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Biophysics and Molecular Biology

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Biophysics and Molecular Biology

Tools and Techniques

Fifth edition

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Chromatography

Chromatography is a physical method for the separation of compounds present in a sample. Tswett, a Russian botanist (referred to as the father of *chromatography*), is credited for developing chromatography. He employed the technique to separate various plant pigments such as chlorophylls and xanthophylls, bypassing 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 a separation process in which the components of a sample are distributed between two phases, one of which is stationary (termed as **stationary phase**) while the other moves (termed as **mobile phase**). The sample to be examined (called the *solute* or *analyte*) is allowed to interact with the mobile phase and stationary phase. These two phases could be a solid and a liquid, or a gas and a liquid or a liquid and another liquid. The stationary phase, which may be a solid or a liquid supported on a solid, does not move. The mobile phase moves the sample through or along the stationary phase in a definite direction. The mobile phase may be a *liquid* (**liquid chromatography**) or a *gas* (**gas chromatography**). In gas chromatography, the term *carrier gas* may be used for the mobile phase. All chromatographic methods involve passing a mobile phase through a stationary phase. Substances separated by a chromatographic system must have different relative affinities for these two phases. Thus, a substance with a relatively higher affinity for the stationary phase moves slowly through the chromatographic system than does a substance with a lower affinity. This difference in mobility ultimately leads to the physical separation of the components present in a sample. The nature of the specific mobile and stationary phases determines which substances travel more quickly or slowly and is how they are separated.

> The international union of pure and applied chemistry (IUPAC) has defined chromatography as: A method, used primarily for the 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.

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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.

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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.

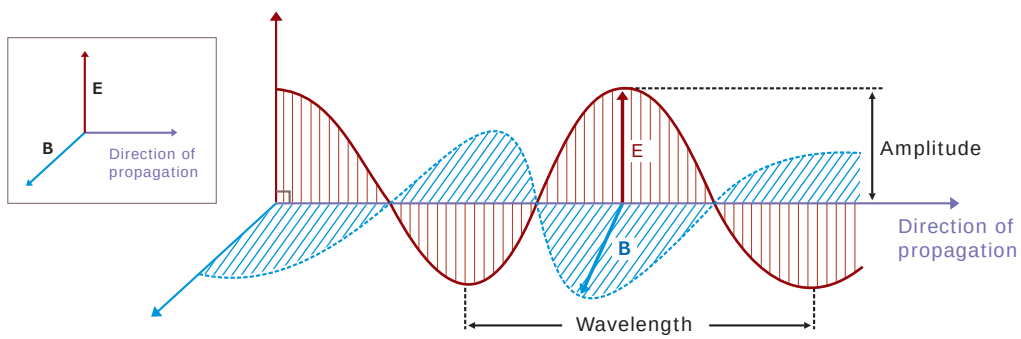

Figure 3.1
A representation of electromagnetic radiation with the electric field (**E**) and the magnetic field (**B**) at right angles to the direction of the wave movement. Both fields oscillate at the same frequency.

In a vacuum, all electromagnetic waves move at the same speed and differ from one another in their frequency. The classification of electromagnetic waves according to frequency or wavelengths is the **electromagnetic spectrum**. Different frequencies correspond to different wavelengths—waves of low frequencies have long wavelengths, and waves of high frequencies have short wavelengths. The electromagnetic spectrum ranges from very short wavelengths

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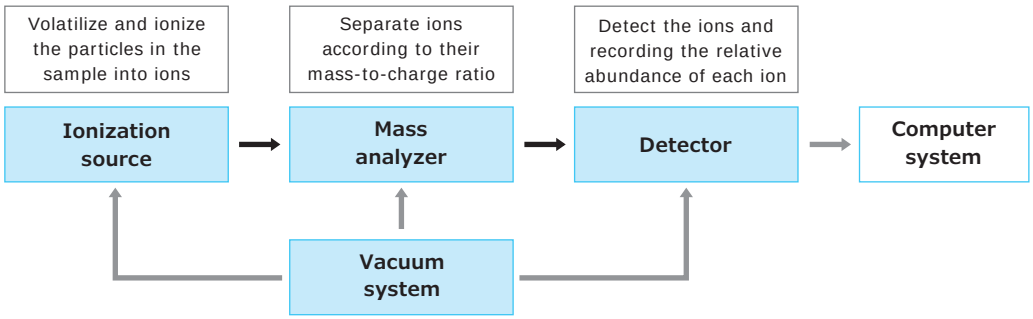
Mass spectrometry

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

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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 force due to gravity (*or* simply, gravity). The force due to gravity 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 force due to gravity 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.

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Microscopy

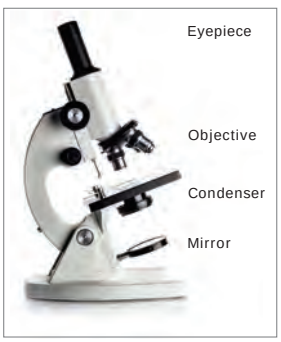
Microscopy is a technique for making very small things visible to the unaided eye. An instrument used to make the small things visible to the naked (unaided) eye is called a **microscope**. There are two fundamentally different types of microscopes based on the source of illumination: the light microscope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

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Flow cytometry

Flow cytometry is a technique used for sorting, counting, and examining cells (or microparticles) suspended in a fluid by their optical properties (light scattering and fluorescence). The term **cytometry** refers to the measurement of cell number and cell characteristics such as cell size, shape, cell cycle stage, DNA content, and more. It simultaneously measures and analyzes multiple physical characteristics of a single cell as they flow through a fluid stream and pass through a beam of light.

Components of a flow cytometer

A *flow cytometer* consists of three main systems: *fluidics*, *optics*, and *electronics*. The **fluidic system** transports the cells in a stream to the light beam for analysis. The **optical system** consists of excitation optics and collection optics. The *excitation optics* include light sources generating scattered and fluorescent light signals, and *collection optics* include lenses that collect light scattered or emitted from the interaction between cells and the light beam. They also include a system of optical mirrors and filters to separate and then direct specific light signals to the appropriate optical detectors. The **electronics system** converts light signals into electronic signals that can be processed by the computer. Essentially, the electronics serve as the brain of the flow cytometer.

Fluidic system

The flow cytometer measures both the physical and chemical characteristics of a cell or microscopic object. This is achieved by introducing cells suspended in a fluid to pass through an *interrogation point* one by one. The location where cells interact with light beam is termed the **interrogation point**. At this point, we interrogate each cell using a specific light beam, and observe the light responses electronically.

The primary function of the fluidics system is to present cells in suspension to the interrogation point for examination one cell at a time. This is achieved by a process known as '*hydrodynamic focusing*'. For this, the sample stream is injected into a faster-moving stream of **sheath fluid** (usually phosphate-buffered saline) in the flow cell (flow chamber). Differences in the pressure, velocity, and density of the two fluids prevent them from mixing. The flow of sheath fluid propels and confines the cells to the center of the sample core, creating a single-file stream of cells.

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X-ray crystallography

X-rays are electromagnetic radiation of short wavelength. Like all electromagnetic radiation, X-rays are absorbed, scattered, and diffracted by matter. The *scattering* and *diffraction* of X-rays in gases, liquids, or disordered solids is caused by interaction with electrons. Electrons in ordered arrays of atoms in crystals scatter X-rays only in particular directions; in other directions, the scattering is negligible. Diffraction is the scattering of X-rays in a few specific directions by crystals. **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

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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**.

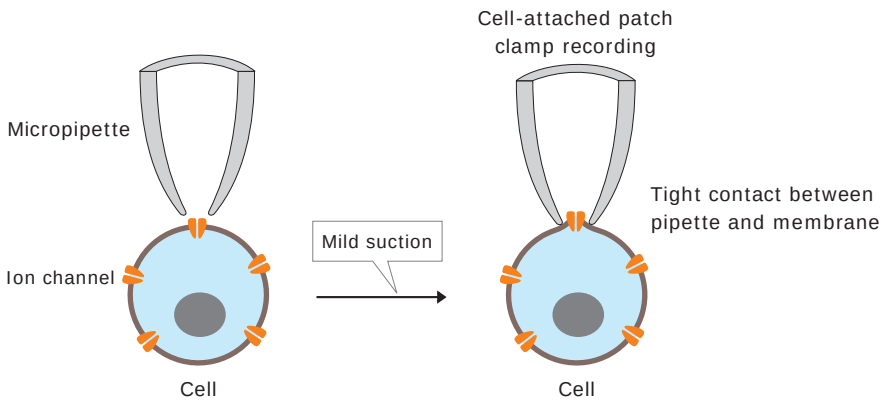

Figure 9.1 Cell-attached patch clamp recording. When the pipette is in closest proximity to the cell membrane, mild suction is applied to gain a tight seal between the pipette and the membrane.

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

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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

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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.

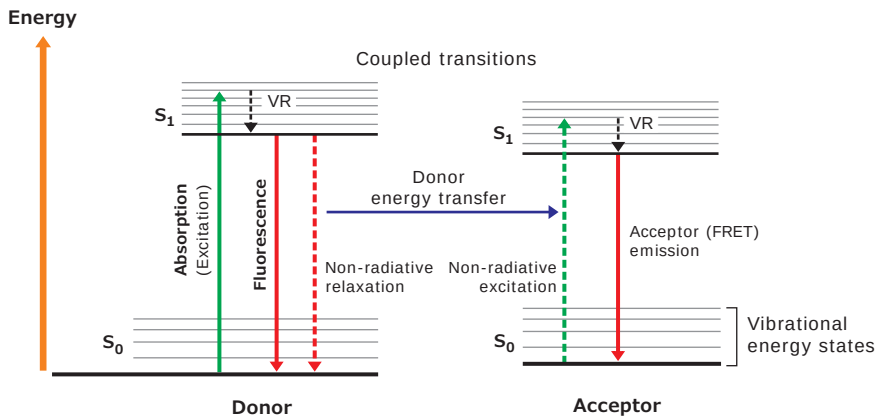

Figure 11.1 A *Jablonski diagram* illustrating the coupled transitions involved between the donor emission and acceptor absorbance in fluorescence resonance energy transfer. In this diagram, the donor molecule is excited by a photon. Under typical fluorescence conditions, relaxation of the electron energy 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.

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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.