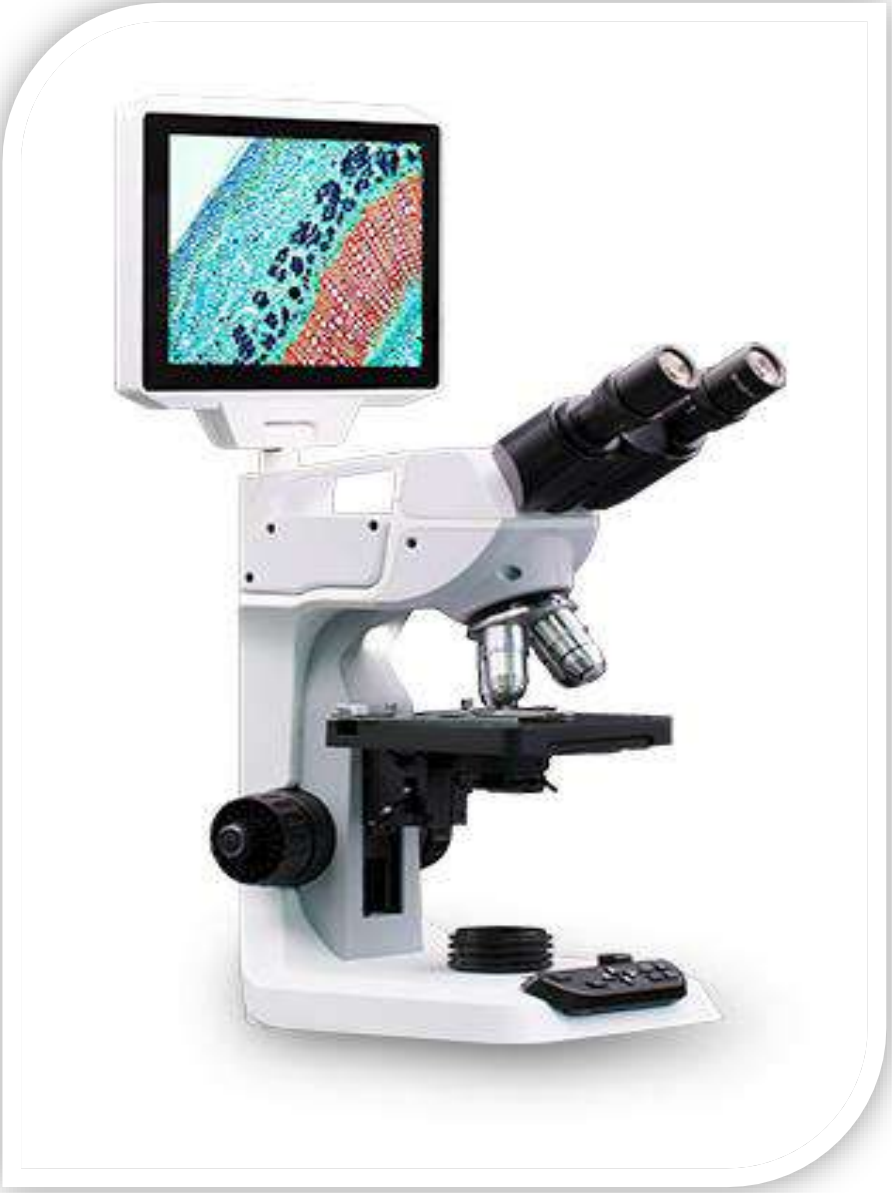
II B. Sc, Zoology (IV Semester)

Subject: BIOLOGICAL TECHNIQUES

Sub Code: 18K4Z07

(Unit 1 to 3)



MICROSCOPE

Introduction

Microscope that produces enlarged images of small objects, allowing the observer an exceedingly close view of minute structures at a scale convenient for examination and analysis.

Although optical microscopes are the subject of this article, an image may also be enlarged by many other wave forms, including acoustic, X-ray, or electron beam, and be received by direct or digital imaging or by a combination of these methods.

The microscope may provide a dynamic image (as with conventional optical instruments) or one that is static (as with conventional scanning electron microscopes).

The magnifying power of a microscope is an expression of the number of times the object being examined appears to be enlarged and is a dimensionless ratio.

It is usually expressed in the form 10× (for an image magnified 10-fold), sometimes wrongly spoken as "ten eks"—as though the × were an algebraic symbol—rather than the correct form, "ten times."

The resolution of a microscope is a measure of the smallest detail of the object that can be observed. Resolution is expressed in linear units, usually micrometers (μm). The most familiar type of microscope is the optical, or light, microscope, in which glass lenses are used to form the image.

Optical microscopes can be simple, consisting of a single lens, or compound, consisting of several optical components in line.

The hand magnifying glass can magnify about 3 to 20×. Single-lensed simple microscopes can magnify up to 300×—and are capable of revealing bacteria while compound microscopes can magnify up to 2,000×.

A simple microscope can resolve below 1 micrometre (μm; one millionth of a metre); a compound microscope can resolve down to about 0.2 μm.

Images of interest can be captured by photography through a microscope, a technique known as photomicrography. From the 19th century this was done with film, but digital imaging is now extensively used instead.

Some digital microscopes have dispensed with an eyepiece and provide images directly on the computer screen. This has given rise to a new series of low-cost digital microscopes with a wide range of imaging possibilities, including time-lapse

micrography, which has brought previously complex and costly tasks within reach of the young or amateur microscopist.

DISSECTION MICROSCOPE

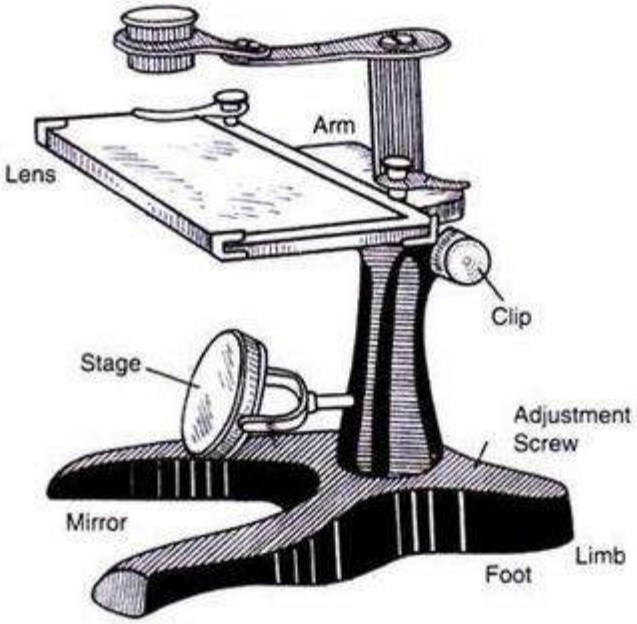


Fig. 6.1 Simple/dissecting microscope

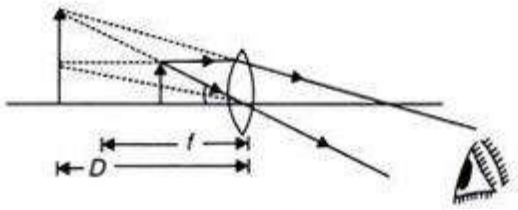


Fig. 6.1

These are also known as stereoscopic microscopes. This is a type of digital optical microscope designed with a low magnification power (5x-250x), by use of light reflected from the surface of the specimen, and not the light reflected the specimen. Its primary role is for dissection of specimens and viewing and qualitatively analyzing the dissected samples.

It was first designed by Cherudin d'Orleans in 1677 by making a small microscope with two separate eyepieces and objective lenses. Later in 1852, an inventor named Charles Wheatstone describe the principle of the stereoscopic visualization and published it with the title 'On Some Remarkable, and Hitherto Unobserved, Phenomena of Binocular Vision'.

It was later advanced by John Leornld Riddell who equally published it on the Journal of Microscopical Science as ‘ On Binocular Microscope’.

Horatio S. Greenough, an American Biologist developed the stereo Microscope, with two separate but identical optical paths and manufactured it with the Carl Zeiss Company, to which the scope of the Dissecting Microscope is built to date.

Principle of Dissection microscope

- The working principle of the dissecting Microscope depends on the two types of light paths used by the microscope’s objectives and eyepiece. Each light path provides a different angle of viewing. They have the top light which is used while dissecting and the bottom light that is used to view the images.
- This lighting is enabled by the construction of two eyepieces (binocular stereoscope) each showing a different type of light pathway, each providing a viewing comfort area.
- Being a digital microscope, the images are viewed live on a computer monitor screen in 3-dimensional visuals. They also offer a very close observation of small specimens such as insects where the image produced is normally larger than the sample size, an effect known as macro-photography. The image is recorded and in complex samples, the topography (surface) is analyzed in 3D.
- The dissecting microscope works with two magnification systems: Fixed (primary) magnification where two objective lenses provide a degree of magnification and the Zoom (pancratic) magnification which offers a continuous magnification at variant ranges, using the auxiliary objectives whose function is to increase total magnification depending on some factors. The variance between the zoom and the fixed magnification can be achieved by changing the eyepiece lenses.
- Between the fixed and the zoom magnification is an optical system known as the Galilean optical system which has fixed-focus lenses that confer fixed magnification for different sets of magnification such as two sets of magnifications offers four-magnifications, three sets offer six-magnifications, etc.

Applications

Like most microscopes, it is used in a wide range of fields including manufacturing, medical, quality control, inspection and biomedical studies like the entomological study of insects and some of its functions include:

1. Studying the topography of solid samples

2. For dissection
3. For microsurgical procedures
4. For the manufacturing of watches, circuit boards, and their inspections
5. Used for inspection of fractures (fractography)
6. Used in forensic engineering

Advantages

1. It can be used in a wide range of fields making it one of the most important microscopic techniques.
2. The use of two light pathways offers great magnification differences for visualizing the image.
3. The attachment of a digital camera allows recording and imaging the image produced.
4. It is easily portable and easy to use.
5. They are used to view whole specimens and not in pieces.

COMPOUND MICROSCOPE

A high power or compound microscope achieves higher levels of magnification than a stereo or low power microscope. It is used to view smaller specimens such as cell structures which cannot be seen at lower levels of magnification. Essentially, a compound microscope consists of structural and optical components. However, within these two basic systems, there are some essential components that every microscopist should know and understand.

Structural Components

The three basic, structural components of a compound microscope are the head, base and arm.

Head/Body houses the optical parts in the upper part of the microscope

- **Base** of the microscope supports the microscope and houses the illuminator
- **Arm** connects to the base and supports the microscope head. It is also used to carry the microscope.
- **Eyepiece or Ocular** is what you look through at the top of the microscope. Typically, standard eyepieces have a magnifying power of 10x. Optional eyepieces of varying powers are available, typically from 5x-30x.

- **Eyepiece Tube** holds the eyepieces in place above the objective lens. Binocular microscope heads typically incorporate a diopter adjustment ring that allows for the possible inconsistencies of our eyesight in one or both eyes. The monocular (single eye usage) microscope does not need a diopter. Binocular microscopes also swivel (Interpupillary Adjustment) to allow for different distances between the eyes of different individuals.
- **Objective Lenses** are the primary optical lenses on a microscope. They range from 4x-100x and typically, include, three, four or five on lens on most microscopes. Objectives can be forward or rear-facing.
- **Nosepiece** houses the objectives. The objectives are exposed and are mounted on a rotating turret so that different objectives can be conveniently selected. Standard objectives include 4x, 10x, 40x and 100x although different power objectives are available.
- **Coarse and Fine Focus knobs** are used to focus the microscope. Increasingly, they are coaxial knobs - that is to say they are built on the same axis with the fine focus knob on the outside. Coaxial focus knobs are more convenient since the viewer does not have to grope for a different knob.
- **Stage** is where the specimen to be viewed is placed. A mechanical stage is used when working at higher magnifications where delicate movements of the specimen slide are required.
- **Stage Clips** are used when there is no mechanical stage. The viewer is required to move the slide manually to view different sections of the specimen.
- **Aperture** is the hole in the stage through which the base (transmitted) light reaches the stage.

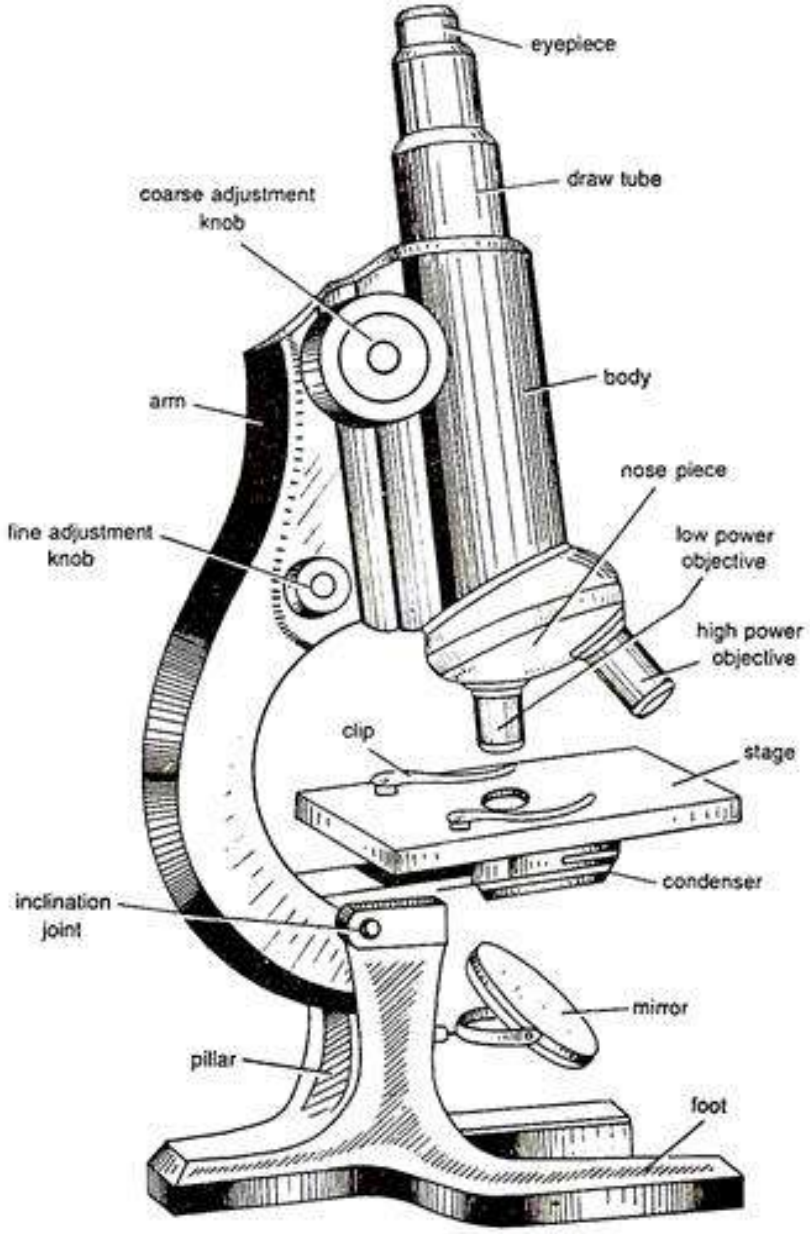


Fig. 278. A compound microscope.

- **Illuminator** is the light source for a microscope, typically located in the base of the microscope. Most light microscopes use low voltage, halogen bulbs with continuous variable lighting control located within the base.
- **Condenser** is used to collect and focus the light from the illuminator on to the specimen. It is located under the stage often in conjunction with an iris diaphragm.
- **Iris Diaphragm** controls the amount of light reaching the specimen. It is located above the condenser and below the stage. Most high quality microscopes include

an Abbe condenser with an iris diaphragm. Combined, they control both the focus and quantity of light applied to the specimen.

- **Condenser Focus Knob** moves the condenser up or down to control the lighting focus on the specimen.

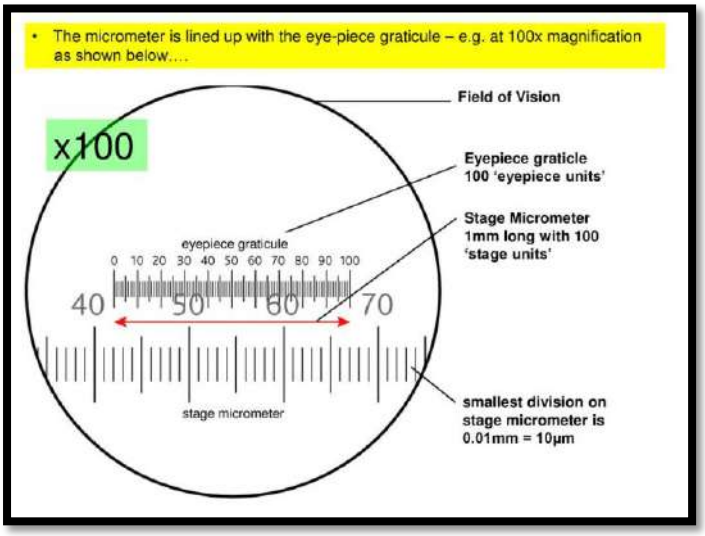
STAGE MICROMETER

A stage micrometer is the term typically referring to a slide (1" x 3" microscope) that comes with a scale on its surface. The sides are mounted with a reticle scale that is used for calibrating the reticles of the eyepiece as well as the objective powers. Although most stage micrometers are made of glass, they are also composed of metal especially for dissecting microscopes.

While microscopes serve the primary function of enlarging objects being viewed, they are also used to make measurements of the objects/specimen. Given that this is not possible with the ordinary ruler, the eyepiece reticle (eyepiece scale) is used to make such measurements.

Stage micrometers are particularly useful given that the objectives and eyepiece reticles of a microscope are often interchanged. For this reason, there is a need to carry out a routine calibration to ensure accuracy when measuring objects/specimen.

*A micrometer may be used by being directly mounted on the object being viewed. However, this is expensive and impractical and thus not common.



Measuring Using a Microscope

Assuming that in a given alignment there are 30 divisions on the stage micrometer that has aligned with 10 on the eyepiece scale, calculating this would give us 3 units. In the event that the calculation gives a number with decimals (e.g. 3.6363) then the number may be rounded off. For instance, 3.6363 can be rounded off to 4 or 3.6. Here, the number obtained from the calculation is the calibration factor and gives the number of units in each division of the eyepiece.

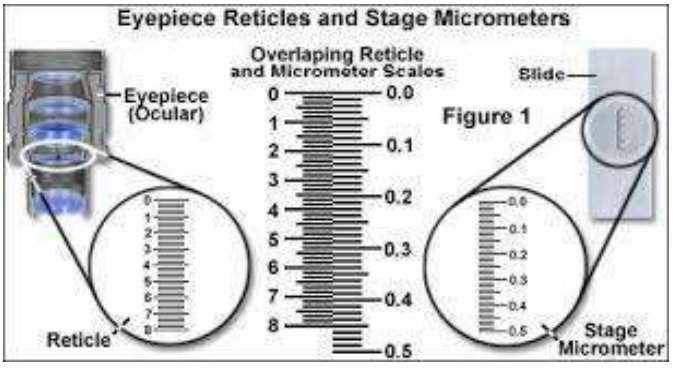
Here, it's worth noting that the conversion factor of each objective is different. For this reason, it's important to calibrate each objective lens individually so as to obtain their respective conversion factor. This becomes important particularly when making measurements when viewing the specimen using different magnifications.

When making measurements, it's particularly important that the right units of measurements are used. Here, 1 mm is equal to 1000 micrometers (um). The figures obtained from the calculations should be converted to micrometers during measurements.

When measuring the size of the object/specimen, it's always important to measure the diameter. Here, the student may measure the longest and shortest diameter of the specimen in the field of view. When in the field of view, the student should calculate the eyepiece divisions, which represents the diameter of the specimen.

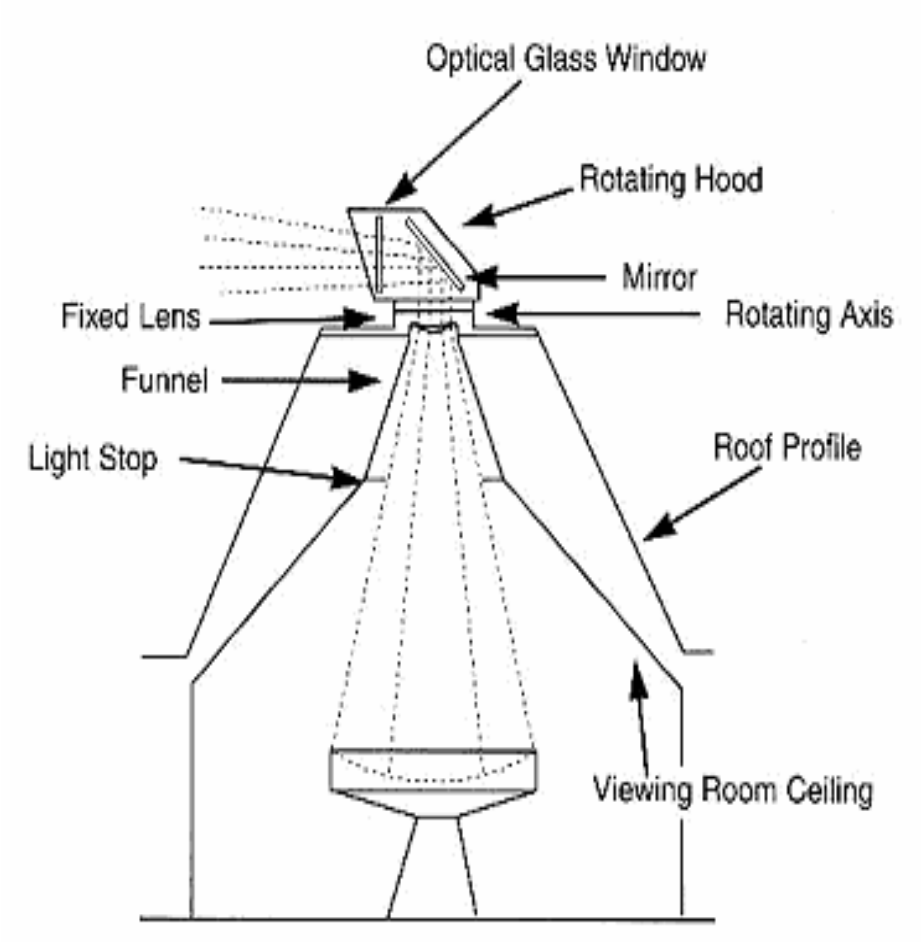
For instance, the diameter of the specimen here may be 12 divisions. This number does not really represent any specific units.

In order to determine the length of the specimen, these units should be multiplied to the conversion factor in order to get the measurements in micrometers. This makes it possible to tell the actually length/width of the specimen/object being observed.



CAMERA LUCIDA

- Camera lucida, (Latin: “light chamber”), optical instrument patented in 1806 by William Hyde Wollaston to facilitate accurate sketching of objects.
- It consists of a four-sided prism mounted on a small stand above a sheet of paper.
- By placing the eye close to the upper edge of the prism so that half the pupil of the eye is over the prism, the observer is able to see a reflected image of an object situated in front of the prism, apparently lying on the paper.
- He can then trace the image with a pencil.
- In its original form the camera lucida was extremely difficult to focus properly, and a weak spectacle lens was added between the prism and the paper.
- A later form, developed about 1880 for use with a microscope, substituted two diagonal mirrors for the prism; one transparent mirror was positioned above the microscope eyepiece and the second at a short distance above the paper.



Camera Lucida

ELECTRON MICROSCOPE

Electron microscopy (EM) is a fantastic tool that enables biologists to capture images of their samples at a greater resolution than with a light microscope.

There are several types of EM and each of these can provide different information about your sample.

The large field of EM is expanding all the time and there are many advanced instruments that I do not describe below.

Magnification and resolution

Often the term magnification is used when discussing the power of a microscope. However, magnification is not the main issue affecting microscopes. It is the resolution. Resolution is the ability to distinguish two objects as separate.

Imagine a car coming towards you at night. Initially you would see a single headlight and at some point you would be able to separate the light into two distinct headlights.

This is the minimum resolvable distance or resolution. In microscopy we refer to the Airy disc, a diffraction pattern created by imaging a point object.

The resolution of two objects in a microscope depends on them being sufficiently separate so that the diffraction patterns do not merge, known as the Rayleigh criterion.

Ernst Abbe (1873) was able to determine the optimum resolution that a microscope can achieve, and the equation for this is the Abbe diffraction limit.

Microscopes are designed to minimize variables so that the main limiting factor is the wavelength used to image the sample.

All electromagnetic radiation (e.g., light, x-rays, radio waves, etc.) has a set wavelength.

Using a form of electromagnetic ration with a smaller wavelength increases the resolution that can be achieved.

Light microscopes can achieve a resolution of 200 nm. Super-resolution light microscopy allows some biologists to go beyond this limit and a few X-ray microscopes are now also available.

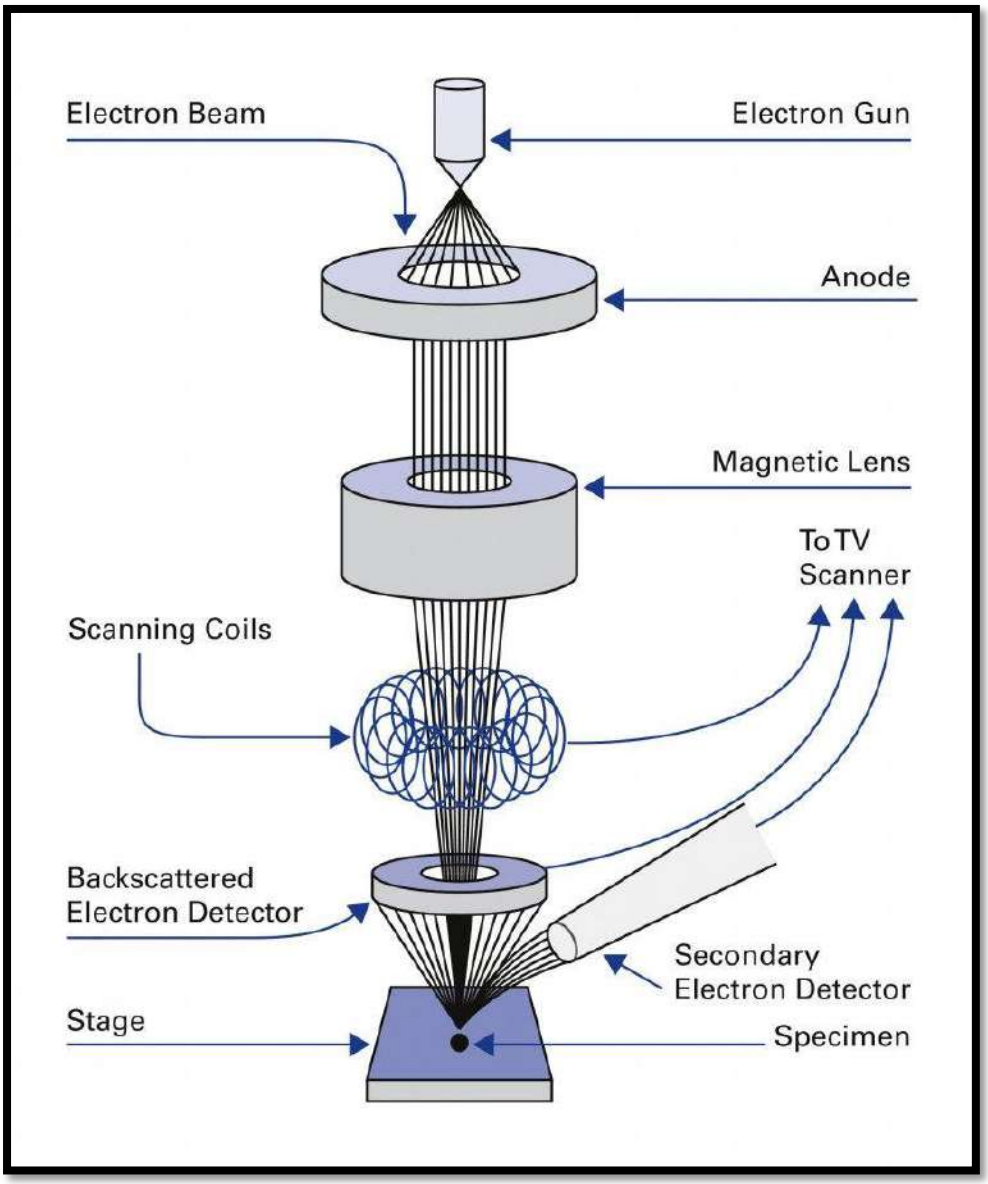
However, EM remains the main technique used by biologists for high resolution imaging of molecules, viruses and cells.

Electrons

Electrons are negatively charged sub-atomic particles that have a wavelength. The wavelength of an electron is determined by the de Broglie wavelength and is linked to the accelerating voltage (AV) used to form the electron beam.

For example, an AV of 100,000 volts results in an electron wavelength of 0.0037 nm and a resolution that is up to 100,000 times smaller than can be achieved with light.

While it is possible to achieve sub-atomic imaging with an electron microscope, it is not possible to image biological samples at this resolution. As we will see, the biological tissue is always the limiting factor.



Schematic of an electron microscope

Instrumentation

There are several different types of EM. These can be split into two main categories, transmission electron microscopes (TEM) and scanning electron microscopes (SEM). The main differences between these are in the optics, how the signal is detected and the type of information you can obtain.

Both types of EM have an electron gun, which contains an electron source (a filament that produces a cloud of electrons), a Wehnelt cylinder (to form the beam) and an anode (to accelerate the beam).

There are three main types of electron source; a tungsten filament, a lanthanum hexaboride (LaB_6) crystal and a field emission filament.

Differences among the filaments are shown in table.

Type		Thermionic	Cold field emission	Schottky field emission
Material	Tungsten (W)	Lanthanum hexaboride (LaB6)	Tungsten (W)	Zirconiated tungsten (ZrO/W)
Beam diameter	1-2 µm	1-2 µm	3-5 nm	10-25 nm
Brightness (A/cm^2sr)	10^6	10^7	10^9	10^8
Energy range (eV)	2.0	1.5	0.2-0.3	0.3-1
Resolution (nm)	<3	<2	<1	<1

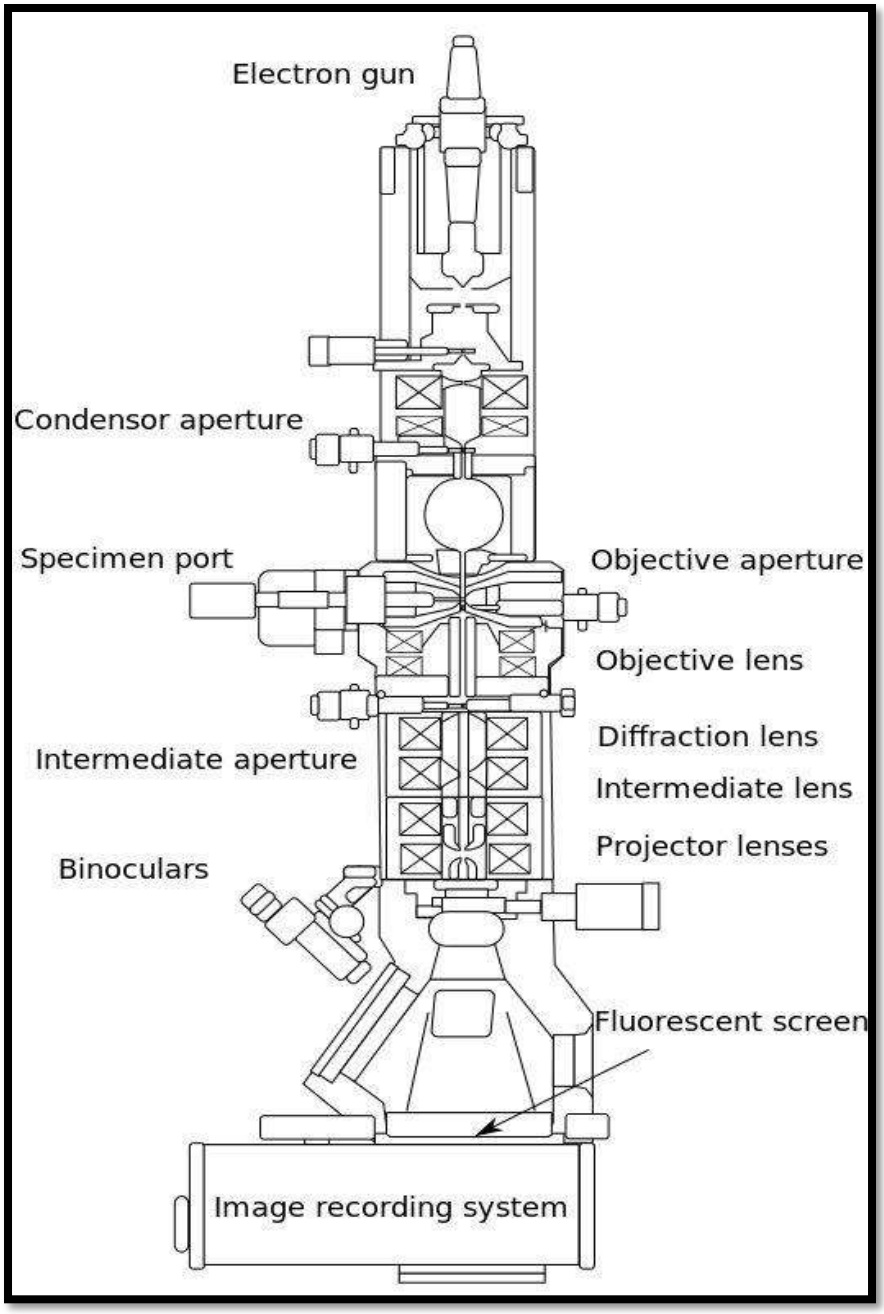
Both TEM and SEM use electromagnetic lenses to focus the beam of electrons. Electrons travel along the magnetic field and can be focused in the same way that light is focused using glass lenses.

Apertures are associated with the lenses and are thin plates of molybdenum with several small bores (usually a range of 10-300 µm in diameter).

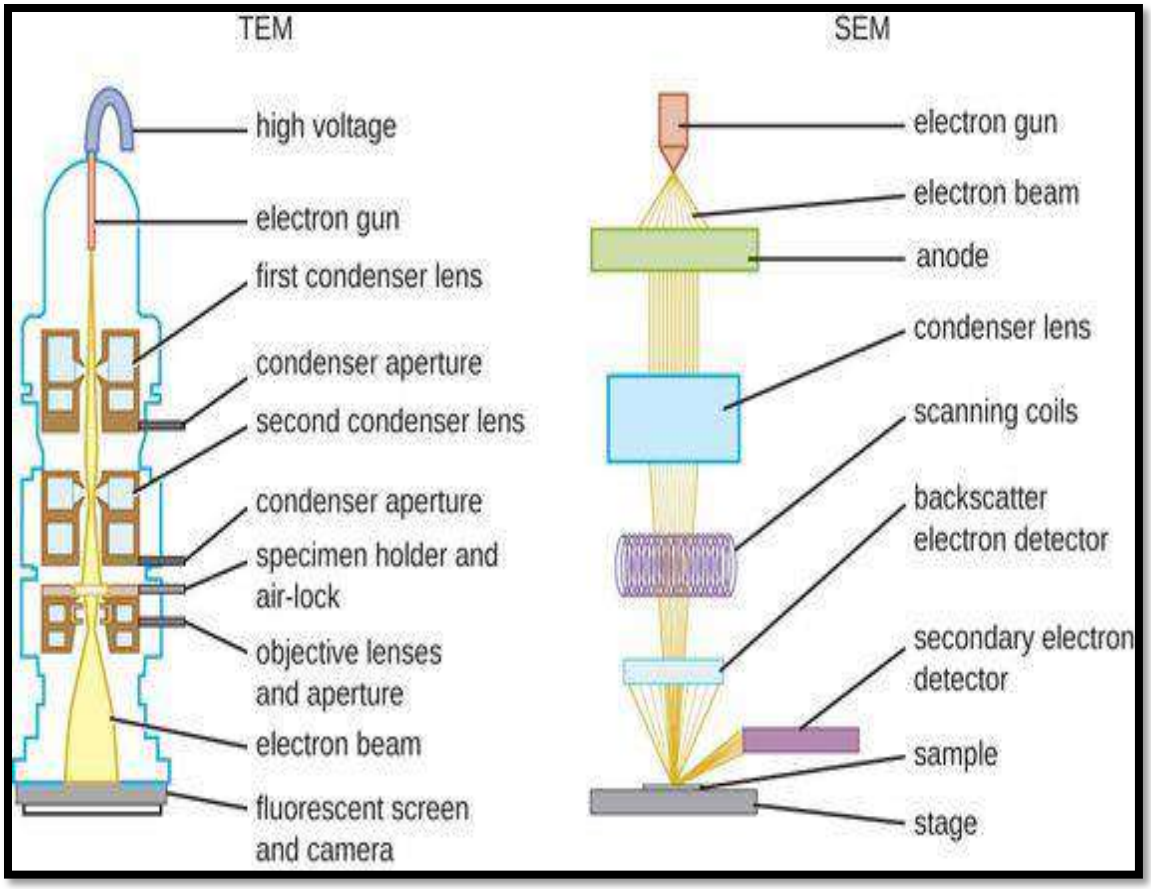
Apertures are used in an EM to control the coherence of the beam, which affects resolution, and the amount of contrast in the signal.

TRANSMISSION ELECTRON MICROSCOPE

- A **TEM** transmits the beam of electrons through a thin sample onto a screen or a camera/detector (Fig. 1). It has a large number of lenses.
- The condenser lenses (2-4 depending on the microscope) are responsible for the amount of illumination that reaches the sample and control beam intensity or brightness.
- The objective lens focuses the beam of electrons onto the sample and applies a small amount of magnification.
- The intermediate and projector lenses magnify the beam and project it onto the camera (CCD or film) or screen to form an image.
- It takes only a few seconds to obtain a micrograph (microscope image). The image is a result of the projected beam intensity:
- Transmitted electrons are detected as light areas in the micrograph; darker areas occur where electrons have been scattered or absorbed by the sample, thus reducing the number of electrons reaching the camera or screen.
- This is known as bright field imaging and is the most common type of imaging for biological samples.
- TEMs are often classified based on the accelerating voltage (AV) they are capable of.
- A routine TEM for biological imaging should be capable of an AV of up to 120 kV. Most thin-section TEM will be conducted using 80-100 kV.
- Advanced TEM techniques may require instruments capable of an AV between 200 kV and 3 MV, which represent a resolution 100,000 to 3 million times smaller than light microscope resolution.

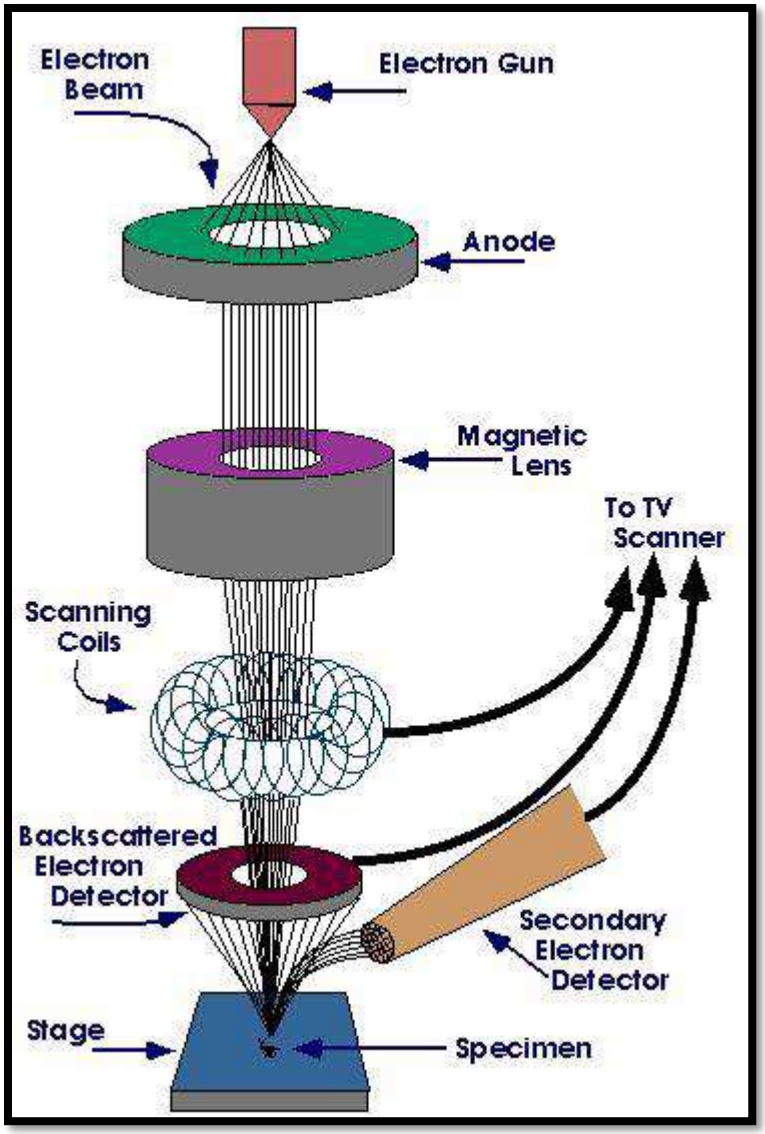


TRANSMISSION ELECTRON MICROSCOPE



SCANNING ELECTRON MICROSCOPE

- A SEM focuses the beam of electrons into a small spot that scans across the surface of a sample.
- The condenser lens assembles the electrons into a fine beam. The objective lens focuses the beam onto the sample.
- Deflection coils cause the beam to move in a rectangular X and Y direction, producing a raster scan across the surface of the sample.
- The signal is transmitted to a computer screen. Reducing the area being scanned results in an increase in magnification).
- An SEM image is formed from signals that are emitted from the sample as a result of the specimen-beam interaction.
- Most biological SEM will generate images using two types of electrons. Secondary electrons (SE) are low energy electrons produced by small energy transfers between electrons from the beam and electrons orbiting atoms in the sample.



SCANNING ELECTRON MICROSCOPE

- The energy transfer causes the orbiting electron to leave the atom and become a secondary electron.
- An outer orbiting electron will then release some energy in order to jump into the gap left by the secondary electron.
- The second type, backscattered electrons (BSE), are high energy electrons that have passed close to an atomic nucleus and been reflected or “back-scattered” out of the specimen.
- In addition, there are a few applications that require the detection of characteristic X-rays (energy dispersive X-ray spectroscopy) or photons (cathodeluminescence).

- There are different types of detectors to collect these signals. Secondary electrons are low energy electrons and only those produced near the surface can be emitted.
- The signal detectors are not cameras and the resolution of an SEM image depends on the spot size of the beam as it hits the sample and the interaction volume between the beam and specimen.
- The interaction volume directly relates to the AV of the beam. Biological SEM typically uses an AV of 1-5 kV for the best resolution.
- The SEM image is inverted compared to the TEM. Bright areas of the image are the result of more electrons being scattered (from topography or heavy element staining). Relatively large biological samples can be imaged using an SEM as we no longer have to transmit the signal through the specimen.

References

Abbe, E., (1873) Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung. *Archiv für mikroskopische Anatomie*, *9*(1), pp.413-418.

Hayat M., (2000) Principles and techniques of electron microscopy, biological applications. 4th Edition. Cambridge: Cambridge University Press.

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தனி நுண்ணோக்கி (simple Microscope)

இவை எளிய நுண்ணோக்கியின் ஒரு இருவில்லை அல்லது பல வில்லைகளின் கூட்டமைப்பு என்றாக தயாரிக்கப்படுகின்றனவாறு அமைந்திருக்கும்.

இவ்வாறு ஒரு வில்லையை கண்ணுக்கும் பொருளுக்கும் இடையே அமைக்கப்படும்போது, கண்ணுக்கு அருகே பொருள் கொண்டு வரப்படும்போது பொருளின் உருவம் பெரிதாக்கப்படுகிறது.

இப்பெரிதாக்கப்பட்ட உருவம் விழித்திரையில் விழுகின்றது. இவ்வாறு தனி நுண்ணோக்கி வழி பெறப்படுகின்ற ஆதை கீழ்க்காணும் வாய்ப்பாடு வழி கண்டறிய கியலும்

$$M = \frac{10}{f} + 1$$

* f - வில்லையுடைய குவிய தூரம்

* 10 = தெளிவான பார்வை தொலைவு இடைத்திருப்பட்ட தூரம்.

எளிய நுண்ணோக்கி வழி பெறப்படும் உருப்பெருக்கத்தின் உச்ச அளவு அமைவு 100 மடங்காகும்.

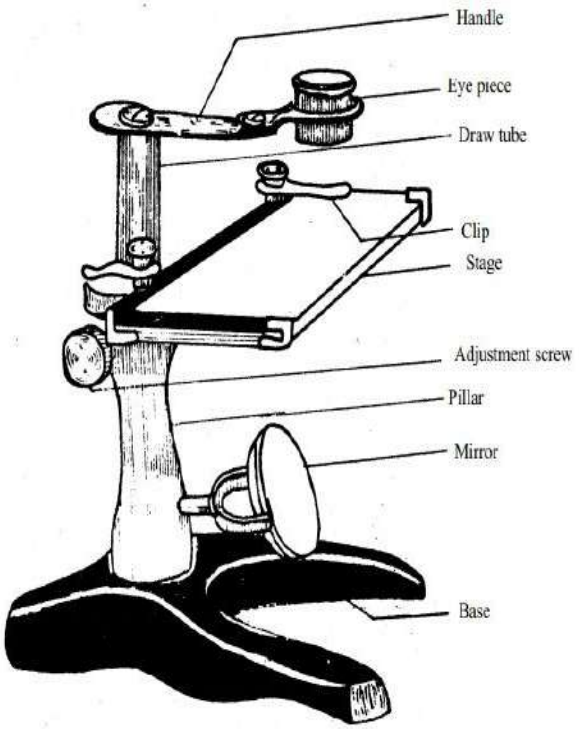


Fig. P.2A Simple Microscope

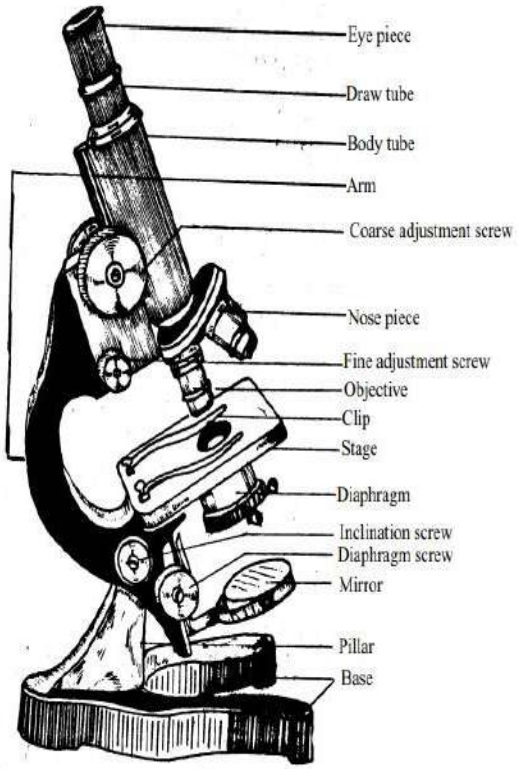
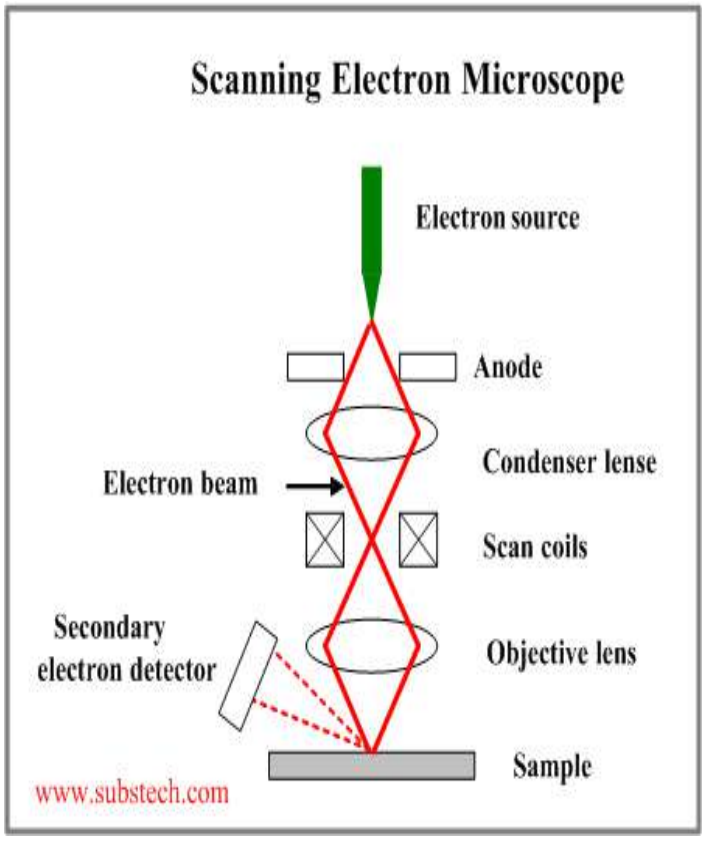
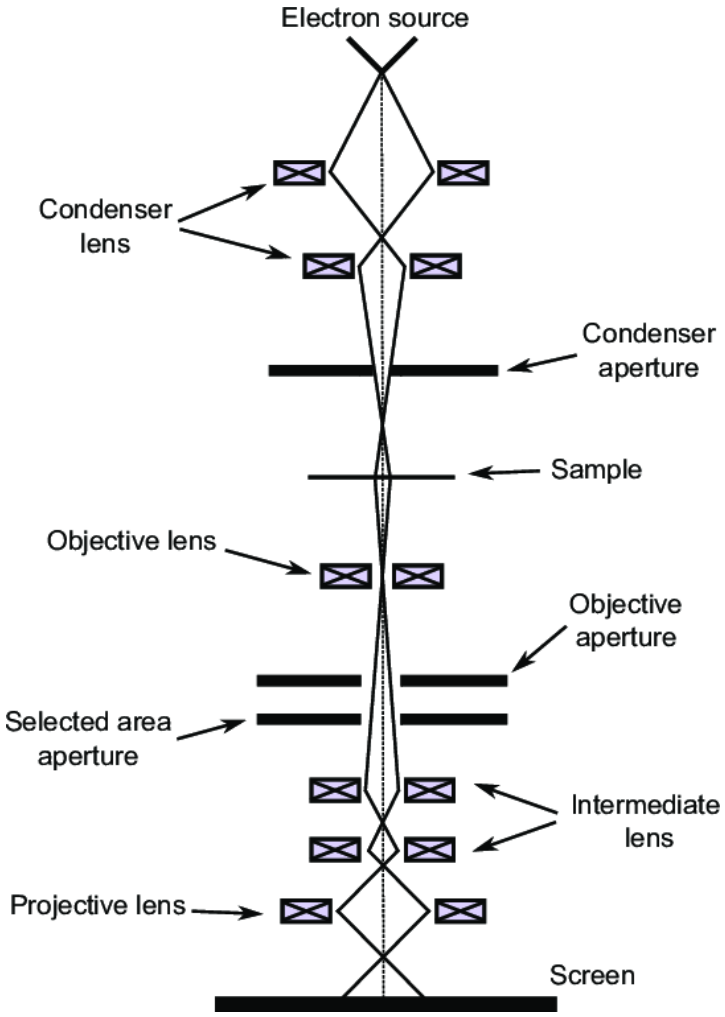
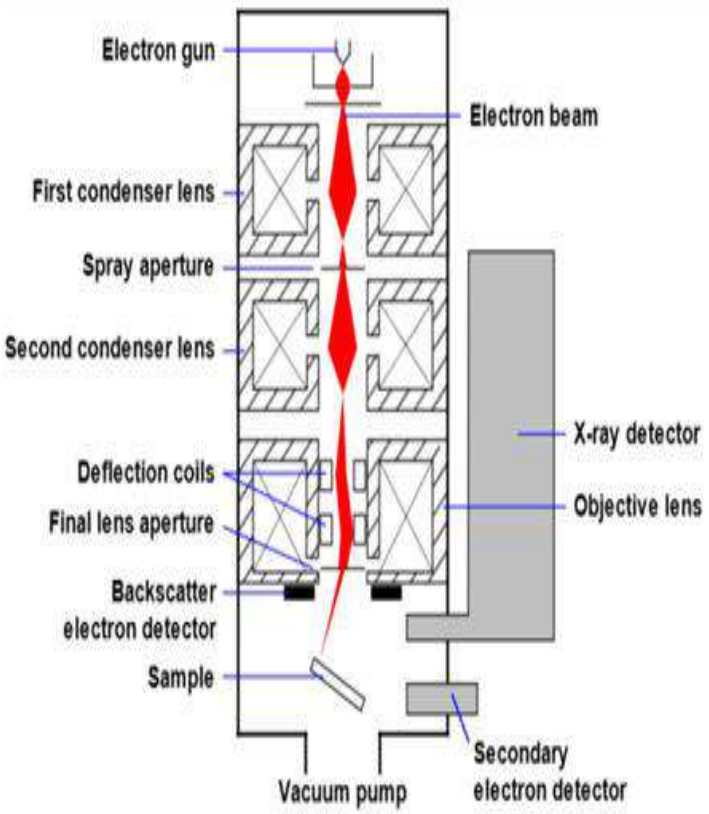


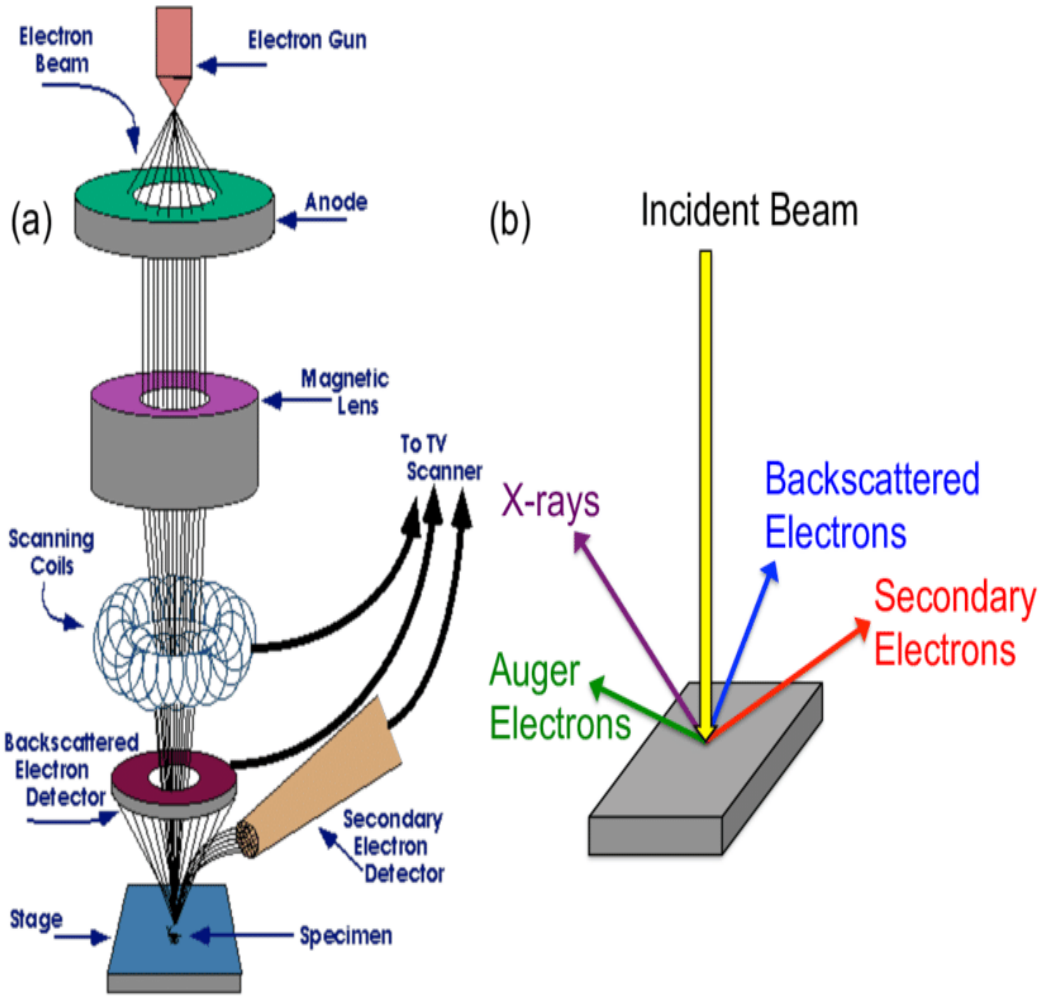
Fig.P.3A Compound Microscope





Transmission electron microscope, TEM


Electron gun
Electron beam
First condenser lens
Spray aperture
Second condenser lens
X-ray detector
Deflection coils
Objective lens
Final lens aperture
Backscatter
electron detector
Sample
Secondary
electron detector
Vacuum pump


Electron Beam
Electron Gun
(a)
Anode
Magnetic Lens
To TV Scanner
Scanning Coils
Backscattered Electron Detector
Secondary Electron Detector
Stage
Specimen
(b)
Incident Beam
X-rays
Backscattered Electrons
Secondary Electrons
Auger Electrons

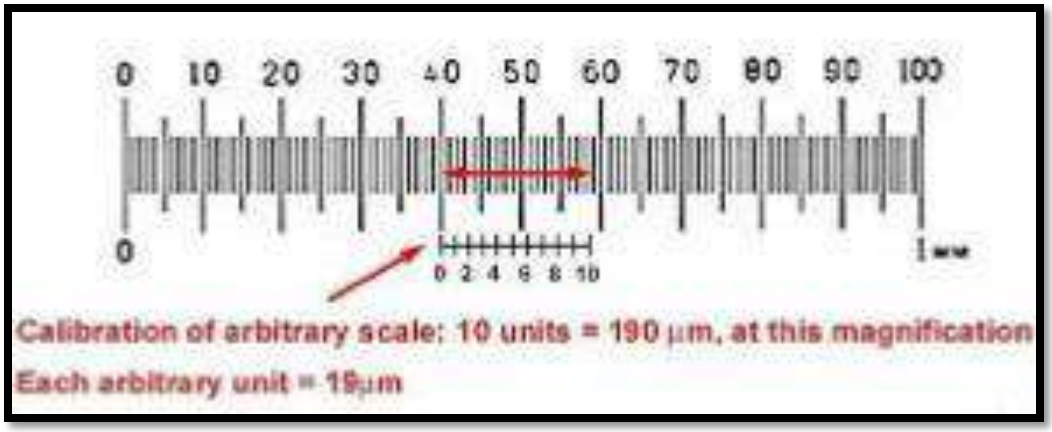
ஒளி நுண்ணோக்கியல் அளவிடும் முறைகள்
(Stage Micrometer) (தள மைக்ரோ மீட்டர்)

எளிய அமைப்புடைய ஒளி நுண்ணோக்கியில் செல்கள் ஆக்குலார் (தனைப்பு) மைக்ரோ மீட்டர் தட்டு, தள (stage) மைக்ரோ மீட்டர் தட்டு இவற்றால் அளக்கப்படுகின்றன. கண்ணாடியாலான ஆன ஆக்குலார் மைக்ரோ மீட்டர் தட்டு ஆக்குலார் டயப்பிரத்தில் வைக்கப்படுகிறது.

அளவீடுகள் செய்வதற்கு முன்பாக மைக்ரோமீட்டர் தட்டு அளவு திருத்தம் (Calibration) செய்யப்படுகிறது. பயன்படுத்தப்படுகின்ற பொருளருகு வில்லை, நுண்ணோக்கி உடல் குழல் (body tube), கண்ணருகு வில்லை ஆகியவையும் அளவு திருத்தம் செய்யப்படுகின்றன.

தள மைக்ரோமீட்டர் (Stage micrometer) பொதுவாக கண்ணாடி தகட்டினால் ஆக்கப்பட்டது. இதன் மீது அறிந்த இடைவெளியுள்ள குறிகள் இடப்படு-கின்றன.

ஆக்குலார் மைக்ரோமீட்டர் தட்டு, மேடை மைக்ரோமீட்டர் ஆகியன ஒரே அலகில் குறிக்கப்பட்டிருப்பின் ஆக்குலார் மைக்ரோமீட்டர் தட்டினை அளவு திருத்தம் செய்வது எளிது. இதனை மேடை மைக்ரோமீட்டரின் மீது குவியம் (focus) செய்து ஆக்குலார் மீட்டர் '0' குறியீடும், தட்டின் மீதுள்ள '0' குறியீடும் ஒன்றாக இருக்கும்படி அமைக்கப்பட்டு அடுத்த அலகுக் குறியீட்டு கோடுகள் நேர் நேராக அமையுமிடம் குறித்துக்கொள்ளப்படுகிறது. நுண்ணோக்கியின் உருப்பெருக்க எண் கொண்டு பொருளின் அளவு அறியப்படுகிறது.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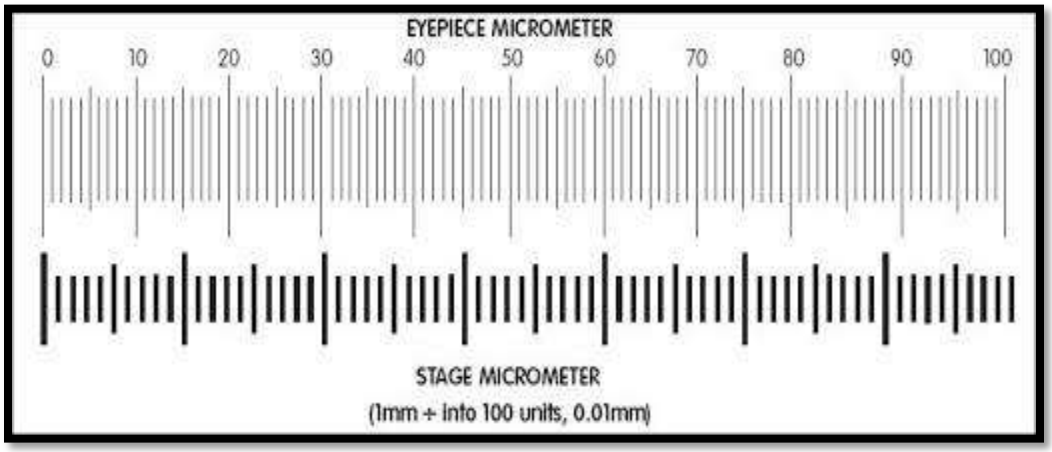
Calibration of arbitrary scale: 10 units = 190 µm, at this magnification
Each arbitrary unit = 19µm


EYEPIECE MICROMETER
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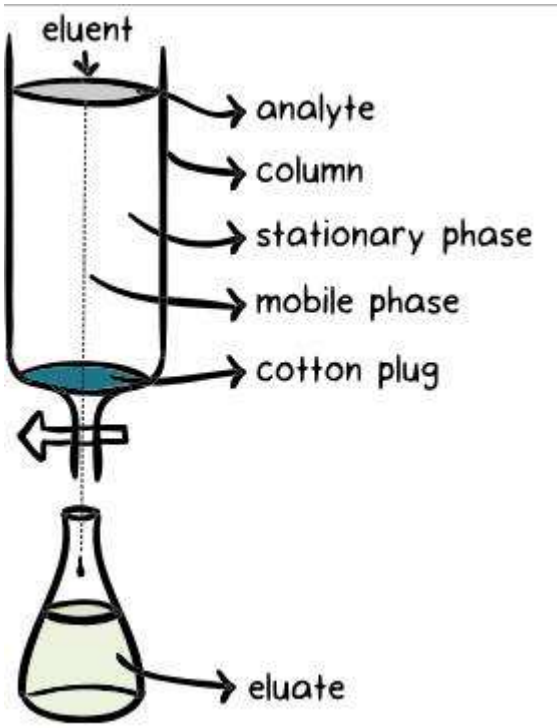
STAGE MICROMETER
(1mm ÷ into 100 units, 0.01mm)

Unit III

CHROMATOGRAPHY- DEFINITION, PRINCIPLE, TYPES, APPLICATIONS

- Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.
- The Russian botanist Mikhail Tswett coined the term chromatography in 1906.
- The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of **fatty acid** mixtures.
- A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties to separate materials.
- It is a powerful separation tool that is used in all branches of science and is often the only means of separating components from complex mixtures.



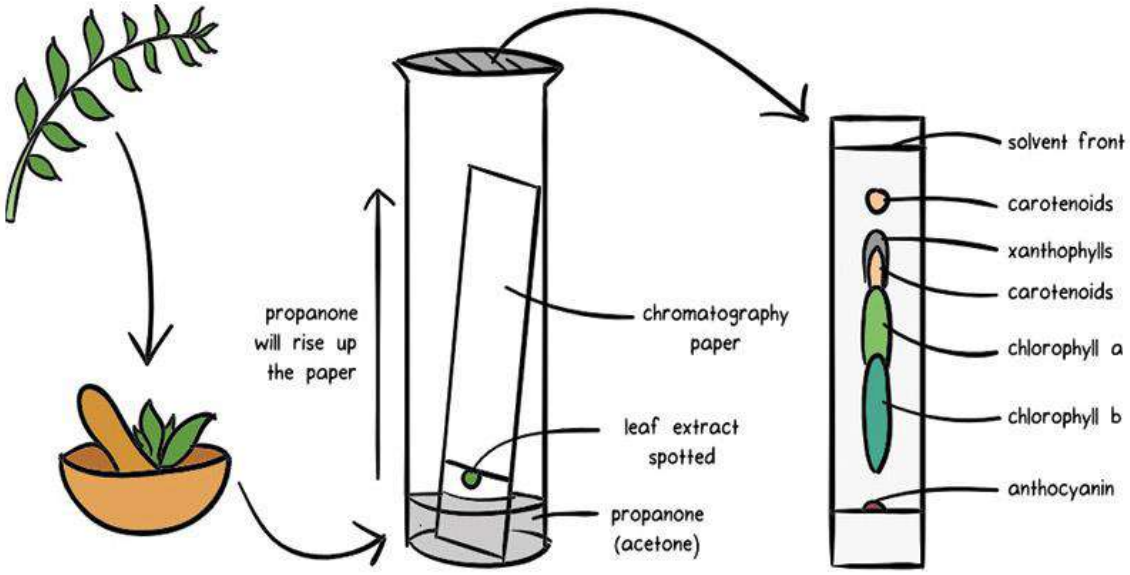
Principle of Chromatography

- Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase.
- The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights.
- Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into the mobile phase, and leave the system faster.

Three components thus form the basis of the chromatography technique.

1. **Stationary phase:** This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface solid support".
2. **Mobile phase:** This phase is always composed of "liquid" or a "gaseous component."
3. **Separated molecules**

The type of interaction between the stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on the separation of molecules from each other.



Types of Chromatography

- Substances can be separated on the basis of a variety of methods and the presence of characteristics such as size and shape, total charge, hydrophobic groups present on the surface, and binding capacity with the stationary phase.
- This leads to different types of chromatography techniques, each with their own instrumentation and working principle.
- For instance, four separation techniques based on molecular characteristics and interaction type use mechanisms of ion exchange, surface adsorption, partition, and size exclusion.
- Other chromatography techniques are based on the stationary bed, including column, thin layer, and paper chromatography.
    - Column chromatography
    - Ion-exchange chromatography
    - Gel-permeation (molecular sieve) chromatography
    - Affinity chromatography
    - Paper chromatography
    - Thin-layer chromatography
    - Gas chromatography (GS)
    - Dye-ligand chromatography
    - Hydrophobic interaction chromatography
    - Pseudoaffinity chromatography
    - High-pressure liquid chromatography (HP)

Applications of Chromatography

Pharmaceutical sector

- To identify and analyze samples for the presence of trace elements or chemicals.
- Separation of compounds based on their molecular weight and element composition.
- Detects the unknown compounds and purity of mixture.
- In drug development.

Chemical industry

- In testing water samples and also checks air quality.
- HPLC and GC are very much used for detecting various contaminants such as polychlorinated biphenyl (PCBs) in pesticides and oils.
- In various life sciences applications

Food Industry

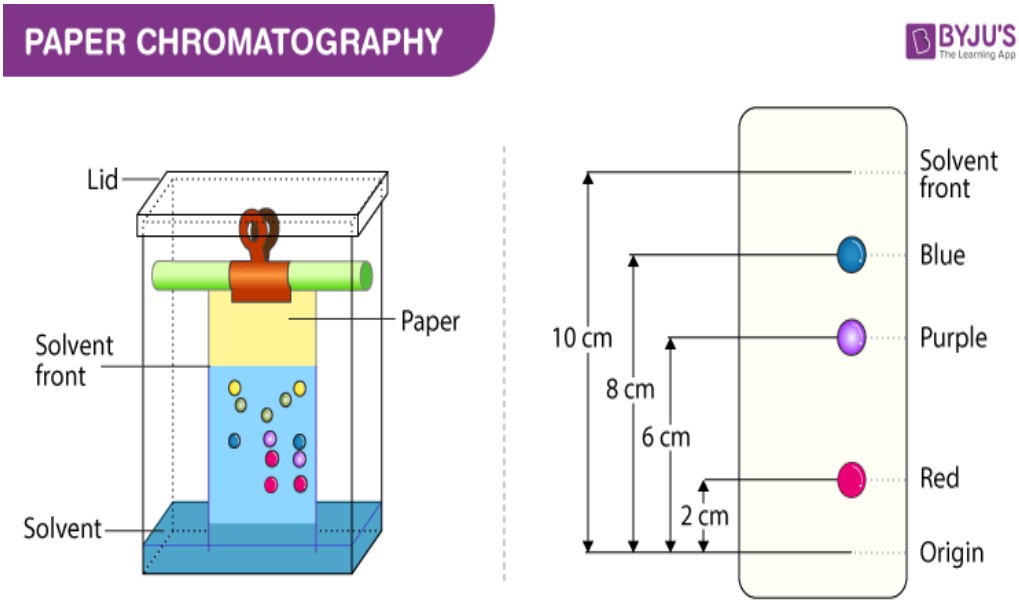
- In food spoilage and additive detection
- Determining the nutritional quality of food

Forensic Science

- In forensic pathology and crime scene testing like analyzing blood and hair samples of crime place.

Molecular Biology Studies

- Various hyphenated techniques in chromatography such as EC-LC-MS are applied in the study of metabolomics and proteomics along with nucleic acid research.
- HPLC is used in Protein Separation like Insulin Purification, Plasma Fractionation, and Enzyme Purification and also in various departments like Fuel Industry, biotechnology, and biochemical processes.



1.

வண்ண வரைகலையியல்
(Chromotography)

வண்ண வரைகலையியல் என்பதை வரைக்க:

மூலக்கூறுகளின் ஒரு கரைசலில் உள்ள மூலக்கூறுகளை தனிமைப்படுத்துவதில் வண்ண வரைகலை நுட்பங்கள் உதவுகின்றன. வடிவம் உருவளவு, பொருண்மை, செறிதிறம் கரைதிறன் மற்றும் மூலக்கூறுகளின் மடிப்பீடு ஆகியவற்றின் இயைபாட்டைப் பொறுத்து இதிதனிமைப்பாடு சாத்தியமாகிறது. வண்ண வரைகலை என்ற சொல் குரோக்கசு சொல்லிலிருந்து தோன்றியதாகும். இதன் பொருள் நிற இற்பெடுத்து என்பதாகும். மிஸ்வெட் என்ற ரஷ்ய தாவரவியல் வல்லுநர் இச்சொல்லினை தென்முதலில் பயன்படுத்தினார்.

வண்ண வரைகலையியல் வகைகள் யாவை?

1. பதி வரைகலையியல்
2. தாள் வரைகலையியல்
3. மெல்லி அடுக்கு வரைகலையியல்
4. வாயு நீரக வரைகலையியல்.

பதி வரைகலையியலின் நெறிமுறைகள், செய்முறை, பயன்பாடுகள் பற்றி விவரிக்க:-

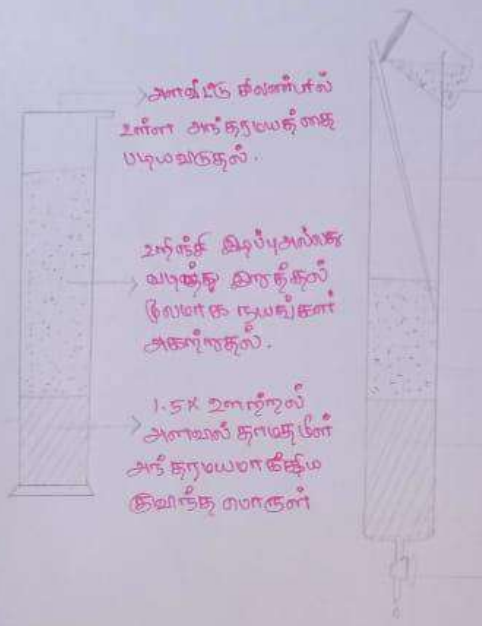
வண்ண வரைகலை பகுதிக்கு பொதுவாக,

கண்ணாடி மயூரை பயன்படுத்தப்படுகிறது. வடிகட்டாக கைட்ரோஜனின், இனாக்ரோபாம், பென்சீன், டொலுவின், கார்பன் டை சல்பைடு, டைக்ளோர மெத்தேன் போன்றவை கரைப்பான்களாகப் பயன்படுத்தப்படுகின்றன.

மயூரைப்பினை பற்றி எனவும் அழைப்பார். தண்ணைப்படுத்தும் பொருட்களின் நல்ல தீர்வு இப்பற்றி வண்ண வரைகலை நிலம் கிடைக்கிறது. தக்க கரைப்பானில் இக்கண்ணாடிப் பற்றி நிரப்பப்படுகிறது. இக்கரைப்பானுடன் சாறு மெதுவாக உறிஞ்சி சேர்க்கப்படுகிறது. இந்த அந்தரமயக் கரைசலை படிய அமைக்கப்படுகிறது. மிகைப்படியான கரைசல் வடிந்தகற்றப்படுகிறது. போதுமான உறிஞ்சி பத்தியானை ஊற்றப்படுகிறது. போதுமான உறிஞ்சி பகுதியானை ஊற்றப்படுகிறது. தேவைப்பட்ட உயரத்தை எட்டும் வரை உறிஞ்சியை ஊற்ற வேண்டும்.

உறிஞ்சியின் புறப்பரப்பிற்கு சாறு மேலாக கரைப்பானின் மட்டம் இருக்குமாறு வைக்கப்பட வேண்டும்.

தனிப்படுத்தப்பட உள்ள நிலக்கூறுகலவையை சிறிதளவாக பீக்கரில் எடுத்து கரைப்பானால் கரைக்க வேண்டும். பத்தியின் மேலே மட்டம் கலன் இணைக்கப்பட்டுள்ளது. கலவை நிலக்கூறுகளுடன் கரைப்பான் மிக வலிமையுடன் செயலைத்ர் செயல்படுகிறது.



உந்தியுடன் திக்கலவை மிக மோசமாக செயலைதிர் தெயலி படுகிறது. எனவே, கலவையுள் காணவற்ற நிலக்கூறுகள் வெகு நிலக்கூற ஊடகொண்ட நிலக்கூறுகள் திலில் கிடி இறங்குகின்றன.

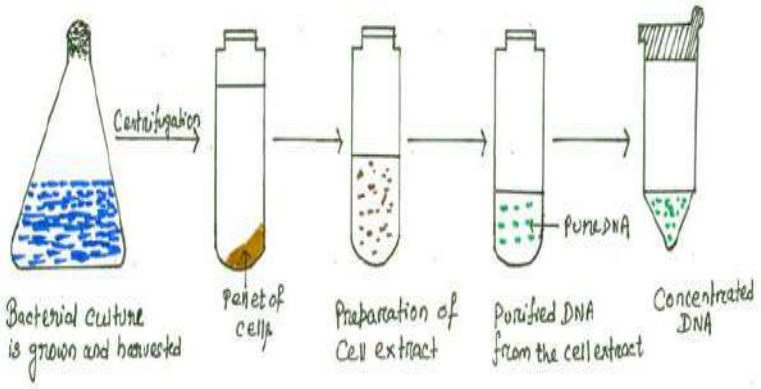
பகுப்பயிருந்து கழிவுநீர் சோதனைக் குழாயில் ஆராய்ச்சியாகச் சேகரிக்கப்படுகிறது. இதனை கைபதனமாக செய்து முடிக்கலாம். அல்லது சிறிய சேகரிப்பு நிலையம் சேகரிக்கலாம். ஏவ்வொரு சிறுகூறும் மண்பு பகுப்பாய்வு செய்யப்படுகிறது.

CENTRIFUGATION- PRINCIPLE, TYPES AND APPLICATIONS

- Centrifugation is a technique of separating substances which involves the application of centrifugal force.
- The particles are separated from a solution according to their size, shape, density, the viscosity of the medium and rotor speed.

Principle of Centrifugation

- In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it floats to the top.
- The greater the difference in density, the faster they move. If there is no difference in density (isopycnic conditions), the particles stay steady.
- To take advantage of even tiny differences in density to separate various particles in a solution, gravity can be replaced with the much more powerful “centrifugal force” provided by a centrifuge.
- A centrifuge is a piece of equipment that puts an object in rotation around a fixed axis (spins it in a circle), applying a potentially strong force perpendicular to the axis of spin (outward).
- The centrifuge works using the sedimentation principle, where the centripetal acceleration causes denser substances and particles to move outward in the radial direction.
- At the same time, objects that are less dense are displaced and move to the center.
- In a laboratory centrifuge that uses sample tubes, the radial acceleration causes denser particles to settle to the bottom of the tube, while low- density substances rise to the top.



Types of Centrifuge

LOW-SPEED CENTRIFUGE

Most laboratories have a standard low-speed centrifuge used for routine sedimentation of heavy particles

2) The low-speed centrifuge has a maximum speed of 4000-5000rpm

3) These instruments usually operate at room temperatures with no means of temperature control. 4) Two types of rotors are used in it,

- Fixed angle
- Swinging bucket.

5) It is used for sedimentation of red blood cells until the particles are tightly packed into a pellet and supernatant is separated by decantation.

HIGH-SPEED CENTRIFUGES

1. High-speed centrifuges are used in more sophisticated biochemical applications, higher speeds and temperature control of the rotor chamber are essential.
2. The high-speed centrifuge has a maximum speed of 15,000 – 20,000 RPM
3. The operator of this instrument can carefully control speed and temperature which is required for sensitive biological samples.

1. Three types of rotors are available for high-speed centrifugation-

- Fixed angle
- Swinging bucket
- Vertical rotors

Types of Centrifugation

1. **Differential Pelleting (differential centrifugation)**

- It is the most common type of centrifugation employed.
- Tissue such as the liver is homogenized at 32 degrees in a sucrose solution that contains buffer.
- The homogenate is then placed in a centrifuge and spun at constant centrifugal force at a constant temperature.
- After some time a sediment forms at the bottom of a centrifuge called pellet and an overlying solution called supernatant.
- The overlying solution is then placed in another centrifuge tube which is then rotated at higher speeds in progressing steps.

2. **Density Gradient Centrifugation**

- This type of centrifugation is mainly used to purify viruses, ribosomes, membranes, etc.
- A sucrose density gradient is created by gently overlaying lower concentrations of sucrose on higher concentrations in centrifuge tubes
- The particles of interest are placed on top of the gradient and centrifuge in ultracentrifuges.
- The particles travel through the gradient until they reach a point at which their density matches the density of surrounding sucrose.
- The fraction is removed and analyzed.

3. **Rate-Zonal Density-Gradient Centrifugation**

- Zonal centrifugation is also known as band or gradient centrifugation
- It relies on the concept of sedimentation coefficient (i.e. movement of sediment through the liquid medium)
- In this technique, a density gradient is created in a test tube with sucrose and high density at the bottom.
- The sample of protein is placed on the top of the gradient and then centrifuged.
- With centrifugation, faster-sedimenting particles in sample move ahead of slower ones i.e. sample separated as zones in the gradient.
- The protein sediment according to their sedimentation coefficient and the fractions are collected by creating a hole at the bottom of the tube.

4. **Isopynic Centrifugation**

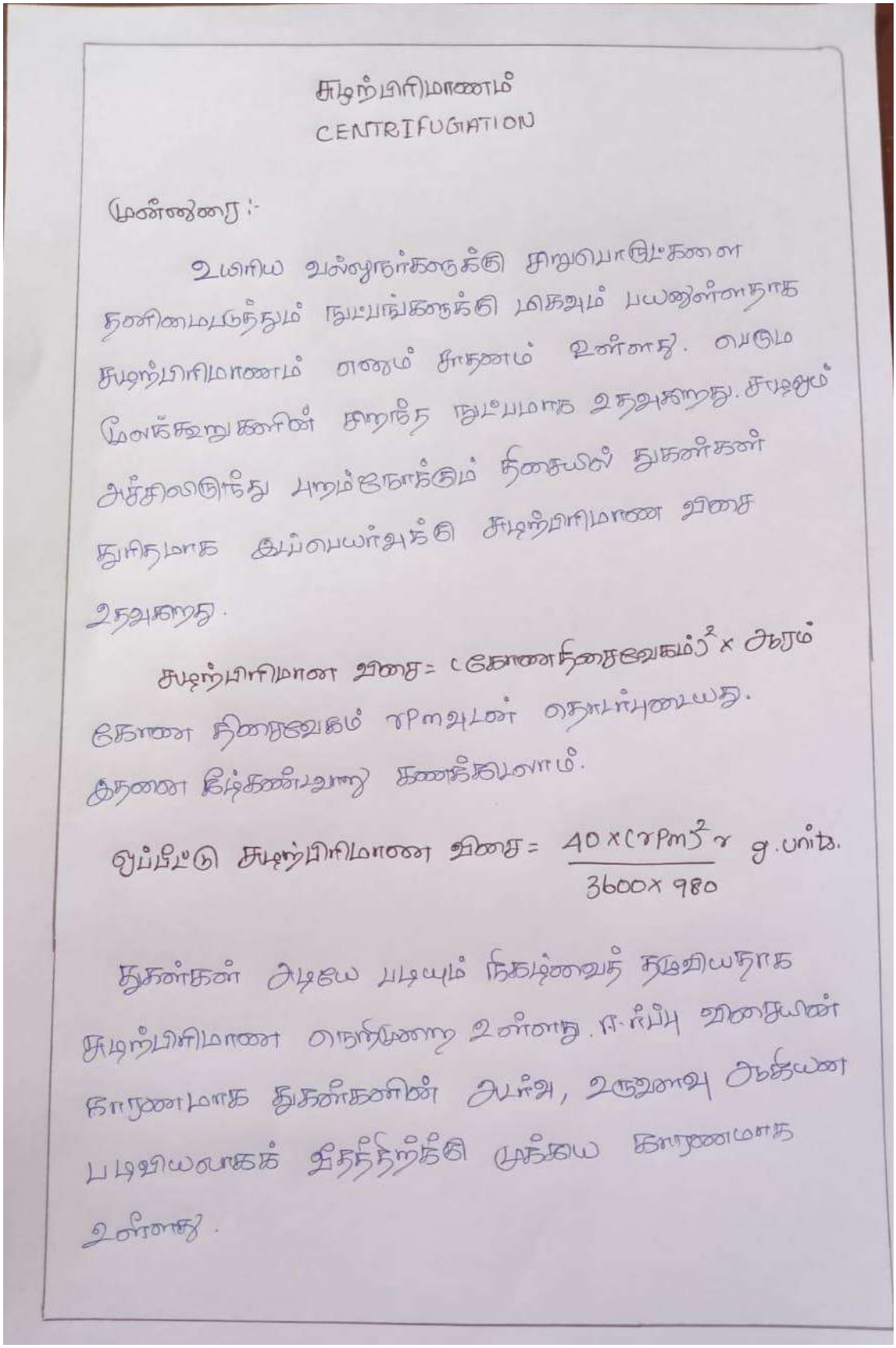
- The sample is loaded into the tube with the gradient-forming solution (on top of or below pre-formed gradient, or mixed in with self-forming gradient)
- The solution of the biological sample and cesium salt is uniformly distributed in a centrifuge tube and rotated in an ultracentrifuge.
- Under the influence of centrifugal force, the cesium salts redistribute to form a density gradient from top to bottom.
- Particles move to point where their buoyant density equals that part of gradient and form bands. This is to say the sample molecules move to the region where their density equals the density of gradient.
- It is a "true" equilibrium procedure since depends on bouyant densities, not velocities

Eg: CsCl, NaI gradients for macromolecules and nucleotides – "self-forming" gradients under centrifugal force.

Applications of Centrifugation

- To separate two miscible substances
- To analyze the hydrodynamic properties of macromolecules
- Purification of mammalian cells
- Fractionation of subcellular organelles (including membranes/membrane fractions) Fractionation of membrane vesicles
- Separating chalk powder from water
- Removing fat from milk to produce skimmed milk
- Separating particles from an air-flow using cyclonic separation
- The clarification and stabilization of wine

- Separation of urine components and blood components in forensic and research laboratories.



சுழற்பிரிமாணம்
CENTRIFUGATION

முன்னுரை :-

உயிரிய வல்லுநர்களுக்கு சிறுபொருட்களை தனிமைபடுத்தும் நுட்பங்களுக்கு மிகவும் பயனுள்ளதாக சுழற்பிரிமாணம் எனும் சாதனம் உள்ளது. பெரும மூலக்கூறுகளின் சிறந்த நுட்பமாக உதவுகிறது. சுழலும் அச்சிலிருந்து புறம்நோக்கும் திசையில் துகள்கள் துரிதமாக இப்பெயர்ச்சிக்கு சுழற்பிரிமாண விசை உதவுகிறது.

சுழற்பிரிமான விசை = (கோணதிசைவேகம்)^2 × அரம்

கோண திசைவேகம் rpm உடன் தொடர்புடையது. அதனை கீழ்க்காண்பதாறு கணக்கிடலாம்.

ஒப்பீட்டு சுழற்பிரிமான விசை = $\frac{40 \times (rpm)^2 \times g.\text{பார}}{3600 \times 980}$

துகள்கள் அடியே படியும் நிகழ்வைத் தடுமியதாக சுழற்பிரிமாண நெறிமுறை உள்ளது. ஈர்ப்பு விசையின் காரணமாக துகள்களின் அடர்த்தி, உருவளவு அதியன படிவியலாகத் தீர்மானிக்கும் முக்கிய காரணமாக உள்ளது.

சுடர்பிரிமாணத்தில் பயன்படுத்தப்படும் சொல் தொகுதி :-

ஒரு சுடர்பிரிமாணத்தில் இரண்டு துணைகள் அல்லது கூரைமங்கனின் இயக்கம், படிவியலாக்கம் எனப்படுகிறது. இவ்வியக்கத்தின் முதற்றை / தலையெழுத்து படிவியலாக்கம் முதம் என அழைப்பர். கலனின் அடியே படியும் துகள்களை / கீறுகள் குறுமணிகள் அல்லது மீதிமதிப்புகள் என அழைக்கப்படுகின்றன. கூறுமணிகளுக்கு மேலாக அமையும் அருகி கூரையின் தளமிதப்பு என அழைப்பர்.

1) ஆய்ந்த சுடர்பிரிமாணம் :

நெல் அங்கங்களின் தனிமையாட்டற்கு இந்நடப்பம் உதவுகிறது. இந்நடப்பதீற்கு இருவகை சுடலிகள் பயன்படுத்தப்படுகின்றன. அவையாவன; 1) அளையாடும் / அளிவாடும் / உள்ளாடும் - வாளி சுடலி 3) நிலையான - கோண சுடலி சுடலியின் அமைப்புக் கலவைகளுடன் உளாக வாளிகளின் ஏறு அணி இணைக்கப்பட்டுள்ளது. சுடலியின் மேற்பகுதியின் பொருத்து கிடைமட்ட நிலைமையில் இந்த வாளி அமைகிறது.

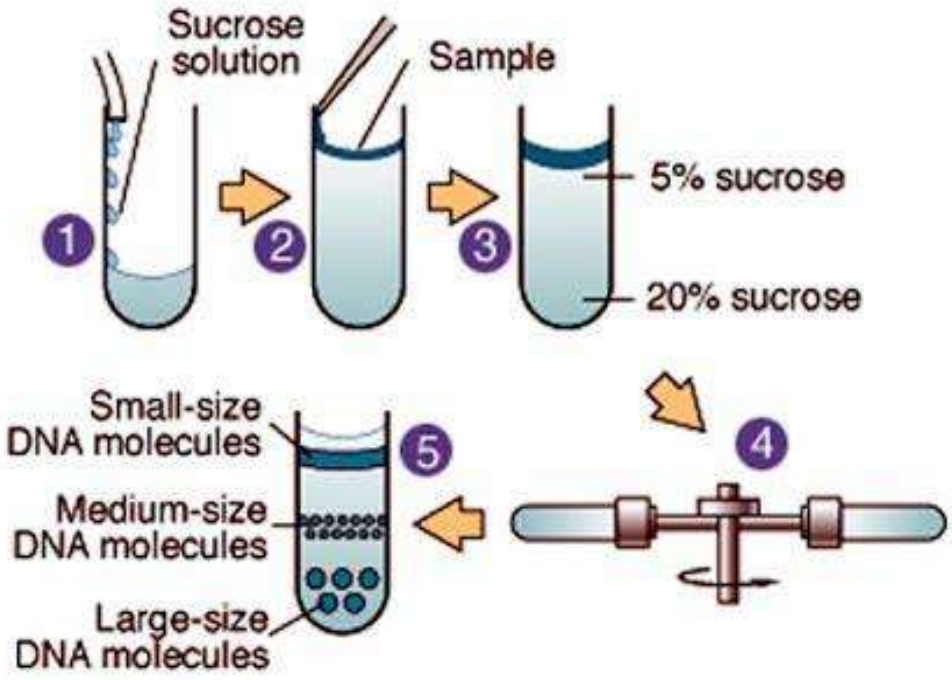
2) வேறுபாட்டு சுழற்பிரிமானம்:

நோபில், வாரிங்டேட், சூல்பர்ட் கொண்டு ஆகியோர் 1940-ல் இந்நுட்பத்தை அறிமுகப்படுத்தினார். ஒரு கரைசலில் உள்ள துகள்கள் அவற்றின் உருவளவு வடிவம், அடர்வு ஆகியவற்றின் அடிப்படையில் படிகின்றன. இம்முறையில் செல் உட் துகள்கள் படியவைக்கப்படுவதுடன் அடுத்தடுத்த உயர் அதிகரிப்பதன் ஒன்றன்பின் ஒன்றாக இத்துகள்கள் அகற்றப்படுகின்றன.

ஒரு கலவையில் உள்ள கரைசல் குறைந்த வேகத்தில் முதலில் சுழற்பிரிமானம் செய்யப்படுகிறது. பெரிய உருவளவுத் துகள்கள் அடியிலே படிகின்றன. இதன் மீதுள்ள தெளிதப்பு, இன்னொரு குழாய்க்கு மாற்றப்படுகிறது. பின்பு இதனையும் உயர் வேகத்தில் வெகு நேரம் சுழற்பிரிமானம் செய்யப்படுகிறது.

எடுத்துக்காட்டாக:

ஈரல் திசுவின் செல் உட் துகள்களின் நிலைமைப்பாடு கீழே கொடுக்கப்பட்டுள்ளது.


Sucrose solution
Sample
1
2
3
5% sucrose
20% sucrose
4
Small-size DNA molecules
Medium-size DNA molecules
Large-size DNA molecules
5

(a)

Chapter 16 | Fixation and Tissue Processing

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Fixation

The structure of a tissue is determined by the shapes and sizes of macromolecules in and around cells. The principal macromolecules inside a cell are proteins and nucleic acids. In vertebrate animals, the macromolecules on the outside surfaces of cells and in the extracellular spaces are glycoproteins and proteoglycans, in which much carbohydrate material is covalently joined to protein molecules. Carbohydrates are hydrophilic: they hold much water in the extracellular space, by hydrogen bonding. There is also, of course, much water inside cells: water accounts for about 60% of the weight of the human body. (Guyton, Arthur C. (1976). *Textbook of Medical Physiology* (5th ed.). Philadelphia: W.B. Saunders. p. 424) In bones and teeth hydroxyapatite, a crystalline mineral containing calcium and phosphate ions, dominates the extracellular domain, together with collagen, a fibrous protein.

An essential part of all histological and cytological techniques is preservation of cells and tissues as they naturally occur. To accomplish this, tissue blocks, sections or smears are usually immersed in a fixative fluid, although in the case of smears, merely drying the preparation acts as a form of preservation. The fixatives employed prevent autolysis by inactivating lysosomal enzymes, and they stabilize the fine structure, both inside and between cells, by making macromolecules resistant to dissolution by water and other liquids. Fixatives also inhibit the growth of bacteria and molds that give rise to putrefactive changes.

In performing their protective role, fixatives denature proteins by coagulation, by forming additive compounds, or by a combination of coagulation and additive processes. A compound that adds chemically to macromolecules stabilizes structure most effectively if it is able to combine with parts of two different macromolecules, an effect known as cross-linking. The result is conformational changes in the structure of proteins and subsequent inactivation of most enzymes. Some lipids, proteins, carbohydrates and minerals are extracted by fixative liquids in which they are soluble. Fixation changes both chemical and antigenic profiles of proteins. The dilemma of fixation has always been that it introduces some artifact in order to have a protective effect. By definition, fixatives change the original chemical and physical compositions of tissues. In addition to altering the chemical nature of the cells and tissues to which they are applied, fixatives also cause physical changes to cellular and extracellular components.

Viable cells have surface membranes that are impermeable to large, hydrophilic molecules. Fixation, especially in organic liquids that dissolve or disrupt the lipids of the cell membrane, allows relatively large molecules to penetrate and escape. Furthermore, the cytoplasm becomes permeable to macromolecules, forming a proteinaceous network sufficiently porous to allow further penetration of large molecules. In this context, "large molecules" include those of paraffin wax, those of antibodies used for immunostaining, and larger dye molecules. Different fixatives result in different degrees of porosity. Coagulants such as mercuric chloride, picric acid, or zinc sulfate result in a larger pore size than do noncoagulant fixatives, such as formaldehyde, glyoxal or glutaraldehyde. Some fixatives are both coagulant and non-coagulant. For example, acetic acid coagulates nuclear chromatin but not proteins. Thus, coagulant fixatives are generally beneficial for subsequent paraffin sections. In addition to facilitating permeation of sections by antibodies, coagulant fixatives can also increase the exposure of antigenic sites, a consequence of the deformation of macromolecular shapes that constitutes coagulation. Cross-linking impedes penetration of paraffin but increases the physical strength of the tissue, especially if frozen sections are needed. Chemical addition of fixative molecules, with or without cross-linking, can modify antigenic sites and suppress immunostaining. This is a well known effect of formaldehyde, often reversible by one of many antigen retrieval procedures.

In attempts to combine the best properties of different compounds used for fixation, many mixtures have been developed. Most contain both coagulant and non-coagulant ingredients. Other substances in fixative solutions have actions such as controlling the osmotic pressure, adjusting the pH, and counteracting shrinkage or swelling caused by another ingredient. Shrinkage or swelling that occurs evenly through a specimen usually does not matter, but severe distortion results from changes in the sizes of some but not all parts of a tissue, as when tubular structures shrink within their surrounding connective tissue, or when artificial spaces are formed around cells.

Embedding and Microtomy

Once the tissue has been processed it is ready to be orientated into a paraffin block and subsequently sectioned. Orientation during embedding is crucial for the representation of proper morphology. Structures in skin, small gastrointestinal biopsies, and vas deferens are among those for which orientation is especially critical. Good microtomy techniques will minimize artifacts that lead to difficult diagnostic interpretation of special stains. One of the most directly correlated factors is the thickness in which a specimen is cut. Routine H&E stained specimens are cut 3–4 µm, but some morphology is best represented otherwise. For example, amyloid deposits are better represented at 8–12 µm, whereas kidney biopsies should be cut at 2 µm for optimal viewing of the structures of glomeruli. Techniques often used to aid in microtomy are water bath adhesive and positively charged slides. However, in some silver impregnation stains, the silver ions are attracted to the coating and produce an overall background to the slide. To avoid this artifact (Figure 3), use clean slides without a coating.

After sectioning, the tissue slide is drained and may be gently heated to evaporate the layer of water between the sections and the glass. When all the water is gone, it is permissible to heat the slide enough to melt the wax, a procedure that may improve adhesion. For optimal melting of paraffin consult the melting temperature and "plastic point" on the manufacturing product insert. The plastic point is usually a few degrees lower than the melting point and represents the lowest temperature at which permanent deformation can occur without fracture.

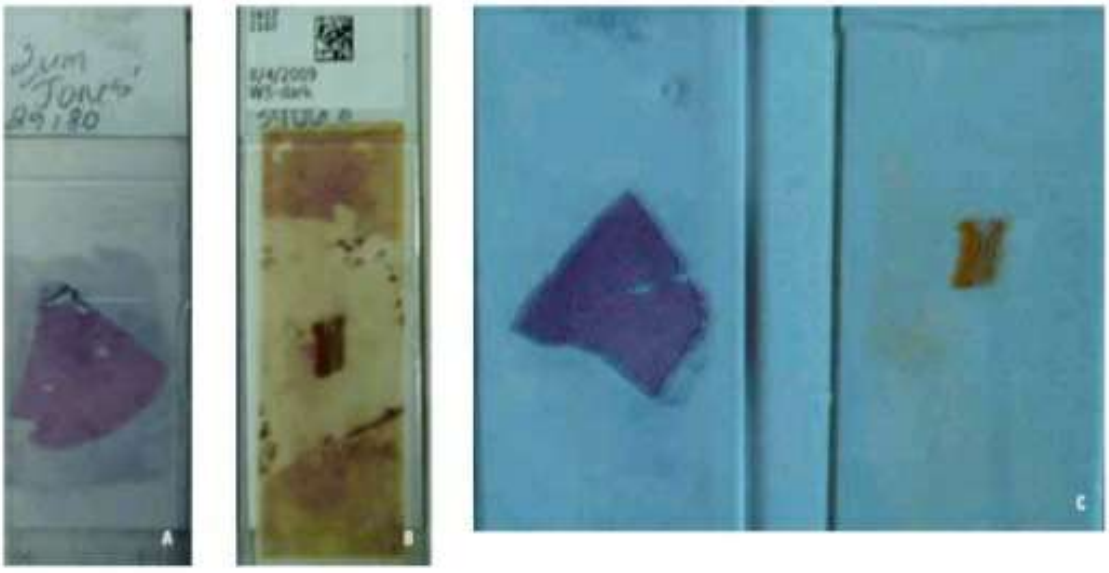

Figure 3. A. Jones' silver method, kidney. 2µm adhesive residue stained by a silver method. B. Warthin Starry 4µm paraffin residue ghost (not stained by reduced silver). C. Jones' and Warthin Starry slides demonstrating no mirroring artifact.

Table 2.

Process	Solution	Time	Retort
b) Conventional processing schedule, 1mm biopsy, 2.5 hrs.			
Dehydrate	Alcohol, 65%	15 min	
Dehydrate	Alcohol, 95%	15 min	
Dehydrate	Alcohol, 95%	15 min	
Dehydrate	Alcohol, absolute	15 min	
Dehydrate	Alcohol, absolute	15 min	
Cleaning agent	Xylene	15 min	
Cleaning agent	Xylene	15 min	
Infiltrate	Paraffin	15 min	
Infiltrate	Paraffin	15 min	
Infiltrate	Paraffin	15 min	
c) Microwave processing schedule, 1mm biopsy, ~45 min.			
Dehydrate	Ethyl alcohol, 100%	---	fill
DRAIN			
Dehydrate	Ethyl alcohol, 100%	5 min	67 °C, microwave
DRAIN			
Dehydrate	Isopropyl alcohol, 100%	3 min	74 °C microwave
DRAIN			
Infiltrate	Liquid paraffin, 60 °C		fill
DRAIN*			
Infiltrate	Liquid paraffin, 60 °C	2 min	65 °C microwave, agitate
	---	5 min	85 °C microwave
DRAIN*			

*Drain to 80 °C container

Keep in mind that individual laboratories must optimize to their specimen types. In general, needle biopsies and bloody specimens should be incubated conservatively, whereas fatty specimens can be processed for longer than average.

In recent years efforts have been made to streamline processing time by using microwave heating techniques. The benefits and risks associated with the technique are being revealed as more laboratories employ the technology and publish their findings. For a list of easily available literature, review the bibliography section. At this time, the concept will be presented with supporting examples.

It has been reported that microwave processing can be achieved ~60% faster than conventional processing time. See Table 2 for a comparison of the two techniques, each using 1-3mm thick biopsy samples.

One critical aspect of using microwave techniques is ensuring the samples have been adequately fixed. Each laboratory should evaluate its fixation control methods to optimize the use of microwave processing. Although the decrease in processing time increases the specimen workflow, there has been some resistance to the implementation of microwave processing technology, partly for staffing reasons. Traditionally histopathology staffs are available in the morning hours when conventional overnight processing yields its highest volumes, whereas microwave processing may keep staff later in the day. Microwave-accelerated processing is as effective as slower traditional processing, and sections stain identically with several methods: Periodic Acid-Schiff's, Van Gieson, Congo red, Masson's trichrome, alcian blue, Mayer's mucicarmine, and silver methods for reticulum. Because staining methods can vary from lab to lab, it is recommended that individual labs validate microwave methods within their environments.

Whether the lab has time initially to validate the technique and train the staff accordingly, long term considerations are prevalent. Converting to the safer chemicals (i.e. less fumes, non-regulated disposal) may include rotating smaller quantities more often, causing a net increase in chemical consumption. The safety benefits of removing undesired regulated waste in addition to calculating net volumes may offer immediate cost savings. Each lab must weigh its benefits to fit its needs.

Table 2.

Process	Solution	Time	Retort
a) Conventional, room temperature processing schedule, 3mm biopsy, 12 hrs.			
Fixation	Formalin, 10%	120 min	---
Fixation	Alcoholic formalin	60 min	---
Fixation	Alcoholic formalin	60 min	---
Dehydrate	Alcohol, 95%	60 min	vacuum
Dehydrate	Alcohol, 95%	45 min	---
Dehydrate	Alcohol, absolute	45 min	vacuum
Dehydrate	Alcohol, absolute	60 min	---
Clearing agent	Xylene	60 min	---
Clearing agent	Xylene	60 min	vacuum
Infiltrate	Paraffin	30 min	---
Infiltrate	Paraffin	60 min	---
Infiltrate	Paraffin	90 min	vacuum

Frozen Sections

For histochemistry, cryostat sections give much faster results than paraffin sections. Additionally, fixative can be used with cryostat sections, allowing the histochemist to select a different and optimal fixative for each stain, all from the same sample. The morphological detail and resolution of frozen sections is usually considerably inferior to tissue that has been embedded in paraffin. In histopathology, frozen sections are commonly cut from muscle and nerve biopsies and from surgically removed tumors. Muscle and nerve biopsies are subdivided into specimens for formalin fixation and paraffin embedding, unfixed snap-frozen for cryostat sections, fixation and resin embedding for electron microscopy (EM) and, in some rare cases, biochemical immunoblotting studies. Multiple fixation processes are required because multiple techniques are to be used. The portion of a specimen portion intended for frozen sectioning should be transported on top of wet ice, on saline-dampened gauze, and rapidly frozen within two hours. Do not allow the tissue to freeze slowly or to soak up excess saline, as these will cause artifacts that can be seen microscopically and interfere with diagnostic interpretation.

It has been suggested that talc powder can alleviate the moisture absorbance of the muscle tissue. This procedure should be evaluated and quality tested before introducing it into a laboratory. Upon receipt in the histology lab, orient in OCT (optimal cutting temperature) compound and snap-freeze in liquid nitrogen/isopentane for optimal results. For complete instruction and illustration, refer to pg 312-314 in Carson & Hladik's textbook. Orientation, size, and expedient flash freezing are critical to obtaining undamaged sections of unfixed muscle fibers. The EM portion of a biopsy will be fixed in a buffered solution of glutaraldehyde and postfixed in osmium tetoxide, usually by a specialist in electron microscopy. In some muscular degenerative disorders, biochemical techniques may also be required.

Smears

Histotechnicans sometimes perform special stains on cytology smears, blood films and cytopreps from other departments within the laboratory. Increasingly, the commonly received cytoprep is that of the "thin prep." These smears are wet-fixed in 95% ethanol immediately after preparation to preserve the fine structure of the chromatin and help in the evaluation of nuclear changes. The May-Grünwald or the Giemsa stain is routinely evaluated, and the more complicated Papanicolaou method is also widely used, especially on samples taken from the vagina and cervix. Air drying is avoided with smears for cytological detection of neoplasia because it changes the appearances of the cells. Slides bearing blood or bone marrow smears, on the other hand, are usually air-dried. Marrow smears are stained in parallel to sections of the bone marrow core biopsy.

Specimen Processing

Staining quality can be depreciated by inadequate fixation and similarly by poor tissue processing. A good technician must evaluate and determine the processing of choice for each purpose, be it special stains on paraffin, frozen or cell smear preparations.

Paraffin processing has evolved and stabilized in the modern histology lab with the use of vacuum infiltration. It remains fundamentally important to remember the basics when processing and troubleshooting. The specimen processing methods presented in this chapter should be considered a brief introduction and do not include all the available procedures.

Tissue Processing

In order to prepare a tissue for embedding, it must be infiltrated with paraffin. Because water and paraffin are not miscible, the specimens must be gradually dehydrated to achieve replacement of water with alcohol before the clearing agent is introduced. The size and penetrability of the tissue dictate how quickly this will occur. Once successfully dehydrated, a clearing agent that is miscible with alcohol and paraffin (i.e. xylene or substitute) is infiltrated through the tissue. Finally, the paraffin is introduced and completes the tissue for embedding. A vacuum automated system improves the efficiency of wax infiltration by speeding up the removal of the clearing agent.

Decalcification

Following fixation, there are techniques available to the histotechnician to improve microtomy and staining quality. Each technique must be evaluated for each assay to preserve morphology and provide the best stain possible (Table 1). The first method, going in chronological order of histology, is decalcification of specimens that may be difficult to cut on a microtome because of calcium carbonate or phosphate deposits. Decalcification can be achieved either by acids or by chelating agents. First, make sure the tissue has been adequately fixed and rinsed well to prevent any undesired reaction with the decalcifying agent. Dilute mineral acids (hydrochloric or nitric) or formic acid can be used effectively if the end point of decalcification is monitored carefully. Nuclear and cytoplasmic detail are compromised if specimens are exposed for too long to acidic decalcifying agents, which can extract RNA and remove the purine and pyrimidine bases from DNA. It is also imperative to wash the acid out of the tissue. If preservation of nuclear DNA is important, or if histochemical methods for nucleic acids or enzyme activities are intended, a chelating agent is preferred to an acid. Usually the disodium salt of EDTA is used, with the pH adjusted to a level between 7 and 8. Decalcification by EDTA much longer than decalcification by acids – weeks rather than days.

Table 1. Stain optimization

Stain Technique	Thickness of Sections	Recommended Fixative	Fixatives to Avoid
AFB	3–4 µm FFPE smear	NBF 95%	
Bielschowsky	3–4 µm FFPE frozen	NBF	
Bodian	3–4 µm	NBF	
Congo Red	8–10 µm FFPE frozen	NBF	
PAS	3–4 µm FFPE frozen 10 µm	NBF	
Jones	2 µm FFPE	NBF	
GMS	3–4 µm FFPE smear	NBF 95%	
Urate Crystals	3–4 µm	alcohol	aqueous, NBF
Iron	3–4 µm smear	NBF air dry	
Reticulin	3–4 µm	NBF	
Snook's			

Mercuric Chloride

Each fixative preserves morphology differently, thus there are multiple options. For some cells and tissues NBF does not provide adequate fixation. Examples are connective tissues, some structures containing mucins, and bone marrow. Mercuric chloride ($HgCl_2$) adds to proteins and brings about rapid coagulation with resulting granularity that is too fine to be visible with light microscopy. Lillie's B5 fixative is 4% aqueous formaldehyde with 0.22M $HgCl_2$ and 0.22M acetic acid. This mixture enhances nuclear detail, which is important for identifying normal and abnormal cell types in bone marrow (hematopoietic tissue) specimens. The coagulation of nuclear chromatin is an effect of the acetic acid. The mercuric chloride ensures rapid structural stabilization and also facilitates bright staining by many of the dyes used in microtechnique. The reasons for this effect on stainability are not understood.

A dirty looking brown crystalline precipitate, probably mercurous chloride (Hg_2Cl_2) forms in all parts of tissues fixed in mixtures containing $HgCl_2$. It is called mercury pigment and must be removed, by sequential treatments with iodine and sodium thiosulfate solutions, before staining. Because it contains mercury, B5 is subject to toxic waste disposal regulations, which apply to the fixative solution and every solution thereafter that has been contaminated with mercury. Records must be kept of all B5 volumes used in the laboratory.

Zinc salts

The toxicity of mercury compounds has discouraged their use in laboratories. Of the other elements in Group 12 (IIb) of the periodic table (Zn, Cd, Hg), zinc is the least toxic. Zinc sulfate has been used for more than a century in astringent lotions and eye drops, which reduce the swelling of inflamed surfaces by coagulating extravasated plasma proteins. The first zinc-formalin fixative was probably the one published by P.A. Fish in 1895, only three years after the discovery that formaldehyde preserved tissue architecture very well. In recent decades several intelligently formulated fixatives have been developed. A few are entirely coagulant, with Zn^{2+} as the only ingredient likely to immobilize proteins. Most zinc-containing fixatives also contain formaldehyde, making them comparable to B5 and other mercury-containing solutions.

Alcoholic Fixatives

NBF is the most widely used fixative, despite not being the best one for every purpose. This is because the shortcomings of NBF are not very serious, provided that the specimens are fixed for sufficient time. Moreover, the appearances of formaldehyde-fixed tissues are familiar to pathologists, who have trained their visual systems to recognize normal and abnormal features in sections cut from routinely fixed materials.

Arguments have been made for changing the routine fixative to one of several non-aqueous mixtures, and strong cases can be made for Clarke's fluid (ethanol and acetic acid, 3:1 by volume), Carnoy's fluid (ethanol, chloroform and acetic acid, 60:30:10) and Puchtler's methacarn (Carnoy with methanol instead of ethanol). These liquids, which fix by coagulation of proteins and chromatin, contain no water. They almost completely dehydrate the tissue, so that the time for processing into paraffin wax is shorter than after NBF or other aqueous fixatives. There are also many mixtures containing an alcohol (usually ethanol), formalin, acetic acid and 10% to 70% water, often called AFA or FAA or named for their inventors, who include Tellyesniczky (around 1900), Bodian (1930s) and Davidson (1940s). In an AFA mixture the chemical reactions of formaldehyde with proteins are not retarded by buffering to a near-neutral pH. All these alcoholic fixatives contain acetic acid, which produces characteristic patterns of coagulated nuclear chromatin, facilitating the recognition of cell types. Nuclei that have been in NBF for a week or more exhibit less pronounced patterns of chromatin.

Alcohol (ethanol or methanol) alone instantly coagulates proteins but causes considerable distortion of the micro-anatomy in pieces of animal tissue. These unwanted changes are opposed by dilution of the alcohol with chloroform (immiscible with water), water, and/or acetic acid (which coagulates chromatin and opposes shrinkage, being miscible with water, alcohol and hydrocarbons). Alcohol alone (methanol is usually preferred) is suitable for fixing thin layer preparations such as blood films or cell cultures. Solid specimens taken from patients with gout are usually fixed in 95% ethanol for subsequent histochemical detection of sodium urate crystals, which can be dissolved out of the tissue by water.

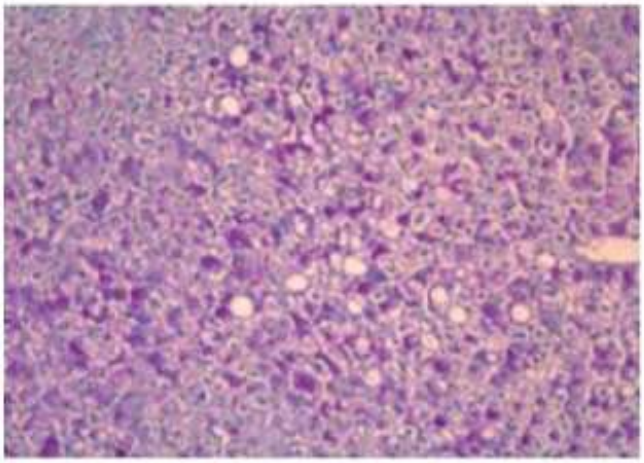

Figure 2. A paraffin section of formaldehyde-fixed liver stained by the periodic acid-Schiff method, illustrating polarization of glycogen (magenta) within the hepatocytes. Nuclei are counterstained with hematoxylin (blue).

Glyoxal

Glyoxal is the simplest dialdehyde, with the formula OHC—CHO. Like formaldehyde, it readily forms hydrates and polymers. Decomposition by way of the Cannizzaro reaction occurs rapidly in neutral or alkaline solutions. Aqueous solutions must be buffered to about pH 4 to be stable, and they must also contain a small proportion of ethanol, which catalyzes the reaction of glyoxal with proteins. Addition to and cross-linking of proteins is quicker than the equivalent reactions of formaldehyde. Adequate fixation of small specimens by glyoxal is achieved in one hour.

The glyoxal-based fixatives available to laboratories are sold as pre-made solutions with trade names and undisclosed compositions. Evidently there are no published recipes for fixatives of proven efficacy that contain glyoxal as the principal ingredient. Proprietary glyoxal fixatives are reported to be useful for general histology and immunohistochemistry. The differences from formaldehyde (NBF) fixation are:

(a) Suppression of staining of arginine-rich proteins by anionic dyes (this matters if you need to stain Paneth cells, eosinophil leukocytes or the tails of spermatozoa), and

(b) Antigen retrieval is less frequently required than after fixation in NBF.

The trade secrecy associated with glyoxal-containing fixatives may preclude their use in research and in diagnostic applications where the pathologist may be interrogated about his competence and knowledge of materials used in the laboratory.

Bouin's Fluid

Pol André Bouin (1870-1962) invented several fixative mixtures in the years 1895-1900; the one most often associated with his name, first published in 1897, contains 10% formaldehyde (25% formalin), 0.9M acetic acid and 0.04M picric acid, in water. Picric acid penetrates tissues rather slowly, coagulating proteins and causing some shrinkage. It also dyes the tissue yellow. The acetic acid coagulates nuclear chromatin and opposes the shrinkage caused by picric acid. The reactions of formaldehyde have already been discussed; at the pH of Bouin's fluid (1.5-2.0) these occur more rapidly than in NBF. The complementary effects of the three ingredients of Bouin's solution work well together to maintain morphology. Specimens are usually fixed in Bouin's for 24 hours. Prolonged storage in this acidic mixture causes hydrolysis and loss of stainable DNA and RNA. Thorough washing after fixation is necessary.

Like mercuric chloride, picric acid enhances subsequent staining, especially with anionic ("acid") dyes. Paraffin sections of formaldehyde-fixed tissues are usually immersed for a few hours in a picric acid solution (Bouin's fluid is commonly used) before staining by the trichrome methods. Trichrome stains use combinations of anionic dyes with phosphotungstic or phosphomolybdic acid to impart contrasting colors to cytoplasm, collagen fibers and other components of tissues.

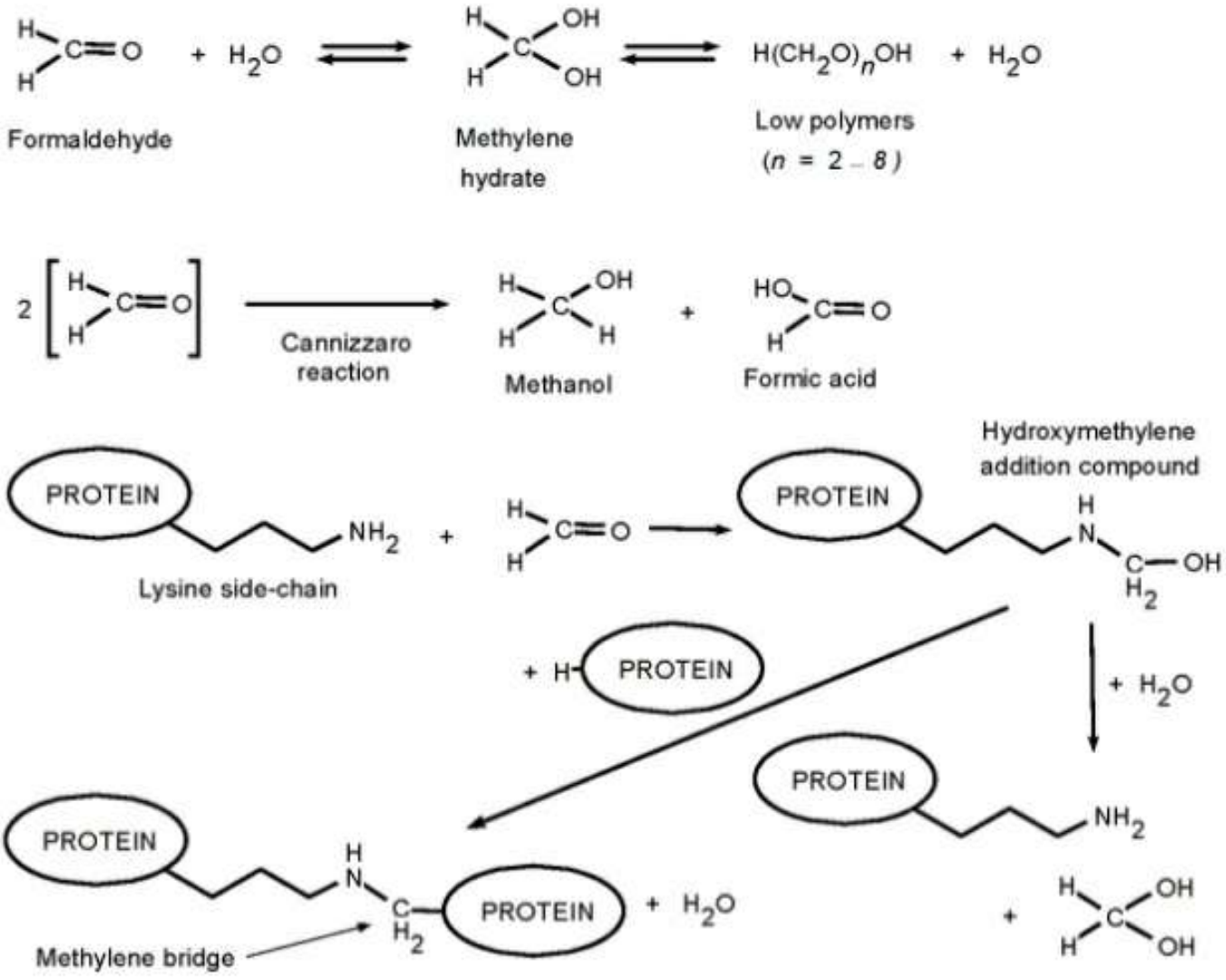

Figure 1. Some reactions of formaldehyde with water, with itself, and with proteins.

Fixation before Paraffin Embedding

Most samples used for staining normal and pathological tissues are embedded in paraffin, and a number of fixatives have been formulated with this in mind. The most commonly used fixatives are discussed here. There is an abundance of specialty fixatives that will not be covered here but may be found in the references given in the bibliography.

Formaldehyde

Formalin is an article of commerce containing 40% w/v (= 40% w/w) formaldehyde (which is a gas) in water. Most of the formaldehyde is present as soluble polymers, which depolymerize on dilution. Formalin also contains about 10% methanol, which is added by the manufacturer to retard the formation of higher polymers, which eventually fall out of solution as paraformaldehyde. Old bottles of formalin, especially after storage in a cold place, often contain deposits of this white powder. Formaldehyde in solution also deteriorates by reacting with itself (Cannizzaro reaction) and changing into methanol and formic acid. Monomeric formaldehyde exists almost entirely as methylene hydrate, an addition compound formed by a reversible reaction with water. Formaldehyde itself is the compound that reacts with proteins; it is present at an extremely low concentration in a "4% formaldehyde" solution, but is produced instantly from methylene hydrate following removal from solution in the reactions of fixation. These chemical properties of formaldehyde are summarized in Figure 1.

The most commonly used fixative 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 pressure 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 peptide linkages. Cross-linking methylene bridges are formed where two formaldehyde binding sites are close together (see Figure 1). 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 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 affects histological and immunohistochemical staining, technicians, pathologists and research workers must therefore 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. The duration of exposure to the fixative must be optimized for each specimen type. For example, it has been noted in a CAP survey that glycogen preservation in liver can be subject to fixation artefacts. As NBF penetrates slowly through the hepatocyte membrane, the glycogen associated with the cytoplasmic protein matrix is displaced to one side of the cell. This observation is referred to as polarization and is considered part of the morphology when discerning the stain result. (Figure 2: glycogen polarization) This is a great example of how specimens that have prolonged exposure to fixatives can demonstrate extreme morphology changes. Glycogen polarization is less marked after fixation in a non-aqueous liquid, in which this polysaccharide is not soluble.

Technical Considerations

There are some technical tips that can apply to all preparative procedures in microtechnique. Make sure glass slides are clean and free from debris. Gentle washing and minimal thickness of cell layers will prevent the cells from detaching during staining. Staining interpretation depends on adequate chemical spread and allocation. Make sure that there are enough sections to make a diagnosis, and that ensure that the reagents have been applied evenly to the slides. If counterstaining is required, be sure to not over-incubate.

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Geiger Müller Counter (GM Counter)

J. W. Geiger was a student of Sir Ernest Rutherford and developed Geiger tube. Later he came up with an advanced version of the tube with his student W. Müller and the same became popular as GM Counter. This is a gas based detector which uses ionization of gas by radiation as the primary tool to detect radiation. The tube is in the form of cylinder and is filled up with noble gas at certain low pressure. A metallic string which runs through the central axis of the cylindrical shape forms the anode whereas the outer shell serves as cathode. One end of the tube has radiation permeable window (mica or beryllium).

Any radiation incident on the tube window will ionize the noble gas. The electrons thus ejected will get attracted towards the anode whereas the heavy ions will move slowly towards cathode. These charges will further ionize more number of atoms thereby setting up an avalanche of ions and electrons which sets up a discharge current. The current causes the voltage between the anode and cathode to drop which the counter detects as a signal.

Since the detection is based on the avalanche, therefore the output pulses are independent of the energy brought in by the incident radiation The pulse height remains the same irrespective of the energy of radiation and therefore GM counter can only be used to detect the presence of radiation but not the type of radiation.

A the positive ions arrive at the cathode, they will produce fresh avalanche. As soon as they reach cathode, they would knock capture electrons from the cathode and would become neutralized. But, in the process, it would emit ultraviolet photons which would set secondary Geiger discharge through ejection of photo electrons. This could run the GM set up in to continuous discharge ultimately causing serious damage to the tube. To counter this, the noble gas is in general mixed with halogen or alcohol. The excited noble gas atoms are neutralized by collision with neutral halogen or alcohol molecules, as a result of which they become ionized. This happens because of higher ionization potential of noble gas than that of halogen or alcohol. Reverse process in forbidden. Now the excited halogen or alcohol molecules dissociate at cathode being neutralized by capture of electrons. This process is known as Quenching or Self Quenching or Internal Quenching.

Unlike alcohol based tubed where the alcohol molecules dissociate in to constituent atoms, the halogen based tubes are long lived as the halogen molecules dissociate in to halogen atoms which recombine to form the same molecule.

Contrary to the above process, in early days, external quenching was used. In this method a very resistance was included in the anode circuit. The potential drop being very high across this resistance used to lower the voltage across the counter tube to such a low value so the gas amplification factor becomes insufficient to trigger second discharge. But, introduction of high resistance would increase the RC time factor which resulted in lower count rate.

The figure1 shows the complete experimental set up for the experiment. The tube is mounted on a structure which have different slots to place the source. The tube is connected to the GM Counter Console which hosts the electronics for amplification and counting. It also provides the necessary power

Figure 1: Experimental set up for GM Counter based experiments. Taken from the company manual http://www.spectrumtechniques.com/resources/instrument-manuals/

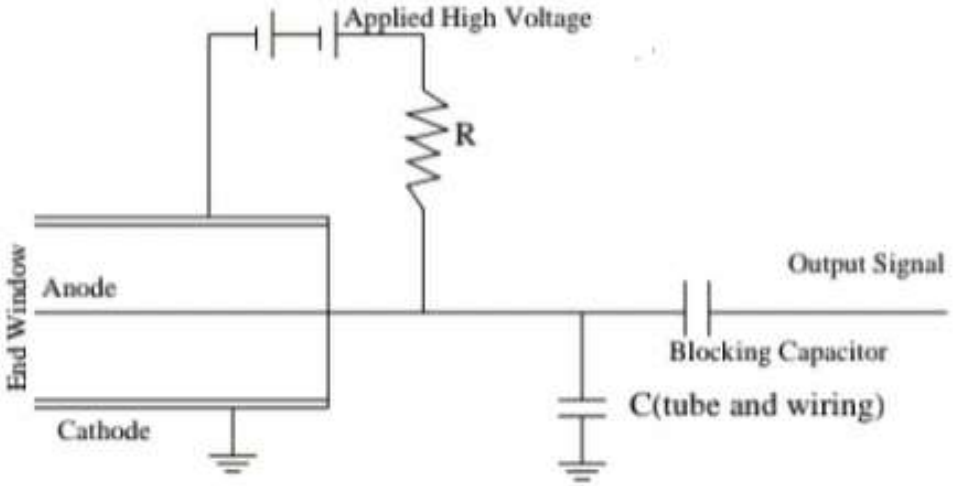

Figure 2: Schematic diagram of GM Counting system.

supply to the tube. For your understanding, a schematic diagram of the set up is given in Fig.2. *By now, you should have wondered how can a single cable be used with GM Tube to provide the power supply as well for the out signal.*

Let us have a look into the schematic diagram. We have learnt in detail about the GM tube operation. The same is depicted in the figure. One can notice a resistance. This is the same resistance, which if increased to a large value would serve the purpose of external quenching. A capacitance has been shown to be connected to the ground. This actually represents all sort of capacitance which can be generated due to the tube, wiring etc. This combination of RC would provide the time constant for the GM set up. This is chosen to be of the order of few microsecond so that only the fast rising components of the signal are preserved. In addition to this, a blocking capacitance is also provided. This serves the purpose of blocking the high voltage and allowing to pass the signal to the subsequent circuitry.

The console can be manually operated as shown in the figure. But, for the present experiment we will be using the computerized interface for all sorts of external control and data acquisition. A demo of how the computer screen would look like is shown in Fig.3. The demo picture is self explanatory. It shows the applied voltage in red block, preset time (time allotted for each count), number of runs (would be required for statistical studies else single run is sufficient), time elapsed (time gradually approaches the preset time and stops) and the counts. The block shown in gray shows the saved files of each run. You can see lots of menus are shown on top of the screen, such as, File, Edit, Set up etc.

1. The File menu can be used to open a file, save a file, print a file and exit from the system.
2. Edit is as usual used for copy and paste.
3. The most useful menu is the Set Up menu. It is used for setting up high voltage through "HV Setting". Here one can also define the steps of increasing voltage and enable the option.
4. Through Preset Menu, the preset time can be set as per the requirement. Here one can also define number of runs through "Runs" command.

Other drop-down menus are not required so often. We can discuss those in the lab. **Now in the next menu line, a green rhombus is shown. This is the button to start the counting. Once run is started, the red button just beside the green one will become active. By pressing the Red Button one can stop the run.**

Exercise : *This type of ionization tubes can be operated in various regions according to the applied voltage. We have just seen one of the regions, i.e.; GM region. Now it is a task for you to find out other regions : ionization region and proportional counter region as well as the applications, advantages and disadvantages over the GM region.*

EXPERIMENT : Operating Plateau for GM Tube

Purpose

The purpose of this experiment is to determine Plateau Characteristics of GM tube and to determine reasonable operating point for the tube.

Underlying Physical Aspects

We have now understood the basic working of GM tube. So, in order to observe the effect of incident radiation on GM tube, we need to increase the voltage between anode and cathode, to a value where the gas amplification sets in. This voltage is called the *Threshold Voltage* or *starting voltage*. Thereafter, the counting rate keeps on increasing till the voltage reaches a *knee value* beyond which the count rate becomes saturated or constant. In this constant region of operation all the primary events are recorded irrespective of the energy. This flat region is called *Plateau of the counter*. If we keep still keep on increasing the voltage, the tube will run in to continuous discharge region which is not desirable. The mid point of the plateau region is taken to be the *Operating Voltage* of the GM tube and once determined, the tube must be operated at this value for subsequent experiments.

Procedure

1. After setting up the GM tube and electronics we can start our experiment. Place the source at a distance of around 6 mm. One can set up a starting voltage say around 650 V with a step voltage of say 20 V. Set the preset time of 30 s.

2. Initially no counts will be registered. Around 750 V, there will be sudden increase in the counts, which would saturate around 800V. Though a small increase in counts will be registered through the experiment.

3. Stop the experiment at around 1200 V or before.

4. As we know that there are background counts in the laboratory. So, whatever counts we have registered, have to be corrected for background counts. One can repeat the steps 1-3, without a source. The process should be repeated twice or thrice and average of the same should be taken to be the background at each voltage. Make sure that no source lies nearby otherwise that might contribute to the background count.

5. Subtract the corresponding background count from each reading and register the correct counts vs voltage in table1.

6. A demo labeled plot of Counts vs Voltage is shown in Fig.4. You should plot Corrected Count Rate vs Voltage.

Inference

- Starting Voltage ..... ±..... [We have least measuring voltage value of 1 V. What error should we mention in the result : either the instrumental value or the graphical one ?]

Table 1: Data Table for GM Plateau

S. No.	Voltage (V)	Counts	Avg. Bkg. Counts	Corrected Counts	Corrected Count rate
1.	650				
2.	670				
3.					
4.					
5.					
6.					

- Knee Voltage ..... ±.....
- Range of Plateau ..... ±..... [While calculating error, keep in consideration that the range is calculated as the difference of two voltage values.]
- Operating Voltage ..... ±.....
- Percentage slope of Plateau = $\frac{\text{Slope of the plateau region}}{\text{Knee Voltage *Voltage Difference of Plateau region}}$*100

Post Lab Questions and Take Home Messages

1. Will the operating voltage remain same if the present tube is replaced by another one ?
2. Will this operating voltage remain constant even after long period of time, say, ten years ?
3. What does the slop of plateau shows ? Why do we have slope, however small it may be ?
4. What will happen if we place the source at the last groove ? We might get the answer in subsequent experiments.
5. For background, we have taken two or three different readings at each voltage and every time we found that we did not get the same reading as previous one. But, the reading varies about certain value. Will it be the same for counts with the sources ? You can check yourself by repeating the counting at operating voltage. And if it varies like the background count, then the data which we have acquired is not correct. What is the remedy to this problem ? Again upcoming experiments may help us to get the answer to our question.

EXPERIMENT : Counting Statistics

Purpose

The radiation emission is statistical in nature. Thus, each event is independent of all previous measurements. But, when data acquisition is repeated for large number times, one can make a wise prediction about the deviation of individual counts about what might be the average of counts in the ensemble. The purpose of the present study is to observe for the particular deviation from the average within a give sample size interval.

Underlying Theory

Let us assume we have ensemble of N independent measurements for the same event :
$x_1, x_2, x_3, \ldots\ldots, x_N$.
Then the total sum of the above data will be

$$\Sigma = \sum_{i=1}^{N} x_i \tag{1}$$

Experimental mean

$$\bar{x} = \Sigma / N \tag{2}$$

Now, since we have large set of data, it is convenient to describe the parameters in terms of frequency of data.

$$F(x) = \text{number of occurrence of value of } x/N \tag{3}$$

then the mean,

$$\bar{x} = \Sigma = \sum_{x=0}^{\infty} xF(x) \tag{4}$$

Since, the data is random and we have large number of data, it is helpful to look at the variance than the deviation of the individual data from the mean.

$$\sigma^2 = \frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N} \tag{5}$$

Or in terms of frequency

$$\sigma^2 = \sum_{x=0}^{\infty}(x - \bar{x})^2 F(x) \tag{6}$$

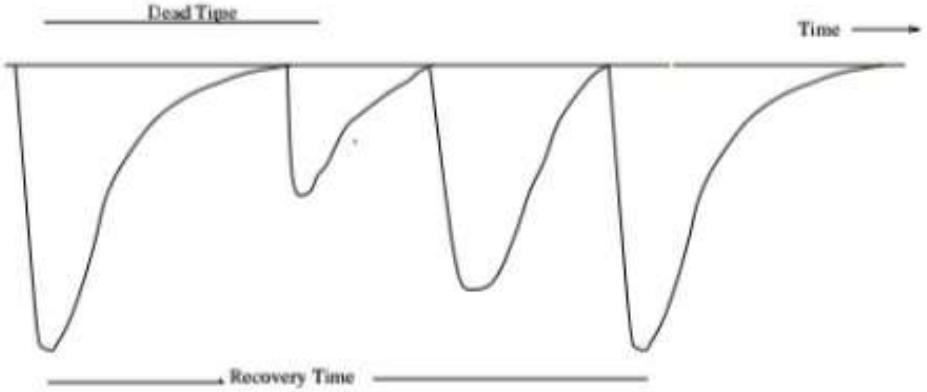

Figure 7: Dead time and Recovery time of GM Tube.

EXPERIMENT : Resolving Time or dead time of GM Counter with two source method

Purpose

To determine the dead time or resolving time of GM counter.

Underlying Physical Aspects

As discussed initially, GM tube consists of noble gases which are ionized by incident radiation generating generating fast moving electrons and slow moving ions. As the field is high nearby the anode the electrons quickly move towards the anode. But, the ions being heavy move slowly towards the cathode and in turn get accumulated near the central wire thereby forming a space charge sheath which reduces the field near the wire and that ultimately lowers the gas multiplication. Once this process continues for a longer period of time, the Geiger discharge stops and GM becomes unable to register any output pulse. The period for which GM tube remains idle is known as **Dead Time or Resolving Time**.

The dead time is of the order of a few hundred microseconds. Thereafter, GM tube once again start registering the incident radiation. However, the pulse height might not rise to the fullest. It takes a little bit longer time for the pulses to grow to its original height. This time span within which the pulses once again regain their original pulse height is called **Recovering Time**. The entire process is pictorially shown in Fig.7. The dead time is inbuilt feature of every detector system. In some cases, dead time might be introduced due to electronics of the system. Dead poses a high threat to the high counting rate events.

There are two models for dead time calculation : *Paralyzable or Extendable* and *Non Paralyzable or Non-Extendable*. This can be understood with the help of figure8. The top figure shows six pulses even though some of them are overlapped. So, we had six original counts. Now in the paralyzable model, if the overlapping pulses are registered, then, the dead time is extended for the subsequent pulses. In the figure, let us say, the dead time is the width of each rectangle. In third and fourth case, since we have overlapping pulses, the dead time will keep on adding up and hence the detector wont register any further pulse. So in this context, Paralyzable Model would only consider three pulses instead of

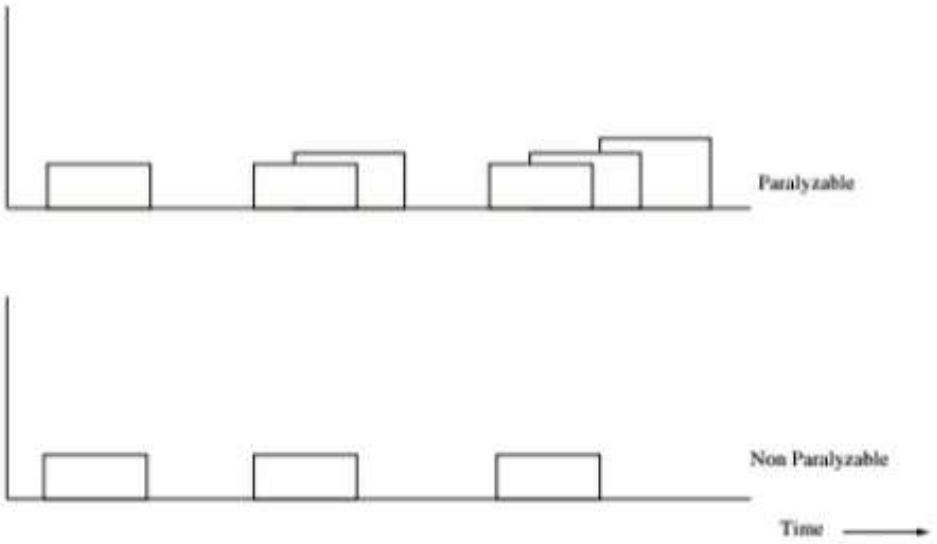

Figure 8: Comparison of paralyzable and non paralyzable models of Dead time.

six. Here if N is the actual rate of arrival of ionizing particles, n is the count rate that counter is able to register and τ be the dead time, then :

$$n = Nexp(-N\tau) \tag{11}$$

This is a transcendental equation and can solved using graphical method. [Refer to Techniques for Nuclear and Particle Physics experiments by Leo.]

For non-paralyzable model, it only consider the dead time of first pulse and would register the next pulse if it lies beyond the dead time limit of the first. So for the first two cases it registers two pulses whereas in the third case where three pulses are overlapped, it does not record the second overlapping pulse but did register the third one since it lies outside the dead time limit of the first pulse. Therefore, it would record four pulses. In the context of this model, let us consider N to be the actual rate of arrival of ionizing particles, n be the count rate that counter is able to register and τ be the dead time. Then, number of particle skipped by the counter per unit time is $Nn\tau$ which should be equal to $N - n$. This implies :

$$N = \frac{n}{1 - n\tau} \tag{12}$$

Based upon this notion, let us consider two sources of approximately same strength and determine the count rates (n_1 and n_2 respectively) for individual sources along with the sum of the count rates (n_t when both the sources are placed. If we solve those three individual equations considering higher powers of τ^2 to be negligible, we obtain :

$$N_t - N_b = N_1 - N_b + N_2 - N_b \tag{13}$$

$$N_{12} + N_b = N_1 + N_2 \tag{14}$$

1. நிலைபதிக்குதல்:

* செல்களை, திசுக்களை திடீரென இறக்கச் செய்தால் செல்களின் புற அமைப்பு, வெளிமுலவி அடங்கு பொருட்கள் பெரும்பாலும் மாறுபடுவதில்லை. என்றாலும் வல்நுண்ணோக்கு திசுக்களை நிலைபதிக்குதல் அவசிய மாகிறது.

* நிலைபேறுத்தப்படவில்லையெனில் திசுக்கள் நுண்ணோக்கு பயனற்றதாகி விடுகின்றன.

நிலைபதிக்கு முறைகள்:

அ) வேதிப்பொருளை கொண்டு நிலைபதிக்குதல்

* செல்லுள் காணப்படும் வேதிப்பொருட்கள் சமமை செயல்படுகின்றன. ரைபோநியூக்ளிக் அமிலம் பற்றி அறிய கார்னாய் கரைசல், போயின் கரைவி போன்ற அமிலத் தன்மையுடைய நிலைபதிக்கும் பொருட்கள் பயன்படுத்தப்படுகின்றன.

பொதுவாக பயன்படுத்தப்படும் நிலைபதிக்கு வேதிப்பொருட்கள் கீழே கொடுக்கப்பட்டுள்ளன.

நிலைபதிக்கி வகைகள்:

நிலைபதிக்கும் வேதிப்பொருட்கள் தனித்து பயன்படுத்தப்படுகிறது. அது தனி நிலைபதிக்கி என்றும் இரண்டிற்கு மேற்பட்ட வேதிப்பொருட்கள் சேர்த்து பயன்படுத்தப்படும் பொது அது கூட்டு நிலைபதிக்கி என்றும் குறிப்பிடப்படுகிறது.

I தனி நிலைபதிக்கிகள்:

பொதுவாகப் பயன்படுத்தப்படும் தனி

நிலைப்படுத்திகள் தேடி நோக்கப்பட்டுள்ளன.
இவற்றையல்லாது பிக்ரிக் அமிலம், குரோமிக் அமிலம்,
மெர்க்குரிக் குளோரைடு ஆகியனவும் பயன்படுத்தப்படுகின்றன

1. அசிட்டிக் அமிலம் (0-0.5%)

அசிட்டிக் அமிலம் நியூக்ளியோ புரதங்களை வீழ்படிவாக்குகின்றது. சைட்டோபிளாச புரதங்களை நிலைக்கச் செய்வதில்லை.

2. பார்மால்டிகைடு (5-10%)

கோல்கை உறுப்பு, மைட்டோகாண்ட்ரியா, நொதிகள் அமைவிடத்தை நிலைப்படுத்தி பார்மால்டிகைடு சில காரத் தன்மையுடைய சாயங்களுடன் சேர்த்து பயன்படுத்தப்படுகிறது.

3. எத்தனால் (70-100%)

நியூக்ளியஸை நிலைப்படுத்துகிறது. எத்தனால் தனித்தோ அல்லது சில காரத்தன்மையுடைய சாயங்களுடனோ சேர்த்து பயன்படுகிறது. இது புரதங்களை வீழ்படிவாக்குவதில்லை.

4. பொட்டாசியம் டை குரோமேட் (2-7%)

பொட்டாசியம் டை குரோமேட் சைட்டோபிளாச உறுப்புகளை, கொழுப்பினை, குரோமோசோம்களை நிலைப்படுத்த பயன்படுத்தப்படுகிறது.

5. ஆஸ்மியம் டெட்ராக்சைடு (0.5-2.0%)

செல்லின் சைட்டோபிளாசம், கோல்கை உறுப்பு, மைட்டோகாண்ட்ரியா, கொழுப்பு ஆகியனவற்றை நிலைப்படுத்துவதற்கு பயன்படுத்தப்படுகின்ற

II கூட்டு நிலைப்படுத்திகள்

1. போயின் கரைசல்

இது பிக்ரிக் அமிலம், 40% பார்மால்டிஹைடு, கிளேசியல் அசிடிக் அமிலம் ஆகியவற்றை முறையே 5:5:1 என்ற விகிதத்தில் கொண்டிருக்கிறது.

2. கார்னாய் திரவம்

எத்தனால், அசிட்டிக் அமிலம் இவையிரண்டின் பண்புகளையும் பெற்றிருக்கிறது. திரவம் மூன்று பகுதிகளையும் கிளேசியல் அசிட்டிக் அமிலம் ஒரு பகுதியும் கொண்ட இது நியூக்ளியோ புரதங்களையும் குரோமோசோம்களையும் நிலைப்படுத்தப் பொதுவாக கார்னாய் திரவம் பயன்படுத்தப்படுகிறது.

3) உறைய வைத்து நிலைப்படுத்தி உலர்த்தும் முறை

எலக்ட்ரான் நுண்ணோக்கி, X-கதிர் நுண்ணோக்கி மூலம் ஆய்வு செய்யப்படும் செல்கள், திசுக்கள் விரைந்து உறைய வைத்து நிலைப்படுத்தப்படுகின்றன.

இத்தகைய திசுக்கள் உயருடன் இஸோ பென்டேன் அல்லது பென்டேன் இவற்றில் திசுக்கள் வைக்கப்பட்டு திரவ நைட்ரஜன் அல்லது ஹீலியத்தால் குளிர்விக்கப்படுகின்றன. 160°C அல்லது -190°C க்கு குளிர்விக்கப்பட்ட திசுக்கள் -30°C முதல் -40°C வரை கொண்டு செல்லப்பட்டு உலர்த்தப்படுகின்றன.

உறைய வைத்து நிலைப்படுத்தும் முறையில் செல்கள் ஒரு திசுக்கள் சுருங்குவதில்லை.

மாதிரியாக நிலைப்படுத்தப்படுகின்றன. கரையும்
பைண்டுகள் எதிரில் மாற்றமடைந்து வெவ்வேறு
நிலைப்படுத்தப்படுகின்றன.

3. நீர்ப்பிரிப்பு - உடலுறுப்புகளும் கட்டமைப்பு அங்கமும் :-

நீர்ப்பிரிப்பு :-

திசுக்கள் தொடர்ந்து ஆல்கஹாலில் அடர் அளவு தோய்ந்து அதிகரிக்கின்றவாறு அகற்றுதல் 10%, 20%, 30%, ... 90%. ஆல்கஹாலுக்கு மாற்றப்படுவதால் நீர் சிறிது சிறிதாக அகற்றப்படுகின்றது.

உடலுறுப்புகளும் கட்டமைப்பு அங்கமும் :-

* நீர் பிரிக்கப்பட்ட திசு, உருகிய மெழுகில் வைப்பதற்போது மெழுகு திசுவின் உள்ளாக உட்புகுகின்றது. திசுவினை பிரதிபலிப்பிட நிலையில் மெழுகில் தொடர்ந்து அமர்த்தி கட்டமைப்பு தயாரிக்கப்படுகிறது.

* மைக்ரோடோமினைப் பயன்படுத்தி கட்டமைப்பு பிரதிபலிப்பிட மைக்ரான் தடிமனுக்கு சன்டாக்கப்படுகிறது. இந்த சன்டாக்கத்தின்போது பெறப்படுகின்ற சன்னங்களின் தொடர்களால் தாடா பெறப்படுகின்றது. மிக மெல்லிய அளவில் மூடை அல்புமன் தடவப்பட்ட கண்ணாடித் தகடுகள் மீது தாடா இடப்படுகிறது.

* இதனால் சன்னங்கள் கண்ணாடித்தகடுகளில் ஒட்டிக்கொள்கின்றன.

* கண்ணாடித்தகடுகளை அடுப்பால் அல்லது வெயிலால் உலர்த்தி, தோய்ந்து வைப்பால் ஆல்கஹாலால் ஏற்பட்ட பலவீனத்தை நீக்கப்படுகின்றது.

4. நிறமேற்றம் :

செல்லுள் காணப்படும் பொருட்கள் அவற்றின் வேறியல் வினைகளுக்கேற்பவும் அவற்றுள் காணப்படும் வேதிப்பொருட்களுக்கேற்பவும் நிறத்தை ஏற்கின்றன. செல்லுள் வெவ்வேறு அளவினதாக வெவ்வேறு அடர்வு பொருட்கள் காணப்படுகின்றன.

இவற்றை அறிவும் நோக்கை செல் வேதியல் எனப்படும். நிறமேற்றம் இப்பொருட்கள் நிறமேற்றிகள் எனப்படும். இந்நிறமேற்றிகளில் கீழ்க்காண்பனவை நிறப்பிடத்தக்கவை.

1. புரதங்களை அறிய

புரதங்களைக் கண்டறிய மெர்குரிக் புரோமோ - பி : னால் நீலம், நாப்தால், மஞ்சள் S போன்றன பயன்படுத்தப்படுகின்றன. இந்நிறமேற்றிகள் லைசின், ஹிஸ்டின், அர்ஜினின் போன்ற காரத் தன்மையான அமினோ -களைக் கொண்ட புரதங்களை நிறமேற்றுகிறது. (உ.ம்) மிலான் நிறமேற்றி வைரோசினை நிறமேற்றம் செய்கின்றது.

2. லிப்பிடுகளை அறிய

செல்லுள் காணப்படும் லிப்பிடுகளை கண்டறிய, நிறமேற்ற கொழுப்பில் கரையக்கூடிய சூடான் கறுப்பு B பயன்படுத்தப்படுகிறது. இது பாஸ்போலிப்பிடு -களையும் கொலஸ்டை உறுப்புகளையும் நிறமேற்றுகிறது. தஸ்மியம் டெட்ராக்ளைடு, சூடான் சிகப்பு போன்றன பிற லிப்பிடுகளை நிறமேற்றுகின்றது.

3. கார்போஹைட்ரேட்டுகளை அறிய

தாவர செல்களிலுள்ள ஸ்டார்ச், செல்லுலோஸ், ஹெமி செல்லுலோஸ், விலங்கு செல்களில் காணப்படும் மியூக்கோ புரதங்கள், கைட்டின் ஆகியன ஷிப்ஸ் வினைப்பொருளினால் நிறமேற்றப்படுகின்றன.

4. RNA, DNA - வை அறிய

· DNA பால்கஜன் கரைசல், மீதைல் பச்சை பைரோனின் கலவையால் நிறமேற்றப்படுகின்றது. மீதைல் பச்சை பைரோனின் DNA - வை பச்சையாகவும், RNA - வை சிகப்பாகவும் மாற்றுகிறது. RNA அஸூர் B யினால் கூட நிறமேற்றப்படுகிறது.

5. வைட்டமின்களை அறிய

வைட்டமின் A, B_1, B_2 ஆகியன பெர்பெரின், அக்ரிடின், ஆரஞ்ச் மற்றும் மஞ்சள் பாஸ்பைன், கோரி பாஸ்பைன் போன்ற ஒளிரும் நிறமேற்றிகளால் நிறமேற்றப்படுகின்றன.

6. நொதிகளை அறிய

மைட்டோகாண்ட்ரியாவில் நிறைந்து காணப்படும் நொதிகள் டெட்ரசோலியம் உப்பினால் அறியப்படுகின்றன. மைட்டோகாண்ட்ரியா ஹைட்ரஜன் நீக்க நொதிகளால் ஆக்ஸிஜனேற்றமடைகின்றன. நிறமடைகின்றன.