F* techniques: FRAP, FLIP, FRET, FLIM, FCS

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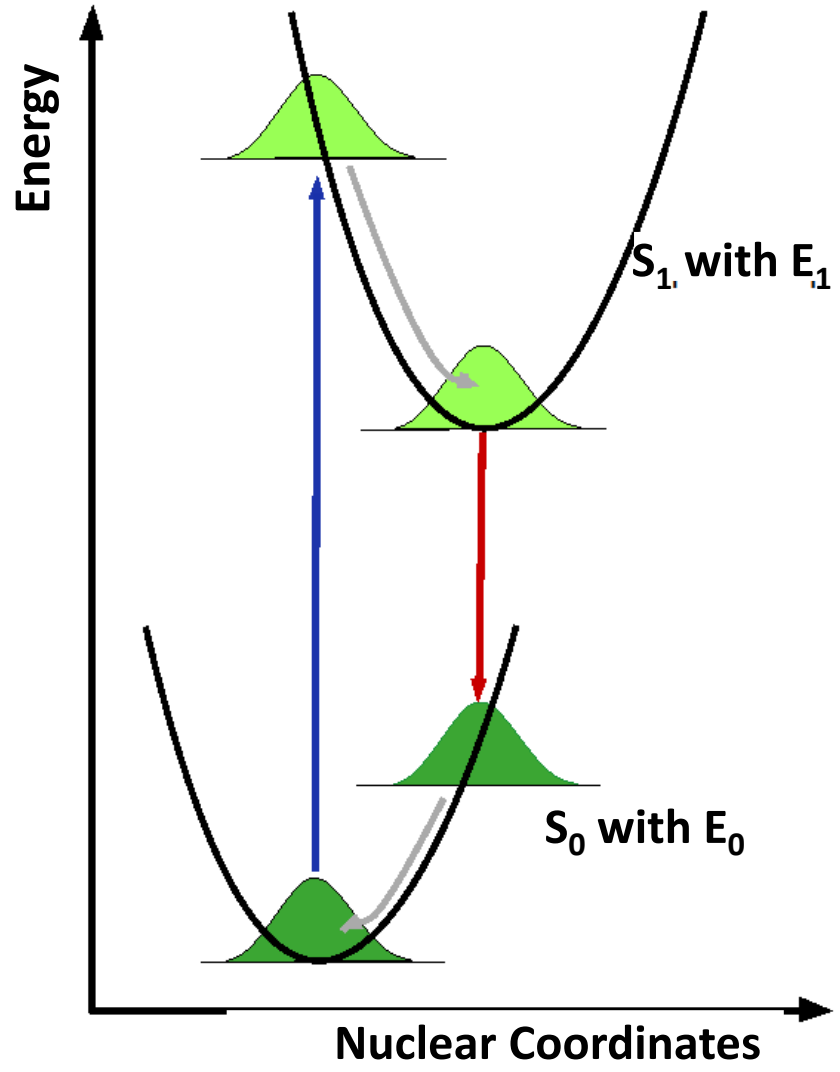
Reminder: Basics of Molecular Spectroscopy

- molecules have electronic, vibrational and rotational energy levels
- Franck Condon Principle = summary of all electronic transition due to absorption of a photon
- emission wavelength is red-shifted due to energy loss in the S_1 state and in the S_0 state

$$E = E_{S_1} - E_{S_0} = \frac{hc}{\lambda}$$

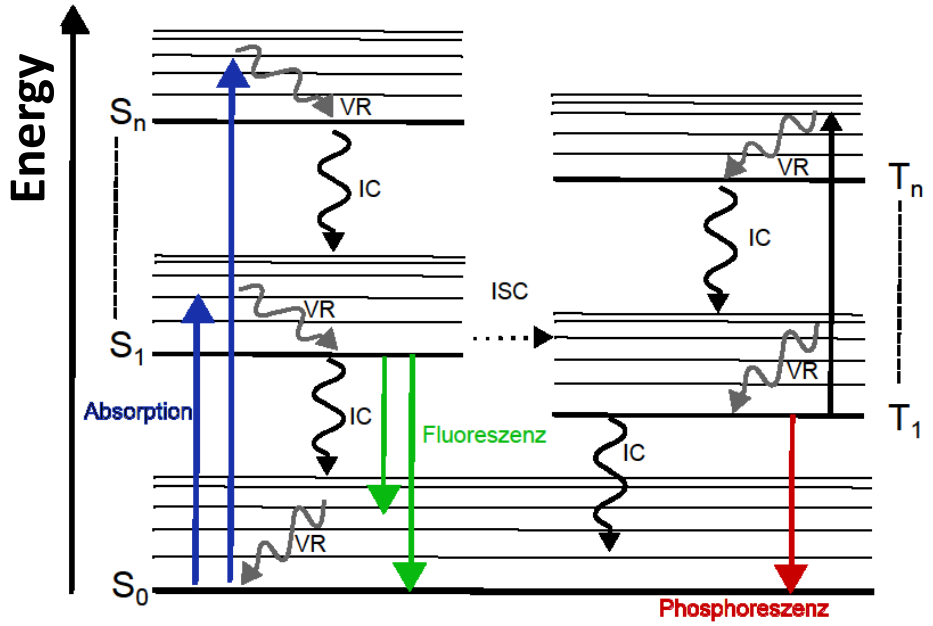


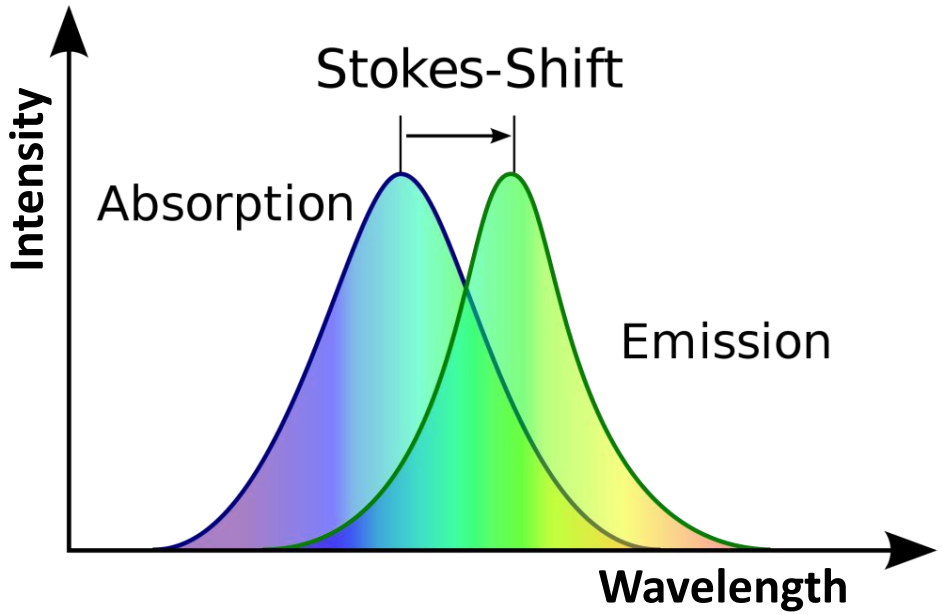
Jablonski Diagramm

- Illustration of the processes that occure between absorption and emission of light

- energy of the emission is typically less than that of absorption. Fluorescence has lower energies/longer wavelengths (Stokes-Shift).

	Name	Time [s]
$S_0 \rightarrow S_1 \dots S_n$	Absorption	10^{-15}
$S_n \rightarrow S_1$	Internal Convesion	$10^{-14} – 10^{-10}$
$S_1 \rightarrow S_1$	Vibrational Relaxation	$10^{-12} – 10^{-10}$
$S_1 \rightarrow S_0$	Fluorescence	$10^{-9} – 10^{-8}$
$S_1 \rightarrow S_1$	Intersystem Crossing	$10^{-9} – 10^{-8}$
$T_1 \rightarrow S_0$	Phosphorescence	$> 10^{-6}$





Fluorescence Techniques

- Advanced fluorescence techniques can be used to measure properties such as dynamics (FRAP, FLIP, FLAP, FSC) or molecular interactions such as (FRET and FCCS)

- most important characteristics of fluorophores:

$$Quantum\ Yield\ Q = \frac{emitted\ photons}{absorbed\ photons} = \frac{k_r}{k_r + k_{nr}}$$

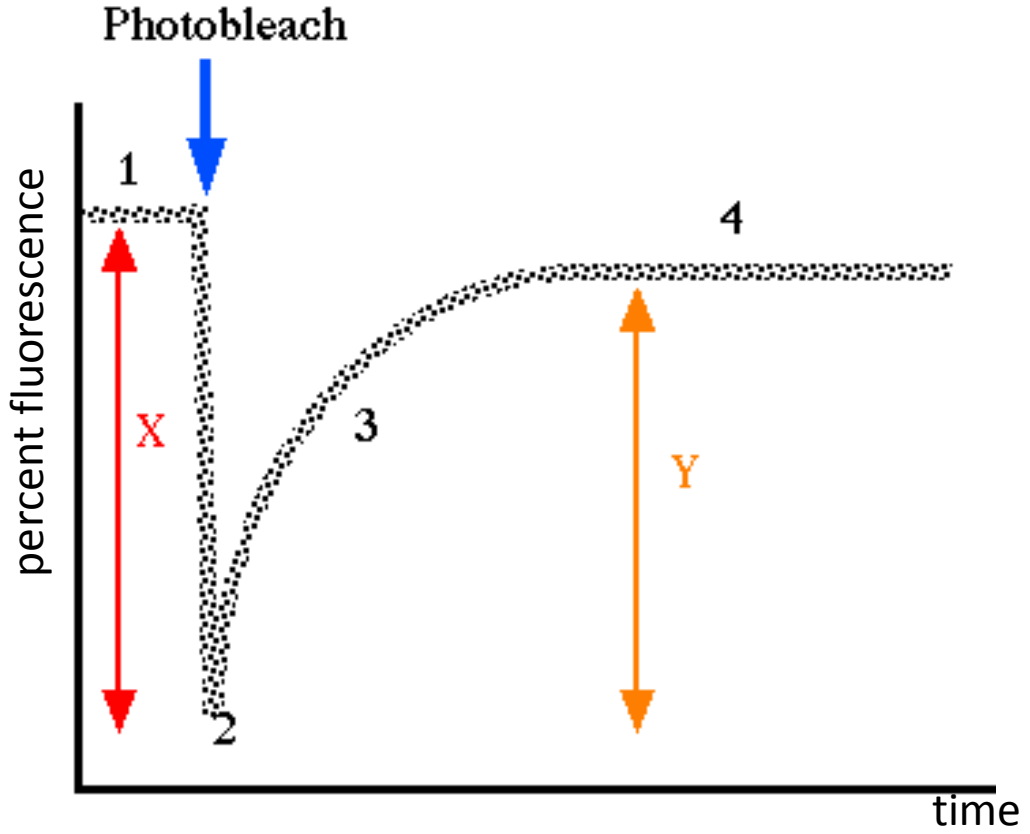
$$Fluorescence\ Lifetime = \frac{1}{k_r + k_{nr}}$$

Photobleaching Techniques

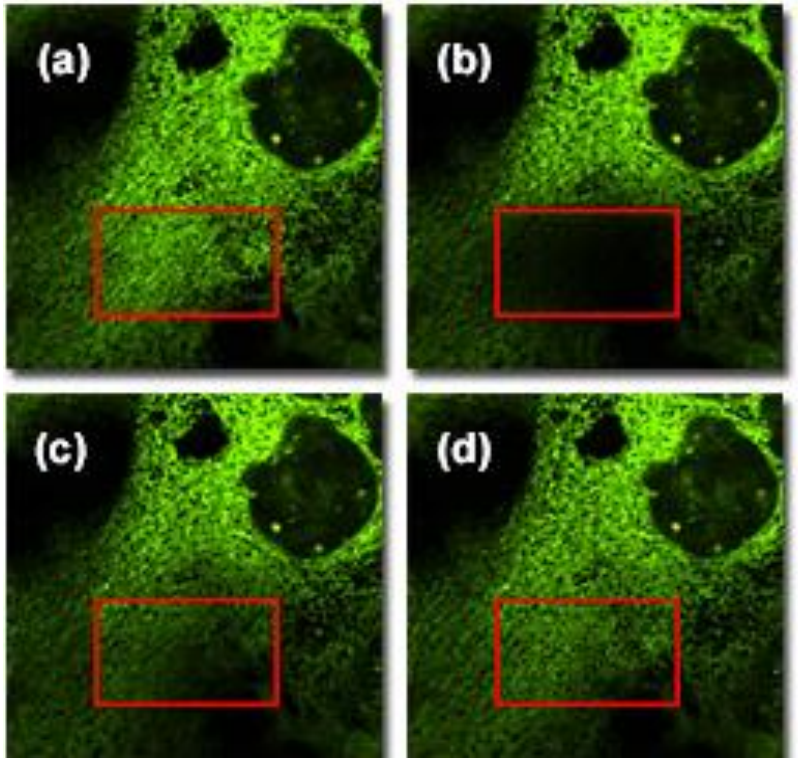
- Illumination with bright light will lead to photobleaching of a fluorophore. Once photobleached the fluorophore is no longer fluorescent.
- Two related techniques of Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Loss in Photobleaching (FLIP) use photobleaching to measure molecular dynamics.
- Laser Scanning Confocal microscopes are good for these photobleaching techniques due to their high intensity laser light sources and their flexible illumination control. They allow selective bleaching of arbitrary regions within the filed of view and can then rapidly switch between bleaching and imaging.
- However, reduced sensitivity and slow speed make a widefield microscope better if it has the ability to photobleach.

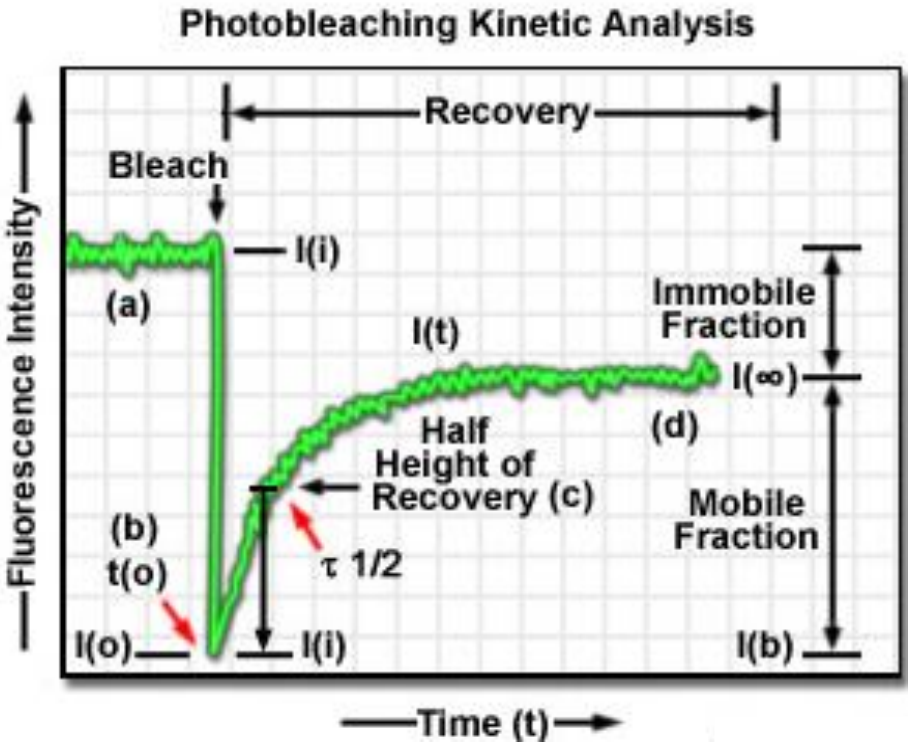
FRAP – Fluorescence Recovery after Photobleaching

- optical technique to quantify 2D lateral diffusion of fluorophores
- a region is rapidly bleached by an intense focused laser beam and the rate of fluorescence recovery is
- recovery occurs by replacement of intact fluorophores in the bleached region by lateral transport from the surrounding surface



FRAP in cells with GFP

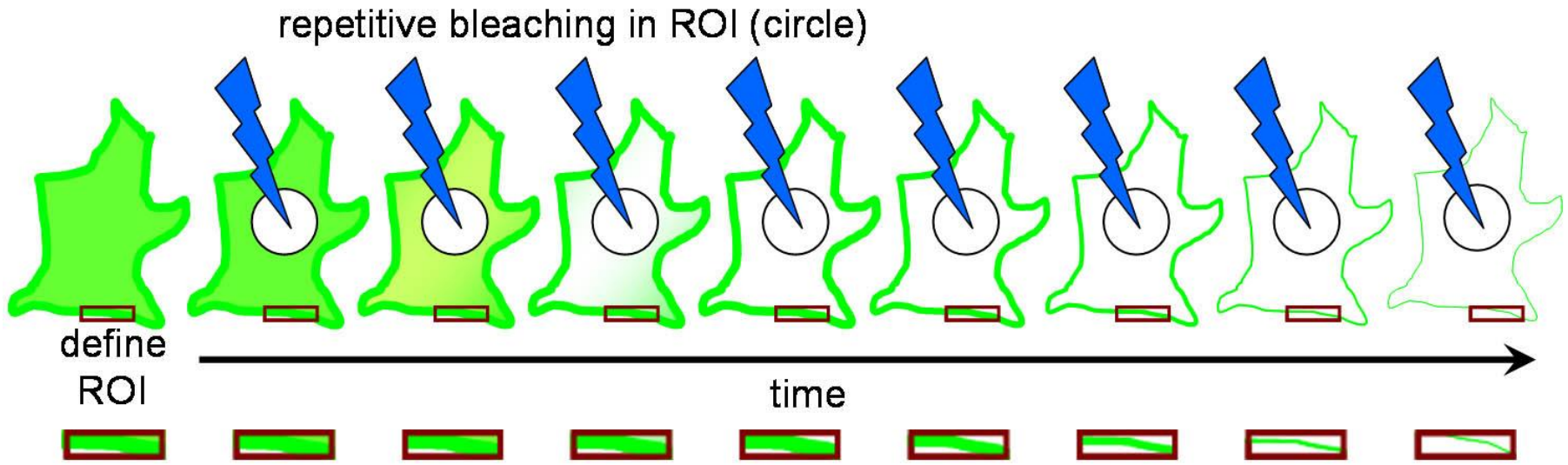




- Small objects – fast diffusion
- Large objects – slow diffusion

FLIP – Fluorescence Loss in Photobleaching

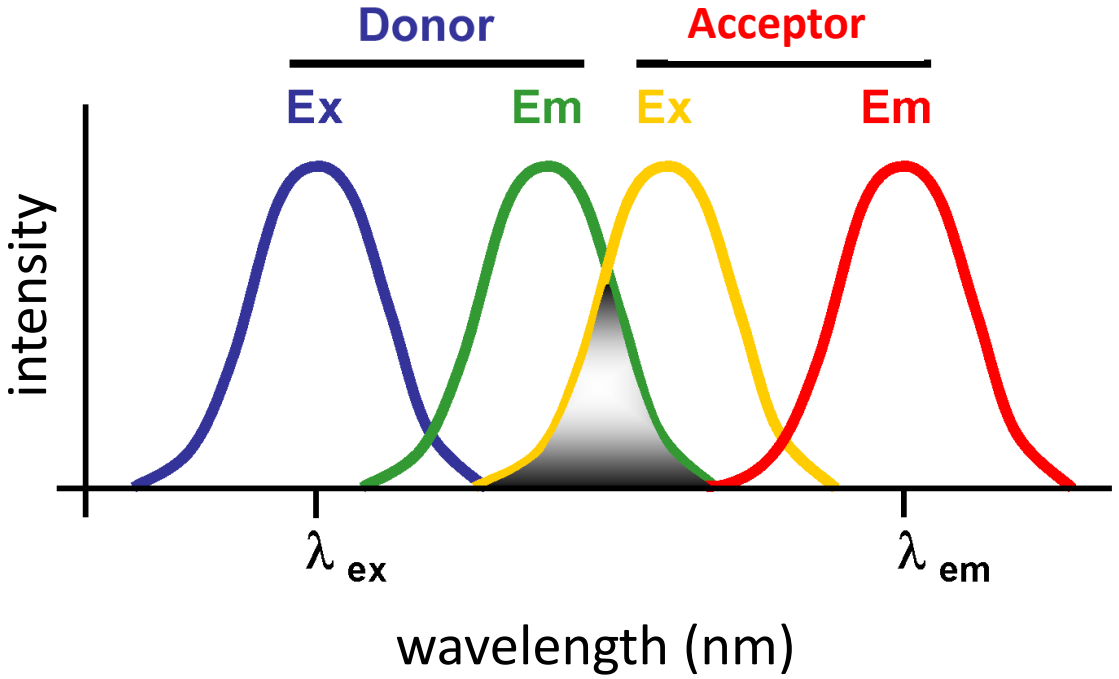
- Related to FRAP, a small region is repeatedly bleached and the loss of fluorescence in another region is measured.
- Useful to show connectivity of compartments or for measuring turnover between compartments.



FRET – Förster Resonance Energy Transfer

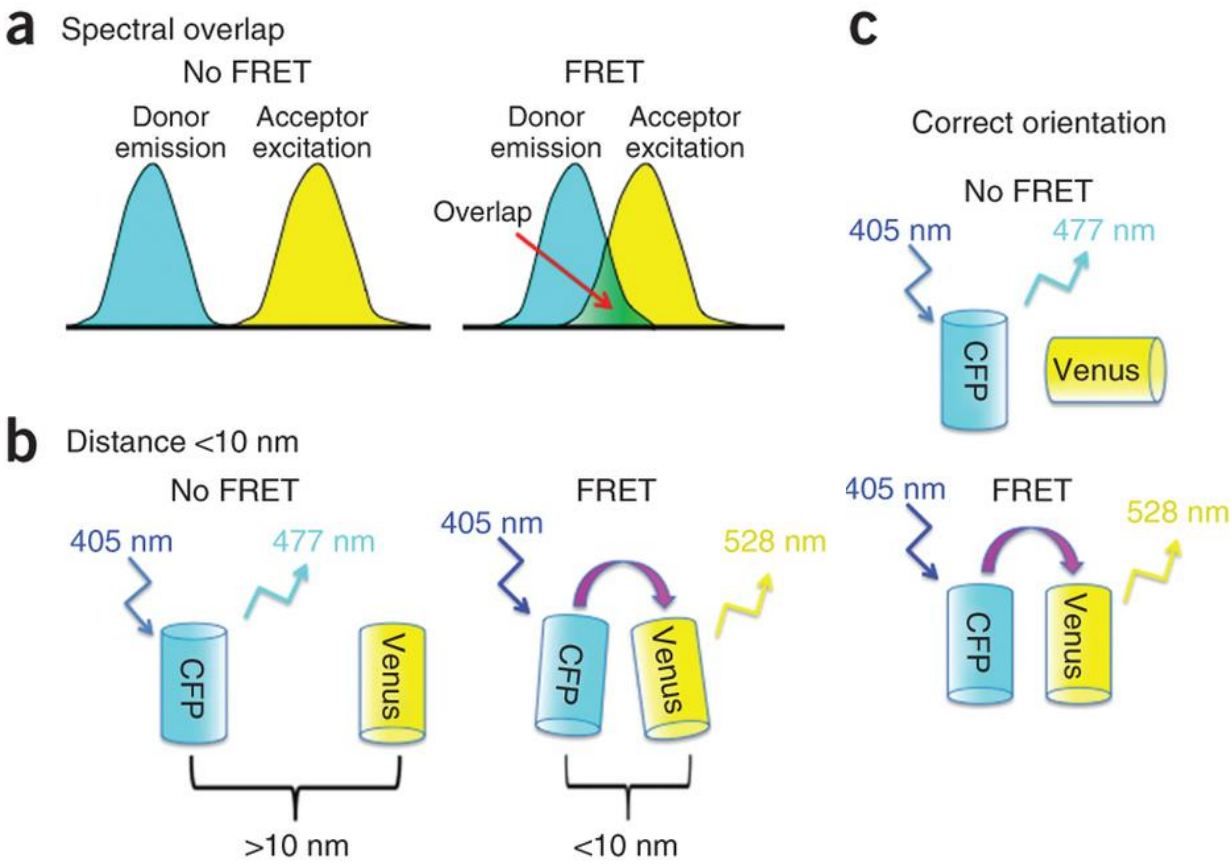
- FRET is an electrodynamic phenomenon between a donor (D) molecule in the excited state and an acceptor (A) molecule in the ground state.

- The donor molecules typically emit at shorter wavelengths that overlap with the absorption spectrum of the acceptor.
- occurs without the appearance of a photon and is the result of longrange dipole–dipole interactions between the donor and acceptor.



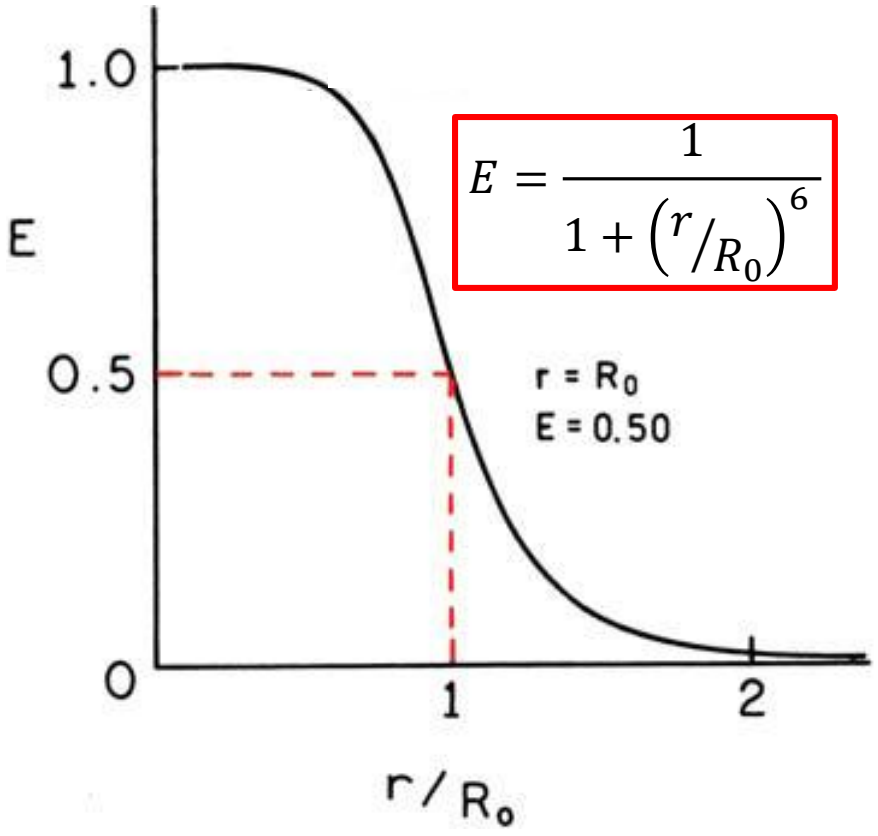
FRET – Förster Resonance Energy Transfer

- depends upon the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor
- the quantum yield of the donor
- the relative orientation of the donor and acceptor transition dipoles
- the distance between the donor and acceptor molecules.



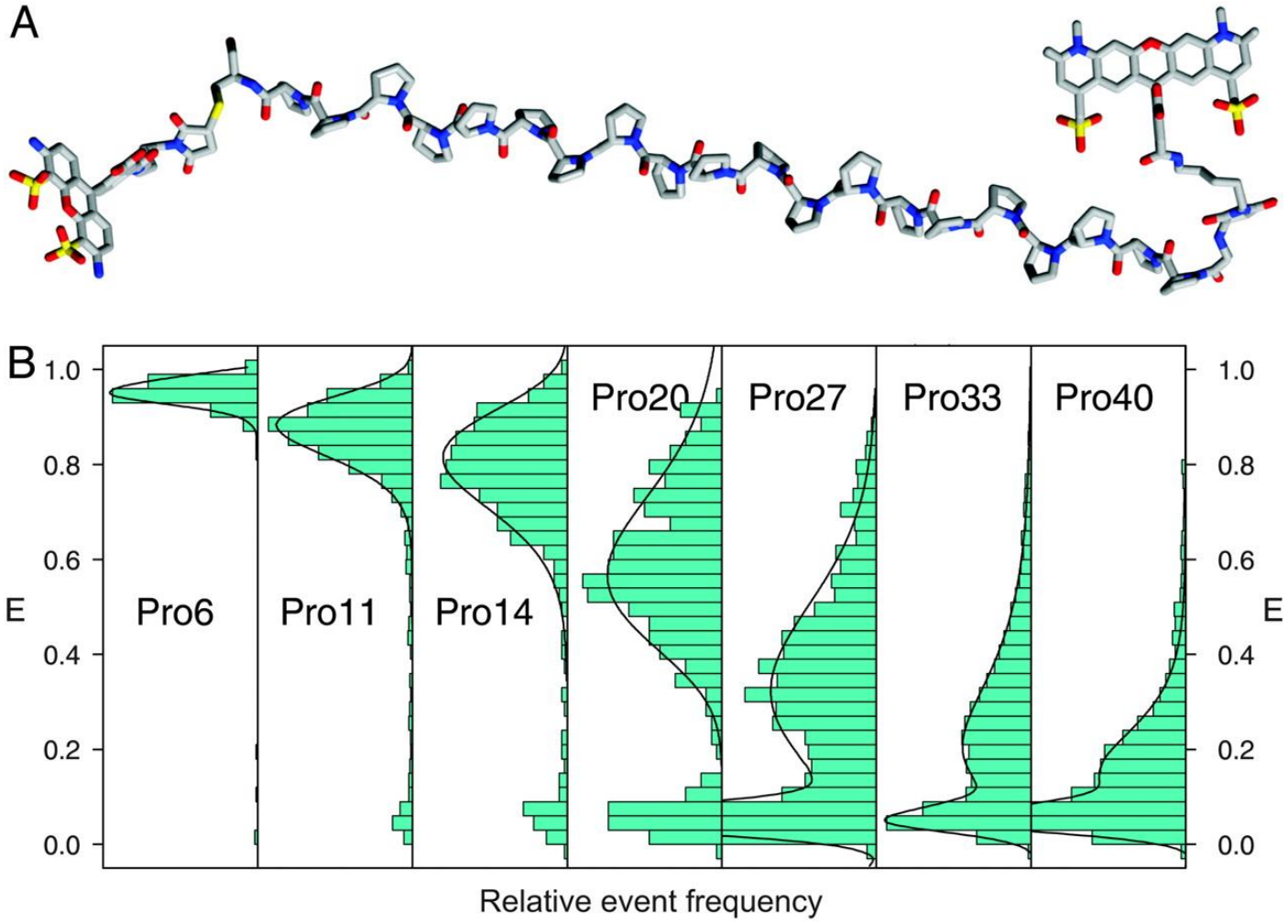
NatProt, 8, 265-281, 2013

FRET - a Molecular Ruler



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

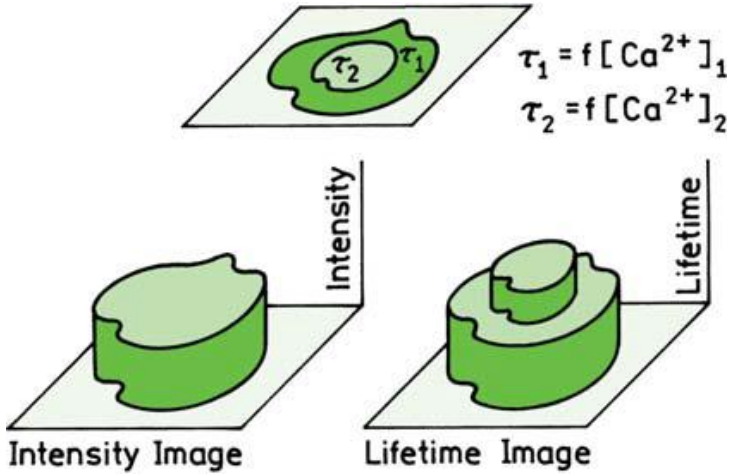
R_0 = 0.5 – 10 nm

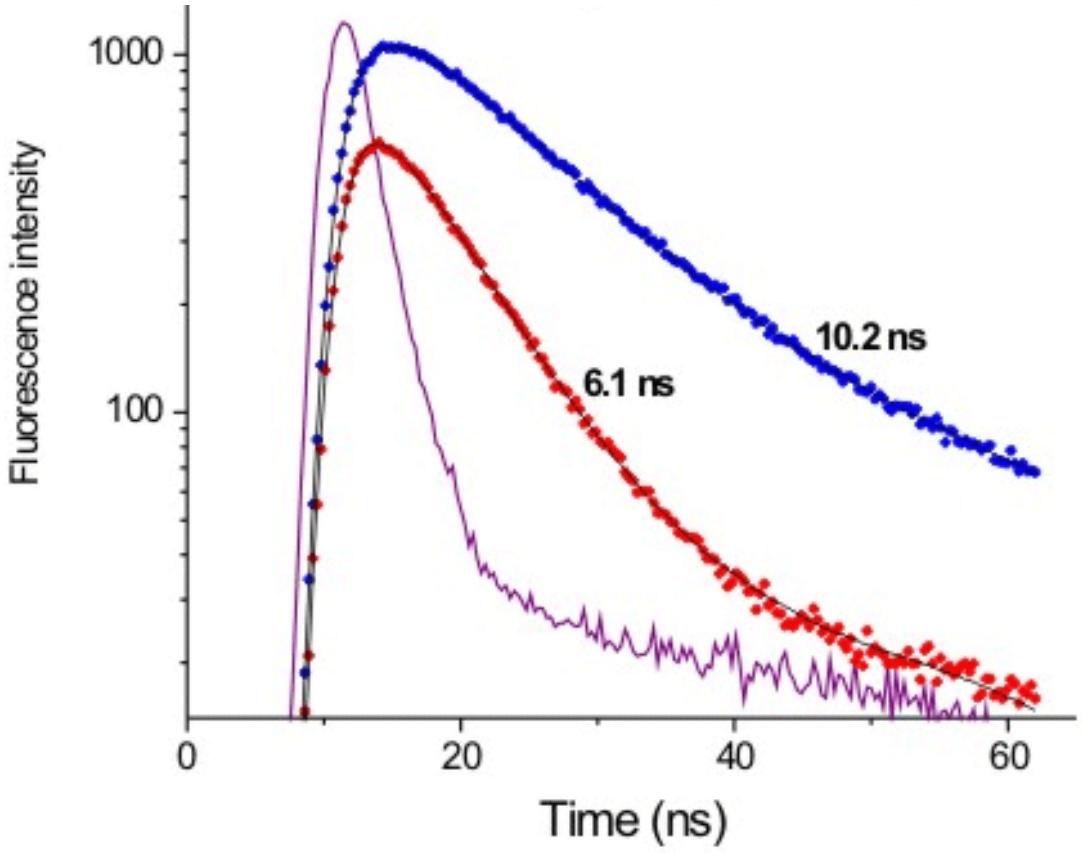


PNA,102,2754-2759, 2005

FLIM – Fluorescence Lifetime Imaging

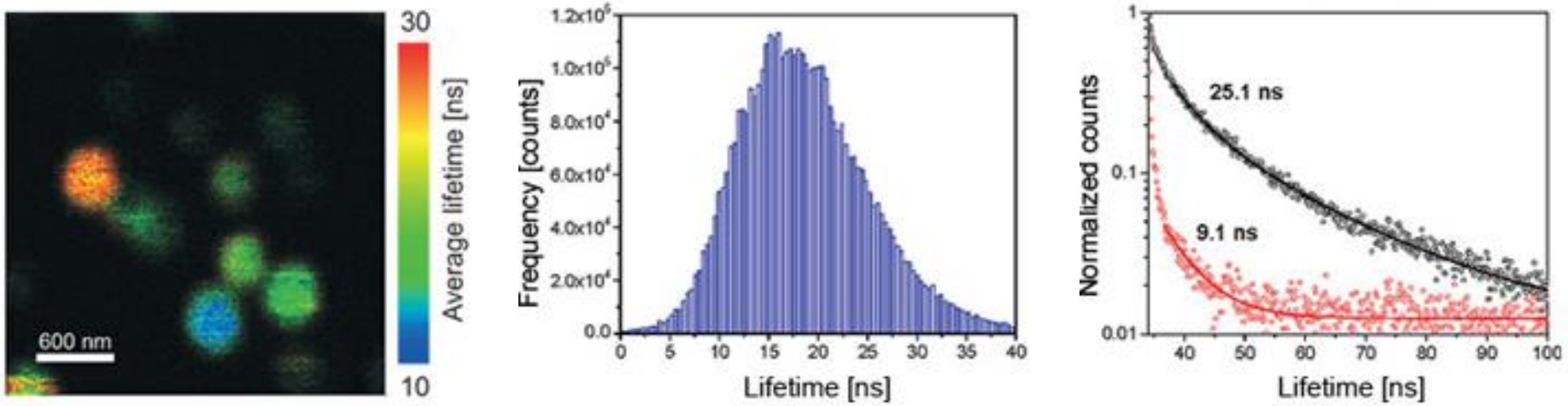
- **…**is an imaging technique for producing an image based on the differences in the exponential decay rate of flourophores.
- The lifetime of the fluorophore signal, rather than its intensity, is used to create the image in FLIM. This has the advantage of minimizing the effect of photon scattering in thick layers of sample.





FLIM Measurements

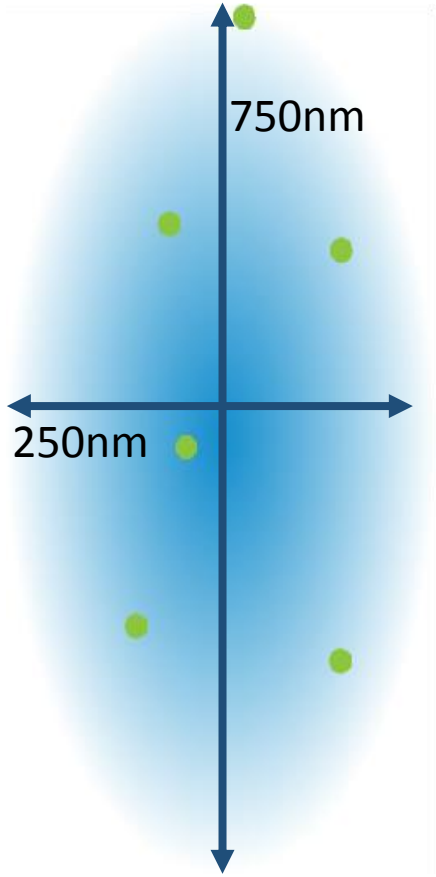
- essential components of a FLIM set-up: pulsed laser source, detector (APD or PMT), dichroic mirror (to separate fluorescence and excitation), objective (to focus the excitation light into the sample and collect fluorescence signal) and a TCSPC unit to measure the time between excitation and fluorescence emission
- the delay times are sorted into a histogram that plots the occurrence of emission over time after the excitation pulse
- line and frame marker signals from the scanner (confocal microscope) are additionally recorded in order to sort the time stream of photons into the different pixels.



FCS – Fluorescence Correlation Spectroscopy

- FCS is used to get time-resolved information about an ensemble with single molecule sensitivity in a small confocal volume

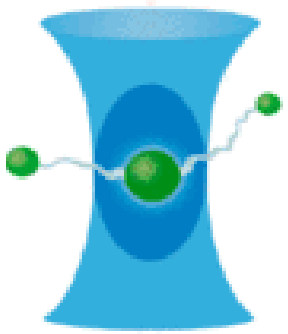
- Widely used technique to obtain quantitative information such as
  - diffusion coefficients
  - hydrodynamic radii
  - average concentrations
  - kinetic chemical reaction rates
  - singlet-triplet dynamics

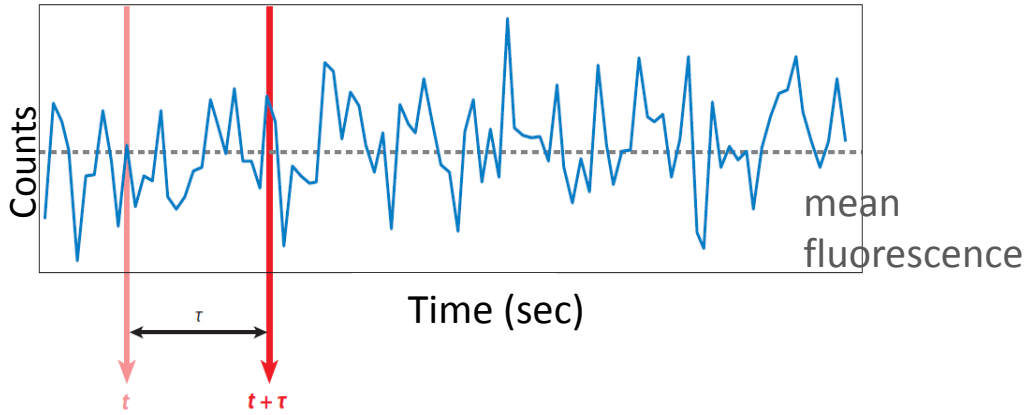


Volume ≈ 10^{-15}l

FCS – Autocorrelation of Fluorecensce Fluctuations

confocal Volume

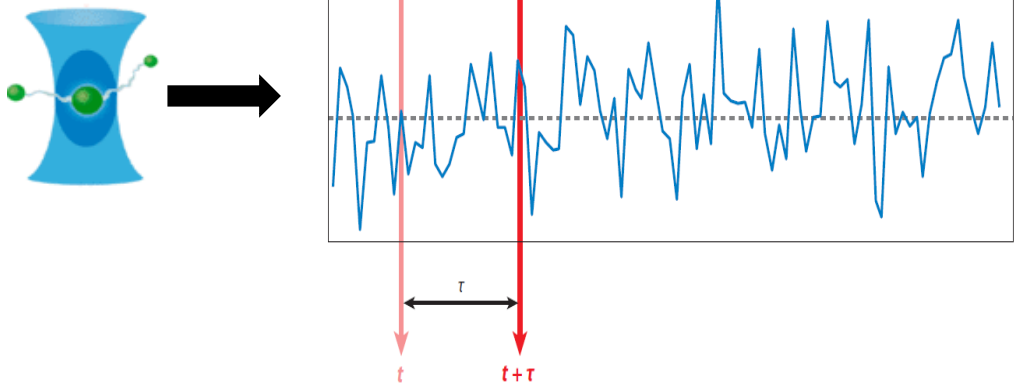


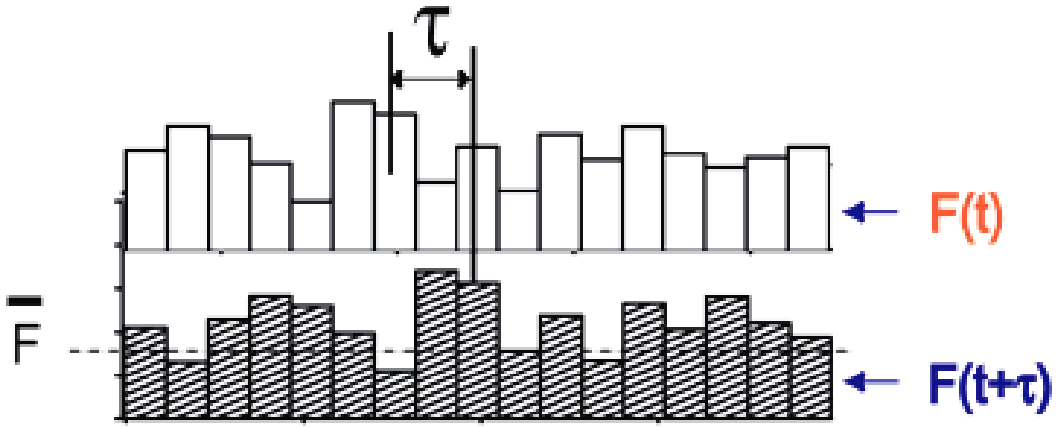


What causes fluctuations in intensity? a) Poison noise, b) diffusion of molecules

Bursts of photons are seen as single fluorophores diffuse in and out of the laser beam

FCS – Autocorrelation of Fluorecensce Fluctuations



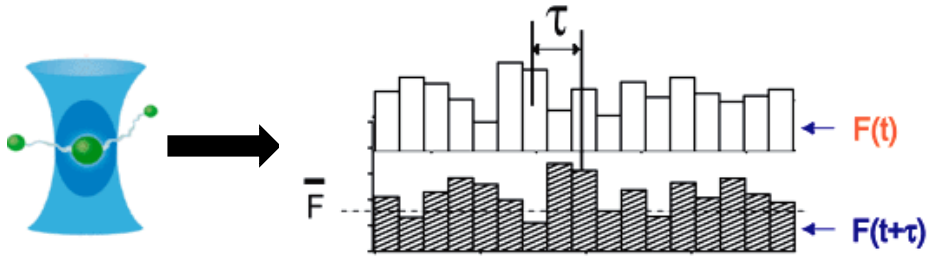


autocorrelation of fluorescence alterations

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle \delta F(t) \rangle^2}$$

$$\delta F(t) = F(t) - \langle F(t) \rangle$$

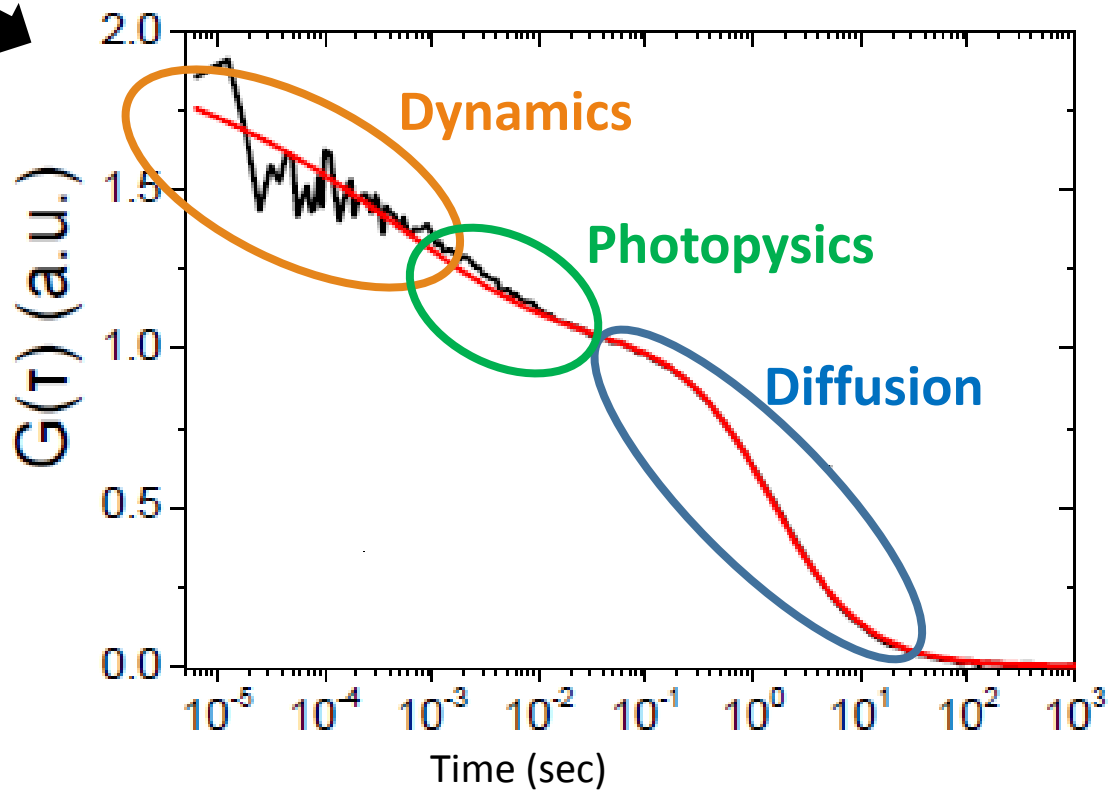
FCS – Autocorrelation of Fluorecensce Fluctuations



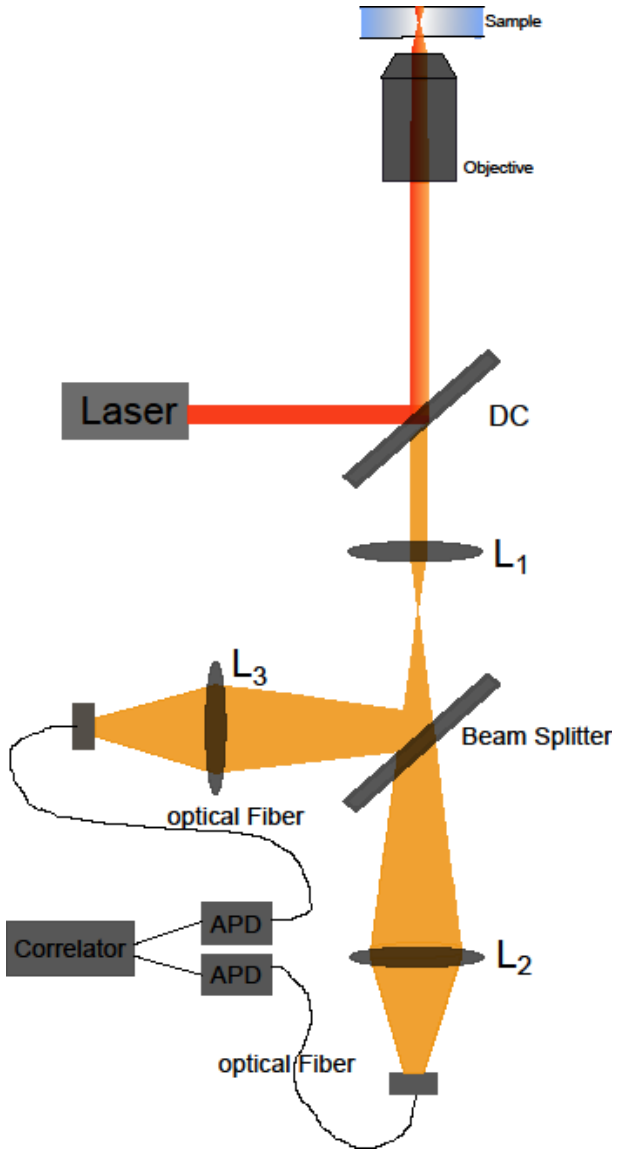
$$G(\tau) = \frac{1}{N}(1+\frac{\tau}{\tau_D})^{-1}(1+A\exp(-k\tau)^{\beta})$$

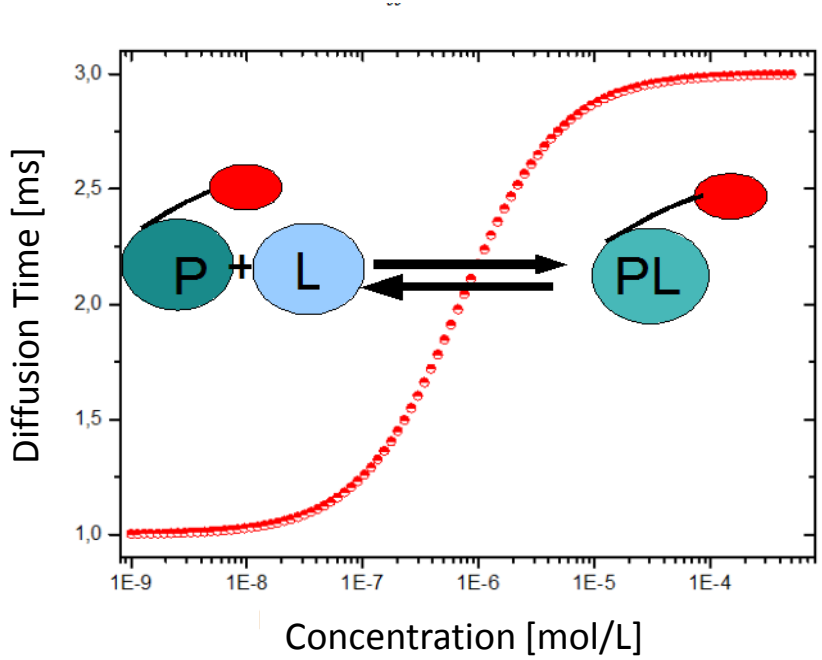
2D Gaussian Model

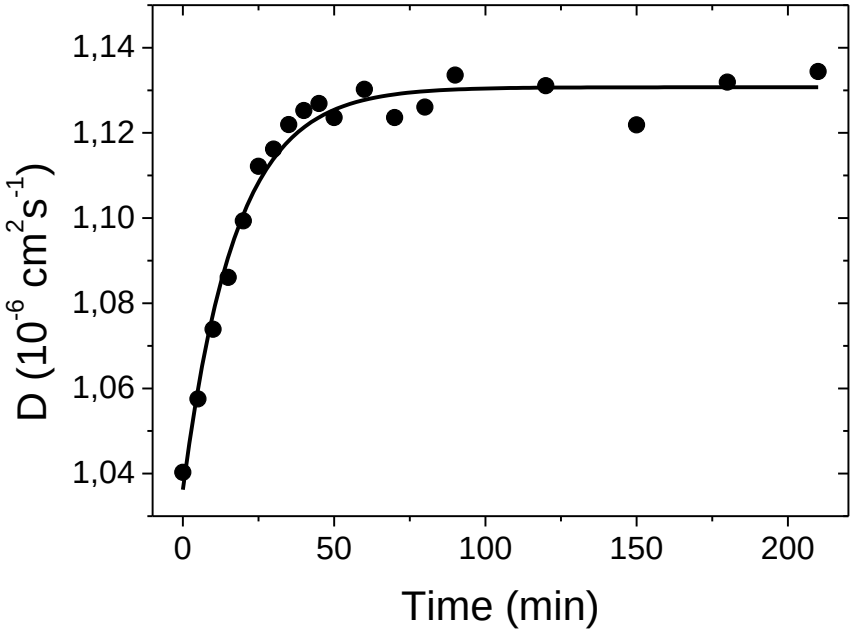
- What Does FCS tell us?
  - Diffusion rates - D ∝ $m^{1/3}$.
  - the number of fluorescent objects in volume and hence concentration.
  - Relaxation rates and folding times



FCS – Binding Kinetics







FCS – Conformational Dynamics

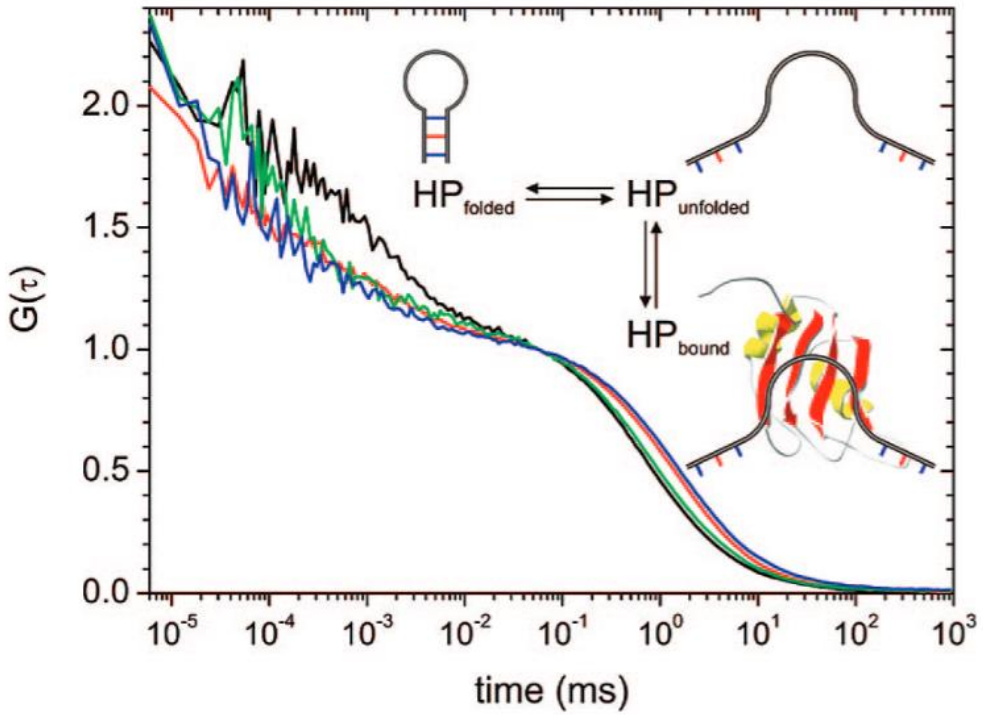
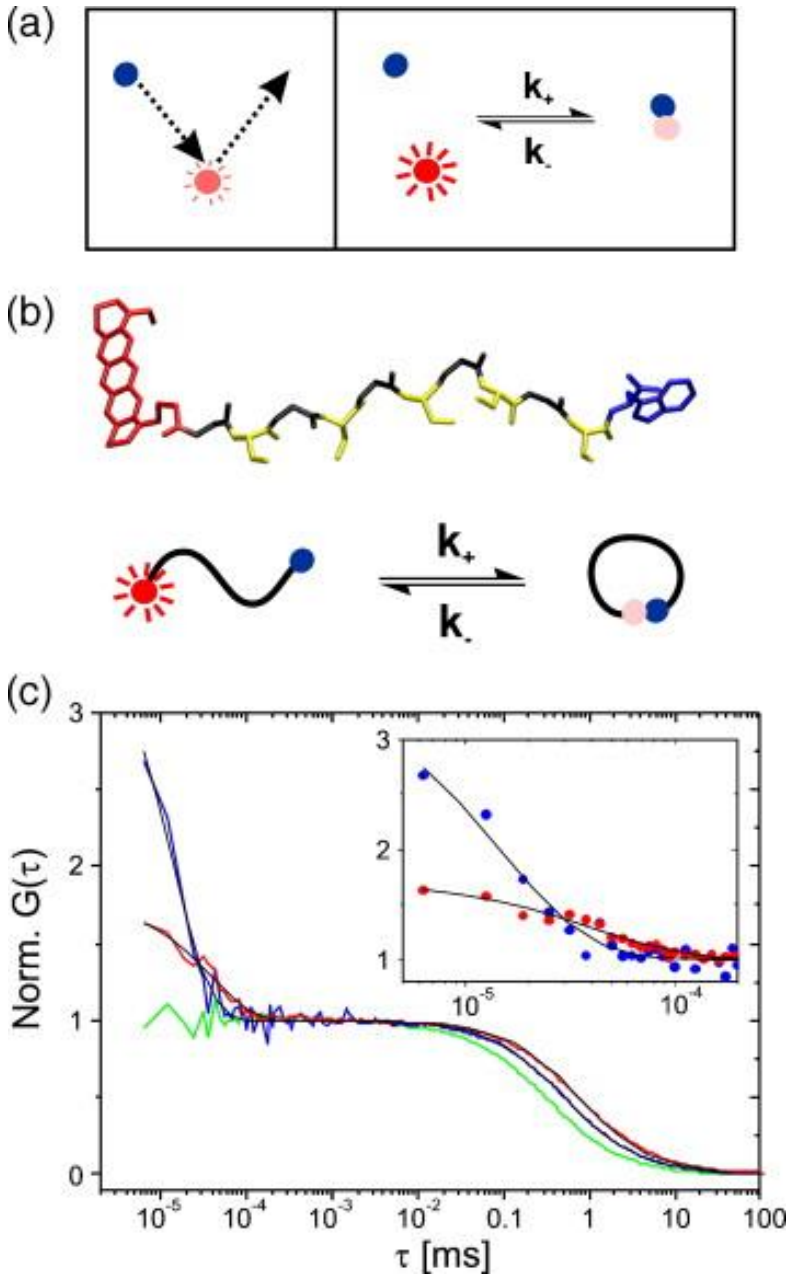


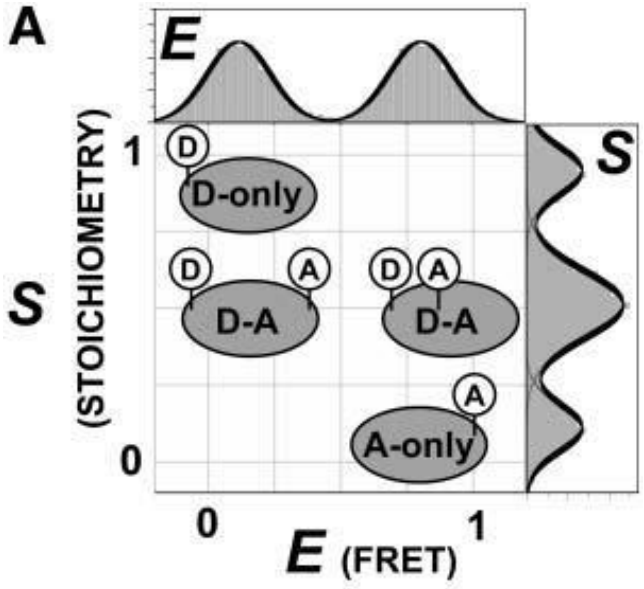
Figure 3. Normalized FCS curves measured for 10^{-9} M solutions of the RNA hairpin HP and the native ORN NT in the absence and presence of *At*GRP7 protein. The fluorescence correlation functions of free HP and NT are shown in black and green, respectively. The curve for HP bound to *At*GRP7 is shown in red and that for NT bound to *At*GRP7 in blue. The proposed model of a two-state hairpin-folding equilibrium whose unfolded state can be bound by the protein[33] is illustrated in the inset.

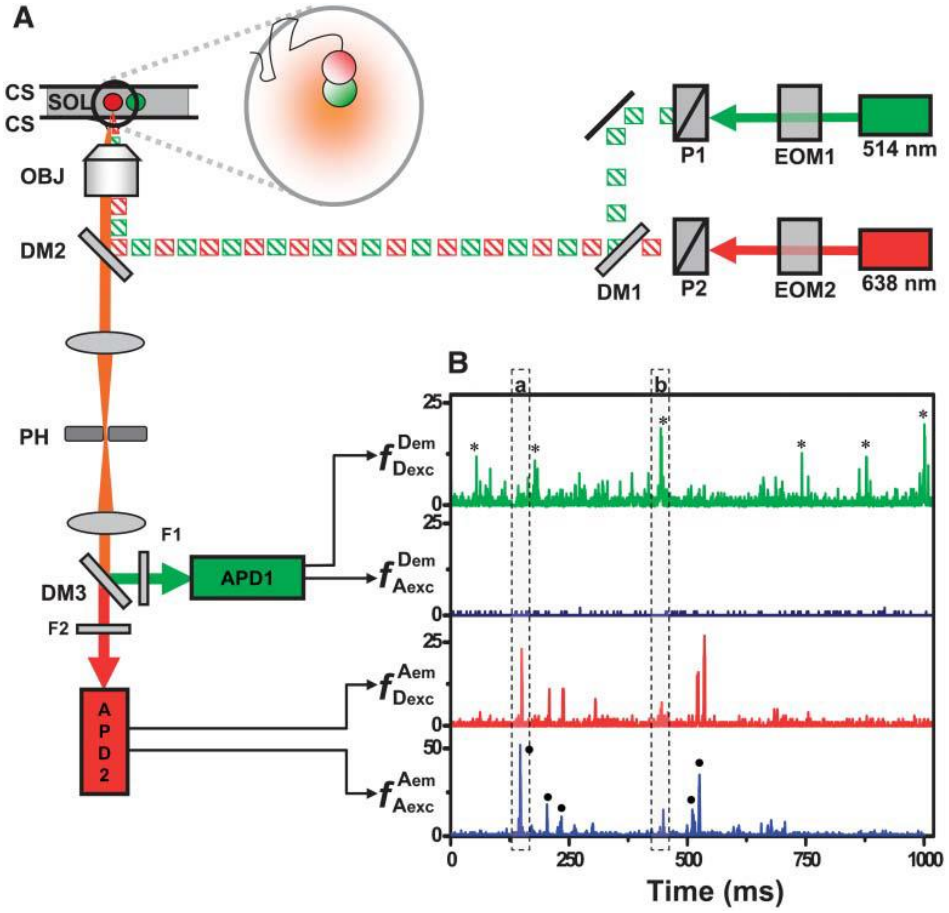


J. Am. Chem. Soc., 2008, 130 (29), pp 9507–9513

ALEX – FCS

- FCS with Alternating Laser Excitation





Summary

- There are a range of fluorescence techniques to probe dynamics and molecular interactions.
- They have different strengths and weaknesses.
- All of them require careful experiment design and data analysis.

Literature

- best book ever about fluorescence: J. Lakowicz, Principles of Fluorescence Spectroscopy
- also the webpages of Olympus, Zeiss and Nikon have really good introductions to the different techniques
- great overview: Molecules 2012, 17(4), 4047-4132

- FRET paper:
  - Nature Protocols, 8, 265-281, 2013
  - PNA,102,2754-2759, 2005
  - J Cell Biol, 3, 2003; 160(5): 629–633
  - FRET and FLIM Techniques, edited by Theodorus W. J. Gadella
- FRAP
  - Biophys J., 1976; 16(9): 1055–1069.
  - Biophys J., 2004; 86(6): 3473–3495
  - J Biomed Opt. Jan 2012; 17(1): 011008
- FCS:
  - J. Am. Chem. Soc., 2008, 130 (29), pp 9507–9513
  - P. Schwille, E. Haustein, FCS- An introduction to its concepts and applications
  - Nature Methods, 4, 963 - 973 (2007)

The End!