SAWA UNIVERSITY

HISTOPATHOLOGY - FIRST COURSE

LAB -34&- Light microscopy & Fixation Tissues

Sawa University — جامعـــــة ســـــــاوة الاهلية

College of health and medical techniques — كليـــــة التقنيـــات الصحيـة والطبيـة

Department of Medical Laboratories — قسم تقنيات المختبرات الطبية

Third Stage — المرحلـــــة الثالثـة

محاضرة رقم 3&4 — الجانب العملي

Lecture No. 3 — Practical

تدريسيين المادة : م.م هبــه صاحب صادق

م.م خلــود عايد حسيــن

The goal of tissue fixation using a light microscope is to preserve its cellular structure and prevent degeneration after removal from the body, allowing for close examination using a light microscope. The fixation process is essential to ensure the stability of cells and tissues. The sample is then processed by cutting it into thin sections, staining it, and examining it under a light microscope.

INTRODUCTION:

Light microscopy

Light microscopy uses a system of lenses and visible light to create magnified images of small objects, such as cells, by bending light through the specimen. It's a versatile technique used to study living cells and their processes, and it provides images that can be viewed directly by eye or captured digitally.

- They use lenses to focus light on the specimen, magnifying it thus producing an image. The specimen is normally placed close to the microscopic lens.

- Microscopic magnification varies greatly depending on the types and number of lenses that make up the microscope. Depending on the number of lenses, there are two types of microscopes i. e Simple light microscope (it has low magnification because it uses a single lens) and the Compound light microscope (it has a higher magnification compared to the simple microscope because it uses at least two sets of lenses, an objective lens, and an eyepiece).
- The lenses are aligned in that, they can be able to bend light for efficient magnification of the image.

- The functioning of the light microscope is based on its ability to focus a beam of light through a specimen, which is very small and transparent, to produce an image. The image is then passed through one or two lenses for magnification for viewing. The transparency of the specimen allows easy and quick penetration of light. Specimens can vary from bacterial to cells and other microbial particles.

How it works

- Light Source: A light source, often at the base of the microscope, illuminates the specimen.
- Condenser and Diaphragm: A condenser lens focuses the light onto the specimen, and a diaphragm controls the amount of light, which helps adjust the image's contrast.
- Specimen: The specimen is placed on a stage, held in place by clips, and must be small and transparent for light to pass through it.

- Objective Lenses: Light passes through the objective lens(es) and the specimen, forming a magnified image.
- Eyepiece Lenses: This magnified image then travels through the microscope's tube to the eyepiece lens(es), which provide further magnification for the observer.
- Focusing: Coarse and fine focus knobs adjust the distance between the lens and the specimen to bring the image into sharp focus.

Features and uses

- Visibility: It makes structures visible that are too small to see with the naked eye.
- Living Cells: Light microscopy allows for the study of living cells and their dynamic behaviors.
- Resolution: While offering well-matched resolution for subcellular structures, it is limited in achieving the high magnifications possible with other microscopy techniques like electron microscopy.

Principle of a light microscope (optical microscope)

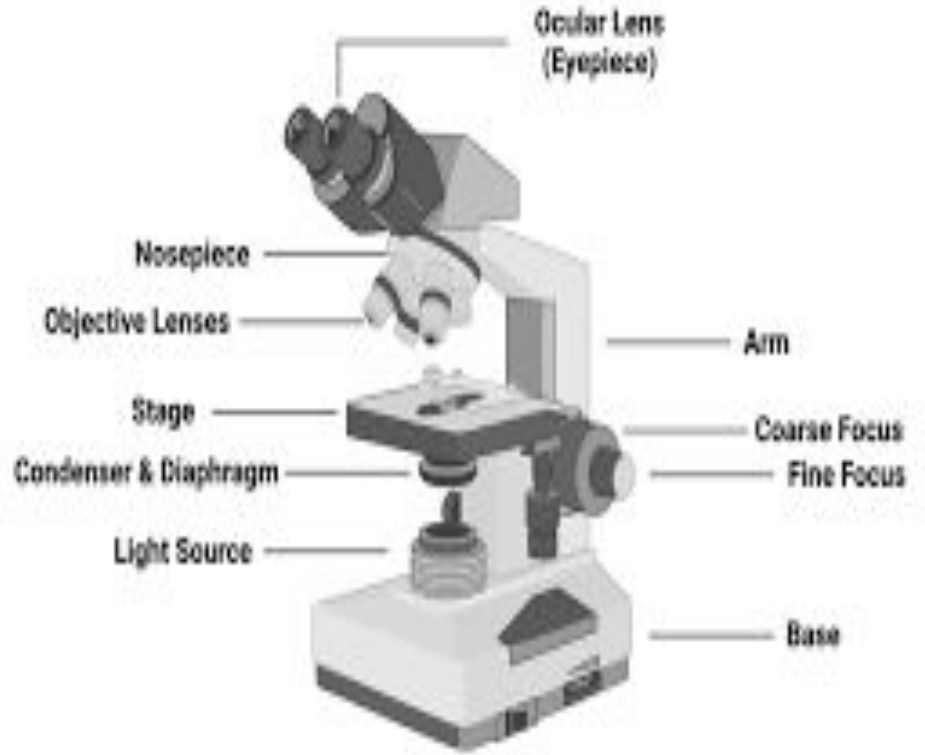
As mentioned earlier, light microscopes visualize an image by using a glass lens, and magnification is determined by, the lens's ability to bend light and focus it on the specimen, which forms an image. When a ray of light passes through one medium into another, the ray bends at the interface causing refraction. The bending of light is determined by the refractive index, which is a measure of how great a substance slows the speed of light. The direction and magnitude of the bending of the light are determined by the refractive indexes of the two mediums that form the interface.

Types of light microscopes (optical microscope)

With the evolved field of Microbiology, the microscopes used to view specimens are both simple and compound light microscopes, all using lenses. The difference is simple light microscopes use a single lens for magnification while compound lenses use two or more lenses for magnifications. This means, that a series of lenses are placed in an order such that, one lens magnifies the image further than the initial lens.

The modern types of Light Microscopes include:

1. **Bright field Light Microscope**
2. **Phase Contrast Light Microscope**
3. **Dark-Field Light Microscope**
4. **Fluorescence Light Microscope**



Fixation :

means placing the specimen in chemical solution called fixative intended to preserve the specimen until it is ready to be cut in to sections for histologic examination .

The aims of fixation :

1. Prevent the processes of autolysis and bacterial attack .
2. Keeping the shape and volume of the tissue during any of subsequent procedures.
3. Keeping the tissues in a condition which subsequently always clear staining.
4. Keeping the tissues as close to their living state as possible.
5. Preserving the microanatomy of tissues well enough to allow a diagnosis to be made in most cases.
6. Hardening the soft tissues of specimen.

The characteristics of a good fixative :

1. It must penetrate the specimen rapidly.
2. It must harden the specimen quickly.
3. It must prevent swollen or shrinkage of the cells.
4. It must kill the cells of the specimen immediately.
5. Modulate of refractive index for some tissue inclusion.
6. Make the tissue resistance the high temperature and improve the ability of tissue to staining.
7. Not harmful.
8. Cheap (low cost).

Fixation methods :

1. Fixation by natural methods :

a. Fixation by heat: The principle of this method is the protein clotting by heating .

b. Fixation by drying in normal temperature: This method is useful when prepare the smear of blood or bone marrow .

c. Fixation by liquid nitrogen: In this method the biopsy cutting to small pecies and freezing it , by liquid nitrogen (-160 c) and dry by special instrument , and immediately embedding inparaffin wax .

It is useful to appearing the enzymes and fats explicitly in the sections.

2. Fixation by chemical methods :

The important characters of chemical fixative is:

- ability to fixation the tissue in highly efficiency by a coagulation of protein
- and preserve the skeleton of the cell to become easy to a penetration for the infiltrated agent but in same time this fixative has a power of penetrating causing the damage of organelles .

Fixation (Types of fixatives):

The purpose of fixation is to preserve tissues permanently in as life-like a state as possible. Fixation should be carried out as soon as possible after removal of the tissues (in the case of surgical pathology) or soon after death (with autopsy) to prevent autolysis. There is no perfect fixative, though formaldehyde comes the closest. Therefore, a variety of fixatives are available for use, depending on the type of tissue present and features to be demonstrated.

There are five major groups of fixatives, classified according to mechanism of action:

1. Aldehydes 2. Mercurials 3. Alcohols 4. Oxidizing agents 5. Picrates

1. Aldehydes :

- include formaldehyde (formalin) and glutaraldehyde.
- Tissue is fixed by cross-linkages formed in the proteins, particularly between lysine residues.
- This cross-linkage does not harm the structure of proteins greatly, so that antigenicity is not lost.
- Therefore, formaldehyde is good for immunoperoxidase techniques.

A. **Formalin :** penetrates tissue well, but is relatively slow.

- The standard solution is 10% neutral buffered formalin.
- A buffer prevents acidity that would promote autolysis and cause precipitation of formol-heme pigment in the tissues.

B. **Glutaraldehyde :** causes deformation of alpha-helix structure in proteins so is not good for immunoperoxidase staining.

- However, it fixes very quickly sois good for electron microscopy.It penetrates very poorly, but gives best overall cytoplasmic and nuclear detail.
- The standard solution is a 2% buffered glutaraldehyde

2. Mercurials :

- fix tissue by an unknown mechanism.
- They contain mercuric chloride and include such well-known fixatives as B-5 and Zenker's.
- These fixatives penetrate relatively poorly and cause some tissue hardness, but are fast and give excellent nuclear detail.

Their best applicationis for fixation of hematopoietic and reticuloendothelial tissues. Since they contain mercury, they must be disposed of carefully.

3. Alcohols :

- including methyl alcohol (methanol) and ethyl alcohol (ethanol).
- are protein denaturants.
- are not used routinely for tissues because they cause too much brittleness and hardne
- However, they are very good for cytologic smears because they act quickly and give good nuclear detail. Spray cans of alcohol fixatives are marketed to physicians doing PAP smears, but cheap hairsprays do just as well.

4. Oxidizing agents :

- include permanganate fixatives (potassium permanganate), dichromate fixatives (potassium dichromate), and osmium tetroxide.
- They cross-link proteins, but cause extensive denaturation.
- Some of them have specialized applications, but are used very infrequently.

5. Picrates :

- include fixatives with picric acid.
- Foremost among these is Bouin's solution.
- It has an unknown mechanism of action.
- It does almost as well as mercurials with nuclear detail but does not cause as much hardness.
- Picric acid is an explosion hazard in dry form.
- As a solution, it stains everything it touches yellow, including skin

Most common Fixatives in common use

The most commonly used fixate ve for histopathology is a 4% aqueous solution of formaldehyde, often called 10% formalin because it is made by tenfold dilution of formalin.

For about 50 years this fixative has also included inorganic salts to maintain a near neutral pH and an osmotic pr essure similar to that of mammalian extracellular fluid.

The solution is called neutral buffered formalin, or NBF. It fixes not by coagulation, but by adding to the side chains of basic amino acids, especially lysine, and to the amide nitrogen atoms of pe ptide linkages. Cross linking methylene bridges are formed where two formaldehyde binding sites are close together. This results in lowered permeability to macromolecules but the structures of protein molecules are not greatly altered.

The small sizes of the methylene glycol and formaldehyde molecules allow rapid penetration. Consequently (thus) this fixative is suitable for large or small specimens. Unfortunately, despite rapid penetration of tissues, the chemical reactions of formaldehyde with tissue proteins, especially the formation of methylene bridges, occur slowly. A commonly made mistake with formalin containing fixatives is to under fix the tissue.

Small (10×10×3 mm) pieces fixed in NBF for 12-24 hours will generally show good cytoplasmic preservation and nuclear detail.

Addition of formaldehyde is largely complete in 24 hours, but cross linking reactions continue for at least two weeks. Large, soft specimens such as whole human brains require 2 -6 weeks in NBF to become firm enough to cut into slices from which samples can be taken for histology. Variations in time and conditions of fixation cause the majority of problems in histochemistry.

Fixation profoundly (strongly) affects histological and immunohistochemical staining, therefore, technicians, pathologists and research workers must decide on the most appropriate method. Aspects to consider are temperature, size of the storage container, volume ratio, salt concentration, pH and incubation time.

Formaldehyde fixation is typically performed at room temperature.

Using a low and wide specimen container to allow for adequate penetration and ease of retrieval by a technician is the best choice for adequate volume ratio. In addition, 1:20 volume ratio of fluid to tissue and 3-4 mm specimen thickness are recommended for good penetration.

To prevent swelling or shrinking of the cells, an isotonic solution buffered to pH 7.2-7.4 is recommended, to maintain ultrastructure and minimize cell distortion. The shorter the time elapsed between removing the sample from the body and immersing it in fixative, the better.

Questions

- What is fixation in light microscopy?
- Why are fixatives used?
- What are types of fixation?
- What is the pH of fixation?

References

1. Grizzle WE. Special symposium: fixation and tissue processing models. Biotech Histochem 2009; 84: 185-193.
2. Junqueira LC, Carneiro J. Basic Histology text & atlas, Nobel Medicine Bookstores, 2006; 1-2 (in Turkey).
3. Jamur MC, Oliver C. Cell fixatives for immunostaining. Methods Mol Biol 2010; 588: 55-61.

Any questons?