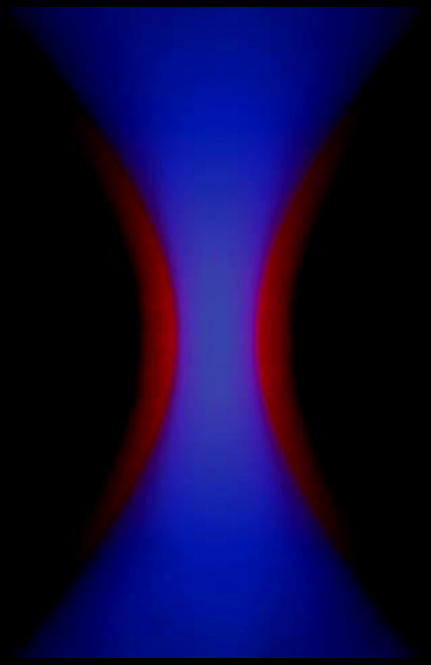

FLUCTUATION MICROSCOPIES

Emmanuel MARGEAT
CNRS
Centre de Biochimie Structurale – Montpellier

FLUCTUATION MICROSCOPIES

- INTRODUCTION
- PRINCIPE OF FCS
- AUTOCORRELATION
- PHOTON COUNTING HISTOGRAM
- CROSS CORRELATION
- EXPERIMENTAL DETAILS
- NUMBER & BRIGHTNESS
- PRACTICAL PART

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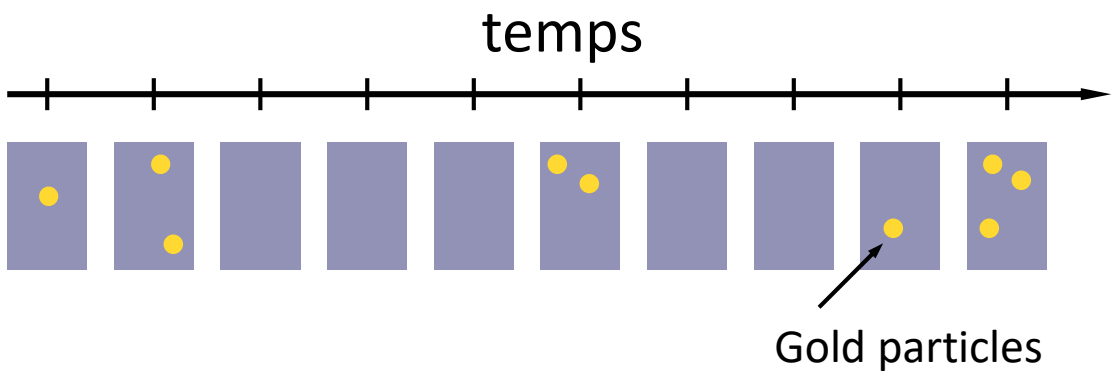
History : 1st measurement of correlation spectroscopy

(Svedberg & Inouye, 1911) *Occupancy Fluctuation*

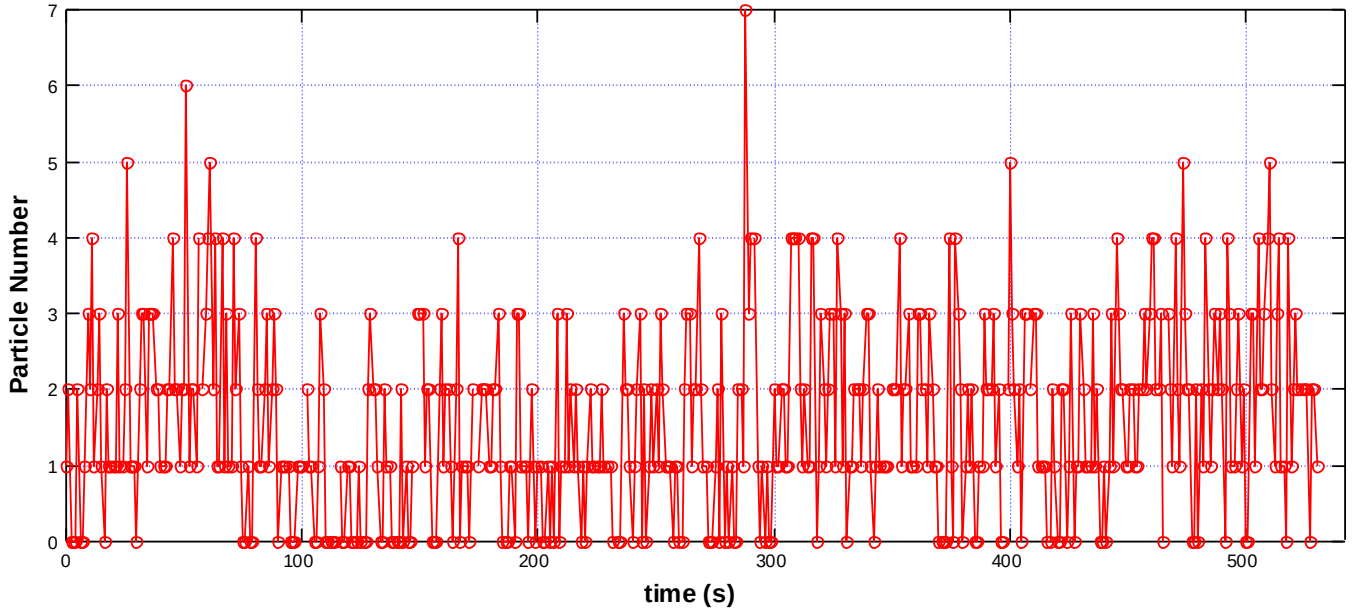


12000200132412310211113112511102331333221112242212261221423452411413114231001004211231232011110001111_211001320000010011000100023221002110000201001_333122000231221024011102_12221122310001103311102101100101030113121210101211112111_10003221012302012121321110110023312242110001203010100221734410101002112211444421211440132123314313011222123310121111222412231113322132110000041043201212001132223120 0_25321203323311110021002201301132113120010131432211221122323442223032142153220020214212323204311231200331422345213411041232222022 1

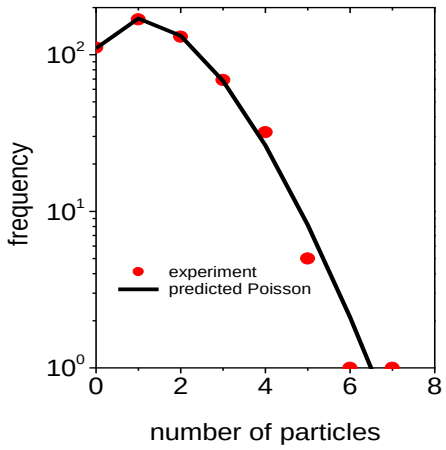
Svedberg and Inouye, *Zeitschr. F. physik. Chemie* 1911, **77**:145

Visual counting of visible gold particles, as seen trough an “ultramicroscope”

Need for statistical analysis



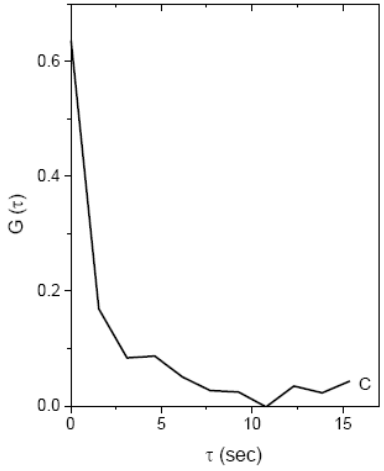
Histogram of particles numbers



- **Poisson law**

$$\langle N \rangle = 1.55$$

***Autocorrelation**

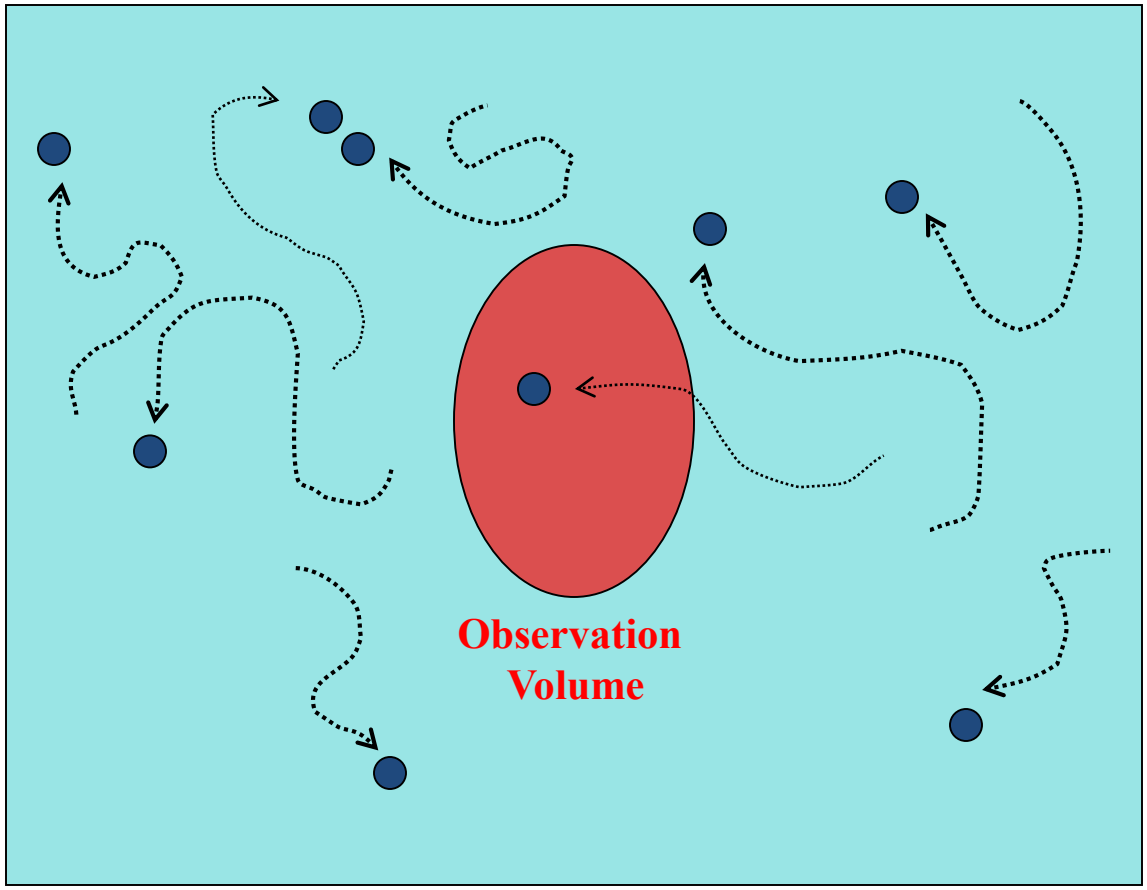


- **Today : easy to calculate**

$$\langle N \rangle = \frac{1}{G(0)} = 1.56$$

Poisson law : decribes independant events, occuring in a fixed time lag, with a fixed average frequency

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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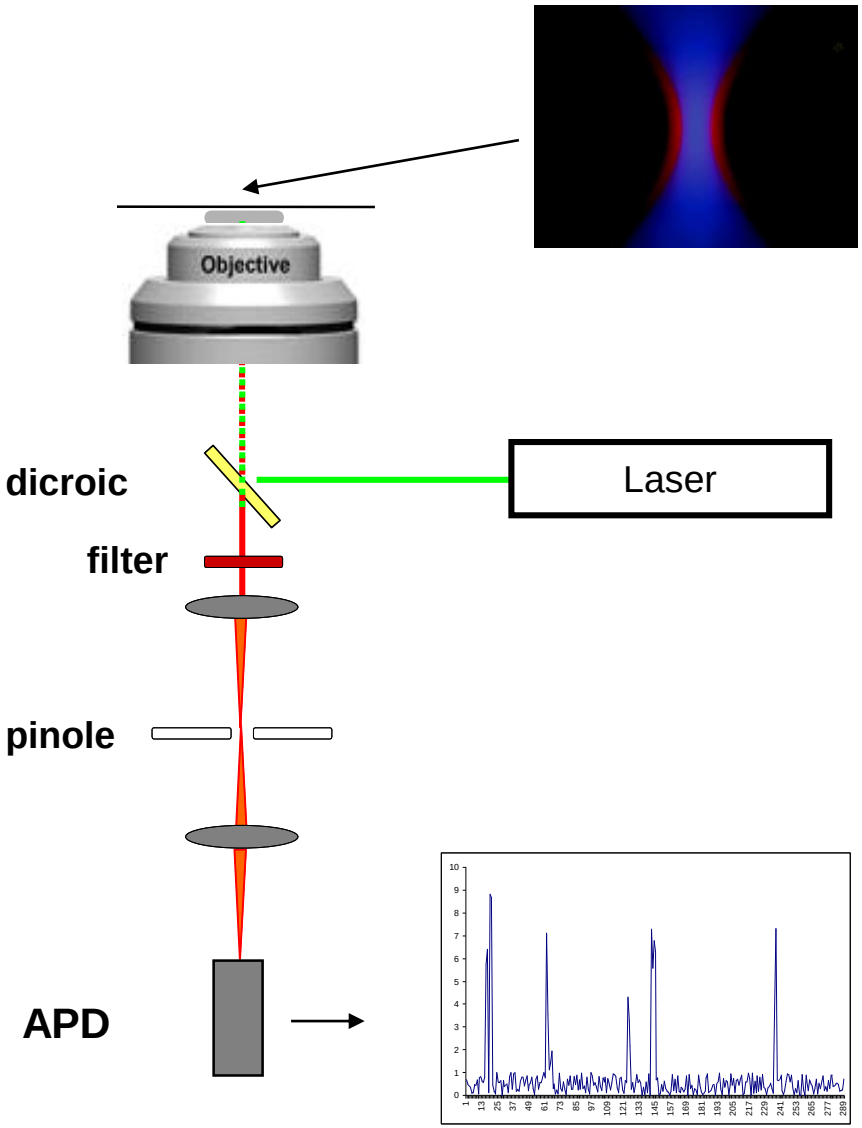
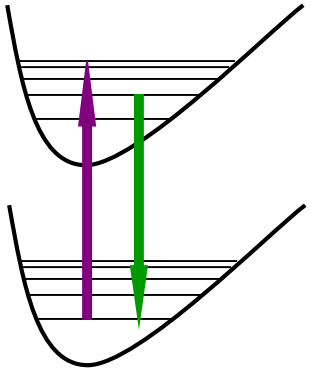
Observing fluctuations : fluorescence microscopy

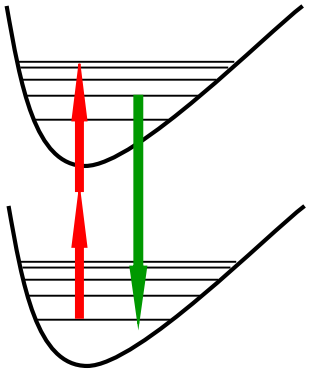


The observation volume
One- & Two-Photon Excitation.

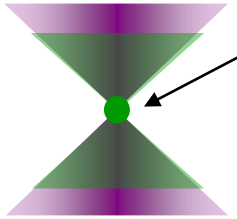
1 - Photon

2 - Photon

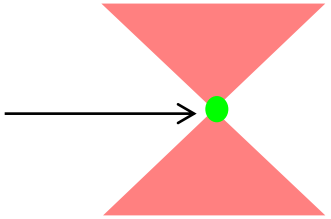




Defined by the size of the pinole, the wavelength, the magnification, and the numerical aperture



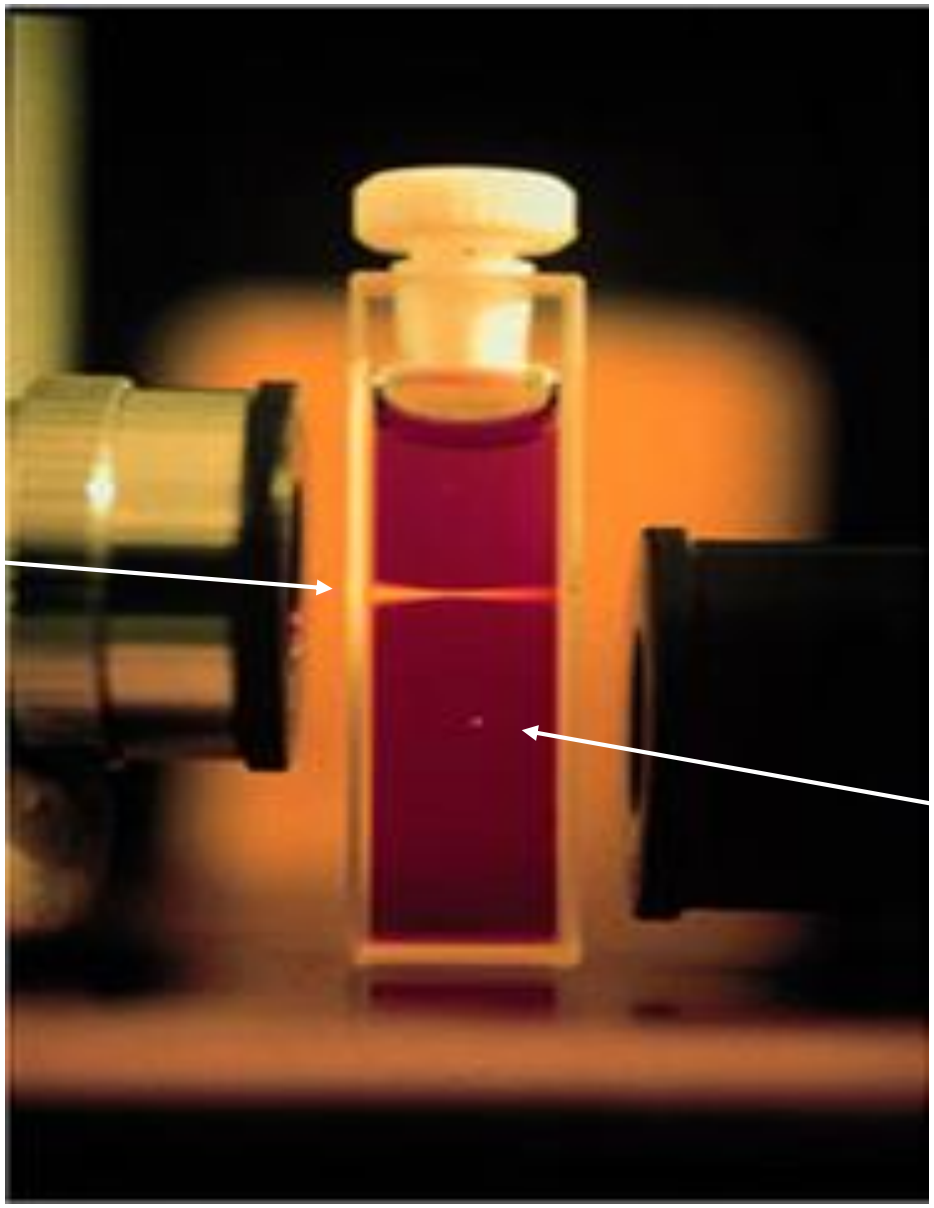
~1 um^3



Defined by the wavelength and the numerical aperture

1-photon

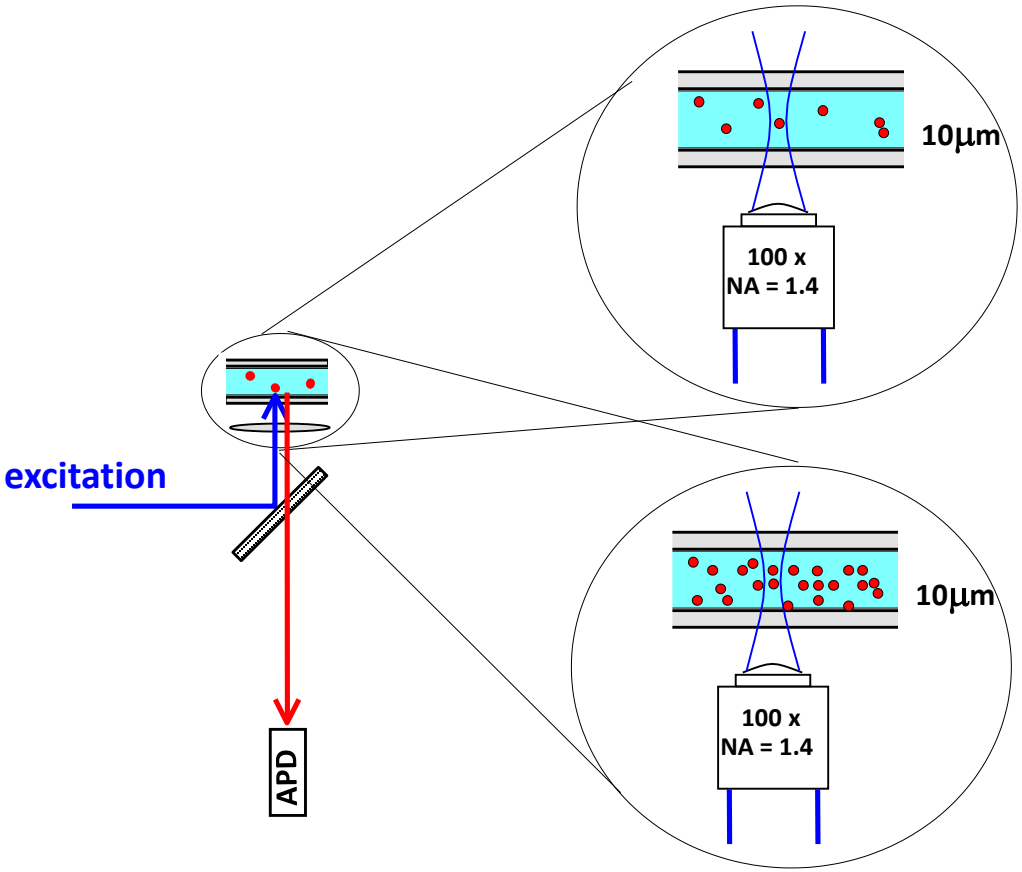
Need a pinhole to define a small volume

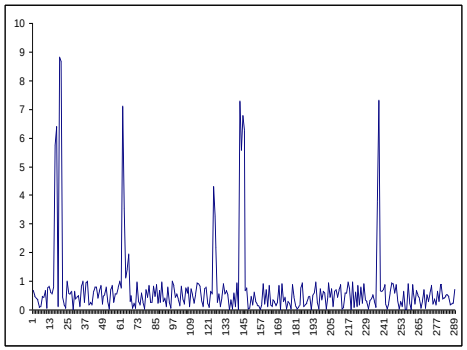


2-photon

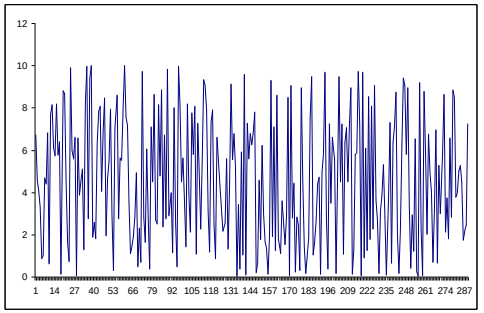
Brad Amos
MRC, Cambridge, UK

Fluorescence correlation spectroscopy





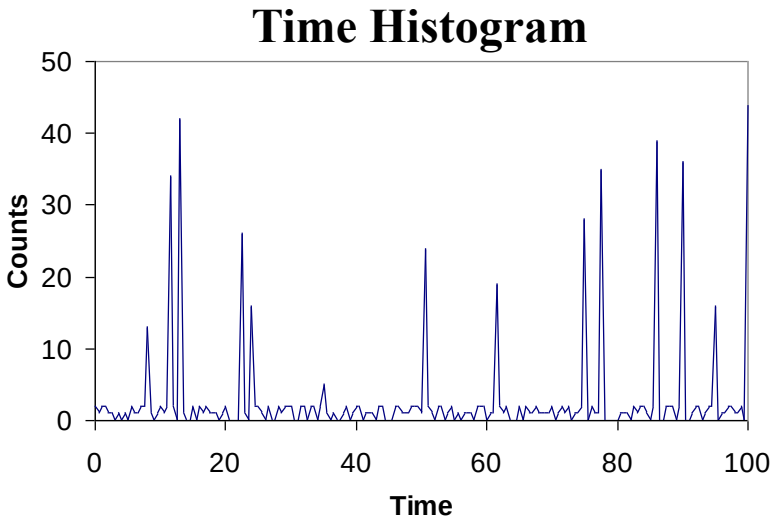
Single molecules regime

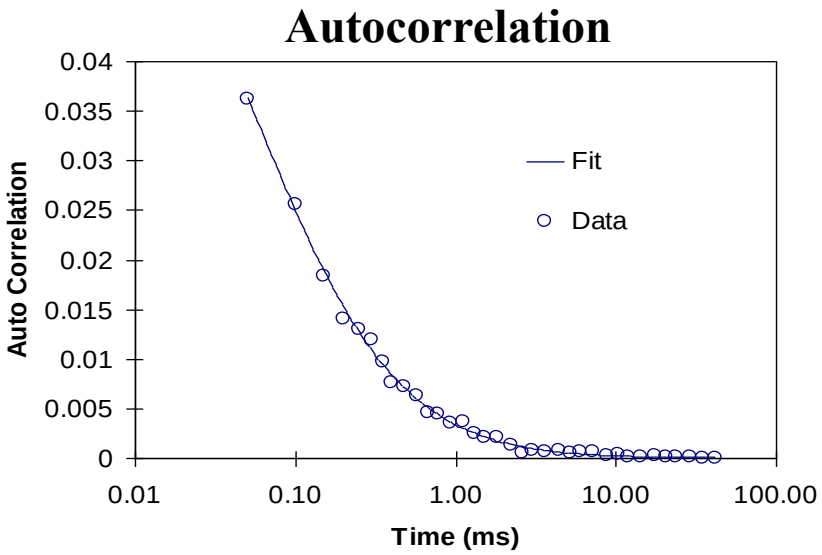


« Small ensemble » regime

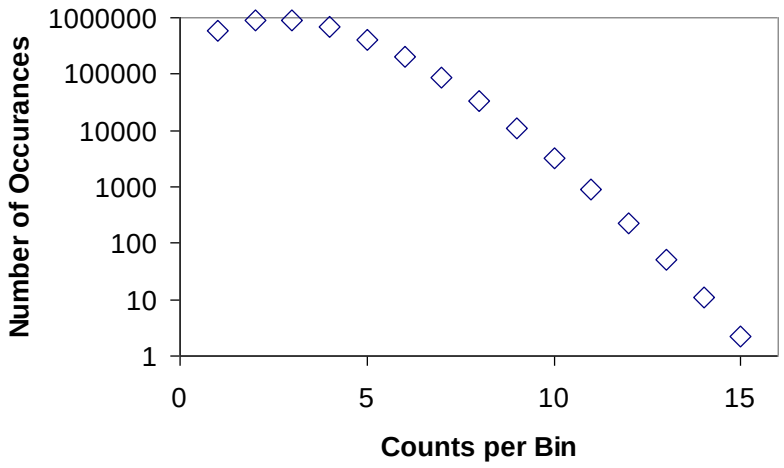
Using FCS, we will analyze these intensity fluctuations

Data Treatment & Analysis





Autocorrelation Parameters: G(0) & k_{action}



PCH Parameters: <N> & ε

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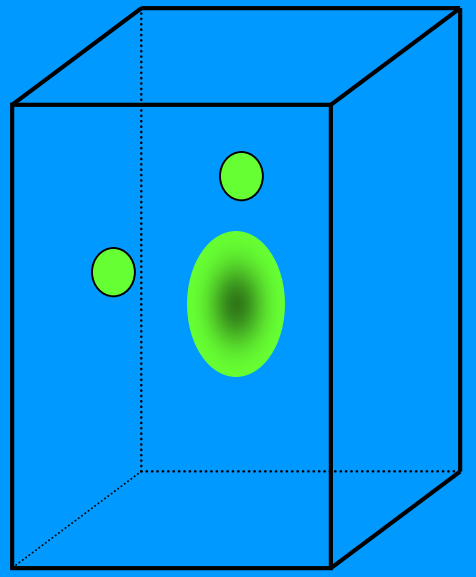
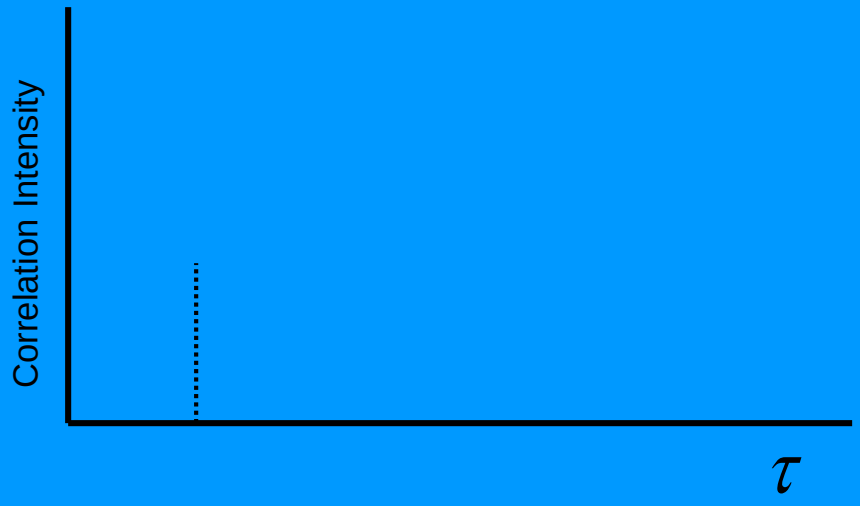
Introduction to correlation function.

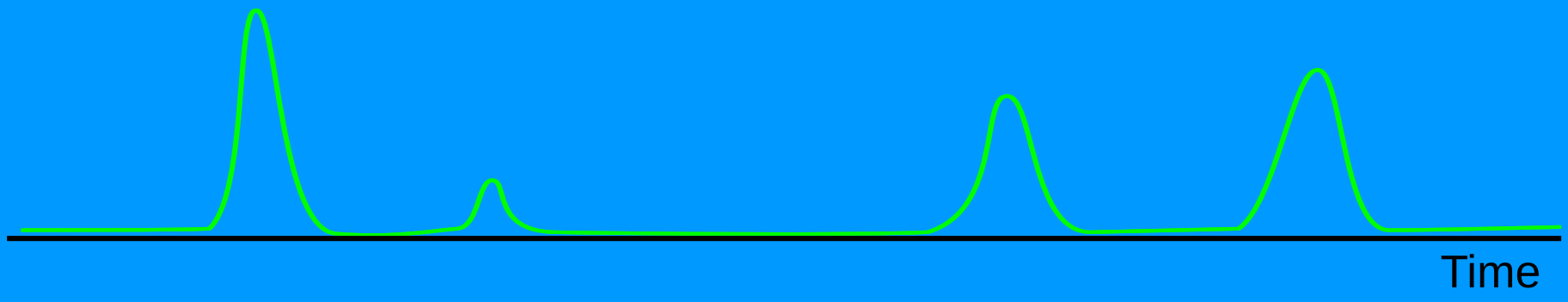


Time

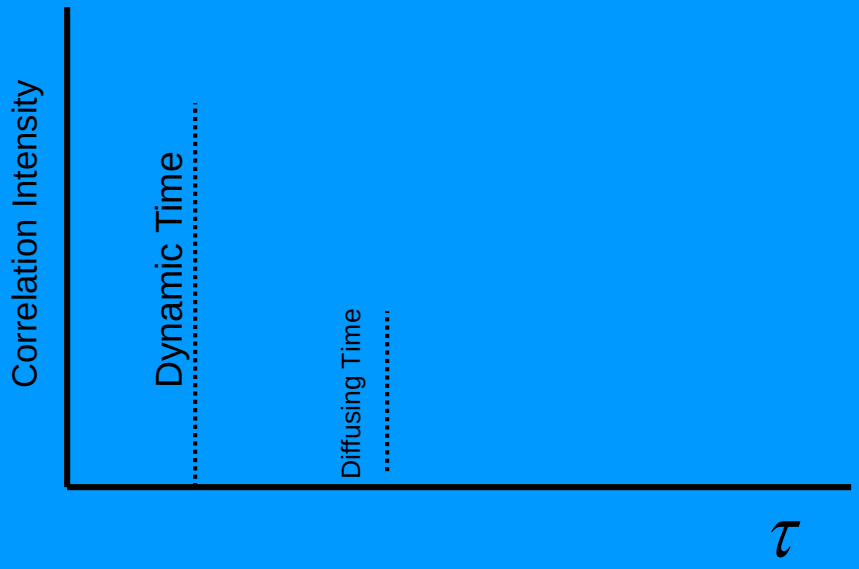
AutoCorrelation. Diffusion only, No Dynamics. “Donor-Donor”

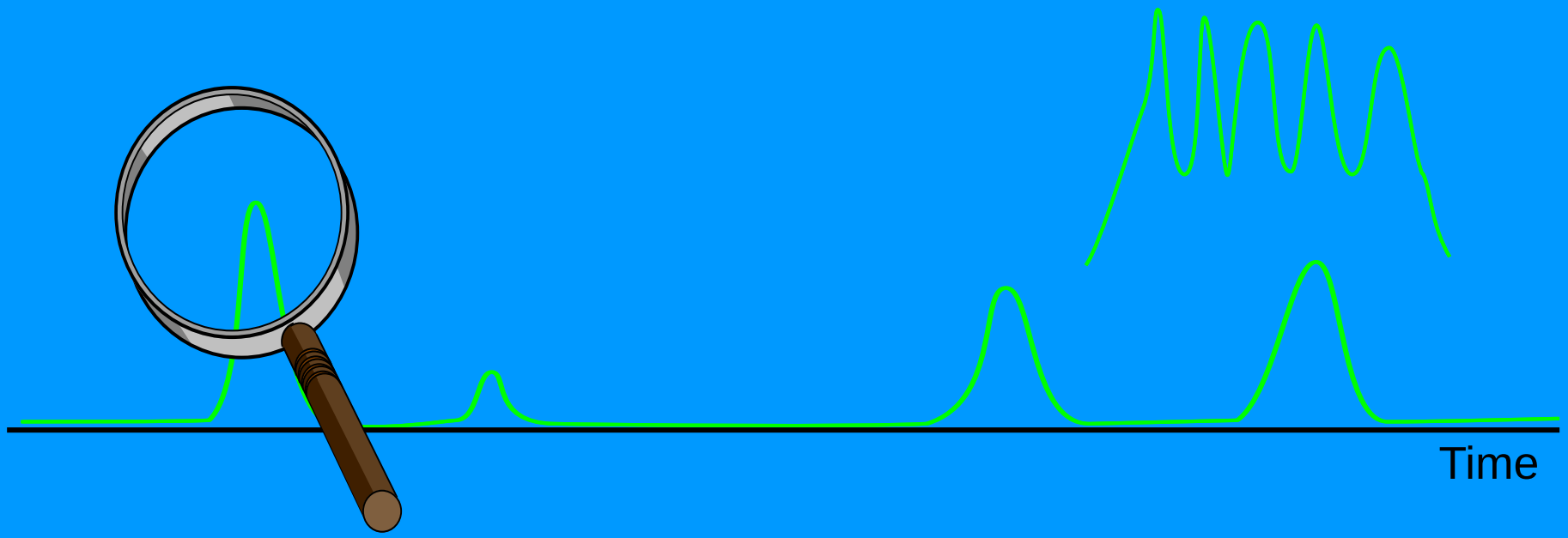


$$G(\tau) = \frac{\langle F(t+\tau) \cdot F(t) \rangle}{\langle F \rangle^2}$$



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 $\tau \rightarrow\rightarrow \infty$

Factors influencing the fluorescence signal:

$$F(t) = \kappa Q \int d\mathbf{r}\, W(\mathbf{r}) C(\mathbf{r}, t)$$

κQ = quantum yield and detector sensitivity (how bright is our probe). This term could contain the fluctuation of the fluorescence intensity due to internal processes

W(r) describes our observation volume

C(r,t) is a function of the fluorophore concentration over time. This is the term that contains the "physics" of the diffusion processes

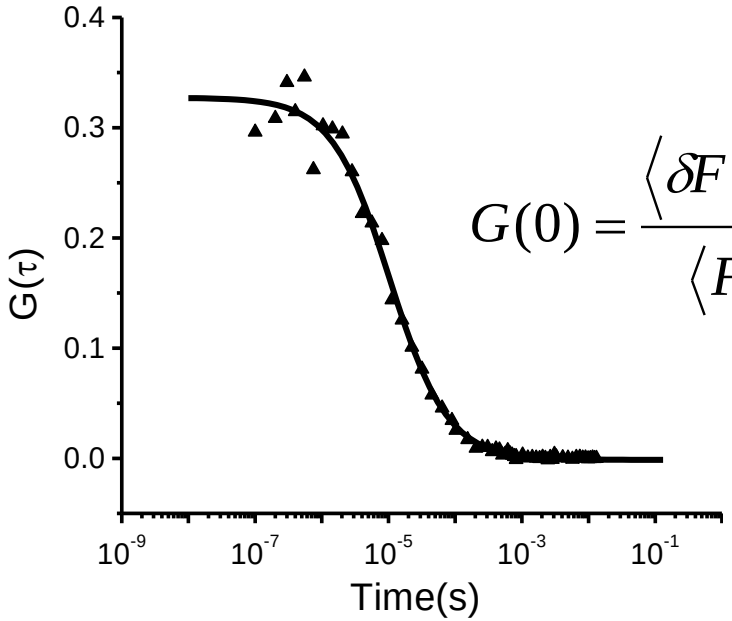
G(0) = 1/Nb of observed particles

In a poissonian systme 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

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



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

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

About the observation volume

$$G(0) = \frac{1}{\langle N \rangle} = \frac{1}{\langle C \rangle V_{eff}} = \frac{\gamma}{\langle C \rangle V_{PSF}}$$

V_{eff} = effective volume
V_{PSF} = volume of the psf

γ = shape factor, that takes into account the non-uniformity of the observation volumes. Practically, $\gamma < 1$

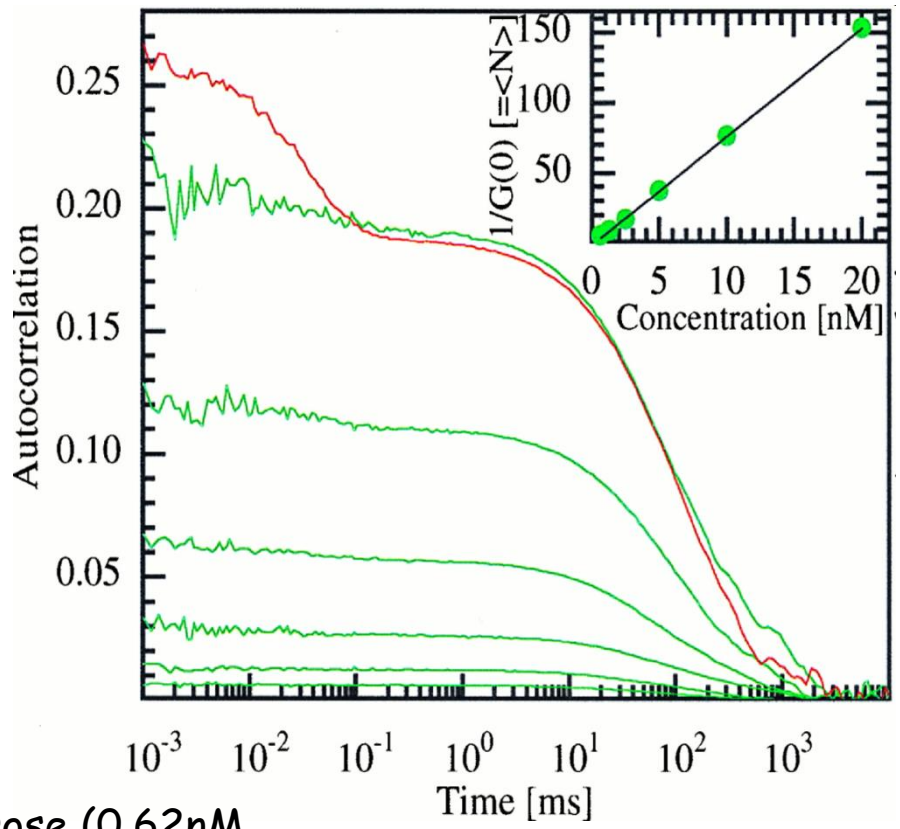
$\gamma = 1/2\sqrt{2}$ 3D Gaussian volume

$\gamma = 3/4\pi^2$ 2D Gaussian – Lorentzien volume

Effect of the concentration

For a single species, no photobleaching

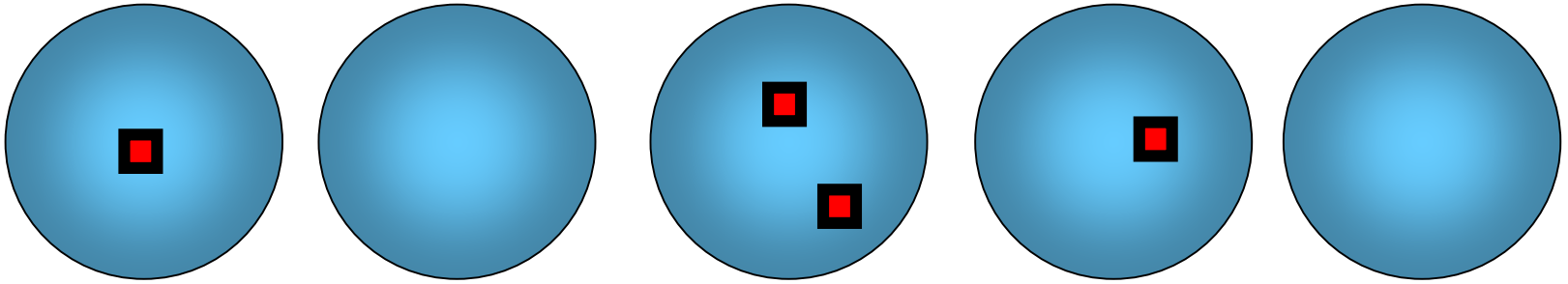
$$G(0) = 1/\langle N \rangle$$



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)

G(0), Particle Brightness and Poisson Statistics



1 0 0 0 0 0 0 0 0 2 0 1 1 1 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 1 0 0 0 1 0 0 1 0 0

Time

Average = 0.275 **Variance = 0.256**

$$\langle N \rangle \propto Average^2 \Big/ Variance = \frac{0.275^2}{0.256} = 0.296$$

Lets increase the particle brightness by 4x:

4 0 0 0 0 0 0 0 0 8 0 4 4 4 0 0 0 0 0 4 0 0 0 0 0 0 0 4 0 4 0 0 0 4 0 0 4 0 0

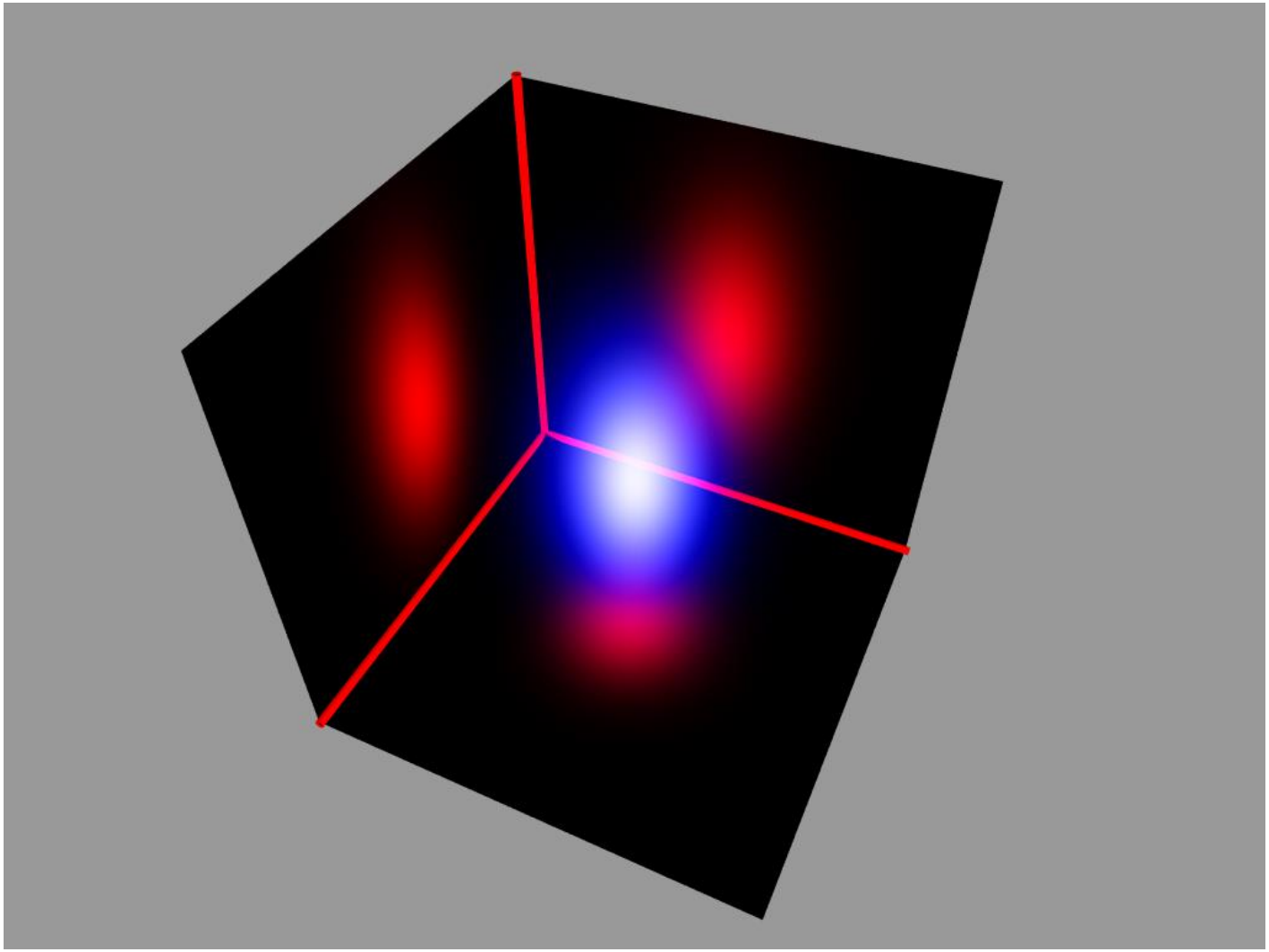
Average = 1.1
Variance = 4.09

$\langle N \rangle \propto$ **0.296**

$F = \varepsilon.N$

Mais ε x4

What about the excitation (or observation) volume shape?



Volume shape and autocorrelation

2-dimensional Gaussian excitation volume:

$$G(\tau) = \frac{\gamma}{N}\left(1 + \frac{4D\tau}{w_{2DG}^2}\right)^{-1}$$

For 2photon excitation, 8Dτ

3-dimensional Gaussian excitation volume:

$$G(\tau) = \frac{\gamma}{N}\left(1 + \frac{4D\tau}{w_{3DG}^2}\right)^{-1}\left(1 + \frac{4D\tau}{z_{3DG}^2}\right)^{-1/2}$$

Triplet :

$$(1 + \frac{T}{1-T}e^{\frac{-\tau}{\tau_T}})$$

..where T is the triplet state amplitude and τ_T is the triplet lifetime.

Orders of magnitude (for 1 μM solution, small molecule, water)

Volume	**Device**	**Size(μm)**	**Molecules**	**Time**
milliliter	cuvette	10000	$6x10^{14}$	10^{4}
microliter	plate well	1000	$6x10^{11}$	10^{2}
nanoliter	microfabrication	100	$6x10^{8}$	1
picoliter	typical cell	10	$6x10^{5}$	10^{-2}
femtoliter	confocal volume	1	$6x10^{2}$	10^{-4}
attoliter	nanofabrication	0.1	$6x10^{-1}$	10^{-6}

The Effects of Particle Size on the Autocorrelation Curve

Diffusion Constants

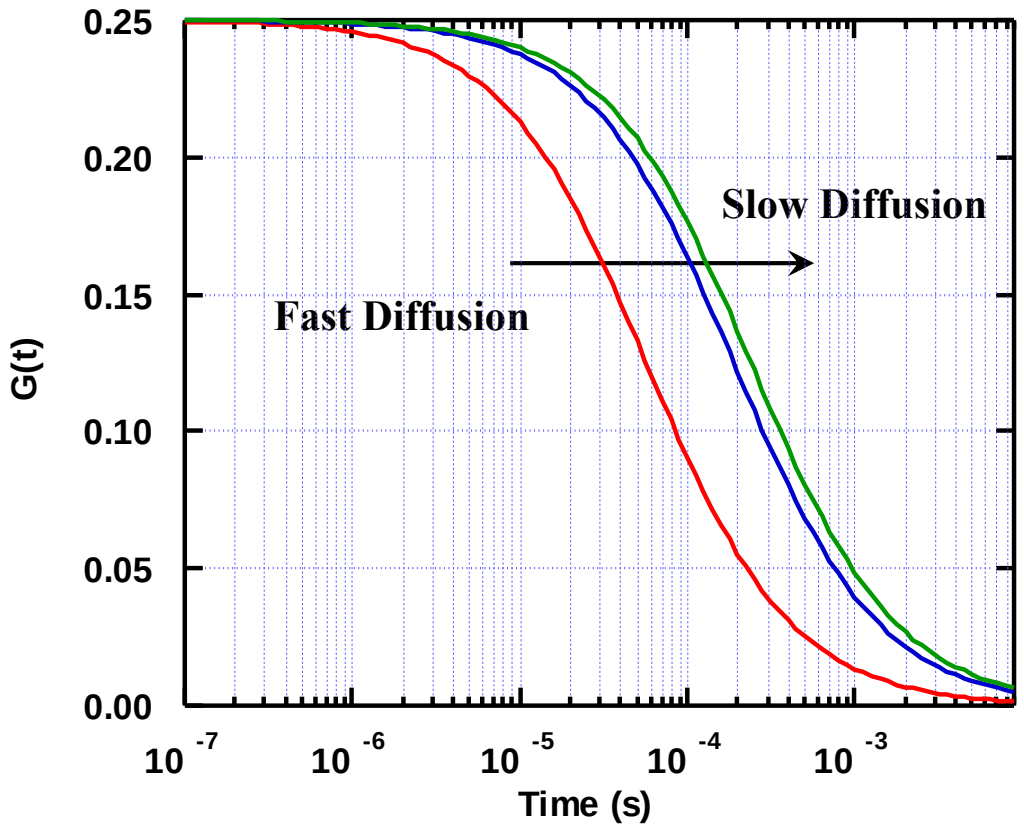
300 um^2/s
90 um^2/s
71 um^2/s

Stokes-Einstein Equation:

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

and

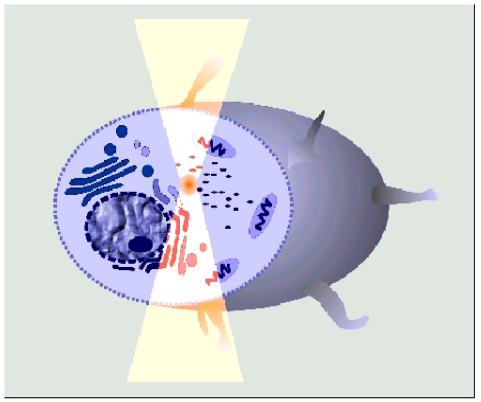
$$MW \propto Volume \propto r^3$$

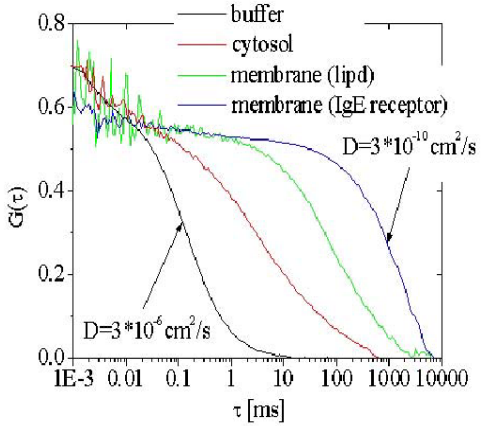


Monomer --> Dimer

Only a change in D by a factor of $2^{1/3}$, or 1.26

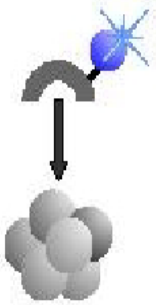
Mobility of a fluorescent macromolecule

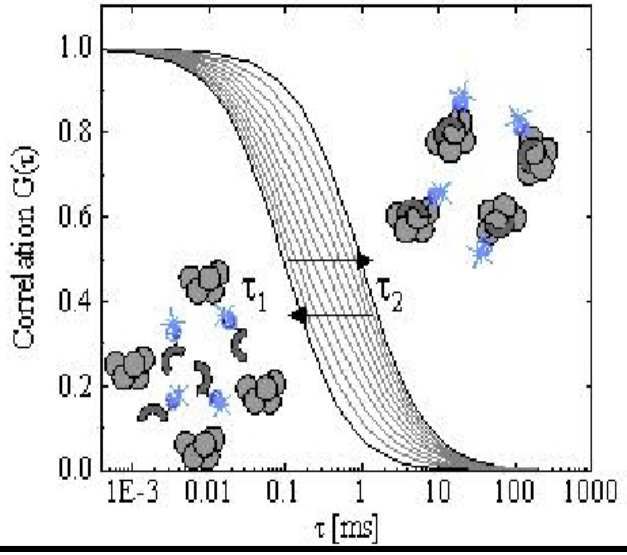




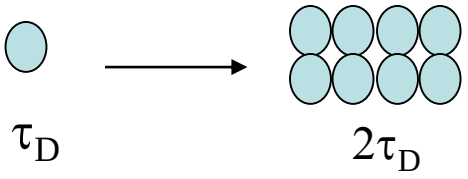
Rhodamine dye in different environments

Measuring molecular interactions





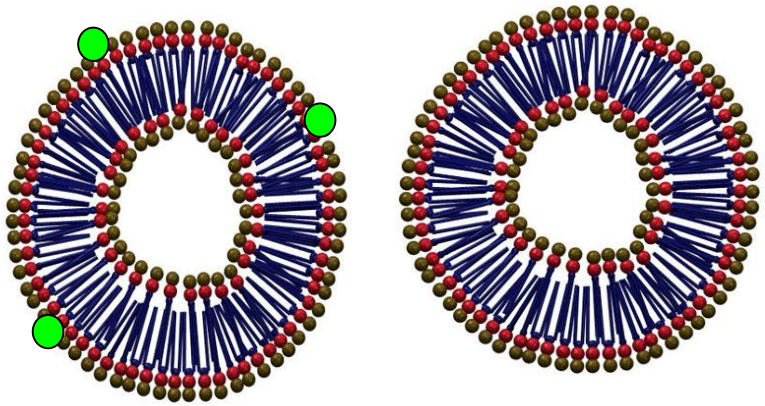
Diffusion time = f (MW) $^{1/3}$



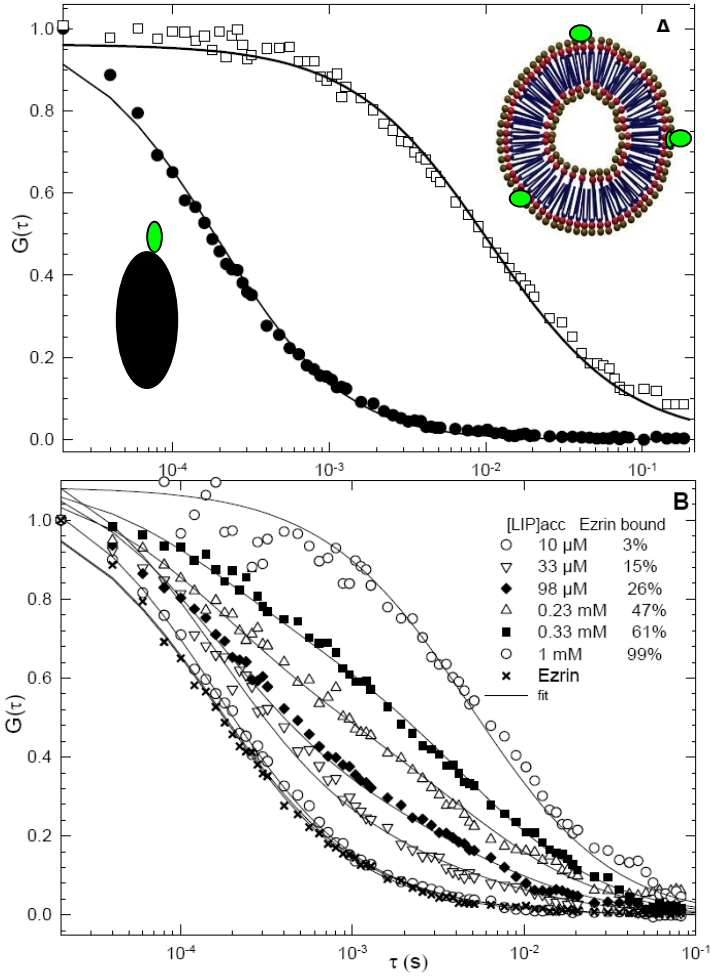
Measurement of the interaction between ezrin and Large Unilamelar Vesicles (LUV)

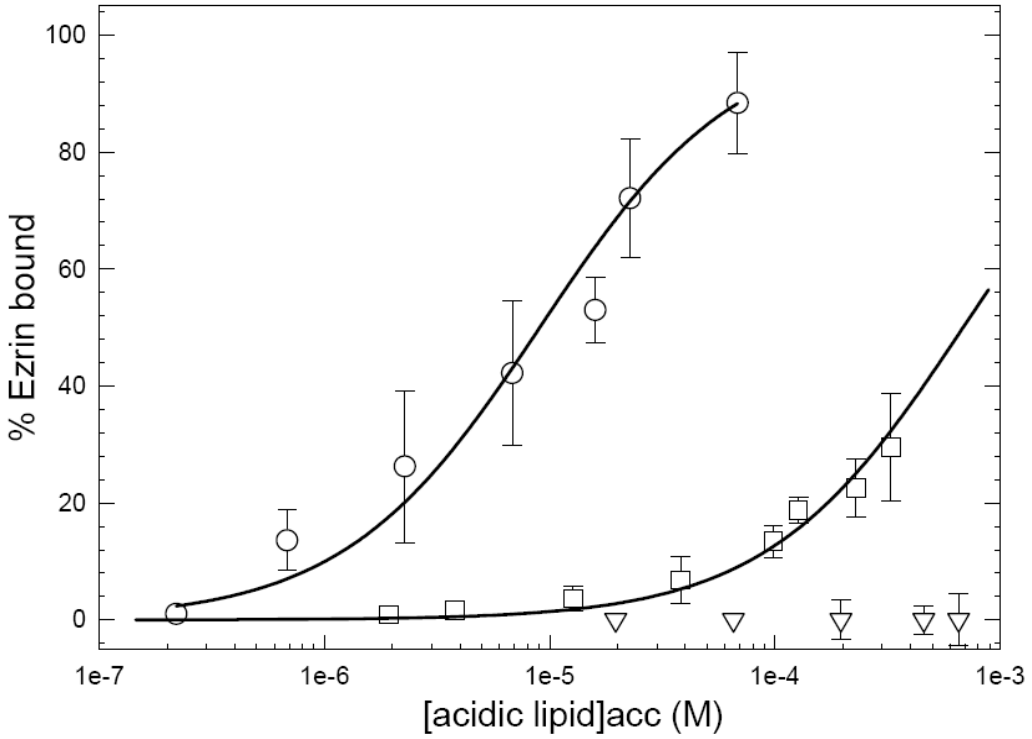
Collaboration with Pr. Picard (UM2)

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

Multiple Species

Case 1: Species vary by a difference in diffusion constant, D.

Autocorrelation function can be used:

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

(2D-Gaussian Shape)

! f_i is the fractional fluorescence intensity of species *i*.

$$\boldsymbol{G(0)_{sample} = \sum f_i^2 \cdot G(0)_i}$$

$G(0)_{sample}$ is no longer γ/N !

Case 2: Species vary by a difference in brightness

assuming that $D_1 \approx D_2$

The quantity G(0) becomes the only parameter to distinguish species, but we know that:

$$G(0)_{sample} = \sum f_i^{\,2} \cdot G(0)_i$$

The autocorrelation function is not suitable for analysis of this kind of data without additional information.

We need a different type of analysis

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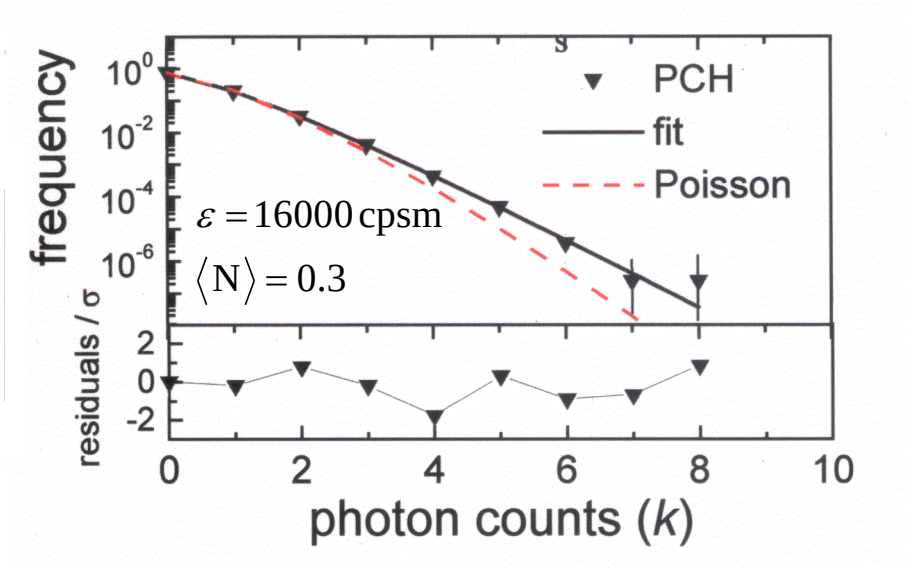
Photon Counting Histogram (PCH)

Aim: To resolve species from differences in their molecular brightness

Molecular brightness ε : The average photon count rate of a single fluorophore

PCH: probability distribution function $p(k)$

where $p(k)$ is the probability of observing k photon counts



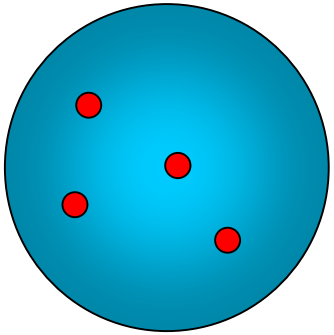
Single Species:

$$p(k) = PCH(\varepsilon, \langle N \rangle)$$

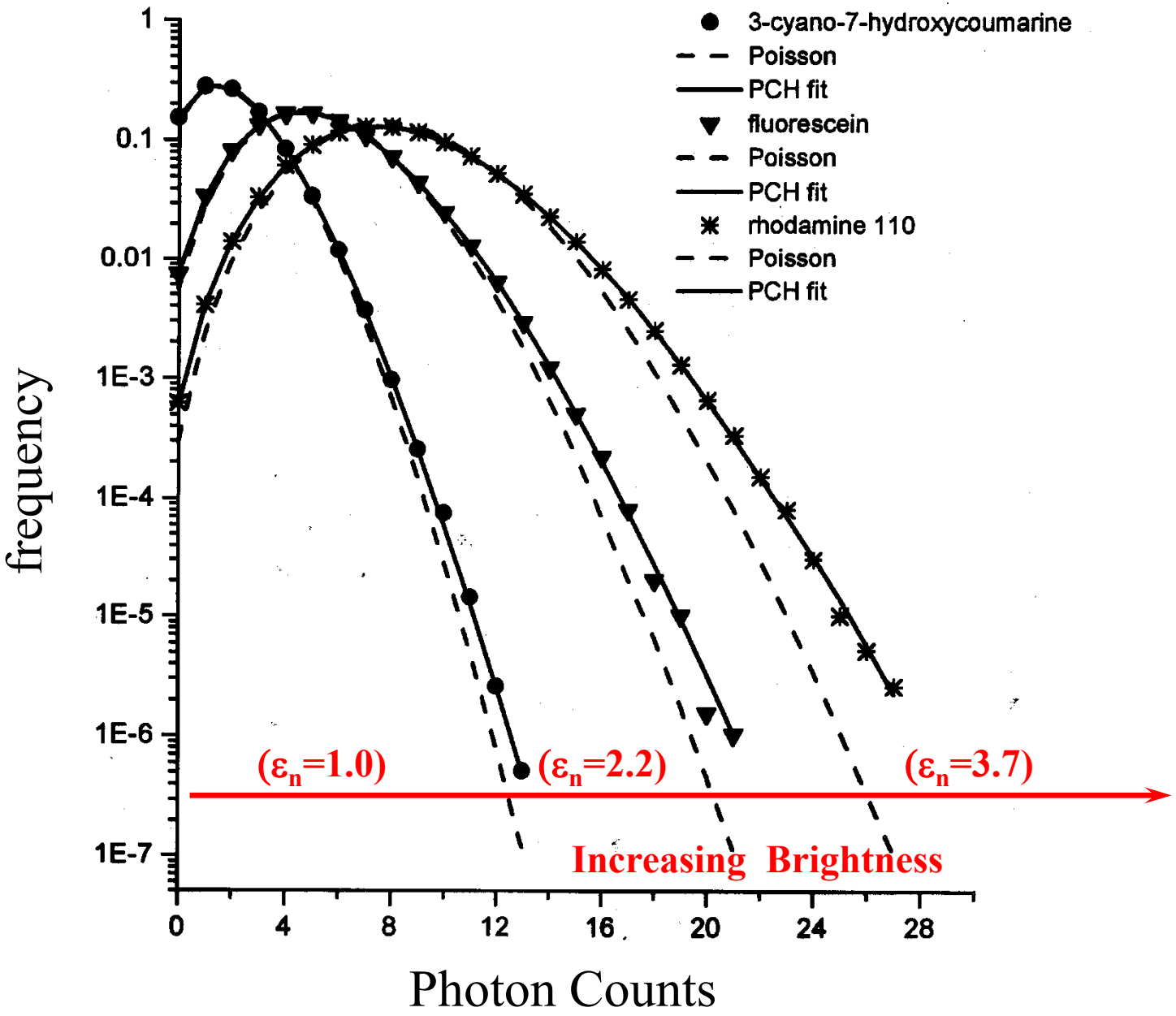
Note: PCH is Non-Poissonian!

Sources of Non-Poissonian Noise

- Detector Noise
- Diffusing Particles in an Inhomogeneous Excitation Beam*
- Particle Number Fluctuations*
- Multiple Species*

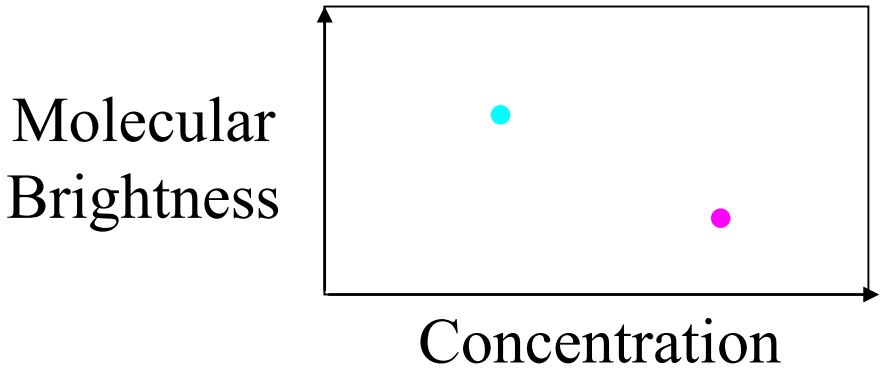


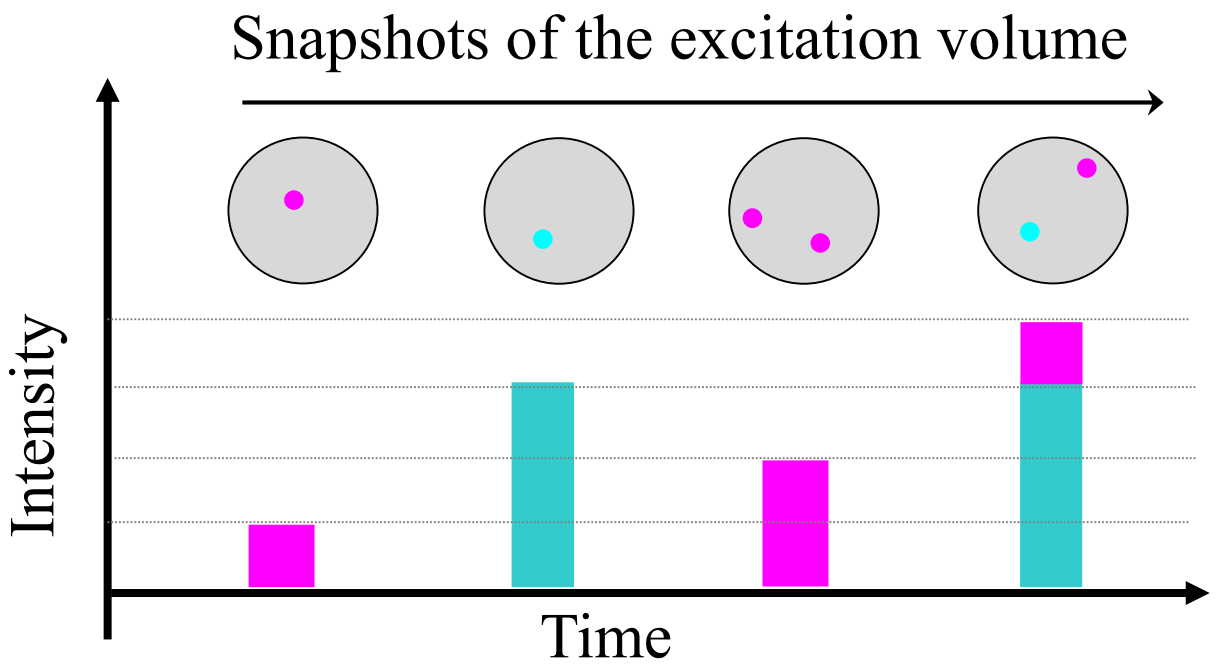
PCH Example: Differences in Brightness



Photon Counting Histogram: Multispecies

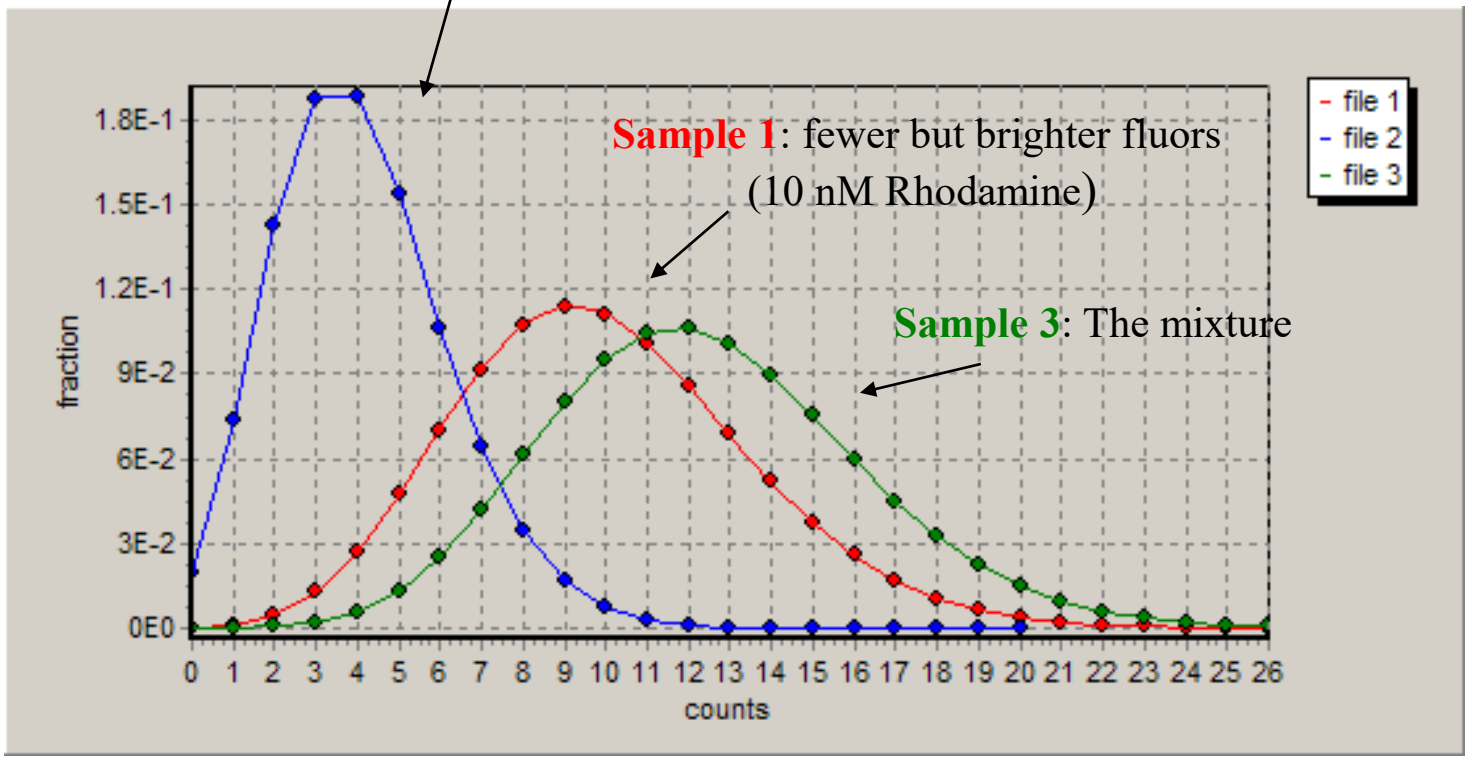
Binary Mixture: $p(k) = PCH(\varepsilon_1, \langle N_1 \rangle) \otimes PCH(\varepsilon_2, \langle N_2 \rangle)$





Photon Counting Histogram: Multispecies

Sample 2: many but dim (23 nM fluorescein at pH 6.3)

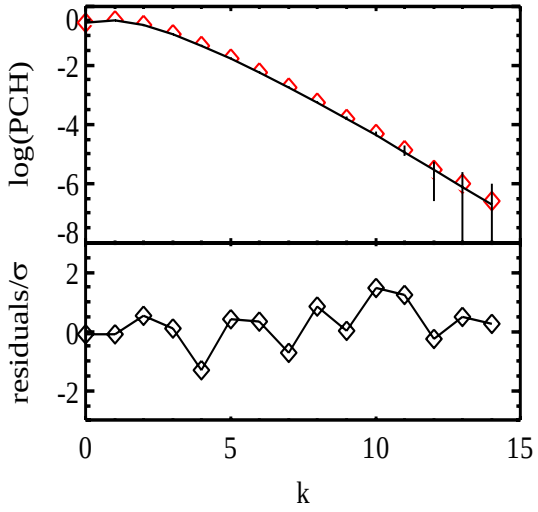


The occupancy fluctuations for each specie in the mixture becomes a convolution of the individual specie histograms. The resulting histogram is then broader than expected for a single species.

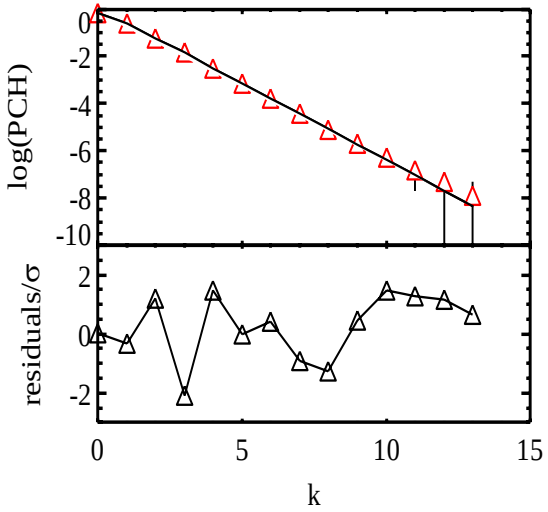
Resolve a protein mixture with a brightness ratio of two

Alcohol dehydrogenase labeling experiments

Singly labeled proteins



Mixture of singly or doubly labeled proteins

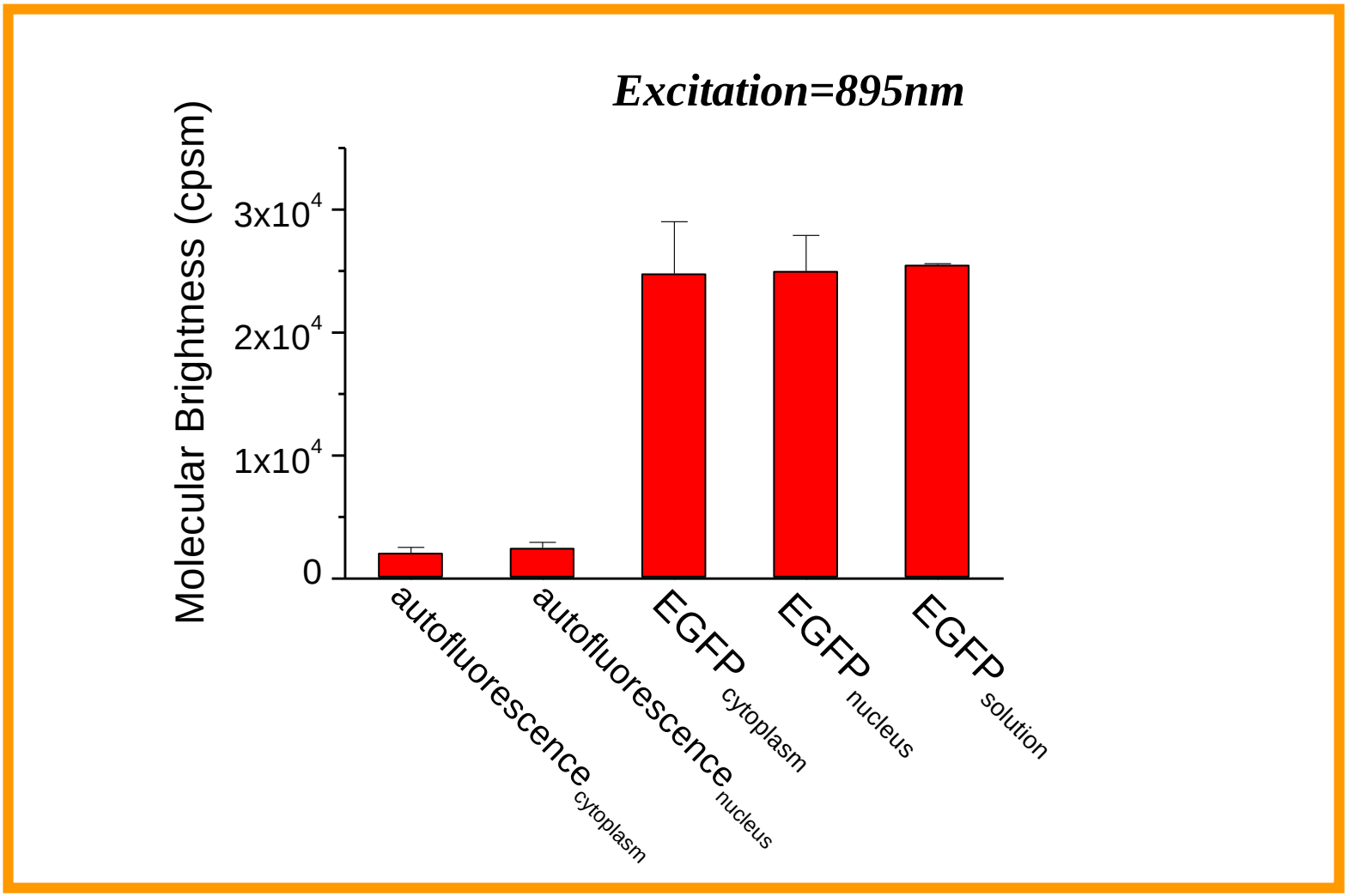


c	ε_1 kcpsm	$\overline{N}_1$	ε_2 kcpsm	$\overline{N}_2$
Sample A	$26.2^{+0.19}_{-0.18}$	$0.540^{+0.004}_{-0.004}$	-----	-----
Sample B	$25.1^{+0.6}_{-1.2}$	$0.155^{+0.007}_{-0.002}$	56^{+10}_{-10}	$0.006^{+0.008}_{-0.003}$

Both species have same

- **color**
- **fluorescence lifetime**
- **diffusion coefficient**
- **polarization**

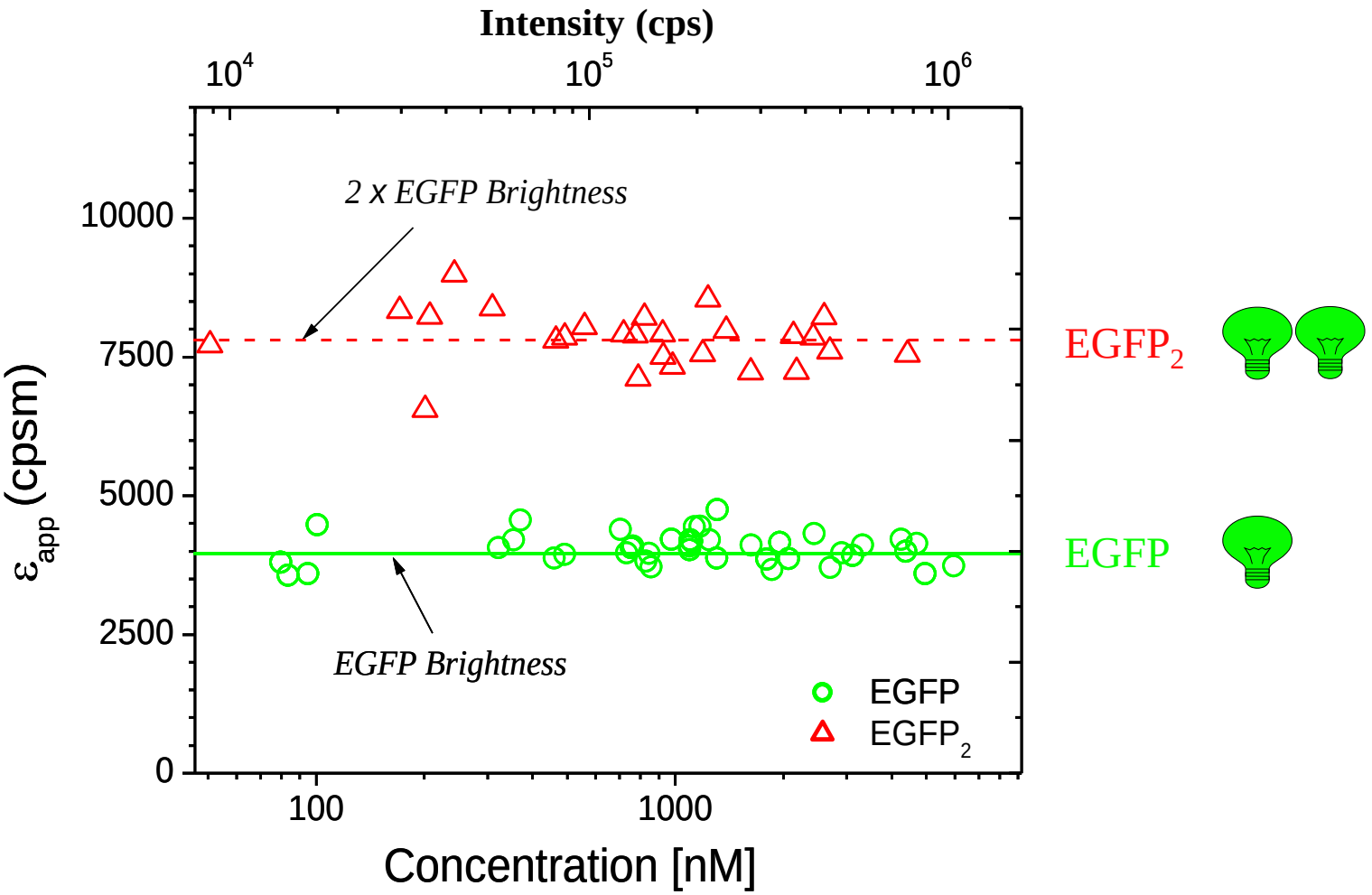
PCH in cells: Brightness of EGFP



The molecular brightness of EGFP is a factor ten higher than that of the autofluorescence in HeLa cells

Chen Y, Mueller JD, Ruan Q, Gratton E (2002) Biophysical Journal, 82, 133 .

Brightness and Stoichiometry



Brightness of $EGFP_2$ is twice the brightness of EGFP

Chen Y, Wei LN, Mueller JD, PNAS (2003) 100, 15492-15497

FLUCTUATION MICROSCOPIES

- INTRODUCTION
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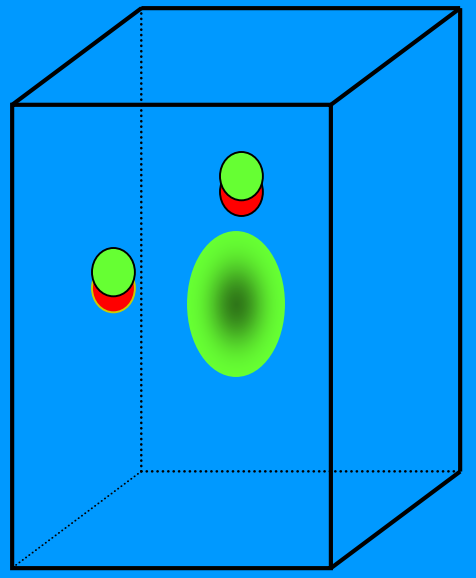
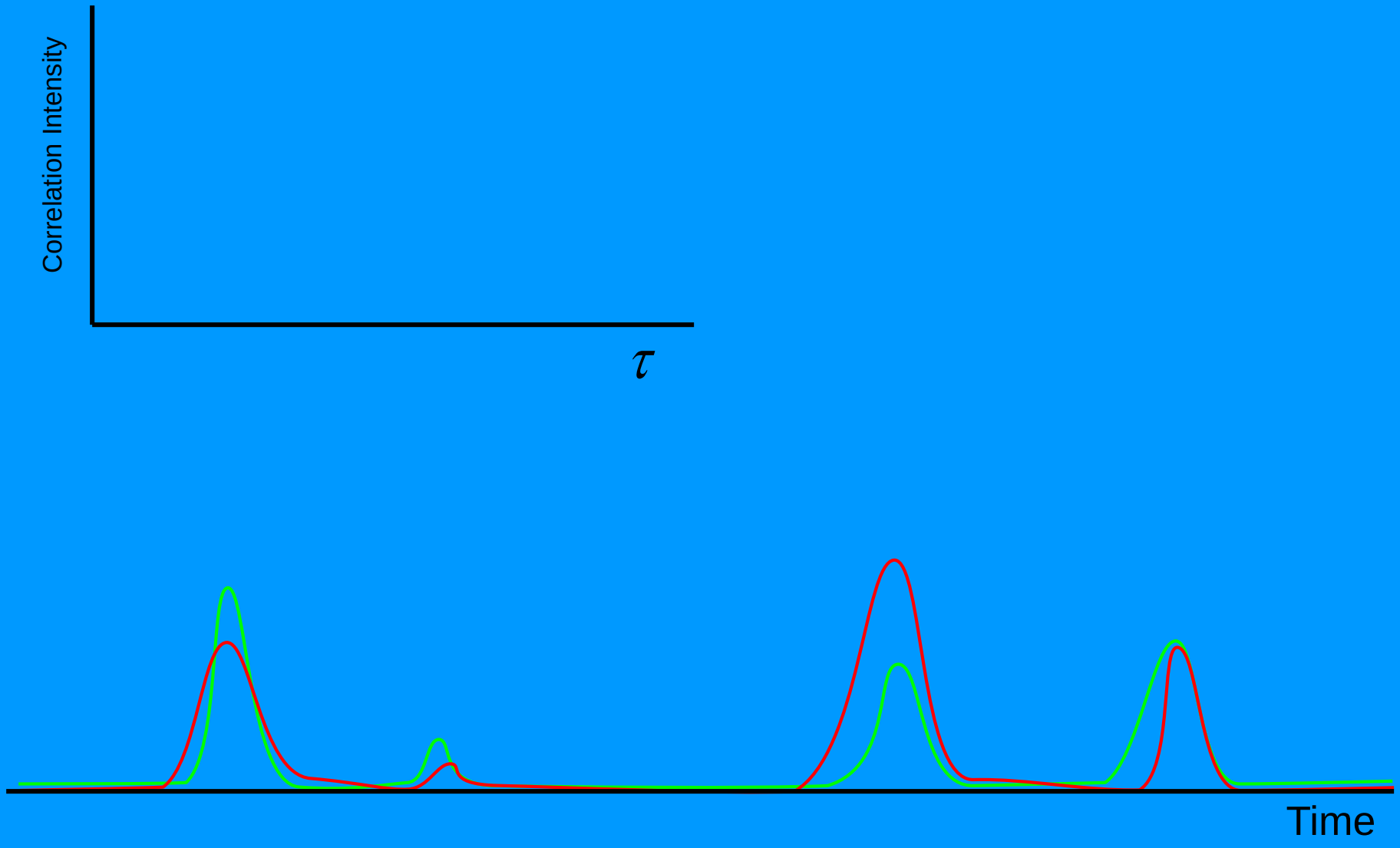
- PHOTON COUNTING HISTOGRAM
- CROSS CORRELATION
- EXPERIMENTAL DETAILS
- NUMBER & BRIGHTNESS
- PRACTICAL PART

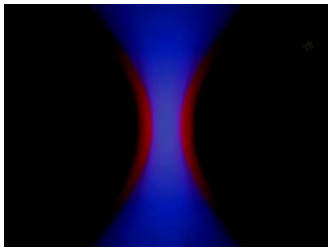
Introduction to correlation function.

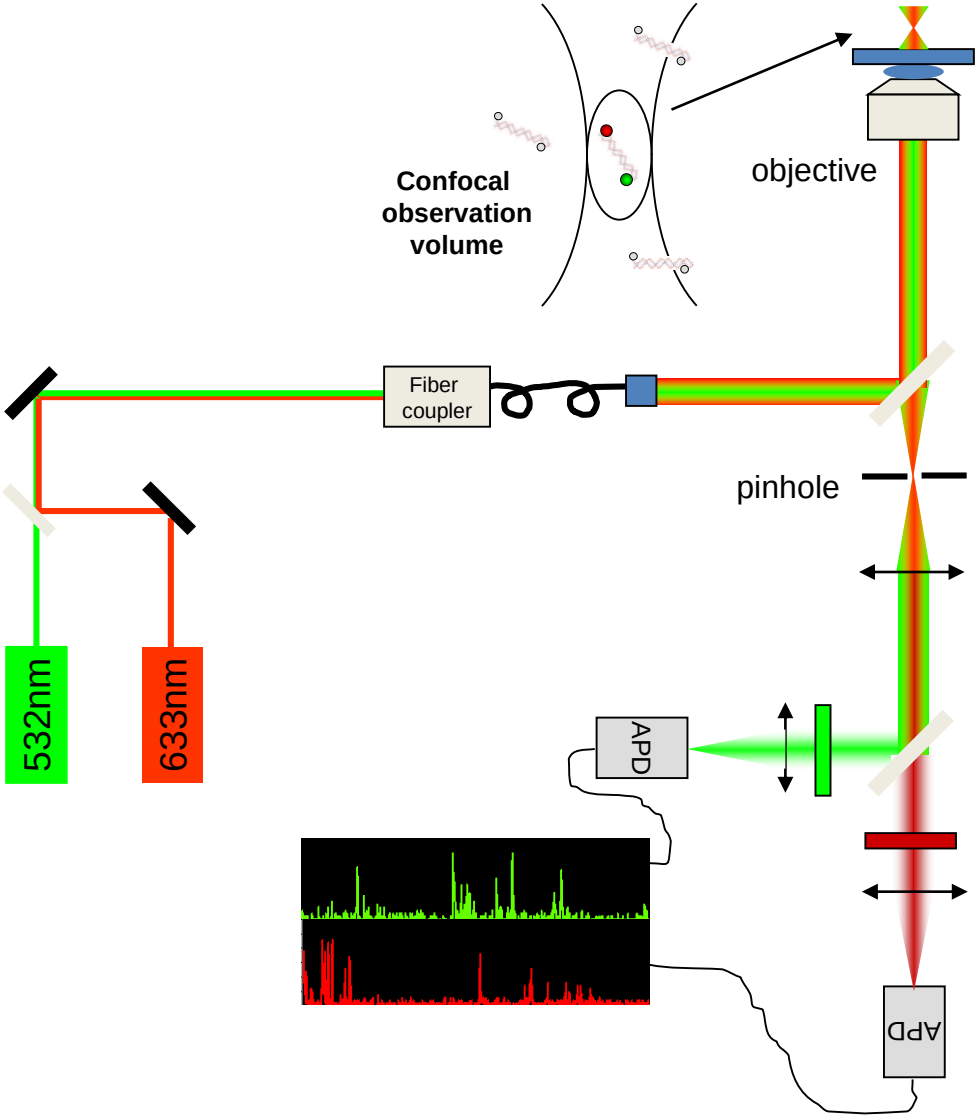


Time

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



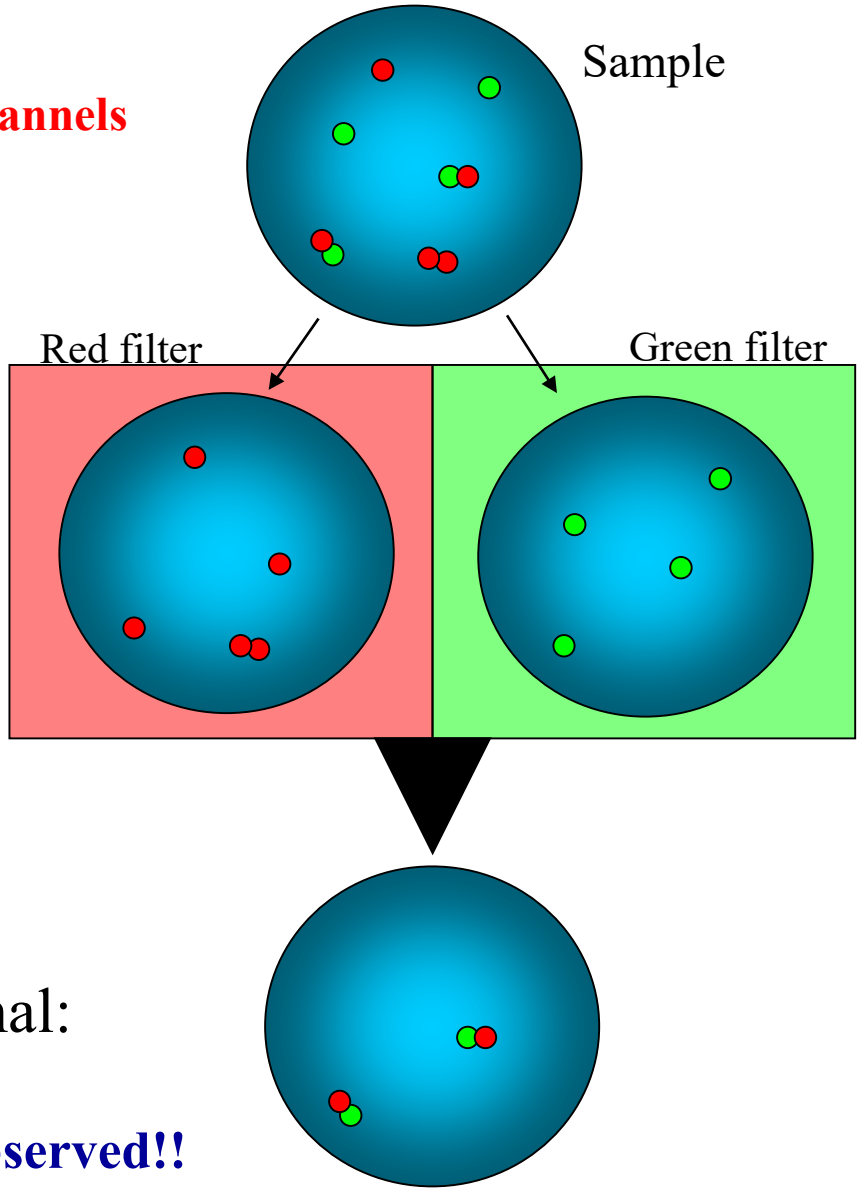



Confocal
observation
volume
objective
Fiber
coupler
pinhole
532nm
633nm
APD
APD

Two-Color Cross-correlation

The cross-correlation
ONLY if particles are observed in both channels

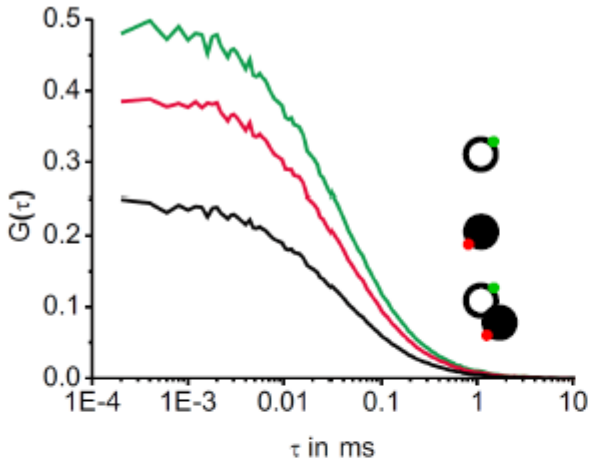
Each detector observes
particles with a particular color



The cross-correlation signal:

Only the green-red molecules are observed!!

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



- 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

Cross-correlation Calculations

One uses the same fitting functions you would use for the standard autocorrelation curves.

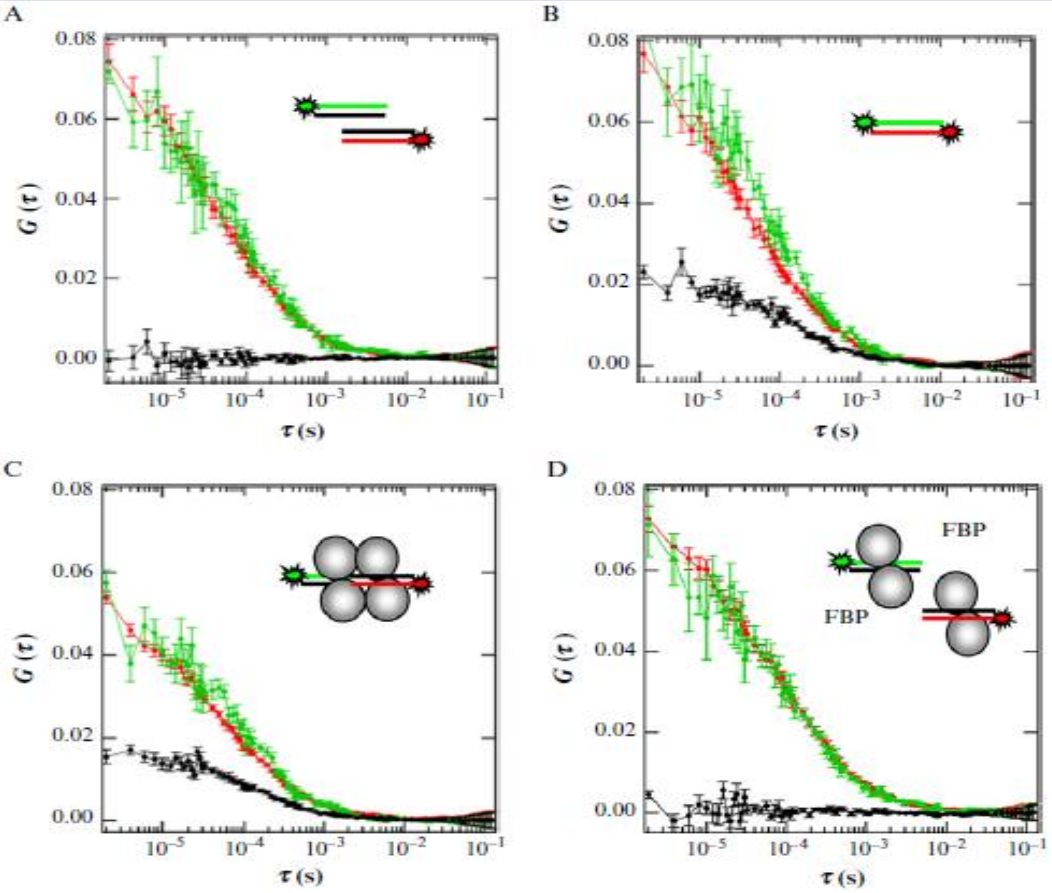
Thus, for a 3-dimensional Gaussian excitation volume one uses:

$$G_{12}(\tau) = \frac{\gamma}{N_{12}} \left(1 + \frac{8D_{12}\tau}{w^2}\right)^{-1} \left(1 + \frac{8D_{12}\tau}{z^2}\right)^{-1/2}$$

G_{12} is commonly used to denote the cross-correlation and G_1 and G_2 for the autocorrelation of the individual detectors. Sometimes you will see $G_x(0)$ or $C(0)$ used for the cross-correlation.

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)

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

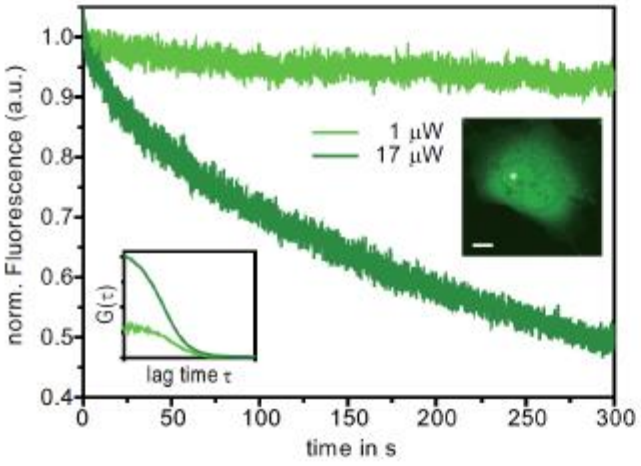
- Correlated (arising from diffusing molecules)

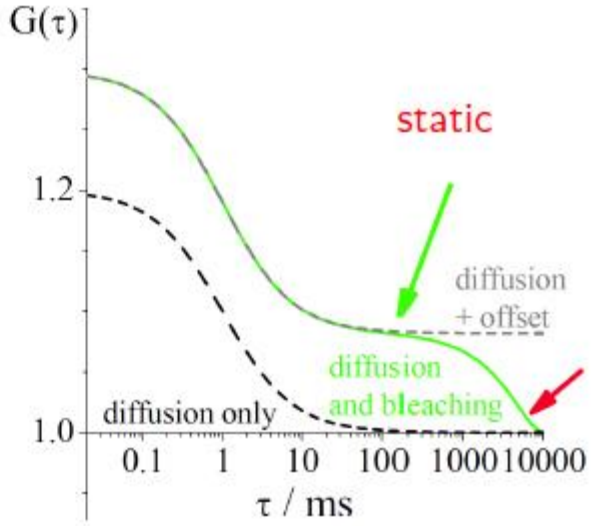
  → Add a new component to the analysis

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

Photobleaching

- Static (photobleaching of quasi immobiles molecules)

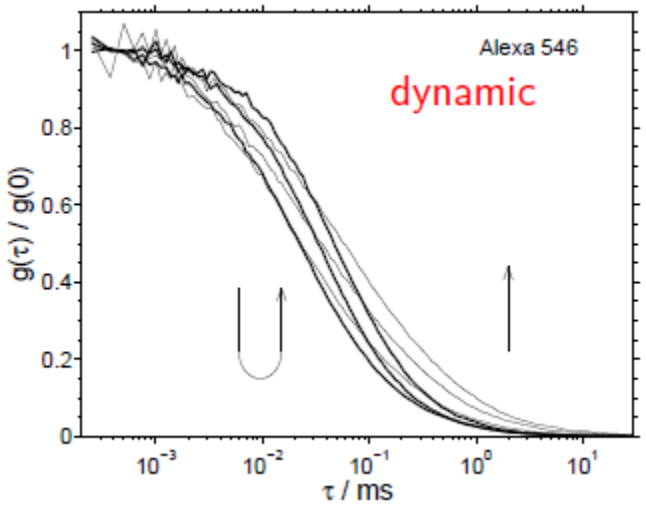




Photobleaching

- Dynamic

→ Diminishes the apparent diffusion coefficient

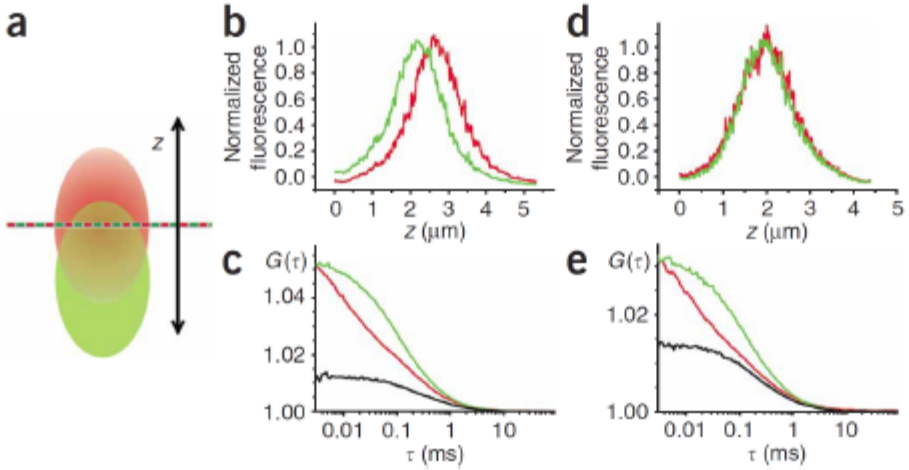


Size and shape of the observation volume

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}: $g(0) = 1/cV_{\text{eff}}$

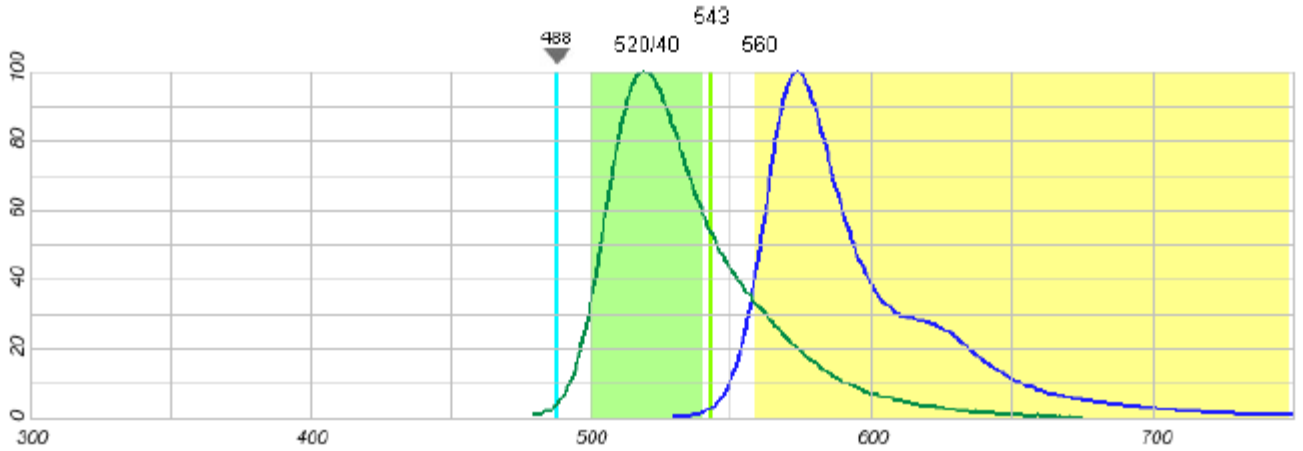
FCCS: in addition to the above, the volume overlap affects $g_\times(\tau)$



— 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

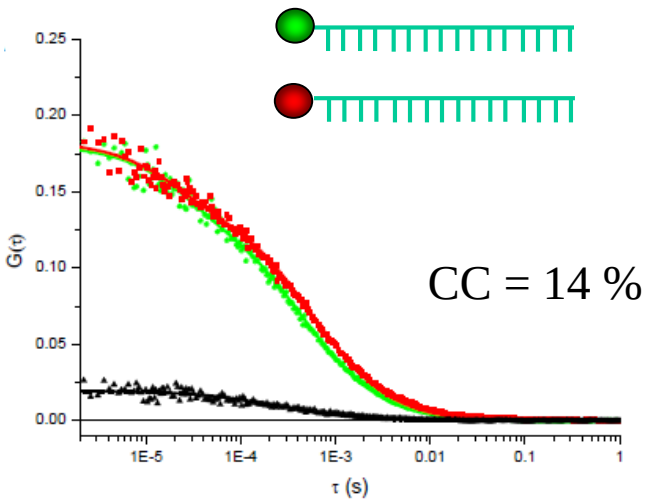
PIE / nsALEX with a supercontinuum source

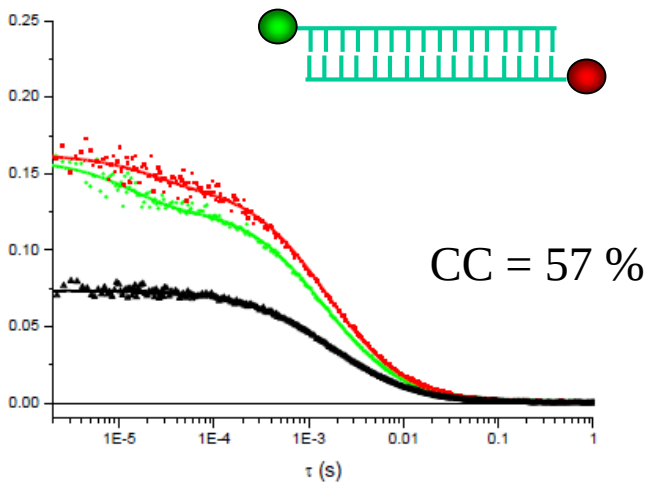
Supercontinuum source
100ps @ 20MHz

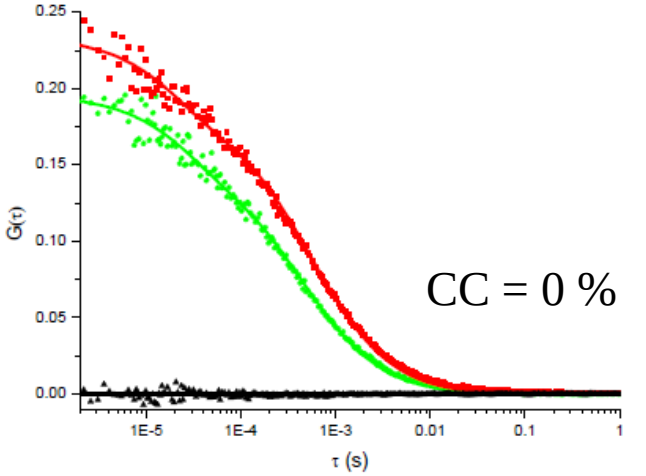
Removal of crosstalk in FCCS

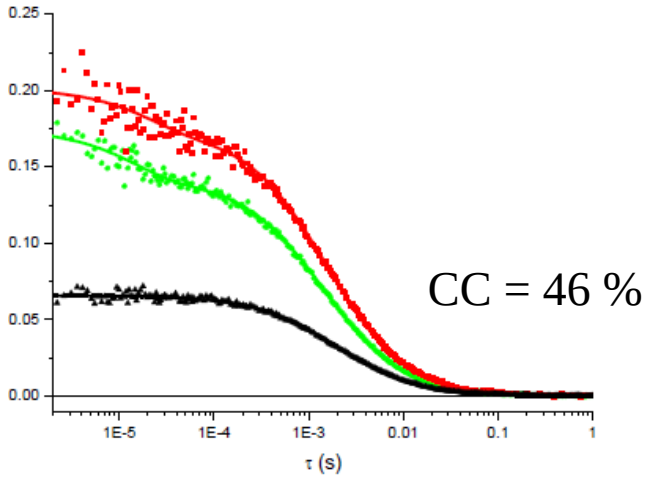
Regular FCCS

PIE FCCS





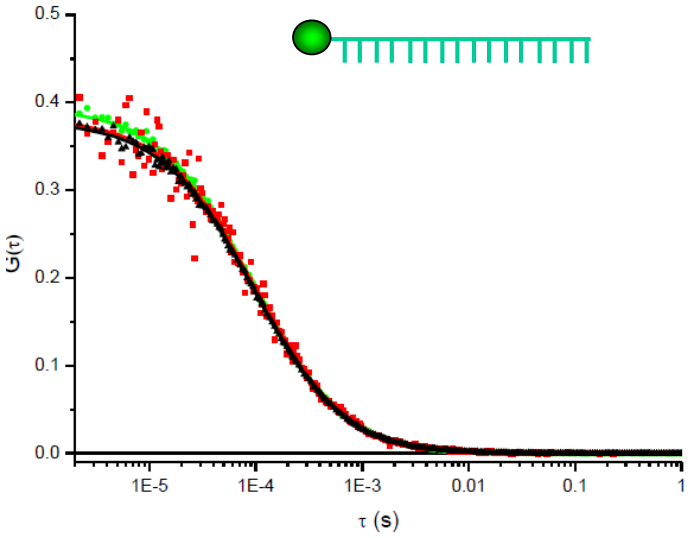


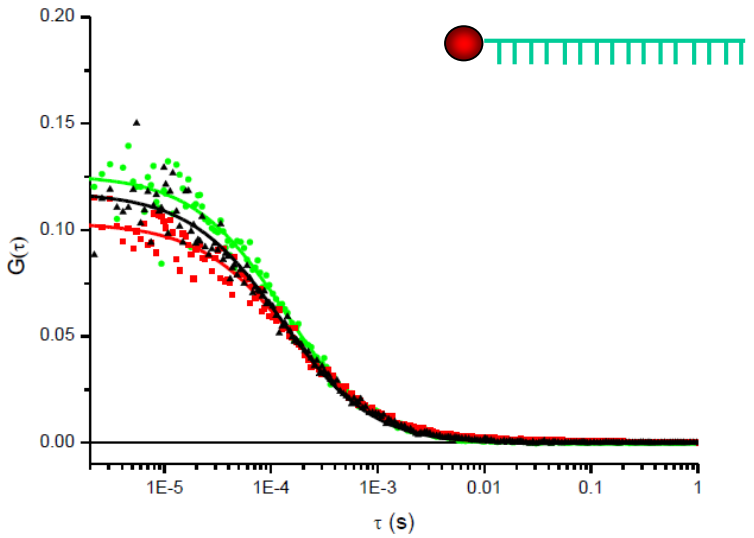


Green AC
Red AC
Green Red CC

Olofsson and Margeat, Optics Express, 2013

Overlap of the excitation and detection volumes





Green excitation

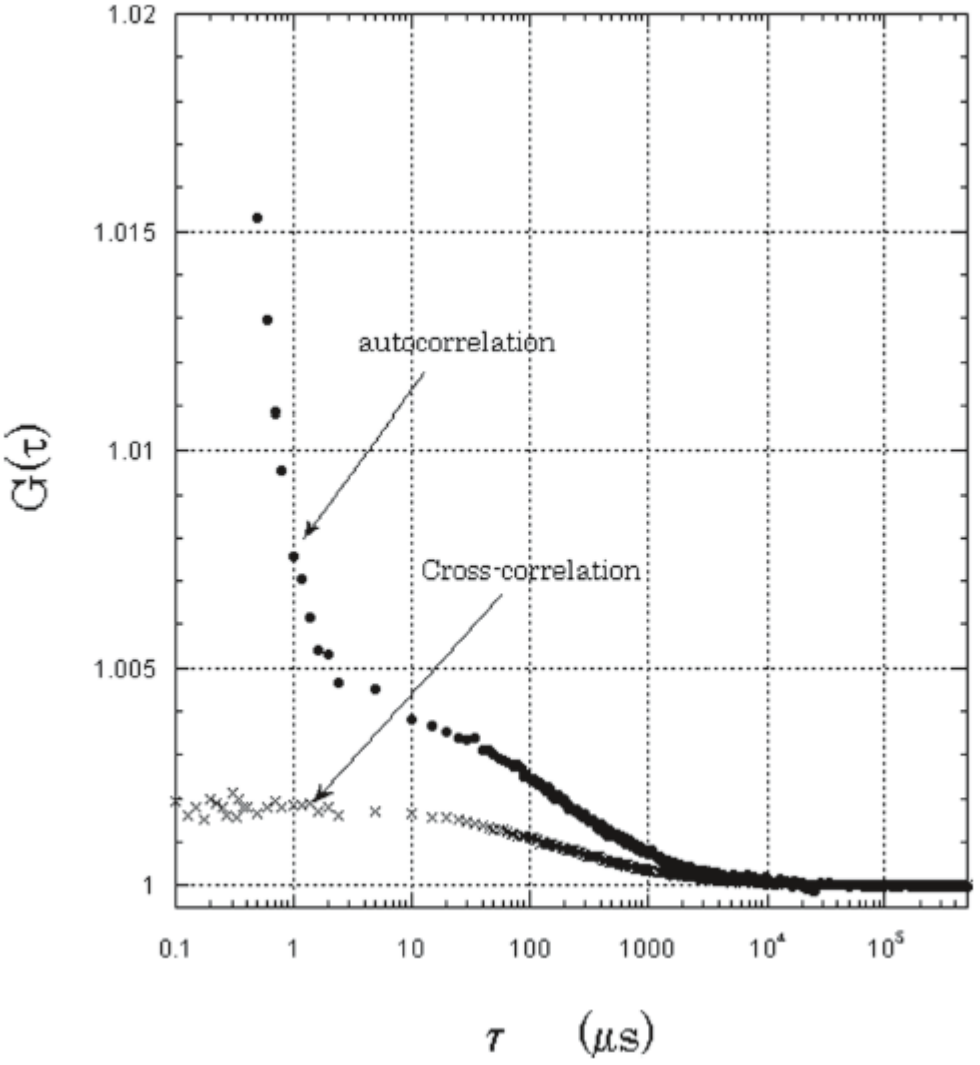
- Green detection AC
- Red detection AC
- Green Red CC

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

Red detection

Olofsson and Margeat, Optics Express, 2013

Afterpulsing



Autocorrelation :
One detector

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