Basic Principles of Fluorescence

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Champaign, 2012

What is Fluorescence?

FLUORESCENCE is the light emitted by an atom or molecule after a finite duration subsequent to the absorption of photons. Specifically, the emitted light arises from the transition of the excited species from its first excited electronic singlet level to its ground electronic level.

What is Fluorescence?

The development of highly sophisticated fluorescent probe chemistries, new lasers 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.

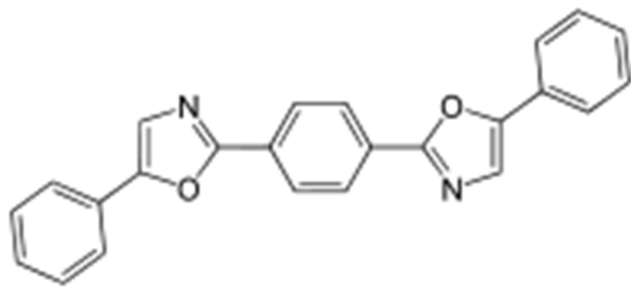
Common Fluorophores

Fluorescein

Rhodamine B

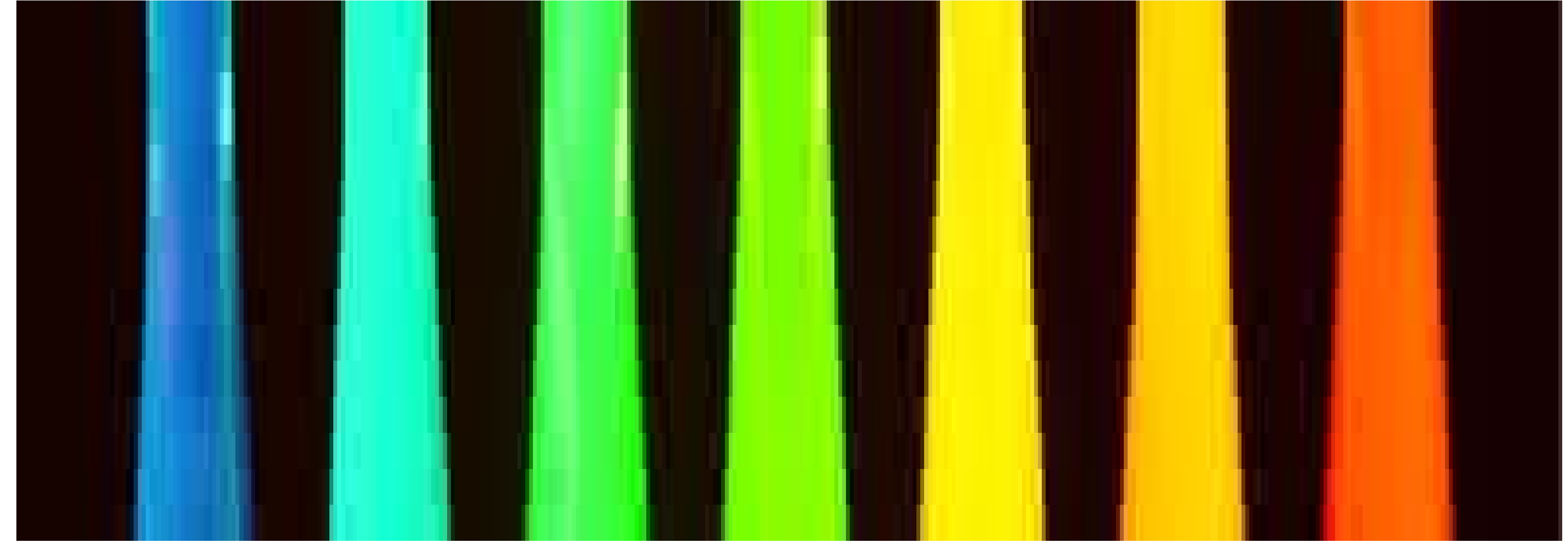
Quinine

Tryptophan



POPOP

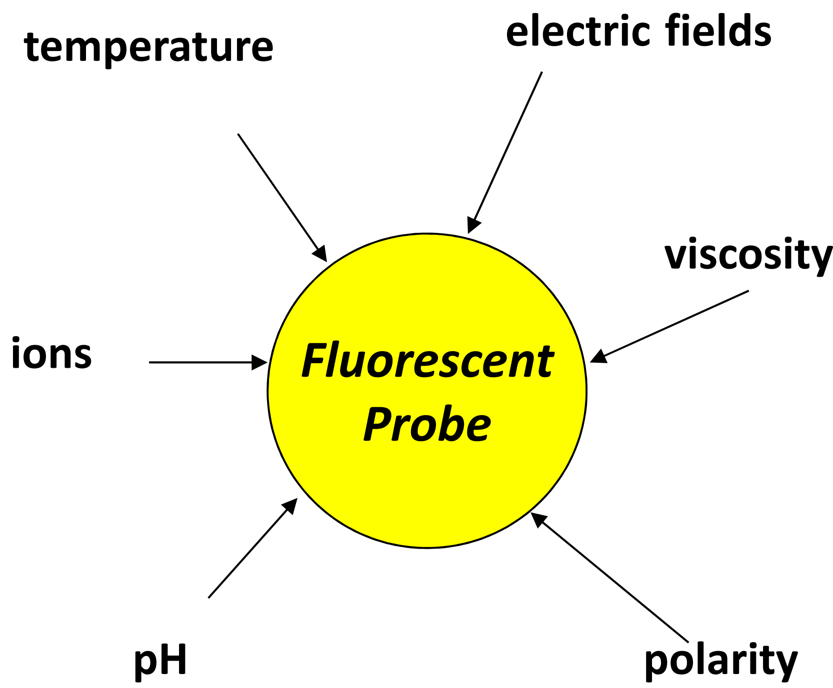
Why Fluorescence?



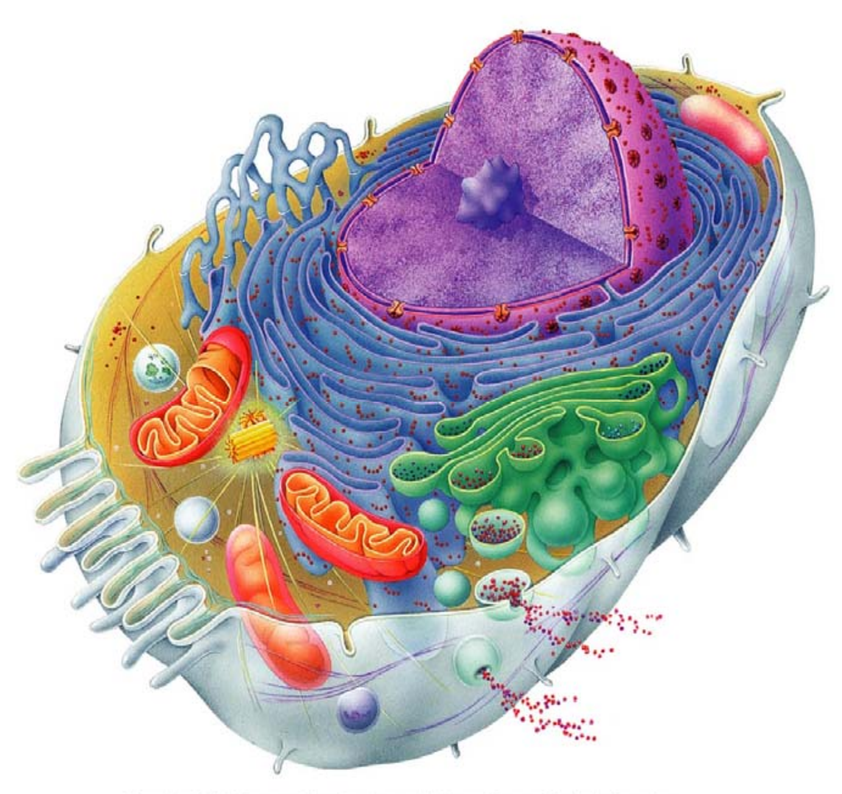
It provides: ***-information on the molecular environment.***

-information on dynamic processes on the nanosecond timescale

Why Fluorescence?



Why Fluorescence?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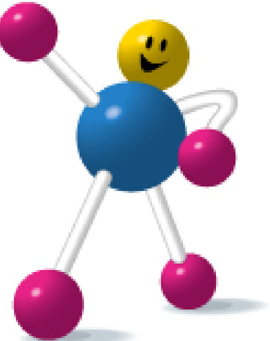
In recent years, due to the availability of multiphoton lasers and GFP probes, fluorescence has been successfully used to monitor processes at the cellular level with detailed spatial resolution.

Why Fluorescence?

Also, fluorescence is very very sensitive!
Applications to solutions with subnanomolar concentrations is fairly common. With proper instrumentation

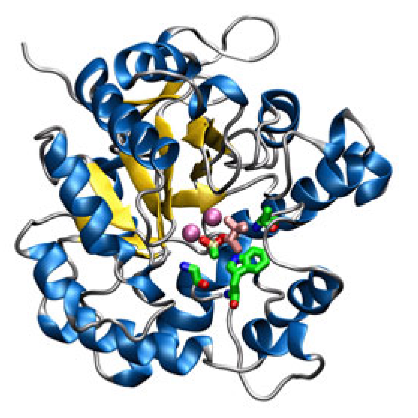
Single molecule detection

has become almost routine

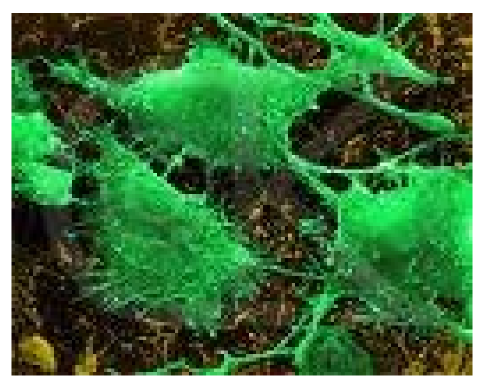


Systems accessible by Fluorescence Techniques

Molecular structure

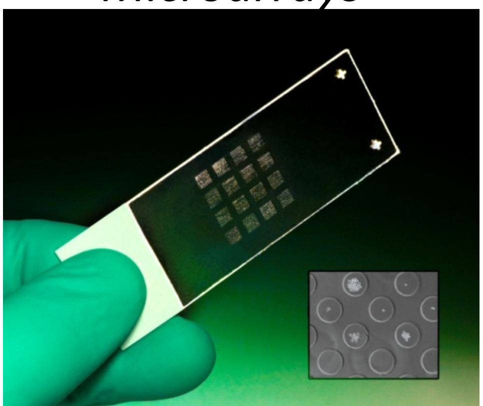


Cell studies



Live animals

Microarrays



Instrumentation

Spectrofluorometers

(courtesy of ISS)

Confocal microscopes

(courtesy of Carl Zeiss)

Instrumentation

Flow Cytometry

(courtesy of ICyt)

Genome Sequencing

(courtesy of Pacific Biosciences)

Instrumentation

Immunoassay Chemistry Analyzer

(courtesy of Abbott Diagnostics)

Microwell Plate Readers

(courtesy of Molecular Devices)

The first records of fluorescence

Nicolás Monardes, a Spanish physician and botanist publishes in 1565 the *“Historia medicinal de las cosas que se traen de nuestras Indias Occidentales”* in which he describes 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.

What is the blue color?

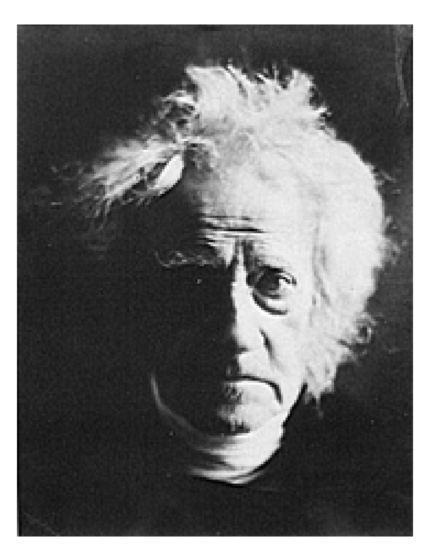
An early Latin translation (1574) of Monardes' work by the influential Flemish botanist **Charles de L'Écluse** (1526-1609), in which the wood's name is given as *Lignum Nephriticum (kidney wood),* helped to extend awareness of its strange optical properties in Europe. This wood was very popular in XVI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

An Englishman, **John Frampton,** translated Monardes description in 1577 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.."***

Fluorescence Fluorescence

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.

Epipolic dispersion



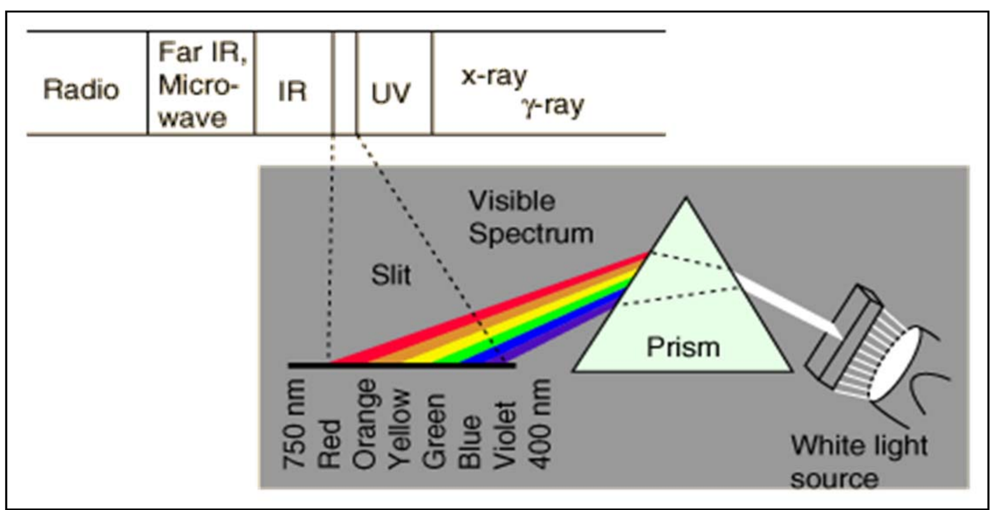
John Herschel (1845) made the first observation of fluorescence from quinine sulfate - he termed this phenomenon "epipolic dispersion".

IV. 'Αμόρφωτα, 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.

Stokes experiment

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.



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.

Understanding the phenomenon

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. He initially used the term "dispersive reflection" to describe the phenomenon presented by quinine sulphate.

* 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 shift

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.**

He also seems to have been the first to propose, in 1864, the use of fluorescence as an analytical tool, in a lecture "*On the application of the optical properties to detection and discrimination of organic substances.”*

Modern Fluorescence

1905 E. Nichols and E. Merrit: **first excitation spectrum of a dye**

1919 Stern and Volmer: **fluorescence quenching**

1923 S.J. Vavilov and W.L. Levshin: **fluorescence polarization of dyes**

1924 S.J. Vavilov: **first determination of fluorescence yield**

1925 F. Perrin: **theory of fluorescence polarization**

1926 E. Gaviola: **first direct measurement of nanosecond lifetime**

1935 A. Jablonski: **diagram**

1948 T. Förster: **QM theory of dipole-dipole interaction**

What are the Parameters measured by Fluorescence?

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**

Perrin-Jablonski diagram

S_2

S_1

K_R

K_{IC}

S_0

Perrin-Jablonski diagram

S_2

T_2

S_1

K_{ISC}

T_1

K_R

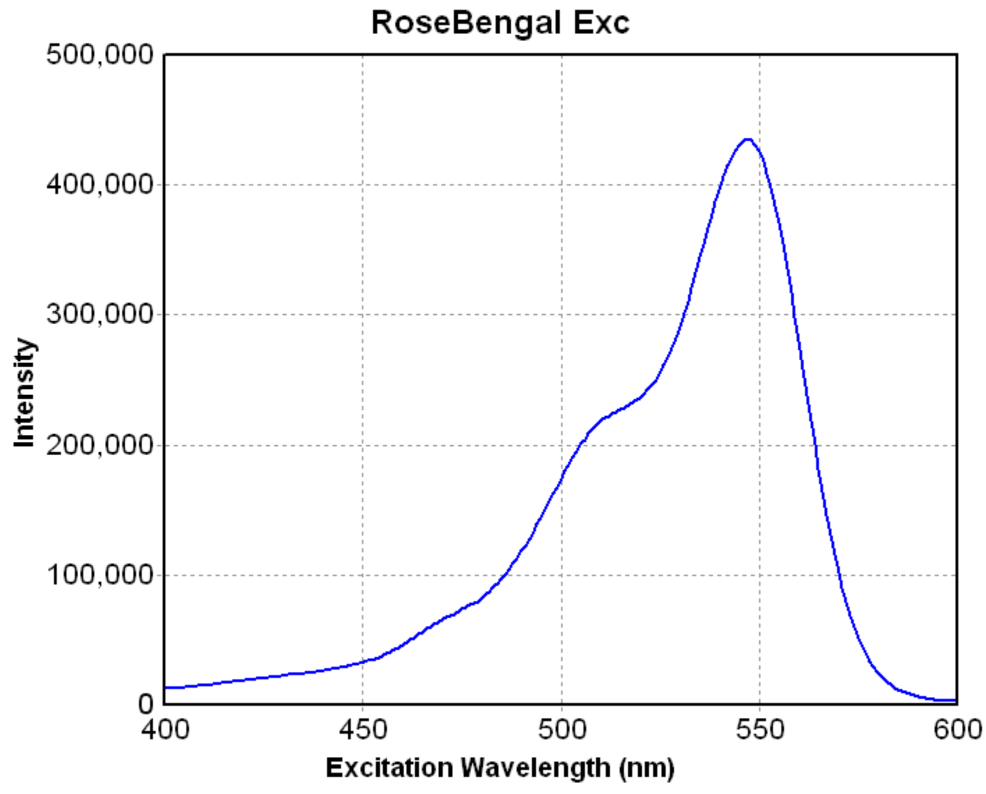
K_{IC}

K_{NR}

S_0

The excitation spectrum

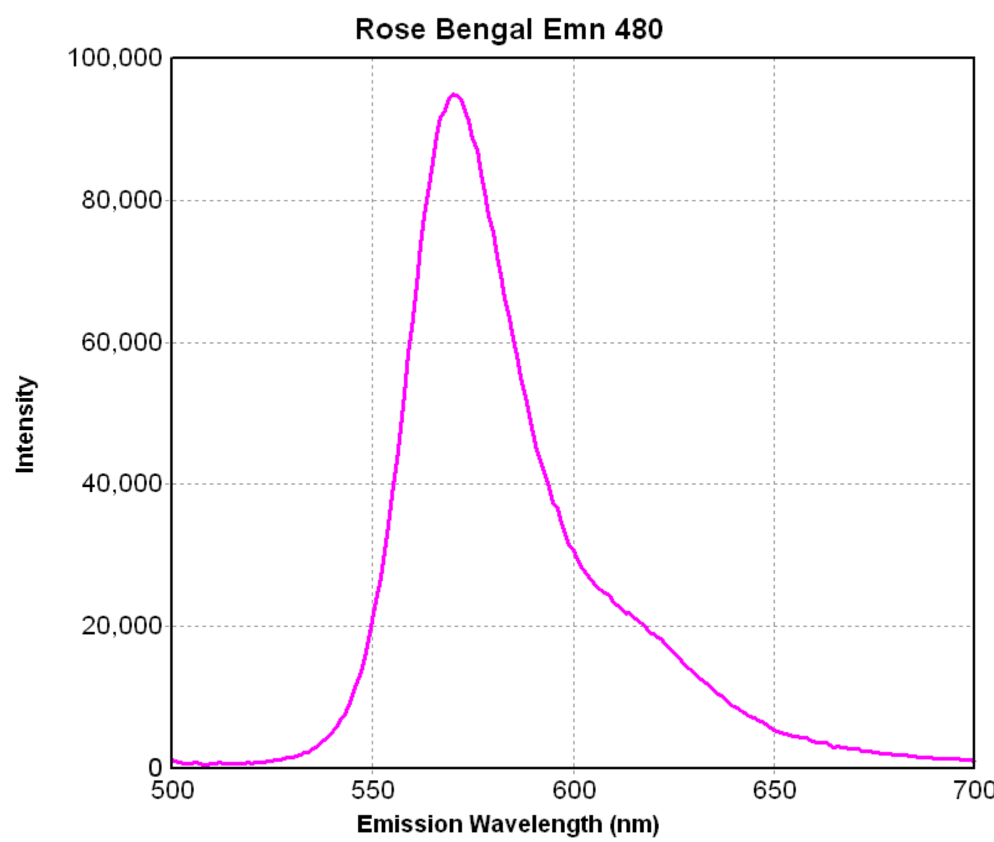
In recording an excitation spectrum, one observes the intensity of emission at a fixed wavelength while scanning the excitation



The excitation spectrum should match the absorption spectrum: companies provide technical corrections to the data

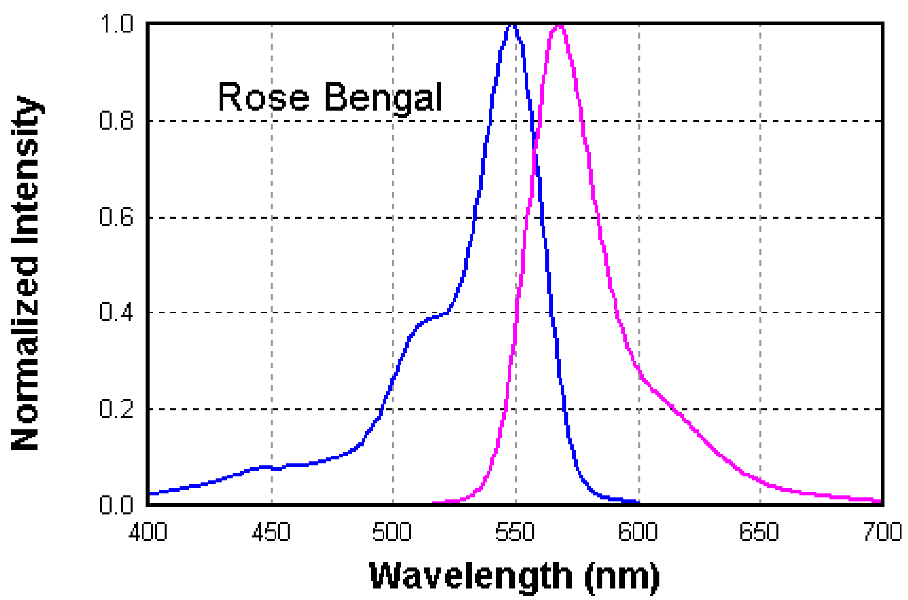
The emission spectrum

In recording an emission spectrum, one keeps the excitation at a fixed wavelength and while scanning the emission.



Some rules about spectra

1)The fluorescence spectrum lies at longer wavelengths than the absorption (Stokes shift)
2) The fluorescence spectrum is, to a good approximation, a mirror image of the absorption band of least frequency.
3) The fluorescence spectrum is invariant, remaining the same independent of the excitation wavelength



Quantum yield

The fluorescence QY is the fraction of excited molecules that return to the ground state with emission of photons.

$$QY = \frac{k_R}{k_R + k_{NR}}$$

Another way of thinking about this parameter is:

$$QY = \frac{no.\ of\ \ photons\ emitted}{no.\ of\ \ photons\ absorbed}$$

List of quantum yields [from Molecular Fluorescence by B. Valeur]

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.), 91S.
10) Magde D., Brannon J. H., Cramers T. L. and Olmsted J. III (1979) *J. Phys. Chem.* 83, 696.

Lifetime

Absorption and emission processes are concepts that involve a population of molecules. In general, if N_1 is the population of the excited level S_1, the population is described by the relation:

$$\frac{dN_1}{dt} = -(k_R + k_{NR})N_1 + f(t)$$

$$N_1 = N_1(0)\, e^{-t/\tau_S} \qquad \tau_S = \frac{1}{k_R + k_{NR}}$$

τ_S is the lifetime of excited state S_1

If a population of fluorophores is excited at time t=0, after a time the number of molecules in τ is decreased to 1/e or to about 36.8%

Quantum yield and lifetime

The fluorescence QY is the fraction of excited molecules that return to the ground state with emission of photons.

$$QY = \frac{k_R}{k_R + k_{NR}} = \frac{\tau_S}{\tau_R}$$

Can the lifetime be calculated?

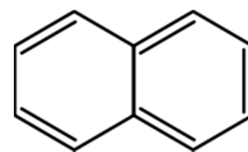
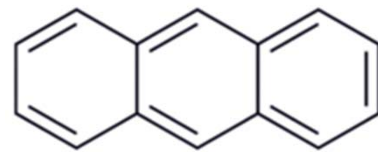
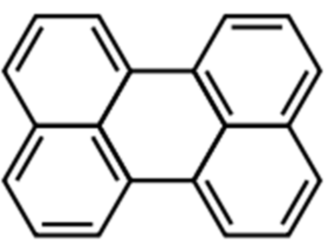
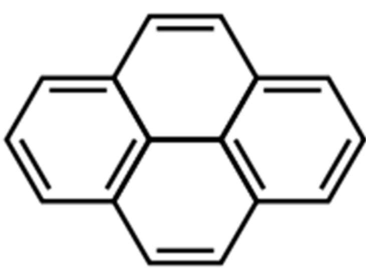
Knowledge of a fluorophore's excited state lifetime is crucial for quantitative interpretations of numerous fluorescence measurements such as quenching, polarization and FRET.

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

$$\frac{1}{\tau_R} = \frac{8\pi \bullet 230 c n^2}{N} \frac{\int F_\nu(\nu_F)\, d\nu_F}{\int \nu_F^{-3} F_\nu(\nu_F)\, d\nu_F} \int \frac{\varepsilon(\nu_A)\, d\nu_A}{\nu_A}$$

F_ν is the fluorescence emission
ε the extinction coefficient
ν the wavenumber

Lifetimes of some aromatic hydrocarbons in ethanol

Naphtalene		*2.7 ns*
Anthracene		*5.1 ns*
Perylene		*4.3 ns*
Pyrene		*410 ns*

Lifetime and the environment

The lifetime and quantum yield for a given fluorophore are often dramatically affected by its environment.

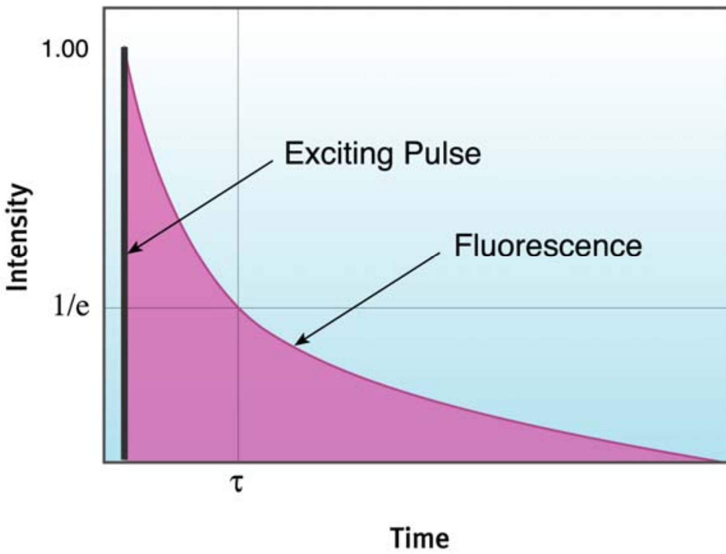
ANS in water is ~100 picoseconds but can be 8 – 10 ns bound to proteins

Ethidium bromide is 1.8 ns in water, 22 ns bound to DNA and 27ns bound to tRNA

The lifetime of tryptophan in proteins ranges from ~0.1 ns to ~ 10 ns

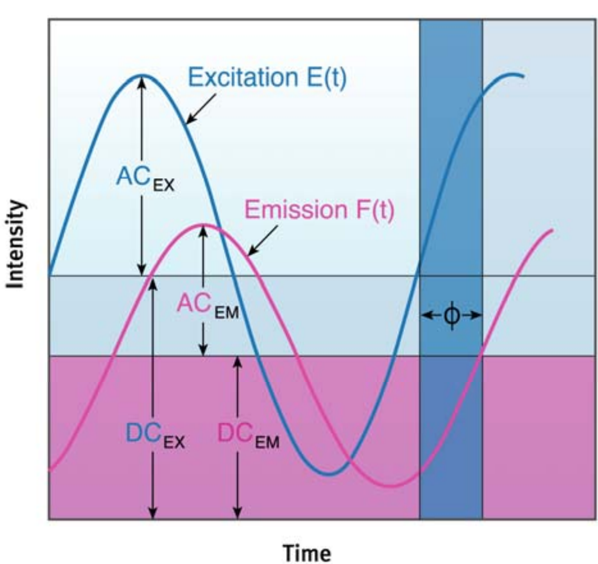
Two ways to measure lifetime

Time domain



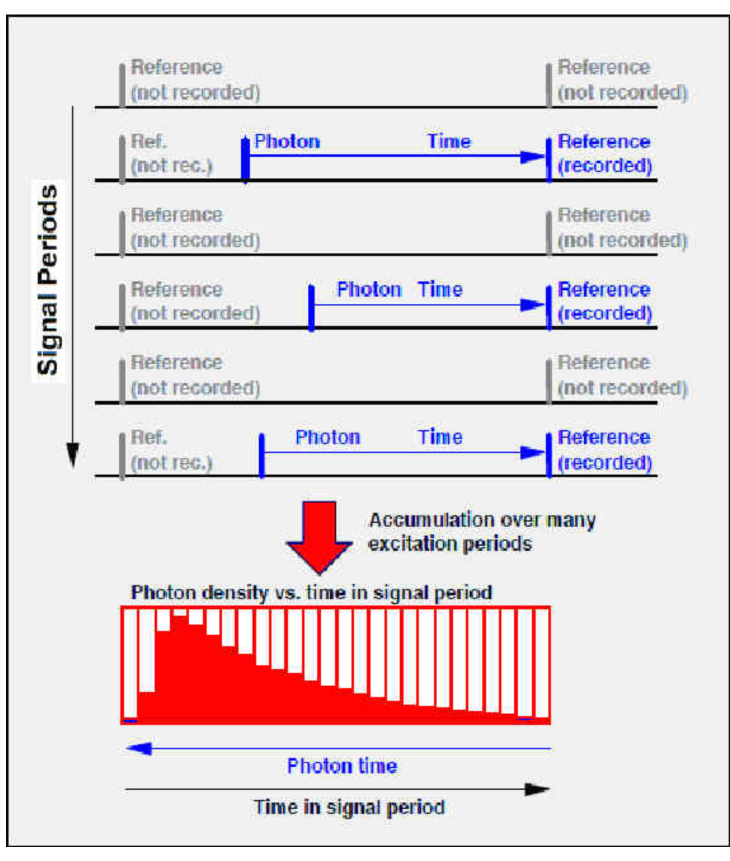
$$I(t) = I_0 \, e^{-t/\tau}$$

Frequency domain



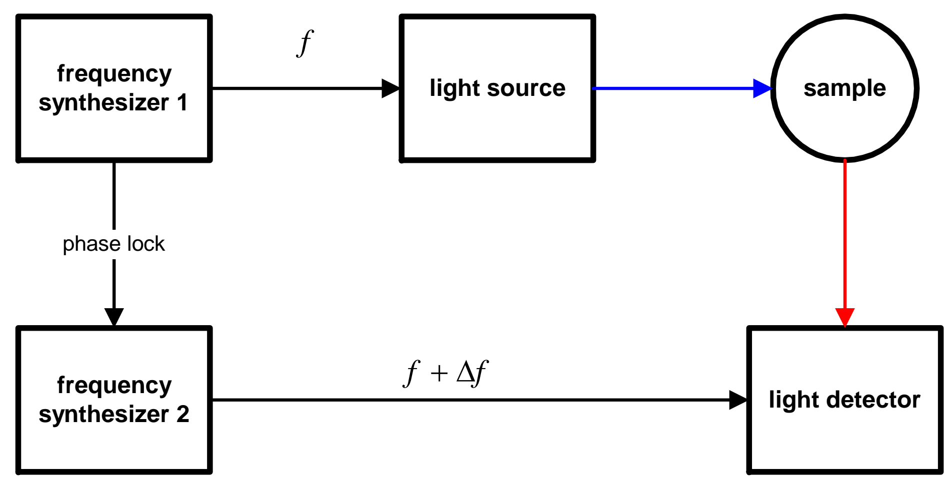
$$\tau_P = \frac{1}{\omega} \tan\phi \qquad \tau_M = \frac{1}{\omega}\sqrt{\frac{1}{m^2} - 1}$$

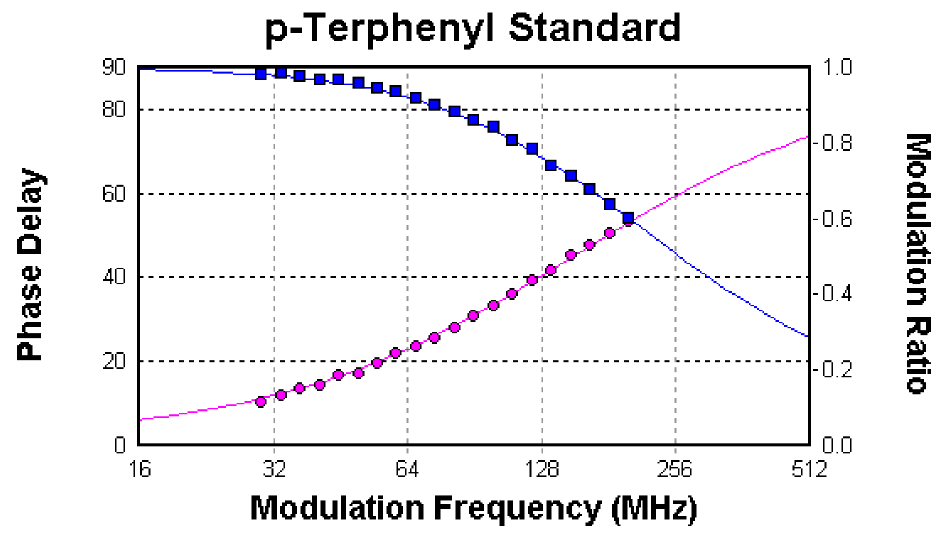
Time Correlated Single Photon Counting



(courtesy of Becker and Hickl)

Analog Frequency Domain

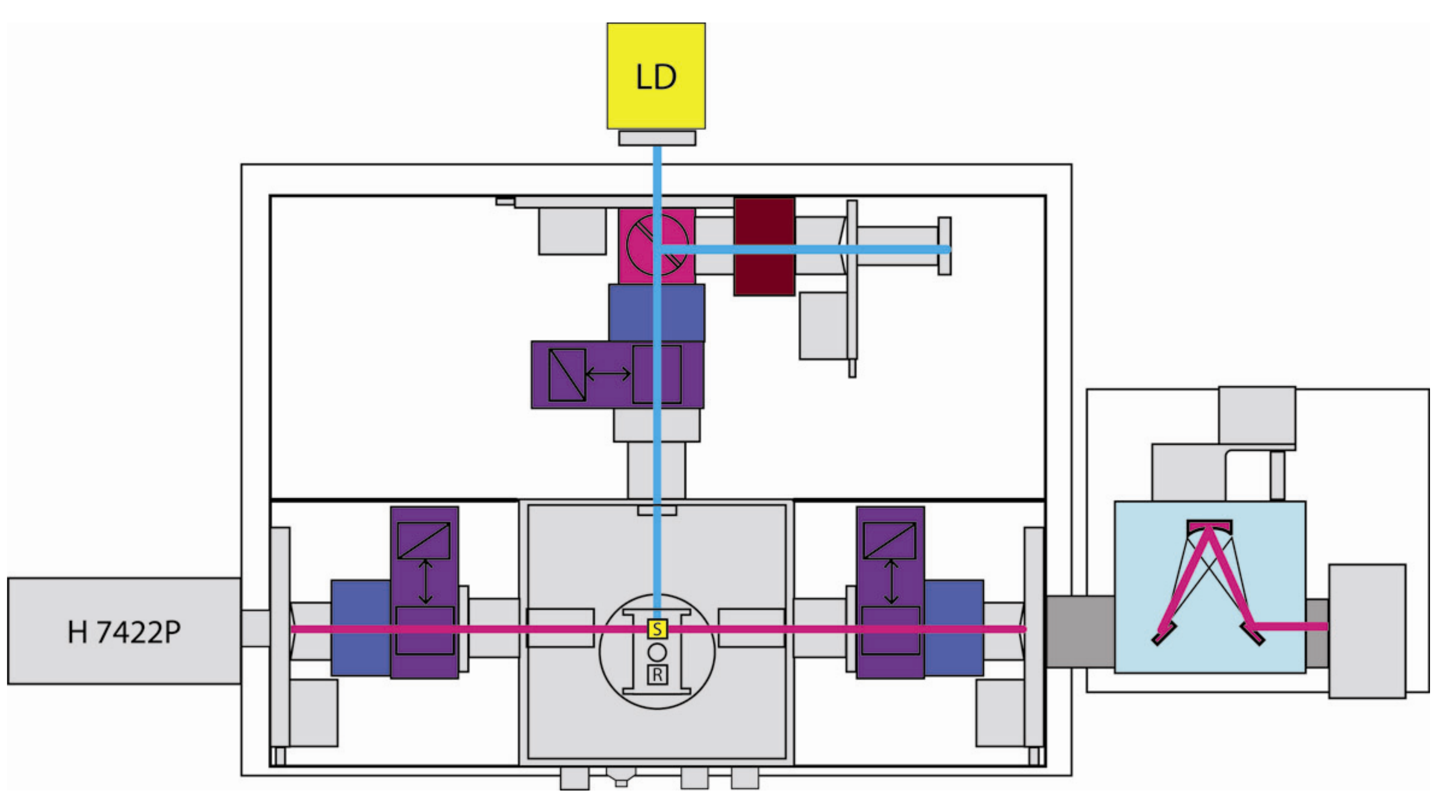




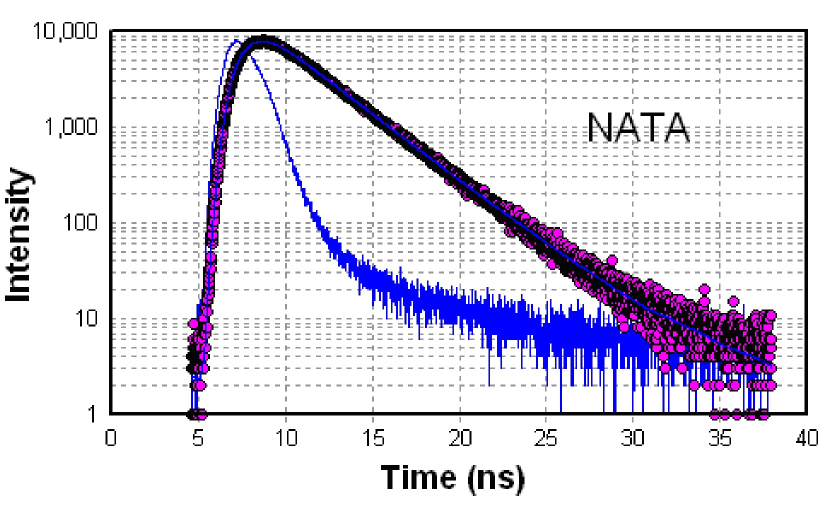
ISS offers both methodologies

ChronosFD and ChronosBH

ISS offers both methodologies



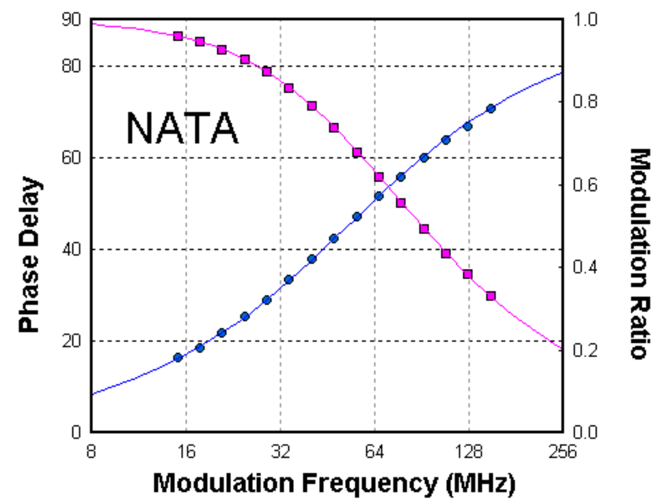
UV Measurements



280 nm pulsed LED

WG 380 LP filter

$\tau = 3.0\, ns$

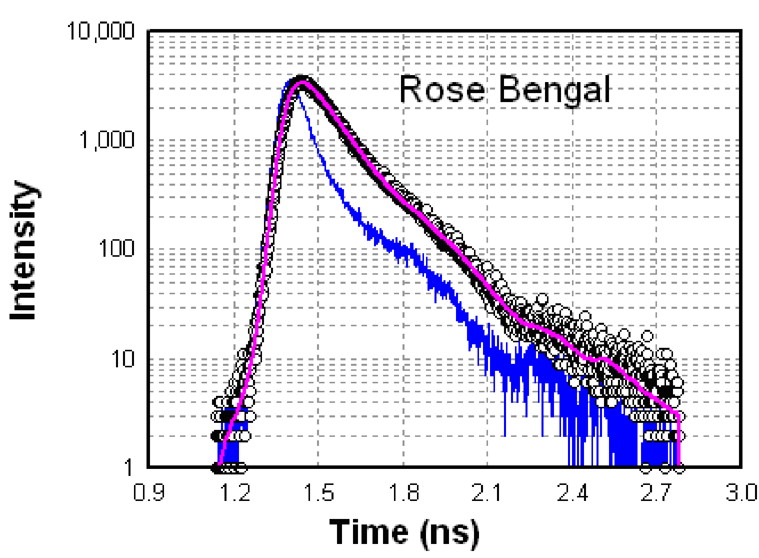


300 nm cw LED

WG 320 LP filter

$\tau = 3.1\, ns$

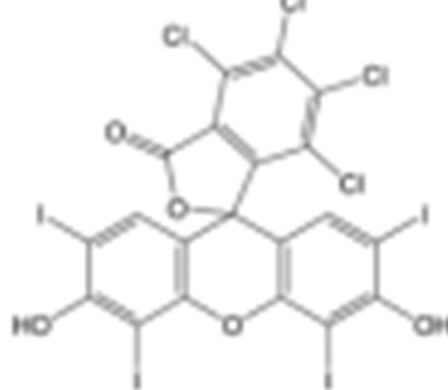
Picoseconds standard

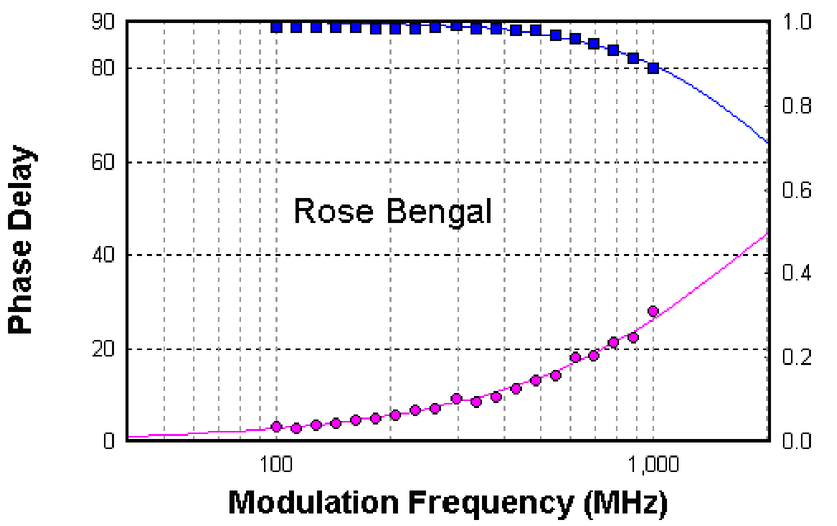


473 nm pulsed laser diode

515 nm LP filter

$$\tau = 77\,ps$$



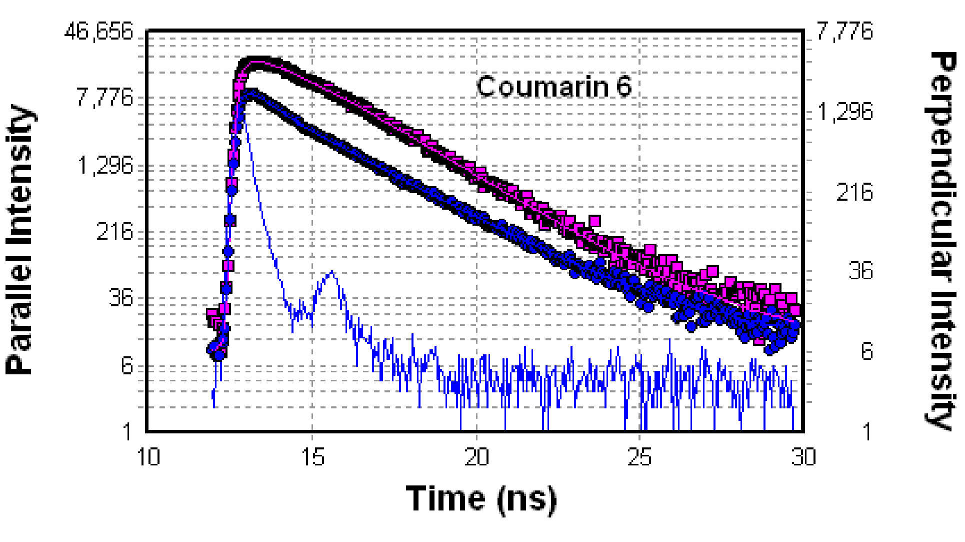


471 nm cw laser diode

OG530 LP filter

$$\tau = 78\,ps$$

Time Resolved Anisotropy

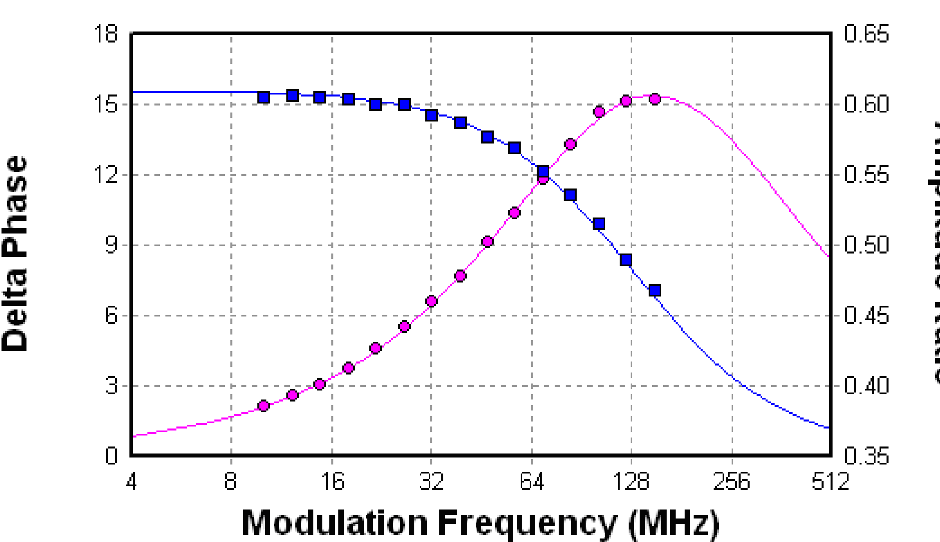


447 nm pulsed laser diode

KV505 LP filter; T=20 ºC

$$\tau = 2.30\ ns$$

$$\Theta = 2.6\ ns \quad R_0 = 0.38$$



473 nm cw laser diode

WG499 LP filter; T=27 ºC

$$\tau = 2.33\ ns$$

$$\Theta = 2.0\ ns \quad R_0 = 0.38$$

Data Analysis

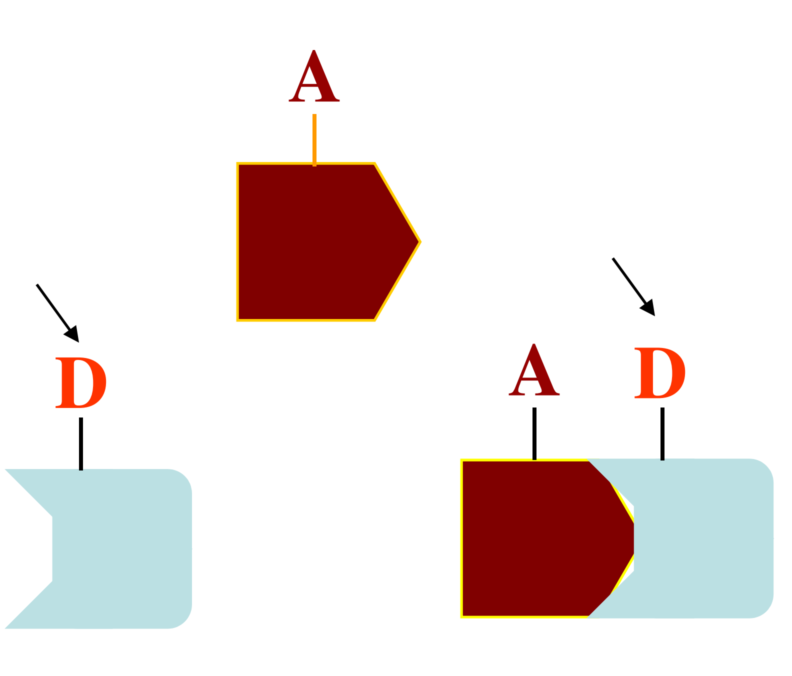
Time domain

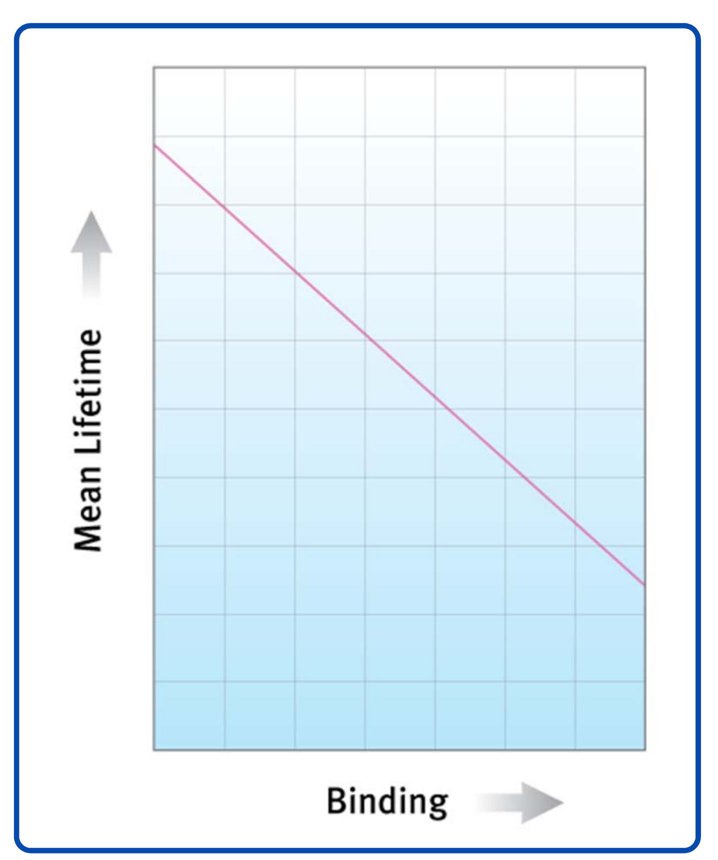
$$\chi^2 = \frac{1}{\nu}\sum_{k=1}^{n}\frac{\left[N(t_k) - N_c(t_k)\right]^2}{\sigma_k^2} = \frac{1}{\nu}\sum_{k=1}^{n}\frac{\left[N(t_k) - N_c(t_k)\right]^2}{N(t_k)}$$

Frequency domain

$$\chi^2 = \frac{1}{\nu}\left\{\sum_{j=1}^{N}\left[\frac{\varphi_\omega - \varphi_{c\omega}}{\sigma_\varphi}\right] + \sum_{j=1}^{N}\left[\frac{M_\omega - M_{c\omega}}{\sigma_M}\right]\right\}$$

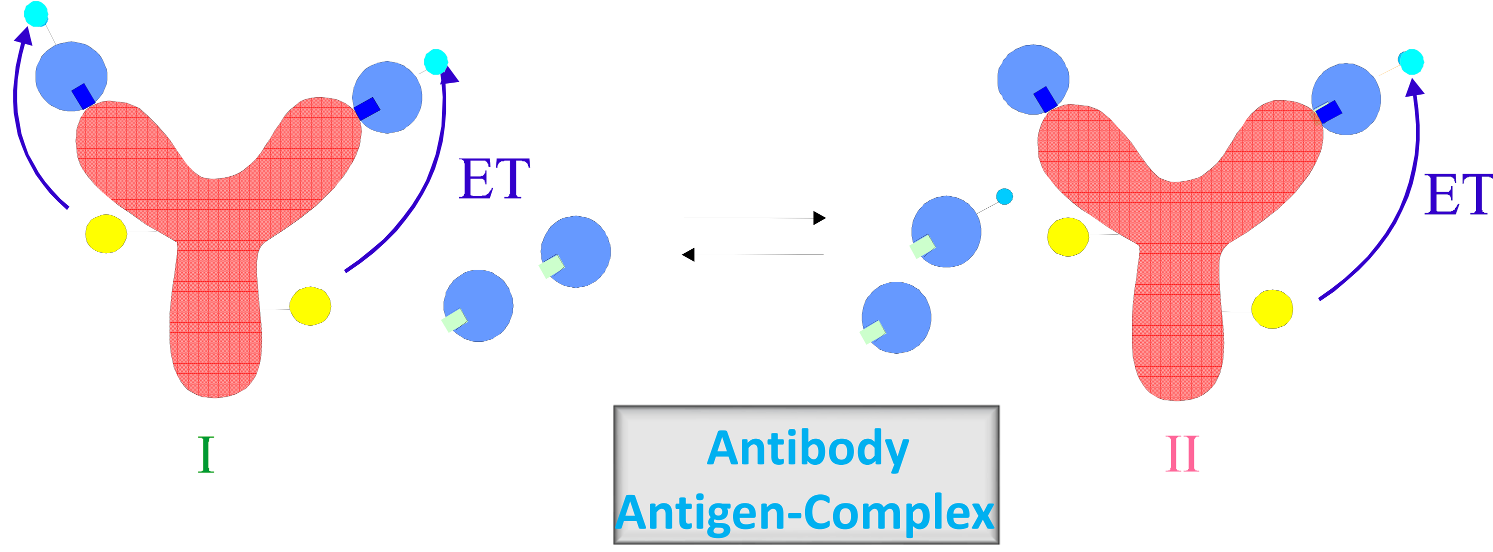
Assays can be designed with Lifetime Readout

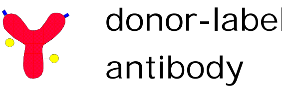




In FRET the Acceptor Shortens the Donor's Lifetime

Competitive Energy Transfer Immunoassay

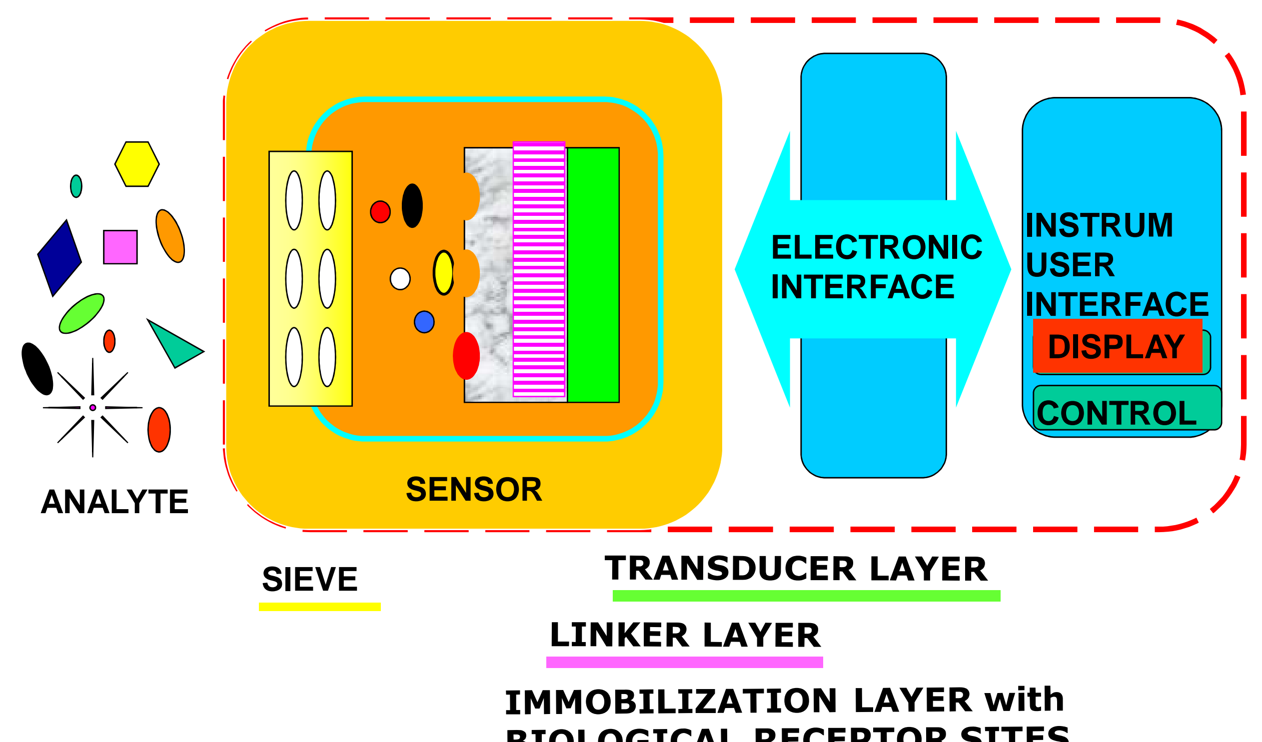




- Fluorescent Donor
- Non-fluorescent Acceptor
- No Need to Separate D and A Signals
- Lifetime independent of Volume, Color- Quenching, etc

$\tau\ (D_{bound}) < \tau\ (D_{free})$

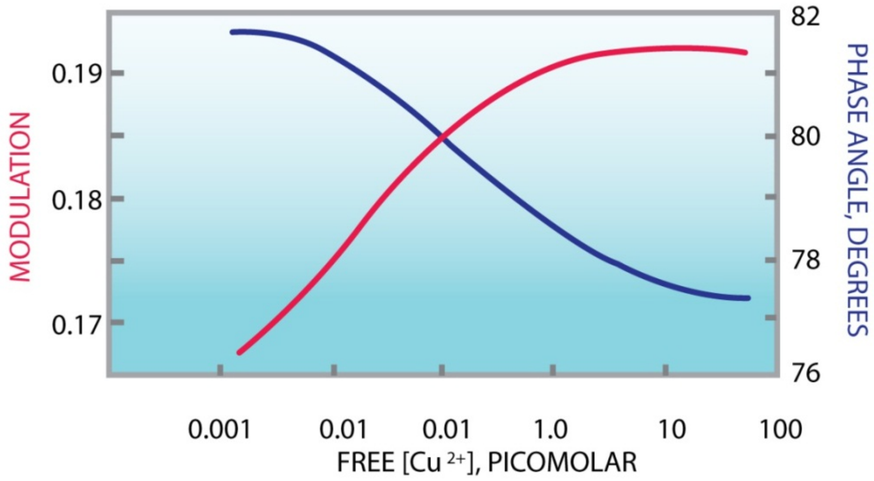
Biosensor System



vandeVen et al. 2002; 5th Intl Weber Symposium

Detection of Picomolar free CU(II) In sea water

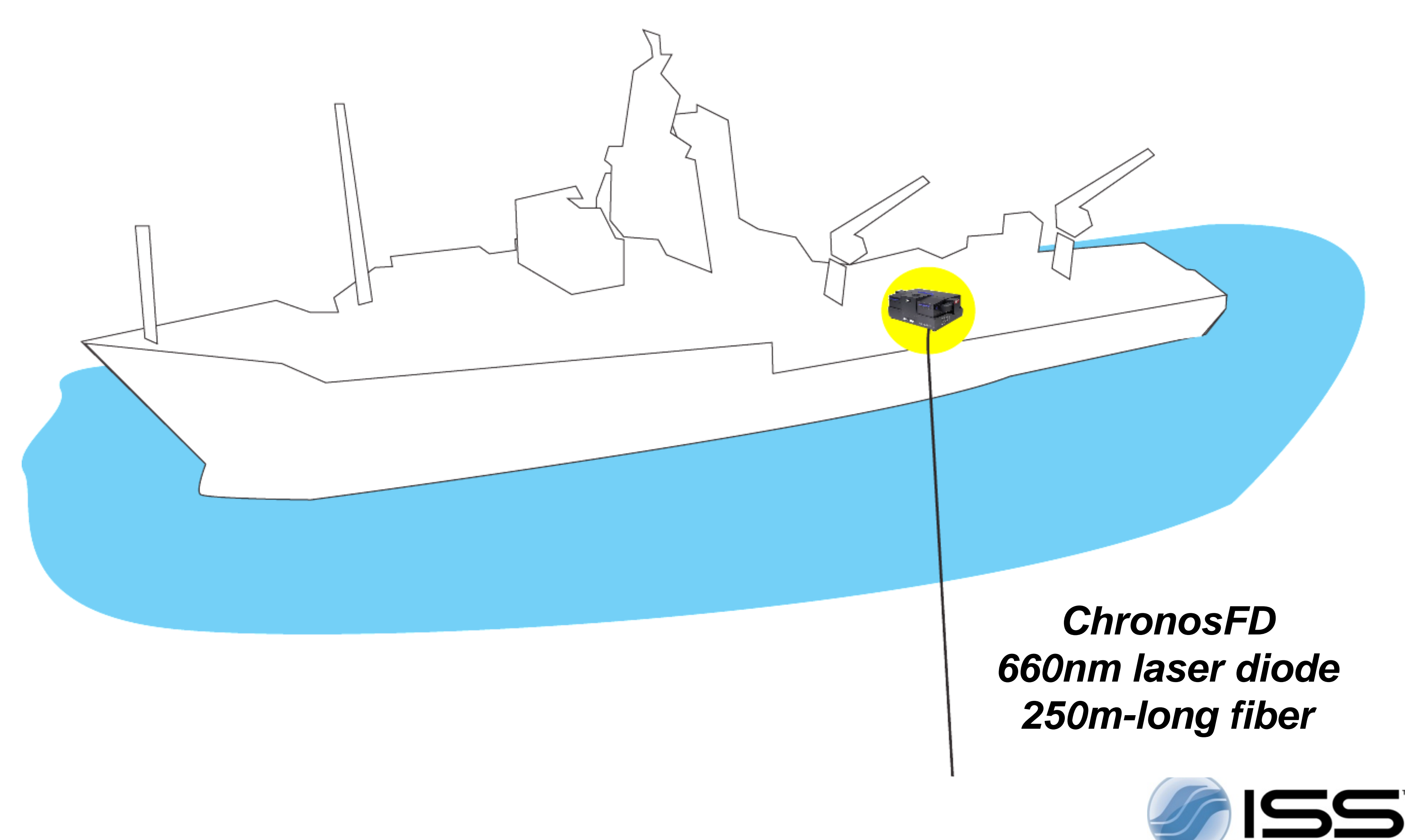
The RV Knorr is the research vessel owned by the U.S. Navy and operated by the Woods Hole Oceanographic Institution.



ChronosFD
660nm laser diode
250m-long fiber

Courtesy of Dr. Richard Thompson

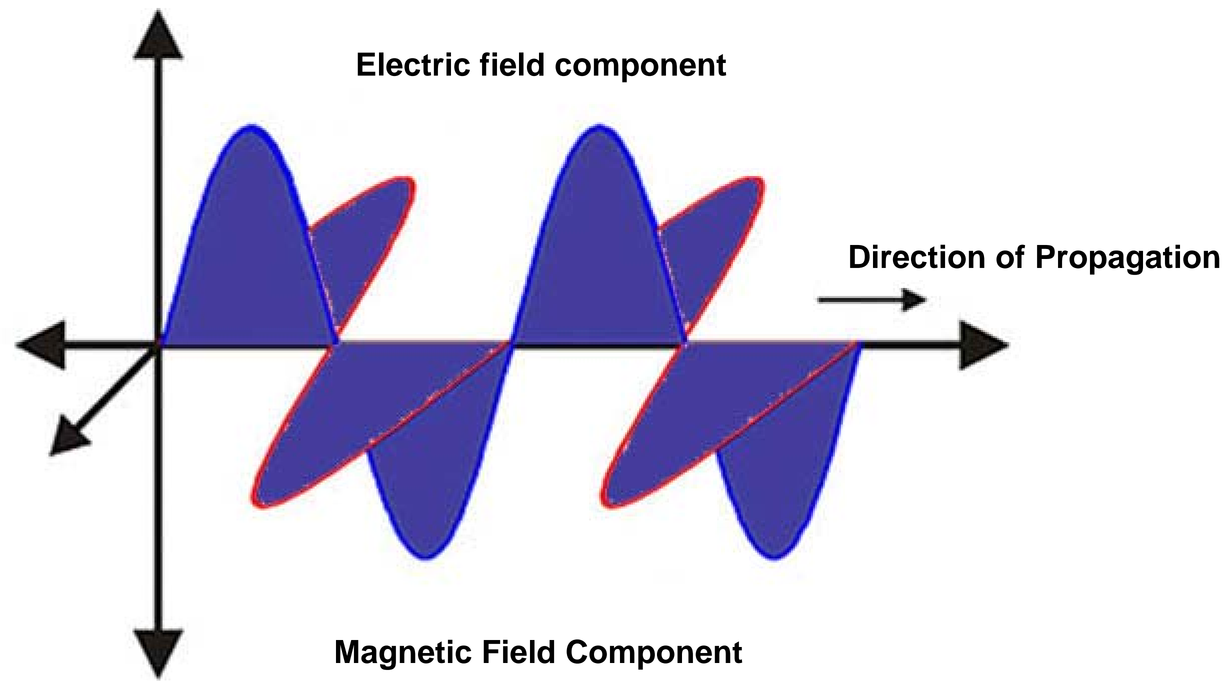
Detection of Picomolar free CU(II) In sea water



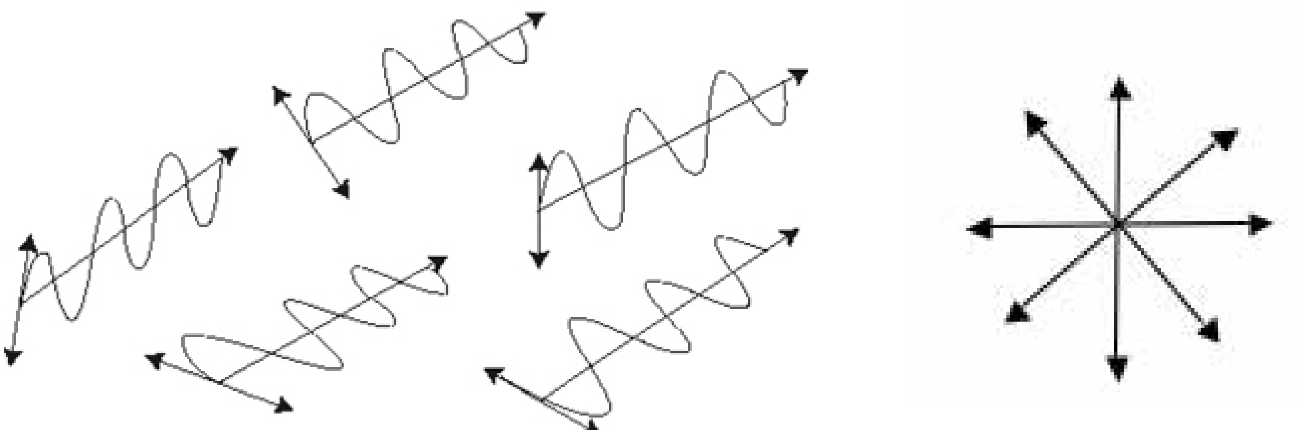
Courtesy of Dr. Richard Thompson

Fluorescence Polarization(Anisotropy)

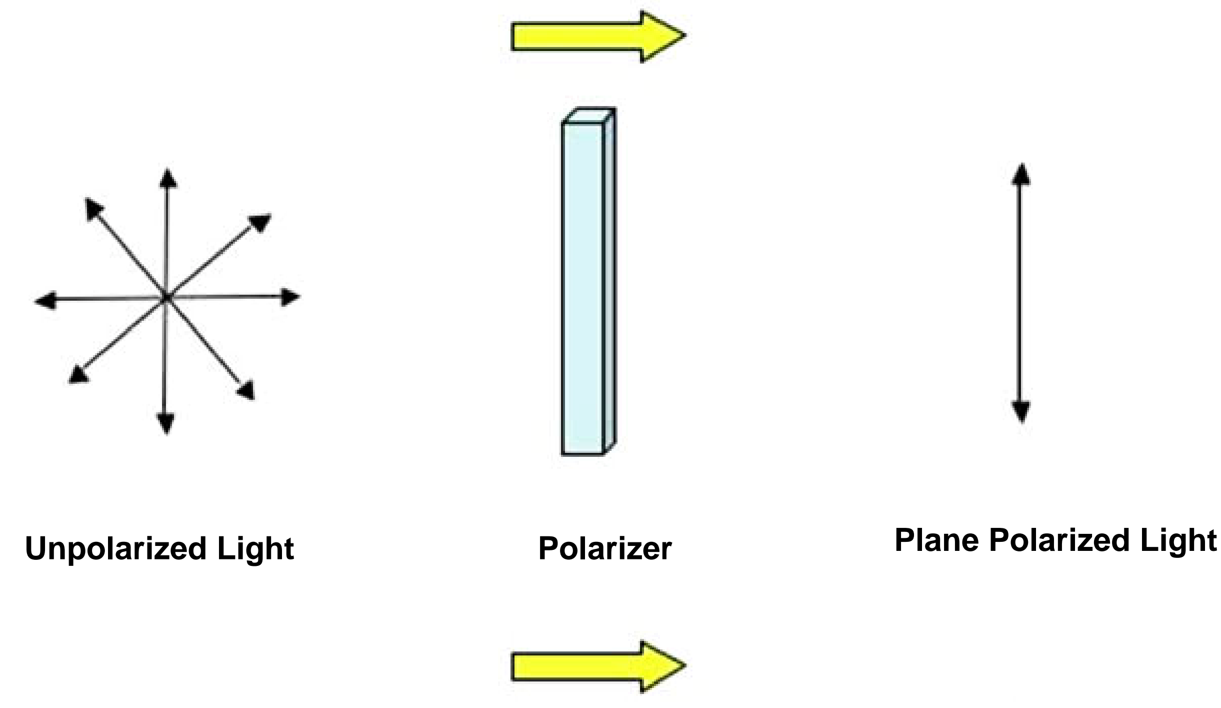
Light is an electromagnetic wave.
The electric field $\vec{E}$ and the magnetic field $\vec{B}$ oscillate perpendicularly to the direction of propagation.



Natural Light



Polarization

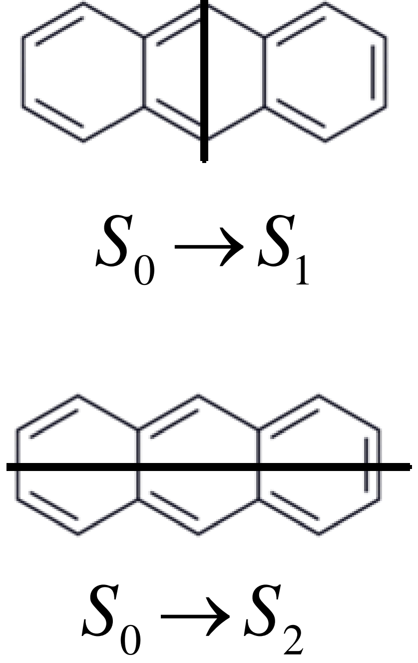


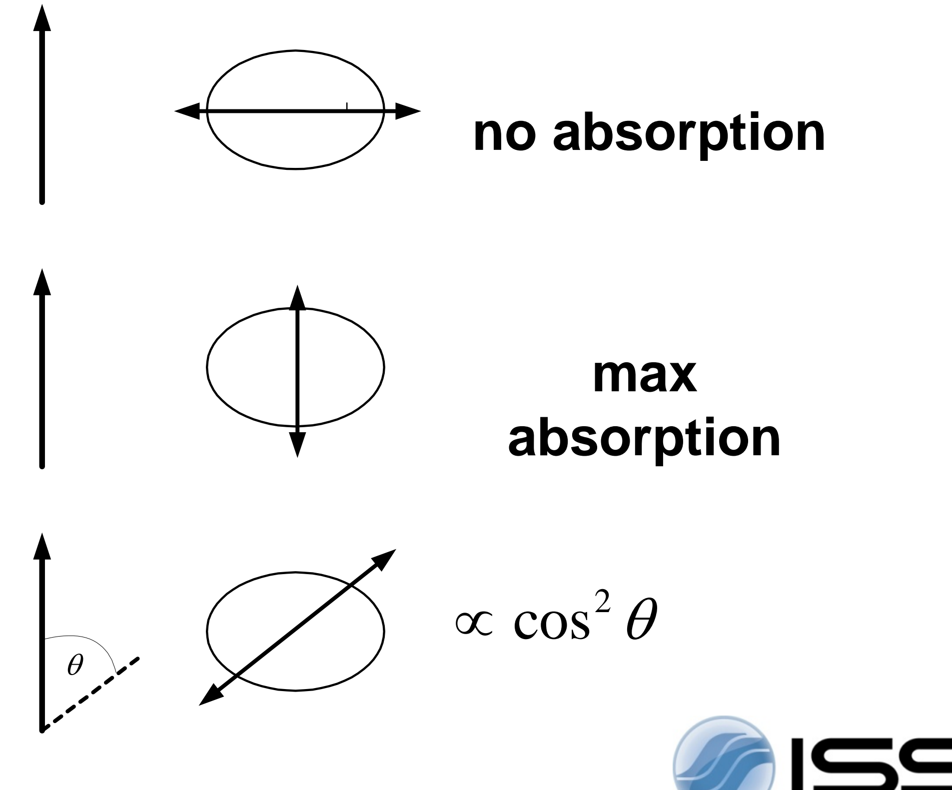
(1) dichroic devices, which operate by effectively absorbing one plane of polarization (e.g., Polaroid type-H sheets based on stretched polyvinyl alcohol impregnated with iodine)

(2) $CaCO_3$ crystal polarizers - which differentially disperse the two planes of polarization

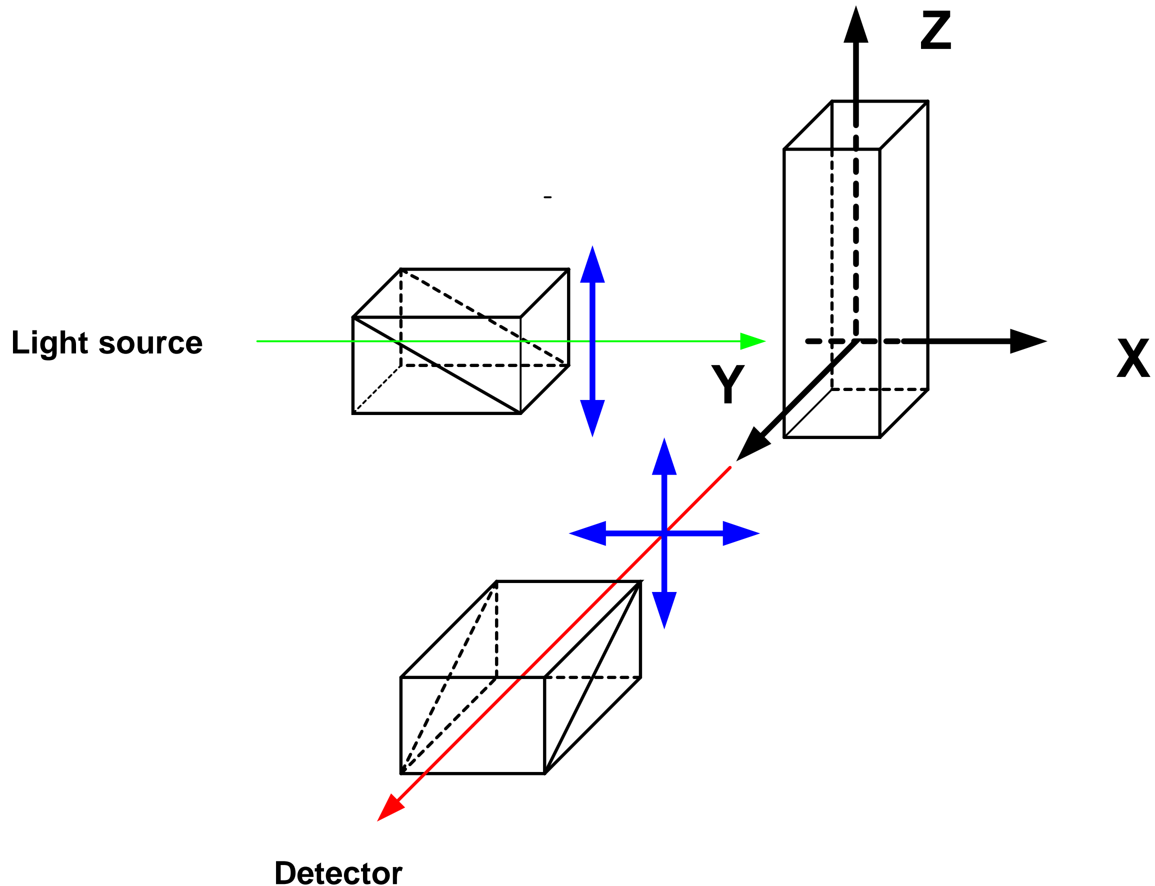
Photoselection

Photoselection: When a population of fluorophores is illuminated by a linearly polarized incident light, the fluorophores with the transition moments oriented in a direction close to that of the electric field are preferentially excited.





Polarization



Anisotropy

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad r = \frac{2P}{3 - P}$$

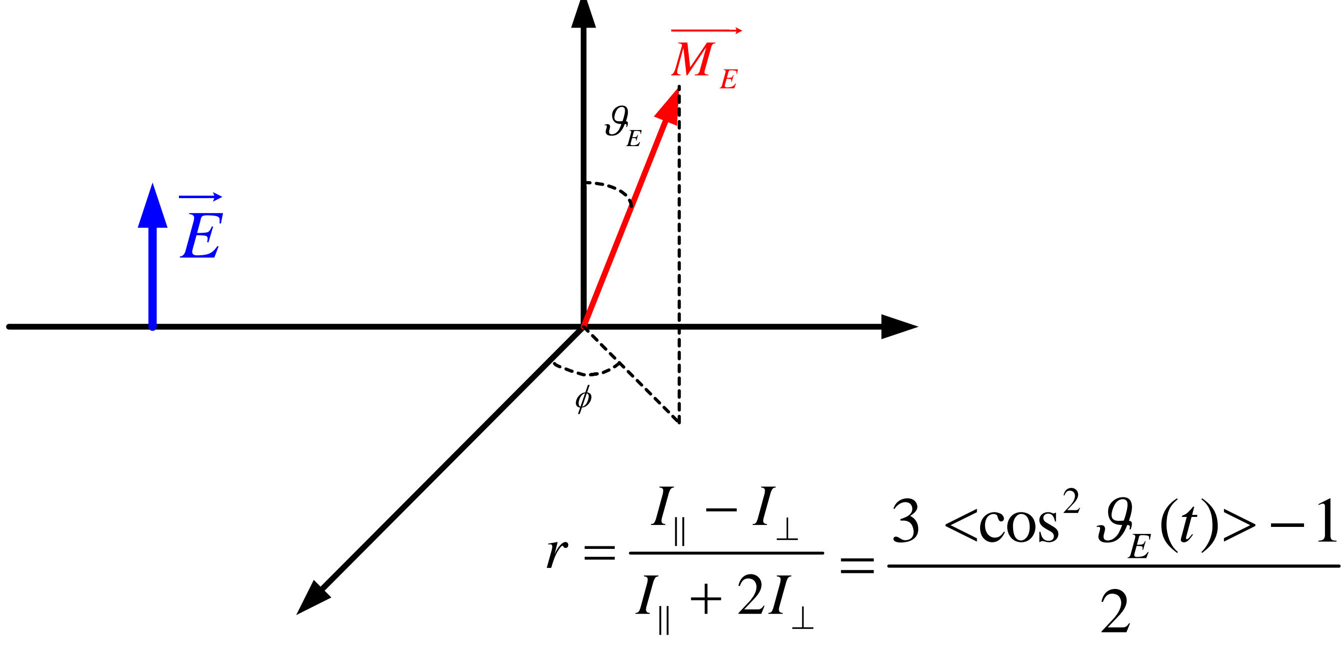
$$-1 \le P \le 1 \qquad -0.5 \le r \le 1 \qquad r = \sum_i f_i \, r_i$$

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

Anisotropy

Let us consider a population of N molecules excited at time 0 by a short pulse of light polarized along z. At time t, the emission transition moments M_E have a certain angular distribution.

The relation between the emission anisotropy and the angular distribution of the emission transition moments is:



Parallel absorption and emission moments: Fundamental Anisotropy

The absorption and emission moments are parallel (excitation to the first singlet state. In this case:

$$<\cos^2 \vartheta> = \frac{3}{5} \qquad \Rightarrow \qquad r_0 = \frac{2}{5} = 0.4$$

This is the theoretical anisotropy in the absence of any motion. The experimental value, called limiting anisotropy, is always a little smaller than the theoretical value.

Non-parallel absorption and emission moments

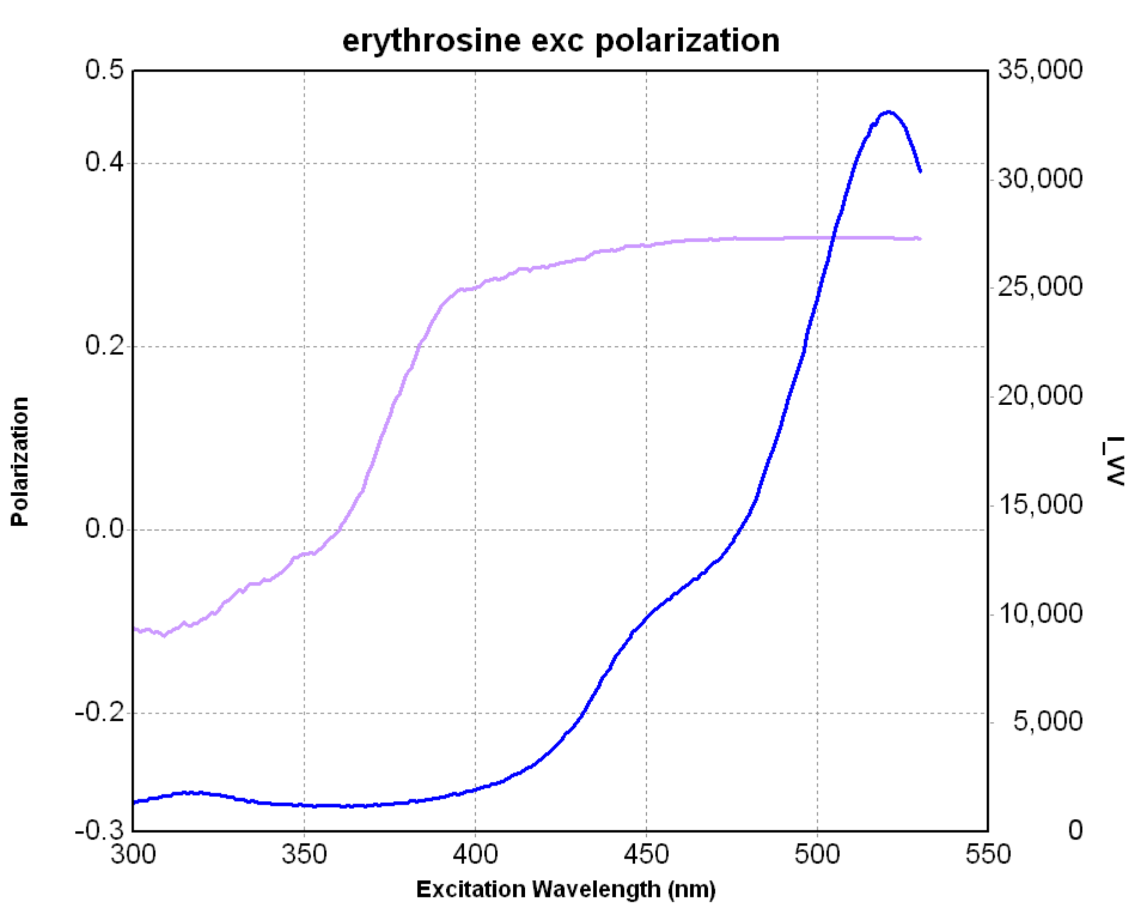
This situation occurs when the excitation brings the fluorophore to an excited state higher than S_1

$$r_0 = \frac{3 < \cos^2 \vartheta_A > -1}{2} * \frac{3 < \cos^2 \alpha > -1}{2} = \frac{2}{5} \frac{3 < \cos^2 \alpha > -1}{2}$$

Where α is the angle between the absorption and emission moments

$$-0.2 \leq r_0 \leq 0.4$$

Anisotropy



Effect of Brownian motion

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

Isotropic molecules $r(t) = r_0 \exp(-6Dt)$ with $D = \frac{RT}{6V\eta}$

a. Time-resolved

b. Steady-state polarization measurements. For single decay:

$$\bar{r} = \frac{1}{\tau} \int_0^\infty r(t) \exp(-6Dt)\, dt$$

$$\frac{1}{\bar{r}} = \frac{1}{r_0}(1 + 6D\tau) = \frac{1}{r_0}\left(1 + \frac{\tau}{\tau_C}\right)$$ ***(Perrin equation)***

Effect of Brownian motion

Anisotropic rotators

$$r(t) = r_0 \sum_i \alpha_i \exp(-t/\tau_{ci})$$

Hindered rotators

$$r(t) = (r_0 - r_\infty)\exp(-t/\tau_c) + r_\infty$$

Some applications of polarization

(from Molecular Fluorescence by B. Valeur)

Spectroscopy	Separation of excited states
Polymers	Local viscosity Molecular orientation Chain dynamics
Immunology	Antigen-antibody reactions immunoassays
Molecular biology	Proteins interactions, denaturation DNA-protein interactions Nucleic acids Biological membranes (fluidity, additives, ..) Micellar systems (microviscosity, ..)

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