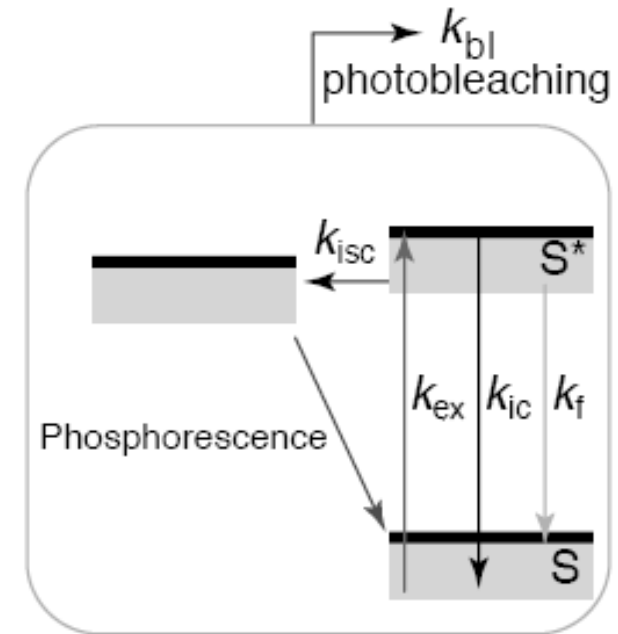
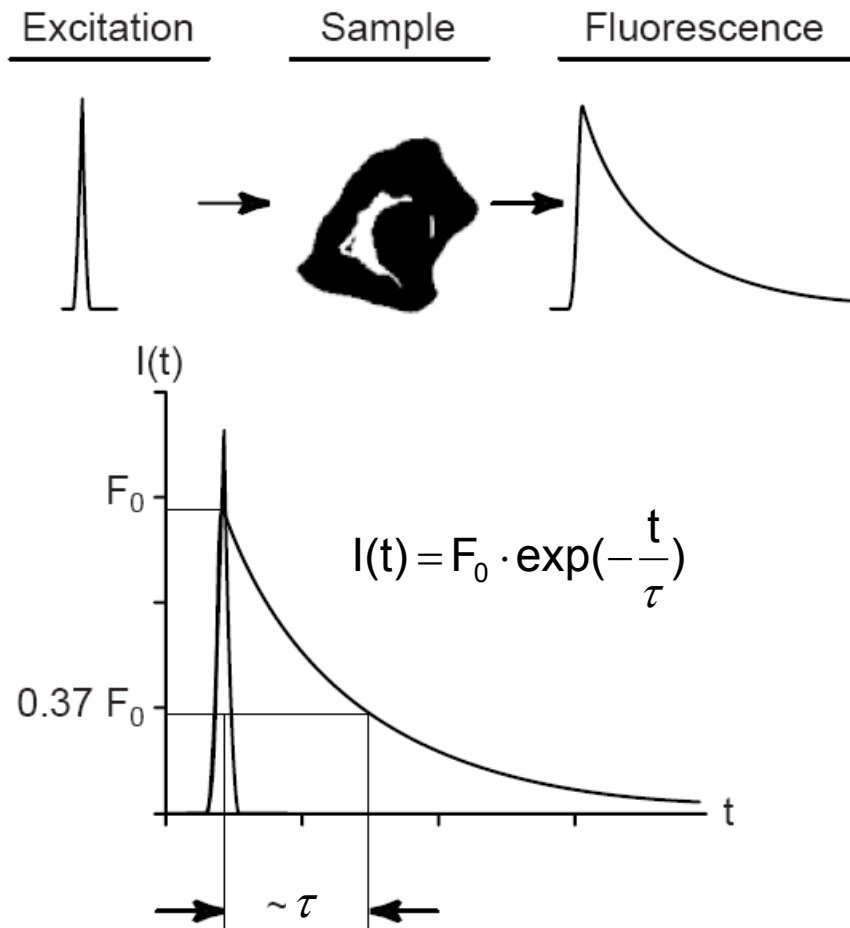


FLIM

Fluorescence Lifetime IMaging

Fluorescence lifetime



$$\frac{1}{\tau} = k_f + k_{nr}$$

$$k_{nr} = k_{ic} + k_{isc} + k_{bl}$$

τ = **fluorescence lifetime (~ns to ps)**
(average residence time of the molecule in the excited state)

k_f = radiative decay rate (photon emission)

k_{nr} = nonradiative decay rate

Note: quantum yield $Q = \frac{k_r}{k_r + k_{nr}}$

Fluorescence lifetime

Fluorescence decay of a fluorophore:

$$\frac{dN_e}{dt} = -(k_{nr} + k_f)N_e$$

where N_e : number of molecules in the excited state ($\propto$ fluorescence intensity)

Solution:

$$F(t) = F_0 e^{-(k_{nr} + k_f)t} = F_0 e^{-t/\tau}$$

where F and F_0 are the instantaneous and initial fluorescence intensity

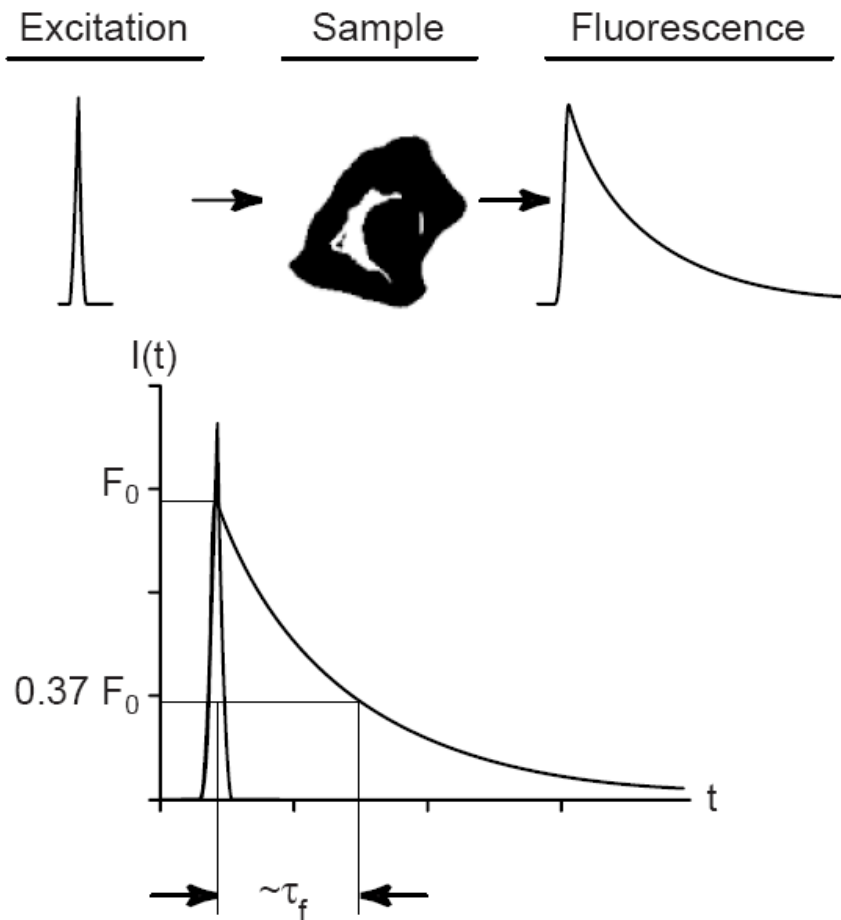
Fluorescence decay (multiple decay pathways and rates):

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

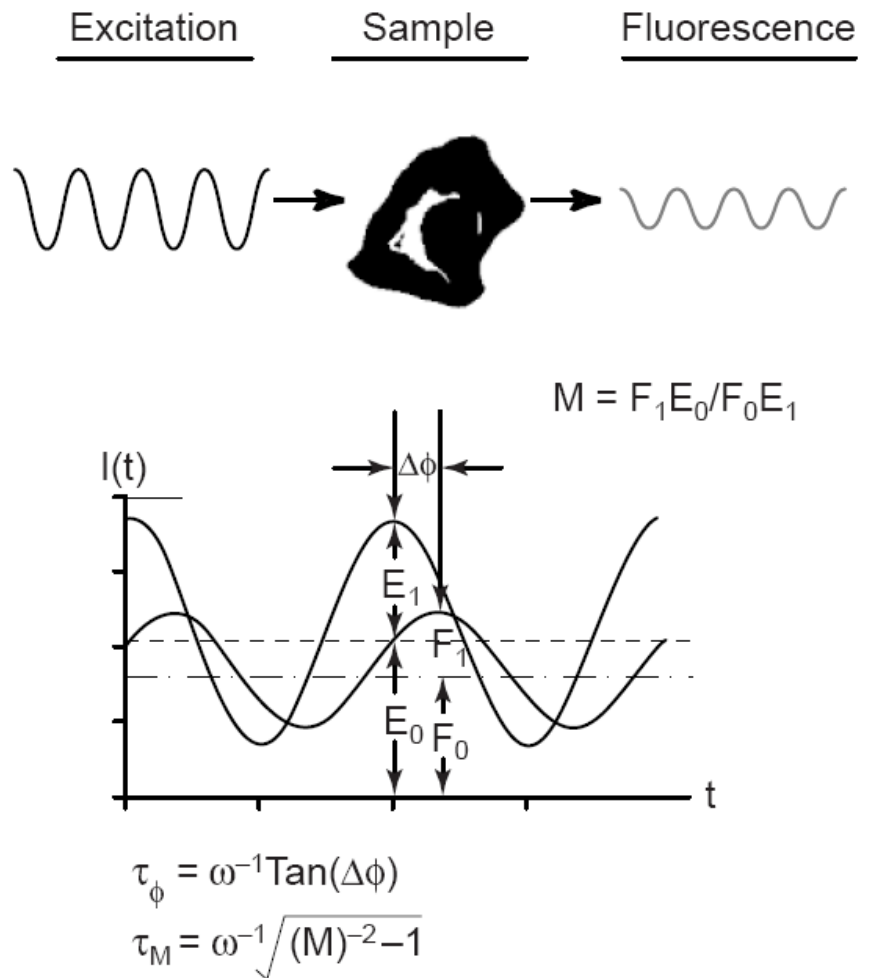
with solution $F(t) = F_0 e^{-\sum_m t/\tau_m}$

FLIM measurement

(a) Time domain



(b) Frequency domain



FLIM to probe the fluorophore local environment

FLIM: non-intensity based chemical sensing!

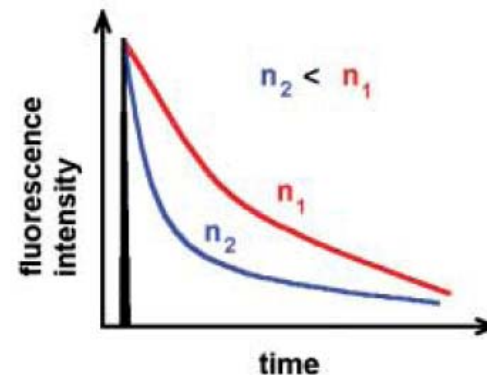
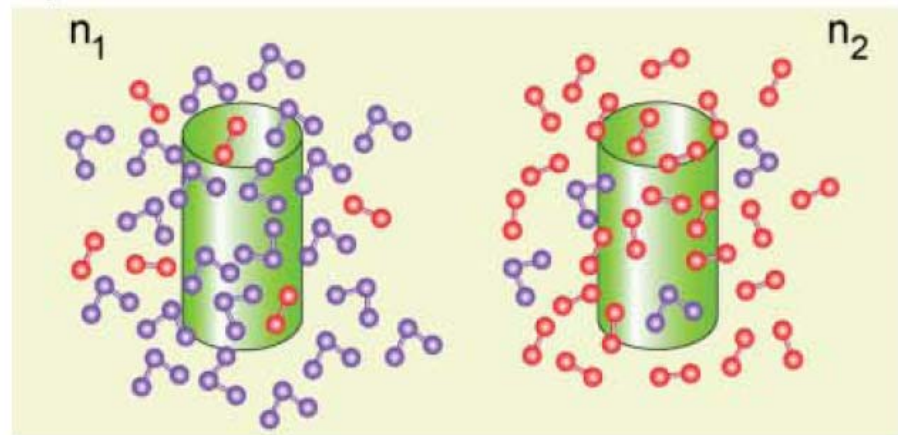
Decay rate can be measured for each pixel of the image

Applications:

- Contrast between fluorophores with different decay rates (even if same emission wavelength).
- Fluorophore local environment
- FLIM combined with FRET

τ sensitive to local environment

a) local environment



FLIM to probe the fluorophore local environment

τ sensitive to local environment

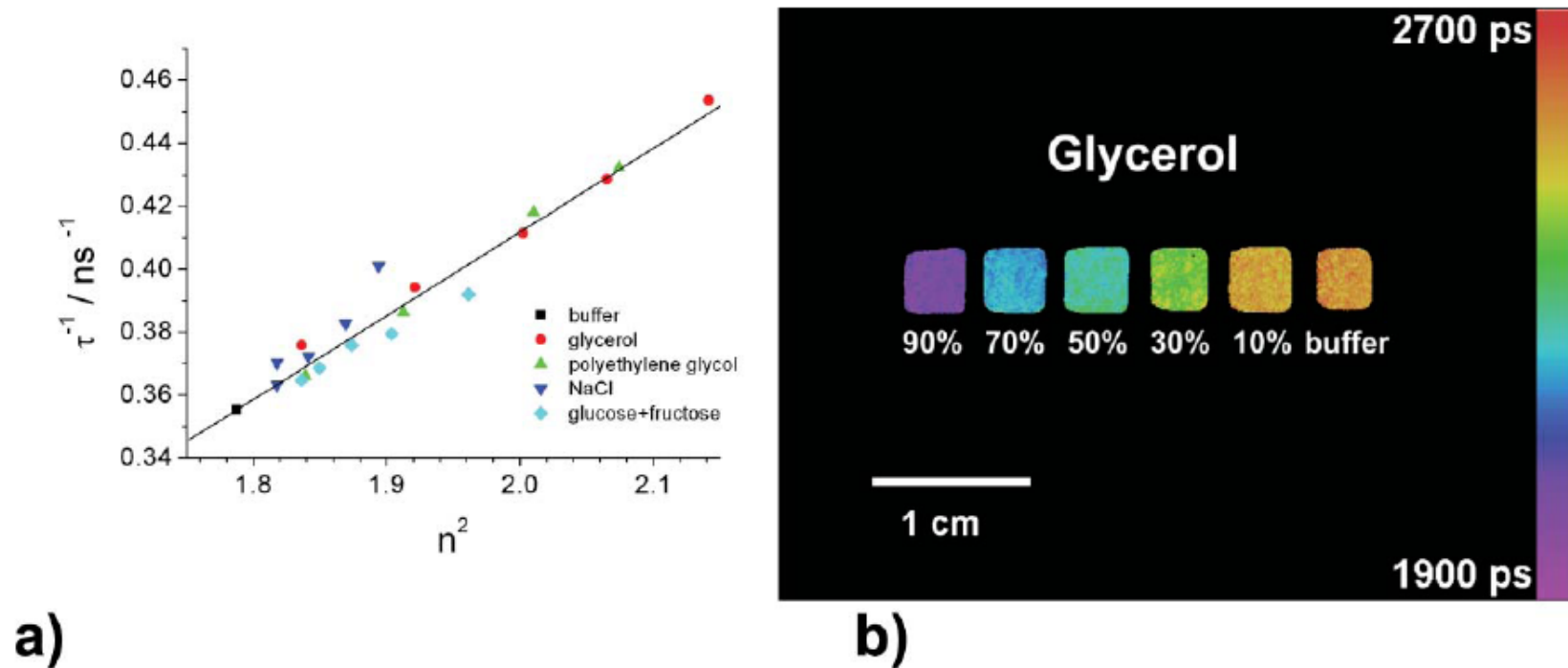


Fig. 3 (a) The inverse average fluorescence lifetime τ_{av}^{-1} of GFP *versus* the square of the refractive index of the solution. τ_{av}^{-1} varies linearly with the square of the refractive index in accordance with the Strickler–Berg formula [eqn. (6)], irrespective of whether the refractive index is increased by adding glycerol, polyethylene glycol, NaCl or glucose and fructose. (b) Fluorescence lifetime image of GFP in mixtures of aqueous buffer and glycerol in a multiwell plate.^{80,144}

FLIM to characterize FRET

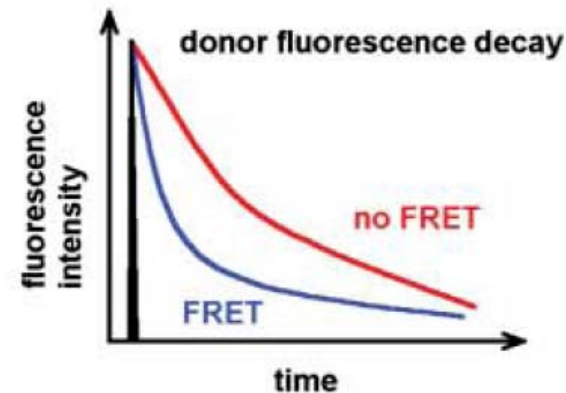
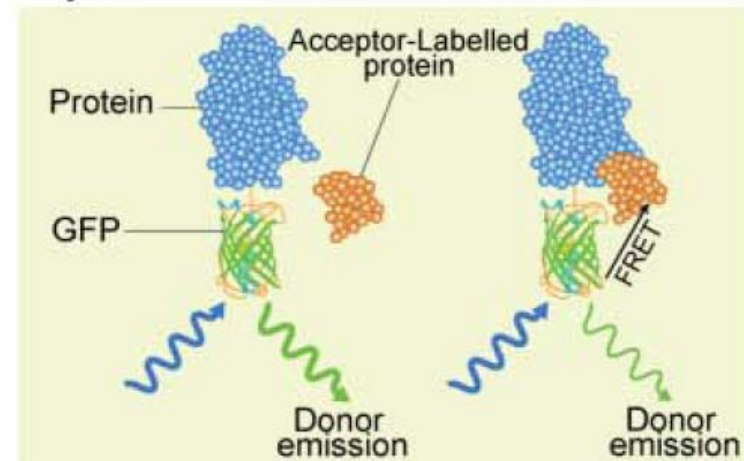
FLIM: non-intensity based chemical sensing!

Decay rate can be measured for each pixel of the image

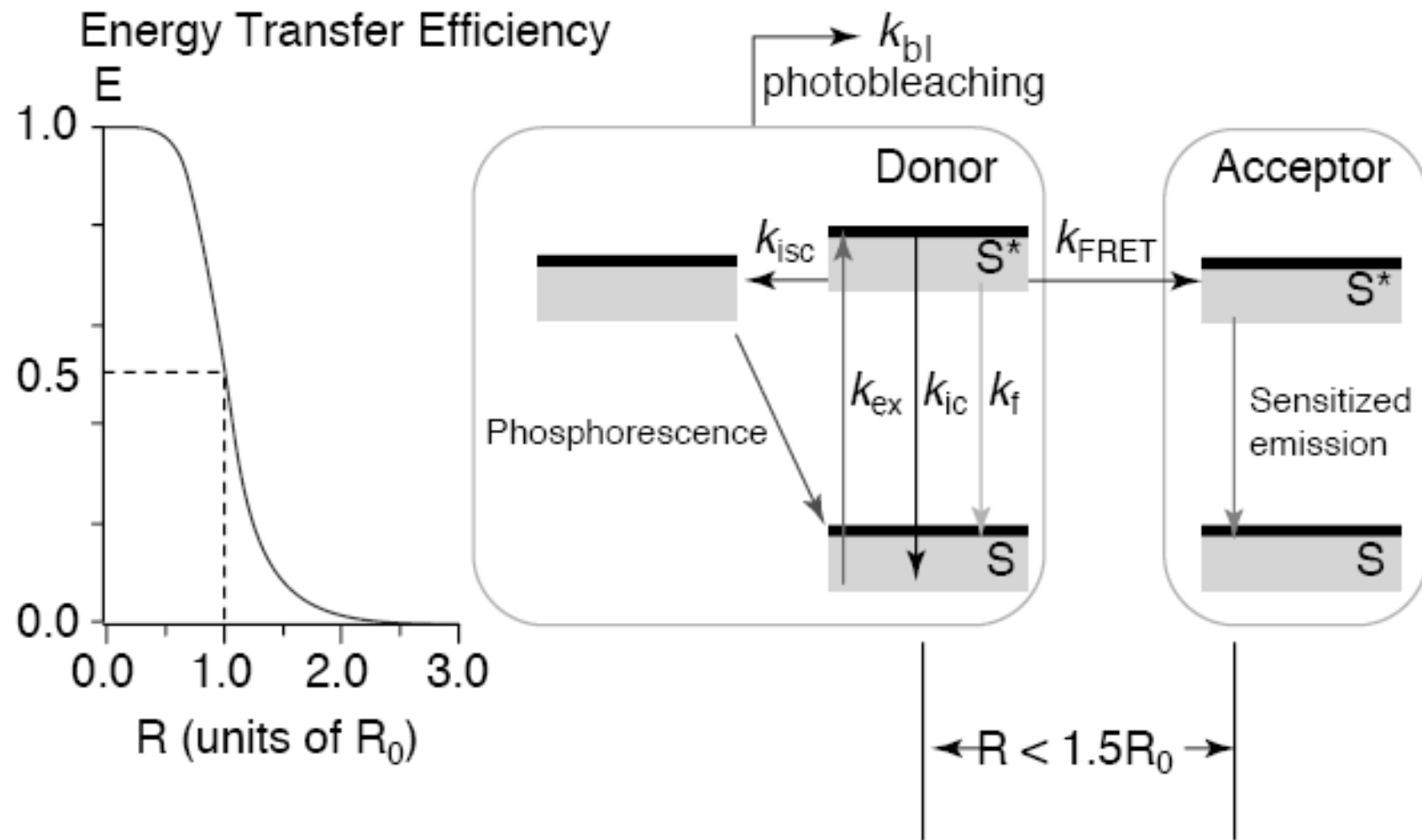
Applications:

- Contrast between fluorophores with different decay rates (even if same emission wavelength).
- Fluorophore local environment
- FLIM combined with FRET

b) FRET



Beyond Jablonski: decay rate

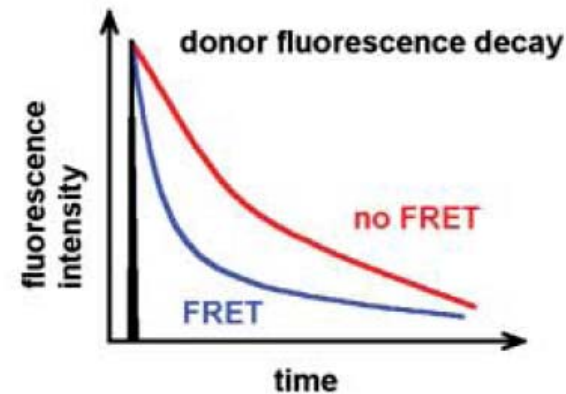
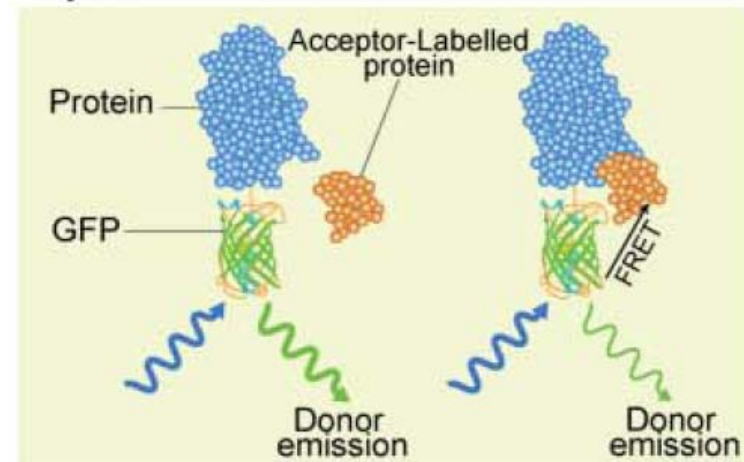


R : distance between fluorophores
 R_0 : Foster radius

FLIM to characterize FRET

FRET decreases the
fluorescence lifetime
of the donor!

b) FRET



FLIM to characterize FRET

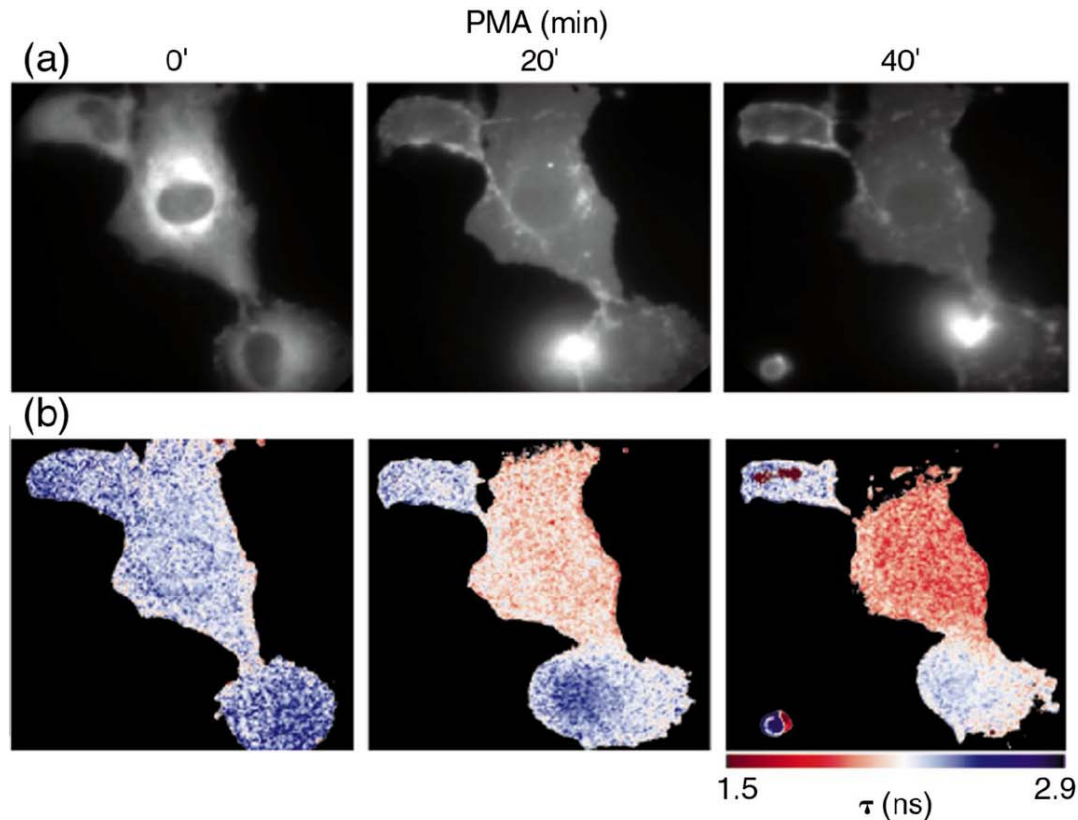
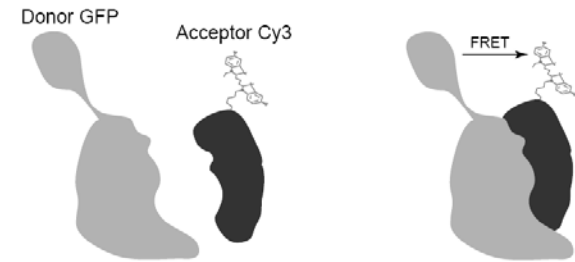


FIGURE 3

Activation of green fluorescent protein (GFP)-tagged protein kinase C alpha (PKC α) in live Cos7 cells measured by autophosphorylation using fluorescence lifetime imaging microscopy (FLIM). Only the middle cell was microinjected with site-specific IgG-Cy3.5. The cells were stimulated with 100 nM phorbol myristate acetate (PMA) and fluorescence lifetime images acquired at the times indicated. (a) Fluorescence images of GFP-PKC α ; (b) fluorescence lifetime images of GFP-PKC α . Note that the lifetimes only decrease in the middle microinjected cell owing to fluorescence resonance energy transfer (FRET) between GFP-PKC α and IgG-Cy3.5.



FRET decreases the fluorescence lifetime of the donor!

FLIM Advantages

FLIM: non-intensity based chemical sensing!

τ is independent of chromophore concentration

τ is directly dependent upon excitation-state reactions

FRAP

Fluorescence Recovery After Photobleaching

Mobile fraction and diffusion constant via FRAP

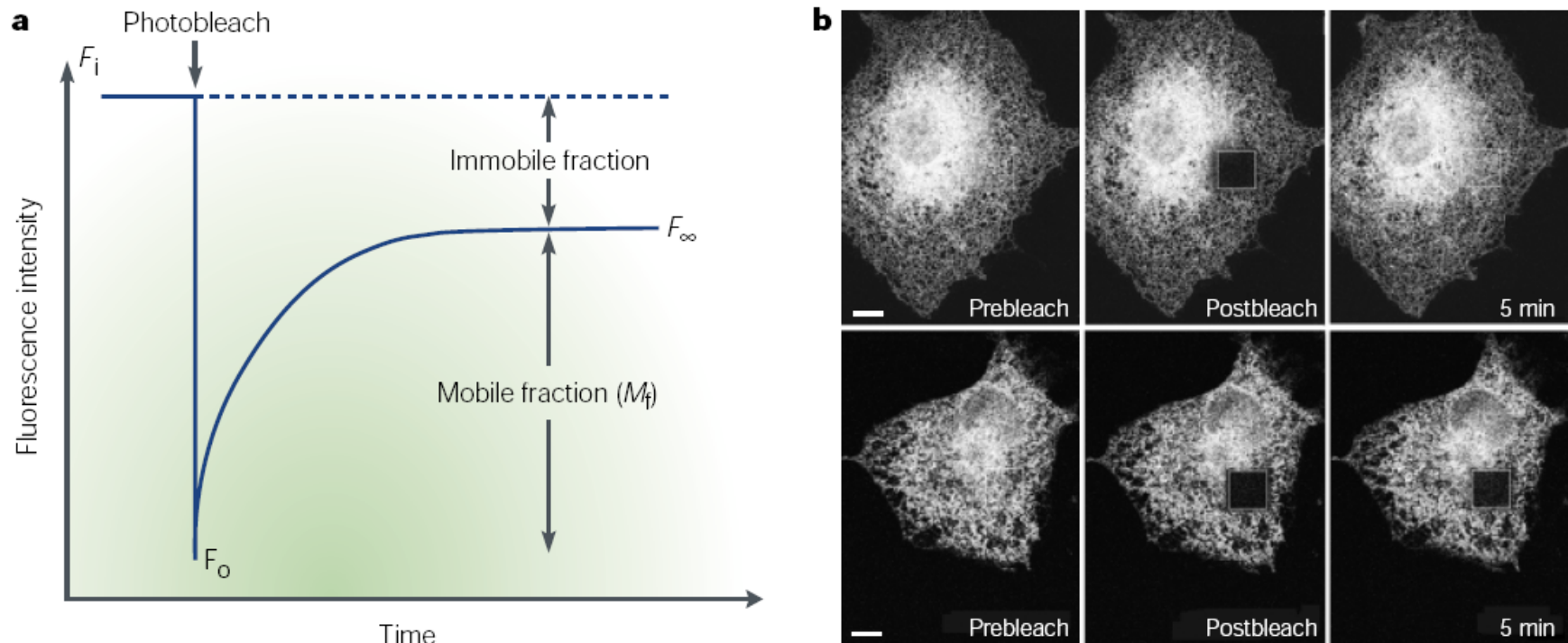


Figure 1 | **Fluorescence recovery after photobleaching.** **a** | Plot of fluorescence intensity in a region of interest versus time after photobleaching a fluorescent protein. The prebleach (F_i) is compared with the asymptote of the recovery (F_∞) to calculate the mobile and immobile fractions. Information from the recovery curve (from F_o to F_∞) can be used to determine the diffusion constant of the fluorescent protein. **b** | Cells expressing VSVG-GFP were incubated at 40 °C to retain VSVG-GFP in the endoplasmic reticulum (ER) under control conditions (top panel) or in the presence of tunicamycin (bottom panel). Fluorescence recovery after photobleaching (FRAP) revealed that VSVG-GFP was highly mobile in ER membranes at 40 °C but was immobilized in the presence of tunicamycin. Adapted with permission from REF. 32 © (2000) Macmillan Magazines Ltd.

Mobile Fraction

$$M_f = \frac{F_\infty - F_o}{F_i - F_o}$$

Lippincott-Schwarz et al, Nature Review in Molecular and Cell Biology, 1999

Diffusion theory

Mobile Fraction M_f : fraction of fluorescent proteins that can diffuse into the bleached region during the time course of the experiment,

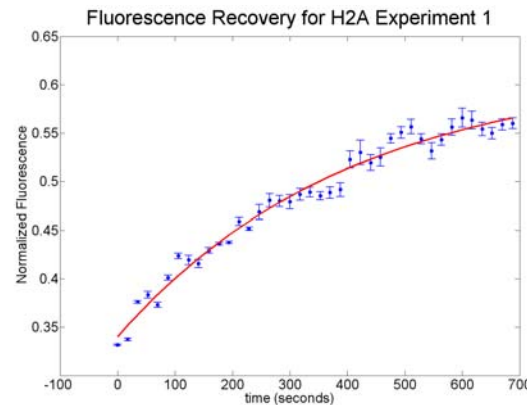
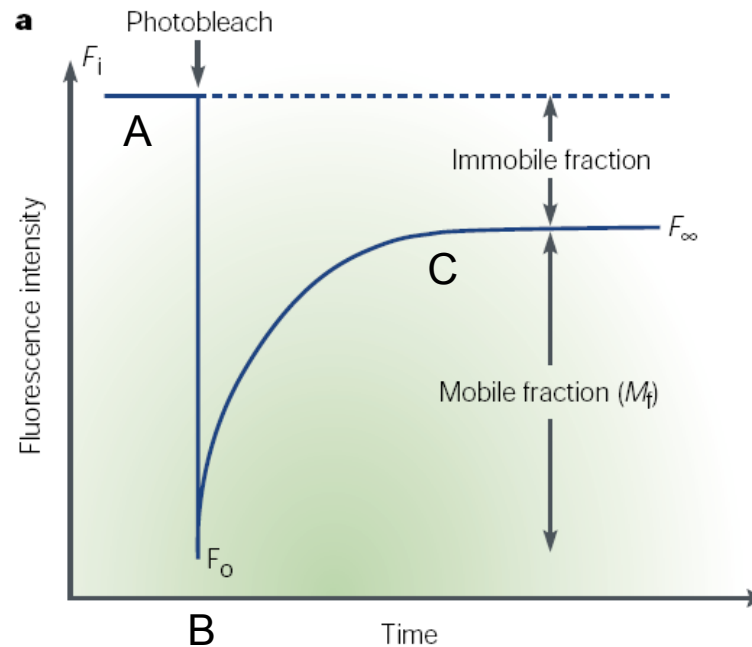
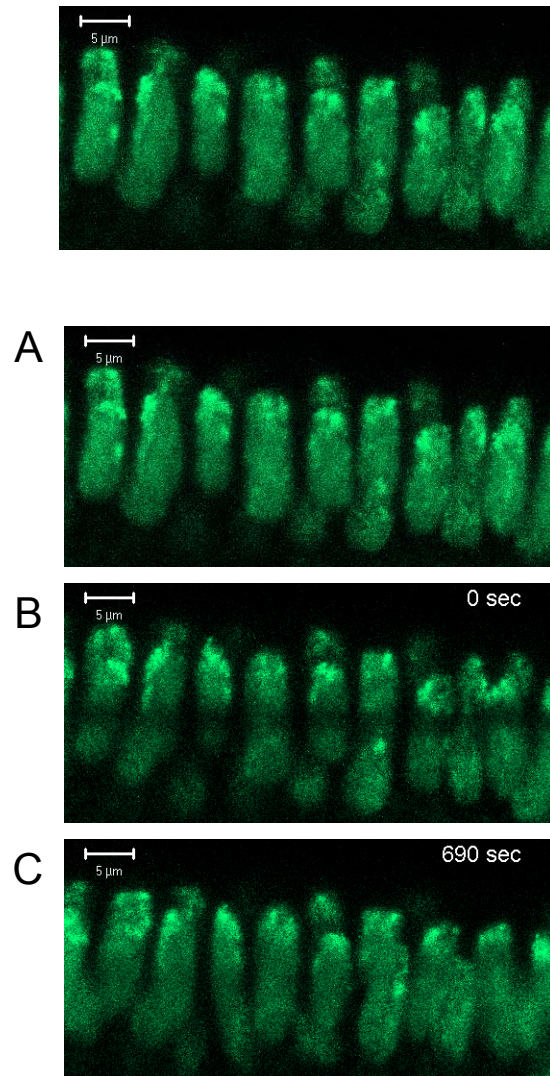
Diffusion constant (particle in a free volume, Stokes-Einstein formula)

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

D : diffusion constant, T : absolute temperature, η : viscosity of the solution k : Boltzmann constant, R : hydrodynamic radius of the particle

= rate of protein movement in the absence of flow or active transport reflects the mean squared displacement that a protein explores through a random walk over time ($\text{cm}^2 \text{s}^{-1}$ or $\mu\text{m}^2 \text{s}^{-1}$)

Mobile fraction and diffusion constant via FRAP



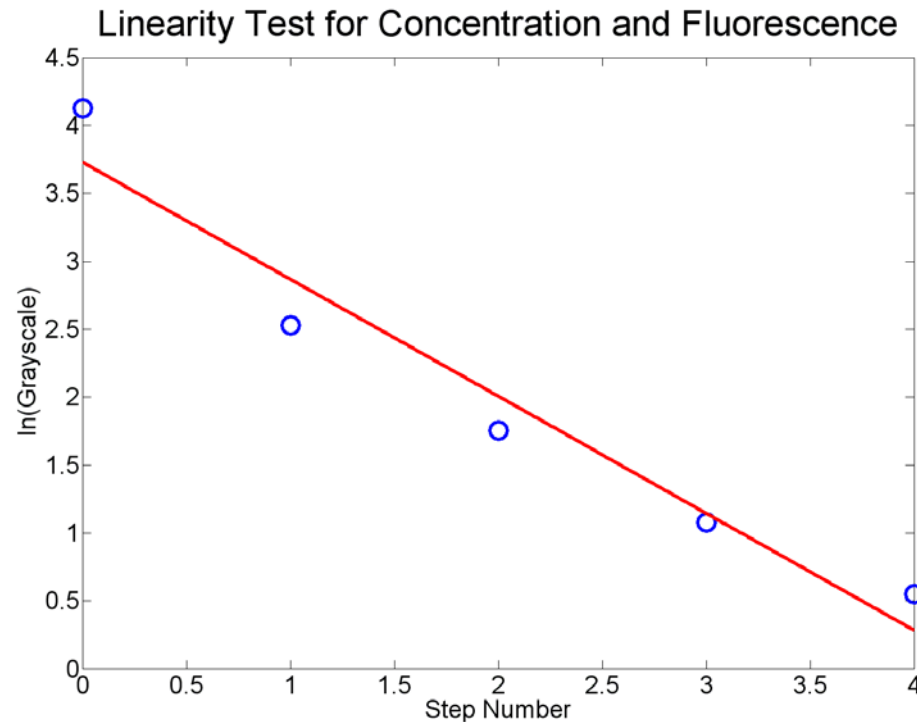
The diffusion coefficient obtained from the recovery curve (depend on the bleaching geometry)

See Axelrod et al, Biophysical Journal, 1976

H2A-GFP:

Diffusion coefficient $\sim 0.01 \mu\text{m}^2.\text{sec}^{-1}$

FRAP Control 1: linearity between [fluorophore] and signal



if linear

$$f_0 = \alpha C_0$$

$$C_n = \gamma^n C_0$$

$$f_n = \alpha C_n$$

$$\ln(f_n) = n \ln(\gamma) + \ln(\alpha C_0)$$

f_0 : fluorescence signal at start

C_0 : unbleached fluorophore concentration at start

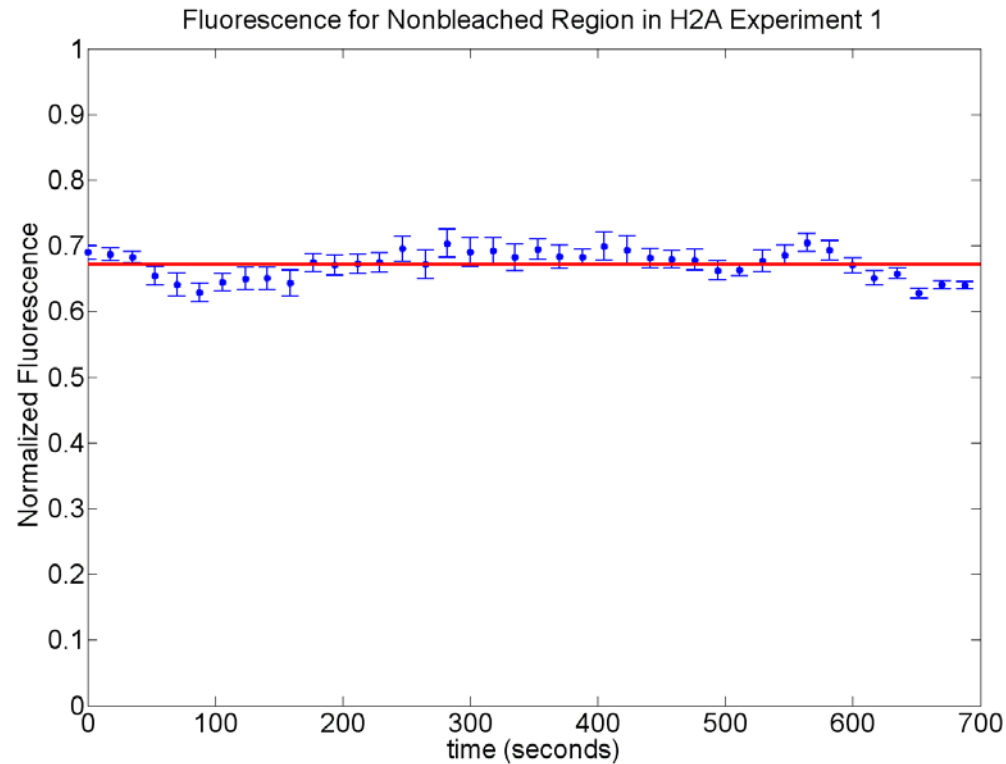
n : bleach step

γ : fraction of fluorophore bleached at each step

FRAP Control 2: bleaching during acquisition

Fluorescence signal in an unbleached region during the time of acquisition.

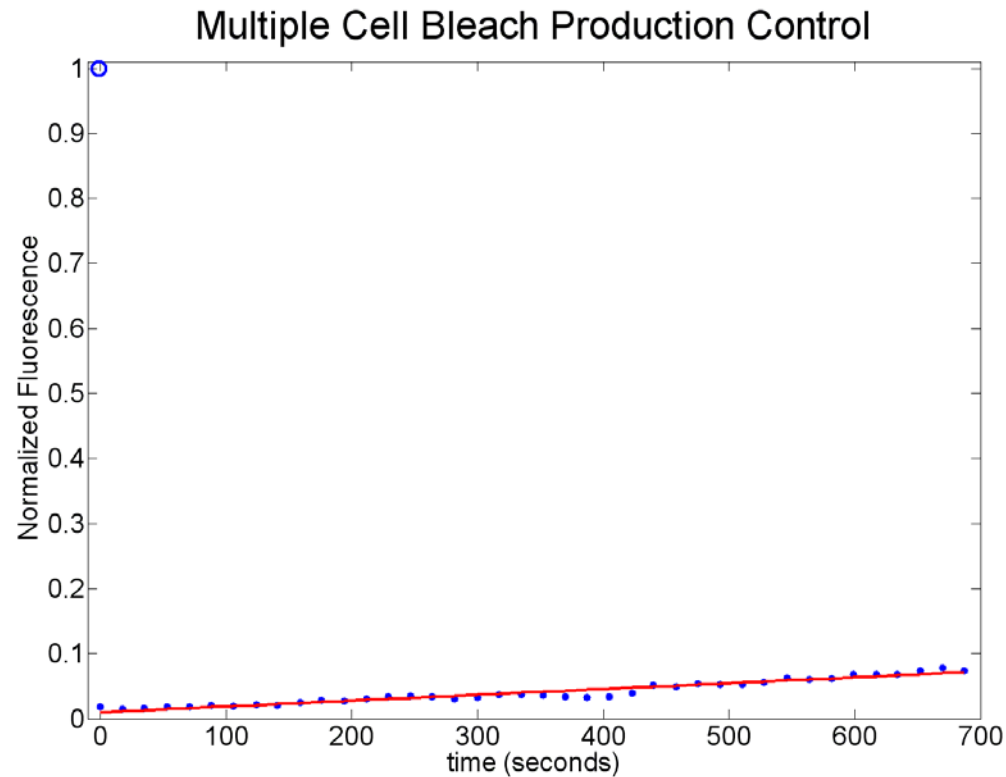
Good: no significant change in fluorescence level



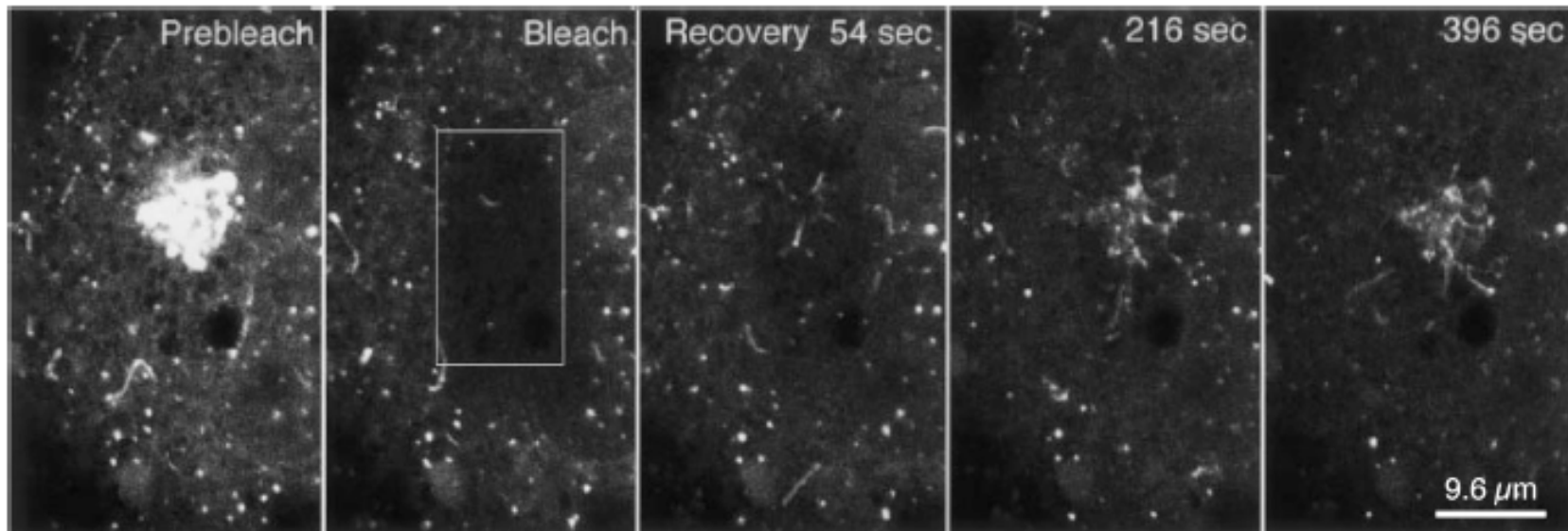
FRAP Control 3: production of GFP during the acquisition time

A large area is bleached.

Good: no recovery = no GFP production



FRAP exemple



ER to Golgi transport of VSVG-GFP

VSVG-GFP-expressing COS cells were incubated for 12 h at 40 °C, shifted to 15 °C for 3h and then warmed to 32 °C. Fluorescence associated with the Golgi complex was photobleached with high-intensity laser light and subsequent inward delivery of VSVG-GFP from pre-Golgi intermediates followed over the time periods indicated.

JF Presley, NB Cole, T Schroer, K Hirschberg, KJM Zaal & J Lippincott-Schwartz, "ER-to-Golgi transport visualized in living cells" *Nature*, **389** (4) 81–86, (1997)

FCS

Fluorescence Correlation Spectroscopy

FCS: spontaneous intensity fluctuations

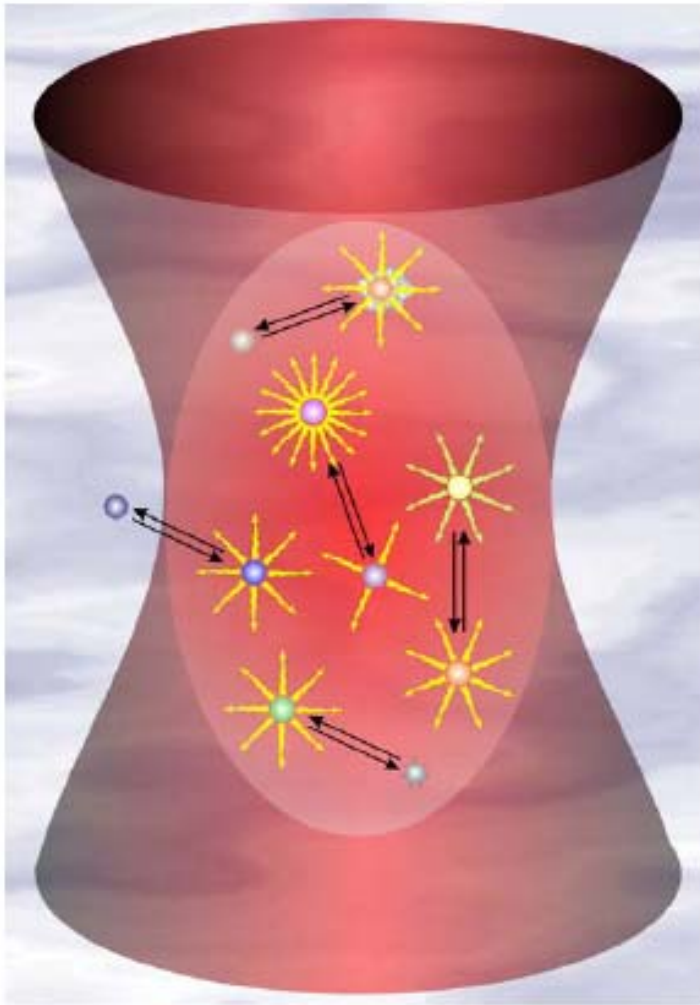


Figure 1: Molecular mechanisms that might give rise to fluorescence fluctuations comprise particle movements, conformational changes, chemical or photophysical reactions.

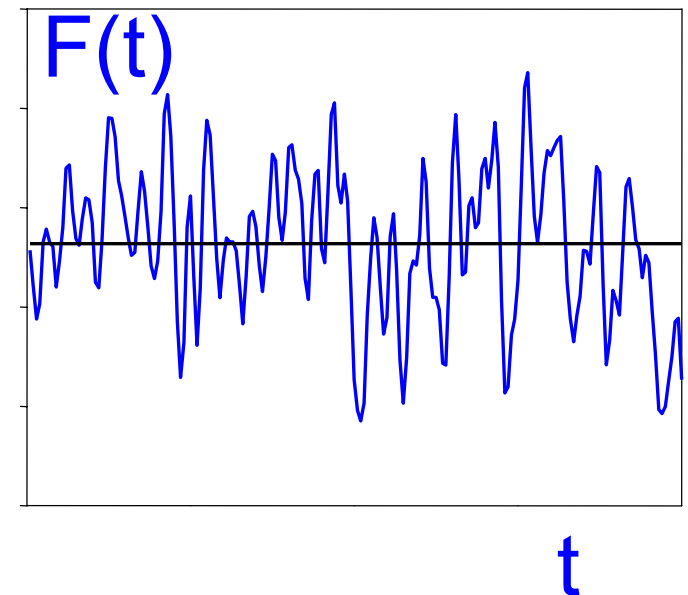
Molecular
dynamics



Number in
focus
fluctuates

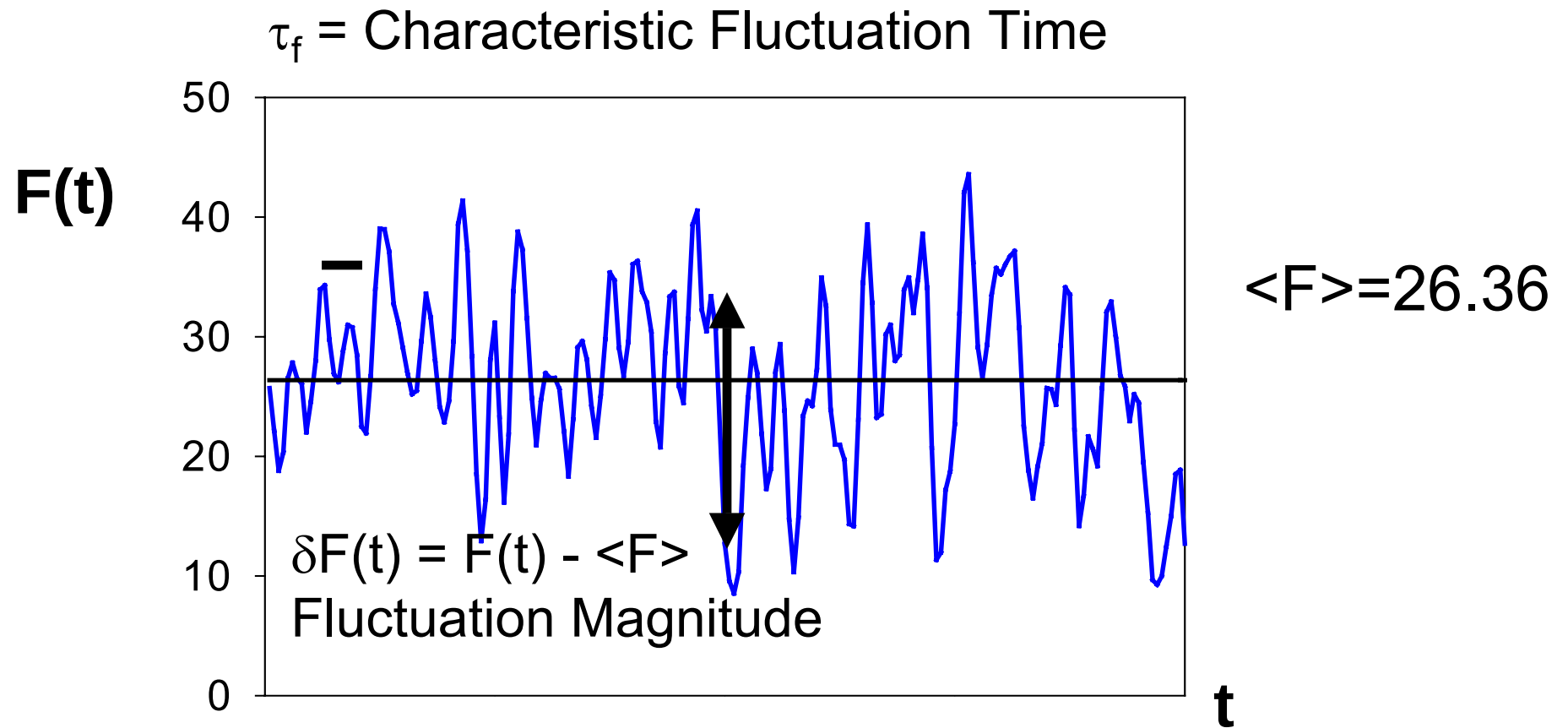


Fluorescence intensity
fluctuations



From Paul Wiseman, McGill University

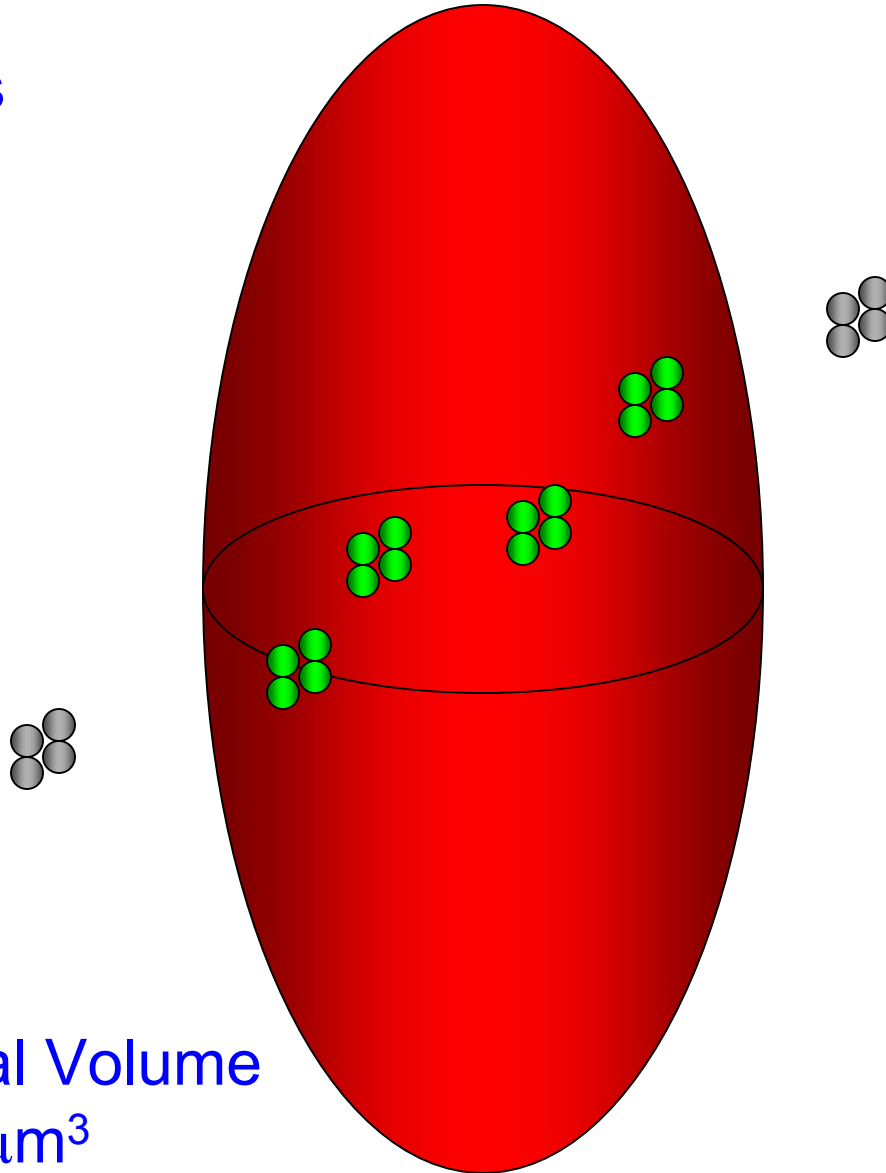
Fluctuation Magnitudes & Fluctuation Times



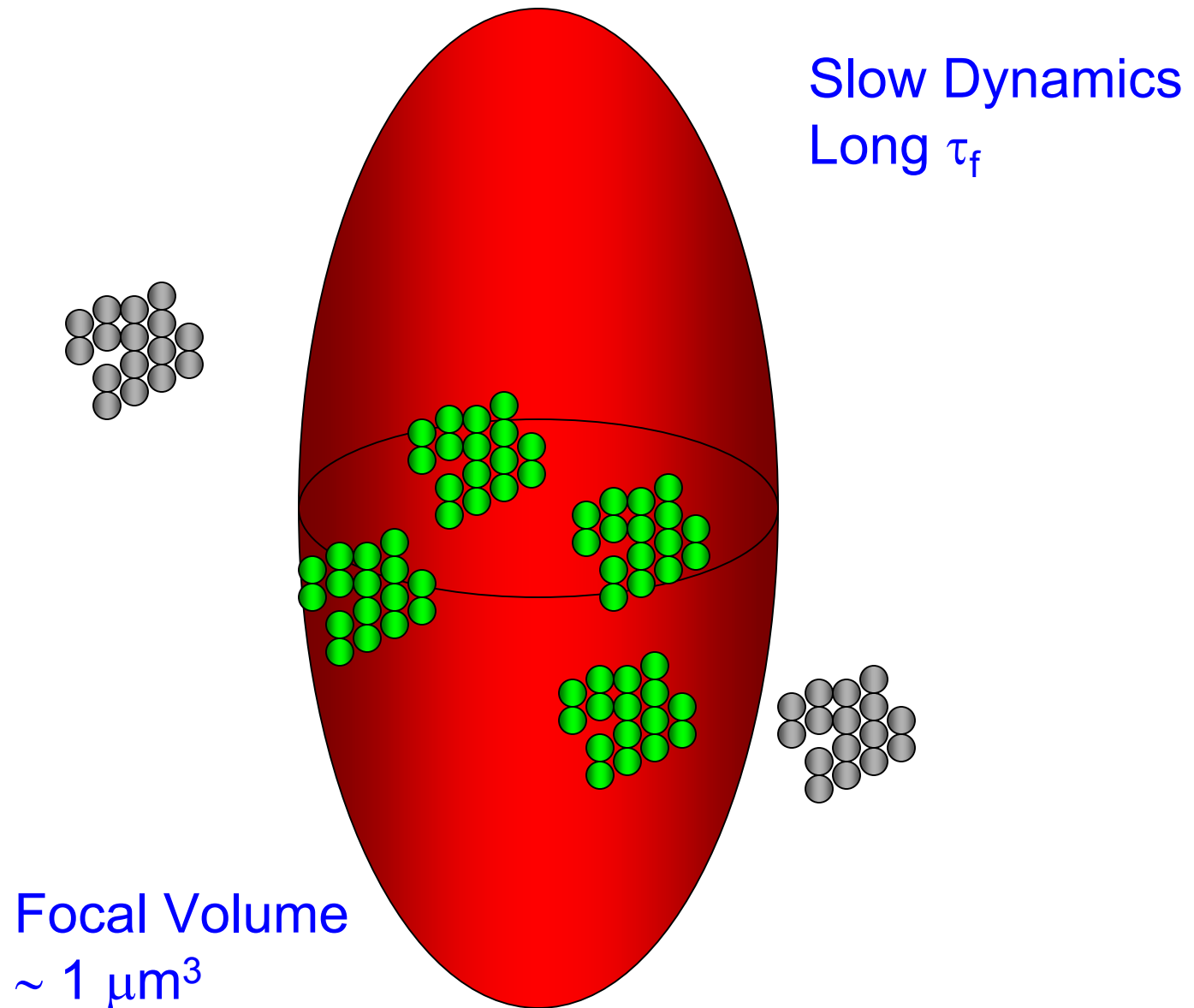
FCS: Fluctuations & Dynamics

Fast Dynamics
Short τ_f

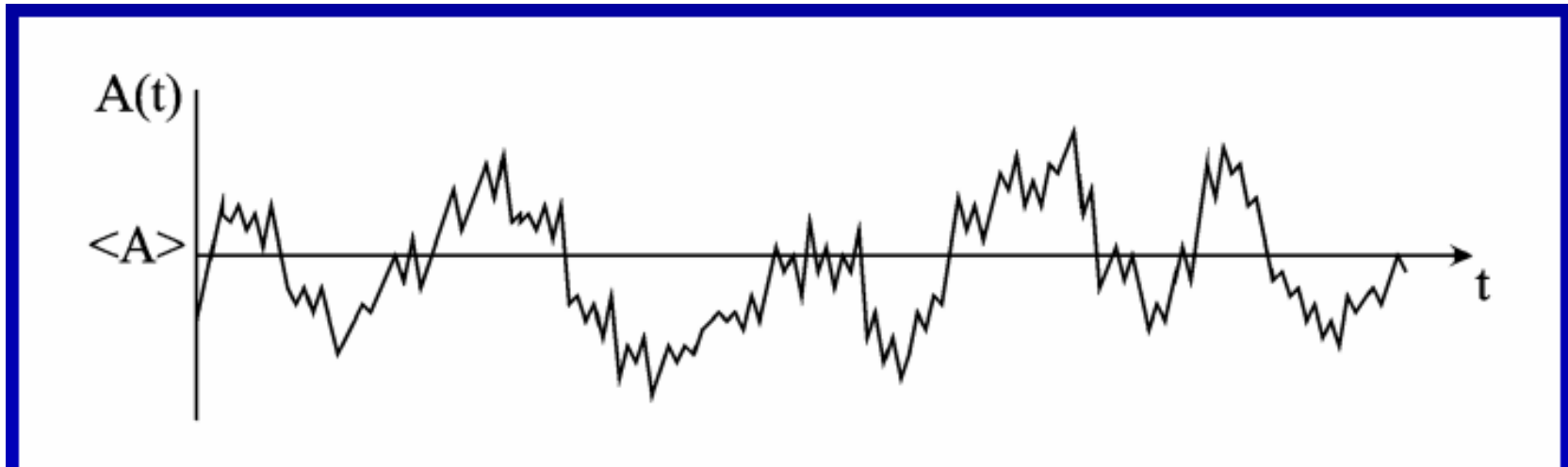
Focal Volume
 $\sim 1 \mu\text{m}^3$



FCS: Fluctuations & Dynamics



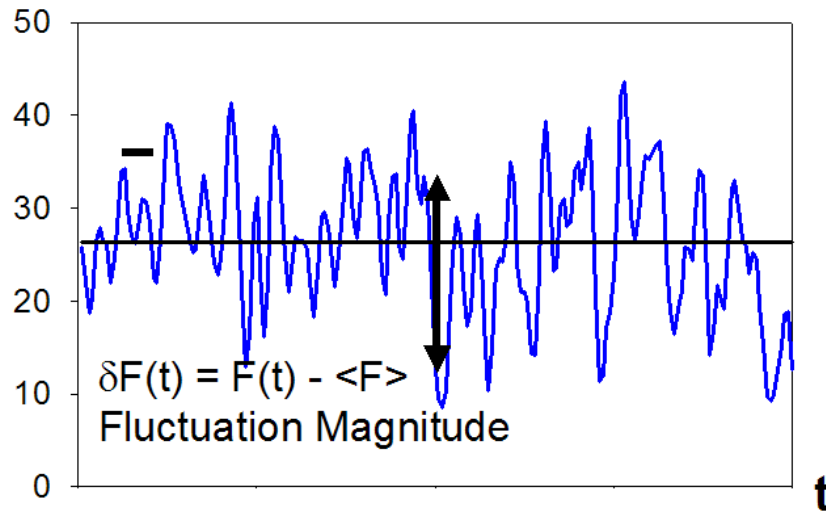
Fluctuation analysis: the auto-correlation function



Definition:
$$G(\tau) = \langle A(t) \cdot A(t + \tau) \rangle = \lim_{T \rightarrow \infty} \frac{1}{T} \int_0^T A(t) \cdot A(t + \tau) dt$$

the correlation function **$G(\tau_c)$** with the characteristic time **τ_c** for the fluctuation period of a timedependent **signal $A(t)$** with a **mean value $\langle A \rangle$** .

Fluctuation analysis: the auto-correlation function



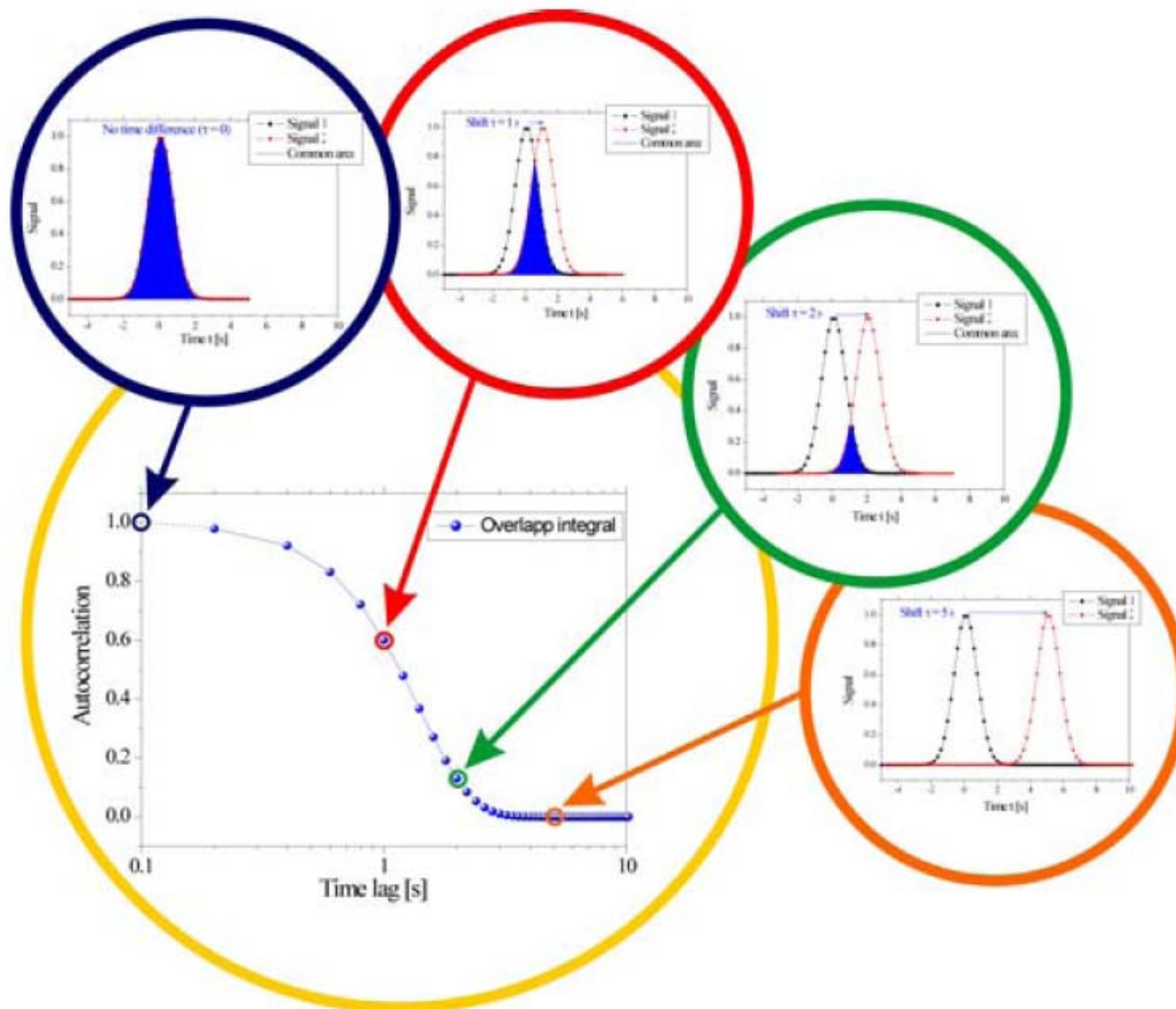
$$\delta F(t) = F(t) - \langle F(t) \rangle$$

$$\langle F(t) \rangle = \frac{1}{T} \int_0^T F(t) dt$$

Normalized auto-correlation function:

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

Fluctuation analysis: the auto-correlation function



Development of an autocorrelation curve.

From <http://www.biophysics.org/education/schwille.pdf>

Fluctuation analysis: the auto-correlation function

pre-condition for measuring $G(\tau_c)$

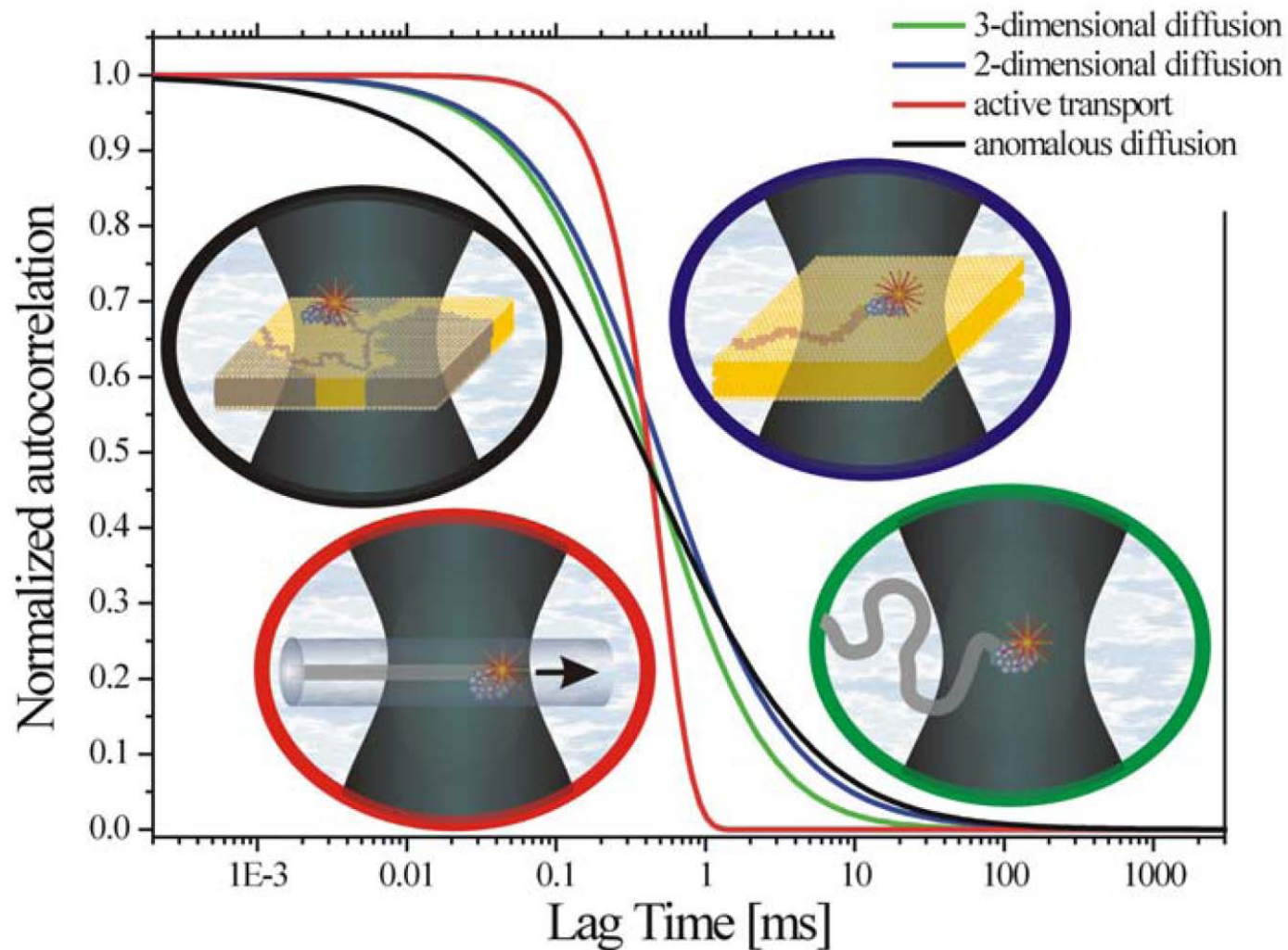
$$\Delta T \ll \tau_c \ll T_{\text{(total)}}$$

Δt : minimal time resolution of the measurement system,
determined by the 'dead times' of the the detector(s)
and the counting device, i.e. the 'binning time'

τ_c : characteristic time of the process
inducing periodic fluctuations

$T_{\text{(total)}}$: overall measurement time

FCS: characterizing particle motion



Model autocorrelation curves for different kinds of particle motion

From <http://www.biophysics.org/education/schwille.pdf>

FCS: probing intramolecular dynamics

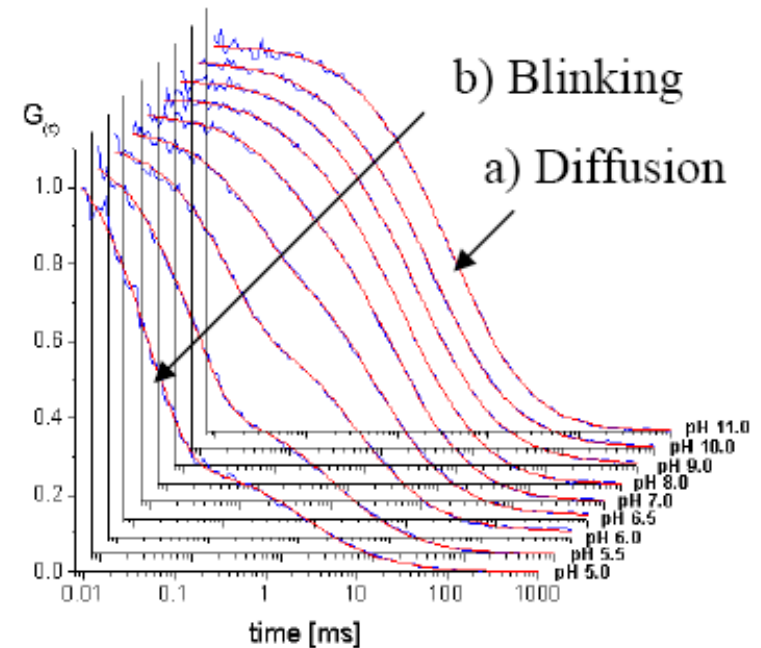
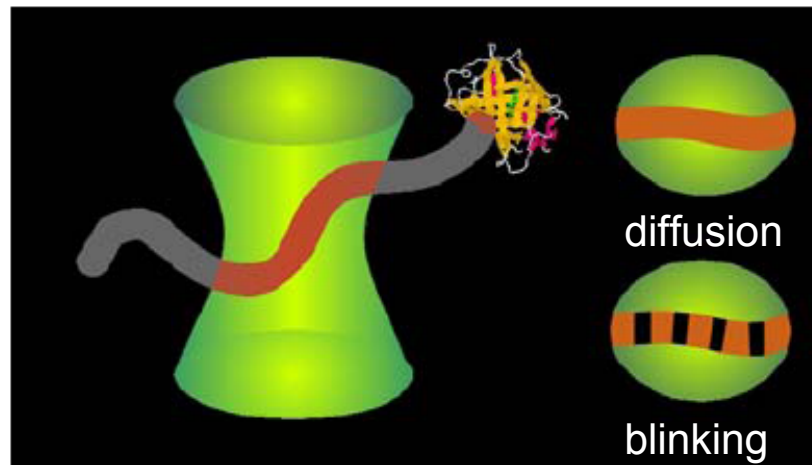
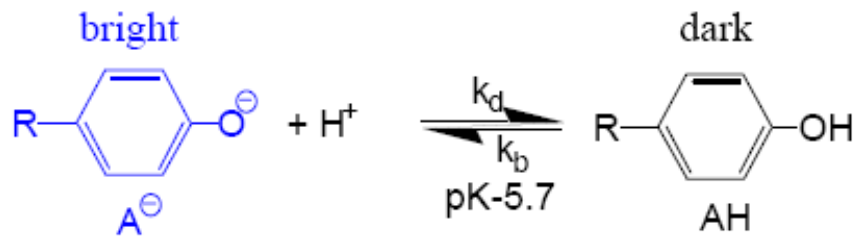
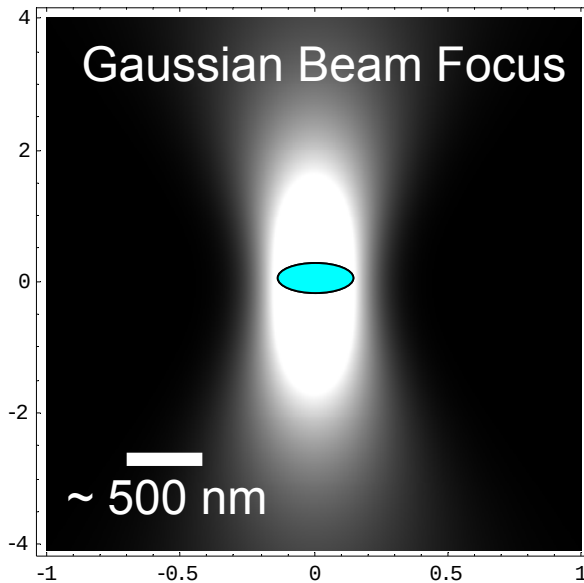


Figure 20: This is the reaction scheme showing the transition from bright (deprotonated) to dark (protonated) state of the dye for excitation at 488 nm. On the right hand side you can see the autocorrelation curves measured in buffer solutions of different pH.

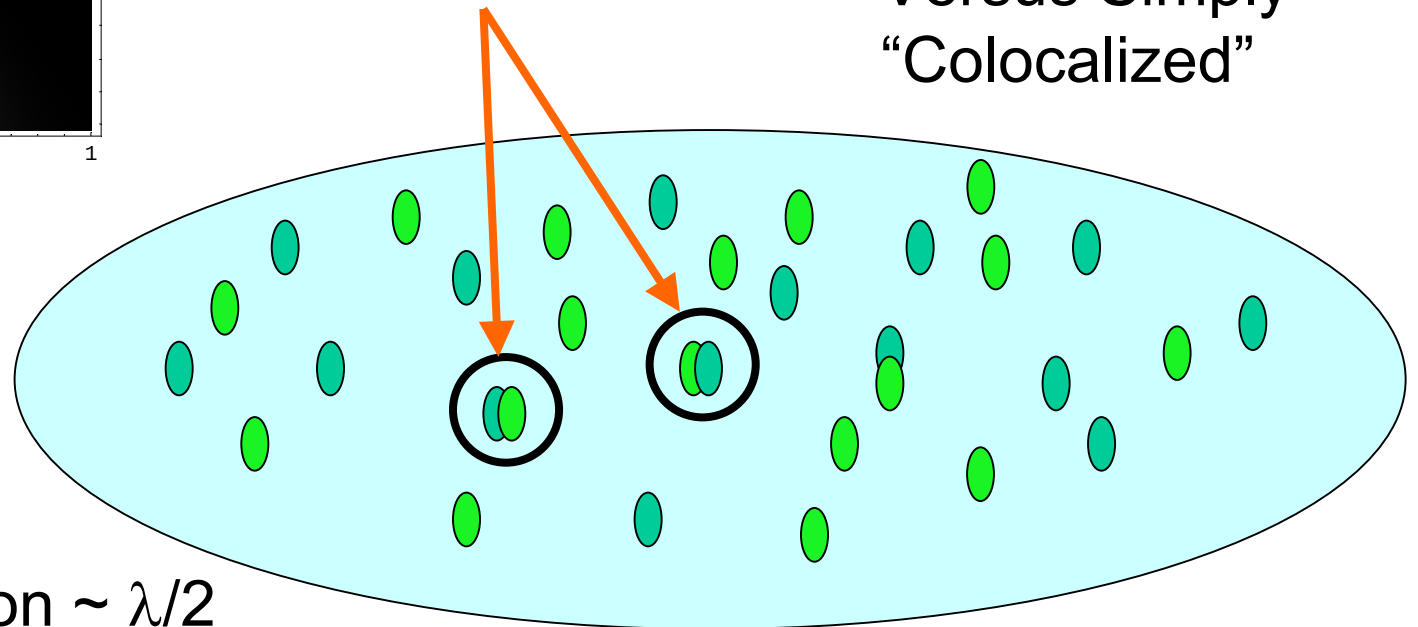
Colocalization vs true molecular interaction



Optical Microscopy
Dynamics at the Price of Spatial Resolution

Truly Interacting Species
Dance Partners

Versus Simply
“Colocalized”



Optical Resolution $\sim \lambda/2$

Macromolecules $\sim \lambda/50$

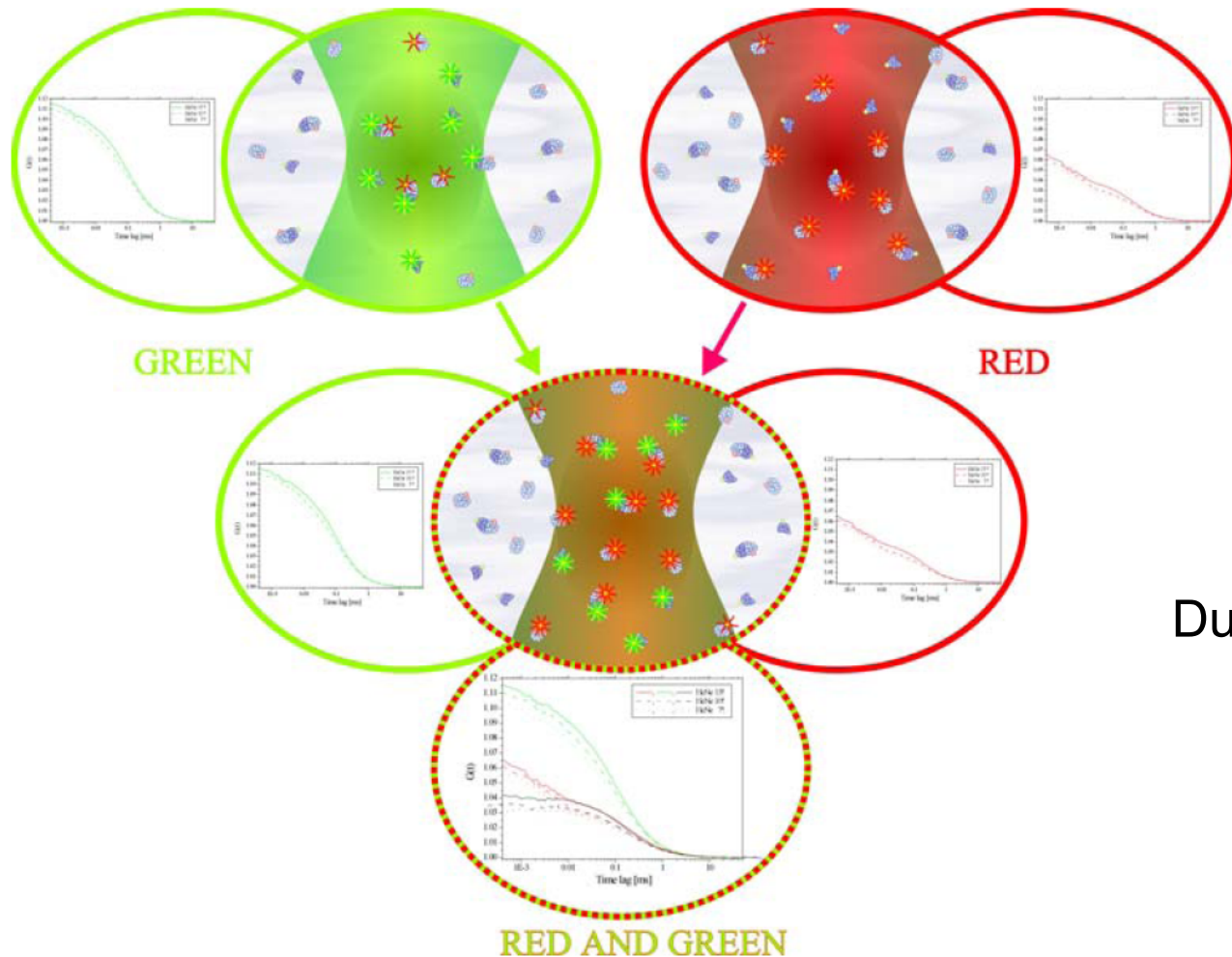
FCS: dual-color cross-correlation inter-molecular interaction

2 fluorophores: cross- instead of auto-correlation

Normalized cross-correlation function:

$$G_{cross}(\tau) = \frac{\langle \delta F_1(t) \cdot \delta F_2(t + \tau) \rangle}{\langle F_1(t) \rangle \langle F_2(t) \rangle}$$

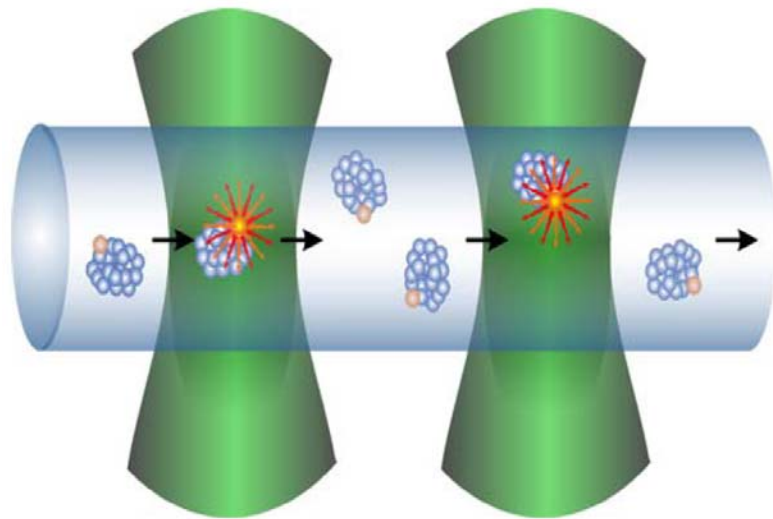
FCS: dual-color cross-correlation inter-molecular interaction



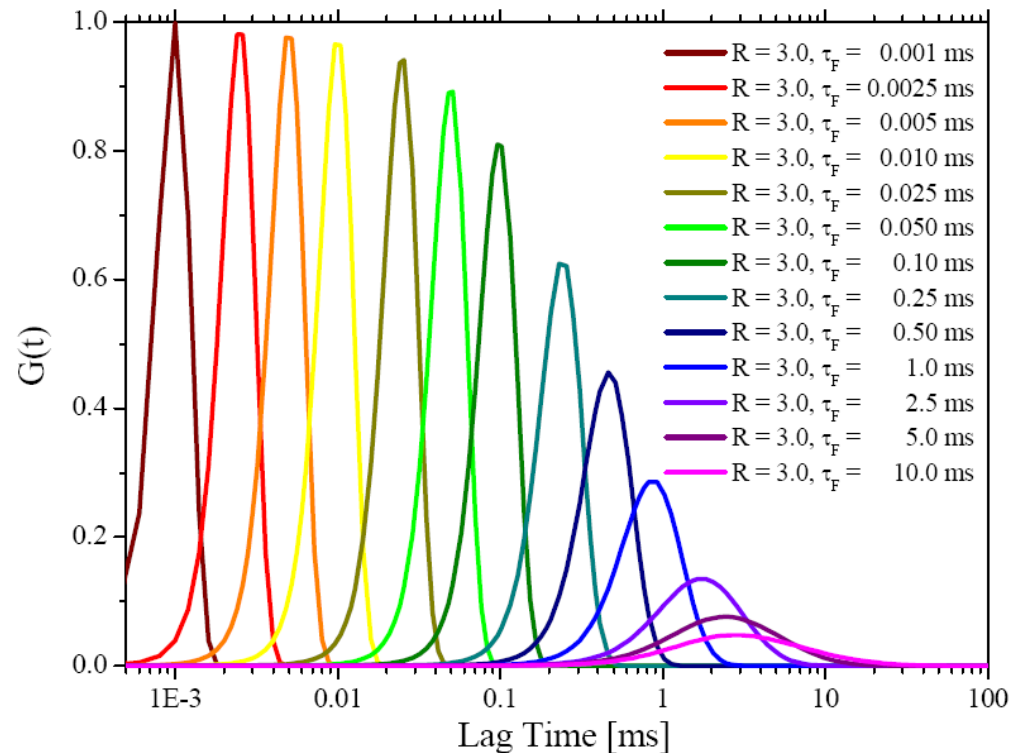
Dual color FCS can
reveal truly
interacting of
molecules!

Figure 14: Using dual-color cross-correlation, more information about double-labeled particles can be received than from both single-color measurements.

FCS: dual-beam cross-correlation measuring velocity



Dual-beam cross-correlation



The flow velocity v is defined as $v = \frac{\text{waist diameter of the focus}}{\text{flow time}} = \frac{r_o}{\tau_{flow}}$

R : distance between the 2 focal volumes

FCS: conclusions

Non-intensity based technique
Sensitive to fluorescence fluctuations
High temporal/spatial resolution

Measure of :

- mobility constant
- intra-molecular dynamics
- inter-molecular dynamics

NOTE: this technique requires a low concentration of fluorophore!

Each molecule has to contribute substantially to the measured signal in order to generate fluorescence fluctuations