SMB5454	Instrumentation methods	L	T	P	Credits	Total Marks
		3	1	0	4	100

COURSE OBJECTIVE:

- To provide basic understanding and sound knowledge on various basic as well as modern instruments and techniques especially which are specific to medical biotechnology. This would help the students to understand the principles of various instruments engaged in the biological research.

UNIT: I PRINCIPLES AND APPLICATION OF SPECTROSCOPY 12 Hrs

Absorption of Radiation, Beers Lambert law, Deviations, Instrumentation, Double beam and single beam spectrometers, sources of radiation detectors, photometric accuracy, spectrophotometer operation, instrumentation optical materials sources, detectors spectrophotometers, Fourier transform, spectrophotometers, calibration and standardisation, atomisation, flame atomisation, sources of radiation, background correction, detection limits, inferences and applications. Spectro-fluorimetry and its applications.

UNIT: II CHARACTERIZATION TECHNIQUES 12 Hrs

Nuclear Magnetic Resonance **(**NMR)**,** Infrared and Raman Spectroscopy and their application. Mass spectrometry, Atomic absorption and atomic emission spectroscopy.

UNIT: III PRINCIPLES AND APPLICATIONS OF X-RAYS12 Hrs

The absorption of x-rays, monochromatic X-ray sources, X-ray detectors, x-ray diffraction, x-ray fluorescence, power and single crystal diffraction methods, comparison of X-ray diffraction and neutron diffraction.

UNIT: IV BIOPHYSICAL TECHNIQUES AND INSTRUMENTATION 12 Hrs

Sedimentation, Ultra Centrifugation, Gradient centrifugation, Electrophoresis – SDS-PAGE and Agarose Gel electrophoresis, Chromatography techniques – TLC, HPLC, GLC and its application. Fluorescent activated cell sorter (FACS).

UNIT: V MICROSCOPY 12 Hrs

Phase contrast microscopy, Electron microscopy (TEM and SEM), Fluorescent Microscopy, Confocal microscopy, Atomic Force microscope (AFM).

Max. 60 Hours

TEXT / REFERENCE BOOKS:

1. Ewing GW, "Instrumental Methods of Chemical Analysis", McGraw Hill Book Company, 1989.
2. Principles of Instrumental Analysis 5th Edn. Skoog.D.A., Thompson, Brooks and Cole.
3. Willard and Merrit, "Instrumental Methods and Analysis ", VI Edition, CBS Publishers and Distributors.
4. Braun H., "Introduction to Chemical Analysis ", McGraw Hill, 1987.

Course Objective:

To provide basic understanding and sound knowledge on various basic as well as modern instruments and techniques especially which are specific to medical biotechnology. This would help the students to understand the principles of various instruments engaged in the biological research.

Course Outcome:

CO1: Explain the theoretical principles of UV and Spectro-fluorimetry.

CO2: Learn basic principles and instrumentation of NMR, Raman, Mass and Atomic emission spectroscopy.

CO3: Learn basic principles involved in principles and applications of X-Rays

CO4: Understand the separation of compounds by various chromatographic and electrophoresis technique.

CO5: Explain theory and instrumentation of different microscopy.

CO6: To learn about the applications of various microscopy.

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DEPARTMENT OF CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES

UNIT – I

INSTRUMENTATION METHODS (SMB5454)

Introduction of UV Spectroscopy

Absorption spectra of chemical species (atoms, molecules, or ions) are generated when a beam of electromagnetic energy (i.e. light) is passed through a sample, and the chemical species absorbs a portion of the photons of electromagnetic energy passing through the sample. A classic example of this phenomenon is responsible for our perception of color. Consider the situation where a beam of white light (i.e. sunlight) passes through a sample solution containing chlorophyll (the compound responsible for the color of leaves). The chlorophyll molecules absorb only a few select photons in the blue and red regions of the visible portion of the electromagnetic spectrum. The energies of these absorbed photons cause electrons in the chlorophyll molecule to be excited, and in the plant cell the energy of these excited electrons is used to drive the conversion of carbon dioxide and water to glucose. More important for our purposes is the fact that when the red and blue photons that chlorophyll absorbs are **subtracted** from white light, the resulting beam of light **leaving the solution** appears green to our eye, and this is why leaves appear green to us.

If we could measure the total number of photons of all colors that enter the sample and compare that with the total number of photons of all colors that leave the sample, we would find fewer photons exiting the sample than entering the sample. This is consistent with the fact that the chlorophyll molecules absorbed some of the photons from the beam of white light that entered the solution.

A spectrophotometer is an instrument designed to make this measurement. Using some very well understood electronics, this device effectively “counts” the number of photons that enters a sample and compares it with the number of photons that exits a sample. In addition, the instrument is able to take white light and separate it into its constituent colors (i.e. somewhat likea prism), allowing the user to examine the absorption of light of individual wavelengths with nearly 1 nm resolution.

In optics, that portion of physics that deals with the properties of light, the measurement of the number of photons delivered at a point in a given unit of time is called the Intensity, I. (Higher intensity could be thought of as “brighter” and lower intensity could be thought of as “dimmer”; hence high intensity light will be bright and low intensity light will be dim.) If we measure the intensity of the beam of light entering our sample (Io) and compare it with the intensity of the beam of light exiting our sample (I) we can take the ratio I/Io to get an indication of what

fraction of the light entering the sample was found exiting the sample. This ratio is called the Transmittance:

Transmittance: T =I/ Io

We can convert this ratio into a percentage by multiplying by 100 to get Percent Transmittance (%T):

% Transmittance: %T = I/ Io x100

Thus, if the intensity of the light exiting our sample is 76 and the intensity of the light entering our sample is 100, then the Transmittance would be 0.76 and the % Transmittance would be 76%, indicating that 76% of the photons entering our sample are finding their way out of our sample.

For our purposes it is mathematically convenient to define a new concept, Absorbance (A):

Absorbance: $A= -\log_{10} I/ Io$

Absorbance is a direct measure of how much light is absorbed by our sample. If you play with the formula in your calculator you will find that absorbance can take on values between 0 (at 100% Transmittance) and about 2.0 (at 1% Transmittance); thus large values of absorbance are associated with very little light passing entirely through the sample and small values of absorbance (i.e. those approaching 0) are associated with most of the light passing entirely through the sample.

The Beer-Lambert Law:

Consider a solution of a chemical species that absorbs light of a particular wavelength. We could imagine two interesting situations. First, if we pass a beam of light of the appropriate wavelength through a fairly dilute solution, we could imagine that the photons will encounter a small number of the absorbing chemical species, so we might expect a high % transmittance and a low absorbance. Alternatively, if we pass the same beam of light through a highly concentrated solution, we could imagine that the photons will encounter a large number of the absorbing chemical species, and we might expect a low % transmittance and a high absorbance. Thus, absorbance is proportional to the concentration of the sample. Secondly, we could imagine that if we allow the beam of light to encounter the solution for a long period of time we might expect to see a low % transmittance and a high absorbance; whereas, if the beam were allowed to encounter the solution for a short period of time we might expect to see a high % transmittance

and a low absorbance. Since light travels at a constant speed, c = 3.0 x 10^8 m/s, this implies that the absorbance should also be proportional to the path length of the beam through the sample. These two considerations allow us to establish the following proportionality:

A α k X l X c, where, k = proportionality constant,
l = path length, and
c = concentration of the absorbing
chemical species
When the path length is measured in centimeters, and the concentration of the absorbing species is measured in Molarity, the proportionality constant is called the Molar Absorptivity, having units of $M^{-1}cm^{-1}$, and our proportionality reduces to the

Beer-Lambert Law:

A = ε X l X c

This technique is used not only by chemists but by scientists of many fields. The Beer-Lambert law allows you, the scientist, to measure the absorbance of a particular sample and to deduce the concentration of the solution from that measurement! In effect, you can measure the concentration of a particular chemical species in a solution as long as you know the species absorbs light of a particular wavelength. Thus, The Beer Lambert law is combined of two laws and each are correlates which state that, the absorbance of light is proportional to the thickness of the sample or absorbance is proportional to the concentration of the sample.

Deviation

Under certain conditions Beer–Lambert law fails to maintain a linear relationship between attenuation and concentration of analyte. These deviations are classified into three categories:

1. Real—fundamental deviations due to the limitations of the law itself.
2. Chemical—deviations observed due to specific chemical species of the sample which is being analyzed.
3. Instrument—deviations which occur due to how the attenuation measurements are made.

There are at least six conditions that need to be fulfilled in order for Beer–Lambert law to be valid. These are:

1. The attenuators must act independently of each other. Electromagnetic coupling must be excluded.
2. The attenuating medium must be homogeneous in the interaction volume.
3. The attenuating medium must not scatter the radiation—no turbidity—unless this is accounted for as in DOAS.
4. The incident radiation must consist of parallel rays, each traversing the same length in the absorbing medium.
5. The incident radiation should preferably be monochromatic, or have at least a width that is narrower than that of the attenuating transition. Otherwise a spectrometer as detector for the power is needed instead of a photodiode which has not a selective wavelength dependence.
6. The incident flux must not influence the atoms or molecules; it should only act as a non-invasive probe of the species under study. In particular, this implies that the light should not cause optical saturation or optical pumping, since such effects will deplete the lower level and possibly give rise to stimulated emission.
7. The wave properties of light must be negligible. In particular interference enhancement or decrease must not occur.

If any of these conditions are not fulfilled, there will be deviations from Beer–Lambert law.

Spectrometer/spectrophotometer

- The simplest spectrophotometer is a single-beam instrument equipped with a fixed-wavelength monochromator,
- Single-beam spectrophotometers are calibrated and used in the same manner as a photometer.
- One common example of a single-beam spectrophotometer is the Spectronic-20
- It has a fixed effective bandwidth of 20 nm.

- Because its effective bandwidth is fairly large, this instrument is more appropriate for a quantitative analysis than for a qualitative analysis.
- Other single-beam spectrophotometers are available with effective bandwidths of 2-3 nm.
- Fixed-wavelength single-beam spectrophotometers are not practical for recording spectra since manually adjusting the wavelength and recalibrating the spectrophotometer is time-consuming.

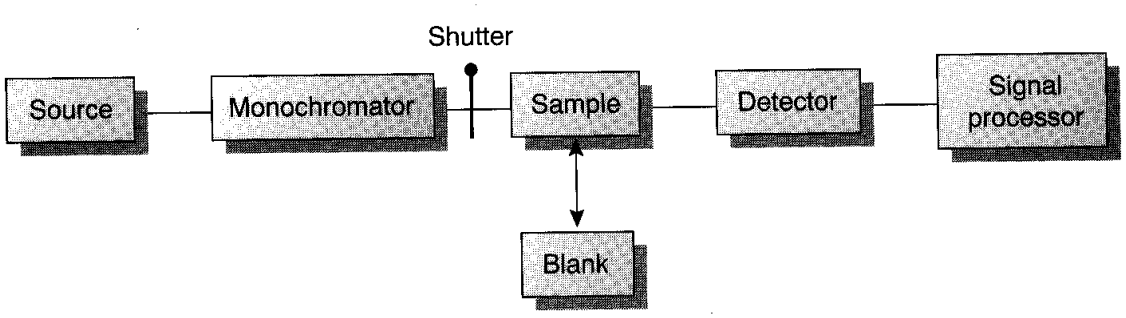

Fig.1: Block diagram for a single beam fixed wavelength spectrophotometer

Double-beam spectrophotometer

- A chopper is used to control the radiation's path, alternating it between the sample, the blank, and a shutter.
- The signal processor uses the chopper's known speed of rotation to resolve the signal reaching the detector into that due to the transmission of the blank (P_o) and the sample (P_T).
- By including an opaque surface as a shutter it is possible to continuously adjust the 0% T response of the detector.
- The effective bandwidth of a double-beam spectrophotometer is controlled by means of adjustable slits at the entrance and exit of the monochromator.
- Effective bandwidths of between 0.2 nm and 3.0 nm are common.
- A scanning monochromator allows for the automated recording of spectra.

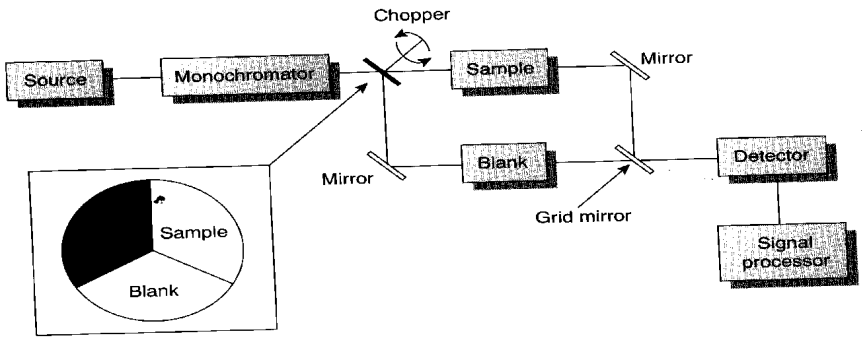

Fig.2: Block diagram for a double beam spectrophotometer

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UNIT – II

INSTRUMENTATION METHODS (SMB5454)

NMR Spectroscopy

In our discussion of spectroscopy, we have shown that absorption of E.M. radiation occurs on resonance: When the frequency of applied E.M. field matches the energy splitting between two quantum states. Magnetic resonance differs from these other methods in the sense that we need to immerse the same in a magnetic field in order to see the levels that we probe with an external (rf or μwave) field. (Two fields: Static magnetic and E.M.) We will be probing the energy levels associated with the spin angular momentum of nuclei and electrons: NMR--nuclear magnetic resonance and ESR/EPR--electron spin resonance.

Angular momentum:

In our treatment of rotational energy levels, we said that the energy levels depended on the rotational angular momentum, *L*, which was quantized:

L2 = 2J (J +1) *J* = 0,1,2… *rot. quant. number*

• Degeneracy of *J* was *mJ* (= 0,…, ±*J*)→(2*J* +1)

• We related L2 to the energy levels

Actually, **all angular momentum is quantized.**

If a particle can spin, it has A.M. and quantized *E* levels.

In particular, we also have to be concerned with the **spin of individual nuclei and electrons.**

Nuclear spin angular momentum

I 2 = 2 *I*(*I* +1) I: nuclear spin quantum number

I z = *mI m I* : − *I*,− *I* + 1,…, *I*

What is *I* ? Each proton/neutron has a spin quantum number of 1/2.

Spin of many nucleons add up vectorily to give *I* . Spins pair up so that even number of spins are paired → *I* = 0 .

1. For even number of protons plus even number of neutrons: I = 0 . 12C, 16O

2. For one unpaired nucleon → *I* = 1

2 *mI* = ± 1

2 degeneracy 2*I* + 1 = 2

1*H*, 13*C*, 15*N*

So the proton and electron are similar—both spin 1

2 particles . We'll talk about

these two particles more specifically…

3. Two unpaired nucleons → $I = 1$ $mI = 0,\pm1$

2 *H*, 14*N*

• For spin ½ particles (I or S = ½) there are two degenerate energy states.

• When you put these in a magnetic field you get a splitting.

 A low energy state aligned with the field, and

 A high energy state aligned against the field

Classical picture:

• Think of our electron or nucleus as a charged particle with angular momentum, M .

• A circulating charge produces a magnetic field.

• This charge possesses a *magnetic* dipole moment μ that can be affected by an applied magnetic field.

$M = m(v \times r)$

$\mu = Q(v \times r) / 2c$

The dipole lies along M and the strength of μ is

proportional to M:

QM M

2mc

$\mu = \equiv \gamma \cdot$

γ is the gyromagnetic ratio

If we immerse this system in a magnetic field, B, which is oriented along the *z* axis

The interaction potential is $E_{int} = -\mu \cdot B$

The spins align along the magnetic field: int z $E = -\mu B$

Since $\mu z = -\gamma e Sz$ and $\mu z = \gamma N Iz$,

using Sz = ms and Iz = mI we have

For electrons: Eint = γe msB s

m 1

2

= ±

For nuclei: int N I E = −γ □m B I

m 1

2

= ±

So, as we increase the magnetic field strength, the two energy levels − originally degenerate – split, one increasing in energy and one decreasing in energy. This is known as the ***Zeeman effect.***

Nuclei Electrons

mI = −1/2 ms = +1/2

N ΔE = −γ □B ΔE = γe□B

mI = +1/2 ms = −1/2

• Now we have a system that can absorb E.M. radiation on resonance: ΔE=hν

• ν is the applied frequency (in the radio frequency range)

• Frequency domain spectrometer: Typically sweep **B** and hold ν constant.

B2γν = π (Larmor frequency)

Typical numbers:

Nuclear Magnetic Resonance Electron Spin Resonance

|μH| = 2.8 × 10-23 · (± ½) erg/gauss |μe| = −1.8 × 10-20 · (± ½) erg/gauss

for B = 10 kG: ν = 42.6 MHz ν = 28 GHz

Typical: 300 MHz/70 kG 9.5 GHz/3.4 kG

Typical population difference ΔN = (N− − N+)/(N− + N+) ≈ 0.008%

The Chemical Shift

Thus far you would think that all 1H absorb at same frequency. Yet, in practice different nuclei absorb at different frequencies.

The resonance frequency depends on the “effective” magnetic field that a proton feels. This can differ for different types of 1H due to local electron currents that counteract the

IR Spectroscopy

Spectroscopy can be defined as the interaction between matter and light. Infrared spectroscopy is a very powerful technique which uses electromagnetic radiation in the infrared region for the determination and identification of molecular structure as well as having various quantitative applications within analytical chemistry (Figure 1).

We do not aim to provide a mechano-quantic description of light and its interaction with atoms, as this is out of the scope of this module. However, it is important to note that atoms can absorb energy from electromagnetic radiation; this absorbed energy alters the state of the atoms within the molecule. These changes are usually manifest in alterations to the frequency and amplitude of molecular vibrations, which may be measured and plotted to produce an infrared spectrum.

Infrared spectrometers use optical devices for dispersing and focusing electromagnetic radiation of IR frequency which is passed through the sample and any changes in absorbance measured against a reference beam.

There are three well defined IR regions (near, mid and far). The boundaries between them are not clearly defined and debate still persists, but broadly they are defined as:

- **Near infrared (12820-4000 cm-1)**: poor in specific absorptions, consists of overtones and combination bands resulting from vibrations in the mid-infrared region of the spectrum.
- **Mid-infrared (4000-400 cm-1)**: provides structural information for most organic molecules.
- **Far Infrared (400-33 cm-1):** has been less investigated than the other two regions; however, it has been used with inorganic molecules.

The low energies, typically encountered within the infrared region, are not sufficient to cause electronic transitions; however, they are large enough to cause changes in the frequency and amplitude of molecular vibrations.

Electromagnetic spectrum

The electromagnetic spectrum is the range of all possible frequencies of electromagnetic radiation, each of which can be considered as a wave or particle travelling at the speed of light, often referred to as a photon. These waves differ from each other in length and frequency.

Frequency ν - the number of wave cycles that pass through a point in one second. Measured in Hertz (Hz).

Wavelength λ - The length of one complete wave cycle (cm).

Frequency and wavelength are inversely related (Equation 1):

Where:

c = speed of light 3 x 1010 cm/sec

The energy of a photon (E in Joules) is related to wavelength and frequency as follows (Equation 2):

Where:

h = Planck's constant 6.6 x 10-34 Joules-sec

Energy is directly proportional to frequency; therefore, high energy radiation will have a high frequency.

Energy is inversely proportional to wavelength, hence, short wavelengths are high energy and vice versa (Figure 2).

Raman Spectroscopy

When light is scattered from a molecule or crystal, most photons are elastically scattered. The scattered photons have the same energy (frequency) and, therefore, wavelength, as the incident photons. However, a small fraction of light (approximately 1 in 107 photons) is scattered at optical frequencies different from, and usually lower than, the frequency of the incident photons. The process leading to this inelastic scatter is termed the Raman effect. Raman scattering can occur with a change in vibrational, rotational or electronic energy of a molecule. If the scattering is elastic, the process is called Rayleigh scattering. If it's not elastic, the process is called Raman scattering.

Raman scattering (or the Raman effect) was discovered in 1928 by V. C. Raman who won the Nobel prize for his work. If the substance being studied is illuminated by monochromatic light, for example from a laser, the spectrum of the scattered light consists of a strong line (the exciting line) of the same frequency as the incident illumination together with weaker lines on either side shifted from the strong line by frequencies ranging from a few to about 3500 cm-1. The lines of frequency less than the exciting lines are called Stokes lines, the others anti-Stokes lines.

Raman spectroscopy is very important practical tool for quickly identifying molecules and minerals.

A Raman spectrometer was deployed on the Viking landers in 1972 and in other missions.

Raman spectroscopy also has important scientific applications in studying molecular structure. In this experiment we will study both kinds of applications.

More on Raman Scattering

The Raman effect arises when a photon is incident on a molecule and interacts with the electric dipole of the molecule. In quantum mechanics the scattering is described as an excitation to a virtual state lower in energy than a real electronic transition with nearly coincident de-excitation and a change in vibrational energy. The scattering event occurs in 10-14 seconds or less. The virtual state description of the scattering is shown in Figure 1.

The energy difference between the incident and scattered photons is represented by the arrows of different lengths in Figure 1. Numerically, the Raman shift in wave numbers (cm-1), is calculated through Eq. 1,

At room temperature the thermal population of vibrational excited states is low, although not zero. Therefore, the initial state is the ground state, and the scattered photon will have lower energy (longer wavelength) than the exciting photon. This Stokes shifted scatter is what is usually observed in Raman spectroscopy. Figure 1(a) depicts Raman Stokes scattering.

A small fraction of the molecules are in vibrationally excited states. Raman scattering from vibrationally excited molecules leaves the molecule in the ground state. The scattered photon appears at higher energy, as shown in Figure 1(b). This anti-Stokes-shifted Raman spectrum is always weaker than the Stokes-shifted spectrum, but at room temperature it is strong enough to be useful for vibrational frequencies less than about 1500 cm-1. The Stokes and anti-Stokes spectra contain the same frequency information. The anti-Stokes spectrum can be used when the Stokes spectrum is not directly observable, for example, because of poor detector response at lower frequencies.

A Bit of Molecular Theory

A complex molecule can absorb and emit energy by interacting with photons that can excite molecules to higher vibrational states. These vibrations are quantized and are called the normal modes of vibration of the molecule. A linear molecule with N atoms has 3N-5 normal modes, while a non-linear molecule has 3N-6 normal modes. The motion that characterizes each normal mode can be:

• Bending motion between three atoms connected by two bonds

• Stretching motion between two bonded atoms

• Out-of-plane deformation modes that change an otherwise planar structure into a nonplanar

One Vibrational Modes

The vibrational spectrum of a molecule is composed of bands representing some active normal vibrations. The spectrum depends on the masses of the atoms in the molecule, the strength of their chemical bands and the atomic arrangement. Consequently, different molecules have different vibrational spectra or different "finger prints." In a first approximation, groups of atoms have certain characteristic vibrations in the IR and Raman spectra.

vibrational spectra are useful in elucidating the molecular conformation/ structure. For example, the stretching frequency of phosphorus-phosphorus bonds range from 460 to 610 to 775 cm-1 for the single, double and triple bonded cases, respectively. Much effort has been devoted to estimation or measurement of force constants. For small molecules, and even for some extended structures such as peptides, reasonably accurate calculations of vibrational frequencies are possible with commercially available software.

Vibrational Raman spectroscopy is not limited to intramolecular vibrations. Crystal lattice vibrations and other motions of extended solids are Raman-active. Their spectra are important in such fields as polymers and semiconductors. In the gas phase, rotational structure is resolvable on vibrational transitions. The resulting vibration/rotation spectra are widely used to study combustion and gas phase reactions generally. Vibrational Raman spectroscopy in this broad sense is an extraordinarily versatile probe into a wide range of phenomena ranging across disciplines from physical biochemistry to materials science.

Raman Selection Rules and Intensities

A simple classical electromagnetic field description of Raman spectroscopy can be used to explain many of the important features of Raman band intensities. The dipole moment, *P*, induced in a molecule by an external electric field, E, is proportional to the field

$P = \alpha E$ (2)

The proportionality constant α is the polarizability of the molecule. The polarizability measures the ease with which the electron cloud around a molecule can be distorted. The induced dipole emits or scatters light at the optical frequency of the incident light wave.

Raman scattering occurs because a molecular vibration can change the polarizability. The change is described by the polarizability derivative, $d\alpha / dQ$, where Q is the normal coordinate of the vibration. The selection rule for a Raman-active vibration, that there be a change in polarizability during the vibration, is

The Raman selection rule is analogous to the more familiar selection rule for an infrared-active vibration, which states that there must be a net change in permanent dipole moment during the vibration. From group theory it is straightforward to show that if a molecule has a center of symmetry, vibrations that are Raman active will be silent in the infrared, and *vice versa*.

Scattering intensity is proportional to the square of the induced dipole moment, *i.e.*, to the square of the polarizability derivative. If a vibration does not greatly change the polarizability, then the polarizability derivative will be near zero, and the intensity of the Raman band will be low. The vibrations of a highly polar moiety, such as the O-H bond, are usually weak. An external electric field can not induce a large change in the dipole moment and stretching or bending the bond does not change this. In organic molecules, particularly, certain frequencies can be associated with typical types of molecular excitations such as C=C triple bond stretching. Some examples are given in Fig. 2.

Mass Spectroscopy

Mass spectrometry's characteristics have raised it to an outstanding position among analytical methods: unequalled sensitivity, detection limits, speed and diversity of its applications. In analytical chemistry, the most recent applications are mostly oriented towards biochemical problems, such as proteome, metabolome, high throughput in drug discovery and metabolism, and so on. Other analytical applications are routinely applied in pollution control, food control, forensic science, natural products or process monitoring. Other applications include atomic physics, reaction physics, reaction kinetics, geochronology, inorganic chemical analysis, ion–molecule reactions, determination of thermodynamic parameters ($G\circ$ f, Ka, etc.), and many others.

Mass spectrometry has progressed extremely rapidly during the last decade, between 1995 and 2005. This progress has led to the advent of entirely new instruments. New atmospheric pressure sources were developed, existing analysers were perfected and new hybrid instruments were realized by new combinations of analysers. An analyser based on a new concept was described:

namely, the orbitrap presented in Chapter 2. This has led to the development of new applications. To give some examples, the first spectra of an intact virus [6] and of very large non-covalent complexes were obtained. New high throughput mass spectrometry was developed to meet the needs of the proteomic metabolomic and other 'omics'.

Principles

The first step in the mass spectrometric analysis of compounds is the production of gasphase ions of the compound, for example by electron ionization:

$M + e^- \longrightarrow M^{\bullet +} + 2e^-$

This molecular ion normally undergoes fragmentations. Because it is a radical cation with an odd number of electrons, it can fragment to give either a radical and an ion with an even number of electrons, or a molecule and a new radical cation. We stress the important difference between these two types of ions and the need to write them correctly:

M·+

EE+

EVEN ION

R·

RADICAL

OE·+

ODD ION

N

MOLECULE

+

+

These two types of ions have different chemical properties. Each primary product ion derived from the molecular ion can, in turn, undergo fragmentation, and so on. All these ions are separated in the mass spectrometer according to their mass-to-charge ratio, and

Diagram of a Mass Spectrometer

A mass spectrometer always contains the following elements, as illustrated in Figure 3: a sample inlet to introduce the compound that is analysed, for example a gas chromatograph or a direct

insertion probe; an ionization source to produce ions from the sample; one or several mass analysers to separate the various ions; a detector to 'count' the ions emerging Mass spectra of isotopic patterns of two alkanes having the molecular formulae C20H42 and C100H202, respectively. The monoisotopic mass is the lighter mass of the isotopic pattern whereas the average mass, used by chemists in stoichiometric calculations, is the balanced mean value of all the observed masses from the last analyser; and finally a data processing system that produces the mass spectrum in a suitable form.

However, some mass spectrometers combine the sample inlet and the ionization source and others combine the mass analyser and the detector.

A mass spectrometer should always perform the following processes:

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

ATOMIC SPECTROSCOPY

The science of atomic spectroscopy has yielded three techniques for analytical use: atomic emission, atomic absorption, and atomic fluorescence. In order to understand the relationship of these techniques to each other, it is necessary to have an understanding of the atom itself and of the atomic process involved in each technique.

The atom is made up of a nucleus surrounded by electrons. Every element has a specific number of electrons which are associated with the atomic nucleus in an orbital structure which is unique to each element. The electrons occupy orbital positions in an orderly and predictable way. The lowest energy, most stable electronic configuration of an atom, known as the ''ground state'', is the normal orbital configuration for an atom. If energy of the right magnitude is applied to an atom, the energy will be absorbed by the atom, and an outer electron will be promoted to a less stable configuration or ''excited state''. As this state is unstable, the atom will immediately and spontaneously return to its ground state configuration. The electron will return to its initial,

stable orbital position, and radiant energy equivalent to the amount of energy initially absorbed in the excitation process will be emitted.

The process is illustrated in Figure 1-1. Note that in Step 1 of the process, the excitation is forced by supplying energy. The decay process in Step 2, involving the emission of light, occurs spontaneously.

Figure 1-1. Excitation and decay processes.

The wavelength of the emitted radiant energy is directly related to the electronic transition which has occurred. Since every element has a unique electronic structure, the wavelength of light emitted is a unique property of each individual element. As the orbital configuration of a large atom may be complex, there are many electronic transitions which can occur, each transition resulting in the emission of a characteristic wavelength of light, as illustrated in Figure 1-2. The process of excitation and decay to the ground state is involved in all three fields of atomic spectroscopy. Either the energy absorbed in the excitation process or the energy emitted in the decay process is measured and used for analytical purposes.

In atomic emission, a sample is subjected to a high energy, thermal environment in order to produce excited state atoms, capable of emitting light. The energy source can be an electrical arc, a flame, or more recently, a plasma. The emission spectrum of an element exposed to such an energy source consists of a collection of the allowable emission wavelengths, commonly called emission lines, because of the discrete nature of the emitted wavelengths. This emission spectrum can be used as a unique characteristic for qualitative identification of the element. Atomic emission using electrical arcs has been widely used in qualitative analysis.

Emission techniques can also be used to determine how much of an element is present in a sample. For a ‘‘quantitative’’ analysis, the intensity of light emitted at the wavelength of the element to be determined is measured. The emission intensity at this wavelength will be greater as the number of atoms of the analyte element increases. The technique of flame photometry is an application of atomic emission for quantitative analysis.

If light of just the right wavelength impinges on a free, ground state atom, the atom may absorb the light as it enters an excited state in a process known as atomic ab- *Figure 1-2. Energy transitions.*

ATOMIC ABSORPTION PROCESS

The quantity of interest in atomic absorption measurements is the amount of light at the resonant wavelength which is absorbed as the light passes through a cloud of atoms. As the number of atoms in the light path increases, the amount of light absorbed increases in a predictable way. By measuring the amount of light absorbed, a quantitative determination of the amount of analyte element present can be made. The use of special light sources and careful selection of wavelength allow the specific quantitative determination of individual elements in the presence of others.

The atom cloud required for atomic absorption measurements is produced by supplying enough thermal energy to the sample to dissociate the chemical compounds into free atoms. Aspirating a solution of the sample into a flame aligned in the light beam serves this purpose. Under the proper flame conditions, most of the atoms will remain in the ground state form and are capable of absorbing light at the analytical wavelength from a source lamp. The ease and speed at which precise and accurate determinations can be made with this technique have made atomic absorption one of the most popular methods for the determination of metals.

A third field in atomic spectroscopy is atomic fluorescence. This technique incorporates aspects of both atomic absorption and atomic emission. Like atomic absorption, ground state atoms created in a flame are excited by focusing a beam of light into the atomic vapor. Instead of looking at the amount of light absorbed in the process, however, the emission resulting from the decay of the atoms excited by the source light is measured. The intensity of this ‘‘fluorescence’’ increases with increasing atom concentration, providing the basis for quantitative determination.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES

UNIT – III

INSTRUMENTATION METHODS (SMB5454)

Introduction

X-Ray photons are electromagnetic radiation with wavelengths typically in the range 0.1 - 100 Å. X Rays used in diffraction experiments have wavelengths of 0.5 - 1.8 Å. X Rays can be produced by conventional generators, by synchrotrons, and by plasma sources. Electromagnetic radiation from nuclear reactions, called γ radiation, can also occur at the same energies as X rays, but γ radiation is differentiated from X ray radiation by the fact that it originates from nuclear reactions.

X rays are sometimes called Röntgen rays after their discoverer, Wilhelm Conrad Röntgen. He called these new rays X rays after the unknown quantity X in mathematics. These new rays had no charge, and were much more penetrating than cathode rays discovered by Johann Hittorf in 1876. X Rays were able to pass through a variety of objects. X rays could expose film. R&oml;ntgen found that X rays could pass through the tissues of a living person and illustrate the bones and other tissues in the body. For this discovery he was awarded the Nobel Prize in physics in 1901.

Röntgen wanted to determine whether X rays were particles or waves. At the time it was known that waves were involved if a stream could be shown to exhibit reflection, refraction, or diffraction. Unfortunately, Röntgen was not able to verify any of these properties of X rays.

From slit measurements, the wavelength of X rays was calculated to be on the order of Angstroms. *Diffraction* can occur when radiation is scattered off of an object with a repeat spacing of approximately the same size as the wavelength of the radiation. Thus, Laue looked for an object with a repeat spacing on the order of Angstroms. Another quick calculation showed that crystals could have the needed lattice spacings. Along with Friedrich and Knipping, Laue showed that X rays could be *diffracted* by a crystal of zinc blende.

Conventional Generators

X Rays are produced in labs by directing an energetic beam of particles or radiation, at a target material. The energetic beam can be electrons, protons, or other X rays. X Rays for crystallographic studies are typically generated by bombarding a metal target with an energetic beam of electrons. The electrons are usually produced by heating a metal filament which then emits electrons. The electrons coming from the filament are then accelerated towards the target by a large applied electrical potential between the filament and the target.

When the beam of electrons hits the target (or anode) a variety of events occur. This rapid deceleration of electrons causes a variety of events including the emission of X-ray radiation, photoelectrons, Auger electrons, and a large amount of heat. Actually two types of X rays are emitted in this process. A continuous band of white radiation is always emitted. If the energy of the electron beam is sufficient then a series of intense, discrete lines that are characteristic of the target material are also observed.

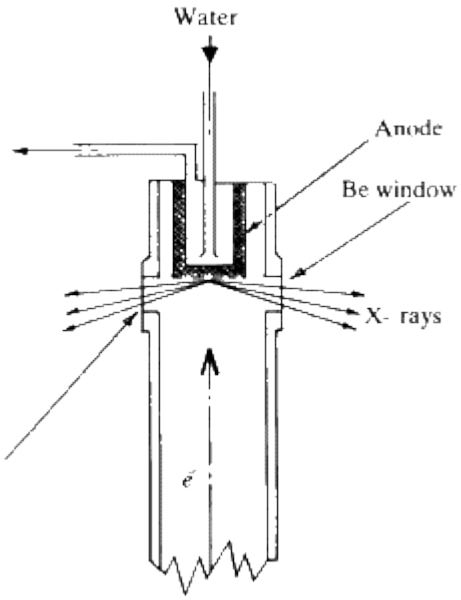


Figure 1. X-ray Tube Schematic.[2]

White Radiation - Bremsstrahlung

Some of the collisions between the thermionic electrons and the target result in the emission of a continuous spectrum of X rays called white radiation or *Bremsstrahlung*. White radiation is believed due to the collision of the accelerated electrons with the atomic nuclei of the target atoms. If all of the kinetic energy carried by an electron is converted into radiation, the energy of the X-ray photon would be given by

$E_{max} = h\nu_{max} = eV$

where h = Plank's constant, ν_{max} = the largest frequency, e = charge of an electron, V = applied voltage. This maximum energy or minimum wavelength is called the Duane-Hunt limit.

$h\nu_{max} = hc/\lambda_{min} = eV$

$\lambda_{min} = hc / eV = 12398. / V$ (volts)

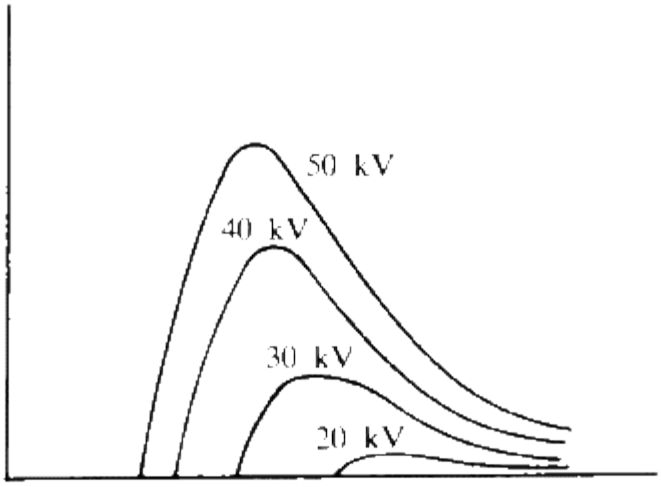


Figure 2. White Radiation from an X-Ray Generator.[2] The intensity of the beam is plotted as a function of the wavelength of the radiation.

The majority of collisions that produce white radiation do not completely dissipate the kinetic energy of the electron in a single collision. Typically, these colliding electrons hit electrons in the target material with a glancing blow dissipating some energy as emitted X-ray photons. Then these photoelectrons hit other electrons in the target material emitting lower energy X-ray photons or hit valence electrons producing heat.

Thus the white radiation spectrum does have a minimum wavelength or maximum energy related to the kinetic energy of the incident radiation beam, and continues to longer wavelengths or lower energies until all of the kinetic energy is absorbed. The highest intensity of emitted white radiation spectrum is obtained at a wavelength that is about 1.5 time the minimum wavelength. The white radiation intensity curve may be fit to an expression of the form:

$I_w = A\ i\ Z\ V^n$, $n \sim 2$

where i is the applied current, Z is the atomic number of the target, V is the applied voltage and A is a proportionality constant. The only type of diffraction experiment that uses white radiation is the Laue experiment.

Characteristic Radiation

When the energy of the electron beam is above a certain threshold value, called the *excitation potential*, an additional set of discrete peaks is observed superimposed on the white radiation curve. The energies of these peaks are characteristic of the type of target material.

These peaks are generated by a two-stage process. First an electron from the filament collides with and removes a core electron from an atom of the target. Then an electron in a higher energy state drops down to fill the lower energy, vacant hole in the atom's structure, emitting an X-ray photon. These emitted X-ray photons have energies that are equal to the difference between the upper and lower energy levels of the electron that filled the core hole. The excitation potential for a material is the minimum energy needed to remove the core electron.

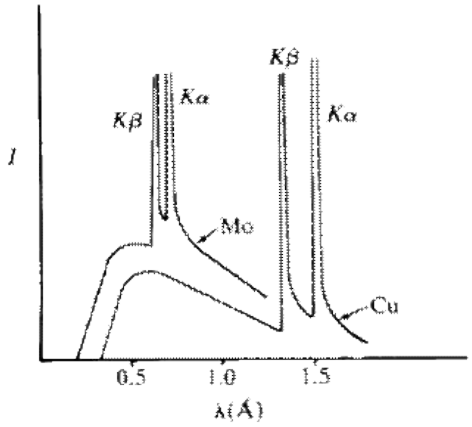


Figure 3. Characteristic radiation from an X-ray generator.[2]

The characteristic lines in an atom's emission spectra are called *K, L, M,* ... and correspond to the n = 1, 2, 3, ... quantum levels of the electron energy states, respectively. When the two atomic energy levels differ by only one quantum level then the transitions are described as α lines (n = 2 to n = 1, or n = 3 to n = 2). When the two levels are separated by one or more quantum levels, the transitions are known as β lines (n = 3 to n = 1 or n = 4 to n = 2).

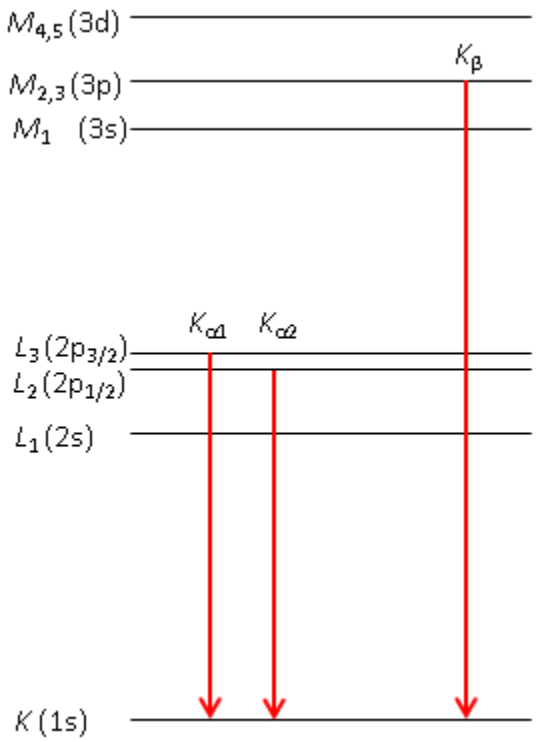


Figure 4. Electronic energy levels of an atom of the anode.

Because all *K* lines (n = 1) arise from a loss of electrons in the n = 1 state, the *K*α and *K*β lines always appear at the same time. The n = 2 and higher energy levels (L, M, N, O) are actually split into multiple energy levels causing the α and β transitions to split into a variety of closely spaced lines at high resolution. Thus, the observed Cu *K*α line can be resolved at high scattering angle (high resolution) into $K\alpha_1$ and $K\alpha_2$ lines with separate wavelengths. The $K\alpha_1$ line is about twice as intense as the $K\alpha_2$ line. At low resolution (lower scattering angle) the *K*α wavelength is considered as a weighted average of the $K\alpha_1$ and $K\alpha_2$ lines with $\lambda(K\alpha_{ave}) = [2*(\lambda(K\alpha_1)) + \lambda(K\alpha_2)]/3$. The *K*α line is about 5 - 10 times as intense as the *K*β line.

The intensity of the *K*α line can be approximately calculated by

$$I_k = B\ i\ (V - V_k)^{1.5}$$

where i = applied current, V_k = excitation potential of the target material, V = applied voltage. It can be shown that the ratio I_k / I_w is a maximum if the accelerating voltage is chosen to be about 4 times the excitation potential of the anode.

The wavelengths of characteristic X-ray lines were found to be inversely related to the atomic number of the atoms of the target material. Moseley found that

$$\sqrt{(f)} = K_1\ [Z - \sigma]$$

where f is the frequency of the radiation, K_1 is a proportionality constant, Z is the atomic number of the target atom type, and σ is the shielding constant that typically has a value of just less than 1. Today this formula is more typically recast as

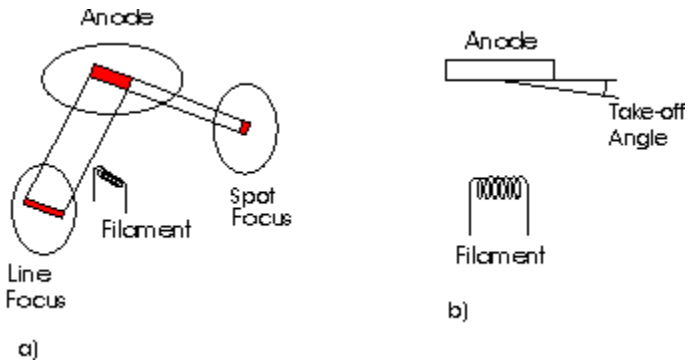
$$1/\lambda = K_2\ [Z - \sigma]^2$$

where λ is the wavelength of the radiation, K_2 is a proportionality constant, Z is the atomic number of the target atoms, and σ is the shielding constant.

The notation for describing the characteristic X-ray lines shown above was first presented by Siegbahn. In 1991, the International Union of Pure and Applied Chemists (IUPAC) recommended that X-ray lines be referred to by writing the initial and final levels separated by a hyphen, e.g. Cu *K*- L_3, rather than using the Siegbahn notation, e.g. Cu $K\alpha_1$, which is based on the relative intensities of the lines.[3] A table of the correspondence between IUPAC and Siegbahn notations is given in the International Tables for Crystallography, Vol. C.[4] The Siegbahn notation remains common in the chemical and crystallographic literature.

The shape of the incident beam depends on the focal projection of the filament onto and the anode material. X-Ray beams that are parallel with wide projection of the filament have a focal shape of a *line*. X-Ray beams that are parallel with the narrow projection of the filament have an approximate focal shape of a square, which is usually labeled as a *spot*. These two focal projections are necessarily about 90 ° apart in the plane normal to the filament-anode axis. The X-ray beams emitted from the anode travel in a variety of angular directions from the anode

surface. As the angle from the anode surface is increased, the intensity of the beam increases, but the spot also becomes less focused. Thus take-off angles are typically selected in the 3 - 6 ° range.



Two cartoons of an X-ray tube. Drawing a) shows the line and spot focus patterns of a typical sealed tube. Drawing b) shows the take-off angle of a tube.

The generation of X rays is very inefficient. In addition to white radiation and characteristic lines, laboratory sources also produce Auger electrons and photo-electrons. However, the vast majority of the power used in generating X rays results in the collision of accelerated electrons with valence electrons of the target material producing heat. A small fraction of the energy applied to the tube actually produces the characteristic radiation used in diffraction experiments.

Sealed-tube X-ray generators use a stationary anode. These tubes are limited in the power that can be applied to the tube by the amount of heat that can be dissipated through water cooling. One way to increase the heat dissipating ability of the system, and thus increase the X-ray beam intensity, is to move or rotate the anode surface so that the beam of electrons continually hits a new region of the anode. These rotating-anode generators typically yield about 5 times the flux of X-rays as is routinely produced by sealed-tube generators with normal-focus X-ray tubes.

Because macromolecular crystallographers need the most intense beam available, they typically use rotating-anode X-ray generators. Rotating-anode generators require a considerable amount of maintenance to replace filaments, and repair or replace the anode bearings as well as vacuum and water seals. To keep from burning the filament, it must remain in a high vacuum. The anode with its constant flow of cooling water must be continuously rotating at speeds of 6000 rpm or more. Special ferro-fluidic seals are used to maintain the vacuum along the rotating shaft of the anode.

Sealed-tube sources with their minimal maintenance requirements are generally quite adequate for most small molecule needs.

Another type of sealed-tube source that produces beam fluxes comparable to rotating-anode systems is a micro-focus generator. Because heat dissipates rather quickly in a metal block, manufacturers have found that when the focal size is reduced to 10-300 μm then the power can be increased to make the beam flux much higher than for normal- or even fine-focus sealed tube sources. One of the great advantages of a micro-focus radiation source is that the electrical power needs are in the range of 30-80 Watts not the 2-3 kWatts that are required of a typical sealed tube generator, or the 3-12 kWatts required by a rotating anode generator.

Other Sources

Synchrotron Radiation Sources

In 1943, Dmitri Ivanenko and Isaak Pomeranchuk predicted that electrons traveling at relativistic speeds when directed through a magnetic field would emit radiation. This prediction was observed in the lab in 1946 by scientists at GE. A synchrotron radiation source is a very intense, tunable source of radiation with wavelengths from hard X-rays through visible wave, to microwaves. Because it is so costly to maintain large quantities of electrons traveling at near the speed of light in a high vacuum storage ring, few of these radiation sources are built.

To make the best use of this type of radiation, synchrotrons are shaped roughly as rings with ports that emit the photons located at nearly each bend of the ring. The radiation from each of these ports is then directed to one or more experimental chambers. Synchrotron radiation is used in crystallography to collect data on biological macromolecules, on tiny small-molecule single crystals, and on various polycrystalline materials. In addition, synchrotron radiation in X-ray energies is used in a variety of scattering and absorption studies as well as a multitude of physics experiments. Aside from the very high flux, synchrotron radiation also has the added benefit of being tunable to a specific wavelength.

In most synchrotrons, electrons are generated by an electron gun, then accelerated first by a linear accelerator, linac, and then transferred to a booster ring where they are accelerated by resonating rf cavities until the energies are 3-6 Gev and the speeds of the electrons are near the

speed of light. These electrons are then directed into the main storage ring. When radiation is emitted, the electrons loose energy. The electrons are reenergized by resonating rf cavities located in the straight parts of the storage ring.

Two types insertion devices, devices to send radiation beams towards an instrument, are used in the straight parts of the storage ring to boost the flux of radiation. Wigglers are a series of electromagnetic plates with opposite charge. Undulators are similar to wigglers with lower energy applied to the plates, but the plates are spaced to give optimum intensity to particular wavelengths and their harmonics.

Fluid Anodes

The main problem limiting brightness in laboratory-based X-ray sources is the removal of heat. A new type of X ray source, that offers a novel solution to this problem, uses a liquid metal, gallium anode.[5] The scientists that developed this source have already reported achieving beam brightnesses greater that modern rotating anodes, with the theoretical capability of increasing this flux by another 3 orders of magnitude. A Swedish company, Excillum, is currently producing these sources.

Carbon-Nanotube Cathodes

A new type of tube that utilizes carbon nanotubes as the cathode are most likely to be developed as portable and miniature X-ray sources.[6] As of this writing, these sources are not commercially available.

Medical X Rays

X Rays for medical use are generally produced by one of two methods. Diagnostic X rays for examining bones and teeth are usually produced by sealed tube equipment with a tungsten target. X Rays for CT (computed tomography) scans and radiation therapy are produced by a linear accelerator, linac. Electrons from an electron gun are accelerated through the linac by a series of charge plates. These electrons then collide with a target giving off Bremsstrahlung. The medical X rays from sealed tube equipment have typical energies of 50-80 kev. The X rays from CT or tomography equipment typically have energies around 4-8 Mev.

Choice of Radiation

Most X-ray tubes used for diffraction studies have targets (anodes) made of copper or molybdenum metal. The characteristic wavelengths and excitation potentials for these materials are shown below. Copper radiation is preferred when the crystals are small or when the unit cells are large. Copper radiation (or softer) is required when the absolute configuration of a compound is needed and the compound only contains atoms with atomic numbers < 10. A copper source is preferred for most types of powder diffraction. Chromium anodes are sometimes used to enhance anomalous scattering effects for some macromolecular samples.

Molybdenum radiation is preferred for larger crystals of strongly absorbing materials and for very high resolution, sin (θ) / λ < 0.6 Å, data. The scintillation point detectors, often used in small molecule diffraction, have somewhat higher quantum efficiencies for molybdenum radiation than for copper radiation. Because the diffraction spots are closer together for molybdenum radiation than for copper radiation, molybdenum is the preferred radiation source when using area detectors to study small molecules. The solid angle coverage of most area detectors is such that with molybdenum radiation, it is usually possible to collect an entire data set with the detector sitting at a single position. However, because a brighter incident beam of X-rays is produced from a copper tube than from a molybdenum tube at the same power level, very small crystals of even strongly absorbing materials will often yield better diffracted intensities from copper radiation than from molybdenum radiation.

Occasionally, other types of target materials, e.g. Cr, Fe, W, or Ag, are chosen for specialized diffraction experiments. Sources with Cr or Fe targets are often chosen when protein crystals are very small or when anomalous differences need to be enhanced. When samples are very strongly absorbing or when extremely high resolution data are needed then X-ray tubes with sources such as W or Ag are usually selected.

Table 1. Selected X-Ray Wavelengths and Excitation Potentials.

	Cr	Fe	Cu	Mo
Z	**24**	**26**	**29**	**42**

$K\alpha_1$, Å	**2.28962**	**1.93597**	**1.54051**	**0.70932**
$K\alpha_2$, Å	**2.29351**	**1.93991**	**1.54433**	**0.71354**
$K\alpha_{ave}$, Å	**2.29092**	**1.93728**	**1.54178**	**0.71073**
$K\beta$, Å	**2.08480**	**1.75653**	**1.39217**	**0.63225**
β filter	**Ti**	**Cr**	**Ni**	**Nb**
Resolution, Å	**1.15**	**0.95**	**0.75**	**0.35**
Excit. Pot. (kV)	**5.99**	**7.11**	**8.98**	**20.0**

Monochromatization and Collimation of X Rays

Nearly all of the data collection experiments require that the energy of the X-ray radiation be limited to as narrow a band of energies (and hence wavelengths) as possible. Using a narrow wavelength band of X rays significantly reduces the fluorescent radiation given off by the sample and makes absorption corrections much simpler to perform. It has been noted that when the applied voltage for *K* excitation occurs, both the *K*α and *K*β lines as well as the white radiation curve are observed. Usually the *K*α band is selected for diffraction experiments because of its greater intensity.

Also, typical data collection methods require that the incident beam be a parallel beam of photons. To assure that the beam is as parallel as possible (lacking divergence), the incident beam path is collimated to produce an incident beam that is about 0.5 mm in diameter for normal focus sources and 0.1-0.3 mm for micro focus sources.

Filters

When the energy of a photon beam is just above the *excitation potential* or *absorption edge* of a material, that material strongly absorbs the given photon beam. If another substance can be found

that has an absorption edge between the *K*α and *K*β lines of the incident photon beam, this other substance can be used to significantly reduce the intensity of the *K*β line relative to the *K*α line. The absorption edges of elements with $Z_{Filter} = Z_{Target} - 1$ (or $- 2$) meet this requirement. The thickness of the filtering material is usually chosen to reduce the intensity of the *K*β line by a factor of 100 while reducing the intensity of the *K*α line by a factor of 10 or less.

The absorption of X rays follows Beer's Law:

$I / I^o = \exp(-\mu \times t)$

where I = transmitted intensity, I^o = incident intensity, t = thickness of material, μ = linear absorption coefficient of the material. The linear absorption coefficient depends on the substance, its density, and the wavelength of radiation. Since μ depends on the density of the absorbing material, it is usually tabulated as the mass absorption coefficient $\mu_m = \mu / \rho$.

Monochromators

An alternative way to produce an X-ray beam with a narrow wavelength distribution is to diffract the incident beam from a single crystal of known lattice dimensions. X-Ray photons of different wavelengths are diffracted from a given set of planes in a crystal at different scattering angles according to Bragg's Law. Therefore a narrow band of wavelengths can be chosen by selecting a particular scattering angle for the monochromator crystal. Crystal monochromators need to have the following properties.

1. The crystal must be mechanically strong and stable in the X-ray beam.
2. The crystal must have a strong diffracted intensity at a reasonably low scattering angle for the wavelength of the radiation being considered.
3. The mosaicity of the crystal, which determines the divergence of the diffracted beam and the resolution of the crystal, should be small.

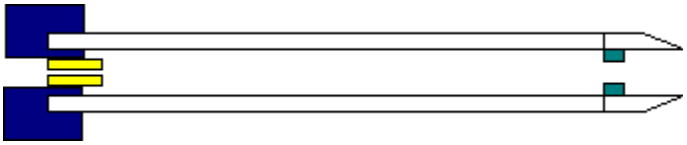
A variety of geometries are possible for crystal monochromators. Most monochromators are cut with one face parallel to a major set of crystal planes. These monochromators are then oriented to diffract *K*α lines from this major set of planes. Some monochromators are cut at an angle to the major set of planes in order to produce a diffracted beam with a smaller divergence. By

properly curving the monochromator crystal, the diffracted beam may be focused onto a very small area. This curving may be achieved either by bending or grinding or both bending and grinding. Curved monochromators are usually reserved for special applications such synchrotrons.

Graphite crystals cut on the (0002) face are the most common crystals used as monochromators in X-ray diffraction laboratories. Other special purpose monochromator materials include germanium and lithium fluoride. In all commercially available single-crystal instruments, the monochromator is placed in the incident beam path. Powder diffraction instruments with a point detector typically place a monochromator in the diffracted beam path to remove any fluorescent radiation from the sample. Crystal monochromators systematically alter the polarization of the incident beam, requiring different geometric corrections be applied to the intensity data.

Collimators

Collimators are objects inserted in the incident- or diffracted-beam path to shape the X-ray beam. Metal tubes are typically used in single-crystal experiments. The inside radius of the collimators is typically chosen to be somewhat larger than the size of the sample so that the sample may be bathed in the incident beam at all times. Incident-beam collimators are usually manufactured with two narrow regions. The region closest to the X-ray source carries out the collimation functions. The second narrow region has a slightly larger diameter than the first and is used to remove the parasitic radiation that takes a bent path due to interaction with the edge of the first narrow region of the collimator. Diffracted beam collimators only function to remove any stray radiation from hitting the detector.



The left end of the collimator shown is mounted on the X-ray tube (or incident beam 34onochromator). The small yellow-colored region at the left is the part of the collimator where the size of the beam is determined. The green region at the right is chosen to have an opening slightly larger than the region drawn in yellow. This green region removes the parasitic radiation.

Recently, manufacturers have been selling metal collimators with a single or multiple glass capillaries. These glass capillaries redirect much of the X-ray beam that would otherwise be blocked by the collimator. Such capillary inserts in a collimator have been shown to increase the intensity of the incident beam by a factor of between two and four.

When a very intense and very small point source is needed, such as in protein crystallography, X-ray mirrors may be used to shape the incident beam. Mirrors are sometimes made from materials that act as beta filters for the radiation in use. Mirrors are primarily used with very bright X-ray sources such as rotating-anode generators or synchrotrons.

Mirrors

X-ray mirrors are sometimes used in the incident beam to shape the beam as is done by a collimator. Even with Cu radiation, the spots in protein diffraction patterns are often very close together. The mirrors act to focus the incident beam into an very small cross section producing very sharp spots in the diffraction pattern. Mirrors are often constructed to absorb more of the $K\beta$ radiation than the $K\alpha$ radiation making the beam approximately monochromatic. Monochromators significantly reduce the intensity of the incident beam; omitting the monochromator maximizes the incident beam flux.

Soller Slits

Powder diffraction experiments usually require a line-shaped incident beam that is produced from a pair of parallel knife edges. A set of Soller slits are used in the beam path after the knife edges to remove parasitic radiation that scatters from the edges of the blades. Soller slits are a set of parallel thin foil sheets that absorb nearly all of the X rays not traveling parallel to the metal sheets.

Multi-Layer Optics

These optics act somewhat as X-ray mirrors that both focus the X-ray beam and selectively absorb the $K\beta$ wavelengths producing an intense beam of $K\alpha$ radiation.

X-Ray Diffraction (XRD)

X-ray diffraction (XRD) relies on the dual wave/particle nature of X-rays to obtain information about the structure of crystalline materials. A primary use of the technique is the identification and characterization of compounds based on their diffraction pattern.

The dominant effect that occurs when an incident beam of monochromatic X-rays interacts with a target material is scattering of those X-rays from atoms within the target material. In materials with regular structure (i.e. crystalline), the scattered X-rays undergo constructive and destructive interference. This is the process of diffraction. The diffraction of X-rays by crystals is described by Bragg's Law, n(lambda) = 2d sin(theta). The directions of possible diffractions depend on the size and shape of the unit cell of the material. The intensities of the diffracted waves depend on the kind and arrangement of atoms in the crystal structure. However, most materials are not single crystals, but are composed of many tiny crystallites in all possible orientations called a polycrystalline aggregate or powder. When a powder with randomly oriented crystallites is placed in an X-ray beam, the beam will see all possible interatomic planes. If the experimental angle is systematically changed, all possible diffraction peaks from the powder will be detected.

The parafocusing (or Bragg-Brentano) diffractometer is the most common geometry for diffraction instruments.

This geometry offers the advantages of high resolution and high beam intensity analysis at the cost of very precise alignment requirements and carefully prepared samples. Additionally, this geometry requires that the source-to-sample distance be constant and equal to the sample-to-detector distance. Alignment errors often lead to difficulties in phase identification and improper quantification. A mis-positioned sample can lead to unacceptable specimen displacement errors. Sample flatness, roughness, and positioning constraints preclude in-line sample measurement. Additionally, traditional XRD systems are often based on bulky equipment with high power requirements as well as employing high powered X-ray sources to increase X-ray flux on the sample, therefore increasing the detected diffraction signals from the sample. These sources also have large excitation areas, which are often disadvantageous for the diffraction analysis of small samples or small sample features.

Polycapillary X-ray optics can be used to overcome many of these drawbacks and constraints to enhance XRD applications. Polycapillary collimating optics convert a highly divergent beam into a quasi-parallel beam with low divergence. They can be used to form a Parallel Beam XRD instrument geometry which greatly reduces and removes many sources of errors in peak position and intensity inherent to the parafocusing geometry, such as sample position, shape, roughness, flatness, and transparency. Polycapillary focusing optics collect X-rays from a divergent X-ray source and direct them to a small focused beam at the sample surface with diameters as small as tens of micrometers for micro X-ray diffraction applications of small samples or small specimen features. Both types of polycapillary optics direct very high X-ray intensities to the sample surface, such that XRD systems employing optics can use low power X-ray sources, reducing instrument size, cost, and power requirements.

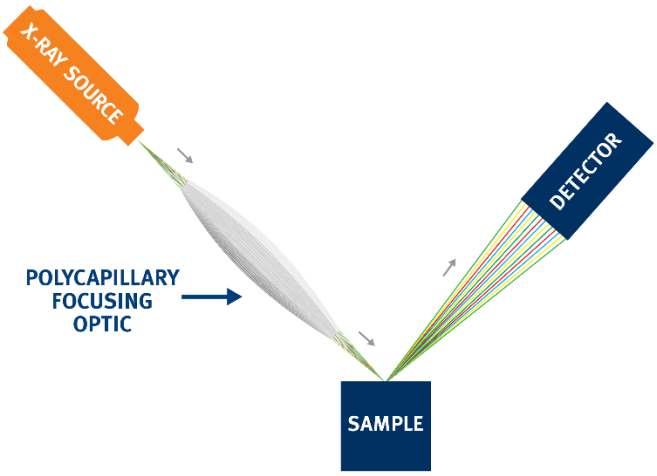
X-ray diffraction using X-ray optics has been applied to many different types of applications including thin film analysis, sample texture evaluation, monitoring of crystalline phase and structure, and investigation of sample stress and strain.

X-Ray Fluorescence (XRF)

X-ray fluorescence (XRF) is a powerful quantitative and qualitative analytical tool for elemental analysis of materials. It is ideally suited to the measurement of film thickness and composition, determination of elemental concentration by weight of solids and solutions, and identification of specific and trace elements in complex sample matrices. XRF analysis is used extensively in many industries including semiconductors, telecommunications, microelectronics, metal finishing and refining, food, pharmaceuticals, cosmetics, agriculture, plastics, rubbers, textiles, fuels, chemicals, and environmental analysis. The method is fast, accurate, non-destructive, and usually requires only minimal sample preparation.

How XRF works

When elements in a sample are exposed to a source of high intensity X-rays, fluorescent X-rays will be emitted from the sample at energy levels unique to those elements.



The basic concept for all XRF spectrometers is a source, a sample, and a detection system. The source irradiates the sample and a detector measures the fluorescence radiation emitted from the sample. In most cases for XRF, the source is an X-ray tube. Alternatives are a radioactive source or a synchrotron. There are two main types of XRF instruments: Energy Dispersive X-ray fluorescence (EDXRF)and Wavelength Dispersive X-ray Fluorescence (WDXRF).

X-ray optics can be used to enhance both types of XRF instrumentation. For conventional XRF instrumentation, typical focal spot sizes at the sample surface range in diameter from several hundred micrometers up to several millimeters. Polycapillary focusing optics collect X-rays from the divergent X-ray source and direct them to a small focused beam at the sample surface with diameters as small as tens of micrometers. The resulting increased intensity, delivered to the sample in a small focal spot, allows for enhanced spatial resolution for small feature analysis and enhanced performance for measurement of trace elements for Micro X-ray Fluorescence applications. Doubly curved crystal optics direct an intense micron-sized monochromatic X-ray beam to the sample surface for enhanced elemental analysis.

X-ray crystallography

Crystallography deals with the study of crystal and its molecular structure. Crystal structure is based on diffraction phenomena caused by the interaction of matter with X-rays, electrons, or

neutrons. X-ray crystallography has application in all branches of science; Physics, Chemistry, Metallurgy and Biology. The study of crystals began to take shape independently in the 17th–18th centuries. In 1611, Johannes Kepler published a short treatise on the six-cornered snowflake, which was the first scientific reference to snow crystals. The origin of the shape and symmetry of snowflakes is due to the internal arrangement of the building elements of water. In 1912, Max von Laue, postulated that atoms in a crystal lattice had a regular, periodic structure with inter atomic distances on the order of 1 Å. This was encouraged by Friedrich and Knipping, who used X-rays for their first diffraction experiment. Later, in 1915 William Henry Bragg and his son William Lawrence Bragg used this technique to determine the structure of a crystal. In 1669, Danish scientist Nicolaus Steno (Niels Stensen) postulated that the angles between corresponding faces of crystals are constant, regardless of the size or shape of the faces. This law is known as Steno's law, or the law of constancy of interfacial angles. This was the first major discovery about the true nature of crystals. In 1895 Wilhelm Ro¨entgen discovered the X- rays and set the stage for the modern science of X-ray crystallography. Later two of his students successfully performed an experiment and obtained the remarkable result that X-rays were indeed diffracted by crystals of copper sulphate. The spread of knowledge stemming from X-ray crystallography was rapid and in recent years it has assumed an important role in the modern sciences, because of its multidisciplinary nature.

X-ray scattering

Electromagnetic radiation, such as an X-ray photon, may be described in terms of its electric and magnetic components. These components are considered to oscillate transversely and sinusoidally in directions that are normal (perpendicular) to the direction of propagation of the photon and normal to each other. When X-ray photons collide with matter, the oscillating electric field of the radiation causes the charged particles of the object to oscillate with the same frequency as the incident radiation. Intensity of each diffraction maximum can be derived in terms of overall electronic distribution within the unit cell. There are two types of scattering is possible when electromagnetic wave interacts with matter, i.e., Thomson scattering and Compton scattering.

Thomson scattering

Thomson scattering is an important phenomenon in plasma physics and was first explained by the physicist J. J. Thomson. Thomson scattering is the scattering of electromagnetic radiation by a free non-relativistic charged particle. The electric and magnetic components of the incident wave accelerate the particle. As it accelerates, in turn, emits radiation and thus, the wave is scattered. The main cause of the acceleration of the particle will be due to the electric field component of the incident wave. The particle will move in the direction of the oscillating electric field, resulting in electromagnetic dipole radiation. The moving particle radiates most strongly in a direction perpendicular to its motion and that radiation will be polarized along the direction of its motion. J. J. Thomson showed that when radiation is scattered by an electron, there is a phase shift of 180° in the radiation. This phase change is same for scattering by all atoms in the crystal, in the absence of unusual effects. Phase relationship between waves scattered from various electrons are well defined. Such scattering is called coherent scattering.

Structure Determination

In order to determine the crystal structure, we need both the magnitudes and phases of the structure factors. Having measured and corrected the intensities of scattered X-rays, electron density distribution within the unit cell has to be determined. This is achieved by the mathematical technique called Fourier synthesis. It requires the value of the structure factor Fhkl both in magnitude and phase. But from the diffraction experiment, we can get the information only of the magnitude, not of the phase. This constitutes the phase problem. To solve the phase problem, two methods are routinely used. First of them is the Patterson method and the second one is the Direct method.

Direct methods

In Direct methods, phase determination involves solely based on the comparison of structure factor magnitudes derived from the study of a single crystal without any reference to prior knowledge of likely structures, or isomorphous crystals. Thus direct methods represent an objective sequence of operations which will give phase information. These mathematical phase determining techniques are of considerable importance in the solution of the structure of small

molecules. The methods used to ensure that the Fourier summation does not give a negative electron-density map are mathematical in nature.

Neutron diffraction

Neutron diffraction or elastic neutron scattering is the application of neutron scattering to the determination of the atomic and/or magnetic structure of a material. A sample to be examined is placed in a beam of thermal or cold neutrons to obtain a diffraction pattern that provides information of the structure of the material. The technique is similar to X-ray diffraction but due to their different scattering properties, neutrons and X-rays provide complementary information: X-Rays are suited for superficial analysis, strong x-rays from synchrotron radiation are suited for shallow depths or thin specimens, while neutrons having high penetration depth are suited for bulk samples.

Instrumental and sample requirements

The technique requires a source of neutrons. Neutrons are usually produced in a nuclear reactor or spallation source. At a research reactor, other components are needed, including a crystal monochromator, as well as filters to select the desired neutron wavelength. Some parts of the setup may also be movable. At a spallation source, the time of flight technique is used to sort the energies of the incident neutrons (higher energy neutrons are faster), so no monochromator is needed, but rather a series of aperture elements synchronized to filter neutron pulses with the desired wavelength.

The technique is most commonly performed as powder diffraction, which only requires a polycrystalline powder. Single crystal work is also possible, but the crystals must be much larger than those that are used in single-crystal X-ray crystallography. It is common to use crystals that are about 1 mm^3.

Summarizing, the main disadvantage to neutron diffraction is the requirement for a nuclear reactor. For single crystal work, the technique requires relatively large crystals, which are usually challenging to grow. The advantages to the technique are many - sensitivity to light atoms, ability to distinguish isotopes, absence of radiation damage, as well as a penetration depth of several cm.

Uses

Neutron diffraction can be used to determine the static structure factor of gases, liquids or amorphous solids. Most experiments, however, aim at the structure of crystalline solids, making neutron diffraction an important tool of crystallography.

Neutron diffraction is closely related to X-ray powder diffraction. In fact, the single crystal version of the technique is less commonly used because currently available neutron sources require relatively large samples and large single crystals are hard or impossible to come by for most materials. Future developments, however, may well change this picture. Because the data is typically a 1D powder diffractogram they are usually processed using Rietveld refinement. In fact, the latter found its origin in neutron diffraction (at Petten in the Netherlands) and was later extended for use in X-ray diffraction.

One practical application of elastic neutron scattering/diffraction is that the lattice constant of metals and other crystalline materials can be very accurately measured. Together with an accurately aligned micro positioner a map of the lattice constant through the metal can be derived. This can easily be converted to the stress field experienced by the material. This has been used to analyse stresses in aerospace and automotive components to give just two examples. The high penetration depth permits measuring residual stresses in bulk components as crankshafts, pistons, rails, gears. This technique has led to the development of dedicated stress diffractometers, such as the ENGIN-X instrument at the ISIS neutron source.

Neutron diffraction can also be employed to give insight into the 3D structure any material that diffracts.

Another use is for the determination of the solvation number of ion pairs in electrolytes solutions.

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DEPARTMENT OF CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES

UNIT – IV

INSTRUMENTATION METHODS (SMB5454)

Sedimentation is the tendency for particles in suspension to settle out of the fluid in which they are entrained and come to rest against a barrier. This is due to their motion through the fluid in response to the forces acting on them: these forces can be due to gravity, centrifugal acceleration, or electromagnetism.

Sedimentation is the process of allowing particles in suspension in water to settle out of the suspension under the effect of gravity. The particles that settle out from the suspension become sediment, and in water treatment is known as sludge.

Centrifugation

Biological centrifugation is a process that uses centrifugal force to separate and purify mixtures of biological particles in a liquid medium. The smaller the particles, the higher the g-forces required for the separation. It is a key technique for isolating and analysing cells, subcellular fractions, supramolecular complexes and, with higher g-force instruments or 'ultra'-centrifuges (up to 60 000 revolutions per minute corresponding to ~ 200 000×g) isolated macromolecules such as proteins or nucleic acids. Such high-speed devices require a vacuum to avoid overheating of samples. The development of the first analytical ultracentrifuge – with a specially designed optical system for monitoring and recording the sedimentation process – by Svedberg in the late 1920s and the technical refinement of the preparative centrifugation technique by Claude and colleagues in the 1940s positioned centrifugation technology at the centre of biological and biomedical research for many decades. Today, centrifugation techniques represent a critical tool for modern biochemistry and are employed in almost all invasive subcellular studies. While analytical ultracentrifugation is mainly concerned with the study of purified macromolecules or isolated supramolecular assemblies, preparative centrifugation methodology is devoted to the actual separation of tissues, cells, subcellular structures, membrane vesicles and other particles of biochemical interest. Most undergraduate students will be exposed to preparative centrifugation protocols during practical classes and might also experience a demonstration of analytical centrifugation techniques.

Types of centrifuges:

Centrifugation techniques take a central position in modern biochemical, cellular and molecular biological studies. Depending on the particular application, centrifuges differ in their overall

design and size. However, a common feature in all centrifuges is the central motor that spins a rotor containing the samples to be separated. Particles of biochemical interest are usually suspended in a liquid buffer system contained in specifi c tubes or separation chambers that are located in specialised rotors. The biological medium is chosen for the specifi c centrifugal application and may differ considerably between preparative and analytical approaches. As outlined below, the optimum pH value, salt concentration, stabilising cofactors and protective ingredients such as protease inhibitors have to be carefully evaluated in order to preserve biological function. The most obvious differences between centrifuges are:

• the maximum speed at which biological specimens are subjected to increased

sedimentation

• the presence or absence of a vacuum

• the potential for refrigeration or general manipulation of the temperature during a

centrifugation run

• the maximum volume of samples and capacity for individual centrifugation tubes.

Many different types of centrifuges are commercially available including:

• large-capacity low-speed preparative centrifuges

• preparative high-speed ultracentrifuges

• refrigerated preparative centrifuges/ultracentrifuges

• analytical ultracentrifuges

• large-scale clinical centrifuges

• small-scale laboratory microfuges.

Some large-volume centrifuge models are quite demanding on space and also generate considerable amounts of heat and noise, and are therefore often centrally positioned in special instrument rooms in biochemistry departments. However, the development of small-capacity

bench-top centrifuges for biochemical applications, even in the case of ultracentrifuges, has led to the introduction of these models in many individual research laboratories.

High-speed refrigerated centrifuges are absolutely essential for the sedimentation of protein precipitates, large intact organelles, cellular debris derived from tissue homogenisation and microorganisms. The initial bulk separation of cellular elements prior to preparative ultracentrifugation is performed by these kinds of centrifuges. They operate at maximum centrifugal fields of approximately 100 000×g. Such centrifugal force is not sufficient to sediment smaller microsomal vesicles or ribosomes, but can be employed to differentially separate nuclei, mitochondria or chloroplasts. In addition, bulky protein aggregates can be sedimented using high-speed refrigerated centrifuges. An example is the contractile apparatus released from muscle fibres by homogenisation, mostly consisting of myosin and actin macromolecules aggregated in filaments. In order to harvest yeast cells or bacteria from large volumes of culture media, high-speed centrifugation may also be used in a continuous flow mode with zonal rotors. This approach does not therefore use centrifuge tubes, but a continuous flow of medium. As the medium enters the moving rotor, biological particles are sedimented against the rotor periphery and excess liquid removed through a special outlet port. Ultracentrifugation has decisively advanced the detailed biochemical analysis of subcellular structures and isolated biomolecules. Preparative ultracentrifugation can be operated at relative centrifugal fields of up to 900 000×g. In order to minimise excessive rotor temperatures generated by frictional resistance between the spinning rotor and air, the rotor chamber is sealed, evacuated and refrigerated. Depending on the type, age and condition of a particular ultracentrifuge, cooling to the required running temperature and the generation of a stable vacuum might take a considerable amount of time. To avoid delays during biochemical procedures involving ultracentrifugation, the cooling and evacuation system of older centrifuge models should be switched on at least an hour prior to the centrifugation run. In contrast, modern ultracentrifuges can be started even without a fully established vacuum and will proceed in the evacuation of the rotor chamber during the initial acceleration process. For safety reasons, heavy armour plating encapsulates the ultracentrifuge to prevent injury to the user in case of uncontrolled rotor movements or dangerous vibrations. A centrifugation run cannot be initiated without proper closing of the chamber system. To prevent unfavourable fluctuations in chamber temperature, excessive vibrations or operation of rotors above their maximum rated speed, newer models of

ultracentrifuges contain sophisticated temperature regulation systems, flexible drive shafts and an over-speed control device

Differential Centrifugation: Cellular and subcellular fractionation techniques are indispensable methods used in biochemical research. Although the proper separation of many subcellular structures is absolutely dependent on preparative ultracentrifugation, the isolation of large cellular structures, the nuclear fraction, mitochondria, chloroplasts or large protein precipitates can be achieved by conventional high-speed refrigerated centrifugation. Differential centrifugation is based upon the differences in the sedimentation rate of biological particles of different size and density. Crude tissue homogenates containing organelles, membrane vesicles and other structural fragments are divided into different fractions by the stepwise increase of the applied centrifugal field. Following the initial sedimentation of the largest particles of a homogenate (such as cellular debris) by centrifugation, various biological structures or aggregates are separated into pellet and supernatant fractions, depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles. To increase the yield of membrane structures and protein aggregates released, cellular debris pellets are often rehomogenised several times and then subjected to further centrifugation. This is especially important in the case of rigid biological structures such as muscular or connective tissues, or in the case of small tissue samples, as is the case with human biopsy material or primary cell cultures.

Density-Gradient Centrifugation: To further separate biological particles of similar size but differing densities, ultracentrifugation with pre-formed or self-establishing density gradients is the method of choice. Both rate separation or equilibrium methods can be used. A mixture of particles, such as is present in a heterogeneous microsomal membrane preparation, is layered on top of a pre-formed liquid density gradient. Depending on the particular biological application, a great variety of gradient materials are available. Caesium chloride is widely used for the banding of DNA and the isolation of plasmids, nucleoproteins and viruses. Sodium bromide and sodium iodide are employed for the fractionation of lipoproteins and the banding of DNA or RNA molecules, respectively. Various companies offer a range of gradient material for the separation of whole cells and subcellular particles, e.g. Percoll ® , Ficoll ® , dextran, metrizamide and Nycodenz ® . For the separation of membrane vesicles derived from tissue homogenates, ultra-

pure DNase-, RNase and protease-free sucrose represents a suitable and widely employed medium for the preparation of stable gradients. If one wants to separate all membrane species spanning the whole range of particle densities, the maximum density of the gradient must exceed the density of the most dense vesicle species. Both step-gradient and continuous-gradient systems are employed to achieve this. If automated gradient-makers are not available, which is probably the case in most undergraduate practical classes, the manual pouring of a stepwise gradient with the help of a pipette is not so time-consuming or difficult. In contrast, the formation of a stable continuous gradient is much more challenging and requires a commercially available gradient-maker. Following pouring, gradients are usually kept in a cold room for temperature equilibration and are moved extremely slowly in special holders so as to avoid mixing of different gradient layers. For rate separation of subcellular particles, the required fraction does not reach its isopycnic position within the gradient. For isopycnic separation, density centrifugation is continued until the buoyant density of the particle of interest and the density of the gradient are equal.

ELECTROPHORESIS

Definition: Electrophoresis is the process of moving charged molecules in solution by applying an electric field across the mixture. Because molecules in an electric field move with a speed dependent on their charge, shape, and size, electrophoresis has been extensively developed for molecular separations.

• Purpose for carrying out electrophoresis:

1. To determine the number, amount and mobility of components in a given sample or to separate them.
2. To obtain information about the electrical double layers surrounding the particles.
3. Determination of molecular weight of proteins and DNA sequencing.

Any charged ion or molecule migrates when placed in an electric field. The rate of migration depend upon its net charge, size, shape and the applied electric current.

• It can be represented by following equation:

$v = Eq/F$

where,

v = velocity of migration of the molecule

E = electric field (in volts/cm)

q = net electric charge on the molecule.

F = frictional co-efficient (depends upon the mass and shape of the molecule).

• The movement of charged particle in an electric field is expressed in terms of electrophoretic mobility denoted by 'μ', where $\mu = v\ E$ or $\mu = q\ F$

• For molecules with similar conformation, F varies with size but not with shape. Thus electrophoretic mobility (μ) of a molecule is directly proportional to the charge density (charge/mass ratio).

GEL ELECTROPHORESIS

• It is a technique used for the separation of Deoxyribonucleic acid, Ribonucleic acid or protein molecules according to their size and electrical charge using an electric current applied to a gel matrix.

• What is a gel?

 Gel is a cross linked polymer whose composition and porosity is chosen based on the specific weight and porosity of the target molecules.

• Types of gels used: Polyacrylamide gel, Agarose gel.

• Types of gel electrophoresis:

1-D GEL ELECTROPHORESIS

Procedure:

 The samples are treated with SDS (sodium dodecyl sulfate), an anionic detergent which denatures the protein by breaking the disulfide bonds and gives negative charge to each protein in proportion to its mass.

 Without SDS, different proteins with similar molecular weights would migrate differently due to differences in folding, as differences in folding patterns would cause some proteins to better fit through the gel matrix than others. SDS linearizes the proteins so that they may be separated strictly by molecular weight.

 The SDS binds to the protein in a ratio of approximately 1.4 g SDS per 1.0 g giving an approximately uniform mass : charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein.
 Proteins may be further treated with reducing agent, such as dithiothreitol (DTT) or TRP (Tributyl phosphine) to break any reformed disulfide bonds and then alkalated with iodoacetamide to prevent reformation of disulfide bonds.
 A tracking dye like bromophenol blue may be added to the protein solution to track the progress of the protein solution through the gel during the electrophoretic run.

POLYACRYLAMIDE GEL

The gel used for SDS-PAGE is made out of acrylamide which form cross-linked polymers of polyacrylamide.

• Standard gels are typically composed of two layers, one top-most layer called the stacking gel and a lower layer called separating or resolving gel.

• The stacking layer contains a low percentage of acylamide and has low pH , while the acrylamide concentration of the separating gel varies according to the samples to be run and has higher pH.

• The difference in pH and acrylamide concentration at the stacking and separating gel provides better resolution and sharper bands in the separating gel.

• This gel material can also withstand high voltage gradients, is amenable to various staining and destaining procedures, and can be digested to extract separated fractions or dried for autoradiography and permanent recording.

STACKING & RESOLVING GELS

• Stacking gel:

 The stacking gel is a large pore PAG (4%T). This gel is prepared with Tris/HCl buffer pH 6.8 of about 2.0 pH units lower than that of electrophoresis buffer (Tris/Glycine).
 This gel is cast over the resolving gel. The height of the stacking gel region is always maintained more than double the height and th volume of the sample to be applied.

• Resolving gel:

 The resolving gel is a small pore polyacrylamide gel (3 -30% acrylamide monomer) typically made using a pH 8.8 Tris/HCl buffer.

 In the resolving gel, macromolecules separate according to their size. Resolving gels have an optimal range of separation that is based on the percent of monomer present in the polymerization reaction.

2-D GEL ELECTROPHORESIS

• Two-dimensional gel electrophoresis, abbreviated as 2-DE or 2-D electrophoresis, is a form of gel electrophoresis commonly used to analyze proteins. Mixtures of proteins are separated by two properties in two dimensions on 2D gels.

• 2-DE was first independently introduced by O'Farrell and Klose in 1975.

• 2-D electrophoresis begins with 1-D electrophoresis but then separates the molecules by a second property in a direction 90 degrees from the first. In 1-D electrophoresis, proteins (or other molecules) are separated in one dimension, so that all the proteins/molecules will lie along a lane but that the molecules are spread out across a 2-D gel. Because it is unlikely that two molecules will be similar in two distinct properties, molecules are more effectively separated in 2-D electrophoresis than in 1-D electrophoresis.

• The two dimensions that proteins are separated into using this technique can be isoelectric point, protein complex mass in the native state, and protein mass.

SDS-PAGE:

- SDS-PAGE is a technique used by many researchers to separate mixtures of proteins by their mass.
- Sodium dodecyl sulfate (SDS) is a detergent that breaks up the interactions between proteins.

- SDS is an anionic detergent that disrupts secondary and non-disulfide-linked tertiary structures and additionally applies a negative charge to each protein in proportion to its mass.

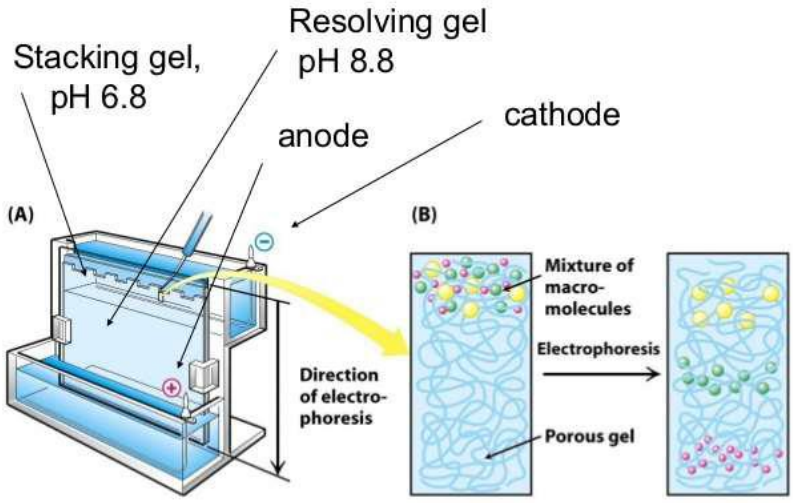

Fig.1: Schematic representation of SDS-PAGE gel electrophoresis

• PRINCIPAL COMPONENTS OF SDS-PAGE:

 The components of an SDS PAGE gel electrophoresis system are the following:

1. A Slab holder for vertical or horizontal gels (thin, flat sheets of many individual lanes)
2. Polyacrylamide or agarose gels (cm x cm x mm); these are poured for each analysis
3. Gel is amended with SDS to dissociate & charge proteins.
4. High voltage power supply (0.1-6 kV)
5. A detection technique (dye staining, fluorescence, or autoradiography to image separated bands)

 SDS-PAGE stands for Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis. Sodium-Dodecyl Sulfate, ("SDS"), is an anionic detergent. It is composed of a hydrophilic group with a net negative charge and a long hydrophobic chain with neutral charge.

• SAMPLE PREPARATION:

 Samples may be any material containing proteins or nucleic acids. These may be biologically derived, for example from prokaryotic or eukaryotic cells, tissues, viruses, environmental samples, or purified proteins.

• Components of SDS-PAGE:

 Chemical buffer: Stabilizes the pH value to the desired value within the gel itself and in the electrophoresis buffer. The buffer should also be unreactive and not modify or react with most proteins. Different buffers may be used as cathode and anode buffers, respectively, depending on the application. Multiple pH values may be used within a single gel, for example in DISC electrophoresis. Common buffers in PAGE include Tris, Bis-Tris, or imidazole.

 Counter-ion: Balance the intrinsic charge of the buffer ion and also affect the electric field strength during electrophoresis. Highly charged and mobile ions are often avoided in SDSPAGE cathode buffers, but may be included in the gel itself, where it migrates ahead of the protein.

 Acrylamide (C3H5NO): When dissolved in water, slow, spontaneous autopolymerization of acrylamide takes place, joining molecules together by head on tail fashion to form long single chain polymers. The presence of a free radical-generating system greatly accelerates polymerization. This kind of reaction is known as Vinyl addition polymerisation. A solution of these polymer chains becomes viscous but does not form a gel, because the chains simply slide over one another. Gel formation requires linking various chains together. Acrylamide is a neurotoxin. It is also essential to store acrylamide in a cool dark and dry place to reduce autopolymerisation and hydrolysis.

 Bisacrylamide (N,N′-Methylenebisacrylamide) (C7H10N2O2): Bisacrylamide is the most frequently used cross linking agent for polyacrylamide gels. Chemically it can be thought of as two acrylamide molecules coupled head to head at their non-reactive ends. Bisacrylamide can crosslink two polyacrylamide chains to one another, thereby resulting in a gel.

 Sodium Dodecyl Sulfate (SDS) (C12H25NaO4S): SDS is a strong detergent agent used to denature native proteins to unfolded, individual polypeptides. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass-charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure.

This is known as Native PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.

 Urea ($CO(NH_2)_2$): Urea is a chaotropic agent that increases the entropy of the system by interfering with intramolecular interactions mediated by non-covalent forces such as hydrogen bonds and van der Waals forces. Macromolecular structure is dependent on the net effect of these forces, therefore it follows that an increase in chaotropic solutes denatures macromolecules,

 Ammonium persulfate (APS) ($N_2H_8S_2O_8$): APS is a source of free radicals and is often used as an initiator for gel formation. An alternative source of free radicals is riboflavin, which generated free radicals in a photochemical reaction.

 TEMED (N, N, N′, N′-tetramethylethylenediamine) ($C_6H_{16}N_2$): TEMED stabilizes free radicals and improves polymerization. The rate of polymerisation and the properties of the resulting gel depend on the concentrations of free radicals. Increasing the amount of free radicals results in a decrease in the average polymer chain length, an increase in gel turbidity and a decrease in gel elasticity. Decreasing the amount shows the reverse effect. The lowest catalytic concentrations that allow polymerisation in a reasonable period of time should be used. APS and TEMED are typically used at approximately equimolar concentrations in the range of 1 to 10 mM.

• Advantages:

1. Migration of the molecules is proportional to their molecular weights.
2. Highly sensitive test, separates molecules that have even a 2% difference in mass.
3. Requires very small amounts of samples.
4. A stable chemically cross-linked gel is used.

• Disadvantages:

1. Poor band resolution due to high alkaline operating pH.
2. Acrylamide gel is a potent neurotoxin chemical.
3. Gel preparation is difficult and takes a long time.
4. Very costly.

Application of SDS-PAGE

SDS-PAGE is used mainly for the following purposes:

1. Measuring molecular weight.
2. Peptide mapping.
3. Estimation of protein size.
4. Determination of protein subunits or aggregation structures.
5. Estimation of protein purity.
6. Protein quantitation.
7. Monitoring protein integrity.
8. Comparison of the polypeptide composition of different samples.
9. Analysis of the number and size of polypeptide subunits.
10. Post-electrophoresis applications, such as Western blotting.
11. Staining of Proteins in Gels with Coomassie G-250 without Organic Solvent and Acetic Acid.
12. Pouring and Running a Protein Gel by reusing Commercial Cassettes.
13. Selective Labelling of Cell-surface Proteins using CyDye DIGE Fluor Minimal Dyes.
14. Detection of Protein Ubiquitination.
15. SDS-PAGE/Immunoblot Detection of Aβ Multimers in Human Cortical Tissue Homogenates using Antigen-Epitope Retrieval.

CHROMATOGRAPHY TECHNIQUES

Definition: Chromatography (from Greek chroma "color and graphein "to write") is the collective term for a set of laboratory techniques for the separation of mixtures. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases.

Principles: Chromatography usually consists of mobile phase and stationary phase.The mobile phase refers to the mixture of substances to be separated dissolved in a liquid or a gas. The

stationary phase is a porous solid matrix through which the sample contained in the mobile phase percolates. The interaction between the mobile phase and the stationary phase results in the separation of the compound from the mixture.

Applications:

- The chromatographic technique is used for the separation of amino acids,proteins & carbohydrates.
- It is also used for the analysis of drugs, hormones, vitamins
- Helpful for the qualitative & quantitative analysis of complex mixtures.
- The technique is also useful for the determination of molecular weight of proteins.

Thin layer chromatography TLC

Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents.

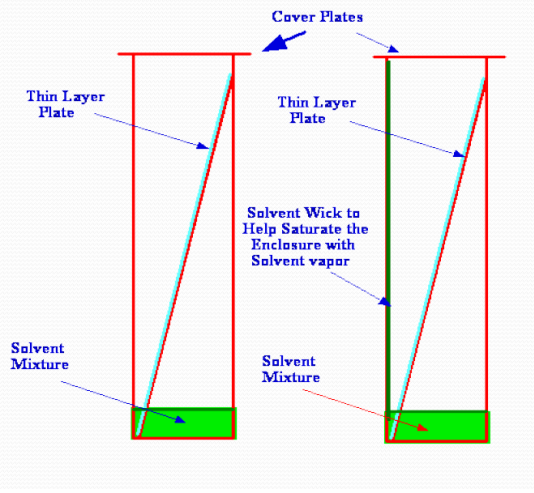

Fig.2: Representation of TLC working principle

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

 Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred to as high performance liquid chromatography (HPLC).

 Since the chromatographic techniques are slow & time consuming, hence the separation can be greatly improved by using high pressure in the range of 5000-10000 psi(pounds per square inch),hence this technique is also referred to as high pressure liquid chromatography.

In HPLC the sample is forced by a liquid at high pressure (the mobile phase) through a column that is packed with a stationary phase composed of irregularly or spherically shaped particles.

 The interaction between the mobile and the stationary phase leads to the separation of the mixture.

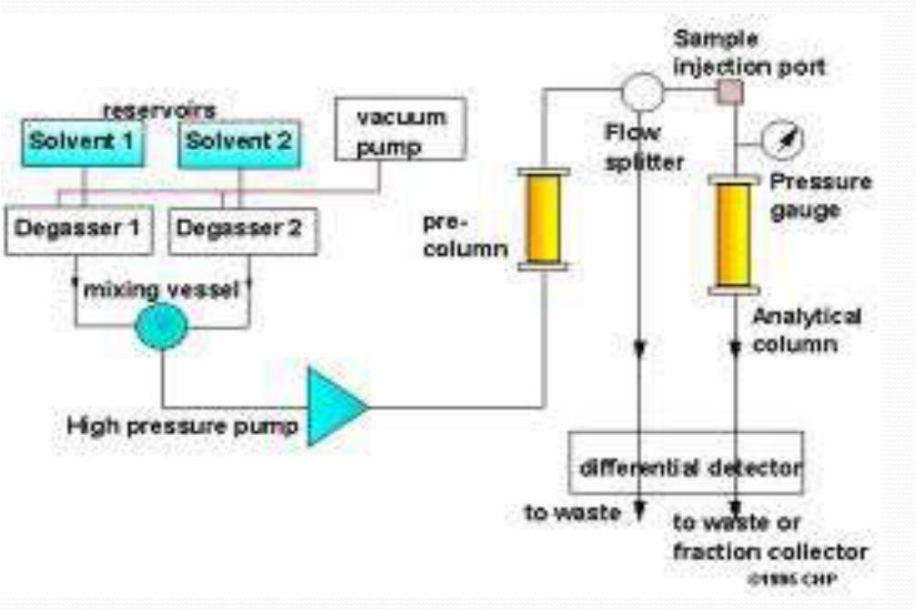

Fig.3: Block diagram of HPLC working principle

GAS-LIQUID CHROMATOGRAPHY

 Gas chromatography (GC), also sometimes known as Gas-Liquid chromatography, (GLC), is a separation technique in which the mobile phase is a gas.It is the method of choice for the separation of volatile substances or the volatile derivatives of certain non-volatile substances.

 Stationary phase is an inert solid material impregnated with a non-volatile liquid.

 In gas chromatography, a sample is rapidly heated and vaporized at the injection port. The sample is transported through the column by a mobile phase consisiting of an inert gas. Sample components are separated based on their boiling points and relative affinity for the stationary phase, which is most often a viscous liquid (wax) within the column. The higher a component's affinity for the stationary phase, the slower it comes off the column. The components are then detected and represented as peaks on a chromatogram.

 The mixture of volatile material is injected into the column along with the mobile phase.

 The separation of the volatile mixture is based on the partition of the components between the mobile phase(gas) and stationary phase (liq.), hence the name GAS-LIQUID CHROMATOGRAPHY.

 It is well suited for use in the petrochemical, environmental monitoring and industrial chemical fields.

 Sensitive, rapid and reliable.

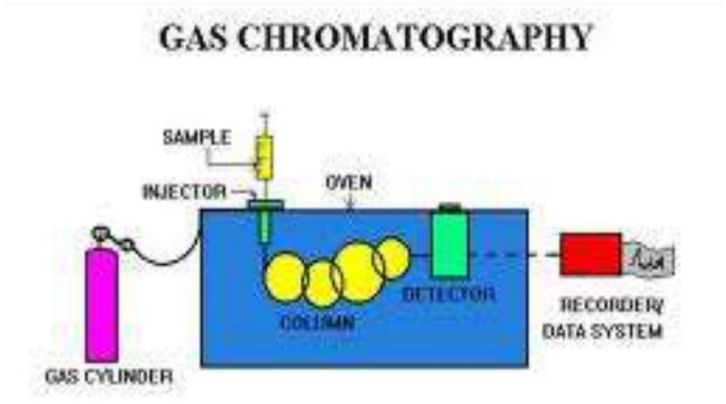

Fig.4: Diagram for a GLC instrumentation

FLUORESCENCE ACTIVATED CELL SORTER (FACS)

Introduction: The FACS (Fluorescence Activated Cell Sorting) is a laboratory technique that allows to examine AND SORT millions of cells, both normal and tumoral cells, in a very short time and receive a lot of information on their biological behaviour. It is a specialized type of flow cytometry, a technique that permits EXAMINATION AND SORTING of cells suspended in a fluid medium. The effectiveness of this particular technique is the ability to measure multiple properties of individual cells at a very fast rate, allowing a detailed qualitative and quantitative analysis. It allows to obtain different information about structures and functions of individual cells through the analysis of some physical parameters (diffraction, refraction, reflection, fluorescence) that characterize a beam of light after it has interacted with every single cell of the test sample.

Principle: The process begins by placing the cells into a flask and letting the cells to enter a small nozzle one at a time (figure). The cells travel down the nozzle which is vibrated at an optimal frequency to produce drops at fixed distance from the nozzle. The system is adjusted so that there is a low probability of more than one cell per droplet. As the cells flow down the stream of liquid, they are scanned by a laser. Some of the laser light is scattered by the cells and this is used to count the cells. This scattered light can also be used to measure the size of the cells. If you wanted to separate a subpopulation of cells, you could do so by tagging those of interest with an antibody linked to a fluorescent dye. The antibody is bound to a protein that is uniquely expressed in the cells you want to separate. The laser light excites the dye which emits a color of light that is detected by the photomultiplier tube, or light detector. By collecting the information from the light (scatter and fluorescence) a computer can determine which cells are to be separated and collected. The final step is sorting the cells, which is accomplished by electrical charge. The computer determines how the cells will be sorted before the drop forms at the end of the stream. As the drop forms, an electrical charge is applied to the stream and the newly formed drop will form with a charge. This charged drop is then deflected left or right by charged electrodes and into waiting sample tubes. Drops that contain no cells are sent into the waste tube. The end result is three tubes with pure subpopulations of cells. The number of cells is each tube is known and the level of fluorescence is also recorded for each cell.

Application: It is extensively used in research for the detection of DNA damage. One of the first applications of FACS in fact was the analysis of the position in the cell cycle, performed by quantification of cellular DNA. This is still one of the major technique in tumor detection. In cancer cells is often seen a change in DNA content, the main consequence of chromosomal and subcromosomal genetic changes, having a key role in the development and course of the disease. In the simplest method, the content of DNA is detected using a fluorescent dye able to bind DNA with high affinity. Another of the major applications of FACS is represented by the analysis (and sorting) of the various populations of blood cells, having an important role in immunophenotyping. Over all in medicine FACS has a broad application varying from the methods explained above to transplantation and prenatal diagnosis.

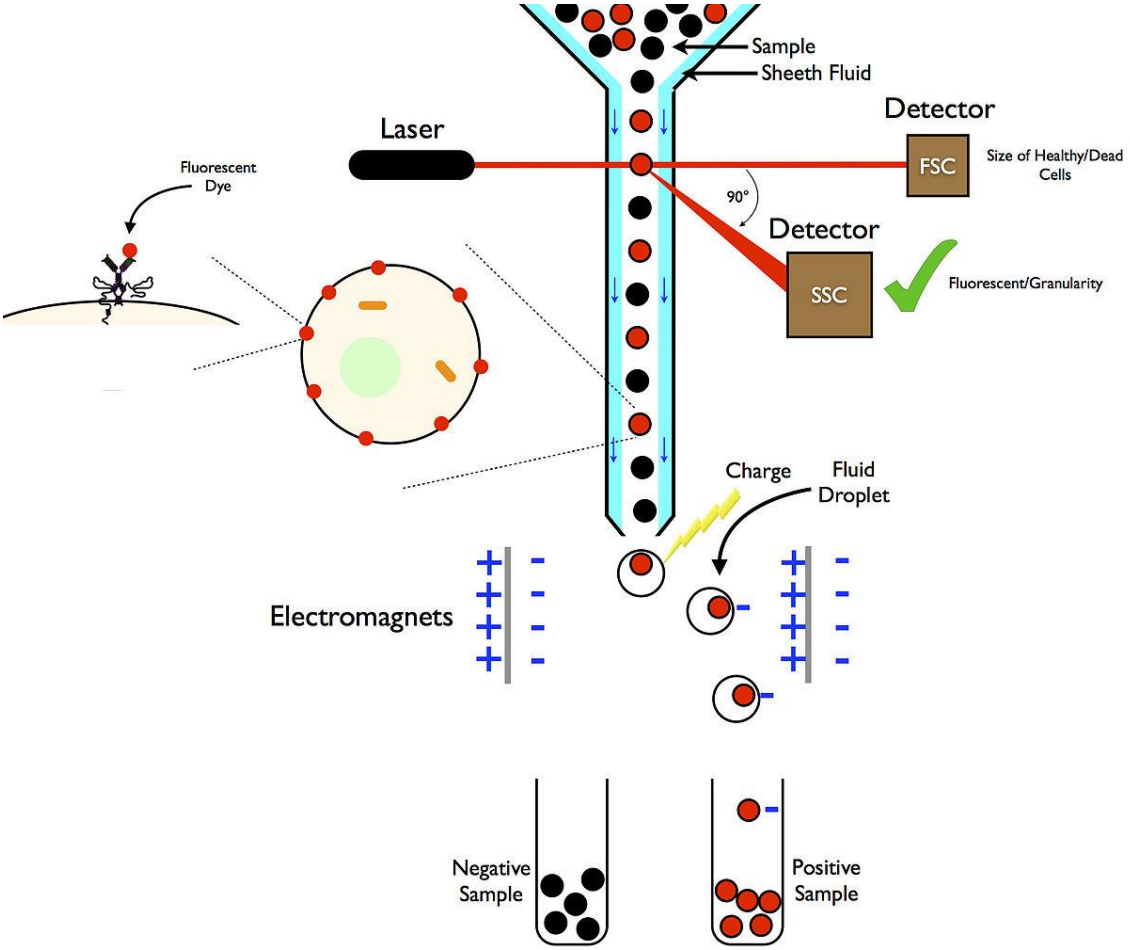

Fig.5: Diagram of FACS machine.

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DEPARTMENT OF CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES

UNIT – V

INSTRUMENTATION METHODS (SMB5454)

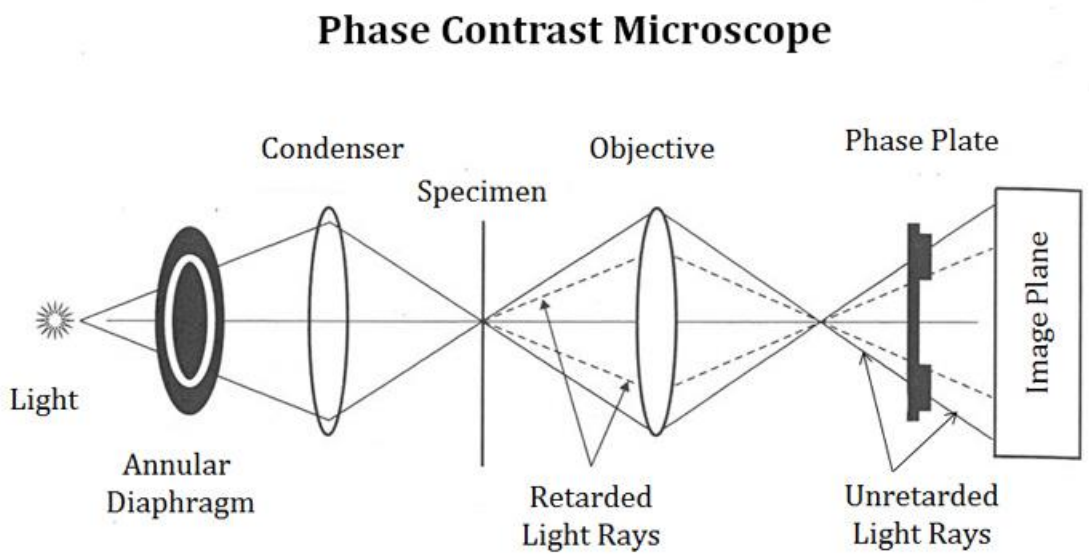
Phase contrast Microscopy

Phase contrast microscopy definition

Unstained living cells absorb practically no light. Poor light absorption results in extremely small differences in the intensity distribution in the image. This makes the cells barely, or not at all, visible in a brightfield microscope. Phase-contrast microscopy is an optical microscopy technique that converts phase shifts in the light passing through a transparent specimen to brightness changes in the image.

It was first described in 1934 by Dutch physicist Frits Zernike.

Principle of Phase-contrast Microscopy



When light passes through cells, small phase shifts occur, which are invisible to the human eye. In a phase-contrast microscope, these phase shifts are converted into changes in amplitude, which can be observed as differences in image contrast.

The Working of Phase contrast Microscopy

Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (labeled condenser annulus) positioned in the substage condenser front focal plane.

Wavefronts passing through the annulus illuminate the specimen and either pass through undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen.

Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by

a phase plate and focused at the intermediate image plane to form the final phase-contrast image observed in the eyepieces.

Parts of Phase contrast Microscopy

Phase-contrast microscopy is basically a specially designed light microscope with all the basic parts in addition to which an annular phase plate and annular diaphragm are fitted.

The annular diaphragm

It is situated below the condenser.

It is made up of a circular disc having a circular annular groove.

The light rays are allowed to pass through the annular groove.

Through the annular groove of the annular diaphragm, the light rays fall on the specimen or object to be studied.

At the back focal plane of the objective develops an image.

The annular phase plate is placed at this back focal plane.

The phase plate

It is either a negative phase plate having a thick circular area or a positive phase plate having a thin circular groove.

This thick or thin area in the phase plate is called the conjugate area.

The phase plate is a transparent disc.

With the help of the annular diaphragm and the phase plate, the phase contrast is obtained in this microscope.

This is obtained by separating the direct rays from the diffracted rays.

The direct light rays pass through the annular groove whereas the diffracted light rays pass through the region outside the groove.

Depending upon the different refractive indices of different cell components, the object to be studied shows a different degree of contrast in this microscope.

Applications of Phase contrast Microscopy

To produce high-contrast images of transparent specimens, such as

living cells (usually in culture),

microorganisms,

thin tissue slices,

lithographic patterns,

fibers,

latex dispersions,

glass fragments, and

subcellular particles (including nuclei and other organelles).

Applications of phase-contrast microscopy in biological research are numerous.

Advantages

Living cells can be observed in their natural state without previous fixation or labeling.

It makes a highly transparent object more visible.

No special preparation of fixation or staining etc. is needed to study an object under a phase-

contrast microscope which saves a lot of time.

Examining intracellular components of living cells at relatively high resolution. eg: The dynamic motility of mitochondria, mitotic chromosomes & vacuoles.

It made it possible for biologists to study living cells and how they proliferate through cell division.

Phase-contrast optical components can be added to virtually any brightfield microscope, provided the specialized phase objectives conform to the tube length parameters, and the condenser will accept an annular phase ring of the correct size.

Limitations

Phase-contrast condensers and objective lenses add considerable cost to a microscope, and so phase contrast is often not used in teaching labs except perhaps in classes in the health professions.

To use phase-contrast the light path must be aligned.

Generally, more light is needed for phase contrast than for corresponding bright-field viewing, since the technique is based on the diminishment of the brightness of most objects.

Scanning Electron Microscope (SEM)

The first Scanning Electron Microscope was initially made by Mafred von Ardenne in 1937 with an aim to surpass the transmission electron Microscope. He used high-resolution power to scan a small raster using a beam of electrons that were focused on the raster. He also aimed at reducing the problems of chromatic aberrations images produced by the Transmission electron Microscopes.

More studies followed by scientists and research institutions such as Cambridge Scientific Instrument Company who eventually developed a fully constructed Scanning electron Microscope, in 1965 and named it a Stereoscan.

Scanning Electron Microscope (SEM) definition

Scanning Electron Microscope (SEM) is a type of electron microscope that scans surfaces of microorganisms that uses a beam of electrons moving at low energy to focus and scan specimens.

The development of electron microscopes was due to the inefficiency of the wavelength of the light microscopes. electron microscopes have vert short wavelengths in comparison to the light

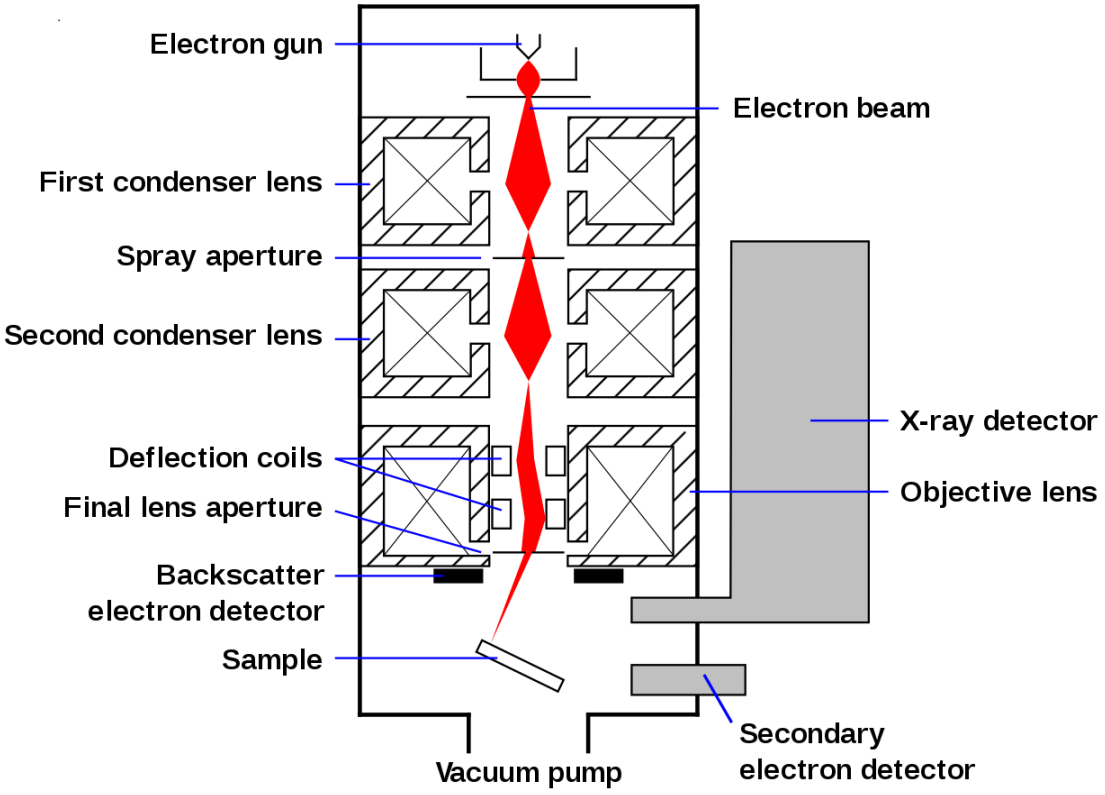
microscope which enables better resolution power.

The Principle of the Scanning Electron Microscope

Unlike the Transmission Electron Microscope which uses transmitted electrons, the scanning electron Microscope used emitted electrons.

The Scanning electron microscope works on the principle of applying kinetic energy to produce signals on the interaction of the electrons. These electrons are secondary electrons, backscattered electrons and diffracted backscattered electrons which are used to view crystallized elements and photons. Secondary and backscattered electrons are used to produce an image. The secondary electrons are emitted from the specimen play the primary role of detecting the morphology and topography of the specimen while the backscattered electrons show contrast in the composition of the elements of the specimen.

How does the Scanning Electron Microscope (SEM) work



The source of the electrons and the electromagnetic lenses are from tungsten filament lamps that are placed at the top of the column and it is similar to those of the transmission electron Microscope.

The electrons are emitted after thermal energy is applied to the electron source and allowed to move in a fast motion to the anode, which has a positive charge.

The beam of electrons activates the emission of primary scattered (Primary) electrons at high energy levels and secondary electrons at low-energy levels from the specimen surface. The beam of electrons interacts with the specimen to produce signals that give information about the surface topography and composition of the specimen.

The specimen does not need special treatment for visualization under the SEM, even air-dried samples can be examined directly. However, microbial specimens need fixation, dehydration, and drying in order to maintain the structural features of the cells and to prevent collapsing of the cells when exposed to the high vacuum of the microscope.

The samples are mounted and coated with thin layer heavy metal elements to allow spatial scattering of electric charges on the surface of the specimen allowing better image production, with high clarity.

Scanning by this microscope is attained by tapering a beam of electrons back and forth over a

thin section of the microscope. When the electrons reach the specimen, the surface releases a tiny staw of electrons known as secondary electrons which are then trapped by a special detector apparatus.

When the secondary electrons reach and enter the detector, they strike a scintillator (a luminescence material that fluoresces when struck by a charged particle or high-energy photon). This emits flashes of light which get converted into an electric current by a photomultiplier, sending a signal to the cathode ray tube. This produces an image that looks like a television picture that can be viewed and photographed.

The quantity of secondary electrons that enter the detector is highly defined by the nature of the specimen i.e raised surfaces receive high quantities of electrons, entering the detector while depressed surfaces have fewer electrons reaching the surface and hence fewer electrons enter the detector.

Therefore raised surfaces will appear brighter on the screen while depressed surfaces appear darker.

Parts of a Scanning Electron Microscope (SEM)

The major components of the Scanning Electron Microscope include;

Electron Source – This is where electrons are produced under thermal heat at a voltage of 1-40kV. the electrons the condense into a beam that is used for the creation of ana image and analysis. There are three types of electron sources that can be used i. e Tungsten filament, Lanthanum hexaboride, and Field emission gun (FEG)

Lenses – it has several condenser lenses that focus the beam of electrons from the source through the column forming a narrow beam of electrons that form a spot called a spot size.

Scanning Coil – they are used to deflect the beam over the specimen surface.

Detector – Its made up of several detectors that are able to differentiate the secondary electrons, backscattered electrons, and diffracted backscattered electrons. The functioning of the detectors highly depends on the voltage speed, the density of the specimen.

The display device (data output devices)

Power supply

Vacuum system

Like the transmission electron Microscope, the Scanning electron microscope should be free from vibrations and any electromagnetic elements.

Applications of the Scanning Electron Microscope (SEM)

It is used in a variety of fields including Industrial uses, nanoscience studies, Biomedical studies, Microbiology

Used for spot chemical analysis in energy-Dispersive X-ray Spectroscopy.

Used in the analysis of cosmetic components which are very tiny in size.

Used to study the filament structures of microorganisms.

Used to study the topography of elements used in industries.

Advantages of the Scanning Electron Microscope (SEM)

They are easy to operate and has user-friendly interfaces.

They are used in a variety of industrial applications to analyze surfaces of solid objects.

Some modern SEMs are able to generate digital data that can be portable.

It is easy to acquire data from the SEM, within a short period of time of about 5 minutes.

Limitations

They are very expensive to purchase

They are bulky to carry

They must be used in rooms that are free of vibrations and free of electromagnetic elements

They must be maintained with a consistent voltage

They should be maintained with access to cooling systems

Transmission Electron Microscope (TEM)

Definition

This is a powerful electron microscope that uses a beam of electrons to focus on a specimen producing a highly magnified and detailed image of the specimen.

The magnification power is over 2 million times better than that of the light microscope, producing the image of the specimen which enables easy characterization of the image in its morphological features, compositions and crystallization information is also detailed.

Early discovery of cathode rays like electrons by Louis de Broglie in the early 1920s, paved way into the development of an electron microscope where they used a beam of electrons creating a

form of wave motion.

Magnetic fields were used as lenses for the electrons. With these discoveries, the first electron microscope was later developed by Ernst Ruska and Max Knolls in 1931 and modified into a Transmission Electron Microscope (TEM) by Ernst Ruska along with the Sieman's company, in 1933.

This TEM microscope has several advantages compared to the light microscope with its efficiency also being very high.

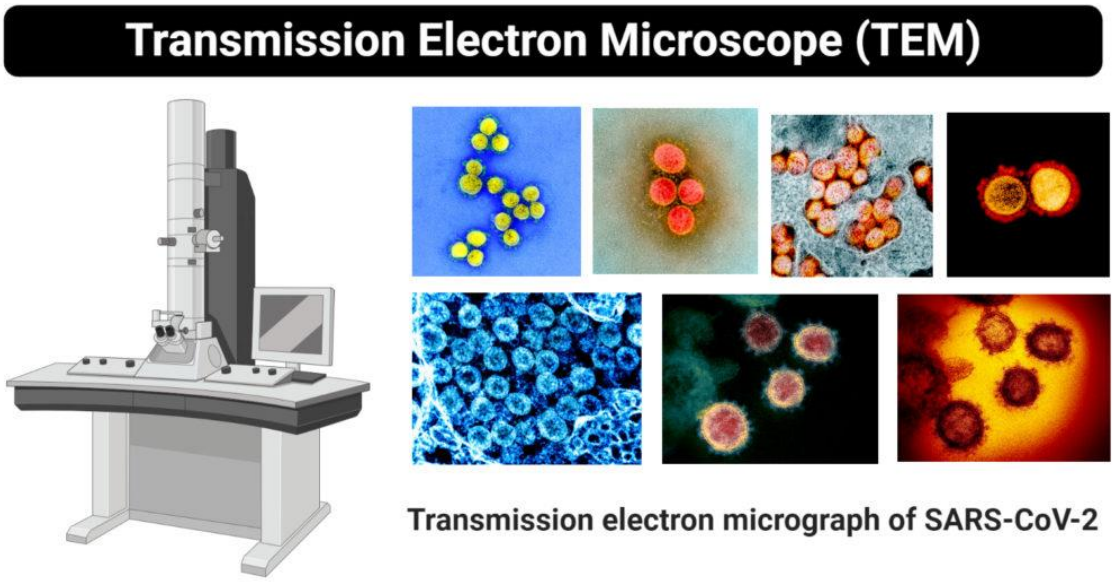
Among all microscopes both light and electron microscopes, TEM are the most powerful microscopes used in laboratories. It can magnify a mall particle of about 2nm, and therefore they have a resolution limit of 0.2um.

Principle of Transmission Electron Microscope (TEM)

The working principle of the Transmission Electron Microscope (TEM) is similar to the light microscope. The major difference is that light microscopes use light rays to focus and produce an image while the TEM uses a beam of electrons to focus on the specimen, to produce an image.

Electrons have a shorter wavelength in comparison to light which has a long wavelength. The mechanism of a light microscope is that an increase in resolution power decreases the wavelength of the light, but in the TEM, when the electron illuminates the specimen, the resolution power increases increasing the wavelength of the electron transmission. The wavelength of the electrons is about 0.005nm which is 100,000X shorter than that of light, hence TEM has better resolution than that of the light microscope, of about 1000times.

This can accurately be stated that the TEM can be used to detail the internal structures of the smallest particles like a virion particle.



Parts of Transmission Electron Microscope (TEM)

Their working mechanism is enabled by the high-resolution power they produce which allows it to be used in a wide variety of fields. It has three working parts which include:

Electron gun

Image producing system

Image recording system

Electron gun

This is the part of the Transmission Electron Microscope responsible for producing electron beams.

Electrons are produced by a cathode that is a tungsten filament that is V-shaped and it is normally heated. The tungsten filament is covered by a control grid known as a Wehnelt cylinder made up of a central hole which lies columnar to the tube. The cathode lies on top of or below the cylindrical column hole. The cathode and the control grid are negatively charged with an end of the anode which is disk-shaped that also has an axial hole.

When electrons are transmitted from the cathode, they pass through the columnar aperture (hole) to the anode at high voltage with constant energy, which is efficient for focusing the specimen to produce an accurately defined image.

It also has the condenser lens system which works to focus the electron beam on the specimen by controlling the energy intensity and the column hole of the electron gun. The TEM uses two condenser lenses to converge the beam of electrons to the specimen. The two condenser lens

each function to produce an image i.e the first lens which has strong magnification, produces a smaller image of the specimen, to the second condenser lens, directing the image to the objectives.

Image- Producing system

Its made up of the objective lens, a movable stage or holding the specimen, intermediate and projector lenses. They function by focusing the passing electrons through the specimen forming a highly magnified image.

The objective has a short focal length of about 1-5mm and it produces an intermediate image from the condenser which are transmitted to the projector lenses for magnification.

The projector lenses are of two types, i.e the intermediate lens which allows great magnification of the image and the projector lens which gives a generally greater magnification over the intermediate lens.

To produce efficient high standard images, the objectives and the projector lenses need high power supplies with high stability for the highest standard of resolution.

Image-Recording System

Its made up of the fluorescent screen used to view and to focus on the image. They also have a digital camera that permanently records the images captured after viewing.

They have a vacuum system that prevents the bombardment or collision of electrons with air molecules disrupting their movement and ability to focus. A vacuumed system facilitates the straight movement of electrons to the image.

The vacuumed system is made up of a pump, gauge, valves and a power supply.

The image that is formed is called a monochromatic image, which is greyish or black and white. The image must be visible to the human eye, and therefore, the electrons are allowed to pass through a fluorescent screen fixed at the base of the microscope.

The image can also be captured digitally and displayed on a computer and stored in a JPEG or TIFF format. During the storage, the image can be manipulated from its monochromatic state to a colored image depending on the recording apparatus eg use of pixel cameras can store the image in color.

The presence of colored images allows easy visualization, identification, and characterization of the images.

How does a Transmission Electron Microscope (TEM) work?

From the instrumentation described, the working mechanism is a sequential process of the parts

of the TEM mentioned above. To mean:

A heated tungsten filament in the electron gun produces electrons that get focus on the specimen by the condenser lenses.

Magnetic lenses are used to focus the beam of electrons of the specimen. By the assistance offered by the column tube of the condenser lens into the vacuum creating a clear image, the vacuum allows electrons to produce a clear image without collision with any air molecules which may deflect them.

On reaching the specimen, the specimen scatters the electrons focusing them on the magnetic lenses forming a large clear image, and if it passes through a fluorescent screen it forms a polychromatic image.

The denser the specimen, the more the electrons are scattered forming a darker image because fewer electron reaches the screen for visualization while thinner, more transparent specimens appear brighter.

Preparation of specimen for visualization by TEM

The specimen to be viewed under the TEM must undergo a special preparation technique to enable visualization and creation of a clear image.

Electrons are easily absorbed and easily scattered on solid elements, showing poor visualization for thick specimens. And therefore, very thin specimens are used for accurate and clear visualization forming a clear image as well. The specimen should be about 20-100nm thin and 0.025-0.1nm diameter, as small as that of a bacterial cell. Thin specimens allow interaction with electrons in a vacuumed space, are able to maintain their innate structure.

To get thin slice specimens, the specimen is first fixed on a plastic material with glutaraldehyde or osmium tetraoxide. These chemical agents stabilize the structure of the cell and maintain its originality. The addition of an organic solvent like alcohol such as ethanol will dehydrate the cell completely for embedding the specimen to the plastics.

The specimen is then permeated by adding an unpolymerized liquid epoxy plastic making it hardened like a solid block. This is where thin sections are cut from using a glass knife with a piece of special equipment known as an ultramicrotome.

The specimen is then stained appropriately (with the appropriate stain) for the uniform scattering of electrons. The thin sections are then soaked in heavy metallic elements such as lead citrate and uranyl acetate allowing the lean and aluminum ions to bind to the cell structures. This forms an opaque layer against the electrons on the cell structures to increase contrast.

The stained thin sections are then mounted on copper grids for viewing.

The primary staining techniques that are applied for viewing under the TEM is Negative staining coupled with heavy metallic elements coating. The metallic coating scatters electrons which appears on the photographic film while uncoated sections and used to study bacterial, viral cell morphologies and structures.

Freeze-itching treatment:

To reduce the possible dangers of artifacts, freeze-itching is used especially for the treatment of microbial cells, unlike chemical fixation, dehydration, and embedding, where most specimens get contaminated.

Microbial cell organelles undergo special treatment known as Freeze-itching whereby the specimens are prepared with liquid nitrogen and then warmed at -100°C in a vacuum chamber.

The sections are then cut with a precooled knife in liquid nitrogen at -196°C. After warming up the sectioned specimen in a high vacuum for about 2 minutes, it can then coated ith platinum and carbon layer forming replicas.

These are then be viewed under the TEM displaying more detailed internal structures of the cell in 3D.

This step of treatment with Liquid nitrogen is known as freeze-itching.

Applications of Transmission Electron Microscope (TEM)

TEM is used in a wide variety of fields From Biology, Microbiology, Nanotechnology, forensic studies, etc. Some of these applications include:

To visualize and study cell structures of bacteria, viruses, and fungi

To view bacteria flagella and plasmids

To view the shapes and sizes of microbial cell organelles

To study and differentiate between plant and animal cells.

Its also used in nanotechnology to study nanoparticles such as ZnO nanoparticles

It is used to detect and identify fractures, damaged microparticles which further enable repair mechanisms of the particles.

Advantages of Transmission Electron Microscope (TEM)

It has a very powerful magnification of about 2 million times that of the Light microscope.

It can be used for a variety of applications ranging from basic Biology to Nanotechnology, to education and industrial uses.

It can be used to acquire vast information on compounds and their structures.

It produces very efficient, high-quality images with high clarity.

It can produce permanent images.

It is easy to train and use the Transmission Electron Microscope

Limitations of Transmission Electron Microscope (TEM)

Generally, the TEMs are very expensive to purchase

They are very big to handle.

The preparation of specimens to be viewed under the TEM is very tedious.

The use of chemical fixations, dehydrators, and embedments can cause the dangers of artifacts.

They are laborious to maintain.

It requires a constant inflow of voltage to operate.

They are extremely sensitive to vibrations and electro-magnetic movements hence they are used in isolated areas, where they are not exposed.

It produces monochromatic images, unless they use a fluorescent screen at the end of visualization.

Fluorescence Microscope

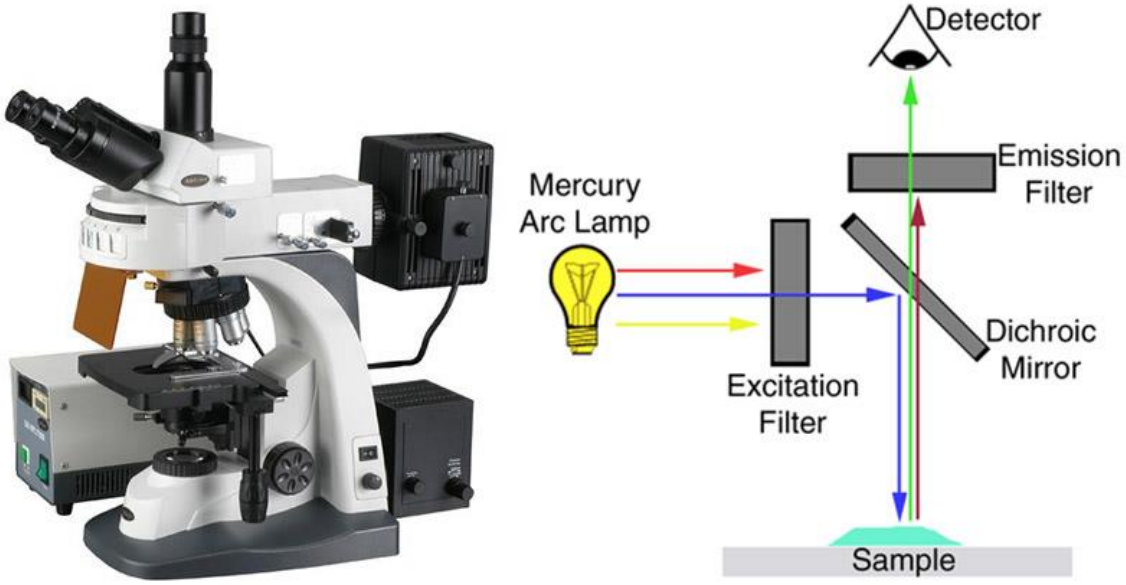
Fluorescence microscope definition

A fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances.

Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation while phosphorescence is a specific type of photoluminescence related to fluorescence.

Unlike fluorescence, a phosphorescent material does not immediately re-emit the radiation it absorbs.

The fluorescence microscope was devised in the early part of the twentieth century by August Köhler, Carl Reichert, and Heinrich Lehmann, among others.



Principle of Fluorescence Microscopy

Most cellular components are colorless and cannot be clearly distinguished under a microscope. The basic premise of fluorescence microscopy is to stain the components with dyes.

Fluorescent dyes, also known as fluorophores or fluorochromes, are molecules that absorb excitation light at a given wavelength (generally UV), and after a short delay emit light at a longer wavelength. The delay between absorption and emission is negligible, generally on the order of nanoseconds.

The emission light can then be filtered from the excitation light to reveal the location of the fluorophores.

Fluorescence microscopy uses a much higher intensity light to illuminate the sample. This light excites fluorescence species in the sample, which then emit light of a longer wavelength.

The image produced is based on the second light source or the emission wavelength of the fluorescent species rather than from the light originally used to illuminate, and excite, the sample.

Working

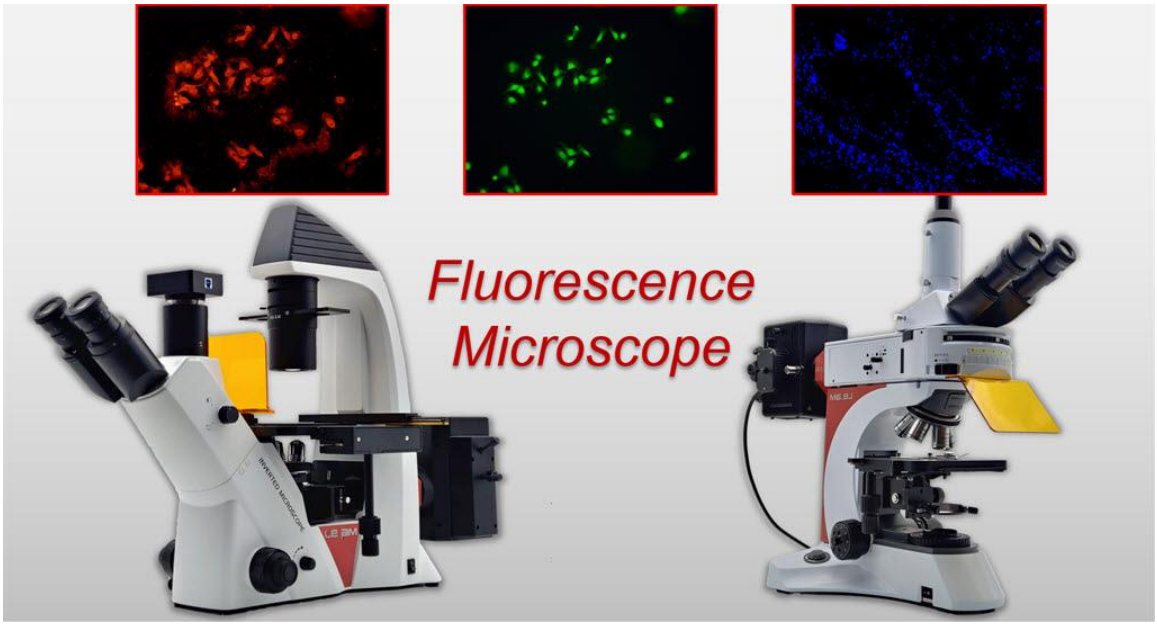
Light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused on the detector by the objective. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light.

Forms

The “fluorescence microscope” refers to any microscope that uses fluorescence to generate an image, whether it is a more simple set up like an epifluorescence microscope, or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescent image.

Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective).

Parts of Fluorescence Microscope



Typical components of a fluorescence microscope are:

Fluorescent dyes (Fluorophore)

A fluorophore is a fluorescent chemical compound that can re-emit light upon light excitation.

Fluorophores typically contain several combined aromatic groups, or plane or cyclic molecules with several π bonds.

Many fluorescent stains have been designed for a range of biological molecules.

Some of these are small molecules that are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst, phalloidin which is used to stain actin fibers in mammalian cells.

A light source

Four main types of light sources are used, including xenon arc lamps or mercury-vapor lamps

with an excitation filter, lasers, and high- power LEDs.

Lasers are mostly used for complex fluorescence microscopy techniques, while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide-field epifluorescence microscopes.

The excitation filter

The exciter is typically a bandpass filter that passes only the wavelengths absorbed by the fluorophore, thus minimizing the excitation of other sources of fluorescence and blocking excitation light in the fluorescence emission band.

The dichroic mirror

A dichroic filter or thin-film filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors.

The emission filter

The emitter is typically a bandpass filter that passes only the wavelengths emitted by the fluorophore and blocks all undesired light outside this band – especially the excitation light.

By blocking unwanted excitation energy (including UV and IR) or sample and system autofluorescence, optical filters ensure the darkest background.

Applications of Fluorescence Microscope

To identify structures in fixed and live biological samples.

Fluorescence microscopy is a common tool for today's life science research because it allows the use of multicolor staining, labeling of structures within cells, and the measurement of the physiological state of a cell.

Advantages of Fluorescence Microscope

Fluorescence microscopy is the most popular method for studying the dynamic behavior exhibited in live-cell imaging.

This stems from its ability to isolate individual proteins with a high degree of specificity amidst non-fluorescing material.

The sensitivity is high enough to detect as few as 50 molecules per cubic micrometer.

Different molecules can now be stained with different colors, allowing multiple types of the molecule to be tracked simultaneously.

These factors combine to give fluorescence microscopy a clear advantage over other optical

imaging techniques, for both in vitro and in vivo imaging.

Limitations of Fluorescence Microscope

Fluorophores lose their ability to fluoresce as they are illuminated in a process called photobleaching. Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence.

Cells are susceptible to phototoxicity, particularly with short-wavelength light. Furthermore, fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect.

Unlike transmitted and reflected light microscopy techniques fluorescence microscopy only allows observation of the specific structures which have been labeled for fluorescence.

Confocal Microscope

Introduction and History of the Confocal Microscope

The concept of confocal microscopy was initially developed by Marvin Minsky in the 1950s, at Harvard University with an aim of viewing the neural network without staining the tissues but it did not bear fruit due to lack of enough light source and a computerized system to store the large data.

The work was later adapted by David Egger M. and Mojmir Petran, forming a multiple-beam confocal microscope in the late 1960s. they used a spinning disk known as Nipkow which they used to examine brain tissues and ganglion cells that were unstained. The technique was later modified and published by Egger forming a mechanical scanned confocal laser microscope, that was able to visualize images of cells.

Later developments in science including development of computers and laser technology and digital manipulations of images using algorithms, grew the advances in confocal microscopy, forming practically usable confocal microscopes by a series of scientists including G. Fred Brakenhoff (1979), Colin Sheppard, Tony Wilson, Brad Amos, and John White (1980s).

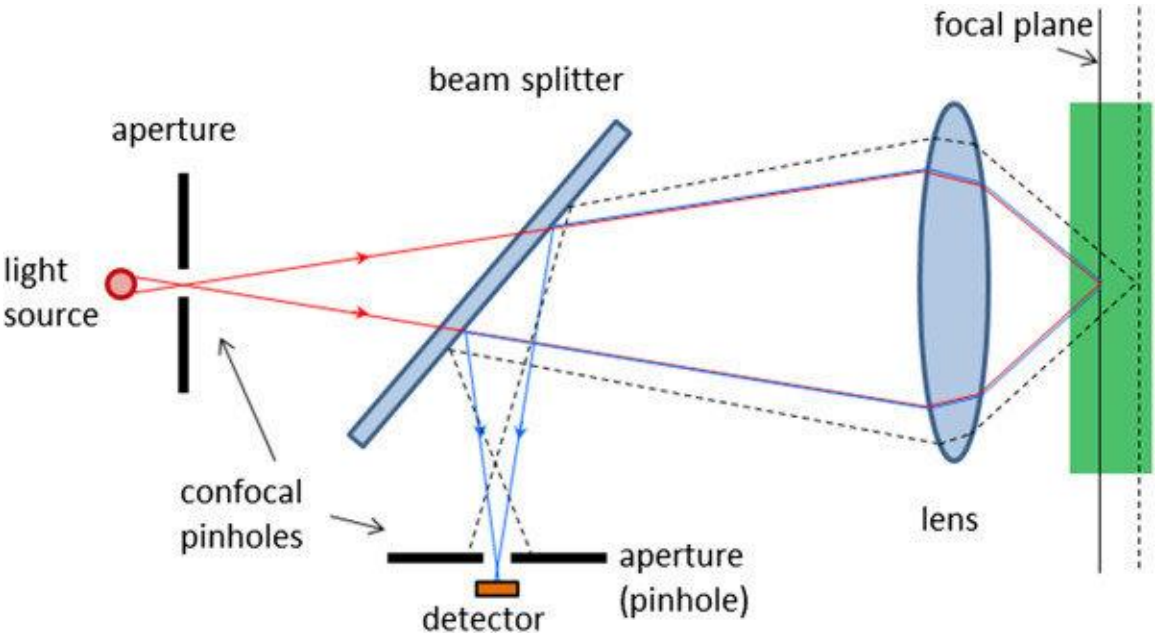
The first commercial confocal microscope was developed in 1987 with improved optics and electronics, powerful lasers with high scanning efficiency.

The modern confocal Microscope has all the possible integration of technology and mechanical components including optical components, which perform the primary function of the configuration by use of electronic detectors, a computer and laser systems.

The functioning of the confocal microscope is a collective role of all its components to produce

an electronic image. To date, these microscopes have been used to investigate molecules, microbial cells, and tissues.

Principle of the Confocal Microscope



Normally a conventional (wide-field) Microscope uses different wavelengths from a light source, to visualize and illuminate a large area of a specimen, forming fuzzy, murky and crowded images, because cell sample images are captured from all directions, without a focal point.

To avoid these issues, a Confocal Microscope is used. In wide-field or Fluorescent microscopes, the whole specimen receives light, receiving complete excitement and emitting light which is

detected by a photodetector on the microscope. However, with the confocal microscope, point illumination is the principle working mechanism.

A specimen is stained with fluorochrome is examined. When a beam of light is focused at a particular point of the fluoro-chromatic specimen, it produces an illumination that is focused by the objective lens to a plane above the objectives. The objective has an aperture on the focal plane located above it, which primarily functions to block any stray light from reaching the specimen.

A measure of the illumination point is about 0.25 to 0.8 um in diameter, determined by the objective numerical aperture and 0.5 to 1.5 um deep, with the brightest intensity.

The specimen normally lies between the camera lens and the perfect point of focus, known as the plane of focus. Using the laser from the microscope, the laser scans over a plane on the specimen (beam scanning0 or by moving the stage (stage scanning). A detector then will measure the illumination producing an image of the optical section. scanning several optical sections, they are collected in a computerized system as data, forming a 3D image. The image can be measured and quantified.

Its outcome is also favored by the aperture found above the objective which blocks stray light.

Images produced by the confocal microscope has a very good contract and resolution capacity despite the thickness of the specimen. Images are stored in the high-resolution 3D image of the cell complexes including its structures.

The main characteristic of the Confocal Microscope is that it only detects what is focused and anything outside the focus point, appears black.

The image of the specimen is formed when the microscope scanner, scans the focused beam across a selected area with the control of two high-speed oscillating mirrors. Their movement is facilitated by galvanometer motors. One mirror moves the beam from left to right on the lateral X-axis while the second mirror translates the beam along the Y-axis. After a scan on the X-axis, the beam moves rapidly back to the starting point to start a new scan, a process known as flyback. No information is collected during the flyback process, therefore the point of focus, which is the area of interest is what is illuminated by the laser scanner.

Parts of the Confocal Microscope

The Confocal Laser Scanning Microscope is made up a few components:

Objective lens

Out-of-focus plane

In-focus plane

Beam splitters

Detector

Confocal pinhole (aperture)

Laser

Oscillator Mirrors

Types of Confocal Microscope

Confocal laser scanning Microscope – It uses several mirrors that scan along the X and Y axes on the specimen, by scanning and descanning, and the image passes through a pinhole into the detector.

Spinning disk, also known as the Nipkow disk, is a type of confocal microscope that uses several movable apertures (pinholes) on a disc to scan for spots of light in a parallel manner over a specified plane, over a long period. The longer the time the less the excitation energy required for illumination, as compared to the Confocal laser scanning microscope. Lessened excitation energy reduces phototoxicity and photobleaching, hence its mainly used to imaging live cells.

Dual spinning Disk or Microlens enhanced confocal Microscope -, it was invented by Yokogawa electric; it works similarly to the spinning disk, the only difference is, it has a second spinning-disk with micro-lenses that is found before the spinning disk that contains the pinholes. The micro-lenses capture broadband of light focusing it into each pinhole, thus increasing the amount of light that is directed into each pinhole, reducing the amount of light that is blocked by the spinning disk. This Confocal Microscopes with enhanced Microlenses are much more sensitive than the spinning disks.

Programmable array Microscope (PAM) – this type of confocal microscope uses a spatial light modulator (SLM – an object that imposes some form of spatially-varying modulation on a beam of light). The SLM has a set of movable apertures (pinholes), with arrays of pixels of opacity, reflectivity or optical rotation. The SLM also has microelectrochemical mirrors that capture the image by a charge-coupled device (CCD) camera.

Each of the confocal microscopes has its advantages and disadvantages, but they all capture the images by recording the images and sometimes they can be programmed to get high-density images, especially the Programmed array Microscope and the Spinning disk confocal Microscope.

Applications of the Confocal Microscope

The Confocal Microscope is used in a wide range of fields including Biomedical sciences, Cells Biology, genetics, Microbiology, Developmental Biology, Spectroscopy, Nanoscience

(nanoimaging) and Quantum Optics.

In Biomedical sciences, it is used in the analysis of eye corneal infections, by quantifying and qualitatively analyzing the endothelial cells of the cornea.

Used to identify the presence of fungal elements in the corneal stroma, during keratomycosis infection, or rapid diagnosis and quick therapeutic response.

It is used in pharmaceutical industries, to ensure the maintenance of thin-film pharmaceuticals, allowing control of the quality and uniformity of drug distribution.

It is used to retrieve data from some 3D optical storage systems. This has helped in quantifying the age of Magdalen papyrus.

Advantages

The advantage of the Confocal microscope is that it improves the outcome of the image because it analyses the image from one optical point to another, therefore there is no interference with scattered light from other parts of the specimen.

They have better resolution and each point of interest is visualized and captured.

It can be used to study live and fixed cells

It can be used to collect serial optical sections.

It illuminates uniformly across the focus points.

The adjust their magnification electronically, without changing the objectives, by a factor known as the zoom factor.

It generates 3D sets of images.

Limitations

They have a limited number of excitation wavelengths, with very narrow bands.

They are expensive to produce the ultraviolet rays used by the Confocal Microscopes

They are also expensive to manufacture and to purchase.

Atomic Force Microscope (AFM)

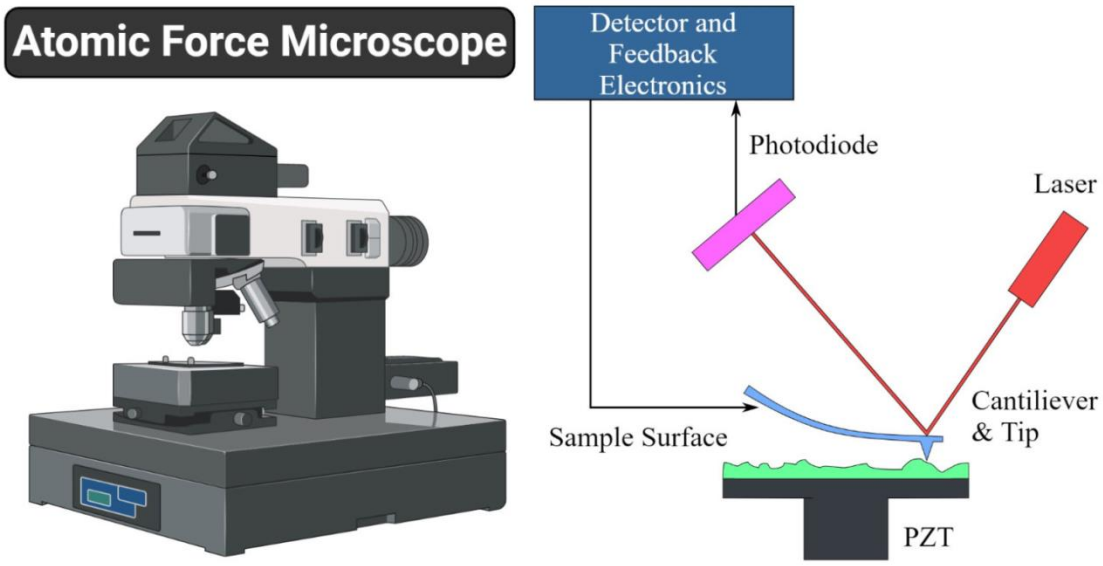
Definition

The atomic force microscope (AFM) is a type of scanning probe microscope whose primary

roles include measuring properties such as magnetism, height, friction.

The resolution is measured in a nanometer, which is much more accurate and effective than the optical diffraction limit. It uses a probe for measuring and collection of data involves touching the surface that has the probe. An image is formed when the scanning probe microscope raster-scans the probe over a section of the sample, measuring its local properties concurrently. They also have piezoelectric elements, which are electric charges that accumulate in selected solid materials like DNA, biological proteins, crystal, etc, to enable tiny accurate and precise movement during scanning upon an electric command.

The Atomic Force Microscope was invented in 1982, by scientists working in IBM, just after the invention of the Scanning tunneling Microscope in 1980 by Gerd Binnig and Heinrich Rohler by IBM Research in Zurich. That is when Binnig later invented the Atomic Force Microscope, and it was first used experimentally in 1986. It was put on the market for commercial sale in 1989.



The working principle of the Atomic Force Microscope

The Atomic Force Microscope works on the principle measuring intermolecular forces and sees atoms by using probed surfaces of the specimen in nanoscale. Its functioning is enabled by three of its major working principles that include Surface sensing, Detection, and Imaging.

The Atomic Force Microscope (AFM) performs surface sensing by using a cantilever (an element that is made of a rigid block like a beam or plate, that attaches to the end of support, from which it protrudes making a perpendicularly flat connection that is vertical like a wall). The cantilever has a sharp tip which scans over the sample surface, by forming an attractive force between the surface and the tip when it draws closer to the sample surface. When it draws very close making contact with the surface of the sample, a repulsive force gradually takes control

making the cantilever avert from the surface.

During the deflection of the cantilever away from the sample surface, there is a change in direction of reflection of the beam and a laser beam detects the aversion, by reflecting off a beam from the flat surface of the cantilever. Using a positive-sensitive photo-diode (PSPD- a component that is based on silicon PIN diode technology and is used to measure the position of the integral focus of an incoming light signal), it tracks these changes of deflection and change in direction of the reflected beam and records them.

The Atomic Force Microscope (AFM) takes the image of the surface topography of the sample by force by scanning the cantilever over a section of interest. Depending on how raised or how low the surface of the sample is, it determines the deflection of the beam, which is monitored by the Positive-sensitive photo-diode (PSDP). The microscope has a feedback loop that controls the length of the cantilever tip just above the sample surface, therefore, it will maintain the laser position thus generating an accurate imaging map of the surface of the image.

Parts of the Atomic Force Microscope

Atomic Force Microscopes have several techniques for measuring force interactions such as van der Waals, thermal, electrical and magnetic force interactions for these interactions done by the AFM, it has the following parts that assist in controlling its functions.

Modified tips which are used to detect the sample surface and undergo deflections

Software adjustments used to image the samples.

Feedback loop control – they control the force interactions and the tip positions using a laser deflector. the laser reflects from the back of the cantilever and the tip and while the tip interacts with the surface of the sample, the laser's position on the photodetector is used in the feedback loop for tracking the surface of the sample and measurement.

Deflection – The Atomic Force Microscope is constructed with a laser beam deflection system. The laser is reflected from the back of the AFM lever to the sensitive detector. They are made from silicon compounds with a tip radius of about 10nm.

Force measurement – the AFM works and depends highly on the force interactions, they contribute to the image produced. The forces are measured by calculation of the deflection lever when the stiffness of the cantilever is known. This calculation is defined by Hooke's law, defined as follows:

F= -kz, where F is the force, k is the stiffness of the lever and z is the distance the lever is bent.

Applications of the Atomic Force Microscope

This type of microscopy has been used in various disciplines in natural science such as solid-

state physics, semiconductor studies, molecular engineering, polymer chemistry, surface chemistry, molecular biology, cell biology, medicine, and physics.

Some of these applications include:

Identifying atoms from samples

Evaluating force interactions between atoms

Studying the physical changing properties of atoms

Studying the structural and mechanical properties of protein complexes and assembly, such as microtubules.

used to differentiate cancer cells and normal cells.

Evaluating and differentiating neighboring cells and their shape and cell wall rigidity.

Advantages of the Atomic Force Microscope

Easy to prepare samples for observation

It can be used in vacuums, air, and liquids.

Measurement of sample sizes is accurate

It has a 3D imaging

It can be used to study living and nonliving elements

It can be used to quantify the roughness of surfaces

It is used in dynamic environments.

Disadvantages of the Atomic Force Microscope

It can only scan a single nanosized image at a time of about 150x150nm.

They have a low scanning time which might cause thermal drift on the sample.

The tip and the sample can be damaged during detection.

It has a limited magnification and vertical range.

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