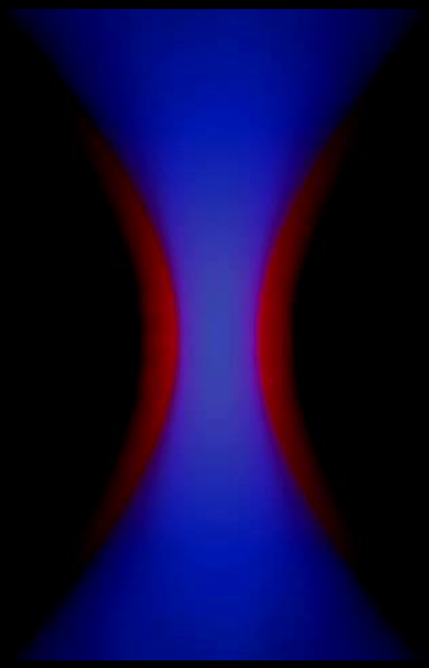

FLUCTUATION MICROSCOPIES

Emmanuel MARGEAT
CNRS
Centre de Biologie Structurale – Montpellier

FLUCTUATION MICROSCOPIES

- INTRODUCTION
- PRINCIPE OF FCS
- AUTOCORRELATION
- CROSS CORRELATION
- EXPERIMENTAL DETAILS
- NUMBER & BRIGHTNESS

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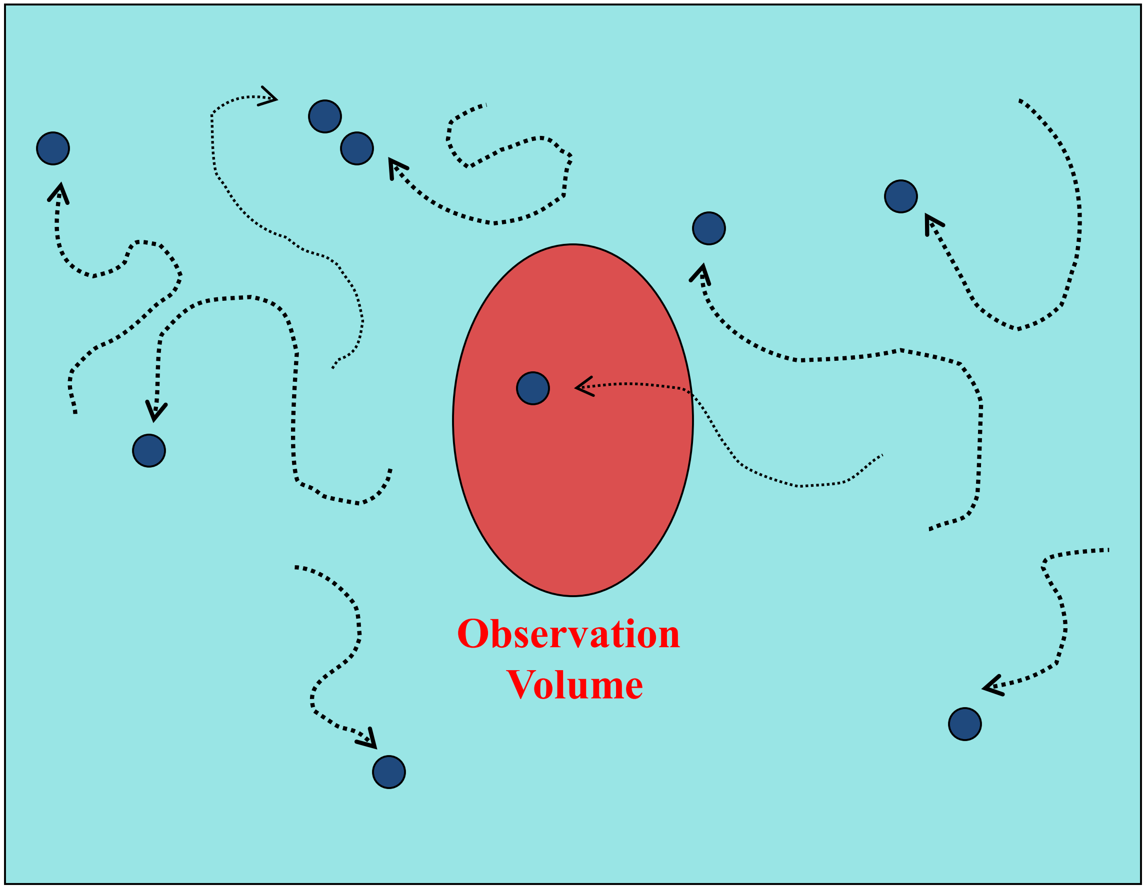
Observation of fluctuations



Sample Space

What can we observe ?

1. Speed of the movement
2. Particles concentration
3. Change of state, and thus of the signal, of the particles

→ Particles interactions

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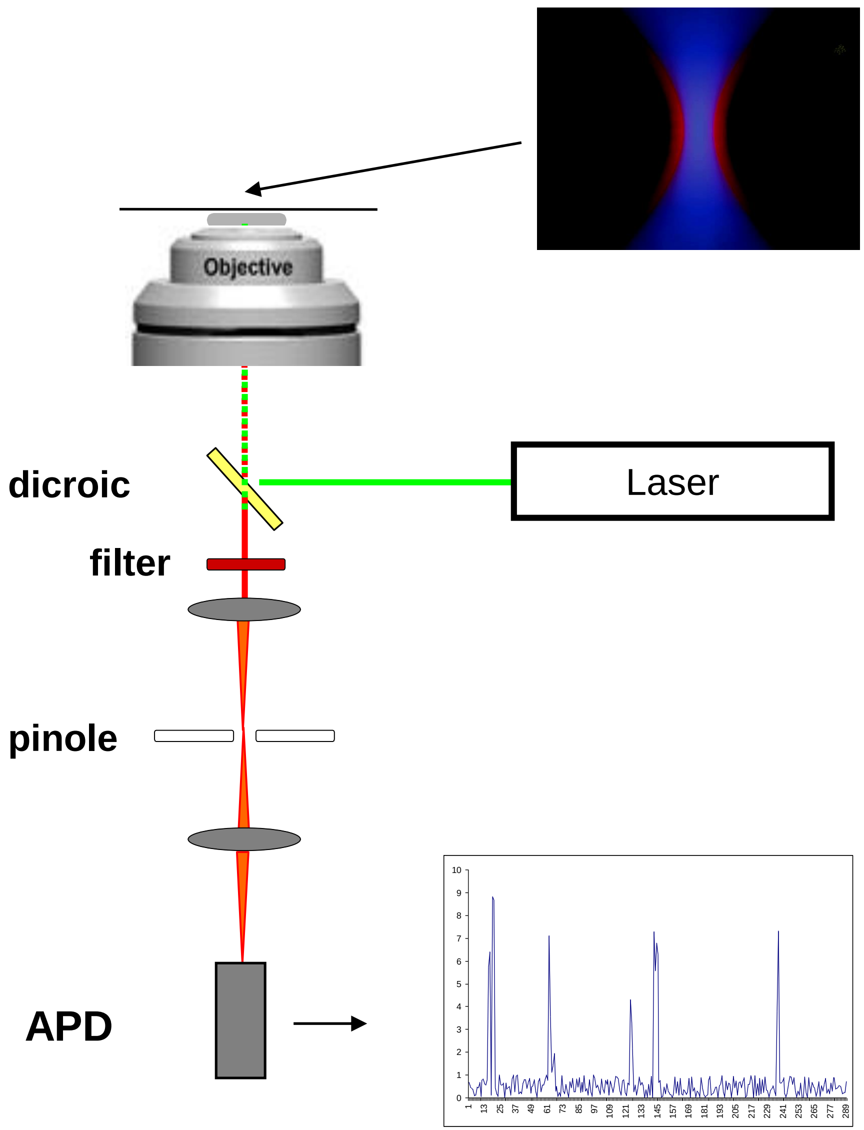
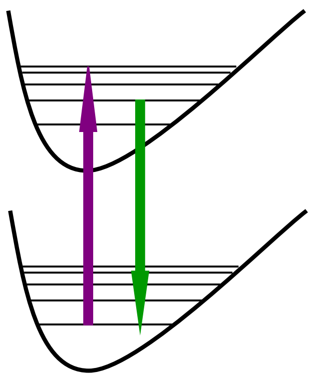
Observing fluctuations : fluorescence microscopy

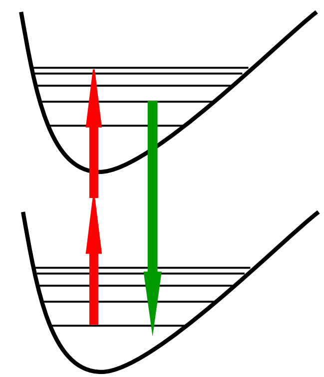


The observation volume
One- & Two-Photon Excitation.

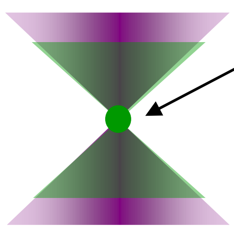
1 - Photon

2 - Photon

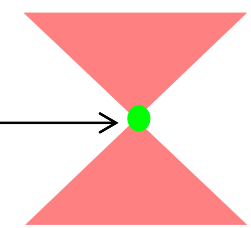




Defined by the size of the pinole, the wavelength, the magnification, and the numerical aperture

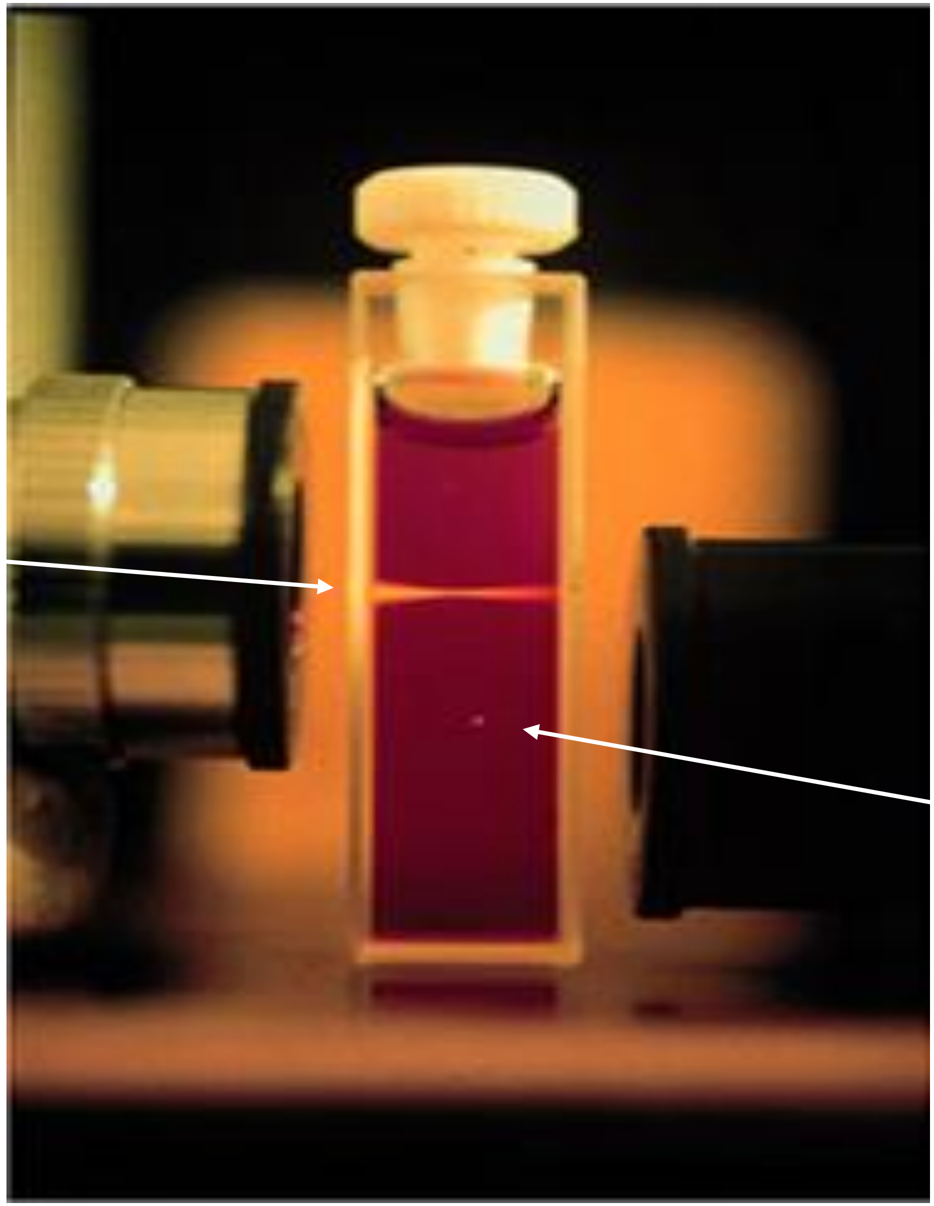


~1 um^3



Defined by the wavelength and the numerical aperture

1-photon

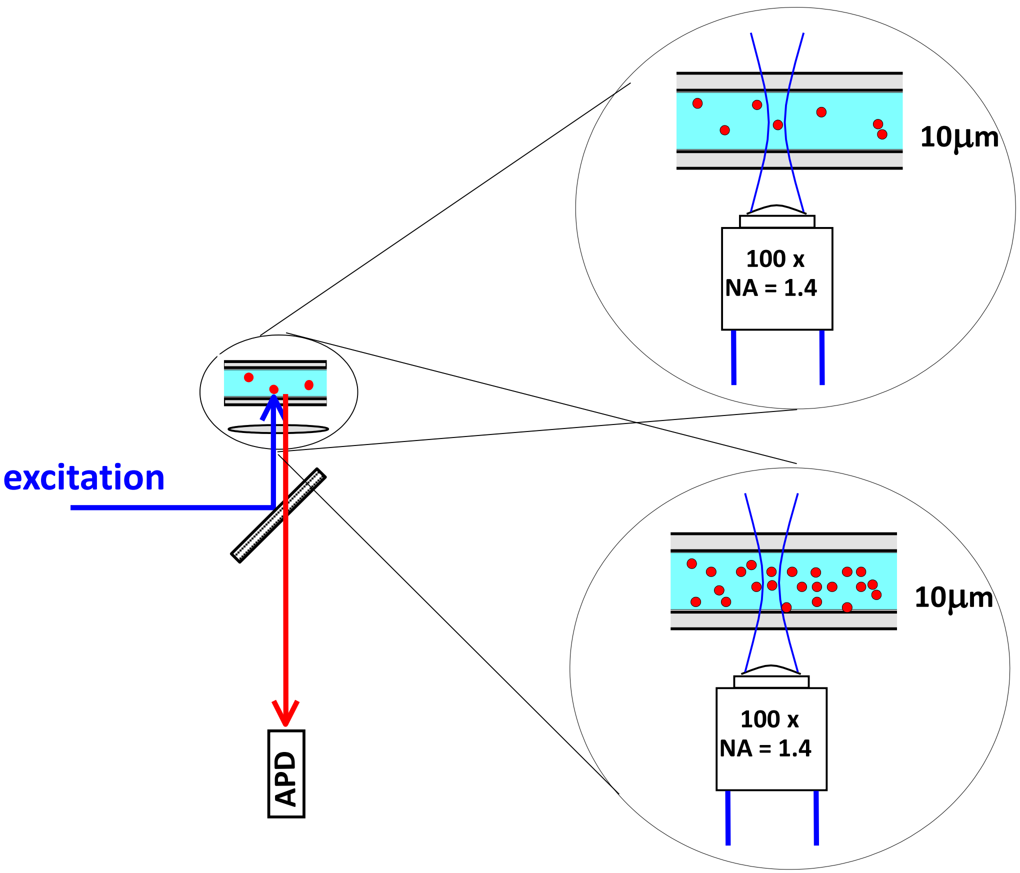


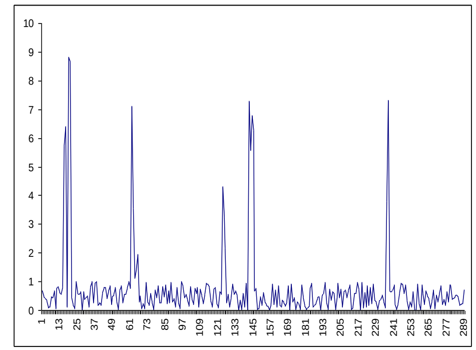
Need a pinhole to
define a small volume

2-photon

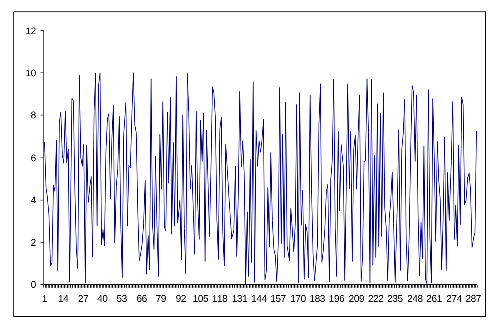
Brad Amos
MRC, Cambridge, UK

Fluorescence correlation spectroscopy





Single molecules regime



« Small ensemble » regime

Using FCS, we will analyze these intensity fluctuations

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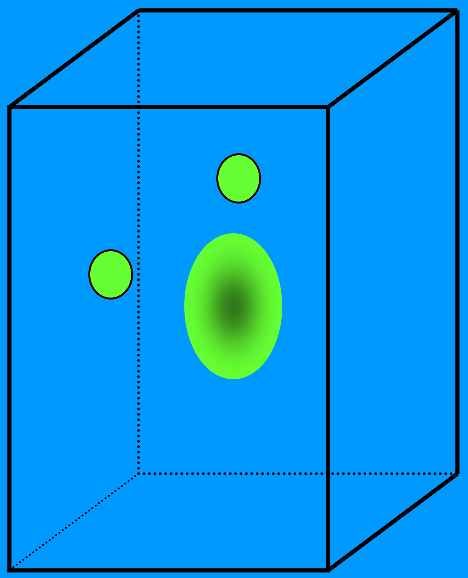
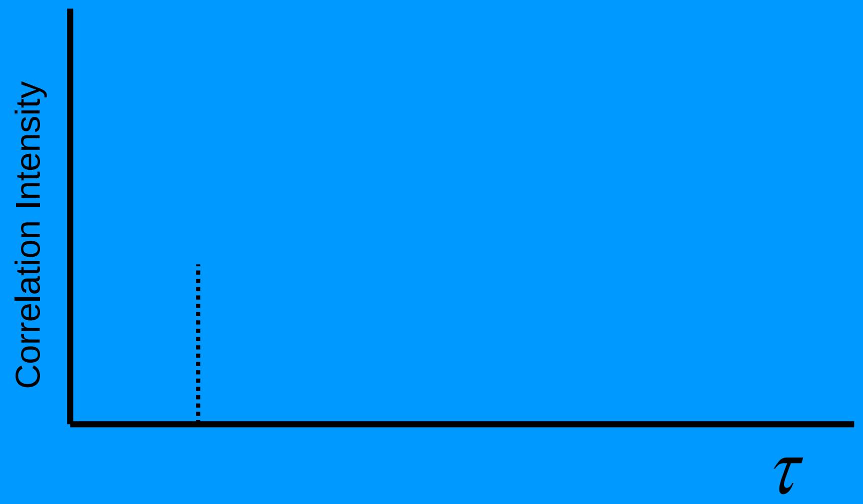
Introduction to correlation function.

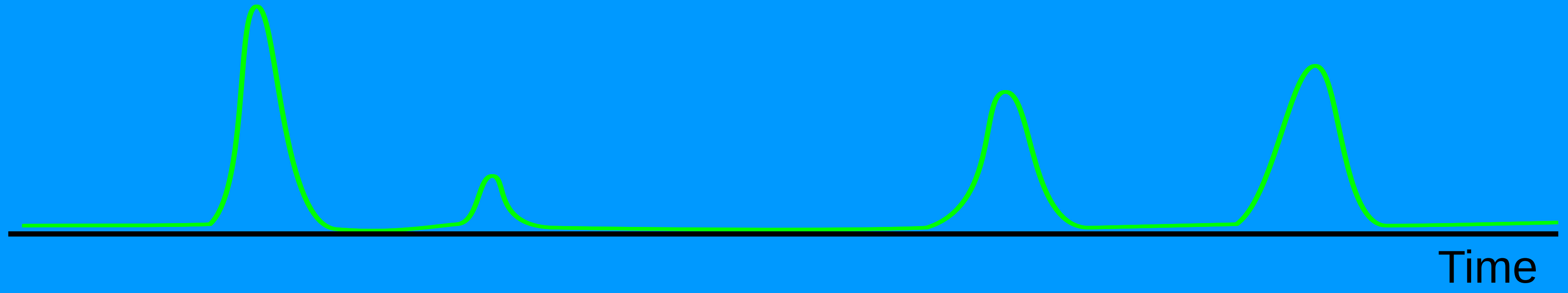


Time

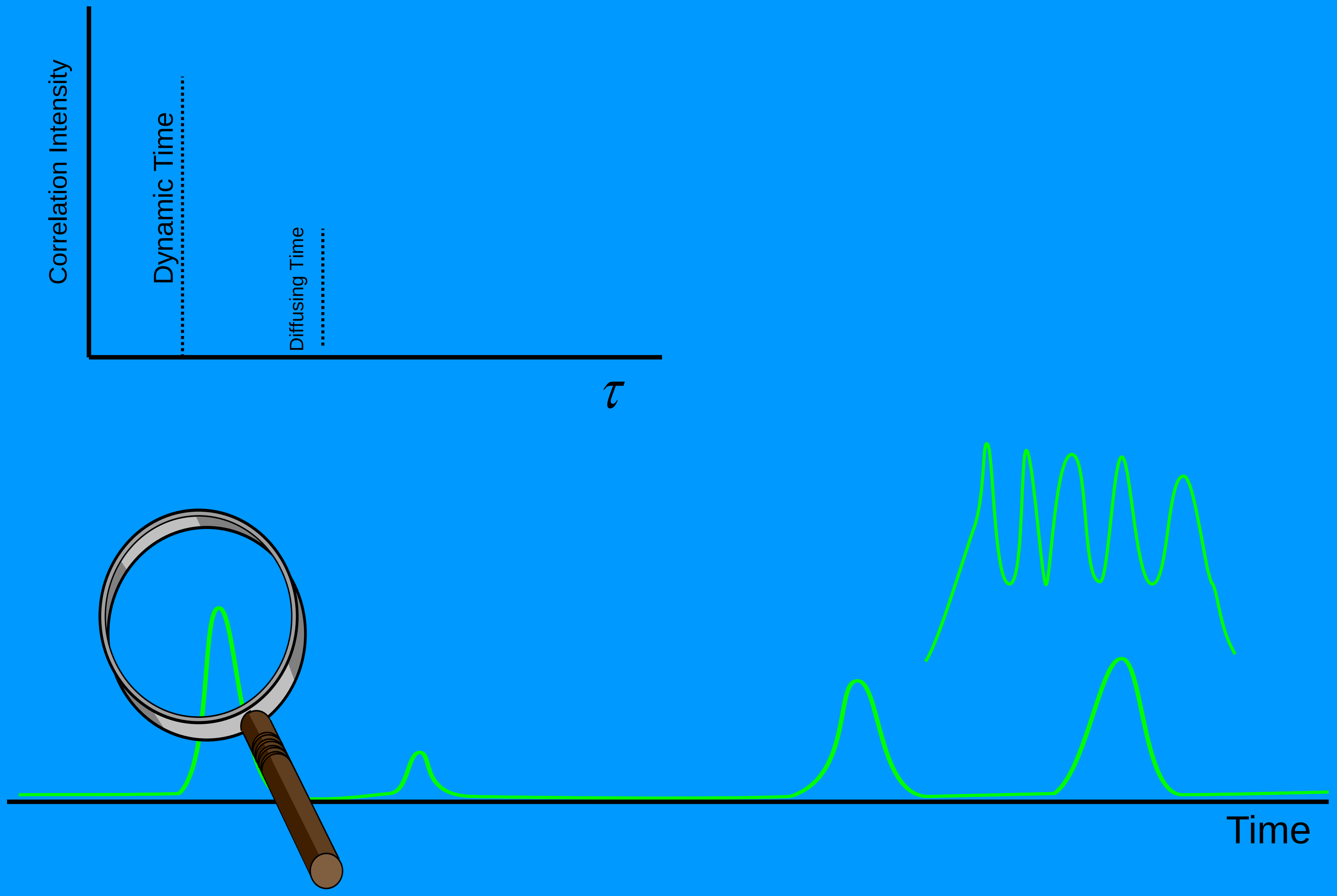
AutoCorrelation. Diffusion only, No Dynamics. "Donor-Donor"



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



AutoCorrelation. Diffusion and Dynamics. “Donor-Donor”



2 definitions for the autocorrelation function

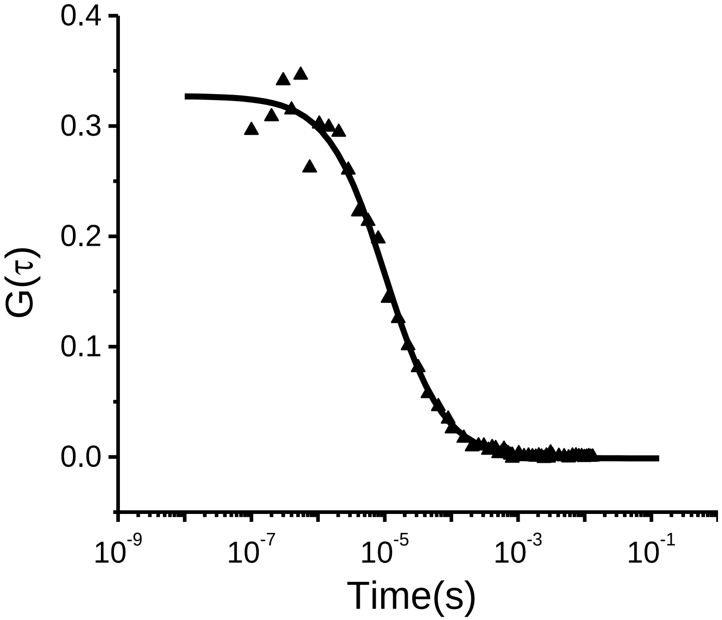
$$G(\tau) = \frac{\langle \delta F(t)\ \delta F(t+\tau)\rangle}{\langle F\rangle^2}$$

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

Decays to 0 for $\tau \rightarrow \infty$

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

Decays to 1 for $\tau \rightarrow \rightarrow \infty$



$$G(\tau) = \frac{1}{N}\left(1+\frac{\tau}{\tau_d}\right)^{-1}\left(1+\frac{r_o^2\tau}{z_o^2\tau_d}\right)^{-1/2}$$

Where r_o and z_o are the lateral and axial radii of the observation volume respectively

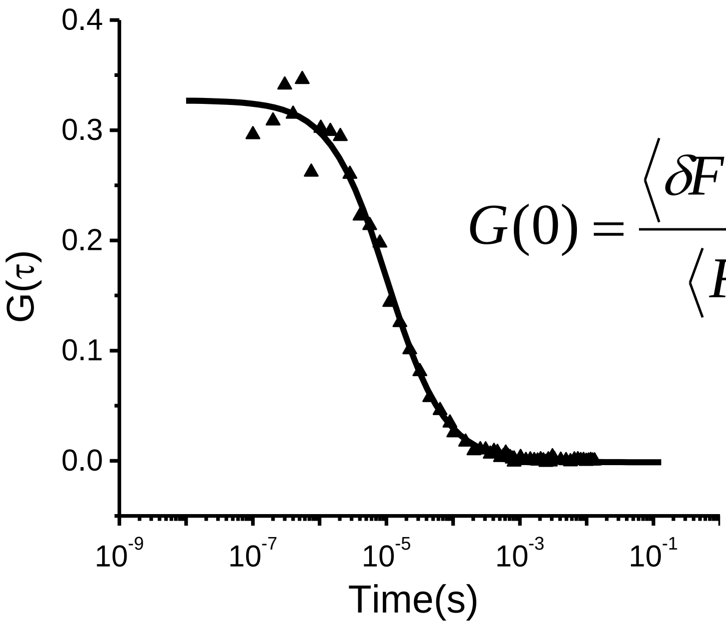
G(0) = 1/Nb of observed particles

In a poissonian system the variance of the number of events is equal to its mean (here the average numer of observed particles)

$$\langle N \rangle = Variance_N = \sigma_N^2$$

$F = \varepsilon . N$, avec ε = molecular brightness

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



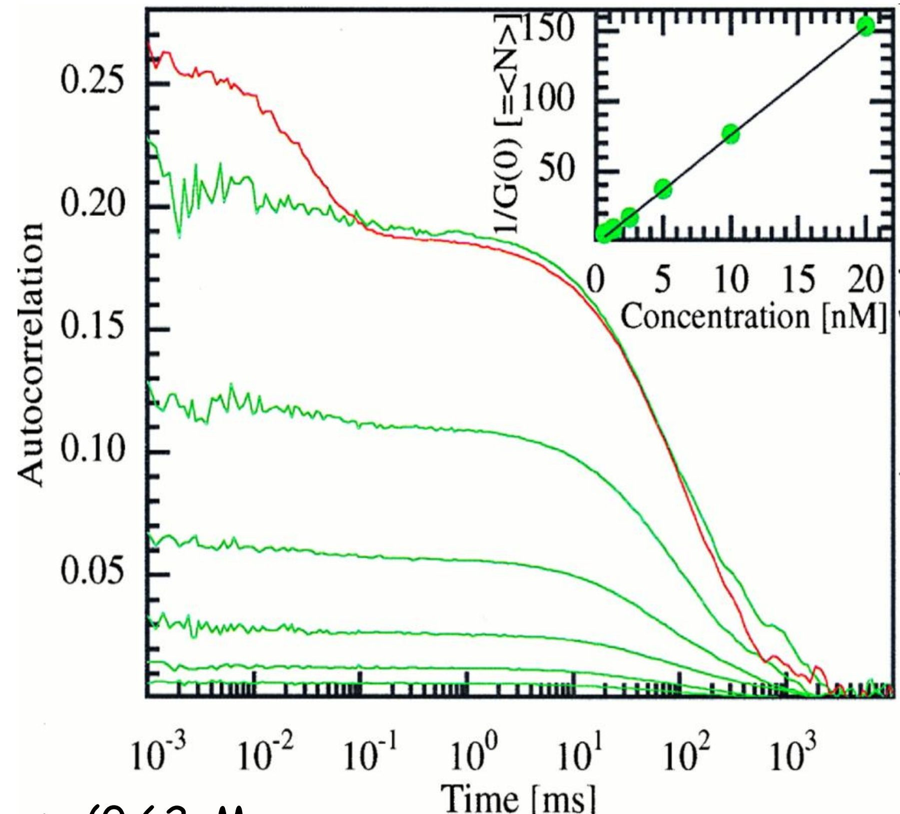
$$G(0) = \frac{\langle \delta F(t)^2 \rangle}{\langle F \rangle^2} = \frac{\left\langle \left(F(t) - \langle F(t) \rangle \right)^2 \right\rangle}{\langle F \rangle^2} = \frac{\sigma_F^2}{\langle F \rangle^2} = \frac{\varepsilon^2 . \sigma_N^2}{\varepsilon^2 . \langle N \rangle^2}$$

$$G(0) = \frac{Variance}{\langle N \rangle^2} = \frac{1}{\langle N \rangle}$$

Effect of the concentration

For a single species, no photobleaching

$$G(0) = \frac{1}{\langle N \rangle}$$



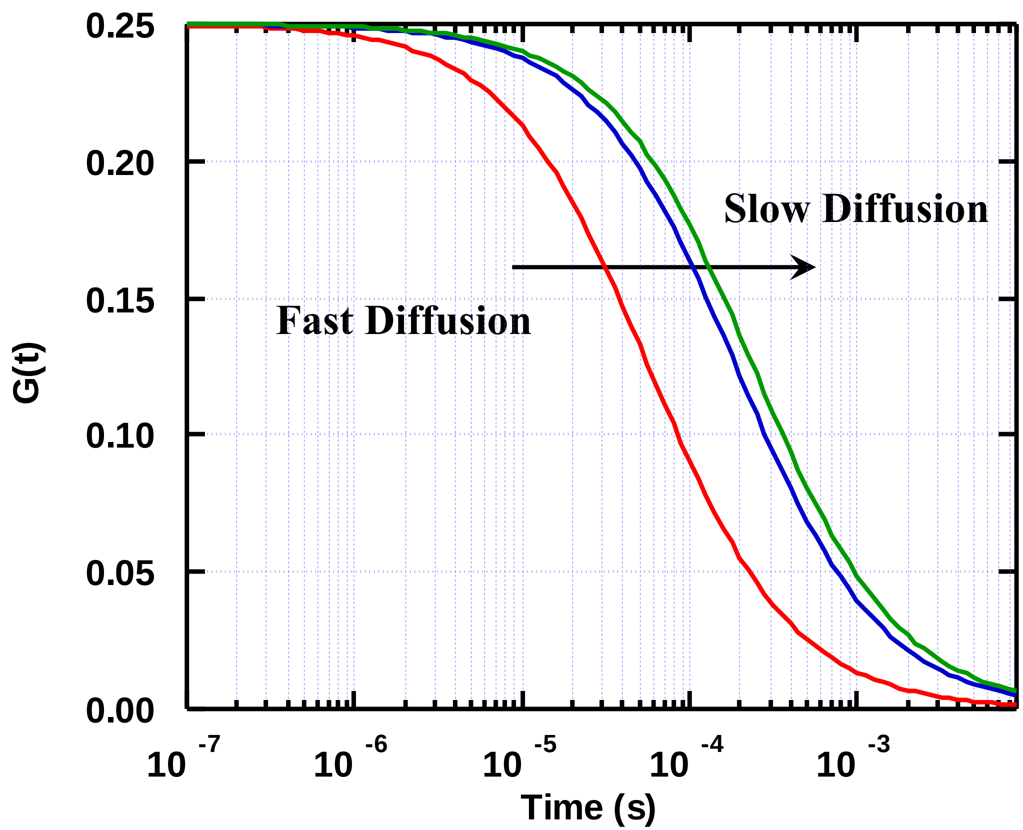
Rhodamine 6G in 70% sucrose (0.62nM, 1.25nM, 2.5nM, 5nM, 10nM, 20nM) (green curves)

0.62nM with a 70 times higher excitation power (red curve)

The Effects of Particle Size on the Autocorrelation Curve

Diffusion Constants

300 um²/s
90 um²/s
71 um²/s



Stokes-Einstein Equation:

$$D = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot r}$$

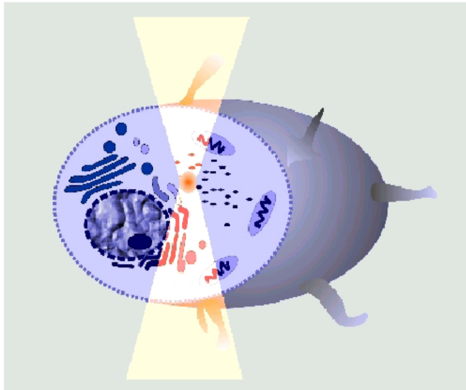
and

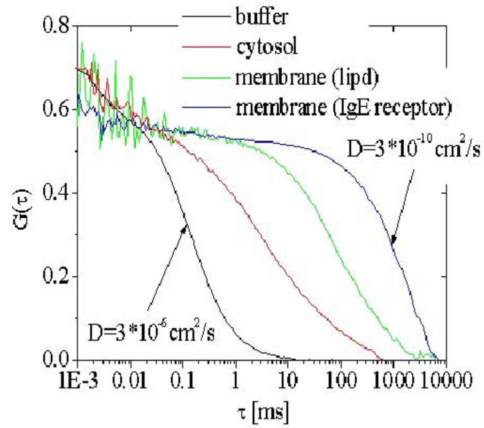
$$MW \propto Volume \propto r^3$$

$$\tau_d = \frac{r_o^2}{4D} \quad \text{and} \quad \tau_d = \frac{r_o^2}{8D}$$

for one and two photon excitation respectively

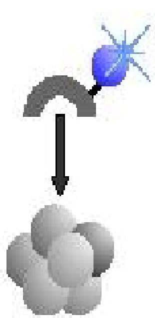
Mobility of a fluorescent macromolecule

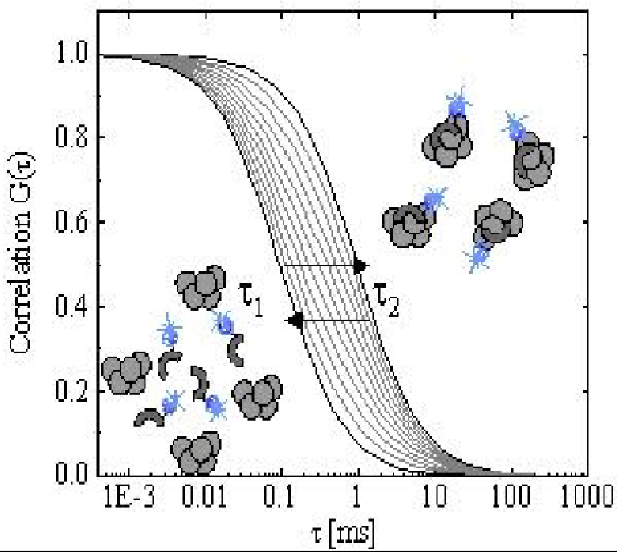




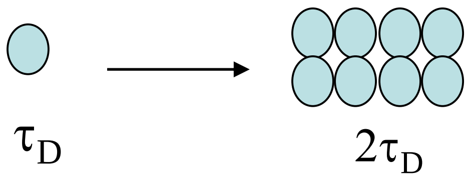
Rhodamine dye in different environments

Measuring molecular interactions



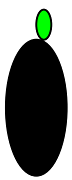


Diffusion time = f (MW) $^{1/3}$

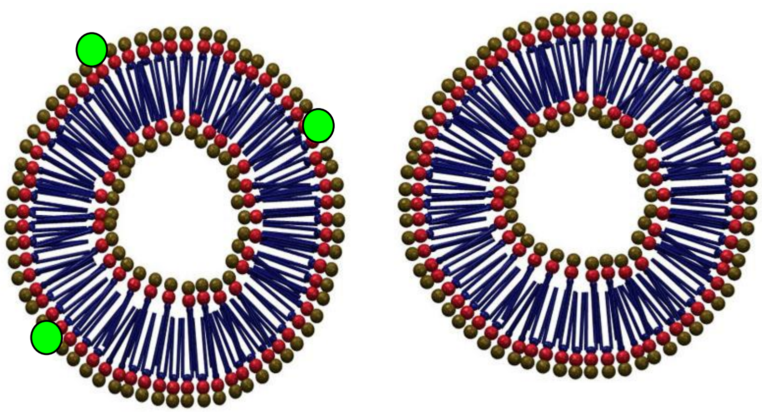


Measurement of the interaction between ezrin and Large Unilamelar Vesicles (LUV)

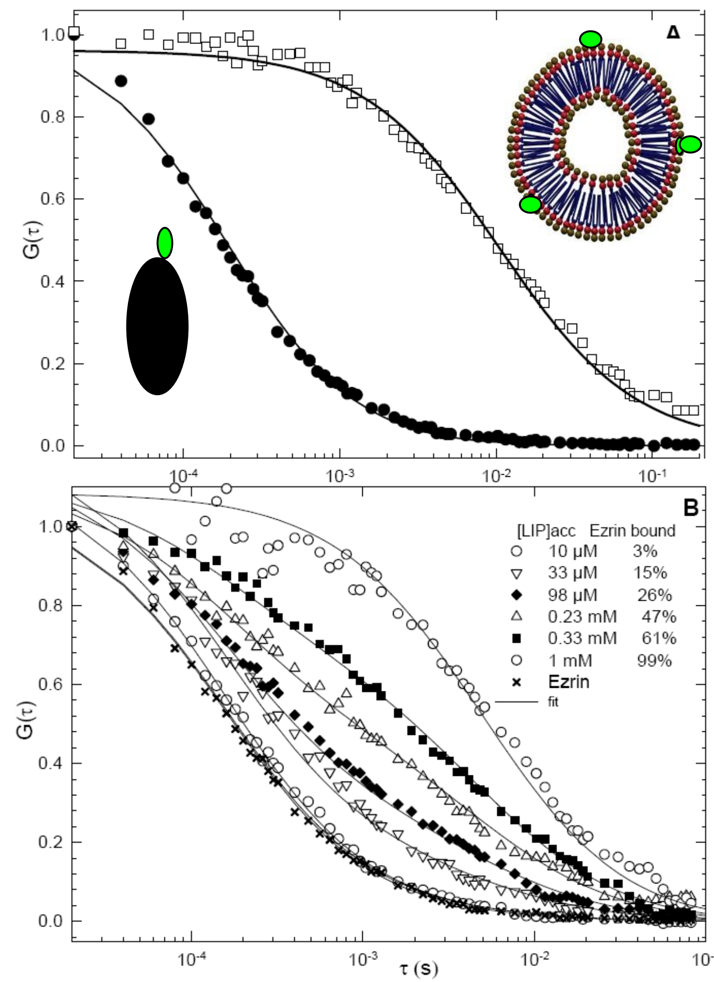
Collaboration with Pr. Picard (UM2)

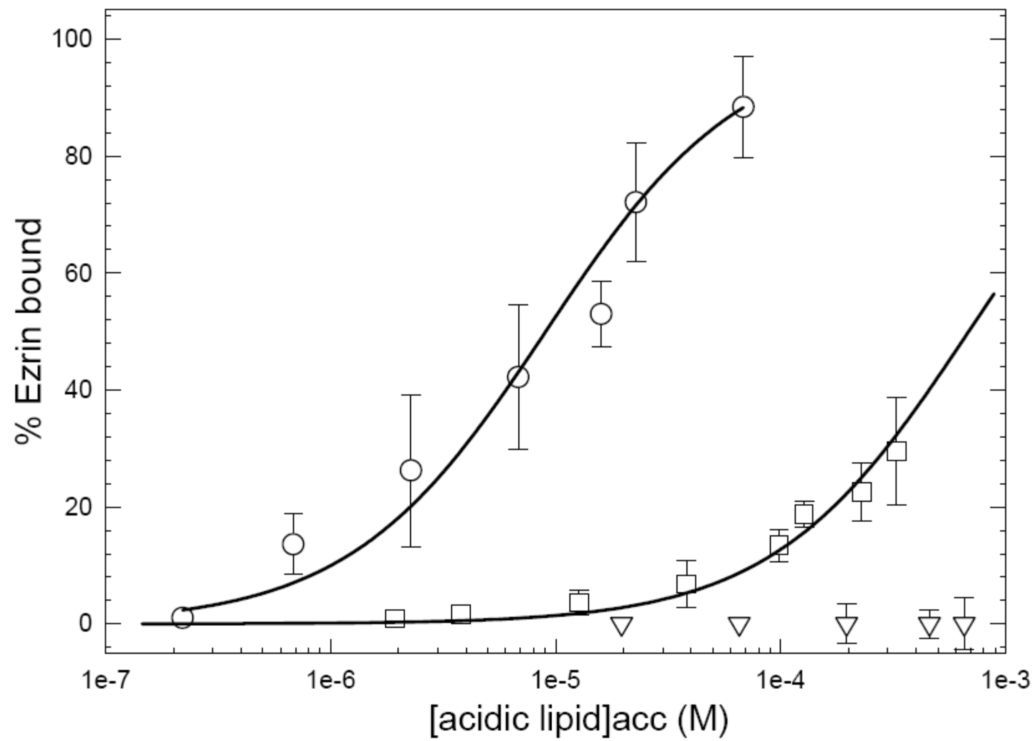


Ezrin : protein interacting with actin and phospholipids
Specific labeling with Alexa488



LUV, labeled or unlabeled





- Titiration curve allowing the determination of the affinity between ezrin and LUV with different lipid compositions

- Demonstration of préférential binding of ezrin to PIP_2-containing vesicles

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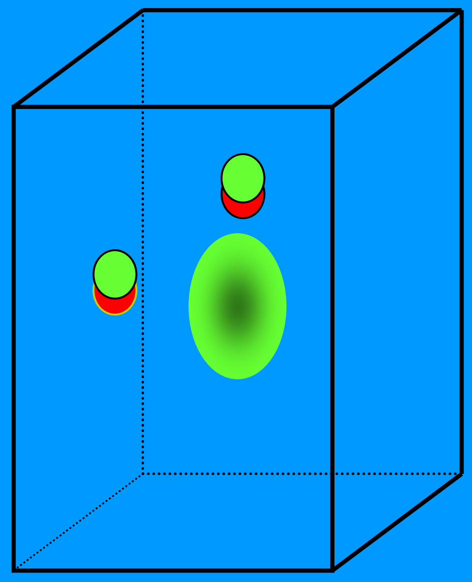
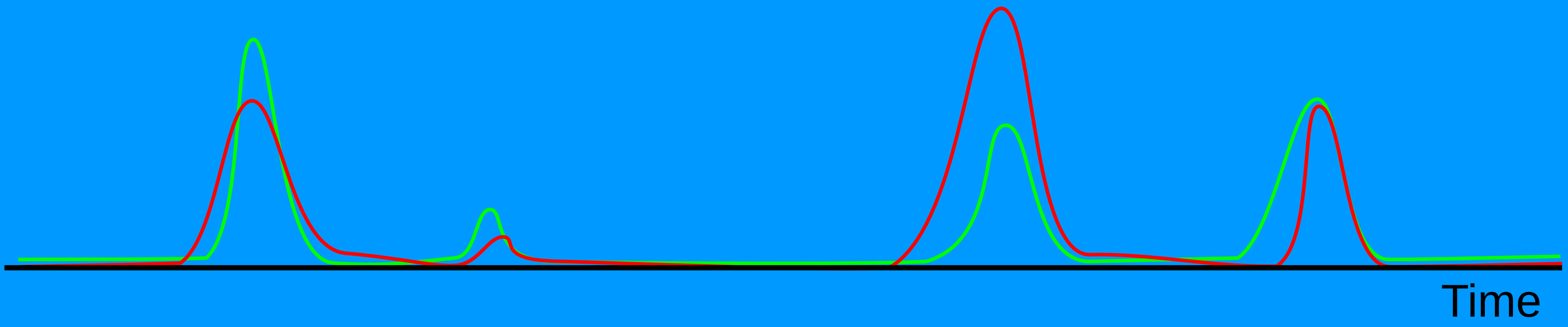
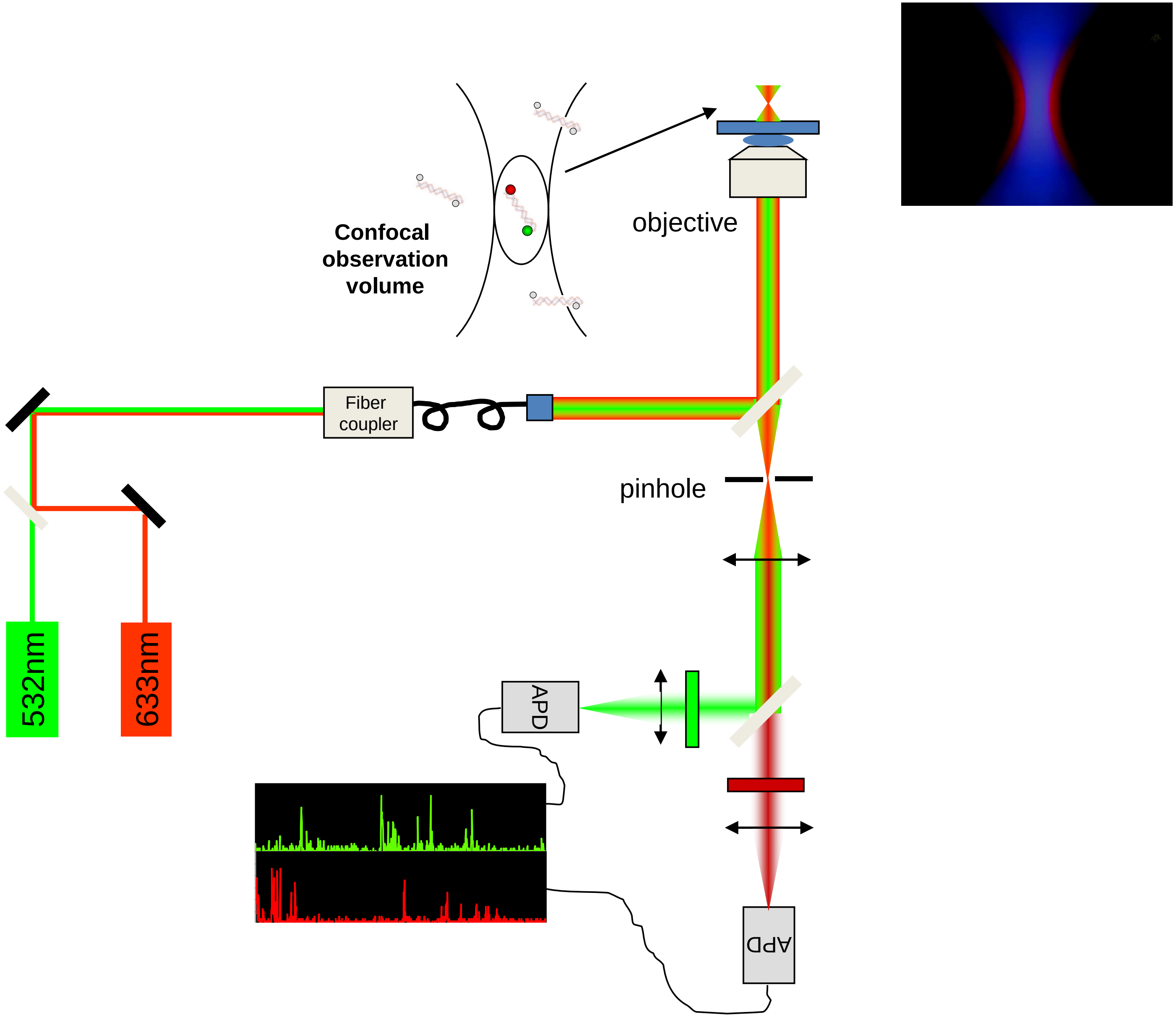
Introduction to correlation function.



Time

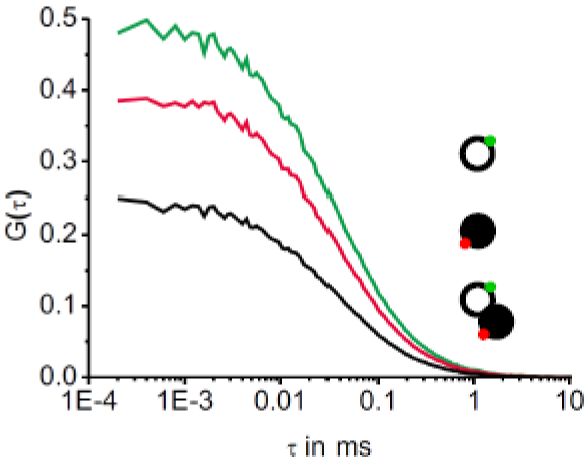
CrossCorrelation. Diffusion only, No Dynamics. “Donor-Acceptor”





Confocal observation volume
objective
Fiber coupler
pinhole
532nm
633nm
APD
APD

$$G_X(\tau) = \frac{\langle \delta F_1(t) . \delta F_2(t+\tau) \rangle}{\langle F_1(t) \rangle \langle F_2(t) \rangle}$$



- n_1: number of green molecules
- n_2: number of red molecules
- n_{12}: number of green-red molecules

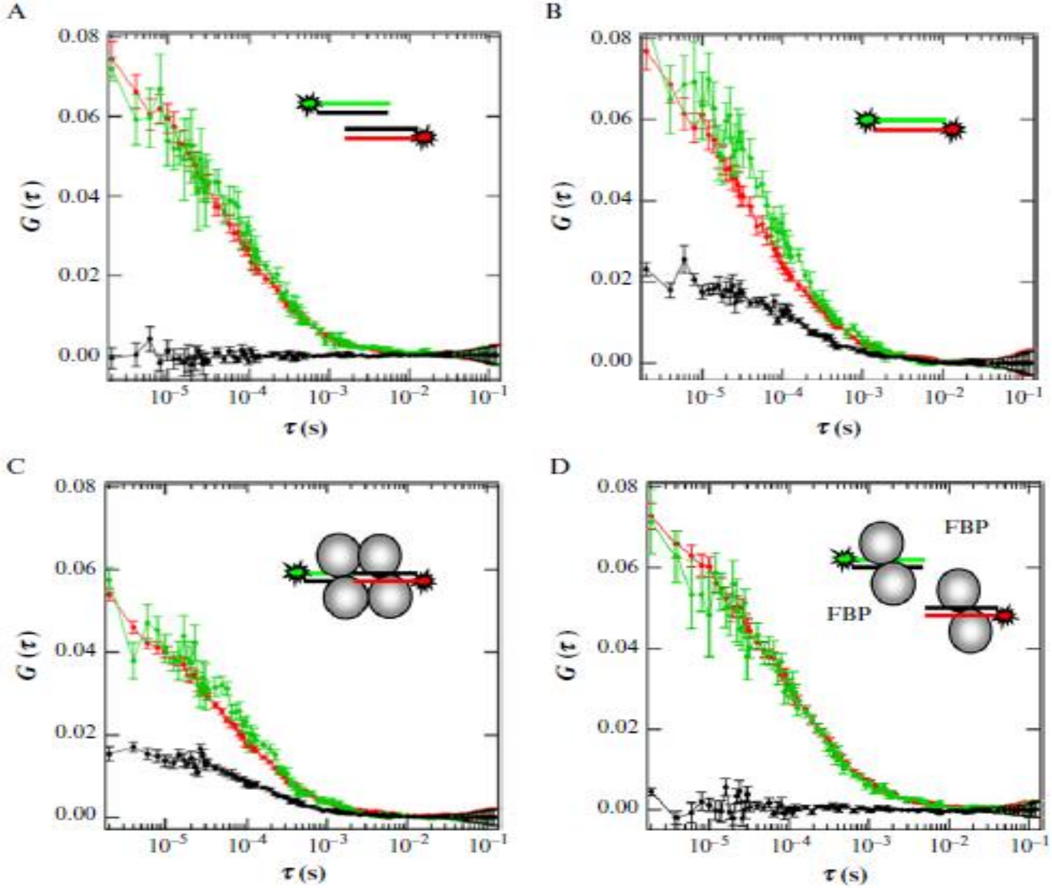
$$g_1(0) = \frac{1}{n_1 + n_{12}}, \quad g_2(0) = \frac{1}{n_2 + n_{12}}, \quad g_\times(0) = \frac{n_{12}}{(n_1 + n_{12})(n_2 + n_{12})}$$

$\frac{g_\times(0)}{g_2(0)} = \frac{n_{12}}{n_1+n_{12}} \rightarrow$ fraction of green in complex with red

$\frac{g_\times(0)}{g_1(0)} = \frac{n_{12}}{n_2+n_{12}} \rightarrow$ fraction of red in complex in complex with green

In vitro interaction between the CGGR repressor and its operator

Nathalie Declerck (CBS)



FBP induces the dissociation of the CGGR tztramer from its operator

FBP: fructose-1,6-bis-phosphate

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Experimental details

- Background
- Photobleaching
- Size and shape of the observation volume
- Cross-talk between detection channels (FCCS)
- Afterpulsing

Background

- Constant, uncorrelated (B)
  → Diminishes the value of G(0)

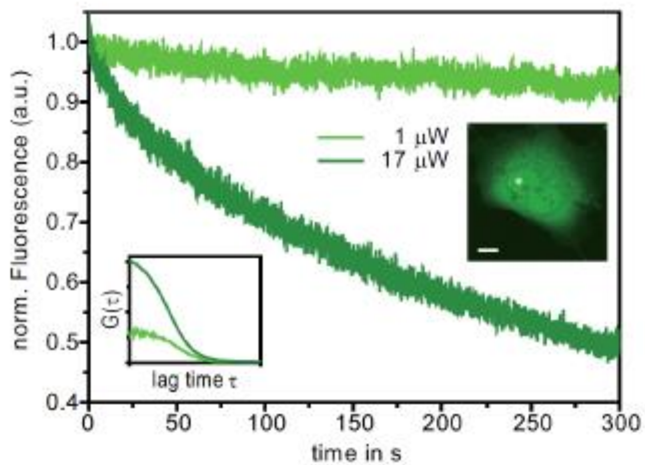
$$g'(\tau) = g(\tau)\left(\frac{\langle F\rangle}{\langle F\rangle + B}\right)^2$$

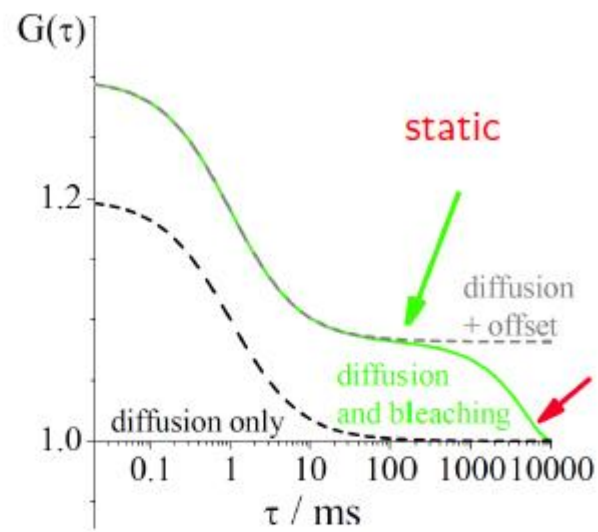
- Correlated (arising from diffusing molecules)
  → Add a new component to the analysis

$$G(\tau) = \sum_{i=1}^{M} f_i^{\,2} \cdot G(0)_i \cdot \left(1 + \frac{8D\tau}{w_{2DG}^2}\right)^{-1} \qquad \text{(2D-Gaussian, 2PE)}$$

Photobleaching

- Static (photobleaching of quasi immobiles molecules)

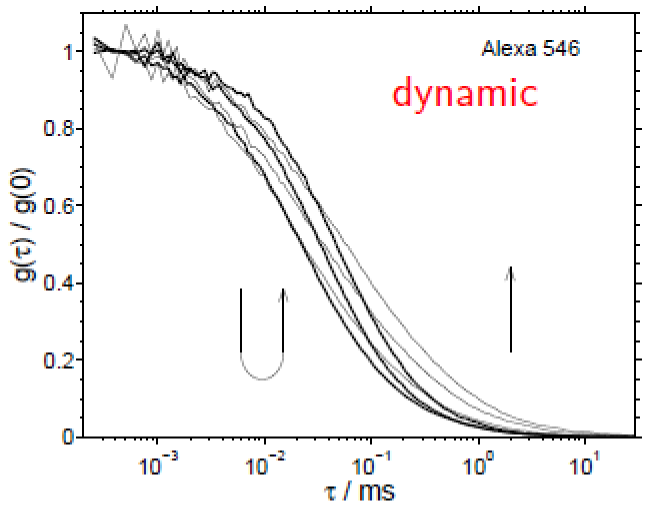




Photobleaching

- Dynamic

→ Diminishes the apparent diffusion coefficient

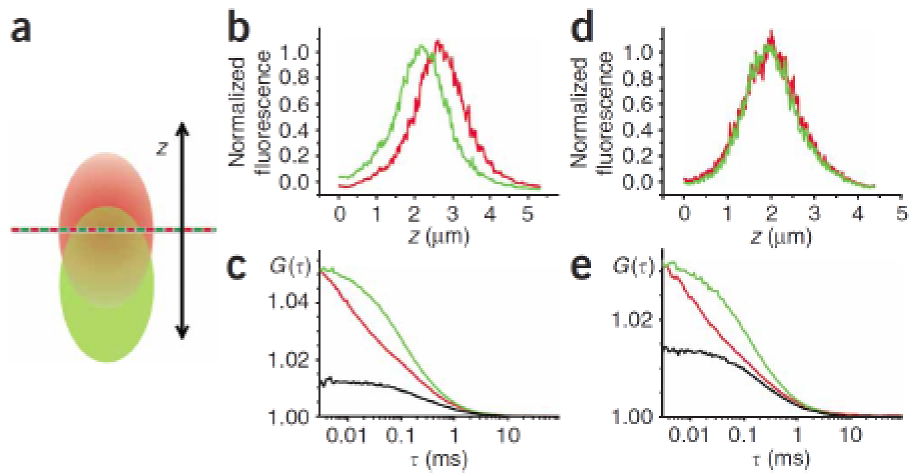


Size and shape of the observation volume

FCS: distortions of the volume size can affect:

- measured diffusion coefficient D via r_0 in $D = r_0^2/\tau_D$
- measured concentration via changed V_{eff}: $g(0) = 1/cV_{\text{eff}}$

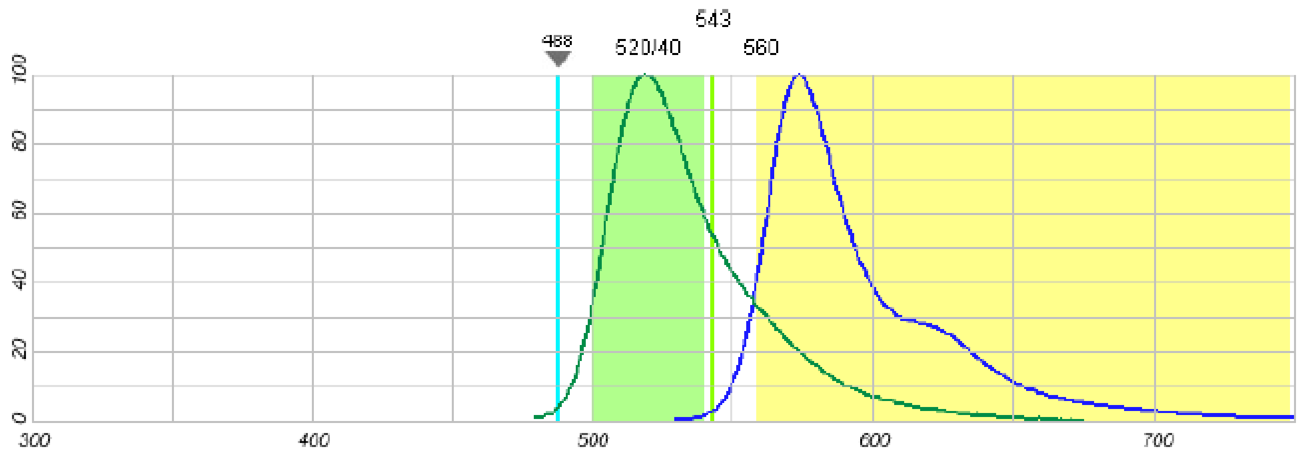
FCCS: in addition to the above, the volume overlap affects $g_\times(\tau)$



— use objective correction collar for coverslip thickness correction

NB : Using 2photon excitation, the excitation volumes are identical

Spectral cross talk (FCCS)

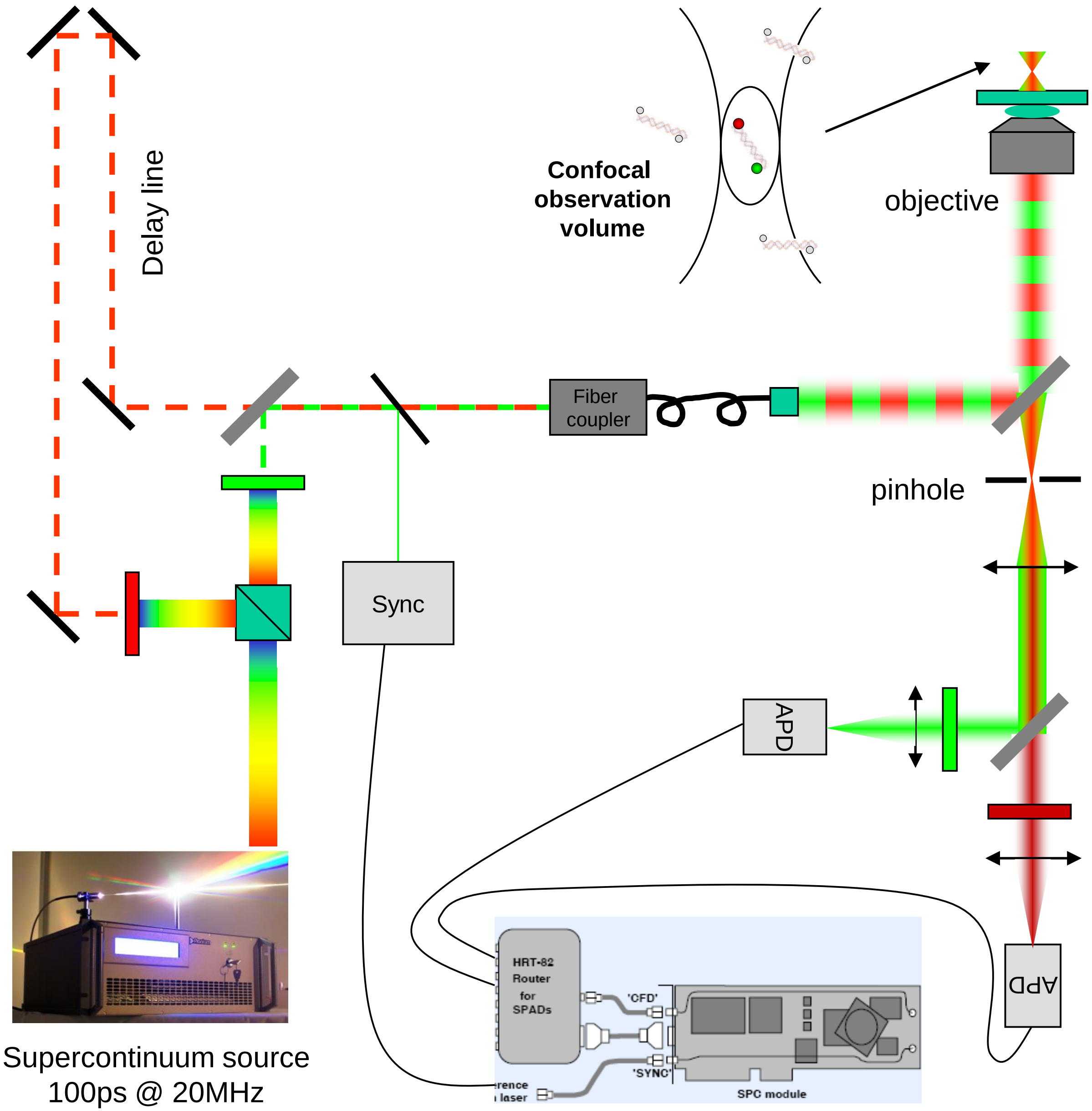


Detection of the « green » fluorophore in the « red » detection channel : → Artifactual cross correlation

→ Use spectrally distincs fluorophores
→ Use alternated laser excitation (nsALEX / PIE)

ALEX : Alternating laser excitation, PIE : Pulsed interleaved excitation

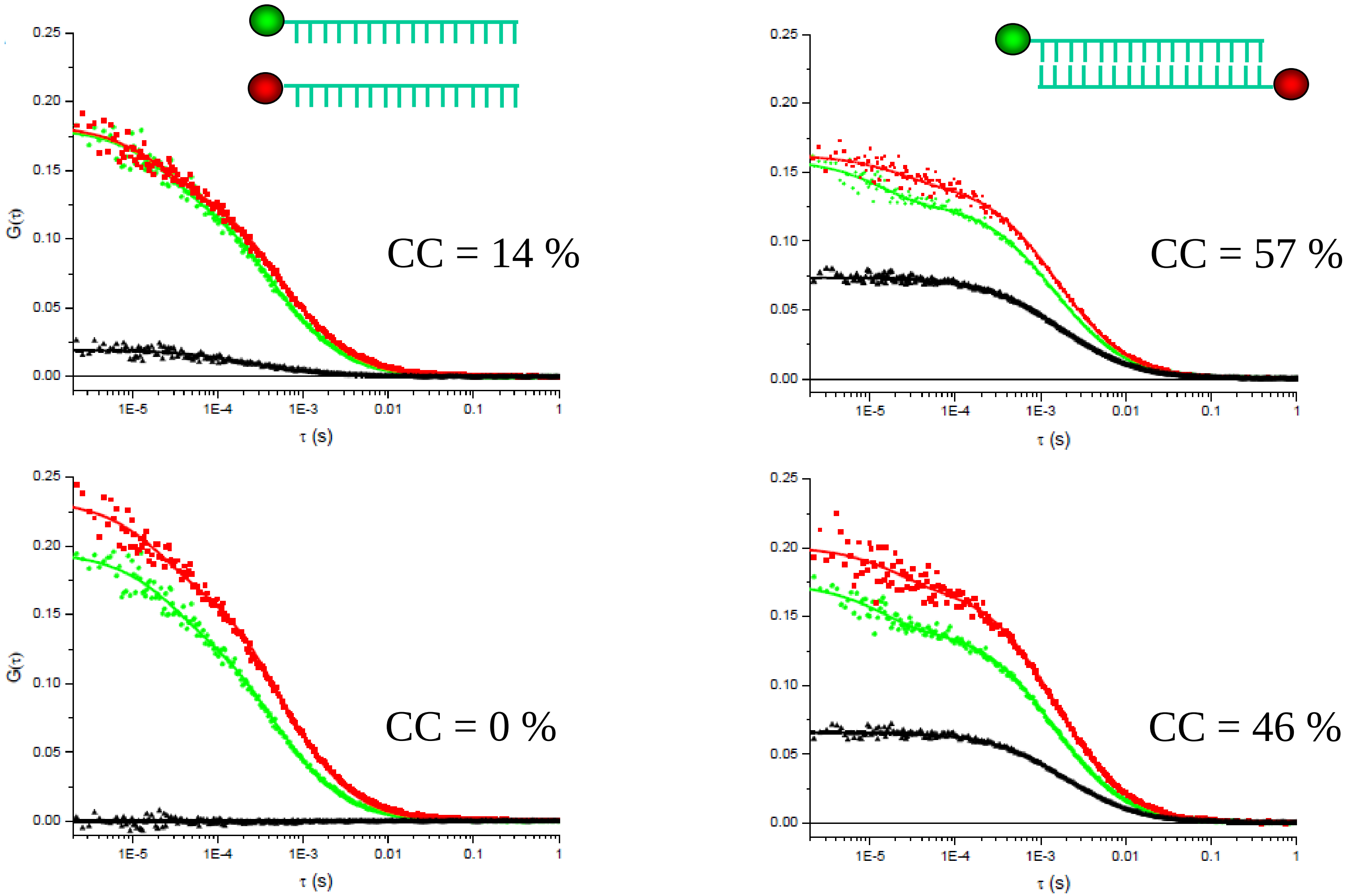
PIE / nsALEX with a supercontinuum source



Removal of crosstalk in FCCS

Regular FCCS

PIE FCCS



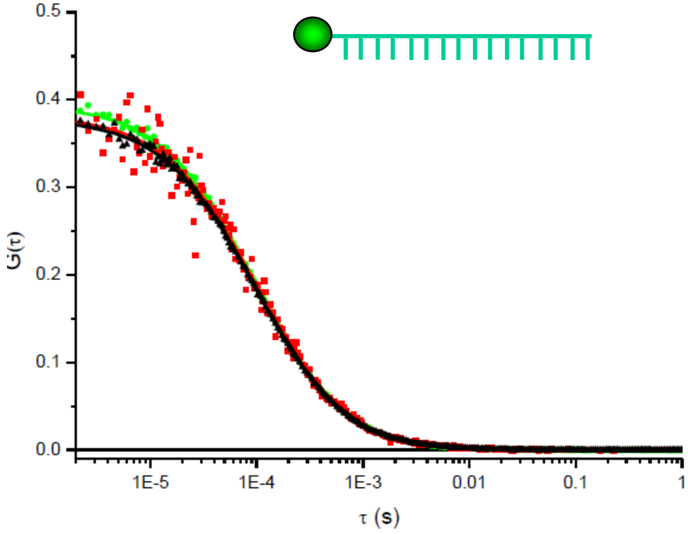
Green AC

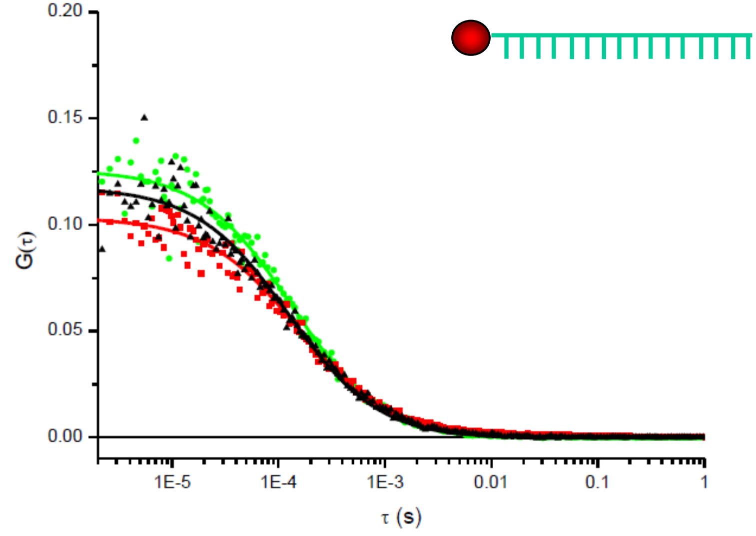
Red AC

Green Red CC

Olofsson and Margeat, Optics Express, 2013

Overlap of the excitation and detection volumes





Green excitation

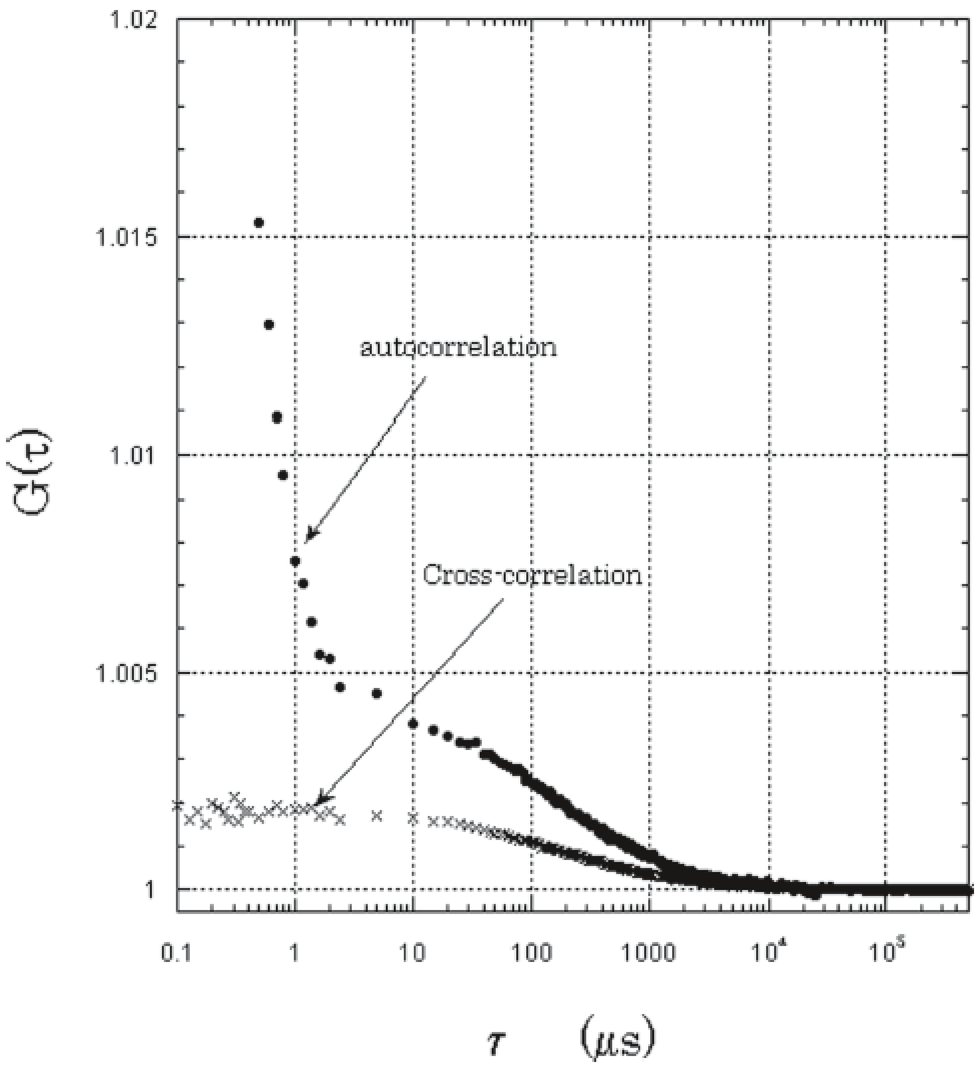
- Green detection AC
- Red detection AC
- Green Red CC

Red detection

- Green excitation AC
- Red excitation AC
- Green Red CC

Olofsson and Margeat, Optics Express, 2013

Afterpulsing



Autocorrelation :
One detector

Cross correlation :
Two spectrally equivalent detectors
50/50 beam splitter

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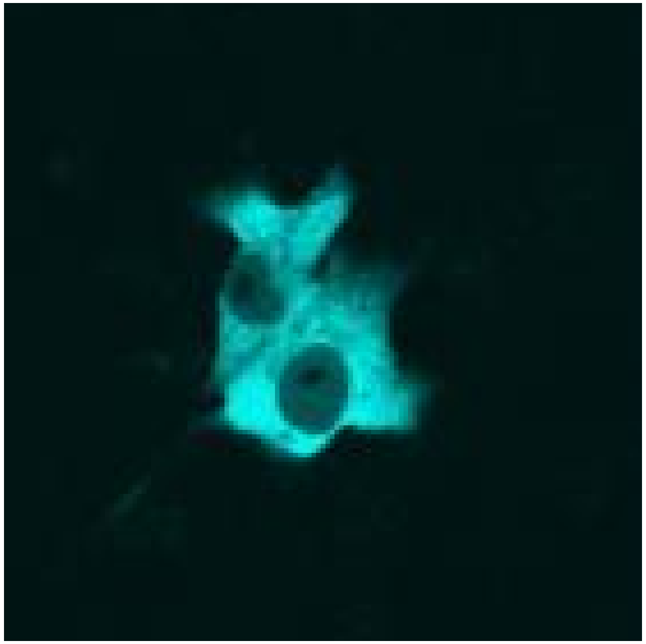
FCS and cell biology



An FCS analysis on all pixels of the image is impossible due to

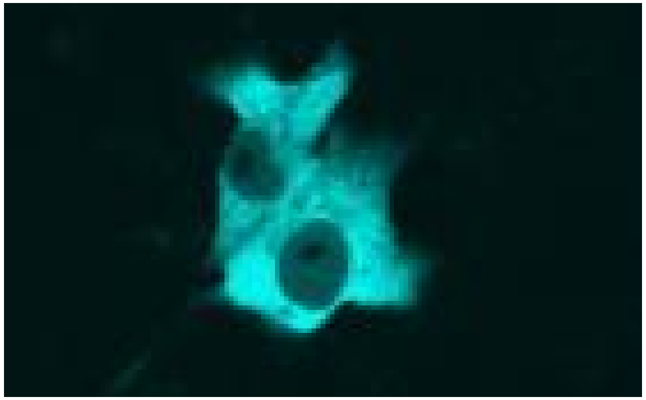
- Photobleaching

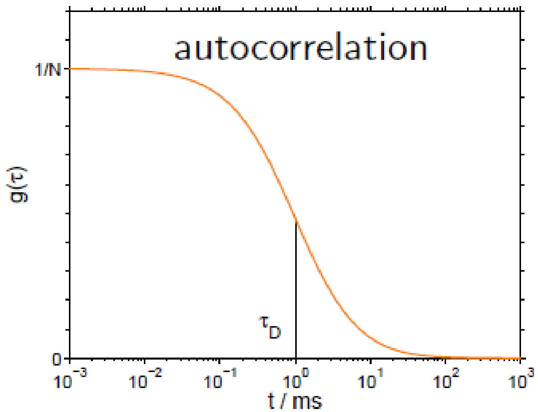
- Measurement time

Une mesure et une analyse en *Number & Brightness (N&B)* va nous permettre de mesurer rapidement les paramètres de
- Concentration (N)
- Oligomerisation / brillance (B)

En cela, l'analyse N&B s'apparente au PCH
→ ne permet pas la determination de D

The number and brigthness (N&B) analysis





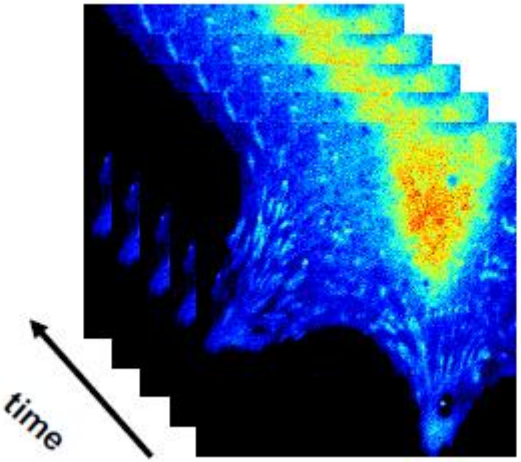
-Building a correlation curve takes time
    -For imaging : 1 curve / pixel
    -Photobleaching becomes rapidly and issue

→ If you are only interested in determining the concentration and brigthness of the molecules, this can be done very fast, in scanning / imaging mode

Number and Brightness

Principle of the N&B analysis

We acquire a stack of K images (at low pixel dwell time) and we define for each pixel :



$$F = \frac{\sum_K F(x,y)}{K} \qquad \sigma_F^2 = \frac{\sum_K \left(F(x,y) - F\right)^2}{K}$$

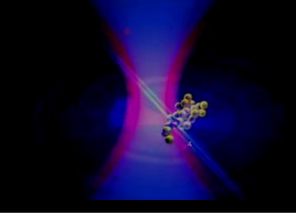
And we can calculate the apparent number and apparent brightness

$$\frac{\sigma_F^2}{\langle F \rangle^2} = \frac{1}{\langle N \rangle} \Rightarrow \boxed{\langle N \rangle = \frac{\langle F \rangle^2}{\sigma_F^2}}$$

$$\langle F \rangle = B \langle N \rangle \Rightarrow \boxed{B = \frac{\sigma_F^2}{\langle F \rangle}}$$

Qian et Elson, Biophysical J., 1990
Digman, … , Gratton, Biophys J, 2006

Principle of the N&B analysis



However, to recover the true number and true brightness we need to take into consideration the various parameters contributing to the variance

n = true number
ε = true brightness

Total variance of the signal $\sigma_F^2 = \sigma_n^2 + \sigma_d^2 = \varepsilon^2 n + \varepsilon n$

$<F> = \varepsilon n$

σ_n^2 The variance in the signal due to variation in n scales with the square of the brigthness

σ_d^2 The variance in the signal due to the detection process scale with the brightness for a photon counting detector

$$B = \frac{\sigma_F^2}{\langle F \rangle} = \frac{\varepsilon^2 n + \varepsilon n}{\varepsilon n} = 1 + \varepsilon$$

$$\langle N \rangle = \frac{\langle F \rangle^2}{\sigma_F^2} = \frac{\varepsilon^2 n^2}{\varepsilon^2 n + \varepsilon n} = \frac{\varepsilon n}{\varepsilon + 1}$$

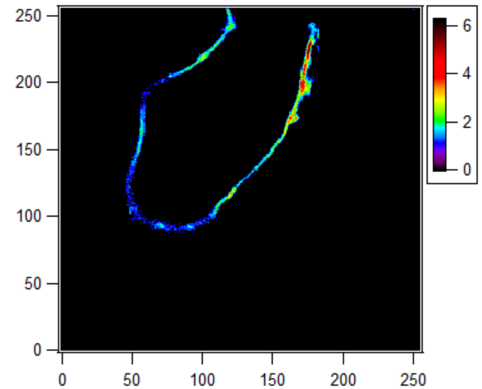
$$\varepsilon = \frac{\sigma_F^2 - \langle F \rangle}{\langle F \rangle}$$

$$n = \frac{\langle F \rangle^2}{\sigma_F^2 - \langle F \rangle}$$

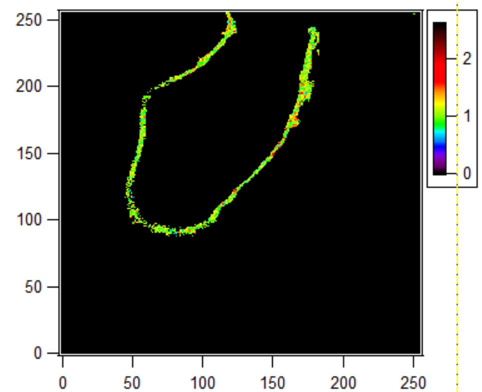
Digman, … , Gratton, Biophys J, 2006

N&B : Data representations

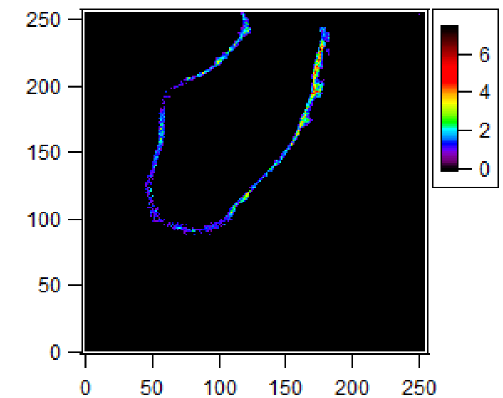
Intensity map

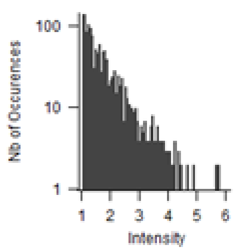


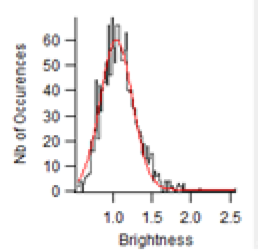
Brightness map

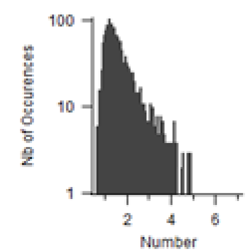


Number map

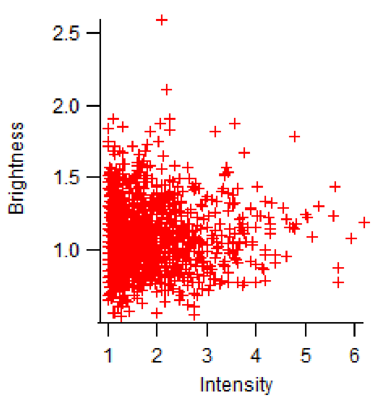


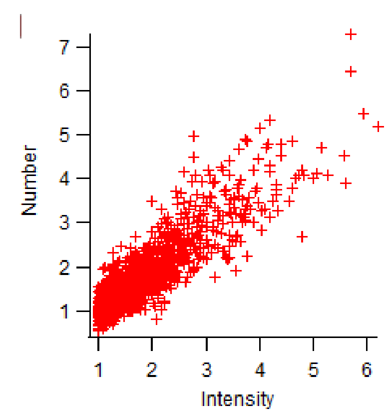




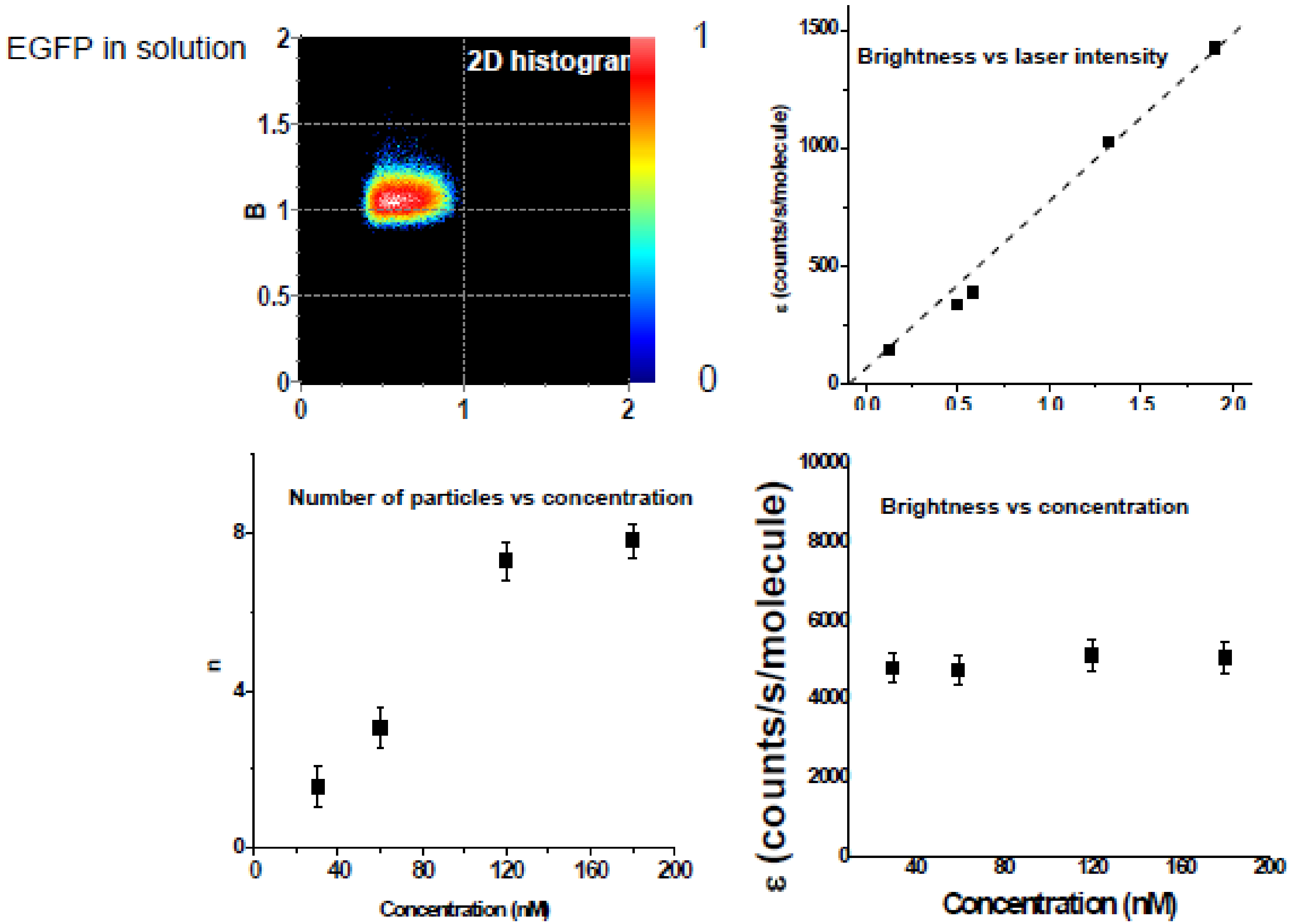


- Home-made software @CBS
- SimFCS by LFD
- Other companies (ISS, …)



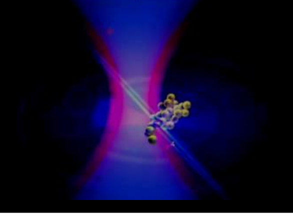


N&B : Demonstration

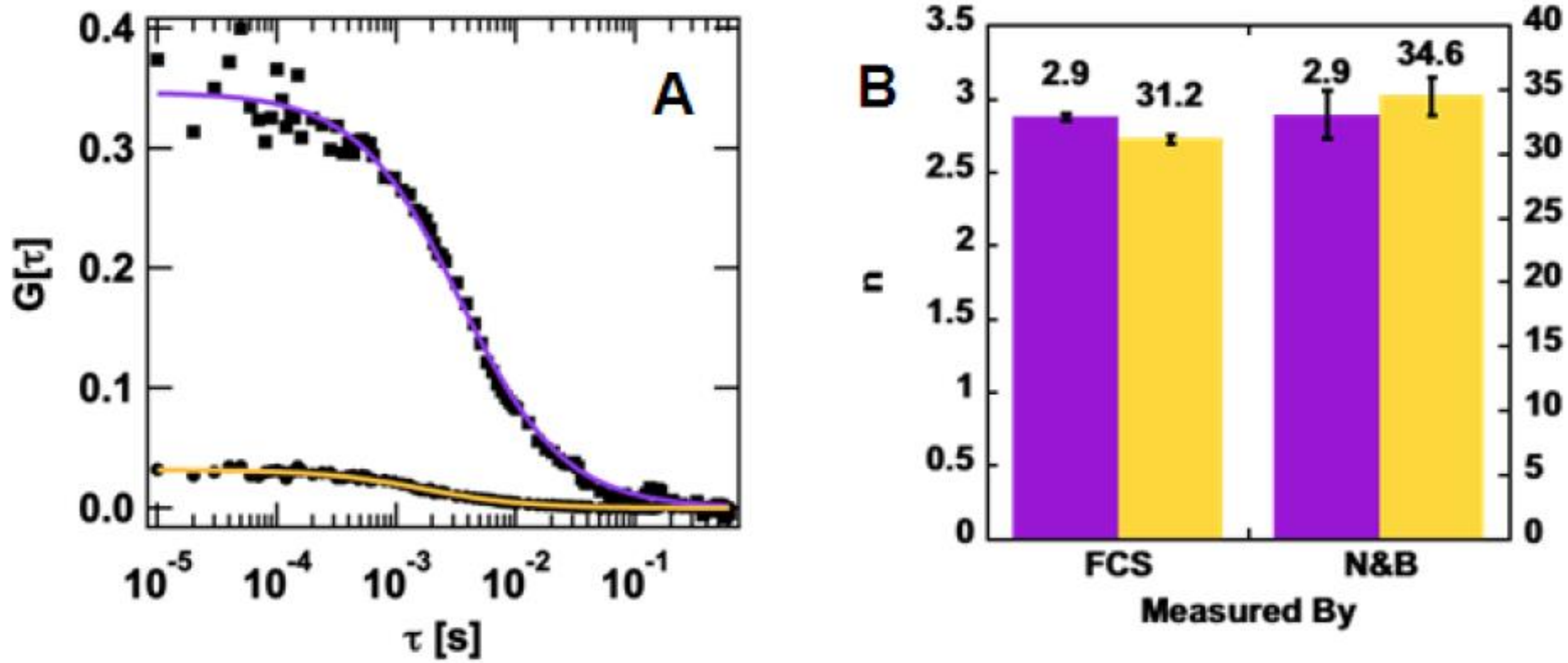


Digman, … , Gratton, Biophys J, 2006

N&B : Demonstration

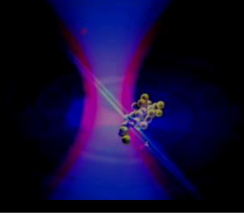


Rhodamine 110 in 75% glycerol at 16nM & 160nM



Ferguson et al., Anal. Biochem. 2011

N&B : the case of immobile particles



For immobile particles, there is no contribution of the number of molecules to the variance

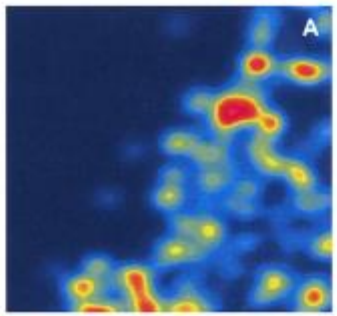
$$B = \frac{\sigma_F^2}{\langle F \rangle} = \frac{\sigma_n^2 + \sigma_d^2}{\langle F \rangle} = \frac{\varepsilon n}{\varepsilon n} = 1$$

In the presence of a mixture of immobile and mobile particles in a pixel, *B* will have an intermediate value between 1 and the value for mobile particles.

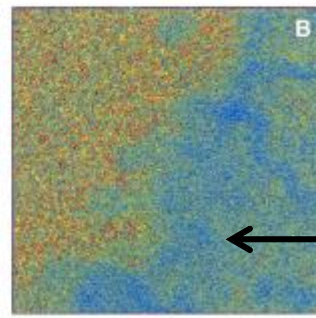
Digman, … , Gratton, Biophys J, 2006

N&B : the case of immobile particles

Sample : fluorescent beads in a 100nM solution of fluorescein

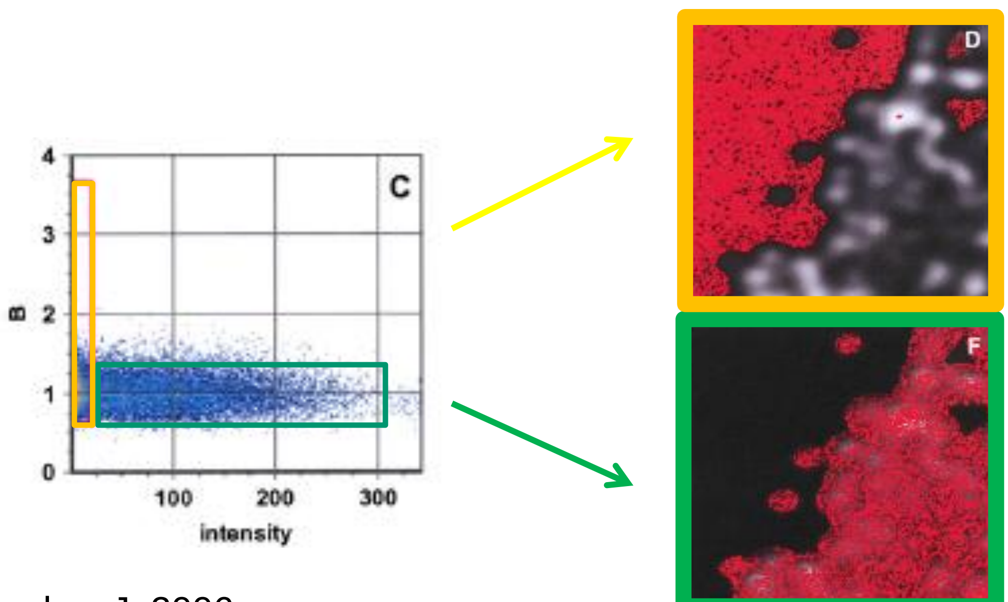


Intensity image



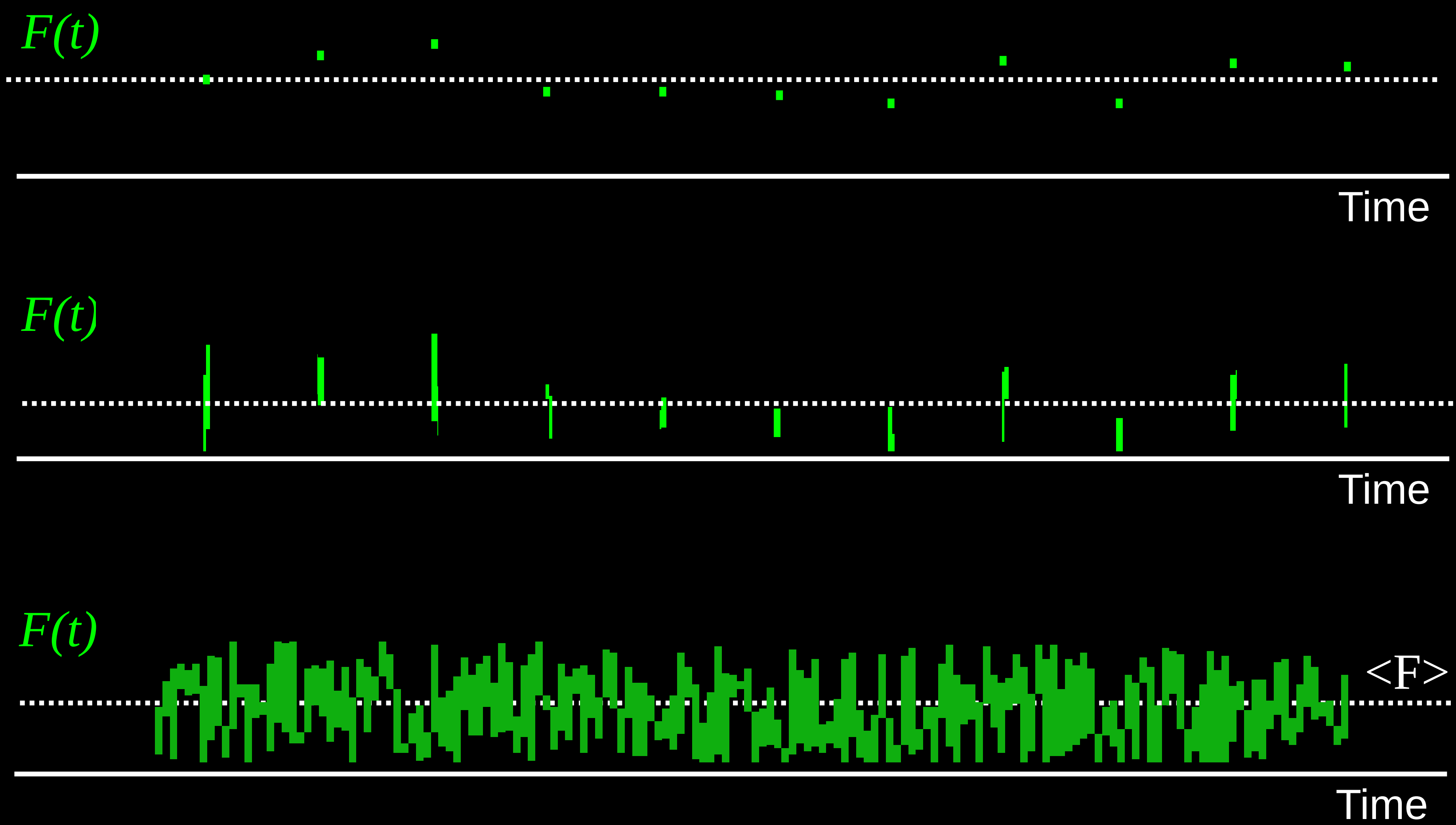
Beads with B=1

Brightness image

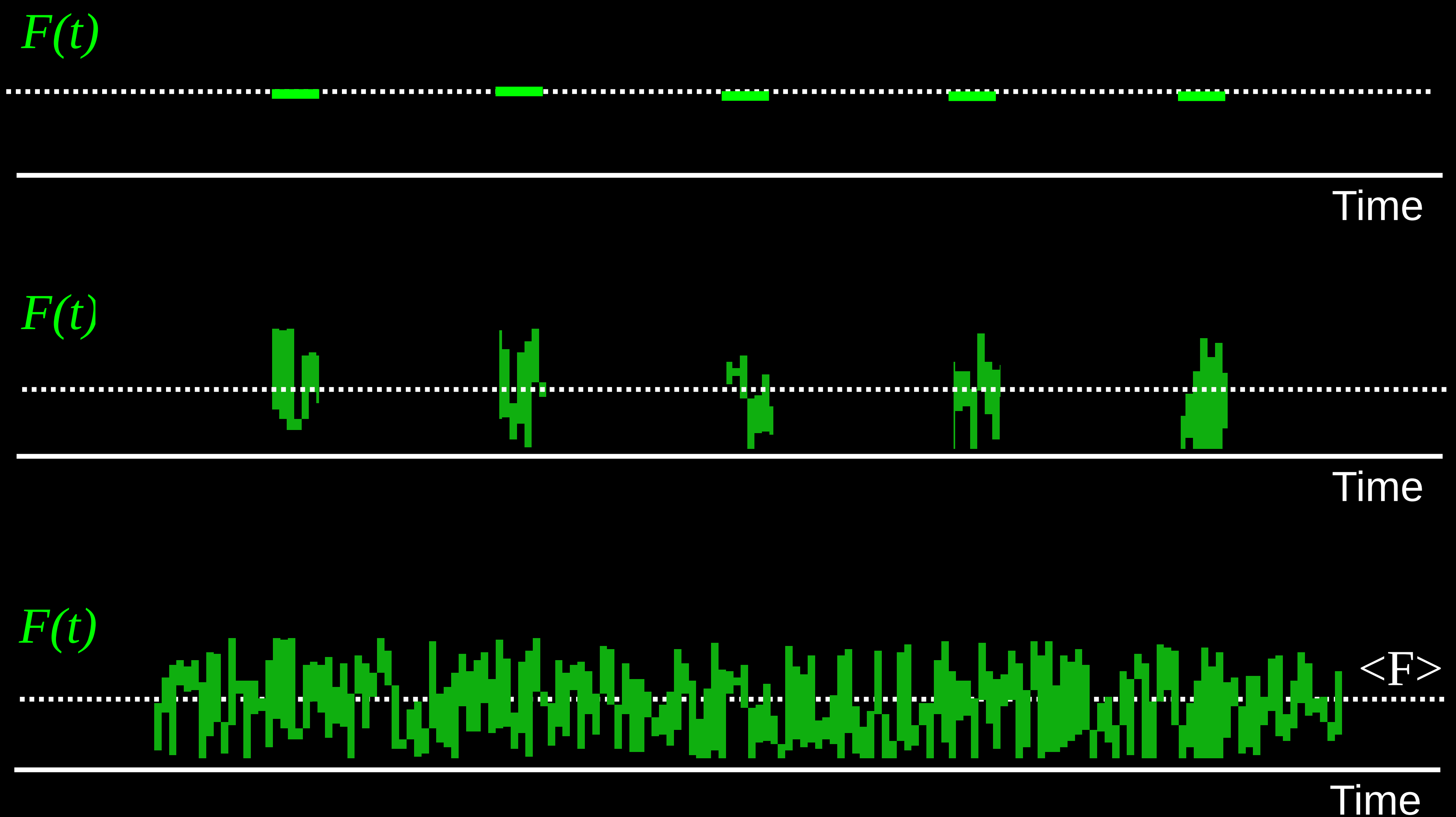


Digman, … , Gratton, Biophys J, 2006

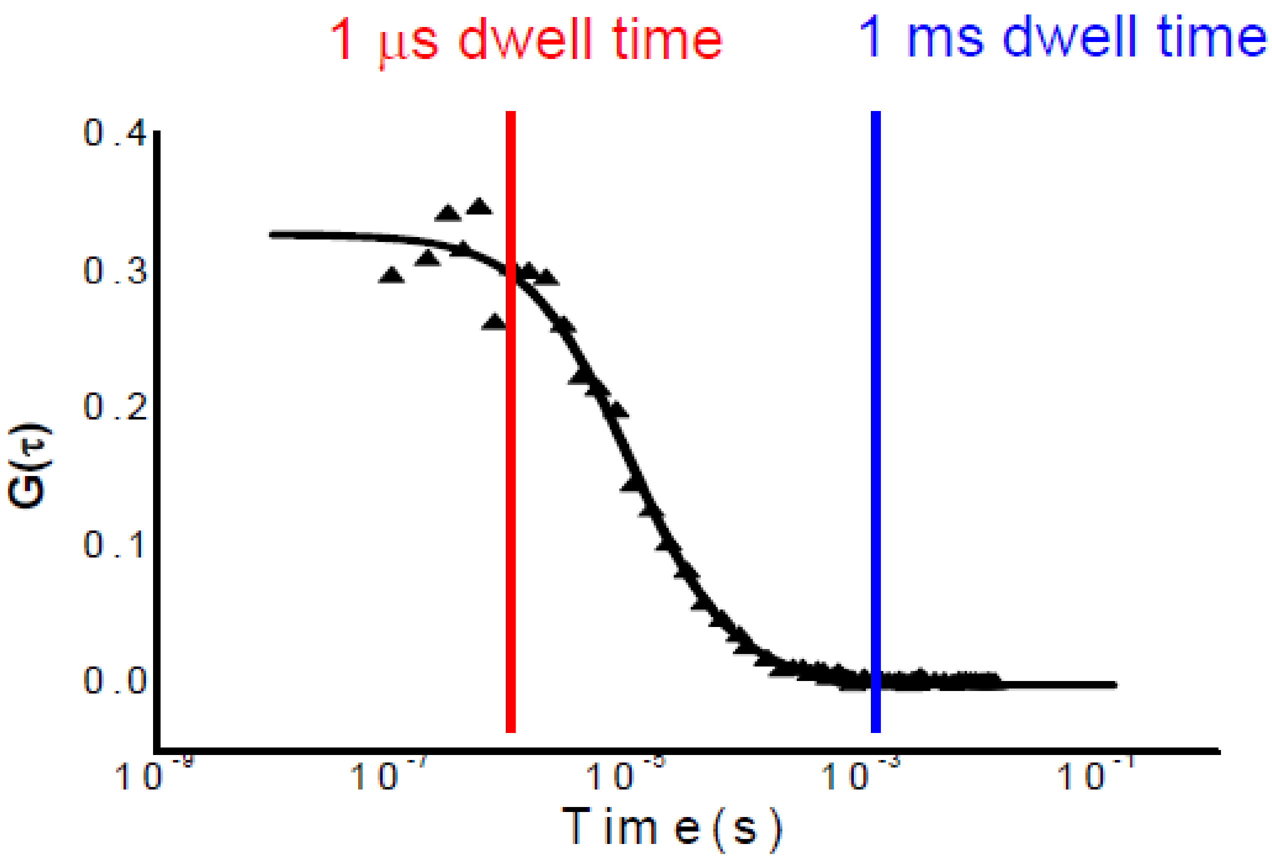
Short dwell time



Long dwell time



N&B : Effect of the dwell time



N&B summary

- N&B quantifies the number of molecules and their brightness for each pixel

  → A map of N&B can be obtained

- Acquisition can be done with a commercial LSM system (APD recommended)

- The immobile fraction can be detected and separated (B=1)

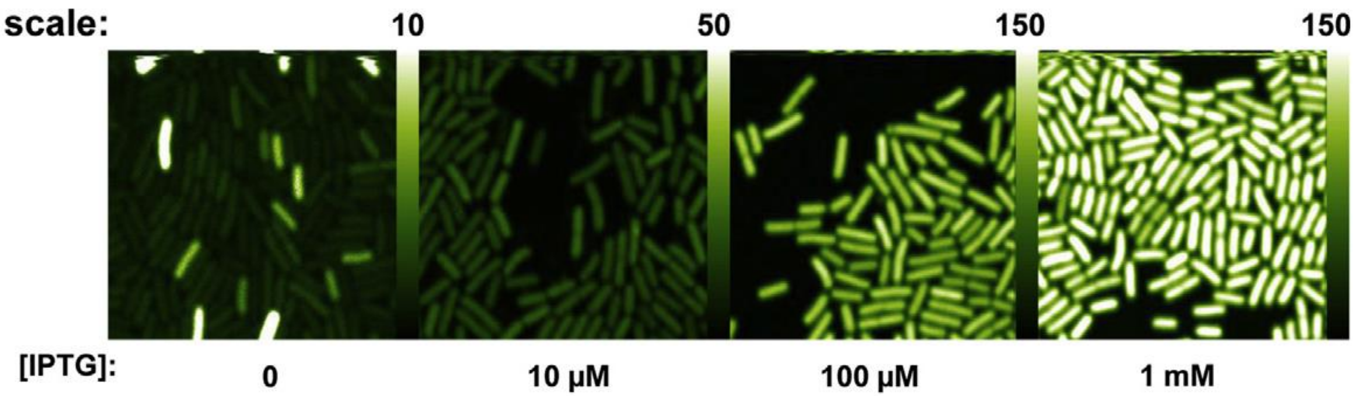
- Photobleaching of the sample is strongly reduced due to fast scanning

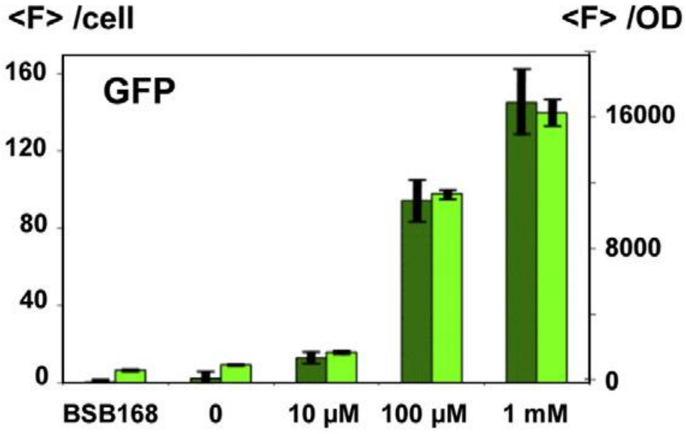
!

- You need fluctuations !!
  - Low concentration of fluorescent species (up to μM)
  - Low background
  - Low photobleaching

Quantification of noise in gene expression in bacteria

Expression of Gfpmut2 under control of an inducible promoter in *B. Subtillis*





Correlation between the expression determined by 2P-microscopy and ensemble fluorescence

Ferguson et al., Anal. Biochem. 2011