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FIRST EDITION **REVISED**

Methods in Biology

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Dr. Aditya Arya
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Module 13

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Drawing Pin Publishing
New Delhi, India.

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Preface

We are glad to introduce this CSIR-NET preparatory module 13, which has been prepared by the extensive efforts of more than three years and about 7500 human hours. Considering the methods in biology as one of the most critical factors for a naive researcher, the importance of this module is well- justified. Although this module has been prepared with a focus of one of well-known competitive examination for research fellowship and lectureship named CSIR-NET, the module will also be highly useful for preparation of other competitive fellowship examinations (ICMR, ICAR, DBT, GRE, etc.) and interviews for various M.Sc and Ph.D. positions. This book will also be a good companion for a beginner in research and lab technicians. We observed the problems faced by research aspirants in finding a suitable text for preparing the methods in biology and thus begun this work long back in 2015. We also understand that the scope and extent of methods in biology are enormous and we cannot limit all the methods in one small book. However, we have tried to cover most of the techniques to utmost clarity and depth in this module. This module comprises of eight chapters subdivided from chapter A through chapter H. Chapter A is quite extensive and includes basics of recombinant DNA technology, which begins from the isolation of nucleic acids and their manipulation, however, the application part of recombinant DNA technology will be included in our upcoming module 12. Other chapters include immunological methods (Chapter B), biophysical methods (Chapter C), statistical methods (Chapter D), radioactivity based methods (Chapter E), microscopic methods (Chapter F), electrophysiological methods (Chapter G) and methods in Field Biology (Chapter H).

In order to improve the clarity of concepts, a number of simplified illustrations have been added to the text. Also, we have made enough emphasis on analysis of data and graphical presentation of data obtained from various techniques, which is the prime requirement of various fellowship exams as well as interview questions. A number of brain teaser questions can also be found flowing with the text. These questions also contain a hint or suitable answers. However, due to the elaboration of text, we have refrained from including the previous year questions on techniques, which are available as a separate booklet (mini book) named detailed analysis of techniques' questions for CSIR-NET.

The strength of this book primarily lies on the expertise of our editors, Dr. Aditya Arya and Dr. Amit Kumar, who not only have a very extensive research experience but also have been trained on most of the techniques in prestigious institutes. The editors have also published a number of research papers which was a critical factor in maintaining the quality of this book for the level of emerging researchers. The passion for writing and contributing the learnings to the budding scientists was one of the driving force behind this book. A more extended version of many techniques will soon be available in the form of biannual review series which will be available in the future. Some “high-value notes” published by us shall also augment the preparation of methods. Additionally, charts, interpret cards shall also be made available to support the understanding of various methods in biology.

We wish our readers and budding researchers good luck and welcome suggestions, critiques for the improvement of this book.

Executive Editor

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PhD Entance Exam (Fellowship Exams) Syllabus Covered in This Module

CSIR-NET Prescribed Syllabus

Unit 13. Methods in Biology

A. Molecular Biology and Recombinant DNA methods: Isolation and purification of RNA , DNA (genomic and plasmid) and proteins, different separation methods. Analysis of RNA, DNA and proteins by one and two dimensional gel electrophoresis, Isoelectric focusing gels. Molecular cloning of DNA or RNA fragments in bacterial and eukaryotic systems. Expression of recombinant proteins using bacterial, animal and plant vectors. Isolation of specific nucleic acid sequences Generation of genomic and cDNA libraries in plasmid, phage, cosmid, BAC and YAC vectors. In vitro mutagenesis and deletion techniques, gene knock out in bacterial and eukaryotic organisms. Protein sequencing methods, detection of post translation modification of proteins. DNA sequencing methods, strategies for genome sequencing. Methods for analysis of gene expression at RNA and protein level, large scale expression, such as micro array based techniques Isolation, separation and analysis of carbohydrate and lipid molecules RFLP, RAPD and AFLP techniques

B. Histochemical and Immunotechniques: Antibody generation, Detection of molecules using ELISA, RIA, western blot, immunoprecipitation, flow-cytometry and immunofluorescence microscopy, detection of molecules in living cells, in situ localization by techniques such as FISH and GISH.

C Biophysical Methods: Molecular analysis using UV/visible, fluorescence, circular dichroism, NMR and ESR spectroscopy Molecular structure determination using X-ray diffraction and NMR, Molecular analysis using light scattering, different types of mass spectrometry and surface plasma resonance methods.

D Statistical Methods: Measures of central tendency and dispersal; probability distributions (Binomial, Poisson and normal); Sampling distribution; Difference between parametric and non-parametric statistics; Confidence Interval; Errors; Levels of significance; Regression and Correlation; t-test; Analysis of variance; X2 test; Basic introduction to Multivariate statistics, etc.

E. Radiolabeling Techniques: Detection and measurement of different types of radioisotopes normally used in biology, incorporation of radioisotopes in biological tissues and cells, molecular imaging of radioactive material, safety guidelines.

F. Microscopic techniques: Visualization of cells and subcellular components by light microscopy, resolving powers of different microscopes, microscopy of living cells, scanning and transmission microscopes, different fixation and staining techniques for EM, freeze-etch and freeze-fracture methods for EM, image processing methods in microscopy.

G. Electrophysiological methods: Single neuron recording, patch-clamp recording, ECG, Brain activity recording, lesion and stimulation of brain, pharmacological testing, PET, MRI, fMRI, CAT .

H. Methods in field biology: Methods of estimating population density of animals and plants, ranging patterns through direct, indirect and remote observations, sampling methods in the study of behaviour, habitat characterization: ground and remote sensing methods.

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DBT –JRF Prescribed syllabus

Unit 2. Methods in Biotechnology

a) Concepts of precision and accuracy in experimental measurements. Concept of signal to noise ratio. b) Biostatistics: Measures of Central Tendency. Fundamental ideas of probability and probability distributions: Binomial, Poisson and Gaussian distributions. Concept of the Central Limit Theorem. Hypothesis testing: Use of Student's t and c 2 tests. Correlation and regression. Basic concepts of design of Experiments **(Chapter D)**. c) Biochemical Methods: Chromatography: Ion exchange, Gel Filtration and Affinity chromatography **(Appendix 5)**. Electrophoresis: Native and SDS-PAGE. Isoelectric focusing. 2D-PAGE and its applications **(Chapter A)**. d) UV/Vis spectrophotometry. Beer-Lambert s law and its use in determination of protein/ nucleic acid concentration. e) Fluorescence Spectroscopy: Basic concepts of excitation and emission. Quenching, Stern-Volmer Plots. Theory and applications of FRET and fluorescence lifetime measurements. f) Fundamentals of CD, IR and Raman spectroscopy and their use in the study of biomolecular conformation **(Chapter C)**. g) Centrifugation: Basic concepts of centrifugation. Calculation of g value from RPM. Density gradient centrifugation. Sedimentation velocity and Sedimentation equilibrium. Separation of sub-cellular components and macromolecules using high speed and ultracentrifugation **(Appendix**

4). h) Microscopy: Bright field, phase contrast, fluorescence, confocal, and electron microscopy **(Chapter F).** i) Fundamentals of X-ray, NMR and cryo-electron microscopy for determination of biomolecular structure **(Chapter C, Online supplement)**.

Unit 5 Recombinant DNA Technology (Chapter A)

a) Enzymes used in Recombinant DNA technology. b) Isolation and purification of DNA (genomic and plasmid) and RNA. Various methods of separation, characterization of nucleic acids including Southern and Northern hybridizations. b) Molecular cloning of DNA or RNA fragments in bacterial and eukaryotic systems. Expression of recombinant proteins using bacterial, animal and plant vectors and their purification. Western blotting. c) Generation of genomic and cDNA libraries. Plasmid, phage, cosmid, BAC and YAC vectors. In vitro mutagenesis and deletion techniques, gene knock out in bacterial and eukaryotic organisms. d) Isolation and amplification of specific nucleic acid sequences, PCR, RT PCR and qRT PCR e) DNA sequencing methods, strategies for genome sequencing. f) Methods for analysis of gene expression at RNA and protein level, large scale expression, such as micro array based techniques **(Chapter A)**. g) Analysis of DNA polymorphism: RFLP, RAPD and AFLP techniques. h) Biosafety regulations and IPR. **(Point f and g are covered in Module 12)**

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ARS-NET (ICAR) prescribed syllabus

Unit 6: Molecular Biology Techniques

Isolation and purification of nucleic acids. Nucleic acids hybridization: Southern, northern and western blotting hybridization. Immune response monoclonal and polyclonal antibodies and ELISA, DNA sequencing. Construction and screening of genomic and C-DNA libraries. Gel electrophoretic techniques. Polymerase chain reactor, **(Chapter A)** spectroscopy **(Chapter C)**, rtPCR ultracentrifugation, chromatography **(Appendix 4,5)**, FISH, RIA etc **(Chapter B)**.

Unit 7: Gene Cloning Restriction enzymes and their uses.

Salient features and uses of most commonly used vectors i.e. plasmids, bacteriophages, phagmids, cosmids, BACs, PACs and YACs, binary vectors, expression vectors. Gene cloning and sub-cloning strategies, chromosome walking, genetic transformation, **(Chapter A)** Basis of animal cloning. Biology. Risk assessment and IPR. **(Covered in module 12).**

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GRE- Biology prescribed syllabus

Methods: Microscopy (e.g., electron, light, fluorescence)- **(Chapter F)** , Separation (e.g., centrifugation, gel filtration, PAGE, fluorescence-activated cell sorting [FACS]), Immunological (e.g., Western Blotting, immunohistochemistry, immunofluorescence) **(Chapter B)** . Recombinant DNA methodology: Restriction endonucleases, Blotting and hybridization, Restriction fragment length polymorphisms, DNA cloning, sequencing, and analysis, Polymerase chain reaction. Whole genome expression (e.g., microarrays) **(Chapter A)**.

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GRE- Biochemistry prescribed syllabus

Biophysical approaches (e.g., spectroscopy, x-ray crystallography, mass spectroscopy), Isotopes, Separation techniques (e.g., centrifugation, chromatography, electrophoresis) **(Chapter C)** , Immunotechniques, Macromolecular structure. Restriction maps and PCR, Nucleic acid blotting and hybridization, DNA cloning in prokaryotes and eukaryotes, Sequencing and analysis Protein-nucleic acid interaction, Transgenic organisms, Microarrays, Proteomics and protein-protein interaction. **(Chapter A)**

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About The Editors

Dr. Aditya Arya, Ph.D.

Dr. Arya is a Ph.D. in the area of nanomedicine from defense research and development organization. Earlier he completed his Master's degree in Biochemistry from Madurai Kamaraj University. He has research experience of more than a decade with a proven track record. He has published more than 25+ research papers in peer-reviewed journals which have attained 250+ citations. He has been trained at some of the most prestigious institutions across the globe, such as Wellcome Trust Sanger Institute, Cambridge, IBRO, Paris, and EMBL, Heidelberg and holds an active membership of several research societies in redox biology and nanomedicine. Besides this, he is also an academician and authored several book chapters in international books and some of the successful books for graduate and postgrads, Concise Biochemistry, Understanding Enzymes to name a few. Dr. Arya has extensive experience in several techniques such as flow cytometry, mass spectrometry, electron microscopy, x-ray diffraction, microarray, radiolabelling, behavioral studies, and gene cloning. He is also a public speaker and science education researcher, as he has developed a number of interesting concepts and analogies in various life sciences subjects. He has delivered a number of scientific lectures at national and international platforms.

Dr. Amit Kumar, Ph.D.

Dr. Amit is a Ph.D. in the area of Marine Molecular Ecology from Stazione Zoologica Anton Dohrn, Naples, Italy. He has published 15+ research papers and book chapters in peer-reviewed international journals/ books. He holds expertise in oxidative and nitrosative stress enzymology of marine plants. Dr. Amit is also the recipient of prestigious international research fellowships and grants in the domain of Marine Ecology. He was trained in various reputed research laboratories in Europe, including Verona University, Antwerp University, Roscoff Marine Station, and the University of Gothenburg. He has delivered several scientific talks at national and international platforms. He is an expert open water diver and participated in several scientific cruise expeditions. He has expertise in several field biology methods such as population estimation, habitat characterization, DNA sequencing, expression analysis, microbial taxonomy, etc. His current research interest is in the domain of microbial ecology of halophiles and the impact of environmental changes on the marine ecosystem.

Free Online Resources

o Additional techniques (one page summary of more than 15 additonal techniques)
o Colour images of some of the figures of this module
o Cited text and further readings

Add-on Resources *

o Set of 8 posters for each chapter
o High value note on restriction digestion
o Audio lectures of author (USB Stick)

* Add on resources are available at extra cost from the publisher.

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Chapter H: Field Biology Methods

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CHAPTER

A

Recombinant-DNA Methods

1. Isolation and Purification of RNA, DNA and Proteins

Isolation of nucleic acids and proteins is one of the primary 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 approaches. In this section, we will mainly discuss the basic principles of methods used for the isolation and purification of DNA, RNA, and proteins.

1.1 Isolation and Purification of DNA

Isolation of nucleic acids i.e. DNA, RNA, and proteins is the primary or initial step towards beginning recombinant DNA technology, molecular biology, transcriptomics, proteomics or genomics. We will consider the isolation of RNA and proteins in the subsequent sections. Basic concepts of cell biology and microbiology provide us the information about cellular structures and we know that cells of various organisms are highly diverse in their external membranes, types of DNA packaging and localization. It is therefore difficult to have a common method of DNA isolation from different sample types. However, some of steps that will be followed in most of the DNA extraction protocol include lysis of cells, removal of cell debris and other components, precipitation of nucleic acids and finally it is washing to remove salts and other impurities. Also, the plasmid DNA and chromosomal DNA vary significantly in their structural organization, size and complexity and therefore different principles of extraction are applied for extraction of genomic or plasmid DNA. Therefore we have sub-categories the DNA isolation based on the sample type and methods involved to enhance learning. General workflow is illustrated below for DNA isolation (particularly for genomic DNA)

Extract sample (cells or tissue) ⇢lysis of cells (mild or harsh) ⇢ removal of debris ⇢removal of other biomolecules ⇢precipitation of DNA ⇢washing and purification of DNA.

As different sources used for the DNA extraction include a variety of cells or tissues such as bacteria, plants animals cells and tissue, the nature of cells is different in each type, hence a variety of methods or sometimes combination of methods are used for the initial step of DNA extraction called cell lysis. The lysis of cells is most commonly performed by physical, chemical or enzymatic means. Crushing of cells or tissues in mortar and pestle in presence of liquid nitrogen is a common physical method to achieve tissue disintegration and cell lysis. Among chemical methods, various lysis buffers with detergents (SDS, triton X) as predominant components are used. Enzymes such as lysozymes are used to lyse bacterial cells, collagenase to disintegrate animal tissues and cellulase to disintegrate plant structures. The debris is removed by centrifugation and cell extract is processed using different methods. Fig A1 outlines common methods of cell lysis.

DNA precipitation is often achieved using dehydration by absolute chilled ethanol or isopropanol and finally salts and extraction reagents are removed by 70% ethanol. Let's now, discuss the removal of other biomolecules in details for different type of samples.

Plasmids are relatively smaller molecules of DNA capable of having an independent existence in the cell. **Alkaline lysis method** is one of the most widespread methods for the extraction of plasmid NA. The basic principle behind the separation using this methods is that larger DNA such as chromatin is not able to renature perfectly and as quickly as smaller DNA like plasmid can do. In this method, a strong alkaline solution consisting of the detergent sodium dodecyl sulfate (SDS) and a strong base such as sodium hydroxide (NaOH) is used to lyse the bacterial cells and denature both chromosomal and plasmid DNA. Later, potassium acetate is added that acidifies the solution and allows the renaturing of plasmid DNA, but not chromosomal DNA, which is precipitated out of solution. Cell debris is removed as pellet along with chromosomal DNA and other biomolecules and the plasmid is then purified from supernatant.

The ratio and procedure of plasmid extraction is slightly modified based on the scale of bacterial culture used and therefore, three common procedures are popularly known as mini-prep, midi-prep and maxi-prep. Mini-prep for extraction using 1-2 ml Eppendorf tubes, midi-prep for extraction in falcon tubes, and maxi-prep for industrial scale extraction.

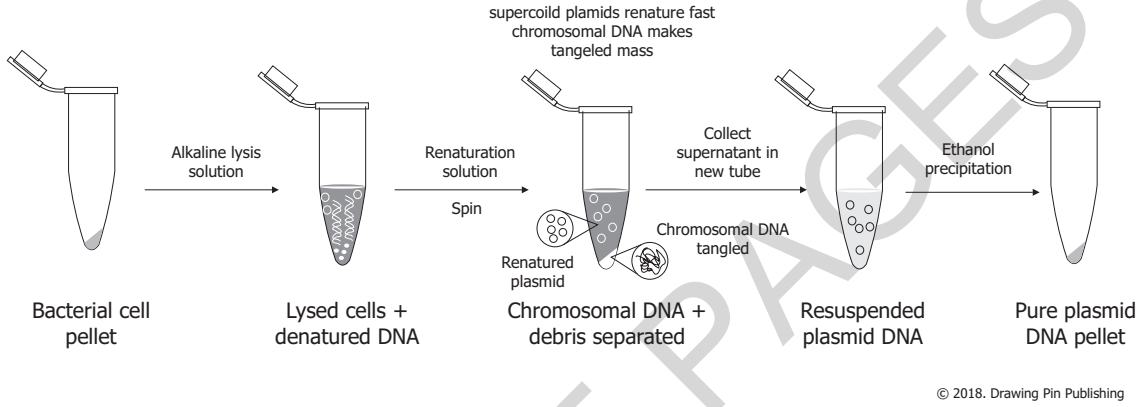


Fig A4. The principle of plasmid DNA extraction from bacterial cells using the alkaline lysis method.

Brain Teaser

How does ethanol cause the precipitation of nucleic acids?

Hint: Ethanol is a dehydrating agent, which removes the water of hydration from the nucleic acids, thereby causing its precipitation in solution. Precipitation is better at low temperatures as the solubility is low.

1.1.5 Other methods of DNA isolation

Besides these conventional methods, some of the non-conventional methods of DNA isolation are also known and used in specific conditions or harnessed by commercial suppliers to prepare kits. One such method is density gradient method. This method is based on the principle that if a salt solution is spin in a centrifuge at a very high speed for a long time, a gradient of concentration is created and various components present in the solution collected at the zone equivalent to their density. Usually, Cesium chloride density gradient is used for the separation of DNA. Density of DNA is 1.7 g/cm^3, while that of RNA is highest (~1.8 g/cm^3) and proteins is very low (~1.6 g/cm^3) creating different positioning of these molecules in density gradient tubes as shown in the figure below. The tubes are made up of plastic and DNA (visible in presence of UV if EtBR was added) can be extracted using a syringe. It is also possible to separate the supercoiled DNA from relaxed DNA by ultracentrifugation in the presence of ethidium bromide. EtBr is an intercalating agent and known to bind more efficiently in open circular or relaxed DNA in comparison to supercoiled DNA. Also, binding of EtBr to DNA reduces the density of DNA. Therefore, due to a difference in the amount of DNA bound to supercoiled and relaxed DNA, the decreases in density is also different, hence the position of bands on density gradient is different as shown in Fig A5.

Some methods have also been developed based on column chromatography and specific binding of DNA to silica beads followed by seaqencial removal of other biomolecules using different salt concentrations and finally eluting the DNA from beads at specific pH and ionic strength. Besides methods stated above, the

isolation of nucleic acids is also performed using solid state matrix in column or affinity chromatography based approaches.

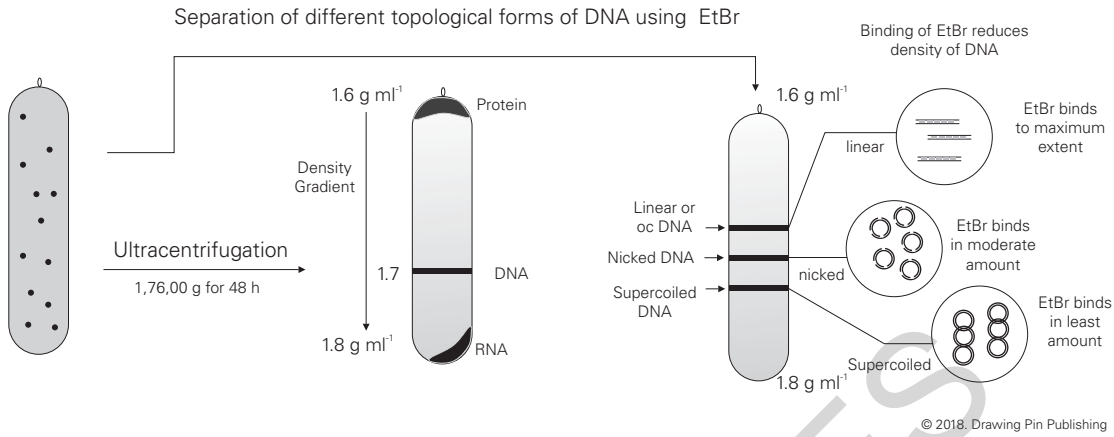


Fig A5. a. Extraction of DNA using density gradient method, b. separation of supercoiled and relaxed DNA using density gradient method.

1.2 Isolation and Purification of RNA

Among various methods available for the isolation of RNA, most widely used method for the isolation of RNA is **trizol method.** Infact, **TRIzol or TriReagent** is the brand name of a solution that is used for RNA extraction. The principle component of Trizol is guanidium thiocyanate, which is a chaotropic agent (similar to urea) that causes denaturation by weakening the hydrogen bonds and other weak interactions within biomolecules, and thereby separating the RNA from rest of the molecules. The debris is removed by centrifugation. Addition of chloroform and to lysed cells containing trizol leads to the phase separation similar to phenol chloroform method for DNA extraction. However, due to the pH difference and nature of the solvent, the middle layer contains DNA and proteins while upper aqueous phase contains RNA. the lower organic phase contains lipids and polysachharides. The final process of RNA precipitation and washing is similar to DNA extraction methods (ethanol precipitation and washing). Fig A6 illustrates the RNA extraction procedure and phase separation principles.

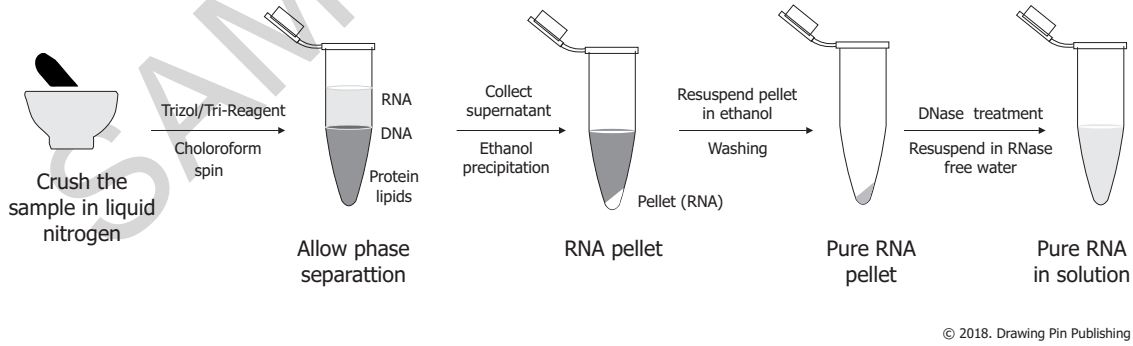


Fig A6. Workflow and procedure of RNA extraction using trizol method, phase separation.

1.2.1 Precautions and inhibition of RNases

Both RNases and DNases are prevalent and cause damage to extracted nucleic acids. They are present everywhere, dust particles, cloths, hands etc. DNase enzyme is dependent on Mg^{2+} ions hence addition of EDTA (a chelating agent) can prevent its activity and protect DNA. This is not true with RNase, hence it is difficult to protect RNA in solution. However, water used in all the buffers of the RNA extraction reagents

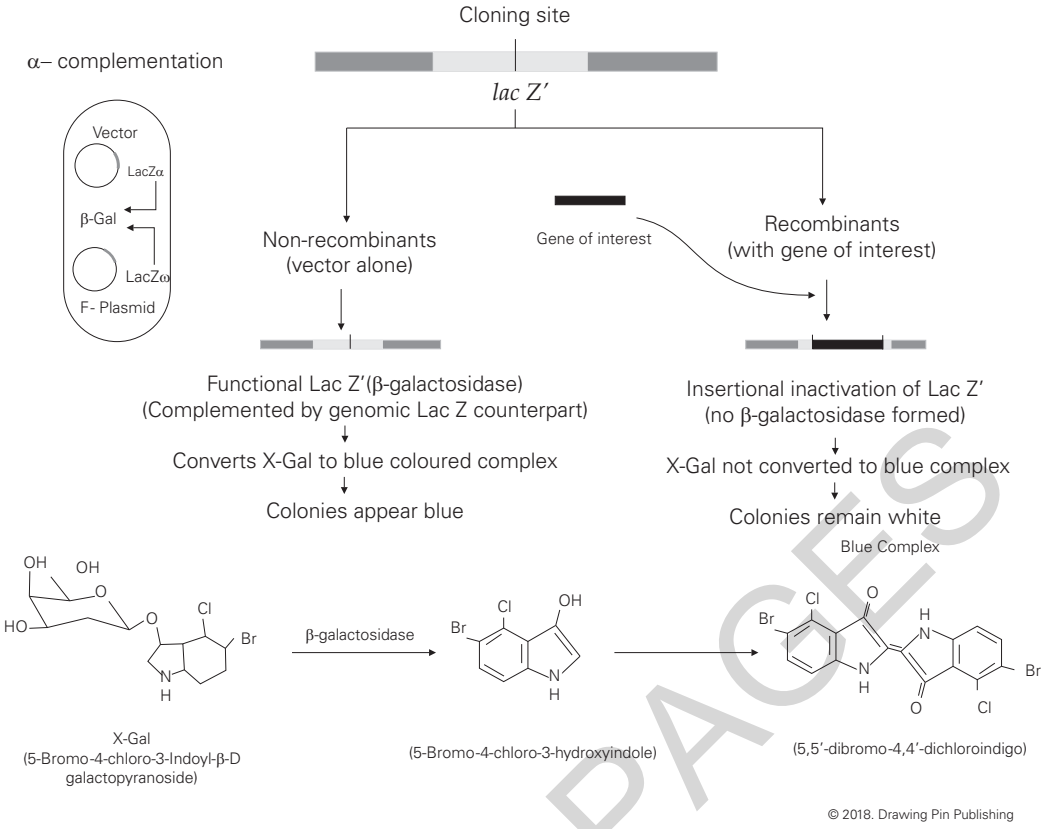


Fig A25. Blue white screening strategy or alpha complementation assay.

d. Turbid and clear plaque selection methods (l phage based vectors)

In some bacteriophage-based vectors another kind of selections method is used, that is also based on scorable marker and visual identification. Two genes cI and cII are involved in the lysogenic cycle of the bacteriophage. High expression of cII promotes the expression of cI which is responsible for maintenance of lysogeny (actually **cI repressor inhibits lytic cycle**, so lysogeny is maintained). In vectors such as **λ**gt 10, cloning site is within the cI gene. Insertion of the gene into this site will inactivate cI and induce a lytic cycle. As noted previously, E. coli containing a lambda provirus (a lambda lysogen) are immune to subsequent phage infection and so can grow in the presence of the virus. This results in a 'cloudy plaque' morphology (cloudy appearance is due to the presence of lysogenic bacteria that continue to grow within the plaque). Recombinant phage carrying a foreign DNA insert are unable to lysogenize (no negative regulator) and therefore have a 'clear plaque' morphology (no lysogenic hosts growing within the plaque). This helps in the identification of cells which contain recombinant DNA. Fig A26 represents cI based selection in cells.

Brain Teaser

Q. Describe the protein structure of cI repressor and its mechanism of lytic repression?

Hint: *cI repressor is a homooctamer that interacts with DNA with HTH motif. cI acts as a transcriptional repressor that allows virus to establish and maintain latency. Prevents both the viral DNA replication and the exit programs. Acts as a transcriptional repressor that allows virus to establish and maintain latency. Prevents both the viral DNA replication and the exit programs. Clamps the two operator OL (operator left made of OL1, OL2 and OL3 sites) and OR (operator right made of OR1, OR2 and OR3 sites) together by binding to them and arranging the intervening DNA in a loop. This step allows repression of lytic pR and pL promoters by binding to OL1, OL2, OR1 and OR2 simultaneously. The binding of cI on OR2 additionally activates the transcription of the cI gene thereby mediating an autoregulatory function to maintain the latent state. Once cI is present in sufficient amount, it can repress its own transcription by binding to OL3 and OR3.*

produced by using transgenic technology (Nagy 2000). Depending on the promoters and other regulatory controls used to construct them, Cre mice can be designed to express Cre recombinase only under certain conditions, including the following: in certain tissues, when a mouse's diet is supplemented with substances such as doxycycline, tetracycline, RU486 and tamoxifen (Brocard et al. 1998; Kellendonk et al. 1999; Utomo et al. 1999) and during certain developmental stages. Depending on the location and orientation of the loxP sites in a Cre-lox mouse, Cre recombinase can initiate deletions, inversions, and translocations of the floxed locus (Nagy 2000).

Usually, Cre and loxP strains are developed independently and then crossed. Many different Cre strains, each containing a Cre transgene under the direction of a different tissue-specific promoter, may be crossed with a single loxP strain. Depending on which strains are mated, a variety of Cre-mediated model systems can be constructed, including transgenics, knockouts, hypomorphs, repairable hypomorphs, chromosome aberrants and diet-induced mutants. In effect, by mixing and matching Cre and loxP strains, an investigator can study a gene's effects in tissue-specific and developmental stage-specific ways that were previously impossible.

Cre-lox reactions are affected by the orientation and location of loxP sites. Paired loxP sites (triangles) have directionality and may be placed in a cis (same DNA strand) or trans (different DNA strands) arrangement. (A) If the loxP sites flank a DNA segment (rectangle) in a cis arrangement and are oriented in the same direction, Cre recombinase mediates excision or circularization of the segment. (B) If the loxP sites flank the DNA segment in a cis arrangement and are oriented in opposite directions, Cre recombinase mediates the inversion of the segment. (C) If the loxP sites are located on different strands of DNA and are oriented in the same direction, Cre recombinase mediates translocation of the segment.

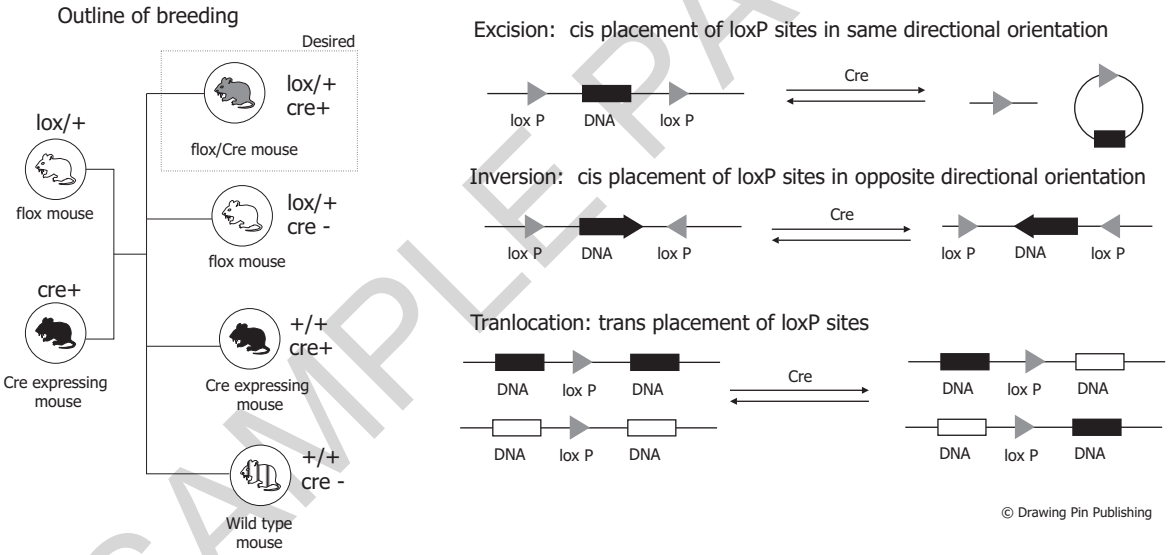


Fig A48. Cre lox strategy for development of knock out organism

Brain Teaser

Q. What do you understand by floxing in the knock-out preparation using cre-lox system?

Hint: In genetics, floxing refers to the sandwiching of a DNA sequence (which is then said to be floxed) between two lox P sites. The terms are constructed upon the phrase "flanking/flanked by LoxP". The mouse that contains the only gene flanked between two lox sites is called flox mice, which is generally bred with Cre+ mice in the beginning and gives rise to lox/+ Cre+ mice.

FLP-FRT strategy

The FLP-FRT system is similar to the Cre-lox system and is becoming more frequently used in mouse-based research. It involves using flippase (FLP) recombinase, derived from the yeast Saccharomyces

G - Methylation of N7 with dimethylsulfate at pH 8.0 renders the C8-C9 bond specifically susceptible to cleavage

A+G - Piperidine formate at pH 2.0 weakens the glycosidic bonds of adenine and guanine residues by protonating nitrogen atoms in the purine rings, resulting in depurination.

C+T - Hydrazine opens pyrimidine rings, which recyclize in a five-membered form that is susceptible to removal

C - In the presence of 1,5 M NaCl. Only cytosine reacts appreciably with hydrazine.

A>C - 1.2 N NaOH at 900C results in strong cleavage at A residues and weaker cleavage at C residues.

The volatile secondary amine piperidine is used to cleave the sugar-phosphate chain of DNA at the sites of base modification. As this will produce DNA fragments of different size with different ends, they can be resolved on acrylamide gels and subsequently sequence may be obtained.

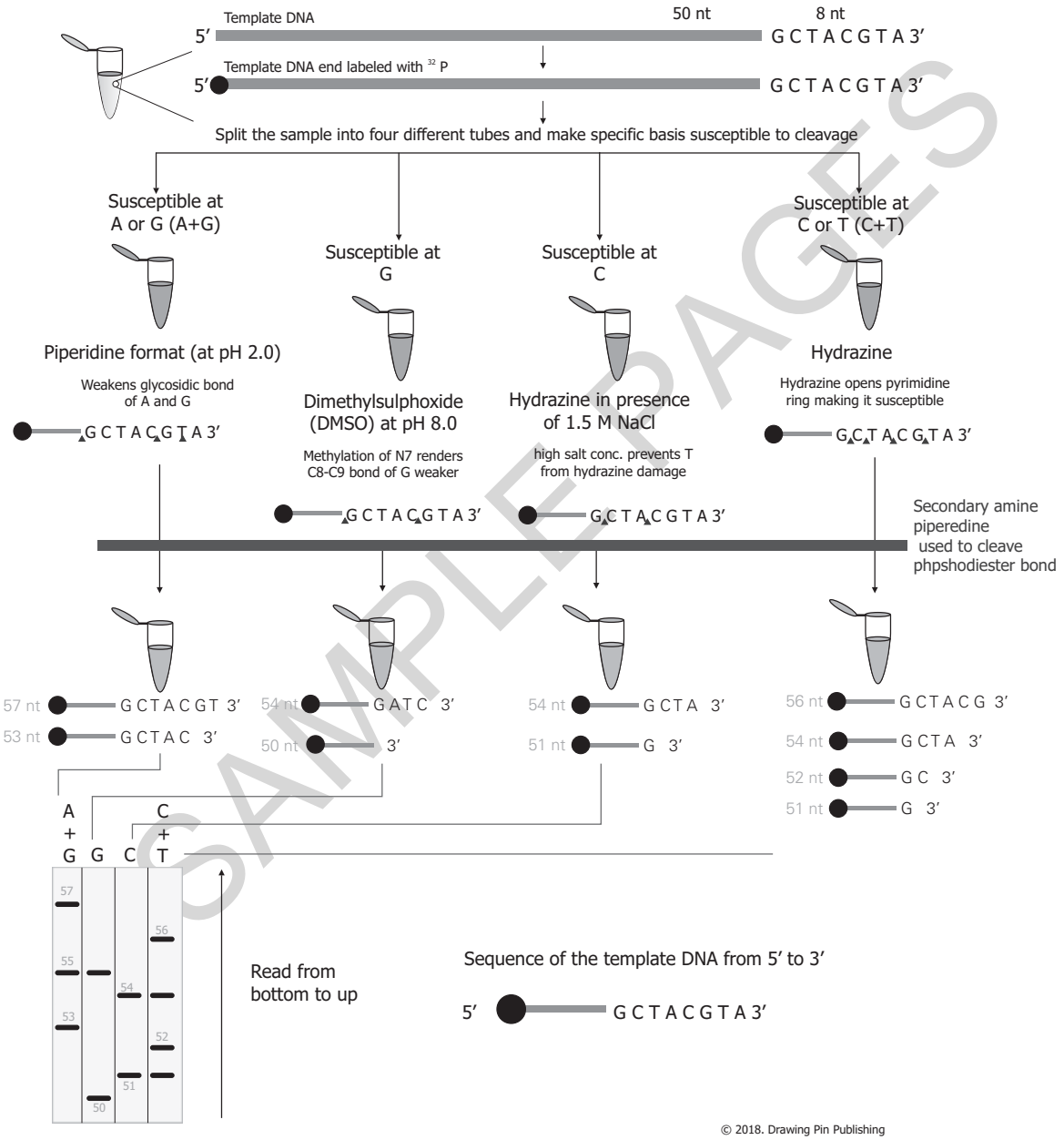


Fig 58. Procedure of Maxam gilbert sequencing

Procedure: In Maxam and Gilbert method the DNA, as stated in the principle, four different set of reaction is set up with template DNA in each tube and one particular type of modifying agent. The next step is then cleavage at the susceptible sites using secondary amine piperidine, which makes all possible type of

Following chart represents the examples and broad categories of NGS technologies, and their specifications on the basis of chemistries involved in the sequencing.[A more detailed version is available as seperate workshop module of NGS from Drawing Pin Publishing]

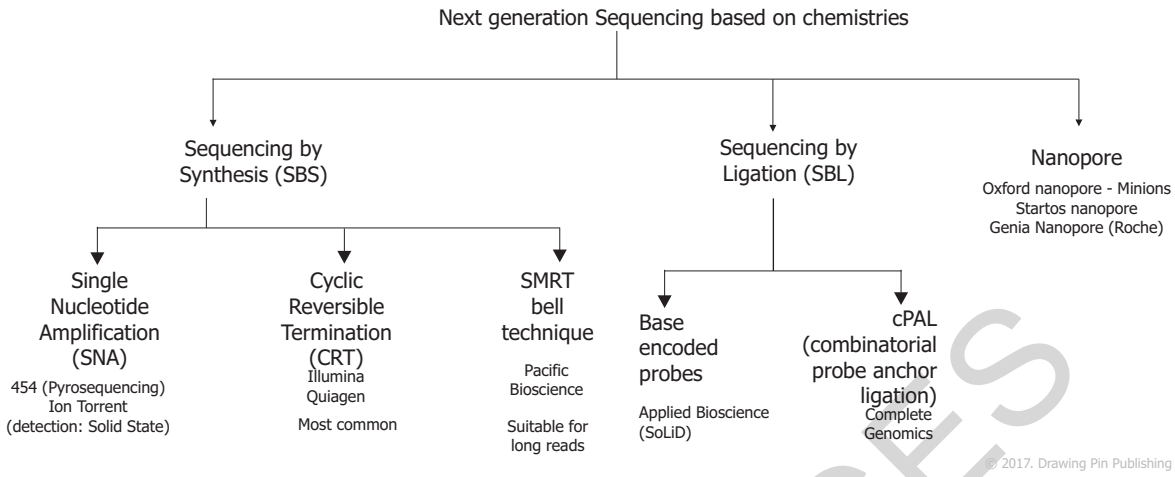


Fig A62. Various platforms of next generation sequencing

9.2 Genome Sequencing Strategies

The first DNA molecules to be sequenced was **cos sites** of the lambda phage DNA by **Wu and Taylor,** while the first organisms to be completely sequenced by **ϕ x 174** bacteriophages by **F. Sanger** in 1971. The major challenge with the sequencing techniques was cost and assembly of smaller reads obtained from machines. With the advancement in the techniques, the Human genome project was one of the major breakthroughs in sequencing projects. Human genome project was carried out by two independent groups Craig Venter Institute. Due to highly competitive research both used different approached to sequence the genome faster. Among the two most common type of genome sequencing strategies, one is hierarchical sequencing that was used by public sector consortium. The second approach was shotgun genome sequencing (used by Craig Venter).

9.2.1 BAC-to-BAC approach

The first approach that was used by public sector consortium is also called top-down, map-based or clone-by-clone sequencing. This strategy was developed in the 1980s for sequencing whole genomes before high throughput techniques for sequencing were available. Individual clones from genomic libraries can be sheared into smaller fragments, usually 500bp to 1000bp, which are more manageable for sequencing. Once a clone from a genomic library is sequenced, the sequence can be used to screen the library for other clones containing inserts which overlap with the sequenced clone. Any new overlapping clones can then be sequenced forming a contig. This technique, called chromosome walking, can be exploited to sequence entire chromosomes. Such type of genome sequencing is also known as **BAC-to BAC cloning.**

9.2.2 Shotgun approach

The second technique is known as whole genome **shotgun sequencing**, that does not require chromosome walking or primer walking techniques, rather, it uses computer algorithms to assemble short sequence reads to cover the entire genome. Genomic libraries are often used in combination with whole genome shotgun sequencing for this reason. A high-resolution map can be created by sequencing both ends of inserts from several clones in a genomic library. This map provides sequences of known distances apart, which can be used to help with the assembly of sequence reads acquired through shotgun sequencing. Nowadays with the advent of next-generation sequencing that has brought rapid sequencing, mostly shotgun sequencing is used followed by computational analysis.

. . . . . .

Brain Teaser

Q. What is the meaning of BAC to BAC approach of genome seqeucning ?

Hint: Melting curve analysis is an assessment of the dissociation characteristics of double-stranded DNA during heating. As the temperature is raised, the double strand begins to dissociate leading to a rise in the absorbance intensity, hyperchromicity. The temperature at which 50% of DNA is denatured is known as the melting temperature.

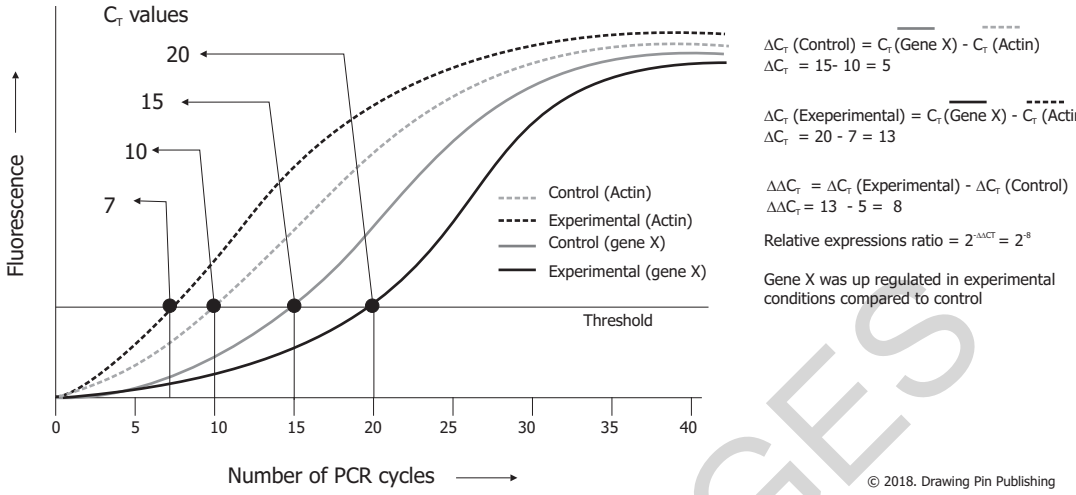


Fig A68. C_T value and its meaning

10.4 Microarray

The microarray is one of the high throughput technique for the large scale analysis of genes and transcripts. It employs hybridization of the known sequence of nucleic acids with that of an unknown pool of genome or cDNA pool which is labelled with certain fluorophores followed by high-resolution scanning and analysis of relative fluorescence. The microarray is defined as hybridization of a nucleic acid sample (target) to a very large set of oligonucleotide probes, which are attached to a solid support, to determine the sequence or to detect variations in a gene sequence or expression or for gene mapping. The process of measuring gene expression via cDNA is called expression analysis or expression profiling. Besides gene expression analysis microarray technology is also used for genetic variation analysis.

10.4.1 Principle of microarray

The core principle behind microarrays is hybridization between two DNA strands, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. A high number of complementary base pairs in a nucleotide sequence means tighter non-covalent bonding between the two strands. After washing off non-specific bonding sequences, only strongly paired strands will remain hybridized. Fluorescently labelled target sequences that bind to a probe sequence generate a signal that depends on the hybridization conditions (such as temperature), and washing after hybridization. Total strength of the signal, from a spot (feature), depends upon the amount of target sample binding to the probes present on that spot. Microarrays use relative quantitation in which the intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position. mRNA is an intermediary molecule which carries the genetic information from the cell nucleus to the cytoplasm for protein synthesis. Whenever some genes are expressed or are in their active state, many copies of mRNA corresponding to the particular genes are produced by a process called transcription. These mRNAs synthesize the corresponding protein by translation. So, indirectly by assessing the various mRNAs, we can assess the genetic information or the gene expression. This helps in the understanding of various processes behind every altered genetic expression. Thus, mRNA acts as a surrogate marker. Since mRNA is degraded easily, it is necessary to convert it into a more stable cDNA form. Labelling of cDNA is done by fluorochrome dyes Cy3 (green) and Cy5 (red). The principle behind microarrays is that complementary sequences will bind to each other.

The unknown DNA molecules are cut into fragments by restriction endonucleases; fluorescent markers are attached to these DNA fragments. These are then allowed to react with probes of the DNA chip. Then the target DNA fragments along with complementary sequences bind to the DNA probes. The remaining DNA

CHAPTER

B

Histo- & Immuno Techniques

1. Antibody Generation

Antibodies are used in almost all laboratories of life sciences particularly for detection of specific molecules, using a variety of techniques. The production of antibodies for commercial use is a tedious and difficult process. The term “antibody production” has two different meanings, one in context of the natural production of antibodies in the biological systems and second in terms of the commercial production process (Readers may find the description on the natural production of antibodies in standard immunology books),here our main focus will remain the commercial production. Commercial antibody generation is the production of antibodies from cells or animals in sufficient quantity to be used as an experimental or therapeutic reagent. Procedures for generating, purifying and modifying antibodies for use as antigen-specific probes were developed by Harlow and Lane during the 1970s and 1980s and have remained relatively unchanged since then, except for some improvements in the commercial efficacy of production.

The ability of animal immune systems to produce antibodies capable of binding to specific antigens can be harnessed to manufacture probes for the detection of molecules of interest in a variety of research and diagnostic applications. No other current technology allows researchers to design and manufacture such highly specific molecular recognition tools. Commercially produced antibodies fall in two different categories, a. polyclonal – antibodies, which as are derived from many types of B-cells and therefore have a mixture of antibodies with different specificity, and b. monoclonal antibodies, that are produced from a single clone of B-cells and are, therefore highly suitable for assays. Polyclonal antibody mixtures are often generated by injecting an animal with an agent that elicits an immune response, while monoclonal antibodies are generated from immortal immune cell lines using hybridoma technology. Table B1 describes the basic differences between monoclonal and polyclonal antibodies.

Table B1. Comparison between monoclonal and polyclonal antibodies

Sr.	Parameter	Monoclonal antibody	Polyclonal antibody
1	B-cells involved	Only single types	Many different types
2	Epitope binding	Bind only to one type of epitope	Bind to many type of epitopes
3	Cross reactivity	None	Yes
4	Specificity	Highly specific	Less specific
5	Cost	Expensive	Less expensive
6	Results	Less background noise	High background noise

Commercial production of antibodies involve some common steps such as immunogen preparation, immunization, collection, screening, isotyping, purification, and labeling for direct use in a particular method. In the more restricted sense, antibody production refers to the steps leading up to antibody generation but does not include various forms of purifying and labeling the antibody for particular uses. The very first step of commercial antibody production is often, preparation of antigen samples and their safe injection into laboratory or farm animals so as to evoke high expression levels of antigen-specific antibodies in the serum, which can then be recovered from the animal. Polyclonal antibodies are recovered directly from serum (bleeds). Monoclonal antibodies are produced by fusing antibody-secret-

B-cells (TK- but HGPRT+) complement each other in trans (hybridoma cells become $TK^{+}/HGPRT^{+}$) and therefore can survive. This process is known as HAT selection, illustrated in Fig B3.

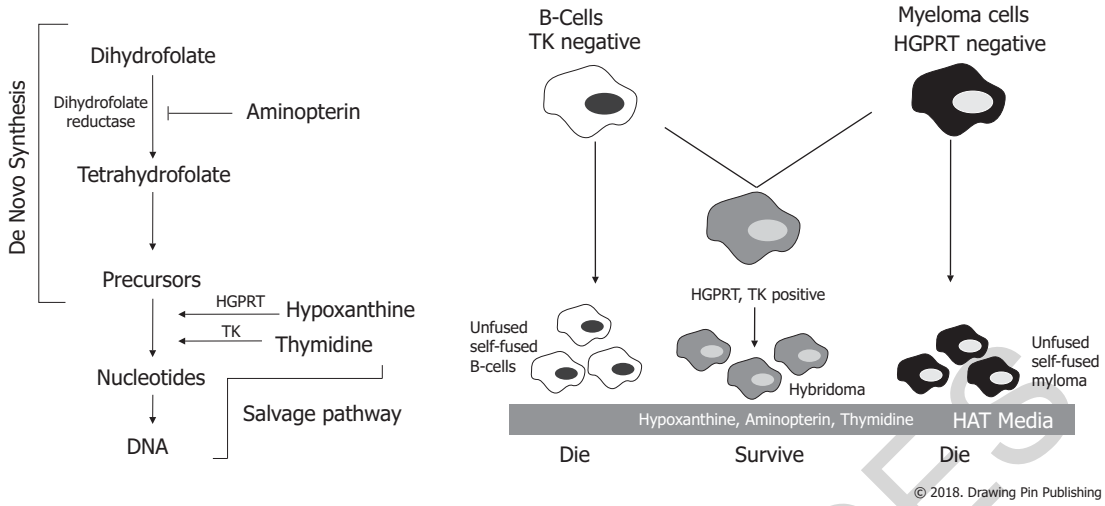


Fig B3. Overview of salvage pathway involving TK and HGPRT and HAT selection strategy

Brain Teaser

What is the average yield of antibodies using hybridoma?

The production MAbs in the culture bottles is rather low (5-10 mg/ml). The yield can be increased by growing the hybrid cells as ascites in the peritoneal cavity of mice. The ascetic fluid contains about 5-20 mg of MAb/ml. This is far superior to the in vitro cultivation techniques.

1.4 Purification and Processing

Crude antibodies produced from the serum (polyclonal antibodies) or hybridoma-cell culture supernatants (monoclonal antibodies) have to be processed through a number of steps for enhancing the commercial applicability and performance. The three main levels of antibody purity are known, **crude antibody preparation**—precipitation of a subset of total serum proteins that includes immunoglobulins, **general antibody preparation**—affinity purification of certain antibody classes (e.g., IgG) without regard to antigen specificity and **specific antibody preparation**—affinity purification of only those antibodies in a sample that bind to a particular antigen molecule. Purification of antibodies is commonly done using affinity chromatography, protein A/G is known to have an affinity to antibodies and therefore can be used to purify them in cost-effective manner. The level of purification required for various applications depends upon the intended purpose for the antibody.

Following purification, antibody characterization is done, this involves three kinds of activities that are usually performed at various stages throughout an entire antibody production and purification project viz., **screening**—identifying antibody samples having antigen-binding specificity, **tittering**—measuring antibody concentration and functional assay titer and **isotyping**—determining a monoclonal antibody class and subclass identity. Sometimes, purified antibodies can be modified for particular uses by several methods including fragmentation into smaller antigen-binding units, conjugation with an enzyme or other detectable markers, and immobilization to solid supports. Most often antibodies are used in a whole-molecule form. However, the performance of some techniques and experiments can be improved by using antibodies whose nonessential portions have been removed. Fab and $F(ab)'_2$ are antibody fragments of IgG that are most frequently created and utilized by researchers.

In order to enable the use of commercially produced antibodies for techniques such as ELISA, immunoblotting, flow cytometry etc., they must be labeled with the suitable enzyme of other conjugates that enables their detection. Moreover, techniques that utilize antibodies for immunoprecipitation or another form of affinity purification depend upon mechanisms for attaching or immobilizing them to chromatography media (e.g., beaded agarose resin). Strategies for accomplishing this involve the same considerations

subsaturating dilution of antibody is reached, whereupon the antigen content of the precipitates progressively decreases. Observation of a constant amount of precipitate at saturating antibody concentrations differs from the precipitin reaction, in which excess antibody actually inhibits the formation of a precipitate.

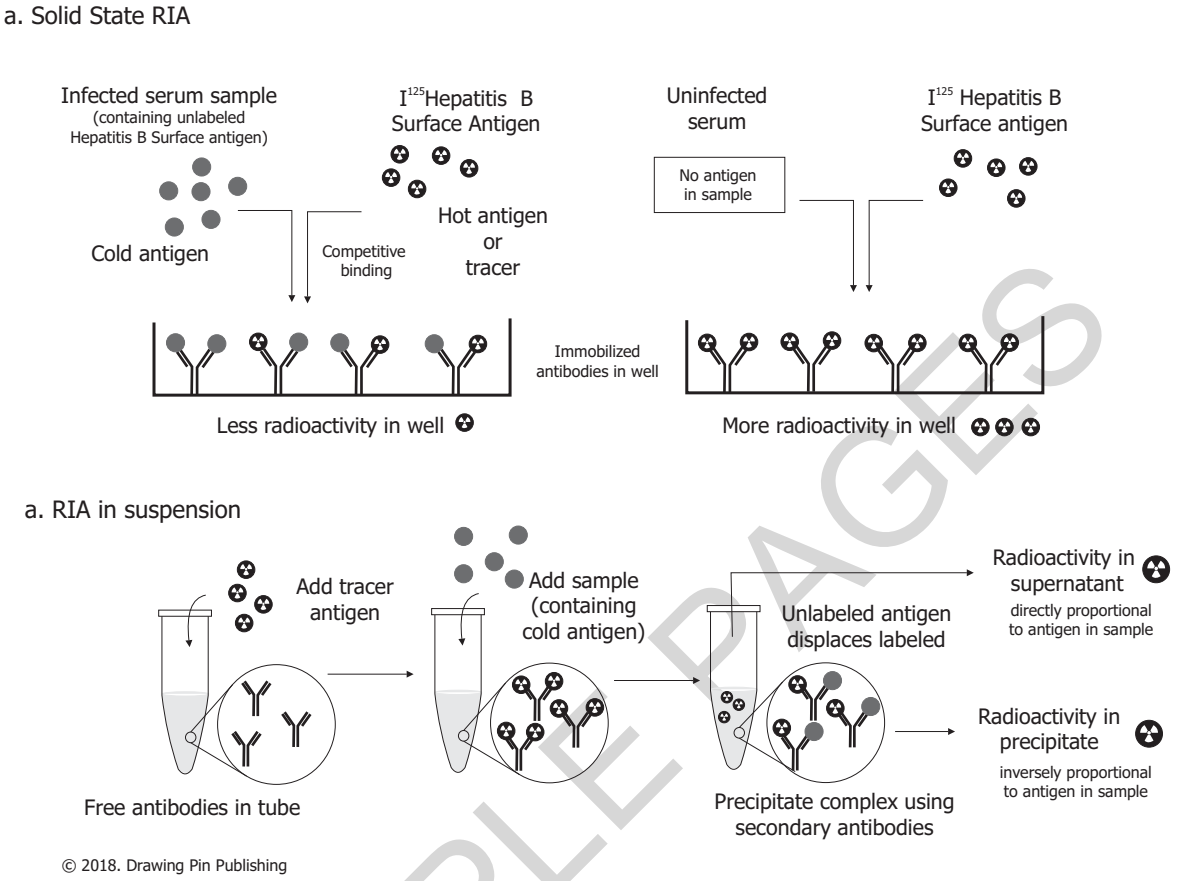


Fig B7. Fundamental principle of RIA, the competition between labelled and unlabeled antigen to bind with immobilized antibodies

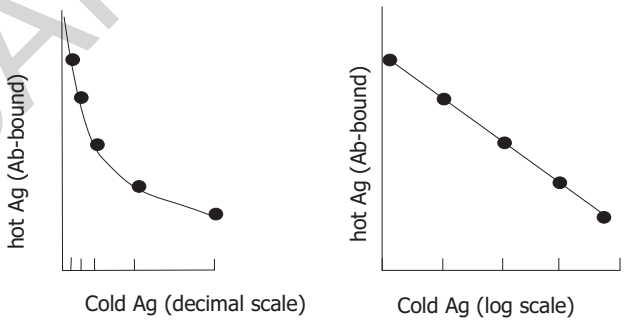


Fig B8. Graphical presentation of RIA data and its comprehension, note that conc. of hot antigen (radioactive) is inversly related to cold antigen (antigen in sample or analyte) in logirthmic manner.

4. Western Blot or Immunoblotting

Gels are known for the separation of biomolecules, however, they are fragile and prone to damage, therefore cannot be used for the analysis of biomolecules. Hence, a technique involving the transfer of proteins

as loading controls. The procedure for loading control is repeated on the same membrane on which a western blot for protein in question was performed. The previously bound complex is washed by a special buffer in a process called stripping. After, stripping the membrane is probed with antibodies of aforesaid proteins and bands are observed. Ideally, the intensity of bands of loading controls should be the same in all the samples, if not, there was some error in loading.

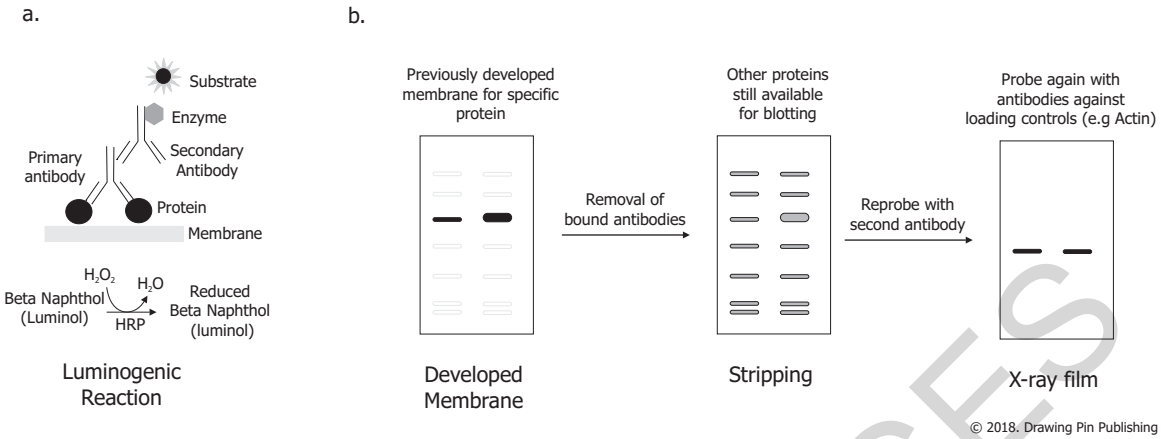


Fig B10. a.A chemical reaction involving light signal generation in immunoblotting and b. The process of stripping and using loading controls.

4.3 Data Comprehension

Most of the western blot data is in the form of dark bands formed by the exposure of X-ray film by light emitted from luminescence substrates (a few may be brown-coloured bands on membrane formed by chromogenic substrate). These images are scanned and analysed using image processing softwares (e.g. ImageJ). The primary goal of image processing to obtain the pixel intensity of specific band, the pixel intensity is directly proportional to the amount of signal and hence the amount of protein present. However, to avoid any loading errors on the gel, a reference blot of housekeeping proteins is also used to normalize the data. Let us understand this with the help of an example: A western blot of a protein of interest from four different samples was carried. We can make some basic conclusion about the level of proteins in the following blot by observing the intensities (size) of the bands. It can be observed that sample 2 has the least protein, followed by sample 1 and sample 3. Maximum protein is present in sample 4. Although this might have also happened due to a difference in the loading or sample preparation, hence to rule out this possibility same membrane is also re-probed with an antibody specific to a protein that carries out housekeeping functions and therefore used as internal controls, such as actin, tubulin etc. As we can observe in the next figure that levels of tubulin are same and therefore the loading was correct and the differences in the image are true representation of protein levels. In order to present these observations in quantitative manner, one has to measure the intensities and these intensities can be plotted on the graph to obtain actual fold change difference in the protein levels.

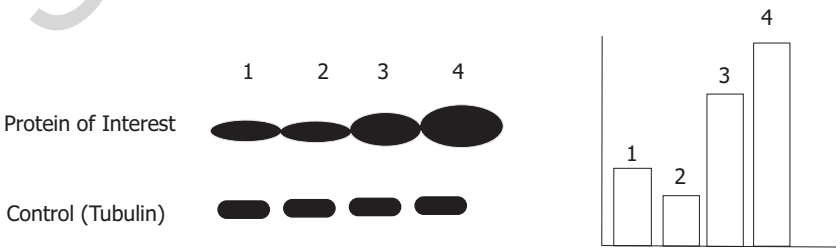


Fig B11. Data comprehension for western blot: density blots and comparison with the control to obtain fold change in the protein levels.

Brain Teaser

Q. If you got different densities of bands of a loading control such as actin or tubulin, what does it mean to you?

Hint: Proteins that are activated in response to tyrosine phosphorylation can be pulled down using an immobilized SH2 domain that targets the phosphorylated tyrosine on a given protein. Additionally, GTPases, which act as molecular switches that regulate cell signaling by cycling between a GTP-bound (active) and GDP-bound (inactive) state, can be pulled down using an immobilized GTPase-binding domain of downstream proteins that are recruited to GTP-bound, activated GTPases

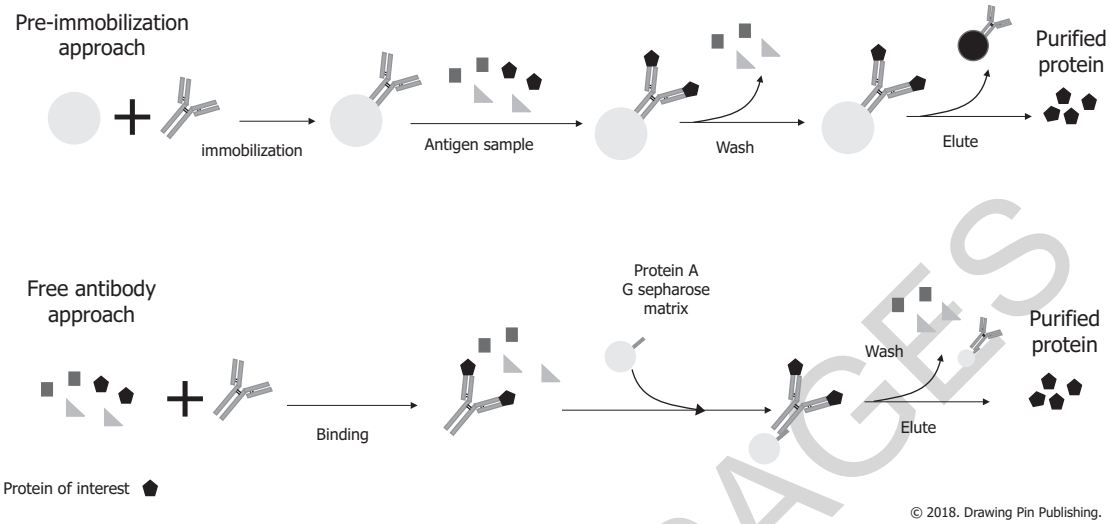


Fig B14. Two different approaches in classical immunoprecipitation or pull down

5.3.4 Co-immunoprecipitation (Co-IP)

Co-immunoprecipitation is an extension of IP that is based on the potential of IP reactions to capture and purify the primary target (i.e., the antigen) as well as other macromolecules that are bound to the target by native interactions in the sample solution. Therefore, an experiment is called an IP or co-IP based on whether the focus of the experiment is the primary target (antigen) or secondary targets (interacting proteins). Because co-immunoprecipitation depends so much on protein-protein interactions in order to detect the bound proteins, the ability to maintain stable physiological interactions throughout the mechanical and chemical stresses of the incubation and washing steps is a critical factor when performing a co-IP reaction. Therefore, low-affinity or transient protein-protein interactions may not be detected by co-IP unless the interaction can be stabilized.

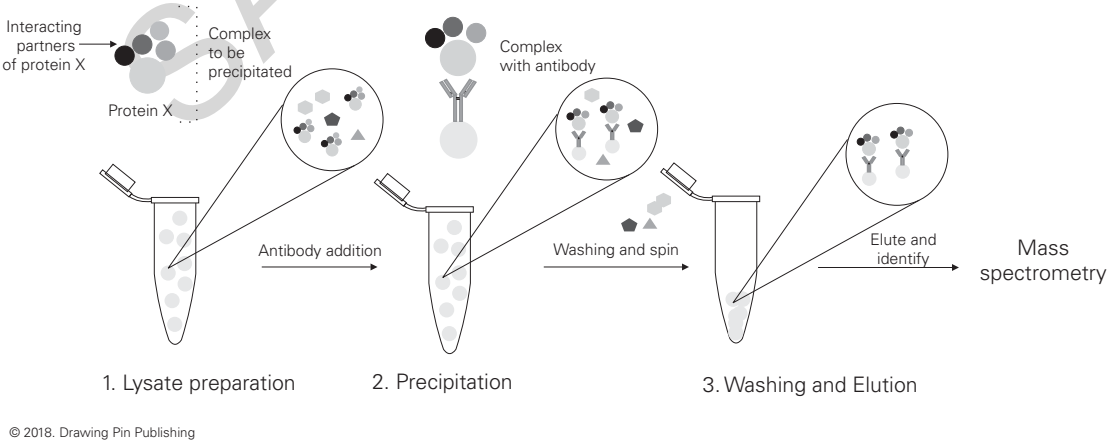


Fig B15. Basic outline of Co-immunoprecipitation method.

Scatter plot provided in Fig B25 represents the data obtained from a typical Annexin V/PI apoptosis assay.

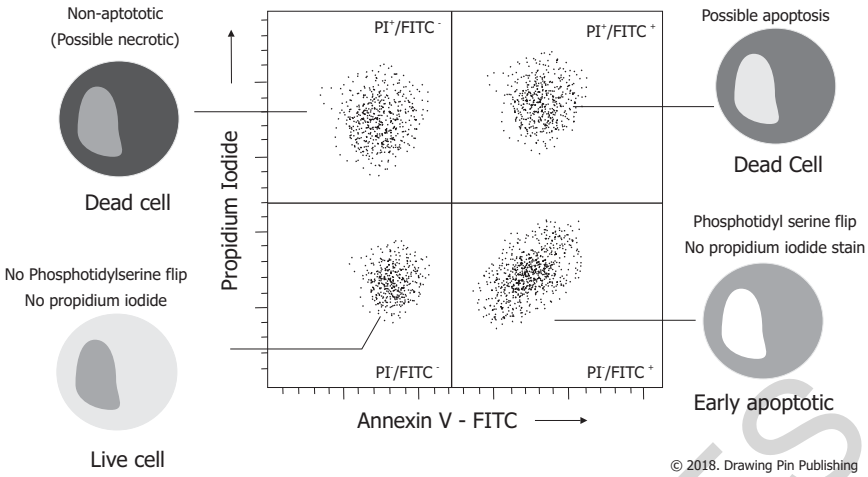


Fig B25. Representative scatter plot of Annexin V-FITC vs PI staining of cells (note the location of live and dead cells on the plot)

6.4.4 Analysis of cell cycle (DNA marker)

DNA content can be measured by flow cytometry in particular phases (G_0/G_1 versus S versus G_2M) of the cell cycle as well as DNA ploidy can also be determined. In most situations DNA ploidy is assessed in hematological or solid tumors; the evidence of aneuploidy by itself is a definitive marker of a presence of the cancerous tumor.

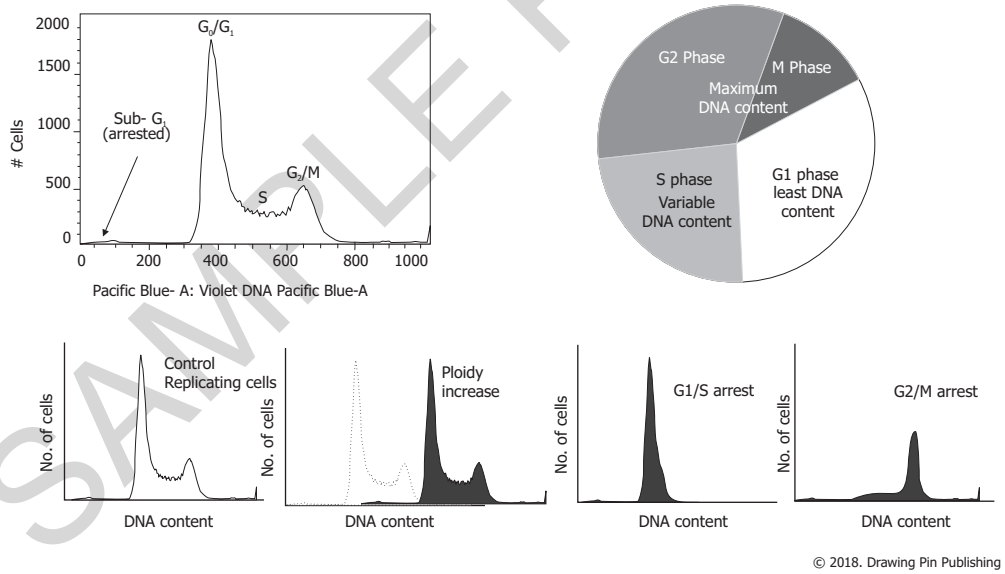


Fig B26. Cell cycle analysis using flow cytometry

Often ploidy is also considered to be a prognostic indicator of tumor progression and outcome of the treatment. To assess DNA ploidy of the tumor sample one has to compare DNA content of the G_0/G_1 cells population of the presumed tumor cells with that of normal (control) cells. Towards this end most frequently the peak value of the integrated fluorescence (peak channel) of $G_0/1$ population of normal cells is being considered to be DI = 1.0 and DNA ploidy of the tumor cells is expressed as a ratio of the peak value (channel) of fluorescence intensity of these cells with respect to that of the normal G_0/G_1 cells.

It is also common to express DI of the tumor as a ratio of modal rather than the peak value of fluorescence intensity representing DNA content of G_0/G_1 population tumor cells to modal value of G0/1 population of normal cells. Some authors still prefer to use the mean values of fluorescence intensity of G_0/G_1 population rather than the peak or modal values to obtain this ratio. In essence, when DNA measurement is done correctly and accurately, either of these approaches is expected to yield similar estimate of

trical field, and is deflected into a tube or plate. Uncharged particles pass into the waste. To prevent the break-off point happening at random distances from the nozzle and to maintain consistent droplet sizes, the nozzle is vibrated at high frequency.

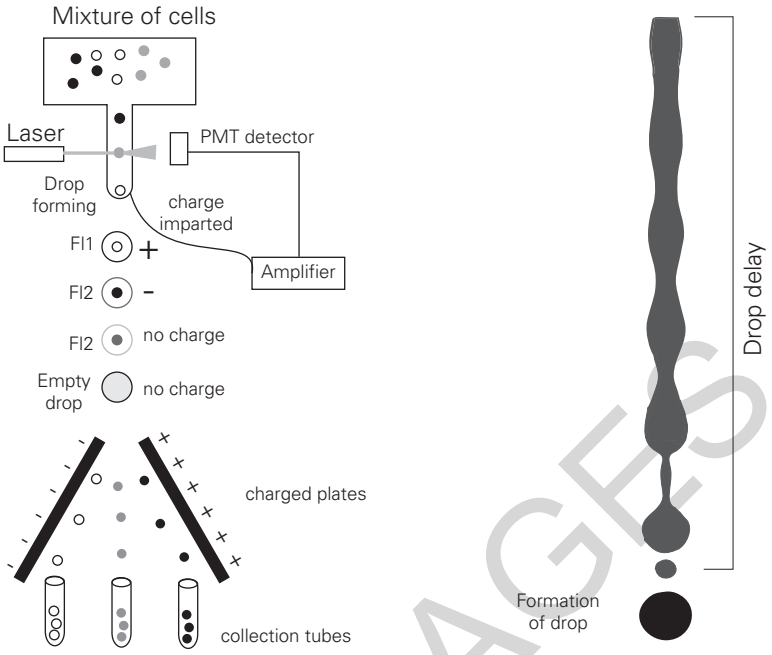


Fig B28. Mechanism of cell sorting in FACS

7. In Situ Localization Techniques

7.1 Introduction

In situ localization refers to the detection of a biomolecule at the actual location of its existence in a cell or a tissue. In order to perform in situ localization, two things are essential, one preservation of original structures until the detection is done, and second availability of highly specific and sensitive detection molecules (probes or labelled antibodies). Among various groups of biomolecules that can be detected using in situ localization methods are proteins, ion, radicals and nucleic acids. In situ localization provides valuable information about the distribution and relative concentration of various target molecules. These techniques are highly valuable in research as well as in clinical diagnosis. Mostly, in situ detection of proteins is performed using **immunohistochemistry** (IHC, for tissues) or **immunocytochemistry** (ICC, for cells), where fluorescent or enzyme labelled antibodies are used to detect the specific molecule at its target site. Later the microscopic images (bright field or fluorescence microscopy) is used to analyse the distribution and relative abundance. Distribution of lipids, modified proteins such as phosphorylated or glycosylated forms can also be achieved by using ICC or IHC techniques. Besides ICC and IHC which are meant for proteins and small metabolites, detection of nucleic acids is performed in situ using methods called **in situ hybridization.** As per the intended purpose of this note we will limit our further discussion to in situ hybridization methods.

Among various other biochemical and molecular techniques, in situ hybridization techniques have their own importance especially due to their ability to detect a molecular change directly at its site of occurrence. In situ hybridization was developed by **Joseph G. Gall and Mary-Lou Pardue** in 1969. In situ hybridization is a type of hybridization of target DNA or RNA with a labeled complementary DNA, RNA or modified nucleic acids strand (commonly known as probe) to localize a specific DNA or RNA sequence in a portion or section of tissue (*in situ*), or, if the tissue is small enough (e.g., plant seeds, Drosophila embryos), in the entire tissue (whole mount ISH), in cells, and in circulating tumor cells (CTCs) etc. Originally, the detection of hybridization was based on radioactive nucleotides and detection of radioactivity, however, with the advent of highly specific and sensitive fluorophore it is now possible to tag probes with fluorophores and therefore the technique is now popular as **Fluorescent in situ hybridization or FISH.**

CHAPTER

C

Biophysical Methods

1. Introduction to Spectroscopic Methods

Spectrometry is derived from the two words, one is 'spectrum' which means a scale of any parameter over a span of its extreme values (if it is light wavelengths – light spectrum, spectrum of m/z ratios – mass spectrum), and the second word 'metry' which means measurement. So, if any physical, chemical or biological parameter is being measured with assessment of spectrum it is called **spectrometry**. A textbook definition of spectroscopy is, "the study of interaction between matter and electromagnetic radiations". These interaction may be of various types and involving different type of radiations and patterns depending on the energy of radiation. As the energy of electromagnetic radiations is directly proportional to their frequency ($E = h\nu$), high energy radiations cause more drastic changes in atoms, as compared to low energy radiations. High energy radiations like X-rays can completely eliminate electrons from matter and ionize them, while UV and visible light has sufficient energy to cause electrons to be excited from low energy state to high energy state. However, low energy radiations like IR and microwaves cannot even drive electronic tranisitions, but have enough energy to excite electrons to next vibrational states or rotational states respectively. Therefore, using different type of radiations, different type of interactions can be studied and various information about atoms and molecules can be derived. UV-vis can be used to determine identity of molecules, their concentration and reaction kinetics, IR spectroscopy can be used to determine type of chemical bonds present in a molecule and microwave spectroscopy can be used for determination of conformational changes based on rotational states. Fig C1 illustrates various types of electromagnetic radiations and their potential use in spectrometry.

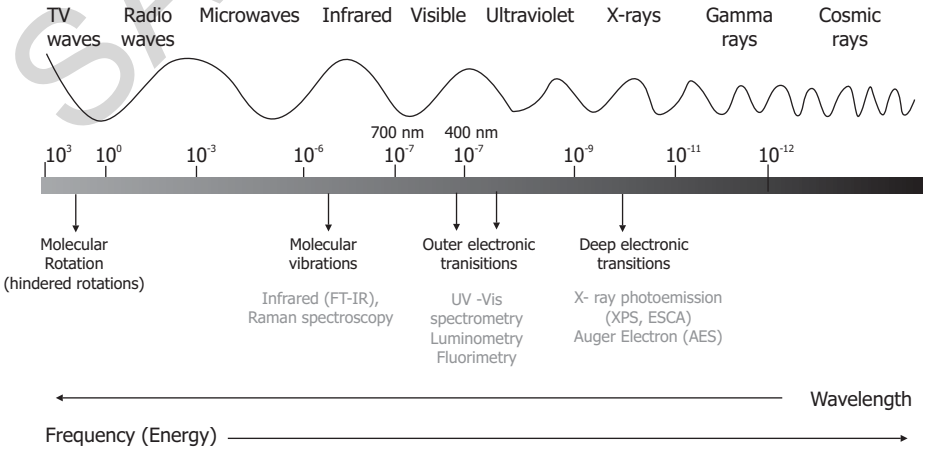


Fig C1. Chart of overview of spectroscopic methods

2.3.2 Beer-Lambert law

In order to perform measurements such as determination of concentration using spectrophotometry, a relationship popularly called as Beer-Lambert law is crucial. This law relates the absorbance with concentration and path length of the solution. **Johann Heinrich Lambert and August Beer** established this law by their experimental evidence. However, there is also evidence of the use of similar law proposed by Pierre Bouguer before 1729. Lambert stated that absorbance of light by any solution is directly proportional to the path length (A α l) and Beer added to this, that absorption is proportional to the concentration of the solution (A α C). This is primarily due to increased number of molecules interacting with the light on increasing concentration or path length (Fig C4).

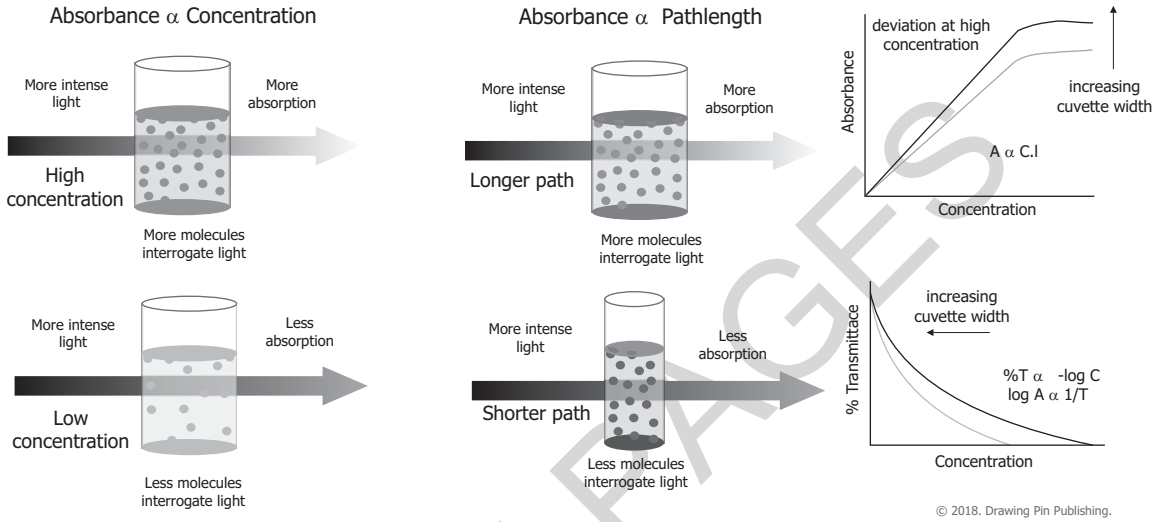
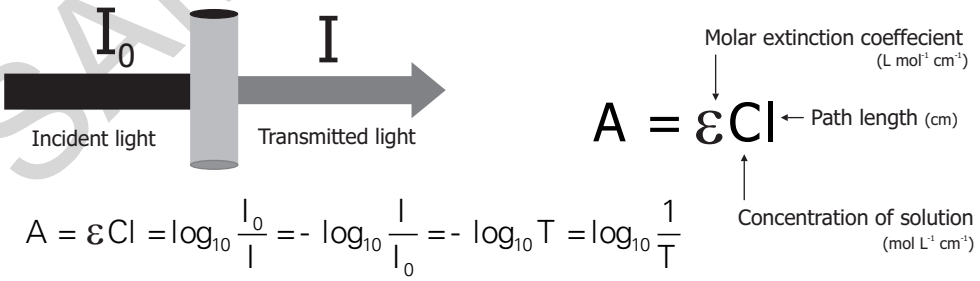


Fig C4. Lambert and Beer law, relating concentration and path length with absorbance

Removing the proportionality sign the formula becomes A= εcl, (where C is the concentration of solution in Moles per Litre and l is path length in centimeter, e is the constant of proportionality and also called as **molar extinction coefficient** or **molar absorptivity**, which is defined as the absorption of light by a solution of concentration 1 mol L^{-1} and path length of 1 cm). Sometimes an alternative term $\varepsilon_{1\%}$ is also used that represent absorption of 1% solution when path length is 1 cm (if $\varepsilon_{1\%}$ is used in numerical problems, the concentration must be in percentage). All the aforesaid mathematical relationships can be represented as follows:



$$A = \varepsilon Cl = \log_{10}\frac{I_0}{I} = -\log_{10}\frac{I}{I_0} = -\log_{10}T = \log_{10}\frac{1}{T}$$

Note: Lambert-Beer law shows a deviation from linear relationship between absorbance and concentration at very high concentrations. (Visit author blog for solved sample problem on UV-Vis spectrophotometry)

2.4 Applications of UV-Vis Spectrophotometry

Spectrophotometry has been used in the analysis of biomolecules for more than a century. The applications of spectrophotometry to biomolecules spans from their qualitative assessment to quantitative estimation, cross-contamination with other biomolecules, the activity of a biomolecule are some of the other applications. We may however, discuss some the applications in life sciences under following sub-headings.

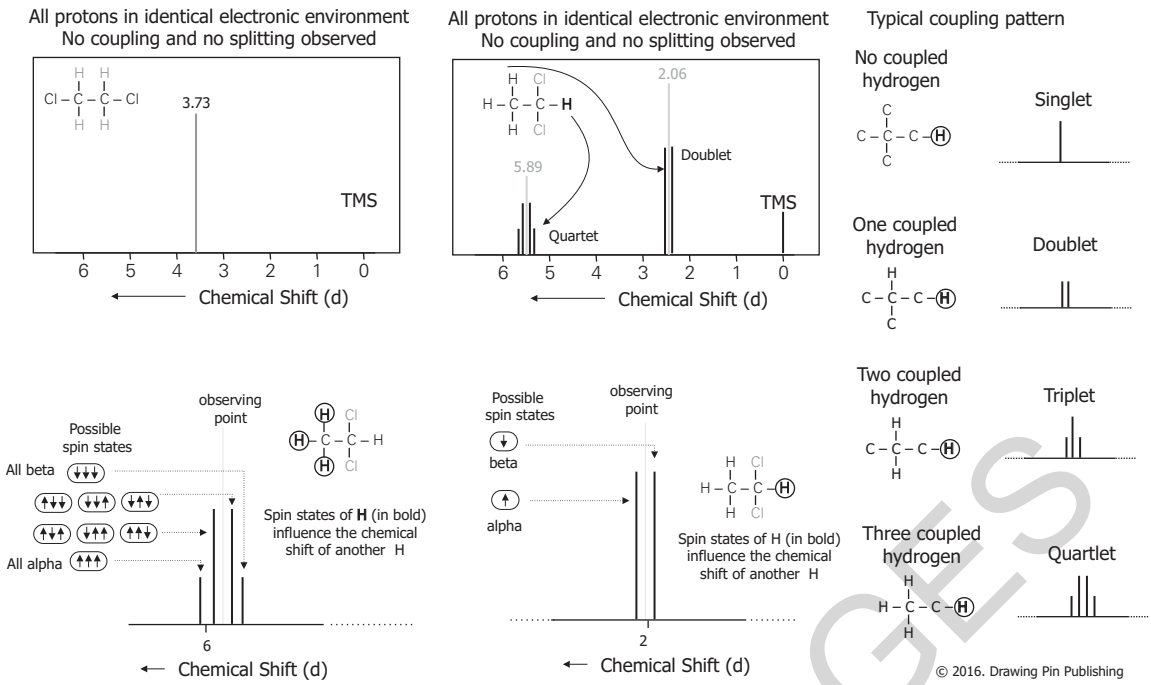


Fig C12. Chemical shift and splitting of peaks due to coupling

5.1.8 Effect of solvent on NMR signals

Most NMR spectra are recorded for compounds dissolved in a solvent. Therefore, signals will be observed for the solvent and this must be accounted for in solving spectral problems. Chloroform-d ($CDCl_3$) is the most common solvent for NMR measurements, thanks to its good solubilizing character and relative unreactive nature (except for 1º and 2º-amines). As noted earlier, other deuterium labeled compounds, such as deuterium oxide (D_2O), benzene-d6 (C_6D_6), acetone-d6 (CD_3COCD_3) and DMSO-d6 (CD_3SOCD_3) are also available for use as NMR solvents. Because some of these solvents have π-electron functions and/or may serve as hydrogen bonding partners, the chemical shifts of different groups of protons may change depending on the solvent being used. The following table gives a few examples, obtained with dilute solutions at 300 MHz. To avoid spectra dominated by the solvent signal, most ^1H NMR spectra are recorded in a deuterated solvent. However, deuteration is not “100%”, so signals for the residual protons are observed. In chloroform solvent ($CDCl_3$), this corresponds to $CHCl_3$, so a singlet signal is observed at 7.26 ppm. For methanol solvent, this corresponds to CHD_2OD, so a 1:2:3:2:1 pentet signal is observed at 3.31 ppm. D-chlorofom alone, gives a peak splited into three in the region of chemical shift between 77.5 to 77.6 ppm.

5.1.9 Commonly used variants of NMR spectroscopy

In general, NMR is performed for one specific type of nuclei at a time (this may be decided by choosing a specific magnetic field or resonance frequency). Most often ^1H, ^3H or ^{13}C are used to perform NMR. Hence, NMR involving detection of one type of atom alone is known as one dimensional NMR (1D-NMR). Such NMR method is useful for the identification of molecules based on the chemical shift or J-coupling. However, for the determination of the structure of biomolecules and larger organic molecules, 1D spectra shall be very crowded (as there will be thousands of hydrogen with different couplings), hence a two dimensional NMR is usually performed. Two-dimensional nuclear magnetic resonance spectroscopy (2D NMR) is a set of nuclear magnetic resonance spectroscopy (NMR) methods which give data plotted in a space defined by two frequency axes rather than one.

Types of 2D NMR include either homonuclear (i.e one types of atoms observed in NMR) or heteronuclear (two type of atoms observed in NMR). Variants of homonuclear 2D NMR include correlation spectroscopy (COSY), J-spectroscopy, exchange spectroscopy (EXSY), and nuclear Overhauser effect spectroscopy (NOESY). COSY-90 is the most common COSY experiment. In COSY-90, the p1 pulse tilts the nuclear spin

This tells us that apparently the two peaks in the spectrum are mathematically related and that the charge state of the 379.2 peak is +1 and the charge state for the 190.1 peak is therefore +2.

Step 4: Use the determined charged states to calculate the mass of the compound. One can think of the different peaks in the spectrum as separate mass measurements of the same peptide. We can then average the two answers to get the final mass.

Brain Teaser

How would post translational modifications change the m/z of various monoprotonated species? Can you detect a PTM if there a specific shift observed?

Hint: Addition or removal of a functional group during PTM, can be easily detected in mass spectrometry, this is represented by a mass shift equivalent to added or removed molecule, under single charged state. Some of the commonly known mass shifts in various PMTs are, phosphorylation - 80 Da, methylation - 14 Da, Acetylation - 42 Da.Glycosylation (mono-hexoses) - 162 Da. Methionine oxidation - 15 Da, Biotinylation - 226 Da.

8.5 Tandem Mass Spectrometry (MS/MS or MS^n)

Conventional mass spectrometry provides a characteristic spectra that is matched with existing database of spectra and proteins are identified based on match. However, if a protein is novel or match does not exist in database, tandem mass spectrometry (MS/MS) is used for identification. It is a also a tool for de novo sequencing of proteins. In this approach, distinct ions of interest are in a quadrupole filter based on their m/z during the first round of MS and are again fragmented by a number of different dissociation methods. One such method involves colliding the ions with a stream of inert gas, which is known as collision-induced dissociation (CID) or higher energy collision dissociation (HCD). Other methods of ion fragmentation include electron-transfer dissociation (ETD) and electron-capture dissociation (ECD). These fragments are then separated based on their individual m/z ratios in a second round of MS. MS/MS is commonly used to sequence proteins and oligonucleotides because the fragments can be used to match predicted peptide or nucleic acid sequences, respectively, that are found in databases such as PI, RefSeq and UniProtKB/Swiss-Prot. These sequence fragments can then be organized *in silico* into full-length sequence predictions.

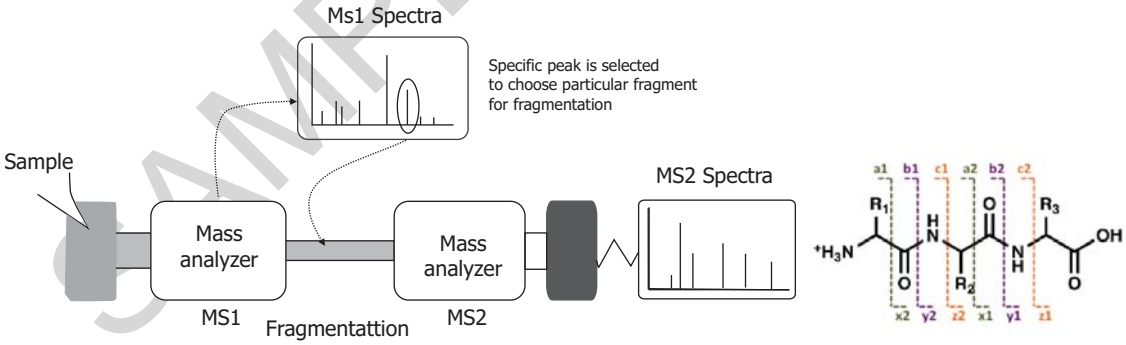


Fig C26. Tandem mass spectrometry or MS/MS enables identification of a peptide with higher score and naming conventions for various type of fragments of a peptide.

Modern Tandem MS instruments include, Typical Tandem MS in space instruments include QqQ, QTOF, and hybrid ion trap/FTMS, etc.QqQ or Triple Quadrupole inlcude Three Quadrupoles (Quad 1, Quad 2, and Quad 3) are lined up in a row. Precursor ions are selected in Quad 1 and sent to Quad 2 for dissociation (fragmentation). The generated product ions are sent to Quad 3 for mass scanning.QTOF or Quadrupole Time-of-flight, where precursor ions are selected in the Quadrupole and sent to the Collision Cell for fragmentation. The generated product ions are detected by time-of-flight (TOF) mass spectrometry and Hybrid Ion Trap/FTMS, where precursor ions are selected and fragmented in an external ion trap. The generated product ions can be detected either in the external trap (lower mass resolution, but faster) by or by FTMS (higher mass accuracy and resolution, but slower).

Brain Teaser

Find the mode from these test results – 90, 80, 77, 86, 90, 91, 77, 66, 69, 65, 43, 65, 75, 43, 90.

a. 43 *b. 77* *c. 65* *d. 90*

2.4 Position and Applicability of Central Tendencies in Various Data Sets

The relative position of the three measures of central tendency (mean, median, and mode) depends on the shape of the distribution. All three measures are identical in a normal distribution.

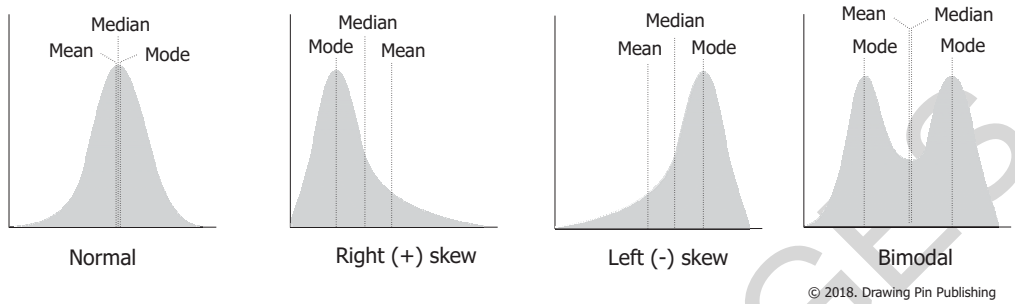


Fig D4. Variation in the measures of central tendencies as per the data type (Right skew is also known as positive skew and Left skew is also known as negative skew, hence in positive skew, Mean > Median > Mode, while in negative skew Mode > Median > Mean).

The applicability of three measures of central tendency is different for different type of data sets. For an interval or ratio data set any measure of central tendency can be used, however it not good to use mean as a measure of central tendencies if there are outliers. Also, with a nominal data only mode is the most suitable. Table D3 compares suitability of various measures of central tendencies.

Table D3. Applicability of measures of central tendency

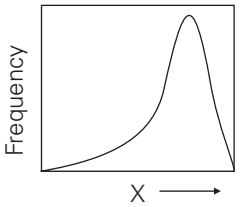
Characteristics	Mean	Median	Mode
Useful with interval, ratio data	Yes	Yes	Yes
Useful with ordinal data	No	Yes	Yes
Useful with nominal data	No	No	Yes
Affected by outliers	Yes	No	No

As mean is always pulled toward the extreme observations, the mean is shifted to the tail in a skewed distribution. Mode is the most frequently occurring score and hence it lies in the hump of the skewed distribution. Median lies in between the mean and the mode in a skewed distribution (Discussed more in Section 4).

Brain Teaser

The figure below shows a frequency histogram for the variable X. Which of these statements is correct for this histogram? [Adapted from TIFR - 2017 entrance exam paper] - similar question asked in CSIR-NET June 18.

a. mean < median < mode *b. mode > mean > median*
c. mode > mean > median *d. mean = mode, mode > median*



Hint: Refer to figure D4, and it can be observed that this data represents a left skewed data, where mean is smallest follwed by median and mode is largest value.

rats and may conclude incorrectly, so we will reject the null hypothesis (that drug does not have any effect) and commit type I error. While in second case where both treated and untreated groups showed different distribution of glucose, if we consider the glucose level of animals at the intersection of data (indicated), we may believe that drug has not caused any change. As some treated and some untreated animals will show similar glucose. So in that case we will commit type II error. Although it's clear from the graph that two outcomes are different, but when experiments are performed, we may commit errors depending on which data we obtained. The data shown in figure may be a result of large number of experiments on many animals, but limited experimentation and choosing wrong data for analysis may lead to an incomplete picture of the data and these errors. Fig D14 below illustrates the two type of errors.

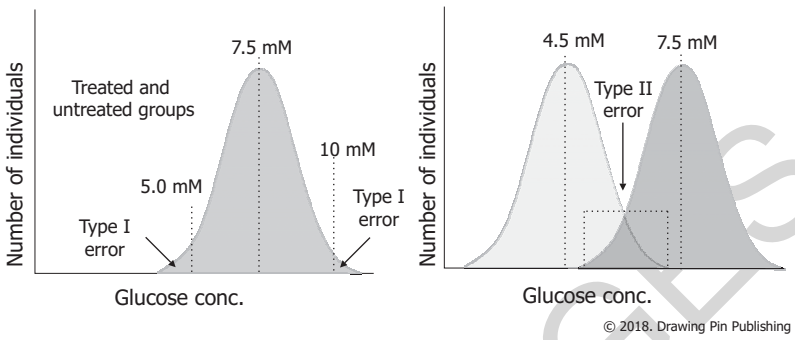


Fig D14. Illustration and comparison of Type I and Type II errors

The risk of type 2 error, is affected by several factors, but the most controllable is sample size. As sample size increases, the risk of Type 2 error decreases. There are formulae for calculating the appropriate sample size to ensure that the risk of Type 2 error is acceptable (e.g. 20%), but these calculations rely on an estimate of effect size and the standard deviation of the sample or population.

Some common differences in two type of errors are highlighted below:

Type I error

- is the incorrect rejection of the null hypothesis
- maximum probability is set in advance as alpha
- is not affected by sample size as it is set in advance
- increases with the number of tests or endpoints

Type II error

- is the incorrect acceptance of the null hypothesis
- probability is beta
- beta depends upon sample size and alpha
- can't be estimated except as a function of the true population effect
- beta gets smaller as the sample size gets larger
- beta gets smaller as the number of tests or endpoints increases

7.3 Confidence Interval and p–Value

Confidence Intervals describe the variability surrounding the sample point estimate (the wider the interval, the less confident we can be about the estimate of the population mean). In general, all things being equal, the larger the sample size the better (more precise) the estimate is, as less variation between sample means is expected. In other words, confidence intervals tell us about how well we have determined the mean. The key point is that the confidence interval tells you about the likely location of the true population parameter. The equation for a 95% confidence interval **(CI)** for the population mean when the population standard deviation is unknown and the sample size is large (over 30) is (discussed previously in central limit theorem).

$$\begin{matrix} L \\ U \end{matrix} = \mu \pm z^* \left(\frac{\sigma}{\sqrt{N}} \right)$$

There is a strong relationship between hypothesis testing and confidence intervals. For example, when carrying out a paired t-test, if the p-value < 0.05, the 95% confidence interval for the paired differences will not contain 0. However, a p-value just concludes whether there is significant evidence of a difference or not. The confidence interval of the difference gives an indication of the size of the difference.

CHAPTER

E

Radiolabelling Techniques

1. Basics of Radioactivity

1.1 Introduction

Radioactivity is continuous emission of some high energy radiations such as alpha (α), beta (b) or gamma (γ), by some atoms until they become stable. Atoms are made up of sub-atomic particles, electrons, protons and neutrons and the ratio of neutrons to protons determines the stability of an atom. Some atoms can have the same number of protons but vary in the number of neutrons (therefore have same atomic no. but a different mass number) are known as **isotopes**, e.g. ^{12}C and ^{14}C (can also be written as Carbon-12 and Carbon -14). Stable isotopes do not emit radiations, but unstable atoms do. Unstable isotopes, if they emit radioactive rays are called **radioisotopes**. Radioisotopes become stable isotopes by the process of radioactive decay. During this process, changes occur in the atomic number or mass and particles and (or) electromagnetic radiations are emitted. Not all the isotopes are radioactive, hence terms isotope and radioactive isotopes are not synonymous. An alternate term **radionuclide** is also used for radioisotopes in chemistry and physics. Radioactive molecules have an enormous range of applications in biology. Readers may also recall that some of the famous hypothesis in molecular biology such as “DNA is a genetic material” and “DNA replicated through semi-conservative replication” were also established by use of radioactive tracers. A large number of biochemical pathways were also deciphered by using radioactive tracers. A number of techniques such as Sanger’s DNA sequencing method, radio-in-situ hybridization (RISH), radioimmunoassay (RIA) were based on the use of radioactive molecules (discussed in chapter A and B).

1.2 Different type of radioactive decays

There are many ways in which a nuclei can become stable by radioactive decay. Some of the commonly known types of radioactive decay are the emission of alpha, beta and gamma rays. Various properties of three kinds of decay and their effects on atomic number and mass number are described in table E1. Don’t you wonder, how the atomic number increases by one in beta emision, when something is emitted out from an atom? Does the number of protons increase? If so, how? In fact, the subatomic particles are also made up of smaller units which can break down and associate. The emerging concepts in sub-atomic particles have revealed that not beyond electrons, protons and, neutrons, there are more elementary particles such as quarks, mesons and Higg’s-boson particles (also known as god particles) have improved the understanding of radioactive decay, origin and interconversion of sub-atomic particles. One such example is splitting of a neutron into a positron and beta particle (negatron) which leads to an increase in charge of a nucleus. Three most common type of emissions is discussed below.

to-back emission. As a result of positron emission the nucleus loses a proton and gains a neutron, the mass number stays the same. An example of an isotope decaying by positron emission is ^{22}Na:

$$_{11}Na^{23} \dashrightarrow {}_{10}Na^{22} + \beta^{+}$$

Positron emitters are detected by the same instruments used to detect γ-radiation. They are used in biological sciences to perform brain scanning with the technique positron emission tomography (PET scanning) used to identify active and inactive areas of the brain (Detailed description in chapter G).

The penetration and ionizing ability of three different types of radiations are illustrated in Fig E1 below.

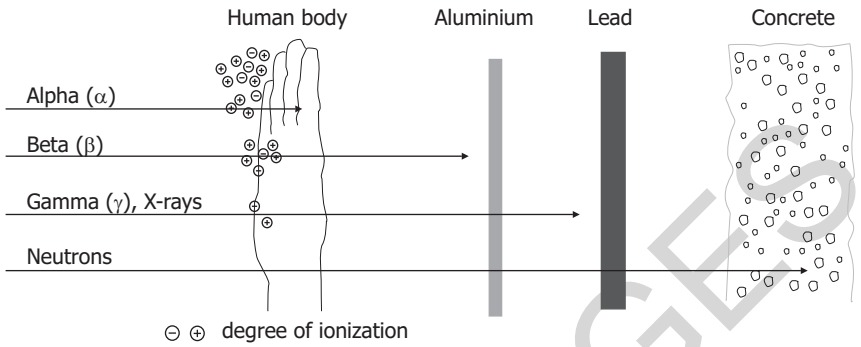


Fig E1. Penetration and the ionizing ability of three types of radiations. It may be noted that high ionization is used for the therapeutic purpose (to kill harmful cells) and high penetrations are preferred for diagnostic and imaging purpose.

1.3 Applications of Radioisotopes in Biology

Radioactivity, although a commonly studied phenomenon in chemistry and physics, has several applications in biology. The span of applications is from biochemical and molecular technique in the lab to diagnosis and therapy of diseases in clinics. Following is the brief description of some widely known applications of radioactivity in life sciences, a more detailed discussion of some applications is provided in the subsequent sections.

1.3.1 Biochemical tracing

Biochemical tracing is a method of tracking the fate of metabolites in the routine metabolic pathways and cycles. Radioactive and non-radioactive tracing using isotopes has been used to decipher most of the biochemical pathways that we see in biochemistry books today. Radioactive carbon was used to trace the metabolic fate of all carbon atoms in glycolysis and TCA cycle. The rapid rate of recycling of ATP and reversible nature of various biochemical reactions was also tested using radioactive tracers.

1.3.2 Nuclear medicine and imaging

Nuclear medicine is another important dimension of biological application of radioactive nuclei. It involves the use of such atoms in various imaging techniques such as PET imaging as well as therapeutic methods such as radiotherapy of cancer and treatment of hyperthyroidism. Gallium-67, for instance, can be ingested by patients. When the patient undergoes an MRI or PET scan, doctors can trace the substance inside the body and see what's going on without invasive surgery. Other radioisotopes, such as Iodine-123 and Iodine-125, help doctors diagnose thyroid disorders and metabolic disorders in a similar way. Cesium-137 and Cobalt-60 are both used to shrink the size of tumors within the bodies of cancer patients. Cobalt-60 is also used to sterilize medical instruments. Some radioisotopes are used to diagnose and treat other disorders, such as Chromium-51, is used to determine the survival rate of red blood cells.

1.3.3 Molecular techniques (blotting and sequencing)

A number of molecular techniques such as detection of nucleic acid of specific sequence after southern transfer and other hybridization techniques such as RISH (radio-in situ hybridization) are based on use of radio labelled nucleic acid probes. Similarly, famous method of DNA sequencing, called chain termination method developed by Sanger was also based on radiolabelling of DNA for its detection using autoradiography. ^{32}P was used in most of the hybridization methods in molecular biology. Sometimes ^{35}S is also used

CHAPTER

F

Microscopic Techniques

1. Visualization of Cells & Subcellular Components

1.1 Introduction

Visualizing cells is not just a matter of curiosity but also a piece of important direct evidence to assess the effect of several biotic and abiotic factors on morphology and functioning of cells. Visualization of cells in situ or in isolation has been an important tool in research as well as clinics. From the diagnosis of malaria to cancer or development of fascinating animal clones like dolly or even the development of some of the potential drugs involved the visualization of cells at some stage. The average size of prokaryotic cells ranges from 1- 10 μm while that of eukaryotic cells ranges from 10 – 100 μm (some of the cells are, however, extremely large such as ostrich egg), and some of the large organelles in the eukaryotic cell such as mitochondria and chloroplast are in the size range of prokaryotic cells (2-5 μm). Considering this average size of the cell and the ability of the human eye to see objects (0.2 mm), it is impossible to see a single cell in the aforesaid size range through naked eyes. Besides Biology, microscopy has been one of the most important technique to enable the visualization of cells. Microscopic techniques are critical to the research of physics, chemistry and even in clinical diagnosis. Apart from an essential tool in research, microscopes have become a teaching and learning companion for both students and teachers. Since the inventions of first microscope by **Hans and Zacharias Janssen** in 1590, microscopes have evolved a lot both in terms of resolution and magnification. The best microscopes today can have a resolution of less than 1 angstrom and a magnification of over 1 million, while our eyes cannot resolve two dots at a distance lesser than 0.2 mm. Increasing resolution and magnification has created enormous opportunities in research and a wider scope of application. Now, microscopes are used in clinical diagnosis, molecular and cell biology studies, nanomaterial research and even determination of the structure of biomolecules.

Microscopy is the technical field of using microscopes to view objects and areas of objects that cannot be seen with the naked eye (objects that are not within the resolution range of the normal eye). There are three well-known branches of microscopy: optical, electron, and scanning probe microscopy. We will be discussing the light microscopy in detail under this topic.

1.2 Classification of Microscopy Techniques

There are a large number of microscopy variants that are based on visualization of cells, broadly grouped into three categories based on the fundamental source of image generation, light, electron beam or scanning probes. Light-based microscopy utilizes the visible light (400 -700 nm), electron microscopy utilizes electron beam (0.01 - 1 nm) and scanning probe microscopy is based on image generation using a highly sensitive tip that scans the surface based on various principles. Fig F1 represents the outline classification of various microscopic techniques. [A poster on detailed classification of microscopes is available from the publisher.]

In the following section, we will discuss more about the sub-types of light based microscopic methods, while other methods are discussed in subsequent sections.

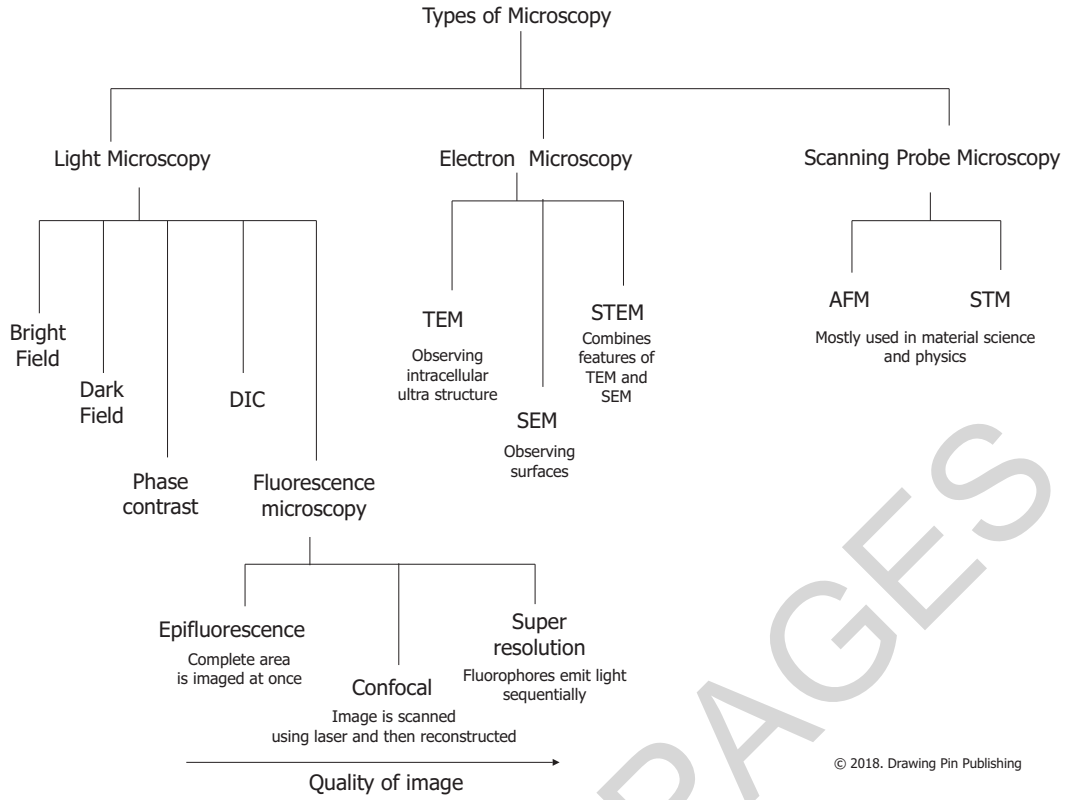


Fig F1. Outline classification of the microscopic techniques

Bright-field microscopy is the simplest of all the optical microscopy illumination techniques. Sample illumination is caused by the transmitted (i.e., illuminated from below and observed from above or vice versa) white light that generates the contrast in the sample mainly due to absorbance or scattering of some of the transmitted light in dense areas of the sample. **Dark field microscopy** (dark ground microscopy) is variant of light microscopy in which the object alone is illuminated while the background remains dark, this helps in better representation and appearance of cellular boundaries. The dark field is achieved using a specific type of disc fitted in the condenser called dark field stop. **Phase contrast microscopy** allows the viewing of unstained specimens by using the light phase amplitude differences within microscopic objects. When an unstained biological specimen is observed in the normal brightfield microscope, it is quite difficult to see because most biological material is uncolored and transparent, providing little contrast to the illuminated background (details in section 3.2). **Differential interference contrast (DIC**) microscopy is much similar in principle to phase contrast with a basic difference that it utilizes polarised light to create phase difference. In **fluorescence microscopy**, the specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. **Confocal microscopy** is an advanced version of fluorescence microscopy to provide much better contrast and clarity by using the scanning method and laser as a light source. It also allows visualization of the sample on the z-axis and therefore generating 3D images. Other types of microscopic techniques that do not involve light are either electron microscopy (discussed in section 4.1) or scanning probe microscopy (not a part of CSIR syllabus- provided in online free supplement).

2. Resolving Power of Different Microscopes

2.1 Understanding Resolving Power

Resolution is defined as the minimum distance between two points that can still be distinguished by the observer or camera system as separate entities. As the definition suggests, for a better resolution the distance should be as small as possible. The resolution is also known as **resolving power** or **the limit**

highly limited depth of focus. Clinically, CLSM is used in the evaluation of various eye diseases, and is particularly useful for imaging, qualitative analysis, and quantification of endothelial cells of the cornea. It is used for localizing and identifying the presence of filamentary fungal elements in the corneal stroma in cases of keratomycosis, enabling rapid diagnosis and thereby early institution of definitive therapy. Research into CLSM techniques for endoscopic procedures (endomicroscopy) is also showing promise. In the pharmaceutical industry, it was recommended to follow the manufacturing process of thin film pharmaceutical forms, to control the quality and uniformity of the drug distribution.

3.4.1 Concept of optical sectioning in confocal microscopy

A very unique feature of confocal microscopy that is not present in epifluorescence micropic techniques is the quality of its images, which has lead to a rapid surge in usage of confocal microscopy. The quality of images depends upon the fact that whole area is not imaged but imaging is done in the form of small parts which are then recombined using computational tools. Another important aspect of confocal microscopy is the ability to reconstruct three dimensional images A conventional epifluorescence microscopy cannot predict that a molecule was present on surface of nuclear membrane or in the interior, but confocal microscopy can do. This is achieved by a process called optical sectioning and then stacking of focal planes by computational approaches.

Optical sectioning: It is possible in confocal microscope that the light (fluorescence) from only one specific plane i.e. in focus is gathered while remaining light from other planes is cut off. This is followed by obtaining images of diffent planes of the same sample. As this is analogous to cutting a section in a microtome, but here done using optics, hence termed as optical sectioning.

Focal plane: A focal plane is represented by one single plane at which the object is being imaged at a time in confocal microscopy, or the place from which the fluorescence is being emitted. Several images of different planes can, therefore, be aquired using a confocal microscope.

3-D reconstruction Several images of an object in seqeuntial manner of ascending or descending focal planes can be utilised to create 3-dimentional structures of cellular components. The array of such images is often known as stacks or z-stacks. These z-stacks are computationally aligned and converted into a single image resembling a three dimensional reconstruction. Hence, it is possible to observe the specific position of fluorophore across different organelles.

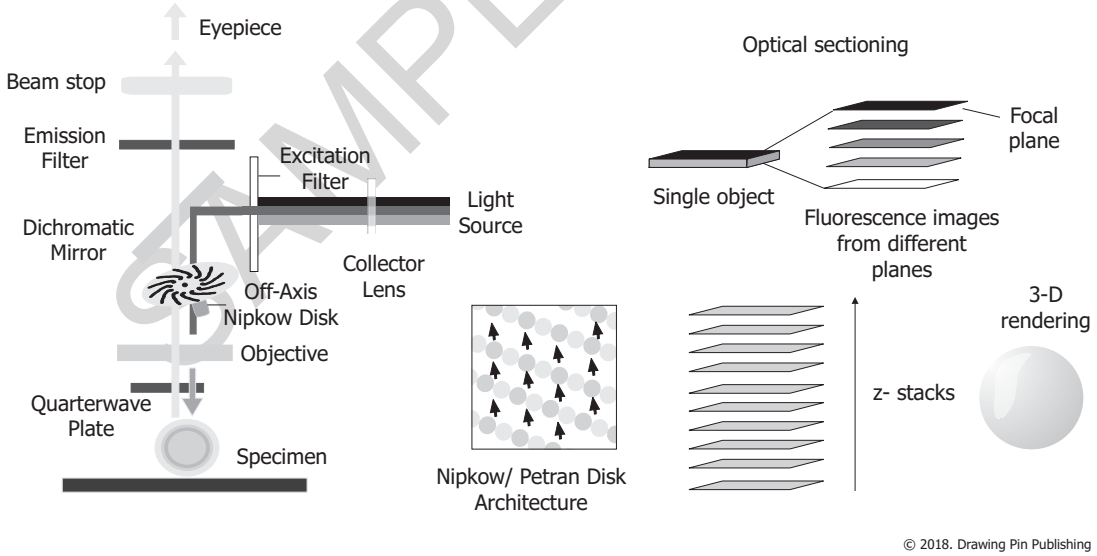


Fig. F8.Optics of confocal microscope, representation of optical sectioning, focal plane and stacks generated using confocal microscopy (This figure depicts spinning disc type model, the other models include laser scanning, multiphoton, TIFRM, and deconvolution confocal microscopy).

CHAPTER

G

Electrophysio-Methods

1. Introduction to Electrophysiological Methods

Since the origin of life, cells have developed various means of communication to promote their survival and evolution. Signals in biological systems can be chemical (hormones and chemokines), mechanical (Juxtacrine), auditory (sound waves) optical (electromagnetic waves) or even electrical (nerve impulse transmission). While most of the lower organisms rely on chemical signalling, multicellular organisms have more complex signalling and involve most of the aforementioned signalling mechanisms. Electrical signals are particularly important due to their dynamic nature and involvement in some of the vital functions of complex life forms, therefore detection of electrophysiological signals has become an important domain in the diagnosis of the functioning of various organs. Generally speaking, almost all the cells, due to the difference in permeability can acquire a Donnan equilibrium with a net charge on the plasma membrane. This results in the net negative charge on most of the plasma membranes, commonly known as resting potential. The net resting potential across two sides of a plasma membrane is close to -70 mV. However, in nerve cells this resting potential is transiently altered by a stimulus causing opening of specific channels and modifying the flux of intracellular and extracellular ions (Sodium ions rapidly move in due to the opening of voltage-gated channels), causing the potential of membrane to turn positive (+30 mV), called depolarised state, which is quickly restored. This mechanism generates an electrical impulse which moves across the length of neurons. At the junctions of neurons, this electrical stimulus is converted into a chemical stimulus (called synaptic signalling). Signals are even stronger and faster when the neurons are covered by a myelin sheath (Refer Module 6 or standard physiology books for the mechanism of nerve impulse transmission).

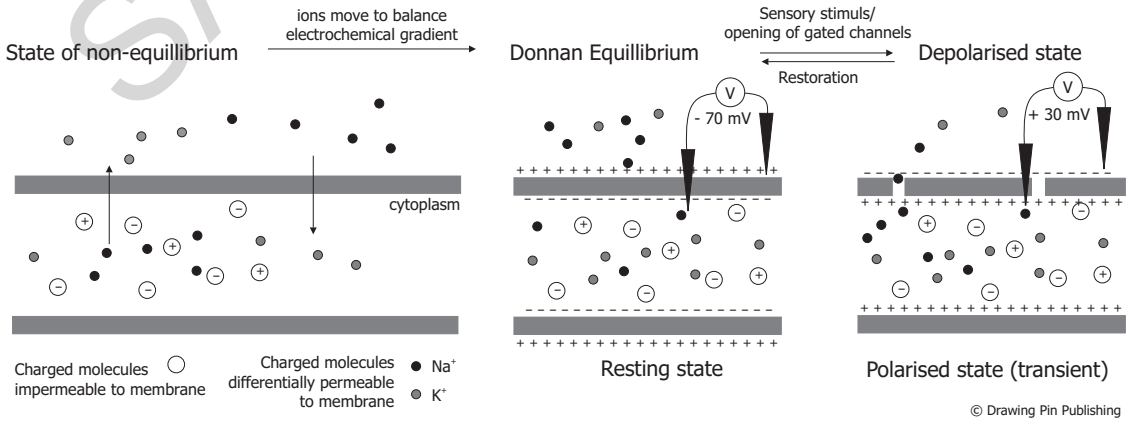


Fig G1. Meaning of resting potential and its generation

Besides neurons, some specialized muscle cells are also capable of generating electric impulse by modulation of ion flux, such as SA node, AV node, Purkinje fibers and Bundle of His in myogenic hearts. The transmission

of an impulse across the tissues generates a potential which can be measured to assess the physiological functions of tissues or organs. Although the magnitude of the electrical signal generated by various means is very small (millivolts), superposition of these small signals to a larger amplitude current can be used for detection. As most of the electrical signals are related with one or more physiological aspect of living organisms such as muscle contraction, nerve impulse transmission, behavior, memory functions, etc., these signals allow the measurement of physiological response and state. Therefore the methods involving the recording of such electrical signals are also known as electrophysiological methods.

1.1 Common Electrophysiological Methods

As mentioned above, brain, heart and skeletal muscles are prime sources of electric and magnetic fields that can be recorded and the resulting patterns can give insight on what ailments the subject may have. These electrophysiological techniques are named according to the data that is measured and sometimes the anatomical location of the sources. Some of the most common methods involve the electrophysiological recording of the brain (electroencephalography), heart (electrocardiography), muscles (electromyography) and retina (Electroretinography). However, a more exhaustive list is provided below in table G1.

Table G1. Various electrophysiological methods and associated body parts.

Method's Name	Abbreviations	Associated body parts
Electrocardiography	ECG or EKG	Heart (cardiac muscle), cutaneous electrodes (noninvasive)
Electroatriography	EAG	Atrial cardiac muscle
Eectroventriculography	EVG	Ventricular cardiac muscle
Intracardiac electrogram	EGM	Heart (cardiac muscle), intra-cardiac electrodes (invasive)
Electroencephalography	EEG	Brain (cortex), with extra-cranial electrodes
Electrocorticography	ECoG or iEEG	Brain (specifically the cerebral cortex)
Electromyography	EMG	Muscles throughout the body (usually skeletal muscles)
Electrooculography	EOG	Eye (entire globe)
Electroretinography	ERG	Retina specifically
Electronystagmography	ENG	Eye via the cornea
Electrogastrography	EGG	Stomach smooth muscle
Electrodermography	EDG	Skin
Electrohysterography	EHG	Uterus
Electroneuronography	ENeG or ENoG	Nerves
Electropneumography	EPG	Lungs (chest movements)
Electrospinography	ESG	Spinal cord

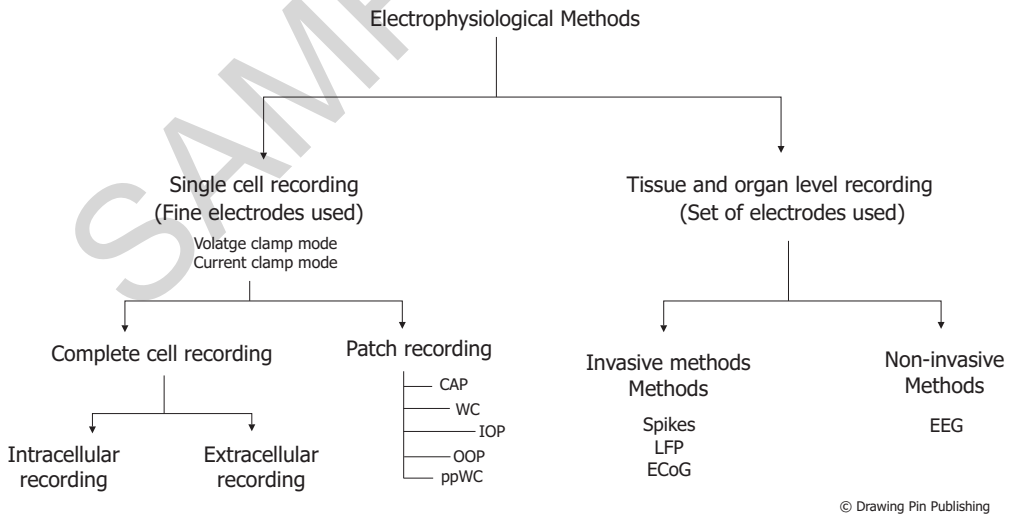


Fig G2. Outline of the electrophysiological methods

Now, besides the target organ, the electrophysiological methods can also be classified on the basis of how the detection is being done, whether whole organ or tissue is being investigated or a single cell is being investigated. And if a cell is being analyzed, whether the recording is being done intracellular or extracellular. Similarly, methods may be categorized on the basis of invasiveness. In clinical terms, an invasive

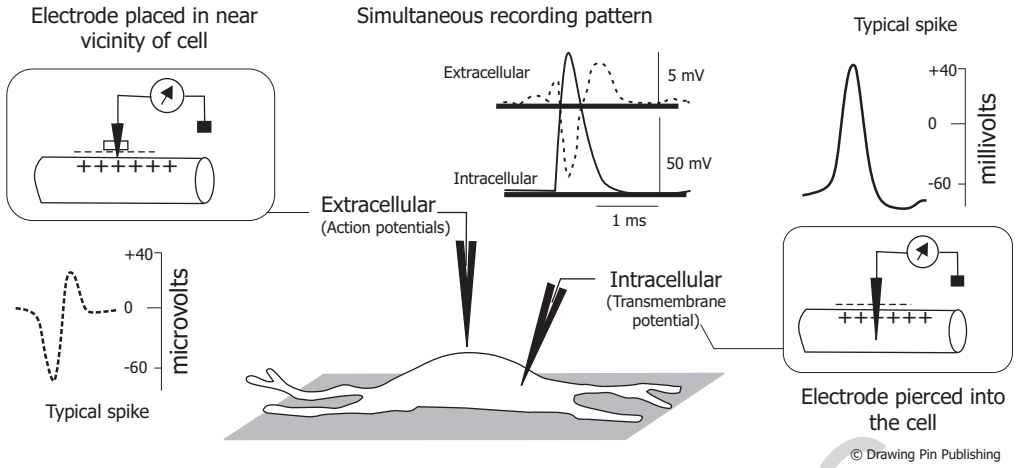


Fig G4. Illustration showing difference between intracelular and extracelluclar recording

2.4 Two formats of Single Cell Recording: Volatge Clamp and Current Clamp

The single cell recording can be performed in two different modes, depending upon whether voltage or current is being kept constant. **Voltage clamp (V-clamp)** is achieved by holding the cell at a predetermined value of membrane potential (e.g. - 70 mV) and the amount of current required from the external source to maintain that value is recorded. Thus voltage remain constant and a graph between time (ms) and current (mA or pA) is obtained. Voltage clamp measurements of current are made possible by the near-simultaneous digital subtraction of transient capacitive currents that pass as the recording electrode and cell membrane are charged to alter the cell's potential. It is suitable for the studies on voltage-dependent channels and most of the patch clamp methods operate in voltage-clamp mode. Voltage clamp has been sometimes criticized for the space clamp problem, i.e., many times the applied potential is not maintained all over the cell. As a disadvantage voltage clamp has the inability to adequately maintain a holding command in distal dendrites and causes washout of cytosolic factors in whole-cell.

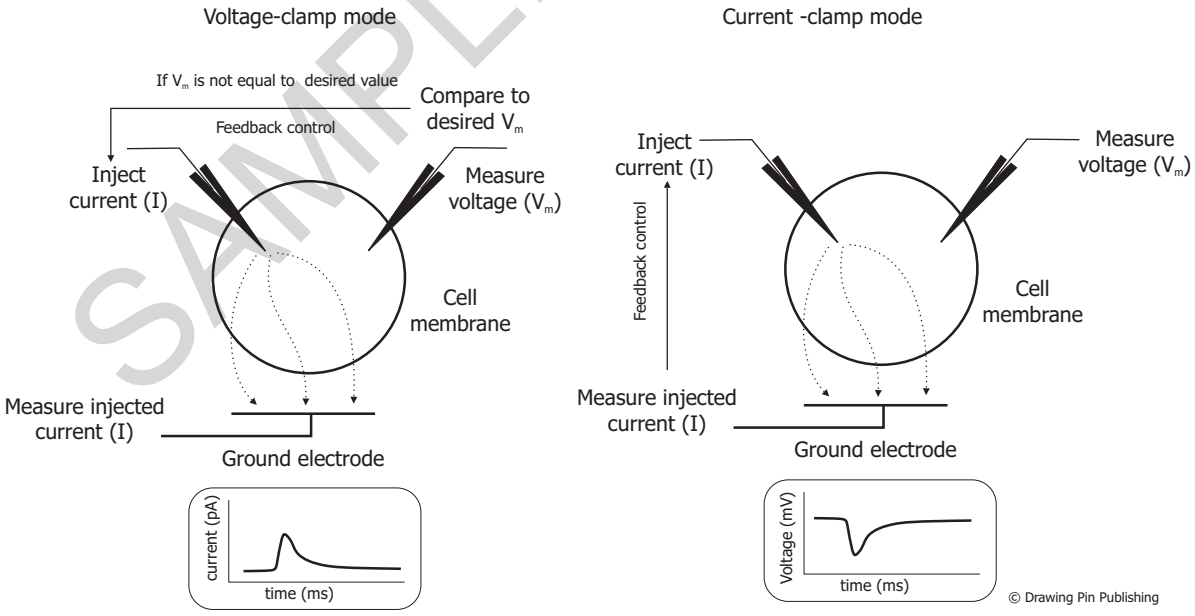


Fig G5. Two different modes of single cell recording: volatge clamp and current clamp.

The current clamp (I-clamp) is achieved by recording the membrane potential by injecting current into a cell through the recording electrode. Unlike in the voltage clamp mode, where the membrane potential is held at a level determined by the experimenter, in "current clamp" mode the membrane potential is free to vary, and the amplifier records whatever voltage the cell generates on its own or as a result of stimu-

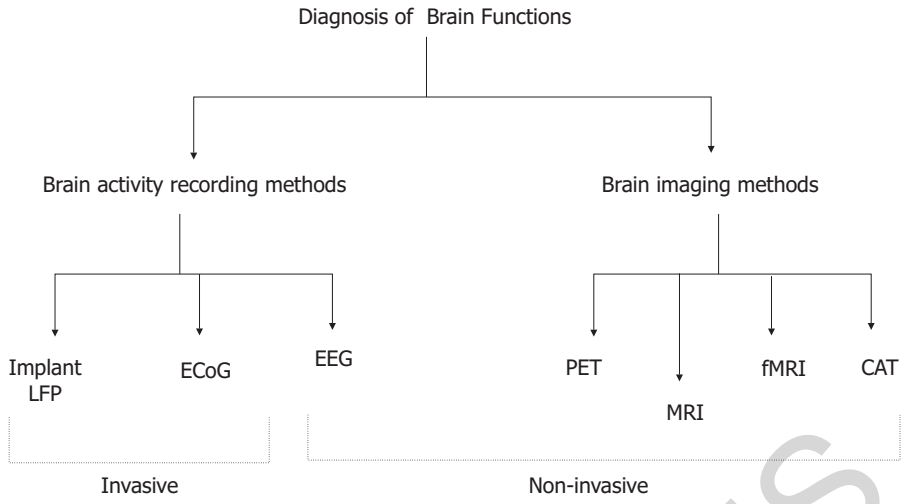


Fig G15. Methods for diagnosis of brain functions

5.1 Scale of Recording Methods and Signal Strength

As discussed above, there are a variety of methods for recording the electrical signals arising from neurons, however, these methods differ in the scale of measurement i.e. difference in a number of neurons involved in signal generation. For very precise results and description about neuron activity, single unit recording or single neuron recording is most appropriate.

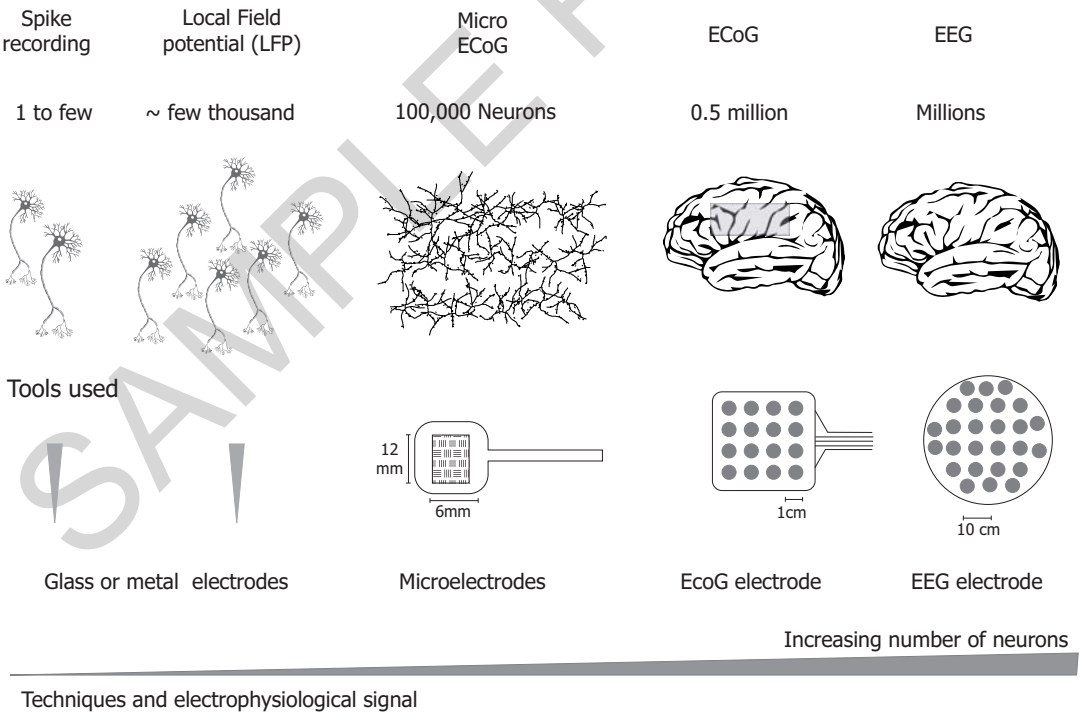


Fig G16. The scale of various recording methods. Only EcoG and EEG can be used on humans while all can be used for experimentation on monkeys (Based on an image provided by Prof. Supratim Ray, IISc Bangalore)

This is important when the objective is to understand the role of different neurons in a different type of cognitive functions. However, these methods require penetration of an electrode at a precise position in the brain and it is highly invasive technique, therefore cannot be used on healthy humans, especially when the purpose is merely diagnosis and not therapy. Moreover, the response of our brain against any stimulus

Advantages

· Allows for direct electrical stimulation of the brain, identifying critical regions of the cortex to be avoided during surgery
· Greater precision and sensitivity than an EEG scalp recording
· Better spatial resolution and high signal-to-noise ratio due to closer proximity to neural activity

Disadvantages

· Sampling time is limited, therefore, seizures (ictal events) may not be recorded during the ECoG recording period
· The field of view is also limited as electrode placement is limited by the area of exposed cortex and surgery time.
· Recording is subject to the influence of anesthetics, narcotic analgesics etc.

5.2.3 Electroencephalography (EEG)

Conventionally, Electroencephalography (EEG) is defined as the measurement of the electrical activity of the brain by recording from electrodes placed on the scalp. The resulting traces are known as an electroencephalogram (EEG) and represent an electrical signal from a large number of neurons.EEGs are frequently used in experimentation because the process is non-invasive to the research subject. The EEG is capable of detecting changes in electrical activity in the brain on a millisecond-level. It is one of the few techniques available that has such high temporal resolution. The discovery of EEG was pioneered from the work of **Hans Berger**, when he discovered in 1929, that electrical signals from the brain can be picked up through the scalp from silver disk electrodes pasted to it. These signals are called EEGs (which stands for electroencephalograms) or, informally, "brain waves." Electrical signals from the brain show different patterns of activity associated with different mental processes. When nerve cells are excited they show more of the impulses each second rather chemical measurements of brain activity. In the past 20 years, several methods have been developed to measure chemical concentration and changes in concentration in small bits of the brains of awake, normally behaving rats and other animal species. The information or pattern obtained from the EEG is known as brain waves or nuronal oscillations.

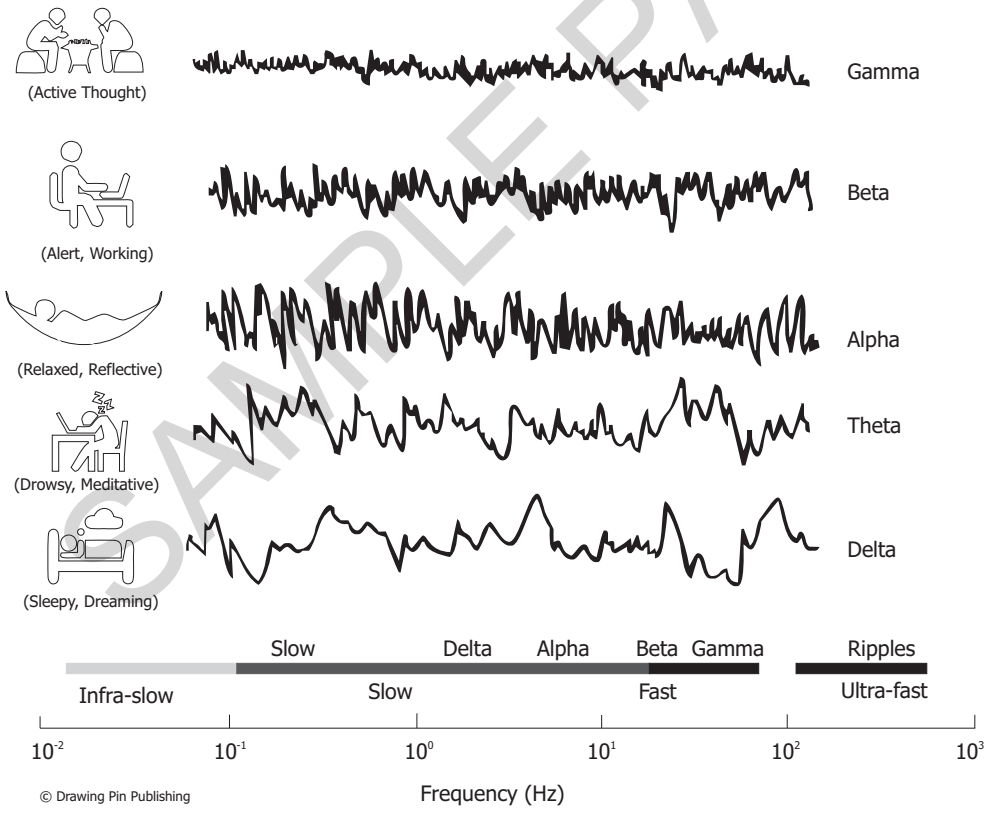


Fig G20. Electrencephalograhy (EEG) signals in various conditions and theri corresponding frequencies

Brain waves or neuronal oscillations are rhythmic or repetitive patterns of neural activity in the central nervous system. Neural tissue can generate oscillatory activity in many ways, driven either by mechanisms within individual neurons or by interactions between neurons. In individual neurons, oscillations can appear either as oscillations in membrane potential or as rhythmic patterns of action potentials, which then produce oscillatory activation of post-synaptic neurons. At the level of neural ensembles, synchronized

CHAPTER

H

Field Biology Methods

1. Introduction to Methods 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.

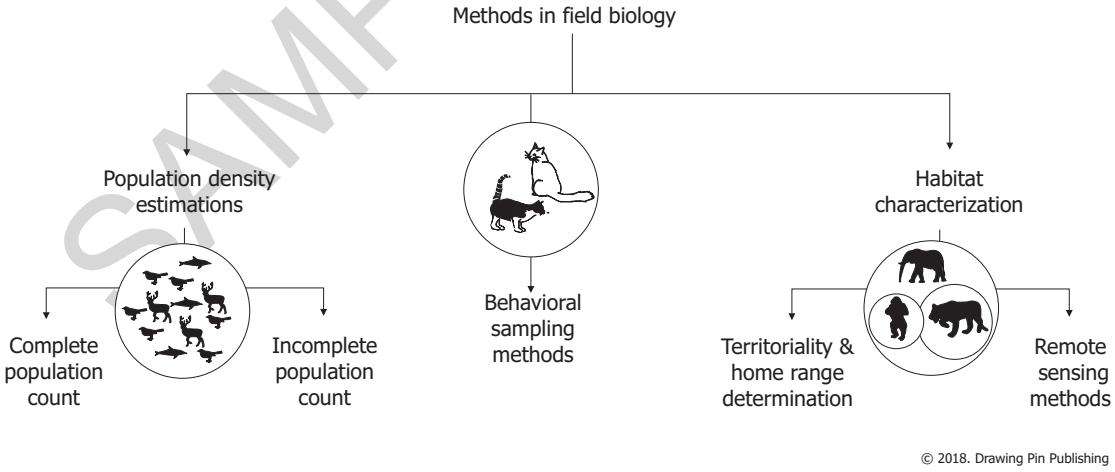


Fig H1. Three important domains of methods in field biology.

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 patterns of various species and habitat characterization. Role of direct and indirect observation coupled with strong statistical analysis is required in field biology to conclude about the ecological aspects of

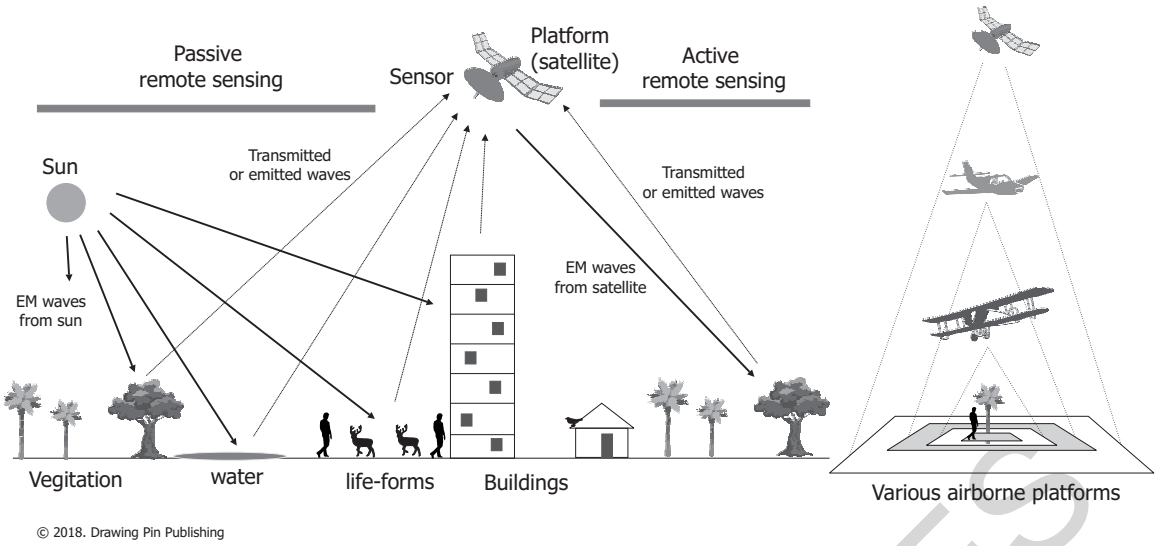


Fig H10. Basic principle of remote sensing and airborne platforms used for carrying remote sensing equipments

Basic steps of a simple remote sensing workflow involve, emission of electromagnetic radiation from a source such as the Sun or an EM radiation source located on the platform. This is followed by the transmission of energy from the source to the object.

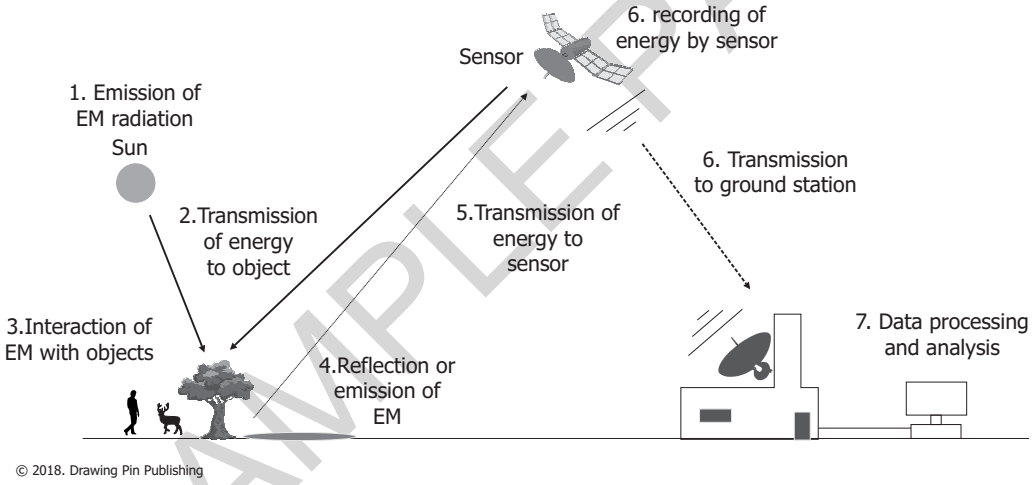


Fig H11. Outline procedure and steps involved in remote sensing

This energy may be three primary types, a. visible and near IR radiation, which primarily results of reflectance from the objects; b. far IR, also known as thermal IR, as it's not reflected radiation but emitted radiation from the objects; c. microwaves, which are either emitted by the object or transmitted by the remote sensing devices and reflected microwaves are studied (radar technique). In the next step, these reflected or emitted radiations are captured by the sensor. Visible rays can be captured by the camera. IR is captured by thermal imaging devices, and microwaves are captured by radiometers (more discussion in section 5.3, on sensors). These signals are recorded on photographic or non-photographic films and these recorded data are sent to the ground station. This data is then processed at the ground station using computational and statistical tools, finally generating information about the landscape or habitats. Fig H11 illustrates the basic outline of the remote sensing process.

5.2.1 Nature of EM 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.

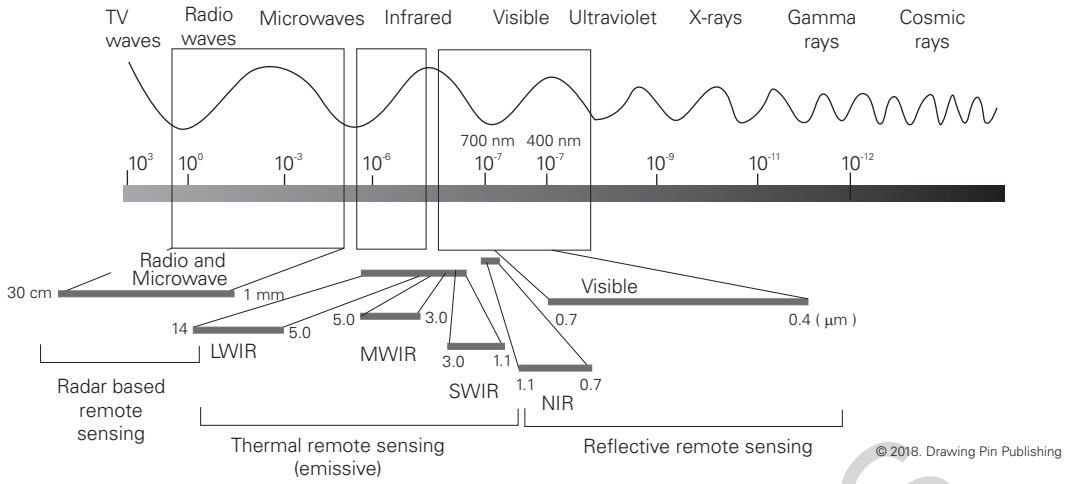


Fig H12. Various electromagentic radiations used in remote sensing

5.2.2 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.

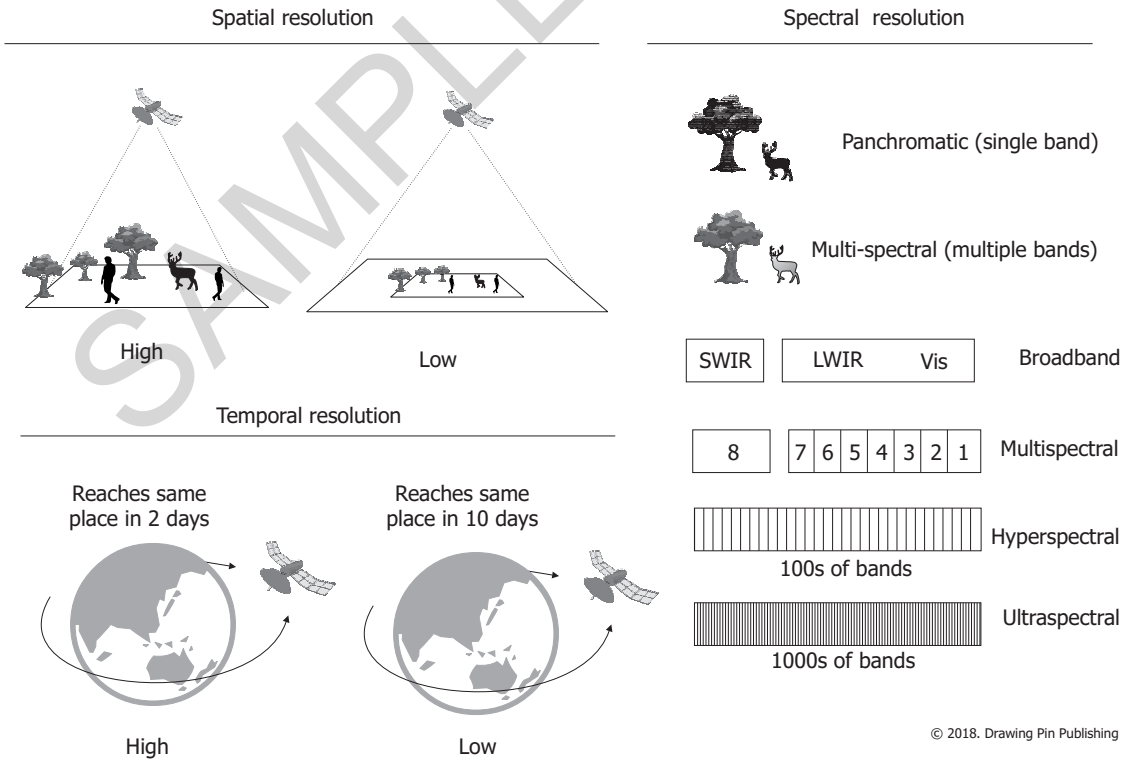


Fig H13. Representation of various types of sensitivity parameters for remote sensing equipments. a. spatial resolution, b. temporal resolution and c. spectral resolution.

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.

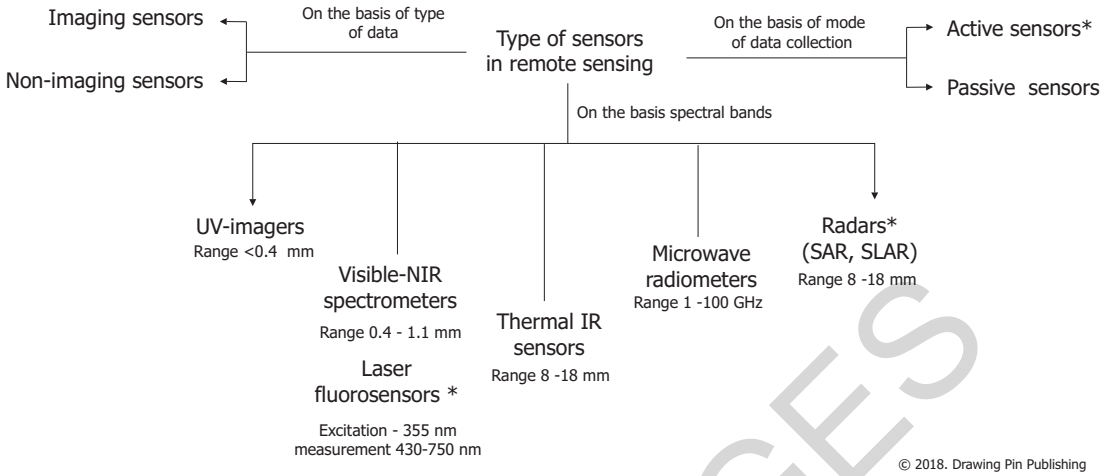


Fig H14. Classification of sensors used in remote sensing

5.4 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.

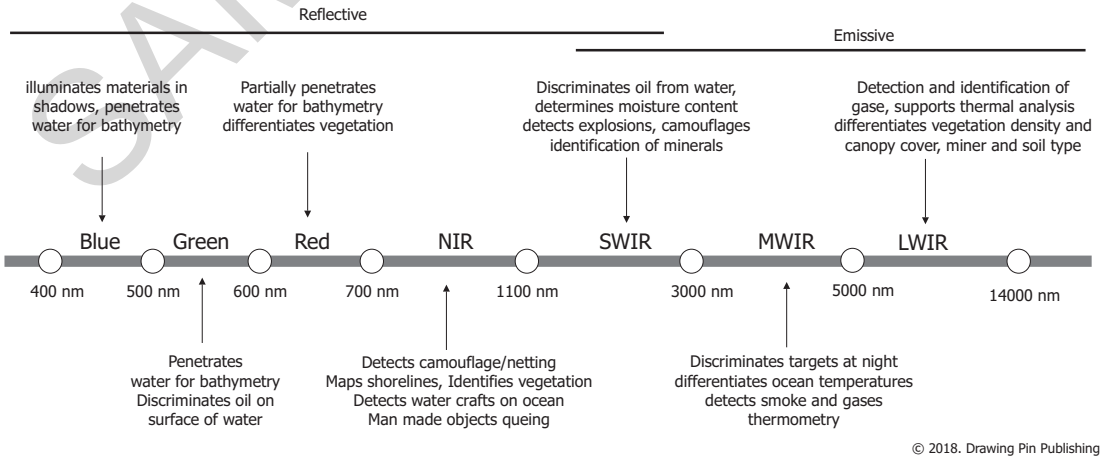


Fig H15. Various spectral bands used in remote sensing and their utility.

Based on the above discussion, it is clear that different features of habitat can be characterized well by the radiation types, the following table represents some spectral bands and their principal applications in habitat characterization. For a different type of habitats, different spectral signatures are used. Such as

Airborne instruments

Aviris	4,20 (m)	2,10	168-224	VNR, SWIR	on demand
CASI-2	5 (m)	1-2.5	48-288	Pan, VNR	on demand
ADAR-5500	0.5 - 3 (m)	1-2.5	4	VNR	on demand

India has been successfully launching satellites of many types since 1975. These satellites have been launched from various vehicles, including American, Russian and European rockets apart from Indian rockets. The organization responsible for India's space program is Indian Space Research Organisation (ISRO) and it shoulders the bulk of the responsibility of designing, building, launching and operating these satellites. The complete list of all India satellites is available at the official website of ISRO as well as Wikipedia. While this chapter was being written, India launched its first hyperspectral satellite HysIS using PSLVC43 (On Nov 29, 2018) and developed anti-satellite missiles (March 2019). The live data and traffic of various satellites over the sky above you can be viewed on the website, https://www.n2yo.com/, and you may track the movements of various satellites above you at any given time.

Practice Questions

1. Compare and contrast the complete count methods and incomplete count methods of estimating population density?

2. How the size of quadrat can be decided? Enlist some computational tools and algoriths for the determination of appropriate quadrat size for a field biology experiment

3. How does mean crowding affect the population estimations? Describe the mathematical formula used for determination of mean crowding in a population?

4. Ennumerate five differences between direct and indirect methods of population estimation with at least three examples from each category?

5. Explain the three common descriptors of remote sensing satellite, Spatial resolution, temporal resolution and spectral resolution?

6. Enlist five Indian and five international remote sensing satellites with multispectral resolution!

APPENDIX

1

Quick Summary

of Methods in Biology

Technique	Abb.	Commonly Used for	Comments
Molecular Biology Methods			
Polymerase chain reaction	PCR	Amplification of DNA	Very long DNA (>5 kb) cannot be amplified.
Restriction Fragment length polymorphism	RFLP	Fingerprinting, gene mapping	Not suitable for very little amount of DNA
Amplified Fragment length polymorphism	AFLP	Fingerprinting, gene mapping, MAS[1]	Additional primer sets needed
Rapid Amplification of Polymorphic DNA	RAPD[2]	Fingerprinting, gene mapping, MAS	Less reproducibility
Western Blot	WB	Detection of protein levels	Not suitable for high throughput analysis
Northern blot	NB	Detection of RNA levels	
Southern blot	SB	Detection of specific DNA	Hybridization probes needed
Eastern Blot	EB	Post translational modification detection	
Edman degradation	ED	Protein sequencing	Chemical method
Microarray	--	High throughput expression	Less reproducibility
DNA sequencing	--	Identification of DNA sequence	Sangers automated method is most common
Next generation sequencing	NGS	Sequencing as well as quantity analysis	Many variants available
Chromosome walking		Used to find, isolate, and clone a particular allele in a gene library	Used when clone is not known
Primer Walking		Method for sequencing of DNA fragments between 1.3 and 7 kilobases	Used when clone is known
Reverse transcription – PCR	RT-PCR	To convert RNA into c-DNA	Require reverse transcriptase
Real time PCR	--	To detect amplification of DNA in real time	

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	Often used for clinical diagnosis
Dot ELISA		Used for detection of antigen	Instead of plate, it is performed on membrane
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.	
Co-immunoprecipitation	Co-IP	Detection of Protein-Protein Interaction	
Chromatin immunoprecipitation	ChIP	Detection of DNA protein interactions	Sequence of DNA interacting is also determined
RNA-Immunoprecipitation	RIP	Detection of RNA-Protein interactions	
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 based on characteristics	Separation of stem cells from bone marrow
Fluorescent in situ hybridization	FISH	Detection of location of a gene in chromosome	Uses complementary labelled DNA
Genome in situ hybridization	GISH	Comparison of genomes of two organisms	Uses complementary labelled DNA
Biophysical Methods			
UV –vis spectroscopy	UV/vis	Detection of purity and concentration of biomolecules, enzyme kinetics	Based on electronic transitions
Circular dichroism Spectroscopy	CD	Detection of conformation of proteins, DNA, RNA and their dynamics	Based on circularly polarized light
Fluorescence spectroscopy	FS	Detection of protein dynamics in solvent, quantification of radicals	High sensitivity, good for assay in biological fluids, cell lysate
Infrared spectroscopy	IR-SP	Detection of chemical bonds in a molecule	Based on change in molecular vibrations
Raman spectroscopy	Raman	Detection of bond type and molecular structure using scattering of light	Based on molecular rotation using microwaves
Nuclear Magnetic Resonance (1 dimensional)	1D NMR	Identification of molecules, such as ingredients of a plant extract.	Atoms with even no of protons and neutrons are not visible in NMR
Nuclear Magnetic Resonance	2D NMR	Detection of structure of biomolecules in their solution	Most commonly used atoms are H^3 and C^{13}

(2 dimensional)		form using nuclear spin (using $1H^{3-}$)	
Electron Spin Resonance	ESR	Identification of free radicals, Lipid-protein interactions	Resonance caused due to electron g-factor.
X-ray diffraction	XRD	Detection of structures of biomolecules (DNA, RNA proteins in crystal state)	Solutions cannot be analyzed. Hydrogen atoms not visible.
Mass spectrometry	MS	Identification of organic molecules (plant extracts, drugs, proteins etc.)	Detection based on m/z ratio
Matrix Assisted Laser Desorption Ionization	MALDI	Variant of MS, used for Identification of a peptide from unknown mixture	Matrix is used to absorb extra high energy of Laser
Liquid Chromatography - Mass spectrometry	LC-MS	A variant of liquid chromatography with liquid chromatography based separation followed by MS.	Separation of proteins from a mixture occurs in machine itself.
Tandem MS	MS^n	MS of specific fragment (peak) again to confirm the peptide (also called as MS/MS)	Sequence of peptide can also be detected using Tandem MS
Surface Plasmon resonance	SPR	Used for the detection of ligand-receptor binding kinetics.	Rate of binding and dissociation can be detected,
Microscopic Methods			
Bright Field Microscopy	BFM	Used for visualizing stained slides (coloured samples)	Mainly used for histopathological analysis
Dark Field Microscopy	DFM	Used for visualizing transparent objects	Only object is illuminated
Phase contrast microscopy	PCM	Used for visualization of live cells (halos are a drawback)	Phase change in light enhances contrast of image
Differential interference contrast microscopy	DIC	Used for visualization of live cells (halos are a not observed)	Polarized light is used instead of normal light
Confocal microscopy	CFM	Used for high resolution florescence imaging. Localization of drug, proteins etc. can be done.	Spinning disc is used to scan the image. z- stacking is possible
Scanning electron micropscy	SEM	Used to visualize surface ultrastructure, cells, or larger specimen like eye of insect, nanomaterial can be studied	Based on detecting scattered electrons
Transmission electron microscopy	TEM	Used for the study of internal ultrastructure of the cells	Resolution upto 1A can be achieved
Cryo-EM	--	Used for detection of ultrastructure of biomolecules, eg. Protein structures	Samples analyzed in frozen state
Freeze-Fracture –Etch	FEM	Used for visualization between the two layers of a plasma membrane	Samples are cut with a sharp knife in frozen stage.
Electrophysiological Methods			
Patch Clamp method	--	Used for study of channels in plasma membrane , electrical activity of membranes	Several variants available based on principles

Local Field Potential	LFP	Used for detection of electrical activity of brain	electric potential recorded in the extracellular space in brain tissue
Electrocorticography	ECoG	Used for detection of electrical activity of brain	Recorded at the surface of the scalp, and with macro-electrodes. skin removed
Electroencephalography	ECG	Used for detection of electrical activity of brain	Recorded on the surface of scalp, non-invasive
Positron emission tomography imaging	PET	Used to image brain for metabolic activity - can detect tumors, cancer	uses trace amounts of short-lived radioactive material
Magnetic Resonance Imaging	MRI	Enables the detection of abnormalities of the brain	Based on phenomenon of nuclear magnetic resonance
Functional MRI	fMRI	Used to produce activation maps (metabolism based) showing brain parts involved in a particular mental process	Based on the uses the blood-oxygen-level dependent
Deep Brain Stimulation	DBS	Some parts of brain are stimulated using artificial electrodes to cure neurdegradative disorders	--
Electrocardiography	ECG	Used for the detection of cardiac cycle and abnormalities in heart.	
Field Biology Methods			
Capture recapture method		Most common method for estimating population	Lincon- Peterson index is used to quantitate.
Quadrant Sampling		Used for the sampling and detection of vegetation diversity in a community	Density , relative density and relative dominance can be detected
Line –Intercept analysis		Used for quantitative estimate of density of individuals area cover and frequency	May not be preferred in hilly terrain
Vertical incidence Sonar Technique		Used for detection for biodiversity on seafloor.	

Footnote

- [1]MAS – Marker assisted selection
- RAPD and AFLP are dominant markers and hence cannot differentiate between heterozygous and homozygous. RFLP is however, a co-dominant technique.

APPENDIX 2

Radioactive Isotopes

Commonly used in Life Sciences

S.No.	Radioisotope	Primary Application	Half Life	Emission
Therapeutic applications				
1	Chromium-51	Used to label red blood cells and quantify gastro-intestinal protein loss.	27.7 days	Gamma
2	Iodine -131	Used to diagnose and treat various diseases associated with the human thyroid	8.02 days	Gamma
3	Iodine -125	nuclear imaging tracers to evaluate the anatomic and physiologic function of the thyroid,	59 Years	Beta
4	Iridium -192	Supplied in wire form for use as an internal radiotherapy source for certain cancers, including those of the head and breast.	73.83 days	Beta
5	Molybdnum-99	Used as the 'parent' in a generator to produce technetium-99m, the most widely used radioisotope in nuclear medicine.	66 hours	Beta
6	Phosphorous-32	Used in the treatment of excess red blood cells.	14.28 days	Beta
7	Samarium- 153	Used to reduce the pain associated with bony metastases of primary tumors.	46.7 days	Beta
8	Technicium-99	Used to image the brain, thyroid, lungs, liver, spleen, kidney, gall bladder, skeleton, blood pool, bone marrow, heart blood pool, salivary and lacrimal glands, and to detect infection	6.01 hours	Beta
9	Yttrium -90	Used for liver cancer therapy.	64 hours	Beta
10	Colbalt-60	Radiotherapy for cancer (gamma knife)	5.27 years	Gamma
11	Carbon -14	Urea breath test for detection of helicobacter pylori infection	5730 years	Beta
PET imaging				
12	F-18 Glucose	Detection of malignant tumors (elevated glucose consumption) Brain activity, cardiac activity etc.	110 min	Gamma
SPECT imaging				
13	Technetium-99m	Bone scan, Myocardial scan, Patathyroid scan, Brain scan	6 hours	Gamma
14	Iodine-123 or Iodine-131	Neurological tumor scan	18 hours	Gamma
15	Indium-111 & Technetium-99m	WBCs scan	67 hours	Gamma
16	99mTc-HMPAO	Cerebral blood flow detection for dementia, Alzhimer's etc.	6 hours	Gamma

APPENDIX

3

Statistical Tables

Z-tables

As discussed in chapter D, section 5.1, z-tables are useful in determination of the location of a data point or area between two z-scores i.e. the percentage of sample coverage. The z score is calculated using following formula,

There are two type of z-score tables, one with negative values and other called positive z-score table with positive values. It may be noted that the probabilities given here in these tables for a specific z-score represent are to the left of z-score (percentage sample) while in order to obtain area to the right of the z-score we use the formula (1-probability for the area to the left of z-score)

Positive z-score table

This z-table (normal distribution table) shows the area to the right hand side of the curve. Use these values to find the area between z=0 and any positive value.

Z	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
0.0	0.0000	0.0040	0.0080	0.0120	0.0160	0.0199	0.0239	0.0279	0.0319	0.0359
0.1	0.0398	0.0438	0.0478	0.0517	0.0557	0.0596	0.0636	0.0675	0.0714	0.0753
0.2	0.0793	0.0832	0.0871	0.0910	0.0948	0.0987	0.1026	0.1064	0.1103	0.1141
0.3	0.1179	0.1217	0.1255	0.1293	0.1331	0.1368	0.1406	0.1443	0.1480	0.1517
0.4	0.1554	0.1591	0.1628	0.1664	0.1700	0.1736	0.1772	0.1808	0.1844	0.1879
0.5	0.1915	0.1950	0.1985	0.2019	0.2054	0.2088	0.2123	0.2157	0.2190	0.2224
0.6	0.2257	0.2291	0.2324	0.2357	0.2389	0.2422	0.2454	0.2486	0.2517	0.2549
0.7	0.2580	0.2611	0.2642	0.2673	0.2704	0.2734	0.2764	0.2794	0.2823	0.2852
0.8	0.2881	0.2910	0.2939	0.2967	0.2995	0.3023	0.3051	0.3078	0.3106	0.3133
0.9	0.3159	0.3186	0.3212	0.3238	0.3264	0.3289	0.3315	0.3340	0.3365	0.3389
1.0	0.3413	0.3438	0.3461	0.3485	0.3508	0.3531	0.3554	0.3577	0.3599	0.3621
1.1	0.3643	0.3665	0.3686	0.3708	0.3729	0.3749	0.3770	0.3790	0.3810	0.3830
1.2	0.3849	0.3869	0.3888	0.3907	0.3925	0.3944	0.3962	0.3980	0.3997	0.4015
1.3	0.4032	0.4049	0.4066	0.4082	0.4099	0.4115	0.4131	0.4147	0.4162	0.4177
1.4	0.4192	0.4207	0.4222	0.4236	0.4251	0.4265	0.4279	0.4292	0.4306	0.4319
1.5	0.4332	0.4345	0.4357	0.4370	0.4382	0.4394	0.4406	0.4418	0.4429	0.4441
1.6	0.4452	0.4463	0.4474	0.4484	0.4495	0.4505	0.4515	0.4525	0.4535	0.4545
1.7	0.4554	0.4564	0.4573	0.4582	0.4591	0.4599	0.4608	0.4616	0.4625	0.4633
1.8	0.4641	0.4649	0.4656	0.4664	0.4671	0.4678	0.4686	0.4693	0.4699	0.4706
1.9	0.4713	0.4719	0.4726	0.4732	0.4738	0.4744	0.4750	0.4756	0.4761	0.4767
2.0	0.4772	0.4778	0.4783	0.4788	0.4793	0.4798	0.4803	0.4808	0.4812	0.4817
2.1	0.4821	0.4826	0.4830	0.4834	0.4838	0.4842	0.4846	0.4850	0.4854	0.4857
2.2	0.4861	0.4864	0.4868	0.4871	0.4875	0.4878	0.4881	0.4884	0.4887	0.4890
2.3	0.4893	0.4896	0.4898	0.4901	0.4904	0.4906	0.4909	0.4911	0.4913	0.4916
2.4	0.4918	0.4920	0.4922	0.4925	0.4927	0.4929	0.4931	0.4932	0.4934	0.4936
2.5	0.4938	0.4940	0.4941	0.4943	0.4945	0.4946	0.4948	0.4949	0.4951	0.4952
2.6	0.4953	0.4955	0.4956	0.4957	0.4959	0.4960	0.4961	0.4962	0.4963	0.4964
2.7	0.4965	0.4966	0.4967	0.4968	0.4969	0.4970	0.4971	0.4972	0.4973	0.4974
2.8	0.4974	0.4975	0.4976	0.4977	0.4977	0.4978	0.4979	0.4979	0.4980	0.4981
2.9	0.4981	0.4982	0.4982	0.4983	0.4984	0.4984	0.4985	0.4985	0.4986	0.4986
3.0	0.4987	0.4987	0.4987	0.4988	0.4988	0.4989	0.4989	0.4989	0.4990	0.4990
3.1	0.4990	0.4991	0.4991	0.4991	0.4992	0.4992	0.4992	0.4992	0.4993	0.4993
3.2	0.4993	0.4993	0.4994	0.4994	0.4994	0.4994	0.4994	0.4995	0.4995	0.4995
3.3	0.4995	0.4995	0.4995	0.4996	0.4996	0.4996	0.4996	0.4996	0.4996	0.4997
3.4	0.4997	0.4997	0.4997	0.4997	0.4997	0.4997	0.4997	0.4997	0.4997	0.4998
3.5	0.4998	0.4998	0.4998	0.4998	0.4998	0.4998	0.4998	0.4998	0.4998	0.4998
3.6	0.4998	0.4998	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999
3.7	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999
3.8	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999	0.4999

APPENDIX

4

Centrifugation

Principles and Applications

1. Introduction

Centrifugation is a technique which involves the application of centrifugal force to separate particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed. This process is used to separate two miscible substances, but also to analyze the hydrodynamic properties of macromolecules. The pioneering work on establishment of centrifugation was done by Theodor Svedberg and his student H. Rinde in 1923, when they successfully analyzed large-grained sols in terms of their gravitational sedimentation. Svedberg developed an analytical centrifuge, equipped with a photographic absorption system, and developed the theory necessary to measure molecular weight. By 1900, when it was clearly understood that proteins were composed of amino acids and the hemoglobin was already studied, its precise molecular weight (68 kDa) calculation were confirmed by centrifugation technique through a series of experiments utilizing the sedimentation equilibrium, suggesting that there are four iron atoms present rather than one. In order to further investigate this phenomenon, a centrifuge with even higher speeds was needed, and thus the ultracentrifuge was created to apply the theory of sedimentation-diffusion. The development of centrifugation was a great advance in experimental protein science. Biological centrifugation is a process that uses centrifugal force to separate and purify mixtures of biological (or even non-biological) particles in a liquid medium.

In biology, it is a key technique for isolating and analyzing cells, subcellular fractions, supra-molecular complexes and isolated macromolecules such as proteins or nucleic acids. Centrifugation techniques represent a critical tool for modern biochemistry and are employed in almost all invasive sub-cellular studies.
Centrifugation is mainly employed in

- The determination of the purity of macromolecules;
- The determination of the relative molecular mass of solutes in their native state;
- The examination of changes in the molecular mass of supra-molecular complexes;
- The detection of conformational changes
- Ligand-binding studies

2. Physics of Centrifugation

Any particle, whether a precipitate, macromolecule, or cell organelle, is subjected to a gravitational pull and therefore gradually progress towards sedimentation in any liquid medium. However, the sedimentation is resisted by two opposing forces buoyancy and friction. Also the constant Brownian motion of particles prevent the sedimentation in all the cases. Hence, under the conditions when the magnitude of gravitational pull exceeds the magnitude of these two forces particles, begin to sediment. As, gravitational pull is not enough to sediment all type of particles, therefore an increment in this force is made by augmenting it with centrifugal force, generated by spinning the samples. This augmentation of gravity or facilitating the sedimentation by rotating a solution is known as centrifugation.

The **centrifugal acceleration 'G'** is given by $G = \omega^2 r$
Therefore the **centrifugal force is F** = $m \omega^2 r$
Where
ω = angular velocity
r = distance of particle from the axis of rotation
m = mass of the particle

When a particle migrates into solution, the net centrifugal force on the particle is reduced due to buoyancy of the particle and frictional force.
The buoyant force is given by relation:

$F = = m_oVd$

Where,
m = solvent displaced by a particle in solution
V = partial specific volume; the volume change occurring when a particle is placed in a large excess or solvent
d = density of the solvent or medium

Additionally, as particles sediment under the influence of the centrifugal field, their movement is countered by a resistance force, the **frictional force.** The frictional force is defined by Equation.

Frictional force = fv

Where,
f = frictional coefficient
v = velocity of the sedimenting particle (sedimentation velocity)

The **frictional coefficient,** depends on the size and shape of the particle, as well as viscosity of the solvent. The frictional force increases with the velocity of the particle until a constant velocity is reached. At this point, the two forces are balanced.

1.1 Svedberg unit: A measure of sedimentation

The sedimentation speed v_t is also known as the terminal velocity. Terminal velocity is the ratio of centrifugal force and the viscous drag. It is constant because the force applied to a particle by gravity or by a centrifuge is cancelled by the viscous resistance of the medium (normally water) through which the particle is moving. The two opposing forces that work to bring sedimentation equilibrium are downward centrifugal force (or gravity if no allowed to settle under gravity) and upward viscous drag (and bouncy).

Centrifugal force = $mr\omega^2$
Here, m is the mass of particle, r is radius of the particle and □□s the angular velocity.

Viscous force = $6\pi\eta rv$
Here, η is the viscosity of the medium, r is the radius of the particle and v is the velocity of the particle. This law applies only for large spheres in an infinite sea of the fluid.

The ratio therefore becomes terminal velocity or sedimentation velocity (v_t)

$v_t = (mr\omega^2)/ 6\pi\eta rv$

Now, another important parameter called sedimentation coefficient (S) is defined as the ratio of a particle's sedimentation velocity (v_t) as calculated above to the acceleration that is applied to it (causing the sedimentation), i.e. s = v/a , where velocity is terminal velocity and a is centrifugal acceleration ($mr\omega^2$).Hence S is given by

$S = v_t / mr\omega^2$, on placing the value of vt from above equation we get

$S = m / 6\pi\eta rv$

The term **S** is most often defined under standard conditions, 20° C and water as the medium, and denoted by S_{20}. The S value is a physical characteristic used to classify biological macromolecules and cell organelles. Sedimentation coefficients are in the range 1 x 10^{-13} to 10,000 x 10^{-13}. For numerical convenience, sedimentation coefficients are expressed in **Svedberg units, S.**

1 S= 1 x 10^{-13} second.

When designing a centrifugation protocol, it is important to keep in mind that:

APPENDIX 5

Chromatography

Fundamental Principles

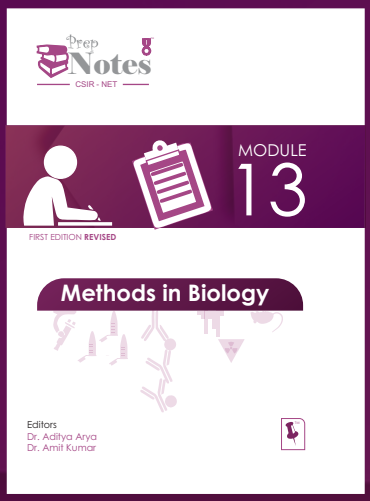
1. Introduction

Chromatography (derived from Greek word chroma which means "color" and graphein "to write") is the collective term for a set of laboratory techniques for the separation of mixtures. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on adsorption, differential partitioning, size, affinity or ion exchange between the mobile and stationary phases. Subtle differences in a compound's partition coefficient, charge, size or affinity result in differential retention on the stationary phase and thus changing the separation. Modern chromatography originated in the late nineteenth and early twentieth centuries from independent work by David T. Day, a distinguished America geologist and mining engineer, and Mikhail Tsvet, a Russian botanist. Day developed procedures for fractionating crude petroleum by passing it through Fuller's earth, and Tsvet used a column packed with chalk to separate leaf pigments into colored bands. Tsvet continued to work with chromatography in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll, carotenes, and xanthophylls. Since these components have different colors (green, orange, and yellow, respectively) they gave the technique its name. Because Tsvet recognized and correctly interpreted the chromatographic processes and named the phenomenon chromatography, he is generally credited with its discovery. New types of chromatography developed during the 1930s and 1940s made the technique useful for many separation processes.

1.1 Common terms in chromatography

- **A chromatograph** is equipment that enables a sophisticated separation, e.g. gas chromatographic or liquid chromatographic separation.
- **Chromatography** is a physical method of separation that distributes components to separate between two phases, one stationary (stationary phase), the other (the mo phase) moving in a definite direction.
- **The eluate** is the mobile phase leaving the column.
- **The eluent** is the solvent that carries the analyte.
- **An eluotropic** series is a list of solvents ranked according to their eluting power.
- **An immobilized phase** is a stationary phase that is immobilized on the support particles, or on the inner wall of the column tubing.
- **The mobile phase** is the phase that moves in a definite direction. It may be a liquid (LC and Capillary Electrochromatography (CEC)),
- a gas (GC), or a supercritical fluid (supercritical-fluid chromatography, SFC). The mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column.
- **The retention time** is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.
- The sample is the matter analyzed in chromatography. It may consist of a single component or it may be a mixture of components.
- **The solute** refers to the sample components in partition chromatography.

About The Book



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