| Semester | Course | Hours | Credit | Sub. Code | Marks | | |
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## ANALYTICAL TECHNIQUES IN PLANT SCIENCES

**UNIT I: IMAGING AND RELATED TECHNIQUES**

Principles and application of microscopy; Light microscopy; Fluorescence microscopy; Confocal microscopy; Transmission and Scanning electron microscopy – sample preparation and staining techniques.

**UNIT II: CELL FRACTIONATION AND RADIOISOTOPES**

Introduction, Basic Principle of Sedimentation, components and different types of centrifuges - Differential and density gradient centrifugation, analytical centrifugation, ultracentrifugation. Basic concept of radio isotope, GM and scintillation counter, autoradiography, Applications in biological science.

**UNIT III:** CHROMATOGRAPHY

Basic principle and biological applications. Paper chromatography; Column chromatography, TLC, GLC, HPTLC, Ion-exchange chromatography; Size exclusion chromatography; Affinity chromatography.

**UNIT IV:** SPECTROPHOTOMETRY

Properties of Electromagnetic radiations; Beer Lambert's Law, Extinction Coefficient, Principle and Applications of UV-Visible light Spectroscopy. Atomic absorption and Flame emission spectroscopic techniques. Mass spectrometry: X-ray diffraction; X-ray crystallography; Principle & biological applications of IR & NMR.

**UNIT V:** ELECTROPHORESIS

Characterization of proteins and nucleic acids; Electrophoresis: AGE, PAGE, SDS-PAGE. Immunoelectrophoresis, Isoelectrofocussing, Capillary Electrophoresis, Polymerase Chain Reaction, DNA sequencing.

**Source of Study Material**


1. Ruzin, S.E. (1999). Plant Microtechnique and Microscopy, Oxford University Press, New York. U.S.A.
2. Willard, H.H., Merritt L.L. Dean J.A. and Settle F.A., "Instrumental Methods of Analysis", 7th Ed., Wadsworth Publishing Co., 1986.
3. Campbell, I.D. and Dwek, R. A., "Biological Spectroscopy", Benjamin Curmmings Publication Co. Inc., 1984.
4. Glasel, J. and Deutscher, M. B., "Introduction to Biophysical Methods for Protein and Nucleic acid Research", Academic Press, 1995.


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## UNIT I: IMAGING AND RELATED TECHNIQUES

### 1.Light microscope

- A light microscope is a biology laboratory instrument or tool, that uses visible light to detect and magnify very small objects, and enlarging them.
- They use lenses to focus light on the specimen, magnifying it thus producing an image. The specimen is normally placed close to the microscopic lens.
- Microscopic magnification varies greatly depending on the types and number of lenses that make up the microscope. Depending on the number of lenses, there are two types of microscopes i. e Simple light microscope (it has low magnification because it uses a single lens) and the Compound light microscope (it has a higher magnification compared to the simple microscope because it uses at least two sets of lenses, an objective lens, and an eyepiece). The lenses are aligned in that, they can be able to bend light for efficient magnification of the image.
- The functioning of the light microscope is based on its ability to focus a beam of light through a specimen, which is very small and transparent, to produce an image. The image is then passed through one or two lenses for magnification for viewing. The transparency of the specimen allows easy and quick penetration of light. Specimens can vary from bacterial to cells and other microbial particles.

Figure: Diagram of Light Microscopes,

Principle of a light microscope(optical micrope)

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

A medium with a lower refractive index such as glass to air, it normally speeds up the light penetration and making light bend away from the normal and when light is passed through a medium with a greater refractive index such as air to glass, it normally slows down and bends towards the normal, perpendicularly to the surface.

If an object is put between these two mediums i.e between water and air, in this case, a prism, the prism will bend the light at an angle. This is how the microscopic lenses work, they bend the light at an angle. The lens (convex) on receiving the light rays, it focuses the rays at a specific point known as the **focal point (F-point)**. The measure of distance from the center of the lens and the focal point is known as the **focal length.**

A microscope uses lenses whose strength is predetermined, in that, the strength of a lens is directly related to the focal length i.e short focal length magnifies objects more than lenses with a long focal length.

Microscopy works strictly with a factor of resolution whereby resolution being the ability of a lens to be able to differentiate small objects that are closely packed together. The resolution of a light microscope is determined by a **numerical aperture** of its lens system and by the wavelength of the light it employs; a numerical aperture a definition of the light wavelengths produced when the specimen is illuminated.

A minimum distance (d) between two objects that distinguishes then to be two separate entities, determined by the wavelengths of the light can be calculated by an Abbe equation using the wavelength of the light that illuminated the specimen (Lambda, **λ**) and the numerical aperture (NA, n sin Θ) i.e. **$d=0.5\ \lambda/n \sin \Theta$**

### Applications in Light Microscopy

Light microscopy has a number of applications in different sectors including in gemmology, metallurgy and chemistry. In terms of biology, it is one of the least invasive techniques for looking at living cells.

The light microscope can be used to provide information about the activity of cells and to look at very small structures such as nanostructures.

Different adaptations can help to enhance images, such as phase contrast microscopy, which provides contrast between cells and the solution they are in. High resolution 3D imaging can also be used to observe organisms over a period of time.

Fluorescence microscopy is also a good technique for observing specimens that fluoresce and emit light of a different colour. The number of fluorescent proteins has increased, expanding the kinds of sample types that can be looked at, from single molecules to whole organisms.

In addition, unwanted side effects have been reduced. However, one limitation of fluorescence microscopy is the overlap of fluorophores, which can make analysis more difficult.

**Molecular imaging**

Microscopy can be used to explore the time- and space-related dynamics of molecules. Localizing single molecules such as RNA and proteins provides insights into how cells and tissues are organized at the molecular level.

**Cell imaging**

- High content screening (HCS) uses microscopy to identify and study substances in cells such as peptides, RNA and small molecules. Automated microscopes can be used to look at thousands of compounds or genetic alterations and the effects these have.
- Information about the structure, heterogeneity, kinetics and more can be obtained from the resulting images.
- HCS can be used by biotechnology and pharmaceutical companies to screen for potential drug candidates. It allows researchers to consider and rule out a number of different molecules in a short period of time.
- This method can also be used to look at genes to find out more about the genome and potentially identify sequences that alter cell phenotype and lead to different diseases.
- The process allows for fast analysis of the genome and can identify molecules that have effects on the majority of the 21,000 gene products found in cells.

**Fluorescence microscope**

**Definition**

- A **fluorescence microscope** is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances.
- Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation while phosphorescence is a specific type of photoluminescence related to fluorescence.
- Unlike fluorescence, a phosphorescent material does not immediately re-emit the radiation it absorbs.
- The fluorescence microscope was devised in the early part of the twentieth century by August Köhler, Carl Reichert, and Heinrich Lehmann, among others.

**Principle of Fluorescence microscopy**

1. Most cellular components are colorless and cannot be clearly distinguished under a microscope. The basic premise of fluorescence microscopy is to stain the components with dyes.
2. Fluorescent dyes, also known as fluorophores or fluorochromes, are molecules that absorb excitation light at a given wavelength (generally UV), and after a short delay emit light at a longer wavelength. The delay between absorption and emission is negligible, generally on the order of nanoseconds.
3. The emission light can then be filtered from the excitation light to reveal the location of the fluorophores.

- Fluorescence microscopy uses a much higher intensity light to illuminate the sample. This light excites fluorescence species in the sample, which then emit light of a longer wavelength.
- The image produced is based on the second light source or the emission wavelength of the fluorescent species rather than from the light originally used to illuminate, and excite, the sample.

**Working**

Light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused on the detector by the objective. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light.

**Forms**

The "fluorescence microscope" refers to any microscope that uses fluorescence to generate an image, whether it is a more simple set up like an epifluorescence microscope, or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescent image.

Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective).

**Typical components of a fluorescence microscope are:**

**Fluorescent dyes (Fluorophore)**

- A fluorophore is a fluorescent chemical compound that can re-emit light upon light excitation.
- Fluorophores typically contain several combined aromatic groups, or plane or cyclic molecules with several π bonds.
- Many fluorescent stains have been designed for a range of biological molecules.
- Some of these are small molecules that are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst, phalloidin which is used to stain actin fibers in mammalian cells.

**A light source**

- Four main types of light sources are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, and high- power LEDs.
- Lasers are mostly used for complex fluorescence microscopy techniques, while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide-field epifluorescence microscopes.

**The excitation filter**

- The exciter is typically a bandpass filter that passes only the wavelengths absorbed by the fluorophore, thus minimizing the excitation of other sources of fluorescence and blocking excitation light in the fluorescence emission band.

**The dichroic mirror**

- A dichroic filter or thin-film filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors.

**The emission filter.**

- The emitter is typically a bandpass filter that passes only the wavelengths emitted by the fluorophore and blocks all undesired light outside this band – especially the excitation light.
- By blocking unwanted excitation energy (including UV and IR) or sample and system autofluorescence, optical filters ensure the darkest background.

**Applications of Fluorescence Microscope**

- To identify structures in fixed and live biological samples.
- Fluorescence microscopy is a common tool for today's life science research because it allows the use of multicolor staining, labeling of structures within cells, and the measurement of the physiological state of a cell.

**Advantages of Fluorescence Microscope**

1. Fluorescence microscopy is the most popular method for studying the dynamic behavior exhibited in live-cell imaging.

2. This stems from its ability to isolate individual proteins with a high degree of specificity amidst non-fluorescing material.
3. The sensitivity is high enough to detect as few as 50 molecules per cubic micrometer.
4. Different molecules can now be stained with different colors, allowing multiple types of the molecule to be tracked simultaneously.
5. These factors combine to give fluorescence microscopy a clear advantage over other optical imaging techniques, for both in vitro and in vivo imaging.
   Confocal microscopy**.**

**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.

Capturing multiple two-dimensional images at different depths in a sample enables the reconstruction of three-dimensional structures (a process known as optical sectioning) within an object. This technique is used extensively in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.
smaller beam of light at one narrow depth level at a time. The CLSM achieves a controlled Light travels through the sample under a conventional microscope as far into the specimen as it can penetrate, while a confocal microscope only focuses a and highly limited depth of focus.

The principle of confocal imaging was patented in 1957 by Marvin Minsky[2] and aims to overcome some limitations of traditional wide-field fluorescence microscopes.[3] In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded evenly in light from a light source. All parts of the sample can be excited at the same time and the resulting fluorescence is detected by the microscope's photodetector or camera including a large unfocused background part.

In contrast, a confocal microscope uses point illumination (see Point Spread Function) and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal – the name "confocal" stems from this configuration. As only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution, particularly in the sample depth direction, is much better than that of wide-field microscopes.

However, as much of the light from sample fluorescence is blocked at the pinhole, this increased resolution is at the cost of decreased signal intensity – so long exposures are often required. To offset this drop in signal after the *pinhole*, the light intensity is detected by a sensitive detector, usually a photomultiplier tube (PMT) or avalanche photodiode, transforming the light signal into an electrical one.[4]

As only one point in the sample is illuminated at a time, 2D or 3D imaging requires scanning over a regular raster (i.e. a rectangular pattern of parallel scanning lines) in the specimen. The beam is scanned across the sample in the horizontal plane by using one or more (servo controlled) oscillating mirrors. This scanning method usually has a low reaction latency and the scan speed can be varied. Slower scans provide a better signal-to-noise ratio, resulting in better contrast.

The achievable thickness of the focal plane is defined mostly by the wavelength of the used light divided by the numerical aperture of the objective lens, but also by the optical properties of the specimen. The thin optical sectioning possible makes these types of microscopes particularly good at 3D imaging and surface profiling of samples.
Successive slices make up a 'z-stack', which can either be processed to create a 3D image, or it is merged into a 2D stack (predominately the maximum pixel intensity is taken, other common methods include using the standard deviation or summing the pixels).[1]

Confocal microscopy provides the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation as well as a marginal improvement in lateral resolution compared to wide-field microscopy.

Biological samples are often treated with fluorescent dyes to make selected objects visible. However, the actual dye concentration can be low to minimize the disturbance of biological systems: some instruments can track single fluorescent molecules. Also, transgenic techniques can create

organisms that produce their own fluorescent chimeric molecules (such as a fusion of GFP, green fluorescent protein with the protein of interest).

Confocal microscopes work on the principle of point excitation in the specimen (diffraction limited spot) and point detection of the resulting fluorescent signal. A pinhole at the detector provides a physical barrier that blocks out-of-focus fluorescence.

Only the in-focus, or central spot of the Airy disk, is recorded. Raster scanning the specimen one point at a time permits thin optical sections to be collected by simply changing the z-focus. The resulting images can be stacked to produce a 3D image of the specimen.

**Scanning transmission electron microscopy**

SEM can stand for either Scanning Electron Microscopy or Scanning Electron Microscope. An SEM is a kind of electron microscope that uses a fine beam of focused electrons to scan a sample's surface. The microscope records information about the interaction between the electrons and the sample, creating a magnified image. SEM has the potential to magnify an image up to 2 million times.

SEM images give insight into a sample's topography and elemental composition. SEM is able to capture 3-D black-and-white images of thin or thick samples. The sample's size is limited only by the size of the electron microscope chamber.

How does SEM work?

To obtain a high-resolution image, an electron source (also known as an electron gun) emits a stream of high-energy electrons towards a sample. The electron beam is focused using electromagnetic lenses. Once the focused stream reaches the sample, it scans its surface in a rectangular. The interaction between the electron beam and the sample creates secondary electrons, backscattered electrons, and X-rays. These interactions are captured to create

A **scanning transmission electron microscope** (**STEM**) is a type of transmission electron microscope (TEM). Pronunciation is [stɛm] or [ɛsti:i:ɛm]. As with a conventional transmission electron microscope (CTEM), images are formed by electrons passing through a sufficiently thin specimen.

However, unlike CTEM, in STEM the electron beam is focused to a fine spot (with the typical spot size 0.05 – 0.2 nm) which is then scanned over the sample in a raster illumination system constructed so that the sample is illuminated at each point with the beam parallel to the optical axis.

The rastering of the beam across the sample makes STEM suitable for analytical techniques such as Z-contrast annular dark-field imaging, and spectroscopic mapping by energy dispersive X-ray (EDX) spectroscopy, or electron energy loss spectroscopy (EELS). These signals can be obtained simultaneously, allowing direct correlation of images and spectroscopic data.

A typical STEM is a conventional transmission electron microscope equipped with additional scanning coils, detectors and necessary circuitry, which allows it to switch between operating as a STEM, or a CTEM; however, dedicated STEMs are also manufactured.

High resolution scanning transmission electron microscopes require exceptionally stable room environments. In order to obtain atomic resolution images in STEM, the level of vibration, temperature fluctuations, electromagnetic waves, and acoustic waves must be limited in the room housing the microscope.[1]

**Applications**

Scanning transmission electron microscopes are used to characterize the nanoscale, and atomic scale structure of specimens, providing important insights into the properties and behaviour of materials and biological cells.

Materials science

Scanning transmission electron microscopy has been applied to characterize the structure of a wide range of material specimens, including solar cells,[12] semiconductor devices, complex oxides, batteries, fuel cells,catalysts,and 2D materials.

Biology

The first application of STEM to the imaging of biological molecules was demonstrated in 1971. The advantage of STEM imaging of biological samples is the high contrast of annular dark-field images, which can allow imaging of biological samples without the need for staining. STEM has been widely used to solve a number of structural problems in molecular biology.

**The transmission electron microscope (TEM)**

- The transmission electron microscope is used to view thin specimens through which electrons can pass generating a projection image.
- The TEM is analogous in many ways to the conventional (compound) light microscope.
- TEM is used, among other things, to image the interior of cells (in thin sections), the structure of protein molecules (contrasted by metal shadowing), the organization of molecules in viruses and cytoskeletal filaments (prepared by the negative staining technique), and the arrangement of protein molecules in cell membranes (by freeze-fracture).

**What is TEM?**

TEM can stand for Transmission Electron Microscopy or Transmission Electron Microscope (TEM). A TEM is a type of electron microscope that uses a broad beam of electrons to create an image of a sample's internal structure. A beam of electrons is transmitted through a sample, creating an image that details a sample's morphology, composition, and crystal structure. Samples must be incredibly thin, often less than 150 nm thick, to allow electrons to pass through them. After the transmission of the electrons through the sample, they arrive at a detector below and a 2-D image is created.

TEMs have an incredible magnification potential of 10-50 million times. The details provided are at the atomic level, the highest resolution of any electron microscope. TEMs are often used to examine molecular and cellular structures.

How does a TEM work?

An electron source sends a beam of electrons through an ultrathin sample. When the electrons penetrate the sample, they pass through lenses below. This data is used to create images directly on a fluorescent screen or onto a computer screen using a charge-coupled device (CCD) camera.

SEM and TEM are both valuable tools in the biological, physical, and chemical sciences. By understanding the differences between these two electron microscopes, scientists can choose the correct type of microscope for their needs.TEM advantages

- Cost less
- Take less time to create an image
- Require less sample preparation
- Accept thicker samples

**Sample Preparation**

**Histology sample preparation** prepares tissue specimens for sectioning, staining and diagnosis. The standard paraffin process (tissue processing) moves specimens through a series of steps so the soft tissue is supported in a medium that allows sectioning.

The standard steps are: **Fixation** that preserves the tissue, **Processing** that dehydrates, clears and infiltrates the tissue with paraffin wax, **Embedding** that allows orientation of the specimen in a "block" that can be sectioned and is easy to store and handle, and **Sectioning** using a microtome to produce very thin sections that are placed on a microscope slide ready for staining.

Frozen sectioning is an alternative preparation technique that quickly freezes tissue to preserve it and provide sufficient hardness so it can be sectioned immediately using a cryostat. This technique is often used during surgery where the surgeon needs to locate a tumor margin to ensure it has all been removed.

**Staining techniques**

There are a number of staining techniques which have been developed over many years for the accurate imaging of specific organisms and cellular structures. Here, we will provide descriptions of the most common and useful. References 1 and 3 provide excellent and exhaustive lists of available stains.

**Simple Stain**

This is a technique that only uses a single stain, and is useful for simply visualizing cells, to determine characteristics like size, shape, and count. In this method, the stain has a basic functionality, causing it to become positively charged in a neutral pH medium. Most cells acquire a negative charge under similar conditions, so the stain selectively attaches to the cell surface.

Examples of stains that can be used for this technique include crystal violet, safranin, and methylene blue.

**Negative Stain**

A negative stain works in the opposite way. These stains are acidic, and therefore become negatively charged at a neutral pH. Instead of staining the cells, they are repelled from the cells and attach and stain the rest of the specimen. When using a negative stain, cells appear bright on a dark background.

**Gram Stain**

Gram staining is a more complex, multi-step technique, but is a powerful method for classifying bacteria. The technique involves exposing the cells to a primary stain, crystal violet, then exposing them to an iodine solution, which forms a complex with the stain. All cells will take up crystal violet and form this complex.

Next, the specimen is exposed to a *decolorizer*, which is either ethanol, or a mixture of acetone and ethanol. In some cells, called 'gram negative' cells, the decolorizer will dissolve the stain-iodine complex and carry it out of the cell. In 'gram positive' cells, the complex cannot pass back through the cell wall, and is trapped inside the cell.

Finally, a second stain is added to re-stain the gram negative cells, typically safranin or carbol fuchsin.

The second stain will also enter gram-positive cells, but it is lighter in color so the staining in those cells is dominated by the darker purple crystal violet-iodine complex. A schematic summary of the Gram staining procedure, with an example of a stained specimen. The staining procedure therefore classifies cells based on the reaction of the cell walls to the decolorizing solvent. Gram positive cells, which show up purple, have a high concentration of sugar-protein complexes in their cell walls, while gram negative cells, which show up pink or red, have a low concentration of these complexes.

The Gram staining procedure can often lead to equivocal results. For example, if the smear is too thick, this can result in incomplete decolorization due to transport limitations of the decolorizing agent.

In other cases, if the culture is too old, results can be variable. Therefore, the most complete guidelines for the staining procedure usually include advice to:

- Use relatively young cultures
- Prepare light, thin films

If possible, repeat the same smearing and Gram staining procedure on two additional control samples- one that is known to be Gram-negative, the other known to be Gram-positive.

**Acid-fast staining**

Acid-fast staining is a similar method to Gram staining, but adapted for organisms with significantly nonpolar and impenetrable cell walls.Examples of such organisms are members of the genus Mycobacterium. The thickness and waxy nature of the cell walls of these organisms make it difficult to stain using the standard Gram technique.

In this procedure, the specimen is first exposed to carbol fuchsin and heated (or alternatively, exposed to a lipid solubilizer).

This stains the cell. In the second step, the specimen is exposed to an acid-alcohol solution. Only cells with a sufficiently impenetrable cell wall will retain the dye in this step, and these cells are said to be *acid fast*. A second counter-stain can then be used to re-stain the decolorized organisms, similar to the Gram staining procedure.

**Flagella stain**

Some microorganisms have one or more small, thin appendages that are used to move the organism around in a liquid. These appendages, called flagella, are far below the resolution limit for optical microscopy, at typically < 30 nm in thickness. Therefore, special staining procedures are necessary to visualize these structures using optical microscopy. One such procedure uses a simple basic stain dissolved in ethyl alcohol.[5] As with other stains for flagella, the technique requires rigorously cleaned microscope slides and otherwise very careful, detail-oriented technique.

An alternative method for visualizing such small structures is the use of electron microscopy.

**Haematoxylin and eosin (H&E) staining**

Microscopic view of a histologic specimen of human lung tissue stained with hematoxylin and eosin.

Haematoxylin and eosin staining is frequently used in histology to examine thin tissue sections.[10] Haematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red.[10] Eosin is strongly absorbed by red blood cells, colouring them bright red. In a skillfully made H&E preparation the red blood cells are almost orange, and collagen and cytoplasm (especially muscle) acquire different shades of pink.

**Silver staining**

Gömöri methenamine silver stain demonstrating histoplasma (illustrated in black).

Silver staining is the use of silver to stain histologic sections. This kind of staining is important in the demonstration of 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.

*Argentaffin cells* 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 (see Golgi's method). A*rgyrophilic cells* reduce silver solution to metallic silver after being exposed to the stain that contains a reductant. An example of this would be hydroquinone or formalin.

**PAS staining**

PAS diastase showing the fungus Histoplasma.

Periodic acid-Schiff is a histology special stain used to mark carbohydrates (glycogen, glycoprotein, proteoglycans). PAS is commonly used on liver tissue where glycogen deposits are made which is done in efforts to distinguish different types of glycogen storage diseases. PAS is important because it can detect glycogen granules found in tumors of the ovaries and pancreas of the endocrine system, as well as in the bladder and kidneys of the renal system. Basement membranes can also show up in a PAS stain and can be important when diagnosing renal disease. Due to the high volume of carbohydrates within the cell wall of hyphae and yeast forms of fungi, the Periodic acid -Schiff stain can help locate these species inside tissue samples of the human body.

1. **Prepare the sample:** Sample preparation includes the dissolution of the sample in a suitable solvent (inert with the sample under analysis) used in making the mobile phase.
2. **Spot the sample on the paper:** Samples should be spotted at a proper position on the paper by using a capillary tube.
3. **Chromatogram development:** Chromatogram development is spotted by immersing the paper in the mobile phase. Due to the capillary action of paper, the mobile phase moves over the sample on the paper.
4. **Paper drying and compound detection**: Once the chromatogram is developed, the paper is dried using an air drier. Also, detecting solution can be sprayed on the chromatogram developed paper and dried to identify the sample chromatogram spots.

# UNIT II: CELL FRACTIONATION AND RADIOISOTOPES

## CELL FRACTIONATION

Cell fractionation is a procedure that allows different parts of a cell to be separated from each other using centrifugation. Once the cells have been fractionated, organelles such as the plasma membrane, nucleus, and mitochondria can be studied separately.

### Principle of Sedimentation

Sedimentation is the tendency for particles in suspension to settle out of the fluid in which they are entrained and come to rest against a barrier. This is due to their motion through the fluid in response to the forces acting on them: these forces can be due to gravity, centrifugal acceleration, or electromagnetism.

### Centrifuge

A centrifuge is a device used to separate components of a mixture on the basis of their size, density, the viscosity of the medium, and the rotor speed. The centrifuge is commonly used in laboratories for the separation of biological molecules from a crude extract. In a centrifuge, the sample is kept in a rotor that is rotated about a fixed point (axis), resulting in strong force perpendicular to the axis.

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

### Components of Centrifuge

Basic centrifuge components include an **electric motor**, a **shaft**and **rotor** heads on which the centrifuge **head** turns, and a**motor**- drive assembly. If the centrifuge is refrigerated, a compressor and associated components are included. The entire system is housed within a chamber.

### Types of centrifuges

### 1) Benchtop Centrifuge

Benchtop centrifuge is a compact centrifuge that is commonly used in clinical and research laboratories.

It is driven by an electric motor where the tubes are rotated about a fixed axis, resulting in force perpendicular to the tubes.

Because these are very compact, they are useful in smaller laboratories with smaller spaces.

Different variations of benchtop centrifuges are available in the market for various purposes.

A benchtop centrifuge has a rotor with racks for the sample tubes and a lid that closes the working unit of the centrifuge.

### 2) Continuous Flow Centrifuge

Continuous flow centrifuge is a rapid centrifuge that allows the centrifugation of large volumes of samples without affecting the sedimentation rates.

This type of centrifuge allows the separation of a large volume of samples at high centrifugal force, thus removing the tedious part of emptying and filling the tubes with each cycle.

They have a shorter pathlength which facilitates the process of pelleting out the solid part out of the supernatant, thus maintaining the speed of the process.

They also have larger capacities which saves time as the sample doesn't have to be load and unloaded over and over again like in traditional centrifuges.

Up to 1 liter of samples can be centrifuged by this centrifuge at a time period of 4 hours or less.

**3) Gas Centrifuge**

A gas centrifuge is a centrifuge explicitly used for the separation of gases based on heir isotopes.

This centrifuge is based on the same principle of centrifugal force as all other centrifuges where the molecules are separated on the basis of their masses.

This centrifuge is used mainly for the extraction and separation of uranium -235 and uranium-238.

The gas centrifuge works on eh design of the continuous flow of gas in and out of the centrifuge, unlike other centrifuge working on batch processing.

These centrifuges are arranged in cascades so that the gases are separated into two units based on their isotopes and then are passed onto the next centrifuge for further processing.

Gas centrifuges have replaced other gaseous diffusion methods as they provide a yield of higher concentration of the gases than the previous techniques.

**4) Hematocrit centrifuges**

Hematocrit centrifuges are specialized centrifuges used for the determination of volume fraction of erythrocytes (RBCs) in a given blood sample.

This centrifuge provides hematocrit values that can be used for testing in biochemistry, immunity, blood test, and other general clinical tests.

Hematocrit centrifuges may be used to help diagnose blood loss, polycythemia (an elevation of the erythrocyte count to above-normal levels), anemia, bone marrow failure, leukemia, and multiple myeloma.

The microhematocrit centrifuge quickly attains speeds of 11,000 rpm and RCFs of up to 15,000 g to spin tube samples.

The components of a hematocrit centrifuge are similar to that of the benchtop centrifuge, but this centrifuge is specialized for the use of blood samples.

**5) High-speed centrifuge**

High-speed centrifuge, as the name suggests, is the centrifuge that can be operated at somewhat larger speeds.

The speed of the high-speed centrifuge can range from 15,000 to 30,000 rpm.

The high-speed centrifuge is commonly used in more sophisticated laboratories with the biochemical application and requires a high speed of operations.

High-speed centrifuges are provided with a system for controlling the speed and temperature of the process, which is necessary for the analysis of sensitive biological molecules.

The high-speed centrifuges come with different adapters to accommodate the sample tubes of various sizes and volumes.

All three types of rotors can be used for the centrifugation process in these centrifuges.

**6) Low-speed centrifuge**

Low-speed centrifuges are the traditional centrifuges that are commonly used in laboratories for the routine separation of particles.

These centrifuges operate at the maximum speed of 4000-5000 rpm.

These are usually operated under room temperature as they are not provided with a system for controlling the speed or temperature of the operation.

Swinging bucket and fixed angle type of rotors can be used in these centrifuges.

These are easy and compact centrifuges that are ideal for the analysis of blood samples and other biological samples.

The low-speed centrifuge works on the same principle as all other centrifuges, but the application is limited to the separation of simpler solutions.

**7) Microcentrifuge**

Microcentrifuges are the centrifuges used for the separation of samples with smaller volumes ranging from 0.5 to 2 µl.

Micro centrifuges are usually operated at a speed of about 12,000-13,000 rpm.

This is used for the molecular separation of cell organelles like nuclei and DNA and phenol extraction.

Microcentrifuges, also termed, microfuge, use sample tubes that are smaller in size when compared to the standard test tubes used in larger centrifuges.

Some microcentrifuges come with adapters that facilitate the use of larger tubes along with the smaller ones.

Microcentrifuges with temperature controls are available for the operation of temperature-sensitive samples.

**8) Refrigerated Centrifuge**

Refrigerated centrifuges are the centrifuges that are provided with temperature control ranging from -20°C to -30°C.

A different variation of centrifuges is available that has the system of temperature control which is essential for various processes requiring lower temperatures.

Refrigerated centrifuges have a temperature control unit in addition to the rotors and racks for the sample tubes.

These centrifuges provide the RCF of up to 60,000 xg that is ideal for the separation of various biological molecules.

These are typically used for collecting substances that separate rapidly like yeast cells, chloroplasts, and erythrocytes.

The chamber of refrigerated centrifuge is sealed off from the outside to meet the conditions of the operations.

**9) Ultracentrifuges**

Ultracentrifuges are the centrifuges that operate at extremely high speeds that allow the separation of much smaller molecules like ribosomes, proteins, and viruses.

It is the most sophisticated type of centrifuge that allows the separation of molecules that cannot be separated with other centrifuges.

Refrigeration systems are present in such centrifuges that help to balance the heat produced due to the intense spinning.

The speed of these centrifuges can reach as high as 150,000 rpm.

It can be used for both preparative and analytical works.

Ultracentrifuges can separate molecules in large batches and in a continuous flow system.

In addition to separation, ultracentrifuges can also be used for the determination of properties of macromolecules like the size, shape, and density.

**10) Vacuum centrifuge**

Vacuum centrifuge utilizes the centrifugal force, vacuum and heat to speed up the laboratory evaporation of samples.

These centrifuges are capable of processing a large number of samples (up to 148 samples at a time).

This type of centrifuge is used in chemical and biological laboratories for the effective evaporation of solvents present in samples, thus concentrating the samples.

These are commonly used in high throughput laboratories for samples that might have a large number of solvents.

A rotary evaporator is used to remove the unnecessary solvents and eliminate solvent bumping.

The centrifuge works by lowering the pressure of the chamber, which also decreases the boiling point of the samples.

This causes the solvents to be evaporated, concentrating the particles to be separated.

**Types of Centrifugation**

i. **Differential centrifugation**
ii. **Density gradient centrifugation**
iii. **Analytical centrifugation**
iv. **Ultracentrifugation**

**i. Differential centrifugation**

Differential centrifugation is a type of centrifugation process in which components are separately settled down a centrifuge tube by applying a series of increasing centrifugal force.

**Principle of Differential centrifugation**

Differential centrifugation is based upon the differences in the sedimentation rate of biological particles of different size and density.

As the increasing centrifugal force is applied, initial sedimentation of the larger molecules takes place.

Further particles settle down depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles.

The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant.

Thus, larger molecules sediment quickly and at lower centrifugal forces whereas the smaller molecules take longer time and higher forces.

In the case of particles that are less dense than the medium, the particles will float instead of settling.

**Steps of Differential centrifugation**

The sample solution is homogenized in the medium containing buffer.

The sample is then placed in the centrifuge tube, which is operated at a particular centrifugal force for a specific time at a particular temperature.

By the end of this operation, a pellet will be formed at the bottom of the tube, which is separated from the supernatant.

The supernatant is added to a new centrifuge tube where it is centrifuged at another speed for a particular time and particular temperature.

Again, the supernatant is separated from the pellets formed.

These steps are continued until all particles are separated from each other.

The particles can then be identified by testing for indicators that are unique to the specific particles.

**Uses of Differential centrifugation**

Differential centrifugation is commonly used for the separation of cell organelles and membranes found in the cell.

It can also be used for low-resolution separation of the nucleus.

As this technique separates particles based on their sizes, this can be used for the purification of extracts containing larger-sized impurities.

**ii) Density Gradient Centrifugation**

Density gradient centrifugation is the separation of molecules where the separation is based on the density of the molecules as they pass through a density gradient under a centrifugal force.

**Principle of Density gradient centrifugation**

Density gradient centrifugation is based on the principle that molecules settle down under a centrifugal force until they reach a medium with the density the same as theirs.

In this case, a medium with a density gradient is employed, which either has to decrease density or increasing density.

Molecules in a sample move through the medium as the sample is rotated creating a centrifugal force.

The more dense molecules begin to move towards the bottom as they move through the density gradient.

The molecules then become suspended at a point in which the density of the particles equals the surrounding medium.

In this way, molecules with different densities are separated at different layers which can then be recovered by various processes.

**Steps of Density gradient centrifugation**

A density gradient of a medium is created by gently laying the lower concentration over the higher concentrations in a centrifuge tube.

The sample is then placed over the gradient, and the tubes are placed in an ultracentrifuge.

The particles travel through the gradient until they reach a point at which their density matches the density of the surrounding medium.

The fractions are removed and separated, obtaining the particles as isolated units.

**Uses of Density gradient centrifugation**

Density gradient centrifugation can be applied for the purification of large volumes of biomolecules.

It can even be used for the purification of different viruses which aids their further studies.

This technique can be used both as a separation technique and the technique for the determination of densities of various particles

**iii) Analytical Centrifugation**

Analytical centrifugation is a separation method where the particles in a sample are separated on the basis of their density and the centrifugal force they experience. Analytical ultracentrifugation (AUC) is a versatile and robust method for the quantitative analysis of macromolecules in solution.

**Principle of Analytical Centrifugation**

Analytical centrifugation is based on the principle that particles that are denser than others settle down faster. Similarly, the larger molecules move more quickly in the centrifugal force than the smaller ones.

Analytical ultracentrifugation for the determination of the relative molecular mass of a macromolecule can be performed by a sedimentation velocity approach or sedimentation equilibrium methodology.

The hydrodynamic properties of macromolecules are described by their sedimentation coefficients. They can be determined from the rate that a concentration boundary of the particular biomolecules moves in the gravitational field.

The sedimentation coefficient can be used to characterize changes in the size and shape of macromolecules with changing experimental conditions.

Three optical systems are available for the analytical ultracentrifuge (absorbance, interference, and fluorescence) that permit precise and selective observation of sedimentation in real-time.

**Steps of Analytical Centrifugation**

Small sample sizes (20-120 $mm^3$) are taken in analytical cells to be placed inside the ultracentrifuge.

The ultracentrifuge is then operated so that the centrifugal force causes a migration of the randomly distributed biomolecules through the solvent radially outwards from the center of rotation.

The distance of the molecules from the center isdetermined through the Schlieren optical system.

A graph is drawn from the solute concentration versus the squared radial distance from the center of rotation, based on which the molecular mass is determined.

**Uses of Analytical Centrifugation**

Analytical centrifugation can be used for the determination of the purity of macromolecules.

It can also be used for the examination of changes in the molecular mass of supramolecular complexes.

Besides, it allows the determination of the relative molecular mass of solutes in their native state.

**iv) Ultracentrifugation**

Ultracentrifuges are the centrifuges that operate at extremely high speeds that allow the separation of much smaller molecules like ribosomes, proteins, and viruses. It is the most sophisticated type of centrifuge that allows the separation of molecules that cannot be separated with other centrifuges. Refrigeration systems are present in such centrifuges that help to balance the heat produced due to the intense spinning. The speed of these centrifuges can reach as high as 150,000 rpm. It can be used for both preparative and analytical works.
Ultracentrifuges can separate molecules in large batches and in a continuous flow system. In addition to separation, ultracentrifuges can also be used for the determination of properties of macromolecules like the size, shape, and density.

## RADIOISOTOPES

Radioisotopes are atoms which have an unstable nucleus, meaning they will undergo radioactive decay. The term radioisotope comes from "radioactive isotope". An isotope is an atom which has the same number of protons, but a different number of neutrons. For example, cobalt-59, with 27 protons and 32 neutrons, and cobalt-60, with 27 protons and 33 neutrons. Isotopes will chemically interact identically, but they have different physical properties.
Radioisotopes emit different forms of radiation when they decay. These are alpha (α), beta (β), and gamma (γ). A stable isotope is a nuclei which does not experience radioactive decay. Natural radioisotopes are radioisotopes which are found in nature, the most well-known being uranium. Artificial radioisotopes are radioisotopes which are artificially manufactured.
Although dangerous if handled without caution, radioisotopes have many applications. The most useful application is in medicine, where they are used to diagnose and treat various disorders, such as tumors.

All elements have **isotopes**. There are**two** main **types of isotopes**: stable and unstable (radioactive). There are 254 known stable **isotopes**. All artificial (lab-made) **isotopes** are unstable and therefore radioactive; scientists call them radioisotopes.

**GM Counter (Geiger–Muller counter)**

A **Geiger counter** is an instrument used for detecting and measuring ionizing radiation. Also known as a **Geiger–Muller counter** (or **Geiger–Müller counter**), it is widely used in applications such as radiation dosimetry, radiological protection, experimental physics, and the nuclear industry.

It detects ionizing radiation such as alpha particles, beta particles, and gamma raysusing the ionization effect produced in a Geiger–Müller tube, which gives its name to the instrument. In wide and prominent use as a hand-held radiation survey instrument, it is perhaps one of the world's best-known radiation detection instruments.

**Working Principle**

The Geiger–Müller tube is filled with an inert gas such as helium, neon, or argon at low pressure, to which a high voltage is applied. The tube briefly conducts electrical charge when a particle or photon of incident radiation makes the gas conductive by ionization. The ionization is considerably amplified within the tube by the Townsend discharge effect to produce an easily measured detection pulse, which is fed to the processing and display electronics. This large pulse

from the tube makes the Geiger counter relatively cheap to manufacture, as the subsequent electronics are greatly simplified.[2] The electronics also generate the high voltage, typically 400–900 volts, that has to be applied to the Geiger–Müller tube to enable its operation. To stop the discharge in the Geiger–Müller tube a little halogen gas or organic material (alcohol) is added to the gas mixture.

**Applications**

To detect **radioactive rocks and minerals** in the course of mineral prospecting or as a mineral collector.
To check for environmental levels of radioactivity near a**nuclear power facility**.
It is widely used in **applications** such as radiation dosimetry, radiological protection, experimental physics, and the nuclear industry.

**Scintillation Counter**

A **scintillation counter** is an instrument for detecting and measuring ionizing radiation by using the excitation effect of incident radiation on a scintillating material, and detecting the resultant light pulses.

It consists of a scintillator which generates photons in response to incident radiation, a sensitive photodetector (usually a photomultiplier tube (PMT), a charge-coupled device (CCD) camera, or a photodiode), which converts the light to an electrical signal and electronics to process this signal.

Scintillation counters are widely used in radiation protection, assay of radioactive materials and physics research because they can be made inexpensively yet with good quantum efficiency, and can measure both the intensity and the energy of incident radiation.

In general, a scintillation detector consists of:

- **Scintillator**. A scintillator generates photons in response to incident radiation.
- **Photodetector**. A sensitive photodetector (usually a photomultiplier tube (PMT), a charge-coupled device (CCD) camera, or a photodiode), which converts the light to an electrical signal and electronics to process this signal.

**Working Principle**

The operation of scintillation counters is summarized in the following points:

- Ionizing radiation enters the **scintillator** and interacts with the scintillator material. This cause electrons to be raised to an **excited state**.
  - For charged particles the track is the path of the particle itself.
  - For gamma rays (uncharged), their energy is converted to an energetic electron via either the photoelectric effect, Compton scattering or pair production.
- The excited atoms of the scintillator material **de-excite**and rapidly **emit a photon** in the visible (or near-visible) light range. The quantity is proportional to the energy deposited by the ionizing particle. The material is said to fluoresce.
- Three classes of phosphors are used:
  - inorganic crystals,
  - organic crystals,
  - plastic phosphors.
- The light created in the scintillator strikes the **photocathode** of a **photomultiplier tube**, releasing at most one photoelectron per photon.
- Using a voltage potential, this group of **primary electrons** is electrostatically accelerated and focused so that they strike the first **dynode** with enough energy to release additional electrons.
- These **secondary electrons** are attracted and strike a second dynode releasing more electrons. This process occurs in the photomultiplier tube.
- Each subsequent dynode impact releases further electrons, and so there is a current amplifying effect at each dynode stage. Each stage is at a higher potential than the previous to provide the accelerating field.
- Primary signal is multiplied and this amplification continues through 10 to 12 stages.

- At the **final dynode**, sufficient electrons are available to produce a **pulse** of sufficient magnitude for further amplification. This pulse carries information about the energy of the original incident radiation. The number of such pulses per unit time also gives information about the intensity of the radiation.

**Applications**

1. Scintillation Counters are widely used in radioactive contamination, radiation survey meters, radiometric assay, nuclear plant safety, and medical imaging, that are used to measure radiation.
2. There are several counters of mounted on helicopters and some pickup trucks for rapid response in case of a security situation due to radioactive waste or dirty bombs.
3. Scintillation counters designed for weighbridge applications, freight terminals, scrap metal yards, border security, contamination monitoring of nuclear waste, and ports.
4. It is widely used in screening technologies, In vivo and ELISA alternative technologies, cancer research, epigenetics, and Cellular research.
5. It also has its applications in Protein interaction and detection, academic research, and Pharmaceutical.
6. Liquid Scintillation Counter is a type of scintillation counter that is used for measuring the beta emission from the nuclides.

**Autoradiography**

Autoradiography is an imaging technique that uses radioactive sources contained *within* the exposed sample. *In vitro* autoradiography methods involve the isolation of cellular components such as DNA, RNA, proteins or lipids, followed by labeling with suitable radioisotopes. In *in vivo* autoradiography, radioisotopes are coupled with radioactive tracers and administered orally or via injection, and the distribution of radiation is evaluated in thin tissue or whole-body cryosections. *In vivo* autoradiography using laboratory animals is widely used in metabolic studies, disease monitoring and new drug development experiments. Common radioisotopes in autoradiography are sulfur-35, hydrogen-3, carbon-14, $^{125}$-iodine or phosphorus-32 ($^{35}S$, $^{3}H$, $^{14}C$, $^{125}I$ and $^{32}P$, respectively) which are used to determine the distribution of the radiolabeled molecules in tissues, cells or cellular organelles, but also in the study of protein modifications and DNA / RNA sequencing

**General Principle of Autoradiography**

The principle of autoradiographic imaging is the precipitation of silver (Ag) atoms, resulting from the ionization of a silver halide (AgX – silver bromide, chloride, iodide or fluoride – AgBr, AgCl, AgI or AgF, respectively) by radiolabeled samples. AgX are light sensitive compounds commonly used in photography. They are generally suspended in a gelatin photographic emulsion. Each AgX molecule is individually encapsulated in the gelatin, and functions as an independent detector of radioactive decay from the radiolabeled sample. Once radioactive particles hit the gelatin emulsion, AgX is reduced resulting in the production of insoluble silver crystals.

Gelatin photographic emulsions are used to coat photographic and X-ray films, which are made of a flexible base (usually cellulose acetate). When a radiolabeled sample is in contact with in a coated X-ray film (exposure), it generates a latent (*hidden*) image corresponding to the radioactivity distribution within the sample. To make the image visible, the exposed photographic / X-ray film must be submerged in a developing reagent, a chemical mixture that converts the silver crystals into metallic silver, darkening the gelatin emulsion. Silver nitrate ($AgNO_3$) is highly efficient in the reduction of AgX molecules and is usually a component of developer solutions. The reaction is then stopped by a fixative reagent, which removes the excess AgX from the photographic / X-ray film. Highly radioactive areas (e.g. areas with higher concentration of a radiolabeled drug, or with higher metabolic activity) reduce more AgX molecules, resulting in higher optical density in the film (darker

areas). Thus, autoradiography must be avoided in samples that are homogeneously labeled. Although it can be quantitative, autoradiography can be a slow process, depending on the half-life of the radioisotopes used.

**Autoradiography: Methods**

Classic autoradiography techniques are performed according to the following general sequential steps:

**In vivo autoradiography**

Radioactive labelling of biological sample. Labeling time depends on the type of radioisotope and the radiotracer molecule.

1. Injection or oral administration of radioactive tracer in laboratory animals.
2. Cryopreservation of euthanized animals and cryosection – whole-body or tissue sections (20-50 µm thick) for microscopy evaluation. Light or electron microscopy can be used, depending on the aim of the study.
3. Whole-body or tissue sections are mounted into glass slides and embedded in photographic emulsion to generate a latent image.
4. Image development. Here, the incubation time in the developer reagent depends only on the radioisotope used.
5. Arrest of image development by exposing the slide to a fixative reagent.

**In vitro autoradiography**

1. Isolation of cellular components such as proteins, DNA or RNA.
2. *In vitro* labeling of isolated cellular components (DNA, RNA, proteins, etc.)
3. Loading the sample in a suitable matrix for image development using a photographic / X-ray film. Radiolabeled proteins can be loaded into acrylamide gels, and transferred to a nitrocellulose membrane, which is then put in contact with the film.
4. Exposure of the photographic / X-ray film to the matrix containing the radiolabeled sample. Exposure time depends of the radioisotope used.
5. Development of the autoradiographic image by emerging the film in a developer reagent solution.
6. Arrest of image development by emerging the film in a fixative reagent to remove the excess silver halide in the photographic emulsion.

In both *in vivo* and *in vitro* autoradiography, the exposure of the radiolabeled sample to AgX-containing photographic emulsion must always be performed in the dark (e.g., in a dark room, or in closed boxes). Because the AgX is sensitive to light and radiation, this ensures that the only AgX molecules reduced are the ones where radioactivity is emitted from the sample. The resolution of the autoradiographic image is proportional to the ionization capacity of the radioisotopes used. Low energy radioisotopes such as $^{3}H$ reduce only neighboring silver halide molecules, producing a very sharp image, while high energy isotopes such as $^{125}I$ can reduce a lot more halide molecules, producing darker images with lower specificity.

**Applications of Autoradiography**

1. To isolate the metabolic activity site in the cell.
2. To find the site and and performance of targeted drug.
3. To locate the metabolic activity site in the cell.
4. To fine the site and performance of targeted drug.
5. Tissue Location of radioactive substance.
6. To fine the location and amoud of particular subtances within a cell including cell organelle, metabolites etc,

7. To find and investigate the various properties of DNA.

## UNIT –III

## CHROMATOGRAPHY

Chromatography technique that uses paper sheets or strips as the adsorbent being the stationary phase through which a solution is made to pass is called paper chromatography. It is an inexpensive method of separating dissolved chemical substances by their different migration rates across the sheets of paper. It is a powerful analytical tool that uses very small quantities of material. Paper chromatography was discovered by Synge and Martin in the year 1943.

### Paper Chromatography Principle

The principle involved can be partition chromatography or adsorption chromatography. Partition chromatography because the substances are partitioned or distributed between liquid phases. The two phases are water held in pores of the filter paper and the other phase is a mobile phase which passes through the paper. When the mobile phase moves, the separation of the mixture takes place. The compounds in the mixture separate themselves based on the differences in their affinity towards stationary and mobile phase solvents under the capillary action of pores in the paper. Adsorption chromatography between solid and liquid phases, wherein the solid surface of the paper is the stationary phase and the liquid phase is the mobile phase.

### Paper Chromatography Applications

- To study the process of fermentation and ripening.
- To check the purity of pharmaceuticals.
- To inspect cosmetics.
- To detect the adulterants.
- To detect the contaminants in drinks and foods.
- To examine the reaction mixtures in biochemical laboratories.
- To determine dopes and drugs in humans and animals.

### Column Chromatography Principle

When the mobile phase along with the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture is at different rates. The components with lower adsorption and affinity to stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first whereas the components that move slowly are eluted out last.

The adsorption of solute molecules to the column occurs in a reversible manner. The rate of the movement of the components is expressed as:

$R_f$ = the distance travelled by solute/ the distance travelled by the solvent

$R_f$ = the retardation factor.

### Column Chromatography Applications

- Column Chromatography is used to isolate active ingredients.
- It is very helpful in Separating compound mixtures.
- It is used to determine drug estimation from drug formulations
- It is used to remove impurities.
- Used to isolation metabolites from biological fluids.

**The principle of thin-layer chromatography (TLC):**

Thin-layer chromatography also relies on the separation principle, like other chromatographic methods. The separation depends on the relative affinity of the analytes against the stationary phase and mobile phase. The analytes in the solvent mixture move over the surface of the stationary phase. The movement occurs in such a way that analytes that have a high affinity towards the stationary phase move slowly while other analytes move quickly that have less affinity. Consequently, separation of the complex mixture is achieved. When the separation process is complete, the individual analytes from the sample mixture visualized as spots at particular levels on the TLC plate.

**Applications of TLC**

TLC is one of the economical, simple, and widely used chromatographic methods in qualitative and quantitative analysis, and is used for separation and analyzes the purity of organic compounds and test the purity of compounds. Thin-layer chromatography is a type of liquid chromatography in which a stationary phase (a thin plate coated with a silica gel or alumina) and a mobile phase (a mixture of solvents) are used to analyze the molecule on a flat surface, and this process works under the capillary action, ambient temperature, and atmospheric pressure.

**Gas – Liquid chromatography -Principle** (GLC) is one of the most useful techniques in analytical chemistry. Claesson published one of the first important accounts of gas liquid chromatography in 1946. Gas – liquid chromatography is a form of partition chromatography in which the stationary phase is a film coated on a solid support and the mobile phase is an inert gas like Nitrogen (N2) called as carrier gas flowing over the surface of a liquid film in a controlled fashion. The sample under analysis is vaporized under conditions of high temperature programming. The components of the vaporized sample are fractionated as a result of partitioning between a mobile gaseous phase and a liquid stationary phase held in a column. Principle: When the vapours of sample mixture move between the stationary phase (liquid) and mobile phase (gas) the different components of a sample mixture will separate according to their partition coefficient between the gas and liquid stationary phase

$$\text{Partition coeff.(Kg)} = \frac{\text{Concn. of solute in liquid (w/cc)}}{\text{Concn of solute in gas (w/cc)}}$$

It is general assumption that if partition coefficient is low the emergence of the component is fast and vice versa. The substances having low boiling point (B.P) i.e. more volatility and higher vapour pressure will have more concentration in the mobile phase and thus will elute or emerge first and so on. For example, lower carbon number compounds have low B.P and higher volatility and vapour pressure will elute first than the higher carbon number compounds e.g. lower chain fatty acids emerge first than long chain ones. Therefore, less polar substances elute fast than polar substances. More polar substances are more retained in the column and therefore move slowly as compared to less polar substances which move at faster rate. In chromatographic analysis there are two terms commonly used (i) Retention Time and (ii)Retention volume.

Retention Time ( tR): It is the time required for the maximum for a solute peak (the peak of that particular component) to reach the detector in a gas chromatographic column. The retention time (tR) is characteristic of that component and the area under the peak is proportional to its quantity. These parameters yield qualitative and quantitative data, respectively. The characterization of mixture in as unknown sample is done through retention time by comparing with those of reference compounds. The relative proportion of varouis components in a mixture is determined by calculating their peak areas and then calculating the percentage of peaks are out of the total area of various peaks obtained. Retention volume (VR) is defined as the volume of the gas required to carry a component

maximum through the column VR = tR Fc Where Fc is the volume flow rate of the gas at outlet. 3.0 Applications of GLC: Gas liquid chromatography is generally used for both qualitative and quantitative analysis of organic compounds. This technique is much sought technique in Agricultural Science, Agriculture Industry, Food industry, Environmental field, Forensic field, Biotechnology field, Perfume and fragrance industry i.e. cosmetic industry and chemical industry. This technique is very useful for the estimation of (i) pesticide and insecticide residues in food and other consumables (ii) estimation of pollutants in water and other food stuff (iii) Banned and controlled drugs in urine, blood, tablets, energy drinks etc.

**High-Performance Thin-Layer Chromatography (HPTLC)**

**Principle**

The HPTLC works on the same principles as TLC such as the principle of separation is adsorption. The mobile phase or solvent flows through the capillary action. The analytes move according to their affinities towards the stationary phase (adsorbent). The higher affinity component travels slower towards the stationary phase. A low-affinity component travels rapidly toward the stationary phase. On a chromatographic plate, then, the components are separated.

**High-Performance Thin-Layer Chromatography (HPTLC) Applications:**

- High-performance thin-layer chromatography is used to analysis of molecules in both qualitative and quantitative terms.
- HPTLC can estimate the concentration of components although TLC can only separate components.
- HPTLC can analyze a complex structure or a very small amount of compounds.
- This method is used in the food industry to evaluate nutrients, beverages, vitamins, and pesticides in fruit, vegetables, and other foodstuffs.
- HPTLC is useful in forensic detection of substances, including adulteration, overdose, counterfeit drugs, and drug misuse.
- To identify the substances including drug abuse, overdose, adulteration, counterfeit drugs it is used forensic dept.
- HPTLC is used in pharmaceuticals for quality control.
- HPTLC is used for the analysis of forced degradation studies, stability testing, and to check the presence of impurities in the drug.

**Ion Exchange chromatography**

**principle**

Exchange of ions is the basic principle in this type of Chromatography. In this process, two types of ion-exchange chromatography. They are i.e., cationic and anionic exchangers can be used.

**Cationic exchangers** possess negatively charged groups, and these will attract positively charged cations. These exchangers are also called "Acidic ion exchange" materials because their negative charges result from the ionization of acidic groups.

**Anionic exchangers** have positively charged groups that will attract negatively charged anions. These are also called "Basic ion exchange" materials.

**Applications of Ion Exchange Chromatography**

1. It is extremely used in the analysis of amino acids. The amino acid "Autoanalyzer" is based on in exchange principle.
2. To determine the base composition of nucleic acids. Chargaff used this technique for established the equivalence of Adenine and Thymine; Guanine and Cytosine.

3. This is the most effective method for water purification. Complete deionization of water (or) a non-electrolyte solution is performed by exchanging solute cations for hydrogen ions and solute anions for hydroxyl ions. This is usually achieved by the method is used for the softening of drinking water.
4. Proteins are also successfully separated by this technique.
5. It is also used for the separation of many vitamins, other biological amines, and organic acids and bases.
6. The selection of molecules on the basis of their molecular size and shape utilizes the molecular sieve properties of a variety of porous materials.
7. Probably the most commonly used of such materials are a group of polymeric organic compounds which possess a three dimensional network of pores which confer gel properties upon them.
8. The general principle of exclusion chromatography is quite simple. A column of gel particles or porous glass granules is in equilibrium with a suitable solvent for the molecules to be separated.
9. Large molecules which are completely excluded from the pores will pass through the interstitial spaces, while smaller molecules will be distributed between the solvent inside and outside the molecular sieve and will then pass through the column at a lower rate.

**Size exclusion chromatography**

**Principle**

The selection of molecules on the basis of their molecular size and shape utilizes the molecular sieve properties of a variety of porous materials. Probably the most commonly used of such materials are a group of polymeric organic compounds which possess a three dimensional network of pores which confer gel properties upon them.

The general principle of exclusion chromatography is quite simple. A column of gel particles or porous glass granules is in equilibrium with a suitable solvent for the molecules to be separated. Large molecules which are completely excluded from the pores will pass through the interstitial spaces, while smaller molecules will be distributed between the solvent inside and outside the molecular sieve and will then pass through the column at a lower rate. Three stages in such a column are represented diagrammatically in the figure 1.13.

**a. Purification:** The main application of exclusion chromatography is in the purification of biological macromolecules. Viruses, proteins, enzymes, hormones, antibodies, nucleic acids, and polysaccharides have all been separated and purified by the use of appropriate gels or glass granules.

**b. Molecular weight determination**: The effluent volumes of globular proteins are largely determined by their molecular weight. It has been shown, that over a considerable molecular weight range, the effluent volume is approximately a linear function of the logarithm of the molecular weight.

**c. Solution concentration**: Solution of high molecular weight substances can be concentrated by the addition of dry sephadex G-25 (coarse). Water and low molecular weight substance remain in solution. After ten minutes the gel is removed by centrifugation, leaving the high molecular material in a solution whose concentration has increased but whose pH and ionic strength are unaltered.

**d. Desalting**: By use of a column of sephadex G-25, solutions of high molecular weight compounds may be desalted. The high molecular weight substances move with the void volume while the low molecular weight components are distributed between the mobile phase and hence move slowly.

**e. Protein building studies**: Exclusion chromatography is one of a number of methods commonly used to study the reversible binding of a ligand to a macromolecular such as proteins including receptor proteins. A sample of t he protein/ligand mixture is applied to a column of a suitable gel (e.g. G-25) which has previously been equilibrated with a solution of the ligand of the same concentration as that in the mixture.

**Principles of Affinity choromatography**

- The stationary phase consists of a support medium, on which the substrate (ligand) is bound covalently, in such a way that the reactive groups that are essential for binding of the target molecule are exposed.
- As the crude mixture of the substances is passed through the chromatography column, substances with binding site for the immobilized substrate bind to the stationary phase, while all other substances is eluted in the void volume of the column.
- Once the other substances are eluted, the bound target molecules can be eluted by methods such as including a competing ligand in the mobile phase or changing the pH, ionic strength or polarity conditions.

**Application of Affinity Choromatography**

- Affinity chromatography is one of the most useful methods for the separation and purification of specific products.
- It is essentially a sample purification technique, used primarily for biological molecules such as proteins.
- Separation of mixture of compounds.
- Removal of impurities or in purification process.
- In enzyme assays
- Detection of substrates
- Investigation of binding sites of enzymes
- In in vitro antigen-antibody reactions

## UNIT-IV : SPECTROPHOTOMETRY

### Electromagnetic Radiation

Electromagnetic radiation can be defined as a form of energy that is produced by the movement of electrically charged particles travelling through a matter or vacuum or by oscillating magnetic and electric disturbance. The magnetic and the electric fields come at 90° to each other, and the combined waves move perpendicular to both electric and magnetic oscillating fields occurring the disturbance.

### Properties of Electromagnetic Radiation

- They can travel through empty space. Waves other than electromagnetic waves have to travel through some substance. For example, sound waves will need either a solid, liquid or gas to pass through.
- The speed of light which is $2.99792458 \times 10^8$ m/s is always constant.
- Wavelength is commonly characterized by the symbol 'λ'. It is the measure between the distance of either troughs or crests.

### Beer Lambert's law

Beer's law, also called Lambert-Beer law or Beer-Lambert la**w**, in spectroscopy, a relation concerning the absorption of radiant energy by an absorbing medium. Formulated by German mathematician and chemist August Beer in 1852, it states that the absorptive capacity of a dissolved substance is directly proportional to its concentration in a solution. The relationship can be expressed as $A = \varepsilon lc$ where $A$ is absorbance, ε is the molar extinction coefficient (which depends on the nature of the chemical and the wavelength of the light used), $l$ is the length of the path light must travel in the solution in centimetres, and $c$ is the concentration of a given solution.

What is the Beer-Lambert Law?

The Beer-Lambert's law is a linear relationship between the absorbance and the concentration, molar absorption coefficient and optical coefficient of a solution.

The molar absorption coefficient is a sample dependent property and is a measure of how strong an absorber the sample is at a particular wavelength of light. The concentration is simply the moles $L^{-1}$ (M) of the sample dissolved in the solution, and the length is the length of the cuvette used for the absorbance measurement and is typically 1 cm.

The Beer-Lambert law states that there is a linear relationship between the concentration and the absorbance of the solution, which enables the concentration of a solution to be calculated by measuring its absorbance. To demonstrate this linear dependence five solutions of Rhodamine B in water were measured using the DS5 Dual Beam Spectrophotometer (Figure 3a) and from these absorption spectra, a linear calibration curve of the absorbance versus concentration was created (Figure 3b). Using this calibration curve the concentration of an unknown Rhodamine B solution can be determined by measuring its absorbance which is the main utility of the Beer-Lambert Law.

UV-**Visible** Spectroscopy

Spectroscopy is the measurement and interpretation of electromagnetic radiation absorbed or emitted when the molecules or atoms or ions of a sample moves from one energy state to another energy state. UV spectroscopy is type of absorption spectroscopy in which light of ultra-violet region (200-400 nm) is absorbed by the molecule which results in the excitation of the electrons from the ground state to higher energy state.

**Principle**

- Basically, spectroscopy is related to the interaction of light with matter.
- As light is absorbed by matter, the result is an increase in the energy content of the atoms or molecules.
- When ultraviolet radiations are absorbed, this results in the excitation of the electrons from the ground state towards a higher energy state.
- Molecules containing π-electrons or non-bonding electrons (n-electrons) can absorb energy in the form of ultraviolet light to excite these electrons to higher anti-bonding molecular orbitals.
- The more easily excited the electrons, the longer the wavelength of light it can absorb. There are four possible types of transitions (π–π*, n–π*, σ–σ*, and n–σ*), and they can be ordered as follows: σ–σ* > n–σ* > π–π* > n–π*
- The absorption of ultraviolet light by a chemical compound will produce a distinct spectrum which aids in the identification of the compound.

**Instrumentation**

**Light Source**

- Tungsten filament lamps and Hydrogen-Deuterium lamps are most widely used and suitable light source as they cover the whole UV region.
- Tungsten filament lamps are rich in red radiations; more specifically they emit the radiations of 375 nm, while the intensity of Hydrogen-Deuterium lamps falls below 375 nm.

**Monochromator**

- Monochromators generally is composed of prisms and slits.
- Most of the spectrophotometers are double beam spectrophotometers.
- The radiation emitted from the primary source is dispersed with the help of rotating prisms.
- The various wavelengths of the light source which are separated by the prism are then selected by the slits such the rotation of the prism results in a series of continuously increasing wavelength to pass through the slits for recording purpose.
- The beam selected by the slit is monochromatic and further divided into two beams with the help of another prism.

**Sample and reference cells**

- One of the two divided beams is passed through the sample solution and second beam is passé through the reference solution.
- Both sample and reference solution are contained in the cells.
- These cells are made of either silica or quartz. Glass can’t be used for the cells as it also absorbs light in the UV region.

**Detector**

- Generally two photocells serve the purpose of detector in UV spectroscopy.
- One of the photocell receives the beam from sample cell and second detector receives the beam from the reference.
- The intensity of the radiation from the reference cell is stronger than the beam of sample cell. This results in the generation of pulsating or alternating currents in the photocells.

**Amplifier**

- The alternating current generated in the photocells is transferred to the amplifier.
- The amplifier is coupled to a small servometer.
- Generally current generated in the photocells is of very low intensity, the main purpose of amplifier is to amplify the signals many times so we can get clear and recordable signals.

**Recording devices**

- Most of the time amplifier is coupled to a pen recorder which is connected to the computer.
- Computer stores all the data generated and produces the spectrum of the desired compound.

**Application**

1. **Detection of Impurities**
   It is one of the best methods for determination of impurities in organic molecules.
   Additional peaks can be observed due to impurities in the sample and it can be compared with that of standard raw material.
   By also measuring the absorbance at specific wavelength, the impurities can be detected.
2. **Structure elucidation of organic compounds**
   It is useful in the structure elucidation of organic molecules, such as in detecting the presence or absence of unsaturation, the presence of hetero atoms.
3. UV absorption spectroscopy can be used for the **quantitative determination of compounds** that absorb UV radiation.
4. UV absorption spectroscopy can characterize those types of compounds which absorbs UV radiation thus used in qualitative determination of compounds. Identification is done by comparing the absorption spectrum with the spectra of known compounds.
5. This technique is used to detect the presence or absence of functional group in the compound. Absence of a band at particular wavelength regarded as an evidence for absence of particular group.
6. Kinetics of reaction can also be studied using UV spectroscopy. The UV radiation is passed through the reaction cell and the absorbance changes can be observed.
7. Many drugs are either in the form of raw material or in the form of formulation. They can be assayed by making a suitable solution of the drug in a solvent and measuring the absorbance at specific wavelength.
8. Molecular weights of compounds can be measured spectrophotometrically by preparing the suitable derivatives of these compounds.
9. UV spectrophotometer may be used as a detector for HPLC.

**Atomic absorption spectroscopy** (**AAS**)

Atomic absorption spectroscopy (AAS) and atomic emission spectroscopy (AES) is a spectroanalytical procedure for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state. Atomic absorption spectroscopy is based on absorption of light by free metallic ions.

In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 70 different elements in solution, or directly in solid samples via electrothermal vaporization and is used in pharmacology, biophysics, archaeology and toxicology research.

**Principle**

The technique makes use of the atomic absorption spectrum of a sample in order to assess the concentration of specific analytes within it. It requires standards with known analyte content to establish the relation between the measured absorbance and the analyte concentration and relies therefore on the Beer-Lambert law.

**Instrumentation**

In order to analyze a sample for its atomic constituents, it has to be atomized. The atomizers most commonly used nowadays are flames and electrothermal (graphite tube) atomizers. The atoms should then be irradiated by optical radiation, and the radiation source could be an element-specific line radiation source or a continuum radiation source. The radiation then passes through a monochromator in order to separate the element-specific radiation from any other radiation emitted by the radiation source, which is finally measured by a detector.

**Flame atomic emission spectroscopy**

- Flame atomic emission spectroscopy (FAES) is a classical method which has been largely displaced by plasma spectroscopies. Plasmas produce higher atomization ratios, but the theory is similar in both flame and the plasmas. FAES is the classical method used as plasmas have taken over as the preferred method due to the higher atomization ratios that occur. Using the flame could be advantageous in a Group I or Group II elemental analysis since less ionization will occur at lower temperatures (compared to a plasma). It is typically not used often, unless sensitivity and cost are possible issues.

- Below is a very simple schematic for a laminar flow burner. The Primary Combustion Zone is where the initial decomposition occurs and molecular fragments are observed. The Interzonal Region is the hottest part of the flame and atomic fragment are observed. The Secondary Combustion Zone is cooler overall and a conversion is seen from atoms back to stable molecules and oxides.
- Fuel (usually acetylene) and air are added mixed with a nebulizer mist. This mixture is then introduced into the flame.
- The advantages to the Laminar flow burner are that it is cheap, simple, relatively stable and can operate at lower temperatures. An issue that complicates flame emission just as it complicates plasma emission is self reversal.

**Applications of Choromatography**

**X-Ray Diffraction**

X-ray diffraction is a powerful nondestructive technique for characterizing crystalline materials. It provides information on structures, phases, preferred crystal orientations (texture), and other structural parameters, such as average grain size, crystallinity, strain, and crystal defects. XRD peaks are produced by constructive interference of a monochromatic beam of X-rays scattered at specific angles from each set of lattice planes in a sample. The peak intensities are determined by the atomic positions within the lattice planes. Consequently, the XRD pattern is the fingerprint of periodic atomic arrangements in a given material. An online search of a standard database for X-ray powder diffraction patterns enables quick phase identification for a large variety of crystalline samples

**X-ray Crystallography**

X-ray crystallography is a tool used for determining the atomic and molecular structure of a crystal. The underlying principle is that the crystalline atoms cause a beam of X-rays to diffract into many specific directions (Fig. 2.10). By measuring the angles and intensities of these diffracted beams, a crystallographer can produce a 3D picture of the density of electrons within the crystal. From this electron density image, the mean positions of the atoms in the crystal can be determined, as well as their chemical bonds, their disorder, and various other information. The method revealed the structure and function of many biological molecules, including vitamins, drugs, proteins, and nucleic acids, such as DNA. Note that the double helix structure of DNA discovered by James Watson

and Francis Crick was revealed by X-ray crystallography. Recent advances in image reconstruction technology have made X-ray crystallography amenable to the structural analysis of much larger complexes, such as virus particles (Fig. 2.11). The major shortcoming of X-ray crystallography is that it is difficult to obtain a crystal of virus particles, which is a prerequisite for X-ray crystallography. Another shortcoming is that X-ray crystallography generally requires placing the samples in nonphysiological environments, which can occasionally lead to functionally irrelevant conformational changes.

**Principle of Infrared Spectroscopy**

The IR spectroscopy theory utilizes the concept that molecules tend to absorb specific frequencies of light that are characteristic of the corresponding structure of the molecules. The energies are reliant on the shape of the molecular surfaces, the associated vibronic coupling, and the mass corresponding to the atoms.

For instance, the molecule can absorb the energy contained in the incident light and the result is a faster rotation or a more pronounced vibration.

**IR Spectroscopy Instrumentation**

The instrumentation of infrared spectroscopy is illustrated below. First, a beam of IR light from the source is split into two and passed through the reference ant the sample respectively.Now, both of these beams are reflected to pass through a splitter and then through a detector. Finally, the required reading is printed out after the processor deciphers the data passed through the detector.

**Applications of IR Spectroscopy**

**Studying the progress of the reaction**

Progress of chemical reaction can be determined by examining the small portion of the reaction mixure withdrawn from time to time. The rate of disappearance of a characteristic absorption band of the reactant group and/or the rate of appearance of the characteristic absorption band of the product group due to formation of product is observed.

**4. Detection of impurities**

IR spectrum of the test sample to be determined is compared with the standard compound. If any additional peaks are observed in the IR spectrum, then it is due to impurities present in the compound.

**5. Quantitative analysis**

The quantity of the substance can be determined either in pure form or as a mixure of two or more compounds. In this, characteristic peak corresponding to the drug substance is chosen and log I0/It of peaks for standard and test sample is compared. This is called base line technique to determine the quantity of the substance.

**Nuclear Magnetic Resonance (NMR) spectroscopy**

**Principle**

The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level (generally a single energy gap).

1. The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency.

2. The signal that matches this transfer is measured in many ways and processed in order to yield an NMR spectrum for the nucleus concerned.

**Application of Nuclear Magnetic Resonance (NMR)**

- It is an analytical chemistry technique used in quality control.
- It is used in research for determining the content and purity of a sample as well as its molecular structure. For example, NMR can quantitatively analyze mixtures containing known compounds.
- NMR spectroscopy is routinely used by chemists to study chemical structure using simple one-dimensional techniques. Two-dimensional techniques are used to determine the structure of more complicated molecules.
- These techniques are replacing x-ray crystallography for the determination of protein structure.
- Time domain NMR spectroscopy techniques are used to probe molecular dynamics in solution.
- Solid state NMR spectroscopy is used to determine the molecular structure of solids.

## UNIT – V : ELECTROPHORESIS

**Agarose Gel Electrophoresis (AGE)**

It 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 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 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. Note however that the size of a circular DNA like plasmids cannot be accurately gauged using standard markers unless it has been linearized by restriction digest, alternatively a supercoiled DNA marker may be used.

**Applications**

- Estimation of the size of DNA molecules following digestion with restriction enzymes, e.g., in restriction mapping of cloned DNA.
- Analysis of products of a polymerase chain reaction (PCR), e.g., 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.

**Polyacrylamide Gel Electrophoresis (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.

The most commonly used form of polyacrylamide gel electrophoresis is the Sodium dodecyl suplhate Polyacrylamide gel electrophoresis (SDS- PAGE) used mostly for the separation of proteins.

**Principle**

The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite sign. The general electrophoresis techniques cannot be used to determine the molecular weight of biological molecules because the mobility of a substance in the gel depends on both charge and size.

**Steps Involved in PAGE**

1. **Sample preparation**

- Samples may be any material containing proteins or nucleic acids.
- 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.
- 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.

2. **Preparation of polyacrylamide gel**

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

3. **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 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.
- After the set amount of time, the biomolecules will 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.

4. **Detection**

- Following electrophoresis, the gel may be stained (for proteins, most commonly with Coomassie Brilliant Blue or autoradiography; for nucleic acids, ethidium bromide; or for either, silver stain), allowing visualization of the separated proteins, or processed further (e.g. Western blot).
- After staining, different species biomolecules appear as distinct bands within the gel.
- It is common to run molecular weight size marker sof 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.

**Applications of PAGE**

- Measuring molecular weight.
- Peptide mapping.
- Estimation of protein size.
- Determination of protein subunits or aggregation structures.
- Estimation of protein purity.
- Protein quantitation.
- Monitoring protein integrity.
- Comparison of the polypeptide composition of different samples.
- Analysis of the number and size of polypeptide subunits.
- Post-electrophoresis applications, such as Western blotting.
- Staining of Proteins in Gels with Coomassie G-250 without Organic Solvent and Acetic Acid.
- Pouring and Running a Protein Gel by reusing Commercial Cassettes.
- Selective Labelling of Cell-surface Proteins using CyDye DIGE Fluor Minimal Dyes.
- Detection of Protein Ubiquitination.

**Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

**Principle**

The concentration of polyacrylamide gels can be prepared as required in two electrophoresis systems —called "continuous system" and "discontinuous system". The biggest feature of "discontinuous system" lies in its greatly improved sample separation resolution. Main features of this electrophoresis are: **(1)** Use of two gel systems with different concentrations; **(2)** Solution composition and pH are different for the preparation of the two gel and are also different from electrophoresis buffer composition and pH in electrophoresis tank. In the experiment, electrophoresis gel is divided into two layers: the upper one is a macroporous gel with low concentration, called stacking gel, buffer for the formulation of this layer is Tris-HCl, pH6.7; the lower one is hole glue with high concentrations, called separating gel or electrophoresis gel , and the buffer for this is Tris-HCl, pH8.9. Electrode buffer in the electrophoresis tank is Tris- glycine, pH8.3. Obviously, the gel concentrations, compositions, pH and the electrophoresis buffer systems are different from each other, thus forming a discontinuous system.

During the experiment, the protein sample was loaded in the stacking gel. To prevent protein sample diffusing in the electrode buffer, adding an equal volume of 40% sucrose or 50% glycerol to increase the density would be a good choice. To observe the mobility of protein samples, it's better to add bromophenol blue dye or some other tracer dyes into the sample. These colored substances can migrate faster than any macromolecules. As long as the dye dose not move out of the gel, there would be no danger for the sample.

**Materials and Reagents**

1. **30% acrylamide**: weigh 29g acrylamide, 1g N, N – methylene bis-acrylamide. Add 60 ml warmed deionized water and heat to 37 ℃. Add deionized water to make a final volume of 100ml; filter; Then we have 30% (w / v) acrylamide stock solution; Acrylamide and bis-acrylamide were transformed slowly into acrylic acid and double acrylic acid during storage, so the pH of the solution should be no more than 7.0 and it should be placed in a brow bottle at 4 ℃.

2. **10% sodium dodecyl sulfate (SDS)**: weigh 10g SDS and 90ml deionized water; heat to 68 ℃ and add a few drops of concentrated hydrochloric acid until the pH becomes 7.2; then water to 100ml; after the whole processes, we have 10% (w/v) SDS.

3. **Stacking gel buffer (1mol / L Tris-HCl pH 6.8)**: dissolve 12.12g Tris in 80ml deionized water. Adjust the pH to 6.8 with concentrated hydrochloric acid; add deionized water to 100ml and store at 4℃.

4. **Resolving gel buffer** (1.5mol / L Tris-HCl pH 8.8): dissolve 18.16g Tris in 80ml deionized water; adjust the pH to 8.8 with concentrated hydrochloric acid; add deionized water to 100ml; store at 4 ℃.

5. **10% ammonium persulfate (AP)**: ammonium persulfate provides the free radical necessary for the catalysis of the Polymerization of Acrylamide and Bis-acrylamide; Use deionized water to prepare a small amount of 10% (w/v) solution and store at 4 ℃. Since ammonium persulfate will decompose slowly, it should be freshly prepared every other week.

6. **TEMED (N, N, N, N – tetramethylethylenediamine)**: by catalyzing ammounium persulfate to form free radicals, TEMED accelerated the polymerization of acrylamide and bis-acrylamide. Since TEMED only functions in a free base form, the polymerization reaction would be inhibited when the pH is low.

7. **Tris- glycine electrophoresis buffer**: weigh 15.1g Tris and 94g glycine; Dissolve in 900ml deionized water; then add 50ml 10% (w/v) SDS and deionized water to 1000ml. Dilute 5-fold when using. The final concentration would be: Tris, 25mmol/L; glycine, 250mmol/L; SDS, 0.1% and the pH of the buffer is 8.3.

8. Polyacrylamide gel electrophoresis tank and electrophoresis power supply.

9. Transfer pipette and tip, etc.

**Operating Method**

1. Assemble glass plate according to the vertical electrophoresis tank instructions; determine the concentration and volume of the separating gel; prepare the desired separating gel according to the ingredients listed for the preparation of Tris- glycine SDS-polyacrylamide gel electrophoresis.
2. Inject the separating gel into the gap of the two glass sheets quickly, leaving space for the infusion of stacking gel (comb teeth length plus 1cm); cover the separating gel with 0.1% SDS carefully( when the concentration of acrylamide ≤ 8%) or isobutanol or water (when the acrylamide concentration ≥10%); the cover layer can prevent the diffusion of oxygen into the gel and inhibit the polymerization of the gel; place the gel vertically at room temperature.

3. When the polymerization of the separating gel completed, pour the cover liquid; wash the top of the gel for several times to remove the acrylamide that were unpolymerized; exclude the liquid on the gels as far as possible.
4. Determine the volume of the stacking gel in need; prepare the desired stacking gel according to the ingredients listed for the preparation of Tris- glycine SDS-polyacrylamide gel electrophoresis of stacking gel; Pipette the stacking gel directly on the separating gel, and insert clean supporting comb immediately, to avoid air bubbles; then add stacking gel solution to fill the gap between comb. Remove the comb after stacking gel polymerization, then the sample hole is formed.
5. Dilute 5-fold of the Tris- glycine electrophoresis buffer stock solution with deionized water; pour the solution into electrophoresis tank; fill the sample hole so that the bubbles in the sample holes can be ruled out through the electrophoresis buffer.

**Immunoelectrophoresis**

Immunoelectrophoresis is a general name for a number of biochemical methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies. All variants of immunoelectrophoresis require immunoglobulins, also known as antibodies, reacting with the proteins to be separated or characterized.

**Method**

Agarose as 1% gel slabs of about 1 mm thickness buffered at high pH (around 8.6) is traditionally preferred for the electrophoresis as well as the reaction with antibodies. The agarose was chosen as the gel matrix because it has large pores allowing free passage and separation of proteins, but provides an anchor for the immunoprecipitates of protein and specific antibodies. The high pH was chosen because antibodies are practically immobile at high pH. An electrophoresis equipment with a horizontal cooling plate was normally recommended for the electrophoresis.

Immunoprecipitates may be seen in the wet agarose gel, but are stained with protein stains like Coomassie Brilliant Blue in the dried gel. In contrast to SDS-gel electrophoresis, the electrophoresis in agarose allows native conditions, preserving the native structure and activities of the proteins under investigation, therefore immunoelectrophoresis allows characterization of enzyme activities and ligand binding etc. in addition to electrophoretic separation.

**Isoelectrofocusing**

Isoelectric focusing (IEF), also known as electrofocusing, is a technique for separating different molecules by differences in their isoelectric point (pI).[1][2] 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 toward 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 toward 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 toward 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.

The method is applied particularly often in the study of proteins, which separate based on their relative content of acidic and basic residues, whose value is represented by the pI. Proteins are introduced into an Immobilized pH gradient gel composed of polyacrylamide, starch, or agarose where a pH gradient has been established. Gels with large pores are usually used in this process to eliminate any "sieving" effects, or artifacts in the pI caused by differing migration rates for proteins of differing sizes. Isoelectric focusing can resolve proteins that differ in pI value by as little as 0.01.[3] Isoelectric focusing is the first step in two-dimensional gel electrophoresis, in which proteins are first separated by their pI value and then further separated by molecular weight through SDS-PAGE.

**Capillary electrophoresis**

Capillary Electrophoresis (CE) is one of the possible methods to analyse complex samples. In High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) the separating force is the difference in affinity of the sample components to a stationary phase, and

or difference in boiling point. With both techniques the most important factor is the polarity of a sample component. In CE the separating force is the difference in charge to size ratio. Not a flow through the column, but the electric field will do the separation.

In Capillary Electrophoresis a capillary is filled with a conductive fluid at a certain pH value. This is the buffer solution in which the sample will be separated. A sample is introduced in the capillary, either by pressure injection or by electrokinetic injection. A high voltage is generated over the capillary and due to this electric field (up to more then 300 V/cm) the sample components move (migrate) through the capillary at different speeds. Positive components migrate to the negative electrode, negative components migrate to the positive electrode. When you look at the capillary at a certain place with a detector you will first see the fast components pass, and later on the slower components.

**Instrumentation**

The instrumentation needed to perform capillary electrophoresis is relatively simple. A basic schematic of a capillary electrophoresis system. The system's main components are a sample vial, source and destination vials, a capillary, electrodes, a high voltage power supply, a detector, and a data output and handling device. The source vial, destination vial and capillary are filled with an electrolyte such as an aqueous buffer solution. To introduce the sample, the capillary inlet is placed into a vial containing the sample. Sample is introduced into the capillary via capillary action, pressure, siphoning, or electrokinetically, and the capillary is then returned to the source vial. The migration of the analytes is initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the high-voltage power supply. In the most common mode of CE, all ions, positive or negative, are pulled through the capillary in the same direction by electroosmotic flow. The analytes separate as they migrate due to their electrophoretic mobility, and are detected near the outlet end of the capillary. The output of the detector is sent to a data output and handling device such as an integrator or computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separated chemical compounds appear as peaks with different retention times in an electropherogram. The technique is often attributed to James W. Jorgensen and Krynn DeArman Lukacs, who first demonstrated the capabilities of this technique. Capillary electrophoresis was first combined with mass spectrometry by Richard D. Smith and coworkers, and provides extremely high sensitivity for the analysis of very small sample sizes. Despite the very small sample sizes (typically only a few nanoliters of liquid are introduced into the capillary), high sensitivity and sharp peaks are achieved in part due to injection strategies that result in concentration of analytes into a narrow zone near the inlet of the capillary. This is achieved in either pressure or electrokinetic injections simply by suspending the sample in a buffer of lower conductivity (*e.g.* lower salt concentration) than the running buffer. A process called field-amplified sample stacking (a form of isotachophoresis) results in concentration of analyte in a narrow zone at the boundary between the low-conductivity sample and the higher-conductivity running buffer.

To achieve greater sample throughput, instruments with arrays of capillaries are used to analyze many samples simultaneously. Such capillary array electrophoresis (CAE) instruments with 16 or 96 capillaries are used for medium- to high-throughput capillary DNA sequencing, and the inlet ends of the capillaries are arrayed spatially to accept samples directly from SBS-standard footprint 96-well plates.

**Advantages of CE include:**

- **DNA fingerprinting -** After the DNA has been amplified, it can be separated by CE. Separation can be at a resolution of one base pair and individual nucleotides can be identified so allowing a high resolution map of the DNA to be created.
- **Drug Analysis -** CE is used in pharma for the analysis of drugs and related compounds as discussed in **Capillary Electrophoresis: an Attractive Technique for Chiral Separations**. Its high selectivity means that it provides a good resolution to separations. When separating certain compounds, amines for example, CE can use a non-reactive capillary surface at a pH chosen by the technician to provide good separation.
- **Characterization of Proteins** - Because of the way CE operates, it can be set up to separate amphoteric molecules such as proteins, thus allowing protein identification. Amphoterics are molecules that can be either acid or basic, and this can be changed by altering the pH of the solution used in CE. Molecules can then be separated by allowing them to migrate to their isoelectric points (where a molecule has no net charge), before mobilizing all the molecules past the detector.

**Polymerase chain reaction**

PCR is shorthand for a simple but very useful procedure in molecular biology called the **P**olymerase **C**hain **R**eaction. It is a technique used to amplify a segment of DNA of interest or produce lots and lots of copies. In other words, PCR enables you to produce millions of copies of a specific DNA sequence from an initially small sample – sometimes even a single copy. It is a crucial process for a range of genetic technologies and, in fact, has enabled the development of a suite of new technologies.

**Step 1: Denaturation**

As in DNA replication, the two strands in the DNA double helix need to be separated.

The separation happens by raising the temperature of the mixture, causing the hydrogen bonds between the complementary DNA strands to break. This process is called denaturation.

**Step 2: Annealing**

Primers bind to the target DNA sequences and initiate polymerisation. This can only occur once the temperature of the solution has been lowered. One primer binds to each strand.

**Step 3: Extension**

New strands of DNA are made using the original strands as templates. A DNA polymerase enzyme joins free DNA nucleotides together. This enzyme is often Taq polymerase, an enzyme originally isolated from a thermophilic bacteria called Thermus aquaticus. The order in which the free nucleotides are added is determined by the sequence of nucleotides in the original (template) DNA strand.

The result of one cycle of PCR is two double-stranded sequences of target DNA, each containing one newly made strand and one original strand.

The cycle is repeated many times (usually 20–30) as most processes using PCR need large quantities of DNA. It only takes 2–3 hours to get a billion or so copies.

**Applications**

- PCR can amplify a single DNA molecule from a complex mixture, largely avoiding the need to use DNA cloning to prepare that molecule. Variants of the technique can similarly amplify a specific single RNA molecule from a complex mixture.
- DNA sequencing has been greatly simplified using PCR, and this application is now common.
- By using suitable primers, it is possible to use PCR to create point mutations, deletions and insertions of target DNA which greatly facilitates the analysis of gene expression and function.
- PCR is exquisitely sensitive and can amplify vanishingly small amounts of DNA. Thus, using appropriate primers, very small amounts of specified bacteria and viruses can be detected in tissues, making PCR invaluable for medical diagnosis.
- PCR is now invaluable for characterizing medically important DNA samples. For example, in screening for human genetic diseases, it is rapidly replacing the use of RFLPs.
- Because of its extreme sensitivity, PCR is now fundamentally important to forensic medicine. It is even possible to use PCR to amplify the DNA from a single human hair or a microscopic drop of blood left at the scene of a crime to allow detailed characterization.

**DNA Sequencing**

DNA sequencing is the process of determining the sequence of nucleotides within a DNA molecule. Every organism's DNA consists of a unique sequence of nucleotides. Determining the sequence can help scientists compare DNA between organisms, which can help show how the organisms are related.

**DNA Sequencing Methods**

There are two main types of DNA sequencing. The older, classical chain termination method is also called the Sanger method**.** Newer methods that can process a large number of DNA molecules

quickly are collectively called High-Throughput Sequencing (HTS) techniques or Next-Generation Sequencing (NGS) methods.

Sanger Sequencing

The Sanger method relies on a primer that binds to a denatured DNA molecule and initiates the synthesis of a single-stranded polynucleotide in the presence of a DNA polymerase enzyme, using the denatured DNA as a template. In most circumstances, the enzyme catalyzes the addition of a nucleotide. A covalent bond, therefore, forms between the 3′ carbon atom of the deoxyribose sugar molecule in one nucleotide and the 5′ carbon atom of the next. This image below shows how this bond is formed.

A sequencing reaction mixture, however, would have a small proportion of modified nucleotides that cannot form this covalent bond due to the absence of a reactive hydroxyl group, giving rise to the term 'dideoxyribonucleotides', i.e., they do not have a 2' or 3' oxygen atom when compared to the corresponding ribonucleotide. This would terminate the DNA polymerization reaction prematurely. At the end of multiple rounds of such polymerizations, a mixture of molecules of varying lengths would be created.

In the earliest attempts at using the Sanger method, the DNA molecule was first amplified using a labeled primer and then split into four test tubes, each having only one type of ddNTP. That is, each reaction mixture would have only one type of modified nucleotide that could cause chain termination. After the four reactions were completed, the mixture of DNA molecules created by chain termination would undergo electrophoresis on a polyacrylamide gel, and get separated according to their length.

A sequencing reaction with ddATP was electrophoresed through the first column. Each line represents a DNA molecule of a particular length, the result of a polymerization reaction that was terminated by the addition of a ddATP nucleotide. The second, third and fourth columns contained ddTTP, ddGTP, and ddCTP respectively.

With time, this method was modified so that each ddNTP had a different fluorescent label. The primer was no longer the source of the radiolabel or fluorescent tag. Also known as dye-terminator sequencing, this method used four dyes with non-overlapping emission spectra, one for each ddNTP.

There is a single reaction mixture carrying all the elements needed for DNA elongation. The reaction mixture also contains small concentrations of four ddNTPs, each with a different fluorescent tag. The completed reaction is run on a capillary gel. The results are obtained through an analysis of the emission spectra from each DNA band on the gel. A software program then analyzes the spectra and presents the sequence of the DNA molecule.

**Advantages of DNA Sequencing**

Traditional, chain-termination technology and HTS methods are used for different applications today. Sanger sequencing is now used mostly for *de novo* initial sequencing of a DNA molecule to obtain the primary sequence data for an organism or gene**.**

This becomes important in evolutionary biology as well as in the detection of mutated genes that can result in disease. For instance, sequence variations in samples from lung adenocarcinoma allowed the detection of rare mutations associated with the disease. The chromatin binding sites for specific nuclear proteins can also be accurately identified using these methods.