Fluorescence Spectroscopy

Steady State and Time Dependent Fluorescence Measurements

Teng, Kai Wen

PHYS 403 Summer 16

EM Spectrum of molecules

Rotational Energy → Infrared

Vibrational Energy → Near Infrared

Electronic Energy → Visible and Ultra-Violet

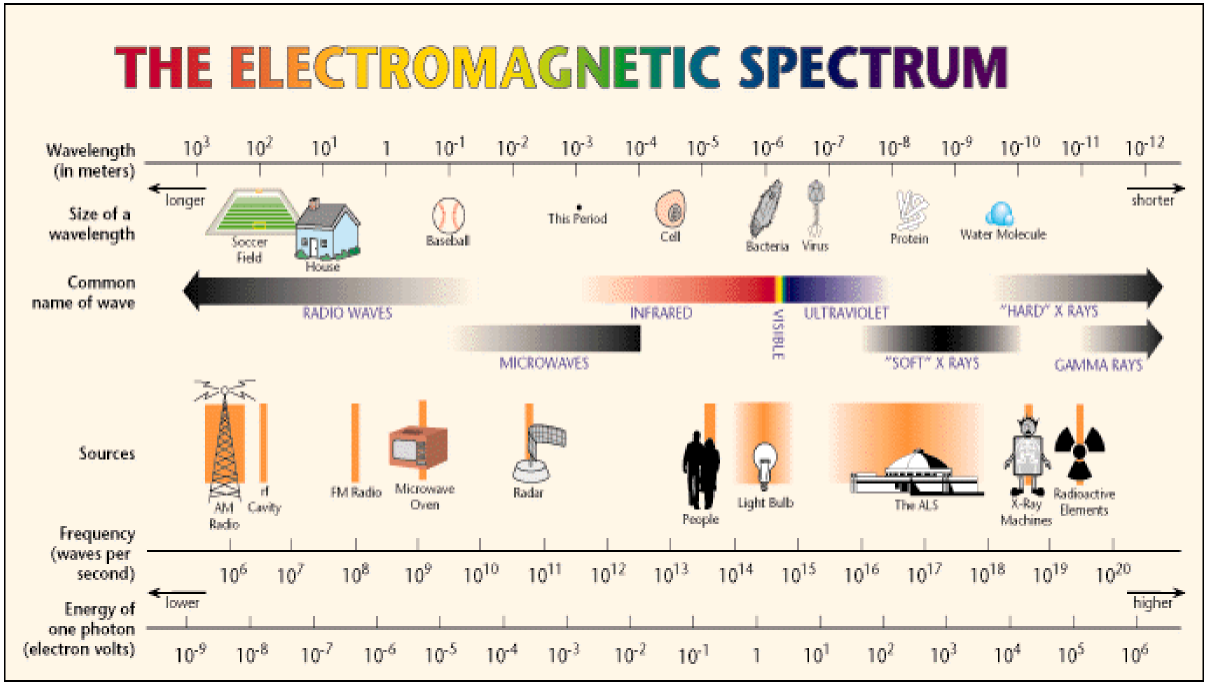


Diagram from http://www.lbl.gov

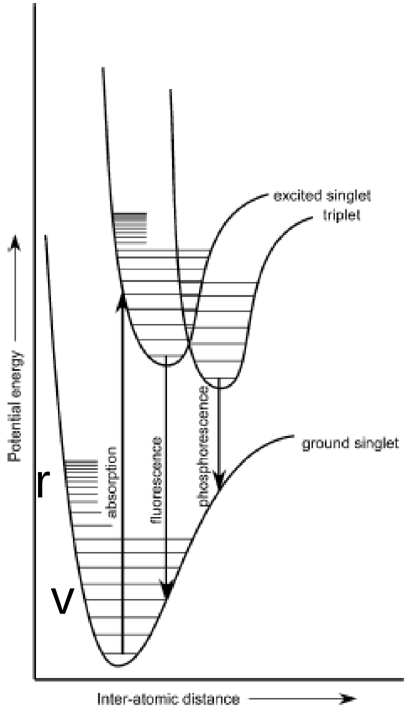
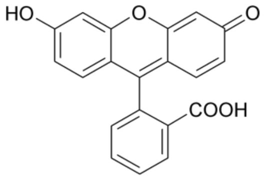


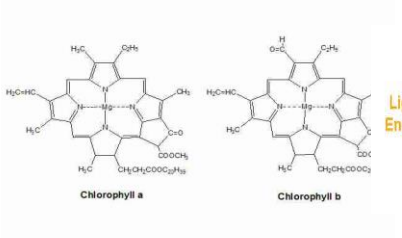
Diagram by Robert Clegg in Photosynth Res. 2009 Aug-Sep;101(2-3):181-94.

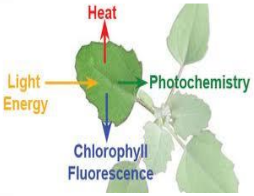
Types of Fluorescent Molecules

Synthetic Organic:
Fluorescein



Naturally Occuring:

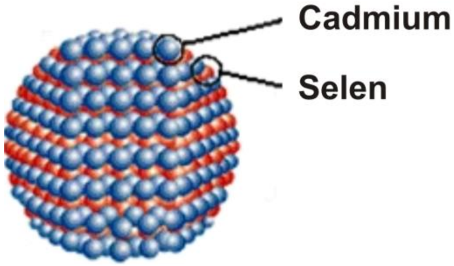


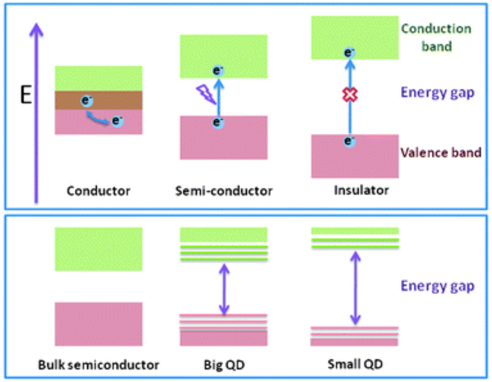


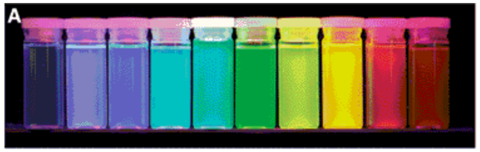
Fluorescent Proteins:

Green Fluorescent Protein

Semiconductor Nanocrystal:







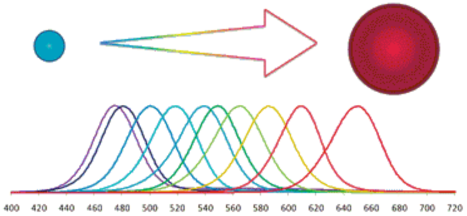
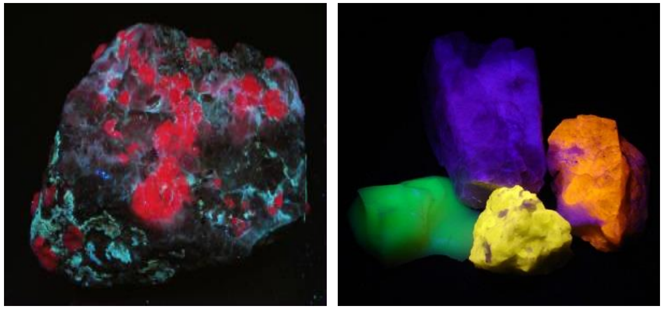


Image from Zrazhevskiy et al. 2010

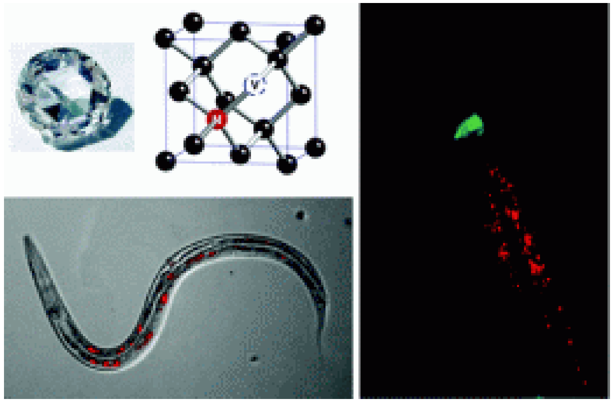
Crystals:



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 (S0, S1 and S2 transitions)

Alexander Jablonski

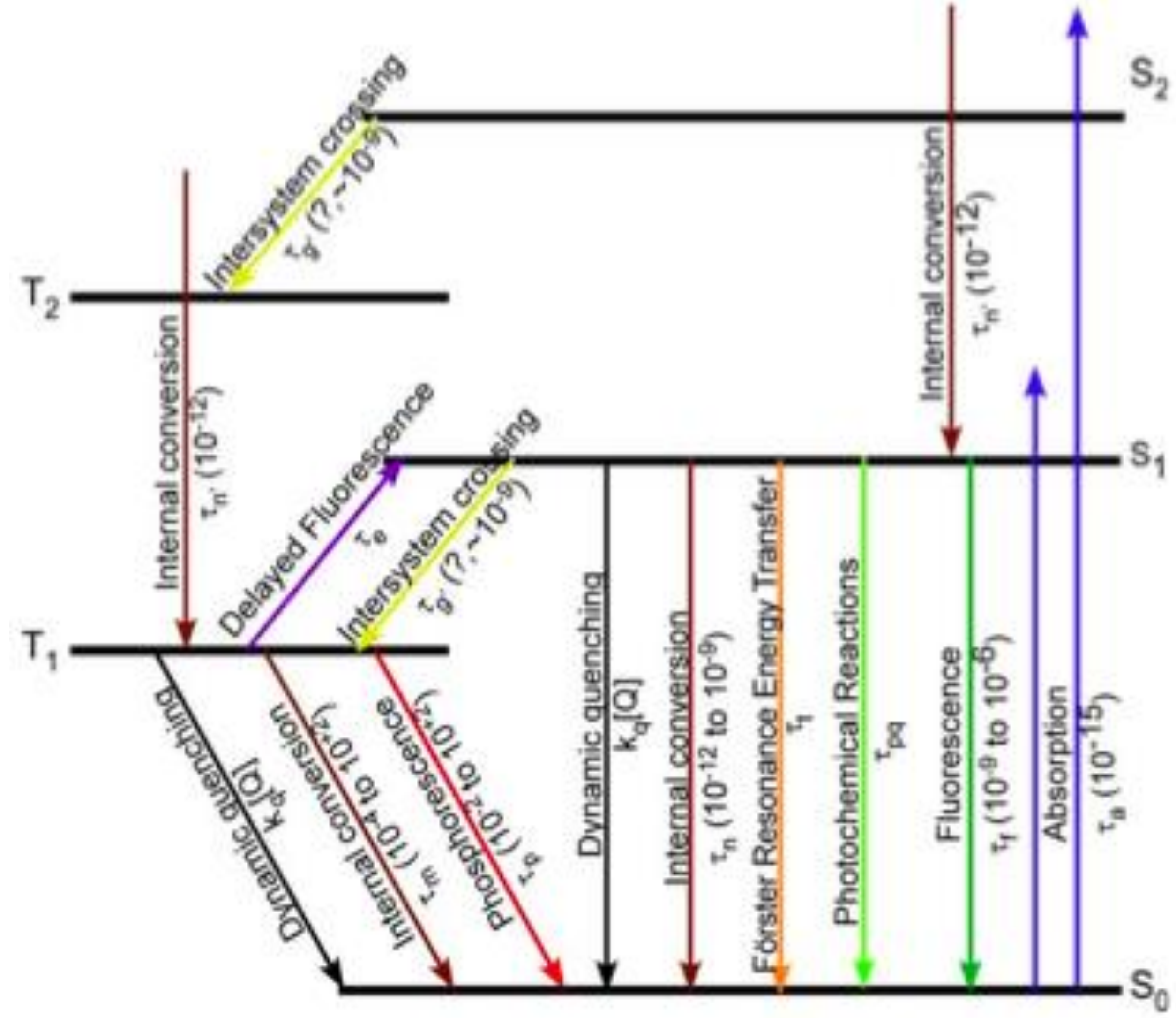
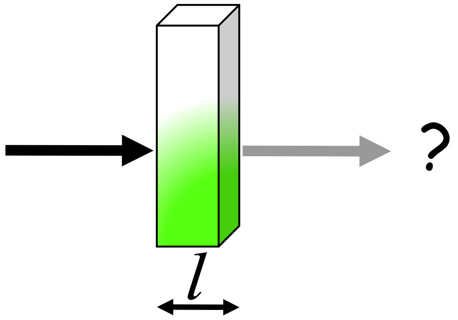


Diagram by Ulai Noomnarm in Photosynth Res. 2009 Aug-Sep;101(2-3):181-94.

Absorption (S_0-S_1)

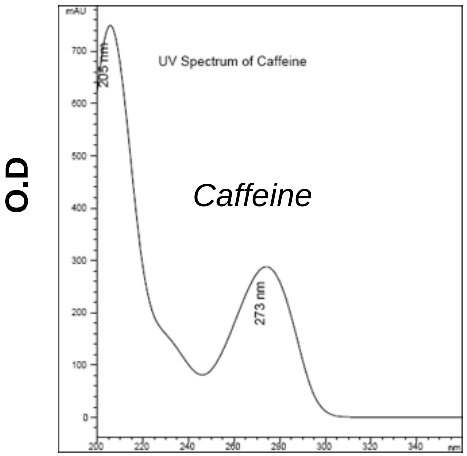


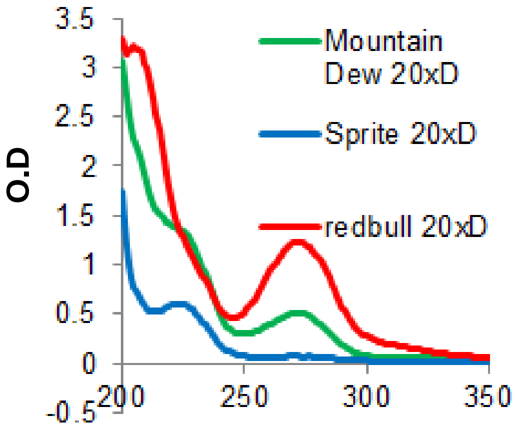
Beer-Lambert's Law

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

Extinction coefficient:

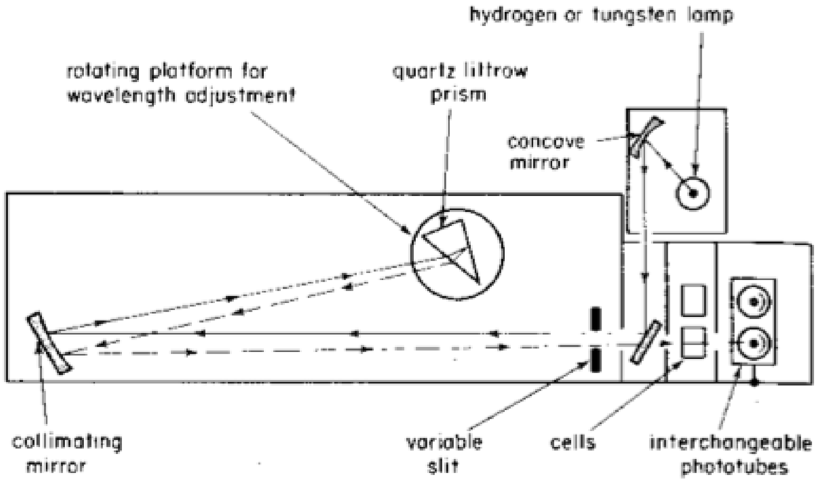
Concentration



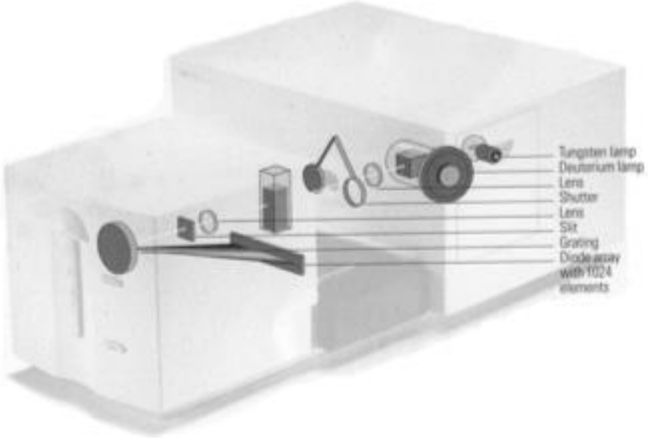


Steady State Measurements: Absorbance

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

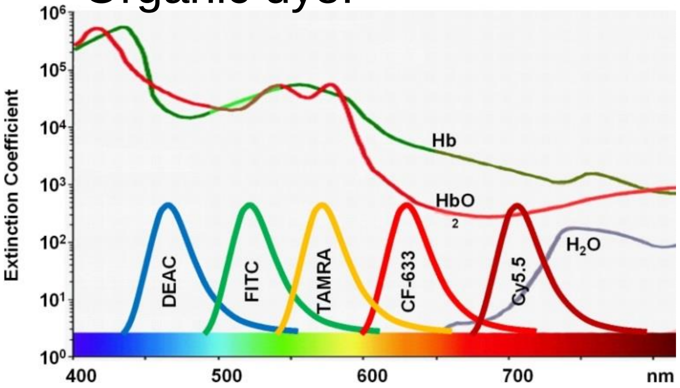


Machine nowadays that utilizes diffraction grating and diode array detector can acquire an absorbance spectra in less than 10 seconds.

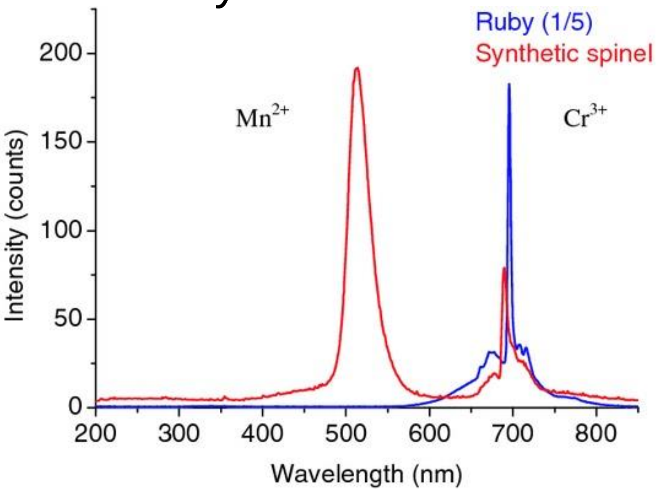


Fluorescence (S_1-S_0)

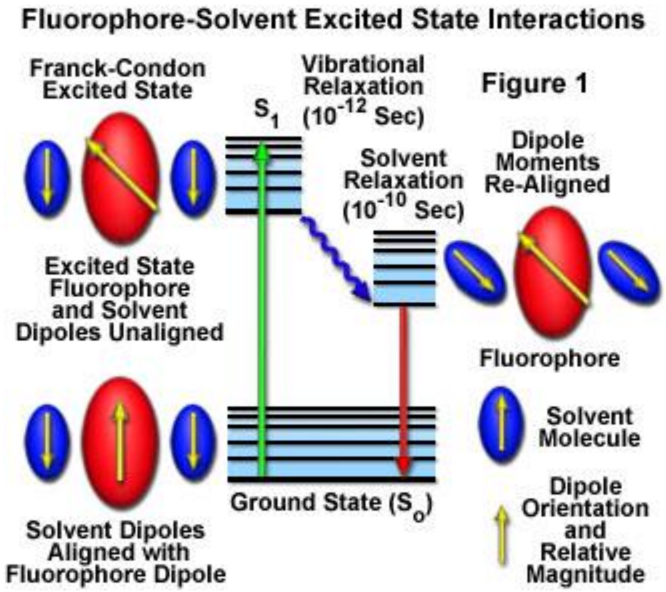
Organic dye:



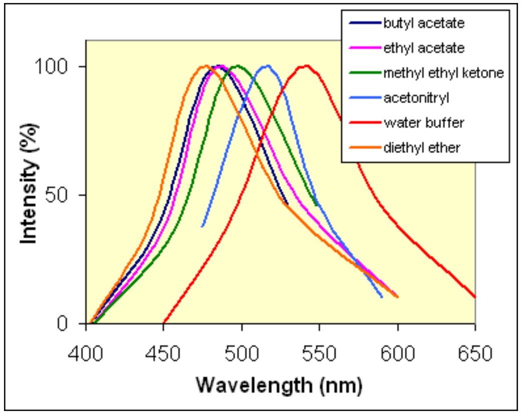
Ruby:



Solvent Effect:



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

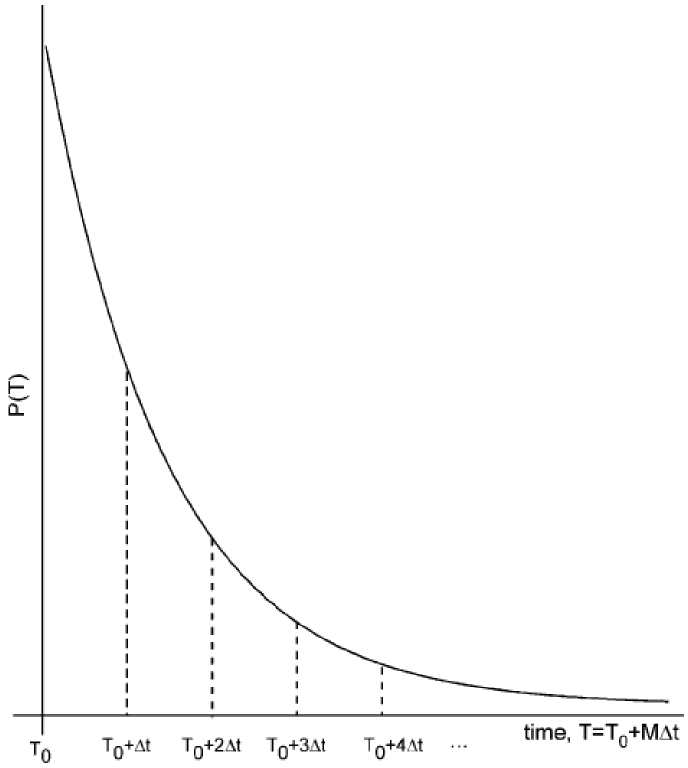
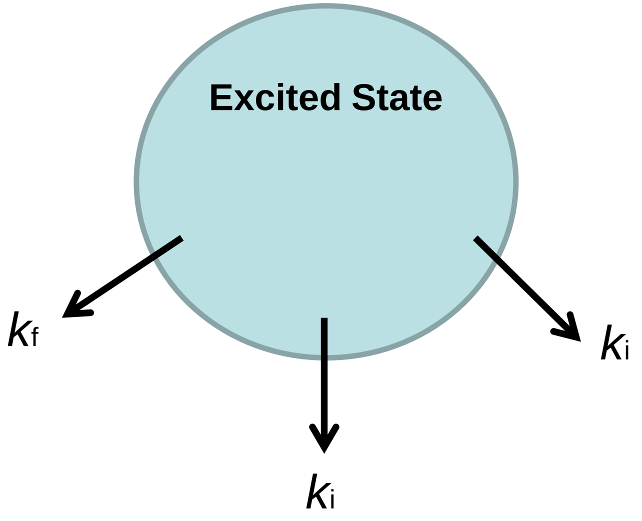


Diagram by Robert Clegg in Photosynth Res. 2009 Aug-Sep;101(2-3):181-94.

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)

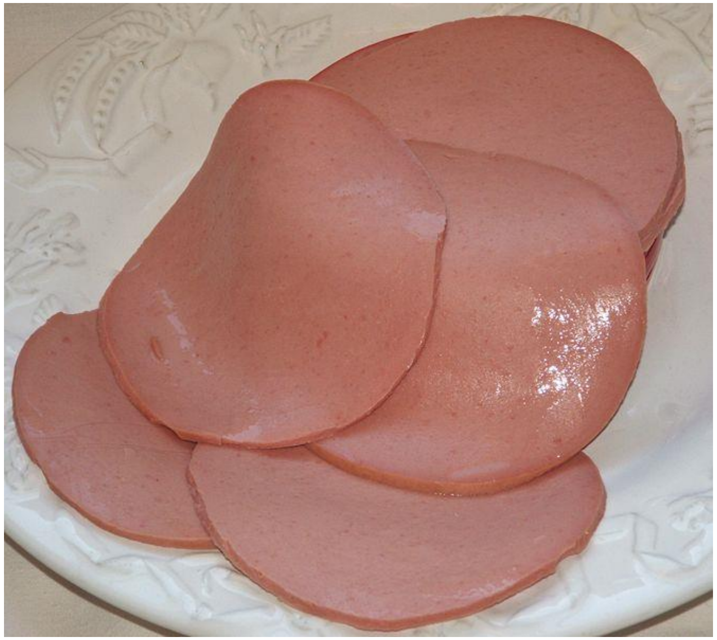


Fluorescence Lifetime: $\tau = \sum_i \frac{1}{k_i}$

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 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 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.

Fig. 1.

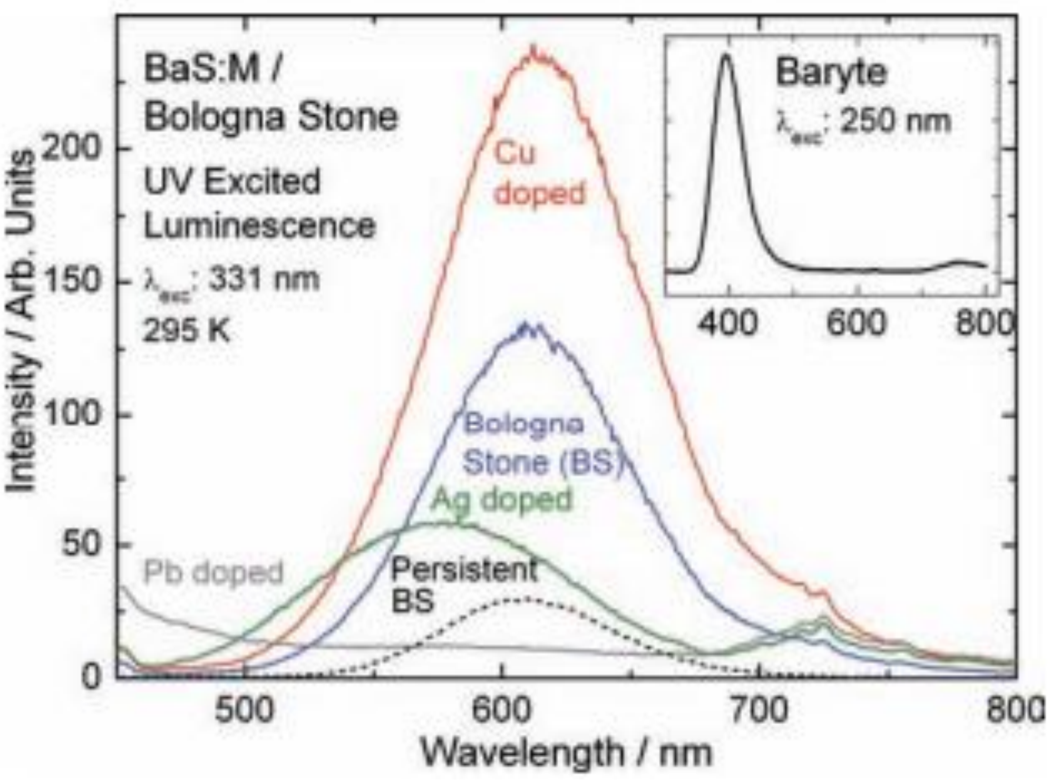
1870.

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

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

Dr. Brand in 1674-5 attempted to distil human urine and in this way discovered phosphorus.

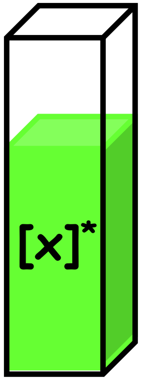
Phosphorus (Greek phosphoros was the ancient name for the planet Venus) was discovered by German alchemist Hennig Brand in 1669 through a preparation from urine. Working in Hamburg, Brand attempted to distill salts by evaporating urine, and in the process produced a white material that glowed in the dark and burned brilliantly.

Misnomer: Phosphorescence of phosphorous is due to slow oxidation

Painting by Joseph Wright of Derby (18thcentury) representing the discovery of the phosphorescence of the phosphorus extracted from urine by Hennig Brand in 1669

Measuring the Depletion of the excited state

$$\left[\# x^{*}\right]=\left[\# x_{o}^{*}\right]e^{-\left(k_{F}+k_{t}\right)t}$$

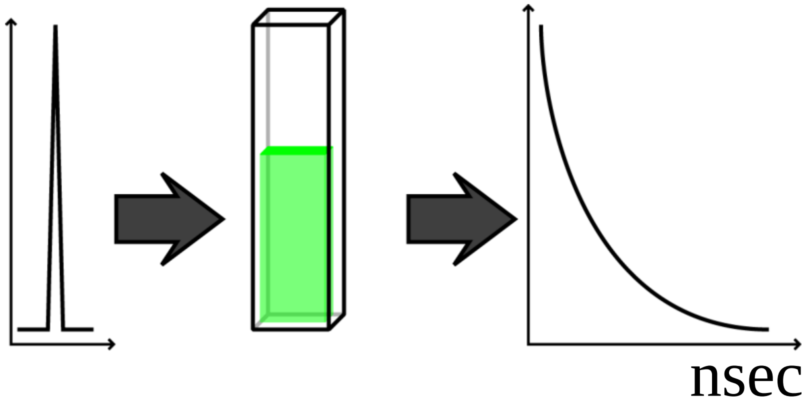


$\left[\# x^{*}\right]\left(k_{F}\right)$ = Intensity that you measure

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?

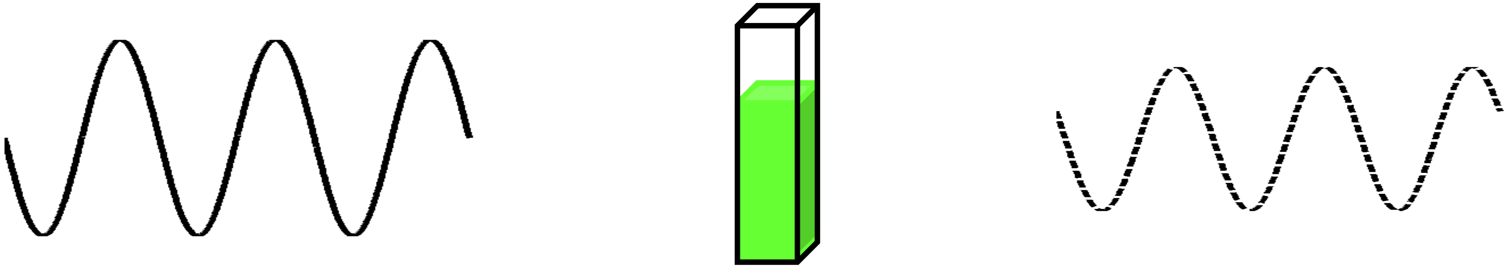
-Collect signal fast enough

-Fitting

Measuring Lifetime: Frequency Domain

$$E(t) = E_o + E_\omega \cos\left(\omega_E t + \varphi_E\right)$$

$$F(t) = F_o + F_\omega \cos\left(\omega_E t + \varphi_E - \varphi\right)$$



$$\tan\left(\varphi\right) = \omega_E \tau_\varphi$$

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

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

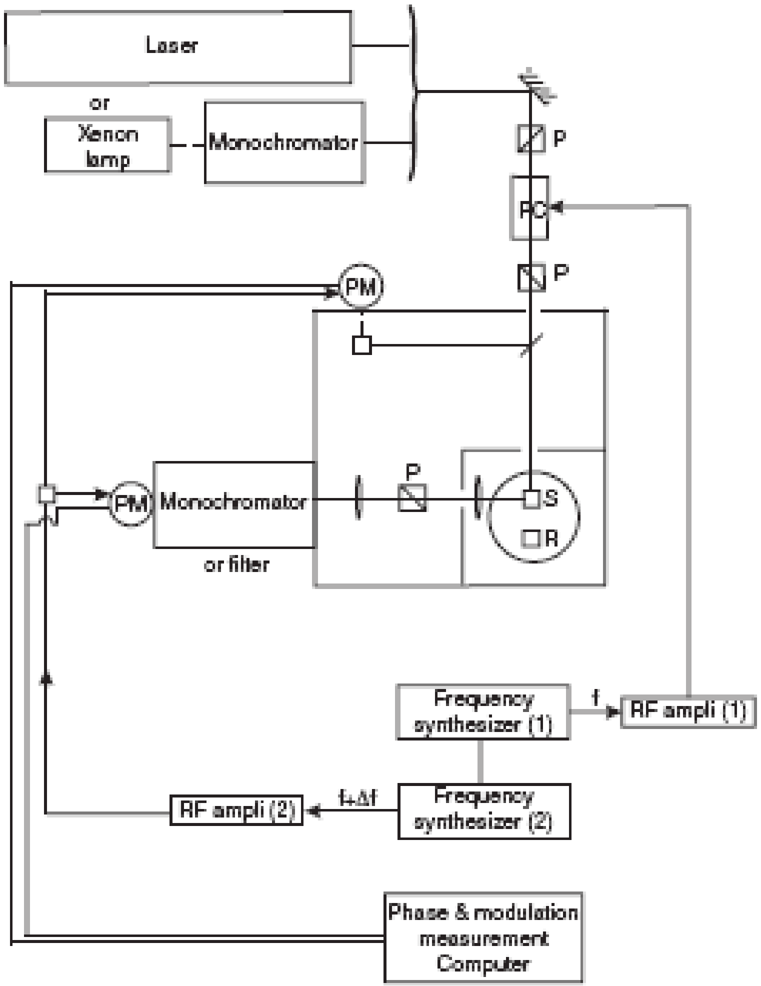
$$F(t) = E_o \sum_i a_i \tau_i + E_\omega \sum_i \frac{a_i \tau_i}{\sqrt{1+(\omega_E \tau_i)^2}} cos(\omega_E t - (\varphi_i - \varphi_E))$$

You still can only measure one $\left(M, \varphi\right)$

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

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

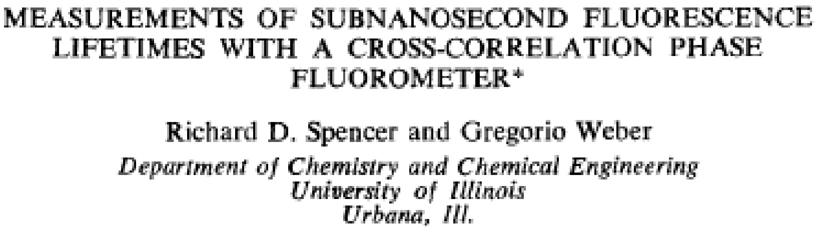
Mixing is used in commercial instruments



$$[G(t)*F(t)] = DC + \boxed{\text{Terms with frequencies } (\omega_E, \omega_G, \omega_E + \omega_G)} + \frac{G_\omega E_\omega}{2} M \left(\cos((\omega_G - \omega_E)t + \varphi_G - \varphi_E + \varphi) \right)$$

Images from Molecular Fluorescence by Bernard Valeur

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

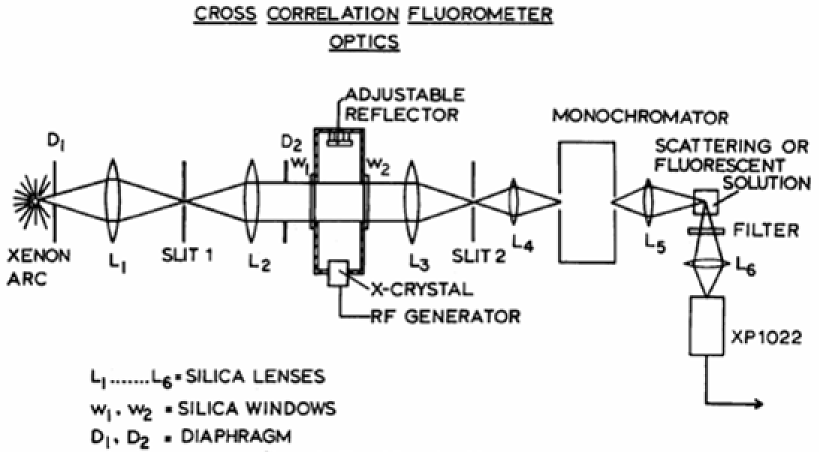


FIGURE 4. Plan of the optics of fluorometer.

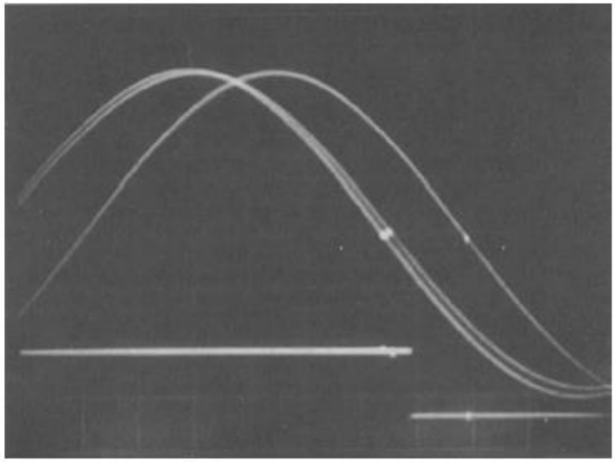


FIGURE 7. The figure shows the cross-correlation photocurrents from a scattering solution (first from left), a solution of NADH in phosphate buffer, pH 7.0, 17° C (second from left), and a solution of fluorescein (1 μgm/ml) in 0.01 M NaOH (right).

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

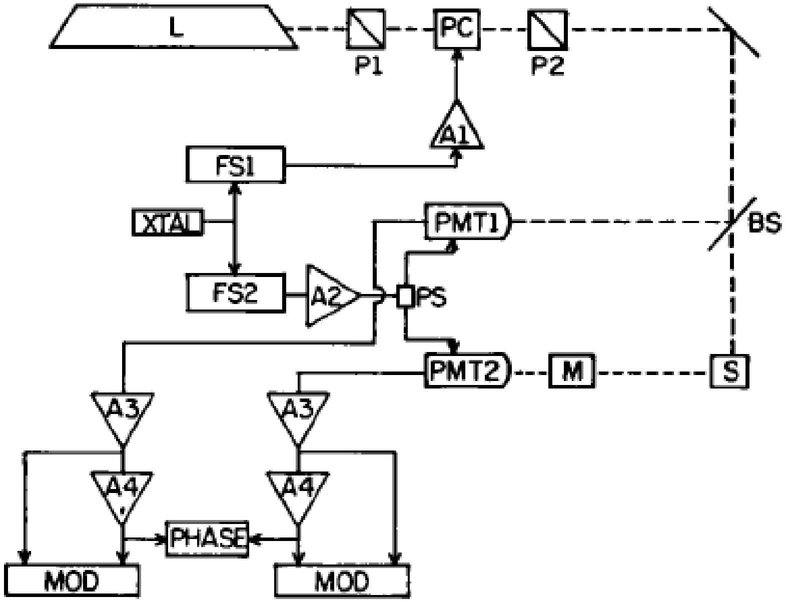
-variations in the intensity modulation caused by temperature

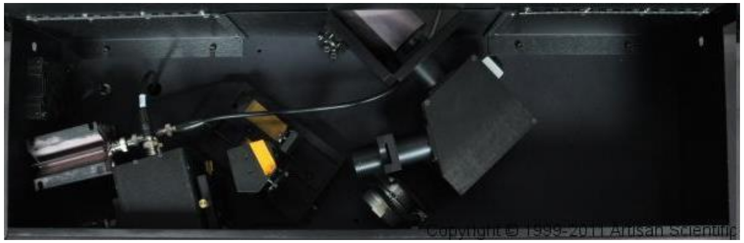
Pockels Cell

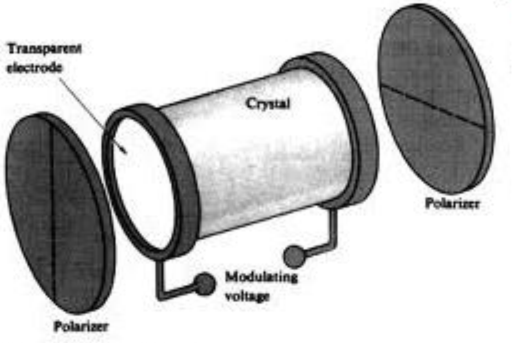
A CONTINUOUSLY VARIABLE FREQUENCY CROSS-CORRELATION PHASE FLUOROMETER WITH PICOSECOND RESOLUTION

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





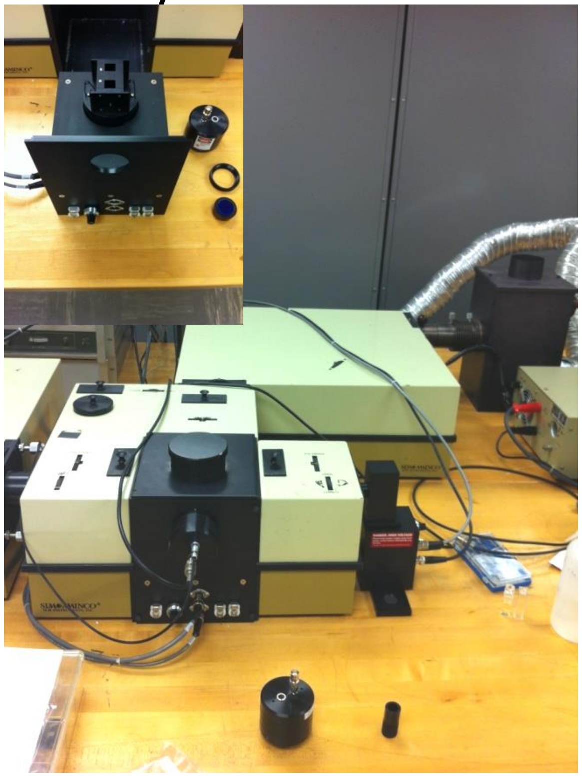


Hecht Optics

Figure 8.57 A Pockels cell.

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

```
SLM SPECTRUM PROCESSOR

For use with the 8000C

Release date 7-26-88

SLM Version 2.3

Copyright (C) SLM INSTRUMENTS INC. 1988
Press <SPACE> to start monochromator calibration or <ESC> to skip
```

New Upgrades

PMT Housing

New Acquisition Box

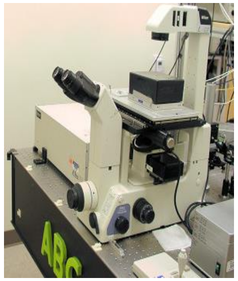
Stepper Motor Controller

- -photon counting
- -Measures nanosecond lifetime

Champaign, Illinois – Domain of FD FLIM

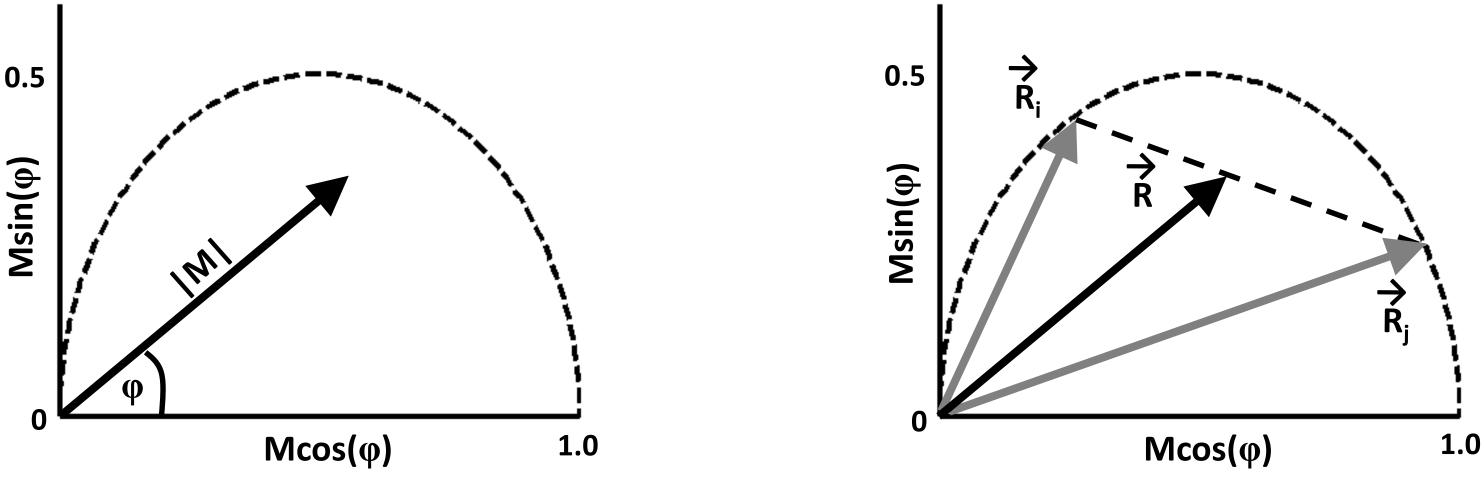
Robert Clegg – UIUC
Full-Field FLIM

Enrico Gratton – UIUC
Scanning Confocal FLIM (FLIMBox)



Beniamino Barbieri – ISS Inc.
Commercialization of FD FLIM

The Polar Plot



Vectors on the Polar Plot

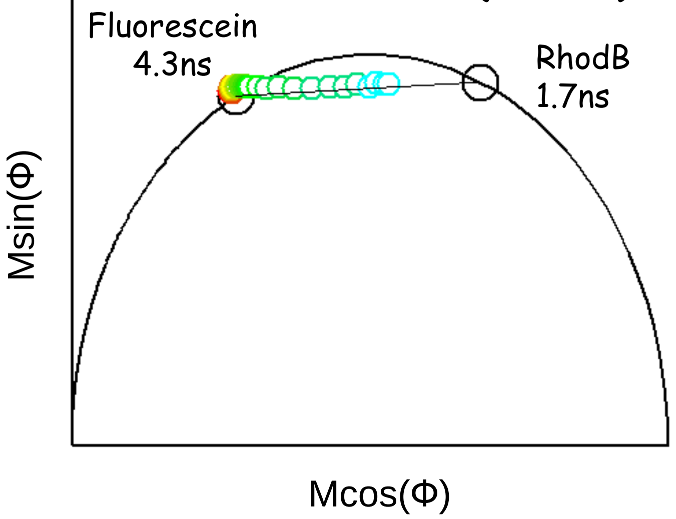
$$\vec{R} = Mcos(\varphi)\hat{x} + Msin(\varphi)\hat{y}$$

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

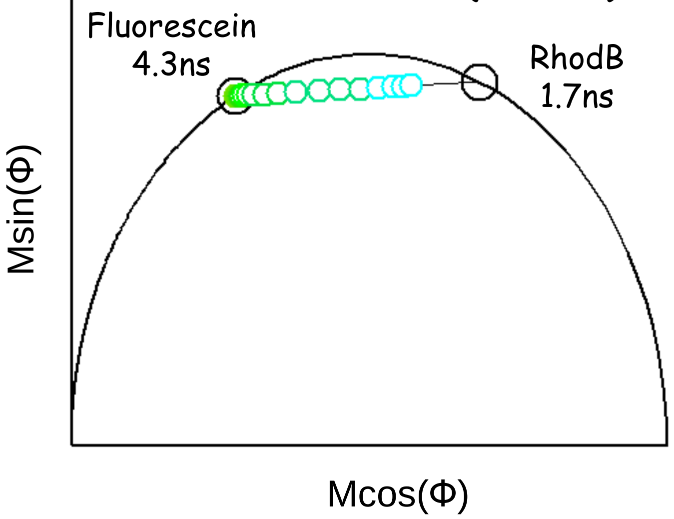
$$\vec{R} = \left(\alpha_i M_i cos(\varphi_i) + \alpha_j M_j cos(\varphi_j)\right)\hat{x} + \left(\alpha_i M_i sin(\varphi_i) + \alpha_j M_j sin(\varphi_j)\right)\hat{y}$$

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

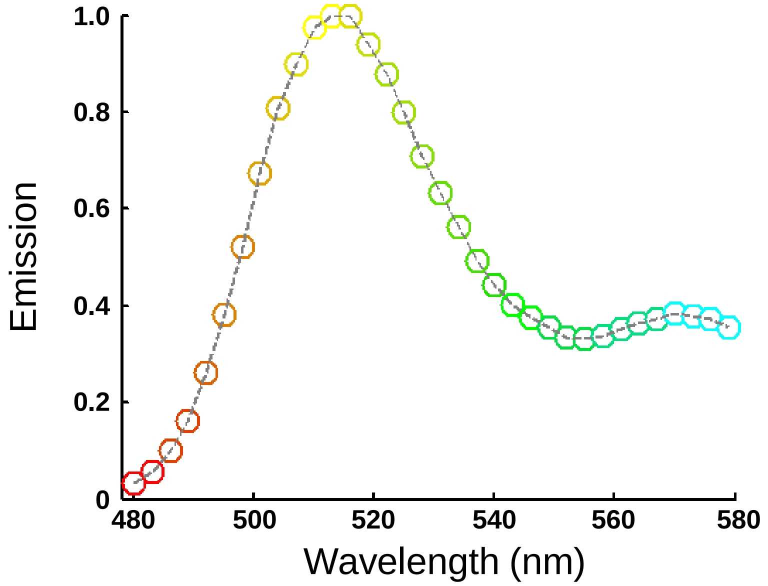
Spectral Analysis on the SLM



Measured Data (60MHz)

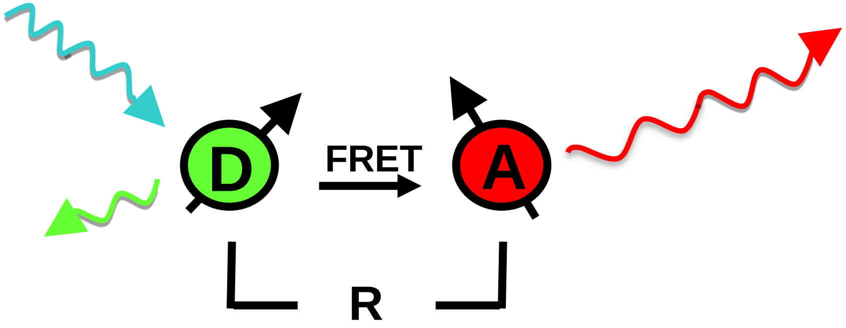


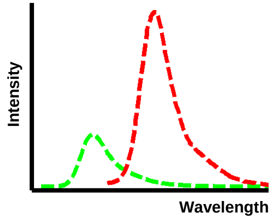
Simulated Data (60MHz)

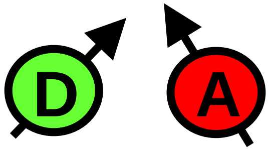


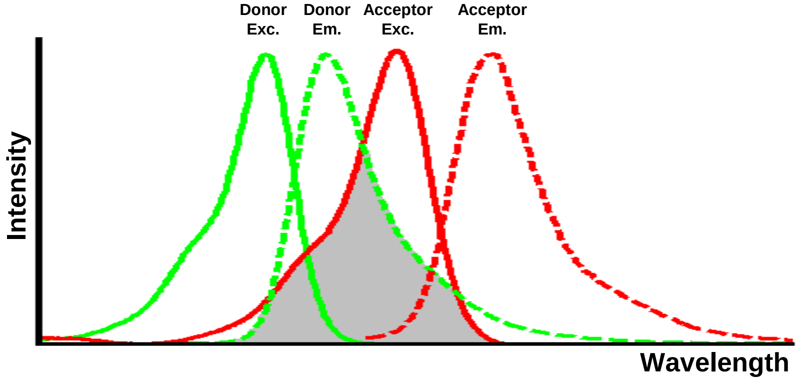
Measured Spectrum

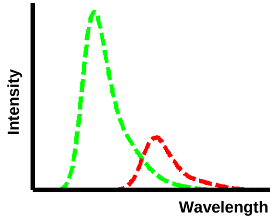
Quantum Yield/FRET

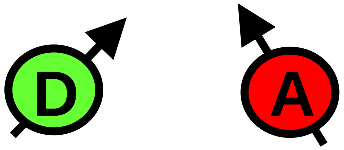










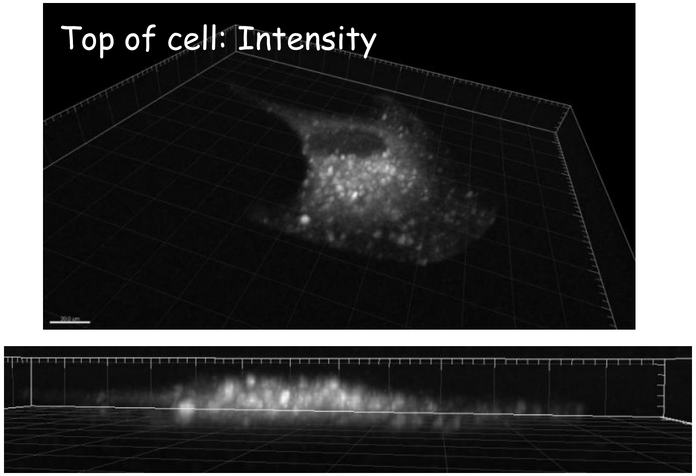


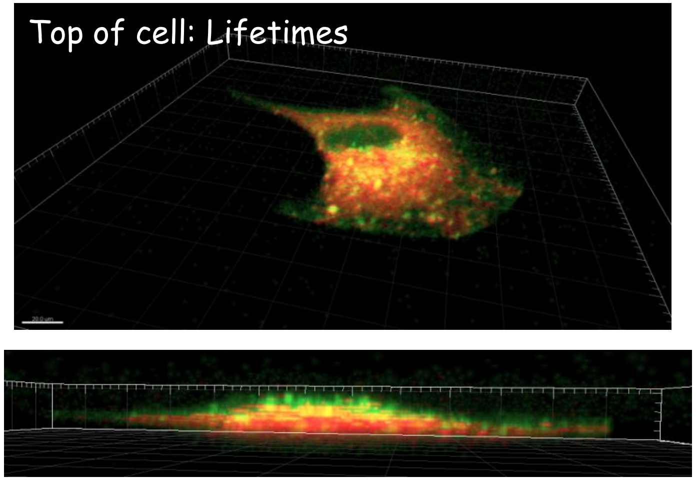
$$\text{Quantum Yield of Energy Transfer } (\mathbf{E}) = \frac{\text{Rate of Energy Transfer}}{\text{Total de-excitation rate}} = \frac{\left(\dfrac{1}{\tau_{DA}}\right) - \left(\dfrac{1}{\tau_D}\right)}{\left(\dfrac{1}{\tau_D}\right)} = 1 - \frac{\tau_{DA}}{\tau_D}$$

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

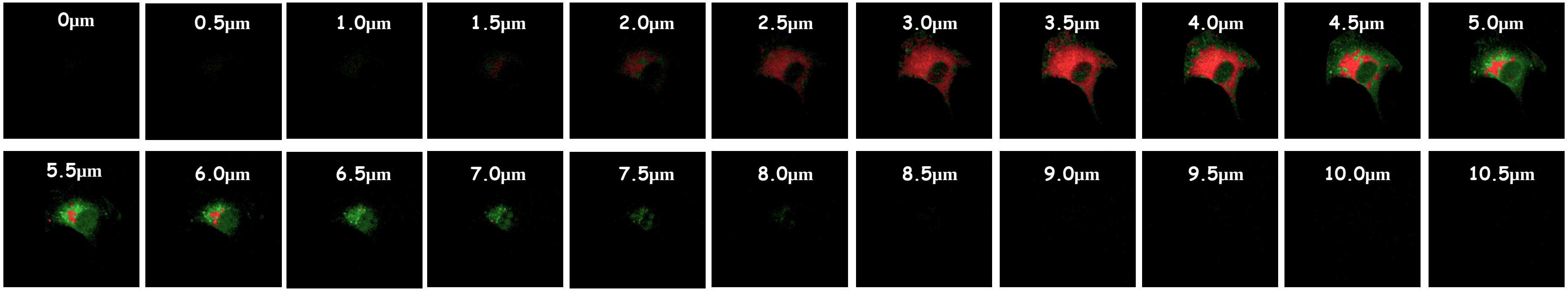
Applications of Fluorescence in Biology

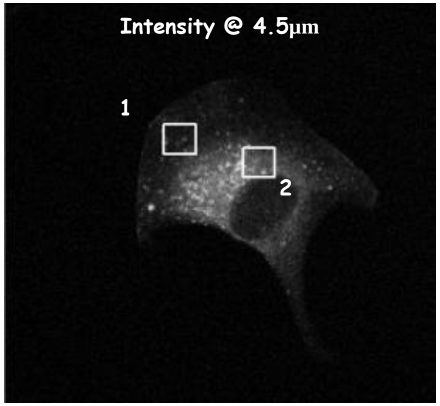
Fluorescence Lifetime Imaging on Live Cells

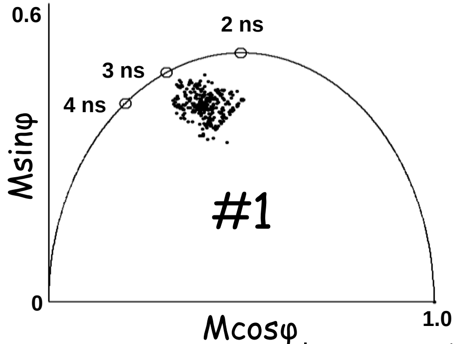


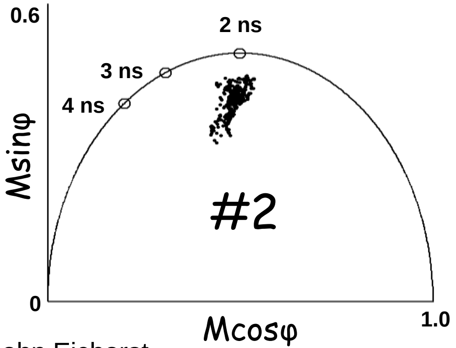


Optical Sections – Rendered by Lifetime



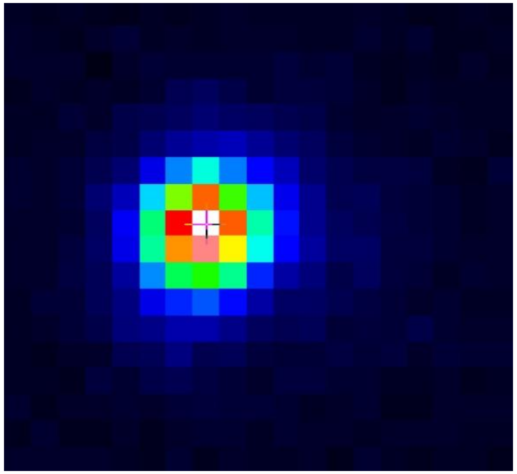


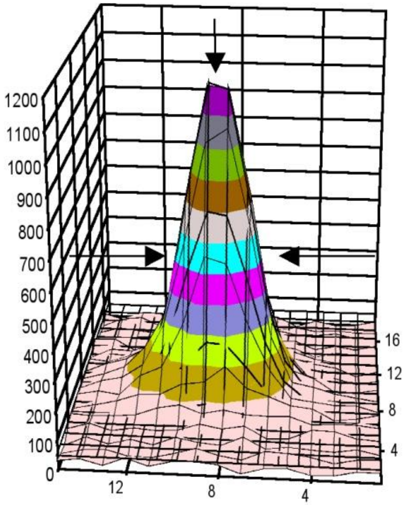


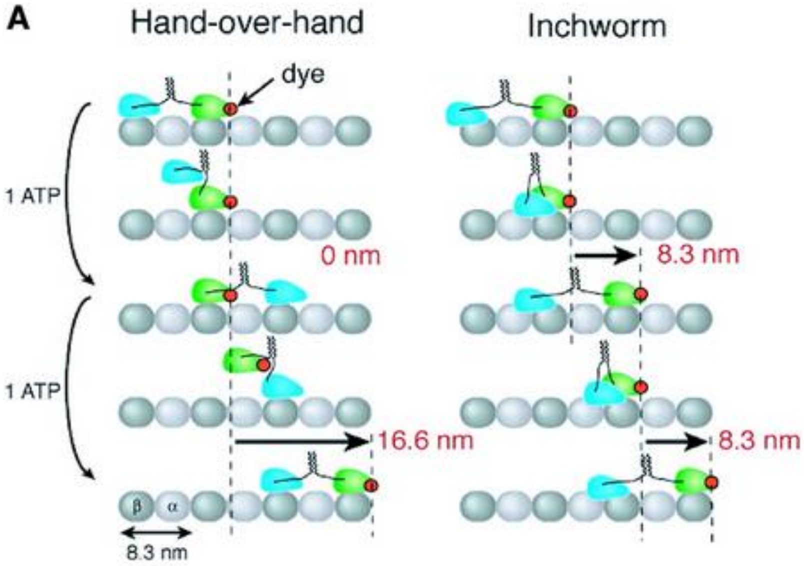


Images courtesy of John Eichorst

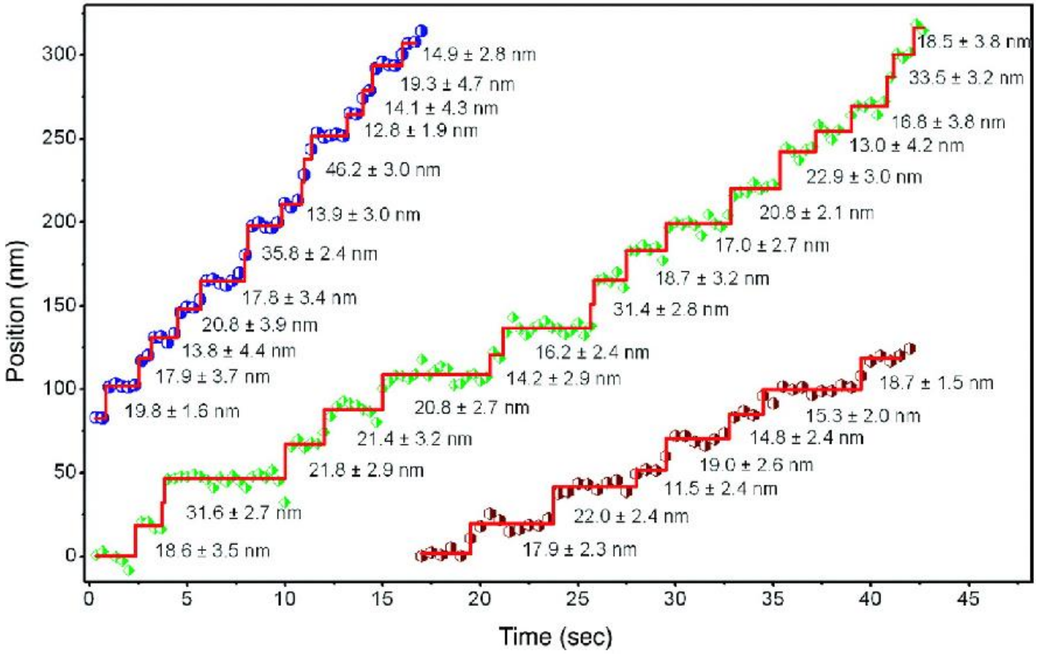
Single Molecule Fluorescence Imaging







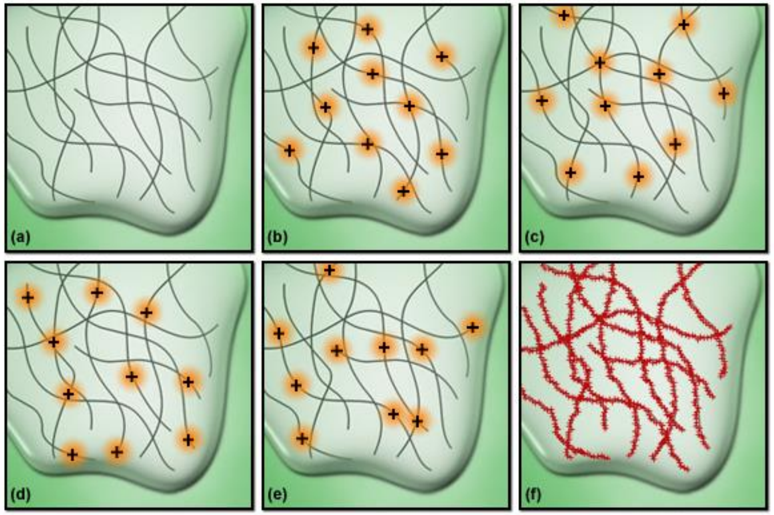
$$\sigma_{\mu_i} = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^4 b^2}{a^2 N^2}\right.}$$



Center of the distribution can be determined in ~1.5 nm accuracy if #N is more than 10^4

Super Resolution Fluorescence Imaging

Basic Principle of STORM Superresolution Imaging



Three-Dimensional Superresolution Imaging with STORM

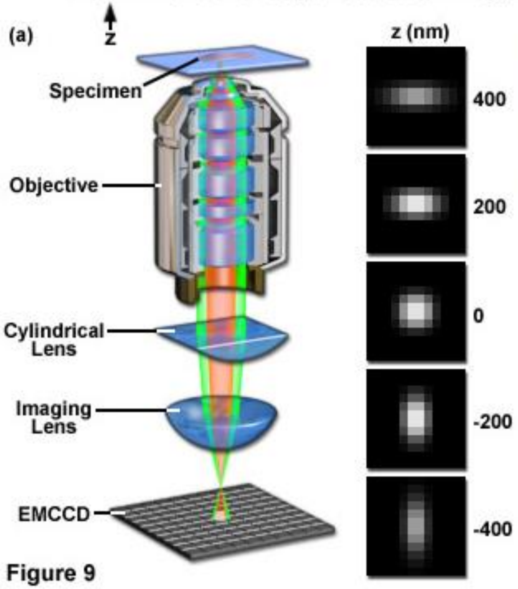
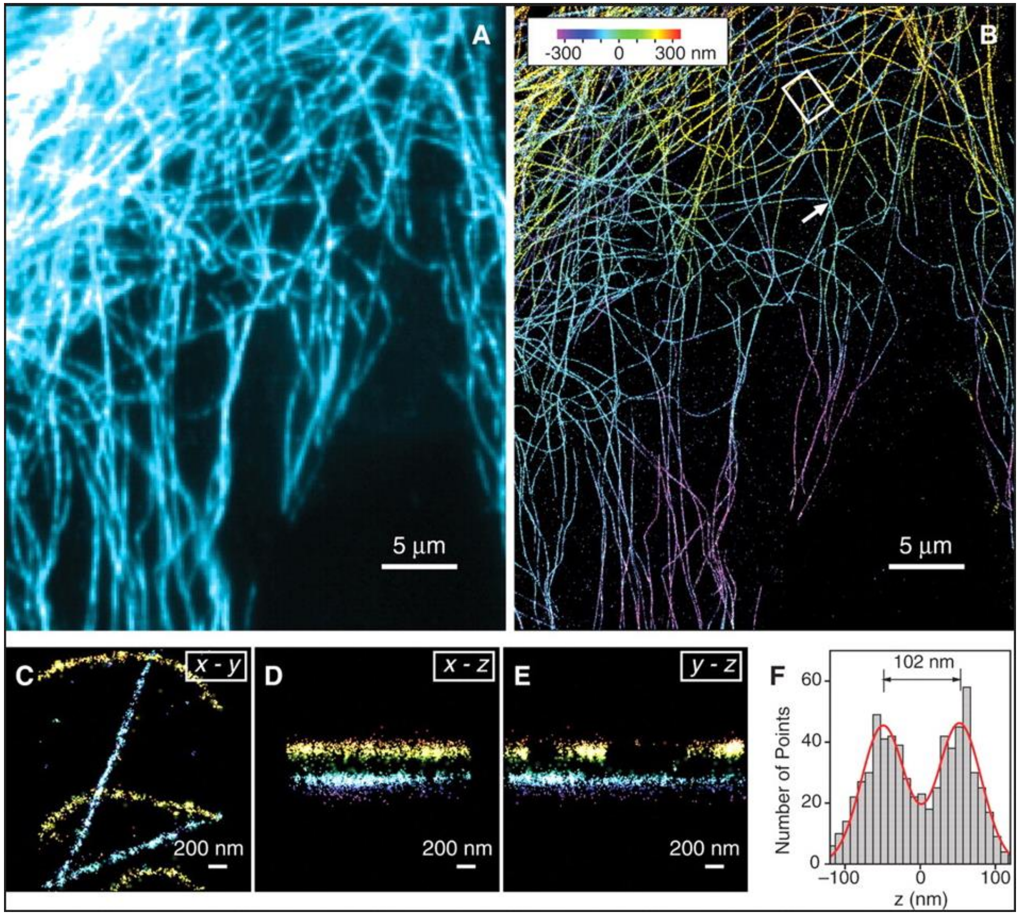


Figure 9



http://www.microscopyu.com/articles/superresolution/stormintro.html
Huang et al Science,2008