



Principles of Fluorescence Techniques
Genova, Italy
June 14-16, 2004

Basic Fluorescence Principles I: David Jameson
**Absorption, Excitation and Emission Spectra,
Quantum Yield, Polarization/Anisotropy**

Why fluorescence?

Also fluorescence is very, very, very sensitive!

Work with subnanomolar concentrations is routine while femtomolar and even SINGLE MOLECULE studies are possible with some effort



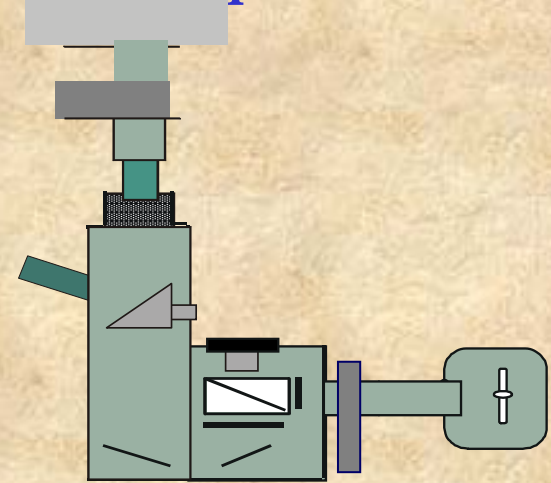
during the excited state lifetime – such as movements of proteins or protein domains

Instrumentation

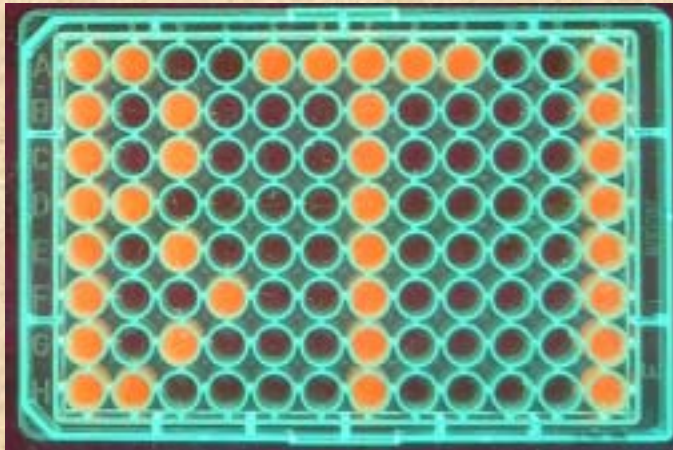
Fluorimeters



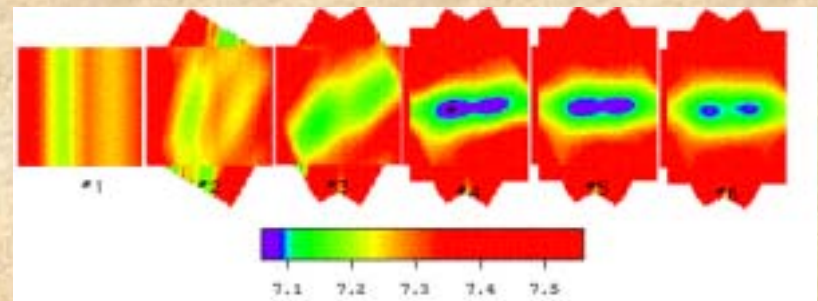
Microscopes



High throughput Plateraders



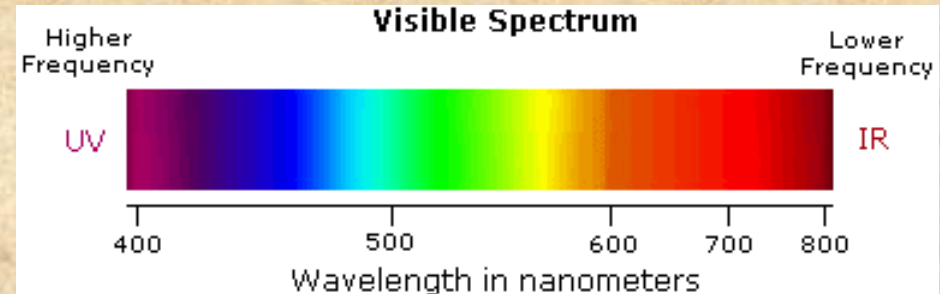
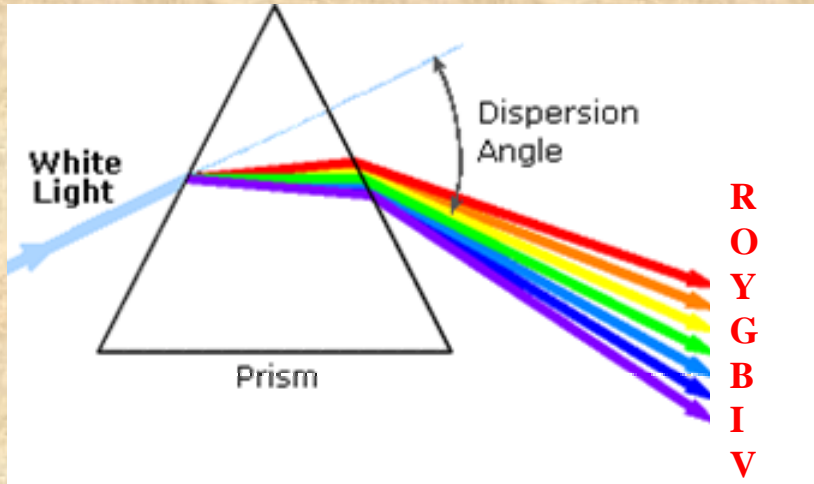
Intravital imaging systems



A very brief history of the study of light

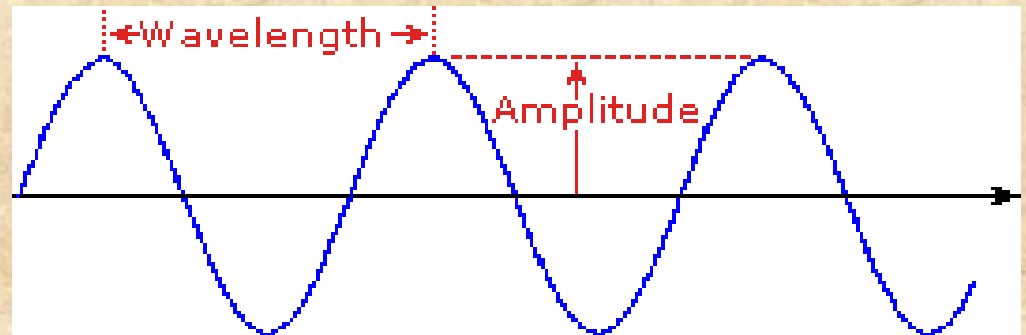
1. Sir Isaac Newton 1672:

Showed that the component colors of the visible portion of white light can be separated through a prism, which acts to bend the light (refraction) in differing degrees according to wavelength. Developed a “corpuscular” theory of light.



2. Christian Huygens 1692:

Developed a wave theory of light



3. Hans Christian Oersted 1820

Showed that there is a magnetic field associated with the flow of electric current

4. Michael Faraday 1831

Showed the converse i.e. that there is an electric current associated with a change of magnetic field

5. James Clark Maxwell: 1865

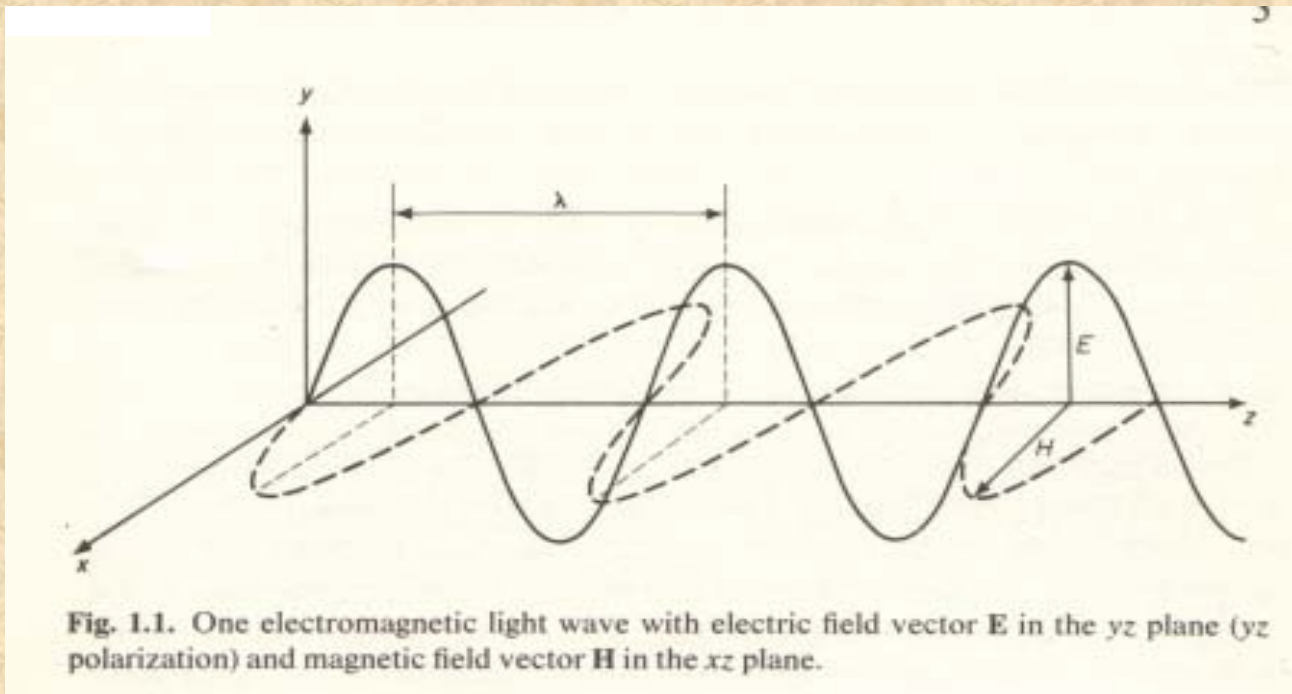
Published his “Dynamical theory of the electromagnetic field” which combined the discoveries of Newton, Young, Foucault, Oersted and Faraday into a unified theory of electromagnetic radiation

Showed that light consists of electromagnetic transverse waves whose frequency of vibration (ν) and wavelength (λ) are related by

$$\nu\lambda = v$$

Where v is the the speed of light in the medium of study

(for a vacuum $v = c$, where $c = 3 \times 10^{10}$ cm/sec) so $\lambda\nu = c$



We need to concern ourselves with how molecules interact with electromagnetic waves.

Absorption: general principles

The Beer Lambert Law

The absorption strength of a molecule can be determined by absorption measurements using

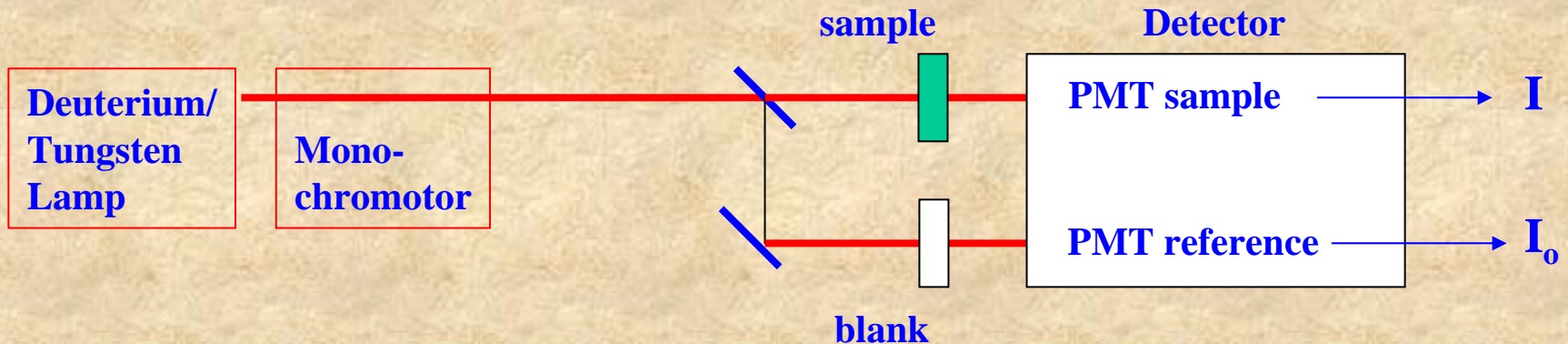
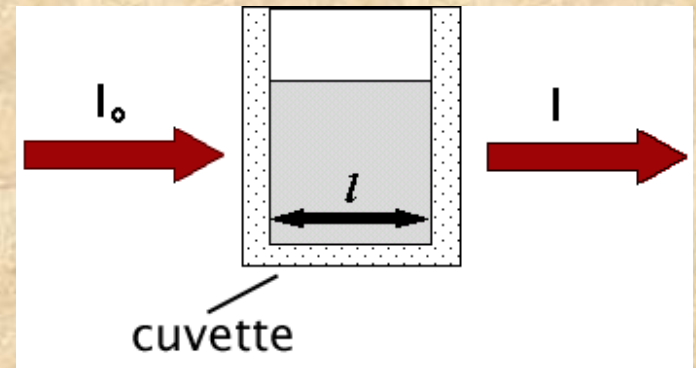
The Beer-Lambert Law, which is expressed as:

$$\text{Absorption (Optical Density)} = \log I_0 / I = \epsilon c l$$

I_0 and I are the intensities entering and leaving the sample respectively

ϵ is the molar extinction coefficient or molar

l is the pathlength of the sample (1 cm)



Dynamic range of absorption values

- ✓ An OD of 1.0 - for every 100 photons entering the sample, 10 leave without being absorbed
- ✓ An OD of 2.0 - for every 100 photons entering the sample, only 1 leaves without being absorbed
- ✓ OD = 3? - measuring the difference between 999 and 1000 photons is difficult!
- ✓ The useful range of absorption is **0.01-2.0 OD units**

Fluorescein

The extinction coefficient of fluorescein is $\sim 72,000 \text{ M}^{-1}\text{cm}^{-1}$

An absorption of 0.022 would correspond to a concentration of $\sim 3 \times 10^{-7} \text{ M}$

An absorption of 2.16 would correspond to a concentration of $3 \times 10^{-5} \text{ M}$

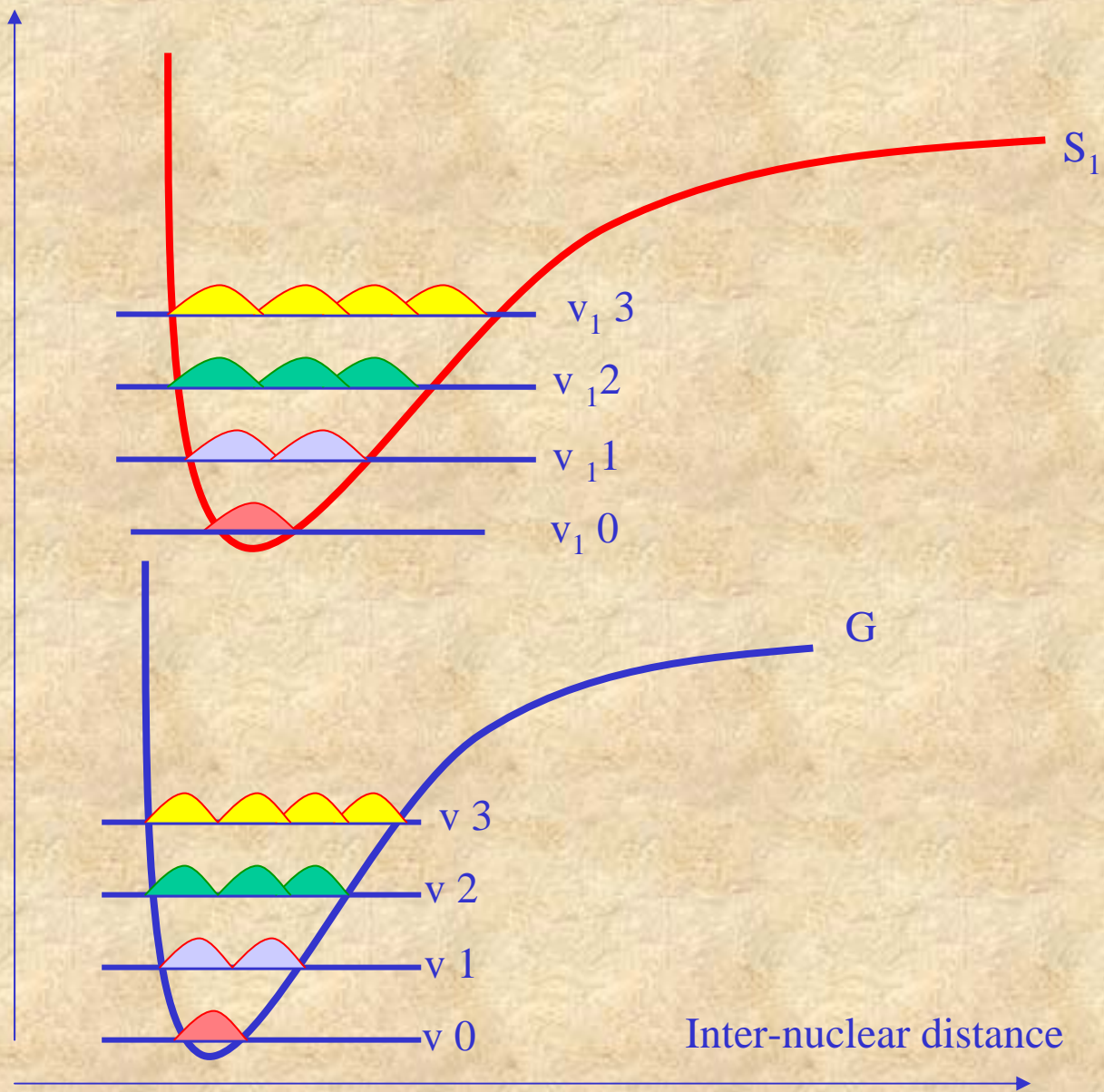
Electronic transitions from the ground state to the excited state

Shaded areas reflects the probability of where the electron would be if it were in that vibrational band

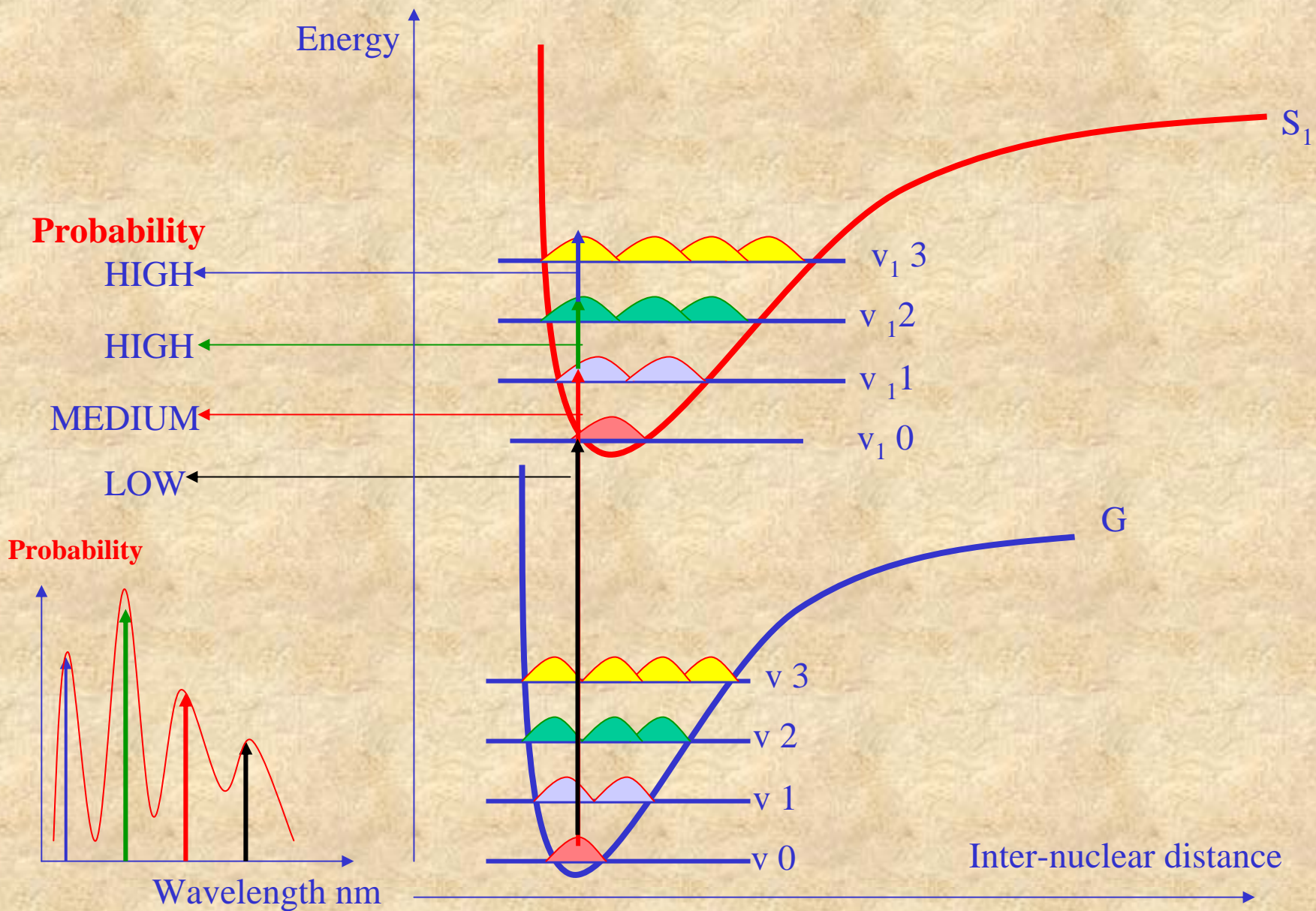
Most favored transitions occur **From** the

Maximum Shaded areas of the ground state

To the maximum shaded areas of the excited state



Electronic transitions from the ground state to the excited state



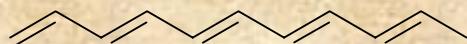
Absorption maxima : The importance of conjugation

The wavelength value of the **absorption maximum**
and the **molar absorptivity**

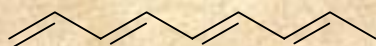
are determined by the degree of **Conjugation of π -bonds**

Increasing the number of double bonds shifts the absorption to lower energy

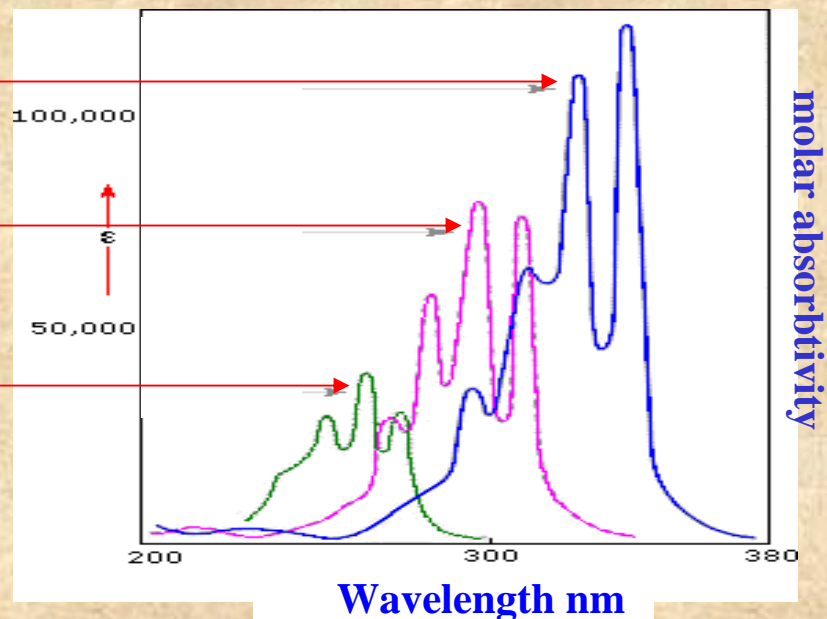
N=5
5 pi-bonds, 10 electrons



N=4
4 pi-bonds, 8 electrons



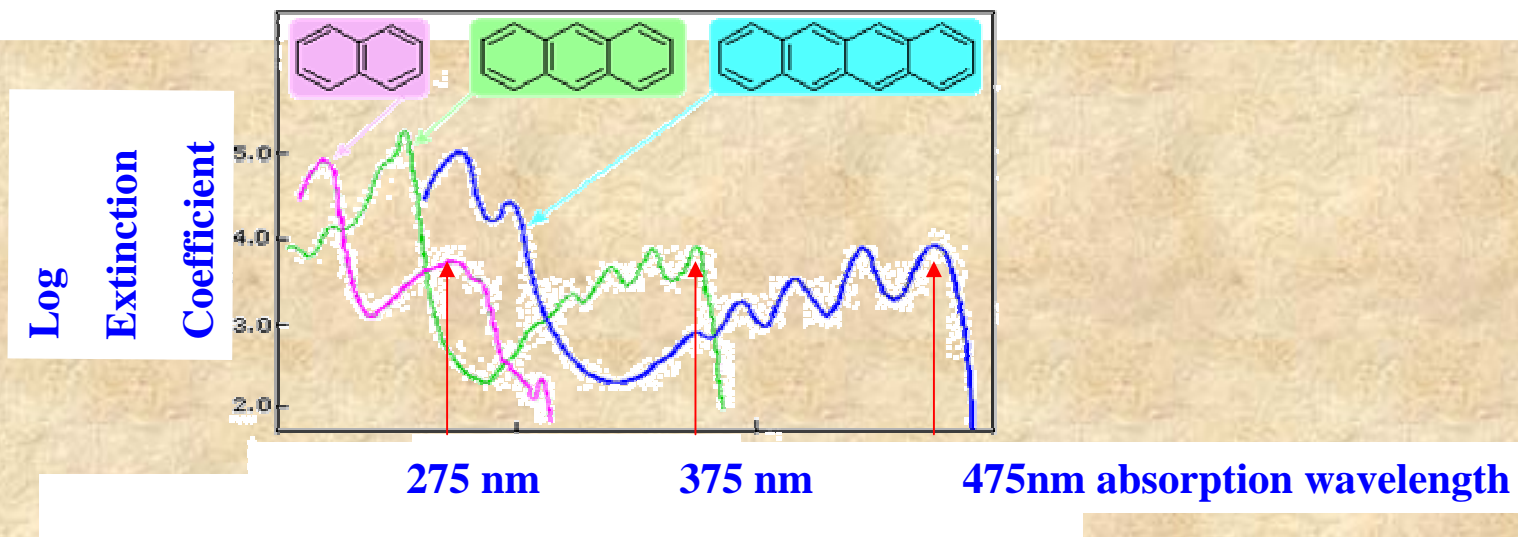
N=3
3 pi-bonds, 6 electrons



Increasing the number of aromatic rings increases the absorption maximum

Benzene < Naphthalene < Anthracene < naphthacene < pentacene

Abs. Max	262nm	275 nm	375 nm	475 nm	580 nm
Log ϵ (Extinction)	3.84	3.75	3.90	4.05	4.20



As the **degree of conjugation increases**

(i.e the number of electrons involved in the delocalized π -orbitals)

the absorption energy decreases ($> \lambda$, the energy between the ground and excited state decreases)

the absorption becomes more intense ($> \epsilon$, increased probability of absorption)

The discovery and characterization of Luminescence

Nicolás Monardes (1577), a Spanish physician and botanist who wrote on medicines of the New World, was the first to describe the bluish opalescence of the water infusion from the wood of a small Mexican tree. *When made into cups and filled with water, a peculiar blue tinge was observed.*

This wood was very popular in XVI - XVII Europe, where it was known as "Lignum nephriticum" (kidney wood), because of its medicinal virtues for treating kidney ailments. An Englishman, John Frampton, translated Mondares description as

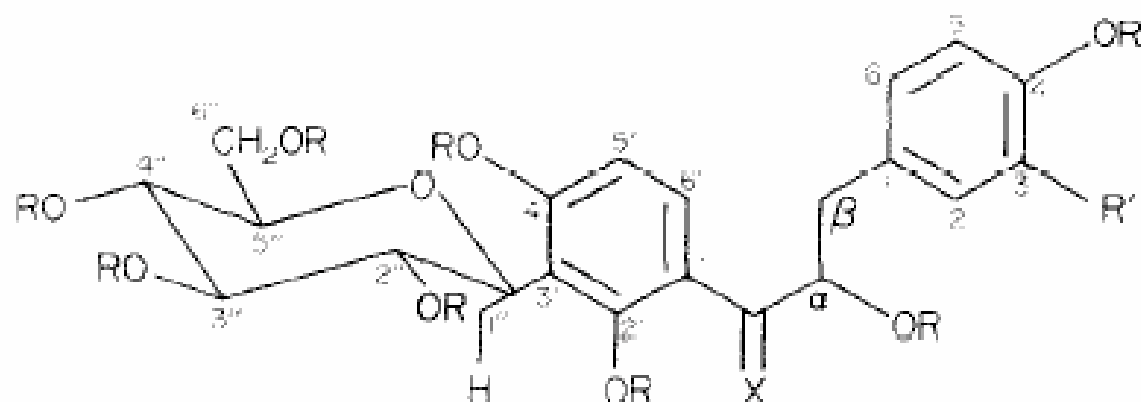
“.. white woodde which gives a blewe color” when placed in water that was good “for them that doeth not pisse liberally and for the pains of the Raines of the stone..”

In the ensuing centuries the wood was no longer used and the botanic identity of the LN was lost in a confusion of several species. Safford, in 1915, succeeded in disentangling the botanic problem and identified the species which produced the Mexican LN as *Eynsemhardtia polystachia*. More recently, several highly fluorescent glucosyl-hydroxichalcones were isolated from this plant.

COATLINE A AND B, TWO C-GLUCOSYL- α -HYDROXYDIHYDROCHALCONES FROM *EYSENHARDTIA POLYSTACHYA*

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- 1a** X = O; R = H; R' = H
1b X = O; R = H; R' = OH
1c X = O; R = Ac; R' = H
1d X = O; R = Ac; R' = OAc
2 X = H,OH; R = Ac; R' = OAc

Robert Boyle (1664) was inspired by Monardes' report and investigated this system more fully. He discovered that after many infusions the wood lost its power to give color to the water and concluded that there was some "essential salt" in the wood responsible for the effect. He also discovered that addition of acid abolished the color and that addition of alkali brought it back. Hence Boyle was the first to use fluorescence as a pH indicator!

Galileo (1612) described the emission of light (phosphorescence) from the famous Bolognian stone, discovered by Vincenzo Casciarolo, a Bolognian shoemaker. Galileo wrote: "It must be explained how it happens that the light is conceived into the stone, and is given back after some time, as in childbirth."

Sir John Herschel (1845) made the first observation of fluorescence from quinine sulfate - he termed this phenomenon "epipolic dispersion"

Sir George Stokes (1852) coined the term "Fluorescence". This name was suggested by Stokes to replace the term "dispersive reflection" which he used in his initial treatise: he wrote "I confess that I do not like the term. I am inclined to coin a term and call it *fluorescence*, from fluor-spar, as the analogous term opalescence is derived from the name of a mineral."

Stokes used a prism to obtain the ultraviolet region of the solar spectrum to illuminate a quinine solution and observed the emission through a stained glass filter. He wrote: "It was certainly a curious sight to see the tube instantaneously light up when plunged into the invisible rays; it was literally darkness visible." This observations led Stokes to proclaim that fluorescence is of longer wavelength than the exciting light, which led to this displacement being called the **Stokes Shift**

Adolph Von Beyer (1871) a German chemist, synthesized Spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one, 3',6'-dihydroxy.

FLUORESC EIN!!!

R. Meyer (1897) used the term “fluorophore” to describe chemical groups which tended to be associated with fluorescence; this word was analogous to “chromophore” which was first used in 1876 by O.N. Witt to describe groups associated with color.

K. Noack (1887) published a book listing 660 compounds arranged according to the color of their fluorescence.

Earliest example of a Molecular Probes catalog!!!

Gregorio Weber (1952) synthesized dansyl chloride for attachment to proteins and used polarization to study protein hydrodynamics - these studies initiated the field of quantitative biological fluorescence.

Shimomura, Johnson and Saiga (1962) discovered Green Fluorescent Protein in the *Aequorea* jellyfish

Fluorescence in the 20th Century

Most of the basic principles of fluorescence were developed during the 1920's and 1930's.

Excited state lifetime (Gaviola)

Quantum yield (Wavilov)

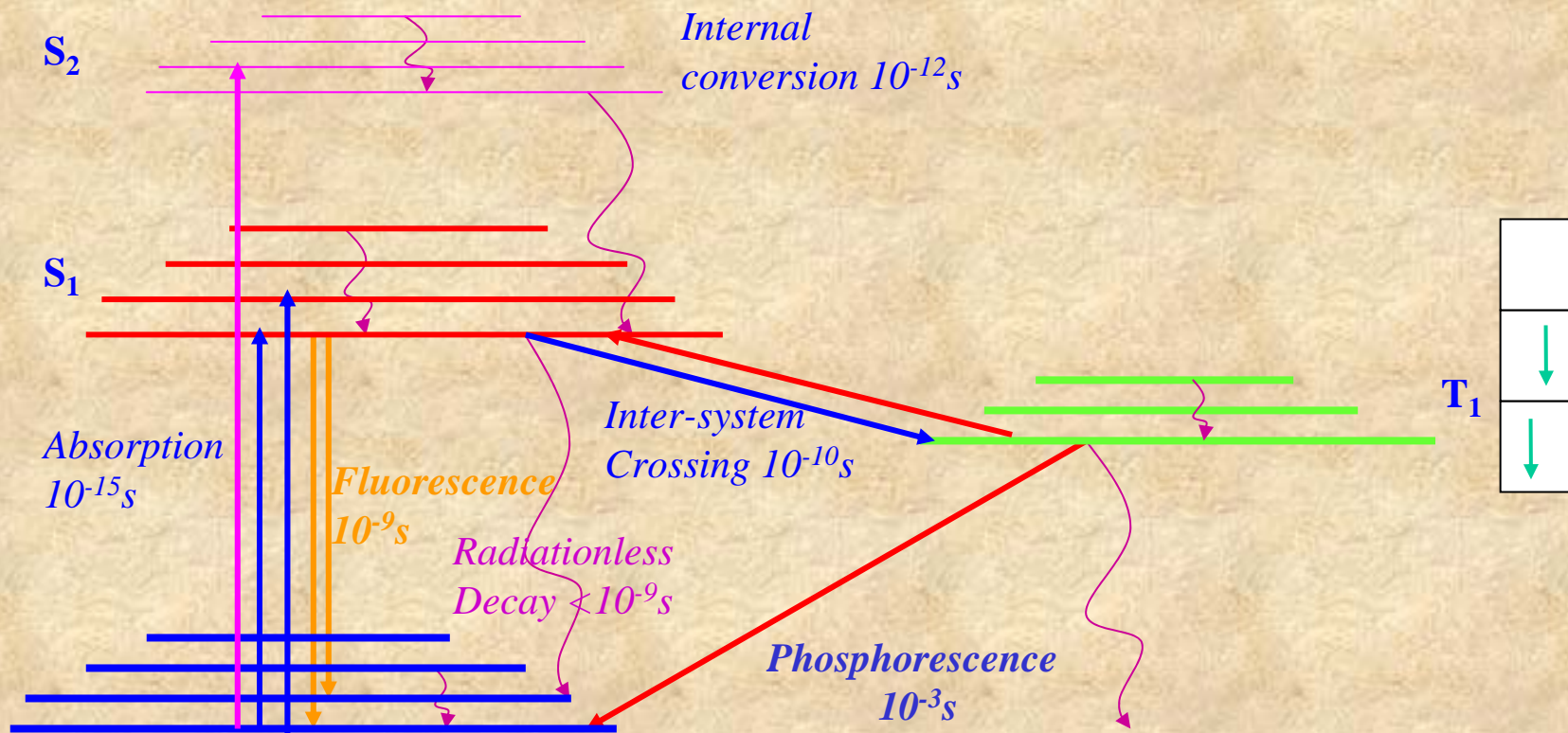
Polarization of fluorescence (Weigert, F. Perrin)

Fluorescence resonance energy transfer (J. and F. Perrin; T. Förster)

Until the second half of the 20th century, however, the use of fluorescence in biology and biochemistry was, *descriptive* in nature and primarily limited to a role in the isolation, purification and quantification of fluorescent substances such as riboflavin and porphyrins. True “quantitative” biological fluorescence began with the pioneering work of Gregorio Weber

The Perrin-Jablonski Diagram

The life history of an excited state electron in a luminescent probe



Key points:

- ✓ Excitation spectra are mirror images of the emission spectra
- ✓ Emission has lower energy compared to absorption
- ✓ Triplet emission is lower in energy compared to singlet emission
- ✓ Most emission/quenching/FRET/chemical reactions occur from the lowest vibrational level of $[S]_1$

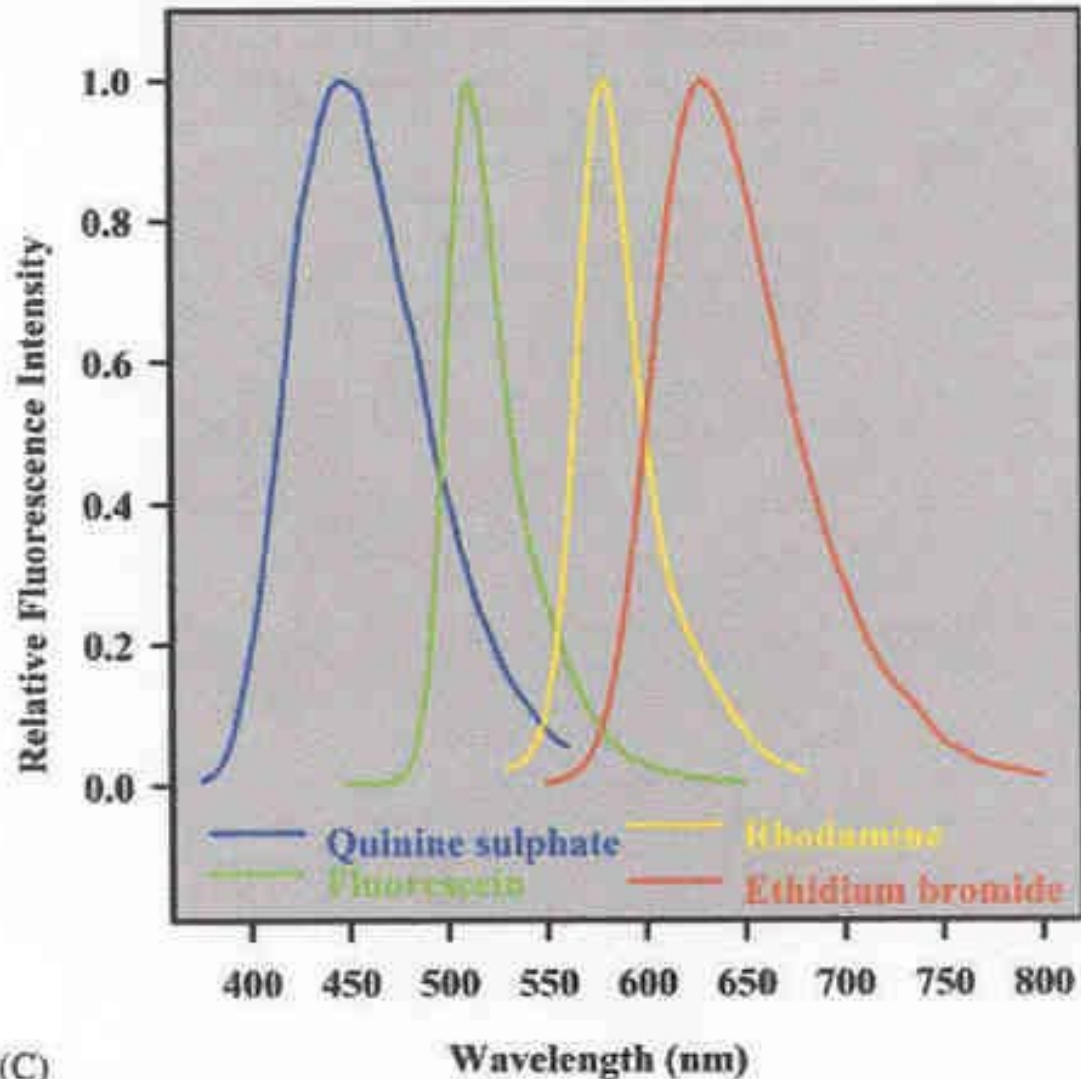
Virtually all fluorescence data required for any research project will fall into one of the following categories.

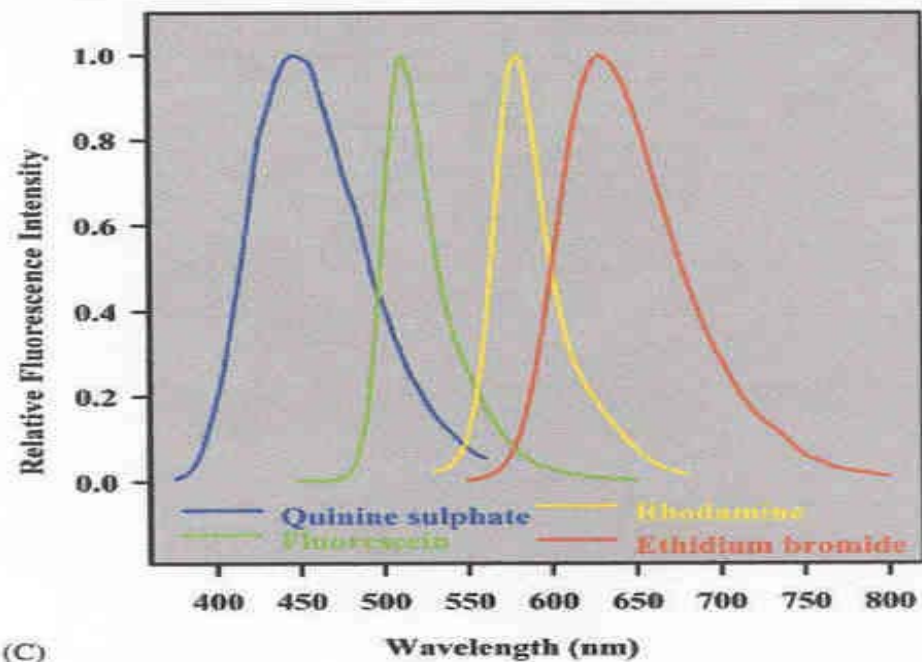
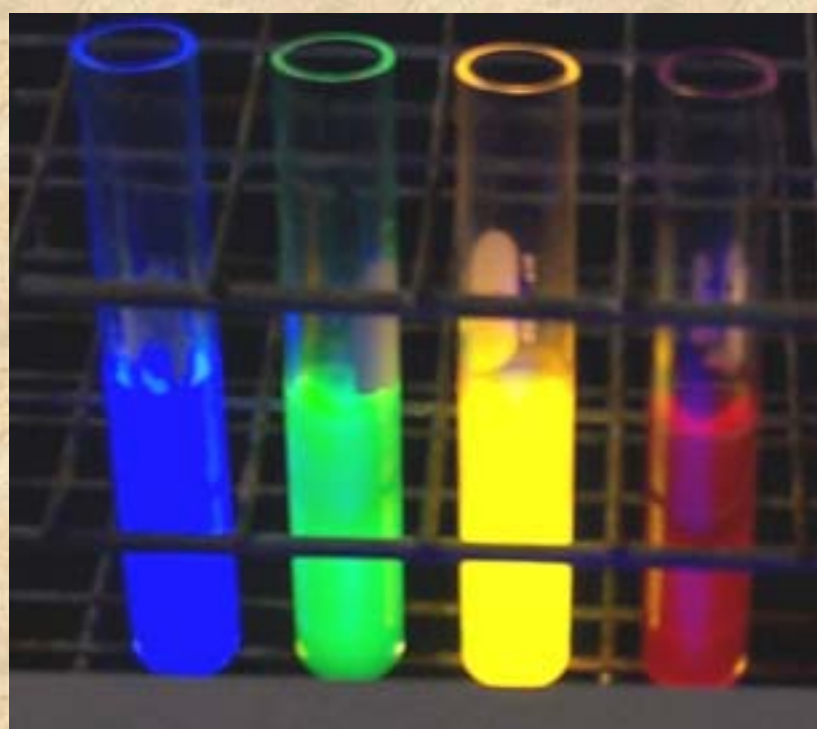
1. The fluorescence emission spectrum
2. The excitation spectrum of the fluorescence
3. The quantum yield
4. The polarization (anisotropy) of the emission
5. The fluorescence lifetime

In these lectures, we examine each of these categories and briefly discuss historical developments, underlying concepts and practical considerations

The fluorescence emission spectrum

In a typical emission spectrum, the excitation wavelength is fixed and the fluorescence intensity versus wavelength is obtained





Early examination of a large number of emission spectra resulted in the formulation of certain general rules:

1) In a pure substance existing in solution in a unique form, the fluorescence spectrum is invariant, remaining the same independent of the excitation wavelength

2) The fluorescence spectrum lies at longer wavelengths than the absorption

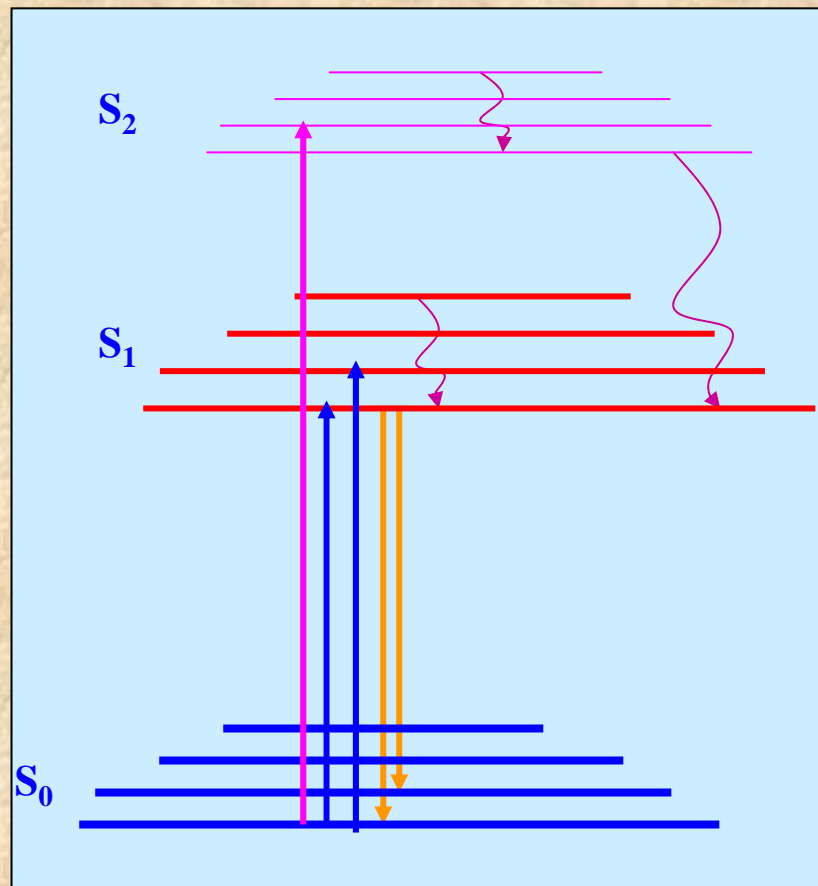
3) The fluorescence spectrum is, to a good approximation, a mirror image of the absorption band of least frequency

These general observations follow from consideration of the Perrin-Jabłoński diagram shown earlier

Specifically, although the fluorophore may be excited into different singlet state energy levels (e.g., S_1 , S_2 , etc) rapid thermalization invariably occurs and emission takes place from the lowest vibrational level of the first excited electronic state (S_1). This fact accounts for the independence of the emission spectrum from the excitation wavelength.

The fact that ground state fluorophores, at room temperature, are predominantly in the lowest vibrational level of the ground electronic state (as required from Boltzmann's distribution law) accounts for the Stokes shift.

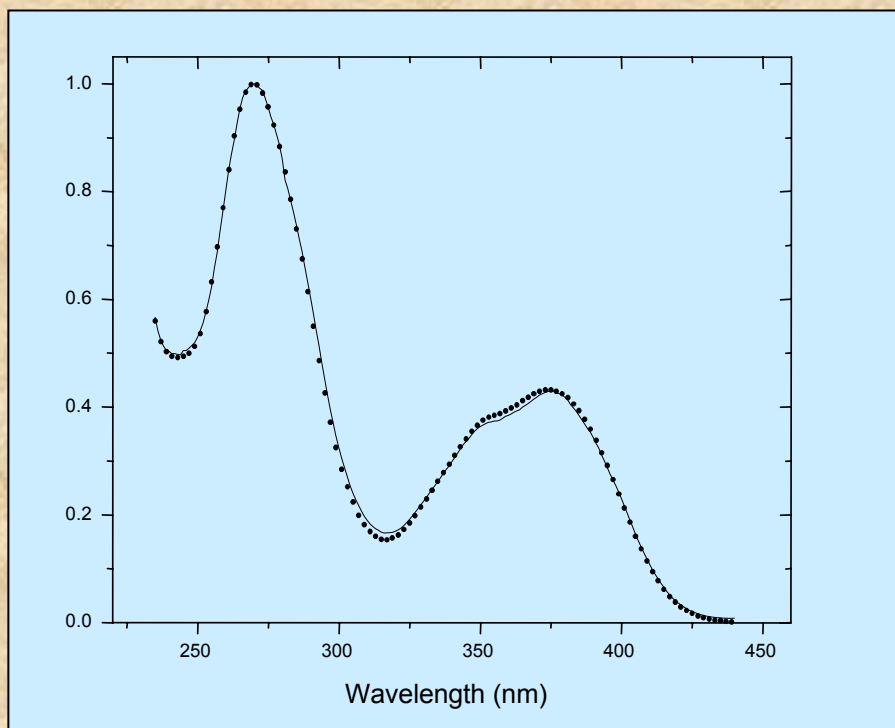
Finally, the fact that the spacings of the energy levels in the vibrational manifolds of the ground state and first excited electronic states are usually similar accounts for the fact that the emission and absorption spectra (plotted in energy units such as reciprocal wavenumbers) are approximately mirror images



The fluorescence excitation spectrum

The relative efficiencies of different wavelengths of incident light to excite fluorophores is determined as the excitation spectrum. In this case, the excitation monochromator is varied while the emission wavelength is kept constant if a monochromator is utilized - or the emitted light can be observed through a filter.

If the system is “well-behaved”, i.e., if the three general rules outlined above hold, one would expect that the excitation spectrum will match the absorption spectrum. In this case, however, as in the case of the emission spectrum, corrections for instrumentation factors are required.



Overlay of Absorption Spectrum and Corrected Excitation Spectrum for ANS in ethanol

Quantum Yield

The quantum yield of fluorescence (QY) is dependent on the **rate of the emission process** divided by the sum of the **rates of all other deactivation processes**

$$QY = k_f / k_f + k_i + k_x$$

k_f is the rate of fluorescence, k_i is the rate of radiationless decay and k_x is the rate of intersystem crossing.

Another way to think about QY is:

QY = Number of emitted photons / Number of absorbed photons

If the rates of the deactivation processes are slow compared to k_f then the QY **is high**

However, if the rates of these other processes are fast compared to k_f then **QY is low**

List of quantum yields from “Molecular Fluorescence” by Bernard Valeur

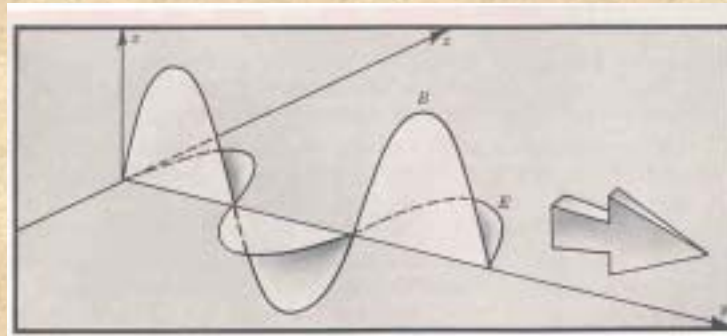
Tab. 6.1. Standards for the determination of fluorescence quantum yields

Range	Compound	Temp. (°C)	Solvent	Φ_F	Ref.
270–300 nm	Benzene	20	Cyclohexane	0.05 ± 0.02	1
300–380 nm	Tryptophan	25	H ₂ O (pH 7.2)	0.14 ± 0.02	2
300–400 nm	Naphthalene	20	Cyclohexane	0.23 ± 0.02	3
315–480 nm	2-Aminopyridine	20	0.1 mol L ⁻¹ H ₂ SO ₄	0.60 ± 0.05	4
360–480 nm	Anthracene	20	Ethanol	0.27 ± 0.03	1, 5
400–500 nm	9,10-diphenylanthracene	20	Cyclohexane	0.90 ± 0.02	6, 7
400–600 nm	Quinine sulfate dihydrate	20	0.5 mol L ⁻¹ H ₂ SO ₄	0.546	5, 7
600–650 nm	Rhodamine 101	20	Ethanol	1.0 ± 0.02	8
				0.92 ± 0.02	9
600–650 nm	Cresyl violet	20	Methanol	0.54 ± 0.03	10

- 1) Dawson W. R. and Windsor M. W. (1968) *J. Phys. Chem.* **72**, 3251.
- 2) Kirby E. P. and Steiner R. F. (1970) *J. Phys. Chem.* **74**, 4480.
- 3) Berlman I. B. (1965) *Handbook of Fluorescence Spectra of Aromatic Molecules*, Academic Press, London.
- 4) Rusakowicz R. and Testa A. C. (1968) *J. Phys. Chem.* **72**, 2680.
- 5) Melhuish W. H. (1961) *J. Phys. Chem.* **65**, 229.
- 6) Hamai S. and Hirayama F. (1983) *J. Phys. Chem.* **87**, 83.
- 7) Meech S. R. and Phillips D. (1983) *J. Photochem.* **23**, 193.
- 8) Karstens T. and Kobs K. (1980) *J. Phys. Chem.* **84**, 1871.
- 9) Arden-Jacob J., Marx N. J. and Drexhage K. H. (1997) *J. Fluorescence* **7(Suppl.)**, 91S.
- 10) Magde D., Brannon J. H., Cramers T. L. and Olmsted J. III (1979) *J. Phys. Chem.* **83**, 696.

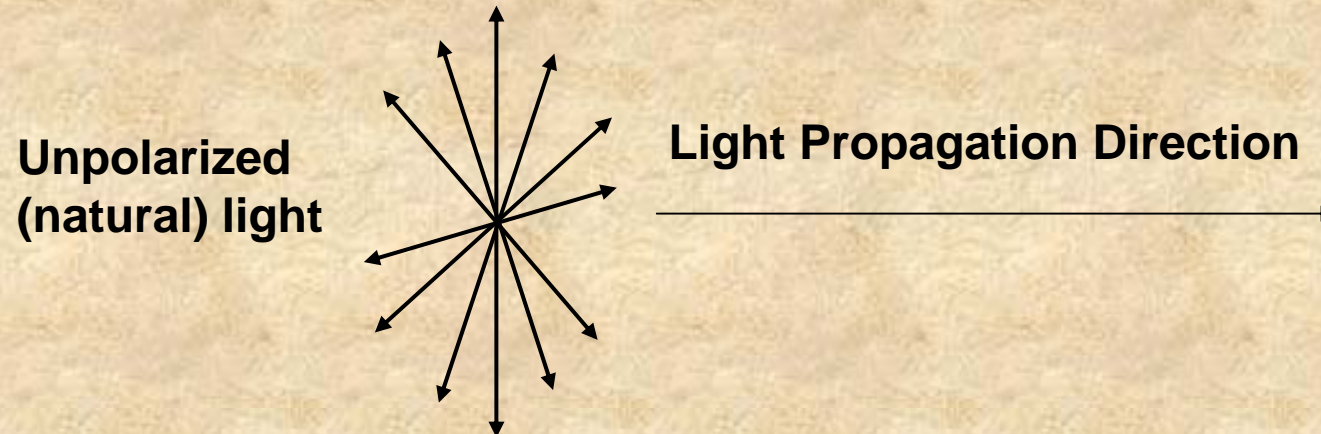
Polarization

As stated earlier, light can be considered as oscillations of an electromagnetic field – characterized by electric and magnetic components - perpendicular to the direction of light propagation.

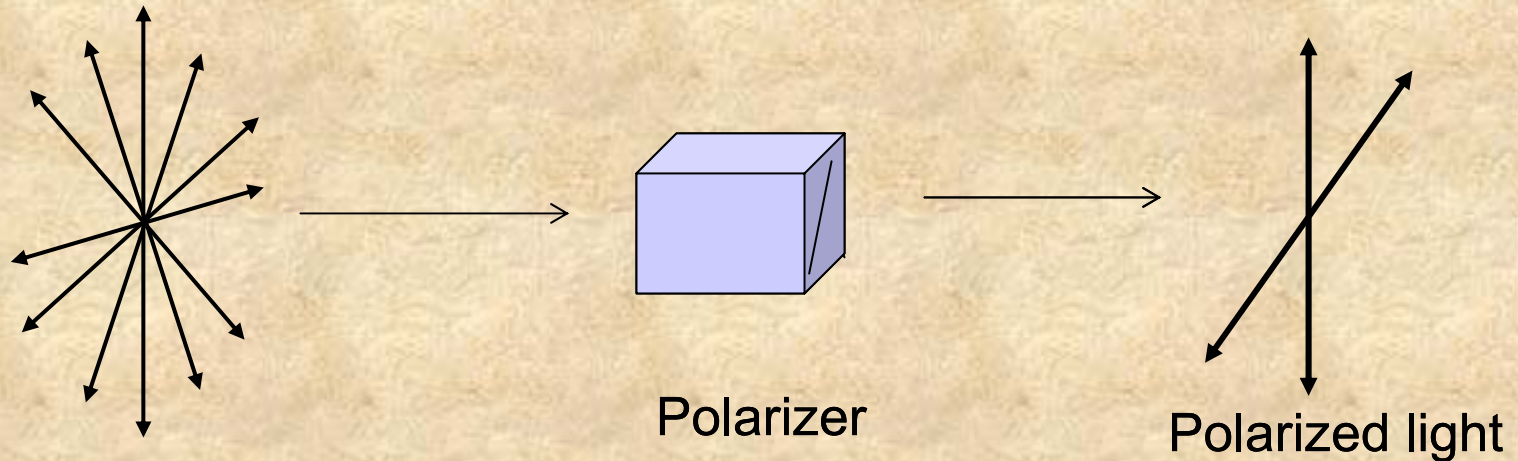


In these lectures we shall be concerned only with the electric component.

In natural light the electric field vector can assume any direction of oscillation perpendicular or normal to the light propagation direction.



Polarizers are optically active devices that can isolate one direction of the electric vector.



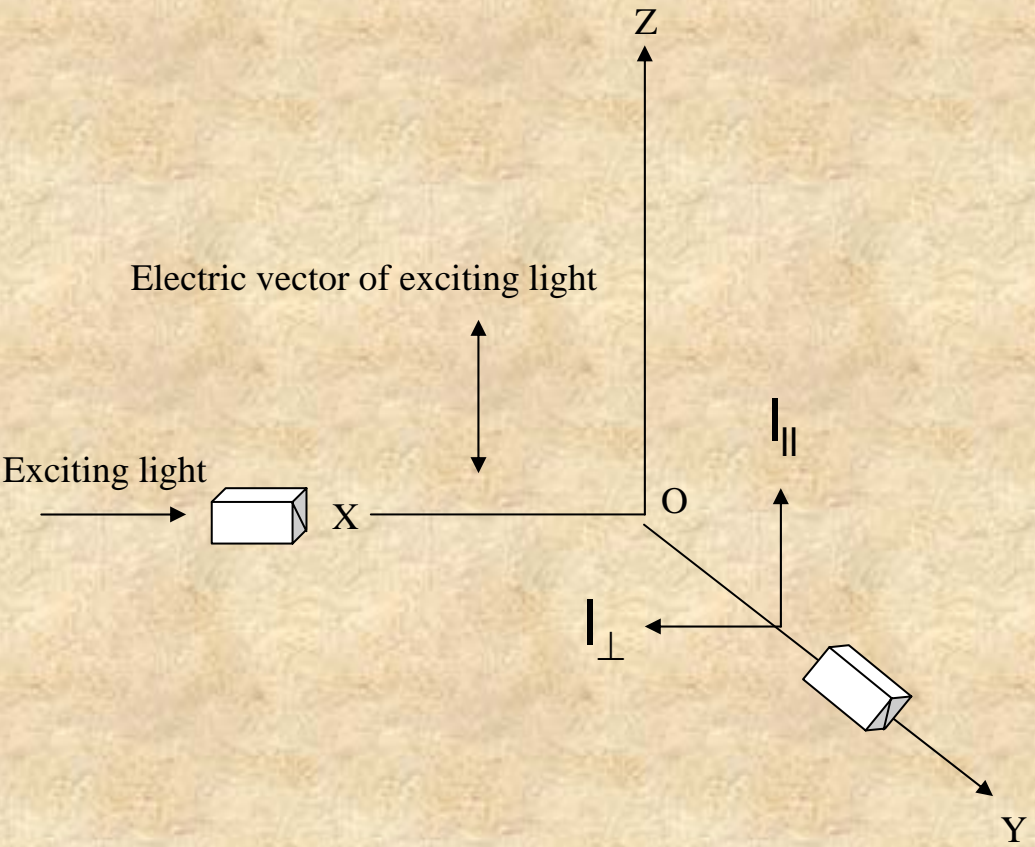
Unpolarized (natural) light

The most common polarizers used today are (1) dichroic devices, which operate by effectively absorbing one plane of polarization (e.g., Polaroid type-H sheets based on stretched polyvinyl alcohol impregnated with iodine) and (2) double refracting calcite (CaCO_3) crystal polarizers - which differentially disperse the two planes of polarization (examples of this class of polarizers are Nicol polarizers, Wollaston prisms and Glan-type polarizers such as the Glan-Foucault, Glan-Thompson and Glan-Taylor polarizers)

Polarizers have, in fact, been in use for a very long time - the Vikings used a “sunstone” (now thought to have been composed of the mineral cordierite, a natural polarizing material) to observe the location of the sun on foggy or overcast days. Since scattered sunlight is highly polarized compared to light coming along the direction to the sun, the distribution of the sky’s brightness could be observed through the sunstone and hence the sun’s position could be localized and, if the time of day were known, the compass directions.

In 1920, F. Weigert discovered that the fluorescence from solutions of dyes was polarized. Specifically, he looked at solutions of fluorescein, eosin, rhodamine and other dyes and noted the effect of temperature and viscosity on the observed polarization. Wiegert discovered that polarization increased with the size of the dye molecule and the viscosity of the solvent, yet decreased as the temperature increased. He recognized that all of these considerations meant that fluorescence polarization increased as the mobility of the emitting species decreased.

Consider an XYZ coordinate framework with a fluorescent solution placed at the origin, as shown below, where XZ is in the plane of the page.



In this system, the exciting light is traveling along the X direction. If a polarizer is inserted in the beam, one can isolate a unique direction of the electric vector and obtain light polarized parallel to the Z axis which corresponds to the vertical laboratory axis.

This exciting light will be absorbed by the fluorophore at the origin and give rise to fluorescence which is typically observed at 90° to the excitation direction, i.e., from along the Y axis.

We initially consider that this fluorescence can have any direction of polarization. The actual direction of the electric vector of the emission can be determined by viewing the emission through a polarizer which can be oriented alternatively in the parallel or perpendicular direction relative to the Z axis or laboratory vertical direction. Polarization is then defined as a function of the observed parallel ($I_{||}$) and perpendicular intensities (I_{\perp}) :

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

If the emission is completely polarized in the parallel direction, i.e., the electric vector of the exciting light is totally maintained, then:

$$P = \frac{1 - 0}{1 + 0} = 1$$

If the emitted light is totally polarized in the perpendicular direction then:

$$P = \frac{0 - 1}{0 + 1} = -1$$

The limits of polarization are thus +1 to -1

Another term frequently used in the context of polarized emission is anisotropy (usually designated as either A or r) which is defined as:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

By analogy to polarization, the limits of anisotropy are +1 to -0.5.

A comment about the difference between polarization and anisotropy:

Given the definition of polarization and anisotropy, one can show that:

$$r = \frac{2}{3} \left(\frac{1}{P} - \frac{1}{3} \right)^{-1} \quad \text{or} \quad r = \frac{2P}{3 - P}$$

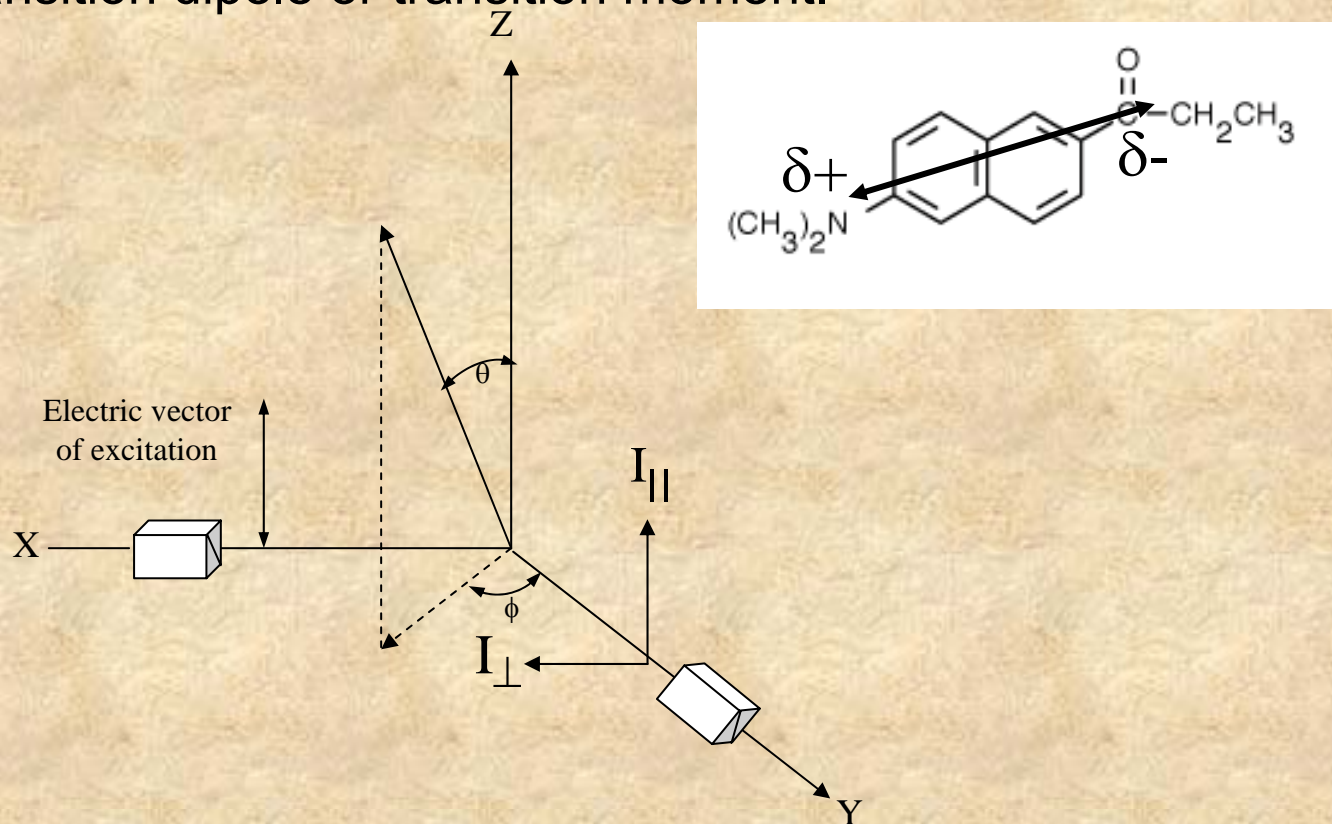
For example:

P	r
0.50	0.40
0.30	0.22
0.10	0.069

Clearly, the information content in the polarization function and the anisotropy function is identical and the use of one term or the other is dictated by practical considerations as will be discussed later.

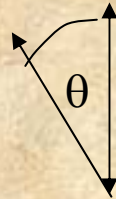
In solution these limits (e.g., +/-1) are not realized. Consider, as shown below, a fluorophore at the origin of our coordinate system.

Upon absorption of an exciting photon a dipole moment is created in the fluorophore (usually of different magnitude and direction from the ground state dipole). The orientation of this dipole moment relative to the nuclear framework, and its magnitude, will be determined by the nature of the substituents on the molecule. This excited state dipole moment is also known as the transition dipole or transition moment.

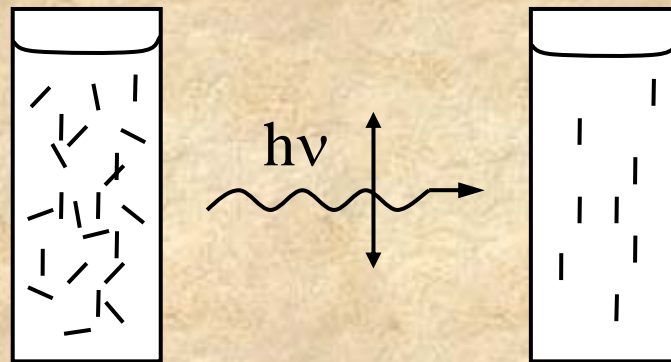


In fact, if light of a particular electric vector orientation (plane polarized light) impinges on a sample, only those molecules which are properly oriented relative to this electric vector can absorb the light.

Specifically, the probability of the absorption is proportional to the cosine squared of the angle θ between the exciting light and the transition dipole ($\cos^2 \theta$).



Hence, when we excite an ensemble of randomly oriented fluorophores with plane-polarized light we are performing a *photoselection* process, creating a population of excited molecules which nominally have their excited dipoles lines up with the polarization direction of the excitation. This process is illustrated below:



Potential dipoles

Excited state dipoles

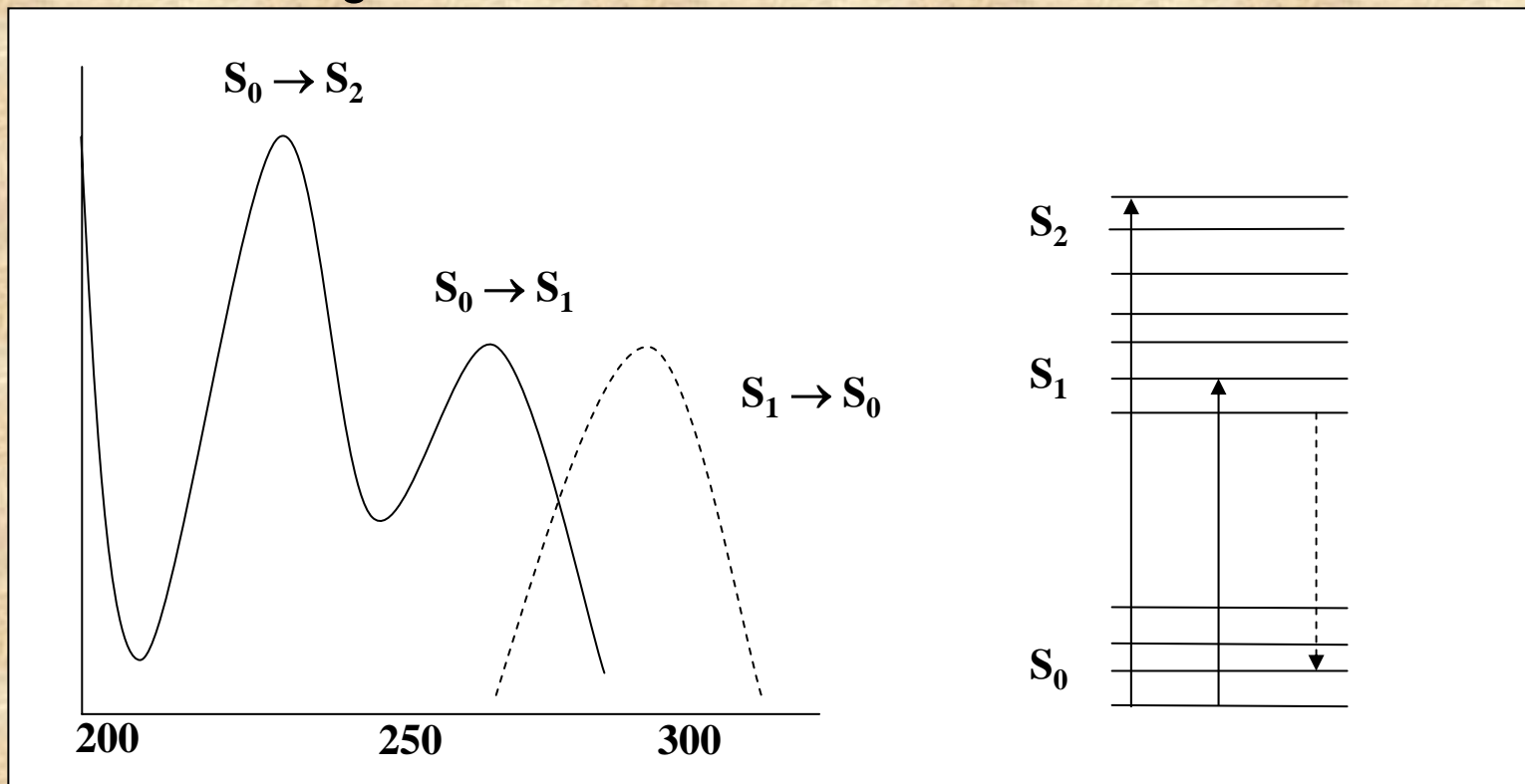
Consider now that the transition dipole corresponding to the emission of light from the excited fluorophore is parallel to the absorption dipole and that the excited fluorophore cannot rotate during the lifetime of the excited state (for example if the fluorophores are embedded in a highly viscous or frozen medium).

If we were to now measure the polarization of the emission it would be less than +1 since some of the dipoles excited will not be exactly parallel to the direction of the exciting light.

In fact, the number of potential dipoles making an angle θ with the vertical axis will be proportional to $\sin \theta$. We can then calculate that the upper polarization limit for such a randomly oriented (but rigidly fixed, i.e., non-rotating) ensemble - with co-linear excitation and emission dipole - will be +1/2 (we note that this limit is exceeded for two-photon excitation processes as will be discussed later).

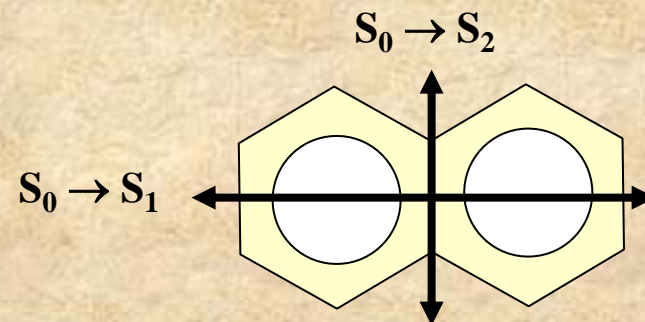
This case, however, assumes that the emission dipole is parallel (co-linear) to the absorption dipole.

Consider the general case shown below:



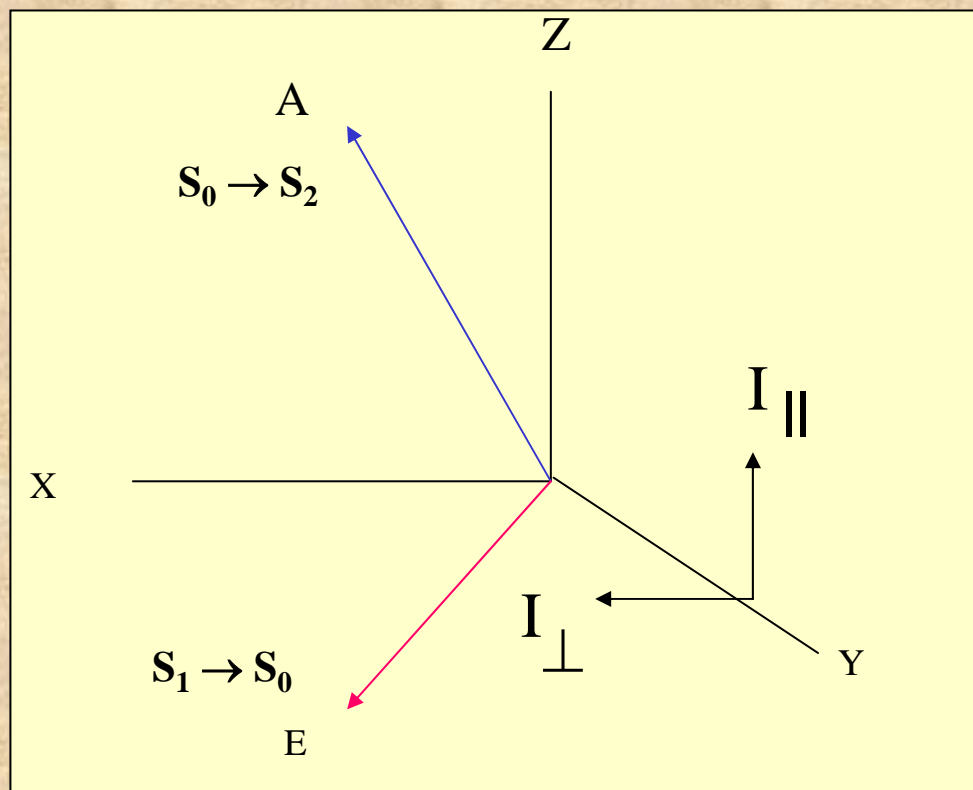
Here are depicted two principle absorption bands for a compound along with the emission band. The energy level diagram corresponding to this system is also depicted.

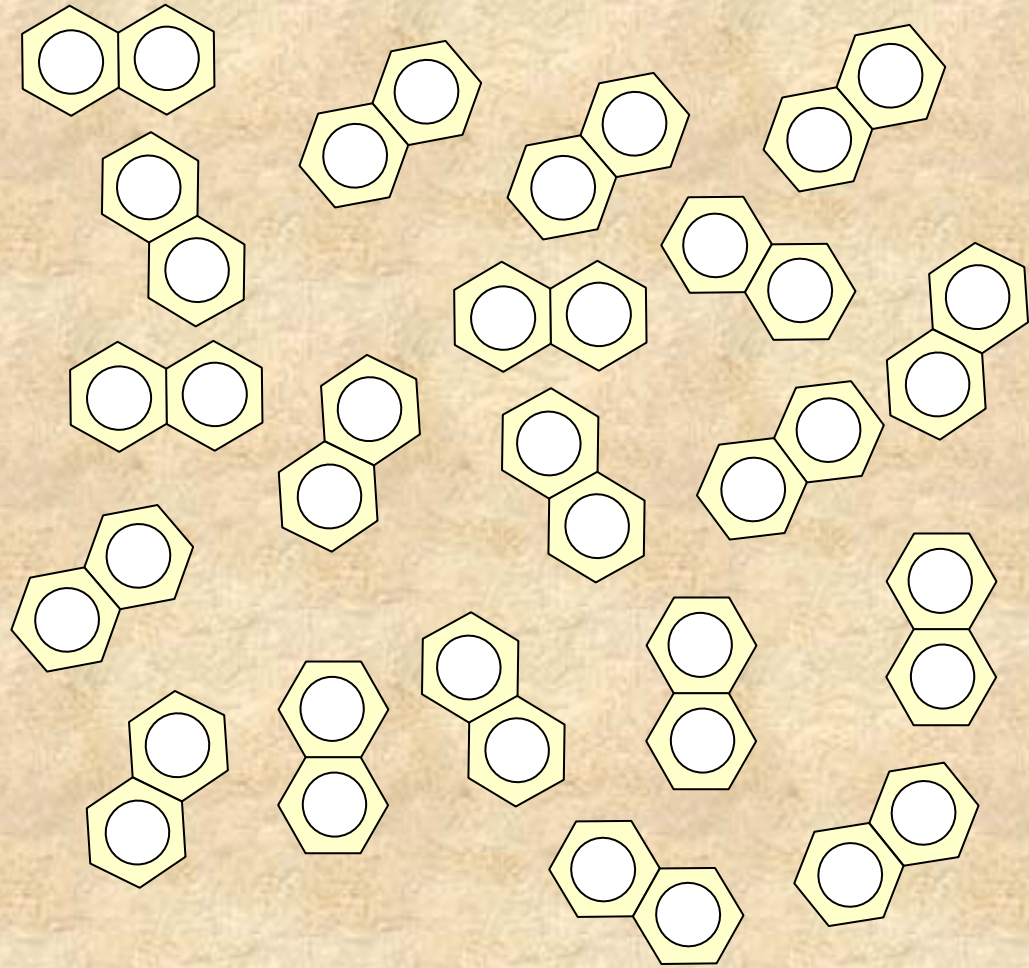
The directions of the absorption dipoles – relative to the nuclear framework – may differ greatly for the two transitions as illustrated on the right.

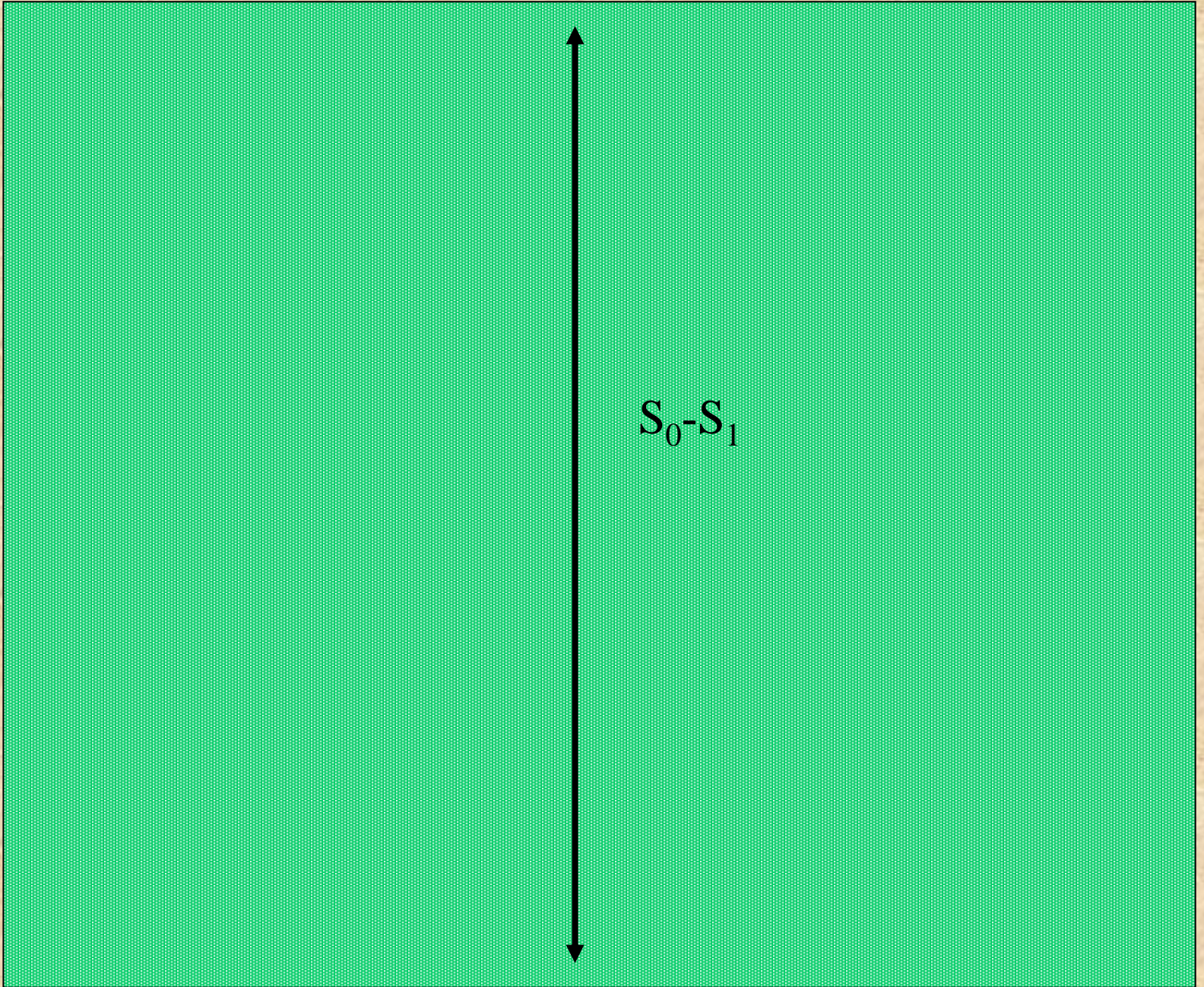


So we see that the two excited dipoles corresponding to the $S_0 \rightarrow S_1$ and the $S_0 \rightarrow S_2$ transitions may be oriented at an arbitrary angle - in the extreme case this angle could be 90° . After the excitation process, however, regardless of whether the absorption process corresponded to the $S_0 \rightarrow S_1$ or the $S_0 \rightarrow S_2$ transition, rapid thermalization leaves the excited fluorophore in the S_1 level. The orientation of the excited dipoles will thus now possess a different average orientation than the absorption dipoles originally photoselected by the exciting light.

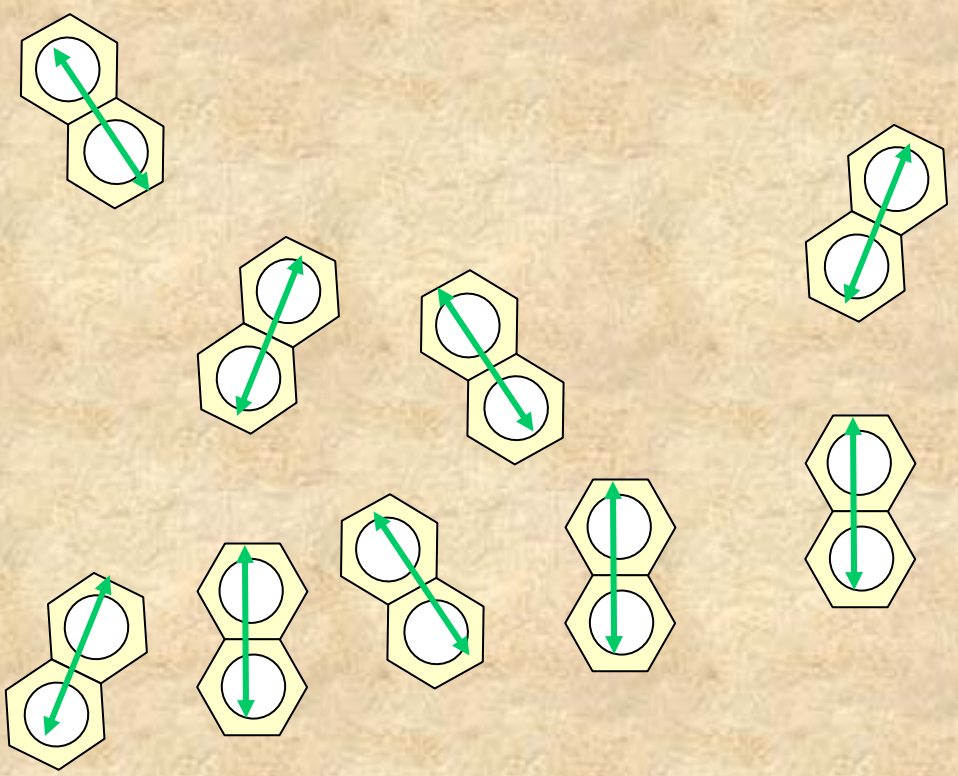
This situation is depicted below:

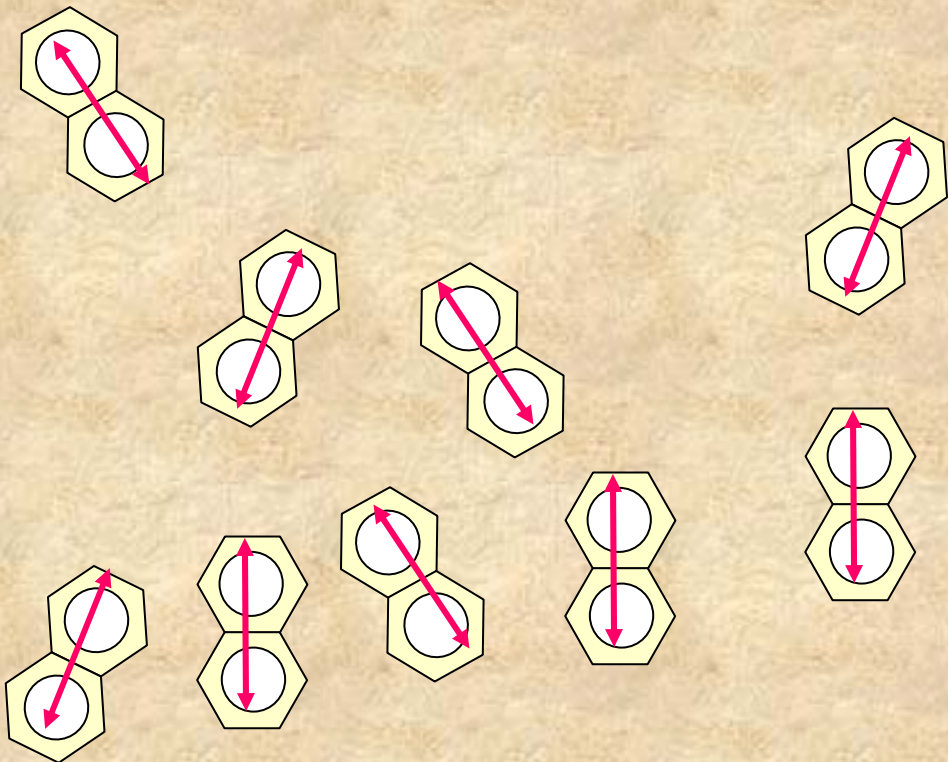




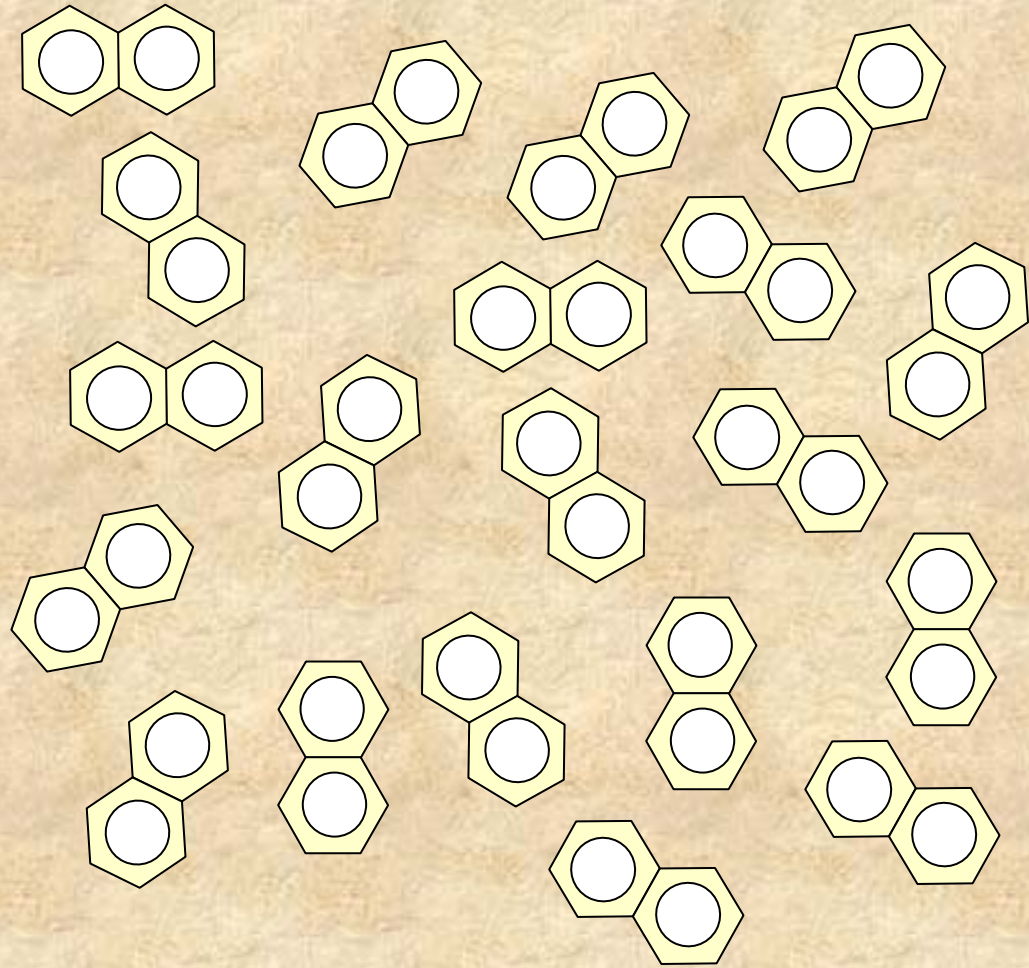


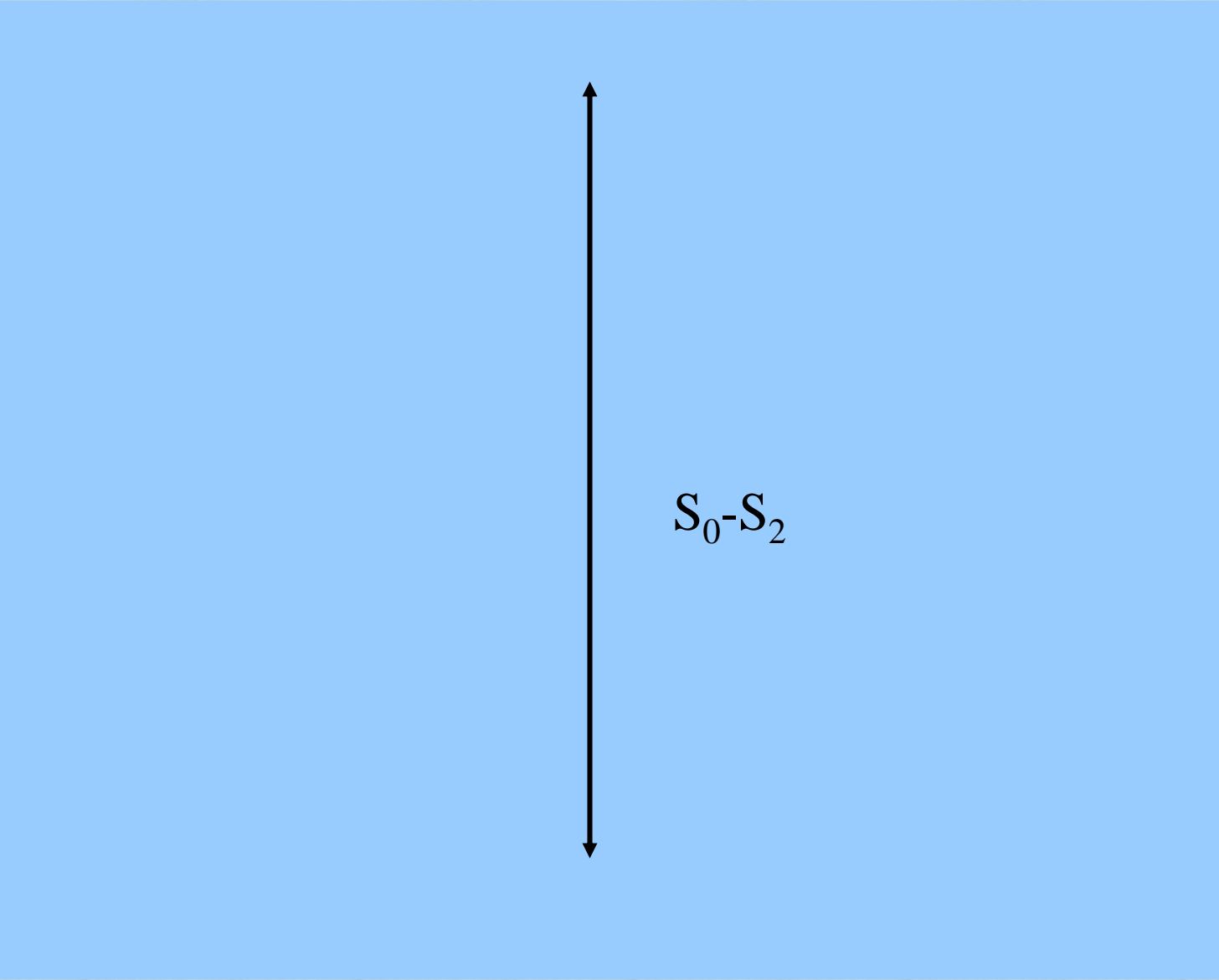
S_0-S_1



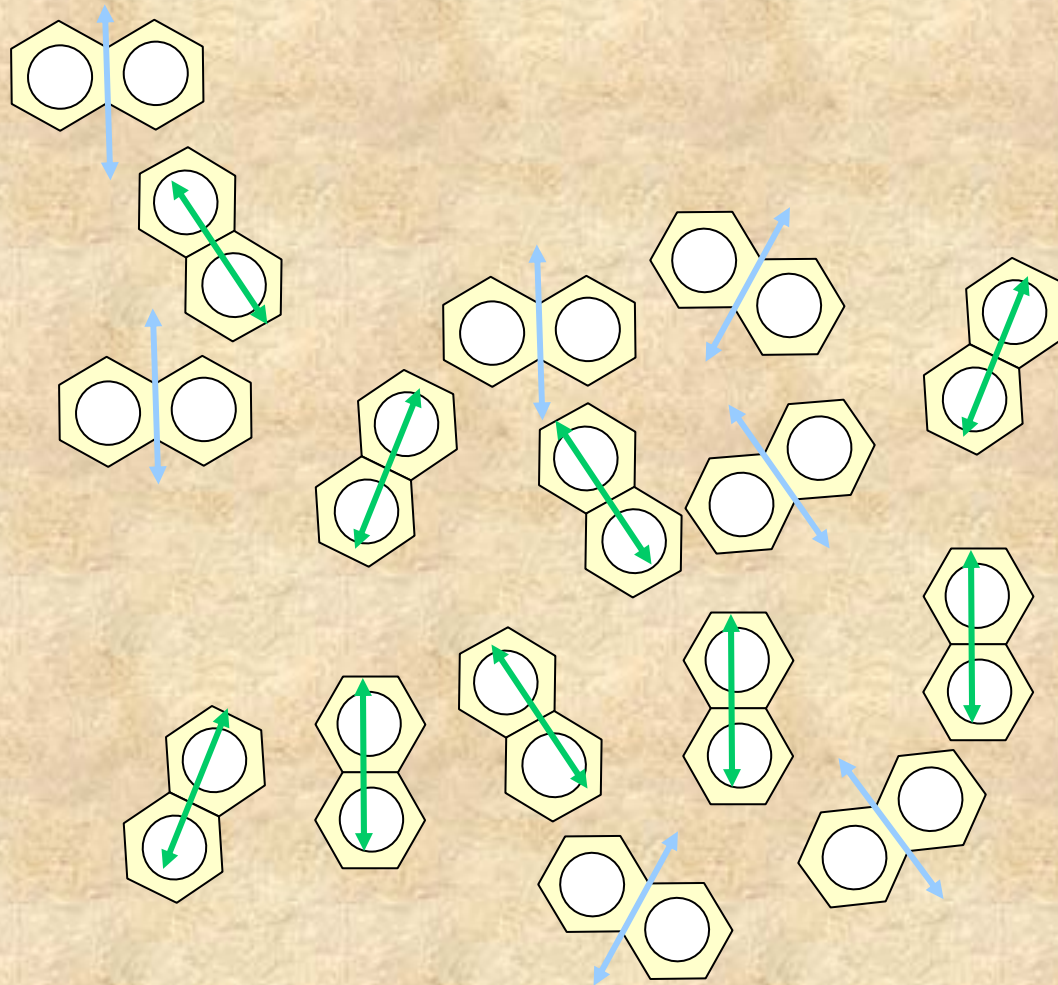


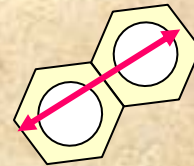
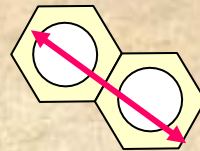
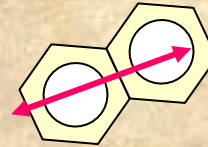
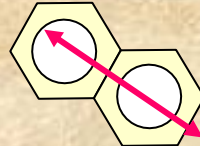
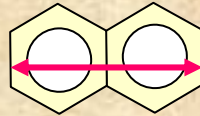
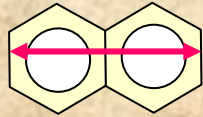
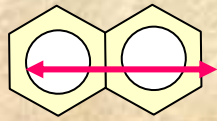
Average direction





S_0-S_2





Average direction

Hence we will observe more emission in the perpendicular direction than in the parallel direction and the resulting polarization will be negative. Considering the same $\cos^2 \theta$ photoselection rule and the $\sin \theta$ population distribution as before we can show that, if the absorption and emission dipoles are at 90° to each other, then $P = -1/3$.

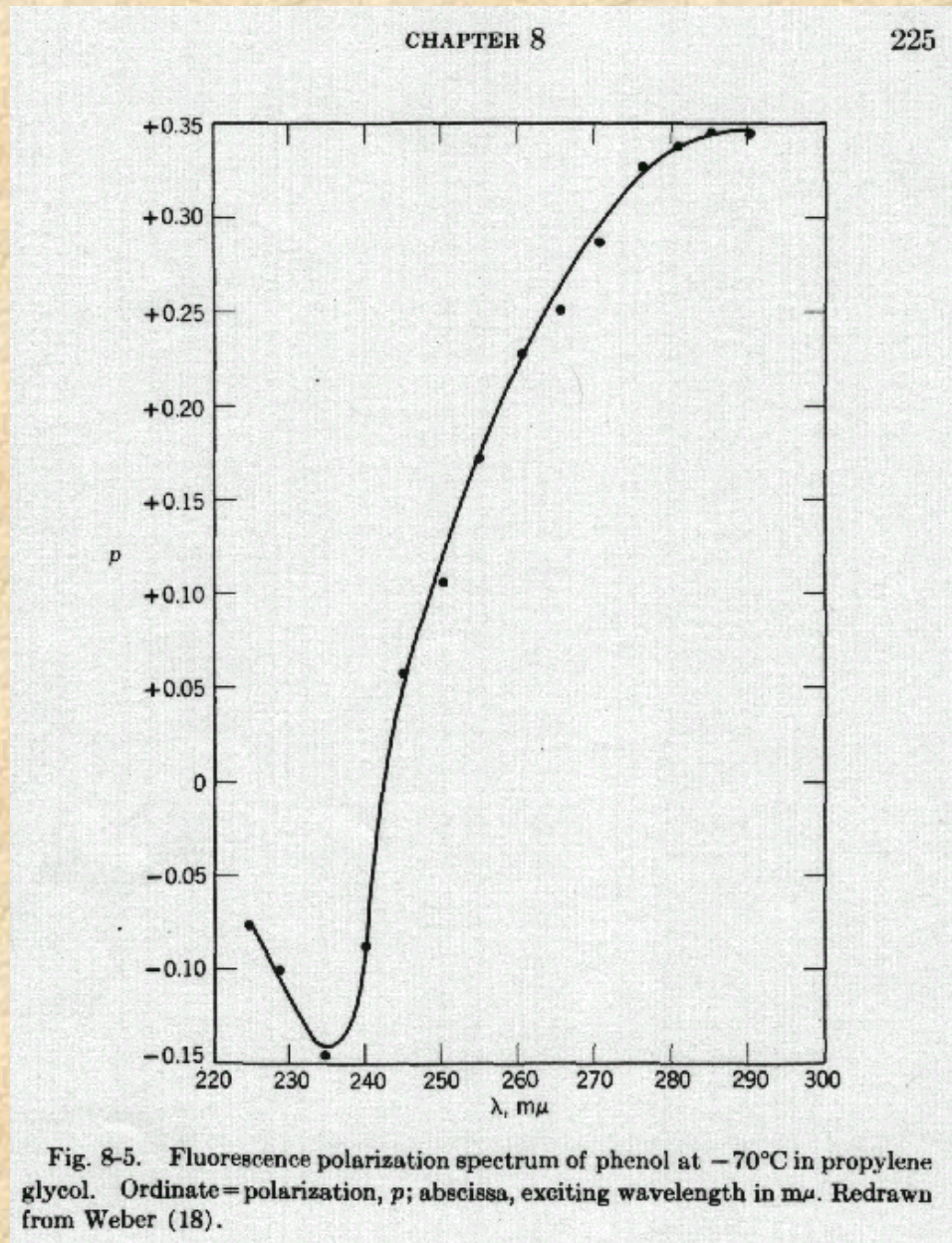
These polarization values, in the absence of rotation, are termed limiting or intrinsic polarizations and are denoted as P_o . In general:

$$\frac{1}{P_o} - \frac{1}{3} = \frac{5}{3} \left(\frac{2}{3 \cos^2 \phi - 1} \right)$$

Where ϕ is the angle between absorption and emission dipoles.

We can then understand that the limiting polarization of a fluorophore will depend upon the excitation wavelength.

Consider the excitation polarization spectrum for phenol (in glycerol at - 70 C).



In cases where there are multiple overlapping absorption bands at various angles, the excitation polarization spectrum can be somewhat complex as shown below for indole.

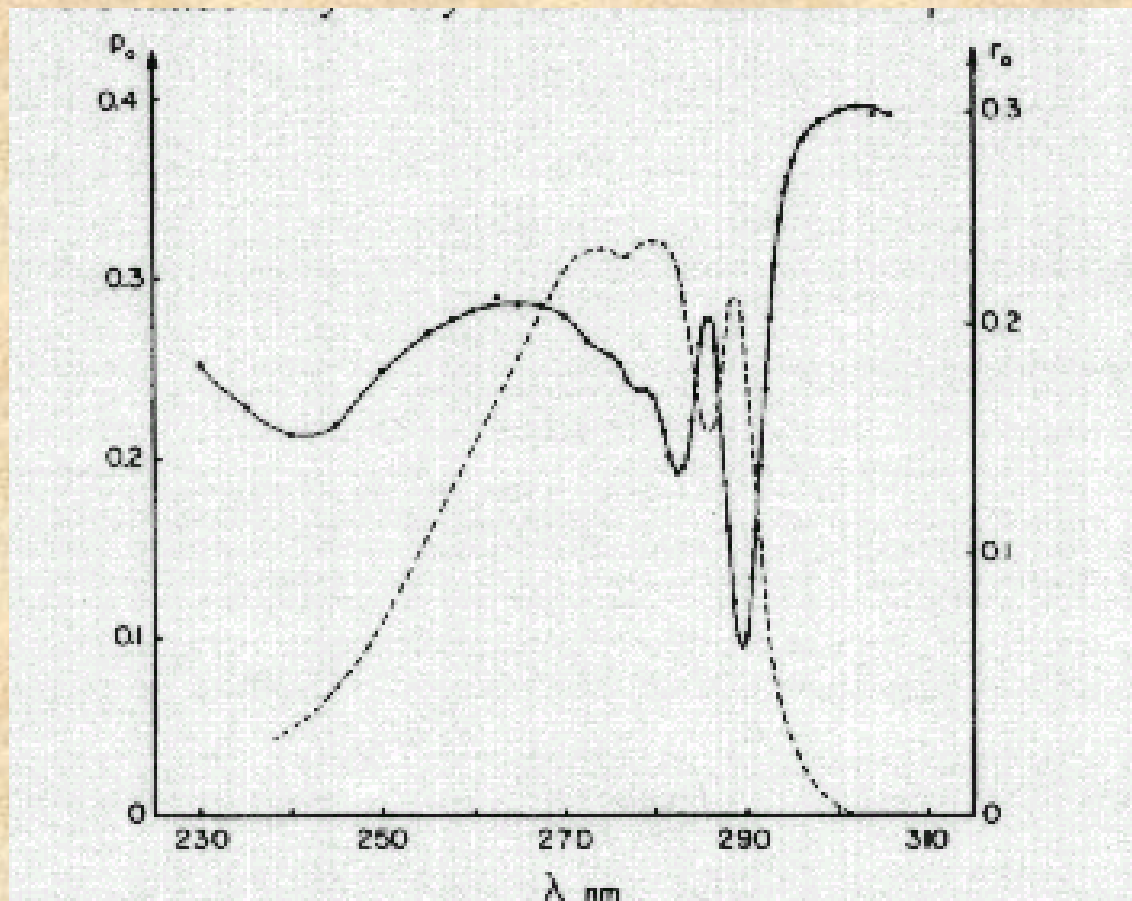
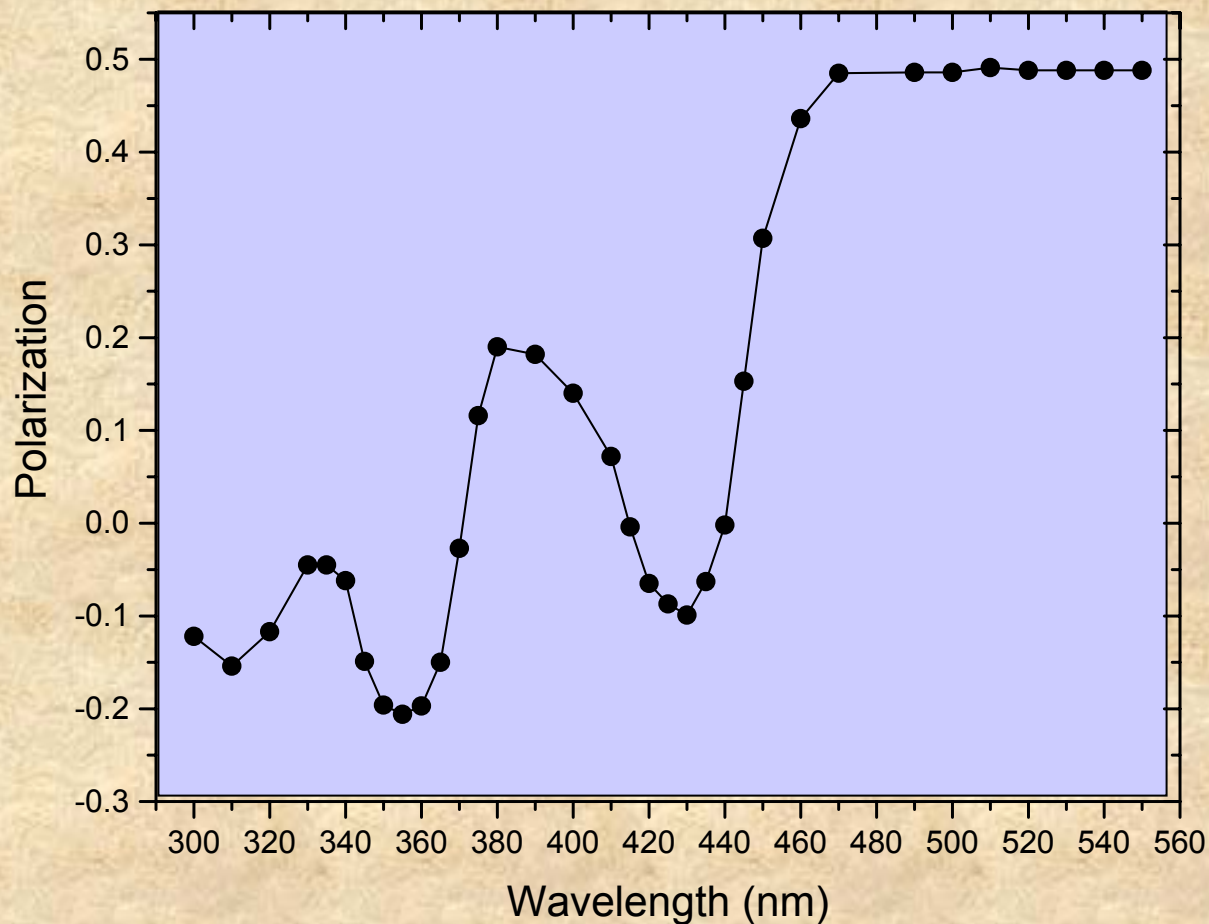
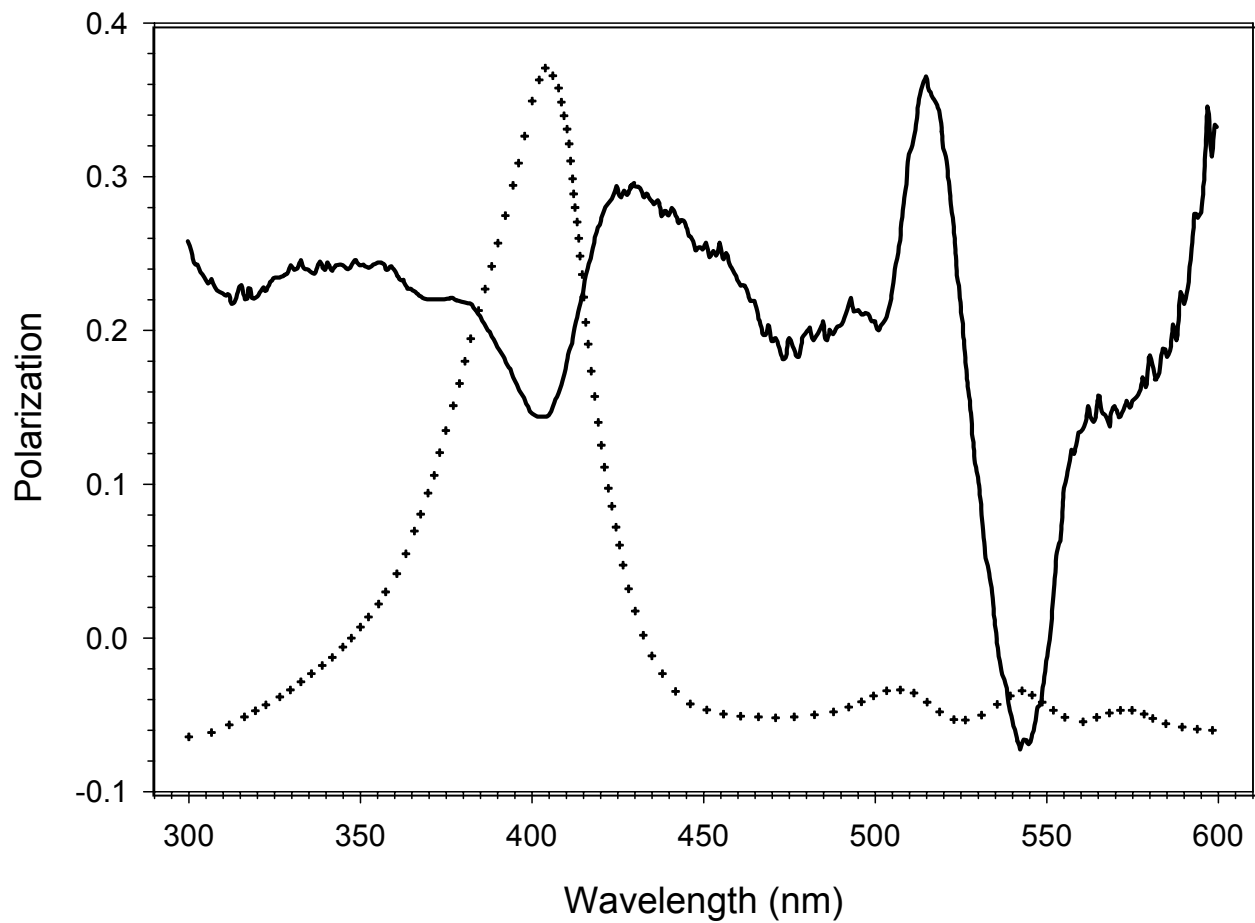


Figure 2. Corrected excitation spectrum (broken line) and excitation polarization spectrum of indole in propylene glycol at -58°C . The fluorescence is observed through a Corning 7-39 filter.

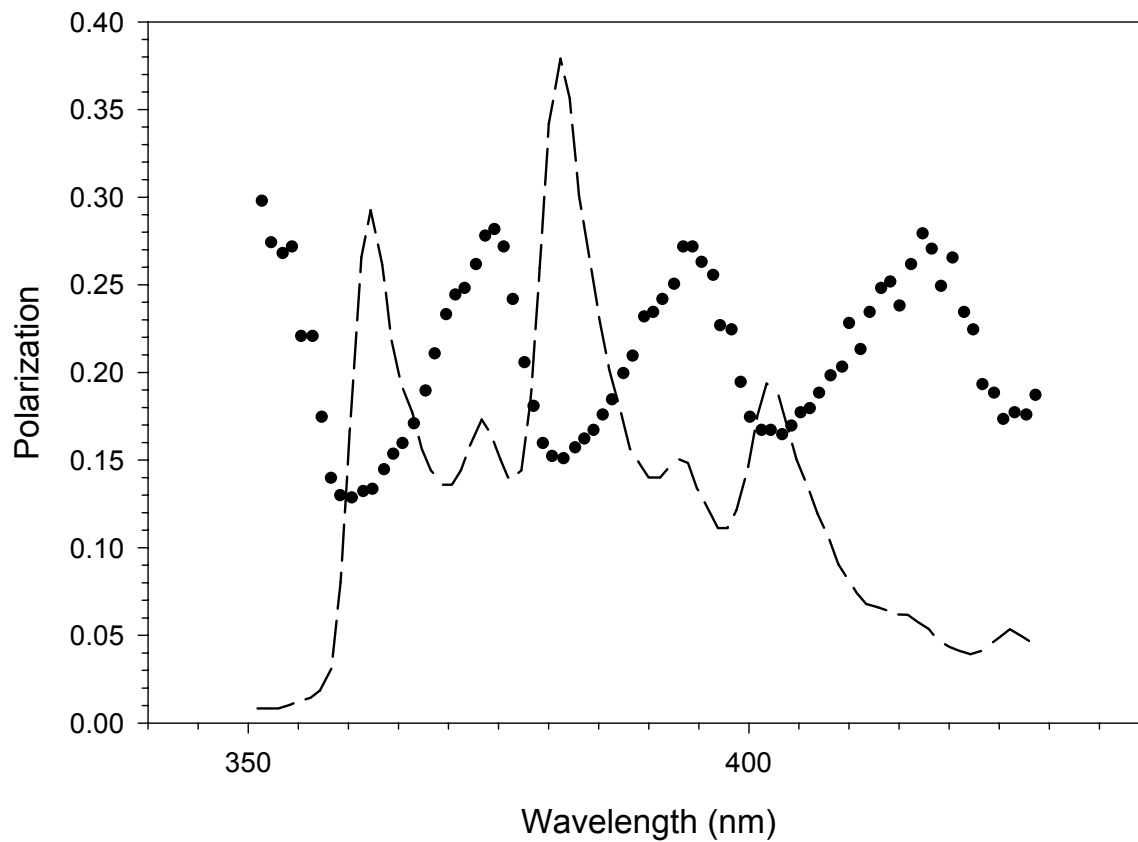
Excitation polarization spectra of rhodamine B embedded in a Lucite matrix at room temperature. Emission was viewed through a cut-on filter passing wavelengths longer than 560nm; slits were ~4nm.



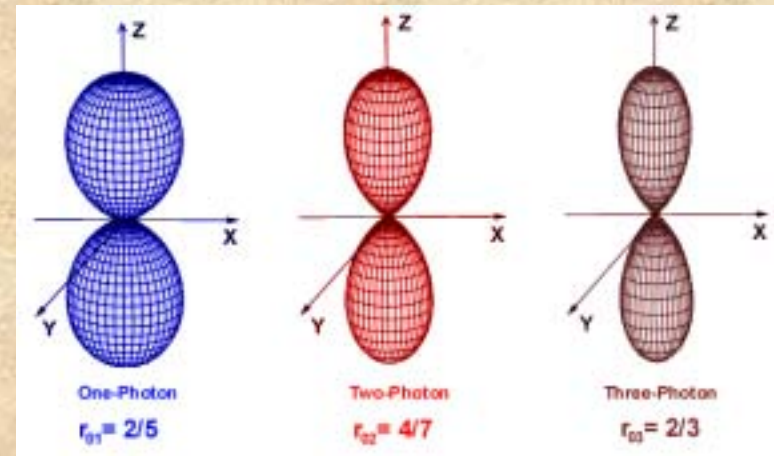
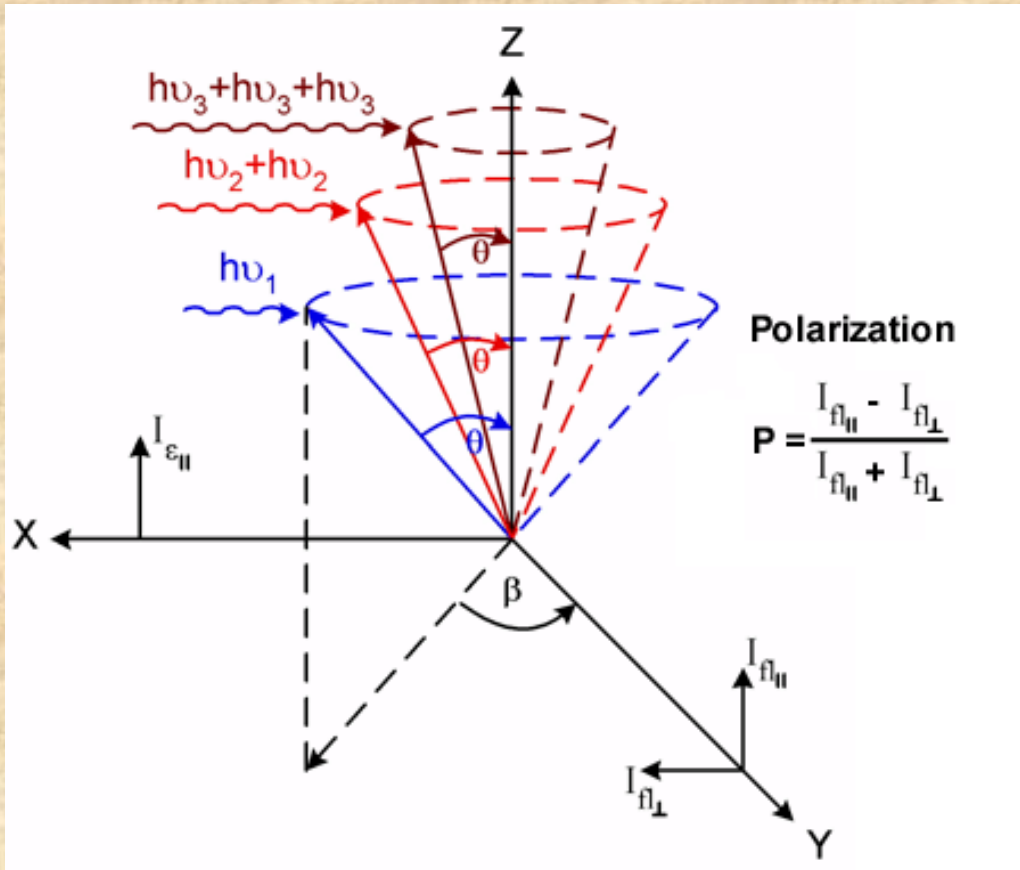
Another example is protoporphyrin IX in glycerol at -20°C



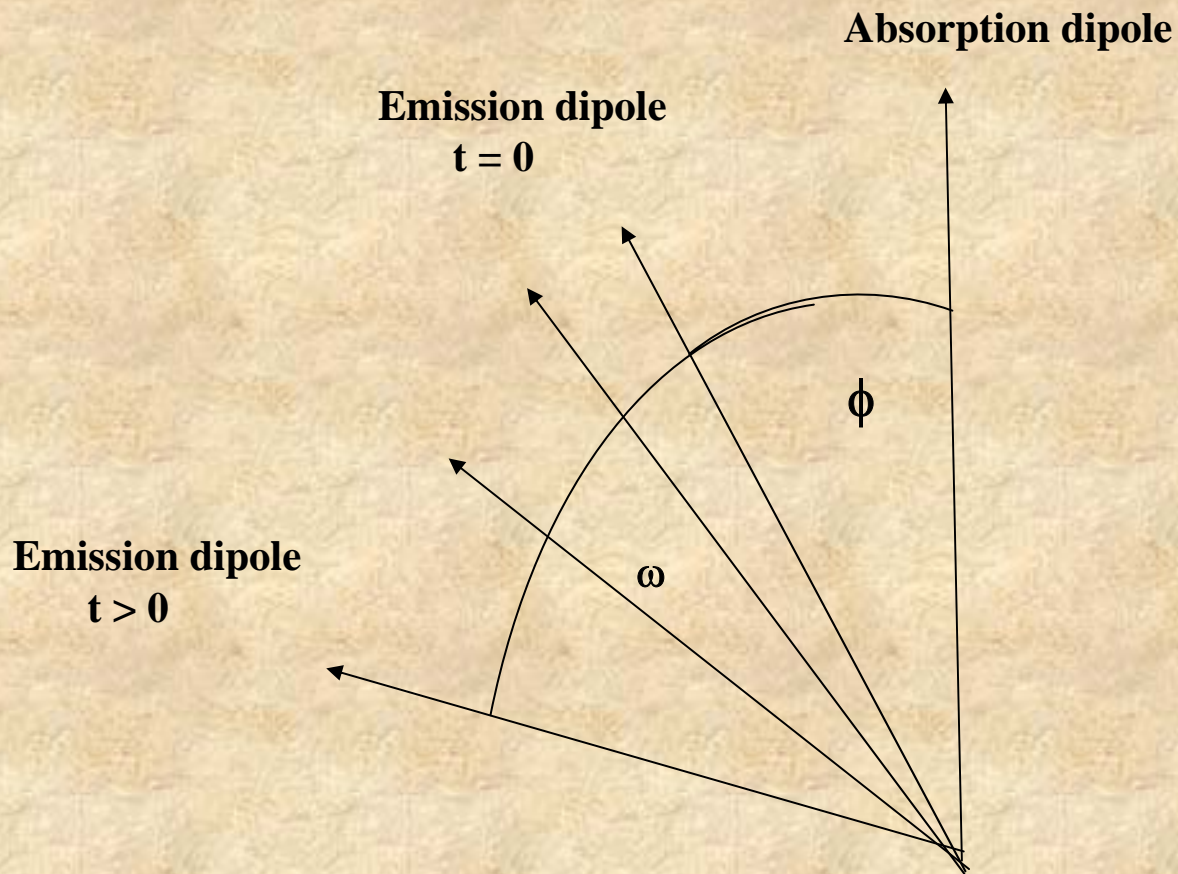
In fact, the limiting polarization can also vary across the emission band, as shown here for chrysenes in glycerol at -60°C

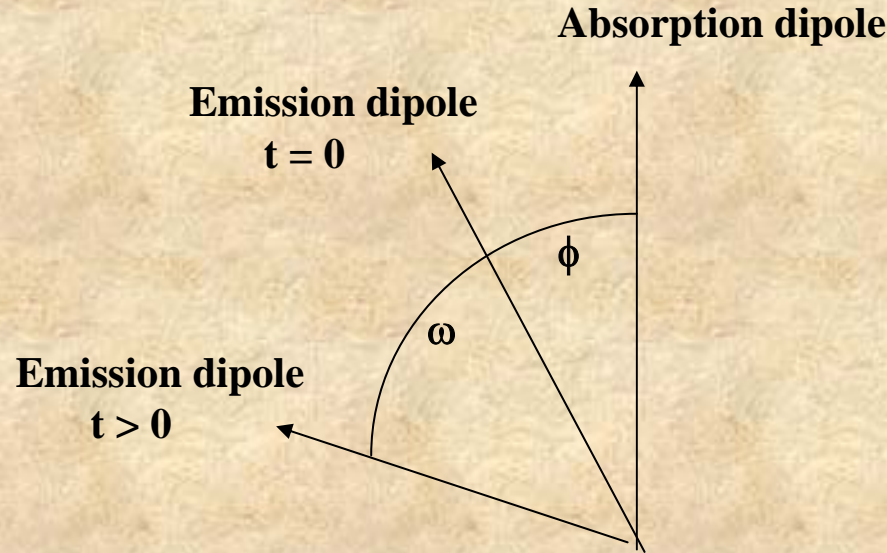


Note: in the case of multi-photon excitation the limits differ



We may now consider the case where the fluorophore is permitted to rotate during the excited state lifetime.





Additional depolarization occurs if the dipole rotates through an angle ω .

In fact:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(\frac{2}{3 \cos^2 \omega - 1} \right)$$

where P is the observed polarization. So the total depolarization is determined by an intrinsic factor (P_0) and an extrinsic factor (ω).

F. Perrin related the observed polarization to the excited state lifetime and the rotational diffusion of a fluorophore: *Perrin, F. 1926. Polarisation de la Lumiere de Fluorescence. Vie Moyene des Molecules Fluorescentes. J. Physique. 7:390-401.*

Specifically:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(1 + \frac{RT}{\eta V} \tau \right)$$

where V is the molar volume of the rotating unit, R is the universal gas constant, T the absolute temperature, η the viscosity and τ the excited state lifetime.

We can rewrite this equation as:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(1 + \frac{3\tau}{\rho} \right)$$

Where ρ is the Debye rotational relaxation time which is the time for a given orientation to rotate through an angle given by the arccos e^{-1} (68.42°).

For a spherical molecule:

$$\rho_0 = \frac{3\eta V}{RT}$$

For a spherical protein,
it follows that:

$$\rho_0 = \frac{3\eta M(\nu + h)}{RT}$$

Where M is the molecular weight, ν is the partial specific volume and h the degree of hydration.

* *Rotational relaxation time versus rotational correlation time.*

We should note that it is not uncommon to see the term “rotational correlation time”, often denoted as τ_c , used in place of the Debye rotational relaxation time. The information content of these terms is similar since $\rho = 3\tau_c$ but we have observed that some people become rather fervently attached to the use of one term or the other.

In the original development of the theories of rotational motion of fluorophores Perrin and others used the rotational relaxation time, as originally defined by Debye in his studies on dielectric phenomena. Only later (in the 1950's) during the development of nuclear magnetic resonance was the term rotational correlation time used by Bloch. It thus seems reasonable for fluorescence practitioners to use ρ but certainly adoption of either term should not lead to confusion. In terms of anisotropy and rotational correlation times, then, the Perrin equation would be:

$$\frac{r_0}{r} = \left(1 + \frac{\tau}{\tau_c} \right)$$

If the molecule is not spherical then the relevant term is the harmonic mean of the rotational relaxation times (ρ_h) about the principle rotational axes

$$\rho_h^{-1} = \left(\frac{\rho_1^{-1} + \rho_2^{-1} + \rho_3^{-1}}{3} \right)$$

A plot of $1/P - 1/3$ versus T/η predicts a straight line, the intercept and slope of which permit determination of P_0 and the molar volume (if the lifetime is known). Shown below is such a plot (termed a Perrin-Weber plot) for protoporphyrin IX associated with apohorseradish peroxidase - the viscosity of the solvent is varied by addition of sucrose.

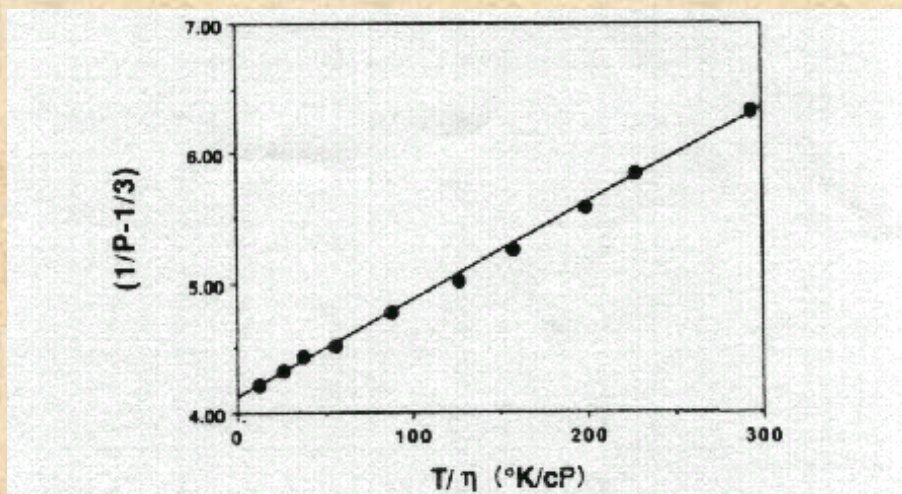
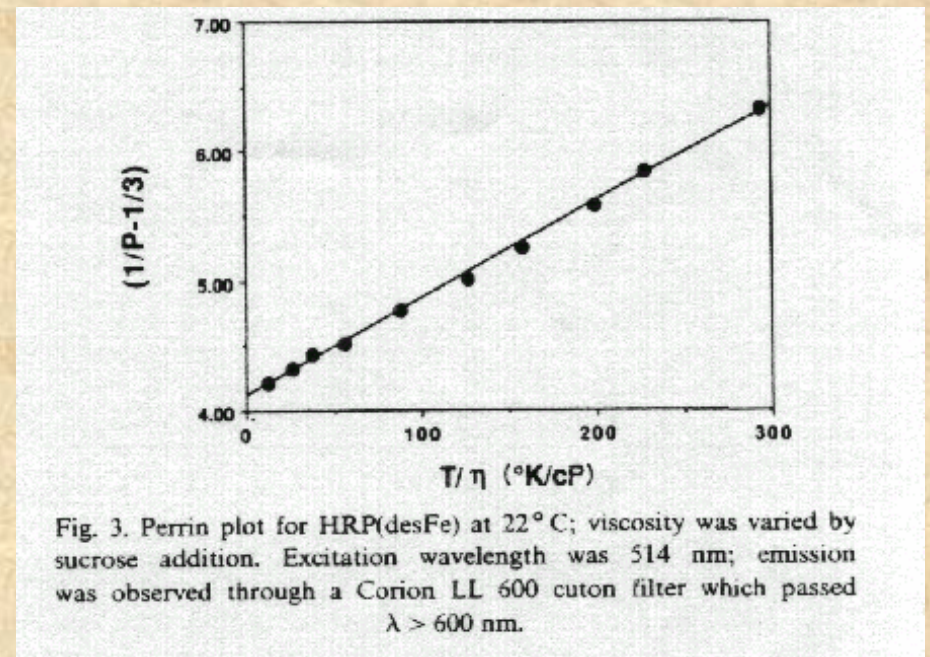


Fig. 3. Perrin plot for HRP(desFe) at 22°C; viscosity was varied by sucrose addition. Excitation wavelength was 514 nm; emission was observed through a Corion LL 600 cuton filter which passed $\lambda > 600$ nm.

The polarization observed in buffer alone was 0.151 while the limiting polarization obtained from the intercept on the Y-axis was 0.225, which is the same value one obtains for upon excitation of protoporphyrin IX in glycerol at low temperatures. From the Perrin equation:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(1 + \frac{3\tau}{\rho} \right)$$



and knowing the lifetime of 16.9 ns, one can calculate a rotational relaxation time of 96 ns for the protein-porphyrin complex:

$$\frac{1}{0.151} - \frac{1}{3} = \left(\frac{1}{0.225} - \frac{1}{3} \right) \left(1 + \frac{3 \times 16.9 \text{ ns}}{\rho} \right)$$

$$\rho = 96 \text{ ns}$$

For a spherical protein of 44,000 daltons and assuming a partial specific volume of 0.74 and 0.3 ml/mg for the hydration, one can then calculate:

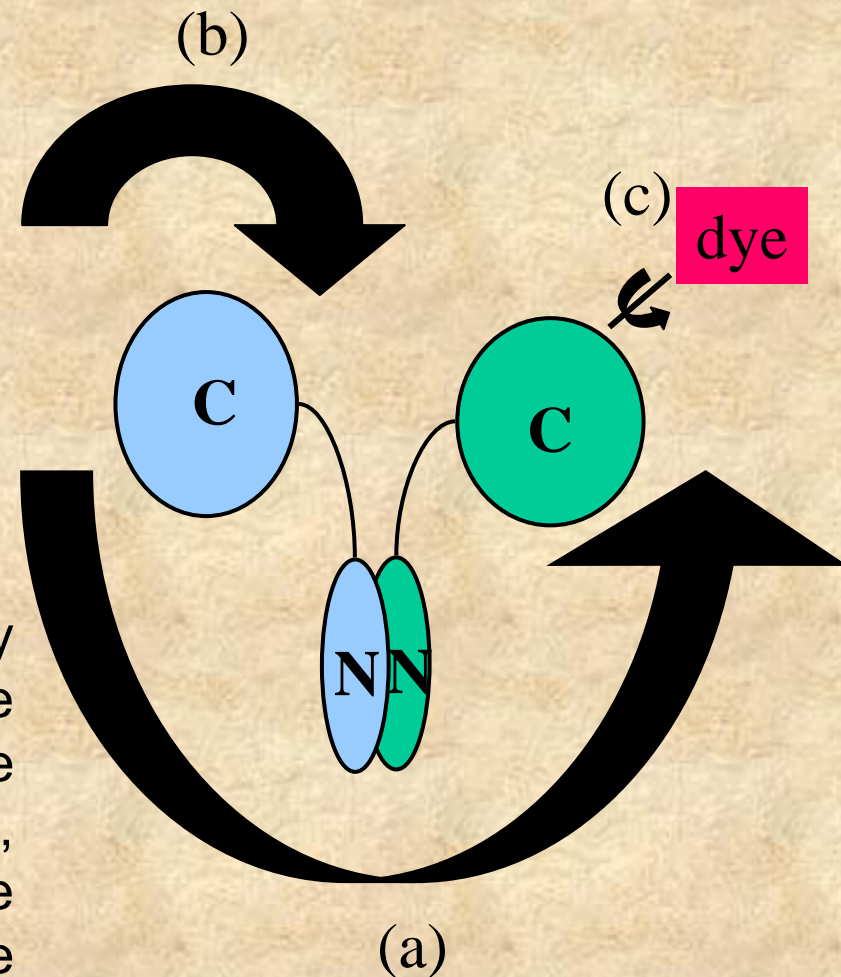
$$\rho_0 = \frac{3\eta M(\nu + h)}{RT}$$

$$\rho_0 = (3)(0.01)(44000)(0.74+0.3)/(8.31 \times 10^7)(293) = \sim 56 \text{ ns}$$

Thus it appears as if this protein is non-spherical

In the case of fluorescence probes associated non-covalently with proteins, (for example porphyrins, FAD, NADH or ANS to give but a few systems), the probe is held to the protein matrix by several points of attachment and hence its “local” mobility, that is, its ability to rotate independent of the overall “global” motion of the protein, is very restricted.

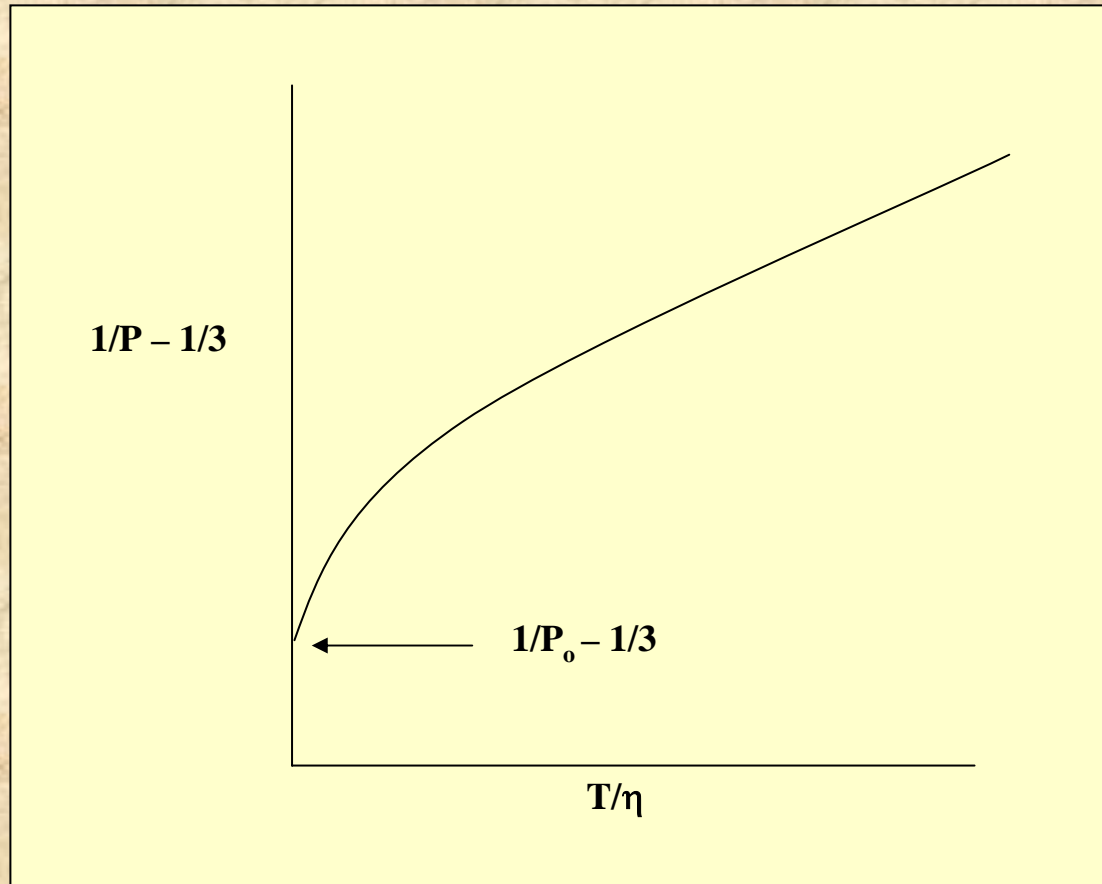
In the case of a probe attached covalently to a protein, via a linkage through an amine or sulfhydryl groups for example, or in the case of tryptophan or tyrosine sidechains, considerable “local” motion of the fluorophore can occur. In addition, the protein may consist of flexible domains which can rotate independent of the overall “global” protein rotation. This type of mobility hierarchy is illustrated on the right for the case of a probe covalently attached to a dimeric protein



Rotational Modalities

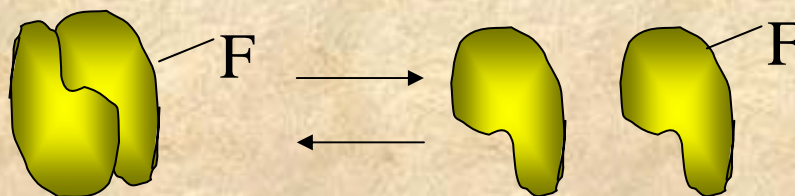
- (a) overall L7/L12 rotation
- (b) movement of one C-domain relative to other domains
- (c) movement of dye molecule around its point of attachment

In such a system one would see a downward curvature in the Perrin-Weber plots as illustrated below:

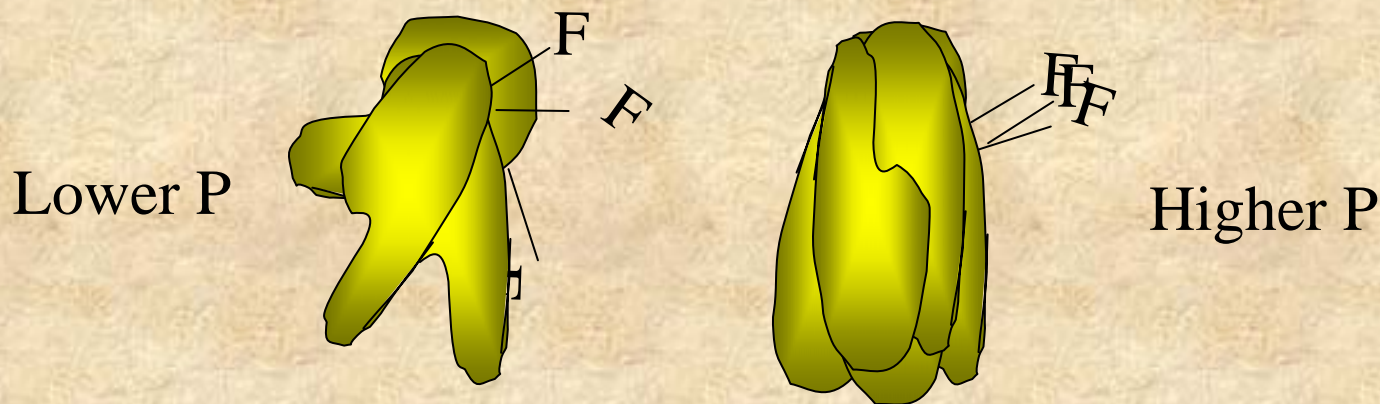


A detailed analysis of the rotational modalities in such a system requires time-resolved measurements, which will be discussed later.

Polarization methods are ideally suited to study the aggregation state of a protein. Consider, for example the case of a protein dimer - monomer equilibrium.



Following either intrinsic protein fluorescence (if possible) or by labeling the protein with a suitable probe one would expect the polarization of the system to decrease upon dissociation of the dimer into monomers since the smaller monomers will rotate more rapidly than the dimers (during the excited state lifetime).



Hence for a given probe lifetime the polarization (or anisotropy) of the monomer will be less than that of the dimer

In the concentration range near the dimer/monomer equilibrium constant, one expects to observe a polarization intermediate between that associated with either dimer or monomer. One can relate the observed polarization to the fraction of dimer or monomer using the additivity of polarizations first described by Weber (1952) namely:

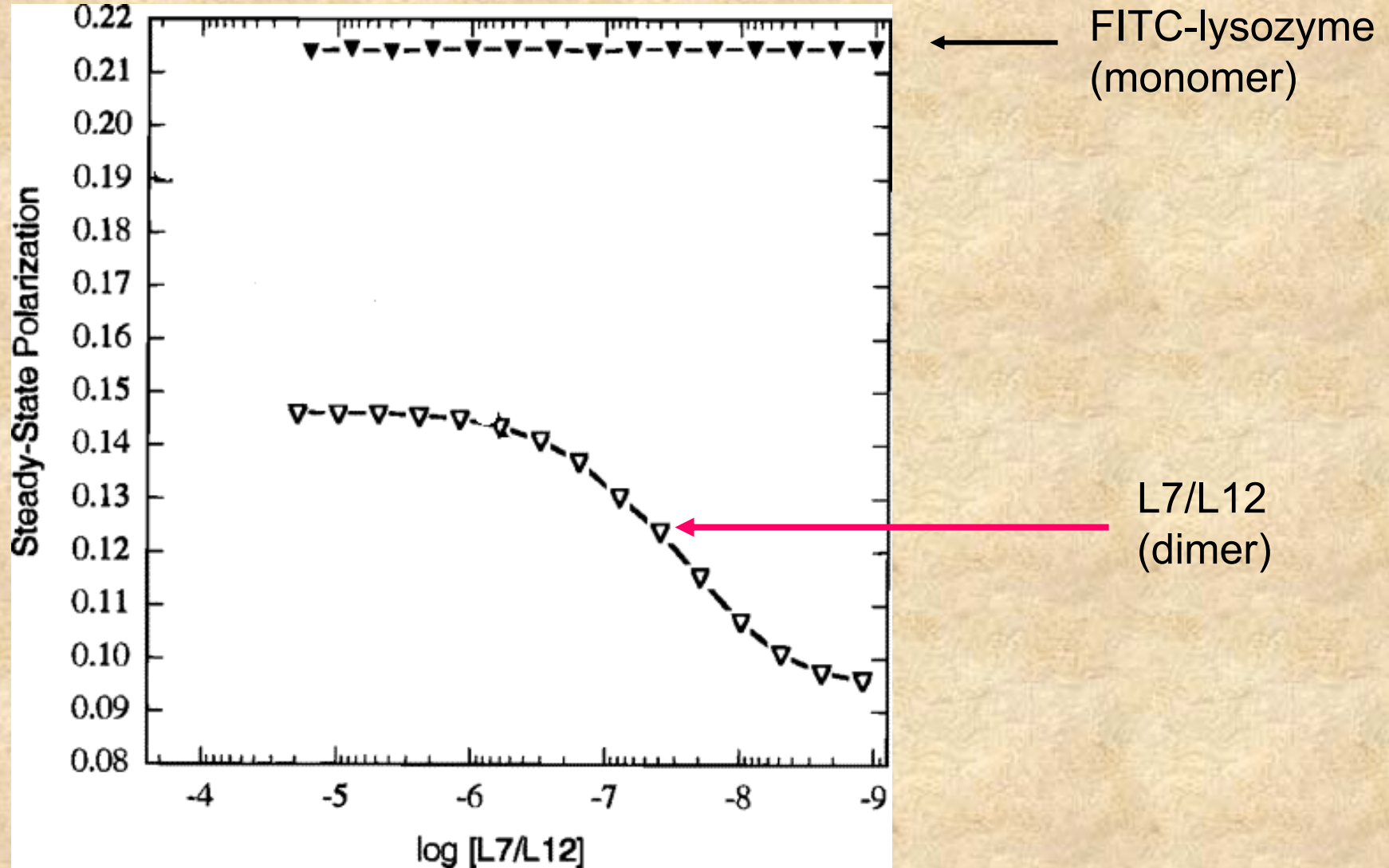
$$\left(\frac{1}{\langle P \rangle} - \frac{1}{3} \right)^{-1} = \sum f_i \left(\frac{1}{P_i} - \frac{1}{3} \right)^{-1}$$

where $\langle P \rangle$ is the observed polarization, f_i is the fractional intensity contributed by the i th component and P_i is the polarization of the i th component. One must then relate the fractional intensity contributions to molar quantities which means that one must take into account any change in the quantum yield of the fluorophore associated with either species.

The anisotropy function is directly additive (owing to the fact that the denominator represents the total emitted intensity) and hence:

$$\langle r \rangle = \sum f_i r_i$$

So to determine the dissociation constant, one can dilute the protein and observe the polarization (or anisotropy) as a function of protein concentration as shown below.



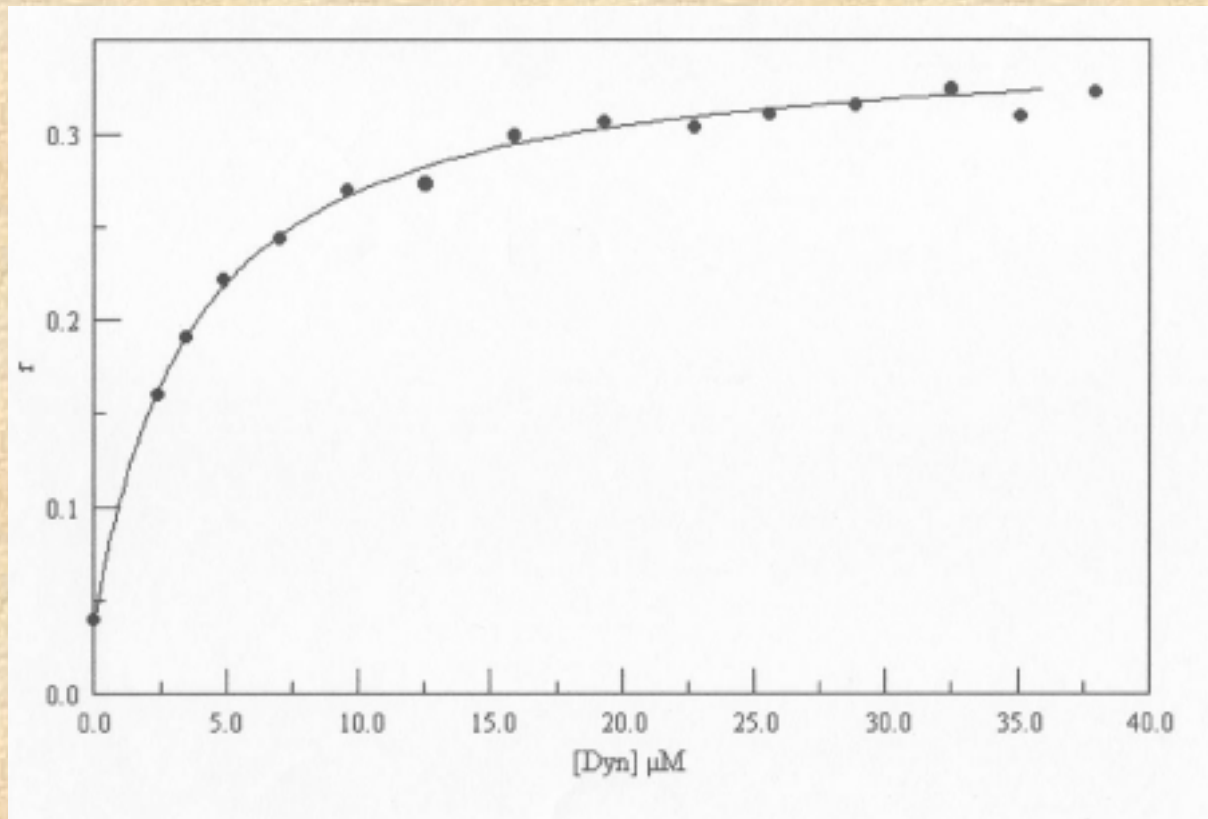
The polarization/anisotropy approach is also very useful to study protein-ligand interactions in general.

The first application of fluorescence polarization to monitor the binding of small molecules to proteins was carried out by D. Laurence in 1952 using Gregorio Weber's instrumentation in Cambridge. Specifically, Laurence studied the binding of numerous dyes, including fluorescein, eosin, acridine and others, to bovine serum albumin, and used the polarization data to estimate the binding constants.

Although many probes (such as fluorescein) do not significantly alter their quantum yield upon interaction with proteins, one should not take this fact for granted and would be well advised to check. If the quantum yield does in fact change, one can readily correct the fitting equation to take the yield change into account. In terms of anisotropy the correct expression relating observed anisotropy (r) to fraction of bound ligand (x), bound anisotropy (r_b), free anisotropy (r_f), and the quantum yield enhancement factor (g) is:

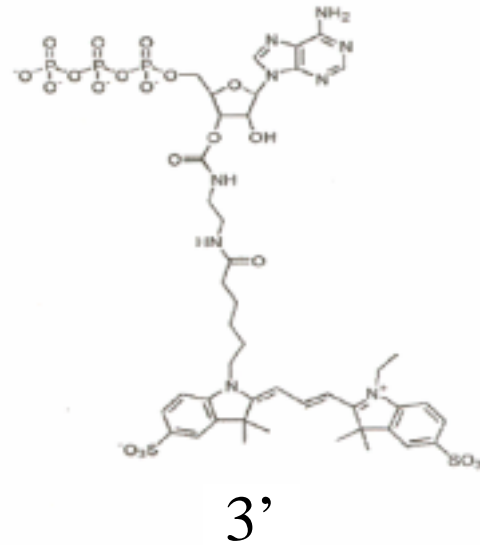
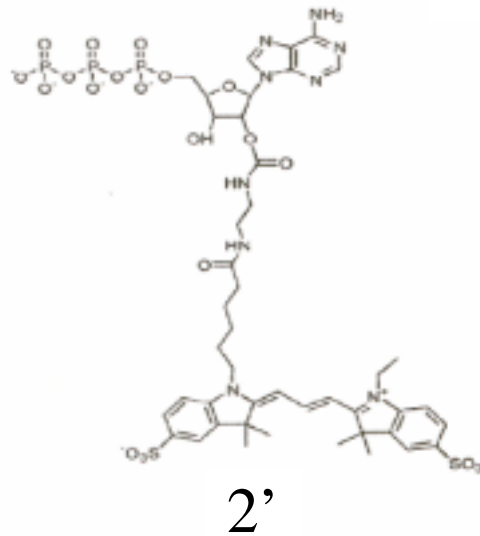
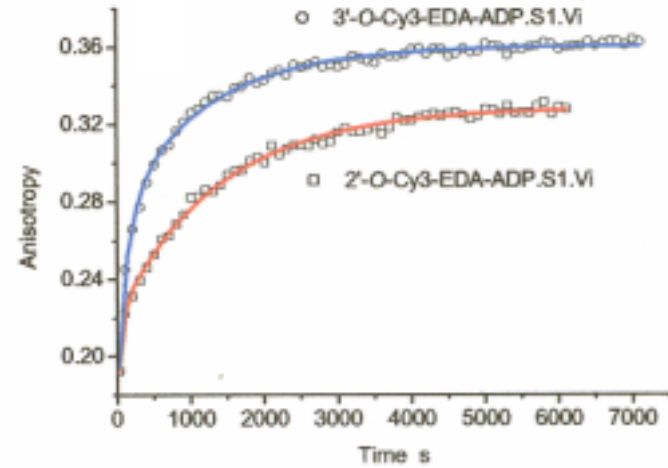
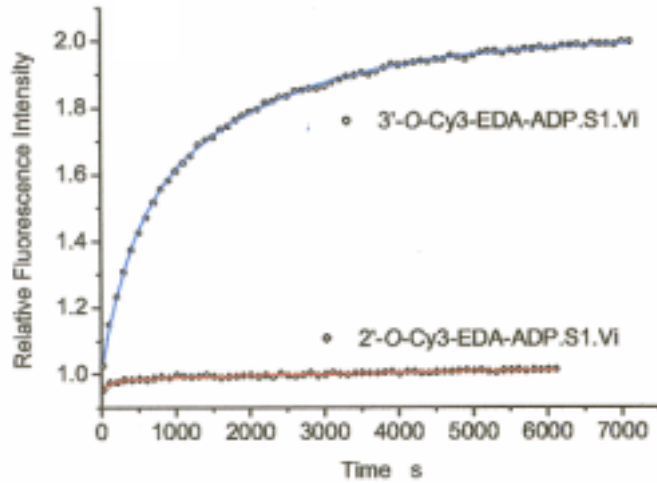
$$X = \frac{r - r_f}{r_b - r_f + (g - 1)(r_b - r)}$$

A typical plot of polarization versus ligand/protein ratio is shown below:



In this experiment, 1 micromolar mant-GTP γ S (a fluorescent, non-hydrolyzable GTP analog) was present and the concentration of the GTP-binding protein, dynamin, was varied by starting at high concentrations followed by dilution. The binding curve was fit to the anisotropy equation (in this case the yield of the fluorophore increased about 2 fold upon binding). A K_d of 8.3 micromolar was found

Another example of the utility of polarization/anisotropy data is shown here for the case of cyanine analogs of ADP binding to myosin subfragment. The 3'-isomer shows increased intensity upon binding while the 2'-isomer does not. But anisotropy data indicate binding of both isomers (from Oiwa et al 2003 Biophys. J. 84:634)



FPIA – Fluorescence Polarization ImmunoAssay

Among the first commercial instruments designed to use a fluorescence polarization immunoassay for clinical diagnostic purposes was the Abbott TDx – introduced in 1981.



The basic principle of a polarization immunoassay is to:

- (1) Add a fluorescent analog of a target molecule – e.g., a drug – to a solution containing antibody to the target molecule
- (2) Measure the fluorescence polarization, which corresponds to the fluorophore bound to the antibody
- (3) Add the appropriate biological fluid, e.g., blood, urine, etc., and measure the decrease in polarization as the target molecules in the sample fluid bind to the antibodies, displacing the fluorescent analogs.

