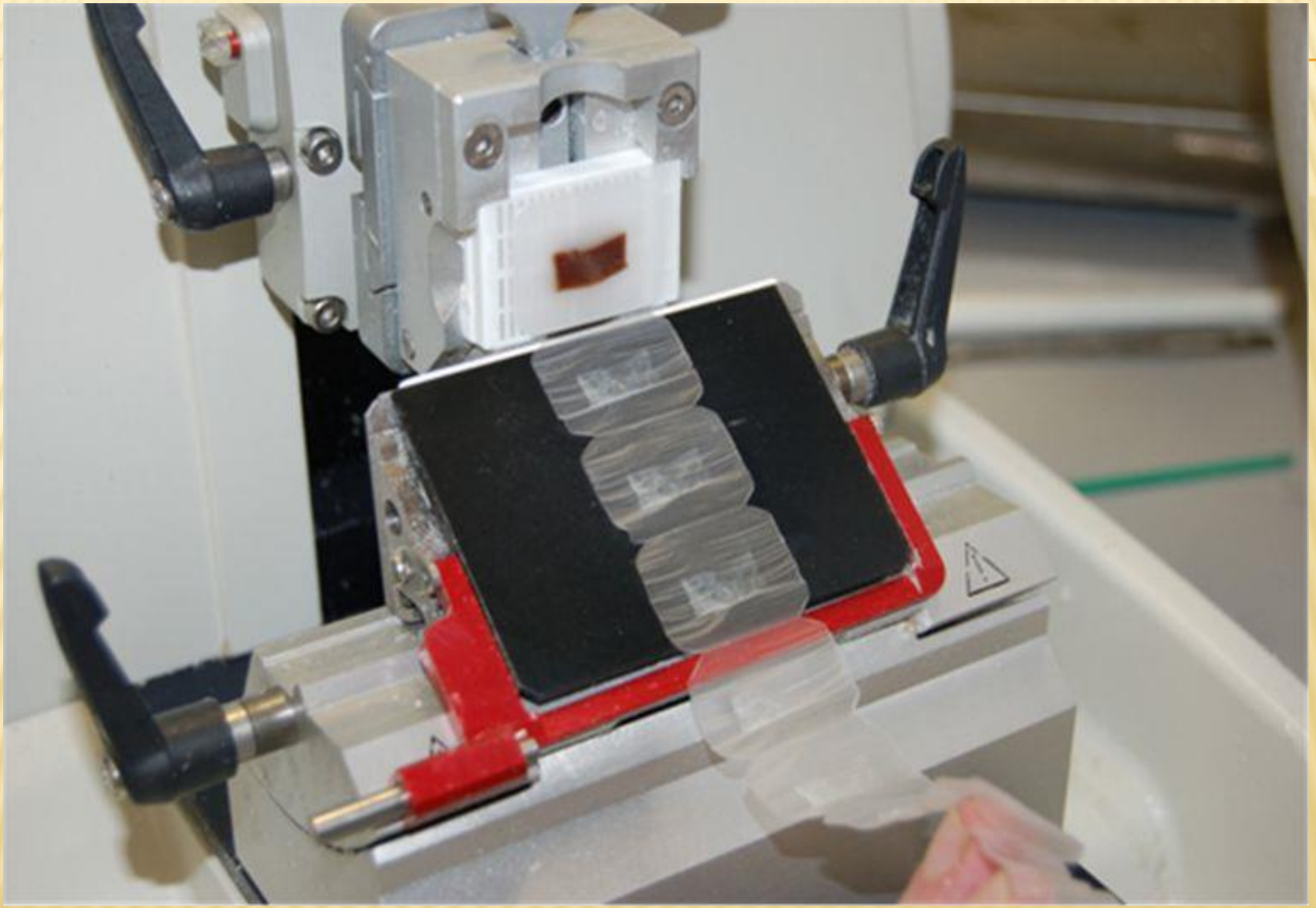
Cutting

TYPES OF MICROTOMES

- Sliding
- Rotary
- Rocking
- Freezing
- Base sledge

Microtome





Rotary Microtome: It is the most commonly used. Also known as Minnot's Rotary microtome. In this the Block holder moves up and down while the knife remains fixed. It is suitable for cutting of small tissues & serial sections can be taken on it.

DIFFICULTIES MOST COMMONLY ENCOUNTERED DURING CUTTING RIBBON

These are found in the table of your book!

Difficulties	Causes
1. failure of block to Ribbon	a. block not parallel to knife edge b. knife dull c. knife tilted too much d. paraffin too hard sections too thick
2. uneven and crocked ribbon trimmed block irregularity in knife edge	a. wedge-shaped or irregularly b. edge of block not parallel to knife edge c. irregularity in knife edge d. paraffin not homogenous
3. compressed , Wrinkled مجعد sections	a. knife dull b. paraffin block too worm c. paraffin on knife edge d. sections too thin e. microtome screws are loose
4. tearing and crumbling تمزيق وانكسارof sections	a. incomplete fixation of tissue b. incomplete dehydration or clearing of tissue c. incomplete infiltration of tissue with paraffin d. paraffin too hot for bath and or embedding

TISSUE FLATTENING

DRYING SLIDES IN DRYING OVEN

Three ways to drying slides!!!!!!!!!

What are the differences between drying slides & fixed slides?

FREEZING SECTION

Commonly, researchers section either frozen tissue with a cryostat, or sliding microtome with freezing stage, or soft tissue with a vibrating microtome.

CRYOSTAT

This cryostat a device that houses a microtome maintained at -20ºC to -30ºC, which is used to cut tissues obtained intraoperatively.

CRYOSECTIONING TISSUES

This section is discussion of the methods that produce sections without the use of dehydrating and clearing solutions , and without embedding media. Frozen sections have many uses including the demonstration of soluble substances, histochemical analysis and intraoperative diagnosis

PROCEDURE

Getting started

1-The cryostat is always on (image at right); before starting, check that the temperature is correct for your tissues.


Front Panel Controls
Storage shelf
All surfaces splashproof and easily wipeable
Side Panel Controls
Mains ON/OFF
Defrost ON/OFF
Defrost timer
UV light socket (optional)
Drive Controls
Motorised rewind /advance (on LS & HS)
Motor drive controls (on HS)
Automatic demist fan upon window closure (shown retracted)
Contoured handle
Contoured surfaces for more comfortable user operation
Footswitch socket

2- Place the tissue block on the cryostat specimen disk. Adjust the positioning of the block to align the block with the knife blade. Cut tissue block until the desired tissue is exposed.

3- Cut sections of the desired thickness (usually 5 µm), place the sections on a Fisher Superfrost slide and dry overnight at room temperature (RT).

4- Cut sections of the desired thickness (usually 5 µm), place the sections on a Fisher Superfrost slide and dry overnight at room temperature (RT).

5- Fix slides by immersion in cold acetone (-20°C) for 2 minutes or other suitable fixative (e.g. alcohol, formal alcohol, formalin, etc.), air dry at RT and proceed to staining.

6- Alternatively, the frozen section slides can be stored for a short period of time at -70°C in a sealed slide box. When ready to stain, remove slides from freezer and warm to -20°C in the cryostat or -20°C freezer, fix for 2 minutes in cold fixative (acetone or other suitable fixative) and allow to come to RT to continue with the staining.

If the chuck is very close to the knife holder, retract it by pressing the rapid retract button to the left, outside the chamber – one of the top buttons shown at right. The top button retracts the chuck fully, the next one retracts it as long as you press the button. The other two buttons advance the chuck towards the knife.

Now place the stub plus tissue into the chuck. The stub is held in place by tightening the black tightening screw sticking out to the left.

Position the stub so the specimen is in the desired orientation for sectioning.

7- There will probably be a cryostat blade in the knife holder, as shown below – it can be used for trimming the frozen block until you reach the tissue.

8- Once the stub is in the holder and oriented, fold back the anti-roll plate for trimming. Set the section thickness to at least 20-30 µm. Close the chamber lid.

9- Bring the knife close to the specimen by pressing the rapid advance buttons outside the chamber. Use the advance handle (wheel on right hand side of cryostat) to raise and lower the specimen as you are positioning the knife close to it.

10- Rotating the advance handle (on right hand side of machine) clockwise will move the tissue up and down past the knife. If the tissue is close enough, a section of tissue will be cut and stay on the knife edge. The chuck will also bring the tissue closer to the knife ready for the next section. Keep the anti-roll bar folded back, and sweep away trimmings until you are ready to collect sections.

The maximum automatic advance is 80 µm – set the advance mechanism to the desired section thickness using dial in front of the knife.

Softer tissues can be sectioned much more thinly – down to a few micrometers.

When you are ready to collect sections, you will need to either move the knife so a fresh zone will cut the tissue, or replace the knife with a new one. For good sections, a sharp knife is essential. Discard blades as soon as they are blunt – they are much cheaper than your precious samples! Follow the specific instrument instructions for knife exchange.

SECTIONING AND COLLECTING SECTIONS

Always close the chamber lid except when you need to reach in to make adjustments or change samples. Sectioning proceeds much more smoothly when the cryostat is not struggling to maintain temperature with the lid open. Keeping the lid closed also prevents buildup of frost inside the chamber and on your sample.

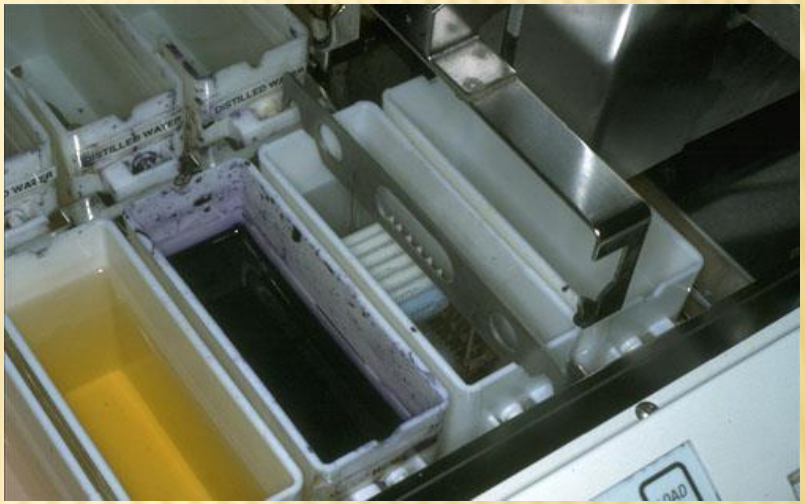
11- When you are ready to collect sections, you need to fold the anti-roll bar in place so it is resting on top of the knife edge.
Now, when you rotate the advance handle, the section should slide under the glass shield and stay flat rather than rolling up. If the section does not do this, use the screw at the back of the anti-roll plate to move it incrementally forwards or backwards until you get good sections. The position of the anti-roll plate is absolutely critical to collecting good, flat sections.

12- Only cut one section at a time.
To collect the sections, lower a sticky slide, sticky side down, onto the section. When the slide gets near the section it will start to melt onto the slide, at which time you can raise the slide and the section should come with it. Depending on how you want to process the section, either air-dry onto the sticky slide, protecting from dust under a large petridish, or allow to dry off slightly, mount in mounting medium (e.g. water) and cover with a coverslip.

13- Once you have finished sectioning, remove specimens from stubs and clean stubs, and place stubs back into machine.
Wipe out any residue left in the machine during sectioning. The chamber must be left as clean as possible for the next user.

Staining

SLIDES STAINING MACHINES



AIM OF STAINING: TO IDENTIFY DIFFERENT TISSUE COMPONENTS BY THEIR COLOR REACTION

Theory of staining:-

1- The physical theory: by osmosis , capillary force , adsorption , absorption , diffusion or by precipitation by acid bases.

2- The chemical theory: had certain affinities for some portions of the protoplasm

STAINING REACTIONS

1- Direct stain

2- Indirect stain

3- Physical stain

4- Chemical stain

What's Mordant?????

Counter stain

The use of one or more additional stains to show the other components of the tissue, these additional stains are called counter stains.

FACTORS INFLUENCING STAINING REACTIONS

1- The components of the fixative used (reaction is intensified with formulas containing picric acid and potassium dichromate, as in Bouine, Hellys and Zenkers fluids)

2- pH of the fixative

3- pH of solutions

4- mordant

5- chemical or reagents which produce oxidation or reduction.

BASIC STAINING RULES

1- keep stains and solutions covered when not in use
2- filter stains before use
3- after the slides are removed from the drying oven, allow them to come to room temperature before placing in xylene (dewax).
4- once the slides have been placed in first xylene to remove the paraffin, do not allow them to dry out.
5- Make certain that the level of any solution used in staining, completely covers the tissue on the slide
6- Re-new water bath after each rack of slides that has been processed .
7- Drain all slides before removing on to the next solution.
8- Use the microscope for quality control.

METHODS OF STAINING

Staining methods may be grouped as follows:

1- Vital staining

2- Routine staining

3- Special staining

PROCEDURE OF STAINING

Every stain is to be used according to a specified method. Staining can be done either manually or in an automatic stained.

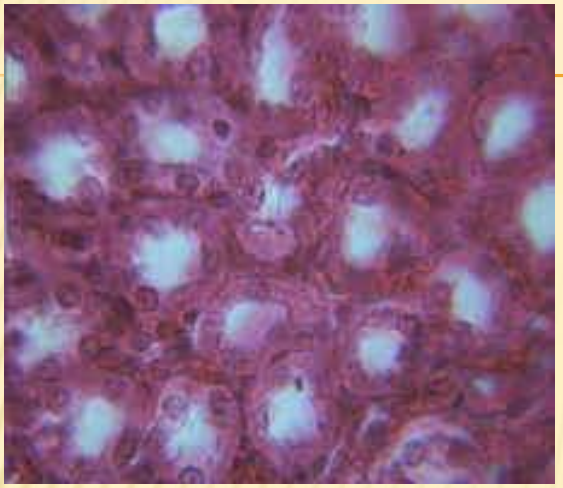
Manual Staining:

In a small laboratory when a few slides are stained daily, this is the method of choice. Although it is time consuming it is economical. Different reagent containers are placed in a special sequence and the slides are removed from one container to another manually.

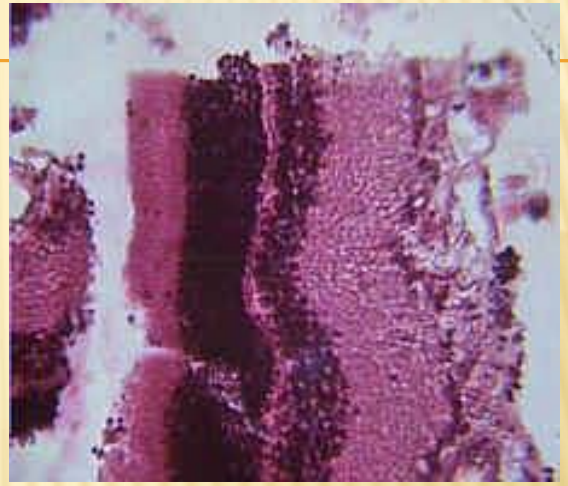
Automatic staining:

STAINING PROCEDURES

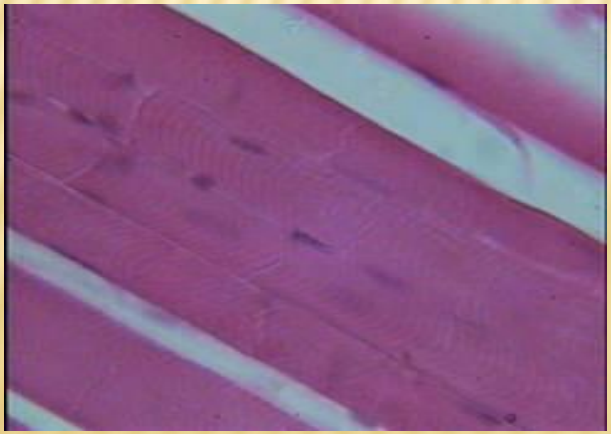
Step	time
Dewax in xylene	
Hydration by series alcohols then bring sections down to water	
Hematoxylin solution.	rinse
Wash in running water	3 min.
Eosin	rinse
D.W	
Dehydrate in ethanol and clear with xylene	
Mount in DPX or Canada balsam	



Rat kidney thin section, showing proximal and distal tubules. 400X magnification, 10 um c.s., hematoxylin-eosin staining.

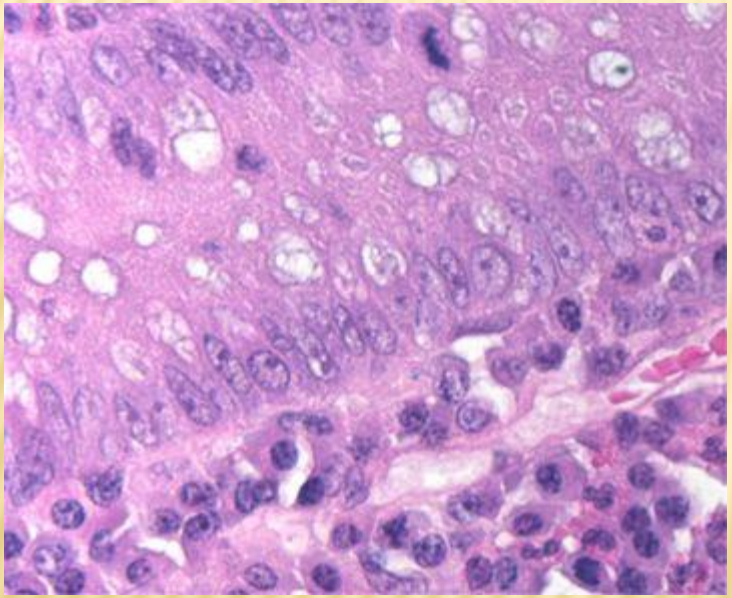


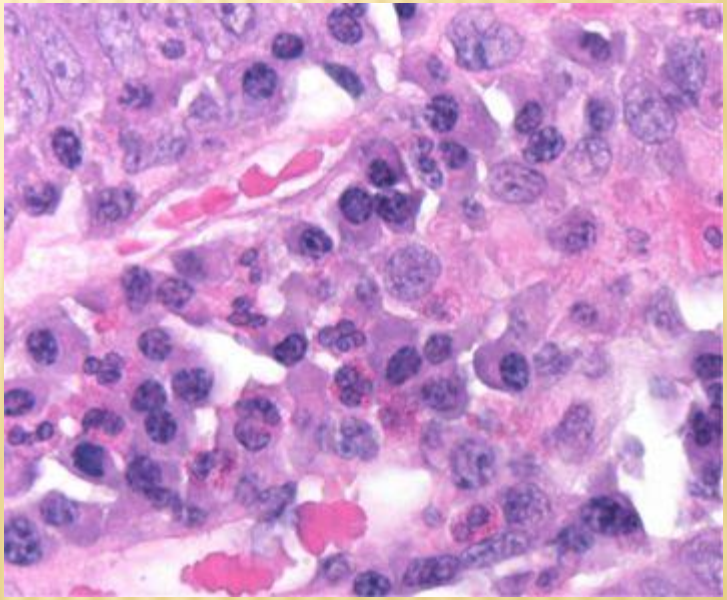
Rat eye thin section, showing the retinal layers. 400X, 10 um c.s., hematoxylin-eosin staining.



Thin section of chicken skeletal muscle showing striations. 1000X, 10 um l.s., hematoxylin-eosin staining

1-THIS SECTION FROM THE MUCOSA OF SMALL INTESTINE 2- LAMINA PROPRIA OF SMALL INTESTINE





PROBLEMS OF STAINING, CAUSES AND SOLVENTS

Problems in staining, causes and solvents

1-Blue—black precipitate on top of the sections. The metallic sheen that develops on most hematoxylin solutions has been picked up on the slide. Filter the hematoxylin solution daily before staining slides.

2-Water bubbles are seen microscopically in the stained sections. The sections were not completely dehydrated, and water is present in the mounted section. Remove the cover glass and mounting medium with xylene. Return the slide to fresh absolute alcohol (several changes). After the sections are dehydrated, clear with fresh xylene and mount with synthetic resin. All dehydrating and clearing solutions should be changed before staining any more sections.

3-Difficulty bringing some areas of the tissue in focus with light microscopy. Mounting medium may be present on top of the cover glass. Remove the cover glass and remount with a clean cover glass. Review the method used for mounting sections, and modify if needed.

4-The mounting medium has retracted from the edge of the cover glass. A. The cover glass is warped. B. The mounting medium has been thinned too much with xylene. A. Remove the cover glass and apply a new cover glass. B. Apply a new cover glass with fresh mounting medium. Keep the mounting medium container tightly capped when not in use. Use a small container for the mounting medium and discard when it becomes too thick.

5-The water and the slides turn milky when the slides are placed in the water following the rehydrating alcohols. Xylene has not been removed completely by the alcohols. Change the alcohols, back the slides up to absolute alcohol, and rehydrate the sections.

6-The slides are hazy or milky in the last xylene rinse prior to coverslipping. Water has not been completely removed from the sections before being placed in the xylene. Change the alcohol solutions, especially the anhydrous or absolute reagents. Redehydrate the sections and clear in fresh xylene.

7- The mounted stained sections do not show the usual transparency and crispness when viewed by light microscopy. The mounting medium may be too thick, causing the cover glass to be held too far above the tissue. Remove the cover glass and mounting medium with xylene. Remount the section with fresh mounting

THANCK YOU