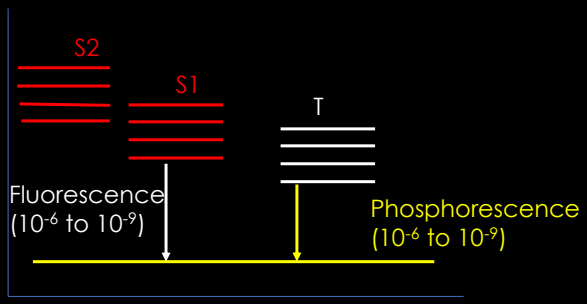
FLOURIMETRY

(Part- 01)

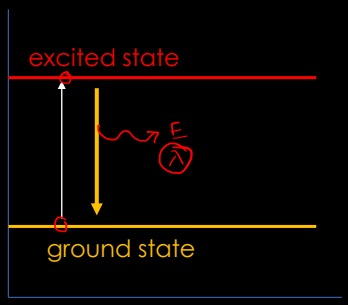
- ✓ Basic Introduction
- ✓ Theory
- ✓ Principle



Spectroscopy
Instrumental Analysis

FLUORIMETRY

- When a molecule absorbs UV-Visible radiation, transit ground state to singlet excited state. The excited state is unstable and deactivation occurs due to:
  a) Internal conversion and internal collisions
  b) External conversion
  c) Re-emission as light (luminescence)
- **Luminescence:** It is a phenomenon of emission (Fluorescence and Phosphorescence) of radiation when the molecules are excited by radiation at certain wavelength and due to above conditions, the molecule emit radiation; when electron undergoes excited state to ground state



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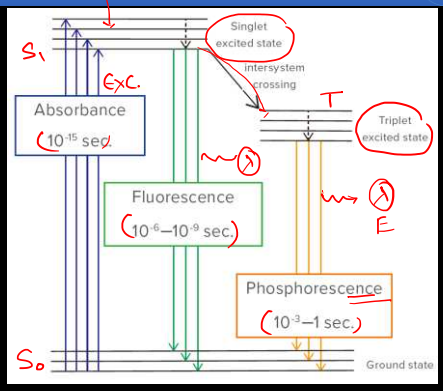
- **Phosphorescence:** It is measurement of emitted radiation; when electron undergoes excited "triplet" state to "ground state". Delayed emission, S2→S1→T→S0
- **Fluorescence:** It is a phenomenon of emission of radiation; when electron undergoes excited "Singlet" state to ground state. Immediate emission, S1→S0
- **Fluorimetry:** It is measurement of fluorescence intensity (emitted radiation; when electron undergoes excited singlet state to ground state) at a particular wavelength with the help of a filter fluorimeter or a spectrofluorometer



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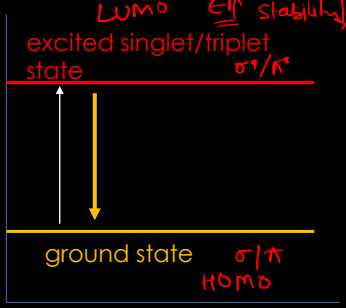
Basic Principle & Theory

- When the molecules absorb radiant energy from a light source, the bonding electrons (HOMO) may be promoted to anti bonding molecular orbital (LUMO). It has more energy and hence less stable.
- The process of promotion of electrons from HOMO to LUMO with absorption of energy is called as excitation.



- **Electronic States:**
  - **Singlet ground state: (↓↑):** All the π electrons in a paired
  - **Doublet State (↑ or ↓):** unpaired π electrons e.g., free radical
  - **Excited Triplet State (↑ ↑):** unpaired π electrons of the same spin
  - **Excited Singlet State (↑ ↓):** unpaired π electrons of the opposite spin

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- When light of appropriate wavelength is absorbed by a molecule the electrons are promoted from singlet ground state to singlet excited state. Once the molecule is in this excited state, relaxation can occur via several processes by emission of radiation. The processes can be the following
- Conversion/Collisional deactivation
- Fluorescence
- Phosphorescence

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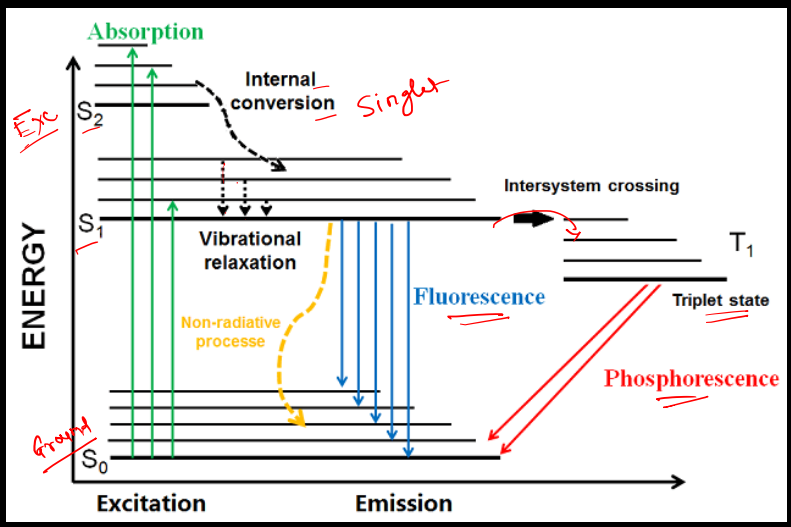
- **Energy level/ JOBLONSKI DIAGRAME:**



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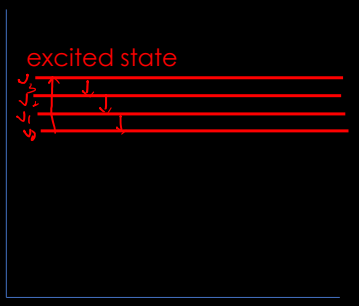
- **Collisional deactivation/Vibrational Relaxation:** In which entire energy lost due to collision de activation and no radiation emitted.
- During the excitation, electron promotes to several vibrational level. The extra energy is lost by collision. The collision of molecules with the excited species and solvent leads to rapid energy transfer (slightly increase the temp.). Vibrational relaxation is so rapid that the life time of a vibrational excited molecule (< 10^{-12} Sec) is less than the lifetime of electronically excited state



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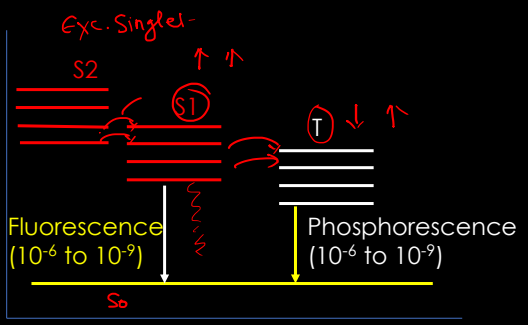
- **Internal conversion**: Intermolecular process by which a molecule passes to a lower energy electronic state without emission of light. Overlap of vibrational energy levels in two electronic energy levels.
- **External conversion**: Deactivation of an excited electronic state by interaction and energy transfer between the excited molecule and solvent or other solutes.
- **Intersystem crossing:** Process in which spin of an excited electron is reversed and change in multiplicity results. Most common when vibrational manifold overlap exists and when the molecule has a heavy atom substituen



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- **Difference between Energy Excited-Ground**:

  ΔE (Absorption) > ΔE (Fluorescence) > ΔE (Phosphorescence)

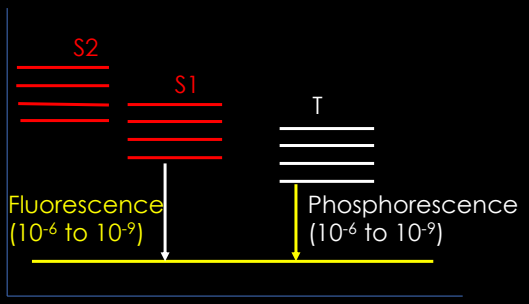
  λ (Absorption) < λ (Fluorescence) < λ (Phosphorescence)

  Ex. Anthracene- λ (Abs; 255 nm < λ (Fl.; 425) < λ (Ph.; 680 nm)

$E = h\nu$

$E = \frac{hc}{\lambda}$

$E \propto \frac{1}{\lambda}$



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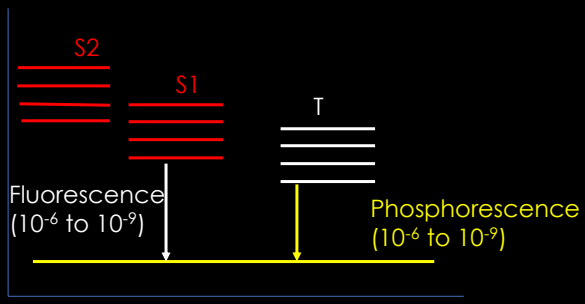
Factors affecting Fluorescence

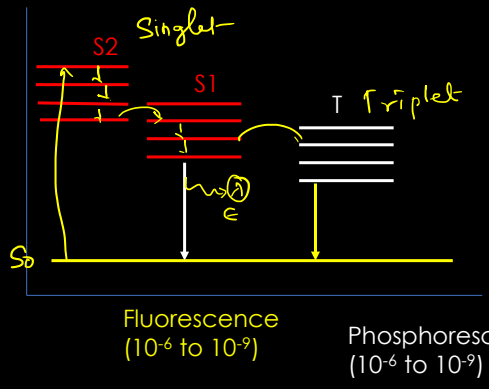
(Part- 02)



Spectroscopy
Instrumental Analysis

FLUORIMETRY

- **FACTORS AFFACTING FLUORESENCE INTENSITY**
- Concentration
- Quantum yield of fluorescence
- Intensity of incident light
- Adsorption
- Oxygen
- Ph
- Temperature& viscosity
- Photodecomposition
- Scatter
- Substituents
- Quenchers



FLUORIMETRY

FACTORS AFFACTING FLUORESENCE INTENSITY

Fluorescence Intensity (F) = $(I_0-I_t)\ \Phi$

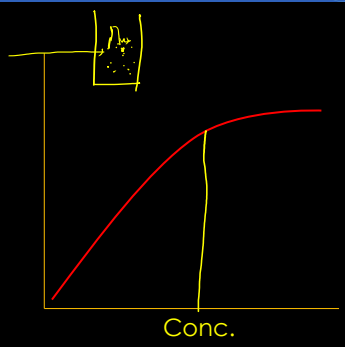
Where Φ = quantum yield of fluorescence (fluorescence Efficiency)

On the mathematical calculation equation for fluorescence becpmes

$$F = 2.3\ I_0\ abc\ \Phi$$

1. Concentration

- As per the equation, Fluorescence intensity is proportional to concentration of substance only when the absorbance is less than 0.02
- Number of molecules absorb more radiation and emit more radiation, but it is true at low conc. (~~0.02~~M).
- At high concentration deviation from linearity occurs due to self quenching or conc quenching. Because emitted radiation before falling to detector, is absorbed and re-emitted by adjacent molecules, which leads to internal circulation of radiation or energy.



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FACTORS AFFACTING FLUORESENCE INTENSITY

2. Quantum yield of fluorescence or Quantum Efficiency (ϕ) :

Ab > emitted
ϕ < 1

- (ϕ) = number of photons emitted/number of photons absorbed
- It is always less than 1.0 since some energy is lost by radiation less pathways (Collisional, Intersystem Crossing, Vibrational Relaxation).
- Highly fluorescent substance have ϕ near to 1
- E.g., 0.1 M NaOH (ϕ = 0.85) and Quinine in 0.05M H_2SO_4 (ϕ = 0.54) at 23 C

3. Intensity of incident light:

- Increase in the intensity of incident light (I_0) on the sample fluorescence intensity also increases.
- I_0 depends on a) Intensity of source, b) property of excitation monochromator and c) Excitation slit width.

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FACTORS AFFACTING FLUORESENCE INTENSITY

4. Adsorption

- Adsorption of sample solution in the container/ sampling tube may leads to a serious problem.
- Fluorometry methods are very sensitive so use 10-100 times more diluted solution than absorption spectroscopy.
- E.g., Quinine may adsorbed on the wall of cell

5. Oxygen

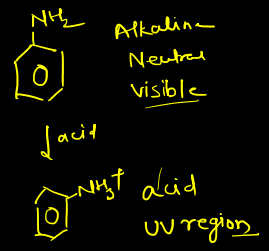
- Presence of oxygen may decrease the fluorescent intensity in two ways a) Oxidation of fluorescent species to a non-fluorescent species and b) quenches fluorescent substance.
- E.g., Anthracene is susceptible for oxidation

FLUORIMETRY

FACTORS AFFACTING FLUORESENCE INTENSITY

6. pH:

- Alteration of pH of a solution will have significant effect on fluorescence. E.g., Aniline in alkali medium (pH 12) and neutral media (pH 7) gives visible fluorescence but in acidic media (pH < 5) gives fluorescence in UV region due to protonation (Anilinium ion)



7. Temperature & viscosity

- Temperature increases can increase the collisional deactivation, and reduce fluorescent intensity.
- If viscosity of solution is more the frequency of collisions are reduced and increase in fluorescent intensity.

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FACTORS AFFACTING FLUORESENCE INTENSITY

8. Scatter

- Scatter is mainly due to colloidal particles in solution.
- Scattering of incident light after passing through the sample leads to decrease in fluorescence intensity.

9. Photodecomposition

- Absorption of intense radiation leads to photochemical decomposition of a fluorescent substance to less fluorescent or non-fluorescent substance

FLUORIMETRY

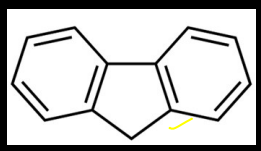
FACTORS AFFACTING FLUORESENCE INTENSITY

10. Substituents

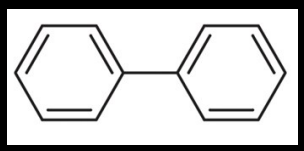
- Electron donating group like –NH2, -OH groups enhance the fluorescence intensity while electron withdrawing groups like –NO2, -COOH reduce the fluorescence intensity.

11. Structure Rigidity

- The molecular rigidity results in an increase in fluorescence



Fluorene (ϕ = 1)



Biphenyl (ϕ = 0.2)

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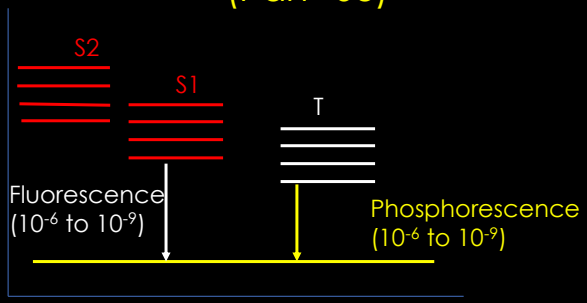
QUENCHING

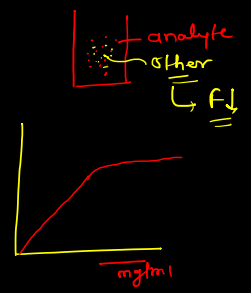
(Part- 03)



Spectroscopy
Instrumental Analysis

FLUORIMETRY

Quenching



- Quenching is the reduction of fluorescence intensity by the presence of substance in the sample other than the fluorescent analyte.
- **Quenching is following types:**

A. Self-quenching or Concentration quenching:

- At low concentration (<0.02M; ug/ml or ng/ml) linearity is observed, at high concentration (mg/ml) of the same substance, proporsnate increase in fluorescent intensity does not occur. This phenomenon is called self-quenching.
- Because emitted radiation before falling to detector, is absorbed and re-emitted by adjacent molecules, which leads to internal circulation of radiation or energy.

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FACTORS AFFACTING FLUORESENCE INTENSITY

B. Inner fluorescent effect:

- Absorption of Incident (UV) light or emitted (fluorescent) light by primary and secondary filters leads to decrease in fluorescence intensity.

C. Collisional quenching:

- Reduce the fluorescence intensity due to increased number of collisions.
- Presence of halide, heavy metals, and increased temp and reduced viscosity may affect.
- E.g., Quinine is highly fluorescent in 0.05M H_2SO_4 while non fluorescent in 0.1N HCl due to presence of halide ion.

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FACTORS AFFACTING FLUORESENCE INTENSITY

D. Static quenching:

- This occurs because of complex formation between the fluorescent molecule and other molecules. Ex: caffeine reduces fluorescence of riboflavin.

E. Chemical quenching:

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- ***Oxygen:*** Presence of oxygen may decrease the fluorescent intensity in two ways a) Oxidation of fluorescent species to a non-fluorescent species and b) quenches fluorescent substance.
- ***Halide/Heavy Metals:*** *Collisional quenching*

FLUORIMETRY

FACTORS AFFACTING FLUORESENCE INTENSITY

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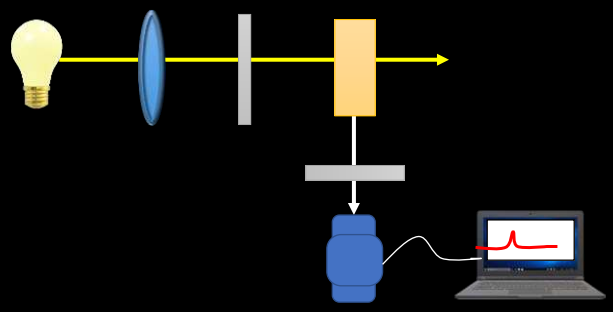
FLOURIMETRY

(Part- 03)

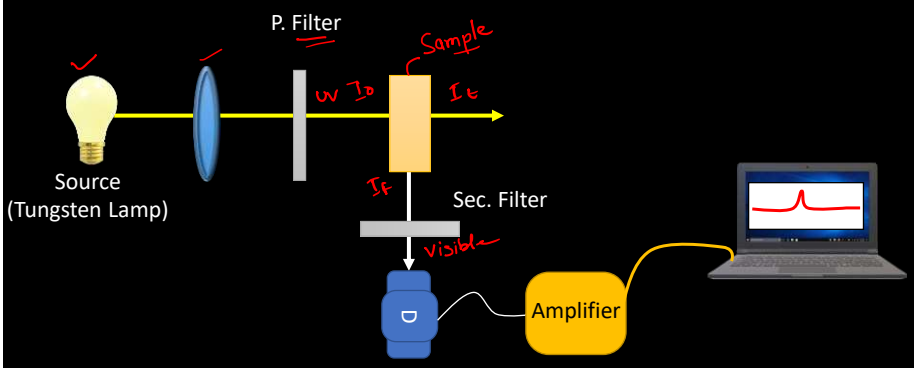
Instrumentation & Applications



Spectroscopy
Instrumental Analysis

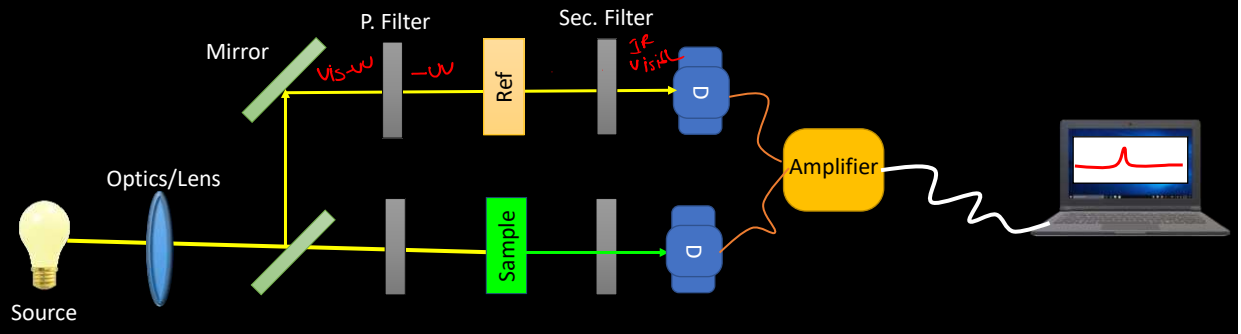
FLUORIMETRY

SINGLE BEAM FILTER FLUORIMETER



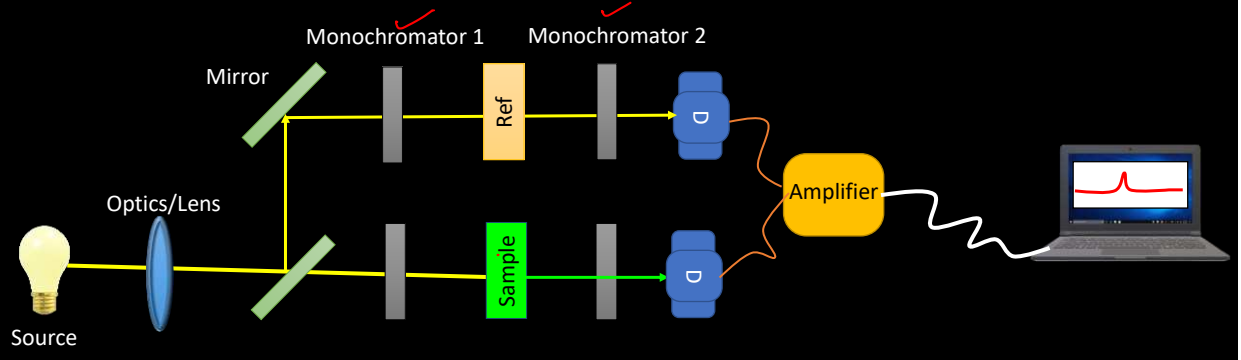
FLUORIMETRY

DOUBLE BEAM FILTER FLUORIMETER



FLUORIMETRY

DOUBLE BEAM SPECTRO-FLUORIMETER



FLUORIMETRY

A. Source of light:

- ***Mercury vapor lamp:*** Mercury vapor at high pressure give intense lines on continuous background above 350 nm. low pressure mercury vapor gives an additional line at 254nm. it is used in filter fluorimeter.
- ***Xenon arc lamp:*** It give more intense radiation than mercury vapor lamp. it is used in spectrofluorimeter.
- ***Tungsten lamp:*** If excitation has to be done in visible region this can be used. It is used in low cost instruments like Single beam filter fluorimeter.

B. Filters and Monochromators:

- ***Filters:*** These are nothing but optical filters work on the principle of absorption of unwanted light and transmitting the required wavelength of light. In inexpensive instruments fluorimeter primary filter and secondary filter are present.

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B. Filters and Monochromators:

- ***Primary filter:*** Absorbs visible radiation and transmit UV radiation.
- ***Secondary filter:*** Absorbs UV radiation and transmit visible radiation.
- **Monochromators:** They convert polychromatic light into monochromatic light. They can isolate a specific range of wavelength or a particular wavelength of radiation from a source.
- *Excitation monochromators:* Provides suitable radiation for excitation of molecule.
- *Emission monochromators*: Isolate only the radiation emitted by the fluorescent molecules.

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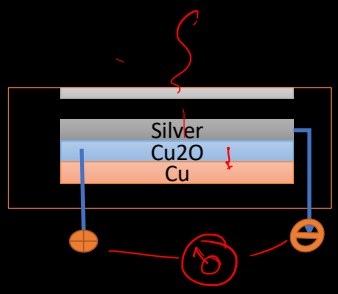
C. Sample cells:

- These are meant for holding liquid samples. These are made up of quartz and can have various shapes ex: cylindrical or rectangular etc

D. Detectors:

- ***Barrier layer /photovoltaic cell***
- It is employed in inexpensive instruments. For ex: Filter Fluorimeter.
- It consists of a copper plate coated with a thin layer of cuprous oxide (Cu_2O). A semi- transparent film of silver is laid on this plate to provide good contact
- When external light falls on the oxide layer, the electrons emitted from the oxide layer move into the copper plate.
- Then oxide layer becomes positive and copper plate becomes negative



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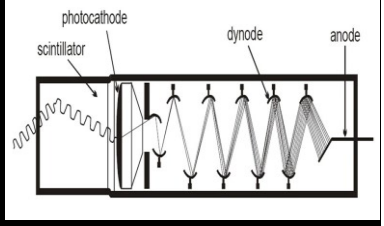
D. Detectors:

2. Photomultiplier tubes (PMT):

- These are incorporated in expensive instruments like spectrofluorimeter. Its sensitivity is high due to measuring weak intensity of light.
- The principle employed in this detector is that, multiplication of photoelectrons by secondary emission of electrons.
- This is achieved by using a photo cathode and a series of anodes (Dyanodes). Up to 10 dyanodes are used. Each dyanode is maintained at 75 - 100V higher than the preceding one.
- At each stage, the electron emission is multiplied by a factor of 4 to 5 due to secondary emission of electrons and hence an overall factor of 106 is achieved.
- PMT can detect very weak signals, even 200 times weaker than that could be done using photovoltaic cell. Hence it is useful in fluorescence measurements



FLUORIMETRY

APPLICATIONS

- Fluorimetric methods are not useful in qualitative analysis and much used in quantitative analysis.
- Determination of inorganic substances. Al^{3+}, Li^{+}, Zn^{2+}
- Determination of thiamine HCl.
- Determination of phenytoin.
- Determination of indoles, phenols, & phenothiazines
- Determination of napthols, proteins, plant pigments and steroids.
- Fluorimetry, nowadays can be used in detection of impurities in nanogram level better than absorbance spectrophotometer with special emphasis in determining components of sample at the end of chromatographic or capillary column.

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APPLICATIONS

- Determination of ruthenium ions in presence of other platinum metals.
- Determination of boron in steel, aluminum in alloys, manganese in steel.
- Estimation of cadmium with 2-(2 hydroxyphenyl) benzoxazole in presence of tartarate.

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