Fluorescence Spectroscopy

Steady State and Time Dependent Fluorescence Measurements

Dr. Teng, Kai Wen

PHYS 403 Fall 16
EM Spectrum of molecules

Rotational Energy $\rightarrow$ Infrared

Vibrational Energy $\rightarrow$ Near Infrared

Electronic Energy $\rightarrow$ Visible and Ultra-Violet

Diagram from http://www.lbl.gov

Types of Fluorescent Molecules

**Synthetic Organic:**
Fluorescein

**Semiconductor Nanocrystal:**
- Cadmium
- Selen

**Naturally Occurring:**
- Ruby and assorted mineral
  - From mineralman.net

**Crystals:**
- Fluorescent Nanodiamonds

**Fluorescent Proteins:**
- Green Fluorescent Protein

*Image from Zrazhevskiy et al. 2010*
Perrin-Jablonski energy diagram (S0, S1 and S2 transitions)

Absorption ($S_0 - S_1$)

Beer-Lambert's Law

\[
\log(I_0) - \log(I) = \varepsilon cl
\]

Extinction coefficient: Concentration

O.D.

Caffeine

OD

Mountain Dew 20xOD

Sprite 20xOD

redbull 20xOD

200 250 300 350

-0.5 0.5 1.5 2 2.5 3 3.5
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:

Solvent Effect:

Ruby:

Ruby (1/5)
Synthetic spinel

http://micro.magnet.fsu.edu

http://www.bio.davidson.edu
Rate constant for leaving excited state while emitting a photon

\[ k_i \]

Rate constant for leaving excited state through other means (i.e., Dynamic quenching, Energy Transfer, etc)

\[ k_i \]

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

\[ \tau = \sum_{i} \frac{1}{k_i} \]

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
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. “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 (18th century) representing the discovery of the phosphorescence of the phosphorus extracted from urine by Hennig Brand in 1669.
Measuring the Depletion of the excited state

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

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

\(K_F\) 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

\[ E(t) = E_o + E_o \cos(\omega_E t + \varphi_E) \]

\[ F(t) = F_o + F_o \cos(\omega_E t + \varphi_E - \varphi) \]

\[ \tan(\varphi) = \omega_E \tau_\varphi \]

\[ M = \frac{F_o / F_o}{E_o / E_o} = \frac{1}{\sqrt{1 + (\omega \tau_{Mod})^2}} \]

What do you need?

- Intensity modulators

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

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 - (\phi_i - \phi_E))

You still can only measure one \((M, \phi)\)

\frac{F(t)}{F_o} = 1 + \frac{E_\omega}{E_o} \sum_i \frac{a_i}{\sqrt{1+(\omega_E \tau_i)^2}} \cos(\omega_E t - (\phi_i - \phi_E))

\frac{F(t)}{F_o} = 1 + \frac{E_\omega}{E_o} M \cos(\omega_E t - (\phi_i - \phi))

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

Samples Described by Multiple Lifetimes
Mixing is used in commercial instruments

\[[G(t)*F(t)] = DC + \frac{G \omega E \omega}{2} M \left( \cos((\omega_G - \omega_E)t + \varphi_G - \varphi_E + \varphi) \right)\]

Terms with frequencies \((\omega_E, \omega_G, \omega_E + \omega_G)\)
-modulation frequency limited by resonance frequency of the acousto-optic cell

-variations in the intensity modulation caused by temperature
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


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

- 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

\[ \vec{R} = M\cos(\phi)\hat{x} + M\sin(\phi)\hat{y} \]

\[ \vec{R} = \alpha_i \vec{R}_i + \alpha_j \vec{R}_j \]

\[ \vec{R} = \left( \alpha_i M_i \cos(\phi_i) + \alpha_j M_j \cos(\phi_j) \right) \hat{x} \]
\[ + \left( \alpha_i M_i \sin(\phi_i) + \alpha_j M_j \sin(\phi_j) \right) \hat{y} \]

- movement of the vector depends on the emitted intensity of each species (i)
Spectral Analysis on the SLM

Measured Data (60MHz)
- Fluorescein: 4.3ns
- RhodB: 1.7ns

Simulated Data (60MHz)
- Fluorescein: 4.3ns
- RhodB: 1.7ns

Measured Spectrum
- Emission vs. Wavelength (nm)
Quantum Yield of Energy Transfer (E) = 
\[ \frac{\text{Rate of Energy Transfer}}{\text{Total de-excitation rate}} \] = \
\[ \frac{\left( \frac{1}{\tau_{DA}} \right) - \left( \frac{1}{\tau_D} \right)}{\tau_D} \] = 
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

Top of cell: Intensity

Top of cell: Lifetimes

Optical Sections - Rendered by Lifetime

Images courtesy of John Eichorst
Single Molecule Fluorescence Imaging

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

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

\[
\sum_{i=1}^{\text{N}} (\text{Position (nm)})
\]
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