Module X.
Biological and Histological Techniques.

- Histological technique deals with the preparation of tissue for microscopic examination.

- The aim of good histological technique to preserve microscopic anatomy of tissue.

FIXATION

- In the fields of histology, pathology, and cell biology, fixation is a critical step in the preparation of histological sections by which biological tissues are preserved from decay, thereby preventing autolysis or putrefaction.

- Fixation terminates any ongoing biochemical reactions, and may also increase the mechanical strength or stability of the treated tissues.

- **Definition:** It is a complex series of chemical events which brings about changes in the various chemical constituents of cell like hardening, however the cell morphology and structural detail is preserved.

- Unless a tissue is fixed soon after the removal from the body it will undergo degenerative changes due to autolysis and putrefaction so that the morphology of the individual cell will be lost.

Principle of fixation

- The fixative brings about crosslinking of proteins which produces denaturation or coagulation of proteins so that the semifluid state is converted into semisolid state; so that it maintains everything in vivo in relation to each other. Thus semisolid state facilitate easy manipulation of tissue.

- For practical purposes fixation aims to prevent or arrest the degenerative processes which commence as soon as a tissue is deprived of its blood supply.

- Autolysis, which results in tissue digestion by intracellular enzymes released when organelle membranes rupture, and bacterial decomposition or putrefaction which is brought about by micro organisms which may already present in the specimen, are processes that must be prevented.

- It does this by stopping enzyme activity, killing microorganisms and hardening the specimen while maintaining sufficient of the molecular structure to enable appropriate staining methods to be applied (including those involving antigen-antibody reactions and those depending on preserving DNA and RNA).

- The sooner fixation is initiated following separation of a specimen from its blood supply the better the result will be.

- The broad objective of tissue fixation is to preserve cells and tissue components and to do this in such a way as to allow for the preparation of thin, stained sections.

- 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.

Aims and Effects of fixation

- If a fresh tissue in kept as such at room, temperature it will become liquefied with a foul odour mainly due to action of bacteria i.e. putrefaction and autolysis so the first and fore most aim of fixation is

1. To preserve the tissue in as lf like manner as possible.

2. To prevent postmortem changes like autolysis and putrefaction.

3. To prevent the loss of cellular constituents.

4. To increase optical differentiation of cellular structures.

5. To increase tissue consistency – especially for slicing

Theoretical basis of fixation

- Fixation may be considered “a complex series of chemical events”.
- Although we can now define some of these “events” our understanding of much of what happens during fixation is still incomplete.

- Some tissue elements will chemically react with the fixative, be stabilised by cross-linking and thus preserved, others may be unaffected by the fixative but trapped within a cell or tissue by other fixed elements.

- Cells and extracellular components contain peptides and proteins, lipids and phospholipids (membranes), carbohydrates and carbohydrate complexes, various types of RNA and DNA and so on.

- Just how these elements will react during fixation will depend on the type of fixation, the fixing agent used and the fixation conditions.

Fixatives classification

- Physical – heat, microwaves
- Chemical:
  - Aldehydes – formaldehyde, glutaraldehyde, acrolein
  - Oxidizing agents – osmium tetraoxide, potassium permanganate, potassium dichromate
  - Protein denaturating agents – acetic acid, methyl alcohol, ethyl alcohol
  - Miscellaneous – mercuric chloride, picric acid, non aldehyde containing fixatives

Qualities of a good fixative

- Destroys microorganisms
- Extracts inactivated autolytic enzymes
- Increases tissue consistancy
- Confers optical differentiation
- Maintains its chemical composition
- Cheap, nontoxic, nonflamable, nonirritant

Qualities of a good fixative

- Good tissue penetration
- Stabilizes the tissue, preserving the character and distribution of cellular components
- Prevents fixation artefacts
- Prevents structure deformation – maintaining shape and volume
- Preservs cellular constituents

Basic factors affecting chemical fixation

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pH (Isoelectric point)

Total ionic strength of reagents

Osmolarity

Temperature

Length of fixation

Method of application of fixative

- The most popular fixing agent is formaldehyde, usually in the form of a phosphate-buffered solution (often referred to as “formalin”).
- Ideally specimens should be fixed by immersion in formalin for six to twelve hours before they are processed.

- Formalin is the most commonly used fixative in histopathology.

- Formaldehyde is a gas (formula HCHO). It dissolves readily into water which is called formalin. Formaldehyde dissolves into water to 37-40% (w/v), and this solution represents 100% formalin.

- When diluting such a formalin solution, the final concentration is expressed based on the 100% formalin reference: 10% formalin is a 1:10 dilution of 100% formalin.

- The terminology 10% formalin is commonly used, i.e. a fixative labelled as 10% formalin (buffered or not) contains actually 4% formaldehyde.

- Paraformaldehyde is another form of formaldehyde. Formaldehyde can be polymerised to big molecules, and this product is called paraformaldehyde which can be obtained as a white powder.

The various important fixing agents used are:

(1) Bouin’s fluid,
(2) Mercuric chloride,
(3) 70% Alcohol,
(4) Acetic acid,
(5) Formaline (formaldehyde),
(6) Potassium dichromate and
(7) Osmic acid (osmium tetra-oxide).

Temporary slides and Permanent Slides

- When using a microscope, slides that are permanent can be examined and stored for a long time, while temporary slides are used for short-term observations. Permanent slides must be properly made for successful long-term storage.

Preparation of Temporary Slides

- This may involve sectioning, staining and mounting. Fresh material may be hand-sectioned with a razor blade.

- A number of stains may be used for staining purpose, and for eg., eosin, methylene blue, leishman's stain,

- The sections are placed in a watch glass containing stain and left there until they are stained to the required depth.

- Sections can also be placed directly on a drop of stain on a slide. A drop of the stain is placed on the slide so that it just touches the edge of the cover slip.

- Fluid is then withdrawn from the opposite side of the cover slip by means of a piece of filter paper or blotting paper. The stain then flows in to replace the fluid taken out.

- The sections should be mounted in a drop of water/stain/saline solution/glycerine on a clean slide and a cover slip applied.
- Temporary slides are usually discarded after observation; however, cover slides can be removed and sterilized for reuse.
- Temporary slides utilize the liquid mounting method the most.

- Another mounting method used is heat fixing the specimen to the slide. Immersion oil is placed on the slide, while heat is used to fix the specimen to the slide and no cover slip is used.

- Example 1 - To Prepare Stained Temporary Mount of Human Cheek Cells

Lab Procedure

1. Gently scrape the inner side of the cheek using a toothpick, which will collect some cheek cells.
2. Place the cells on a glass slide that has water on it.
3. Mix the water and the cheek cells using a needle & spread them.
4. Take a few drops of Methylene blue solution using a dropper and add this to the mixture on the slide.
5. After 2-3 minutes remove any excess water & stain from the slide using a blotting paper.

6. Take a few drops of glycerine using a dropper and add this to the test mixture.
7. Take a clean cover slip and lower it carefully on the mixture with the aid of a needle.
8. Using a brush and needle, press the cover slip gently to spread the epithelial cells.
9. Remove any extra liquid around the cover slip using a blotting paper.
10. Place this glass side on the stage of the compound microscope and view it.

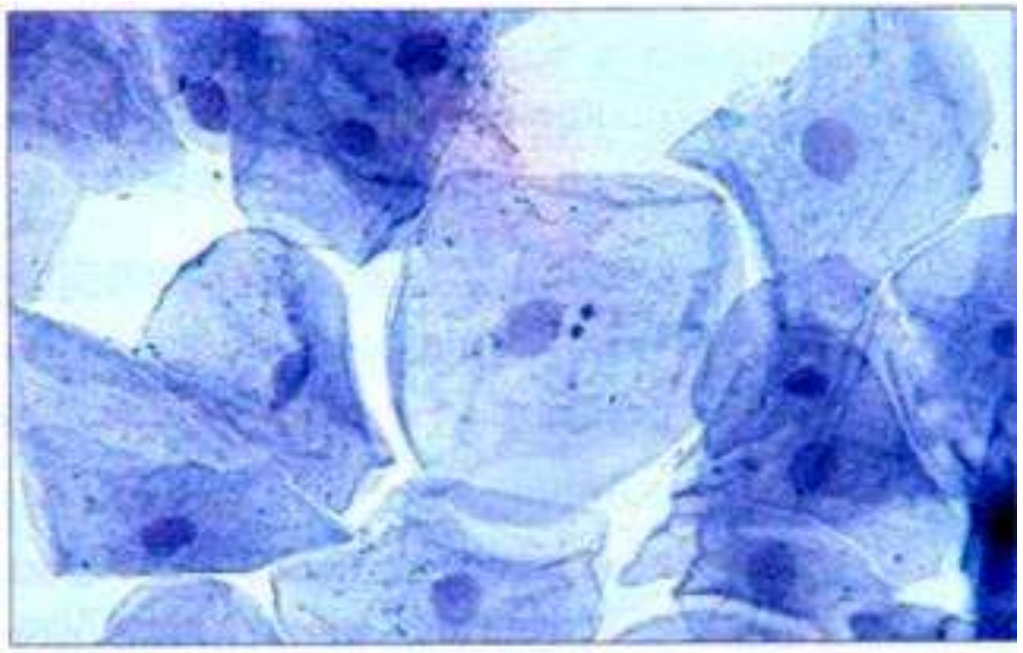
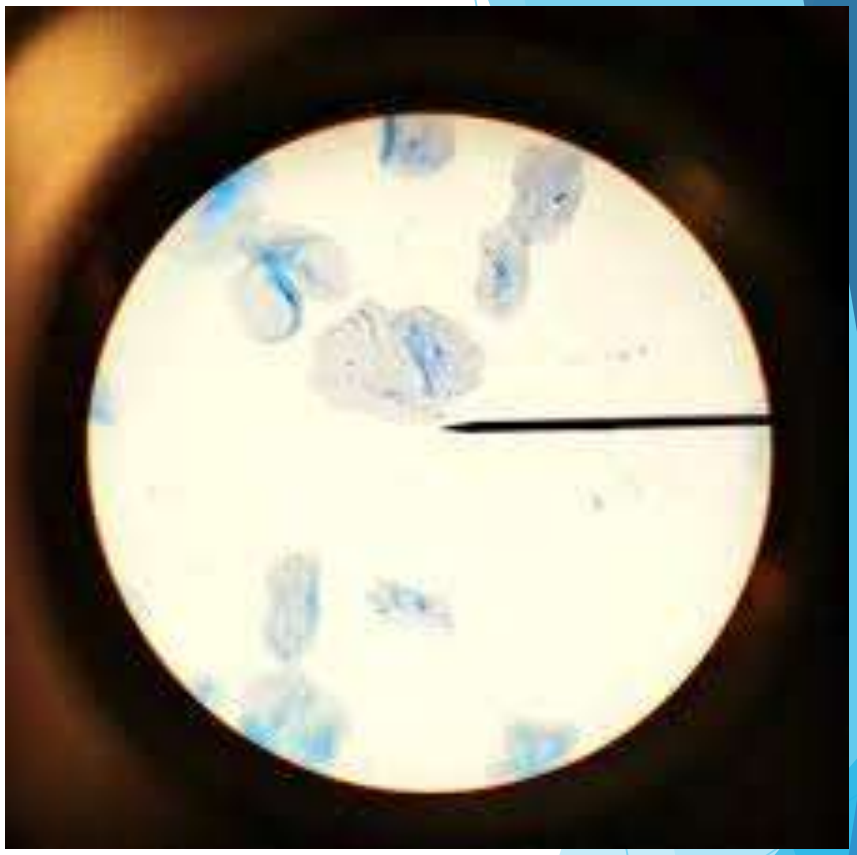


Fig. 2.4 Cheek cells do not have a cell wall.



Permanent slide preparation

- **Microscopic animals**."permanent preparation" refers to the preparation of a microscopic slide for long term storage.

- The following points highlight the seven main processes involved in preparation of permanent slides. The processes are:

- 1. Killing
- 2. Fixing and Hardening
- 3. Staining
- 4. Dehydration
- 5. Clearing
- 6. Mounting
- 7. Labelling.

1. Killing:

It is the first step in the preparation of permanent mounts and is of prime importance. By killing we mean the instantaneous stoppage of all the activities of life in their respective original state without giving the tissue enough opportunity to undergo any post-mortem changes. This can only be achieved by not allowing any change in the form of the tissue through use of reagents which are quick acting. The best killing agents are: 0.1% osmic acid and ether.

Process of Killing:

(1) The material to be killed is placed on a slide on which a very thin film of Mayer's albumen has been spread uniformly by rubbing a drop of albumen on surface of slide vigorously with index finger.

(2) (a) The slide is either inverted for few seconds on the mouth of the bottle in which osmic acid crystals have been put in distilled water.

Or (b) A few drops of ether or absolute alcohol are placed

on the slide over the object (material) and are allowed to evaporate.

2. Fixing and Hardening:

The fixing and hardening is the second major step in slide preparation. In case of minute living organisms it is usually attained by the killing agents alone but in case of sections of larger organisms or tissues it may be done through various fixing agents.

The process of fixing has multifarious importance i.e.,

1. Fixing stops any alteration in the form of tissues.
2. It hardens the tissue and makes them fit for extensive subsequent treatment.
3. It makes the tissue suitable and susceptive for the action of stains and various other reagents.
4. Fixing agents make various constituents and components of a tissue optically differentiated by changing their refractive indices.

- **After fixing in any of the aforesaid fixatives thorough washing of material is very essential otherwise the tissue will be completely spoiled, various mediums used for washing are:**

(1) 70% Alcohol (for Bouin's)
(2) Iodine+ 70% alcohol, (for mercuric chloride)
(3) 50% alcohol (for acetic acid)
(4) 70% alcohol (tor formaldehyde)
(5) Water and 0.12% chloral hydrate (for $K_2Cr_2O_7$) or
(6) Water (for osmic acid and $K_2C_2O_7$)

3. Staining:

- The process of colouring of various components and parts of a tissue for purpose of clear and absolute differentiation through use of different dyes (strains) is called staining.

- The nature of dyes may be acidic, basic or neutral.

- Usually the acidic dyes stain (colour) the cytoplasmic part and basic dyes colour the nuclear part.

4. Dehydration:

- The process by which the traces of water present in the tissue are removed and replaced by alcohol, in which clearing agent and solvent of mounting medium are readily soluble, is called dehydration.

- It is done because water is un-miscible with mounting medium, its solvent (usually Xylene) and the clearing agent (usually Xylene or Benzene).

- The dehydration is achieved by passing the tissue through gradually increasing percentage of alcohol.

- Otherwise, if we directly put the material in absolute alcohol it will shrink because of sudden loss of water.

- The material is thus passed through 30%, 50%, 70%, 90% and 100% or absolute alcohol. To achieve proper dehydration the material after absolute alcohol, should once again be placed in fresh absolute alcohol for 3-5 minutes.

Advantages of the graded alcohol series

1. During dehydration the water within the tissue is driven out and the dehydrating agent diffuses into the tissue. The diffusion is directly related to the conc. of the diffusing medium., i.e, alcohol. Because of progressive increase in the conc. of alcohol, diffusion is very gradual. Therefore tissues do not shrink and maintain its original form.

2. Dehydration prevents putrefaction of the material.

3. The graded series of alcohol not only prevents the cellular distortion but the cell membranes which are prone to damage in an aqueous medium are also protected from damage.

4. It is often necessary to preserve specimens for a long period of time. A good preservative prevents disintegration, swelling, and shrinkage of the tissues giving a firm consistency without causing them to become hard or brittle.

5. Clearing (Dealcoholization):

- The substitution of dehydrating agent by the solvent of mounting medium is called clearing.

- The term clearing is also used because of the fact that the solvent or clearing agent imparts transparency to tissue.
- The best clearing agents are Cedar wood oil and Clove oil but the most commonly used reagent is Xylene.

- In its place Benzene may also be used Xylene makes the tissue hard and brittle and also causes its shrinkage.

- As such it may be avoided if possible and should be replaced by Cedar wood oil or Clove oil.

Procedure:

- The material after absolute alcohol is placed in xylene or any other clearing agent. If the clearing agent turns turbid or white, it shows that dehydration is not complete. Put the material back in absolute alcohol for 5 minutes and then in clearing agent for 5 minutes or till it becomes transparent. Still if turbidity comes give the material 2-3 changes in clearing agent.

6. Mounting:

- The material after it has been made transparent is transferred to a drop of mounting medium which is placed in the centre of slide and is covered by a cover slip.
- The mounting medium should be of the same refractive index as crown glass (R.I. 1.5).

The best mounting mediums are:

- (1) Canada balsam dissolved in xylene (1.4 refractive index) or
- (2) Euparol (1.4 refractive index)

7. Labelling:

Now, write the name of the specimen on the slide put it under microscope for examination.

Whole mounts, Smears, Squashes and Sections

1. Whole mounts

- The practice of placing an entire organism on a microscope slide for examination
- Whole mount preparation is otherwise called “Micropreparation” or “Microslide” preparation

Preparation of whole mount

Single staining with Borax carmine:

1. Formalin preserved material should be washed through two or three changes of water till the fixative is washed out completely. In case the material is preserved in alcohol, omit this step.

2. After washing, dehydrate the material by transferring it through 30, 50 and 70 parcent alcohol. Leave for 5 minutes in each solution.

In case of alcohol preserved tissues transfer the material directly from 70% alcohol to Borax carmine stain.

3. Stain in Borax carmine and leave for 10 to 15 minutes depending on the thickness of the material.

4. Wash the material, if over stained then remove the excess stain by acid alcohol (1 % HCL in 70% alcohol).

5. Wash the material in 90% and then to absolute alcohol and keep for 10 minutes in each.

6. Remove alcohol by clearing in xylol for 10 minutes.

Mounting:

1.The material is then temporarily or permanently mounted on a slide.

- For temporary preparation the material is kept on the glycerine placed on a clear grease free slide and is covered with a cover-slip.

- After placing cover slip slight pressure is applied on the cover-slip so that excess glycerine and air bubbles come out.

- If still air bubbles remain present under the cover slip, slides should be placed on Hot plate for few minutes to make it air free.

2. For permanent mounting the above mentioned procedure is applied. But instead of glycerine Canada Balsam or DPX is used.

1. Smears

- a sample of tissue or other material taken from part of the body, spread thinly on a microscope slide for examination.

- The basic approach in smear technique is to spread the loose cells in a single layer on the surface of a clean microslide, kill and fix them all instantly without distortion.

- Fortunately all the cells that can be smeared will stick to the surface of the slide. Therefore there is no need for using a cementing agent.

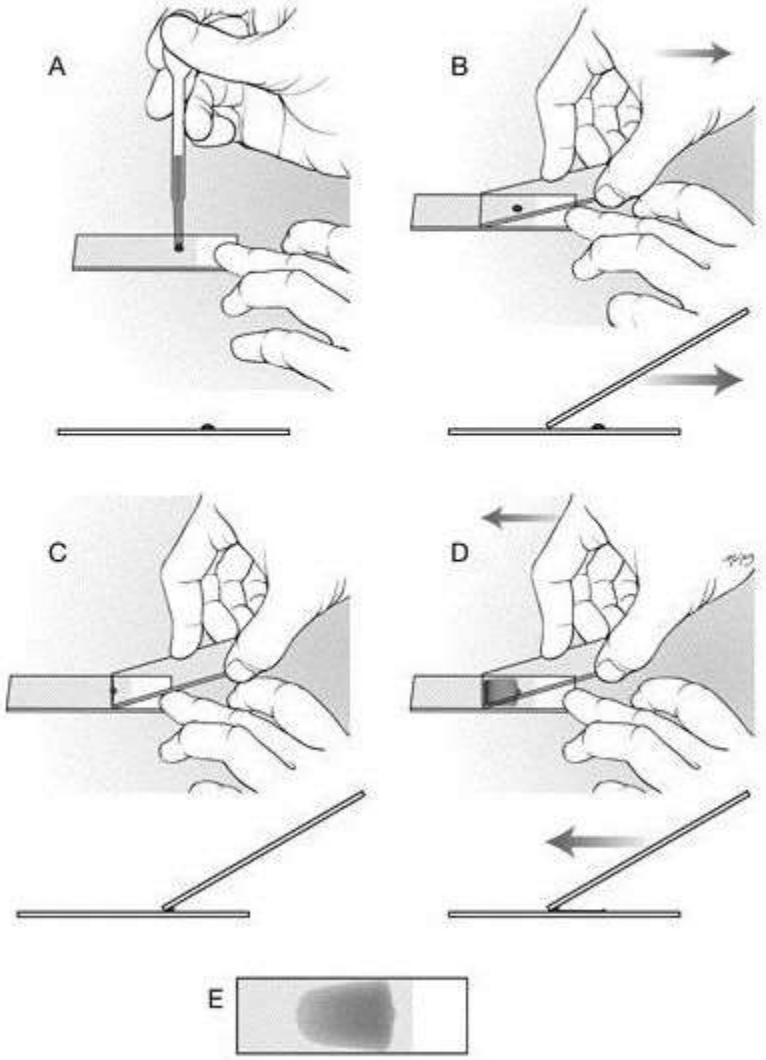
- The smear method is limited to study of loose cells that are not held to each other by middle lamella.

Making and staining a blood film

Small drop of blood is placed near the end of the microscope slide

The spreader is held at an angle of about 30° and pushed along the slide, spreading the drop of blood as a smear

The slide is fixed with alcohol to preserve the cells



The end of another slide is placed on the sample slide

The slide is labelled and allowed to dry in the air, so the cells stick to the slide

Slide is stained using Romanowsky stain (Leishman's stain). This is poured on and left for 2 minutes before being washed off with water

Characteristics of a Good Smear

1) Thick at one end, thinning out to a smooth rounded feather edge.
2) Should occupy 2/3 of the total slide area.
3) Should not touch any edge of the slide.
4) Should be margin free, except for point of application.
5) thickness - thin enough to allow a single layer of cells & a large enough reading area
6) spread - smooth & even; no waves, streaks, ridges, bubbles, holes

2. Squashes

- As the name literally means, the specimen placed on a clean slide is crushed under a coverslip and examined.
- This method is applicable to soft tissues/organs when it is desirable to separate the constituent cells.

- In smears the cells which are already loose and not attached to each other are spread/smeared over the surface of slide. But in case of squashes, soft tissues or organs are squashed on the slide to separate constituent cells.

- In squash cells are broken by applying pressure to the slide, intracellular components are liberated. Eg., chromosome.

Steps

- Place a drop of water on the slide and then a small piece of the specimen into the water.
- Carefully position the specimen in the centre and place the cover glass on top, as if making a regular wet mount.

- Using a soft round object, such as an eraser, carefully press down on the coverslip without horizontal movement, which would introduce shearing forces. This is the testing stage, to check if the specimen is sufficiently soft. The cover glass may break otherwise. This is, why you should use an eraser, to protect yourself.

- If the sample is sufficiently soft, you can press down with more force.

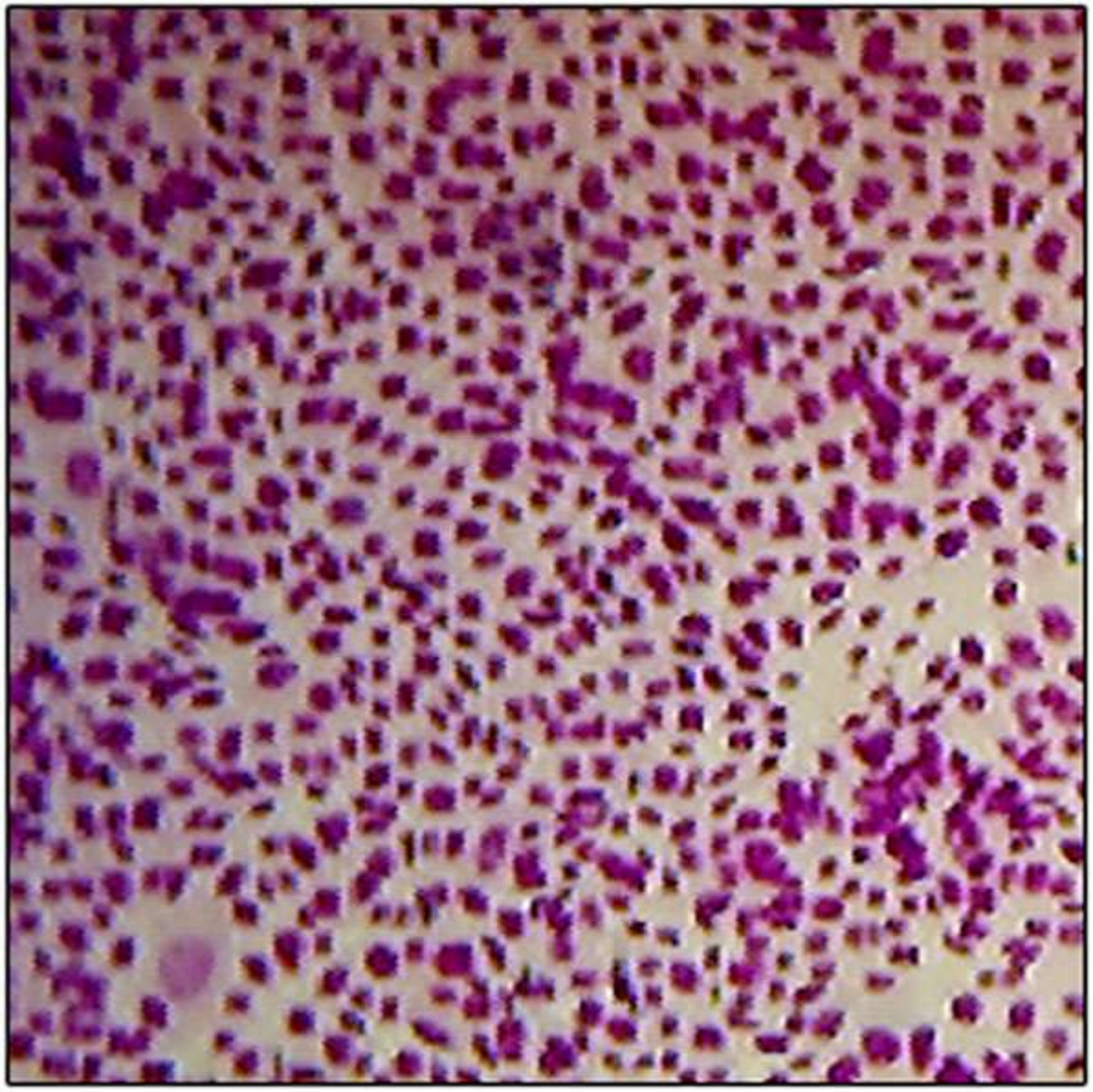
- Samples that are too solid need to be softened first. Some plant material can be made softer by boiling, but this may not be enough to soften the cellulose of the cell walls.

- The cellulose of the cell walls can be made softer by heating with an acid, such as diluted HCl or acetate. Rinse the specimen after acid treatment with water and compress it.

- Staining the samples should take place before squashing the specimen, as it is otherwise difficult for the stain to reach the cells.
- Suitable specimens include soft fruits and fungi. Squashing may introduce artifacts.
- The cells are separated from each other and it is not possible to see the original place of the cells.

- If you want to see the arrangement of cells, as they occur naturally, then you need to resort to microtoming. The advantage of squashing is, that it is a fast and easy method to obtain very thin specimen samples.

- The sample should form a thin, almost transparent film between coverslip and slide. Again, do not introduce a horizontal movement.
- Excess water should be soaked up with tissue paper.
- You can use any objective to observe under the microscope.



Squash preparation of onion root tip

3. Sections

- In order to study the internal arrangement of cells, the given biological material is to be cut into very thin slices of around 0.1mm or less. Such thin slices of tissue are called microsections.

- The section may be taken for one time observation in the class or practical examination which requires only temporary and semi-permanent micropreparations.

- Depending on their nature some specimens are sectioned directly without being subjected to prior treatment.

- For eg., stiff specimens, which offer resistance to the cutting edge of the razor blade such as stems, roots, leaves, petioles of many tracheophytes, etc. can be sectioned as such with the help of a razor blade, while holding the specimen between the thumb and index finger.

- Flat and pliable organs such as leaves, sporophills and cones of pteridopytes, fruit bodies of certain basidiomycetes, etc. needed to be supported in some kind of soft-solid material like pieces of carrot, pieces of thermocols etc.

- The support that is used for sectioning should be soft enough that the razor blade cuts across it with ease. These sections obtained are then processed individually and finally mounted on the slide.

- Some specimens may be too soft to section as such and these require some processing prior to sectioning so that they are rendered hard enough to stand against the cutting edge of the razor blade or knife.

- Such specimens are dehydrated, cleared, and embedded in paraffin wax and the wax blocks are held and sectioned.

Manual sectioning has the following disadvantages.

- Sections are of uneven thickness
- Very thin sections are not possible
- More often the specimen is cut obliquely.

➢ Mechanical devices employed for taking very thin sections are called microtomes.

Specimen preparation for TEM, SEM

- Specimen preparation for TEM, refer microscopy ppt

Specimen preparation for SEM

1. Specimen Killing, Fixation, and Dehydration

- Living specimens being prepared for scanning electron microscopy first need to be killed and fixed.
- This is usually done using a chemical fixative, such as glutaraldehyde and osmium.
- When applied in combination, typically glutaraldehyde fixation is used first.

- Concentrations of glutaraldehyde typically vary between 2% and 5% and are usually mixed with a buffer that operates at physiological pH.

- Two common choices, are phosphate buffers and cacodylate buffers, which are typically used at a concentration of approximately 0.05M and a pH of between 6.8 and 7.4.

- The goal of buffering the fixative is to provide an isotonic solution and to protect the biological tissue from becoming acidic, which frequently occurs during glutaraldehyde cross-linking.

- Killing may occur within the first several minutes of incubation in glutaraldehyde, but cross-linking of proteins through covalent bonds typically requires several hours to reach saturation in; therefore overnight fixation is not unusual.

- The specimen is then typically rinsed several times in their respective buffer, and transferred to buffered osmium tetroxide.
- Typically the same buffer and pH is used.
- Osmium fixation is usually conducted on ice or in the refrigerator for a shorter period of time, typically two hours.

- Use of warm osmium over a period of several hours can actually be used to extract some materials and expose internal surfaces by removing membranes. Following osmium fixation, water is chemically extracted from the specimen using a graded series of ethanol.

- Typically, the concentrations of ethanol used begin at 30% and proceed at 20% steps up to 70% followed by 10% changes to 100% ethanol. The final changes of ethanol must be conducted using anhydrous ethanol. Typically 3 changes of anhydrous ethanol are used.

2. Specimen Drying

- In order to prevent damage to the specimens during air drying, the critical point drying technique is frequently employed.

- A critical point drier (CPD) is used to replace all of the ethanol with liquid carbon dioxide under pressure.

- The pressure and temperature are raised in the CPD until the specimen is above the triple point at which time it is safe to decrease the temperature and release the pressure.

- The volume of liquid carbon dioxide is replaced several times until ethanol is no longer present in the purge line.

- One or two additional changes of liquid carbon dioxide are typically used in order to assure that no ethanol is present during the drying stage.

- Once the dried material is removed, it needs to be stored in a desiccated environment until viewing.

3. Specimen Mounting

- Specimens are mounted on stubs appropriate for the particular scanning electron microscope being used. A convenient adhesive is double-stick, electrically-conductive carbon tape.

- In order to assure electrical continuity of the specimen with the stub, a small drop of silver paint may be added.

4. Specimen Coating

- Specimens viewed using a conventional scanning electron microscope need to be coated with electrically conductive material.

- Typically this is applied using a sputter coater. Sputter coater's are plasma chambers with low discharge capability that he radiates a target made of heavy metal with argon atoms.

- Typically, the target consists of 60% gold and 40% palladium.

- Gold is preferred because it has a theory high electron output for secondary electrons, whereas palladium is added, because it provides a more contiguous surface of metal.

Microphotography

- Microphotographs are photographs shrunk to microscopic scale – not to be confused with photomicrographs, which are photographs of microscopic things.

The Royal Microscopical Society defines:

1) Microphotography - "Photography, especially of documents, arranged to produce small images which cannot be studied without magnification. Not to be confused with photomicrography."

- Microphotographs are photographs which are reduced to a minute or even microscopic size. They have many applications in science and industry, the most extensive being the microphotographic reproduction of documents.

- The advantages of the use of photographic reduction in recording documents are so obvious that it is not surprising that the first microphotograph was made shortly after the invention of photography

- All one needs is a method to connect the camera to the microscope. For decades, the 35mm Single Lens Reflex (SLR) camera was the choice for microphotography