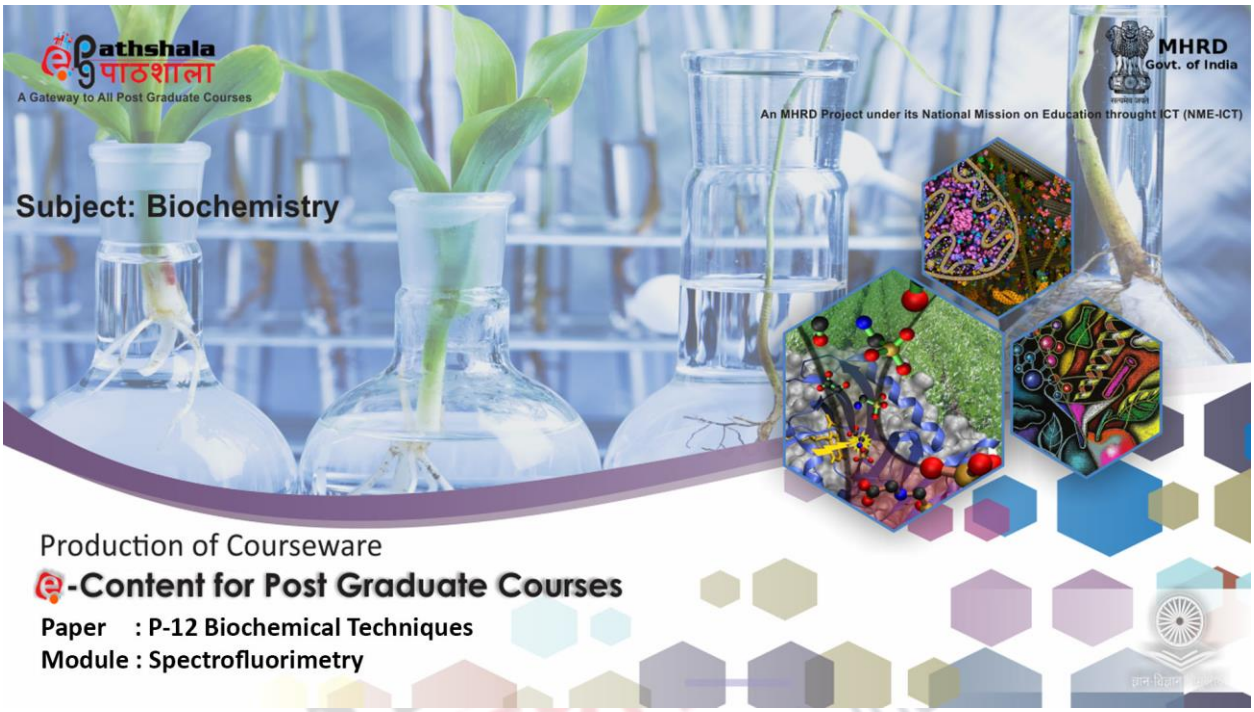


athshala पाठशाला

MHRD Govt. of India

Development Team

Principal Investigator	Dr. Sunil Kumar Khare, Professor, Department of Chemistry, IIT-Delhi
Content Writer:	Dr. Y.S. Rajput and Dr. Sunita Meena
Content Reviewer:	Dr. T.K. Choudhuri, Professor School of Biological Sciences, Indian Institute of Technology, Delhi
Paper Coordinator:	Dr. Y.S. Rajput, Emeritus Scientist, Animal Biochemistry Division, National Dairy Research Institute, Karnal

Description of Module	
Subject Name	Biochemistry
Paper Name	12 Biochemical Techniques
Module Name/Title	18 Spectrofluorimetry

A Gateway to All Post Graduate Courses

1. Objectives

1.1 To understand technique of Spectrofluorimetry .

1.2 To explain instrumentation design

1.3 What are applications of Spectrofluorimetry?

Fluorescence spectroscopy or 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.

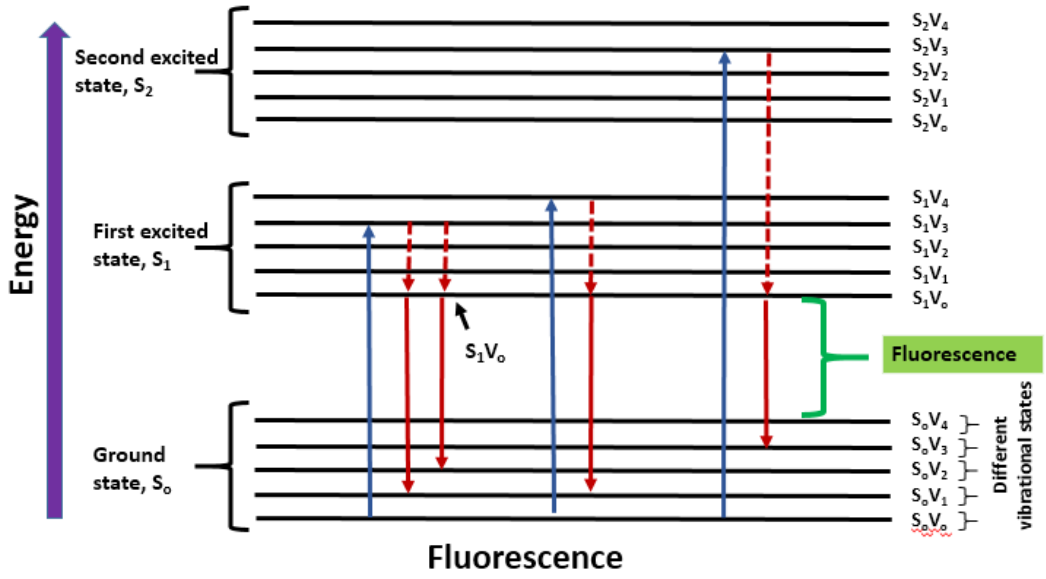
Chromophores which exhibit the phenomenon of fluorescence are called fluors or fluorophores. Fluorophores are organic molecules of 20-100 Daltons. Fluorescent molecules absorb the electromagnetic radiation in visible region and emit the radiation at a higher wavelength in the visible. Example: ethidium 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.



Quantum efficiency (Q) = quanta fluoresce/quanta absorbed **or** number of photons emitted/number of photons absorbed. It is independent of the excitation wavelength.

Spectrofluorimetry exhibits accuracy with fluorescent samples present at low concentration. At low concentrations, the intensity of fluorescence (I_f) is related to the intensity of the incident radiation (I_0) by:

$I_f = 2.3\ I_0\ \varepsilon_\gamma c l Q$., i.e. $I_f \alpha c$

Where,

c=concentration of the fluorescing solution (molar)
l = light path in fluorescing solution (cm)
ε_γ = molar extinction coefficient for the absorbing material at wavelength γ ($dm^3\ mol^{-1}\ cm^{-1}$)

Depending upon the final vibrational and rotational energy levels of electrons in ground state, the emitted radiation will be of many closely related wavelengths and will exhibit as band spectra. Aliphatic molecules are usually flexible and tend to photo-dissociate rather than fluorescence, whereas aromatic compounds with delocalized π-electrons sometimes fluoresce.

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

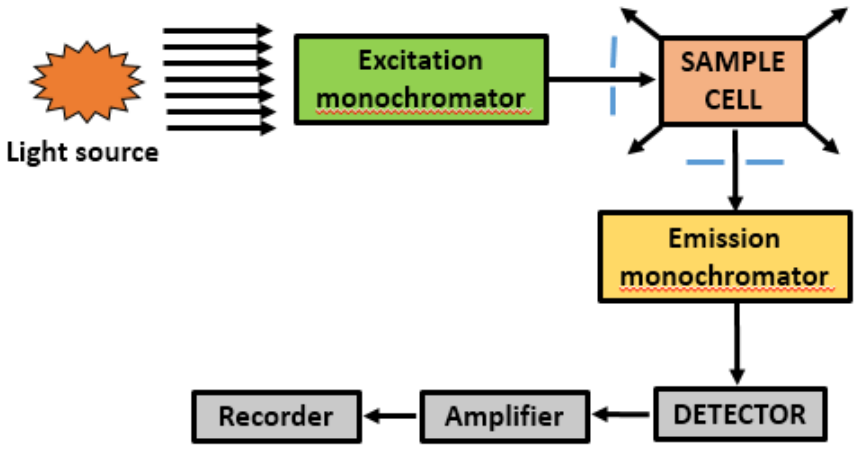
Light source: Mercury lamp emits light near peak wavelengths. Xenon arc exhibits continuous emission spectrum with constant intensity in 300-800 nm range, but can also used for just above 200 nm.

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:

(i) 90° illumination (as discussed above)



Pre-filter effects arises due to the absorption of radiation before reaching to the fluorescent molecule and **post-filter effects** arises due to the decrease in fluorescence emitted by the fluorescent molecule before escaping the cuvette. These effects increase with the increase in sample concentration. The use of microcuvettes alleviates this effect to some extent.

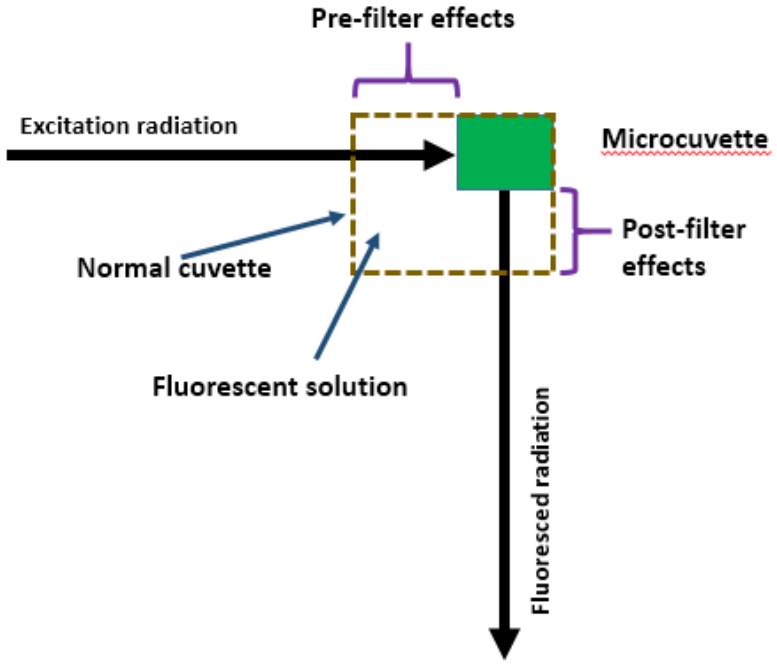
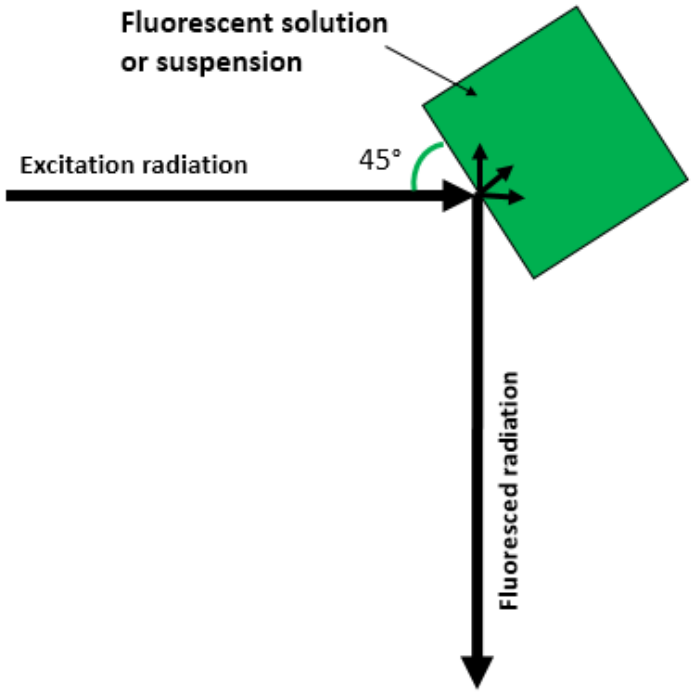


DIAGRAM SHOWING PRE AND POST FILTER EFFECTS

(ii) **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.



FRONT FACE ILLUMINATION

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.

7. **Fluorescence activated cell sorter (FACS)**

FACS is a type of flow cytometry. It is the method of physical separation or sorting of cells from a mixture of cells (cell suspension) into different compartments which is based on the fluorescence and light scattering emitted by the cells tagged with different fluor. A cell suspension is allowed to pass through a narrow nozzle in a stream of liquid. This fast flowing stream of liquid is broken in to droplets, each droplet containing a single cell and is achieved by vibration. One cell in one droplet then pass through the fluorescence detection apparatus, which senses the fluorescence characteristic of the particular cell. Additionally, each droplet also passes through an electric charging ring which give the charges to the droplet, the charges depends upon the fluorescence emitted by the cell. These charged droplets then pass through an electrostatic deflection system, which senses the charges on the droplet and physically separate/deflects the droplets in to sequentially arranged containers. This techniques is used in the counting the cells, separation of cells, purification of cells from a mixture.

8. **Microspectrofluorimetry**

A spectrofluorimter is equipped with microscope which enables to observe the binding of antibody to a single bacterial cell or subcellular organelle and also helps in the identification of cancerous cells from normal cell as they express different set of protein for which fluoresce labelled specific antibody can be applied. Since some malignant cells have more nucleic acid content than do normal cell have, therefore these malignant cells take up more acridine orange dye which binds to the DNA and can be differentiated from the normal cells

A Gateway to All Post Graduate Courses