FCS, Autocorrelation; PCH, Cross-correlation

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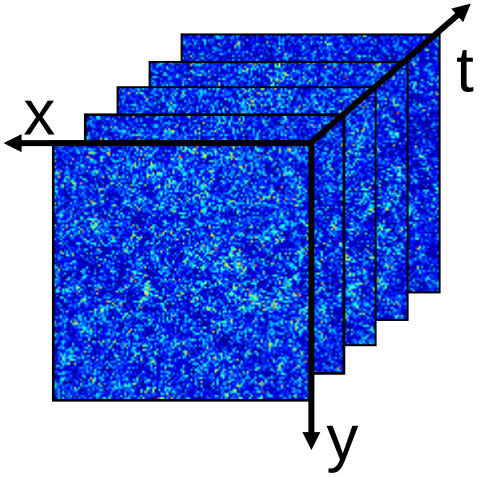

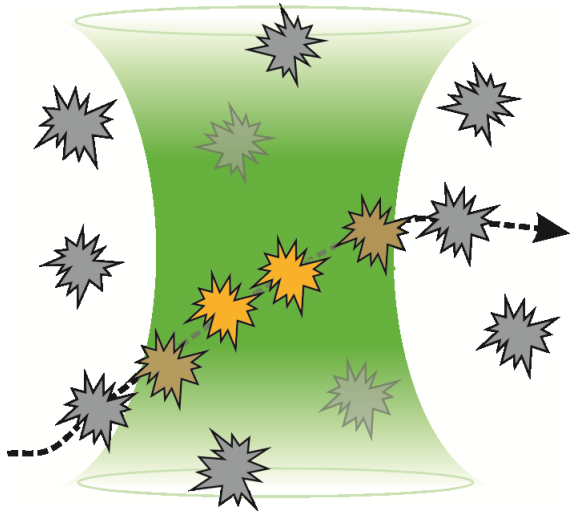

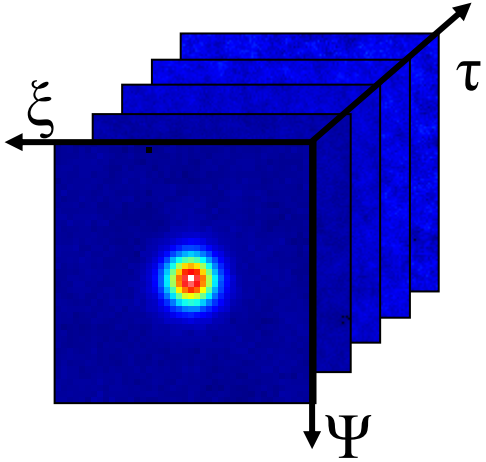

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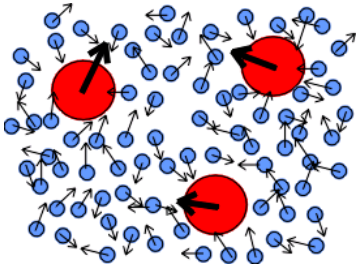
Brownian Motion

- In 1827 the English botanist Robert Brown noticed that pollen grains suspended in water jiggled about under the lens of the microscope, following a zigzag path. Even more remarkable was the fact that pollen grains that had been stored for a century moved in the same way.
- In 1889 G.L. Gouy found that the "Brownian" movement was more rapid for smaller particles (no Brownian movement of cars, bricks, or people).
- In 1900 F.M. Exner undertook the first quantitative studies, measuring how the motion depended on temperature and particle size.
- The first good explanation of Brownian movement was advanced by Desaulx in 1877: "In my way of thinking the phenomenon is a result of thermal molecular motion in the liquid environment (of the particles)." This is indeed the case. A suspended particle is constantly and randomly bombarded from all sides by molecules of the liquid. If the particle is very small, the hits it takes from one side will be stronger than the bumps from other side, causing it to jump. These small random jumps are what make up Brownian motion.

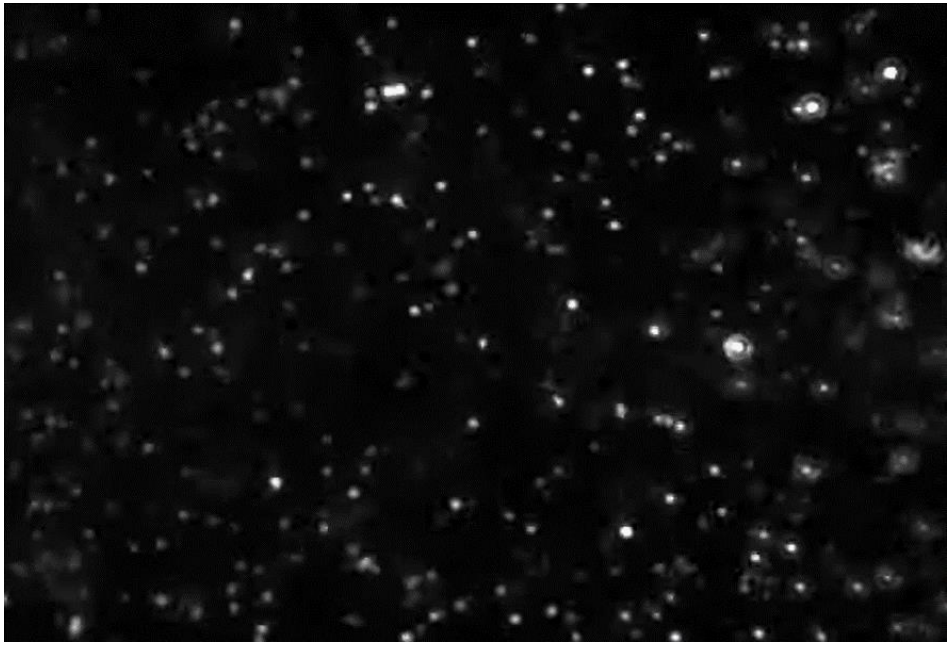


- In 1905 A. Einstein explained Brownian motion using energy equipartition: the kinetic theory of gases developed by Boltzmann and Gibbs could explain the randomness of the motion of large particles without contradicting the Second Principle of Thermodynamics. This was the first “convincing” proof of the particle nature of matter as declared by the adversaries of atomism.

Fluorescence Fluctuation Spectroscopy

How can we measure the molecular exploration of space and time?

-> Label and observe with fluorescence microscope!



https://youtu.be/cDcprgWiQEY
Brownian Motion - nanoparticles in water

Fluorescence Fluctuation Spectroscopy

How does it look in the cellular environment?

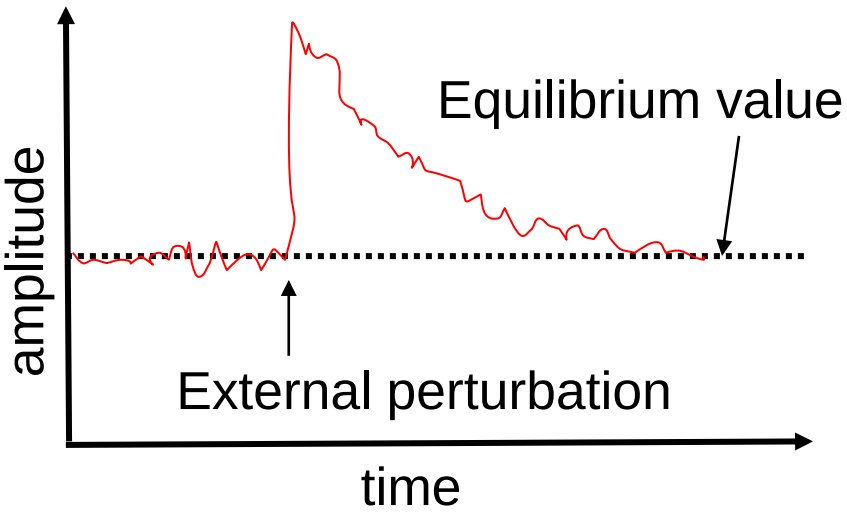
- Crowded environment, dim molecules!
- Further limited by spatial and temporal resolution of the microscope.

-> Fluorescence Fluctuation Spectroscopy!

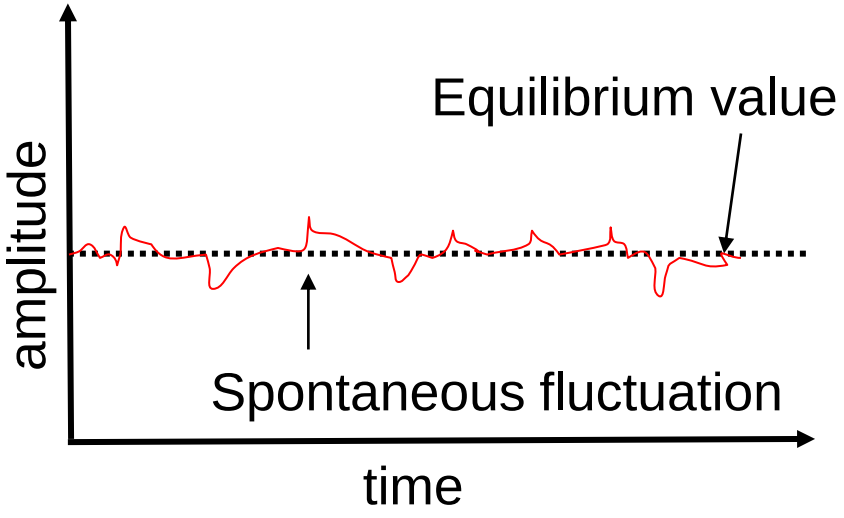
The Fluctuation-Dissipation Principle

If we **perturb** a system from **equilibrium**, it returns to the average value with a characteristic time that depends on the process responsible for returning the system to equilibrium.

Spontaneous energy fluctuations in a part of the system, can cause the system to locally go out of equilibrium. These spontaneous fluctuations **dissipate** with the same time constant as if we had externally perturbed the equilibrium of the system.



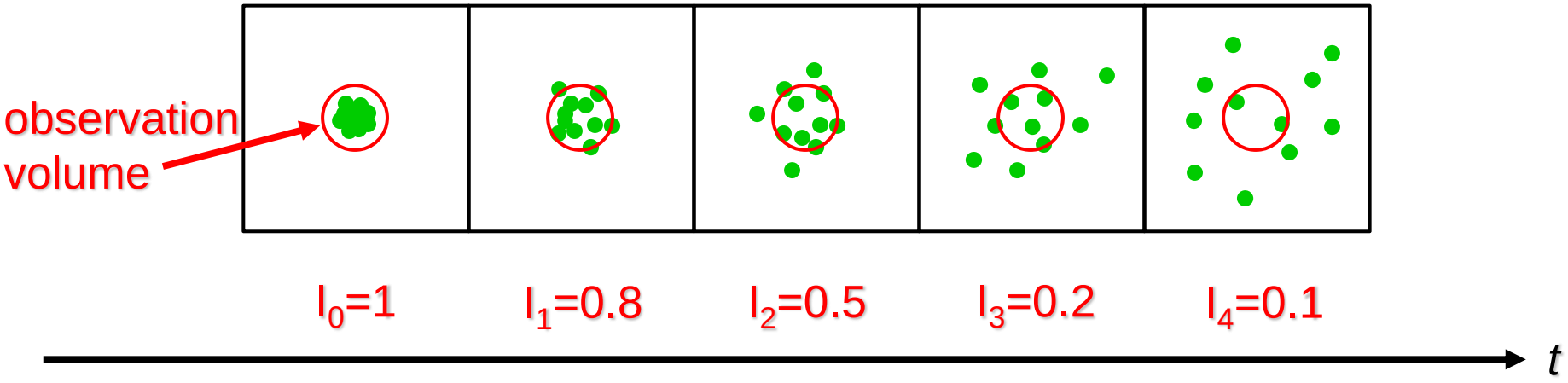
Synchronized



Non-synchronized

The Fluctuation-Dissipation Principle

Follow the intensity from a static observation volume over time:



Information of particle dynamics can be obtained by the change in fluorescence intensity over time.

-> Fluorescence Recovery after Photobleaching (FRAP)

The Fluctuation-Dissipation Principle

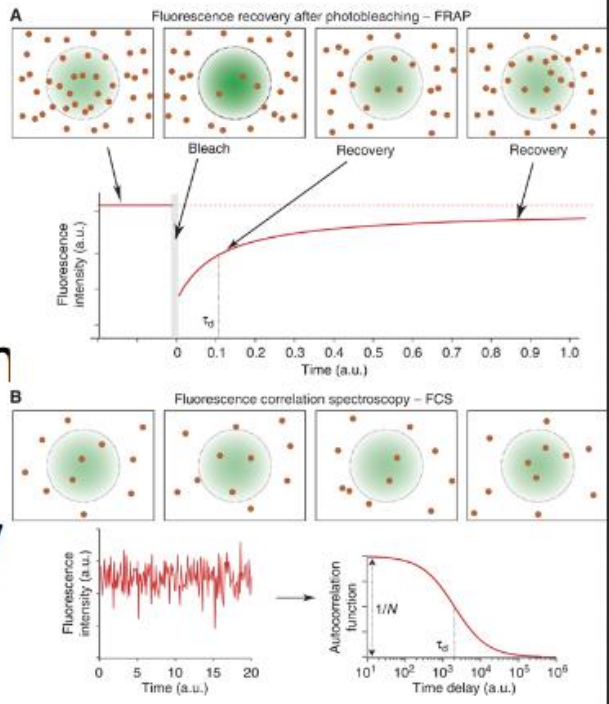
Methods based on perturbation

Typically FRAP (fluorescence recovery after photobleaching)

Methods based on fluctuations

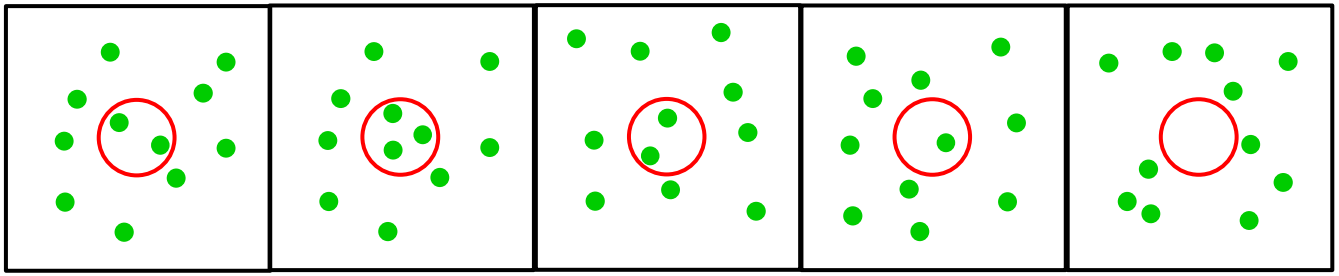
Typically FCS and dynamic ICS methods

There is a fundamental difference between the two approaches, although they are related as to the physical phenomena they report.



The Fluctuation-Dissipation Principle

In equilibrium the particles are already dispersed:



Measure fluctuations in intensity!

-> Fluorescence Correlation Spectroscopy (FCS)

First Application of Fluctuation Spectroscopy

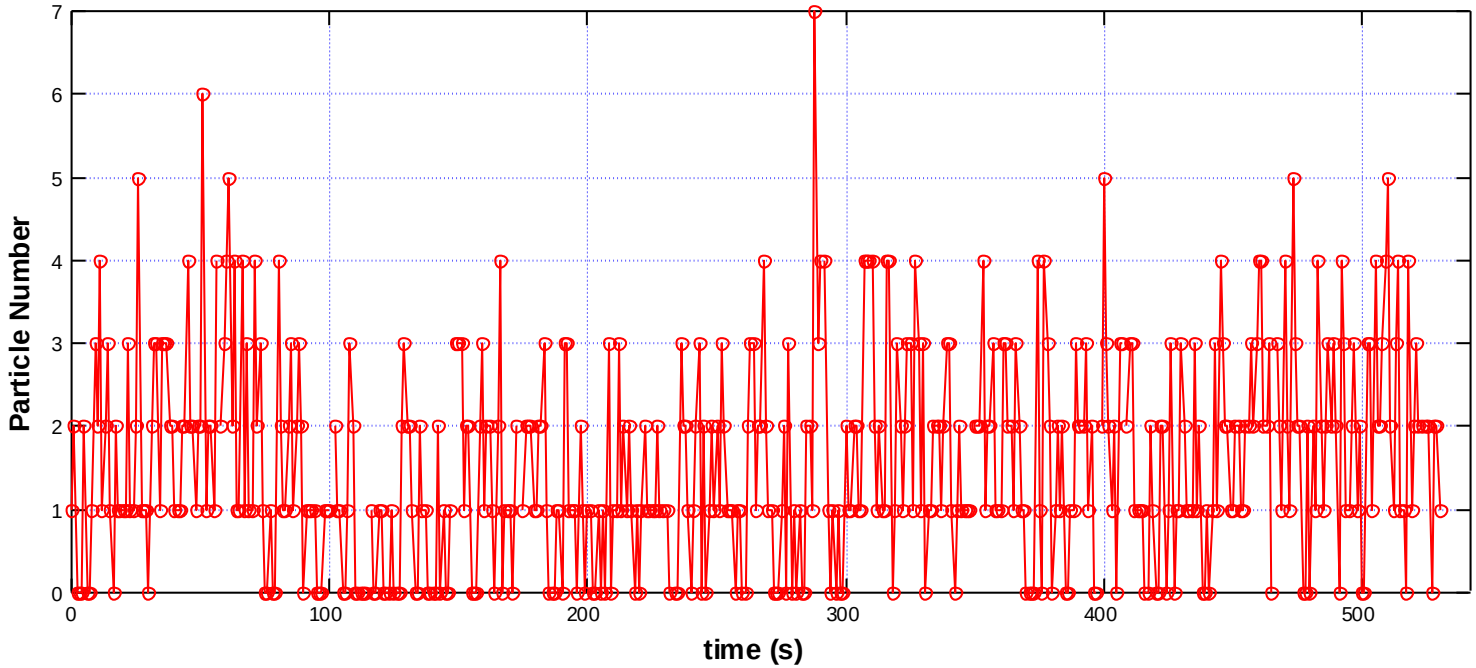
(Svedberg & Inouye, 1911) *Occupancy Fluctuation*

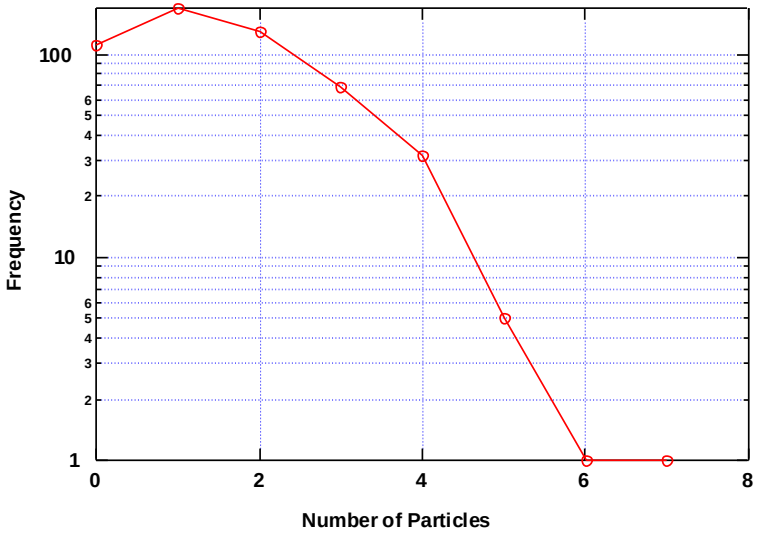
Experimental data on colloidal gold particles:

```
120002001324123102111131125111023313322111224221226122 14
234524114131142310010042112312320111100011 1_2110013200000
100110001000232210021100002010 01_33312200023122102401110 2_
122211223100011033111021011001010301131212101012111121 1_10
003221012302012121321110110023312242110001203010100221734
410101002112211444421211440132123314313011222123310121111
222412231113322132110000410432012120011322231200_253212033
233111100210022013011321113120010131432211221122323442230
321421532200202142123232043112312003314223452134110412322
220221
```

Collected data by counting (by visual inspection) the number of particles in the observation volume as a function of time

Particle Fluctuation



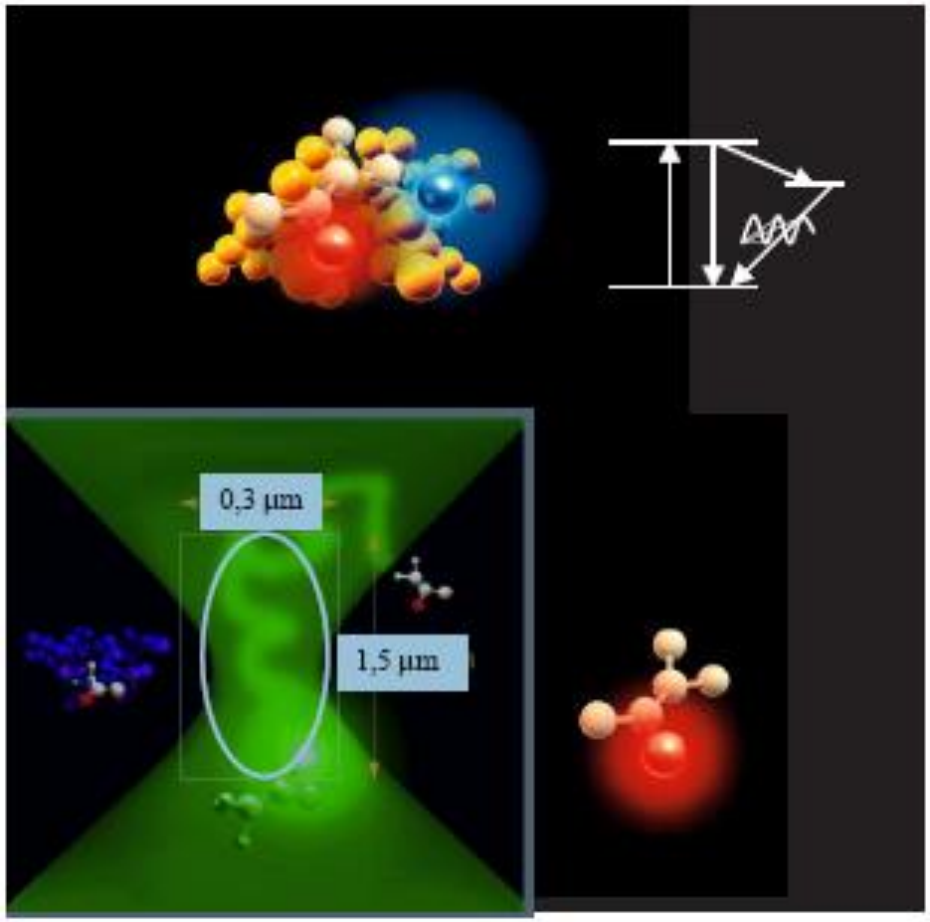


*Histogram of particle counts

*Poisson behavior

*Autocorrelation not available

Generating Fluctuations By Motion



What is Measured?

Diffusion

Enzymatic Activity

Phase Fluctuations

Conformational Dynamics

Rotational Motion

Protein Folding

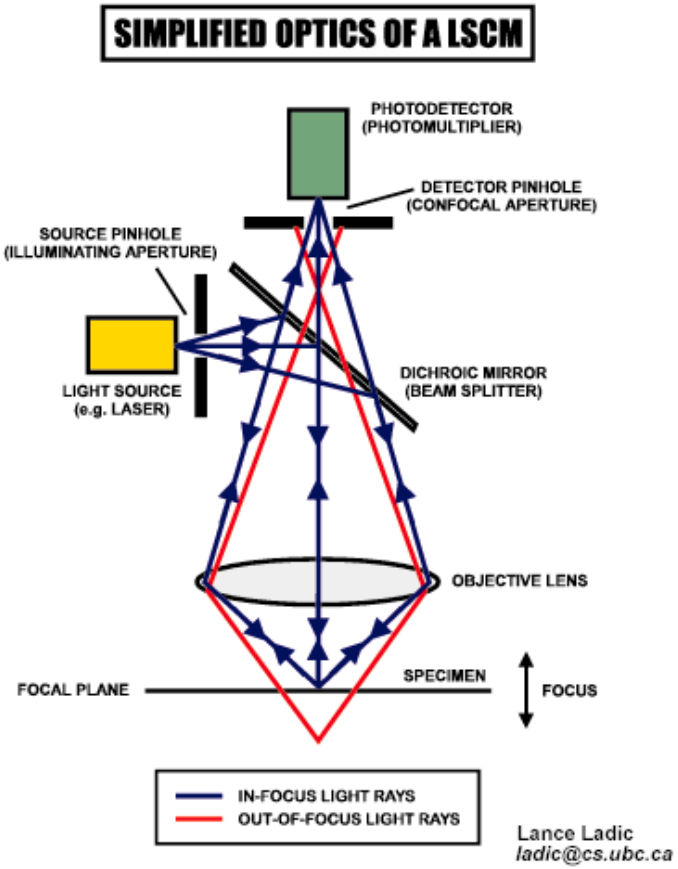
Methods to produce a small volume

(limited by the wavelength of light to about 0.1 fL)

- Confocal pinhole
- Multiphoton effects
  - 2-photon excitation (TPE)
  - Second-harmonic generation (SGH)
  - Stimulated emission
  - Four-way mixing (CARS)

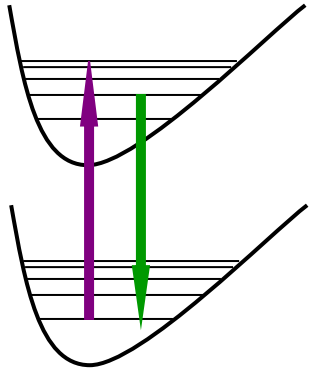
(not limited by wavelength not applicable to cells)

- Nanofabrication
- Local field enhancement
- Near-field effects

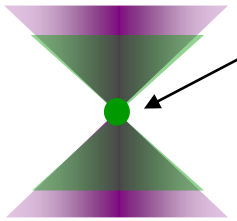


Defining Our Observation Volume: One- & Two-Photon Excitation.

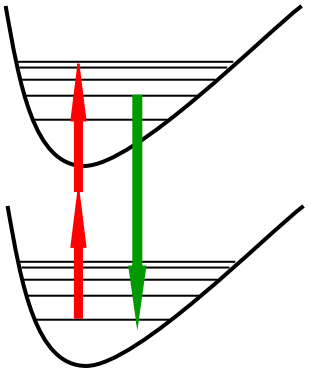
1 - Photon



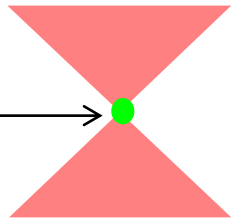
Defined by the pinhole size, wavelength, magnification and numerical aperture of the objective



2 - Photon

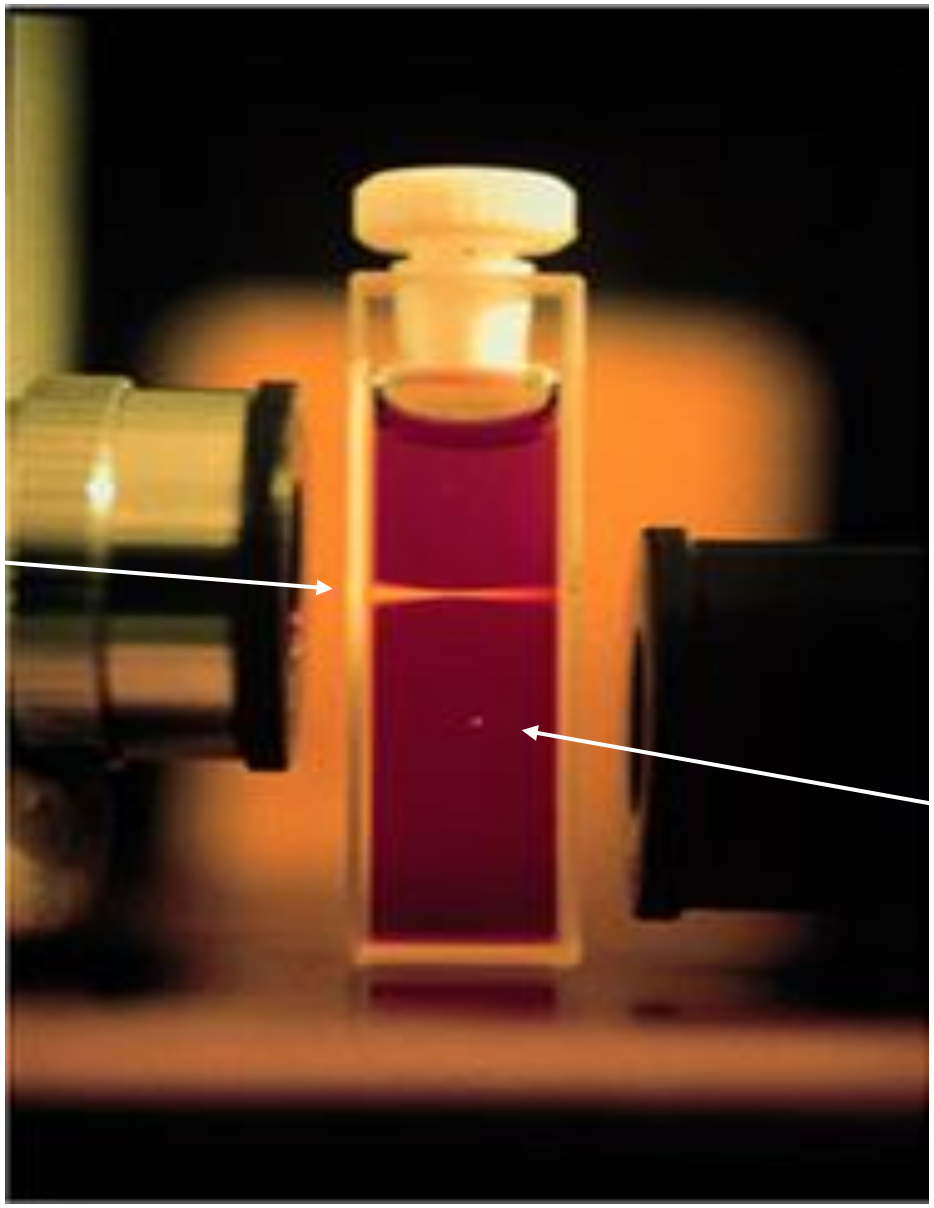


Approximately 1 um^3



Defined by the wavelength and numerical aperture of the objective

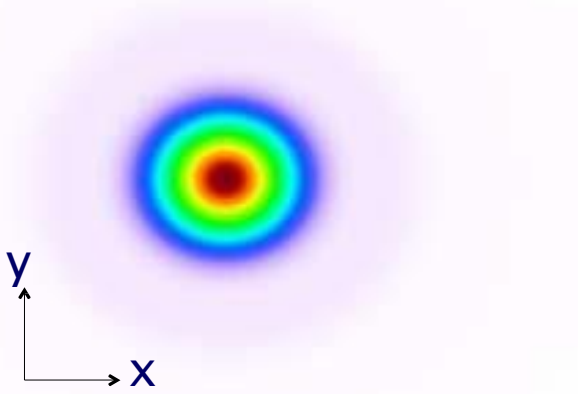
1-photon



2-photon

Brad Amos
MRC, Cambridge, UK

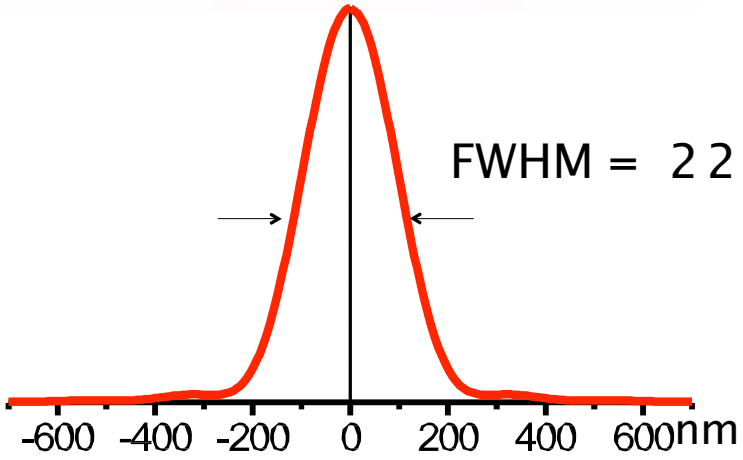
The lateral size of the PSF



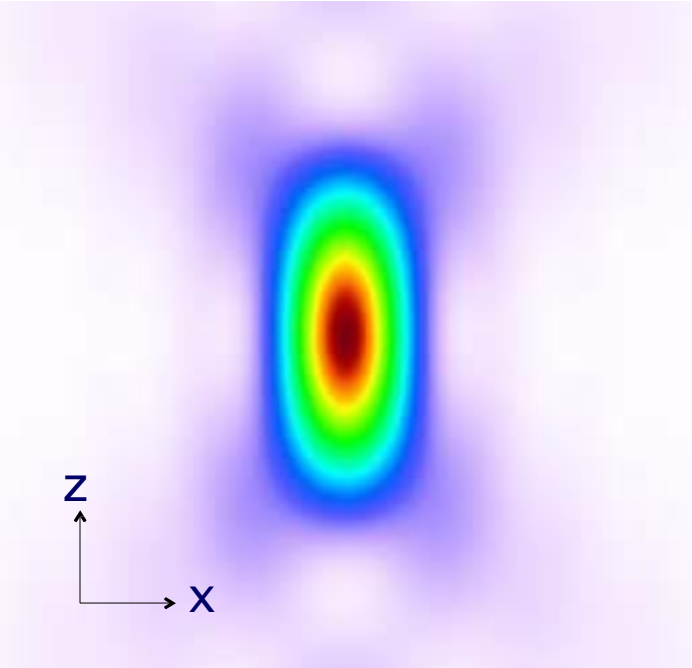
Lateral resolution ≈ $r = \frac{1.22\lambda}{2n\sin\theta} = \frac{0.61\lambda}{\mathrm{NA}}$

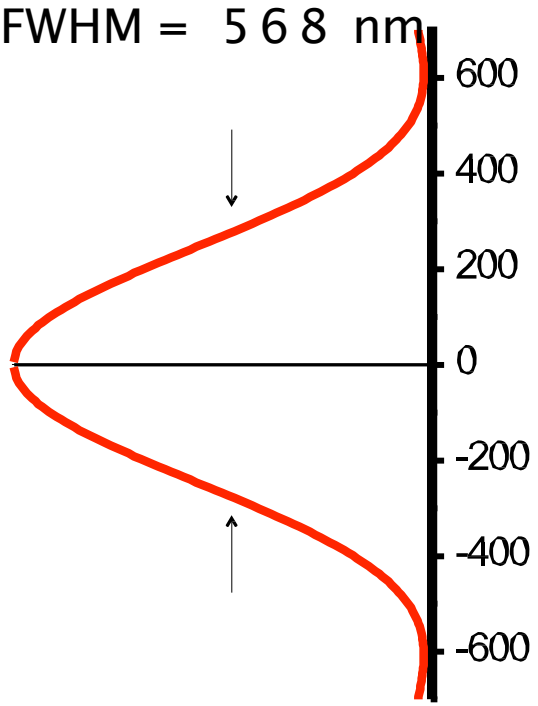
(N.A. = $n\sin\theta$)

Example: 1.4 NA objective at 550 nm
$\Delta_{xy} \approx 240$ nm



The axial size of the PSF



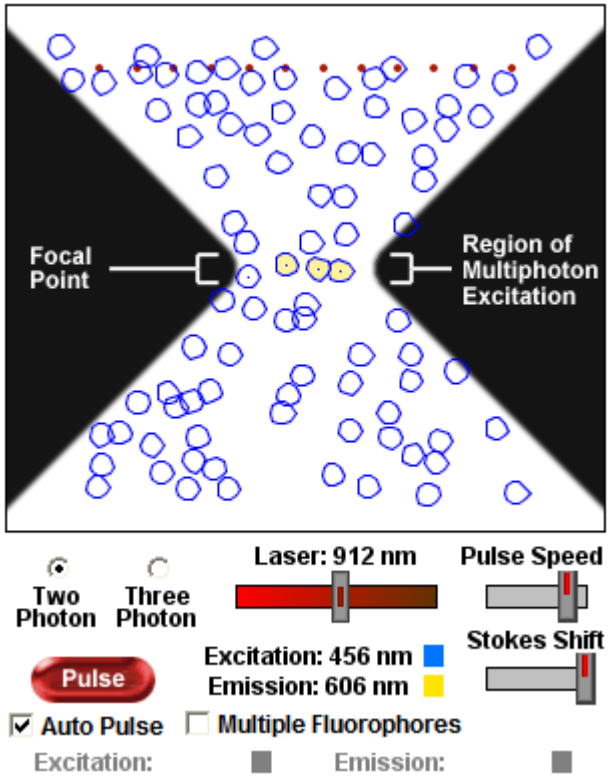


Axial resolution $\approx \frac{2n\lambda}{NA^2}$

(works only for low NA system)

Example: 1.4 NA objective at 550 nm

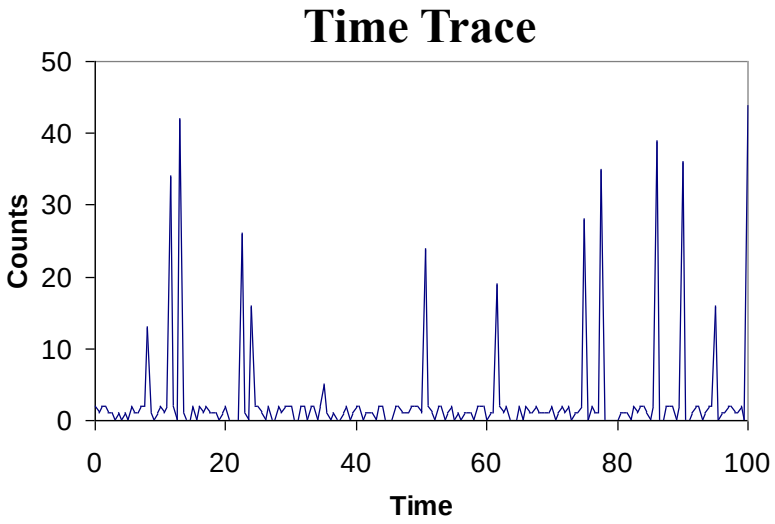
$\Delta_z \approx 850$ nm

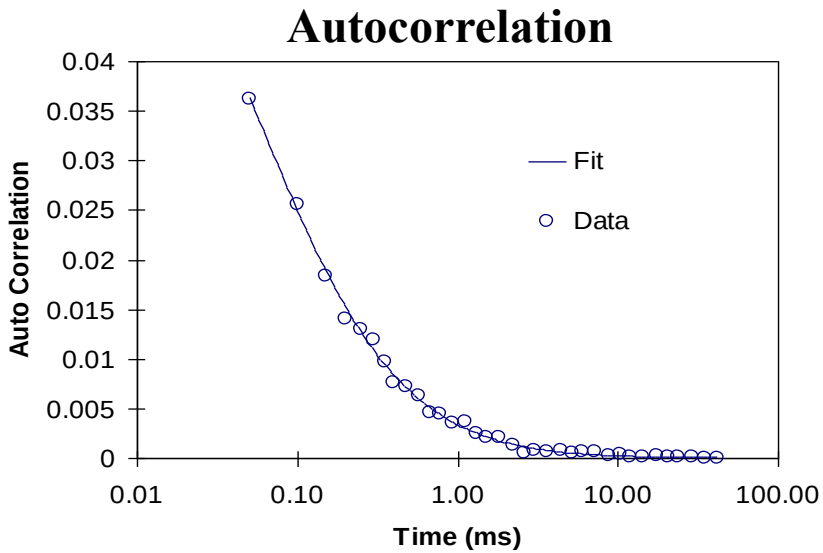


- Typically Used Ti:Sapphire (titanium-sapphire laser)
  - Mode-locked oscillator
  - Tunable 650-1100nm Wavelength (red to near infrared)
  - 10-1000 fs pulse duration (ultrashort pulses)

- Short Pulses means:
  - High photon Density but low average energy
  - High Bandwidth (many wavelengths in laser line)

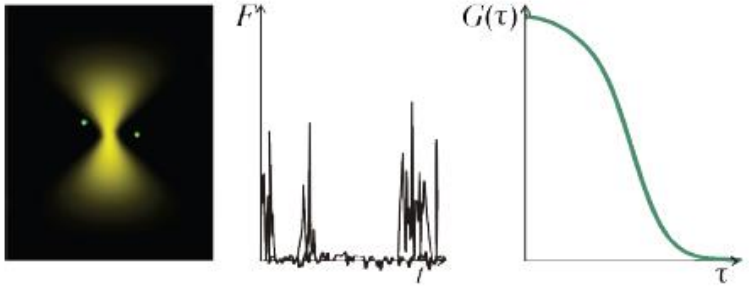
Data Treatment & Analysis



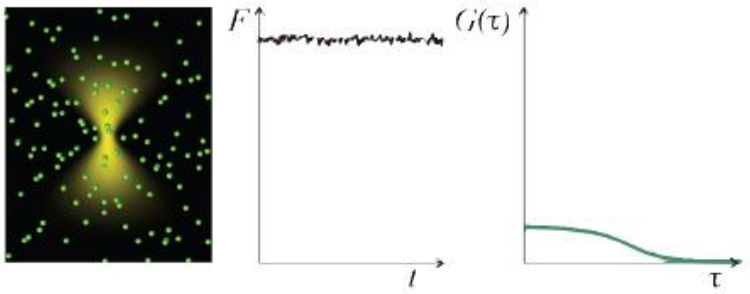


Autocorrelation Parameters:
G(0) & k_{action}

Low concentration



High concentration



How to extract the information about the fluctuations and their characteristic time?

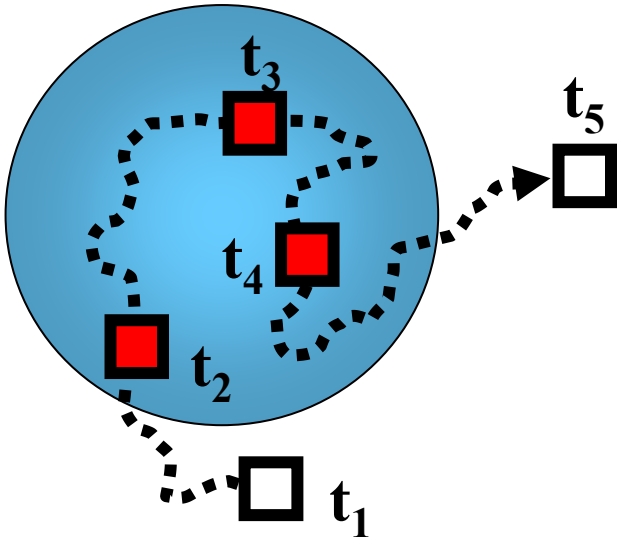
Distribution of the **amplitude** of the fluctuations

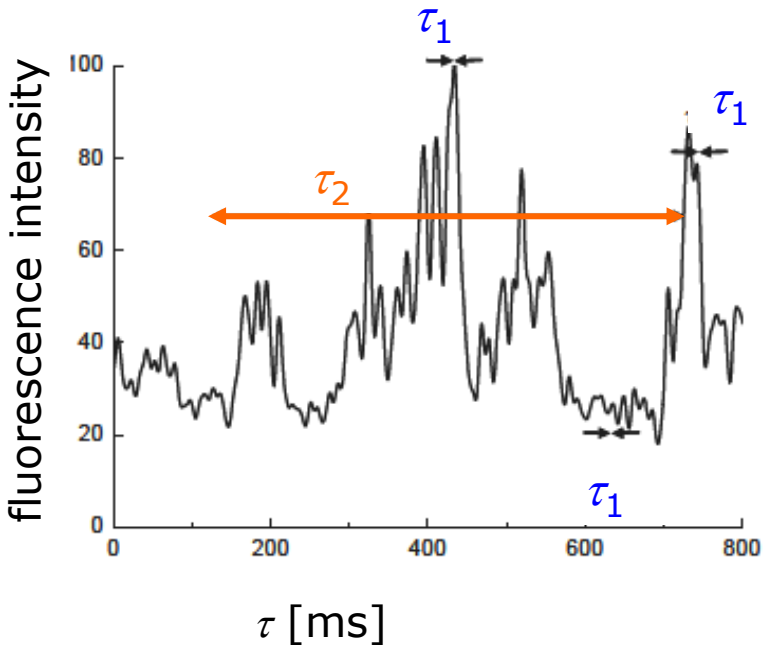
Distribution of the **duration** of the fluctuations

To extract the distribution of the duration of the fluctuations we use a math based on calculation of the **correlation function**

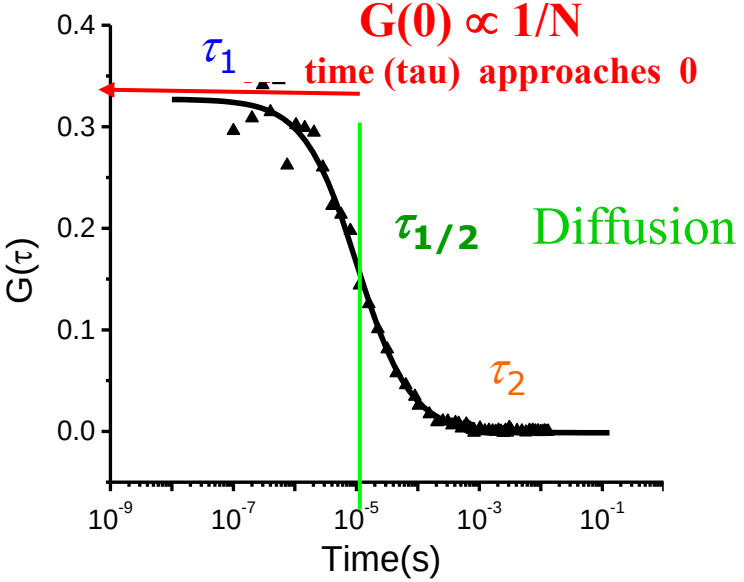
To extract the distribution of the amplitude of the fluctuations, we use a math based on the **PCH distribution**

The Autocorrelation Function





$$G(\tau) = \frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2} - 1$$

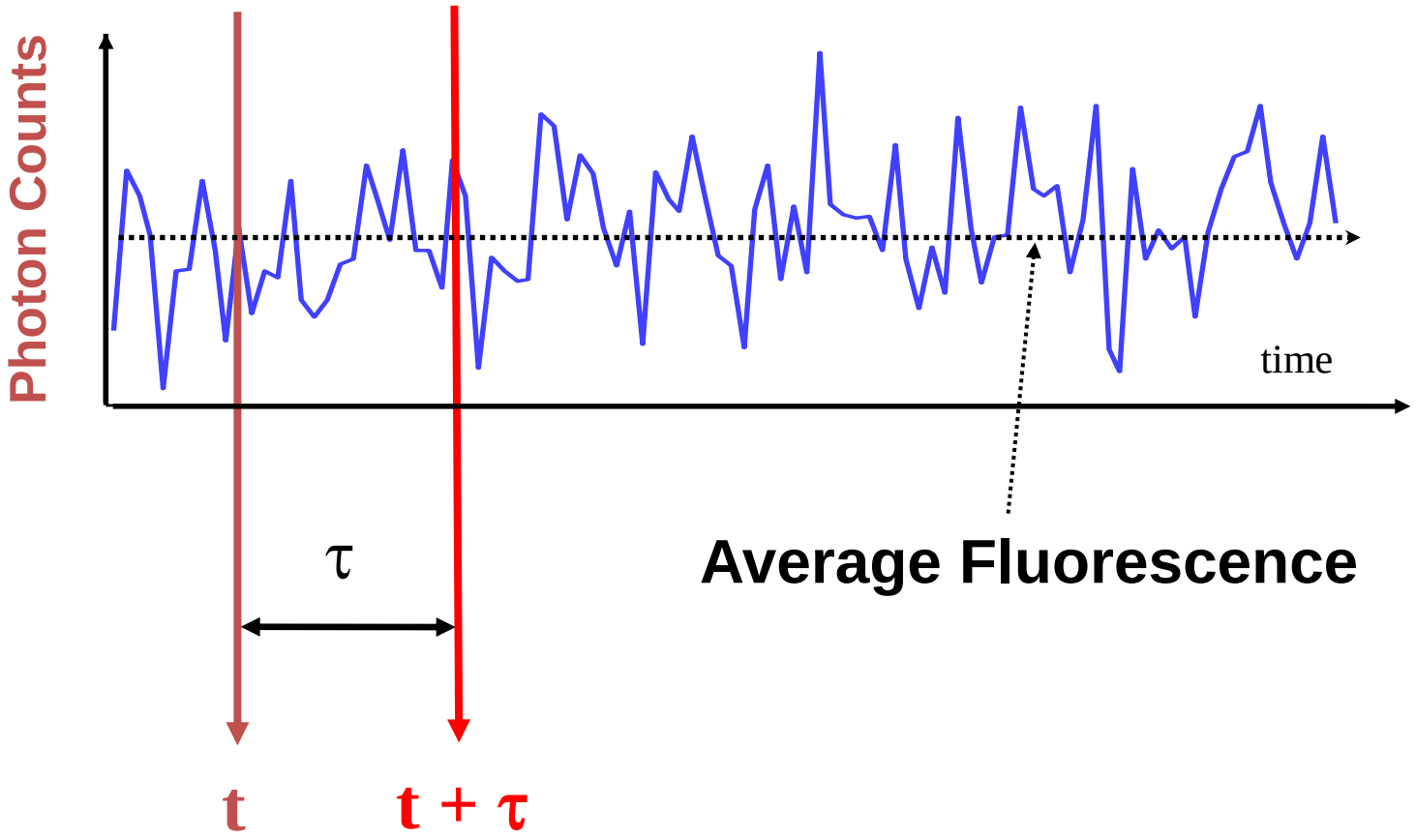


$\tau_{1/2}$ – characteristic timescale of the fluctuations

Definition of the Autocorrelation Function

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

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



Autocorrelation Function

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

The autocorrelation amplitude G(0) is therefore merely the normalized variance of the fluctuating fluorescence signal δF(t).

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

Factors influencing the fluorescence signal:

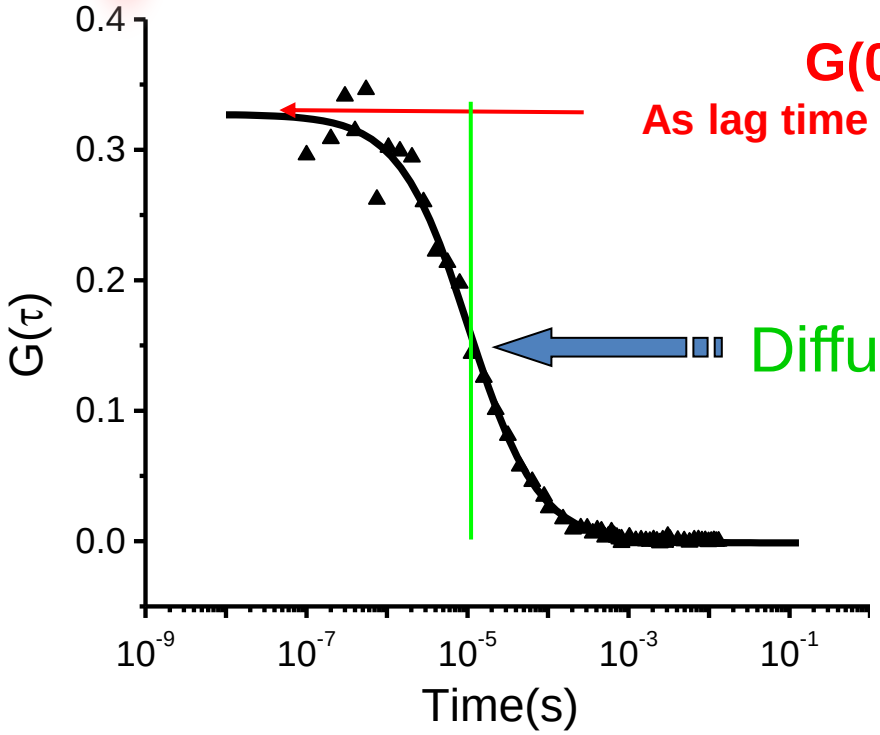
$$F(t) = \kappa Q \int d\mathbf{r}\, W(\mathbf{r}) C(\mathbf{r}, t)$$

κQ = quantum yield and detector sensitivity (how bright is our probe)

W(r) describes our observation volume

C(r,t) is a function of the fluorophore concentration over time

The Autocorrelation Function



G(0) ∝ 1/N

As lag time (tau) approaches zero

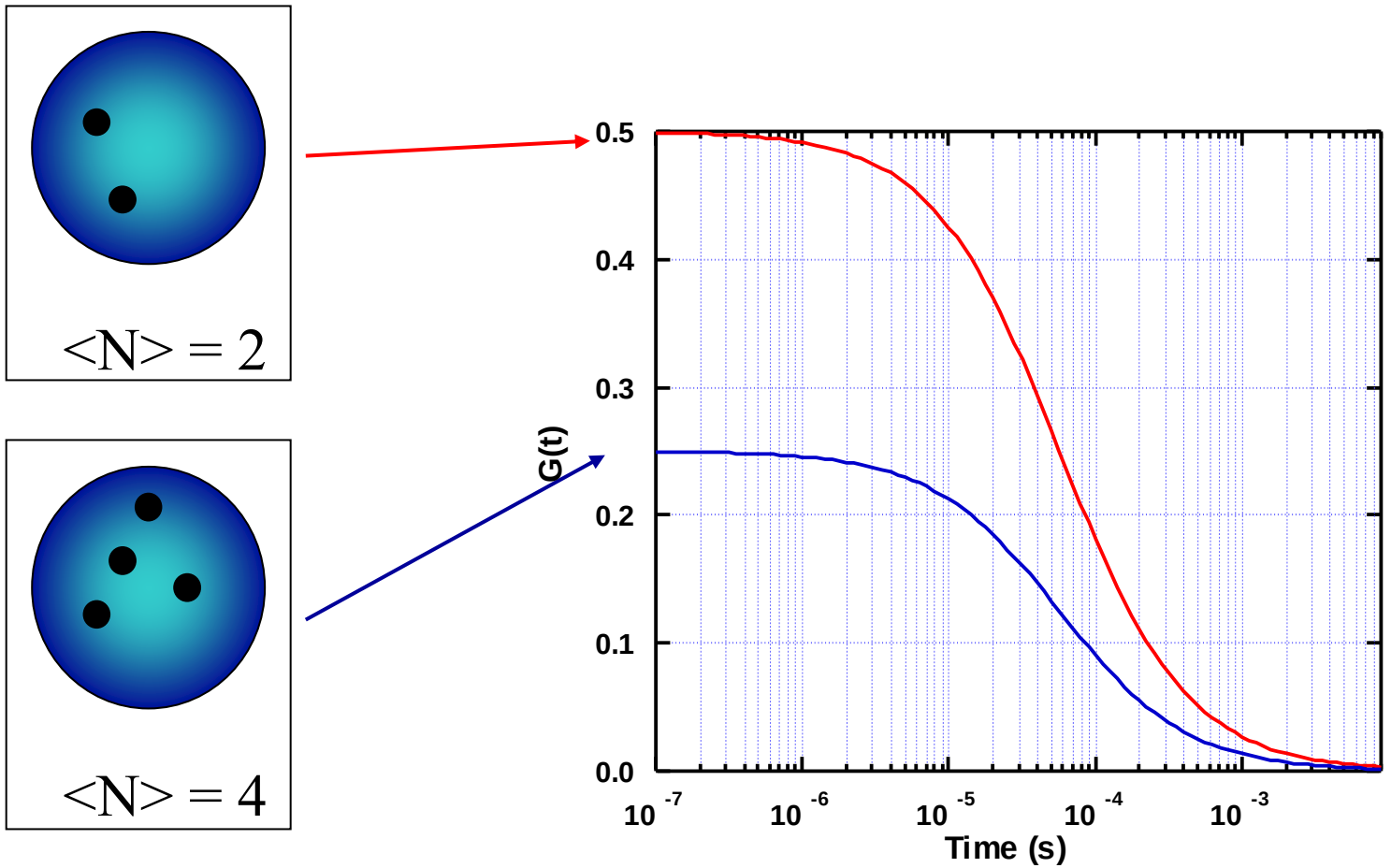
Diffusion

For a 3-dimensional Gaussian excitation volume:

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

In the simplest case, two parameters define the autocorrelation function: the amplitude of the fluctuation G(0) and the characteristic relaxation time of the fluctuation.

The Effects of Particle Concentration on the Autocorrelation Curve



<ΔF(t)^2> / <F(t)>^2

can be simplified as follows:

First, let's define some terms for clarity:

- ΔF(t) represents the fluctuation or variation of F(t) at a particular time t from its average value.
- <F(t)> represents the average or mean value of F(t) over time.

Now, let's simplify the original expression step by step:

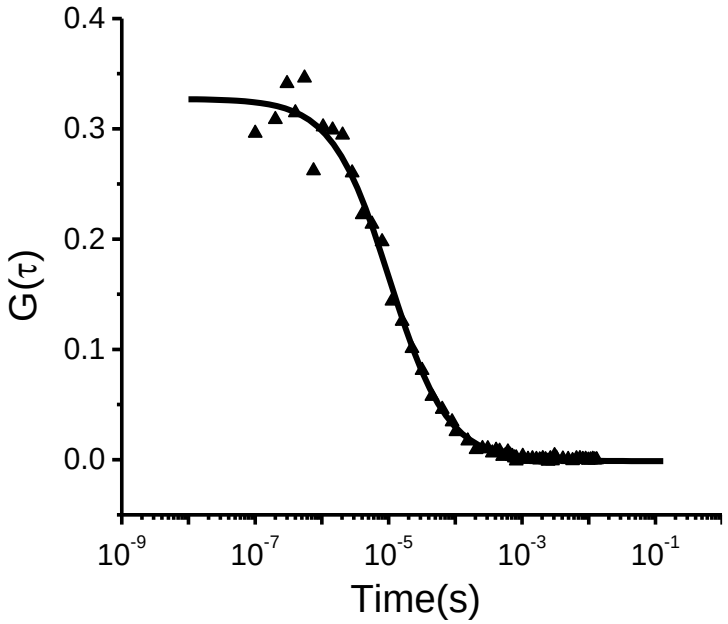
1. Start with the expression:
   <ΔF(t)^2> / <F(t)>^2
2. Expand ΔF(t)^2 as (F(t) - <F(t)>)^2:
   <(F(t) - <F(t)>)^2> / <F(t)>^2
3. Apply the properties of variance and mean:
   The variance of a random variable X, Var(X), is defined as:
   Var(X) = <(X - <X>)^2>
   So, <(F(t) - <F(t)>)^2> is simply the variance of F(t), or Var(F(t)).
   So, the expression becomes:
   Var(F(t)) / <F(t)>^2
4. Now, you can rewrite Var(F(t)) as:
   Var(F(t)) = <F(t)^2> - <F(t)>^2
   This is the definition of variance.
5. Substitute this back into the expression:
   (<F(t)^2> - <F(t)>^2) / <F(t)>^2
6. Distribute the denominator (<F(t)>^2) across both terms in the numerator:
   <F(t)^2> / <F(t)>^2 - <F(t)>^2 / <F(t)>^2
7. Finally, simplify by canceling the common factor <F(t)>^2:
   1 - 1
   This simplifies to 0.

So, <ΔF(t)^2> / <F(t)>^2 is indeed equal to 0, as demonstrated in the simplification steps. This indicates that there is no relative variation or fluctuation (ΔF(t)) in the dataset when compared to its mean (<F(t)>).

Why Is G(0) Proportional to 1/Particle Number?

A Poisson distribution describes the statistics of particle occupancy fluctuations. In a Poissonian system the variance is proportional to the average number of fluctuating species:

$$\langle Particle\_Number \rangle = Variance$$

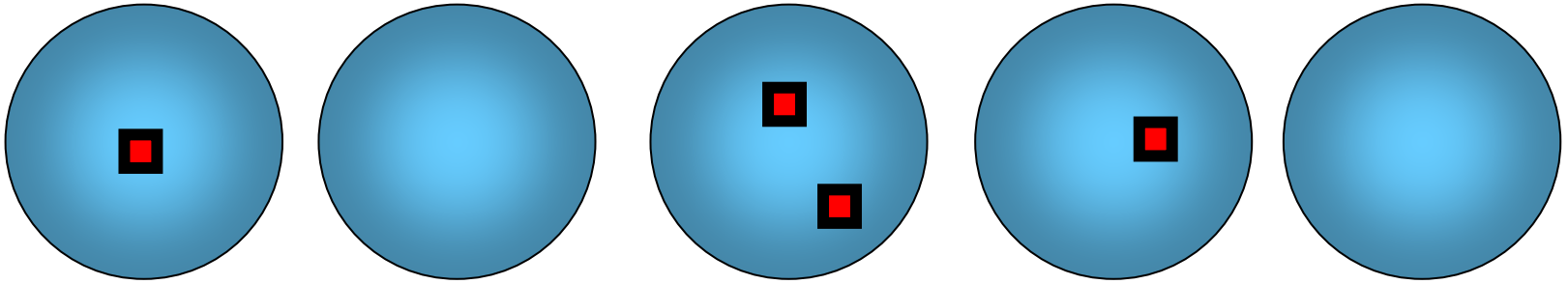


$$G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

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

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

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**

Effect of Shape on the Autocorrelation Functions:

For a 3-dimensional Gaussian excitation volume:

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

Additional Equations:

3D Gaussian Confocor analysis:

$$G(\tau) = 1 + \frac{1}{N}\left(1 + \frac{\tau}{\tau_D}\right)^{-1} \cdot \left(1 + S^2 \cdot \frac{\tau}{\tau_D}\right)^{-\frac{1}{2}}$$

... where N is the average particle number, τ_D is the diffusion time (related to D, $\tau_D = w^2/8D$, for two photon and $\tau_D = w^2/4D$ for 1-photon excitation), and S is a shape parameter, equivalent to w/z in the previous equations.

were N stand for the number of molecules and s describes the ratio between the elongation of the spot in xy and z direction.

$$N, s = \frac{\omega_{xy}}{\omega_z}$$

tDdescribes the time the molecule needs to diffuse through the light spot.

$$\tau_D \approx \frac{\omega_{xy}}{4D}$$

The diffusion coefficient of the molecule is related to its hydrodynamical radius and thus its mass.

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

The Effects of Particle Size on the Autocorrelation Curve

Diffusion Constants

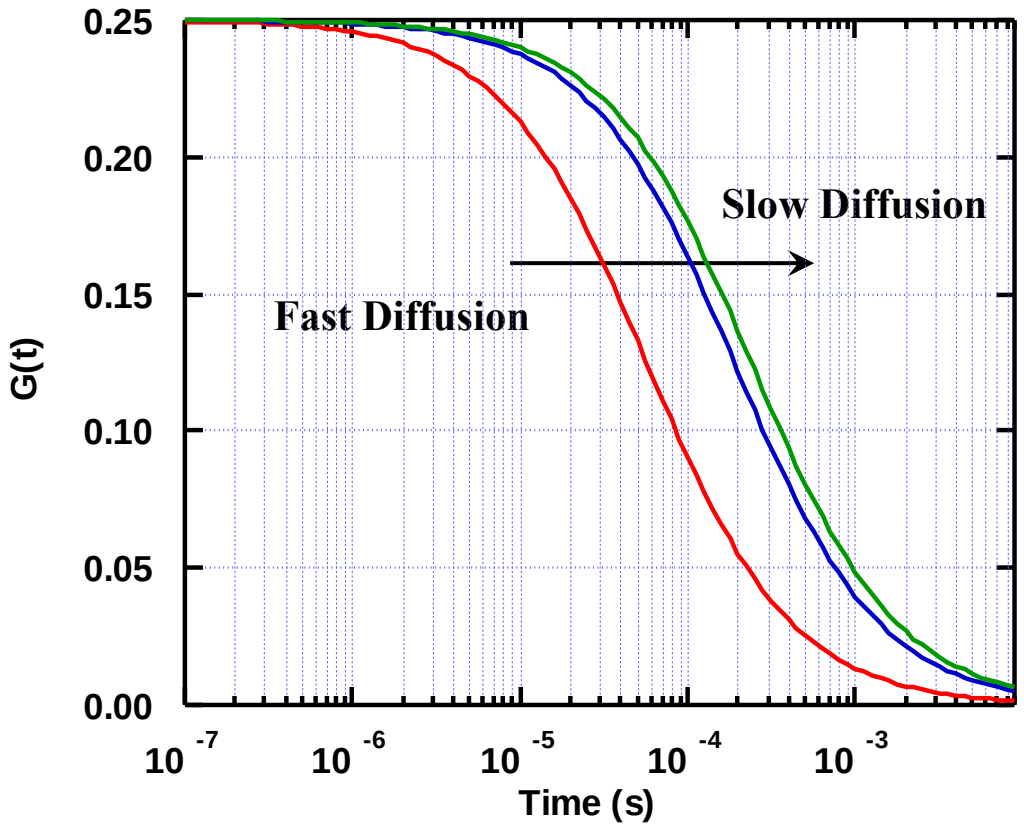
300 um^2/s
90 um^2/s
71 um^2/s

Stokes-Einstein Equation:

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

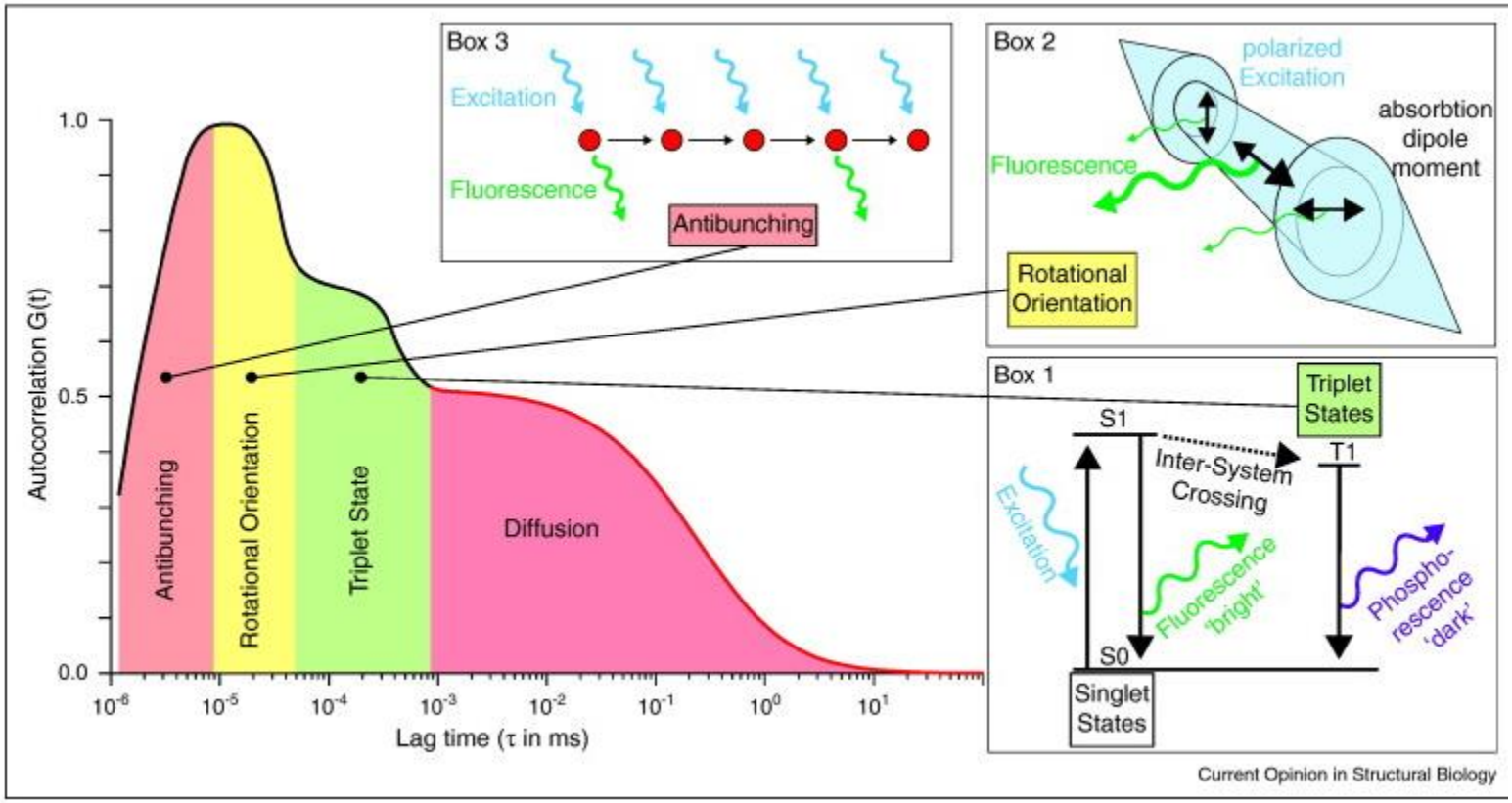
and

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



Monomer --> Dimer

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

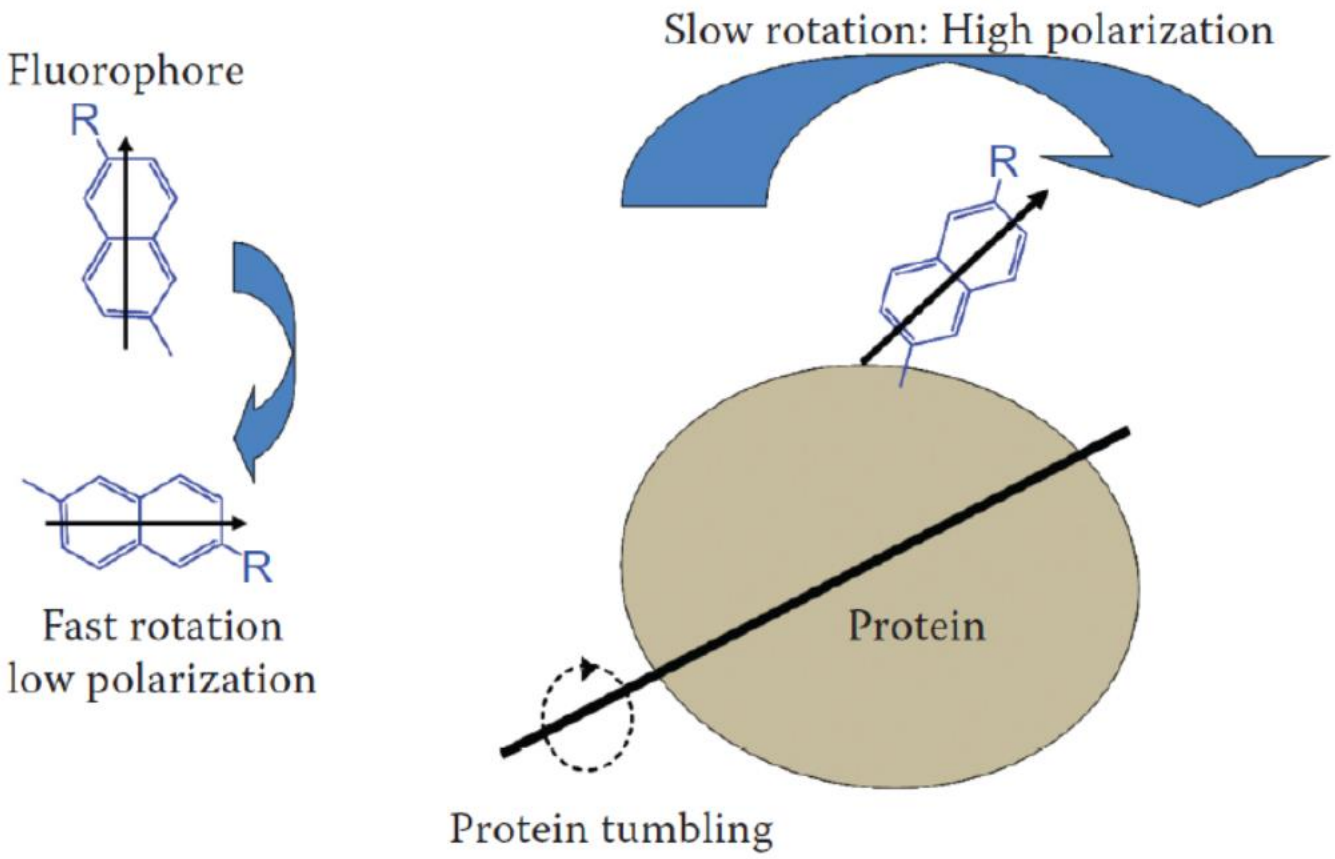


Antibunching: The fastest process that can be recorded is the probability for fluorescence emission itself. This event, termed 'antibunching' simply describes the finite probability for a fluorophore to emit a photon at a given time *t* after the previous one was emitted at time *t* = 0.

Rotational motion: When a fluorophore absorbs excitation light, it does so preferentially when that light is polarized parallel to its absorption dipole moment, that is, those whose dipoles are oriented correctly will be 'photoselected' for excitation (Box 2). Consequently, the emission photon will be polarized in the direction of the emission dipole of the fluorophore. If the excitation light is linearly polarized, or the detection is polarization dependent, changes in the rotational orientation of the fluorophore can lead to observable fluctuations in the fluorescence signal.

Triplet: Inter-system crossing of the fluorophore to a triplet state, also known as blinking (Box 1). During these intervals the fluorophore cannot emit any photons and is considered 'optically dark'.

The degree of the polarization increases with increasing molecular size, with increasing viscosity of the medium and with decreasing temperature, that is with the reduction of the mobility of the single particles

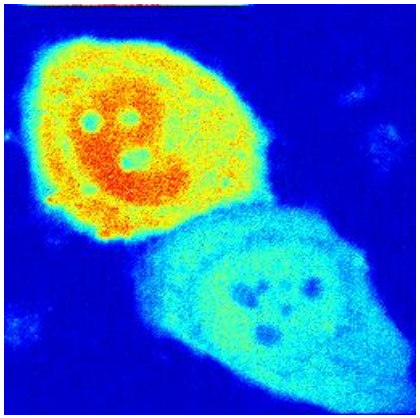


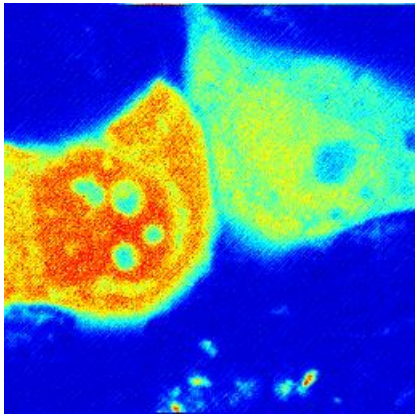
Typical Diffusion times of molecules

Orders of magnitude for 10uM solution, small molecule, water

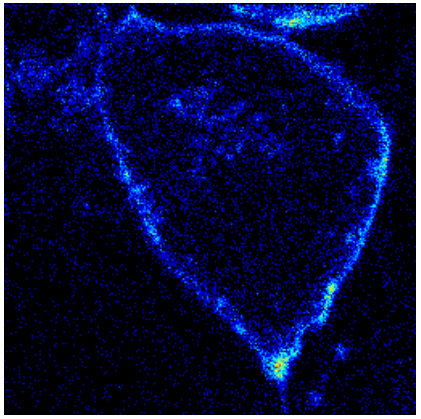
Volume	Device	Size (μm)	Molecules	Diffusion Time (s)
Milliliter	Cuvette	10000	$6x10^{12}$	10^{4}
Microliter	Plate well	1000	$6x10^{9}$	10^{2}
Nanoliter	microfabrication	100	$6x10^{6}$	1
Picoliter	Typical cell	10	$6x10^{3}$	10^{-2}
Femtoliter	Confocal volume	1	$6x10^{0}$	10^{-4}
Attoliter	nanofabriacation	0.1	$6x10^{-3}$	10^{-6}

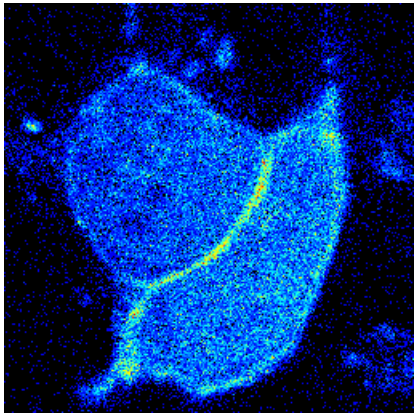
Autocorrelation Adenylate Kinase -EGFP Chimeric Protein in HeLa Cells





Examples of different *Hela* cells transfected with AK1-EGFP



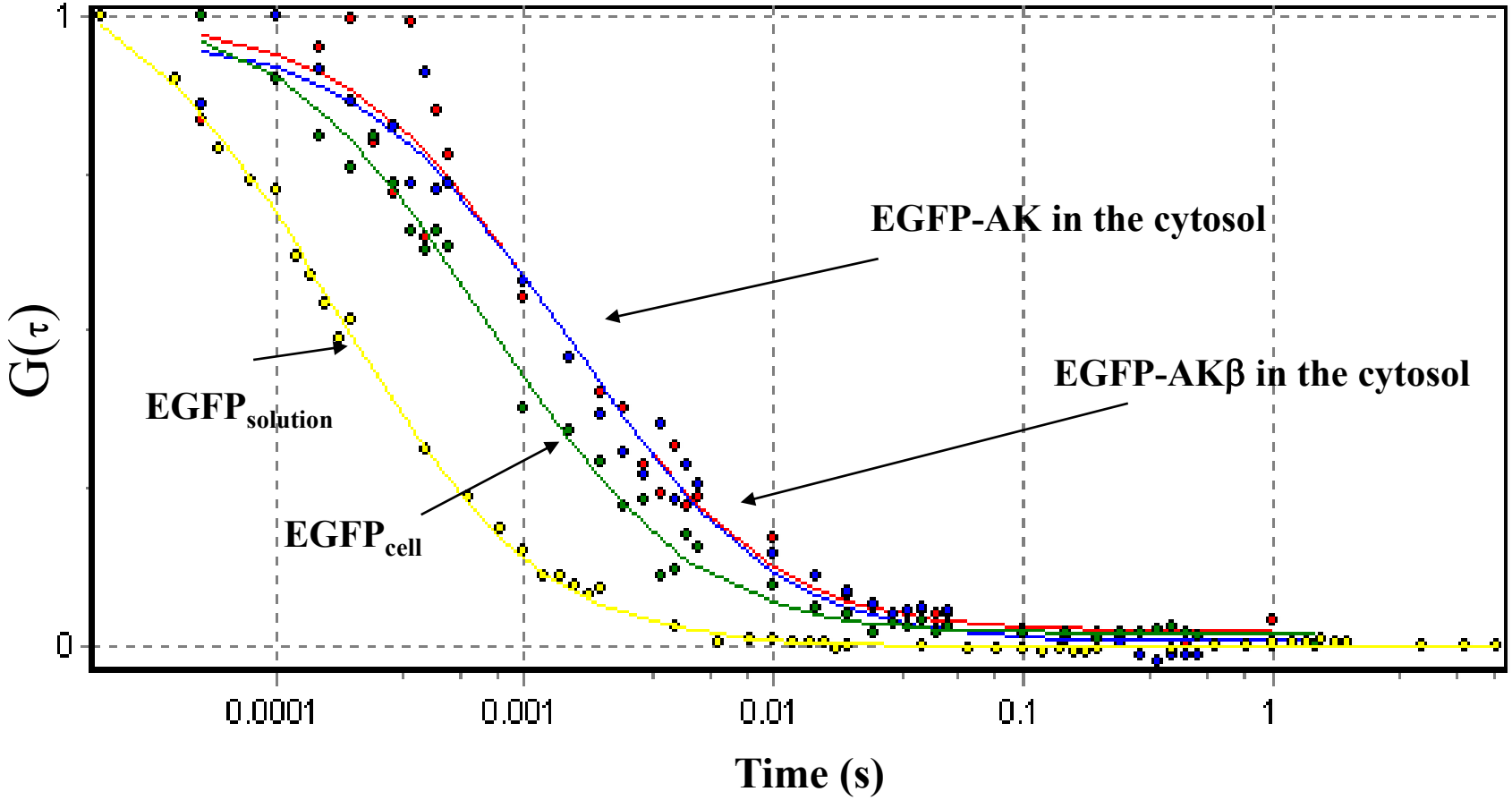


Examples of different *Hela* cells transfected with AK1β -EGFP

Fluorescence Intensity

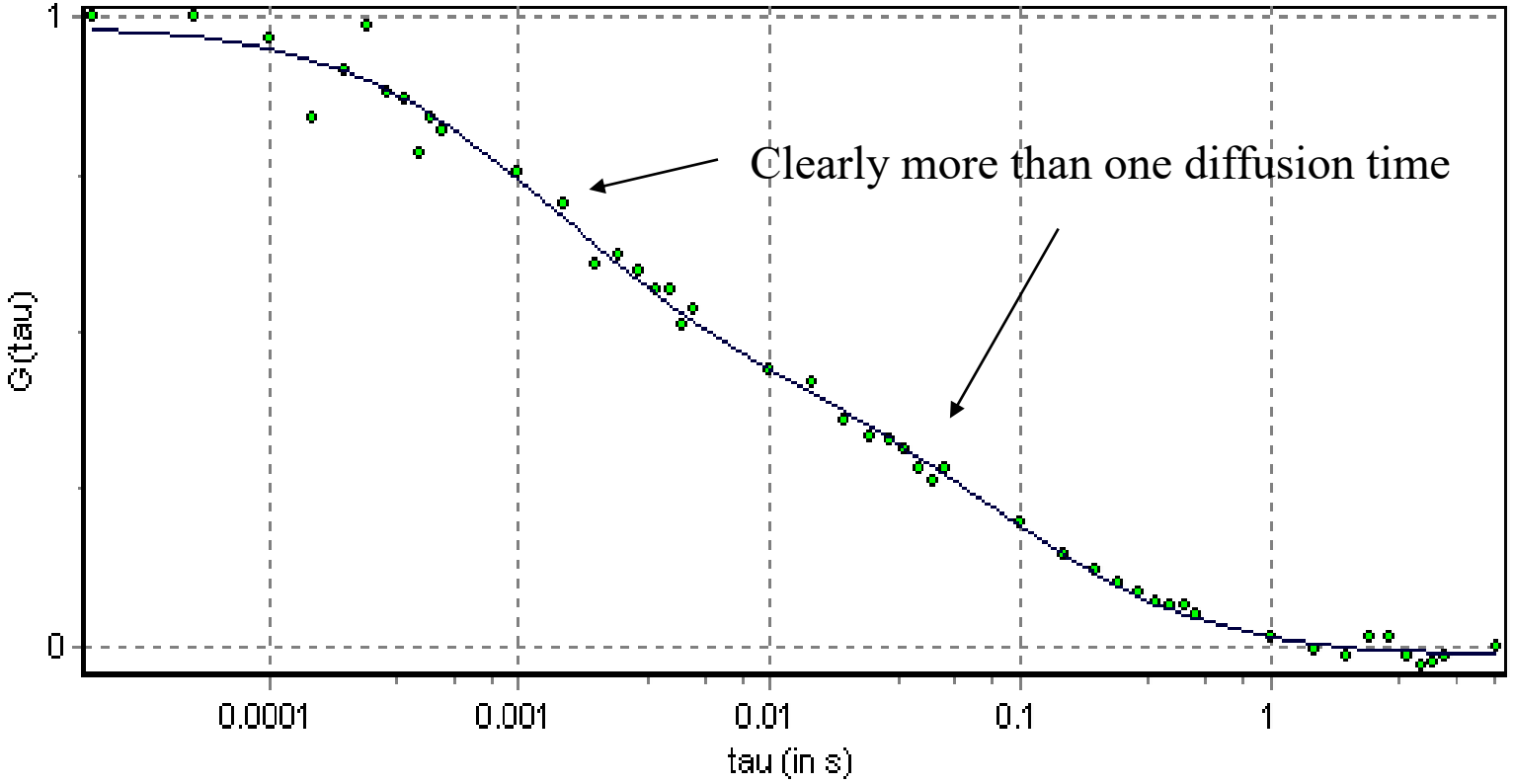
Qiao Qiao Ruan, Y. Chen, M. Glaser & W. Mantulin Dept. Biochem & Dept Physics- LFD Univ Il, USA

Autocorrelation of EGFP & Adenylate Kinase -EGFP



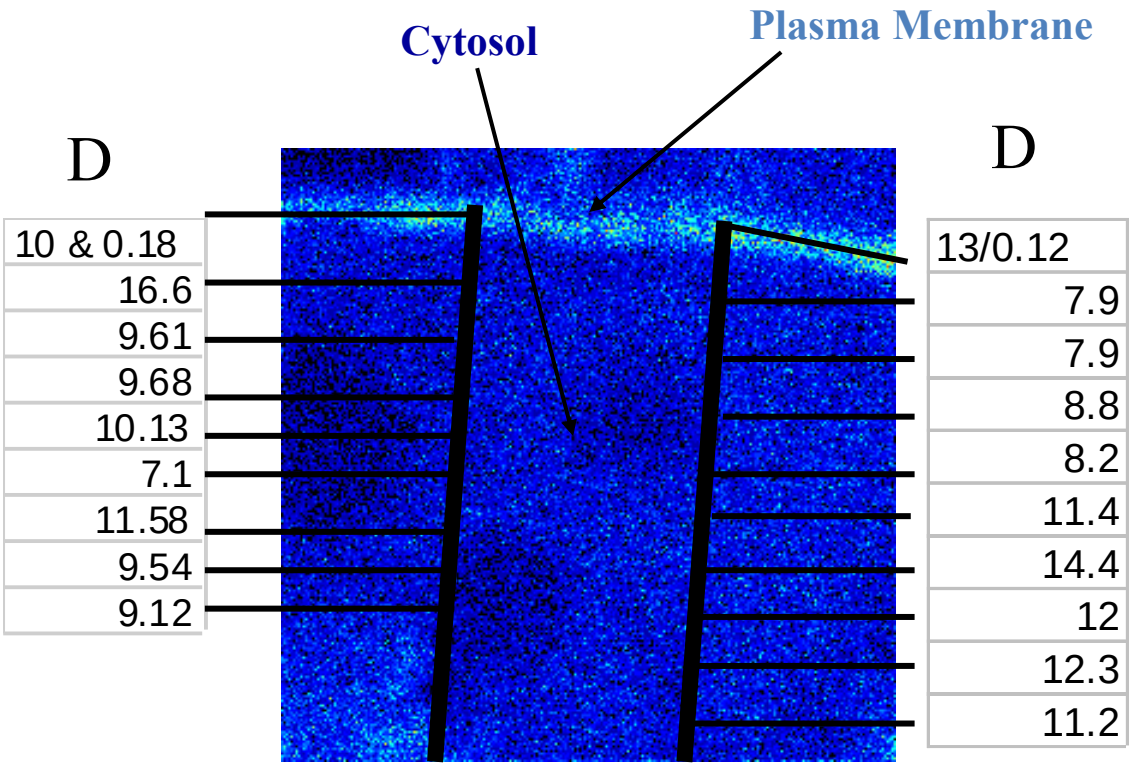
Normalized autocorrelation curve of EGFP in solution (•), EGFP in the cell (•), AK1-EGFP in the cell(•), AK1β-EGFP in the cytoplasm of the cell(•).

Autocorrelation of Adenylate Kinase –EGFP on the Membrane



A mixture of AK1b-EGFP in the cytoplasm and membrane of the cell.

Autocorrelation Adenylate Kinaseβ -EGFP



Diffusion constants (um^2/s) of AK EGFP-AKβ in the cytosol -EGFP in the cell (HeLa). At the membrane, a dual diffusion rate is calculated from FCS data. Away from the plasma membrane, single diffusion costants are found.

Fluorescence Correlation Spectroscopy

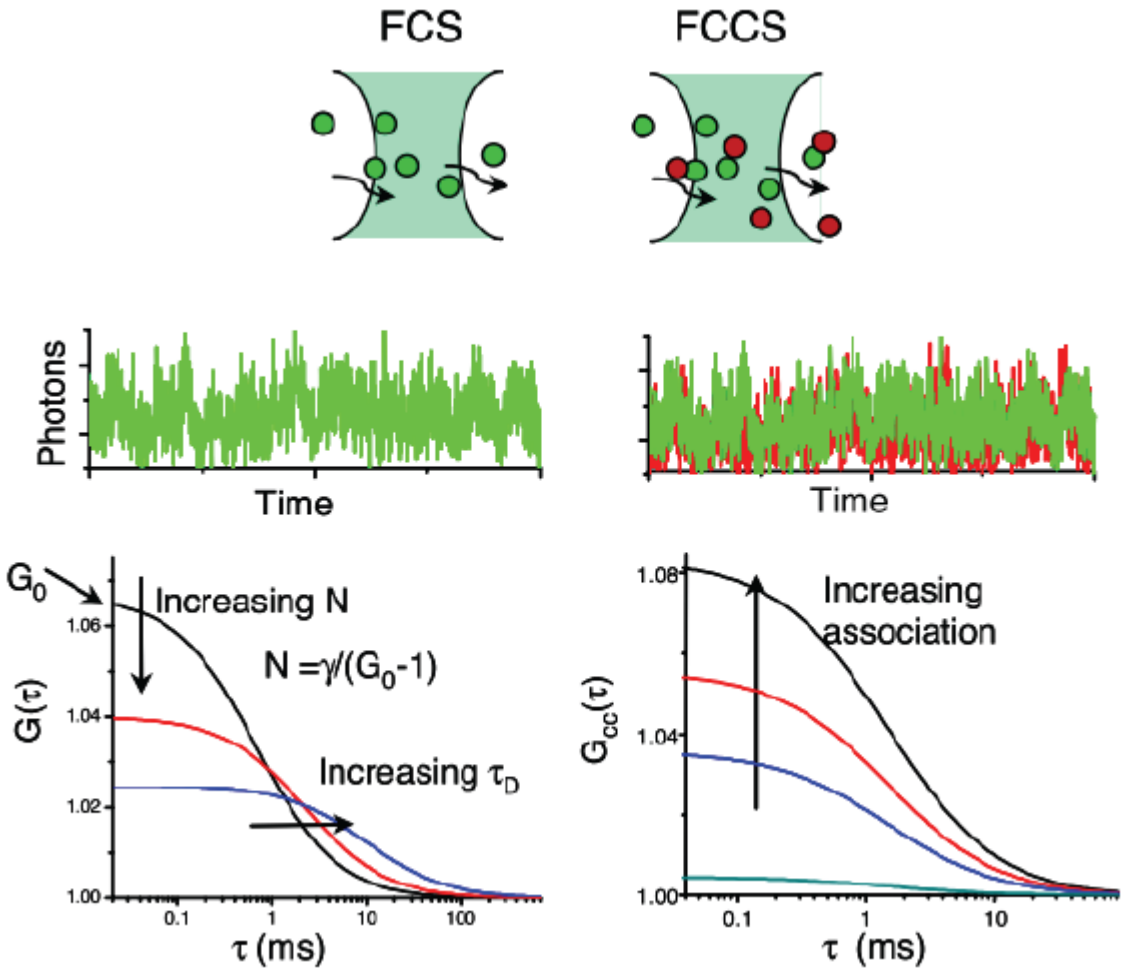
Benefits:

- High temporal resolution, even rotational diffusion can be measured (nanosecond timescale).
- Smallest possible observation volume (diffraction limited or smaller with STED-FCS).

Challenges:

- No image information during measurement.
- Immobile fraction results in bleaching curve.
- Correlation curves can be difficult to interpret with regards to type of motion.

Cross-Correlation FCS for protein-protein interaction

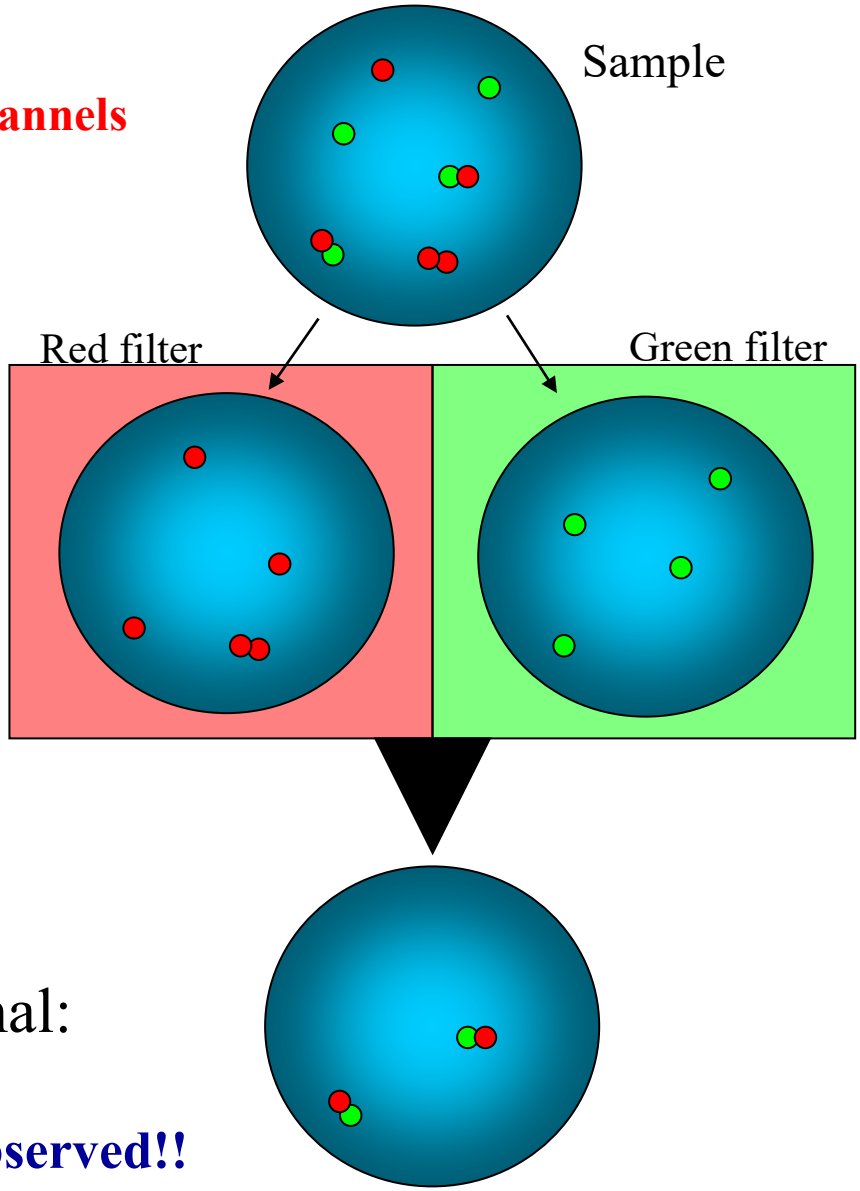


- Interacting molecules must be labeled with spectrally shifted fluorophores
- Fluorescence fluctuations between two detectors are only correlated if the two molecules are interacting

Two-Color Cross-correlation

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

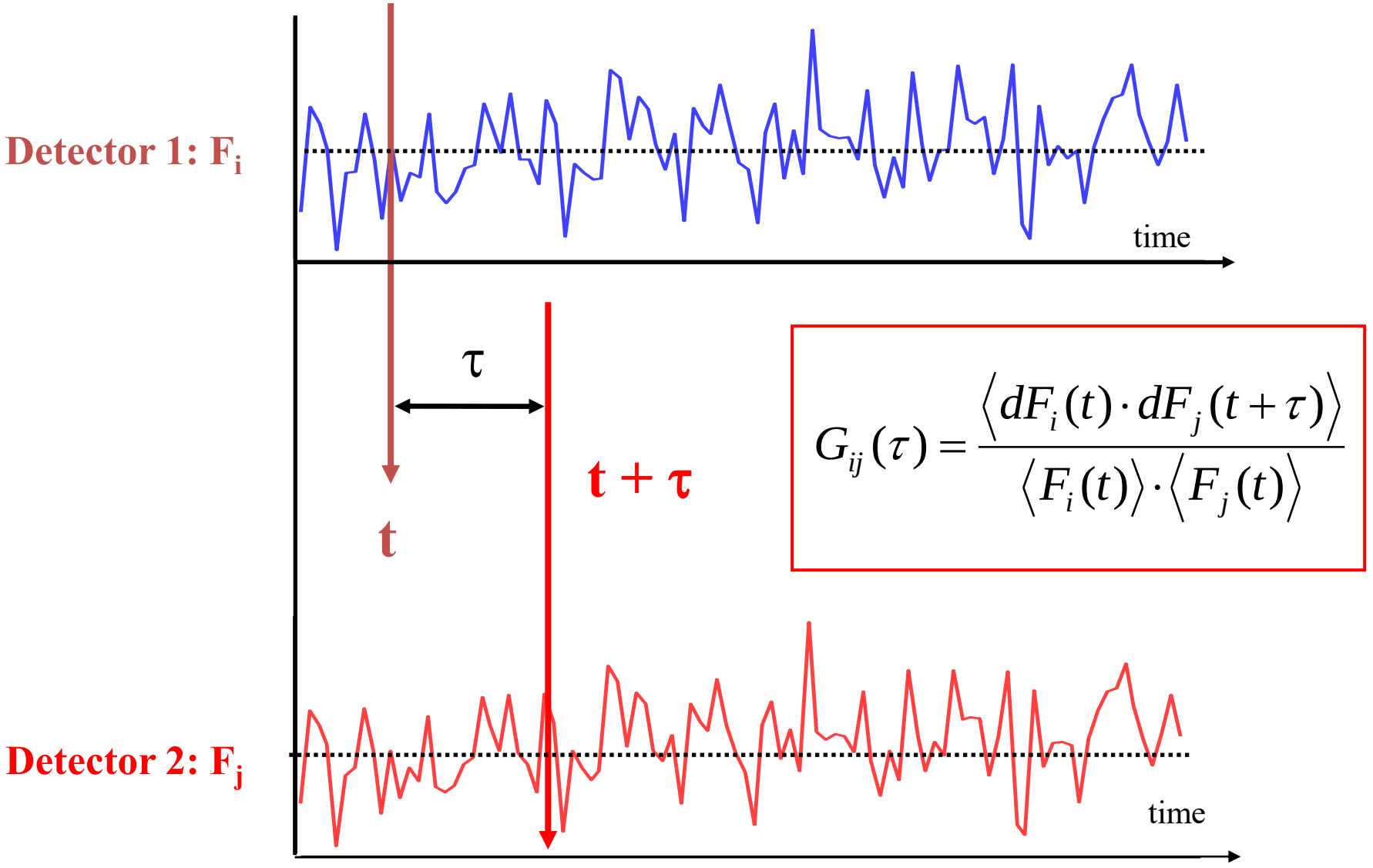
Each detector observes
particles with a particular color



The cross-correlation signal:

Only the green-red molecules are observed!!

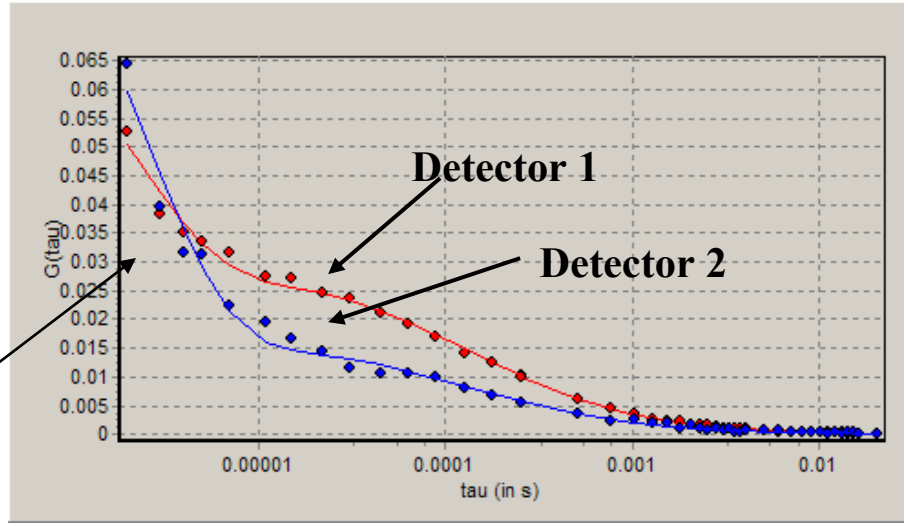
Calculating the Cross-correlation Function



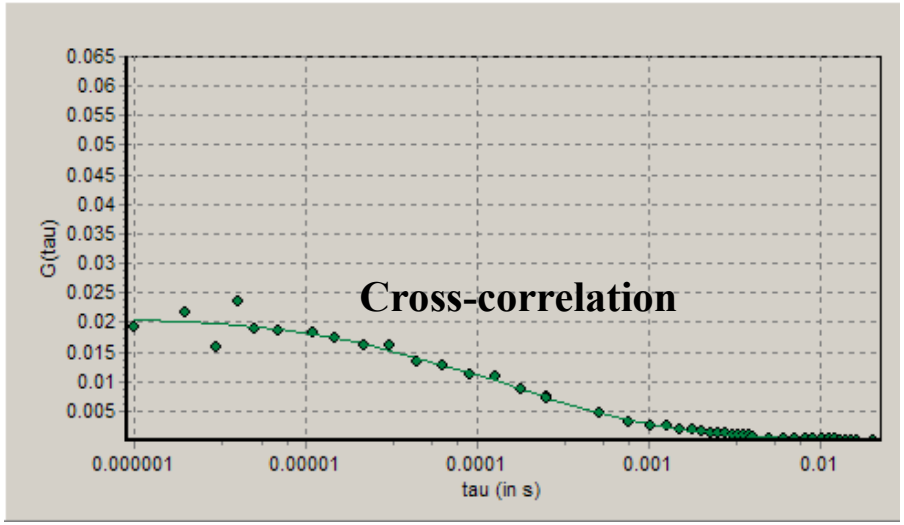
$$G_{ij}(\tau) = \frac{\langle dF_i(t) \cdot dF_j(t+\tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle}$$

Removal of Detector Noise by Cross-correlation

11.5 nM Fluorescein



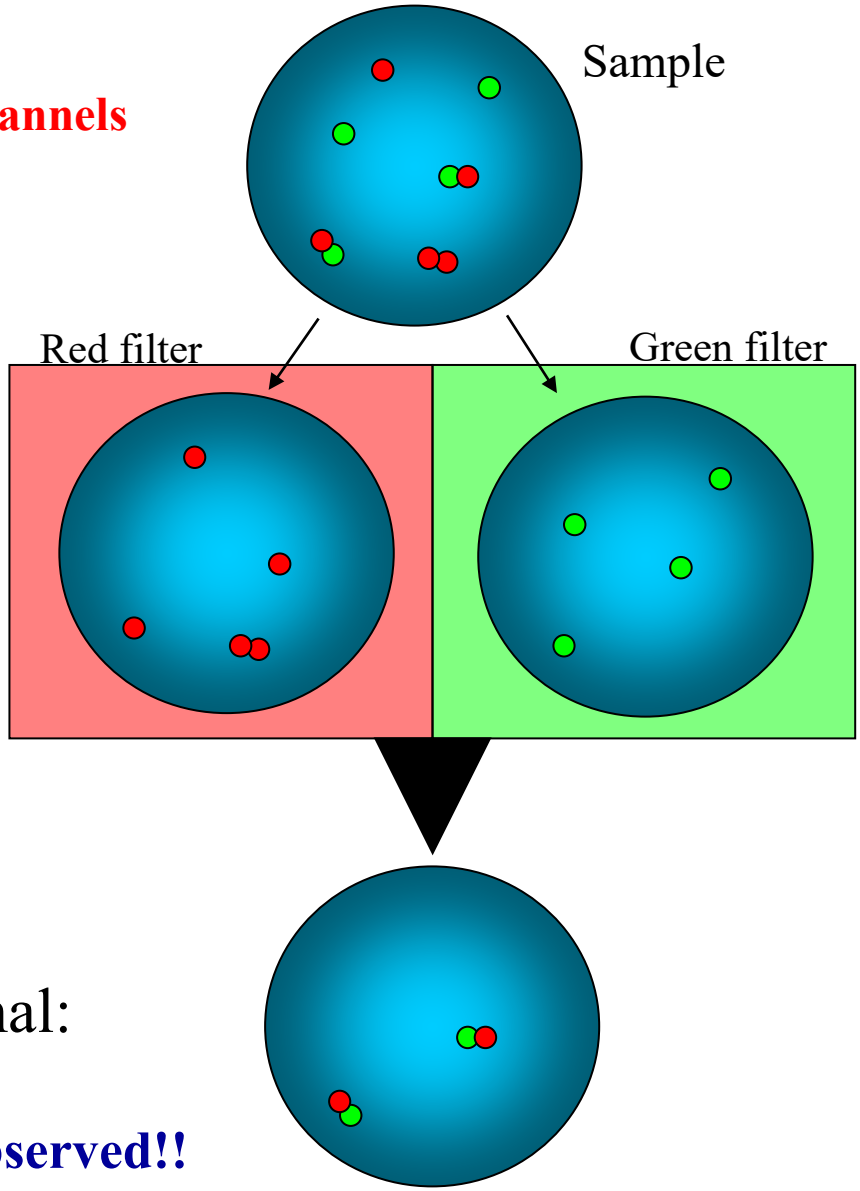
Detector after-pulsing



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!!

Two-color Cross-correlation

Equations are similar to those for the cross correlation using a simple beam splitter:

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

Information Content	Signal
Correlated signal from particles having **both colors**.	$G_{12}(\tau)$
Autocorrelation from channel 1 on the **green particles**.	$G_1(\tau)$
Autocorrelation from channel 2 on the **red particles**.	$G_2(\tau)$

Experimental Concerns: Excitation Focusing & Emission Collection

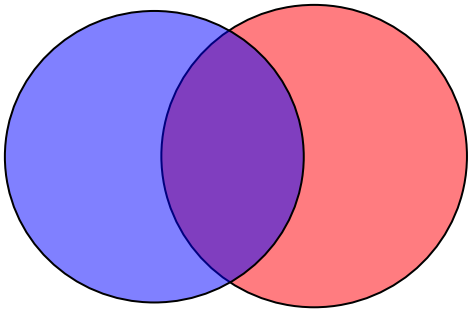
We assume exact match of the observation volumes in our calculations which is difficult to obtain experimentally.

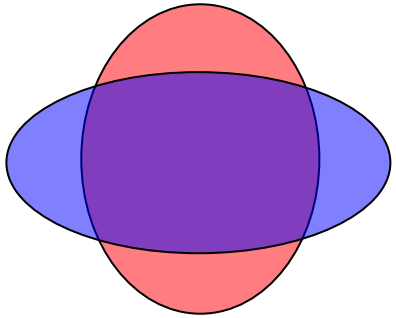
Excitation side:

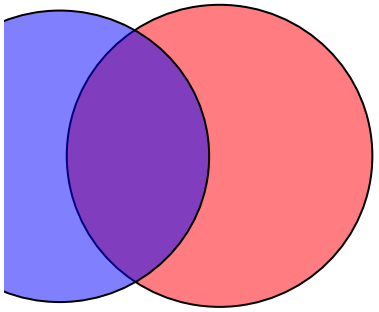
(1) Laser alignment
(2) Chromatic aberration
(3) Spherical aberration

Emission side:

(1) Chromatic aberrations
(2) Spherical aberrations
(3) Improper alignment of detectors or pinhole
(cropping of the beam and focal point position)







Application to FCS and Cross-Correlation

Experiment

Video:

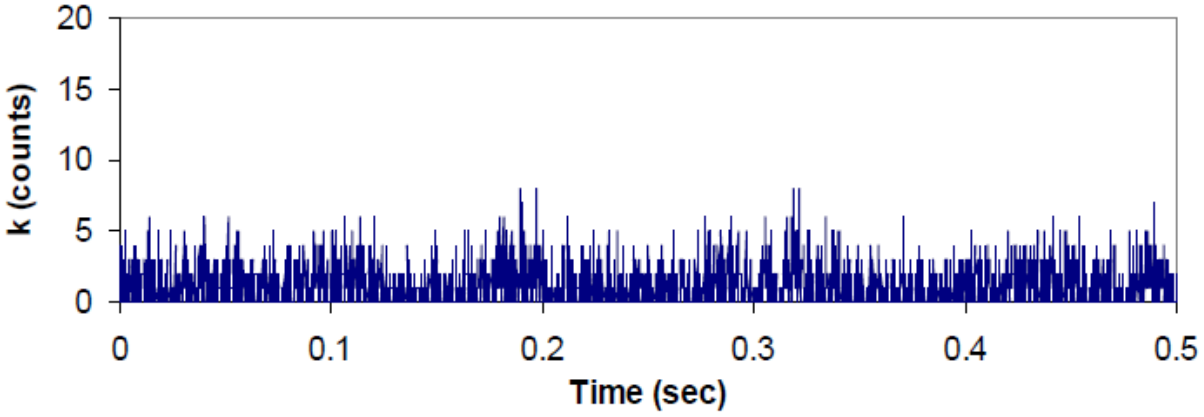
http://www.jove.com/video/3513/determination-lipid-raft-partitioning-fluorescently-tagged-probes

Transition from FCS

- The Autocorrelation function only depends on fluctuation duration and fluctuation density (i.e. the number of molecules and the time it takes for the molecule to move which is independent of excitation power)

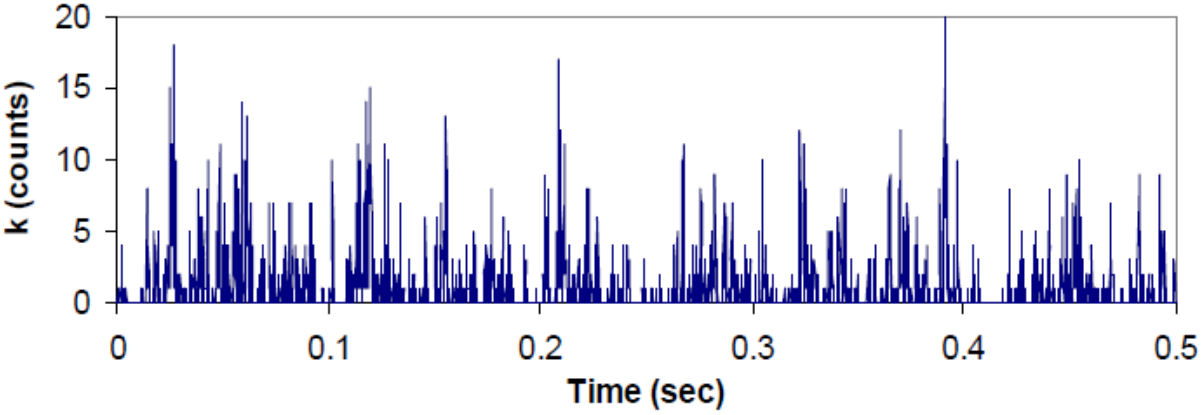
- PCH: distribution of intensity (independent of time)

Fluorescence Trajectories



Fluorescent Monomer:

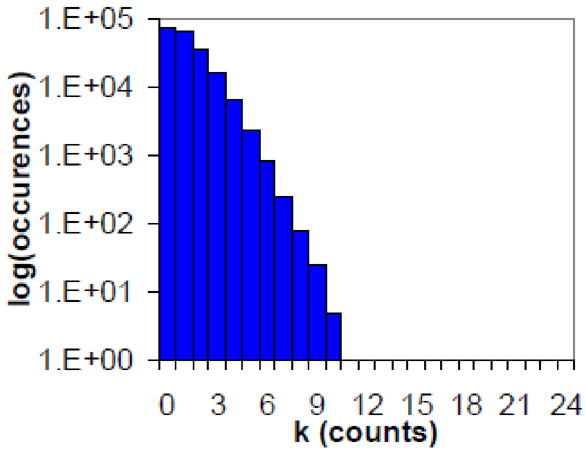
Intensity = 115,000 cps

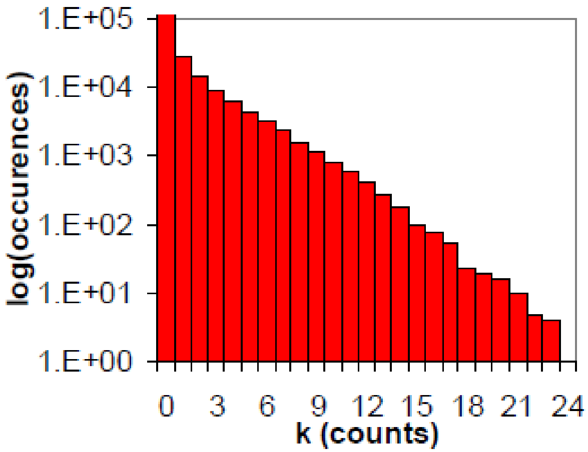


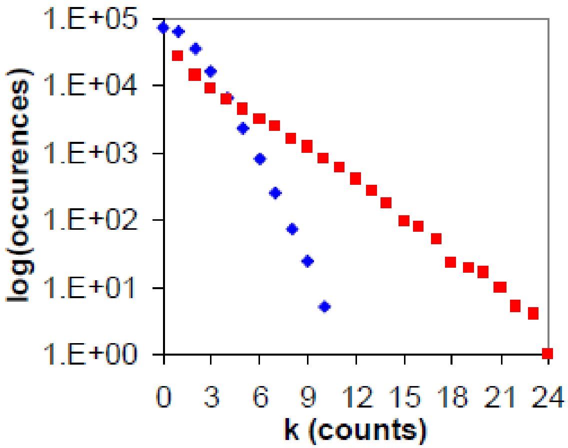
Aggregate:

Intensity = 111,000 cps

Plotting the raw data into a histogram





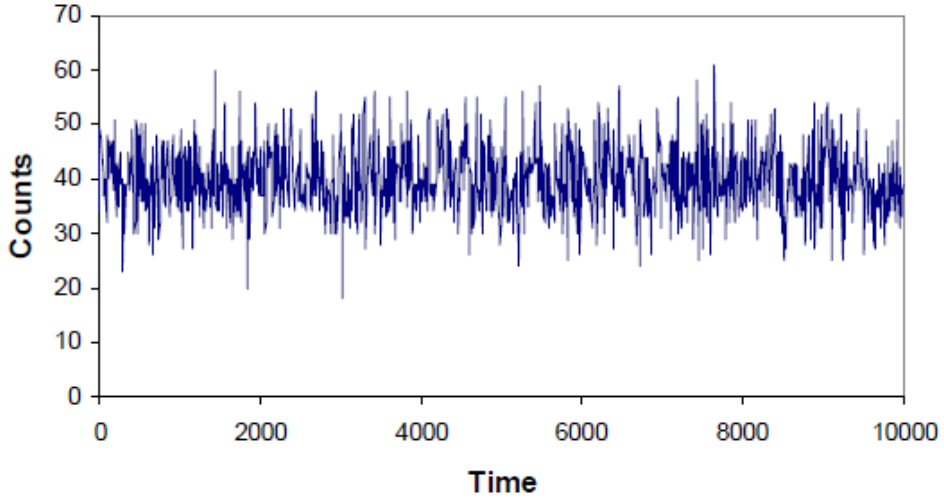


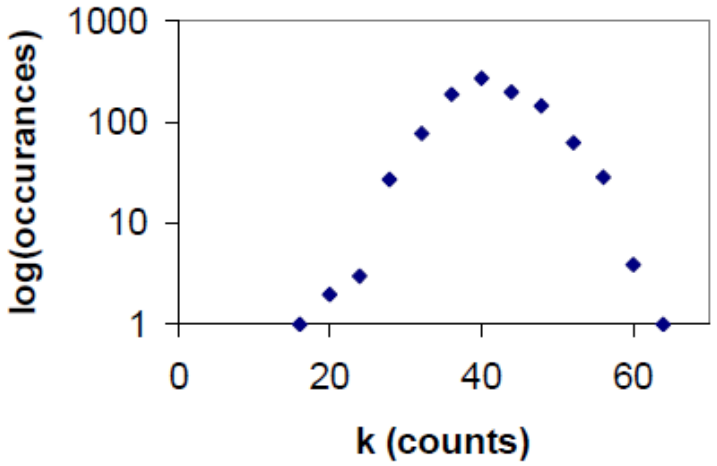
Can we quantitate this?

What contributes to the distribution of intensities?

Contribution from the detector noise

Fixed Particle Noise (Shot Noise)





Noise follows the Poisson distribution -> average=variance

$$Poi(k,\langle k\rangle) = \frac{\langle k\rangle^{k}}{k!}\exp\left(-\langle k\rangle\right)$$

Photon counting statistics, Mandel's formula

$$\mathrm{p}(k,t,T) = \int_0^\infty \frac{(\eta_w W)^k e^{-\eta W}}{k!}\,\mathrm{p}(W)\;dW\;.$$

The probability p(k,t,T) to observe *k* photoelectron events at time *t* depends on the statistical properties of the light reaching the detector, the detection efficiency η_w and the integration time *T*.
The energy of light falling upon the detector surface is given by the light intensity I(r,t) integrated over the time period *T* and the detector area *A*,

$$W = \int_t^{t+T}\int_{\mathbf{A}} I(r,t)\;d\mathbf{A}\,dt$$

The photon counting distribution p(k,t,T) is thus the Poisson transformation of the energy distribution p(W). From a mathematical point of view p(k,t,T) constitutes a doubly stochastic Poisson point process based on the two sources of randomness encountered: 1) The quantum intrinsic statistics of the photoelectron process and 2) the noise distribution of the light source

As a consequence of the convolution of the source fluctuations with the detector “shot noise” the resulting distribution is characterized by a variance which is larger than the “Poisson” variance due to the photoelectric effect. Sometimes we refer to this situation as a “**super-poissonian**” distribution.

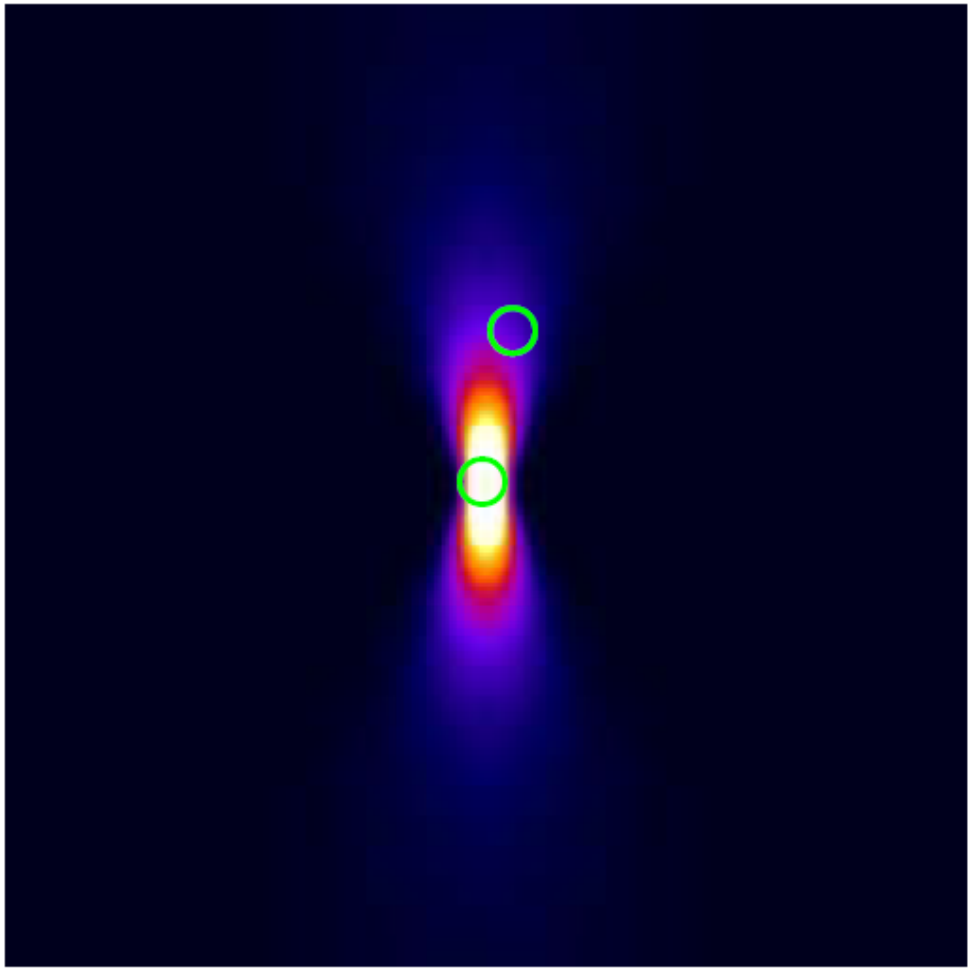
It is possible to have a **sub-poissonian** distribution. This occur when the convolution is with sources which are correlated, rather than independent.

The full-description of the statistics requires quantum-mechanical considerations including squeezed states of light (Walls, 1983, reducing the noise in either the phase or the amplitude of the light at the expense of the other), and photon anti bunching (Kimble et al., 1977). We only consider the semi-classical treatment.

In the PCH approach, we have super-poissonian distributions

Contribution from the profile of illumination

The Point Spread Function (PSF)



One Photon Confocal:

$$I_{3DG}(r,z) = \exp\left(-\frac{2r^2}{\omega_0^2} - \frac{2z^2}{z_0^2}\right)$$

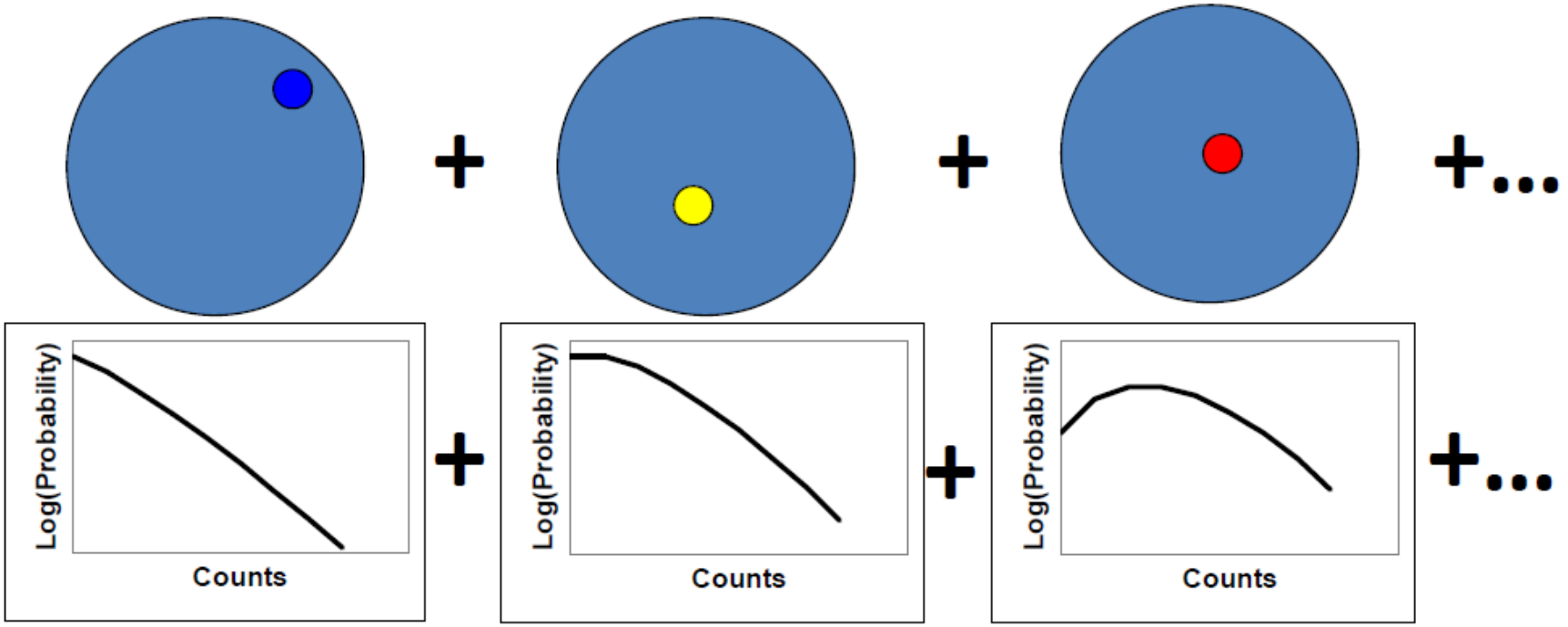
Two Photon:

$$I_{GL^2}(r,z) = \frac{4\omega_0^4}{\pi^2\omega^4(z)}\exp\left(-\frac{4r^2}{\omega^2(z)}\right)$$

$$\omega^2(z) = \omega_0^2\left(1+\left(\frac{z}{z_R}\right)^2\right)$$

$$z_R = \frac{\pi\omega_0^2}{\lambda}$$

Single Particle PCH



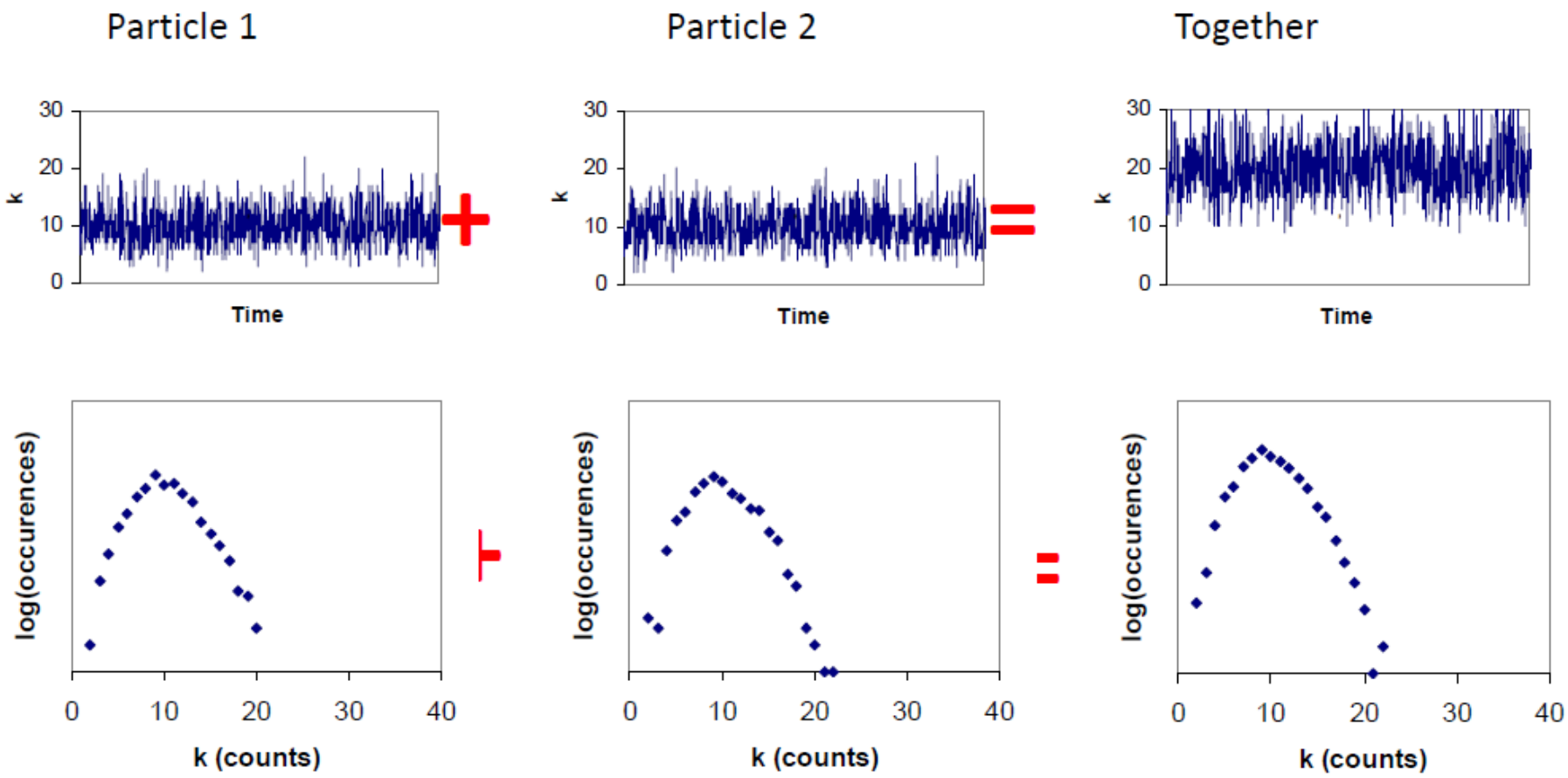
Have to sum up the poissonian distributions for all possible positions of the particle within the PSF

$$p^{(1)}(k) = \frac{1}{V_0} \int_{V_0} Poi\left(k, \varepsilon \overline{PSF}(\vec{r})\right) d\vec{r}$$

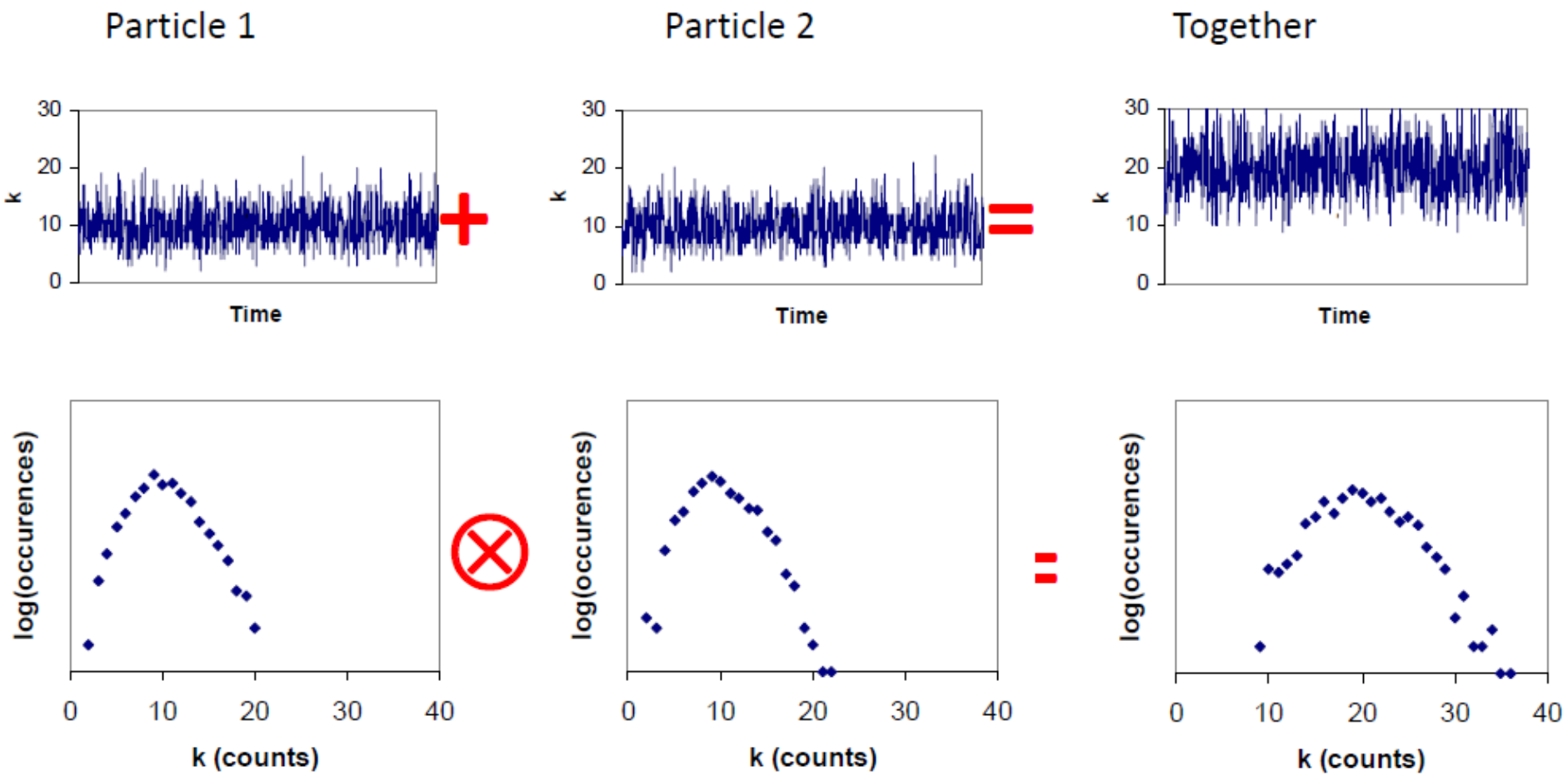
- What if I have two particles in the PSF?
- Have to calculate every possible position of the second particle for each possible position of the first!

Contribution from several particles of same brightness

Combining Distributions

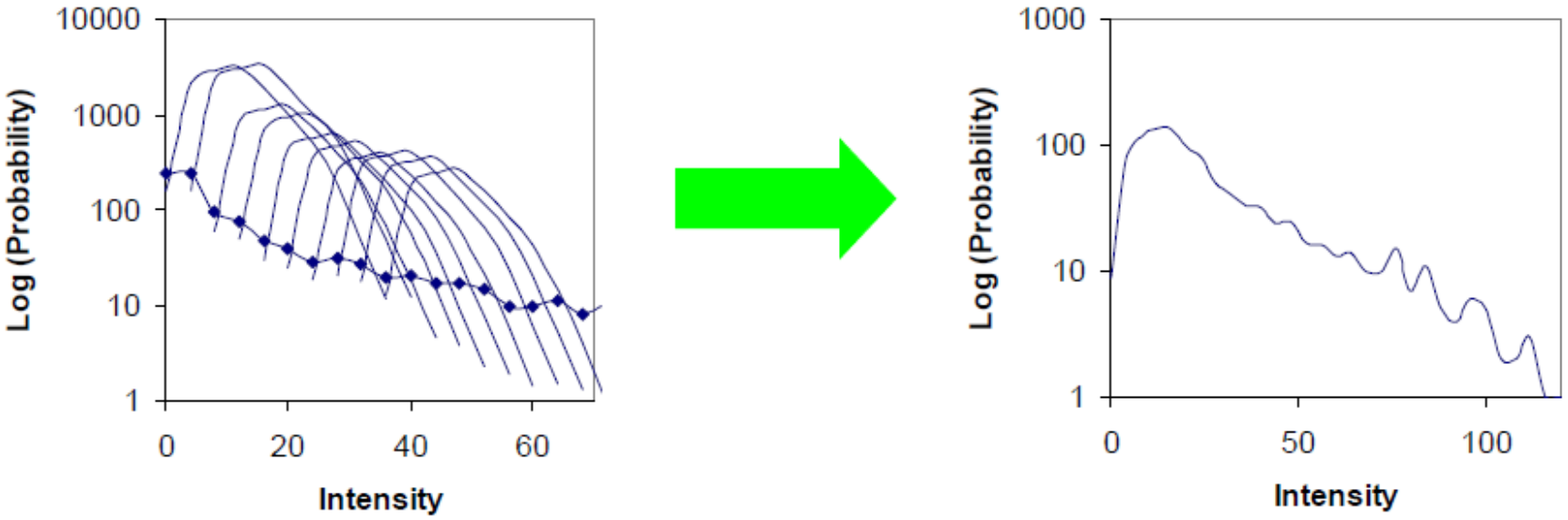


Combining Distributions



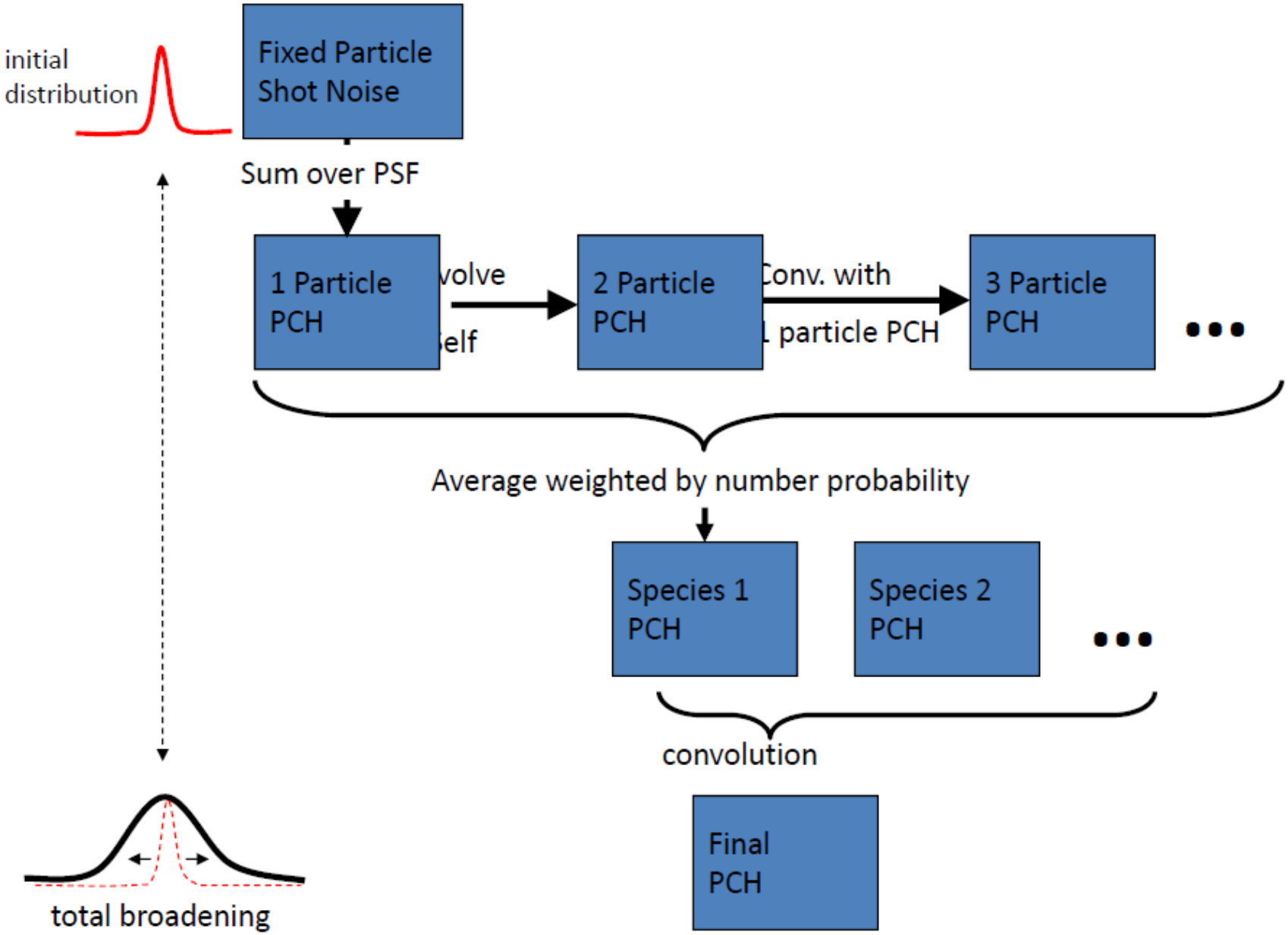
Convolution

- Sum up all combinations of two probability distributions (joint probability distribution)
- Distributions (particles) must be independent



$$p^{(1+2)}(k) = \sum_{r=0}^{r=k} p^{(1)}(k-r) \cdot p^{(2)}(r)$$

Recap: Factors that contribute to the final broadening of the PCH



Method

- Sum up Poisson distributions from all possible arrangements and number of fluorophores in excitation volume (PSF)
  - Intensity weighted sum of all possible single particle histograms (Poisson functions)
  - Convolution to get multiple particle histograms
  - Number probability weighted sum of multiple particle histograms
  - Convolution to get multi-species histograms

Chen et al., *Biophys. J.*, **1999,** *77,* 553.

Fitting

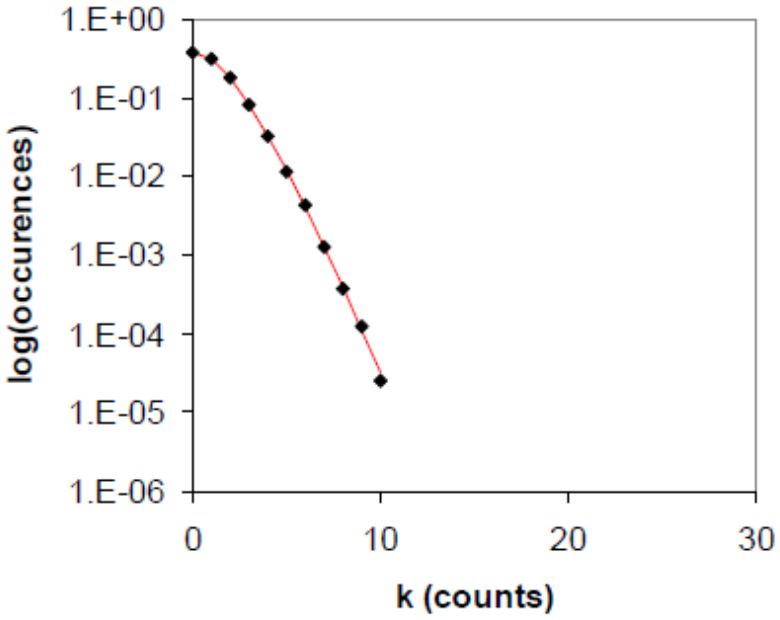
$$\chi^2 = \frac{\sum_k \left(M \frac{PCH_{model}(k) - PCH_{observed}(k)}{\sqrt{M \cdot PCH_{observed}(k) \cdot (1 - PCH_{observed}(k))}} \right)^2}{k_{\max} - d}$$

M is number of observations

d is number of fitting parameters

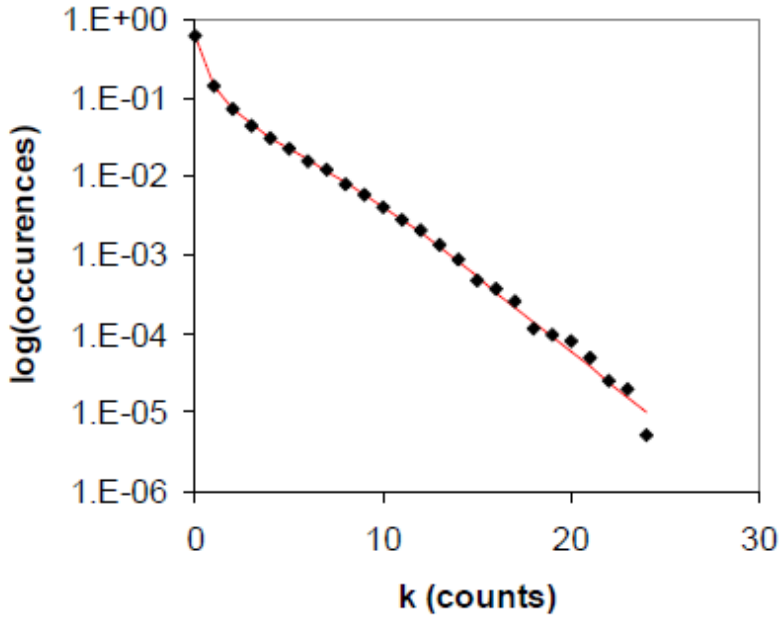
Chen et al., *Biophys. J.*, **1999,** *77,* 553.

Model Test



ε = 9,030 cpsm

N = 1.28



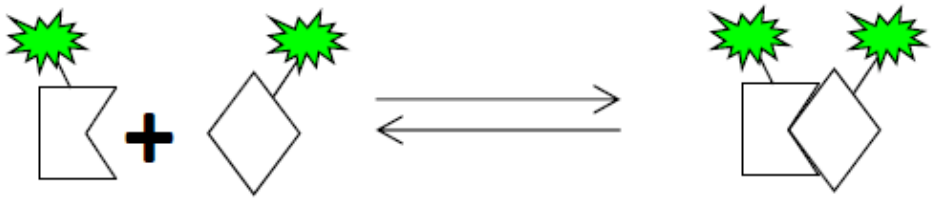
ε = 91,330 cpsm

N = 0.12

Hypothetical situation: Protein Interactions

- 2 proteins are labeled with a fluorophore
- Proteins are soluble
- How do we assess interactions between these proteins?

Dimer has double the brightness

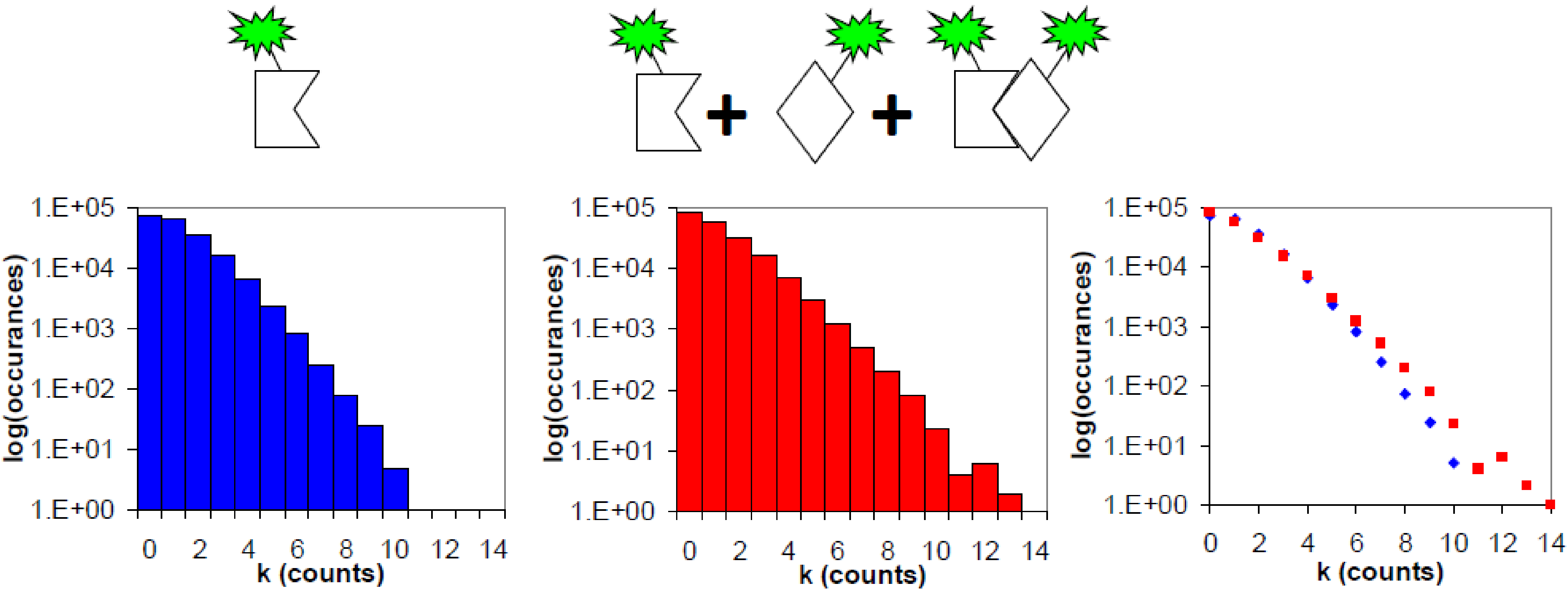


$\varepsilon = \varepsilon_{monomer}$ $\varepsilon = 2 \times \varepsilon_{monomer}$

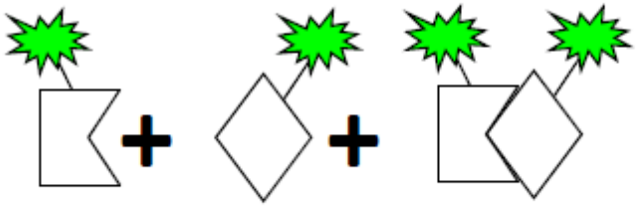
All three species are present in equilibrium mixture

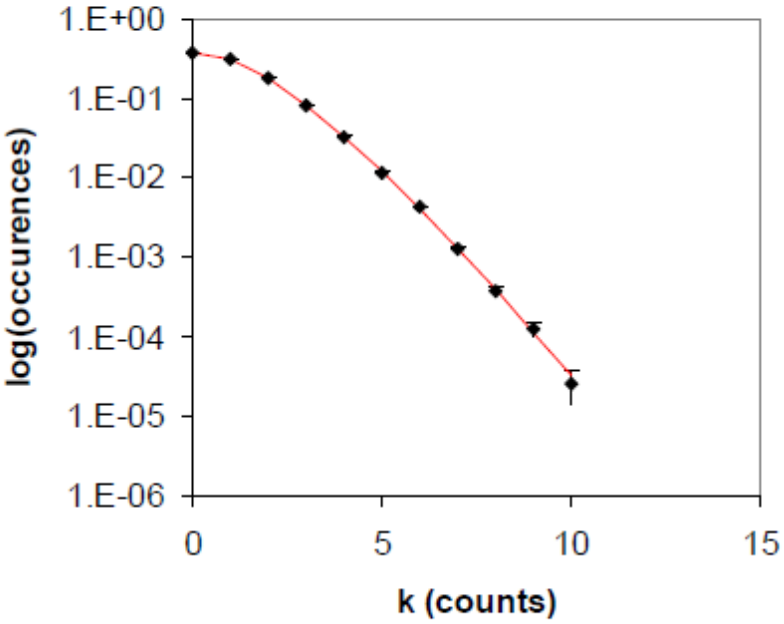
Typical one photon $\varepsilon_{monomer}$ = 10,000 cpsm

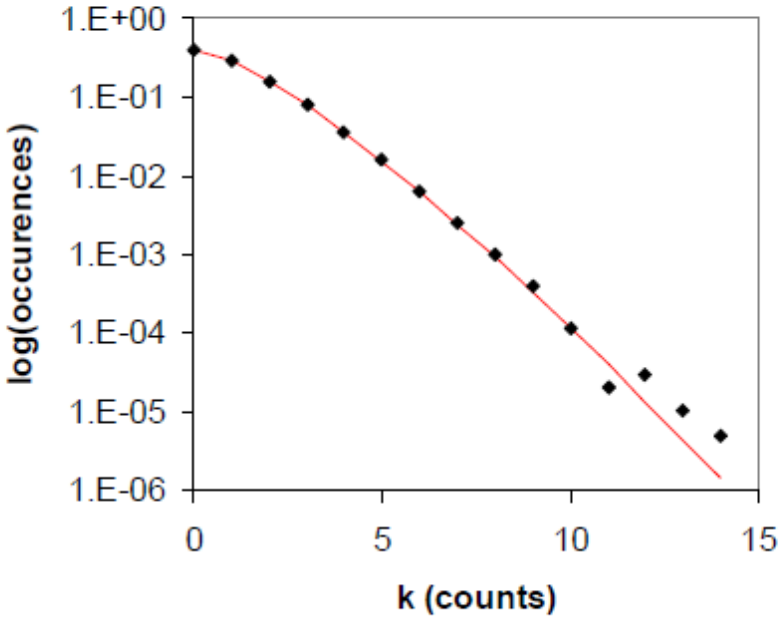
Photon Count Histogram (PCH)



Simulation Solution







ε = 9,000 cpsm

N = 1.3

ε = 16,000 cpsm

N = 0.73

What we measure is the number of particles in the PSF. How Do We Get Concentrations?

- N is defined relative to PSF volume
- One photon:

$$V_{3DG} = w_0^2 z_0 (\pi / 2)^{3/2} \qquad V_{PSF} = \int PSF(\vec{r}) d\vec{r}$$

- Two photon:

$$V_{GL2} = \frac{\pi w_0^4}{\lambda}$$

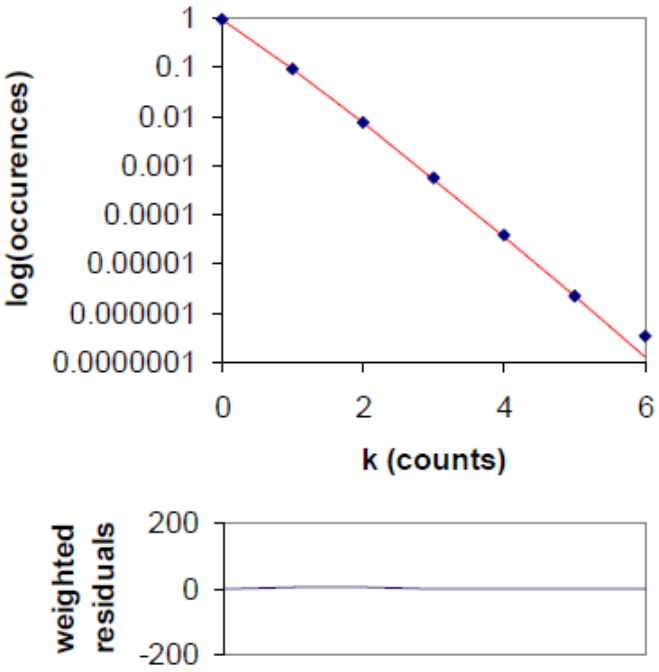
- Definition is same as for FCS
- Can use FCS to determine w_0 (and maybe z_0)

w_0 = 0.21 um, z_0 = 1.1 um, V_{PSF} = 0.091 um^3, C = 23 nM

Saturation Effect

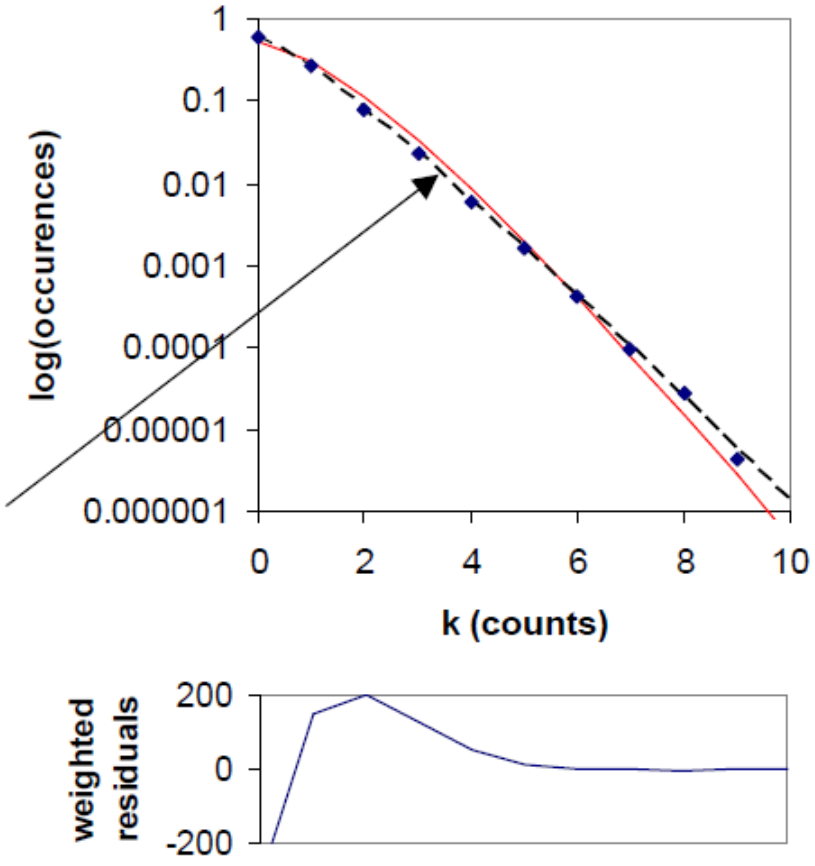
Rhodamine 110 on the Zeiss Confocor 3

10 uW laser



Multi-Species Fit

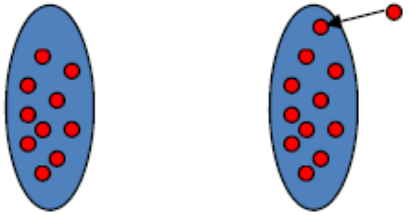
60 uW laser



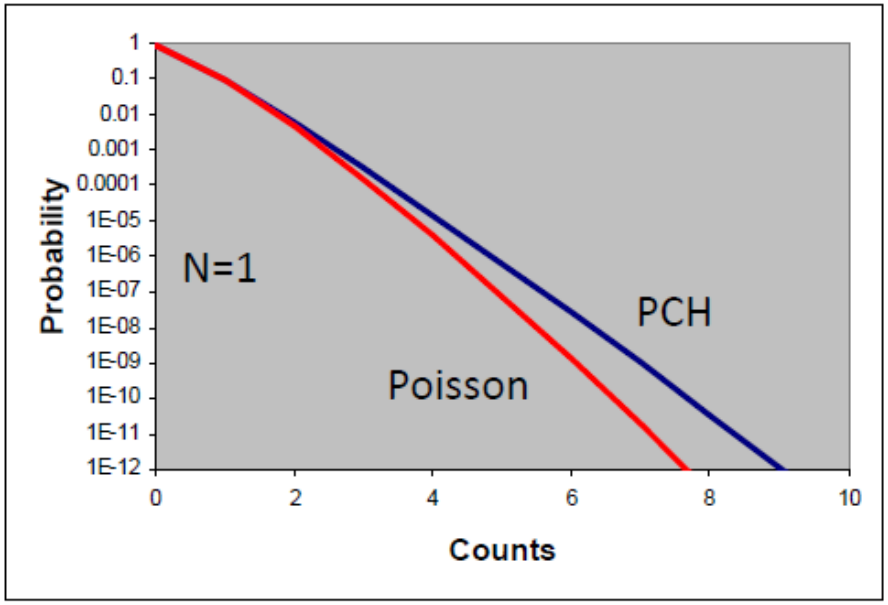
Laser power is not an infinite source of brightness!

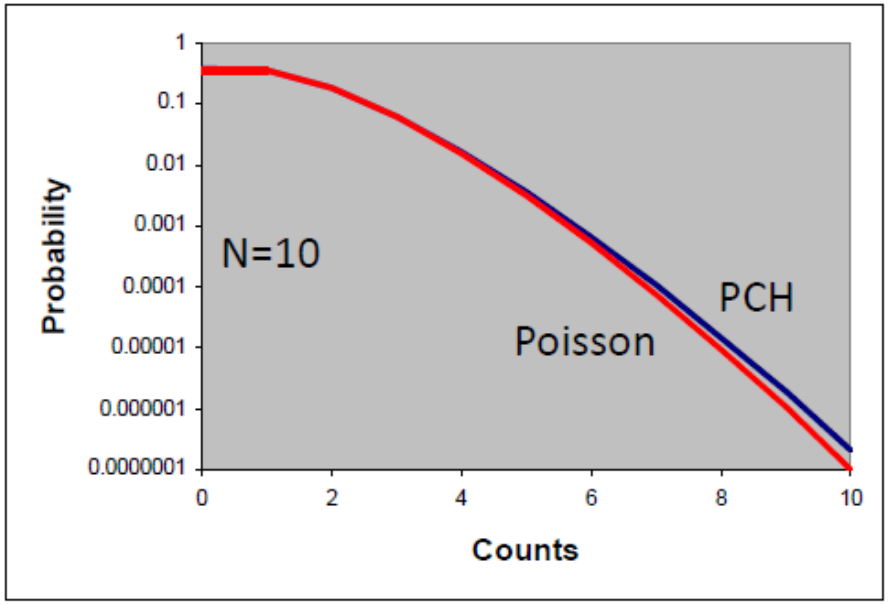
Concentration Effect

Brightness increases
by 100%



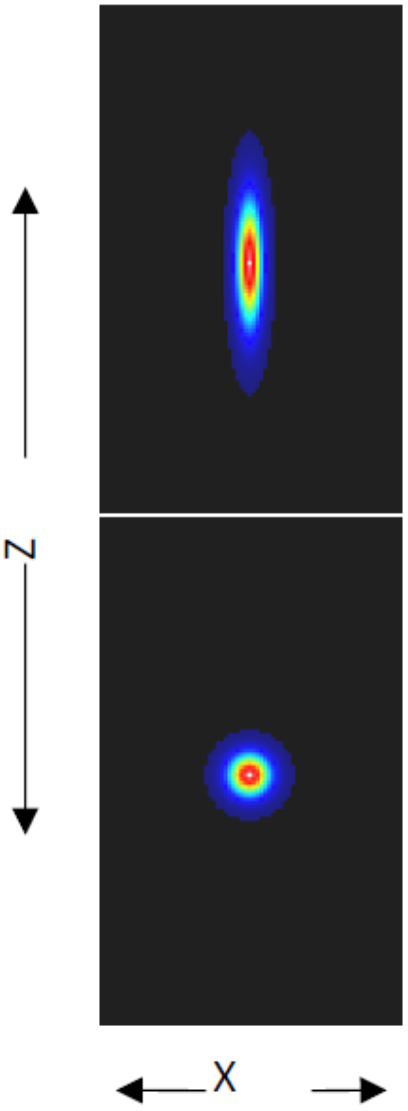
Brightness increases
by 10%





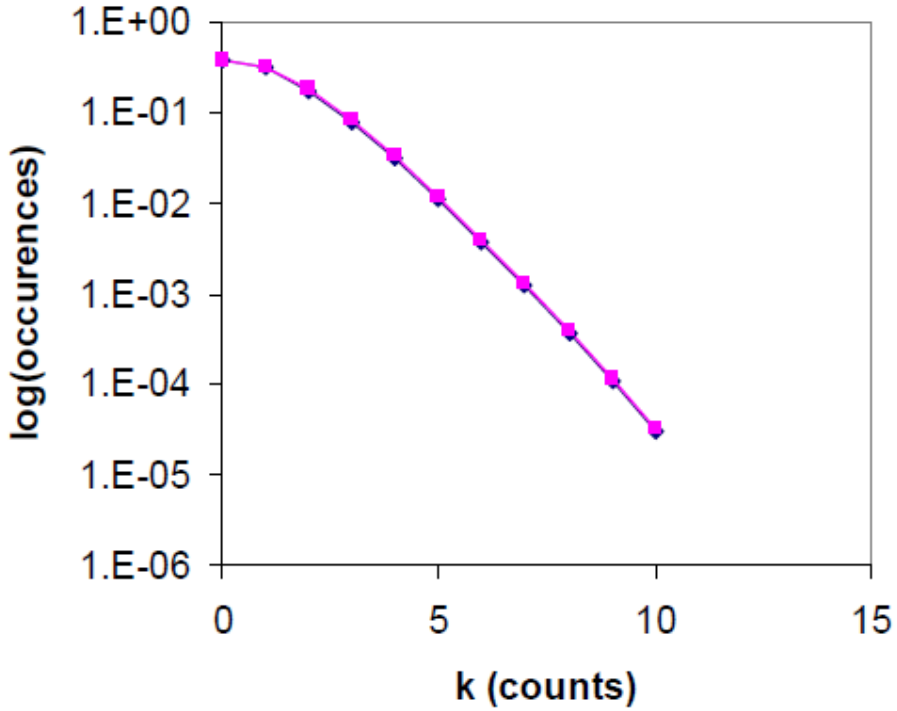
Note: if N is too low, experiment becomes photon limited

PSF X,Y, and Z Dimensions Don't Matter

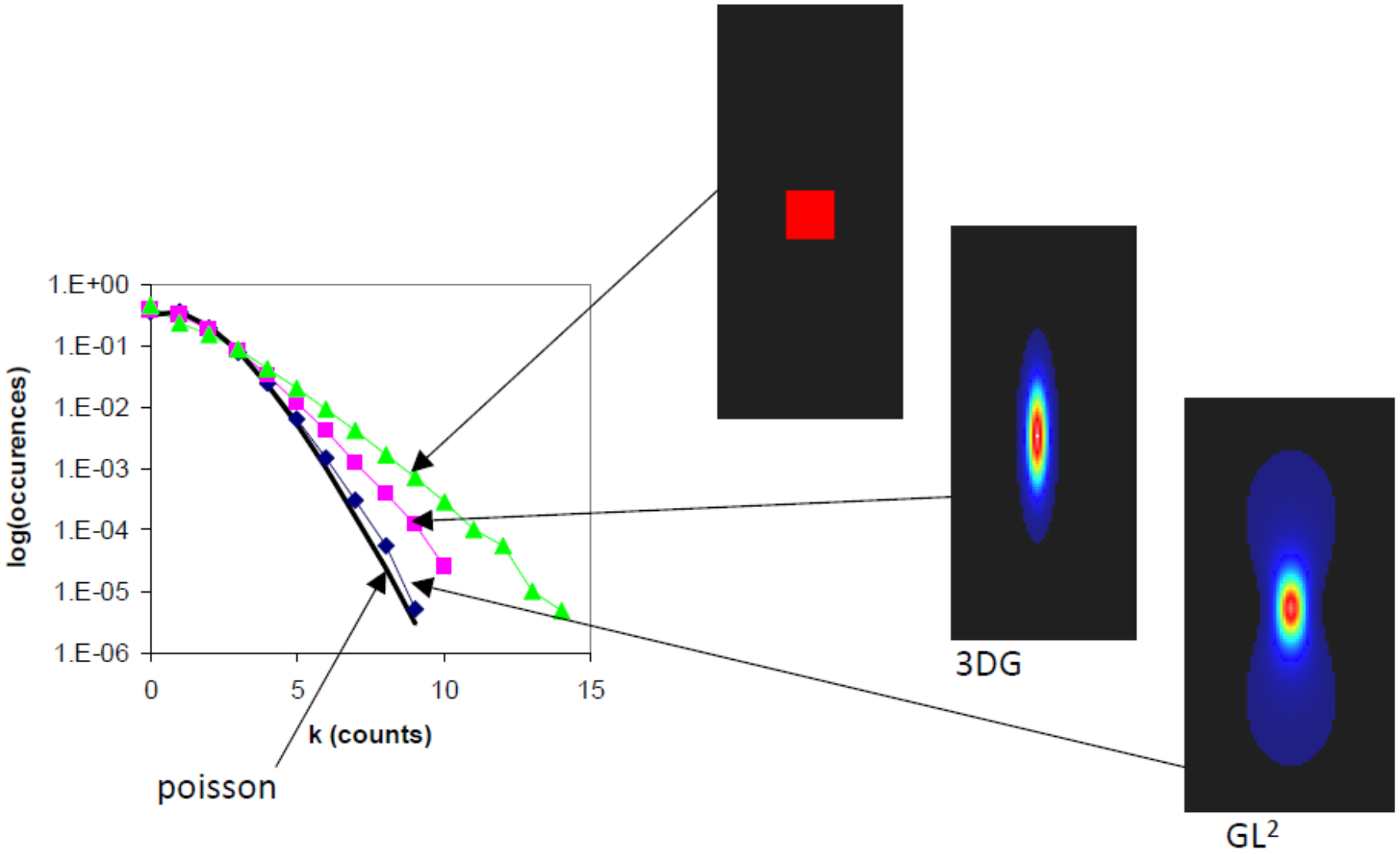


V_{PSF} = 0.08 fL

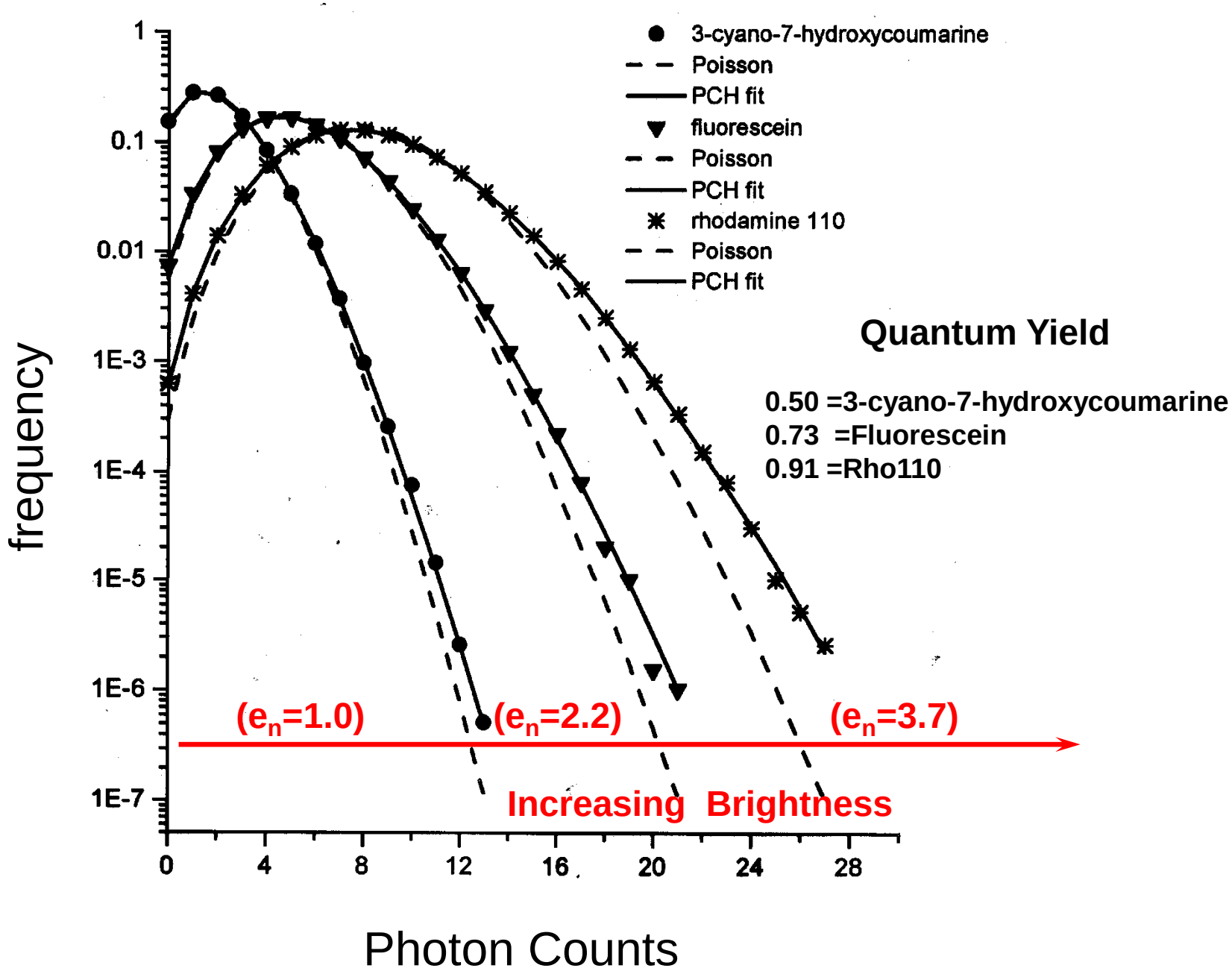
V_{PSF} = 0.08 fL



Functional Form DOES Matter



PCH Example: Differences in Brightness



Point Spread Function Effects

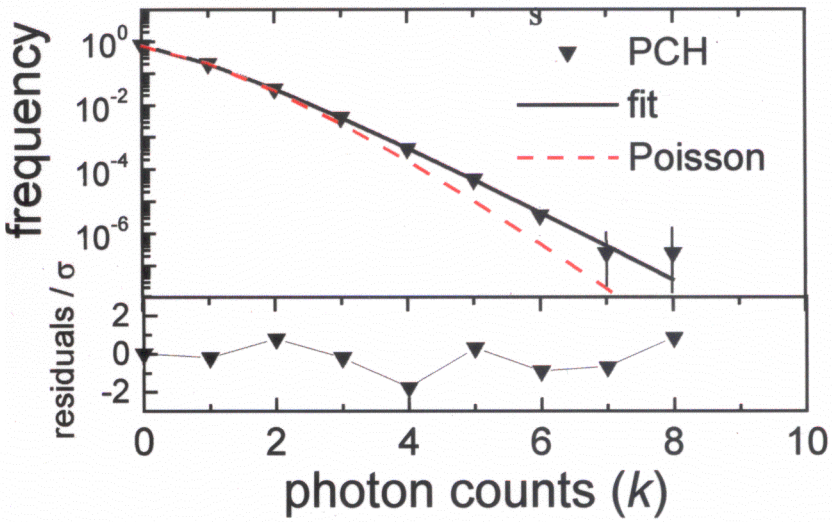
$$p^{(1)}(k) = \frac{1}{V_0} \int_{V_0} Poi\left(k, \varepsilon \overline{PSF}(\vec{r})\right) d\vec{r}$$

This equation will work for ANY PSF shape.

Single Species PCH: Concentration

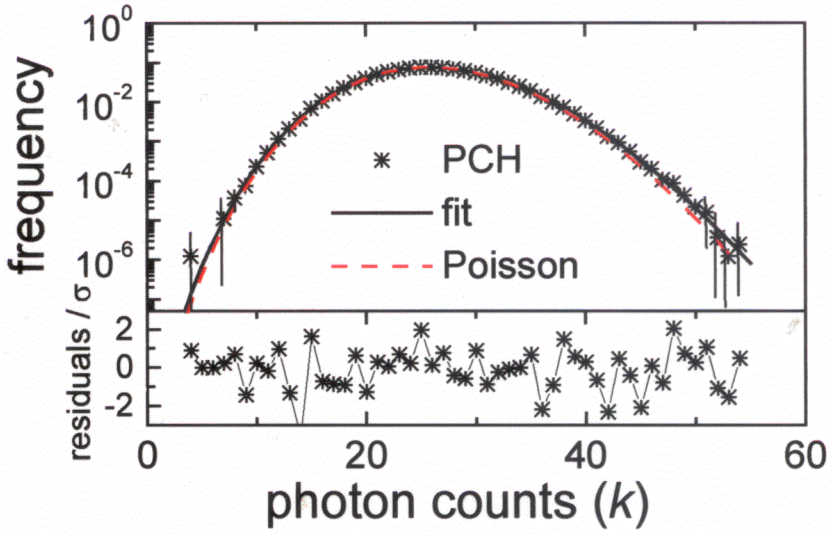
5.5 nM Fluorescein

Fit:
e = 16,000 cpsm
N = 0.3



550 nM Fluorescein

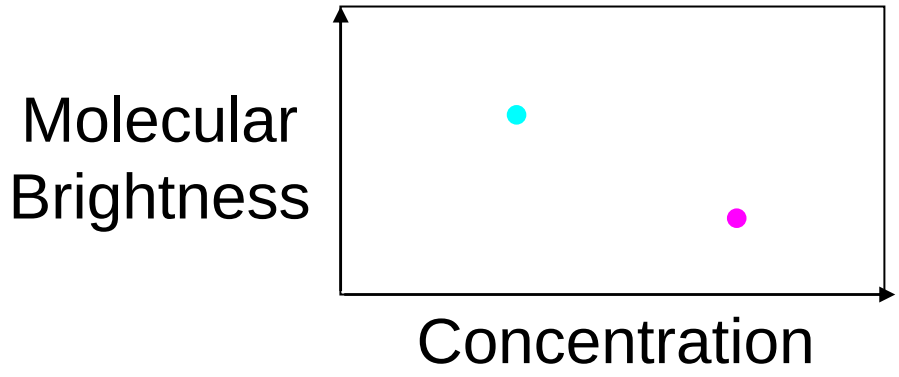
Fit:
e = 16,000 cpsm
N = 33

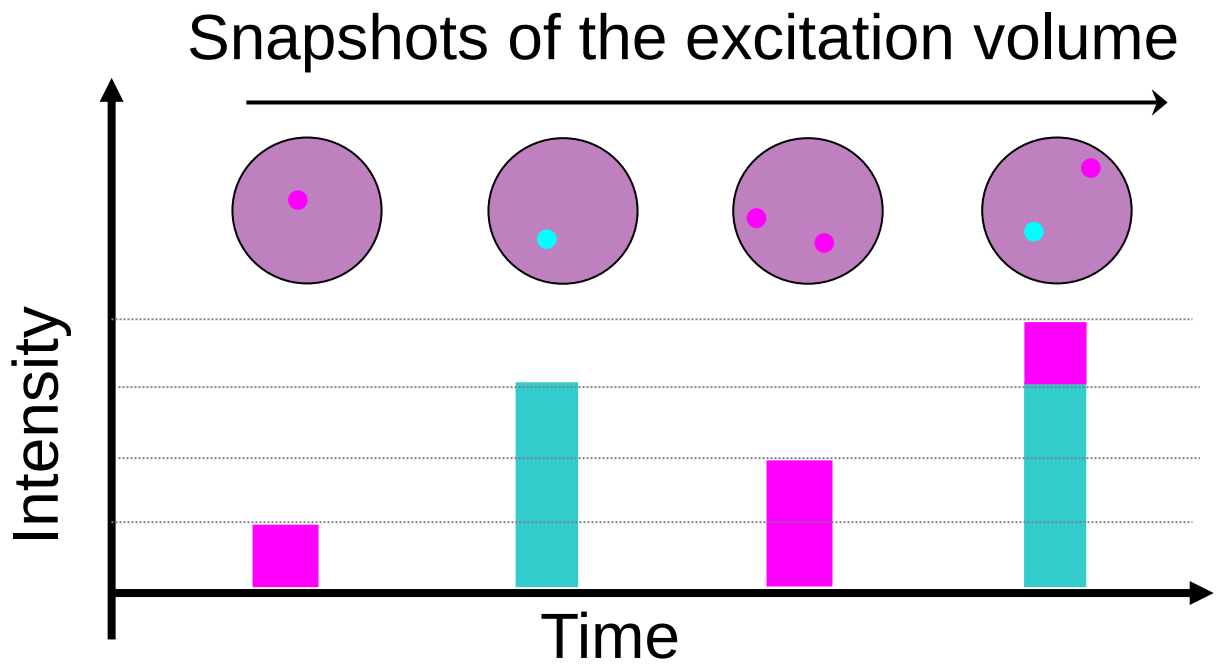


As particle concentration increases the PCH approaches a Poisson distribution

Photon Counting Histogram: Multispecies

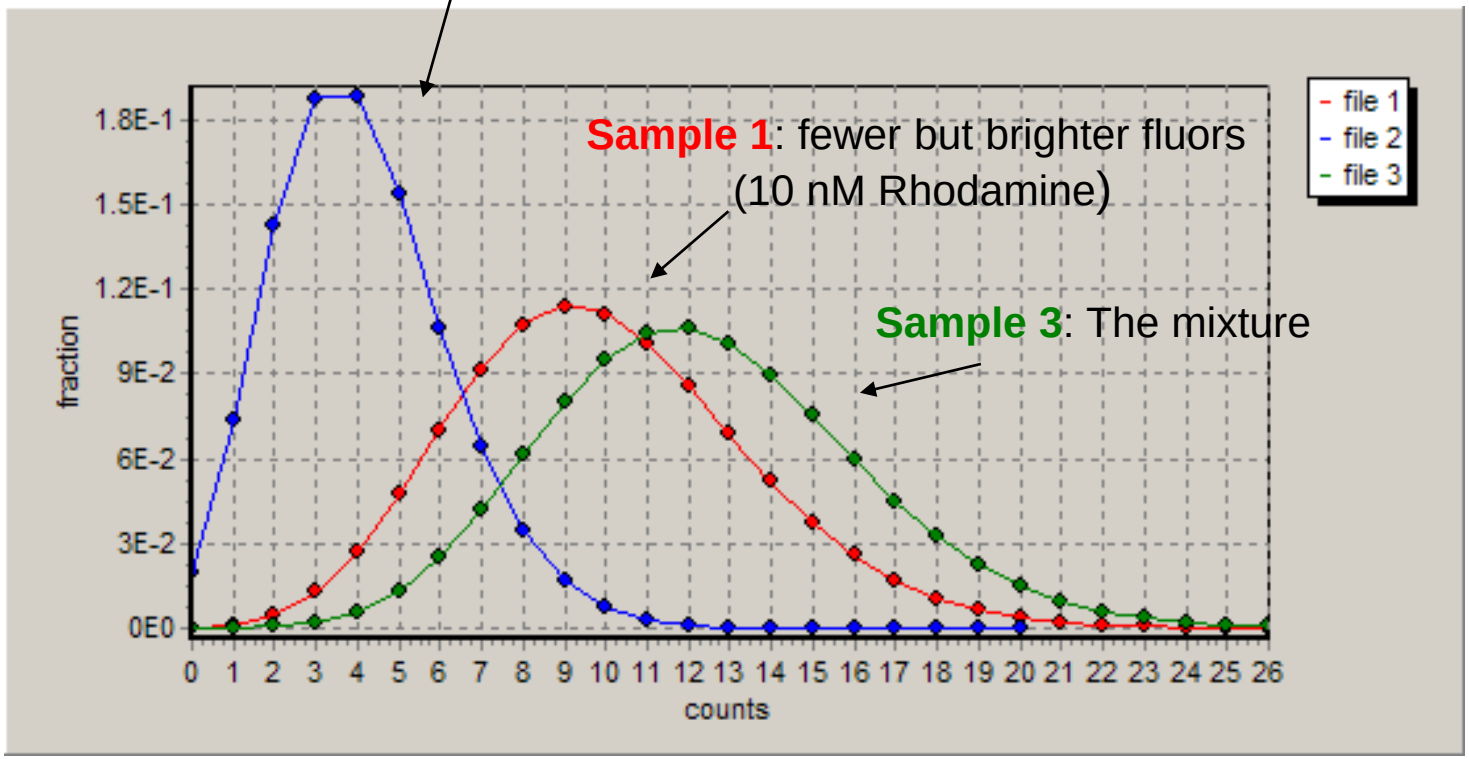
Binary Mixture: $p(k) = PCH(\varepsilon_1, \langle N_1 \rangle) \otimes PCH(\varepsilon_2, \langle N_2 \rangle)$





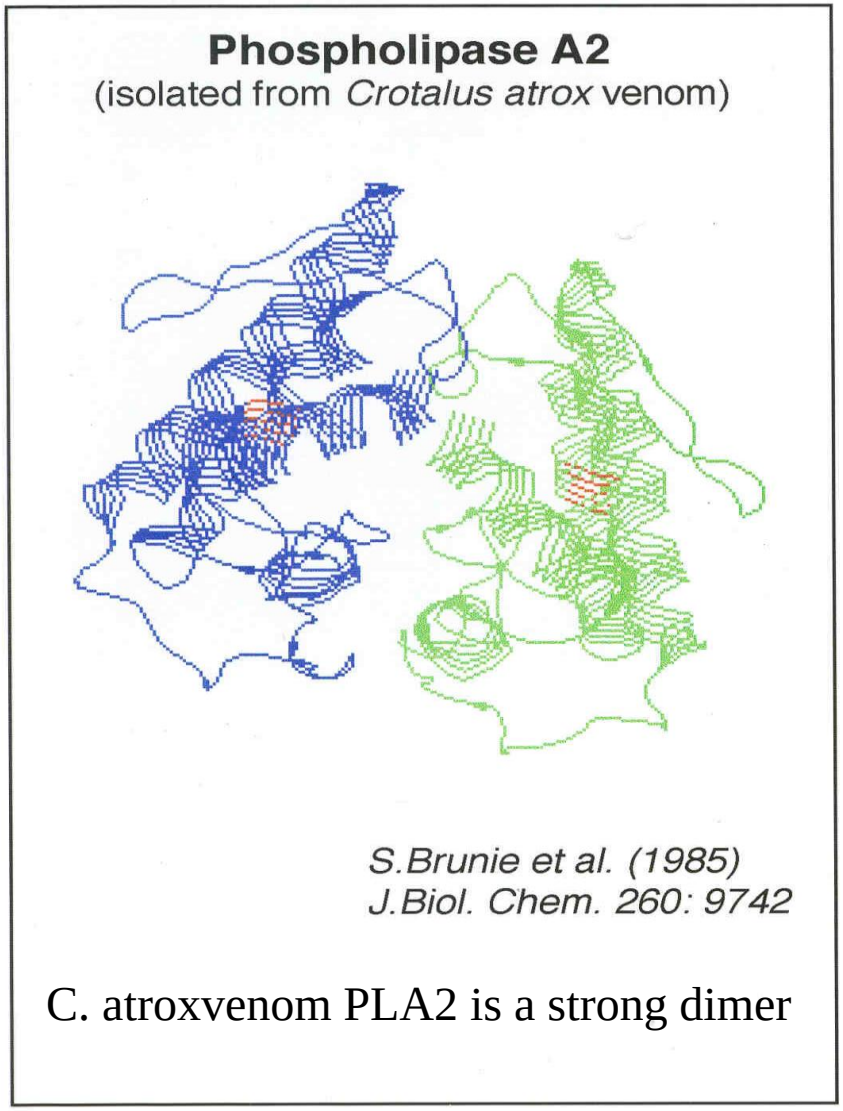
Photon Counting Histogram: Multispecies

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.

Examination of a Protein Dimer with FCS: Secreted Phospholipase A_2



C. atroxvenom PLA2 is a strong dimer

- It would appear that the monomer contains all of the necessary structure for fullenzymatic activity.
- However, the existence of tight dimerPLA2s such as the PLA2 from Crotalus atrox venom begs the question as to the role that the dimer plays in PLA2 function?

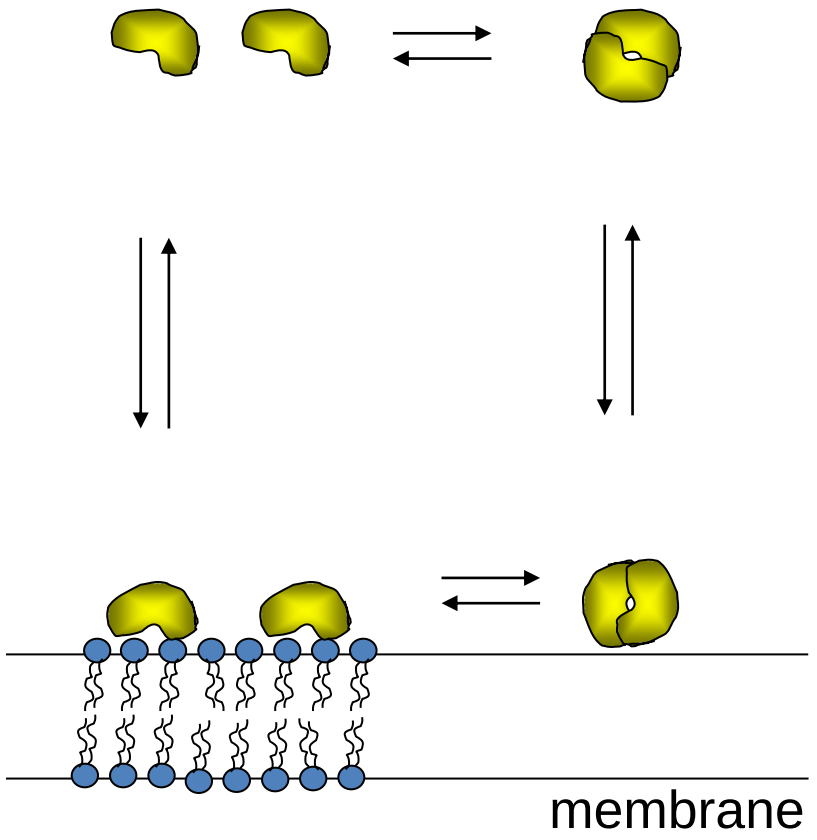
Sanchez, S. A., Y. Chen, J. D. Mueller, E. Gratton, T. L. Hazlett. (2001) Biochemistry, 40, 6903-6911.

$sPLA_2$ Interfacial Binding

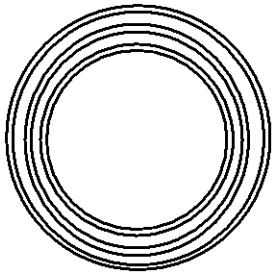
$sPLA_2$ Self-Association

$sPLA_2$ Membrane Binding

Interfacial $sPLA_2$Self-Association

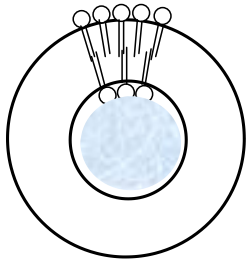


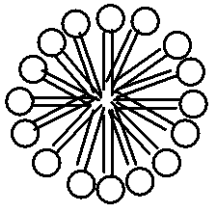
Lipid Interfaces



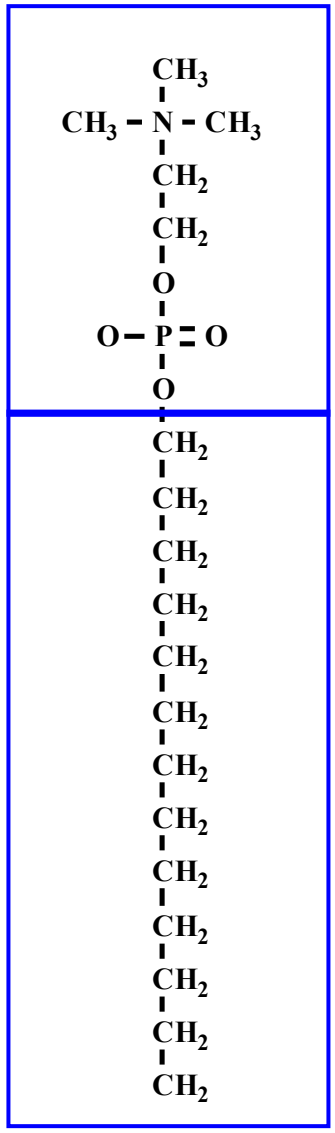
Multibilayers
(MLVs)

Vesicles
(SUVs, LUVs
& GUVs)





Micelles



Choline Group

12 Carbon Tail

Dodecylphosphocholine (DPC)
Micellar Lipid Analog (CMC = 1.1 mM)

In Solution: Fluorescein-sPLA$_2$ +/- Urea

1. Autocorrelation

sPLA$_2$

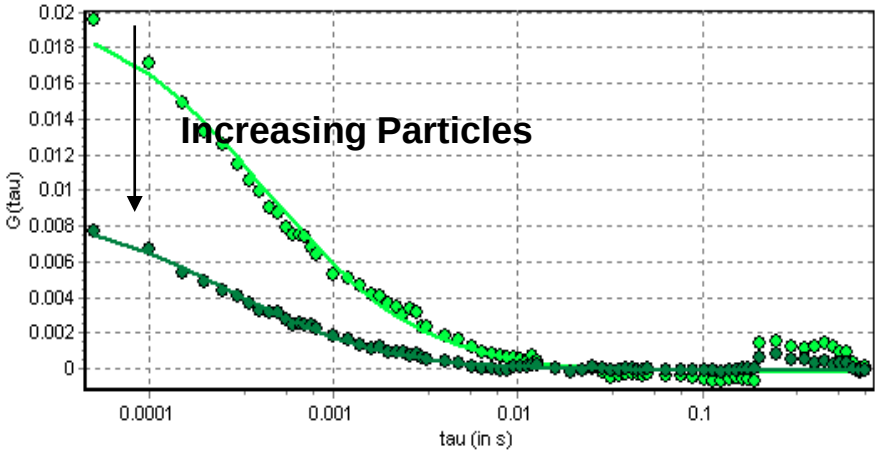
G(0)=0.021

D = 72 um^2/s

sPLA$_2$ + 3M Urea

G(0)=0.009

D = 95 um^2/s



2. PCH analysis

sPLA$_2$

ε = 0.6

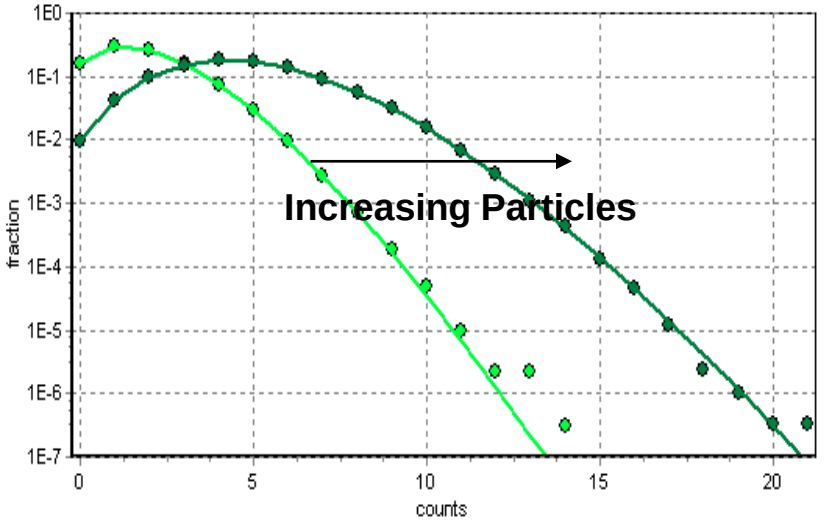
N = 3.29

sPLA$_2$ + 3M Urea

ε= 0.6

N = 8.48

Adjusted for viscosity differences



Change in number of particles, little change in brightness

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- Professor Enrico Gratton
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- Yan Chen
- Joachim Mueller
- Susana Sanchez
- Chip Hazlett

Hybrid-Symposium: Frontiers in Biological Fluorescence 2024

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