Fluorescence Spectroscopy

- Fluorescence spectroscopy or fluorimetry or spectro-fluorimetry is a technique 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 electro-magnetic 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.

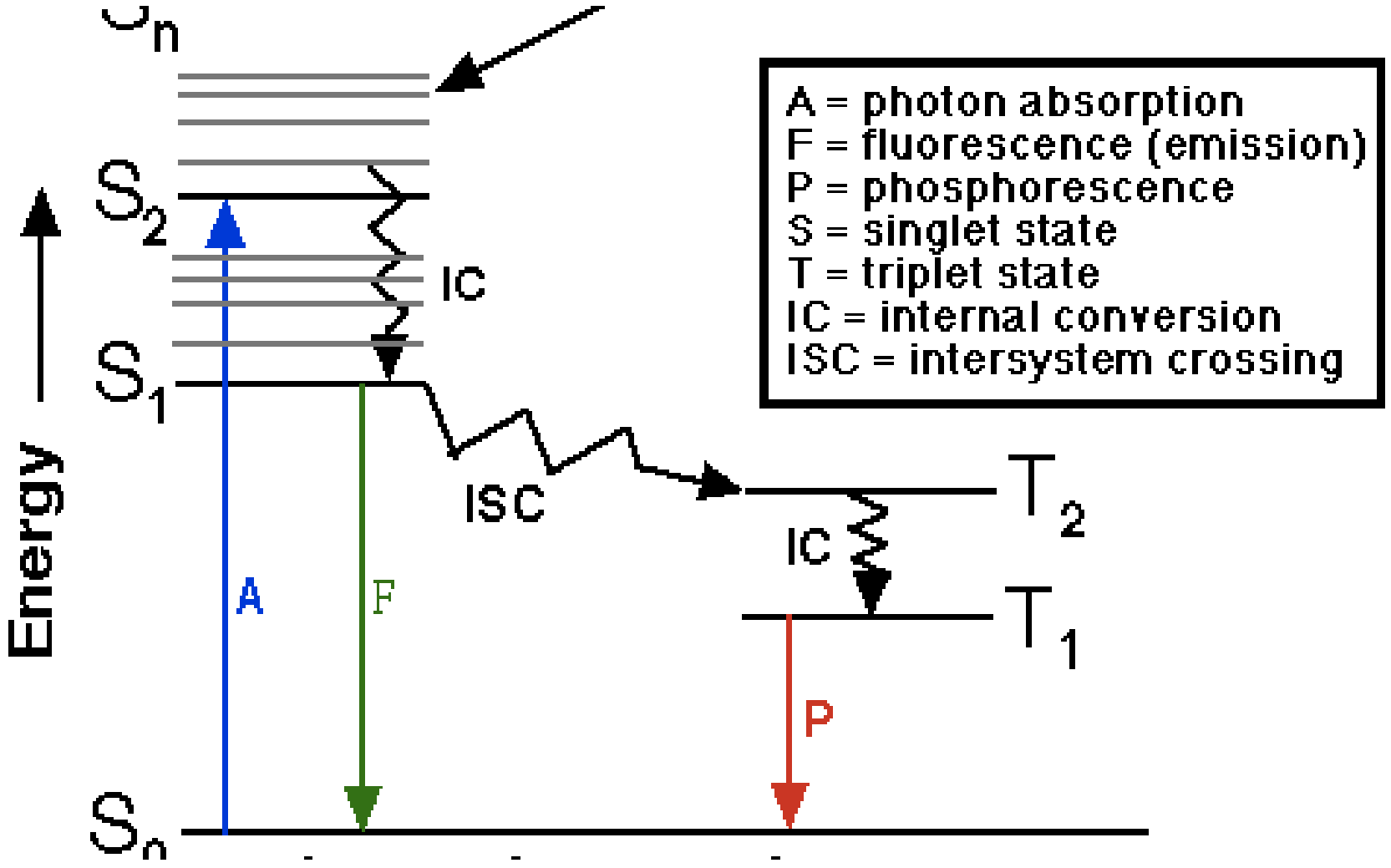
Absorbance/Fluorescence Spectroscopy

- Fluorescence is more sensitive to environment than absorbance
- Smaller amounts of material are required for fluorescence spectroscopy
- Hence Fluorescence spectroscopy is of greater value than Absorbance spectroscopy.
- Absorbance spectroscopy is simpler to perform

Information obtained from Fluorescence Spectra

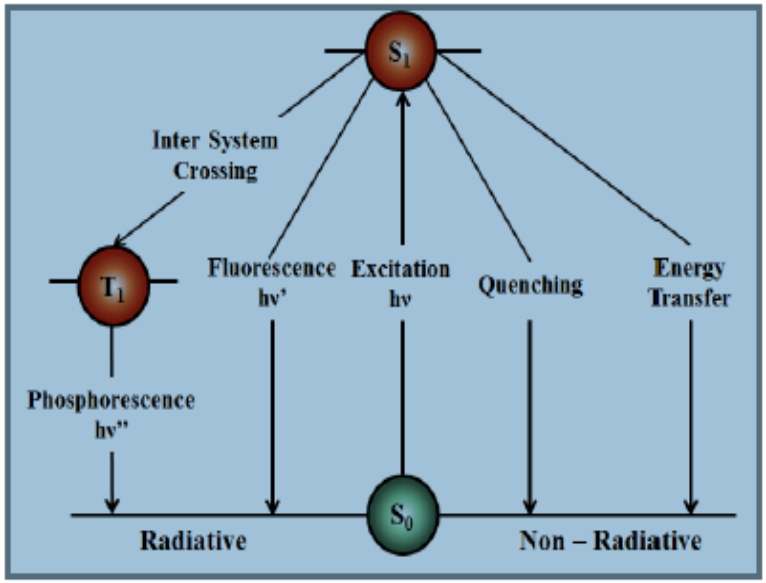
- Conformation
- Binding sites
- Solvent interactions
- Degree of flexibility
- Intermolecular distance
- Rotational diffusion coefficient of macromolecules
- With living cells fluorescence can be used to localize otherwise undetectable substance

Jablonski diagram of Fluorescence excitation, radiative decay and nonradiative decay



Theory of Fluorescence:

- When a molecule absorbs light, an electron is promoted to a higher excited state (generally a singlet state but may also be a triplet state). The excited state can get depopulated in several ways.
- The molecule can lose its energy non –radiatively by giving its energy to another absorbing species in its immediate vicinity (energy transfer) or by collisions with other species in the medium.
- If an excited state triplet overlaps with the exited state singlet, the molecule can cross over into this triplet state This is known as inter system crossing



If the molecule then returns to the ground state singlet (T_1--S_0) by emitting light, the process is known as **phosphorescence.**

The molecule can partially dissipate its energy by undergoing conformational changes and relaxed to the lowest vibrational level of the excited state in a process called **vibrational relaxation**.

If the molecule is rigid and cannot vibrationally relax to the ground state, it then returns to the ground state (S_1-- S_0) by emitting light, the process is known **as fluorescence.**

Stokes Shift:

- The difference between the wavelength of absorption maxima and the emission maxima.

- Wavelength of absorbed radiation (having low wavelength, higher energy) is denoted by (a) wavelength of emitted (fluorescence) radiation (having higher wavelength values and lower energy) 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.

Fluorophores:

- Chromophores which exhibit the phenomenon of fluorescence are called fluors or fluorophores (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 e.g., 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).

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

Fluorophores (Contd.)

- **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).

Disadvantages of Fluorescence Spectroscopy

- 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
- To increase the sensitivity and accuracy of spectrofluorometer, 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

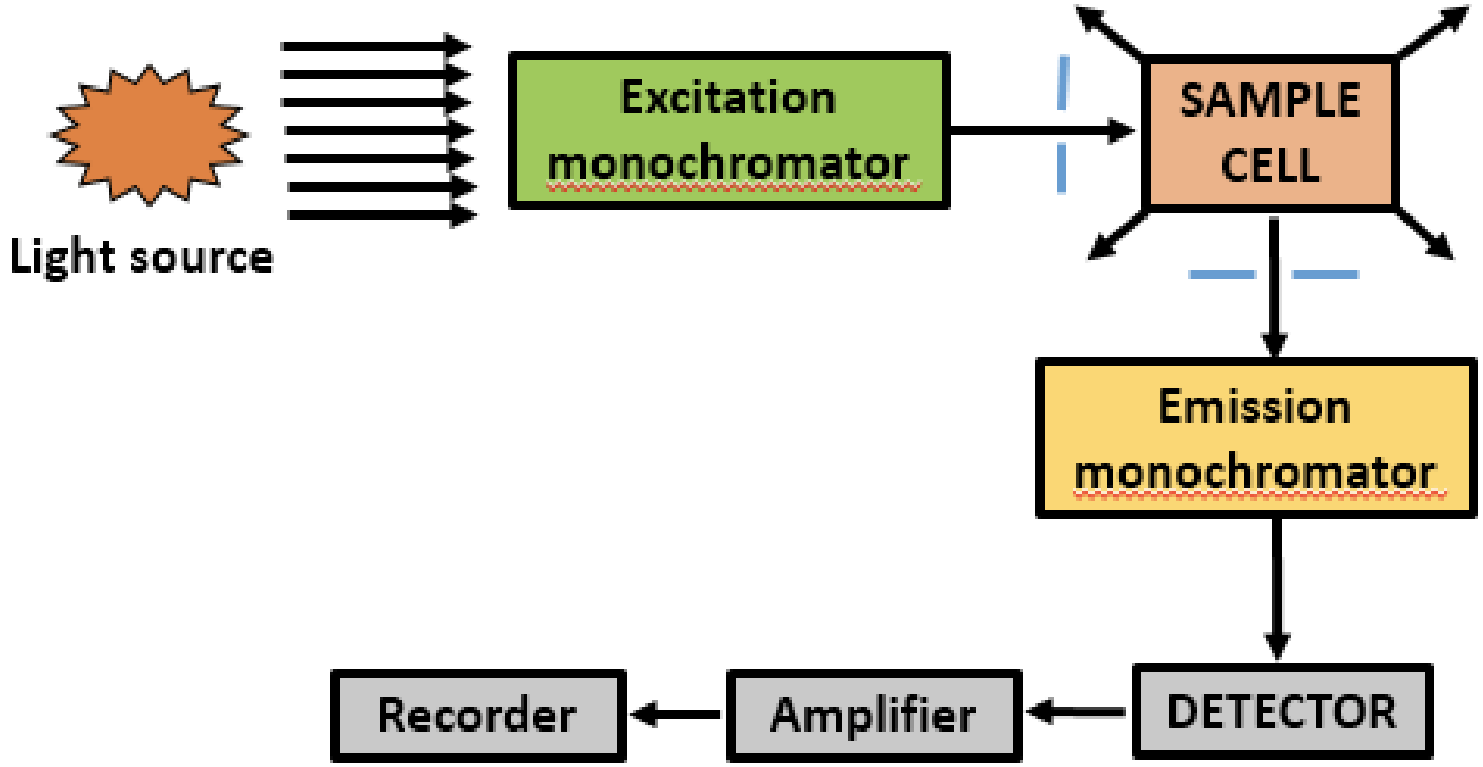
Quantum efficiency

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

Spectrofluorimeter Instrumentation

- Light Source: A high intensity light beam: Xenon Arc lamp , LASER
- Monochromator: Diffraction grating is most common, Two monochromators are used one is excitation and another is emission
- Detector: A sensitive Photocell. Single Channel (detects one wavelength at a time) or multichannel (detects all emitted wavelengths)

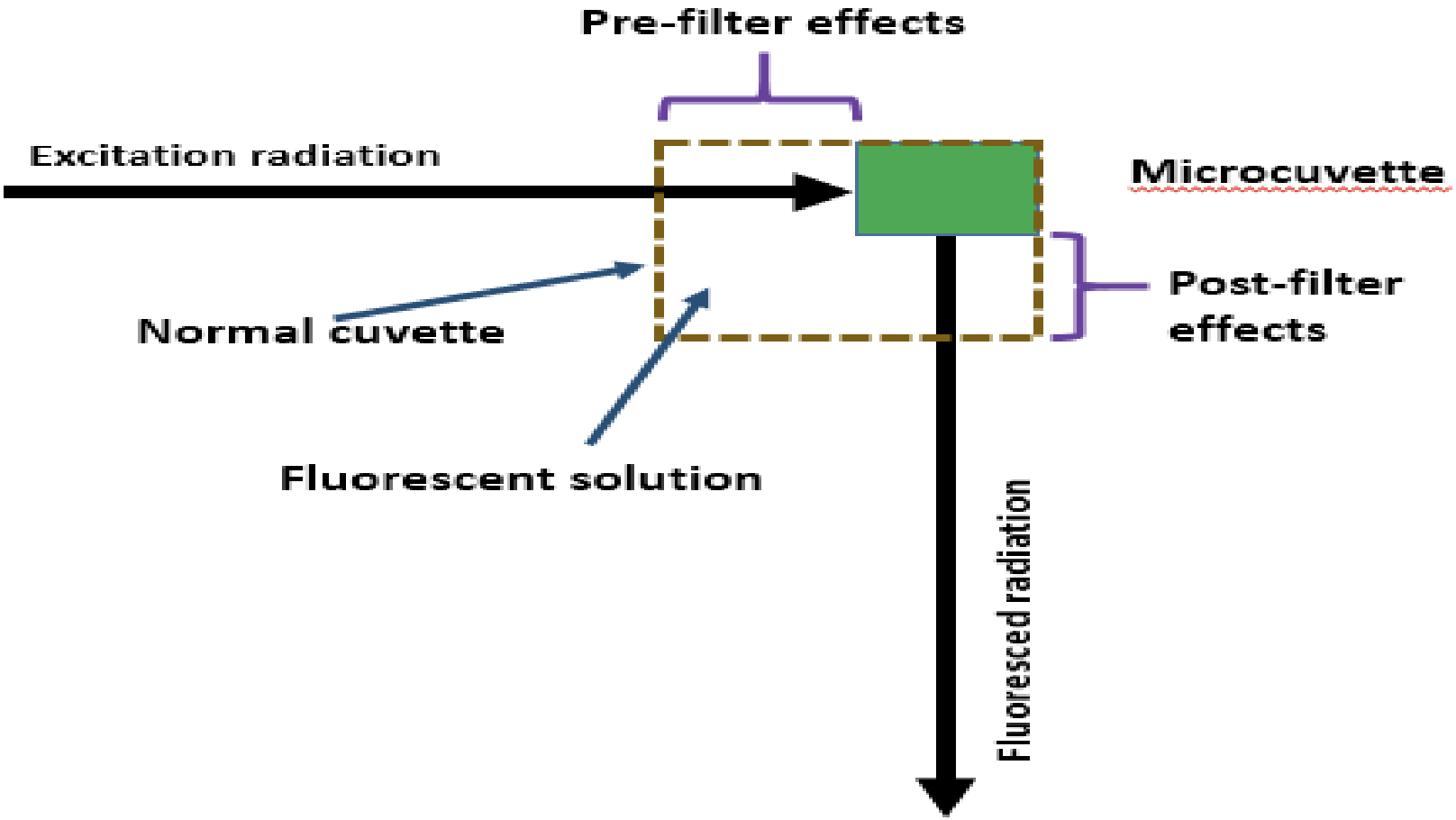
Spectrofluorimeter Instrumentation
90^0 Illumination



Pre and Post Filter Effects

- **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.

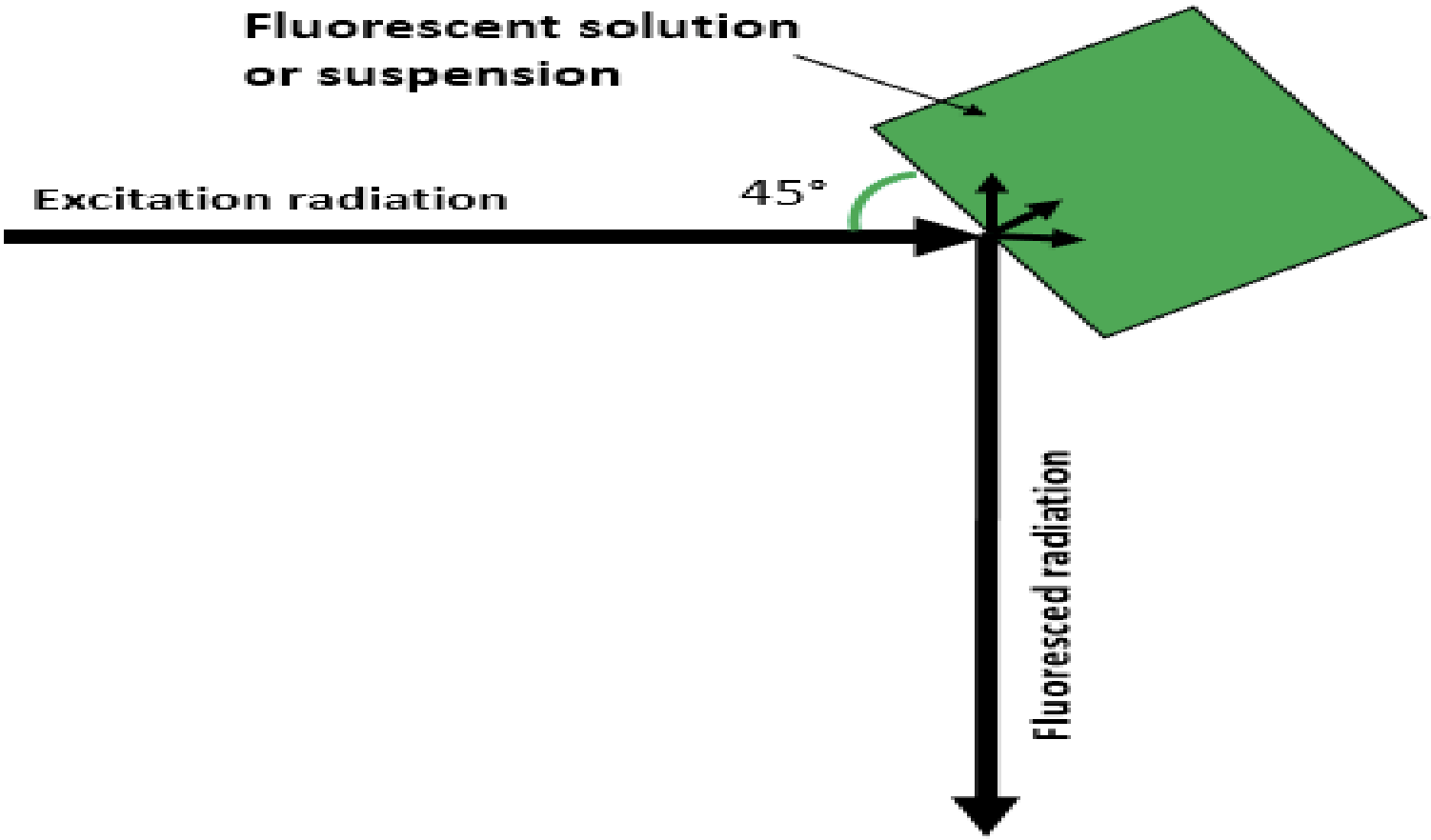
Pre-and post Filter Effect



Front face illumination

- This type of illumination set up removes pre and post filter effects.
- In front face illumination, cuvette with one optical face is used and the excitation and emission occur at the same face.
- This set up is less sensitive than 90^0 illumination

Front Face illumination



Applications

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

Protein and peptide structure

- The intrinsic fluors such Trp, Tyr and Phe are present in the protein.
- 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, Trp exhibits strong fluorescence whereas Tyr and Phe exhibits less fluorescence.
- The emission fluorescence of Trp is solvent dependent.
- As the polarity of the solvent surrounding the Trp decreases, the fluorescence intensity of the Trp increase.

Protein and peptide structure (Contd)

- **Tyr** emits fluorescence less than the **Trp** and its fluorescence is quenched by the **Trp** present in its vicinity.
- **Phe** gives weak fluorescence and its fluorescence is only observed when both **Tyr** and **Trp** 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 conformation and thus changes the fluorescence spectra.
- It can also be used to study the denaturation and aggregation of protein and peptides.

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 sulphonate) probe can be used to monitor the changes in the mitochondrial membranes during energy transduction.
- Hydrophilic 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.

Membrane Fluidity

For polarization measurement, polarizers are inserted into the excitation and emission light paths. With the excitation polarizer fixed, the emission polarizer can be rotated to measure the perpendicular ($I_{\underline{i}}$) and parallel(I_{ii}) components of the fluorescence emission. The steady state polarization is defined as:

$$P= I_{\underline{i}} - I_{ii}/I_{\underline{i}} + I_{ii}$$

and an equivalent measure is the steady state anisotropy:

$$r= I_{\underline{i}} - I_{ii}/I_{\underline{i}} + 2I_{ii}$$

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 labeled and fluorescence in measured in sample and image is observed and captured with the help of optical microscope equipped with the timeline camera.
- Light source is focused on the small patch of the sample and exposed to high intensity illumination (radiation) which causes photo bleaching of fluorescent probes.
- Photo bleaching 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 into 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).

FRAP (Contd)

- This technique is very useful for studying the diffusion, fluorescently labeled 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.

Lipids and Many Membrane Proteins Diffuse Rapidly in the Plane of the Membrane

- Lipids and many membrane proteins are constantly in lateral motion, a process called lateral diffusion
- The rapid lateral movement of membrane proteins has been visualized by means of fluorescence microscopy through the use of the technique of fluorescence recovery after Photobleaching.
- The rate of recovery of fluorescence depends on the lateral mobility of the fluorescence-labeled component, which can be expressed in terms of a diffusion coefficient, D.
- The average distance s traversed in time t depends on D according to the expression

$$s = (4Dt)^{1/2}$$

- Proteins vary markedly in their lateral mobility. Some proteins are nearly as mobile as lipids, whereas others are virtually immobile.

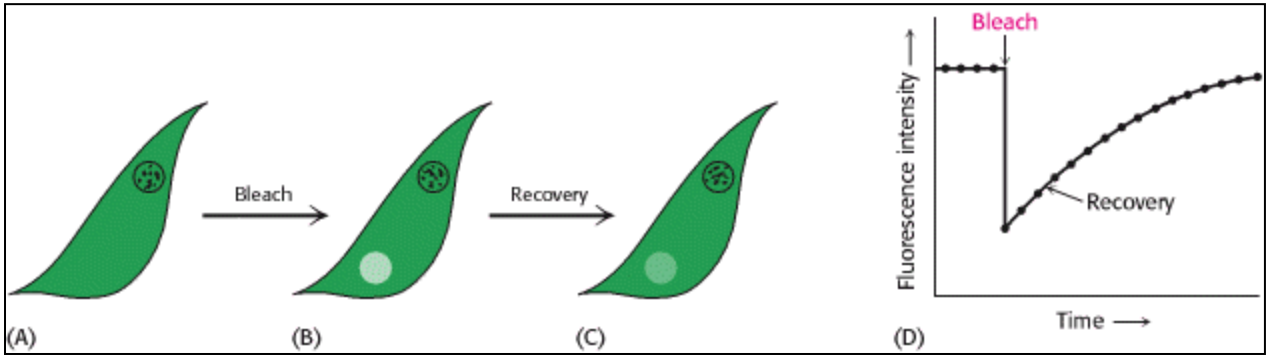


Figure: Fluorescence Recovery After Photobleaching (FRAP) Technique. (A) The cell-surface fluoresces because of a labeled surface component. (B) The fluorescent molecules of a small part of the surface are bleached by an intense light pulse. (C) The fluorescence intensity recovers as bleached molecules diffuse out of the region and unbleached molecules diffuse into it. (D) The rate of recovery depends on the diffusion coefficient.

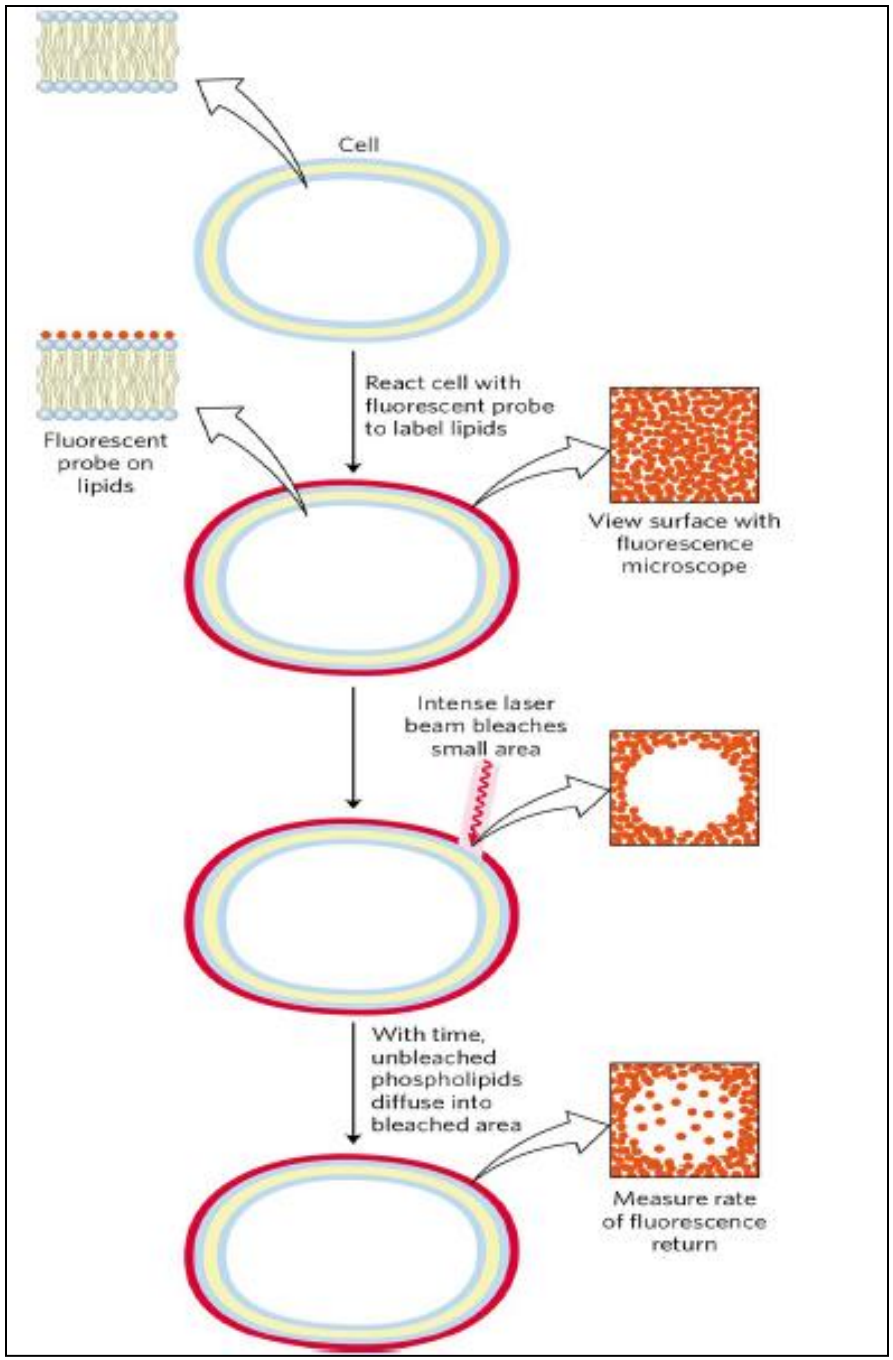


FIGURE: Measurement of lateral diffusion rates of lipids by fluorescence recovery after photobleaching (FRAP). Lipids in the outer leaflet of the plasma membrane are labeled by reaction with a membrane-impermeant fluorescent probe (red) so that the surface is uniformly labeled when viewed with a fluorescence microscope. A small area is bleached by irradiation with an intense laser beam and becomes nonfluorescent. With the passage of time, labeled lipid molecules diffuse into the bleached region, and it again becomes fluorescent. Researchers can track the time course of fluorescence return and determine a diffusion coefficient for the labeled lipid. The diffusion rates are typically high; a lipid moving at this speed could circumnavigate an *E. coli* cell in one second. (The FRAP method can also be used to measurelateral diffusion of membrane proteins.)

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.

FRET (Contd)

- 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

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.

Fluorescence activated cell sorter (FACS)

- FACS is a type of flow cytometer.
- It is sorting of cells from a mixture of cells (cell suspension) into different compartments on the basis of 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 into droplets, each droplet containing a single cell and is achieved by vibration.

FACS (Contd.)

- 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 depend 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.

Micro-spectrofluorimetry

- A spectrofluorimter is equipped with microscope which enables to observe the binding of antibody to a single bacterial cell or subcellular organelle
- 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