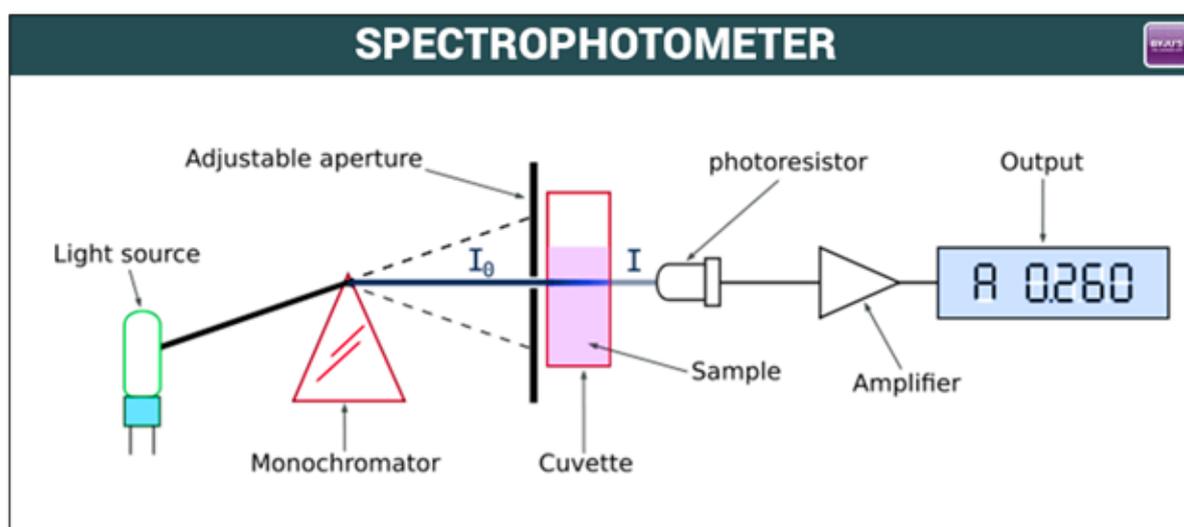## SPECTROPHOTOMETER

A spectrophotometer measures the amount of light that a sample absorbs when a beam of light is made to pass through it. The intensity of light is measured by a detector that is placed after the sample. The beam of light consists of a stream of photons that have chance of getting absorbed by the sample, thus reducing the number of photons in the beam of light, thereby reducing the intensity of the light beam that reaches the detector.

A spectrophotometer consists of two instruments, namely a spectrometer for producing light of any selected wavelength, and a photometer for measuring the intensity of light. The instruments are arranged so that sample liquid in a cuvette can be placed between the spectrometer beam and the photometer. The amount of light passing through the tube is measured by the photometer. The photometer converts light into a voltage signal to a display device, normally a galvanometer. The signal changes as the amount of light absorbed by the liquid changes.

There are two major kinds of spectrophotometers- **single beam** spectrophotometer and **double beam** spectrophotometer. A singe beam spectrophotometer measures the ratio of absolute light intensity and the double beam spectrophotometer measures the ratio of the light intensity of two different light paths. Although ratio measurements are easier, single beam instruments have advantages for they can have a larger range.

Single beam spectrophotometer:

To use a **single beam spectrophotometer**, the machine is zeroed first, the wavelength is set, the blank is adjusted and then the sample is inserted and read. The wavelength is then adjusted by some determined interval, the zero is checked, the blank re-inserted and adjusted, and the sample re-inserted and read. This procedure continues until all wavelengths to be scanned have been read. In this procedure, the sample remains the same, but the wavelength is adjusted. Compounds have differing absorption coefficients for each wavelength. Thus, each time the wavelength is altered, the instrument must be recalibrated.

Dual beam spectrophotometer:

A **dual beam spectrophotometer** divides the light into two paths. One beam is used to pass through a blank, while the remaining beam passes through the sample. Thus, the machine can monitor the

difference between the two as the wavelength is altered. These instruments usually come with a motor driven mechanism for altering the wavelength, or scanning the sample.

The newer version of an instrument scans a blank, and places the digitalized information in computer memory. It then rescans a sample and compares the information from the sample scan to the information obtained from the blank scan. Since the information is digitalized, manipulation of the data is possible. These instruments usually have direct ports for connection to personal computers, and often have built in temperature controls as well. In these the voltage meter scale has given way to a CRT display, complete with graphics and built in functions for statistical analysis.

The most common spectrophotometer is used in the UV and visible regions of the spectrum and some of these instruments also operate into the near-infrared region. One major factor is the type of photo sensors that are available for different spectral regions. Usually, spectrophotometers use a monochromator to analyse the desired spectrum but there are also spectrophotometers that can use an array of photosensors.

The steps required in a spectrophotometer are as follows:

- The light is projected into a monochromator.

- A particular wavelength is selected and beamed at the sample in a cuvette.

- The sample absorbs light.

- The photo detector behind the sample responds to the light stimulus and converts it into an electronic current which is then transformed into a usable format.

- The results are either plotted straight away or fed into a computer and manipulated in different presentable forms.

Infrared spectrometer:

An **infrared spectrometer** directs infrared radiation through a sample and records the relative amount of energy absorbed by the sample as a function of the wavelength or frequency of the infrared radiation. The method is applicable particularly to organic materials, because the vibrational frequencies of the constituent groups within the molecules coincide with the electromagnetic frequencies of the infrared radiation. Therefore, the infrared radiation is selectively absorbed by the material to produce an absorption spectrum.

The spectrum produced is compared with correlation spectra from known substances. A sample cell for infrared spectrophotometry comprises a sample holder for holding a sample to be analysed by infrared spectrophotometry, a cool air passageway and a vortex tube. The sample holder includes a primary optical surface through which infrared radiation is directed to a sample contained in the holder, and the cool air passageway is adjacent to the primary optical surface of the sample holder for directing a cool air stream across the primary optical surface. The vortex tube has a cool air outlet connected to the cool air passageway for supplying cool air to the passageway. Infrared spectrophotometry is most commonly used in studying the molecular structures of complex organic compounds.

UV-Visible spectrophotometer:

The **UV-Visible spectrophotometer** uses two light sources, a deuterium (D2) lamp for ultraviolet light and a tungsten lamp for visible light. After bouncing off a mirror, the light beam passes through a slit

and hits a diffraction grating. The grating can be rotated allowing for a specific wavelength to be selected. At any specific orientation of the grating, only monochromatic (single wavelength) successfully passes through a slit. A filter is used to remove unwanted higher diffractions. The light beam hits a second mirror before it gets split by a half mirror in which half of the light is reflected, while the other half passes through.

One of the beams is allowed to pass through a reference cuvette which contains the solvent only, while the other beam passes through the sample cuvette. The intensities of the light beams are then measured at the end. UV/Visible spectroscopy is routinely used in the quantitative determination of solutions of transition metal ions and highly conjugated organic compounds. Organic compounds, especially those with a high degree of conjugation, also absorb light in the UV or visible regions of the electromagnetic spectrum. The solvents for these determinations are often water for water soluble compounds, or ethanol for organic-soluble compounds. Ultraviolet spectrophotometry is particularly useful in detecting colourless substances in solution and measuring their concentration.
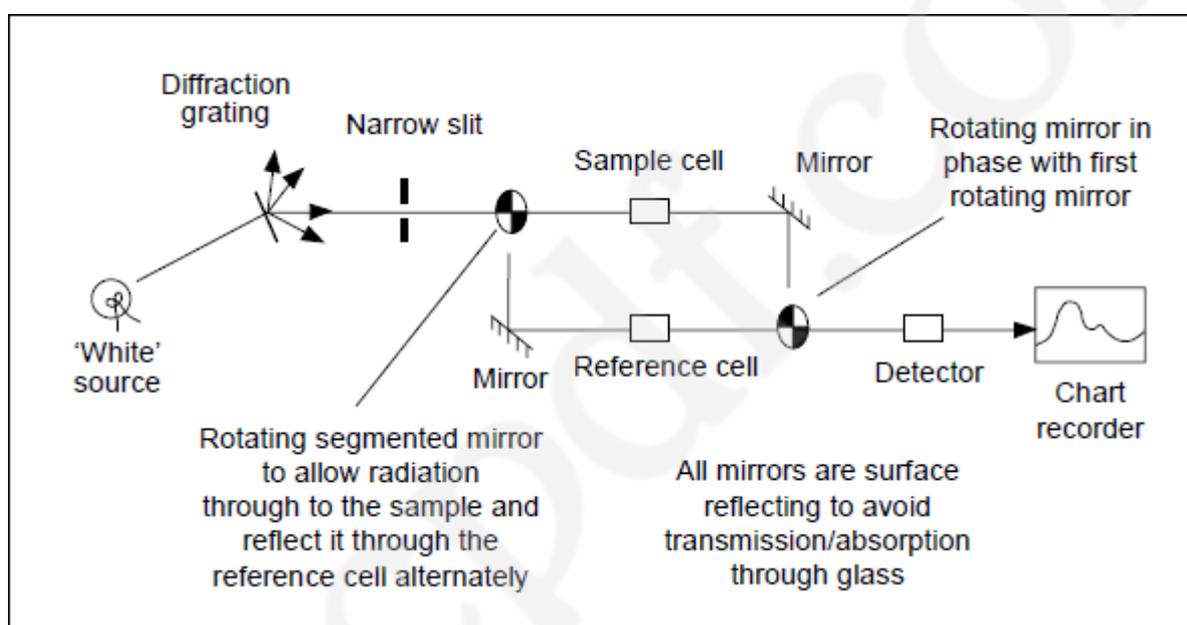
Laws of absorption of energy:

Two laws express the relationship between the absorption of radiant energy and the absorbing medium. According to **Bouguer's (or Lambert's) law,** each layer of equal thickness of the medium absorbs an equal fraction of the energy traversing it. According to **Beer's law,** the absorptive capacity of a dissolved substance is directly proportional to its concentration in a solution.

The change in the intensity of light after passing through a sample should be proportional to the following:

- Path length – the longer the path, more photons should be absorbed.

- Concentration of sample – more molecules absorbing means more photons absorbed.

- Intensity of the incident light – more photons means more opportunity for a molecule to see a photon.

The intensity of light, symbolized as I_0, is a measure of the number of photons per second. When the light is passed through the blank solution, it does not absorb light and is symbolized as (I). Another important factors are Absorbance (A) and Transmittance (T).

$T = I/I_0$

$A = -\log 10\ T$

The transmittance and absorption relation is:

Absorbance (A) = $-\log(T) = -\log(I/I_0)$

The transmittance of an unknown sample can be calculated using the formula given below.

Transmittance (T)= $I_{t/}\ I_0$

Here,

I_t= Light intensity after passing via cuvette

I_0 = Light intensity before passing via cuvette

Applications of Spectrophotometer:

1. Qualitative Analysis:

The visible and UV spectrophotometer may be used to identify classes of compounds in both the pure state and in biological preparations. This is done by plotting absorption spectrum curves. Absorption by a compound in different regions gives some hint about its structure.

2. Quantitative Analysis:

Spectrophotometer is used in the Quantitative analysis for determining an unknown concentration of a given compounds by absorption spectrometry. Most of the organic compounds of biological interest absorb light in the UV-visible range of the spectrum. Thus, a number of important classes of biological compounds may be measured semi-quantitatively using the UV-visible spectrophotometer. Nucleic acids at 254nm, protein at 280nm provide good examples of such use. The absorbance at 280nm by proteins depends on their "Tyrosine" and "Tryptophan" content.

3. Enzyme Assay:

This is the basic application of spectrophotometry. An assay is carried out most quickly and conveniently when the substrate or the product is coloured or absorbs light in the UV range e.g. Lactate Dehydrogenase (LDH) assay, Pyruvate Kinase assay.

4. Molecular Weight determination:

Molecular weights of amine picrates, sugars and many aldehyde and ketone compounds have been determined by this method. Molecular weight of only small molecules may be determined by this method.

    a. **Study of Cis-Trans Isomerism:** Geometrical isomers differ in the spatial arrangement of groups about a plane, the absorption spectra of the isomers also differs. The trans-isomer is usually more elongated than its cis counterpart. Absorption spectrometry can be utilized to study Cis-Trans isomerism.

b. **Control of Purification:** Impurities in a compound can be detected very easily by spectrophotometric studies. "Carbon disulphide" impurity in carbon tetrachloride can be detected easily by measuring absorbance.

5. Physiochemical Studies:

Spectrophotometry (UV-VIS) has been used to study the following physiochemical phenomena:

- Determination of empirical formulae

- Formation constants of complexes in solution

- Hydration equilibrium of carbonyl compounds

- Association constants of weak acids and bases in organic solvents

- Protein-dye interactions

- Chlorophyll-Protein complexes

- Determination of reaction rates

ELECTROPHORESIS

In the olden days, DNA fragments were laboriously separated by using gravity. In the 1970s, the powerful tool called DNA gel electrophoresis was developed, in which electricity was used to separate DNA fragments by size as they migrate through a porous gel matrix.

Gel electrophoresis is now used to sort out strands of DNA, RNA or protein molecules by size, by using agarose gel and electrical current. This is achieved by moving negatively charged DNA molecules through an agarose matrix to which an electric field is applied (electrophoresis). Shorter molecules move faster and migrate farther than the longer ones, thus separating them by size. DNA Gel electrophoresis is generally used after amplification of DNA using PCR technique. It is done in the following steps:

- To prepare gel, agarose powder is mixed with electrophoresis buffer to the desired concentration and then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (0.5 ug/ml) to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature. Gel casting trays are available in a variety of sizes and are made of UV-transparent plastic.

- After the gel has solidified, the comb is removed. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with buffer. The buffer conducts the electric current and keeps the gel from drying out. Electrophoresis buffer is usually Tri-acetate-EDTA (TAE) or Tri-borate-EDTA (TBE).

- Loading buffer that contains dye is added to the DNA sample. Micropipette is used to load DNA sample mixed with the loading buffer into the first well of the agarose gel. Loading buffer contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.

- Micropipette is used to load DNA size standard into the next well of the agarose gel. The DNA size standard contains strands of known length.

- Electrophoresis chamber or box is connected to the electric current. The negative (black) end of the current must be placed on the end closest to the wells.

- Upon switching the electric current, the DNA strands move away from the negative current. The short strands move through the gel more quickly than the long strands. This can be observed due to the blue dye of the loading buffer.

- You can confirm that the current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually coloured red.

- Current is turned off and the gel mould is removed from the electrophoresis box.

- The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively.

- Gel is placed in the DNA staining solution – ethidium bromide. This binds to DNA and can be viewed under fluorescent light. Ethidium bromide is a fluorescent dye used for staining nucleic acids so that they become visible.

- Gel is removed from ethidium bromide after about 30 minutes and placed on UV light box called transilluminator. The DNA bands will be visible for both the DNA sample and DNA size standard.
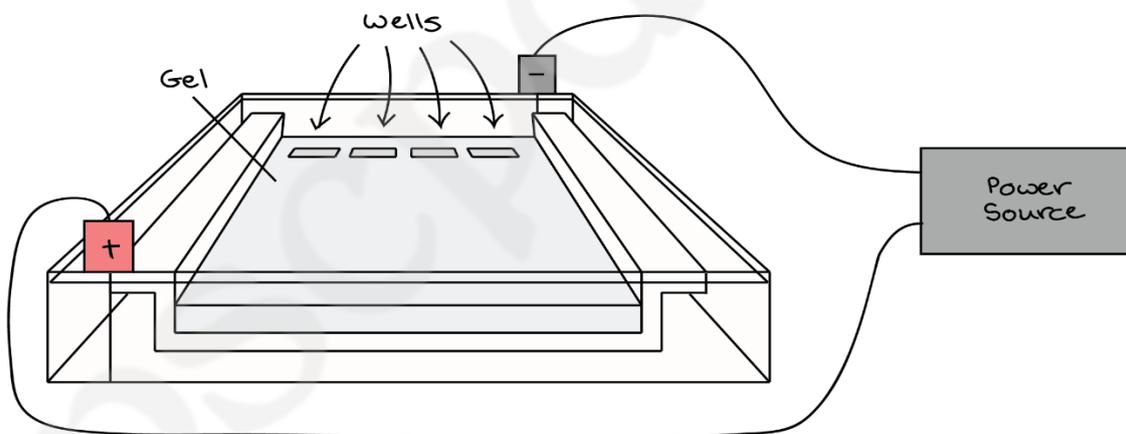
Migration of DNA Fragments in Agarose

Fragments of linear DNA migrate through the agarose gels with a mobility that is inversely proportional to the log10 of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the log10 of either their molecular weights or number of base pairs, a roughly straight line will appear.

Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass. Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized. Additionally, most preparations of uncut plasmid contain at least two topologically different forms of DNA, corresponding to super coiled forms and nicked circles.

Agarose Concentration

By using gels with different concentrations of agarose, one can resolve different sizes of DNA fragments. Higher concentrations of agarose facilitate separation of small DNA fragments, while low agarose concentrations allow resolution of larger DNA fragments.

Voltage

As the voltage applied to a gel is increased, larger fragments migrate proportionally faster than smaller fragments. For that reason, the best resolution of fragments larger than about 2 kb is attained by applying no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).

Electrophoresis Buffer

Several different buffers have been recommended for electrophoresis of DNA. The most commonly used for duplex DNA are **TAE** (Tri-acetate-EDTA) and **TBE** (Tri-borate-EDTA). DNA fragments will migrate at somewhat different rates in these two buffers due to differences in ionic strength. Buffers not only establish a pH, but provide ions to support conductivity. If you mistakenly use water instead of buffer, there will be essentially no migration of DNA in the gel. Conversely, if you use concentrated buffer (e.g. a 10X stock solution), enough heat may be generated in the gel to melt it.

Effects of Ethidium Bromide

Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels. It can be incorporated into agarose gels or added to samples of DNA before loading to enable visualization of the fragments within the gel. As might be expected, binding of ethidium bromide to DNA alters its mass and rigidity, and therefore its mobility.

Applications:

Agarose gel electrophoresis technique was extensively used for investigating the DNA cleavage efficiency of small molecules and as a useful method to investigate various binding modes of small molecules to supercoiled DNA. This was mainly due to the importance of DNA cleavage in drug designing.
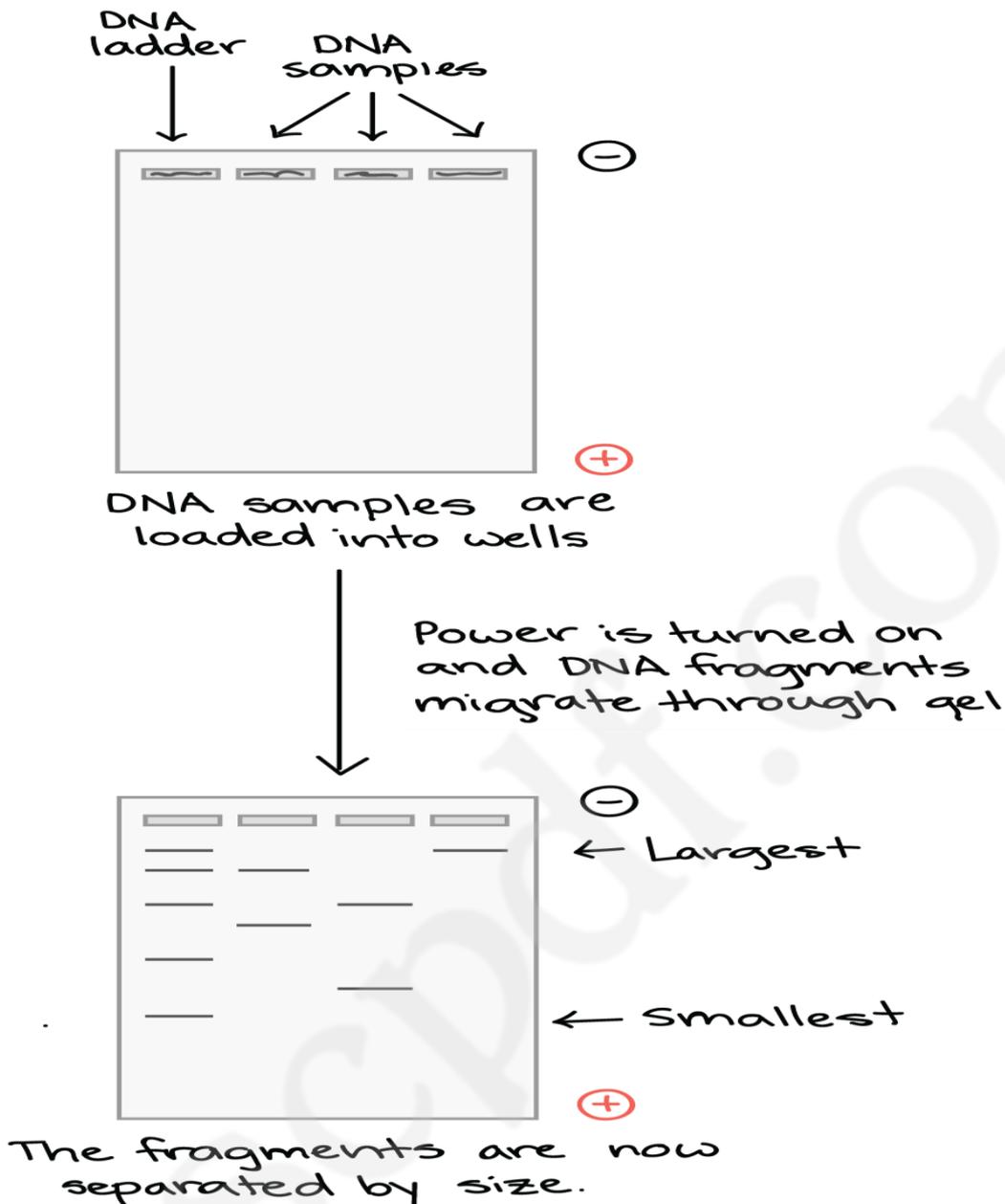
DNA can be separated by electrophoresis to:

1. Visualize bands of a molecular marker to genotype individual plants
2. Verify amplification by PCR or sequencing reactions
3. Check the quality and quantity of genomic DNA after DNA extraction
4. Separate DNA fragments to clone a specific band

Gel electrophoresis can also be used for DNA fingerprinting for forensics.

Advantage: easily processed and sample can be recovered without harm; DNA is not denatured

Disadvantage: may melt when electric current is passed, genetic material can change shape

DNA ladder

DNA samples

DNA samples are loaded into wells

Power is turned on and DNA fragments migrate through gel

← Largest

← Smallest

The fragments are now separated by size.

PAGE (Polyacrylamide Gel Electrophoresis)

PAGE (Polyacrylamide Gel Electrophoresis), is an analytical method used to separate components of a protein mixture based on their size. 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. To overcome this, the biological samples needs to be treated so that they acquire uniform charge, then the electrophoretic mobility depends primarily on size. For this different protein molecules with different shapes and sizes, needs to be denatured (done with the aid of SDS) so that the proteins lose their secondary, tertiary or quaternary structure .The proteins being covered by SDS are negatively charged and when loaded onto a gel and placed in an electric field, will migrate towards the anode (positively charged electrode) and are separated by a molecular sieving effect based on size. After the visualization by a staining

(protein-specific) technique, the size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder (marker).

Separation of charged molecules in an electric field is based on the relative mobility of charged species which is related to frictional resistance.

PAGE works upon the principle in which, the charged molecule will migrate towards the oppositely charged electrode through highly cross linked matrix. Separation occurs due to different rates of migration which occurs by the magnitude of charge and frictional resistance related to the size.

Relative Mobility:

$$R_f = \frac{ZE}{F}$$

Where,

Z = charge on the molecule

E = Voltage applied

f = frictional resistance

Rf is measured by:

$$R_f = \frac{\text{Distance protein band moves}}{\text{Distance dye front moves.}}$$

Direction of movement is determined from Z

if Z < 0, then →+

if Z > 0, then → -

if Z = 0, then no movement

The gel used is divided into an upper "stacking" gel of low percentage (with large pore size) and low pH (6.8), where the protein bands get squeezed down as a thin layer migrating toward the anode and a resolving gel (pH 8.8) with smaller pores. Cl⁻ is the only mobile anion present in both gels. When electrophoresis begins, glycine present in the electrophoresis buffer, enters the stacking gel, where the equilibrium favours zwitterion form with zero net charge. The glycine front moves through the stacking gel slowly, lagging behind the strongly charged, Cl- ions. Since these two current carrying species separate, a region of low conductivity, with high voltage drop, is formed between them. This zone sweeps the proteins through the large pores of the stacking gel, and depositing it at the top of the resolving gel as a narrow band.

Stacking gel interactions:

Stacking occurs by the differential migration of ionic species, which carry the electric current through the gel. When an electrical current is applied to the gel, the negatively charged molecules start migrating to the positively charged electrode. Cl- ions, having the highest charge/mass ratio move faster, being depleted and concentrated at anode end. SDS coated proteins has a higher charge/mass ratio than glycine so it moves fast, but slower than Cl-. When protein encounters resolving gel it slows the migration because of increased frictional resistance, allowing the protein to stack in the gel.

Resolving Gel Interactions:

When glycine reaches resolving gel it becomes negatively charged and migrates much faster than protein due to higher charge/mass ratio. Now proteins are the main carrier of current and separate according to their molecular mass by the sieving effect of pores in gel.

TRANSMISSION ELECTRON MICROSCOPE

In transmission electron microscope (TEM), a beam of electrons is transmitted through the section of a specimen and an image is formed by the interaction of the electrons transmitted through the specimen. The image is magnified and focused onto an imaging device, such as a fluorescent screen or a photographic film, or to the computer screen.

TEM operates on the same basic principles as the light microscope but uses electrons instead of light, which makes it possible to get a resolution a thousand times better than with a light microscope. It was developed by **Max Knoll** and **Ernst Ruska** in Germany in 1931. Reinhold Rudenberg, the scientific director of Siemens, had patented the electron microscope in 1931, stimulated by family illness to make the poliomyelitis virus particle visible. Siemens produced the first commercial Transmission Electron Microscope (TEM) in 1939, but the first practical electron microscope had already been built at the University of Toronto in 1938, by Eli Franklin Burton, Cecil Hall, James Hillier and Albert Prebus.

By another analogy, a TEM works like a slide projector. A projector shines a beam of light through the slide and as the light passes through, it is affected by the object on the slide. This transmitted beam is then projected onto the viewing screen, forming an enlarged image of the slide.

Electron microscopes have much greater resolving power than light microscopes and use electromagnetic radiation that can obtain much higher magnifications of up to 2 million times, while the best light microscopes are limited to magnifications of 2000 times. Unlike Scanning Electron Microscope (SEM) that bounces electrons off the surface of a sample to produce an image, Transmission Electron Microscopes (TEM) shoots the electrons completely through the sample.

A source at the top of the microscope emits electrons that travel through vacuum in the column of the microscope. Instead of glass lenses focusing is done by electromagnetic lenses and the electrons are focussed into a very thin beam. The electron beam then travels through the specimen you want to study. Depending on the density of the material present, some electrons are scattered and disappear from the beam and un-scattered electrons hit a fluorescent screen, which produces the image of the specimen with its different parts displayed in varied darkness according to their density. The image can be studied directly by the operator or photographed with a camera.

TEM WORKING

- The "Virtual Source" at the top represents the electron gun, producing a stream of monochromatic electrons. Electrons are charged particles, and because collision with molecules of air will absorb and deflect electrons and distort the beam, the optical system of an electron microscope must be evacuated of air. The electron source is produced by heating a tungsten filament at voltages usually ranging from 6,000 to 10,000 Volts. Because electron beams are invisible to the eye, the images they form are revealed on a fluorescent screen and can then be photographed.

- This stream is focused to a narrow beam by the use of condenser lenses. The first lens largely determines the "spot size"; the general size range of the final spot that strikes the sample.

The second lens is controlled by the brightness knob and actually changes the size of the spot on the sample; changing it from a wide dispersed spot to a pinpoint beam.

- The beam is restricted by the condenser aperture, knocking out high angle electrons.

- The beam strikes the specimen and parts of it are transmitted. The specimen must be extremely thin for the electrons to pass through and create an image. Ultra-thin sections are approximately 0.01um (100nm) thick, and are cut on an ultra-microtome. Because ultra-thin sections have little contrast, they must be stained with electron-absorbing heavy metal salts to provide contrast necessary to reveal details of the cells ultra-structure.

- This transmitted portion is focused by the objective lens into an image.

- Optional Objective and selected area metal apertures can restrict the beam, the objective aperture enhancing contrast by blocking out high-angle diffracted electrons, the selected area aperture enabling the user to examine the periodic diffraction of electrons by ordered arrangements of atoms in the sample.

- The image is passed down the column through the intermediate and projector lenses and is enlarged all the way.

- The image strikes the phosphorescent image screen and light is generated, allowing the user to see the image. The darker areas of the image represent those areas of the sample that fewer electrons were transmitted through, meaning that they are thicker or denser. The lighter areas of the image represent those areas of the sample that more electrons were transmitted through, meaning that they are thinner or less dense. Fluorescent viewing screen is coated with a phosphor or scintillator material such as zinc sulphide.
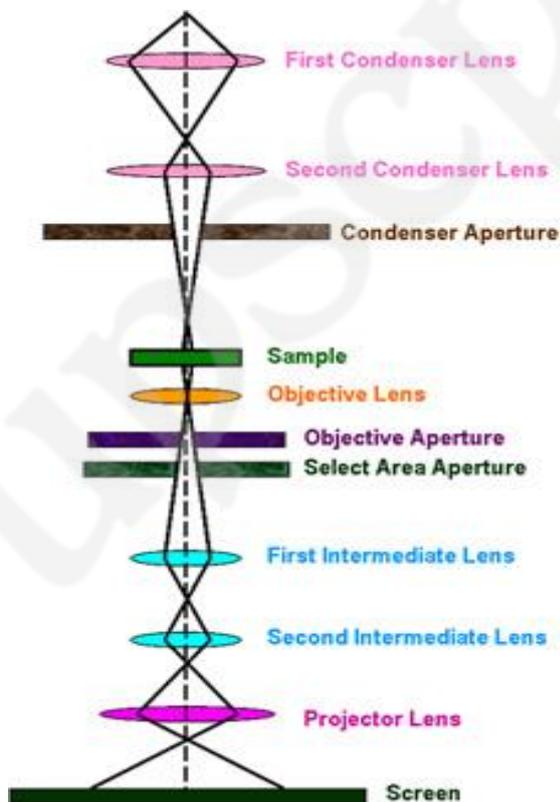
SAMPLE PREPARATION

1. Fixation: The first step in sample preparation has the aim of preserving tissue in its original state. Fixatives must be buffered to match the pH and osmolality of the living tissue. Glutaraldehyde is the most commonly used primary fixative. It penetrates rapidly and stabilizes proteins by forming cross links, but does not fix lipids. Osmium tetroxide is used as a secondary fixative, reacting with lipids and acting as a stain. Following each fixation step, excess fixative must be washed out of the tissue.

2. Dehydration: Biological material contains large quantities of water. Since the TEM works in vacuum, the water must be removed. This is carried out using a graded ethanol series. To avoid disruption as a result of the loss of water, you preserve the tissue with different fixatives. These cross-link molecules with each other and trap them together as stable structures. The tissue is then dehydrated in alcohol or acetone.

3. Infiltration and Embedding in resin: The sample is infiltrated with a resin before being placed in an embedding mould, which is then polymerised in an oven at 60°C.

4. Sections of Embedded Material: Specimen can be embedded in plastic that polymerize into a solid hard plastic block. The block is cut into thin sections by a diamond knife in an instrument called ultra-microtome. Each section is only 50-100 nm thick. The thin sections of your sample is placed on a copper grid and stained with heavy metal

5. Negative Staining of Isolated Material: The isolated material that can be a solution with bacteria, is spread onto a support grid coated with plastic. A solution of heavy metal salt is added. The metal salt solution does not bind to the material but forms a "shadow" around it on the grid. The specimen will appear as a negative picture when viewing it in the TEM.

TEM Applications

- A Transmission Electron Microscope is ideal for a number of different fields such as life sciences, nanotechnology, medical, biological and material research, forensic analysis, gemology and metallurgy as well as industry and education.
- TEMs provide topographical, morphological, compositional and crystalline information.
- The images allow researchers to view samples on a molecular level, making it possible to analyse structure and texture. This information is useful in the study of crystals and metals, and also has industrial applications.
- TEMs can be used in semiconductor analysis and production and the manufacturing of computer and silicon chips.
- Technology companies use TEMs to identify flaws, fractures and damages to micro-sized objects; this data can help fix problems and/or help to make a more durable, efficient product.
- Colleges and universities can utilize TEMs for research and studies.

Advantages

- TEMs offer the most powerful magnification, potentially over one million times or more

- TEMs have a wide-range of applications and can be utilized in a variety of different scientific, educational and industrial fields

- TEMs provide information on element and compound structure

- Images are high-quality and detailed

- TEMs are able to yield information of surface features, shape, size and structure

- They are easy to operate with proper training

Disadvantages

- TEMs are large and very expensive

- Laborious sample preparation

- Operation and analysis requires special training

- Samples are limited to those that are electron transparent, able to tolerate the vacuum chamber and small enough to fit in the chamber

- TEMs require special housing and maintenance

- Images are black and white

- Electron microscopes are sensitive to vibration and electromagnetic fields and must be housed in an area that isolates them from possible exposure

- A Transmission Electron Microscope requires constant upkeep including maintaining voltage, currents to the electromagnetic coils and cooling water

SCANNING ELECTRON MICROSCOPE

The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The SEM's job is to use an electron beam to trace over the object, creating an exact replica of the original object on a monitor. As the electron beam traces over the object, it interacts with the surface of the object, dislodging secondary electrons from the surface of the specimen in a unique pattern.

A secondary electron detector attracts those scattered electrons and, depending on the number of electrons that reach the detector, registers different levels of brightness on a screen. The scanning electron microscope has many advantages over traditional microscopes.

The SEM has a large depth of field, which allows more of a specimen to be in focus at one time, producing strikingly clear images. The SEM also has much higher resolution, so closely spaced specimens can be magnified at much higher levels. Because the SEM uses electromagnets rather than lenses, the researcher has much more control over the degree of magnification.

The first SEM image was obtained by **Max Knoll,** who in 1935 obtained an image of silicon steel showing electron channelling contrast. Subsequently **M. von Ardenne** (1938) constructed a scanning transmission electron microscope by adding scan coils to a transmission electron microscope. The SEM was further developed by Professor Sir **Charles Oatley** and **Gary Stewart** in 1965. The first SEM used to examine the surface of a solid specimen was described by **Zworykin** et al. (1942), who was working in the RCA Laboratories in the United States.

**Principles of Scanning Electron Microscopy**

Accelerated electrons in an SEM carry significant amounts of kinetic energy, and this energy is dissipated as a variety of signals produced by electron-sample interactions when the incident electrons are decelerated in the solid sample. These signals include:

a) secondary electrons that produce SEM images
b) backscattered electrons (BSE) and diffracted backscattered electrons (EDS) that are used to determine crystal structures and orientation of minerals
c) photons - characteristic X-rays that are used for elemental analysis and continuum X-rays
d) visible light (cathodoluminescence – CL)
e) and heat

Secondary electrons and backscattered electrons are commonly used for imaging samples: secondary electrons are most valuable for showing morphology and topography on samples and backscattered electrons are most valuable for illustrating contrasts in composition in multiphase samples. X-ray generation is produced by inelastic collisions of the incident electrons with electrons in discrete shells of atoms in the sample. As the excited electrons return to lower energy states, they yield X-rays that are of a fixed wavelength. SEM analysis is considered to be "non-destructive"; that is, X-rays generated by electron interactions do not lead to volume loss of the sample, so it is possible to analyse the same materials repeatedly.

The SEM can produce very high-resolution images of a sample surface that can be magnified up to 300,000 times the size of the object, revealing details about 1 to 5 nm in size. Due to the way these images are created, SEM micrographs have a very large depth of field yielding a characteristic three-dimensional appearance useful for understanding the surface structure of a sample but SEMs cannot produce colour images.

COMPONENTS OF A SCANNING ELECTRON MICROSCOPE
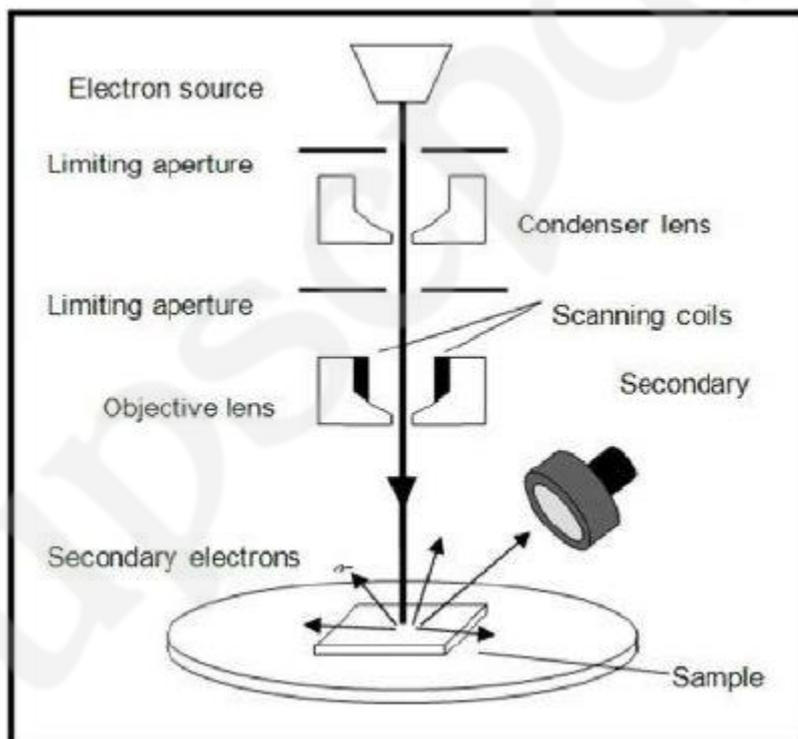
Electron gun produces steady stream of electrons necessary for SEMs to operate. Electron guns are typically one of the two types: **Thermionic guns**, which apply thermal energy to a filament (usually made of tungsten) to detach electrons away from the gun and toward the specimen. **Field emission guns**, on the other hand, create a strong electrical field to pull electrons away from the atoms they

are associated with. The anode, which is positive with respect to the filament, forms powerful attractive forces for electrons. This causes electrons to accelerate toward the anode.

When a SEM is used, the column must always be at a vacuum. There are many reasons for this. If the sample is in a gas filled environment, an electron beam cannot be generated or maintained because of a high instability in the beam. Gases could react with the electron source, causing it to burn out, or cause electrons in the beam to ionize, which produces random discharges and leads to instability in the beam. The transmission of the beam through the electron optic column would also be hindered by the presence of other molecules.

Lenses: SEMs use lenses to produce clear and detailed images but the lenses work differently and they are made of magnets capable of bending the path of electrons. By doing so, the lenses focus and control the electron beam, ensuring that the electrons end up precisely where they are needed.

Sample chamber: The sample chamber of an SEM is where specimen is placed in a vacuum. Because the specimen must be kept extremely still for the microscope to produce clear images, the sample chamber must be very sturdy and insulated from vibration. In fact, SEMs are so sensitive to vibrations that they're often installed on the ground floor of a building. They also manipulate the specimen, placing it at different angles and moving it so that researchers don't have to constantly remount the object to take different images.

Detectors: These devices detect the various ways that the electron beam interacts with the sample object. For instance, **Everhart-Thornley detectors** register secondary electrons, which are electrons dislodged from the outer surface of a specimen. These detectors are capable of producing the most detailed images of an object's surface. Other detectors, such as **backscattered electron detectors** and **X-ray detectors**, can tell researchers about the composition of a substance.

Vacuum chamber: SEMs require a vacuum to operate. Without a vacuum, the electron beam generated by the electron gun would encounter constant interference from air particles. Not only would these particles block the path of the electron beam, they would also be knocked out of the air and onto the specimen, which would distort the surface of the specimen.

Scanning coils create a magnetic field using fluctuating voltage, to manipulate the electron beam. The scanning coils are able to move the beam precisely back and forth over a defined section of an object. If a researcher wants to increase the magnification of an image, he or she simply sets the electron beam to scan a smaller area of the sample.

SAMPLE PREPARATION

Sample preparation can be minimal or elaborate for SEM analysis, depending on the nature of the samples and the data required. Minimal preparation includes acquisition of a sample that will fit into the SEM chamber and some accommodation to prevent charge build-up on electrically insulating samples. Most electrically insulating samples are coated with a thin layer of conducting material, commonly carbon, gold, or some other metal or alloy. Carbon is most desirable if elemental analysis is a priority, while metal coatings are most effective for high resolution electron imaging applications. Alternatively, an electrically insulating sample can be examined without a conductive coating in an instrument capable of "low vacuum" operation.

The **sputtercoater** uses argon gas and a small electric field. The sample is placed in a small chamber which is at vacuum. Argon gas is then introduced and an electric field is used to cause an electron to be removed from the argon atoms to make the atoms ions with a positive charge. The Argon ions are then attracted to a negatively charged piece of gold foil. The Argon ions act like sand in a sandblaster,

knocking gold atoms from the surface of the foil. These gold atoms now settle onto the surface of the sample, producing a gold coating.

Conductive materials in current use for specimen coating include gold, gold/palladium alloy, platinum, osmium, iridium, tungsten, chromium and graphite. Coating prevents the accumulation of static electric charge on the specimen during electron irradiation.

SCANNING PROCESS

The electron beam, which typically has an energy ranging from a few hundred eV to 40 keV, is focused by one or two condenser lenses to a spot about 0.4 nm to 5 nm in diameter. The beam passes through pairs of scanning coils or pairs of deflector plates in the electron column, typically in the final lens, which deflect the beam in the x and y axes so that it scans in a raster fashion over a rectangular area of the sample surface.

The energy exchange between the electron beam and the sample results in the reflection of high-energy electrons by elastic scattering, emission of secondary electrons by inelastic scattering and the emission of electromagnetic radiation, each of which can be detected by specialized detectors. The beam current absorbed by the specimen can also be detected and used to create images of the distribution of specimen current.

The raster scanning of the CRT display is synchronised with that of the beam on the specimen in the microscope, and the resulting image is therefore a distribution map of the intensity of the signal being emitted from the scanned area of the specimen.

Unlike optical and transmission electron microscopes, image magnification in the SEM is not a function of the power of the objective lens. SEMs may have condenser and objective lenses, but their function is to focus the beam to a spot, and not to image the specimen. In an SEM, magnification results from the ratio of the dimensions of the raster on the specimen and the raster on the display device. Magnification is therefore controlled by the current supplied to the x,y scanning coils, and not by objective lens power.

A scanning device near the bottom of the vacuum chamber controls the movement of the electron beam across the specimen, row by row. As the electron beam sweeps the surface, it excites electrons present in the atomic structure of molecules, causing some of them to escape from the surface. These escaping electrons, known as deflected secondary electrons, have specific energies that can be measured. As they are released from each area of the sample, they are collected and counted by a detector that sends their amplified signals. The various electronic energies produced are analysed by computer software, and the resulting image is displayed on a computer monitor.

SEM Applications

SEMs have a variety of applications in a number of scientific and industry-related fields, especially where characterizations of solid materials is beneficial.

In addition to topographical, morphological and compositional information, a Scanning Electron Microscope can detect and analyse surface fractures, provide information in microstructures, examine surface contaminations, reveal spatial variations in chemical compositions, provide qualitative chemical analyses and identify crystalline structures.

SEMs can be as essential research tool in fields such as life science, biology, gemology, medical and forensic science, and metallurgy.

In addition, SEMs have practical industrial and technological applications such as semiconductor inspection, production line of miniscule products and assembly of microchips for computers.

SEM Advantages

- Advantages of a Scanning Electron Microscope include its wide-array of applications, the detailed three-dimensional and topographical imaging and the versatile information garnered from different detectors.
- SEMs are also easy to operate with the proper training and advances in computer technology and associated software make operation user-friendly.
- This instrument works fast, often completing SEI, BSE and EDS analyses in less than five minutes. In addition, the technological advances in modern SEMs allow for the generation of data in digital form.
- Although all samples must be prepared before placed in the vacuum chamber, most SEM samples require minimal preparation actions.

SEM Disadvantages

- SEMs are expensive, large and must be housed in an area free of any possible electric, magnetic or vibration interference.
- Maintenance involves keeping a steady voltage, currents to electromagnetic coils and circulation of cool water.
- Special training is required to operate an SEM as well as prepare samples.
- The preparation of samples can result in artefacts. The negative impact can be minimized with knowledgeable experienced researchers being able to identify artefacts from actual data as well as preparation skill. There is no absolute way to eliminate or identify all potential artefacts.
- In addition, SEMs are limited to solid, inorganic samples small enough to fit inside the vacuum chamber that can handle moderate vacuum pressure.
- Finally, SEMs carry a small risk of radiation exposure associated with the electrons that scatter from beneath the sample surface.
- The sample chamber is designed to prevent any electrical and magnetic interference, which should eliminate the chance of radiation escaping the chamber. Even though the risk is minimal, SEM operators and researchers are advised to observe safety precautions.

PHASE CONTRAST MICROSCOPY

Phase contrast microscopy, first described in 1934 by Dutch physicist Frits Zernike, is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens, such as living cells (usually in culture), microorganisms, thin tissue slices, lithographic patterns, fibres, latex dispersions, glass fragments, and subcellular particles (including nuclei and other organelles).

In effect, the phase contrast technique employs an optical mechanism to translate minute variations in phase into corresponding changes in amplitude, which can be visualized as differences in image contrast. One of the major advantages of phase contrast microscopy is that living cells can be examined in their natural state without previously being killed, fixed, and stained. As a result, the dynamics of ongoing biological processes can be observed and recorded in high contrast with sharp clarity of minute specimen detail.

Figure 4 - Phase Contrast Microscope Optical Train
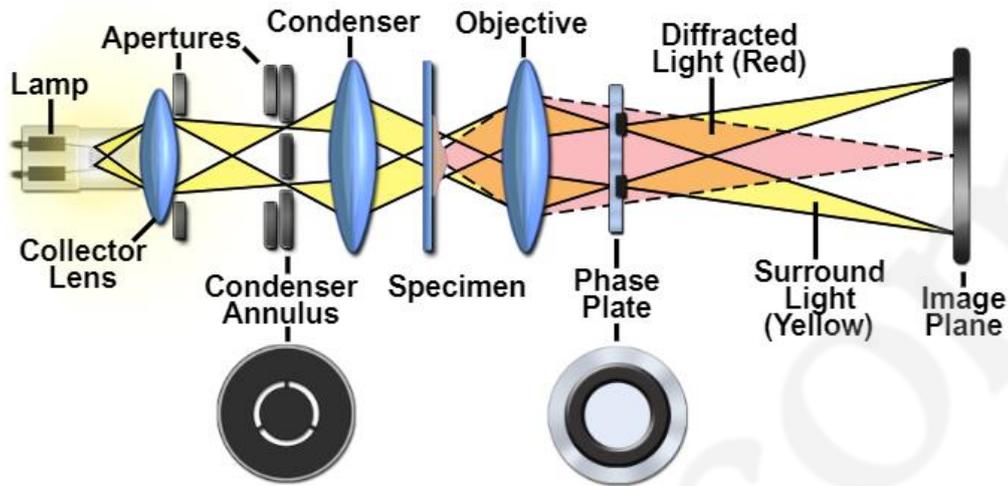
Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (condenser annulus). Wave-fronts passing through the annulus illuminate the specimen and either pass through undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen. Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a phase plate and focused at the intermediate image plane to form the final phase contrast image observed in the eyepieces.

Prior to the invention of phase contrast techniques, transmitted bright-field illumination was one of the most commonly utilized observation modes in optical microscopy, especially for fixed, stained specimens or other types of samples having high natural absorption of visible light. Collectively, specimens readily imaged with bright-field illumination are termed amplitude objects (or specimens) because the amplitude or intensity of the illuminating wave-fronts is reduced when light passes through the specimen.

The addition of phase contrast optical accessories to a standard bright-field microscope can be employed as a technique to render a contrast-enhancing effect in transparent specimens that is reminiscent of optical staining.

Light waves that are diffracted and shifted in phase by the specimen (termed a phase object) can be transformed by phase contrast into amplitude differences that are observable in the eyepieces. Large, extended specimens are also easily visualized with phase contrast optics due to diffraction and scattering phenomena that occur at the edges of these objects. The performance of modern phase contrast microscopes is so refined that it enables specimens containing very small internal structures, or even just a few protein molecules, to be detected when the technology is coupled to electronic enhancement and post-acquisition image processing.

Modern phase contrast objectives, designed and produced by Nikon and other optical manufacturers, are capable of operating in combination with auxiliary contrast-enhancing techniques, such as differential interference contrast, fluorescence, and polarized light. These objectives are available with internal phase plates that have varying levels of absorption and phase displacement of the surround (undiffracted) illumination to produce a wide spectrum of specimen contrast and background intensity choices for phase contrast microscopy.

Interaction of Light Waves with Phase Specimens

An incident wavefront present in an illuminating beam of light becomes divided into two components upon passing through a phase specimen. The primary component is an undeviated (or undiffracted; zeroth-order) planar wavefront, commonly referred to as the surround (S) wave, which passes through and around the specimen, but does not interact with it. In addition, a deviated or diffracted spherical wavefront (D-wave) is also produced, which becomes scattered over a wide arc (in many directions) that passes through the full aperture of the objective. After leaving the specimen plane, surround and diffracted light waves enter the objective front lens element and are subsequently focused at the intermediate image plane where they combine through interference to produce a resultant particle wave (often referred to as a P-wave). The mathematical relationship between the various light waves generated in phase contrast microscopy can be described simply as:

P = S + D

Detection of the specimen image depends on the relative intensity differences, and therefore on the amplitudes, of the particle and surround (P and S) waves. If the amplitudes of the particle and surround waves are significantly different in the intermediate image plane, then the specimen acquires a considerable amount of contrast and is easily visualized in the microscope eyepieces. Otherwise, the specimen remains transparent and appears as it would under ordinary brightfield conditions (in the absence of phase contrast or other contrast-enhancing techniques).

In terms of optical path variations between the specimen and its surrounding medium, the portion of the incident light wavefront that traverses the specimen (D-wave), but does not pass through the surrounding medium (S-wave), is slightly retarded. For arguments in phase contrast microscopy, the role of the specimen in altering the optical path length (in effect, the relative phase shift) of waves passing through is of paramount importance. In classical optics, the optical path length (OPL) through an object or space is the product of the refractive index (n) and the thickness (t) of the object or intervening medium as described by the relationship:

Optical Path Length (OPL) = n × t

When light passes from one medium into another, the velocity is altered proportionally to the refractive index differences between the two media. Thus, when a coherent light wave emitted by the focused microscope filament passes through a phase specimen having a specific thickness (**t**) and refractive index (**n**), the wave is either increased or decreased in velocity. If the refractive index of the specimen is greater than that of the surrounding medium, the wave is reduced in velocity while passing through the specimen and is subsequently retarded in relative phase when it emerges from the specimen. In contrast, when the refractive index of the surrounding medium exceeds that of the specimen, the wave is advanced in phase upon exiting the specimen. The difference in location of an emergent wavefront between the specimen and surrounding medium is termed the **phase shift** (δ).

$\delta = 2\pi\Delta/\lambda$

Optical Path Difference (OPD) = Δ = (n2 - n1) × t

Where, n2 is the refractive index of the specimen and n1 is the refractive index of the surrounding medium

The Phase Contrast Microscope

The most important concept underlying the design of a phase contrast microscope is the segregation of surround and diffracted wavefronts emerging from the specimen, which are projected onto

different locations in the objective rear focal plane (the diffraction plane at the objective rear aperture). In addition, the amplitude of the surround (undeviated) light must be reduced and the phase advanced or retarded (by a quarter wavelength) in order to maximize differences in intensity between the specimen and background in the image plane. The mechanism for generating relative phase retardation is a two-step process, with the diffracted waves being retarded in phase by a quarter wavelength at the specimen, while the surround waves are advanced (or retarded) in phase by a phase plate positioned in or very near the objective rear focal plane. Only two specialized accessories are required to convert a brightfield microscope for phase contrast observation. A specially designed annular diaphragm, which is matched in diameter and optically conjugate to an internal phase plate residing in the objective rear focal plane, is placed in the condenser front focal plane.

Positive and negative phase contrast

Figure 6 - Positive and Negative Phase Contrast Systems
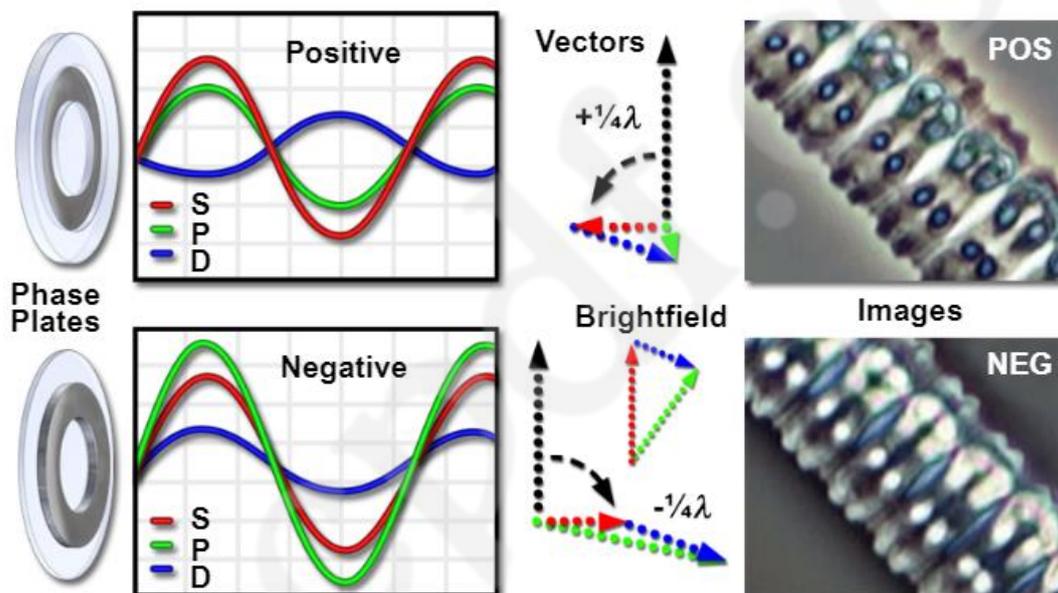
Positive phase contrast plates advance the surround wave by a quarter-wavelength due to the etched ring in the glass plate that reduces the physical path taken by the waves through the high refractive index plate. Because the diffracted specimen rays (D waves) are retarded by a quarter-wavelength when interacting with the specimen, the optical path difference between the surround and diffracted waves upon emergence from the phase plate is one-half wavelength. The net result is a 180-degree optical path difference between the surround and diffracted waves, which results in destructive interference for a high refractive index specimen at the image plane. The amplitude of the resultant particle (P) wave is lower than the surround (S) wave, causing the object to appear relatively darker than the background.

In negative phase contrast, the surround (S) wave is retarded (rather than being advanced) by a quarter-wavelength relative to the diffracted (D) wave. The result is that specimens having high refractive indices appear bright against a darker gray background. In negative phase contrast, the objective phase plate contains an elevated ring that retards the phase (rather than advancing the phase as in positive phase contrast) of the zeroth-order surround wave by a quarter-wavelength relative to the phase of the diffracted wave. Because the diffracted wave has already been retarded

by a quarter-wavelength when passing through the specimen, the optical path difference between the surround and diffracted waves is eliminated, and constructive interference occurs for a high refractive index specimen at the image plane. Note that the resulting particle (P) wave is higher in amplitude than the surround (S) wave in negative phase contrast.

Uses of phase contrast microscopy

- Phase contrast is an excellent method for enhancing the contrast of thin, transparent specimens without loss of resolution, and has proven to be a valuable tool in the study of dynamic events in living cells.
- Examine living cells, tissues, and microorganisms that are transparent
- Widely employed in diagnosis of tumor cells and the growth, dynamics, and behavior of a wide variety of living cells in culture
- Tissue culture investigation
- Applications in hematology, virology, bacteriology, parasitology, paleontology, and marine biology
- Industrial and chemical applications for phase contrast include mineralogy, crystallography, and polymer morphology investigations
- Colorless microcrystals, powders, particulate solids, and crystalline polymers, having a refractive index that differs only slightly from that of the surround immersion liquid, are often easily observed using phase contrast microscopy
- Obtain refractive index values
- Scrutinize clays, fats, oils, soaps, paints, pigments, foods, drugs, textiles, and other fibres
- Useful for examination of surfaces, including integrated circuits, crystal dislocations, defects, and lithography. A good example is the stacking faults in silicon epitaxial wafers, which are of tremendous significance to the semiconductor industry
- Phase contrast is often utilized with fluorescence imaging to determine the locations of fluorophores, and shows promise for enhancing contrast in scanning optical microscopy.

FLUORESCENCE MICROSCOPY

The absorption and subsequent re-radiation of light by organic and inorganic specimens is typically the result of well-established physical phenomena described as being either fluorescence or phosphorescence. The emission of light through the fluorescence process is nearly simultaneous with the absorption of the excitation light due to a relatively short time delay between photon absorption and emission, ranging usually less than a microsecond in duration. When emission persists longer after the excitation light has been extinguished, the phenomenon is referred to as phosphorescence.

The technique of fluorescence microscopy has become an essential tool in biology and the biomedical sciences, as well as in materials science due to attributes that are not readily available in other contrast modes with traditional optical microscopy. The application of an array of fluorochromes has made it possible to identify cells and sub-microscopic cellular components with a high degree of specificity amid non-fluorescing material. In fact, the fluorescence microscope is capable of revealing the presence of a single molecule. Through the use of multiple fluorescence labeling, different probes can simultaneously identify several target molecules simultaneously. Although the fluorescence microscope cannot provide spatial resolution below the diffraction limit of specific specimen features, the detection of fluorescing molecules below such limits is readily achieved.

A variety of specimens exhibit autofluorescence (without the application of fluorochromes) when they are irradiated, a phenomenon that has been thoroughly exploited in the fields of botany, petrology, and the semiconductor industry. In contrast, the study of animal tissues and pathogens is often complicated with either extremely faint or bright, nonspecific autofluorescence. Of far greater value for the latter studies are added fluorochromes (also termed fluorophores), which are excited by specific wavelengths of irradiating light and emit light of defined and useful intensity. Fluorochromes are stains that attach themselves to visible or sub-visible structures, are often highly specific in their attachment targeting, and have a significant quantum yield (the ratio of photon absorption to emission). The widespread growth in the utilization of fluorescence microscopy is closely linked to the development of new synthetic and naturally occurring fluorophores with known intensity profiles of excitation and emission, along with well-understood biological targets.

Fundamentals of Excitation and Emission

The basic function of a fluorescence microscope is to irradiate the specimen with a desired and specific band of wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light. In a properly configured microscope, only the emission light should reach the eye or detector so that the resulting fluorescent structures are superimposed with high contrast against a very dark (or black) background. The limits of detection are generally governed by the darkness of the background, and the excitation light is typically several hundred thousand to a million times brighter than the emitted fluorescence.

In a fluorescence vertical illuminator, light of a specific wavelength (or defined band of wavelengths), often in the ultraviolet, blue or green regions of the visible spectrum, is produced by passing multispectral light from an arc-discharge lamp or other source through a wavelength selective excitation filter. Wavelengths passed by the excitation filter reflect from the surface of a dichromatic (also termed a dichroic) mirror or beamsplitter, through the microscope objective to bath the specimen with intense light. If the specimen fluoresces, the emission light gathered by the objective passes back through the dichromatic mirror and is subsequently filtered by a barrier (or emission) filter, which blocks the unwanted excitation wavelengths. It is important to note that fluorescence is the only mode in optical microscopy where the specimen, subsequent to excitation, produces its own light. The emitted light re-radiates spherically in all directions, regardless of the excitation light source direction.

Epi-fluorescence illumination is the overwhelming choice of techniques in modern microscopy, and the reflected light vertical illuminator is interposed between the observation viewing tubes and the nosepiece housing the objectives. The illuminator is designed to direct light onto the specimen by first passing the excitation light through the microscope objective (which in this configuration, acts as a **condenser**) on the way toward the specimen, and then using that same objective to capture the emitted fluorescence. This type of illuminator has several advantages. The fluorescence microscope objective serves first as a well-corrected condenser and secondly as the image-forming light gatherer. Being a single component, the objective/condenser is always in perfect alignment. A majority of the excitation light reaching the specimen passes through without interaction and travels away from the objective, and the illuminated area is restricted to that which is observed through the eyepieces (in most cases). Unlike the situation in some contrast enhancing techniques, the full numerical aperture of the objective is available when the microscope is properly configured for Köhler illumination. Finally, it is possible to combine with or alternate between reflected light fluorescence and transmitted light observation and the capture of digital images.

Stokes' Shift

Vibrational energy is lost when electrons relax from the excited state back to the ground state. As a result of the energy loss, the emission spectrum of an excited fluorophore is usually shifted to longer wavelengths when compared to the absorption or excitation spectrum (note that wavelength varies inversely to radiation energy). This well-documented phenomenon is known as **Stokes' Law** or **Stokes' shift**. As Stokes' shift values increase, it becomes easier to separate excitation from emission light through the use of fluorescence filter combinations.

In order to achieve maximum fluorescence intensity, a fluorophore (often termed a **dye**) is usually excited at wavelengths near or at the peak of the excitation curve, and the widest possible range of emission wavelengths that include the emission peak are selected for detection. The selection of excitation and emission wavelengths is typically based on interference filters

The effective separation and detection of excitation and emission wavelengths is achieved in fluorescence microscopy through the proper selection of filters to block or pass specific wavelength bands in the ultraviolet, visible, and near-infrared spectral regions. Fluorescence vertical illuminators are designed with the purpose of controlling the excitation light through the application of readily interchangeable filter (neutral density and interference excitation balancers) insertions into the light path on the way toward the specimen, and again in the path between the specimen and the observation tubes or camera detector system. Perhaps the most important criteria, in view of relatively low fluorescence emission intensities (see discussion above), is that the light source utilized for excitation be of sufficient brightness so that the weak emission light can be maximized, and that the fluorochromes possess adequate absorption properties and emission quantum yields.

The efficiency with which a particular fluorophore absorbs a photon of the excitation light is a function of the molecular cross-section, and the likelihood of absorption is known as the **extinction coefficient**. Larger extinction coefficients indicate that the absorption of a photon (or quantum) in a given wavelength region is more likely. The quantum yield denotes the ratio of the number of quanta emitted compared to those absorbed (and is usually a value between 0.1 and 1.0). Quantum yield values below 1 are the result of the loss of energy through non-radiative pathways, such as heat or a photochemical reaction, rather than the re-radiative pathway of fluorescence. Extinction coefficient, quantum yield, mean luminous intensity of the light source, and fluorescence lifetime are all important factors that contribute to the intensity and utility of fluorescence emission.

Fading, Quenching, and Photobleaching

A wide spectrum of conditions often come into play that ultimately affect the re-radiation of fluorescence emission and thus reduce the intensity. The general term for a reduction of fluorescence emission intensity is **fading**, a catch-all category that is usually further subdivided into **quenching** and **photobleaching** phenomena for more precise descriptions. Photobleaching is the irreversible decomposition of the fluorescent molecules in the excited state because of their interaction with molecular oxygen before emission. The occurrence of photobleaching is exploited in a technique known as fluorescence recovery after photobleaching (**FRAP**), a very useful mechanism for investigating the diffusion and motion of biological macromolecules. The method is based upon photobleaching a sharply defined region of the specimen by an intense burst of laser light, accompanied by the subsequent observation of the rates and pattern of fluorescence recovery in the photobleached area. A related technique, known as fluorescence loss in photobleaching (**FLIP**), is employed to monitor the decrease of fluorescence in a defined region lying adjacent to a photobleached area. Similar to FRAP, the latter technique is useful in the investigation of molecular mobility and dynamics in living cells.

The excited state relaxation process of quenching results in reduced fluorescence intensity through a variety of mechanisms involving non-radiative energy loss and frequently occurs as a result of oxidizing agents or the presence of salts or heavy metals or halogen compounds. In some cases, quenching results from the transfer of energy to another molecule (termed the **acceptor**), which resides physically close to the excited fluorophore (the **donor**), a phenomenon known as fluorescence resonance energy transfer (**FRET**). This particular mechanism has become the basis for a useful technique involving the study of molecular interactions and associations at distances far below the lateral resolution of the optical microscope.

Detecting Single Molecules

Under ideal conditions, it is often possible to detect the fluorescence emission from a single molecule, provided that the optical background and detector noise are sufficiently low. As discussed above, a single fluorescein molecule could emit as many as 300,000 photons before it is destroyed by photobleaching. Assuming a 20-percent collection and detection efficiency, about 60,000 photons would be detected. Using avalanche photodiode or electron multiplying CCD detectors for these experiments, investigators have been able to monitor the behavior of single molecules for many seconds and even minutes. The major problem is adequate suppression of the optical background noise. Because many of the materials utilized in construction of microscope lenses and filters display some level of autofluorescence, efforts were initially directed toward the manufacture of very low fluorescence components. However, it soon became evident that fluorescence microscopy techniques utilizing total internal reflection (TIR) provided the desired combination of low background and high excitation light flux.

Total internal reflection fluorescence microscopy (TIRFM) takes advantage of the evanescent wave that is developed when light is totally internally reflected at the interface between two media having dissimilar refractive indices.

In this technique, a beam of light (usually an expanded laser beam) is directed through a prism of high refractive index, such as glass or sapphire, which abuts a lower refractive index medium of glass or aqueous solution. If the light is directed into the prism at higher than the critical angle, the beam will be totally internally reflected at the interface. The reflection phenomenon develops an evanescent wave at the interface by the generation of an electromagnetic field that permeates about 200 nanometers or less into the lower refractive index space. The light intensity in the evanescent wave is sufficiently high to excite the fluorophores within it, but because of its shallow depth, the volume excited is very small. The result is an extremely low-level background because so little of the specimen is exposed to the excitation light (only that portion within a 200-nanometer distance of the interface).

Applications

The increasing application of electro-optics in fluorescence microscopy has led to the development of optical tweezers capable of manipulating sub-cellular structures or particles, the imaging of single molecules, and a wide range of sophisticated spectroscopic applications.

- Labelling multiple antibodies with different fluorophores allows visualization of multiple targets within a single image (multiple channels). DNA microarrays are a variant of this.
- Immunology: An antibody is first prepared by having a fluorescent chemical group attached, and the sites (e.g., on a microscopic specimen) where the antibody has bound can be seen, and even quantified, by the fluorescence.
- FLIM (Fluorescence Lifetime Imaging Microscopy) can be used to detect certain bio-molecular interactions that manifest themselves by influencing fluorescence lifetimes.

CHROMOSOME PAINTING

Chromosome painting involves the use of fluorescent-tagged chromosome specific DNA sequences to visualize specific chromosomes or chromosome segments by in situ DNA hybridization and fluorescence microscopy. Chromosome painting refers to the hybridization of fluorescently labelled chromosome-specific, composite probes to cytological preparations.

Chromosome painting allows the visualization of individual chromosomes in metaphase or interphase stages and the identification of both numerical and structural chromosomal aberrations with high sensitivity and specificity. The simultaneous hybridization of multiple chromosome painting probes, each tagged with a specific light-emitting fluorochrome has resulted in the differential colour display of human and mouse chromosomes, which is also called colour karyotyping.

Fluorescent in situ hybridization (FISH) has been used to detect the location of specific genomic targets using probes that are labelled with specific fluorochromes. That is the reason chromosome painting is also called **M-FISH** or **multicolour FISH**. The technique allowed detection of simple and complex chromosomal rearrangements. In addition, complex chromosomal abnormalities could also be identified that could not be detected by the conventional cytogenetic banding techniques.

Almost a decade ago, chromosome painting was developed independently by research teams at Lawrence Livermore National Laboratories and at Yale University. Both groups had taken advantage of the availability of cloned DNA libraries that were derived from flow-sorted human chromosomes. The first generation of probes, based on chromosome-specific **phage libraries,** were rather cumbersome to use, due to low insert-to-vector ratios, which frequently resulted in a relatively high background staining. Some of these limitations were overcome with the availability of **plasmid libraries**, where an improved insert-to-vector ratio and easier probe generation enhanced the painting quality considerably.

Chromosome painting has improved the efficiency of screening cells for chromosome abnormalities, in testing chemicals for mutagenacity and for rearrangements associated with tumours. Painting probes detect chromosome rearrangements. Use of the same chromosome paints for chromosomes of different species reveals the extent of chromosome rearrangements since divergence of the species.

Chromosome painting probes are now also available for an ever increasing number of species, most notably for the mouse and the rat, allowing the expansion of chromosome painting analyses to animal models for human diseases. FISH techniques have been developed and applied to identify the origin of the markers and other structural chromosomal aberrations. The use of chromosome painting probes in one, two or three colour FISH experiments has significantly improved the definitive diagnosis of chromosomal aberrations.

The introduction of chromosome painting to the field of comparative cytogenetics has added significantly to the understanding of chromosome changes that occurred during the evolution of species. Chromosome painting can be used to identify homologous chromosome segments in different species and to map probes of different complexities and chromosome rearrangements in a single experiment. In recent years, the complete karyotypes of various mammals including primates, carnivores and artiodactyls have been analyzed by chromosome painting.

Chromosome Painting Probes, available in liquid format, are directly labelled in either a red or green fluorophore. They can be mixed together to label a number of chromosomes in a single reaction. The probes come in the form of ready to use hybridization solution in a five-test kit format, and are

supplied complete with DAPI counter stain. The protocol is rapid and simple and follows simultaneous co-denaturation of the FISH probe and target DNA.

The origin of marker chromosomes that were unidentifiable by standard banding techniques could be verified by **reverse chromosome painting.** This technique includes micro-dissection, followed by *in vitro*DNA amplification and fluorescence *in situ* hybridization (FISH). The chromosomal material is amplified by a degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR). The resulting PCR products are labelled by nick-translation with biotin-11-dUTP and used as probes for FISH.

Homologies between the chromosomes of different species can be detected by chromosome painting. A more detailed description of how this method of chromosome painting works is given below:

· Initially suspensions of chromosomes from dividing cells are sorted by a method known as flow cytometry.

· DNA from one chromosome is then labelled with a fluorescent dye using a technique called fluorescence in situ hybridisation **(FISH).** This labelled DNA paints the chromosome and allows homologous regions of DNA of other species, from great apes to mice, to be identified. Homologous regions show up in the same colour on the chromosome charts.

· If you take a paint made from human chromosome **2** and hybridise it onto the chromosomes of another species, then you'll see segments of paint on different chromosomes, each being homologous to part of human chromosome **2**, so with the gibbon karyotype, regions on chromosome **2** in human can be tracked to 5 different chromosomes in gibbon. The **fluorescent-labelled DNAs** will attach to the analogous chromosomes from which they were derived. DNA fragments with the same base sequences have the characteristic of attaching to each other.

· This tells us that the lineage that produced gibbon and the lineage that produced human have diverged over several million years and during this time rearrangements have occurred between the two species which can be tracked using the painting technique.

· If a part of a painted chromosome (yellow, for example) had undergone an exchange with another, non-painted chromosomes (stained red), it is possible to detect the aberration as reciprocal translocation, because the aberrant chromosome contains both yellow and red segments. Usually, a pair of bi-coloured chromosomes can be detected in one metaphase, because two chromosomes typically exchange a part of their DNA. Reciprocal translocations are difficult to detect by simple staining technique that stains the entire set of chromosomes with a single material such as with Giemsa.

· When human chromosome probes are hybridised to the chromosomes from other species, the same set of blocks, dispersed across multiple human chromosomes, are often located together on one chromosome in other species. For example, parts of human chromosome 3 and 21, or 14 and 15, or 12 and 22, tend to be located together on one chromosome in other animals. This is evidence that an ancestral block of genes has been split apart and moved to different chromosomes during human evolution.

· Collating all these patterns of linked regions of chromosome across various mammalian species has enabled to assemble a picture of genomic commonalities across multiple species.
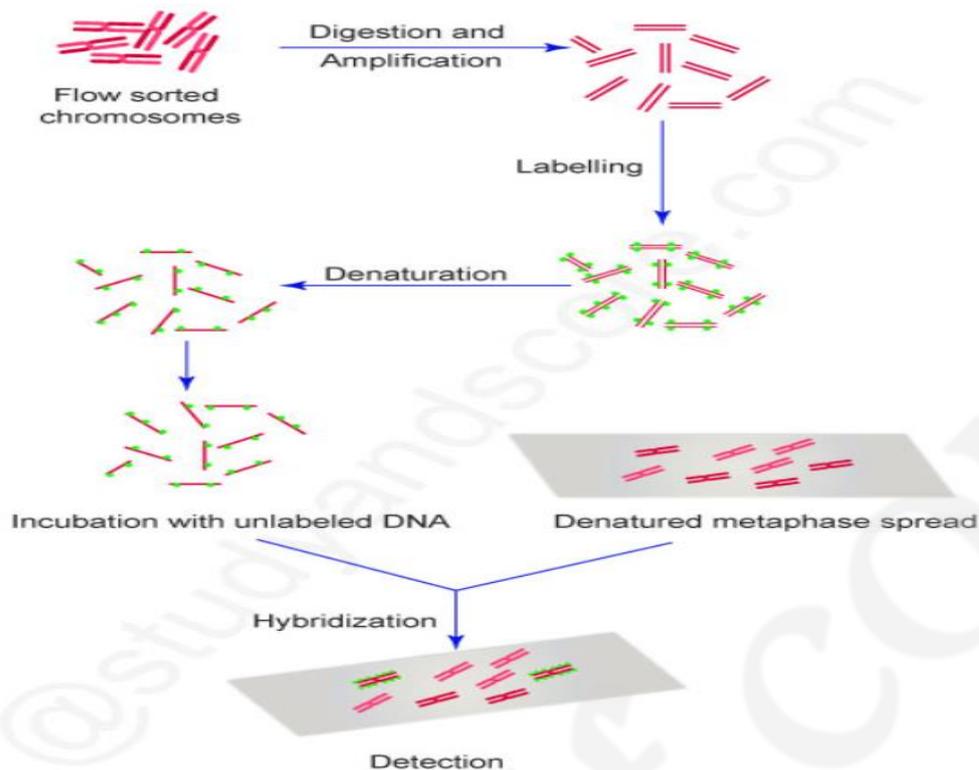
Chromosome Walking

A technique that helps to identify overlapping cloned DNA fragments from one continuous segment of a chromosome. These fragments are generated by random shearing or by partial digestion with a restriction endonuclease. A series of colony hybridisations is carried out, starting with a specific cloned fragment that has already been identified and which is known to be in the region encompassed by the overlapping clones. This identified fragment is used as probe to identify clones containing adjacent sequences. These are in turn used as probes to identify other clones carrying sequences adjacent to them and so on. With each round of hybridisation, it is possible to "walk" further along the chromosome from the initial fragment. This technique was developed in the late 1980s and is a powerful method to detect translocations.

Applications of chromosome painting

1. With the help of chromosome painting, we can get information about the divergence of lineages which has occurred over several million tears. Also the rearrangements which took place between species can be tracked.

2. Chromosome painting helps in visualization of individual chromosomes in metaphase or interphase stages.

3. Chromosome painting can identify both numerical and structural chromosomal aberrations with high sensitivity and specificity

4. Chromosome painting is highly sensitive and specific.

5. The application of chromosome painting in the field of comparative cytogenetics helps in understanding and determining the chromosomal changes that have occurred during the evolution of species.

6. Chromosome painting identifies homologous chromosome segments in different species.

7. This technique can map probes of different complexities and chromosome rearrangements in one shot-single experiment.

8. In recent years, the complete karyotypes of various mammals including primates, carnivores and artiodactyls have been analysed by chromosome painting.

FISH

FISH is a technique that is used to detect the presence of specific DNA sequences on chromosomes out of large number of fragments of similar size. It uses fluorescent probes that bind to those parts of chromosome which have similar base sequences. The detection of base sequences on a combed DNA molecule is done by first hybridizing the known base sequences **(the probes)** with the combed DNA **(matrixDNA** or **target DNA).**
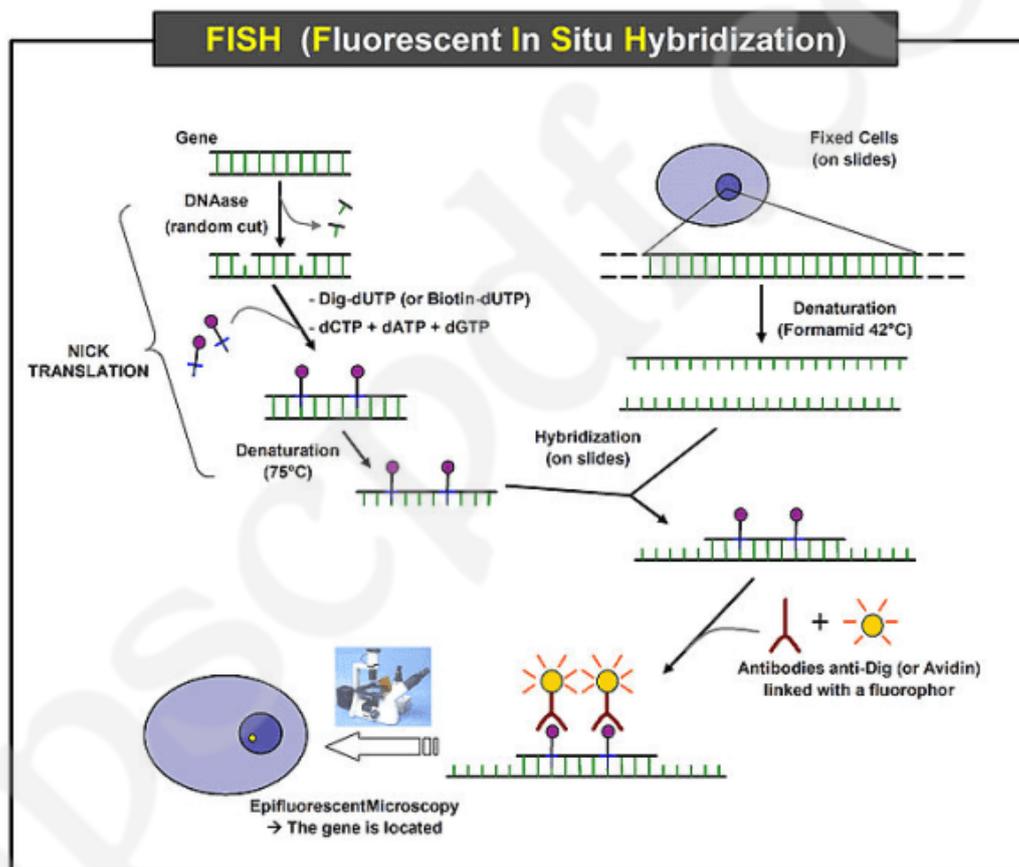
The method comprises of three basic steps: fixation of a specimen on a microscope slide, hybridization of labelled probe to homologous fragments of genomic DNA, and enzymatic detection fluorescent detection of the tagged target hybrids. Normal hybridization requires the isolation of DNA or RNA, separating it on a gel, blotting it onto nitrocellulose and probing it with a complementary sequence.

The most common tissue sections used with in situ hybridization are:

a) Frozen sections. Fresh tissue is frozen rapidly and then embedded in a special support medium for cryosectioning. The sections are rapidly fixed in 4% paraformaldehyde prior to hybridization.

b) Paraffin embedded sections. Sections are fixed in formalin and then embedded in wax paraffin before being cut into sections.

c) Cells in suspension. Cells can be cytospun onto glass slides and fixed with methanol.

PROBES

Probes are fragments of cloned DNA that are isolated, purified, and amplified for use in the hybridization. Probes are complimentary sequences of nucleotide bases to the specific mRNA sequence of interest. These probes can be as small as 20-40 base pairs or be up to 1000 bp. These fragments should not be so large as to impede the hybridization process. Probes of different types can be used to detect distinct DNA types.

There are essentially four types of probes that can be used in performing in situ hybridization:

1. **Oligonucleotide probes** are produced synthetically by readily available deoxynucleotides but the specific nucleotide sequence you wish to prepare should be known. These probes have the advantages of being stable, resistant to RNases and are small, generally around 40-50 base-pairs and hence allow easy penetration into the cells or tissue of interest. They are available for purchase and faster and less expensive to use. Unlike RNA probes, oligonucleotide probes can be designed to selectively recognize members of closely related gene families.

2. **Single stranded DNA probes.** These are much larger, probably in the 200-500 bp range. They can be produced by reverse transcription of RNA or by amplified primer extension of a PCR-generated fragment in the presence of a single antisense primer. They require time to prepare, need expensive reagents and a good molecular skills.

3. **Double stranded DNA probes.** These can be produced by the inclusion of the sequences of interest into bacterial cell, which is replicated, lysed and the DNA extracted, purified and the sequence of interest is excised with restriction enzymes. On the other hand, if the sequence is known then by designing appropriate primers one can produce the relevant sequence very rapidly by PCR. Because the probe is double stranded, denaturation or melting has to be carried out prior to hybridization.

4. **RNA probes (cRNA probes or riboprobes).** RNA probes have the advantage that RNA-RNA hybrids are very thermostable and are resistant to digestion by RNAses. This allows the possibility of post-hybridization digestion with RNAses to remove non-hybridized RNA and therefore reduces the possibility of background staining. These probes however can be very difficult to work with as they are very sensitive to RNases (ubiquitous RNA degrading enzymes).

LABELLING

To see where the probe has hybridized within the tissue section or within cells, you must attach to the probe with an easily detectable substance or **"label"** before hybridization. Classically oligonucleotide probes have been either 5' or 3' end-labelled or 3' tailed with modified nucleotides that have a "label" attached that can be detected after the probe has hybridized to its target.

Traditionally oligonucleotide probes have been radiolabelled, for instance 35Sulphur (35S) is the most commonly used radioisotope because its high activity is necessary to detect transcripts present in low amounts. Both radiolabels and non-radioactive labels are "attached" to the single stranded oligonucleotide probe by using the enzyme terminal transferase to add a tail of labelled dioxy

nucleotides to the 3' end of the oligonucleotide. Radiolabelled probes are visualized by exposure of the tissue section or cells against photographic film which is then developed.

In contrast to adding the labeled nucleotides to either end of the oligonucleotide probe it is also possible to have labels incorporated into the oligonucleotide when it is being synthesized, for example by adding biotin- or FITC-labeled dATP in place of non-labeled dATP during synthesis so that a label or "tag" appears every time that the ATP nucleotide appears in the probe sequence.

HYBRIDIZATION

Hybridization involves mixing the single strand probes with the denatured target DNA. Denaturation of the DNA is obtained by heating, which separates the two strands, and allows access of the single strand probes to their respective complementary combed single strand.

The factors that influence the hybridization of the oligonucleotide probes to the target mRNAs are: Temperature, pH, monovalent cation concentration and presence of organic solvents.

The following is a typical hybridization solution at temperature of 37oC and an overnight incubation period.

Dextran sulphate. This is added because it becomes strongly hydrated and therefore effectively increases the probe concentration in solution resulting in higher hybridization rates.

Formamide and DTT (dithiothreitol). These are organic solvents which reduce the thermal stability of the bonds allowing hybridization to be carried out at a lower temperature.

SSC (NaCl + Sodium citrate). Monovalent cations interact mainly with the phosphate groups of the nucleic acids decreasing the electrostatic interactions between the two strands.

EDTA. This is a chelator and removes free divalent cations from the hybridization solution, because they strongly stabilize duplex DNA.

Following hybridization the material is washed to remove unbound probe or probe which has loosely bound to imperfectly matched sequences.

OBSERVATION

Observation of the hybridized sequences is done using epifluorescence microscopy. White light from a source lamp is filtered so that only the relevant wavelengths for excitation of the fluorescent molecules reach the sample. The light emitted by fluorochromes is generally of larger wavelengths, which allows the distinction between excitation and emission light by means of a second optical filter. Therefore, it is possible to see bright coloured signals on a dark background. It is also possible to distinguish between several excitation and emission bands, thus between several fluorochromes, which allows the observation of many different probes on the same target.

As mentioned, radiolabeled probes are detectable using either photographic film or photographic emulsion.

The fluorescent labels described above are detectable by using a fluorescent microscope to examine the tissue on which the labeled oligonucleotide probe has hybridized. The use of fluorescent labels with *in situ* hybridization has come to be known as **FISH(fluorescent in situ hybridization)** and one advantage of these fluorescent labels is that two or more different probes can be visualized at one time.

In contrast both Biotin and DIG labeled oligonucleotide probes generally require an intermediate before detection of the probe can occur and they are thus detected indirectly like a typical immunocytochemistry protocol. Biotin is the common compound used in the labelling of oligonucleotide probes. Linked to ATP it can be detected with antibodies but more often with a glygoprotein **Avidin** from egg white or **Streptavidin**from the fungi *Streptomyces avidinii*, as they have a high binding capacity.

Applications

Medical application:

- Often parents of children with a developmental disability want to know more about their child's conditions before choosing to have another child. These concerns can be addressed by analysis of the parents' and child's DNA. In cases where the child's developmental disability is not understood, the cause of it can potentially be determined using FISH and cytogenetic techniques.
- In medicine, FISH can be used to form a diagnosis, to evaluate prognosis, or to evaluate remission of a disease, such as cancer. Treatment can then be specifically tailored.
- FISH can also be used to detect diseased cells more easily than standard Cytogenetic methods, which require dividing cells and requires labor and time-intensive manual preparation and analysis of the slides by a technologist.

Species identification:

FISH is often used in clinical studies. If a patient is infected with a suspected pathogen, bacteria, from the patient's tissues or fluids, are typically grown on agar to determine the identity of the pathogen. Many bacteria, however, even well-known species, do not grow well under laboratory conditions. FISH can be used to detect directly the presence of the suspect on small samples of patient's tissue.

Comparative genomic hybridization can be described as a method that uses FISH in a parallel manner with the comparison of the hybridization strength to recall any major disruptions in the duplication process of the DNA sequences in the genome of the nucleus.

ELISA

ELISA stands for "enzyme-linked immunosorbent assay" that is an immunochemical test involving an enzyme and an antibody or antigen. ELISA combines the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily assayed enzyme. ELISA tests are utilized to detect substances that have antigenic properties, primarily proteins. Some of these include hormones, bacterial antigens and antibodies.

In ELISA an unknown amount of antigen is affixed to a surface and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added so that the enzyme can convert to some detectable signal. In the case of fluorescence ELISA, when light of the appropriate wavelength is projected upon the sample, it will glow and thus detected.

HISTORY

The **enzymeImmunoassay (EIA)** and **Enzyme–linkedImmunosorbentAssay (ELISA)** were developed independently and simultaneously by the research group of **PeterPerlmann** and **EvaEngvall** at

Stockholm University, Sweden and by the research group of **Anton Schuurs** and **Bauke van Weemen** in The Netherlands. Both techniques are based on the principle of immunoassay with an **enzyme** rather than radioactivity as the reporter label.

In the early 1970s, the idea of using enzyme labels was met with scepticism and incredulity. How could so bulky and large a molecule as an enzyme be attached to an antigen or antibody without hindering the immunochemical reaction between the antigen and antibody? However, between 1966 and 1969, the group in Villejuif reported their successful results of coupling antigens or antibodies with enzymes such as alkaline phosphatase glucose oxidase etc. Their purpose was to use the enzyme-labelled antigens and antibodies to detect antibodies or antigens by immunofluorescence.

Engvall and Perlmann published their first paper on ELISA in 1971 and demonstrated quantitative measurement of **IgG** in rabbit serum with alkaline phosphatase as the reporter label. Perlmann's further research included cytotoxicity of human lymphocytes and immunogen selection and epitope mapping for malaria vaccine development. Engvall's group applied the ELISA measurement tool to malaria and trichinosis, microbiology and oncology.

Engvall then focussed her scientific interests on the biochemistry of fibronectin, laminin, integrins and muscular dystrophies. In 1976, Organon Teknika developed and marketed a highly successful EIA system for the **hepatitis B** surface antigen featuring a 96-well microtiter plate format. This test became the first commercially available EIA. Other microbiological and virologic diagnostic tests soon followed, e.g., for hepatitis B e-antigens, *Rubella* antibodies, *Toxoplasma* antibodies, and in 1980s for detection of human immunodeficiency virus antibodies.

In the **rabies** field, ELISA was initially developed for the titration of rabies virus-neutralizing antibodies. The technique was applied to the quantification of rabies antigen by Atanasiu et al. using fluorescein-labelled IgG to the purified nucleocapsid. Subsequently, Perrin et al. developed an ELISA called rapid rabies enzyme immunodiagnosis **(RREID),** which was based upon the detection of rabies virus nucleocapsid antigen in brain tissue. In this test microplates were coated with purified IgG and an IgG-peroxidase conjugate was used to react with immunocaptured antigen.

Sandwich Elisa
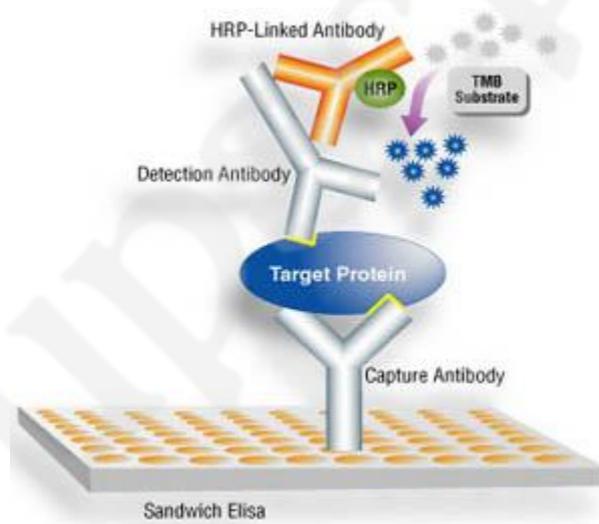
ELISA PROCEDURE

1. Coating of Wells with Antibody

100 μL of antibody diluted in buffer A is added to each well. Pipette 0.2 ml of the diluted capture antibody to each well of a microtiter plate. The antibody should be directed against the antigen to be

determined. Cover the plate with plastic film or aluminium foil and incubate at 4°C overnight. Use the immunoglobulin fraction of the antiserum for coating, not whole, unprocessed antiserum. The protein concentration of diluted antibody should be about 10 mg per L. Capture antibodies are typically plated at 0.2-10.0 μg/ml. Incubate the plate for 1 hour at 37 °C.

2. Washing

Wash the wells 4 times with buffer B using a micro plate washer. Alternatively, wash manually. The 0.1% Tween 20 in the washing buffer reduces the background and should be preferred for 0.05% Tween 20, which is commonly used.

Empty the plate by inversion over a sink. Tap the inverted plate against some layers of soft paper tissue to remove residual liquid. Wash the plate by filling the wells by immersion in buffer B. Leave on the table for 3 minutes. Empty the plate as described above and repeat washing two more times.

3. Incubation with Test Samples

100 μL of test sample or standard diluted in buffer B is added per well. Cover the plate and incubate at room temperature for 2 hours. Generally, biological samples should be diluted at least 1+1 to reduce the risk of non-specific reactions. Using the given general procedure, the detection limit is about 1 to 5 μg of antigen per litre of test sample. The sensitivity of a given antigen/antibody system might be improved if the sample is made 3% in polyethylene glycol. Further, the sensitivity will be improved by prolonging the incubation time.

4. Wash as described in step 2.

5. Incubation with Peroxidase-Conjugated Antibody

100 μL of peroxidase-conjugated antibody diluted in buffer B is added to each well. Cover the plate and incubate at room temperature for 1 hour. The peroxidase-conjugated antibody should be directed against the antigen to be determined. It might speed up the reaction and improve the sensitivity if the peroxidase-conjugated antibody is made 3% in polyethyleneglycol. In addition, the sensitivity might be improved if the incubation time is prolonged to 1 or 2 hours.

6. Wash as described in step 2.

When washing manually it is mandatory at this step that the washing buffer in the reservoir is totally exchanged after the first of the 3 washes. If this is not done, a high background staining will occur.

7. Colour Development

100 μL of **chromogenic substrate C** is added to each well. Cover the plate and incubate for 15 minutes, or until a suitable colour has developed. The plate should preferably be protected against light during this incubation.

Popular enzymes are those that convert a colourless substrate to a coloured product, e.g., pnitrophenylphosphate (pNPP), which is converted to the yellow p-nitrophenol by alkaline phosphatase. Substrates used with peroxidase include 2,2'-azo-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), o-phenylenediamine (OPD) and 3,3'5,5'- tetramethylbenzidine base (TMB), which yield green, orange and blue colours, respectively.

Alkaline phosphatase yellow (pNPP) liquid substrate can be used as dye for the ELISA . This product is supplied as a ready-to-use buffered alkaline phosphatase substrate p-nitro-phenylphosphate (pNPP). Prior to the reaction with alkaline phosphatase, the substrate should appear as a colourless to pale

yellow solution. It will develop a yellow reaction product when reacted with alkaline phosphatase in microwell applications and can be read at 405 nm. For the end-point assays, the reaction can be stopped with 0.05 ml/well of 3 N NaOH for every 0.2 ml of substrate reaction

8. Stopping the Colour Development

The reaction can be stopped by adding reagent D (100ul 0.5 M H_2SO_4) to each well.

9. Reading of Results

Read results directly through the bottom of the microwell plate using an automated or semi automated ELISA-reader. Read the plate at 490 nm. The subtraction of the absorbance at a reference wavelength (between 620 and 650 nm) is recommended, but not essential. Alternatively, read results within 3 hours in a photometer at 490 nm using a cuvette requiring no more than a 200 µL volume. The cuvette can conveniently be emptied by a piece of plastic tubing connected via a reservoir to a vacuum pump or to water suction.

An ELISA plate reader is used to measure the intensity of the colour formed in each well. A high-intensity lamp passes light to the microtiter well and the light emitted by the reaction happening in the microplate well is quantified by a detector. Common detection modes for microplate assays are absorbance, fluorescence intensity, luminescence, time-resolved fluorescence, and fluorescence polarization.

10. Plot the standard curve on semi logarithmic paper with A490 nm as ordinate and log10 concentration o standard as abscissa.

HIV ELISA

An HIV ELISA, sometimes called an **HIV enzyme immunoassay (EIA)** is the most basic test to determine if an individual is positive for a selected pathogen, such as HIV. The test is performed in an 8 cm x 12 cm plastic plate which contains an 8 x 12 matrix of 96 wells, each of which is about 1 cm high and 0.7 cm in diameter.

When the body is infected with a virus, the immune system responds by producing antibodies– proteins that circulate in the blood and attempt to destroy the virus. If antibodies against HIV are present in a blood sample, they will stick to the plate coated with fragments of the virus. The ELISA reagent will detect the presence of the bound antibodies and will change colour giving a positive result.

Partially purified, inactivated HIV antigens are coated onto an ELISA plate. Patient's blood serum which contains antibodies to HIV is added and those antibodies will bind to the HIV antigens on the plate. Anti-human immunoglobulin is coupled to an enzyme. This is the second antibody that binds to human antibodies. Chromogen or substrate which changes colour when cleaved by the enzyme is attached to the second antibody.

In most cases if the ELISA tests are positive, the patient will be retested by the western blotting analysis again for confirmation.

TYPES OF ELISA

Generally there are 5 types of ELISA: Direct ELISA; Indirect ELISA; Sandwich ELISA; Competitive ELISA and Multiplex ELISA. The two common types of ELISA are indirect ELISA (that detect antibodies in sample) and sandwich ELISA (that detect antigen in sample).

DIRECT ELISA

The direct ELISA uses the method of directly labelling the antibody itself. Microwell plates are coated with a sample containing the target antigen, and the binding of labelled antibody is quantitated by a colorimetric, chemiluminescent or fluorescent end-point. Since the secondary antibody step is omitted, the direct ELISA is relatively quick, and avoids potential problems of cross-reactivity of the secondary antibody with components in the antigen sample.

INDIRECT ELISA

The indirect ELISA is a two-step method that uses a labelled secondary antibody for detection. Apply a sample of known antigen of known concentration to the well of a microtiter plate. A concentrated solution of non-interacting protein, such as bovine serum albumin or casein, is added to all plate wells for blocking, because the serum proteins block non-specific adsorption of other proteins to the plate.

The plate wells are then coated with serum samples of unknown antigen concentration, diluted into the same buffer used for the antigen standards. The plate is washed, and a detection antibody specific to the antigen of interest is applied to all plate wells. This antibody will only bind to immobilized antigen on the well surface, not to other serum proteins or the blocking proteins. Secondary antibodies, which will bind to any remaining detection antibodies, are added to the wells. Wash the plate, so that excess unbound enzyme-antibody conjugates are removed.

Apply a substrate which is converted by the enzyme to elicit a chromogenic or fluorogenic or electrochemical signal.

View the result using a spectrophotometer, spectrofluorometer, or other optical or electrochemical devices.

The enzyme acts as an amplifier; even if only few enzyme-linked antibodies remain bound, the enzyme molecules will produce many signal molecules.

SANDWICH ELISA

The sandwich ELISA measures the amount of antigen between two layers of antibodies. The antigens to be measured must contain at least two antigenic sites, capable of binding to the antibodies, since at least two antibodies act in the sandwich. To utilize this assay, one antibody (the "capture" antibody) is purified and bound to a solid phase typically attached to the bottom of a plate well. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash and a labelled second antibody (the "detection" antibody) is allowed to bind to the antigen, thus completing the "sandwich".

Prepare a surface to which a known quantity of capture antibody is bound. Block any non specific binding sites on the surface by adding serum proteins. Apply the antigen-containing sample to the plate. Wash the plate, so that unbound antigen is removed. Apply primary antibodies that bind specifically to the antigen. Apply enzyme-linked secondary antibodies which are specific to the primary antibodies. Wash the plate, so that the unbound antibody-enzyme conjugates are removed. Apply a chemical which is converted by the enzyme into a colour or fluorescent or electrochemical signal. Measure the absorbance or fluorescence or electrochemical signal of the plate wells to determine the presence and quantity of antigen.

COMPETITIVE ELISA

When two "matched pair" antibodies are not available for a target, another option is the competitive ELISA. The advantage of competitive ELISA is that non-purified primary antibodies may be used. Although there are several different configurations for competitive ELISA, one reagent must be conjugated to a detection enzyme, such as horseradish peroxidase. The enzyme may be linked to either the antigen or the primary antibody. In this type of ELISA, there is an inverse relationship between the signal obtained and the concentration of the analyte in the sample, due to the competition between the free analyte and the ligand-enzyme conjugate for the antibody coating the microplate, i.e. the more analyte the lower the signal.

An unlabelled purified primary antibody is coated onto the wells of a 96 well microtiter plate. This primary antibody is then incubated with unlabeled standards. After this reaction is allowed to go to equilibrium, conjugated antigen is added. This conjugate will bind to the primary antibody wherever its binding sites are not already occupied by unlabelled antigen. Thus, the more unlabelled antigens in the sample or standard, the lower the amount of conjugated antigen bound. The plate is then developed with substrate and colour change is measured.

MULTIPLEX ELISA

In this ELISA a protein array format that allows simultaneous detection of multiple analytes at multiple array addresses within a single well. Antigens are measured by coating or printing capture antibodies in an array format within a single well to allow for the construction of "sandwich" ELISA quantification assays. Generally, multiplex ELISA can also be achieved through antibody array, where different primary antibodies can be printed onto the glass plate to capture corresponding antigens in a biological sample such as plasma, cell lysate, or tissue extracts. Detection method can be direct or indirect, sandwich or competitive, labelling or non-labelling, depending upon antibody array technologies.

PCR

The polymerase chain reaction is a technique for cloning a particular piece or amplifying a single or few copies of a piece of DNA, generating millions or more copies of that particular DNA sequence. One can make virtually unlimited copies of a single DNA molecule even though it may initially be present in a mixture containing many different DNA molecules.

In PCR, the enzyme DNA polymerase assembles a new DNA strand from nucleotides, by using single-stranded DNA as a template and DNA primers for initiation of DNA synthesis. Developed in 1984 by **Kary Mullis**, PCR is now a common technique used in biological research.

MATERIAL REQUIRED

A basic PCR requires the following components:

- **DNAtemplate** that contains the DNA region called target to be amplified.

- Two **primers** complementary to the DNA regions at the 5' or 3' end of DNA.

- The enzyme, **Taq polymerase** or another DNA polymerase with a temperature optimum at around 70°C.

- Deoxynucleoside triphosphates **(dNTPs)** that are building blocks for DNA synthesis, e.g. dATP, dGTP, dCTP and dTTP.

- **Buffer solution**, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

- **Divalent cations**, magnesium or manganese ions; generally Mg2+ is used but Mn2+ can be utilized for PCR-mediated DNA mutagenesis.

- **Monovalent cation** potassium ions.

THE PROCEDURE

In order to perform PCR, you must know at least a portion of the sequence of the DNA molecule that you wish to replicate. You must then synthesize primers that are short oligonucleotides, containing about two dozen nucleotides that are precisely complementary to the sequence at the 3' end of each strand of the DNA you wish to amplify.

The DNA sample is heated to separate its strands and mixed with the primers.

If the primers find their complementary sequences in the DNA, they bind to them.

Synthesis begins in 5' – 3' direction, using the original strand as template.

The reaction mixture must contain all four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) and a DNA polymerase that is not denatured by the high temperature. Almost all PCRs employ a heat-stable DNA polymerase, such as **Taq polymerase**, an enzyme originally isolated from the bacterium *Thermus aquaticus.* The PCR usually consists of a series of 20 to 40 repeated temperature changes called cycles.

The first step consists of heating the reaction to a temperature of 94-98°C, which is held for 20-30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

In the second step, the reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

In the third step, the temperature is raised commonly to 72°C and for *Taq polymerase* to 75-80°C. DNA polymerase now synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent strand

Fourth step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended. Polymerization continues until each newly-synthesized strand has proceeded far enough to contain the site recognized by the other primer.

In the final step temperature is lowered at 4-15°C for an indefinite period for short-term storage of the reaction.

Now you have two DNA molecules identical to the original molecule.

You take these two molecules, heat them to separate their strands, and repeat the process. Each cycle doubles the number of DNA molecules. Using automated equipment, each cycle of replication can be

completed in less than 5 minutes. After 30 cycles, a single molecule of DNA has been amplified to more than a billion copies.

Applications

1. Selective DNA isolation

PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many ways, such as generating hybridization probes for Southern or northern hybridization and DNA cloning, which require larger amounts of DNA, representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material.

Some PCR 'fingerprints' methods have high discriminative power and can be used to identify genetic relationships between individuals, such as parent-child or between siblings, and are used in paternity testing (Fig. 4). This technique may also be used to determine evolutionary relationships among organisms when certain molecular clocks are used (i.e., the 16S rRNA and recA genes of microorganisms).

Electrophoresis of PCR-amplified DNA fragments. (1) Father. (2) Child. (3) Mother. The child has inherited some, but not all of the fingerprint of each of its parents, giving it a new, unique fingerprint.

2. Amplification and quantification of DNA

Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence.

PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals.

Quantitative PCR or Real Time Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample—a technique often applied to quantitatively determine levels of gene expression. Quantitative PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

3. Medical applications

Prospective parents can be tested for being genetic carriers, or their children might be tested for actually being affected by a disease.

PCR analysis is also essential to preimplantation genetic diagnosis, where individual cells of a developing embryo are tested for mutations.

PCR can also be used as part of a sensitive test for tissue typing, vital to organ transplantation.

Many forms of cancer involve alterations to oncogenes. By using PCR-based tests to study these mutations, therapy regimens can sometimes be individually customized to a patient.

4. Infectious disease applications

PCR allows for rapid and highly specific diagnosis of infectious diseases, including those caused by bacteria or viruses.

5. Forensic applications

In its most discriminating form, genetic fingerprinting can uniquely discriminate any one person from the entire population of the world. Minute samples of DNA can be isolated from a crime scene, and compared to that from suspects, or from a DNA database of earlier evidence or convicts.

6. Research applications

PCR has been applied to many areas of research in molecular genetics

A common application of PCR is the study of patterns of gene expression. Tissues (or even individual cells) can be analyzed at different stages to see which genes have become active, or which have been switched off. This application can also use quantitative PCR to quantitate the actual levels of expression

Advantages

It is fairly simple to understand and to use, and produces results rapidly.

The technique is highly sensitive with the potential to produce millions to billions of copies of a specific product for sequencing, cloning, and analysis.

Limitations

One major limitation of PCR is that prior information about the target sequence is necessary in order to generate the primers that will allow its selective amplification. This means that, typically, PCR users must know the precise sequence(s) upstream of the target region on each of the two single-stranded templates in order to ensure that the DNA polymerase properly binds to the primer-template hybrids and subsequently generates the entire target region during DNA synthesis.

Like all enzymes, DNA polymerases are also prone to error, which in turn causes mutations in the PCR fragments that are generated.

Another limitation of PCR is that even the smallest amount of contaminating DNA can be amplified, resulting in misleading or ambiguous results.

RADIOACTIVE TRACER

Radioactive tracers are substances that contain a radioactive atom to allow easier detection and measurement. (Radioactivity is the property possessed by some elements of spontaneously emitting energy in the form of particles or waves by disintegration of their atomic nuclei.) For example, it is possible to make a molecule of water in which one of the two hydrogen atoms is a radioactive tritium (hydrogen-3) atom. This molecule behaves in almost the same way as a normal molecule of water. The main difference between the tracer molecule containing tritium and the normal molecule is that the tracer molecule continually gives off radiation that can be detected with a Geiger counter or some other type of radiation detection instrument.

One application for the tracer molecule described above would be to monitor plant growth by watering plants with it. The plants would take up the water and use it in leaves, roots, stems, flowers, and other parts in the same way it does with normal water. In this case, however, it would be possible to find out how fast the water moves into any one part of the plant. One would simply pass a Geiger counter over the plant at regular intervals and see where the water has gone.

Applications

Industry and research. Radioactive tracers have applications in medicine, industry, agriculture, research, and many other fields of science and technology. For example, a number of different oil companies may take turns using the same pipeline to ship their products from the oil fields to their refineries. How do companies A, B, and C all know when their oil is passing through the pipeline? One way to solve that problem is to add a radioactive tracer to the oil. Each company would be assigned a different tracer. A technician at the receiving end of the pipeline can use a Geiger counter to make note of changes in radiation observed in the incoming oil. Such a change would indicate that oil for a different company was being received.

Another application of tracers might be in scientific research on plant nutrition. Suppose that a scientist wants to find out how plants use some nutrient such as phosphorus. The scientist could feed a group of plants fertilizer that contains radioactive phosphorus. As the plant grows, the location of the phosphorus could be detected by use of a Geiger counter. Another way to trace the movement of the phosphorus would be to place a piece of photographic film against the plant. Radiation from the phosphorus tracer would expose the film, in effect taking its own picture of its role in plant growth.

Medical applications: Some of the most interesting and valuable applications of radioactive tracers have been in the field of medicine. For example, when a person ingests (takes into the body) the element iodine, that element goes largely to the thyroid gland located at the base of the throat. There the iodine is used in the production of various hormones (chemical messengers) that control essential body functions such as the rate of metabolism (energy production and use).

Suppose that a physician suspects that a person's thyroid gland is not functioning properly. To investigate that possibility, the patient can be given a glass of water containing sodium iodide (similar to sodium chloride, or table salt). The iodine in the sodium iodide is radioactive. As the patient's body takes up the sodium iodide, the path of the compound through the body can be traced by means of a Geiger counter or some other detection device. The physician can determine whether the rate and location of uptake is normal or abnormal and, from that information, can diagnose any problems with the patient's thyroid gland.

ULTRACENTRIFUGE

The ultracentrifuge is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as 1 000 000 g (approx. 9 800 km/s²). There are two kinds of ultracentrifuges, the preparative and the analytical ultracentrifuge. Both classes of instruments find important uses in molecular biology, biochemistry, and polymer science.

Centrifugation is a technique used for the separation of particles using a centrifugal field. The particles are suspended in liquid medium and placed in a centrifuge tube. The tube is then placed in a rotor and spun at a definitive speed. Rotation of the rotor about a central axis generates a centrifugal force upon the particles in the suspension.

Two forces counteract the centrifugal force acting on the suspended particles:

- Buoyant force: This is the force with which the particles must displace the liquid media into which they sediment.

- Frictional force: This is the force generated by the particles as they migrate through the solution.

Particles move away from the axis of rotation in a centrifugal field only when the centrifugal force exceeds the counteracting buoyant and frictional forces resulting in sedimentation of the particles at a constant rate.

Particles which differ in density, size or shape sediment at different rates. The rate of sedimentation depends upon:

1. The applied centrifugal field

2. Density and radius of the particle.

3. Density and viscosity of the suspending medium.

Analytical ultracentrifuge

In an analytical ultracentrifuge, a sample being spun can be monitored in real time through an optical detection system, using ultraviolet light absorption and/or interference optical refractive index sensitive system. This allows the operator to observe the evolution of the sample concentration versus the axis of rotation profile as a result of the applied centrifugal field. With modern instrumentation, these observations are electronically digitized and stored for further mathematical analysis. Two kinds of experiments are commonly performed on these instruments: sedimentation velocity experiments and sedimentation equilibrium experiments.

Sedimentation velocity experiments aim to interpret the entire time-course of sedimentation, and report on the shape and molar mass of the dissolved macromolecules, as well as their size-distribution. The size resolution of this method scales approximately with the square of the particle radii, and by adjusting the rotor speed of the experiment size-ranges from 100 Da to 10 GDa can be covered. Sedimentation velocity experiments can also be used to study reversible chemical equilibria between macromolecular species, by either monitoring the number and molar mass of macromolecular complexes, by gaining information about the complex composition from multi-signal analysis exploiting differences in each components spectroscopic signal, or by following the composition dependence of the sedimentation rates of the macromolecular system, as described in Gilbert-Jenkins theory.

Sedimentation equilibrium experiments are concerned only with the final steady-state of the experiment, where sedimentation is balanced by diffusion opposing the concentration gradients, resulting in a time-independent concentration profile. Sedimentation equilibrium distributions in the centrifugal field are characterized by Boltzmann distributions. This experiment is insensitive to the shape of the macromolecule, and directly reports on the molar mass of the macromolecules and, for chemically reacting mixtures, on chemical equilibrium constants.

The kinds of information that can be obtained from an analytical ultracentrifuge include the gross shape of macromolecules, the conformational changes in macromolecules, and size distributions of macromolecular samples. For macromolecules, such as proteins, that exist in chemical equilibrium with different non-covalent complexes, the number and subunit stoichiometry of the complexes and equilibrium constant constants can be studied.

Analytical ultracentrifugation has recently seen a rise in use because of increased ease of analysis with modern computers and the development of software, including a National Institutes of Health supported software package, SedFit.

Preparative ultracentrifuge

Preparative ultracentrifuges are available with a wide variety of rotors suitable for a great range of experiments. Most rotors are designed to hold tubes that contain the samples. Swinging bucket rotors allow the tubes to hang on hinges so the tubes reorient to the horizontal as the rotor initially accelerates.[citation needed] Fixed angle rotors are made of a single block of material and hold the tubes in cavities bored at a predetermined angle. Zonal rotors are designed to contain a large volume of sample in a single central cavity rather than in tubes. Some zonal rotors are capable of dynamic loading and unloading of samples while the rotor is spinning at high speed.

Preparative rotors are used in biology for pelleting of fine particulate fractions, such as cellular organelles (mitochondria, microsomes, ribosomes) and viruses. They can also be used for gradient separations, in which the tubes are filled from top to bottom with an increasing concentration of a dense substance in solution. Sucrose gradients are typically used for separation of cellular organelles. Gradients of caesium salts are used for separation of nucleic acids. After the sample has spun at high speed for sufficient time to produce the separation, the rotor is allowed to come to a smooth stop and the gradient is gently pumped out of each tube to isolate the separated components.

Hazards

The tremendous rotational kinetic energy of the rotor in an operating ultracentrifuge makes the catastrophic failure of a spinning rotor a serious concern. Rotors conventionally have been made from lightweight metals, aluminum or titanium. The stresses of routine use and harsh chemical solutions eventually cause rotors to deteriorate. Proper use of the instrument and rotors within recommended limits and careful maintenance of rotors to prevent corrosion and to detect deterioration is necessary to mitigate this risk.

More recently some rotors have been made of lightweight carbon fiber composite material, which are up to 60% lighter, resulting in faster acceleration/deceleration rates. Carbon fiber composite rotors also are corrosion-resistant, eliminating a major cause of rotor failure.