6Th Semester Theory

DSE4T : Research Methodology and Design

Tools techniques for research in Physiology and Health Science :

Microscopy : An Introduction

- **OPTICAL COMPONENTS OF THE LIGHT MICROSCOPE**
  A *compound light microscope* is an optical instrument that uses visible light to produce a magnified image of an object (or specimen) that is projected onto the retina of the eye or onto an imaging device. The word *compound* refers to the fact that two lenses, the objective lens and the eyepiece (or ocular), work together to produce the final magnification *M* of the image such that

$$M_{final} = M_{obj} * M_{oc}.$$

- Two microscope components are of critical importance in forming the image: (1) the objective lens, which collects light diffracted by the specimen and forms a magnified real image at the real intermediate image plane near the eyepieces or oculars, and (2) the condenser lens, which focuses light from the illuminator onto a small area of the specimen.The arrangement of these and other components is shown in Figure 1-1. Both the objective and condenser contain multiple lens elements that perform close to their theoretical limits and are therefore expensive. As these optics are handled frequently, they require careful attention. Other components less critical to image formation are no less deserving of care, including the tube and eyepieces, the lamp collector and lamp socket and its cord, filters, polarizers, retarders, and the microscope stage and stand with coarse and fine focus dials.

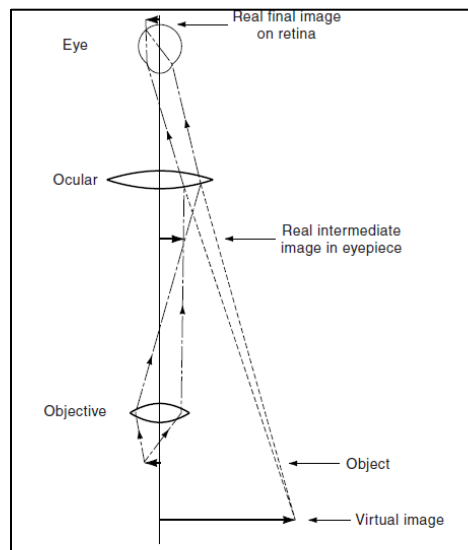


Figure 1-2
Perception of a magnified virtual image of a specimen in the microscope. The objective lens forms a magnified image of the object (called the real intermediate image) in or near the eyepiece; the intermediate image is examined by the eyepiece and eye, which together form a real image on the retina.

- The magnifying power of a microscope is an expression of the number of times the object being examined appears to be enlarged and is a dimensionless ratio. It is usually expressed in the form 10× (for an image magnified 10-fold), sometimes wrongly spoken as “ten eks”—as though the × were an algebraic symbol—rather than the correct form, “ten times.” The resolution of a

microscope is a measure of the smallest detail of the object that can be observed. Resolution is expressed in linear units, usually micrometres (μm).

- The most familiar type of microscope is the optical, or light, microscope, in which glass lenses are used to form the image. Optical microscopes can be simple, consisting of a single lens, or compound, consisting of several optical components in line. The hand magnifying glass can magnify about 3 to 20×. Single-lensed simple microscopes can magnify up to 300×—and are capable of revealing bacteria—while compound microscopes can magnify up to 2,000×. A simple microscope can resolve below 1 micrometre (μm; one millionth of a metre); a compound microscope can resolve down to about 0.2 μm.
- There are three structural parts of the microscope i.e. head, base, and arm.
    1. **Head** – This is also known as the body, it carries the optical parts in the upper part of the microscope.
    2. **Base** – It acts as microscopes support. It also carriers the microscopic illuminators.
    3. **Arms** – This is the part connecting the base and to the head and the eyepiece tube to the base of the microscope. It gives support to the head of the microscope and it also used when carrying the microscope. Some high-quality microscopes have an articulated arm with more than one joint allowing more movement of the microscopic head for better viewing.
- The optical parts of the microscope are used to view, magnify, and produce an image from a specimen placed on a slide. These parts include:
    1. **Eyepiece** – also known as the ocular. this is the part used to look through the microscope. Its found at the top of the microscope. Its standard magnification is 10x with an optional eyepiece having magnifications from 5X – 30X.
    2. **Eyepiece tube** – its the eyepiece holder. It carries the eyepiece just above the objective lens. In some microscopes such as the binoculars, the eyepiece tube is flexible and can be rotated for maximum visualization, for variance in distance. For monocular microscopes, they are none flexible.
    3. **Objective lenses** – These are the major lenses used for specimen visualization. They have a magnification power of 40x-100X. There are about 1- 4 objective lenses placed on one microscope, in that some are rare facing and others face forward. Each lens has its own magnification power.
    4. **Nose piece** – also known as the revolving turret. It holds the objective lenses. It is movable hence it cal revolve the objective lenses depending on the magnification power of the lens.
    5. **The Adjustment knobs** – These are knobs that are used to focus the microscope. There are two types of adjustment knobs i.e fine adjustment knobs and the coarse adjustment knobs.
    6. **Stage** – This is the section on which the specimen is placed for viewing. They have stage clips hold the specimen slides in place. The most common stage is a mechanical stage, which allows the control of the slides by moving the slides using the mechanical knobs on the stage instead of moving it manually.

7. **Aperture** – This is a hole on the microscope stage, through which the transmitted light from the source reaches the stage.
8. **Microscopic illuminator** – This is the microscopes light source, located at the base. It is used instead of a mirror. it captures light from an external source of a low voltage of about 100v.
9. **Condenser** – These are lenses that are used to collect and focus light from the illuminator into the specimen. They are found under the stage next to the diaphragm of the microscope. They play a major role in ensuring clear sharp images are produced with a high magnification of 400X and above. The higher the magnification of the condenser, the more the image clarity. More sophisticated microscopes come with an Abbe condenser that has a high magnification of about 1000X.
10. **Diaphragm** – its also known as the iris. Its found under the stage of the microscope and its primary role is to control the amount of light that reaches the specimen. Its an adjustable apparatus, hence controlling the light intensity and the size of the beam of light that gets to the specimen. For high-quality microscopes, the diaphragm comes attached with an Abbe condenser and combined they are able to control the light focus and light intensity that reaches the specimen.
11. **Condenser focus knob** – this is a knob that moves the condenser up or down thus controlling the focus of light on the specimen.
12. **Abbe Condenser** – this is a condenser specially designed on high-quality microscopes, which makes the condenser to be movable and allows very high magnification of above 400X. The high-quality microscopes normally have a high numerical aperture than that of the objective lenses.
13. **The rack stop** – It controls how far the stages should go preventing the objective lens from getting too close to the specimen slide which may damage the specimen. It is responsible for preventing the specimen slide from coming too far up and hit the objective lens.

SPECTROPHOTOMETERY : An Introduction

- **Spectrophotometry** is a process where we measured absorption and transmittance of monochromatic light in terms of ratio or a function of the ratio, of the radiant power of the two beams as a functional of spectral wave length. These two beams may be separated in time, space or both.
- **The basic introduction of electromagnetic spectrum :** The absorption and the emission of energy in the electromagnetic spectrum occur in discrete packets or photons. The various regions in the electromagnetic spectrum are shown in figure-1 along with the nature of the changes brought about by the radiation.

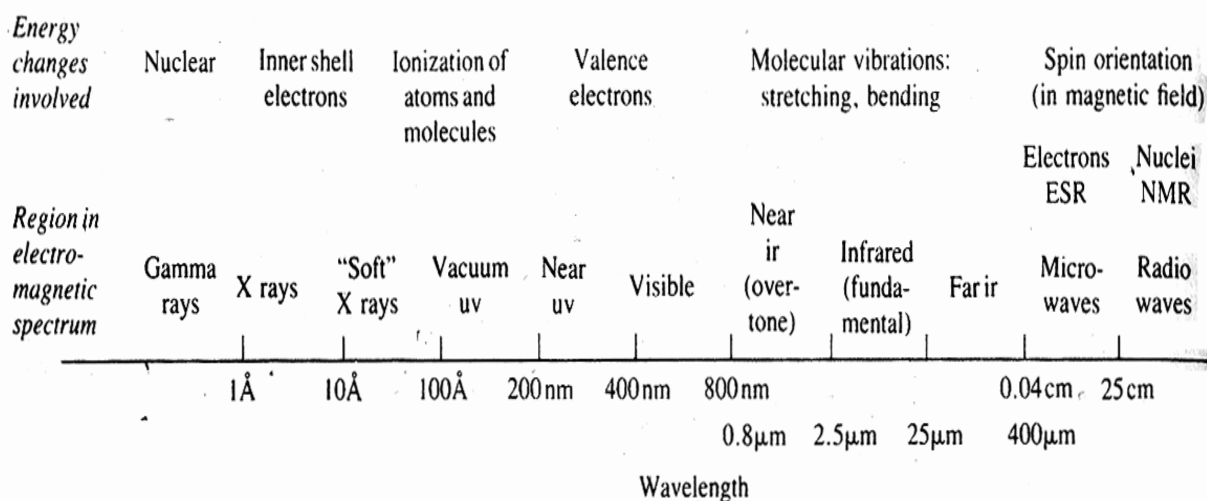


Figure-1 : Schematic diagram of electromagnetic spectrum. Wavelength scale is nonlinear.

- **THEORY**

It is possible for a ray of light to be absorbed by some material and simply pass through others without being affected. When a molecule absorbs light, energy is transferred from the ray of light to the molecule. If the frequency of the electronic and magnetic fields of a ray of light match the frequency at which molecules will vibrate, then light will be absorbed, if the frequency does not match, then the light will pass straight through unaltered. Inert molecules whether solid or liquid appear colored due to the way they modify light illuminating the object. Thus different objects absorb some wavelengths and reflect others. For example, if a white light passes through a yellow solution, it absorbs all colors except yellow.

- **BEER'S LAW**

The light transmitted through a solution changes as an inverse logarithmic relationship to the sample concentration.

Transmission T = (It / Io)

Optical density O.D = log (Io / It)

Where It = Intensity of light passing through the sample
Io = Intensity of light falling on the sample

Absorbance A = a b c

Where a = absorbity constant of the specimen
b = light path length
c = concentration

- **DEFINATION OF VISIBLE SPECTROPHOTOMETER**

The visible spectrophotometer is single – beam ratio-indicating instrument with a wavelength range of 340-600nm (Nanometer). The basic wavelength range may be converted to 340-950 nm by using accessory phototube and filters.

- **MAIN PARTS**

Visible spectrophotometer essentially consists of following basic components as shown in the figure below

- Radiation Source (Light)
- Optical System (Monochromatic)
- Sample Section
- Detector
- Filters
- Read Out

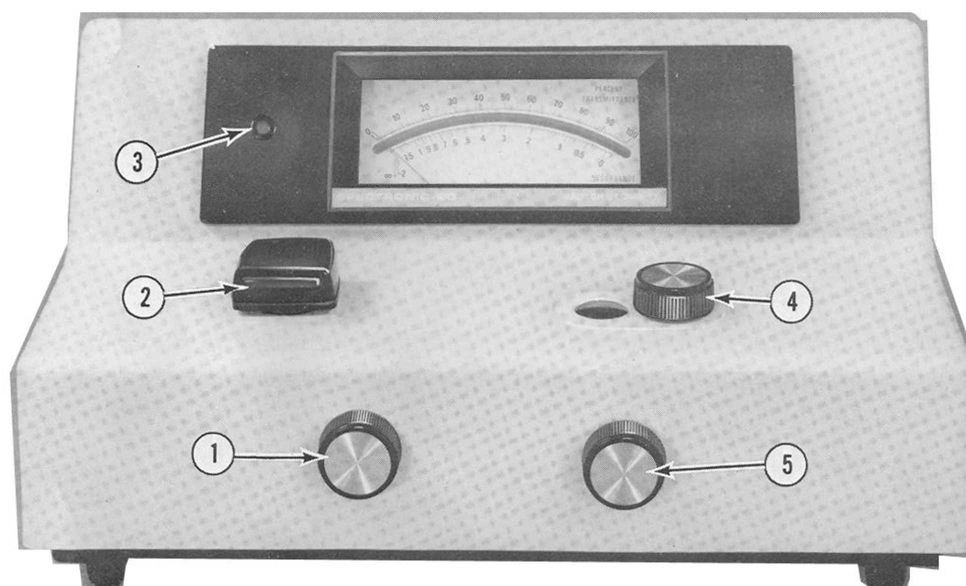


Figure-3(a) view of instrument : 1. Power Switch / Zero Control 3. Pilot Lamp
2. Sample Holder 4. Wavelength

Fluorescence spectroscopy :

Fluorimetry or spectrofluorimetry is a techniqiue to detect and analyze the fluorescence in the sample. Fluorescence is the emission of light by a substance (fluor) that has absorbed light or other electromagnetic radiation. In this emission phenomenon, a beam of light (usually UV light) excites the electron in a molecule which moves from ground state to higher energy excited state. When the electron falls back to the ground state, it emits fluorescence. Fluorescence spectroscopy is mainly concerned with electronic (ground state and excited state) and vibrational states.

(i) In molecular species, energy transition may occur in different vibrational levels of a particular excited state because the energy of the vibrational level of excited state matches with the energy of vibrational level of ground state and therefore in such energy transition, some energy is lost as heat (also known a non-radiative transition) until it reaches the lowest vibrational level of the excited state. After losing some energy as non-radiative transition and reaching the lowest vibrational level of the excited state, the electrons follow radiative transition. Radiative transition occurs when electrons falls back from higher energy excited state to lower energy ground state within the molecule, then energy emitted is measured as light. Therefore, in most cases, the emitted light has a longer wavelength (lower energy) than the absorbed radiation (higher energy). During radiative transition, the electrons or molecules may descend into any of several vibrational levels in the ground state, as a result the emitted photons will have different energies, and thus different frequencies. It is a form of luminescence when the emitted light is in the visible range. A fascinating example of fluorescence is when the absorbed radiation is in the ultraviolet region (invisible to the human eye) of the electromagnetic spectrum and the emitted light is in the visible region.

(ii) Similar phenomenun occurs in some atomic or molecular species. There are some chromophores which are inflexible and rigid molecules and therefore, may have limited range of vibrational energy levels. In such molecules, the vibrational energy level of the excited state often does not overlap with those of the ground state. When chromophores of this type

absorb light, it is not possible for them to return to the ground by simply losing their excess energy as heat. Instead, they undergo a radiative transition in which the absorbed energy is reemitted as light with the same frequency. This process of re-emitting the absorbed photon is “resonance fluorescence” and this is seen in molecular fluorescence.

- **Stokes Shift:** Stokes shift is named after Irish physicist George G. It is the difference between the wavelength of absorption maxima and the emission maxima.

Wavelength of absorbed radiation (having low wavelength units and higher energy) is denoted by **a**

Wavelength of emitted (fluorescence) radiation (having higher wavelength units values and lower energy is denoted by **b**

Stokes shift = b-a.

Good results are achieved with the compounds having the greater Stokes shift. Greater the Stokes shift, lesser will be the interference as the excitation and the emission spectra do not overlap.

Chromophores which exhibit the phenomenon of fluorescence are called fluors or fluorophores. Fluorophores are organic molecules of 20-100 Daltons. Fluorescent molecules absorb the electromagnetic radiation in visible region and emit the radiation at a higher wavelength in the visible. Example: ethidium bromide (493 nm/620 nm). Most commonly fluorescent molecules absorb the electromagnetic radiation in the UV range and emits in visible range. Example: green fluorescent protein (360 nm/508 nm).

- **Intrinsic fluors:** The native compound exhibits the property due to the presence of aromatic groups in amino acid side-chains in the case of proteins for example tyrosine, tryptophan and phenylalanine. Cofactors such as FMN, FAD and NAD also exhibit fluorescence.
- **Extrinsic fluors:** Non-fluorescent compounds can be detected by coupling a fluorescent probe (or fluor). Examples are 1- Anilino-8-naphthalene suphonate, fluorescein (for protein), ethidium bromide and acridine orange (for DNA).

Fluors have characteristics emission spectrum (fluorescence) or as well as characteristic absorbance spectrum which depends upon its structure and chemical environment.

Most electrons will occupy the ground state and lowest vibrational level (S_0V_0) at room temperature.

- Electrons are elevated to the high energy excitation state S_1, S_2, etc by the absorption of photons provided by the electromagnetic radiation. The excitation occurs in less than 10^{-15} s
- The life time of excited state is very short, ranging from 0.5 to 8 ns (0.5 to 8 x 10^{-9} s) or in some cases it may range up to 2s (this situation can arise as a consequence of a phenomenon associated with electrons called magnetic spin)
- Non radiative transition of electrons leads to a rapid loss of energy in the form of heat. This occurs by the collision degradation resulting in the lowest vibrational energy in the lowest excited state (S_1V_0).
- Electrons after reaching the lowest vibrational level of the excited state return to the ground state in less than 10^{-8} s and the emitted energy is stated as fluorescence.
- In fluorescence emission measurement, the excitation wavelength is fixed but the detection wavelength varies. In fluorescence excitation measurement, the detection is fixed but the emission wavelength varies.

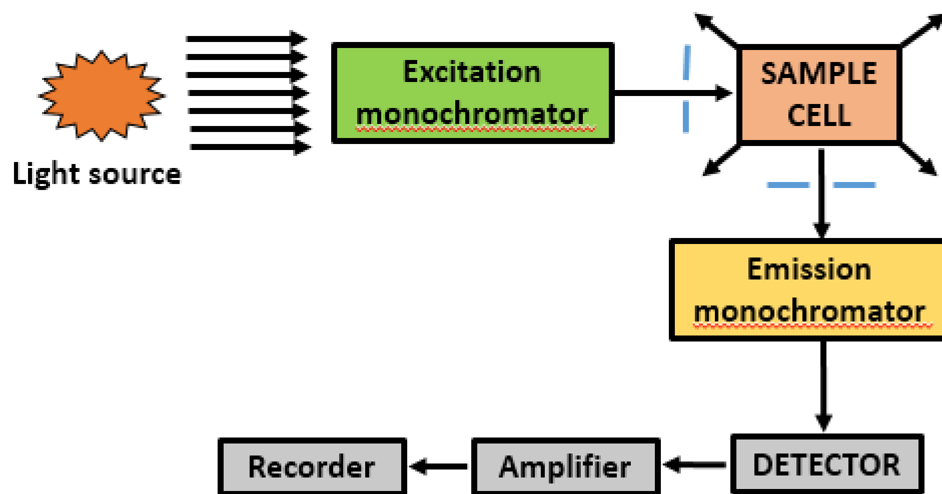
Disadvantages

• Fluorescence is susceptible to pH, temperature and solvent polarity.

• Whether a particular compound will fluoresce or not is the main problem being encountered.

• Fluorescence quenching. This occurs when emitted fluorescence is lost to other molecules by collision interaction. Quenching is more in concentrated samples and therefore assay is used for concentrated solutions. To increase the sensitivity and accuracy of spectrofluorimeter, very low concentrated samples are used which decrease the collisions and hence the quenching.

• Many interfering materials such as detergents, filter paper and some tissues material may affect the fluorescence



- **INSTRUMENTATION**
- **Light source:** Mercury lamp emits light near peak wavelengths. Xenon arc exhibits continuous emission spectrum with constant intensity in 300-800 nm range, but can also used for just above 200 nm.
- **Monochromators:** Most common type of monochromators utilizes a diffraction grating. Two monochromators are used. One monochromator (excitation monochromator) is used for the selection of the excitation wavelength from incident beam. Fluorescent sample will emit the fluorescence in all the directions. Angle of 90° is chosen for emitted florescence and second monochromator (emission monochromator) is used for determination of fluorescence spectrum. The excitation wavelengths which are frequently being selected are in the ultraviolet region and the emission wavelength is in the visible region.
- **Detector:** Detector can be a single-channeled (detects the one wavelength at a time) or multi-channeled (detects all emitted wavelength) both having advantages and disadvantages. Detector is a sensitive photocell, (eg: red sensitive photomultiplier for wavelengths greater than 500nm).
- There are two setup for the illumination of the sample:
  - (a) **90° illumination** (as discussed above)
  - (b) **Front face illumination:** This type of illumination setup removes pre and post-filter effects. In front face illumination, cuvette with one optical face is used and the excitation and the emission occur at the same face. This set up is less sensitive than 90° illumination.

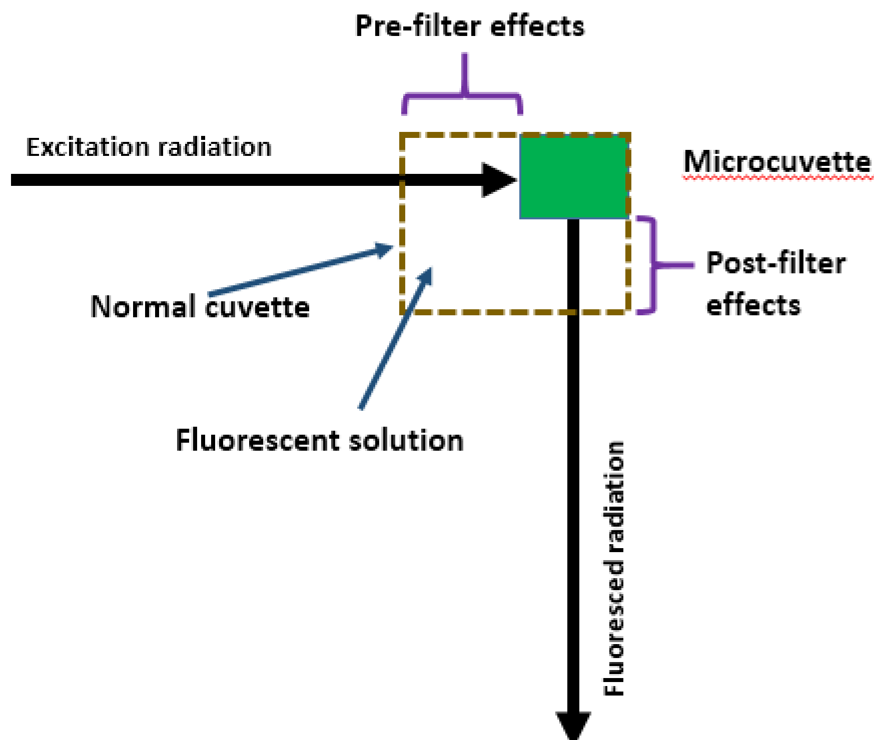


Figure : Pre and Post filter effects.

APPLICATIONS

1. Fluorescent probes: Probes are useful in both qualitative and quantitative detection. It helps in the detection of biological compound which is present in very low concentration in a mixture. They are applied to characterize folding intermediates and surface hydrophobicity.

2. Protein and peptide structure: The intrinsic fluors such tryptophan, tyrosine and phenylalanine present in the protein are responsible for the fluorescence exhibited by the proteins. Proteins are generally excited at 280 nm and fluorescence is measured at 295 nm. The fluorescence of folded protein is contributed by all individual aromatic amino acids present in it. Among these, tryptophan exhibits strong fluorescence whereas tyrosine and phenylalanine exhibits less fluorescence. The emission fluorescence of tyrosine is solvent dependent. As the polarity of the solvent surrounding the tryptophan decreases, the fluorescence intensity of the tryptophan increase.
Tyrosine emits fluorescence less than the tryptophan and its fluorescence is quenched by the tryptophan present in its vicinity. Phenylalanine gives weak fluorescence and its fluorescence is only observed when both tyrosine and tryptophan are absent. Any conformational change in the protein therefore changes the absorbance. Cofactors such as FMN, FAD, NAD exhibits the fluorescence and are also applied in the protein structural studies. The binding and release of cofactors, inhibitors, substrates at sites close to the fluor, cause changes in the conformational change and thus changes the fluorescence spectra. It can also be used to study the denaturation and aggregation of protein and peptides.

3. Membrane Structure: The intensity fluorescence of a fluorescently labelled molecule is dependent upon the solvent/environment in which it is present. Changes in the pH or solvent polarity affects the conformation and therefore structure changes can be monitored by the changes in the fluorescence. Extrinsic fluor, ANS (1-Anilino-8-naphthalene suphonate) probe can be used to monitor the changes in the mitochondrial membranes during energy transduction. Hydrophillic and hydrophobic probes can be used for the membrane structure studies as they can orient themselves in hydrophilic and hydrophobic regions of the membrane and gives the information regarding the properties of the membrane and its surface. Phospholipids containing 12-(9-anthroanoyl)-stearic acid and 2-(9-anthroanoyl)-palmitic acid into membranes yields the information about the thickness of the membrane. 12-(9-anthroanoyl)-stearic acid and 2-(9-anthroanoyl)-palmitic acid when present in the membranes indicates the regions 0.5nm and 1.5 nm, respectively, from the phosphate head groups of the lipid bilayer.

4. Fluorescence recovery after photobleaching (FRAP)
FRAP technique is used for measuring the lateral diffusion in layers or thin membrane by fluorescent probes. The sample under the study is fluorescently labelled and fluorescence in measured in sample and image is observed and captured with the help of optical microscope equipped with the time line camera. Light source is focused on the small patch of the sample and exposed to high intensity illumination (radiation) which causes photobleaching of fluorescent probes. Photobleaching permanently lose the ability of fluor to fluoresce. This turns the patch in to dark color, fluorescence intensity in this area decreases and the image of the sample is continuously observed in the microscope. With time, the adjacent and nearby fluorescing probes will slowly

diffuse in to the dark patch as Brownian motion proceeds. Depending upon the speed of diffusion and time, the dark patch will fluoresce again as the fluorescent probes moved in to the bleached area of non-fluorescent probes (beached probes). This technique is very useful for studying the diffusion, fluorescently labelled phospholipids or proteins may be incorporated into a biological membrane and subjected to the similar treatment. The motion of these phospholipids or proteins in the membrane can be studied by monitoring with low intensity radiation. FRAP can also be used to study the protein binding in cell membrane, cell surface characterization, studying free energy in phospholipid layer.

5. Fluorescence resonance energy transfer (FRET) : Energy may be transferred from donor to acceptor flour through FRET or electronic energy transfer or dipole-dipole coupling. For this to happen, the distance between the donor and acceptor is critical and both the fluors must be situated closely, there must be overlap between the donor fluorescence spectrum and acceptor fluorescence spectrum. When the donor fluor is present alone, it will fluoresce. Placing the acceptor fluor in the vicinity of donor fluor, quenches the fluorescence emitted by the donor flour. This emitted radiation is sufficient for the electronic transitions in the acceptor flour, and thus emits the fluorescence of different intensity.
This technique detects very small changes in distance, detects molecular interactions in different systems, localization of metals in metalloproteins, detects the interaction the between the proteins, measurement of conformational changes during binding of enzymes with substrate and receptors with ligand, used to measure the distance between the two domains in the same protein, gives information about lipid rafts in the cell membranes.

6. Fluorescence immunoassay (FIA) : FIA is a sophisticated technique and is used to detect the antigen and antibody interactions by using the fluorescent probes to label either antigen or antibody. Antigen is detected by the binding of primary antibody. Excess of the primary antibody can be removed by washing. The antigen-antibody complex is then detected by the secondary antibody labelled with the fluor. Excess of the secondary antibody can be removed by washing. The fluor is excited at a particular wavelength and the fluorescence is detected by the spectrofluorimetry. High background fluorescence is the major disadvantage of this technique. Two approaches are followed to reduce the background fluorescence and increases the sensitivity. First, fluors having large stokes shifts should be preferred, example: europium chelates. And secondly well designed fluorimeters, which delays the detection of emitted light and mean while the background fluorescence declines.