Luminescence - emission of light by substance when e returns to its ground state
phosphorescent → delayed fluorescence. is phosp.

* Fluorometry.

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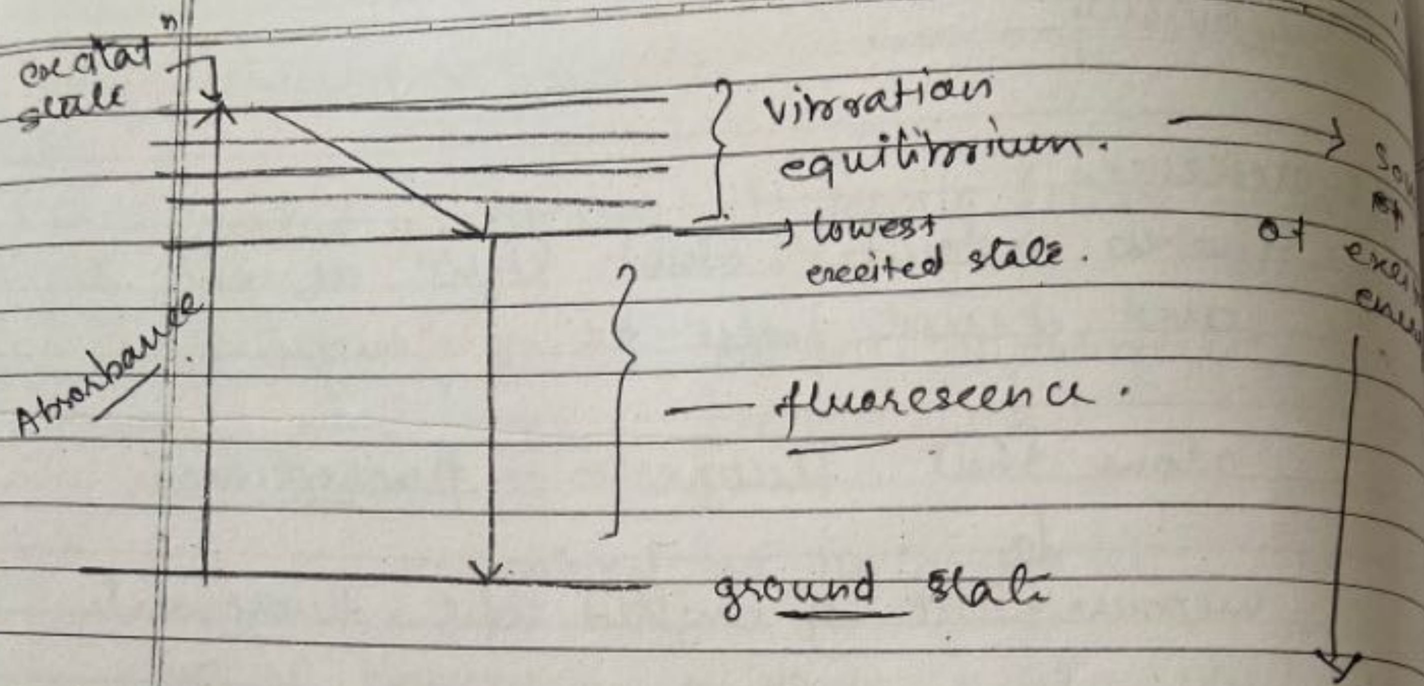
· Fluorescence
↳ when molecule absorb light at one λ and reemit light at a longer λ.
↓
atom that fluoresces → fluorophore
↓
measurement of emitted fluorescent light = fluorometry.
- light exiting sample is not measured, so stray light effect is reduced, → accurate and sensitive

* Principle :-

Molecule contain series of closely spaced energy level
↓
when light energy is passed on molecule
↓
Absorptⁿ of light energy by molecule
↓
Transition of electron from ground state to one of excited state
↓
Once excited, ē returns to original ground state by several ways

- Radiation-less vibratⁿ equilibrium → as heat
- emission of radiant light → fluorescence
- quenching → trapping of energy.



Thus, emitted fluorescent light is of less energy (higher λ) than excitation energy.

↓

Difference b/w max λ of excitation light and emitted light

↓

Stokes shift (constant)

(measure of loss of energy during lifetime of excited state (Vibration equilibrium) before returning to ground state..

⇒ Time relationship of fluorescence emission.

There is considerable delay time between the

① Absorption of light energy

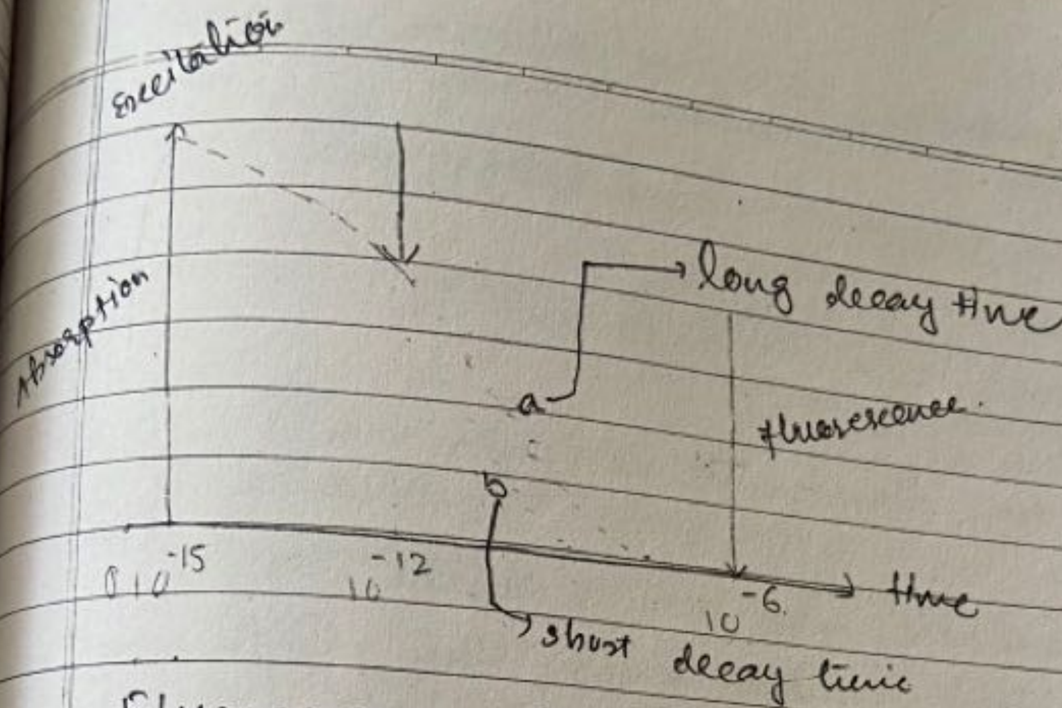
② Vibration equilibrium.

③. emission of fluorescence.

See behind.

↓

shown in figure.



Fluorescence decay process.

⇒ Relationship of concentratⁿ and fluorescence intensity

- Fluorescence intensity directly ∝ to concentratⁿ of fluorophore.
  - Magnitude of intensity determined by
    ① conc.
    ② length path
    ③ Intensity of light source.

- Fluorescence measurement is 100 - 2000 time more sensitive than abs. measurement d/t ① more intense light
  ② Digital filtering tech.
  ③. Sensitive emission photometer.

- Time Relationship of fluorescence emission
- Time req. for molecule to absorb energy and to be excited = 10^{-15} sec
- length of vibratⁿ equilibrium to lowest excited state = 10^{-12} sec.
- time req. for fluorescence emission = 10^{-7}

→ There is considerable time delay b/w ① Absorbtⁿ of energy ② return to lower energy state ③ flo. emission

→ Fluorescence decay time = time required for the emitted light to reach 1/e where e = 2.178 (Naperian base) of its initial intensity is called fluorescence decay time / average life time of excitate state molecule.

↓

this time delay is used in fluorometry instrument → time resolved fluorometry

↓

pulse of light (xenon lamp / laser) is used

↓

fluorescence is measured in time following pulse of light.

↓

Advantage → ① ↓ effect of stray light and ② no light scattering of incident light

↓

↓ background interference.

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(2) Monochromator :- excitation / emission.

- Interference filter
  - all - dielectric multicavity filter
  - Combine c̄ sharp cut off filter → ~~single filter~~ package
    ↓
    - remove undesired transmission.
    - provide narrow bandwidth, higher peak transmission

- Coloured glass.
  - used for both excitatⁿ & emission filter.
  - more chances of stray light transmission
    ↓
    unwanted fluorescence.

- Grating :- → provide selectivity of excitatⁿ and emission λ required when working with new fluorophore.

(3) Cuvette

- square / rectangular.
- material allows excitation and emission light to pass
  ↓
  glass / plastic - for visible light.
  quartz - UV light

* Relation of concentration and fluorescent intensity
. derived from beer's -lambert's low.
↓.
fluorescence intensity ∝ concentration
∝ intensity of incident light
↓
↑ concentration } → ↑ fluorescence.
↑ lamp intensity }

→ Inner filter effects

→ Fluorescence measurement are express-ed in relative intensity unit
↳ which are ~~arbitary~~ arbitrary unit decided by manufacturer.
↓
Intensity measured is not the absolute quantity, but a small part of total fluorescence emission
↓
its magnitude is defined by instrument slit width, excitation intensity & detector sensitivity
(instrument-related variables)

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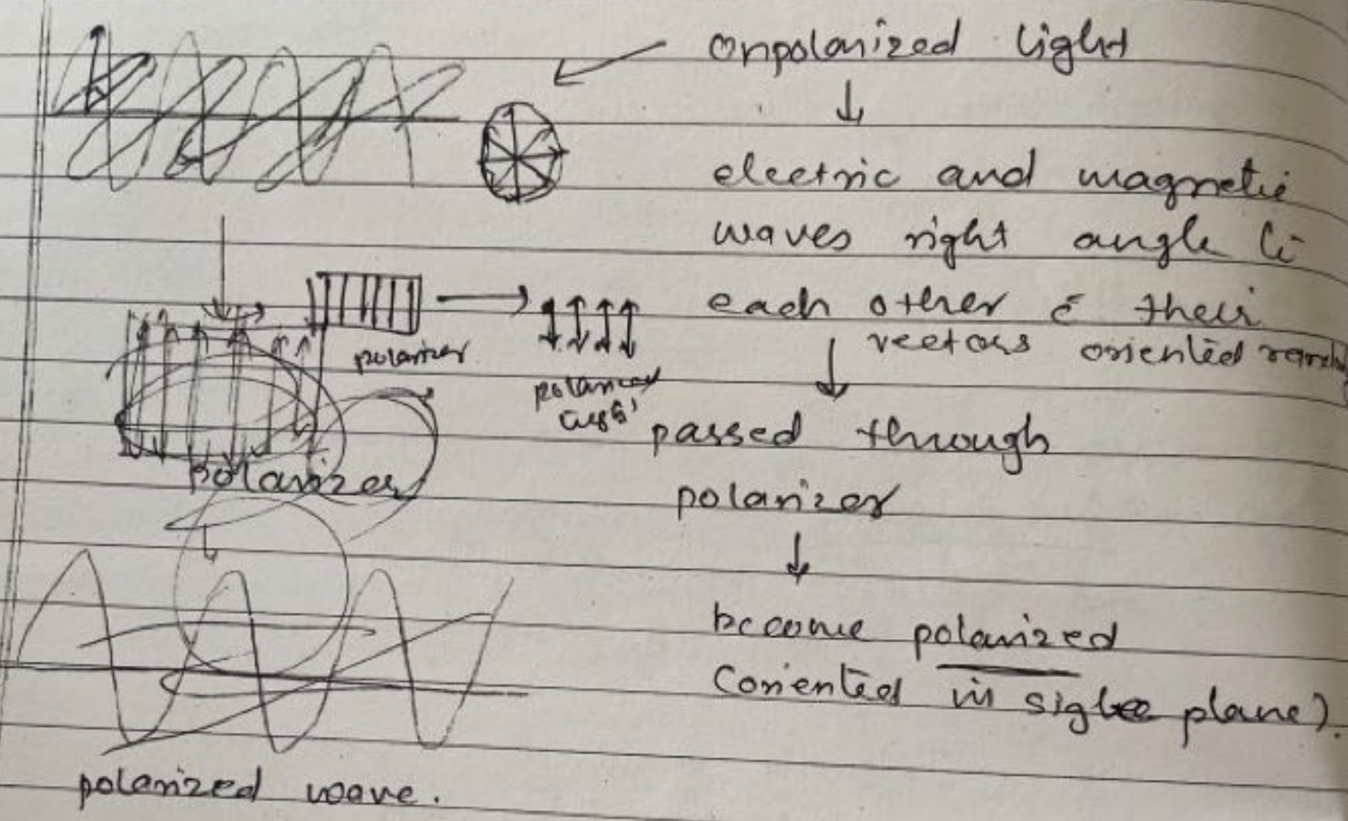
* Fluorosence polarizatⁿ :-

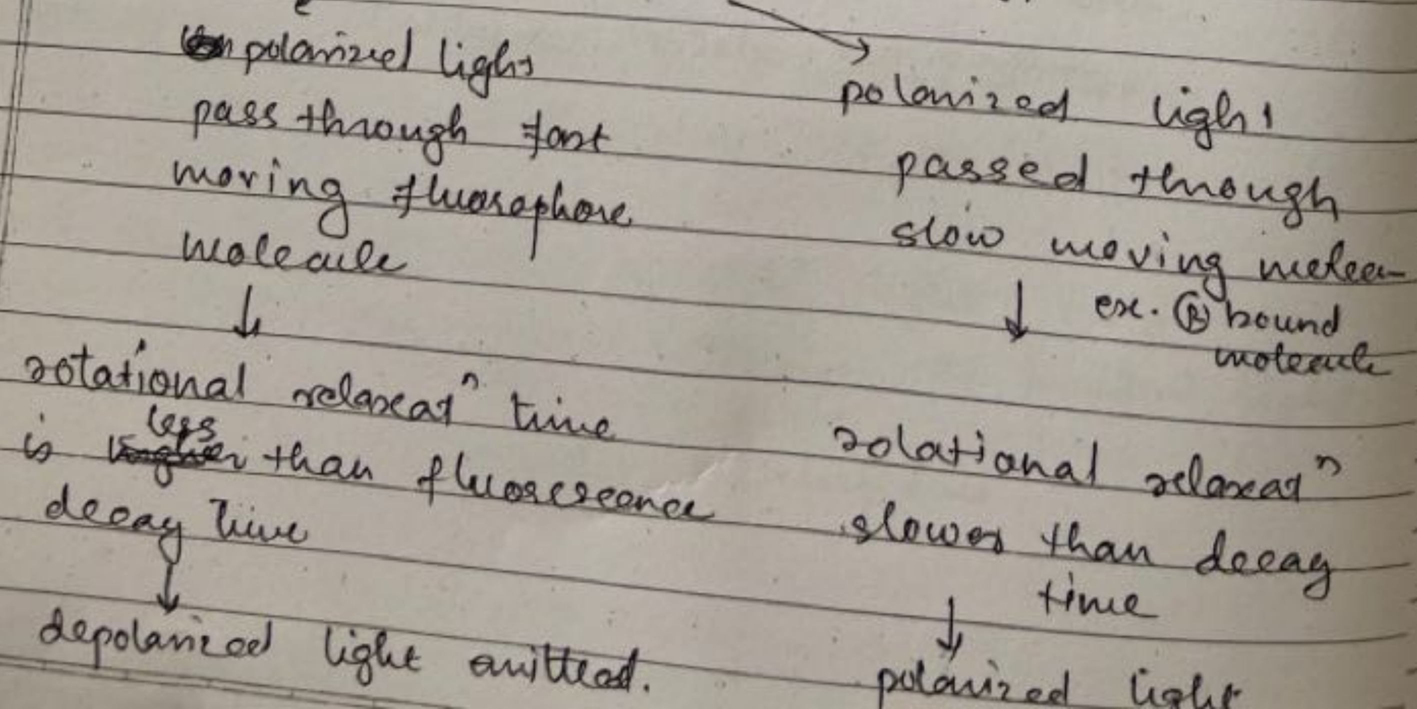
→ light is composed of electric and magnetic waves at right angle to each other.



unpolarized light
↓
electric and magnetic waves right angle to each other with their vectors oriented randomly
↓
passed through polarizer
↓
become polarized (oriented in single plane).

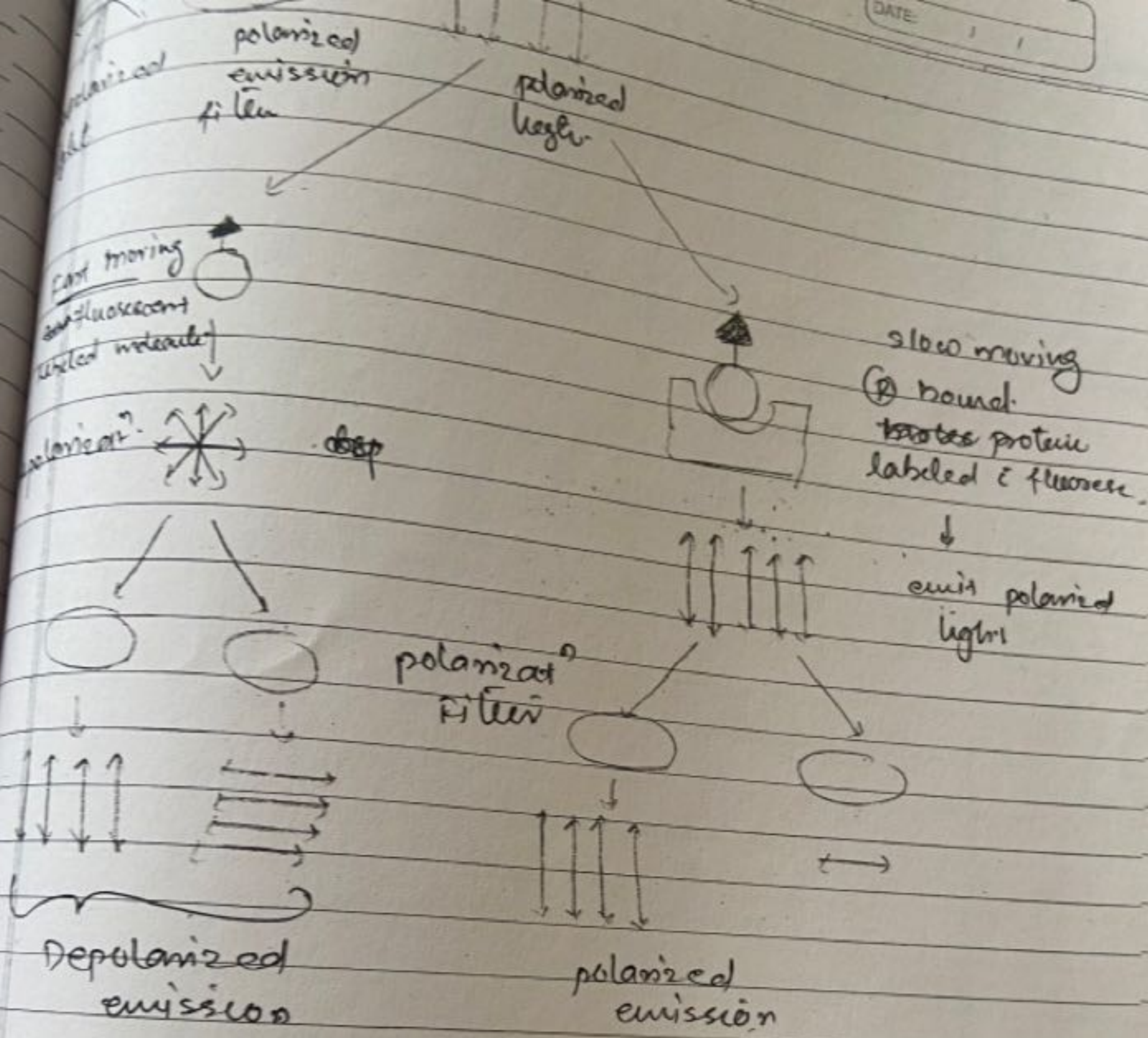
polarized wave.

→ Fluorophore absorbs light most efficiently in plane of electronic energy.



unpolarized light pass through fast moving fluorophore molecule
↓
rotational relaxatⁿ time is less than fluorescence decay time
↓
depolarized light emitted.

polarized light passed through slow moving molecule
(ex. bound molecule)
↓
rotational relaxatⁿ slower than decay time
↓
polarized light



Use of fluorescence polarizatⁿ :-

↓

quantitative analyte by change in fluorescence polarizatⁿ f/b immunological reaction

↓

labeled ~~Ab~~ Analyte bind to Ab

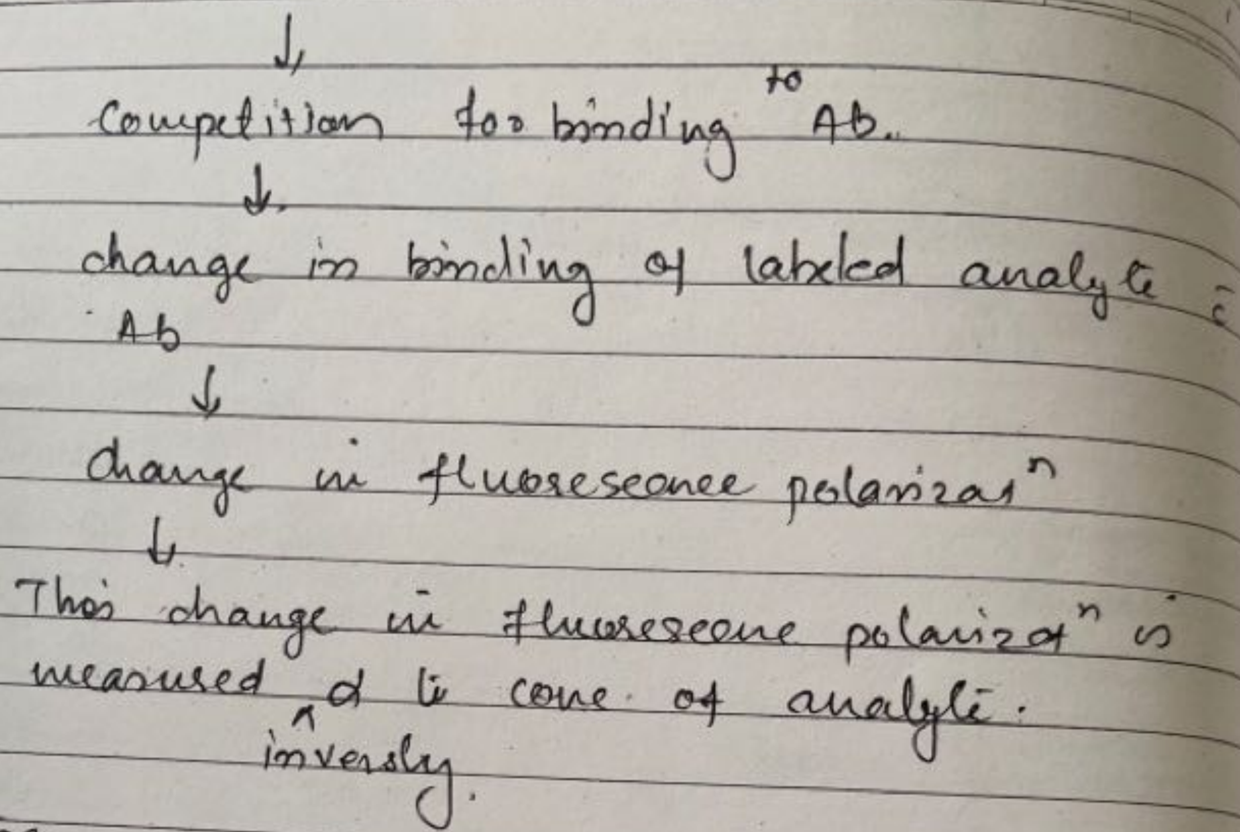
↓

emission of polarized light

↓

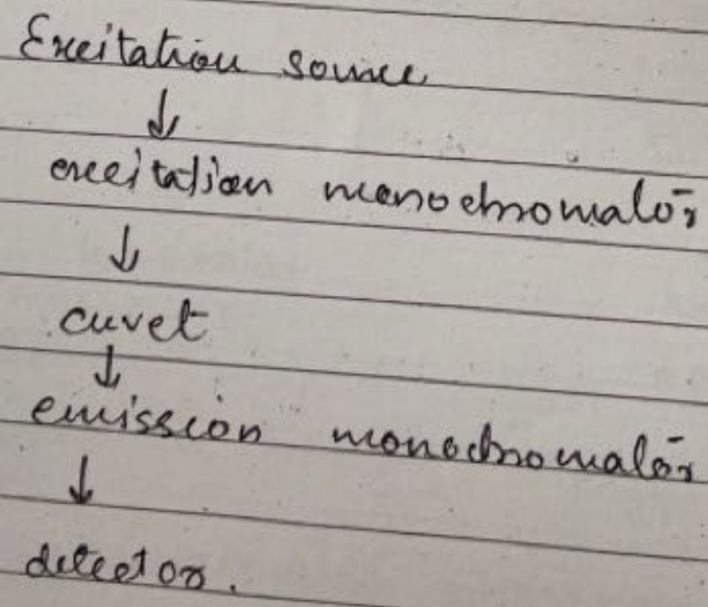
Addition of nonlabeled molecules e.g. drugs in serum sample.

↓



- Advantage - Homogenous immunoassay → Automatn ↓TAT

* Instrumentations:



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(I) Excitation source:-

- Absorption spectra of most of fluorophore = 300-700 nm
- Fluorescence emission ∝ (1) initial excitatⁿ energy
  (2) Conc. of analyte

↓

Intense lamp capable of emitting radiant energy of over large spectrum desirable

ex → ① Xenon lamp.
② Mercury arc lamp
③ Laser.

(ii) Xenon lamp. - mc used.
- high intensity radiant energy → 280 to 800 nm
- stable lamp flashes.
- high UV and visible spectrum output.

Disadvantage → arc wandering and flickering by fluctuatⁿ in current
↓
prevented by current-stabilized power supply

(iii) Laser:-
used in fluorescence applicatⁿ where high Intensity, well focused and monochromatic light is req.

applicatⁿ - time resolved
Flow cytometry

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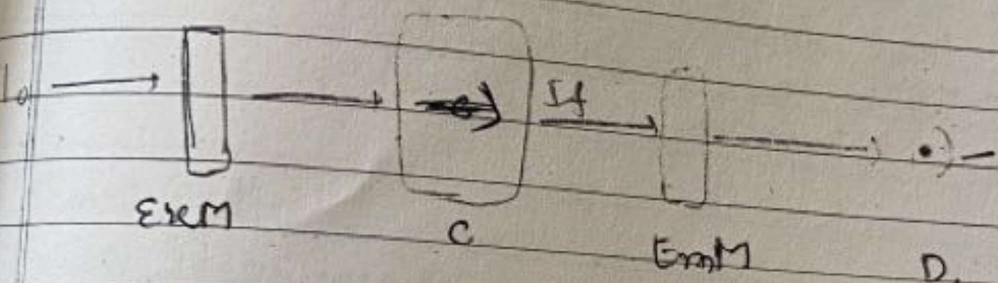
Geometric arrangements of fluorometers :-

Fluorescence light is emitted in all directions from molecule
↓
several geometry are used to measure fluorescence.

① End-on geometry :

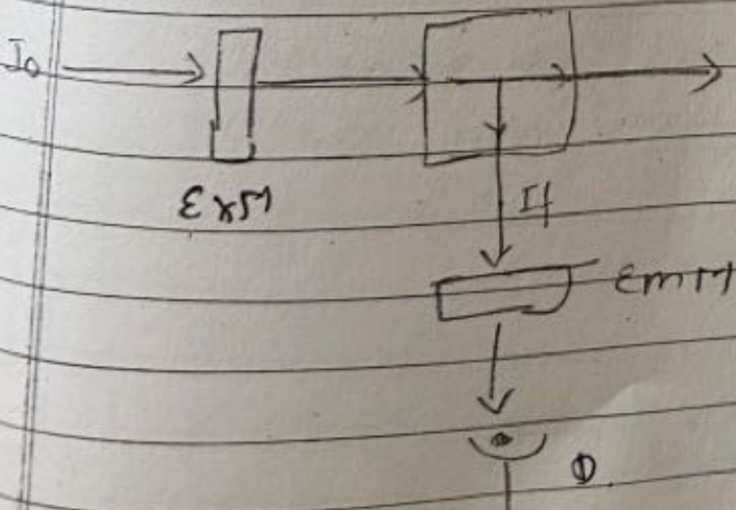


ExM = Excitation monochro
C = cuvet
EmM = Emission
D = detector
I_0 = initial excitation energy
If = fluorescence energy.

- Not used widely
- limited sensitivity d/t
  ① quality of excitation and emission filter pair
  ② Overlapping of ex and em spectral band
  ③ Inner filter effect.

- Advantage - can be combined c̄ spectrophotometer

② Right angle geometry.



- Most commonly used
- ↓ background signals
  ↓ scattering.
  ↓
  ↑ Analytical sensitivity

Disadv = ~~high~~ ↑ inner filter effect

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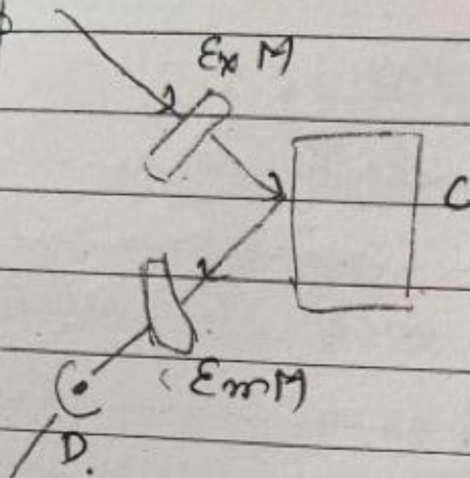
③ Front- surface geometry



- minimise inner effect

↓

↑ linearity over range of conc.

- Similar sensitivity to right angle. but more susceptible to background light sd scattering.

* Limitation of fluorescence measurement :

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Factors

1. Concentratⁿ effects → Inner filler effect
   ↘ Concentratⁿ quenching

2. Background effects → dt scattering

3. Solvent effects → Interfering non specific fluorescence, quenching from solvent

4. Sample effect → light scattering
   ↘ Interfering fluorescence
   ↘ Sample adsorption.

5. temp. effects

6. photo decomposition of sample

(I) Inner filler effect :-

fluorophore itself and other non-fluorescent molecules absorb exciting light or emission light

↓

loss of excitation & emission intensity across the cuvet path length

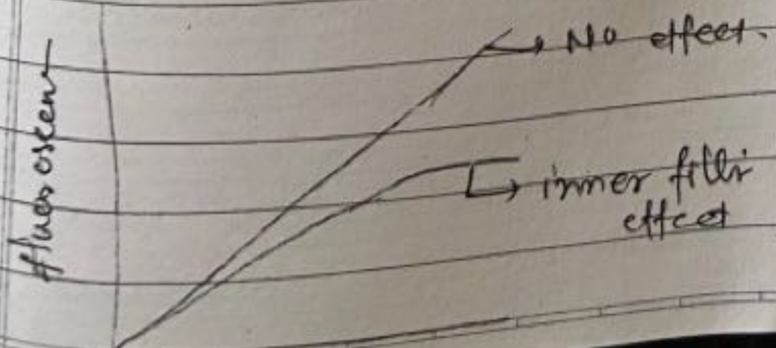
↓

relationship b/w concentratⁿ and fluorescence becomes non linear (if abs. > 2%)

↓

problem is more common in right angle fluor... emission slit is set to center of sample cell.

↑ absorption



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→ problem solution

(1) use of front-surface fluorescence instrument
↓
emission slit is located near the front edge of sample cell.

(2) Dilution of Solution

(II) Concentration quenching

(i) Detectⁿ of macro-molecules eg Ab
↓
heavily labeled c̄ fluorophore.

OR (ii) in flow cytometry / laser induced fluorescence
↓
where ↑ed density of fluorescein label.

↓

when excited, fluoroscence lables are in a close proximity
↓
radiation-less energy transfer
↓
quenching
↓
Resulting fluorescence is much lower than expected.

sky is blue bcz of Rayleigh scattering of short
rays → blue by molecules having size less than λ

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(3) light scattering:-

Rayleigh scattering
- scattering without change in λ.
- more common in fluorophores with excitation and emission spectra overlap (small stokes shift) → background light scatter
- minimised by use of excitation and emission interference filter or use of polarizer.

Raman scattering
- with lengthening of wavelength
- Independent of excitation λ and its a property of solvent
- minimised by setting excitation and emission λ far enough to prevent Raman scattering & narrowing slit width.

(4) Cuvet material and solvent effects:-

certain Cuvet materials — quartz glass / plastic
↓
contain UV absorbers
↓
give fluoresce.

certain solvent → e.g. ethanol
↓
give fluorescence.

- Cuvet and solvent with minimal fluorescence emission are commercially available.
↳ ↓ background fluorescence