Rice/TCU REU on Computational Neuroscience

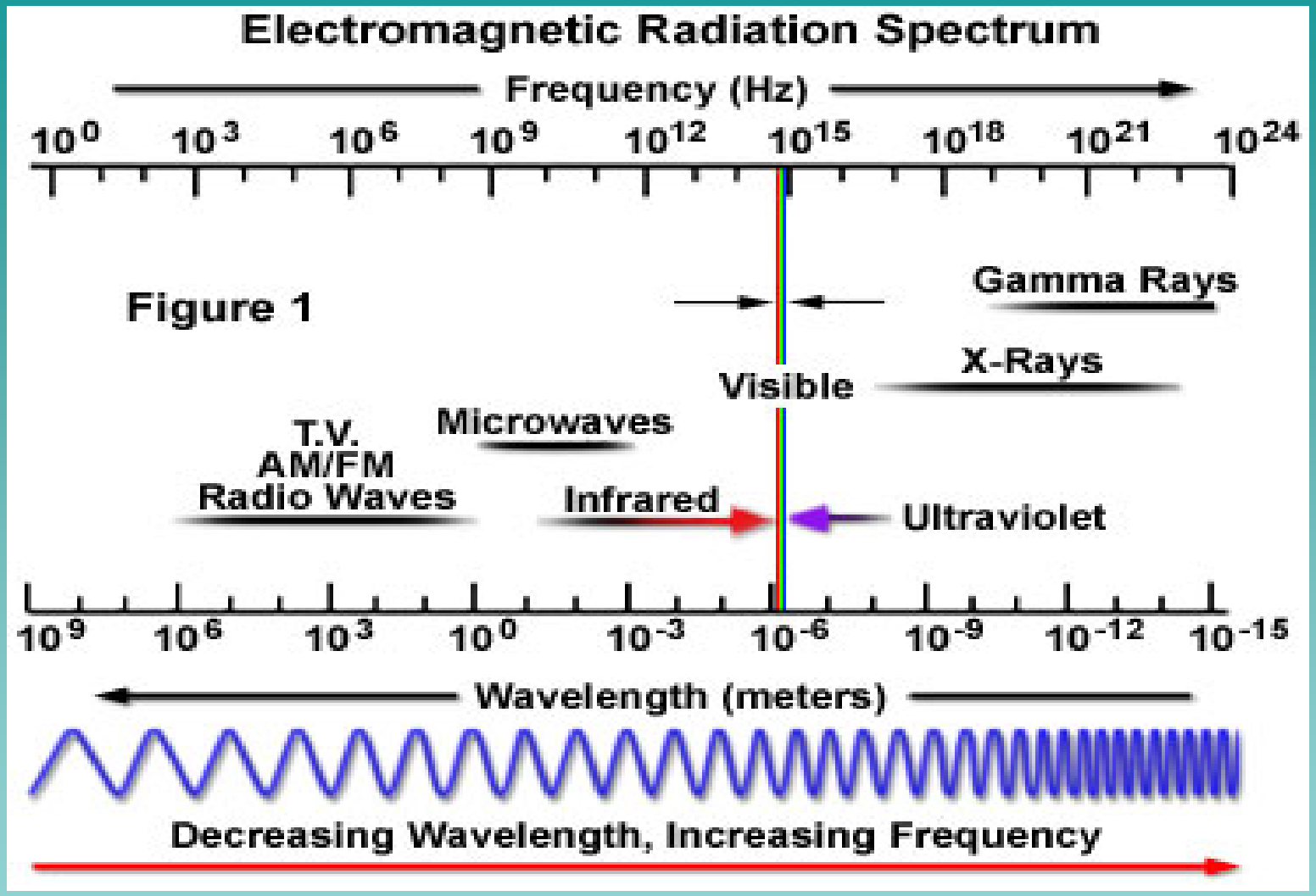
Fundamentals of Molecular Imaging

June 3, 2008

Neal Waxham
713-500-5621
m.n.waxham@uth.tmc.edu

Objectives

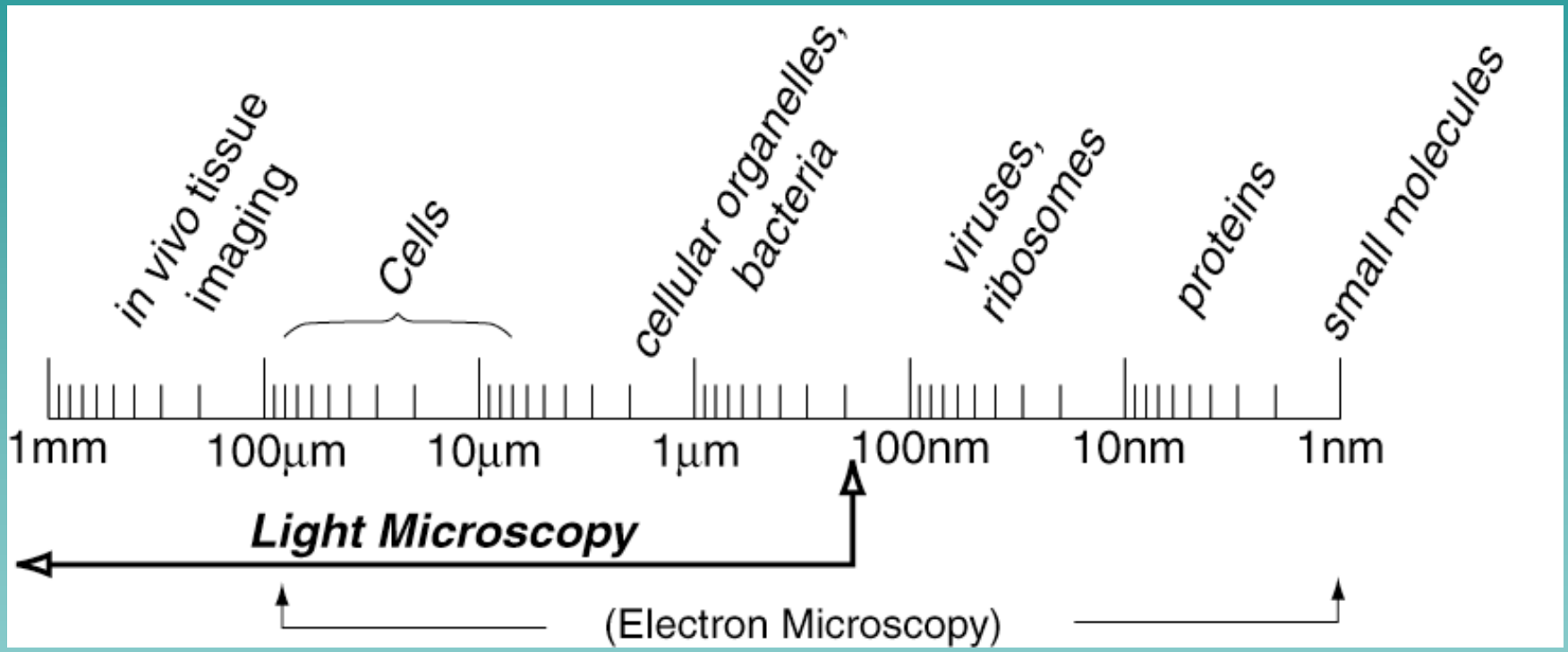
- Brief discussion of optical resolution and lasers as excitation sources
- Multiphoton excitation-advantages/disadvantages
- Molecules inside cells
- Applications of MPE to the study of intracellular diffusion and biochemistry
- Photobleaching Recovery
- The concept of single molecule analysis
- Fluorescence Correlation Spectroscopy
- Fluorescence Cross-Correlation Spectroscopy



Resolution = 0.61λ / NA

λ = wavelength of electromagnetic radiation

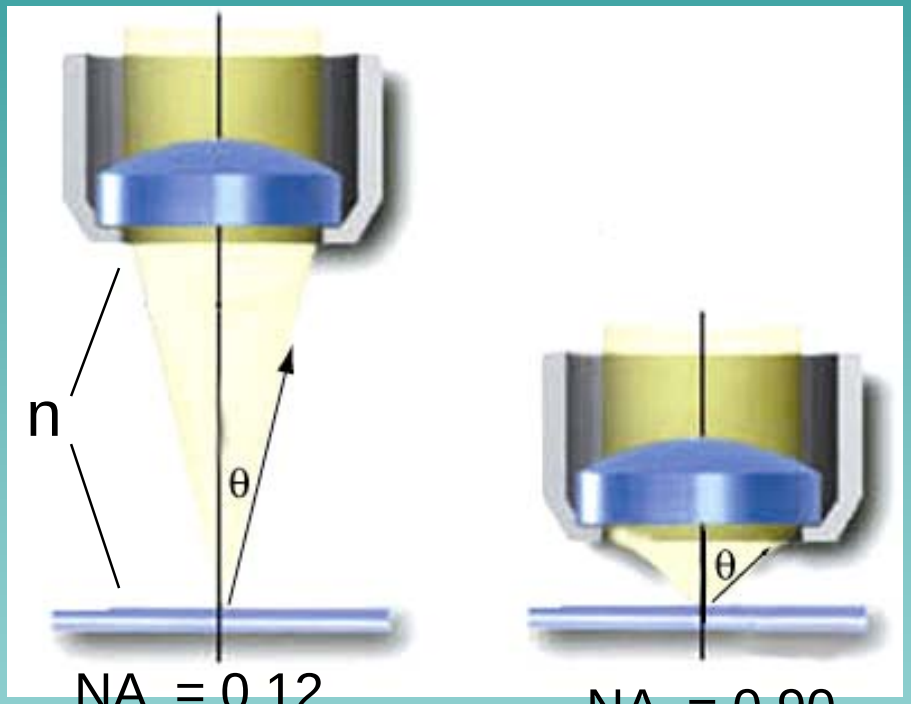
Spatial and Temporal Scales of Microscopy



Temporal scales - picoseconds to months

What Limits Resolution in Microscopy?
Numerical Aperture (NA)

NA = n sin(θ), where n is the index of refraction and θ the half angle of the illumination cone.



NA = 0.12

θ ~ 7°

Long working distances

NA = 0.90

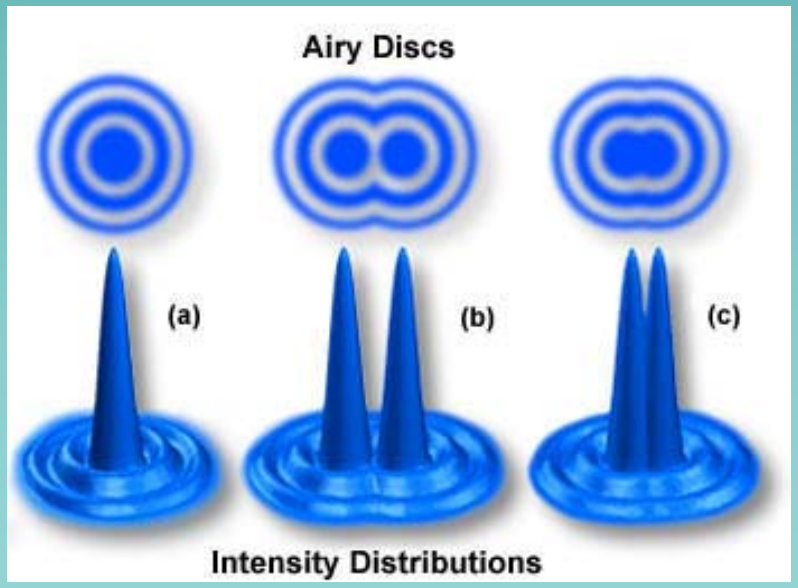
θ ~ 64°

Short working distances

Rayleigh criterion:

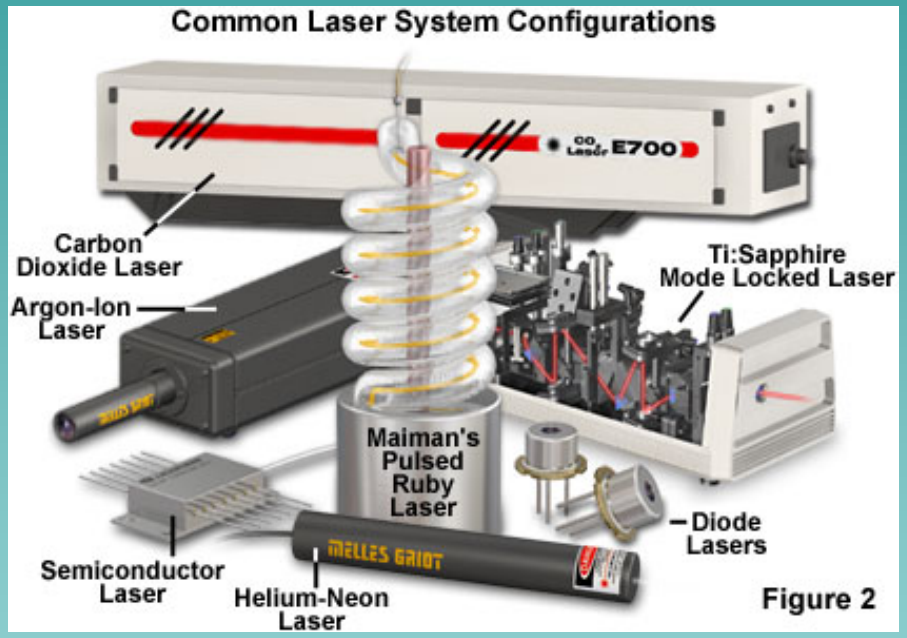
Resolution = 0.61λ / NA

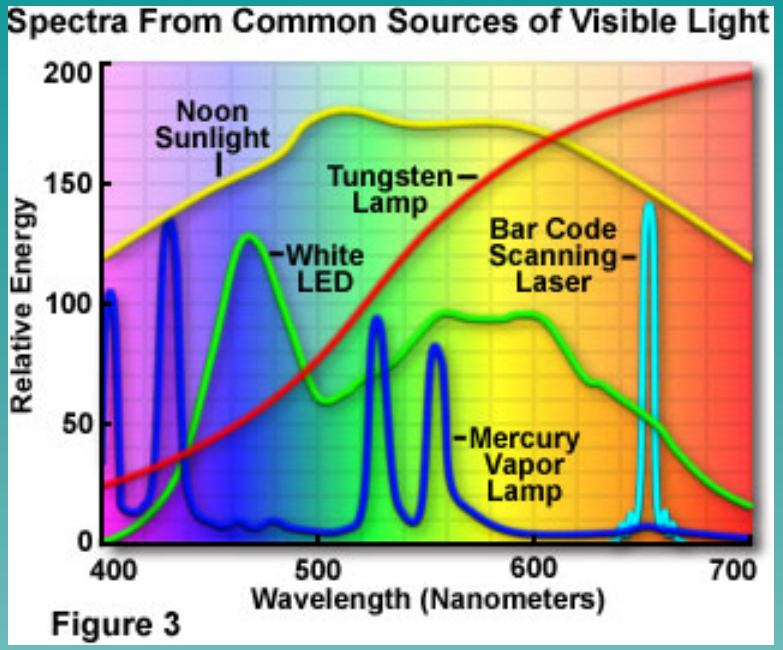
$R = 1.22\lambda / (NA_{obj} + NA_{cond})$

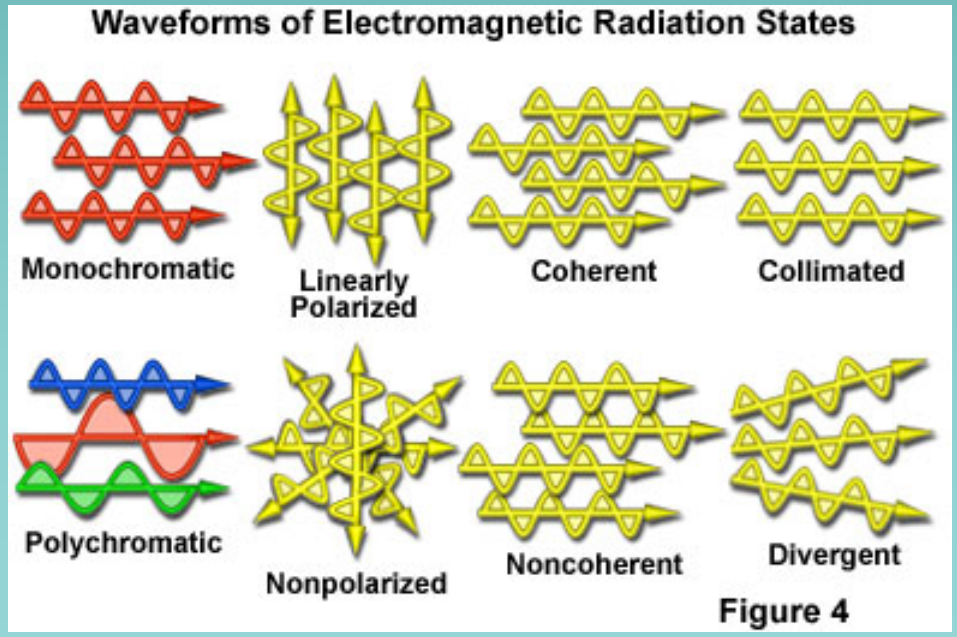


Destructive Interference

Characteristics of Light from Lasers

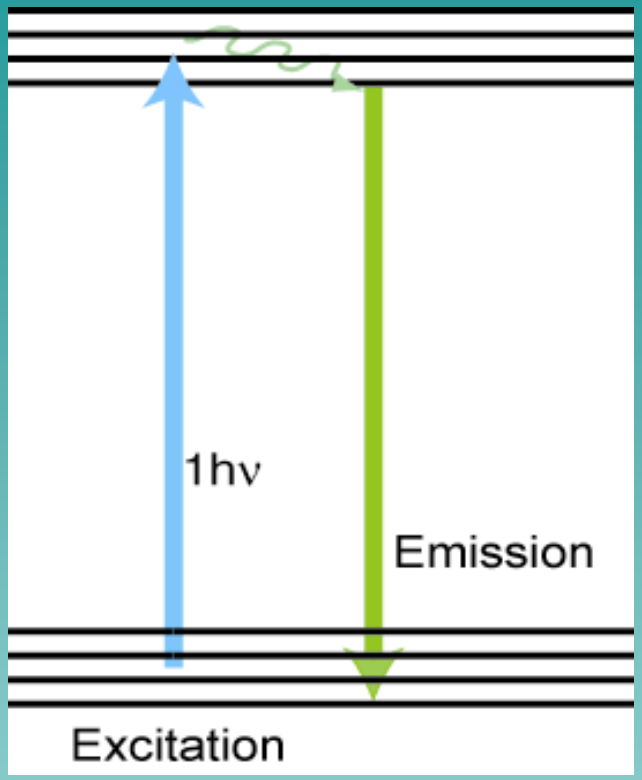


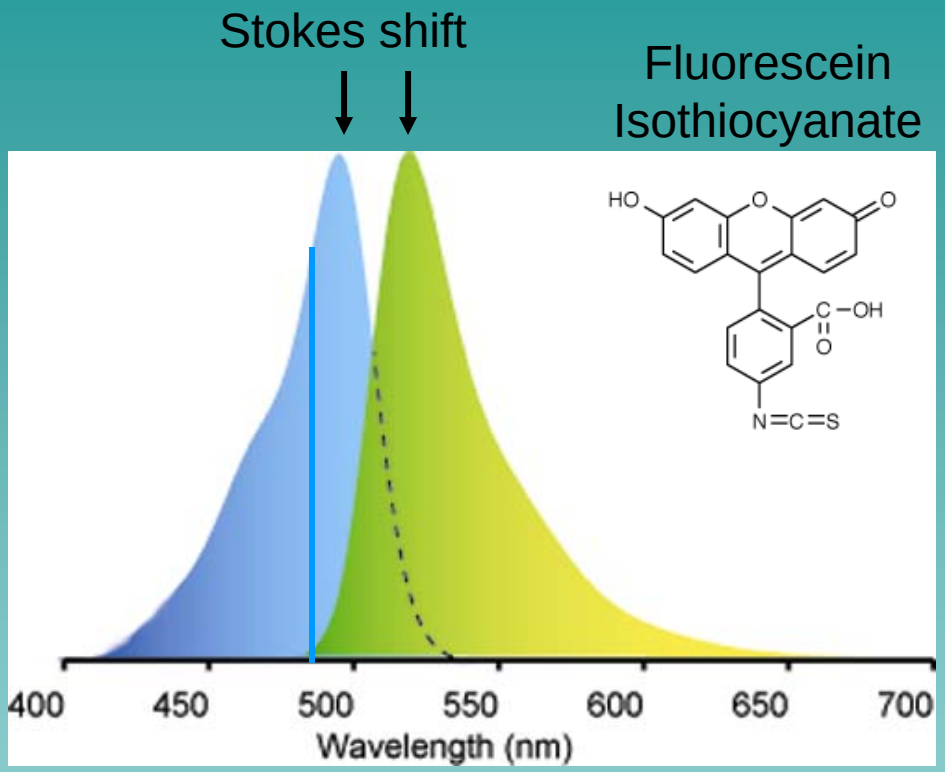




Light **A**mplification by **S**timulated **E**mission of **R**adiation

Fluorescence



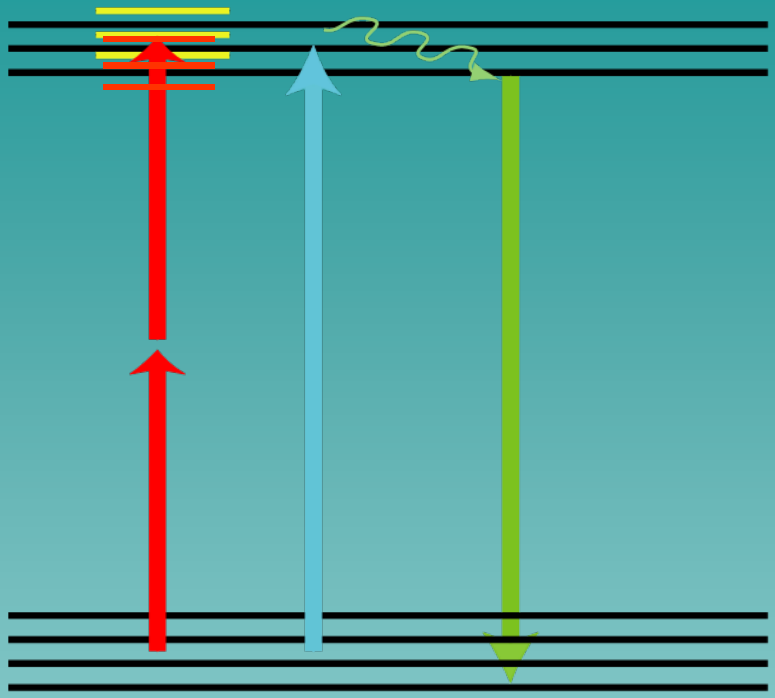


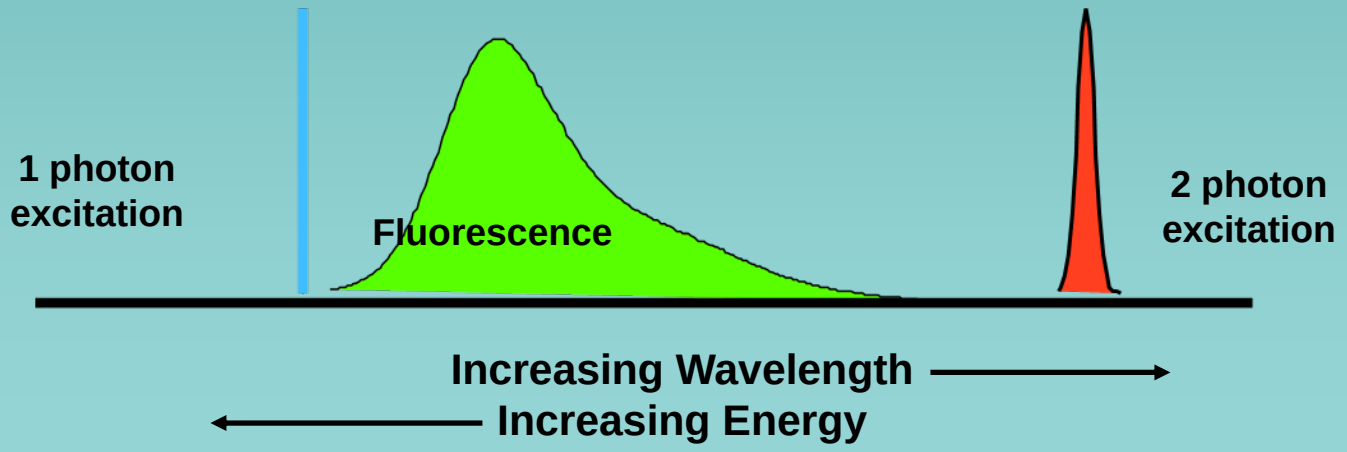
Following absorption, a number of vibrational levels of the excited state are populated. Molecules in these higher vibrational levels then relax to the lowest vibrational level of the excited state (vibrational relaxation). From the lowest vibrational level, there can be an emission of photon of lower energy (fluorescence)

What is Two Photon Excited Florescence?

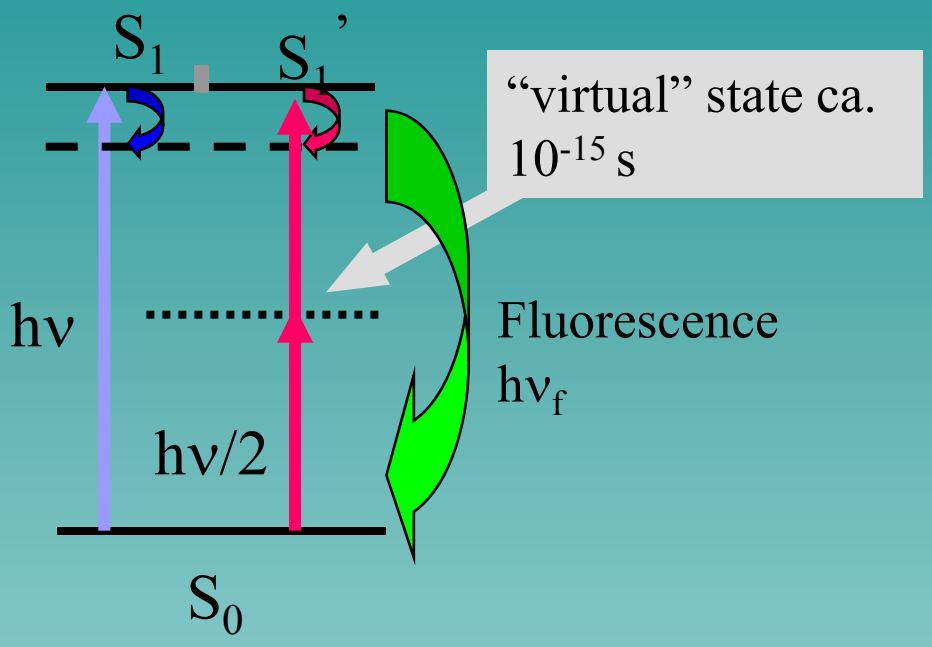
Two (or more) photons can interact simultaneously with a molecule adding their energies to produce an excitation equal to the sum of their individual energies.

i.e. 2 red photons <u>can</u> = 1 blue photon





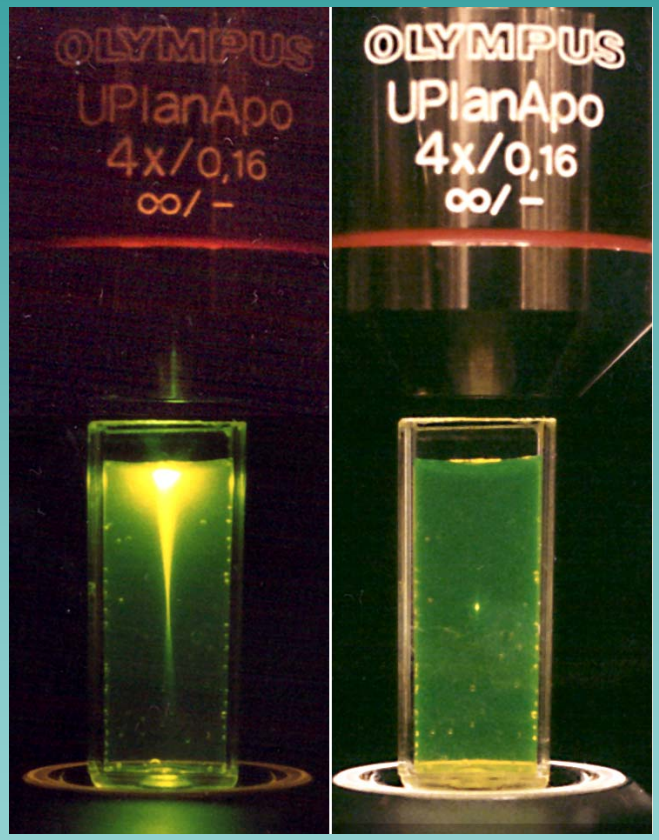
Two/(Multi)-Photon-Excitation



Idea:
Simultaneous (10^{-15}s) absorption of
n photons of wavelength

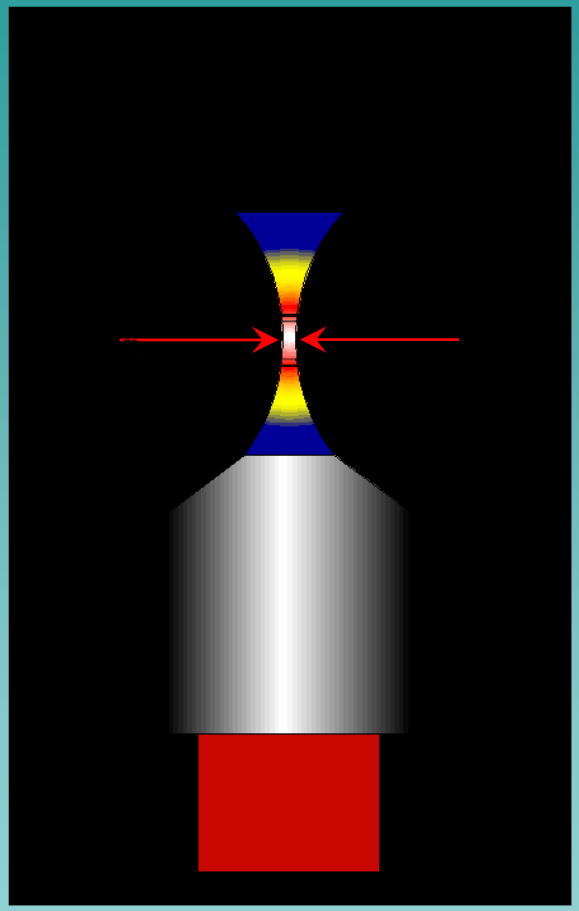
Major advantage:
Inherent spatial sectioning by I^n -
dependency of excitation probability.
Excitation only in vicinity of focal spot

Single photon excitation (488 nm) | **Two photon excitation (900 nm)**

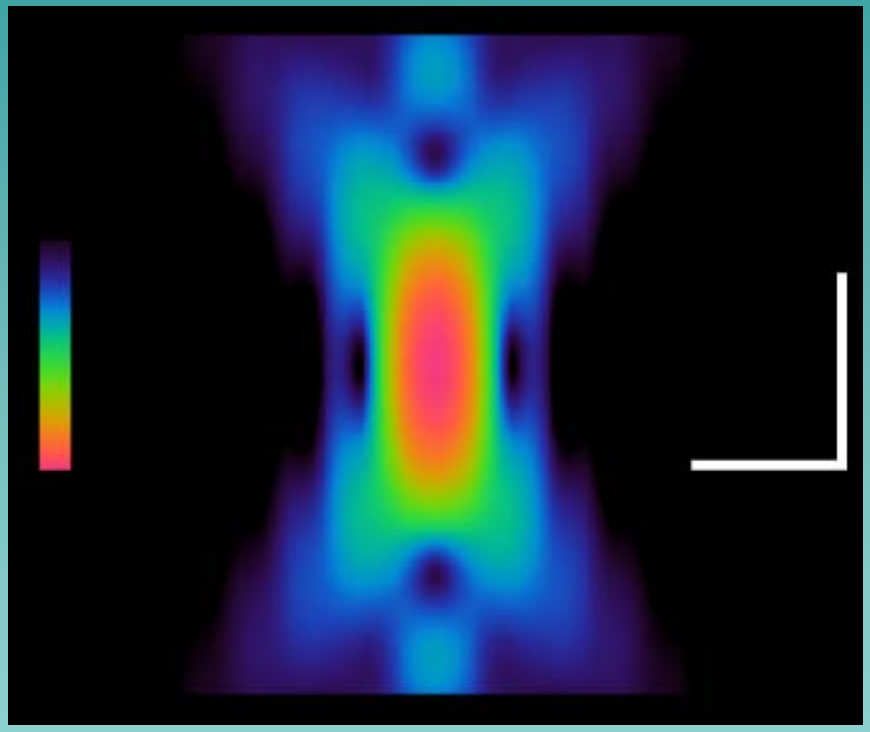


Pulsed excitation = (100 fs, 80 MHz)

MPE is inherently localized to the focus of a high NA objective

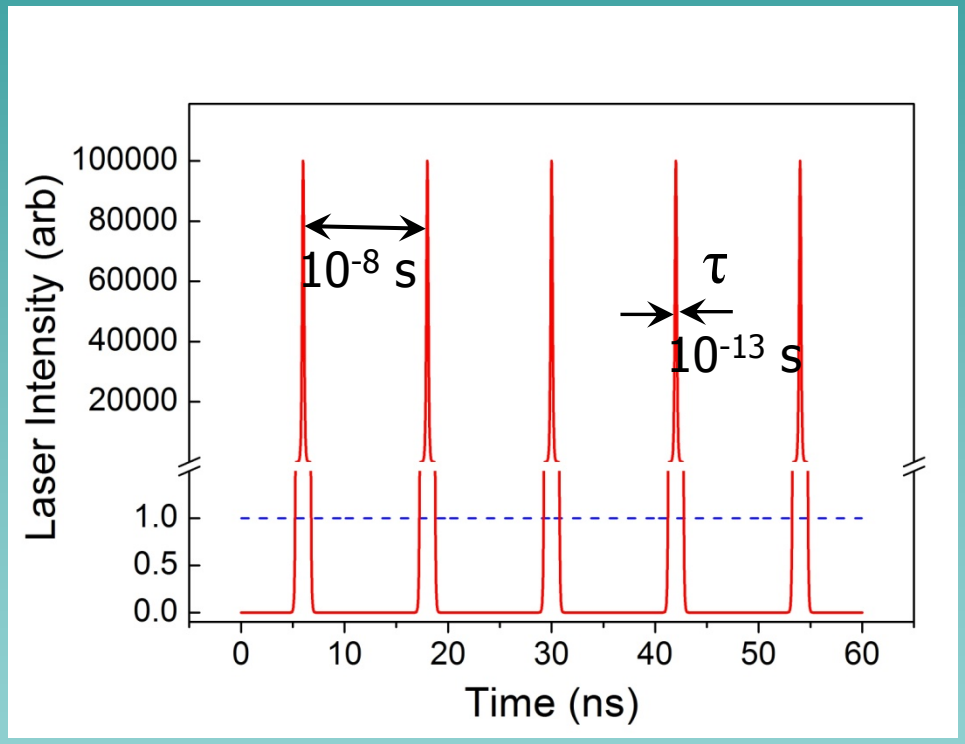


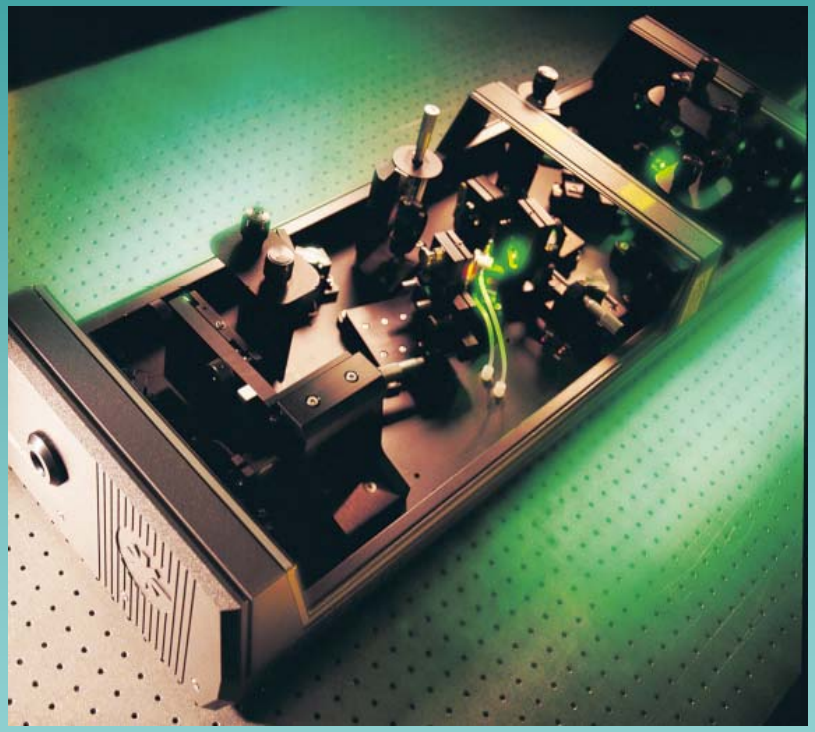
The intensity (squared) declines from z (red arrows) as z^{-4}



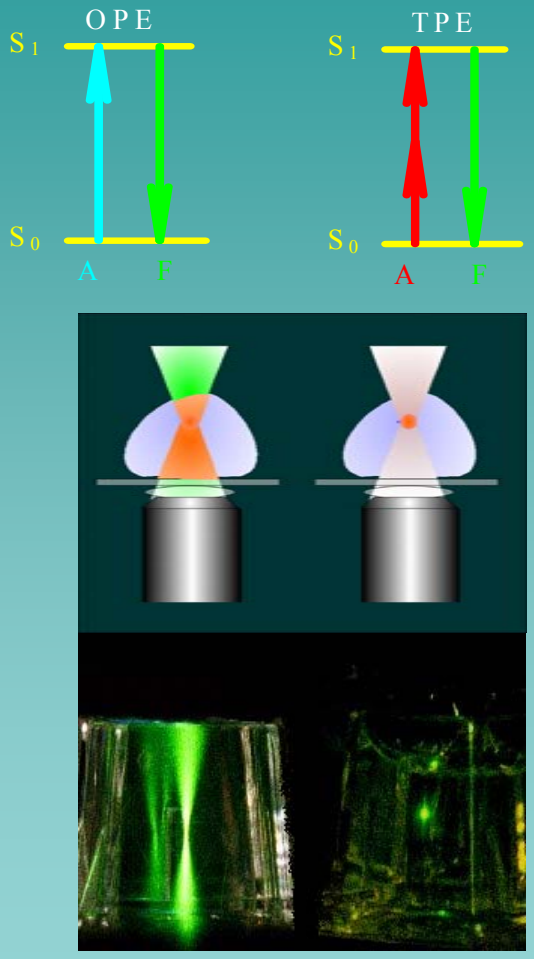
Calculated intensity of 740 nm light near focus of 1.2 NA objective

Pulsed laser excitation enhances two-photon absorption





Two Photon Excitation (2PE)



Advantages

For intracellular work:

1. **Small focal volume**
2. **Decreased photobleaching**
3. **Decreased phototoxicity**
4. **Increased viability**
5. **Increased focus depth**

For cross-correlation work:

6. **Single laser line**
7. **No pinhole necessary**
8. **Good S/N ratio**

Disadvantages

1. **Greater average Illumination intensities**
2. **Loss of resolution**
3. **High cost of pulse laser**

Fluorescent Probes

Uses of fluorescent molecules:

1. Labels - free dyes that may partition to a specific region of a cell or tissue, or fluorescent molecules that are bound to antibodies, receptor proteins or other biomolecules of interest.

2. Indicators dyes - the probes dynamically bind an ion (Ca^{++}, H^{+}, Mg^{++}) and then change in either fluorescence intensity, emission or excitation spectrum.

3. Fluorescent proteins such as GFP, that are produced by the organism after the DNA for GFP, or more commonly a GFP fusion protein, is introduced into the cell.

Problems with Fluorescence - Photobleaching and Blinking

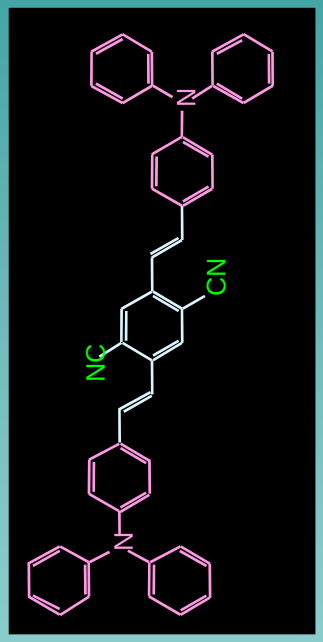
Molecular fluorophores do not emit fluorescence photons indefinitely - they have a limited lifetime that depends on their chemical structure and the chemical environment they're in.

For example, a single rhodamine molecule will emit 10^5 - 10^6 fluorescence photons before it becomes irreversibly photobleached. **Some intrinsically fluorescent biological molecules such as tryptophan (UV excitation) emit, on average 1 photon before the molecule is irreversibly photodamaged**.

Reversible photobleaching (triplet state, other dark states) can also occur, which limits the photon yield per unit time since the molecule spends a percentage its time in an non-excitable state. (Example - eGFP)

Fluorescent Probes

molecules

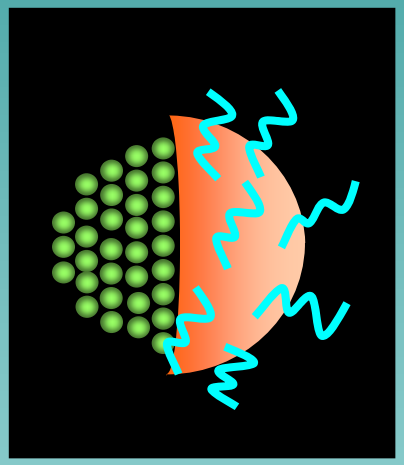


1 nm

EGFP

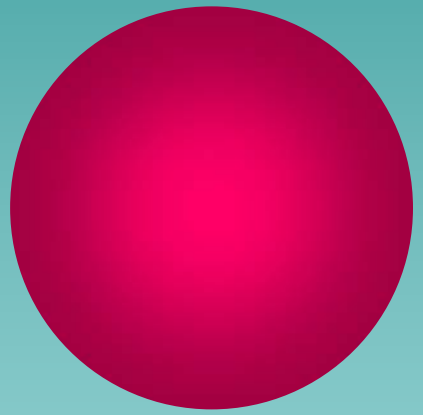
3 nm

quantum dot



6 nm

silica nanoparticle
(rhodamine)

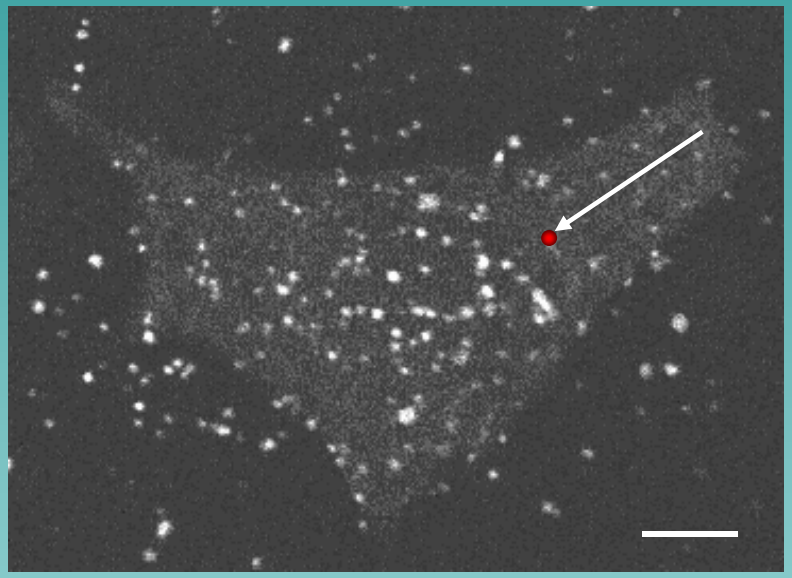


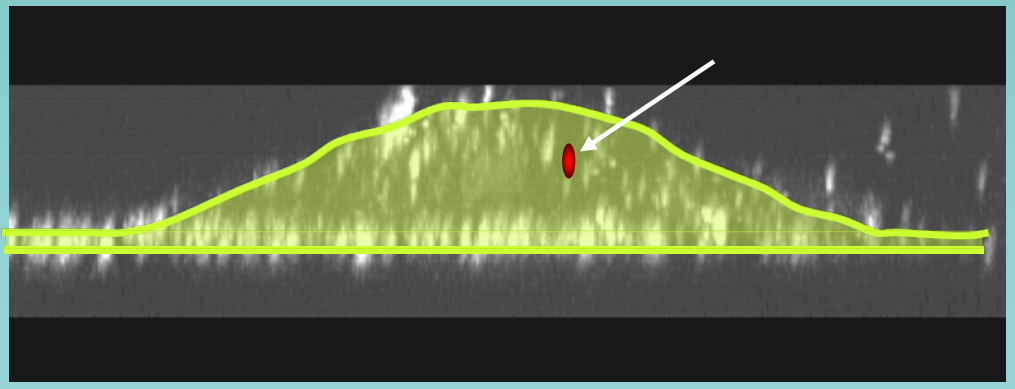
50 nm

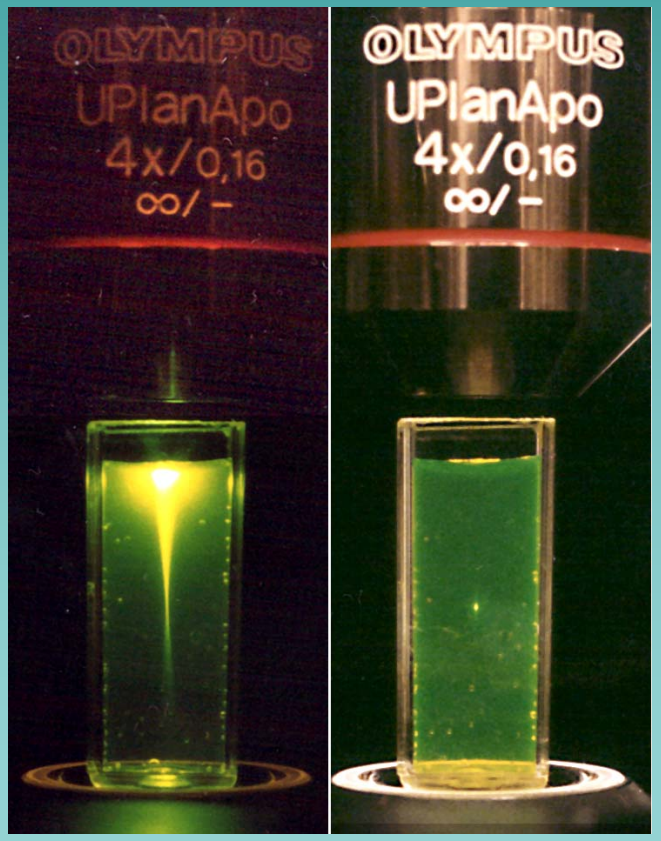
Single fluorophores

Multiple fluorophores

Two Photon Laser Scanning Microscopy Coupled to Spectroscopy Techniques

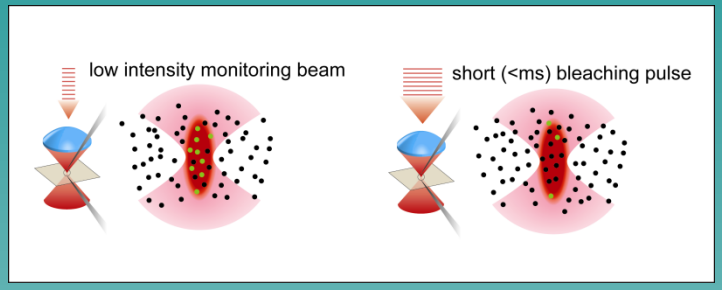




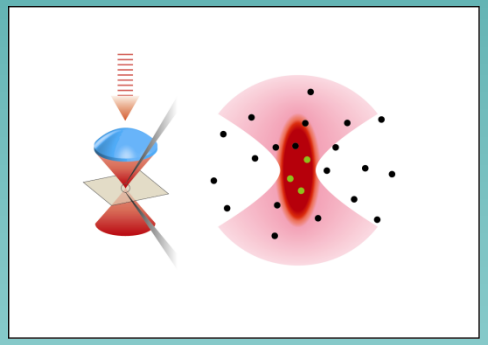


Optical Methods Applied to Study Protein Dynamics

1. Two-Photon Fluorescence Photobleaching Recovery (TPFPR)



2. Two-Photon Fluorescence Correlation Spectroscopy (TPFCS)



3. Two-Photon Fluorescence Dual-Color Cross-Correlation (TPCCS)

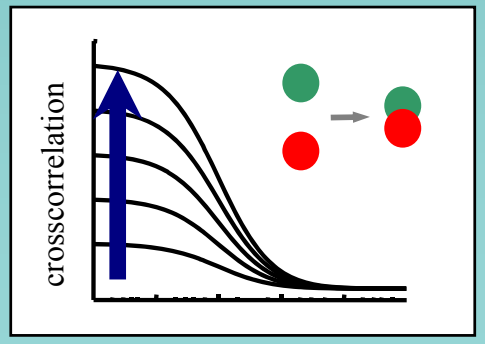


Table 1
Physical Properties of a 100 kDa protein

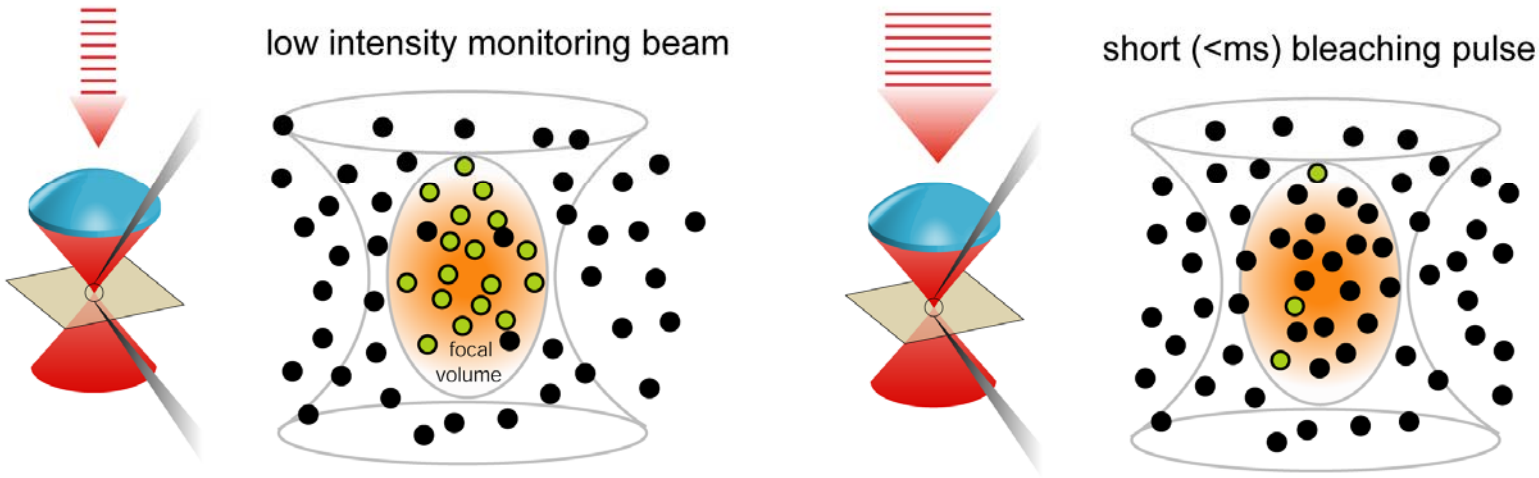
Property	Value	Comment
Mass	166×10^{-24} kg	Mass of one mole/Avogadro's constant
Density	1.38×10^{3} kg/m^3	1.38 times the density of water
Volume	120 nm^3	Mass/density
Radius	3 nm	Assuming a spherical shape
Drag Coefficient (in water @ 20^{o}C)	60 pN.s/m	From Stoke's Law
Diffusion Coefficient (in water @ 20^{o}C)	67 μm^2/s	From the Stoke's-Einstein relationship
Average Speed	8.6 m/s	From the Equipartion principle

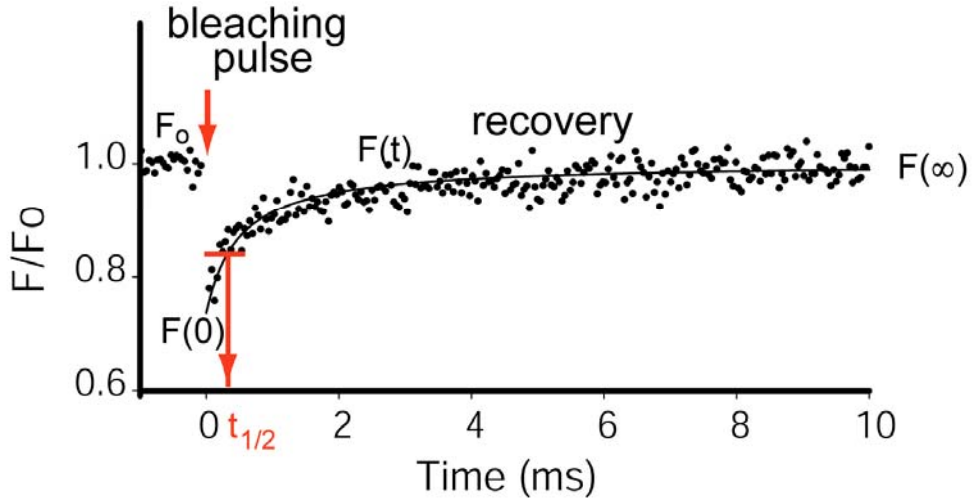
Distance/Time Relationship for One-Dimensional Diffusion of Different Sized Objects in Water

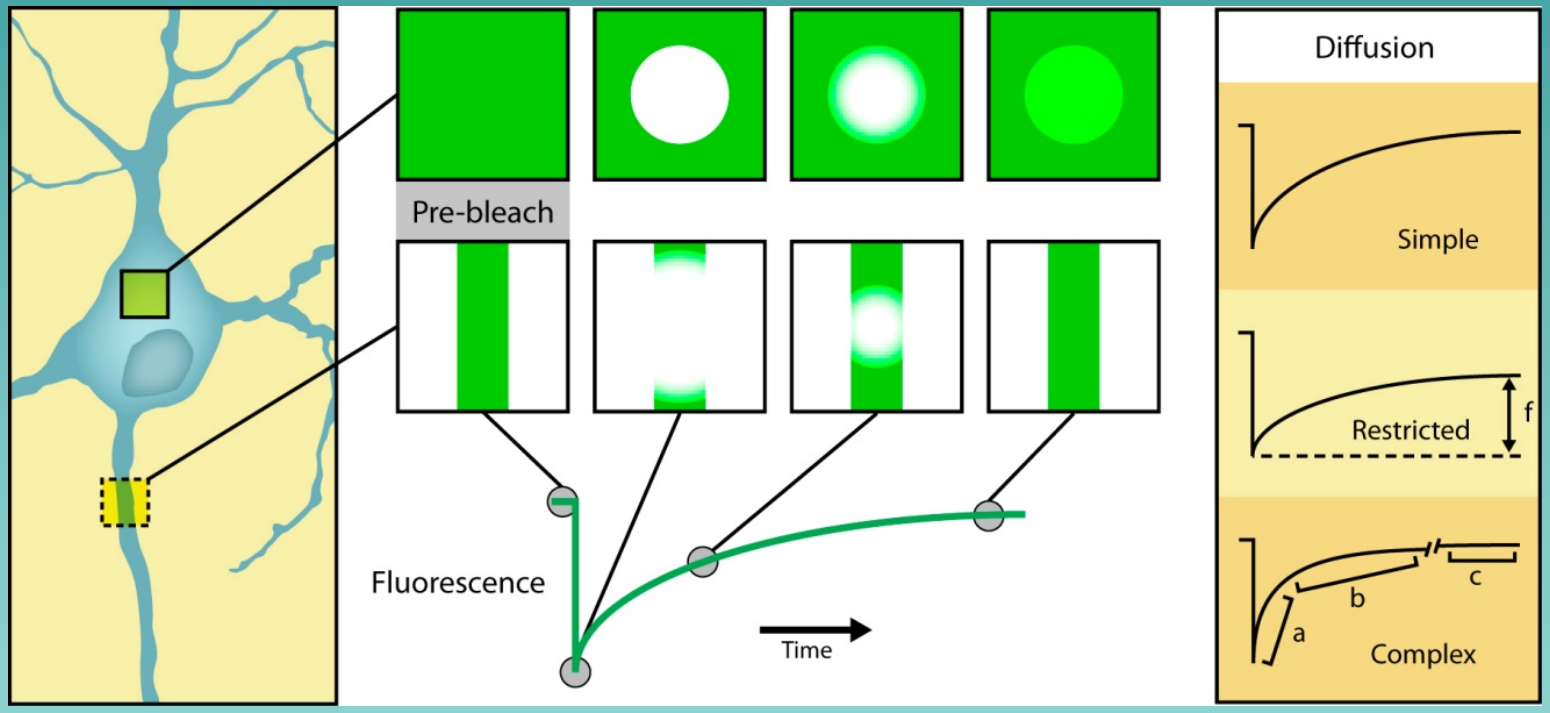
Distance Traveled				
Object	**1 μm**	**100 μm**	**10 mm**	**1 m**
K^+	0.25 ms	2.5 s	2.5×10^4 s (7 hours)	2.5×10^8s (8 years)
Protein (3 nm radius)	5 ms	50 s	5×10^5 s (6 days)	5×10^9 s 150 years
Organelle (0.5 μm radius)	1 s	10^4 s (3 hr)	10^8 s (3 years)	10^{12} s (30 million years)

$\langle x^2 \rangle = nDt$ *n = 2, 4 or 6 for one, two and three dimensional diffusion*

Two-Photon Fluorescence Photobleaching Recovery (TPFPR)






Pre-bleach
Fluorescence
Time
Diffusion
Simple
Restricted
f
a
b
c
Complex

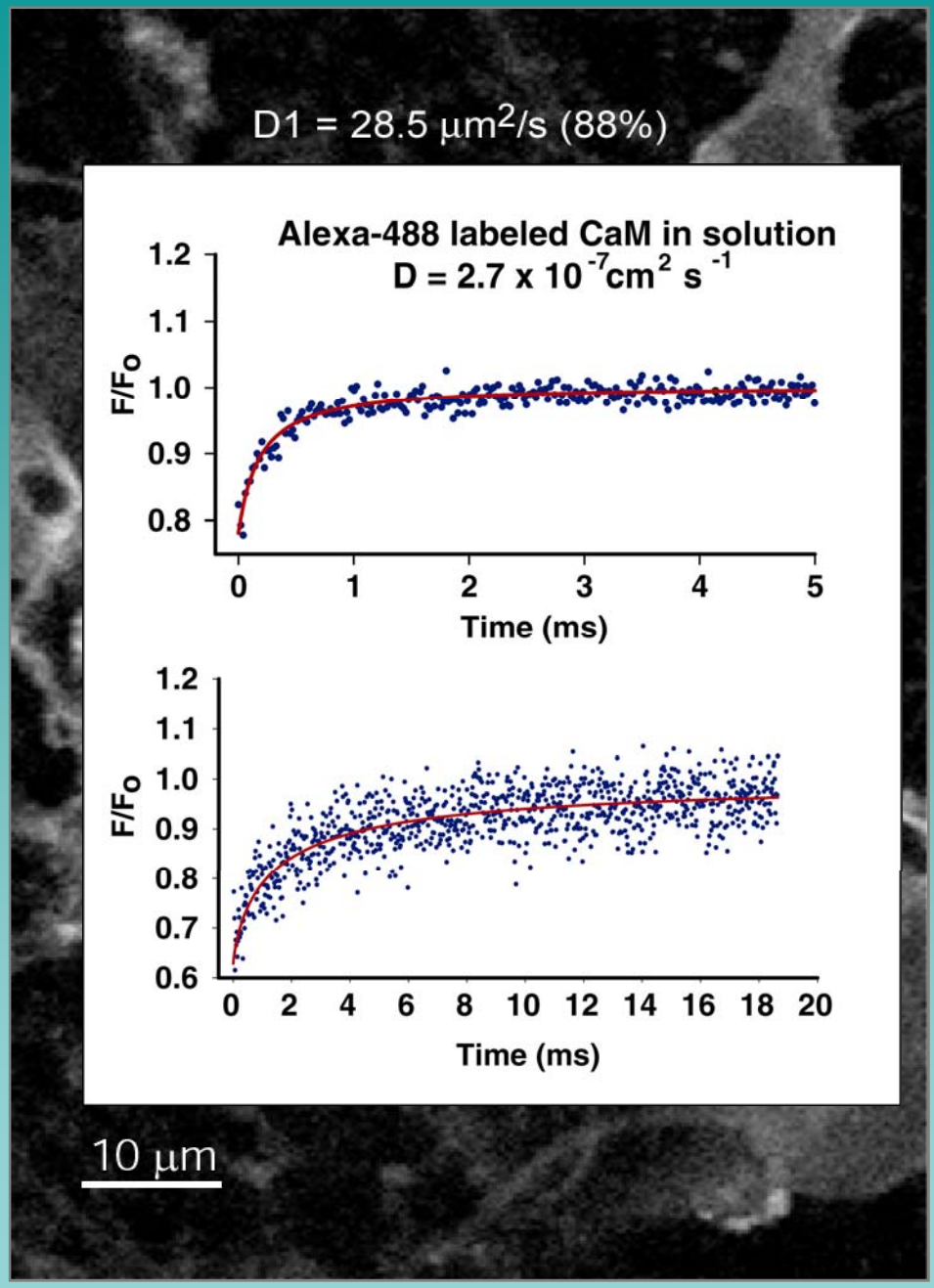
Diffusion Mapping of Alexa-488-Labeled Calmodulin in Neurons Using MPFPR

Alexa-488-CaM in solution

D(t) = 54 µm^2/sec

D(t) of faster diffusing species

Species	Soma	Neurite
10 kD dextran	29.2	29.0
Alexa-488-CaM	28.5	22.3



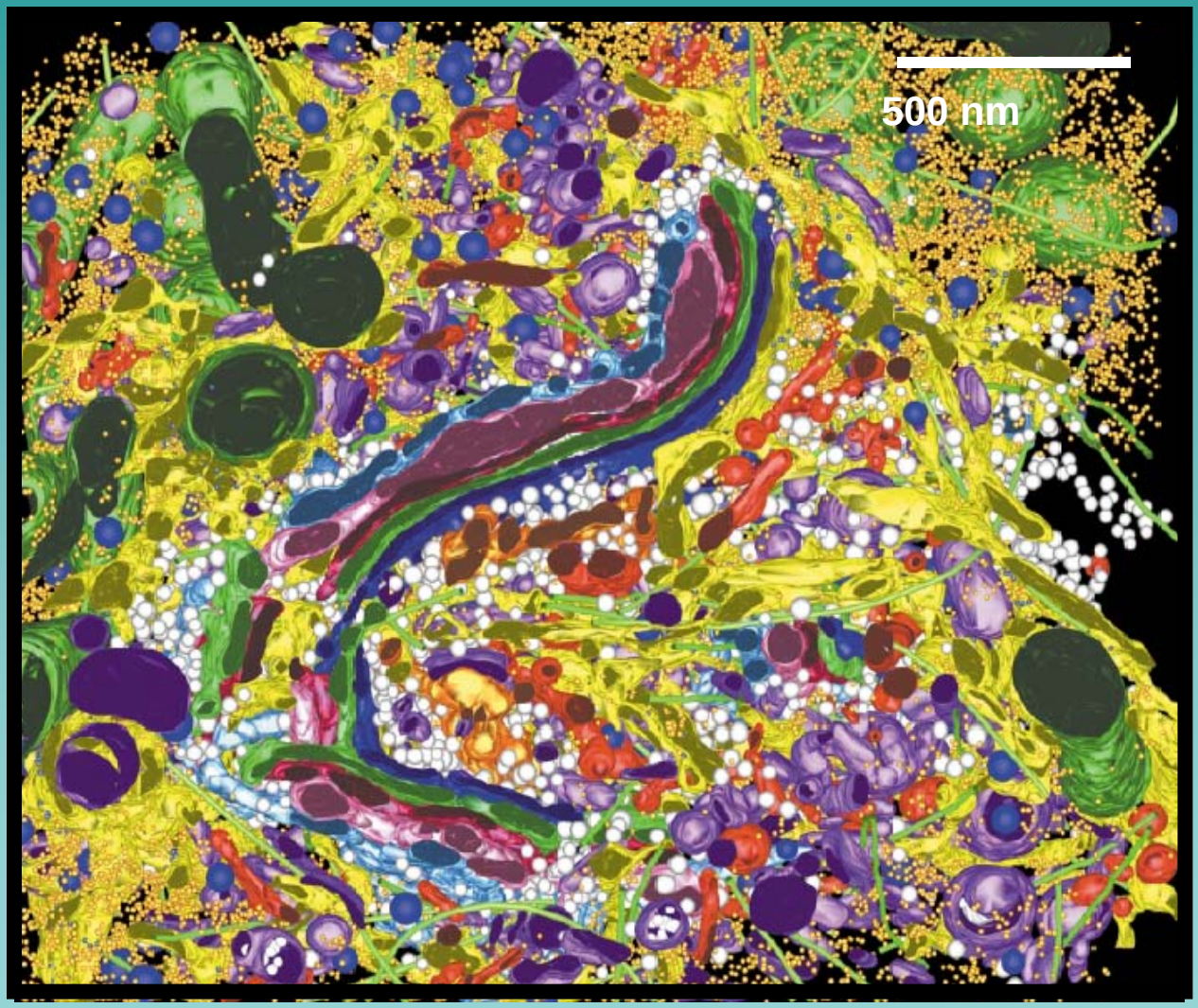
Comparison of Diffusion Coefficients from *in Vitro* and *in Situ* FPR Measurements

Protein	Radius (nm)	D_s (in solution)	D_c (in cytoplasm	D_c/D_s	% mobile
Calmodulin	2.1	102	<4	0.039	81
GFP	2.5	87	27	0.31	82
BSA	3.2	67	6.8	0.1	77
Creatine kinase	3.3	65	<4.5	0.07	50-80
Enolase	3.8	56	13.5	0.24	100
IgG	4.7	46	6.7	0.15	54

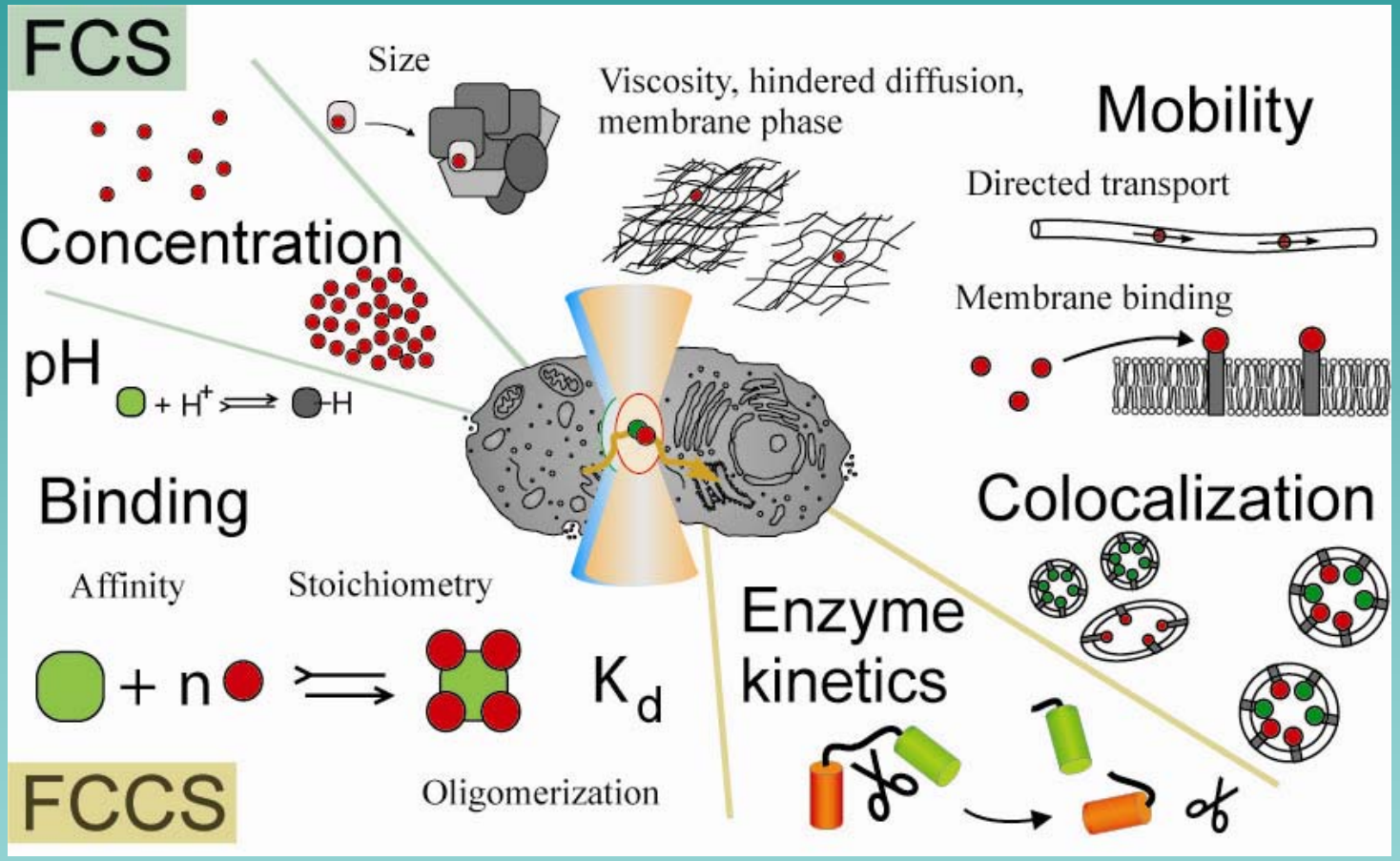
D= diffusion coefficients ($\mu m^2/s$); modified from Luby-Phelps, 2001

$$D = \frac{kT}{6\pi\eta r}$$

Intracellular Diffusion: Far from Simple



Applications of Single Molecule Approach to Biochemistry and Cell Biology

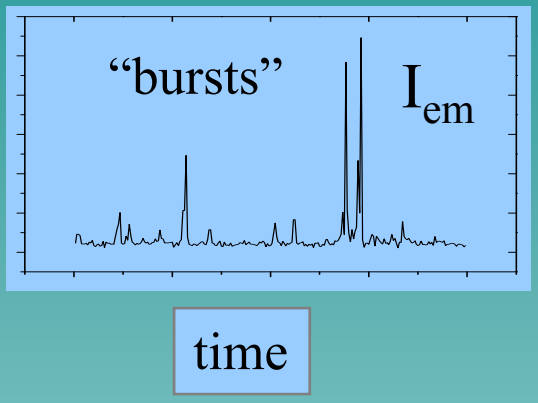


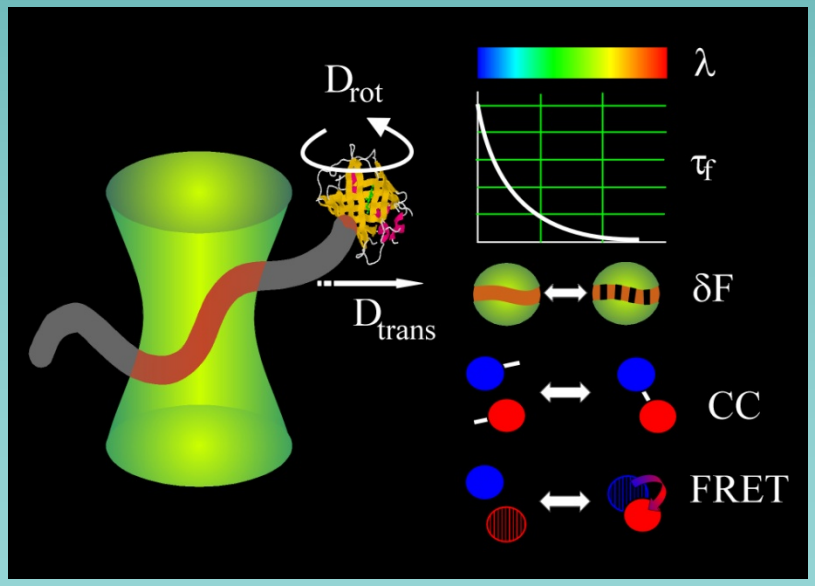
Fluorescence Correlation Spec./Fluorescence Cross Correlation Spec.

How to detect single molecules ?

Low concentrations of fluor (<10^{-9} M)

Small volume elements achieved through confocal or multiphoton optics

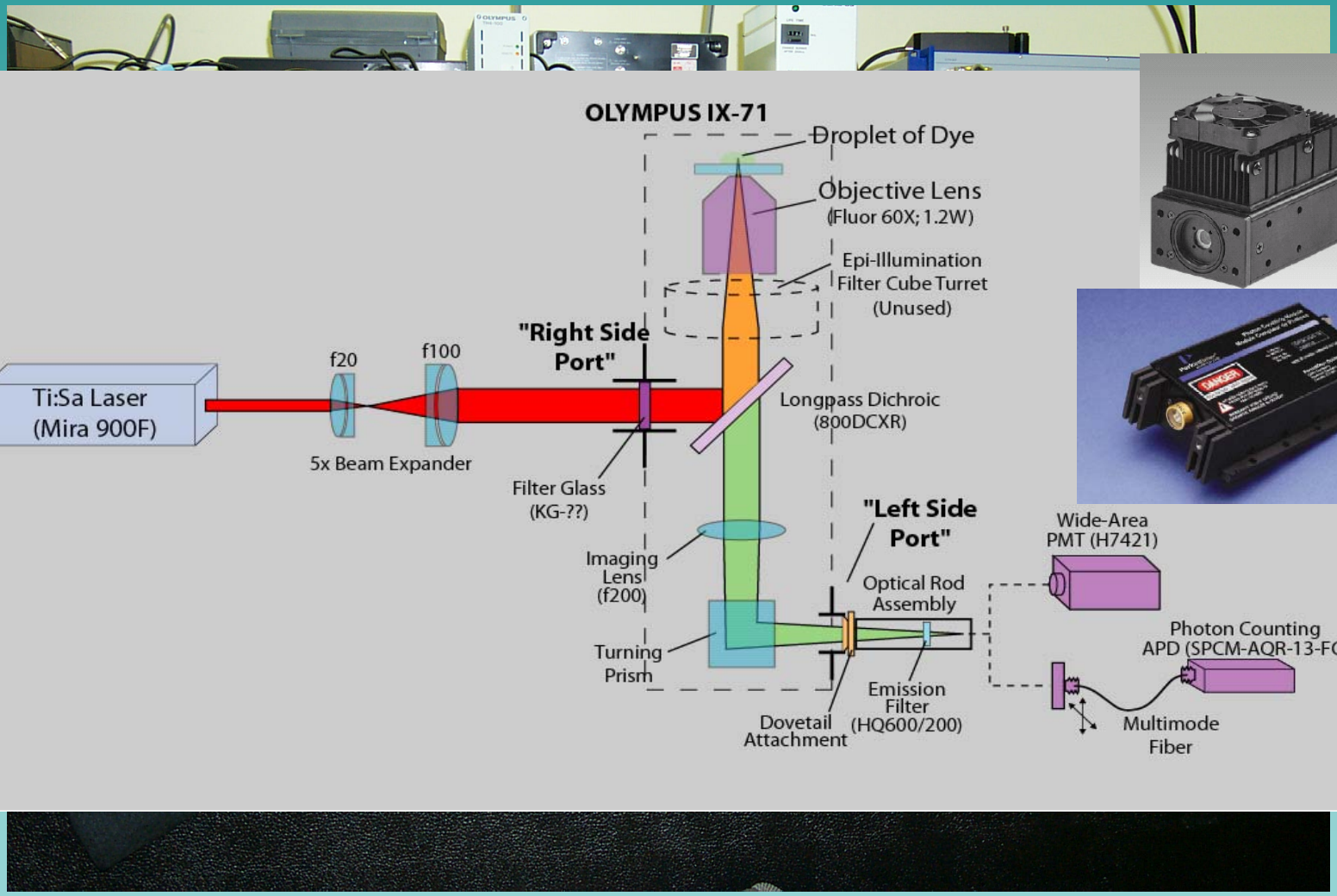




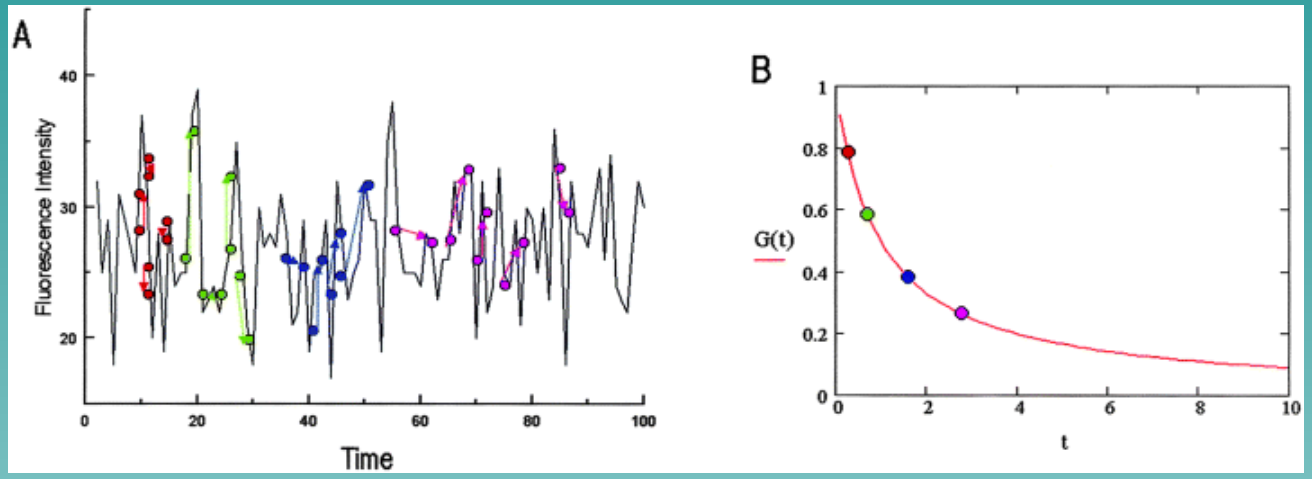
Primary measurement parameter is *signal fluctuations* induced by:

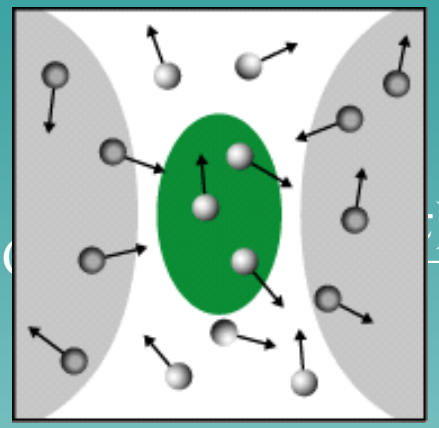
- Diffusion of molecules through the open measurement volume
- Intramolecular dynamics which affect the fluorescence emission

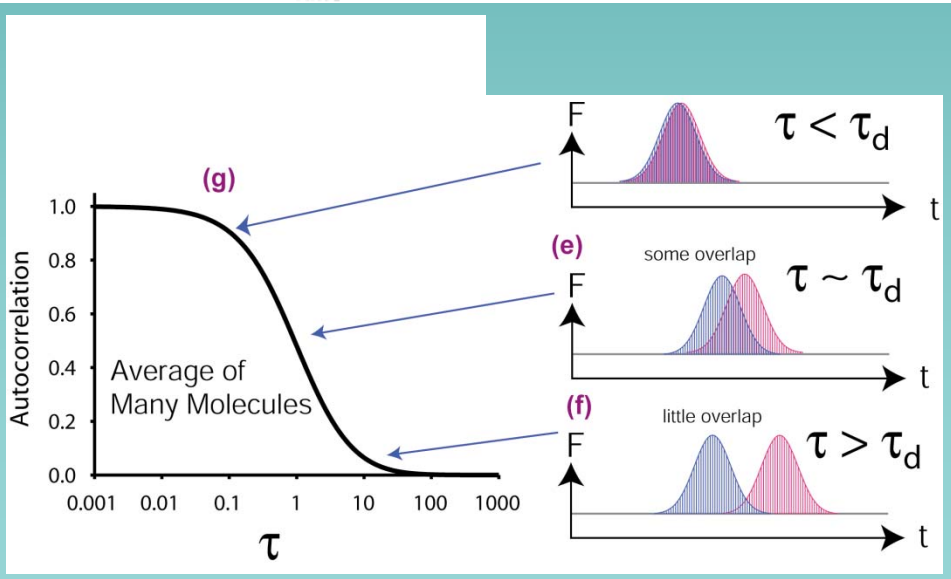
Experimental Apparatus



Analysis of Fluorescence Fluctuations





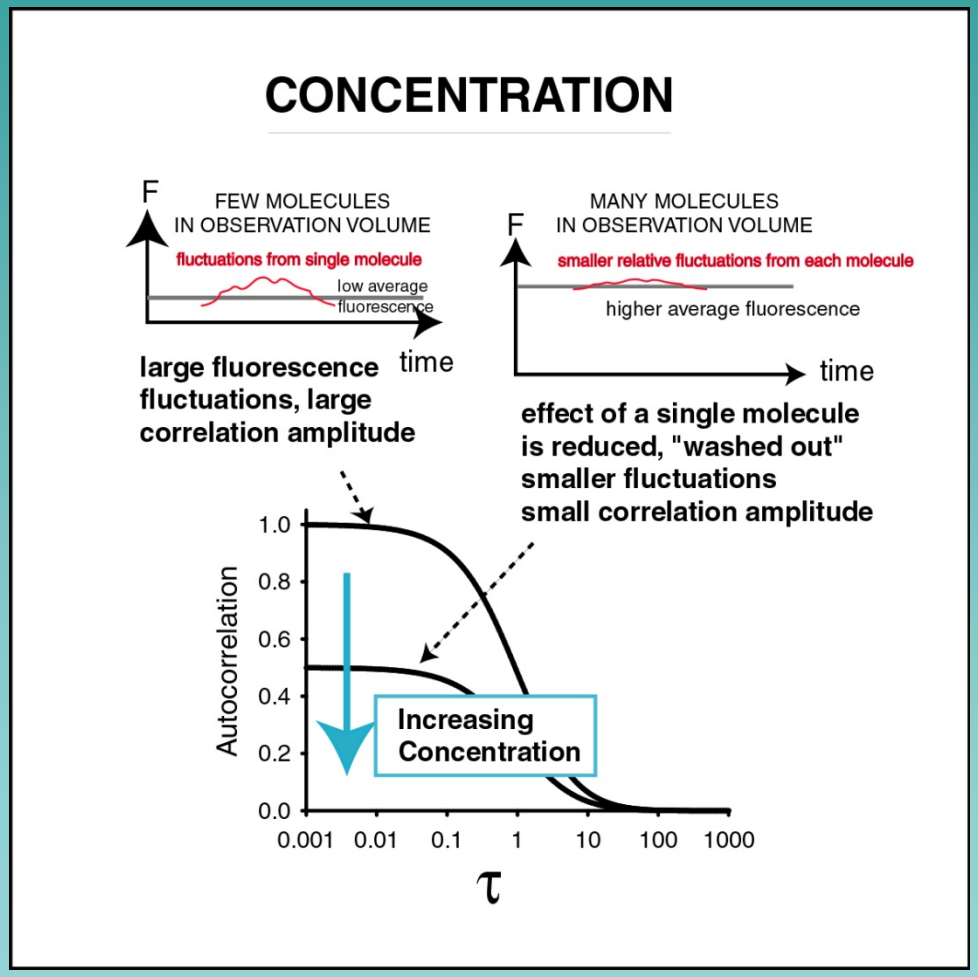


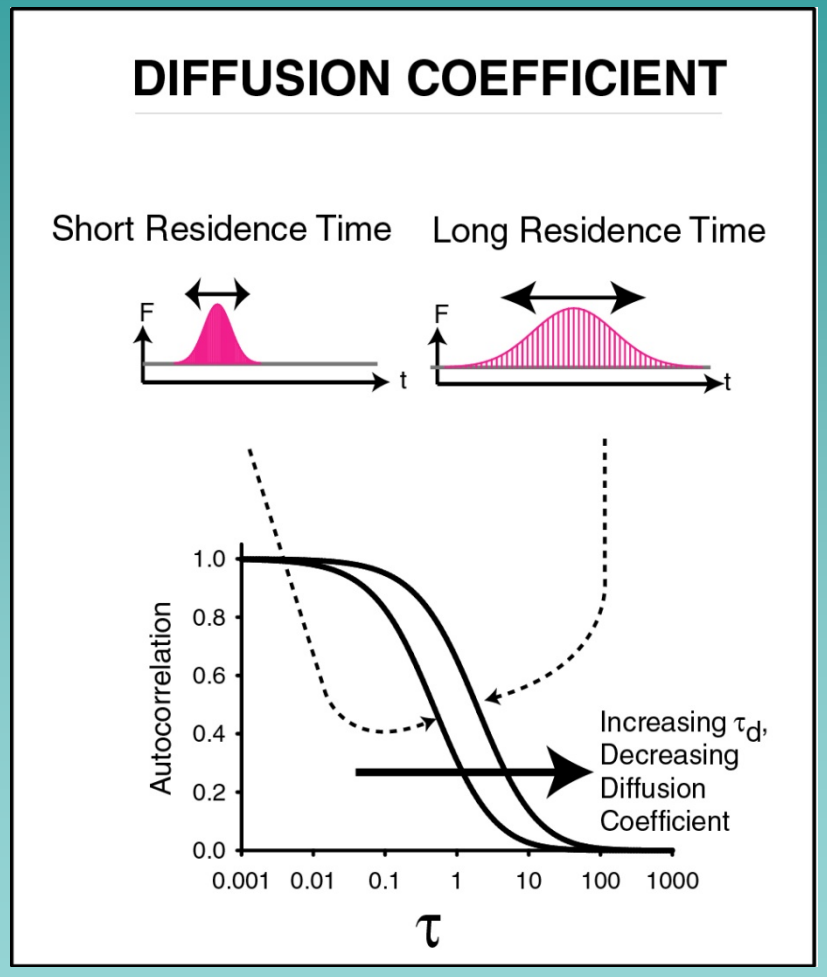
Temporal analysis of spontaneous fluorescence fluctuations -δF-

Signal fluctuations are induced by:

- Diffusion of molecules through the open measurement volume
- Intramolecular dynamics which affect the fluorescence emission

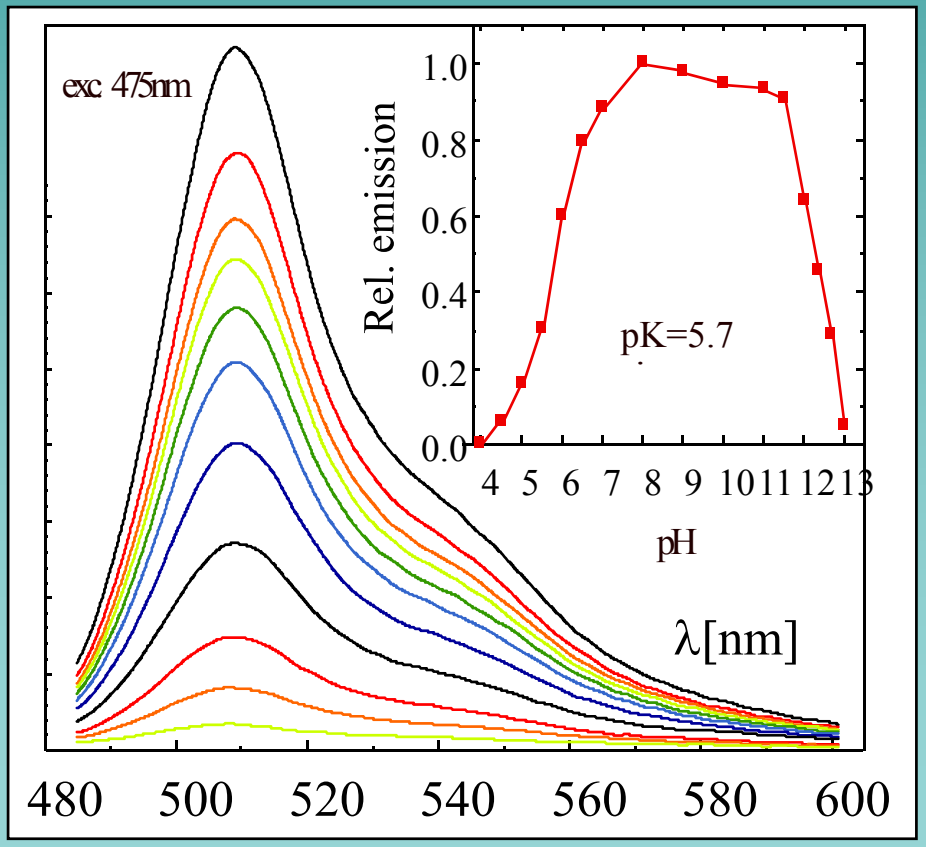
Parameters Provided by Fluorescence Correlation Spectroscopy (FCS)

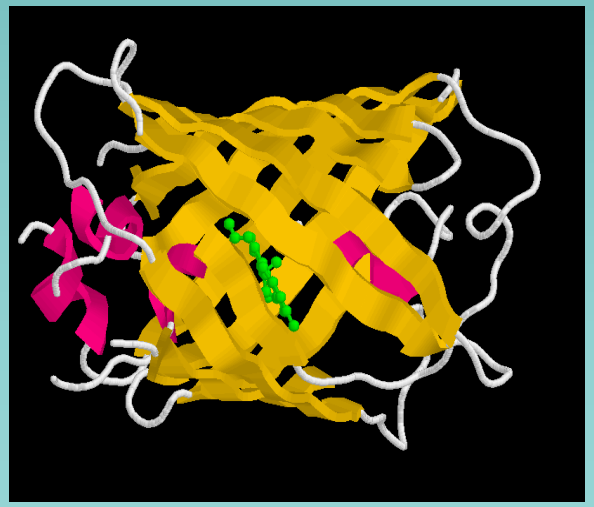




Examples for fast internal dynamics: “flickering”

Molecules under study: GFP Green Fluorescent Protein) and its mutants:
many of them show *pH-dependent emission*

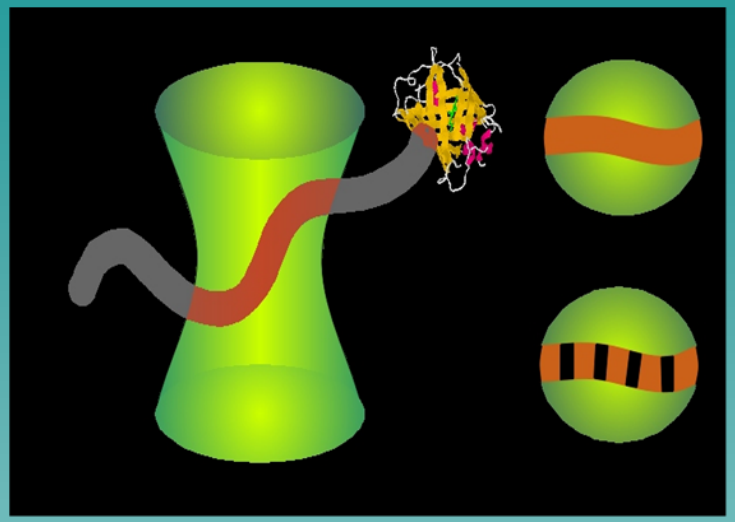


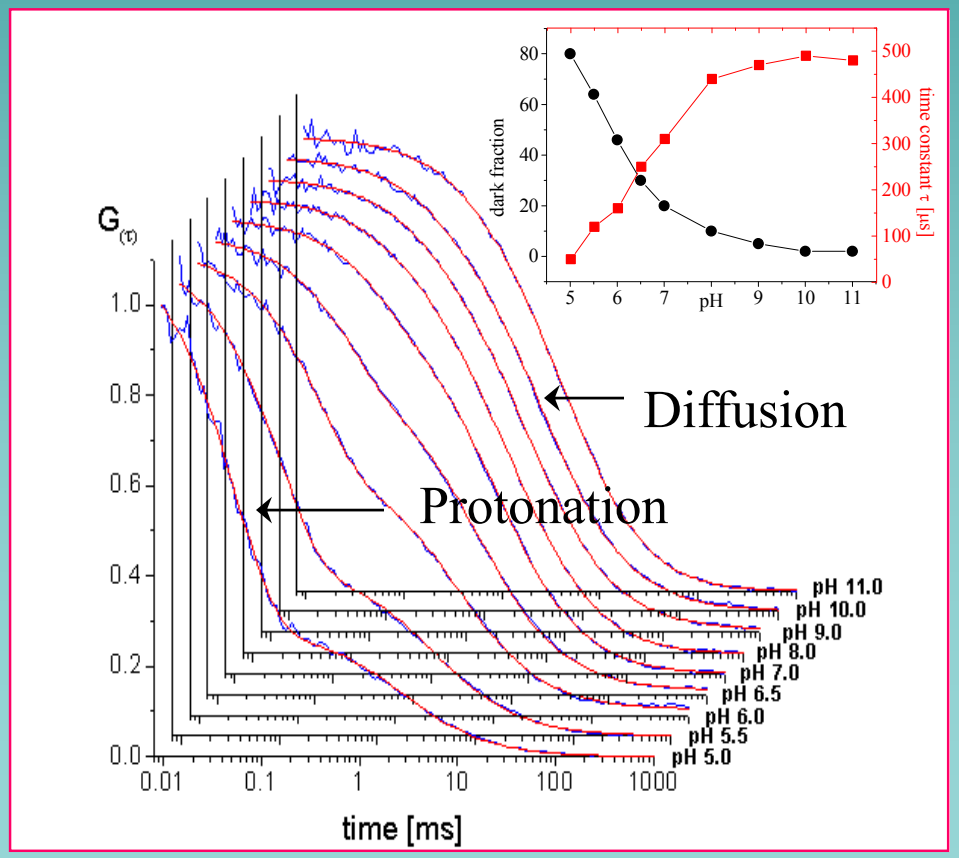


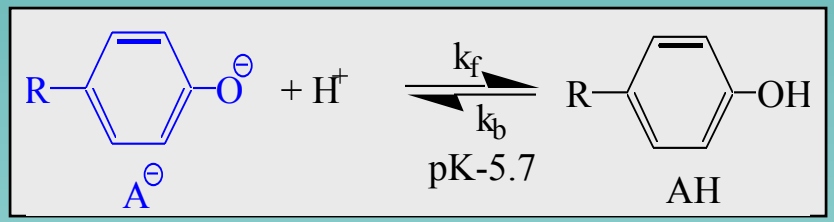
FCS measurements of GFP-a pH sensor

GFP "blinks" on a single molecule scale. Fast dynamics are strongly pH dependent

➡ reversible protonation





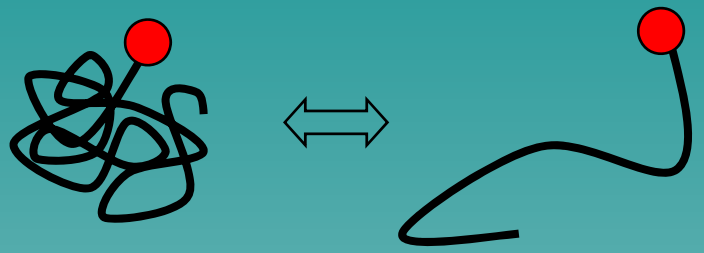


$\lambda_{abs,deprot}$ = 488nm

$\lambda_{abs,prot}$ = 400 nm

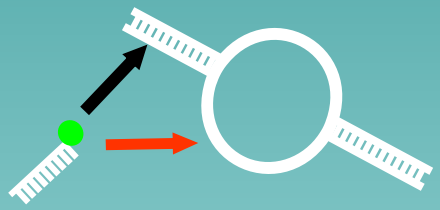
GFP can be employed as single molecule pH meter!

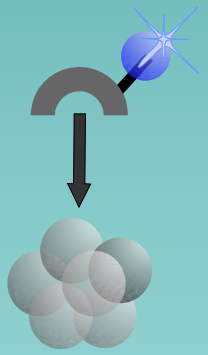
What to determine by diffusion analysis?

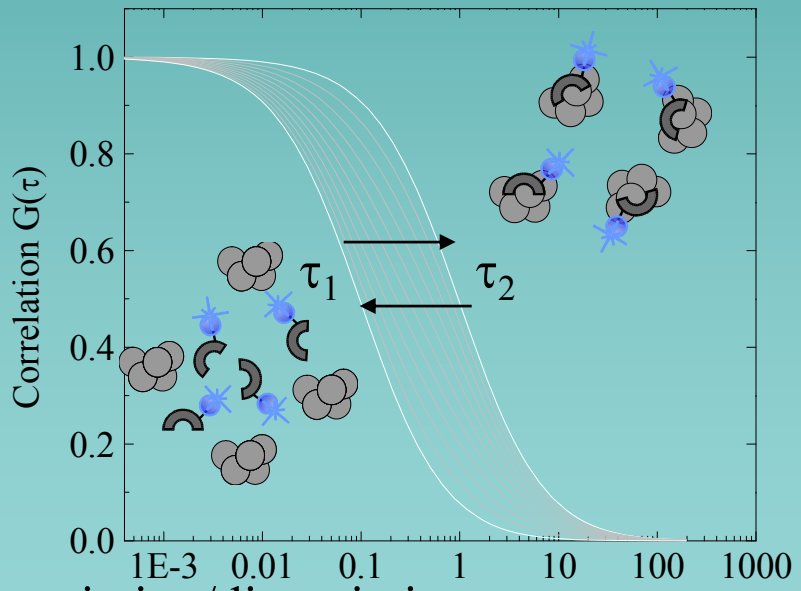


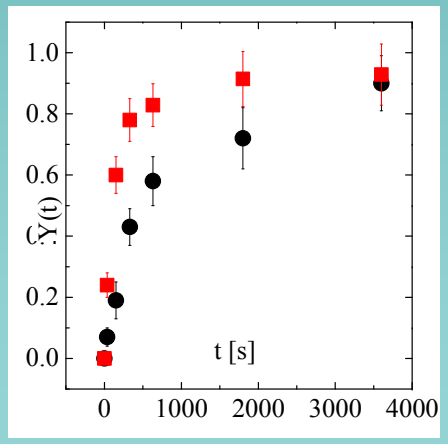
$$D = \frac{kT}{6\pi\eta R_h}$$

Analysis of molecular structure: diffusion properties depend on hydrodynamic radius





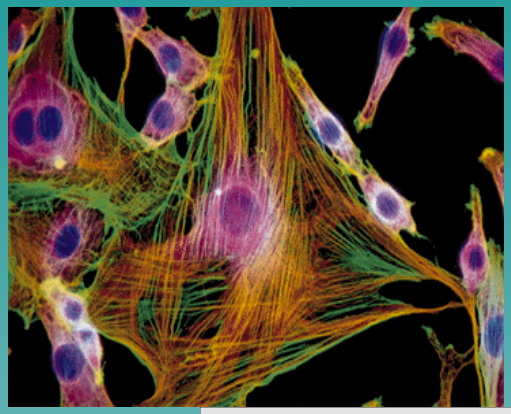


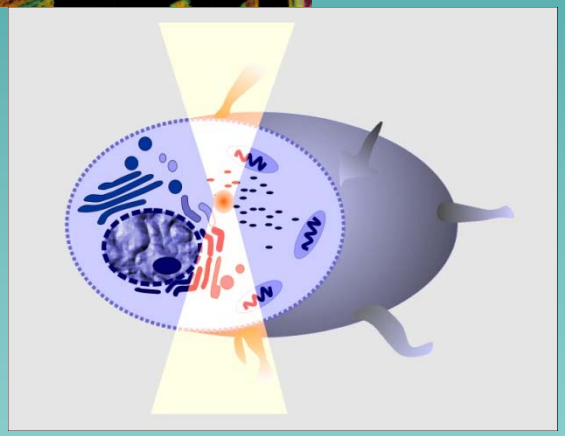


Analysis of association/dissociation processes by change in molecular mass

$k_{ass}=10^4\ M^{-1}s^{-1}$ to $10^6\ M^{-1}s^{-1}$

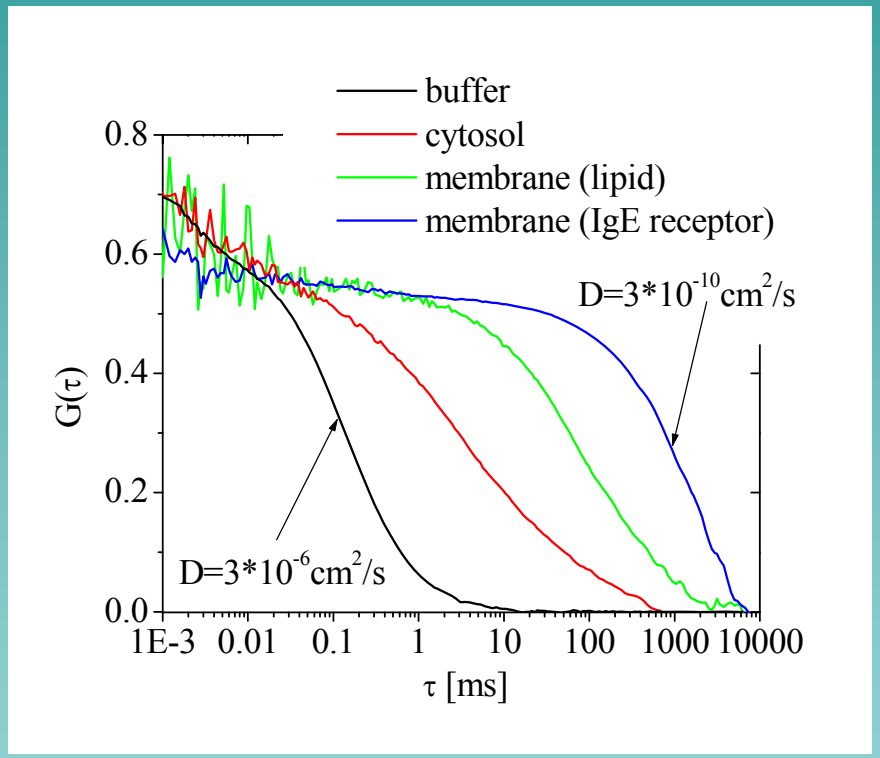
Assessing molecular mobility in different cellular compartments

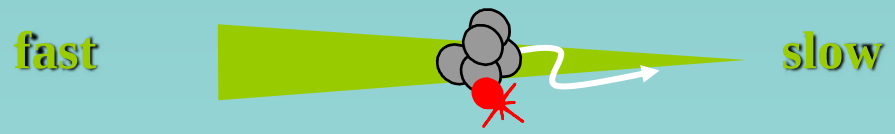




Requirement: specific labeling of regions of interest

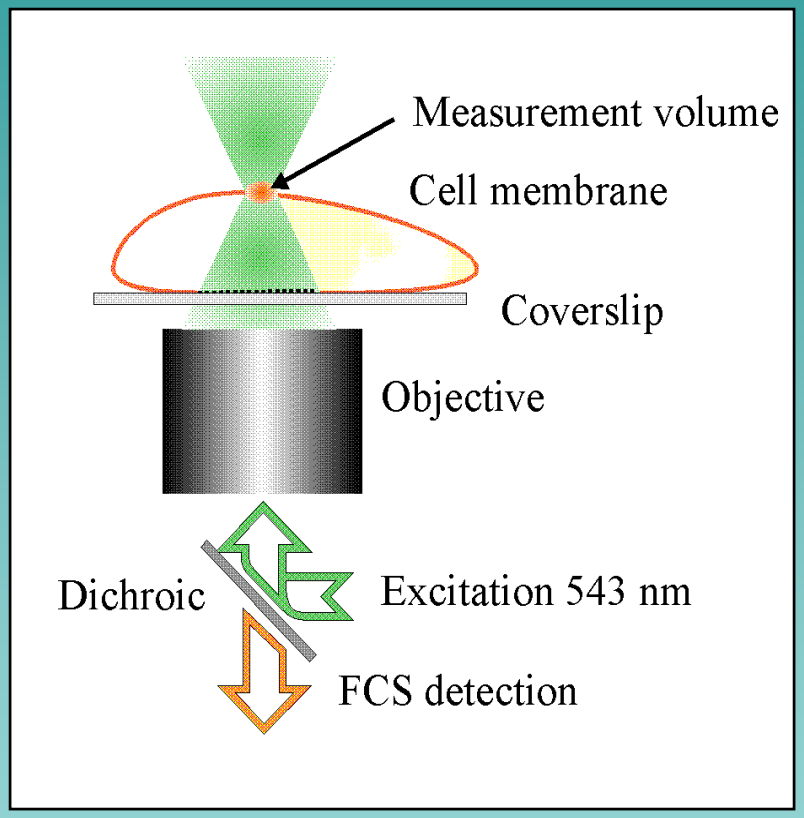
Precision: 0.3 um in XY
1.0 um in Z

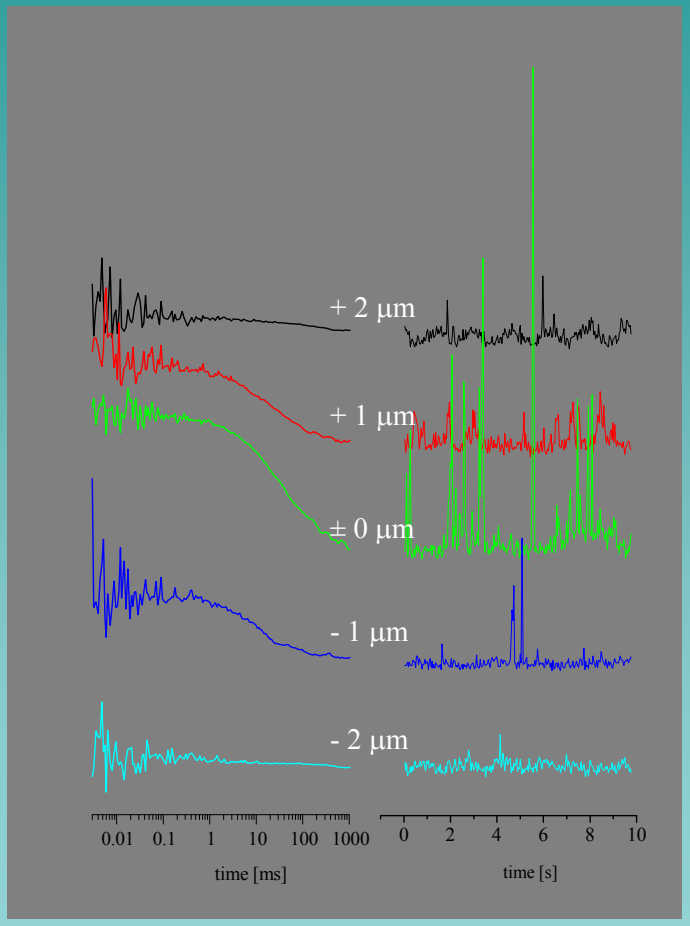




Determination of "molecular speed"

Detection of single molecules in membranes





⇒ Only labeled regions contribute to the measured signal

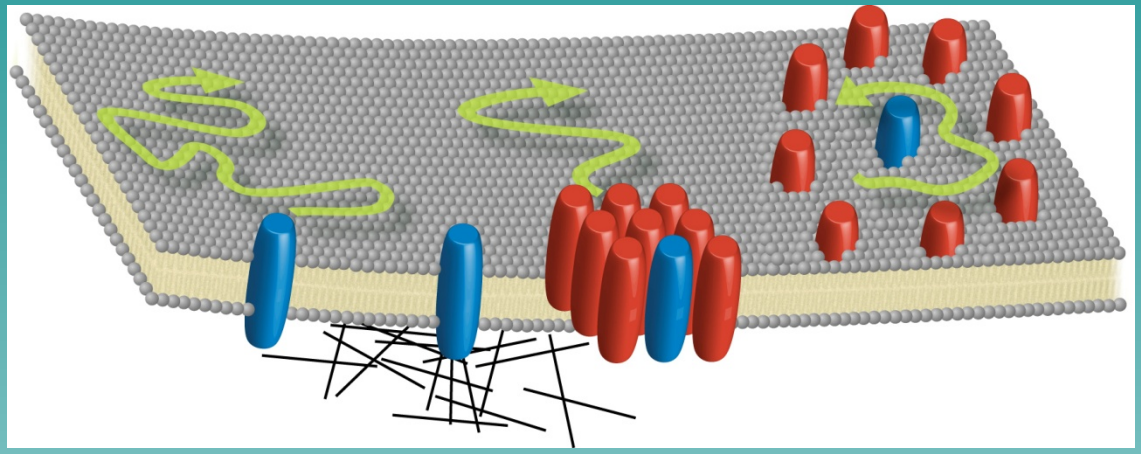
Diffusion of GFP and GFP-Fusion Proteins by FPR

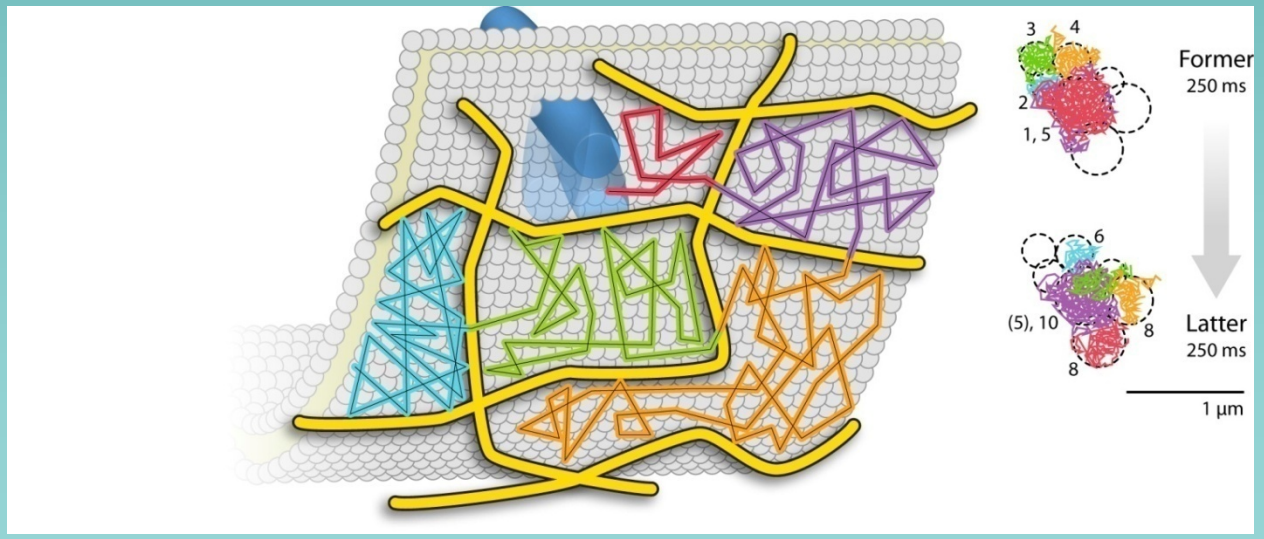
Molecule	**D ($\mu m^2/s$)**
GFP in water	87
GFP in cytoplasm	25
GFP in the ER lumen	5-10
GFP in the mitochondrial matrix	20-30
ER Membrane	
GFP-VSV G-protein	0.45
GFP-signal recognition particle	0.26
Golgi Membrane	
GFP-galactosyltransferase	0.54
Nucleoplasm	
GFP-fibrillarin	0.53
GFP-ERCC1/XPF	15
Plasma Membrane	
GFP-cadherin	0.03-0.04

The viscosity of the membrane has been likened to that of olive oil, some 50-100 times that of water

$$D = \frac{kT}{6\pi\eta r}$$

Diffusion in Membranes





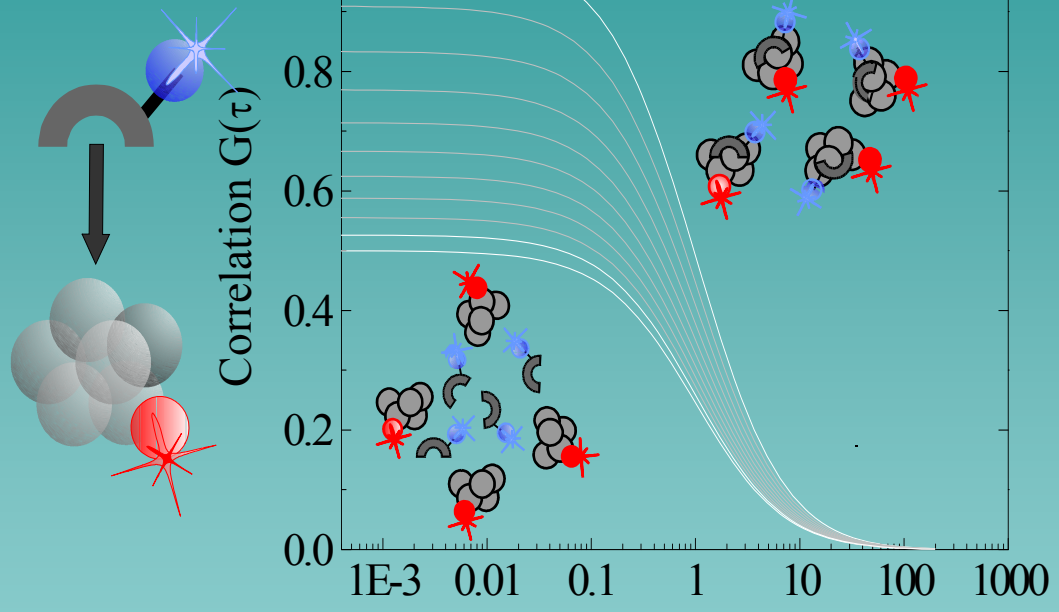
Dual-color cross-correlation analysis FCCS

Advantage: mobility independent analysis of molecular interactions

$$D = \frac{kT}{6\pi\eta R_h}$$

RECALL

$R_h \sim (MW)^{1/3}$



Principle: only doubly labeled species contributes to cross-correlation signal

Red channel: +

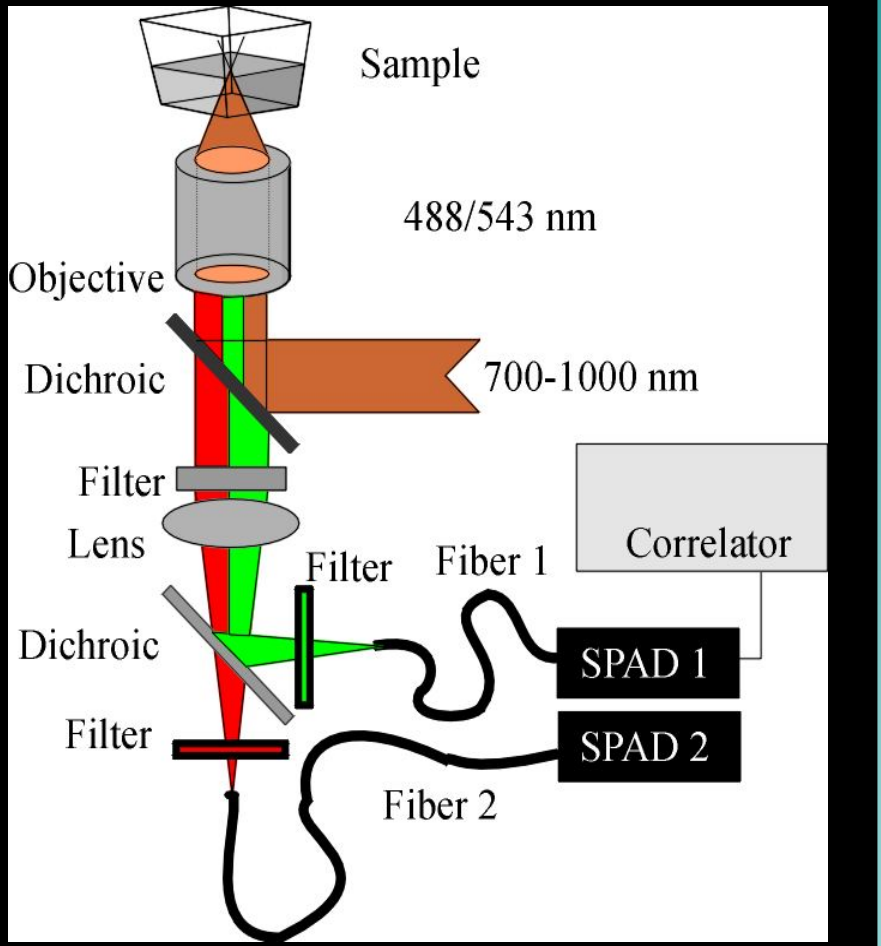
Blue channel: +

cross-c.

$$G_{ij}(\tau) = \frac{\langle \delta F_i(t) \cdot \delta F_j(t+\tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle}$$

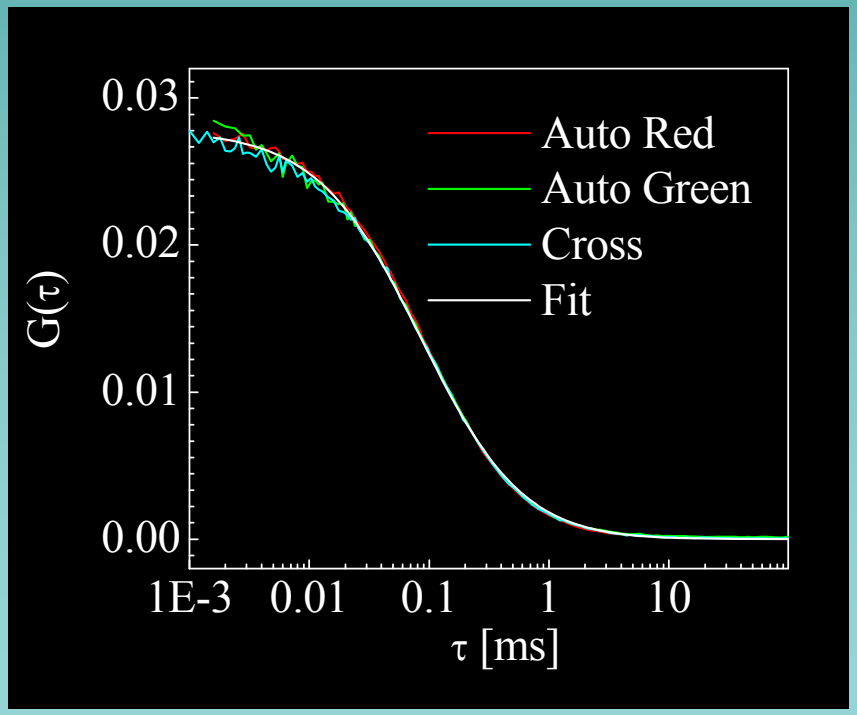
Denominator Numerator

Experimental setup for TPCCS



Inherent overlap of excitation volumes

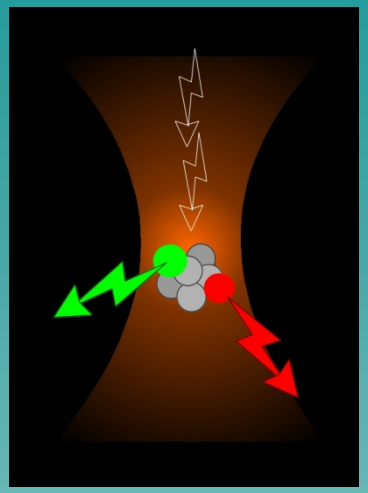
Simplified alignment of detection volumes (no pinholes required)

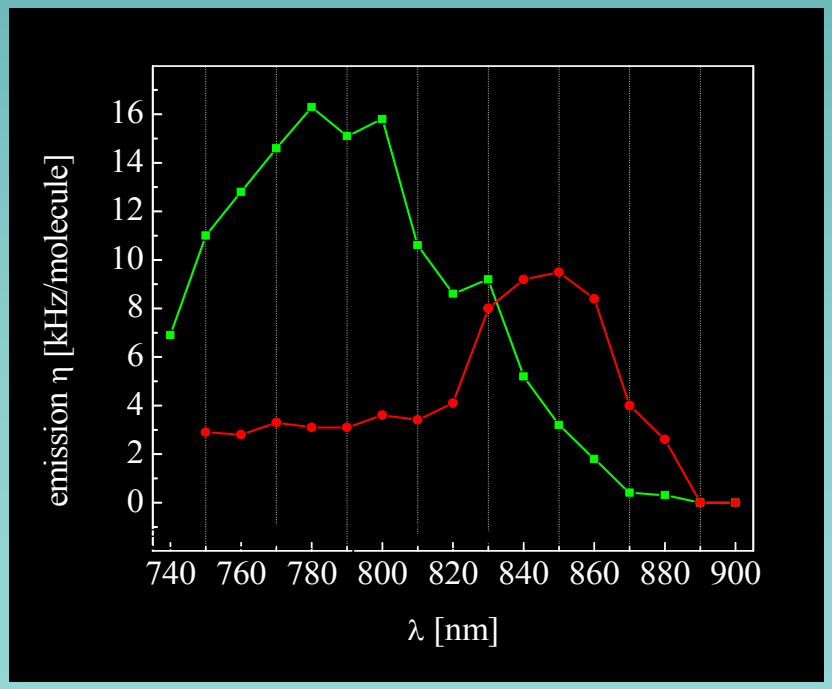


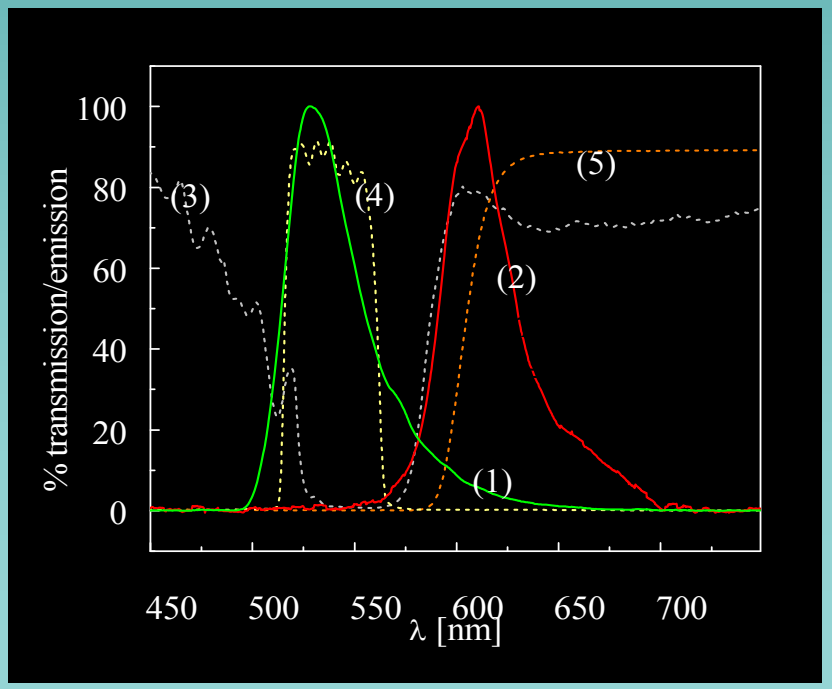
Dual-color two-photon cross-correlation (TPCCS)

Concept: Excitation of spectrally separable fluorophores with a single IR line

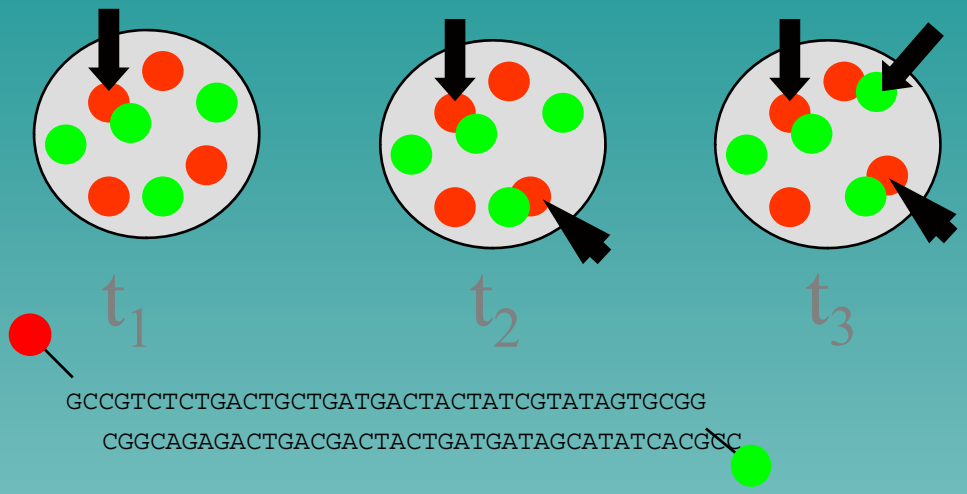
Requirement: both dyes show similar emission on a single molecule scale



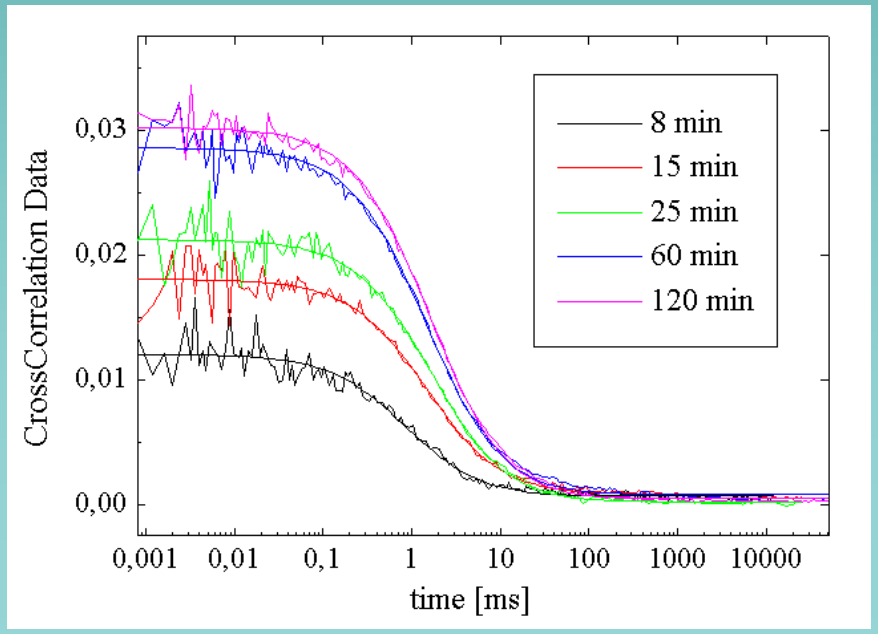


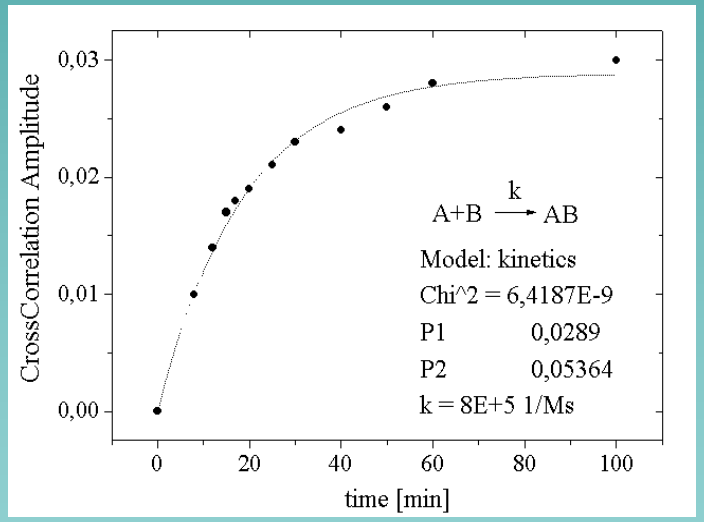


Analysis of DNA-DNA association



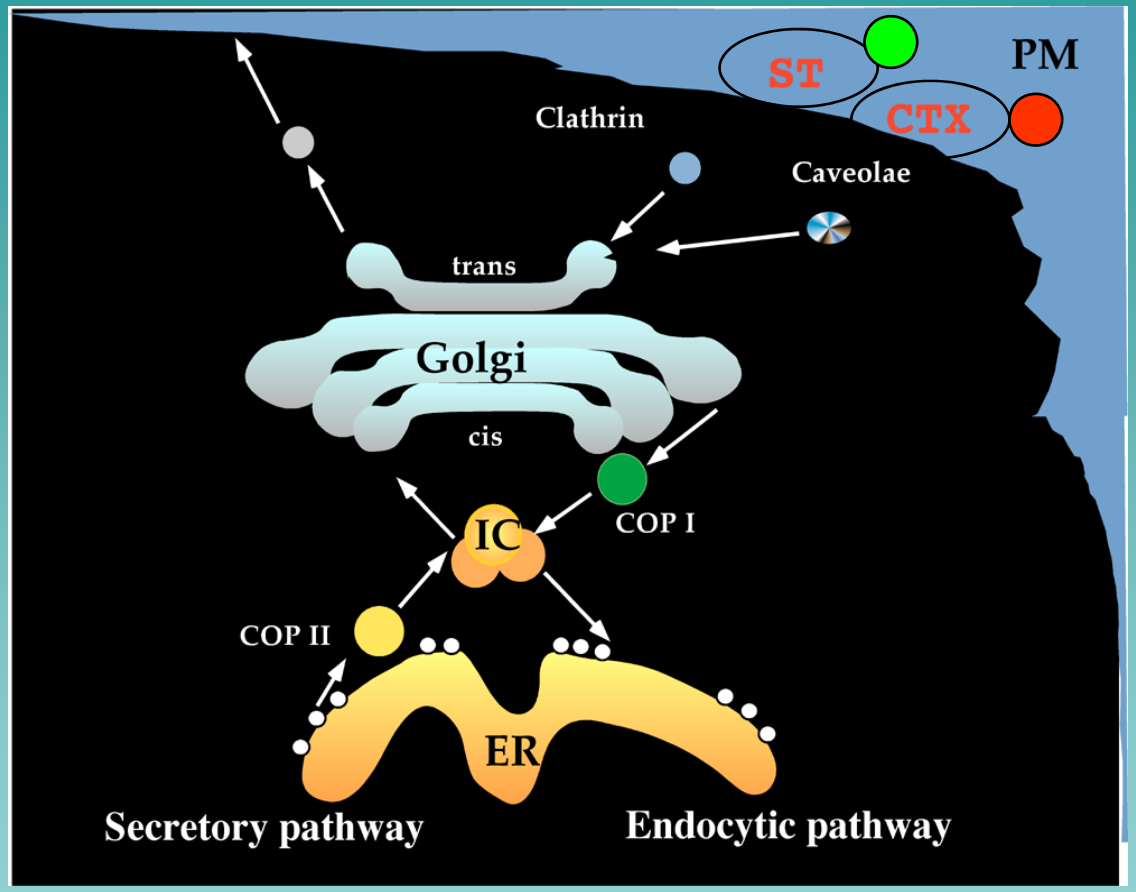
$$G^{\times}_{RB}(\tau) = \frac{C_{RB} Diff_{BR}}{\left[V_{eff}\left(C_R + C_{RB}\right)\left(C_B + C_{RB}\right)\right]}$$





Greater specificity for reaction product observation

Intracellular FCCS applications: The toxin system

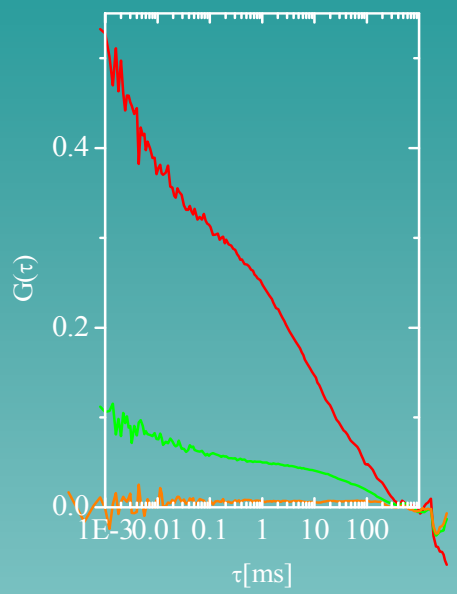


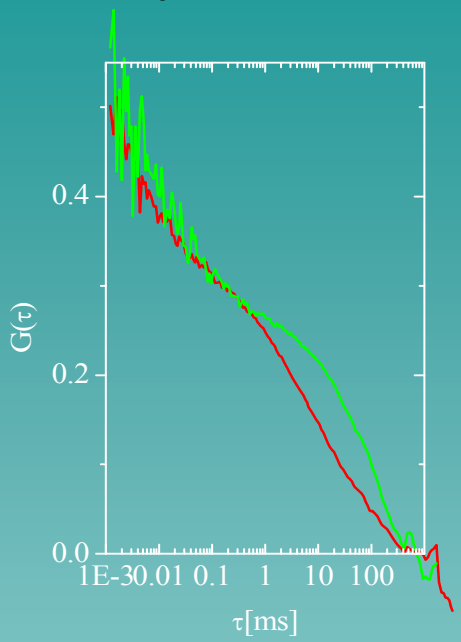
CTX-*Cholera*
ST-*Shigella*

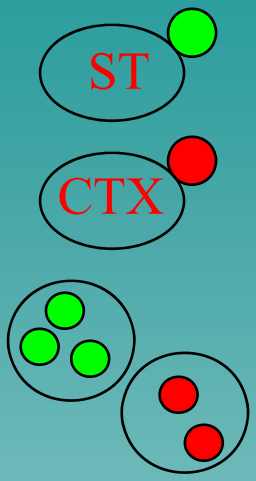
Bacia et al., 2002, Biophy. J.

System: Bacterial protein toxins entering the cell in a retrograde fashion
Objective: to simultaneously study the endocytic trafficking of Cholera (red label) and Shiga (green label) Toxin

Comparing Endocytic Pathways for CTX and ST

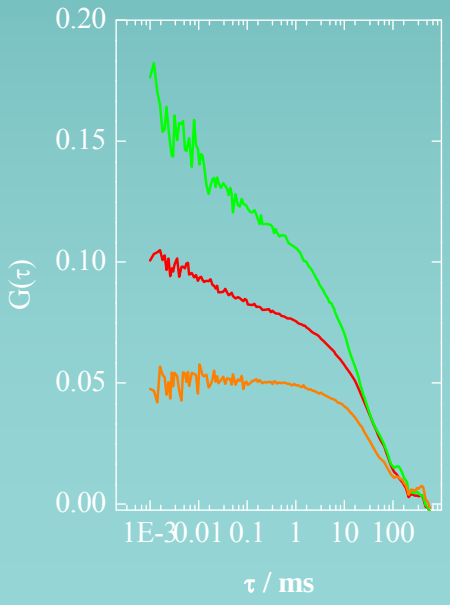


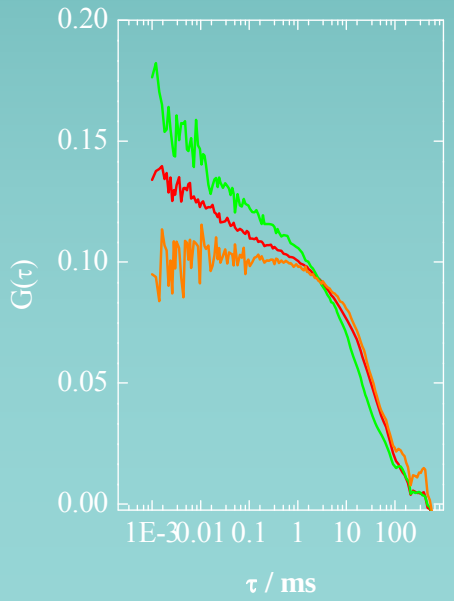


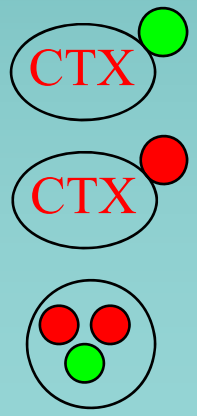


Distinct autocorrelations, no cross-correlation: different pathways

CTX-*Cholera*
ST-*Shigella*

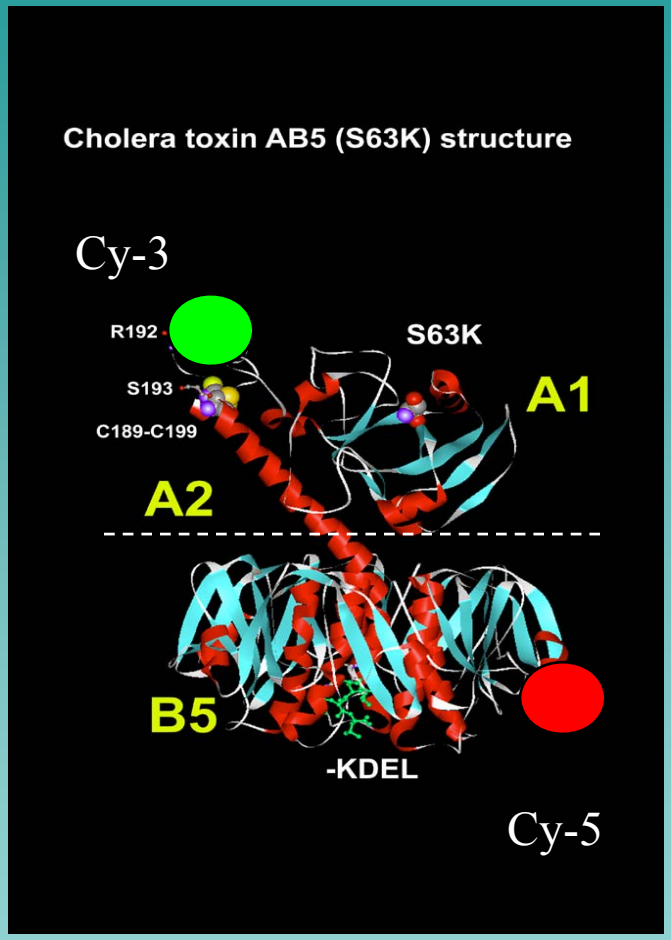




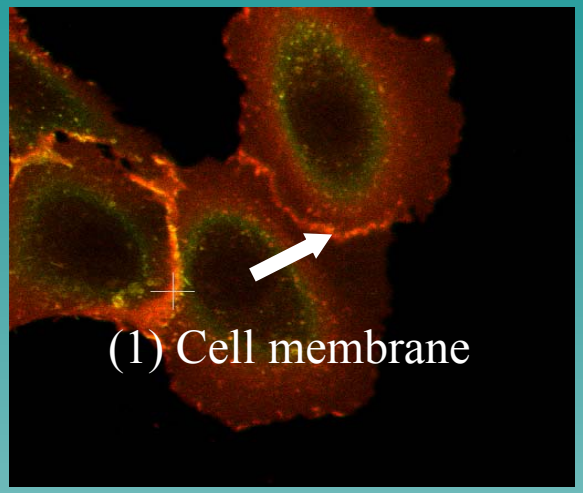


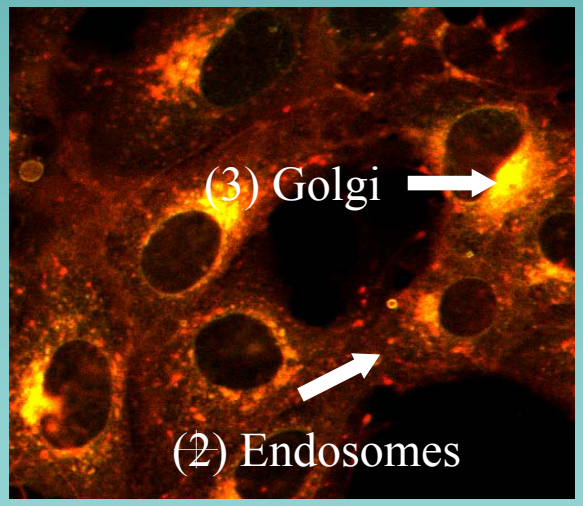
Comparable autocorrelations, existing cross-correlation: same pathway

FCCS reveals where the subunits dissociate

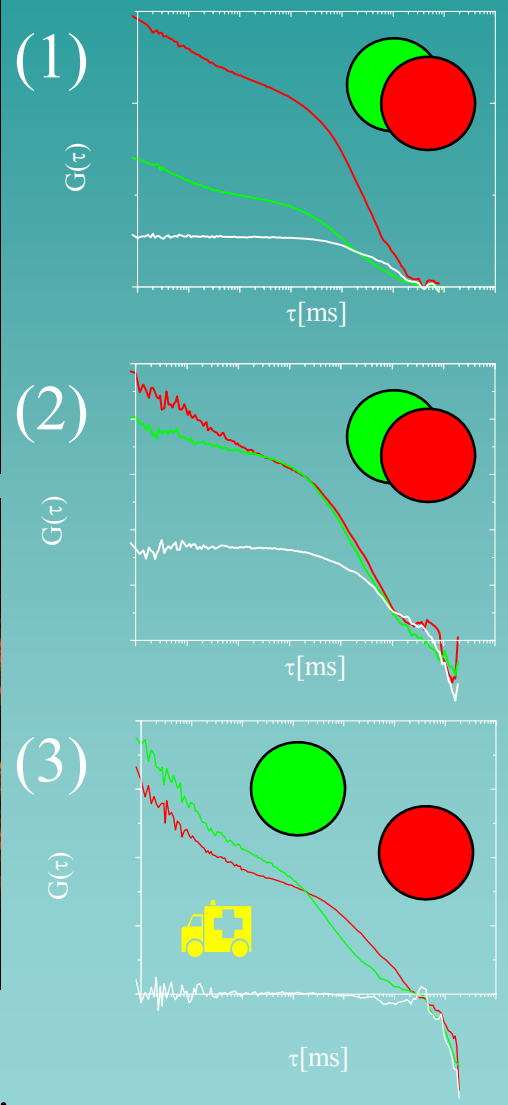


Dissociation of A and B_5 subunits required to induce toxicity of A

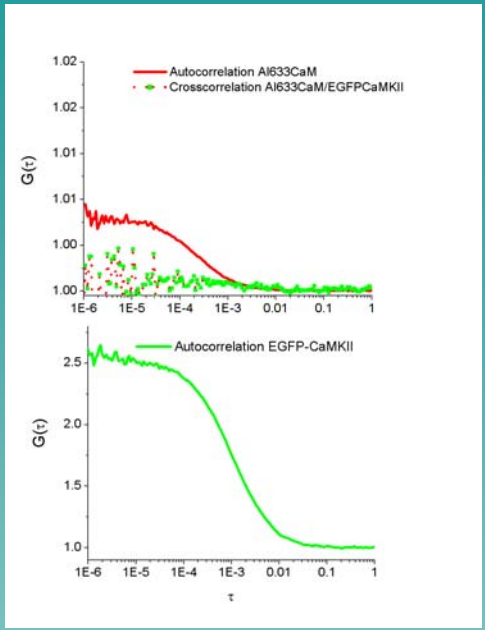


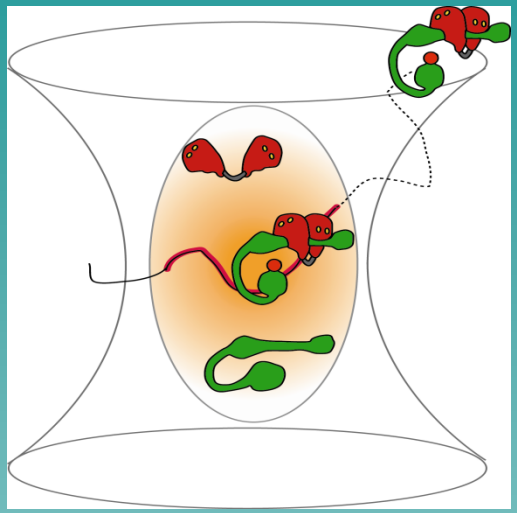


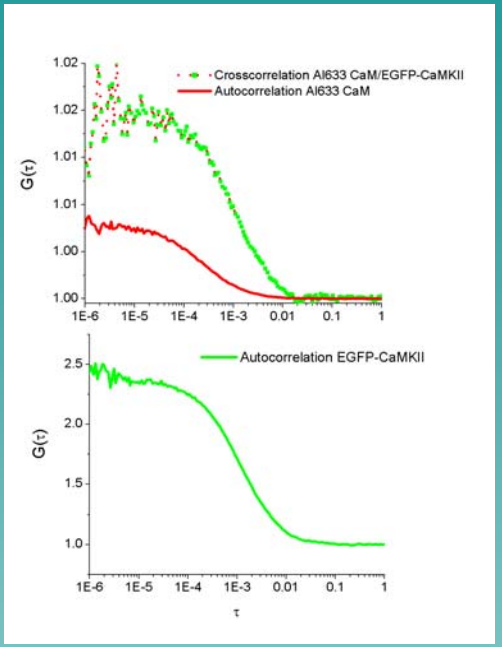
Cross-correlation finally decays to zero in the Golgi



Assessing Binding Ratios of CaM and CaMKII







***1/G(0) = N* : absolute particle number**

	+ EGTA	**+ MgATP**
eGFP-CaMKII	***N* = 0.73**	***N* = 0.73**
	cpms = 13.7	**cpms = 12.5**
Alexa 633CaM	***N* = 130**	***N* = 128**
	cpms = 3.6	**cpms = 4.1**
Cross-Correlation (KK)	**~0**	**251.1%**
(KK/AK$_2$)		

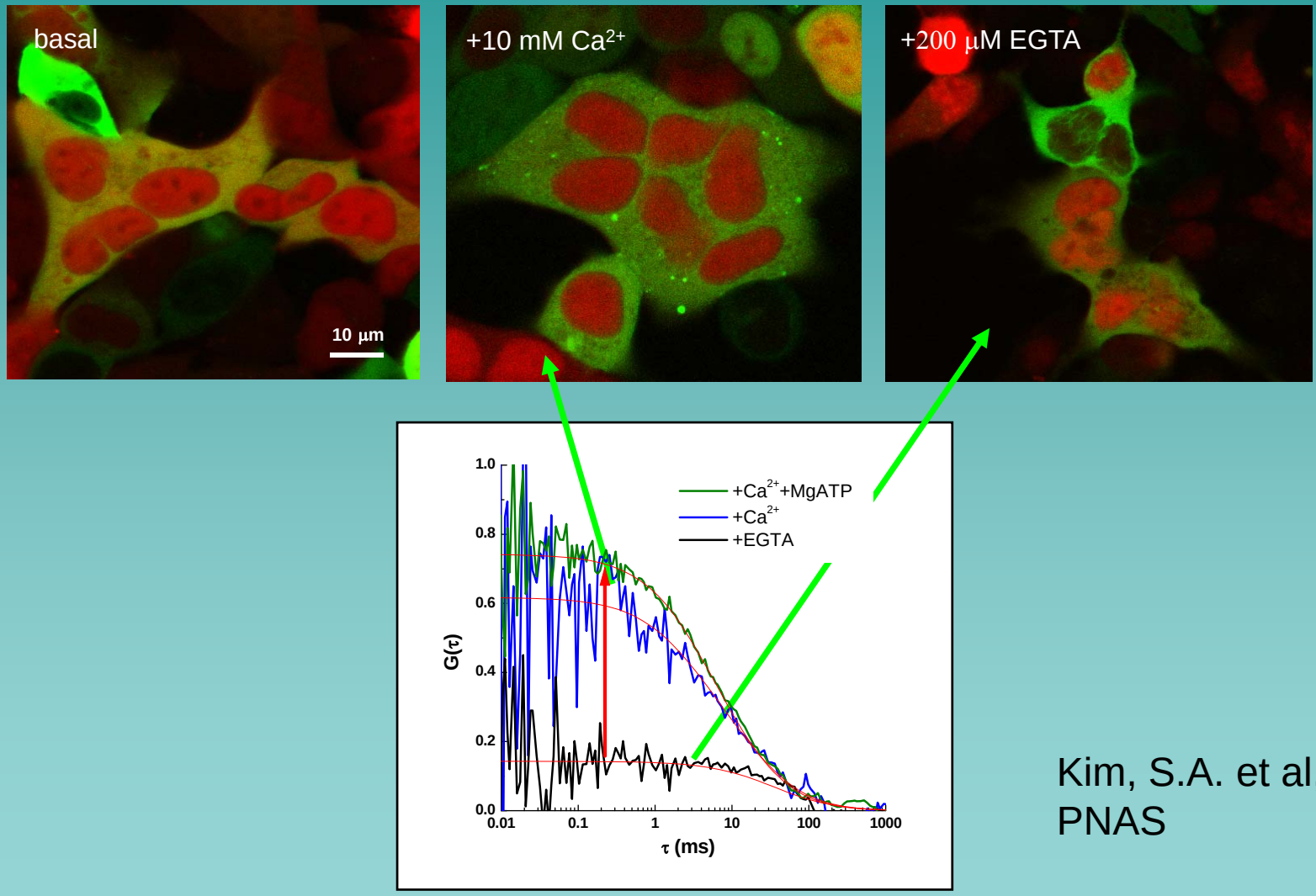
$$\frac{KK}{AK_2} = \frac{n \cdot C_{20}}{C_{20} + n(n-1)C_{10}} = \frac{n \cdot 130}{130 + n(n-1)0.73}$$

For

n = 1 -> KK = 100 %
n = 2 -> KK = 198 %
n= 3 -> KK = 296.7 %

Therefore, experimental data means an average of 2.5 Alexa 633 CaMs are bound to 1 eGFP-CaMKII.

Protein Signaling Analyzed with Cross-Correlation



Kim, S.A. et al, 2004
PNAS

Other Applications of MPE

- Uncaging of fluorescent compounds: inherent spatial localization provides excellent spatial selectivity for uncaging

- In vivo imaging over long time scales (months):

  e.g., watching the development of amyloid plaques develop over months in the brain of a living animal