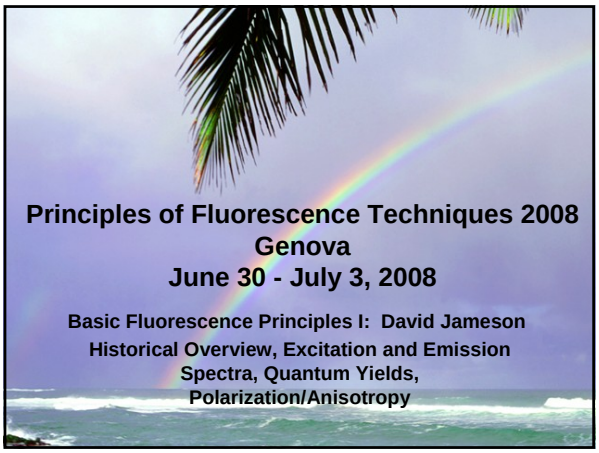


Principles of Fluorescence Techniques 2008
Genova
June 30 - July 3, 2008

Basic Fluorescence Principles I: David Jameson

Historical Overview, Excitation and Emission Spectra, Quantum Yields, Polarization/Anisotropy

What is fluorescence?

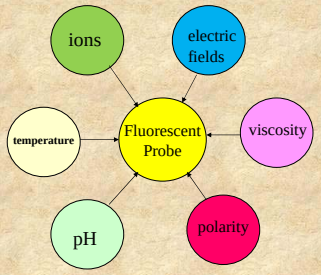
FLUORESCENCE is the light emitted by an atom or molecule after a finite duration subsequent to the absorption of electromagnetic energy.

Specifically, the emitted light arises from the transition of the excited species from its first excited electronic singlet level to its ground electronic level.

The development of highly sophisticated fluorescent probe chemistries, new laser and microcopy approaches and site-directed mutagenesis has led to many novel applications of fluorescence in the chemical, physical and life sciences. Fluorescence methodologies are now widely used in the biochemical and biophysical areas, in clinical chemistry and diagnostics and in cell biology and molecular biology.

Why fluorescence?

- its pretty!
- it provides information on the molecular environment
- it provides information on dynamic processes on the nanosecond timescale



Fluorescence Probes are essentially molecular stopwatches which monitor *dynamic* events which occur during the excited state lifetime – such as movements of proteins or protein domains

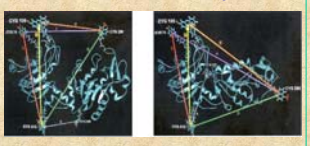
Coupled with modern fluorescence microscopy (confocal, multiphoton, etc) and fluorescent proteins (such as GFP, etc) fluorescence is also providing extremely detailed spatial information in living cells – as well as information on the dynamics of cellular components

Also fluorescence is very, very, very sensitive!

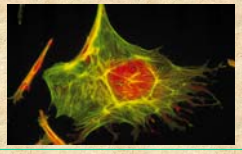
Work with subnanomolar concentrations is routine while femtomolar and even SINGLE MOLECULE studies are possible with some effort

Experimental Systems Accessible to Fluorescence

Molecular structure and dynamics

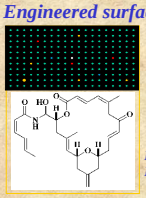


Cell organization and function



Live Animals

Engineered surfaces



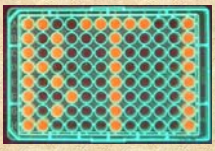
High throughput Drug discovery

Instrumentation

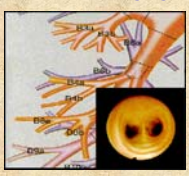
Fluorimeters/Fluorometers

Microscopes

High throughput Platereaders



Intravital Imaging



The discovery and characterization of Fluorescence

Nicolás Monardes (1577), a Spanish physician and botanist who wrote on medicines of the New World, is usually credited as being 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.*

Actually, Bernardino de Sahagún, a Franciscan missionary, independently described the wood – called "coatli" by the Aztecs.

I am indebted to Ulises Acuna for this picture and for information about these early studies.

Coatli .....patli, yoan aquixtiloni, matlatic iniayo axixpatli..
"it is a medicine, and makes the water of blue color, its juice is medicinal for the urine"

Sahagún, Florentine Codex Vol. III f. 266; CM-RAH, f. 203v.

An early Latin translation (1574) by the influential Flemish botanist Charles de L'Écluse (1526-1609), in which the wood's name is given as *Lignum Nephriticum (kidney wood)*, helped to extend awareness of its strange optical properties in Europe. This wood was very popular in XVI - XVII Europe, 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.."

The German Jesuit priest Athanasius Kircher, among his numerous achievements, wrote a book in 1646 called *Ars Magna Lucis et Umbrae* in which he described his observation of the wood extract *Lignum nephriticum*. Light passing through an aqueous infusion of this wood appeared more yellow while light reflected from the solution appeared blue.

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.

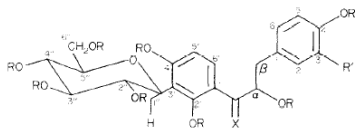
Recent studies by Ulises Acuna indicate that the original blue emission observed by the Aztecs was probably due to the conversion of Coatline B, under mildly alkaline conditions, to a strongly blue-emitting compound - resembling fluorescein - with an emission maximum near 466nm and with a quantum yield near 0.8

Phytochemistry, Vol. 21, No. 12, pp. 2931–2935, 1982.
Printed in Great Britain.

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

EVELYN BELTRAMI, MARIA DE BERNARDI*, GIOVANNI FRONZA†, GIORGIO MELLERIO, GIOVANNI VIDARI* and PAOLA VITA-FINZI*

Istituto di Chimica Organica dell'Università, Via Taramelli 10, 27100 Pavia, Italy; †Centro del CNR per la Chimica delle Sostanze Organiche Naturali, Politecnico, Piazza Leonardo da Vinci 32, 20133 Milano, Italy



Ia X = O, R = H, R' = H
Ib X = O, R = H, R' = OH
Ic X = O, R = Ac, R' = H
Id X = O, R = Ac, R' = OAc
2 X = H_2OH, 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 Galilei (1612) described the emission of light (phosphorescence) from the famous Bolognian stone, discovered in 1603 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."

David Brewster (1833) described that when a beam of white light passed through an alcohol solution of leaves a red beam could be observed from the side (which was of course chlorophyll fluorescence).

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

IV. *Ἀμόρφωσις*, No. I.—*On a Case of Superficial Colour presented by a homogeneous liquid internally colourless. By* Sir JOHN FREDERICK WILLIAM HERSCHEL, *Bart., K.H., F.R.S., &c. &c.*

Received January 28, 1845,—Read February 13, 1845.

an extremely vivid and beautiful celestial blue colour

XXX. *On the Change of Refrangibility of Light.* By G. G. Stokes, *M.A., F.R.S., Fellow of Pembroke College, and Lucasian Professor of Mathematics in the University of Cambridge.*

Received May 11,—Read May 27, 1852.

1. The following researches originated in a consideration of the very remarkable phenomenon discovered by Sir John Herschel in a solution of sulphate of quinine, and described by him in two papers printed in the Philosophical Transactions for 1845, entitled 'On a Case of Superficial Colour presented by a Homogeneous Liquid internally colourless,' and 'On the Epipolic Dispersion of Light.' The solution of quinine, though it appears to be perfectly transparent and colourless, like water, when viewed by transmitted light, exhibits nevertheless in certain aspects, and under certain incidences of the light, a beautiful celestial blue colour. It appears from the experiments of Sir John Herschel that the blue colour comes only from a stratum of fluid of small but finite thickness adjacent to the surface by which the light enters.

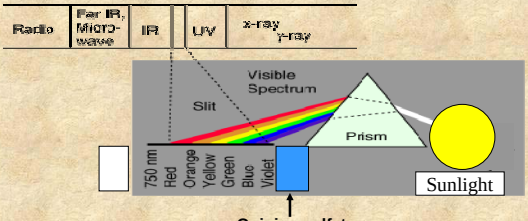
George Gabriel Stokes (1852) published his massive treatise "On the Change of Refrangibility of Light" – more than 100 pages.

In a this work he initially using the term "dispersive reflection" to describe the phenomenon presented by quinine sulphate.

Fortunately for all of us today, however, he then wrote:

* I confess I do not like this term. I am almost inclined to coin a word, and call the appearance *fluorescence*, from fluor-spar, as the analogous term *opalescence* is derived from the name of a mineral.

Stokes used a prism to disperse the solar spectrum and illuminate a solution of quinine. He noted that there was no effect until the solution was placed in the ultraviolet region of the spectrum.

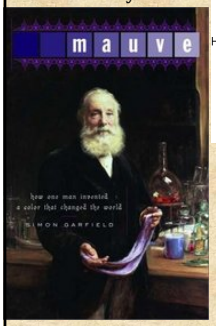


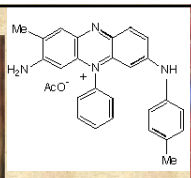
He wrote:

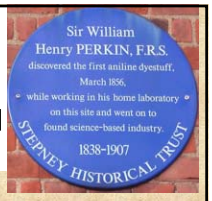
It was certainly a curious sight to see the tube instantaneously lighted up when plunged into the invisible rays: it was literally *darkness visible*. Altogether the phenomenon had something of an unearthly appearance.

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

William Henry Perkin







In 1856, at the age of 18, William Henry Perkin set out with idea of making *quinine* by oxidizing *allytoluidine* – instead he accidentally produced the synthetic dye, *mauve,* a derivative of coal tar with an aniline base.

Fortunately for him Queen Victoria loved it!
Not long afterward Perkin produced a green and a violet, and soon the canal outside his factory was turning a different color every week.

Although others – including Friedlieb Runge and Robert Rumney – had synthesized synthetic dyes, Perkins was the first to recognize the potential for commercialization and really started the synthetic dye industry.
Histologists started using the dyes to stain samples within a decade of Perkin's discovery

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

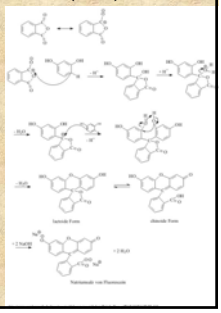
FLUORESCEIN!!!

Adolf Baeyer: Ueber eine neue Klasse von Farbstoffen.
(Vorgetr. vom Verf.)

Berichte der Deutschen Chemischen Gesellschaft, (1871) 4:555-558.

He apparently coined the name "fluorescein", from "fluo" and resorcin, (resorcinol) which he reacted with phthalic anhydride

In 1905 he was awarded the Nobel Prize in Chemistry "in recognition of his services in the advancement of organic chemistry and the chemical industry, through his work on organic dyes and hydroaromatic compounds".



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FLUORESCEIN!!!

One of the first uses of fluorescein was in 1877 in a major ground-water tracing experiment in southern Germany.

Ueber die hydrographischen Beziehungen zwischen der Donau und der Aachquelle im Badischen Oberlande.

Von

Dr. A. Knop.

The results of this experiment showed that the River Danube actually flowed to the North Sea (east) rather than into the Black Sea (west) when most of its flow disappeared into its bed near the town of Tuttlingen.

10 Kilograms of fluorescein were used!

Fig. 4 The Danube at the Immendingen weir with sinkholes on the right bank and the well-stratified Oxfordian limestone behind

If this experiment were repeated today with Alexa 488 it would cost ~$2,210,000,000. Unless Molecular Probes offered a bulk discount.

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

FLUORESCEIN!!!

Every year on St. Patrick's Day, the Chicago river is dyed green with about 40 pounds of fluorescein.

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FLUORESCEIN!!!

Paul Erlich (1882) used uranin (the sodium salt of fluorescein) to track secretion of the aqueous humor in the eye. First *in vivo* use of fluorescence.

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Earliest example of a Molecular Probes catalog!!!

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Earliest example of a Molecular Probes catalog!!!

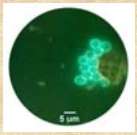
R. Meyer (1897) used the term "fluorophore" to describe chemical groups which tended to be associated with fluorescence; this word was analogous to "chromophore" which was first used in 1876 by O.N. Witt to describe groups associated with color.

Otto Heimstaedt and Heinrich Lehmann (1911-1913) developed the first fluorescence microscopes as an outgrowth of the UV microscope (1901-1904). the instrument was used to investigate the autofluorescence of bacteria, protozoa, plant and animal tissues, and bioorganic substances such as albumin, elastin, and keratin.

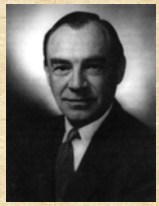
Stanislav Von Prowazek (1914) employed the fluorescence microscope to study dye binding to living cells.

PROWAZEK, S. VON, 1914. 'Über Fluoreszenz der Zellen.' Kleinwelt, 6, 30 and 37.

Albert Coons (1941) labeled antibodies with FITC, thus giving birth to the field of immunofluorescence.



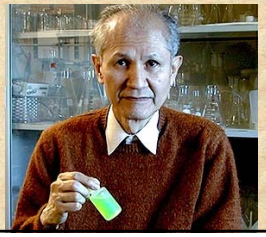
Cryptosporidium oocytes labeled with FITC tagged antibody



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 victoria*** jellyfish

Osamu Shimomura in the lab in the basement of his home. He is holding a sample of GFP isolated from Aequorea victorea, not produced by bacteria.



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

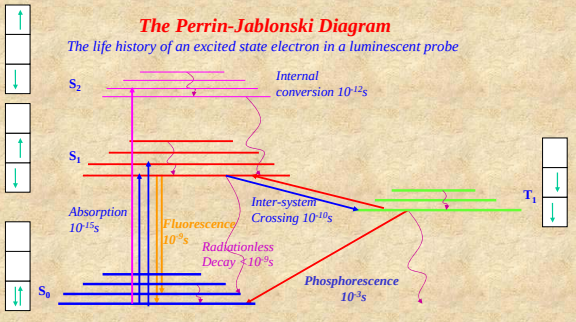
Virtually all fluorescence data required for any research project will fall into one of the following categories.

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 Perrin-Jablonski Diagram

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



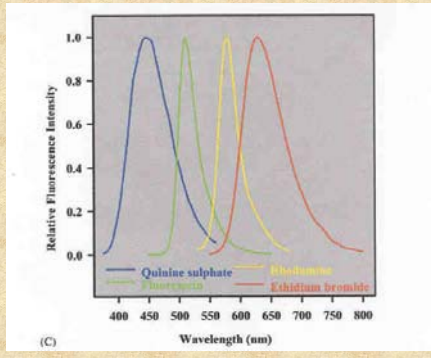
Key points:

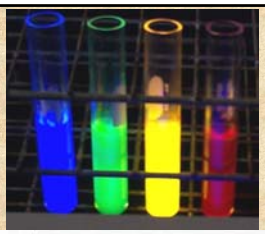
- ✓ *Excitation spectra are mirror images of the emission spectra*
- ✓ *Emission has lower energy compared to absorption*
- ✓ *Triplet emission is lower in energy compared to singlet emission*
- ✓ *Most emission/quenching/FRET/chemical reactions occur from the lowest vibrational level of* $[S]_1$

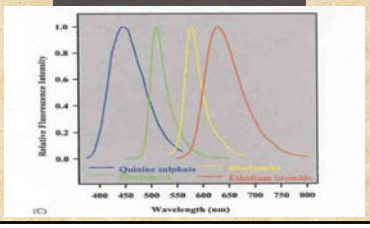
GM

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:

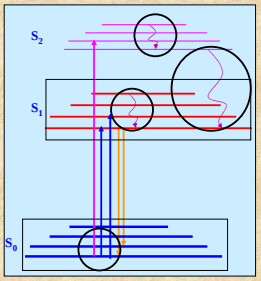
1) In a pure substance existing in solution in a unique form, the fluorescence spectrum is invariant, remaining the same independent of the excitation wavelength

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.



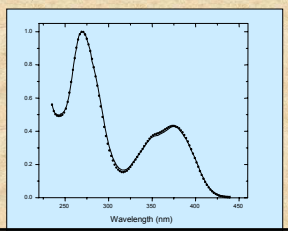
The fact that ground state fluorophores, at room temperature, are predominantly in the lowest vibrational level of the ground electronic state (as required from Boltzmann's distribution law) accounts for the Stokes shift.

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

GM

List of quantum yields from "Molecular Fluorescence" by Bernard Valeur

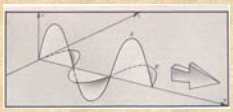
Tab. 6.1. Standards for the determination of fluorescence quantum yields

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_2O (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_2SO_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_2SO_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.)**, 915.
10) Magde D., Brannon J. H., Cramers T. L. and Olmsted J. III (1979) *J. Phys. Chem.* **83**, 696.

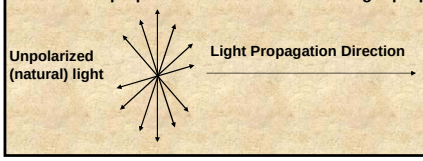
Polarization

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

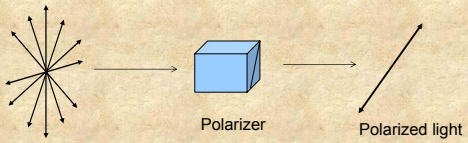


In these lectures we shall be concerned only with the electric component.

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.



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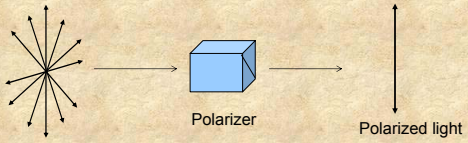
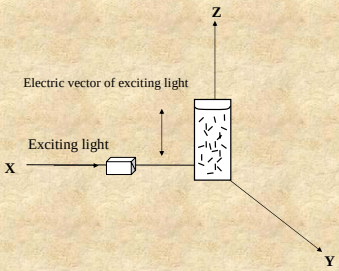


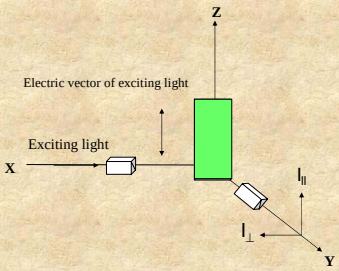
The most common polarizers used today are (1) dichroic devices, which operate by effectively absorbing one plane of polarization (e.g., Polaroid type-H sheets based on stretched polyvinyl alcohol impregnated with iodine) and (2) double refracting calcite ($CaCO_3$) crystal polarizers - which differentially 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)

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.



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 ($I_{\parallel}$) and perpendicular intensities ($I_{\perp}$) :

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

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:

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

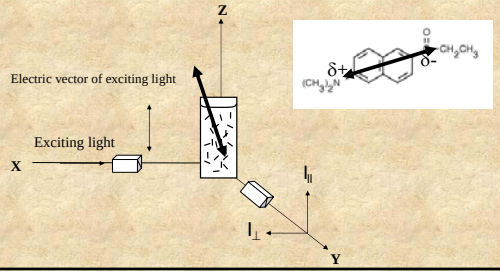
For example:

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.

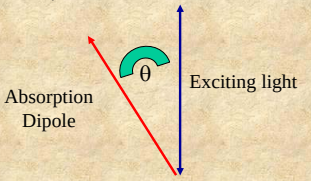
In solution these limits (e.g., +/-1) are not realized. Consider, as shown below, fluorophores at the origin of our coordinate system.

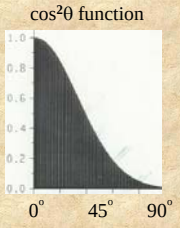
Upon absorption of an exciting photon a dipole moment is created in the fluorophore (usually of different magnitude and direction from the ground state dipole). The orientation of this dipole moment relative to the nuclear framework, and its magnitude, will be determined by the nature of the substituents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



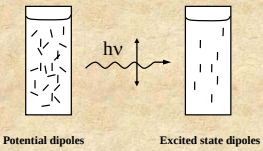
In fact, if light of a particular electric vector orientation (plane polarized light) impinges on a sample, only those molecules which are properly oriented relative to this electric vector can absorb the light.

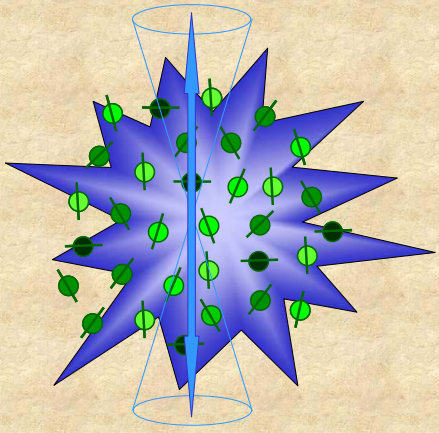
Specifically, the probability of the absorption is proportional to the cosine squared ($\cos^2\theta$) of the angle **θ** between the exciting light and the transition dipole.



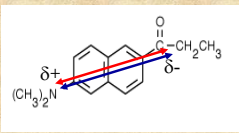


Hence, when we excite an ensemble of randomly oriented fluorophores with plane-polarized light we are performing a *photoselection* process, creating a population of excited molecules which nominally have their excited dipoles lined up with the polarization direction of the excitation. This process is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

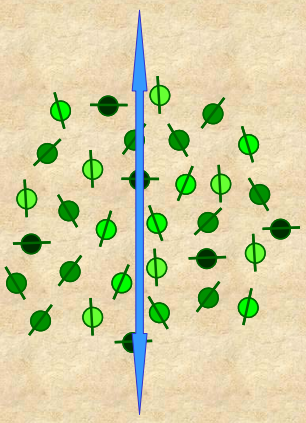




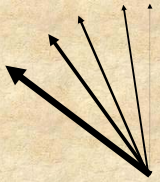
Consider now that the transition dipole corresponding to the emission of light from the excited fluorophore is parallel to the absorption dipole and that the excited fluorophore cannot rotate during the lifetime of the excited state (for example if the fluorophores are embedded in a highly viscous or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



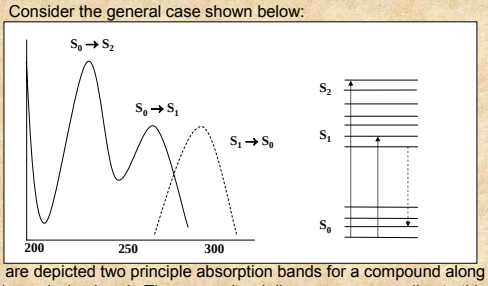
In fact, the number of potential dipoles making an angle θ with the vertical axis will be proportional to sin θ.



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

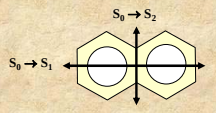
This case, however, assumes that the emission dipole is parallel (co-linear) to the absorption dipole.

Consider the general case shown below:



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

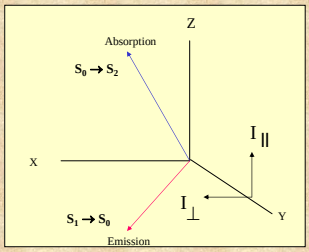
The directions of the absorption dipoles – relative to the nuclear framework – may differ greatly for the two transitions as illustrated on the right.



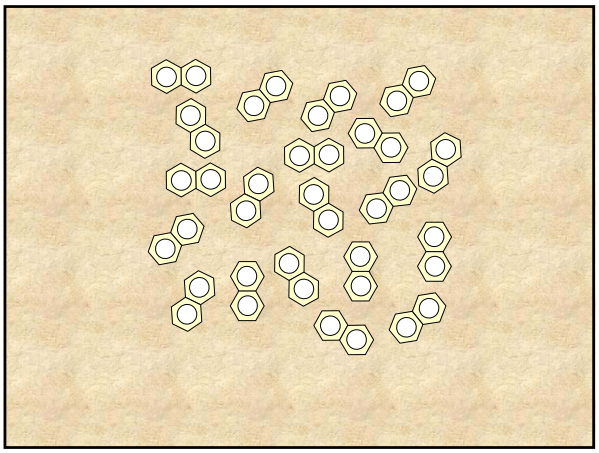
So we see that the two excited dipoles corresponding to the $S_0 \rightarrow S_1$ and the $S_0 \rightarrow S_2$ transitions may be oriented at an arbitrary angle - in the extreme case this angle could be 90°.

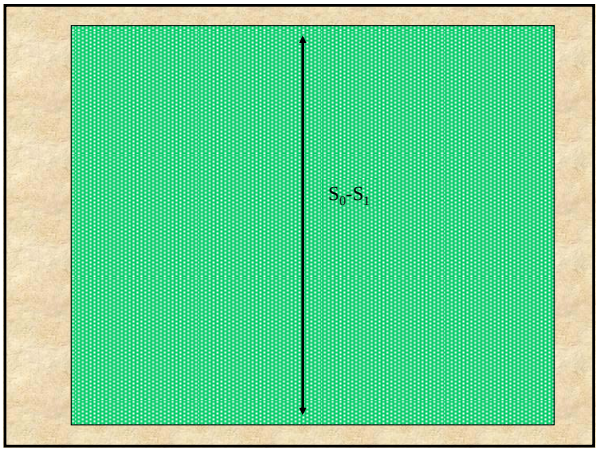
After the excitation process, however, regardless of whether the absorption process corresponded to the S0 → S1 or the S0 → S2 transition, rapid thermalization leaves the excited fluorophore in the S1 level.

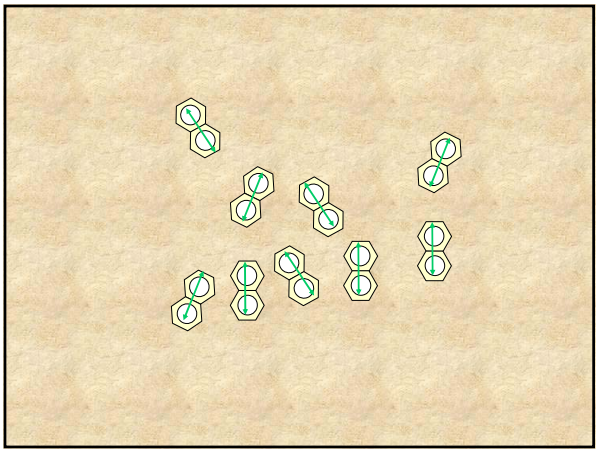
This situation is depicted below:

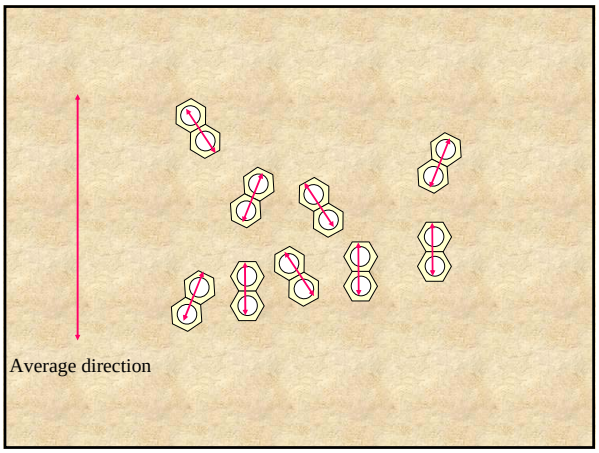


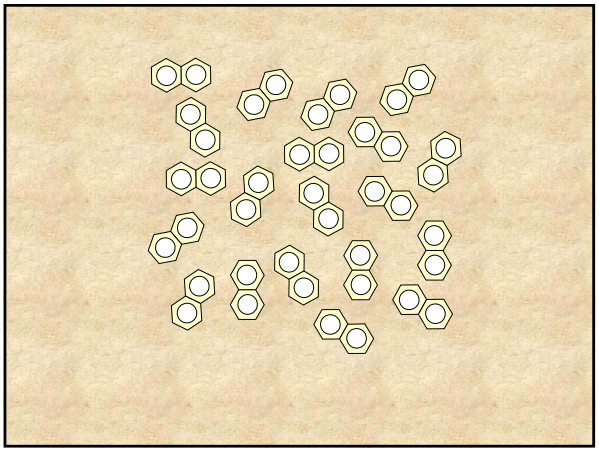
The orientation of the excited dipoles will thus now possess a different average orientation than the absorption dipoles originally photoselected by the exciting light.

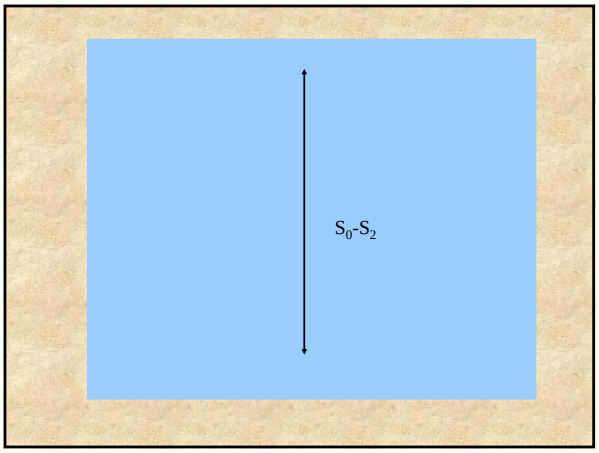


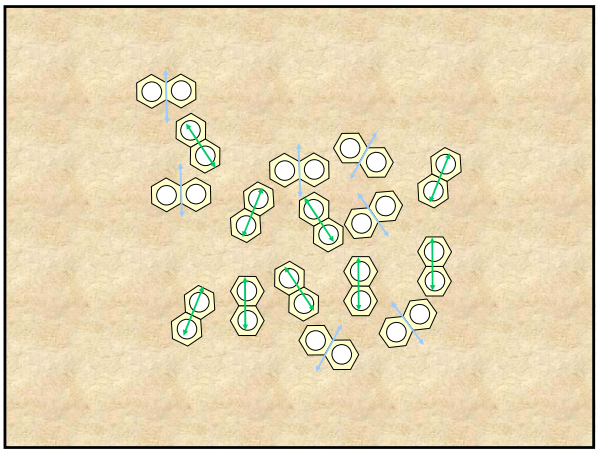

S_0-S_1

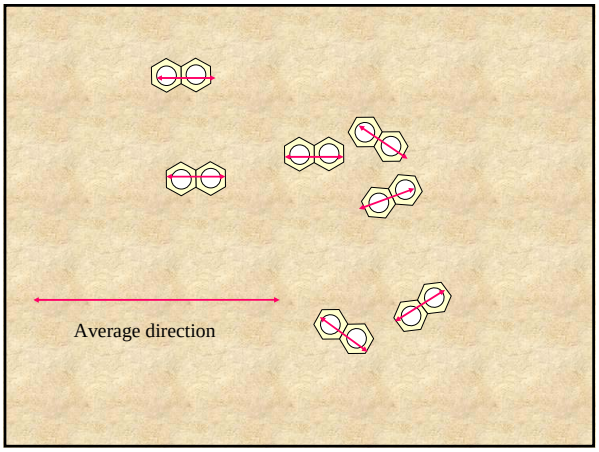



Average direction




S_0-S_2





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).

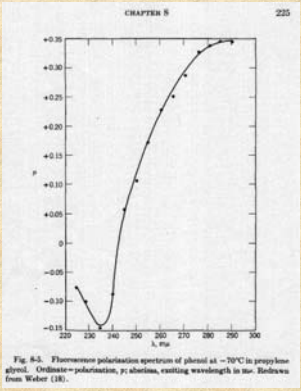


Fig. 8-3. Fluorescence polarization spectrum of phenol at −70°C in propylene glycol. Ordinate = polarization, p; abscissa, emitting wavelength in mμ. Redrawn from Weber (18).

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.

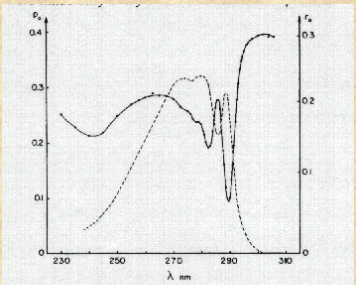
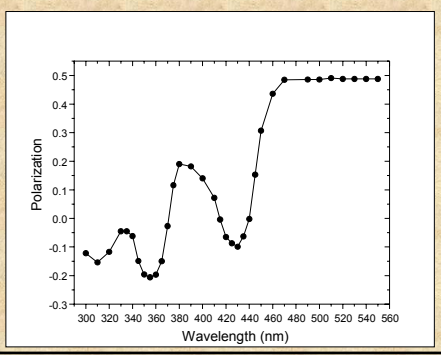
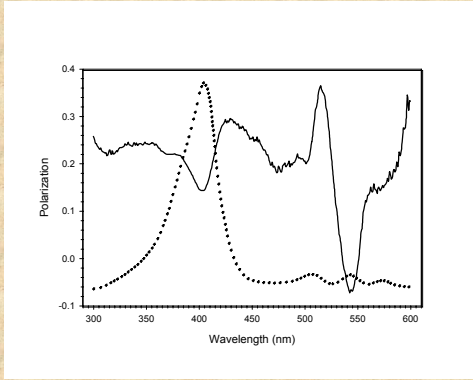


Figure 2. Corrected excitation spectrum (broken line) and excitation polarization spectrum of indole in propylene glycol at −58°. The fluorescence is observed through a Corning 7-39 filter.

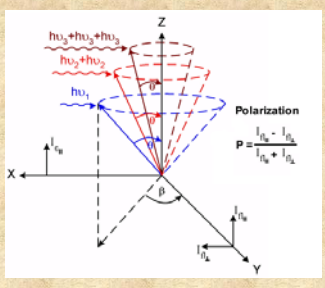
Excitation polarization spectra of rhodamine B embedded in a Lucite matrix at room temperature. Emission was viewed through a cut-on filter passing wavelengths longer than 560nm; slits were ~4nm.

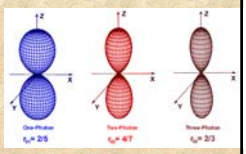


Another example is protoporphyrin IX in glycerol at –20C

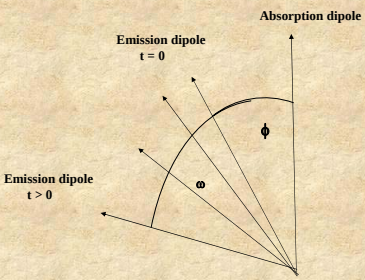


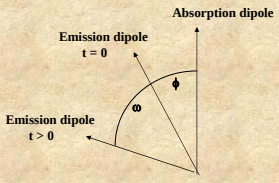
Note: in the case of multi-photon excitation the limits differ





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

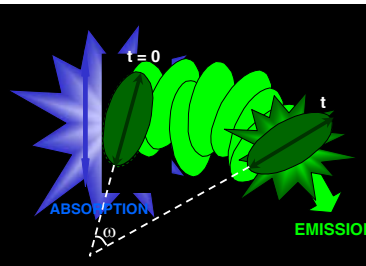




Additional depolarization occurs if the dipole rotates through an angle **ω**.

In fact: $$\frac{1}{P}-\frac{1}{3}=\left(\frac{1}{P_o}-\frac{1}{3}\right)\left(\frac{2}{3\cos^2\omega-1}\right)$$

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



- **ORIENTATION AUTOCORRELATION FUNCTION**
  probability that a molecule having a certain orientation at time zero is oriented at angle ω with respect to its initial orientation

$$\frac{\overline{3\cos^2\omega(t)}-1}{2}=\frac{r(t)}{r_0}$$

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

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

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:

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

For a spherical protein, it follows that:

$$\rho_o = \frac{3\eta M(\upsilon + h)}{RT}$$

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*.

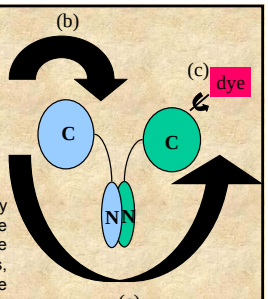
We should note that it is not uncommon to see the term "rotational correlation time", often denoted as τ_c, used in place of the Debye rotational relaxation time. The information content of these terms is similar since $\rho = 3\tau_c$ but we have observed that some people become rather fervently attached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

$$\frac{r_o}{r} = \left(1 + \frac{\tau}{\tau_C}\right)$$

In the case of fluorescence probes associated non-covalently with proteins, (for example porphryins, 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

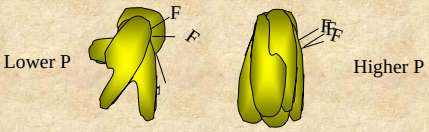
(a) overall dimer rotation

(b) movement of one C-domain relative to other domains

(c) movement of dye molecule around its point of attachment

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:

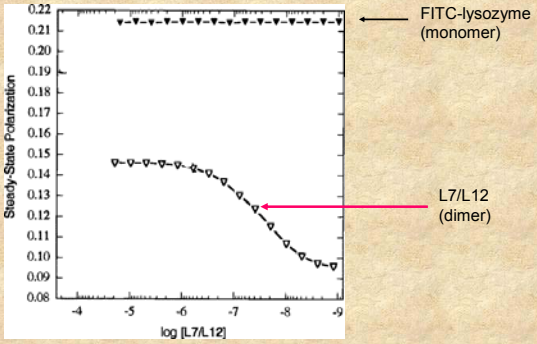
$$\left(\frac{1}{<P>}-\frac{1}{3}\right)^{-1}=\sum f_i\left(\frac{1}{P_i}-\frac{1}{3}\right)^{-1}$$

where <P> is the observed polarization, f_i is the fractional intensity contributed by the ith component and P_i is the polarization of the ith 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:

$$<r>=\sum f_i r_i$$

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.



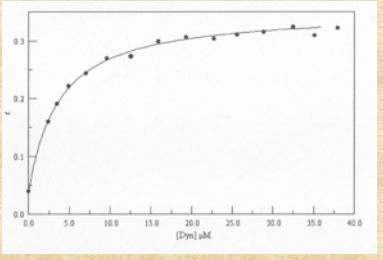
The polarization/anisotropy approach is also very useful to study protein-ligand interactions in general.

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:

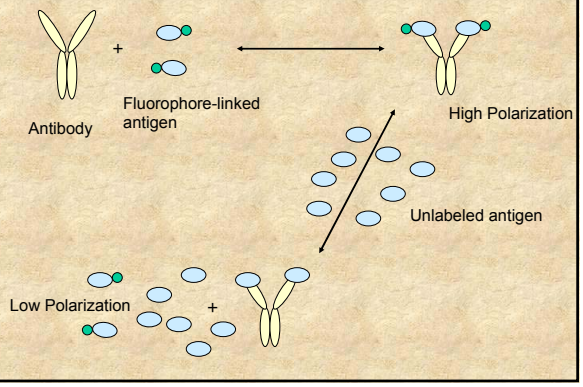
$$x = \frac{r - r_f}{r_b - r_f + (g-1)(r_b - r)}$$

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



In this experiment, 1 micromolar mant-GTPγS (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

7. Dandliker, W. B., Kelly, R. J., Dandliker, J., et al., Fluorescence polarization immunoassay. Theory and experimental method. *Immunochemistry* 10, 219–227 (1973).



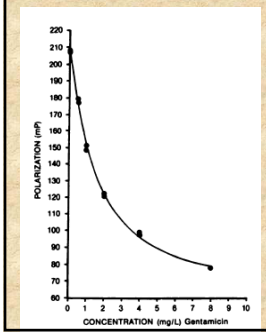
In the mid-1970's, Abbott Laboratories consulted with Gregorio Weber about the development of a polarization instrument for clinical assays.

The result was the introduction in 1981 of the TD_x instrument which has become one of the leading clinical instruments - more than 30,000 TD_x instruments are presently in the field.

CLIN. CHEM. 27/7, 1190–1197 (1981)

Fluorescence Polarization Immunoassay I. Monitoring Aminoglycoside Antibiotics in Serum and Plasma

Michael E. Jolley, Stephen D. Stroupe, Chao-Huei J. Wang, Helen N. Panas, Candace L. Keegan, Robert L. Schmidt, and Kathryn S. Schwenzer



Special Apparatus

A microprocessor-controlled fluorometer was constructed in our laboratories (*19*). The instrument was designed to measure precisely the polarization of fluorescence emitted from the sample contained in a standard 12 × 75 mm disposable culture tube. Each determination was performed in 10 s, to a precision of ±0.001 polarization unit. The instrument determined fluorescence polarization (in arbitrary units) according to the equation (*7*):

$$P = \frac{I_{parallel} - I_{perpendicular}}{I_{parallel} + I_{perpendicular}}$$

where I represents light intensity. For convenience, results are reported in millipolarization units (mP), where 1 mP = 0.001 P.

Time for a coffee!!!!!!