FLOURIMETRY

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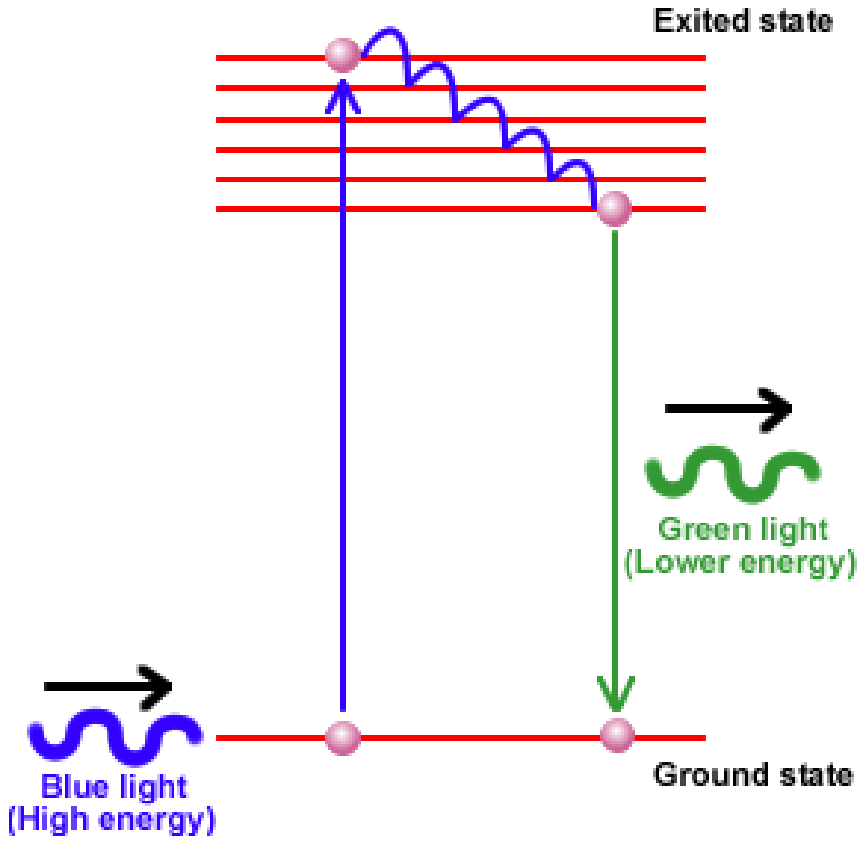
FLOURIMETRY



CONTENTS

INTRODUCTION

Luminescence is the emission of light by a substance. It occurs when an electron returns to the electronic ground state from an excited state and loses its excess energy as a photon.

It is of 3 types.

- **Fluorescence spectroscopy.**
- **Phosphorescence spectroscopy.**
- **Chemiluminescence spectroscopy**

FLUORESCENCE

When a beam of light is incident on certain substances they emit visible light or radiations. This is known as fluorescence.

Fluorescence starts immediately after the absorption of light and stops as soon as the incident light is cut off.

The substances showing this phenomenon are known as flourescent substances.

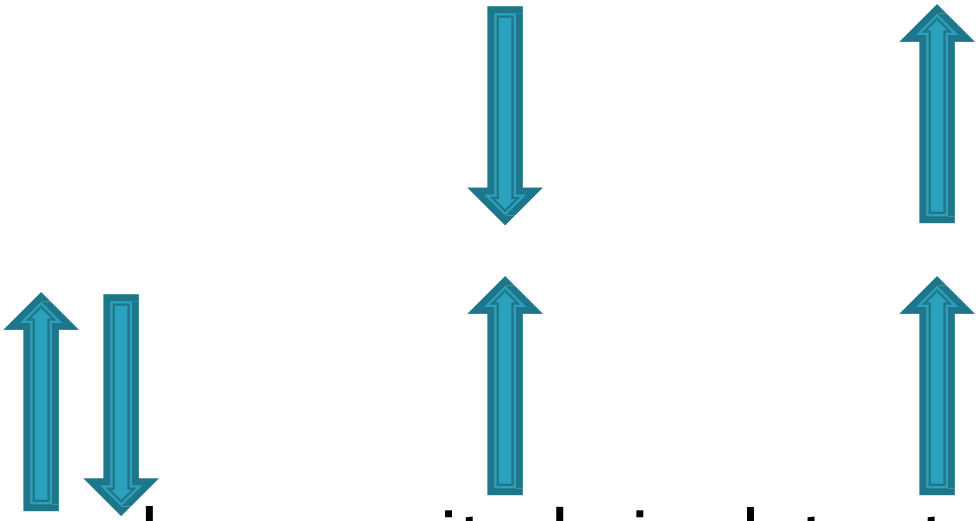
PHOSPHORESCENCE

- **When light radiation is incident on certain substances they emit light continuously even after the incident light is cut off.**
- **This type of delayed fluorescence is called phosphorescence.**
- **Substances showing phosphorescence are phosphorescent substances.**

THEORY OF FLOURESCENCE AND PHOSPHORESCENCE

- A molecular electronic state in which all of the electrons are paired are called singlet state.
- In a singlet state molecules are diamagnetic.
- Most of the molecules in their ground state are paired.
- When such a molecule absorbs uv/visible radiation, one or more of the paired electron raised to an excited singlet state /excited triplet state.

SINGLET/TRIPLET STATE



Ground singlet states

excited singlet state

spin paired

no net mag.field

triplet state

spins unpaired

net mag.field

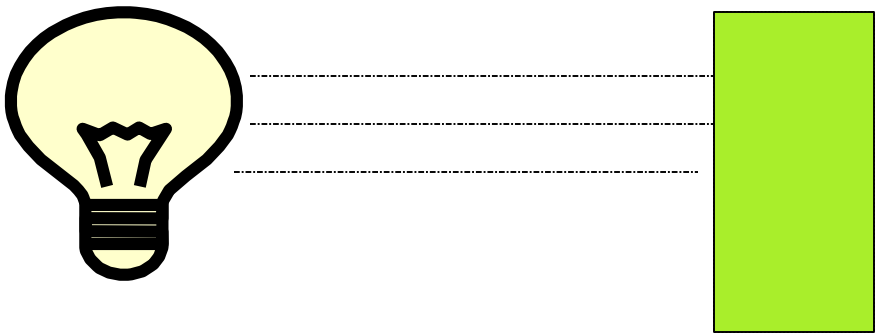
From the excited singlet state one of the following phenomenon occurs

- Fluorescence
- Phosphorescence
- Radiation less processes

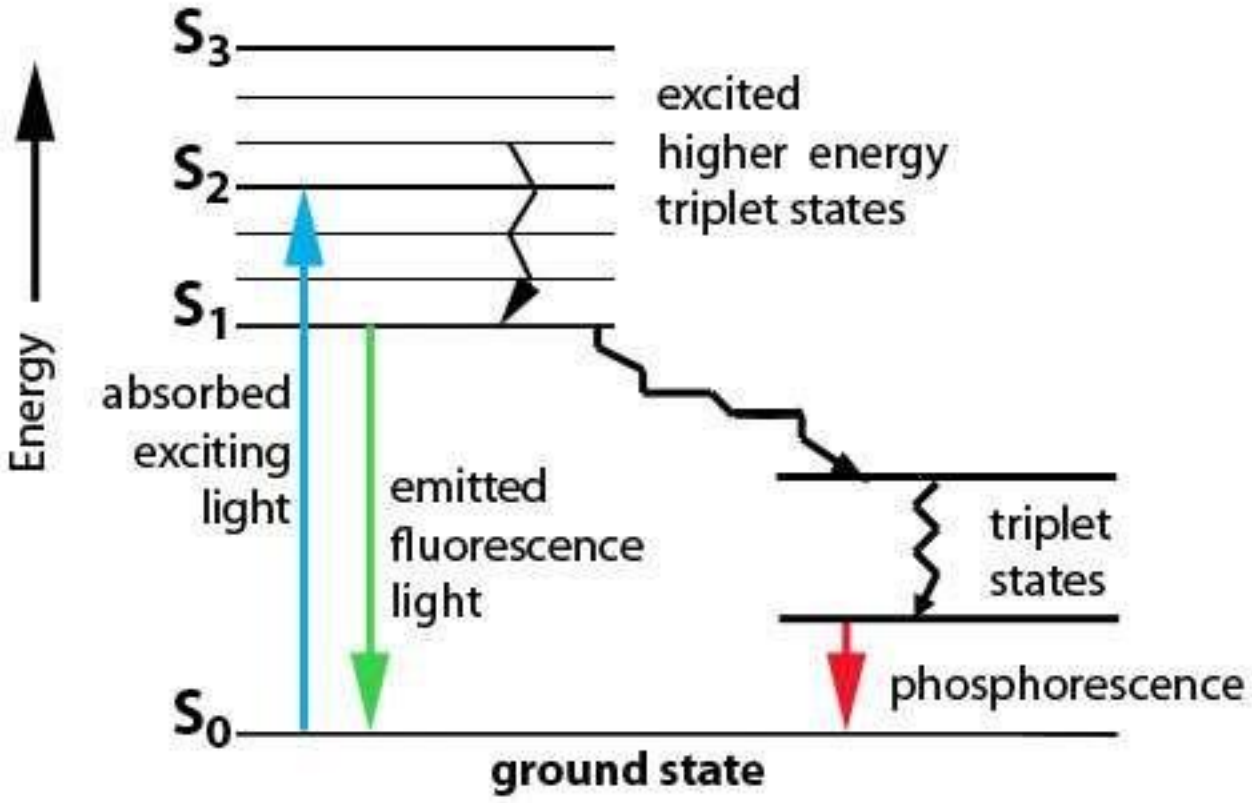
  - Vibration relaxation
  - Internal conversion
  - External conversion
  - Intersystem crossing

FLUORESCENCE

LIGHT EMITING AT ONCE SOURCE STARTS & STOPS WHEM SOURCE STOPS



FLOURESCENCE &PHOSPHORESCENCE



- **PRINCIPLE:-** Molecule contains σelectrons,Π electrons and non bonding (n) electron.
- The electrons may be present in bonding molecular orbital. It is called as highest occupied molecular orbital (HOMO).It has lest energy and more stable.
- When the molecules absorbs radiant energy from a light source, the bonding electrons 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.
- **Singlet state**:-a state in which all the electrons in a molecule are paired ↓↑
- **Doublet state**:- a state in which un paired electrons is present ↓ or ↑
- **Triplet state**:- a state in which unpaired electrons of same spin present↑ ↑
- **Singlet excited state**:- a state in which electrons are unpaired but of opposite spin like↑ ↓ (un paired and opposite spin)

- 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 process. For ex by emission of radiation . The process can be the following
- 1) Collisional deactivation
- 2)Fluorescence
- 3)Phosphorescence.

- **Collisional de activation** :- In which entire energy lost due to collision de activation and no radiation emitted.
- **Fluorescence:**-excited singlet state is highly unstable. Relaxation of electrons from excited singlet to singlet ground state with emission of light.
- **Phosphorescence:**-At favorable condition like low temperature and absence of oxygen there is transition from excited singlet state to triplet state which is called as inner system crossing. The emission of radiation when electrons undergo transition from triplet state to singlet ground state is called as phosphorescence.

- The basis of fluorimetry is the measurement of fluorescence. Drugs which are intrinsically fluorescent, are determined fluorimetrically.

e.g. Quinine sulfate in 0.1 N sulfuric acid; Ergometrine in 1% tartaric acid etc.

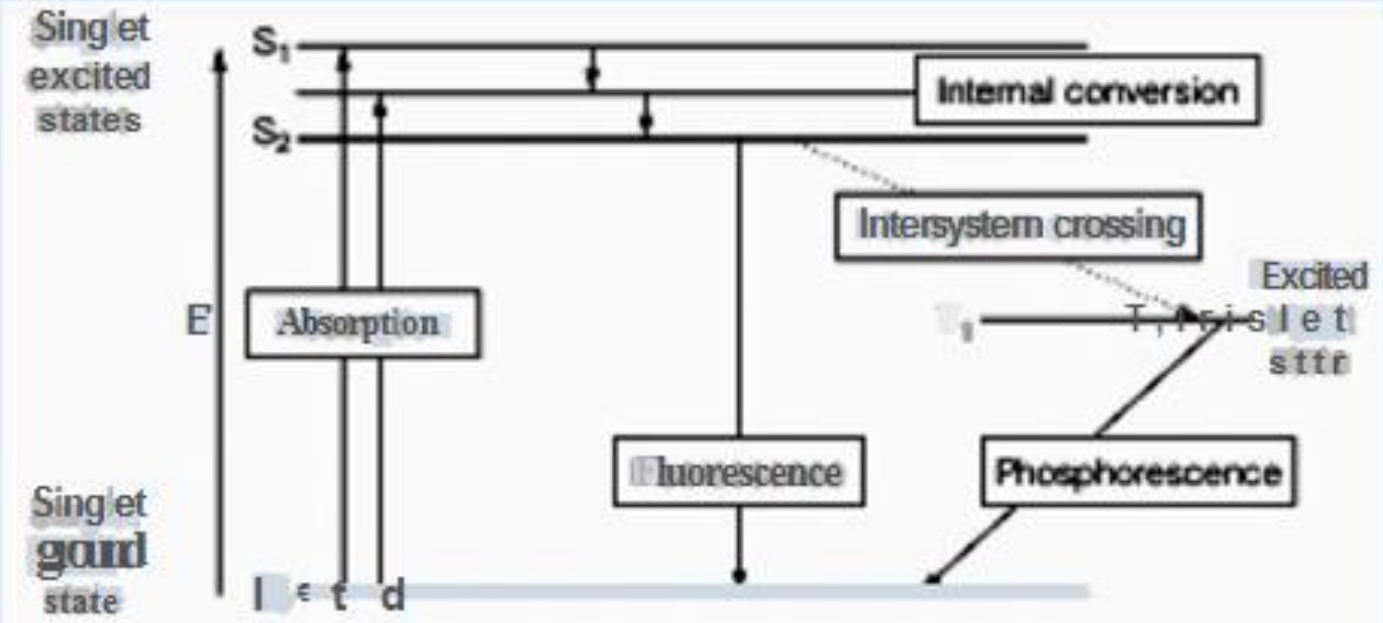


Fig.2. Molecular energy levels associated with fluorescence and phoenphorescence.

JABLONSKI ENERGY DIAGRAM

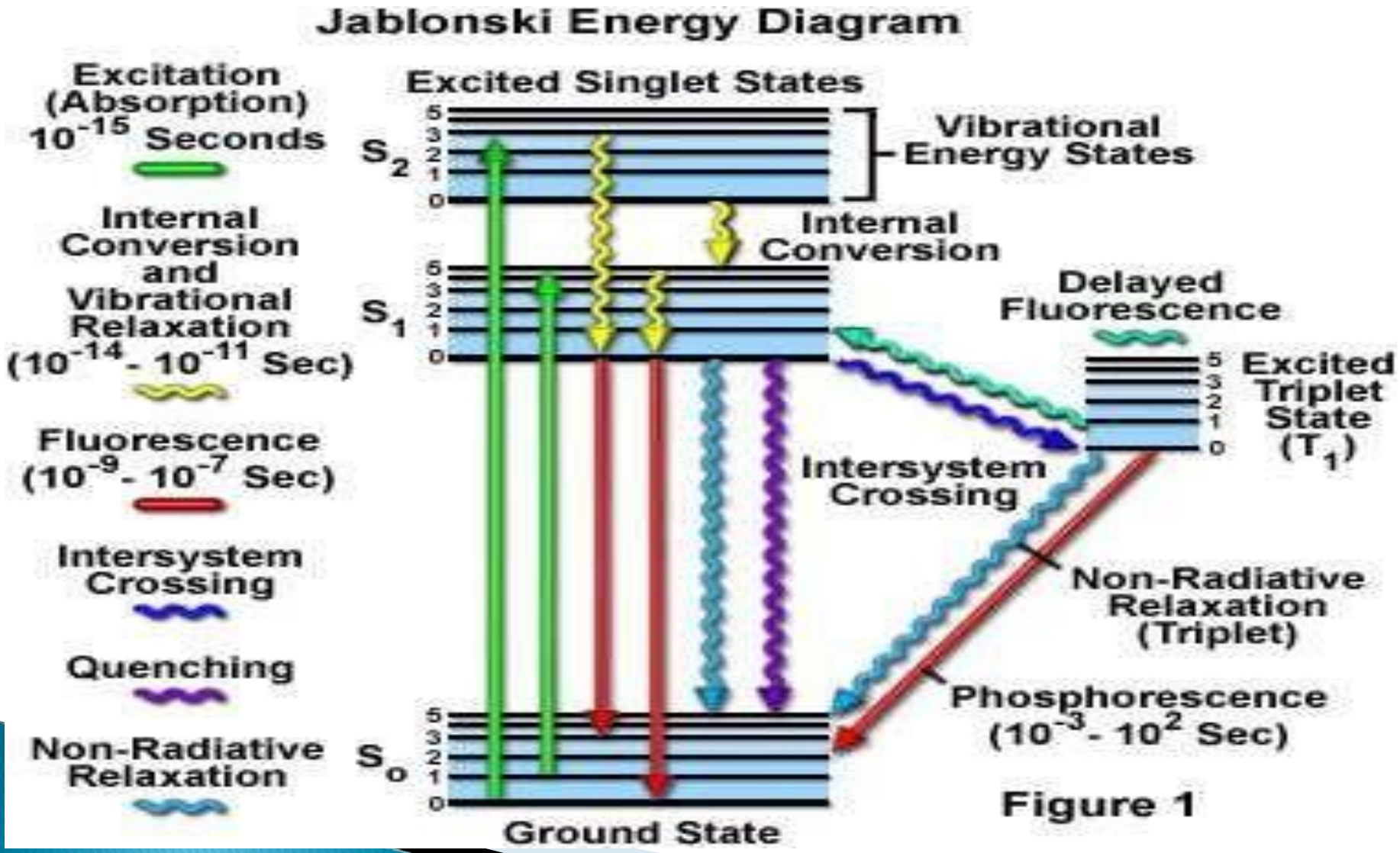


Figure 1

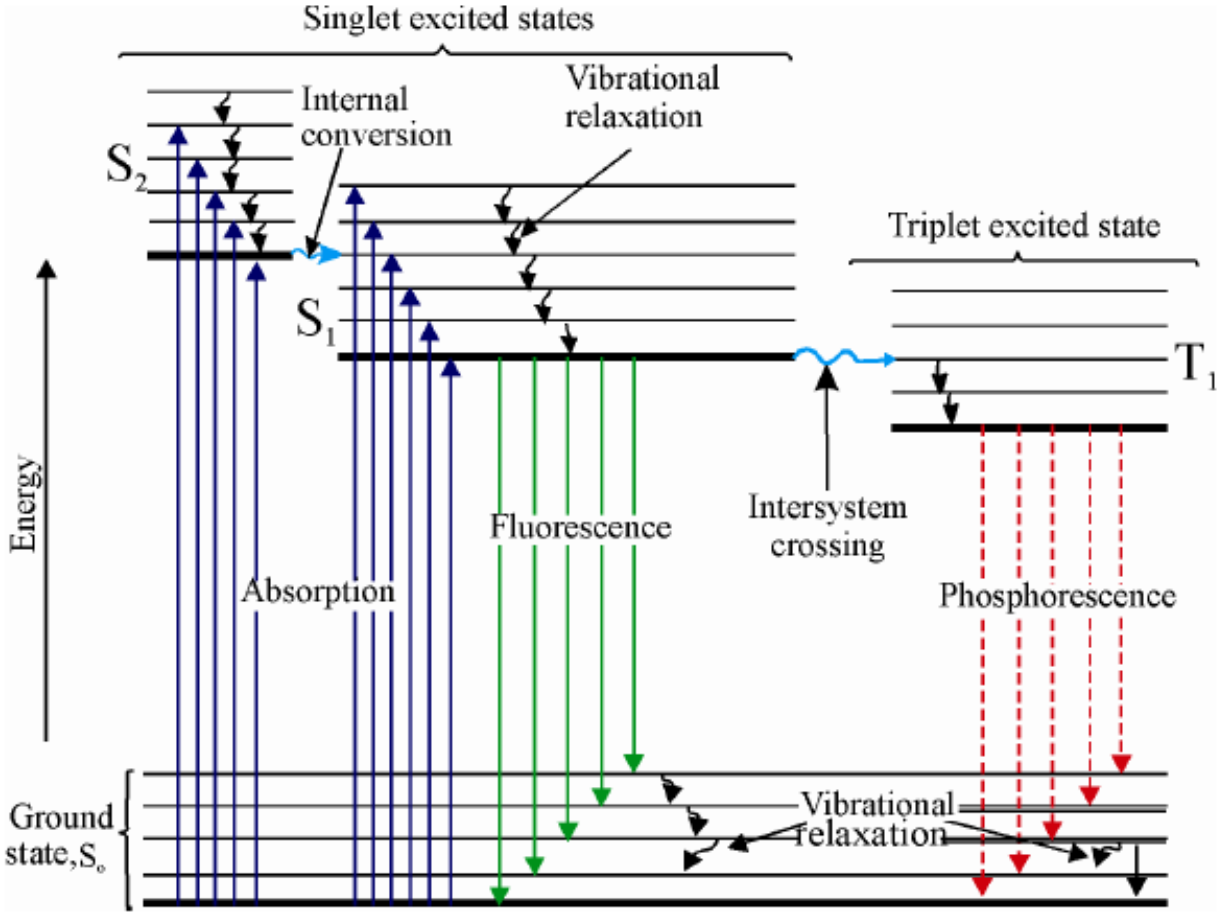
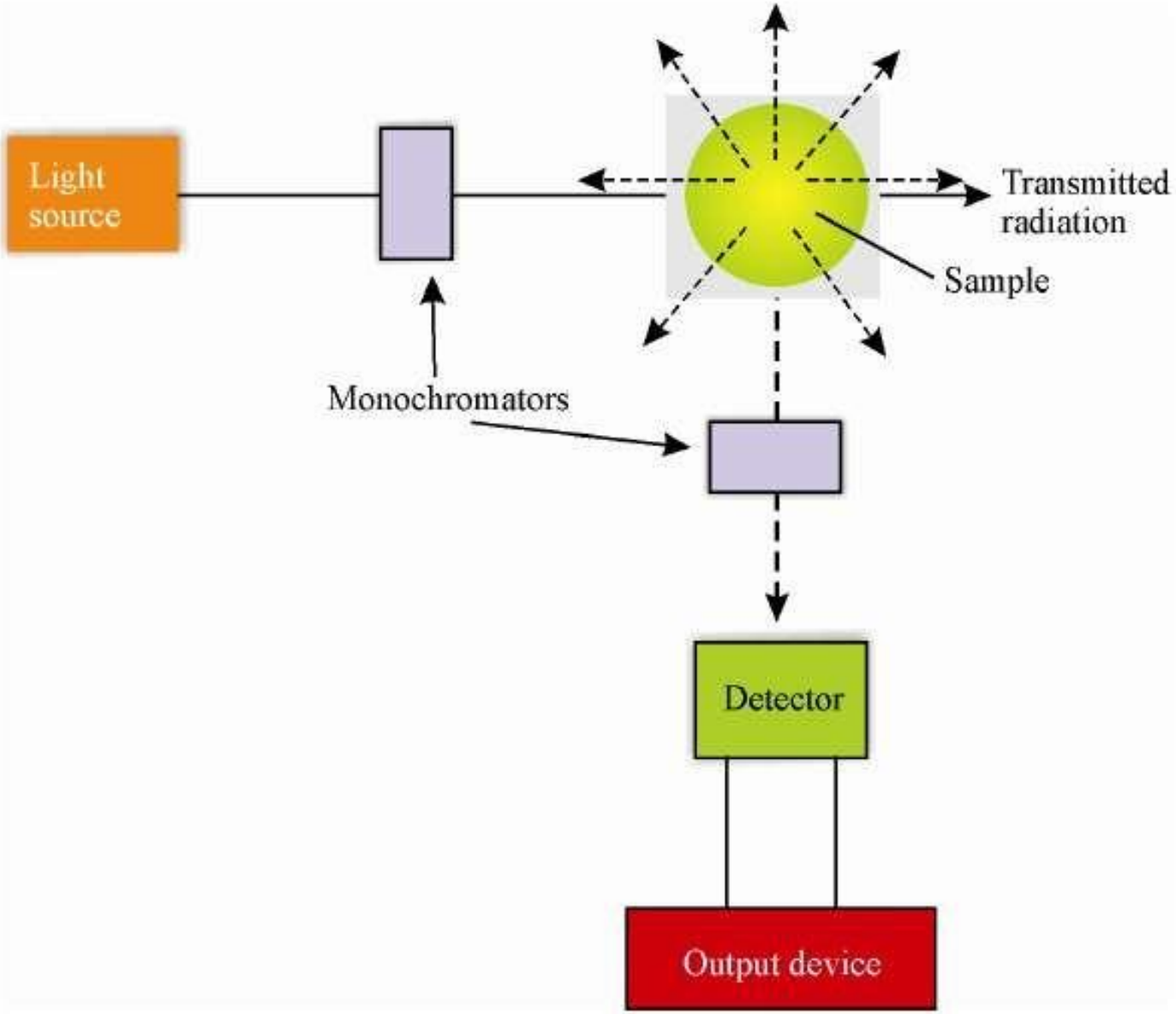


Fig. 5.1: The Jablonski diagram showing the phenomena of fluorescence and phosphorescence


Light source
Transmitted radiation
Sample
Monochromators
Detector
Output device

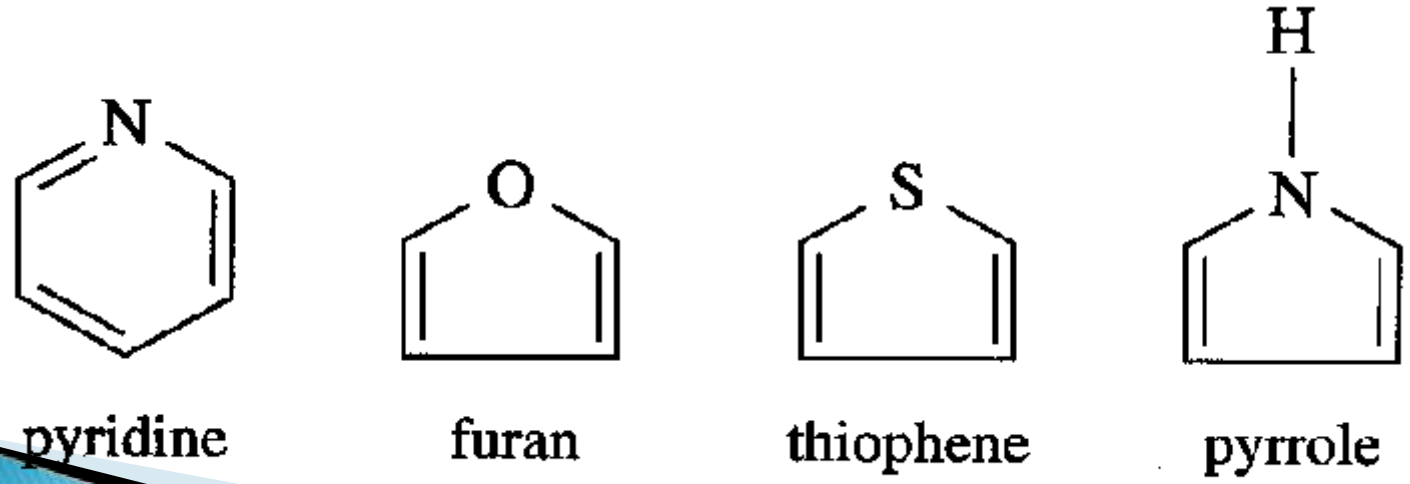
FLUORESCENCE AND CHEMICAL STRUCTURE

- **Fluorescence is most commonly observed in compounds containing aromatic functional groups with low energy.**

- **Most unsubstituted aromatic hydrocarbons show fluorescence - quantum efficiency increases with the no: of rings and degree of condensation.**

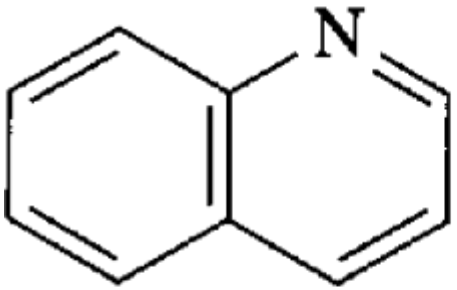
CONTD…

- **Simple heterocyclic do not exhibit fluorescence.**
- **The n - π*singlet quickly converts to the n - π* triplet and prevents fluorescence.**

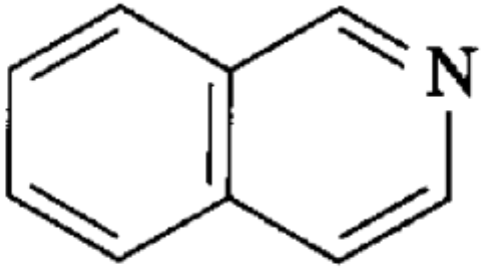


CONTD..

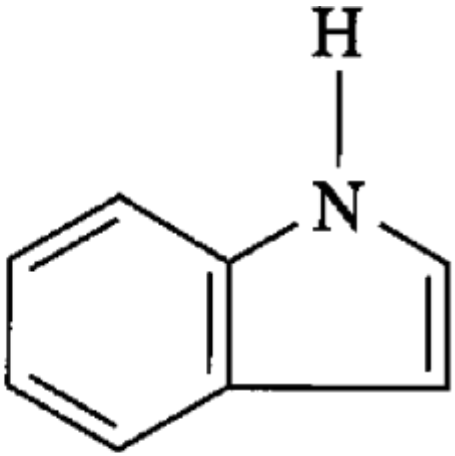
- **Fusion of heterocyclic nucleus to benzene ring increases fluorescence.**



quinoline



isoquinoline

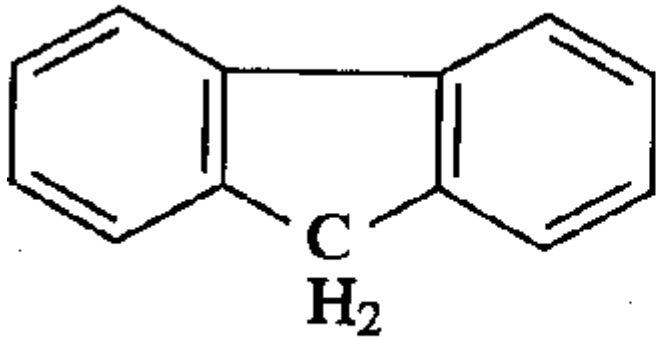


indole

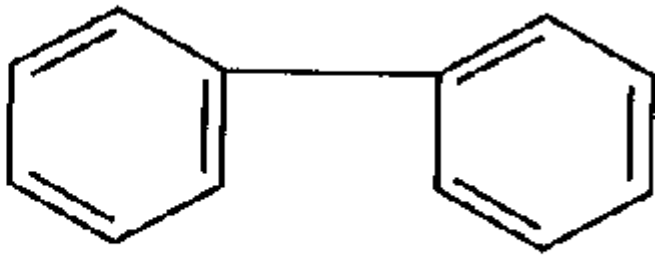
CONTD..

- **Substitution on the benzene ring shifts wavelength of absorbance maxima and corresponding changes in fluorescence peaks**
  - **Fluorescence decreases with increasing atomic no: of the halogen.**
  - **Substitution of carboxylic acid or carboxylic group on aromatic ring inhibits fluorescence.**

STRUCTURAL RIGIDITY



fluorene



biphenyl

- **Fluorescence is favored in molecules with structural rigidity.**

- **organic chelating agents complexed with metal ion increases fluorescence.**

FACTORS AFFECTING FLUORESCENCE INTENSITY

- Nature of molecule
- Nature of substituent
- Effect of concentration
- Adsorption, Light
- Oxygen,ph
- Photodecomposition
- Temp . &viscosity
- Quantum yield
- Intensity of incident light
- Path length

nature of molecules

- All the molecules cannot show the phenomenon of fluorescence.
- Only the molecules absorbs uv/visible radiation can show this phenomenon.
- Greater the absorbency of the molecule the more intense its fluorescence.

nature of substituent

- **Electron donating group enhances fluorescence – e.g.:NH_2,OH etc.**
- **Electron withdrawing groups decrease or destroy fluorescence. e.g.:COOH,NO_2, N=N etc.**
- **High atomic no: atom introduced into π electron system decreases fluorescence.**

EFFECT OF CONCENTRATION

- Fluorescence is directly proportional to concentration.

Contd…

$$FI = Q \times I_a$$

i.e, $F = QI_Oact$

Q = Constant for a particular substance

I_O = Constant for an instrument

a = Molecular extinction coefficient

t = Path length

C = Concentration of the substance

F = KC Where K represents all constants

$FI \propto$ Concentration.

ADSORPTION

- **Extreme sensitiveness of the method requires very dilute solution.**
- **Adsorption of the fluorescent substances on the container wall create serious problems.**
- **Hence strong solutions must be diluted.**

LIGHT

- Monochromatic light is essential for the excitation of fluorescence because the intensity will vary with wavelength.

OXYGEN

The presence of oxygen may interfere in 2 ways.

1] by direct oxidation of the fluorescent substances to non fluorescent.

2] by quenching of fluorescence.

PH

- Alteration of the ph of the solution will have significant effect on fluorescence.
- Fluorescent spectrum is different for ionized and un-ionized species.

TEMPERATURE & VISCOSITY

- Increase in temperature/decrease in viscosity will decrease fluorescence.

fluorescence quantum yield:

K_f = fluorescence
k_{ec} = external conversion
k_{ic} = internal conversion
k_{isc} = intersystem crossing
k_{pd} = pre dissociation
K_d = dissociation

➢ QUANTUM YIELD OF FLUORESCENCE:-(∅)

(∅)=

NUMBER OF PHOTONS EMITTED\NUMBER OF PHOTONS ABSORBEDS

It Is Always Less Than 1.0 Since Some Energy Is Lost By Radiation less Pathways (Collisional, Intersystem Crossing, Vibrational Relaxation)

INTENSITY OF INCIDENT LIGHT

- **Increase in intensity of light incident on sample increases fluorescence intensity.**
- **The intensity of light depends upon**
  - **1)light emitted from the lamp.**
  - **2)Excitation monochromaters**
  - **3)Excitation slit width**

PATH LENGTH

- **The effective path length depends on both the excitation and emission slit width.**
- **Use of microcuvette does not reduce the fluorescence.**
- **Use of microcell may reduce interferences and increases the measured fluorescence**

QUENCHING

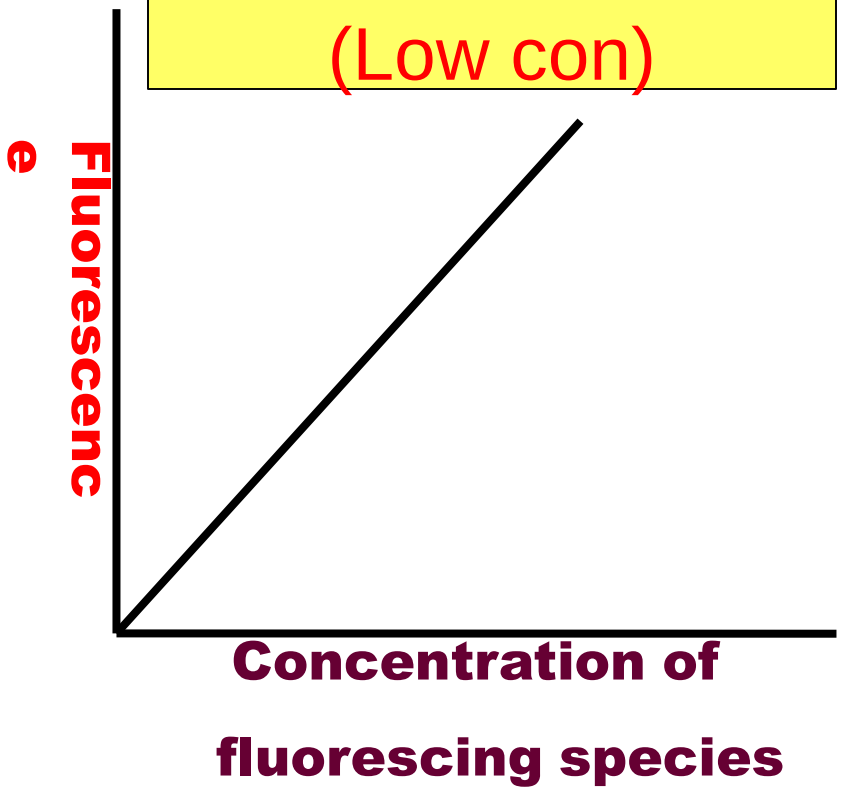
- **Decrease in fluorescence intensity due to specific effects of constituents of the solution.**
- **Due to concentration, ph, pressure of chemical substances, temperature, viscosity, etc.**

Types of quenching

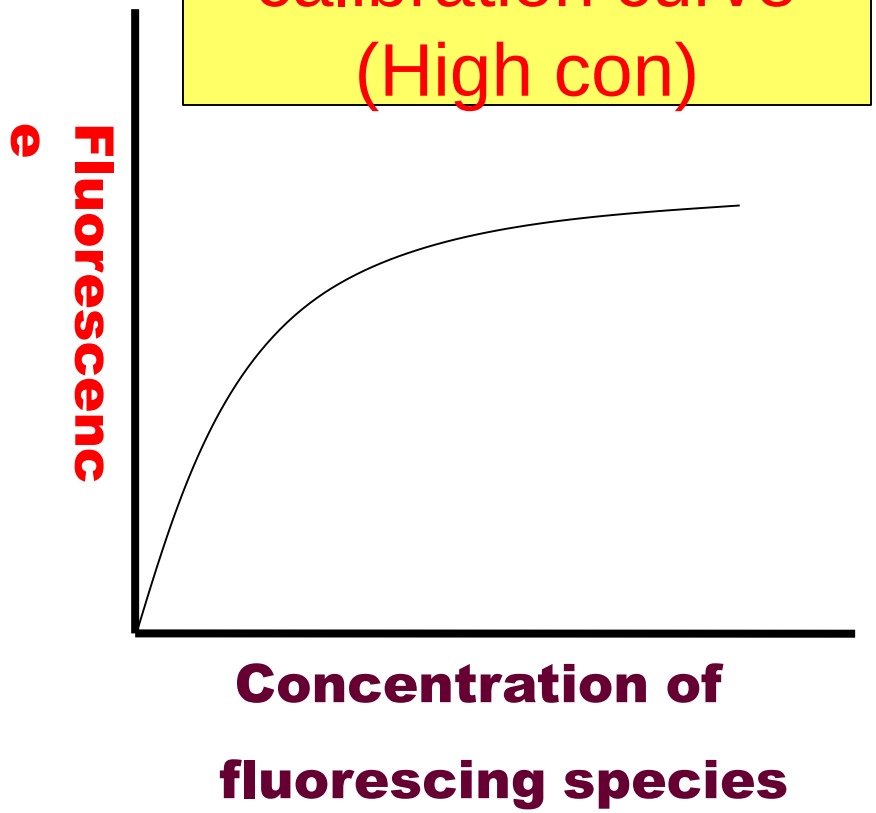
Self quenching
Chemical quenching
Static quenching
Collision quenching

SELF QUENCHING/CONC.QUENCHING

Calibration curve (Low con)



calibration curve (High con)



Deviations at higher concentrations can be attributed to self-quenching or self-absorption.

CHEMICAL QUENCHING

- Here decrease in fluorescence intensity due to the factors like change in ph,presence of oxygen, halides &heavy metals.
- ph- aniline at ph 5-13 gives fluorescence but at ph <5 &>13 it does not exhibit fluorescence.
- halides like chloride,bromide,iodide & electron withdrawing groups like no2,cooH etc. leads to quenching.
- Heavy metals leads to quenching, because of collisions of triplet ground state.

STATIC QUENCHING

- This occurs due to complex formation.
  e.g.. caffeine reduces the fluorescence of riboflavin by complex formation.

COLLISIONAL QUENCHING

- It reduces fluorescence by collision. where no. of collisions increased hence quenching takes place.

INSTRUMENTATION

COMPONENTS OF FLUORIMETERS AND SPECTROFLUORIMETERS

- SOURCE OF LIGHT
- FILTERS AND MONOCHROMATORS
- SAMPLE CELLS
- DETECTORS

SOURCE OF LIGHT

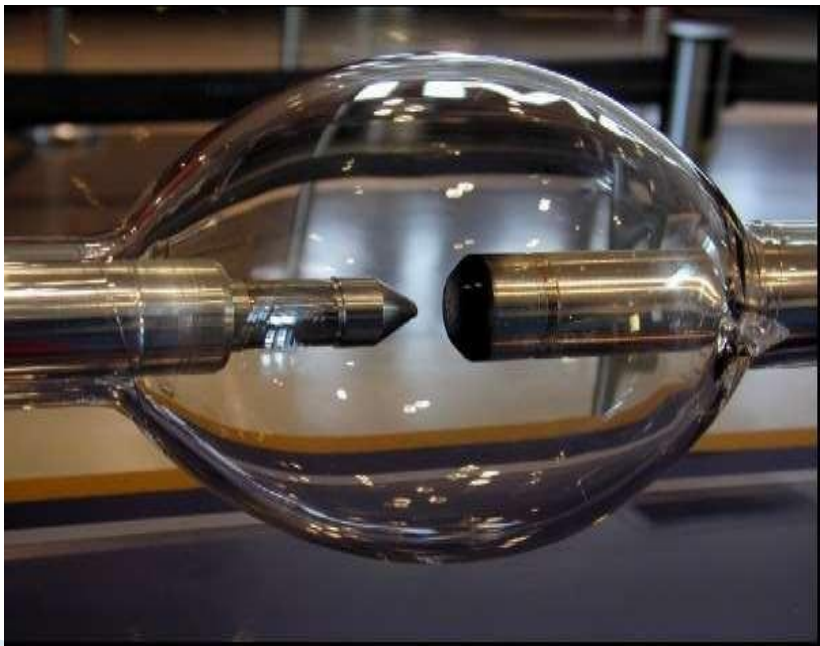
- MERCURY ARC LAMP.
- XENON ARC LAMP.
- TUNGSTEN LAMP.
- TUNABLE DYE LASERS.

MERCURY ARC LAMP

- **Produce intense line spectrum above 350nm.**
- **High pressure lamps give lines at 366,405, 436, 546,577,691,734nm.**
- **Low pressure lamps give additional radiation at 254nm.**

XENON ARC LAMP

- **Intense radiation by passage of current through an atmosphere of xenon.**
- **Spectrum is continuous over the range between over 250-600nm,peak intensity about 470nm.**



TUNGSTEN LAMP

- **Intensity of the lamp is low.**
- **If excitation is done in the visible region this lamp is used.**
- **It does not offer UV radiation.**

TUNABLE DYE LASERS

- **Pulsed nitrogen laser as the primary source.**
- **Radiation in the range between 360 and 650 nm is produced.**

❖ 2) FILTERS AND MONOCHROMATORS:-

- **Filters:** these are nothing but optical filters works 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.

Primary filter:-absorbs visible radiation

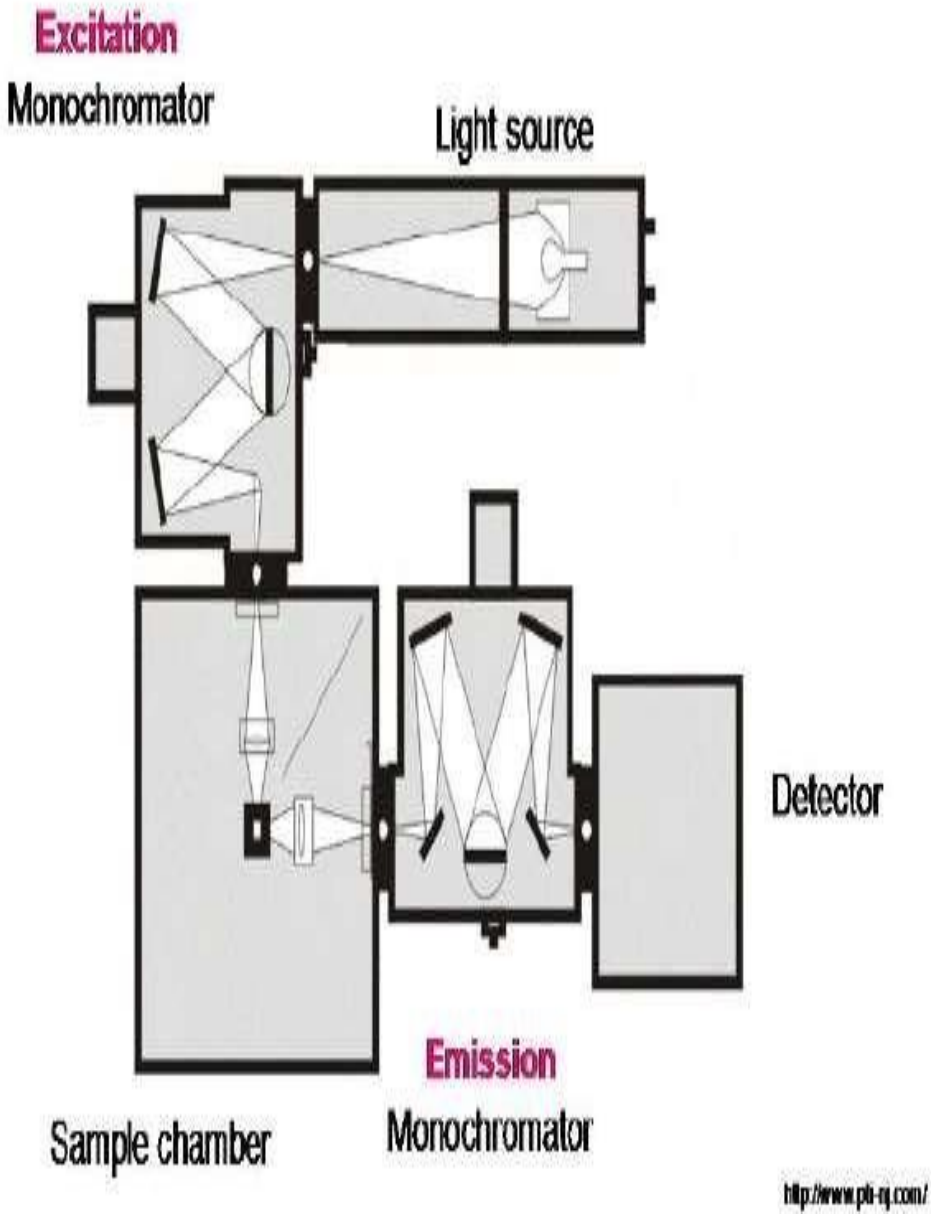
- and transmit UV radiation.

Secondary filter:-absorbs UV radiation and transmit visible radiation.

FIGURE 6

- **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.



FILTERS & MONOCHROMATORS

FILTERS

Primary filter–absorbs visible light & transmits uv light.

Secondary filter–absorbs uv radiations & transmits visible light.

MONOCHROMATORS

Exitation monochromaters–isolates only the radiation which is absorbed by the molecule.

Emission monochromaters–isolates only the radiation emitted by the molecule.

SAMPLE AND SAMPLE HOLDER

- **The majority of fluorescence assays are carried out in solution.**
- **Cylindrical or rectangular cells fabricated of silica or glass used.**
- **Path length is usually 10mm or 1cm.**
- **All the surfaces of the sample holder are polished in fluorimetry.**

DETECTORS

- **PHOTOVOLTAIC CELL**
- **PHOTO TUBE**
- **PHOTOMULTIPLIER TUBES – Best and accurate.**

PHOTOMULTIPLIER TUBE

- **Multiplication of photo electrons by secondary emission of radiation.**
- **A photo cathode and series of dynodes are used.**
- **Each cathode is maintained at 75-100v higher than the preceding one.**
- **Over all amplification of 10^6 is obtained.**

DETECTOR –
PHOTOMULTIPLIER TUBE

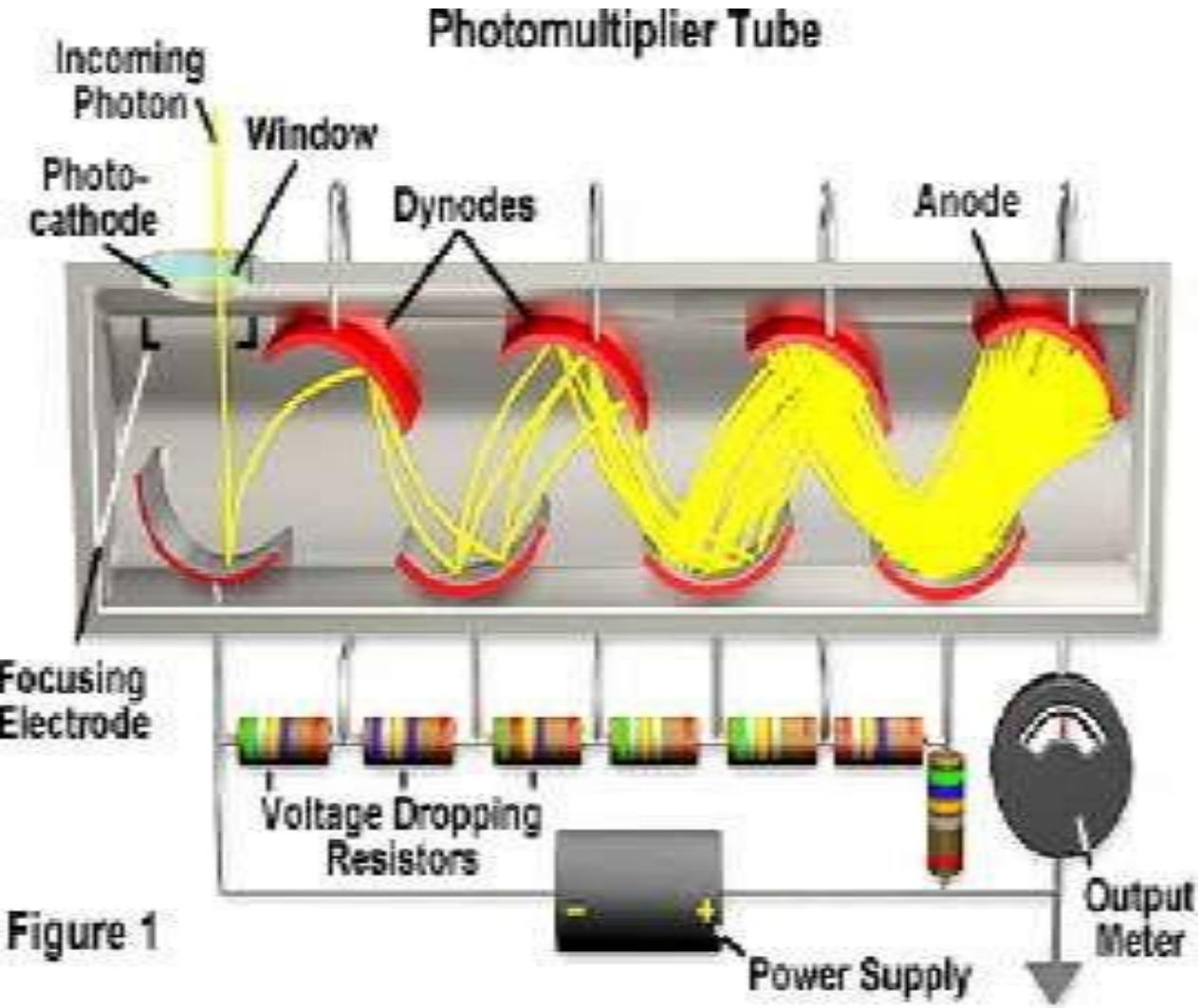
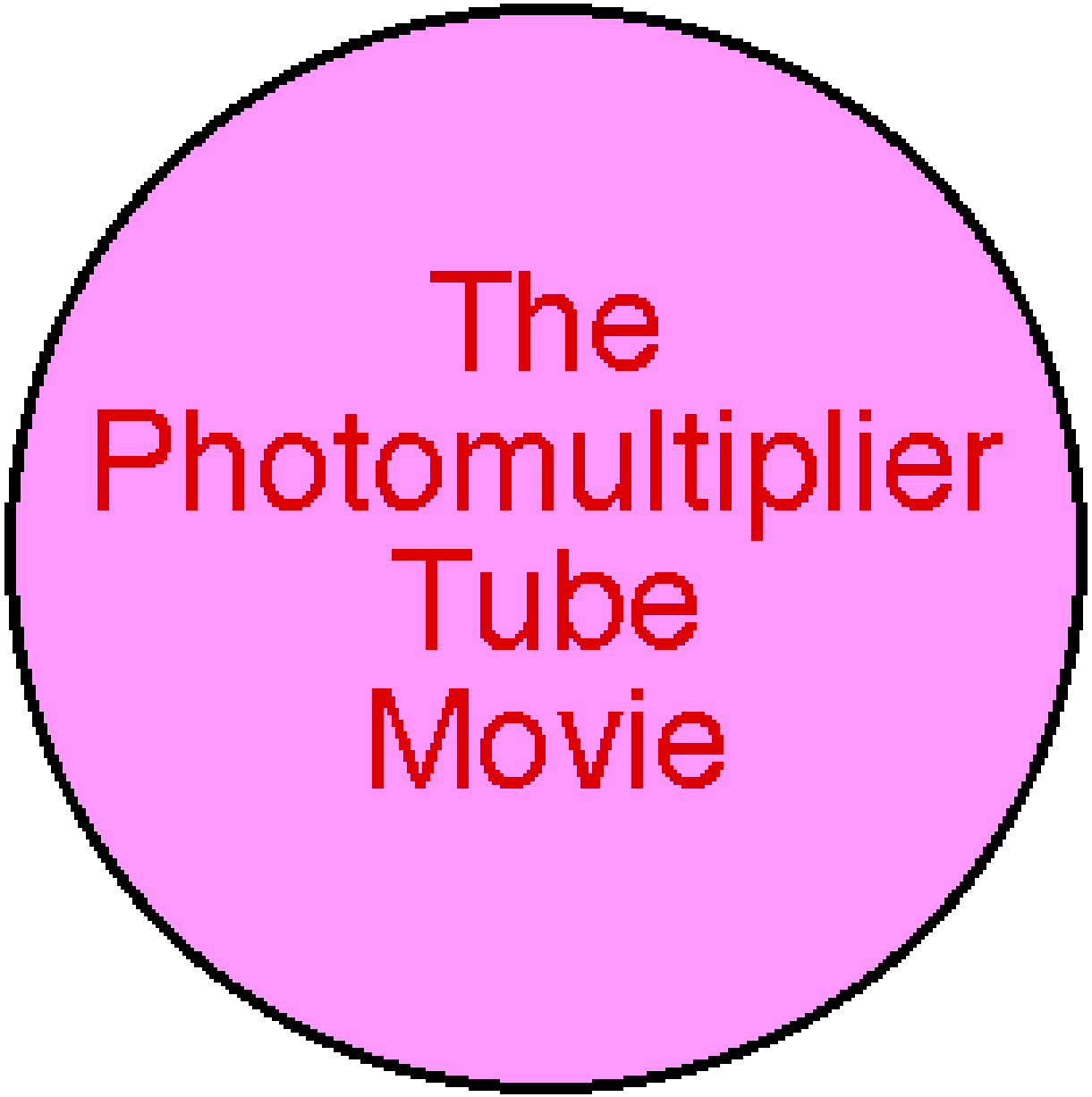
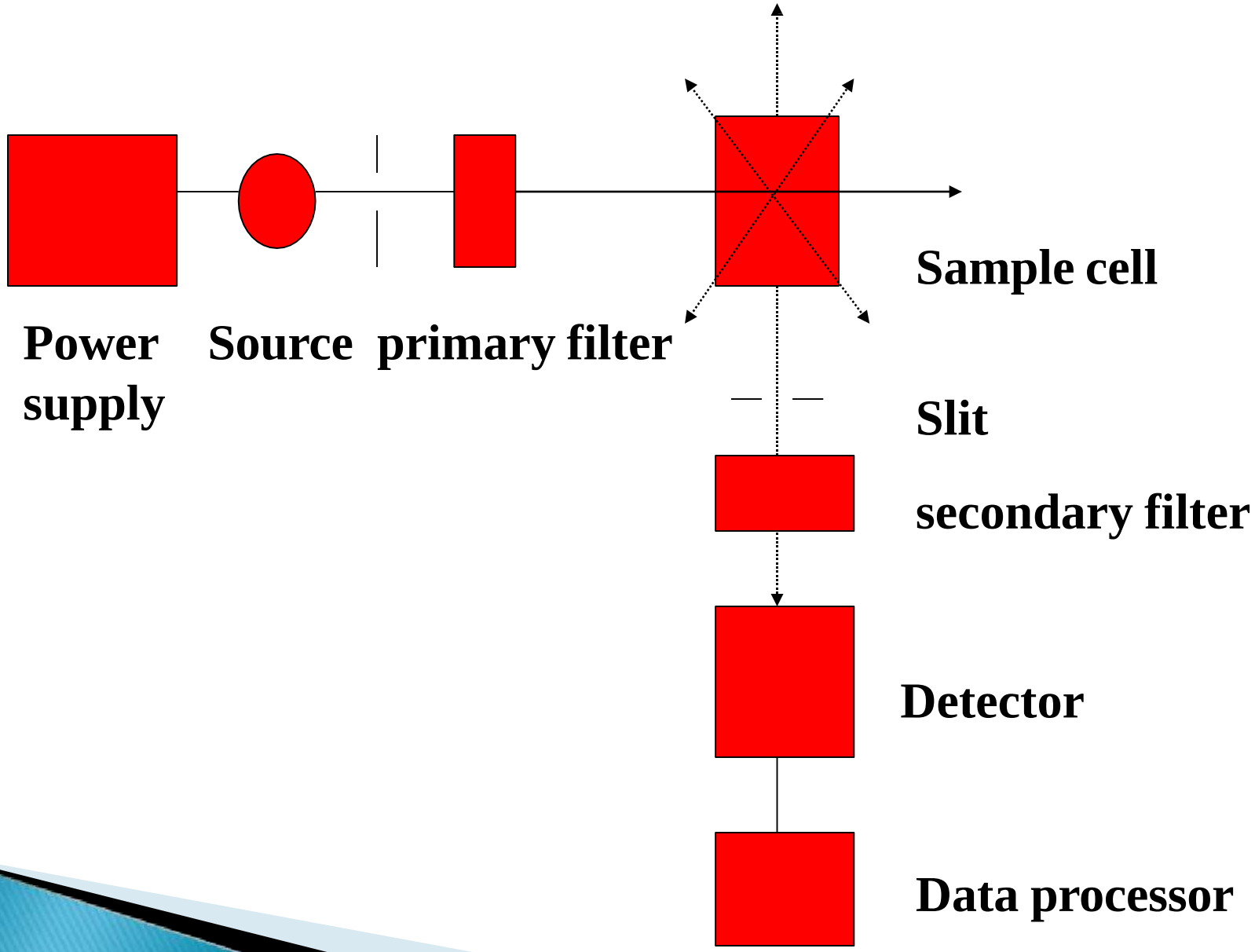


Figure 1


The
Photomultiplier
Tube
Movie

INSTRUMENT DESIGNS

GENERAL LAYOUT OF FLUORIMETER



SINGLE BEAM FLUORIMETER

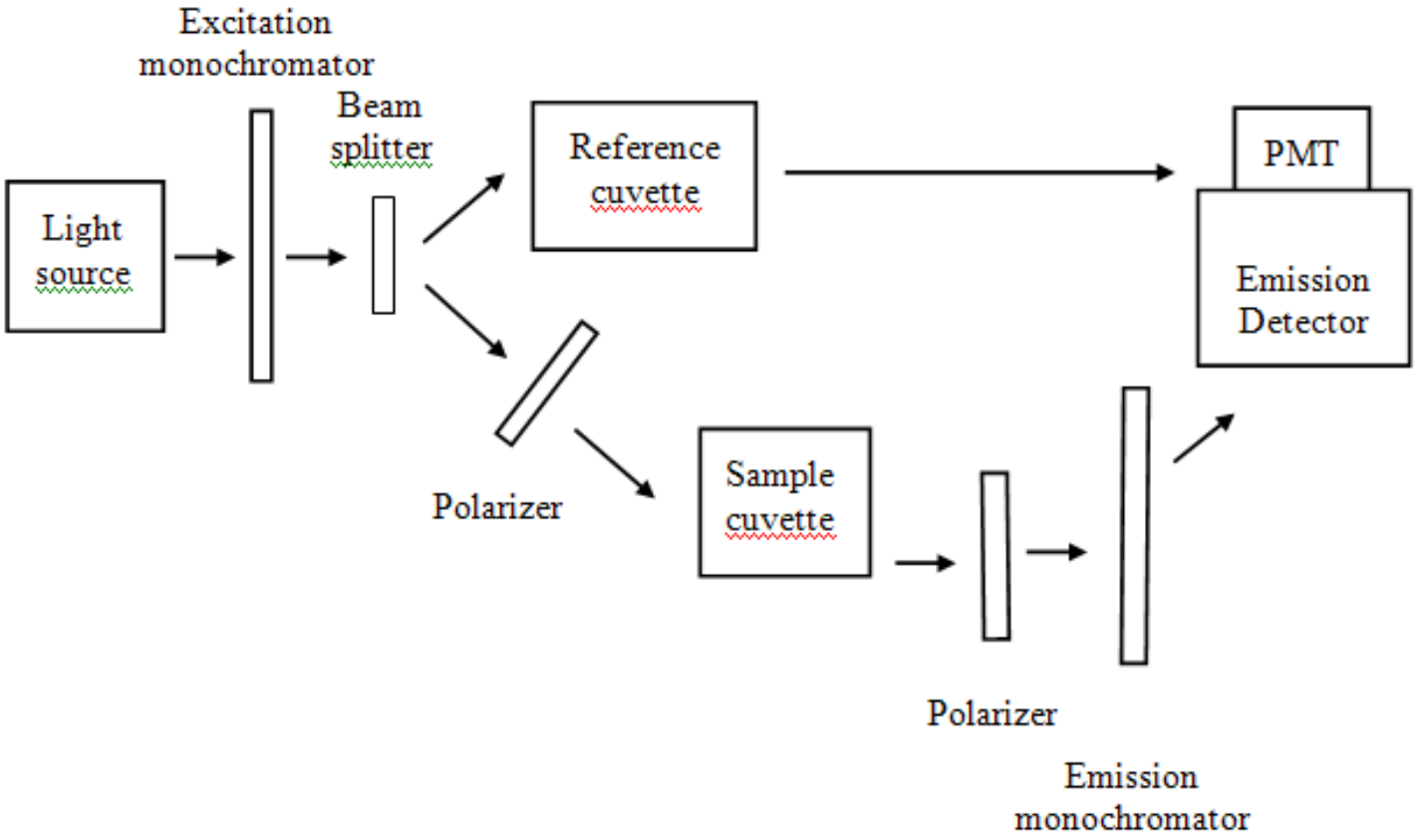
- **Tungsten lamp as source of light.**
- **The primary filter absorbs visible radiation and transmits uv radiation.**
- **Emitted radiation measured at 90^{o} by secondary filter.**
- **Secondary filter absorbs uv radiation and transmits visible radiation.**

Advantages

- Simple in construction
- Easy to use.
- Economical

disadvantages

- It is not possible to use reference solution & sample solution at a time.
- Rapid scanning to obtain Exitation & emission spectrum of the compound is not possible.


Excitation monochromator
Beam splitter
Reference cuvette
PMT
Light source
Emission Detector
Polarizer
Sample cuvette
Polarizer
Emission monochromator

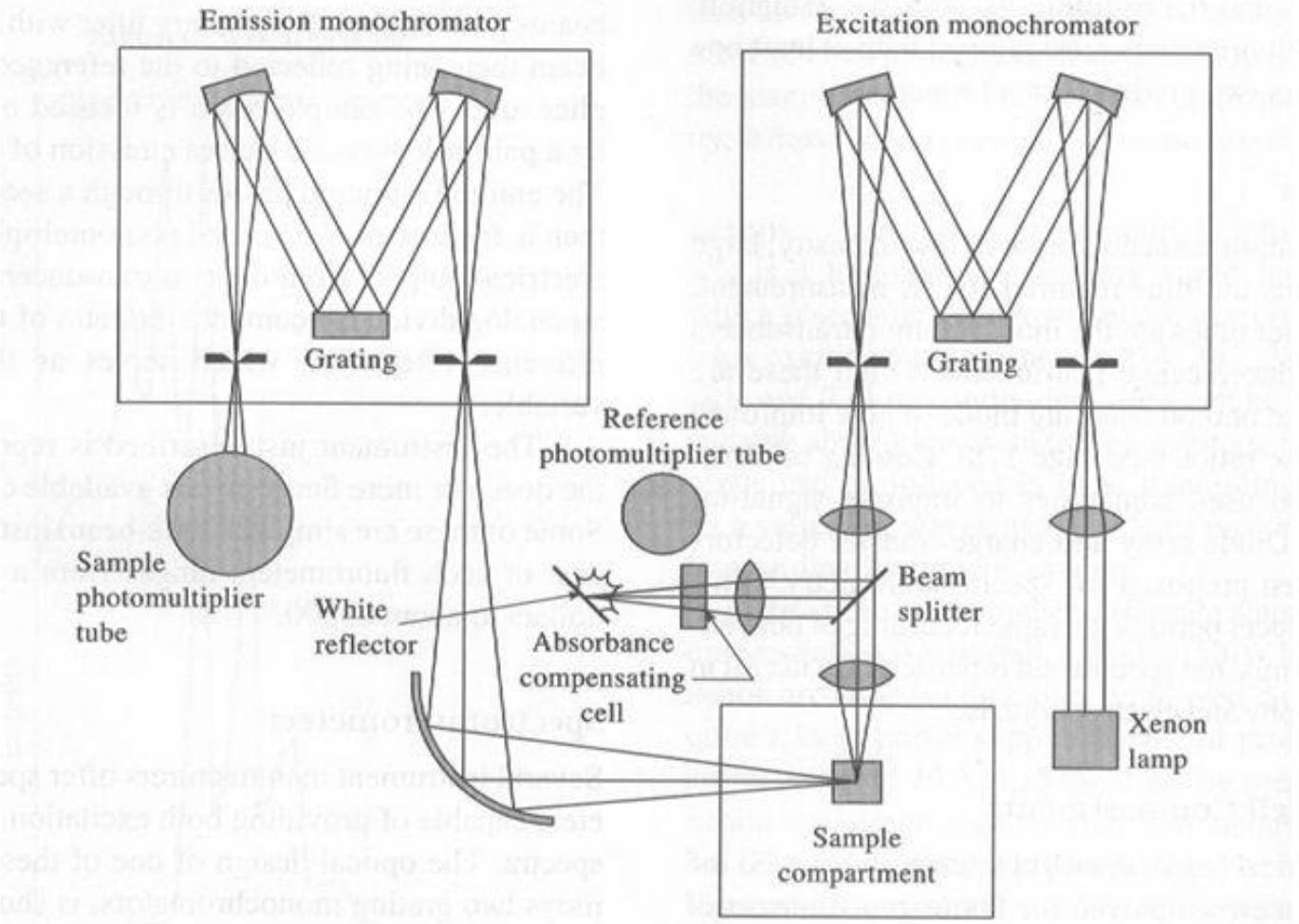


Figure 15-7 A spectrofluorometer. *(Courtesy of SLM Instruments, Inc., Urbana, IL.)*

DOUBLE BEAM FLUORIMETER

- **Similar to single beam instrument.**
- **Two incident beams from light source pass through primary filters separately and fall on either sample or reference solution.**
- **The emitted radiation from sample or reference pass separately through secondary filter.**

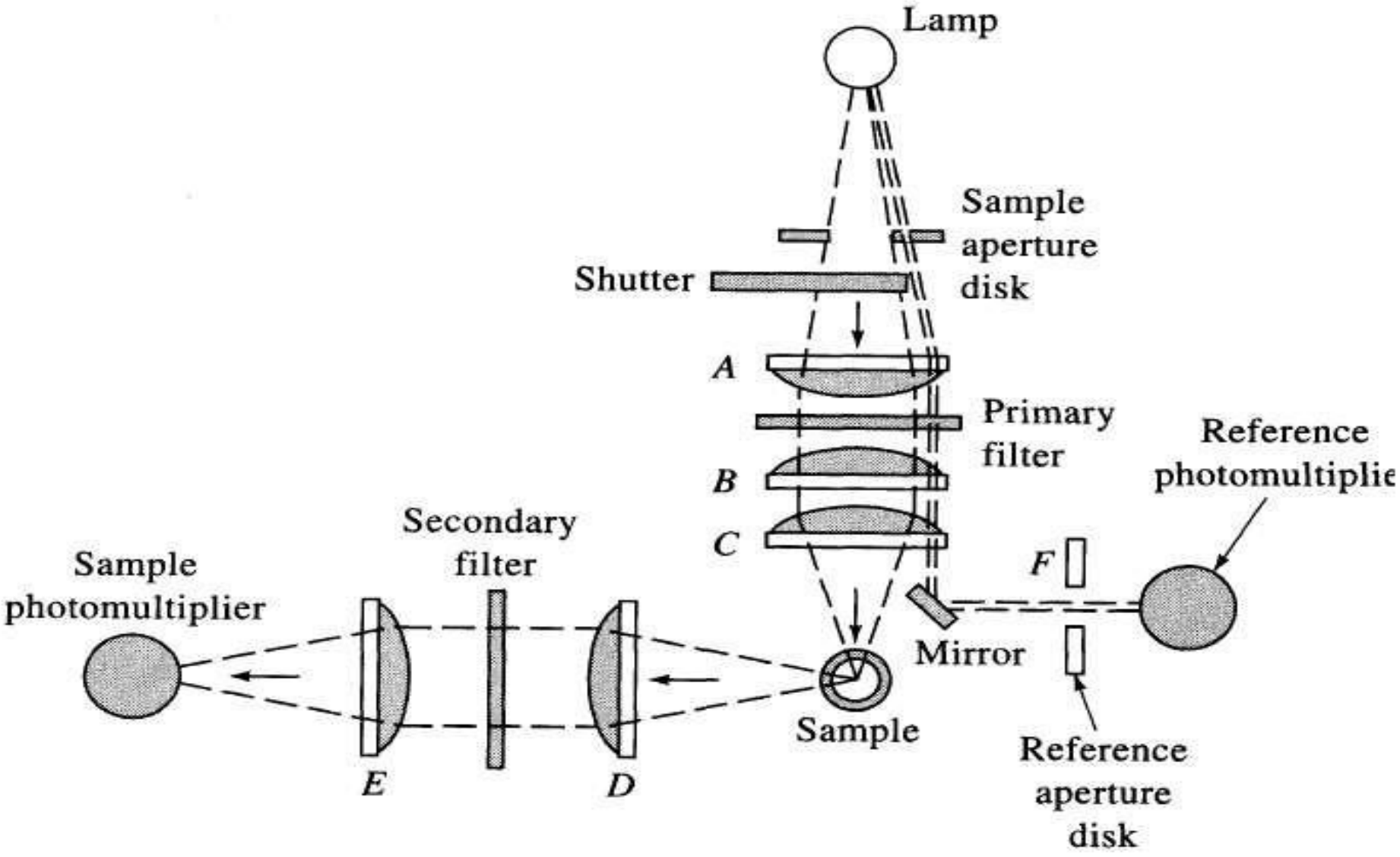
Advantages

- Sample & reference solution can be analyzed simultaneously.

disadvantage

- Rapid scanning is not possible due to use of filters.

SCHEMATIC DIAGRAM OF FLUORIMETER



APPLICATIONS OF FLUORIMETRY

1] Determination of inorganic substances

- **Determination of ruthenium ions in presence of other platinum metals.**
- **Determination of aluminum (III) in alloys.**
- **Determination of boron in steel by complex formed with benzoin.**
- **Estimation of cadmium with 2-(2 hydroxyphenyl) benzoxazole in presence of tartarate.**

2]Nuclear research

- Field determination of uranium salts.

3]fluorescent indicators

Mainly used in acid-base titration.

e.g.:

eosin- colorless-green.

Fluorescein:colourless-green.

Quinine sulphate: blue-violet.

Acridine: green-violet

4] Fluorometric reagent

- **Aromatic structure with two or more donor functional groups**

Reagent	Ion	Fluorescence wavelength	Sensitivity
Alizarin garnet B	Al^{3+}	500	0.007
Flavanol	Sn^{4+}	470	0.1
8-Hydroxy quinoline	Li^{2+}	580	0.2

5] organic analysis

- **Qualitative and quantitative analysis of organic aromatic compounds present in cigarette smoke, air pollutants, automobile exhausts etc.**

6] pharmaceutical analysis

compound	reagent	excitation wavelength	fluorescence
hydrocortisone	**75%v/v H_2SO_4 in ethanol**	**460**	**520**
nicotinamide	**cyanogen chloride**	**250**	**430**

7] Liquid chromatography

- **Fluorescence is an imp method of determining compounds as they appear at the end of chromatogram or capillary electrophoresis column.**

8]determination of vitamin B1 &B2.

REFERENCES

- **Douglas ASkoog, Principles of instrumental analysis**
- **H:\UV-Vis Luminescence Spectroscopy - Theory.mht**
- **Dr.B.K.Sharma, Instrumental methods of chemical analysis**
- **Gurdeep RChatwal, Instrumental methods of chemical analysis**

Application

1. Analysis of medicinal compound:

- A number of drugs can be estimated using fluorimetry.
- Fluorescence is made to produce from non-fluorogenic drugs by following ways:

(a) Some drugs are capable of exhibiting fluorescence in an appropriate solvent.

e.g. Quinine in 0.1 N Sulfuric acid, Riboflavin in 1% tartaric acid, Aminocrine in 0.1 N HCl.

(b) Organic and inorganic compounds can be made fluorogenic by chemical change such as oxidation. E.g. Diphenylhydantoin (phenytoin) is oxidised by alkaline $KMnO_4$ to form benzophenone which exhibits flurescence.

(c) Organic and inorganic compounds are complexed with suitable reagents to make them fluorogenic.

(d) When two or more drugs are present, each drug can be estimated individually by adopting suitable method like;

- Conversion of acidic to alkaline solution or vice versa.
- Conversion of ionic to non-ionic compound or vice versa.
- Selection of wavelength of excitation for each drug.
- Extraction of any one drug from the mixture and analysing it.

(e) Preparation of fluorogenic derivative from non-fluorogenic drug. Some of the examples include;

- Complex of atropine with eosin is soluble in chloroform and exhibit fluorescence.
- Other non-fluorogenic drugs which can be analysed are morphine and codeine.

- http://en.wikipedia.org/wiki/Fluorescence
- http://images.google.co.in/imghp?oe=UTF-8&hl=en&tab=wi&q=fluorescence
- http://www.bertholdtech.com/ww/en pub/bioanalytik/biomethods/fluor.cfm

THANK YOU