جامعة المستقبل
AL MUSTAQBAL UNIVERSITY

كلية العلـــوم
قـســـــــم الادلة الجنائية

المحاضرة السابعة

principles of Fluorescence

المادة : تحليلية
المرحلة : الثانية
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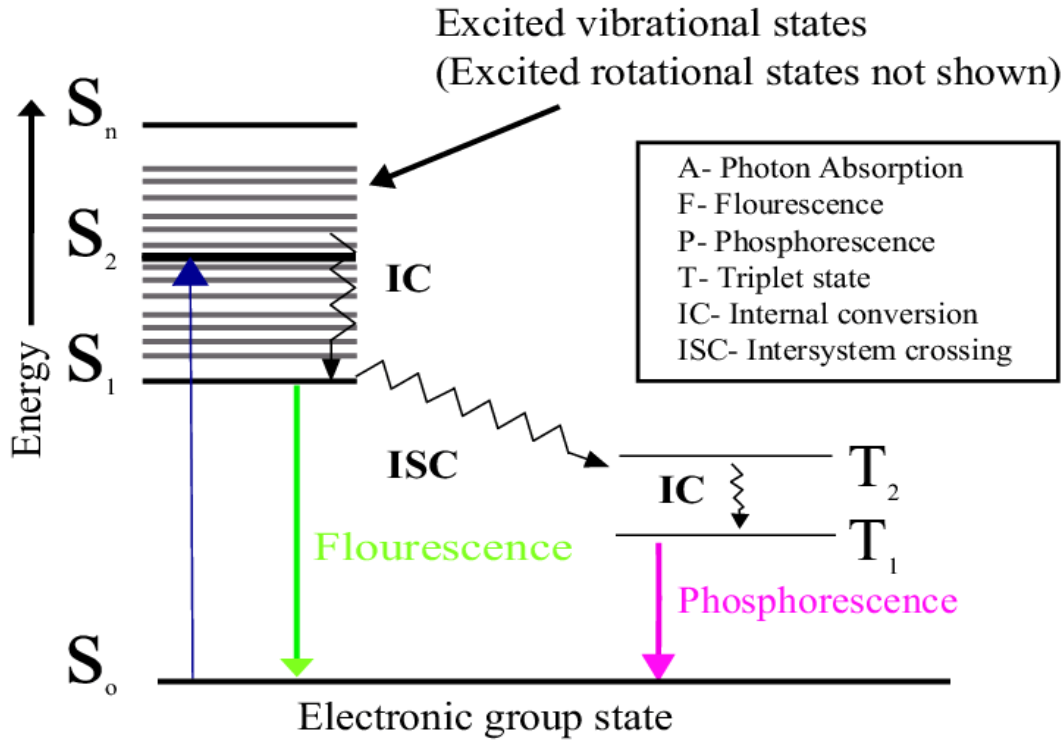
principles of Fluorescence

- ✓ Fluorescence is an emission phenomenon, the energy transition from a higher to a lower state within the molecule concerned being measured by the detection of this emitted radiation rather than the absorption.
- ✓ A molecule absorbs light at one wavelength and emits light at a longer wavelength.
- ✓ An atom or molecule that fluoresces is termed a fluorophore.
- ✓ Fluorometry is defined as the measurement of emitted fluorescent light.

Types of emission

- **Fluorescence** – return from excited singlet state to ground state; does not require a change in electron spin orientation (more common form of relaxation).
- **Phosphorescence** – return from a triplet excited state to a ground state; electron requires a change in its spin orientation.

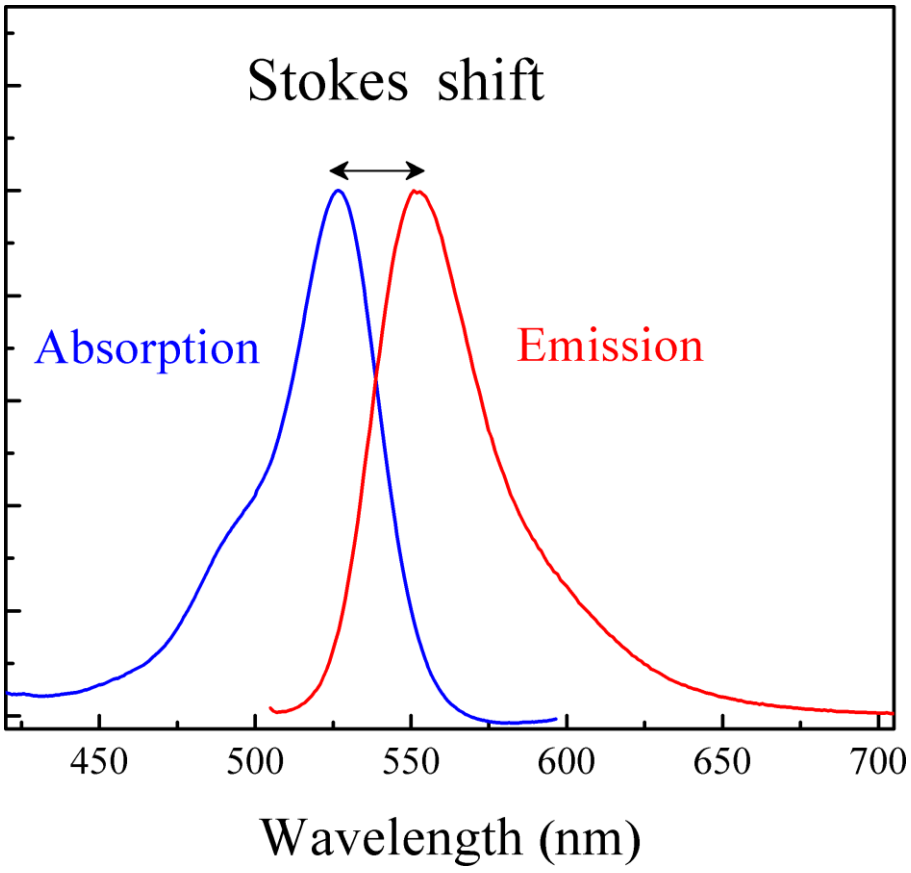


Internal conversion Vs Intersystem Crossing

Internal conversion is a transition occurring between states of the same multiplicity and it takes place at a time scale of 10^{-12} s (faster than that of fluorescence process).

Intersystem Crossing

- **Intersystem crossing** refers to a non-radiative transition between states of different multiplicity.
- It occurs via inversion of the spin of the excited electron, resulting in two unpaired electrons with the same spin orientation. This creates a state with Spin=1 and a multiplicity of 3 (triplet state).



- The difference between the maximum wavelength of the excitation light and the maximum wavelength of the emitted fluorescence light is a constant referred to as the **Stokes shift**.
- It is a measure of the energy lost during the lifetime of the excited state (through radiation-less vibrational deactivation) before returning to the ground singlet level (via fluorescence emission).
- The best results are obtained from compounds involving large shifts.

Theory

- Fluorescence spectroscopy is a **fast**, **simple**, **and inexpensive** method for determining the concentration of an analyte in a solution.
- It is used for the **quantitative analysis** of known compounds to find their concentration.
- Its main application is for measuring compounds **in solution**.

In fluorescence spectroscopy:

- A beam of light with a wavelength ranging between 180 and ~800 nm passes through a solution in a cuvette.
- The light emitted by the sample is then measured from an angle.

Measurements:

- **Excitation spectrum:** (the light absorbed by the sample).
- **And/or emission spectrum:** (the light emitted by the sample).

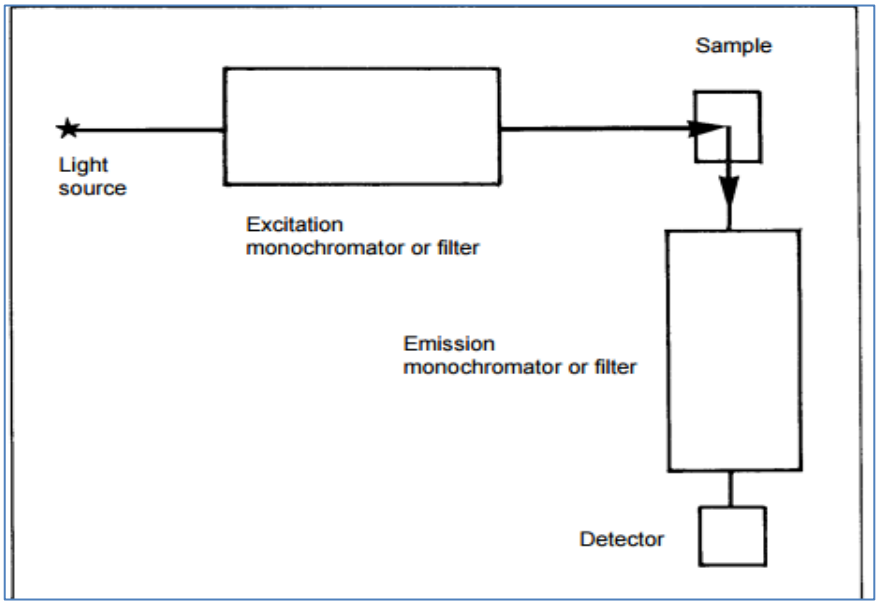
Key Principle:

- The concentration of the analyte is **directly proportional** to the intensity of the emission.

Factors influencing the emission spectrum:

The intensity and shape of the emission spectrum depend on:

- The excitation wavelength.
- The concentration of the analyte and the solvent.
- The path length of the cuvette.
- The self-absorption of the sample.

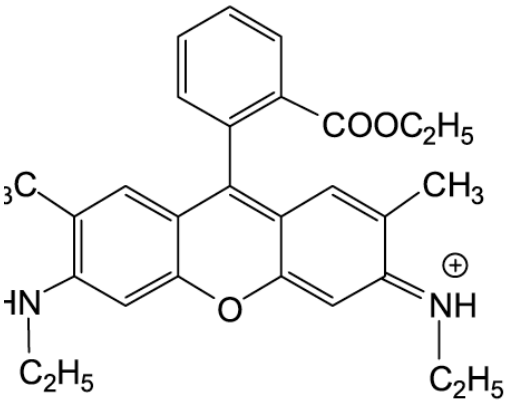


Mechanism of a Spectrofluorometer

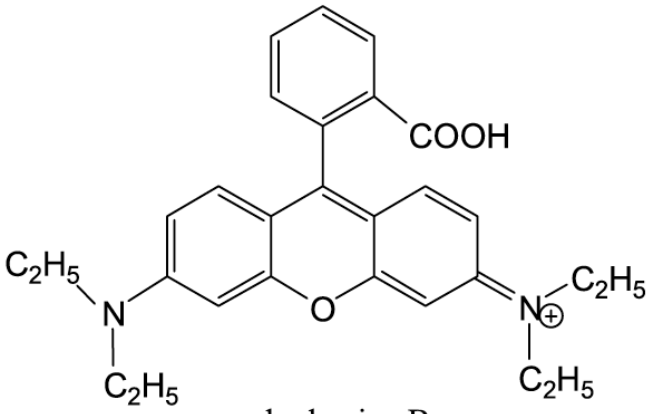
✓ The light from an excitation source passes through a filter or monochromator (1), and passes through the sample. Here some of it is absorbed, making some of the molecules in the sample fluoresce.

- ✓ A part of the fluorescent light is then focused on a filter or monochromator (2), which often is placed at a 90° angle to the excitation light.
- ✓ The light is then detected by a detection device.
- ✓ Various light sources may be used as excitation sources, such as: lasers, photodiodes, xenon arcs, and mercury-vapour lamps.

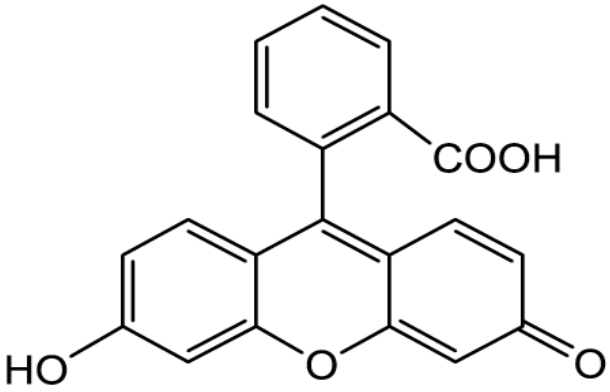
Common Fluorophores



rhodamine 6G



rhodamine B



fluorescein

Fluorescence – Concentration relationship :

- **The fluorescence intensity "F" is proportional to the radiant power of the excitation beam that absorbed by the fluorescent species :**

$$F = K (Po - P)$$

where K is constant dependant on quantum efficiency

- **From Beer;s law**

$$A = \log Po/P = abc$$

$$P/Po = 10^{-abc}$$

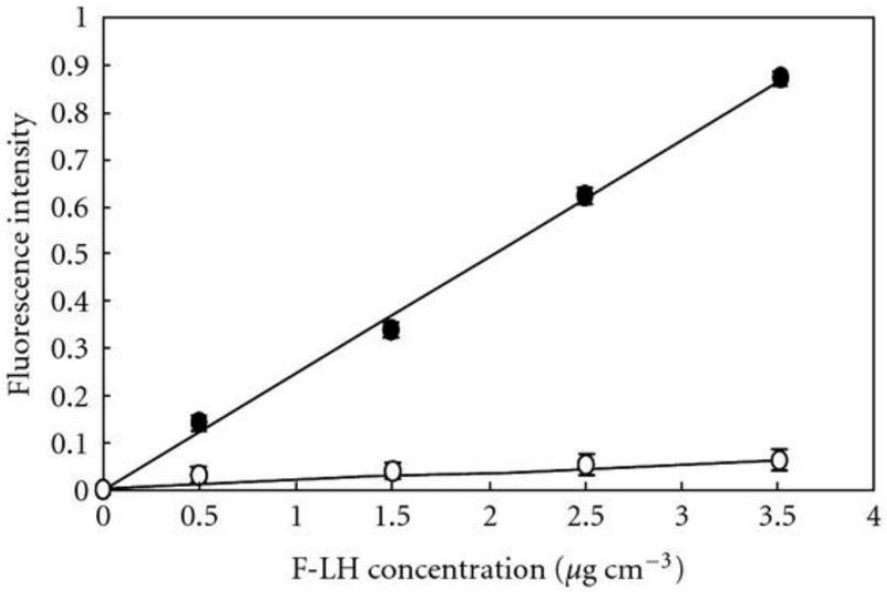
$$F = k\,Po\,(1 - 10^{-abc})$$

$$F = 2.3\,K\,abc\,Po \quad (\text{at dilute solution , } A < 0.05)$$

- **At constant Po**

$$F = KC$$

- **Plot of fluorescence intensity F of a solution versus concentration of the emitted species , C , should be linear (at low concentration , where A ‹ 0.05)**



Factors that influence fluorescence measurements include:

- **Concentration effects**
  - Inner filter effects, concentration quenching
- **Background effects**
  - due to Rayleigh and Raman scattering
- **Solvent effects**
  - Interfering nonspecific fluorescence, quenching from the solvent
- **Sample effects**
  - Light scattering, interfering fluorescence, sample adsorption
- **Temperature effects**
- **Photodecomposition (bleaching) of the sample.**

Advantage of fluorescence spectroscopy

Sensitivity:

- It is more sensitive when the concentration is low, such as µg/ml or ng/ml.

Precision:

- Accuracy of up to 1% can be achieved.

Specificity:

- More specific than the absorption method, where two compounds may have the same absorption maxima.

Range Of Application:

  - Even non-fluorescent compounds can be converted into fluorescent compounds using chemical agents.

Disadvantages:

1. Not useful for identification.
2. Not all compounds fluoresce.
3. Contamination can quench the fluorescence and hence give false or no results.

Applications

Analytical Chemistry:

- To detect compounds from HPLC flow.
- TLC plates can be visualized if the compounds or a coloring reagent is fluorescent.
- Plant pigments, steroids, proteins, naphthols, etc., can be determined at low concentrations.

Biochemistry:

- Generally used as a non-destructive way of tracking or analyzing biological molecules (e.g., proteins).
- Enables direct or indirect analysis of aromatic amino acids (phenylalanine, tyrosine, tryptophan).

- Fingerprints can be visualized with fluorescent compounds such as ninhydrin.

Medicine

- Blood and other substances are sometimes detected using fluorescent reagents, particularly when their location is not previously known.
- There has also been a report of its use in differentiating malignant skin tumors from benign ones.

Pharmacy

Possible direct or indirect analysis of drugs such as:

- **Vitamins:** Vitamin A, Vitamin B2, Vitamin B6, Vitamin B12, Vitamin E, Folic Acid.
- **Catecholamines:** Dopamine, Norepinephrine.
- **Other Drugs:** Quinine, Salicylic Acid, Morphine, Barbiturates, Lysergic Acid Diethylamide (LSD).
- To measure the amount of impurities present in a sample.

Fluorescence vs. Phosphorescence

Property	Fluorescence	Phosphorescence
Electronic transition	S1 → S0	T1 → S0
Spin state	Singlet → Singlet	Triplet → Singlet
Lifetime	10^{-9} – 10^{-7} s (nanoseconds)	10^{-3} s to hours
Quantum rule	Allowed transition	Spin-forbidden transition
Afterglow	Stops immediately when excitation ends	Continues glowing after excitation
Energy	Higher	Lower
Wavelength	Shorter	Longer
Effect of oxygen	Low quenching	Strongly quenched
Common examples	Fluorescent dyes, GFP	Glow-in-the-dark materials