Fluorescence and fluorophores:

Introduction:

When a compound absorbs light (and hence energy) electrons are raised from the ground state to an excited state. Electrons return to the ground state by a variety of transitions which may involve the emission of a quantum of light (radiative transition). This effect is termed fluorescence .The emitted light will always be of lower energy, and hence longer wavelength, than the exciting light. The energy may also be lost by nonradiative processes and eventually dissipated as heat. Fluorescence is the luminescence emitted when photons are absorbed and released by molecules known as fluorochromes (fluorophores).



**Figure:** The absorption and emission of light during fluorescence. After excitation by light absorption, the electron moves to an excited state of lower energy (red arrow). When it drops to the ground state (green arrow) light is emitted.

**Fluorophores:**

Fluorophores are typically polyaromatic compounds having a conjugated π-electron system. Fluorophores in biological applications can be broadly divided into two main categories: intrinsic and extrinsic.

**Intrinsic fluorophores:** These are the ones that occur naturally and include aromatic amino acids, nicotinamide adenine dinucleotide (NADH), flavins, and derivatives of pyridoxal and chlorophyll. Intrinsic protein fluorescence arises from the aromatic amino acids tryptophan, tyrosine, and phenylalanine. Most intrinsic fluorophores require excitation by short wavelength ultraviolet and blue light which is often hazardous for live cells. Further, the brightness and quantum yield of intrinsic fluorophores is quite low for most practical applications.

**Extrinsic fluorophores:** Fluorescent molecules with desirable fluorescent properties such as absorption at longer wavelengths, higher quantum yield, and photostability, which are added to the sample to provide fluorescence. Such extrinsic fluorophores can be bioconjugated to the molecule of interest by various chemical strategies. However, the use of extrinsic fluorophores for in vivo studies presents experimental disadvantages. Bioconjugation with an exogenous fluorophore may lead to conformational changes and loss in activity of the molecule of interest. Example: fluorescein, rhodamine etc.

**Fluoresecent Dyes:**

Synthetic organic dyes were the first fluorochromes applied to biological research. Fluorescent dyes are commonly used for observing plant cells today, as they are convenient and easily applied to both living and chemically fixed tissues. The majority of fluorescent dyes are small (~ 0.5 nm) organic molecules that can passively enter the cell or be taken up via active transport. Due to their small size, common fluorescent dyes such as fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) can also be conjugated to other biomolecules such as antibodies, without interfering in their biological functions.

Several common fluorescent stains that are routinely used to facilitate microscopic observation of plant structures.

1. **Calcofluor white (Fluorescent Brightener 28)**: Primary cell walls are mainly composed of cellulose, hemicellulose, and pectin. Unlike the heavily lignified secondary walls of wood tissues which produce endogenous fluorescence, primary cell walls do not typically autofluoresce. For live-cell imaging of primary walls, calcofluor white (Fluorescent Brightener 28) is one of the most widely used fluorescent dyes.
2. **3,3′-dihexyloxacarbocyanine iodide (DiOC6)**: DiOC6 is a potential membrane-sensitive, cationic fluorescent dye. It is the dye’s hydrophilic groups that allow it to strongly accumulate in intracellular membranes where it absorbs blue light and emits a strong green fluorescence signal. DiOC6 stains both living and glutaraldehyde-fixed cells.
3. **Rhodamine 123 (R123)**: It is another potential membrane-sensitive, cationic green fluorescent dye that is readily sequestered by active mitochondria. As a result of electric potential across the mitochondrial membranes, R123 selectively accumulates in the mitochondria.
4. **Acridine orange**: One commonly used nuclear dye is acridine orange, a cationic dye that binds to both DNA and RNA via electrostatic attractions. When bound to double-stranded DNA, acridine orange has an excitation and emission maxima of 502 and 525 nm (green), respectively, but it has an excitation of 460 nm and emission of 650 nm (red) when bound to the phosphate groups of single-stranded DNA or RNA. Acridine orange is readily taken up by both living and fixed cells, and its detection of both RNA and DNA makes it ideal for cell cycle studies.

### Green Fluorescent protein:

For live-cell imaging, the expression of biological fluorochromes has become a valuable and powerful alternative to immunofluorescence. In 1994, green fluorescent protein (GFP) was cloned from jellyfish ( *Aequorea victoria*) and used as a fluorescent reporter. Biological fluorochromes can be introduced to a large variety of species and expressed alone or fused to a protein of interest allowing protein function to be investigated. A recent revolution in the use of fluorescence for studying biological systems came with the development and use of naturally fluorescent proteins as fluorescent probes.

 Green fluorescent protein (GFP) is an autofluorescent protein isolated from the bioluminescent jellyfish *Aequorea victoria*. Emission of blue light by aequorin protein leads to the excitation of its companion protein GFP, thereby resulting in the characteristic green fluorescence of the species. GFP is a ~27 kDa protein consisting of 238 amino acids. The fluorescent emission wavelength in the green portion of the visible spectrum is due to a chromophore formed from a maturation reaction of three specific amino acids at the center of the protein (Ser65, Tyr66, and Gly67). The chromophore forms spontaneously and without additional co-factors, substrates, or enzymatic activity – it only requires the presence of oxygen during maturation. This means that the protein could be taken directly from *A. Victoria* and expressed in any organism while still maintaining fluorescence. The fastest and most cost effective method to monitor GFP expression is by direct fluorescence microscopy.

GFP's main advantage over conventional fluorescent dyes of the time was the fact that it was non-toxic and could be expressed in living cells, enabling the study of dynamic, physiological processes.