

Fluorescence Spectroscopy

Steady State and Time Dependent Fluorescence Measurements

Kai Wen Teng

PHYS 403 Fall 15

EM Spectrum of molecules

Rotational Energy $\longrightarrow$ Infrared

Vibrational Energy $\longrightarrow$ Near Infrared

Electronic Energy $\longrightarrow$ Visible and Ultra-Violet

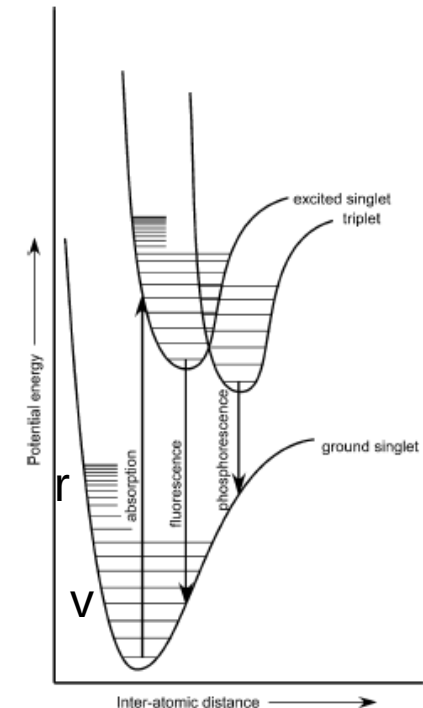
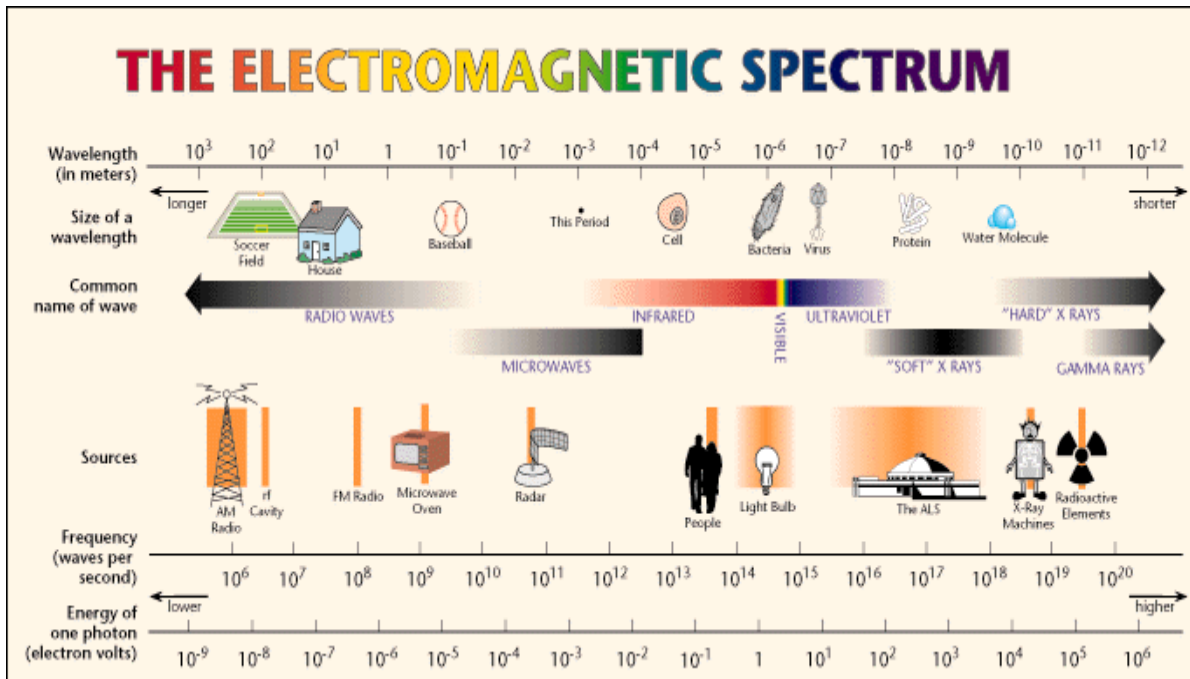
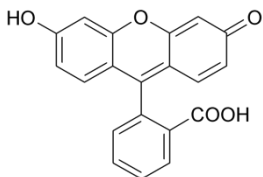
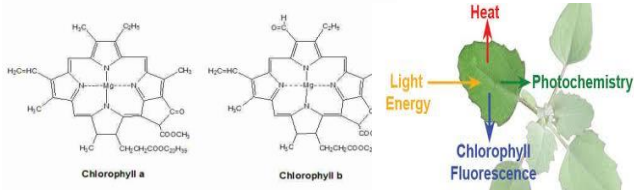


Diagram by Robert Clegg in Photosynth Res. 2009 Aug-Sep;101(2-3):181-94.

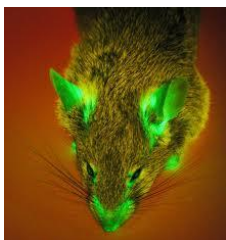
Synthetic Organic: Fluorescein



Naturally Occuring:



Fluorescent Proteins:



Green Fluorescent Protein

Semiconductor Nanocrystal:

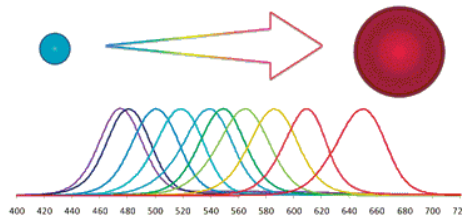
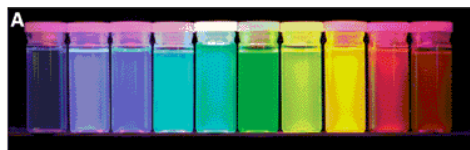
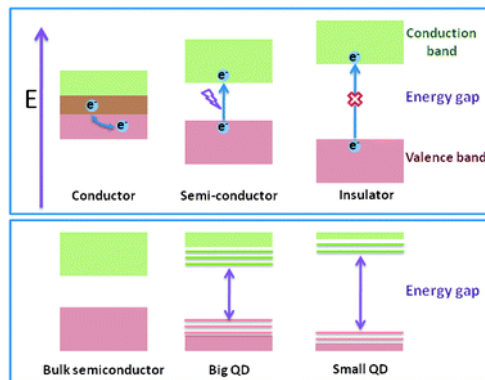
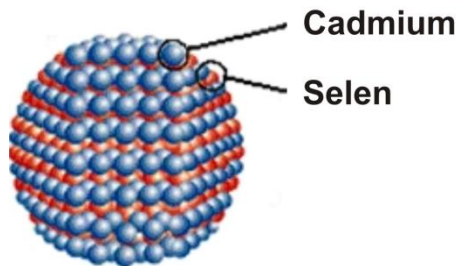
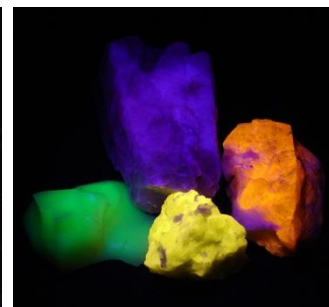
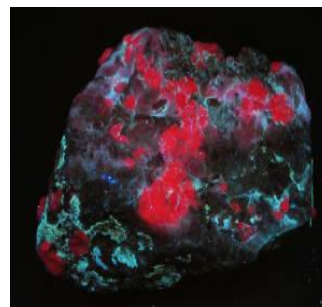


Image from Zrazhevskiy et al. 2010

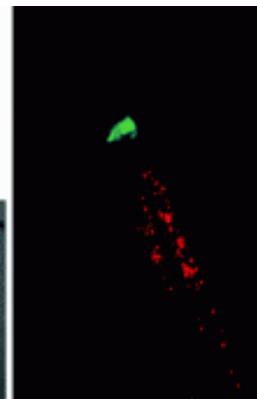
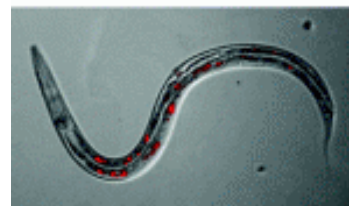
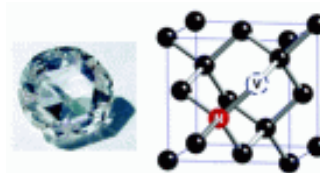
Crystals:



Ruby and assorted mineral

From mineralman.net

Fluorescent Nanodiamonds

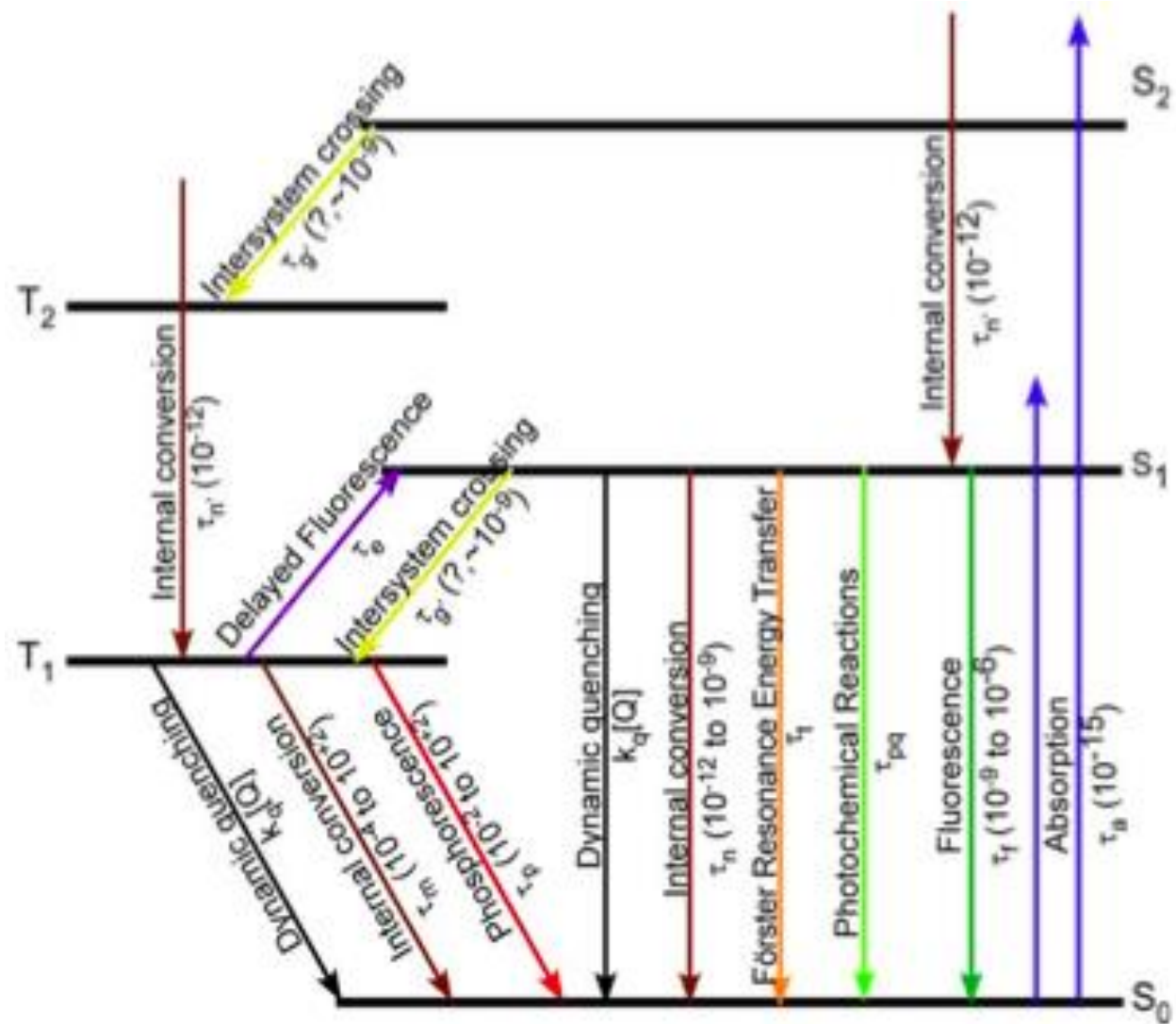


Nano Lett., 2010, 10 (9), pp 3692-3699. DOI:
10.1021/nl1021909

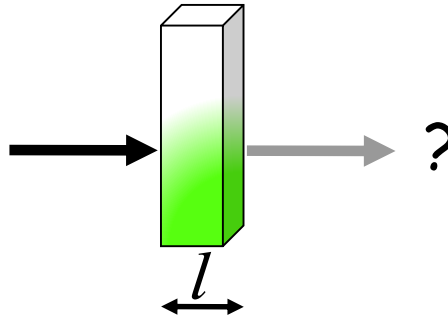
Perrin-Jablonski energy diagram (S_0 , S_1 and S_2 transitions)



Alexander Jablonski



Absorption ($S_0 - S_1$)

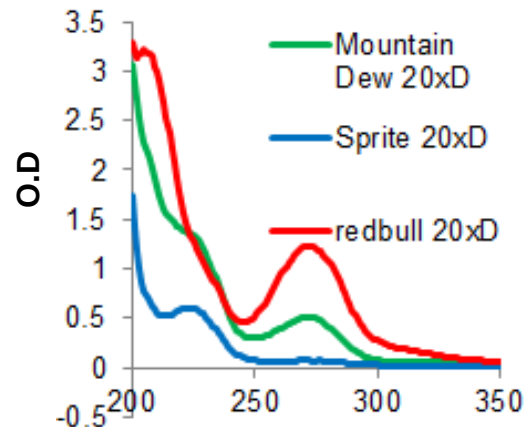
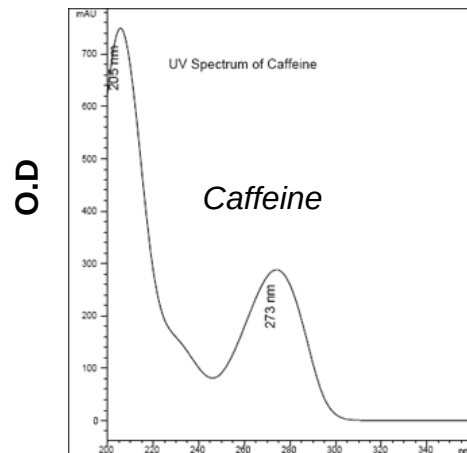


Beer-Lambert's Law

$$\log(I_0) - \log(I) = \epsilon c l$$

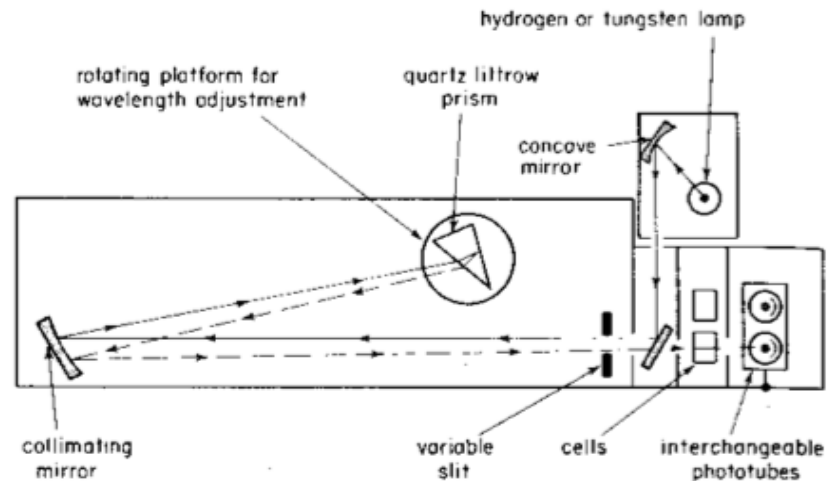
Extinction coefficient:

Concentration

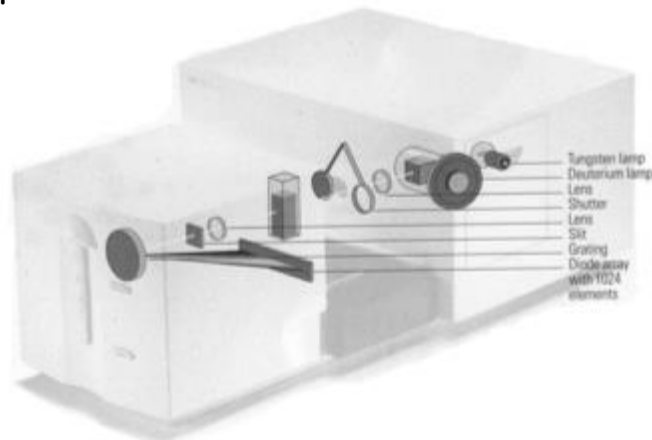


Steady State Measurements: Absorbance

One of the very first commercially available instrument that measures absorbance was the Beckman DU



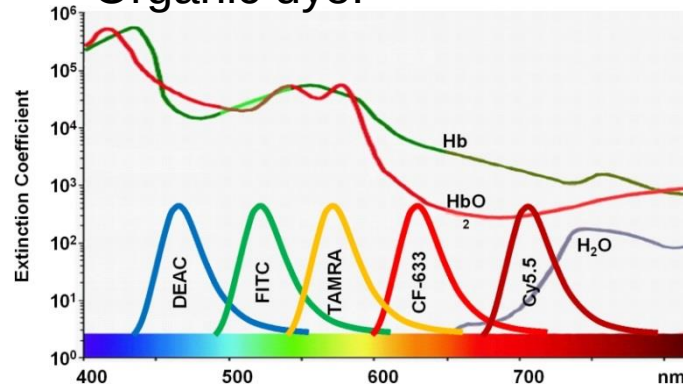
Machine nowadays that utilizes diffraction grating and diode array detector can acquire an absorbance spectra in less than 10 seconds.



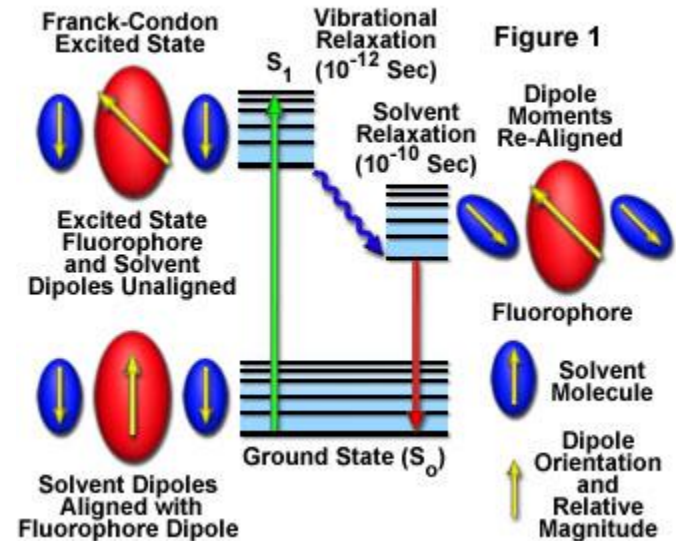
Fluorescence (S_1-S_0)

Solvent Effect:

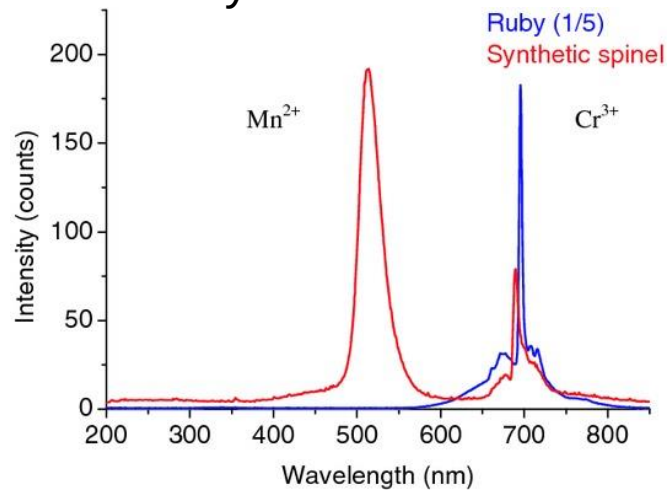
Organic dye:



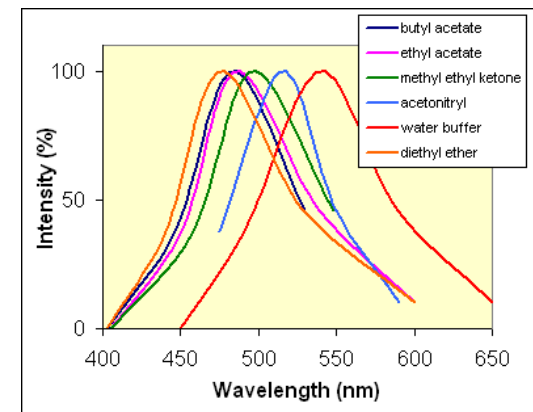
Fluorophore-Solvent Excited State Interactions



Ruby:



<http://micro.magnet.fsu.edu>

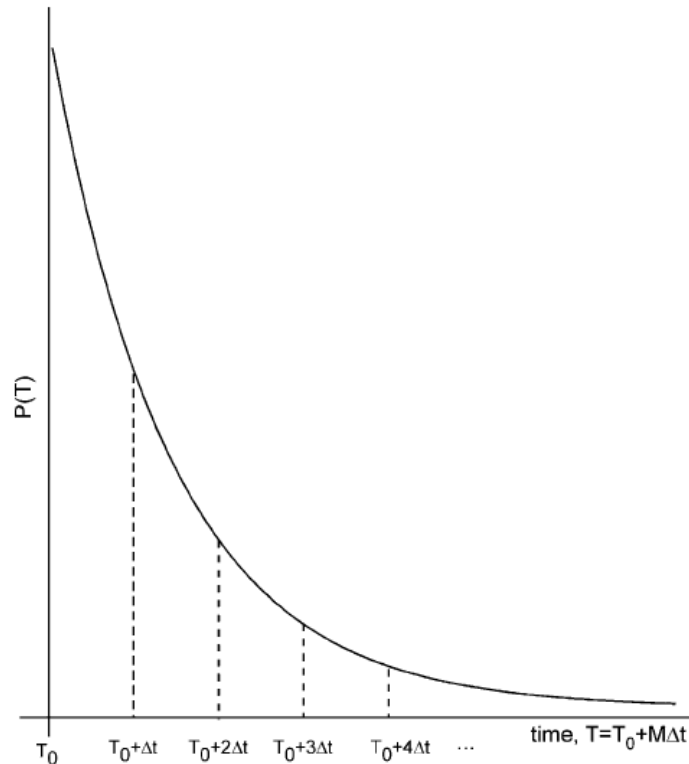


<http://www.bio.davidson.edu>

Time-Dependent Fluorescence: Fluorescence Lifetime

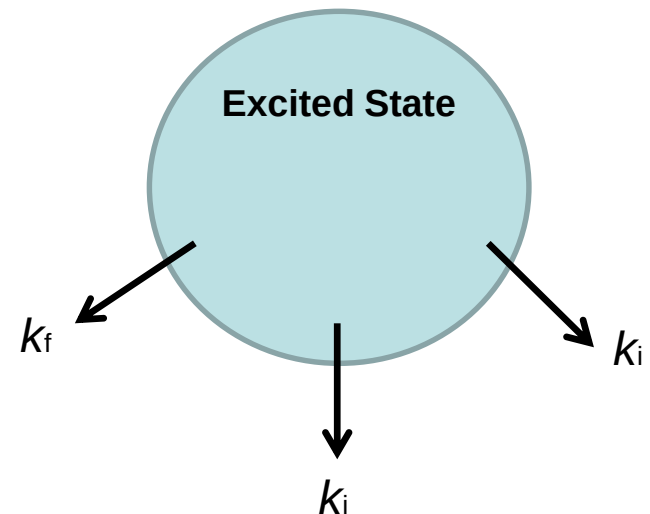
Fluorescence Lifetime: The average amount of time a molecule stays in excited state

Probability of being in the excited state



k_f = rate constant for leaving excited state while emitting a photon

k_i = rate constant for leaving excited state through other means (ie. Dynamic quenching, Energy Transfer, etc)



Fluorescence Lifetime:
$$\tau = \sum_i \frac{1}{k_i}$$

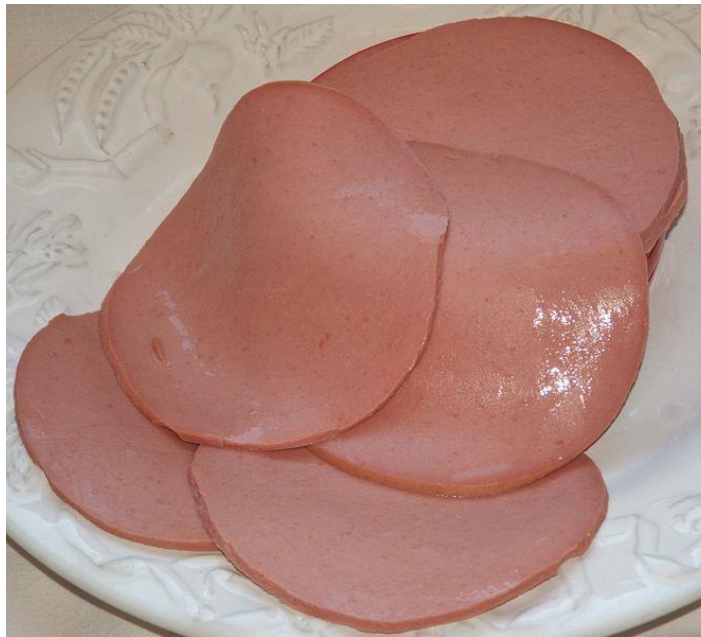
Lifetime is sensitive to other decaying pathway present!

The Bolognian Stone

http://www.isbc.unibo.it/Files/10_SE_BoStone.htm



MarcAntonioCellio
(1680)
representing the
light emission of
heated barite



It is now a long time since the cobbler of Bologna, in Italy, astonished and amused his friends with a peculiar substance since known as *Bologna phosphorus*, *Bologna stone*, or *Solar phosphorus*, which shines brightly in the dark after having been placed in the sunlight for some time. This substance is sulphuret of barium. The cobbler prepared it by heating red-hot with charcoal a piece of *sulphate of baryta*, or *Barytine*, (Fig. 1,) a stone which he



Fig. 1.

picked up in the secondary strata of the Monte Paterno, where he found it in lumps of considerable weight.* The German chemist, Marggraf, used to prepare solar phosphorus by powdering down the stone, and making it into thin cakes, with a mixture of flour and water, before submitting it to calcination. This "Bologna phosphorus" was the first substance known to become phosphorescent after insolation, and, consequently, it has been

1870.

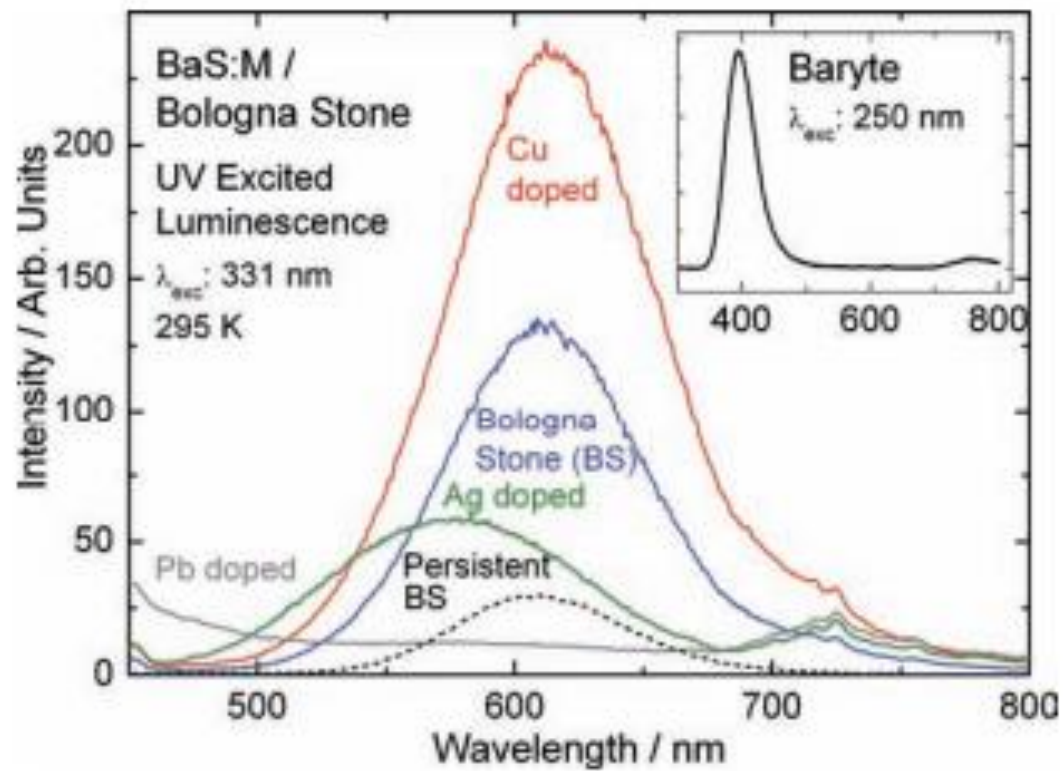
T. L. PHIPSON, PH.D., F.C.S.

submitted to many and varied experiments. It is best obtained by the calcination of pulverized sulphate of baryta, made into a firm paste with common gum. It should be preserved in a bottle which closes hermetically with a glass stopper.

It will be easily understood what is meant by the term *Phosphorescence*, when we remind our readers that phosphorus, which shines so curiously in the dark, and which enters into the composition of our common lucifer matches, is the most remarkable of all phosphorescent bodies. The word "phosphorus," which signifies a substance that bears or emits a light, has frequently been applied to various other substances besides the non-metallic element termed *phosphorus* in chemistry, on account of the property these substances possess likewise of shining in the dark.

First mention of lifetimes?

"The Bologna stone, when placed in the sun attracts the rays, and retains them so long as to give light a considerable time after it is removed into the dark." *Goethe* "The Sorrows of Werter"

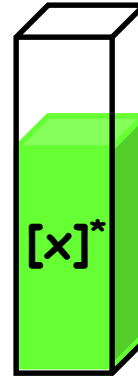


Lastusaari et al. 2011

Measuring the Depletion of the excited state

$$[\# x^*] = [\# x_o^*] e^{-(k_F + k_t)t}$$

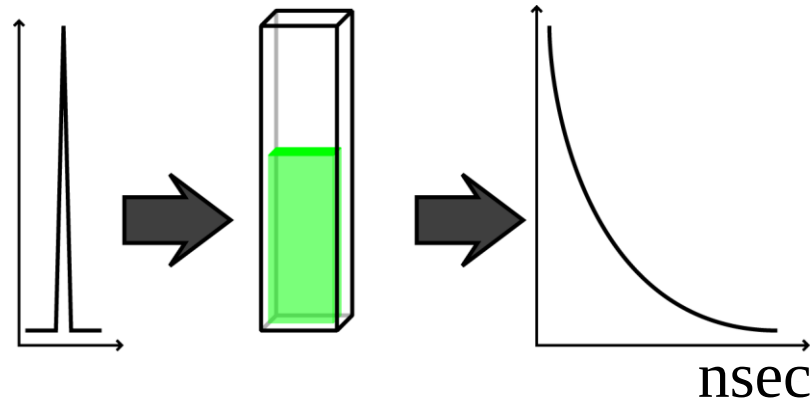
$$[\# x^*](k_F) = \text{Intensity that you measure}$$



K_F is rate constant of fluorescence

Intensity measured is proportional to the # of molecules in the excited state!

Measuring Lifetime: Time Domain

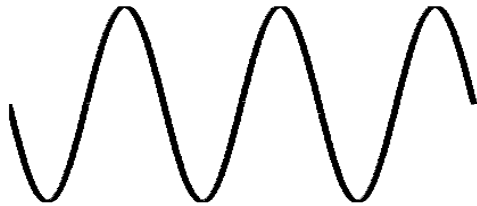


What do you need?

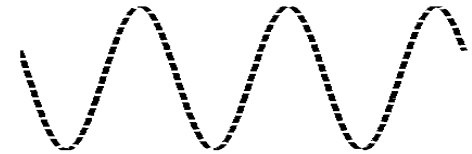
- Collect signal fast enough
- Fitting

Measuring Lifetime: Frequency Domain

$$E(t) = E_o + E_\omega \cos(\omega_E t + \phi_E)$$



$$F(t) = F_o + F_\omega \cos(\omega_E t + \phi_E - \phi)$$



$$\tan(\phi) = \omega_E \tau_\phi$$

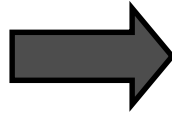
$$M = \frac{F_\omega / F_o}{E_\omega / E_o} = \frac{1}{\sqrt{1 + (\omega \tau_{Mod})^2}}$$

What do you need?

- Intensity modulators
- Synchronization

Samples Described by Multiple Lifetimes

$$I(t) = \sum_i a_i e^{-t/\tau_i}$$



Some Examples:

- ECFP (Enhanced Cyan Fluorescent Protein (FP)), FPs
- Ruby Rhodamine Mixture
- Crystals

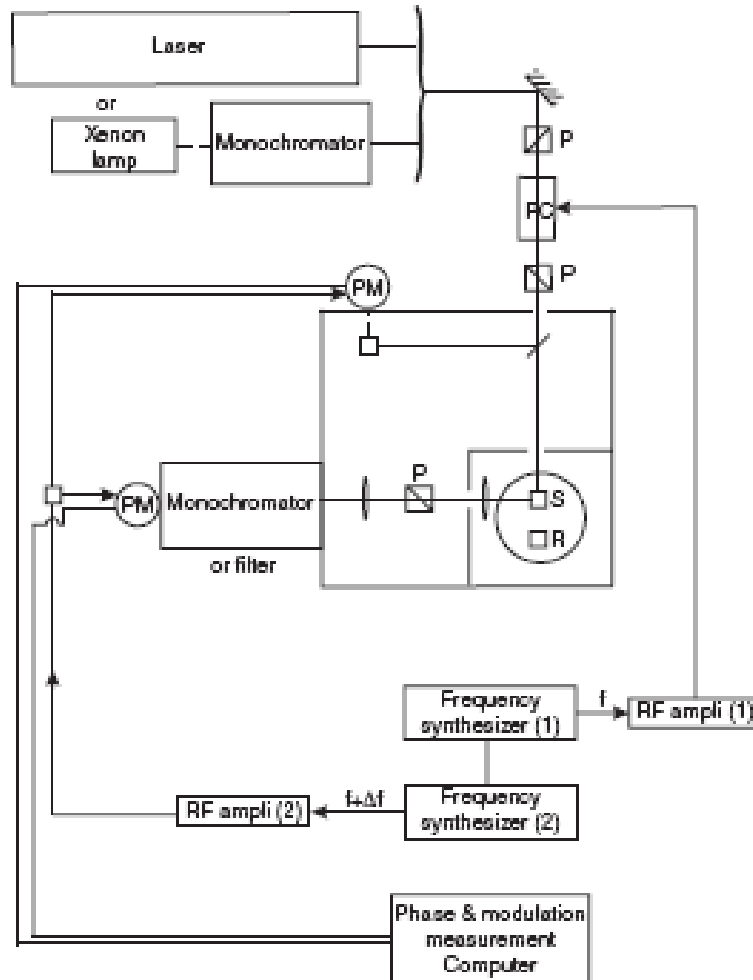
$$F(t) = E_o \sum_i a_i \tau_i + E_\omega \sum_i \frac{a_i \tau_i}{\sqrt{1 + (\omega_E \tau_i)^2}} \cos(\omega_E t - (\varphi_i - \varphi_E))$$

You still can only measure one (M, φ)

$$\frac{F(t)}{F_o} = 1 + \frac{E_\omega}{E_o} \sum_i \frac{\alpha_i}{\sqrt{1 + (\omega_E \tau_i)^2}} \cos(\omega_E t - (\varphi_i - \varphi_E))$$

$$\frac{F(t)}{F_o} = 1 + \frac{E_\omega}{E_o} M \cos(\omega_E t - (\varphi_i - \varphi))$$

Mixing is used in commercial instruments

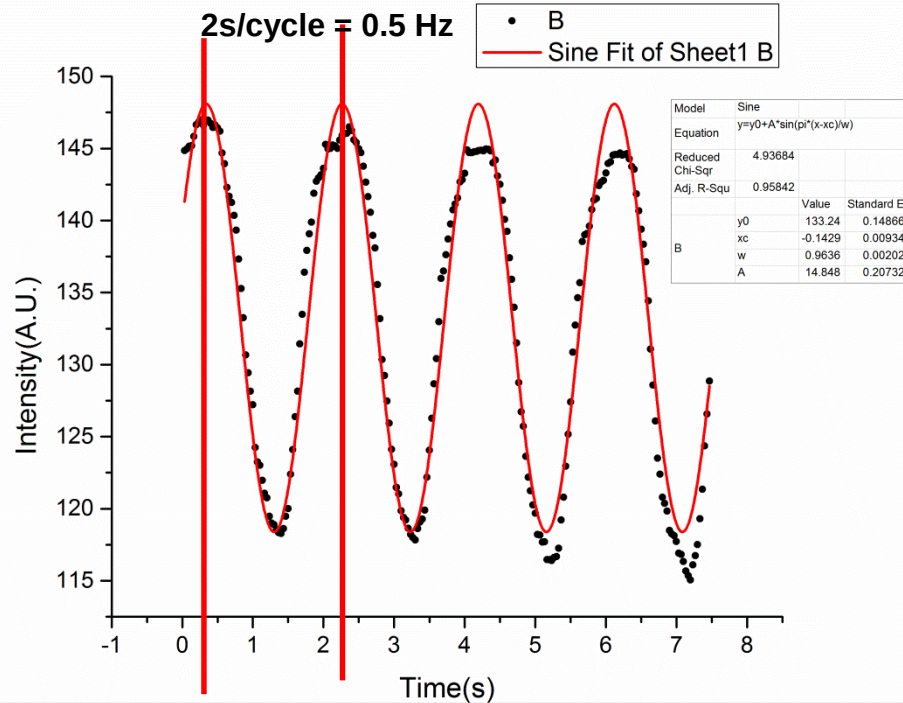
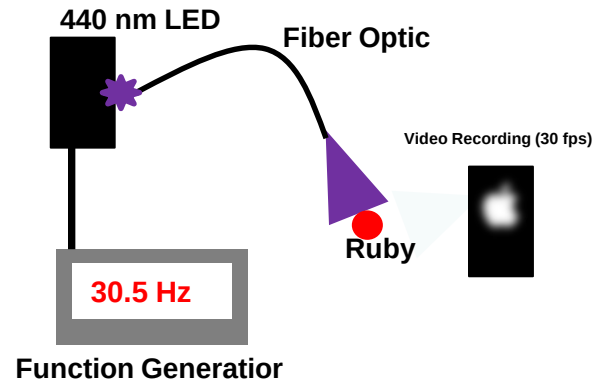


$$[G(t)*F(t)] = DC +$$

Terms with
frequencies
 $(\omega_E, \omega_G, \omega_E + \omega_G)$

$$+ \frac{G_{\omega} E_{\omega}}{2} M(\cos((\omega_G - \omega_E)t + \varphi_G - \varphi_E + \varphi))$$

Example of Heterodyning – Lifetime Imaging With Your Phone



$$[G(t)*F(t)] = DC +$$

Terms with
frequencies
($\omega_E, \omega_G, \omega_E + \omega_G$)

$$+ \frac{G_{\omega} E_{\omega}}{2} M \left(\cos((\omega_G - \omega_E)t + \varphi_G - \varphi_E + \varphi) \right)$$

AOMs - Intensity Modulator

MEASUREMENTS OF SUBNANOSECOND FLUORESCENCE LIFETIMES WITH A CROSS-CORRELATION PHASE FLUOROMETER*

Richard D. Spencer and Gregorio Weber
*Department of Chemistry and Chemical Engineering
 University of Illinois
 Urbana, Ill.*

Annals of the New York Academy of Sciences Vol. 158 pp 361-376, 1969

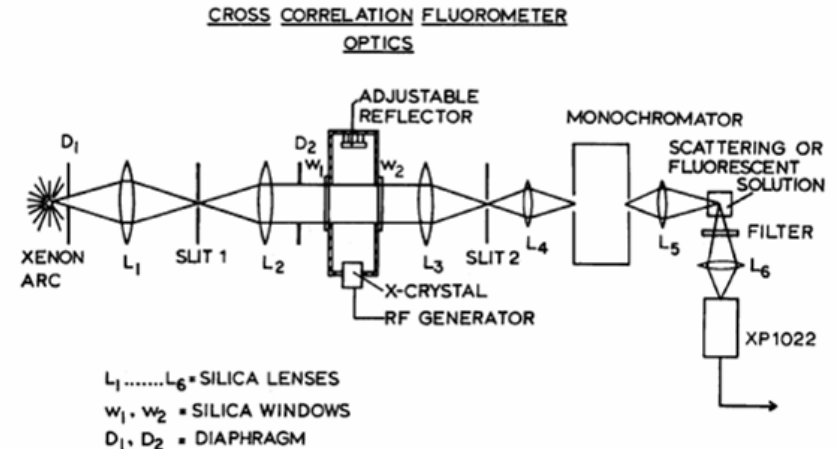


FIGURE 4. Plan of the optics of fluorometer.

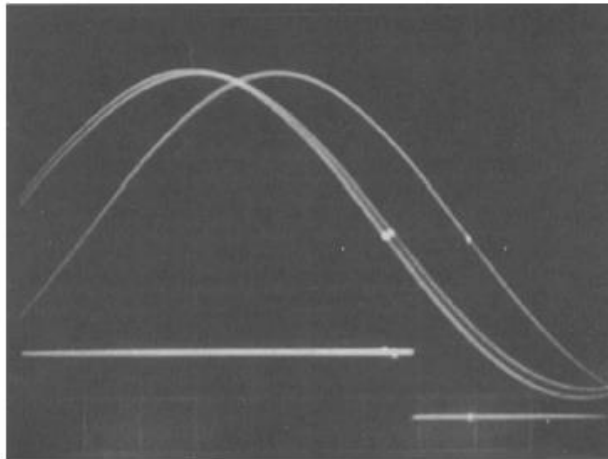


FIGURE 7. The figure shows the cross-correlation photocurrents from a scattering solution (first from left), a solution of NADH in phosphate buffer, pH 7.0, 17° C (second from left), and a solution of fluorescein (1 $\mu\text{gm/ml}$) in 0.01 M NaOH (right).

-modulation frequency limited by resonance frequency of the acousto-optic cell

-variations in the intensity modulation caused by temperature

Pockels Cell

A CONTINUOUSLY VARIABLE FREQUENCY CROSS-CORRELATION PHASE FLUOROMETER WITH PICOSECOND RESOLUTION

E. GRATTON AND M. LIMKEMAN

Department of Physics, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Biophysical Journal Vol. 44 (1983) pp 315-324

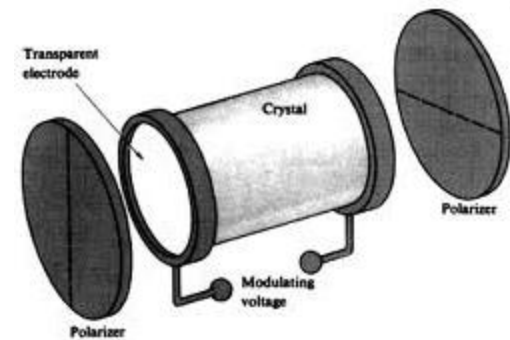
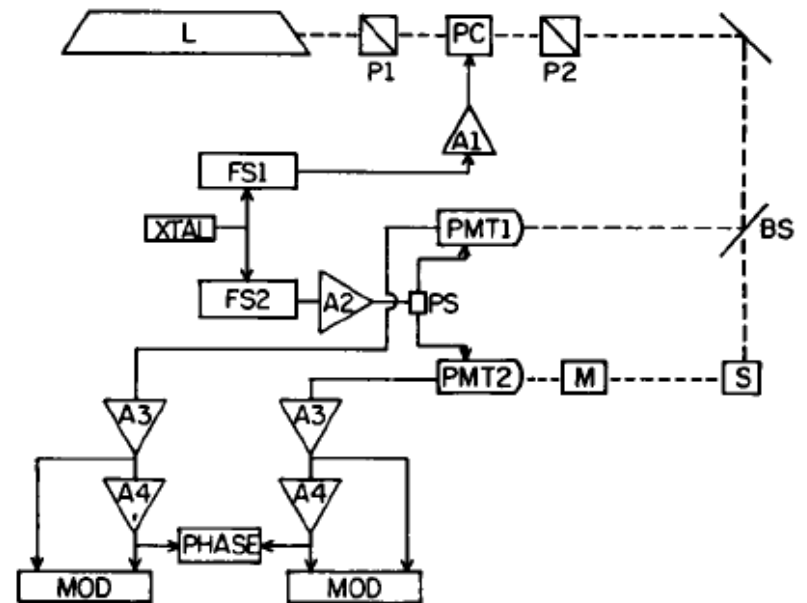


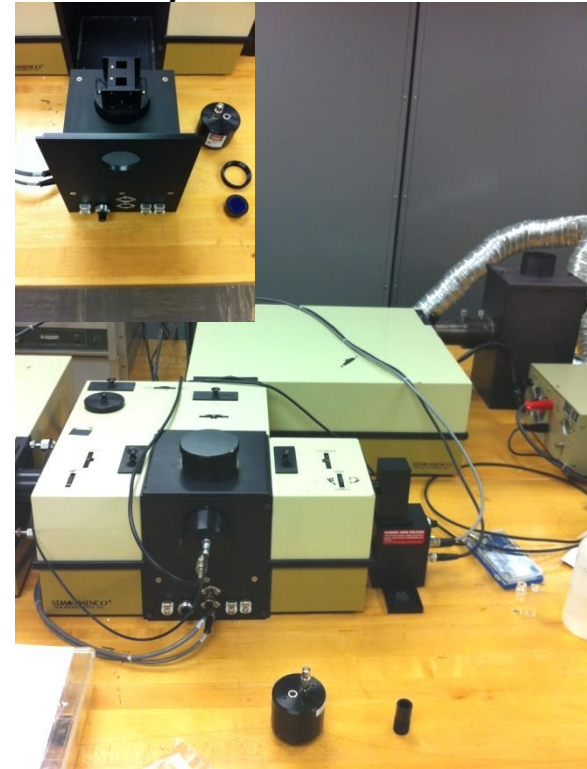
Figure 8.57 A Pockels cell.

Hecht Optics

Directly Modulated Diode



System in ESB

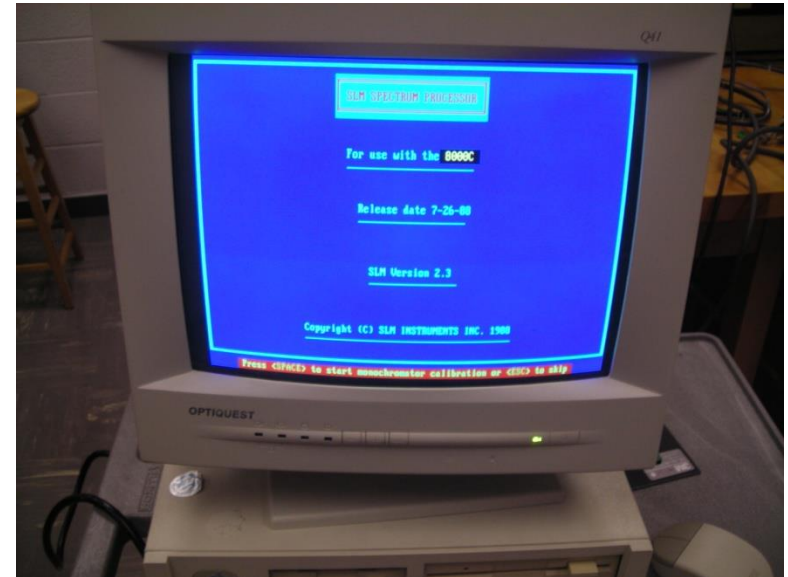


Laser Diodes -> (405nm,436nm,473nm,635nm,690nm,780nm,830nm)

LEDs -> (280nm,300nm,335nm,345nm,460nm,500nm,520nm)

ISS SLM Phoenix Upgrade

Original System



New Upgrades

PMT Housing



New Acquisition Box



Stepper Motor Controller



-photon counting

-Measures
nanosecond
lifetime

Champaign, Illinois - Domain of FD FLIM

Robert Clegg - UIUC
Full-Field FLIM



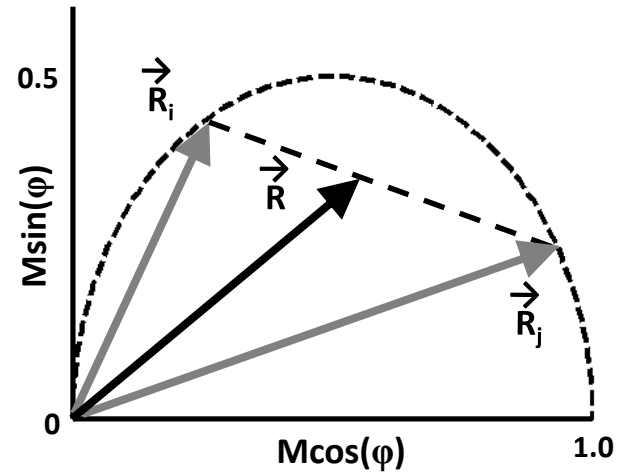
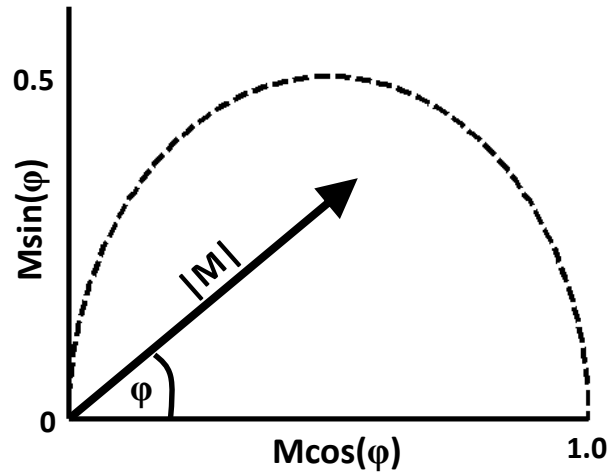
Enrico Gratton - UIUC
Scanning Confocal FLIM (FLIMBox)



Beniamino Barbieri - ISS Inc.
Commercialization of FD FLIM



The Polar Plot



Vectors on the Polar Plot

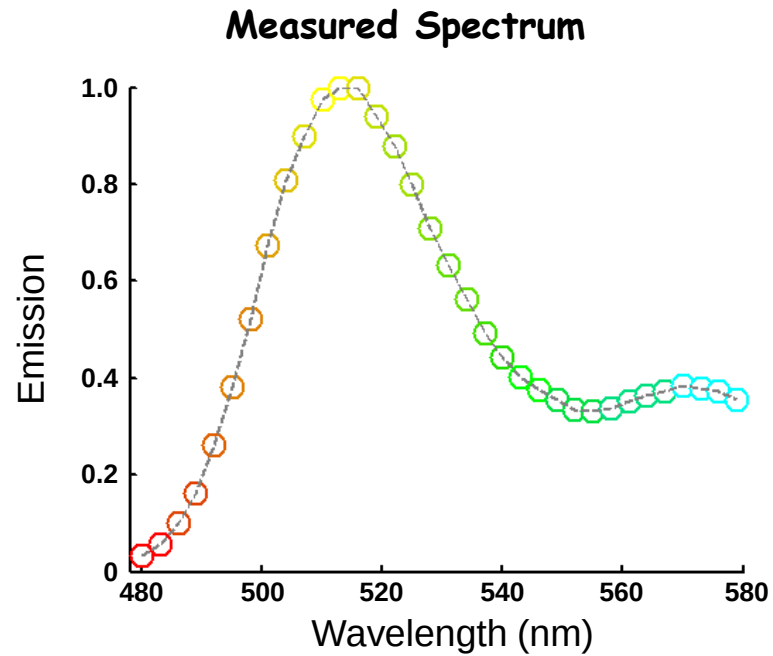
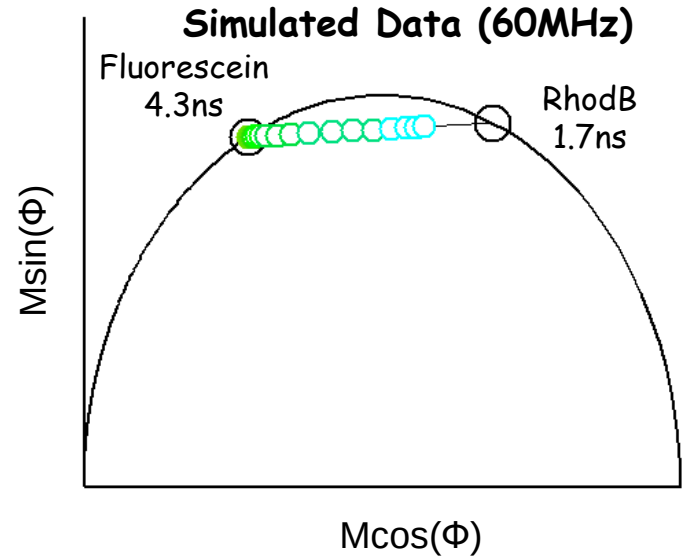
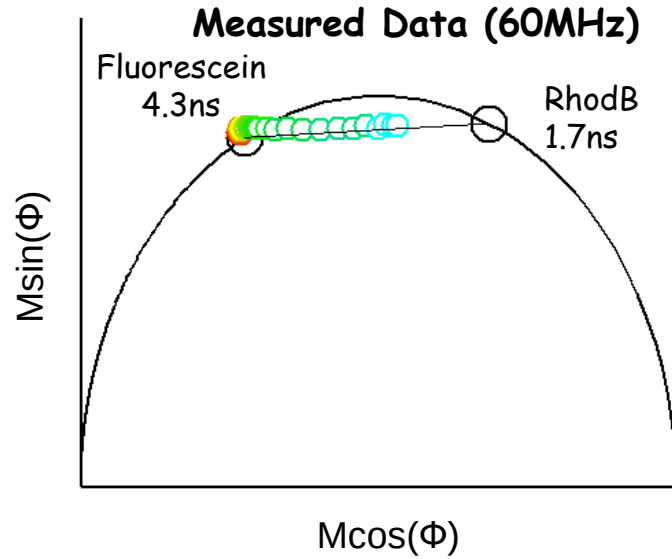
$$\vec{R} = M\cos(\varphi)\hat{x} + M\sin(\varphi)\hat{y}$$

$$\vec{R} = \alpha_i \vec{R}_i + \alpha_j \vec{R}_j$$

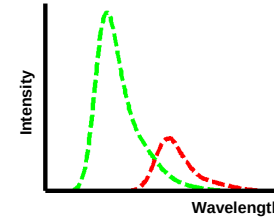
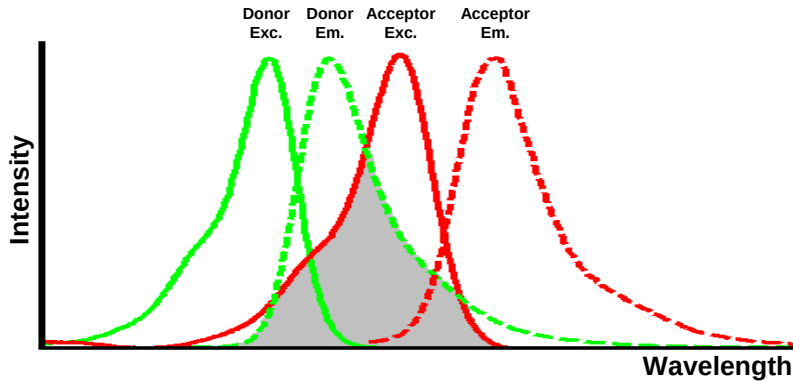
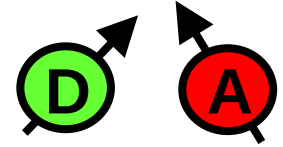
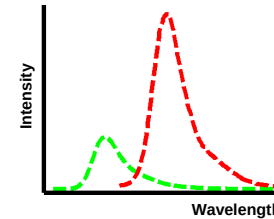
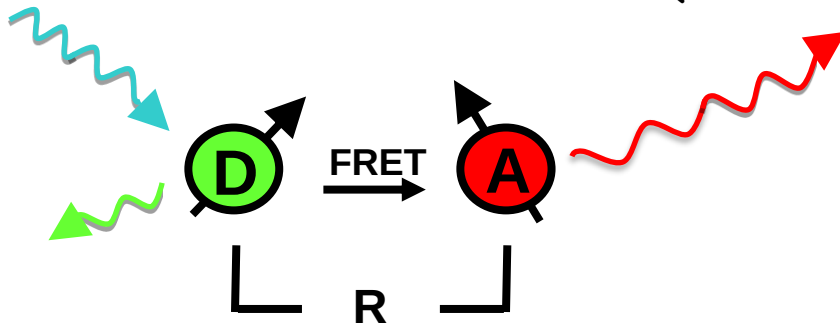
$$\begin{aligned} \vec{R} = & \left(\alpha_i M_i \cos(\varphi_i) + \alpha_j M_j \cos(\varphi_j) \right) \hat{x} \\ & + \left(\alpha_i M_i \sin(\varphi_i) + \alpha_j M_j \sin(\varphi_j) \right) \hat{y} \end{aligned}$$

-movement of the vector depends on the emitted intensity of each species (*i*)

Spectral Analysis on the SLM



Quantum Yield/FRET



Quantum Yield of Energy Transfer (E)

=

Rate of Energy Transfer

Total de-excitation rate

=

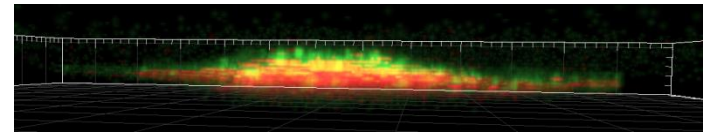
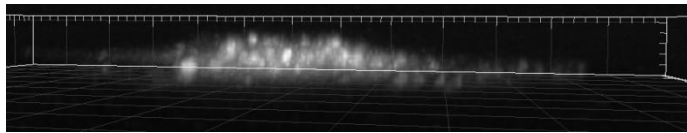
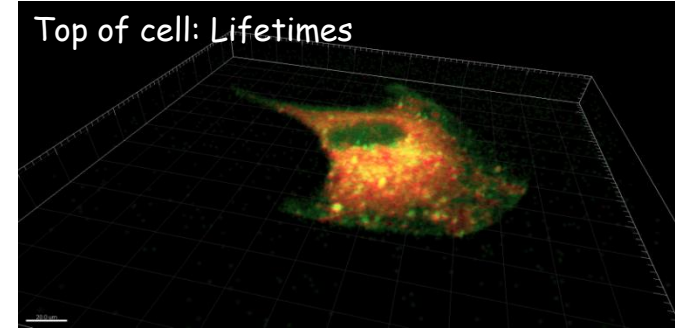
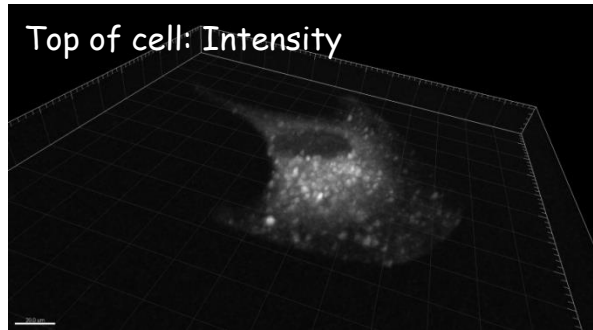
$$\frac{\left(\frac{1}{\tau_{DA}}\right) - \left(\frac{1}{\tau_D}\right)}{\left(\frac{1}{\tau_D}\right)}$$

$$= 1 - \frac{\tau_{DA}}{\tau_D}$$

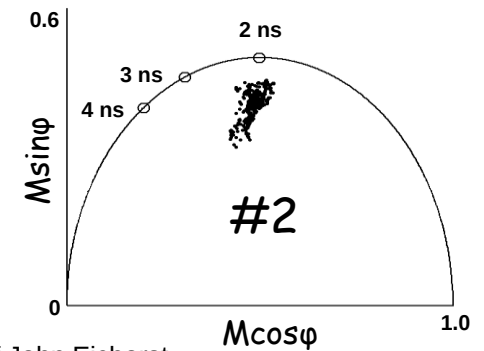
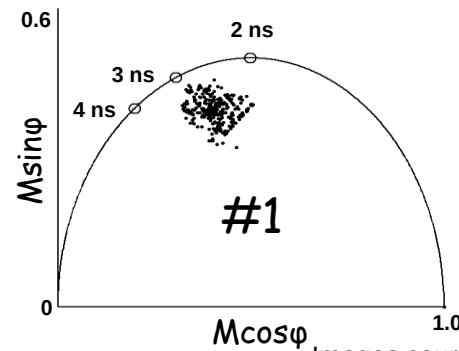
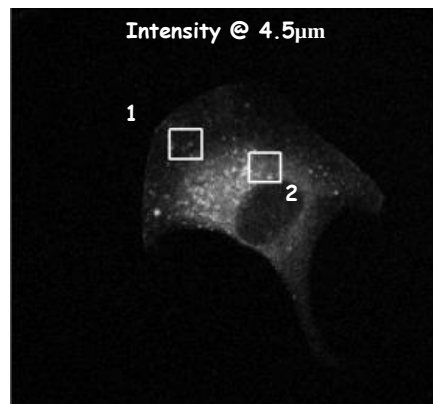
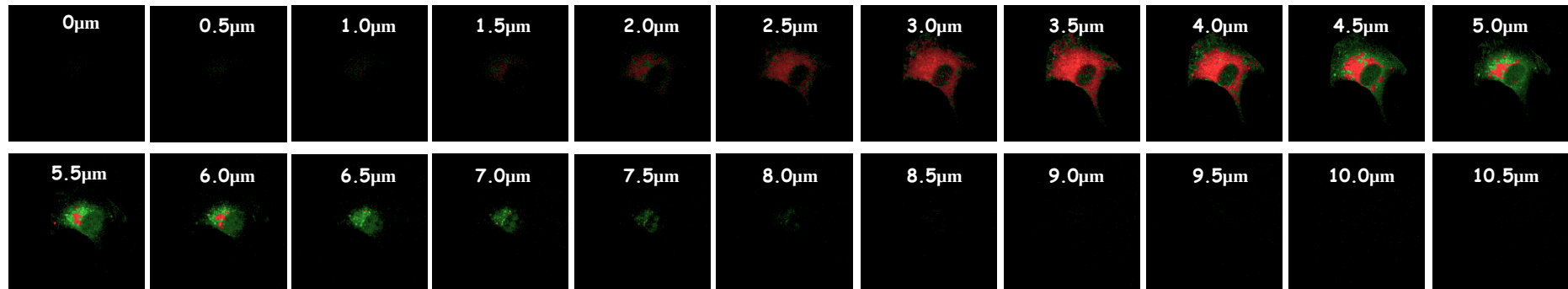
Theoretically: $E = \frac{1}{1 + (r/R_0)^6}$

Applications of Fluorescence in Biology

Fluorescence Lifetime Imaging on Live Cells

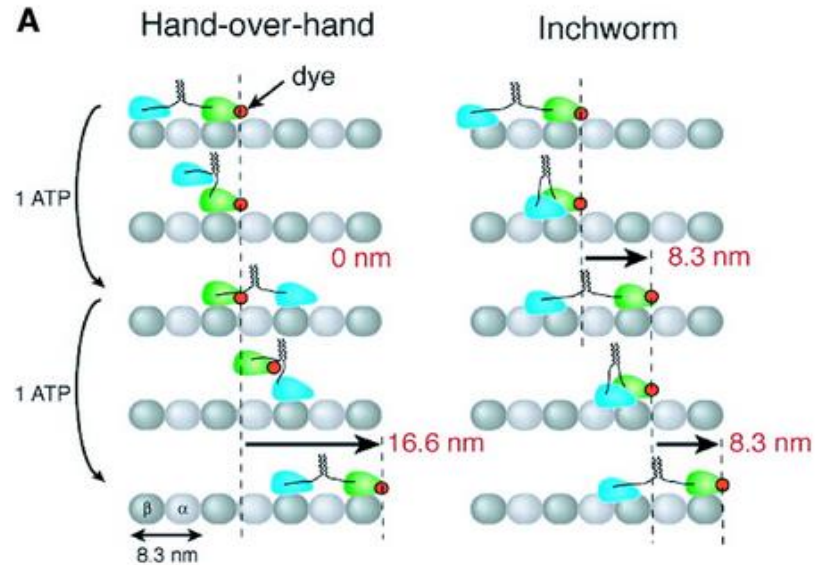
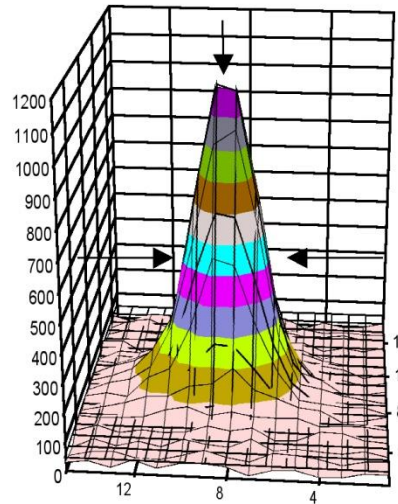
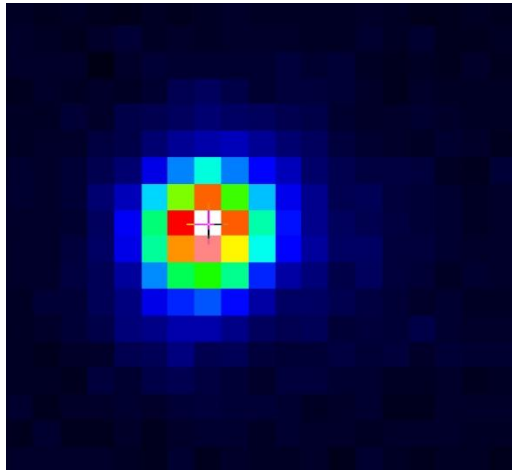


Optical Sections - Rendered by Lifetime



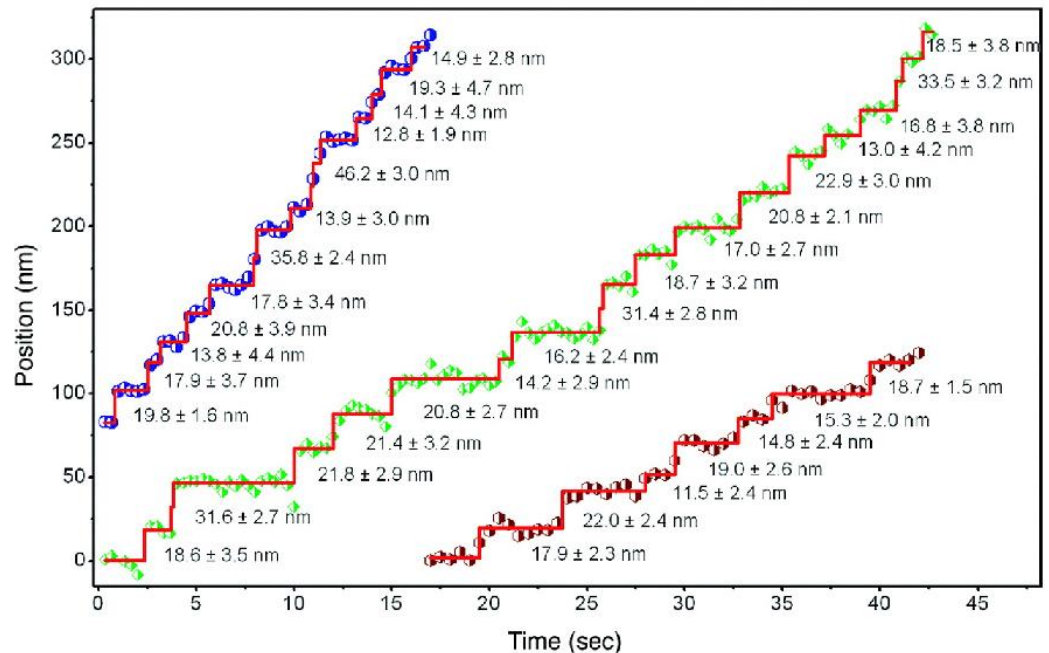
Images courtesy of John Eichorst

Single Molecule Fluorescence Imaging



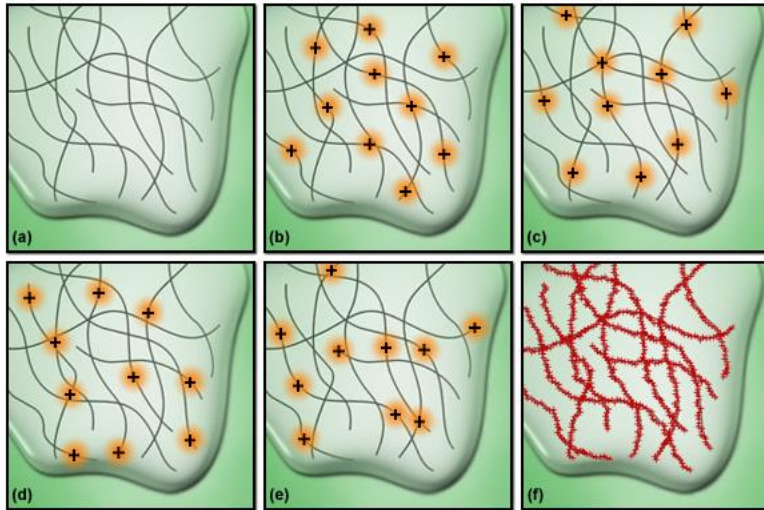
$$\sigma_{\mu_i} = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^4 b^2}{a^2 N^2}\right)}$$

Center of the distribution can be determined in ~1.5 nm accuracy if #N is more than 10⁴



Super Resolution Fluorescence Imaging

Basic Principle of STORM Superresolution Imaging



Three-Dimensional Superresolution Imaging with STORM

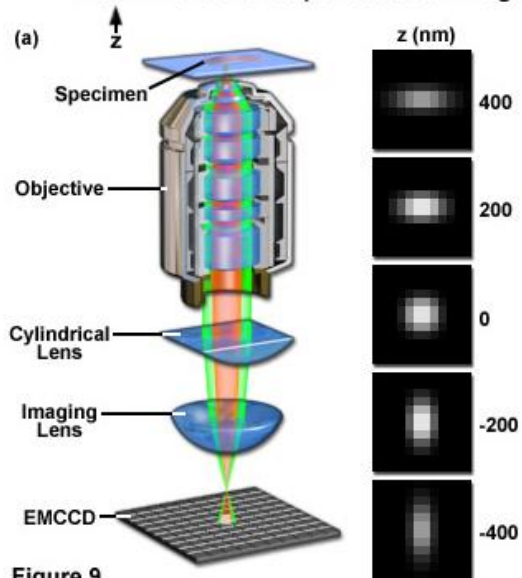
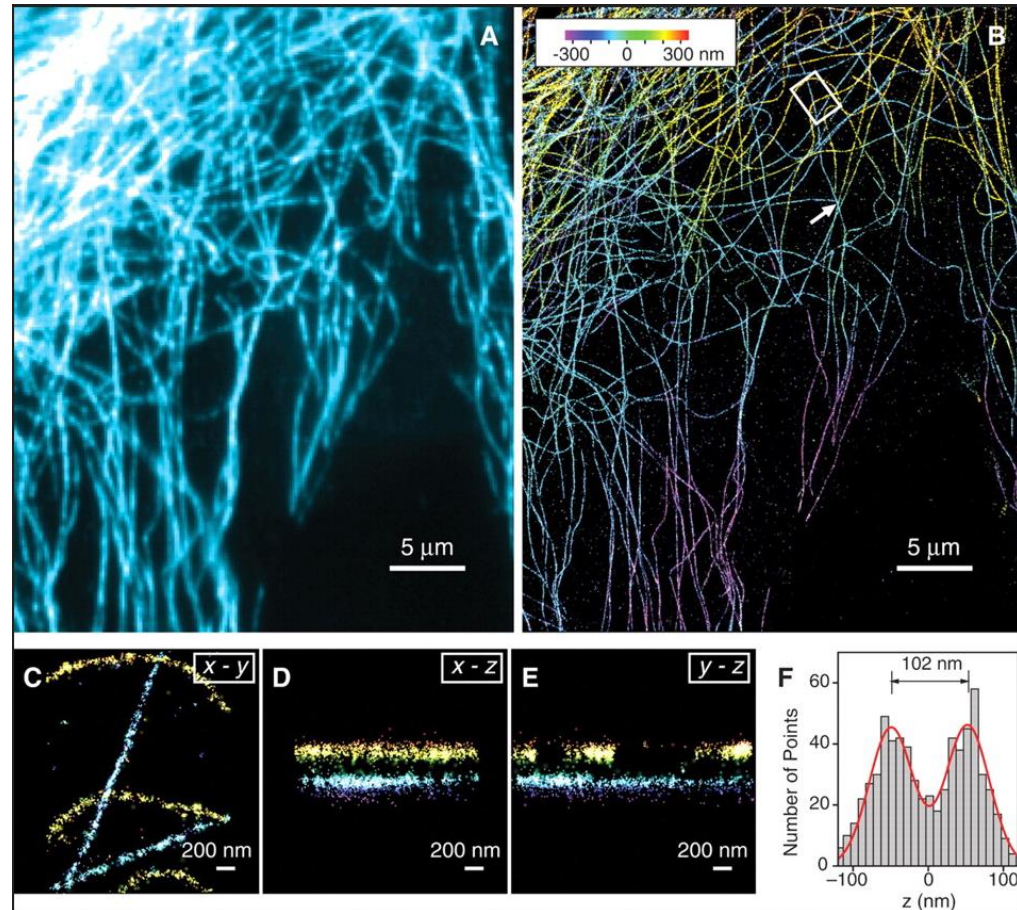


Figure 9



<http://www.microscopyu.com/articles/superresolution/stormintro.html>

Huang et al Science, 2008