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Biophysics and Molecular Biology

Tools and Techniques

Pranav Kumar

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Fourth edition

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Former faculty,
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Preface

The field of biophysical and molecular biology continues to be one of the most exciting and dynamic areas of science. Over the past few decades, the spectacular progress in this field has occurred due to the conceptual synthesis of ideas from biology, physics, chemistry, mathematics, statistics and computer science.

This textbook has the primary goal to teach students about theoretical principles and applications of the key biophysical and molecular methods used in biochemistry and molecular biology. I have tried to present the subject from a conceptual perspective. A substantial theoretical basis has been covered to understand key experimental techniques so that students can make appropriate choices and efficient use of techniques. There have been a number of major advances in molecular biology in the past few years. But I have covered selected topics that provide the basic principles for understanding the structure and functional relationships of molecular biology.

The most significant feature of this book is its clear, up-to-date and accurate explanations of mechanisms, rather than the mere description of facts and events. The question of what to include and what to omit is, for today's authors, crucially important. With such a broad array of potential topics and techniques available, it is difficult to select those that students should experience and master. However, there are techniques and concepts that most of us would agree form a 'core' in biophysics and molecular biology. I have tried to resist the temptation to describe more and more techniques, adding detail but not increasing understanding of the basic concepts. I hope that this text book will prove useful both to teachers and students. Finally, I have provided a concise list of selected references (research papers, reviews and books) so that curious readers can trace fundamentals and ideas to their roots. These are arranged in alphabetical order.

Although the chapters of this book can be read independently of one another, they are arranged in a logical sequence. Each page is carefully laid out to place related text, figures and tables near one another, minimizing the need for page turning while reading a topic. I have given equal importance to text and illustrations as well.

Acknowledgements

There is an old proverb that says that you never really learn a subject until you teach it. We now know that you learn a subject even better when you write about it. Preparing this text has provided me with a wonderful opportunity to share my knowledge with students. Thanks go first and foremost to our students. In preparing this book, I have relied heavily on and benefited greatly from the advice and constructive criticism of numerous colleagues. I am particularly grateful to Ajay Kumar for his enthusiastic editing of the complete manuscript. I would also like to thank Prakash Vardhan and Harleen Kaur for invaluable contribution. This book is a team effort, and producing it would be impossible without the outstanding people of Pathfinder Publication. It was a pleasure to work with many other dedicated and creative people of Pathfinder Publication during the production of this book, especially Pradeep Verma.

Reviewers

The followings, in alphabetical order, have reviewed one or more chapters, correcting errors of fact or interpretation and helping to ensure they have the appropriate balance and emphasis. Their thoughtful comments, suggestions, and encouragement have been of immense help to me in writing this book.

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Contents

Biophysics

Molecular Biology

Units, Conversion factors and Physical constants

Base units

Length	meter (m)
Mass	kilogram (kg)
Time	second (s)
Electric current	ampere (A)
Temperature	kelvin (K)
Amount of substance	mole (mol)
Luminous intensity	candela (cd)

Conversion factors

Multiplication factor	Prefix	Symbol
10^{12}	tera	T
10^{9}	giga	G
10^{6}	mega	M
10^{3}	kilo	k
10^{2}	hecto	h
10	deka	da
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f

Length units

Millimeters (mm)	Centimeters (cm)	Meters (m)	Kilometers (km)
1	0.1	0.001	0.000001
10	1	0.01	0.00001
1000	100	1	0.001
1000000	100000	1000	1

Volume units

Cubic centimeter (cm^3)	Cubic meter (m^3)	Liter (ltr)
1	0.000001	0.001
1000000	1	1000
1000	0.001	1

Conversion units

1 revolution = 2π radians = 360 degrees

1 degree = 60 minutes

π radians = 3.1416 radians = 180 degrees

1 Hz = 1 cycle/sec.

1 rpm (revolutions per minute) = 60 rps (revolutions per second) = 60Hz

1 Hz (Hertz) = 1 s^{-1}

1 N (Newton) = 1 kgm/s^2 = 100,000 dyne

1 dyne = 10^{-5} Newton

1 Pascal (Pa) = 1 N/m^2 = 6.895 kPa

1 atm (metric atmosphere) = 760 mm Hg at 0°C = 1.0132×10^5 N/m^2

1 microbar = 0.1 N/m^2

1 angstrom (Å) = 10^{-10} m

Physical constants

Ideal gas law constant (R) = 1.987 cal/mole K

Boltzmann's constant (K) = 1.3×10^{-16} erg/K = 1.3×10^{-23} J/K

Planck's constant (h) = 6.62×10^{-27} erg-sec = 6.62×10^{-34} J.sec

Avogadro's number = 6.02×10^{23} mol^{-1}

Density of water = 1 g/cm^3

Electron charge = 1.60×10^{-19} coulombs

Electron rest mass = 9.11×10^{-31} kg

Proton rest mass = 1.67×10^{-27} kg

Speed of light (c) = 3.00×10^8 m/sec

Gravitational constant (G) = 6.67×10^{-11} Nm^2/kg^2

Acceleration due to gravity (g) = 9.8 m/s^2

Chapter 1

Chromatography

Chromatography is a physical method for separation of compounds. Tswett, Russian botanist (referred as the *father of chromatography*) is credited for the development of chromatography. He employed the technique to separate various plant pigments such as chlorophylls and xanthophylls by passing solutions of these compounds through a glass column packed with finely divided calcium carbonate. The separated species appeared as colored bands on the column, which accounts for the name he chose for the method (Greek *chroma* meaning 'color' and *graphein* meaning 'writing').

Chromatography is based on a very simple principle. The sample to be examined (called the *solute* or *analyte*) is allowed to interact with two immiscible phases – a **mobile phase** and a **stationary phase**. The two immiscible phases could be a solid and a liquid, or a gas and a liquid or a liquid and another liquid. The stationary phase (i.e. *sorbent*), which may be a solid or a liquid supported on a solid, does not move. The mobile phase moves the sample through the stationary phase. The mobile phase may be a *liquid* (**liquid chromatography**) or a *gas* (**gas chromatography**). All chromatographic methods involve passing a mobile phase through a stationary (immobile) phase. The two phases are chosen so that the components of the sample distribute themselves between the mobile and stationary phases to varying degrees.

The **International Union of Pure and Applied Chemistry** (IUPAC) has defined chromatography as: A method, used primarily for separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while the other moves. The stationary phase may be a solid, or a liquid supported on a solid, or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc.; in these definitions chromatographic bed is used as a general term to denote any of the different forms in which the stationary phase may be used. The mobile phase may be gas or liquid.

Classification of chromatographic methods

Chromatographic methods can be classified in three fundamental ways:

- Based on the shape of the chromatographic bed.
- Based on the physical state of the mobile and stationary phases.
- Based on the mechanism of separation.

Based on the shape of the chromatographic bed

Based on the shape of the chromatographic bed, there are two types of chromatography — *planar* chromatography and *column* chromatography.

In **planar chromatography**, the stationary phase is spread on a flat, planar surface. The plane can be a paper impregnated by a substance acting as a stationary phase (**paper chromatography, PC**) or a thin layer of a substance acting as a stationary phase spread on a glass, metal or plastic plate (**thin layer chromatography, TLC**). Planar chromatography is also termed **open-bed chromatography**. In thin layer chromatography, the stationary phase is a thin layer of silica gel or alumina on a glass, metal or plastic plate. Most commonly, silica gel is used

It is the partition coefficient, σ, which describes how much of the internal volume is available for the solute ($0 < \sigma < 1$). When σ is compared with the values measured for solutes of known size, it provides information about the molecular size of an unknown solute. If a series of solutes of known size is subjected to size exclusion chromatography, a linear relationship between partition coefficient and size is observed.

1.3 Ion exchange chromatography

Ion exchange chromatography is applicable for the separation of charged molecules. In this chromatographic technique, ionic solutes display reversible electrostatic interactions with a charged stationary phase. The stationary solid phase commonly consists of an insoluble matrix with covalently attached anions or cations (called **ion exchangers**). Solutes entering the column may be negatively charged, positively charged, or neutral under the experimental conditions. Solute ions of the opposite charge in the mobile liquid phase bind reversibly to the ion exchanger by electrostatic interactions. The strength of interactions depends on the size of the charge and the charge density (amount of charge per unit volume) of the solute. The greater the charge or the charge density, the stronger the interaction. Neutral solutes show little or no affinity for the stationary phase and move with the eluting buffer. The bound solutes can be released by eluting the column with a buffer of increased ionic strength or pH. An increase in buffer ionic strength releases bound solutes by displacement. Increasing the buffer pH decreases the strength of the interaction by reducing the charge on the solute or on the resin.

Ion exchanger

Ion exchangers are made up of two parts – an insoluble matrix and chemically bonded charged groups within and on the surface of the matrix. An ion exchanger is classified as *cationic* or *anionic* depending on whether it exchanges cations or anions.

Cation exchanger (also called *acidic ion exchanger*): It is used for cation separation.

Anion exchanger (also called *basic ion exchanger*): It is used for anion separation.

Each type of exchanger is also classified as *strong* or *weak* according to the ionizing strength of the functional group. An exchanger with a quaternary amino group is, therefore, a strongly basic anion exchanger, whereas primary or secondary aromatic or aliphatic amino groups would lead to a weakly basic anion exchanger. A strongly acidic cation exchanger contains the sulfonic acid group.

Table 1.2 Commonly used ion exchangers

Name	Type	Functional group
Anion exchanger		
DEAE-cellulose	Weakly basic	Diethylaminoethyl (DEAE)
QAE-Sephadex	Strongly basic	Quaternary aminoethyl (QAE)
Q-Sepharose	Strongly basic	Quaternary ammonium (Q)
Cation exchanger		
CM-cellulose	Weakly acidic	Carboxymethyl (CM)
SP-Sepharose	Strongly acidic	Sulfopropyl (SP)
SOURCE S	Strongly acidic	Methylsulphate (S)

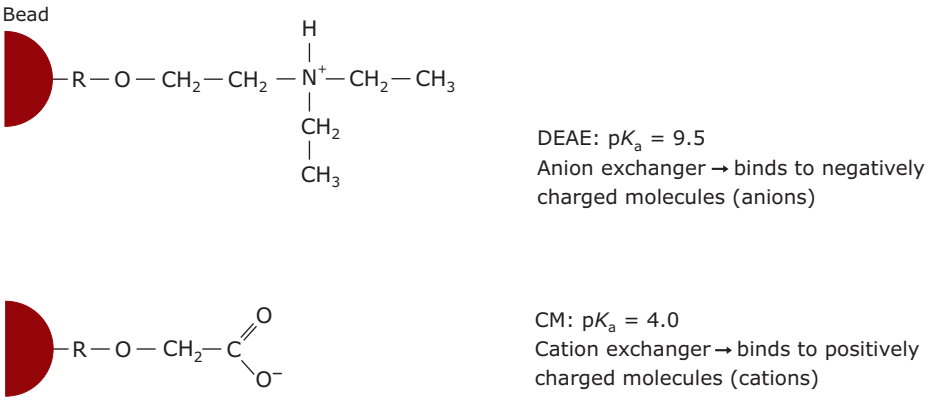


Figure 1.3 Ion exchangers – diethylaminoethyl (DEAE) and carboxymethyl (CM). The positive charge of DEAE attracts negatively charged molecules. CM is suitable for binding with positively charged molecules.

A solute in a given sample, which has the opposite charge to that of the charged group of the ion exchanger binds to the column. Separation of charged solutes occurs because different solutes have different degree of interaction with the ion-exchanger due to differences in their charges, charge density and distribution of charge on their surfaces. If a solute has a net positive charge at pH 7, it will usually bind to ion exchanger (cation exchanger) containing carboxylate groups, whereas a negatively charged protein will not.

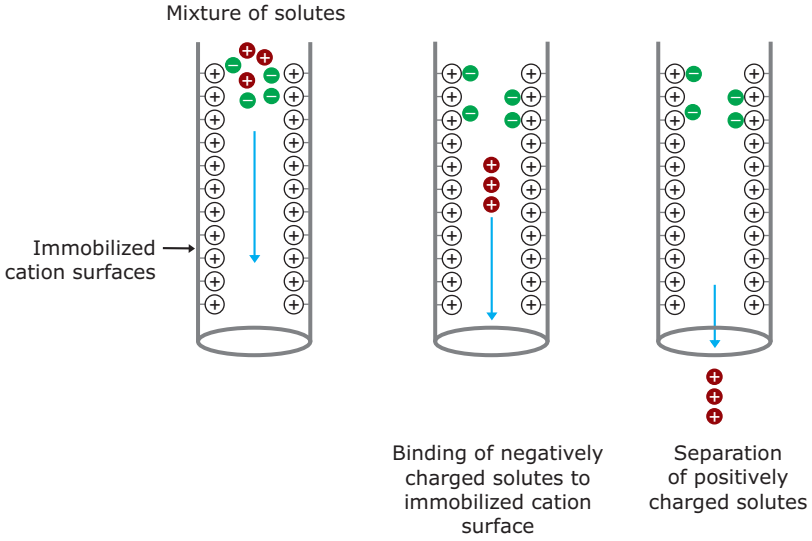


Figure 1.4 Column materials used for ion exchange chromatography contain charged groups covalently linked to the surface of an insoluble matrix. The charged groups of the matrix can be positively or negatively charged. Anion exchangers have positively charged groups that will attract negatively charged anions. When a mixture of solutes is loaded into the anion exchanger, negatively charged solutes bind to the exchanger.

The bound molecules can be eluted by altering the pH of the eluting buffer or by increasing the salt concentration of the eluting buffer. A positively charged protein bound to cation exchanger can be eluted by increasing the salt concentration in the eluting buffer because cations present in the buffer compete with positively charged groups on the protein for binding to the ion exchanger. Proteins that have a low density of net positive charge will tend to emerge first, followed by those having a higher charge density.

Selection of the ion exchanger

Before a proper choice of ion exchanger can be made, the nature of the solutes to be separated must be considered. The choice of ion exchanger (whether to use a cationic or anionic exchanger) for the purification of a biomolecule largely depends on the isoelectric point, pI, of the biomolecule.

If the solute molecule has only one type of charged group, the choice is simple. A solute that has a positive charge will bind to a cationic exchanger and vice versa. However, many solutes

have more than one type of ionizing group and may have both negatively and positively charged groups. The net charge on such molecules depends on pH. At the isoelectric point, the solute has no net charge and would not bind to any type of ion exchanger. At a pH value above the pI of a solute, it will have a net negative charge and adsorb to an anion exchanger. Below the pI, the solute has a net positive charge and will adsorb to a cation exchanger.

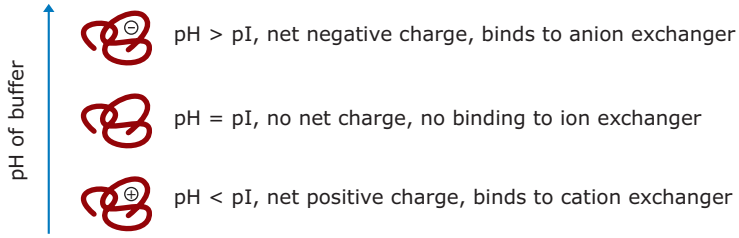


Figure 1.5 The net charge on a protein is influenced by the pH of its solvent. At pH=pI, the protein has zero net charge and, therefore, will not bind to a cation exchanger or an anion exchanger. Adjusting the pH above or below the pI of the protein will lead to a net charge and protein binding to either an anion exchanger (pH > pI) or a cation exchanger (pH < pI).

In principle, solutes with both negatively and positively charged groups should bind to both anionic and cationic exchangers. However, when one is dealing with large biomolecules, the pH *range of stability* must also be evaluated. The range of stability refers to the pH range in which the biomolecule is not denatured. For example, if the pI of a protein is 4 then, in most cases, it is advisable to choose an ion exchanger which binds to the protein at a pH > 4. Since at pH > 4 this protein is negatively charged, the ion exchanger has to be an anion exchanger, e.g. DEAE. One could also use a pH < 4 and a cation exchanger, but many proteins are not stable or aggregate under these conditions. If, in contrast, the protein we want to purify has a pI = 10, it is positively charged at a pH around 7. Thus, in general for this protein type we have to choose a cation exchanger, which is negatively charged at neutral pH.

Ion exchange chromatography can also be used to separate DNA from a cell extract. It is based on the interaction between anion exchanger (DEAE) and negatively charged phosphates of the DNA backbone. The anion-exchange resin consists of silica beads with a high charge density. When the cell extract passes through the column, all the negatively charged molecules bind to the resin and retained in the column. If the salt solution of gradually increasing concentration is passed through the column, the different types of molecule will elute in the sequence protein, RNA and finally DNA.

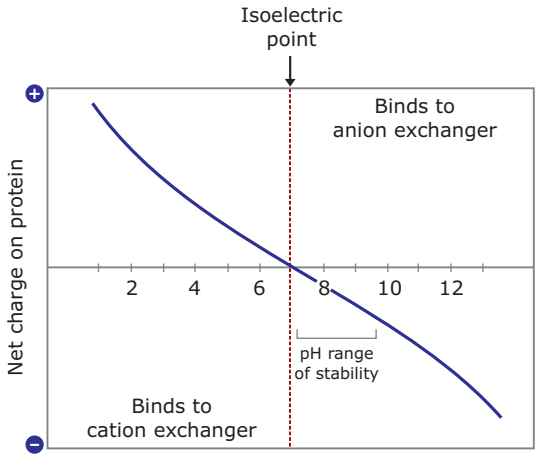


Figure 1.6 Diagram shows how the net charge of a hypothetical protein changes as a function of pH. Below the isoelectric point, the molecule has a net positive charge and would be bound to a cation exchanger. Above the isoelectric point, the net charge is negative, and the protein would bind to an anion exchanger. Superimposed on this graph is the pH range of stability for the hypothetical protein. The range of stability refers to the pH range in which the biomolecule is not denatured. Because it is stable in the range of pH 7.0-9.0, the ion exchanger of choice is an anionic exchanger. In most cases, the isoelectric point of the protein is not known. The type of ion exchanger must be chosen by trial and error.

In most cases, the isoelectric point of the protein is not known. The type of ion exchanger must be chosen by trial and error as follows. Small samples of the solute mixture in buffer are equilibrated for 10 to 15 minutes in separate test tubes, one with each type of ion exchanger. The tubes are then centrifuged or let stand to sediment the ion exchanger. Check each supernatant for the presence of solute. If a supernatant has a relatively low level of added solute, that ion exchanger would be suitable for use. This simple test can also be extended to find conditions for elution of the desired macromolecule from the ion exchanger. The ion exchanger charged with the macromolecule is treated with buffers of increasing ionic strength or changing pH. The supernatant after each treatment is analyzed as before for release of the macromolecule.

Choice of buffer

This decision includes not just the buffer substance but also the pH and the ionic strength. Buffer ions will, of course, interact with ion-exchange resins. Buffer ions with a charge opposite to that on the ion exchanger compete with solute for binding sites and greatly reduce the capacity of the column. Cationic buffers should be used with anionic exchangers; anionic buffers should be used with cationic exchangers.

The pH chosen for the buffer depends first of all on the range of stability of the macromolecule to be separated. Second, the buffer pH should be chosen so that the desired macromolecule will bind to the ion exchanger. In addition, the ionic strength should be relatively low to avoid damping of the interaction between solute and ion exchanger. Buffer concentrations in the range 0.05 to 0.1 M are recommended.

Problem

Suppose, you have determined that your partially purified protein is stable and active between pH 5 and pH 7.5. On either side of that pH range, the protein is no longer active. Now, you want to do a quick experiment to determine proper pH to do the ion-exchange chromatography. You mixed a bit of the crude preparation with a small amount of the ion-exchange resin DEAE-Sepharose in a series of buffer solutions that have a pH between 5 and 7.5. Next, you pelleted the resin and assayed the supernatant for the presence of your protein. Finally, you chosen this information to pick the appropriate pH to do the ion-exchange chromatography. You have completed the first two steps and have obtained the results shown in figure 1.7.

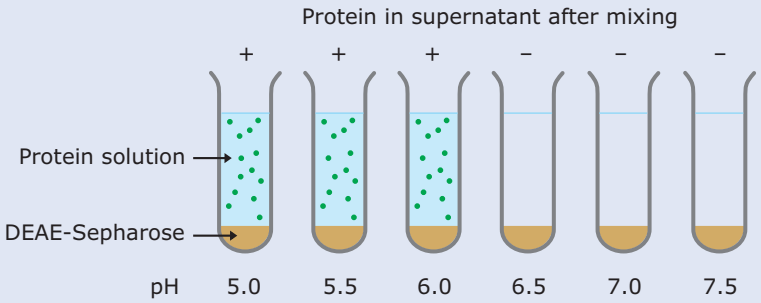


Figure 1.7 Samples of the protein were mixed with DEAE-Sepharose beads in buffers at a range of pH values and then the mixtures were centrifuged to pellet the beads. The presence of the protein in the supernatant is indicated by (+) and its absence is indicated by (–).

a. For the chromatography, should you pick a pH at which the protein binds to the beads (pH 6.5 to 7.5) or a pH where it does not bind (pH 5 to 6)?

b. Should you pick a pH close to the boundary (i.e. pH 6 or 6.5) or far away from the boundary (i.e. pH 5 or pH 7.5)?

Solution

a. You want to pick a pH at which your protein binds to the DEAE-Sepharose beads. If it does not bind to the beads, the protein will pass through the column with all the other proteins that do not bind. Ion-exchange chromatography is carried out under conditions in which the protein interacts with the beads.

b. In general, you want to pick a pH at which the protein of interest binds to the column, but does not bind too strongly. Thus, pH 6.5 would be the best choice for your initial studies. If you pick too high a pH, the protein may bind to the column too strongly, requiring harsh conditions to remove it.

1.4 Affinity chromatography

Affinity chromatography is a technique enabling purification of a biomolecule with respect to biological function or individual chemical structure. The substance to be purified is specifically and reversibly adsorbed to a *ligand* (binding substance), immobilized by a covalent bond to a chromatographic bed material (i.e. matrix). Samples are applied under favourable conditions for their specific binding to the ligand. Substances of interest are consequently bound to the ligand while unbound substances are washed away. Recovery of substances of interest can be achieved by changing experimental conditions to favour desorption. The operation of affinity chromatography involves the following steps:

- Choice of an appropriate ligand.
- Immobilization of the ligand onto a support matrix.
- Binding of the molecules of interest with the ligand.
- Removal of non-specifically bound molecules.
- Elution of the molecules of interest in a purified form.

A biospecific ligand that can be attached to a chromatography matrix covalently is one of the requirements for successful affinity purification. The binding between the ligand and molecules of interest must be reversible to allow the molecules to be removed in an active form. After washing away the contaminants, the coupled ligand must retain its specific binding affinity for the molecules of interest. Some examples of types of ligands that are usually used in affinity chromatography are listed in table 1.3.

Table 1.3 Typical biological interactions used in affinity chromatography

Types of ligand	**Target molecules** or **molecules of interest**
Enzyme	Substrate analogue, inhibitor, cofactor
Antibody	Antigen
Lectin	Polysaccharide, glycoprotein, cell surface receptor, cell
Nucleic acid	Complementary base sequence, nucleic acid binding protein
Avidin	Biotin
Calmodulin	Calmodulin-binding molecule
Poly(A)	RNA containing poly(U) sequences
Glutathione	Glutathione-S-transferase or GST fusion proteins
Proteins A and G	Immunoglobulins

Chapter 2

Electrophoresis

Electrophoresis (*Electro* refers to the energy of electricity and *Phoresis*, from the Greek verb *phoros*, means to carry across) is a technique for separating or resolving charged molecules (such as amino acids, peptides, proteins, nucleotides, and nucleic acids) in a mixture under the influence of an applied electric field. Charged molecules in an electric field move or migrate, at a speed determined by their *charge to mass* ratio. According to the laws of electrostatics, an ion with charge 'Q' in an electric field of strength 'E' will experience an electric force, $F_{electrical}$

$$F_{electrical} = Q.E$$

The resulting migration of the charged molecule through the solution is opposed by a frictional force, $F_{frictional}$

$$F_{frictional} = V.f$$

where, V is the rate of migration of charged molecule and f is its *frictional coefficient*

The **frictional coefficient** measures the resistance encountered by the molecule in moving through the solvent. It depends on the size and shape of the migrating molecule and the viscosity of the medium. For a spherical molecule, it is given by the Stokes' law;

$$f = 6\pi\eta r$$

where, η is the viscosity of the solvent and r is the radius of the molecule

In the constant electric field, the force on charged molecule balances each other;

$$Q.E = V.f$$

so that each charged molecule moves with a constant characteristic velocity.

The migration of the charged molecule in the electric field is generally expressed in terms of **electrophoretic mobility** (μ), which is the ratio of the migration rate of a charged molecule to the applied electric field:

$$\mu = \frac{V}{E} = \frac{Q}{f}$$

The SI unit of electrophoretic mobility is $m^2\ s^{-1}\ V^{-1}$.

So according to the above equation, electrophoretic mobility is directly proportional to the charge and inversely proportional to the viscosity of the medium, size and shape of the molecule.

Electrophoresis is of two types – *moving boundary* (or free boundary) *electrophoresis* and *zone electrophoresis.*

Moving boundary electrophoresis is the electrophoresis in a free solution, without a supporting media. It was developed by Tiselius in 1937. To separate the different charged molecules present in a mixture, the sample (dissolved in a buffer solution that serves as an electrolyte and maintains the desired pH) is placed in a glass tube connected to electrodes. When an electrical potential is applied across the tube, the charged molecules migrate toward one or the other electrode. Because different charged molecules migrate at different rates, a number of *interfaces* or *boundaries* are formed between the leading edge of each charged molecules and the remaining mixture.

In **zone electrophoresis**, a sample is constrained to move in some kind of inert matrix such as filter paper moistened with buffer (**paper electrophoresis**) or a gel (**gel electrophoresis**). A matrix is required because the electric current passing through the electrophoresis solution generates heat, which causes diffusion and convective mixing of the bands in the absence of a supporting matrix. The kind of supporting matrix used depends on the type of molecules to be separated and on the desired basis for separation.

2.1 Gel electrophoresis

Gel electrophoresis is a simple, rapid and sensitive analytical tool for separating charged molecules. Under the influence of an electrical field charged molecules migrate in the direction of the electrode bearing the opposite charge. Most biomolecules carry a net charge at any pH other than at their isoelectric point. These charged biomolecules have different electrophoretic mobilities due to their differences in charge, shape and size. In gel electrophoresis, gel serves as porous matrix and behave like a molecular sieve. Typically, a gel matrix provides resistance to the migration of the charged molecules that are being subjected to the electrical force. There are two basic types of materials used to make gels: *agarose* and *polyacrylamide*.

Agarose is a natural colloid extracted from seaweed. It is a linear polysaccharide made up of the basic repeating unit **agarobiose**, which comprises alternating units of *galactose* and *3,6-anhydrogalactose*. Agarose gels have a relatively large pore size and are used primarily to separate larger molecules with a molecular mass greater than 200 kDa. Agarose is usually used at concentrations between 1% and 3%. Agarose gels are used for the electrophoresis of both proteins and nucleic acids. Because the pores of an agarose gel are large, agarose is used to separate macromolecules such as nucleic acids, large proteins and protein complexes.

In gel electrophoresis separation of charged molecules depends on:

- Size, shape and charge of the molecules
- Properties of the gel
- Strength of the electric field
- Features of the buffer
- Temperature

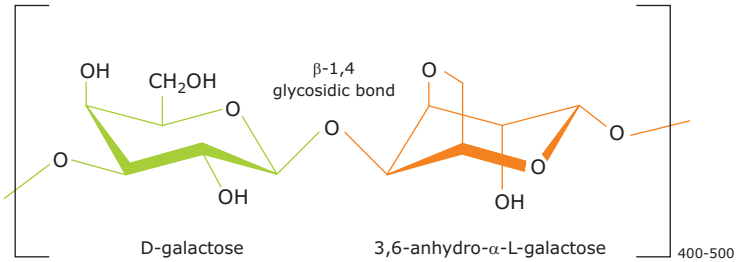


Figure 2.1 Agarose is a linear polysaccharide made up of the basic repeat unit *agarobiose*, which comprises alternating units of galactose and 3,6-anhydro-α-L-galactose.

In discontinuous PAGE, discontinuity is based on four parameters:

- the gel structure
- the pH value of the buffer
- the ionic strength of the buffer
- the nature of the ions in the gel and in the electrode buffer

Discontinuous PAGE can be native or denaturing. In discontinuous native PAGE, proteins are prepared in nonreducing, nondenaturing sample buffer, and electrophoresis is also performed in the absence of denaturing and reducing agents. In discontinuous denaturing PAGE, electrophoresis is performed in the presence of denaturing detergent sodium dodecyl sulfate (**SDS**), called **SDS-PAGE**.

2.2.1 SDS–PAGE

Electrophoretic separation of proteins is most commonly performed in polyacrylamide gels. The relative movement of proteins through a polyacrylamide gel depends on the *charge density* (charge per unit of mass), *mass* (or size) and *shape* of the molecules. If two proteins have the same mass and shape, the one with the greater charge density will move faster through the gel. Similarly, if two proteins having the same charge density and shape, the one of smaller mass or size will migrate faster than the large size protein. The shape is also a factor because compact globular proteins move more rapidly than elongated fibrous proteins of comparable mass.

In **SDS-PAGE**, proteins are treated with the negatively charged anionic detergent **sodium dodecyl sulfate** (also called *sodium lauryl sulfate*) before and during gel electrophoresis. SDS imparts a large net negative charge on protein. Proteins treated with SDS have similar charge-to-mass ratio i.e. binds to proteins to make them uniformly negatively charged. This is because the amount of SDS bound per unit weight of the protein is constant ~1.4 g of SDS per gram of protein (a stoichiometry of about one SDS molecule per two amino acids). The amount of SDS in the complex depends only on the size of the protein, not on charge or sequence. The negative charge acquired by protein due to binding of SDS is usually much greater than the charge on native protein; this native charge thus becomes insignificant. If the protein itself has a very large positive or negative charge, this charge may not be negligible compared with the charge produced by the bound SDS. A specific example is histone H1 which has a molecular mass of about 21 kDa, but behaves in gel electrophoresis like a molecule of 30 kDa, because of its strong positive charge.

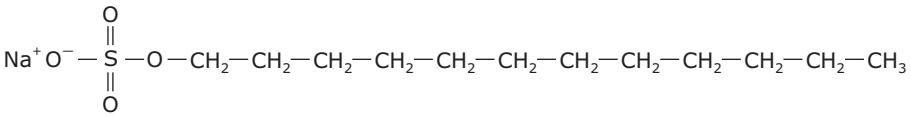


Figure 2.5 Structure of sodium dodecyl sulfate (SDS).

SDS bound polypeptides are flexible rod-shaped with a uniform negative charge per unit length. In SDS-PAGE, migration is, therefore, determined not by the intrinsic electric charge of polypeptides but by molecular weight because SDS treatment eliminates the effect of differences in charge density and shape. Thus, when a current is applied, all SDS-bound proteins in a sample will migrate through the gel toward the positively charged electrode. Proteins with less mass travel more quickly through the gel than those with greater mass because of the sieving effect of the gel matrix.

Reducing and non-reducing SDS-PAGE: SDS denatures proteins, causing multimeric proteins to dissociate into their subunits, and all polypeptide chains are forced into extended conformations. In **reducing SDS-PAGE**, along with SDS, protein is also treated with **reducing agents** such as β-mercaptoethanol, dithiothreitol to break intrachain or interchain disulfide bonds between cysteine residues. In a multimeric protein, subunits are dissociated when treated with SDS and a reducing agent. Thus, reducing SDS-PAGE gives monomeric mass. A protein shows a band corresponding to 100kDa in native-PAGE but 25kDa in reducing SDS-PAGE suggest the protein is an oligomeric protein with four identical subunits. In **nonreducing SDS-PAGE**, reducing agents are not used.

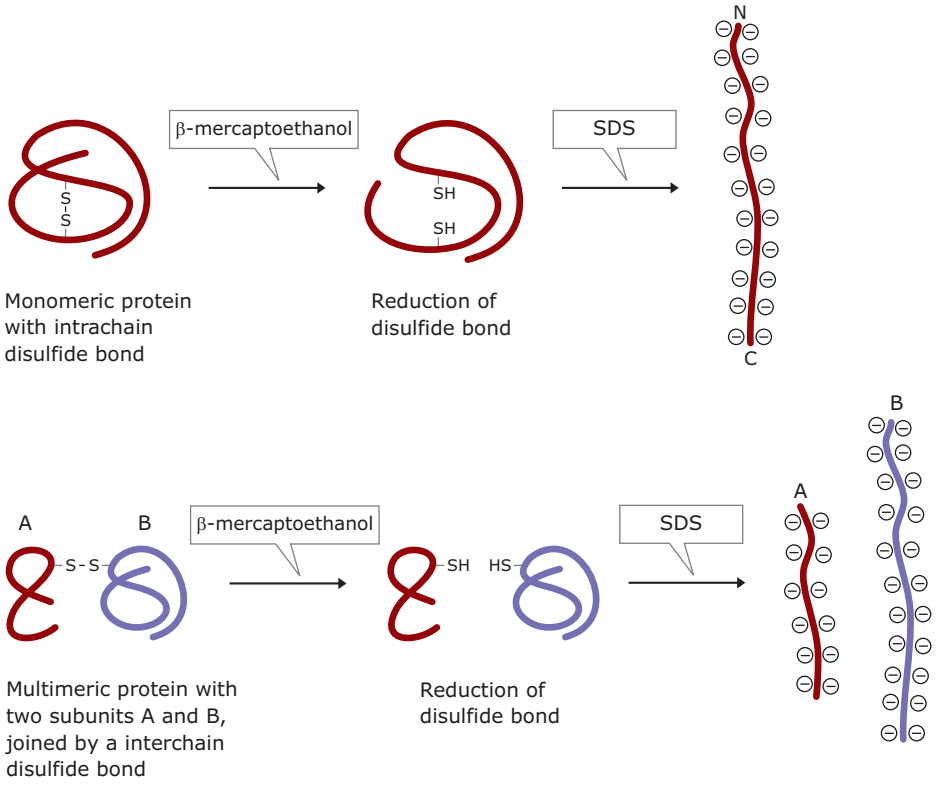


Figure 2.6 Effect of SDS on the conformation and charge of a protein. The protein is denatured and coated with a molecule with a uniform charge per unit length. The intrinsic charge of the protein is neutralized. Addition of reducing agents such as β-mercaptoethanol reduces any disulfide bonds.

2.2.2 Native PAGE

In SDS-PAGE, the gel is cast in a buffer containing SDS, an anionic detergent. The SDS denatures proteins in the sample. Usually, a *reducing agent* such as β-mercaptoethanol, dithiothreitol is also added to break intrachain and interchain disulfide bonds and ensure that no tertiary or quaternary protein structure remains. Consequently, when these samples are electrophoresed, proteins separate according to mass alone.

In native PAGE, proteins are separated according to the net charge, size and shape of their native structure. Most protein molecules carry a net charge at any pH other than their isoelectric point and hence electrophoretic migration occurs because most proteins carry a net negative charge in alkaline running buffers. The higher the negative charge density, the faster a protein will migrate. At the same time, the frictional force of the gel matrix creates a sieving effect,

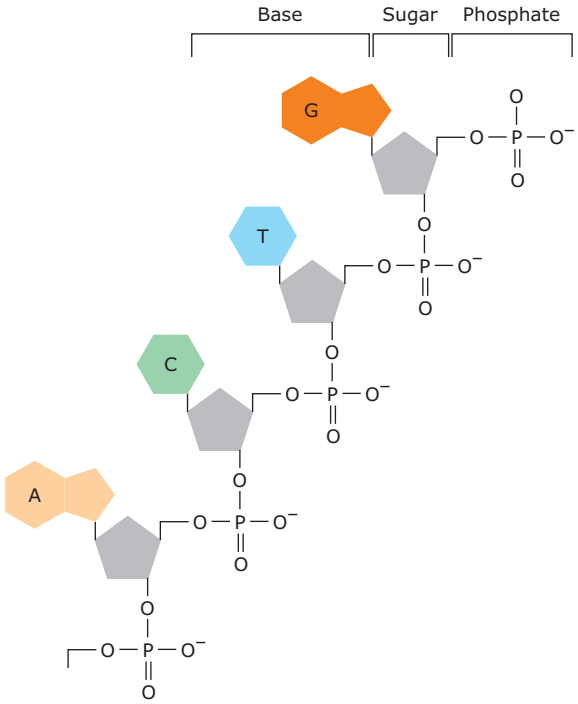


Figure 2.12 The sugar-phosphate backbone of nucleic acid composed of alternating sugar and phosphate groups is negatively charged at neutral to basic pH and hydrophilic.

Sequence and conformation of nucleic acid molecules

Nucleic acid molecules have different electrophoretic mobility when they are of different sizes. The mobility of nucleic acid molecules in gel electrophoresis is determined mainly on the basis of size. However, nucleic acids with the same number of nucleotides i.e. same size but different sequence composition and conformation (shape) may have different mobilities during electrophoresis.

Sequence: AT-rich nucleic acid may migrate more slowly than GC-rich nucleic acid of the same size and shape. The anomalous migration is likely due to sequence composition affecting their molecular conformation.

Conformation: Electrophoresis separates nucleic acid molecules, not only according to their size, but also according to their conformation (*or* shape). The conformations of nucleic acids can be circular and supercoiled, circular and relaxed, and linear. The migration of DNA molecules of the same sequence but differing conformations is affected by the compactness of each conformation. The more compact the conformation of DNA, the more easily it is able to migrate through the gel. Thus, a relaxed circular DNA migrates more slowly than a highly supercoiled form of same circular DNA.

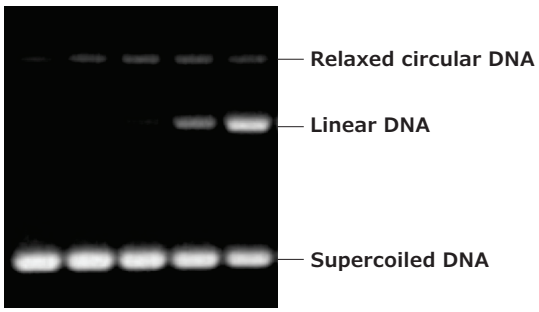


Figure 2.13 DNA molecules having different conformations move through the gel at different rates. Supercoiled covalently closed circular DNA, because of its compact conformation, moves through the gel fastest, followed by a linear DNA fragment of the same size, with the relaxed circular form traveling the slowest. The basis of this separation is that the greater the supercoiling, the more compact the conformation of a covalently closed circular DNA (cccDNA). The more compact the DNA, the more easily it is able to migrate through the gel.

toxicity than EtBr, but they are considerably more expensive. Moreover, all of the alternative dyes either cannot be or do not work well when added directly to the gel, therefore the gel will have to be post stained after electrophoresis.

UV shadowing

In place of staining with a dye, nucleic acids may be indirectly visualized by a method call **UV shadowing**, taking advantage of UV absorption by nucleic acids. For detection by UV shadowing, nanograms to micrograms of samples are needed, and a thin and transparent gel-like polyacrylamide should be used to ensure UV absorption and transmission. In a UV shadowing protocol, the gel is removed from the cassette after electrophoresis to maximize detection, wrapped in clear plastic film for protection, and then placed on a UV-fluorescent thin layer chromatography (TLC) plate. When the gel is exposed to UV radiation, absorption by the nucleic acid bands casts shadows on the TLC plate. The shadowy areas of the gel of desired sizes are cut out for further processing.

2.3.2 Quantification

Qualitative analysis of gels for the presence or absence of a band or relative mobilities of two bands can easily be performed by visual inspection. Answering 'How much?' and 'What size?' questions require additional work.

Amount: The amount of material in a band can be determined by a number of methods. The simplest is to visually compare the intensity of a band, either stained or autoradiographic, to standards of known quantity on the same gel. More accurate answers can be determined by using a densitometer to scan the stained gel or photograph/autoradiograph of the gel.

Size: The migration distances of nucleic acids in gel electrophoresis generally display a predictable correlation with their sizes, enabling calculation of the size of nucleic acids in a given sample. For linear double-stranded DNA fragments, migration distance is inversely proportional to the log of the molecular weight (expressed in base pairs), within a certain range. For approximate sizing, migration distances are compared to molecules of known sizes (called '**molecular weight standards**'). For a given gel concentration, usually, there will be some molecular weight range in which log of the molecular weight and migration distance are approximately linearly related. The DNA standard contains a mixture of DNA fragments of known sizes that can be compared against the unknown DNA samples. The exact sizes of separated DNA fragments can be determined by plotting the log of the molecular weight for the different bands of a DNA standard against the distance traveled by each band. Because shape affects the mobility of a molecule through a sieving gel, all the molecules in one gel must have similar shapes for valid comparisons. This does not present a problem for double-stranded DNA, because the shape of the molecules is virtually sequence-independent. Single-stranded nucleic acids, however, must be denatured to ensure similar shapes. For RNA or single-stranded DNA, denaturants added to the buffer may include formamide, urea, formaldehyde, or methylmercury hydroxide. Nucleic acids can also be denatured by treatment with glyoxal before electrophoresis.

2.4 Pulsed–field gel electrophoresis

The sizes of the DNAs that can be separated by conventional gel electrophoresis are limited to ~50 kb in size. Very large DNA fragments are unable to penetrate the pores in an agarose gel and thus cannot readily be resolved. However, larger DNA fragments can be resolved

Chapter 3

Spectroscopy

Spectroscopy is the study of the interaction between electromagnetic radiation and matter as a function of the wavelength or frequency of the radiation. The matter can be atoms, molecules or ions. The nature of the interaction between radiation and matter may include – absorption, emission or scattering. It is the absorption, emission or scattering of radiation by matter that is used to quantitatively or qualitatively study the matter or a physical process. A study of the radiation absorbed or emitted by an atom or a molecule will give information about its identity and this technique is known as **qualitative spectroscopy**. Measurement of the total amount of radiation will give information about the number of absorbing or emitting atoms or molecules and is called **quantitative spectroscopy**.

3.1 Electromagnetic radiation

Electromagnetic radiation is a form of energy and has both electrical and magnetic characteristics. A representation of electromagnetic radiation with *electric field* (E) and the *magnetic field* (B) – at right angle to the direction of the wave – is depicted in the figure 3.1. The electric and magnetic fields in an electromagnetic wave oscillate along directions perpendicular to the propagation direction of the wave.

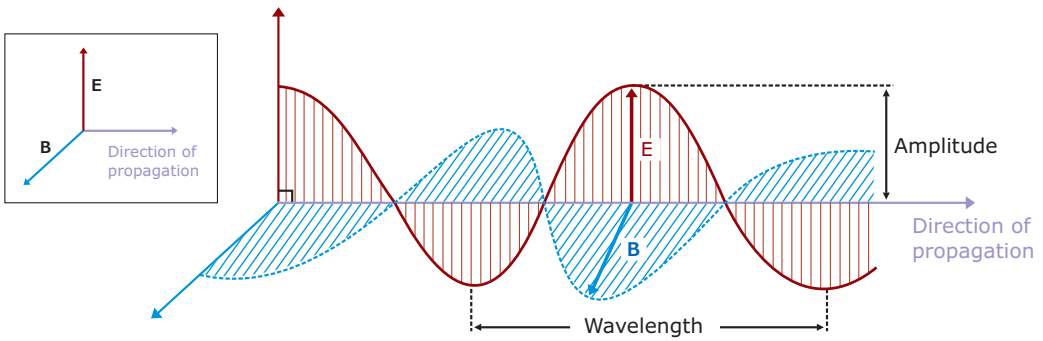


Figure 3.1
A representation of electromagnetic radiation with the electric field (**E**) and the magnetic field (**B**) at right angles to the direction of the wave movement. Both fields oscillate at the same frequency.

Electromagnetic spectrum ranges from very short wavelengths (such as gamma rays) to very long wavelengths (radio waves). The visible region of the spectrum extends approximately over the wavelength range 400–700 nm, the shorter wavelengths being the blue end of the spectrum and the longer wavelengths the red. The wavelengths between 400 and 200 nm make

up the near ultraviolet region of the spectrum and wavelengths above 700 nm to approximately 2000 nm (2 μm) the infrared region. The boundaries describing the electromagnetic spectrum are not rigid, and an overlap between spectral regions is possible.

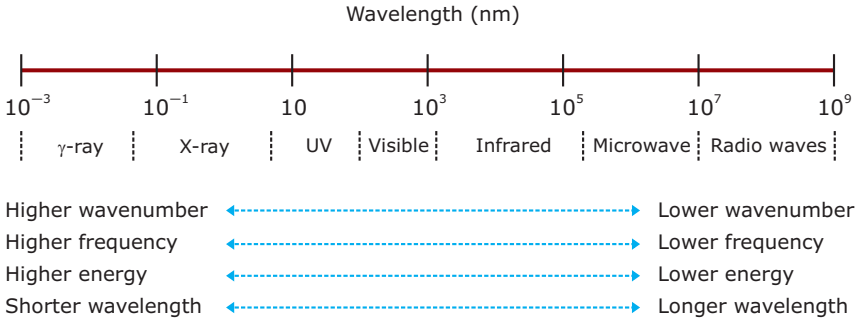


Figure 3.2 Range of electromagnetic radiation.

An electromagnetic radiation is characterized by several fundamental properties such as its velocity, amplitude, frequency, energy and polarization. The energy associated with a given segment of the spectrum is related to its frequency and wavelength. **Frequency** (ν) is the number of wave cycles that pass through a point in one second. It is measured in Hz, where 1 Hz = 1 cycle/sec. The **wavelength** (λ) is the length of one complete wave cycle. It is often measured in centimeters. It is inversely proportional to the frequency (ν) and is governed by the relationship;

$$\nu = \frac{c}{\lambda}$$ where, c = speed of light.

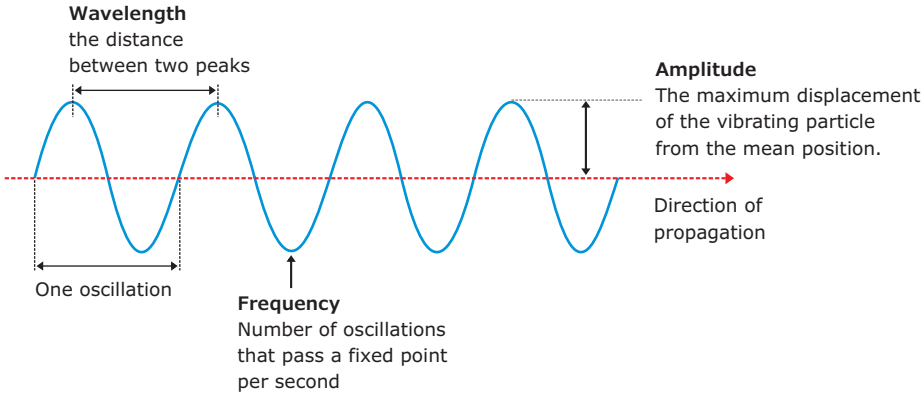
The energy is directly proportional to frequency and inversely proportional to wavelength. It is related to wavelength and frequency by the following equation:

$$E = h\nu = \frac{hc}{\lambda}$$ where, h is the Planck's constant (6.6×10^{-34} joules-sec).

The radiation in the infrared region of the electromagnetic spectrum is also expressed in terms of **wavenumber**, rather than wavelength. It is the reciprocal of the wavelength. It is denoted by $\bar{\nu}$. Wavenumber is expressed in units of per centimeter (cm^{-1}).

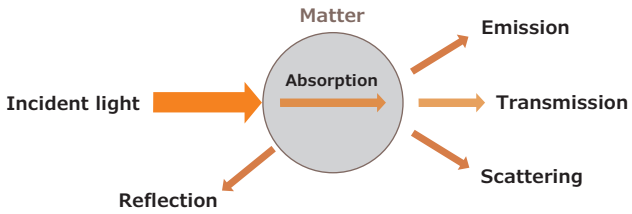
$$\bar{\nu} = \frac{1}{\lambda}$$ where, $\bar{\nu}$ is in units of cm^{-1} and λ is in units of cm.

The main reason chemists prefer to use wavenumbers as units is that they are directly proportional to energy (*a higher wavenumber corresponds to a higher energy*).



3.2 Types of spectroscopy

Spectroscopy is the collective term for a diverse group of techniques in which the interactions of electromagnetic radiation with a matter of interest are studied. When electromagnetic radiation meets matter, the radiation is either reflected off, scattered, emitted, transmitted or absorbed. This gives rise to three principal branches of spectroscopy: *absorption spectroscopy*, *emission spectroscopy* and *scattering spectroscopy*.



Absorption spectroscopy

Absorption spectroscopy studies radiation *absorbed* at various wavelengths. When a beam of electromagnetic radiation passes through a sample, much of the radiation passes through the sample without a loss in intensity. However, at selected wavelengths, the radiation's intensity is attenuated (decrease in the number of photons). This process of attenuation is called **absorption**. A plot of the amount of light absorbed by a sample versus the wavelength of the light is called an **absorption spectrum**. Through absorption spectroscopy, one can find both *qualitative* and *quantitative* information about the sample. In absorption spectroscopy, electromagnetic radiation is absorbed by an atom or molecule, which undergoes a transition from a lower-energy state to a higher energy or excited state. Absorption occurs only when the energy of radiation matches the difference in energy between two energy levels. The type of transition depends on the energy of electromagnetic radiation. Absorption spectroscopy can be categorized into different spectroscopic types (X-ray spectroscopy, UV-Vis spectroscopy, infrared spectroscopy) based on the region of the electromagnetic spectrum absorbed by an atom or molecule.

Table 3.1 Major types of absorption spectroscopy

Region of the electromagnetic spectrum	Spectroscopic type
X-ray	X-ray spectroscopy
UV–Vis	UV-Vis spectroscopy
Infrared (IR)	Infrared spectroscopy
	Raman spectroscopy
Microwave	Microwave spectroscopy
Radio wave	Electron spin resonance spectroscopy
	Nuclear magnetic resonance spectroscopy

Mass spectrometry is different from *spectroscopy*. It does not measure the absorption or emission of radiation. It determines the mass of the molecule.

Effect of interaction between electromagnetic radiation and matter

Atomic and molecular spectroscopies are mainly related to the absorption, emission, or scattering of electromagnetic radiation and the changes taking place in those systems due to the energy of the radiation. In absorption spectroscopy, the effect of electromagnetic radiation depends on the energy associated with the radiation. The absorption of very energetic radiations

Jablonski diagram

The processes that occur between the absorption and emission of light are usually illustrated by the Jablonski diagram. A typical Jablonski diagram (basically an energy diagram) is shown in the figure. The singlet ground, first and second excited electronic energy states are depicted by S_0, S_1 and S_2, respectively. A molecular electronic energy state in which all electron spins are paired is called a *singlet state*. Each of the electronic energy states (ground or excited) has a number of vibrational energy levels, depicted by 0, 1, 2, etc. The lowest vibrational energy level for each electronic energy state is designated as 0, and the levels above it are successively 1, 2, etc. Each of these vibrational energy levels can be subdivided even further into rotational energy levels.

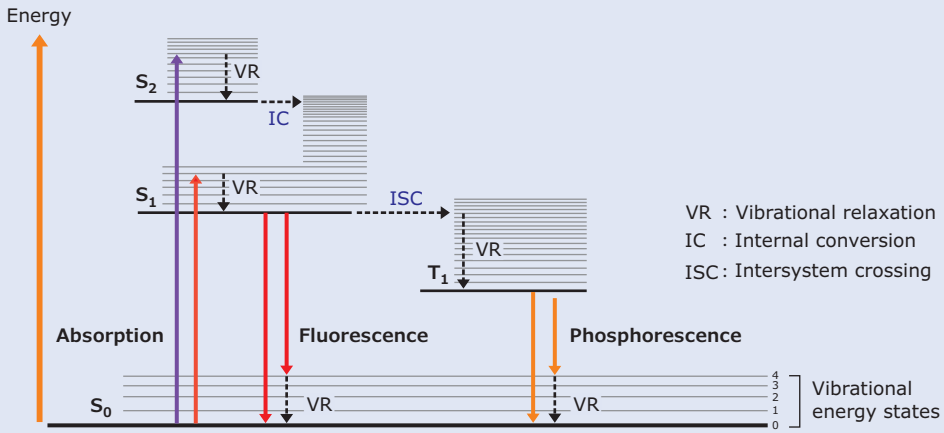


Figure 3.5 Jablonski diagram. The lowest thick horizontal line represents the ground electronic energy states of the molecule, which is normally a singlet state and is labeled S_0. The upper thick lines are energy levels for the ground vibrational states of three excited electronic states. The two lines on the left represent the first (S_1) and second (S_2) electronic singlet states. The one on the right (T_1) represents the energy of the first electronic triplet state. Numerous vibrational energy levels are associated with each of the four electronic states as represented by the thin horizontal lines. An excited molecule in a singlet or triplet excited energy state can undergo various modes of nonradiative as well as radiative decay. The light emitted by the radiative, singlet excited-to-ground state transition is called fluorescence (emission between states of same spin). The light emitted by the radiative, triplet-to-ground state transition is called phosphorescence (emission between states of different spin).

The absorbance of light of a particular wavelength by the molecule of interest causes transition of an electron from a lower electronic energy state to a higher electronic energy state. Only certain wavelengths of light are absorbed, that is, wavelengths that have energies that correspond to the energy difference between two different energy states of the particular molecule. The promotion of electrons to an excited energy state is called *excitation*. It is possible to excite the molecule to higher vibrational levels within the excited electronic states, so there are many possible absorption transitions.

Once an electron is in excited state, there are several ways that energy may be dissipated. The first is through a nonradiative process called **vibrational relaxation**. This is indicated on the Jablonski diagram as a dotted arrow between vibrational energy levels. This relaxation occurs between vibrational energy levels of one electronic energy state. Electrons will not change the electronic energy states.

However, if vibrational energy levels strongly overlap electronic energy state, a possibility exists that the excited electron can move from a vibration energy level of an excited electronic

energy state to another vibration energy level in a lower energy state. This process is called **internal conversion**, a non-radiative process. It is mechanistically identical to vibrational relaxation but occurs between two vibrational energy levels in different electronic energy states. Internal conversion occurs because of the overlap of vibrational and electronic energy states.

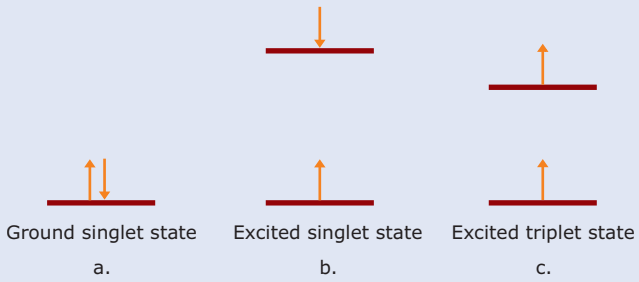


Figure 3.6 Electronic spin states of molecules. In **a**, the ground electronic state is shown. In the ground state, the spins are always paired, and the state is said to be a singlet state. In **b** and **c**, excited electronic states are shown. If the spins remain paired in the excited state, the molecule is in an excited singlet state **b**. If the spins become unpaired, the molecule is in an excited triplet state **c**.

An excited state may return to the ground state by emitting light. The emission of light may occur during the transition of electrons from a singlet first excited energy state (S_1) to a singlet ground state (S_0). This radiative process is called **fluorescence** (emission involving states of the same spin multiplicity). The energy of fluorescent light is always less than that of the exciting light. This difference is because in the excited state, some energy is always lost by non-radiative processes (such as transitions between vibrational states). Therefore, the energy of the emitted light is always less than that of the absorbed light, and hence, greater wavelengths than the absorbed light. The difference between the absorption (excitation) and emission wavelengths is called the **Stokes shift**.

Molecules in the singlet first excited energy state (S_1) can also undergo a spin conversion to the first triplet state, T_1. When one electron of a pair of electrons of a molecule is excited to a higher energy level, a singlet or a triplet state is formed. In the excited singlet state, the spin of the promoted electron is still paired with the ground state electron. In the triplet state, however, the spins of the two electrons have become unpaired and are thus parallel. Conversion of S_1 to T_1 or vice versa is called **intersystem crossing**, a nonradiative process. It is a spin-dependent internal conversion process where the electron changes spin multiplicity. Intersystem crossing leads to several interesting routes back to the singlet ground energy state (S_0). One direct transition is **phosphorescence** (emission involving states of different spin multiplicity), where a radiative transition from T_1 to S_0 occurs. This is also a very slow, forbidden transition. Another possibility is delayed fluorescence, the transition back to the S_1, leading to the radiative transition to the S_0.

Emission involving two states of the same spin is called fluorescence, while emission between states of different spin is phosphorescence. Emission from a triplet excited electronic state to a singlet ground state is thus phosphorescence.

Stokes shift

When a molecule absorbs a photon, it gains energy and enters an excited state. One way for the molecule to relax is to emit a photon, thus losing its energy. When the emitted photon has less energy than the absorbed photon, this energy difference is the Stokes shift. It is the difference (in wavelength units) between positions of the band maxima of the absorption and emission spectra of the same electronic transition.

The **atomic spectroscopy** refers to the study of the electromagnetic radiation absorbed and emitted by atoms whereas the **molecular spectroscopy** refers to the study of the electromagnetic radiation absorbed and emitted by molecules.

to an unoccupied orbital of greater potential energy. Generally, the transition of electrons occurs from the *highest occupied molecular orbital* (**HOMO**) to the *lowest unoccupied molecular orbital* (**LUMO**).

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

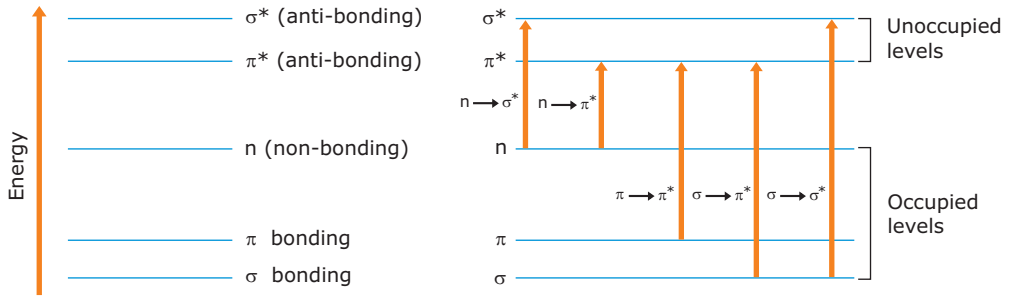


Figure 3.8 The possible electron jumps that light might cause are shown here. In each possible case, an electron is excited from a full orbital into an empty anti-bonding orbital. Each jump takes energy from the light, and a big jump obviously needs more energy than a small one. Each wavelength of light has a particular energy associated with it. If that particular amount of energy is just right for making one of these energy jumps, then that wavelength will be absorbed – its energy will be used in promoting an electron. The energy required to bring about transitions from the highest occupied energy level (HOMO) in the ground state to the lowest unoccupied energy level (LUMO) is less than the energy required to bring about a transition from a lower occupied energy level.

When electromagnetic radiation (light) passes through a compound, energy from the radiation is used to promote an electron from a bonding or non-bonding orbital into one of the empty anti-bonding orbitals. The possible electron jumps that electromagnetic radiation might cause are shown in figure 3.8. In each possible case, an electron is excited from a full orbital into an empty anti-bonding orbital. The energy gaps between these levels determine the wavelength of the electromagnetic radiation absorbed, and these gaps will be different in different compounds. The larger the gap between the energy levels, the greater the energy required to promote the electron to the higher energy level; resulting in light of higher frequency, and therefore shorter wavelength, being absorbed. Each wavelength of electromagnetic radiation has a particular energy associated with it. If that particular amount of energy is just right for making one of these energy jumps, then that wavelength will be absorbed – its energy will have been used in promoting an electron. An absorption of electromagnetic radiation in UV and visible region (200 to 700 nm) cause only a limited number of the possible electron jumps. These jumps are from π bonding orbitals to π anti-bonding orbitals (π to π*) and from non-bonding orbitals, n, to π anti-bonding orbitals (n to π*). This means that in order to absorb electromagnetic radiation in the UV and visible region, the molecule must contain either π bonds or atoms with non-bonding orbitals. Both *n* to π* and π to π* transitions require the presence of an unsaturated functional group to provide the p-orbitals. Molecules containing such functional groups and capable of absorbing UV and visible radiation are called **chromophores**, Greek words meaning 'to bear color'.

Chromophore
The bonds and functional groups that give rise to the absorption of ultraviolet and visible radiation.

Molecules that show increasing degrees of conjugation require less energy for excitation and as a result absorb radiation of longer wavelengths. Molecules that contain conjugated systems,

Electronic transitions

Transition	*Wavelength*
$\sigma \rightarrow \sigma^*$	<200 nm
$n \rightarrow \sigma^*$	160–260 nm
$\pi \rightarrow \pi^*$	200–500 nm
$n \rightarrow \pi^*$	250–600 nm

i.e. alternating single and double bonds, will have their electrons *delocalized* due to overlap of the p-orbitals in the double bonds. As the amount of delocalization in the molecule increases, the energy gap between the π bonding orbitals and π anti-bonding orbitals gets smaller; and therefore, light of lower energy, and longer wavelength, is absorbed.

3.4 IR absorption spectroscopy

The term **infrared** (IR) covers the range of the electromagnetic spectrum between 1 micrometer to 100 micrometers. It is commonly divided into three sub-regions – near-IR, mid-IR and far-IR. Each frequency (the number of wavelengths that pass a point per unit time) of infrared radiation has a specified amount of energy. If a particular frequency is being absorbed as it passes through the compound being investigated, it must mean that its energy is being transferred to the compound. As with other types of electromagnetic radiation absorption, molecules are excited to a higher energy state when they absorb IR radiation. A molecule absorbs only selected frequencies of IR radiation. The absorption of IR radiation corresponds to energy changes in the order of 8 to 40 kJ/mole. IR radiation does not have enough energy to induce electronic transitions as seen with UV and visible radiations. The energies of IR radiation correspond to the energies involved in bond vibrations.

Physical basis of IR spectroscopy

IR spectroscopy is one type of **vibrational spectroscopy**. At temperatures above absolute zero, the bonds within molecules all vibrate. There are two main types of bond vibrations – stretching and bending. A **stretching** vibration occurs along the line of the chemical bond whereas a **bending** vibration is any vibration that does not occur along the line of the chemical bond. Different bonds vibrate with characteristic frequencies.

Consider, for example, the C–H bonds in a typical organic compound. These bonds undergo various stretching and bending vibrations. Let us understand the situation by considering the C–H stretching bond vibration. This vibration takes place with a certain frequency ν; that is, it occurs a certain number of times per second. Suppose that a C–H bond has a stretching frequency of 9×10^{13} times per second. A wave of electromagnetic radiation can transfer its energy to the vibrational wave motion of the C–H bond only if *there is an exact match between the frequency of the radiation and the frequency of the vibration*. Thus, if a C–H vibration has a frequency of 9×10^{13} times per second, then it will absorb energy from radiation with the same frequency. When radiation of this frequency interacts with a vibrating C–H bond, energy is absorbed and both the frequency and the intensity of the bond vibration increase. That is, after absorbing energy, the bond vibrates with a greater frequency and with a larger amplitude (a larger stretch and tighter compression). This absorption gives rise to the peak in the IR spectrum. Eventually, the bond returns to its normal vibration frequency, energy is released in the form of heat.

Thus, when the natural frequency of bond vibration in a molecule is equal to the frequency of the IR radiation directed on the molecule, the molecule absorbs the radiation. Different bonds have different vibrational frequencies and therefore different IR absorption frequencies. When IR radiation is absorbed, both the frequency and the intensity of the bond vibration increase. That is, after absorbing energy, the bond vibrates with a greater frequency and with a larger amplitude.

A normal mode of vibration that gives rise to an oscillating dipole in the IR spectral range is said to be **IR-active**. Conversely, a normal mode of vibration that does not give rise to an oscillating dipole moment (e.g. the stretching of a homonuclear diatomic molecule) cannot lead to IR absorption and is said to be **IR-inactive**.

Dipole moment depends on the variation in distribution of electrons along the bond and also its length. For bonds between unlike atoms, the larger the difference in electronegativity, the greater the dipole moment.

Not all bonds of a molecule are capable of absorbing IR radiation, even if the frequency of the IR radiation exactly matches that of the bond vibrational frequency. To absorb IR radiation, the molecule must have a **dipole moment** and that must change during vibration. A polar chemical bond has a bond dipole. If a polar bond vibrates with a particular frequency, its bond dipole vibrates with the same frequency. The magnitude of the bond dipole is proportional not only to the amount of charge on each bonded atom but also to the distance between the atoms, that is, the *bond length*. Therefore, as the bond stretches, the bond dipole increases, and, as the bond compresses, the bond dipole decreases. When the frequencies of the IR radiation and the vibrating bond dipole match, the bond dipole absorbs energy from the IR radiation.

Thus, a bond must have a dipole moment that is changing at the same frequency as the incoming radiation for energy to be transferred. Molecular vibrations that occur but do not give rise to IR absorptions are said to be **infrared-inactive**. In contrast, any vibration that gives rise to an IR absorption is said to be **infrared-active**.

Diatomic molecules such as H_2, N_2 and O_2 (having two identical atoms) have zero dipole moment and vibration of the bonds also do not produce it. These molecules are said to be infrared-inactive. For diatomic molecules, having two different atoms, usually have a permanent dipole moment due to a difference in the electronegativity of the bonded atoms. A greater electronegativity difference between the two atoms usually leads to a larger dipole moment for that bond. However, all molecules having a permanent dipole moment are also not infrared-active. In order to be infrared-active, the bond vibration (stretching or bending) must cause a change in the dipole moment of the molecule.

It is also possible for a molecule that has a zero dipole moment but a specific molecular vibration creates a temporary dipole moment in the molecule. Consider the CO_2 molecule as an example. It is a linear symmetric molecule in which the three atoms are arranged linearly with a partial positive charge on carbon and partial negative charge on oxygens. Its equilibrium dipole moment is zero. In the case of **symmetrical stretching**, both bonds are compressed or stretched to the same extent. As a result dipole moment remains zero. Thus, symmetric stretching vibrations do not absorb IR radiation and said to be *infrared-inactive*. But in **asymmetrical stretching**, one of the bonds is compressed while the other is stretched. Hence, there is change in bond length and dipole moment. So it is *infrared-active*. Bending vibration is also allowed to this molecule. This also makes molecule infrared-active.

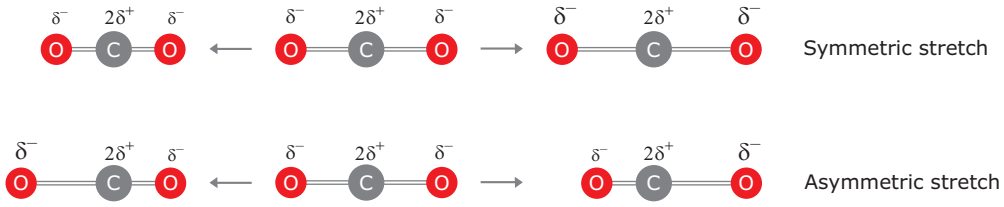


Figure 3.9 Symmetric and asymmetric stretching vibration of the carbon dioxide molecule. In the symmetrical stretch, the two C=O bonds stretched (or compressed) at the same time so that the molecule maintains its symmetry. In the asymmetrical stretch, one C=O bond shortens when the other lengthens. In case of the symmetrical stretch, both bonds are increased; but, because they are exactly equal and oppose each other, the dipole moment remains zero. Hence, the symmetrical stretching vibration is infrared-inactive. In an asymmetrical stretch, one C=O bond is reduced in length while the other is increased. Because the 'long' C=O bond has a greater bond dipole than the 'short' C=O bond, the two bond dipoles no longer cancel. Thus, the asymmetrical stretch imparts a temporary dipole moment to the CO_2 molecule. Consequently, this vibration is infrared-active—it gives rise to an IR absorption.

Infrared spectra

An infrared spectrum, like any absorption spectrum, is a record of the IR radiation absorbed by a compound as a function of wavelength. The instrument that determines the IR absorption spectrum for a compound is called an **infrared spectrometer**. An infrared spectrum provides information about what functional groups are present in a compound.

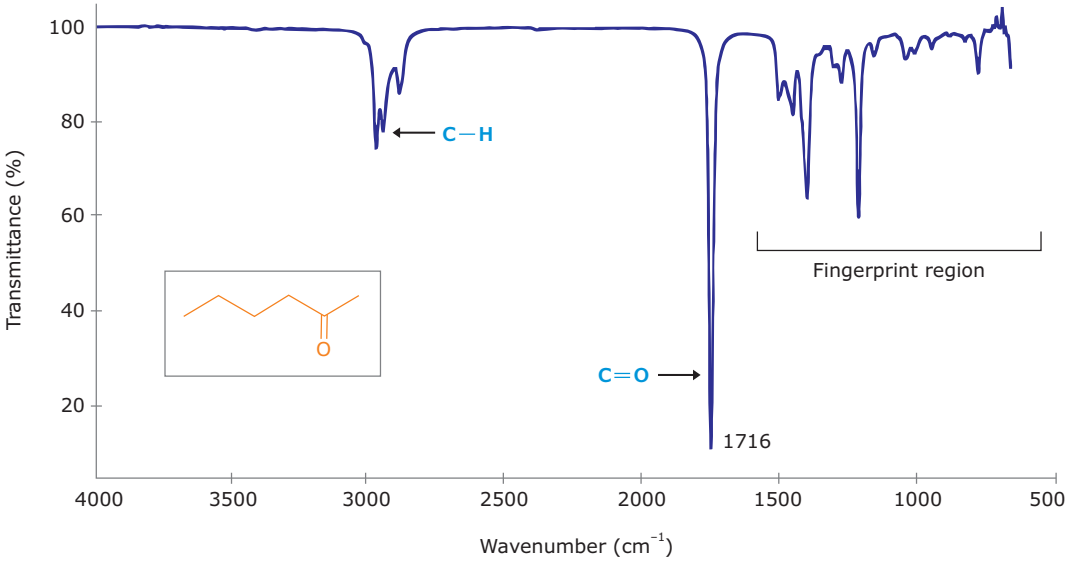


Figure 3.10 Infrared spectrum of 2-hexanone. The light transmitted through a sample of 2-hexanone is plotted as a function of wavelength or wavenumber. The wavenumber is simply the inverse of the wavelength. Absorptions are indicated by the inverted peaks. Each peak in the IR spectrum of a molecule corresponds to the absorption of energy by the vibration of a particular bond or group of bonds. A larger value of absorbance is associated with a stronger peak and a smaller value of absorbance with a weaker peak.

Two aspects of IR spectra are particularly important. First is the *position* of the peak—the wavenumber or wavelength at which it occurs. Second is the *intensity* of the peak—how strong it is.

IR absorption position

The energy involved in bond vibration (i.e. position of the peak in an infrared spectrum) depends on:

Strength of the bond: A simple measure of bond strength is the energy required to break the bond, which is the *bond dissociation energy*. *The higher the bond dissociation energy, the stronger the bond*. Thus, *the IR absorptions of stronger bonds (e.g. bonds with greater bond dissociation energies) occur at higher wavenumber*. Triple bonds have higher stretching frequencies than corresponding double bonds, which in turn have higher frequencies than single bonds.

Masses of the atoms involved in the bond: *The vibration frequency for a bond between two atoms of different mass depends more on the mass of the lighter object than on the mass of the heavier one.* Bonds to lighter atoms (such as hydrogen atom) have higher stretching frequencies than those to heavier atoms.

Type of vibration being observed: The absorption frequency also depends on the type of vibration. The two general types of vibrations in molecules are stretching vibrations and bending vibrations.

In general, bending vibrations occur at lower frequencies (higher wavelengths) than stretching vibrations of the same groups. It is easier to bend a bond than to stretch or compress it.

Values chiefly affected by mass of atoms (*lighter atom*, *higher frequency*)			
C–H	C–D	C–O	C–Cl
3000 cm^{-1}	2200 cm^{-1}	1100 cm^{-1}	700 cm^{-1}
Values chiefly affected by bond strength (*stronger atom*, *higher frequency*)			
C≡O	C=O	C–O	
2143 cm^{-1}	1715 cm^{-1}	1100 cm^{-1}	

D, deuterium, has twice the mass of H, and Cl has about twice the mass of O.

This means that each different bond will vibrate in a different way, involving different amounts of energy. The amount of energy it needs to do this will vary from bond to bond, and so each different bond will absorb a different frequency (and hence energy) of IR radiation.

IR absorption intensity

The different peaks in an IR spectrum typically have very different intensities. The intensity of an IR absorption depends on:

Concentration of molecules in the sample: The greater the number of molecules per unit volume, the greater is the intensity of IR absorption.

Magnitude of dipole moment: The intensity of IR absorption varies with the change of dipole moment. *The greater the change in the dipole moment, the stronger will be the absorption.* If the bond is perfectly symmetrical, there is no change in dipole moment and there is no IR absorption. As we know, the magnitude of the bond dipole is proportional not only to the amount of charge on each bonded atom but also to the distance between the atoms—that is, the bond length.

In IR spectroscopy, spectroscopists normally use **wavenumbers** instead of frequency. A wavenumber is the inverse of the wavelength. It has units of cm^{-1}. It is directly proportional to the frequency and the energy of the radiation–IR radiation with a high wavenumber has higher frequency and energy than IR radiation with a lower wavenumber. Because wavenumber and frequency are directly proportional to one another, it is common for the two terms to be used almost interchangeably.

Infrared absorption and chemical structure

The most common application of IR spectroscopy is perhaps to identify the functional groups. This is possible because different functional groups vibrate at different frequencies allowing their identification. *In all compounds, a given type of functional group absorbs IR radiation in the same general region of the IR spectrum*. The frequency of vibration, however, depends on additional factors such as delocalization of electrons, H-bonding, and substitutions at the nearby groups.

An IR spectrum usually extends from radiation around 4000 cm^{-1} to 650 cm^{-1} and can be split into the **functional group region** and the **fingerprint region**. Many functional groups absorb infrared radiation at about the same frequency, regardless of the structure of the rest of the molecule. The absorption bands in the 4000 – 1500 cm^{-1} region allow identification of functional groups; this region therefore is also termed the *functional group region* of the IR spectrum. For example, C–H stretching vibrations usually appear between 3200 and 2800 cm^{-1}; and carbonyl (C=O) stretching vibrations usually appear between 1800 and 1600 cm^{-1}. This makes these bands diagnostic markers for the presence of a functional group in a sample. These types of infrared bands are called *group frequencies* because they tell us about the presence or absence of specific functional groups in a sample.

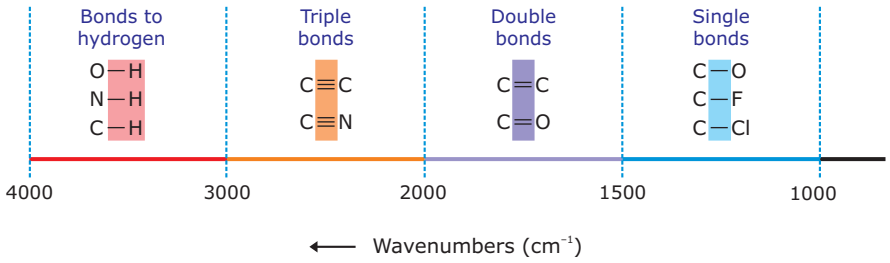


Figure 3.11 Infrared spectrum. The approximate regions where various common types of bonds absorb for stretching vibrations only. The first region, from 4000 to 2500 cm^{-1} is the region for C–H, N–H, and O–H bond stretching. The triple bond region from about 2500 to 2000 cm^{-1}. Double bonds appear about 2000–1500 cm^{-1} and single bonds come below 1500 cm^{-1}.

The region of the infrared spectrum from 1200 to 700 cm^{-1} is called the **fingerprint region**. This region usually contains a very complicated series of absorptions. Many different vibrations, including C—O, C—C and C—N single bond stretches, C—H bending vibrations, and some bands due to benzene rings are found in this region. The fingerprint region is often the most complex and confusing region to interpret. This region is different for each molecule just like a fingerprint is different for each person. Two different molecules may have similar functional group regions because they have similar functional groups, but they will always have a different fingerprint region. Since every type of bond has a different natural frequency of vibration, and since two of the same types of bonds in two different compounds are in two slightly different environments, no two molecules of different structure have exactly the same infrared absorption pattern or infrared spectrum. By comparing the infrared spectra of two substances, one can establish whether they are identical or non-identical. A second use of the infrared spectrum is to determine structural information about a molecule. The absorptions of each type of bond (N—H, C—H, O—H, C—O, C—C and other) are found only in certain small portions of the infrared region. Thus, a small range of absorption can be defined for each type of bond.

In **infrared spectra:**

Position of band depends on	Mass of atoms – **Light** atoms give **high** frequency. Bond strength – **Strong** bonds give **high** frequency.
Strength of band depends on	Change in dipole moment A **large** change in dipole moment gives **strong** absorption.
Width of band depends on	Hydrogen bonding **Strong** H-bond gives **wide** peak.

3.5 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is a spectroscopic technique that involves a change in nuclear spin energy in the presence of an external magnetic field. It is based on the magnetic properties of nuclei that result from a property called *nuclear spin*. NMR allows us to detect atomic nuclei and say what sort of environment they are in, within their molecule. For example, the hydrogen of the hydroxyl group in propanol is different from the hydrogens of its carbon

skeleton. With the help of a proton NMR, one can easily distinguish between these two sorts of hydrogens. '^{1}H NMR' and 'proton NMR' are interchangeable terms. The hydrogen atom is denoted by ^{1}H. Likewise, **carbon NMR** (^{13}C NMR) can easily distinguish between the three different carbon atoms in propanol.

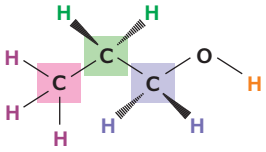


Figure 3.12 ^{1}H NMR distinguishes the coloured hydrogens and ^{13}C NMR distinguishes the boxed carbons.

NMR spectroscopy is used to detect nuclei, but only those nuclei that have a magnetic property as a result of nuclear spin. Spin comes in multiples of 1/2 and can be + or −. Not all atomic nuclei have *nuclear spin*. The rules for determining the net spin of a nucleus are as follows;

- If the number of neutrons and the number of protons are both *even*, then individual spins are paired and the overall spin becomes zero (i.e. nuclei have no spin).
- If the number of neutrons and the number of protons are both *odd*, then the nucleus has an integer spin (i.e. 1, 2, 3).
- If the number of neutrons plus the number of protons is *odd*, then the nucleus has a half-integer spin (i.e. 1/2, 3/2, 5/2).

Table 3.2 Number of protons, number of neutrons and nuclear spin quantum number of some elements

Number of protons	**Number of neutrons**	**Spin quantum number** (I)	**Element**
Even	Even	0	^{12}C, ^{16}O
Odd	Odd	Integer (1,2,...)	^{14}N
Even	Odd	Half-integer (1/2, 3/2,...)	^{13}C
Odd	Even	Half-integer (1/2, 3/2,...)	^{19}F, ^{15}N

For each nucleus with spin, the number of allowed spin states – it may adopt – is determined by its **nuclear spin quantum number** (I). A nucleus of spin quantum number I has $2I + 1$ allowed spin states. For example, ^{1}H has the nuclear spin quantum number $I = 1/2$ and has two allowed spin states [2(1/2) + 1 = 2] for its nucleus, −1/2 and +1/2. For the chlorine nucleus, $I = 3/2$ and there are four allowed spin states [2(3/2) + 1 = 4] for its nucleus, −3/2, −1/2, +1/2 and +3/2.

Table 3.3 Spin quantum numbers and number of spin states of some common element

Element	$^{1}_{1}$H	$^{2}_{1}$H	$^{12}_{6}$C	$^{13}_{6}$C	$^{14}_{7}$N	$^{16}_{8}$O	$^{17}_{8}$O	$^{19}_{9}$F
Spin quantum number	$^{1}/_{2}$	1	0	$^{1}/_{2}$	1	0	$^{5}/_{2}$	$^{1}/_{2}$
Number of spin states	2	3	0	2	3	0	6	2

The hydrogen nucleus ^{1}H has a nuclear spin that can assume either of two spin states, −1/2 and +1/2. The ^{12}C nucleus has no spin whereas ^{13}C nucleus has spin that can assume either of two spin states, −1/2 and +1/2.

Physical basis of NMR

NMR spectroscopy is based on the magnetic properties of nuclei that result from a property called *nuclear spin.* All nucleus with spin act like a tiny magnet. We can compare the behaviour of a nucleus acting like a tiny magnet with magnetic compass placed in an external magnetic field. Imagine for a moment that we were able to 'switch off' the Earth's magnetic field. Compass needle (made of a magnetic material) will point randomly in any direction. However, as soon as we switched on the Earth's magnetic field back, the needle would point North—their *lowest* energy state. If we wanted needle to point South, we would have to apply force (energy). But after removal of external force, the needle would return to its lowest energy state, pointing North. How hard it is to turn the compass needle depends on how strong the magnetic field is and also on how well the needle is magnetized. If the needle is not magnetized at all, it is free to rotate.

Nevertheless, there is an important difference between a compass needle and the atomic nucleus acting like a tiny magnet. A real compass needle can rotate through 360° and have a virtually infinite number of different energy levels, all higher in energy than the lowest energy state (pointing North). When atomic nuclei acting like tiny magnets are placed in an external magnetic field, they have different energy levels. Fortunately, the number of energy levels an atomic nucleus can adopt is very less. For example, a ^{1}H or C^{13} nucleus in a magnetic field can have two energy levels.

Let's take an example of the hydrogen nuclei in a chemical sample. In the discussion, the word 'proton' is used for '^{1}H nuclei'. In the absence of an external magnetic field, the nuclear magnetic poles are oriented randomly. When an external magnetic field is applied to ^{1}H nuclei, they can either align themselves parallel to the field, which would be the lowest energy state, or they can align themselves antiparallel to the field, which is higher in energy. The magnetic poles of nuclei with a spin of +½ are oriented *parallel* to the applied field, and those nuclei with a spin of -½ are oriented *antiparallel* to the applied field.

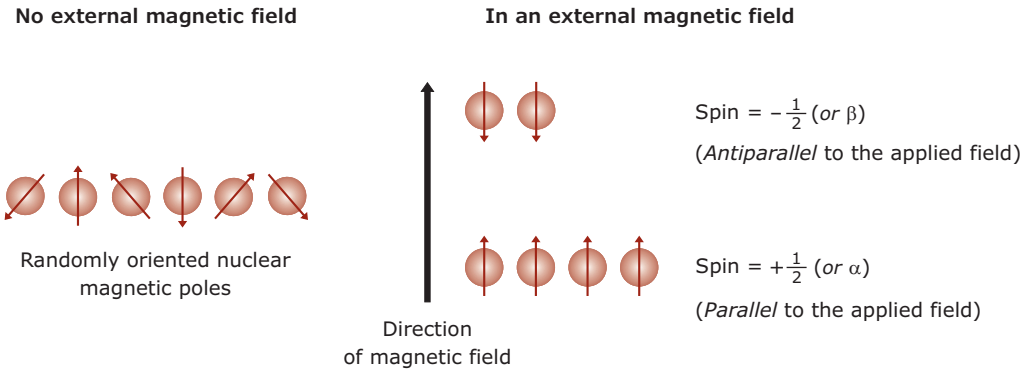


Figure 3.13 In the absence of an external magnetic field, the nuclear magnetic poles are oriented randomly (with arrows indicating their magnetic, north-south, polarity). In an external magnetic field or applied magnetic field, the spin state +½ is aligned parallel to the field, while the spin state −½ is aligned antiparallel to the applied field. More nuclei are oriented along with the applied field because this arrangement is lower in energy.

In the absence of an external magnetic field, the two spin states have the *same* energy. But when an external magnetic field is applied, the two spin states have *different* energies: the +½ spin state has lower energy than the -½ spin state. The energy difference between two spin states is a function of the strength of the applied magnetic field. The stronger the applied magnetic field, the higher the energy difference between the possible spin states. However, the energy difference between the two nuclear spin states is very small—so small that a very, very strong magnetic field is required to see any difference at all.

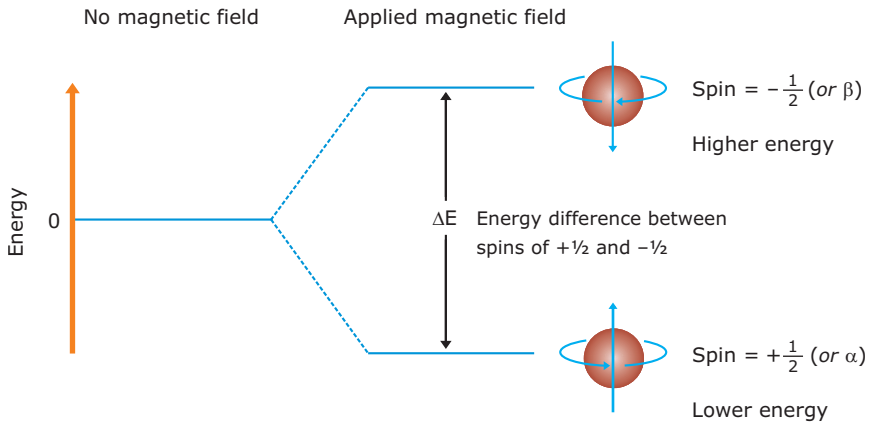


Figure 3.14 Effect of increasing magnetic field at a ^{1}H nucleus on the energy difference between its +½ and -½ spin states. The two spin states have identical energies when the external magnetic field is absent. The difference in energy between the two spin states depends on how strong the applied external magnetic field is, and also on the properties of the nucleus itself. The energy difference between the two spin states grows with increasing field. The stronger the magnetic field, the higher the energy difference between the two states.

Thus, for a nucleus in an external magnetic field, the difference in energy between two spin states in an applied magnetic field depends on:

- How strong the magnetic field is, and
- The magnetic properties of the nucleus itself.

The energy difference between the two spin states is given by the fundamental equation of NMR:

$$\Delta E = \frac{h\gamma_H}{2\pi} B_P$$

where, h is Planck's constant,

B_P is the magnitude of the magnetic field at the H^1 nucleus (proton), in gauss; and

γ_H is a fundamental constant of the proton, called the **gyromagnetic ratio**. The value of this constant is 26,753 radians gauss^{-1} s^{-1}.

This equation shows that when the external magnetic field is zero, there is no energy difference between the spin states and as the magnetic field is increased, the energy difference between the two spin states increases.

When the ^{1}H nuclei in a chemical sample are subjected in a magnetic field; each ^{1}H nucleus is in one of two spin states that differ in energy by an amount ΔE, and; a small excess of ^{1}H nuclei have spin +½. Even at a very strong magnetic field, the difference in the populations of the two spin energy states is very small. If the sample is now subjected to electromagnetic radiation with energy *exactly equal* to ΔE, this energy is absorbed by some of the ^{1}H nuclei in the +½ spin state. The absorbed energy causes these protons to 'flip' their spins and assume a more energetic state with a spin −½. Since the energy difference between the two states even in a very very strong external magnetic field is so small, the amount of energy needed to flip the nuclei can be provided by electromagnetic radiation of radio wave frequency. Radio waves flip the nucleus from the lower energy state to the higher state. This absorption phenomenon, called **nuclear magnetic resonance**, can be detected in a type of absorption spectrometer called an **NMR spectrometer**.

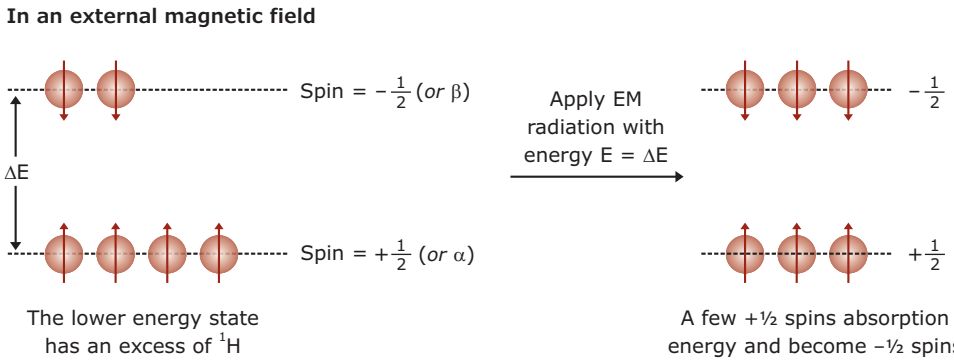


Figure 3.15 A chemical sample is subjected in a magnetic field; each ^{1}H nucleus is in one of two spin states that differ in energy by an amount ΔE, and; a small excess of ^{1}H nuclei have spin +½. The lower energy level contains slightly more nuclei than the higher level. The amount of energy needed to flip the nucleus can be provided by electromagnetic radiation (EM) of radio wave frequency. Radio waves flip the nuclei from the lower energy state to the higher state.

Chemical shift

To understand the chemical shift, let us analyze the **proton NMR** (^{1}H NMR) spectrum of **dimethoxymethane**. There are two absorption peaks in the protons NMR spectrum. Each peak represents a different kind of protons (hydrogen nuclei): each one absorbs energy at a different frequency. But why should protons present in the same molecule be different? We might expect all protons to resonate at one particular frequency. But they don't. In an applied magnetic field, not all protons in a molecule resonate at exactly the same frequency. This variability is due to the fact that the hydrogens in a molecule exist in slightly different electronic environments from one another.

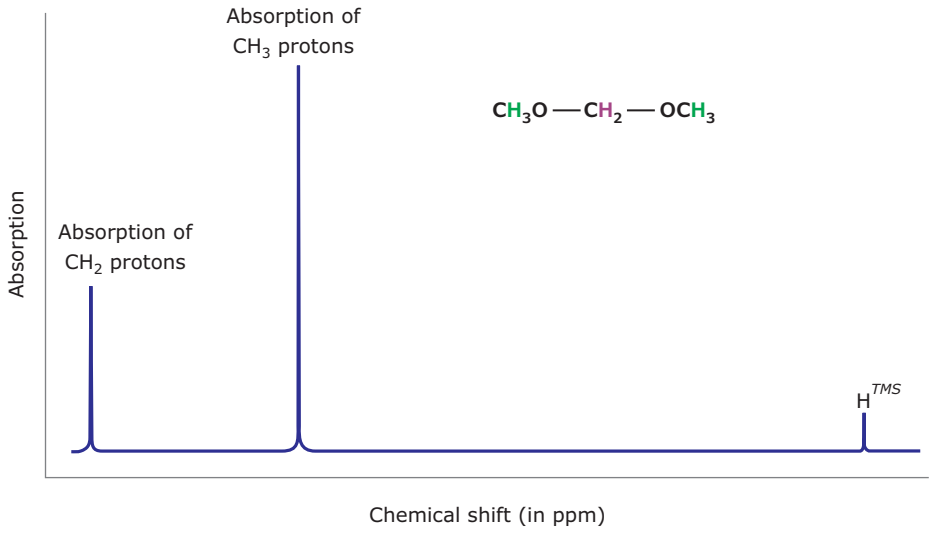


Figure 3.16 The proton NMR (^{1}H NMR) spectrum of dimethoxymethane. This spectrum is a plot of energy absorption on the y-axis versus relative frequency of the radiation from which energy is absorbed on the x-axis. Peaks in an NMR spectrum are called *absorptions*, or *lines*. The position of absorption on the horizontal axis is called its *chemical shift*. We usually express the peak positions in ppm. The small peak at the far right is from the protons of tetramethylsilane (TMS), a reference standard.

Each nucleus is surrounded by electrons. The valence-shell electron densities vary from one hydrogen to another. In an applied magnetic field, the valence-shell electrons are caused to circulate. This circulation, called a **local diamagnetic current**, generates own magnetic field that opposes the applied magnetic field (B_0). Circulation of electrons around a nucleus can be viewed as being similar to the flow of an electric current in an electric wire. In physics, we know that the flow of a current through a wire induces a magnetic field. In an atom, the local

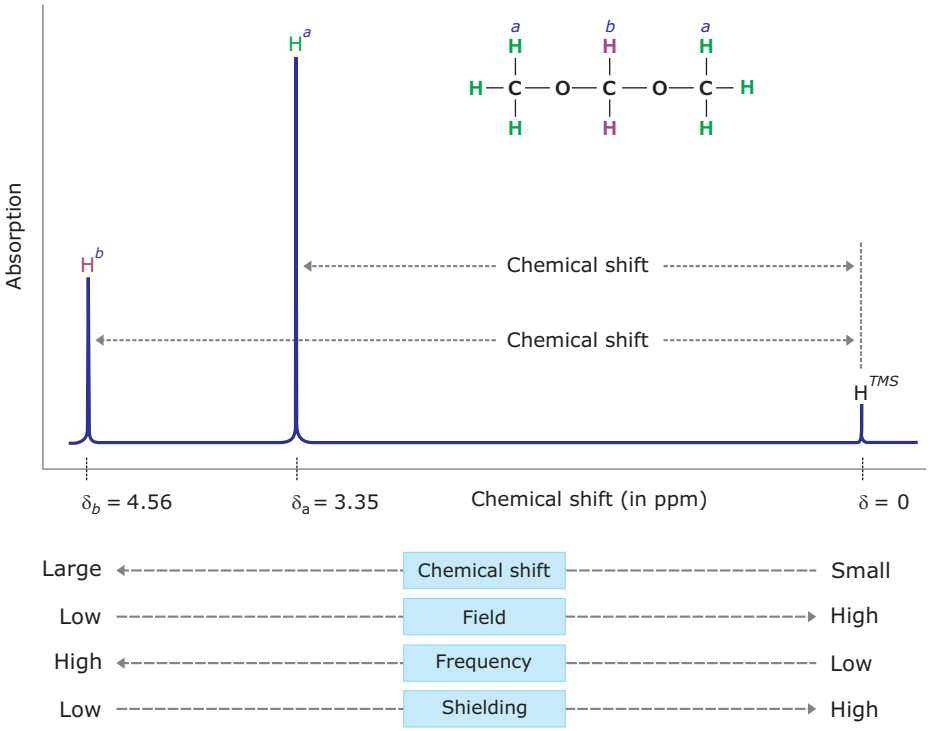
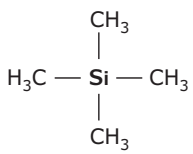


Figure 3.17 The proton NMR spectrum of dimethoxymethane. This spectrum is a plot of energy absorption on the y-axis versus relative frequency of the radiation from which energy is absorbed on the x-axis. Peaks in an NMR spectrum are called absorptions or lines. The position of an absorption peak on the horizontal axis is called its chemical shift. We express the peak positions in parts per million or ppm. The chemical shift of an absorption is written with a δ followed by the numerical value of the peak position. The absorption position of TMS (a reference standard) defines the δ = 0 position on the x-axis of the spectrum. The chemical shift scale runs to the left from zero (where TMS resonates). The other two peaks—the ones at δ = 3.35 and δ = 4.56—are the NMR absorptions of the protons of dimethoxymethane. We can say that the spectrum contains peaks at 0, 3.35 and 4.56 ppm. Two absorptions (δ = 3.35 and δ = 4.56) indicate that there are two chemically distinguishable sets of protons in dimethoxymethane, the CH_2 protons and the CH_3 protons. The two peaks have different sizes because different numbers of protons contribute to each absorption. The resonance at δ = 3.35 is larger because more protons (six) contribute to this resonance than to the resonance at δ = 4.56 (two).

In summary, proton NMR provides four types of information:

1. the number of sets of chemically nonequivalent protons
2. the chemical environments of each set of protons (chemical shift)
3. the number of protons within each set
4. the number of protons in adjacent sets

The reference sample — Tetramethylsilane



Tetramethylsilane

Unlike IR and UV/Vis spectroscopy, where the signals are fixed at particular frequencies or wavelengths, in NMR, the signal is dependent on the field strength. Since no two magnets have the same field, the frequency at which signals are obtained would vary correspondingly. Therefore, there was a need to characterize and specify the location of the signals. In order to avoid this situations, it has been decided to eliminate the problems by keeping a standard reference with respect to which a numerical value can be assigned. The compound we use as a reference sample in ^1H and ^{13}C NMR is usually **tetramethylsilane**, TMS. This is silane (SiH_4)

with each of the hydrogen atoms replaced by methyl groups to give $Si(CH_3)_4$. Because of molecular symmetry, all 12 protons of TMS absorb at the same frequency and all 4 carbons absorb at the same frequency. The frequency of absorption for a nucleus of interest relative to the frequency of absorption of a standard is called the *chemical shift* of the nucleus. The chemical shift of the ^1H nuclei in the ^1H NMR spectrum or ^{13}C nuclei in the ^{13}C NMR spectrum of TMS appear at 0 ppm. Typically, it increases from 0 on the right-hand side of the spectrum to 10 ppm on the left hand side of an ^1H NMR spectrum or from 0 on the right hand side to 200 ppm on the left hand side of a ^{13}C NMR spectrum. The reason frequencies of absorption are recorded on the δ-scale relative to those of a standard molecule. It makes the position of absorption independent of the spectrometer used to record the spectrum, in particular independent of the strength of the magnetic field of the spectrometer.

3.6 Circular Dichroism

Light is a transverse electromagnetic wave. The electric and magnetic fields in an electromagnetic wave oscillate along directions perpendicular to the propagation direction of the wave. Light can be *unpolarized* or *polarized* depending on how the electric field is oriented. In **unpolarized light**, the electric field vector oscillates in more than one plane; all perpendicular to the direction of propagation. If the electric field vector oscillates in single plane perpendicular to the direction of propagation, it is called **linearly** or **plane polarized light**. The orientation of a linearly polarized light is defined by the direction of the electric field vector. Linear polarization is obtained by passing unpolarized light through a polarizer that absorbs all electric field vectors not lying along a particular plane.

If the electric field vector rotates around the propagation axis maintaining a constant magnitude, it is called **circularly polarized light**. Circularly polarized light is the antithesis of linearly polarized light. In linear polarized light, the direction of the vector stays constant and the magnitude oscillates whereas in circularly polarized light, the magnitude stays constant while the direction oscillates. Circularly polarized light is obtained by superimposing two plane polarized light of same wavelengths and amplitudes but having a phase difference of 90°. A phase difference of 90° means that when one wave is at its peak, then the other one is just crossing the zero line.

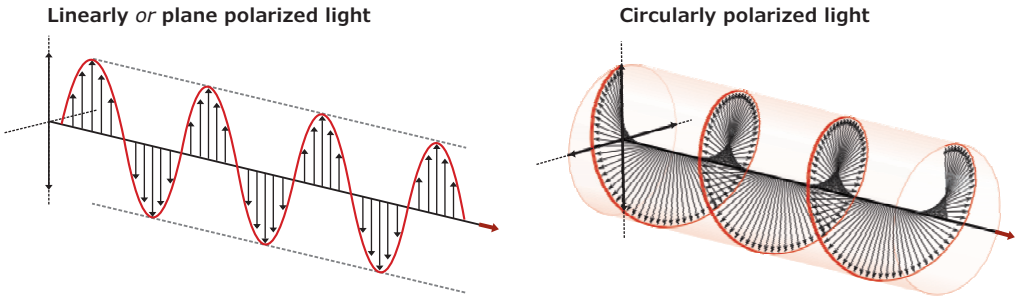
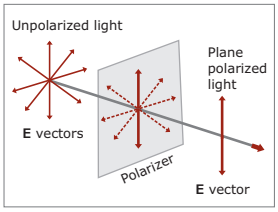


Figure 3.18 Linearly polarized light results when the direction of the electric field vector is restricted to a plane perpendicular to the direction of propagation while its magnitude oscillates. In circularly polarized light, the magnitude of the oscillation is constant and the direction oscillates.

A circularly plane polarized light may be right or left-handed circularly polarized depending on the rotation direction. For **left circularly polarized light** with propagation towards the observer,



Generation of plane polarized light.

the electric field vector rotates counter-clockwise. For **right circularly polarized light**, the electric field vector rotates clockwise. The superposition of left and right circularly polarized light beams of equal amplitudes can result in linearly polarized light. Thus, linear polarized light can be viewed as a superposition of opposite circular polarized light of equal amplitude and phase.

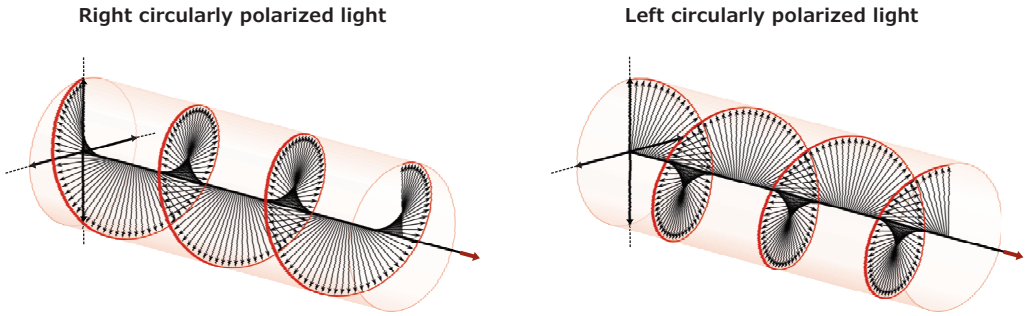


Figure 3.19 Schematic representation of right circularly polarized and left circularly polarized light. In both cases, the length of the vector remains constant. In linearly polarized light, the electric vector stays in the same plane but its length changes.

Optical rotatory dispersion (ORD) and **circular dichroism** (CD) are two phenomena that result when *chiral molecules* (molecules that have no plane and center of symmetry) interact with polarized light. Most of biological molecules are chiral molecules and thus they are **optically active**. An optically active compound shows a phenomenon called **optical rotation**. It means that the plane of polarization of a linearly polarized light rotates as it passes through an optically active medium. The technique of ORD measures the ability of optically active compounds to rotate plane polarized light as a function of the wavelength (i.e. wavelength dependence of optical rotation).

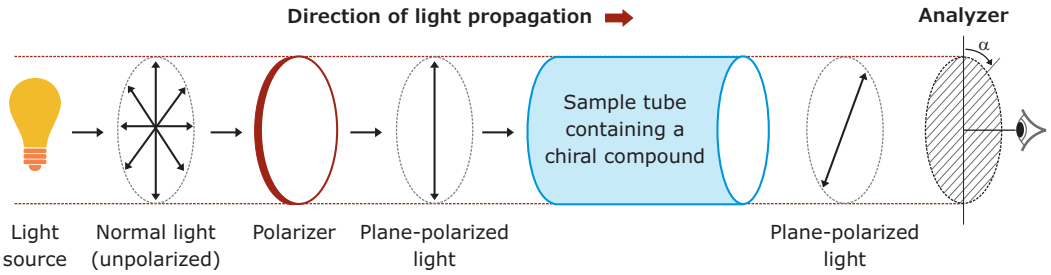


Figure 3.20 When plane-polarized light is passed through a solution containing an optically active compound, there is net rotation of the plane polarized light. The plane-polarized light is rotated either clockwise (dextrorotatory) or counterclockwise (levorotatory) by an angle.

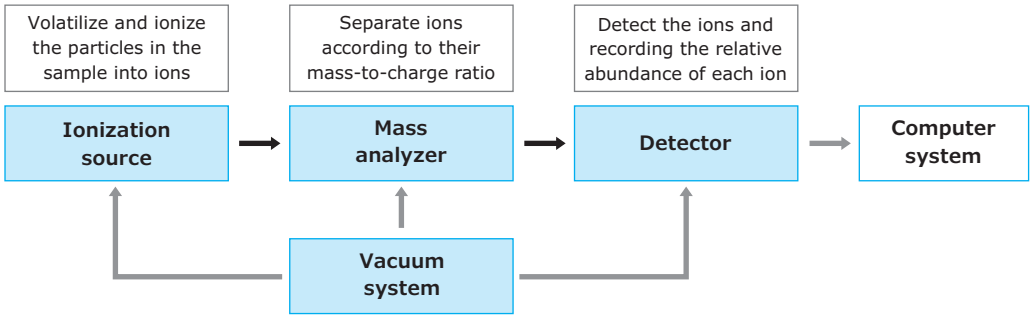
Chiral molecules
Most of chiral molecules contain one or more *chiral center*(s). Any tetrahedral carbon atom that has four different substituents is a *chiral center*. A molecule is not chiral (even if it has chiral centers) if it has a plane or center of symmetry. To be a chiral molecule there should be no plane and center of symmetry.

Circular Dichroism is an absorption spectroscopy. Dichroism is a word derived from Greek which means two-colors, because the sample under analysis has one color if illuminated with the right polarized light and a different color if illuminated with the left one. The color, in fact, depends on light absorption. Circular dichroism is observed when optically active matter absorbs left and right circularly polarized light slightly different. In fact, circular dichroism is the absorption difference between left and right circularly polarized light at a given wavelength. Absorption is quantitated by the **molar extinction coefficient** (ε). Optically active samples have distinct molar extinction coefficients for left (ε_L) and right (ε_R) circularly polarized light. The difference in absorbance of left and right circularly polarized light is a measure of circular dichroism.

Chapter 4

Mass Spectrometry

Mass spectrometry is an analytical technique that determines the mass of an ionic species by measuring the mass-to-charge (m/z) ratio. In mass spectrometry, an instrument called a **mass spectrometer** is used to determines the m/z ratio by performing four essential steps – *ionization*, *acceleration*, *deflection* or *separation*, and *detection*. In the process of *ionization*, a sample is first converted into gas-phase ions. Once the gas-phase ions are formed, the ions are accelerated so that they all have the same kinetic energy (*acceleration*). A magnetic field then deflects the ions according to their masses (*deflection*). Finally, the beam of ions passing through the machine is detected electrically (*detection*). A mass spectrometer has three basic components: the **ionization source** (generates gas-phase ions), the **mass analyzer** (causes separation of ions) and the **detector** (detects the relative number of ions of each mass).



The operation of the *mass spectrometer* involves the following steps:

1. The particles in the sample (atoms or molecules) are converted in to gas-phase ions.
2. The ions are *accelerated* so that they all have the same kinetic energy.
3. Different ions are *deflected* or *separated* by the magnetic field. The amount of deflection depends on: the *mass* of the ions (lighter ions are deflected more than heavier ones) and the *charge* on the ions. These two factors are combined into the mass-to-charge ratio.
4. Detection of the relative number of ions of each mass.

Ionization process

The particles in the sample (atoms or molecules) studied by mass spectrometer must be converted to gas phase-charged particles by the ionization process before they can be analyzed and detected. However, the term *ionization* is misleading because most ionization processes

do not perform the ionization of sample particles per se. Instead, the term ionization relates to the transfer to the gas-phase of a sample while maintaining its charge and/or acquiring a charge from the sample environment, typically in the form of a proton. Several methods are used for converting the sample to ions. These methods include – electron ionization, chemical ionization, desorption ionization and electrospray ionization. All these ionization processes are fundamentally different techniques, but they achieve essentially the same end result — the generation of gas-phase ions via non-destructive vaporization and ionization.

Electron ionization

In electron ionization, a beam of high-energy electrons strikes the molecules. The high-energy electrons cause the ejection of an electron from the molecule, creating a radical cation. For example, if CH_4 is treated with high electrons, it loses an electron from one of the C–H bonds.

Chemical ionization

In chemical ionization, the sample molecules are combined with an ionized reagent gas. When the sample molecules collide with the ionized reagent gas, some of the sample molecules are ionized by various mechanisms like proton transfer, electron transfer and adduct formation.

Desorption ionization

In desorption ionization, the sample to be analyzed is dissolved in a matrix and placed in the path of high energy beam of ions or high intensity photons. In case of high intensity photons, it is termed as **MALDI** (matrix-assisted laser desorption/ionization). In MALDI, analytes are placed in a light-absorbing solid matrix. A matrix is used to protect the analytes from being destroyed by direct laser beam. Solid matrix strongly absorbs the laser radiation and acts as a receptacle for energy deposition. With a short pulse of laser light, the analytes are ionized and then desorbed from the matrix into the vacuum system. The exact mechanism of the origin of ions in MALDI is still not completely understood. Commonly used matrix materials are aromatic compounds that contain carboxylic acid functional groups. The aromatic ring of the matrix acts as a chromophore for the absorption of laser irradiation leading to the desorption of matrix and analytes into the gas phase.

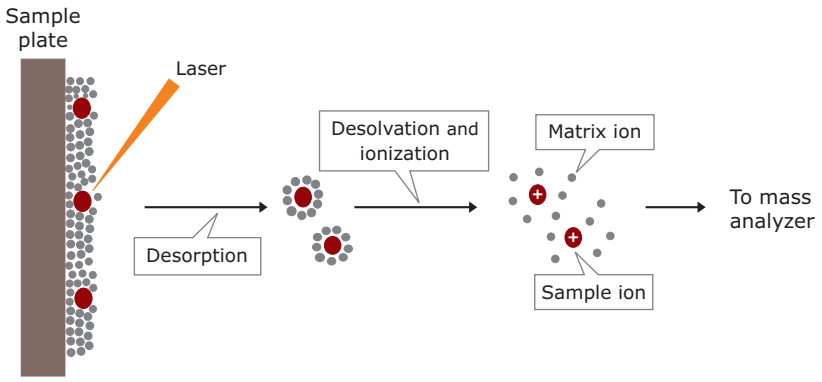


Figure 4.1 Schematic representation of MALDI. It is a mass spectrometry ionization method for directly vaporizing and ionizing non-volatile samples. This method involves three steps. First, the heat-sensitive sample is mixed with the matrix and applied to the metal plate. Secondly, a pulsed laser is used to evaporate the matrix and the sample into the gas phase. Finally, ionization takes place in the gas phase. This is achieved by proton transfer, leading to predominantly $[M + H]^+$ species being produced.

Electrospray ionization

During standard electrospray ionization, the sample is dissolved in a polar, volatile solvent and pumped through a narrow, stainless steel capillary. A high voltage of 3 or 4 kV is applied to the tip of the capillary. As a consequence of this strong electric field, the sample emerging from the tip is dispersed into an aerosol of highly charged droplets. The charged droplets diminish in size by solvent evaporation, assisted by a flow of drying gas. Eventually charged sample ions, free from solvent, are released from the droplets.

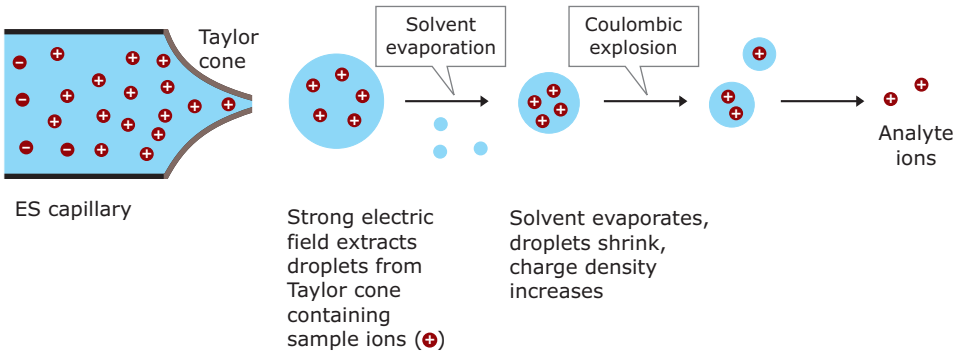


Figure 4.2 Electrospray ionization: The liquid effluent containing the analytes is electrostatically dispersed. This generates highly charged droplets, which are normally positively charged. Once the droplets are airborne, the solvent evaporates, which decreases the size and increases the charge density of the droplets. Desolvated ions are generated by the desorption of sample ions from the droplet surface due to high electrical fields and/or the formation of very small droplets due to repetitive droplet fission until each droplet contains, on average, only one sample ion.

Mass analysis

Once the sample has been ionized, the beam of ions is *accelerated* by an electric field and then passes into the mass analyzer, the region of the mass spectrometer where the ions are separated according to their mass-to-charge ratio. Just like ionization methods, there are several types of mass analyzer. Some examples include **quadrupoles**, **ion traps**, **orbitraps**, **Fourier transform** and **time-of-flight**. The most common type of mass analyzer is **time-of-flight** (TOF) mass analyzer. The TOF mass analyzer measures ion flight time. It is based on the simple idea that velocities of two ions with the same kinetic energy will vary depending on the mass of an ion – the lighter ion will have higher velocity. Because the ions have different velocities, the ions reach the detector at different times. The smaller ions reach the detector first because of their greater velocity as compared to the larger ions. Hence, the analyzer is called **TOF** because the mass is determined from the ion's time-of-flight.

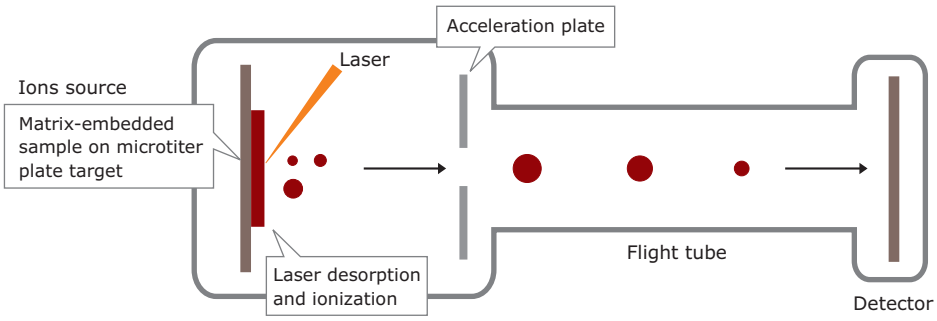


Figure 4.3 Schematic representation of a MALDI/TOF mass spectrometer.

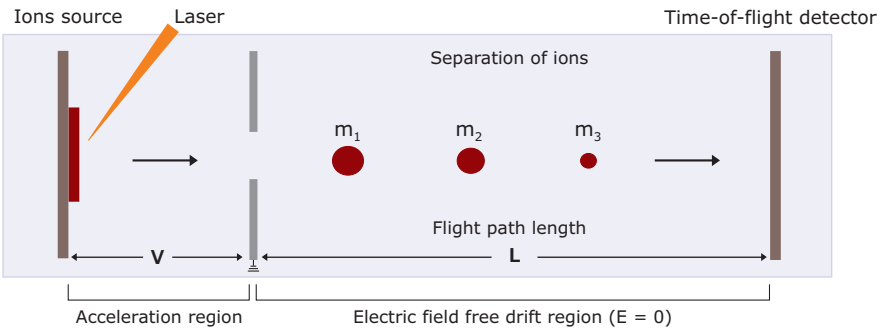


Figure 4.4 In MALDI-TOF mass spectrometry, first desorption and ionization of the sample occurs. The ions formed are then accelerated by an electric field, so that they all have the same kinetic energy. Accelerated ions pass through electric field free drift region. Ions with smaller m/z value move faster through the drift region. Consequently, the time of ions flight differs according to the mass-to-charge ratio of the ions. TOF mass analyzer measures the time these ions take to reach a detector.

Quantification of m/z: Mass-to-charge ratios are determined by measuring the time ions take to move through a field-free region between the source and the detector. Based on classical physics, ions with lower m/z will travel the fastest and arrive at the detector first, while ions with larger m/z will travel the slowest and arrive at the detector last. Once the ion source generates gas-phase ions, these ions are accelerated so that they all have the same kinetic energy. Thus, the potential energy given to ions in the accelerated regions is converted to kinetic energy for all ions.

Potential energy of a charged particle in the electric field = zeV

where, z is the number of charges, e is the amount of charge on an electron and V is the voltage

When an ion is accelerated into a flight tube (or TOF tube) by the voltage V, its potential energy is converted to kinetic energy.

Kinetic energy = $\frac{1}{2}$ mv^2

where, v = velocity of sample ion and m = mass of sample ion

The potential energy is converted to kinetic energy.

Hence, $zeV = \frac{1}{2}$ mv^2

The velocity of the sample ion, $v = \frac{\text{Flight path length (L)}}{\text{Time required to reach the detector (t)}}$

$zeV = \frac{1}{2}$ $mv^2 = \frac{1}{2}$ $m\,(L/t)^2$

$$\frac{m}{z} = 2eV\left[\frac{t}{L}\right]^2$$

Because V and L are known, the m/z of an ion can be determined from the time the ion takes to reach the detector.

Absolute masses
A mass spectrometer does not measure absolute mass, *M*. The instrument needs to be calibrated with standard compounds, whose *M* values are known very accurately.

Application

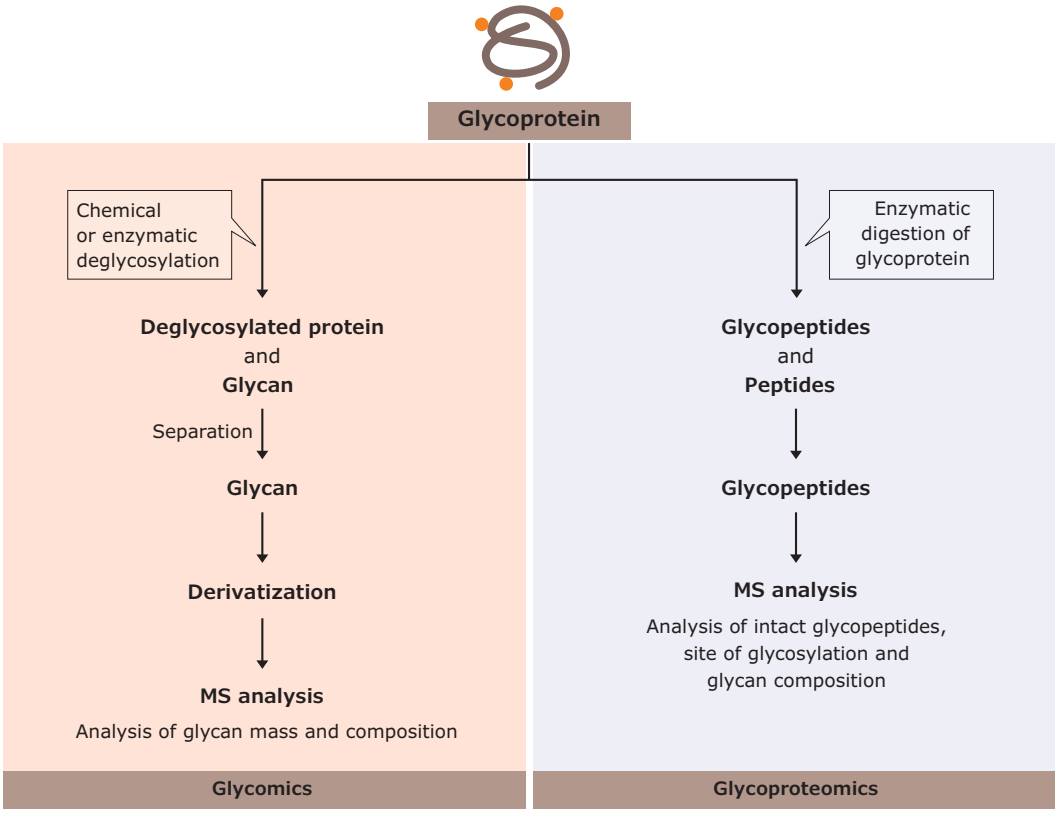
Mass spectrometry is a powerful analytical technique used to determine the molecular mass of a compound. As an essential analytical tool, it has both qualitative and quantitative uses. It is used for three purposes: 1. to determine the mass of an unknown compound, 2. to determine the structure (or a partial structure) of an unknown compound and 3. to confirm the

Glycan

According to IUPAC, the term glycan is used for polysaccharides meaning a compound consists of a large number of covalently linked monosaccharides. However, in general use, this term may also be used to refer to the carbohydrate portion of a glycoconjugate, such as a glycoprotein or a glycolipid, even if the carbohydrate is only an oligosaccharide.

Numerous approaches are used for the characterization of glycoprotein through mass spectrometry. However, two approaches are most common:

- Removal of carbohydrates from glycoprotein by chemical or enzymatic treatment and analysis of the deglycosylated protein and the released carbohydrates separately. The differences in protein–sugar linkage chemistry of *N*- and *O*-glycans require different approaches for the release of covalently-linked carbohydrates.
- Proteolytic digestion of the glycoprotein and analysis of the resulting glycopeptides.



Chapter 5

Centrifugation

Centrifugation is a technique used to separate or concentrate materials suspended in a liquid medium by applying centrifugal force. The separation of particles is based on their differences in sedimentation rate under the centrifugal field. The sedimentation rate of particles depends on a number of different factors, including size, density, and shape. A device that generates centrifugal force by spinning the fluid at high speed and separate particles is called a **centrifuge**. In addition to the separation of molecules, it is also used to measure the physical properties (such as molecular weight, density, and shape). If centrifugation is used to separate one type of material from others, it is termed **preparative centrifugation**; whereas if it is used for measurement of physical properties of materials, then termed **analytical centrifugation**.

Principle of centrifugation

Particles suspended in a solution are pulled downward by Earth's gravitational force. Gravitational force depends only on the mass of the particles and not on charge, shape, and chemical composition. Because the Earth's gravitational field is weak, a solution containing particles of very small masses usually remains suspended due to random thermal motion. Hence, forces much larger than Earth's gravitational force are required to cause appreciable sedimentation of such small masses. Such forces can be obtained by subjecting particles to centrifugation. By applying centrifugal forces, the sedimentation of these particles can be enhanced. A centrifuge does the same thing. It increases the sedimentation by generating centrifugal forces as great as 10,00,000 times the force of gravity.

Let us understand this principle by considering a solution being spun in a centrifuge tube. The centrifugal force acting on a solute particle of mass m,

Centrifugal force = $m\omega^2 r$

where, ω is the angular velocity in radians per second,

r is the distance from the center of rotation to the particle, and

$\omega^2 r$ is the centrifugal acceleration.

A particle will move through a liquid medium when subjected to a centrifugal force. Hence, we must also consider the particle's buoyancy due to the displacement of the solvent molecules by the particle. This buoyancy reduces the force on the particle by $\omega^2 r$ times the mass of the displaced solvent.

Buoyant force = $m_0\omega^2 r$

where, m_0 is the mass of fluid displaced by the particle

$m_0 = m\bar{\nu}\rho$

Where, ρ is the density of the solution (g/mL) and $\bar{\nu}$ is the *partial specific volume* of the particle. As measuring the volume of a very small particle is difficult, for convenience we use a term called partial specific volume. It is the volume in mL that each gram of the particle occupies in solution. Thus, the net force acting on the particle downward is given by,

Net force = Centrifugal force – Buoyant force

$= m\omega^2 r - m_0\omega^2 r$

$= m\omega^2 r - m\bar{\nu}\rho\ \omega^2 r$

When particles move downward through the solution, the motion is also opposed by the frictional force. The frictional force is equal to the product of the *frictional coefficient*, f ($Nm^{-1}s$) and the sedimentation velocity, v. It acts in the opposite direction to the net force.

$F_{friction} = f.v$

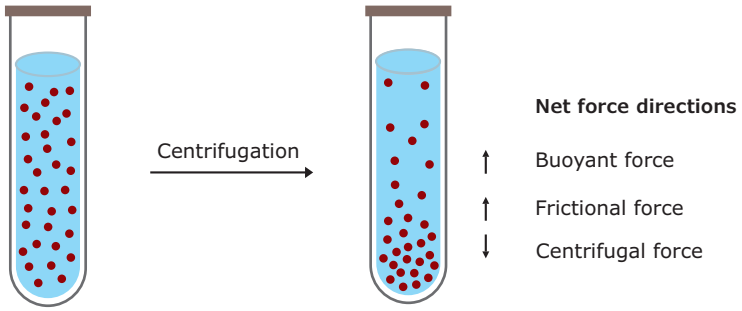


Figure 5.1 Direction of different forces and distribution of solute particles during centrifugation.

At steady state, then, the *frictional force* is equal to the net force and the molecule moves with velocity v downward:

$fv = m\ \omega^2 r - m\bar{\nu}\rho\ \omega^2 r$

$fv = m\ \omega^2 r\ (1 - \bar{\nu}\rho)$

Since, m (mass of a single particle in grams) = M/N.

Where, M is the molar weight of the solute in g/mol and N is the Avogadro's number.

$$fv = \frac{M}{N}\ \omega^2 r\ (1 - \bar{\nu}\rho)$$

$$v = \frac{M\ \omega^2 r\ (1 - \bar{\nu}\rho)}{Nf}$$

Now, the *sedimentation coefficient*,

$$s = \frac{v}{\omega^2 r} = \frac{M}{N}\ \frac{(1 - \bar{\nu}\rho)}{Nf}$$

The **sedimentation coefficient** is the ratio of a velocity to the centrifugal acceleration. The sedimentation coefficient (s) has units of second. A sedimentation coefficient of 1×10^{-13} second is defined as one **Svedberg**, S, ($1S = 10^{-13}$ second). This unit is named for The Svedberg, a pioneer in the field of centrifugation.

RCF describes and compares the strength of the fields generated by different-sized rotors and different operating speeds. A centrifuge with a higher radius of rotation will spin samples at a higher RCF. For example, when revolving at 2000 rpm, a larger centrifuge with a higher radius of rotation will spin samples at a higher RCF than a smaller centrifuge with a shorter radius of rotation.

Fixed-angle or **swing-bucket rotors**

Two types of rotors are commonly used in the laboratory – *fixed-angle* and *swing-bucket* rotors. However, these rotors are fundamentally different in their geometry and consequently in their sedimentation properties. In the following diagram, major differences between these rotors have been shown.

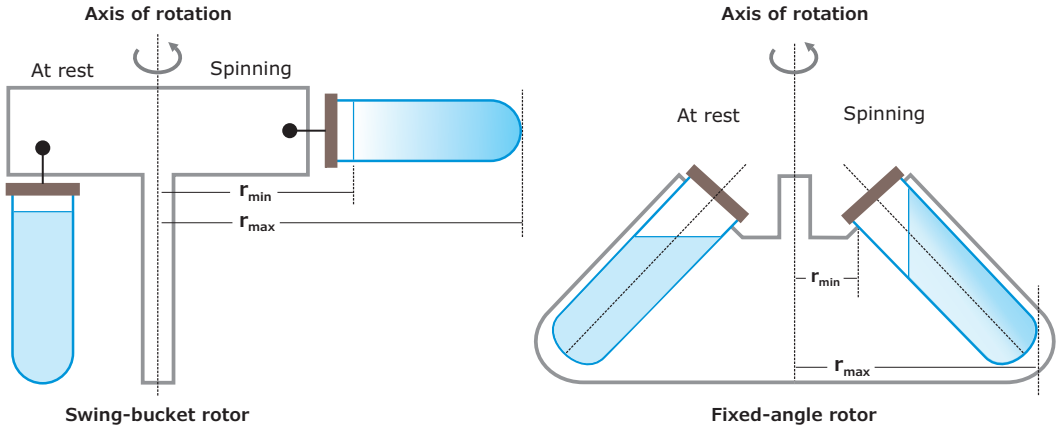


Figure 5.2 Schematic sketches of the two types of rotors: *swinging bucket* and *fixed angle* rotors.

Differential centrifugation

Differential centrifugation is the simplest form of centrifugation. Particles of different densities or sizes present in a suspension will sediment at different rates, with the largest and most dense particles sedimenting fast followed by less dense and smaller particles. These sedimentation rates can be increased by using centrifugal force. This centrifugation process is used mainly for the separation of sub-cellular components.

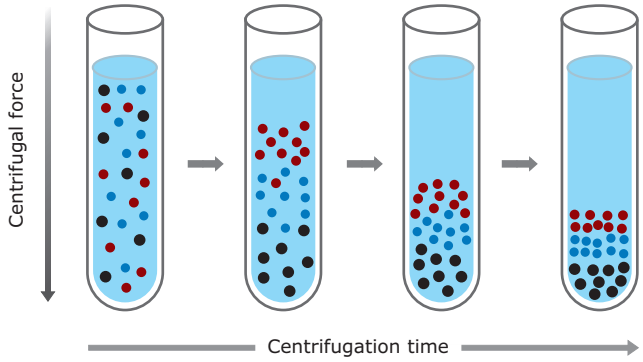


Figure 5.3 Differential centrifugation separates particles based on differences in sedimentation rate, which reflect differences in size or density. Particles which are large or dense sediment rapidly, those that are intermediate in size or density sediment less rapidly, and the smallest or least dense particles sediment very slowly. Eventually, all of the particles reach the bottom of the tube.

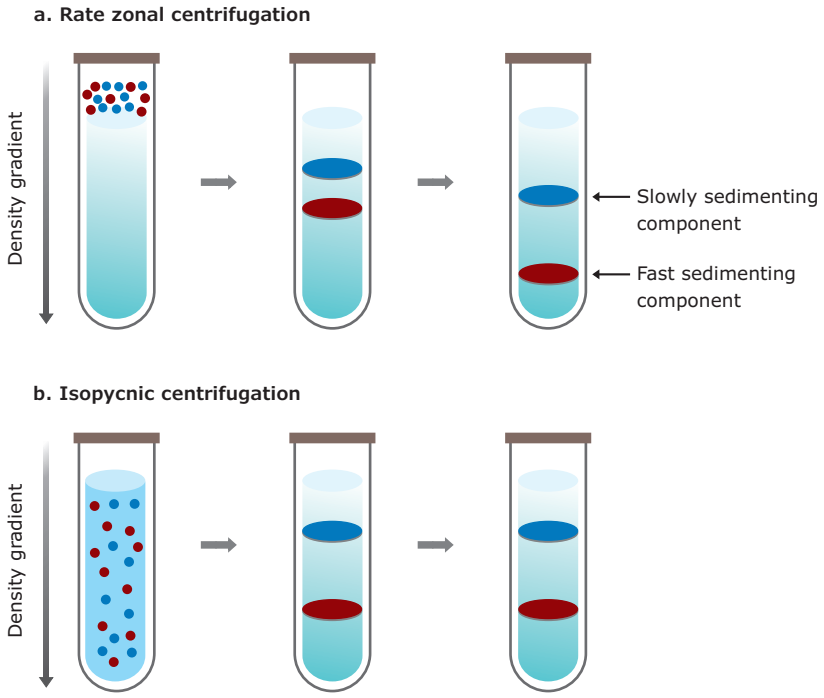


Figure 5.5 (a) Rate-zonal centrifugation is a variation of differential centrifugation in which the sample for fractionation is placed as a thin layer on top of a gradient of solute. The gradient consists of an increasing concentration of solute—and therefore density—from the top of the tube to the bottom. When subjected to a centrifugal force, particles differing in size or density move downward as discrete bands, that migrate at different rates. Because of the gradient of solute in the tube, the particles at the leading edge of each zone continually encounter a slightly denser solution and are, therefore, slightly impeded. As a result, each zone remains very compact, maximizing the resolution of different particles.
(b) Isopycnic centrifugation also includes a gradient of solute that increases in concentration and density, but in this case the solute is concentrated so that the density gradient spans the range of densities of the particles about to be separated. During centrifugation, the particles move into the gradient until each reaches its equilibrium (or buoyant) density—the point in the gradient at which the density of the particle is exactly equal to the density of the gradient.

When macromolecules present in the CsCl solution are centrifuged, they form bands at distinct points in the gradient and exactly where a particular macromolecule forms band will depend on its buoyant density. DNA has a buoyant density of about 1.70 g/cm^3, and therefore it will migrate to the point in the gradient where the CsCl density is also 1.70 g/cm^3. In contrast, protein molecules have much lower buoyant densities and so float at the top of the tube, whereas RNA forms a pellet at the bottom of the tube due to high buoyant density. Isopycnic centrifugation can therefore separate DNA, RNA and protein.

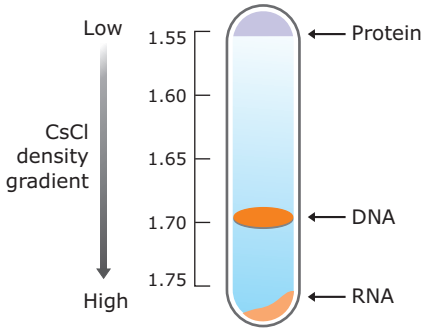


Figure 5.6 CsCl equilibrium density gradient centrifugation. Separation of protein, DNA and RNA in a density gradient.

Chapter 6

Microscopy

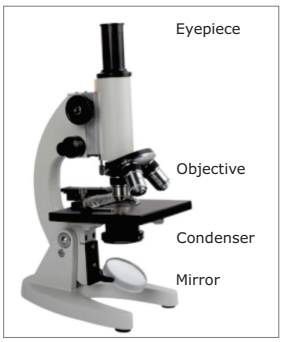
Microscopy is a technique for making very small things visible to the unaided eye. An instrument used to make the small things visible to the naked (unaided) eye is called a **microscope**. There are two fundamentally different types of microscopes based on the source of illumination: the light microscope and the electron microscope.

6.1 Light microscope

Light or *optical microscope* uses visible light as a source of illumination. Because the light travels through the specimen, this instrument can also be called a *transmission light microscope*.

The simplest form of light microscope consists of a single lens, a magnifying glass. Microscope made up of more than one glass lens in combination is termed **compound microscope**. Compound microscope includes the *condenser lens*, the *objective lens* and the *eyepiece lens*. **Condenser lens** focuses the light from the light source at the specimen. The one facing the object is called the **objective** and the one close to the eye is called the **eyepiece**. The objective has a smaller aperture and smaller focal length than those of the eyepiece (also referred to as the **ocular**). A compound microscope with a single eyepiece is said to be **monocular** and one with two eyepieces is said to be **binocular**. The word 'compound' refers to the fact that two lenses, the objective lens and the eyepiece, work together to produce the final magnified image.

The objective lens is responsible for producing the magnified image. It is available in different varieties (4x, 10x, 20x, 40x, 60x, 100x). The power of a lens is described with a number followed by the letter 'x'. For example, if through a microscope one can see something 25 times larger than actual size, its magnification power is 25x. The eyepiece works in combination with the objective lens to further magnify the image. Eyepieces usually magnify by 10x, since an eyepiece of higher magnification merely enlarges the image, with no improvement in resolution.



Compound microscope

In *compound microscope*, the objective lens forms a real and inverted magnified image of the object (called the *real intermediate image*) in the focal plane of eyepiece. This image works as an object for the eyepiece. It magnifies the intermediate image. The image produced by the eyepiece is a magnified virtual image. It is erect with respect to the first image and hence, inverted with respect to the object. The eye views the virtual image created by the eyepiece, which serves as the object for the lens in the eye. The virtual image formed by the eyepiece is well outside the focal length of the eye, so the eye forms a real image on the retina.

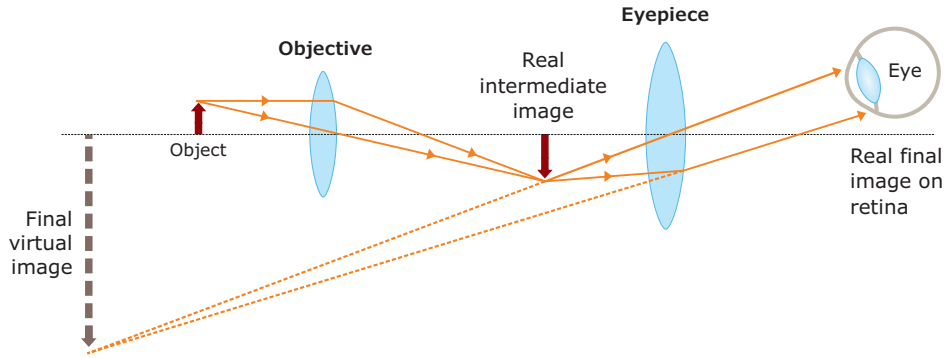
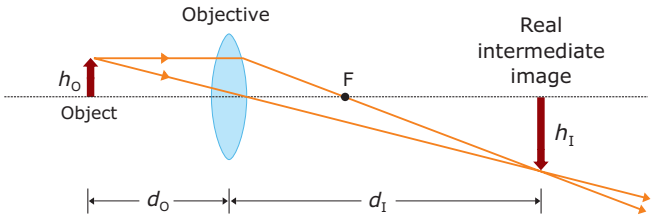


Figure 6.1 Compound microscope and the ray diagram for image formation. It consists of two converging lenses arranged coaxially. The one facing the object is called the objective and the other close to eye is called the eyepiece.

Magnification

The magnification of a compound microscope is the product of the magnification of the objective and the eyepiece. The magnification produced by the *objective lens* is called the **linear magnification** of the compound microscope, because it is measured in linear dimensions. It is the ratio of the image size to the object size.

Linear magnification by objective lens



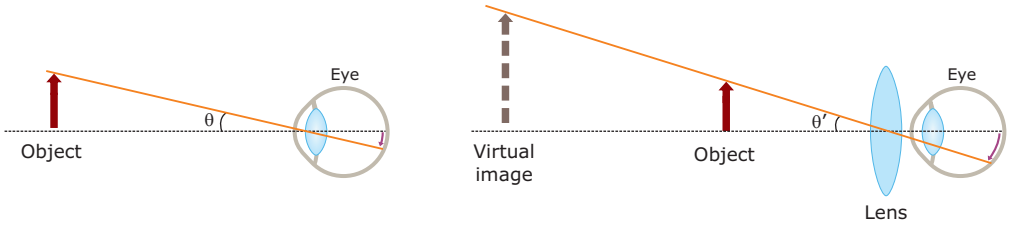
$$\text{Linear magnification} = \frac{\text{Image size}}{\text{Object size}} = \frac{h_I}{h_O} = \frac{d_I}{d_O}$$

The magnification produced by the *eyepiece* is called the **angular magnification**. This is also known as the **magnifying power**. It is the ratio of the angle subtended by the image on the eye when the microscope is used and the angle subtended by the object without microscope (i.e. naked eye).

Magnification means how large the image is as compared to the actual specimen size. In contrast, the **resolution** is the smallest distance below, which we will see two discrete objects as one. Magnification is a function of the number of lenses. Resolution is a function of the ability of a lens to gather light.

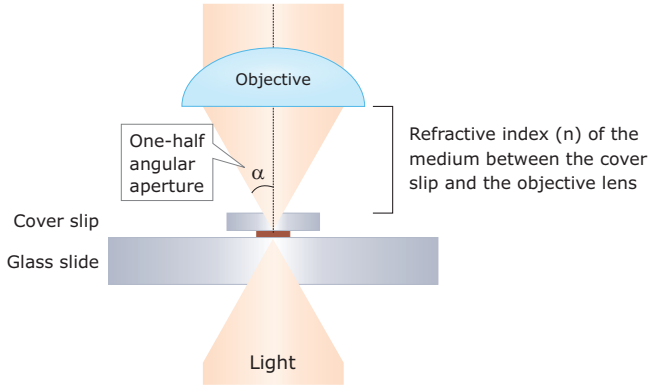
The *maximum magnification* of the compound light microscopes is usually 1500x and has a limit of resolution of about 0.2 μm.

Angular magnification by eyepiece



$$\text{Angular magnification} = \frac{\text{Angle subtended by image } (\theta')}{\text{Angle subtended by object } (\theta)}$$

The overall magnification is the product of the linear magnification of the objective lens and the angular magnification of the eyepiece with the first image at the focal length.



For small value of limit of resolution, the numerator of the equation should be as small as possible (i.e. light of the shortest wavelength) and the denominator should be as large as possible (i.e. maximum possible value of numerical aperture). Hence, resolution can be improved by:

- Increasing the *one-half angular aperture* (α)
- Increasing the *refractive index* (n) of medium between the objective lens and the specimen
- Shortening the *wavelength* (λ) of the illuminating light

Angular aperture

The angular aperture is the angle of the cone of light collected by the objective lens. It varies with the size and shape of the illumination cone entering the objective lens. As the light cones grow larger, the angular aperture increases. The light cone can be increased either by shortening the distance between the objective lens and the specimen or by increasing the diameter of the lens.

Theoretically, the highest angular aperture possible with a standard microscope objective lens would be 180°, resulting in a value of 90° for the one-half angular aperture. Thus, maximum value of $\sin\alpha$ will be 1. The working value of one-half angular aperture for the best objective lenses is about 70°. Hence, the maximum value for $\sin\alpha$ is about 0.94. Since the refractive index of air is about 1.0, no lens working in the air can have a numerical aperture greater than 1.0.

Figure 6.4 Angular aperture and working distance. The higher value of the one-half angular aperture (α) can be obtained by shortening the distance between the objective lens and the specimen.

Refractive index

The refractive index of air is about 1.0, so for a lens designed for use in air, the maximum numerical aperture is about 1.0. To increase the numerical aperture some microscope lenses are designed to be used with a layer of **immersion oil** between the objective lens and the specimen. Immersion oil has a higher refractive index (about 1.5) than air and, therefore,

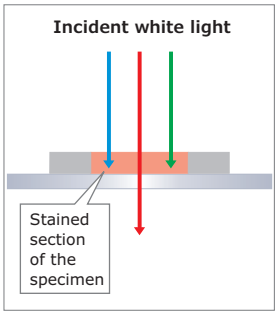
6.2 Types of light microscope

Bright-field microscopy

It is the original and most commonly used form of microscopy. When a specimen is illuminated with light, the transmitted light may undergo change in amplitude, phase and polarization. In bright-field microscopy, illuminating light is transmitted through the specimen and the contrast is generated due to differential absorption of light. In this microscopy, stained specimens or specimens that have natural pigmentation, give color contrast due to differential absorption of illuminating light. Absorption of light may also occur if specimens are thick enough to absorb a significant amount of light despite being colorless (*brightness contrast*). Absorption of light causes decrease in the amplitude of the transmitted light, resulting in **amplitude contrast**.

Dark-field microscopy

A dark-field microscope is a type of microscope in which objects are illuminated at a very low angle from the side so that the background appears dark and the objects show up against this dark background. It is a technique for improving the contrast of unstained, transparent specimens. Central circular disk stop that prevents direct condenser rays from entering the objective lens. To view a specimen in a dark field, a carefully aligned light source is used to minimize the quantity of directly transmitted light and collecting only the light scattered by the specimen. To achieve this an opaque disc is placed underneath the condenser lens, that blocks rays coming from condenser to enter the objective lens directly. Only those rays that have been scattered by the specimen enter the objective lens to generate the final image.



Color contrast in light microscopy. The stained portion of the cell absorbs light of specific wavelengths, depending on the stain types and allow other wavelengths to pass through it. As a result, a colored image of the cell is obtained that is visible in the normal bright-field light microscope.

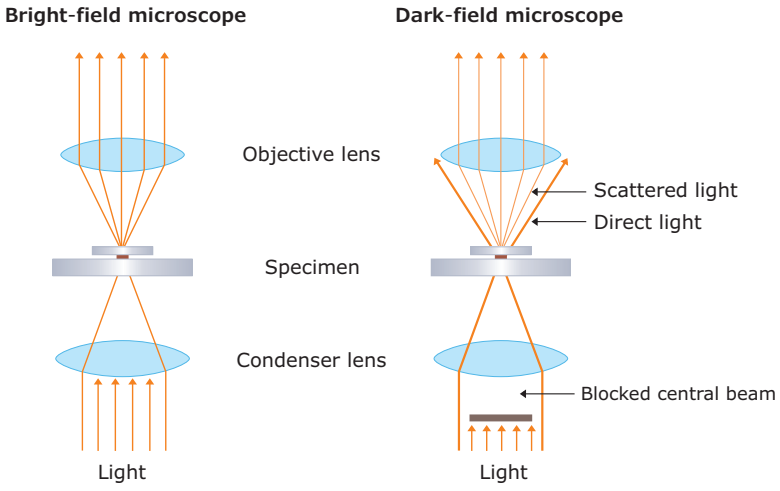


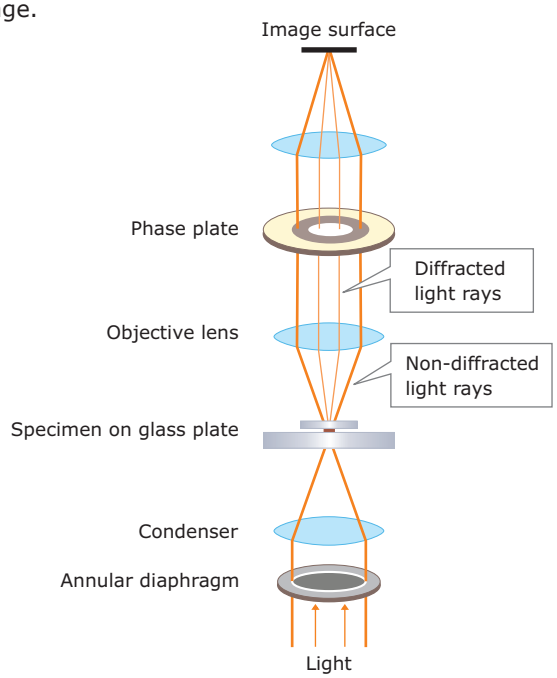
Figure 6.6 In bright-field microscope, the specimen is evenly illuminated by light directly coming from condenser. Light is transmitted through the specimen and the contrast is generated by the differential absorption of light. It forms a dark image against a brighter background. A dark-field microscope uses a dark-field condenser that contains an opaque disk, placed between light source and condenser. Due to presence of opaque disk, light can only pass through the outer edge of the condenser at a wide-angle. Thus, direct light is prevented from passing through the specimen. The specimen is illuminated by oblique light rays. Light passing through the specimen is scattered and only these scattered light reaches the objective lens for visualization. Direct light rays do not enter the objective thus the specimen is brightly illuminated on a dark background. Because there is no direct background light, the specimen appears light against a black background-the dark field.

Phase–contrast microscopy

Phase-contrast microscopy is used to study transparent and colourless specimens. This microscopic technique does not require cells to be killed, fixed or stained. When light passes through a living cell, the phase of the light wave changes due to variations in thickness and density within the cell. Light passing through a relatively thick or dense part of the cell, such as the nucleus, is retarded; its phase, consequently, is shifted relative to light that has passed through an adjacent thinner region of the cytoplasm.

Let us understand this by considering two beams of light from same source, one passing through an optically transparent material denser than air and other simply through air. When one beam of light passes through optically transparent material, its speed decreases and magnitude of change depends on both the density (as determined by the refractive index) and thickness of the material. The speed of second beam of light passing through air remain unchanged. Hence, these two beams, which was in phase are now out of phase; this difference is referred to as the phase difference.

When transparent unstained specimens are used for phase contrast imaging, they do not absorb light and are called **phase objects**. However, when light passes through the specimens, diffraction or scattering of light occurs. The diffracted and the non-diffracted beam of light have phase difference. Non-diffracted light is referred to as direct light as it continues unchanged through the sample. In phase-contrast microscopy additional phase differences is introduced between the non-diffracted and diffracted light. Interference of the two sets of beams leads to overall differences in the amplitude, which can be detected by the eye as differences in brightness. The specimen shows different degrees of brightness and contrast. Thus, phase contrast microscopy transforms differences in the relative phase of light waves to amplitude differences in the image.



Phase contrast micrograph of a human cheek cell.

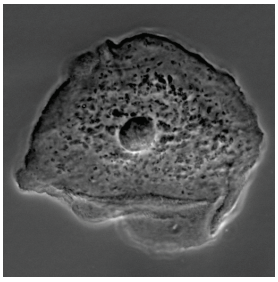


Figure 6.7 Phase contrast microscopy (ray diagram). When light passes through a transparent and colourless specimen, the phase of the light wave changes due to variations in thickness and density within the specimen. The thick lines represent the non-diffracted beams, while the diffracted beams are shown by thin lines. The diffracted light represents only a small part of the total light. This diffracted light beam arrives at the detector out of phase with the non-diffracted light. Phase plate introduces additional phase shift between the non-diffracted and diffracted light. These phase shifts are converted into changes in amplitude and can be observed as differences in image contrasts.

6.3 Electron microscope

The fundamental principles of electron microscopy are similar to those of light microscopy except one major difference of using electromagnetic lenses, rather than optical lenses to focus a high-velocity electron beam instead of visible light. The relationship between the limit of resolution and the wavelength of the illuminating radiation hold true for both – a beam of light or a beam of electrons. Due to the short wavelength of electrons, the resolving power of the electron microscope is very high.

Electron microscopes are of two basic types: *transmission electron microscope* and *scanning electron microscope*. The most commonly used type of electron microscope is called the transmission electron microscope (**TEM**), because it forms an image from electrons that are transmitted through the specimen being examined. Scanning electron microscope (**SEM**) is fundamentally different from TEM, because it produces image from electrons deflected from a specimen's outer surface (rather than electrons transmitted through the specimen).

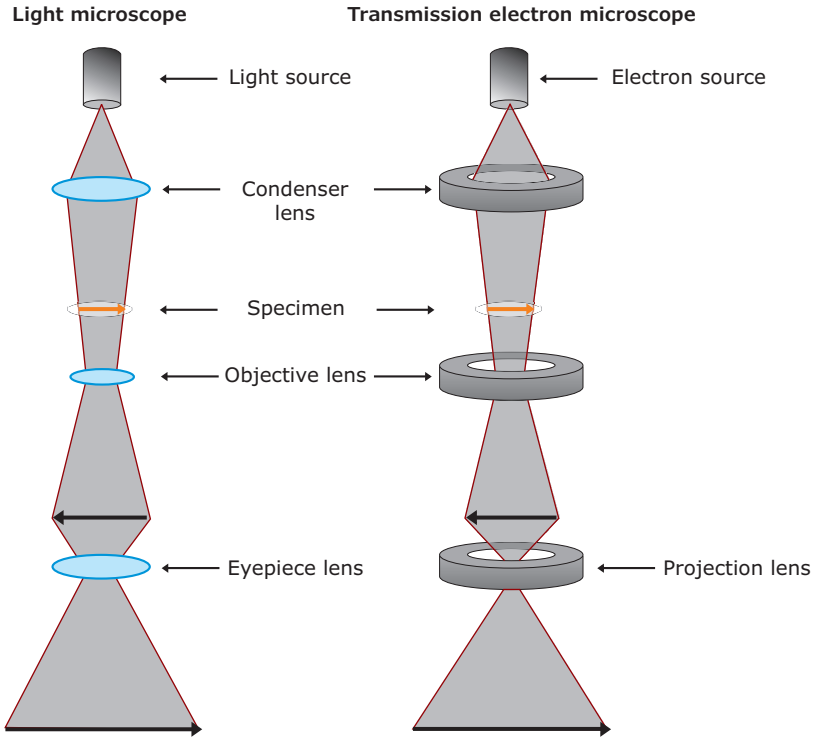


Figure 6.10 Light and electron microscopy.

Electron microscopes consist largely of a tall, hollow cylindrical column through which the electron beam passes. The top of the column contains the cathode, a tungsten wire filament that is heated to provide a source of electrons. Electrons are accelerated as a fine beam by the high voltage applied between the cathode and anode.
Just as a beam of light rays can be focused by a glass lens in light microscopes, a beam of negatively charged electrons can be focused by electromagnetic lenses. The strength of the magnets is controlled by the current provided them. Air is pumped out of the column, producing a vacuum through which the electrons travel. If the air were not removed, electrons would be prematurely scattered by collision with air molecules.

Table 6.1 Differences between light and electron microscopes

Feature	**Light microscope**	**Electron microscope** (**TEM**)
Highest practical magnification	About 1,000-1,500	Over 100,000
Best resolution	0.2 μm	0.5 nm
Radiation source	Visible light	Electron beam
Medium of travel	Air	High vacuum
Type of lens	Glass	Electromagnet
Source of contrast	Differential light absorption	Scattering of electrons
Specimen mount	Glass slide	Metal grid (usually copper)

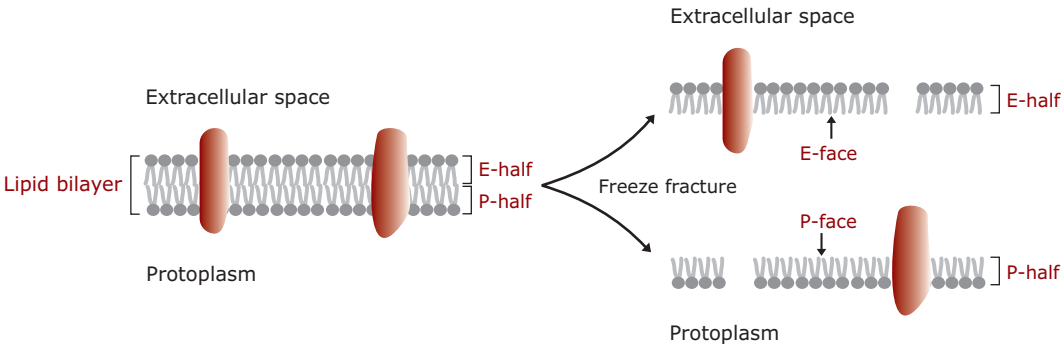


Figure 6.14 Nomenclature for describing the aspects of the plasma membrane revealed by freeze fracture and etching. The membrane comprises a lipid bilayer with intercalated proteins. The half-membrane leaflet adjacent to the extracellular space is termed the E-half and that adjacent to the protoplasm is termed the P-half. The interior views revealed when the membrane is split by freeze fracture are termed faces to distinguish them from the true surfaces. The P-face is the fracture face of the P-half of the membrane and will always have protoplasm beneath it. The E-face is the fracture face of the E-half of the membrane and has extracellular space beneath it.

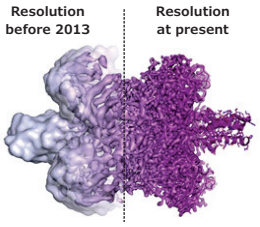
6.6 Cryo–electron microscopy: Resolution revolution

Cryo-electron microscopy (**Cryo-EM**) is a high-resolution structure determination technique of biomolecules in solution. It is an electron microscope imaging of frozen biological samples kept at cryogenic temperatures.

As we know, transmission electron microscopes (**TEMs**) use a beam of electrons to examine the structures of biomolecules such as proteins at the atomic scale. As the beam is transmitted through a biological sample, it interacts with the samples, which projects an image of the sample onto the detector. Image formation depends on the differential scattering of electrons by different parts of the sample. Due to the short wavelength of the electron beam, the resolving power of the electron microscope is very high. But biological samples are not compatible with the high-vacuum conditions and high-energy electron beams used in TEMs. There are two reasons for this. *First*, the high-energy electron beam burns and destroys the biological sample. *Second*, the water that surrounds the biological sample evaporates and the vapour from the sample destroys the vacuum required to run the microscope. To overcome this, the biological sample could be dried out before being examined, but this can alter their structure.

Cryo-EM uses frozen samples and low-energy electron beams to overcome these problems. But low-energy electron beam would result in fuzzier images. This problem has been overcome by the use of a powerful image-analysis algorithm for merging multiple fuzzy 2D images from an electron microscope to create a 3D image of a molecule. This involves taking thousands of images of randomly oriented molecules and sorting them into groups of similar images. Each group is then processed to create a set of much sharper images. The spatial relationships between the groups are then calculated, leading to the assembly of a high-resolution 3D image.

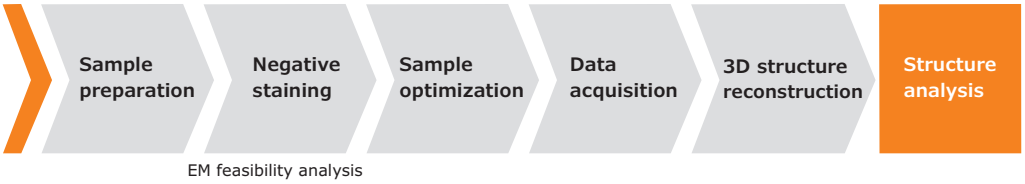
Since liquid water evaporates in the case of TEM, thus to obtain an image using cryo-EM, biological samples are rapidly frozen at –190°C. Liquid nitrogen-cooled ethane freezes the thin film of water on the sample so quickly that the water molecules don't have time to arrange into a crystalline lattice. Due to rapid freezing, the water forms a disordered glass rather than crystalline ice. This **vitrification** of water is crucial because it does not cause electron diffraction and also allows the samples to retain their natural shape and organization. If the sample



Protein structure: shapeless blobs to atomic resolution. © Martin Högbom, The Royal Swedish Academy of Sciences.

is frozen too slowly, then ice crystals form and the ordered ice crystals would strongly diffract the electron beam. Once the cryo-EM sample has been frozen, a focused beam of electrons reveals the shape of these very small, nanometer-sized 'vitrified' biological samples. Each image contains all the information required to determine the 3D structure. Specialized computer analysis then combines hundreds of thousands of individual, 2D snapshots from different angles into a composite that can be viewed in 3D.

Steps involved in structure determination by single particle cryo-EM



Cryo–EM Vs X–ray crystallography

X-ray crystallography gives very high-resolution structures of biomolecules. X-ray crystallography uses X-ray to determine the position and arrangement of atoms in a crystal. It is based on **X-ray diffraction**. When a beam of X-ray strikes a crystal, the beam may be diffracted. From the angles and intensities of these diffracted beams, a three-dimensional picture of the density of electrons within the crystal can be derived. But to get an x-ray diffraction structure, the biomolecule such as protein should be crystallised. Many proteins won't crystallise at all. And in some cases, the process of crystallisation alters the structure, so it's not representative of what the molecule looks like in real life. Cryo-EM doesn't require crystals, and it also enables scientists to see how biomolecules move and interact as they perform their functions.

Cryo–EM Vs NMR

NMR can also give very detailed structures, and investigate conformational changes or binding of small biological molecules. But it's limited to relatively small molecular mass (<30 kDa) and usually soluble intracellular molecules, rather than those membrane bound. If someone want to study high molecular mass proteins, membrane-bound receptors, or complexes of several biomolecules together, cryo-EM is where it's at.

6.7 Scanning probe microscopes

Scanning probe microscopes (**SPMs**) are a family of microscopes used to map topographical features of the sample surface at atomic level (resolution in nanometer scale). In all SPMs, a physical probe is used to scan the surface of a sample. During the scanning process, interactions of the probe tip with the surface of a sample are measured and data are used to generate an image of the surface. Various types of interaction between the probe tip and the surface are exploited in different types of SPMs. Hence, there are several types of SPMs. **Scanning tunneling microscope** (**STM**) and **Atomic force microscope** (**AFM**) are two major types of SPMs. AFMs measure the interactive forces between the probe tip and the sample whereas STMs measure the tunneling current flowing between the probe tip and the conducting sample. Application of the STM for surface imaging requires conductive sample. Hence, most biological samples due to poor electron conductivity are not suitable for STM imaging. Unlike STMs, the AFM does not need a conducting sample.

Scanning tunneling microscope

Historically, the first microscope in the family of SPMs is the scanning tunneling microscope (STM). It was invented by Binning and Rohrer in 1981. STM is based on the concept of electrons tunneling through a narrow potential barrier between a metal tip and a conducting sample in an external electric field. If the probe tip and sample are connected to a voltage source and the tip is close enough to the sample surface and the sample is electrically conductive, electrons will begin to leak or tunnel across the gap between the probe and the sample. This current can be measured, and the magnitude depends on the distance between the tip and the surface. The high spatial resolution of the STM is due to the exponential dependence of the tunneling current on the tip-sample distance.

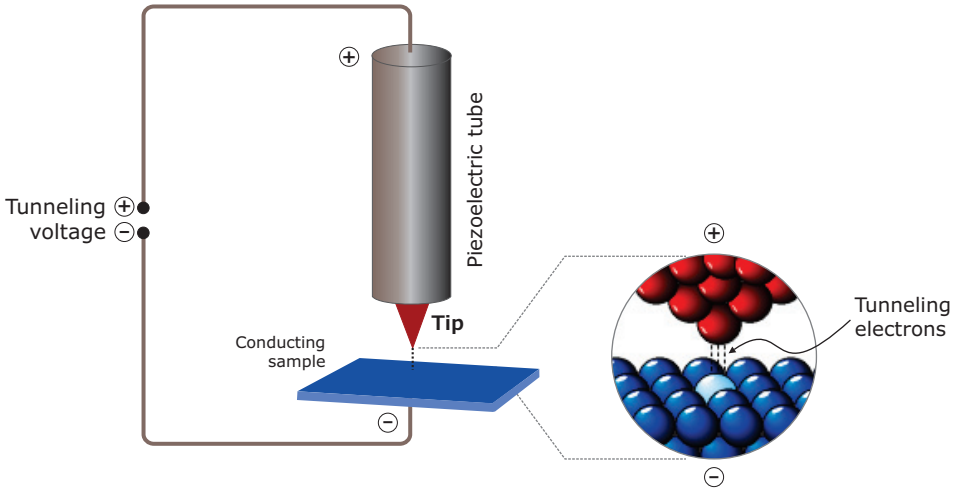


Figure 6.15 The metal probe tip and the sample are both connected to a voltage supply. The sample is scanned by a very fine metallic probe tip mounted on a piezoelectric tube. A tunnelling current starts to flow when the tip is close to the sample. This feeble tunneling current is amplified and measured.

Atomic–force microscope

Atomic-force microscope (**AFM**) is based on the principle of the measurement of the interactive forces between a probe tip and a sample surface. The interactive forces (like capillary forces, electrostatic forces, van der Walls forces, etc.) have been classified as short- or long-range forces, attractive or repulsive forces. As the tip scans the surface, the interactions between the tip and the sample experience attractive or repulsive forces. This causes the tip to move up and down, which in turn causes the entire **cantilever** to deflect. The attractive force between the surface and the tip causes the cantilever to deflect towards the surface whereas repulsive force causes the cantilever to deflect away from the surface. By measuring the deflection of the cantilever, the topographical features of the surface can be mapped out.

Atomic force microscopy does not use lenses to form an image, but instead uses a sharp probe tip at the end of a flexible cantilever to scan and sense the topography of a sample.

The methods used in AFM to acquire images can be categorized in three groups: *contact mode, tapping* or *intermittent mode* and *non-contact mode*. In **contact mode**, the probe tip directly touches the sample surface during scanning and the force acting between the atoms of probe tip and sample is counterbalanced by the elastic force. The probe tip predominately experiences repulsive forces. The contact mode may be carried out either at *constant force* (fixing the tip–sample interaction force through a feedback loop) or at *constant distance* between probe tip and sample (fixing the height of the scanner). A drawback of contact modes is the

direct mechanical interaction of the probe tip with the sample. The **tapping mode** is similar to contact mode. However, in this mode intermittent contact occurs between the probe tip and the sample surface during scanning. The probe tip experiences both attractive and repulsive forces. The cantilever is oscillated at its resonant frequency. *Resonant frequency* is a natural frequency of vibration determined by the physical parameters of the vibrating object. By maintaining a constant oscillation amplitude, a constant tip-sample interaction is maintained and an image of the surface is obtained. In **non-contact mode**, probe tip does not contact the sample surface, but oscillates above the surface during scanning. In this mode, the probe tip predominately experiences attractive forces.

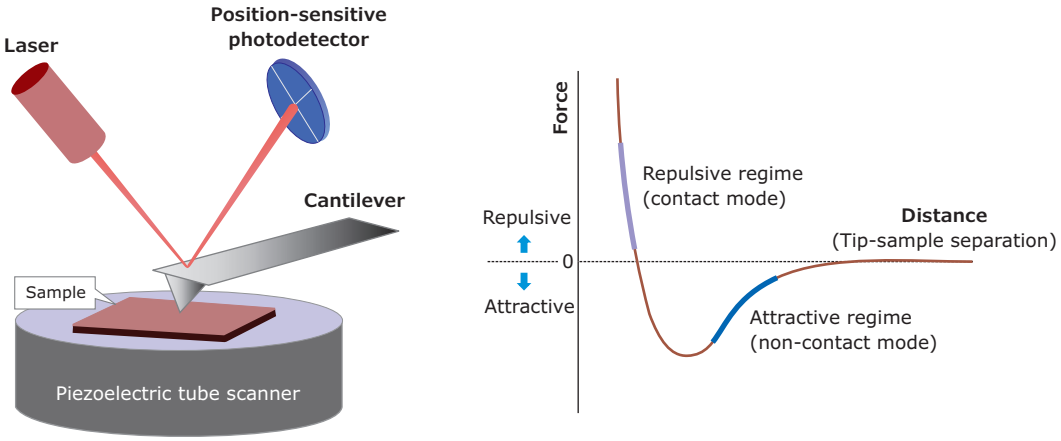


Figure 6.16 **A.** Schematic diagram of an AFM. The essential elements of an AFM are: a probe tip attached to a cantilever; a laser deflection system to measure deflections of the cantilever; a piezoelectric tube scanner for moving the sample and probe relative to each other. The deflection of the cantilever in response to interactions between probe tip and surface is detected by a laser deflection system. It measures the position of a laser on the photodetector which is reflected from the back of a cantilever. **B.** Tip–sample interaction force curve. Graph shows different force regimes which correspond to inter-atomic forces. As seen in this figure, the atoms are separated by a large distance on the right side of the curve. As the atoms are brought close, they attract each other. This attraction increases until they come so close together that their electrons begin to repel each other electrostatically. This repulsive electrostatic force exceeds the attractive force as the inter-atomic distance decreases.

References

Alberts B et al (2008), *Molecular Biology of the Cell*, 5th ed. Garland Science Publishing.

Bai XC, Yan C, Yang G et al (2015), An atomic structure of human γ-secretase, Nature, 525, 212.

Becker WM, Kleinsmith LJ and Hardin J (2006), *The world of the cell*, 6th ed. Pearson Education Inc.

Behrmann E, Loerke J, Budkevich TV et al (2015), Structural snapshots of actively translating human ribosomes, Cell, 161, 845.

Bozzola JJ and Russell LD (1998), *Electron Microscopy: Principles and Techniques for Biologists*, 2nd ed. Jones and Bartlett Publishers, Inc.

Hoppert M (2003), *Microscopic Techniques in Biotechnology*, Wiley-VCH Verlag.

Lodish H, Berk A, Kaiser CA et al (2008), *Molecular Cell Biology*, 6th ed. W.H. Freeman and Company.

Merk A, Bartesaghi A, Banerjee S et al, (2016), Breaking Cryo-EM Resolution Barriers to Facilitate Drug Discovery, Cell 165, 1698.

Pawley J (2006), *Handbook of Biological Confocal Microscopy*, 3rd ed. Springer.

Sirohi D, Chen Z, Sun L et al (2016), The 3.8 Å resolution cryo-EM structure of Zika Virus, Science, 352, 467.

Verma HC (1999), *Concept of Physics*, Bharati Bhawan, India.

Chapter 7

Flow cytometry

Flow cytometry is a technique for counting, examining, and sorting microscopic objects suspended in a fluid based on their optical properties (scattering and fluorescence). It simultaneously measures and then analyzes multiple physical characteristics of single object (usually cell) as they flow in a fluid stream through a beam of light.

A flow cytometer is made up of three main systems: fluidics, optics, and electronics. The *fluidics system* transports cells in a stream to the laser beam for interrogation. The *optics system* consists of lasers to illuminate the cells in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors. The *electronics system* converts the detected light signals into electronic signals that can be processed by the computer.

Fluidics system

Flow cytometer measures the physical and chemical characteristics of a cell. This can be done by introducing cells present in suspension to go through an observation point one at a time. At this observation point, using a specific light beam, we interrogate each cell and observe the light responses electronically. Flow cytometers use the principle of hydrodynamic focusing for presenting cells one at a time to a light source.

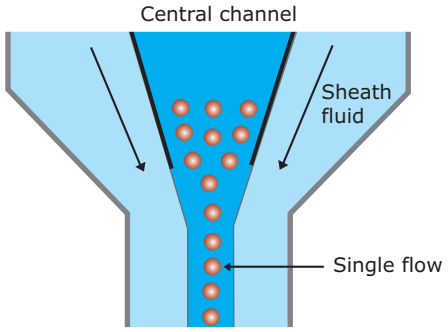


Figure 7.1 Hydrodynamic focusing produces a single stream of particles.

The fluidics system consists of a central channel through which the sample is injected, enclosed by an outer sheath that contains faster flowing fluid. As the sheath fluid moves, it creates a massive drag effect on the narrowing central chamber. This alters the velocity of the central fluid whose flow front becomes parabolic with the greatest velocity at its center and

zero velocity at the wall. The effect creates a single stream of particles or cells and is called *hydrodynamic focusing*. Under laminar flow conditions, the fluid in the central chamber will not mix with the sheath fluid.

Optics

After hydrodynamic focusing, each cell passes through a beam of light. Light scattering or fluorescence emission (if the particle is labeled with a fluorochrome) provides information about the cell's properties. Scattered and emitted light from cells are converted to electrical pulses by optical detectors.

Flow cytometers routinely measure light scattered at two different angles (forward and side). Forward scatter is measured in the same direction as the laser beam is moving. Side scatter is measured at the 90° relative to the primary laser beam.

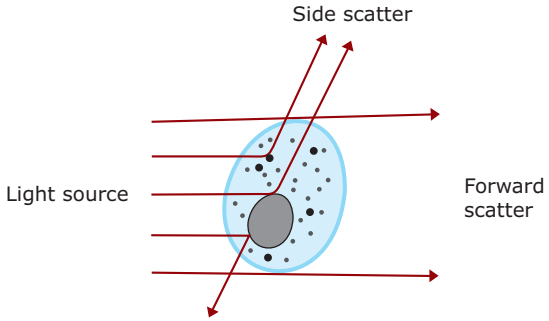


Figure 7.2 Diagram of forward and side scatter. The amount of light scattered in a forward direction is an indicator of the relative size of a cell. This is referred to as *forward scatter.* Likewise, light scattered at higher angles gives rise to information about internal complexity and granularity of a cell, such as cytoplasmic granules, multi-lobed nucleus, cytoplasmic vacuoles, etc. This is referred to as *side scatter*.

Light that is scattered by an object is detected by different detectors. One detector is placed in line with the light beam to measure the **forward scatter** (FSC) from the objects. Detectors perpendicular to the beam measure **side scatter** (SSC) and fluorescence. Both FSC and SSC are unique for every object, and a combination of the two may be used to differentiate different objects in a heterogeneous sample. Forward scatter is based on two properties - size and refractive index.

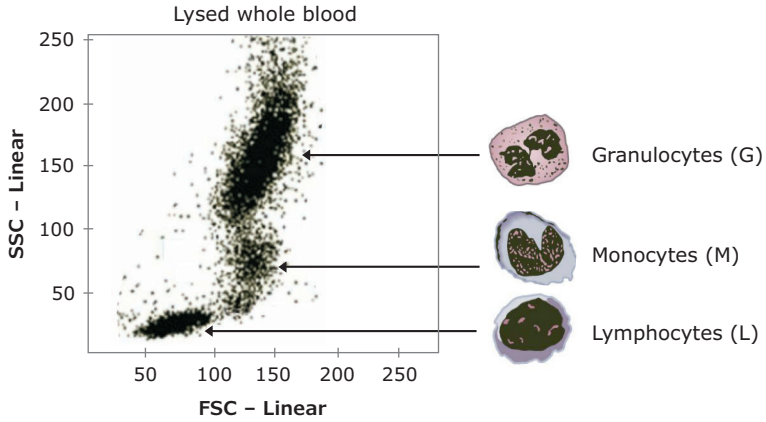


Figure 7.3 The different physical properties of granulocytes, monocytes and lymphocytes allow them to be distinguished from each other and from cellular contaminants. Forward and side scatter can be used to distinguish different cell types in human blood. In this plot, three blood cell populations can be identified based on their light scattering. Each dot represents a cell, and its location in the graph corresponds to the amount of light scattered by the cell.

References

Alberts B et al (2008), *Molecular Biology of the Cell*, 5th ed. Garland Science Publishing.

Baumgarth N and Roederer M (2000), A practical approach to multicolor flow cytometry for immunophenotyping. *J Immunol Methods* 243:77-97.

Nunez R (2001), DNA measurement and cell cycle analysis by flow cytometry. *Curr. Issues Mol. Biol* 3(3): 67-70.

Ormerod MG (2000), *Flow Cytometry. A practical approach*. 3rd ed. Oxford University Press.

Riccardi C and Nicoletti I (2006), Analysis of apoptosis by propidium iodide staining and flow cytometry, *Nature Protocols* 1458-1461.

Shapiro HM (2003), *Practical Flow Cytometry*, John Wiley and Sons, Inc.

Watson JV (2005), *Flow Cytometry Data Analysis: Basic Concepts and Statistics,* Cambridge University Press.

Chapter 8

X–ray crystallography

X-ray crystallography is a method of determining the arrangement of atoms within a crystal. This technique is based on **X-ray diffraction**, a nondestructive technique. When a beam of X-ray strikes a crystal, the beam may be diffracted. From the angles and intensities of these diffracted beams, a three-dimensional picture of the density of electrons within the crystal can be derived. From this electron density, the mean positions of the atoms in the crystal can be determined. This method acts as an atomic microscope, using X-rays instead of visible light to determine the three-dimensional structure of crystals. X-rays cannot be focused by lenses to form an image of a molecule. Instead, the X-rays are diffracted from a single crystal. This technique requires three distinct steps:

1. growing a crystal,
2. collecting the X-ray diffraction pattern from the crystal, and
3. constructing and refining a structural model to fit the X-ray diffraction pattern.

When X-rays interact with a single particle, it scatters the incident beam uniformly in all directions. However, when X-rays interact with a solid material, the scattered beams can add together in a few directions and reinforce each other to yield *diffraction*. The regularity of the material is responsible for the diffraction of the beams. Hence, for X-ray crystallography, molecule must be crystallized. A **crystal** is built up of many billions of small identical units called **unit cells**. The *unit cell* is the smallest and simplest volume element that is completely representative of the whole crystal. Each unit cell may contain one or more molecules. A *crystal* is a stacking of unit cells repeated in three dimensions to build a *lattice*, leaving no space between the unit cells.

The terms **diffraction** and **scattering** are often used interchangeably and are considered to be almost synonymous. Scattering simply refers to the ability of objects to change the direction of a wave. Diffraction describes a specialized case of light scattering in which an object with regularly repeating features produces an orderly pattern.

Theory of X–ray diffraction

A molecular structure resolved at atomic level means that the positions of each atom can be distinguished from those of all other atoms in three-dimensional space. The closest distance between two atoms in space is the length of a covalent bond, and a typical length of a covalent bond is approximately 0.12 nm. If we require to resolve the atoms of a macromolecule, the wavelength of light required for our atomic microscope would necessarily be <0.24 nm. This falls into the X-ray range of the electromagnetic spectrum. However, X-rays cannot be focused and thus cannot form an image of an object in the same manner as a light microscope. We rely on the constructive and destructive interference caused by scattering radiation from the regular and repeating lattice of a single crystal to determine the structure of macromolecules.

A good way to understand X-ray diffraction is to draw an analogy with visible light. Light has certain properties that is best described by considering wave nature. Whenever wave phenomena occur in nature, interaction between waves can occur. If waves from two sources are in same phase with one another, their total amplitude is additive (**constructive interference**); and if they are out of phase, their amplitude is reduced (**destructive interference**). This effect can be seen in figure 8.1 when light passes through two pinholes in a piece of opaque material and then falls onto a white surface. Interference patterns result, with dark regions where light waves are out of phase and bright regions where they are in phase.

If the wavelength of the light (λ) is known, one can measure the angle α between the original beam and the first diffraction peak and then calculate the distance d between the two holes with the formula

$$d = \frac{\lambda}{\sin\alpha}.$$

The same approach can be used to calculate the distance between atoms in crystals. Instead of visible light, which has longer wavelength to interact with atoms, we can use a beam of X-rays. X-rays, like light, are a form of electromagnetic radiation, but they have a much smaller wavelength. The wavelengths of X-rays (typically around 0.1 nm) are of the same order of magnitude as the distances between atoms or ions in a molecule or crystal. If a narrow beam of X-rays is directed at a crystalline solid, most of the X-rays will pass straight through it. A small fraction, however, scatters by the atoms in the crystal. The electrons of an atom are primarily responsible for the scattering of X-rays. The number of electrons in a given volume of space (the *electron density*) determines how strongly an atom scatters X-rays. The interference of the scattered X-rays leads to the general phenomenon of *diffraction*.

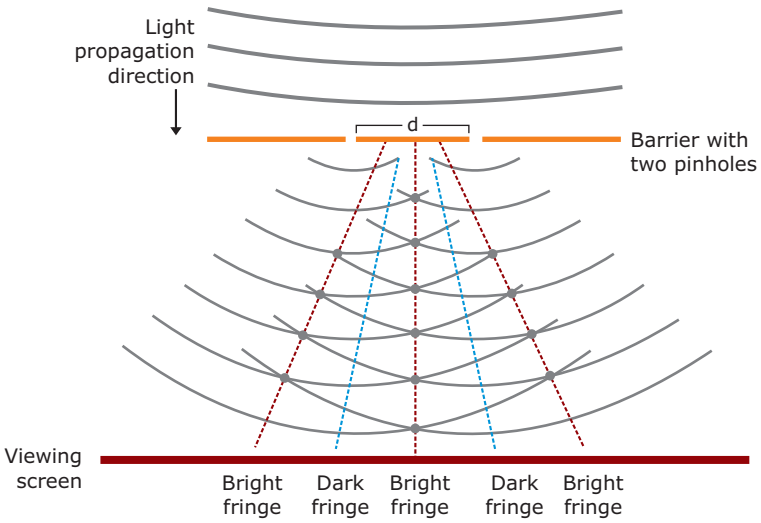


Figure 8.1 Diffraction patterns. Any energy in the form of waves will produce interference patterns if the waves from two or more sources are superimposed in space. One of the simplest patterns can be seen when monochromatic light passes through two neighbouring pinholes and is allowed to fall on a screen. When the light passes through the two pinholes, the holes act as light sources, with waves radiating from each and falling on a white surface. When the waves are in the same phase, a bright fringe appears (*constructive interference*), but when the waves are out-of-phase they cancel each other out, producing dark fringe (*destructive interference*).

Refraction refers to the bending of light rays due to differences in refractive indices when it moves from one medium to another, while **diffraction** is a phenomenon that is often described as 'waves bending around corners'.

Chapter 9

Patch clamp techniques

The introduction of the patch clamp technique has revolutionized the study of cellular physiology by providing a method of observing the function of individual ion channels in a variety of cell types. It permits high resolution recording of the ionic currents flowing through a cell's plasma membrane.

The patch clamp technique has been invented by Sakmann and Neher in the 1976, for which they received the Nobel Prize in Physiology and Medicine in 1991. This technique is based on a very simple idea. A micropipette with a very small opening is used to make tight contact with a small area, or patch, of cell membrane. After the application of a small amount of suction to the back of the pipette, the contact between pipette and membrane becomes so tight that no ions can flow between the pipette and the membrane. Thus, all the ions that flow when a single ion channel opens must flow into the pipette. The resulting electrical current, though small, can be measured with an ultra-sensitive electronic amplifier connected to the pipette. Based on the geometry involved, this arrangement usually is called the **cell-attached patch clamp recording**.

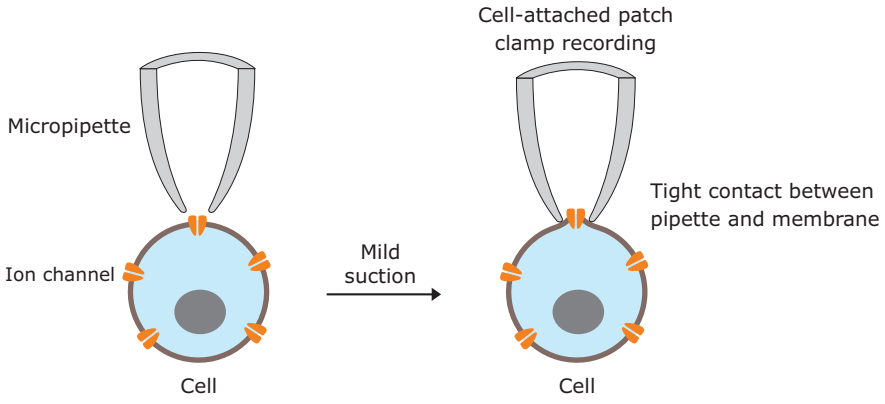


Figure 9.1 Cell-attached patch clamp recording. When the pipette is in closest proximity to the cell membrane, mild suction is applied to gain a tight seal between the pipette and the membrane.

In order to form the cell-attached mode, a pipette tip is placed on the surface of the cell, forming a low resistance contact (seal) with its membrane. Slight suction applied to the upper end of the pipette results in formation of a tight seal. Such a seal with a resistance in the range of gigaohms is called '**giga-seal**'. In the cell-attached mode, recordings are made from the membrane area under the pipette, while the structure of the cell remains intact. A metal

electrode inside the glass pipette, containing a salt solution resembling the fluid normally found within the cell, accomplishes transduction of the ionic current into the electrical current while another one in the bath solution serves as ground. Pt and Ag/AgCl electrodes are especially used for their low junction potentials and weak polarization. The tight seal between pipette and cell membrane isolates the membrane patch electrically, which means that all ions fluxing the membrane patch flow into the pipette and are recorded by an electrode connected to a highly sensitive electronic amplifier. A bath electrode is used to set the zero level.

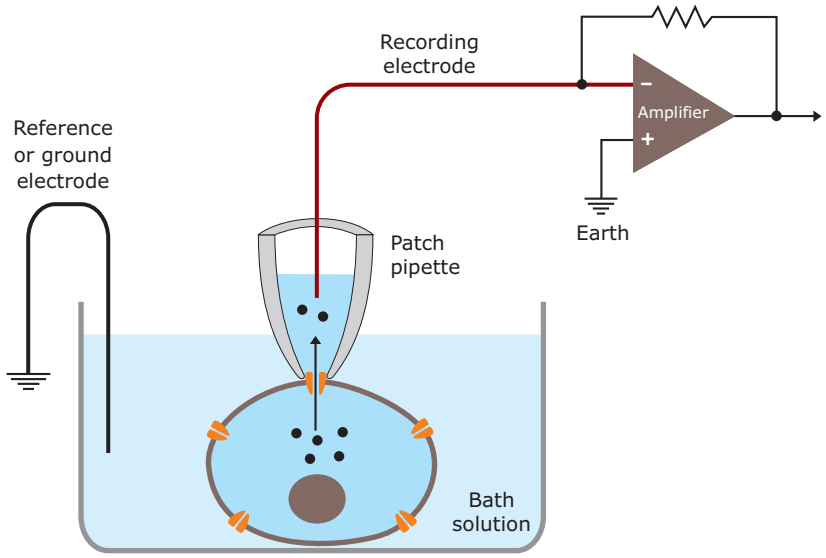


Figure 9.2 General principle of patch-clamp recordings. A micropipette containing electrolyte solution is tightly sealed onto the cell membrane and, thus, isolates a membrane patch electrically. Currents fluxing through the channels in this patch, hence, flow into the pipette and can be recorded by an electrode that is connected to a highly sensitive differential amplifier. In the voltage-clamp configuration, a current is injected into the cell via a negative feedback loop to compensate changes in membrane potential. Recording of this current allows conclusions about the membrane conductance.

Recording mode

Patch clamp technique can be operated in one of two modes – *current clamp* and *voltage clamp* modes. **Current clamp** mode applies a constant current at the tip of the pipette, and measures the membrane potential over time. In contrast, **voltage clamp** mode holds a cell (or patch of membrane) at a specified potential, and measures the current required to maintain that set voltage. The selection of voltage clamp or current clamp depends on the type of information the investigator wishes to gather. Broadly, voltage clamp is ideal for characterizing individual ion channel properties whereas, the current clamp is more commonly used to study how a cell responds when electrical current enters in it. The current clamp mode allows to monitor different forms of cell activity, for example, action potentials, excitatory and inhibitory post-synaptic potentials as well as changes in membrane potentials due to activation of electrogenic membrane transporters.

Most patch-clamp measurements are performed in a *voltage clamp mode*. Voltage clamp allows an experimenter to 'clamp' the cell membrane potential (voltage) at a chosen value. This makes it possible to measure voltage specific activity of ion channels. The voltage-gated channels are primarily studied in this mode. Since the introduction of the patch clamp technique by Neher and Sakmann in 1976, patch clamp most often means voltage clamp of a membrane patch.

Chapter 10

Immunotechniques

10.1 Immunoprecipitation reaction

Antigen-antibody interaction is highly specific and occurs in a similar way as a bimolecular association of an enzyme and a substrate. The binding between antigen (Ag) and antibody (Ab) involves weak and reversible non-covalent interactions consists mainly of van der Waals forces, electrostatic forces, H-bonding and hydrophobic forces. The smallest unit of antigen that is capable of binding with antibodies is called an *antigenic determinant* (or **epitope**). The corresponding area on the antibody molecule combining with the epitope is called **paratope**. The number of epitopes on the surface of an antigen is its **valence**. The valence determines the number of antibody molecules that can combine with the antigen at one time. If one epitope is present, the antigen is **monovalent**. Most antigens, however, have more than one copy of the same epitope and are termed **polyvalent**.

Immunoprecipitation reaction results from the interaction of a *soluble* antibody with a *soluble* antigen to form an *insoluble* complex. Antibodies that aggregate soluble antigens are called **precipitins**. Formations of an antigen-antibody lattice depend on the valency of both antibody and antigen. The antibody must be bivalent for precipitation reaction to occur. Monovalent Fab fragments cannot form precipitate with antigen. Similarly, the antigen must be either bivalent or polyvalent. If the antigen is bi- or polyvalent, it can bind with multiple antibodies. Eventually, the resulting cross-linked complex becomes so large that it falls out of solution as a precipitate. Immunoprecipitation reaction can be performed in *solution* or in *gel.*

Immunoprecipitation reaction in solution

Precipitation occurs maximally only when there are optimal proportions of the two reacting substances – antigen and antibody. Hence, an insoluble antigen-antibody complex formation occurs within a narrow concentration range known as the **zone of equivalence**. This represents the conditions under which antigen-antibody complexes are formed that are sufficiently large to be precipitated. Outside the equivalence concentration, conditions known as *antigen* or *antibody excess* occur, which result in the formation of small, soluble complexes. When increasing concentrations of antigen are added to a series of tubes that contain a constant concentration of antibodies, variable amounts of precipitate form. If the amount of the precipitate is plotted against the amount of antigen added, a **precipitin curve**, as shown in the

figure 10.1, is obtained. This immunoprecipitation reaction can be used to remove particular antigens from a solution.

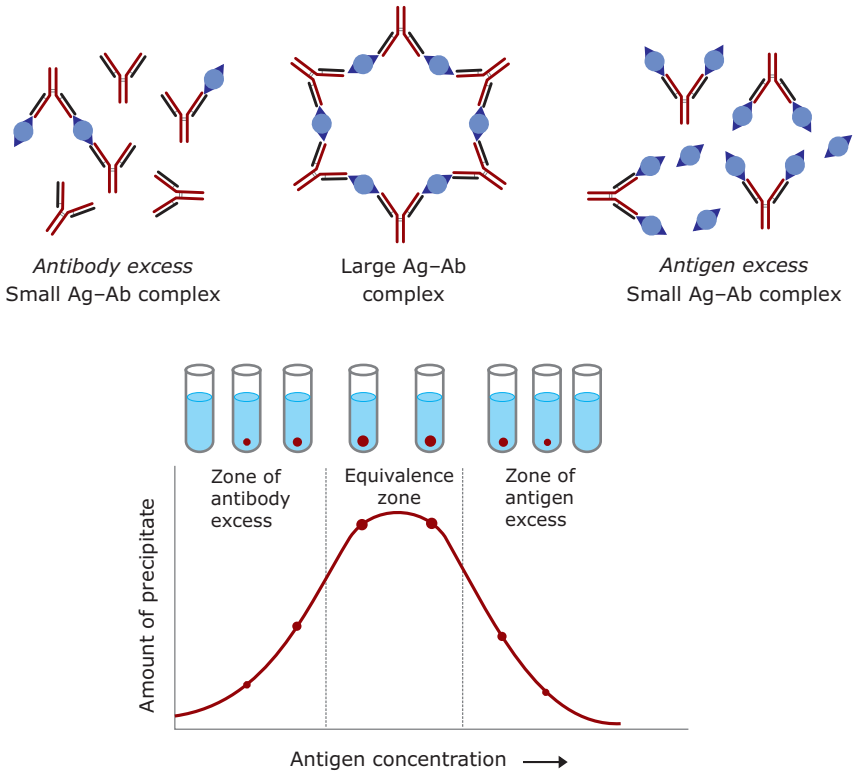


Figure 10.1 A precipitation reaction can be performed by placing a constant amount of antibody (Ab) in a series of tubes and adding increasing amounts of antigen (Ag) to the tubes. After the precipitate forms, each tube is centrifuged to pellet the precipitate, the supernatant is poured off, and the amount of precipitate is measured. Plotting the amount of precipitate against increasing antigen concentrations yields a precipitin curve. Under conditions of antibody excess or antigen excess, extensive lattices do not form and precipitation is inhibited. In the equivalence zone, the ratio of antibody to antigen is optimal. As a result, a large multimolecular lattice is formed at equivalence, the complex increases in size and precipitates out of solution.

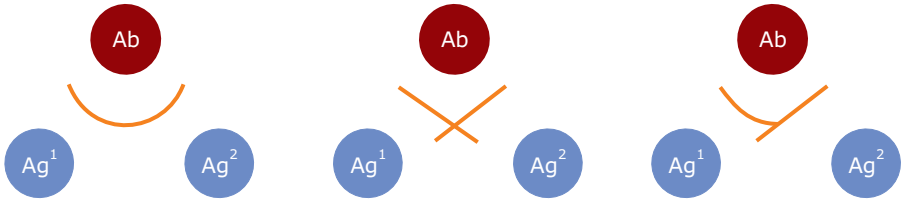
Immunoprecipitation reactions in gels

Immunoprecipitation reactions carried out in agar gels are referred to as *immunodiffusion reactions*. When antigen and antibody diffuse toward one another in gel, or when an antibody is incorporated into the gel and antigen diffuses into the antibody-containing matrix, a visible line of precipitation (**precipitin line**) will form. Visible precipitation occurs in the region of equivalence. No visible precipitate forms in regions of antibody excess or antigen excess. Two types of immunodiffusion reactions can be used to determine the relative concentrations of antibodies or antigens as well as the identity of antigens. These immunodiffusion techniques are *radial immunodiffusion* and *double immunodiffusion*.

Radial immunodiffusion (Mancini method)

The relative concentration of an antigen can be determined by a simple quantitative assay in which an antigen sample is placed in a well and allowed to diffuse into agar gel containing antibody. In agar gel, antibody is uniformly distributed. At the region of equivalence, a precipitation ring forms around the well. The diameter of the ring is proportional to the log of the concentration of antigen since the amount of antibody is constant.

- **Partial identity** occurs when two antigens share some epitopes but one of the other has a unique epitope. The antiserum forms a line of identity with the common epitope and a curved spur with a unique epitope.



A. Identity **B**. Non-identity **C**. Partial identity

Figure 10.3 Ouchterlony double immunodiffusion patterns. Antibody that is a mixture of antibody-1 and antibody-2 is placed in the central well. Unknown antigens are placed in the outside wells. **A**. *Identity*: The arc indicates that the two antigens (Ag^1 and Ag^2) are identical. **B**. *Non-identity*: Two crossed lines represent two different precipitation reactions. The antigens share no common epitopes. **C**. *Partial identity*: Ag^1 and Ag^2 share some epitopes.

The Ouchterlony method can also be used to estimate the relative concentration of antigens. When an antigen has a relatively higher concentration, the equivalent zone will be formed a little bit away from the antigen well. When an antigen has a relatively lower concentration, the equivalent zone will be formed a little bit closer to the antigen well.

Agglutination reactions

Agglutination is the visible clumping of a particulate antigen when mixed with antibodies specific for the particulate antigens. Antibodies that produce such reactions are called **agglutinins**. The general term agglutinin is used to describe antibodies that agglutinate particulate antigens. When the antigen is an erythrocyte, the term *hemagglutination* is used. All antibodies can theoretically agglutinate particulate antigens; but IgM, due to its high valence, is a particularly good agglutinin.

Zeta potential
The surfaces of certain particulate antigens may possess an electrical charge, as, for example, the net negative charge on the surface of erythrocytes caused by the presence of sialic acid. When such charged particles are suspended in saline solution, an electrical potential, termed the zeta potential, is created between particles, preventing them from getting very close to each other. This introduces a difficulty in agglutination of charged particles by antibodies.

Agglutination reactions are similar, in principle, to precipitation reactions. Just as an excess of antibody inhibits precipitation reactions, such excess can also inhibit agglutination reactions; this inhibition is called the **prozone effect**. In the case of antigen excess, the **postzone effect** occurs.

The agglutination reactions can be *direct* (active) or *indirect* (passive). When the antigen is an integral part of the surface of a cell or other insoluble particle, the agglutination reaction is referred to as **direct agglutination**. The agglutination test only works with particulate antigens. However, it is possible to coat cell or other insoluble particles with a soluble antigen (e.g. viral antigen, a polysaccharide or a hapten) and use the coated cells in an agglutination test for antibody to the soluble antigen. This is called **passive agglutination**.

Coombs test

When antibodies bind to erythrocytes, they do not always result in agglutination. This can result from the antigen-antibody ratio (either antigen excess or antibody excess); or in some cases, **zeta potential** on the erythrocytes preventing the effective cross-linking of the cells. In order to detect the presence of non-agglutinating antibodies on erythrocytes, one simply adds a second antibody directed against the antibodies attached to their respective epitopes on erythrocytes. This anti-immunoglobulin can now cross-link the erythrocytes and result in agglutination. This test is known as the *Coombs test* (anti-immunoglobulin test).

Direct ELISA

This is the simplest ELISA technique. The antigen present in the sample is first immobilized to the wall of the wells of a microtiter plate. The wells are then washed thoroughly, leaving only the adsorbed antigen. An enzyme-linked antibody, complementary to the antigen of interest, is then added to the wells where it binds to the antigen. The well is again washed. This leaves a bound antigen-antibody complex on the surface of the well. A substrate is then added, which will be converted by the enzyme-linked with antibodies into a detectable product. Detection may be based on color, fluorescence, or luminescence. This method has the advantage of being quicker and simpler than the other ELISA methods, with fewer steps, and just one antibody. It does, however, have some limitations. In complex samples containing a range of different antigens, there will be a variety of antigens adsorbed onto the well that is not the antigen of interest. This proves problematic when the antigen of interest is in low abundance as the sensitivity of the test is reduced. Also, conjugating the antibody with an enzyme has the potential to reduce the affinity of the antibody to the antigen and thus reduces sensitivity.

Indirect ELISA

Sample containing the antigen of interest is adsorbed onto the microtiter well. A complementary antibody (**primary antibody**) is then added, which binds to the antigen forming a complex. After any free primary antibody is washed away, the presence of antibody bound to the antigen is detected by adding an enzyme-linked **secondary antibody**, which binds to the primary antibody. Any free secondary antibody then is washed away, and a substrate for the enzyme is added. The amount of colored reaction products that forms is measured by specialized spectrophotometric plate readers, which can measure the absorbance of all of the wells.

This method differs from the direct method in that the antibody binding to the antigen does not have attached to it an enzyme. Instead, the purpose of this antibody is to act as a bridge between the antigen and an enzyme-linked secondary antibody. The indirect ELISA is used to detect the presence of antibodies against HIV. In this test, the antigens are adsorbed to the bottom of a well. Then antibodies (act as primary antibodies) from a patient are added to the coated well and allowed to bind to the antigen. Finally, the enzyme-linked secondary antibodies to the human antibodies are allowed to react in the well, and unbound antibodies are removed by washing. A substrate is then applied. An enzyme reaction suggests that the enzyme-linked antibodies were bound to human antibodies, which in turn, implies that the patient had antibodies to the viral antigen.

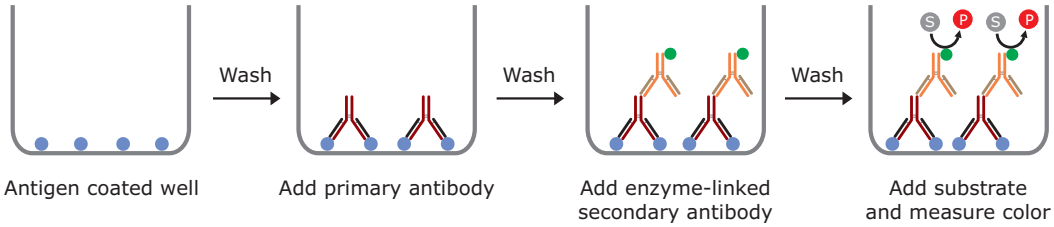


Figure 10.9 Indirect ELISA. The purified antigen of interest is first immobilized on the bottom of a well. Test antiserum is added and allowed to incubate. If any antibody in the test antiserum has bound to the immobilized antigen, their presence is detected by adding an enzyme-conjugated secondary antibody. Enzyme substrate (S) is then added and the amount of colored reaction product (P) that forms is measured.

Sandwich ELISA

An antigen can be detected or measured by a sandwich ELISA. In this ELISA, the antibody (rather than the antigen) is immobilized on a microtiter well. Immobilized antibodies (act as *capture antibodies*) are able to bind to specific antigen present in the sample with high affinity. A separate enzyme-linked antibody that recognizes a different epitope from that recognized by the immobilized first antibody is then used to detect the bound antigen. Thus, in this case, two antigen-specific antibodies are required, and they need to bind to different epitopes of the same antigen. To measure the presence and amount of specific antigen, a sample containing antigen is added and allowed to react with the immobilized antibody. After the well is washed, a second enzyme-linked antibody specific to a different epitope on the antigen is added and allowed to react with the bound antigen. Any free secondary antibodies are then washed away, and a substrate for the enzyme is added. Finally, the amount of colored reaction products that form is measured.

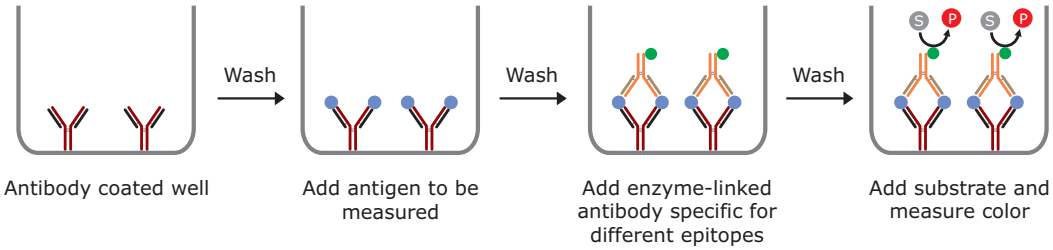


Figure 10.10 Sandwich ELISA. Antibody to a particular antigen is first immobilized on the bottom of a well. Next, the sample containing antigen is added to the well, which binds to the antibody. Finally, a second, different antibody to the antigen is added. This antibody is enzyme-linked. After any free second antibody is removed by washing, substrate (S) for enzyme-linked with antibody is added, and the colored reaction product (P) is measured. The extent of reaction is directly proportional to the amount of antigen present.

Competitive ELISA

The competitive ELISA is perhaps the most complex of all the ELISA techniques. It involves the use of **inhibitor antigen**, so competitive ELISA is also known as **inhibition ELISA**. In fact, each of the three formats, direct, indirect, and sandwich, can be adapted to the competitive format. In competitive ELISA, the inhibitor antigen and the antigen of interest compete for binding to the primary antibody i.e. two antigens to compete with each other for binding to antibodies.

In this ELISA, the unlabeled primary antibody is first incubated with the sample containing the antigen of interest, leading to the formation of antigen-antibody complex. Since the antibody is excessive compared with the antigen, so there are free antibodies left.

In the next step, the antigen-antibody mixture is added to the plate coated with inhibitor antigen that can also bind to the primary antibody. The free primary antibody in the mixture binds to the inhibitor antigen on the plate, while the antigen-antibody complexes in the mixture do not and are therefore washed off. The enzyme-labeled secondary antibody is added to the plate and binds to the primary antibody bound to the inhibitor antigen on the plate. Finally, a substrate is added to react with the enzyme and emit a visible signal for detection.

Through this procedure, one can find that the final signal is inversely associated with the amount of the antigen of interest in the sample, meaning that the more antigen in the sample, the weaker the final signal. This is because primary antibodies bound to sample antigen will be washed off, while free primary antibodies left will be captured by inhibitor antigen

Chapter 11

FRET and FRAP

11.1 FRET

FRET (Fluorescence Resonance Energy Transfer) is a phenomenon in which an excited donor molecule transfers energy (not an electron) to an acceptor molecule through a non-radiative process. It is a highly distance-dependent radiationless energy transfer process. In this energy transfer process, two molecules interact with each other in which one acts as donor and other as acceptor. The donor is a *fluorophore* that initially absorbs the energy and the acceptor is the *fluorophore* or *chromophore* to which the energy is subsequently transferred. A pair of molecules that interact in such a manner that FRET occurs is often referred to as a donor-acceptor pair.

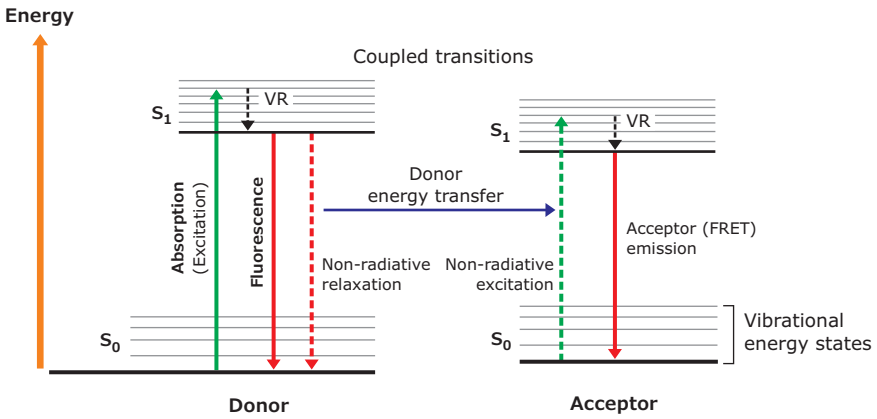


Figure 11.1 A *Jablonski diagram* illustrating the coupled transitions involved between the donor emission and acceptor absorbance in fluorescence resonance energy transfer. In this diagram, the donor molecule is excited by a photon. Under typical fluorescence conditions, relaxation of the electron energy would result in the emission of a photon (*fluorescence*) by the donor fluorophore. However, if a suitable acceptor molecule is within a certain distance (*Förster distance*) then donor energy can experience the transfer of energy to the acceptor, resulting in acceptor fluorescence. The transfer of energy between molecules is nonradiative, and that the distances involved are much less than the wavelength of light. In the presence of a suitable acceptor, the donor fluorophore can transfer excited state energy directly to the acceptor without emitting a photon.

Resonance energy transfer, a photophysical process in which the excited state energy from a donor fluorophore is transferred via a non-radiative mechanism to a ground state acceptor chromophore via weak long-range dipole–dipole coupling.

FRET is a non-radiative quantum mechanical process that does not require a collision and does not involve the production of heat. When energy transfer occurs, the acceptor molecule quenches the donor molecule fluorescence, and if the acceptor is itself a fluorophore, increased or sensitized fluorescence emission is observed.

There are some criteria that must be satisfied in order for FRET to occur. The process of resonance energy transfer can take place when a donor fluorophore in an electronically excited state transfers its excitation energy to a nearby fluorophore, the acceptor. The primary condition is the distance between the donor and the acceptor. The donor and acceptor molecules must be in close proximity to one another for FRET to occur.

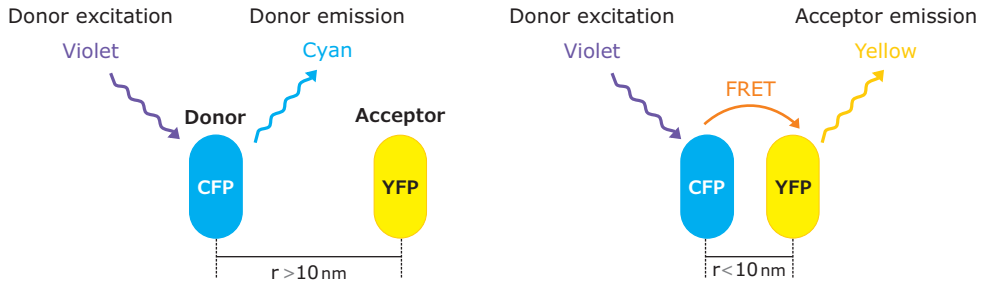


Figure 11.2 The acceptor and donor fluorophores must be very close for the FRET to occur. FRET is a process by which radiationless transfer of energy occurs from an excited state donor fluorophore to an acceptor fluorophore in close proximity. Because the range over which the energy transfer can take place is limited to approximately 10 nanometers, and the efficiency of transfer is extremely sensitive to the separation distance between fluorophores. In this example, CFP (Cyan fluorescent protein) is a donor fluorophore and YFP (Yellow fluorescent protein) is a acceptor fluorophore.

The extent of energy transfer for a single donor-acceptor pair at a fixed distance depends on the inverse sixth power of the distance between the donor and acceptor pair (r) and is given by:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

Where R_0 is the **Förster radius** at which half of the excitation energy of donor is transferred to the acceptor chromophore. Therefore, Förster radius is referred to as the distance at which the efficiency of energy transfer is 50% and is characteristic of the donor-acceptor pair, the relative orientation of their transition dipoles, and the medium between them. Förster distances are typically in the range of 15 to 60 Å.

FRET relies on the distance dependent transfer of energy from the donor fluorophore to an acceptor fluorophore. In FRET, a donor fluorophore is excited by incident light, and if an acceptor is in close proximity, the excited-state energy from the donor can be transferred. The process is non-radiative (not mediated by a photon). The donor molecule must have an emission spectrum that overlaps the absorption spectrum of the acceptor molecule. In most applications, both donor and acceptor are fluorophores.

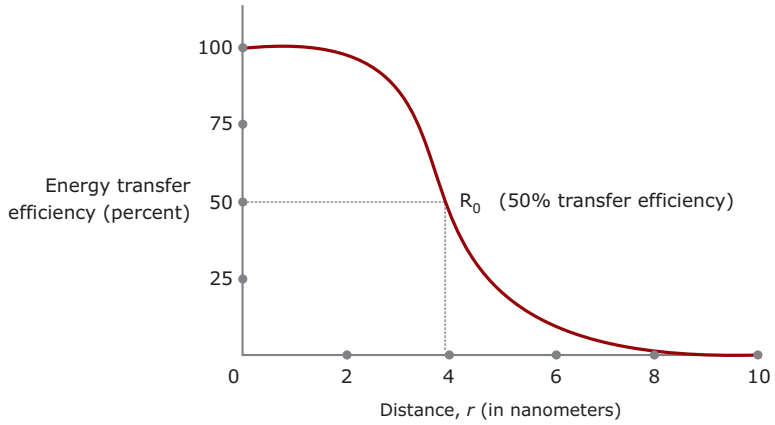


Figure 11.3 Distance and energy transfer efficiency. Energy transfer efficiency is most sensitive to distance between the donor-acceptor molecules. The graph illustrates the exponential relationship between transfer efficiency and the distance separating the donor and the acceptor. The efficiency rapidly increases to 100% as the separation distance decreases below R_0 and conversely, decreases to zero when r is greater than R_0. Because of the strong (sixth-power) dependence of transfer efficiency on distance, measurements of the donor-acceptor separation distance are only reliable when the donor and acceptor radius lies within the Förster distance by a factor of two.

Chapter 12

Molecular Biology Techniques

12.1 Nucleic acid extraction

The extraction of nucleic acid is a crucial step for biochemical and diagnostic processes. It is a series of steps to obtain pure nucleic acid samples that are suitable for different downstream applications. Different extraction methods result in the difference in yield, purity and extraction time.

DNA extraction

The choice of the DNA extraction method depends on the types of DNA (chromosomal and plasmid), source organism (bacteria, fungi, plant or animal), starting material (organ, tissue, cell, etc.) and desired results (molecular weight of the desired DNA, purity, extraction time required, etc.).

The extraction of DNA from biological material requires: cell lysis (cell disruption), inactivation of cellular nucleases, removal of biomolecules other than DNA, purification and quantification of the DNA. Common lysis procedures include **mechanical disruption** (for example, grinding, ultrasonication hypotonic lysis, osmotic lysis) and **chemical lysis** (for example, detergent lysis, alkali treatment). Lytic enzymes, chaotropic agents, and different types of detergents are the main components of chemical lysis.

Common DNA extraction methods

Nucleic acid extraction methods can be widely characterized into two different types: solution-based methods and solid-phase based methods. In **solution-based extraction methods**, cell extracts are mixed with chemical solutions devised to purify nucleic acid. **Solid-phase extraction methods** work by causing nucleic acids to bind to solid supports, such as magnetic beads coated with silica or other materials. Different extraction methods result in different yields and purity of DNA.

Organic extraction

A traditional method that can be used to obtain highly pure DNA. This method involves organic extraction (e.g. phenol:chloroform) followed by ethanol precipitation. In this method, cells are lysed and cell debris is usually removed by centrifugation. The remaining soluble material is then mixed with organic solvents such as phenol, or 1:1 mixture of phenol and chloroform.

A phenol-chloroform organic solvent is used to concentrate DNA in a hydrophilic phase. Phenol dissociates proteins bound to DNA while chloroform denatures proteins and lipids. Three distinct phases will form: the *aqueous phase*, the *interphase* and the *organic phase*. Of these, the aqueous phase contains the DNA, whereas the proteins and lipids remain in the other two phases. The aqueous phase is then separated and treated with ethanol to precipitate the DNA (termed **ethanol precipitation**). The precipitated DNA can then be pelleted by centrifugation and dissolved in a buffer of choice for use in downstream reactions.

If the protein content in cell extract is very high, then proteases such as pronase or proteinase K are used to degrade proteins before phenol extraction. RNA is digested from the preparation by treatment with RNase.

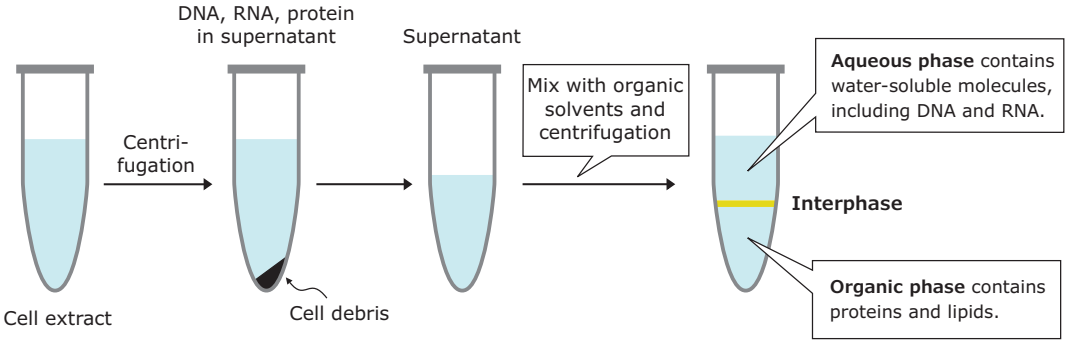


Figure 12.1 Organic extraction method.

Silica-based method

It is a solid-phase DNA extraction method. Silicates have a high binding affinity for DNA under alkaline conditions and high salt concentration. The mechanism involved in this technique is the affinity between negatively charged DNA and positively charged silica material, resulting in selective binding of nucleic acids to the silica matrices (consist of silica material, in the form of either gel or glass particle). As a final step, DNA can be eluted from the silica matrix by any hypoosmotic solution, such as nuclease-free water or buffers such as alkaline Tris-EDTA.

Magnetic bead-based method

Magnetic beads are paramagnetic materials with a uniform particle sizes that have been coated with a functional group that interacts with DNA. Under optimized conditions, DNA selectively binds to an appropriately-coated bead surface, leaving contaminants in solution. A magnetic field is used to separate magnetic beads from a suspension. Hence after DNA binding, beads are separated from other contaminating cellular components by placing a magnet outside of the tube to create a strong external magnetic field. This approach removes the need for vacuum or centrifugation, which minimizes stress or shearing forces on the target molecules.

Anion exchange method

Just like silica matrices, anion exchange resins are also widely used in DNA extraction. DNA extraction by anion exchange chromatography is based on the specific interaction between negatively charged phosphates of the nucleic acid and positively charged surface molecules on the substrate. Unlike silicate negative charge, anion exchange resin makes use of the positively charged diethylaminoethyl (DEAE) cellulose to attract the negatively charged phosphate of nucleic acid. DNA binds specifically to the substrate in the presence of low salt, contaminants are removed by wash steps using a low or medium salt buffer, and purified DNA is eluted using

Qualitative and quantitative analysis of nucleic acid

Nucleic acid quantitation is an important and necessary step prior to most nucleic acid analysis methods. Common methods used for determining quality and quantity of nucleic acid include absorbance, fluorescence and qPCR.

Absorbance based quantification method is performed at 260 nm (based on the fact that the heterocyclic rings of nucleotides absorb UV light with an absorption maximum at around 260 nm). Absorbance method is simple, quick and has a wide detection range. This method does not require any reagents. But this method is less sensitive than fluorescence or qPCR based methods and also cannot distinguish between ssDNA, dsDNA or RNA.

Nucleic acid can be quantified by measuring the absorption at 260 nm. For dsDNA, 1 absorbance unit is equivalent to 50 µg DNA (i.e. a solution of dsDNA with a concentration of 50 µg/mL will have an absorbance at 260 nm equal to 1); for ssDNA, it is equivalent to 33 µg DNA; for ssRNA, it is equivalent to 40 µg RNA.

Absorbance is also used to measure purity of nucleic acids present in the sample. The ratio of absorbance at 260 nm and absorbance at 280 nm (A_{260}/A_{280}) and the ratio of absorbance at 260 nm and absorbance at 230 nm (A_{260}/A_{230}) are used to check nucleic acids purity. For a pure DNA sample, A_{260}/A_{280} ratios is ~1.8 and for a pure RNA sample, the ratio of A_{260}/A_{280} is ~2. A ratio of < 1.8 indicates the DNA sample is contaminated with protein or an organic solvent such as phenol. Typically, protein contamination can be detected by a reduction of this ratio; RNA contamination can be detected by an increase of this ratio.

The A_{260}/A_{230} values for 'pure' nucleic acid are commonly in the range of 2.0–2.2. The A_{260}/A_{230} is a sensitive indicator of contaminants that absorb at 230 nm. These contaminants are significantly more numerous than those absorbing at 280 nm and include chaotropic salts such as guanidine thiocyanate, EDTA, non-ionic detergents like Triton X-100 and phenol. Substances like polysaccharides also show absorbance at this wavelength, but will have a weaker effect.

Fluorescence-based quantification methods use fluorescence dyes that preferentially bind a given species of nucleic acid (e.g. dsDNA, ssDNA or RNA). When a dsDNA binding dye, for example, is excited by a given wavelength of light, only dye in the dsDNA-bound state will fluoresce. As the dye binds to the target nucleic acid, fluorescent quantum yield increases as a function of shift in fluorophore molecular geometry. The intensity of fluorescent signal relates the amount of nucleic acid present. Fluorescence detection methods are comparatively more sensitive than absorbance.

12.2 Polymerase chain reaction

Polymerase chain reaction (PCR) is a rapid and versatile *in vitro* method for amplifying defined target DNA sequences present within the source of DNA. This technique was formulated by *Kary Mullis* in 1983. Usually, the method is designed to permit *selective amplification* of a specific target DNA sequence(s) within a heterogeneous collection of DNA molecules (e.g. total genomic DNA or a complex cDNA population). To permit such selective amplification, some prior DNA sequence information from the target sequences is required. This information is used to design two oligonucleotide primers (amplimers), which are specific to the target sequence and are often about 15–25 nucleotides long. After the primers are added to denatured template DNA, they bind specifically to complementary DNA sequences at the target site. In the presence of a suitably heat-stable DNA polymerase and DNA precursors (the four

deoxynucleoside triphosphates, dATP, dCTP, dGTP and dTTP), primer initiates the synthesis of new DNA strands which are complementary to the individual DNA strands of the target DNA segment and will overlap each other.

Essential components of PCRs:

- Thermostable DNA polymerase
- Primers
- dNTP (dATP, dCTP, dGTP and dTTP)
- Divalent cations (usually Mg^{2+}) as a cofactor for activity of DNA polymerase
- Buffer, provides a suitable chemical environment for activity of DNA polymerase
- Template DNA

Primer designing

In PCR, primer designing is the most important aspect for selective amplification. For primer design, some prior DNA sequence information from the target DNA is required. The information is used to design two primers, which are specific to sequences flanking the target DNA sequence. So, for most PCR reactions, it is very important to reduce the chance of the primers binding to other locations in the DNA than the desired one. Hence, certain rules for primer design are important to consider. These rules follow:

Primer length

Length of the primers should not be very short or long. If the primers are too short, they might hybridize to non-target sites and give undesired amplification products.

To illustrate this point, imagine a DNA molecule of 10 Mbp is used in a PCR experiment with a pair of primers eight nucleotides in length. The attachment sites for these primers are expected to occur, on average, once every 4^8 = 65,536 bp. This means that it would be very unlikely that a pair of 8 nucleotides long primers would give a single, specific amplification product. If the primer length will be 15 nucleotides, then the expected frequency of attachment site for the primer will be once every 4^{15} = 1073741824 bp. This figure is higher than the length of the DNA molecule, so 15 nucleotides long primer would be expected to have just one attachment site. But we cannot take very long primer because the long primer influences the rate at which it hybridizes to the template DNA; long primers hybridize at a slower rate. It is generally accepted that the optimal length of primers is 18–20 nucleotides. This length is long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature.

Calculation of length of primers: The longer the primer, the higher its specificity for a particular target. The following equation can be used to calculate the probability that a sequence exactly complementary to the primer sequence will occur by chance in a DNA molecule that consists of a random sequence of nucleotides.

$$K = [g/2]^{G+C} \times [(1-g)/2]^{A+T}$$

where, K is the expected frequency of occurrence of target sequence in a DNA molecule,
g is the relative G + C content of the DNA molecule and
G, C, A and T are the number of specific nucleotides in the primer.

For a double-stranded genome of size N (in nucleotides), the expected number (n) of sites complementary to the primer is $n = 2NK$.

General recommendations on designing PCR primers:

- 15–30 nucleotides long
- T_m 55–70°C (within 5°C, for two primers)
- 40–60% GC (with uniform distribution)
- One C or G at 3′ end

Quantitative Real–Time PCR

Quantitative Real-Time PCR is based on the general principle of PCR, which is used to amplify and simultaneously quantify a target DNA molecule. This is called Real-Time PCR because it allows the scientist to actually view the increase in the amount of DNA as it is amplified. Real-Time PCR systems rely upon the detection and quantitation of a fluorescent reporter, whose signal increases in direct proportion to the amount of PCR product in a reaction. These fluorescent reporter molecules include dyes that bind to the dsDNA (i.e. SYBR Green) or sequence-specific probes. **SYBR Green** binds to the minor groove of the dsDNA only. In the solution, the unbound dye exhibits very little fluorescence. This fluorescence is substantially enhanced when the dye is bound to dsDNA. SYBR Green has, however, a limitation that includes preferential binding to G.C rich sequences.

SYBR Green monitors the total amount of double-stranded DNA, but cannot distinguish between different sequences. To be sure that the correct target sequence is being amplified, a sequence-specific fluorescent probe is needed. An example is the **TaqMan probe** which is oligonucleotide designed to hybridize to an internal region of a PCR product.

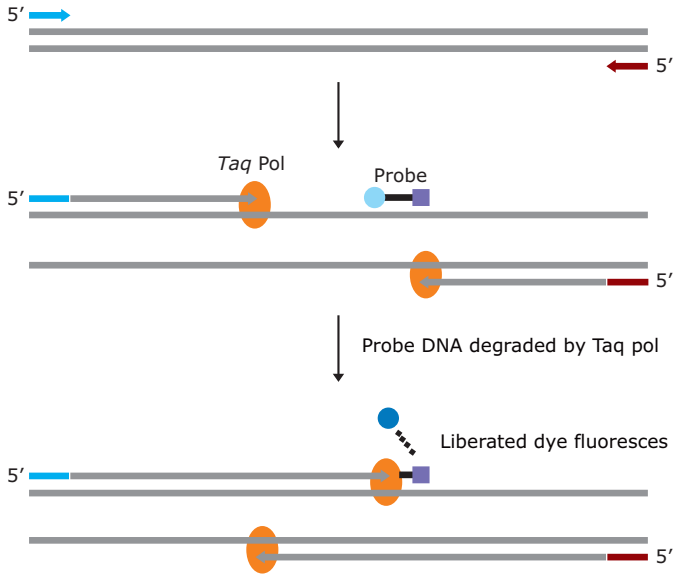


Figure 12.8 Real-time PCR with TaqMan probe. The TaqMan probe binds to the target sequence after the denaturation step that separates the two DNA strands. As the *Taq* polymerase extends the primer during the next PCR cycle, it will eventually bump into the TaqMan probe. The *Taq* polymerase has a 5′ to 3′ exonuclease activity that degrades the DNA strand of the probe. This breaks the linkage between the fluorophore and quencher. The fluorophore is now free from quenching and its fluorescence increases.

Quenchers are substances capable of absorbing radiation from a fluorophore and re-emitting much of that energy as either heat (in case of **dark quenchers**) or visible light (in case of **fluorescence quenchers**).

The TaqMan probe has three elements: a short-wavelength fluorophore on one end, a sequence that is specific for the target DNA, and a quencher at the other end. As long as the fluorophore and the quencher are close to each other, fluorescence is quenched and no fluorescent light is emitted. This probe is designed to anneal to the center of the target DNA. When *Taq* polymerase elongates the second complementary strand during PCR, its 5′ to 3′ exonuclease activity cuts the probe into single nucleotides. This removes the close proximity between the fluorophore and the quencher and abolishes quenching. The short-wavelength fluorophore can now fluoresce and a signal will be detected that is proportional to the number of newly synthesized strands.

RT–PCR

RT-PCR (Reverse Transcription PCR) is a method used to amplify, isolate or identify a known sequence from a cellular or tissue RNA. The PCR reaction is preceded by a reaction using reverse transcriptase to convert RNA to cDNA. Some thermostable DNA polymerase can use RNA templates as substrate. An example of this is the recombinant form of **Tth polymerase** from *Thermus thermophilus*, which can catalyze high-temperature reverse transcription of RNA in the presence of $MnCl_2$. It has both intrinsic reverse transcriptase and thermostable DNA-dependent DNA polymerase activities. This has led to the development of protocols for single-enzyme reverse transcription and PCR amplification.

Inverse PCR

Standard PCR is used to amplify a segment of DNA that lies between two inward-pointing primers. In contrast, inverse PCR (also known as inverted or inside-out PCR) is used to amplify unknown DNA sequences that flank one end of a known DNA sequence and for which no primers are available. The inverse PCR method involves a series of restriction digestion and ligation, resulting in a circular DNA that can be primed for PCR from a single section of known sequence. It involves isolating a restriction fragment that contains the known sequence plus flanking sequences. The restriction fragments circularize to form circular DNA under very low concentrations in the presence of DNA ligase. To perform PCR, two primers that bind specifically to the known sequence but they are oriented in opposite directions. Successful PCR with these primers produces a linear product in which central unknown region remains flanked by two short known sequences.

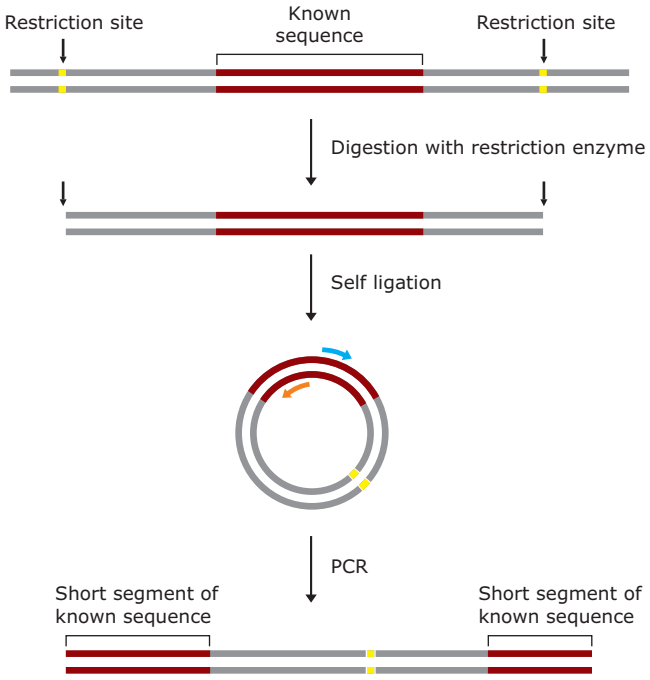


Figure 12.9 Inverse PCR. A region of DNA in which part of the sequence is known. If the areas of interest lie outside the known region, inverse PCR can be used to amplify the flanking region. It involves the digestion of genomic DNA with appropriate restriction endonucleases, intramolecular ligation to circularize the DNA fragments and PCR amplification. First, the DNA is cut with a restriction enzyme that does not cut within the region of known sequence. This generates a fragment of DNA containing the known sequence flanked by two regions of unknown sequence. Under low DNA concentrations, self-ligation is induced to give a circular DNA product. Finally, PCR is performed on the circular fragments of DNA. Two primers are used that face outwards from the known DNA sequence. PCR amplification gives linear products.

In the *exchange reaction*, polynucleotide kinase first transfers the phosphate from the 5′-end of DNA to ADP, forming ATP and leaving a dephosphorylated target. Then enzyme performs a forward reaction and transfers a labeled gamma phosphate from ATP onto the target DNA.

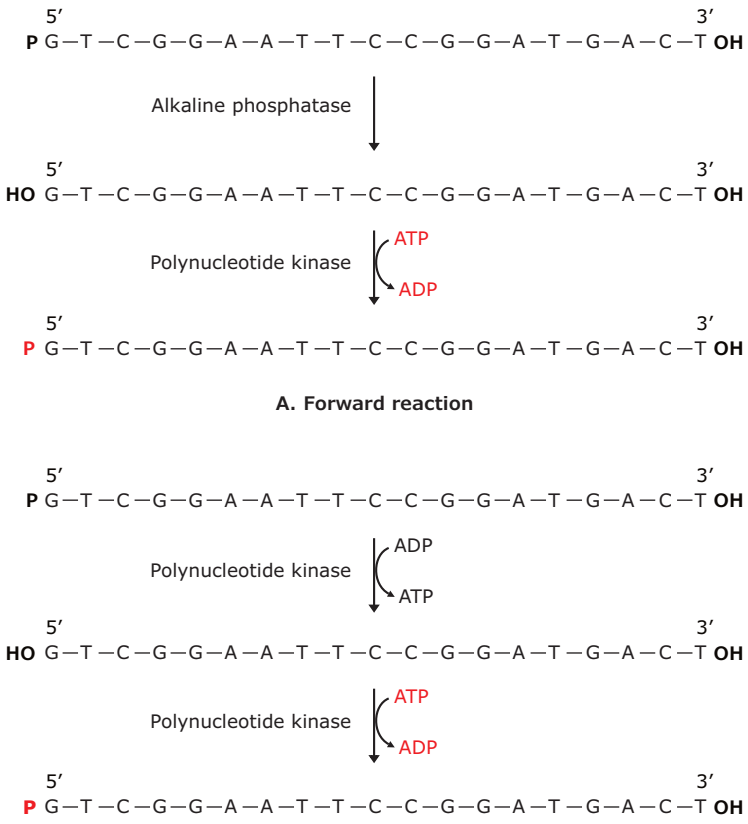


Figure 12.17 5′-end labeling. **A.** In the *forward reaction*, polynucleotide kinase transfers the gamma phosphate from ATP to the 5′-end of a polynucleotide (DNA or RNA). **B.** In the *exchange reaction*, target DNA or RNA that has a 5′ phosphate is incubated with an excess of ADP. Polynucleotide kinase first transfers the phosphate from the nucleic acid onto an ADP, forming ATP and leaving a dephosphorylated target. Polynucleotide kinase then performs a forward reaction and transfers a phosphate from ATP onto the target nucleic acid.

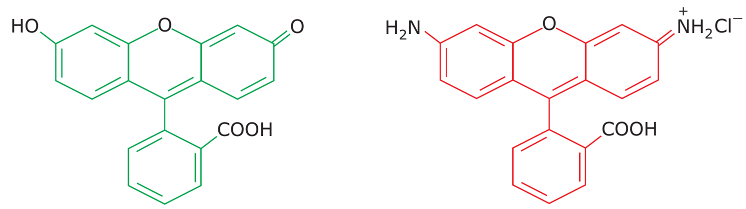
Nonisotopic labeling

Compared to radioactive labels, the use of nonradioactive labels have several advantages:

- Safety.
- Higher stability of probe.
- Efficiency of the labeling reaction.
- *In situ* detection.
- Less time taken to detect the signal.

Nonisotopic labeling systems involve the use of non-radioactive probes. Two types of non-radioactive labeling are conducted – direct and indirect. Direct labeling strategies utilize probes that are directly conjugated to a dye or an enzyme, which generates the detection signal. Indirect labeling systems utilize probes that contain a hapten that will bind to a secondary agent generating the detection signal; the probe itself does not generate signal.

Direct nonisotopic labeling, where a nucleotide which contains the label that will be detected is incorporated. Often such systems involve incorporation of modified nucleotides containing a fluorophore, a chemical group which can fluoresce when exposed to light of a certain wavelength. Most commonly used fluorophores for direct labeling are **fluorescein**, a pale green fluorescent dye and **rhodamine**, a red fluorescent dye.



Fluorescein

Rhodamine, core structure

Indirect nonisotopic labeling, usually featuring the chemical coupling of a modified **reporter molecule** to a nucleotide precursor. After incorporation into DNA, the reporter groups can be specifically bound by an **affinity molecule**, a protein or other ligand which has a very high affinity for the reporter group. Conjugated to the latter is a marker molecule or group which can be detected in a suitable assay. The reporter molecules on modified nucleotides need to protrude sufficiently far from the nucleic acid backbone to facilitate their detection by the affinity molecule and so a **spacer** of 4-16 carbon atoms long is required to separate the nucleotide from the reporter group.

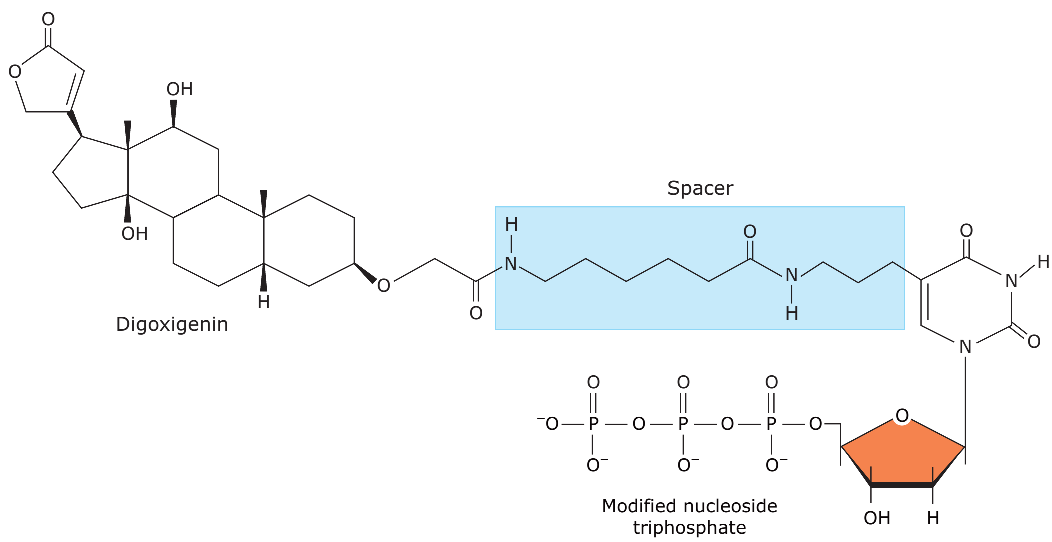


Figure 12.18 The base of the nucleoside triphosphate depicted is an analog of thymine in which the methyl group has been replaced by a spacer arm linked to the plant steroid digoxigenin. The digoxigenin is detected by a specific antibody coupled to a visible marker such as a fluorescent dye.

Two indirect nonisotopic labeling systems are widely used:

The **biotin-streptavidin system** utilizes the extremely high affinity of two ligands – **biotin** (vitamin H or B7) which acts as the *reporter*; and the bacterial protein **streptavidin** which is the *affinity molecule*. Biotin and streptavidin bind together extremely tightly with a dissociation constant in the order of 10^{-14} mol/litre, one of the strongest known in biology. Although both **avidin** and **streptavidin** bind to biotin with very high affinity, but avidin in some applications show high non-specific binding due to the presence of the sugars and high pI. The *streptavidin-biotin* system can be incorporated into virtually every immunoassay, whereby an antibody is conjugated to biotin (**biotinylated antibodies**) and then detected with streptavidin.

Digoxigenin is a plant steroid (obtained from *Digitalis* species) to which a specific antibody has been raised. The digoxigenin-specific antibody permits detection of nucleic acid molecules which have incorporated nucleotides containing the digoxigenin *reporter molecule*.

Both biotin and digoxigenin are linked to uracil, which is normally a component of RNA not DNA. Therefore, to label DNA, uracil must be incorporated into the DNA instead of thymine. If deoxyUTP labeled with biotin or digoxigenin is added to the polymerization reaction, DNA polymerase will incorporate the labeled uridine where thymidine would normally be inserted. The biotin or digoxigenin tags stick out from the DNA without disrupting its structure.

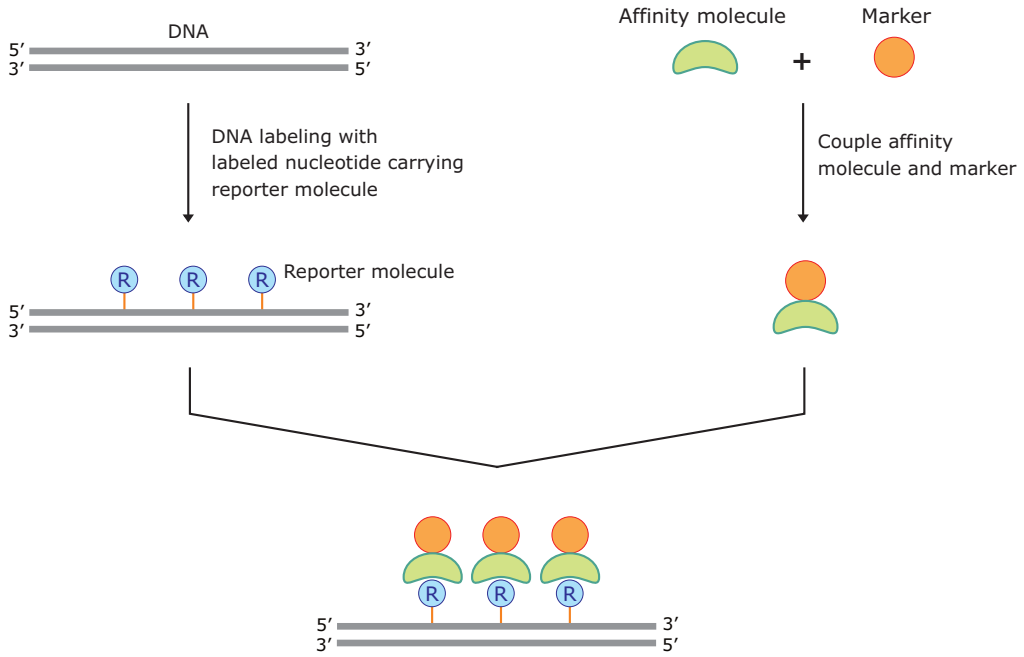


Figure 12.19 Indirect non-isotopic labeling involves chemical linkage between a reporter molecule and a nucleotide. When this modified nucleotide is incorporated into DNA, then it binds specifically to an affinity molecule which has high affinity against the reporter molecule. A long spacer is introduced between nucleotide and reporter molecule so as to reduce steric hindrances for binding of affinity molecule.

Molecular beacons

The *molecular beacon* is a structured fluorescent probe. A structured probe contains stem-loop structure regions that confer enhanced target specificity when compared with a traditional linear probe. Molecular beacons form a stem-loop structure, where the central-loop-sequence is complementary to the target of interest and the stem arms are complementary to each other. It is an oligonucleotide (about 25 nucleotides long) that contains both a fluorophore and a quenching group at opposite ends. Its detection mechanism relies on the principle of FRET, in which a fluorophore in the excited state, can transfer energy to acceptor fluorophore, with subsequent emission of a fluorescent signal from the acceptor, or to a quencher dye, which dissipates the energy without emission of a detectable fluorescence signal. In order for this energy transfer to take place, the donor and acceptor molecules must be situated in close physical proximity.

Dot blot assay

In a dot blot assay, a specified volume of nucleic acid mixture is spotted onto a small area of a nylon membrane. In this technique, the nucleic acid molecules are not first separated by electrophoresis. Instead, a specimen containing nucleic acid mixture to be detected is applied directly on a membrane as a small dot. The dot is treated with an alkaline solution to denature DNA molecules. Finally, the dot-blot membrane is allowed to hybridize with a labeled probe. The membranes are processed and exposed to film. If the dot of DNA contains a sequence similar to the probe, the film will turn dark in that area. If no dark spot appears on the film, it can be inferred that no DNA hybridization has occurred.

Slot blot is fundamentally similar to dot blot. The difference between dot and slot blot procedures is in the way that the nucleic acid mixture is blotted onto the membrane. In dot blot, the nucleic acids are blotted as circular blots, whereas in slot blot they are blotted in rectangular slots.

12.6 Phage display

Phage display is a molecular technique that allows expression of foreign polypeptides or peptides on the surface of phage particles. This method was first described by George Smith in 1985. In this technique, the DNA encoding the protein of interest is fused with a gene encoding one of the proteins that forms the viral coat. Genetic engineering techniques are used to insert foreign DNA fragments into a suitable phage coat protein gene. Phage display involved the use of filamentous phages such as fd, f1, M13, where the foreign gene was incorporated into a gene specifying a minor coat protein. Filamentous phage M13 is the most popular choice for phage display. M13 is a filamentous phage contains 6.4 kb single-stranded circular DNA. M13 enters *E. coli* through the bacterial sex pilus, a protein appendage that permits the transfer of DNA between bacteria. The single-stranded DNA in the virus particle [called the (+) strand] is replicated through an intermediate circular double-stranded replicative form containing (+) and (–) strands. Only the (+) strand is packaged into new virus particles.

Protein-protein interaction
Study of interaction between different proteins is very useful as it yields information about the tentative function of a protein through its interaction with well characterized protein with known role or function. Phage display and yeast two hybrid system are two well known and most useful methods for studying protein-protein interactions.

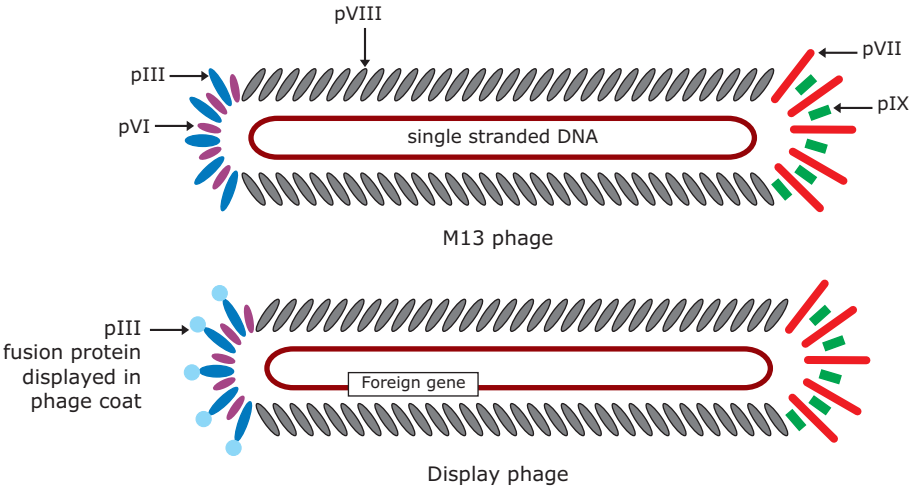


Figure 12.23 Phage display. In order to display a peptide on the surface of a bacteriophage, the DNA sequence encoding the peptide must be fused to the gene for a bacteriophage coat protein (either to the pVIII gene or the pIII gene). The coat protein is expressed as a fusion protein on the virion surface without disturbing the infectivity of the phage.

12.13 DNA sequencing

The term DNA sequencing encompasses biochemical methods for determining the order of the nucleotide bases, adenine, guanine, cytosine and thymine, in a DNA molecule. The methodologies for DNA sequencing are as follows:

Chain termination method

The enzymatic chain termination method (also dideoxy method) was developed by Frederick Sanger and coworkers in year 1977. The method is based on the DNA polymerase-dependent synthesis of a complementary DNA strand in the presence of natural deoxynucleotides (dNTPs) and dideoxynucleotides (ddNTPs) that serve as terminators. The DNA synthesis reaction is randomly terminated whenever a ddNTP is added to the growing oligonucleotide chain, resulting in truncated products of varying lengths with an appropriate ddNTP at their 3'-terminus. The products are separated by size using polyacrylamide gel electrophoresis and the terminal ddNTPs are used to reveal the DNA sequence of the template strand.

Chemical degradation method

The chemical degradation method was introduced by Allan Maxam and Walter Gilbert in year 1977. In this method, the sequence of a dsDNA molecule is determined by treatment with chemicals that cut the molecule at specific nucleotide positions.

Pyrosequencing method

The pyrosequencing method was developed by Mostafa Ronaghi and Pal Nyrén at the Royal Institute of Technology in Stockholm in 1996. In this method, the addition of a deoxynucleotide to the end of the growing strand is detectable because it is accompanied by the release of a flash of light.

Chain termination method

Chain termination method relies on the use of dideoxyribonucleoside triphosphates (ddNTP), derivatives of the normal deoxyribonucleoside triphosphates that lack the 3' hydroxyl group. Purified DNA is synthesized in vitro in a mixture that contains single-stranded molecules of the DNA to be sequenced, the enzyme DNA polymerase, a short primer DNA to enable the polymerase to start DNA synthesis, and the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP: A, C, G and T). If a dideoxyribonucleotide analog of one of these nucleotides is also present in the nucleotide mixture, it can become incorporated into a growing DNA chain. Because this chain now lacks a 3' OH group, the addition of the next nucleotide is blocked, and the DNA chain terminates at that point.

To determine the complete sequence of a DNA fragment, the double-stranded DNA is first separated into its single strands and one of the strands is used as the template for sequencing. Four different chain-terminating dideoxyribonucleoside triphosphates (ddATP, ddCTP, ddGTP and ddTTP) are used in four separate DNA synthesis reactions on copies of the same single-stranded DNA template.

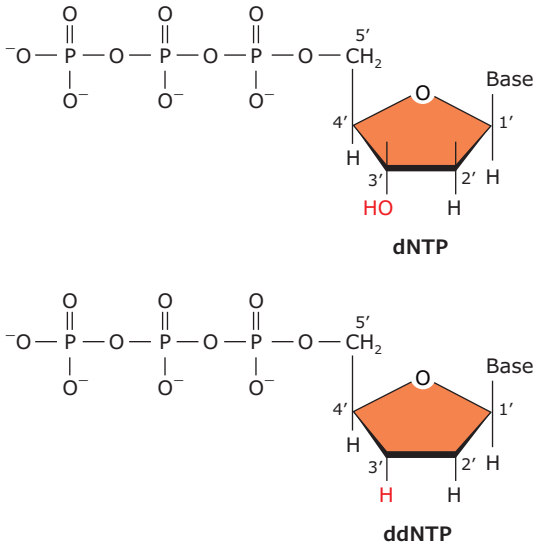


Figure 12.38 The structures of dNTP and ddNTP differ in the number and location of hydroxyl groups on the 2′ and 3′ carbons. DNA polymerase cannot add another nucleotide to a chain ending in dideoxyribose because its 3′ carbon does not have a hydroxyl group.

Each reaction produces a set of DNA copies that terminate at different points in the sequence. The products of these four reactions are separated by electrophoresis in four parallel lanes of a polyacrylamide sequencing gel. Sequencing gels are run in the presence of denaturing agents, urea and formamide. They routinely contain 6–20% polyacrylamide and 7 mol/l urea.

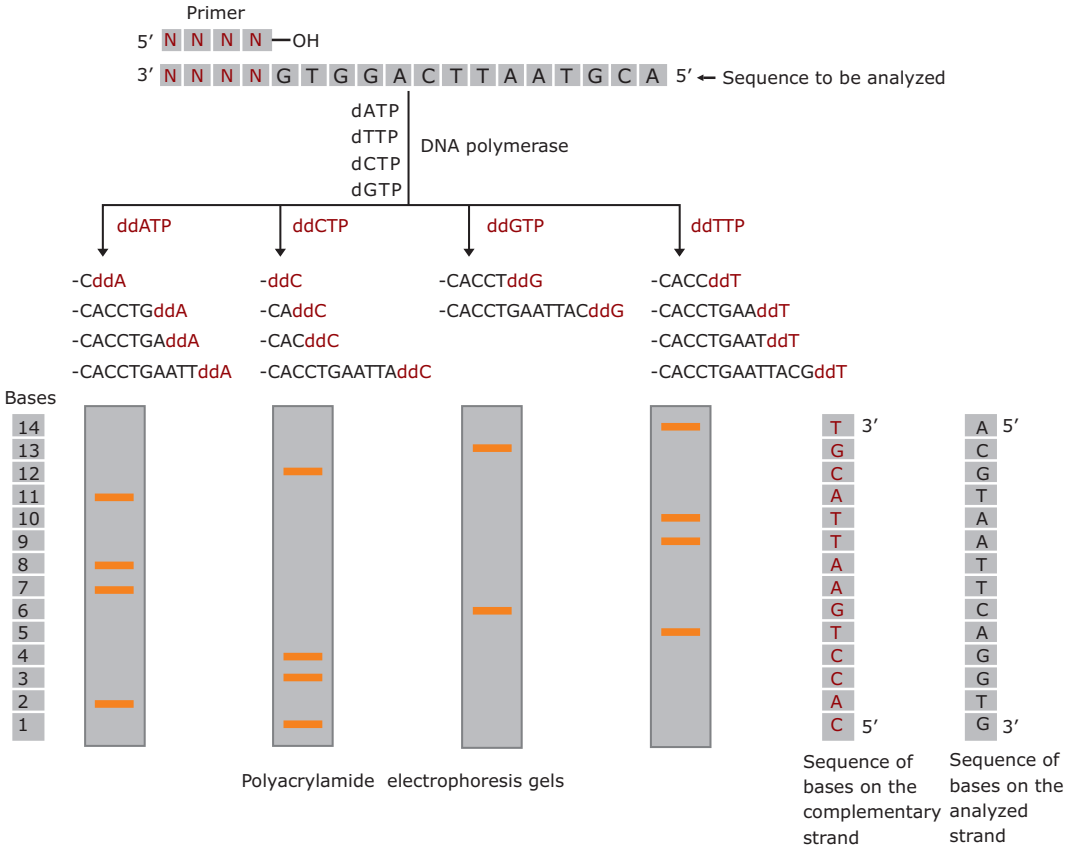


Figure 12.39 DNA sequencing by chain termination method (Sanger method).

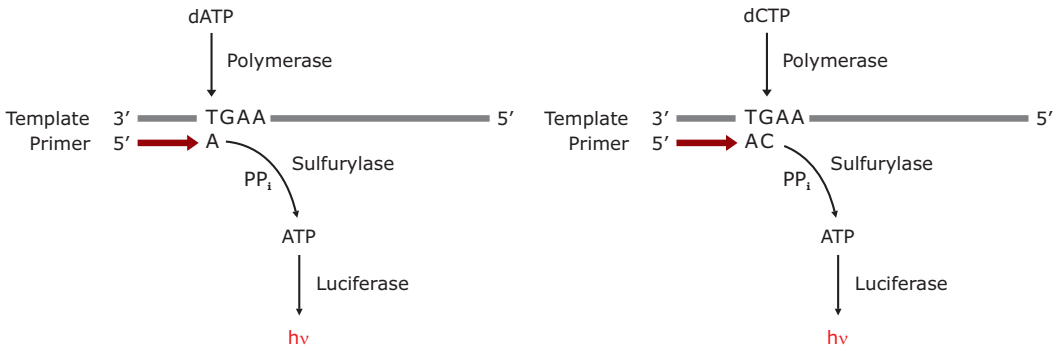


Figure 12.43 Schematic representation of the pyrosequencing enzyme system. If the added dNTP forms a base pair with the template, DNA polymerase incorporates it into the growing DNA strand and pyrophosphate is released. ATP sulfurylase converts the PP_i into ATP which serves as substrate for the light producing enzyme luciferase. The produced light is detected as evidence of that nucleotide incorporation has taken place.

Next–generation sequencing technologies

Nucleic acid sequencing is a method for determining the exact order of nucleotides present in a given DNA or RNA molecule. The chain-termination method (also commonly referred to as Sanger or dideoxy sequencing method), published in 1977, has remained the most commonly used DNA sequencing technique to date. The Human Genome Project, led by the International Human Genome Sequencing Consortium and Celera Genomics, was accomplished with *first-generation* Sanger sequencing. Since completion of the first human genome sequence, demand for cheaper and faster sequencing methods has increased greatly. This demand has driven the development of *second-generation* sequencing methods (or next-generation sequencing).

First generation sequencing technologies include sequencing by synthesis developed by Sanger and sequencing by cleavage pioneered by Maxam and Gilbert. *Next-generation sequencing* refers to non-Sanger-based high-throughput DNA sequencing technologies. Millions or billions of DNA strands can be sequenced in parallel, yielding substantially more throughput and minimizing the need for the fragment-cloning methods that are often used in Sanger sequencing of genomes. The four main advantages of next-generation sequencing over classical Sanger sequencing are:

- speed
- cost
- sample size
- accuracy

Next-generation sequencing platforms perform massively parallel sequencing, during which millions of fragments of DNA from a single sample are sequenced in unison. Massively parallel sequencing technology facilitates high-throughput sequencing, which allows an entire genome to be sequenced in less than one day. In the past decade, several next-generation sequencing platforms have been developed.

To illustrate how next-generation sequencing process works, consider a single genomic DNA (gDNA) sample. The gDNA is first fragmented into a library of small segments that can be uniformly and accurately sequenced in millions of parallel reactions. The newly identified strings of bases, called *reads*, are then reassembled using a known reference genome as a scaffold (resequencing), or in the absence of a reference genome (*de novo* sequencing). The full set of aligned reads reveals the entire sequence of each chromosome in the gDNA sample.

Illumina (Solexa) sequencing

This system was initially developed in 2007 by Solexa and was subsequently acquired by Illumina, Inc. Illumina next-generation sequencing utilizes a fundamentally different approach from the classic Sanger chain-termination method. It is based on sequencing by synthesis (SBS) technology – tracking the addition of labeled nucleotides as the DNA chain is copied – in a massively parallel fashion. Illumina sequencing systems can deliver data output ranging from 300 kilobases up to 1 terabase in a single run, depending on instrument type and configuration.

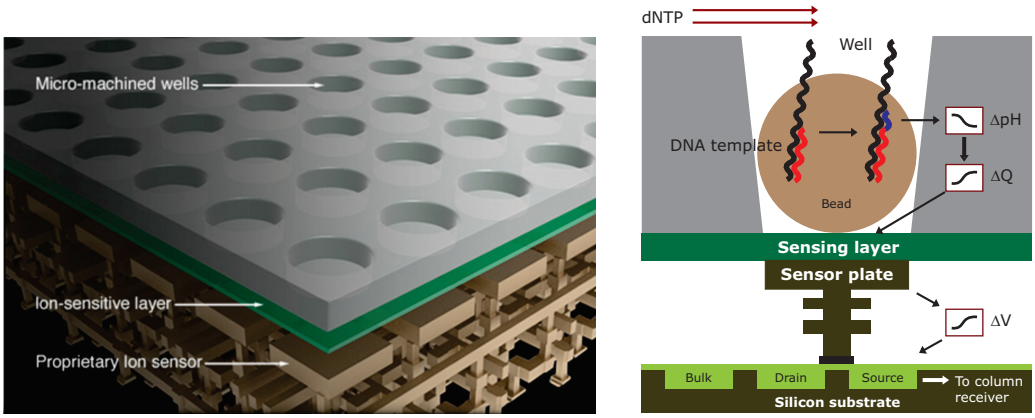


Figure 12.44 Ion Torrent technology- Sensor, well and chip architecture. An image of a well, a bead containing DNA template, and the underlying sensor and electronics. Cross section view of a single well that houses ion sphere particles with a clonal amplified DNA template. A hydrogen ion (proton) is released when a nucleotide is incorporated by DNA polymerase. The proton is then detected by the sensing layer due to the change of pH, therefore translating the chemical signal to a digital input.

12.14 Biosensors

A *biosensor* is a measuring device which is used to detect chemical compounds by converting a biological response into an electrical signal. The electrical signal it produces carries the necessary information about the process under investigation. The most widely accepted definition of a biosensors is: '*a self-contained analytical device that incorporates a biologically active material in contact with an appropriate transducer for the purpose of detecting the concentration or activity of chemical species in any type of sample*.'

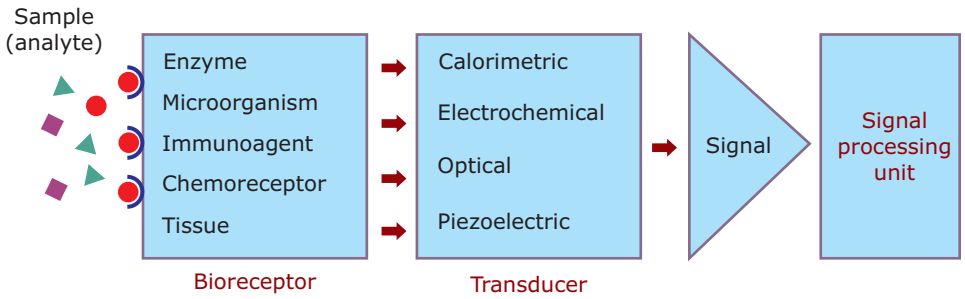


Figure 12.45 Schematic diagram showing the main components of a biosensor. The bioreceptor – interacts with the specific analyte of interest to produce signal; the transducer – transforms the signal resulting from the interaction of the analyte with the bioreceptor into another signal (i.e. transduces) that can be more easily measured and quantified; and signal processing unit – converts the signal into a workable form (amplified, processed and displayed).

There are three main parts of a biosensor:

1. Biological detection system, a *bioreceptor*. Bioreceptor may be an enzyme, an antibody or similar binding molecule, a living cell or organelles.
2. A *transducer*, which transforms the signal resulting from the interaction of the analyte with the bioreceptor into a measurable signal.
3. A signal processing system, which converts the signal into a workable form.

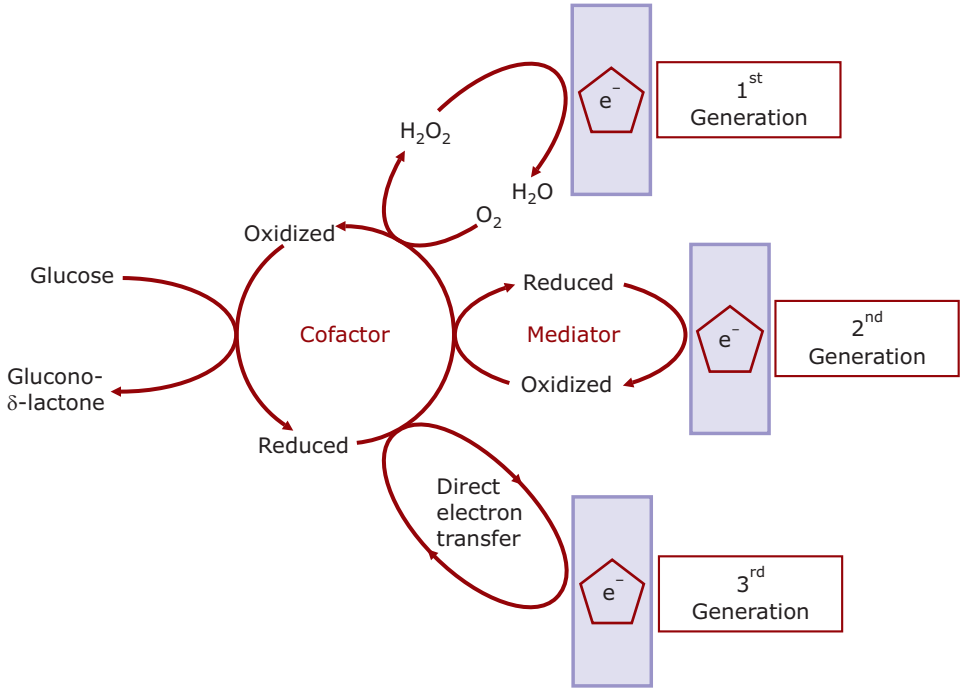


Figure 12.47 The evolution from 1st to 3rd generation electrochemical biosensors. The figure highlights modifications in the biosensor layout with each generation using glucose sensors as an example.

12.15 CRISPR/Cas systems

CRISPR/Cas systems are highly diverse adaptive microbial immune systems used by most archaea (~90%) and many eubacteria (~40%) to protect themselves from invading viruses and plasmids. These systems allow the cell to recognize and distinguish incoming 'foreign' DNA from 'self' DNA. **CRISPR/Cas** systems consist of two general parts: **CRISPRs** (clustered regularly interspaced short palindromic repeats) and **Cas** (CRISPR-associated) proteins.

CRISPRs consist of highly conserved short repeated sequences separated by similarly sized short **spacers sequences**. The size of CRISPR repeats and spacers varies between 23 to 47 bp and 21 to 72 bp, respectively. The bacterial genome may contain more than one CRISPR locus. The CRISPR loci have highly diverse and hypervariable spacer sequences, even between closely related strains. Spacers are unique sequences originating from viral or plasmid DNA. These sequences are used as recognition elements for invading viral or plasmid DNA. By adding new spacers in their own genome, bacteria able to recognize new matching viral or plasmid genomes.

Another feature associated with CRISPR loci is the presence of a conserved sequence, called **leader**, located upstream of the CRISPR with respect to the direction of transcription. CRISPR activity requires the presence of a set of CRISPR-associated (**cas**) genes, usually found adjacent to the CRISPR, that code for **Cas proteins** essential to the immune response. The Cas proteins with perform a variety of functions such as nucleases, helicases and polymerases. The CRISPR immune system works through the cooperation of many diverse Cas proteins, resulting in CRISPR-Cas systems currently being grouped into two classes, six types, and over 30 subtypes.

Emmanuelle Charpentier and Jennifer Doudna are awarded the Nobel Prize in Chemistry 2020 for discovering the CRISPR/Cas9 system, a method for genome editing.

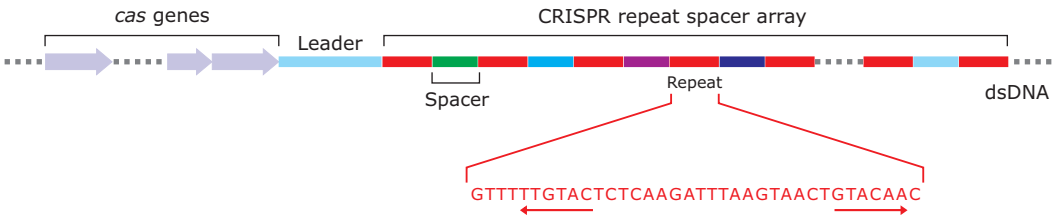


Figure 12.48 Locus organization: CRISPR loci consist of 23 to 47 bp palindromic repeat sequences (in red), separated by 21 to 72 bp spacer sequences. The spacers have no common features in their sequences. The *cas* genes are located close to the CRISPR loci. The number of CRISPR loci, their sequences, and the type of *cas* gene vary among species.

General mechanism

A simple analogy to understand the CRISPR mechanism is to imagine the design of a personalized weapon. Suppose, earth is attacked by an alien species and none of the earthly weapons are working on it, so someone would go and pick up the body parts of alien and based on that a new weapon specific for that alien would be developed. Also, some of the body parts will be stored for future memory. Similar to this, a bacterium, when attacked by bacteriophage, is not aware of any defence mechanism. Hence, a small Cas protein picks the part of DNA of phage and bring it close to the CRISPR loci, where it is integrated into the CRISPR site. A **crRNA** (CRISPR RNA) is synthesized from that loci which along with other proteins then recognize the complementary sequence present in the invading DNA of phage and degrade it. The CRISPR/Cas system works in a similar manner.

The general mechanism of CRISPR works in three distinct phases: **adaptation** (or *acquisition*), **expression and maturation** (or *biogenesis*) and **interference** (or *targeting*).

Adaptation phase

Adaptation is the first step in the process of developing a weapon against foreign invading DNA (viral or plasmid DNA). During adaptation, Cas proteins capture short segments of foreign nucleic acids (termed **protospacers**) and integrate them as 'spacers' into the CRISPR locus in order to record a molecular memory of past invaders. The invading DNAs are directionally integrated, as new CRISPR spacers, into a CRISPR array that is separated by repeat sequences. Some CRISPR/Cas systems employ an alternative mechanism of adaptation — namely, spacer acquisition from RNA, via reverse transcription by a reverse transcriptase encoded at the CRISPR/Cas locus.

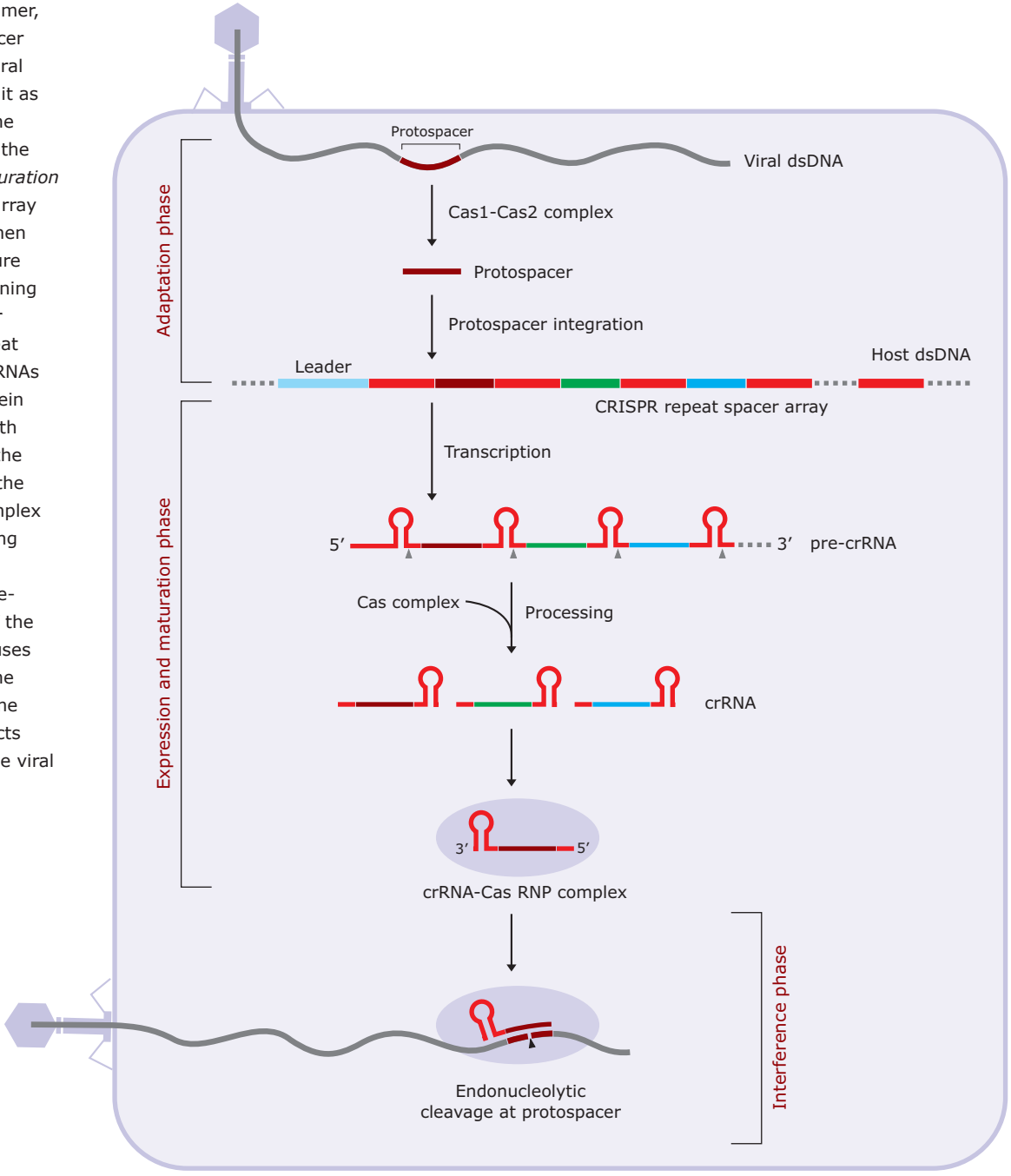
Expression and maturation phase

Once the desired sequence (i.e. protospacer) from the invading DNA is integrated into the loci of CRISPR, the CRISPR locus is transcribed into a pre-CRISPR RNA (**pre-crRNA**). CRISPR loci are transcribed from an upstream promoter located in the AT-rich leader sequence. The pre-crRNA is then processed into mature **crRNAs**, each containing a transcribed spacer sequence joined to the partial repeat sequence. The pre-crRNA processing differs in different CRISPR/Cas variants. It may be mediated by a complex composed of multiple Cas proteins or a single multidomain Cas protein or by non-Cas host RNases.

Figure 12.49 The CRISPR/Cas system has three phases. 1. In the *adaptation stage*, the Cas1–Cas2 complex, which comprises two Cas1 dimers and a single Cas2 dimer, acquires a protospacer from the invading viral DNA and integrates it as a new spacer into the CRISPR array. 2. In the *expression and maturation stage*, the CRISPR array is transcribed and then processed into mature crRNAs, each containing a transcribed spacer and part of the repeat sequence. These crRNAs form ribonucleoprotein (RNP) complexes with Cas proteins. 3. In the *interference stage*, the crRNA–Cas RNP complex identifies the invading target DNA through complementary base-pairing. Cleavage of the target sequence causes the destruction of the invading viral genome and therefore protects the bacteria from the viral infection.

Interference phase

The crRNA forms a complex with Cas proteins. The crRNA of the crRNA-Cas complex makes base pairing with the protospacer of the invading DNA. Finally, crRNA-directed cleavage of invading DNA occurs by Cas proteins at **protospacer**, a site complementary to the crRNA spacer sequence.



Types of CRISPR/Cas systems

The diversity of Cas proteins and the presence of multiple CRISPR loci make classification a complex task. According to the current classification scheme, all CRISPR/Cas systems are

divided into two distinct classes, based on the differences between the interference complexes – *class 1 systems* and *class 2 systems*.

The **class 1 systems** involve multi-subunit effector complexes (composed of multiple Cas proteins— Cas3, Cas5–Cas8, Cas10 and Cas11). By contrast, **class 2 systems** involve single subunit effectors (composed of Cas9, Cas12 or Cas13).

The two CRISPR-Cas class systems are divided into three types each,

Class 1 systems: types I, III and IV

Class 2 systems: type II (Cas9), type V (Cas12) and type VI (Cas13)

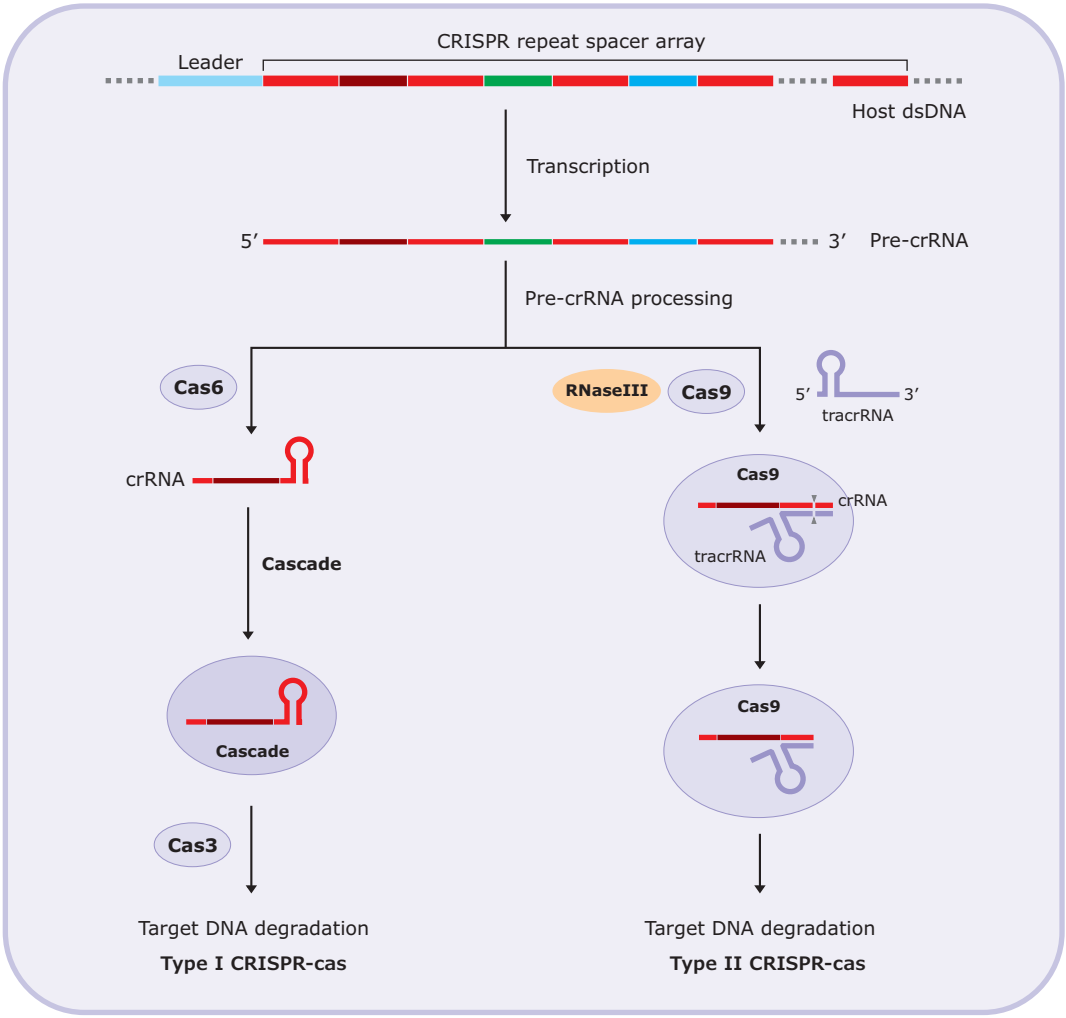
As we discussed above that the general mechanism of CRISPR/Cas system based adaptive immunity is staged into three phases. First, in the *adaptation phase*, Cas proteins integrate short regions of the invading viral or plasmid genome into the CRISPR array as new spacers. During crRNA biogenesis, the repeats and spacers are transcribed into a long pre-crRNA, which is subsequently processed to generate mature crRNAs that each contain a single spacer sequence. Mature crRNAs combine with one or more Cas proteins to form an effector complex, which during *interference*, senses and cleaves protospacers, complementary to the crRNA. Although all CRISPR/Cas systems adhere to this general pathway, they exhibit striking diversity in their processing and interference steps.

Type I, II and V systems recognize and cleave DNA, type VI can edit RNA, and type III edits both DNA and RNA. How the effect of type IV system on DNA or RNA is still unknown. Nearly all characterized CRISPR-Cas systems (except type III) mediate both adaptation and interference to the foreign genetic elements by specifically recognizing **PAM** (protospacer-adjacent motif). The PAM is a short sequence located adjacent to the protospacer on the invading DNA. It is essential for recognition (by Cas1 and Cas2 proteins) and the distinction between self and non-self DNA. The absence of the PAM sequence at the CRISPR locus in the host genome protects it from self-cleavage.

Type I systems are the most frequent and widespread systems, which target invading DNA in a PAM dependent manner. This system employs **cascade** (CRISPR-associated complex for antiviral defence) – a multiprotein complex containing mature crRNA and Cas proteins– Cas6, Cas5, Cas7, Cas8, and Cas11, to target DNA recognition and cleavage. Cascade first recognizes the PAM sequence on the target DNA, and promotes the pairing of crRNA. During the interference stage, triggers the recruitment of the endonuclease Cas3, which initiates degradation of the invading target DNA.

Similar to type I systems, type III systems employ cascade for antiviral defense and target both DNA and RNA substrates. Type III systems work in PAM-independent manner and are categorized into four subtypes named A–D. The interference target of type III-A is mRNA, while the interference target of type III-B is the same as that of type I and II CRISPR-Cas systems, which is DNA. However, the interference targets of types III-C and D are unclear.

Type II systems (also type V and VI systems) only employing a single protein and crRNA in the RNP complex against invaders. The type II systems comprise of Cas9 protein, crRNA and tracrRNA (trans-activating crRNA) and work in a PAM dependent manner. TracrRNA is a hairpin RNA transcribed from a repeat region. Cas9 binds and stabilizes the tracrRNA-crRNA duplex. tracrRNA is responsible for activating RNase III to promote the pre-crRNA processing. Mature crRNA combines with tracrRNA and Cas9 activates cleavage. Type II systems include three subtypes, II-A, II-B, and II-C.



Double-stranded breaks (DSBs) induce endogenous DNA repair mechanisms. Within a cell, double-stranded breaks in DNA can be repaired by non-homologous end joining (**NHEJ**) or homology-directed repair (**HDR**). NHEJ is a process by which the cell can repair strand breaks without the need for a homologous template. NHEJ often leads to insertions or deletions of nucleotides (termed indels) at the site of repair. In contrast, HDR performs DNA repair using homologous DNA as a template.

Figure 12.50 Comparison of mode of action of types I and II CRISPR-Cas systems. These two systems differ in their processing and interference steps. During the expression stage, CRISPR loci are transcribed and the resulting pre-crRNA is processed into short crRNAs by cleavage in the repeat sequences. In type I system, pre-crRNA cleavage is carried out by Cas6 endoribonucleases. In type II systems, this process involves the expression of a tracrRNA, which base-pairs with the repeats in the pre-crRNA transcript. The resulting duplexes are cleaved in the repeat sequences by RNase III in a Cas9-dependent reaction. Thus, in both CRISPR–Cas systems, cleavage of the pre-crRNA occurs in the repeat sequences, hence, mature crRNA consists of a spacer flanked by partial repeats. In type II systems, the tracrRNA remains bound to the crRNA and the mature crRNA–tracrRNA duplexes are complexed with Cas9. In type I systems, mature crRNA is bound by a Cas protein complex (i.e. Cascade). During the interference stage crRNAs function as guides for the Cas proteins, as they recognize and bind to complementary sequences in invading genome.

CRISPR/Cas9 and targeted genome editing

The CRISPR/Cas bacterial immune system is used as a simple, RNA-guided platform for highly efficient and targeted genome editing. CRISPR genome editing technology takes advantage of DNA repair. crRNAs guide Cas9 proteins to target sequences, which are recognized through their complementarity to the crRNA. The Cas9 nuclease then introduces a double-strand break in the target DNA. This break can be repaired through two pathways— non-homologous end-

joining (NHEJ) or homology-directed repair (HDR). The double-strand break repair by NHEJ may result in small insertions or deletions that can inactivate the target gene. By using two crRNAs, larger deletions can be generated, and if sites on two different chromosomes are targeted, NHEJ can result in chromosomal translocations and inversion. Alternatively, if a donor template with homology to the targeted locus is supplied, the double strand break may be repaired by the homology-directed repair (HDR) pathway allowing for precise replacement mutations to be made.

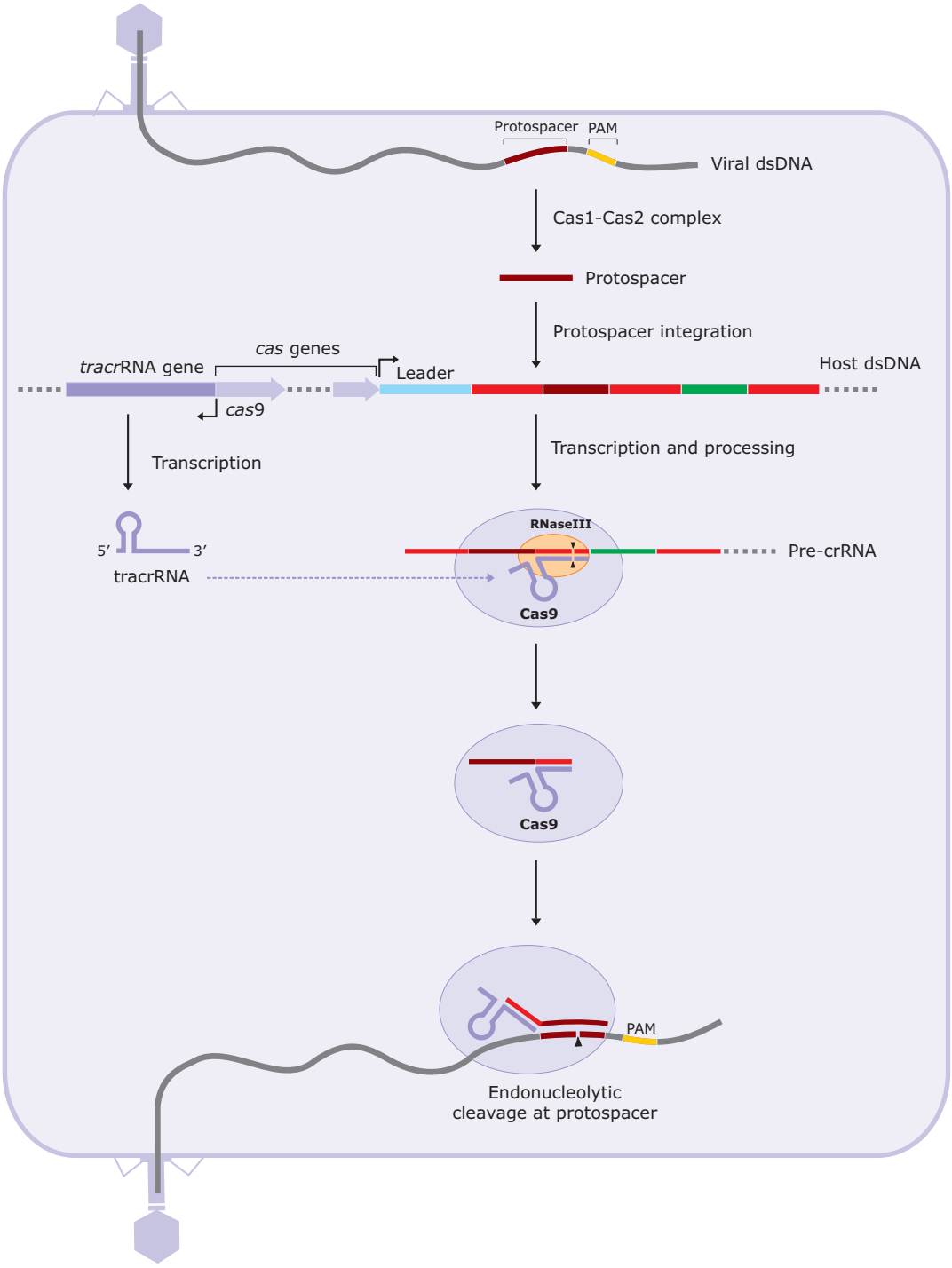


Figure 12.51 Type II CRISPR/Cas system. In the acquisition step, invading DNA is directionally integrated, as new CRISPR spacer, into a CRISPR array that is separated by repeat sequences, thus creating a memory of invading genetic element. In the expression and maturation step, the CRISPR locus is transcribed into a pre-crRNA, which is then processed into mature crRNAs that contain spacer sequences joined to partial repeats. The CRISPR locus also encodes a tracrRNA that has complementarity to the repeat regions of crRNA transcripts. In the interference stage, a crRNA–tracrRNA hybrid is formed through binding of the complementary repeat region sequences, and this RNA hybrid guides the Cas nuclease towards complementary DNA sequences, which leads to the targeting and cleaving of invading genetic elements.

Tools for programmable genome editing

Scientists can probe the function of a gene or open reading frame by mutating or deleting a locus of interest and observing the resulting phenotype. In the past decade, researchers hypothesized that by exploiting endogenous, cellular DNA repair pathways, one could create mutations or precise edits at a desired location in the genome, termed *genome editing*. Double-strand breaks are lethal to cells, thus organisms evolved mechanisms to repair these lesions. Scientists proposed that by generating a targeted, double-strand break at a site of interest, then during the repair process errors may occur, resulting in a mutation at a desired site. Additionally, endogenous double-strand break repair pathways could also stimulate the incorporation of exogenous DNA, creating very specific researcher-designed edits. Thus, nuclease-mediated approaches have been extensively explored for site-specific gene editing.

Meganucleases (or *homing nucleases*), are among the first classes of nucleases that were engineered to target specific genomic sites for gene editing purposes. Meganucleases are a group of nucleases that recognize long nucleotide sequences and induce a double-stranded break at their targeted site. The long recognition sequence of meganucleases may occur only once within a genome, thereby facilitating its use for site-specific genome editing. Meganucleases can be reengineered to target novel sequences through strategies such as protein engineering, structure-based design, and molecular evolution, although the procedure is usually labor-intensive.

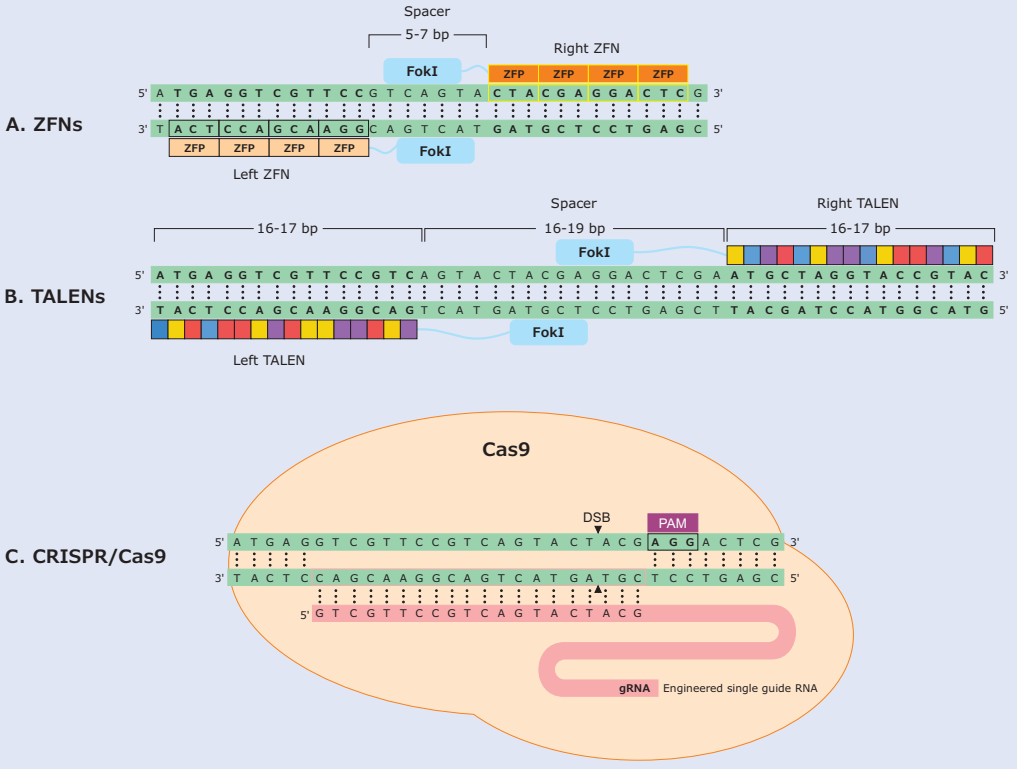


Figure 12.52 Schematic representation of components of ZFNs, TALENs and CRISPR/Cas9 systems. **A**. ZFN enzyme comprise a DNA-binding domain formed by zinc finger modules (ZF), each recognizing a unique 3-base pair sequence on the target DNA, and a DNA-cleaving domain composed of FokI. Two ZFN are designed to recognize DNA sequences that flank the desired cleavage site. In the example, each ZFN comprises four ZF which recognize 12 bp on opposite strands. Upon interaction of ZF with the target site, a FokI dimer catalyzes a targeted double-strand break. **B**. Targeted DNA cleavage is achieved by a pair of TALEN molecules. Each TALEN is comprised by a TALE and a FokI. **C**. CRISPR/Cas9 systems.

Other examples of programmable genome editing machines include *zinc-finger nucleases* (**ZFNs**) and *transcription activator-like effector nucleases* (**TALENs**), in which the DNA-binding domains of transcription factors have been fused with the nuclease domain of the restriction enzyme **FokI**, an obligate dimer. When targeted to paired adjacent sequences, the FokI domains of these programmable, site-specific nucleases form a dimer that activates the nuclease activity, thus creating a double-stranded break near their binding sites. Researchers can exploit the cell's endogenous DNA repair pathways to create mutations at the desired double-stranded break sites. However, because these tools function through protein–DNA interactions, targeting to a new site requires engineering and cloning a new protein, which precludes ZFNs and TALENs from being used for high-throughput applications.

In contrast to most known DNA-binding proteins, **Cas9** is an RNA-guided nuclease whose sequence specificity largely arises from Watson–Crick base pairing between its guide RNA and the target DNA site, in addition to a direct interaction between Cas9 and a short protospacer-adjacent motif (**PAM**) of DNA. Thus, Cas9 can be programmed to target new sites simply by changing its guide RNA sequence, making it an ideal platform for high-throughput sequence-specific gene editing, as well as other applications. Its natural endonuclease activity has been co-opted for sequence-specific editing of the genome in a wide range of organisms, including bacteria, fungi, plants and animals. To enable sequence-specific genomic regulation, nuclease-deactivated Cas9 (dCas9) has been engineered and can be fused to a variety of effectors, such as transcriptional activators, repressors, and epigenetic modifiers. In addition to applications in genome editing and regulation, DNA-binding proteins, such as ZFs, TALEs, and dCas9, have been fused to fluorescent proteins (FPs) to allow direct imaging of genomic loci in living cells.

References

Alberts B, et al (2008), *Molecular Biology of the Cell*, 5th ed. Garland Science.

Bakker E (2004), *Electrochemical sensors*, Analytical Chemistry 2004, 76 (12), 3285–3298.

Berk AJ (1989), Characterization of RNA molecules by S1 nuclease analysis. *Methods in Enzymology*, 180, 334–347

Brown TA (2010), *Gene cloning and DNA analysis*, 6th ed. Wiley-Blackwell.

Carrigan PE, Ballar P, Tuzmen S (2011), Site-directed mutagenesis, *Methods Mol Biol*. 700:107-24.

Divan A and Royds J (2013), *Tools and techniques in biomolecular science*, Oxford University Press.

Doudna J and Charpentier E (2014), The new frontier of genome engineering with CRISPR-Cas9. Science, Vol 346 Issue 6213.

Eggins B (2002) *Chemical sensors and biosensors*, Analytical Techniques in the Sciences. John Wiley & Sons, West Sussex.

Ekins R and Chu FW (1999), Microarrays: their origins and applications. *Trends Biotechnol.* 17(6):217–18

Erlich HA. Gelfand D and Sninsky JJ (1991), Recent advances in the polymerase chain reaction. *Sciences,* 252: 1643-1651.

Fields S and Sternglanz R (1994), The two hybrid system: an assay for protein-protein interactions, *Trends in Genetics*, 10, 286–292.

Fields, S and Song OK (1989), A novel genetic system to detect protein-protein interactions. *Nature,* 340: 245-246.

Freeman WM, Walker SJ and Vrana KE (1999), Quantitative RT-PCR: Pitfalls and potential. *BioTechniques,* 26: 112-125.

Galas DJ and Schmitz A (1978), DNase footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids research*, 5, 3157-3170.

Gilmartin PM and Bowler DChris (2002), *Molecular Plant Biology: A Practical Approach*, Volume 2, Oxford university press.

Horvath P and Barrangou R (2010), CRISPR/Cas, the Immune System of Bacteria and Archaea. Science, 327, 167.

Jiang W and Marraffini L (2015), CRISPR-Cas: New Tools for Genetic Manipulations from Bacterial Immunity Systems. Annu. Rev. Microbiol, 69:209–28.

Khudyakov YE and Fields HA (2010), *Artificial DNA: Methods and Applications*, CRC Press.

Leanna CA and Hannink M (1996), The reverse two-hybrid system: a genetic scheme for selection against specific protein/protein interactions. *Nucl. Acids Res,* 24: 3341-3347.

Li Y and Peng N (2019), Endogenous CRISPR-Cas System-Based Genome Editing and Antimicrobials: Review and Prospects, Front. Microbiol.

Liu Z, Dong H, Cui Y, Cong L and Zhang D (2020). Application of different types of CRISPR/Cas-based systems in bacteria, Microbial cell factories volume 19, Article number: 172.

Lodish H, Berk A, Kaiser CA, et al (2008), Molecular Cell Biology, 6th ed. New York: WH Freeman.

Makarova K, Haft D, Barrangou R and Brouns S (2011), Evolution and classification of the CRISPR–Cas systems. Nature Reviews Microbiology 9, 467-477

Makarova KS et al (2019), Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants. Nature Reviews, Microbiology.

Mardis ER (2008), Next-Generation DNA Sequencing Methods, Annu. Rev. Genomics Hum. Genet, 9:387-402.

Mardis ER (2013), Next-Generation Sequencing Platforms, Annu. Rev. Anal. Chem 6:287-303.

McPherson M and Moller S (2006), *PCR – The basics*, 2nd ed. Taylor and Francis group.

Metzker ML (2010), Sequencing technologies — the next generation, Nature Reviews Genetics 11, 31-46.

Miesfeld RL (1999), *Applied Molecular Genetics*, Wiley-Liss, Inc.

Nicholl ST (2002), *An Introduction to Genetic Engineering*, 3rd ed. Cambridge University Press.

Parra I and Windle B (1993), *High resolution visual mapping of stretched DNA by fluorescent hybridization*. Nature Genetics 5, 17–21.

Puschnik A, Majzoub K, Ooi Y and Carette J (2017), A CRISPR toolbox to study virus–host interactions. Nature Reviews Microbiology 15, 351–364.

Ried T, Schröck E, Ning Y, et al. (1998), Chromosome painting: a useful art. *Hum Mol Genet* 7:1619–1626.

Ronaghi M, Ehleen M and Nyrn P (1998), A sequencing method based on real-time pyrophosphate. *Science*, 281, 363–365.

Schena M, Shalon D, Davis RW and Brown PO (1995), Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science,* 270 (5235): 467-70.

Southern EM (2001), DNA microarrays: History and overview. *Methods Mol Biol* 170:1–15

Speicher MR and Carter NP (2005), The new cytogenetics: Blurring the boundaries with molecular biology. Nature Reviews Genetics 6, 782–792.

Stoughton RB (2005), Applications of DNA microarrays in biology. Annu Rev Biochem, 74:53–82.

Strachan T and Read AP (2004), *Human Molecular Genetics*, 3rd ed. Garland Publishing.

VanGuilder HD, Vrana KE and Freeman WM (2008), Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques*, 44, 619–624.

Wang H, Russa ML and Qi LS (2016), *CRISPR/Cas9 in Genome Editing and Beyond*, Annual Review of Biochemistry, Vol. 85:227-264.

Weissensteiner T, Nolan T, Bustin SA, Griffin HG and Griffin A (2004), *PCR Technology: Current Innovations*, 2nd ed. CRC Press.

Self Test

Question 1

A biochemist is attempting to separate a DNA binding proteins (X) in a solution. Only three other proteins (A, B and C) are present. The proteins have the following properties:

	Isoelectric point	Size	Bind to DNA
Protein A	7.4	82,000	Yes
Protein B	3.8	21,500	Yes
Protein C	7.9	23,000	No
Protein X	7.8	22,000	yes

What type of protein separation techniques might she use to separate?

P. Protein X can be separated from protein A by size-exclusion chromatography.

Q. Protein X can be separated from protein B by ion-exchange chromatography.

R. Protein X can be separated from protein A by isoelectric focusing.

S. Protein X can be separated from protein C by affinity chromatography.

a. P and Q
b. Q and S
c. P, Q and S
d. R and S

Question 2

A mixture of amino acids can be analyzed by first separating the mixture into its components through ion exchange chromatography. Amino acids placed on a cation exchange resin containing sulfonate groups flow down the column at different rates because of two factors that influence their movement:

1. Ionic attraction between the sulfonate residues on the column and positively charged functional groups on the amino acids.
2. Hydrophobic interactions between amino acid side chains and the strongly hydrophobic backbone of the polystyrene resin. For each pair of amino acids listed, determine which will be eluted first from the cation-exchange column by a pH 7 buffer.

P. Asp and Lys

Q. Glu and Val

R. Gly and Leu

S. Ser and Ala

a. P–Asp, Q–Glu, R–Gly, S–Ser
b. P–Asp, Q–Val, R–Leu, S–Ala
c. P–Lys, Q–Glu, R–Leu, S–Ser
d. P–Lys, Q–Val, R–Gly, S–Ala

Question 3

A protein has a molecular mass of 400 kDa when measured by gel filtration chromatography. When subjected to gel electrophoresis in the presence of SDS, the protein gives three bands with molecular masses of 180, 160 and 60 kDa. When electrophoresis is carried out in the presence of SDS and β-mercaptoethanol, three bands are again formed, this time with molecular masses of 160, 90 and 60 kDa. What is the subunit composition of the protein?

P. The protein has four subunits – 160, 90, 90 and 60 kDa.

Q. Two 90 kDa subunits are linked by disulfide bonds.

R. The protein has three subunits – 180, 160 and 60 kDa.

S. Two 60 kDa subunits are linked by disulfide bonds.

a. P and Q

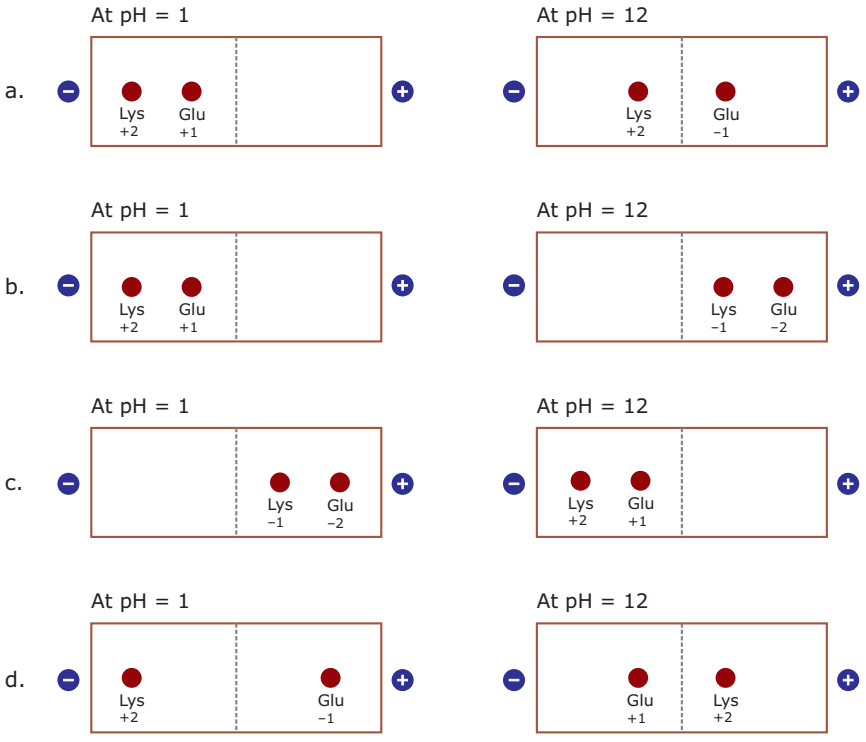
b. P and S

c. Q and R

d. R and S

Question 4

A mixture of glutamic acid and lysine is separated by paper electrophoresis in two experiments, one at pH 1 and other at pH 12. After the separation, the papers are treated with ninhydrin to reveal the location of the amino acids (colored spots). The results are shown below. Which of the following represent the *correct* result?



Question 5

From the data given below, identify the protein pair that would (P) give the least mobility band on a SDS polyacrylamide gel electrophoresis experiment and (Q) elute last on an anion exchange e.g. DEAE column respectively:

Protein	pI	Subunit MW	Native MW
A	9	10,000	20,000
B	8	35,000	35,000
C	6	15,000	90,000
D	5	20,000	80,000
E	3	30,000	30,000

a. P–protein C ; Q–protein A
b. P–protein A ; Q–protein E
c. P–protein B ; Q–protein E
d. P–protein B ; Q–protein A

Question 6

You have a mixture of proteins with the following properties:

Protein 1 : molecular mass – 12 kDa and pI = 10

Protein 2 : molecular mass – 62 kDa and pI = 4

Protein 3 : molecular mass – 28 kDa and pI = 8

Protein 4 : molecular mass – 09 kDa and pI = 5

Predict the order of emergence of these proteins when a mixture of the four is chromatographed in DEAE-cellulose at pH 7.0

a. 1, 3, 4, 2
b. 2, 4, 3, 1
c. 4, 1, 3, 2
d. 5, 8, 4, 10

Question 7

A mixture of four proteins of pIs 11, 7, 5 and 3 are loaded on DEAE anion-exchange column equilibrated with low ionic strength buffer of pH 8. Which of the four proteins would be expected to be retained on the column?

a. Protein with pI 11 but not the others
b. Proteins with pIs 11 and 7 but not 5 and 3
c. Proteins with pIs 7, 5 and 3
d. Protein with pI 7 but not the others

Question 8

The void volume of a gel filtration column, V_0 = 30 mL. A monomeric protein with a known molecular mass of 25 kDa elutes at a volume of 45 mL. The protein that you are trying to purify elutes at a volume of 35 mL. Which of the following is a valid conclusion?

a. Your protein is repelled by the gel filtration material
b. Your protein molecular mass = 25 kDa
c. Your protein molecular mass > 25 kDa
d. Your protein molecular mass < 25 kDa

Index

Biophysics and Molecular Biology

Tools and Techniques

This textbook covers essential fundamentals and their applications of current techniques and methodologies. It is presented in a sharply focused manner without overwhelming or excessive detail. Each chapter contains information in a systematic and logically organized manner. It is a perfect introductory textbook for any reader needing to learn the basics of the subject, assuming they have little prior knowledge of the subject.

In this revised and updated version, I focused on building on the strengths of the previous editions to present biophysics in an even more precise and streamlined manner, as well as incorporating exciting new advances from the field. Throughout the book, we have updated explanations of basic concepts and bolstered them with examples from new research.

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