COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH
UNIVERSITY GRANTS COMMISSION

LIFE SCIENCE CODE: 03

13. METHODS IN BIOLOGY

AT A GLANCE:

Molecular Biology Methods		
Technique	**Abbreviation**	**Commonly used for**
Polymerase chain reaction	PCR	Amplification
Restriction Fragment Length Polymorphism	RFLP	Fingerprinting, gene mapping
Amplified Fragment Length Polymorphism	AFLP	Fingerprinting, gene mapping
Western Blot	WB	Detection of protein levels
Northern blot	NB	Detection of RNA levels
Southern blot	SB	Detection of Specific DNA
Eastern Blot	EB	Post translational modification detection
Microarray	-	High throughout expression
DNA sequencing	-	Identification of DNA sequence
Next generation sequencing	-	Sequencing as well as quantity analysis
Reverse transcription -PCR	RT-PCR	To convert RNA into c-DNA
Histology and Immunological Methods		
Hybridoma	-	Used for generation of fusion cells for antibody production
HAT selection	-	A method for selecting hybridoma cells
Sandwich ELISA	-	Used for detection of antigen
Competitive ELISA	-	Used for detection of antigen/ Antibody Labelled antigen is premixed with analyte
Radioimmunoassay	RIA	Used for detection of antigen More sensitive than ELISA
Immunoprecipitation	IP	Purification of specific type of protein using antibodies
Flow Cytometry	-	Analysis of various parameters of cells, identification, cell cycle analysis Also used in diagnosis
Fluorescence assisted cell sorting	FACS	Used for sorting cells

Fluorescent in situ hybridization	FISH	Location of a gene in chromosome
Genome in situ hybridization	GISH	Comparison of genomes

Biophysical Methods		
Technique	**Abbreviation**	**Commonly used for**
UV –vis spectroscopy	UV/vis	Detection of purity and concentration of biomolecules, enzyme kinetics
Circular dichroism Spectroscopy	CD	Detection of conformation of proteins, DNA, RNA and their dynamics
Fluorescence spectroscopy	FS	Detection of protein dynamics in solvent, quantification of radicals
Infrared spectroscopy	IR-SP	Detection of chemical bonds in a molecule
Raman spectroscopy	Raman	Detection of bond type and molecular structure using scattering of light
Nuclear Magnetic Resonance (1 dimensional)	1D NMR	Identification of molecules, such as ingredients of a plant extract.
Nuclear Magnetic Resonance (2 dimensional)	2D NMR	Detection of structure of biomolecules in their solution form using nuclear spin
X-ray diffraction	XRD	Detection of structures of biomolecules (DNA, RNA proteins in crystal state)
Mass spectrometry	MS	Identification of organic molecules (plant extracts, drugs, proteins etc.)
Matrix Assisted Laser Desorption Ionization	MALDI	Variant of MS, used for Identification of a peptide from unknown mixture
Surface Plasmon resonance	SPR	Used for the detection of ligand receptor binding kinetics.
Microscopic Methods		
Bright Field Microscopy	BFM	Used for visualizing stained slides (coloured samples)
Dark Field Microscopy	DFM	Used for visualizing transparent objects

Confocal microscopy	CFM	Used for high resolution florescence imaging. Localization of drug, proteins etc. can be done.
Scanning electron micropscy	SEM	Used to visualize surface ultrastructure, cells, or larger specimen like eye of insect, nanomaterial can be studied
Transmission electron microscopy	TEM	Used for the study of internal ultrastructure of the cells
Freeze-Fracture –Etch	FEM	Used for visualization between the two layers of a plasma membrane

Electrophysiological Methods		
Technique	**Abbreviation**	**Commonly used for**
Patch Clamp method	-	Used for study of channels in plasma membrane, electrical activity of membranes Several
Electrocorticography	ECoG	Used for detection of electrical activity of brain
Electroencephalography ECG	ECG	Used for detection of electrical activity of brain
Positron emission tomography imaging	PET	Used to image brain for metabolic activity can detect tumors, cancer
Magnetic Resonance Imaging	MRI	Enables the detection of abnormalities of the brain
Functional MRI	fMRI	Used to produce activation maps (metabolism based) showing brain parts involved in a particular mental process Based
Field Biology Methods		
Capture recapture method		Most common method for estimating population
Quadrant Sampling		Used for the sampling and detection of vegetation diversity in a community
Line –Intercept analysis		Used for quantitative estimate of density of individuals area cover and frequency
Vertical incidence Sonar Technique		Used for detection for biodiversity on seafloor.

IMPORTANT TOPICS OF THIS UNIT FOR CSIR-NET

1. **RECOMBINANT DNA TECHNOLOGY**
2. **1D AND 2D GEL ELECTROPHORESIS**
3. **ISOELECTRIC FOCUSING GELS**
4. **RFLP, RAPD, AND AFLP TECHNIQUES**
5. **ELISA, RIA,**
6. **WESTERN BLOT, IMMUNOPRECIPITATION**
7. **FLOW CYTOMETRY, IMMUNOFLUORESCENCE MICROSCOPY,**
8. **FLUORESCENCE SPECTROSCOPY**
9. **PATCH-CLAMP RECORDING**
10. **BRAIN ACTIVITY RECORDING**
11. **PET, MRI, FMRI, CAT**
12. **GENE KNOCK OUT**
13. **MICROARRAYS**
14. **NMR**
15. **COSMID, BAC AND YAC VECTORS**
16. **CIRCULAR DICHROISM**

13.1. Molecular Biology and Recombinant DNA methods

13.1.1. Isolation and purification of RNA, DNA (genomic and plasmid) and proteins, different separation methods.

Isolation of nucleic acids and proteins is one of the principal steps in molecular biology and recombinant DNA technology. Yield and the quality of these processes largely decide the downstep processes such as manipulation of DNA, PCR, sequencing or other proteomics and transcriptomic methods. In this section, we will mainly discuss the basic principles of methods used for the isolation and purification of DNA, RNA, and proteins.

Isolation of DNA:

- The use of DNA for analysis or manipulation usually requires that it is isolated and purified to a certain extent. DNA is recovered from cells by the gentlest possible method of cell rupture to prevent the DNA from fragmenting by mechanical shearing. This is usually in the presence of EDTA which chelates the Mg^{2+} ions needed for enzymes that degrade DNA termed DNase. Ideally, cell walls, if present, should be digested enzymatically (e.g. lysozyme treatment of bacteria), and the cell membrane should be solubilised using detergent. If physical disruption is necessary, it should be kept to a minimum, and should involve cutting or squashing of cells, rather than the use of shear forces. Cell disruption (and most subsequent steps) should be performed at 4^0C, using glassware and solutions that have been autoclaved to destroy DNase activity.

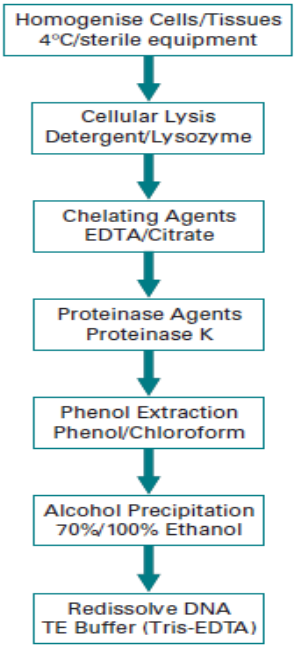

Figure: General steps involved in extracting DNA from cells or tissues.

- After release of nucleic acids from the cells, RNA can be removed by treatment with ribonuclease (RNase) that has been heat-treated to inactivate any DNase contaminants; RNase is relatively stable to heat as a result of its disulphide bonds, which ensure rapid renaturation of the molecule on cooling. The other major contaminant, protein, is removed by shaking the solution gently with water-saturated phenol, or with a phenol/chloroform mixture, either of which will denature proteins but not nucleic acids.

- Centrifugation of the emulsion formed by this mixing produces a lower, organic phase, separated from the upper, aqueous phase by an interface of denatured protein. The aqueous solution is recovered and deproteinised repeatedly, until no more material is seen at the interface.
- Finally, the deproteinised DNA preparation is mixed with two volumes of absolute ethanol, and the DNA allowed to precipitate out of solution in a freezer. After centrifugation, the DNA pellet is redissolved in a buffer containing EDTA to inactivate any DNases present. This solution can be stored at 4^0C for at least a month. DNA solutions can be stored frozen although repeated freezing and thawing tends to damage long DNA molecules by shearing.
- If the DNA from a specific organelle or viral particle is needed, it is best to isolate the organelle or virus before extracting its DNA, since the recovery of a particular type of DNA from a mixture is usually rather difficult. Where a high degree of purity is required DNA may be subjected to density gradient ultracentrifugation through caesium chloride which is particularly useful for the preparation of plasmid DNA.
- It is possible to check the integrity of the DNA by agarose gel electrophoresis and determine the concentration of the DNA by using the fact that 1 absorbance unit equates to 50 µg ml^{-1} of DNA. Contaminants may also be identified by scanning UV spectrophotometry from 200 nm to 300 nm. A ratio of 260nm : 280nm of approximately 1.8 indicates that the sample is free of protein contamination, which absorbs strongly at 280 nm.

Isolation of RNA:

- The methods used for RNA isolation are very similar to those described above for DNA; however, RNA molecules are relatively short, and therefore less easily damaged by shearing, so cell disruption can be rather more vigorous. RNA is, however, very vulnerable to digestion by RNases which are present endogenously in various concentrations in certain cell types and exogenously on fingers. Gloves should therefore be worn, and a strong detergent should be included in the isolation medium to immediately denature any RNases.
- Subsequent deproteinisation should be particularly rigorous, since RNA is often tightly associated with proteins. DNase treatment can be used to remove DNA, and RNA can be precipitated by ethanol. One reagent in particular which is commonly used in RNA extraction is guanadinium thiocyanate which is both a strong inhibitor of RNase and a protein denaturant.

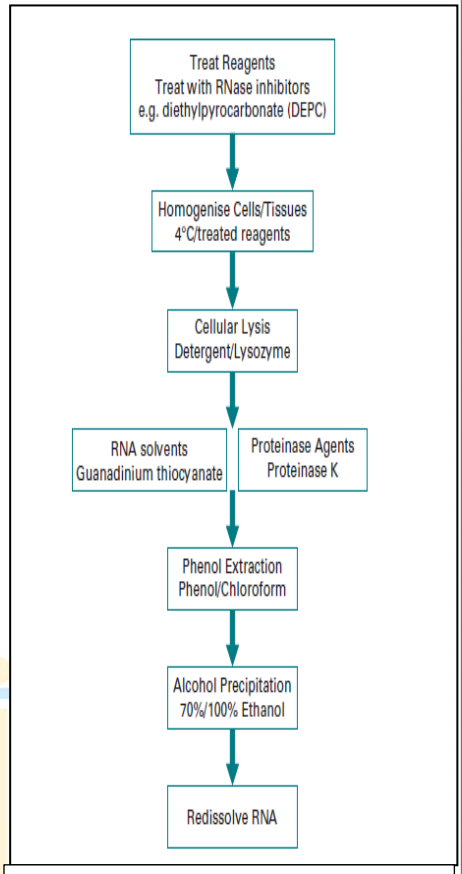

Figure: General steps involved in extracting RNA from cells or tissues.

- It is possible to check the integrity of an RNA extract by analysing it by agarose gel electrophoresis. The most abundant RNA species, the rRNA molecules 23S and 16S for prokaryotes and 18S and 28S for eukaryotes, appear as discrete bands on the agarose gel and thus indicate that the other RNA components are likely to be intact. This is usually carried out under denaturing conditions to prevent secondary structure formation in the RNA.

13.1.2. Different separation methods:

Chromatography:

The first detailed description of chromatography is generally credited to Michael Tswett. A Russian biochemist. who separated chlorophyll from a mixture of plant pigments in 1906. Because of the nature of the pigments in the sample. each band had a distinctive color. Thus,

the name of the process was coined from the Greek words for color (chromo) and to write" (graphy).

If the individual components of a mixture have extensively dissimilar physical and chemical properties, it is very easy to separate one from another. But as the individual components of a mixture get more and more similar in physical and chemical properties, it becomes increasingly difficult to separate them from one another. For example. most amino acids resemble one another rather closely in physio-chemical properties. It is impossible to separate a given amino acid from a mixture of several by conventional separation methods such as fractional crystallization. A separation might. however. be achieved readily using chromatography.

- **Column chromatography:**

Since proteins have difference characteristic features as size, shape, net charge, stationary phase used, and binding capacity, each one of these characteristic components can be purified using chromatographic methods. Among these methods, most frequently column chromatography is applied. This technique is used for the purification of biomolecules.

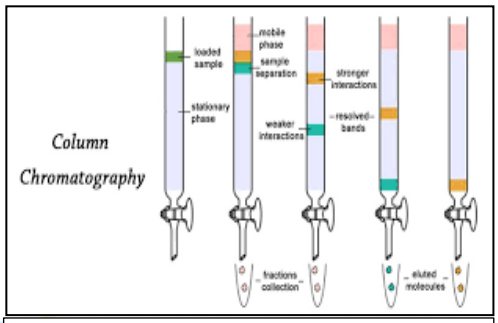

Figure: Column chromatography

On a column (stationary phase) firstly the sample to be separated, then wash buffer (mobile phase) are applied. Their flow through inside column material placed on a fiberglass support is ensured. The samples are accumulated at the bottom of the device in a time, and volume-dependent manner.

- **Ion- exchange chromatography:**

Ion- exchange chromatography is based on electrostatic interactions between charged protein groups, and solid support material (matrix). Matrix has an ion load opposite to that of the protein to be separated, and the affinity of the protein to the column is achieved with ionic ties.

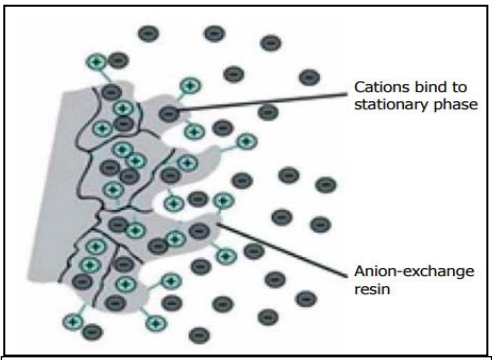

Figure: Ion-exchange chromatography

Proteins are separated from the column either by changing pH, concentration of ion salts or ionic strength of the buffer solution. Positively charged ion- exchange matrices are called anion-exchange matrices, and adsorb negatively charged proteins.

While media bound with negatively charged groups are known as cation-exchange matrices, and adsorb positively charged proteins.

- **Affinity chromatography:**

This chromatography technique is used for the purification of enzymes, hormones, antibodies, nucleic acids, and specific proteins. A ligand which can make a complex with specific protein (dextran, polyacrylamide, cellulose etc.) binds the filling material of the column. The specific protein which makes a complex with the ligand is attached to the solid support (matrix), and retained in the column, while free proteins leave the column.

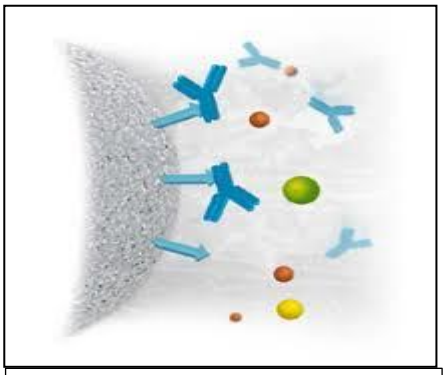

Figure: Affinity chromatography

Then the bound protein leaves the column by means of changing its ionic strength through alteration of pH or addition of a salt solution.

- **Paper chromatography:**

In paper chromatography support material consists of a layer of cellulose highly saturated with water. In this method a thick filter paper comprised the support, and water drops settled in its apertures made up the stationary "liquid phase." Mobile phase consists of an appropriate fluid placed in a developing tank. Paper chromatography is a "liquid-liquid" chromatography.

- **Thin-layer chromatography:**

Thin-layer chromatography is a "solid-liquid adsorption" chromatography. In this method stationary phase is a solid adsorbent substance coated on glass plates. As adsorbent material all solid substances used. in column chromatography (alumina, silica gel, cellulose) can be utilized. In this method, the mobile phase travels upward through the stationary phase. The solvent travels up the thin plate soaked with the solvent by means of capillary action. During this procedure, it also drives the mixture priorly dropped on the lower parts of the plate with a pipette upwards with different flow rates. Thus, the separation of analytes is achieved. This upward travelling rate depends on the polarity of the material, solid phase, and of the solvent.
In cases where molecules of the sample are colorless, florescence, radioactivity or a specific chemical substance can be used to produce a visible colored reactive product so as to identify their positions on the chromatogram. Formation of a visible color can be observed under room light or UV light. The position of each molecule in the mixture can be measured by calculating the ratio between the distances travelled by the molecule and the solvent. This measurement value is called relative mobility, and expressed with a symbol R_f. R_f. value is used for qualitative description of the molecules.

- **High-pressure liquid chromatography (HPLC):**

Using this chromatography technique it is possible to perform structural, and functional analysis, and purification of many molecules within a short time, This technique yields perfect results in the separation, and identification of amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, and other biologically active molecules, In HPLC, mobile phase passes through columns under 10–400 atmospheric pressure, and with a high (0.1–5 cm/sec) flow rate. In this method, use of small particles, and application of high pressure on the rate of solvent flow increases separation power, of HPLC and the analysis is completed within a short time. Essential components of a HPLC device are solvent depot, high- pressure pump, commercially prepared column, detector, and recorder. Length of separation is controlled with the aid of a computerized system, and material is accrued.

13.1.3. Analysis of RNA, DNA and proteins by one- and two-dimensional gel:

Agarose Gel Electrophoresis:

- Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA, RNA or proteins in a matrix of agarose. Agarose is a natural linear polymer extracted from seaweed that forms a gel matrix by hydrogen-bonding when heated in a buffer and allowed to cool.

  They are the most popular medium for the separation of moderate and large-sized nucleic separation.

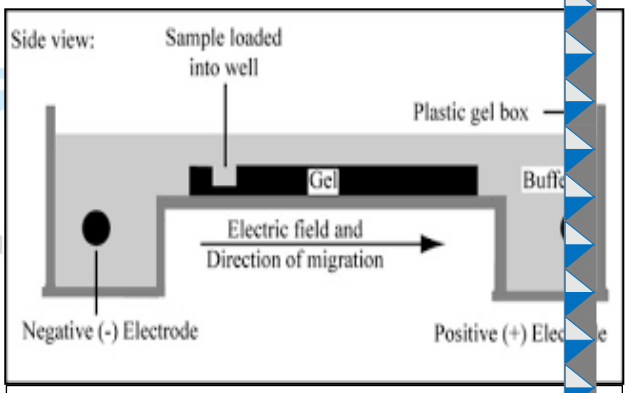

Figure: Agarose gel electrophoresis system

- Gel electrophoresis separates DNA fragments by size in a solid support medium such as an agarose gel. Sample (DNA) are pipetted into the sample wells, followed by the application of an electric current at the anodal, negative end which causes the negatively-charged DNA to migrate (electrophorese) towards the bottom (cathodal, positive) end. The rate of migration is proportional to size: smaller fragments move more quickly, and wind up at the bottom of the gel.
- DNA is visualized by including in the gel an intercalating dye, ethidium bromide. DNA fragments take up the dye as they migrate through the gel. Illumination with ultraviolet light causes the intercalated dye to fluoresce.

- The larger fragments fluoresce more powerfully. Although each of the fragments of a single class of molecule are present in equimolar proportions, the smaller fragments include less mass of DNA, take up less dye, and therefore fluoresce less intensely. A "ladder" set of DNA fragments of known size can be run simultaneously and used to estimate the sizes of the other unknown fragments.

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis:

- SDS PAGE or Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis is a technique used for the separation of proteins based on their molecular weight. It is a technique widely used in forensics, genetics, biotechnology and molecular biology to separate the protein molecules based on their electrophoretic mobility.
- The principle of SDS-PAGE states that a charged molecule migrates to the electrode with the opposite sign when placed in an electric field. The separation of the charged molecules depends upon the relative mobility of charged species.
- The smaller molecules migrate faster due to less resistance during electrophoresis. The structure and the charge of the proteins also influence the rate of migration. Sodium dodecyl sulphate and polyacrylamide eliminate the influence of structure and charge of the proteins, and the proteins are separated based on the length of the polypeptide chain.
- SDS is a detergent present in the SDS-PAGE sample buffer. SDS along with some reducing agents functions to break the disulphide bonds of proteins disrupting the tertiary structure of proteins.

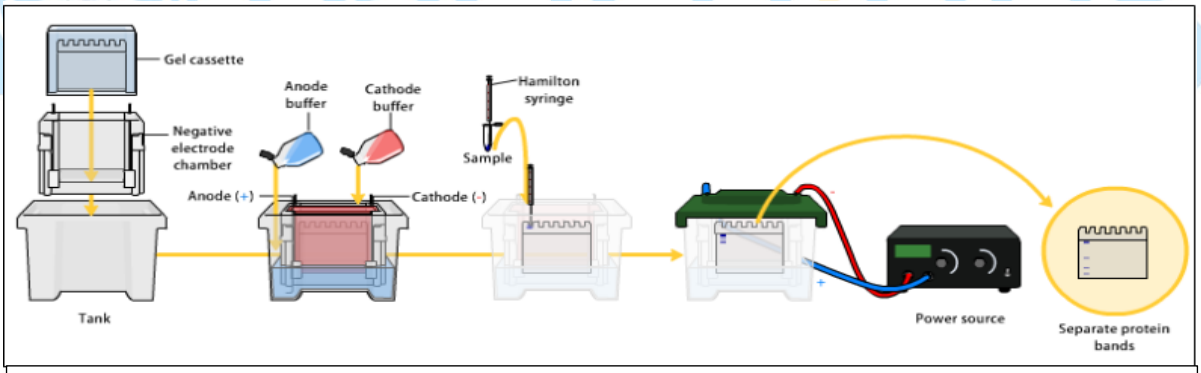

Figure: Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis

Isoelectric focussing gels:

This method is ideal for the separation of amphoteric substances such as proteins because it is based on the separation of molecules according to their different isoelectric points. The method has high resolution, being able to separate proteins that differ in their isoelectric points by as little as 0.01 of a pH unit. The most widely used system for IEF utilizes horizontal gels on glass plates or plastic sheets. Separation is achieved by applying a potential difference across a gel that contains a pH gradient. The pH gradient is formed by the introduction into the gel of compounds known as ampholytes, which are complex mixtures of synthetic polyaminopolycarboxylic acids.

However, the introduction of thin-layer IEF gels, which are only 0.15mm thick and which are prepared using a layer of electrical insulation tape as the spacer between the gel plates, has considerably reduced the cost of preparing IEF gels, and such gels are now commonly used.

Since this method requires the proteins to move freely according to their charge under the electric field, IEF is carried out in low percentage gels to avoid any sieving effect within the gel. Polyacrylamide gels (4%) are commonly used.

To prepare a thin-layer IEF gel, carrier ampholytes, covering a suitable pH range, and riboflavin are mixed with the acrylamide solution, and the mixture is then poured over a glass plate (typically 25 cm 10 cm), which contains the spacer. The second glass plate is then placed on top of the first to form the gel cassette, and the gel polymerised by photopolymerisation by placing the gel in front of a bright light.

Once the gel has set, the glass plates are prised apart to reveal the gel stuck to one of the glass sheets. Electrode wicks, which are thick (3 mm) strips of wetted filter paper (the anode is phosphoric acid, the cathode sodium hydroxide) are laid along the long length of each side of the gel and a potential difference applied. Under the effect of this potential difference, the ampholytes form a pH gradient between the anode and cathode. The power is then turned off and samples applied by laying on the gel small squares of filter paper soaked in the sample. A voltage is again applied for about 30 min to allow the sample to electrophorese off the paper and into the gel, at which time the paper squares can be removed from the gel. Depending on which point on the pH gradient the sample has been loaded, proteins that are initially at a pH region below their isoelectric point will be positively charged and will initially migrate towards the cathode. As they proceed, however, the surrounding pH will be steadily increasing, and therefore the positive charge on the protein will decrease correspondingly until eventually the protein arrives at a point where the pH is equal to its isoelectric point. The protein will now be in the zwitterion form with no net charge, so further movement will cease.

Following electrophoresis, the gel must be stained to detect the proteins. However, this cannot be done directly, because the ampholytes will stain too, giving a totally blue gel. The gel is therefore first washed with fixing solution (e.g. 10% (v/v) trichloroacetic acid). This precipitates the proteins in the gel and allows the much smaller ampholytes to be washed out. The gel is stained with Coomassie Brilliant Blue and then destained.

IEF is a highly sensitive analytical technique and is particularly useful for studying microheterogeneity in a protein. For example, a protein may show a single band on an SDS gel, but may show three bands on an IEF gel. This may occur, for example, when a protein exists in mono-, di- and tri-phosphorylated forms. The difference of a couple of phosphate groups has no significant effect on the overall relative molecular mass of the protein, hence a single band on SDS gels, but the small charge difference introduced on each molecule can be detected by IEF.

Two-dimensional polyacrylamide gel electrophoresis:

This technique combines the technique of IEF (first dimension), which separates proteins in a mixture according to charge (pI), with the size separation technique of SDS–PAGE (second dimension). The combination of these two techniques to give two-dimensional (2-D) PAGE provides a highly sophisticated analytical method for analysing protein mixtures. To maximise separation, most workers use large format 2-D gels, although the mini gel system can be used to provide useful separation in some cases. For large-format gels, the first dimension (isoelectric focussing) is carried out in an acrylamide gel that has been cast on a plastic strip. The gel contains ampholytes (for forming the pH gradient) together with 8M urea and a non-ionic detergent, both of which denature and maintain the solubility of the proteins being analysed. The denatured proteins therefore separate in this gel according to their isoelectric points. The IEF strip is then incubated in a sample buffer containing SDS (thus binding SDS to the denatured proteins) and then placed between the glass plates of, and on top of, a previously prepared 10% SDS–PAGE gel. The IEF gels are provided as dried strips and need rehydrating overnight. The first dimension IEF run takes 6-8 hrs, the equilibration step with SDS sample buffer takes about 15 min, and then the SDS–PAGE step takes about 5 h. Using this method one can routinely

resolve between 1000 and 3000 proteins from a cell or tissue extract and in some cases workers have reported the separation of between 5000 and 10 000 proteins.

13.1.5. Molecular cloning of DNA or RNA fragments in bacterial and eukaryotic systems.

- **Vectors**

Vectors can be defined as an autonomously replicating DNA sequence that can be used to carry foreign DNA fragments. Commonly used vectors are bacteriophages and plasmids. Vector that is used predominantly for reproducing DNA segment 1s referred to as cloning vectors and vectors used for expressing a gene contained within the cloned DNA is referred to as expression vectors.

- **Characteristics of Vector**

a) Ability to self-replicate.
b) Selectable characteristic so that transformed cells can be recognized from non-transformed cells
c) Should have origin of replication.
d) Selectable markers.
e) Small size.
f) Plasmid high copy number.
g) Restriction site.

- **Cloning Vectors:**

Cloning vectors are DNA molecules that are used to "transport" cloned sequences between biological hosts and the test tube. A vector is used to amplify a single molecule of DNA into many copes.

- **Characteristics of Cloning Vector**

a) Ability to replicate
b) Contain a genetic marker for selection
c) Unique restriction sites to facilitate cloning of insert DNA
d) Minimum amount of nonessential DNA to optimize cloning

- **Plasmid:**

These are naturally occurring extra-chromosomal DNA fragments that are stably inherited from one generation extra-chromosomal state. Size is 1500 bp to over 300 kbp. Shape Closed circular ds DNA molecule that confer particular phenotype onto bacterial cell in which they can replicated. Plasmids often carry a gene that codes resistance to either antibiotics or heavy metals, or that produces DNA restriction and modification enzyme, that bacterium normally does not produce. First vector constructed was pSC101.

The selection is done by selectable markers called antibiotic resistance genes. When foreign gene is inserted in the gene, insertional inactivation occurs. The disadvantage of this vector is that its selection is not easy.

- **Components of the plasmid:**

1. Ori Origin of replication, to ensure high plasmid copy number. It can be increased by chloramphenicol amplification.
2. Antibiotic Resistance Genes Two genes; a selectable marker either ampicillin or tetracycline can be used. Each marker includes restriction sites used for cloning.
3. Cloning Sites Plasmid carries number of unique restriction enzyme recognition sites. These are located in Antibiotic resistance genes.
4. Selection There is a direct selection of recombinants in process called insertional inactivation.

- **Bacteriophage Vectors (M13):**

A M13 are filamentous E. coli bacteriophages. It is a male specific lysogenic phage with a circular single stranded DNA genome of 6407 bp in length. It infects bacteria that harbor F-pilus. The phage particle absorbs in end to the F-pilus and the single stranded phage DNA enters the bacterium and very rapidly ssDNA gets converted to dsDNA by the synthesis of complimentary DNA strand using bacteria DNA polymerase. Transcription of viral genes occurs to produce proteins required for assembly of new viral particles. Products of viral encoded single stranded binding protein forces asymmetric replication of dsDNA. This results in only one viral strand being synthesized. These single stranded DNA molecules ensemble into the viral particles and are released from the end without cell lysis. M13 phage infection does not result in bacterial cell death and M13 infections appear as turbid plaques. The M13 ori has two overlapping but distinct DNA sequences that act to control the DNA synthesis. These sites, the F1 initiator and F1 terminator-single, are the beginning and end of DNA replication respectively. After transcription, rolling circle replication occurs to form new strands. This results in phage assembly and finally phage is released from the bacterium.

- **Phagemid:**

These are the plasmids that contain F1 phage origin of replication for production of ssDNA. These are generally small plasmids so that they have the capability to accept larger DNA inserts than M13-based vectors. It is a type of cloning vector developed as a hybrid of filamentous phage M13 and plasmids to produce a vector that can grow as a plasmid and also be packaged as single strand DNA in viral particles that contains ori for λ_t 's replication and an F1 ori to enable single stranded replication and packaging into phage particles. Phagemid can also be used to clone DNA fragments and be introduced into a bacterial host by range of techniques However infection of bacteria most containing a phagemid (with a helper phage) Provides necessary viral components to enable ssDNA replication and packaging of the phagemid DNA into phage particles. These are secreted through cell wall and released into the medium. F1 ori is composed of an initiator and terminator.

- **Yeast artificial chromosomes (YACs):**
  1. Yeast artificial chromosomes (YACs) are genetically engineered chromosomes derived from the DNA of the yeast.
  2. It is a human-engineered DNA molecule used to clone DNA sequences in yeast cells.
  3. They are the products of a recombinant DNA cloning methodology to isolate and propagate very large segments of DNA in a yeast host.

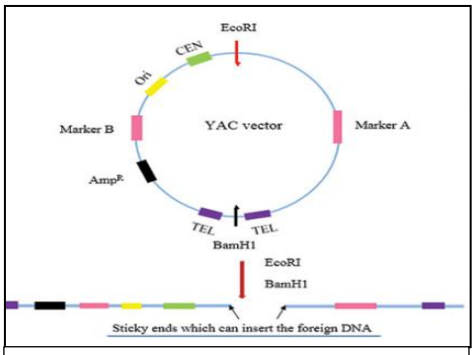

Figure: Yeast artificial chromosomes

  4. By inserting large fragments of DNA, the inserted sequences can be cloned and physically mapped using a process called chromosome walking.
  5. The amount of DNA that can be cloned into a YAC is, on average, from 200 to 500 kb.
  6. However, as much as 1 Mb (mega, 106) can be cloned into a YAC.

- **Bacterial Artificial Chromosomes (BACs):**

The F (fertility) factor is a plasmid that can be mobilized from F+ male bacteria and F- female bacteria. The gene transfer from one to another bacterial cell is called conjugation. The F factor controls its own replication. It has two origins of replication: oriV is the origin for bidirectional replication; oriS is the origin for unidirectional replication. The F factor also has genes that regulate DNA synthesis so that its copy number is kept at a low level; and, genes that regulate the partition into the daughter cells after E. coli divides.

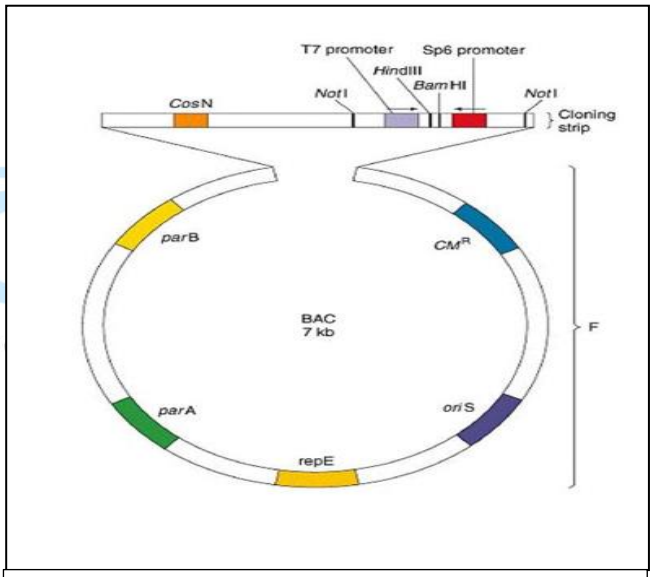

Figure: Bacterial Artificial Chromosomes

A modification on BAC vectors is to add a second replication origin to the vectors. The second origin, the oriV, will generate multiple copies of BACs, if a trans-acting factor, the TRF, is present. The expression of the trf gene, which has been integrated into the chromosome of the bacteria is controlled by a tightly-regulated inducible promoter.

13.1.6. Isolation of specific nucleic acid sequences

- **Polymerase Chain Reaction (PCR):**

The polymerase chain reaction technique is carried out in vitro and is used for the amplification of DNA. It was developed by Kary Mullis in 1983. Through this technique billion copies of the desired DNA or RNA can be made in a matter of few hours, The PCR reaction mix contains genomic DNA having the target sequence, two oligonucleotide primers- forward ,and reverse primer that are complementary to the borders of the two strands of the desired DNA segment, four deoxynucleoside triphosphates i.e. dTTP (deoxythymidine triphosphate), dCTP (deoxycytidine, triphosphate), dATP (deoxyadenosine triphosphate) and dGTP (deoxy guanosine triphosphate) and Taq polymerase, MgC12 and Buffer.

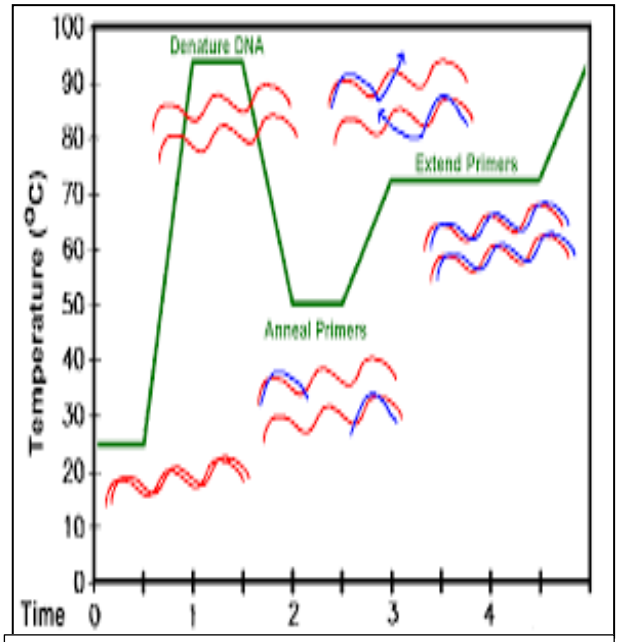

Figure: PCR Reaction cycle:

- **Procedure of PCR/ Reaction cycle:**

➢ **Denaturation:** During denaturation step, the reaction mixture is first heated to a temperature between 90-98°C that ensures DNA denaturation. The duration of this step in the first cycle of PCR is usually 2 min at 94°C.

➢ **Annealing:** During annealing, the mixture is cooled to a temperature of 40-60°C so that annealing of primer to the complementary sequences in the DNA take place. The duration of annealing step is usually 1 minute. Primer-template hybrid formation is greatly favoured over reannealing of the template strand**.**

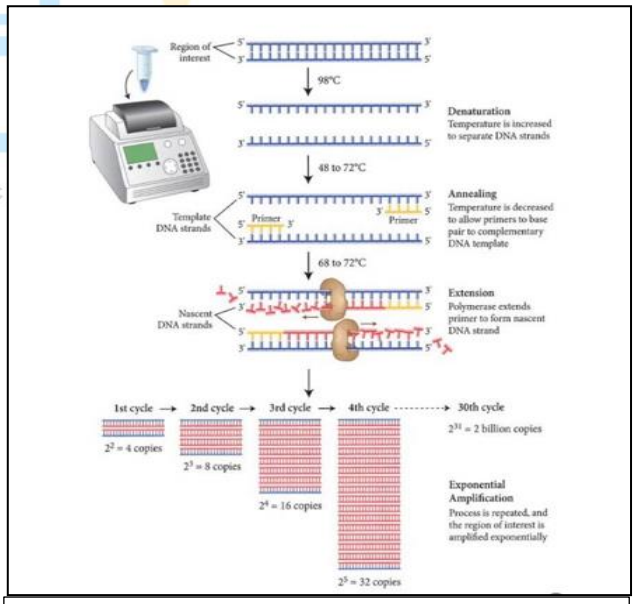

Figure: Procedure of PCR

➢ **Primer Extension:** By utilizing 3'-OH of the primers, primer extension is done. The duration of primer extension is usually 2 minute at 72°C. The primers are extended towards each other so that the DNA segment lying between the two primers is copied. Taq polymerase catalyses the extension of DNA segment. The optimum temperature for working of Taq polymerase is 72-74°C. These cycles are repeated 20-30 times to

get million copies of desired gene segment as after each cycle there is (2^n) exponential increase in the copies of DNA segment.

- **Applications of PCR**

Infectious disease diagnosis, progression and response to therapy
Diagnosis of genetic diseases
Genetic counselling
Forensic science
Research in Molecular Biology

Types of PCR

a). Inverse PCR:

Inverse PCR can be used to amplify the sequences flanking a segment of the border sequences which are known. This is done by using prime oligonucleotides complementary to the 5'ends of the desired segment. This orients the free 3' OH of the primers outward of the sequence, as a result the newly synthesized chain grows away from the borders of the concerned segment.

The procedure involves- (a) Restriction of the target DNA with a restriction enzyme that produces sticky ends and that cuts at unknown sites on either side of the known region. (b) The restriction fragment is allowed to circularize, that is unknown region of interest. (c) The circularized fragment is ligated and then opened with an enzyme that cuts only within the known region. (d) The resulting linear fragment now has the known region on both its ends which can therefore be exponentially amplified using the primers specific to the known region.

b). RT-PCR (Reverse Transcription-PCR):

RT-PCR is used to amplify RNA sequences into DNA. RT-PCR is used to selectively amplify the desired cDNA molecule from a mixture of cDNAs. One must know the base sequence just preceding the 3'-poly-A tail. An oligonucleotide having this base sequence is used as a gene-specific primer in the PCR. The steps involved in RT-PCR are; firstly, the total mRNA is used to produce the cDNA duplex. secondly an oligo-c primer and the gene-specific primer are used for PCR amplification of the desired cDNA present among the mixture of cDNAs. Alternatively, the gene-specific primer (complimentary to the 3'-end of the desired mRNA) is itself used to prime reverse transcription to generate the cDNA single-strand, which is copied using a primer specific to the 5'- end of the mRNA.

The two specific primers are used to prime the PCR for amplifying the cDNA. The use of 5'-specific primer eliminates the risk of amplification of partial cDNAs. A modification of RT-PCR is called RACE (Rapid Amplification of cDNA Ends).

c). Thermal Cycle Sequencing PCR:

In thermal cycle sequencing PCR, only one primer is used in combination with dideoxynucleotide. The primer is either radio or fluorescence labeled. The product of PCR reaction is subjected to PAGE for determining the base sequence of the template DNA.

d). Touchdown PCR:

This type of PCR is initiated at very high annealing temperatures which allow only perfectly matched primer-template DNA hybrids to form and support amplification. The annealing temperature is dropped in a step. wise fashion with each cycle of PCR. This variation of PCR has been devised to increase the specificity of PCR without lowering the efficiency.

e). Hot start PCR:

In hot start PCR, a critical component like Me or Taq polymerase, is left out from the reaction mixture. Once the reaction mixture is heated to the denaturation temperature, this reagent is added to the PCR tubes by opening their tops. This is done in order to check the activity of Taq polymerase which shows a degree of activity at room with amplification of spurious sequences.

f). Nested PCR:

Nested PCR is used in situations in which it is necessary to increase the sensitivity and/or specificity of PCR. For example, when amplifying a particular member of a polymorphic gene family or when amplifying a cDNA copy of an mRNA present at very low abundance in a clinical specimen containing several different cell types (a heterogeneous population of cells). This PCR increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets (instead of one pair) of primers are used in two successive PCRs. In the first reaction, on pair of primers outer pair is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments.

13.1.7. In vitro mutagenesis and deletion techniques

In-vitro Mutagenesis In this technique, mutation is produced in a segment of DNA which is cloned. This mutated DNA is then inserted into a cell in order to study mutations. Due to this technique, it is possible to alter (efficiently and systematically) nucleotide sequences in DNA. It can be categorised into various categories like restructuring of DNA segments, localized random mutagenesis, oligonucleotides directed mutagenesis and gene disruption.

- **Oligonucleotides directed mutagenesis:**

In oligonucleotide directed mutagenes, an oligonucleotide is a short stretch of synthetic DNA of desired sequence, is made chemically and a specific point in a sequenced gene is pinpointed for mutation. These synthetic nucleotides can be used to construct mutant alleles of a cloned gene with base substitutions, insertions or deletions. For example, the oligonucleotide might have adenine in one specific location instead of guanine. This oligonucleotide is hybridized to the complementary strand of the cloned gene; it will hybridize despite the one base pair mismatch. Various enzymes are added to allow the oligonucleotide to prime the synthesis of a complete strand within the vector. When the vector is introduced into a bacterial cell and replicates, the mutated strand acts as a template for a complementary strand that is also mutant and thus a fully mutant molecule is obtained. This fully mutant cloned molecule is then reintroduced into the donor organism and the mutant DNA replaces the resident gene.

- **Gene knockout in bacterial and eukaryotic organisms:**

A gene knockout is a genetically engineered organism that carries one or more genes in its chromosomes that have been made deleted or inoperative. This is done for research purposes. Also known as knockout organisms or simply knockouts, they are used in learning about a gene that has been sequenced, but which has an unknown or incompletely known function. Knockout is accomplished through a combination of techniques. beginning in the test tube with a plasmid, a bacterial artificial chromosome or other DNA construct and proceeding to cell culture. Individual cells are genetically transformed with the construct and for knockouts in multi-cellular organisms; ultimately fused with a stem cell from a nascent embryo. The construct is engineered to recombine with the target gene, which is accomplished by incorporating sequences from the gene itself into the construct. Recombination then occurs in the region of that sequence within the gene. resulting in the insertion of a foreign sequence to disrupt the gene. With its sequence interrupted, the altered gene in most cases will be translated into a nonfunctional protein, if it is translated at all. A conditional knockout allows gene deletion in a tissue specific manner. Because recombination is a rare event in the case of most cells and most constructs, the foreign sequence chosen for insertion usually is a, reporter. This enables easy selection of cells or individuals in which knockout was successful. In diploid organisms, which contain two alleles for most genes and may as well contain several related genes that collaborate in the same role, additional rounds of transformation and selection are performed until every targeted gene is knocked out. Knock-in is similar to knock-out. but instead it replaces a gene with another instead of deleting it.

13.1.8. DNA sequencing methods:

DNA sequencing is a process used to map out was first discovered in 1975 and has now become a powerful technique in molecular biology, allowing analysis of genes at the nucleotide level. For this reason, this tool has been applied to many areas of research.

- **Maxam and Gilbert method of sequencing:**

The Maxam-Gilbert method of nucleotide sequence determination is based on preferential. base-specific methylation followed by chemical cleavage to generate a set of end-labeled derivatives. In 1976-1977. Allan imam and Walter Gilbert developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases. Maxam Gilbert sequencing rapidly became more popular. Since purified DNA could be used directly, while the initial Sanger method required that each read start be cloned, for production of single-stranded DNA.

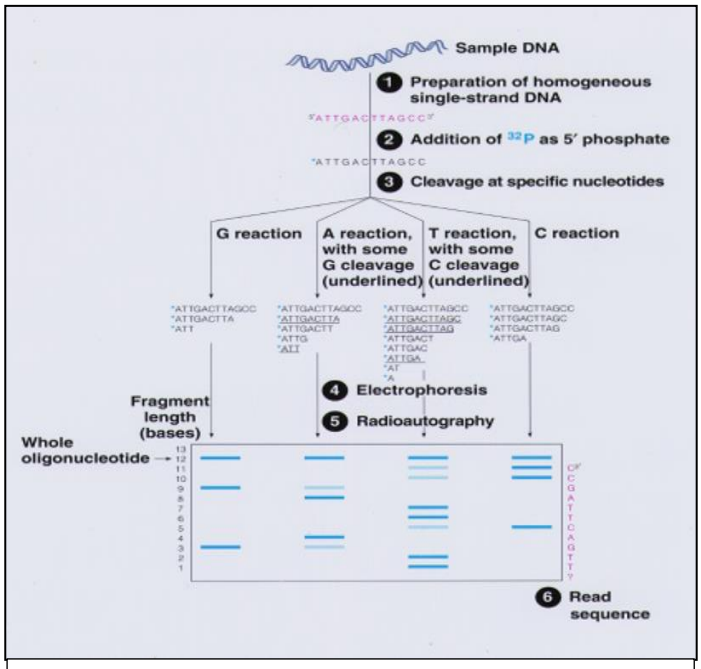

Figure: Maxam and Gilbert method of sequencing

However, with the Improvement of the chain-termination method Maxam-Gilbert sequencing has fallen out of favour due to its technical complexity prohibiting its use in standard molecular biology kits. Extensive use of hazardous chemicals and difficulties with scale-up. The method requires radioactive labelling at one end and purification of the DNA fragment to be sequenced. Chemical treatment generates breaks at a small proportion of one or two of the four nucleotide bases in each of four reactions (G, A+G, C and C+ T). Thus, a series of labelled fragments is generated, from the radiolabeled end to the for size first cut site in each molecule. The fragments in the four reactions are arranged side by side in gel electrophoresis separation. To visualize the fragments, the gel is exposed to X-ray film for autoradiography, yielding a series of ark bands each corresponding to a radiolabeled DNA fragment, from which the sequence may be inferred. It’s also known as 'chemical sequencing' and was originated in the study of DNA-protein interactions (footprinting), nucleic acid structure and epigenetic modifications to DNA.

- **Sanger method of DNA sequencing:**

This technique utilizes 2', 3'- dideoxynucleotide triphosphates (ddNTP's), molecules that differ from deoxynucleotides having a hydrogen atom attached to the 3' carbon rather than an -OH group. These molecules terminate DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide. The first step is to convert double stranded DNA into single stranded DNA which is done by denaturing the double stranded DNA with NaOH.

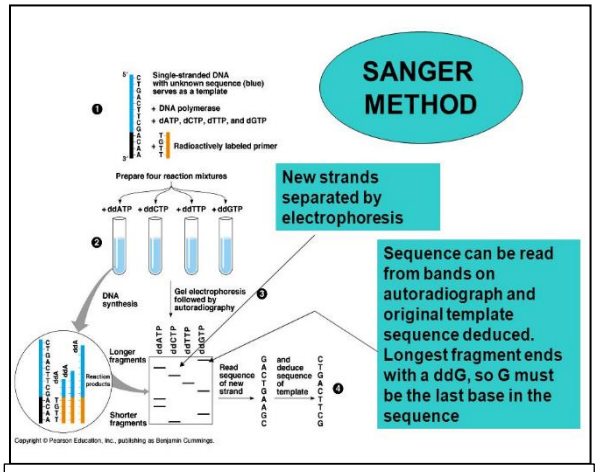

Figure: Sanger method of DNA sequencing:

A Sanger reaction consists of the following: a strand to be sequenced (one of the single strands which was denatured using NaOH), DNA primers (short pieces of DNA that are both complementary to the strand which is to be sequenced and radioactively labelled at the 5' end), a mixture of a particular ddNTP labeled with fluorescent dyes (such as ddATP) with its normal dNTP (dATP in this case) and the other three dNTPs (dCTP, dGTP and dTTP). The concentration of ddATP should be 1% of the concentration of dATP. This reaction is performed four times using a different ddNTP for each reaction. Automated sequencers with multiple capillaries working in parallel can read up to 96 different sequences in a two hours period, which means that with an average of 750 bp per individual experiments, 864 kb of information can be generated per machine per day. This, of course, requires round the clock technical support, ideally with robotic devices used to prepare the sequencing reactions and to load the reaction products into the sequencers.

13.1.9. Methods for analysis of gene expression at RNA and protein level, large scale expression, such as micro array-based techniques:

- **Microarray based technology:**

Microarray technology involves hybridization of a nucleic acid sample to a very large set of oligonucleotide probes which are attached to a solid support. This is done to determine sequence or to detect variations in a gene sequence or expression for gene mapping. The two major applications of microarray technology are gene expression analysis and genetic variation analysis. Microarray technology has various applications like identification of common regulatory elements shared by co-regulated genes, studying different diseases, including heart disease, mental illness, infectious diseases genetic diseases (e.g. for detection of mutant alleles causing cystic fibrosis, mutant alleles of gene BRCA), studying gene expression pattern of an organism as affected by the environment, usage of DNA chips for the detection of SNPs.

Working of DNA Microarray DNA microarrays arc created by robotic machines that arrange minuscule amounts of hundreds or thousands of gene sequences on a single microscope slide. First in determining which genes are turned on and which are turned off in a given cell one should collect the messenger RNA molecules present in that cell. Second step is labelling each mRNA molecule by using a reverse transcriptase enzyme that generates a complementary cDNA to the m-RNA. Fluorescein nucleotides arc attached to the cDNA. The tumour and the normal samples arc labelled with different fluorescent dyes. Labelled cDNAs arc placed onto a DNA microarray slide.

- **Type of Microarrays:**

Microarrays are basically of two types- DNA microarrays and Antibody microarrays.

- **DNA microarrays:**

A DNA microarray (DNA chip or bio-chip) is a collection of microscopic DNA spots attached to a solid surface. It is used to measure the expression levels of large number of gene or to study the genotype of multiple regions of a genome. Each DNA spot contains a probe or reporters or oligos that consist of picomoles of specific DNA sequence. DNA microarrays are of further divided into two types- Spotted microarrays and Oligonucleotide microarrays.

- **Spotted DNA microarrays:**

In this type, DNA sequences representing different genes of an organism are spotted onto the slides and are used to determine genes being transcribed in the cell concerned that is their transcriptome (total mRNA content of a cell).

- **Oligonucleotide Microarrays:**

DNA chips are oligonucleotide microarrays. These are thin wafers of silicon glass carrying many different oligonucleotides synthesized at a very high density directly on the wafer. Each oligonucleotide has the sequence of a different gene present in the genome. The oligonucleotide synthesis is based on two techniques called photolithography and solid-phase DNA synthesis. The DNA chips are inverted and mounted in a temperature-controlled hybridization chamber into which fluorescently labelled cDNA preparation is injected and allowed to hybridize with the oligonucleotides. Laser excitation enters through the back of the glass support focused at the interface of the array surface and target solution. Fluorescent emission is collected by a lens and passed on to a sensitive detector and a quantitative assay of hybridization intensity is obtained.

- **Antibody Microarrays:**

Antibody microarrays, also referred to as antibody arrays or antibody chips, are specific protein microarrays consisting of a collection of capture antibodies fixed on a solid surface such as glass, plastic or silicon, for the purpose of detecting antigens. These arrays are often used for detecting protein expressions from cell lysates in general research and special biomarkers from serum or urine for diagnostic applications, identifying biomarkers and developing drug compounds. Arrays allow protein expression profiling, screening and comparison between

normal, diseased or treated samples. Arrays are available in one or two slides, one slide is for a control sample, and the other is for a treated sample. Hormone antibodies, cell cycle antibodies and stem cell antibodies are examples of the types of arrays available for high throughput screening. Factors to consider when selecting an antibody array are sample type (e.g. cells, tissue or serum), detectable change in expression levels, protein labeling, method of detection and type of analysis. A busy lab has the options of sending sample out for analysis or conducting an in-house analysis with a quality, commercially produced antibody microarray. The fact that lipids are soluble in organic solvents, but insoluble in water, provides the food analyst with a convenient method of separating the lipid components in foods from water soluble components, such as proteins, carbohydrates and minerals. In fact, solvent extraction techniques are one of the most commonly used methods of isolating lipid from foods.

13.1.10. RFLP, RAPD and AFLP techniques:

- **RFLP (Restriction Fragment Length Polymorphism):**

Restriction analysis of DNA by its digestion with restriction endonucleases (RE) in specific restriction sites. Restriction endonucleases enzymes that cleave DNA molecules at specific nucleotide sequences. Restriction enzymes are isolated from a wide variety of bacterial genera. For example, HindII enzyme cuts at GTGCAC or GTTAAC. Variation in the DNA sequence of a genome detected by breaking DNA into pieces with restriction enzymes.

- **RFLP analysis technique:**

RFLP refers to a variation in a sequence of DNA that has a restriction site on each end with a target sequence in between. A target sequence is any segment of DNA that binds to a probe by forming complementary base pairs. A probe is a sequence of single-stranded DNA that has been to with radioactivity or an enzyme so that the probe can be detected. When a probe base Pairs to its target the investigator can detect this binding and know where the target sequence is, as the probe is detectable. SSR and RFLP molecular marker are co-dominant in nature.

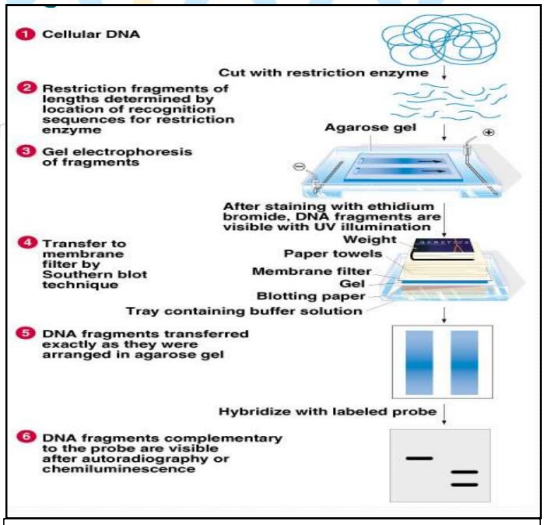

Figure: RFLP analysis technique

RFLP produces a series of bands when a Southern blot is Performed with a particular combination of restriction enzyme and probe sequence. RFLP can be used for fingerprinting.

- **RAPD (Randomly Amplified Polymorphic DNA):**

It is a type of PCR reaction, but the segments of DNA that are amplified are random. RAPD creates several short primers (8–12 nucleotides), then proceeds with the PCR using a large

template of genomic DNA, the fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction.

RAPD is a PCR based technique for identifying genetic variation. It involves use of single arbitrary primer in a PCR reaction, resulting in amplification of many discrete DNA. RAPD technology provides a quick and efficient screen for DNA sequence-based polymorphism at a very large number of loci. It is different from conventional PCR as it need one primer for amplification. The size of primer is shorter (10 nucleotides) therefore less specific. The primers can be designed without the experimenter having any genetic information for the organism being tested. Genomic DNA normally has complimentary sequences to RAPD primers at many locations. The RAPD technology has provided a quick and efficient screen for DNA-sequence polymorphisms at a very large no of loci. Normally, a few (3-20) loci can be amplified by one single RAPD primer.

- **RAPD analysis technique:**

1.Extraction of DNA

2. Selection of Primers

The standard RAPD technology utilizes short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR**.**

3. PCR Amplification

The polymerase Chain Reaction (PCR) is a relatively simple but powerful technique that amplifies a DNA template to produce multiple copies of specific DNA fragment in vitro.

PCR amplification consists of following 3 steps:

Denaturation

Annealing

Extension

- **AFLP (Amplified Fragment Length Polymorphism):**

The AFLP technique is based on the principle of selectively amplifying a subset of restriction fragments from a complex mixture of DNA fragments obtained after digestion of genomic DNA with restriction endonucleases.

- **AFLP analysis technique:**

Following steps are involve in AFLP Digestion, adaptor Ligation, amplification, electrophoresis

Two different restriction endonucleases are used in digestion. One is 4-base cutter (MseI- 5'TTAA3') and the other one is 6-base cutter (EcoRI- 5'GAATTC3'). Two different adaptors (short double stranded DNA sequences with sticky end) are ligated to the digested fragments.

One adaptor will complement to the MseI cut end, the other will complement to the EcoRI cut end. DNA fragments with MseI-EcoRI ends with be selected as DNA template

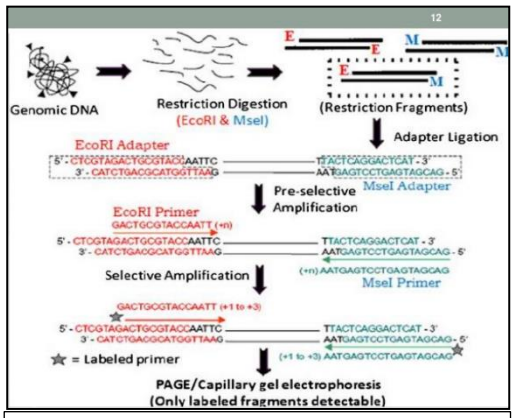

Figure: AFLP analysis technique

for amplication, two PCR primers complementary to the two adaptors are used in amplification. The PCR primers are labelled with radioactive or fluorescence dye for detection of DNA bands on gels, polyacrylamide gel is used for separating DNA bands. Normally, 30-100 DNA bands can be detected by AFLP on polyacrylamide gel.

13.2. Histochemical and Immunotechniques

13.2.1. Radioimmunoassay (RIA):

Radioimmunoassay is a very sensitive in vitro assay technique used to measure concentrations of antigens (for example, hormone levels in the blood) by use of antibodies. As such, it can be seen as the inverse of a radio binding assay, which quantifies an antibody by use of corresponding antigens. Although the RIA technique is extremely sensitive and extremely specific, requiring specialized equipment, it remains among the least expensive methods to perform such measurements. It requires special precautions and licensing, since radioactive substances are used.

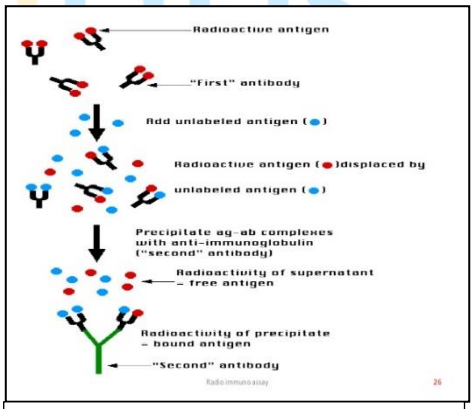

Figure: Radioimmunoassay

The technique was introduced in 1960 by Berson and Yalow as an assay for the concentration of insulin in plasma. It represented the first time that hormone levels in the blood could be detected by an in vitro assay.

13.2.2. Enzyme-linked immunosorbent assay (ELISA):

The enzyme-linked immunosorbent assay (ELISA) is an immunological assay commonly used to measure antibodies, antigens, proteins and glycoproteins in biological samples. Some examples include: diagnosis of HIV infection, pregnancy tests, and measurement of cytokines or soluble receptors in cell supernatant or serum. ELISA assays are generally carried out in 96 well plates, allowing multiple samples to be measured in a single experiment. These plates need to be special absorbant plates to ensure the antibody or antigen sticks to the surface. Each ELISA measures a specific antigen, and kits for a variety of antigens are widely available.
There are three types of enzyme-linked immunosorbent assay (ELISA)

- **Direct ELISA:** A target protein (or a target antibody) is immobilized on the surface of microplate wells and incubated with an enzyme-labeled antibody to the target protein (or a specific antigen to the target antibody). After washing, the activity of the microplate well-bound enzyme is measured.

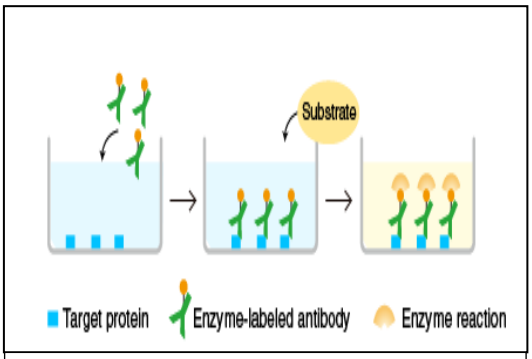

Figure: Direct ELISA

- **Indirect ELISA:** A target protein is immobilized on the surface of microplate wells and incubated with an antibody to the target protein (the primary antibody), followed by a secondary antibody against the primary antibody. After washing, the activity of the microplate well-bound enzyme is measured. Although indirect ELISA requires more steps than direct ELISA, labeled secondary antibodies are commercially available, eliminating the need to label the primary antibody.

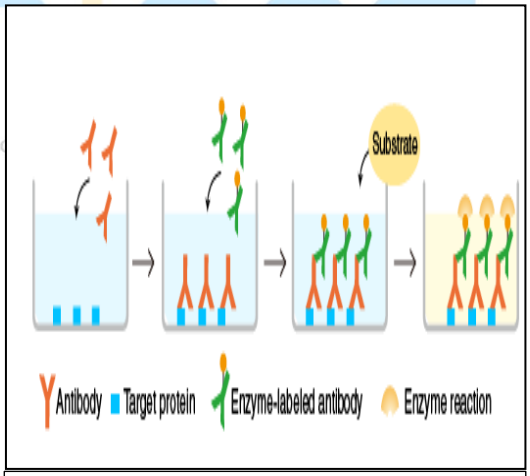

Figure: Indirect ELISA

- **Competitive ELISA:** An antibody specific for a target protein is immobilized on the surface of microplate wells and incubated with samples containing the target protein and a known amount of enzyme-labeled target protein. After the reaction, the activity of the microplate well-bound enzyme is measured.

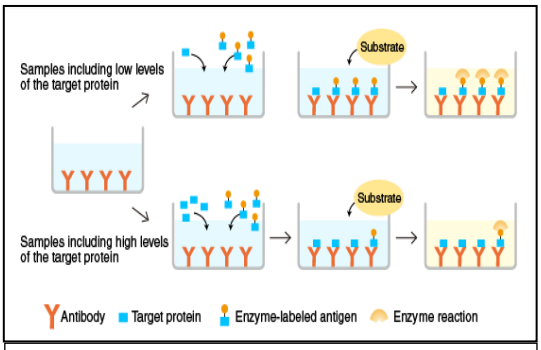

Figure: Competitive ELISA

When the antigen level in the sample is high, the level of antibody-bound enzyme-labeled antigen is lower and the color is lighter. Conversely, when it is low, the level of antibody-bound enzyme-labeled antigen is higher and the color, darker. The graph above illustrates the correlation between absorption and antigen levels in samples.

When a target antigen is a small molecule, such as histamine, pesticide, and dioxin, two antibodies cannot simultaneously bind to the antigen in sandwich ELISA. Competitive ELISA is useful for the measurement of low molecular weight targets.

13.2.3. Western Blotting:

The term Western Blotting is based on a play of words. The southern blot, which is a method to detect specific DNA sequences, is named after Ed Southern, who first described this procedure. The western blot (immunoblot), as well as the northern blot (for RNA detection), play on the meaning of this name.

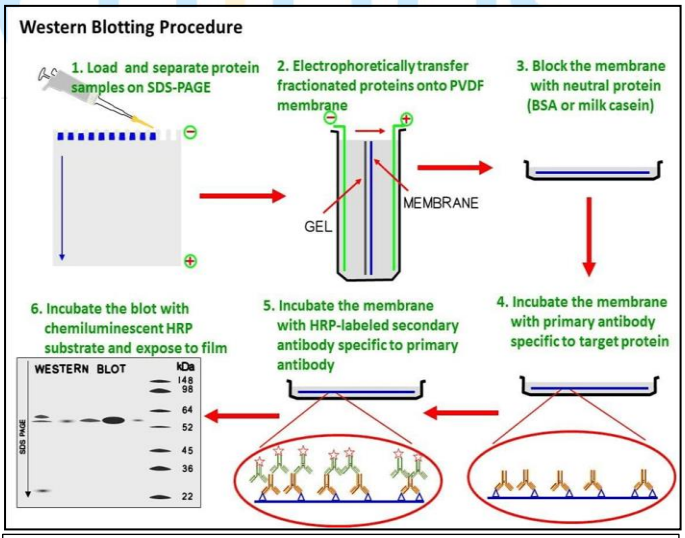

Figure: Western Blotting

Western Blotting (also called immunoblotting) is a technique used for analysis of individual proteins in a protein mixture (e.g. a cell lysate). In Western blotting (immunoblotting) the protein mixture is applied to a gel electrophoresis in a carrier matrix (SDS-PAGE, native PAGE, isoelectric focusing, 2D gel electrophoresis, etc.) to sort the proteins by size, charge, or other differences in individual protein bands. The separated protein bands are then transferred

to a carrier membrane (e.g. nitrocellulose, nylon or PVDF). This process is called blotting. The proteins adhere to the membrane in the same pattern as they have been separated due to interactions of charges. The proteins on this immunoblot are then accessible for antibody binding for detection.

Antibodies are used to detect target proteins on the western blot (immunoblot). The antibodies are conjugated with fluorescent or radioactive labels or enzymes that give a subsequent reaction with an applied reagent, leading to a coloring or emission of light, enabling detection.

13.2.4. Immuno-precipitation:

The immune-precipitation technique allows isolation of antigen of interest and provides a sensitive assay for the presence of 4 particular antigen in given cell or tissue type. The sails or tissues are disrupted to form an extract that is mixed with an antibody against the antigen of interest to from a precipitate. The formation of the precipitate is dependent on the antigen concentration. If the antigen concentration is too low, it may take hours or days for precipitate formation and the immunoprecipitate formed is in small amount which is difficult to isolate.

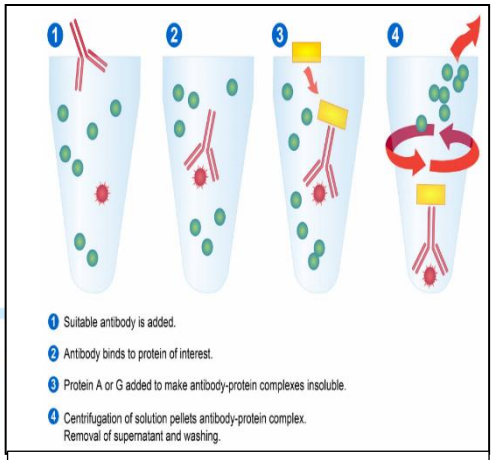

Figure: Immuno-precipitation

These limitations can be circumvented by attaching the antibody to a solid support such as synthetic bead. This allows collecting the antigen-antibody complex by centrifugation.
Adding a secondary antibody specific for the primary antibody to bind antigen-antibody complexes. If secondary antibody is attached to bead and then the immune precipitations are collected by pairing a magnet against side of the tube, the immune complexes can be collected by centrifugation.

13.2.5. Immunofluorescence Microscopy:

In 1944, Albert Coons showed that antibodies having property of fluorescence could be labeled with molecules. Fluorescent molecules absorb light of one wavelength (excitation) and emit light of another wavelength. In this technique, the antibody molecules are labeled with a fluorescent dye or fluorochrome, immune complexes containing fluorescently labeled antibodies (FA) which can be detected by colored light emission when excited by light of appropriate wavelength. Antibody molecules bound to antigens in cells or tissue sections can be visualized similarly. The emitted light can be viewed with a fluorescence microscope, which is equipped with a UV light source. Fluorescein and rhodamine are in common use, but other,

such as phycoerythrin, an intensely colored and highly fluorescent pigment obtained from algae is also used. These molecules are conjugated to the Fc region of an antibody molecule without affecting the specificity of the antibody. Fluorescent-antibody staining of cell membrane molecules or tissue sections can be direct or indirect. In direct technique specific antibody (the primary antibody) is directly conjugated with fluorescein; in indirect staining, the primary antibody unlabeled and is detected with an additional fluorochrome-labeled reagent. Indirect immunofluorescence involves fluorescent labeled with immunoglobulin-specific antibodies. Any type of variation in the indirect immunofluorescence testing is generally used to detect circulating autoantibodies in immunobullous diseases.

13.2.6. Flow Cytometry:

The flow cytometer is used for the analysis and separation of cells stained with fluorescent antibody. It uses a laser beam and light detector to count single intact cells in suspension. Every time a cell passes the laser beam, light is deflected from the detector and this interruption of the laser signal is recorded. Those cells having a fluorescently tagged antibody bound to their cell surface antigens are excited by the laser and emit light that is recorded by a second detector system located at a right angle to the laser beam.

Flow cytometry measures single cells flowing through a detector system. The process begins with the selection of fluorescent-labeled antibodies specific to cell-surface markers used to characterize the cell population of interest. These cell surface markers are usually glycoproteins called cluster of differentiation (CD) markers and they help differentiate cell subpopulations (e.g., $CD3^+$ $CD8^+$ for cytotoxic T cells). Flow cytometry can be performed on a variety of tissues, including peripheral blood, bone marrow aspirates, skin biopsies and tissue culture cell lines.

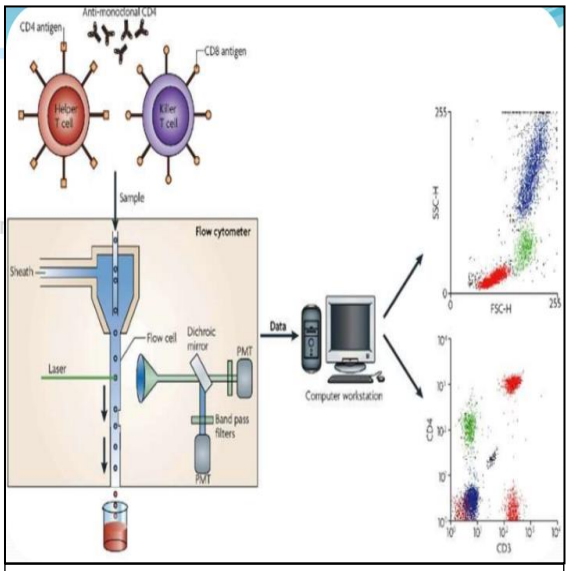

Figure: Flow Cytometry

The sample is processed, for example, with enzymatic degradation, centrifugation and/or filtration to isolate the cells of interest and the resulting cellular suspension is stained with fluorescent antibodies. The single cell suspension is then introduced into the flow cytometer into a cell-free buffer solution called the sheath fluid, which flows toward a laser aimed at the solution's path. Because the flow of the liquid through the tubing is laminar, or sheet-like and the diameter of the tubing narrows along its path, the cells are forced to line up single file as they approach the laser. The fluorescent chemical bound to the antibody, called a fluorophore, is chosen based on the specific wavelength of laser present in each flow cytometer. If cells have

the selected marker on the surface, the bound antibody—fluorophore will absorb the laser energy and subsequently release it in the form of a specific wavelength of light as the cells pass through the laser.

The emitted light is detected by an optical system that is sensitive to various wavelengths, allowing for information on multiple surface markers to be read simultaneously and collected by an adjoined computer. Specialized software then can graphically represent the distribution of the labeled cell populations in One, two or three- dimensional formats.

13.2.7. FISH (Fluorescent in situ hybridization):

FISH is a powerful technique used in the detection of chromosomal abnormalities. The high Sensitivity and specificity of FISH and the speed with which the assays can be performed have made FISH a pivotal cytogenetic technique that has provided significant advances in both the research and diagnosis of hematological malignancies and solid tumors, From a medical perspective, FISH can be applied to detect genetic abnormalities such as characteristic gene fusions, aneuploidy, loss of a chromosomal region or a whole chromosome or to monitor the Progression of an aberration serving as a technique that can help in both the diagnosis of a genetic disease or suggesting prognostic outcome.

FISH is based on DNA probes annealing to specific target sequence of sample DNA. Attached to the probes are fluorescent reporter molecules which under fluorescence microscopy to confirm the presence or absence of a particular genetic aberration when viewed under fluorescence microscopy. The technique has recently evolved to allow screening of the whole genome simultaneously through multicolor whole genome probe technique has techniques such as multiplex FISH or spectral karyotyping, or through an array-based method whole-chromosome probe hybridization. This simple, yet effective, technique has revolutionized cytogenetics and has become well established in its potential as a diagnostic and discovery tool in the fight against cancer.

13.3. Biophysical Method

- **UV/VIS absorption spectroscopy**

When electromagnetic radiation passes through a transparent material, a portion of radiation may be absorbed. As a result of energy absorption, atoms or molecules pass from a state of low energy (ground state) to a state of higher energy (excited state). The electromagnetic radiation that is absorbed has energy exactly equal to the energy difference between the excited and ground states. UV/Vis absorption spectroscopy is based on the transitions of electrons from one molecular orbital to another due to the absorption of electromagnetic radiation of UV and visible region. As a molecule absorbs energy, an electron is promoted from an occupied orbital to an unoccupied orbital of greater potential energy. Generally, the transition of electrons occurs from the highest occupied molecular orbital to the lowest unoccupied molecular orbital.

Molecular orbitals with lowest energy are the α-orbitals. The α-orbitals lie at high energy levels and non-bonding orbitals lie at even higher energies. The non-bonding orbitals contain a lone pair of electrons and they are stable, filled orbitals. Anti-bonding orbitals are normally empty and have higher energy than bonding or non-bonding orbitals.

- **Fluorescence spectroscopy:**

Fluorescence spectroscopy works most accurately at very low concentrations of emitting fluorophores. UV/Vis spectroscopy, in contrast, is least accurate at such low concentrations. One major factor adding to the high sensitivity of fluorescence applications is the spectral selectivity. Due to the Stokes shift, the wavelength of the emitted light is different from that of the exciting light. Another feature makes use of the fact that fluorescence is emitted in all directions. By placing the detector perpendicular to the excitation pathway, the background of the incident beam is reduced.

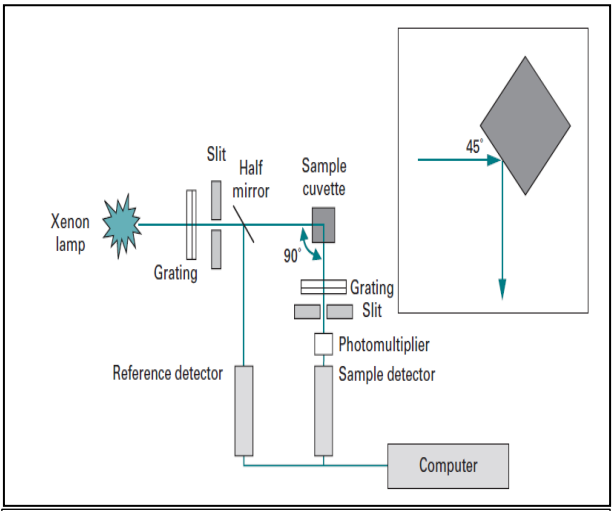

Figure: Schematics of a spectrofluorometer

Two monochromators are used, one for tuning the wavelength of the exciting beam and a second one for analysis of the fluorescence emission. Due to the emitted light always having a lower energy than the exciting light, the wavelength of the excitation monochromator is set at a lower wavelength than the emission monochromator. The better fluorescence spectrometers in laboratories have a photon-counting detector yielding very high sensitivity. Temperature control is required for accurate work as the emission intensity of a fluorophore is dependent on the temperature of the solution.

Two geometries are possible for the measurement, with the 90^0 arrangement most commonly used. Pre- and post-filter effects can arise owing to absorption of light prior to reaching the fluorophore and the reduction of emitted radiation. These phenomena are also called inner filter effects and are more evident in solutions with high concentrations. As a rough guide, the absorption of a solution to be used for fluorescence experiments should be less than 0.05. The use of microcuvettes containing less material can also be useful. Alternatively, the front-face illumination geometry can be used which obviates the inner filter effect. Also, while the 90^o geometry requires cuvettes with two neighbouring faces being clear (usually, fluorescence cuvettes have four clear faces), the front-face illumination technique requires only one clear face, as excitation and emission occur at the same face. However, front-face illumination is less sensitive than the 90^o illumination.

- **CIRCULAR DICHROISM SPECTROSCOPY:**

The basic layout of a circular dichroism (CD) spectrometer follows that of a single-beam UV absorption spectrometer. Owing to the nature of the measured effects, an electro-optic modulator, as well as a more sophisticated detector are needed, though. Generally, left and right circularly polarised light passes through the sample in an alternating fashion. This is achieved by an electro-optic modulator which is a crystal that transmits either the left- or right-handed polarised component of linearly polarised light, depending on the polarity of the electric field that is applied by alternating currents.

The photomultiplier detector produces a voltage proportional to the ellipticity of the resultant beam emerging from the sample. The light source of the spectrometer is continuously flushed with nitrogen to avoid the formation of ozone and help to maintain the lamp.

Circular dichroism spectrometry involves measuring a very small difference between two absorption values which are large signals. The technique is thus very susceptible to noise and measurements must be carried out carefully. Some practical considerations involve having a clean quartz cuvette, and using buffers with low concentrations of additives. While this is sometimes tricky with protein samples, reducing the salt concentrations to values as low as 5mM helps to obtain good spectra. Also, filtered solutions should be used to avoid any turbidity of the sample that could produce scatter. Saturation of the detector must be avoided, this becoming more critical with lower wavelengths. Therefore, good spectra are obtained in a certain range of protein concentrations only where enough sample is present to produce a good signal and does not saturate the detector.

- **Raman spectroscopy:**

Raman spectroscopy is a spectroscopic technique used to observe vibrational, rotational and other low-frequency modes in a system. It relies on inelastic scattering or Raman scattering, of monochromatic light, usually from a laser in the visible, sear infrared, or near ultraviolet range. The laser light interacts with molecular vibrations, photons or other excitations in the system, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the vibrational modes in the system. A sample is illuminated with a laser beam. Electromagnetic radiation from the illuminated spot is collected with a lens and sent through a monochromator.

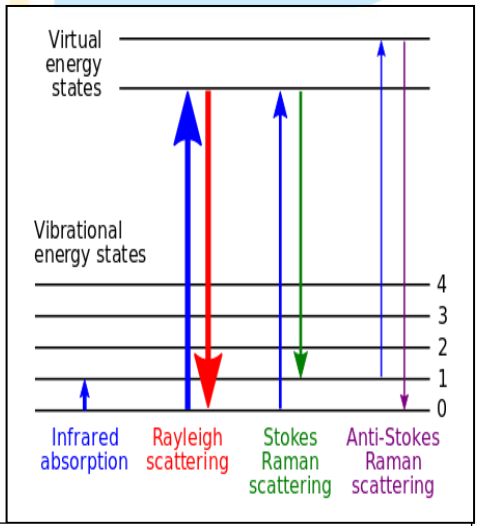

Figure: Energy level diagram of Raman Spectroscopy

Elastic scattered radiation at the wavelength corresponding to the laser line, called elastic Rayleigh scattering is filtered out while the rest of the collected light is dispersed onto a detector

by either a notch filter or band pass filter. The difference in energy between the original rovibronic state and this resulting rovibronic state leads to a shift in the emitted photons frequency away from the excitation wavelength, the so-called Rayleigh line.

If the final vibrational state of the molecule is more energetic than the initial State, the inelastically scattered photons will be shifted to a lower frequency for the total energy of the system to remain balanced. This shift in frequency is designated as stroke shift. If the final vibrational state is less energetic than the initial state, then the inelastically higher frequency and this is designated as an anti-stokes shift. scattered photon will be shifted to an anti-stroke shift.

- **Infrared spectroscopy Infrared spectroscopy:**

Infrared spectroscopy (IR- spectroscopy) is the spectroscopy that deals, with the infrared region electromagnetic spectrum that is light with longer Wavelength and lower frequency than visible light. It covers a range of technique mostly based absorption spectroscopy. As with all spectroscopic technique, it can be used to identify and study chemicals.

A basic IR spectrum is essentially a graph of infrared light absorbance on the vertical axis vs frequency or wavelength on the horizontal axis. Typical units of frequency used in IR spectra are reciprocal centimeter. Units of IR wavelength are commonly given in microns.

- **Nuclear Magnetic Resonance Spectroscopy:**

Just as electrons spin about the axis it is believed that protons and neutrons present in nuclei of atom also win about then axis. NMR involves the protons of species like ^{1}H, ^{13}C, ^{15}N, ^{31}P, ^{18}O atomic nuclei cannot he probed by nuclear magnetic resonance spectroscopy. The. resonance conditions in NMR arc satisfied in external magnetic field with the absorption occurring in the region of radio wave frequency and there is change in state from E_l to E_2. The molecular environment at a proton decides the value of applied external field at which the nucleus resonates, this is recorded as chemical shill and is measured relative to the standard i.e. TMS (Tetra methyl silane) whose structure contains 12 identical protons. The chemical shift arises from applied field inducing the secondary fields at the proton by interacting with adjacent bonding electrons. If the induced field opposes the applied field, the protons are said to be shielded. So, the high of energy is required for resonance to occur. But if the induced field is reinforced along the applied field the protons feel higher field strength and such a proton said to be deshielded. Difference in absorption position of proton with respect to TMS signal is called chemical shift.

The type of proton may be identified by the absorption peak position i.e. its chemical shift and the area under each peak is directly proportional to the number of such protons in a particular group. High resolution NMR gives information about the structure which is obtained by hyperfine splitting which is due to spin -spin interactions.

Molecular structure determination using X- ray diffraction and NMR

❖ Nuclear Magnetic Resonance Spectroscopy

This spectroscopy uses radiofrequency radiation to induce transitions between different nuclear spin state of samples in a magnetic field. NMR spectroscopy is the absorption of the radiofrequency radiations by a nucleus in a strong magnetic field. It is based on transitions between the spin states of the spinning nuclei which produce magnetic fields along the spin axis of the nucleus. The energies of the spin states of a nucleus are degenerated in the absence of magnetic field. This degeneracy of the spin does not occur under the influence of an external magnetic field when the nucleus is placed in a magnetic field of strength H.

Molecular structure determination by NMR Study of molecular structure, conformational changes and certain types of kinetic investigation is the main use of NMR in biological field. The molecular structure determination can be done by studying NMR spectra.

❖ X-ray diffraction:

X-rays were first discovered by the German physicist Roentgen. X-rays are invisible rays which are more penetrating than ordinary light. X-rays diffraction are electromagnetic radiation with wavelengths in the range 0.5-2.5 A°. Since this is of the same order of magnitude as the interatomic distances in solids, X-rays are frequently used to study the internal (crystalline) structure of materials. An X-ray beam impinging on a crystal will be scattered in all directions by the atoms of the crystal.

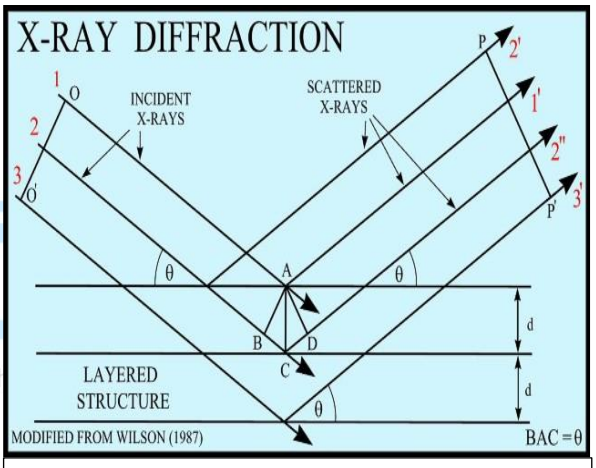

Figure: X-ray diffraction

In some directions, an increased intensity is observed due to the constructive interference of the scattered waves. The conditions for constructive interference are easily derived from the simple geometrical picture for the scattering of an X-ray beam by planes of atoms in a crystal. X-rays can pass through the human body and affect photograph film forming radiograph which is used by physicians. In the year 1912, the X-ray diffraction by crystals was discovered. This discovery provided a new method for investigating the fine structure of matter. X-rays have shorter wavelength which make them ideal to calculate interatomic distances which are also of the order as that of X-rays. Diffraction refers to phenomenon associate with the bending of waves they interact with obstacle in their path.

❖ Molecular analysis using light scattering

As light is passed through a material it can give rise to interactions like- transmission, fluorescence, absorption and scattering. Out of these interactions, the light scattering can be used for molecular analysis as it is a non-invasive and very sensitive technique. The scattering intensity of light depends upon the molecular weight and concentration of the sample. This technique gives information about the size, mass and charge of a protein sample.

- **Static Light Scattering (SLS):**

SLS can be used to determine molecular weight of the sample. In this technique the total amount of light is scattered by the sample is measured by a sensitive detector at a particular scattering angle. This technique does not require calibration as the technique always provide absolute values. The intensity of scattered light that a macromolecule produces is directly a to as product of molecular weight and concentration of the macromolecule.

- **Dynamic Light Scattering (DLS):**

This type of light scattering technique is used for measuring the size of molecules and nanoparticle. DLS measure time dependent fluctuations in the scattering intensity to determine the translational diffusion coefficient and subsequent hydrodynamic size is measured. The intensity fluctuations are the result of Brownian motion due to bombardment of solvent molecules that surrounds them. These fluctuations can be correlated with particle diffusion coefficient and size.

❖ Mass Spectrometer

Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances.

This technique basically studies the effect of ionizing energy on molecules. It depends upon chemical reactions in the gas phase in which sample molecules are consumed during the formation of ionic and neutral species.

Basic Principle:

A mass spectrometer generates multiple ions from the sample under investigation, it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type.

The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance

versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

Components of instruments:

The instrument consists of three major components:

- **Ion Source:** For producing gaseous ions from the substance being studied.
- **Analyzer:** For resolving the ions into their characteristics mass components according to their mass-to-charge ratio.
- **Detector System:** For detecting the ions and recording the relative abundance of each of the resolved ionic species.

In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10-6 to 10-8 mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.

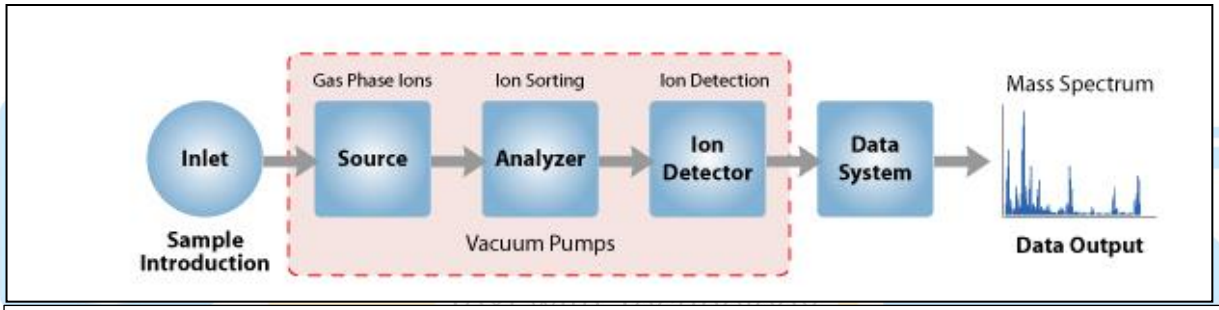

Figure: Components of a Mass Spectrometer

With all the above components, a mass spectrometer should always perform the following processes:

- Produce ions from the sample in the ionization source.
- Separate these ions according to their mass-to-charge ratio in the mass analyzer.
- Eventually, fragment the selected ions and analyze the fragments in a second analyzer.
- Detect the ions emerging from the last analyzer and measure their abundance with the detector that converts the ions into electrical signals.
- Process the signals from the detector that are transmitted to the computer and control the instrument using feedback.

❖ Tandem Mass Spectrometry:

Tandem Mass Spectrometry A tandem mass spectrometer is one capable of multiple rounds of mass spectrometry. Tandem mass spectrometry (MS/M%5) involves multiple steps of mass selection or analysis, usually separated by some form of fragmentation. For example, one mass analyzer can isolate one peptide from many entering a mass spectrometer. A second mass analyzer then stabilizes the peptide ions while they collide with a gas, causing them to fragment by collision-induced dissociation (CID). A third mass analyzer then catalogs the fragments produced from the peptides. Tandem MS can also be done in a single mass analyzer over time as in a quadrupole ion trap. There are various methods for fragmenting molecules for tandem MS, including collision-induced-dissociation (CID), electron capture dissociation (ECD), electron transfer dissociation (PTD), infrared multiphoton dissociation (IRMPD) and blackbody infrared radiative dissociation (BIRD). The important application using tandem mass spectrometry is in protein identification.

❖ Surface plasmon resonance (SPR):

Surface plasmon resonance (SPR) is a phenomenon occurring at metal surfaces (typically gold and silver) when an incident light beam strikes the surface at a particular angle. Depending on the thickness of a molecular layer at the metal surface, the SPR phenomenon results in a graded reduction in intensity of the reflected light. Biomedical applications take advantage of the exquisite sensitivity of SPR to the refractive index of the medium next to the metal surface, which makes it possible to measure accurately the adsorption of molecules on the metal surface and their eventual interactions with specific ligands. The last ten years have seen a tremendous development of SPR use in biomedical applications. The technique is applied not only to the measurement in real-time of the kinetics of ligand-receptor interactions and to the screening of lead compounds in the pharmaceutical industry, but also to the measurement of DNA hybridization, enzyme-substrate interactions, in polyclonal antibody characterization, epitope mapping, protein conformation studies and label-free immunoassays. Conventional SPR is applied in specialized biosensing instruments. These instruments use expensive sensor chips of limited reuse capacity and require complex chemistry for ligand or protein immobilization.

13.4. Statisitcal Methods

Statistics in Biology:

Every scientific experiment involves a set of observations and whether these observations are truly meaningful and significant or mere chance, can only be decided by using statistical methods. A poor knowledge or improper use of statistics may give wrong inferences out of scientific data. For example, if a researcher used an extract of a plant to cure cancer and fortunately the cancer was cured itself (placebo effect), Researcher cannot make a conclusion that the plant has anti-cancer properties unless the valid statistics has been applied on the experimental data and a significant number of observations have been made. When analyzing data, our goal is to make the strongest possible conclusion from limited amounts of data. To

do this, we need to overcome two problems, first, important findings can be obscured by biological variability and experimental imperfection and second, human brain excels at finding patterns, even in random data. Our natural inclination (especially with our own data) is to conclude that the differences are real and to minimize the contribution of random variability. Statistics is used to prevent us from making such mistakes.

In order to understand basics of statistics, one need to know about various components of statistics such as types of variables (or data type), the pattern of a data distribution based on the probability or sampling, measures of dispersion and central tendencies, and finally the statistical test to define significance.

13.5. Radiolabeling techniques

13.5.1. Detection and measurement of different types of radioisotopes normally used in biology:

Radiation is an energy form spreading out from a Centre. Radio waves, light, infrared light and microwaves are all examples of radiation. These types of radiation are all related, they are members of the electromagnetic spectrum and all travel at the speed of light, 3×10^8 ms^{-1}.

- **Atom:** An atom is fundamental piece of matter. An atom itself is made up of three tiny kinds of particles called subatomic particles: protons, neutrons and electrons. The protons and neutrons make up the center of the atom called the nucleus. The electrons fly around above the nucleus in a small cloud. Protons are positively charged while electrons are negatively charged sub atomic particles. The number of protons present in the nucleus is known as the atomic number (Z). Neutrons are uncharged particles with a mass approximately equal to that of a proton. The sum of the protons (z)and neutrons(N) in a given nucleus is the mass number (A).

$$A=Z+N$$

- **Isotopes:** Atoms of a given element with different mass numbers (i.e. different numbers of neutrons) are called isotopes. Different isotopes of the same element have the same number of protons in their atomic nuclei but differing numbers of neutrons. A nuclear species is represented by a subscript number for the atomic number, and a superscript number for the mass number (atomic weight) followed by the symbol of element.

$^{16}{}_{8}O$ (8= atomic number of oxygen atom, 16= atomic mass or mass number of oxygen atom) The number of isotopes of a given element varies: there are three isotopes of hydrogen ($_1{}^1H$, $^2{}_1H$, $^3{}_1H$), seven of carbon ($^{10}{}_6C$, $^{16}{}_6C$) etc.

- **Radioisotopes:** Radioisotopes are radioactive isotopes of an element. They can also be defined as atoms that contain an unstable combination of neutrons and protons, or excess energy in their nucleus. The radioactive isotopes or radioisotopes, are isotopes of an element having an unstable nucleus that decays (emitting alpha, beta, gamma rays) until stability is reached. The stable end product is a nonradioactive isotope of

another element. More than 3000 radioisotopes are known, of which only about 84 are seen in nature.

There are three natural a type of radiation from nuclear decay are

- **α radiation:** $_2^4$He - a helium atom nucleus. This is a "slow" moving particle, with a short range in air. An alpha particle is a particle which is extremely dangerous because of their high ionization potential inside the body but not very dangerous outside as they cannot penetrate the skin. The speed of α radiation is about 0.1 of the speed of light. The radiation has two elementary positive charges and the particle has considerable mass.
- **β radiation:** an electron '$_{-1}^0$e' or β particle is ejected from the nucleus when a neutron changes spontaneously to a proton. These are moving fast, about 0.9 of the speed of light, they can get through skin and have a reasonably long range in air (about one metre). These have only a little mass and are negatively charged. β-radiation is dangerous if ingested.
- **γ radiation:** This is a very energetic form of electromagnetic radiation. Compared to light, each bit (photon) has 1 million time as much energy or | thousand time more energy than an X-ray photon. They travel at the speed of light. They travel easily through air. They are dangerous to humans even when not ingested due to their penetrative ability. Gamma radiation has no charge associated with it.

13.5.1. 1. Radioactivity measurement:

Radioactivity is physical, not a biological phenomenon. Simply stated, the radioactivity of a sample can be measured by counting how many atoms are spontaneously decaying each second. This can be done with instruments designed to detect the particular type of radiation emitted with each "decay" or disintegration. The actual number of disintegrations per second may be quite large. Scientists have agreed upon common unit to use as a form of shorthand. Thus, a curie (abbreviated 'Ci') and named after Pierre and Marie Curie, the discoverers of radium is simply a shorthand way of writing 37,000,000,000 disintegrations per second", the rate of disintegration occurring in 1 gram of radium. The more modern International System of Measurements (SI) unit for the same type of measurement is the Becquerel (abbreviated Bq and named after Henn Becquerel, the discoverer of radioactivity), which is simply a shorthand for "1 disintegration per second".

- **Half-life:** The rate at which a radioactive isotope decays is measured in half life. The term half-life is defined as the time it takes for one-half of the atoms of radioactive material to disintegrate. In other word we can say that Half-life is the time required for a quantity to reduce to half of its initial value.

13.5.1.2. Radioactive decay chain:

Stability may be achieved in a single decay, or a nucleus may decay through a series of states before it reaches a truly stable configuration, a bit like a slinky toy stepping down a set of stairs. Each state or step will have its own unique characteristics of half-life and type of radiation to be emitted as the move is made to the next state. Much scientific effort has been devoted to unraveling these decay chains, not only to achieve a basic understanding of nature, but also to design nuclear weapons and nuclear reactors. The unusually complicated decay of uranium-238, for example the primary source of natural radioactivity on earth proceeds as follow.

U-238 emits an alpha
Thorium-234 emits a beta
Protactintum-234 emits a beta
Uranium-234 emits an alpha
Thorium-230 emits an alpha
Radium-226 emits an alpha
Radon-222 emits an alpha
Polonium-218 emits an alpha

13.5.1.3. Analytical Methods:

The specific detection of radioactive isotopes is straightforward with liquid scintillation counting (LSC) being the main method by which long-lived radiolabels are quantitatively detected. LSC uses a photomultiplier tube to detect light emissions from the Fluor. A fluor is a fluorescent molecule that undergoes excitation by the absorption of radiation and releases light when it relaxes to the ground state. The amount of light emitted by a specified amount of radioactive material can be directly correlated to the amount of radioactivity present. Accelerator mass spectrometry has also been used to detect low levels of radioactivity in biological samples but is infrequently used due to the expense of the equipment and the difficulty in sample preparation. Scintillation based methods have typically been preferred for the detection of radiolabels both due to the sensitivity and also due to the difficulties in handling Contamination from sample spillage inside an instrument such as an NMR spectrometer.

GM Counter detects radiation such as alpha particles, beta particles and gamma rays using the ionization produced in a Geiger-Muller tube, which gives its name to the instrument. The Geiger counter consists of two main elements; the Geiger-Muller tube which detects the radiation and the processing and display electronics. The Geiger-Muller tube is filled with an inert gas such as helium, neon or argon at low pressure, which briefly conducts electrical charge when a particle or photon of incident radiation makes the gas conductive by ionization. The ionization current is greatly amplified within the tube by the Townsend avalanche effect to produce an easily measured detection pulse.

13.5.2. Incorporation of Radioisotopes in Biological Tissues and Cells:

13.5.2.1. Radioisotopes and Their Biomedical Applications

Radioisotopes are widely used for a number of purposes following are some major applications of radioisotope.

13.5.2.2. Autoradiography:

The use of radioactive radiations to obtain the photographic film of the test material incorporated with the radioactive tracer is called autoradiography and the film obtained is called autoradiograph. After development, the irradiated areas appear on the film as dark areas corresponding to the distribution of the tracer. Radioactivity can be detected either directly with the scintillation counter or indirectly via their effect on photographic film. At the light microscopic level, the autoradiography is based on the principle that if photographic emulsion is brought into contact with radioactive material, the ionic radiation will convert the emulsion as dark spots of silver at certain points.

13.5.2.3. Investigating aspects of metabolism Metabolic pathways:

Radioisotopes are frequently used for tracing metabolic pathways. This involves adding a radioactive substrate and detecting them chromatographically with the help of radioactivity detectors. These isotopes are also used to confirm the mode of operation of a metabolic pathway involved during glucose catabolism. The two important metabolic pathways involved are pentose phosphate pathway and Krebs cycle. The C of glucose molecule is labeled and their contribution to the metabolic pathway could be subsequently studied.

13.5.2.4. Cancer studies Some of the isotopes are used in the treatment of cancer.

Isotopes are used in elucidation of reaction mechanism by using isotopic effects. Because gamma rays can kill living cells, they are used to kill cancer cells without having a need to difficult surgery. This is called Radiotherapy and cancer cells fail to repair themselves when damaged by gamma rays, whereas healthy cells can. Some of the isotopes are used to study the proper functioning of internal organs.

13.5.2.5. Clinical diagnostic:

Positron Emission Tomography (PET) and PETCT make use of radionuclides emitting positron particle that is injected in to the target. cell or tissue. Radionuclide decay release positron particles which interact with the nearby negatively charged particle resulting in the emission of gamma rays which is detected by a PET or gamma camera to give an exact image of the target.

13.5.2.6. Pharmacological Studies:

Another field where radioisotopes are widely used is in the development of new drugs. This is a particularly complicated process, because, besides showing whether a drug has a desirable effect, much more must be ascertained before it can be used in the treatment of clinic al conditions. For instance, the site of drug accumulation, the rate of accumulation, the rate of metabolism and the metabolic products must all be determined. In each of these areas of study, radiotracers are extreme useful, if not indispensable. For instance, autoradiography on whole sections of experimental anima yields information on the site and rate of accumulation, while typical techniques used in metabolic studies can be used to follow the rate and products of metabolism.

13.5.2.2. Safety Guidelines:

❖ **Safety and Precautions:**

- **Loss of label:**

Except in specific case where an exchange reaction is being studied, the isotopic marker should not be in a labile functional group that is easily exchanged under normal conditions, such as deuterium (^{2}H) or tritium (^{3}H) in place of an alcoholic, phenolic or acidic OH, or ^{15}O,^{17}O,or ^{18}O as a carbonyl oxygen in an aqueous environment, since the label will be readily washed out. Although isotopic labeling is used extensively to study biochemical reactions, certain unanticipated bioreactions can lead to an unexpected loss of label.

- **Storage:**

Storage Compounds or reagents enriched with stable isotopes can be stored in the same manner as the unenriched materials. By definition, radio labeled compounds and reagents emit radiation and this can accelerate various decomposition processes.

- **Disposal:**

The disposal of radioactive waste poses a challenge and must be handled separately by appropriately proper practice. Radioactive waste materials should not be mixed with general laboratory waste. The disposal of mixed waste such as flammable or highly toxic radioactive waste such as mercury or lead waste can be extremely expensive. Potentially contaminated items should be checked with an appropriate radiation detector.

❖ **General guidelines to follow include**

- Protection from light and storage under N_2; or Ar.
- Storage at low temperatures (<-80 °C).
- Storage as solution: Alcohols, especially ethanol and acetonitrile are good solvents, water should be avoided.
- When a compound is stored as a solid, it should be in crystalline form and not amorphous.

13.6. Microscopic techniques

With the advent of high-resolution microscopes modern microbiologists have access to microscope. That produces images with high clarity & magnification. The Leeuwenhoek's single lens microscope has been transformed into a high-resolution multi-lens combination with magnification up to two thousand time. Further electron microscopes with magnification up to one lakh times. Used to study fine structure of cells and sub- cellular components.

- **Resolving Power:** It is the ability to distinguish two closely placed objects as Separate objects. Magnification without resolution is not beneficial. Resolving power is a function of wavelength of light used and numerical aperture of lens system.
- **Numerical Aperture (NA = nsinθ):** It is the measure of the resolving power of a microscope objective, equal to the product of the refractive index of the medium in front of the objective and the sin of the angle between the outermost ray entering the objective and the optical axis

  For Dry Objectives, NA= 1

  For Immersion Oil, NA = 1.56

13.6.1. Different Types of Microscopy

➢ **Bright field microscopy:**

Bright field microscopy is obtained in the normal method of illuminating the object, when thin, more or less transparent samples (such as cells or bacteria) are being viewed. The light is transmitted through the sample and the image is formed by the absorption of this light. Thus, the image will appear darker than the background which is the bright field. In other words, the image is formed due to the contrast in the amplitude of the light waves coming from the object, and those that are transmitted unchanged through the object. This imaging technique may be called amplitude contrast.

➢ **Dark field microscopy:**

In the case of dark field microscopy, the background illumination is cut off completely, leaving the field of the image dark. An extra condenser lens is added so that the rays of light fall obliquely on the object. The direct beam then passes straight through and is cut out by means of a diaphragm. Only the light scattered by the object is collected by the objective and sent on to the eyepiece. The light coming from the source itself has its central portion cut off and only an annular ring of light falls on the condenser lens. This technique requires a very powerful source of illumination since only the scattered light is used in the image formation. Nevertheless, it is one of the oldest kinds of microscopy and still finds application when viewing very small objects. Bacterial flagella, for example, with diameters of only about 20nm can be followed in motion using a very strong source of light and dark field microscopy.

This is also a form of amplitude contrast microscopy. Every wave has a characteristic phase. In a microscope if the phase of the rays scattered from the sample can be made to have a different phase from the unscattered rays, then the image of the specimen will be different in

light intensity as compared to the background and will be more clearly visible. This is because when two waves (e.g. the scattered rays and unscattered rays) are in phase, they reinforce one another and the total amplitude is larger. Against this, when the two waves are out of phase, they combine together destructively and give a smaller amplitude. This effect is used in a phase contrast microscope to image thin transparent specimens. The basic principle of the method is that the unscattered beam in the direction of the incident light has its phase shifted (or changed) inside the microscope before being allowed to recombine with the scattered light. The phase shifting is done as follows. The illumination of the specimen is made obliquely using a condenser lens as in the case of dark field microscopy. Instead of a diaphragm cutting off the direct beam, however, a phase plate is placed in the focal plane of the objective lens. The phase plate consists of a transparent disc in which there is an annulus of smaller optical path than the rest of the plate. Since the medium of the plate is dense, light passing through it will be retarded. The direct beam will be less retarded than the scattered light, thus introducing a phase difference.

This enables the image to be formed clearly in a bright field.

Two effects usually accompany phase contrast microscopy. First, some thick objects may show less contrast than thinner ones and may even reverse contrast so that they appear brighter than the background. Second, every object is surrounded by a diffuse halo of light, and does not represent the real structure. These effects are due to the fact that, apart from the phase plate, the objects themselves introduce a shift in phase.

In the case of the thick objects the shift in phase may be such that when the rays recombine, the scattered and the unscattered light combine constructively, thus producing reverse contrast. The halo effects appear because some of the direct beam, instead of passing through the retarding portion of the phase plate, passes through the other portion. This creates the effect of a bright, blurred, low-resolution image of the object appearing superimposed on the sharper actual image. The phase contrast microscope is used only with bright field illumination. However, by changing the shape of the phase plate such that, instead of a ring of reduced thickness for the unscattered beam, we have a ring of enhanced thickness, it is possible to have reverse contrast for thin objects instead of positive contrast.

- **Fluorescence microscopy:**

Fluorescence is the phenomenon by which certain substances absorb light of a particular wavelength. After a very short interval of time the light is re-emitted with its wavelength altered. If the delay between absorption and emission is less than 10 seconds, the effect is called fluorescence. Longer delays lead to the phenomenon of phosphorescence. Both go under the general name of luminescence.

In general, the wavelength of the emitted light is longer than the wavelength of the absorbed light.

Fluorescence microscopy uses this property to specifically image materials of interest While performing the experiment, filters are used for both the incident light and the scattered light so

that only a narrow band of wavelengths is passed in each case. The incident light filter allows only those wavelengths that will be absorbed to impinge on the specimen.

The filter on the scattered light, also known as the barrier filter allows only the emitted wavelengths to pass through. Since the emitted wavelengths are different from the absorbed wavelength, all the background is filtered out and only the image of the sample is allowed through. Many natural materials exhibit fluorescence. The amino acid tryptophan, for example, is an ubiquitous component of proteins and fluoresces in the 250 to 400 run wavelength region. Such an auto–fluorescer, however, is of limited value. Of much greater value is the use of flourochromes. These are small fluorescent molecules, which may be linked chemically to materials of interest. Fluorescein isothiocyanate (FITC) absorbs blue light and emits green light. Rhodamine compounds absorb green light and emit red light. These compounds are especially useful in immuno–fluorescence microscopy. In this technique fluorescent dyes are linked to antibodies raised against the molecules of interest. The labeled antibodies are then cross-reacted with the sample containing the antigens. The fluorescent image of the sample will then clearly indicate the distribution, and even the size and shape of the antigen.

In clinical medicine, fluorescence microscopy is a powerful technique to identify the presence of particular viruses in the sample. In biology, it enables the mapping of the distribution of even fairly small cell components. The antibodies are raised directly against the material extracted from similar cells and purified biochemically. Another method is to attach the fluorochrome to an anti-antibody, i.e. a general-purpose antibody which will attach to the antibodies against the particular materials. By this method, the process of attaching the ‘stain’ to the antibody each time is avoided as the non-specific, labeled anti-antibody can be produced commercially and stored.

- **Polarising microscopy:**

As electromagnetic radiation, such as light, propagates, its electric field oscillates perpendicular to

the direction of propagation. If the electric field is confined to one plane (say, the vertical ‘up-down’plane) the light is said to be plane polarised. Light can be plane polarised by crystals or by special materials such as Polaroid. When a beam of unpolarised light falls on a sheet of Polaroid, only light polarised in one particular direction passes through. The rest is absorbed. Thus, when a beam of polarised light falls on such a sheet, whether the light passes through or not depends on the relative polarisation directions of both the beam and the sheet. A device that polarises the light is called a polariser. A device used to analyse the direction of polarisation of the beam is called an analyser. Both the polariser and the analyser are normally the same material such as a nicol prism or a sheet of Polaroid.

In a polarising microscope, light polarised by the polariser is allowed to fall on the object. Light scattered from the object is then viewed through an analyser. A polarising microscope helps to determine the structures of ordered domains within the sample. This is because such ordered domains possess the property of birefringence. In stretched biological fibres, for example, most of the chemical bonds will be oriented in a particular direction and the electric field of the

incident light interacts very strongly if it is polarised in the same direction. In effect the fibres act as a kind of polariser. This property is called birefringence. If the direction of oscillation is along the fibre axis, the light travels slower than when the electric field oscillation is perpendicular to the fibre axis. This is called positive birefringence. For negative birefringence, the long film axis is the fast axis and the shorter axis is the slow axis. If therefore a beam of plane polarised light is incident on a birefingent object the effect is to turn the plane of polarisation by an angle. If now the object is viewed through the analyser placed perpendicular to the polariser, all the direct light is cut off and only the light from the object will pass through (actually only a component of this light will pass through), and the birefringent object is clearly visible. By rotating the sample and observing the change in the intensity, it is possible to measure very precisely the direction of orientation of the birefringent portions of the sample.
Typical applications of the polarising microscope in biology include the study of cell spindle formation and the behaviour of the components of a contracting muscle. Polarisation measurements can also be made in conjunction with fluorescence microscopy and information about the orientation of the marker dyes is obtained.

- **Confocal Microscopy:** It is an optical imaging technique that is used for increasing the optical resolution and contrast of 2 micrograph by adding special pinhole placed at the confocal plane of the lens to eliminate out-of-focus light. It enables the reconstruction of 3D structures from the obtained images. This technique was developed to overcome the limitations of the fluorescence microscope.
- **Electron Microscopy:** A beam of electrons is used instead of light to produce an image. Electron microscope has much higher resolution than any type of light microscope because of very short wave length of electrons.
  - Scanning electron microscopy
  - Transmission electron microscopy
- **Scanning Electron Microscope (SEM):**

The SEM is a microscope that uses electrons instead of light to form an image. 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. The SEM also has much higher resolution, so closely spaced specimens can be magnified at much higher levels. SEM uses electromagnets rather than lenses. Working the SEM is an instrument that produces a largely magnified image by using electrons instead of light to form an image. A beam of electrons is produced at the top of the microscope by an electron gun. The electron beam follows a vertical path through the microscope, which is held within a vacuum. The beam travels through electromagnetic fields and lenses, which focus the beam down toward the sample. Once the beam hits the sample, electrons and X-rays are ejected from the sample. Detectors collect these X-rays, backscattered electrons and secondary electrons and convert them into a signal that is sent to a screen similar to a television screen. This produces the final

image. In SEM, there is a scanner which scans the defracted electrons for the surface SEM gives the surface image of specimen.

- **Transmission Electron Microscope (TEM):**

The source of illumination is a beam of electrons of very short wavelength, emitted from a tungsten filament at the top of a cylindrical column of about 2 m high. The whole optical system of the microscope is enclosed in vacuum. Air must be evacuated from the column to create a vacuum so that the collision of electrons with air molecules and hence the scattering of electrons is avoided. Along the column, at specific intervals magnetic coils are placed. Just as the light is focused by the glass lenses in a light microscope, these magnetic coils in the electron microscope focus the electron beam. The magnetic coils placed at specific intervals in the column acts as an electromagnetic condenser lens system. The specimen is stained with an electron dense material and placed in the vacuum. The electron beam passes through the specimen and is scattered by the internal structures.

13.6.2. Different fixation and staining techniques for EM

13.6.2.1. Fixation: Fixation is process by which the internal and external structure of cells and microorganisms are preserved and fixed in position. It inactivates enzymes that might disrupt cell morphology during staining and observation.

13.6.2.2. Methods of fixation: Fixation of tissues can be achieved by chemical or physical method.

- Physical methods include heating, micro-waving and cryo-preservation. Heat fixation is rarely used on tissue specimens, its application being confined to smears of microorganisms. However, microwave fixation, which can regard as a form heat fixation. Cryo-preservation is usually done in the form of freeze drying. It is done by immersing the tissue in liquid nitrogen at -196°C. It has application in histochemistry but is not usually applied to diagnostic tissue specimens.
- Chemical fixation is usually achieved by immersing the specimen in the fixative or in the case of small animals or some whole organs such as a lung by perfusing the vascular system with fixative (perfusion fixation).

13.6.2.3. Staining techniques:

Although secondary fixation in osmium tetroxide provides some areas of electron density. this usually not sufficient to provide high contrast, high definition images. A number of staining techniques are available to enhance the contrast of areas of interest.

- **Positive stains:** They deposit electron dense material on the area of interest, so that it stands out as a dark area on a light background. Uranyl acetate is used as a positive stain for electron microscope. Uranyl ions react strongly with phosphate and amino

groups, staining DNA and some proteins. Organelles composed of membranes are not stained well. Lead citrate may also be employed as a positive stain.

- **Negative stains:** They penetrate and darken the interstices between areas of interest, which appears light on a dark background. Negative staining is most often used to highlight surface features on individual particles, such as virions. bacteria or cell fragments.

13.6.3. Freeze- etch and freeze -fracture methods for Electron Microscope:

13.6.3.1. Freeze-etching:

Freeze-etching is used to see the shape of organelles within organisms. Cells are rapidly frozen in liquid nitrogen and then warmed to -100° C in a vacuum chamber. A knife that has been precooled with liquid nitrogen at -196^{o} C fractures the frozen cell, which are very brittle and break along lines of greatest weakness, usually down the middle of internal membranes. The specimen is left in the high vacuum for a minute or more so that some of the ice can sublimate away and uncover more structural detail.

Finally, the exposed surfaces are shadowed and coated with layers of platinum over carbon to form a replica of the surface. After the specimen has been removed chemically. this replica is studied in the TEM and provides a replica of the surface. After the specimen has been removed chemically, detailed, three-dimensional view of intracellular structure. An advantage of freeze-etching is that it minimizes the danger of o artifacts because the cells are frozen quickly rather than being subjected to chemical fixation, dehydration, and embedding.

13.6.3.2. Freeze fracture technique:

It is a technique which is used to look at membranes that reveal the pattern of integral membrane proteins.

Cells are quickly frozen in liquid nitrogen (-196°C), which immobilized components instantly. Block of frozen cells is fractured. This fracture is irregular and occurs along lines of weakness like the plasma membrane surface of organelles. Surface ice is removed by vacuum. A thin layer of carbon is evaporated vertically onto the surface to produce a carbon replica which provides strength to metal cast.

Organic material is digested away by acid, leaving a replica. Put on a grid and examined by a transmission electron microscope.

13.7. Electrophysiological methods

13.7.1. Single neuron recording:

For most of the studies related to the disorders and for other intracranial recordings EEG is usually performed. Clinical intracranial recordings are often done in the medial structures which comprise hippocampus, amygdala, entorhinal cortex and parahippocampal cortex. These areas are involved in very important processes such as memory formation that is why

these areas are topic of interest for most of neurologist to study how neurons in the MLT behave in different tasks and conditions.

To understand deeply about how neuron, encode information these areas one needs to measure the activity of single neurons directly which cannot be achieved by EEG.

In single neuron recording microelectrodes system is used to record electrical current flowing through the neuron. When action potential propagates through the cell, the electrical current flows in and out of the axons at excitable membrane regions.

13.7.2. Patch-clamp recording

The patch clamp technique is a laboratory technique in electrophysiology that allows the study of single or multiple ion channels in cells. Sakmann and Neher - develop the patch clamp technique in 1970s and early 1980s. Received the Nobel prize for this high scientific work in1991.

Principle and procedure:

The principle of the method is to isolate a patch of membrane electrically from the external solution and to record current flowing into the patch. This is achieved by pressing a fire-polished glass pipette, which has been filled with a suitable electrolyte solution, against the surface of a cell and applying light suction. The generation of an action potential in heart muscle cells depends on the opening and closing of ion selective channels in the plasma membrane.

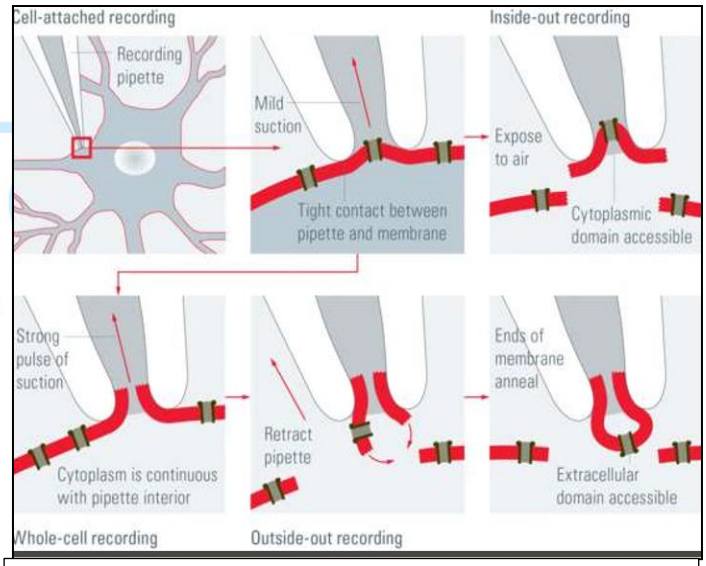

Figure: Four recording methods

The patch-clamp technique enables the investigation of drug interactions with ion-channel. The Isolated cells are ready for experiment. Glass micro-pipette - a tip opening of about 1 µm, is placed onto the cell. The patch-pipette is filled with either high NaCl or KCl solution and is mounted on a micro manipulator. A chlorided silver wire connects the pipette solution to the head stage of an electronical amplifier. A second chlorided silver wire is inserted into the bath and serves a ground electrode. This high input resistance enables the recording of small electrical currents in the range of Picosiemens (10–12 S), which are flowing through channel-forming proteins situated in the membrane patch. The electrical current is driven by applying

an electrical potential across the membrane patch, and/or by establishing an appropriated chemical gradient for the respective ion species.

13.7.3. ECG (Electrocardiogram):

The electrical currents generated in and transmitted through the heart spread throughout the body and can be detected with a device called an electrocardiograph. To record an ECG, recording electrodes (typically 12 leads) are placed at various sites on the body surface. Three electrodes are bipolar leads that measure the voltage difference either between the arms or between an arm and a leg, and nine are unipolar leads. Together the 12 leads provide a comprehensive picture of the heart's electrical activity.

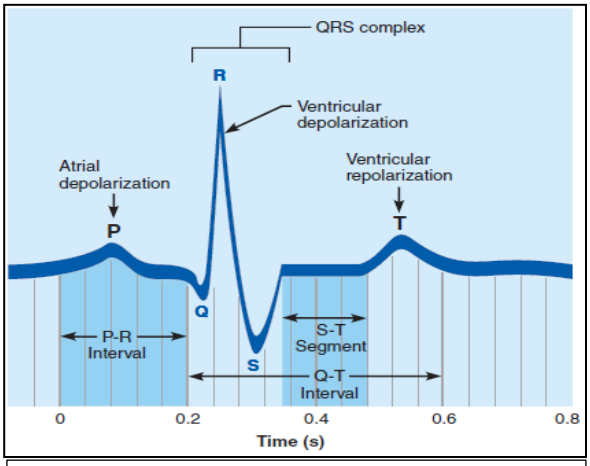

Figure: Electrocardiogram (ECG)

A typical ECG has three almost immediately distinguishable waves or deflections: the P wave, the QRS complex, and the T wave.

P wave: The first, the small P wave, lasts about 0.08 s and results from movement of the depolarization wave from the SA node through the atria. Approximately 0.1 s after the P wave begins, the atria contract.

QRS complex: The large **QRS complex** results from ventricular depolarization and precedes ventricular contraction. It has a complicated shape because the paths of the depolarization waves through the ventricular walls change continuously, producing corresponding changes in current direction. Additionally, the time required for each ventricle to depolarize depends on its size relative to the other ventricle. Average duration of the QRS complex is 0.08 s.

T wave: The T wave, caused by ventricular repolarization, typically lasts about 0.16 s. Repolarization is slower than depolarization, so the T wave is more spread out and has a lower amplitude (height) than the QRS complex. Because atrial repolarization takes place during the period of ventricular excitation, the wave representing atrial repolarization is normally obscured by the large QRS complex being recorded at the same time.

13.7.4. Brain activity recording

EEG (Electroencephalogram):

Normal brain function involves continuous electrical activity of neurons. An electroencephalogram, or EEG, records some aspects of this activity An EEG is made by placing electrodes on the scalp and connecting the electrodes to an apparatus that measures voltage differences between various cortical areas. The patterns of neuronal electrical activity

recorded, called brain waves, are generated by synaptic activity at the surface of the cortex, rather than by action potentials in the white matter.

Each of us has a brain wave pattern that is as unique as our fingerprints. For simplicity, however, we can group brain waves into the four frequency classes. Each wave is a continuous train of peaks and troughs, and the wave frequency, expressed in hertz (Hz), is the number of peaks in one second. A frequency of 1 Hz means that one peak occurs each second.

The amplitude or intensity of any wave is represented by how high the wave peaks rise and how low the troughs dip. The amplitude of brain waves reflects the synchronous activity of many neurons and not the degree of electrical activity of individual neurons. Usually, brain waves are complex and low amplitude. During some stages of sleep, neurons tend to fire synchronously,

producing similar, high-amplitude brain waves.

- **Alpha waves** (8–13 Hz) are relatively regular and rhythmic, low-amplitude, synchronous waves. In most cases, they indicate a brain that is "idling" a calm, relaxed state of wakefulness.
- **Beta waves** (14–30 Hz) are also rhythmic, but less regular than alpha waves and with a higher frequency. Beta waves occur when we are mentally alert, as when concentrating on some problem or visual stimulus.

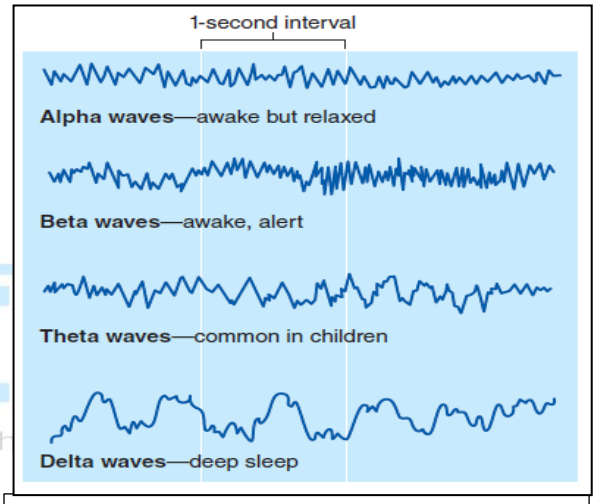

Figure: Brain waves shown in EEGs fall into four general classes.

- **Theta waves** (4–7 Hz) are still more irregular. Though common in children, theta waves are uncommon in awake adults but may appear when concentrating.
- **Delta waves** (4 Hz or less) are high-amplitude waves seen during deep sleep and when the reticular activating system is damped, such as during anesthesia. In awake adults, they indicate brain damage.

13.7.5. Lesion and stimulation of brain:

Brain lesion is the area of brain that has been damaged through the injury or disease. These brain lesions can vary from small to large or from relatively harmless to life threating lesions. Brain lesions can be caused due to injury, infection, exposure to certain chemicals. The symptoms of brain lesion can be headache, nausea, neck pain, mood swings, memory loss, fever. Arteriovenous malformations (AVMs), Cerebral infarction, Abscesses these are different type of brain lesion.

13.7.6. Pharmacologic Testing:

Pharmacological testing is done to evaluate biological activeness of any substance. Pharmacological testing plays very important role in demonstrating pros and cons associated with a substance. Many pharmacological tests aim to understand the mechanisms behind illnesses, their causes and cures. Pharmacological tests range from laboratory tests to animal testing Animal testing is mainly used in the drug development as absorption, decomposition and damaging effects of a drug must be studied in a whole organism before making it available for humans. One can also determine the effect of a substance on enzymes, human or animal cells by using a test tube like products to be used for treating cancer are first examined in test tubes on cultivated cancer cells from tumors then they are tested on other systems. To minimize the number of animals to be used for testing, ethical codes are needed to be implemented in order to ensure that animals are treated well. Pharmacokinetics is one important term that comes under pharmacological testing. It is the study of how fast and in which ways a drug is both absorbed into and excreted from the body. The drugs taken by human beings after entering the human organs get fragmented into various ae which effects body organs differently and these effects depend upon the time for which product remains in the body.

13.7.7. Positron Emission Tomographic Scanning (PET):

Positron emission tomography is an imaging modality for obtaining in vivo cross-sectional images of positron-emitting isotopes that demonstrate biological function, physiology or pathology. In this technique, a chemical compound with the desired biological activity is labelled with a radioactive isotope that decays by emitting a positron, or positive electrons. The emitted positron almost immediately combines with an electron and the two are mutually annihilated with the emission of two gamma rays. The two gamma ray photons travel in almost opposite directions, penetrate the surrounding tissue and are recorded outside the subject by a circular array of detectors.

The positron(β^+) is emitted from a proton-rich nucleus with a variable amount of kinetic energy, the maximum amount being the endpoint energy ($E\beta^+$), given for various isotopes (^{15}O, ^{11}C, ^{13}N, ^{18}F).

13.7.8. Magnetic Resonance Imaging (MRI)

Nuclear magnetic resonance (NMR) tomography has emerged as a powerful imaging technique in the medical field because of its high-resolution capability and potential for chemical specific imaging. Although similar to the X-ray computerized tomography (CT), it uses magnetic fields and radio frequency signals to obtain anatomical information about the human body as cross-sectional images in any desired direction and can easily discriminate between healthy and diseased tissue. NMR images are essentially a map of the distribution density of hydrogen nuclei and parameters reflecting their motion, in cellular water and lipids. The total avoidance of ionizing radiation, its lack of known hazards and the penetration of bone and air without

attenuation make it a particularly attractive non-invasive imaging technique. CT provides details about the bone and tissue structure of an organ whereas NMR highlights the liquid-like areas on those organs and can also be used to detect flowing liquids, like blood. A conventional X-ray scanner can produce an image only at right angles to the axis of the body, whereas the NMR scanner can produce any desired cross-section, which offers a distinct advantage to and is a big boon for the radiologist.

13.7.9. Functional MRI (f-MRI):

Functional magnetic resonance imaging (f-MRI) measures the small changes in blood flow that occur with brain activity. It may be used to examine the brain's functional anatomy, (determine which parts of the brain are handling critical functions), evaluate the effects of stroke or other disease, or to guide brain treatment. F- MRI may detect abnormalities within the brain that cannot be found with other imaging techniques.

fMRI is becoming the diagnostic method of choice for learning how a normal, diseased or injured brain is working, as well as for assessing the potential risks of surgery or other invasive treatments of the brain.

Physicians perform fMRI to:

- examine the functional anatomy of the brain.
- determine which part of the brain is handling critical functions such as thought, speech, movement and sensation, which is called brain mapping.
- help assess the effects of stroke, trauma, or degenerative disease (such as Alzheimer's) on brain function.
- monitor the growth and function of brain tumors.
- guide the planning of surgery, radiation therapy, or other invasive treatments for the brain.

13.7.10. Computed Tomographic Scanning (CT scan)

A British physicist Godfrey Hounsfield in 1972 developed a new method forming image from X-rays which now called as computed tomography or computerized axial tomography.

The technique combines the use of X-rays with computer technology to produce a two three-dimensional clear cross-sectional image of an area. In computed tomography, X-rays from a finely collimated source are made to pass through a slice of the object or patient from a variety of directions. For directions along which the path length through-tissue is longer, fewer X-rays are transmitted as compared to directions where there is less tissue attenuating the X-ray beam. In addition to the length of the tissue traversed, structures in the patient such as bone, may attenuate X-rays more than a similar volume of less dense soft tissue. In principle, computed tomography involves the determination of attenuation characteristics for each small volume of tissue in the patient slice, which constitute the transmitted radiation intensity recorded from various irradiation directions.

13.8. Methods in field biology:

13.8.1. Basic concepts in field biology:

Biodiversity is the vast diversification of flora and fauna in the biological world. Its relationship to the different environmental factors represents the very basis of human existence. Sustainable management of natural resources has become a key issue for the survival of the human's abode. Field biology is the branch of ecological sciences that involves direct or indirect observations about the real-life scenarios of the ecosystem. This helps in determination of biodiversity its assessment and time-based reports, which helps in the development of various conservation policies, strategies for enhanced productivity and maintenance of ecological balance. The most appropriate method to measure the biodiversity would be to assess ecological sustainability by way of understanding the ecosystem or landscape complexities and their uniqueness. Landscape elements have been found to be very useful to generate scientific basis and understanding for biodiversity characterization. Remote sensing and geographic information system (GIS) have proven to be very effective tools to analyze landscape level elements to characterize biodiversity.

Scientific characterization provides knowledge about the quantity and quality of cover, the physical settings, and impacts of human interventions. Quality of habitat is generally reflected in the status of vegetational cover and seasonal variation. Remote sensing data helps to study land cover, vegetation types, physiography and human interventions at fine to coarse spatial scales. Although they can be measured on a landscape level interpretation will be more effective through remotely sensed data with verification from careful ground truthing. A number of domains are available in field biology studies such as estimation of the population, population density, ranging generating four-point quarter sections. This method is very efficient when it is easy to divide the area around at random into four quadrants accurately.

13.8.2. Capture-Recapture Method:

These methods are used extensively to estimate populations of fish, game animals, and many non-game animals. The approach was first used by Petersen (1896) to study European plaice in the Baltic Sea and later proposed by Lincoln (1930) to estimate numbers of ducks. Petersen's and Lincoln's method are often referred to as the Lincoln Petersen Index, even though it is not an index but a method to estimate actual population sizes. Their method involves capturing a number of animals, marking them, releasing them back into the population, and then determining the ratio of marked to unmarked animals in the population.

- Mark and recapture method is based on some assumptions as follows:
- Mortality is the same for marked and unmarked animals
- Marked individuals do not lose their marks
- Marked individuals are caught at the same rate as unmarked individuals (no trap-happy or trap-shy animals)
- The population has no significant recruitment, or ingress (births or immigration)

- The population has no significant egress (deaths or emigration);
- Marked animals mixed randomly with unmarked animals
- Each trapping session captures a representative sample of various age and sex categories from within the population.

13.8.3. Nature of Electromagnetic radiations used in remote sensing:

Electromagnetic radiations are the primary source for the generation for remote sensing data. Electromagnetic radiation is one that contains an electric field and magnetic field pulsating in sinusoidal (like sine-wave) perpendicular to each other. The EM radiations may span from cosmic rays to microwaves or radio and TV waves as per their increasing wavelengths.

13.8.4. Sensitivity parameters of remote sensing methods:

Various types of resolutions are used to assess the ability of remote sensing, these include spatial resolution, temporal resolution and spectral resolution.

- **Spatial resolution:** The ability of the sensor to detect the smallest single object in the Earth surface is referred to as Spatial Resolution. Extraction of details from the image highly depends upon the spatial resolution. The spatial resolution of the sensor highly depends on their Instantaneous Field of View (IFOV). IFOV is the angular cone of visibility of the sensor. It determines the area of the Earth's surface which is "seen" from a given altitude at a particular moment of time. The size of the area viewed is determined by multiplying IFOV by the distance from the ground to the sensor. It defines the nature of the study.
- **Temporal resolution:** Like other resolution properties, temporal resolution is also important in remote sensing studies. Temporal resolution means the revisit of the satellite over the particular area at the same interval of time. The temporal resolutions of the satellites are usually several days which enable to capture the images of an area regularly and can monitor well. The images collected over a given interval are called multi-temporal data, which is the main advantage.
- **Spectral resolution:** Different objects have a different spectral signature. Multispectral remote sensing data commonly consist of 4 to 7 broad spectral bands in the visible (VIS) and near-infrared (NIR) regions of the electromagnetic spectrum. With the development of hyperspectral remote sensing technologies, researchers have benefited from significant improvements in the spectral and spatial properties of the data, allowing for more detailed plant and environmental studies. These technologies acquire many hundreds of spectral bands across the As energy hits the detector, a signal proportional to the incoming irradiance is processed to either a digital or analog output that can be recorded. Detectors for radiometers have been devised to measure wavelengths from 0.4 to 14 micrometers. Radiometers that measure more than one waveband are called multispectral radiometers. For this type of radiometer, the light

must be separated into discrete wavebands so that multiple waveband or multichannel readings can be taken. This separation can be done using filters, prisms or other sophisticated techniques. Non-imaging radiometers are commonly used as research tools to better understand how light interacts with objects, for spectral characterization of a variety of surfaces, and for atmospheric measurements. Another common use is to measure the quantity and quality of solar energy. These measurements can, in turn, be used to correct other imaging and non-imaging measurements for atmospheric effects.

13.8.5. Spectral ranges for Habitat Characterization and Territoriality:

Use of remote sensing in habitat characterization is widespread. In order to characterize a habitat, differences in the spectral emittance or spectral reflectance are used, as different components of a habitat such as grass, trees, water bodies, soil have significant differences. The overall spectrum of wavelengths emitted or transmitted from different objects is known as spectral emittance and spectral transmittance respectively. Some of the commonly known examples of reflectance, are IR reflectance from chlorophyll containing features such as vegetation. Different plant species also have differences in their IR emittance and hence, they can also be distinguished. While reflective radiations are being used for characterization, mainly visible and near IR is used for detection, while in case of emissive radiations, primarily various regions of IR are used for characterization. Depending on the sensitivity of sensors, the details of habitat can be gathered. A multichromatic and hyperspectral sensor can provide much finer details of habitat compared to para chromatic or broadband sensors. The following figure outlines the use of reflective and emissive strategies.