Introduction to Fluorescence
23-25 March 2009
Steady State Fluorescence Principles:
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Absorption, 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 relaxation of the excited species from its first excited electronic singlet level to its ground electronic level. *(usually)*

The development of highly sophisticated fluorescent probe chemistries, new laser and microscopy 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?

- It's 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.
• Spatial Information
  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

• Sensitivity !!!

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

High throughput Platereaders

Microscopes

Intravital imaging systems
The discovery and characterization of Fluorescence

Quiz: When was the first report on “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 aqujxtiloni,*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.
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.
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”.


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

an extremely vivid and beautiful celestial blue colour.

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 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.

<table>
<thead>
<tr>
<th>Radio</th>
<th>Far IR, Microwave</th>
<th>IR</th>
<th>UV</th>
<th>x-ray</th>
<th>γ-ray</th>
</tr>
</thead>
</table>

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.
Adolph Von Beyer (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.)


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

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


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 victoria*, not produced by bacteria.
Fluorescence in the 20\textsuperscript{th} 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 20\textsuperscript{th} 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 porphyrs. 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 fluorescence lifetime
5. The polarization (anisotropy) of the emission

In these lectures, we examine each of these categories and briefly discuss historical developments, underlying concepts and practical considerations
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]$_1$
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.
Absorption maxima: The importance of conjugation
Increasing the number of aromatic rings increases the absorption maximum

The wavelength value of the absorption maximum and the molar absorbtivity are determined by the degree of Conjugatation of \( \pi \)-bonds

Benzene < Naphthalene < Anthracene < naphthacene < pentacene

<table>
<thead>
<tr>
<th></th>
<th>Abs. Max</th>
<th>262 nm</th>
<th>275 nm</th>
<th>375 nm</th>
<th>475 nm</th>
<th>580 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log ( \varepsilon )</td>
<td>3.84</td>
<td>3.75</td>
<td>3.90</td>
<td>4.05</td>
<td>4.20</td>
<td></td>
</tr>
</tbody>
</table>

(Extinction)

As the degree of conjugation increases
(i.e. the number of electrons involved in the delocalized \( \pi \)-orbitals)
the absorption energy decreases (>\( \lambda \), the energy between the ground and excited state decreases)
the absorption becomes more intense (>\( \varepsilon \), increased probability of absorption)
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 emission 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.
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
The fluorescence emission spectrum

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

The quantum yield of fluorescence (QY) is defined as the rate of the emission process divided by the sum of the rates of all deactivation processes:

$$QY = \frac{k_f}{k_f + k_{nr} + k_{ic}}$$

$k_f$ is the rate of fluorescence, $k_{nr}$ is the rate of radiationless decay and $k_{ic}$ is the rate of intersystem crossing.

*Another way to think about QY is:*

$$QY = \frac{\text{Number of emitted photons}}{\text{Number of absorbed photons}}$$

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

<table>
<thead>
<tr>
<th>Range</th>
<th>Compound</th>
<th>Temp. (^{\circ}\text{C})</th>
<th>Solvent</th>
<th>(\Phi_F)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>270–300 nm</td>
<td>Benzene</td>
<td>20</td>
<td>Cyclohexane</td>
<td>0.05 (\pm 0.02)</td>
<td>1</td>
</tr>
<tr>
<td>300–380 nm</td>
<td>Tryptophan</td>
<td>25</td>
<td>(\text{H}_2\text{O}) (pH 7.2)</td>
<td>0.14 (\pm 0.02)</td>
<td>2</td>
</tr>
<tr>
<td>300–400 nm</td>
<td>Naphthalene</td>
<td>20</td>
<td>Cyclohexane</td>
<td>0.23 (\pm 0.02)</td>
<td>3</td>
</tr>
<tr>
<td>315–480 nm</td>
<td>2-Aminopyridine</td>
<td>20</td>
<td>0.1 mol L(^{-1}) (\text{H}_2\text{SO}_4)</td>
<td>0.60 (\pm 0.05)</td>
<td>4</td>
</tr>
<tr>
<td>360–480 nm</td>
<td>Anthracene</td>
<td>20</td>
<td>Ethanol</td>
<td>0.27 (\pm 0.03)</td>
<td>1, 5</td>
</tr>
<tr>
<td>400–500 nm</td>
<td>9,10-diphenylanthracene</td>
<td>20</td>
<td>Cyclohexane</td>
<td>0.90 (\pm 0.02)</td>
<td>6, 7</td>
</tr>
<tr>
<td>400–600 nm</td>
<td>Quinine sulfate dihydrate</td>
<td>20</td>
<td>0.5 mol L(^{-1}) (\text{H}_2\text{SO}_4)</td>
<td>0.546</td>
<td>5, 7</td>
</tr>
<tr>
<td>600–650 nm</td>
<td>Rhodamine 10I</td>
<td>20</td>
<td>Ethanol</td>
<td>1.0 (\pm 0.02)</td>
<td>8</td>
</tr>
<tr>
<td>600–650 nm</td>
<td>Cresyl violet</td>
<td>20</td>
<td>Methanol</td>
<td>0.92 (\pm 0.02)</td>
<td>9</td>
</tr>
</tbody>
</table>

Effect of Solvent

- Effect varies depending on the fluorophore
- Highly solvent dependent – eg, ANS, bodipy
- Low solvent dependence – eg. Rhodamines, Alexa 488
- Solvent properties
  - Viscosity eg. Rhodamine B, Thioflavin T
  - Refractive index
  - Polarity eg. ANS, Bis-ANS, EtBr
  - Protic eg. Tryptophan
  - pH eg. BCECF, SNARF
  - Temperature eg. tryptophan
• Local environment can affect any or all of the fluorescence properties of the fluorophore
  – Excitation/emission spectrum
  – Quantum yield
  – Lifetime
  – Often affects more than one, eg ANS in EtOH Vs H₂O results in blue shift in the excitation and emission spectrum, increase in lifetime and quantum yield.
Corrected Emission Spectra

Correct for:

• Emission monochromator efficiency
• Detector wavelength dependence
• Polarization dependence of the monochromator - Woods anomaly
Monochromator Polarization Bias

Tungsten Lamp Profile Collected on an SLM Fluorometer

Wood’s Anomaly

No Polarizer

Parallel Emission

Perpendicular Emission

Corrected Excitation Spectra

Correct for:

• Lamp curve
• Excitation monochromator efficiency

Typically conducted using conc (~10mM Rh B in EtOH) in short path length cuvette

Not a trivial measurement !!
Excitation Correction

Absorption (dotted line) and Excitation Spectra (solid line) of ANS in Ethanol

(from Jameson, Croney and Moens, Methods in Enzymology, 360:1)
What is meant by the “lifetime” of a fluorophore???

Although we often speak of the properties of fluorophores as if they are studied in isolation, such is not usually the case.

In general, the behavior of an excited population of fluorophores is described by a familiar rate equation:

$$\frac{dn^*}{dt} = -n^* \Gamma + f(t)$$

where $n^*$ is the number of excited elements at time $t$, $\Gamma$ is the rate constant of emission and $f(t)$ is an arbitrary function of the time, describing the time course of the excitation. The dimensions of $\Gamma$ are sec$^{-1}$ (transitions per molecule per unit time).
If excitation occurs at $t = 0$, the last equation, takes the form:

$$\frac{dn^*}{dt} = -n^* \Gamma$$

and describes the decrease in excited molecules at all further times. Integration gives:

$$n^*(t) = n^*(0) \exp(-\Gamma t)$$

The lifetime, $\tau$, is equal to $\Gamma^{-1}$

If a population of fluorophores are excited, the lifetime is the time it takes for the number of excited molecules to decay to $1/e$ or 36.8% of the original population according to:

$$\frac{n^*(t)}{n^*(0)} = e^{-t/\tau}$$
In pictorial form:

\[ \frac{n^*(t)}{n^*(0)} = e^{-t/\tau} \]
Knowledge of a fluorophore’s excited state lifetime is crucial for quantitative interpretations of numerous fluorescence measurements such as quenching, polarization and FRET.

In most cases of interest, it is virtually impossible to predict a priori the excited state lifetime of a fluorescent molecule. The true molecular lifetime, i.e., the lifetime one expects in the absence of any excited state deactivation processes – can be approximated by the Strickler-Berg equation (1962, J. Chem. Phys. 37:814).

\[
\tau_m^{-1} = 2.88 \times 10^{-9} n^2 \left\langle \nu_f^{-3} \right\rangle \int_{\Delta\nu_e} \varepsilon(\nu) d\ln\nu
\]

where

\[
\left\langle \nu_f^{-3} \right\rangle = \frac{\int_{\Delta\nu_e} F(\nu) d\nu}{\left(\int_{\Delta\nu_a} F(\nu) \nu^{-3} d\nu\right)^2}
\]

\(\tau_m\) is the molecular lifetime, \(n\) is the refractive index of the solvent, \(\Delta\nu_e\) and \(\Delta\nu_a\) correspond to the experimental limits of the absorption and emission bands (\(S_0 - S_1\) transitions), \(\varepsilon\) is the molar absorption and \(F(\nu)\) describes the spectral distribution of the emission in photons per wavelength interval.

How well do these equations actually work?

Not very well – sometimes off by factors of 2 – 5 fold.
Polarization

Polarizers have been in use for a very long time - the Vikings used a “sunstone” (thought to have been composed either of the mineral cordierite or Iceland spar – calcite – both of which are naturally polarizing materials) 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.
He published his findings in 1809: “Sur une propriété de la lumière réfléchie par les corps diaphanes” (Bull. Soc. Philomat. I:16)

Malus also derived an expression for calculating the transmission of light as a function of the angle ($\theta$) between two polarizers. This equation (Malus’ Law) is now written as: $I_\theta = I_0 (\cos^2 \theta)$
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.

Unpolarized (natural) light

Polarized light
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)
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:

\[ r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \]

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:

<table>
<thead>
<tr>
<th>P</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>0.40</td>
</tr>
<tr>
<td>0.30</td>
<td>0.22</td>
</tr>
<tr>
<td>0.10</td>
<td>0.069</td>
</tr>
</tbody>
</table>

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 $\theta$ 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 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.

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
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 \( 90^\circ \).

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.
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 $90^\circ$ 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 $\phi$ 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. 8-5. Fluorescence polarization spectrum of phenol at −70°C in propylene glycol. Ordinate = polarization, p; abscissa, exciting wavelength in m. 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°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.
Excitation Polarization Spectrum of GFP
Emission 507nm

GFP-SNAP Concentration (EF/verts)

2.4 nm

4.2 nm

RT 26-27C
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 $\omega$.

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 ($\omega$).

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, \(\eta\) the viscosity and \(\tau\) 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 \(\rho\) 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:

\[ \rho_o = \frac{3\eta M(\nu + h)}{RT} \]

Where \( M \) is the molecular weight, \( \nu \) 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 $\tau_c$, 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 $\rho$ 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:

$$\frac{r_0}{r} = \left(1 + \frac{\tau}{\tau_c}\right)$$
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 sulphydryl 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
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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\(_\gamma\)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
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.
Antibody + Fluorophore-linked antigen → High Polarization

Unlabeled antigen → Low Polarization +

**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):

\[
P = \frac{I_{\text{parallel}} - I_{\text{perpendicular}}}{I_{\text{parallel}} + I_{\text{perpendicular}}}
\]

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