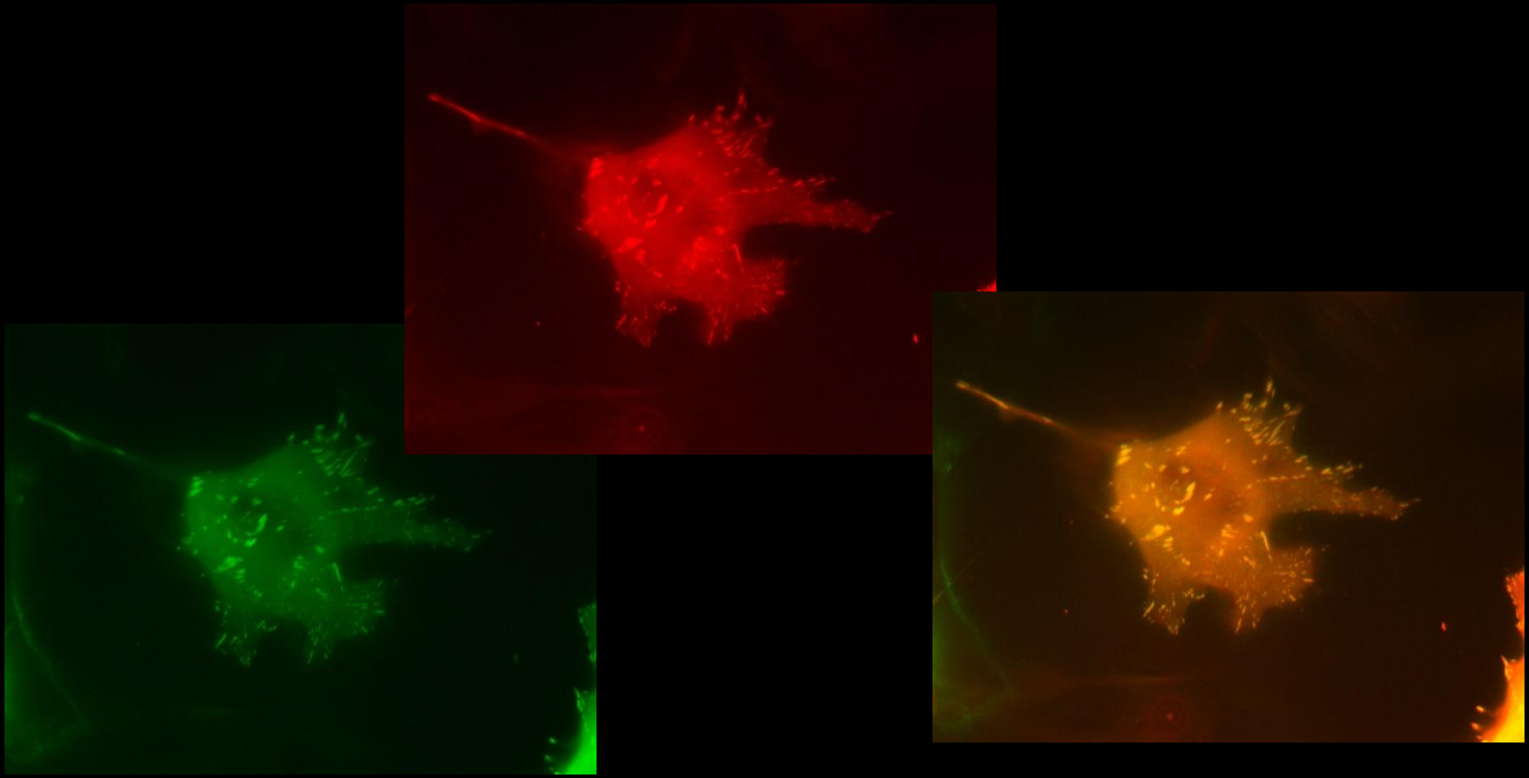
Fluorescence Spectroscopy III



Advanced fluorescence measurement

Fluorescence lifetime

Fluorescence lifetime is a complimentary measurement to spectral measurement. Most fluorophores has a signature lifetime as well as spectral. More important, some fluorophores have lifetimes tha are more sensitive to environmental factors than their spectra.

The fluorescence decay of a fluorophore is governed by the following equation:

$$\frac{dN_e}{dt} = -(k + \Gamma)N_e$$

where N_e is the number of molecules in the excited state which is proportional to the fluorescence intensity. This differential equation can be easily solved:

$$F = F_0 e^{-(k+\Gamma)t} = F_0 e^{-t/\tau}$$

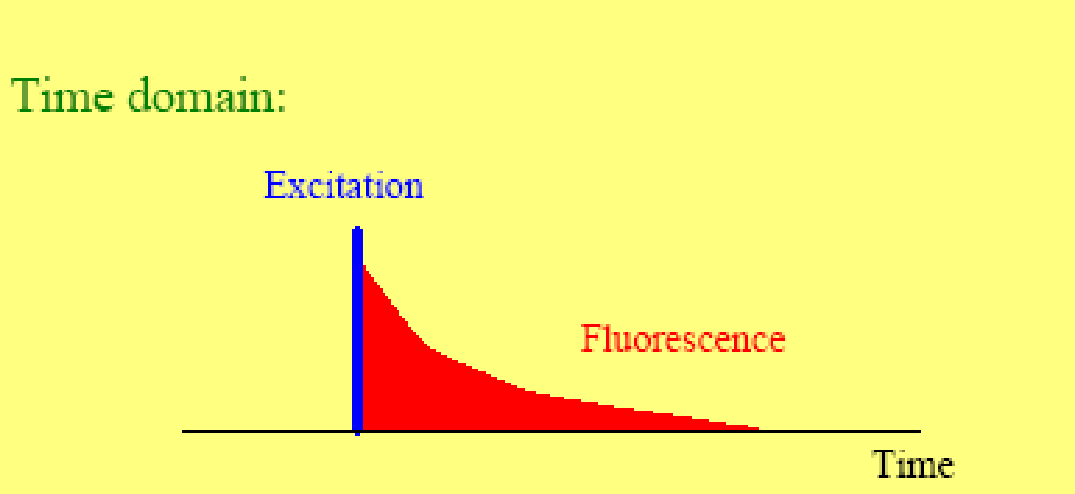
where F and F_0 are the instantaneous and initial fluorescence intensity. Therefore, we can see that fluorescence emission is a statistical process that is characterized by exponential decays. What if there are multiple decay pathways and multiple rates? In this case, the fluorescence decay will be multiple exponential:

$$\frac{dN_e}{dt} = -(\frac{1}{\tau_1} + \frac{1}{\tau_2} + ...)N_e$$

$$F = F_0 e^{-\Sigma t/\tau_i}$$

As a matter of fact, fluorescence decay of most fluorophores in biological system often has multiple exponential decay that is characteristic both of the probe and its environment.

While fluorescence lifetime is a very useful parameter, it is however difficult to measure. As we have discussed before, fluorescence lifetime is typically on the time scale of nanoseconds. We therefore require very fast optics electronic to measure these events. Conceptually, the measurement can be done in the following way:

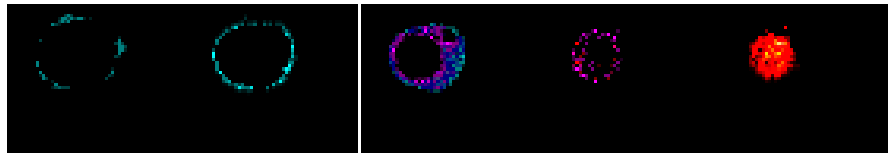


We will use a laser that can generate pulses that are very short compared with the fluorescence decay time (fs or ps). The fluorescence lifetime can then be measured by determining the time lapse between the excitation light and the first emission photon detected. One important catch to this scheme is that the photon detected for each excitation pulse has to be less than one.

Lifetime imaging and biological functions

(1) Distinguish cellular organelles by multiple lifetime imaging

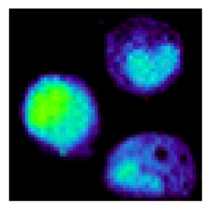
Lifetime Sections



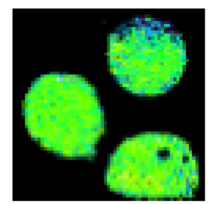
1.6-2.4 2.4-4 4-8 8-9 9-19

(2) Monitor metabolite concentration (Ca, pH etc)

Intensity

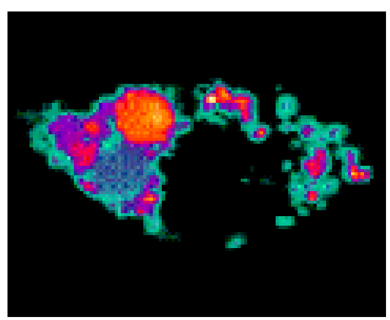


Lifetime

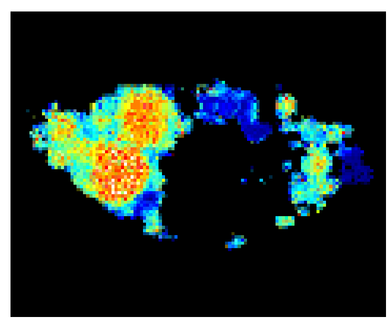


(3) Monitor cellular processes such as proteolytic activity

Intensity



Lifetime



Fluorescence Quenching

It is often very useful to measure the diffusion of small metabolites in a biological system; oxygen is a good example. As it turns out, a number of fluorophore, such as pyrene, with sufficiently long lifetime can be quenched by the presence of metabolite such as oxygen due to molecular collision. Upon collision, the fluorophore is de-excited non-radiatively.

The collision frequency is proportional to the concentration of the quencher and the rate equation in the presence of the quencher can be expressed as:

$$\frac{dN_e}{dt} = -(k_o[Q] + \Gamma)N_e$$

where [Q] is the concentration of the quencher and k_0 is a proportionality constant related to the diffusivity of the reactants.

$$k_0 \propto (R_f + R_q)(D_f + D_q)$$

where R_f, R_q are the "collision" radii of the fluorophore and the quencher and D_f, D_q are the diffusion coefficients of the fluorophore and the quencher

Therefore, we have:

$$\tau^{-1} = k_0[Q] + \Gamma = k_0[Q] + \tau_0^{-1} = \tau_0^{-1}(1 + k_0\tau_0[Q])$$

Therefore, by measuring fluorescence lifetime, we can determine quencher concentration as long as the natural lifetime and the proportionality constant k can be calibrated.

The effect of quencher can also be studied by monitoring the steady state fluorescence emission. We will add a constant illumination term, I, to the fluorescence rate equation:

$$\frac{dN_e}{dt} = -(k_o[Q] + \Gamma)N_e + I$$

In the steady state, $\frac{dN_e}{dt} = 0$, and we have the fluorescence, F, signal:

$$(k_o[Q] + \Gamma)F = I$$

We can re-write this equation in the absence of quencher. The steady state fluorescence in the absence of quencher, F_0, is:

$$\Gamma F_0 = I$$

Combining the last two equations, we get the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_o \tau_0 [Q]$$

For dynamic (collision) quenching process, the steady state fluorescence intensity is a linear function of quencher concentration.

The quenching process that we have described previously is called dynamic quenching where a fluorophore is de-excited by collision process in the excited state. For dynamic quenching, both the steady state fluorescence intensity and the fluorescence lifetime changes linearly with quencher concentration.

A molecule can also be quenched by a ground state process where the molecule is chemically bound to a quencher to form a "dark complex" – a reaction product that do not fluoresce. The ground state reaction can be described by the standard chemical kinetic rate equation where Ks is the association constant, [F] is the concentration of the un-complexed fluorophores, [F-Q] is the concentration of the complexes.

$$K_S = \frac{[F-Q]}{[F][Q]}$$

The total concentration of fluorophore, $[F]_0$, is given by:

$$[F]_0 = [F] + [F-Q]$$

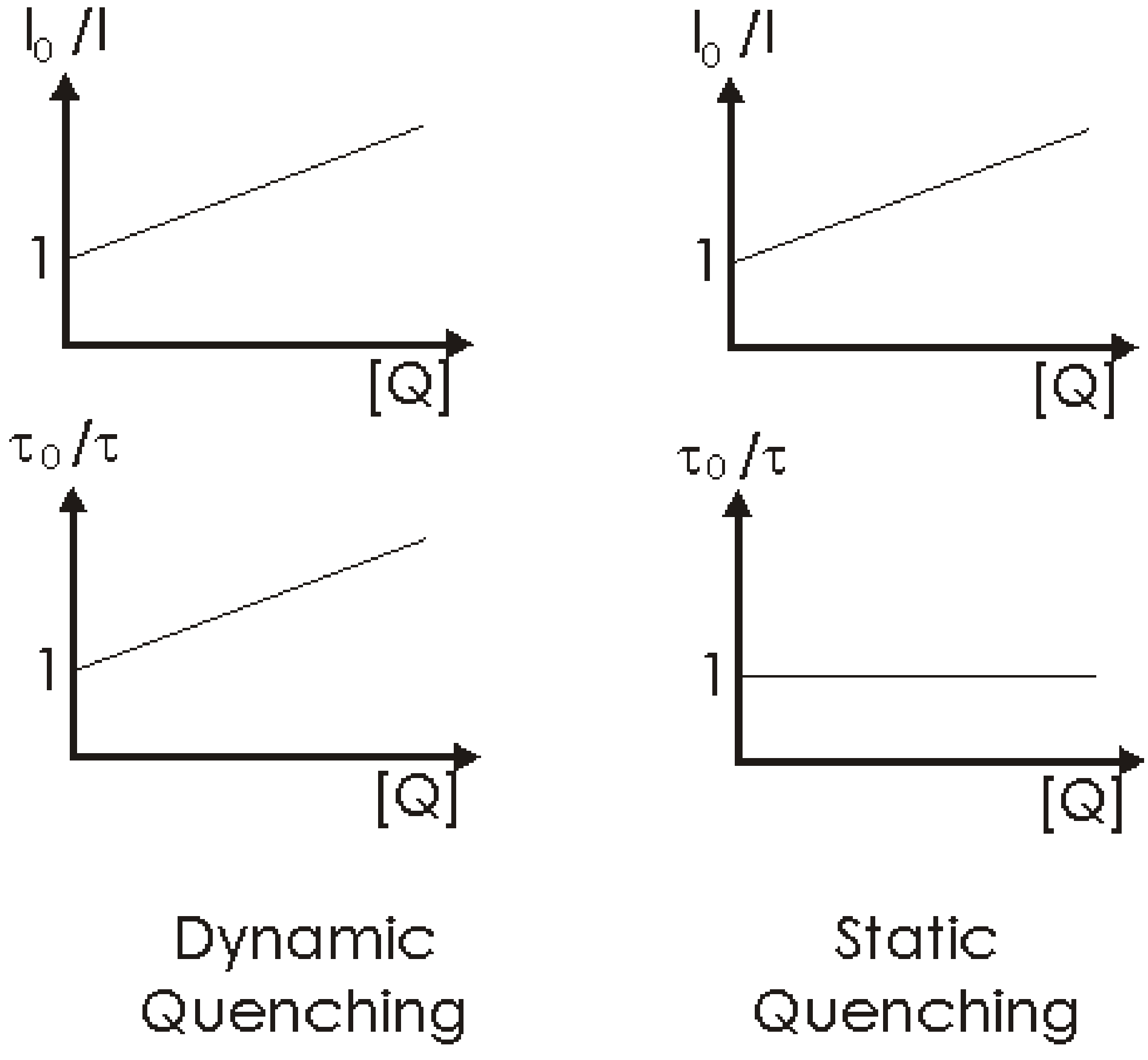
$$K_S = \frac{[F]_0 - [F]}{[F][Q]} = \frac{[F]_0}{[F][Q]} - \frac{1}{[Q]}$$

$$\frac{F_0}{F} = \frac{[F]_0}{[F]} = 1 + K_S[Q]$$

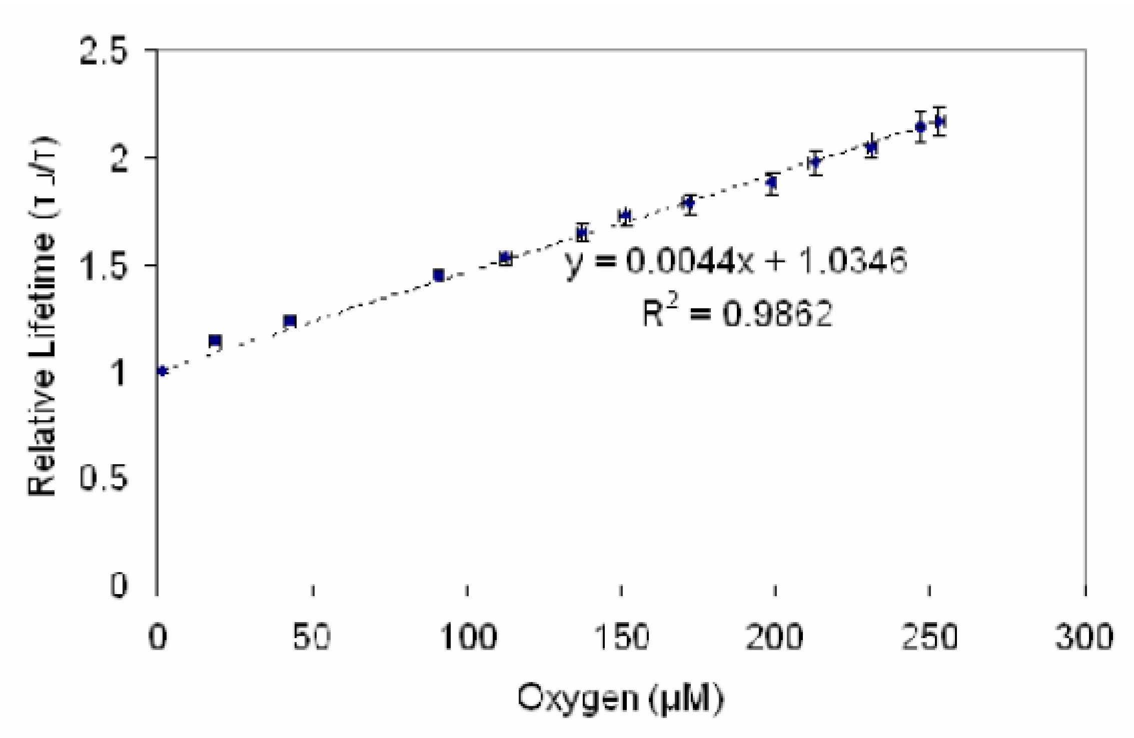
Therefore, in static quenching, the steady state fluorescence again decreases linearly with quencher concentration.

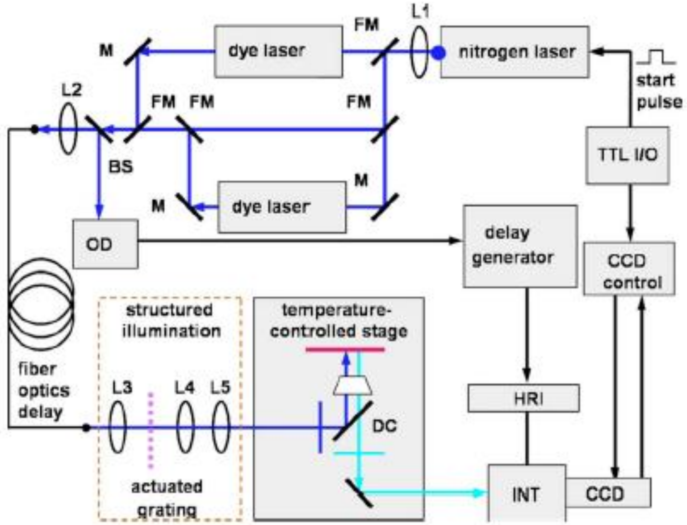
However, it is important to note that steady state quenching does NOT affect fluorescence lifetime as it does not affect the excited state and its effect is mainly the reduction of available fluorophores to be excited.

A summary of dynamic vs static quenching



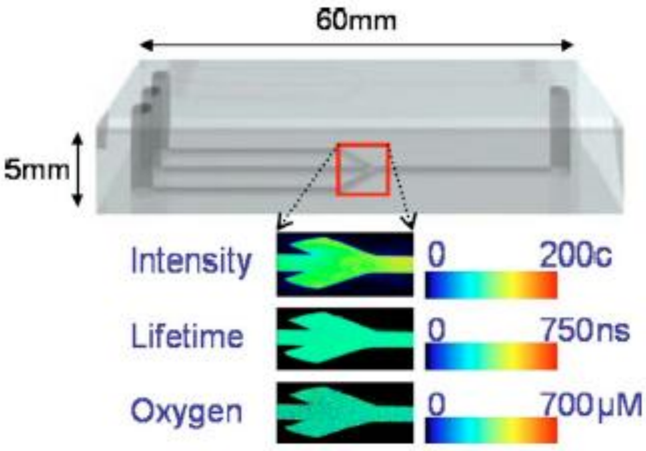
Imaging Oxygen Consumption in Microfluidic Devices

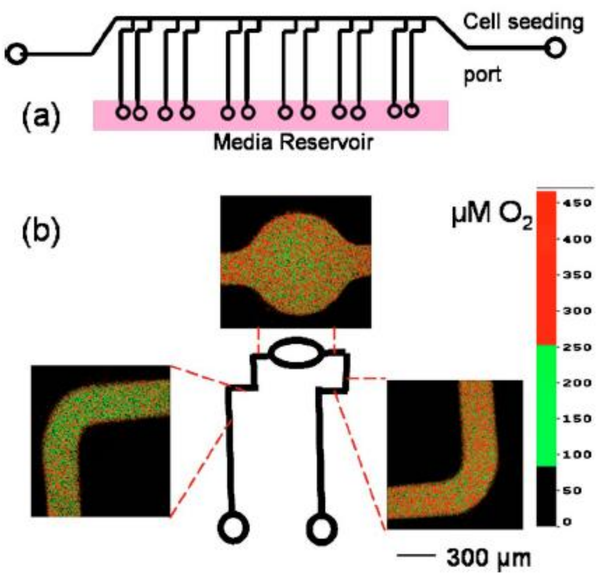


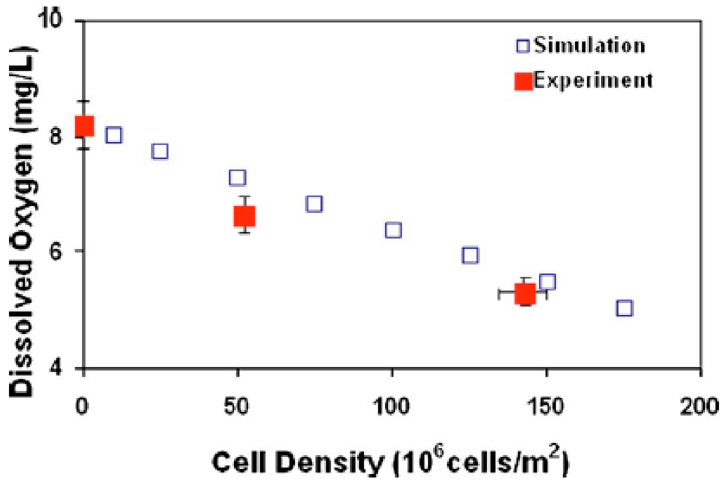


From Sud, OE 2006, Sub JBO Letters 2006

Imaging Oxygen Consumption in Microfluidic Devices





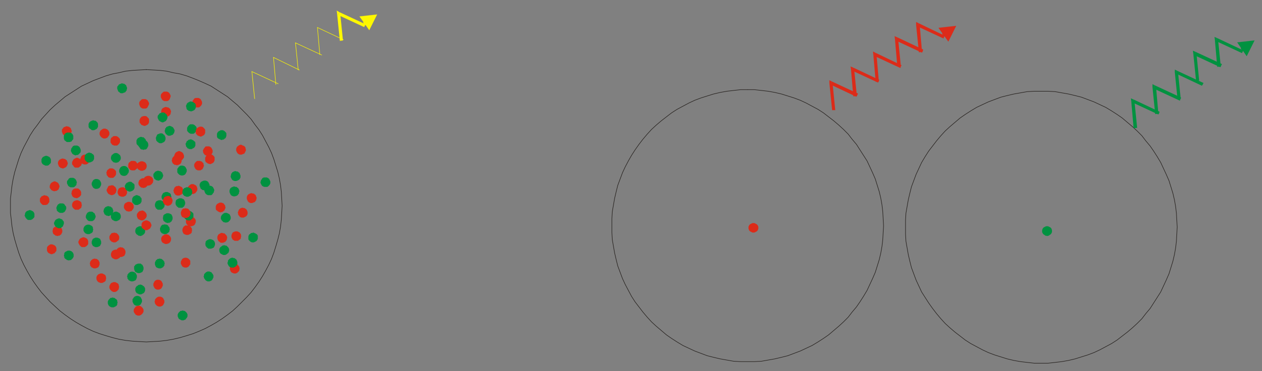


From Sud, OE 2006, Sub JBO Letters 2006

FLUORESCENCE CORRELATION SPECTROSCOPY

WHAT ARE THE BASIS OF THESE TECHNIQUE?

*Information are lost in an ensemble average
*In a dilute system with few, or one, particles, molecular information can be extracted.



WHAT DO THESE TECHNIQUES HAVE TO OFFER?

Molecular properties

* Number density
* Intrinsic brightness
* Diffusion rate (translation and rotation)
* Structural changes (protein folding)

Inter-molecular / molecule-environment interactions

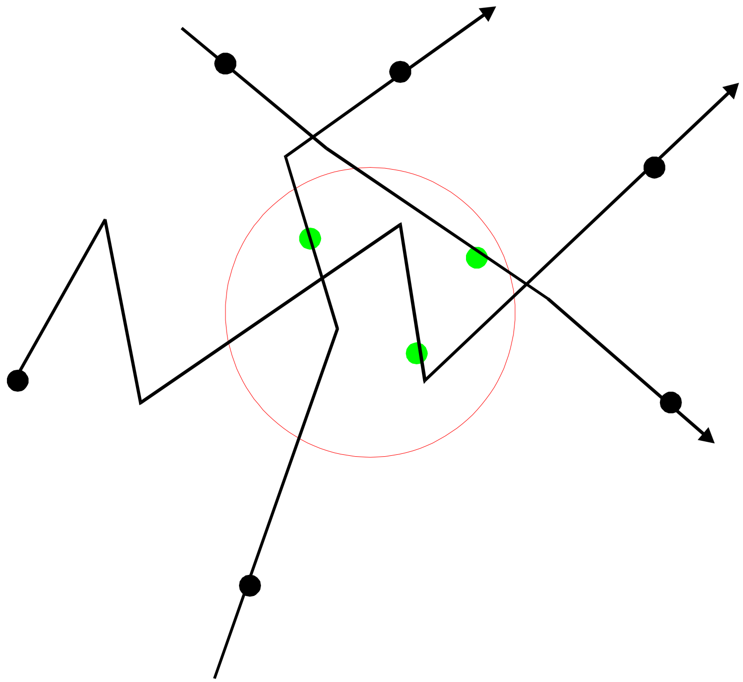
* Oligomerization
* Biochemical reaction rate
* Flow processes

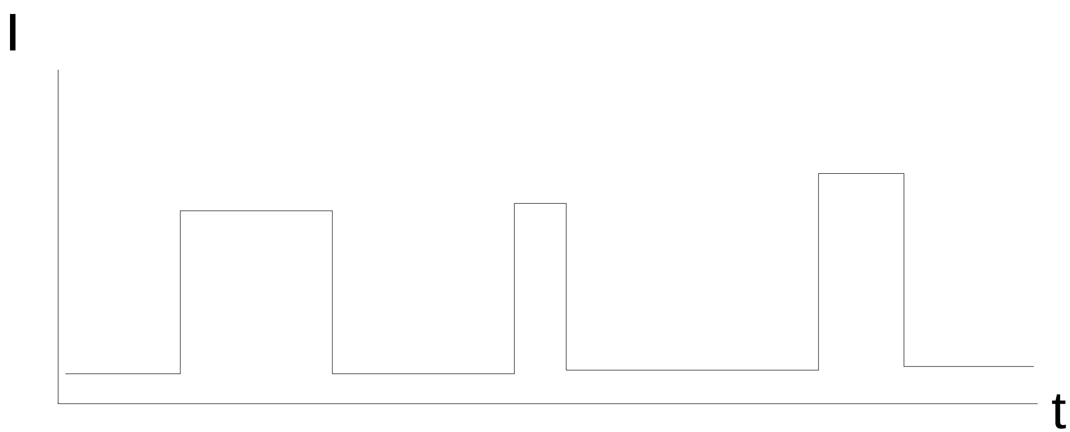
BASIC IDEA: LOOK AT "NOISE"

If you look into a small enough volume, molecule will move in and out of it. If these molecules are tagged with a fluorophore, the detected signal with blink on and off. The temporal statistics of the blinking gives information of the molecular diffusion.

Fluorescence Correlation Spectroscopy

Temporal "correlation" provides
the mean transition time of the molecule
across a small excitation region.

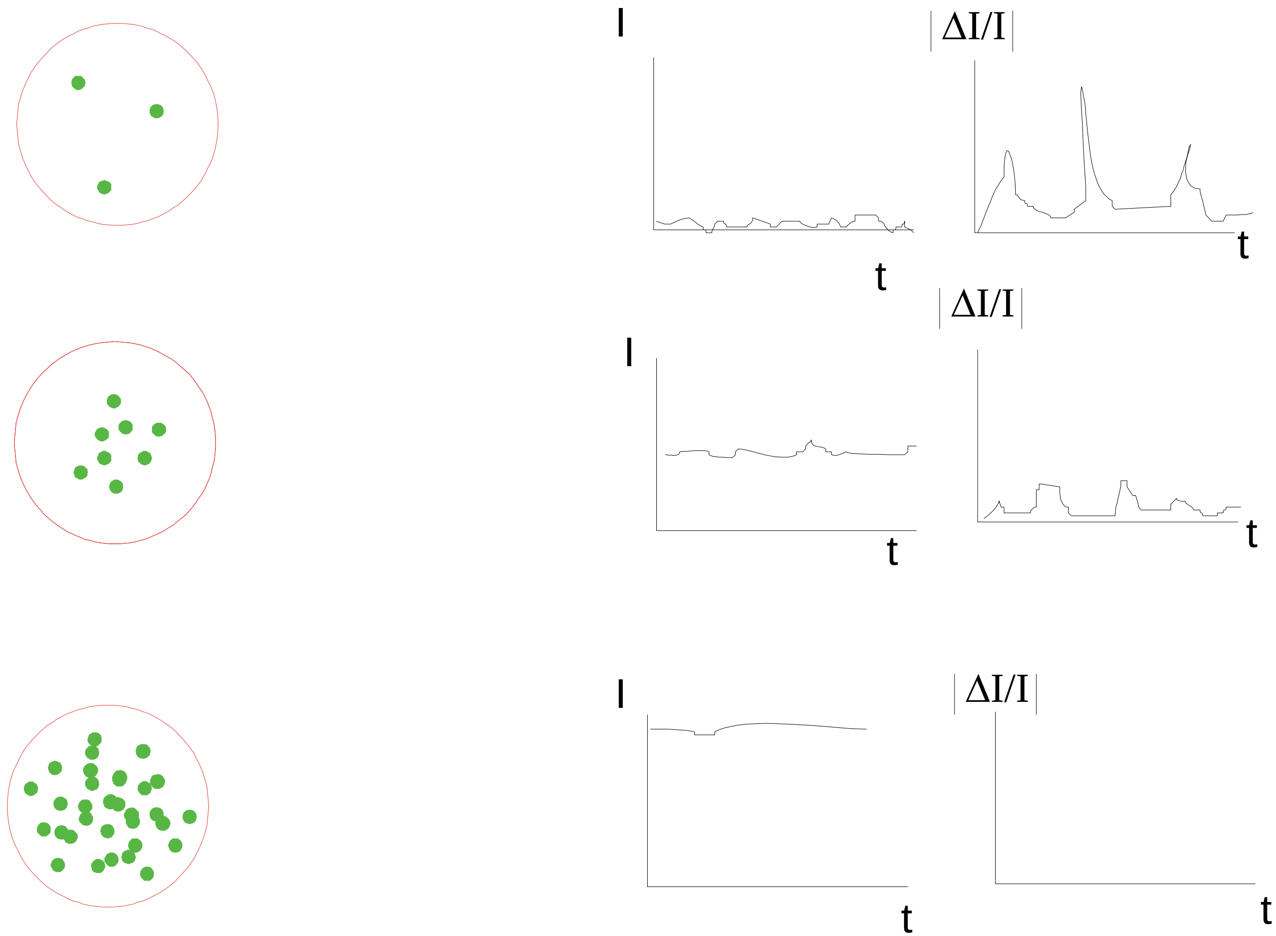




Fluorescence Correlation Spectroscopy

What else can we find out by looking at noise?

What does Poisson statistics tell us?



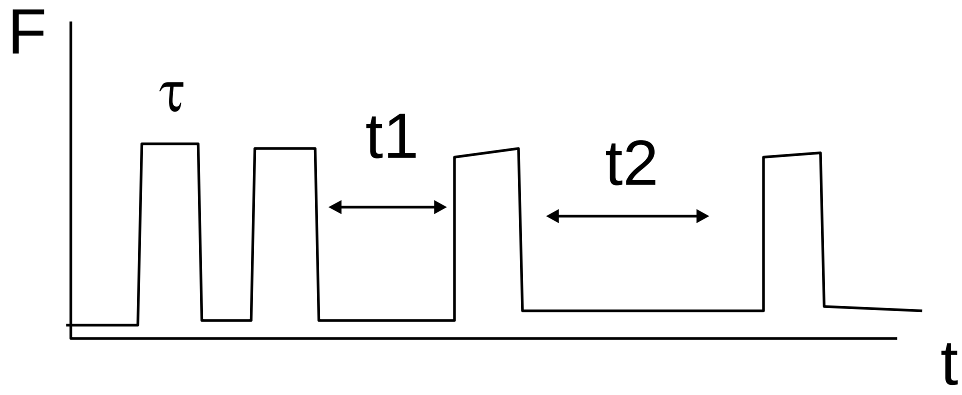
Fluorescence Correlation Spectroscopy

Mathematical Formulation

Intensity fluctuation is typically analyzed using the autocorrelation function:

$$g(\tau) = \frac{< F(t)F(t+\tau) > - < F(t) >^2}{< F(t)^2 >}$$

What does it mean? It is a measure of this: if you are measuring a high intensity at a given moment, what is the chance that you will still measure a high intensity some time τ away.



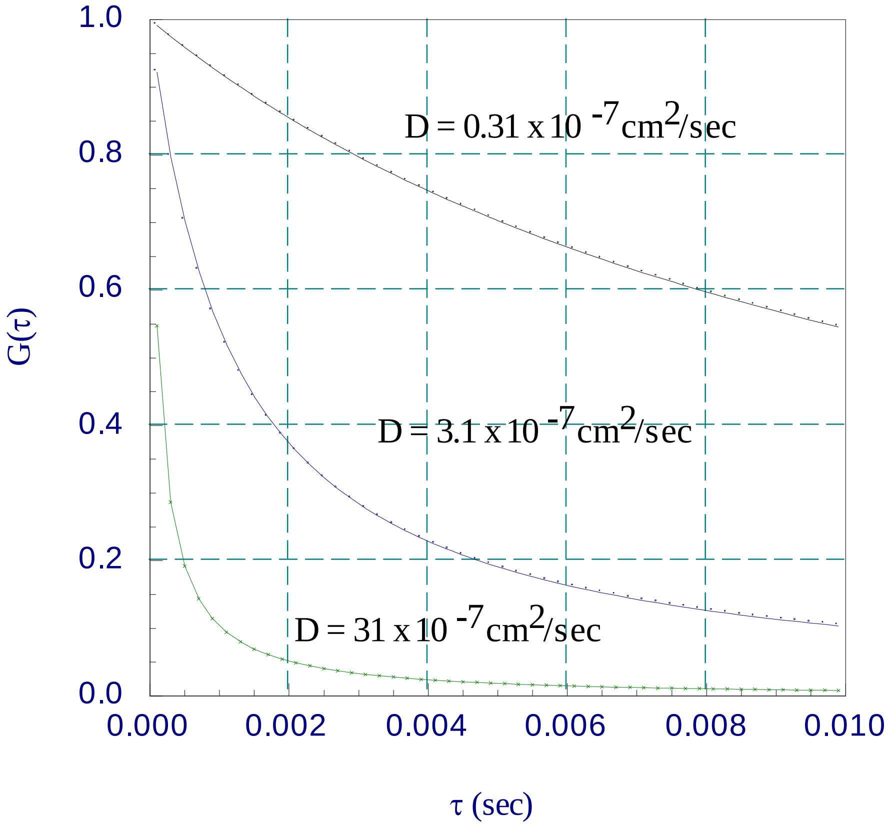
Typical Autocorrelation Function

For pure diffusion and simple excitation geometry profile (2D gaussian beam), autocorrelation function has a simple closed form:

$$g(\tau) = \frac{g(0)}{1 + (\frac{\tau}{s^2 / 4D})}$$

D is the diffusion coefficient

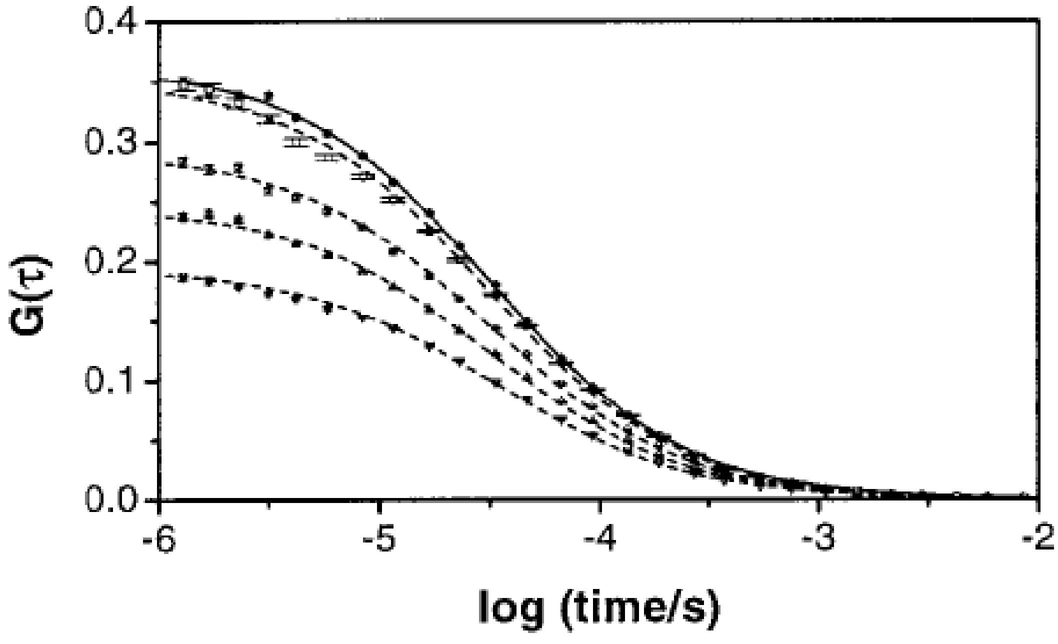
S is the “radius” of the laser beam



From Dr. K. Berland, Ph.D. Thesis

Logarithmic representation of the autocorrelation function

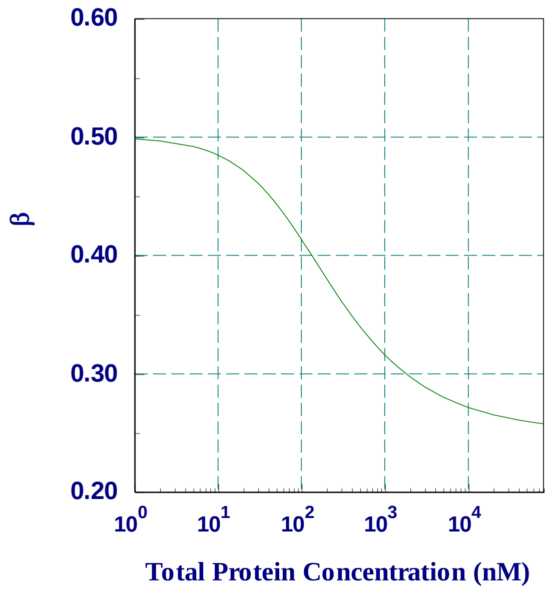
Fitting data over multiple time scales allow much better determination of experimental parameters



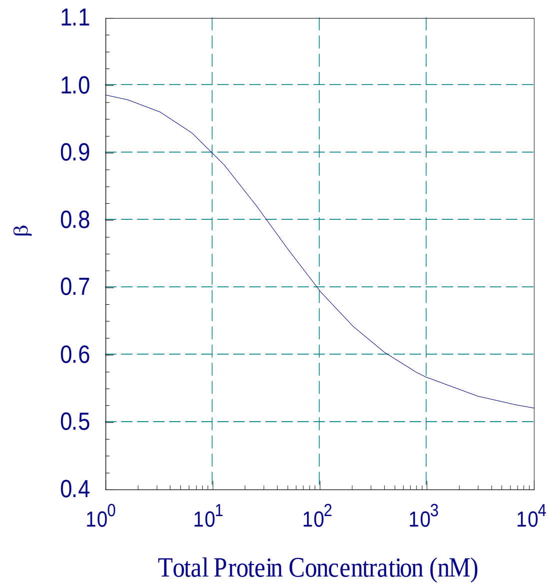
Lamb et al. Biophys. J., 2000

C. Dissociation of Oligomeric Proteins upon Dilution

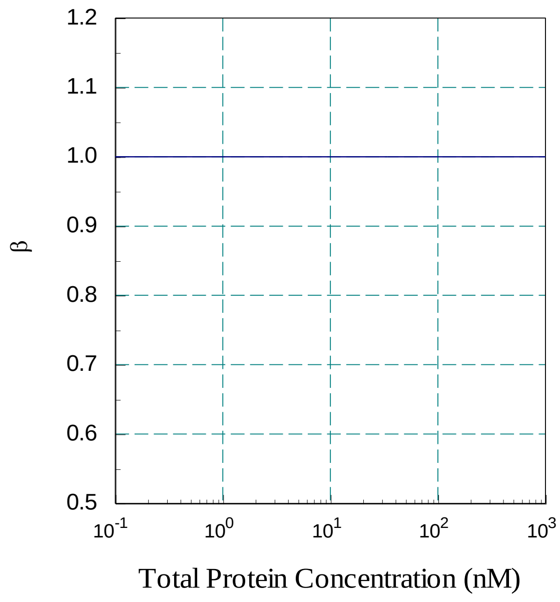
Glycogen Phosphorylase A



Melate Dehydorgenase



β Phycoerythrin

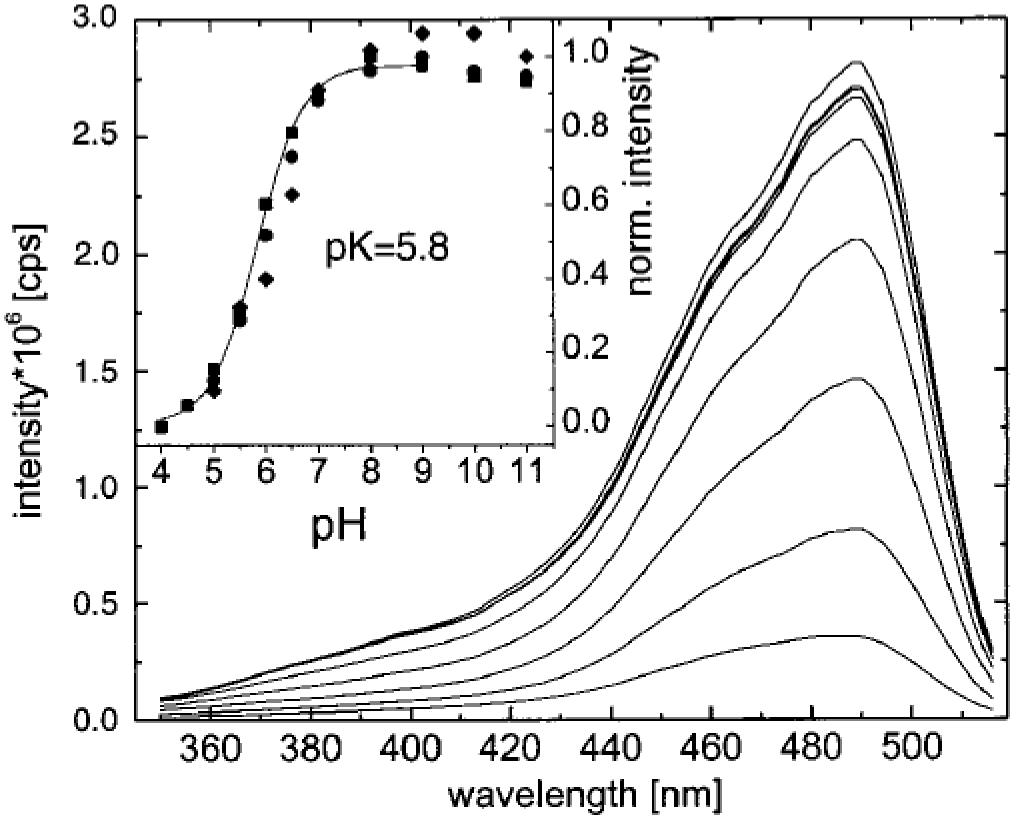


β is defined as the particle to monomer ratio

Berland et al., Biophys. J, 1996

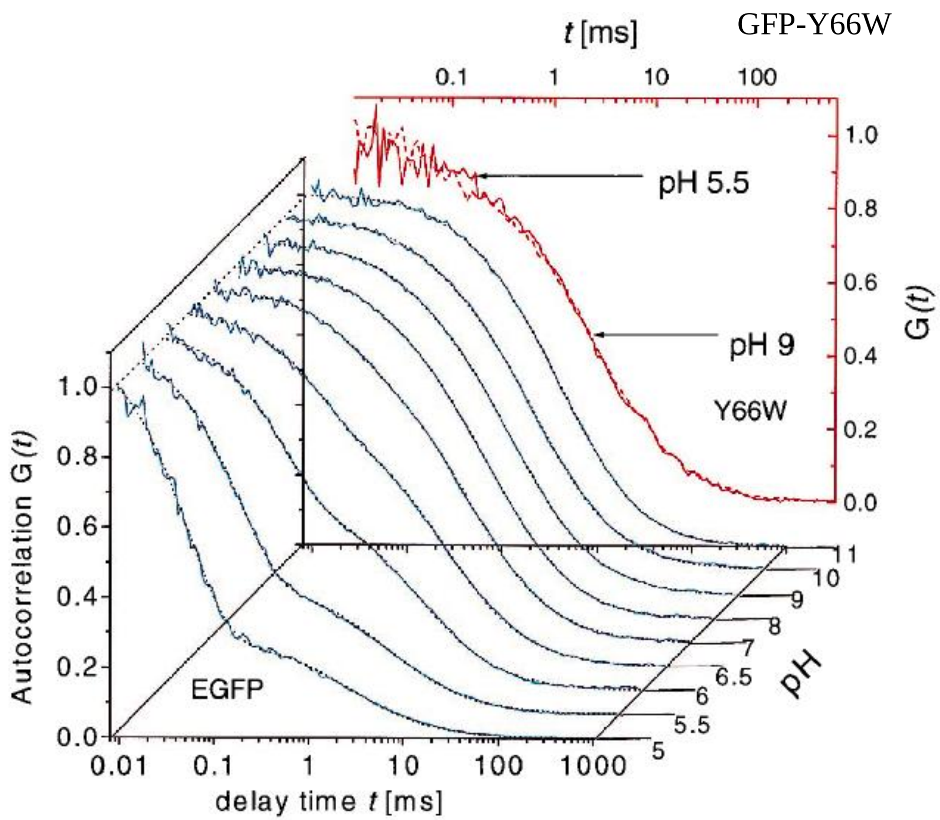
FCS Monitoring of Protein Conformation States Upon Protonation

Excitation Spectrum of EGFP as a function of pH

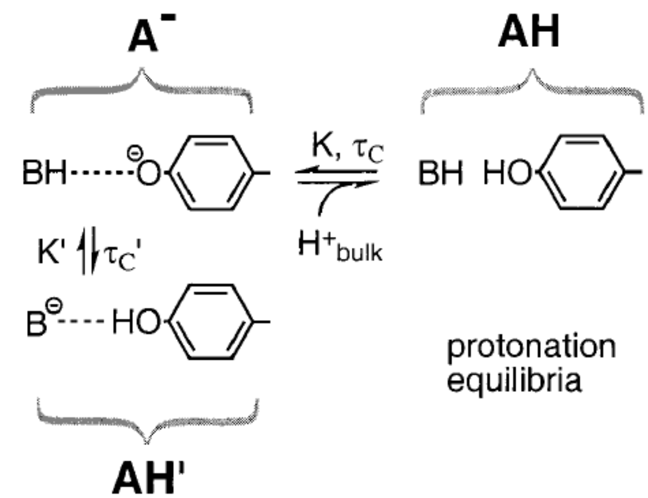


Haupts, PNAS, 1998

EGFP Protonation States Resolved Based on FCS



EGFP



$\tau_C \approx 10-200\ \mu s$ *pH dependent*

$\tau_C' \cong 450\ \mu s$ *pH independent*

Haupts, PNAS, 1998