ALAGAPPA UNIVERSITY

[Accredited with 'A+' Grade by NAAC (CGPA:3.64) in the Third Cycle and Graded as Category–I University by MHRD-UGC]

KARAIKUDI – 630 003

DIRECTORATE OF DISTANCE EDUCATION

M.Sc. [Botany]
346 13

BIOLOGICAL TECHNIQUES IN BOTANY

I - Semester

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(A State University Established by the Government of Tamil Nadu)

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BIOLOGICAL TECHNIQUES IN BOTANY

Author:

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Units (1-14)

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Unit-2: Transmission and Scanning electron microscopes - Confocal Microscopy, Phase Contrast Microscopy. Photomicrography- Digital imaging.	**Unit 2:** Electron Microscope, Confocal Microscopy and Photomicrography **(Pages 11-36);**
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Unit-5: Material preparation techniques for microtome sectioning - fixatives, dehydrating agents, killing, fixing methods.	**Unit 5:** Microtome Sectioning **(Pages 62-74);**
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INTRODUCTION

NOTES

Biological techniques are methods or procedures that are used to study living things. They include experimental and computational methods, approaches, protocols and tools for biological research.

Botany, is a branch of biology that deals with the study of plants, including their classification of plants, structure, properties, biochemical processes and study of plant diseases, and role of the environment in the growth of plants. The principles and findings of botany have provided the base for such applied sciences as agriculture, horticulture, and forestry. Characteristically, plants are one of the most fascinating and important groups of organisms living on Earth. They serve as the channel of energy into the biosphere, provide food, and shape our environment. The fundamental biology of plants is similar to our own as they use the same genetic code, share many homologous genes, and even many regulatory mechanisms, basic biochemical pathways, and fundamental processes in cell biology. However, their form and lifestyle are fundamentally different. Earlier, the biologists could study about living things, only from what they could see with the naked eye. New tools and techniques were invented which helped in the study of finer structure of various kinds of organisms and their parts. Microscope not only revealed a world of minute organisms but also minute details of internal structure of organisms. In the course of history of biology, various new tools and techniques were developed, like microscopy, paper chromatography, etc.

This book, *Biological Techniques in Botany*, is divided into four blocks, which is further divided into fourteen units which will help you understand about the basic principles of image formation and applications of light, polarizing microscopy, transmission and scanning electron microscopes, microscopic measurements (micrometers, ocular, stage), microtomes, rocking, rotary, sledge and their uses, material preparation techniques for microtome sectioning (fixatives, dehydrating agents, killing, fixing methods), stains (uses and staining of plant tissues), histochemical techniques for staining of proteins, carbohydrates, lipids and enzymes, microslide preparation, sectioning of biological specimens, dewaxing and staining of the sections, fixing coverslips and ringing, maceration technique (PAGE, SDS – PAGE and Agarose Gel Electrophoresis), separation techniques, ultracentrifugation (TLC, HPLC, HPTLC, FPLC), tracer techniques, principles and applications of radioactive isotopes, autoradiography and liquid scintillation spectrometry, blotting techniques (southern, northern and western blotting techniques, and hybridization), principles and applications of PCR, RFLP, RAPD, AFLP and DNA fingerprinting.

The book follows the self-instruction mode or the SIM format wherein each unit begins with an 'Introduction' to the topic followed by an outline of the 'Objectives'. The content is presented in a simple and structured form interspersed with 'Check Your Progress' questions and answers for better understanding. A list of 'Key Words' along with a 'Summary' and a set of 'Self Assessment Questions and Exercises' is provided at the end of the each unit for effective recapitulation.

BLOCK - I

MICROSCOPY

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UNIT 1 MICROSCOPY: AN INTRODUCTION

Structure

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1.0 INTRODUCTION

Microscopy refers to any method used to acquire images of nearby objects at resolutions that greatly exceed the resolving ability of the unaided human eye. Object visualization may be mediated by light or electron beams using optical or magnetic lenses respectively, or through the use of a physical scanning probe that measures one of a wide range of different sample characteristics. It is the technical field of using microscopes to view objects and areas of objects that cannot be seen with the naked eye, objects that are not within the resolution range of the normal eye. There are three well-known branches of microscopy: optical, electron, and scanning probe microscopy, along with the emerging field of X-ray microscopy.

In this unit, you will study about the principles, image formation and applications of light, polarizing microscopy, etc.

1.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand the basic principles of microscopy
- Analyse the mechanism of image formation
- Discuss the characteristics and application of light
- Explain about polarizing microscopy

1.2 BASIC PRINCIPLES OF MICROSCOPY

NOTES

Microscopy is necessary to evaluate the integrity of samples and to correlate structure with function. Microscopy serves two independent functions of enlargement or magnification and improved resolution or rendering of two objects as separate entities.

Light microscopes employ optical lenses to sequentially focus the image of objects, whereas electron microscope uses electromagnetic lenses.

Light and electron microscopes (Refer Figure 1.1) work either in transmission or scanning mode depending on whether the light or electron beam either passes through the specimen and is diffracted or deflected by specimen surface. Polarized light microscopes detect optically active substances in cells; for example, particles of silica or asbestos in lung tissue.

Phase contrast microscopes are often used to improve image contrast of unstained material which is caused either by diffraction by the specimen or even by differences in thickness of the specimen. At their point of focus, the converging light rays shows interference, resulting in either increase or decrease in the amplitude of the resultant wave (constructive or destructive interference, respectively), which the eye detects as differences in brightness.

Confocal microscopy is an imaging technique used to increase micrograph contrast and/or to reconstruct three-dimensional images by using a spatial pinhole to eliminate out-of-focus light or flare in specimens that are thicker than the focal plane. This technique has been gaining popularity in the scientific and industrial communities.

Typical applications include life sciences and semiconductor inspection. In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded in light from a light source. Due to the conservation of light intensity transportation, all parts of specimen throughout the optical path will be excited and the fluorescence will be detected by a photo detector or a camera.

In contrast, a confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information. Only the light within the focal plane can be detected, so the image quality is much better than that of wide-field images.

As only one point is illuminated at a time in confocal microscopy, 2D or 3D imaging requires scanning over a regular raster (i.e., a rectangular pattern of parallel scanning lines) in the specimen. The thickness of the focal plane is defined mostly by the square of the numerical aperture of the objective lens, and also by the optical properties of the specimen and the ambient index of refraction.

Microscopes using visible light will magnify approximately 1500 times and have a resolution limit of about 0.2 mm whereas a transmission electron microscope

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is capable of magnifying approximately 2,00,000 times and has a resolution limit for biological specimens of about 1 nm. This capability of TEM is largely a function of the very short wavelength of electrons accelerated under the influence of an applied electric field. (An accelerating voltage of 100 kV produces a wavelength of 4×10^{-3} nm.)

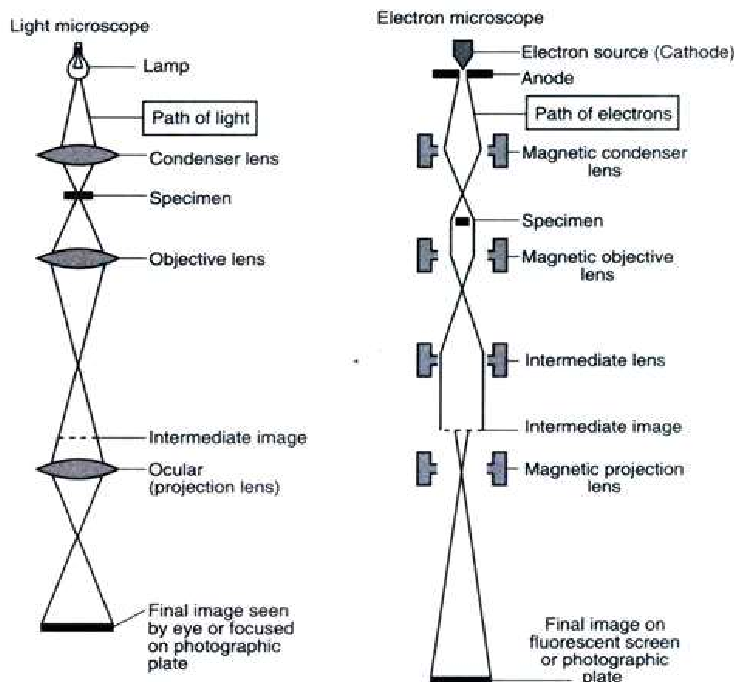

Fig. 1.1 *Schematic Diagram of Light Microscope and Electron Microscope*

Scanning electron microscopes (SEM) use a fine beam of electrons to scan back and forth across the metal coated specimen surface. The secondary electrons are generated from this surface collected by a scintillation crystal, which converts each electron impact into a flash of light.

Each light flash inside the crystal is amplified by a photomultiplier and used to build up an image on a fluorescent screen. The principle application of SEM is the study of surfaces such as those of cells. The resolution limit of scanning electron microscope is about 6 nm.

Check Your Progress

1. Define the term microscopy.
2. What is a light microscope?
3. What is electron microscope?
4. Define confocal microscopy.

1.3 IMAGE FORMATION AND APPLICTION OF LIGHT

NOTES

The objective collects a fan of rays from each object point and images the ray bundle at the front focal plane of the eyepiece. The conventional rules of ray tracing apply to the image formation. In the absence of aberration, geometric rays form a point image of each object point. In the presence of aberrations, each object point is represented by an indistinct point. The eyepiece is designed to image the rays to a focal point at a convenient distance for viewing the image. In this system, the brightness of the image is determined by the sizes of the apertures of the lenses and by the aperture of the pupil of the eye. The focal length and resulting magnification of the objective should be chosen to attain the desired resolution of the object at a size convenient for viewing through the eyepiece. Image formation in the microscope is complicated by diffraction and interference that take place in the imaging system and by the requirement to use a light source that is imaged in the focal plane (Refer Figure 1.2).

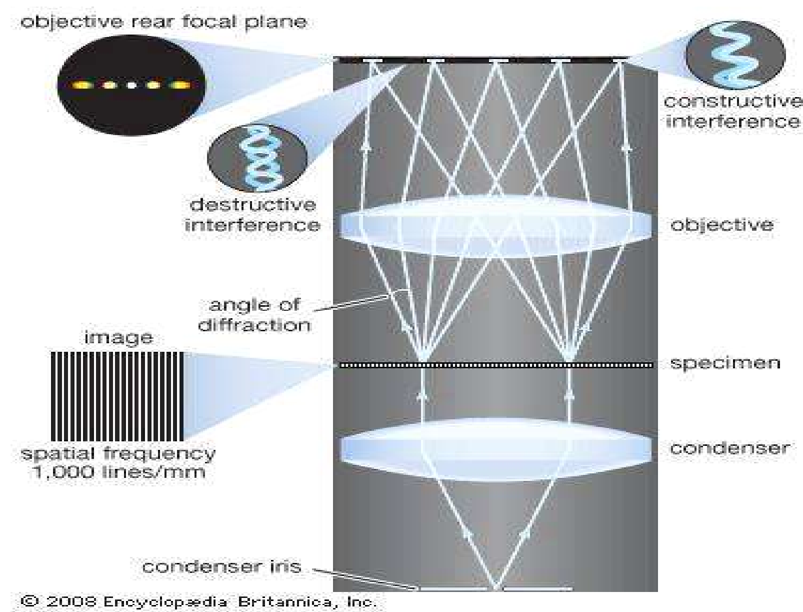

Fig. 1.2 *Image Formation in a Microscope*

Image formation in a microscope, according to the Abbe theory. Specimens are illuminated by light from a condenser. This light is diffracted by the details in the object plane: the smaller the detailed structure of the object, the wider the angle of diffraction. The structure of the object can be represented as a sum of sinusoidal components. The rapidity of variation in space of the components is defined by the period of each component, or the distance between adjacent peaks in the sinusoidal function. The spatial frequency is the reciprocal of the period. The finer the details, the higher the required spatial frequency of the components that

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represent the object detail. Each spatial frequency component in the object produces diffraction at a specific angle dependent upon the wavelength of light. Here, for example, a specimen with structure that has a spatial frequency of 1,000 lines per millimeter produces diffraction with an angle of 33.6°. The microscope objective collects these diffracted waves and directs them to the focal plane, where interference between the diffracted waves produces an image of the object. *Encyclopædia Britannica, Inc.*

The modern theory of image formation in the microscope was founded in 1873 by the German physicist Ernst Abbe. The starting point for the Abbe theory is that objects in the focal plane of the microscope are illuminated by convergent light from a condenser. The convergent light from the source can be considered as a collection of many plane waves propagating in a specified set of directions and superimposed to form the incident illumination. Each of these effective plane waves is diffracted by the details in the object plane: the smaller the detailed structure of the object, the wider the angle of diffraction.

The structure of the object can be represented as a sum of sinusoidal components. The rapidity of variation in space of the components is defined by the period of each component, or the distance between adjacent peaks in the sinusoidal function. The spatial frequency is the reciprocal of the period. The finer the details, the higher the required spatial frequency of the components that represent the object detail. Each spatial frequency component produces diffraction at a specific angle dependent upon the wavelength of light. As an example, spatial frequency components having a period of 1ìm would have a spatial frequency of 1,000 lines per millimeter. The angle of diffraction for such a component for visible light with a wavelength of 550 nanometers (nm; 1 nanometer is 10^{-9} meter) will be 33.6°. The microscope objective collects these diffracted waves and directs them to an image plane, where interference between the diffracted waves produces an image of the object.

Because the aperture of the objective is limited, not all the diffracted waves from the object can be transmitted by the objective. Abbe showed that the greater the number of diffracted waves reaching the objective, the finer the detail that can be reconstructed in the image. He designated the term numerical aperture (NA) as the measure of the objective's ability to collect diffracted light and thus also of its power to resolve detail. On this basis it is obvious that the greater the magnification of the objective, the greater required NA of the objective. The largest NA theoretically possible in air is 1.0, but optical design constraints limit the NA that can be achieved to around 0.95 for dry objectives.

For the example above of a specimen with a spatial frequency of 1,000 lines per millimeter, the required NA to collect the diffracted light would be 0.55. Thus, an objective of 0.55 NA or greater must be used to observe and collect useful data from an object with details spaced 1ìm apart. If the objective has a lower NA, the details of the object will not be resolved. Attempts to enlarge the image detail by use of a high-power eyepiece will yield no increase in resolution. This latter condition is called empty magnification.

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The wavelength of light is shortened when it propagates through a dense medium. In order to resolve the smallest possible details, immersion objectives are able to collect light diffracted by finer details than can objectives in air. The NA is multiplied by the index of refraction of the medium, and working NA of 1.4 are possible. In the best optical microscopes, structures with spatial frequency as small as 0.4 ìm can be observed. Note that the single lenses made by Leeuwenhoek have been shown to be capable of resolving fibrils only 0.7 ìm in thickness.

1.4 POLARIZING MICROSCOPES

Polarizing microscopes are conventional microscopes with additional features that permit observation under polarized light. The light source of such an instrument is equipped with a polarizing filter, the polarizer, so that the light it supplies is linearly polarized (i.e., the light waves vibrate in a given direction rather than randomly in all directions as in ordinary light). When this linearly polarized light passes through the object under examination, it may be unaffected or, if the object is birefringent, it may be split into two beams with different polarizations. A second filter, a polarization analyzer, is fitted to the eyepiece, where it blocks out all but one polarization of the light. The analyzer can be rotated to obtain maximum contrast in the image, and so the direction of polarization of the light transmitted through the object can be determined. The eyepiece can also be equipped with a polarization retarder, which shifts the phase of the light between selected polarization directions and which can be rotated to measure the amount of elliptic polarization produced by the specimen.

Many precautions must be taken in the design and construction of a polarizing microscope to avoid using optical components that introduce undesirable polarization retardation in the beam of light after it has left the object. There is a basic limitation placed upon the use of objectives with high NA wherein the necessary high angles of incidence on the surface produce some depolarization. Specialized microscope objectives that minimize this effect have been designed and produced. Polarizing microscopes are primarily used to examine the nature of crystals in geologic samples and to analyze the details of birefringence and stress in biological structures. They have been of crucial importance in the detection and monitoring of asbestos fibers.

Check Your Progress

5. What is an eyepiece?
6. Define the term numerical aperture.
7. What is empty magnificence?
8. When was modern theory of image formation in the microscope founded?

1.5 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

NOTES

1. Microscopy refers to any method used to acquire images of nearby objects at resolutions that greatly exceed the resolving ability of the unaided human eye.
2. A light microscope (LM) is an instrument that uses visible light and magnifying lenses to examine small objects not visible to the naked eye, or in finer detail than the naked eye allows.
3. An electron microscope is a type of microscope that uses electrons to illuminate a specimen and create an enlarged image.
4. Confocal microscopy, most frequently confocal laser scanning microscopy (CLSM) or laser confocal scanning microscopy (LCSM), is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of using a spatial pinhole to block out-of-focus light in image formation.
5. An eyepiece, or ocular lens, is a type of lens that is attached to a variety of optical devices such as telescopes and microscopes. It is so named because it is usually the lens that is closest to the eye when someone looks through the device.
6. The Numerical Aperture (NA) is a measure of how much light can be collected by an optical system such as an optical fiber or a microscope lens.
7. A microscope's total magnification is a combination of the eyepieces and the objective lens. For example, a biological microscope with 10x eyepieces and a 40x objective has 400x magnification. There are however, a few limits to the amount of total magnification that can be reached before empty magnification comes into play. Empty magnification occurs when the image continues to be enlarged, but no additional detail is resolved.
8. The modern theory of image formation in the microscope was founded in 1873 by the German physicist Ernst Abbe.

1.6 SUMMARY

- Microscopy is necessary to evaluate the integrity of samples and to correlate structure with function.
- Microscopy serves two independent functions of enlargement or magnification and improved resolution or rendering of two objects as separate entities.
- Light microscopes employ optical lenses to sequentially focus the image of objects, whereas electron microscope uses electromagnetic lenses.

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- Light and electron microscope work either in transmission or scanning mode depending on whether the light or electron beam either passes through the specimen and is diffracted or deflected by specimen surface.
- Polarized light microscopes detect optically active substances in cells; for example, particles of silica or asbestos in lung tissue.
- Phase contrast microscopes are often used to improve image contrast of unstained material which is caused either by diffraction by the specimen or even by differences in thickness of the specimen.
- Confocal microscopy is an imaging technique used to increase micrograph contrast and/or to reconstruct three-dimensional images by using a spatial pinhole to eliminate out-of-focus light or flare in specimens that are thicker than the focal plane. This technique has been gaining popularity in the scientific and industrial communities.
- Typical applications include life sciences and semiconductor inspection. In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded in light from a light source.
- Due to the conservation of light intensity transportation, all parts of specimen throughout the optical path will be excited and the fluorescence will be detected by a photo detector or a camera.
- In contrast, a confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information.
- The thickness of the focal plane is defined mostly by the square of the numerical aperture of the objective lens, and also by the optical properties of the specimen and the ambient index of refraction.
- Microscopes using visible light will magnify approximately 1500 times and have a resolution limit of about 0.2 mm whereas a transmission electron microscope is capable of magnifying approximately 2,00,000 times and has a resolution limit for biological specimens of about 1 nm.
- Scanning electron microscopes (SEM) use a fine beam of electrons to scan back and forth across the metal coated specimen surface.
- The secondary electrons are generated from this surface collected by a scintillation crystal, which converts each electron impact into a flash of light.
- Each light flash inside the crystal is amplified by a photomultiplier and used to build up an image on a fluorescent screen.
- The principle application of SEM is the study of surfaces such as those of cells. The resolution limit of scanning electron microscope is about 6 nm.
- The objective collects a fan of rays from each object point and images the ray bundle at the front focal plane of the eyepiece.

- The conventional rules of ray tracing apply to the image formation. In the absence of aberration, geometric rays form a point image of each object point. In the presence of aberrations, each object point is represented by an indistinct point.
- The eyepiece is designed to image the rays to a focal point at a convenient distance for viewing the image. In this system, the brightness of the image is determined by the sizes of the apertures of the lenses and by the aperture of the pupil of the eye.
- The focal length and resulting magnification of the objective should be chosen to attain the desired resolution of the object at a size convenient for viewing through the eyepiece.

NOTES

1.7 KEY WORDS

- **Microscope:** A microscope is an instrument used to see objects that are too small to be seen by the naked eye.
- **Microscopy:** Microscopy refers to any method used to acquire images of nearby objects at resolutions that greatly exceed the resolving ability of the unaided human eye.
- **Light microscope:** A light microscope (LM) is an instrument that uses visible light and magnifying lenses to examine small objects not visible to the naked eye, or in finer detail than the naked eye allows
- **Electron microscope:** An electron microscope is a type of microscope that uses electrons to illuminate a specimen and create an enlarged image.
- **Confocal microscopy:** Confocal microscopy, most frequently confocal laser scanning microscopy (CLSM) or laser confocal scanning microscopy (LCSM), is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of using a spatial pinhole to block out-of-focus light in image formation.
- **Focal point:** The point on the axis of a lens or mirror to which parallel rays of light converge or from which they appear to diverge after refraction or reflection.
- **Eyepiece:** An eyepiece, or ocular lens, is a type of lens that is attached to a variety of optical devices such as telescopes and microscopes. It is so named because it is usually the lens that is closest to the eye when someone looks through the device.
- **Numerical aperture:** The Numerical Aperture (NA) is a measure of how much light can be collected by an optical system such as an optical fiber or a microscope lens.

1.8 SELF ASSESSMENT QUESTIONS AND EXERCISES

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Short Answer Questions

1. Write a short note on basic principles of microscopy.
2. What is confocal microscopy?
3. What is empty magnification?
4. What are polarizing microscopes?

Long Answer Questions

1. Discuss about the basics of microscope, microscopy and its related terms, providing a general introduction of all.
2. Explain about how image formation occurs in light and electron microscope.
3. Write about the applications of light.
4. Give a detailed overview about the polarizing microscope.

1.9 FURTHER READINGS

Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

Mortin, R. 1996. *Gel Electrophoresis: Nucleic Acids* (*Introduction to Biotechniques*). England: Garland Science/BIOS Scientific Publishers.

Sameer, A. S. 2011. *Molecular Biology and Biotechniques*. Riga (Europe): VDM Verlag Dr. Müller.

Dewan, S.K. 2012. *Organic Spectroscopy* (*NMR IR MASS and UV*). New Delhi: CBS Publishers & Distributors Pvt. Ltd.

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UNIT 2 ELECTRON MICROSCOPE, CONFOCAL MICROSCOPY AND PHOTOMICROGRAPHY

Structure

2.0 Introduction
2.1 Objectives
2.2 Transmission and Scanning Electron Microscope
2.2.1 Scanning Electron Microscope
2.3 Introduction to Confocal Microscopy
2.4 Phase Contrast Microscopy
2.5 Photomirography- Digital Imaging
2.6 Answers to Check Your Progress Questions
2.6 Summary
2.7 Key Words
2.8 Self Assessment Questions and Exercises
2.9 Further Readings

2.0 INTRODUCTION

The Transmission Electron Microscope or TEM is a very powerful tool for material science. A high energy beam of electrons is shone through a very thin sample, and the interactions between the electrons and the atoms can be used to observe features, such as the crystal structure and the structure like dislocations and grain boundaries. Chemical analysis can also be performed. TEM can be used to study the growth of layers, their composition and defects in semiconductors. High resolution can be used to analyze the quality, shape, size and density of quantum wells, wires and dots. The TEM operates on the same basic principles as the light microscope but uses electrons instead of light.

In this unit, you will study about the concept of transmission and scanning electron microscopes, confocal microscopy, phase contrast microscopy, photomicrography, digital imaging, etc.

2.1 OBJECTIVES

After going through this unit you will be able to:

- Understand the concept of transmission and scanning electron microscopes
- Discuss about confocal microscopy
- Explain what is phase contrast microscopy

- Discuss what is photomicrography
- Understand about digital imaging

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2.2 TRANSMISSION AND SCANNING ELECTRON MICROSCOPE

There are four main components to a Transmission Electron Microscope (TEM) an electron optical column, a vacuum system, the necessary electronics (lens supplies for focusing and deflecting the beam and the high voltage generator for the electron source), and control software. A modern TEM typically comprises an operating console surmounted by a vertical column and containing the vacuum system, and control panels conveniently placed for the operator. The microscope may be fully enclosed to reduce interference from environmental sources, and operated remotely. Figure 2.1 shows the image for electron microscope.

Fig. 2.1 *Electron Microscope*

The electron column includes elements analogous to those of a light microscope. The light source of the light microscope is replaced by an electron gun, which is built into the column. The glass lenses are replaced by electromagnetic lenses. Unlike glass lenses, the power (focal length) of magnetic lenses can be changed by changing the current through the lens coil. The eyepiece or ocular is replaced by a fluorescent screen and/or a digital camera. The electron beam emerges from the electron gun, and passes through a thin specimen, transmitting electrons which are collected, focused, and projected onto the viewing device at the bottom of the column. The entire electron path from gun to camera must be under vacuum.

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2.2.1 Scanning Electron Microscope

A scanning electron microscope (SEM), like a transmission electron microscope, consists of an electron optical column, a vacuum system, electronics, and software. The column is considerably shorter because the only lenses needed are those above the specimen used to focus the electrons into a fine spot on the specimen surface. The specimen chamber, however, is larger because the SEM technique does not impose any restriction on specimen size other than chamber size.

The electron gun at the top of the column produces an electron beam that is focused into a fine spot as small as 1 nm in diameter on the specimen surface. This beam is scanned in a rectangular raster over the specimen and the intensities of various signals created by interactions between the beam electrons and the specimen are measured and stored in computer memory. The stored values are then mapped as variations in brightness on the image display. The secondary electron (SE) signal is the most frequently used signal. It varies with the topography of the sample surface much like an aerial photograph: edges are bright, recesses are dark. The ratio of the size of the displayed image to the size of the area scanned on the specimen gives the magnification.

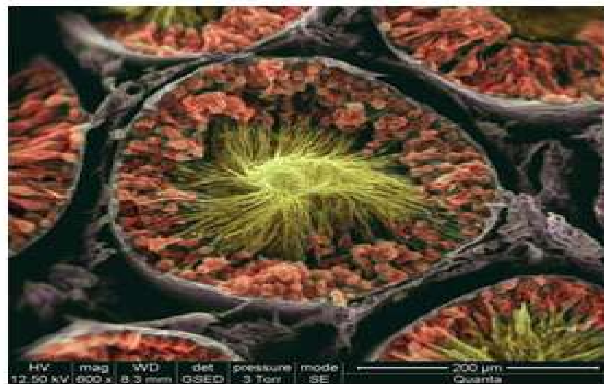

Fig 2.2 *Sperm Tails Tangled up in a Seminiferous Tubule*

Figure 2.2 shows the sperm tails tangled up in a seminiferous tubule, magnified 600x on an FEI Quanta™ Scanning Electron Microscope

The electron gun and lenses are similar to those of a transmission electron microscope. The most important differences between a transmission electron microscope and a scanning electron microscope are

- Rather than the broad static beam used in TEM, the SEM beam is focused to a fine point and scans line by line over the sample surface in a rectangular raster pattern.
- The accelerating voltages are much lower than in TEM because it is no longer necessary to penetrate the specimen; in a SEM they range from 50 to 30,000 volts.
- The specimen need not be thin, greatly simplifying specimen preparation.

The interactions between the beam electrons and sample atoms are similar to those described for a transmission electron microscope

- The specimen itself emits secondary electrons.
- Some of the primary electrons are reflected backscattered electrons (BSE). These backscattered electrons can also cause the emission of secondary electrons as they travel through the sample and exit the sample surface.
- If the sample is thin, the SEM may be operated in STEM mode with a detector located below the sample to collect transmitted electrons.

All these phenomena are interrelated and all of them depend to some extent on the topography, the atomic number, and the chemical state of the specimen.

Check Your Progress

1. What are the main components of TEM?
2. What does a TEM comprises of?
3. How can the power of magnetic lens changed?
4. From where does electron beam emerges?
5. Which is the most frequently used signal?

2.3 INTRODUCTION TO CONFOCAL MICROSCOPY

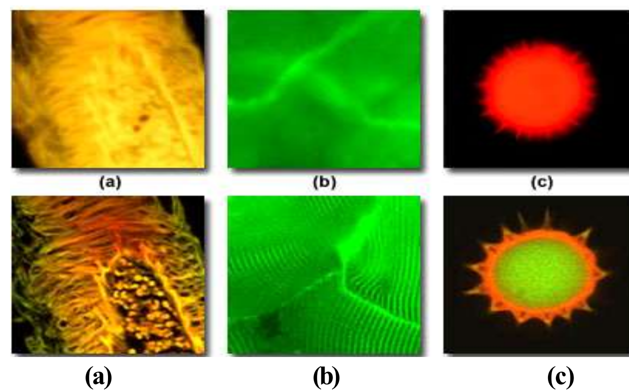

Fig. 2.3 *Confocal and Widefield Fluorescence Microscopy*

In a conventional widefield optical epifluorescence microscope, secondary fluorescence emitted by the specimen often occurs through the excited volume and obscures resolution of features that lie in the objective focal plane. The problem is compounded by thicker specimens (greater than 2 micrometers), which usually exhibit such a high degree of fluorescence emission that most of the fine detail is lost. Confocal microscopy provides only a marginal improvement in both axial (**z**; along the optical axis) and lateral (x and y; in the specimen plane) optical resolution, but is able to exclude secondary fluorescence in areas removed from the focal

plane from resulting images. Even though resolution is somewhat enhanced with confocal microscopy over conventional widefield techniques, it is still considerably less than that of the transmission electron microscope. In this regard, confocal microscopy can be considered a bridge between these two classical methodologies.

Presented in Figure 2.3 are a series of images that compare selected viewfields in traditional widefield and laser scanning confocal fluorescence microscopy. A thick section of fluorescently stained human medulla in widefield fluorescence exhibits a large amount of glare from fluorescent structures above and below the focal plane (Refer Figure 2.3 a). When imaged with a laser scanning confocal microscope (Refer Figure 2.3 d), the medulla thick section reveals a significant degree of structural detail. Likewise, widefield fluorescence imaging of whole rabbit muscle fibers stained with fluorescein produce blurred images (Refer Figure 2.3 b) lacking in detail, while the same specimen field (Refer Figure 2.3 e) reveals a highly striated topography in confocal microscopy. Autofluorescence in a sunflower pollen grain produces an indistinct outline of the basic external morphology (Refer Figure 2.3c), but yields no indication of the internal structure. In contrast, a thin optical section of the same grain (Refer Figure 2.3f) acquired with confocal techniques displays a dramatic difference between the particle core and the surrounding envelope.

Historical Perspective

The basic concept of confocal microscopy was originally developed by Marvin Minsky in the mid-1950s (patented in 1957) when he was a postdoctoral student at Harvard University. Minsky wanted to image neural networks in unstained preparations of brain tissue and was driven by the desire to image biological events at they occur in living systems. Minsky's invention remained largely unnoticed, due most probably to the lack of intense light sources necessary for imaging and the computer horsepower required to handle large amounts of data. Following Minsky's work, M. David Egger and Mojmir Petran fabricated a multiple-beam confocal microscope in the late 1960s that utilized a spinning (Nipkow) disk for examining unstained brain sections and ganglion cells. Continuing in this arena, Egger went on to develop the first mechanically scanned confocal laser microscope, and published the first recognizable images of cells in 1973. During the late 1970s and the 1980s, advances in computer and laser technology, coupled to new algorithms for digital manipulation of images, led to a growing interest in confocal microscopy.

Fortuitously, shortly after Minsky's patent had expired, practical laser scanning confocal microscope designs were translated into working instruments by several investigators. Dutch physicist G. Fred Brakenhoff developed a scanning confocal microscope in 1979, while almost simultaneously, Colin Sheppard contributed to the technique with a theory of image formation. Tony Wilson, Brad Amos, and John White nurtured the concept and later (during the late 1980s) demonstrated the utility of confocal imaging in the examination of fluorescent biological specimens. The first commercial instruments appeared in 1987. During

the 1990s, advances in optics and electronics afforded more stable and powerful lasers, high-efficiency scanning mirror units, high-throughput fiber optics, better thin film dielectric coatings, and detectors having reduced noise characteristics. In addition, fluorochromes that were more carefully matched to laser excitation lines were beginning to be synthesized. Coupled to the rapidly advancing computer processing speeds, enhanced displays, and large-volume storage technology emerging in the late 1990s, the stage was set for a virtual explosion in the number of applications that could be targeted with laser scanning confocal microscopy.

Modern confocal microscopes can be considered as completely integrated electronic systems where the optical microscope plays a central role in a configuration that consists of one or more electronic detectors, a computer (for image display, processing, output, and storage), and several laser systems combined with wavelength selection devices and a beam scanning assembly. In most cases, integration between the various components is so thorough that the entire confocal microscope is often collectively referred to as a digital or video imaging system capable of producing electronic images. These microscopes are now being employed for routine investigations on molecules, cells, and living tissues that were not possible just a few years ago.

Principles of Confocal Microscopy

The confocal principle in epifluorescence laser scanning microscopy is diagrammatically presented in Figure 2.4. Coherent light emitted by the laser system (excitation source) passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector (a photomultiplier tube). As the laser is reflected by a dichromatic mirror and scanned across the specimen in a defined focal plane, secondary fluorescence emitted from points on the specimen (in the same focal plane) pass back through the dichromatic mirror and are focused as a confocal point at the detector pinhole aperture.

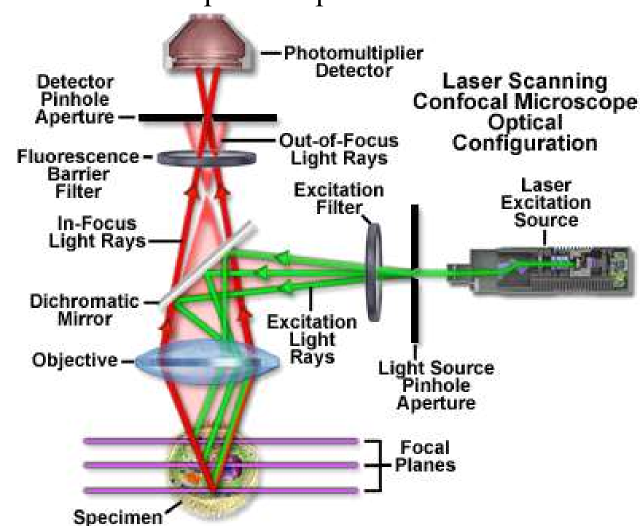

Fig. 2.4 *Laser Scanning Confocal Microscope Optical Configuration*

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The significant amount of fluorescence emission that occurs at points above and below the objective focal plane is not confocal with the pinhole (termed out-of-focus light rays in Figure 2.4) and forms extended Airy disks in the aperture plane. Because only a small fraction of the out-of-focus fluorescence emission is delivered through the pinhole aperture, most of this extraneous light is not detected by the photomultiplier and does not contribute to the resulting image. The dichromatic mirror, barrier filter, and excitation filter perform similar functions to identical components in a widefield epifluorescence microscope. Refocusing the objective in a confocal microscope shifts the excitation and emission points on a specimen to a new plane that becomes confocal with the pinhole apertures of the light source and detector.

In traditional widefield epifluorescence microscopy, the entire specimen is subjected to intense illumination from an incoherent mercury or xenon arc-discharge lamp, and the resulting image of secondary fluorescence emission can be viewed directly in the eyepieces or projected onto the surface of an electronic array detector or traditional film plane. In contrast to this simple concept, the mechanism of image formation in a confocal microscope is fundamentally different. As discussed above, the confocal fluorescence microscope consists of multiple laser excitation sources, a scan head with optical and electronic components, electronic detectors (usually photomultipliers), and a computer for acquisition, processing, analysis, and display of images.

The scan head is at the heart of the confocal system and is responsible for rasterizing the excitation scans, as well as collecting the photon signals from the specimen that are required to assemble the final image. A typical scan head contains inputs from the external laser sources, fluorescence filter sets and dichromatic mirrors, a galvanometer-based raster scanning mirror system, variable pinhole apertures for generating the confocal image, and photomultiplier tube detectors tuned for different fluorescence wavelengths. The general arrangement of scan head components is presented in Figure 3 for a typical commercial unit.

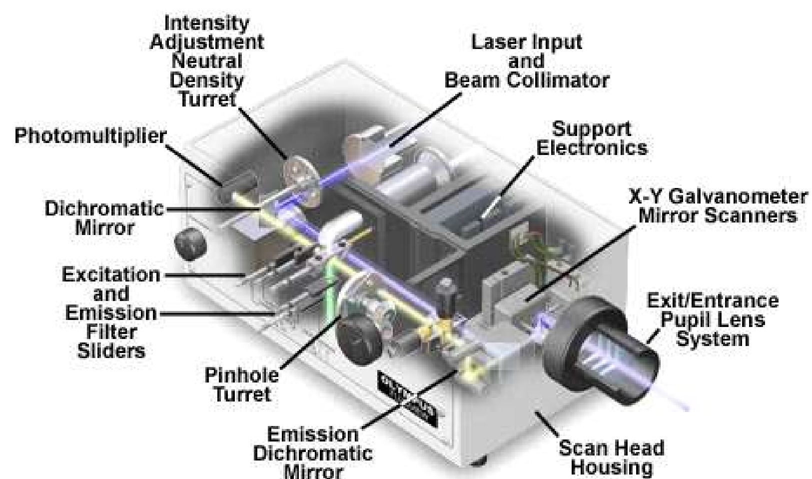

Fig. 2.5 *Confocal Microscopy Scanning Unit*

In epi-illumination scanning confocal microscopy, the laser light source and photomultiplier detectors are both separated from the specimen by the objective, which functions as a well-corrected condenser and objective combination. Internal fluorescence filter components (such as the excitation and barrier filters and the dichromatic mirrors) and neutral density filters are contained within the scanning unit (Refer Figure 2.5). Interference and neutral density filters are housed in rotating turrets or sliders that can be inserted into the light path by the operator. The excitation laser beam is connected to the scan unit with a fiber optic coupler followed by a beam expander that enables the thin laser beam wrist to completely fill the objective rear aperture (a critical requirement in confocal microscopy). Expanded laser light that passes through the microscope objective forms an intense diffraction-limited spot that is scanned by the coupled galvanometer mirrors in a raster pattern across the specimen plane (point scanning).

One of the most important components of the scanning unit is the pinhole aperture, which acts as a spatial filter at the conjugate image plane positioned directly in front of the photomultiplier. Several apertures of varying diameter are usually contained on a rotating turret that enables the operator to adjust pinhole size (and optical section thickness). Secondary fluorescence collected by the objective is descanned by the same galvanometer mirrors that form the raster pattern, and then passes through a barrier filter before reaching the pinhole aperture. The aperture serves to exclude fluorescence signals from out-of-focus features positioned above and below the focal plane, which are instead projected onto the aperture as Airy disks having a diameter much larger than those forming the image. These oversized disks are spread over a comparatively large area so that only a small fraction of light originating in planes away from the focal point passes through the aperture. The pinhole aperture also serves to eliminate much of the stray light passing through the optical system. Coupling of aperture-limited point scanning to a pinhole spatial filter at the conjugate image plane is an essential feature of the confocal microscope.

When contrasting the similarities and differences between widefield and confocal microscopes, it is often useful to compare the character and geometry of specimen illumination utilized for each of the techniques. Traditional widefield epi-fluorescence microscope objectives focus a wide cone of illumination over a large volume of the specimen, which is uniformly and simultaneously illuminated (as illustrated in Figure 2.6). A majority of the fluorescence emission directed back towards the microscope is gathered by the objective (depending upon the numerical aperture) and projected into the eyepieces or detector. The result is a significant amount of signal due to emitted background light and autofluorescence originating from areas above and below the focal plane, which seriously reduces resolution and image contrast.

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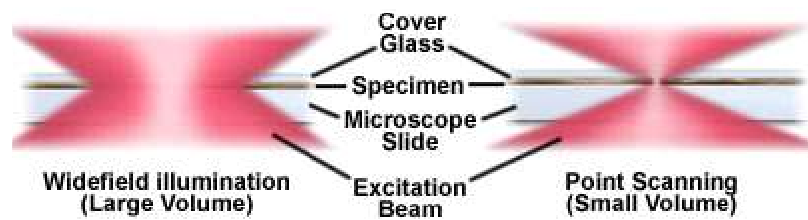

Fig. 2.6 *Widefield vs. Confocal Point Scanning of Specimens*

The laser illumination source in confocal microscopy is first expanded to fill the objective rear aperture, and then focused by the lens system to a very small spot at the focal plane (Refer Figure 2.6). The size of the illumination point ranges from approximately 0.25 to 0.8 micrometers in diameter (depending upon the objective numerical aperture) and 0.5 to 1.5 micrometers deep at the brightest intensity. Confocal spot size is determined by the microscope design, wavelength of incident laser light, objective characteristics, scanning unit settings, and the specimen. Presented in Figure 2.6 is a comparison between the typical illumination cones of a widefield and point scanning confocal microscope at the same numerical aperture. The entire depth of the specimen over a wide area is illuminated by the widefield microscope, while the sample is scanned with a finely focused spot of illumination that is centered in the focal plane in the confocal microscope.

In laser scanning confocal microscopy, the image of an extended specimen is generated by scanning the focused beam across a defined area in a raster pattern controlled by two high-speed oscillating mirrors driven by galvanometer motors. One of the mirrors moves the beam from left to right along the x lateral axis, while the other translates the beam in the **y** direction. After each single scan along the x axis, the beam is rapidly transported back to the starting point and shifted along the **y** axis to begin a new scan in a process termed flyback. During the flyback operation, image information is not collected. In this manner, the area of interest on the specimen in a single focal plane is excited by laser illumination from the scanning unit.

As each scan line passes along the specimen in the lateral focal plane, fluorescence emission is collected by the objective and passed back through the confocal optical system. The speed of the scanning mirrors is very slow relative to the speed of light, so the secondary emission follows a light path along the optical axis that is identical to the original excitation beam. Return of fluorescence emission through the galvanometer mirror system is referred to as descanning. After leaving the scanning mirrors, the fluorescence emission passes directly through the dichromatic mirror and is focused at the detector pinhole aperture. Unlike the raster scanning pattern of excitation light passing over the specimen, fluorescence emission remains in a steady position at the pinhole aperture, but fluctuates with respect to intensity over time as the illumination spot traverses the specimen producing variations in excitation.

Fluorescence emission that is passed through the pinhole aperture is converted into an analog electrical signal having a continuously varying voltage (corresponding to intensity) by the photomultiplier. The analog signal is periodically sampled and converted into pixels by an Analog-to-Digital (A/D) converter housed in the scanning unit or the accompanying electronics cabinet. The image information is temporarily stored in an image frame buffer card in the computer and displayed on the monitor. It is important to note that the confocal image of a specimen is reconstructed, point by point, from emission photon signals by the photomultiplier and accompanying electronics, yet never exists as a real image that can be observed through the microscope eyepieces.

Laser Scanning Confocal Microscope Configuration

Basic microscope optical system characteristics have remained fundamentally unchanged for many decades due to engineering restrictions on objective design, the static properties of most specimens, and the fact that resolution is governed by the wavelength of light. However, fluorescent probes that are employed to add contrast to biological specimens and, and other technologies associated with optical microscopy techniques, have improved significantly. The explosive growth and development of the confocal approach is a direct result of a renaissance in optical microscopy that has been largely fueled by advances in modern optical and electronics technology. Among these are stable multi-wavelength laser systems that provide better coverage of the ultraviolet, visible, and near-infrared spectral regions, improved interference filters (including dichromatic mirrors, barrier, and excitation filters), sensitive low-noise wide band detectors, and far more powerful computers. The latter are now available with relatively low-cost memory arrays, image analysis software packages, high-resolution video displays, and high quality digital image printers. The flow of information through a modern confocal microscope is presented diagrammatically in Figure 2.7.

Although many of these technologies have been developed independently for a variety of specifically-targeted applications, they have been gradually been incorporated into mainstream commercial confocal microscopy systems. In current microscope systems, classification of designs is based on the technology utilized to scan specimens. Scanning can be accomplished either by translating the stage in the x, y, and z directions while the laser illumination spot is held in a fixed position, or the beam itself can be raster-scanned across the specimen. Because three-dimensional translation of the stage is cumbersome and prone to vibration, most modern instruments employ some type of beam-scanning mechanism.

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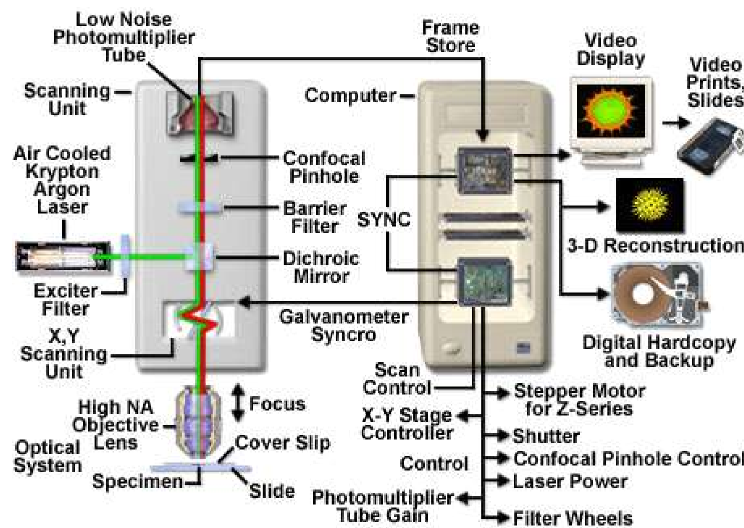

Fig. 2.7 *Confocal Microscopy Information Flow Schematic Diagram*

In modern confocal microscopes, two fundamentally different techniques for beam scanning have been developed. Single-beam scanning, one of the more popular methods employed in a majority of the commercial laser scanning microscopes, uses a pair of computer-controlled galvanometer mirrors to scan the specimen in a raster pattern at a rate of approximately one frame per second. Faster scanning rates (to near video speed) can be achieved using acousto-optic devices or oscillating mirrors. In contrast, multiple-beam scanning confocal microscopes are equipped with a spinning Nipkow disk containing an array of pinholes and microlenses. These instruments often use arc-discharge lamps for illumination instead of lasers to reduce specimen damage and enhance the detection of low fluorescence levels during real time image collection. Another important feature of the multiple-beam microscopes is their ability to readily capture images with an array detector, such as a Charge-Coupled Device (CCD) camera system.

All laser scanning confocal microscope designs are centered around a conventional upright or inverted research-level optical microscope. However, instead of the standard tungsten-halogen or mercury arc-discharge lamp, one or more laser systems are used as a light source to excite fluorophores in the specimen. Image information is gathered point by point with a specialized detector such as a photomultiplier tube or avalanche photodiode, and then digitized for processing by the host computer, which also controls the scanning mirrors and/or other devices to facilitate the collection and display of images. After a series of images (usually serial optical sections) has been acquired and stored on digital media, analysis can be conducted utilizing numerous image processing software packages available on the host or a secondary computer.

Advantages and Disadvantages of Confocal Microscopy

The primary advantage of laser scanning confocal microscopy is the ability to serially produce thin (0.5 to 1.5 micrometer) optical sections through fluorescent

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specimens that have a thickness ranging up to 50 micrometers or more. The image series is collected by coordinating incremental changes in the microscope fine focus mechanism (using a stepper motor) with sequential image acquisition at each step. Image information is restricted to a well-defined plane, rather than being complicated by signals arising from remote locations in the specimen. Contrast and definition are dramatically improved over widefield techniques due to the reduction in background fluorescence and improved signal-to-noise. Furthermore, optical sectioning eliminates artifacts that occur during physical sectioning and fluorescent staining of tissue specimens for traditional forms of microscopy. The non-invasive confocal optical sectioning technique enables the examination of both living and fixed specimens under a variety of conditions with enhanced clarity.

With most confocal microscopy software packages, optical sections are not restricted to the perpendicular lateral (x-y) plane, but can also be collected and displayed in transverse planes. Vertical sections in the x-z and y-z planes (parallel to the microscope optical axis) can be readily generated by most confocal software programs. Thus, the specimen appears as if it had been sectioned in a plane that is perpendicular to the lateral axis. In practice, vertical sections are obtained by combining a series of x-y scans taken along the **z** axis with the software, and then projecting a view of fluorescence intensity as it would appear should the microscope hardware have been capable of physically performing a vertical section.

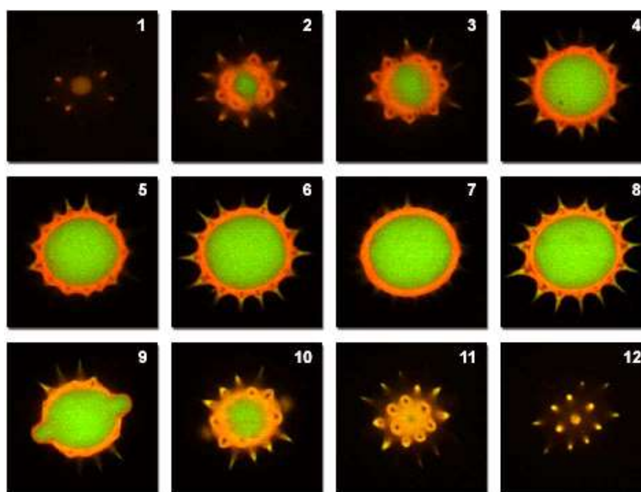

Fig. 2.8 *Pollen Grain Serial Optical Sections by Confocal Microscopy*

A typical stack of optical sections (often termed a z-series) through a sunflower pollen grain revealing internal variations in autofluorescence emission wavelengths is illustrated in Figure 2.8. Optical sections were gathered in 0.5-micrometer steps perpendicular to the z-axis (microscope optical axis) using a dual argon-ion (488 nanometer; green fluorescence) and green helium/neon (543 nanometer; red fluorescence) laser system. Pollen grains of from this species range between 20 and 40 micrometers in diameter and yield blurred images in widefield

fluorescence microscopy (Refer Figure 2.8), which lack information about internal structural details. Although only 12 of the over 48 images collected through this series are presented in the figure, they represent individual focal planes separated by a distance of approximately 3 micrometers and provide a good indication of the internal grain structure.

In specimens more complex than a pollen grain, complex interconnected structural elements can be difficult to discern from a large series of optical sections sequentially acquired through the volume of a specimen with a laser scanning confocal microscope. However, once an adequate series of optical sections has been gathered, it can be further processed into a three-dimensional representation of the specimen using volume-rendering computational techniques. This approach is now in common use to help elucidate the numerous interrelationships between structure and function of cells and tissues in biological investigations. In order to ensure that adequate data is collected to produce a representative volume image, the optical sections should be recorded at the appropriate axial (z-step) intervals so that the actual depth of the specimen is reflected in the image.

Most of the software packages accompanying commercial confocal instruments are capable of generating composite and multi-dimensional views of optical section data acquired from z-series image stacks. The three-dimensional software packages can be employed to create either a single three-dimensional representation of the specimen (Refer Figure 2.9) or a video (movie) sequence compiled from different views of the specimen volume. These sequences often mimic the effect of rotation or similar spatial transformation that enhances the appreciation of the specimen's three-dimensional character. In addition, many software packages enable investigators to conduct measurements of length, volume, and depth, and specific parameters of the images, such as opacity, can be interactively altered to reveal internal structures of interest at differing levels within the specimen.

Typical three-dimensional representations of several specimens examined by serial optical sectioning are presented in Figure 2.9. The pollen grain optical sections illustrated in Figures 2.3 and 2.8 were combined to produce a realistic view of the exterior surface (Refer Figure 2.9(a)) as it might appear if being examined by a scanning electron microscope. The algorithm utilized to construct the three-dimensional model enables the user to rotate the pollen grain through 360 degrees for examination. The tissue culture cells in Figure 2.9 (b) are derived from the Chinese hamster ovary (CHO) line and were transfected with a chimeric plasmid vector containing the green fluorescent protein and a human immunodeficiency virus (HIV) protein that is expressed in the nucleus (thus, labeling the nuclear region). Thick tissue sections are also easily viewed in three-dimensions constructed from optical sections. The mouse intestine section illustrated in Figure 2.9(c) was labeled with several fluorophores and created from a stack of 45 optical sections.

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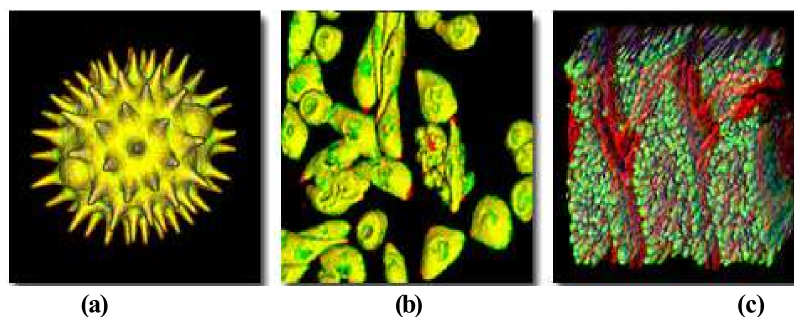

Fig. 2.9 *Three-dimensional Volume Readers from Confocal Optical Sections*

In many cases, a composite or projection view produced from a series of optical sections provides important information about a three-dimensional specimen than a multi-dimensional view. For example, a fluorescently labeled neuron having numerous thin, extended processes in a tissue section is difficult (if not impossible) to image using widefield techniques due to out-of-focus blur. Confocal thin sections of the same neuron each reveal portions of several extensions, but these usually appear as fragmented streaks and dots and lack continuity. Composite views created by flattening a series of optical sections from the neuron will reveal all of the extended processes in sharp focus with well-defined continuity. Structural and functional analysis of other cell and tissue sections also benefits from composite views as opposed to, or coupled with, three-dimensional volume rendering techniques.

Advances in confocal microscopy have made possible multi-dimensional views of living cells and tissues that include image information in the x, y, and **z** dimensions as a function of time and presented in multiple colors (using two or more fluorophores). After volume processing of individual image stacks, the resulting data can be displayed as three-dimensional multicolour video sequences in real time. Note that unlike conventional widefield microscopy, all fluorochromes in multiply labeled specimens appear in register using the confocal microscope. Temporal data can be collected either from time-lapse experiments conducted over extended periods or through real time image acquisition in smaller frames for short periods of time. The potential for using multi-dimensional confocal microscopy as a powerful tool in cellular biology is continuing to grow as new laser systems are developed to limit cell damage and computer processing speeds and storage capacity improves.

Additional advantages of scanning confocal microscopy include the ability to adjust magnification electronically by varying the area scanned by the laser without having to change objectives. This feature is termed the zoom factor, and is usually employed to adjust the image spatial resolution by altering the scanning laser sampling period. Increasing the zoom factor reduces the specimen area scanned and simultaneously reduces the scanning rate. The result is an increased number of samples along a comparable length, which increases both the image spatial resolution and display magnification on the host computer monitor. Confocal

zoom is typically employed to match digital image resolution with the optical resolution of the microscope when low numerical aperture and magnification objectives are being used to collect data.

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Digitization of the sequential analog image data collected by the confocal microscope photomultiplier (or similar detector) facilitates computer image processing algorithms by transforming the continuous voltage stream into discrete digital increments that correspond to variations in light intensity. In addition to the benefits and speed that accrue from processing digital data, images can be readily prepared for print output or publication. In carefully controlled experiments, quantitative measurements of spatial fluorescence intensity (either statically or as a function of time) can also be obtained from the digital data.

Disadvantages of confocal microscopy are limited primarily to the limited number of excitation wavelengths available with common lasers (referred to as laser lines), which occur over very narrow bands and are expensive to produce in the ultraviolet region. In contrast, conventional widefield microscopes use mercury or xenon based arc-discharge lamps to provide a full range of excitation wavelengths in the ultraviolet, visible, and near-infrared spectral regions. Another downside is the harmful nature of high-intensity laser irradiation to living cells and tissues (an issue that has recently been addressed by multiphoton and Nipkow disk confocal imaging). Finally, the high cost of purchasing and operating multi-user confocal microscope systems, which can range up to an order of magnitude higher than comparable widefield microscopes, often limits their implementation in smaller laboratories. This problem can be easily overcome by cost-shared microscope systems that service one or more departments in a core facility. The recent introduction of personal confocal systems has competitively driven down the price of low-end confocal microscopes and increased the number of individual users.

Check Your Progress

6. Who developed the basic concept of confocal microscopy?
7. What is flyback?
8. Define descanning.
9. What happens to the fluorescence emission that is passed through the pinhole aperture?
10. Give a key feature of multiple-beam microscope.
11. How is image formation gathered?

2.4 PHASE CONTRAST MICROSCOPY

The search was still on in the 1930's to find a way of using both direct and diffracted light from all azimuths to yield good contrast images of unstained objects that do not absorb light. Research by Frits Zernike during this period uncovered phase

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and amplitude differences between zeroth order and deviated light that can be altered to produce favorable conditions for interference and contrast enhancement.

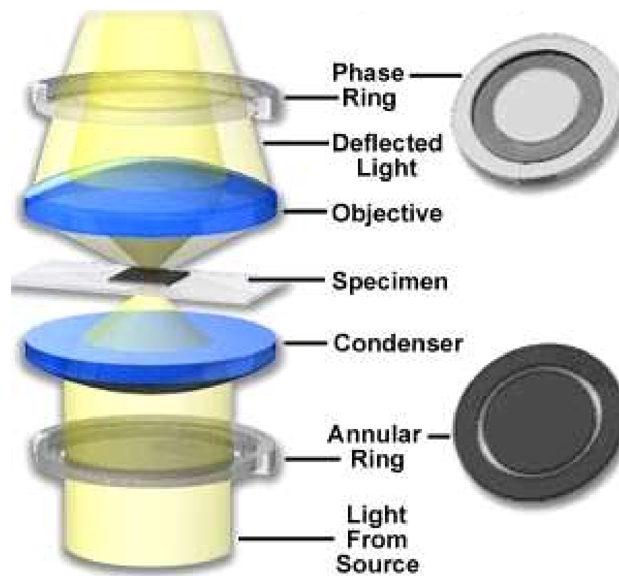

Fig. 2.10 *Phase Contrast Light Pathway*

Unstained specimens that do not absorb light are called phase objects because they slightly alter the phase of the light diffracted by the specimen, usually by retarding such light approximately 1/4 wavelength as compared to the undeviated direct light passing through or around the specimen unaffected. Unfortunately, our eyes as well as camera film, are unable to detect these phase differences. To reiterate, the human eye is sensitive only to the colors of the visible spectrum (variations in light frequency) or to differing levels of light intensity (variations in wave amplitude).

In phase specimens, the direct zeroth order light passes through or around the specimen undeviated. However, the light diffracted by the specimen is not reduced in amplitude as it is in a light-absorbing object, but is slowed by the specimen because of the specimen's refractive index or thickness (or both). This diffracted light, lagging behind by approximately 1/4 wavelength, arrives at the image plane out of step (also termed out of phase) with the undeviated light but, in interference, essentially undiminished in intensity. The result is that the image at the eyepiece level is so lacking in contrast as to make the details almost invisible.

Zernike succeeded in devising a method—now known as Phase Contrast microscopy—for making unstained, phase objects yield contrast images as if they were amplitude objects. Amplitude objects show excellent contrast when the diffracted and direct light are out of step (display a phase difference) by 1/2 of a wavelength. Zernike's method was to speed up the direct light by 1/4 wavelength so that the difference in wavelength between the direct and deviated light for a phase specimen would now be 1/2 wavelength. As a result, the direct and diffracted

light arriving at the image level of the eyepiece would be able to produce *destructive interference* (see the section on image formation for absorbing objects previously described). Such a procedure results in the details of the image appearing darker against a lighter background. This is called dark or positive phase contrast. A schematic illustration of the basic phase contrast microscope configuration is illustrated in Figure 2.11.

Another possible course, much less often used, is to arrange to slow down the direct light by 1/4 wavelength so that the diffracted light and the direct light arrive at the eyepiece in step and can interfere constructively. This arrangement results in a bright image of the details of the specimen on a darker background, and is called negative or bright contrast.

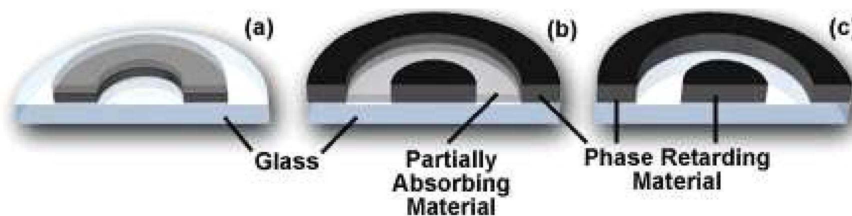

Fig. 2.11 *Annular Phase Plates*

Phase contrast microscopy was very successful and ultimately gained widespread application, resulting in Zernike's award of the prestigeous Nobel prize in physics in 1953. The phase contrast technique has hailed as the greatest advance in microscopy in a century. Phase contrast, by 'converting' phase specimens such as living material into amplitude specimens, allowed scientists to see details in unstained and/or living objects with a clarity and resolution never before achieved.

The Zernike method involves the separation of the direct zeroth order light from the diffracted light at the rear focal plane of the objective. To do this, a ring annulus is placed in position directly under the lower lens of the condenser at the front focal plane of the condenser, conjugate to the objective rear focal plane. As the hollow cone of light from the annulus passes through the specimen undeviated, it arrives at the rear focal plane of the objective in the shape of a ring of light. The fainter light diffracted by the specimen is spread over the entire rear focal plane of the objective.

If this combination were allowed, as is, to proceed to the image plane of the eyepiece, the diffracted light would be approximately 1/4 wavelength behind the direct light. At the image plane, the phase of the diffracted light would be out of phase with the direct light, but the amplitude of their interference would be almost the same as that of the direct light. This would result in very little specimen contrast.

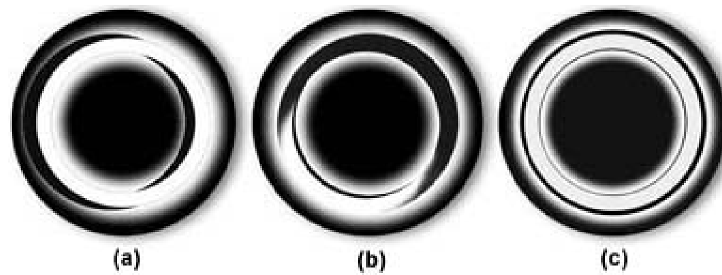

Fig. 2.12 *Phase Plate and Light Annulus Alignment*

To speed up the direct undeviated zeroth order light, a phase plate is installed with a ring shaped phase shifter attached to it at the rear focal plane of the objective. The narrow area of the phase plate is optically thinner than the rest of the plate. As a result, undeviated light passing through the phase ring travels a shorter distance in traversing the glass of the objective than does the diffracted light.

Now, when the direct undeviated light and the diffracted light proceed to the image plane, they are 1/2 wavelength out of phase with each other. The diffracted and direct light can now interfere destructively so that the details of the specimen appear dark against a lighter background (just as they do for an absorbing or amplitude specimen). This is a description of what takes place in positive or dark phase contrast.

If the ring phase-shifter area of the phase plate were to be made thicker than the rest of the plate, direct light is slowed by 1/4 wavelength. In this case, the zeroth order light arrives at the image plane in step (or in phase) with the diffracted light, and constructive interference takes place. The image would appear bright on a darker background. The image appears bright on a darker background. This type of phase contrast is described as negative or bright contrast.

Because the undeviated light of the zeroth order is much brighter than the faint diffracted light, a thin absorptive transparent metallic layer is deposited on the ring to bring the direct and diffracted light into better balance of intensity in order to increase contrast. Also, because the speeding up or slowing down of the direct light is calculated on a 1/4 wavelength of green light, the phase image will appear best when a green filter is placed in the light path (a green interference filter is preferable). Such a green filter also helps achromatic objectives produce their best images, since achromats are spherically corrected for green light.

The accessories needed for phase contrast work are a sub stage phase contrast condenser equipped with annuli and a set of phase contrast objectives, each of which has a phase plate installed. The condenser usually has a brightfield position with an aperture diaphragm and a rotating turret of annuli (each phase objective of different magnification requires an annulus of increasing diameter as the magnification of the objective increases). Each phase objective has a darkened ring on its back lens. Such objectives can also be used for ordinary brightfield transmitted light work with only a slight reduction in image quality.

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The phase outfit, as supplied by the manufacturer, usually includes a green filter and a phase telescope. The latter is used to enable the microscopist to alight the condenser annulus to superimpose it onto the ring of the phase plate. A set of centering screws in the sub stage condenser allows manipulation of the annulus to align it while observing the back focal plane of the objective with the phase telescope (or through a Bertrand lens).

To set up a phase microscope (cheek lining cells are a readily available test material), focus the specimen with the 10X phase objective. Next, configure the microscope for Köhler illumination using the brightfield (0) position of the condenser. This critical step is to assure the proper alignment of the microscope's objective, condenser, and field diaphragm. After the microscope is properly aligned, open up the condenser aperture diaphragm and swing the turret of the condenser into the 10 position (this usually automatically opens the aperture diaphragm). Place the green filter in the light path, and remove one of the eyepieces. Insert the phase telescope and, while observing the back of the objective, use the Annulus Centering Screws to center the annulus to the ring of the plate. A Bertrand lens or a pinhole eyepiece, if available, will allow a view of the back focal plane of the objective. Centering the phase annulus is often easier to do if the specimen is temporarily removed from the light path. After alignment of the phase ring with the annulus, reinsert the eyepiece and place the specimen back into its proper place on the microscope stage in the optical path.

Repeat the same procedure for each objective, making sure that the turret is rotated so the appropriate phase annulus is positioned to match the objective magnification. Some manufacturers provide individual push-in, centering, annuli that can be inserted into the lower part of the common Abbe condenser. Such inexpensive, simple devices do well with the 10X, 20X, and 40X phase objectives, but the condenser can receive only one at a time.

Phase microscopy continues to be a widely used and important tool, particularly for the microscopist studying living and/or unstained material, such as cells and tissues in culture. The method is also currently used simultaneously with reflected light fluorescence to reveal areas of a specimen that do not fluoresce. Phase microscopy techniques are particularly useful with specimens that are thin and scattered in the field of view.

There are some limitations of phase contrast microscopy:

- Phase images are usually surrounded by halos around the outlines of details. Such halos are optical artifacts, which sometimes obscure the boundaries of details.
- The phase annuli do limit the working numerical aperture of the optical system to a certain degree, thus reducing resolution.
- Phase contrast does not work well with thick specimens because of shifts in phase occur from areas slightly below or slightly above the plane that is in focus. Such phase shifts confuse the image and distort image detail.

- Phase images appear gray if white light is used and green if a green filter is used. In the past, many microscopists restricted their film to black and white when performing photomicrography on phase specimens. Today, many color films reproduce black, white, and grayscales very effectively, especially the tungsten-balanced transparency films from Fuji, Kodak, and Agfa.

Phase microscopy is another exemplification of how the manipulation of light at the sub stage condenser lower lens level and at the objective rear focal plane level has significant effect upon the image that is observed through the eyepiece.

2.5 PHOTOMIROGRAPHY- DIGITAL IMAGING

The use of photography is to capture images in a microscope dates back to the invention of the photographic process. Early photomicrographs were remarkable for their quality, but the techniques were laborious and burdened with long exposures and a difficult process for developing emulsion plates. The primary medium for photomicrography was film until the past decade when improvements in electronic camera and computer technology made digital imaging cheaper and easier to use than conventional photography. This section will address photomicrography both on film and with electronic analog and digital imaging systems.

The quality of a photomicrograph, either digital or recorded on film, depends upon the quality of the microscopy. Film is a stern judge of how good the microscopy has been prior to capturing the image. It is essential that the microscope be configured using Köhler illumination, and that the field and condenser diaphragms are adjusted correctly and the condenser height is optimized. When properly adjusted, the microscope will yield images that have even illumination over the entire field of view and display the best compromise of contrast and resolution.

Almost all microscopists will, at some point, have the need or desire to record the images seen through the microscope. The main mechanism, for many years, of producing such photomicrographs was through the use of film, although in recent years most scientists have begun to capture images by means of electronic cameras. The main purpose of this tutorial is to enable the microscopist to record the observed images on film or digital media, and to do so with accuracy of image reproduction and with fidelity of color when color film is being used. The further aim is to empower the photomicrographer to secure excellent pictures without having to struggle through the already existing, far more complex reference literature.

The Digital Image Sensor

Whilst it is not essential to have an understanding of how digital cameras work, a basic knowledge can be beneficial in making informed decisions regarding selection

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of a camera, and also in understanding why some post-capture image processing is necessary in photomicrography. For a fuller treatment on the digital image process, the reader is referred to Michael Freeman's book as one of the clearest introductions to the subject.

At the heart of digital imaging is the image sensor. This consists of an array of light-sensitive receptors, embedded into a microchip containing the wiring and circuitry necessary to record light levels captured from each receptor. The receptors, termed pixels (an abbreviation of picture elements) generally consist of photodiodes embedded in a well. The photodiodes convert photons of light striking the sensor into electrons in a proportional relationship (the more photons striking, the more electrons generated). The charge generated is measured by the microchip circuitry, converted to a digital signal and processed by in-camera software. The photodiodes are only responsive to the intensity of light, and not its colour. Colour information is introduced into the digital signal in one of two ways. In conventional digital cameras, a transparent filter mask (termed a Bayer mask, after its inventor) is located immediately in front of the sensor; this mask has a matrix of red, green and blue filters, with one colour located above each individual pixel.

Digital imaging has made the recording of high-quality photomicrographs accessible to all at low cost. Cameras have now evolved to a stage where virtually any digital camera will produce an image suitable for printing at A3 size or greater, and at high resolution. A range of software is now available to the amateur that enables images to be generated that previously were not possible, and all at relatively low cost. Whilst the field continues to develop rapidly, the 'pixel-war' of ever-increasing sensor resolution has probably reached its peak. However, new software will continue to be developed that further increases the possibilities for digital imaging with the microscope.

Each pixel therefore records light intensity, and its location under the Bayer mask is used by camera software to determine the colour of light at that position in the image. The Bayer mask is generally not an even distribution of red, green and blue filters; green locations are predominant to produce an image that more closely approximates human vision and 'typical' subjects such as landscapes. In dedicated photomicrographic cameras, a colour filter wheel is often provided in front of the sensor. Three separate images are recorded with the red, green and blue filters rotated in place in succession; the three images are then combined to produce the final colour image. This provides better colour fidelity, but cannot be used for motile or dynamic subjects.

There are a number of different sensor types. The first generally available, and best known, is the Charge Coupled Device (CCD). The CCD is still the most common type of sensor in professional photomicrographic cameras, but has largely been replaced in consumer cameras by the Complementary Metal Oxide Sensor (CMOS). The CMOS sensor is a greater range of consumer devices; mass production means that the CMOS sensor is significantly cheaper than the equivalent

CCD sensor. Although the CMOS sensor requires more processing by the camera software to reduce noise in the image, the image quality from the current generation of CMOS sensors cannot be distinguished from images created by CCD sensors.

NOTES

Check Your Progress

12. What does photomicrography mean?
13. Define digital imaging.
14. What are the factors on which the quality of photomicrograph depend?
15. Name the different types of sensors.

2.6 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. There are four main components to a transmission electron microscope (TEM): an electron optical column, a vacuum system, the necessary electronics (lens supplies for focusing and deflecting the beam and the high voltage generator for the electron source), and control software.
2. A modern TEM typically comprises an operating console surmounted by a vertical column and containing the vacuum system, and control panels conveniently placed for the operator.
3. The power (focal length) of magnetic lenses can be changed by changing the current through the lens coil.
4. The electron beam emerges from the electron gun, and passes through a thin specimen, transmitting electrons which are collected, focused, and projected onto the viewing device at the bottom of the column.
5. The secondary electron (SE) signal is the most frequently used signal. It varies with the topography of the sample surface much like an aerial photograph: edges are bright, recesses are dark.
6. The basic concept of confocal microscopy was originally developed by Marvin Minsky in the mid-1950s (patented in 1957) when he was a postdoctoral student at Harvard University.
7. After each single scan along the x axis, the beam is rapidly transported back to the starting point and shifted along the **y** axis to begin a new scan in a process termed flyback.
8. Return of fluorescence emission through the galvanometer mirror system is referred to as descanning.
9. Fluorescence emission that is passed through the pinhole aperture is converted into an analog electrical signal having a continuously varying voltage (corresponding to intensity) by the photomultiplier.

10. Another important feature of the multiple-beam microscopes is their ability to readily capture images with an array detector, such as a charge-coupled device (CCD) camera system.
11. Image information is gathered point by point with a specialized detector such as a photomultiplier tube or avalanche photodiode, and then digitized for processing by the host computer, which also controls the scanning mirrors and/or other devices to facilitate the collection and display of images.
12. Photomicrography, photography of objects under a microscope. Such opaque objects as metal and stone may be ground smooth, etched chemically to show their structure, and photographed by reflected light with a metallurgical microscope.
13. Digital imaging or digital image acquisition is the creation of a digitally encoded representation of the visual characteristics of an object, such as a physical scene or the interior structure of an object. The term is often assumed to imply or include the processing, compression, storage, printing, and display of such images. A key advantage of a digital image, versus an analog imagesuch as a film photograph, is the ability make copies and copies of copies digitally indefinitely without any loss of image quality.
14. The quality of a photomicrograph, either digital or recorded on film, depends upon the quality of the microscopy. Film is a stern judge of how good the microscopy has been prior to capturing the image. It is essential that the microscope be configured using Köhler illumination, and that the field and condenser diaphragms are adjusted correctly and the condenser height is optimized. When properly adjusted, the microscope will yield images that have even illumination over the entire field of view and display the best compromise of contrast and resolution.
15. The different sensor types are
    - Charge Coupled Device (CCD)
    - Complementary Metal Oxide Sensor (CMOS)

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2.6 SUMMARY

- There are four main components to a Transmission Electron Microscope (TEM): an electron optical column, a vacuum system, the necessary electronics (lens supplies for focusing and deflecting the beam and the high voltage generator for the electron source), and control software.
- A modern TEM typically comprises an operating console surmounted by a vertical column and containing the vacuum system, and control panels conveniently placed for the operator.
- The microscope may be fully enclosed to reduce interference from environmental sources, and operated remotely.

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- The electron column includes elements analogous to those of a light microscope.
- A Scanning Electron Microscope (SEM), like a transmission electron microscope, consists of an electron optical column, a vacuum system, electronics, and software.
- The Secondary Electron (SE) signal is the most frequently used signal. It varies with the topography of the sample surface much like an aerial photograph: edges are bright, recesses are dark.
- Confocal microscopy offers several advantages over conventional widefield optical microscopy, including the ability to control depth of field, elimination or reduction of background information away from the focal plane (that leads to image degradation), and the capability to collect serial optical sections from thick specimens.
- The basic key to the confocal approach is the use of spatial filtering techniques to eliminate out-of-focus light or glare in specimens whose thickness exceeds the immediate plane of focus.
- The basic concept of confocal microscopy was originally developed by Marvin Minsky in the mid-1950s (patented in 1957) when he was a postdoctoral student at Harvard University.
- Minsky's invention remained largely unnoticed, due most probably to the lack of intense light sources necessary for imaging and the computer horsepower required to handle large amounts of data.
- Following Minsky's work, M. David Egger and Mojmir Petran fabricated a multiple-beam confocal microscope in the late 1960s that utilized a spinning (Nipkow) disk for examining unstained brain sections and ganglion cells.
- Continuing in this arena, Egger went on to develop the first mechanically scanned confocal laser microscope, and published the first recognizable images of cells in 1973.
- During the late 1970s and the 1980s, advances in computer and laser technology, coupled to new algorithms for digital manipulation of images, led to a growing interest in confocal microscopy.
- Basic microscope optical system characteristics have remained fundamentally unchanged for many decades due to engineering restrictions on objective design, the static properties of most specimens, and the fact that resolution is governed by the wavelength of light.
- The explosive growth and development of the confocal approach is a direct result of a renaissance in optical microscopy that has been largely fueled by advances in modern optical and electronics technology.
- The use of photography to capture images in a microscope dates back to the invention of the photographic process.

- Early photomicrographs were remarkable for their quality, but the techniques were laborious and burdened with long exposures and a difficult process for developing emulsion plates.
- The primary medium for photomicrography was film until the past decade when improvements in electronic camera and computer technology made digital imaging cheaper and easier to use than conventional photography.

2.7 KEY WORDS

- **Photomicrograph:** A light micrograph or photomicrograph is a micrograph prepared using an optical microscope, a process referred to as photomicroscopy.
- **Backscatter:** Backscatter or backscattering is the reflection of waves, particles, or signals back to the direction from which they came. It is a diffuse reflection due to scattering, as opposed to specular reflection as from a mirror.
- **Fluorescein:** Fluorescein is a fluorophore commonly used in microscopy, in a type of dye laser as the gain medium, in forensics and serology to detect latent blood stains, and in dye tracing.
- **Autofluorescence:** Autofluorescence is the natural emission of light by biological structures.
- **Photomultiplier:** Photomultiplier tubes or PMTs for short are the members of the class of vacuum tubes, and more specifically vacuum phototubes, are extremely sensitive detectors of light in the ultraviolet, visible, and near-infrared ranges of the electromagnetic spectrum.
- **Flyback:** A flyback transformer (FBT), also called a line output transformer (LOPT), is a special type of electrical transformer and was initially designed to generate high voltage sawtooth signals at a relatively high frequency.
- **Microlens:** A microlens is a small lens, generally with a diameter less than a millimetre (mm) and often as small as 10 micrometres.
- **Photodiode:** A photodiode is a semiconductor device that converts light into an electrical current. The current is generated when photons are absorbed in the photodiode.
- **Photomicrography:** Photomicrography refers to the study of, photography of objects under a microscope.
- **Digital imaging:** Digital imaging or digital image acquisition is the creation of a digitally encoded representation of the visual characteristics of an object, such as a physical scene or the interior structure of an object.

2.8 SELF ASSESSMENT QUESTIONS AND EXERCISES

NOTES

Short Answer Questions

1. What is transmission and scanning of electron microscope?
2. What is phase contrast microscopy?
3. Write a short note on confocal microscopy.
4. What is photomicrography?
5. Write a short note on digital imaging.

Long Answer Questions

1. Give a detailed overview on scanning of an electron microscope.
2. Discuss about confocal microscopy and its advantages and disadvantages in detail.
3. Explain about phase contrast microscopy.
4. Discuss about photomicrography explaining about it in detail.
5. Write about digital imaging and explain all the key points in detail.

2.9 FURTHER READINGS

Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

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UNIT 3 MICROSCOPIC MEASUREMENTS

NOTES

Structure

3.0 Introduction
3.1 Objectives
3.2 Micrometers
3.2.1 Stage Micrometer
3.2.2 Ocular Meter
3.3 Haemocytometer and Camera Lucida
3.4 Answers to Check Your Progress Questions
3.5 Summary
3.6 Key Words
3.6 Self Assessment Question and Exercises
3.7 Further Readings

3.0 INTRODUCTION

When we measure everyday items most of us grab a ruler in order to make a measurement. When using a microscope, generally magnification is higher and a ruler will not work unless you are using a very low power stereo microscope. When using a compound high power microscope an eyepiece reticle is used to make measurements. Most measurements that are made with a compound microscope are between 0.2µm to 25mm.

Micrometer is a unit of measure for the core in optical fiber, for which the most common diameter is 62.5 micrometers. It is also used to measure the line width on a microchip. Micrometry is the science in which we have some measurement of the dimensions of an object being observed under the microscope. The method employs some special types of measuring devices which are so oriented that these can well be attached to or put into the microscope and observed.

In this unit, you will study about microscopic measurements, mircometers, ocular, stage, haemocytometer and camera lucida in detail.

3.1 OBJECTIVES

After going through this unit you will be able to:

- Understand about microscopic measurements
- Discuss about mircometers
- Explain what ocular, stage, haemocytometer and camera lucida is

3.2 MICROMETERS

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Micrometer is a unit of measure for the core in optical fiber, for which the most common diameter is 62.5 micrometers. It is also used to measure the line width on a microchip. Micrometry is the science in which we have some measurement of the dimensions of an object being observed under the microscope. The method employs some special types of measuring devices which are so oriented that these can well be attached to or put into the microscope and observed. The object, to be measured, is calibrated against these scales.

Once we are observing an object under a microscope by the 5X objective and the 10X eyepiece we say that the image that we are able to perceive is $5 \times 10 = 50$ times of the object.

We get the magnified view no doubt and also that it is the perfect coordination of the dimensions, but to find out the exact size of the object will need precision and that is achieved through the application of some small scales called micrometers.

Types of Micrometer

There are usually two types of micrometers

- Stage micrometer (Refer Figure 3.1)
- Ocular meter or ocular micrometer (Refer Figure 3.2)

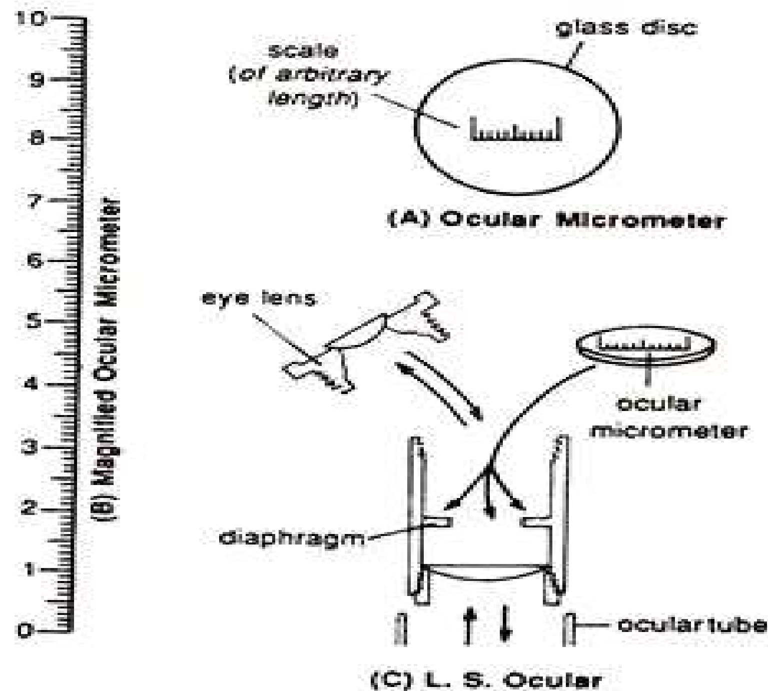

Fig. 3.1 Stage Micrometer

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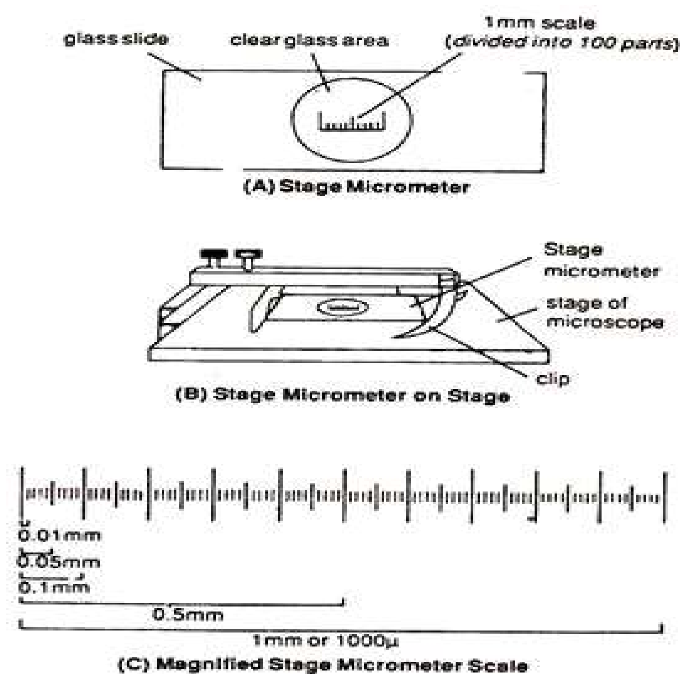

Fig. 3.2 *Ocular Meter or Ocular Micrometer*

3.2.1 Stage Micrometer

Stage Micrometer- a microscope slide (generally 1" x 3") that has a ruler etched on it. It is either made of glass (for transmitted light) or metal (for reflected light). The stage micrometer is used to calibrate an eyepiece reticle when making measurements with a microscope.

As is clear from its name it is for the measurement on the stage of the microscope where an object is to be kept. This micrometer is of a slide's shape and size and has a mount of very finely graduated scale. The scale measures only 1 mm and has a least count of 0.01 mm, i.e., 1 mm region is divided into 100 divisions. As 1 mm has 1000μ, one division of stage micrometer is equivalent to 10μ.

3.2.2 Ocular Meter

This micrometer is used inside the eyepiece. The upper eye lens is unscrewed and the ocular meter is put into the tube of eyepiece, and the eye lens is again replaced in its original position. There are usually 50 or 100 divisions in the ocular meter which are engraved on the glass.

Experiment No. 1

Object

To measure the dimensions of common microorganisms by calibration and standardization of microscope using stage micrometer and ocular micrometer.

Requirements

Microscope, stage micrometer, ocular meter, slide of the microorganism to be measured.

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Procedure

To work out the measurements per ocular divisions the stage micrometer is kept under low power of microscope and is observed through the eyepiece having ocular meter. Suppose we have 10X objective and 5X eyepiece fitted in the microscope with a tube of 170 mm length.

At this magnification the number of ocular divisions coinciding the stage micrometer are observed (Refer Figure 3.3) and thence calculated for microns per ocular divisions, for example, let us take that 5 ocular divisions coincide with 7 divisions of stage micrometer.

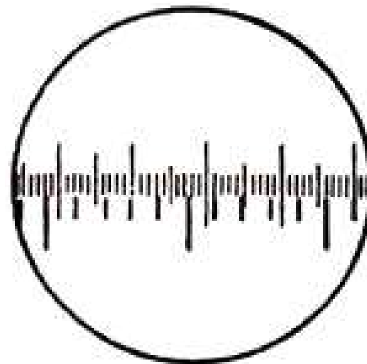

Fig. 3.3 *Scales of Stages and Ocular Micrometers Super-imposed*

Therefore, 5 ocular divisions = 7 stage micrometer divisions, or 5 ocular divisions = 0.07 mm (since 1 division = 0.01 mm).

1 ocular division = $0.07/5\text{mm} = 0.07 \times 1000/5\mu$

$= 70/5\mu = 14\mu$

Or, it can also be calculated by following formula:

One division of ocular = Number of stage micrometer divisions/Number of ocular meter divisions × 10

In the case mentioned above it will be

$= 7 \times 10/5\mu = 14\mu$.

In this way the microscope is calibrated for different combinations of eyepieces and objective lenses and is kept for record. It is to note that this calibration will be just only of the tried lenses on this particular microscope.

Take three readings in this way, and the mean value of these readings will be the actual value of one part of ocular meter.

NOTES

Record your data in the following table:

Observations :

S. No.	Number of divisions of stage micrometer	Number of divisions of ocular meter	Value of one division of oculometer $= \frac{stage}{ocular} \times 10\ \mu$	Mean/ Result
1.	7	5	$= \frac{7}{5} \times 10 = 14\mu$	
2.				
3.				
4.				

Measurement of the Microorganisms

When the microscope is calibrated, then the object or organism to be measured is kept on the sage of the microscope and is observed through the eyepiece with ocular. The object is measured in the particular magnification by ocular divisions and then is changed into microns by multiplying ocular divisions with calibrated value of one ocular division in that particular magnification.

Suppose the diameter of an object (Refer Figure 3.4) is observed to be equal to 6 divisions of ocular, so the diameter of this object in microns will be

$6 \times 14\mu = 84\mu$

In this way the object is measured in different magnifications.

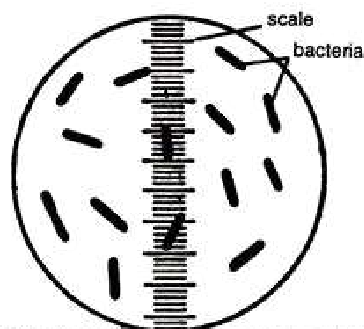

Fig. 3.4 *Diagram Showing an Ocular Micrometer for measuring the Dimensions of Bacterial Cells*

Check Your Progress

1. Define the term micrometer.
2. What is micrometry?
3. How many types of micrometer are there? Name them.
4. What is ocular meter?
5. Define stage micrometer.

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3.3 HAEMOCYTOMETER AND CAMERA LUCIDA

Haemocytometer

The simplest, most convenient and cheapest means of accurately determining the numbers of cells in a sample is to use a Haemocytometer and a microscope. A Haemocytometer is a specialised slide that has a counting chamber with a known volume of liquid.

The Haemocytometer consists of a heavy glass slide with two counting chambers, each of which is divided into nine large 1 mm squares, on an etched and silvered surface separated by a trough.

A coverslip sits on top of the raised supports of the 'H' shaped toughs enclosing both chambers. There is a 'V' or notch at either end where the cell suspension is loaded into the Haemocytometer. When loaded with the cell suspension it contains a defined volume of liquid.

The engraved grid on the surface of the counting chamber ensures that the number of particles in a defined volume of liquid is counted.

Camera Lucida

For an exact drawing of the image a camera Lucida, a drawing prism or a drawing head (Refer Figure 3.4) can be used. All the three instruments have the same working principle. The drawing prism is most commonly used. It has a prism which can reflect the image downwards at 45° angle.

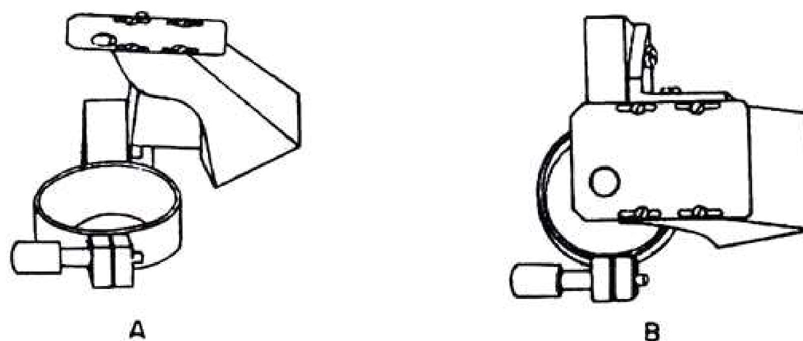

Fig. 3.4 *Drawing Prism - A. Side View, B. Top View*

The object is properly focused under the microscope, using the high power or oil lens — as is suitable. The eye-piece is removed and the hollow ring of the drawing prism is slid into the draw tube of the microscope. The eye-piece is replaced, the prism is brought over the eye-piece and is adjusted in such a way that it remains horizontal and just touches the eye-piece.

The hollow ring is now tightened with the metallic screw so that the prism does not move. The left eye is now placed over the eye-piece and the drawing paper on the table near the right hand side of the microscope. The viewer can see

the image on the paper. A sharply pointed pencil held with the right hand is held over the paper.

Now both the image and the pencil can be simultaneously seen and an exact drawing is made. To have a distinct image it is necessary to adjust the light. The microscope light can be regulated by adjusting the level of the condenser and by adjusting its aperture. Light falling on the drawing paper can be regulated by placing an opaque body in front of it.

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A microscope with a built-in light system is quite handy for this type of camera-Lucida drawing. A drawing head is more handy. It is simply placed over the eye-piece and the drawing is made. The camera Lucida is the oldest of the three models. It has a mirror fitted at the end of a handle which can be manually adjusted to reflect the image.

Magnification of the Camera Lucida Drawing

A camera Lucida drawing becomes more magnified than the image seen directly by the eye because of angular deviation. As already stated, the camera Lucida image is reflected downward at 45° angle and this deviation further magnifies the image.

To find out the exact magnification, the slide is removed and a stage micrometer is placed under the microscope using the same combination of lenses. A stage micrometer has a scale where one mm is divided into 100 equal segments, so that one stage division is equal to 10 μ.

The stage micrometer is focused at the center of the field of observation and a few divisions are drawn with the help of the drawing prism. To get a more accurate result, the stage micrometer should be focused at the centre, at the left side and at the right side of the field of observation and three drawings should be made, because magnification varies according to the position of the stage micrometer.

It is greatest at the left end lowest at the right end of the field of observation. These divisions are now measured by a mm scale and the mean magnification of a stage micrometer division is found out by simple arithmetic.

Illustration

1 stage micrometer division is magnified to 5 mm

i.e., 10 μ is magnified to 5 × 1,000 = 5,000 μ

1 μ is magnified 5,000/10 = 500 μ

Magnification is 500 times.

Measurements

For measuring the actual size of a cell or a microscopic plant organ a stage micrometer and an ocular scale or micrometer are necessary. The first step is standardization of the ocular micrometer.

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The ocular scale is placed within the eye-piece and the stage micrometer on the stage. The two scales are so adjusted that they lie one above the other in a parallel manner. A number of readings are now taken indicating how many ocular divisions coincide with how many stage divisions.

The mean is then found out and calculations are made in the following manner

Suppose

10 ocular divisions coincide with 25 stage divisions

1 ocular division = 2.5 stage divisions

$= 2.5 \times 10\ \mu$ (1 stage div. $= 10\ \mu$)

$= 25\mu$

Now the stage micrometer is removed and the object is placed there. The object is measured by the ocular scale and the actual size is calculated in terms of micron according to the standardize value of the ocular scale.

Example

Suppose the mean length and breadth of a cell are 4.2 and 2.5 ocular divisions.

- The length of a cell $= 4.2 \times 25\ \mu = 105\ \mu$
- And the breadth of a cell $= 2.5 \times 25\ \mu = 62.5\ \mu$.
- The size of a cell is $105\ \mu \times 62.5\ \mu$

Check Your Progress

6. Define the term hemocytometer.
7. Who invented hemocytometer?
8. What is lucida?
9. What is the composition of hemocytometer?

3.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Micrometer is a unit of measure for the core in optical fiber, for which the most common diameter is 62.5 micrometers.
2. Micrometry is the science in which we have some measurement of the dimensions of an object being observed under the microscope.
3. There are usually two types of micrometers
   - Stage micrometer
   - Ocular meter or ocular micrometer

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4. An ocular micrometer is a glass disk that fits in a microscope eyepiece that has a ruled scale, which is used to measure the size of magnified objects. The physical length of the marks on the scale depends on the degree of magnification.
5. A Stage Micrometer is simply a microscope slide with a finely divided scale marked on the surface. The scale is of a known true length and is used for the calibration of optical systems with eyepiece graticule patterns.
6. The hemocytometer or haemocytometer is a counting-chamber device originally designed and usually used for counting blood cells.
7. The hemocytometer was invented by Louis-Charles Malassez and consists of a thick glass microscope slide with a rectangular indentation that creates a chamber.
8. Lucida is an extended family of related typefaces designed by Charles Bigelow and Kris Holmes and released from 1984 onwards. The family is intended to be extremely legible when printed at small size or displayed on a low-resolution display.
9. The Haemocytometer consists of a heavy glass slide with two counting chambers, each of which is divided into nine large 1 mm squares, on an etched and silvered surface separated by a trough.

3.5 SUMMARY

- Micrometer is a unit of measure for the core in optical fiber, for which the most common diameter is 62.5 micrometers.
- It is also used to measure the line width on a microchip. Micrometry is the science in which we have some measurement of the dimensions of an object being observed under the microscope.
- The method employs some special types of measuring devices which are so oriented that these can well be attached to or put into the microscope and observed. The object, to be measured, is calibrated against these scales.
- Stage Micrometer -a microscope slide (generally 1" x 3") that has a ruler etched on it. It is either made of glass (for transmitted light) or metal (for reflected light). The stage micrometer is used to calibrate an eyepiece reticle when making measurements with a microscope.
- As is clear from its name it is for the measurement on the stage of the microscope where an object is to be kept. This micrometer is of a slide's shape and size and has a mount of very finely graduated scale.
- The scale measures only 1 mm and has a least count of 0.01 mm, i.e. 1 mm region is divided into 100 divisions. As 1 mm has 1000μ, one division of stage micrometer is equivalent to 10μ.

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- This micrometer is used inside the eyepiece. The upper eye lens is unscrewed and the ocular meter is put into the tube of eyepiece, and the eye lens is again replaced in its original position.
- There are usually 50 or 100 divisions in the ocular meter which are engraved on the glass.
- When the microscope is calibrated, then the object or organism to be measured is kept on the sage of the microscope and is observed through the eyepiece with ocular.
- The object is measured in the particular magnification by ocular divisions and then is changed into microns by multiplying ocular divisions with calibrated value of one ocular division in that particular magnification.
- A camera Lucida drawing becomes more magnified than the image seen directly by the eye because of angular deviation. As already stated, the camera Lucida image is reflected downward at 45° angle and this deviation further magnifies the image.
- The simplest, most convenient and cheapest means of accurately determining the numbers of cells in a sample is to use a Haemocytometer and a microscope. A Haemocytometer is a specialised slide that has a counting chamber with a known volume of liquid.
- The Haemocytometer consists of a heavy glass slide with two counting chambers, each of which is divided into nine large 1 mm squares, on an etched and silvered surface separated by a trough.
- For measuring the actual size of a cell or a microscopic plant organ a stage micrometer and an ocular scale or micrometer are necessary. The first step is standardization of the ocular micrometer.
- The ocular scale is placed within the eye-piece and the stage micrometer on the stage. The two scales are so adjusted that they lie one above the other in a parallel manner. A number of readings are now taken indicating how many ocular divisions coincide with how many stage divisions.

3.6 KEY WORDS

- **Hemocytometer:** The hemocytometer or haemocytometer is a counting-chamber device originally designed and usually used for counting blood cells.
- **Lucida:** Lucida is an extended family of related typefaces designed by Charles Bigelow and Kris Holmes and released from 1984 onwards. The family is intended to be extremely legible when printed at small size or displayed on a low-resolution display.
- **Ocular meter:** An ocular micrometer is a glass disk that fits in a microscope eyepiece that has a ruled scale, which is used to measure the size of magnified

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objects. The physical length of the marks on the scale depends on the degree of magnification.

- **Stage micrometer:** A Stage Micrometer is simply a microscope slide with a finely divided scale marked on the surface. The scale is of a known true length and is used for the calibration of optical systems with eyepiece graticule patterns.
- **Coverslip:** Coverslip a very thin piece of glass placed over a specimen on a glass slide that is to be examined under a microscope.
- **Camera lucida:** A camera lucida is an optical device that allows you to see what you wanted to paint or draw as if reflected on your piece of paper.

3.6 SELF ASSESSMENT QUESTION AND EXERCISES

Short Answer Questions

1. Brief about microscopic measurements.
2. What are micrometers?
3. Give a key difference between stage micrometer and ocular micrometer.
4. What is camera lucida?
5. Write briefly about hemocytometer.

Long Answer Questions

1. Write about micrometers and its types in detail.
2. Discuss about a experiment conducted to measure the dimensions of common microorganisms by calibration and standardization of microscope using stage micrometer and ocular micrometer.
3. Explain about haemocytometer.
4. Discuss about camera lucida also the magnification of the camera lucida drawing.

3.7 FURTHER READINGS

Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

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BLOCK - II
MICROTOMY

NOTES

UNIT 4 MICROTOMES

Structure

4.0 Introduction
4.1 Objectives
4.2 Microtomes
  4.2.1 Cambridge Rocking Microtome
  4.2.2 Rotary Microtome
  4.2.3 Sledge Microtome
  4.2.4 Ultramicrotome
4.3 Answers to Check Your Progress Questions
4.4 Summary
4.5 Key Words
4.6 Self Assessment Question and Exercises
4.7 Further Readings

4.0 INTRODUCTION

Microtome is a mechanical instrument used to cut biological specimens into very thin segments for microscopic examination. Biological specimens can be presented in many ways. But more often, these specimens are embedded in paraffin wax blocks and the commonest way of sectioning these specimens can be achieved by the microtome. The earliest form of the microtome enabled free hand sectioning of fresh or fixed material using a sharp razor. Modern microtomes are precision instruments designed to cut uniformly thin sections of a variety of materials for detailed microscopic examination. Central to the production of good sections is the microtome knife. Microtomy virtually begins and ends with a sharp, blemish-free cutting edge. The introduction of disposable blades has made easier the production of good quality, thin sections, but they are often unsatisfactory for sectioning harder tissues, especially bone. A sharp knife edge free from imperfections is essential for the production of good sections. Since many types of microtomes are commercially available in the market, choosing the right microtome is essential for producing the best result as required. A classification is proposed that unifies and organizes the various microtomes based on the mode of operation.

In this unit, you will study about microtomes and its related terms like rocking, rotary, sledge and ultra microtomes and their uses.

4.1 OBJECTIVES

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After going through this unit, you will be able to:

- Understand what microtomes is
- Discuss about different terms related to microtomes like rocking, rotary, sledge and ultra microtomes
- Analyse about the uses and advantages of microtomes

4.2 MICROTOMES

A microtome (from the Greek mikros, meaning 'small', and temnein, meaning 'to cut') is a tool used to cut extremely thin slices of material, known as sections. Important in science, microtomes are used in microscopy, allowing for the preparation of samples for observation under transmitted light or electron radiation. Microtomes use steel, glass, or diamond blades depending upon the specimen being sliced and the desired thickness of the sections being cut. Steel blades are used to prepare sections of animal or plant tissues for light microscopy histology. Glass knives are used to slice sections for light microscopy and to slice very thin sections for electron microscopy. Industrial grade diamond knives are used to slice hard materials such as bone, teeth and plant matter for both light microscopy and for electron microscopy. Gem quality diamond knives are used for slicing thin sections for electron microscopy.

Microtomy is a method for the preparation of thin sections for materials such as bones, minerals and teeth, and an alternative to electropolishing and ion milling. Microtome sections can be made thin enough to section a human hair across its breadth, with section thickness between 50 nm and 100 μm.

History

In the beginnings of light microscope development, sections from plants and animals were manually prepared using razor blades. It was found that to observe the structure of the specimen under observation it was important to make clean reproducible cuts on the order of 100 μm, through which light can be transmitted. This allowed for the observation of samples using light microscopes in a transmission mode.

One of the first devices for the preparation of such cuts was invented in 1770 by George Adams, Jr. (1750–1795) and further developed by Alexander Cummings. The device was hand operated, and the sample held in a cylinder and sections created from the top of the sample using a hand crank.

In 1835, Andrew Prichard developed a table based model which allowed for the vibration to be isolated by affixing the device to the table, separating the operator from the knife.

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Occasionally, attribution for the invention of the microtome is given to the anatomist Wilhelm His, Sr. (1865), In his Beschreibung eines Mikrotoms (German for Description of a Microtome), Wilhelm wrote: The apparatus has enabled a precision in work by which I can achieve sections that by hand I cannot possibly create. Namely it has enabled the possibility of achieving unbroken sections of objects in the course of research.

Other sources further attribute the development to a Czech physiologist Jan Evangelista Purkynì. Several sources describe the Purkyne model as the first in practical use.

The obscurities in the origins of the microtome are due to the fact that the first microtomes were simply cutting apparatuses, and the developmental phase of early devices is widely undocumented.

At the end of the 1800s, the development of very thin and consistently thin samples by microtomy, together with the selective staining of important cell components or molecules allowed for the visualisation of microscope details.

Today, the majority of microtomes are a knife-block design with a changeable knife, a specimen holder and an advancement mechanism. In most devices the cutting of the sample begins by moving the sample over the knife, where the advancement mechanism automatically moves forward such that the next cut for a chosen thickness can be made. The section thickness is controlled by an adjustment mechanism, allowing for precise control.

Applications

The most common applications of microtomes are

- **Traditional Histology Technique:** Tissues are hardened by replacing water with paraffin. The tissue is then cut in the microtome at thicknesses varying from 2 to 50 μm. From there the tissue can be mounted on a microscope slide, stained with appropriate aqueous dye(s) after prior removal of the paraffin, and examined using a light microscope.
- **Frozen Section Procedure:** Water-rich tissues are hardened by freezing and cut in the frozen state with a freezing microtome or microtome-cryostat; sections are stained and examined with a light microscope. This technique is much faster than traditional histology (5 minutes vs 16 hours) and is used in conjunction with medical procedures to achieve a quick diagnosis. Cryosections can also be used in immunohistochemistry as freezing tissue stops degradation of tissue faster than using a fixative and does not alter or mask its chemical composition as much.
- **Electron Microscopy Technique:** After embedding tissues in epoxy resin, a microtome equipped with a glass or gem grade diamond knife is used to cut very thin sections (typically 60 to 100 nanometer). Sections are stained with an aqueous solution of an appropriate heavy metal salt and examined with a transmission electron microscope. This instrument is often called an

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ultramicrotome. The ultramicrotome is also used with its glass knife or an industrial grade diamond knife to cut survey sections prior to thin sectioning. These survey sections are generally 0.5 to 1 µm thick and are mounted on a glass slide and stained to locate areas of interest under a light microscope prior to thin sectioning for the TEM. Thin sectioning for the TEM is often done with a gem quality diamond knife. Complementing traditional TEM techniques ultramicrotomes are increasingly found mounted inside an SEM chamber so the surface of the block face can be imaged and then removed with the microtome to uncover the next surface for imaging. This technique is called Serial Block-Face Scanning Electron Microscopy (SBFSEM).

- **Botanical Microtomy Technique:** Hard materials like wood, bone and leather require a sledge microtome. These microtomes have heavier blades and cannot cut as thin as a regular microtome.
- **Spectroscopy (especially FTIR or Infrared spectroscopy) Technique:** Thin polymer sections are needed in order that the infra-red beam will penetrate the sample under examination. It is normal to cut samples to between 20 and 100 µm in thickness. For more detailed analysis of much smaller areas in a thin section, FTIR microscopy can be used for sample inspection.
- **Fluorescence Microscopy:** Samples can be made into thin slices to be viewed under a fluorocent microscope.

A recent development is the laser microtome, which cuts the target specimen with a femtosecond laser instead of a mechanical knife. This method is contact-free and does not require sample preparation techniques. The laser microtome has the ability to slice almost every tissue in its native state. Depending on the material being processed, slice thicknesses of 10 to 100 µm are feasible.

Microtome Types

- Cambridge Rocking Microtome
- Rotary Microtome
- Sledge Microtome
- Ultramicrotome

4.2.1 Cambridge Rocking Microtome

The instrument is so named because the arm has to move in a rocking motion while cutting the sections. The instrument was invented by Sir Horace Darwin in 1881 was developed by Cambridge company hence it is called the Cambridge rocking microtome. It is a simple machine in which the knife is held by means of microtome thread. The rocking microtome was designed primarily for cutting paraffin wax sections but in an emergency use frozen section by inserting a wooden block in which the tissue is frozen.

Parts of Rocking Microtomes

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- Knife Holder
- Block Holder or Chuck
- Upper Arm
- Screw
- Lever
- Pawl
- Ratchet Wheel
- Mil head Microtome Screw
- Sleeve
- Lower Arm
- Scale

It cuts the sections between 1 to 20 microns. The knife is fixed with the edge, while the object is moved against this knife circularly, producing a sharply curved surface to the block with each stroke the tissue holder automatically moves vertically towards the life. Cutting stroke is Spring operated and is easy to handle. The microtome must be placed on a solid non-slippery surface to allow a better hold.

Advantages of Cambridge Rocking Microtomes

- The cost of knife and microtome is low.
- Celloidin embedded tissues can be sectioned easily.

4.2.2 Rotary Microtome

A rotary microtome of older construction is a common microtome design. This device operates with a staged rotary action such that the actual cutting is part of the rotary motion. In a rotary microtome, the knife is typically fixed in a horizontal position. The Rotary microtome is so called because of a Rotary action of the hand wheel responsible for the cutting moment. The block holder is mounted on a steel carriage, which makes up and down in groves this type of instrument is the most ideal for routine and research work it is excellent for cutting serial sections.

Principle of Sample Movement for making a cut on a Rotary Microtome

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

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

Parts of Rotary Microtomes

- Block Holder
- Knife Clamp Screw
- Knife Clamps
- Block Adjustment
- Thickness Gauge
- Angle of Tilt Adjustment
- Operating Handle

Here the feed mechanism is activated by turning a wheel on one side of the machine. The knife is fixed with its edge fixed upwards and the object is moved against the knife rising and falling vertically.

One rotation of the operating wheel produces a complete cycle downwards cutting stroke and an upward return stroke and activation of the advanced mechanism. It is often modified to cut ultrathin sections between 50Å – 200Å

The wheel may be electrically operated or manually. In the former case the hands may be made free for tissue maintenance, makes it available for incorporation in automated cryostats.

Advantages of the Rotary Microtome

- Heavy and Stable
- Ideal for Serial Sections in Large Numbers
- Paraffin-Embedded Tissues are Cut by a Rotary Microtome
- The Knife Holder is Movable
- The Sections are Cut are Flat
- It is Useful for Routine and Research Papers

4.2.3 Sledge Microtome

A sledge microtome is a device where the sample is placed into a fixed holder (shuttle), which then moves backwards and forwards across a knife. Modern sled microtomes have the sled placed upon a linear bearing, a design that allows the microtome to readily cut many coarse sections. By adjusting the angles between

the sample and the microtome knife, the pressure applied to the sample during the cut can be reduced. Typical applications for this design of microtome are of the preparation of large samples, such as those embedded in paraffin for biological preparations. Typical cut thickness achievable on a sledge microtome is between 1 and 60 µm.

This is a large heavy instrument with a fixed knife beneath which the object moves mounted on a heavy sliding base containing the feed mechanism used primarily for cutting the sections of cellulose nitrate embedded tissues with an obliquely set knife.

Parts of Base-Sledge Microtome

- Angular Tilt Adjustment
- Knife Clamps
- Block Holder
- Coarse Feed Adjustment
- Operating Handle
- Thickness Gauge
- Adjustment Locking Nut
- Block Adjustment Screw
- Split Nut Clasp

The blocks holder is mounted on a steel carriage which slides backward and forwards on groups against fixed horizontal knife this microtome is heavy and very stable. The block is raised towards the knife at a predetermined thickness. This type of microtome is designed for cutting sections of very large blocks of tissues for example whole brain, this microtome has become popular for routine use.

Advantages of Base-Sledge Microtome

- It is useful for cutting extremely hard blocks and large sections.
- The microtome is heavy and stable.
- The knife used is sledge shaped which requires less honing.

4.2.4 Ultramicrotome

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

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

NOTES

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

Ultramicrotomy Process

Ultra-thin sections of specimens are cut using a specialized instrument called an ultramicrotome. The ultramicrotome is fitted with either a diamond knife, for most biological ultra-thin sectioning, or a glass knife, often used for initial cuts. There are numerous other pieces of equipment involved in the ultramicrotomy process. Before selecting an area of the specimen block to be ultra-thin sectioned, the technician examines semi thin or thick sections range from 0.5 to 2 µm. These thick sections are also known as survey sections and are viewed under a light microscope to determine whether the right area of the specimen is in a position for thin sectioning. Ultra-thin sections from 50 to 100 nm thick are able to be viewed in the TEM.

Tissue sections obtained by ultramicrotomy are compressed by the cutting force of the knife. In addition, interference microscopy of the cut surface of the blocks reveals that the sections are often not flat. With Epon or Vestopal as embedding medium the ridges and valleys usually do not exceed 0.5 µm in height, i.e., 5–10 times the thickness of ordinary sections.

A small sample is taken from the specimen to be investigated. Specimens may be from biological matter, like animal or plant tissue, or from inorganic material such as rock, metal, magnetic tape, plastic, film, etc. The sample block is first trimmed to create a block face 1 mm by 1 mm in size. Thick sections (1 ìm) are taken to be looked at on an optical microscope. An area is chosen to be sectioned for TEM and the block face is re-trimmed to a size no larger than 0.7 mm on a side. Block faces usually have a square, trapezoidal, rectangular, or triangular shape. Finally, thin sections are cut with a glass or diamond knife using an ultramicrotome and the sections are left floating on water that is held in a boat or trough. The sections are then retrieved from the water surface and mounted on a copper, nickel, gold, or other metal grid. Ideal section thickness for transmission electron microscopy with accelerating voltages between 50kV and 120kV is about 30–100 nm.

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Advances of Cryo Ultramicrotomy

In 1952 Fernandez-Moran introduced cryo ultramicrotomy, which is a similar technique but done at freezing temperatures between 20 and 150°C. Cryo ultramicrotomy can be used to cut ultra-thin frozen biological specimens. One of the advantages over the more traditional ultramicrotomy process is speed, since it should be possible to freeze and section a specimen in 1 to 2 hours.

Microtome Knives

A diamond knife blade used for cutting ultrathin sections (typically 70 to 350 nm) for transmission electron microscopy.

The selection of microtome knife blade profile depends upon the material and preparation of the samples, as well as the final sample requirements (for example, cut thickness and quality).

Knife Design and Cut Types

Profiles of Microtome Knives

Generally, knives are characterized by the profile of the knife blade, which falls under the categories of planar concave, wedge shaped or chisel shaped designs.

Planar concave microtome knives are extremely sharp, but are also very delicate and are therefore only used with very soft samples. The wedge profile knives are somewhat more stable and find use in moderately hard materials, such as in epoxy or cryogenic sample cutting. Finally, the chisel profile with its blunt edge, raises the stability of the knife, whilst requiring significantly more force to achieve the cut.

For ultramicrotomes, glass and diamond knives are required, the cut breadth of the blade is therefore on the order of a few millimetres and is therefore significantly smaller than for classical microtome knives. Glass knives are usually manufactured by the fracture of glass bars using special knife-maker fracturing devices. Glass knives may be used for initial sample preparations even where diamond knives may be used for final sectioning. Glass knives usually have small troughs, made with plastic tape, which are filled with water to allow the sample to float for later collection. Diamond blades may be built into such an existing trough, allowing for the same collection method.

Sectioning

Prior to cutting by microtome, biological materials are usually placed in a more rigid fixative, in a process known as embedding. This is achieved by the inflow of a liquid substance around the sample, such as paraffin (wax) or epoxy, which is placed in a mold and later hardened to produce a block which is readily cut.

The declination is the angle of contact between the sample vertical and knife blade. If the knife blade is at right angles (declination=90) the cut is made directly

using a pressure based mode, and the forces are therefore proportionally larger. If the knife is tilted, however, the relative motion of the knife is increasingly parallel to sample motion, allowing for a slicing action. This behavior is very important for large or hard samples

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The inclination of the knife is the angle between the knife face and the sample. For an optimal result, this angle must be chosen appropriately. The optimal angle depends upon the knife geometry, the cut speed and many other parameters. If the angle is adjusted to zero, the knife cut can often become erratic, and a new location of the knife must be used to smooth this out.

If the angle is too large, the sample can crumple and the knife can induce periodic thickness variations in the cut. By further increasing the angle such that it is too large one can damage the knife blade itself.

Check Your Progress

1. Define the term microtome.
2. What does microtome use?
3. How thin can a microtome section be made?
4. What is Cambridge rocking microtome and why is it named so?
5. Name the parts of rocking microtome.
6. Give any two advantages of Cambridge rocking microtome.
7. Give advantages of rotary microtome.
8. What is sledge and how is it used?
9. Define ultramicrotome .

4.3 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Microtome is a mechanical instrument used to cut biological specimens into very thin segments for microscopic examination. Biological specimens can be presented in many ways.
2. Microtomes use steel, glass, or diamond blades depending upon the specimen being sliced and the desired thickness of the sections being cut. Steel blades are used to prepare sections of animal or plant tissues for light microscopy histology. Glass knives are used to slice sections for light microscopy and to slice very thin sections for electron microscopy. Industrial grade diamond knives are used to slice hard materials such as bone, teeth and plant matter for both light microscopy and for electron microscopy.
3. Microtome sections can be made thin enough to section a human hair across its breadth, with section thickness between 50 nm and 100 μm.

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4. Cambridge rocking microtome is named because the arm has to move in a rocking motion while cutting the sections. The instrument was invented by Sir Horace Darwin in 1881 was developed by Cambridge company hence it is called the Cambridge rocking microtome. It is a simple machine in which the knife is held by means of microtome thread. The rocking microtome was designed primarily for cutting paraffin wax sections but in an emergency use frozen section by inserting a wooden block in which the tissue is frozen.
5. Parts of rocking microtomes are:
   - Knife holder
   - Block holder or chuck
   - Upper arm
   - Screw
   - Lever
   - Pawl
   - Ratchet wheel
   - Mil head microtome screw
   - Sleeve
   - Lower Arm
   - Scale
6. Advantages of Cambridge rocking microtomes are:
   - The cost of knife and microtome is low.
   - Celloidin embedded tissues can be sectioned easily.
7. Advantages of the rotary microtome are:
   - Heavy and stable
   - Ideal for serial sections in large numbers
   - Paraffin-embedded tissues are cut by a rotary microtome
   - The knife holder is movable
   - The sections are cut are flat
   - It is useful for routine and research paper
8. A sledge microtome is a device where the sample is placed into a fixed holder (shuttle), which then moves backwards and forwards across a knife. Modern sled microtomes have the sled placed upon a linear bearing, a design that allows the microtome to readily cut many coarse sections. By adjusting the angles between the sample and the microtome knife, the pressure applied to the sample during the cut can be reduced. Typical applications for this design of microtome are of the preparation of large samples, such as those embedded in paraffin for biological preparations.

Typical cut thickness achievable on a sledge microtome is between 1 and 60 μm.

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

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4.4 SUMMARY

- A microtome (from the Greek mikros, meaning 'small', and temnein, meaning 'to cut') is a tool used to cut extremely thin slices of material, known as sections.
- Important in science, microtomes are used in microscopy, allowing for the preparation of samples for observation under transmitted light or electron radiation.
- Microtomes use steel, glass, or diamond blades depending upon the specimen being sliced and the desired thickness of the sections being cut.
- Steel blades are used to prepare sections of animal or plant tissues for light microscopy histology.
- Glass knives are used to slice sections for light microscopy and to slice very thin sections for electron microscopy.
- Industrial grade diamond knives are used to slice hard materials such as bone, teeth and plant matter for both light microscopy and for electron microscopy.
- Gem quality diamond knives are used for slicing thin sections for electron microscopy.
- Microtomy is a method for the preparation of thin sections for materials such as bones, minerals and teeth, and an alternative to electropolishing and ion milling.
- Microtome sections can be made thin enough to section a human hair across its breadth, with section thickness between 50 nm and 100 μm.
- One of the first devices for the preparation of such cuts was invented in 1770 by George Adams, Jr. (1750–1795) and further developed by Alexander Cummings.
- The device was hand operated, and the sample held in a cylinder and sections created from the top of the sample using a hand crank.
- Cambridge rocking microtome is so named because the arm has to move in a rocking motion while cutting the sections. The instrument was invented by

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Sir Horace Darwin in 1881 was developed by Cambridge company hence it is called the Cambridge rocking microtome.

- In a rotary microtome, the knife is typically fixed in a horizontal position. The Rotary microtome is so called because of a Rotary action of the hand wheel responsible for the cutting moment.
- A sledge microtome is a device where the sample is placed into a fixed holder (shuttle), which then moves backwards and forwards across a knife. Modern sled microtomes have the sled placed upon a linear bearing, a design that allows the microtome to readily cut many coarse sections.
- Ultra-thin sections of specimens are cut using a specialized instrument called an ultramicrotome. The ultramicrotome is fitted with either a diamond knife, for most biological ultra-thin sectioning, or a glass knife, often used for initial cuts.
- Planar concave microtome knives are extremely sharp, but are also very delicate and are therefore only used with very soft samples. The wedge profile knives are somewhat more stable and find use in moderately hard materials, such as in epoxy or cryogenic sample cutting.

4.5 KEY WORDS

- **Electropolishing:** Electropolishing is an electrochemical process similar to, but the reverse of, electroplating. The electropolishing process smooths and streamlines the microscopic surface of a metal object such as 304, 316, and the 400 series stainless steel.
- **Microtomy:** Microtomy is a method for the preparation of thin sections for materials such as bones, minerals and teeth, and an alternative to electropolishing and ion milling.
- **Immunohistochemistry:** Immunohistochemistry (IHC) is a method for detecting antigens or haptens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues.
- **Ultramicrotomy:** Ultramicrotomy is a method for cutting specimens into extremely thin slices, called ultra-thin . The ultramicrotome is fitted with either a diamond knife, for most biological ultra-thin sectioning, or a glass knife, often used for initial cuts.
- **Cryostat:** A cryostat is a device used to maintain low cryogenic temperatures of samples or devices mounted within the cryostat.
- **Ultramicrotome:** Ultra-thin sections of specimens are cut using a specialized instrument called an ultramicrotome.

4.6 SELF ASSESSMENT QUESTION AND EXERCISES

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Short Answer Questions

1. Write a short note on microtome and name its different types.
2. What are the applications of microtome?
3. What is rotary microtome?
4. Write a short note on sledge microtome.
5. Write about ultramicrotomy process.

Long Answer Question

1. Write a short note on microtome and describe its different types in detail.
2. Write about cambridge rocking microtome describing its parts and applications.
3. Describe about rotary microtome, its parts, applications and advantages.
4. Explain about sledge microtome giving a detailed overview about its parts and advantages.
5. Discuss about the ultramicrotome and ultramicrotomy process giving a detailed overview of its applications as well.

4.7 FURTHER READINGS

Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

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UNIT 5 MICROTOME SECTIONING

NOTES

Structure

5.0 Introduction
5.1 Objectives
5.2 Microtome Section and Section Cutting Process
5.3 Material Preparation Techniques for Microtome Sectioning
    5.3.1 Fixation
    5.3.2 Dehydration
    5.3.3 Fixing Agent
5.4 Answers to Check Your Progress Questions
5.5 Summary
5.6 Key Words
5.7 Self Assessment Questions and Answers
5.8 Further Readings

5.0 INTRODUCTION

A microtome is a tool used to cut extremely thin slices of material, known as sections. Important in science, microtomes are used in microscopy, allowing for the preparation of samples for observation under transmitted light or electron radiation. It is a mechanical instrument used to cut biological specimens into very thin segments for microscopic examination. Biological specimens can be presented in many ways. But more often, these specimens are embedded in paraffin wax blocks and the commonest way of sectioning these specimens can be achieved by the microtome.

Microtome includes various steps like embedding, sectioning, mounting, stretching, drying, etc., which you will study in the unit later. In this unit, you will also study about the different material preparation techniques for microtome sectioning, various methods like fixatives, dehydrating agents, killing, fixing are also described in detail.

5.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand what material preparation techniques are in microtome sectioning
- Discuss about the different material preparation techniques
- Explain different material preparation techniques like fixatives, dehydrating agents, killing, fixing methods, etc.

5.2 MICROTOME SECTION AND SECTION CUTTING PROCESS

Rotary microtomes are commonly used for section-cutting. Move the block holding the socket backward as far as possible with the help of the backward movement handle, which usually lies at the far side of the microtome. Adjust the thickness of sections.

Ordinarily, flower buds are cut at 14 μ and root tips at 12 μ thickness. Cut out one block from the large one cast previously. Trim it with a scalpel and/or blade, holding it gently with the left hand and obtain a perfect square or rectangle with the material at the centre. Now hold the trimmed block gently with the thumb and forefinger of the left hand, heat a scalpel and place it flatly on the block-holder resting the block on the scalpel and remove the scalpel. Prick the four sides of the block where it joins the holder with a hot needle and submerge the block in cold water for a few seconds.

Fix the block-holder in the block-holder socket with the help of its screws and razor in its socket, both parallel to each other having razor tip slightly tilted inwardly at 8° angle. Now bring the block and razor close without touching each other and cut sections by moving the front wheel in a clockwise direction.

If the ribbon slips over the razor support it with a needle. When the material is exhausted, dislodge the ribbon with a brush and place on a half foolscap piece of blotting paper or on a glossy and stiff paper.

Precautions

1. The block-holding socket is moved backward as far as possible because, if the microtome gets stuck midway while section cutting, the ribbon will be disturbed. Resetting it may cause the loss of a portion of the material with the result that the entire material is not present in sections.
2. Thickness of sections depends upon the size of the cells. The idea is to get sections one cell in thickness.
3. Trimming the block is a very critical step, if trimmed into odd shapes the ribbon will not be straight which causes difficulty in joining of the sections to form the ribbon having excess paraffin on one side but, should not be cut in one stroke.

   The four sides should be trimmed in such a way that the material finally lies at the centre. Longitudinal sections are normally made in case of flower buds, and transverse sections in case of root tips.
4. Add sufficient paraffin on the block-hold to ensure proper fixing of the block and, at the same time, reduce the chance of the metallic holder rubbing against the razor. While fixing the block, the scalpel method should be

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followed. Pricking with a hot needle makes fixing doubly sure. It is all the more necessary because often the block gets detached while section cutting.

5. Note carefully before section cutting that the block and the razor are perfectly parallel to each other; otherwise the ribbon will not be straight. Very often the ribbon becomes curved or dentate. This is due to faulty trimming or not keeping the block parallel to the razor.
6. The razor should be placed slightly inward to avoid rubbing of the block against the lower side of the razor.
7. As the block strikes the razor a section is produced and the friction against the razor produces a little heat energy. When sections are cut in quick succession this energy is sufficient to join the very thin sections end to end to produce a ribbon, so paraffin of suitable melting point has to be taken while casting blocks.
8. For flower buds and L.S. (longitudinal section) of root tips cut the entire material into sections. While making T. S. of root tips, cut sections to fill one slide; divisions are not found beyond this region.
9. Never touch the edge of the razor with a needle or scalpel. Dislodge the ribbon at the end of section cutting with a brush. Friction with metal may cause minute dentation or folding of the edge of the razor.

Mounting of Sections

Cut the ribbon into 1½” (4 cm) long strips. Put a small drop of Meyer’s fixative (white of egg 50 ml, Glycerol 50 ml; Sodium salicylate 1 gm.) on a clean grease-free slide. Rub it gently with the little finger all over the slide and drain off the excess fixative, if any. Flood the slide with a thin film of water with the help of a dropper. Dip the tip of a scalpel in cold water and touch the ribbon with it. As the ribbon will adheres to it, free the other end of the ribbon with a needle or scalpel and place it on the slide.

Stretching of Sections

Transfer the slide on to a hot plate kept at 37-43°C, on a thin film of water. Wait for a few seconds. The ribbons will automatically stretch on being warmed. Take two needles and further stretch the ribbons by pulling in opposite directions. Dip the needles in cold water to keep cool.

Drying of Sections

Take out the slide from the hot plate. Hold it with the thumb and forefinger of the left hand without touching the ribbons, push the ribbons to one side of the slide and tilt the slide after placing the needle below them to drain off the excess water.

Adjust the ribbons in such a way that on one side of the slide about 2 cm open space and on the opposite side about 2-3 mm open space are left and keep it on the hot plate for 3 hours to overnight for drying.

Precautions

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1. The size of the slide is 33 × 13 (75 × 25 mm) and that of a microtome cover-glass 23 × 13 (approx.) (50 × 25 mm). Hence, if the ribbon is cut into 1½" (37 mm) long pieces, after stretching they will be 23 (50 mm) long (approx.) to fill the cover glass completely.
2. Rub Meyer's fixative with the little finger and keep the slide flooded with water to keep the ribbons floating, so that they can stretch when warmed.
3. Transfer the ribbons to the slide maintaining their continuity. In root-tips the ribbon-pieces are mounted as soon as the material comes in the ribbon, and in flower buds the initial and final sections are discarded and only the middle portion of the ribbon is taken having anther sections, spore mother cells.
4. The lower surface of the ribbon which remains in contact with the razor is glossy. While mounting, this surface should come in contact with the slide for better adherence to it.
5. Full stretching of the ribbon is of utmost importance. As the ribbon stretches, the sections also stretch, eliminating the possibility of minute folds remaining in the sections.

   Direct heating over a burner should never be made, for it may melt away all the paraffin and the sections may get displaced and distorted due to over-heating. Heating over an alcohol flame may, should be done carefully.
6. The ribbons are mounted on a thin film of water on the slide to keep them floating, so that they can stretch freely. A thin film of water is kept in-between the slide and the hot plate surface for intimate contact between the slide and the hot plate both of which are solid.
7. If the ribbons are found to be curved after mounting, touch the two inner edges of the curve with two needles and pull them upwards while stretching.
8. The final size of the ribbons is limited by the length of the microtome cover glass, which is about 23 (5 cm). At one side of the slide, 2-3 mm of empty space is left so that the ribbons or sections do not get damaged by friction with the slide box.

Staining the Sections

Microtome sections are stained in cylindrical or rectangular staining Jars or Coplin Jars provided with lids. Each jar should be filled up to about 2½" (6 cm) with the reagent so that the fingers are not dipped in the reagent while staining. Transferring a slide from one jar to another should be done slowly to keep the mixing of the reagents at a minimum.

Both root tips and flower buds are stained in 1% aqueous crystal violet solution. As the sections are embedded in paraffin, the first step is removal of this

paraffin by xylol. Then xylol is replaced by alcohol and alcohol by water. The slides should be kept in xylol and abs. alcohol as these reagents make the sections brittle.

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The slides are stained after reaching down to water and then are mordanted in KI and iodine mordant for a few seconds. Mordanting increases the stain-retaining capacity of the chromosomes. Mordanting after staining is called post-mordanting. Mordanting is followed by quick dehydration in abs. alcohol and then differentiation in clove oil.

Crystal violet is highly soluble in alcohol, so dehydration is done as quickly as possible or else the stain may wash out. After differentiation, clove oil is cleared by xylol and, finally, the slides are mounted in Canada balsam.

Schedule of Staining the Section

Pass the slide with paraffin sections through the following grades: Xylol I (Down), Xylol II (Down), Xylol III (Down) — 1 hr. in each Xylol — absolute ethyl alcohol mixture (1:1) — 1 hr.; absolute ethyl alcohol, 95%, 90%, 80%, 70%, 50% and 30% alcohol — 30 mins. in each. If required, the slides can be kept for overnight in 70% alcohol. Finally, keep in water at least 15 mins.

Stain in 1% aq. crystal violet solution for 30 mins. Keep in Pot. Iodine mordant (1 gr KI and 1 gm. iodine crystals dissolved in 100 ml 80% alcohol) for 45 seconds. Pas through abs. ethyl alcohol I (UP), II (UP) and III (UP) — 2 to 3 dips in each.

At this stage a piece of blotting paper should be kept nearby and, after 2 to 3 rapid dips in each alcohol jar, the slides should be rapidly jerked against the wall of the reagent jar and then on the blotting paper.

If cytoplasm and nucleus cannot be differentiated, put the slide back in clove oil I and again observe under microscope. After differentiation, keep the slide in clove oil II (10-15 min.).

Pass through xylolI (UP), II (UP) and III (UP) — 1 hr. in each. If required, the slides can be kept in xylol III (UP) for overnight. Mount in Canada balsam. Put 3 drops of Canada balsam on the slide immediately after taking it out of xylol III (UP). Dip the cover- glass in xylol and place it gradually on the slide. Press the cover-glass gently with a needle to bring out excess balsam and dry the slide on a hot plate.

Check Your Progress

1. Where are rotary microtomes used?
2. What is post-mordanting?
3. What does mordanting do?
4. Why the razor should be kept inwards in section cutting?

5.3 MATERIAL PREPARATION TECHNIQUES FOR MICROTOME SECTIONING

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A microtome is a tool used to cut extremely thin slices of material, known as sections. Important in science, microtomes are used in microscopy, allowing for the preparation of samples for observation under transmitted light or electron radiation. It is a mechanical instrument used to cut biological specimens into very thin segments for microscopic examination. Biological specimens can be presented in many ways. But more often, these specimens are embedded in paraffin wax blocks and the commonest way of sectioning these specimens can be achieved by the microtome. The earliest form of the microtome enabled free hand sectioning of fresh or fixed material using a sharp razor. Modern microtomes are precision instruments designed to cut uniformly thin sections of a variety of materials for detailed microscopic examination. Central to the production of good sections is the microtome knife. Microtomy virtually begins and ends with a sharp, blemish-free cutting edge.The introduction of disposable blades has made easier the production of good quality, thin sections, but they are often unsatisfactory for sectioning harder tissues, especially bone. A sharp knife edge free from imperfections is essential for the production of good sections.

5.3.1 Fixation

Fixation is the first step in any procedure in which tissue is to be preserved for histological study. Fixatives kill the tissue, as well as any bacteria that are present that otherwise would cause the tissue to rot. They also coagulate or cross-link proteins, making them insoluble. All fixatives distort tissue to a certain extent, but in general, proteins and cellular structure are preserved. Normally you choose a fixative containing several ingredients that balance out each others ill-effects. For example, alcohol shrinks tissue and causes excessive hardening. You can counter these effects by adding an acid such as acetic acid, which swells tissue and prevents overhardening.

One of the safest fixatives to use, which will not leave any toxic residues behind, is Carnoy's fixative, a mixture of alcohol and acetic acid. It is not an ideal fixative. The addition of formaldehyde, for example, would give better preservation of cytological detail. Formaldehyde, however, and most other fixing agents leave highly toxic residues that are virtually impossible to remove from instruments and glassware. If you were making whole mounts, where cytological detail is not critical, or you cannot risk contaminating your work area for future live material, Carnoy's is ideal for use.

Glacial means 100% acetic acid; vinegar is 5% acetic acid. Ethanol is the type of alcohol that people get arrested for driving under the influence of; hard liquor such as whiskey is about 40%–50% ethanol. Rubbing alcohol is 70% isopropyl alcohol—don't drink it, it's toxic. If you were out in the boondocks with

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no scientific supplies and found the perfect specimen you needed to preserve, what makeshift fixative would you devise?

A commonly used fixative that is better at preserving structure than Carnoy's is FAA, made of formaldehyde, alcohol, and acetic acid. (Formaldehyde is a gas, which is sold in solution as formalin, which is about 40% formaldehyde in water).

A fixative that is widely used for embryological material, since it will not overharden yolky material, is Bouin's fluid. It has the advantage that tissues may be stored indefinitely in it. Its major disadvantage is that the picric acid, though an excellent fixative for lipid-rich tissues that doesn't harden the tissues, also stains the tissue yellow. The yellow color must be removed before other stains can be applied. Usually, this is accomplished after sectioning as the slides descend through the alcohol series. Just leave the slides in 70% alcohol for a longer period of time if the yellow color doesn't disappear using the normal dehydration schedule.

5.3.2 Dehydration

The first stage in tissue processing is dehydration (the removal of water). In tissues, water is present in both free and bound forms and needs to be removed before processing can continue. Dehydration is usually carried out using alcohols (such as ethanol) but these can dissolve certain cellular components such as lipids. Although dehydration can also cause tissue shrinkage, the stage is necessary in all infiltration methods, except where tissues are supported by an aqueous embedding medium (such as water-soluble waxes).

In paraffin wax processing, dehydration from aqueous fixatives such as formalin is usually initiated in 70% alcohol before progressing through 90%-95% to absolute alcohol before proceeding to the clearing stage. However, direct transfer to 95% alcohol is often performed if tissues are adequately fixed. Duration of dehydration is dependent on tissue thickness; the thicker the block, the longer the time. Generally, blocks 1 mm thick should receive up to 30 minutes while blocks 5 mm thick require up to 90 minutes or longer in each change.

Types of Dehydrating Agents

Acetone $(CH_3)_2CO$: This is a colourless flammable liquid with a characteristic odour, low toxicity and is freely miscible with water and organic solvents. Acetone is fast and effective as a dehydrant and may also act as a coagulant secondary fixative. Acetone is ideal for fatty tissue samples and they can be transferred directly from acetone into paraffin wax.

Alcohols: These are clear, colourless, flammable and hydrophilic liquids that are miscible with water and most of the organic solvents. In addition to their role as dehydrants, alcohols also act as secondary coagulant fixatives during tissue processing. The most commonly used alcohol used in tissue processing is ethanol.

Butanol C_4H_9OH: This alcohol is mainly used for plant and animal tissues. n-butanol causes less hardening and shrinkage than ethanol but is poorly miscible

with water and paraffin wax so longer times are required. It is flammable, has a penetrating odour and is an eye irritant. Iso-butanol has similar properties but is a less costly substitute for n-butanol.

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Cellosolve: Also known as ethylene glycol or 2-ethoxyethanol, it is a colourless, almost odourless flammable liquid. The reagent is miscible with water and most other organic solvents. As a dehydrating agent, it is especially used for preceding ester wax embedding. In order to avoid severe tissue shrinkage, they are transferred from aqueous fixative or 70% ethanol into full strength cellosolve.

Dimethoxypropane (DMP) and Diethoxypropane (DEP)

These are flammable, miscible with wax and are used for the chemical dehydration of tissues. They are suitable for rapid manual processing or machine processing and are comparable to conventional dehydration for tissue morphology and staining reactions.

Dioxane: Also known as diethylene dioxide, this is a colourless, flammable liquid that produces less shrinkage and hardening than with ethanol. The liquid is miscible with water, most organic solvents and paraffin wax and is excellent for tissues that have been excessively hardened by conventional processing. Dioxane has a rapid but gentle action and tissues are able to remain in it for long periods without harm.

Ethanol (Ethyl Alcohol): This is a rapid and efficient dehydrant and is the most commonly used. Dehydration is usually initiated in 75% alcohol with progress through 90%-95% ethanol before several changes of absolute ethanol to complete dehydration. Progressive removal of bound water from carbohydrates and proteins during prolonged immersion in absolute ethanol causes tissues to harden excessively and become brittle. Colloid, blood, collagen and yolky tissues are particularly affected. Ethanol is usually supplied as absolute ethanol or Industrial Methylated Spirits (IMS) which is also known as 74 O.P (over proof) spirit.

Proof spirit is defined as that which, at a temperature of 510 F, weighs exactly 12/13 of an equal volume of distilled water. Proof spirit is the standard, contains 57% ethanol and is referred to as 100 degrees. Therefore, 74 O.P spirit equals 174 degrees. Thus it contains:

$$\frac{57 \times 174}{100} = 99.9\% \text{ Ethanol}$$

Isopropanol: This is completely miscible with water and most organic solvents and is fully miscible with molten paraffin wax. Isopropanol shrinks and hardens tissues and is used to dehydrate hard, dense tissues. It is less severe than ethanol but tissues may be transferred from 60%-70% to absolute isopropanol to minimize shrinkage. Isopropanol can be used as a xylene substitute.

Methanol: This reagent is a good ethanol substitute but is rarely used because it is volatile, flammable and costly. Methanol tends to harden tissues more than ethanol and is a poor lipid solvent.

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Phenol: This consists of clear hygroscopic crystals and is also available in a liquefied form. Phenol is soluble in water, alcohol and most organic solvents. However, phenol develops a pink discolour on exposure to air and light so containers must be protected from light and tightly sealed.

Polyethylene Glycols: These are used to dehydrate and embed tissues that are affected by the solvents and heat of the paraffin wax method. They are clear, viscous liquids or solids of low toxicity. At low molecular weight, the polyethylene glycols are liquid and can be used to dehydrate the tissues as they pass through glycols of increasing molecular weight and viscosity. However, at room temperature, high molecular weight polyethylene glycols are solid and can be used for embedding tissues.

Tetrahydrofuran: This is a colourless, highly volatile and flammable solvent with an offensive smell. The solution is completely miscible with water, most organic solvents, paraffin wax and mounting media. It dehydrates rapidly causing little shrinkage or hardening and is possibly the best of the universal solvents.

5.3.3 Fixing Agent

The Corrosive Sublimate and 5 per cent. Acetic solution is an excellent fixative for general purposes. It can be used with fresh or salt water and either hot or cold. It gives good results for histological preparations. Very delicate specimens need only a few seconds, while large objects should be given from some minutes up to some hours, according to their size and permeability. For small fresh-water forms, it is best to dilute the solution of corrosive sublimate with an equal volume of water. For marine animals the concentrated solution is required, and for such things, as, for example, coral polyps, it is best to use a heated solution. Always wash out with 50 per cent and then 70 per cent. alcohol, and test with a solution of Iodine in 70 per cent. alcohol, to make sure that all the corrosive sublimate is removed.

Various other fixing agents are in use for different purposes, as for example:

Flemming's Solution (Strong)

1 Per Cent Chromic Acid - 15 cc.

2 Per Cent Osmic Acid - 4 cc

Glacial Acetic Acid - 1 cc

This solution is good for fixing nuclei and chromosomes, but it does not keep well and penetrates badly, so that only small pieces of tissue or small animals can be fixed with it. The specimen should be given from 1 to 24 hours in the fixing agent (12 hours is the usual time), washed in running water for some hours, and then put into 30 per cent alcohol.

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Bouin's Solution

Saturated Solution of Picric Acid in Water – 75 cc.

40 per cent Formalin and, at the time of using - 25 cc

Glacial Acetic Acid - 5 cc

This is a good fixing agent and penetrates well. The specimen must not be left in it longer than 18 hours (the usual time is from 6 to 12 hours).

Check Your Progress

5. What is fixation?
6. What are fixatives?
7. What does dehydration mean?
8. Name some o the dehydrating agents.
9. What is acetone?
10. What is tetrahydrofuran?
11. What is methanol?

5.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Rotary microtomes are commonly used for section-cutting.
2. Mordanting after staining is called post-mordanting.
3. Mordanting increases the stain-retaining capacity of the chromosomes.
4. The razor should be placed slightly inward to avoid rubbing of the block against the lower side of the razor.
5. Fixation is the process of fixing a tissue. Fixation kills the tissue, as well as any bacteria present, and coagulates (cross-links) proteins.
6. Fixative are anything that fixes a living organism so that it can't be degraded by bacteria or other microorganisms.
7. Dehydration means the removal of water. In histological procedures, a specimen is usually dehydrated through a graded series of alcohol prior to embedding or mounting.
8. Some of the dehydrating agents are-
   - Acetone
   - Alcohols
   - Butanol
   - Cellosolve
   - Dioxane
   - Methanol

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9. Acetone (propanone) is the organic compound with the formula (CH3)2CO. It is a colorless, volatile, flammable liquid, and is the simplest and smallest ketone. Acetone is miscible with water and serves as an important solvent in its own right, typically for cleaning purposes in laboratories.
6. Tetrahydrofuran is a colourless, highly volatile and flammable solvent with an offensive smell. The solution is completely miscible with water, most organic solvents, paraffin wax and mounting media. It dehydrates rapidly causing little shrinkage or hardening and is possibly the best of the universal solvents.
7. Methanol is a good ethanol substitute but is rarely used because it is volatile, flammable and costly. Methanol tends to harden tissues more than ethanol and is a poor lipid solvent.

5.5 SUMMARY

- A microtome is a tool used to cut extremely thin slices of material, known as sections.
- Important in science, microtomes are used in microscopy, allowing for the preparation of samples for observation under transmitted light or electron radiation.
- It is a mechanical instrument used to cut biological specimens into very thin segments for microscopic examination.
- The earliest form of the microtome enabled free hand sectioning of fresh or fixed material using a sharp razor.
- Modern microtomes are precision instruments designed to cut uniformly thin sections of a variety of materials for detailed microscopic examination.
- Microtomy virtually begins and ends with a sharp, blemish-free cutting edge.
- The introduction of disposable blades has made easier the production of good quality, thin sections, but they are often unsatisfactory for sectioning harder tissues, especially bone.
- A sharp knife edge free from imperfections is essential for the production of good sections.
- Fixation is the first step in any procedure in which tissue is to be preserved for histological study.
- Fixatives kill the tissue, as well as any bacteria that are present that otherwise would cause the tissue to rot. They also coagulate or cross-link proteins, making them insoluble.
- All fixatives distort tissue to a certain extent, but in general, proteins and cellular structure are preserved.

NOTES

- Normally you choose a fixative containing several ingredients that balance out each others ill-effects.
- One of the safest fixatives to use, which will not leave any toxic residues behind, is Carnoy's fixative, a mixture of alcohol and acetic acid.
- Formaldehyde, however, and most other fixing agents leave highly toxic residues that are virtually impossible to remove from instruments and glassware.
- If you were making whole mounts, where cytological detail is not critical, or you cannot risk contaminating your work area for future live material, Carnoy's is ideal for use.
- In tissues, water is present in both free and bound forms and needs to be removed before processing can continue.
- Dehydration is usually carried out using alcohols (such as ethanol) but these can dissolve certain cellular components such as lipids.
- Although dehydration can also cause tissue shrinkage, the stage is necessary in all infiltration methods, except where tissues are supported by an aqueous embedding medium (such as, water-soluble waxes).
- In paraffin wax processing, dehydration from aqueous fixatives such as formalin is usually initiated in 70% alcohol before progressing through 90%-95% to absolute alcohol before proceeding to the clearing stage.

5.6 KEY WORDS

- **Carnoy's solution:** Carnoy's solution is a fixative composed of 60% ethanol, 30% chloroform and 10% glacial acetic acid,1gm of ferric chloride.
- **Dehydrating:** The removal of water. In histological procedures, a specimen is usually dehydrated through a graded series of alcohol prior to embedding or mounting.
- **Fixation:** The process of fixing a tissue. Fixation kills the tissue, as well as any bacteria present, and coagulates (cross-links) proteins.
- **Fixative:** Anything that fixes a living organism so that it can not be degraded by bacteria or other microorganisms.
- **Acetone:** Acetone (propanone) is the organic compound with the formula $(CH_3)_2CO$. It is a colorless, volatile, flammable liquid, and is the simplest and smallest ketone.
- **Carnoy's solution:** Carnoy's solution is a fixative composed of 60% ethanol, 30% chloroform and 10% glacial acetic acid,1gm of ferric chloride.
- **Butanol:** n-Butanol or n-butyl alcohol or normal butanol is a primary alcohol with a 4-carbon structure and the chemical formula C_4H_9OH.

5.7 SELF ASSESSMENT QUESTIONS AND ANSWERS

NOTES

Short Answer Questions

1. What is microtome and microtome sectioning?
2. What is fixation and what are fixatives?
3. What is dehydration?
4. Name few dehydrants and explain them briefly.
5. Write in brief about fixing agents.

Long Answer Questions

1. Explain about microtomy and sectioning of microtome in detail.
2. Discuss about how the material preparation techniques for microtome sectioning done.
3. Explain what is fixation in detail.
4. Discuss about dehydration and its different dehydrating agents.
5. What are fixing agents? Explain about the various fixing agents in detail.

5.8 FURTHER READINGS

Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

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UNIT 6 STAINS AND THEIR USES

Structure

6.0 Introduction
6.1 Objectives
6.2 Stain and Staining - Staining of a Plant Tissues
 6.2.1 Clearing (Dealcoholization)
 6.2.2 Mounting
 6.2.3 Mountants
6.3 Answers to Check Your Progress Questions
6.4 Summary
6.5 Key Words
6.6. Self Assessment Questions and Exercises
6.7 Further Readings

6.0 INTRODUCTION

Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues highlighting, for example, muscle fibers or connective tissue, cell populations classifying different blood cells, for instance, or organelles within individual cells.

In biochemistry it involves adding a class-specific, such as DNA, proteins, lipids, carbohydrates dye to a substrate to qualify or quantify the presence of a specific compound. Staining and fluorescent tagging can serve similar purposes. Biological staining is also used to mark cells in flow cytometry, and to flag proteins or nucleic acids in gel electrophoresis. Staining is not limited to biological materials, it can also be used to study the morphology of other materials, for example the lamellar structures of semi-crystalline polymers or the domain structures of block copolymers.

In this unit, you will study about stains and staining and their different uses, different staining techniques are also described in the unit like clearing, mounting and mountants.

6.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand what stain and staining is
- Discuss about the various stains and their uses
- Explain about staining of plant tissues
- Discuss about what clearing, mounting and mountants are

6.2 STAIN AND STAINING - STAINING OF A PLANT TISSUES

NOTES

Cellulose is the main component of all plant cell walls, and is the most abundant organic compound in most plants. Lignin, another polysaccharide, is the next most abundant. Lignin forms a major support system by providing rigidity to xylem, the water transport structure of plants. The vascular tissues of plants can be distinguished by their structure and molecular composition using stains that react differentially with cellulose and lignin.

In the microscopic analysis of animal and plant tissues, thin sections of tissue are required to achieve a slice with only one or a few layers of cells. If the section is too thick, the light of the microscope cannot penetrate the specimen. To achieve this, an instrument called a microtome is used. Tissue is usually fixed (preserved), embedded in wax or a similar material, then passed across a sharp blade to slice off very thin sections.

In this below practical a 'hand microtome' is used, which replicates the principle on which a standard laboratory microtome works. Thin sections of plant stem of dicotyledons or monocotyledons are prepared. The sections are then stained with differential stains to help distinguish between cell walls made of cellulose and lignin, and thus identify phloem and xylem.

Common Stains for Slide Preparation

Microscope Slide Stains

Since most biological structures are transparent, we need a technique to distinguish the parts as we observe under the microscope. This is possible by some means by which contrast between different structures can be obtained. Sometimes adjusting light helps but the most common method is staining.

Stains, such as methylene blue in low concentrations does not harm the tissues and so can be safely used on living materials. Such stains are called vital stains. For making temporary slides stains, such as methylene blue, idodine, aniline hydrochloride, safranin etc., are used.

Given below are some common stains and their uses and the colour they show staining:

- Iodine: Stains carbohydrates in plant and animal specimens brown or blue-black. Stains glycogen red.
- Methylene Blue: Stains acidic cell parts (like nucleus) blue. Used on animal, bacteria and blood specimens. Can be used as a substitute for Janis B green.
- Eosin Y: Stains alkaline cell parts (like cytoplasm) pink. Used on plants,animals and blood. Can be used as a substitute for Congo Red and Carmine.

- Safranin : Mainly used for sections of plant tissues, stains red.
- Toluidene Blue: Stains acidic cell parts (like nucleus) dark blue. Used to show mitosis in plant cells.
- Wright's Stain: Stains red blood cells pink/red.
- Leishman's Stain: Stains nucleus of WBC blue and blood cells pink.
- Crystal Violet: Stains bacteria purple.
- Aceto-Orcein: Biological stain for chromosomes and connective tissues.
- Sudan III: Biological stain used as a lipid indicator.

Materials Required

- Hand Microtomes (Nut and Bolt)
- 10 ml 70% Ethanol
- Plant stem, for example thyme, Geranium
- 10 ml 95% Ethanol
- Melted Paraffin Wax
- 10 ml Distilled Water
- Forceps/Tweezers – Fine Point
- Filter Paper (Cut into Squares/Arcs)
- Razor – Single Edge
- Waste Beaker & Paper Towel
- Multiwell Dish or Small Petri Dish

Stains in Dropper Bottles

- 4 transfer pipettes (ethanol and wash)
- 0.5% safranin O in 50% ethanol
- Microscope slides and coverslips
- 0.5% methyl green
- Mountant (glycerol:water 1:1)
- 0.025% toluidine blue

Method – Thin Sections With a Hand Microtome

1. Choose a stem 2 to 5mm diameter. The stem tissue should be firm but not woody. Cut a piece about 1cm long (make sure its longer than the depth of the nut of your hand microtome. The tissue can be used freshly cut from the plant, or cut and placed in 70% Ethanol up to a day before sectioning.
2. Loosen the nut so it is barely secured to the end of the bolt. This creates a 'well' in the nut.

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3. Stand the bolt up so its head is on the table.
4. Perform this step quickly so that the tissue is embedded uniformly; the wax starts to set rapidly in small volumes at room temperature. Place some molten wax in the well formed in the nut. Immediately stand the stem in the molten wax. When the stem can stand up by itself, add more melted wax around the tissue to form a mound around the protruding stem. Allow the wax to completely harden (about 10 minutes).
5. Safety: Blade motion away from you Hold the head of the bolt flat on the table with one hand. Hold the blade with the other hand. With the razor blade, carefully shave off the mound of wax and stem from above the top of the nut. Run the blade over the nut's surface to make the wax completely smooth. This portion is discarded. Alternatively, place the nut bolt horizontally on a cutting board, hold the nut firmly (keeping fingers out of the way) then slice downwards against the edge of the nut.
6. Turn the bolt clockwise about 1/12th of a turn to make the wax cylinder protrude slightly. Again holding the microtome and blade slice the blade over the nut's surface to make a shaving of the wax surface through the stem. A smooth quick slicing motion is the best. Continue the procedure to get several thin sections of the stem.
7. For staining, choose the 2 4 thinnest sections (the most transparent sections). Stain 2 sections with each of the staining protocols below.

Staining Method 1 Double Stain – Safranin O and Methyl Green

Cell walls composed mostly of cellulose will appear thin and blue or blue green (stained with methyl green) while those containing lignin will appear thicker and red orange or red (stained with Safranin O).

Double stain – Safranin-O & Methyl green				
Stain	**Colour of stained structure**	**Molecule stained**	**Structure stained**	**Other features**
Safranin-O	Red-orange, red	lignin	Xylem	thick
Methyl green	Blue, blue-green	cellulose	Phloem, pith, epidermis	thin

1. Using forceps, gently transfer the sections into a small petri dish or well (separated them from the wax as you do this) and immediately add a drop of safranin stain to cover the sample. Let the section sit in the stain for 3 minutes.
2. Remove safranin by blotting it with filter paper and immediately add 70 % ethanol. Let the section sit in 70% ethanol for 1 minutes. Remove the ethanol wash, add more 70% ethanol for another 1 to 2 minutes to completely remove excess safranin from the section.

NOTES

3. Remove the remaining 70% ethanol by blotting and immediately add methyl green and let it sit for 1 minute. Remove methyl green by blotting.
4. Immediately add 95% ethanol and allow it to sit with gentle agitation for 1 minute. Remove the ethanol wash. Add more 95% ethanol for another 1 to 2 minutes to remove excess of both stains. Blot out the ethanol. Do not leave the section for too long in ethanol as it may remove too much of the stain. The section should appear translucent to blue. If it appears red, then the excess stain has not been effectively removed (this happens when the sections are too thick).
5. Gently transfer the section to a microscopic slide and with filter paper blot out excess ethanol.
6. Immediately add 1 to 2 drops of mountant (Glycerol: Water, 1:1) and add a coverslip over the section. Water can be used for mounting.
7. Label the slide and examine under low power (4x objective) and higher power (10x objective) of your microscope.

Staining Method 2 Toluidine Blue

Toluidine blue is a polychromatic stain, meaning that different components of the cell wall stain different colours. Cellulose stains pink/purple, lignin stains blue/blue green.

Toluidine blue Polychromatic – different components of cell wall stain different colour			
Tissue	**Colour seen under microscope**	**Molecules stained**	**Other features**
Phloem	Pink-purple	Cellulose (and other pectic substances, but not lignin)	Thin cell wall
Xylem	Blue, blue-green	Lignin	Thick cell wall

1. Gently transfer the sections into the Petri dish (if the tissue was fixed in Ethanol before sectioning, sit in dH_2O for 3 minutes, then remove the water before staining)
2. Add a drop of Toluidine blue stain. Let the section sit in the stain for 5 minutes.
3. Remove stain by blotting with filter paper. Add distilled water to the dish, swirl and remove water. Repeat until excess stain washes out.
4. Gently transfer the section to a microscopic slide and with filter paper blot out excess water.
5. Immediately add 1 to 2 drops of mountant (Glycerol: Water, 1:1) and add a coverslip over the section. Water can be used for mounting.
6. Label the slide and examine under low (4x objective) and high (10x objective) powers of your microscope.

Preparation and Procedural Notes

NOTES

- This procedure can be done with fresh stem tissue. It can also be done with tissue placed in 70% ethanol for fixation prior to the practical.
- Stains: easiest if you can purchase the solution ready made. Otherwise follow the instructions with the powder for dissolution. Safranin O and toluidine blue in solution are classed non hazardous. Methyl green powder has hazard associated with handling. Dispense stains into dropper bottles and keep in the dark.
- Safranin O is in 50% ethanol. Apart from dissolving the stain, the ethanol also also fixes the tissue sections.
- Toluidine Blue – purchase 0.05% solution. Dilute 1:1 in dH_2O for stem staining procedure, for example 10mL of 0.05% Toluidine blue + 10mL dH_2O.
- Only small volumes of stain (1 to 2 drops) are needed when using small stems (3 to 5mm diameter. Best to have stains in dropper bottles.
- To save some class time, stems may be mounted in wax in the 'microtome' before class. Mount the stem in wax not too long before class to prevent too much drying out of the stem. Make sure there is at least 5mm excess stem above the top of the nut, so this will be trimmed off to reach good fresh tissue.
- The sections can be removed from the surrounding wax (handle them carefully if doing this before staining), or they can remain surrounded by wax until mounting on the slide, then remove the wax.
- Apply the stain directly to the tissue section. Use multiwell dishes/trays or small (for example 35mm) petri dishes. Small wells need less ethanol volume
- 10mL of 70% and 95% ethanol (in capped tubes or bottles, dispensed with transfer pipettes or Pasteur pipettes and bulbs) should be sufficient for the destaining/washing steps as long as excess stain is not used and the procedure times are adhered to. Longer staining makes it harder to remove the excess stain. The result is lack of colour differentiation of the tissues.
- Mountant: many protocols suggest 50% glycerol in water (1 part glycerol: 1 part dH_2O). To prepare 50mL of 50% glycerol, pour 25mL glycerol into a capped tube or other vessel with a well sealed cap or stopper. Add 25 ml of dH_2O. Cap or stopper the tube and mix by inversion. Alternatively, mix equal volumes into a beaker with a magnetic stir bar, stir gently until uniformly mixed. Dispense into small volumes, 1 to 2 ml per group. Glycerol does not dry out as occurs with water mounts, so stains can be done one day and viewed in a subsequent class. It also prevents microbial growth. If students prepare really good sections and stains, they can be kept for a long period in glycerol mountant. The edges of the coverslip can be sealed with nail polish to keep it in place.

NOTES

- Bubbles may appear from the stem tissue after mounting it seems air gets trapped in the tissue sections. The bubbles could be misleading for students, who may misinterpret them as cells.
- Plant tissue may be fixed in 70 % ethanol before sectioning. Otherwise, if you do not want the students to do the sectioning, fresh tissue sections can be fixed in 50-70% ethanol immediately upon sectioning, then distributed to students in small dishes.
- These plant staining procedures produce variations in colour depending on the plant and tissue stained. It can become confusing and may vary depending on the thickness of the tissue section and whether the tissue was fixed in ethanol prior to staining. Focus on the main elements for the vascular tissue, xylem and phloem.

6.2.1 Clearing *(*Dealcoholization*)*

The substitution of dehydrating agent (alcohol or cello solve) by the solvent of mounting medium is called clearing. The term clearing is also used because of the fact that the solvent or clearing agent imparts transparency to tissue.

The best clearing agents are Cedar wood oil and Clove oil but the most commonly used reagent is Xylene. In its place Benzene may also be used Xylene makes the tissue hard and brittle and also causes its shrinkage. As such it may be avoided if possible and should be replaced by Cedar wood oil or Clove oil.

Dehydration is done is graded alcohols or acetones from 70% to absolute alcohol or acetone. Dehydrating alcohol and acetones can remove some of the stains. Time has to be suitably modified to minimize fading of stains. Since alcohol and acetone are miscible in xylol, it is used for clearing the sections. Any sections from which water has not been completely removed would give a milky appearance after the first xylol. Such sections should be returned to absolute. alcohol and the process repeated. Mounting is done after 2nd or 3rd xylol.

Procedure

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

6.2.2 Mounting

The material after it has been made transparent is transferred to a drop of mounting medium which is placed in the centre of slide and is covered by a cover slip.

The mounting medium should be of the same Refractive Index as crown glass (R.I. 1.5).

NOTES

The Best Mounting Mediums are

1. Canada Balsam dissolved in Xylene (1.4 Refractive Index)
2. Euparol (1.4 Refractive Index)

Procedure

With a glass rod put a small drop of anada balsam in the centre of slide. Transfer the material from Xylene to this drop with a brush. Take a cover slip and put its one edge on the slide touching the balsam. The other end of cover slip should be held obliquely through a needle as shown in Figure 6.1.

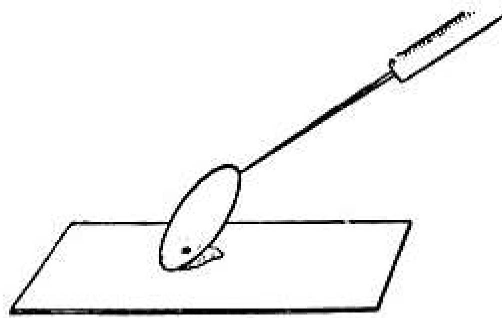

Fig. 6.1 *Method of Holding the Coverslip on Slide*

Now, bring down slowly the edge which is held with needle as shown in Figure 6.2. This will prevent the air bubble from entering in between the balsam and coverslip. Clean the oozing balsam with the help of a blotting paper.

Fig. 6.2 *Method of Putting the Coverslip*

1. There should be no air bubble in balsam.
2. The material should be in the centre of coverslip.
3. The coverslip should be in the centre of slide.
4. The Canada balsam should not ooze out of coverslip.

6.2.3 Mountants

Histological sections which need to be examined for any length of time or to be stored, must be mounted under a cover-slip.

In histology or a pathology laboratory, mounting is the last procedure in the series that ends with a permanent histological preparation on the table, well after the tissue processing and staining, i.e., fixing, paraffin embedding, sectioning, staining, dehydrating, and clearing operations.

The mounting medium is the solution in which the specimen is embedded, generally under a cover glass. It may be liquid, gum or resinous, soluble in water, alcohol or other solvents and be sealed from the external atmosphere by non-soluble ringing media. The main purpose of mounting media is to physically protect the specimen; the mounting medium bonds specimen, slide and coverslip together with a clear durable film. The medium is important for the image formation as it affects the specimen's rendition.

Mounting media should ideally have a Refractive Index (RI) as close as possible to that of the fixed protein (tissue) . As light passes from one medium to another, it changes speed and bends. An example of this is the apparent bending of a stick when placed in water. Light travels fastest in a vacuum and in all other media light travels more slowly. The RI of a medium is the ratio of the speed of light in a vacuum to the speed of light in the medium.

A mounting medium with an RI close to that of the fixed tissue will therefore render it transparent, with only the stained tissue elements visible. This is where the term 'clearing' comes from-xylene, for instance, has an RI very close to that of fixed tissue; therefore, inducing a certain amount of transparency.

A mounting medium with an RI too far either side of 1.53 will provide poor clarity and contrast. This can be demonstrated practically by viewing a tissue section with no mounting medium since air has a RI of 1.0. A mounting medium should be chosen that will not fade the particular stains used; for example, basic aniline dyes should be mounted in non-acid containing mountants. Preparations showing the Prussian blue reaction should be mounted in non-reducing media.

Properties of an Ideal Mounting Media (Mountant)

- RI should be as close as possible to that of glass, i.e., 1.5.
- It should be colorless and transparent.
- It should not cause stain to diffuse or fade.
- It should be dry to a non-stick consistency and harden relatively quickly.
- It should not shrink back from the edge of cover-glass.
- It should be able to completely permeate and fill tissue interstices.
- It should have no adverse effect on tissue components.
- It should be resistant to contamination (particularly microorganism growth).
- It should protect the section from physical damage and chemical activity (oxidation and changes in pH).
- It should be completely miscible with dehydrant or clearing agent.
- It should set without crystallizing, cracking or shrinking (or otherwise deform the material being mounted) and not react with, leach or induce fading in stains and reaction products (including those from enzyme histochemical, hybridization, and immunohistochemical procedures).

- Finally, once set, the mountant should remain stable (in terms of the features listed above).

There are following two types of mounting media:

NOTES

1. Aqueous Media - Used for material which is unstained, stained for fat, or mechanically stained.
2. Resinous Media - For routine staining.

Aqueous Mounting Media

Aqueous mounting medium are used for mounting sections from distilled water when the stains would be decolorized or removed by alcohol and xylene as would be the case with most of the fat stains (Sudan methods). These media are of three types: The syrups, Gelatin media, and Gum Arabic media.

Some of the metachromatic stains tend to diffuse from the sections into mounting media shortly after mounting: this may be prevented by using fructose syrup. This can be avoided by using Highman's medium. Aqueous mounting media require the addition of bacteriostatic agents such as phenol, crystal of thymol or sodium merthiolate to prevent the growth of fungi.

- Water (RI = 1.333)
- Glycerine Jelly (RI = 1.47)
- Glycerine-Glycerol (RI = 1.47)
- Apathy's Medium (RI = 1.52)
- Farrant's Medium (RI = 1.43)
- Highman's Medium (RI = 1.52)
- Fructose Syrup (RI = 1.47)
- Polyvinyl Alcohol

Resinous Media

These are natural or synthetic resins dissolved in benzene, toluene or xylene and are used when a permanent mount is required and frequently used in routine H and E staining procedures.

In general, adhesives harden through solvent evaporation and thereby fix the accompanying coverslip to the slide. During this process the RI of the medium alters, moving away from that of the solvent and toward that of the dry mountant. The exact RI of the applied medium cannot therefore be known. Nevertheless, as the RI of hydrophobic (adhesive) mountants usually approximates that of tissue proteins (fixed) and they provide firm adhesion of the coverslip, these mountants are the type most frequently used.

Natural resinous media are:

- Canada Balsam

- Phenol Balsam
- Variant of Canada Balsam)
- Dammar Balsam
- Euparal

NOTES

Check Your Progress

1. What is staining?
2. What is the use of stains and dyes?
3. Where else can staining be used except in the biological materials?
4. Define cellulose.
5. What is lignin?
6. How can the vascular tissues of plants can be distinguished?
7. What is clearing?

6.3 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image.
2. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues highlighting, for example, muscle fibers or connective tissue, cell populations classifying different blood cells, for instance, or organelles within individual cells.
3. Staining is not limited to biological materials, it can also be used to study the morphology of other materials for example the lamellar structures of semi-crystalline polymers or the domain structures of block copolymers.
4. Cellulose is the main component of all plant cell walls, and is the most abundant organic compound in most plants.
5. Lignin, another polysaccharide, is the next most abundant. Lignin forms a major support system by providing rigidity to xylem, the water transport structure of plants.
6. The vascular tissues of plants can be distinguished by their structure and molecular composition using stains that react differentially with cellulose and lignin.
7. The substitution of dehydrating agent (alcohol or cello solve) by the solvent of mounting medium is called clearing. The term clearing is also used because of the fact that the solvent or clearing agent imparts transparency to tissue.

6.4 SUMMARY

NOTES

- Cellulose is the main component of all plant cell walls, and is the most abundant organic compound in most plants.
- Lignin, another polysaccharide, is the next most abundant. Lignin forms a major support system by providing rigidity to xylem, the water transport structure of plants.
- The vascular tissues of plants can be distinguished by their structure and molecular composition using stains that react differentially with cellulose and lignin.
- In the microscopic analysis of animal and plant tissues, thin sections of tissue are required to achieve a slice with only one or a few layers of cells.
- Tissue is usually fixed (preserved), embedded in wax or a similar material, then passed across a sharp blade to slice off very thin sections.
- The substitution of dehydrating agent (alcohol or cellosolve) by the solvent of mounting medium is called clearing.
- The term clearing is also used because of the fact that the solvent or clearing agent imparts transparency to tissue.
- Dehydration is done is graded alcohols or acetones from 70% to absolute alcohol or acetone. Dehydrating alcohol and acetones can remove some of the stains.
- Histological sections which need to be examined for any length of time or to be stored, must be mounted under a cover-slip.
- The mounting medium is the solution in which the specimen is embedded, generally under a cover glass.
- It may be liquid, gum or resinous, soluble in water, alcohol or other solvents and be sealed from the external atmosphere by non-soluble ringing media.
- The main purpose of mounting media is to physically protect the specimen; the mounting medium bonds specimen, slide and coverslip together with a clear durable film.
- Mounting media should ideally have a Refractive Index (RI) as close as possible to that of the fixed protein (tissue) .
- As light passes from one medium to another, it changes speed and bends. An example of this is the apparent bending of a stick when placed in water.
- Light travels fastest in a vacuum and in all other media light travels more slowly.
- The RI of a medium is the ratio of the speed of light in a vacuum to the speed of light in the medium.

- A mounting medium with an RI close to that of the fixed tissue will therefore render it transparent, with only the stained tissue elements visible.
- A mounting medium with an RI too far either side of 1.53 will provide poor clarity and contrast. This can be demonstrated practically by viewing a tissue section with no mounting medium since air has a RI of 1.0.
- A mounting medium should be chosen that will not fade the particular stains used; for example, basic aniline dyes should be mounted in non-acid containing mountants.

6.5 KEY WORDS

- **Staining:** Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image.
- **Cellulose:** Cellulose is the main component of all plant cell walls, and is the most abundant organic compound in most plants.
- **Lignin:** Lignin is a class of complex organic polymers that form key structural materials in the support tissues of vascular plants and some algae.
- **Clearing:** The substitution of dehydrating agent (alcohol or cello solve) by the solvent of mounting medium is called clearing.
- **Mountant:** Any substance in which a specimen is suspended between a slide and a cover glass for microscopic examination.

6.6. SELF ASSESSMENT QUESTIONS AND EXERCISES

Short Answer Questions

1. Brief about stains and staining.
2. What is Toluidine Blue?
3. What is Dealcoholization?
4. What is the procedure for clearing?
5. What are the properties of an ideal mounting media?

Long Answer Questions

1. Describe stain and staining along with their uses and methods.
2. Explain the significant Double Stain, Safranin O, Methyl Green and Toluidine Blue.
3. Describe about clearing and its procedure in detail.

4. What is mounting? Describe its procedure in detail.
5. Write a note on mountants explaining about the properties of mounting media and its types.
6. Explain about aqueous mounting media and resinous media.

NOTES

6.7 FURTHER READINGS

Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

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BLOCK - III
HISTOCHEMICAL STUDIES

NOTES

UNIT 7 HISTOCHEMICAL TECHNIQUES

Structure

7.0 Introduction
7.1 Objectives
7.2 Histochemical Techniques: An Introduction
  7.2.1 Lipids Staining
  7.2.2 Protein and Amino Acids
  7.2.3 PAS Reaction (Periodic Acid Schiff)
  7.2.4 Enzyme
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7.0 INTRODUCTION

Histochemistry combines the techniques of biochemistry and histology in the study of the chemical constitution of cells and tissues. The importance of histochemistry has decreased as IHC methods have developed. However, the breadth of knowledge for many histochemical methods is such that they are still used in decision-making, including in our laboratories. The availability of commercial staining kits for many established histochemical methods is a draw to continue using these methods. However, some complex stains may require contracting out the staining to specialized laboratories. Finally, histochemical stains often provide high contrast, which allows for image analysis.

Since histochemical reactions take place over time and are subject to enzyme kinetics, it is also possible, with sufficient knowledge of the pharmacokinetics of a given reaction, to perform quantitative histochemistry by applying morphometric analysis to an experimental tissue and comparing to an appropriate control. Quantitative histochemistry requires the use of specialized microscopic setups that use a photometer or fluorometer to standardize light emission or fluorescence between samples, and to objectively quantitate outcomes based on mathematic models of emission; hence, it is not to be undertaken lightly. Nevertheless, when applied to a specific and localizable enzymic reaction or cell component, quantitative histochemical studies may potentially add greatly to an investigator's

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understanding of how cellular homeostatic reactions are affected by drugs or xenobiotic chemicals.

In this unit, you will study about various histochemical techniques and its various methods that includes proteins, carbohydrates, lipids and enzymes.

7.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand what histochemical techniques are
- Explain about histochemical techniques
- Discuss different types of histochemical techniques, such as proteins, carbohydrates, lipids and enzymes

7.2 HISTOCHEMICAL TECHNIQUES: AN INTRODUCTION

Histochemistry is an important technique that is used for the visualization of biological structures. As such, it is concerned with the identification and distribution of various chemical components of tissues through the use of stains, indicators as well as microscopy.

Essentially, identification and distribution of chemical constituents of tissues is achieved through the exploitation of unique chemical environments in cells, heterologous expression techniques as well as enzymatic activities.

The various methods for histochemical techniques includes the staining of:

- Proteins
- Carbohydrates
- Lipids
- Enzymes

7.2.1 Lipids Staining

This technique is dependent on dyes that are soluble in lipids. Some of the most common dyes used include:

- Sudan VI
- Sudan Black
- Oil Red O
- Nile Blue

Lipid staining is a useful technique that is used for demonstrating intracellular lipids in various tissue sections.

Principle

For this technique, the dye is more soluble in the lipid, which allows it to be more demonstrated than in the vehicular solvent. The dyes used in this technique are all interchangeable, which means that they can be substituted for each other for the staining process.

Staining Procedure

Requirements/Reagents

- ORO (Oil Red O) solution
- Glycerine jelly mounting medium

Procedure

- Cut the sample to obtain sections of between 8 and 10 microns and air dry
- Rinse the section with 60 percent isopropanol
- Stain the section with the Oil Red O working solution for about 15 minutes
- Rinse the specimen with 60 percent isopropanol
- Dip the section in Alum hematoxylin a few times in order to stain the nuclei
- Rinse in distilled water
- Mount the specimen in water or in glycerin jelly

Observation

Red color indicates the lipid while blue coloration indicates the nuclei.

Lipid staining technique is useful for showing the normal distribution of lipids as well as disease-related lipid accumulation.

7.2.2 Protein and Amino Acids

Some of the methods used for specific amino acids include:

- Millon's Reaction
- Sakaguchi Reaction
- Tetrazotized Benzidine Reaction

Millon's Reagent

Millon's reagent is used for detecting amino acid tyrosine:

Principle

In this technique, the mercurous and mercuric nitrate (components of the reagent) reacts with hydroxybenzene radicals to form a compound that is red in color. Tyrosine contains the phenolic group, which forms the red coloration in the presence of Millon's reagent. The compound formed through this reaction is called mercuric fumarate.

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Millon's Method Procedure

- Add about 2ml of the protein solution into a test-tube.
- Add a few drops of the reagent (Millon's reagent) in to the test-tube that contains the original solution.
- Using a tube holder, hold the test-tube over boiling water or flame and boil for about half a minute.

Observation

If the solution turns reddish in color after boiling, then tyrosine is present in the solution.

Sakaguchi Test

The Sakaguchi reaction test involves the use of the Sakaguchi reagent. This reagent is composed of 1-naphthol and sodium hypobromite and forms a reddish compound when mixed with the sample containing arginine.

The test is positive for any amino acid that contains the guanidine group in Arginine. Therefore, the Guanidine group in an amino acid will react with the α-Naphthol and alkaline hypobromite in the reagent to give of a red-colored complex indicating the presence of such amino acids.

Sakaguchi's Test Procedure

- Add 1 ml of the protein solution in to a test-tube
- Add 2 or 3 drops of 40 percent of sodium hydroxide, 2 drops of ethanolic α-Naphthol and 5 drops of bromide water.
- Mix the contents in the test-tube well.
- If a red-color complex forms when shaking the contents, then it is positive that the solution contains Arginine or a protein containing Arginine.

Tetrazotized Benzidine Reaction

Although it has been shown to be less effective, Tetrazotized benzidine is used in histochemistry to detect non-collagen proteins. The procedure involves the coupling of Tetrazotized benzidine with beta naphthol or Hyaluronic acid.

The method, commonly referred to as Tetrazotized benzidine helps in the detection of such non-collagen proteins as tyrosine, histidine as well as tryptophan.

Nucleic Acids and DNA

Feulgen's Reaction

This is a relatively new technique that is used for demonstrating DNA in tissue sections. It is a sensitive means of detecting aldehydes, which makes it the ideal method for detecting the presence of DNA. Here, the section is treated with dilute hydrochloric acid in order to remove the bases.

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The sugar part that remains reacts as an aldehyde ultimately forming a visible color. Therefore, this method can be said to be divided in to two main parts:

1. The first part of the procedure is the hydrolysis phase that involves the use of 5N HCl, ambient temperature for 40 minutes. This step is aimed at separately selecting 2 purine bases (Adenine and Guanine) which are removed from the DNA molecule.
2. The second step is the staining phase. The reagent used is preferred because it is highly selective for DNA rather than RNA. Here, RNA does not react because of the presence of hydroxyl on carbon 2 of ribose, which prevents the acid (HCl) from hydrolyzing sugar. The reaction is also precise for the localization of DNA given that deoxyribose radicals are bound to phosphoric acid of the apurinic acid molecule following the removal of purine bases.

Some of the stains used for both DNA and RNA include,

- Methyl Green Pyronin Stain
- Acridine Orange

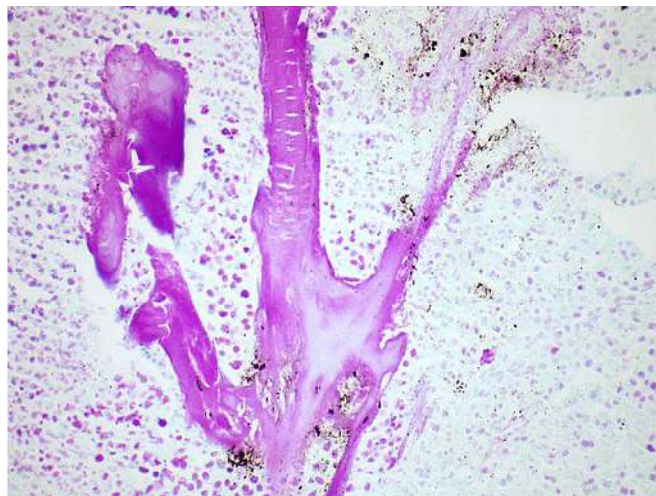

Fig. 7.1 *DNA Straining*

7.2.3 PAS Reaction (Periodic Acid Schiff)

This is one of the most popular histochemical techniques for the detection of glycogen. It has been shown to be one of the best techniques for demonstrating carbohydrates in tissue. In this technique, the periodic acid oxidizes tissue carbohydrates to produce aldehyde groups. This group then condenses with the reagent to form a bright red coloration to demonstrate the tissue component with carbohydrate attachments.

The diastase and a-amylase in the reagent act on the glycogen and depolymerize it into smaller sugar units (maltose and glucose) which are then washed out of the section.

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Procedure

- Deparaffinize and hydrate the slide using distilled water
- Place the section in preheated diastate solution (at 37 degrees centigrade) for about an hour
- Wash the sample in running water for about 5 minutes
- Place the sections in 0.5 per cent periodic acid solution for about 5 minutes
- Wash the section in distilled water
- Place the section in Schiff reagent for about 15 minutes
- Wash the section for about a minute in 0.55 per cent potassium metabisulfite in order to remove excess stains
- wash in running tap water for about 10 minutes
- Counterstain using Harris's hematoxylin with acetic acid for half a minute
- Wash with running water
- Dehydrate with two changes using absolute alcohol, clear with xylene and mount to view

Some of the other stains used for staining sacchrides include,

- Lectins
- Ruthenium Red
- Alcian Blue

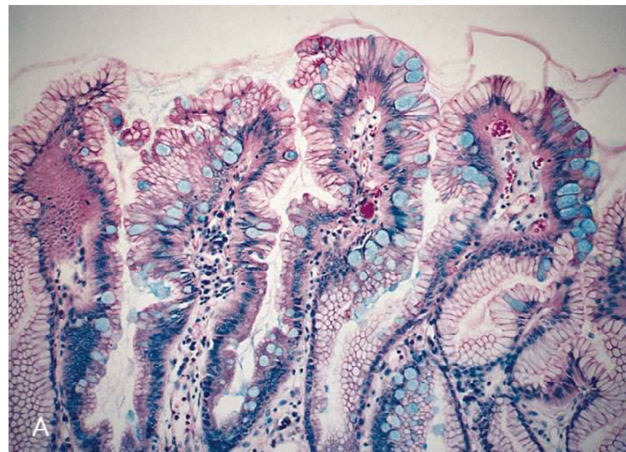

***Fig.* 7.2** *Detection of Glycogen*

Immunochemistry

Whereas histochemistry includes a number of techniques used for the visualization of various chemical components in tissues, immunochemistry involves the study of identities and functions of components of the immune system (particularly antibodies).

Essentially, immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies. This allows for these methods to be used to quantify antigens or antibodies.

All the immunochemical methods/techniques depend on a highly specific and sensitive reaction between antigens and antibodies. There are a number of immunochemistry techniques based on the type of reaction, reagents and samples that are used which includes

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Particle Methods - This is the technique where the antigen-antibody interaction is observed. It includes a number of methods such as Immunoprecipitation, Immunoelectrophoresis, Immunofixation.

Label Methods - With label methods, either the antigen or the antibody is labeled allowing for the antigen-antibody reaction to be observed. Immunoassay and competitive binding are examples of label methods. Some of the other methods include:

- Immunofluorescence
- Immunoelectron Microscopy

7.2.4 Enzyme

Enzyme histochemistry serves as a link between biochemistry and morphology. It is based on metabolization of a substrate provided to a tissue enzyme in its orthotopic localization. Visualization is accomplished with an insoluble dye product. It is a sensitive dynamic technique that mirrors even early metabolic imbalance of a pathological tissue lesion, combined with the advantage of histotopographic enzyme localization. With the advent of immunohistochemistry and DNA-oriented molecular pathology techniques, the potential of enzyme histochemistry currently tends to be underrecognized. This review aims to draw attention to the broad range of applications of this simple, rapid and inexpensive method. Alkaline phosphatase represents tissue barrier functions in brain capillaries, duodenal enterocyte and proximal kidney tubule brush borders. Decrease in enzyme histochemical alkaline phosphatase activity indicates serious functional impairment. Enzyme histochemical increase in lysosomal acid phosphatase activity is an early marker of ischemic tissue lesions. Over the last four decades, acetylcholinesterase enzyme histochemistry has proven to be the gold standard for the diagnosis of Hirschsprung disease and is one of the most commonly applied enzyme histochemical methods today. Chloroacetate esterase and tartrate-resistant phosphatase are both resistant to formalin fixation, EDTA decalcification and paraffin embedding. Early enzyme histochemical insight into development of a pathologic tissue lesion and evaluation of function and vitality of tissue enhance our understanding of the pathophysiology of diseases. In this process, enzyme histochemistry constitutes a valuable complement to conventional histology, immunohistochemistry and molecular pathology for both diagnostic and experimental pathology.

NOTES

Check Your Progress

1. What is histochemistry?
2. What are the various methods for histochemical techniques used in staining?
3. Name some of the common dyes used in lipid staining.
4. What is Millon's reagent is used for?
5. Define PAS reaction.
6. What does the term immunoelectrophoresis mean?
7. Define immunochemistry.

7.3 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Histochemistry is an important technique that is used for the visualization of biological structures.
2. The various methods for histochemical techniques includes the staining of -
    - Proteins
    - Carbohydrates
    - Lipids
    - Enzymes
3. Some of the most common dyes used in lipid staining include:
    - Sudan VI
    - Sudan Black
    - Oil Red O
    - Nile Blue
4. Millon's reagent is used for detecting amino acid tyrosine.
5. PAS reaction (Periodic Acid Schiff) is one of the most popular histochemical techniques for the detection of glycogen. It has been shown to be one of the best techniques for demonstrating carbohydrates in tissue.
6. Immunoelectrophoresis also called gamma globulin electrophoresis, or immunoglobulin electrophoresis, is a method of determining the blood levels of three major immunoglobulins: immunoglobulin M (IgM), immunoglobulin G (IgG), and immunoglobulin A (IgA).
7. Immunochemistry is a branch of chemistry that involves the study of the molecular mechanisms underlying the function of the immune system, especially the nature of antibodies, antigens and their interactions.

7.4 SUMMARY

NOTES

- Histochemistry is an important technique that is used for the visualization of biological structures.
- It is concerned with the identification and distribution of various chemical components of tissues through the use of stains, indicators as well as microscopy.
- Identification and distribution of chemical constituents of tissues is achieved through the exploitation of unique chemical environments in cells, heterologous expression techniques as well as enzymatic activities.
- Millon's reagent is used for detecting amino acid tyrosine. In this technique, the mercurous and mercuric nitrate (components of the reagent) reacts with hydroxybenzene radicals to form a compound that is red in color.
- Tyrosine contains the phenolic group, which forms the red coloration in the presence of Millon's reagent. The compound formed through this reaction is called mercuric fumarate.
- The Sakaguchi reaction test involves the use of the Sakaguchi reagent. This reagent is composed of 1-naphthol and sodium hypobromite and forms a reddish compound when mixed with the sample containing arginine.
- The test is positive for any amino acid that contains the guanidine group in Arginine. Therefore, the Guanidine group in an amino acid will react with the á-Naphthol and alkaline hypobromite in the reagent to give of a red-colored complex indicating the presence of such amino-acids.
- Tetrazotized benzidine is used in histochemistry to detect non-collagen proteins. The procedure involves the coupling of Tetrazotized benzidine with beta naphthol or Hyaluronic acid.
- The method, commonly referred to as Tetrazotized benzidine helps in the detection of such non-collagen proteins as tyrosine, histidine as well as tryptophan.
- Feulgen's Reaction is a relatively new technique that is used for demonstrating DNA in tissue sections. It is a sensitive means of detecting aldehydes, which makes it the ideal method for detecting the presence of DNA. Here, the section is treated with dilute hydrochloric acid in order to remove the bases.
- PAS reaction (Periodic Acid Schiff) is one of the most popular histochemical techniques for the detection of glycogen. It has been shown to be one of the best techniques for demonstrating carbohydrates in tissue.
- The diastase and a-amylase in the reagent act on the glycogen and depolymerize it into smaller sugar units (maltose and glucose) which are then washed out of the section.
- Immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies. This allows for these methods to be used to quantify antigens or antibodies.

NOTES

- All the immunochemical methods/techniques depend on a highly specific and sensitive reaction between antigens and antibodies.
- Particle methods is a technique where the antigen-antibody interaction is observed. It includes a number of methods such as Immunoprecipitation, Immunoelectrophoresis, Immunofixation.

7.5 KEY WORDS

- **Morphometrics:** Morphometrics refers to the quantitative analysis of form, a concept that encompasses size and shape.
- **Fluorometer**: A fluorometer or fluorimeter is a device used to measure parameters of fluorescence: its intensity and wavelength distribution of emission spectrum after excitation by a certain spectrum of light.
- **Xenobiotic**: A xenobiotic is a chemical substance found within an organism that is not naturally produced or expected to be present within the organism. It can also cover substances that are present in much higher concentrations than are usual.
- **Hematoxylin**: Hematoxylin is a natural product extracted from the heartwood of the logwood tree. Hematoxylin is relatively colorless and without further modifications has little or no value as a biological stain.
- **Tyrosine**: Tyrosine is an amino acid. Amino acids are the building blocks of protein. The body makes tyrosine from another amino acid called phenylalanine.
- **Tryptophan:** It is an essential amino acid that serves several important purposes, like nitrogen balance in adults and growth in infants. It also creates niacin, which is essential in creating the neurotransmitter serotonin.
- **Hydroxyl:** A hydroxy or hydroxyl group is the entity with the formula OH. It contains oxygen bonded to hydrogen.
- **Immunochemistry:** Immunochemistry is a branch of chemistry that involves the study of the molecular mechanisms underlying the function of the immune system, especially the nature of antibodies, antigens and their interactions.
- **Immunoprecipitation**: Immunoprecipitation (IP) is the technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein.
- **Immunoelectrophoresis**: Immunoelectrophoresis also called gamma globulin electrophoresis, or immunoglobulin electrophoresis, is a method of determining the blood levels of three major immunoglobulins: immunoglobulin M (IgM), immunoglobulin G (IgG), and immunoglobulin A (IgA).
- **Immunofixation**: Immunofixation permits the detection and typing of monoclonal antibodies or immunoglobulins in serum or urine.

7.6 SELF ASSESSMENT QUESTIONS AND EXERCISES

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Short Answer Questions

1. Brief about histochemical techniques.
2. What is lipid staining?
3. What is Millon's reagent?
4. Brief about Feulgen's reaction.
5. Define immunochemistry.
6. Differentiate between label method and particle method.

Long Answer Questions

1. Explain about histochemistry and various histochemical techniques.
2. Explain in detail about lipid staining.
3. Write about PAS reaction.
4. Discuss in detail about Sakaguchi reaction test.
5. Write about enzymes in detail.

7.7 FURTHER READINGS

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UNIT 8 MICRO SLIDE PREPARATION

Structure

8.0 Introduction
8.1 Objectives
8.2 Micro Slide Preparation
8.2.1 Preparation Techniques: Dry Mounts, Wet Mount, Squash, Staining
8.2.2 Whole Mounts
8.3 Answers to Check Your Progress Questions
8.4 Summary
8.5 Key Words
8.6 Self Assessment Questions and Exercices
8.7 Further Readings

8.0 INTRODUCTION

Microscope slides are pieces of transparent glass or plastic that support a sample so that it can be viewed using a light microscope. There are different types of microscopes and also different types of samples, so there is more than one way to prepare a microscope slide. Three of the most common methods are wet mounts, dry mounts, and smears.

A microscope slide is a thin flat piece of glass, typically 75 by 26 mm- 3 by 1 inches and about 1 mm thick, used to hold objects for examination under a microscope. Typically the object is mounted on the slide, and then both are inserted together in the microscope for viewing. This arrangement allows several slide-mounted objects to be quickly inserted and removed from the microscope, labeled, transported, and stored in appropriate slide cases or folders, etc.

Microscope slides are often used together with a cover slip or cover glass, a smaller and thinner sheet of glass that is placed over the specimen. Slides are held in place on the microscope's stage by slide clips, slide clamps or a cross-table which is used to achieve precise, remote movement of the slide upon the microscope's stage, such as in an automated / computer operated system, or where touching the slide with fingers is inappropriate either due to the risk of contamination or lack of precision.

In this unit, you will study how to prepare the micro slides and the different methods of preparing the slides which includes - wet mounts, dry mounts, and smears, squashes. Along with this you will also study about whole mounts.

8.1 OBJECTIVES

After going through this unit, you will be able to

- Understand how micro slides are prepared
- Discuss about whole mounts
- Explain the procedure of smears and squashes

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8.2 MICRO SLIDE PREPARATION

Objects magnified under compound microscopes are mounted onto microscope slides. Made of glass or plastic, slides are approximately 1x3 inches and between 1mm-1.2 mm thick.

Multiple methods of preparation allow for advanced viewing of inorganic and organic objects.

Flat and Concave Styles

The most basic of all microscope slides is a flat rectangular piece of soda lime glass, borosilicate cover glass or plastic, with ground edges. All corners are a sharp 90-degrees and, along with a rough outer edge, can cause minor finger cuts if not handled with care. The top and/or bottom edges of a slide can be frosted, enabling easy marking for sample identification and/or orientation. The etched frosting keeps all pen marks safely away from the sample and a selection of frosted colors provides additional means of categorization.

Rounded safety corners to prevent accidental cuts as well as beveled edges with clipped corners ideal for blood samples are options available for both generic and frosted slides.

Concave microscope slides contain one or more surface depressions ideal for liquid solutions and larger specimens. These more expensive microscope slides can be used without a cover. Some manufacturers produce plastic chambers with a predetermined number of slides with covers. Filled calibrated wells or flasks are viewed quickly without preparing or clipping individual slides to the microscope stage, making this especially useful in sediment studies, such as urine analysis; in addition, some tray designs can be placed in an incubator or refrigerator, allowing for the study of cultured samples.

Additional Features

Some cells and tissues cannot adhere to a plain glass surface and require a positive charge or surface modifications. Saving time and money, electrostatic charged slides are a popular choice for researchers of histology, cytology and pathology. Surfaces treated with biological reagents can make a slide water-proof, resistant to certain chemicals and reduce instances of cross-contamination.

Additional variations to microscope slides include,

Etched Grid System or Graticule

- Enables researcher to monitor and communicate area(s) of interest
- Aids in hand sketching
- Helps geographical plotting
- Estimates size and scale

Dual Concavity

- Side-by-side comparisons, including sample to control
- Reduces risk of cross-contamination

Transparent Mica

- Rarely used substitute for glass
- Less prone to dust, scratching
- Prevents glare

Cover Slips

Almost always made of borosilicate or silicate glass, cover slips hold samples in place and protect them from inadvertent movement and contamination.

It also protects the microscope, preventing direct contact between the sample and lens as well as accidental leakage of water-based preparations.

The thin, transparent cover glass is usually square and available in types number 1 and 2. Suited for high-resolution microscopy and oil immersion preparations, Number 1 covers are .13-.17mm thick. Number 2 covers, .17-.25 mm, are designed for general purpose. Less frequent characteristics include rectangular shapes, alternative materials such as quartz and certain types of plastic, etched lines or grids and additional thicknesses.

If not creating a permanent slide with glue and/or sealant, cover slips can be removed and sterilized for multiple reuses.

8.2.1 Preparation Techniques: Dry Mounts, Wet Mount, Squash, Staining

The main methods of placing samples onto microscope slides are wet mount, dry mount, smear, squash and staining.

Dry Mount

The dry mount is the most basic technique: simply position a thinly sliced section on the center of the slide and place a cover slip over the sample.

Dry mounts are ideal for observing hair, feathers, airborne particles such as pollens and dust as well as dead matter such as insect and aphid legs or antennae. Opaque specimens require very fine slices for adequate illumination.

Since they are used for primarily inorganic and dead matter, dry mounts can theoretically last indefinitely.

Wet Mount

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Used for aquatic samples, living organisms and natural observations, wet mounts suspend specimens in fluids such as water, brine, glycerin and immersion oil. A wet mount requires a liquid, tweezers, pipette and paper towels.

To prepare the slide

- Place a drop of fluid in the center of the slide
- Position sample on liquid, using tweezers
- At an angle, place one side of the cover slip against the slide making contact with outer edge of the liquid drop
- Lower the cover slowly, avoiding air bubbles
- Remove excess water with the paper towel

Although wet mounts can be used to prepare a significantly wide range of microscope slides, they provide a transitory window as the liquid will dehydrate and living specimens will die.

Organisms such as protozoa may only live 30 minutes under a wet mount slide; applying petroleum jelly to the outer edges of the cover slip creates a seal that may extend the life of the slide up to a few days.

In addition, larger protozoan such as paramecium may be too large and/or move too quickly under the wet mount.

In these circumstances, adding ground pieces of cover glass to the water before the slip layer will create added space and chemicals or strands of cotton can be added to slow the movement of paramecium, amoeba and ciliates.

Smear Slides

Smear slides require two or more flat, plain slides, cover slips, pipette and tissue paper

- Pipe a liquid sample such as blood or slime onto a slide
- Using the edge of the second slide, slowly smear the sample creating a thin, even coating
- Put a cover slip over the sample, careful not to trap air bubbles
- Remove excess liquid

Ideally, smears should dry naturally in an environment of moderate, steady temperature.

The angle of the smearing slide determines the length of the smear; a steeper angle creates a shorter smear. For samples such as blood, begin by backing the smearing slide into the sample and then push across the slide, pulling the blood in the opposite direction to create a smooth layer.

A thicker slide can be created with two drops, but only with the blood of mammals as the erythrocytes lack a nucleus allowing cells to be amassed in multiple layers.

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Squash Slides

Designed for soft samples, squash slides begin by preparing a wet mount; place lens tissue over the cover glass; gently press down, careful not to destroy the sample or break the cover glass, and squash the sample; remove excess water.

Staining

A variety of methods exist for staining microscope slides, including non-vital or in vitro stains of non-living cells and vital or in-vivo stains of living tissue. Staining provides contrast through color that reveals structural details undetected in other slide preparations.

Staining solutions such as iodine, methylene blue and crystal violet can be added to wet or dry mounts.

A Simple Staining Method

- Add a drop of staining solution on the edge of one side of the cover slip
- Position the edge of a paper towel on the opposite end
- Allow dye to be pulled across the specimen

Stains are especially useful in the fields of histology, virology and pathology, allowing researchers to study and diagnose diseases, identify gram positive and negative bacteria as well as examine detailed attributes of a variety of cells.

8.2.2 Whole Mounts

Whole mounts is the preparation of an entire organism or structure, which is small enough or thin enough to be placed directly onto a microscope slide (e.g. a small unicellular or multi-cellular organism or a membrane that can be stretched thinly on to a slide).

Whole mount staining is the staining of small pieces of tissue – usually embryos – without sectioning.

Whole mount staining is very similar to immunocytochemistry (ICC) or staining of cryosections. If an antibody has been used successfully on cryosections (this does not include paraffin-embedded sections), then the antibody should work for a whole mount embryo.

The difference is that the sample being stained is much larger and thicker than a normal section on a slide. Therefore, incubations for fixative, blocking buffer, antibody, wash buffer, permeabilization and substrate color development will need to be much longer to allow for permeabilization right into the center of the sample.

Based on the purpose and length of use the whole mounts are divided into

- Temporary Whole Mount
- Semi- permanent Whole Mount
- Permanent Whole Mount

Temporary Whole Mount

- Mostly for classwork purpose.
- The plant sample is mounted is water.

Semi-Permanent Whole Mount

- The materials preparation is kept for little longer (few hours to a fortnight).
- The plant sample is mounted in Glycerine (mounting medium)
- Pure glycerine or Aqueous dilution of Glycerine or Glycerine jelly are used for making semi-permanent mounts. Kaiser's glycerine jelly
  (a) Pure gelatin – 1 part
  (b) Water – 6 part
- Advantage – the glycerine preserve the natural colours of the plant specimens.
- In semi permanent preparation the slides are sealed by Canada balsam.

The Semi-Permanent Preparation Whole Mount is Ideal for

- Unicellular and Colonial Algae
- Mosses Protonemata
- Fungal Spores
- Ferns – Prothalli

Permanent Whole Mount Preparation

Several methods and available but two methods often used for the whole mount preparation are

- Hygrobutol Method
- Glycerine – Xylol Method.

Hygrobutol Method

- Kill and fix the specimen in Chrome – acetic (fixative)
- Place the specimens in watch glass and periodically remove the fluid come out from the material.
- Wash the material in running water.

- Staining – Suitable stains are
  - (a) Harris Haematoxylin
  - (b) Iron Haematoxylin
  - (c) Mayer's Carmalum
- Wash the materials in differentiating solution (agents)-15%,30%,50%,and 70% ethyl alcohol at least 20 min in each solution –(Note-to be closed in order to avoid the evaporation of differentiating agents).
- Leave the specimen in 85% alcohol for 18 hrs.
- Counter stain is dissolved in 95% alcohol and methyl cellosolve.
- A complete wash in 95% alcohol.
- Add hygrobutol gradually (a solution – contain 90 parts hygrobutol and 10 parts ethyl alcohol)
- Place the specimens in Butyl Balsam.
- The medium in specimens (Watch glass) – gradually allowed for evaporation – (2 hrs).
- The specimens transferred to mounting medium.
- Finally, Covered, Sealed and Used as a "Permanent Whole Mount". Preparation – Store for long period.

Glycerine – Xylol Method

- Simple and inexpensive method and often used for permanent whole mount preparation.
- Kill and fix – Chrome Acetic (fixation)
- Wash material – thoroughly in running water
- Stain – in Haematoxylin solution (a basic stain)
- Transfer the stained specimen in 10% aqueous glycerine – use watch glass / petri dish.
- Keep the specimen (container) in undisturbed, dust free condition.
- Leave the specimen for few days until the diluted glycerine attains the consistency to pure glycerine.
- If required, the specimen container to kept in the hot-air over to accelerate the process of evaporation.
- Remove the Glycerine by using 95% Alcohol.
- Now, the specimen need posed to counterstaining, if not pass on to the next process.
- To complete the process of dehydration the absolute alcohol is used.

- De-alcholize the specimen by gradual replacement in – Alcohol : xylol = 9 : 1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2:8, 1 : 9 and finally pure alcohol. Allow the specimen in 5 to 10 minutes in each solution.
- The specimen is placed in Xylol.
- Replace the Xylol with Balsam.
- Finally mount the specimen with coverslip on mounting medium and used as 'Permanent Whole Mount' Preparation.

Check Your Progress

1. Define staining.
2. What is whole mount?
3. Give a difference between dry and wet mount.
4. How is wet mount slide prepared?
5. In how many parts whole mount divided?
6. What are methods that are used in permanent whole mount preparation?

8.3 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes.
2. Whole-mounts is the preparation of an entire organism or structure, which is small enough or thin enough to be placed directly onto a microscope slide.
3. In a dry mount, the specimen is placed directly on the slide. A cover slip may be used to keep the specimen in place and to help protect the objective lens. Dry mounts are suitable for specimens such as samples of pollen, hair, feathers or plant materials. In a wet mount, a drop of water is used to suspend the specimen between the slide and cover slip. Place a sample on the slide. Using a pipette, place a drop of water on the specimen.
4. Here are the steps to prepare wet mount slide
   - Place a drop of fluid in the center of the slide
   - Position sample on liquid, using tweezers
   - At an angle, place one side of the cover slip against the slide making contact with outer edge of the liquid drop
   - Lower the cover slowly, avoiding air bubbles
   - Remove excess water with the paper towel

5. Based on the purpose and length of use the whole mounts are divided into
   - Temporary Whole Mount
   - Semi- Permanent Whole Mount
   - Permanent Whole Mount
6. Several methods and available but two methods often used in permanent whole mount preparation are
   - Hygrobutol Method
   - Glycerine – Xylol method.

8.4 SUMMARY

- Objects magnified under compound microscopes are mounted onto microscope slides. Made of glass or plastic, slides are approximately 1x3 inches and between 1mm-1.2 mm thick.
- Multiple methods of preparation allow for advanced viewing of inorganic and organic objects.
- Rounded safety corners to prevent accidental cuts as well as beveled edges with clipped corners ideal for blood samples are options available for both generic and frosted slides.
- Concave microscope slides contain one or more surface depressions ideal for liquid solutions and larger specimens. These more expensive microscope slides can be used without a cover.
- Some manufacturers produce plastic chambers with a predetermined number of slides with covers.
- Some cells and tissues cannot adhere to a plain glass surface and require a positive charge or surface modifications. Saving time and money, electrostatic charged slides are a popular choice for researchers of histology, cytology and pathology.
- Surfaces treated with biological reagents can make a slide water-proof, resistant to certain chemicals and reduce instances of cross-contamination.
- Almost always made of borosilicate or silicate glass, cover slips hold samples in place and protect them from inadvertent movement and contamination.
- The dry mount is the most basic technique: simply position a thinly sliced section on the center of the slide and place a cover slip over the sample.
- Dry mounts are ideal for observing hair, feathers, airborne particles such as pollens and dust as well as dead matter such as insect and aphid legs or antennae. Opaque specimens require very fine slices for adequate illumination.

NOTES

- Used for aquatic samples, living organisms and natural observations, wet mounts suspend specimens in fluids such as water, brine, glycerin and immersion oil. A wet mount requires a liquid, tweezers, pipette and paper towels.
- Designed for soft samples, squash slides begin by preparing a wet mount; place lens tissue over the cover glass; gently press down, careful not to destroy the sample or break the cover glass, and squash the sample; remove excess water.
- A variety of methods exist for staining microscope slides, including non-vital or in vitro stains of non-living cells and vital or in-vivo stains of living tissue. Staining provides contrast through color that reveals structural details undetected in other slide preparations.
- Staining solutions such as iodine, methylene blue and crystal violet can be added to wet or dry mounts.
- Whole-mounts is the preparation of an entire organism or structure, which is small enough or thin enough to be placed directly onto a microscope slide.

8.5 KEY WORDS

- **Ciliate:** The ciliates are a group of protozoans characterized by the presence of hair-like organelles called cilia, which are identical in structure to eukaryotic flagella, but are in general shorter and present in much larger numbers, with a different undulating pattern than flagella.
- **Staining:** Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image.
- **Whole mounts:** Whole mounts is the preparation of an entire organism or structure, which is small enough or thin enough to be placed directly onto a microscope slide.
- **Dry mount:** In a dry mount, the specimen is placed directly on the slide and cover slip may be used to keep the specimen in place and to help protect the objective lens.
- **Wet mount:** In a wet mount, a drop of water is used to suspend the specimen between the slide and cover slip.
- **Immunocytochemistry:** Immunocytochemistry (ICC) is a common laboratory technique that is used to anatomically visualize the localization of a specific protein or antigen in cells by use of a specific primary antibody that binds to it.
- **Cryosection:** This is the frozen section procedure is a pathological laboratory procedure to perform rapid microscopic analysis of a specimen. It is used most often in oncological surgery.

NOTES

- **Protonema;** A protonema is a thread-like chain of cells that forms the earliest stage of a bryophyte life cycle.
- **Prothallium:** A prothallium or prothallus is usually the gametophyte stage in the life of a fern or other pteridophyte. Occasionally the term is also used to describe the young gametophyte of a liverwort or peat moss as well.

8.6 SELF ASSESSMENT QUESTIONS AND EXERCICES

Short Answer Questions

1. What are flat and concave styles?
2. What are cover slips and how are they used?
3. What is dry mount and how is it used?
4. Write a short note on wet mounts and the steps to prepare wet mount slide.
5. What is staining ?
6. What is whole mount?

Long Answer Questions

1. Give a general introduction how micro slides are prepared and what are the different equipments needed.
2. Explain in detail about dry mounts.
3. Discuss about wet mounts also how the slides are prepared.
4. Distinguish between smear and squash slides.
5. Write a detailed note on whole mounts.
6. Explain in detail about hygrobutol method.
7. Discuss about the glycerine – xylol method.

8.7 FURTHER READINGS

Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

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NOTES

UNIT 9 SECTIONING OF BIOLOGICAL SPECIMENS

Structure

9.0 Introduction
9.1 Objectives
9.2 Sectioning of Biological Specimens: Basic Concepts
 9.2.1 Free Hand Sectioning
 9.2.2 Hand Microtome
 9.2.3 Rotary Microtome Sectioning
 9.2.4 Sledge Microtome
9.3 Embedding Methods
9.4 Answers to Check Your Progress Questions
9.5 Summary
9.6 Key Words
9.7 Self Assessment Questions and Exercises
9.8 Further Readings

9.0 INTRODUCTION

Sectioning is the first step to prepare a slide of the biological material for microscopic investigation. Fresh or preserved materials are cut into thin sections at suitable plane. It is essential to cut section thin enough to observe the details at the required level. Hand sectioning is carried out with sharp razor.Uniform section of given thickness can be obtained by special section cutting machines called microtome. Prior to microtome section cutting, material is processed which involves dehydration, fixation and embedding of the tissue material in wax blocks. Ultra microtomes are used to obtain extremely thin sections (20-100 nm) required for observation under electron microscope.

In this unit, you will study about sectioning of biological specimens, free hand, hand microtome, sludge, rotary microtome sectioning is also described, embedding methods are also explained in detail.

9.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand about sectioning of biological specimens
- Explain what free hand, hand microtome, sludge is
- Discuss about rotary microtome sectioning
- Describe about various embedding methods

9.2 SECTIONING OF BIOLOGICAL SPECIMENS: BASIC CONCEPTS

NOTES

There are several methods to prepare biological specimens depending on the nature of the specimen and the purpose of the study. In this unit we will discuss about some commonly used methods. Fundamentally, for routine microscopic examination, it is generally accepted that specimens should be thin, dry and contain molecules which diffract electrons. Biological specimens, which are large and consist of large amounts of water, also do not diffract electrons and are therefore difficult to examine and analyse under the microscope. While preparing biological specimens for the any microscope, one must keep in mind the structural morphology of the material. Small or very thin objects can be examined directly by mounting them onto a support film and introducing them directly into the electron beam.

Ultra-microtomy is a method for cutting specimens into extremely thin slices, called ultra-thin sections that can be studied and documented at different magnifications in a microscope. It is used mostly for biological specimens, but sections of plastics and soft metals can also be prepared. Ultra-thin sections of specimens are cut using a specialized instrument called an 'ultra-microtome'.

9.2.1 Free Hand Sectioning

Free hand sectioning is a micro technique or method of preparing portions of the media to be studied by manual cutting. It is one of the simplest methods in preparing specimens for microscopic viewing. It often provides an adequate method for rapid and inexpensive microscopic observation of plant internal structure. Basically it involves the use of sharp blade to slice a very thin section of the media. Free hand sectioning is commonly restricted to plant specimens that are easy to cut. Moreover, this very simple technique that when precisely executed, often results in high quality images.

In free-hand sections, the thickness of the section cannot be regulated, it may be thick or thin, as also oblique. Moreover, the entire material cannot be obtained in sections, as the defective sections have to be thrown away.

9.2.2 Hand Microtome

This hand held microtome is just the tool for making own prepared slides. It's designed to securely hold specimens and expose them in tiny increments so that they can be thinly sliced for slide preparation. Includes a calibrated microtome with a razor knife for slicing sections.

To use the microtome, open it as wide as it can go by turning the round screw dial on the side. One can see the clamp open or close by looking into the big hole on the black end. Put the specimen to view into the hole, and slide it in until it hits the bottom. Screw the round dial to tighten it against the specimen to

hold it secure inside the microtome. Take the razor and slice off any excess specimen by cutting against the black bottom.

Now the specimen is flush with the black surface. Turn the back knob of the microtome, which will push out the specimen inside the microtome, to the desired thickness. Take the razor blade and cut again, just like before, to get a thin cross section of your specimen. Take the thin slice and put on the slide and it is ready for viewing. Adjust the back knob to easily make thinner or thicker slices.

Then squeeze 1-2 drops of water over the specimen section to make a 'wet mount' slide, and the cover it with a slide coverslip. Figure 9.1 shows the image for hand microtome.

Fig. 9.1 *Hand Microtome*

9.2.3 Rotary Microtome Sectioning

The rotary microtome is the most common instrument found in a histology laboratory. Although most microtomes are manual, some are automatic or semi-automatic, where the advancement of the block and speed of cutting are controlled by a foot pedal or a digital keypad at one's fingertips. Automatic and semi-automatic microtomes greatly improve ergonomics by reducing repetitive stress on joints. Microtomes have become more precise and easier to use since the first versions. Although a good microtome can last decades, most laboratories are equipped with modern microtomes with current design innovations. Figure 9.2 shows the rotary microtome sectioning.

Fig. 9.2 *Rotary Microtome Sectioning*

NOTES

Microtomes are very heavy, weighing 40 to 60 pounds. This is to reduce vibration during microtomy, in which stability is critical during sectioning to prevent undulations (wash boarding) in the paraffin sections. Daily cleaning from paraffin debris and yearly preventive maintenance will keep a microtome cutting optimally for many years.

The main components of a rotatory microtome are described below. Although some microtomes have more bells and whistles, the standard microtome remains relatively simple to operate.

1. **Microtome Base Plate or Stage:** A platform which has rails that secure the knife holder base.
2. **Knife Holder Base:** A part that anchors the knife holder to the microtome stage. The knife holder base can be moved toward or away from the block, but must be stationary and locked during microtomy.
3. **Knife Holder:** This part is comprised of several components including the blade clamp that holds the blade, the knife tilt for adjusting the knife angle, and the face plate that guides that ribbons away from the blade and towards the operator.
4. **Cassette Clamp or Block Holder:** Holds the paraffin block in place. Typically, the block moves up and down with each revolution while the blade is stationary. The block holder may have knobs that allow the user to manipulate the block face in various directions to bring the tissue in alignment with the blade.
5. **Coarse Handwheel:** Moves the block holder either toward the knife or away from the knife.
6. **Advancement Handwheel:** Turns in one direction and advances the block toward the knife at the specified microns. Most handwheels are equipped with a safety lock to prevent the wheel from releasing and having the block holder come down towards the blade while a block is inserted or removed. The safety lock should be used anytime the microtomist is not actively sectioning paraffin blocks.
7. **Micron Adjustment:** Micron settings for section thickness can range from 1 to 60 microns on most microtomes.

9.2.4 Sledge Microtome

A sledge microtome is a device where the sample is placed into a fixed holder (shuttle), which then moves backwards and forwards across a knife. Modern sled microtomes have the sled placed upon a linear bearing, a design that allows the microtome to readily cut many coarse sections. By adjusting the angles between the sample and the microtome knife, the pressure applied to the sample during the cut can be reduced. Typical applications for this design of microtome are of the preparation of large samples, such as those embedded in paraffin for biological

preparations. Typical cut thickness achievable on a sledge microtome is between 1 and 60 μm. Figure 9.3 shows the image for sledge microtome.

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Fig. 9.3 *Image for Sledge Microtome.*

9.2.5 Ultra-Microtomy

Ultra-microtomy is a method for cutting specimens into extremely thin slices, called ultra-thin sections that can be studied and documented at different magnifications in a Transmission Electron Microscope (TEM). It is used mostly for biological specimens, but sections of plastics and soft metals can also be prepared. Sections must be very thin because the 50 to 125 kV electrons of the standard electron microscope cannot pass through biological material much thicker than 150 nm. For best resolutions, sections should be from 30 to 60 nm. This is roughly the equivalent to splitting a 0.1 mm-thick human hair into 2,000 slices along its diameter, or cutting a single red blood cell into 100 slices.

Ultra-Microtomy Process: Ultra-thin sections of specimens are cut using a specialized instrument called an 'ultra-microtome' which is fitted with either a diamond knife, for most biological ultra-thin sectioning, or a glass knife, often used for initial cuts. Before selecting an area of the specimen block to be ultra-thin sectioned, the specialist examines semi-thin or thick sections ranging from 0.5 to 2 μm. These thick sections are also known as survey sections and are viewed under a light microscope to determine whether the right area of the specimen is in a position for thin sectioning. 'Ultra-thin' sections from 50 to 100 nm thick are able to be viewed in the TEM.

Tissue sections obtained by ultramicrotomy are compressed by the cutting force of the knife. In addition, interference microscopy of the cut surface of the blocks reveals that the sections are often not flat. With Epon or Vestopal as embedding medium the ridges and valleys usually do not exceed 0.5 μm in height, i.e., 5–10 times the thickness of ordinary sections.

NOTES

Check Your Progress

1. What is free hand sectioning?
2. Give a drawback for free hand sectioning.
3. What is hand held microscope used for?
4. What does coarse handwheel do?
5. What is micron adjustment used for?

9.3 EMBEDDING METHODS

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

The choice of embedding media depends upon

- Type of Microscope
- Type of Microtome
- Type of Tissue, for example hard tissue like bone or soft tissue like liver biopsy

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

Other types of embedding media include

- **Carbowax**: It is a water soluble wax. Therefore tissues are directly transferred to water soluble wax after fixation and washing.
- **Methacrylate**: It is easily miscible with alcohol and gives a clear and hard block when polymerised Polymerization takes place in the presence of a catalyst. Any trace of water causes uneven polymerization and formation of bubbles in the block around the tissue.
- **Epoxy Resin (Araldite)**: Epoxy polymers of araldite is used in higher resolution work and to see greater details. Epoxy resins are used for electron microscopy. Epoxy polymers of araldite differ from methacrylate in that they are cross-linked causing the cured solid block of araldite to be insoluble in any solvent. Longer filtration is required because the viscosity of resin is greater than methacrylate.
- **Agar Embedding**: It is mainly used in double embedding. Multiple fragments and friable tissue may be impregnated in one block when sectioning on the cryostat. Another use of agar embedding is for FNAC specimens.

NOTES

- **Celloidin Media**: Celloidin is a purified form of nitrocellulose. It is used for cutting hard tissues.
- **Gelatin**:Its melting point is less than the melting point of agar.Gelatin may be used when frozen sections are required on friable and necrotic tissues.

Types of Moulds

A variety of moulds are used for embedding. These may be LEUCKHARD embedding moulds (L mould) (Refer Figure 9.4) paper blocks, plastic moulds. Most of the laboratories use L moulds. L moulds are made up of metal, easy to procure, reusable and may be adjusted to make different size of blocks. One limb of the L is longer than the other. The two Ls are jointed to form a sides of the rectangular box that act as a cast to make the mould.

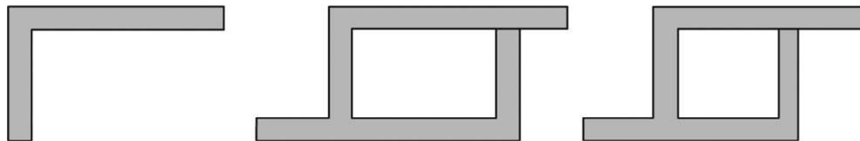

Fig. 9.4 *L Moulds*

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

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

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

Advantages

- Since the cassette is processed with the tissues and afterwards used for embedding, the writing has to be done once.
- Cassettes are thin so less wax is required.
- The space required for filing the blocks is less.

NOTES

Disadvantages

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

Paraffin Wax Additives

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

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

Commonly Used Additives

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

Devices for Tissue Embedding

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

Tissue Embedding Machine

All the blocking steps can be performed with the help of tissue embedding machine. The embedding machine contains the following parts (Refer Figure 9.5).

- Mould warmer, cassette bath, working surface warmer with a nozzle for pouring the wax, forceps well and cold plate.
- The cold plate is of high efficiency refrigeration system having temperature control ranging from different freezing points to 4 or 5 degree C. It can occupy about 50-60 blocks.
- Large3-5 litre capacity paraffin reservoir with adjustable temperature of 45- 75 degree C.

NOTES

- Optional vacuum lids, which allows for vacuum infiltration of tissues.
- It has a forceps warmer convenient drain for excess wax.
- The embedding machines are available with many other features.

Fig. 9.5 *Tissue Embedding Machine*

Method of Embedding

- Open the tissue cassette, check requisition form entry to ensure the correct number of tissue pieces is present.
- Select the mould; there should be sufficient room for the tissue with allowance for at least a 2 mm surrounding margin of wax.
- Leuckhart mould method-This is the traditional embedding method. The 'L moulds are adjusted according to the shape and size of the tissue. Glycerine may be applied to the L pieces and also to the metal or glass plate on which the moulds are placed for embedding. Simple glossed wall or floor tiles may also be used in place of glass plate'.
- Fill the mould with paraffin wax.
- Using warm forceps select the tissue, taking care that it does not cool in the air; at the same time.
- Place the tissue in the mould according to the side to be sectioned. This side should be facing down against the mould. A small amount of pressure may be used in order to have more even embedding.
- Chill the mould on the cold plate, orienting the tissue and firming it into the wax with warmed forceps. This ensures that the correct orientation is maintained and the tissue surface to be sectioned is kept flat.
- Insert the identifying label or place the labelled embedding ring or cassette base onto the mould.
- Add more paraffin into the mould to fill the cassette and mould.
- Cool the block on the cold plate.

NOTES

- Remove the block from the mould.
- Cross check block, label and requisition form.

Orientation of Different Tissue

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

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

Check Your Progress

6. What is embedding?
7. On what does the choice of embedding media depends?
8. What is paraffin wax?
9. What is Epoxy polymers?
10. Give any two methods of embedding.

9.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Free hand sectioning is a micro technique or method of preparing portions of the media to be studied by manual cutting. It is one of the simplest methods in preparing specimens for microscopic viewing.
2. In free-hand sections, the thickness of the section cannot be regulated, it may be thick or thin, as also oblique. Moreover, the entire material cannot be obtained in sections, as the defective sections have to be thrown away.
3. This hand held microtome is just the tool for making own prepared slides. It's designed to securely hold specimens and expose them in tiny increments so that they can be thinly sliced for slide preparation. Includes a calibrated microtome with a razor knife for slicing sections.
4. Coarse handwheel moves the block holder either toward the knife or away from the knife.
5. Micron adjustment is the settings for section thickness can range from 1 to 60 microns on most microtomes.
6. Embedding is the process in which the tissues or the specimens are enclosed in a mass of the embedding medium using a mould.

NOTES

7. The choice of embedding media depends upon
   - Type of microscope
   - Type of microtome
   - Type of tissue, for example hard tissue like bone or soft tissue like liver biopsy
8. Paraffin wax with a higher melting point (56 to 62°C) is used for embedding. The molten wax is filtered inside the oven through a course filter paper into another container. This will protect the knife edge.
9. Epoxy polymers of araldite is used in higher resolution work and to see greater details. Epoxy resins are used for electron microscopy. Epoxy polymers of araldite differ from methacrylate in that they are cross-linked causing the cured solid block of araldite to be insoluble in any solvent. Longer filtration is required because the viscosity of resin is greater than methacrylate.
10. Two method of embedding are -
    1. Open the tissue cassette, check requisition form entry to ensure the correct number of tissue pieces is present.
    2. Select the mould; there should be sufficient room for the tissue with allowance for at least a 2 mm surrounding margin of wax.

9.5 SUMMARY

- Free hand sectioning is a micro technique or method of preparing portions of the media to be studied by manual cutting. It is one of the simplest methods in preparing specimens for microscopic viewing.
- Free hand sectioning is commonly restricted to plant specimens that are easy to cut. Moreover, this very simple technique that when precisely executed, often results in high quality images.
- In free-hand sections, the thickness of the section cannot be regulated, it may be thick or thin, as also oblique. Moreover, the entire material cannot be obtained in sections, as the defective sections have to be thrown away.
- The rotary microtome is the most common instrument found in a histology laboratory.
- Although most microtomes are manual, some are automatic or semi-automatic, where the advancement of the block and speed of cutting are controlled by a foot pedal or a digital keypad at one's fingertips.
- Automatic and semi-automatic microtomes greatly improve ergonomics by reducing repetitive stress on joints.
- Microtomes have become more precise and easier to use since the first versions. Although a good microtome can last decades, most laboratories are equipped with modern microtomes with current design innovations.

NOTES

- A sledge microtome is a device where the sample is placed into a fixed holder (shuttle), which then moves backwards and forwards across a knife.
- Modern sled microtomes have the sled placed upon a linear bearing, a design that allows the microtome to readily cut many coarse sections.
- A variety of moulds are used for embedding. These may be LEUCKHARD embedding moulds (L mould) paper blocks, plastic moulds. Most of the laboratories use L moulds.
- L moulds are made up of metal, easy to procure, reusable and may be adjusted to make different size of blocks. One limb of the L is longer than the other. The two Ls are jointed to form a sides of the rectangular box that act as a cast to make the mould.
- During embedding the orientation of tissue is important. Correct orientation of tissue in a mould is the most important step in embedding.

9.6 KEY WORDS

- **Free hand sectioning:** it is a micro technique or method of preparing portions of the media to be studied by manual cutting.
- **Sledge microtome:** A sledge microtome is a device where the sample is placed into a fixed holder (shuttle), which then moves backwards and forwards across a knife.
- **Celloidin:** A semisolid solution of pyroxylin in ether and alcohol. Used to embed specimens for microscopy before they are sectioned and placed on slides. Quotations
- **Methacrylate:** It is a monocarboxylic acid anion that is obtained by removal of a proton from the carboxylic acid group of methacrylic acid.
- **Embedding:** Embedding is the process in which the tissues or the specimens are enclosed in a mass of the embedding medium using a mould.
- **Mould:** A mould is a fungus that grows in the form of multicellular filaments called hyphae. In contrast, fungi that can adopt a single-celled growth habit are called yeasts.

9.7 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short Answer Questions

1. Write a short note on free hand sectioning.
2. What is hand microtome?

3. What are the main components of rotatory microtome?
4. Write in brief about sledge microtome.
5. What are embedding methods?

NOTES

Long Answer Questions

1. Write about the sectioning of biological specimens?
2. What are different types of sectioning methods? Explain in detail.
3. Discuss about hand held microtome and free hand sectioning.
4. Describe in detail about rotary microtome.
5. Explain about embedding methods in detail.

9.8 FURTHER READINGS

Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

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UNIT 10 DEWAXING AND STAINING

NOTES

Structure

10.0 Introduction
10.1 Objectives
10.2 Dewaxing and Staining of the Sections
10.2.1 Dewaxing
10.3 Staining
10.3.1 In-Vivo vs In-Vitro
10.3.2 Specific Techniques
10.3.3 Common Biological Stains
10.4 Fixing Coverslips and Ringing
10.5 Answers to Check Your Progress Questions
10.6 Summary
10.7 Key Words
10.8 Self Assessment Questions and Exrcises
10.9 Further Readings

10.0 INTRODUCTION

Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes.

In their natural state, most of the cells and microorganisms that we observe under the microscope lack color and contrast. This makes it difficult, if not impossible, to detect important cellular structures and their distinguishing characteristics, hence certain techniques involving stains and fluorescent dyes. Indeed, numerous methods have been developed to identify specific microbes, cellular structures, DNA sequences, or indicators of infection in tissue samples, under the microscope.

In this unit, you will study about dewaxing and staining, and different staining methods and techniques used, fixing coverslips and ringing is also described in the unit. You will learn that how paraffin wax is removed from the sections and the tissue is first stained with Ehrilich's Haematoxylin and then Eosin.

10.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand what dewaxing is
- Discuss about staining and staining methods
- Explain the procedure of fixing of the coverslips
- Discuss about ringing method

10.2 DEWAXING AND STAINING OF THE SECTIONS

Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelleswithin individual cells. In their natural state, most of the cells and microorganisms that we observe under the microscope lack color and contrast. This makes it difficult, if not impossible, to detect important cellular structures and their distinguishing characteristics without artificially treating specimens. We have already alluded to certain techniques involving stains and fluorescent dyes, and in this section we will discuss specific techniques for sample preparation in greater detail. Indeed, numerous methods have been developed to identify specific microbes, cellular structures, DNA sequences, or indicators of infection in tissue samples, under the microscope. Here, we will focus on the most clinically relevant techniques.

After removing the paraffin wax of the sections, the tissue is first stained with Ehrilich's Haematoxylin and then with Eosin. The first stain is a basic dye staining nuclear material while the second is an acidic dye staining cytoplasmic contents.

10.2.1 Dewaxing

Dewaxing is a process in which wax is removed from a material or a surface. The slides first have to be dewaxed. Traditionally, this is done by immersing in two different containers of Xylene, not less than 10 minutes in each. Though Hystoclear is also considered as an original and safer alternative to Xylene and is used in the same way.

After removing the paraffin wax of the sections, the tissue is first stained with Ehrilich's Haematoxylin and then with Eosin. The first stain is a basic dye staining nuclear material while the second is an acidic dye staining cytoplasmic contents.

Paraffin sections are usually rehydrated before staining. It is possible to apply aqueous dye solutions without first removing the wax. Staining then occurs more slowly, and only if the embedding medium has not melted or become unduly soft after cutting. To avoid this problem, sections are flattened on water no hotter than 45°C and dried overnight at 40°C. Minor technical modifications to the staining procedures are needed. Mercury deposits are removed by iodine, and a 3% solution of sodium thiosulfate in 60% ethanol is used to remove the iodine from paraffin sections. At room temperature, progressive staining takes 10-20 times longer for sections in paraffin than for hydrated sections; at 45°C, this can be shortened to

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about three times the regular staining time. After staining, the slides are rinsed in water, air dried, dewaxed with xylene, and coverslipped in the usual way. Nuclear staining in the presence of wax was achieved with toluidine blue, O, alum-hematoxylin and Weigert's iron-hematoxylin. Eosin and van Gieson's picric acid-acid fuchsine were effective anionic counterstains. A one-step trichrome mixture containing 3 anionic dyes and phosphomolybdic acid was unsuitable for sections in wax because it imparted colors that were uninformative and quite different from those obtained with hydrated sections. Advantages of staining in the presence of wax include economy of solvents, reduced risk of overstaining and strong adhesion of sections to slides.

Sections must be free of wax to allow aqueous solutions to penetrate.

- Xylene x2 (2-3mins)
- Absolute ethanol x2 (2-3mins)
- Water wash.

Cryostat and free floating sections may need defatting prior to immuno-histochemical staining. This must be optimised by the user.

Check Your Progress

1. Define staining?
2. Where are stains and dyes used?

10.3 STAINING

Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells. In biochemistry it involves adding a class-specific (DNA, proteins, lipids, carbohydrates) dye to a substrate to qualify or quantify the presence of a specific compound. Staining and fluorescent tagging can serve similar purposes. Biological staining is also used to mark cells in flow cytometry, and to flag proteins or nucleic acids in gel electrophoresis.

Simple staining is staining with only one stain/dye. There are various kinds of multiple staining, many of which are examples of counterstaining, differential staining, or both, including double staining and triple staining. Staining is not limited to biological materials, it can also be used to study the morphology of other materials for example the lamellar structures of semi-crystalline polymers or the domain structures of block copolymers.

10.3.1 In-Vivo vs In-Vitro

In vivo staining (also called vital staining or intravital staining) is the process of dyeing living tissues—in vivo means 'in life' (compare with in vitro staining). By causing certain cells or structures to take on contrasting colour(s), their form (morphology) or position within a cell or tissue can be readily seen and studied. The usual purpose is to reveal cytological details that might otherwise not be apparent; however, staining can also reveal where certain chemicals or specific chemical reactions are taking place within cells or tissues.

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In-vitro staining involves colouring cells or structures that have been removed from their biological context. Certain stains are often combined to reveal more details and features than a single stain alone. Combined with specific protocols for fixation and sample preparation, scientists and physicians can use these standard techniques as consistent, repeatable diagnostic tools. A counterstain is stain that makes cells or structures more visible, when not completely visible with the principal stain. For example, crystal violet stains only Gram-positive bacteria in Gram staining. A safranin counterstain is applied that stains all cells, allowing identification of Gram-negative bacteria.

While ex-vivo, many cells continue to live and metabolize until they are 'fixed'. Some staining methods are based on this property. Those stains excluded by the living cells but taken up by the already dead cells are called vital stains (for example, trypan blue or propidium iodide for eukaryotic cells). Those that enter and stain living cells are called supravital stains (for example, New Methylene Blue and brilliant cresyl blue for reticulocyte staining). However, these stains are eventually toxic to the organism, some more so than others. Partly due to their toxic interaction inside a living cell, when supravital stains enter a living cell, they might produce a characteristic pattern of staining different from the staining of an already fixed cell (for example, reticulocyte look versus diffuse polychromasia). To achieve desired effects, the stains are used in very dilute solutions ranging from 1:5000 to 1:500000 (Howey, 2000).

In-Vitro Methods

Preparation

The preparatory steps involved depend on the type of analysis planned; some or all of the following procedures may be required.

Fixation–which may itself consist of several steps–aims to preserve the shape of the cells or tissue involved as much as possible. Sometimes heat fixation is used to kill, adhere, and alter the specimen so it accepts stains. Most chemical fixatives (chemicals causing fixation) generate chemical bonds between proteins and other substances within the sample, increasing their rigidity. Common fixatives include formaldehyde, ethanol, methanol, and/or picric acid. Pieces of tissue may be embedded in paraffin wax to increase their mechanical strength and stability and to make them easier to cut into thin slices.

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Permeabilization involves treatment of cells with (usually) a mild surfactant. This treatment dissolves cell membranes, and allows larger dye molecules into the cell's interior.

Mounting usually involves attaching the samples to a glass microscope slide for observation and analysis. In some cases, cells may be grown directly on a slide. For samples of loose cells (as with a blood smear or a pap smear) the sample can be directly applied to a slide. For larger pieces of tissue, thin sections (slices) are made using a microtome; these slices can then be mounted and inspected.

Standardization

Most of the dyes commonly used in microscopy are available as BSC-certified stains. This means that samples of the manufacturer's batch have been tested by an independent body, the Biological Stain Commission (BSC), and found to meet or exceed certain standards of purity, dye content and performance in staining techniques. These standards are published in the Commission's journal Biotechnic & Histochemistry. Many dyes are inconsistent in composition from one supplier to another. The use of BSC-certified stains eliminates a source of unexpected results.

Some vendors sell stains certified by themselves rather than by the Biological Stain Commission. Such products may or may not be suitable for diagnostic and other applications.

Negative Staining

A simple staining method for bacteria that is usually successful, even when the 'positive staining' methods detailed below fail, is to use a negative stain. This can be achieved by smearing the sample onto the slide and then applying nigrosin (a black synthetic dye) or India ink (an aqueous suspension of carbon particles). After drying, the microorganisms may be viewed in bright field microscopy as lighter inclusions well-contrasted against the dark environment surrounding them. Note: negative staining is a mild technique that may not destroy the microorganisms, and is therefore unsuitable for studying pathogens.

10.3.2 Specific Techniques

Gram Staining

Gram staining is used to determine gram status to classify bacteria broadly. It is based on the composition of their cell wall. Gram staining uses crystal violet to stain cell walls, iodine as a mordant, and a fuchsin or safranin counterstain to mark all bacteria. Gram status is important in medicine; the presence or absence of a cell wall changes the bacterium's susceptibility to some antibiotics.

Gram-positive bacteria stain dark blue or violet. Their cell wall is typically rich with peptidoglycan and lacks the secondary membrane and lipopolysaccharide layer found in Gram-negative bacteria.

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On most Gram-stained preparations, Gram-negative organisms appear red or pink because they are counterstained. Because of presence of higher lipid content, after alcohol-treatment, the porosity of the cell wall increases, hence the CVI complex (crystal violet – iodine) can pass through. Thus, the primary stain is not retained. Also, in contrast to most Gram-positive bacteria, Gram-negative bacteria have only a few layers of peptidoglycan and a secondary cell membrane made primarily of lipopolysaccharide.

Endospore Staining

Endospore staining is used to identify the presence or absence of endospores, which make bacteria very difficult to kill. This is particularly useful for identifying endospore-forming bacterial pathogens such as Clostridium difficile.

Ziehl-Neelsen Stain

Ziehl-Neelsen staining is used to stain species of Mycobacterium tuberculosis that do not stain with the standard laboratory staining procedures such as Gram staining.

The stains used are the red coloured Carbol fuchsin that stains the bacteria and a counter stain such as Methylene blue

Haematoxylin and Eosin (H and E) Staining

Haematoxylin and eosin staining protocol is used frequently in histology to examine thin sections of tissue. Haematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. Eosin is strongly absorbed by red blood cells, colouring them bright red. In a skilfully made H & E preparation the red blood cells are almost orange, and collagen and cytoplasm (especially muscle) acquire different shades of pink. When the staining is done by a machine, the subtle differences in eosinophilia are often lost. Hematoxylin stains the cell nucleus and other acidic structures (such as RNA-rich portions of the cytoplasm and the matrix of hyaline cartilage) blue. In contrast, eosin stains the cytoplasm and collagen pink.

Papanicolaou Staining

Papanicolaou staining, or Pap staining, is a frequently used method for examining cell samples from various bodily secretions. It is frequently used to stain Pap smear specimens. It uses a combination of haematoxylin, Orange G, Eosin Y, Light Green SF yellowish, and sometimes Bismarck Brown Y.

PAS Staining

Periodic acid-Schiff staining is used to mark carbohydrates (glycogen, glycoprotein, and proteoglycans). It is used to distinguish different types of glycogen storage diseases.

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Masson's Trichrome

Masson's trichrome is (as the name implies) a three-colour staining protocol. The recipe has evolved from Masson's original technique for different specific applications, but all are well-suited to distinguish cells from surrounding connective tissue. Most recipes produce red keratin and muscle fibers, blue or green staining of collagen and bone, light red or pink staining of cytoplasm, and black cell nuclei.

Romanowsky Stains

The Romanowsky stains are all based on a combination of eosinate (chemically reduced eosin) and methylene blue (sometimes with its oxidation products Azure a and Azure B). Common variants include Wright's stain, Jenner's stain, May-Grünwald stain, Leishman stain and Giemsa stain.

All are used to examine blood or bone marrow samples. They are preferred over H and E for inspection of blood cells because different types of leukocytes (white blood cells) can be readily distinguished. All are also suited to examination of blood to detect blood-borne parasites such as malaria.

Silver Staining

Silver staining is the use of silver to stain histologic sections. This kind of staining is important especially to show proteins (for example type III collagen) and DNA. It is used to show both substances inside and outside cells. Silver staining is also used in temperature gradient gel electrophoresis.

Some cells are argentaffin. These reduce silver solution to metallic silver after formalin fixation. This method was discovered by Italian Camillo Golgi, by using a reaction between silver nitrate and potassium dichromate, thus precipitating silver chromate in some cells. Other cells are argyrophilic. These reduce silver solution to metallic silver after being exposed to the stain that contains a reductant, for example hydroquinone or formalin.

Sudan Staining

Sudan staining is the use of Sudan dyes to stain sudanophilic substances, usually lipids. Sudan III, Sudan IV, Oil Red O, Osmium tetroxide, and Sudan Black B are often used. Sudan staining is often used to determine the level of fecal fat to diagnose steatorrhea.

Conklin's Staining

Special technique designed for staining true endospores with the use of malachite green dye, once stained, they do not decolourize.

10.3.3 Common Biological Stains

Different stains react or concentrate in different parts of a cell or tissue, and these properties are used to advantage to reveal specific parts or areas. Some of the

most common biological stains are listed below. Unless otherwise marked, all of these dyes may be used with fixed cells and tissues; vital dyes (suitable for use with living organisms) are noted.

Acridine Orange

Acridine orange (AO) is a nucleic acid selective fluorescent cationic dye useful for cell cycle determination. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions. When bound to DNA, it is very similar spectrally to fluorescein. Like fluorescein, it is also useful as a non-specific stain for backlighting conventionally stained cells on the surface of a solid sample of tissue (fluorescence backlighted staining).

Bismarck Brown

Bismarck brown (also Bismarck brown Y or Manchester brown) imparts a yellow colour to acid mucins.

Carmine

Carmine is an intensely red dye used to stain glycogen, while Carmine alum is a nuclear stain. Carmine stains require the use of a mordant, usually aluminium.

Coomassie Blue

Coomassie blue (also brilliant blue) nonspecifically stains proteins a strong blue colour. It is often used in gel electrophoresis.

Cresyl Violet

Cresyl violet stains the acidic components of the neuronal cytoplasm a violet colour, specifically nissl bodies. Often used in brain research.

Crystal Violet

Crystal violet, when combined with a suitable mordant, stains cell walls purple. Crystal violet is the stain used in Gram staining.

DAPI

DAPI is a fluorescent nuclear stain, excited by ultraviolet light and showing strong blue fluorescence when bound to DNA. DAPI binds with A=T rich repeats of chromosomes. DAPI is also not visible with regular transmission microscopy. It may be used in living or fixed cells. DAPI-stained cells are especially appropriate for cell counting.

Eosin

Eosin is most often used as a counterstain to haematoxylin, imparting a pink or red colour to cytoplasmic material, cell membranes, and some extracellular structures. It also imparts a strong red colour to red blood cells. Eosin may also be used as a

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counterstain in some variants of Gram staining, and in many other protocols. There are actually two very closely related compounds commonly referred to as eosin. Most often used is eosin Y (also known as eosin Y ws or eosin yellowish); it has a very slightly yellowish cast. The other eosin compound is eosin B (eosin bluish or imperial red); it has a very faint bluish cast. The two dyes are interchangeable, and the use of one or the other is more a matter of preference and tradition.

Ethidium Bromide

Ethidium bromide intercalates and stains DNA, providing a fluorescent red-orange stain. Although it will not stain healthy cells, it can be used to identify cells that are in the final stages of apoptosis – such cells have much more permeable membranes. Consequently, ethidium bromide is often used as a marker for apoptosis in cells populations and to locate bands of DNA in gel electrophoresis. The stain may also be used in conjunction with Acridine Orange (AO) in viable cell counting. This EB/AO combined stain causes live cells to fluoresce green whilst apoptotic cells retain the distinctive red-orange fluorescence.

Acid Fuchsine

Acid fuchsine may be used to stain collagen, smooth muscle, or mitochondria. Acid fuchsine is used as the nuclear and cytoplasmic stain in Mallory's trichrome method. Acid fuchsine stains cytoplasm in some variants of Masson's trichrome. In Van Gieson's picro-fuchsine, acid fuchsine imparts its red colour to collagen fibers. Acid fuchsine is also a traditional stain for mitochondria (Altmann's method).

Haematoxylin

Haematoxylin (hematoxylin in North America) is a nuclear stain. Used with a mordant, haematoxylin stains nuclei blue-violet or brown. It is most often used with eosin in H&E (haematoxylin and eosin) staining—one of the most common procedures in histology.

Hoechst Stains

Hoechst is a bis-benzimidazole derivative compound that binds to the minor groove of DNA. Often used in fluorescence microscopy for DNA staining, Hoechst stains appear yellow when dissolved in aqueous solutions and emit blue light under UV excitation. There are two major types of Hoechst: Hoechst 33258 and Hoechst 33342. The two compounds are functionally similar, but with a little difference in structure. Hoechst 33258 contains a terminal hydroxyl group and is thus more soluble in aqueous solution, however this characteristics reduces its ability to penetrate the plasma membrane. Hoechst 33342 contains an ethyl substitution on the terminal hydroxyl group (i.e., an ethylether group) making it more hydrophobic for easier plasma membrane passage

Iodine

Iodine is used in chemistry as an indicator for starch. When starch is mixed with iodine in solution, an intensely dark blue colour develops, representing a starch/iodine complex. Starch is a substance common to most plant cells and so a weak iodine solution will stain starch present in the cells. Iodine is one component in the staining technique known as Gram staining, used in microbiology. Lugol's solution or Lugol's Iodine (IKI) is a brown solution that turns black in the presence of starches and can be used as a cell stain, making the cell nuclei more visible. Iodine is also used as a mordant in Gram's staining, it enhances dye to enter through the pore present in the cell wall/membrane.

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Malachite Green

Malachite green (also known as diamond green B or victoria green B) can be used as a blue-green counterstain to safranin in the Gimenez staining technique for bacteria. It can also be used to directly stain spores.

Methyl Green

Methyl green is used commonly with bright-field, as well as fluorescence microscopes to dye the chromatin of cells so that they are more easily viewed.

Methylene Blue

Methylene blue is used to stain animal cells, such as human cheek cells, to make their nuclei more observable. Also used to stain the blood film and used in cytology.

Neutral Red

Neutral red (or toluylene red) stains Nissl substance red. It is usually used as a counterstain in combination with other dyes.

Nile Blue

Nile blue (or Nile blue A) stains nuclei blue. It may be used with living cells.

Nile Red

Nile red (also known as Nile blue oxazone) is formed by boiling Nile blue with sulfuric acid. This produces a mix of Nile red and Nile blue. Nile red is a lipophilic stain; it will accumulate in lipid globules inside cells, staining them red. Nile red can be used with living cells. It fluoresces strongly when partitioned into lipids, but practically not at all in aqueous solution.

Osmium Tetroxide (Formal Name: Osmium Tetraoxide)

Osmium tetraoxide is used in optical microscopy to stain lipids. It dissolves in fats, and is reduced by organic materials to elemental osmium, an easily visible black substance.

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Propidium Iodide

Propidium iodide is a fluorescent intercalating agent that can be used to stain cells. Propidium iodide is used as a DNA stain in flow cytometry to evaluate cell viability or DNA content in cell cycle analysis, or in microscopy to visualize the nucleus and other DNA-containing organelles. Propidium Iodide cannot cross the membrane of live cells, making it useful to differentiate necrotic, apoptotic and healthy cells. PI also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining

Rhodamine

Rhodamine is a protein specific fluorescent stain commonly used in fluorescence microscopy.

Safranine

Safranine (or Safranine O) is a red cationic dye. It binds to nuclei (DNA) and other tissue polyanions, including glycosaminoglycans in cartilage and mast cells, and components of lignin and plastids in plant tissues. Safranine should not be confused with saffron, an expensive natural dye that is used in some methods to impart a yellow colour to collagen, to contrast with blue and red colours imparted by other dyes to nuclei and cytoplasm in animal (including human) tissues.

Stainability of Tissues

Tissues which take up stains are called chromatic. Chromosomes were so named because of their ability to absorb a violet stain.

Positive affinity for a specific stain may be designated by the suffix -philic. For example, tissues that stain with an azure stain may be referred to as azurophilic. This may also be used for more generalized staining properties, such as acidophilic for tissues that stain by acidic stains (most notably eosin), basophilic when staining in basic dyes, and amphophilic when staining with either acid or basic dyes. In contrast, chromophobic tissues do not take up coloured dye readily.

Electron Microscopy

As in light microscopy, stains can be used to enhance contrast in transmission electron microscopy. Electron-dense compounds of heavy metals are typically used.

Phosphotungstic Acid

Phosphotungstic acid is a common negative stain for viruses, nerves, polysaccharides, and other biological tissue materials.

Osmium Tetroxide

Osmium tetroxide is used in optical microscopy to stain lipids. It dissolves in fats, and is reduced by organic materials to elemental osmium, an easily visible black

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substance. Because it is a heavy metal that absorbs electrons, it is perhaps the most common stain used for morphology in biological electron microscopy. It is also used for the staining of various polymers for the study of their morphology by TEM. OsO 4 is very volatile and extremely toxic. It is a strong oxidizing agent as the osmium has an oxidation number of +8. It aggressively oxidizes many materials, leaving behind a deposit of non-volatile osmium in a lower oxidation state.

Ruthenium Tetroxide

Ruthenium tetroxide is equally volatile and even more aggressive than osmium tetraoxide and able to stain even materials that resist the osmium stain, for example, polyethylene.

Other chemicals used in electron microscopy staining include: ammonium molybdate, cadmium iodide, carbohydrazide, ferric chloride, hexamine, indium trichloride, lanthanum nitrate, lead acetate, lead citrate, lead(II) nitrate, periodic acid, phosphomolybdic acid, potassium ferricyanide, potassium ferrocyanide, ruthenium red, silver nitrate, silver proteinate, sodium chloroaurate, thallium nitrate, thiosemicarbazide, uranyl acetate, uranyl nitrate, and vanadyl sulfate.

10.4 FIXING COVERSLIPS AND RINGING

Formulations of mounting media that can add favorable properties such as optimizing the refractive index to match that of glass, preventing photobleaching, or preserving samples for long-term storage are widely available. Keep in mind that hard-setting mountants require time to 'cure' or harden.

Try to limit the formation of bubbles in your mounting medium. Some bubbles are inevitable, and you can image around them as long as there are only a few.

To avoid bubbles

- Do not shake or invert your bottle of mounting medium.
- Clear bubbles from the tip of your applicator (i.e., from the tip of the pipette or dropper bottle) by squeezing a little bit of mounting medium onto a lab tissue before applying mounting medium to your slide
- Clean the surface of the non-sample side of the coverslip prior to imaging. Fingerprints, residual salts, and other contaminants can diminish image quality.

Steps to Mount a Coverslip

1. Apply a small amount of mounting medium to the surface of the slide; try to use an amount that will just fill the space under the coverslip.
2. Remove coverslip containing the sample from the buffer.
3. Blot excess buffer or solvent from the non-sample surface of the coverslip (or allow it to air-dry and then remove the salt residue before imaging).

4. Slowly tip the coverslip onto the mounting medium, and avoid creating bubbles as you lower it into place.
5. Follow manufacturer's directions for curing time
6. Seal the coverslip if desired.
7. Image under microscope.

In order to mount most animals on slides they must be cleared, dehydrated, embedded in a hardening resin and covered with a cover slip. As usual there are many techniques, some more permanent than others, that can be used. Two of the simpler techniques are described below:

Dry Mount

In a dry mount, the simplest kind of mounting, the object is merely placed on the slide. A cover slip may be placed on top to protect the specimen and the microscope's objective and to keep the specimen still and pressed flat. This mounting can be successfully used for viewing specimens like pollen, feathers, hairs, etc. It is also used to examine particles caught in transparent membrane filters (for example, in analysis of airborne dust).

Permanent Mount

1. Clearing: opaque specimens must first be cleared to facilitate identification.
   (a) Place specimens in a 10% Potassium Hydroxide or Sodium Hydroxide solution overnight. If the specimen is dry it must first be wetted by soaking it in a detergent solution before clearing. (If additional clearing is needed it may require physically cleaning out the specimen's internal contents using small needles and forceps.)
   (b) After initial clearing place in acetic acid to neutralize the alkali.
   (c) Transfer to oil of cloves to complete clearing. Keep in oil until specimen becomes transparent (up to 60 minutes).
2. Dehydration: transfer to xylol to dehydrate and remove oil.
3. Slide Mounting: Place a drop of Canada balsam on a clean glass slide.
   (a) With fine forceps or lifting pin transfer specimen to slide and arrange appendages to an extended position. Make sure each is clearly visible since it may be needed for identification.
   (b) Carefully place coverslip on slide. Avoid trapping air bubbles by first placing the coverslip at an angle and then slowly lowering its other side
   (c) Label slide
   (d) Place slide on a stable horizontal surface until the balsam dries; this will take several week at room temperature. if available place slide in drying oven to speed up the process (up to one week).

Semi-Permanent Mount

1. Once the animal has been killed and fixed transfer it to a watch glass containing 5% glycerin in 50% alcohol or 5% formalin.
2. Cover loosely for about a week and allow the alcohol to evaporate.
3. Melt the glycerine jelly and place a drop on the slide.
4. Transfer the animal with a transfer needle to the drop of glycerine jelly and carefully arrange it to show important anatomical features.
5. Carefully place a coverslip over the specimen, avoid trapping air bubbles on the slide.
6. Allow to stand and set for several hours
7. Trim the excess glycering jelly from the slide and apply several coats of clear nail polish to seal the slide.

Strew Mounting

Strew mounting describes the production of palynological microscope slides by suspending a concentrated sample in distilled water, placing the samples on a slide, and allowing the water to evaporate.

Check Your Progress

3. What is in-vitro staining?
4. What happens in ex-vivo?
5. Define negative staining?
6. What is the use of Gram staining?
7. Give a use of endosperm staining.
8. Where is Ziehl-Neelsen staining used?
9. What is Papanicolaou staining?
10. Define Sudan staining.
11. What is DAPI?
12. Where is acid fuchsine used?

10.5 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image.
2. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different

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microscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells.

3. In vitro staining involves colouring cells or structures that have been removed from their biological context. Certain stains are often combined to reveal more details and features than a single stain alone. Combined with specific protocols for fixation and sample preparation, scientists and physicians can use these standard techniques as consistent, repeatable diagnostic tools.
4. In ex-vivo, many cells continue to live and metabolize until they are "fixed". Some staining methods are based on this property. Those stains excluded by the living cells but taken up by the already dead cells are called vital stains (for example, rypan blue or propidium iodide for eukaryotic cells). Those that enter and stain living cells are called supravital stains. Negative staining is a simple staining method for bacteria that is usually successful, even when the positive staining methods detailed below fail, is to use a negative stain. This can be achieved by smearing the sample onto the slide and then applying nigrosin (a black synthetic dye) or India ink (an aqueous suspension of carbon particles).
5. Gram staining is used to determine gram status to classify bacteria broadly. It is based on the composition of their cell wall. Gram staining uses crystal violet to stain cell walls, iodine as a mordant, and a fuchsin or safranin counterstain to mark all bacteria. Gram status is important in medicine; the presence or absence of a cell wall changes the bacterium's susceptibility to some antibiotics.
6. Endospore staining is used to identify the presence or absence of endospores, which make bacteria very difficult to kill. This is particularly useful for identifying endospore-forming bacterial pathogens such as Clostridium difficile.
7. Ziehl-Neelsen staining is used to stain species of Mycobacterium tuberculosis that do not stain with the standard laboratory staining procedures such as Gram staining.
8. Papanicolaou staining, or Pap staining, is a frequently used method for examining cell samples from various bodily secretions. It is frequently used to stain Pap smear specimens. It uses a combination of haematoxylin, Orange G, eosin Y, Light Green SF yellowish, and sometimes Bismarck Brown Y.
9. Sudan staining is the use of Sudan dyes to stain sudanophilic substances, usually lipids. Sudan III, Sudan IV, Oil Red O, Osmium tetroxide, and Sudan Black B are often used. Sudan staining is often used to determine the level of fecal fat to diagnose steatorrhea.

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10. DAPI is a fluorescent nuclear stain, excited by ultraviolet light and showing strong blue fluorescence when bound to DNA. DAPI binds with A=T rich repeats of chromosomes. DAPI is also not visible with regular transmission microscopy. It may be used in living or fixed cells. DAPI-stained cells are especially appropriate for cell counting.
11. Acid fuchsine may be used to stain collagen, smooth muscle, or mitochondria. Acid fuchsine is used as the nuclear and cytoplasmic stain in Mallory's trichrome method. Acid fuchsine stains cytoplasm in some variants of Masson's trichrome. In Van Gieson's picro-fuchsine, acid fuchsine imparts its red colour to collagen fibers. Acid fuchsine is also a traditional stain for mitochondria (Altmann's method).

10.6 SUMMARY

- Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes.
- Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells.
- Staining is not limited to biological materials, it can also be used to study the morphology of other materials for example the lamellar structures of semi-crystalline polymers or the domain structures of block copolymers.
- In vivo staining (also called vital staining or intravital staining) is the process of dyeing living tissues—in vivo means 'in life' (compare with in vitro staining). By causing certain cells or structures to take on contrasting colour(s), their form (morphology) or position within a cell or tissue can be readily seen and studied.
- In vitro staining involves colouring cells or structures that have been removed from their biological context. Certain stains are often combined to reveal more details and features than a single stain alone. Combined with specific protocols for fixation and sample preparation, scientists and physicians can use these standard techniques as consistent, repeatable diagnostic tools.
- In ex-vivo, many cells continue to live and metabolize until they are "fixed". Some staining methods are based on this property. Those stains excluded by the living cells but taken up by the already dead cells are called vital stains (e.g. trypan blue or propidium iodide for eukaryotic cells). Those that enter and stain living cells are called supravital stains (for example, New Methylene Blue and brilliant cresyl blue for reticulocyte staining).

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- Gram staining is used to determine gram status to classify bacteria broadly. It is based on the composition of their cell wall. Gram staining uses crystal violet to stain cell walls, iodine as a mordant, and a fuchsin or safranin counterstain to mark all bacteria.
- Gram-positive bacteria stain dark blue or violet. Their cell wall is typically rich with peptidoglycan and lacks the secondary membrane and lipopolysaccharide layer found in Gram-negative bacteria.
- Endospore staining is used to identify the presence or absence of endospores, which make bacteria very difficult to kill. This is particularly useful for identifying endospore-forming bacterial pathogens such as Clostridium difficile.
- Haematoxylin and eosin staining protocol is used frequently in histology to examine thin sections of tissue. Haematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. Eosin is strongly absorbed by red blood cells, colouring them bright red.
- Strew mounting describes the production of palynological microscope slides by suspending a concentrated sample in distilled water, placing the samples on a slide, and allowing the water to evaporate.

10.7 KEY WORDS

- **Supravital staining:** Supravital staining is a method of staining used in microscopy to examine living cells that have been removed from an organism.
- **Brilliant cresyl blue:** Brilliant cresyl blue is a supravital stain used for counting reticulocytes and is classified as an oxazine dye.
- **Polychromasia:** Polychromasia also known as polychromatophilia is a disorder where there is an abnormally high number of immature red blood cells found in the bloodstream as a result of being prematurely released from the bone marrow during blood formation.
- **Nigrosin:** Nigrosin (CI 50415, Solvent black 5) is a mixture of synthetic black dyes made by heating a mixture of nitrobenzene, aniline, and hydrochloric acid in the presence of copper or iron.
- **Ziehl–Neelsen stain:** The Ziehl–Neelsen stain, also known as the acid-faststain, was first described by two German doctors: the bacteriologist Franz Ziehl (1859–1926) and the pathologist Friedrich Neelsen (1854–1898).
- **Carbol fuchsin:** Carbol fuchsin, carbol-fuchsin, or carbolfuchsin, is a mixture of phenol and basic fuchsin, used in bacterial staining procedures. as it has an affinity for the mycolic acids found in their cell membranes.

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- **Haematoxylin:** Haematoxylin or hematoxylin, also called natural black 1 or C.I. 75290, is a compound extracted from the heartwood of the logwood tree (Haematoxylum campechianum).
- **Papanicolaou stain:** Papanicolaou stain (also Papanicolaou's stain and Pap stain) is a multichromatic staining cytological technique developed by George Papanikolaou, the father of cytopathology.
- **Romanowsky stains:** Romanowsky stains are neutral stains composed of a mixture of oxidized methylene blue (azure) dyes and Eosin Y.
- **Argentaffin:** Argentaffin refers to cells which take up silver stain. Enteroendocrine cells are sometimes also called argentaffins, because they take up this stain.
- **Nissl:** A Nissl body, also known as Nissl substance and Nissl material, is a large granular body found in neurons and made up of Rough Endoplasmic Reticulum (RER) with rosettes of free ribosomes, and are the site of protein synthesis.
- **Glycosaminoglycans:** Glycosaminoglycans (GAGs) or muco-polysaccharides are long unbranched polysaccharides consisting of a repeating disaccharide unit.
- **Phosphotungstic acid:** Phosphotungstic acid is a common negative stain for viruses, nerves, polysaccharides, and other biological tissue materials.
- **Phosphorus trichloride:** Phosphorus trichloride is a chemical compound of phosphorus and chlorine, having the chemical formula PCl_3. It has a trigonal pyramidal shape.

10.8 SELF ASSESSMENT QUESTIONS AND EXRCISES

Short Answer Questions

1. Write a short note on dewaxing.
2. What is staining and what are its uses?
3. Write a short note on in-vitro methods.
4. Brief about Gram staining.
5. Write the steps to mount a coverslip.
6. Give a brief note on dry mount.

Long Answer Questions

1. Explain about staining in detail.
2. Discuss about the specific techniques used in staining.

3. Distinguish between in-vitro and on-vivo.
4. Explain about some of the common biological stains.
5. Discuss about fixing of coverslips and ringing in detail.

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10.9 FURTHER READINGS

Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

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BLOCK - IV
MACERATION, SEPARATION TRACER AND BLOTTING TECHNIQUES

NOTES

UNIT 11 MACERATION TECHNIQUE

Structure

11.0 Introduction
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11.0 INTRODUCTION

Maceration is the winemaking process where the phenolic materials of the grape—tannins, coloring agents (anthocyanins) and flavor compounds—are leached from the grape skins, seeds and stems into the must. To macerate is to soften by soaking, and maceration is the process by which the red wine receives its red color, since raw grape juice (with the exceptions of teinturiers) is clear-grayish in color. In the production of white wines, maceration is either avoided or allowed only in very limited manner in the form of a short amount of skin contact with the juice prior to pressing. This is more common in the production of varietals with less natural flavor and body structure like Sauvignon blanc and Sémillon.

An extraction process that consists of maintaining contact between the plant and a liquid (solvent) for a period of time. Maceration is an extractive technique that is conducted at room temperature. It consists of immersing a plant in a liquid like water, oil, alcohol, etc., inside an airtight container, for a variable time based on the plant material and liquid used. Before being processed, the plant must be properly washed and separated from foreign material, such as topsoil, pebbles or rocks, weeds, and materials non-suitable for extraction. The plant material can be used fresh or dry based on the desired product.

In this unit, you will study about maceration techniques, PAGE, SDS – PAGE and agarose gel electrophoresis.

11.1 OBJECTIVES

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After going through this unit, you will be able to:

- Understand about maceration techniques
- Explain about PAGE, SDS-PAGE
- Discuss about agarose gel
- Explain what electrophoresis is

11.2 MACERATION TECHNIQUE - PAGE, SDS –PAGE AND AGAROSE GEL ELECTROPHORESIS

Maceration is the winemaking process where the phenolic materials of the grape—tannins, coloring agents (anthocyanins) and flavor compounds—are leached from the grape skins, seeds and stems into the must. To macerate is to soften by soaking, and maceration is the process by which the red wine receives its red color, since raw grape juice (with the exceptions of teinturiers) is clear-grayish in color. In the production of white wines, maceration is either avoided or allowed only in very limited manner in the form of a short amount of skin contact with the juice prior to pressing. This is more common in the production of varietals with less natural flavor and body structure like Sauvignon blanc and Sémillon. For Rosé, red wine grapes are allowed some maceration between the skins and must, but not to the extent of red wine production.

The process of maceration begins, to varying extent, as soon as the grapes' skins are broken and exposed to some degree of heat. Temperature is the guiding force, with higher temperatures encouraging more breakdown and extraction of phenols from the skins and other grape materials. Maceration continues during the fermentation period, and can last well past the point when the yeast has converted all sugars into alcohol. The process itself is a slow one with compounds such as the anthocyanins needing to pass through the cell membrane of the skins to come into contact with the wine. During fermentation, higher temperatures and higher alcohol levels can encourage this process with the alcohol acting as a solvent to assist in the breakdown of the organic compounds within the grape materials. This process seems to slow once the wine reaches an alcohol level of 10%.

11.2.1 PAGE

PolyAcrylamide Gel Electrophoresis (PAGE) is a technique widely used in biochemistry, forensic chemistry, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility. Electrophoretic mobility is a function of the length, conformation and charge of the molecule. Polyacrylamide gel electrophoresis is a

powerful tool used to analyze RNA samples. When polyacrylamide gel is denatured after electrophoresis, it provides information on the sample composition of the RNA species.

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Hydration of acrylonitrile results in formation of acrylamide molecules (C_3H_5NO) by nitrile hydratase. Acrylamide monomer is in a powder state before addition of water. Acrylamide is toxic to the human nervous system, therefore all safety measures must be followed when working with it. Acrylamide is soluble in water and upon addition of water it polymerizes resulting in formation of polyacrylamide. It is useful to make polyacrylamide gel via acrylmide hydration because pore size can be regulated. Increased concentrations of acrylamide result in decreased pore size after polymerization. Polyacrylamide gel with small pores helps to examine smaller molecules better since the small molecules can enter the pores and travel through the gel while large molecules get trapped at the pole openings.

As with all forms of gel electrophoresis, molecules may be run in their native state, preserving the molecules' higher-order structure. This method is called native-PAGE. Alternatively, a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured molecule whose mobility depends only on its length (because the protein-SDS (Sodium Dodecyl Sulphate) complexes all have a similar mass-to-charge ratio). This procedure is called SDS-PAGE. Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) is a method of separating molecules based on the difference of their molecular weight. At the pH at which gel electrophoresis is carried out the SDS molecules are negatively charged and bind to proteins in a set ratio, approximately one molecule of SDS for every 2 amino acids. In this way, the detergent provides all proteins with a uniform charge-to-mass ratio. By binding to the proteins the detergent destroys their secondary, tertiary and/or quaternary structure denaturing them and turning them into negatively charged linear polypeptide chains. When subjected to an electric field in PAGE, the negatively charged polypeptide chains travel toward the anode with different mobility. Their mobility, or the distance travelled by molecules, is inversely proportional to the logarithm of their molecular weight. By comparing the relative ratio of the distance travelled by each protein to the length of the gel (RF) one can make conclusions about the relative molecular weight of the proteins, where the length of the gel is determined by the distance traveled by a small molecule like a tracking dye.

For nucleic acids, urea is the most commonly used denaturant. For proteins, Sodium Dodecyl Sulfate (SDS) is an anionic detergent applied to protein samples to coat proteins in order to impart two negative charges (from every SDS molecule) to every two amino acids of the denatured protein. 2-Mercaptoethanol may also be used to disrupt the disulfide bonds found between the protein complexes, which helps further denature the protein. In most proteins, the binding of SDS to the polypeptide chains impart an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Proteins

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that have a greater hydrophobic content — for instance, many membrane proteins, and those that interact with surfactants in their native environment — are intrinsically harder to treat accurately using this method, due to the greater variability in the ratio of bound SDS. Procedurally, using both Native and SDS-PAGE together can be used to purify and to separate the various sub units of the protein. Native-PAGE keeps the oligomeric form intact and will show a band on the gel that is representative of the level of activity. SDS-PAGE will denature and separate the oligomeric form into its monomers, showing bands that are representative of their molecular weights. These bands can be used to identify and assess the purity of the protein.

Procedure

Sample Preparation

Samples may be any material containing proteins or nucleic acids. These may be biologically derived, for example from prokaryotic or eukaryotic cells, tissues, viruses, environmental samples, or purified proteins. In the case of solid tissues or cells, these are often first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), by sonicator or by using cycling of high pressure, and a combination of biochemical and mechanical techniques – including various types of filtration and centrifugation – may be used to separate different cell compartments and organelles prior to electrophoresis. Synthetic biomolecules such as oligonucleotides may also be used as analytes.

The sample to analyze is optionally mixed with a chemical denaturant if so desired, usually SDS for proteins or urea for nucleic acids. SDS is an anionic detergent that denatures secondary and non–disulfide–linked tertiary structures, and additionally applies a negative charge to each protein in proportion to its mass. Urea breaks the hydrogen bonds between the base pairs of the nucleic acid, causing the constituent strands to anneal. Heating the samples to at least 60°C further promotes denaturation.

In addition to SDS, proteins may optionally be briefly heated to near boiling in the presence of a reducing agent, such as Dithiothreitol (DTT) or 2-Mercaptoethanol (Beta-mercaptoethanol/BME), which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (Oligomeric sub units). This is known as reducing SDS-PAGE.

A tracking dye may be added to the solution. This typically has a higher electrophoretic mobility than the analytes to allow the experimenter to track the progress of the solution through the gel during the electrophoretic run.

Preparing Acrylamide Gels

The gels typically consist of acrylamide, bisacrylamide, the optional denaturant (SDS or urea), and a buffer with an adjusted pH. The solution may be degassed

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under a vacuum to prevent the formation of air bubbles during polymerization. Alternatively, butanol may be added to the resolving gel (for proteins) after it is poured, as butanol removes bubbles and makes the surface smooth. A source of free radicals and a stabilizer, such as ammonium persulfate and TEMED are added to initiate polymerization. The polymerization reaction creates a gel because of the added bisacrylamide, which can form cross-links between two acrylamide molecules. The ratio of bisacrylamide to acrylamide can be varied for special purposes, but is generally about 1 part in 35. The acrylamide concentration of the gel can also be varied, generally in the range from 5% to 25%. Lower percentage gels are better for resolving very high molecular weight molecules, while much higher percentages of acrylamide are needed to resolve smaller proteins. The average pore diameter of polyacrylamide gels is determined by the total concentration of acrylamides (% T with T = Total concentration of Acrylamide and Bisacrylamide) and the concentration of the cross-linker bisacrylamide (%C with C = Bisacrylamide concentration). The pore size is reduced reciprocally to the %T. Concerning %C, a concentration of 5% produces the smallest pores, since the influence of Bisacrylamide on the pore size has a parabola-shape with a vertex at 5%.

Gels are usually polymerized between two glass plates in a gel caster, with a comb inserted at the top to create the sample wells. After the gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

Electrophoresis

Various buffer systems are used in PAGE depending on the nature of the sample and the experimental objective. The buffers used at the anode and cathode may be the same or different.

An electric field is applied across the gel, causing the negatively charged proteins or nucleic acids to migrate across the gel away from the negative electrode (which is the cathode being that this is an electrolytic rather than galvanic cell) and towards the positive electrode (the anode). Depending on their size, each biomolecule moves differently through the gel matrix: small molecules more easily fit through the pores in the gel, while larger ones have more difficulty. The gel is run usually for a few hours, though this depends on the voltage applied across the gel; migration occurs more quickly at higher voltages, but these results are typically less accurate than at those at lower voltages. After the set amount of time, the biomolecules have migrated different distances based on their size. Smaller biomolecules travel farther down the gel, while larger ones remain closer to the point of origin. Biomolecules may therefore be separated roughly according to size, which depends mainly on molecular weight under denaturing conditions, but also depends on higher-order conformation under native conditions. The gel mobility is defined as the rate of migration traveled with a voltage gradient of 1V/cm and has units of cm^2/sec/V. For analytical purposes, the relative mobility of biomolecules, RF, the ratio of the distance the molecule traveled on the gel to the

total travel distance of a tracking dye is plotted versus the molecular weight of the molecule (or sometimes the log of MW, or rather the Mr, molecular radius). Such typically linear plots represent the standard markers or calibration curves that are widely used for the quantitative estimation of a variety of biomolecular sizes.

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Certain glycoproteins, however, behave anomalously on SDS gels. Additionally, the analysis of larger proteins ranging from 250,000 to 600,000 Da is also reported to be problematic due to the fact that such polypeptides move improperly in the normally used gel systems.

Further Processing

Fig. 11.1 *Two SDS-PAGE-Gels After a Completed Run*

Following electrophoresis, the gel may be stained (for proteins, most commonly with Coomassie Brilliant Blue R-250 or autoradiography; for nucleic acids, ethidium bromide; or for either, silver stain), allowing visualization of the separated proteins, or processed further (for example Western blot). After staining, different species biomolecules appear as distinct bands within the gel. It is common to run molecular weight size markers of known molecular weight in a separate lane in the gel to calibrate the gel and determine the approximate molecular mass of unknown biomolecules by comparing the distance traveled relative to the marker. (Refer Figure 11.1)

For proteins, SDS-PAGE is usually the first choice as an assay of purity due to its reliability and ease. The presence of SDS and the denaturing step make proteins separate, approximately based on size, but aberrant migration of some proteins may occur. Different proteins may also stain differently, which interferes with quantification by staining. PAGE may also be used as a preparative technique for the purification of proteins. For example, quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE) is a method for separating native metalloproteins in complex biological matrices.

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Chemical Ingredients and Their Roles

PolyAcrylamide Gel (PAG) had been known as a potential embedding medium for sectioning tissues as early as 1964, and two independent groups employed PAG in electrophoresis in 1959. It possesses several electrophoretically desirable features that make it a versatile medium. It is a synthetic, thermo-stable, transparent, strong, chemically relatively inert gel, and can be prepared with a wide range of average pore sizes. The pore size of a gel and the reproducibility in gel pore size are determined by three factors, the total amount of acrylamide present (%T) (T = Total concentration of acrylamide and bisacrylamide monomer), the amount of Cross-linker (% C) (C = Bisacrylamide concentration), and the time of polymerization of acrylamide (cf. QPNC-PAGE). Pore size decreases with increasing % T; with cross-linking, 5%C gives the smallest pore size. Any increase or decrease in %C from 5% increases the pore size, as pore size with respect to % C is a parabolic function with vertex as 5% C. This appears to be because of non-homogeneous bundling of polymer strands within the gel. This gel material can also withstand high voltage gradients, is amenable to various staining and destaining procedures, and can be digested to extract separated fractions or dried for autoradiography and permanent recording.

Components

1. Polyacrylamide gels are composed of a stacking gel and separating gel. Stacking gels have a higher porosity relative to the separating gel, and allow for proteins to migrate in a concentrated area. Additionally, stacking gels usually have a pH of 6.8, since the neutral glycine molecules allow for faster protein mobility. Separating gels have a pH of 8.8, where the anionic glycine slows down the mobility of proteins. Separating gels allow for the separation of proteins and have a relatively lower porosity. Here, the proteins are separated based on size (in SDS-PAGE) and size/ charge (Native PAGE).
2. Chemical buffer stabilizes the pH value to the desired value within the gel itself and in the electrophoresis buffer. The choice of buffer also affects the electrophoretic mobility of the buffer counterions and thereby the resolution of the gel. The buffer should also be unreactive and not modify or react with most proteins. Different buffers may be used as cathode and anode buffers, respectively, depending on the application. Multiple pH values may be used within a single gel, for example in DISC electrophoresis. Common buffers in PAGE include Tris, Bis-Tris, or imidazole.
3. Counterion balance the intrinsic charge of the buffer ion and also affect the electric field strength during electrophoresis. Highly charged and mobile ions are often avoided in SDS-PAGE cathode buffers, but may be included in the gel itself, where it migrates ahead of the protein. In applications such as DISC SDS-PAGE the pH values within the gel may vary to change the average charge of the counterions during the run to improve resolution.

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Popular counterions are glycine and tricine. Glycine has been used as the source of trailing ion or slow ion because its pKa is 9.69 and mobility of glycinate are such that the effective mobility can be set at a value below that of the slowest known proteins of net negative charge in the pH range. The minimum pH of this range is approximately 8.0.

4. Acrylamide (C3H5NO; mW: 71.08) when dissolved in water, slow, spontaneous auto-polymerization of acrylamide takes place, joining molecules together by head on tail fashion to form long single-chain polymers. The presence of a free radical-generating system greatly accelerates polymerization. This kind of reaction is known as vinyl addition polymerisation. A solution of these polymer chains becomes viscous but does not form a gel, because the chains simply slide over one another. Gel formation requires linking various chains together. Acrylamide is carcinogenic, a neurotoxin, and a reproductive toxin. It is also essential to store acrylamide in a cool dark and dry place to reduce auto-polymerisation and hydrolysis.
5. Bisacrylamide (N,N_2 -Methylenebisacrylamide) ($C_7H_{10}N_2O_2$; mW: 154.17) is the most frequently used cross linking agent for polyacrylamide gels. Chemically it can be thought of as two acrylamide molecules coupled head to head at their non-reactive ends. Bisacrylamide can crosslink two polyacrylamide chains to one another, thereby resulting in a gel.
6. Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}NaO_4S$; mW: 288.38) (only used in denaturing protein gels) is a strong detergent agent used to denature native proteins to individual polypeptides. This denaturation, which is referred to as reconstructive denaturation, is not accomplished by the total linearization of the protein, but instead, through a conformational change to a combination of random coil and á helix secondary structures. When a protein mixture is heated to 100 °C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g SDS/g of polypeptide.
7. Urea ($CO(NH_2)_2$; mW: 60.06) is a chaotropic agent that increases the entropy of the system by interfering with intramolecular interactions mediated by non-covalent forces such as hydrogen bonds and van der Waals forces. Macromolecular structure is dependent on the net effect of these forces, therefore it follows that an increase in chaotropic solutes denatures macromolecules.
8. Ammonium persulfate (APS) ($N_2H_8S_2O_8$; mW: 228.2) is a source of free radicals and is often used as an initiator for gel formation. An alternative source of free radicals is riboflavin, which generated free radicals in a photochemical reaction.
9. TEMED (N, N, N_2, N_2 -Tetramethylethylenediamine) ($C_6H_{16}N_2$; mW: 116.21) stabilizes free radicals and improves polymerization. The rate of polymerisation and the properties of the resulting gel depend on the

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concentrations of free radicals. Increasing the amount of free radicals results in a decrease in the average polymer chain length, an increase in gel turbidity and a decrease in gel elasticity. Decreasing the amount shows the reverse effect. The lowest catalytic concentrations that allow polymerisation in a reasonable period of time should be used. APS and TEMED are typically used at approximately equimolar concentrations in the range of 1 to 10 mM.

Chemicals for Processing and Visualization

The following chemicals and procedures are used for processing of the gel and the protein samples visualized in it.

Tracking dye; as proteins and nucleic acids are mostly colorless, their progress through the gel during electrophoresis cannot be easily followed. Anionic dyes of a known electrophoretic mobility are therefore usually included in the PAGE sample buffer. A very common tracking dye is Bromophenol blue (BPB, 3',3", 5',5" Tetrabromophenolsulfonphthalein). This dye is coloured at alkali and neutral pH and is a small negatively charged molecule that moves towards the anode. Being a highly mobile molecule it moves ahead of most proteins. As it reaches the anodic end of the electrophoresis medium electrophoresis is stopped. It can weakly bind to some proteins and impart a blue colour. Other common tracking dyes are xylene cyanol, which has lower mobility, and Orange G, which has a higher mobility.

Loading aids; most PAGE systems are loaded from the top into wells within the gel. To ensure that the sample sinks to the bottom of the gel, sample buffer is supplemented with additives that increase the density of the sample. These additives should be non-ionic and non-reactive towards proteins to avoid interfering with electrophoresis. Common additives are glycerol and sucrose.

Coomassie Brilliant Blue R-250 (CBB)($C_{45}H_{44}N_3NaO_7S_2$; mW: 825.97) is the most popular protein stain. It is an anionic dye, which non-specifically binds to proteins. The structure of CBB is predominantly non-polar, and it is usually used in methanolic solution acidified with acetic acid. Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated into the gel can be removed by destaining with the same solution without the dye. The proteins are detected as blue bands on a clear background. As SDS is also anionic, it may interfere with staining process. Therefore, large volume of staining solution is recommended, at least ten times the volume of the gel.

Ethidium Bromide (EtBr) is a popular nucleic acid stain. EtBr allows one to easily visualize DNA or RNA on a gel as EtBr fluoresces an orange color under UV light. Ethidium bromide binds nucleic acid chains through the process of Intercalation. While Ethidium bromide is a popular stain it is important to exercise caution when using EtBr as it is a known carcinogen. Because of this fact, many researchers opt to use stains, such as SYBR Green and SYBR Safe which are safer alternatives to EtBr. EtBr is used by simply adding it to the gel mixture. Once the gel has run, the gel may be viewed through the use of a photo-documentation system.

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Silver staining is used when more sensitive method for detection is needed, as classical Coomassie Brilliant Blue staining can usually detect a 50 ng protein band. Silver staining increases the sensitivity typically 10-100 fold more. This is based on the chemistry of photographic development. The proteins are fixed to the gel with a dilute methanol solution, then incubated with an acidic silver nitrate solution. Silver ions are reduced to their metallic form by formaldehyde at alkaline pH. An acidic solution, such as acetic acid stops development.

Autoradiography, also used for protein band detection post gel electrophoresis, uses radioactive isotopes to label proteins, which are then detected by using X-ray film.

Western blotting is a process by which proteins separated in the acrylamide gel are electrophoretically transferred to a stable, manipulable membrane such as a nitrocellulose, nylon, or PVDF membrane. It is then possible to apply immunochemical techniques to visualise the transferred proteins, as well as accurately identify relative increases or decreases of the protein of interest.

11.2.2 SDS –PAGE

SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis) is a variant of polyacrylamide gel electrophoresis, an analytical method in biochemistry for the separation of charged molecules in mixtures by their molecular masses in an electric field. It uses sodium dodecyl sulfate (SDS) molecules to help identify and isolate protein molecules. (Refer Figure 11.2).

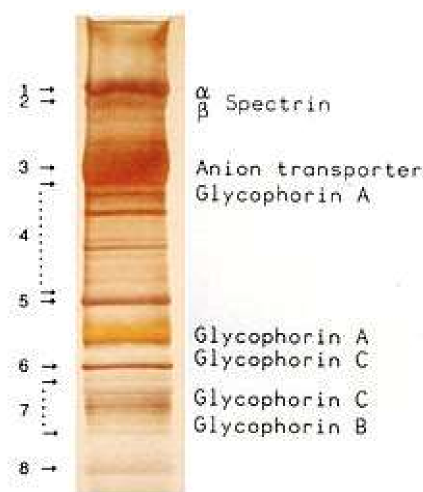

***Fig. 11.2** Proteins of the Erythrocyte Membrane Separated by SDS-PAGE According to Their Molecular Masses*

SDS-PAGE is a discontinuous electrophoretic system developed by Ulrich K. Laemmli which is commonly used as a method to separate proteins with molecular masses between 5 and 250 KDa. The publication describing it is the most frequently cited paper by a single author, and the second most cited overall.

Procedure

The SDS-PAGE method is composed of gel preparation, sample preparation, electrophoresis, protein staining or western blotting and analysis of the generated banding pattern.

Gel Production

When using different buffers in the gel (discontinuous gel electrophoresis), the gels are made up to one day prior to electrophoresis, so that the diffusion does not lead to a mixing of the buffers. The gel is produced by radical polymerisation in a mold consisting of two sealed glass plates with spacers between the glass plates. In a typical mini-gel setting, the spacers have a thickness of 0.75 mm or 1.5 mm, which determines the loading capacity of the gel. For pouring the gel solution, the plates are usually clamped in a stand which temporarily seals the otherwise open underside of the glass plates with the two spacers. For the gel solution, acrylamide is mixed as gel-former (usually 4% V/V in the stacking gel and 10-12 % in the separating gel), methylenebisacrylamide as a cross-linker, stacking or separating gel buffer, water and SDS. By adding the catalyst TEMED and the radical initiator ammonium persulfate (APS) the polymerisation is started. The solution is then poured between the glass plates without creating bubbles. Depending on the amount of catalyst and radical starter and depending on the temperature, the polymerisation lasts between a quarter of an hour and several hours. The lower gel (separating gel) is poured first and covered with a few drops of a barely water-soluble alcohol (usually buffer-saturated butanol or isopropanol), which eliminates bubbles from the meniscus and protects the gel solution of the radical scavenger oxygen. After the polymerisation of the separating gel, the alcohol is discarded and the residual alcohol is removed with filter paper. After addition of APS and TEMED to the stacking gel solution, it is poured on top of the solid separation gel. Afterwards, a suitable sample comb is inserted between the glass plates without creating bubbles. The sample comb is carefully pulled out after polymerisation, leaving pockets for the sample application. For later use of proteins for protein sequencing, the gels are often prepared the day before electrophoresis to reduce reactions of unpolymerised acrylamide with cysteines in proteins.

By using a gradient mixer, gradient gels with a gradient of acrylamide (usually from 4 to 12%) can be cast, which have a larger separation range of the molecular masses. Commercial gel systems (so-called pre-cast gels) usually use the buffer substance Bis-tris methane with a pH value between 6.4 and 7.2 both in the stacking gel and in the separating gel. These gels are delivered cast and ready-to-use. Since they use only one buffer (continuous gel electrophoresis) and have a nearly neutral pH, they can be stored for several weeks. The more neutral pH slows the hydrolysis and thus the decomposition of the polyacrylamide. Furthermore, there are fewer acrylamide-modified cysteines in the proteins. Due to the constant pH in collecting and separating gel there is no stacking effect.

Proteins in BisTris gels cannot be stained with ruthenium complexes. This gel system has a comparatively large separation range, which can be varied by using MES or MOPS in the running buffer.

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Sample Preparation

During sample preparation, the sample buffer, and thus SDS, is added in excess to the proteins, and the sample is then heated to 95°C for five minutes, or alternatively 70°C for ten minutes. Heating disrupts the secondary and tertiary structures of the protein by disrupting hydrogen bonds and stretching the molecules. Optionally, disulfide bridges can be cleaved by reduction. For this purpose, reducing thiols, such as α-mercaptoethanol (α-ME, 5% by volume), Dithiothreitol (DTT, 10 millimolar) or Dithioerythritol (DTE, 10 millimolar) are added to the sample buffer. After cooling to room temperature, each sample is pipetted into its own well in the gel, which was previously immersed in electrophoresis buffer in the electrophoresis apparatus.

Electrophoresis

Electrophoresis chamber after a few minutes of electrophoresis. In the first pocket a size marker was applied with bromophenol blue, in the other pockets, the samples were added bromocresol green

For separation, the denatured samples are loaded onto a gel of polyacrylamide, which is placed in an electrophoresis buffer with suitable electrolytes. Thereafter, a voltage (usually around 100 V, 10-20 V per cm gel length) is applied, which causes a migration of negatively charged molecules through the gel in the direction of the positively charged anode. The gel acts like a sieve. Small proteins migrate relatively easily through the mesh of the gel, while larger proteins are more likely to be retained and thereby migrate more slowly through the gel, thereby allowing proteins to be separated by molecular size. The electrophoresis lasts between half an hour to several hours depending on the voltage and length of gel used.

The fastest-migrating proteins (with a molecular weight of less than 5 KDa) form the buffer front together with the anionic components of the electrophoresis buffer, which also migrate through the gel. The area of the buffer front is made visible by adding the comparatively small, anionic dye bromophenol blue to the sample buffer. Due to the relatively small molecule size of bromophenol blue, it migrates faster than proteins. By optical control of the migrating colored band, the electrophoresis can be stopped before the dye and also the samples have completely migrated through the gel and leave it.

The most commonly used method is the discontinuous SDS-PAGE. In this method, the proteins migrate first into a collecting gel with neutral pH, in which they are concentrated and then they migrate into a separating gel with basic pH, in which the actual separation takes place. Stacking and separating gels differ by different pore size (4-6 % T and 10-20 % T), ionic strength and pH values (pH

6.8 or pH 8.8). The electrolyte most frequently used is an SDS-containing Tris-glycine-chloride buffer system. At neutral pH, glycine predominantly forms the zwitterionic form, at high pH the glycines lose positive charges and become predominantly anionic. In the collection gel, the smaller, negatively charged chloride ions migrate in front of the proteins (as leading ions) and the slightly larger, negatively and partially positively charged glycinate ions migrate behind the proteins (as initial trailing ions), whereas in the comparatively basic separating gel both ions migrate in front of the proteins. The pH gradient between the stacking and separation gel buffers leads to a stacking effect at the border of the stacking gel to the separation gel, since the glycinate partially loses its slowing positive charges as the pH increases and then, as the former trailing ion, overtakes the proteins and becomes a leading ion, which causes the bands of the different proteins (visible after a staining) to become narrower and sharper - the stacking effect. For the separation of smaller proteins and peptides, the TRIS-Tricine buffer system of Schägger and von Jagow is used due to the higher spread of the proteins in the range of 0.5 to 50 KDa.

Gel Staining

Coomassie-stained 10% Tris/Tricine Gel. In the left lane, a molecular weight size marker was used to estimate the size (from top to bottom: 66, 45, 35, 24, 18 and 9 kDa). In the remaining lanes purified yeast proteins were separated.

At the end of the electrophoretic separation, all proteins are sorted by size and can then be analyzed by other methods, for example protein staining such as Coomassie staining (most common and easy to use), silver staining (highest sensitivity), stains all staining, Amido black 10B staining, Fast green FCF staining, fluorescent stains such as epicocconone stain and SYPRO orange stain, and immunological detection such as the Western Blot. The fluorescent dyes have a comparatively higher linearity between protein quantity and color intensity of about three orders of magnitude above the detection limit, i. e., the amount of protein can be estimated by color intensity. When using the fluorescent protein dye trichloroethanol, a subsequent protein staining is omitted if it was added to the gel solution and the gel was irradiated with UV light after electrophoresis.

Analysis

Protein staining in the gel creates a documentable banding pattern of the various proteins. Glycoproteins have differential levels of glycosylations and adsorb SDS more unevenly at the glycosylations, resulting in broader and blurred bands. Membrane proteins, because of their transmembrane domain, are often composed of the more hydrophobic amino acids, have lower solubility in aqueous solutions, tend to bind lipids, and tend to precipitate in aqueous solutions due to hydrophobic effects when sufficient amounts of detergent are not present. This precipitation manifests itself for membrane proteins in a SDS-PAGE in 'tailing' above the band of the transmembrane protein. In this case, more SDS can be used (by using more or more concentrated sample buffer) and the amount of protein in the sample

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application can be reduced. An overloading of the gel with a soluble protein creates a semi-circular band of this protein (e. g. in the marker lane of the image at 66 KDa), allowing other proteins with similar molecular weights to be covered. A low contrast (as in the marker lane of the image) between bands within a lane indicates either the presence of many proteins (low purity) or, if using purified proteins and a low contrast occurs only below one band, it indicates a proteolytic degradation of the protein, which first causes degradation bands, and after further degradation produces a homogeneous color ('smear') below a band. The documentation of the banding pattern is usually done by photographing or scanning. For a subsequent recovery of the molecules in individual bands, a gel extraction can be performed.

Archiving

After protein staining and documentation of the banding pattern, the polyacrylamide gel can be dried for archival storage. Proteins can be extracted from it at a later date. The gel is either placed in a drying frame (with or without the use of heat) or in a vacuum dryer. The drying frame consists of two parts, one of which serves as a base for a wet cellophane film to which the gel and a one percent glycerol solution are added. Then a second wet cellophane film is applied bubble-free, the second frame part is put on top and the frame is sealed with clips. The removal of the air bubbles avoids a fragmentation of the gel during drying. The water evaporates through the cellophane film. In contrast to the drying frame, a vacuum dryer generates a vacuum and heats the gel to about 50°C.

Molecular Mass Determination

The proteins of the size marker (black X) show an approximately straight line in the representation of log M over RF. The molecular weight of the unknown protein (red X) can be determined on the y-axis.

For a more accurate determination of the molecular weight, the relative migration distances of the individual protein bands are measured in the separating gel. The measurements are usually performed in triplicate for increased accuracy. The relative mobility (called Rf value or Rm value) is the quotient of the distance of the band of the protein and the distance of the buffer front. The distances of the bands and the buffer front are each measured from the beginning of the separation gel. The distance of the buffer front roughly corresponds to the distance of the bromophenol blue contained in the sample buffer. The relative distances of the proteins of the size marker are plotted semi-logarithmically against their known molecular weights. By comparison with the linear part of the generated graph or by a regression analysis, the molecular weight of an unknown protein can be determined by its relative mobility. Bands of proteins with glycosylations can be blurred. Proteins with many basic amino acids can lead to an overestimation of the molecular weight or even not migrate into the gel at all, because they move slower in the electrophoresis due to the positive charges or even to the opposite direction.

Accordingly, many acidic amino acids can lead to accelerated migration of a protein and an underestimation of its molecular mass.

Applications

The SDS-PAGE in combination with a protein stain is widely used in biochemistry for the quick and exact separation and subsequent analysis of proteins. It has comparatively low instrument and reagent costs and is an easy-to-use method. Because of its low scalability, it is mostly used for analytical purposes and less for preparative purposes, especially when larger amounts of a protein are to be isolated.

Additionally, SDS-PAGE is used in combination with the western blot for the determination of the presence of a specific protein in a mixture of proteins - or for the analysis of post-translational modifications. Post-translational modifications of proteins can lead to a different relative mobility (i.e. a band shift) or to a change in the binding of a detection antibody used in the western blot (i.e., a band disappears or appears).

In mass spectrometry of proteins, SDS-PAGE is a widely used method for sample preparation prior to spectrometry, mostly using in-gel digestion. In regards to determining the molecular mass of a protein, the SDS-PAGE is a bit more exact than an analytical ultracentrifugation, but less exact than a mass spectrometry or, ignoring post-translational modifications, a calculation of the protein molecular mass from the DNA sequence.

In medical diagnostics, SDS-PAGE is used as part of the HIV test and to evaluate proteinuria. In the HIV test, HIV proteins are separated by SDS-PAGE and subsequently detected by Western Blot with HIV-specific antibodies of the patient, if they are present in his blood serum. SDS-PAGE for proteinuria evaluates the levels of various serum proteins in the urine, for example, Albumin, Alpha-2-Macroglobulin and IgG.

Variants

SDS-PAGE is the most widely used method for gel electrophoretic separation of proteins. Two-dimensional gel electrophoresis sequentially combines isoelectric focusing or BAC-PAGE with a SDS-PAGE. Native PAGE is used if native protein folding is to be maintained. For separation of membrane proteins, BAC-PAGE or CTAB-PAGE may be used as an alternative to SDS-PAGE. For electrophoretic separation of larger protein complexes, agarose gel electrophoresis can be used, for example the SDD-AGE. Some enzymes can be detected via their enzyme activity by zymography.

Alternatives

While being one of the more precise and low-cost protein separation and analysis methods, the SDS-PAGE denatures proteins. Where non-denaturing conditions are necessary, proteins are separated by a native PAGE or different

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chromatographic methods with subsequent photometric quantification, for example affinity chromatography (or even tandem affinity purification), size exclusion chromatography, ion exchange chromatography. Proteins can also be separated by size in a tangential flow filtration or a ultrafiltration. Single proteins can be isolated from a mixture by affinity chromatography or by a pull-down assay. Some historically early and cost effective but crude separation methods usually based upon a series of extractions and precipitations using kosmotropic molecules, for example the ammonium sulfate precipitation and the polyethyleneglycol precipitation.

11.2.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules, such as DNA or proteins in a matrix of agarose, one of the two main components of agar. The proteins may be separated by charge and/or size (isoelectric focusing agarose electrophoresis is essentially size independent), and the DNA and RNA fragments by length. Biomolecules are separated by applying an electric field to move the charged molecules through an agarose matrix, and the biomolecules are separated by size in the agarose gel matrix.

Agarose gel is easy to cast, has relatively fewer charged groups, and is particularly suitable for separating DNA of size range most often encountered in laboratories, which accounts for the popularity of its use. The separated DNA may be viewed with stain, most commonly under UV light, and the DNA fragments can be extracted from the gel with relative ease. Most agarose gels used are between 0.7–2.0% dissolved in a suitable electrophoresis buffer.

Properties of Agarose Gel

Fig. 11.5 *An Agarose Gel Cast in Tray Used for Gel Electrophoresis*

Agarose gel is a three-dimensional matrix formed of helical agarose molecules in supercoiled bundles that are aggregated into three-dimensional structures with channels and pores through which biomolecules can pass. The 3-D structure is held together with hydrogen bonds and can therefore be disrupted by heating back to a liquid state. The melting temperature is different from the gelling temperature, depending on the sources, agarose gel has a gelling temperature of 35–42°C and a melting temperature of 85–95 °C. Low-melting and low-gelling agaroses made through chemical modifications are also available. (Refer Figure 11.5).

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Factors Affecting Migration of Nucleic Acid in Agarose Gel

Gels of plasmid preparations usually show a major band of supercoiled DNA with other fainter bands in the same lane. Note that by convention DNA gel is displayed with smaller DNA fragments nearer to the bottom of the gel. This is because historically DNA gels were run vertically and the smaller DNA fragments move downwards faster.

A number of factors can affect the migration of nucleic acids: the dimension of the gel pores (gel concentration), size of DNA being electrophoresed, the voltage used, the ionic strength of the buffer, and the concentration of intercalating dye such as ethidium bromide if used during electrophoresis.

Smaller molecules travel faster than larger molecules in gel, and double-stranded DNA moves at a rate that is inversely proportional to the logarithm of the number of base pairs. This relationship however breaks down with very large DNA fragments, and separation of very large DNA fragments requires the use of Pulsed Field Gel Electrophoresis (PFGE), which applies alternating current from two different directions and the large DNA fragments are separated as they reorient themselves with the changing current.

For standard agarose gel electrophoresis, larger molecules are resolved better using a low concentration gel while smaller molecules separate better at high concentration gel. High concentrations gel however requires longer run times (sometimes days).

The movement of the DNA may be affected by the conformation of the DNA molecule, for example, supercoiled DNA usually moves faster than relaxed DNA because it is tightly coiled and hence more compact. In a normal plasmid DNA preparation, multiple forms of DNA may be present. Gel electrophoresis of the plasmids would normally show the negatively supercoiled form as the main band, while nicked DNA (open circular form) and the relaxed closed circular form appears as minor bands. The rate at which the various forms move however can change using different electrophoresis conditions, and the mobility of larger circular DNA may be more strongly affected than linear DNA by the pore size of the gel.

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Ethidium bromide which intercalates into circular DNA can change the charge, length, as well as the superhelicity of the DNA molecule, therefore its presence in gel during electrophoresis can affect its movement. For example, the positive charge of ethidium bromide can reduce the DNA movement by 15%. Agarose gel electrophoresis can be used to resolve circular DNA with different supercoiling topology.

DNA damage due to increased cross-linking will also reduce electrophoretic DNA migration in a dose-dependent way.

Migration Anomalies

- Smiley Gels - This edge effect is caused when the voltage applied is too high for the gel concentration used.
- Overloading of DNA - Overloading of DNA slows down the migration of DNA fragments.
- Contamination - Presence of impurities, such as salts or proteins can affect the movement of the DNA.

Mechanism of Migration and Separation

The negative charge of its phosphate backbone moves the DNA towards the positively charged anode during electrophoresis. However, the migration of DNA molecules in solution, in the absence of a gel matrix, is independent of molecular weight during electrophoresis. The gel matrix is therefore responsible for the separation of DNA by size during electrophoresis, and a number of models exist to explain the mechanism of separation of biomolecules in gel matrix. A widely accepted one is the Ogston model which treats the polymer matrix as a sieve. A globular protein or a random coil DNA moves through the interconnected pores, and the movement of larger molecules is more likely to be impeded and slowed down by collisions with the gel matrix, and the molecules of different sizes can therefore be separated in this sieving process.

The Ogston model however breaks down for large molecules whereby the pores are significantly smaller than size of the molecule. For DNA molecules of size greater than 1 kb, a reptation model (or its variants) is most commonly used. This model assumes that the DNA can crawl in a 'snake-like' fashion through the pores as an elongated molecule. A biased reptation model applies at higher electric field strength, whereby the leading end of the molecule become strongly biased in the forward direction and pulls the rest of the molecule along. Real-time fluorescence microscopy of stained molecules, however, showed more subtle dynamics during electrophoresis, with the DNA showing considerable elasticity as it alternately stretching in the direction of the applied field and then contracting into a ball, or becoming hooked into a U-shape when it gets caught on the polymer fibres.

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General Procedure

Casting of Gel

The gel is prepared by dissolving the agarose powder in an appropriate buffer, such as TAE or TBE, to be used in electrophoresis. The agarose is dispersed in the buffer before heating it to near-boiling point, but avoid boiling. The melted agarose is allowed to cool sufficiently before pouring the solution into a cast as the cast may warp or crack if the agarose solution is too hot. A comb is placed in the cast to create wells for loading sample, and the gel should be completely set before use.

The concentration of gel affects the resolution of DNA separation. For a standard agarose gel electrophoresis, a 0.8% gives good separation or resolution of large 5–10kb DNA fragments, while 2% gel gives good resolution for small 0.2–1kb fragments. 1% gels is often used for a standard electrophoresis. The concentration is measured in weight of agarose over volume of buffer used (g/ml). High percentage gels are often brittle and may not set evenly, while low percentage gels (0.1-0.2%) are fragile and not easy to handle. Low-melting-point (LMP) agarose gels are also more fragile than normal agarose gel. Low-melting point agarose may be used on its own or simultaneously with standard agarose for the separation and isolation of DNA. PFGE and FIGE are often done with high percentage agarose gels

Loading of samples

Once the gel has set, the comb is removed, leaving wells where DNA samples can be loaded. Loading buffer is mixed with the DNA sample before the mixture is loaded into the wells. The loading buffer contains a dense compound, which may be glycerol, sucrose, or Ficoll, that raises the density of the sample so that the DNA sample may sink to the bottom of the well. If the DNA sample contains residual ethanol after its preparation, it may float out of the well. The loading buffer also includes colored dyes such as xylene cyanol and bromophenol blue used to monitor the progress of the electrophoresis.

Electrophoresis

Agarose gel slab in electrophoresis tank with bands of dyes indicating progress of the electrophoresis. The DNA moves towards anode. Agarose gel electrophoresis is most commonly done horizontally in a submarine mode whereby the slab gel is completely submerged in buffer during electrophoresis. It is also possible, but less common, to perform the electrophoresis vertically, as well as horizontally with the gel raised on agarose legs using an appropriate apparatus. The buffer used in the gel is the same as the running buffer in the electrophoresis tank, which is why electrophoresis in the submarine mode is possible with agarose gel.

For optimal resolution of DNA greater than 2 kb in size in standard gel electrophoresis, 5 to 8 V/cm is recommended (the distance in cm refers to the

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distance between electrodes, therefore this recommended voltage would be 5 to 8 multiplied by the distance between the electrodes in cm). Voltage may also be limited by the fact that it heats the gel and may cause the gel to melt if it is run at high voltage for a prolonged period, especially if the gel used is LMP agarose gel. Too high a voltage may also reduce resolution, as well as causing band streaking for large DNA molecules. Too low a voltage may lead to broadening of band for small DNA fragments due to dispersion and diffusion.

Since DNA is not visible in natural light, the progress of the electrophoresis is monitored using colored dyes. Xylene cyanol (light blue color) comigrates large DNA fragments, while Bromophenol blue (dark blue) comigrates with the smaller fragments. Less commonly used dyes include Cresol Red and Orange G which migrate ahead of bromophenol blue. A DNA marker is also run together for the estimation of the molecular weight of the DNA fragments.

Staining and Visualization

DNA as well as RNA are normally visualized by staining with ethidium bromide, which intercalates into the major grooves of the DNA and fluoresces under UV light. The intercalation depends on the concentration of DNA and thus, a band with high intensity will indicate a higher amount of DNA compared to a band of less intensity. The ethidium bromide may be added to the agarose solution before it gels, or the DNA gel may be stained later after electrophoresis. Destaining of the gel is not necessary but may produce better images. Other methods of staining are available; examples are SYBR Green, GelRed, methylene blue, brilliant cresyl blue, Nile blue sulphate, and crystal violet. SYBR Green, GelRed and other similar commercial products are sold as safer alternatives to ethidium bromide as it has been shown to be mutagenic in Ames test, although the carcinogenicity of ethidium bromide has not actually been established. SYBR Green requires the use of a blue-light transilluminator. DNA stained with crystal violet can be viewed under natural light without the use of a UV transilluminator which is an advantage, however it may not produce a strong band.

When stained with ethidium bromide, the gel is viewed with an ultraviolet (UV) transilluminator. The UV light excites the electrons within the aromatic ring of ethidium bromide, and once they return to the ground state, light is released, making the DNA and ethidium bromide complex fluoresce. Standard transilluminators use wavelengths of 302/312-nm (UV-B), however exposure of DNA to UV radiation for as little as 45 seconds can produce damage to DNA and affect subsequent procedures, for example reducing the efficiency of transformation, in vitro transcription, and PCR. Exposure of the DNA to UV radiation therefore should be limited. Using a higher wavelength of 365 nm (UV-A range) causes less damage to the DNA but also produces much weaker fluorescence with ethidium bromide. Where multiple wavelengths can be selected in the transillumintor, the shorter wavelength would be used to capture images,

while the longer wavelength should be used if it is necessary to work on the gel for any extended period of time.

Downstream Procedures

The separated DNA bands are often used for further procedures, and a DNA band may be cut out of the gel as a slice, dissolved and purified. Contaminants however may affect some downstream procedures such as PCR, and low melting point agarose may be preferred in some cases as it contains fewer of the sulphates that can affect some enzymatic reactions. The gels may also be used for blotting techniques.

Buffers

In general, the ideal buffer should have good conductivity, produce less heat and have a long life. There are a number of buffers used for agarose electrophoresis; common ones for nucleic acids include Tris/Acetate/EDTA (TAE) and Tris/Borate/EDTA (TBE). The buffers used contain EDTA to inactivate many nucleases which require divalent cation for their function. The borate in TBE buffer can be problematic as borate can polymerize, and/or interact with cis diols such as those found in RNA. TAE has the lowest buffering capacity, but it provides the best resolution for larger DNA. This means a lower voltage and more time, but a better product.

Many other buffers have been proposed, for example lithium borate (LB), iso electric histidine, pK matched goods buffers, etc.; in most cases the purported rationale is lower current (less heat) and or matched ion mobilities, which leads to longer buffer life. Tris-phosphate buffer has high buffering capacity but cannot be used if DNA extracted is to be used in phosphate sensitive reaction. LB is relatively new and is ineffective in resolving fragments larger than 5 kbp; However, with its low conductivity, a much higher voltage could be used (up to 35 V/cm), which means a shorter analysis time for routine electrophoresis. As low as one base pair size difference could be resolved in 3% agarose gel with an extremely low conductivity medium (1 mM lithium borate).

Applications

- Estimation of the size of DNA molecules following digestion with restriction enzymes, for example, in restriction mapping of cloned DNA.
- Analysis of products of a Polymerase Chain Reaction (PCR), for example in molecular genetic diagnosis or genetic fingerprinting
- Separation of DNA fragments for extraction and purification.
- Separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer.
- Separation of proteins, for example, screening of protein abnormalities in clinical chemistry.

Isoelectric Focusing

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IsoElectric Focusing (IEF), also known as electrofocusing, is a technique for separating different molecules by differences in their isoelectric point (pI). It is a type of zone electrophoresis, usually performed on proteins in a gel, that takes advantage of the fact that overall charge on the molecule of interest is a function of the pH of its surroundings.

Procedure

IEF involves adding an ampholyte solution into immobilized pH gradient (IPG) gels. IPGs are the acrylamide gel matrix co-polymerized with the pH gradient, which result in completely stable gradients except the most alkaline (>12) pH values. The immobilized pH gradient is obtained by the continuous change in the ratio of immobilines. An immobiline is a weak acid or base defined by its pK value.

A protein that is in a pH region below its isoelectric point (pI) will be positively charged and so will migrate towards the cathode (negatively charged electrode). As it migrates through a gradient of increasing pH, however, the protein's overall charge will decrease until the protein reaches the pH region that corresponds to its pI. At this point it has no net charge and so migration ceases (as there is no electrical attraction towards either electrode). As a result, the proteins become focused into sharp stationary bands with each protein positioned at a point in the pH gradient corresponding to its pI. The technique is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands.

Molecules to be focused are distributed over a medium that has a pH gradient (usually created by aliphatic ampholytes). An electric current is passed through the medium, creating a positive anode and negative cathode end. Negatively charged molecules migrate through the pH gradient in the medium toward the positive end while positively charged molecules move toward the negative end. As a particle moves towards the pole opposite of its charge it moves through the changing pH gradient until it reaches a point in which the pH of that molecules isoelectric point is reached. At this point the molecule no longer has a net electric charge (due to the protonation or deprotonation of the associated functional groups) and as such will not proceed any further within the gel. The gradient is established before adding the particles of interest by first subjecting a solution of small molecules such as polyampholytes with varying pI values to electrophoresis.

Scheme of Isoelectric Focusing

Living Cells

According to some opinions, living eukaryotic cells perform isoelectric focusing of proteins in their interior to overcome a limitation of the rate of metabolic reaction by diffusion of enzymes and their reactants, and to regulate the rate of particular

biochemical processes. By concentrating the enzymes of particular metabolic pathways into distinct and small regions of its interior, the cell can increase the rate of particular biochemical pathways by several orders of magnitude. By modification of the isoelectric point (pI) of molecules of an enzyme by, for example, phosphorylation or dephosphorylation, the cell can transfer molecules of the enzyme between different parts of its interior, to switch on or switch off particular biochemical processes.

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Microfluidic Chip Based

Microchip based electrophoresis is a promising alternative to capillary electrophoresis since it has the potential to provide rapid protein analysis, straightforward integration with other microfluidic unit operations, whole channel detection, nitrocellulose films, smaller sample sizes and lower fabrication costs.

Multi-Junction

The increased demand for faster and easy-to-use protein separation tools has accelerated the evolution of IEF towards in-solution separations. In this context, a multi-junction IEF system was developed to perform fast and gel-free IEF separations. The multi-junction IEF system utilizes a series of vessels with a capillary passing through each vessel. Part of the capillary in each vessel is replaced by a semipermeable membrane. The vessels contain buffer solutions with different pH values, so that a pH gradient is effectively established inside the capillary. The buffer solution in each vessel has an electrical contact with a voltage divider connected to a high-voltage power supply, which established electrical field along the capillary. When a sample (a mixture of peptides or proteins) is injected in the capillary, the presence of the electrical field and the pH gradient separates these molecules according to their isoelectric points. The multi-junction IEF system has been used to separate tryptic peptide mixtures for two-dimensional proteomics and blood plasma proteins from Alzheimer's disease patients for biomarker discovery.

2D Electrophoresis

Two-dimensional gel electrophoresis, abbreviated as 2-DE or 2-D electrophoresis, is a form of gel electrophoresis commonly used to analyze proteins. Mixtures of proteins are separated by two properties in two dimensions on 2D gels. 2-DE was first independently introduced by O'Farrell and Klose in 1975.

Basis for Separation

2-D electrophoresis begins with electrophoresis in the first dimension and then separates the molecules perpendicularly from the first to create an electropherogram in the second dimension. In electrophoresis in the first dimension, molecules are separated linearly according to their isoelectric point. In the second dimension, the molecules are then separated at 90 degrees from the first electropherogram

according to molecular mass. Since it is unlikely that two molecules will be similar in two distinct properties, molecules are more effectively separated in 2-D electrophoresis than in 1-D electrophoresis.

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The two dimensions that proteins are separated into using this technique can be isoelectric point, protein complex mass in the native state, or protein mass.

Separation of the proteins by isoelectric point is called isoelectric focusing (IEF). Thereby, a gradient of pH is applied to a gel and an electric potential is applied across the gel, making one end more positive than the other. At all pH values other than their isoelectric point, proteins will be charged. If they are positively charged, they will be pulled towards the more negative end of the gel and if they are negatively charged they will be pulled to the more positive end of the gel. The proteins applied in the first dimension will move along the gel and will accumulate at their isoelectric point; that is, the point at which the overall charge on the protein is 0 (a neutral charge).

For the analysis of the functioning of proteins in a cell, the knowledge of their cooperation is essential. Most often proteins act together in complexes to be fully functional. The analysis of this sub organelle organisation of the cell requires techniques conserving the native state of the protein complexes. In native polyacrylamide gel electrophoresis (native PAGE), proteins remain in their native state and are separated in the electric field following their mass and the mass of their complexes respectively. To obtain a separation by size and not by net charge, as in IEF, an additional charge is transferred to the proteins by the use of Coomassie Brilliant Blue or lithium dodecyl sulfate. After completion of the first dimension the complexes are destroyed by applying the denaturing SDS-PAGE in the second dimension, where the proteins of which the complexes are composed of are separated by their mass.

Detecting Proteins

The result of this is a gel with proteins spread out on its surface. These proteins can then be detected by a variety of means, but the most commonly used stains are silver and Coomassie Brilliant Blue staining. In the former case, a silver colloid is applied to the gel. The silver binds to cysteine groups within the protein. The silver is darkened by exposure to ultra-violet light. The amount of silver can be related to the darkness, and therefore the amount of protein at a given location on the gel. This measurement can only give approximate amounts, but is adequate for most purposes. Silver staining is 100x more sensitive than Coomassie Brilliant Blue with a 40-fold range of linearity.

Molecules other than proteins can be separated by 2D electrophoresis. In supercoiling assays, coiled DNA is separated in the first dimension and denatured by a DNA intercalator (such as ethidium bromide or the less carcinogenic chloroquine) in the second. This is comparable to the combination of native PAGE /SDS-PAGE in protein separation.

Common Techniques

IPG-DALT

A common technique is to use an Immobilized pH gradient (IPG) in the first dimension. This technique is referred to as IPG-DALT. The sample is first separated onto IPG gel (which is commercially available) then the gel is cut into slices for each sample which is then equilibrated in SDS-mercaptoethanol and applied to an SDS-PAGE gel for resolution in the second dimension. Typically IPG-DALT is not used for quantification of proteins due to the loss of low molecular weight components during the transfer to the SDS-PAGE gel.

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IEF SDS-PAGE

Isoelectric focusing (IEF), also known as electrofocusing, is a technique for separating different molecules by differences in their isoelectric point (pI). It is a type of zone electrophoresis, usually performed on proteins in a gel, which takes advantage of the fact that overall charge on the molecule of interest is a function of the pH of its surroundings. IEF involves adding an ampholyte solution into immobilized pH gradient (IPG) gels. IPGs are the acrylamide gel matrix co-polymerized with the pH gradient, which result in completely stable gradients except the most alkaline (>12) pH values. The immobilized pH gradient is obtained by the continuous change in the ratio of immobilines. An immobiline is a weak acid or base defined by its pK value.

2D Gel Analysis Software

In quantitative proteomics, these tools primarily analyze bio-markers by quantifying individual proteins, and showing the separation between one or more protein 'spots' on a scanned image of a 2-DE gel. Additionally, these tools match spots between gels of similar samples to show, for example, proteomic differences between early and advanced stages of an illness. Software packages include Delta2D, ImageMaster, Melanie, PDQuest, Progenesis and REDFIN – among others. While this technology is widely utilized, the intelligence has not been perfected. For example, while PDQuest and Progenesis tend to agree on the quantification and analysis of well-defined well-separated protein spots, they deliver different results and analysis tendencies with less-defined less-separated spots.

Check Your Progress

1. What is maceration?
2. What is PAGE?
3. What is SDS-PAGE?
4. Define electrophoresis.
5. What are surfactants?
6. What are biomolecules?

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11.3 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Maceration is the winemaking process where the phenolic materials of the grape—tannins, coloring agents (anthocyanins) and flavor compounds—are leached from the grape skins, seeds and stems into the must.
2. PolyAcrylamide Gel Electrophoresis (PAGE) is a technique widely used in biochemistry, forensic chemistry, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility.
3. SDS-PAGE (Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis) is a variant of polyacrylamide gel electrophoresis, an analytical method in biochemistry for the separation of charged molecules in mixtures by their molecular masses in an electric field. It uses Sodium Dodecyl Sulfate (SDS) molecules to help identify and isolate protein molecules.
4. Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field.
5. Surfactants are compounds that lower the surface tension (or interfacial tension) between two liquids, between a gas and a liquid, or between a liquid and a solid. Surfactants may act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants.
6. Biomolecules are organic molecules especially macromolecules like carbohydrates, proteins in living organisms. All living forms bacteria, algae, plant and animals are made of similar macromolecules that are responsible for life. All the carbon compounds we get from living tissues can be called biomolecules.

11.4 SUMMARY

- Maceration is the winemaking process where the phenolic materials of the grape—tannins, coloring agents (anthocyanins) and flavor compounds—are leached from the grape skins, seeds and stems into the must.
- The process of maceration begins, to varying extent, as soon as the grapes' skins are broken and exposed to some degree of heat. Temperature is the guiding force, with higher temperatures encouraging more breakdown and extraction of phenols from the skins and other grape materials.
- Maceration continues during the fermentation period, and can last well past the point when the yeast has converted all sugars into alcohol. The process itself is a slow one with compounds such as the anthocyanins needing to pass through the cell membrane of the skins to come into contact with the wine.

- During fermentation, higher temperatures and higher alcohol levels can encourage this process with the alcohol acting as a solvent to assist in the breakdown of the organic compounds within the grape materials. This process seems to slow once the wine reaches an alcohol level of 10%.

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- PolyAcrylamide Gel Electrophoresis (PAGE) is a technique widely used in biochemistry, forensic chemistry, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility.
- Electrophoretic mobility is a function of the length, conformation and charge of the molecule. Polyacrylamide gel electrophoresis is a powerful tool used to analyze RNA samples.
- When polyacrylamide gel is denatured after electrophoresis, it provides information on the sample composition of the RNA species.
- Hydration of acrylonitrile results in formation of acrylamide molecules (C_3H_5NO) by nitrile hydratase.
- Acrylamide is toxic to the human nervous system, therefore all safety measures must be followed when working with it. Acrylamide is soluble in water and upon addition of water it polymerizes resulting in formation of polyacrylamide.
- An electric field is applied across the gel, causing the negatively charged proteins or nucleic acids to migrate across the gel away from the negative electrode (which is the cathode being that this is an electrolytic rather than galvanic cell) and towards the positive electrode (the anode).
- Smaller biomolecules travel farther down the gel, while larger ones remain closer to the point of origin. Biomolecules may therefore be separated roughly according to size, which depends mainly on molecular weight under denaturing conditions, but also depends on higher-order conformation under native conditions.
- SDS-PAGE (Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis) is a variant of polyacrylamide gel electrophoresis, an analytical method in biochemistry for the separation of charged molecules in mixtures by their molecular masses in an electric field. It uses Sodium Dodecyl Sulfate (SDS) molecules to help identify and isolate protein molecules.

11.5 KEY WORDS

- **Polyacrylamide gel electrophoresis:** PolyAcrylamide Gel Electrophoresis (PAGE) is a technique widely used in biochemistry, forensic chemistry, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility.

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- **Agarose:** Agarose is a polysaccharide, generally extracted from certain red seaweed. It is a linear polymer made up of the repeating unit of agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose.
- **Anthocyanins:** Anthocyanins are a type of flavonoid, a class of compounds with antioxidant effects. Found naturally in a number of foods, anthocyanins are the pigments that give red, purple, and blue plants their rich coloring.
- **Electrophoresis:** Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field.
- **Acrylamide:** Acrylamide is a chemical used mainly in certain industrial processes, such as in making paper, dyes, and plastics, and in treating drinking water and wastewater.
- **Sodium dodecyl sulfate:** Sodium Dodecyl Sulfate (SDS), synonymously Sodium Lauryl Sulfate (SLS), or sodium laurilsulfate, is a synthetic organic compound with the formula $CH_3(CH_2)_{11}SO_4$ Na. It is an anionic surfactant used in many cleaning and hygiene products.
- **Surfactants:** Surfactants are compounds that lower the surface tension (or interfacial tension) between two liquids, between a gas and a liquid, or between a liquid and a solid.
- **Biomolecules:** Biomolecules are organic molecules especially macromolecules like carbohydrates, proteins in living organisms. All living forms bacteria, algae, plant and animals are made of similar macromolecules that are responsible for life.
- **Polymerisation:** Polymerisation is the chemical process of monomers joining together to form polymers, often it takes many thousands of monomers to make a single polymer.

11.6 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short Answer Questions

1. Write a short note on maceration technique?
2. What is PAGE?
3. Write a short note on preparing acrylamide gels
4. Write a short note on electrophoresis.
5. Give a brief account of few components used in electrophoresis.

Long Answer Questions

1. Discuss about maceration techniques in detail.
2. What is polyacrylamide gel electrophoresis? Expalin in detail.
3. Discuss about electrophoresis in detail also about the components used.
4. Expalin about the chemicals for processing and visualization in electrophoresis.
5. Discuss in detail about SDS-PAGE in detail.
6. Expalin in detail about agarose gel electrophoresis.
7. Discuss in detail about the mechanism of migration and separation.

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11.7 FURTHER READINGS

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UNIT 12 SEPARATION TECHNIQUE

NOTES

Structure

12.0 Introduction
12.1 Objectives
12.2 Separartion Process
12.3 Ultracentrifuge
 12.3.1 Thin-Layer Chromatography (TLC)
 12.3.2 High-Performance Liquid Chromatography
 12.3.3 High-Performance Thin-Layer Chromatography
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12.0 INTRODUCTION

In our daily life we come across so many substances that need to be separated from other substances in a mixture. There are various techniques that are used for the separation of substances from other substances. Separation technique is a method in which mass transfer takes place and converts a mixture of substances into two or more substances. Two or more substances which are separated using various methods of separation can be useful or useless. Segregation of substance is also used to remove harmful substances from a useful substance. The separation of solutions and mixtures into their single components is an operation of great importance for the chemical, petrochemical, and oil industries. Almost all chemical processes need preliminary raw material purification or the separation of primary from secondary products.

In this unit, you will study about separation technique, ultracentrifugation, TLC, HPLC, HPTLC, FPLC, GC, MS, MALDI Tof. in detail.

12.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand about separation technique
- Expalin ultracentrifugation
- Understand the significance of ultracentrifugation and its different types
- Discuss about TLC, HPLC, HPTLC, FPLC, GC, MS, MALDI Tof.

12.2 SEPARARTION PROCESS

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A separation process is a method that converts a mixture or solution of chemical substances into two or more distinct product mixtures. At least one of results of the separation is enriched in one or more of the source mixture's constituents. In some cases, a separation may fully divide the mixture into pure constituents. Separations exploit differences in chemical properties or physical properties (such as, size, shape, mass, density, or chemical affinity) between the constituents of a mixture.

Processes are often classified according to the particular differences they use to achieve separation. If no single difference can be used to accomplish a desired separation, multiple operations can often be combined to achieve the desired end. With a few exceptions, elements or compounds exist in nature in an impure state. Often these raw materials must go through a separation before they can be put to productive use, making separation techniques essential for the modern industrial economy.

The purpose of a separation may be analytical, can be used as a lie components in the original mixture without any attempt to save the fractions, or may be preparative, i.e., to 'prepare' fractions or samples of the components that can be saved. The separation can be done on a small scale, effectively a laboratory scale for analytical or preparative purposes, or on a large scale, effectively an industrial scale for preparative purposes, or on some intermediate scale.

Complete and Incomplete Separation

In some cases, separations require total purification, as in the electrolysis refining of bauxite ore for aluminum metal, but a good example of an incomplete separation technique is oil refining. Crude oil occurs naturally as a mixture of various hydrocarbons and impurities. The refining process splits this mixture into other, more valuable mixtures such as natural gas, gasoline and chemical feed stocks, none of which are pure substances, but each of which must be separated from the raw crude. In both of these cases, a series of separations is necessary to obtain the desired end products. In the case of oil refining, crude is subjected to a long series of individual distillation steps, each of which produces a different product or intermediate.

Separating Liquids

Separators are used to divide liquids. Vertically supported centrifuges are built with flying bearings. A separator is a continues sedimentation centrifuge. Both exit streams are continuously discharged, using a pump (under pressure) or pressure free. The solid material can be discharged discontinuously (chamber drum, solid walled disc drum), pseudo continuously (self-cleaning disc drum) or continuously (nozzle drum). The drum is the centerpiece of the separator, in which the separation

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process takes place. There are two types of drums: the chamber drum (known as chamber separators) and the disc drum (known as disc separators). The power transmission on the spindle and thereby on the drum can take place by using one of the three drive motors: helical gears, a belt drive or direct drive, via a special motor. The sealing of the separators is differentiated into four types: open, semi-closed, hydro-hermetic (sealing of the product space) or fully hermetic (absolute airtight).

List of Separation Techniques

- Adsorption, adhesion of atoms, ions or molecules of gas, liquid, or dissolved solids to a surface
- Capillary Electrophoresis
- Centrifugation and cyclonic separation, separates based on density differences
- Chelation
- Chromatography separates dissolved substances by different interaction with (i.e., travel through) a material
  - o High-Performance Liquid Chromatography (HPLC)
  - o Thin-Layer Chromatography (TLC)
  - o Counter Current Chromatography (CCC)
  - o Droplet Countercurrent Chromatography (DCC)
  - o Paper chromatography
  - o Ion chromatography
  - o Size-exclusion chromatography
  - o Affinity chromatography
  - o Centrifugal partition chromatography
  - o Gas chromatography and Inverse gas chromatography
- Crystallization
- Decantation
- Demister (vapor), removes liquid droplets from gas streams
- Distillation, used for mixtures of liquids with different boiling points
- Drying, removes liquid from a solid by vaporisation
- Electrophoresis, separates organic molecules based on their different interaction with a gel under an electric potential (i.e., different travel)
- Electrostatic separation, works on the principle of corona discharge, where two plates are placed close together and high voltage is applied. This high voltage is used to separate the ionized particles.

- Elutriation
- Evaporation
- Extraction
  - o Leaching
  - o Liquid-liquid extraction
  - o Solid phase extraction
  - o Supercritical fluid extraction
- Field flow fractionation
- Flotation
  - o Dissolved air flotation, removes suspended solids non-selectively from slurry by bubbles that are generated by air coming out of solution
  - o Froth flotation, recovers valuable, hydrophobic solids by attachment to air bubbles generated by mechanical agitation of an air-slurry mixture, which float, and are recovered
  - o Deinking, separating hydrophobic ink particles from hydrophilic paper pulp in paper recycling
- Flocculation, separates a solid from a liquid in a colloid, by use of a flocculant, which promotes the solid clumping into flocs
- Filtration – Mesh, bag and paper filters are used to remove large particulates suspended in fluids (for example, fly ash) while membrane processes including microfiltration, ultrafiltration, nanofiltration, reverse osmosis, dialysis (biochemistry) utilising synthetic membranes, separates micrometre-sized or smaller species
  - Fractional distillation
  - Fractional freezing
- Oil-water separation, gravimetrically separates suspended oil droplets from waste water in oil refineries, petrochemical and chemical plants, natural gas processing plants and similar industries
- Magnetic separation
- Precipitation
- Recrystallization
- Scrubbing, separation of particulates (solids) or gases from a gas stream using liquid.
- Sedimentation, separates using vocal density pressure differences
- Sieving
- Stripping
- Sublimation

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- Vapor-liquid separation, separates by gravity, based on the Souders-Brown equation
- Winnowing
- Zone refining

Check Your Progress

1. What is separation process?
2. List few of the separation techniques.
3. Define decantation.
4. What is elutriation?
5. What does the term fractional distillation mean?

12.3 ULTRACENTRIFUGE

The ultracentrifuge is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as 1,000,000 g (approx. 9 800 km/s^2). There are two kinds of ultracentrifuges, the preparative and the analytical ultracentrifuge. Both classes of instruments find important uses in molecular biology, biochemistry, and polymer science.

History

Theodor Svedberg invented the analytical ultracentrifuge in 1925, and won the Nobel Prize in Chemistry in 1926 for his research on colloids and proteins using the ultracentrifuge.

The vacuum ultracentrifuge was invented by Edward Greydon Pickels in the Physics Department at the University of Virginia. It was his contribution of the vacuum which allowed a reduction in friction generated at high speeds. Vacuum systems also enabled the maintenance of constant temperature across the sample, eliminating convection currents that interfered with the interpretation of sedimentation results.

In 1946, Pickels cofounded Spinco (Specialized Instruments Corp.) to market analytical and preparative ultracentrifuges based on his design. Pickels considered his design to be too complicated for commercial use and developed a more easily operated, “foolproof” version. But even with the enhanced design, sales of analytical centrifuges remained low, and Spinco almost went bankrupt. The company survived by concentrating on sales of preparative ultracentrifuge models, which were becoming popular as workhorses in biomedical laboratories. In 1949, Spinco introduced the Model L, the first preparative ultracentrifuge to reach a maximum speed of 40,000 rpm. In 1954, Beckman Instruments, now Beckman Coulter, purchased the company, forming the basis of its Spinco centrifuge division.

Analytical Ultracentrifuge

In an analytical ultracentrifuge, a sample being spun can be monitored in real time through an optical detection system, using ultraviolet light absorption and/or interference optical refractive index sensitive system. This allows the operator to observe the evolution of the sample concentration versus the axis of rotation profile as a result of the applied centrifugal field. With modern instrumentation, these observations are electronically digitized and stored for further mathematical analysis. Two kinds of experiments are commonly performed on these instruments: sedimentation velocity experiments and sedimentation equilibrium experiments.

Sedimentation velocity experiments aim to interpret the entire time-course of sedimentation, and report on the shape and molar mass of the dissolved macromolecules, as well as their size-distribution. The size resolution of this method scales approximately with the square of the particle radii, and by adjusting the rotor speed of the experiment size-ranges from 100 Da to 10 GDa can be covered. Sedimentation velocity experiments can also be used to study reversible chemical equilibria between macromolecular species, by either monitoring the number and molar mass of macromolecular complexes, by gaining information about the complex composition from multi-signal analysis exploiting differences in each components spectroscopic signal, or by following the composition dependence of the sedimentation rates of the macromolecular system, as described in Gilbert-Jenkins theory.

Preparative Ultracentrifuge

Preparative ultracentrifuges are available with a wide variety of rotors suitable for a great range of experiments. Most rotors are designed to hold tubes that contain the samples. Swinging bucket rotors allow the tubes to hang on hinges so the tubes reorient to the horizontal as the rotor initially accelerates. Fixed angle rotors are made of a single block of material and hold the tubes in cavities bored at a predetermined angle. Zonal rotors are designed to contain a large volume of sample in a single central cavity rather than in tubes. Some zonal rotors are capable of dynamic loading and unloading of samples while the rotor is spinning at high speed.

Preparative rotors are used in biology for pelleting of fine particulate fractions, such as cellular organelles (mitochondria, microsomes, ribosomes) and viruses. They can also be used for gradient separations, in which the tubes are filled from top to bottom with an increasing concentration of a dense substance in solution. Sucrose gradients are typically used for separation of cellular organelles. Gradients of caesium salts are used for separation of nucleic acids. After the sample has spun at high speed for sufficient time to produce the separation, the rotor is allowed to come to a smooth stop and the gradient is gently pumped out of each tube to isolate the separated components.

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Hazards

The tremendous rotational kinetic energy of the rotor in an operating ultracentrifuge makes the catastrophic failure of a spinning rotor a serious concern. Rotors conventionally have been made from high strength-to-weight metals such as aluminum or titanium. The stresses of routine use and harsh chemical solutions eventually cause rotors to deteriorate. Proper use of the instrument and rotors within recommended limits and careful maintenance of rotors to prevent corrosion and to detect deterioration is necessary to mitigate this risk.

More recently some rotors have been made of lightweight carbon fiber composite material, which are up to 60% lighter, resulting in faster acceleration/ deceleration rates. Carbon fiber composite rotors also are corrosion-resistant, eliminating a major cause of rotor failure.

12.3.1 Thin-Layer Chromatography (TLC)

After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. The mobile phase has different properties from the stationary phase. For example, with silica gel, a very polar substance, non-polar mobile phases such as heptane are used. The mobile phase may be a mixture, allowing chemists to fine-tune the bulk properties of the mobile phase.

After the experiment, the spots are visualized. Often this can be done simply by projecting ultraviolet light onto the sheet; the sheets are treated with a phosphor, and dark spots appear on the sheet where compounds absorb the light impinging on a certain area. Chemical processes can also be used to visualize spots; anisaldehyde, for example, forms colored adducts with many compounds, and sulfuric acid will char most organic compounds, leaving a dark spot on the sheet.

To quantify the results, the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase. (The mobile phase must not be allowed to reach the end of the stationary phase.) This ratio is called the Retardation Factor (RF). In general, a substance whose structure resembles the stationary phase will have low RF, while one that has a similar structure to the mobile phase will have high retardation factor.

Thin-layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Specific examples of these applications include: analyzing ceramides and fatty acids, detection of pesticides or insecticides in food and water, analyzing the dye composition of fibers in forensics, assaying the radiochemical purity of radiopharmaceuticals, or identification of medicinal plants and their constituents.

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Plate Preparation

TLC plates (Refer Figure 12.1) are usually commercially available, with standard particle size ranges to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate (gypsum) and water. This mixture is spread as a thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic. The resultant plate is dried and activated by heating in an oven for thirty minutes at 110°C. The thickness of the absorbent layer is typically around 0.1 – 0.25 mm for analytical purposes and around 0.5 – 2.0 mm for preparative TLC.

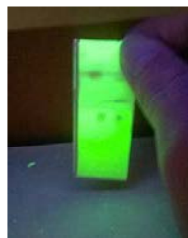

Fig. 12.1 *Fluorescent TLC Plate Under an Ultraviolet (UV) Light*

Technique

The process is similar to paper chromatography with the advantage of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products. Plates can be labeled before or after the chromatography process using a pencil or other implement that will not interfere or react with the process.

To run a thin layer chromatography plate, the following procedure is carried out. Using a capillary, a small spot of solution containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge. The solvent is allowed to completely evaporate off to prevent it from interfering with sample's interactions with the mobile phase in the next step. If a non-volatile solvent was used to apply the sample, the plate needs to be dried in a vacuum chamber. This step is often repeated to ensure there is enough analyte at the starting spot on the plate to obtain a visible result. Different samples can be placed in a row of spots the same distance from the bottom edge, each of which will move in its own adjacent lane from its own starting point.

A small amount of an appropriate solvent (eluent) is poured into a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter. A strip of filter paper (wick) is put into the chamber so that its bottom touches the solvent and the paper lies on the chamber wall and reaches almost to the top of the container. The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the chamber.

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Separation Process and Principle

Different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase and because of differences in solubility in the solvent. By changing the solvent, or perhaps using a mixture, the separation of components (measured by the Rf value) can be adjusted. Also, the separation achieved with a TLC plate can be used to estimate the separation of a flash chromatography column. (A compound elutes from a column when the amount of solvent collected is equal to 1/RF.) Chemists often use TLC to develop a protocol for separation by chromatography and use TLC to determine which fractions contain the desired compounds.

Surface of a freshly cut plank of Eucalyptus camaldulensis displaying thin-layer chromatography. The horizontal blue strip is from a reaction between the iron bandsaw supports and the acidic timber

Separation of compounds is based on the competition of the solute and the mobile phase for binding sites on the stationary phase. For instance, if normal-phase silica gel is used as the stationary phase, it can be considered polar. Given two compounds that differ in polarity, the more polar compound has a stronger interaction with the silica and is, therefore, better able to displace the mobile phase from the available binding sites. As a consequence, the less polar compound moves higher up the plate (resulting in a higher RF value).

Analysis

As the chemicals being separated may be colorless, several methods exist to visualize the spots:

- fluorescent analytes like quinine may be detected under blacklight (366 nm)
- Often a small amount of a fluorescent compound, usually manganese-activated zinc silicate, is added to the adsorbent that allows the visualization of spots under UV-C light (254 nm). The adsorbent layer will thus fluoresce light-green by itself, but spots of analyte quench this fluorescence.
- Iodine vapors are a general unspecific color reagent
- Specific color reagents into which the TLC plate is dipped or which are sprayed onto the plate exist.
    - o Potassium permanganate - oxidation
    - o Bromine
- In the case of lipids, the chromatogram may be transferred to a PVDF membrane and then subjected to further analysis, for example mass spectrometry, a technique known as Far-Eastern blotting.

Applications

Characterization

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In organic chemistry, reactions are qualitatively monitored with TLC. Spots sampled with a capillary tube are placed on the plate: a spot of starting material, a spot from the reaction mixture, and a cross-spot with both. A small (3 by 7 cm) TLC plate takes a couple of minutes to run. The analysis is qualitative, and it will show if the starting material has disappeared, i.e., the reaction is complete, if any product has appeared, and how many products are generated (although this might be underestimated due to co-elution). Unfortunately, TLCs from low-temperature reactions may give misleading results, because the sample is warmed to room temperature in the capillary, which can alter the reaction—the warmed sample analyzed by TLC is not the same as what is in the low-temperature flask. One such reaction is the DIBALH reduction of ester to aldehyde.

In one study TLC has been applied in the screening of organic reactions, for example in the fine-tuning of BINAP synthesis from 2-naphthol. In this method, the alcohol and catalyst solution (for instance iron(III) chloride) are placed separately on the base line, then reacted, and then instantly analyzed.

Isolation

Since different compounds will travel a different distance in the stationary phase, chromatography can be used to isolate components of a mixture for further analysis. The separated compounds each occupying a specific area on the plate, they can be scraped off (along with the stationary phase particles) and dissolved into an appropriate solvent. As an example, in the chromatography of an extract of green plant material (for example spinach) shown in 7 stages of development, Carotene elutes quickly and is only visible until step 2. Chlorophyll A and B are halfway in the final step and lutein the first compound staining yellow. Once the chromatography is over, the carotene can be removed from the plate, extracted into a solvent and placed into a spectrophotometer to determine its spectrum. The quantities extracted are small and a technique such as column chromatography is preferred to separate larger amounts.

Examining Reactions

TLC is also used for the identification of the completion of any chemical reaction. To determine this it is observed that at the beginning of a reaction the entire spot is occupied by the starting chemicals or materials on the plate. As the reaction starts taking place the spot formed by the initial chemicals starts reducing and eventually replaces the whole spot of starting chemicals with a new product present on the plate. The formation of an entirely new spot determines the completion of a reaction.

12.3.2 High-Performance Liquid Chromatography

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High-Performance Liquid Chromatography (HPLC; formerly referred to as high-pressure liquid chromatography) is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column.

HPLC has been used for manufacturing (for example, during the production process of pharmaceutical and biological products), legal (for example, detecting performance enhancement drugs in urine), research (for example, separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and medical (for example, detecting vitamin D levels in blood serum) purposes.

Chromatography can be described as a mass transfer process involving adsorption. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with adsorbent, leading to the separation of the sample components. The active component of the column, the adsorbent, is typically a granular material made of solid particles (for example, silica, polymers, etc.), 2–50 ìm in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles. The pressurized liquid is typically a mixture of solvents (for example, water, acetonitrile and/or methanol) and is referred to as a 'mobile phase'. Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination.

HPLC is distinguished from traditional 'low pressure' liquid chromatography because operational pressures are significantly higher (50–350 bar), while ordinary liquid chromatography typically relies on the force of gravity to pass the mobile phase through the column. Due to the small sample amount separated in analytical HPLC, typical column dimensions are 2.1–4.6 mm diameter, and 30–250 mm length. Also HPLC columns are made with smaller adsorbent particles (2–50 ìm in average particle size). This gives HPLC superior resolving power (the ability to distinguish between compounds) when separating mixtures, which makes it a popular chromatographic technique.

Operation

The sample mixture to be separated and analyzed is introduced, in a discrete small volume (typically microliters), into the stream of mobile phase percolating through the column. The components of the sample move through the column at different

velocities, which are a function of specific physical interactions with the adsorbent (also called stationary phase). The velocity of each component depends on its chemical nature, on the nature of the stationary phase (column) and on the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called its retention time. The retention time measured under particular conditions is an identifying characteristic of a given analyte.

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Many different types of columns are available, filled with adsorbents varying in particle size, and in the nature of their surface ('surface chemistry'). The use of smaller particle size packing materials requires the use of higher operational pressure ('back pressure') and typically improves chromatographic resolution (i.e., the degree of separation between consecutive analytes emerging from the column). Sorbent particles may be hydrophobic or polar in nature.

Common mobile phases used include any miscible combination of water with various organic solvents (the most common are acetonitrile and methanol). Some HPLC techniques use water-free mobile phases (see Normal-phase chromatography below). The aqueous component of the mobile phase may contain acids (such as formic, phosphoric or trifluoroacetic acid) or salts to assist in the separation of the sample components. The composition of the mobile phase may be kept constant ('isocratic elution mode') or varied ('gradient elution mode') during the chromatographic analysis. Isocratic elution is typically effective in the separation of sample components that are very different in their affinity for the stationary phase. In gradient elution the composition of the mobile phase is varied typically from low to high eluting strength.

History and Development

Prior to HPLC scientists used standard liquid chromatographic techniques. Liquid chromatographic systems were largely inefficient due to the flow rate of solvents being dependent on gravity. Separations took many hours, and sometimes days to complete. Gas chromatography (GC) at the time was more powerful than liquid chromatography (LC), however, it was believed that gas phase separation and analysis of very polar high molecular weight biopolymers was impossible. GC was ineffective for many biochemists because of the thermal instability of the solutes. As a result, alternative methods were hypothesized which would soon result in the development of HPLC.

Following on the seminal work of Martin and Synge in 1941, it was predicted by Cal Giddings, Josef Huber, and others in the 1960s that LC could be operated in the high-efficiency mode by reducing the packing-particle diameter substantially below the typical LC (and GC) level of 150 ìm and using pressure to increase the mobile phase velocity. These predictions underwent extensive experimentation and refinement throughout the 60s into the 70s. Early developmental research began to improve LC particles, and the invention of Zipax, a superficially porous particle, was promising for HPLC technology.

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The 1970s brought about many developments in hardware and instrumentation. Researchers began using pumps and injectors to make a rudimentary design of an HPLC system. Gas amplifier pumps were ideal because they operated at constant pressure and did not require leak free seals or check valves for steady flow and good quantitation. Hardware milestones were made at Dupont IPD (Industrial Polymers Division) such as a low-dwell-volume gradient device being utilized as well as replacing the septum injector with a loop injection valve.

Types

Partition Chromatography

Partition chromatography was one of the first kinds of chromatography that chemists developed. The partition coefficient principle has been applied in paper chromatography, thin layer chromatography, gas phase and liquid–liquid separation applications. The 1952 Nobel Prize in chemistry was earned by Archer John Porter Martin and Richard Laurence Millington Synge for their development of the technique, which was used for their separation of amino acids. Partition chromatography uses a retained solvent, on the surface or within the grains or fibers of an 'inert' solid supporting matrix as with paper chromatography; or takes advantage of some coulombic and/or hydrogen donor interaction with the stationary phase. Analyte molecules partition between a liquid stationary phase and the eluent. Just as in Hydrophilic Interaction Chromatography (HILIC; a sub-technique within HPLC), this method separates analytes based on differences in their polarity. HILIC most often uses a bonded polar stationary phase and a mobile phase made primarily of acetonitrile with water as the strong component. Partition HPLC has been used historically on unbonded silica or alumina supports. Each works effectively for separating analytes by relative polar differences. HILIC bonded phases have the advantage of separating acidic, basic and neutral solutes in a single chromatographic run.

Normal–Phase Chromatography

Normal–phase chromatography was one of the first kinds of HPLC that chemists developed. Also known as normal-phase HPLC (NP-HPLC) this method separates analytes based on their affinity for a polar stationary surface such as silica, hence it is based on analyte ability to engage in polar interactions (such as hydrogen-bonding or dipole-dipole type of interactions) with the sorbent surface. NP-HPLC uses a non-polar, non-aqueous mobile phase (for example, Chloroform), and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity. The interaction strength depends not only on the functional groups present in the structure of the analyte molecule, but also on steric factors. The effect of steric hindrance on interaction strength allows this method to resolve (separate) structural isomers.

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Partition- and NP-HPLC fell out of favor in the 1970s with the development of reversed-phase HPLC because of poor reproducibility of retention times due to the presence of a water or protic organic solvent layer on the surface of the silica or alumina chromatographic media. This layer changes with any changes in the composition of the mobile phase (for example, moisture level) causing drifting retention times.

Recently, partition chromatography has become popular again with the development of Hilic bonded phases which demonstrate improved reproducibility, and due to a better understanding of the range of usefulness of the technique.

Displacement Chromatography

The basic principle of displacement chromatography is: A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities. There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings.

Reversed-Phase Chromatography (RPC)

Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is a silica which has been surface-modified with RMe2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With such stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily (early in the analysis). An investigator can increase retention times by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent. RP-HPLC is so commonly used that it is often incorrectly referred to as 'HPLC' without further specification. The pharmaceutical industry regularly employs RP-HPLC to qualify drugs before their release.

RP-HPLC operates on the principle of hydrophobic interactions, which originates from the high symmetry in the dipolar water structure and plays the most important role in all processes in life science. RP-HPLC allows the measurement of these interactive forces. The binding of the analyte to the stationary phase is

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proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand on the stationary phase. This solvophobic effect is dominated by the force of water for 'cavity-reduction' around the analyte and the C18-chain versus the complex of both. The energy released in this process is proportional to the surface tension of the eluent (water: 7.3×10"6 J/cm^2, methanol: 2.2×10"6 J/cm^2) and to the hydrophobic surface of the analyte and the ligand respectively.

Size-Exclusion Chromatography

Size-Exclusion Chromatography (SEC), also known as gel permeation chromatography or gel filtration chromatography, separates particles on the basis of molecular size (actually by a particle's Stokes radius). It is generally a low resolution chromatography and thus it is often reserved for the final, 'polishing' step of the purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins. SEC is used primarily for the analysis of large molecules such as proteins or polymers. SEC works by trapping these smaller molecules in the pores of a particle. The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules therefore flow through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time.

Ion-Exchange Chromatography

In ion-Exchange Chromatography (IC), retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Solute ions of the same charge as the charged sites on the column are excluded from binding, while solute ions of the opposite charge of the charged sites of the column are retained on the column. Solute ions that are retained on the column can be eluted from the column by changing the solvent conditions (for example, increasing the ion effect of the solvent system by increasing the salt concentration of the solution, increasing the column temperature, changing the pH of the solvent, etc.).

Types of ion exchangers include polystyrene resins, cellulose and dextran ion exchangers (gels), and controlled-pore glass or porous silica. Polystyrene resins allow cross linkage which increases the stability of the chain. Higher cross linkage reduces swerving, which increases the equilibration time and ultimately improves selectivity. Cellulose and dextran ion exchangers possess larger pore sizes and low charge densities making them suitable for protein separation.

Bioaffinity Chromatography

This chromatographic process relies on the property of biologically active substances to form stable, specific, and reversible complexes. The formation of these complexes involves the participation of common molecular forces such as the Van der Waals interaction, electrostatic interaction, dipole-dipole interaction, hydrophobic interaction, and the hydrogen bond. An efficient, biospecific bond is

formed by a simultaneous and concerted action of several of these forces in the complementary binding sites.

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Aqueous Normal-phase Chromatography

Aqueous Normal-Phase Chromatography (ANP) is a chromatographic technique which encompasses the mobile phase region between reversed-phase chromatography (RP) and organic normal phase chromatography (ONP). This technique is used to achieve unique selectivity for hydrophilic compounds, showing normal phase elution using reversed-phase solvents.

Isocratic and Gradient Elution

A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic (meaning constant composition). (The example of these the percentage of methanol throughout the procedure will remain constant, i.e., 10%) The word was coined by Csaba Horvath who was one of the pioneers of HPLC.

The mobile phase composition does not have to remain constant. A separation in which the mobile phase composition is changed during the separation process is described as a gradient elution. One example is a gradient starting at 10% methanol and ending at 90% methanol after 20 minutes. The two components of the mobile phase are typically termed 'A' and 'B'; A is the 'weak' solvent which allows the solute to elute only slowly, while B is the 'strong' solvent which rapidly elutes the solutes from the column. In reversed-phase chromatography, solvent A is often water or an aqueous buffer, while B is an organic solvent miscible with water, such as acetonitrile, methanol, THF, or isopropanol.

Parameters

Theoretical

HPLC separations have theoretical parameters and equations to describe the separation of components into signal peaks when detected by instrumentation such as by a UV detector or a mass spectrometer. The parameters are largely derived from two sets of chromatagraphic theory: plate theory (as part of Partition chromatography), and the rate theory of chromatography / Van Deemter equation. Of course, they can be put in practice through analysis of HPLC chromatograms, although rate theory is considered the more accurate theory.

They are analogous to the calculation of retention factor for a paper chromatography separation, but describes how well HPLC separates a mixture into two or more components that are detected as peaks (bands) on a chromatogram. The HPLC parameters are the: efficiency factor(N), the retention factor (kappa prime), and the separation factor (alpha). Together the factors are variables in a resolution equation, which describes how well two components' peaks separated or overlapped each other. These parameters are mostly only

used for describing HPLC reversed phase and HPLC normal phase separations, since those separations tend to be more subtle than other HPLC modes (for example, ion exchange and size exclusion).

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Void volume is the amount of space in a column that is occupied by solvent. It is the space within the column that is outside of the column's internal packing material. Void volume is measured on a chromatogram as the first component peak detected, which is usually the solvent that was present in the sample mixture; ideally the sample solvent flows through the column without interacting with the column, but is still detectable as distinct from the HPLC solvent. The void volume is used as a correction factor.

Efficiency factor (N) practically measures how sharp component peaks on the chromatogram are, as ratio of the component peak's area ('retention time') relative to the width of the peaks at their widest point (at the baseline). Peaks that are tall, sharp, and relatively narrow indicate that separation method efficiently removed a component from a mixture; high efficiency. Efficiency is very dependent upon the HPLC column and the HPLC method used. Efficiency factor is synonymous with plate number, and the 'number of theoretical plates'.

Internal Diameter

The Internal Diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded onto the column. Larger columns are usually seen in industrial applications, such as the purification of a drug product for later use. Low-ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

Larger ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity.

Analytical scale columns (4.6 mm) have been the most common type of columns, though smaller columns are rapidly gaining in popularity. They are used in traditional quantitative analysis of samples and often use a UV-Vis absorbance detector.

Narrow-bore columns (1–2 mm) are used for applications when more sensitivity is desired either with special UV-vis detectors, fluorescence detection or with other detection methods like liquid chromatography-mass spectrometry

Capillary columns (under 0.3 mm) are used almost exclusively with alternative detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

Particle Size

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5 µm beads being the most common. Smaller particles

generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.

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This means that changing to particles that are half as big, keeping the size of the column the same, will double the performance, but increase the required pressure by a factor of four. Larger particles are used in preparative HPLC (column diameters 5 cm up to >30 cm) and for non-HPLC applications such as solid-phase extraction.

Pore Size

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics, especially for larger analytes. For example, a protein which is only slightly smaller than a pore might enter the pore but does not easily leave once inside.

Pump Pressure

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible volumetric flow rate. Pressure may reach as high as 60 MPa (6000 lbf/in2), or about 600 atmospheres. Modern HPLC systems have been improved to work at much higher pressures, and therefore are able to use much smaller particle sizes in the columns (<2 μm). These ultra high performance liquid chromatography' systems or UHPLCs can work at up to 120 MPa (17,405 lbf/in2), or about 1200 atmospheres. The term 'UPLC' is a trademark of the Waters Corporation, but is sometimes used to refer to the more general technique of UHPLC.

Detectors

HPLC detectors fall into two main categories: universal or selective. Universal detectors typically measure a bulk property (for example, refractive index) by measuring a difference of a physical property between the mobile phase and mobile phase with solute while selective detectors measure a solute property (for example, UV-Vis absorbance) by simply responding to the physical or chemical property of the solute. HPLC most commonly uses a UV-Vis absorbance detector, however, a wide range of other chromatography detectors can be used. A universal detector that complements UV-Vis absorbance detection is the Charged Aerosol Detector (CAD). A kind of commonly utilized detector includes refractive index detectors, which provide readings by measuring the changes in the refractive index of the effluent as it moves through the flow cell. In certain cases, it is possible to use multiple detectors, for example LCMS normally combines UV-Vis with a mass spectrometer.

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Autosamplers

Large numbers of samples can be automatically injected onto an HPLC system, by the use of HPLC autosamplers. In addition, HPLC autosamplers have an injection volume and technique which is exactly the same for each injection, consequently they provide a high degree of injection volume precision.

Applications

Manufacturing

HPLC has many applications in both laboratory and clinical science. It is a common technique used in pharmaceutical development, as it is a dependable way to obtain and ensure product purity. While HPLC can produce extremely high quality (pure) products, it is not always the primary method used in the production of bulk drug materials. According to the European pharmacopoeia, HPLC is used in only 15.5% of syntheses. However, it plays a role in 44% of syntheses in the United States pharmacopoeia.

Legal

This technique is also used for detection of illicit drugs in urine. The most common method of drug detection is an immunoassay. This method is much more convenient. However, convenience comes at the cost of specificity and coverage of a wide range of drugs. As HPLC is a method of determining (and possibly increasing) purity, using HPLC alone in evaluating concentrations of drugs is somewhat insufficient. With this, HPLC in this context is often performed in conjunction with mass spectrometry. Using liquid chromatography instead of gas chromatography in conjunction with MS circumvents the necessity for derivitizing with acetylating or alkylation agents, which can be a burdensome extra step.

Research

Similar assays can be performed for research purposes, detecting concentrations of potential clinical candidates like anti-fungal and asthma drugs. This technique is obviously useful in observing multiple species in collected samples, as well, but requires the use of standard solutions when information about species identity is sought out. It is used as a method to confirm results of synthesis reactions, as purity is essential in this type of research. However, mass spectrometry is still the more reliable way to identify species.

Medical

Medical use of HPLC can include drug analysis, but falls more closely under the category of nutrient analysis. While urine is the most common medium for analyzing drug concentrations, blood serum is the sample collected for most medical analyses with HPLC. Other methods of detection of molecules that are useful for clinical studies have been tested against HPLC, namely immunoassays. In one example

of this, competitive protein binding assays (CPBA) and HPLC were compared for sensitivity in detection of vitamin D.

12.3.3 High-Performance Thin-Layer Chromatography

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High-Performance Thin-Layer Chromatography (HPTLC) is an enhanced form of Thin-Layer Chromatography (TLC). A number of enhancements can be made to the basic method of thin-layer chromatography to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements.

Automation is useful to overcome the uncertainty in droplet size and position when the sample is applied to the TLC plate by hand. One recent approach to automation has been the use of piezoelectric devices and inkjet printers for applying the sample.

The spot capacity (analogous to peak capacity in HPLC) can be increased by developing the plate with two different solvents, using two-dimensional chromatography. The procedure begins with development of sample loaded plate with first solvent. After removing it, the plate is rotated 90° and developed with a second solvent.

12.3.4 Fast protein liquid chromatography

Fast Protein Liquid Chromatography (FPLC), is a form of liquid chromatography that is often used to analyze or purify mixtures of proteins. As in other forms of chromatography, separation is possible because the different components of a mixture have different affinities for two materials, a moving fluid (the 'mobile phase') and a porous solid (the stationary phase). In FPLC the mobile phase is an aqueous solution, or 'buffer'. The buffer flow rate is controlled by a positive-displacement pump and is normally kept constant, while the composition of the buffer can be varied by drawing fluids in different proportions from two or more external reservoirs. The stationary phase is a resin composed of beads, usually of cross-linked agarose, packed into a cylindrical glass or plastic column. FPLC resins are available in a wide range of bead sizes and surface ligands depending on the application.

In the most common FPLC strategy, ion exchange, a resin is chosen that the protein of interest will bind to the resin by a charge interaction while in buffer A (the running buffer) but become dissociated and return to solution in buffer B (the elution buffer). A mixture containing one or more proteins of interest is dissolved in 100% buffer A and pumped into the column. The proteins of interest bind to the resin while other components are carried out in the buffer. The total flow rate of the buffer is kept constant; however, the proportion of Buffer B (the 'elution' buffer) is gradually increased from 0% to 100% according to a programmed change in concentration (the 'gradient'). At some point during this process each of the bound proteins dissociates and appears in the eluant. The eluant passes through

two detectors which measure salt concentration (by conductivity) and protein concentration (by absorption of ultraviolet light at a wavelength of 280nm). As each protein is eluted it appears in the eluant as a 'peak' in protein concentration and can be collected for further use.

FPLC was developed and marketed in Sweden by Pharmacia in 1982 and was originally called fast performance liquid chromatography to contrast it with HPLC or high-performance liquid chromatography. FPLC is generally applied only to proteins; however, because of the wide choice of resins and buffers it has broad applications. In contrast to HPLC the buffer pressure used is relatively low, typically less than 5 bar, but the flow rate is relatively high, typically 1-5 ml/min. FPLC can be readily scaled from analysis of milligrams of mixtures in columns with a total volume of 5ml or less to industrial production of kilograms of purified protein in columns with volumes of many liters. When used for analysis of mixtures the eluant is usually collected in fractions of 1-5 ml which can be further analyzed.

FPLC System Components

A typical laboratory FPLC consist of one or two high-precision pumps, a control unit, a column, a detection system and a fraction collector. Although it is possible to operate the system manually, the components are normally linked to a personal computer or, in older units, a microcontroller.

1. **Pumps:** Majority of systems utilize two two-cylinder piston pumps, one for each buffer, combining the output of both in a mixing chamber. Some simpler systems use a single peristaltic pump which draws both buffers from separate reservoirs through a proportioning valve and mixing chamber. In either case the system allows the fraction of each buffer entering the column to be continuously varied. The flow rate can go from a few milliliters per minute in bench-top systems to liters per minute for industrial scale purifications. The wide flow range makes it suitable both for analytical and preparative chromatography.
2. **Injection Loop:** A segment of tubing of known volume which is filled with the sample solution before it is injected into the column. Loop volume can range from a few microliters to 50ml or more.
3. **Injection Valve:** A motorized valve which links the mixer and sample loop to the column. Typically the valve has three positions for loading the sample loop, for injecting the sample from the loop into the column, and for connecting the pumps directly to the waste line to wash them or change buffer solutions.
4. **Column:** The column is a glass or plastic cylinder packed with beads of resin and filled with buffer solution. It is normally mounted vertically with the buffer flowing downward from top to bottom. A glass frit at the bottom of the column retains the resin beads in the column while allowing the buffer and dissolved proteins to exit.

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5. **Flow Cells:** The eluant from the column passes through one or more flow cells to measure the concentration of protein in the eluant (by UV light absorption at 280nm). The conductivity cell measures the buffer conductivity, usually in millisiemens/cm, which indicates the concentration of salt in the buffer. A flow cell which measures pH of the buffer is also commonly included.
6. **Monitor/Recorder:** The flow cells are connected to a display and/or recorder. On older systems this was a simple chart recorder, on modern systems a computer with hardware interface and display is used. This permits the experimenter to identify when peaks in protein concentration occur, indicating that specific components of the mixture are being eluted.
7. **Fraction Collector:** The fraction collector is typically a rotating rack that can be filled with test tubes or similar containers. It allows samples to be collected in fixed volumes, or can be controlled to direct specific fractions detected as peaks of protein concentration, into separate containers.

FPLC Columns

The columns used in FPLC are large [mm id] tubes that contain small [μ] particles or gel beads that are known as stationary phase. The chromatographic bed is composed by the gel beads inside the column and the sample is introduced into the injector and carried into the column by the flowing solvent. As a result of different components adhering to or diffusing through the gel, the sample mixture gets separated.

Columns used with an FPLC can separate macromolecules based on size, charge distribution (ion exchange), hydrophobicity, reverse-phase or biorecognition (as with affinity chromatography). For easy use, a wide range of pre-packed columns for techniques such as ion exchange, gel filtration (size exclusion), hydrophobic interaction, and affinity chromatography are available. FPLC differs from HPLC in that the columns used for FPLC can only be used up to maximum pressure of 3-4 MPa (435-580 psi). Thus, if the pressure of HPLC can be limited, each FPLC column may also be used in an HPLC machine.

Optimizing Protein Purification

Combinations of chromatographic methods can be used to purify a target molecule. The purpose of purifying proteins with FPLC is to deliver quantities of the target at sufficient purity in a biologically active state to suit its further use. The quality of the end product varies depending the type and amount of starting material, efficiency of separation, and selectivity of the purification resin. The ultimate goal of a given purification protocol is to deliver the required yield and purity of the target molecule in the quickest, cheapest, and safest way for acceptable results. The range of purity required can be from that required for basic analysis (SDS-PAGE or ELISA, for example), with only bulk impurities removed, to pure enough for structural analysis (NMR or X-ray crystallography), approaching >99% target molecule.

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Purity required can also mean pure enough that the biological activity of the target is retained. These demands can be used to determine the amount of starting material required to reach the experimental goal. If the starting material is limited and full optimization of purification protocol cannot be performed, then a safe standard protocol that requires a minimum adjustment and optimization steps are expected. This may not be optimal with respect to experimental time, yield, and economy but it will achieve the experimental goal. On the other hand, if the starting material is enough to develop more complete protocol, the amount of work to reach the separation goal depends on the available sample information and target molecule properties. Limits to development of purification protocols many times depends on the source of the substance to be purified, whether from natural sources (harvested tissues or organisms, for example), recombinant sources (such as using prokaryotic or eukaryotic vectors in their respective expression systems), or totally synthetic sources.

No chromatographic techniques provide 100% yield of active material and overall yields depend on the number of steps in the purification protocol. By optimizing each step for the intended purpose and arranging them that minimizes inter step treatments, the number of steps will be minimized.

12.3.4 Gas Chromatography

Gas Chromatography (GC) is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture (the relative amounts of such components can also be determined). In some situations, GC may help in identifying a compound. In preparative chromatography, GC can be used to prepare pure compounds from a mixture.

In gas chromatography, the mobile phase (or 'moving phase') is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. Helium remains the most commonly used carrier gas in about 90% of instruments although hydrogen is preferred for improved separations. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column (an homage to the fractionating column used in distillation). The instrument used to perform gas chromatography is called a gas chromatograph (or aerograph, gas separator).

The gaseous compounds being analyzed interact with the walls of the column, which is coated with a stationary phase. This causes each compound to elute at a different time, known as the retention time of the compound. The comparison of retention times is what gives GC its analytical usefulness.

Gas chromatography is in principle similar to column chromatography (as well as other forms of chromatography, such as HPLC, TLC), but has several notable differences. First, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase, whereas in

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column chromatography the stationary phase is a solid and the mobile phase is a liquid. (Hence the full name of the procedure is Gas–liquid chromatography, referring to the mobile and stationary phases, respectively.) Second, the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography (typically) has no such temperature control. Finally, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas.

Gas chromatography is also similar to fractional distillation, since both processes separate the components of a mixture primarily based on boiling point (or vapor pressure) differences. However, fractional distillation is typically used to separate components of a mixture on a large scale, whereas GC can be used on a much smaller scale (i.e., microscale).

Gas chromatography is also sometimes known as Vapor-Phase Chromatography (VPC), or Gas–Liquid Partition Chromatography (GLPC). These alternative names, as well as their respective abbreviations, are frequently used in scientific literature.

History

Chromatography dates to 1903 in the work of the Russian scientist, Mikhail Semyonovich Tswett. German physical chemist Erika Cremer developed solid state gas chromatography in 1947 together with Austrian graduate student Fritz Prior. Archer John Porter Martin, who was awarded the Nobel Prize for his work in developing liquid–liquid (1941) and paper (1944) chromatography, laid the foundation for the development of gas chromatography and he later produced liquid-gas chromatography (1950).

A gas chromatograph is a chemical analysis instrument for separating chemicals in a complex sample. A gas chromatograph uses a flow-through narrow tube known as the column, through which different chemical constituents of a sample pass in a gas stream (carrier gas, mobile phase) at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the stationary phase. As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (retention time). Other parameters that can be used to alter the order or time of retention are the carrier gas flow rate, column length and the temperature.

GC Analysis

In a GC analysis, a known volume of gaseous or liquid analyte is injected into the entrance (head) of the column, usually using a microsyringe (or, solid phase microextraction fibers, or a gas source switching system). As the carrier gas sweeps the analyte molecules through the column, this motion is inhibited by the adsorption of the analyte molecules either onto the column walls or onto packing materials in

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the column. The rate at which the molecules progress along the column depends on the strength of adsorption, which in turn depends on the type of molecule and on the stationary phase materials. Since each type of molecule has a different rate of progression, the various components of the analyte mixture are separated as they progress along the column and reach the end of the column at different times (retention time). A detector is used to monitor the outlet stream from the column; thus, the time at which each component reaches the outlet and the amount of that component can be determined. Generally, substances are identified (qualitatively) by the order in which they emerge (elute) from the column and by the retention time of the analyte in the column.

Physical Components

Autosamplers

The autosampler provides the means to introduce a sample automatically into the inlets. Manual insertion of the sample is possible but is no longer common. Automatic insertion provides better reproducibility and time-optimization.

Different kinds of autosamplers exist. Autosamplers can be classified in relation to sample capacity (auto-injectors vs. autosamplers, where auto-injectors can work a small number of samples), to robotic technologies (XYZ robot vs. rotating robot – the most common), or to analysis:

- o Liquid
- o Static head-space by syringe technology
- o Dynamic head-space by transfer-line technology
- o Solid Phase MicroExtraction (SPME)

Inlets

The column inlet (or injector) provides the means to introduce a sample into a continuous flow of carrier gas. The inlet is a piece of hardware attached to the column head.

Common inlet types are:

1. S/SL (Split/Splitless) injector; a sample is introduced into a heated small chamber via a syringe through a septum – the heat facilitates volatilization of the sample and sample matrix. The carrier gas then either sweeps the entirety (splitless mode) or a portion (split mode) of the sample into the column. In split mode, a part of the sample/carrier gas mixture in the injection chamber is exhausted through the split vent. Split injection is preferred when working with samples with high analyte concentrations (>0.1%) whereas splitless injection is best suited for trace analysis with low amounts of analytes (<0.01%).
2. On-column inlet; the sample is here introduced directly into the column in its entirety without heat, or at a temperature below the boiling point of

the solvent. The low temperature condenses the sample into a narrow zone. The column and inlet can then be heated, releasing the sample into the gas phase.

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3. PTV injector; Temperature-programmed sample introduction was first described by Vogt in 1979. Originally Vogt developed the technique as a method for the introduction of large sample volumes (up to 250 µL) in capillary GC. Vogt introduced the sample into the liner at a controlled injection rate. The temperature of the liner was chosen slightly below the boiling point of the solvent. The low-boiling solvent was continuously evaporated and vented through the split line.
4. Gas source inlet or gas switching valve; gaseous samples in collection bottles are connected to what is most commonly a six-port switching valve. The carrier gas flow is not interrupted while a sample can be expanded into a previously evacuated sample loop.
5. P/T (Purge-and-Trap) system; An inert gas is bubbled through an aqueous sample causing insoluble volatile chemicals to be purged from the matrix. The volatiles are 'trapped' on an absorbent column (known as a trap or concentrator) at ambient temperature. The trap is then heated and the volatiles are directed into the carrier gas stream. Samples requiring preconcentration or purification can be introduced via such a system, usually hooked up to the S/SL port.

Detectors

The most commonly used detectors are the Flame Ionization Detector (FID) and the Thermal Conductivity Detector (TCD). Both are sensitive to a wide range of components, and both work over a wide range of concentrations. While TCDs are essentially universal and can be used to detect any component other than the carrier gas (as long as their thermal conductivities are different from that of the carrier gas, at detector temperature), FIDs are sensitive primarily to hydrocarbons, and are more sensitive to them than TCD. However, a FID cannot detect water. Both detectors are also quite robust. Since TCD is non-destructive, it can be operated in-series before a FID (destructive), thus providing complementary detection of the same analytes.

Other detectors are sensitive only to specific types of substances, or work well only in narrower ranges of concentrations. They include:

- Thermal Conductivity detector (TCD), this common detector relies on the thermal conductivity of matter passing around a tungsten -rhenium filament with a current traveling through it. In this set up helium or nitrogen serve as the carrier gas because of their relatively high thermal conductivity which keep the filament cool and maintain uniform resistivity and electrical efficiency of the filament.

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- Flame Ionization Detector (FID), in this common detector electrodes are placed adjacent to a flame fueled by hydrogen / air near the exit of the column, and when carbon containing compounds exit the column they are pyrolyzed by the flame.
- Catalytic Combustion Detector (CCD), which measures combustible hydrocarbons and hydrogen.
- Discharge Ionization Detector (DID), which uses a high-voltage electric discharge to produce ions.
- Dry Electrolytic Conductivity Detector (DELCD), which uses an air phase and high temperature (v. Coulsen) to measure chlorinated compounds.
- Electron Capture Detector (ECD), which uses a radioactive beta particle (electron) source to measure the degree of electron capture. ECD are used for the detection of molecules containing electronegative / withdrawing elements and functional groups like halogens, carbonyl, nitriles, nitro groups, and organometalics.
- Flame Photometric Detector (FPD), which uses a photomultiplier tube to detect spectral lines of the compounds as they are burned in a flame. Compounds eluting off the column are carried into a hydrogen fueled flame which excites specific elements in the molecules, and the excited elements (P,S, Halogens, Some Metals) emit light of specific characteristic wavelengths.
- Atomic Emission Detector (AED), a sample eluting from a column enters a chamber which is energized by microwaves that induce a plasma. The plasma causes the analyte sample to decompose and certain elements generate an atomic emission spectra.
- Hall Electrolytic Conductivity Detector (ElCD)
- Helium Ionization Detector (HID)
- Nitrogen–Phosphorus Detector (NPD), a form of thermionic detector where nitrogen and phosphorus alter the work function on a specially coated bead and a resulting current is measured.
- Alkali Flame Detector, AFD or Alkali Flame Ionization Detector, AFID. AFD has high sensitivity to nitrogen and phosphorus, similar to NPD. However, the alkaline metal ions are supplied with the hydrogen gas, rather than a bead above the flame.
- InfraRed Detector (IRD)
- Mass Spectrometer (MS), also called GC-MS; highly effective and sensitive, even in a small quantity of sample.
- Photo-Ionization Detector (PID)

- The Polyarc reactor is an add-on to new or existing GC-FID instruments that converts all organic compounds to methane molecules prior to their detection by the FID. This technique can be used to improve the response of the FID and allow for the detection of many more carbon-containing compounds.
- Pulsed Discharge Ionization Detector (PDD)
- Thermionic Ionization Detector (TID)
- Vacuum Ultraviolet (VUV) represents the most recent development in Gas Chromatography detectors. Most chemical species absorb and have unique gas phase absorption cross sections in the approximately 120–240 nm VUV wavelength range monitored.

Method

The method is the collection of conditions in which the GC operates for a given analysis. Method development is the process of determining what conditions are adequate and/or ideal for the analysis required.

Conditions which can be varied to accommodate a required analysis include inlet temperature, detector temperature, column temperature and temperature program, carrier gas and carrier gas flow rates, the column's stationary phase, diameter and length, inlet type and flow rates, sample size and injection technique. Depending on the detector(s) installed on the GC, there may be a number of detector conditions that can also be varied. Some GCs also include valves which can change the route of sample and carrier flow. The timing of the opening and closing of these valves can be important to method development.

Carrier Gas Selection and Flow Rates

Typical carrier gases include helium, nitrogen, argon, hydrogen and air. Which gas to use is usually determined by the detector being used, for example, a DID requires helium as the carrier gas. When analyzing gas samples, however, the carrier is sometimes selected based on the sample's matrix, for example, when analyzing a mixture in argon, an argon carrier is preferred, because the argon in the sample does not show up on the chromatogram. Safety and availability can also influence carrier selection, for example, hydrogen is flammable, and high-purity helium can be difficult to obtain in some areas of the world. As a result of helium becoming more scarce, hydrogen is often being substituted for helium as a carrier gas in several applications.

The purity of the carrier gas is also frequently determined by the detector, though the level of sensitivity needed can also play a significant role. Typically, purities of 99.995% or higher are used. The most common purity grades required by modern instruments for the majority of sensitivities are 5.0 grades, or 99.999% pure meaning that there is a total of 100ppm of impurities in the carrier gas that could affect the results. The highest purity grades in common use are 6.0 grades,

but the need for detection at very low levels in some forensic and environmental applications has driven the need for carrier gases at 7.0 grade purity and these are now commercially available. Trade names for typical purities include 'Zero Grade', 'Ultra-High Purity (UHP) Grade', '4.5 Grade' and '5.0 Grade'.

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Stationary Compound Selection

The polarity of the solute is crucial for the choice of stationary compound, which in an optimal case would have a similar polarity as the solute. Common stationary phases in open tubular columns are cyanopropyl phenyl dimethyl polysiloxane, carbowax polyethylene glycol, bis-cyanopropyl cyanopropyl phenyl polysiloxane and diphenyl dimethyl polysiloxane. For packed columns more options are available.

Inlet Types and Flow Rates

The choice of inlet type and injection technique depends on if the sample is in liquid, gas, adsorbed, or solid form, and on whether a solvent matrix is present that has to be vaporized. Dissolved samples can be introduced directly onto the column via a COC injector, if the conditions are well known; if a solvent matrix has to be vaporized and partially removed, a S/SL injector is used (most common injection technique); gaseous samples (for example, air cylinders) are usually injected using a gas switching valve system; adsorbed samples (for example, on adsorbent tubes) are introduced using either an external (on-line or off-line) desorption apparatus such as a purge-and-trap system, or are desorbed in the injector (SPME applications).

Sample Size and Injection Technique

Sample Injection

The real chromatographic analysis starts with the introduction of the sample onto the column. The development of capillary gas chromatography resulted in many practical problems with the injection technique. The technique of on-column injection, often used with packed columns, is usually not possible with capillary columns. The injection system in the capillary gas chromatograph should fulfil the following two requirements:

1. The amount injected should not overload the column.
2. The width of the injected plug should be small compared to the spreading due to the chromatographic process. Failure to comply with this requirement will reduce the separation capability of the column. As a general rule, the volume injected, Vinj, and the volume of the detector cell, Vdet, should be about 1/10 of the volume occupied by the portion of sample containing the molecules of interest (analytes) when they exit the column.

   Some general requirements which a good injection technique should fulfill are:

- It should be possible to obtain the column's optimum separation efficiency.
- It should allow accurate and reproducible injections of small amounts of representative samples.
- It should induce no change in sample composition. It should not exhibit discrimination based on differences in boiling point, polarity, concentration or thermal/catalytic stability.
- It should be applicable for trace analysis as well as for undiluted samples.

However, there are a number of problems inherent in the use of syringes for injection:

- Even the best syringes claim an accuracy of only 3%, and in unskilled hands, errors are much larger
- The needle may cut small pieces of rubber from the septum as it injects sample through it. These can block the needle and prevent the syringe filling the next time it is used. It may not be obvious that this has happened.
- A fraction of the sample may get trapped in the rubber, to be released during subsequent injections. This can give rise to ghost peaks in the chromatogram.
- There may be selective loss of the more volatile components of the sample by evaporation from the tip of the needle.

Column Selection

The choice of column depends on the sample and the active measured. The main chemical attribute regarded when choosing a column is the polarity of the mixture, but functional groups can play a large part in column selection. The polarity of the sample must closely match the polarity of the column stationary phase to increase resolution and separation while reducing run time. The separation and run time also depends on the film thickness (of the stationary phase), the column diameter and the column length.

Column Temperature and Temperature Program

The column(s) in a GC are contained in an oven, the temperature of which is precisely controlled electronically.The rate at which a sample passes through the column is directly proportional to the temperature of the column. The higher the column temperature, the faster the sample moves through the column. However, the faster a sample moves through the column, the less it interacts with the stationary phase, and the less the analytes are separated.

In general, the column temperature is selected to compromise between the length of the analysis and the level of separation.

A method which holds the column at the same temperature for the entire analysis is called 'isothermal' . Most methods, however, increase the column

temperature during the analysis, the initial temperature, rate of temperature increase (the temperature 'ramp'), and final temperature are called the 'temperature program'.

A temperature program allows analytes that elute early in the analysis to separate adequately, while shortening the time it takes for late-eluting analytes to pass through the column.

Data Reduction and Analysis

Qualitative Analysis

Generally chromatographic data is presented as a graph of detector response (y-axis) against retention time (x-axis), which is called a chromatogram. This provides a spectrum of peaks for a sample representing the analytes present in a sample eluting from the column at different times. Retention time can be used to identify analytes if the method conditions are constant. Also, the pattern of peaks will be constant for a sample under constant conditions and can identify complex mixtures of analytes.

Quantitative analysis

The area under a peak is proportional to the amount of analyte present in the chromatogram. By calculating the area of the peak using the mathematical function of integration, the concentration of an analyte in the original sample can be determined. Concentration can be calculated using a calibration curve created by finding the response for a series of concentrations of analyte, or by determining the relative response factor of an analyte.

In most modern GC-MS systems, computer software is used to draw and integrate peaks, and match MS spectra to library spectra.

Applications

In general, substances that vaporize below 300 °C (and therefore are stable up to that temperature) can be measured quantitatively. The samples are also required to be salt-free; they should not contain ions. Very minute amounts of a substance can be measured, but it is often required that the sample must be measured in comparison to a sample containing the pure, suspected substance known as a reference standard.

Various temperature programs can be used to make the readings more meaningful; for example to differentiate between substances that behave similarly during the GC process.

Gas Chromatography is used extensively in forensic science. Disciplines as diverse as solid drug dose (pre-consumption form) identification and quantification, arson investigation, paint chip analysis, and toxicology cases, employ GC to identify and quantify various biological specimens and crime-scene evidence.

12.3.5 Matrix-Assisted Laser Desorption/Ionization

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In mass spectrometry, Matrix-Assisted Laser Desorption/Ionization (MALDI) is an ionization technique that uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation. It has been applied to the analysis of biomolecules (biopolymers such as DNA, proteins, peptides and sugars) and large organic molecules (such as, polymers, dendrimers and other macromolecules), which tend to be fragile and fragment when ionized by more conventional ionization methods. It is similar in character to electrospray ionization (ESI) in that both techniques are relatively soft (low fragmentation) ways of obtaining ions of large molecules in the gas phase, though MALDI typically produces far fewer multi-charged ions.

MALDI methodology is a three-step process. First, the sample is mixed with a suitable matrix material and applied to a metal plate. Second, a pulsed laser irradiates the sample, triggering ablation and desorption of the sample and matrix material. Finally, the analyte molecules are ionized by being protonated or deprotonated in the hot plume of ablated gases, and then they can be accelerated into whichever mass spectrometer is used to analyse them.

History

The term Matrix-Assisted Laser Desorption Ionization (MALDI) was coined in 1985 by Franz Hillenkamp, Michael Karas and their colleagues. These researchers found that the amino acid alanine could be ionized more easily if it was mixed with the amino acid tryptophan and irradiated with a pulsed 266 nm laser. The tryptophan was absorbing the laser energy and helping to ionize the non-absorbing alanine. Peptides up to the 2843 Da peptide melittin could be ionized when mixed with this kind of 'matrix'. The breakthrough for large molecule laser desorption ionization came in 1987 when Koichi Tanaka of Shimadzu Corporation and his co-workers used what they called the 'ultra fine metal plus liquid matrix method' that combined 30 nm cobalt particles in glycerol with a 337 nm nitrogen laser for ionization. Using this laser and matrix combination, Tanaka was able to ionize biomolecules as large as the 34,472 Da protein carboxypeptidase-A. Tanaka received one-quarter of the 2002 Nobel Prize in Chemistry for demonstrating that, with the proper combination of laser wavelength and matrix, a protein can be ionized. Karas and Hillenkamp were subsequently able to ionize the 67 kDa protein albumin using a nicotinic acid matrix and a 266 nm laser. Further improvements were realized through the use of a 355 nm laser and the cinnamic acid derivatives ferulic acid, caffeic acid and sinapinic acid as the matrix. The availability of small and relatively inexpensive nitrogen lasers operating at 337 nm wavelength and the first commercial instruments introduced in the early 1990s brought MALDI to an increasing number of researchers. Today, mostly organic matrices are used for MALDI mass spectrometry.

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Matrix

The matrix consists of crystallized molecules, of which the three most commonly used are 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxycinnamic acid (α-CHCA, alpha-cyano or alpha-matrix) and 2,5-Dihydroxybenzoic Acid (DHB). A solution of one of these molecules is made, often in a mixture of highly purified water and an organic solvent, such as Acetonitrile (ACN) or Ethanol. A counter ion source such as Trifluoroacetic acid (TFA) is usually added to generate the [M+H] ions. A good example of a matrix-solution would be 20 mg/mL sinapinic acid in ACN:water:TFA

The identification of suitable matrix compounds is determined to some extent by trial and error, but they are based on some specific molecular design considerations. They are of a fairly low molecular weight (to allow easy vaporization), but are large enough (with a low enough vapor pressure) not to evaporate during sample preparation or while standing in the mass spectrometer. They are often acidic, therefore act as a proton source to encourage ionization of the analyte. Basic matrices have also been reported. They have a strong optical absorption in either the UV or IR range, so that they rapidly and efficiently absorb the laser irradiation. This efficiency is commonly associated with chemical structures incorporating several conjugated double bonds, as seen in the structure of cinnamic acid. They are functionalized with polar groups, allowing their use in aqueous solutions. They typically contain a chromophore.

The matrix solution is mixed with the analyte (for example, protein-sample). A mixture of water and organic solvent allows both hydrophobic and water-soluble (hydrophilic) molecules to dissolve into the solution. This solution is spotted onto a MALDI plate (usually a metal plate designed for this purpose). The solvents vaporize, leaving only the recrystallized matrix, but now with analyte molecules embedded into MALDI crystals. The matrix and the analyte are said to be co-crystallized. Co-crystallization is a key issue in selecting a proper matrix to obtain a good quality mass spectrum of the analyte of interest.

In analysis of biological systems, Inorganic salts, which are also part of protein extracts, interfere with the ionization process. The salts can be removed by solid phase extraction or by washing the dried-droplet MALDI spots with cold water. Both methods can also remove other substances from the sample.

Instrumentation

There are several variations of the MALDI technology and comparable instruments are today produced for very different purposes. From more academic and analytical, to more industrial and high throughput. The MS field has expanded into requiring ultrahigh resolution mass spectrometry such as the FT-ICR instruments as well as more high-throughput instruments. As many MALDI MS instruments can be bought with an interchangeable ionization source (Electrospray ionization, MALDI, Atmospheric pressure ionization, etc.) the technologies often overlap

and many times any soft ionization method could potentially be used. For more variations of soft ionization methods go to Soft laser desorption or Ion source.

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Laser

MALDI techniques typically employ the use of UV lasers such as nitrogen lasers (337 nm) and frequency-tripled and quadrupled Nd:YAG lasers (355 nm and 266 nm respectively).

Infrared laser wavelengths used for infrared MALDI include the 2.94 µm Er:YAG laser, mid-IR optical parametric oscillator, and 10.6 µm carbon dioxide laser. Although not as common, infrared lasers are used due to their softer mode of ionization. IR-MALDI also has the advantage of greater material removal (useful for biological samples), less low-mass interferences, and compatibility with other matrix-free laser desorption mass spectrometry methods.

Time of Flight

The type of a mass spectrometer most widely used with MALDI is the TOF (Time-Of-Flight mass spectrometer), mainly due to its large mass range. The TOF measurement procedure is also ideally suited to the MALDI ionization process since the pulsed laser takes individual 'shots' rather than working in continuous operation. MALDI-TOF instrument or reflectron is equipped with an "ion mirror" that reflects ions using an electric field, thereby doubling the ion flight path and increasing the resolution. Today, commercial reflectron TOF instruments reach a resolving power m/Äm of well above 20,000 FWHM (full-width half-maximum, Äm defined as the peak width at 50% of peak height).

MALDI has been coupled with IMS-TOF MS to identify phosphorylated and non-phosphorylated peptides.

MALDI-FT-ICR MS has been demonstrated to be a useful technique where high resolution MALDI-MS measurements are desired.

Atmospheric Pressure

Atmospheric Pressure (AP) matrix-assisted laser desorption/ionization (MALDI) is an ionization technique (ion source) that in contrast to vacuum MALDI operates at normal atmospheric environment.

The main difference between vacuum MALDI and AP-MALDI is the pressure in which the ions are created. In vacuum MALDI, ions are typically produced at 10 mTorr or less while in AP-MALDI ions are formed in atmospheric pressure. In the past the main disadvantage of AP MALDI technique compared to the conventional vacuum MALDI has been its limited sensitivity; however, ions can be transferred into the mass spectrometer with high efficiency and attomole detection limits have been reported.

AP-MALDI is used in mass spectrometry (MS) in a variety of applications ranging from proteomics to drug discovery. Popular topics that are addressed by

AP-MALDI mass spectrometry include: proteomics; mass analysis of DNA, RNA, PNA, lipids, oligosaccharides, phosphopeptides, bacteria, small molecules and synthetic polymers, similar applications as available also for vacuum MALDI instruments. The AP-MALDI ion source is easily coupled to an ion trap mass spectrometer or any other MS system equipped with ESI (electrospray ionization) or nano-ESI source.

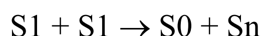
Ionization

The laser is fired at the matrix crystals in the dried-droplet spot. The matrix absorbs the laser energy and it is thought that primarily the matrix is desorbed and ionized (by addition of a proton) by this event. The hot plume produced during ablation contains many species: neutral and ionized matrix molecules, protonated and deprotonated matrix molecules, matrix clusters and nanodroplets. Ablated species may participate in the ionization of analyte, though the mechanism of MALDI is still debated. The matrix is then thought to transfer protons to the analyte molecules (for example, protein molecules), thus charging the analyte.

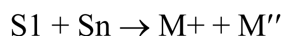
An ion observed after this process will consist of the initial neutral molecule [M] with ions added or removed. This is called a quasimolecular ion, for example [M+H]+ in the case of an added proton, [M+Na]+ in the case of an added sodium ion, or [M-H]" in the case of a removed proton. MALDI is capable of creating singly charged ions or multiply charged ions ([M+nH]n+) depending on the nature of the matrix, the laser intensity, and/or the voltage used. Note that these are all even-electron species. Ion signals of radical cations (photoionized molecules) can be observed, for example, in the case of matrix molecules and other organic molecules.

The gas phase proton transfer model, implemented as the Coupled Physical and Chemical Dynamics (CPCD) model, of UV laser MALDI postulates primary and secondary processes leading to ionization. Primary processes involve initial charge separation through absorption of photons by the matrix and pooling of the energy to form matrix ion pairs. Primary ion formation occurs through absorption of a UV photon to create excited state molecules by

$$S0 + hí \rightarrow S1$$



$$S1 + S1 \rightarrow S0 + Sn$$



$$S1 + Sn \rightarrow M+ + M''$$

where S0 is the ground electronic state, S1 the first electronic excited state, and Sn is a higher electronic excited state. The product ions can be proton transfer or electron transfer ion pairs, indicated by M+ and M″ above. Secondary processes involve ion-molecule reactions to form analyze ions.

In the lucky survivor model, positive ions can be formed from highly charged clusters produced during break-up of the matrix- and analyte-containing solid.

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The lucky survivor model (cluster ionization mechanism) postulates that analyte molecules are incorporated in the matrix maintaining the charge state from solution. Ion formation occurs through charge separation upon fragmentation of laser ablated clusters. Ions that are not neutralized by recombination with photoelectrons or counter ions are the so-called lucky survivors.

Applications

Biochemistry

In proteomics, MALDI is used for the rapid identification of proteins isolated by using gel electrophoresis: SDS-PAGE, size exclusion chromatography, affinity chromatography, strong/weak ion exchange, Isotope Coded Protein Labelling (ICPL), and two-dimensional gel electrophoresis. Peptide mass fingerprinting is the most popular analytical application of MALDI-TOF mass spectrometers. MALDI TOF/TOF mass spectrometers are used to reveal amino acid sequence of peptides using post-source decay or high energy collision-induced dissociation.

Loss of sialic acid has been identified in papers when DHB has been used as a matrix for MALDI MS analysis of glycosylated peptides. Using sinapinic acid, 4-HCCA and DHB as matrices, S. Martin studied loss of sialic acid in glycosylated peptides by metastable decay in MALDI/TOF in linear mode and reflector mode. A group at Shimadzu Corporation derivatized the sialic acid by an amidation reaction as a way to improve detection sensitivity and also demonstrated that ionic liquid matrix reduces a loss of sialic acid during MALDI/TOF MS analysis of sialylated oligosaccharides. THAP, DHAP and a mixture of 2-aza-2-thiothymine and phenylhydrazine have been identified as matrices that could be used to minimize loss of sialic acid during MALDI MS analysis of glycosylated peptides.

It has been reported that a reduction in loss of some post-translational modifications can be accomplished if IR MALDI is used instead of UV MALDI.

In molecular biology, a mixture of 5-methoxysalicylic acid and spermine can be used as a matrix for oligonucleotides analysis in MALDI mass spectrometry, for instance after oligonucleotide synthesis.

Organic Chemistry

Some synthetic macromolecules, such as catenanes and rotaxanes, dendrimers and hyperbranched polymers, and other assemblies, have molecular weights extending into the thousands or tens of thousands, where most ionization techniques have difficulty producing molecular ions. MALDI is a simple and fast analytical method that can allow chemists to rapidly analyze the results of such syntheses and verify their results.

Polymers

In polymer chemistry MALDI can be used to determine the molar mass distribution. Polymers with polydispersity greater than 1.2 are difficult to characterize with

MALDI due to the signal intensity discrimination against higher mass oligomers.

A good matrix for polymers is dithranol or AgTFA. The sample must first be mixed with dithranol and the AgTFA added afterwards; otherwise the sample would precipitate out of solution.

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Microbiology

MALDI/TOF spectra are used for the identification of micro-organisms such as bacteria or fungi. A portion of a colony of the microbe in question is placed onto the sample target and overlaid with matrix. The mass spectra generated are analyzed by dedicated software and compared with stored profiles. Species diagnosis by this procedure is much faster, more accurate and cheaper than other procedures based on immunological or biochemical tests. MALDI/TOF is becoming a standard method for species identification in medical microbiological laboratories.

One main advantage over other microbiological identification methods is its ability to rapidly and reliably identify, at low cost, a wide variety of microorganisms directly from the selective medium used to isolate them. The absence of the need to purify the suspect or 'presumptive' colony allows for a much faster turn-around times.

Another advantage is the potential to predict antibiotic susceptibility of bacteria. A single mass spectral peak can predict methicillin resistance of Staphylococcus aureus MALDI can also detect carbapenemase of carbapenem-resistant enterobacteriaceae, including Acinetobacter baumannii and Klebsiella pneumoniae.

Medicine

MALDI/TOF spectra are often utilized in tandem with other analysis and spectroscopy techniques in the diagnosis of diseases. MALDI/TOF is a diagnostic tool with much potential because it allows for the rapid identification of proteins and changes to proteins without the cost or computing power of sequencing nor the skill or time needed to solve a crystal structure in X-ray crystallography.

One example of this is Necrotizing EnteroColitis (NEC), which is a devastating disease that affects the bowels of premature infants. The symptoms of NEC are very similar to those of sepsis, and many infants die awaiting diagnosis and treatment. MALDI/TOF was used to quickly analyze fecal samples and find differences between the mutant and the functional protein responsible for NEC.

Another example of the diagnostic power of MALDI/TOF is in the area of cancer. Pancreatic cancer remains one of the most deadly and difficult to diagnose cancers. Impaired cellular signaling due to mutations in membrane proteins has been long suspected to contribute to pancreatic cancer. MALDI/TOF has been used to identify a membrane protein associated with pancreatic cancer and at one point may even serve as an early detection technique.

MALDI/TOF can also potentially be used to dictate treatment as well as diagnosis. MALDI/TOF serves as a method for determining the drug resistance of bacteria, especially to â-lactams (Penicillin family). The MALDI/TOF detects the presence of carbapenemases, which indicates drug resistance to standard antibiotics.

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12.3.6 Mass Spectroscopy

Mass Spectrometry (MS) is an analytical technique that ionizes chemical species and sorts the ions based on their mass-to-charge ratio. In simpler terms, a mass spectrum measures the masses within a sample. Mass spectrometry is used in many different fields and is applied to pure samples as well as complex mixtures.

A mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio. These spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules and other chemical compounds.

History

In 1886, Eugen Goldstein observed rays in gas discharges under low pressure that traveled away from the anode and through channels in a perforated cathode, opposite to the direction of negatively charged cathode rays (which travel from cathode to anode). Goldstein called these positively charged anode rays 'Kanalstrahlen'; the standard translation of this term into English is 'canal rays'. Wilhelm Wien found that strong electric or magnetic fields deflected the canal rays and, in 1899, constructed a device with perpendicular electric and magnetic fields that separated the positive rays according to their charge-to-mass ratio (Q/m). Wien found that the charge-to-mass ratio depended on the nature of the gas in the discharge tube. English scientist J.J. Thomson later improved on the work of Wien by reducing the pressure to create the mass spectrograph.

The word spectrograph had become part of the international scientific vocabulary by 1884. Early spectrometry devices that measured the mass-to-charge ratio of ions were called mass spectrographs which consisted of instruments that recorded a spectrum of mass values on a photographic plate. A mass spectroscope is similar to a mass spectrograph except that the beam of ions is directed onto a phosphor screen. A mass spectroscope configuration was used in early instruments when it was desired that the effects of adjustments be quickly observed. Once the instrument was properly adjusted, a photographic plate was inserted and exposed. The term mass spectroscope continued to be used even though the direct illumination of a phosphor screen was replaced by indirect measurements with an oscilloscope.

Modern techniques of mass spectrometry were devised by Arthur Jeffrey Dempster and F.W. Aston in 1918 and 1919 respectively.

Sector mass spectrometers known as calutrons were developed by Ernest O. Lawrence and used for separating the isotopes of uranium during the Manhattan

Project. Calutron mass spectrometers were used for uranium enrichment at the Oak Ridge, Tennessee Y-12 plant established during World War II.

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In 1989, half of the Nobel Prize in Physics was awarded to Hans Dehmelt and Wolfgang Paul for the development of the ion trap technique in the 1950s and 1960s.

In 2002, the Nobel Prize in Chemistry was awarded to John Bennett Fenn for the development of electrospray ionization (ESI) and Koichi Tanaka for the development of soft laser desorption (SLD) and their application to the ionization of biological macromolecules, especially proteins.

Parts

A mass spectrometer consists of three components: an ion source, a mass analyzer, and a detector. The ionizer converts a portion of the sample into ions. There is a wide variety of ionization techniques, depending on the phase (solid, liquid, gas) of the sample and the efficiency of various ionization mechanisms for the unknown species. An extraction system removes ions from the sample, which are then targeted through the mass analyzer and into the detector. The differences in masses of the fragments allows the mass analyzer to sort the ions by their mass-to-charge ratio. The detector measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present. Some detectors also give spatial information, for example, a multichannel plate.

Creating Ions

The ion source is the part of the mass spectrometer that ionizes the material under analysis (the analyte). The ions are then transported by magnetic or electric fields to the mass analyzer.

Techniques for ionization have been key to determining what types of samples can be analyzed by mass spectrometry. Electron ionization and chemical ionization are used for gases and vapors. In chemical ionization sources, the analyte is ionized by chemical ion-molecule reactions during collisions in the source. Two techniques often used with liquid and solid biological samples include electrospray ionization (invented by John Fenn) and matrix-assisted laser desorption/ionization (MALDI, initially developed as a similar technique 'Soft Laser Desorption (SLD)' by K. Tanaka for which a Nobel Prize was awarded and as MALDI by M. Karas and F. Hillenkamp.

Hard Ionization and Soft Ionization

In mass spectrometry, ionization refers to the production of gas phase ions suitable for resolution in the mass analyser or mass filter. Ionization occurs in the ion source. There are several ion sources available; each has advantages and disadvantages for particular applications. For example, electron ionization (EI) gives a high degree of fragmentation, yielding highly detailed mass spectra which when skilfully analysed can provide important information for structural elucidation/characterisation and

facilitate identification of unknown compounds by comparison to mass spectral libraries obtained under identical operating conditions.

Hard ionization techniques are processes which impart high quantities of residual energy in the subject molecule invoking large degrees of fragmentation (i.e., the systematic rupturing of bonds acts to remove the excess energy, restoring stability to the resulting ion).

Soft ionization refers to the processes which impart little residual energy onto the subject molecule and as such result in little fragmentation. Examples include Fast Atom Bombardment (FAB), Chemical Ionization (CI), Atmospheric-Pressure Chemical Ionization (APCI), Electrospray Ionization (ESI), and Matrix-Assisted Laser Desorption/Ionization (MALDI).

Inductively Coupled Plasma

Inductively Coupled Plasma (ICP) sources are used primarily for cation analysis of a wide array of sample types. In this source, a plasma that is electrically neutral overall, but that has had a substantial fraction of its atoms ionized by high temperature, is used to atomize introduced sample molecules and to further strip the outer electrons from those atoms. The plasma is usually generated from argon gas, since the first ionization energy of argon atoms is higher than the first of any other elements except He, O, F and Ne, but lower than the second ionization energy of all except the most electropositive metals. The heating is achieved by a radio-frequency current passed through a coil surrounding the plasma.

Photoionization

Photoionization can be used in experiments which seek to use mass spectrometry as a means of resolving chemical kinetics mechanisms and isomeric product branching. In such instances a high energy photon, either X-ray or UV, is used to dissociate stable gaseous molecules in a carrier gas of He or Ar. In instances where a synchrotron light source is utilzed, a tuneable photon energy can be utilized to acquire a photoionization efficiency curve which can be used in conjunction with the charge ratio m/z to fingerprint molecular and ionic species.

Other Ionization Techniques

Others include glow discharge, Field Desorption (FD), Fast Atom Bombardment (FAB), Thermospray, Desorption/Ionization on Silicon (DIOS), Direct Analysis in Real Time (DART), Atmospheric Pressure Chemical Ionization (APCI), Secondary Ion Mass Spectrometry (SIMS), Spark Ionization and Thermal Ionization (TIMS).

Applications

Mass spectrometry has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its

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fragmentation. Other uses include quantifying the amount of a compound in a sample or studying the fundamentals of gas phase ion chemistry (the chemistry of ions and neutrals in a vacuum). MS is now commonly used in analytical laboratories that study physical, chemical, or biological properties of a great variety of compounds.

As an analytical technique it possesses distinct advantages such as: Increased sensitivity over most other analytical techniques because the analyzer, as a mass-charge filter, reduces background interference, Excellent specificity from characteristic fragmentation patterns to identify unknowns or confirm the presence of suspected compounds, Information about molecular weight, Information about the isotopic abundance of elements, Temporally resolved chemical data.

Isotope Ratio MS: Isotope Dating and Tracing

Mass spectrometry is also used to determine the isotopic composition of elements within a sample. Differences in mass among isotopes of an element are very small, and the less abundant isotopes of an element are typically very rare, so a very sensitive instrument is required. These instruments, sometimes referred to as Isotope Ratio Mass Spectrometers (IR-MS), usually use a single magnet to bend a beam of ionized particles towards a series of Faraday cups which convert particle impacts to electric current. A fast on-line analysis of deuterium content of water can be done using flowing afterglow mass spectrometry, FA-MS. Probably the most sensitive and accurate mass spectrometer for this purpose is the Accelerator Mass Spectrometer (AMS). This is because it provides ultimate sensitivity, capable of measuring individual atoms and measuring nuclides with a dynamic range of ~1015 relative to the major stable isotope. Isotope ratios are important markers of a variety of processes. Some isotope ratios are used to determine the age of materials for example as in carbon dating. Labeling with stable isotopes is also used for protein quantification.

Trace Gas Analysis

Several techniques use ions created in a dedicated ion source injected into a flow tube or a drift tube: selected ion flow tube (SIFT-MS), and proton transfer reaction (PTR-MS), are variants of chemical ionization dedicated for trace gas analysis of air, breath or liquid head space using well defined reaction time allowing calculations of analyte concentrations from the known reaction kinetics without the need for internal standard or calibration.

Atom Probe

An atom probe is an instrument that combines time-of-flight mass spectrometry and field-evaporation microscopy to map the location of individual atoms.

Pharmacokinetics

Pharmacokinetics is often studied using mass spectrometry because of the complex nature of the matrix (often blood or urine) and the need for high sensitivity to observe low dose and long time point data. The most common instrumentation used in this application is LC-MS with a triple quadrupole mass spectrometer. Tandem mass spectrometry is usually employed for added specificity. Standard curves and internal standards are used for quantitation of usually a single pharmaceutical in the samples. The samples represent different time points as a pharmaceutical is administered and then metabolized or cleared from the body. Blank or t=0 samples taken before administration are important in determining background and ensuring data integrity with such complex sample matrices. Much attention is paid to the linearity of the standard curve; however it is not uncommon to use curve fitting with more complex functions such as quadratics since the response of most mass spectrometers is less than linear across large concentration ranges.

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Protein Characterization

Mass spectrometry is an important method for the characterization and sequencing of proteins. The two primary methods for ionization of whole proteins are Electrospray Ionization (ESI) and Matrix-Assisted Laser Desorption/Ionization (MALDI). In keeping with the performance and mass range of available mass spectrometers, two approaches are used for characterizing proteins. In the first, intact proteins are ionized by either of the two techniques described above, and then introduced to a mass analyzer. This approach is referred to as 'top-down' strategy of protein analysis. The top-down approach however is largely limited to low-throughput single-protein studies. In the second, proteins are enzymatically digested into smaller peptides using proteases such as trypsin or pepsin, either in solution or in gel after electrophoretic separation.

Glycan Analysis

Mass Spectrometry (MS), with its low sample requirement and high sensitivity, has been predominantly used in glycobiology for characterization and elucidation of glycan structures. Mass spectrometry provides a complementary method to HPLC for the analysis of glycans. Intact glycans may be detected directly as singly charged ions by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) or, following permethylation or peracetylation, by fast atom Bombardment Mass Spectrometry (FAB-MS). Electrospray Ionization Mass Spectrometry (ESI-MS) also gives good signals for the smaller glycans. Various free and commercial software are now available which interpret MS data and aid in Glycan structure characterization.

Space Exploration

As a standard method for analysis, mass spectrometers have reached other planets and moons. Two were taken to Mars by the Viking program. In early 2005 the Cassini–Huygens mission delivered a specialized GC-MS instrument aboard the Huygens probe through the atmosphere of Titan, the largest moon of the planet Saturn. This instrument analyzed atmospheric samples along its descent trajectory and was able to vaporize and analyze samples of Titan's frozen, hydrocarbon covered surface once the probe had landed. These measurements compare the abundance of isotope(s) of each particle comparatively to earth's natural abundance. Also on board the Cassini–Huygens spacecraft was an ion and neutral mass spectrometer which had been taking measurements of Titan's atmospheric composition as well as the composition of Enceladus' plumes. A Thermal and Evolved Gas Analyzer mass spectrometer was carried by the Mars Phoenix Lander launched in 2007.

Mass spectrometers are also widely used in space missions to measure the composition of plasmas. For example, the Cassini spacecraft carried the Cassini Plasma Spectrometer (CAPS), which measured the mass of ions in Saturn's magnetosphere.

Respired Gas Monitor

Mass spectrometers were used in hospitals for respiratory gas analysis beginning around 1975 through the end of the century. Some are probably still in use but none are currently being manufactured.

Found mostly in the operating room, they were a part of a complex system, in which respired gas samples from patients undergoing anesthesia were drawn into the instrument through a valve mechanism designed to sequentially connect up to 32 rooms to the mass spectrometer. A computer directed all operations of the system. The data collected from the mass spectrometer was delivered to the individual rooms for the anesthesiologist to use.

Preparative Mass Spectrometry

The primary function of mass spectrometry is as a tool for chemical analyses based on detection and quantification of ions according to their mass-to-charge ratio. However, mass spectrometry also shows promise for material synthesis. Ion soft landing is characterized by deposition of intact species on surfaces at low kinetic energies which precludes the fragmentation of the incident species. The soft landing technique was first reported in 1977 for the reaction of low energy sulfur containing ions on a lead surface.

Check Your Progress

6. What is ultracentrifuge?
7. What is HPCL?
8. What is NP-HPCL?
9. Define HPTLC.
10. What is GS?
11. Define atmospheric pressure.

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12.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. A separation process is a method that converts a mixture or solution of chemical substances into two or more distinct product mixtures.
2. Some of the Separation Techniques are-
   - Adsorption, adhesion of atoms, ions or molecules of gas, liquid, or dissolved solids to a surface
   - Capillary electrophoresis
   - Centrifugation and cyclonic separation, separates based on density differences
   - Chelation
   - Chromatography separates dissolved substances by different interaction with (i.e., travel through) a material
3. Decantation is a process for the separation of mixtures of immiscible liquids or of a liquid and a solid mixture such as a suspension.
4. Elutriation is a process for separating particles based on their size, shape and density, using a stream of gas or liquid flowing in a direction usually opposite to the direction of sedimentation.
5. Fractional distillation is the separation of a mixture into its component parts, or fractions. Chemical compounds are separated by heating them to a temperature at which one or more fractions of the mixture will vaporize.
6. The ultracentrifuge is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as 1,000,000 g (approx. 9800 km/s^2). There are two kinds of ultracentrifuges, the preparative and the analytical ultracentrifuge. Both classes of instruments find important uses in molecular biology, biochemistry, and polymer science.
7. High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography) is a technique in analytical chemistry

used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material.

8. Normal–phase chromatography was one of the first kinds of HPLC that chemists developed. Also known as Normal-Phase HPLC (NP-HPLC) this method separates analytes based on their affinity for a polar stationary surface such as silica, hence it is based on analyte ability to engage in polar interactions (such as hydrogen-bonding or dipole-dipole type of interactions) with the sorbent surface.
9. High-Performance Thin-Layer Chromatography (HPTLC) is an enhanced form of Thin-Layer Chromatography (TLC). A number of enhancements can be made to the basic method of thin-layer chromatography to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements.
10. Gas Chromatography (GC) is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition.
11. Atmospheric Pressure (AP) Matrix-Assisted Laser Desorption/Ionization (MALDI) is an ionization technique (ion source) that in contrast to vacuum MALDI operates at normal atmospheric environment.

12.5 SUMMARY

- A separation process is a method that converts a mixture or solution of chemical substances into two or more distinct product mixtures. At least one of results of the separation is enriched in one or more of the source mixture’s constituents.
- In some cases, a separation may fully divide the mixture into pure constituents. Separations exploit differences in chemical properties or physical properties (such as size, shape, mass, density, or chemical affinity) between the constituents of a mixture.
- The purpose of a separation may be analytical, can be used as a lie components in the original mixture without any attempt to save the fractions, or may be preparative, i.e., to prepare fractions or samples of the components that can be saved.
- The separation can be done on a small scale, effectively a laboratory scale for analytical or preparative purposes, or on a large scale, effectively an industrial scale for preparative purposes, or on some intermediate scale.
- Crude oil occurs naturally as a mixture of various hydrocarbons and impurities. The refining process splits this mixture into other, more valuable mixtures such as natural gas, gasoline and chemical feed stocks, none of

which are pure substances, but each of which must be separated from the raw crude.

- The ultracentrifuge is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as 1,000,000 g (approx. 9800 km/s^2). There are two kinds of ultracentrifuges, the preparative and the analytical ultracentrifuge. Both classes of instruments find important uses in molecular biology, biochemistry, and polymer science.
- In an analytical ultracentrifuge, a sample being spun can be monitored in real time through an optical detection system, using ultraviolet light absorption and/or interference optical refractive index sensitive system. This allows the operator to observe the evolution of the sample concentration versus the axis of rotation profile as a result of the applied centrifugal field.
- Theodor Svedberg invented the analytical ultracentrifuge in 1925, and won the Nobel Prize in Chemistry in 1926 for his research on colloids and proteins using the ultracentrifuge.
- The vacuum ultracentrifuge was invented by Edward Greydon Pickels in the Physics Department at the University of Virginia. It was his contribution of the vacuum which allowed a reduction in friction generated at high speeds. Vacuum systems also enabled the maintenance of constant temperature across the sample, eliminating convection currents that interfered with the interpretation of sedimentation results.
- Thin-Layer Chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase.
- Size-Exclusion Chromatography (SEC), also known as gel permeation chromatography or gel filtration chromatography, separates particles on the basis of molecular size (actually by a particle's Stokes radius).
- The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules therefore flow through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time.
- In Ion-Exchange chromatography (IC), retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Solute ions of the same charge as the charged sites on the column are excluded from binding, while solute ions of the opposite charge of the charged sites of the column are retained on the column.
- Aqueous Normal-Phase chromatography (ANP) is a chromatographic technique which encompasses the mobile phase region between Reversed-Phase Chromatography (RP) and Organic Normal Phase Chromatography

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(ONP). This technique is used to achieve unique selectivity for hydrophilic compounds, showing normal phase elution using reversed-phase solvents.

- The parameters are largely derived from two sets of chromatagraphic theory: plate theory (as part of Partition chromatography), and the rate theory of chromatography / Van Deemter equation. Of course, they can be put in practice through analysis of HPLC chromatograms, although rate theory is considered the more accurate theory.

12.6 KEY WORDS

- **Separation process:** A separation process is a method that converts a mixture or solution of chemical substances into two or more distinct product mixtures.
- **Chelation:** Chelation is a chemical process in which a substance is used to bind molecules, such as metals or minerals, and hold them tightly.
- **Decantation:** Decantation is a process for the separation of mixtures of immiscible liquids or of a liquid and a solid mixture such as a suspension.
- **Elutriation:** Elutriation is a process for separating particles based on their size, shape and density, using a stream of gas or liquid flowing in a direction usually opposite to the direction of sedimentation.
- **Extraction:** Extraction in chemistry is a separation process consisting in the separation of a substance from a matrix. It includes Liquid-liquid extraction, and Solid phase extraction.
- **Supercritical fluid:** A SuperCritical Fluid (SCF) is any substance at a temperature and pressure above its critical point, where distinct liquid and gas phases do not exist.
- **Flocculation:** Flocculation refers to the process by which fine particulates are caused to clump together into a floc. The floc may then float to the top of the liquid (creaming), settle to the bottom of the liquid (sedimentation), or be readily filtered from the liquid.
- **Microfiltration:** Microfiltration is a type of physical filtration process where a contaminated fluid is passed through a special pore-sized membrane to separate microorganisms and suspended particles from process liquid.
- **Ultrafiltration:** UltraFiltration (UF) is a pressure-driven barrier to suspended solids, bacteria, viruses, endotoxins and other pathogens to produce water with very high purity and low silt density.
- **Gravimetric:** Gravimetric analysis describes a set of methods used in analytical chemistry for the quantitative determination of an analyte (the ion being analyzed) based on its mas

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- **Recrystallization:** Recrystallization is defined as the process in which grains of a crystal structure come in a new structure or new crystal shape.
- **Radiopharmaceuticals:** Radiopharmaceuticals, or medicinal radiocompounds, are a group of pharmaceutical drugs which have radioactivity.
- **Spectrophotometer:** The spectrophotometer is an optical instrument for measuring the intensity of light relative to wavelength.

12.7 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short Answer Questions

1. Write a note on separation techniques and different separation techniques used.
2. What is analytical ultracentrifuge?
3. Write a note on preparative ultracentrifuges.
4. Write about separation process and principle of TLC.
5. What is Partition chromatography?
6. What is normal–phase chromatography?
7. Write a short note on basic principle of displacement chromatography.

Long Answer Questions

1. Write a note on separation process and explain the different types of separation techniques used
2. Expalin about ultracentrifugation.
3. Explain about TLC, writing about its technique, separation process and principle and applications.
4. What is HPLC? Expalin the types of HPLC and its applications.
5. Give a detailed note on HPTLC.
6. What is FPLC? Explain about FPLC system components, columns, optimizing protein purification.
7. Discuss about GS in detail explaining about its history, analysis and components in detail.
8. Write a detailed note on MALDI .
9. Expalin about MS in detail.

12.8 FURTHER READINGS

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Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

Mortin, R. 1996. *Gel Electrophoresis: Nucleic Acids* (*Introduction to Biotechniques*). England: Garland Science/BIOS Scientific Publishers.

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UNIT 13 TRACER TECHNIQUES

NOTES

Structure

13.0 Introduction
13.1 Objectives
13.2 Tracer Techniques
13.3 Principles and Applications of Radioactive Isotopes
13.4 Autoradiography
13.5 Liquid Scintillation Spectrometry
13.6 Answers to Check Your Progress Questions
13.7 Summary
13.8 Key Words
13.9 Self Assessment Questions and Exercises
13.10 Further Readings

13.0 INTRODUCTION

In radioactive tracer technique, radioactive nuclides are used to follow the behavior of elements or chemical species in chemical and other processes. This is realized by means of radioactivity measurement. In 1913, Hevesy and Paneth succeeded in determining the extremely low solubility of lead salts by using naturally occurring 210Pb as a radioactive tracer. As various radioactive nuclides became artificially available, this technique has been widely employed in studies of chemical equilibrium and reactions as well as in chemical analysis. It is also an essential technique in biochemical, biological, medical, geological, and environmental studies. Medical diagnosis and industrial process control are the fields of its most important practical application. In this unit, fundamental ideas concerning radioactive tracers will be described followed by their applications with the help of examples.

An isotope is a form of an element which has the same atomic number of electrons as the common form of the element but it differs in atomic weight. The difference of atomic weight is due to difference of number of neutrons in its nucleus. An isotope may be stable or radioactive depending on the relative number of protons and neutrons in its nucleus.

In this unit, you will study about tracer techniques, its principles and applications of radioactive isotopes, autoradiography and liquid scintillation spectrometry are also described in the unit.

13.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand about tracer techniques

- Discuss about the principles and applications of radioactive isotopes
- Explain what is autoradiography and its importance
- Discuss what liquid scintillation spectrometry is

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13.2 TRACER TECHNIQUES

For the study of biological pathway and mechanism Tracer techniques has its importance. Tracer techniques involve use of isotopically labelled molecules and detection of the isotopes for the study (Refer Figure 13.1).

An isotope is a form of an element which has the same atomic number of electrons as the common form of the element but it differs in atomic weight. The difference of atomic weight is due to difference of number of neutrons in its nucleus. An isotope may be stable or radioactive depending on the relative number of protons and neutrons in its nucleus.

Both stable and radioactive isotopes of an element are identical in chemical properties, and thus they undergo all the physical and chemical changes like the ordinary form of the element. Moreover, the can be detected at any time by atomic weight or radioactivity. By Ginger-Muller counter or other sensitive detectors the radioactive isotopes can be detected by their radioactivity even when it present in very small quantity. Detection of stable isotopes can be done by their atomic weight through a mass spectrograph. Stable or radioactive isotopes used for studying the fate of a molecule in physical, chemical or biological processes are called tracer element and the methods for such studies are called tracer techniques . Commonly used radioactive tracers in the study of biology are C^{14}, P^{12}, H^{3} etc. An important stable isotope used as tracer in biology is O^{18}.

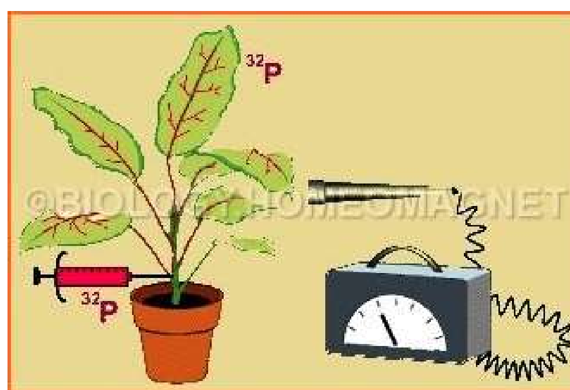

Fig. 13.1 *A Image of Tracer Technique*

At specific positions in their molecules inorganic and organic compounds can be prepared with isotopes. Such compound containing an isotope in its molecule is used as a tracer. In the tracer technique the isotope element in the molecule is said 'tagged 'or 'labelled'. When an isotopically labelled compound is administrated

to an animal or a plant or incubated with tissue preparations, it undergoes same fate as the unlabelled form of the compound and the isotopically labelled products can be detected. In this way, this source, metabolic pathway and end products of bio-molecules can be studied with the use of isotopically tagged tracers.

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In the process of Tracer techniques tracers are also used for determining the following

- Metabolic turnover of a substance.
- Relative proportion of a substance being catabolised through different pathways.
- Intestinal absorption of the nutrient.
- Volume of body fluids.
- Blood level of a hormone.
- Mechanism and site of action of a hormone.
- Cardiac output.
- Flow of blood through an organ.
- Intracellular distribution, i.e., autoradiography.

The most common radioactive tracer used in metabolic studies is C^{14}. P^{32}, which is a radioactive tracer is mainly used to study the phosphorylation reactions. H^3 or tritium is also a radioactive isotope which is used as a tracer in the form of tritium oxide (THO) for determination of total body fluid volume. O^{18} is a stable isotope and it is used to trace the source of O_2 liberated in photosynthesis. When O^{18} labelled water (H_2O^{18}) is used in photosynthesis, $O^{18}{}_2$ is liberated. It is thus proved that the water is the source of oxygen liberated in photosynthesis.

Check Your Progress

1. What is tracer technique used for?
2. Define isotope.
3. Give one property of isotope.
4. Name few of the areas where tracer techniques are used.

13.3 PRINCIPLES AND APPLICATIONS OF RADIOACTIVE ISOTOPES

A radioactive tracer, or radioactive label, is a chemical compound in which one or more atoms have been replaced by a radionuclide so by virtue of its radioactive decay it can be used to explore the mechanism of chemical reactions by tracing the path that the radioisotope follows from reactants to products. Radiolabeling is thus the radioactive form of isotopic labeling.

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Radioisotopes of hydrogen, carbon, phosphorus, sulfur, and iodine have been used extensively to trace the path of biochemical reactions. A radioactive tracer can also be used to track the distribution of a substance within a natural system such as a cell or tissue, or as a flow tracer to track fluid flow. Radioactive tracers are also used to determine the location of fractures created by hydraulic fracturing in natural gas production. Radioactive tracers form the basis of a variety of imaging systems, such as PET scans, SPECT scans and technetium scans. Radiocarbon dating uses the naturally occurring carbon-14 isotope as an isotopic label.

Methodology

Isotopes of a chemical element differ only in the mass number. For example, the isotopes of hydrogen can be written as 1H, 2H and 3H, with the mass number at top left. When the atomic nucleus of an isotope is unstable, compounds containing this isotope are radioactive. Tritium is an example of a radioactive isotope.

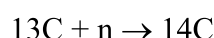
The principle behind the use of radioactive tracers is that an atom in a chemical compound is replaced by another atom, of the same chemical element. The substituting atom, however, is a radioactive isotope. This process is often called radioactive labeling. The power of the technique is due to the fact that radioactive decay is much more energetic than chemical reactions. Therefore, the radioactive isotope can be present in low concentration and its presence detected by sensitive radiation detectors such as Geiger counters and scintillation counters. George de Hevesy won the 1943 Nobel Prize for Chemistry 'for his work on the use of isotopes as tracers in the study of chemical processes'.

There are two main ways in which radioactive tracers are used

1. When a labeled chemical compound undergoes chemical reactions one or more of the products will contain the radioactive label. Analysis of what happens to the radioactive isotope provides detailed information on the mechanism of the chemical reaction.
2. A radioactive compound is introduced into a living organism and the radio-isotope provides a means to construct an image showing the way in which that compound and its reaction products are distributed around the organism.

Production

The commonly used radioisotopes have short half-lives and so do not occur in nature. They are produced by nuclear reactions. One of the most important processes is absorption of a neutron by an atomic nucleus, in which the mass number of the element concerned increases by 1 for each neutron absorbed. For example,



$$13C + n \rightarrow 14C$$

In this case the atomic mass increases, but the element is unchanged. In other cases the product nucleus is unstable and decays, typically emitting protons, electrons (beta particle) or alpha particles. When a nucleus loses a proton the atomic number decreases by 1. For example,

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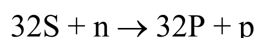


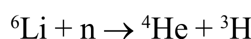
$$32S + n \rightarrow 32P + p$$

Neutron irradiation is performed in a nuclear reactor. The other main method used to synthesize radioisotopes is proton bombardment. The protons are accelerated to high energy either in a cyclotron or a linear accelerator.

Tracer Isotopes

Hydrogen

Tritium is produced by neutron irradiation of ^{6}Li



$$^{6}Li + n \rightarrow {}^{4}He + {}^{3}H$$

Tritium has a half-life 4,500±8 days (approximately 12.32 years), and it decays by beta decay. The electrons produced have an average energy of 5.7 keV. Because the emitted electrons have relatively low energy, the detection efficiency by scintillation counting is rather low. However, hydrogen atoms are present in all organic compounds, so tritium is frequently used as a tracer in biochemical studies.

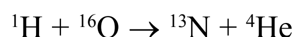
Carbon

^{11}C decays by positron emission with a half-life of ca. 20 min. ^{11}C is one of the isotopes often used in positron emission tomography.

^{14}C decays by beta-decay, with a half-life of 5730 y. It is continuously produced in the upper atmosphere of the earth so it occurs at a trace level in the environment. However, it is not practical to use naturally-occurring ^{14}C for tracer studies. Instead it is made by neutron irradiation of the isotope ^{13}C which occurs naturally in carbon at about the 1.1% level. ^{14}C has been used extensively to trace the progress of organic molecules through metabolic pathways.

Nitrogen

^{13}N decays by positron emission with a half-life of 9.97 min. It is produced by the nuclear reaction



$$^{1}H + {}^{16}O \rightarrow {}^{13}N + {}^{4}He$$

^{13}N is used in positron emission tomography (PET scan).

Oxygen

^{15}O decays by positron emission with a half-life of 122 sec. It is used in positron emission tomography

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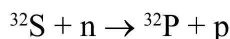
Fluorine

^{18}F decays by emission with a half-life of 109 min. It is made by proton bombardment of 18O in a cyclotron or linear particle accelerator. It is an important isotope in the radiopharmaceutical industry. It is used to make labeled fluorodeoxyglucose (FDG) for application in PET scans.

Phosphorus

^{32}P is made by neutron bombardment of ^{32}S



$^{32}S + n \rightarrow {}^{32}P + p$

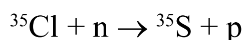
It decays by beta decay with a half-life of 14.29 days. It is commonly used to study protein phosphorylation by kinases in biochemistry.

^{33}P is made in relatively low yield by neutron bombardment of ^{31}P. It is also a beta-emitter, with a half-life of 25.4 days. Though more expensive than ^{32}P, the emitted electrons are less energetic, permitting better resolution in, for example, DNA sequencing

Both isotopes are useful for labeling nucleotides and other species that contain a phosphate group.

Sulfur

^{35}S is made by neutron bombardment of ^{35}Cl



$^{35}Cl + n \rightarrow {}^{35}S + p$

It decays by beta-decay with a half-life of 87.51 days. It is used to label the sulfur-containing amino-acids methionine and cysteine. When a sulfur atom replaces an oxygen atom in a phosphate group on a nucleotide a thiophosphate is produced, so ^{35}S can also be used to trace a phosphate group.

Technetium

^{99m}Tc is a very versatile radioisotope, and is the most commonly used radioisotope tracer in medicine. It is easy to produce in a technetium-99m generator, by decay of ^{99}Mo.

$^{99}Mo \rightarrow {}^{99m}Tc + e^- + \nu e$-

The molybdenum isotope has a half-life of approximately 66 hours (2.75 days), so the generator has a useful life of about two weeks. Most commercial ^{99m}Tc generators use column chromatography, in which ^{99}Mo in the form of molybdate, $MoO_4^{2"}$ is adsorbed onto acid alumina (Al_2O_3). When the ^{99}Mo decays it forms pertechnetate TcO4", which because of its single charge is less tightly bound to the alumina. Pulling normal saline solution through the column of immobilized ^{99}Mo elutes the soluble ^{99m}Tc, resulting in a saline solution containing the ^{99m}Tc as the dissolved sodium salt of the pertechnetate. The pertechnetate is treated with a reducing agent such as Sn^{2+} and a ligand. Different ligands form

coordination complexes which give the technetium enhanced affinity for particular sites in the human body.

^{99m}Tc decays by gamma emission, with a half-life: 6.01 hours. The short half-life ensures that the body-concentration of the radioisotope falls effectively to zero in a few days.

Iodine

^{123}I is produced by proton irradiation of 124Xe. The caesium isotope produced is unstable and decays to ^{123}I. The isotope is usually supplied as the iodide and hypoiodate in dilute sodium hydroxide solution, at high isotopic purity. ^{123}I has also been produced at Oak Ridge National Laboratories by proton bombardment of ^{123}Te.

^{123}I decays by electron capture with a half-life of 13.22 hours. The emitted 159 keV gamma ray is used in Single Photon Emission Computed Tomography (SPECT). A 127 keV gamma ray is also emitted.

^{125}I is frequently used in radioimmunoassays because of its relatively long half-life (59 days) and ability to be detected with high sensitivity by gamma counters.

^{129}I is present in the environment as a result of the testing of nuclear weapons in the atmosphere. It was also produced in the Chernobyl and Fukushima disasters. ^{129}I decays with a half-life of 15.7 million years, with low-energy beta and gamma emissions. It is not used as a tracer, though its presence in living organisms, including human beings, can be characterized by measurement of the gamma rays.

Other Isotopes

Many other isotopes have been used in specialized radiopharmacological studies. The most widely used is ^{67}Ga for gallium scans. ^{67}Ga is used because, like ^{99m}Tc, it is a gamma-ray emitter and various ligands can be attached to the Ga^{3+} ion, forming a coordination complex which may have selective affinity for particular sites in the human body.

An extensive list of radioactive tracers used in hydraulic fracturing can be found below.

Application

In metabolism research, Tritium and ^{14}C-labeled glucose are commonly used in glucose clamps to measure rates of glucose uptake, fatty acid synthesis, and other metabolic processes. While radioactive tracers are sometimes still used in human studies, stable isotope tracers such as ^{13}C are more commonly used in current human clamp studies. Radioactive tracers are also used to study lipoprotein metabolism in humans and experimental animals.

In medicine, tracers are applied in a number of tests, such as ^{99m}Tc in autoradiography and nuclear medicine, including Single Photon Emission Computed Tomography (SPECT), Positron Emission Tomography (PET) and scintigraphy.

NOTES

The urea breath test for helicobacter pylori commonly used a dose of ^{14}C labeled urea to detect h. pylori infection. If the labeled urea was metabolized by h. pylori in the stomach, the patient's breath would contain labeled carbon dioxide. In recent years, the use of substances enriched in the non-radioactive isotope ^{13}C has become the preferred method, avoiding patient exposure to radioactivity.

In hydraulic fracturing, radioactive tracer isotopes are injected with hydraulic fracturing fluid to determine the injection profile and location of created fractures. Tracers with different half-lives are used for each stage of hydraulic fracturing. In the United States amounts per injection of radionuclide are listed in the US Nuclear Regulatory Commission (NRC) guidelines. According to the NRC, some of the most commonly used tracers include antimony-124, bromine-82, iodine-125, iodine-131, iridium-192, and scandium-46. A 2003 publication by the International Atomic Energy Agency confirms the frequent use of most of the tracers above, and says that manganese-56, sodium-24, technetium-99m, silver-110m, argon-41, and xenon-133 are also used extensively because they are easily identified and measured.

Check Your Progress

5. What is a radioactive tracer?
6. What are the main two ways where radioactive tracers are used?
7. How does 18F decays ?
8. How does 14C decays?

13.4 AUTORADIOGRAPHY

Autoradiography is the use of X-ray (or occasionally photographic) film to detect radioactive materials. It produces a permanent record of the positions and relative intensities of radiolabeled bands in a gel or blot. Typically, biomolecules are labeled with ^{32}P or ^{35}S, and detected by overnight film exposure.

Autoradiograph

An autoradiograph is an image on an x-ray film or nuclear emulsion produced by the pattern of decay emissions (for example, beta particles or gamma rays) from a distribution of a radioactive substance. Alternatively, the autoradiograph is also available as a digital image (digital autoradiography), due to the recent development of scintillation gas detectors or rare earth phosphorimaging systems. The film or emulsion is apposed to the labeled tissue section to obtain the autoradiograph (also called an autoradiogram). The auto- prefix indicates that the radioactive substance is within the sample, as distinguished from the case of historadiography or microradiography, in which the sample is X-rayed using an external source. Some autoradiographs can be examined microscopically for localization of silver grains (such as on the interiors or exteriors of cells or organelles) in which the

process is termed micro-autoradiography. For example, micro-autoradiography was used to examine whether atrazine was being metabolized by the hornwort plant or by epiphytic microorganisms in the biofilm layer surrounding the plant.

NOTES

Applications

In biology, this technique may be used to determine the tissue (or cell) localization of a radioactive substance, either introduced into a metabolic pathway, bound to a receptor or enzyme, or hybridized to a nucleic acid.

The use of radiolabeled ligands to determine the tissue distributions of receptors is termed either in vivo or in vitro receptor autoradiography if the ligand is administered into the circulation (with subsequent tissue removal and sectioning) or applied to the tissue sections, respectively. The ligands are generally labeled with ^{3}H (tritium) or ^{125}I (radioiodine). The distribution of RNA transcripts in tissue sections by the use of radiolabeled, complementary oligonucleotides or ribonucleic acids (riboprobes) is called in-situ hybridization histochemistry. Radioactive precursors of DNA and RNA, [^{3}H]-thymidine and [^{3}H]-uridine respectively, may be introduced to living cells to determine the timing of several phases of the cell cycle. RNA or DNA viral sequences can also be located in this fashion. These probes are usually labeled with ^{32}P, ^{33}P, or ^{35}S. In the realm of behavioral endocrinology, autoradiography can be used to determine hormonal uptake and indicate receptor location; an animal can be injected with a radiolabeled hormone, or the study can be conducted in vitro.

Rate of DNA Replication

The rate of DNA replication in a mouse cell growing in vitro was measured by autoradiography as 33 nucleotides per second. The rate of phage T4 DNA elongation in phage-infected E. coli was also measured by autoradiography as 749 nucleotides per second during the period of exponential DNA increase at 37 °C.

Detection of Protein Phosphorylation

Phosphorylation means the posttranslational addition of a phosphate group to specific amino acids of proteins, and such modification can lead to a drastic change in the stability or the function of a protein in the cell. Protein phosphorylation can be detected on an autoradiograph, after incubating the protein in vitro with the appropriate kinase and ã-32P-ATP. The radiolabbeled phosphate of latter is incorporated into the protein which is isolated via SDS-PAGE and visualized on an autoradiograph of the gel.

Other Techniques

This autoradiographic approach contrasts to techniques such as PET and SPECT where the exact 3-dimensional localization of the radiation source is provided by careful use of coincidence counting, gamma counters and other devices.

NOTES

Krypton-85 is used to inspect aircraft components for small defects. Krypton-85 is allowed to penetrate small cracks, and then its presence is detected by autoradiography. The method is called krypton gas penetrant imaging. The gas penetrates smaller openings than the liquids used in dye penetrant inspection and fluorescent penetrant inspection.

Unintentional Exposure

The task of radioactive decontamination following the Baker nuclear test at Bikini Atoll during Operation Crossroads in 1946 was far more difficult than the U.S. Navy had prepared for. Though the task's futility became apparent and the danger to cleanup crews mounted, Colonel Stafford Warren, in charge of radiation safety, had difficulty persuading Vice Admiral William H. P. Blandy to abandon the cleanup and with it the surviving target ships. On August 10, Warren showed Blandy an autoradiograph made by a surgeonfish from the lagoon that was left on a photographic plate overnight. The film was exposed by alpha radiation produced from the fish's scales, evidence that plutonium, mimicking calcium, had been distributed throughout the fish. Blandy promptly ordered that all further decontamination work be discontinued.

13.5 LIQUID SCINTILLATION SPECTROMETRY

Different to alpha particles and gamma radiation, beta particles do not have discrete energies because the decay energy is randomly transferred to a beta particle and an antineutrino. Instead, beta particles are characterised by a mean and a maximum energy, which are superimposed for different nuclides. Therefore, identification of nuclides with beta spectrometry is limited.

Liquid scintillation counting is the measurement of activity of a sample of radioactive material which uses the technique of mixing the active material with a liquid scintillator (for example, Zinc sulfide) , and counting the resultant photon emissions. The purpose is to allow more efficient counting due to the intimate contact of the activity with the scintillator. It is generally used for alpha and beta particle detection.

The analysis with liquid scintillation counting (LSC) is a diverse method and is suitable to detect alpha and beta particles. For measurement with LSC the nuclides are homogenously dissolved in a cocktail or are surrounded by it. The energy of the ionising particles is transferred to a solvent molecule and then to the scintillator. The scintillator emits this energy as light, which is detected with two opposite photomultipliers. Because the pulse shapes of alpha and beta particles are different, the discrimination of alpha and beta radiation is possible via pulse shape analysis. Assertions on particle energy can be provided from the number of detected photons. The detection limit of a LSC is in the range of 5 to 20 mBq.

NOTES

Liquid Scintillation Counter

Samples are dissolved or suspended in a "cocktail" containing a solvent (historically aromatic organics such as xylene or toluene, but more recently less hazardous solvents are used), typically some form of a surfactant, and small amounts of other additives known as fluors or scintillators. Scintillators can be divided into primary and secondary phosphors, differing in their luminescence properties.

Beta particles emitted from the isotopic sample transfer energy to the solvent molecules: the ð cloud of the aromatic ring absorbs the energy of the emitted particle. The energized solvent molecules typically transfer the captured energy back and forth with other solvent molecules until the energy is finally transferred to a primary scintillator. The primary phosphor will emit photons following absorption of the transferred energy. Because that light emission may be at a wavelength that does not allow efficient detection, many cocktails contain secondary phosphors that absorb the fluorescence energy of the primary phosphor and re-emit at a longer wavelength.

The radioactive samples and cocktail are placed in small transparent or translucent (often glass or plastic) vials that are loaded into an instrument known as a liquid scintillation counter. Newer machines may use 96-well plates with individual filters in each well. Many counters have two photo multiplier tubes connected in a coincidence circuit. The coincidence circuit assures that genuine light pulses, which reach both photo multiplier tubes, are counted, while spurious pulses (due to line noise, for example), which would only affect one of the tubes, are ignored.

Counting efficiencies under ideal conditions range from about 30% for tritium (a low-energy beta emitter) to nearly 100% for phosphorus-32, a high-energy beta emitter. Some chemical compounds (notably chlorine compounds) and highly colored samples can interfere with the counting process. This interference, known as quenching, can be overcome through data correction or through careful sample preparation.

High-energy beta emitters, such as phosphorus-32, can also be counted in a scintillation counter without the cocktail, instead using an aqueous solution. This technique, known as Cherenkov counting, relies on the Cherenkov radiation being detected directly by the photomultiplier tubes. Cherenkov counting in this experimental context is normally used for quick, rough measurements, since the geometry of the sample can create variations in the output.

Check Your Progress

9. What is autoradiography?
10. Define mass spectrometry.
11. What is radiopharmaceutical?
12. What is RIA?

13.6 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

NOTES

1. Tracer techniques involve use of isotopically labelled molecules and detection of the isotopes for the study
2. An isotope is a form of an element which has the same atomic number of electrons as the common form of the element but it differs in atomic weight.
3. An isotope may be stable or radioactive depending on the relative number of protons and neutrons in its nucleus.
4. In the process of Tracer techniques tracers are also used for determining the following
   - Metabolic turnover of a substance.
   - Relative proportion of a substance being catabolised through different pathways.
   - Intestinal absorption of the nutrient.
   - Volume of body fluids.
5. A radioactive tracer, or radioactive label, is a chemical compound in which one or more atoms have been replaced by a radionuclide so by virtue of its radioactive decay it can be used to explore the mechanism of chemical reactions by tracing the path that the radioisotope follows from reactants to products.
6. There are two main ways in which radioactive tracers are used
   - When a labeled chemical compound undergoes chemical reactions one or more of the products will contain the radioactive label. Analysis of what happens to the radioactive isotope provides detailed information on the mechanism of the chemical reaction.
   - A radioactive compound is introduced into a living organism and the radio-isotope provides a means to construct an image showing the way in which that compound and its reaction products are distributed around the organism.
7. ^{18}F decays by emission with a half-life of 109 min. It is made by proton bombardment of 18O in a cyclotron or linear particle accelerator. It is an important isotope in the radiopharmaceutical industry. It is used to make labeled fluorodeoxyglucose (FDG) for application in PET scans.
8. ^{14}C decays by beta-decay, with a half-life of 5730 y. It is continuously produced in the upper atmosphere of the earth so it occurs at a trace level in the environment. However, it is not practical to use naturally-occurring ^{14}C for tracer studies. Instead it is made by neutron irradiation of the isotope ^{13}C which occurs naturally in carbon at about the 1.1% level. ^{14}C has been used extensively to trace the progress of organic molecules through metabolic pathways.

9. Autoradiography is the use of X-ray (or occasionally photographic) film to detect radioactive materials. It produces a permanent record of the positions and relative intensities of radiolabeled bands in a gel or blot.
10. Mass spectrometry, an analytical technique that measures the mass-to-charge ratio of charged particles. Rutherford backscattering spectrometry, an analytical technique used to determine the structure and composition of materials by measuring the back-scattering of a beam of high energy ions impinging on a sample.
11. A Radiopharmaceutical is a drug that can be used either for diagnostic or therapeutic purposes. It is composed of a radioisotope bond to an organic molecule. The organic molecule conveys the radioisotope to specific organs, tissues or cells. The radioisotope is selected for its properties.
12. A RadioImmunoAssay (RIA) is an immunoassay that uses radiolabeled molecules in a stepwise formation of immune complexes. An RIA is a very sensitive in vitro assay technique used to measure concentrations of substances, usually measuring antigen concentrations (for example, hormone levels in blood) by use of antibodies.

NOTES

13.7 SUMMARY

- For the study of biological pathway and mechanism Tracer techniques has its importance. Tracer techniques involve use of isotopically labelled molecules and detection of the isotopes for the study
- An isotope is a form of an element which has the same atomic number of electrons as the common form of the element but it differs in atomic weight.
- The difference of atomic weight is due to difference of number of neutrons in its nucleus. An isotope may be stable or radioactive depending on the relative number of protons and neutrons in its nucleus.
- By Ginger-Muller counter or other sensitive detectors the radioactive isotopes can be detected by their radioactivity even when it present in very small quantity.
- Detection of stable isotopes can be done by their atomic weight through a mass spectrograph. Stable or radioactive isotopes used for studying the fate of a molecule in physical, chemical or biological processes are called tracer element and the methods for such studies are called tracer techniques . Commonly used radioactive tracers in the study of biology are C^{14}, P^{12}, H^{3} etc. An important stable isotope used as tracer in biology is O^{18}.
- Radioisotopes of hydrogen, carbon, phosphorus, sulfur, and iodine have been used extensively to trace the path of biochemical reactions. A radioactive tracer can also be used to track the distribution of a substance within a natural system such as a cell or tissue, or as a flow tracer to track fluid flow.

NOTES

- Isotopes of a chemical element differ only in the mass number. For example, the isotopes of hydrogen can be written as 1H, 2H and 3H, with the mass number at top left. When the atomic nucleus of an isotope is unstable, compounds containing this isotope are radioactive. Tritium is an example of a radioactive isotope.
- Autoradiography is the use of X-ray (or occasionally photographic) film to detect radioactive materials. It produces a permanent record of the positions and relative intensities of radiolabeled bands in a gel or blot. Typically, biomolecules are labeled with ^{32}P or ^{35}S, and detected by overnight film exposure.
- Phosphorylation means the posttranslational addition of a phosphate group to specific amino acids of proteins, and such modification can lead to a drastic change in the stability or the function of a protein in the cell.
- Protein phosphorylation can be detected on an autoradiograph, after incubating the protein in vitro with the appropriate kinase and ã-32P-ATP. The radiolabbeled phosphate of latter is incorporated into the protein which is isolated via SDS-PAGE and visualized on an autoradiograph of the gel.

13.8 KEY WORDS

- **Nuclide:** A nuclide (or nucleide, from nucleus, also known as nuclear species) is an atomic species characterized by the specific constitution of its nucleus, i.e., by its number of protons Z, its number of neutrons N, and its nuclear energy state
- **Autoradiography:** Autoradiography is the use of X-ray (or occasionally photographic) film to detect radioactive materials.
- **Isotopes:** Isotopes are variants of a particular chemical element which differ in neutron number, and consequently in mass number.
- **Mass spectrometry:** Mass spectrometry, is an analytical technique that measures the mass-to-charge ratio of charged particles.
- **Spectrograph:** A spectrograph is an instrument that separates light by its wavelengths and records this data.
- **Isotopic tracer:** Isotopic tracer, one any radioactive atom detectable in a material in a chemical, biological, or physical system and used to mark that material for study, to observe its progress through the system, or to determine its distribution.
- **Radiopharmaceutical:** A Radiopharmaceutical is a drug that can be used either for diagnostic or therapeutic purposes.
- **Radioimmunoassay:** A radioimmunoassay (RIA) is an immunoassay that uses radiolabeled molecules in a stepwise formation of immune complexes.
- **Lipoproteins:** Lipoproteins are basically a core full of fat and cholesterol, along with a lipid membrane that contains proteins called apolipoproteins.

NOTES

- **Historadiography:** Historadiography is a technique formerly utilized in the fields of histology and cellular biology to provide semi- quantitative information regarding the density of a tissue sample.
- **Ribonucleic acid:** Ribonucleic acid (RNA) is a polymeric molecule essential in various biological roles in coding, decoding, regulation and expression of genes.
- **Cherenkov radiation:** Cherenkov radiation is an electromagnetic radiation emitted when a charged particle (such as an electron) passes through a dielectric medium at a speed greater than the phase velocity of light in that medium.

13.9 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short Answer Questions

1. What is tracer technique and where is it used?
2. Write a short note on applications of radioactive isotopes.
3. Write about few of the tracer isotopes.
4. What are the applications of tracer isotopes?
5. Write a short note on autoradiography.

Long Answer Questions

1. Write about tracer techniques and its applications in various areas in detail.
2. Discuss about principles and applications of radioactive isotopes.
3. Explain in detail about tracer isotope.
4. Discuss about the applications of tracer isotopes.
5. What is autoradiography? Explain in detail about its applications and the areas where it is used.
6. Explain in detail about liquid scintillation counter.

13.10 FURTHER READINGS

Singh, D.K. 2013. *Principles and Techniques in Histology, Microscopy and Photomicrography*. New Delhi: CBS Publishers & Distributors Pvt. Ltd.

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UNIT 14 BLOTTING TECHNIQUES

NOTES

Structure

14.0 Introduction
14.1 Objectives
14.2 Blotting Techniques
  14.2.1 Southern Blotting
  14.2.2 Northern Blotting
14.3 Principles and Applications of PCR, RFLP, RAPD, AFLP and DNA Fingerprinting
  14.3.1 PCR
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14.0 INTRODUCTION

Biochemistry studies molecules, such as DNA, RNA and proteins. Blotting techniques are what scientists use to separate these types of molecules. In cells, they exist as a mixture. Blotting allows researchers to find one protein among many, like a needle in a haystack. Blotting is generally done by letting a mixture of DNA, RNA or protein flow through a slab of gel. This gel allows small molecules to move faster than bigger ones. The separated molecules are then pressed against a membrane, which helps move the molecules from the gel onto the membrane. The molecules stick to the membrane, but stay in the same location, apart from each other, as if they were still in the gel.

Blotting is a common technique which is widely used in the field of molecular biology. These methods, such as southern, western, northern and eastern are applicable for different types of macromolecules like lipids, RNA, DNA and proteins. Each technique depends upon the following factors, such as the size of molecule and their binding ability to the solid support. Finally, by using probe we have to detect the molecule of interest.

In this unit, you will study about blotting techniques, principle and techniques of southern, northern and western blotting techniques and hybridization, principles and application of PCR, RFLP, RAPD AFLP and DNA fingerprinting is also described in detail.

14.1 OBJECTIVES

NOTES

After going through this unit, you will be able to:

- Discuss the significance of blotting technique
- Understand various principles and techniques of southern, northern and western blotting techniques and hybridization
- Explain about principles and applications of PCR, RFLP, RAPD, AFLP
- Understand what DNA fingerprinting is

14.2 BLOTTING TECHNIQUES

Blotting is a common technique which is widely used in the field of molecular biology. These methods such as southern, western, northern and eastern are applicable for different types of macromolecules like lipids, RNA, DNA and proteins. Each technique depends upon the following factors such as the size of molecule and their binding ability to the solid support. Finally, by using probe we have to detect the molecule of interest.

Blotting is technique in which nucleic acids, i.e., RNA and DNA or proteins are transferred onto a specific membrane. This membrane may be nitrocellulose PVDF or nylon membrane. This process can be done just after the gel electrophoresis, by transferring the molecules from the gel onto the surface of blotting membrane. But sometimes it can be done by directly transferring the molecules onto the membrane. And then we can visualize these transferring molecules by using staining. Examples: Ethidium bromide, Crystal violet, Safranine and Osmium tetroxide, etc.

Types of Blotting

There are basically following 4 types of blotting

- Southern Blotting
- Western Blotting
- Northern Blotting
- Eastern Blotting

14.2.1 Southern Blotting

A Southern blot is a method used for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization. The method is named after its inventor, the British biologist Edwin Southern.

NOTES

Procedure

High molecular weight DNA strands are cut into smaller fragments by restriction endonucleases. The DNA fragments are separated by size by agarose gel electrophoresis and then transferred to a nitrocellulose membrane which is placed on the top of the gel. In Southern blotting, before transfer, DNA is usually denatured with alkali for denaturation of the double stranded DNA. The denaturation in an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, separating it into single DNA strands for later hybridization to the probe, and destroys any residual RNA that may still be present in the DNA. After transfer of the DNA fragments to the nitrocellulose membrane which is done by capillary action or may be by electro transfer, vacuum transfer or centrifugation, the membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours to permanently attach the transferred DNA to the membrane. The membrane is then exposed to a hybridization probe (a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined). The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye. After hybridization, excess probe is washed from the membrane, and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe, or by development of color on the membrane if a chromogenic detection method is used.

The hybridization and washing conditions are critical. If the probe and target are 100% identical in sequence, then a high stringency hybridization can be carried out. The stringency is determined by the hybridization temperature and the salt concentration in the hybridization buffer. For probes that don't match the target completely, the stringency must be reduced to a level that allows imperfect hybrids to form. If the stringency of the hybridization is too low, then the probe may bind to too many sequences to be useful. Formamide can be included in the hybridization buffer to reduce the actual hybridization temperature by about 25°C, from the usual 68°C to the more convenient 43°C.

Applications

Southern hybridization can also be used to locate the exact position of a cloned gene within a recombinant DNA molecule. This is important as often the cloned DNA fragment is relatively large (40 kb for a cosmid vector) whereas the gene of interest, contained somewhere in the cloned fragment, may be less than 1kb in size. Southern blots of cloned genomic DNA fragments can be probed with cDNA molecules to find which parts of the genomic clone correspond to the cDNA fragment. If the Southern blot contains genomic DNA fragments from the whole genome, the probe will give information about the size of the fragment the gene is on the genome and how many copies of the gene are present in the genome.

14.2.2 Northern Blotting

Northern blotting, the name was extrapolated from Southern blotting. The northern blot is a technique used to study gene expression by detection of RNA (or isolated mRNA) in a sample.

Procedure

The nucleic acid molecules (RNA samples) are separated by agarose gel electrophoresis and then transferred to a nitrocellulose membrane but for RNA in Northern blotting, alkali denaturation is not necessary and would in any case hydrolyze the molecules. A nylon membrane with a positive charge is the most effective for use in northern blotting since the negatively charged nucleic acids have a high affinity for them. The transfer buffer used for the blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction, thus preventing RNA degradation by high temperatures. Once the RNA has been transferred to the membrane, it is immobilized through covalent linkage to the membrane by UV light or heat. After a probe has been labeled, it is hybridized to the RNA on the membrane. Experimental conditions that can affect the efficiency and specificity of hybridization include ionic strength, viscosity, duplex length, mismatched base pairs, and base composition. The membrane is washed to ensure that the probe has bound specifically and to avoid background signals from arising. The hybrid signals are then detected by X-ray film.

Applications

Northern blots give information about the size of the mRNA and any precursors, and can be useful to determine whether a cDNA clone used as a probe is full-length or whether it is one of a family of related transcripts. Northern blots can help to identify whether a genomic clone has regions that are transcribed and, if the RNA on the blot is made from different tissues, where these transcripts are made. With northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions.

14.2.3 Western Blotting and Hybridization

Identification of a specific protein in a complex mixture of proteins can be done by a technique known as western blotting. Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin, et al. in 1979 and is now a routine technique for protein analysis. The procedure for Western blotting is given below.

Gel Electrophoresis

In Western blotting, first a protein mixture is separated by electrophoresis on an SDS-polyacrylamide gel (SDS-PAGE), a slab gel infused with Sodium Dodecyl Sulphate (SDS), a dissociating agent. Proteins are commonly separated using

Polyacrylamide Gel Electrophoresis (PAGE) to characterize individual proteins in a complex sample or to examine multiple proteins within a single sample. When combined with Western blotting, PAGE is a powerful analytical tool providing information on the mass, charge, purity or presence of a protein. Several forms of PAGE exist and can provide different types of information about the protein(s). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (for example, disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilodaltons, kDa). The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration the better the resolution of higher molecular weight proteins. Proteins travel only in one dimension along the gel for most blots. Samples are loaded into wells in the gel. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (different electrophoretic mobilities) separate into bands within each lane.

Transfer of Proteins to a Membrane

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). There are a variety of methods that have been used for this process, including diffusion transfer, capillary transfer, heat-accelerated convectional transfer, vacuum blotting transfer and electroelution. In capillary transfer, the membrane is placed on top of the gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. But this method of transfer is very time consuming. The transfer method that is most commonly used for proteins is electroelution or electrophoretic transfer because of its speed and transfer efficiency. Electrophoretic transfer of proteins involves placing a protein-containing polyacrylamide gel in direct contact with a piece of nitrocellulose or other suitable, protein-binding support and 'sandwiching' this between two electrodes submerged in a conducting solution. When an electric field is applied, the proteins move out of the polyacrylamide gel and onto the surface of the membrane, where the proteins become tightly attached. The result is a membrane with a copy of the protein pattern that was originally in the polyacrylamide gel. The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Coomassie Brilliant Blue or Ponceau S dyes.

Blocking Non-specific Sites

After the transfer of the proteins from the gel, the remaining surface of the membrane is blocked to prevent non-specific binding of the detection antibodies during subsequent steps. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically 3-5% Bovine Serum Albumin (BSA) or non-fat dry milk in Tris-Buffered Saline (TBS), with a minute percentage of detergent such as Tween 20 or Triton X-100. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces 'noise' in the final product of the western blot, leading to clearer results, and eliminates false positives.

Detection

During the detection process the membrane is 'probed' for the protein of interest with a modified antibody which is linked to a reporter enzyme; when exposed to an appropriate substrate this enzyme drives a colorimetric reaction and produces a colour.

Incubation with the Primary Antibody

Western blotting is typically performed by probing the blocked membrane with a primary antibody that recognizes a specific protein or epitope on a group of proteins (i.e., SH2 domain or phosphorylated tyrosine). The choice of a primary antibody for a Western blot will depend on the antigen to be detected and what antibodies are available to that antigen.

After blocking, a dilute solution of primary antibody is incubated with the membrane under gentle agitation. Typically, the solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. If incubating in blocking buffer overnight, it is imperative to incubate at 4°C or contamination will incur and thus destruction of the protein (especially phospho groups). Agitation of the antibody is recommended to enable adequate homogenous covering of the membrane and prevent uneven binding.

Incubation with Secondary Antibody

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. A wide variety of labeled secondary detection reagents can be used for Western blot detection. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

NOTES

Methods of Detection

Enzymatic labels are most commonly used for Western blotting and, although they require extra steps, can be extremely sensitive when optimized with an appropriate substrate. Alkaline Phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes used most extensively as labels for protein detection. The high activity rate, good stability, low cost and wide availability of substrates make HRP the enzyme of choice for most applications. After binding of the enzyme-antibody conjugate, addition of a chromogenic substrate that produces a highly coloured and insoluble product causes the appearance of a coloured band at the site of the target antigen. The site of the protein of interest can be determined with much higher sensitivity if a chemiluminescent compound along with suitable enhancing agents is used to produce light at the antigenic site.

The second method of secondary antibody detection utilizes a near-infrared (NIR) fluorophore-linked antibody. Light produced from the excitation of a fluorescent dye is static, making fluorescent detection a more precise and accurate measure of the difference in signal produced by labeled antibodies bound to proteins on a western blot. Proteins can be accurately quantified because the signal generated by the different amounts of proteins on the membranes is measured in a static state, as compared to chemiluminescence, in which light is measured in a dynamic state. The use of fluorophore-conjugated antibodies in immunoassays requires fewer steps because there is no substrate development step in the assay. This method requires special equipment in order to detect and document the fluorescent signal due to the need for an excitation light source.

Applications

- Western blotting can be used to identify a specific antibody in a mixture. In this case, known antigens of well-defined molecular weight are separated by SDS-PAGE and blotting onto nitrocellulose. The separated bands of known antigens are then probed with the sample suspected of containing antibody specific for one or more of these antigens. Reaction of an antibody with a band is detected by using either radiolabeled or enzyme linked secondary antibody that is specific for the species of the antibodies in the test sample. The most widely used application of this procedure is in confirmatory testing for HIV, where Western blotting is used to determine whether the patient has antibodies that react with one or more viral proteins.
- A western blot is also used as the definitive test for mad cow disease.
- Western blot can also be used as a confirmatory test for Hepatitis B infection.

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Check Your Progress

1. What is blotting?
2. Where is blotting used?
3. Name the different types of blotting.
4. What is southern blotting?
5. What happens in northern blotting?
6. Why is western blotting done?

14.3 PRINCIPLES AND APPLICATIONS OF PCR, RFLP, RAPD, AFLP AND DNA FINGERPRINTING

Following are the principles and applications of PCR, RFLP, AFLP and DNA finger printing.

14.3.1 PCR

Polymerase Chain Reaction (PCR) is a method widely used in molecular biology to make many copies of a specific DNA segment. Using PCR, a single copy (or more) of a DNA sequence is exponentially amplified to generate thousands to millions of more copies of that particular DNA segment. PCR is now a common and often indispensable technique used in medical laboratory and clinical laboratory research for a broad variety of applications including biomedical research and criminal forensics. PCR was developed by Kary Mullis in 1983 while he was an employee of the Cetus Corporation. He was awarded the Nobel Prize in Chemistry in 1993 (along with Michael Smith) for his work in developing the method.

The vast majority of PCR methods rely on thermal cycling. Thermal cycling exposes reactants to repeated cycles of heating and cooling to permit different temperature-dependent reactions—specifically, DNA melting and enzyme-driven DNA replication. PCR employs two main reagents - primers (which are short single strand DNA fragments known as oligonucleotides that are a complementary sequence to the target DNA region) and a DNA polymerase. In the first step of PCR, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is lowered and the primers bind to the complementary sequences of DNA. The two DNA strands then become templates for DNA polymerase to enzymatically assemble a new DNA strand from free nucleotides, the building blocks of DNA. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the original DNA template is exponentially amplified.

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Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the thermophilic bacterium Thermus aquaticus. If the polymerase used was heat-susceptible, it would denature under the high temperatures of the denaturation step. Before the use of Taq polymerase, DNA polymerase had to be manually added every cycle, which was a tedious and costly process.

Applications of PCR technique include DNA cloning for sequencing, gene cloning and manipulation, gene mutagenesis; construction of DNA-based phylogenies, or functional analysis of genes; diagnosis and monitoring of hereditary diseases; amplification of ancient DNA; analysis of genetic fingerprints for DNA profiling (for example, in forensic science and parentage testing); and detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases.

Principles

PCR amplifies a specific region of a DNA strand (the DNA target). Most PCR methods amplify DNA fragments of between 0.1 and 10 kilo base pairs (kbp) in length, although some techniques allow for amplification of fragments up to 40 kbp. The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses.

A basic PCR set-up requires several components and reagents, including

- A DNA template that contains the DNA target region to amplify
- A DNA polymerase, an enzyme that polymerizes new DNA strands; heat-resistant Taq polymerase is especially common, as it is more likely to remain intact during the high-temperature DNA denaturation process
- Two DNA primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strands of the DNA target (DNA polymerase can only bind to and elongate from a double-stranded region of DNA; without primers there is no double-stranded initiation site at which the polymerase can bind); specific primers that are complementary to the DNA target region are selected beforehand, and are often custom-made in a laboratory or purchased from commercial biochemical suppliers
- Deoxynucleoside triphosphates, or dNTPs (sometimes called deoxynucleotide triphosphate; nucleotides containing triphosphate groups), the building blocks from which the DNA polymerase synthesizes a new DNA strand
- A buffer solution providing a suitable chemical environment for optimum activity and stability of the DNA polymerase
- Bivalent cations, typically magnesium (Mg) or manganese (Mn) ions; Mg^{2+} is the most common, but Mn^{2+} can be used for PCR-mediated DNA mutagenesis, as a higher Mn^{2+} concentration increases the error rate during DNA synthesis

Procedure

Typically, PCR consists of a series of 20–40 repeated temperature changes, called thermal cycles, with each cycle commonly consisting of two or three discrete temperature steps. The cycling is often preceded by a single temperature step at a very high temperature (>90 °C (194 °F)), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters, including the enzyme used for DNA synthesis, the concentration of bivalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers. The individual steps common to most PCR methods are as follows:

- Initialization: This step is only required for DNA polymerases that require heat activation by hot-start PCR. It consists of heating the reaction chamber to a temperature of 94–96 °C (201–205°F), or 98 °C (208°F) if extremely thermostable polymerases are used, which is then held for 1–10 minutes.
- Denaturation: This step is the first regular cycling event and consists of heating the reaction chamber to 94–98°C (201–208°F) for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.
- Annealing: In the next step, the reaction temperature is lowered to 50–65°C (122–149 °F) for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand.

It is critical to determine a proper temperature for the annealing step because efficiency and specificity are strongly affected by the annealing temperature. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should bind only to a perfectly complementary part of the strand, and nowhere else. If the temperature is too low, the primer may bind imperfectly. If it is too high, the primer may not bind at all. A typical annealing temperature is about 3–5°C below the Tm of the primers used. Stable hydrogen bonds between complementary bases are formed only when the primer sequence very closely matches the template sequence. During this step, the polymerase binds to the primer-template hybrid and begins DNA formation.

Extension/elongation: The temperature at this step depends on the DNA polymerase used; the optimum activity temperature for the thermostable DNA polymerase of Taq (Thermus aquaticus) polymerase is approximately 75–80 °C (167–176°F), though a temperature of 72 °C (162°F) is commonly used with this

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enzyme. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand.

The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is 2n, where n is the number of cycles. Thus, a reaction set for 30 cycles results in 230, or 1073741824, copies of the original double-stranded DNA target region.

- Final elongation: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.
- Final hold: The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and may be employed for short-term storage of the PCR products.

To check whether the PCR successfully generated the anticipated DNA target region (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis may be employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder, a molecular weight marker which contains DNA fragments of known size run on the gel alongside the PCR products.

Applications

Selective DNA Isolation

PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many ways, such as generating hybridization probes for Southern or northern hybridization and DNA cloning, which require larger amounts of DNA, representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material.

Other applications of PCR include DNA sequencing to determine unknown PCR-amplified sequences in which one of the amplification primers may be used in Sanger sequencing, isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid, phage, or cosmid (depending on size) or the genetic material of another organism. Bacterial colonies (such as, *E. coli*) can be rapidly screened by PCR for correct DNA vector constructs. PCR may also be used for genetic fingerprinting; a forensic

technique used to identify a person or organism by comparing experimental DNAs through different PCR-based methods.

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Amplification and Quantification of DNA

Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian tsar and the body of English King Richard III.

Quantitative PCR or Real Time Quantitative PCR (RT-qPCR) methods allow the estimation of the amount of a given sequence present in a sample—a technique often applied to quantitatively determine levels of gene expression. Quantitative PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

RT-qPCR allows the quantification and detection of a specific DNA sequence in real time since it measures concentration while the synthesis process is taking place. There are two methods for simultaneous detection and quantification. The first method consists of using fluorescent dyes that are retained non-specifically in between the double strands. The second method involves probes that code for specific sequences and are fluorescently labeled. Detection of DNA using these methods can only be seen after the hybridization of probes with its complementary DNA takes place.

Medical Applications

Prospective parents can be tested for being genetic carriers, or their children might be tested for actually being affected by a disease. DNA samples for prenatal testing can be obtained by amniocentesis, chorionic villus sampling, or even by the analysis of rare fetal cells circulating in the mother's bloodstream. PCR analysis is also essential to preimplantation genetic diagnosis, where individual cells of a developing embryo are tested for mutations.

PCR can also be used as part of a sensitive test for tissue typing, vital to organ transplantation. As of 2008, there is even a proposal to replace the traditional antibody-based tests for blood type with PCR-based tests.

Many forms of cancer involve alterations to oncogenes. By using PCR-based tests to study these mutations, therapy regimens can sometimes be individually customized to a patient. PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a

sensitivity that is at least 10,000 fold higher than that of other methods. PCR is very useful in the medical field since it allows for the isolation and amplification of tumor suppressors.

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Infectious Disease Applications

PCR allows for rapid and highly specific diagnosis of infectious diseases, including those caused by bacteria or viruses. PCR also permits identification of non-cultivatable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.

Characterization and detection of infectious disease organisms have been revolutionized by PCR in the following ways:

The human immunodeficiency virus (or HIV), is a difficult target to find and eradicate. The earliest tests for infection relied on the presence of antibodies to the virus circulating in the bloodstream. However, antibodies don't appear until many weeks after infection, maternal antibodies mask the infection of a newborn, and therapeutic agents to fight the infection don't affect the antibodies. PCR tests have been developed that can detect as little as one viral genome among the DNA of over 50,000 host cells. Infections can be detected earlier, donated blood can be screened directly for the virus, newborns can be immediately tested for infection, and the effects of antiviral treatments can be quantified.

Viral DNA can be detected by PCR. The primers used must be specific to the targeted sequences in the DNA of a virus, and PCR can be used for diagnostic analyses or DNA sequencing of the viral genome. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease. Such early detection may give physicians a significant lead time in treatment. The amount of virus ('viral load') in a patient can also be quantified by PCR-based DNA quantitation techniques.

Forensic Applications

The development of PCR-based genetic (or DNA) fingerprinting protocols has seen widespread application in forensics:

In its most discriminating form, genetic fingerprinting can uniquely discriminate any one person from the entire population of the world. Minute samples of DNA can be isolated from a crime scene, and compared to that from suspects, or from a DNA database of earlier evidence or convicts. Simpler versions of these tests are often used to rapidly rule out suspects during a criminal investigation. Evidence from decades-old crimes can be tested, confirming or exonerating the people originally convicted.

Forensic DNA typing has been an effective way of identifying or exonerating criminal suspects due to analysis of evidence discovered at a crime scene. The

human genome has many repetitive regions that can be found within gene sequences or in non-coding regions of the genome. Specifically, up to 40% of human DNA is repetitive. There are two distinct categories for these repetitive, non-coding regions in the genome. The first category is called variable number tandem repeats (VNTR), which are 10-100 base pairs long and the second category is called short tandem repeats (STR) and these consist of repeated 2-10 base pair sections. PCR is used to amplify several well-known VNTRs and STRs using primers that flank each of the repetitive regions. The sizes of the fragments obtained from any individual for each of the STRs will indicate which alleles are present. By analyzing several STRs for an individual, a set of alleles for each person will be found that statistically is likely to be unique. Researchers have identified the complete sequence of the human genome. This sequence can be easily accessed through the NCBI website and is used in many real-life applications.

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Research Applications

PCR allows rapid production of short pieces of DNA, even when not more than the sequence of the two primers is known. This ability of PCR augments many methods, such as generating hybridization probes for Southern or northern blot hybridization. PCR supplies these techniques with large amounts of pure DNA, sometimes as a single strand, enabling analysis even from very small amounts of starting material.

The task of DNA sequencing can also be assisted by PCR. Known segments of DNA can easily be produced from a patient with a genetic disease mutation. Modifications to the amplification technique can extract segments from a completely unknown genome, or can generate just a single strand of an area of interest.

PCR has numerous applications to the more traditional process of DNA cloning. It can extract segments for insertion into a vector from a larger genome, which may be only available in small quantities. Using a single set of 'vector primers', it can also analyze or extract fragments that have already been inserted into vectors. Some alterations to the PCR protocol can generate mutations (general or site-directed) of an inserted fragment.

Sequence-tagged sites is a process where PCR is used as an indicator that a particular segment of a genome is present in a particular clone. The Human Genome Project found this application vital to mapping the cosmid clones they were sequencing, and to coordinating the results from different laboratories.

A common application of PCR is the study of patterns of gene expression. Tissues (or even individual cells) can be analyzed at different stages to see which genes have become active, or which have been switched off. This application can also use quantitative PCR to quantitate the actual levels of expression

Advantages

PCR has a number of advantages. It is fairly simple to understand and to use, and produces results rapidly. The technique is highly sensitive with the potential to

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produce millions to billions of copies of a specific product for sequencing, cloning, and analysis. QRT-PCR shares the same advantages as the PCR, with an added advantage of quantification of the synthesized product. Therefore, it has its uses to analyze alterations of gene expression levels in tumors, microbes, or other disease states.

PCR is a very powerful and practical research tool. The sequencing of unknown etiologies of many diseases are being figured out by the PCR. The technique can help identify the sequence of previously unknown viruses related to those already known and thus give us a better understanding of the disease itself. If the procedure can be further simplified and sensitive non radiometric detection systems can be developed, the PCR will assume a prominent place in the clinical laboratory for years to come.

Limitations

One major limitation of PCR is that prior information about the target sequence is necessary in order to generate the primers that will allow its selective amplification. This means that, typically, PCR users must know the precise sequence(s) upstream of the target region on each of the two single-stranded templates in order to ensure that the DNA polymerase properly binds to the primer-template hybrids and subsequently generates the entire target region during DNA synthesis.

Like all enzymes, DNA polymerases are also prone to error, which in turn causes mutations in the PCR fragments that are generated.

Another limitation of PCR is that even the smallest amount of contaminating DNA can be amplified, resulting in misleading or ambiguous results. To minimize the chance of contamination, investigators should reserve separate rooms for reagent preparation, the PCR, and analysis of product. Reagents should be dispensed into single-use aliquots. Pipettors with disposable plungers and extra-long pipette tips should be routinely used.

PCR Analysis

Developed by Kary Mullis in 1983, a process was reported by which specific portions of the sample DNA can be amplified almost indefinitely (Saiki et al. 1985, 1985) The process, polymerase chain reaction (PCR), mimics the biological process of DNA replication, but confines it to specific DNA sequences of interest. With the invention of the PCR technique, DNA profiling took huge strides forward in both discriminating power and the ability to recover information from very small (or degraded) starting samples.

PCR greatly amplifies the amounts of a specific region of DNA. In the PCR process, the DNA sample is denatured into the separate individual polynucleotide strands through heating. Two oligonucleotide DNA primers are used to hybridize to two corresponding nearby sites on opposite DNA strands in such a fashion that the normal enzymatic extension of the active terminal of each primer (that is, the 3' end) leads toward the other primer. PCR uses replication enzymes that are tolerant

of high temperatures, such as the thermostable Taq polymerase. In this fashion, two new copies of the sequence of interest are generated. Repeated denaturation, hybridization, and extension in this fashion produce an exponentially growing number of copies of the DNA of interest. Instruments that perform thermal cycling are readily available from commercial sources. This process can produce a million-fold or greater amplification of the desired region in 2 hours or less.

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14.3.2 RFLP

In molecular biology, Restriction Fragment Length Polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated. In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis. Although now largely obsolete due to the rise of inexpensive DNA sequencing technologies, RFLP analysis was the first DNA profiling technique inexpensive enough to see widespread application. RFLP analysis was an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing.

Technology

The basic technique for the detection of RFLPs involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest. The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis, and transferred to a membrane via the Southern blot procedure. Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which are complementary to the probe. An RFLP occurs when the length of a detected fragment varies between individuals. Each fragment length is considered an allele, and can be used in genetic analysis.

Procedure

The first methods for finding out genetics used for DNA profiling involved RFLP analysis. DNA is collected from cells and cut into small pieces using a restriction enzyme (a restriction digest). This generates DNA fragments of differing sizes as a consequence of variations between DNA sequences of different individuals. The fragments are then separated on the basis of size using gel electrophoresis.

The separated fragments are then transferred to a nitrocellulose or nylon filter; this procedure is called a Southern blot. The DNA fragments within the blot are permanently fixed to the filter, and the DNA strands are denatured. Radiolabeled probe molecules are then added that are complementary to sequences in the genome that contain repeat sequences. These repeat sequences tend to vary in length among different individuals and are called variable number tandem repeat sequences or

VNTRs. The probe molecules hybridize to DNA fragments containing the repeat sequences and excess probe molecules are washed away. The blot is then exposed to an X-ray film. Fragments of DNA that have bound to the probe molecules appear as fluorescent bands on the film.

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Applications

Analysis of RFLP variation in genomes was a vital tool in genome mapping and genetic disease analysis. If researchers were trying to initially determine the chromosomal location of a particular disease gene, they would analyze the DNA of members of a family afflicted by the disease, and look for RFLP alleles that show a similar pattern of inheritance as that of the disease (see Genetic linkage). Once a disease gene was localized, RFLP analysis of other families could reveal who was at risk for the disease, or who was likely to be a carrier of the mutant genes.

RFLP analysis was also the basis for early methods of genetic fingerprinting, useful in the identification of samples retrieved from crime scenes, in the determination of paternity, and in the characterization of genetic diversity or breeding patterns in animal populations.

Alternatives

The technique for RFLP analysis is, however, slow and cumbersome. It requires a large amount of sample DNA, and the combined process of probe labeling, DNA fragmentation, electrophoresis, blotting, hybridization, washing, and autoradiography could take up to a month to complete. A limited version of the RFLP method that used oligonucleotide probes was reported in 1985. The results of the Human Genome Project have largely replaced the need for RFLP mapping, and the identification of many single-nucleotide polymorphisms (SNPs) in that project (as well as the direct identification of many disease genes and mutations) has replaced the need for RFLP disease linkage analysis. The analysis of VNTR alleles continues, but is now usually performed by polymerase chain reaction (PCR) methods. For example, the standard protocols for DNA fingerprinting involve PCR analysis of panels of more than a dozen VNTRs.

RFLP is still a technique used in marker assisted selection. Terminal restriction fragment length polymorphism (TRFLP or sometimes T-RFLP) is a molecular biology technique initially developed for characterizing bacterial communities in mixed-species samples. The technique has also been applied to other groups including soil fungi.

TRFLP works by PCR amplification of DNA using primer pairs that have been labeled with fluorescent tags. The PCR products are then digested using RFLP enzymes and the resulting patterns visualized using a DNA sequencer. The results are analyzed either by simply counting and comparing bands or peaks in the TRFLP profile, or by matching bands from one or more TRFLP runs to a database of known species. The technique is similar in some aspects to DGGE or TGGE.

RFLP Analysis

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14.3.3 RAPD

RAPD (pronounced as 'rapid') stands for 'Random Amplification of Polymorphic DNA'. It is a type of PCR, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8–12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from an RAPD reaction.

No knowledge of the DNA sequence of the targeted genome is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared (it is not suitable for forming a cDNA databank). Because it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species-specific DNA comparison methods, such as short tandem repeats. In recent years, RAPD has been used to characterize, and trace, the phylogeny of diverse plant and animal species.

Procedure

RAPD markers are decamer (10 nucleotides long) DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way. It is used to analyse the genetic diversity of an individual by using random primers. Due to problems in experiment reproducibility, many scientific journals do not accept experiments

merely based on RAPDs anymore. RAPD requires only one primer for amplification.

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Unlike traditional PCR analysis, RAPD does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. For example, no fragment is produced if primers annealed too far apart or 3' ends of the primers are not facing each other. Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel.

Limitations

- Nearly all RAPD markers are dominant, i.e., it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Codominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely.
- PCR is an enzymatic reaction, therefore, the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome. Thus, the RAPD technique is notoriously laboratory dependent and needs carefully developed laboratory protocols to be reproducible.
- Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product. Thus, the RAPD results can be difficult to interpret.

Developing Locus-specific, Co-dominant Markers from RAPDs

- The polymorphic RAPD marker band is isolated from the gel.
- It is amplified in the PCR reaction.
- The PCR product is cloned and sequenced.
- New longer and specific primers are designed for the DNA sequence, which is called the Sequenced Characterized Amplified Region Marker (SCAR).

14.3.4 AFLP

AFLP-PCR or just AFLP is a PCR-based tool used in genetics research, DNA fingerprinting, and in the practice of genetic engineering. Developed in the early 1990s by Keygene, AFLP uses restriction enzymes to digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments is then selected to be amplified. This selection is achieved by using primers complementary to the adaptor sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments. The amplified fragments are separated and visualized on denaturing on agarose gel electrophoresis,

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either through autoradiography or fluorescence methodologies, or via automated capillary sequencing instruments.

Although AFLP is commonly referred to as 'Amplified Fragment Length Polymorphism', the resulting data are not scored as length polymorphisms, but instead as presence-absence polymorphisms.

AFLP-PCR is a highly sensitive method for detecting polymorphisms in DNA. The technique was originally described by Vos and Zabeau in 1993. In detail, the procedure of this technique is divided into three steps:

1. Digestion of total cellular DNA with one or more restriction enzymes and ligation of restriction half-site specific adaptors to all restriction fragments.
2. Selective amplification of some of these fragments with two PCR primers that have corresponding adaptor and restriction site specific sequences.
3. Electrophoretic separation of amplicons on a gel matrix, followed by visualisation of the band pattern.

Applications

The AFLP technology has the capability to detect various polymorphisms in different genomic regions simultaneously. It is also highly sensitive and reproducible. As a result, AFLP has become widely used for the identification of genetic variation in strains or closely related species of plants, fungi, animals, and bacteria. The AFLP technology has been used in criminal and paternity tests, also to determine slight differences within populations, and in linkage studies to generate maps for Quantitative Trait Locus (QTL) analysis.

There are many advantages to AFLP when compared to other marker technologies including Randomly Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), and microsatellites. AFLP not only has higher reproducibility, resolution, and sensitivity at the whole genome level compared to other techniques, but it also has the capability to amplify between 50 and 100 fragments at one time. In addition, no prior sequence information is needed for amplification (Meudt & Clarke 2007). As a result, AFLP has become extremely beneficial in the study of taxa including bacteria, fungi, and plants, where much is still unknown about the genomic makeup of various organisms.

14.3.5 DNA Fingerprinting

DNA profiling (also called DNA fingerprinting) is the process of determining an individual's DNA characteristics, which are as unique as fingerprints. DNA analysis intended to identify a species, rather than an individual, is called DNA barcoding.

DNA profiling is a forensic technique in criminal investigations, comparing criminal suspects' profiles to DNA evidence so as to assess the likelihood of their involvement in the crime. It is also used in parentage testing, to establish immigration eligibility, and in genealogical and medical research. DNA profiling has also been

used in the study of animal and plant populations in the fields of zoology, botany, and agriculture.

Procedure

The process begins with a sample of an individual's DNA (typically called a reference sample). Reference samples are usually collected through a buccal swab. When this is unavailable (for example, when a court order is needed but unobtainable) other methods may be needed to collect a sample of blood, saliva, semen, vaginal lubrication, or other fluid or tissue from personal use items (for example, a toothbrush, razor) or from stored samples (for example, banked sperm or biopsy tissue). Samples obtained from blood relatives can indicate an individual's profile, as could previous profiled human remains. A reference sample is then analyzed to create the individual's DNA profile using one of the techniques discussed below. The DNA profile is then compared against another sample to determine whether there is a genetic match.

DNA Extraction

When a sample such as blood or saliva is obtained, the DNA is only a small part of what is present in the sample. Before the DNA can be analyzed, it must be extracted from the cells and purified. There are many ways this can be accomplished, but all methods follow the same basic procedure. The cell and nuclear membranes need to be broken up to allow the DNA to be free in solution. Once the DNA is free, it can be separated from all other cellular components. After the DNA has been separated in solution, the remaining cellular debris can then be removed from the solution and discarded, leaving only DNA. The most common methods of DNA extraction include organic extraction (also called phenol chloroform extraction), Chelex extraction, and solid phase extraction. Differential extraction is a modified version of extraction in which DNA from two different types of cells can be separated from each other before being purified from the solution.

DNA Family Relationship Analysis

Using PCR technology, DNA analysis is widely applied to determine genetic family relationships such as paternity, maternity, siblingship and other kinships.

During conception, the father's sperm cell and the mother's egg cell, each containing half the amount of DNA found in other body cells, meet and fuse to form a fertilized egg, called a zygote. The zygote contains a complete set of DNA molecules, a unique combination of DNA from both parents. This zygote divides and multiplies into an embryo and later, a full human being.

At each stage of development, all the cells forming the body contain the same DNA—half from the father and half from the mother. This fact allows the relationship testing to use all types of all samples including loose cells from the cheeks collected using buccal swabs, blood or other types of samples.

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There are predictable inheritance patterns at certain locations (called loci) in the human genome, which have been found to be useful in determining identity and biological relationships. These loci contain specific DNA markers that scientists use to identify individuals. In a routine DNA paternity test, the markers used are short tandem repeats (STRs), short pieces of DNA that occur in highly differential repeat patterns among individuals.

Each person's DNA contains two copies of these markers—one copy inherited from the father and one from the mother. Within a population, the markers at each person's DNA location could differ in length and sometimes sequence, depending on the markers inherited from the parents.

The combination of marker sizes found in each person makes up his/her unique genetic profile. When determining the relationship between two individuals, their genetic profiles are compared to see if they share the same inheritance patterns at a statistically conclusive rate.

Y-chromosome Analysis

Recent innovations have included the creation of primers targeting polymorphic regions on the Y-chromosome (Y-STR), which allows resolution of a mixed DNA sample from a male and female or cases in which a differential extraction is not possible. Y-chromosomes are paternally inherited, so Y-STR analysis can help in the identification of paternally related males. Y-STR analysis was performed in the Sally Hemings controversy to determine if Thomas Jefferson had sired a son with one of his slaves. The analysis of the Y-chromosome yields weaker results than autosomal chromosome analysis. The Y male sex-determining chromosome, as it is inherited only by males from their fathers, is almost identical along the patrilineal line. This leads to a less precise analysis than if autosomal chromosomes were testing, because of the random matching that occurs between pairs of chromosomes as zygotes are being made.

Mitochondrial Analysis

For highly degraded samples, it is sometimes impossible to get a complete profile of the 13 CODIS STRs. In these situations, mitochondrial DNA (mtDNA) is sometimes typed due to there being many copies of mtDNA in a cell, while there may only be 1-2 copies of the nuclear DNA. Forensic scientists amplify the HV1 and HV2 regions of the mtDNA, and then sequence each region and compare single-nucleotide differences to a reference. Because mtDNA is maternally inherited, directly linked maternal relatives can be used as match references, such as one's maternal grandmother's daughter's son. In general, a difference of two or more nucleotides is considered to be an exclusion. Heteroplasmy and poly-C differences may throw off straight sequence comparisons, so some expertise on the part of the analyst is required. mtDNA is useful in determining clear identities, such as those of missing people when a maternally linked relative can be found. mtDNA

testing was used in determining that Anna Anderson was not the Russian princess she had claimed to be, Anastasia Romanov.

NOTES

mtDNA can be obtained from such material as hair shafts and old bones/ teeth. Control mechanism based on interaction point with data. This can be determined by tooled placement in sample.

Check Your Progress

7. What is PCR?
8. Who developed PCR?
9. What are few common applications of PCR?
10. What is initialization?
11. What is denaturation?
12. What does final hold mean?

14.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Blotting is technique in which nucleic acids, i.e., RNA and DNA or proteins are transferred onto a specific membrane. This membrane may be nitrocellulose PVDF or nylon membrane. This process can be done just after the gel electrophoresis, by transferring the molecules from the gel onto the surface of blotting membrane.
2. Blotting is a common technique which is widely used in the field of molecular biology.
3. There are basically following 4 types of blotting:
   - Southern Blotting
   - Western Blotting
   - Northern Blotting
   - Eastern Blotting
4. A Southern blot is a method used for detection of a specific DNA sequence in DNA samples.
5. Northern blotting, the name was extrapolated from Southern blotting. The northern blot is a technique used to study gene expression by detection of RNA (or isolated mRNA) in a sample.
6. Identification of a specific protein in a complex mixture of proteins can be done by a technique known as western blotting. Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin, et al. in 1979 and is now a routine technique for protein analysis.

NOTES

7. Polymerase Chain Reaction (PCR) is a method widely used in molecular biology to make many copies of a specific DNA segment.
8. PCR was developed by Kary Mullis in 1983 while he was an employee of the Cetus Corporation. He was awarded the Nobel Prize in Chemistry in 1993 (along with Michael Smith) for his work in developing the method.
9. Applications of PCR technique include DNA cloning for sequencing, gene cloning and manipulation, gene mutagenesis; construction of DNA-based phylogenies, or functional analysis of genes; diagnosis and monitoring of hereditary diseases; amplification of ancient DNA; analysis of genetic fingerprints for DNA profiling (for example, in forensic science and parentage testing); and detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases.
10. Initialization is the step which is only required for DNA polymerases that require heat activation by hot-start PCR. It consists of heating the reaction chamber to a temperature of 94–96 °C (201–205°F), or 98 °C (208°F) if extremely thermostable polymerases are used, which is then held for 1–10 minutes.
11. Denaturation is the first step that regular cycling event and consists of heating the reaction chamber to 94–98°C (201–208°F) for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.
12. Final hold is the final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and may be employed for short-term storage of the PCR products.

14.5 SUMMARY

- Blotting is a common technique which is widely used in the field of molecular biology.
- These methods such as southern, western, northern and eastern are applicable for different types of macromolecules like lipids, RNA, DNA and proteins.
- Each technique depends upon the following factors such as the size of molecule and their binding ability to the solid support. Finally, by using probe we have to detect the molecule of interest.
- Blotting is technique in which nucleic acids, i.e., RNA and DNA or proteins are transferred onto a specific membrane. This membrane may be nitrocellulose PVDF or nylon membrane. This process can be done just after the gel electrophoresis, by transferring the molecules from the gel onto the surface of blotting membrane.

NOTES

- A Southern blot is a method used for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization. The method is named after its inventor, the British biologist Edwin Southern.
- If the Southern blot contains genomic DNA fragments from the whole genome, the probe will give information about the size of the fragment the gene is on the genome and how many copies of the gene are present in the genome.
- Northern blotting, the name was extrapolated from Southern blotting. The northern blot is a technique used to study gene expression by detection of RNA (or isolated mRNA) in a sample.
- The nucleic acid molecules (RNA samples) are separated by agarose gel electrophoresis and then transferred to a nitrocellulose membrane but for RNA in Northern blotting, alkali denaturation is not necessary and would in any case hydrolyze the molecules.
- In capillary transfer, the membrane is placed on top of the gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. But this method of transfer is very time consuming.
- Polymerase Chain Reaction (PCR) is a method widely used in molecular biology to make many copies of a specific DNA segment. Using PCR, a single copy (or more) of a DNA sequence is exponentially amplified to generate thousands to millions of more copies of that particular DNA segment.
- PCR was developed by Kary Mullis in 1983 while he was an employee of the Cetus Corporation. He was awarded the Nobel Prize in Chemistry in 1993 (along with Michael Smith) for his work in developing the method.
- In molecular biology, Restriction Fragment Length Polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated.
- In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis.
- RAPD (pronounced as rapid) stands for 'Random Amplification of Polymorphic DNA'. It is a type of PCR, but the segments of DNA that are amplified are random.
- AFLP-PCR or just AFLP is a PCR-based tool used in genetics research, DNA fingerprinting, and in the practice of genetic engineering. Developed

in the early 1990s by Keygene, AFLP uses restriction enzymes to digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments.

- DNA profiling (also called DNA fingerprinting) is the process of determining an individual's DNA characteristics, which are as unique as fingerprints. DNA analysis intended to identify a species, rather than an individual, is called DNA barcoding.
- At each stage of development, all the cells forming the body contain the same DNA—half from the father and half from the mother. This fact allows the relationship testing to use all types of all samples including loose cells from the cheeks collected using buccal swabs, blood or other types of samples.

14.6 KEY WORDS

- **Cosmid**: A cosmid is a type of hybrid plasmid that contains a Lambda phage cos sequence. Cosmids (cos sites + plasmid = cosmids) DNA sequences are originally from the lambda phage.
- **Polyvinylidene**: Polyvinylidene fluoride or polyvinylidene difluoride (PVDF) is a highly non-reactive thermoplastic fluoropolymer produced by the polymerization of vinylidene difluoride.
- **Electroelution**: Electroelution is a method used to extract a nucleic acid or a protein sample from an electrophoresis gel by applying a negative current in the plane of the smallest dimension of the gel, drawing the macromolecule to the surface for extraction and subsequent analysis.
- **Chemiluminescence**: Chemiluminescence is the production of light from a chemical reaction.
- **Deoxynucleotide triphosphates**: Deoxynucleotide triphosphates (dNTPs) are the essential building blocks of nucleic acid molecules, and as such are necessary components of PCR mixes as no new (amplified) DNA could be generated without them.
- **Amplimer**: Amplimer is a piece of DNA formed as the products of natural or artificial amplification events, as in a polymerase chain reaction.
- **Amplicon:** Amplicon is a piece of DNA or RNA formed as the products of natural or artificial amplification events, as in a polymerase chain reaction.
- **Polymerase chain reaction:** Polymerase Chain Reaction (PCR) is a method widely used in molecular biology to make many copies of a specific DNA segment.
- **Taq polymerase**: Taq polymerase is a thermostable DNA polymerase named after the thermophilic bacterium Thermus aquaticus from which it was originally isolated by Chien *et al.* in 1976.

NOTES

- **Minisatellite**: A minisatellite is a tract of repetitive DNA in which certain DNA motifs (ranging in length from 10–60 base pairs) are typically repeated 5-50 times.
- **Mitochondrial DNA:** Mitochondrial DNA (mtDNA or mDNA) is the DNA located in mitochondria, cellular organelles within eukaryotic cells that convert chemical energy from food into a form that cells can use, adenosine triphosphate (ATP).
- **Heteroplasmy**: Heteroplasmy is the presence of more than one type of organellar genome (mitochondrial DNA or plastid DNA) within a cell or individual.

14.7 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short Answer Questions

1. Write a short note on blotting and giving a brief description about its types.
2. What are the applications of northern blotting?
3. Write a short note on incubation with the primary antibody.
4. What are the principles of PCR?
5. What are infectious diseases applications of PCR?
6. What are the alternatives of RFLP?
7. Brief about Y-chromosome analysis.

Long Answer Questions

1. What is blotting and various blotting techniques?
2. Explain in detail about northern, western and southern blotting.
3. Discuss about PCR in detail giving its applications, principle, procedure and analysis.
4. What is RFLP? Explain bout RFLP mentioning its procedure, application and principle.
5. Explain about RAPD in detail.
6. What is AFLP? Discuss in detail.
7. Write a note on DNA fingerprinting.

NOTES

14.8 FURTHER READINGS

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