Rice/TCU REU on Computational Neuroscience

Fundamentals of Molecular Imaging

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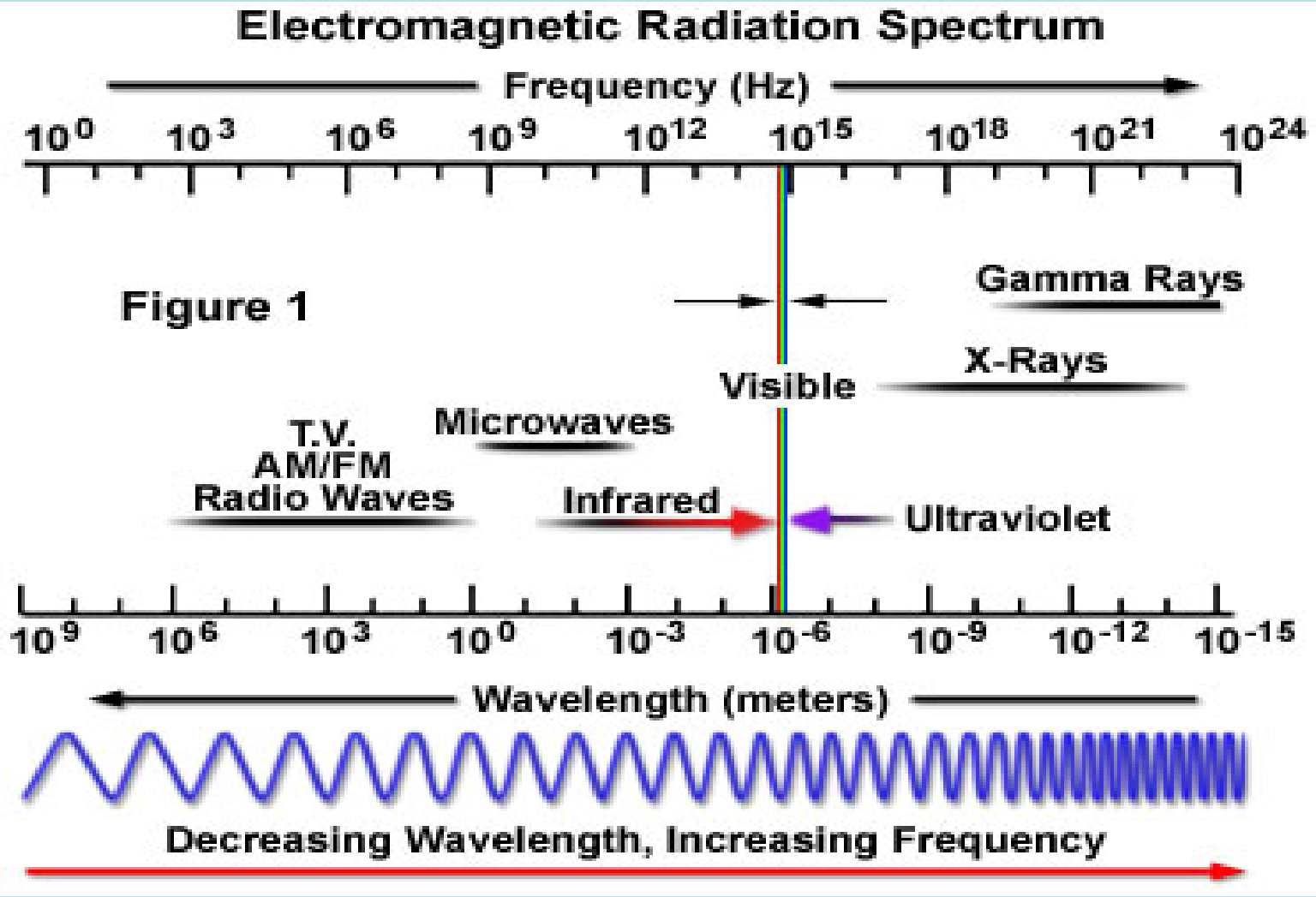
Objectives

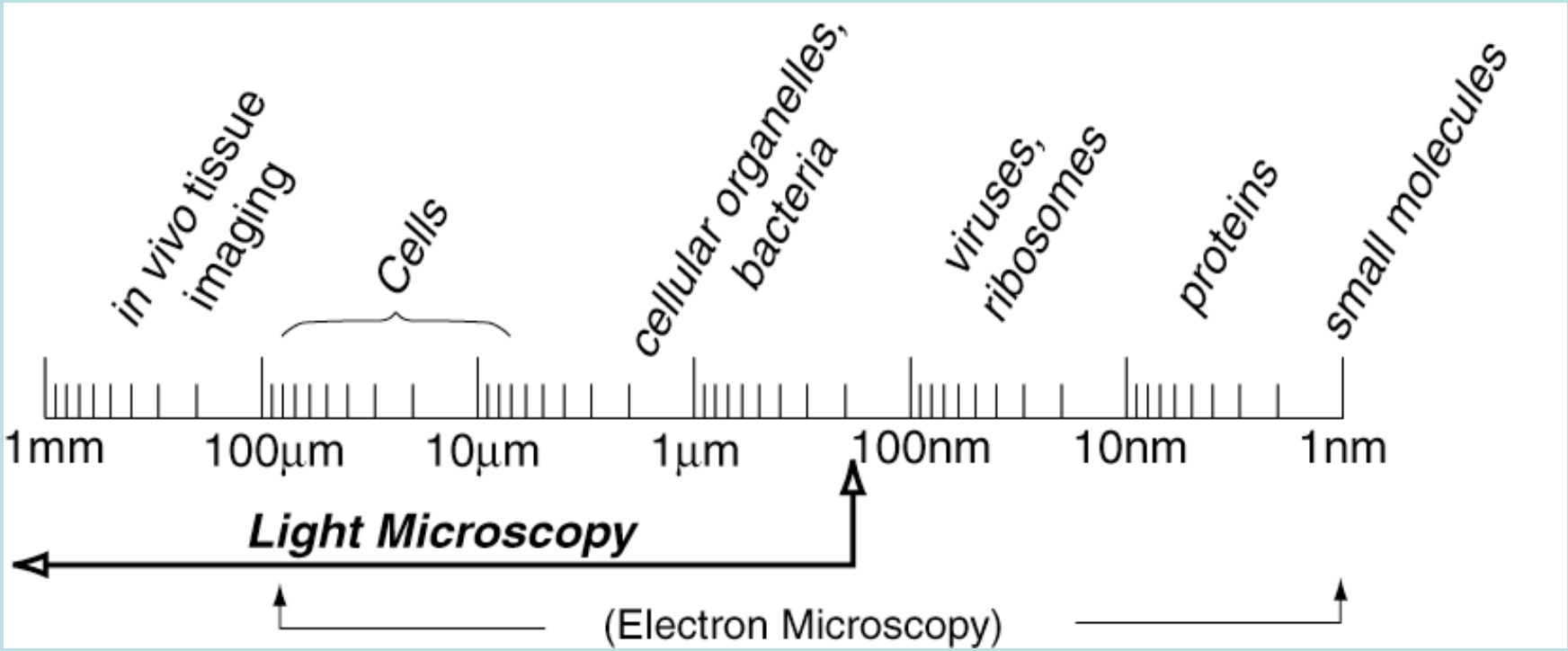
- Introduction to resolution in light microscopy
- Brief discussion of lasers as excitation sources
- Multiphoton excitation-What is it?
  advantages/disadvantages
- Fluorescent Probes-uses and characteristics
- Applications of MPE to the study of intracellular biochemistry
- The concept of single molecule analysis



Resolution = 0.61λ / NA

λ = wavelength of electromagnetic radiation

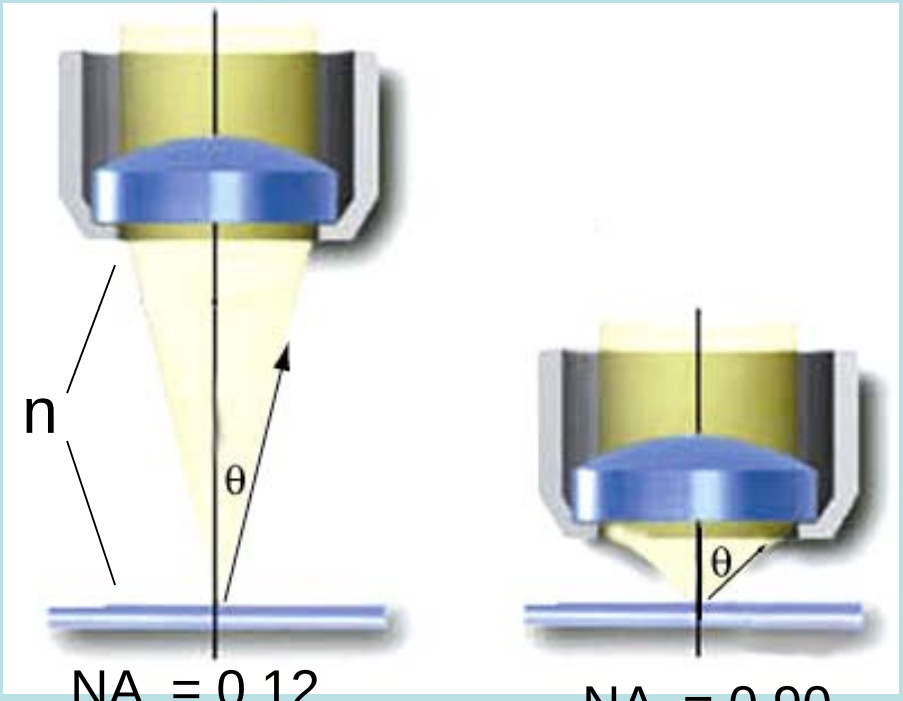
Spatial and Temporal Scales of Microscopy



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

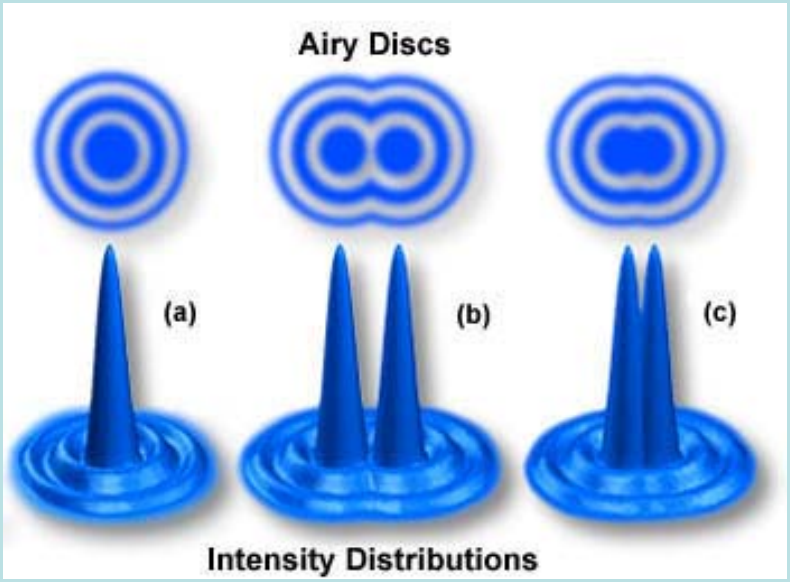
θ ~ 64°

Short working distances

Rayleigh criterion:

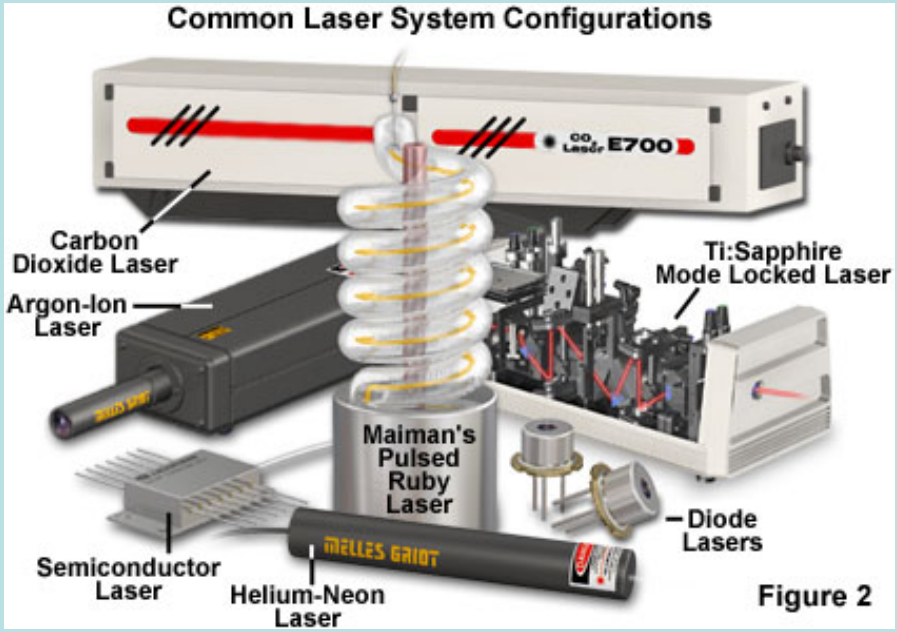
Resolution = 0.61λ / NA

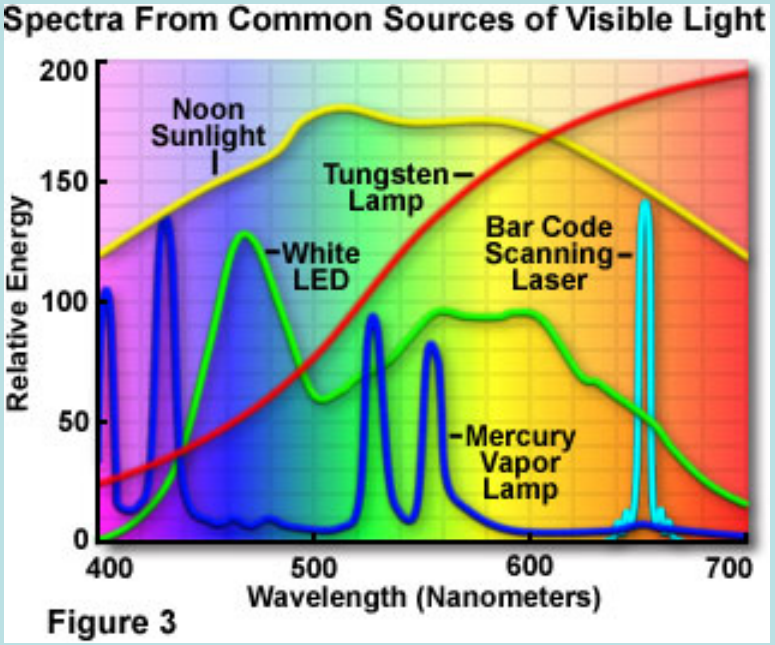
$R = 1.22\lambda / (NA_{obj} + NA_{cond})$

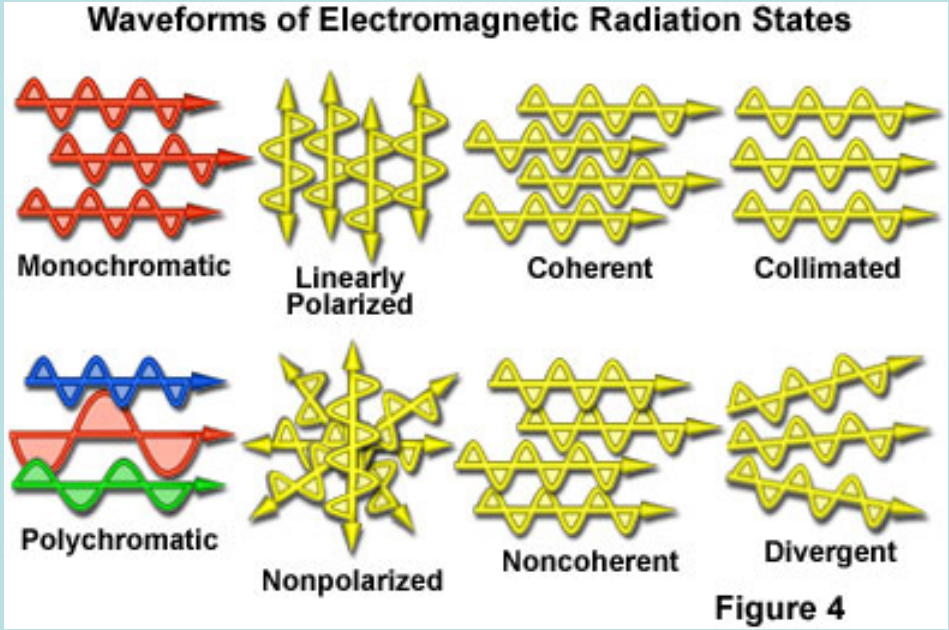


Destructive Interference

Characteristics of Light from Lasers

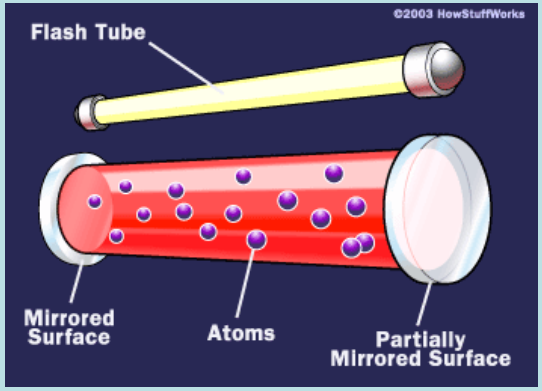


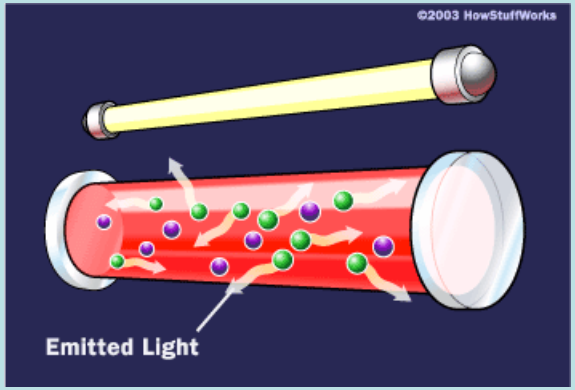


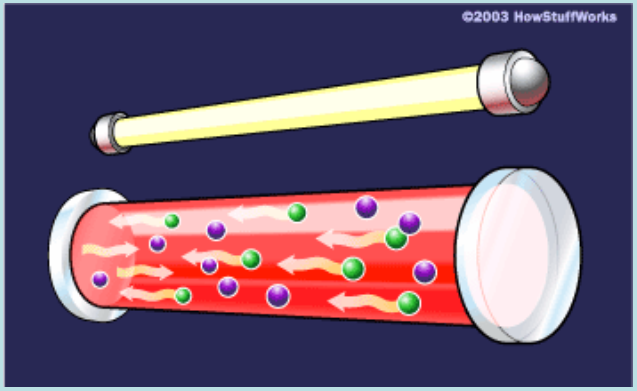


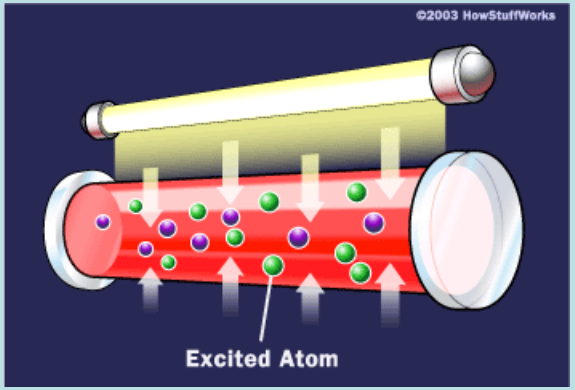
Light **A**mplification by **S**timulated **E**mission of **R**adiation

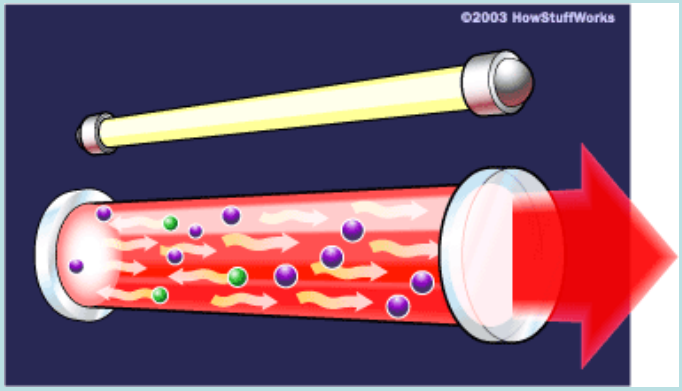
How Lasers Work



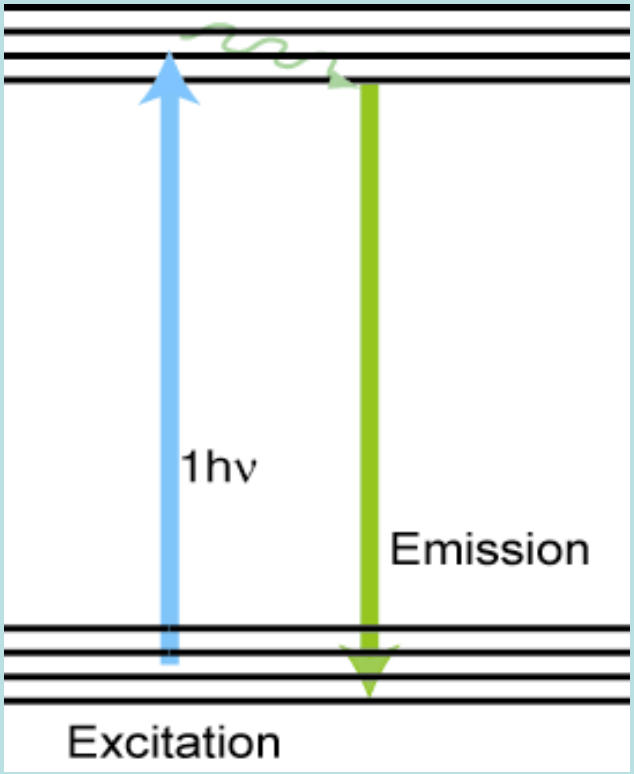


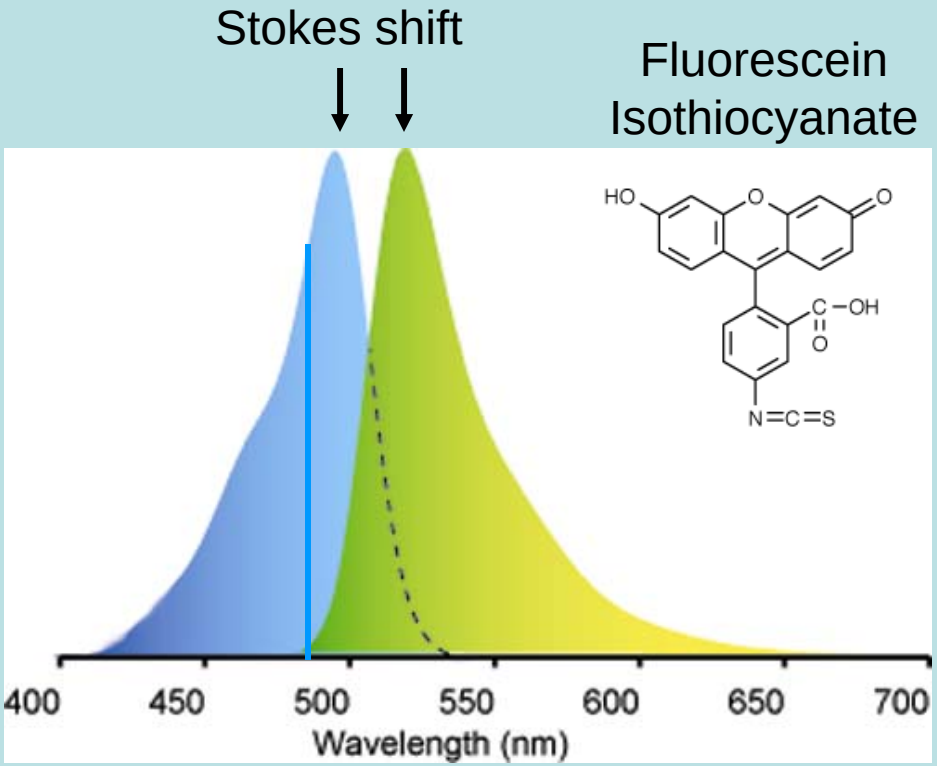






Fluorescence



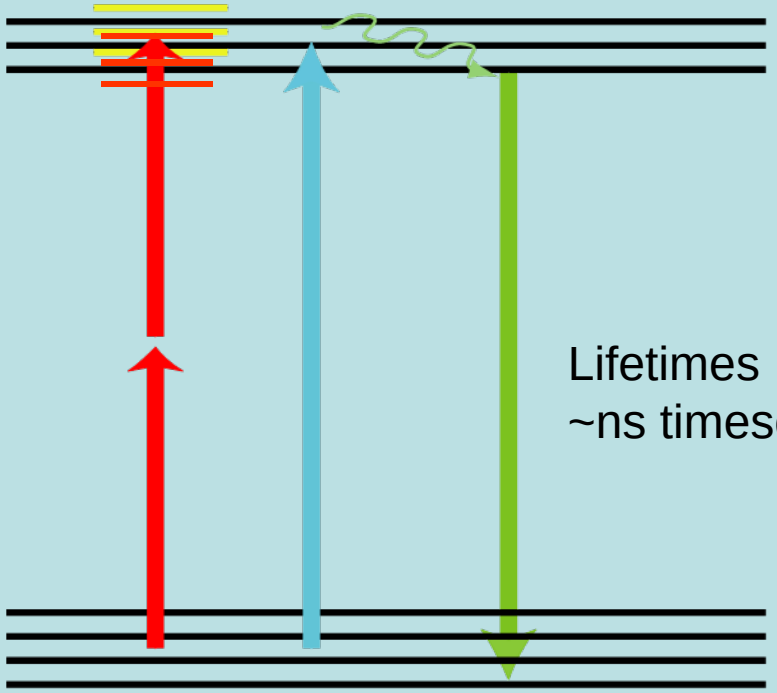


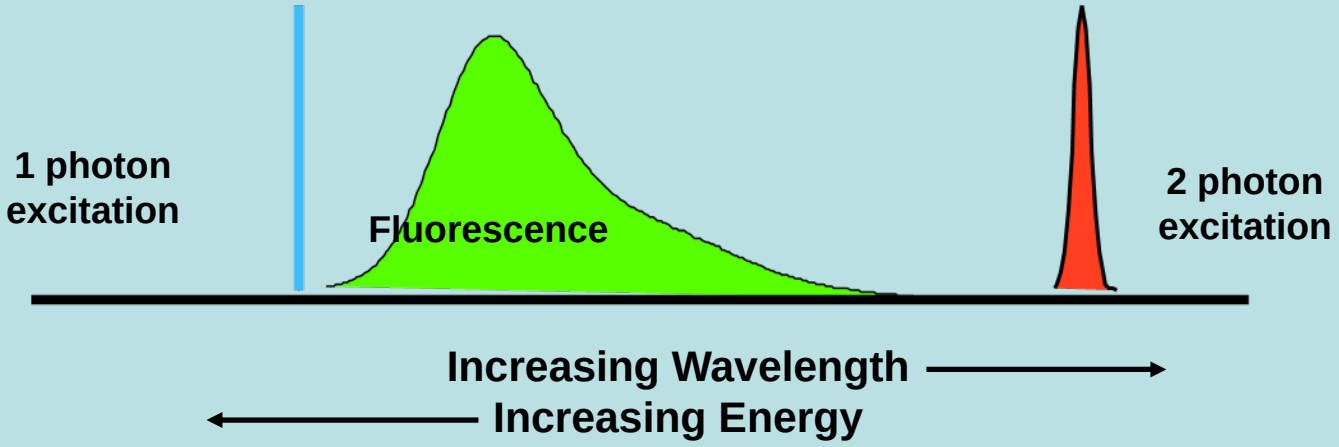
Following absorption, a number of vibrational levels of the excited state are populated. Molecules in these higher vibrational levels then relax to the lowest vibrational level of the excited state (vibrational relaxation). From the lowest vibrational level, there can be an emission of photon of lower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

One Photon vs. Two Photon Florescence?

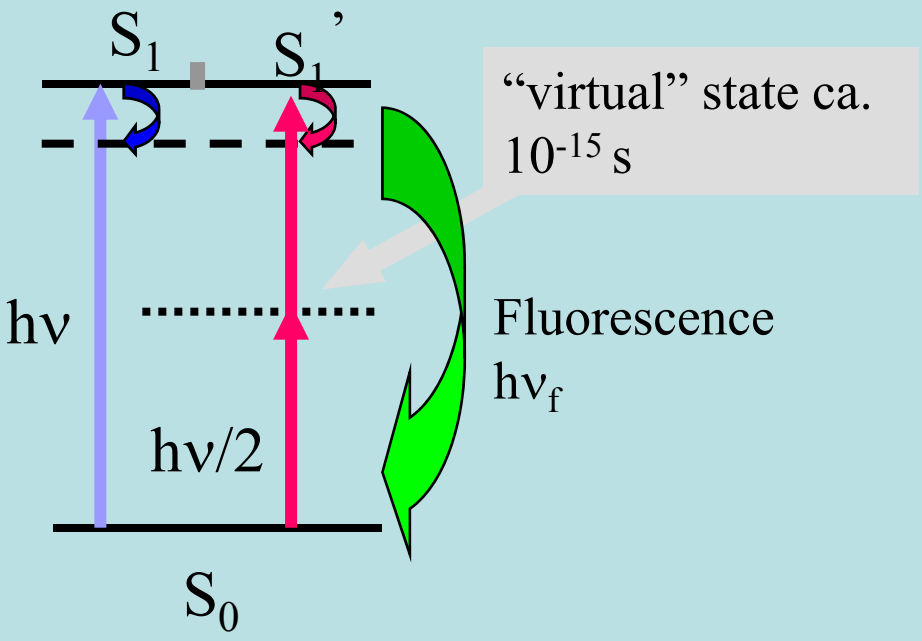
Two (or more) photons can interact simultaneously with a molecule adding their energies to produce an excitation equal to the sum of their individual energies.

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





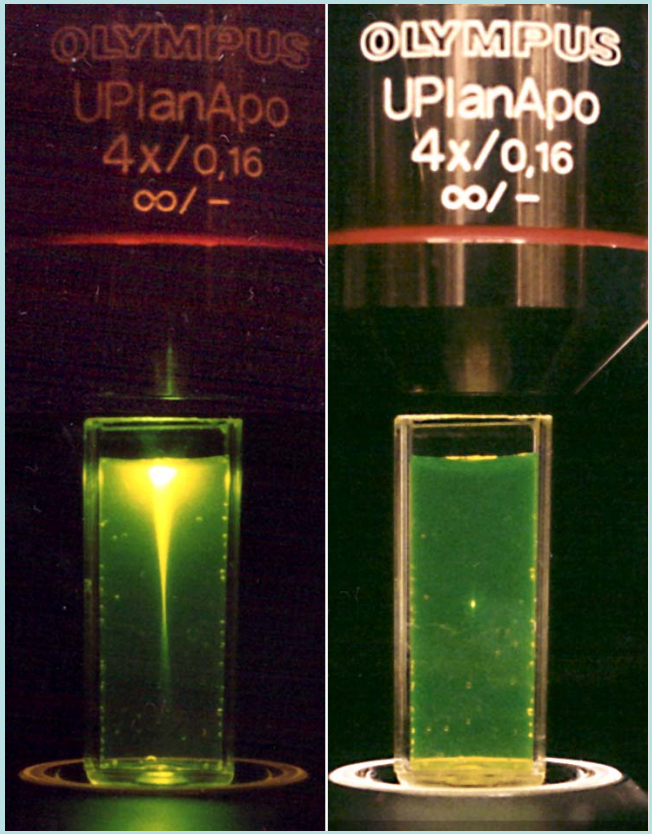
Two/(Multi)-Photon-Excitation



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

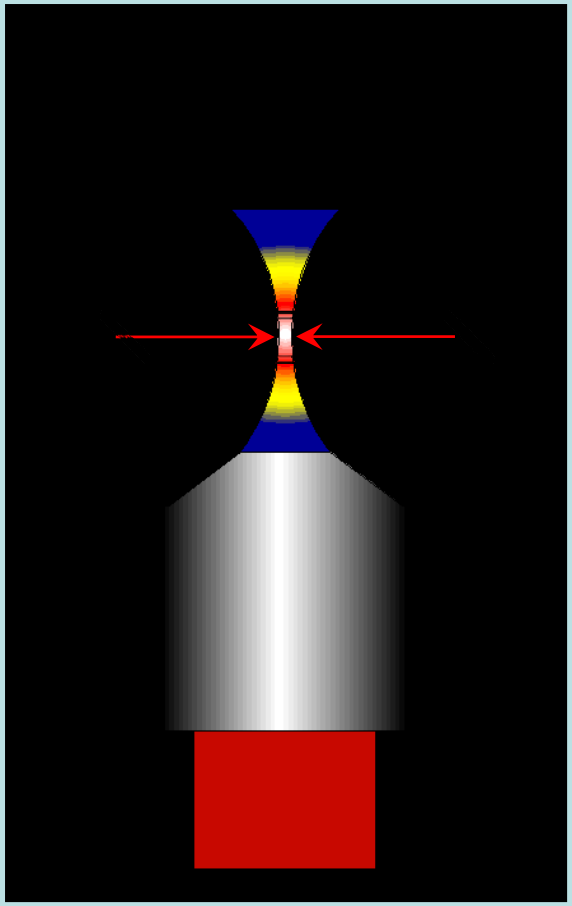
Major advantage:
Inherent spatial sectioning by I^n -
dependency of excitation probability.
Excitation only in vicinity of focal spot

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

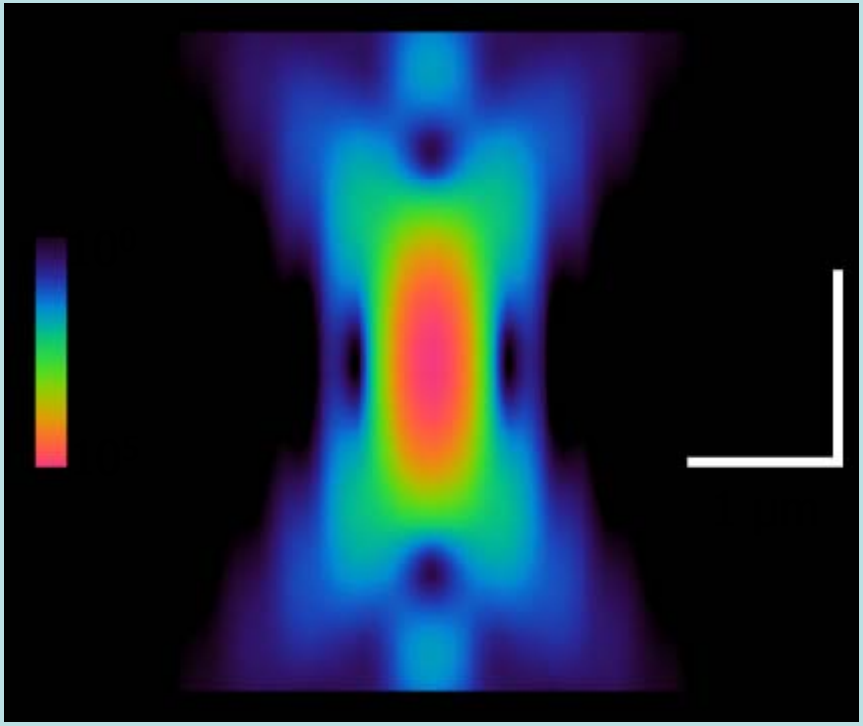


Pulsed excitation = (100 fs, 80 MHz)

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

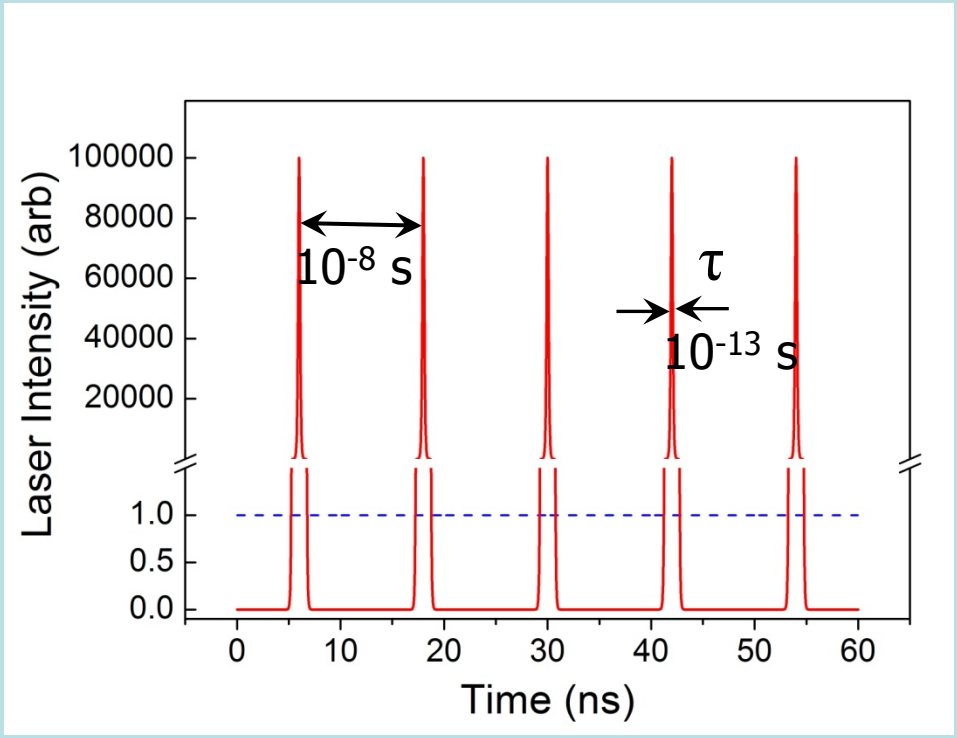


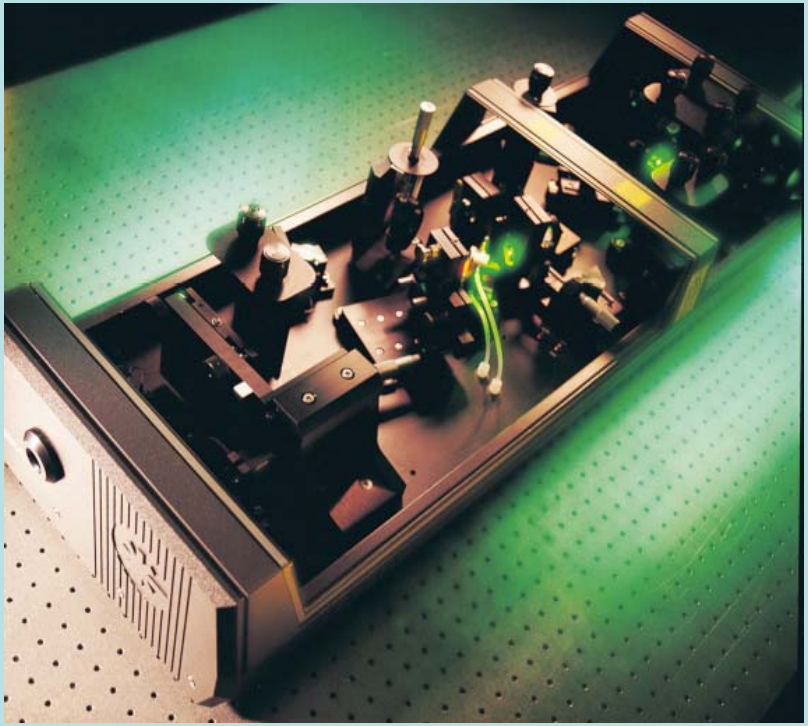
The intensity (squared) declines from z (red arrows) as z^{-4}



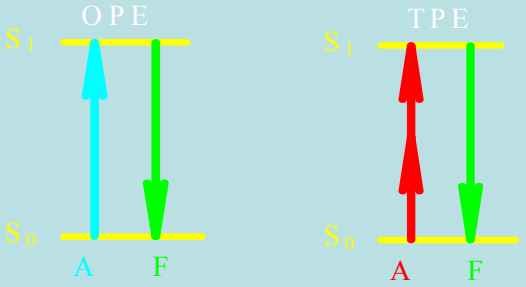
Calculated intensity of 740 nm light near focus of 1.2 NA objective

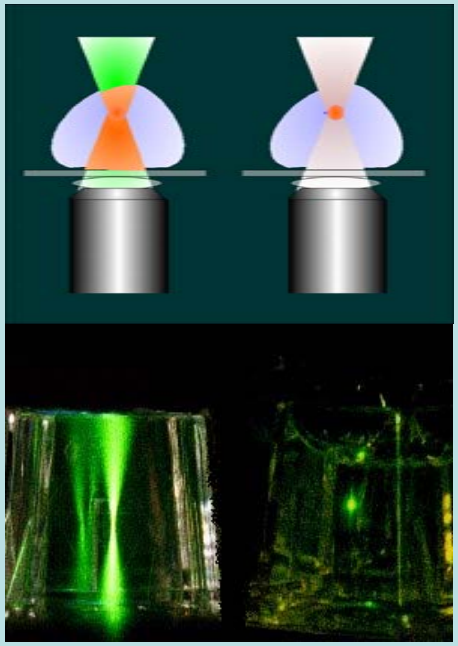
Pulsed laser excitation enhances two-photon absorption





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:

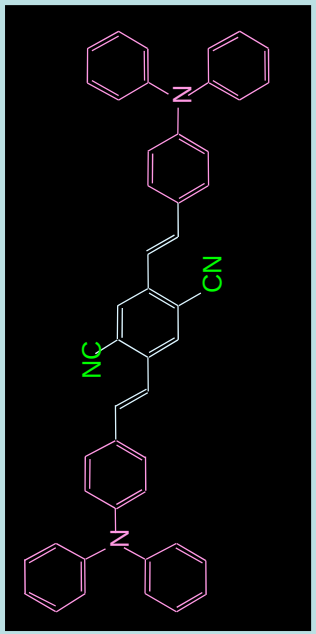
1. Labels - free dyes that may partition to a specific region of a cell or tissue, or fluorescent molecules that are bound to antibodies, receptor proteins or other biomolecules of interest.

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.

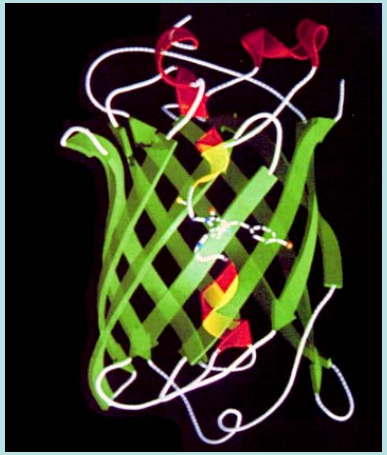
Fluorescent Probes

molecules



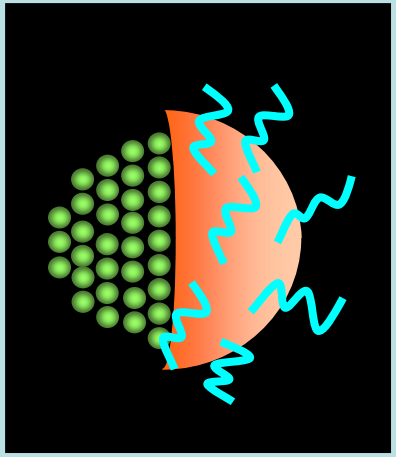
1 nm

EGFP



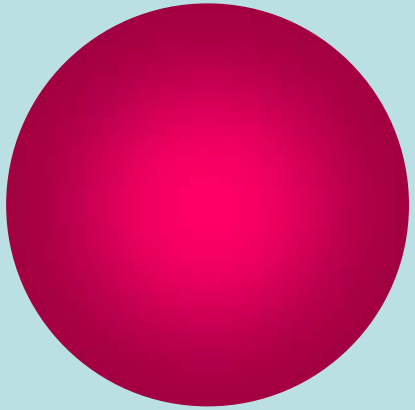
3 nm

quantum dot



6 nm

silica nanoparticle
(rhodamine)

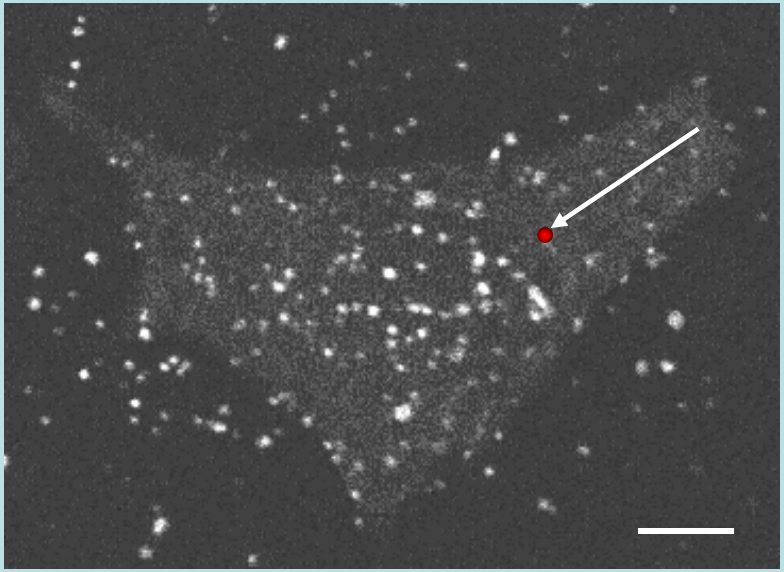


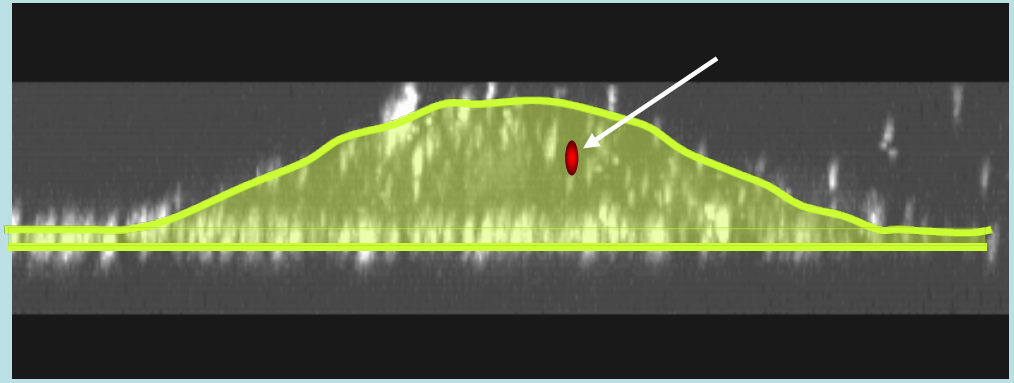
50 nm

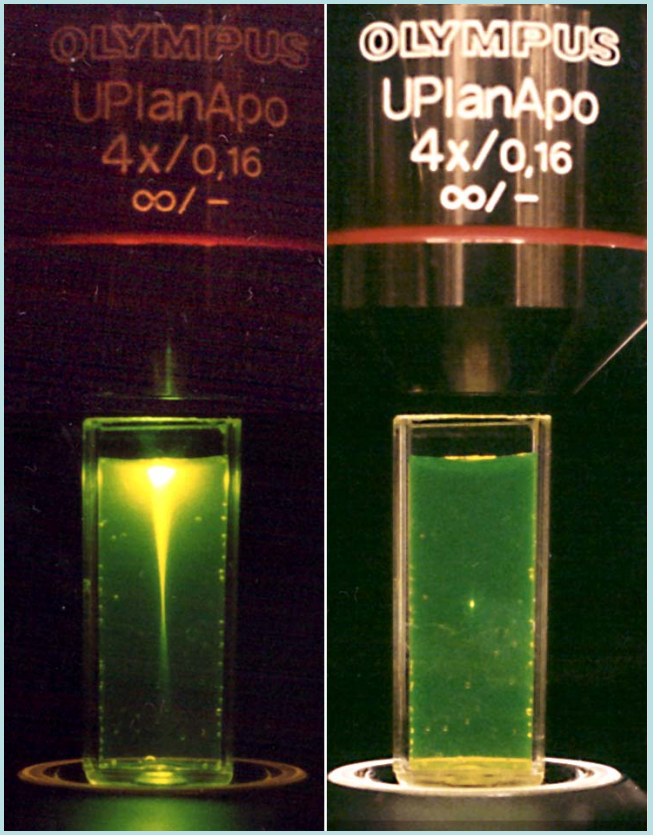
Single fluorophores

Multiple fluorophores

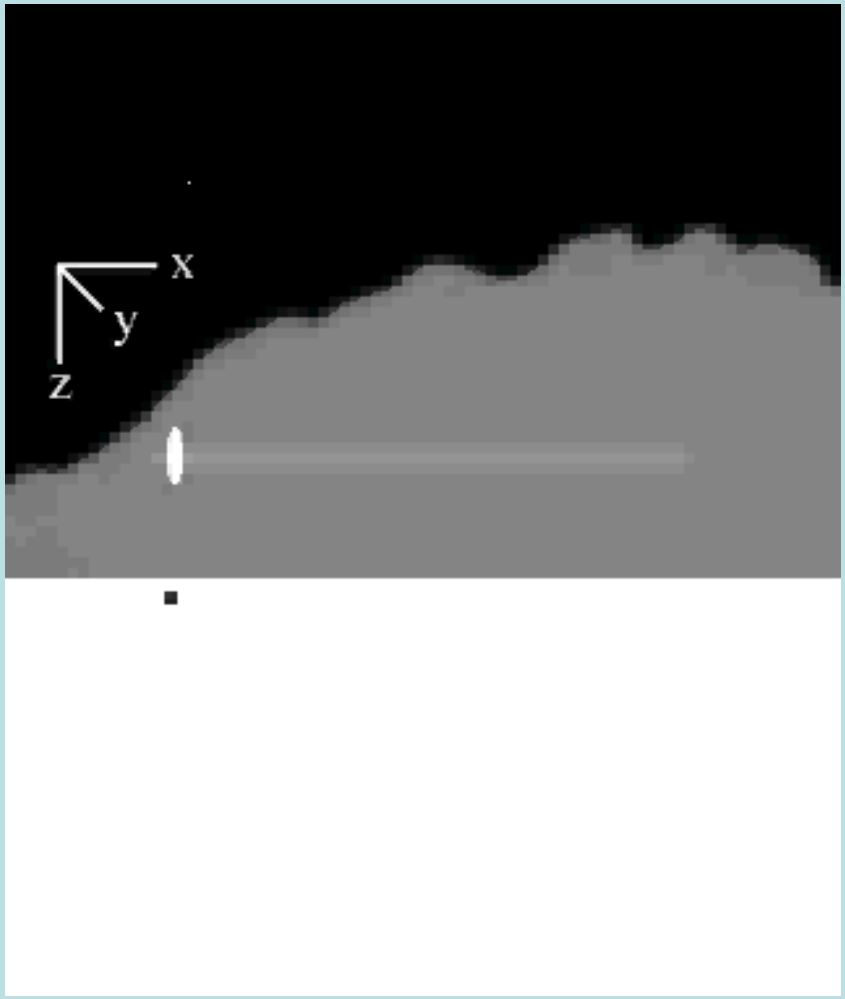
Two Photon Laser Scanning Microscopy Coupled to Spectroscopy Techniques







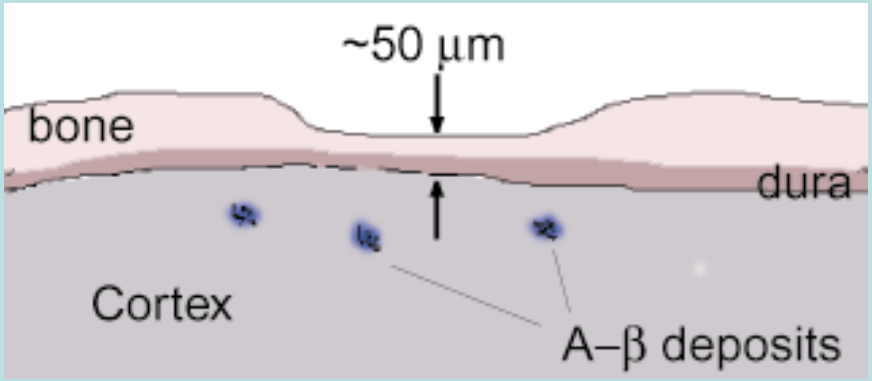
How do you generate an image?

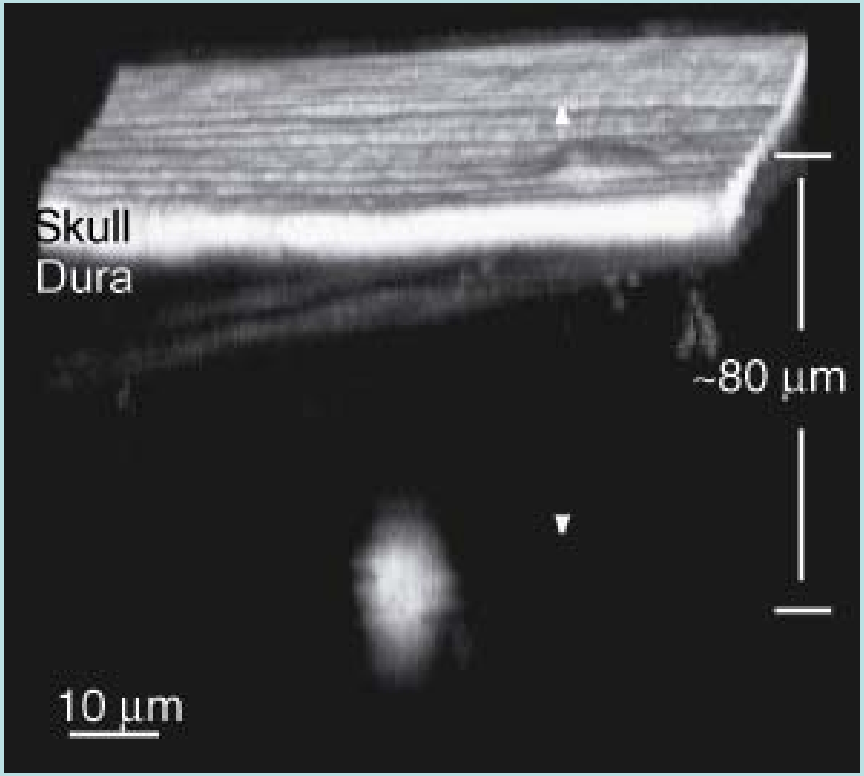


Scanning mirrors move the laser beam back and forth across the sample

A detector collects the photons that come out of a single area and map them onto an X-Y Image as pixels.

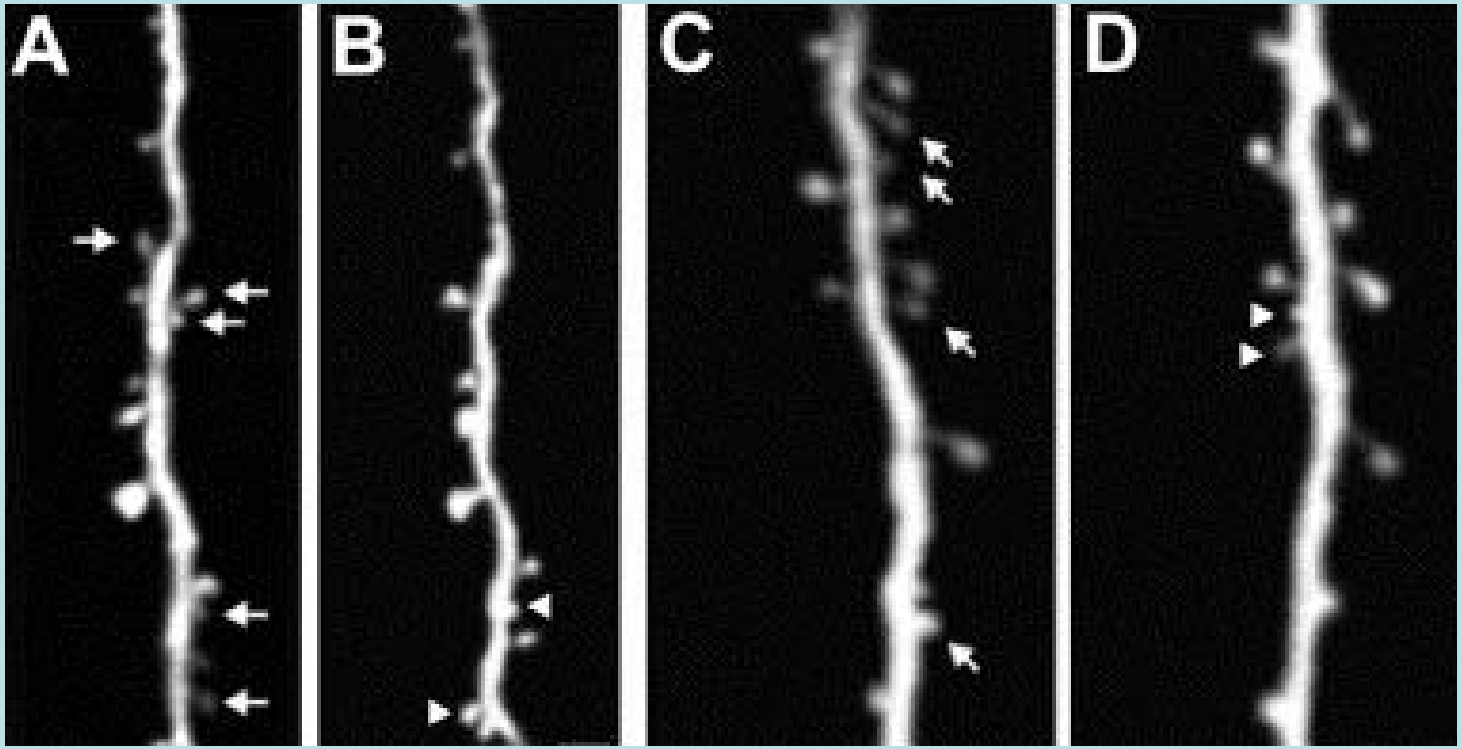
In vivo imaging - example: transgenic mouse models of Alzheimer's disease.





β amyloid plaque stained with Thio-S, excitation at 760 nm

Two-photon Imaging in Live Animals

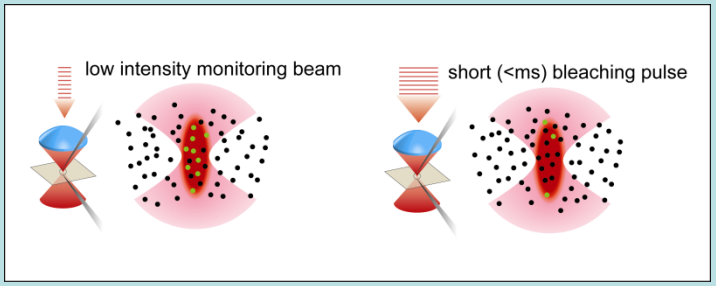


Neurons imaged in the brain of a live animal 18 months apart
Arrows-spines eliminated; Arrowheads, spines formed

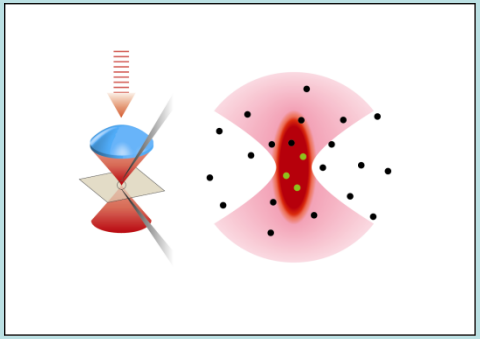
From Zuo et al., Neuron 2005

Optical Methods Applied to Study Protein Dynamics

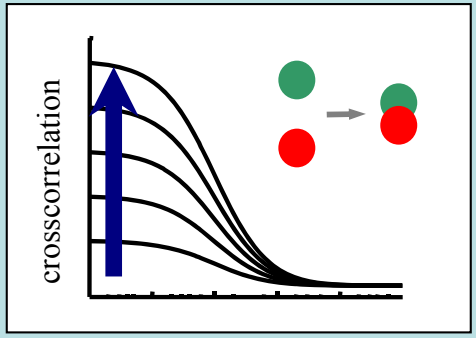
1. Two-Photon Fluorescence Photobleaching Recovery (TPFPR)



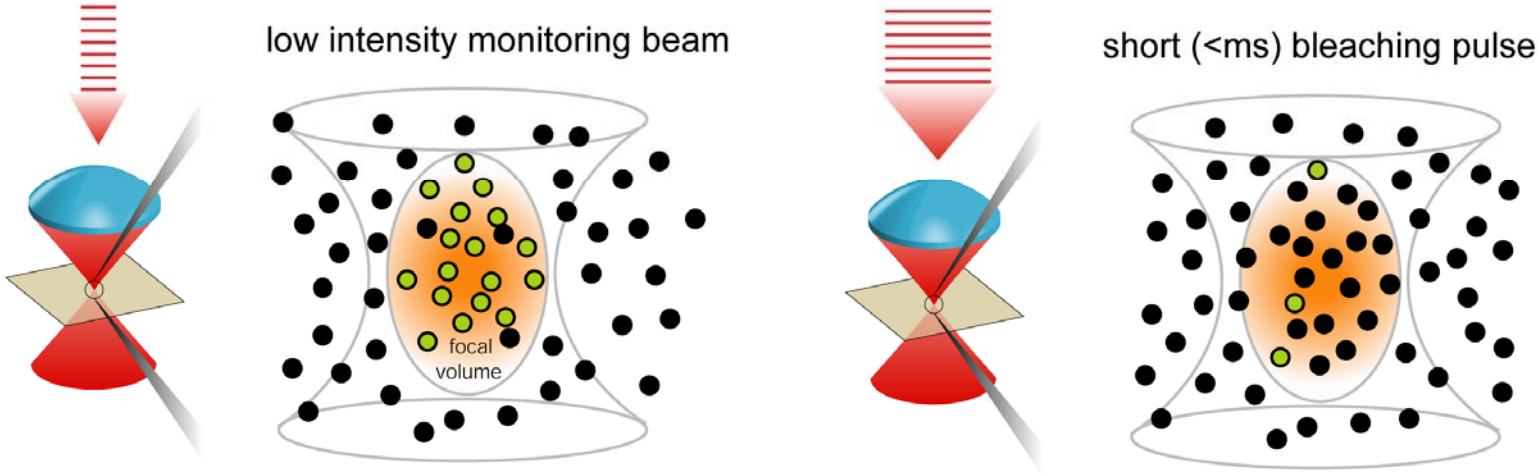
2. Two-Photon Fluorescence Correlation Spectroscopy (TPFCS)

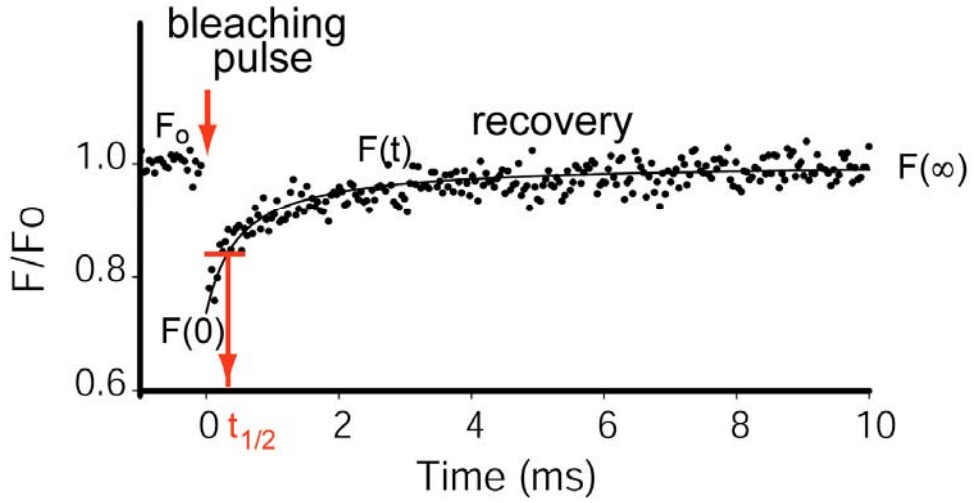


3. Two-Photon Fluorescence Dual-Color Cross-Correlation (TPCCS)



Two-Photon Fluorescence Photobleaching Recovery (TPFPR)





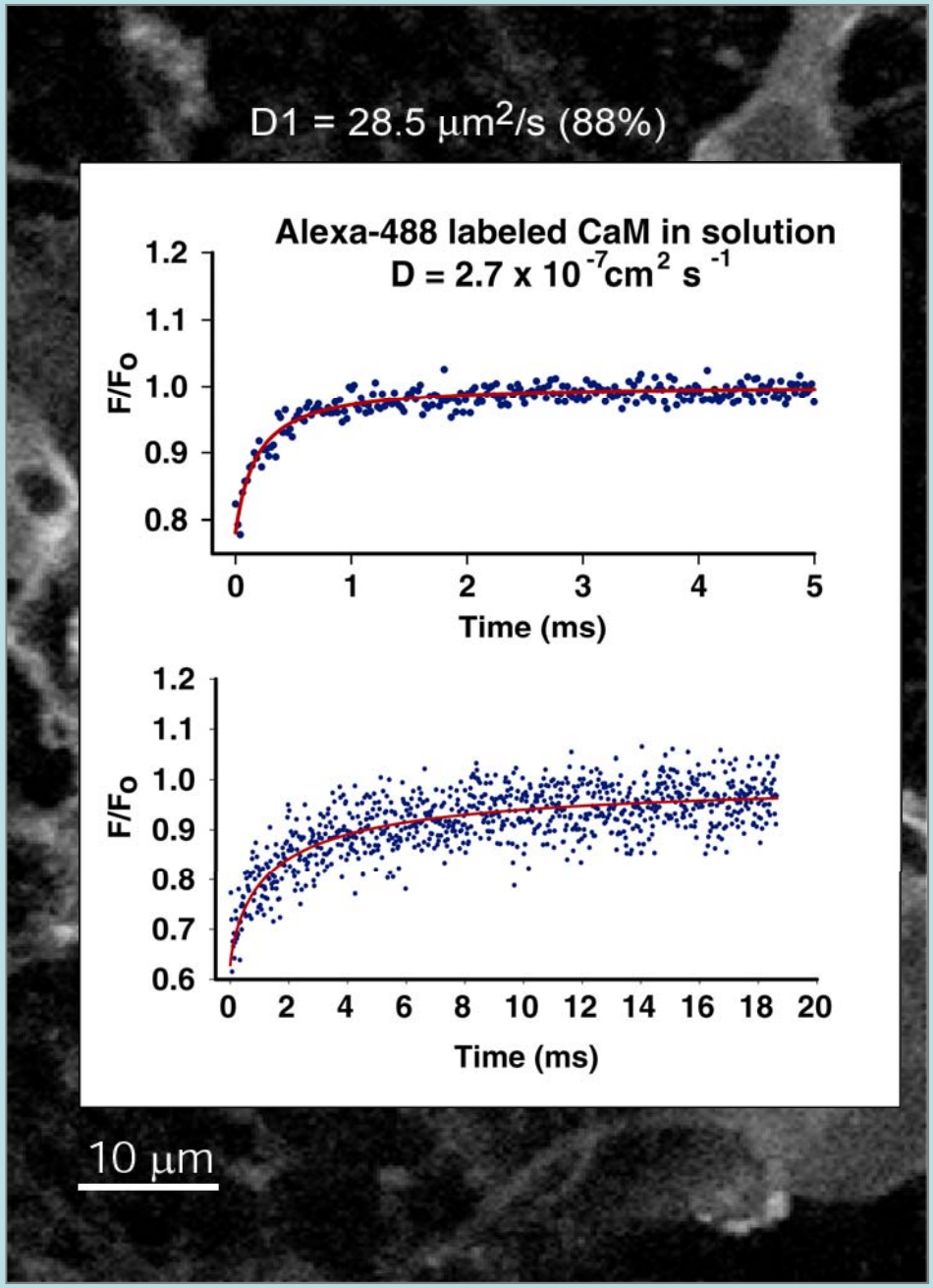
Diffusion Mapping of Alexa-488-Labeled Calmodulin in Neurons Using MPFPR

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



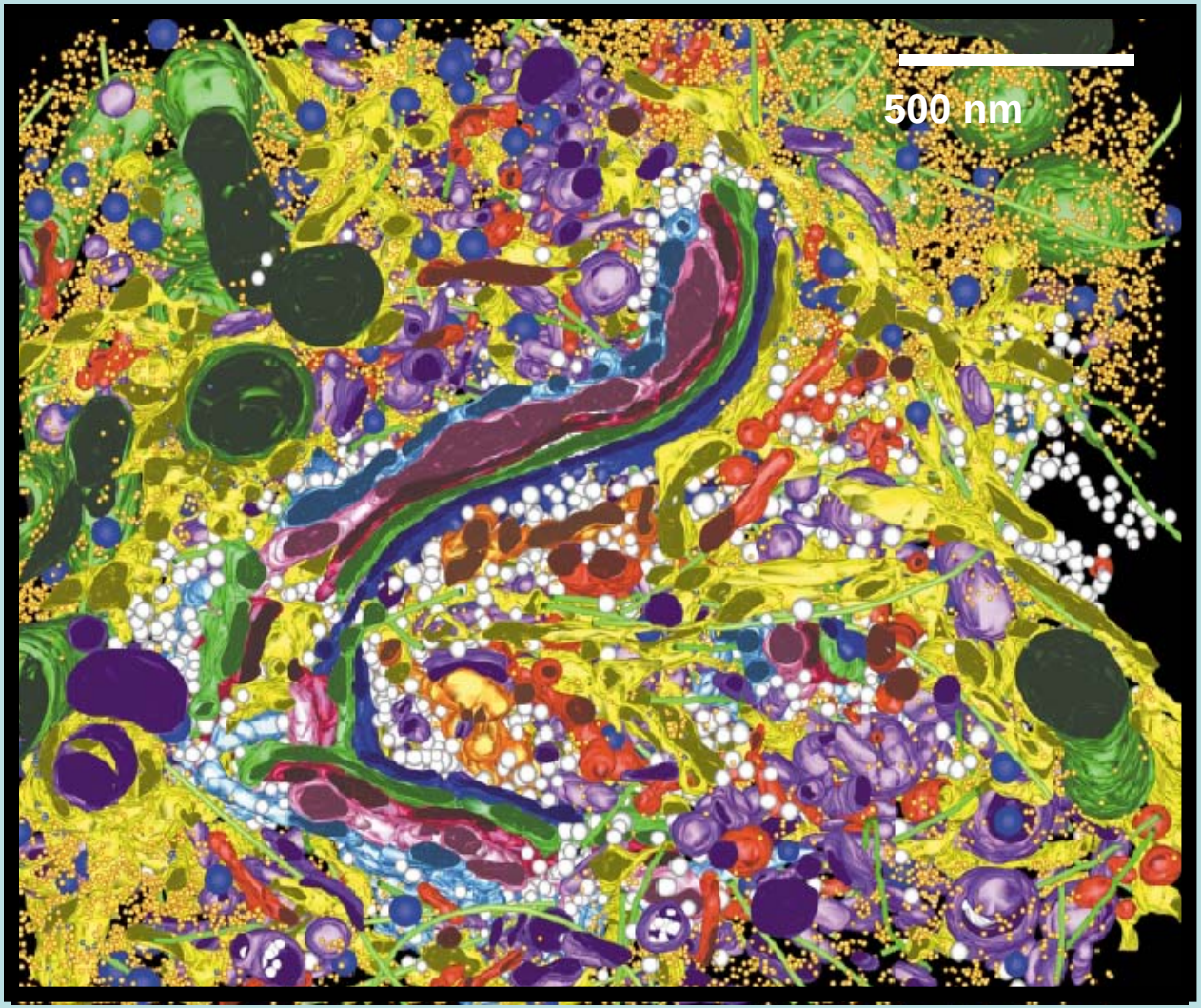
Comparison of Diffusion Coefficients from *in Vitro* and *in Situ* FPR Measurements

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

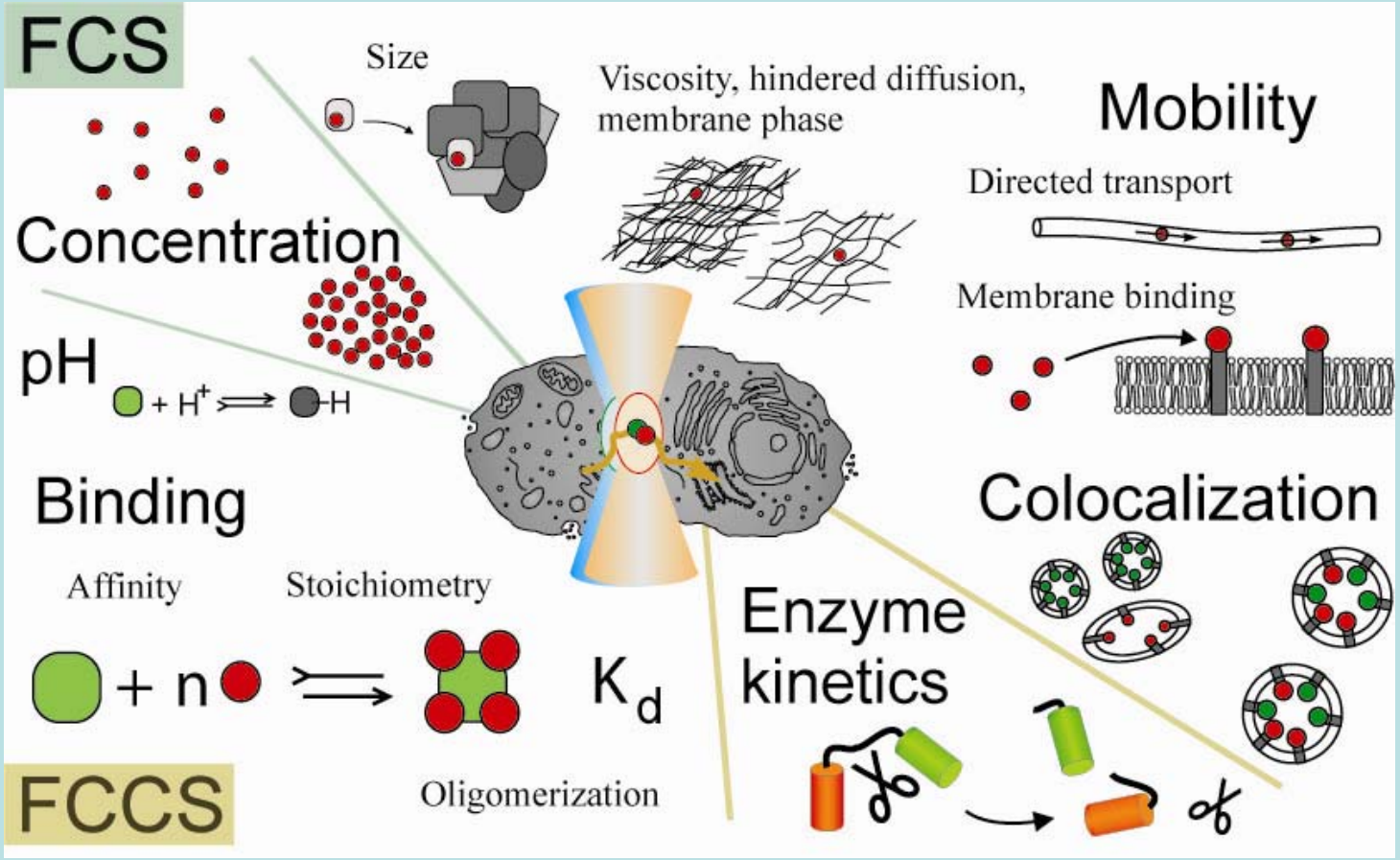
D= diffusion coefficients ($\mu m^2/s$); modified from Luby-Phelps, 2001

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

Intracellular Diffusion: Far from Simple



Applications of Single Molecule Approach to Biochemistry and Cell Biology

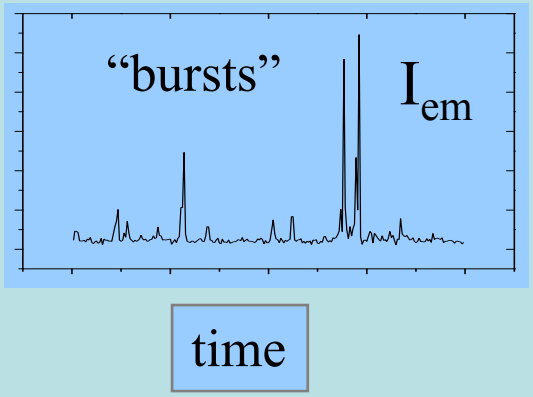


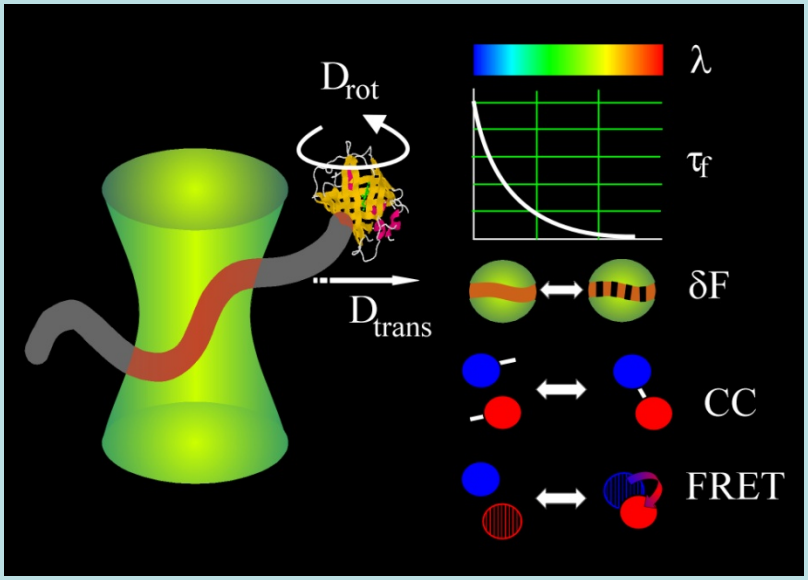
Fluorescence Correlation Spec./Fluorescence Cross Correlation Spec.

How to detect single molecules ?

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

Small volume elements achieved through confocal or multiphoton optics

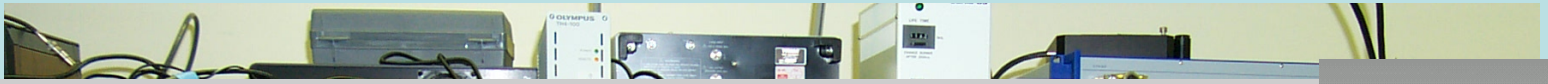


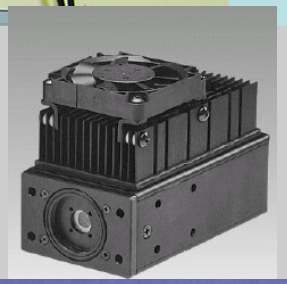


Primary measurement parameter is *signal fluctuations* induced by:

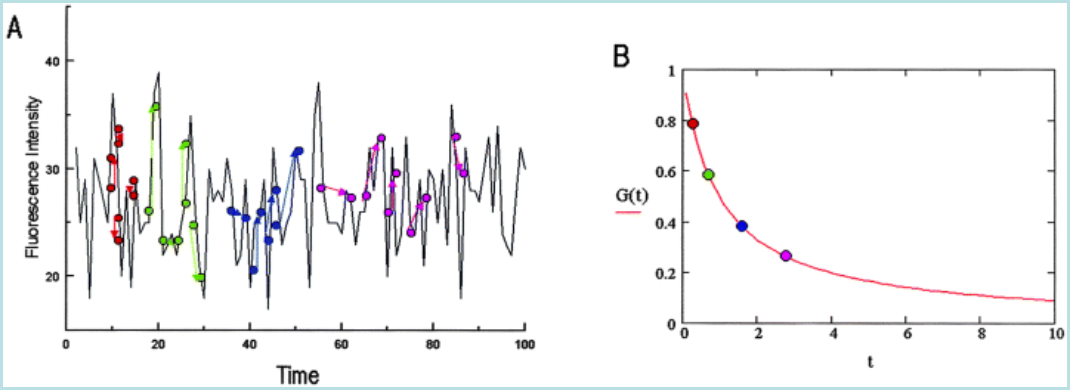
- Diffusion of molecules through the open measurement volume
- Intramolecular dynamics which affect the fluorescence emission

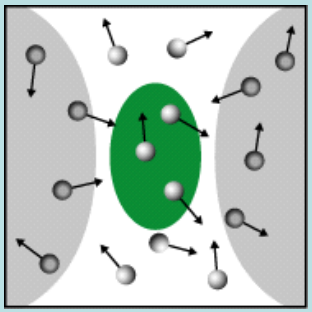
Experimental Apparatus



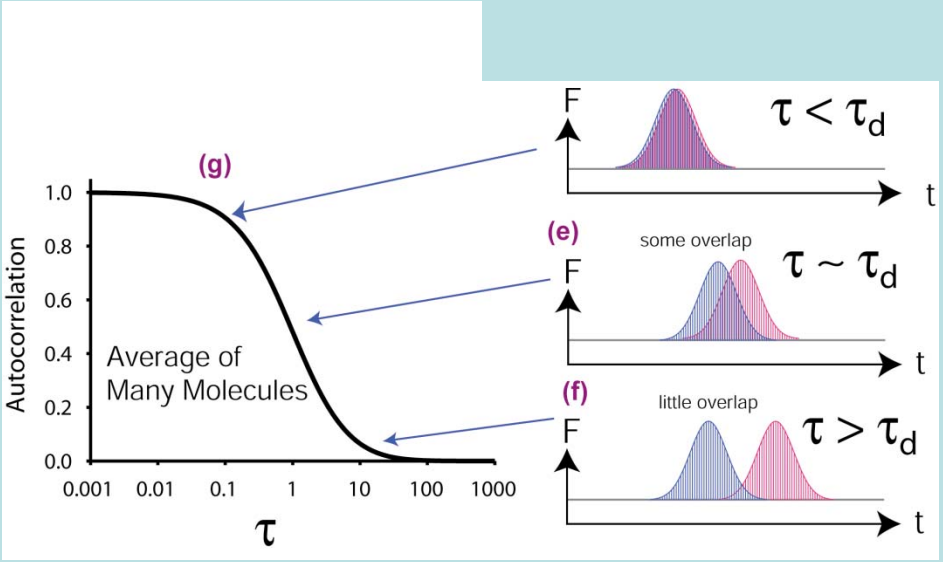


Analysis of Fluorescence Fluctuations





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

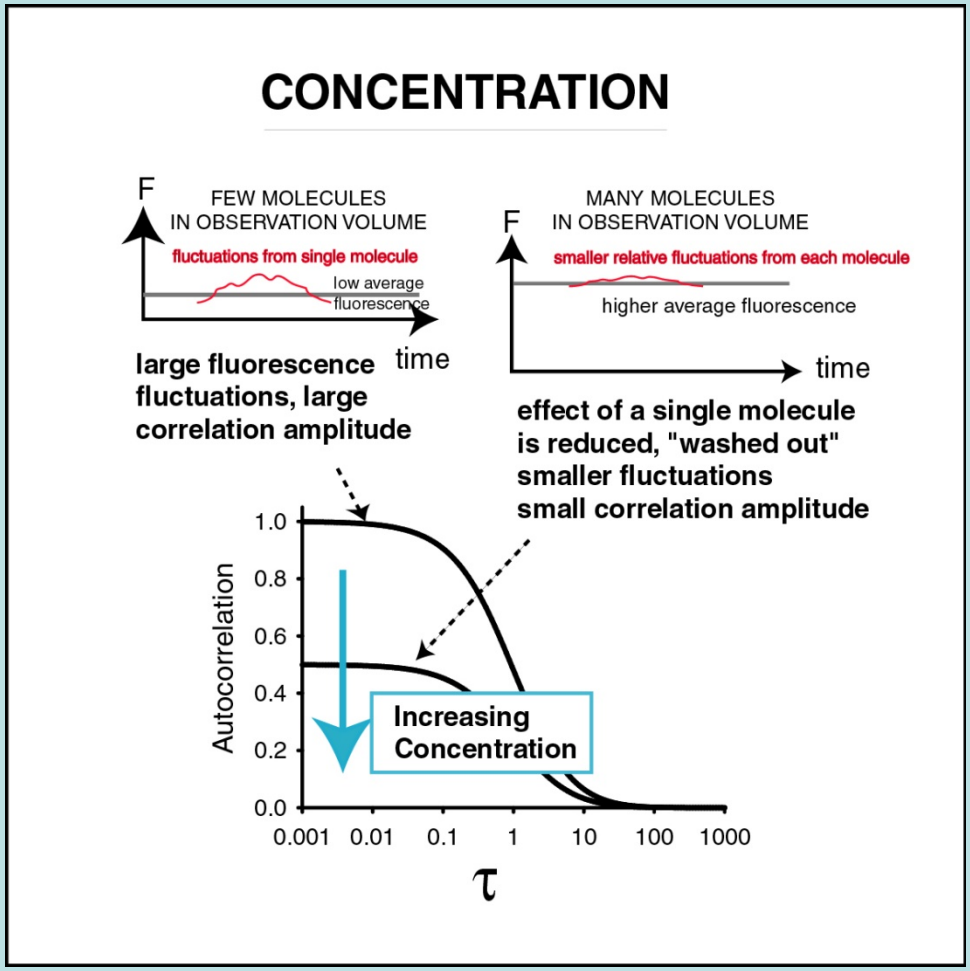


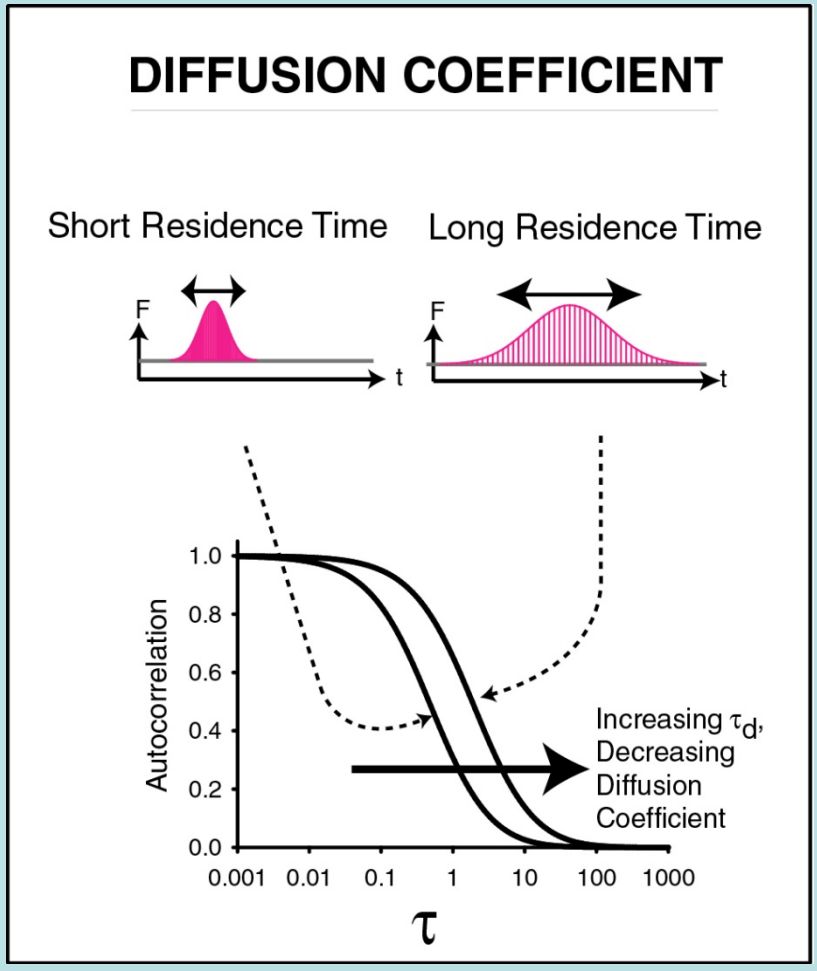
Temporal analysis of spontaneous fluorescence fluctuations -δF-

Signal fluctuations are induced by:

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

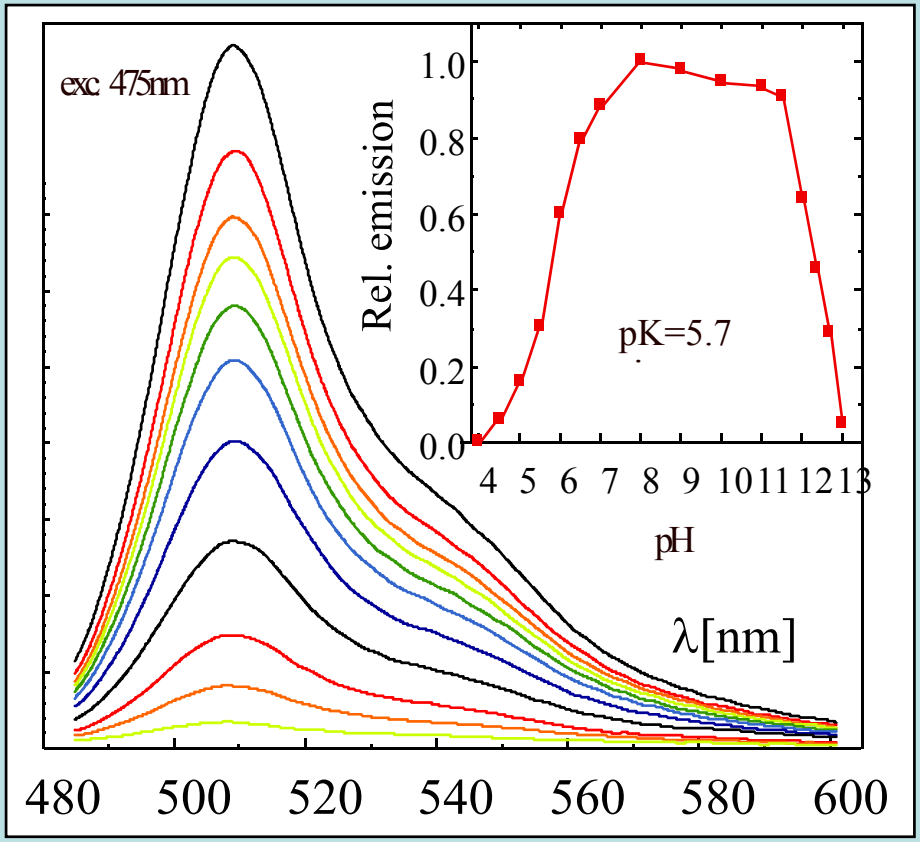
Parameters Provided by Fluorescence Correlation Spectroscopy (FCS)

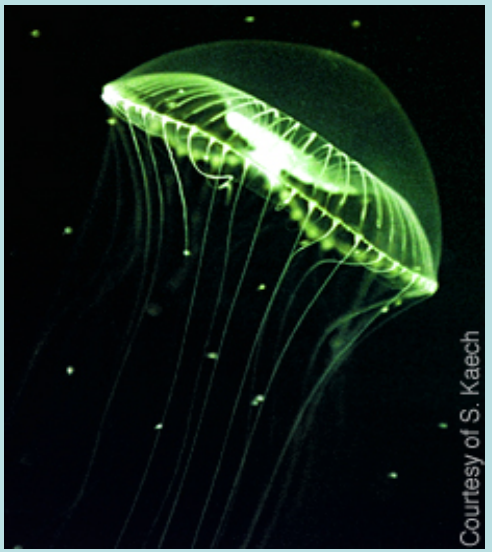


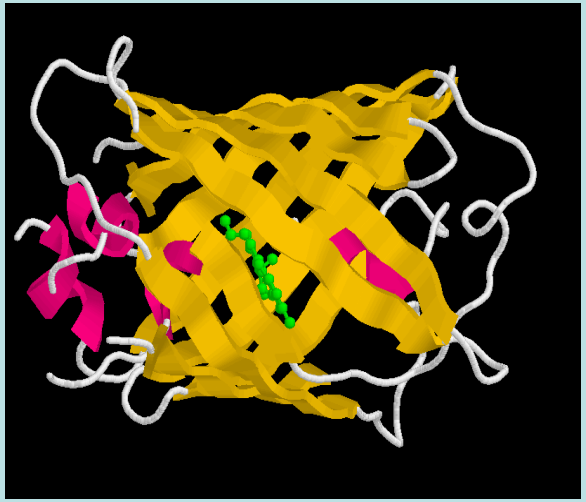


Examples for fast internal dynamics: “flickering”

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



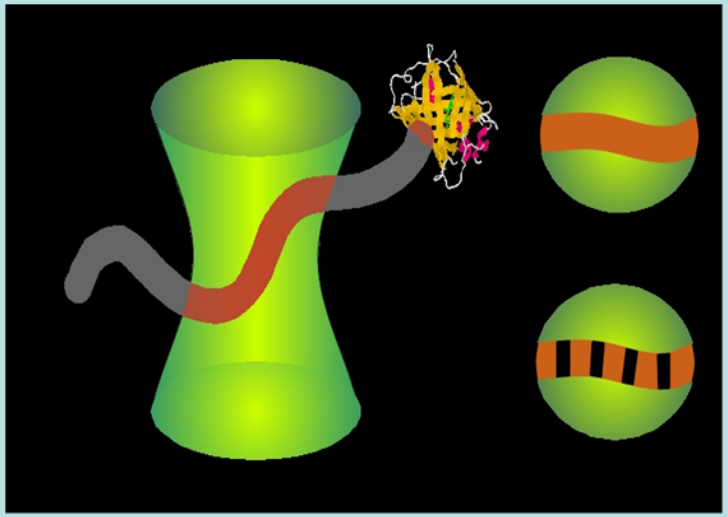


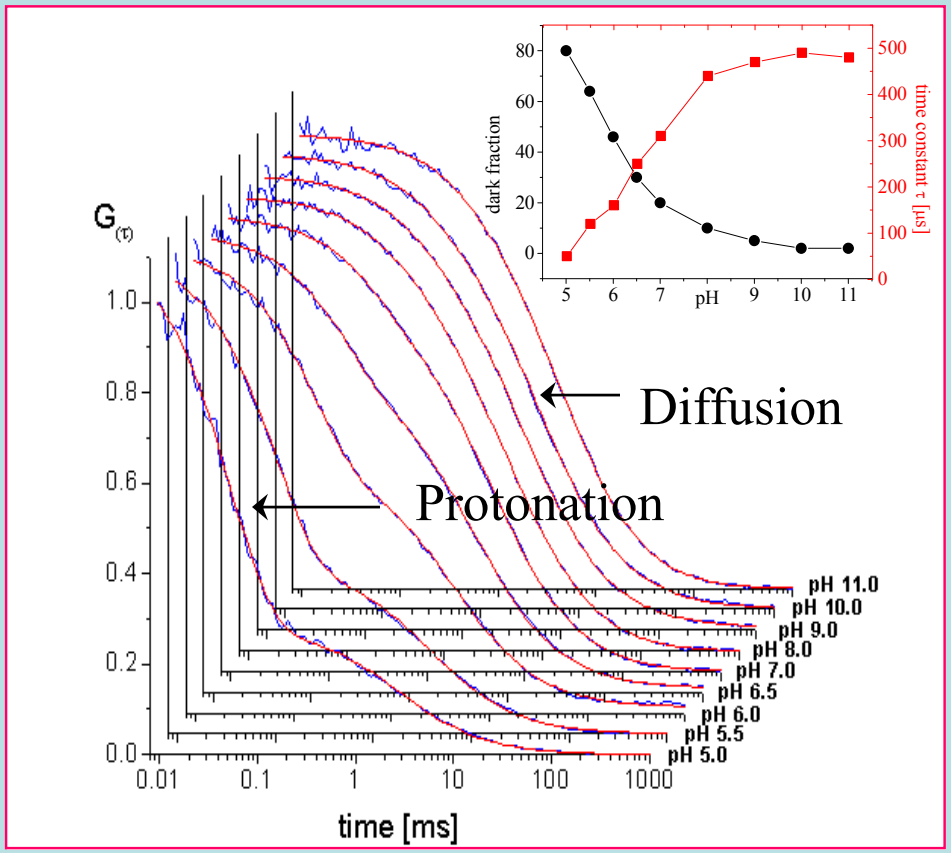


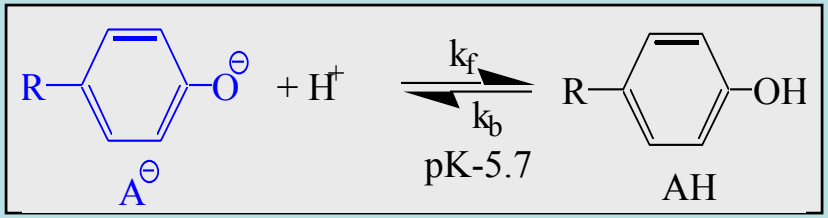
FCS measurements of GFP-a pH sensor

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

⟹ reversible protonation





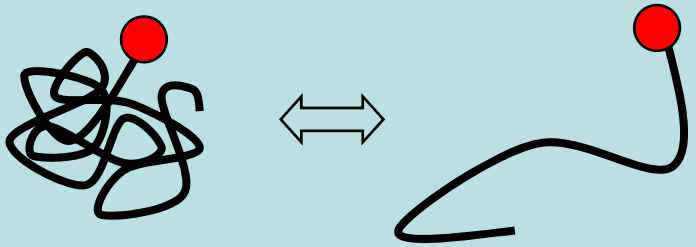


$\lambda_{abs,deprot}$ = 488nm

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

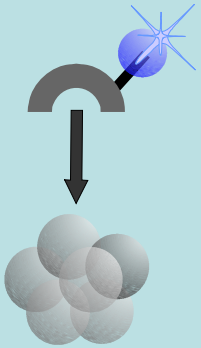
GFP can be employed as single molecule pH meter!

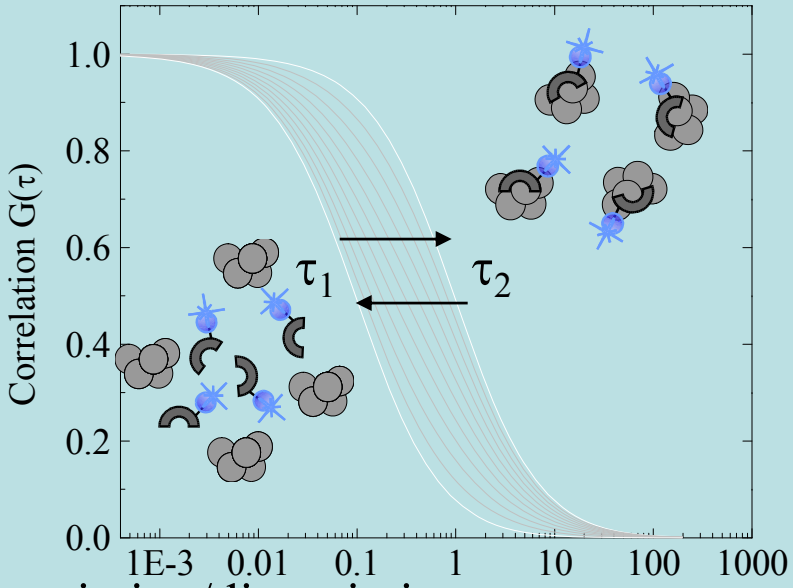
What to determine by diffusion analysis?



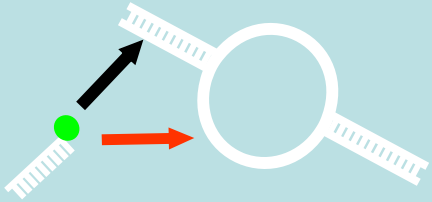
$$D = \frac{kT}{6\pi\eta R_h}$$

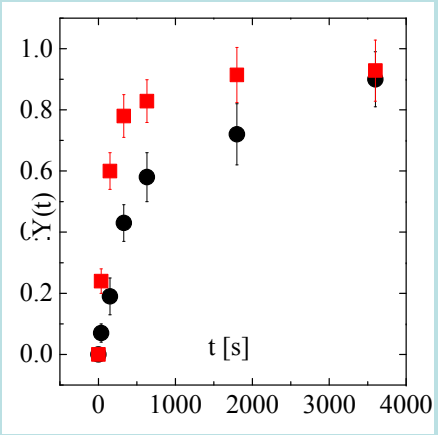
Analysis of molecular structure: diffusion properties depend on hydrodynamic radius





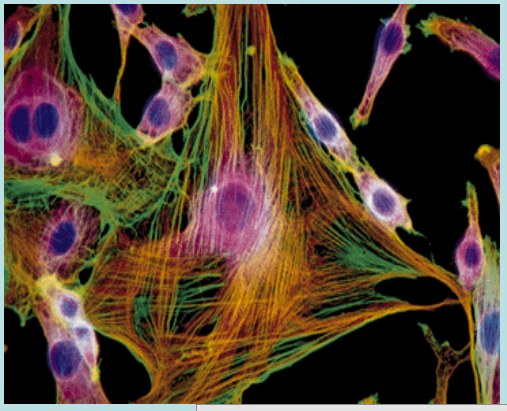
Analysis of association/dissociation processes by change in molecular mass

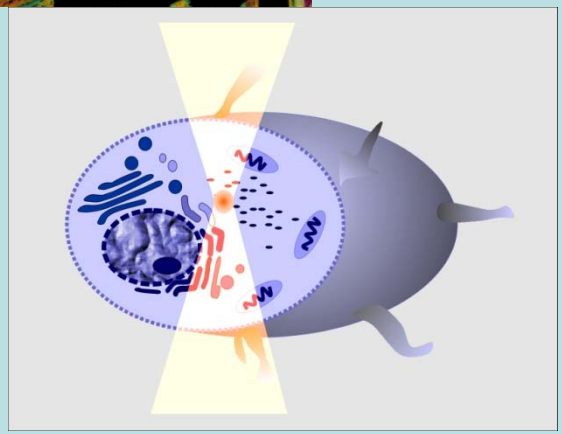




k_{ass}=10^4 $M^{-1}s^{-1}$ to 10^6 $M^{-1}s^{-1}$

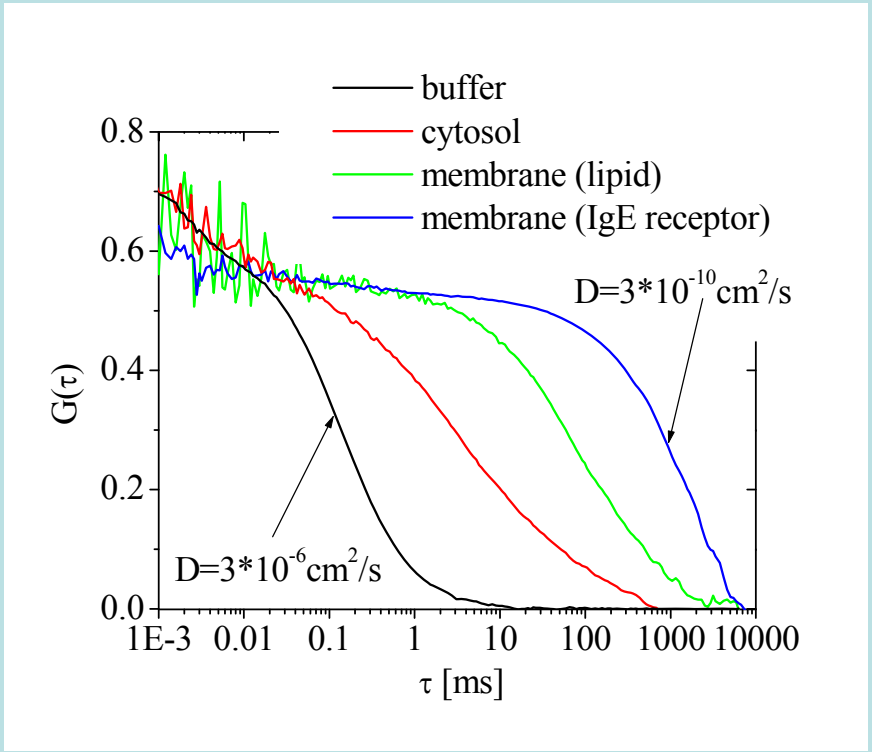
Assessing molecular mobility in different cellular compartments

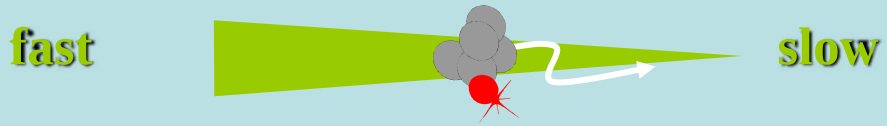




Requirement: specific labeling of regions of interest

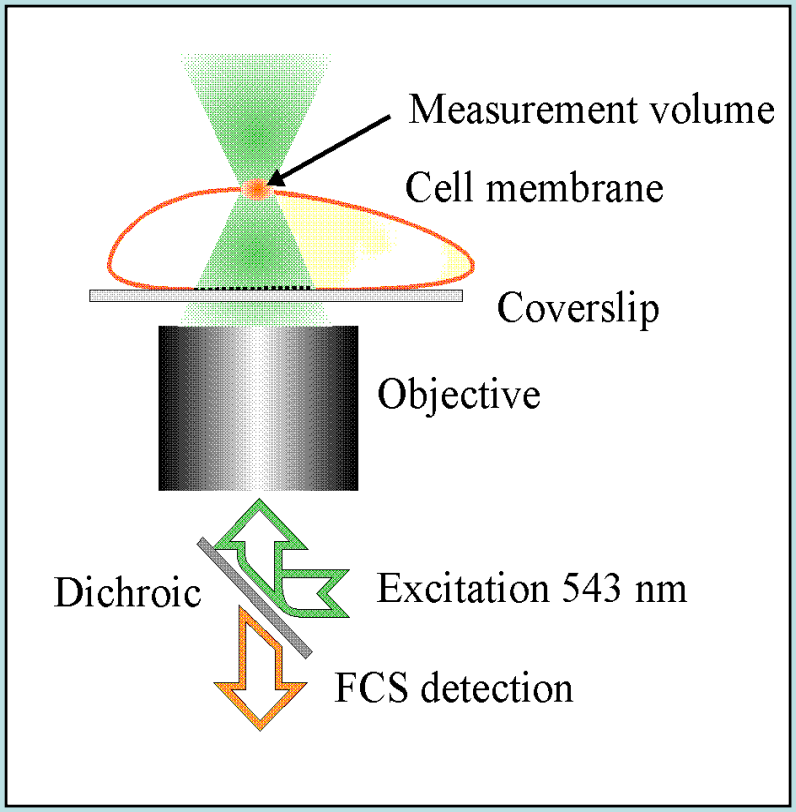
Precision: 0.3 um in XY
1.0 um in Z

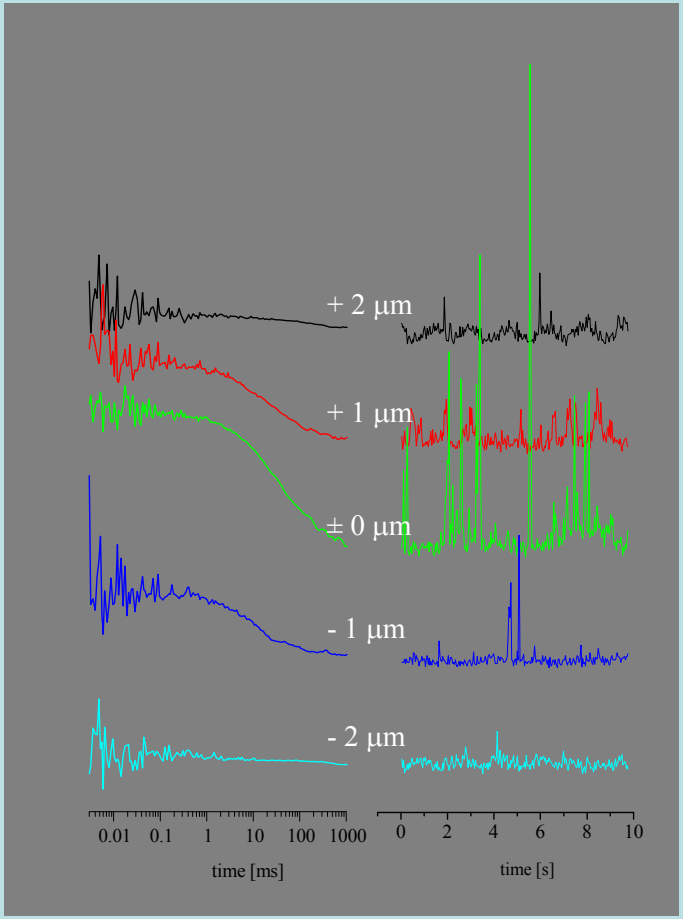




Determination of "molecular speed"

Detection of single molecules in membranes





⇒ Only labeled regions contribute to the measured signal

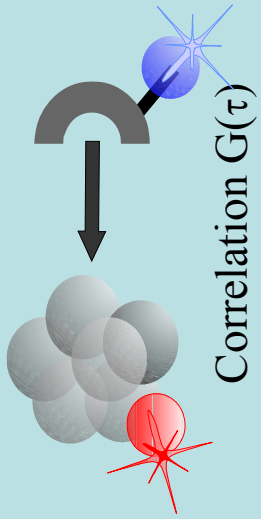
Dual-color cross-correlation analysis FCCS

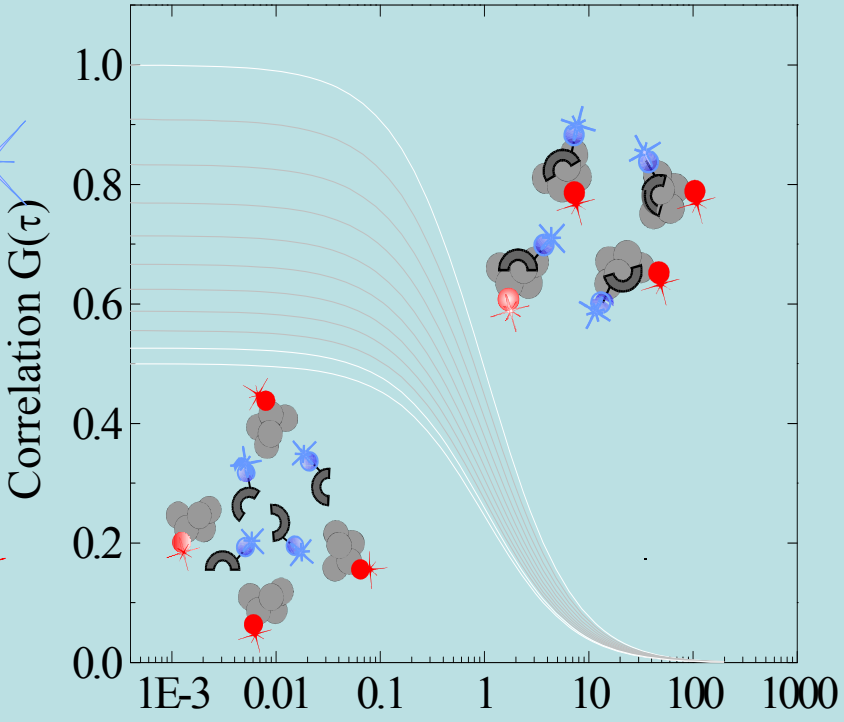
Advantage: mobility independent analysis of molecular interactions

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

RECALL

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





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

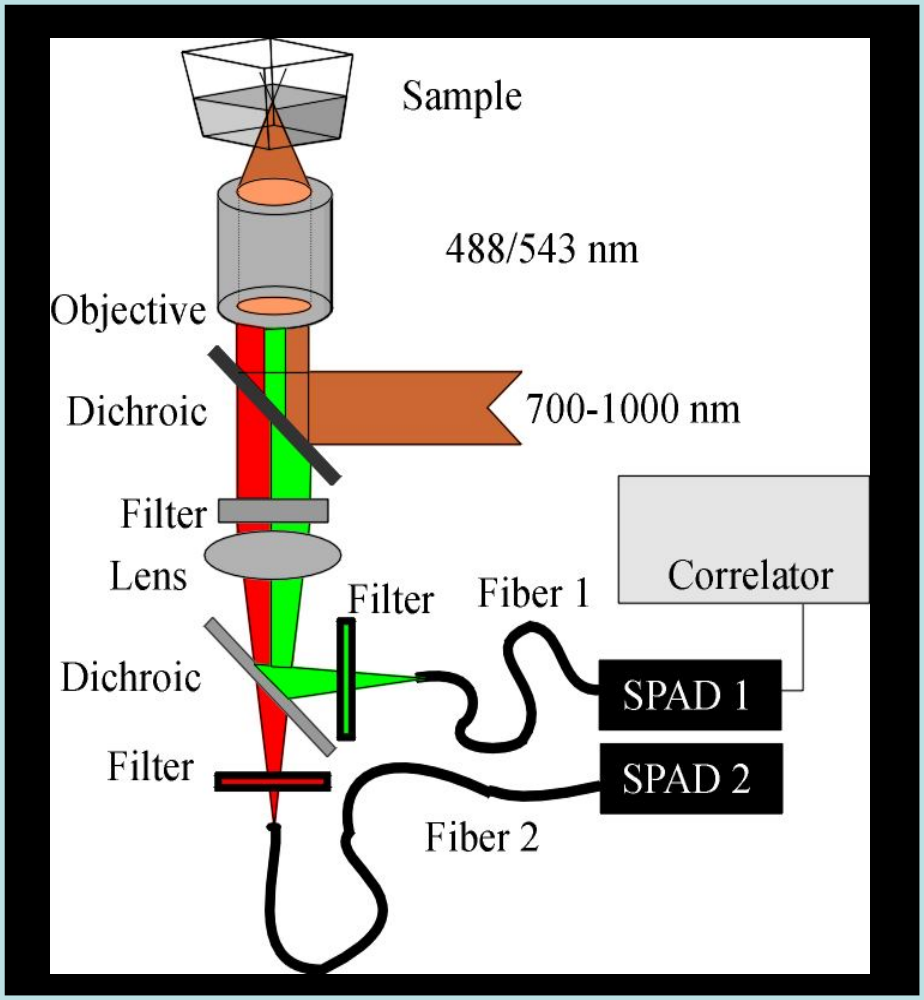
Red channel: + cross-c.

Blue channel: +

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

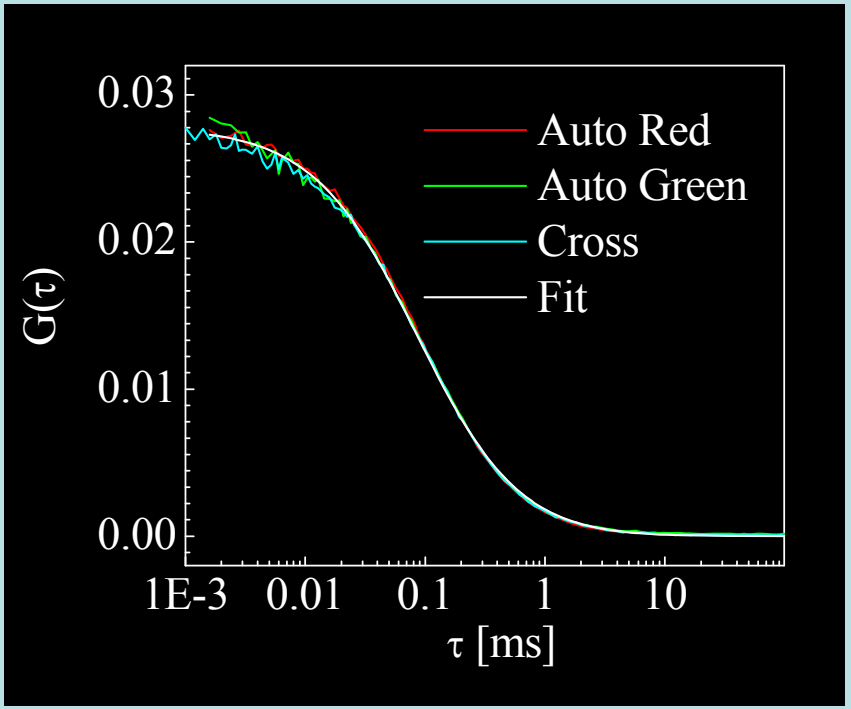
Denominator Numerator

Experimental setup for TPCCS



Inherent overlap of excitation volumes

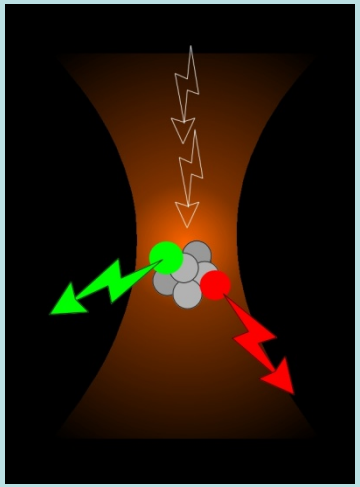
Simplified alignment of detection volumes (no pinholes required)

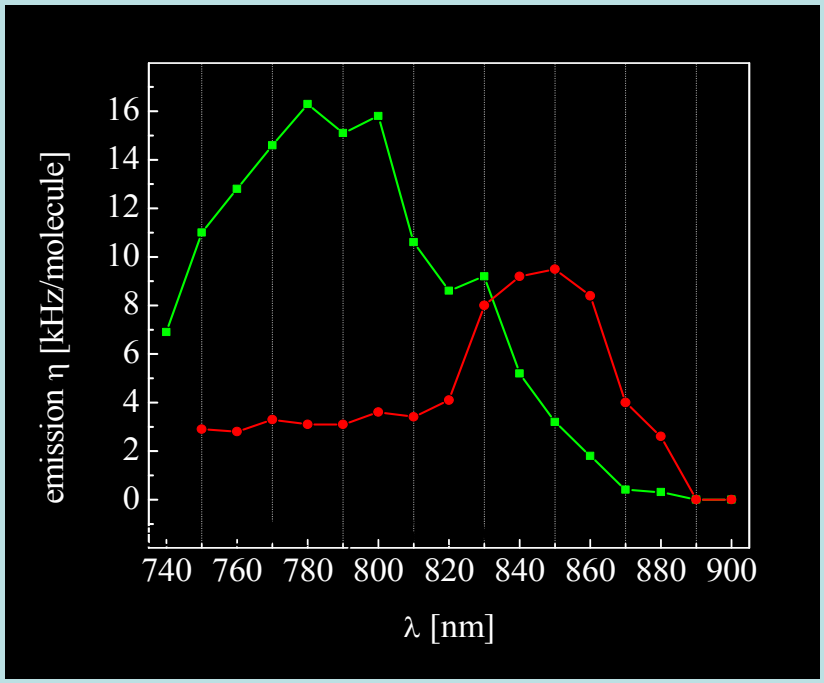


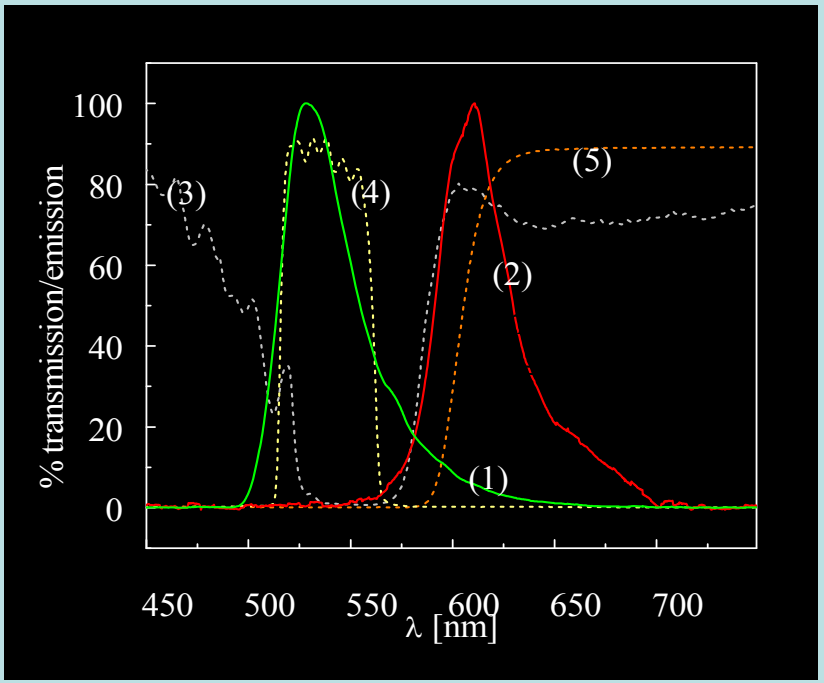
Dual-color two-photon cross-correlation (TPCCS)

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

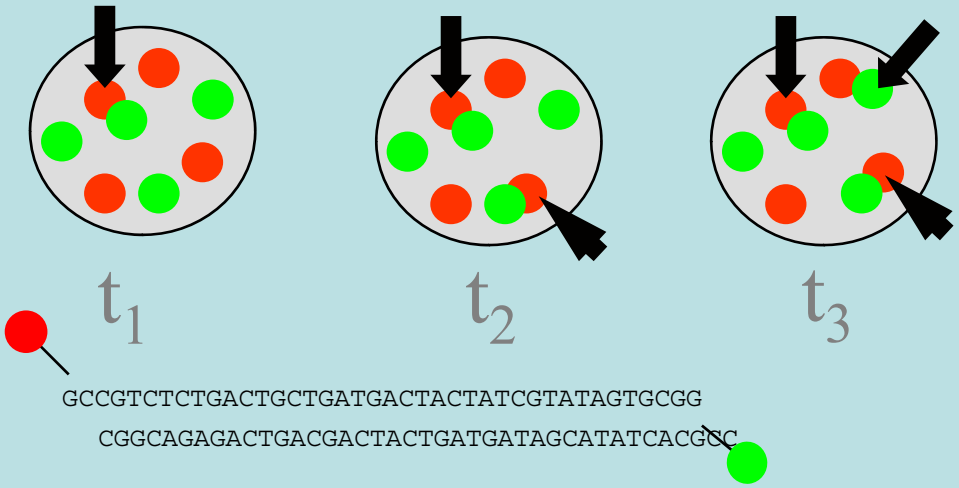
Requirement: both dyes show similar emission on a single molecule scale



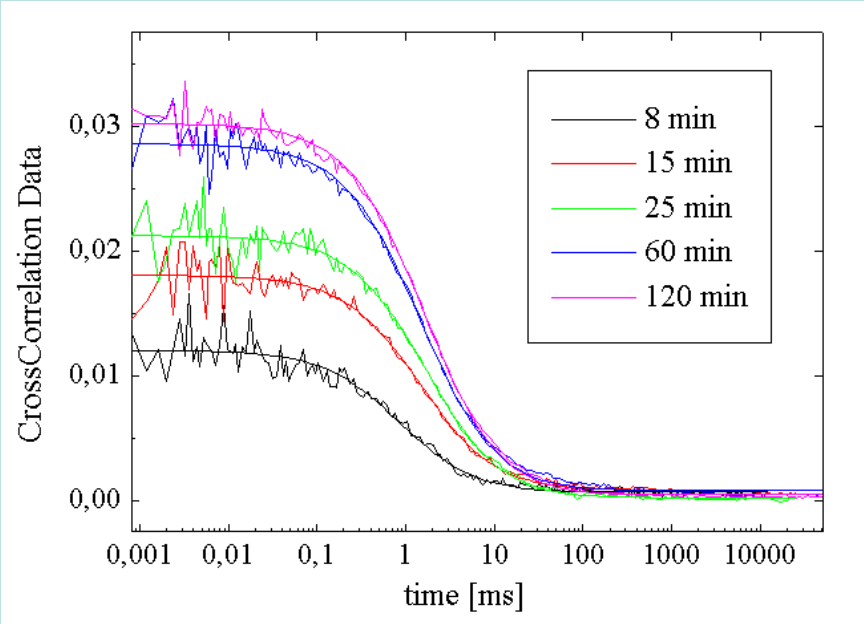


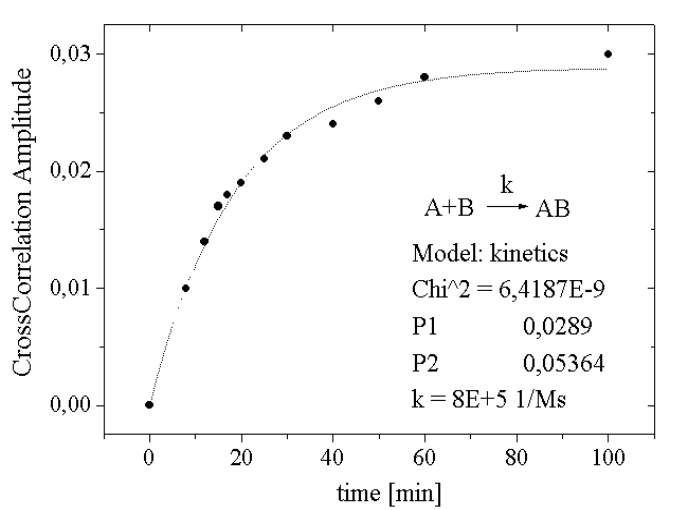


Analysis of DNA-DNA association



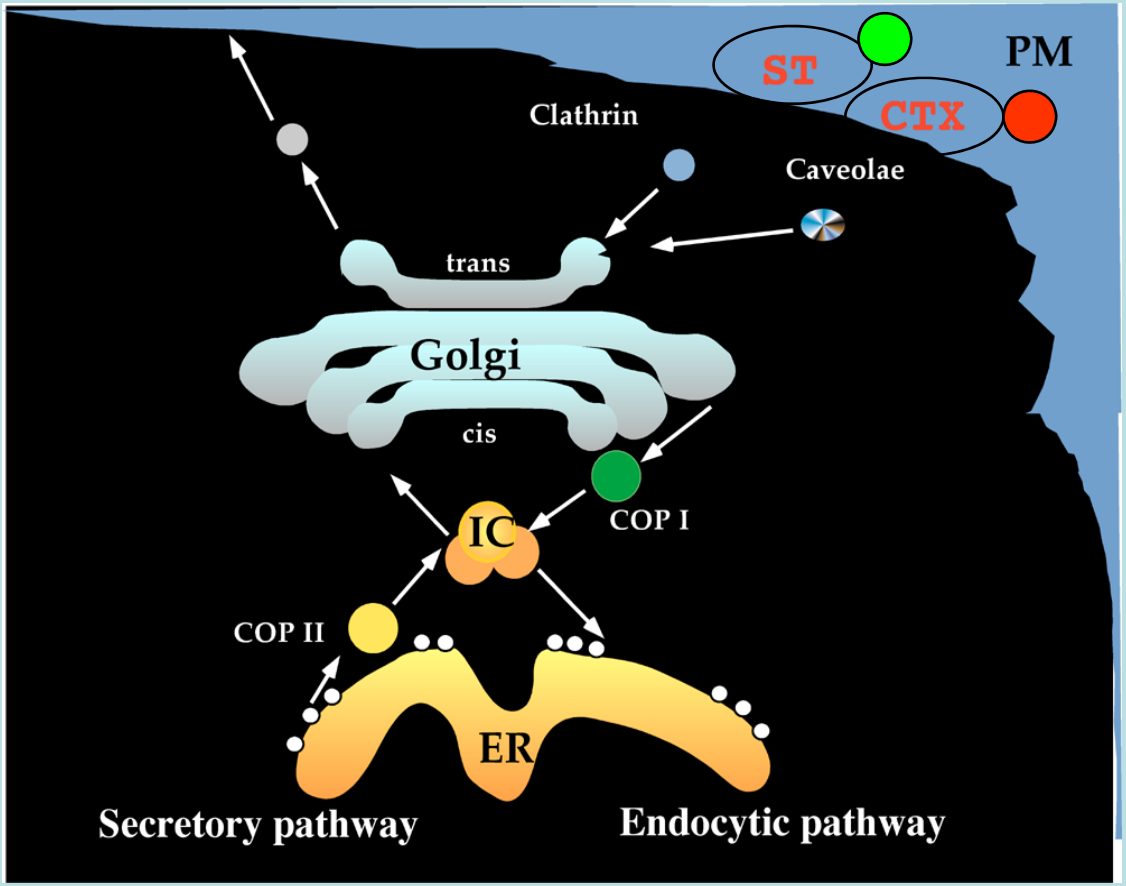
$$G^{\times}_{RB}(\tau) = \frac{C_{RB} Diff_{BR}}{\left[V_{eff}\left(C_R + C_{RB}\right)\left(C_B + C_{RB}\right)\right]}$$





Greater specificity for reaction product observation

Intracellular FCCS applications: The toxin system

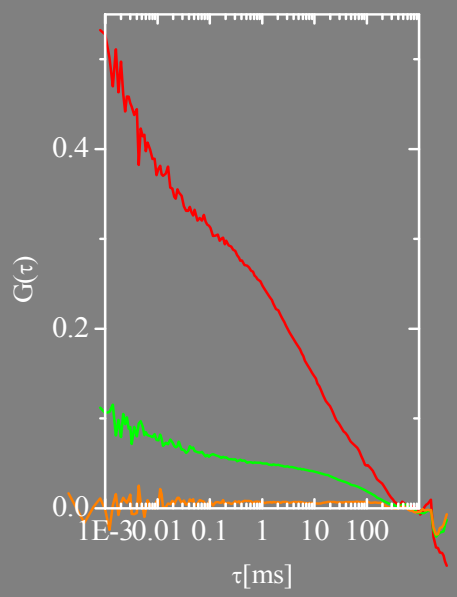


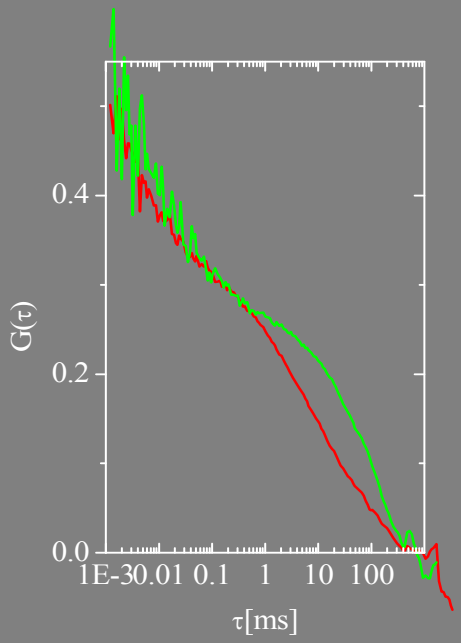
CTX-*Cholera*
ST-*Shigella*

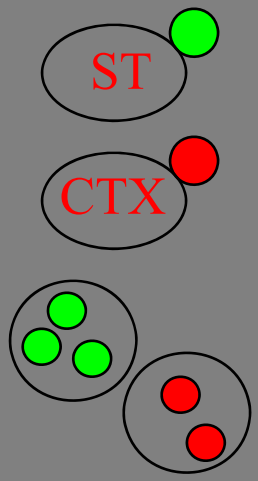
Bacia et al., 2002, Biophy. J.

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

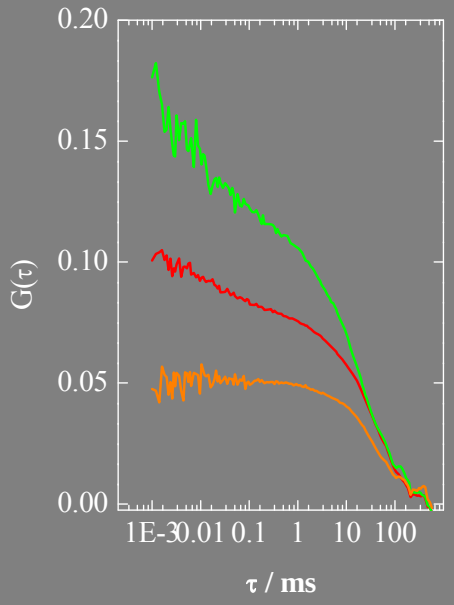


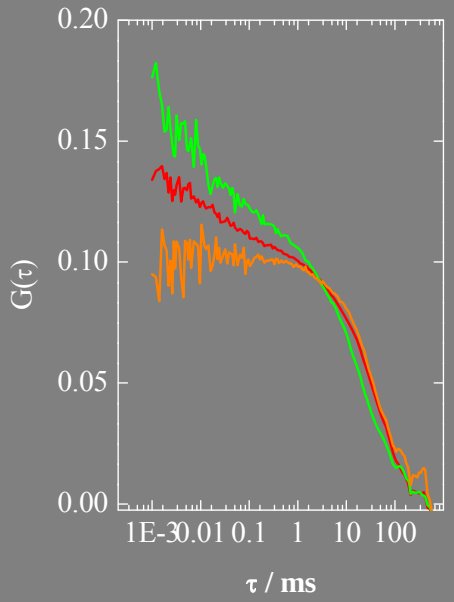


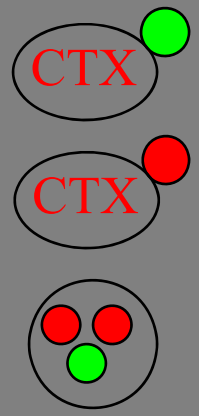


Distinct autocorrelations, no cross-correlation: different pathways

CTX-*Cholera*
ST-*Shigella*

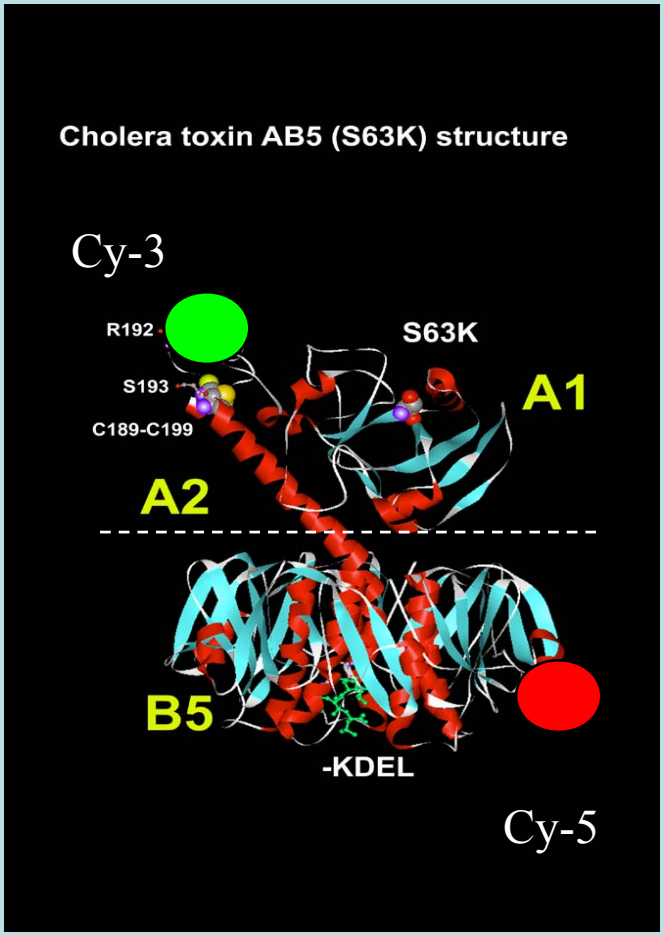




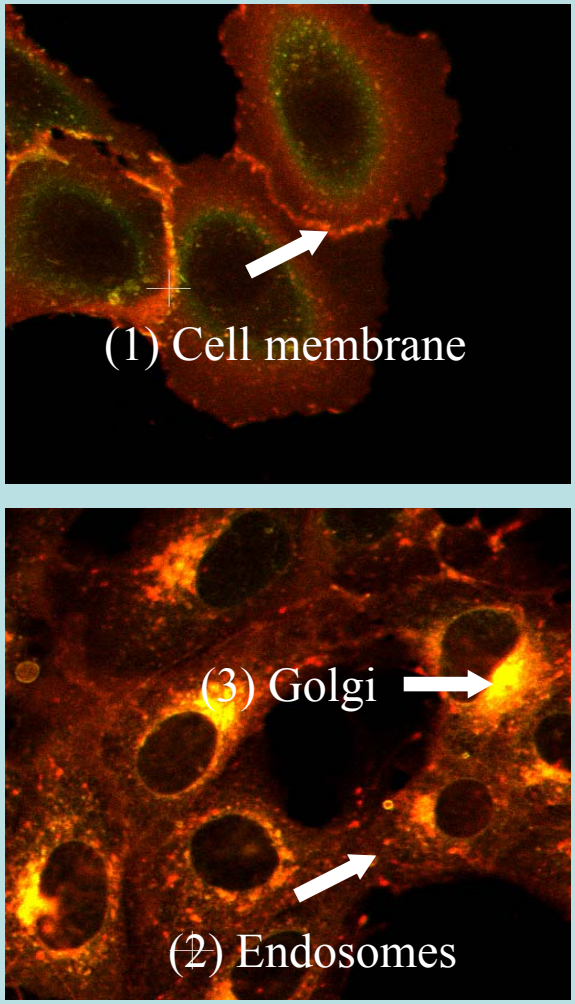


Comparable autocorrelations, existing cross-correlation: same pathway

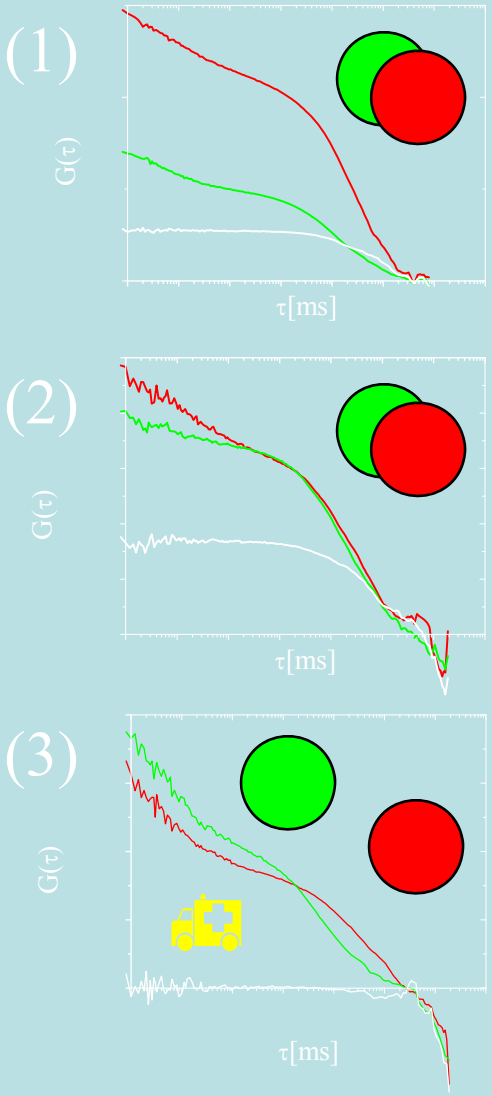
FCCS reveals where the subunits dissociate



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



Cross-correlation finally decays to zero in the Golgi



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) deep in living tissue