Introduction to FCS
PCH analysis
Cross-correlation

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From cuvette to the microscope

1. Excitation & Emission Spectra
    - Local environment polarity, fluorophore concentration
2. Anisotropy & Polarization
    - Rotational diffusion
3. Quenching
    - Solvent accessibility
    - Character of the local environment
4. Fluorescence Lifetime
    - Dynamic processes (nanosecond timescale)
5. Resonance Energy Transfer
    - Probe-to-probe distance measurements
6. Fluorescence microscopy
    - localization

7. **Fluorescence Correlation Spectroscopy**
    - **Translational & rotational diffusion**
    - **Concentration**
    - **Dynamics**

In the microscope, the spatial location matters: spatial correlations and distributions are a component of the experiment

Why we need FCS to measure the internal dynamics in cell??

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 on.

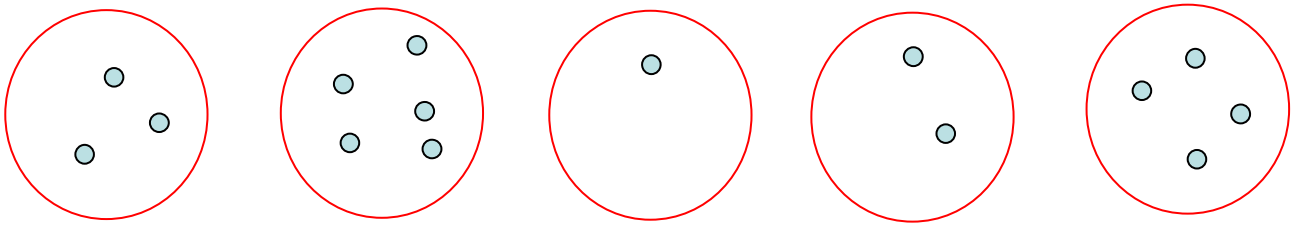
Introduction to “number” fluctuations

In any open volume, the number of molecules or particles fluctuate according to a Poisson statistics (if the particles are not-interacting)

The average number depends on the concentration of the particles and the size of the volume

The variance is equal to the number of particles in the volume

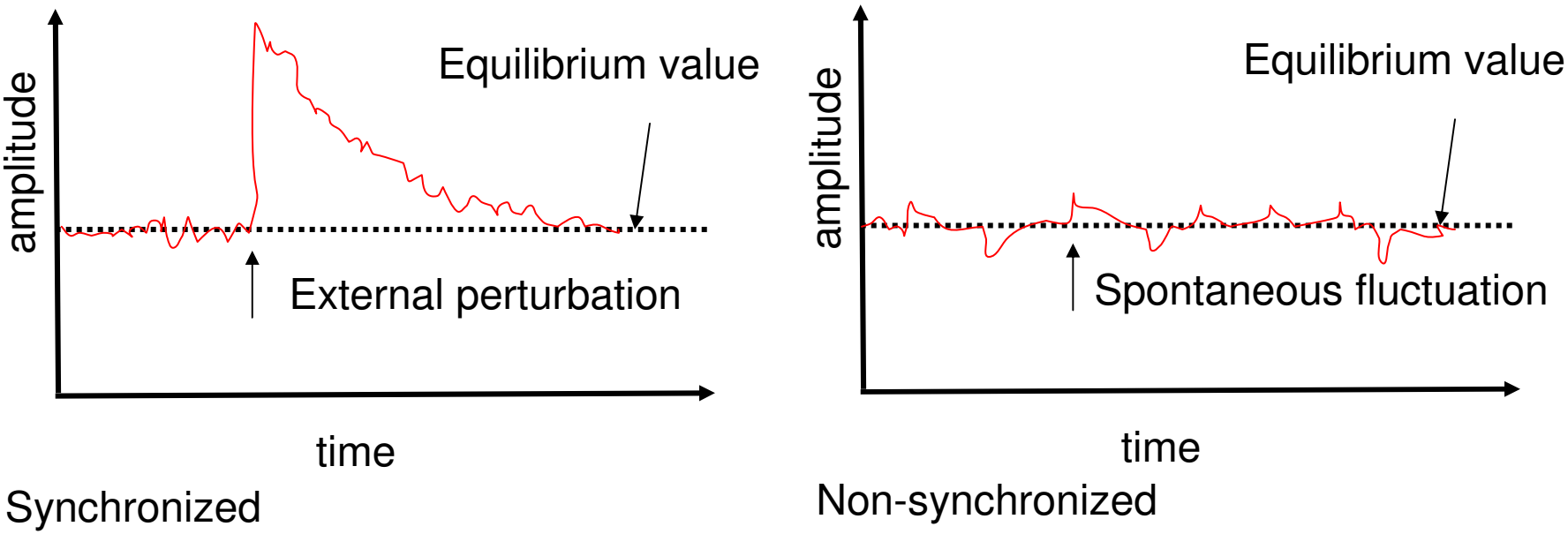
This principle does not tell us anything about the time of the fluctuations



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.



First Application of Correlation Spectroscopy

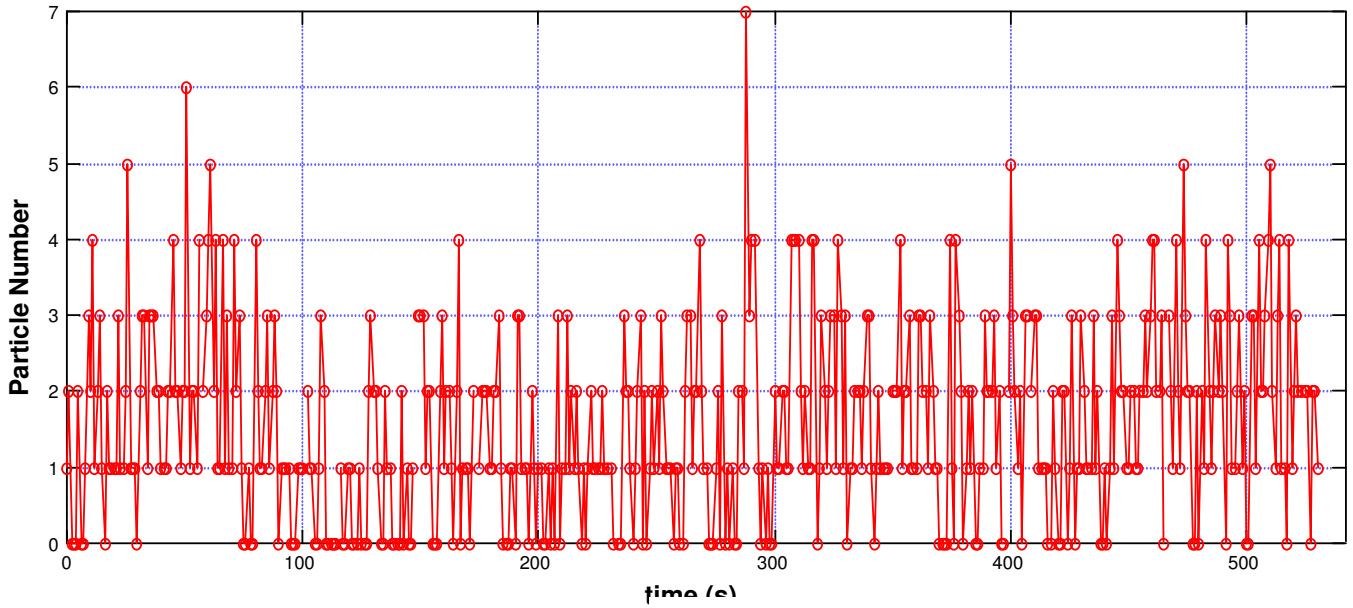
(Svedberg & Inouye, 1911) *Occupancy Fluctuation*

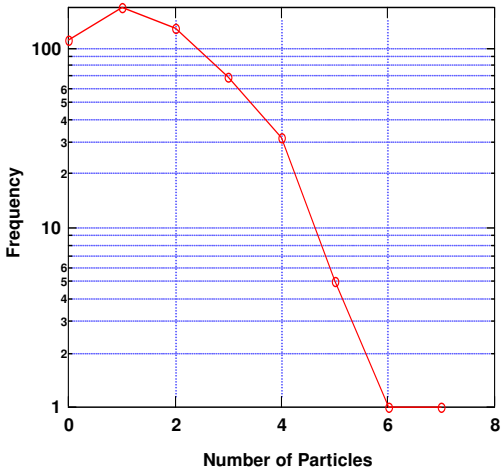
Experimental data on colloidal gold particles:

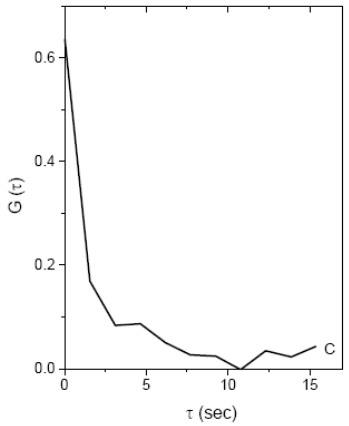
120002001324123102111131125111023313332211122422122612214
234524114131142310010042112312320111100011 1_2110013200000
100110001000232210021100002010 01_33312200023122102401110 2_
122211223100011033111021011001010301131212101012111121 1_10
003221012302012121321110110023312242110001203010100221734
410101002112211444421211440132123314313011222123310121111
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 using a “ultra microscope”

Particle Correlation







***Histogram of particle counts**

***Poisson behavior**

***Autocorrelation not available in the original paper. It can be easily calculated today.**

Comments to this paper conclude that scattering will not be suitable to observe single molecules, but fluorescence could

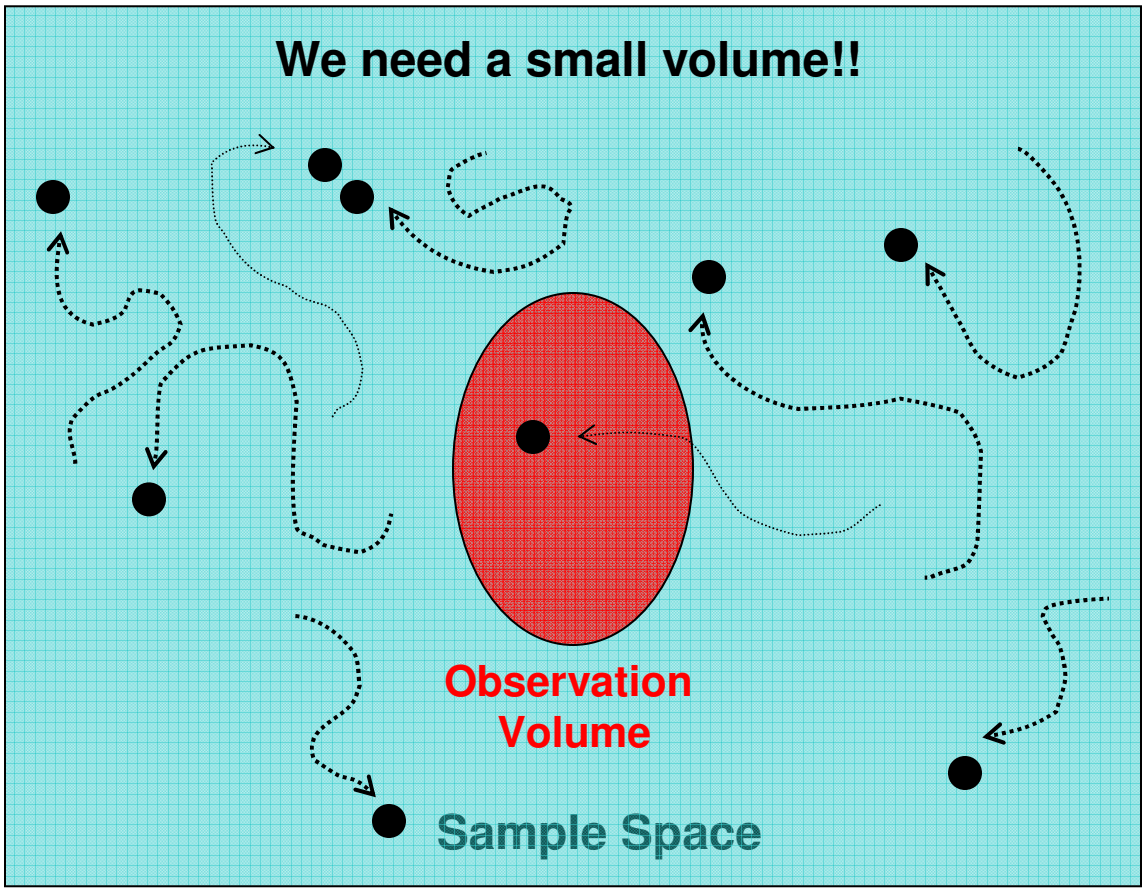
What can cause a fluctuation in the fluorescence signal???

- **Number of fluorescent molecules in the volume of observation, diffusion or binding**
- **Conformational Dynamics**
- **Rotational Motion if polarizers are used either in emission or excitation**
- **Protein Folding**
- **Blinking**
- **And many more**

Example of processes that could generate fluctuations

Each of the above processes has its own dynamics. FCS can recover that dynamics

Generating Fluctuations By Motion



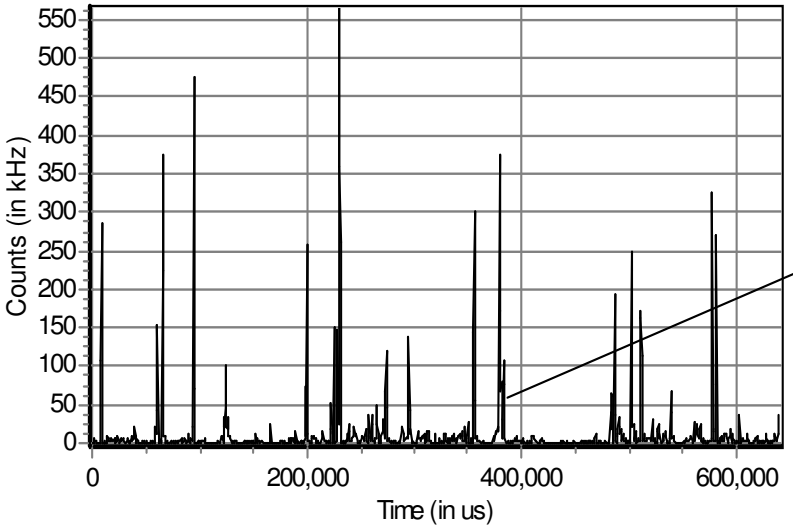
What is Observed?

1. The Rate of Motion

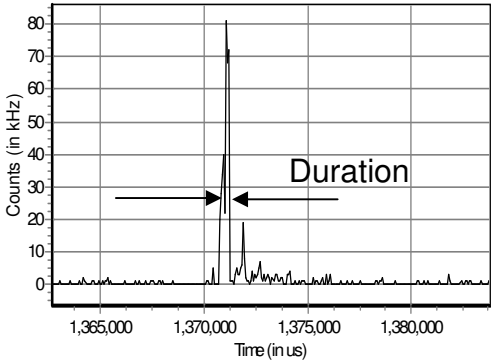
2. The Concentration of Particles

3. Changes in the Particle Fluorescence while under Observation, for example conformational transitions

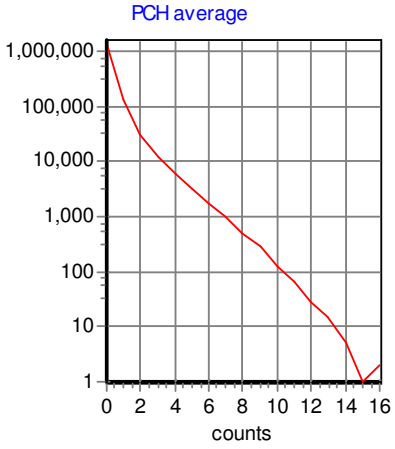
Data presentation and Analysis



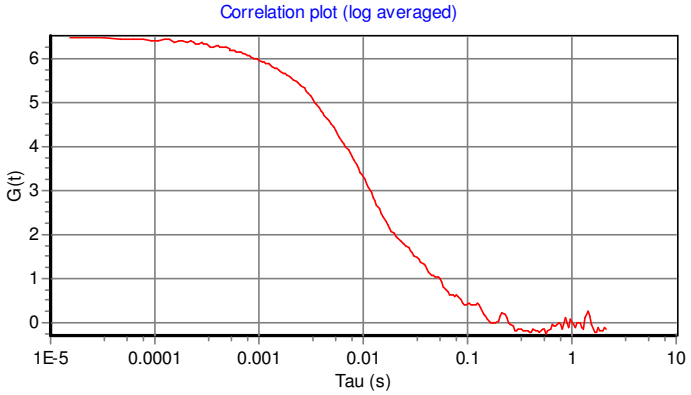
The time series



Detail of one time region



The histogram of the counts in a given time bin (PCH). N and brightness



The autocorrelation function
N and relaxation time of the fluctuation

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

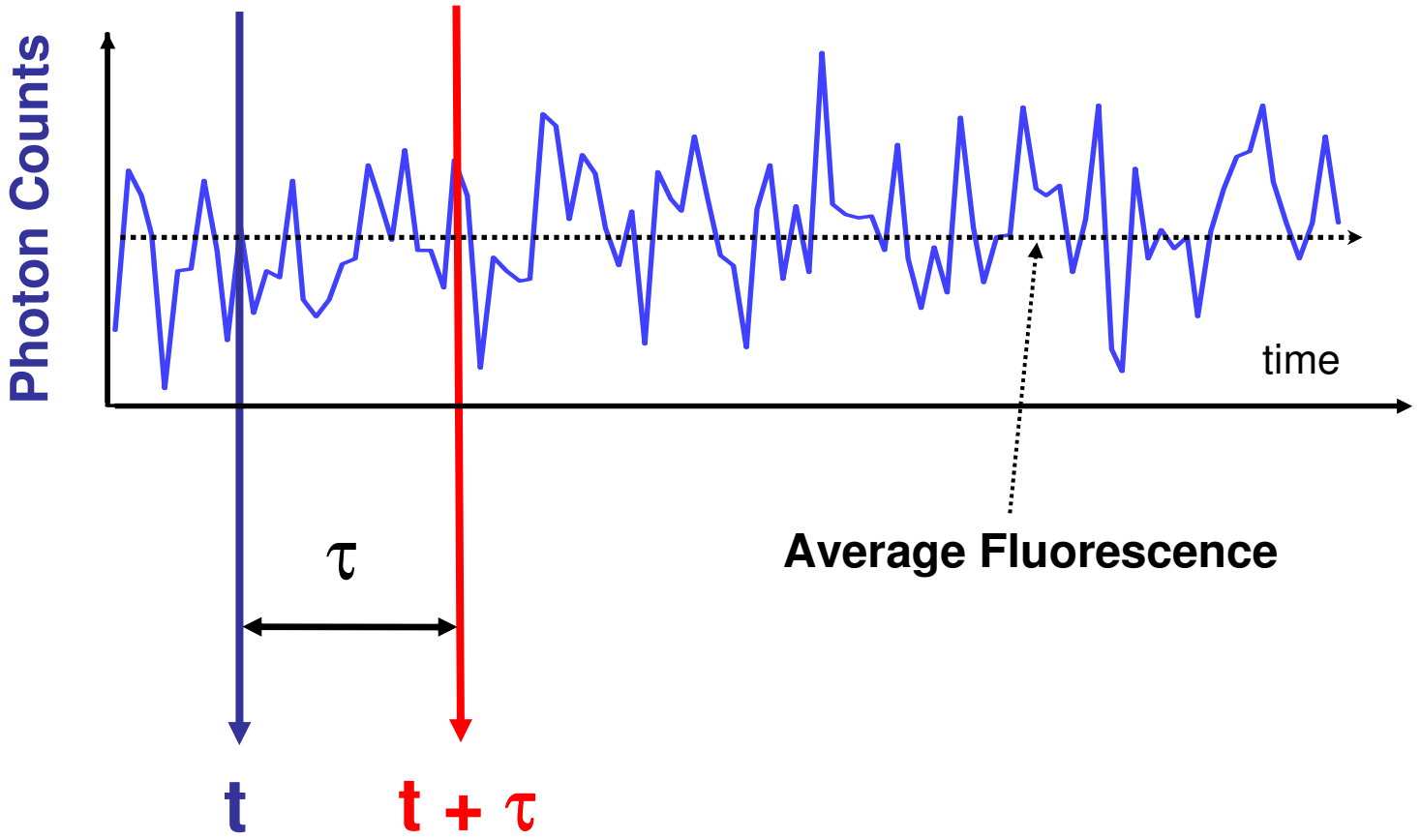
To extract the distribution of the duration of the fluctuations we use a math based on calculation of the **correlation function**

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

The definition of the Autocorrelation Function

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

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



What determines the intensity of the fluorescence signal??

This is the fundamental equation in FCS

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

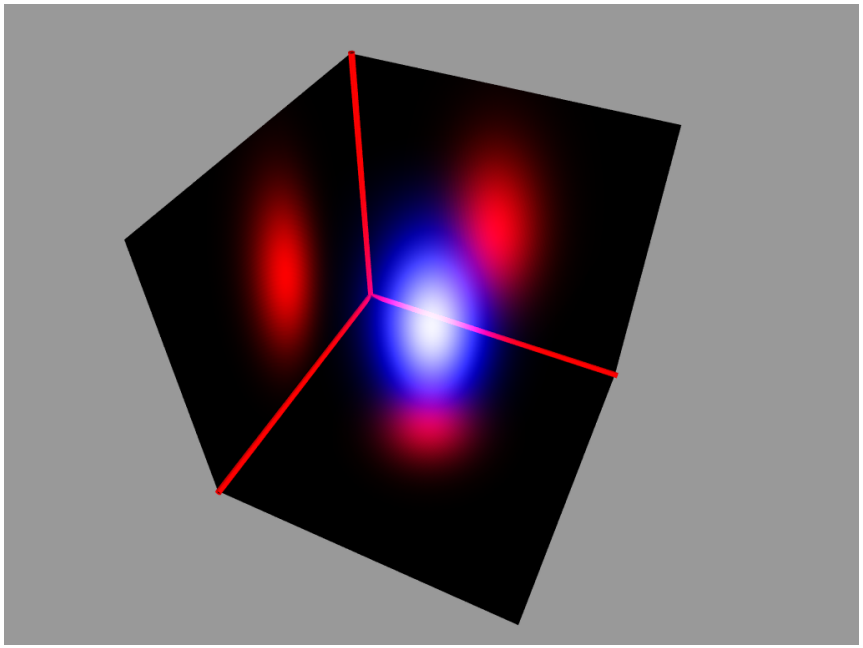
kQ = quantum yield and detector sensitivity (how bright is our probe). This term could contain the fluctuation of the fluorescence intensity due to internal processes

W(r) describes the profile of illumination

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

The value of F(t) depends on the profile of illumination!

What about the excitation (or observation) volume shape?



$$F(x,y,z) = I_0 I(z) e^{-\frac{2(x^2+y^2)}{w_0^2}}$$

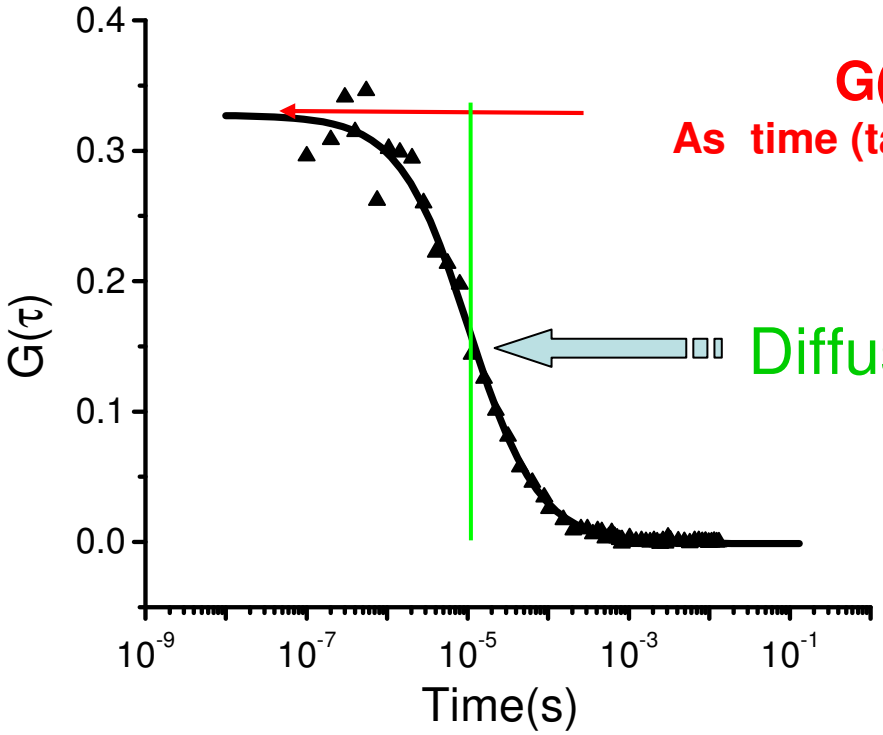
$$I(z) = Exp\left[-\frac{2z^2}{w_{0z}^2}\right]$$ Gaussian z

$$I(z) = \frac{1}{1+(\frac{z}{w_{oz}})^2}$$ Lorentzian z

More on the PSF in Jay's lecture

For the 2-photon case, these expression must be squared

The Autocorrelation Function



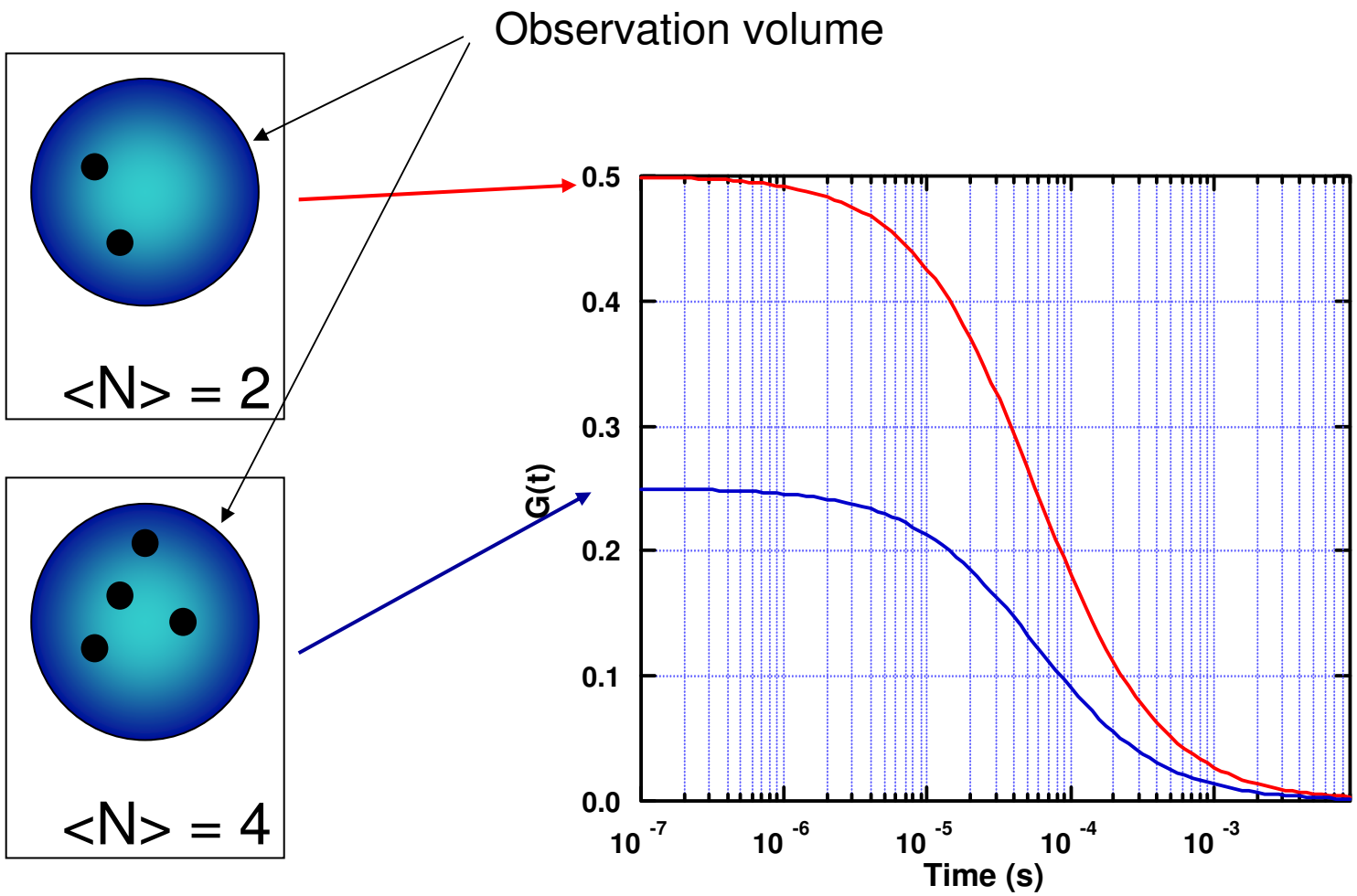
G(0) ∝ 1/N

As time (tau) approaches 0

Diffusion

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

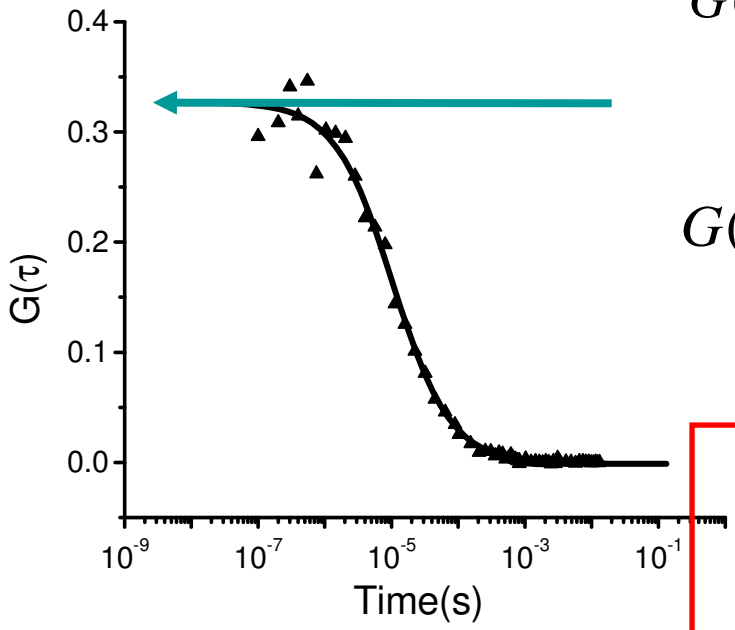
The Effects of Particle Concentration on the Autocorrelation Curve



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

A Poisson distribution describes the statistics of particle occupancy fluctuations. For a Poisson distribution the variance is proportional to the average:

$$< N >= \langle Particle\_Number \rangle = Variance$$

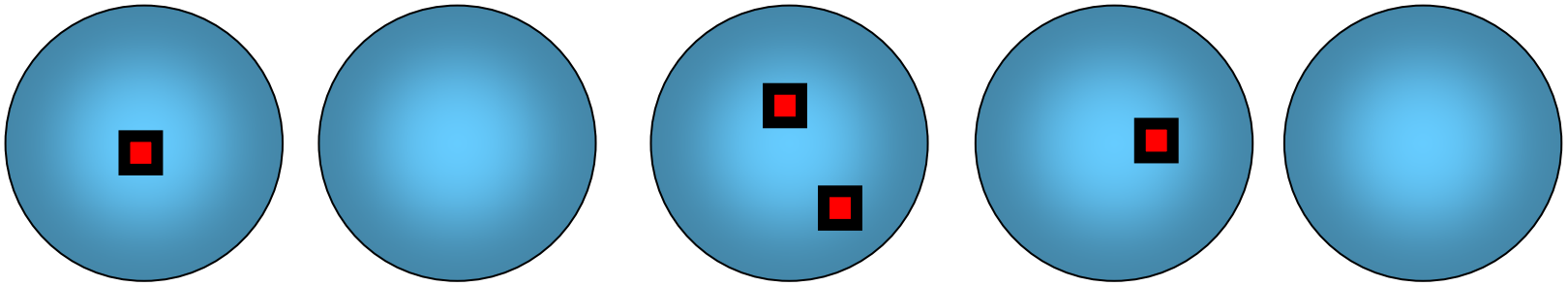


$$G(\tau) = \frac{\langle \delta F(t)\, \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$ Definition

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

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

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 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 (Two-Photon) Autocorrelation Functions:

For a 2-dimensional Gaussian excitation volume:

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

2-photon equation contains a 8, instead of 4

For a 3-dimensional Gaussian excitation volume:

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

3D Gaussian "time" analysis: with $\tau_D = w^2/4D$ and $S = w/z$

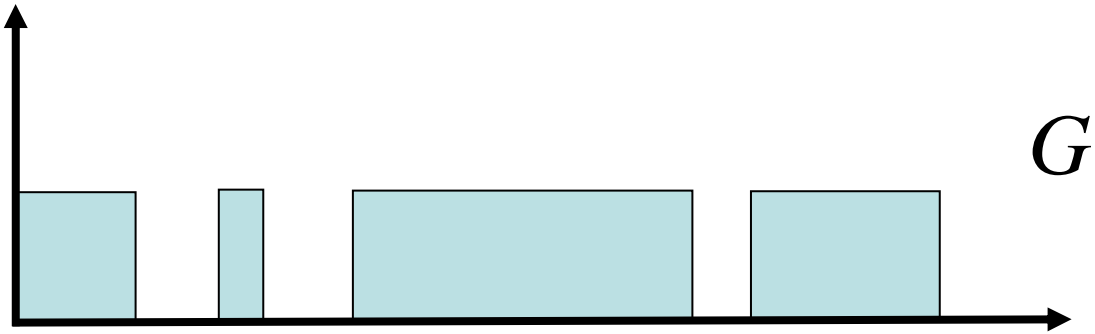
$$G(\tau) = \frac{\gamma}{N}\left(1 + \frac{\tau}{\tau_D}\right)^{-1} \cdot \left(1 + S^2 \cdot \frac{\tau}{\tau_D}\right)^{-\frac{1}{2}}$$

Blinking or other exponential processes:

If the particle blinks during the times it goes through the illumination volume, an additional term appears in the fluctuation amplitude.

How to account for this process??

Reasoning: let us assume that the particle is **not moving** and it is at the center of the PSF.
The intensity will turn **ON** and **OFF**.
The **OFF** time depends on the characteristic blinking time (triplet state lifetime).
The **ON** time depends on the laser intensity. The larger the laser intensity, the lesser is the **ON** time.



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

Triplet state term:

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

Blinking and binding processes

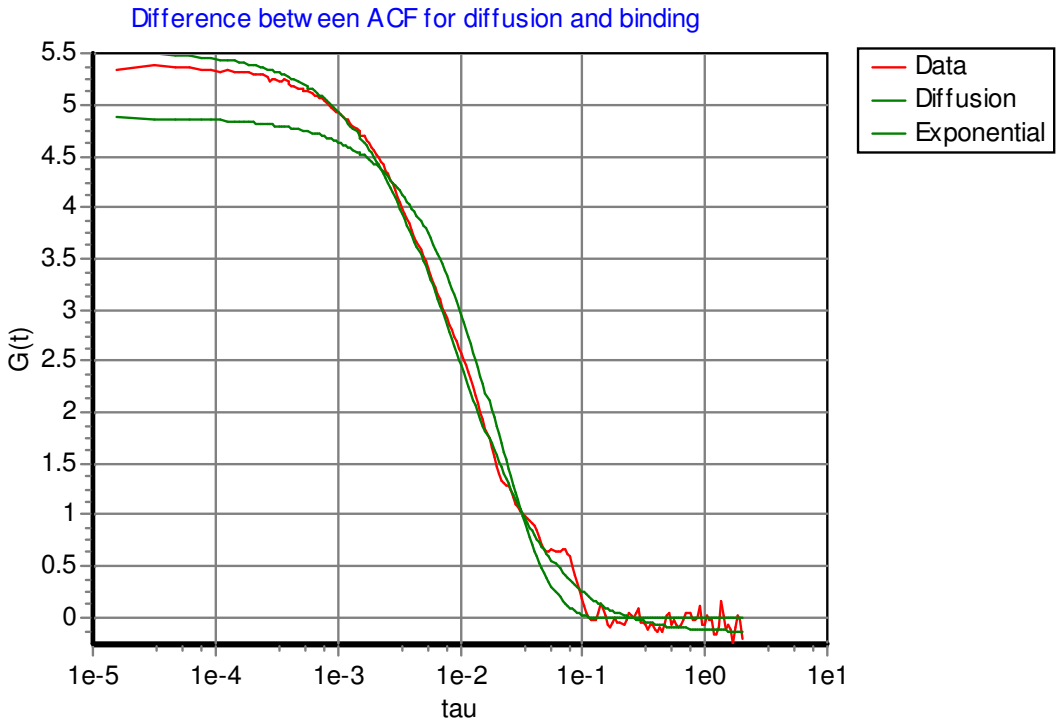
Until now, we assumed that the particle is **not moving**. If we assume that the blinking of the particle is **independent on its movement**, we can use a general principle that states that the correlation function splits in the product of the two independent processes.

$$G_{Total}(\tau) = G_{Blinking}(\tau) \cdot G_{Diffusion}(\tau)$$

$$G_{Binding}(\tau) = \left[1 + K \left(f_A - \frac{f_B}{K} \right)^2 e^{-\lambda\tau} \right]$$

$K = k_f / k_b$ is the equilibrium coefficient; $\lambda = k_f + k_b$ is the apparent reaction rate coefficient; and *fj* is the fractional intensity contribution of species *j*

How different is G(binding) from G(diffusion)?



With good S/N it is possible to distinguish between the two processes.
Most of the time diffusion and exponential processes are combined

Table of characteristic times for diffusion

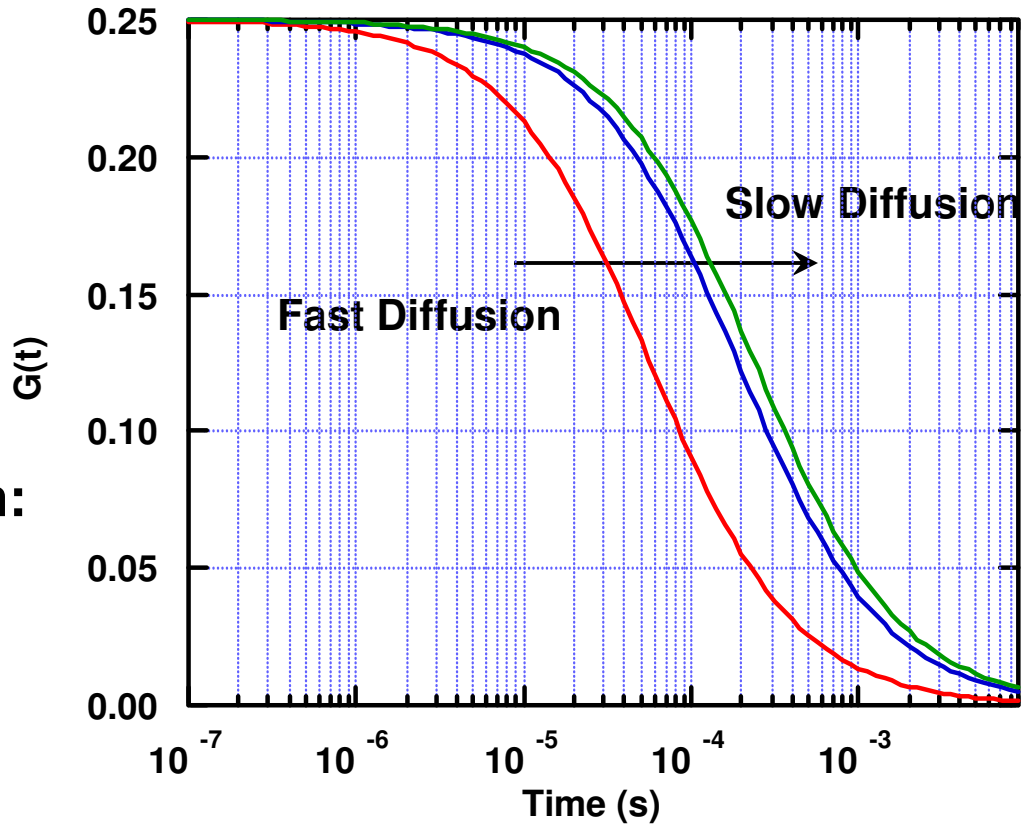
Orders of magnitude (for 1 μM solution, small molecule, water)

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:

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

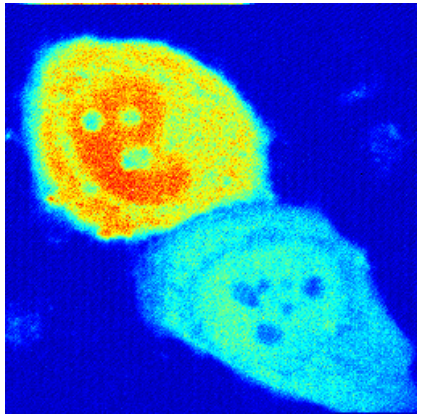
and

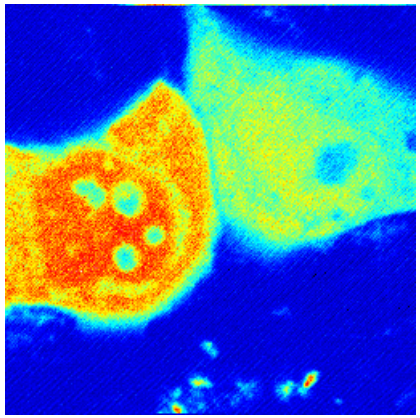
$$MW \propto Volume \propto r^3$$

Monomer --> Dimer

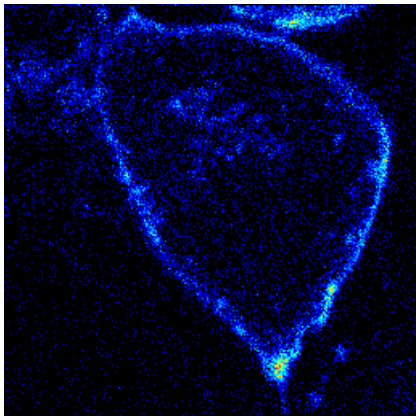
Only a change in D by a factor of $2^{1/3}$, or 1.26

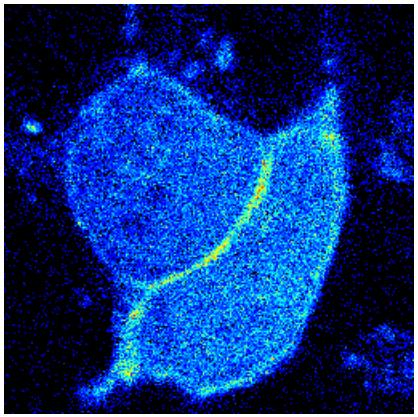
Autocorrelation Adenylate Kinase -EGFP Chimeric Protein in HeLa Cells





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



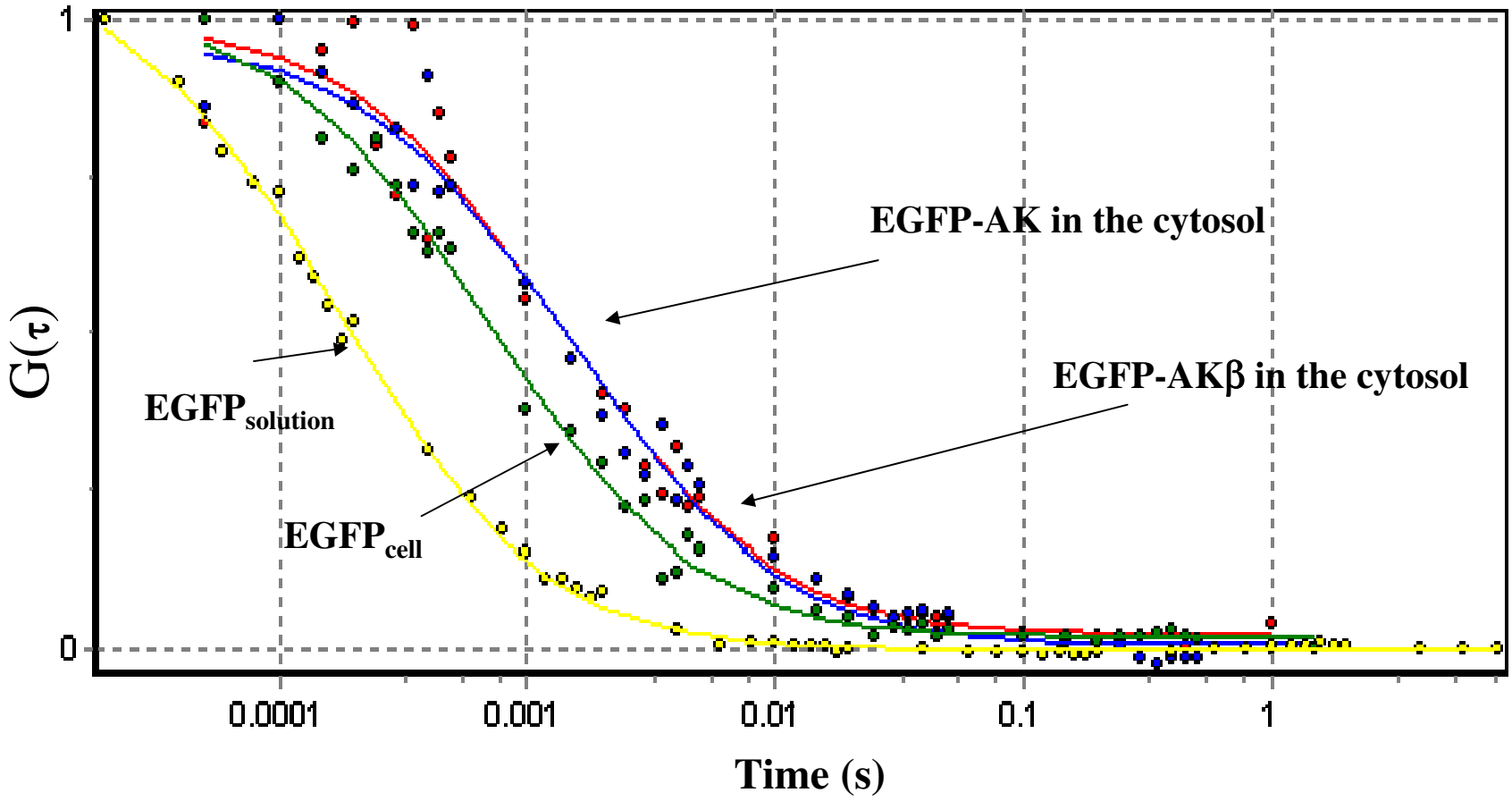


Examples of different *Hela* cells transfected with AK1β -EGFP

Fluorescence Intensity

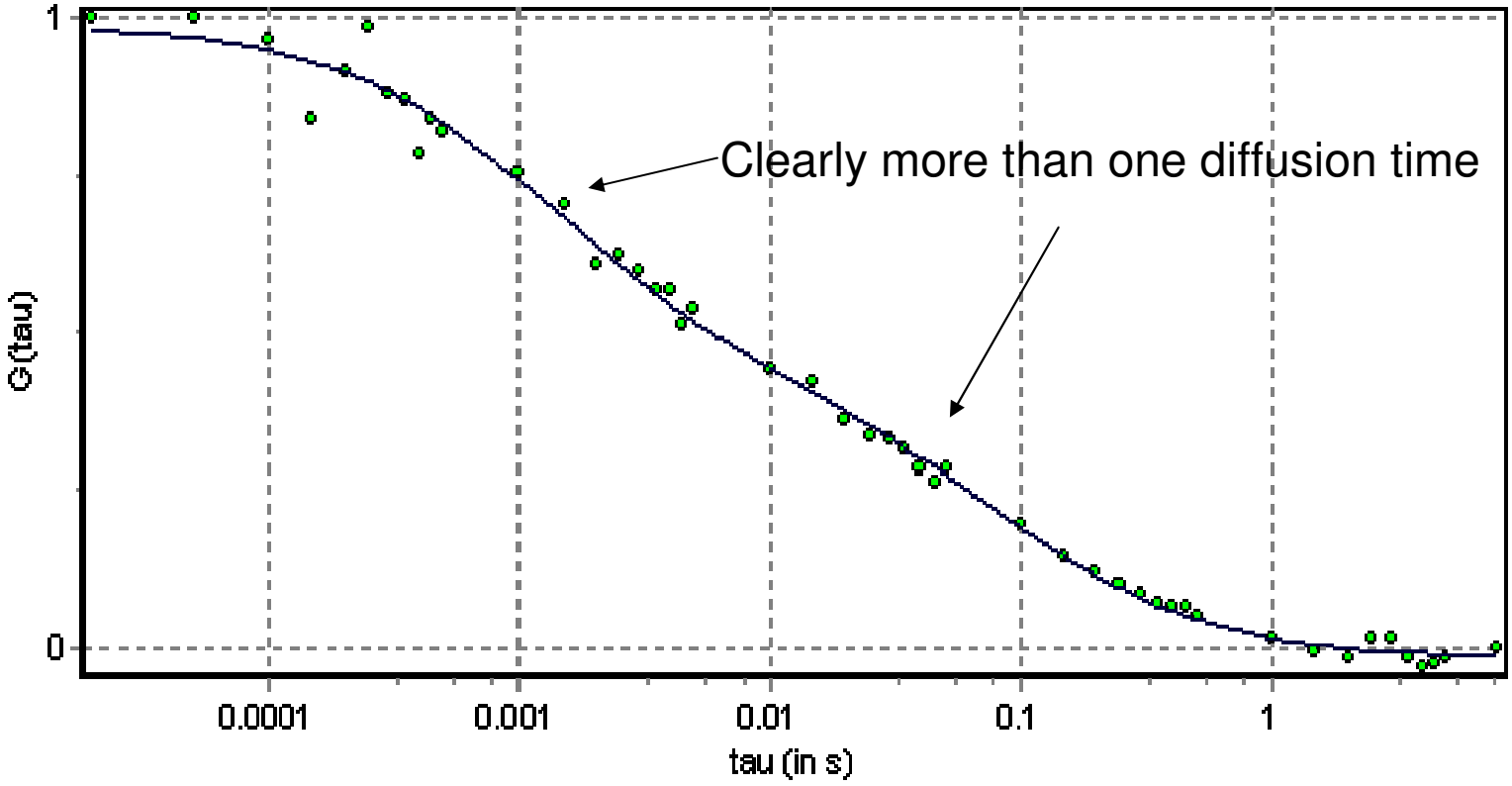
Qiao Qiao Ruan, Y. Chen, M. Glaser & W. Mantulin Dept. Biochem & Dept Physics- LFD Univ Il, USA

Autocorrelation of EGFP & Adenylate Kinase -EGFP



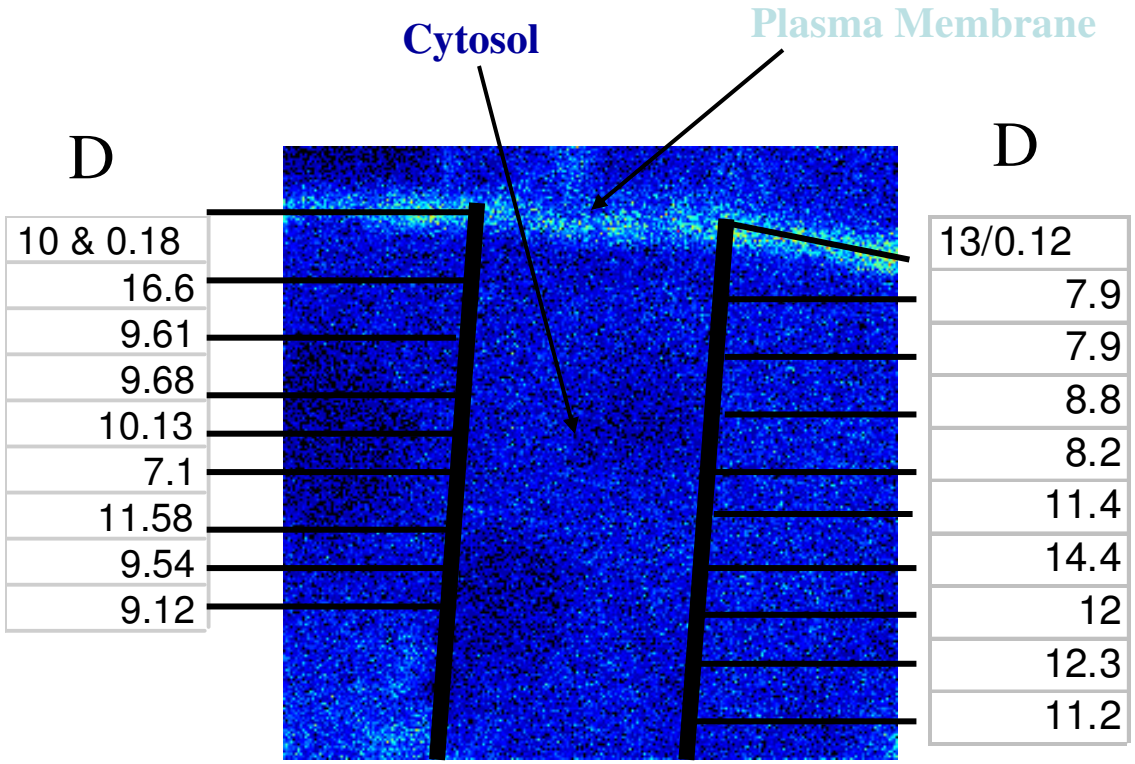
Normalized autocorrelation curve of EGFP in solution (•), EGFP in the cell (•), AK1-EGFP in the cell(•), AK1β-EGFP in the cytoplasm of the cell(•).

Autocorrelation of Adenylate Kinase –EGFP on the Membrane



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

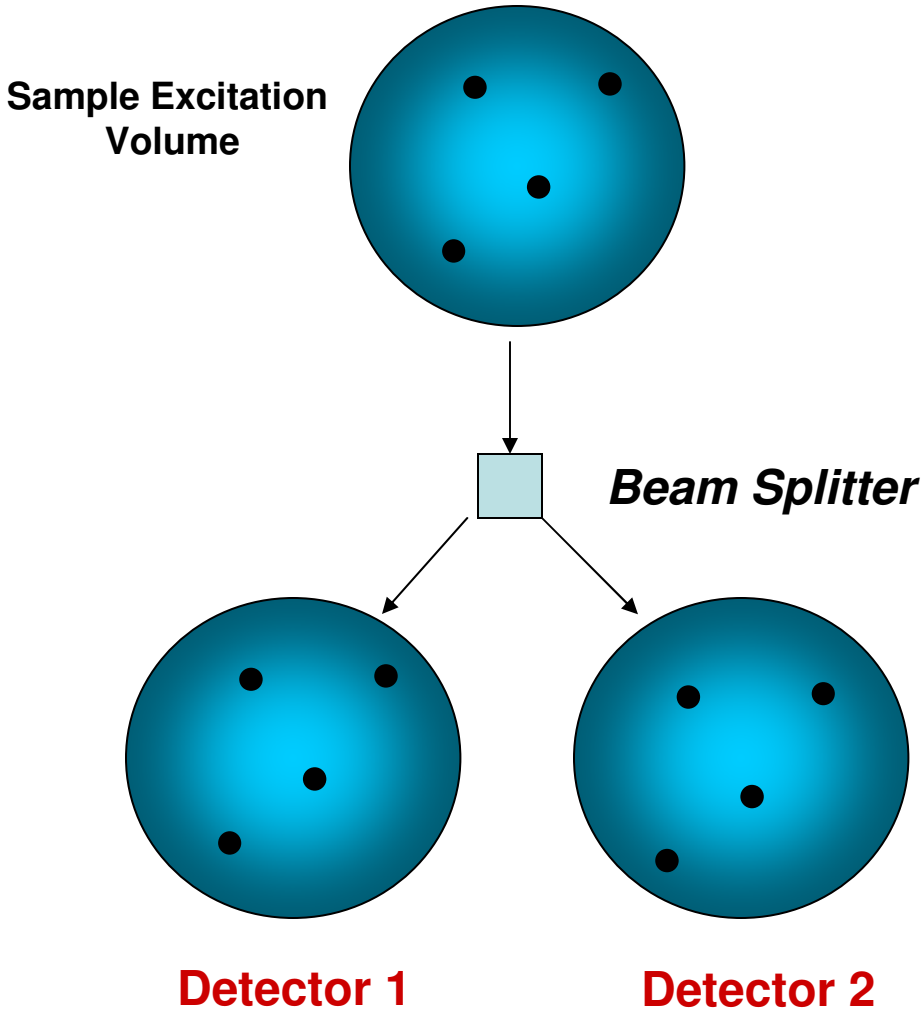
Autocorrelation Adenylate Kinaseβ -EGFP



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 constants are found.

Two Channel Detection: Cross-correlation

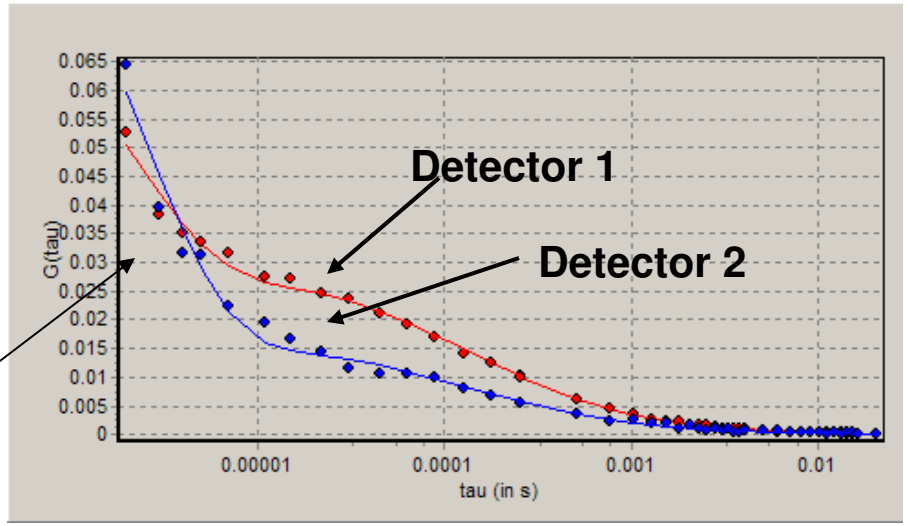
1. Increases signal to noise by isolating correlated signals.
2. Corrects for PMT noise



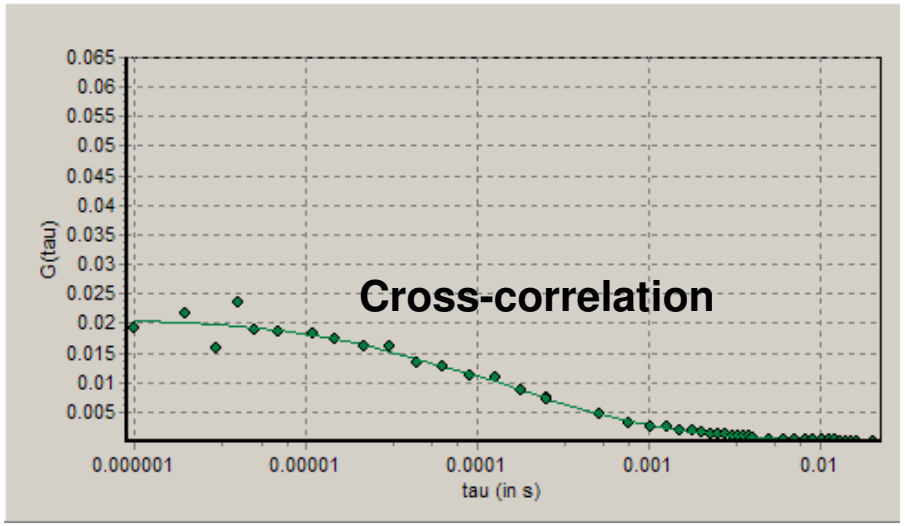
Each detector observes the same particles

Removal of Detector Noise by Cross-correlation

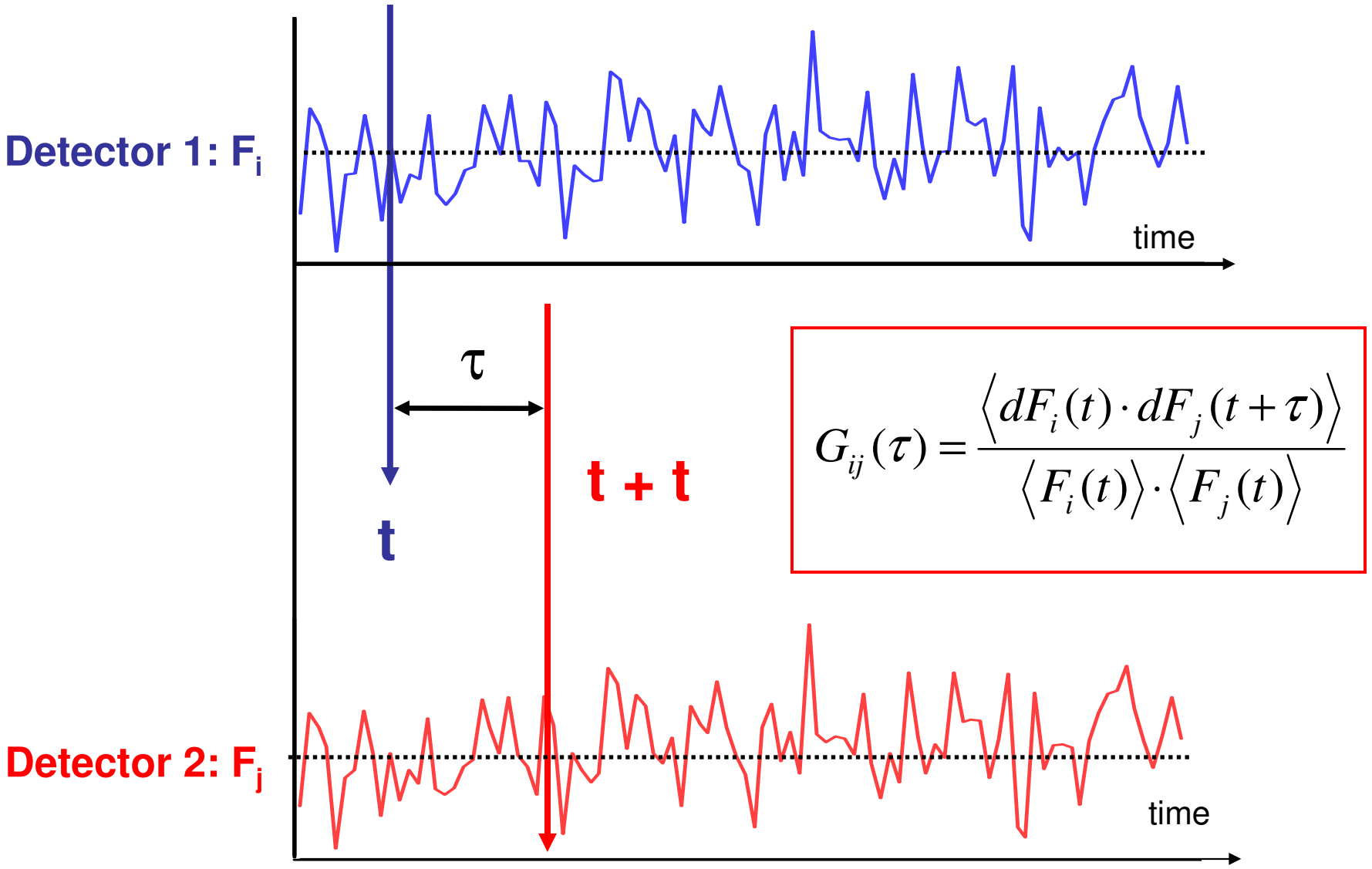
11.5 nM Fluorescein



Detector after-pulsing



Calculating the Cross-correlation Function



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

Cross-correlation calculations

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

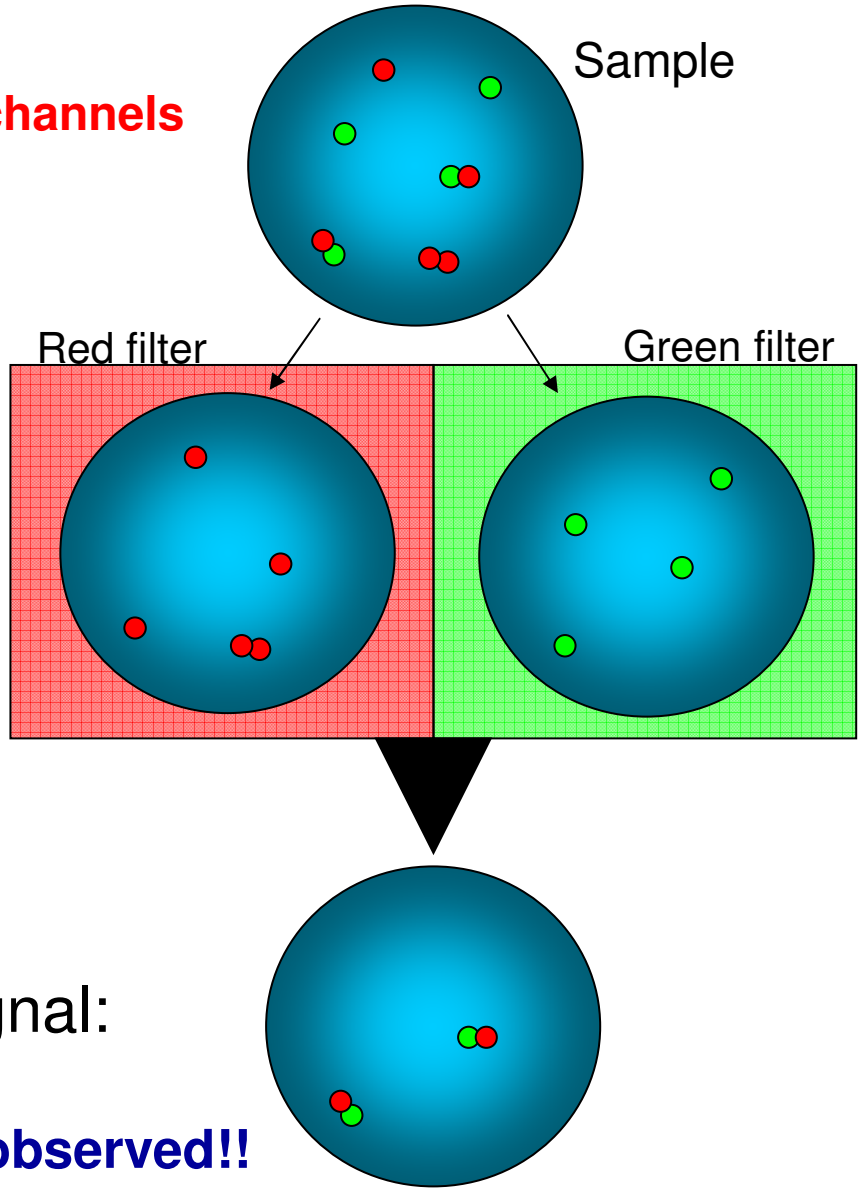
Thus, for a 3-dimensional Gaussian excitation volume one uses:

$$G_{12}(\tau) = \frac{\gamma}{N_{12}}\left(1+\frac{4D_{12}\tau}{w^2}\right)^{-1}\left(1+\frac{4D_{12}\tau}{z^2}\right)^{-1/2}$$

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.

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

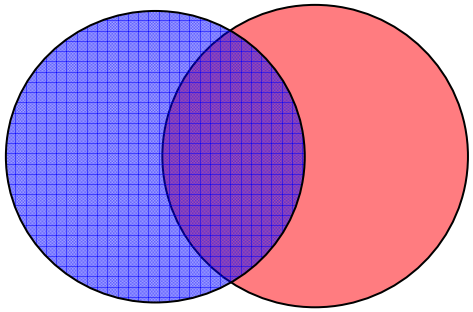
We assume exact match of the observation volumes in our calculations which is difficult to obtain experimentally.

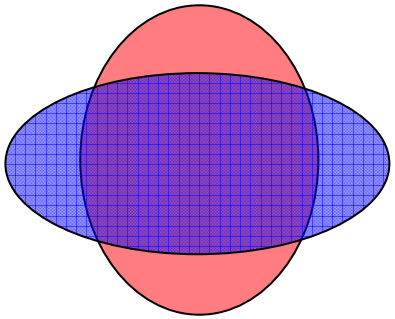
Excitation side:

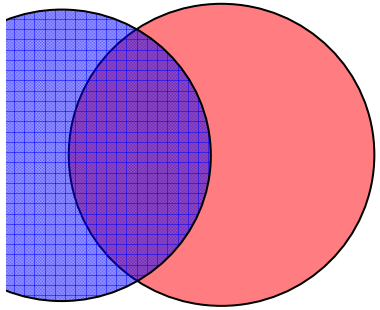
(1) Laser alignment
(2) Chromatic aberration
(3) Spherical aberration

Emission side:

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

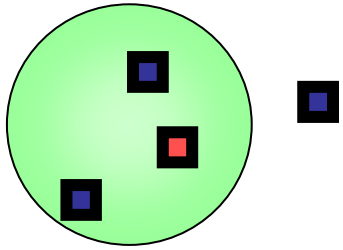






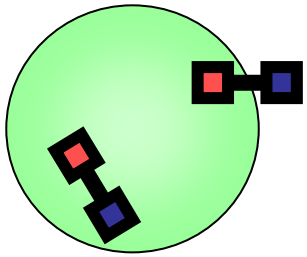
Two-Color Fluctuation Correlation Spectroscopy

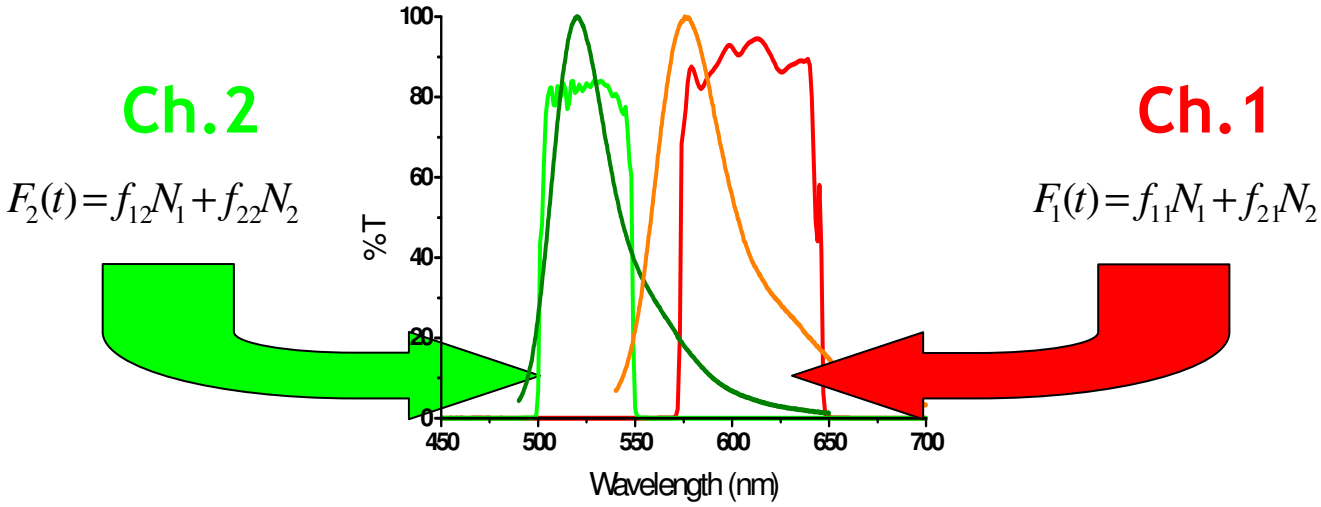
Uncorrelated



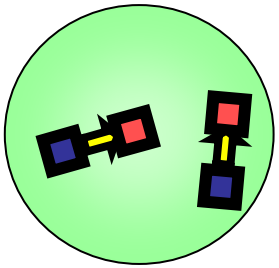
$$G_{ij}(\tau) = \frac{< F_i(t) F_j(t+\tau) >}{< F_i(t) >< F_j(t) >} - 1$$

Correlated





Interconverting



For two ***uncorrelated*** species, the amplitude of the cross-correlation is proportional to:

$$G_{12}(0) \propto \left[\frac{f_{11}f_{12}\langle N_1 \rangle + f_{21}f_{22}\langle N_2 \rangle}{f_{11}f_{12}\langle N_1 \rangle^2 + (f_{11}f_{22} + f_{21}f_{12})\langle N_1 \rangle\langle N_2 \rangle + f_{21}f_{22}\langle N_2 \rangle^2} \right]$$

Applications: Cross-correlation

Ramesh C Patel, Ujendra Kumar, Don C Lamb, John S Eid, Magalie Rocheville, Michael Grant, Aruna Rani, Theodore L Hazlett, Shutish C Patel, Enrico Gratton, and Yogesh C Patel.

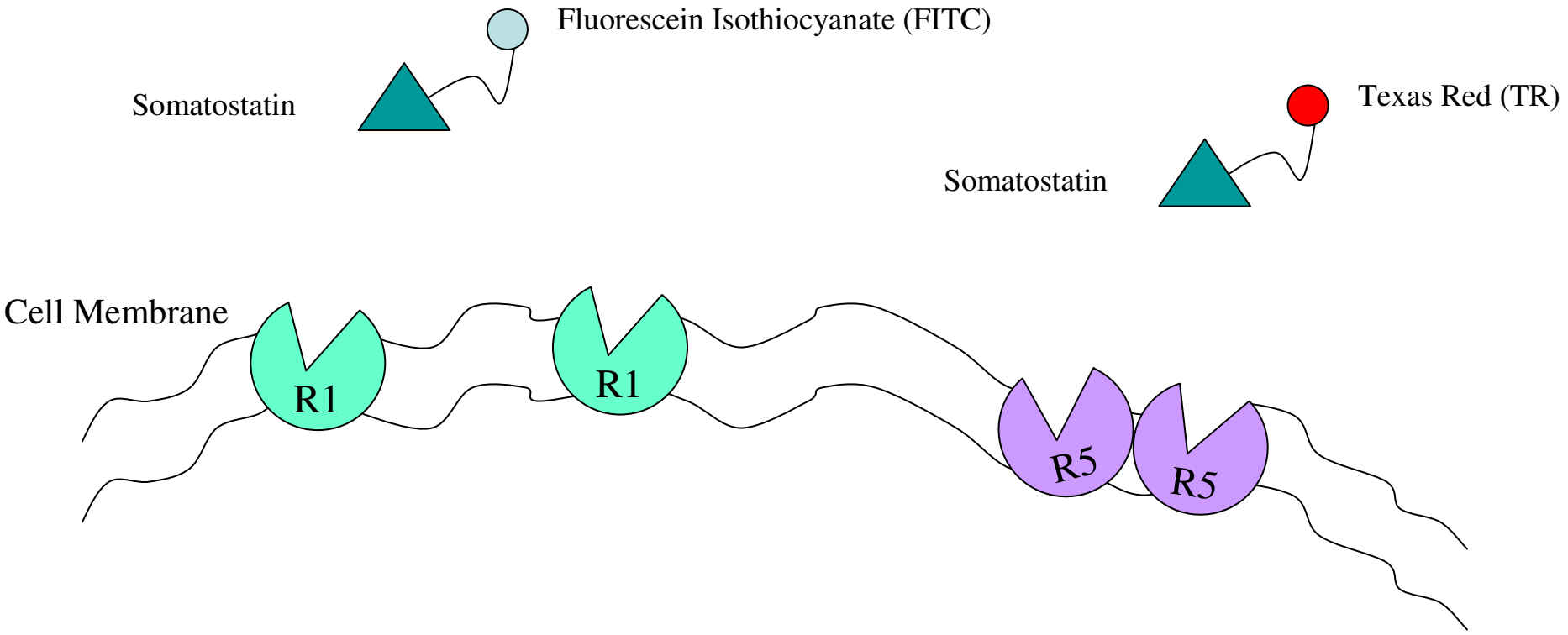
Ligand binding to somatostatin receptors induces receptor-specific oligomer formation in live cells.

Proc Natl Acad Sci USA. 2002; 99(5): 3294-9. PMCID: PMC122512

Does SSTR1 exist as a monomer after ligand binding while SSTR5 exists as a dimer/oligomer?

Collaboration with Ramesh Patel*† and Ujendra Kumar*

*Fraser Laboratories, Departments of Medicine, Pharmacology, and Therapeutics and Neurology and Neurosurgery, McGill University, and Royal Victoria Hospital, Montreal, QC, Canada H3A 1A1; †Department of Chemistry and Physics, Clarkson University, Potsdam, NY 13699

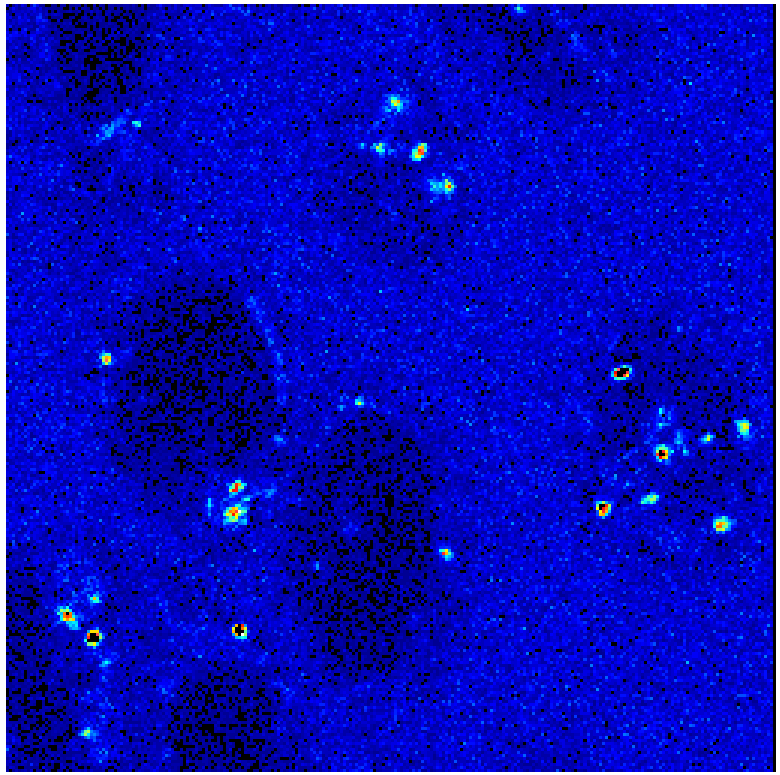


Three Different CHO-K1 cell lines: wt R1, HA-R5, and wt R1/HA-R5

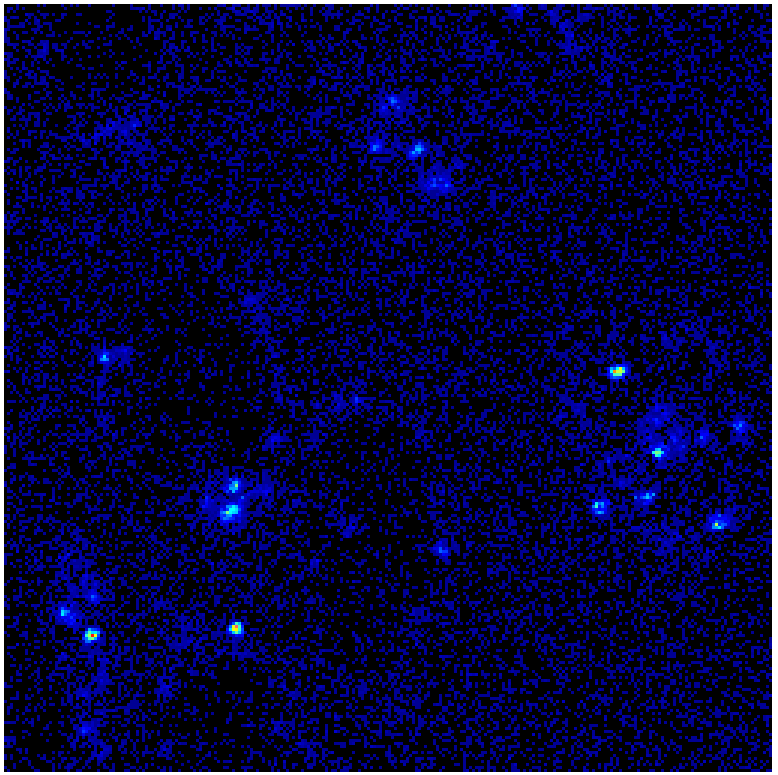
Hypothesis: R1- monomer ; R5 - dimer/oligomer; R1R5 dimer/oligomer

SSTR1 CHO-K1 cells with SST-fitc + SST-tr

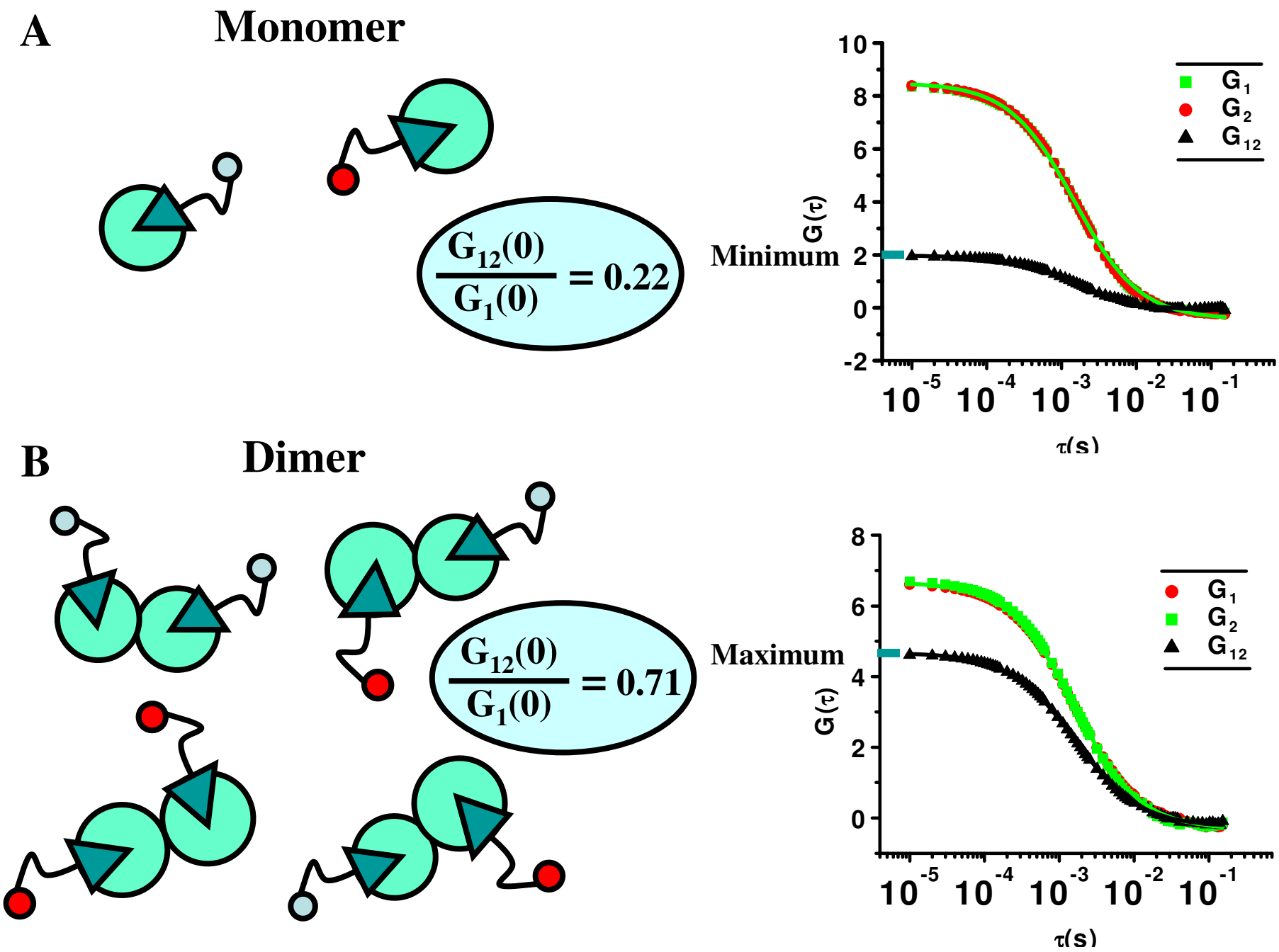
Green Ch.



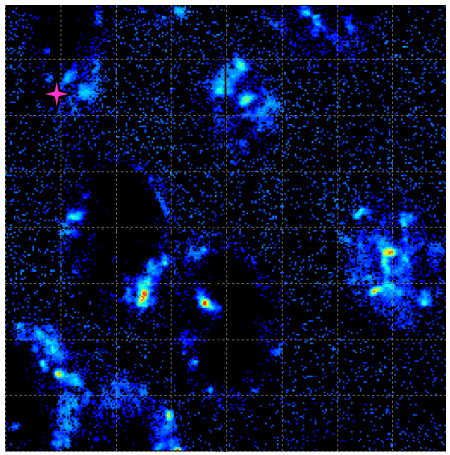
Red Ch.

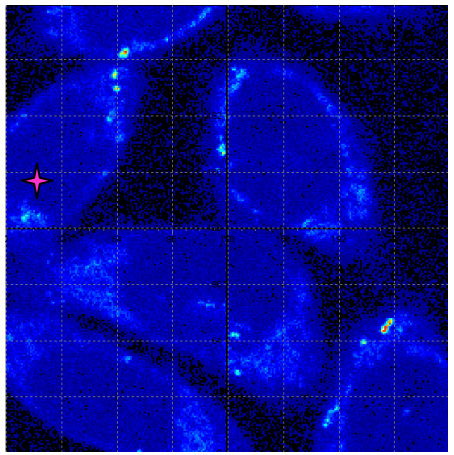


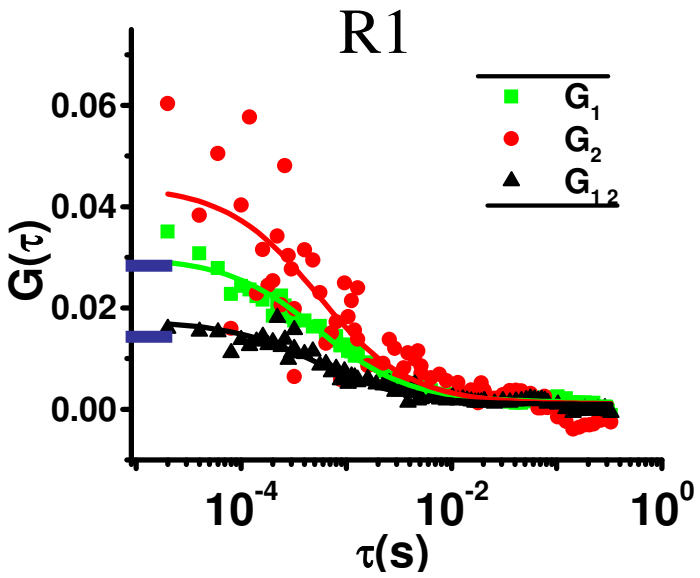
- Very little labeled SST inside cell nucleus
- Non-homogeneous distribution of SST
- Impossible to distinguish co-localization from molecular interaction

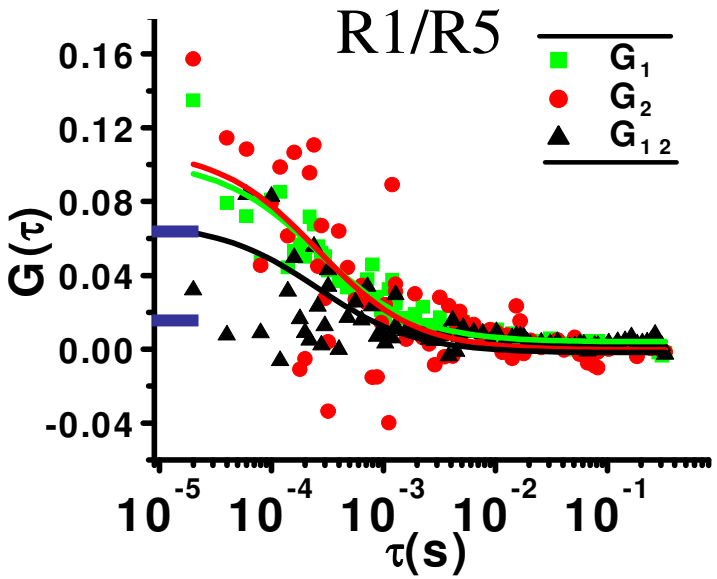

A
Monomer
$\frac{G_{12}(0)}{G_1(0)} = 0.22$
Minimum
G(τ)
τ(s)
G_1
G_2
G_{12}
B
Dimer
$\frac{G_{12}(0)}{G_1(0)} = 0.71$
Maximum
G(τ)
τ(s)
G_1
G_2
G_{12}

Experimentally derived auto- and cross-correlation curves from live R1 and R5/R1 expressing CHO-K1 cells using dual-color two-photon FCS.









The R5/R1 expressing cells have a greater cross-correlation relative to the simulated boundaries than the R1 expressing cells, indicating a higher level of dimer/oligomer formation.

Patel, R.C., *et al.*, *Ligand binding to somatostatin receptors induces receptor-specific oligomer formation in live cells.* PNAS, 2002. **99**(5): p. 3294-3299

Discussion

1. The PSF: how much it affects our estimation of the processes?
2. Models for diffusion, anomalous?
3. Binding?
4. FRET (dynamic FRET)?
5. Bleaching?

6. ……and many more questions

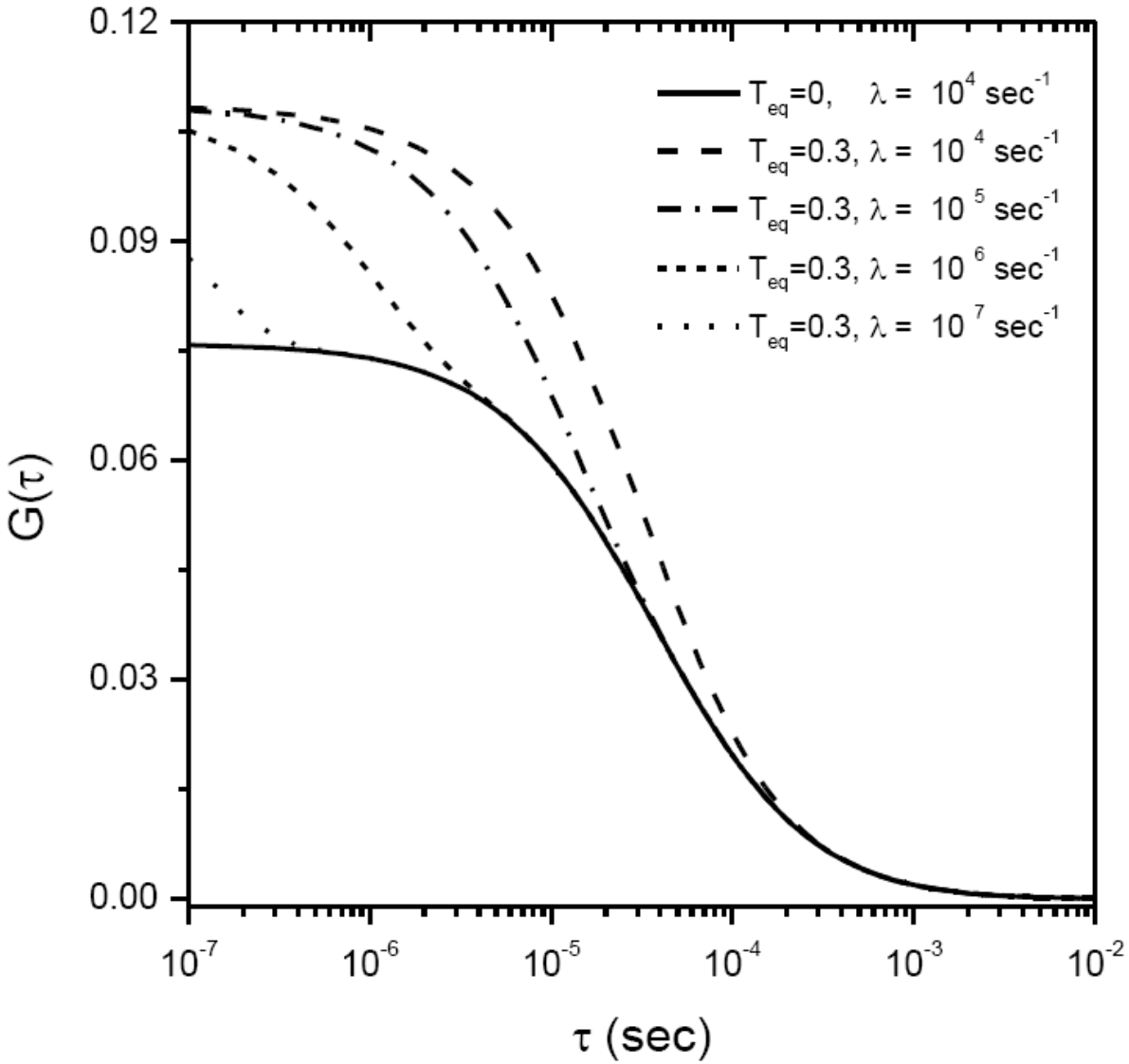
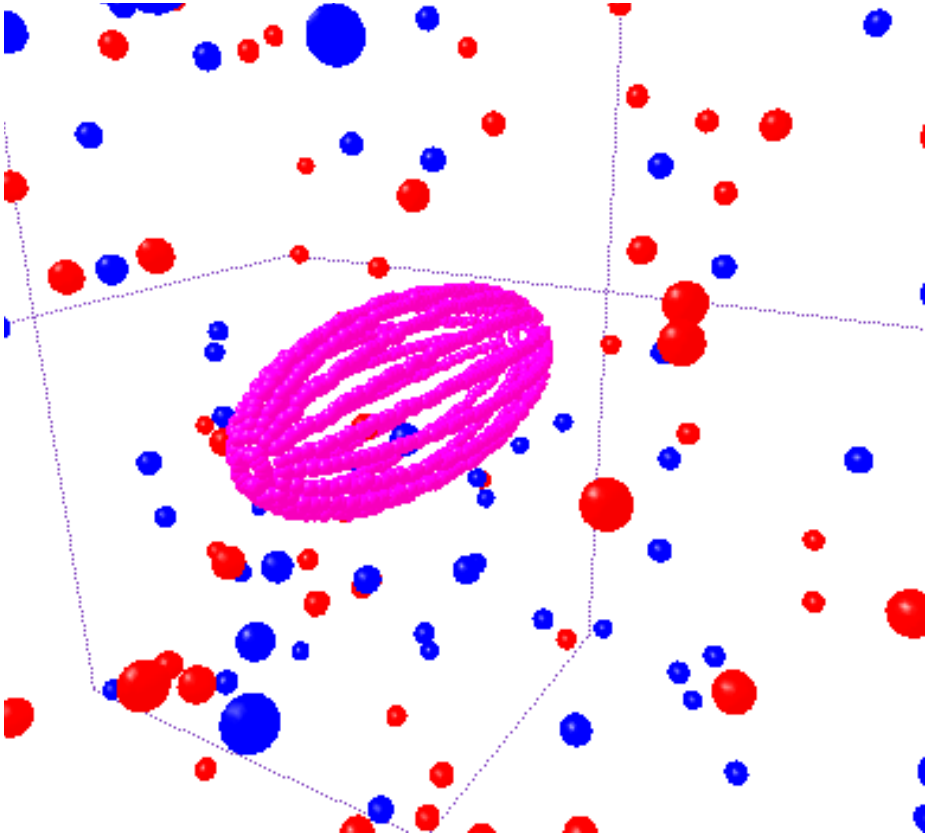


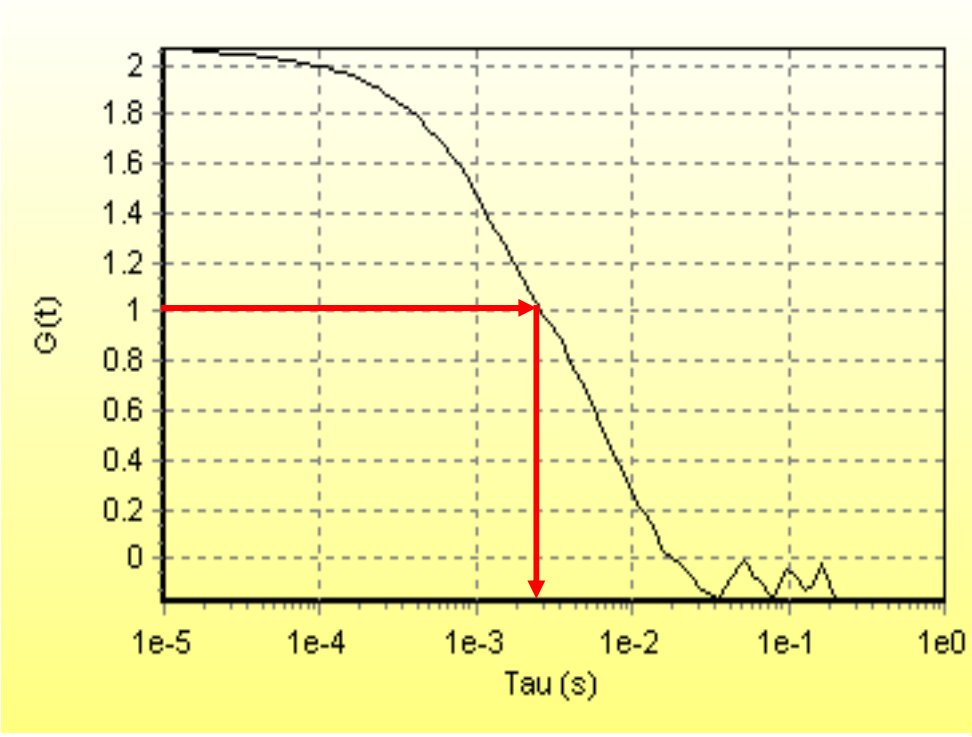
Figure 4.2 Simulation of autocorrelation functions using equation (4.12). The diffusion coefficient used is $300\,\mu m^2/\sec$, $w_{3DG} = 0.3\mu m$, $z_{3DG} = 1.5\mu m$.



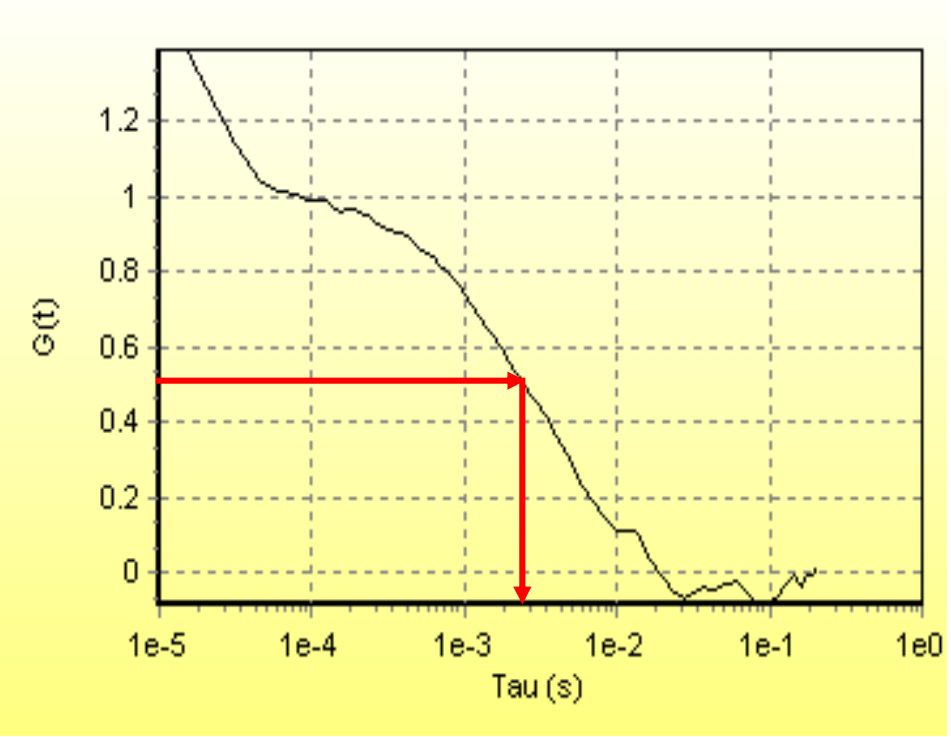
Box size=6.4 μm
Diffusion coefficient D=23 μm^2/s
Periodic boundary conditions

$\tau_D = w^2/8D = 2.6$ ms

100 red and 100 blue particles in the box. The detector is sensitive only to the blue particles. The particles perform a random motion in 3D. At random times after excitation, the blue particle (in the singlet state) can convert into the red particle (in the triplet state). After about 10^{-5}s, the triplet state decays and the particle returns to be blue (singlet state). The particle is only detected when inside the illumination volume (in pink). The intensity is properly weighted according to a 3-D Gaussian intensity model



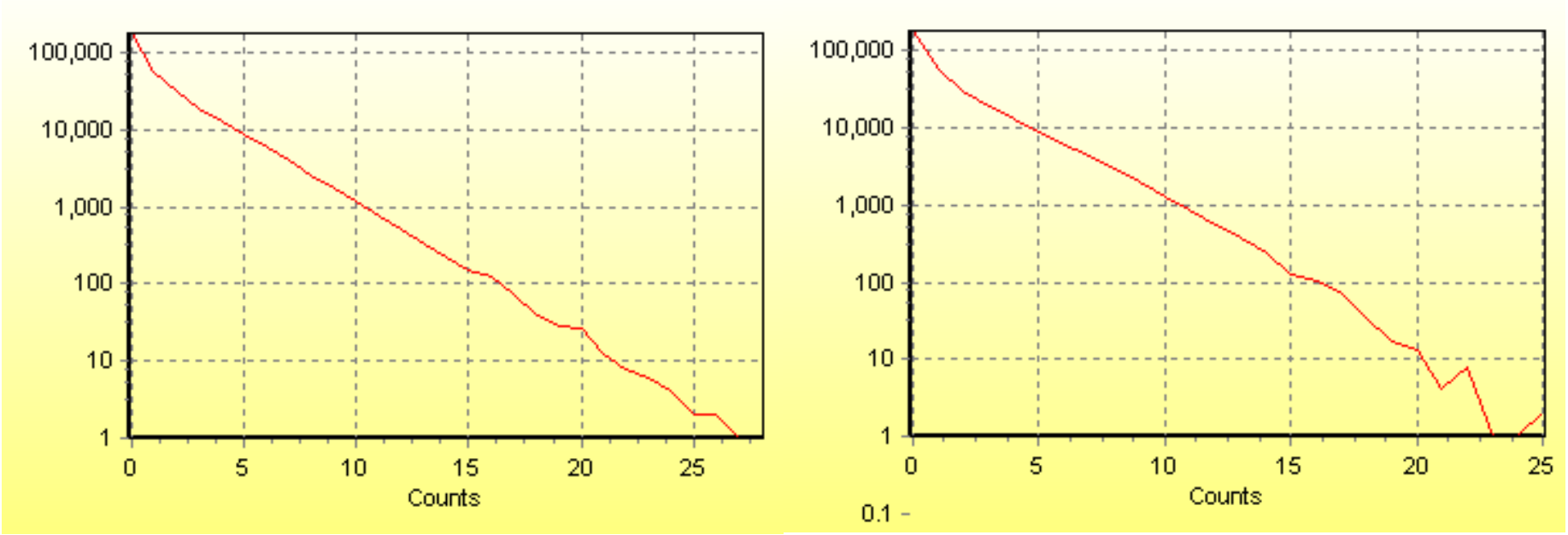
Correlation function for **pure diffusion**



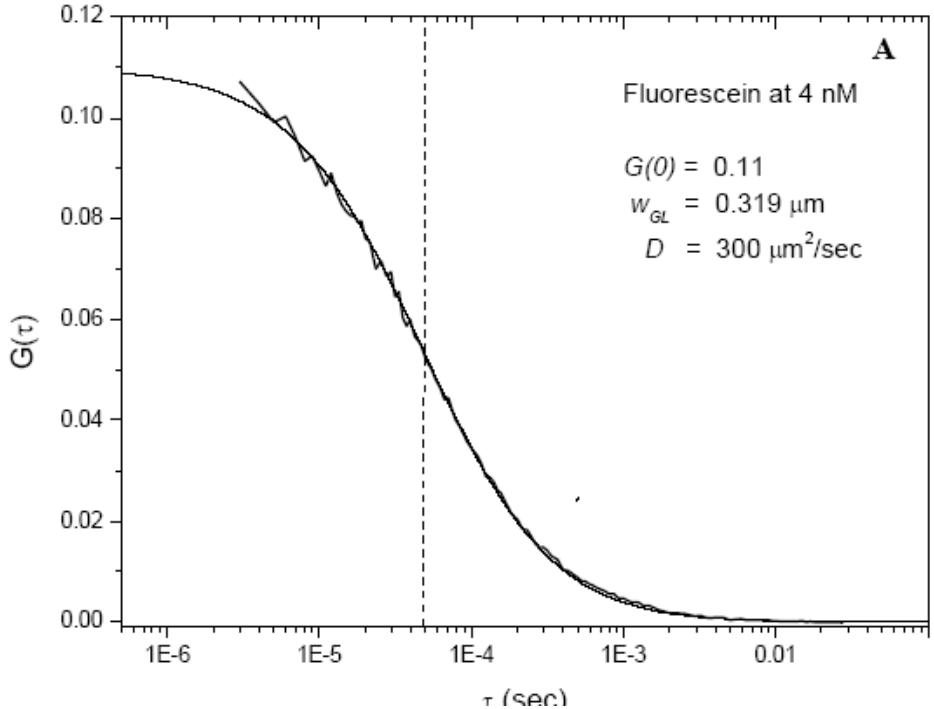
Correlation function for **diffusion and excited-state reaction** (triplet state)

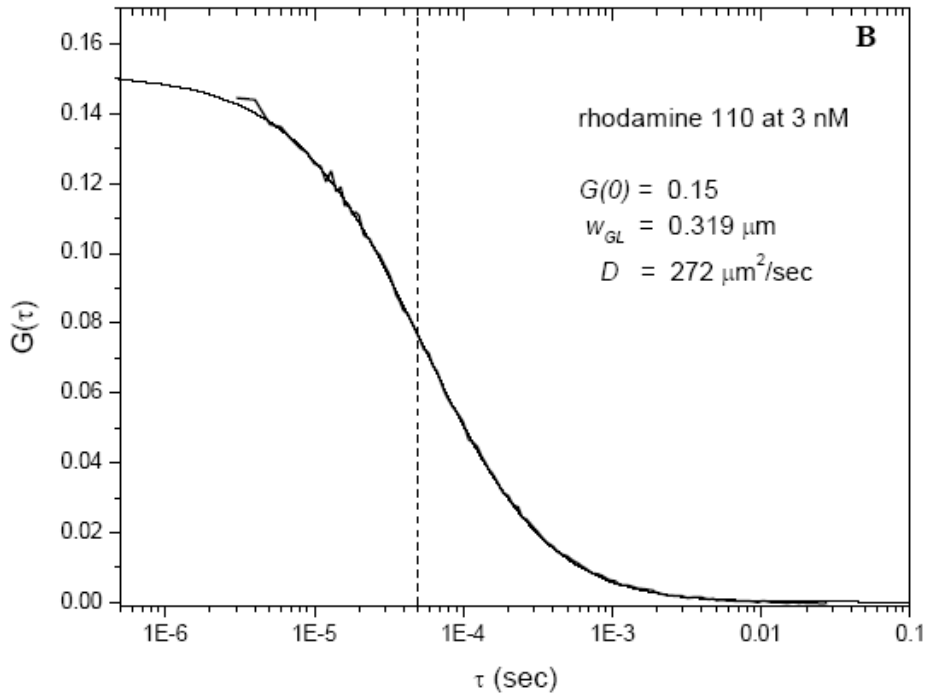
Panel 1: 100 particles in a box of approximately 6.4 μm side and a PSF of 0.5 μm waist and 1.5 μm axial waist.

Panel 2: 200 particles in a box. All particles undergo an excited state reaction with a decay rate of 10^{-5}s. The system is at equilibrium with half the particles in the triplet excited state. What is the apparent G(0) in panel 2? Why are the two correlation functions different?



Photon counting histogram for the sample with 100 particles in a box (panel 1) and with 200 particles (panel 2) undergoing an excited state reaction at a rate of 10^{-5}s. The system is at equilibrium and half of the particles are in the triplet excited state. Why are the two histograms identical (within noise)?


A
Fluorescein at 4 nM
G(0) = 0.11
w_{GL} = 0.319 μm
D = 300 μm²/sec
G(τ)
τ (sec)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1E-6
1E-5
1E-4
1E-3
0.01


B
rhodamine 110 at 3 nM
G(0) = 0.15
w_{GL} = 0.319 μm
D = 272 μm²/sec
G(τ)
τ (sec)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1E-6
1E-5
1E-4
1E-3
0.01
0.1

Multiple Species

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

Autocorrelation function can be used:

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

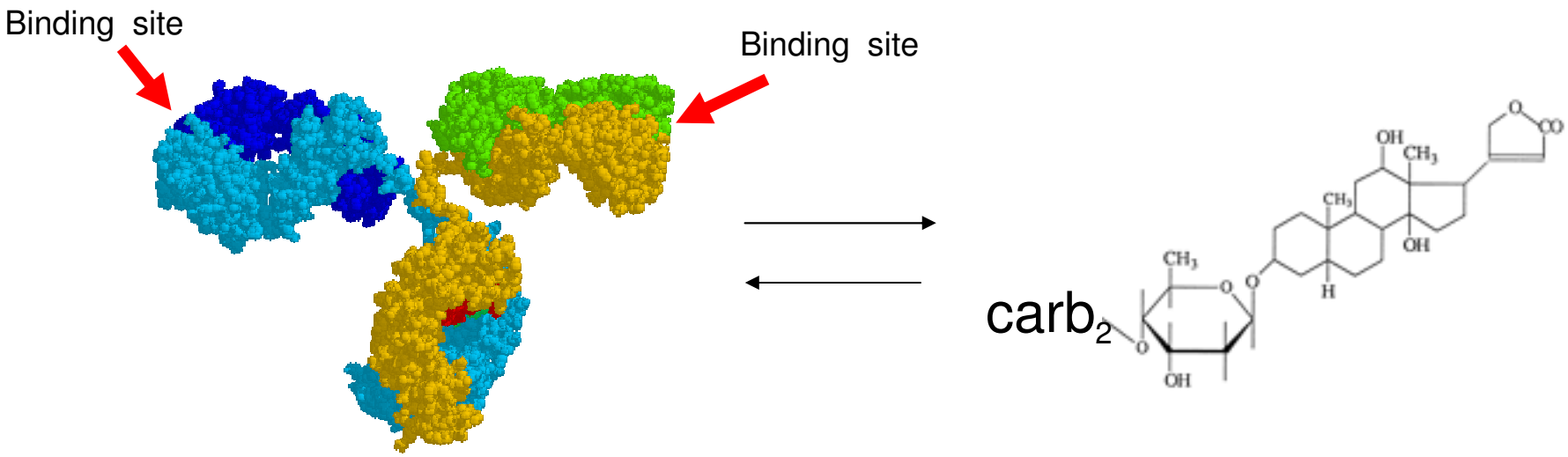
(2D-Gaussian Shape)

!

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

$G(0)_{sample}$ is no longer g/N !

Antibody - Hapten Interactions

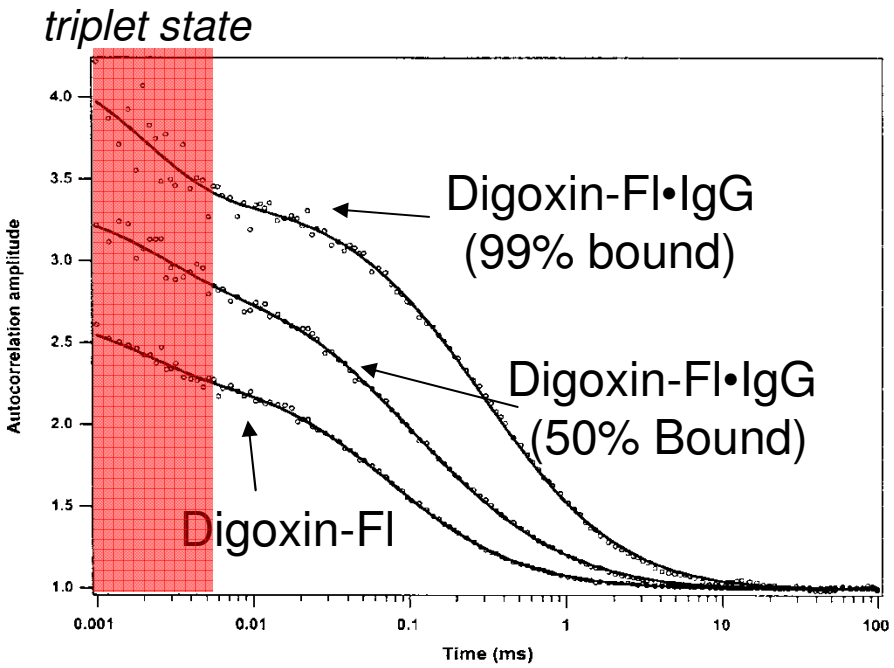


Mouse IgG: The two heavy chains are shown in yellow and light blue. The two light chains are shown in green and dark blue..*J.Harris, S.B.Larson, K.W.Hasel, A.McPherson, "Refined structure of an intact IgG2a monoclonal antibody", Biochemistry 36: 1581, (1997)*.

Digoxin: a cardiac glycoside used to treat congestive heart failure. Digoxin competes with potassium for a binding site on an enzyme, referred to as potassium-ATPase. Digoxin inhibits the Na-K ATPase pump in the myocardial cell membrane.

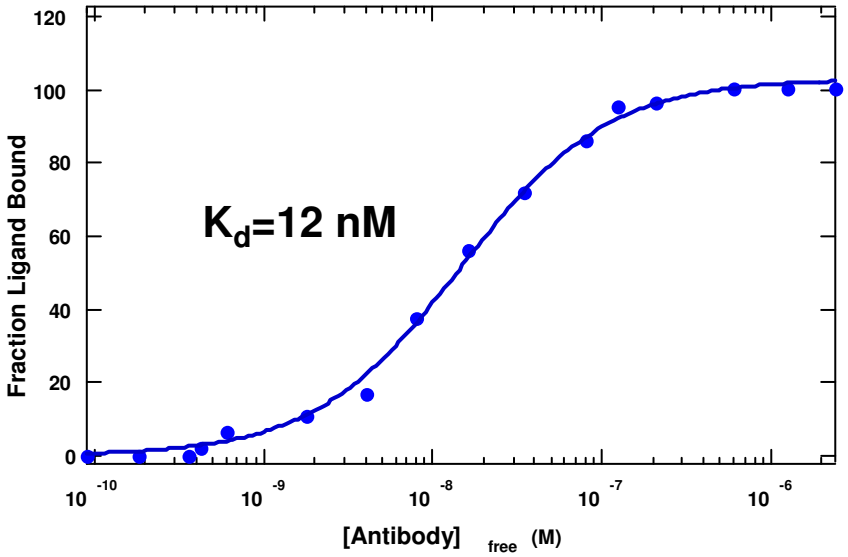
Anti-Digoxin Antibody (IgG) Binding to Digoxin-Fluorescein

Autocorrelation curves:



Binding titration from the autocorrelation analyses:

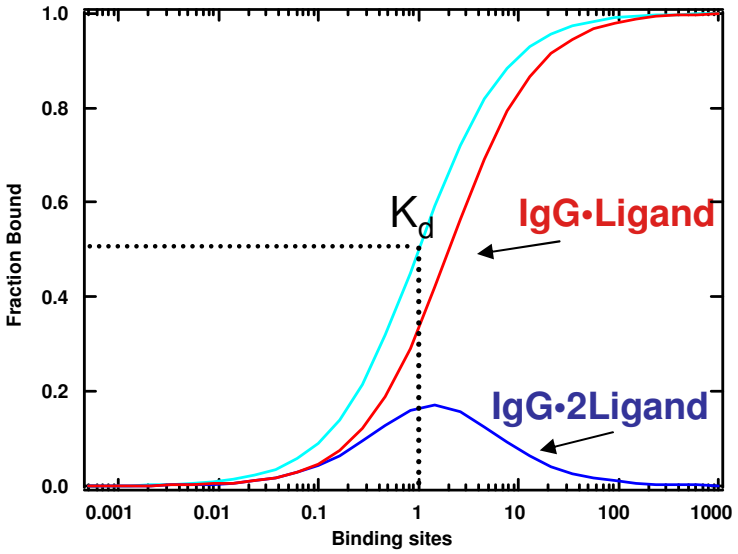
$$F_b = \frac{m \cdot S_{free}}{K_d + S_{free}} + c$$

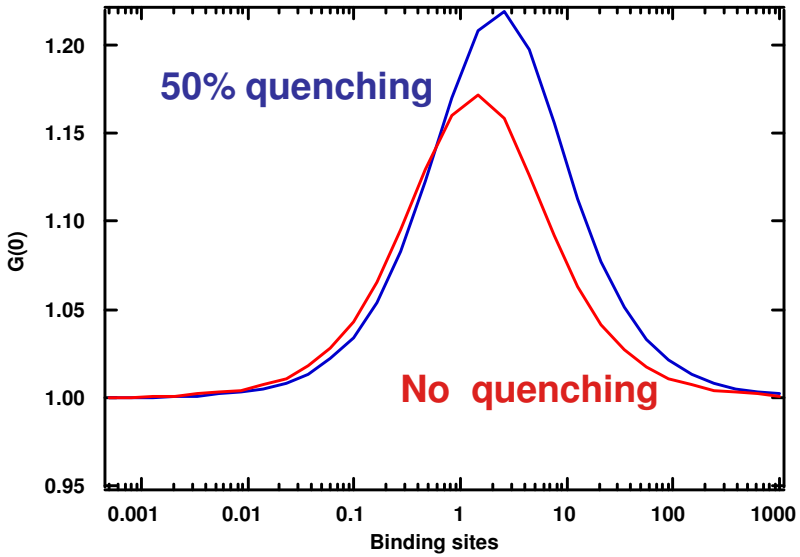


S. Tetin, K. Swift, & , E, Matayoshi , 2003

Two Binding Site Model

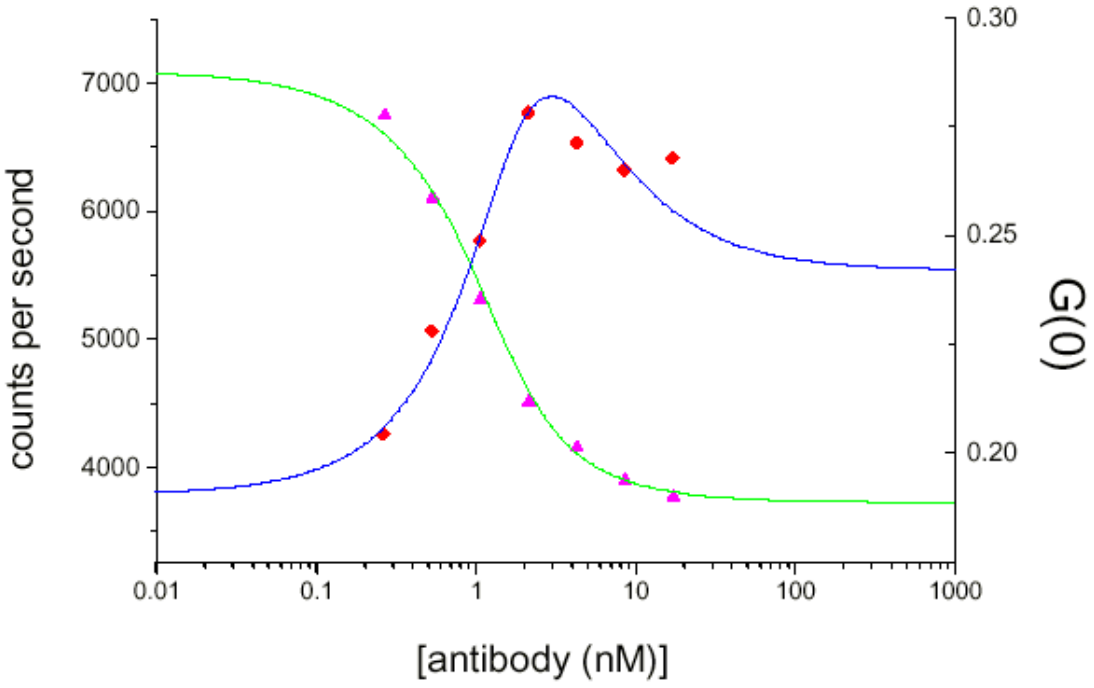
IgG + 2 Ligand-Fl ⟷ IgG•Ligand-Fl + Ligand-Fl ⟷ IgG•2Ligand-Fl





[Ligand]=1, G(0)=1/N, K_d=1.0

Digoxin-FL Binding to IgG: G(0) Profile



	Lifetime (nsec)	molecular fraction (lifetime)	*cpsm*	Molecular fraction ($G(0)$)
Digoxin	4.01	100%	29000	100%
Ligated Digoxin(C_1)	4.03	53.6%	23600	52%
Ligated Digoxin(C_2)	1.25	46.4%	7100	48%

Y. Chen , Ph.D. Dissertation; Chen et. al., Biophys. J (2000) 79: 1074

Case 2: Species vary by a difference in brightness

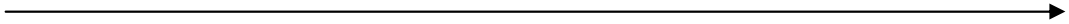
assuming that $D_1 \approx D_2$

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

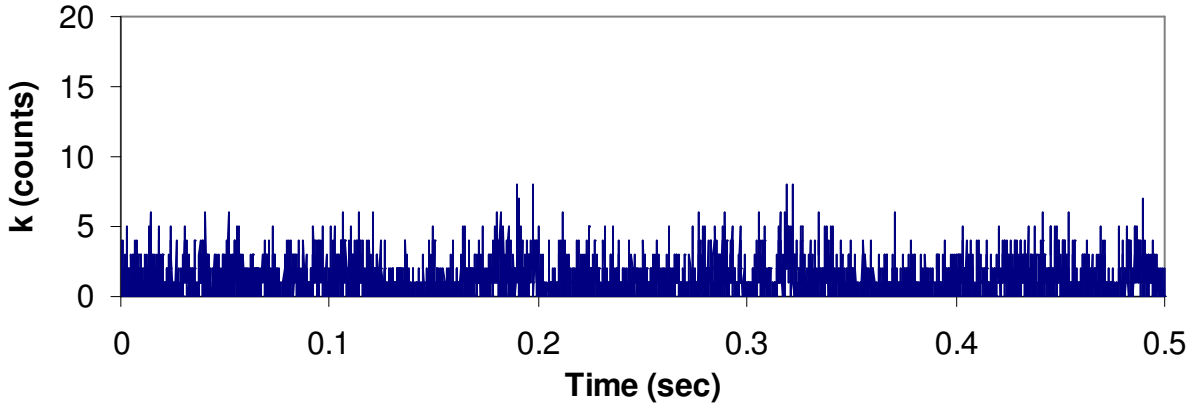


The Photon Counting Histogram: Statistical Analysis of Single Molecule Populations

Transition from FCS

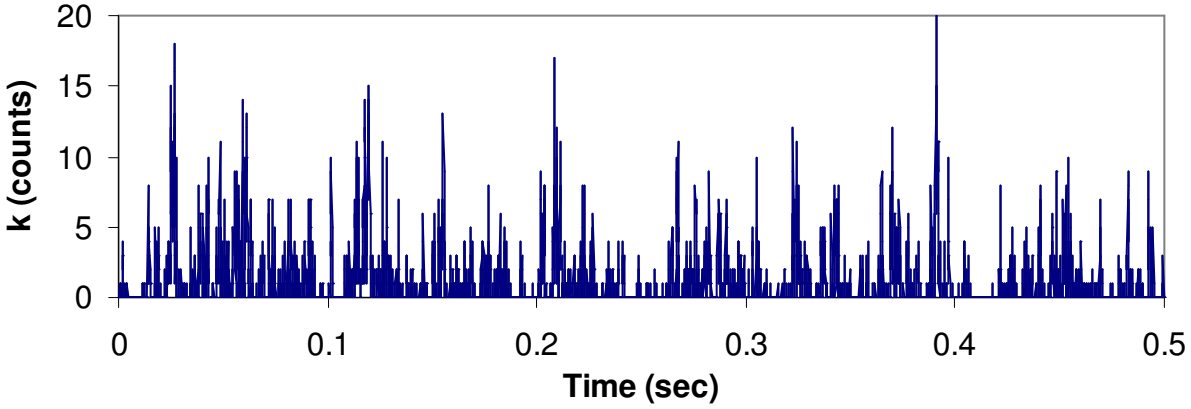
- The Autocorrelation function only depends on fluctuation duration and fluctuation density (independent of excitation power)
- PCH: distribution of intensities (independent of time)

Fluorescence Trajectories



Fluorescent Monomer:

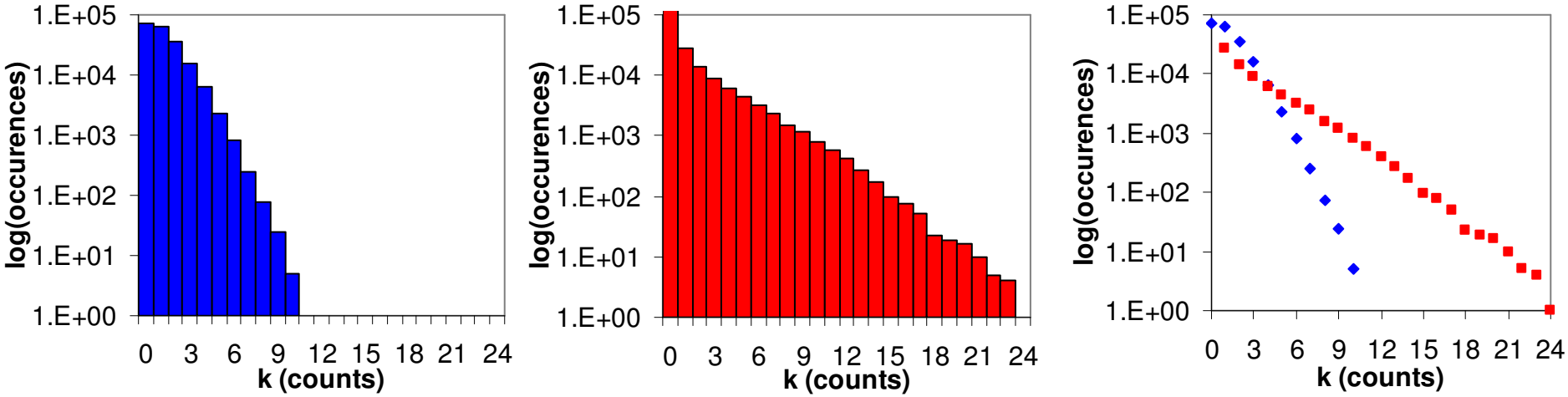
Intensity = 115,000 cps



Aggregate:

Intensity = 111,000 cps

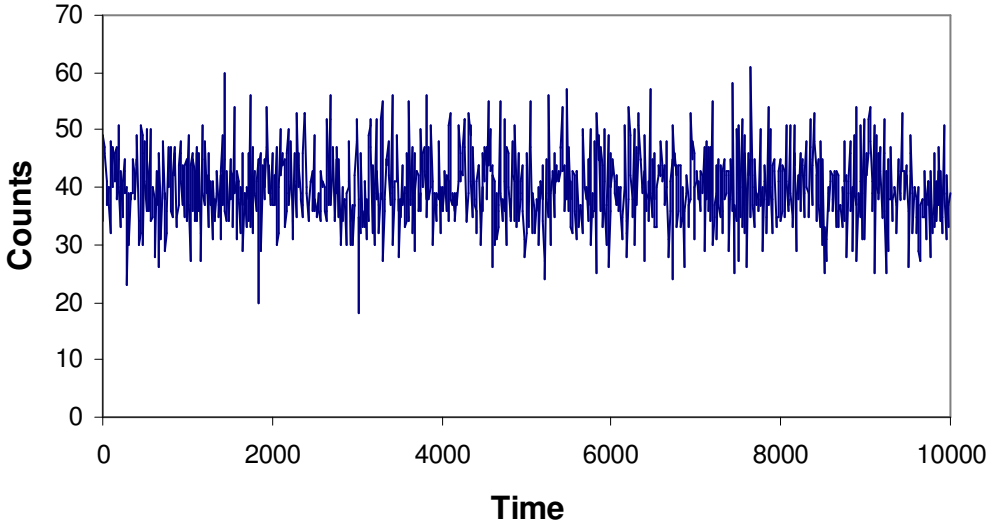
Photon Count Histogram (PCH)

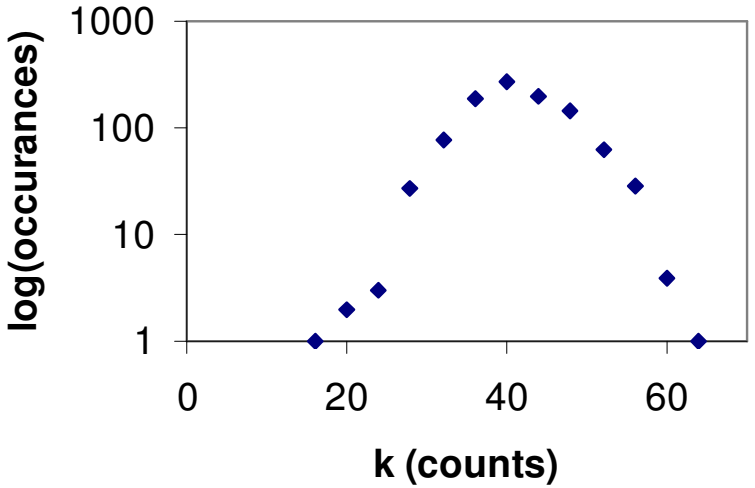


Can we quantitate this?

What contributes to the distribution of intensities?

Contribution from the detector noise Fixed Particle Noise (Shot Noise)



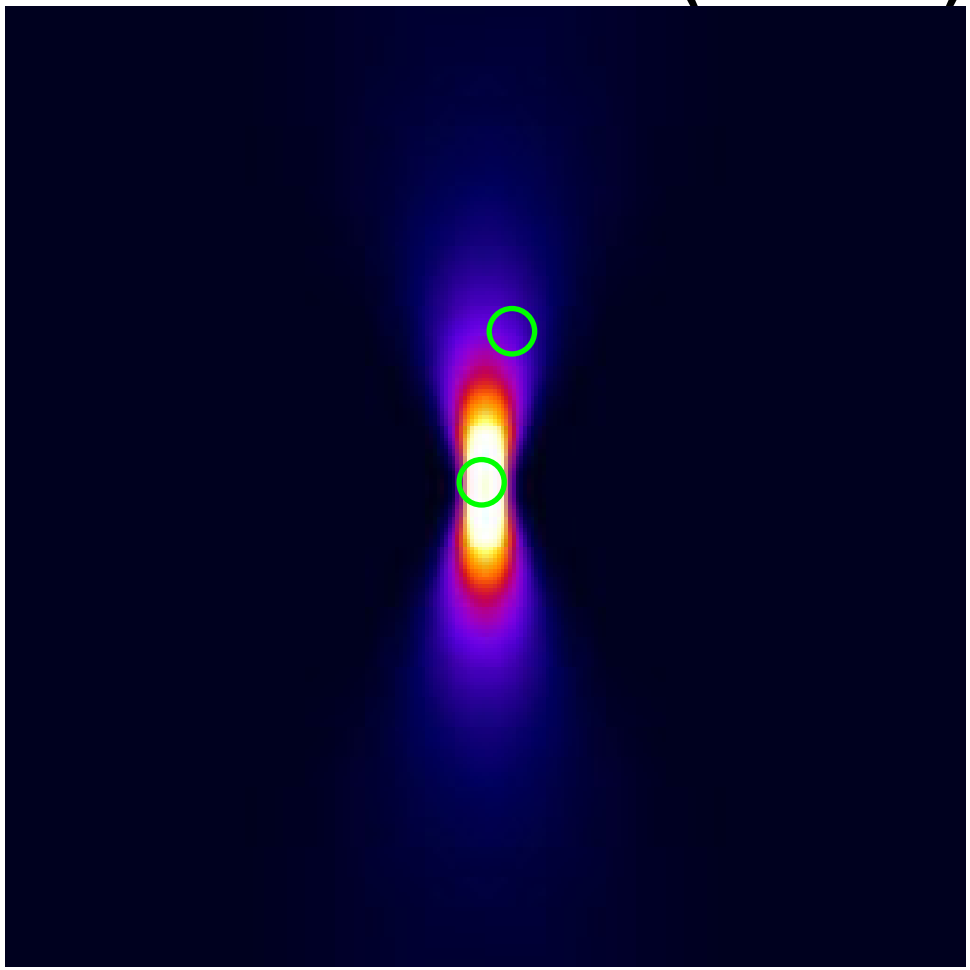


Noise is Poisson

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

Contribution from the profile of illumination

The Point Spread Function (PSF)



One Photon Confocal:

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

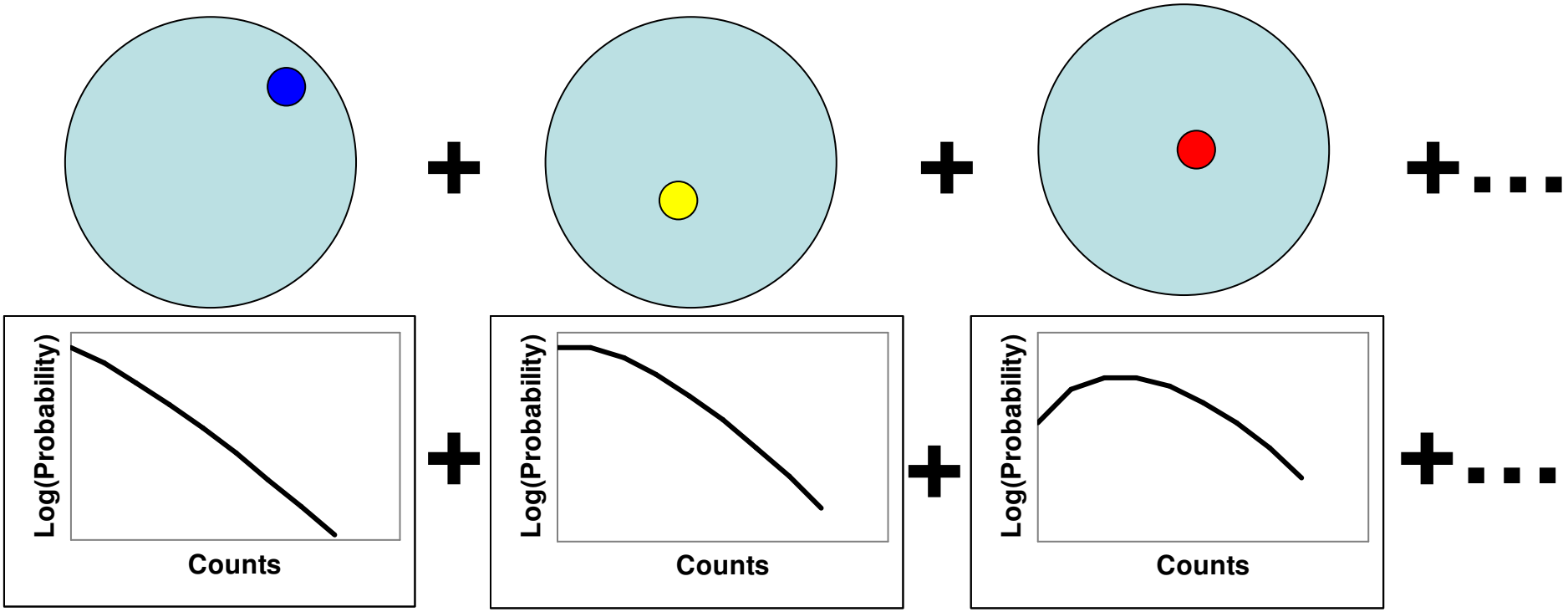
Two Photon:

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

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

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

Single Particle PCH



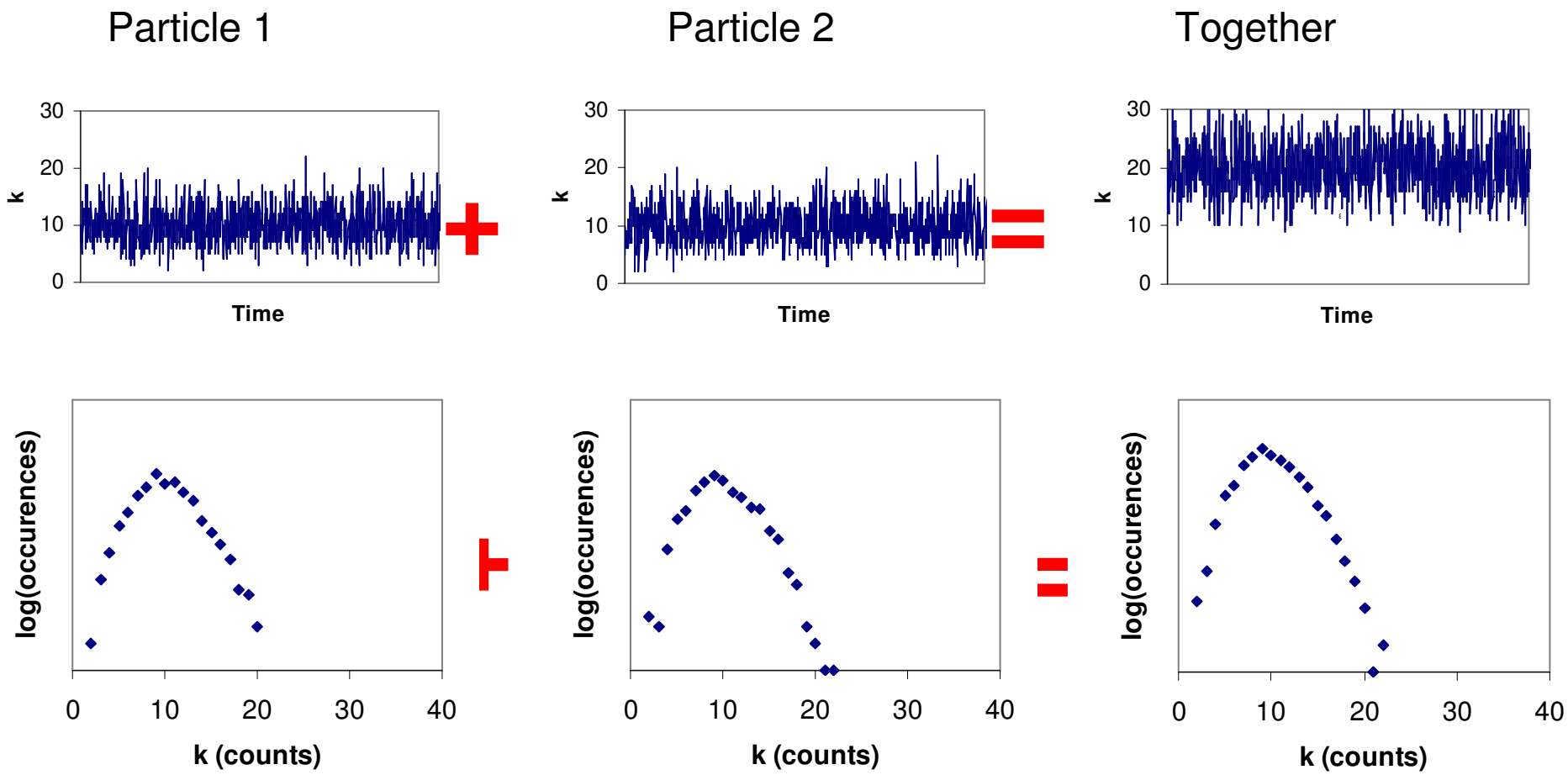
Have to sum up the poissonian distributions for all possible positions of the particle within the PSF

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

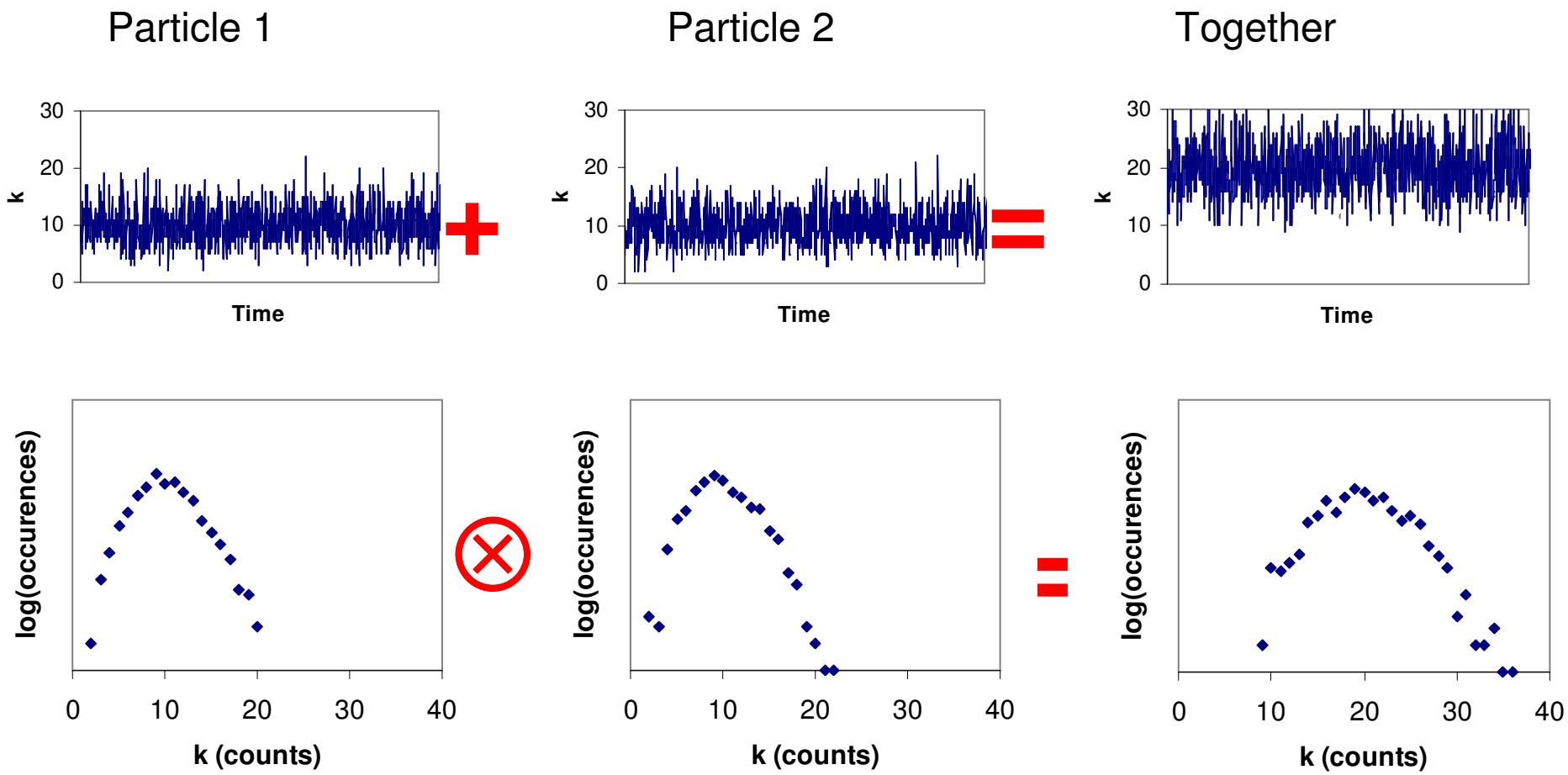
- What if I have two particles in the PSF?
- Have to calculate every possible position of the second particle for each possible position of the first!

Contribution from several particles of same brightn

Combining Distributions

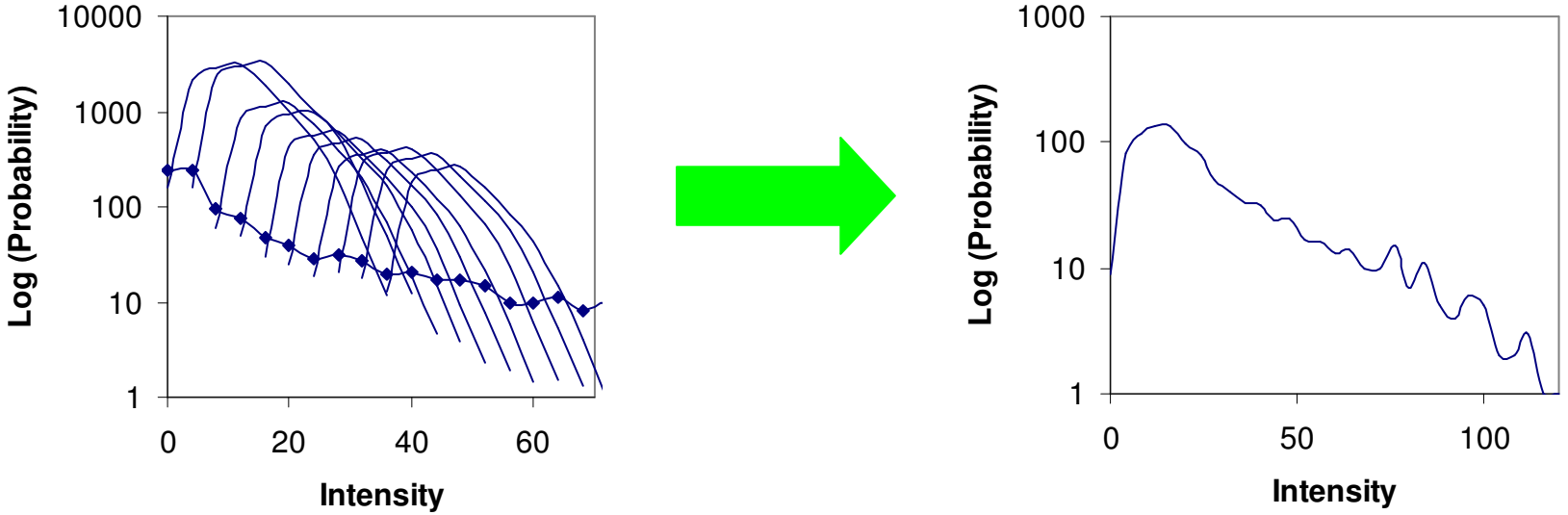


Combining Distributions



Convolution

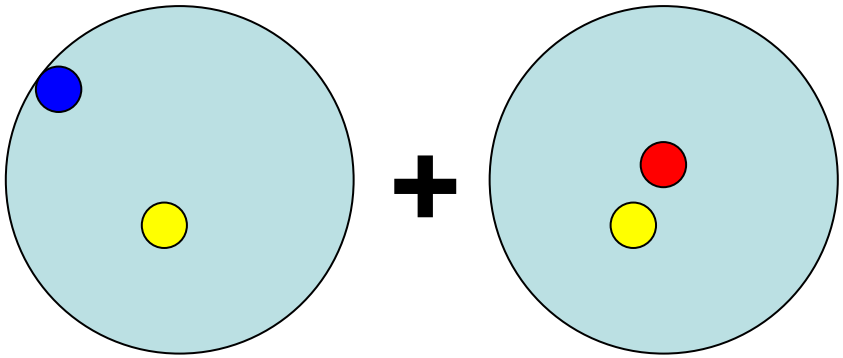
- Sum up all combinations of two probability distributions (joint probability distribution)
- Distributions (particles) must be independent



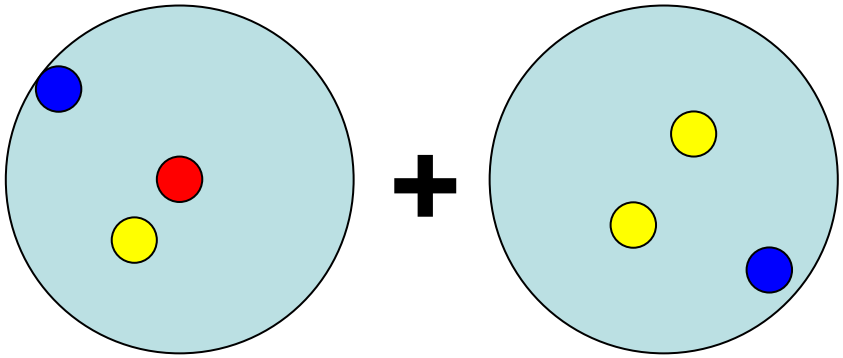
$$p^{(1+2)}(k) = \sum_{r=0}^{r=k} p^{(1)}(k-r) \cdot p^{(2)}(r)$$

Contribution from particles of different brightn

More Particles



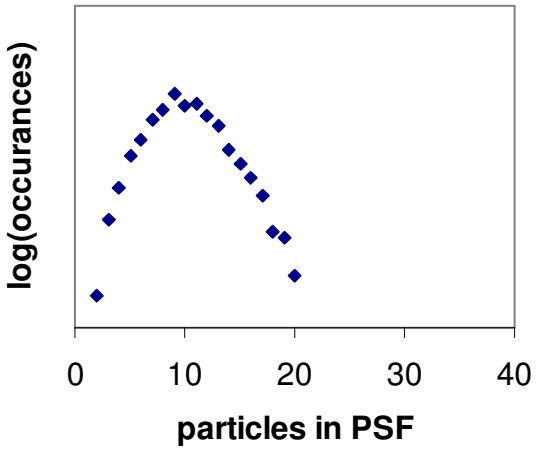
$$p^{(2)}(k) = p^{(1)}(k) \otimes p^{(1)}(k)$$



$$p^{(3)}(k) = p^{(1)}(k) \otimes p^{(2)}(k)$$

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

How Many Particles Do We Have in the PSF?



$$P(n,N) = Poi(n,N)$$

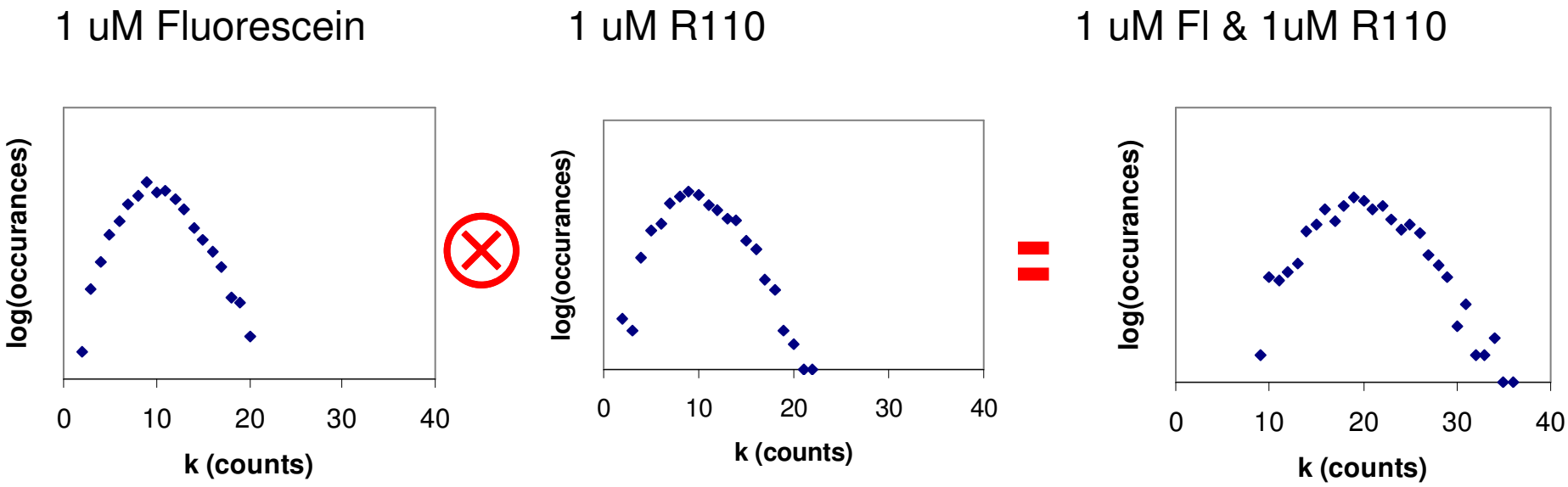
Particle occupation fluctuates around average, N with a poissonian distribution

Calculate poisson weighted average of n particle distributions

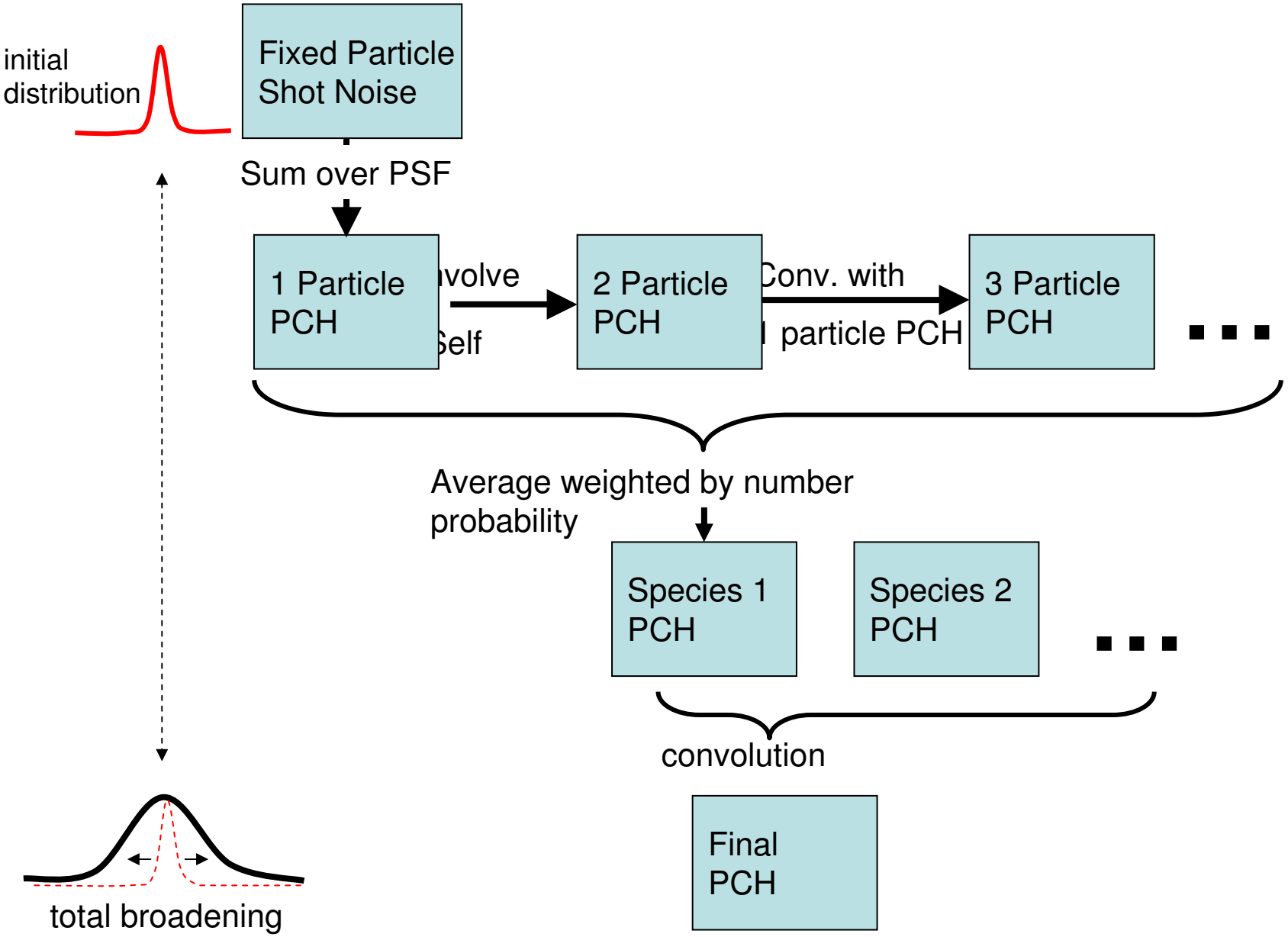
$$PCH(k,N) = \sum_{n} p^{(n)}(k) \cdot P(n,N)$$

Multiple Species

- Species are independent so just convolute!



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

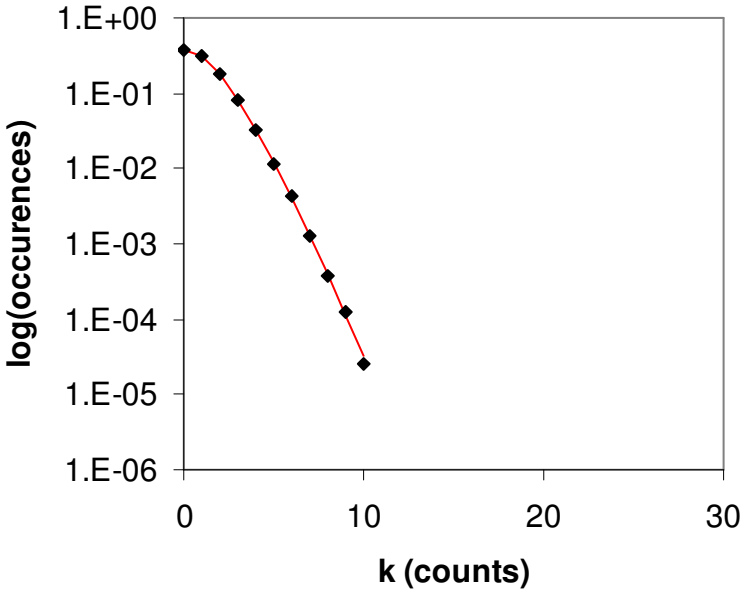
$$\chi^2 = \frac{\sum_k \left(M \frac{PCH_{model}(k) - PCH_{observed}(k)}{\sqrt{M \cdot PCH_{observed}(k) \cdot (1 - PCH_{observed}(k))}} \right)^2}{k_{\max} - d}$$

M is number of observations

d is number of fitting parameters

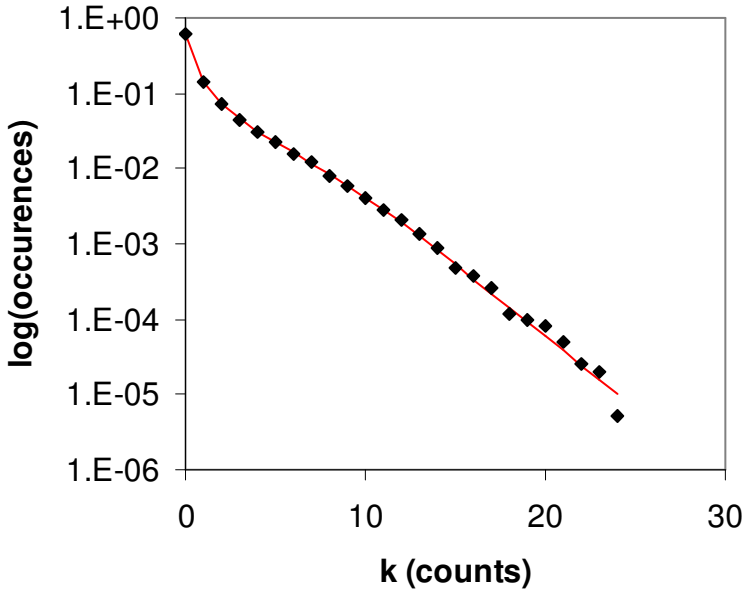
Chen et al., *Biophys. J.*, **1999,** *77,* 553.

Model Test



ε = 9,030 cpsm

N = 1.28



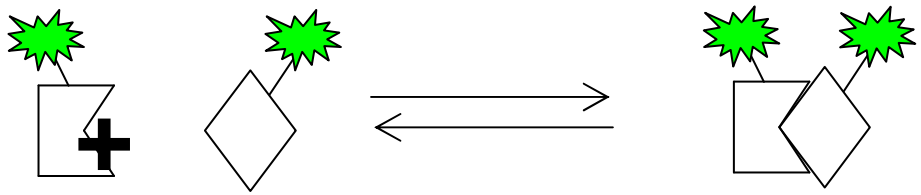
ε = 91,330 cpsm

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

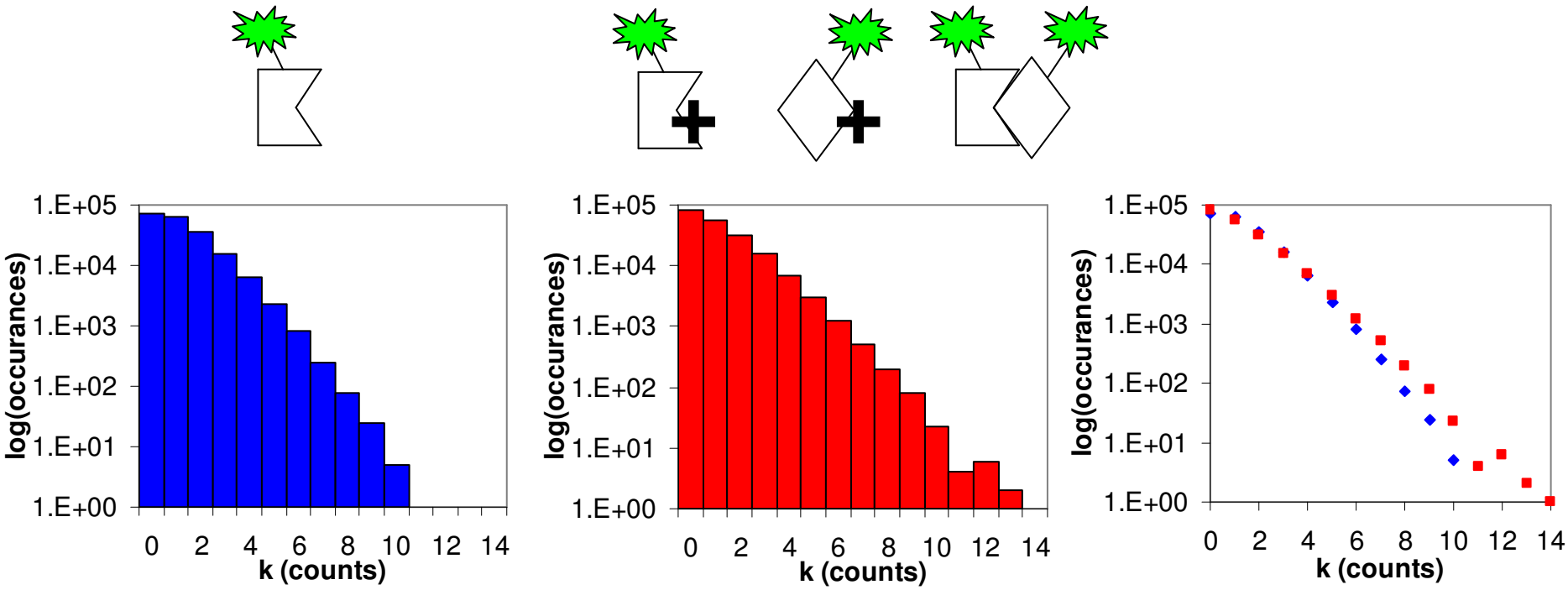


$\varepsilon = \varepsilon_{monomer}$ $\varepsilon = 2 \times \varepsilon_{monomer}$

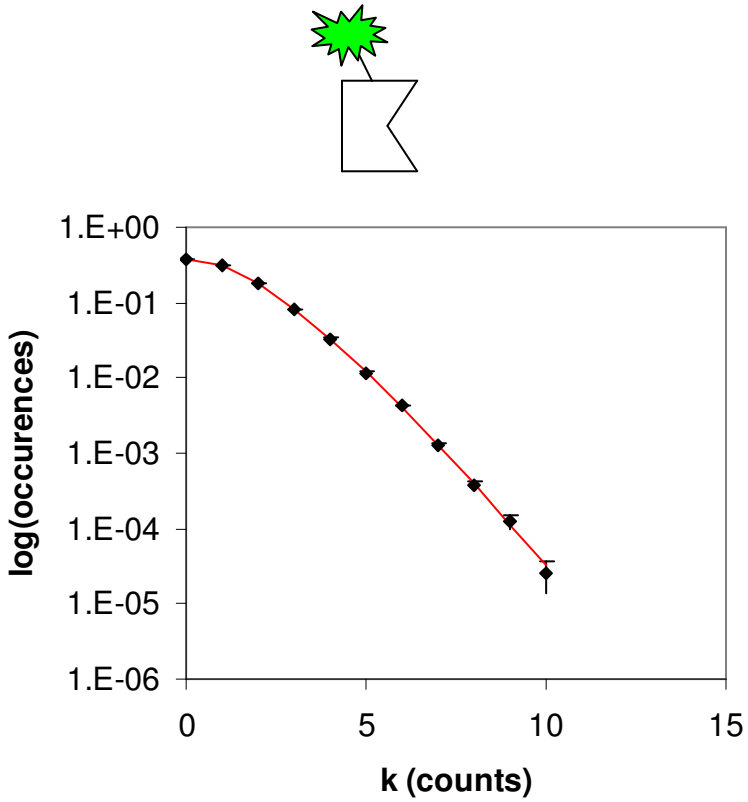
All three species are present in equilibrium mixture

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

Photon Count Histogram (PCH)

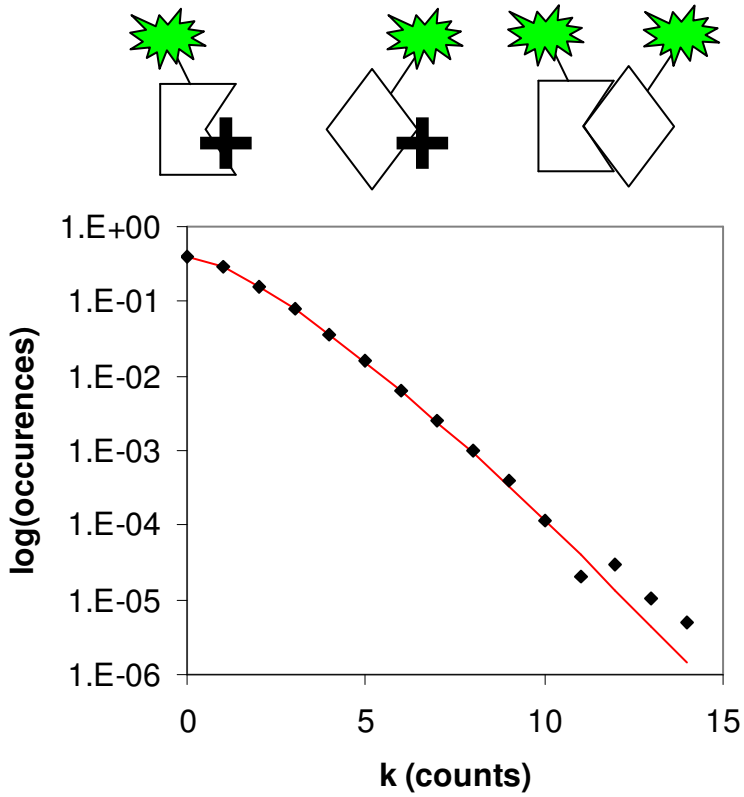


Simulation Solution



ε = 9,000 cpsm

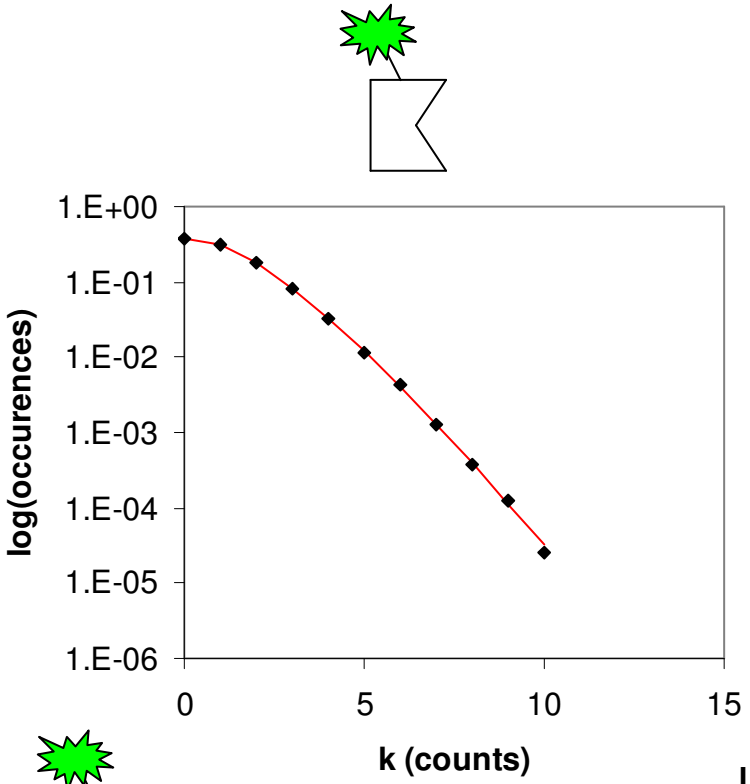
N = 1.3

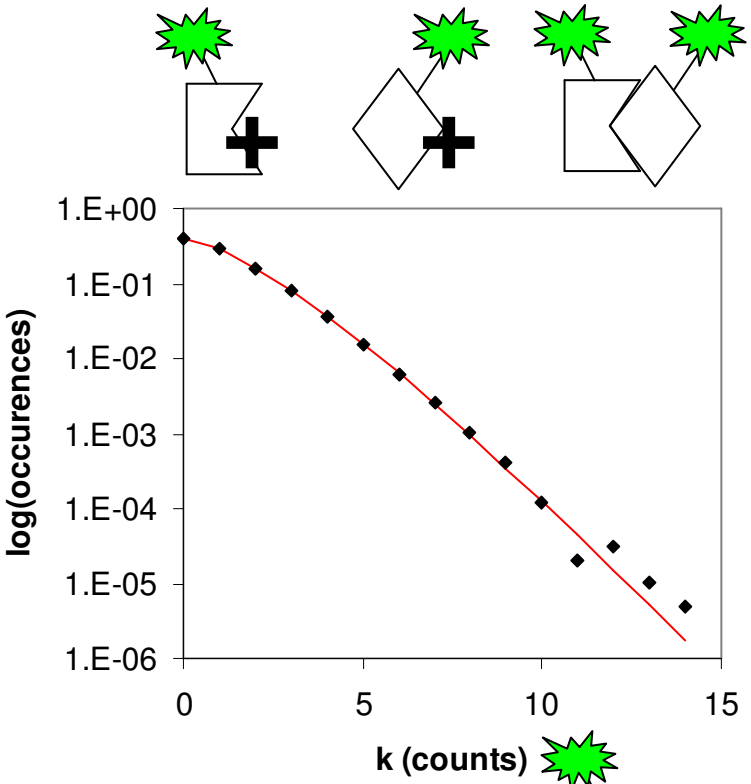


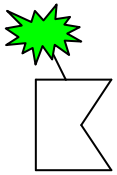
ε = 16,000 cpsm

N = 0.73

Global Fitting: Fit Data Sets Simultaneously



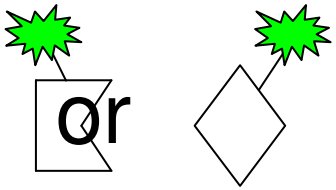


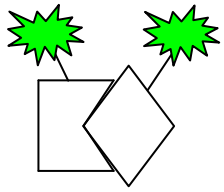


ε = 9,000 cpsm N = 1.3

Link

ε_1 = 9,000 cpsm N_1 = 0.29 ε_2 = 18,100 cpsm N_2 = 0.50





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

- N is defined relative to PSF volume $V_{PSF} = \int PSF(\vec{r})d\vec{r}$
- One photon:

$$V_{3DG} = w_0^2 z_0 (\pi / 2)^{3/2}$$

- Two photon:

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

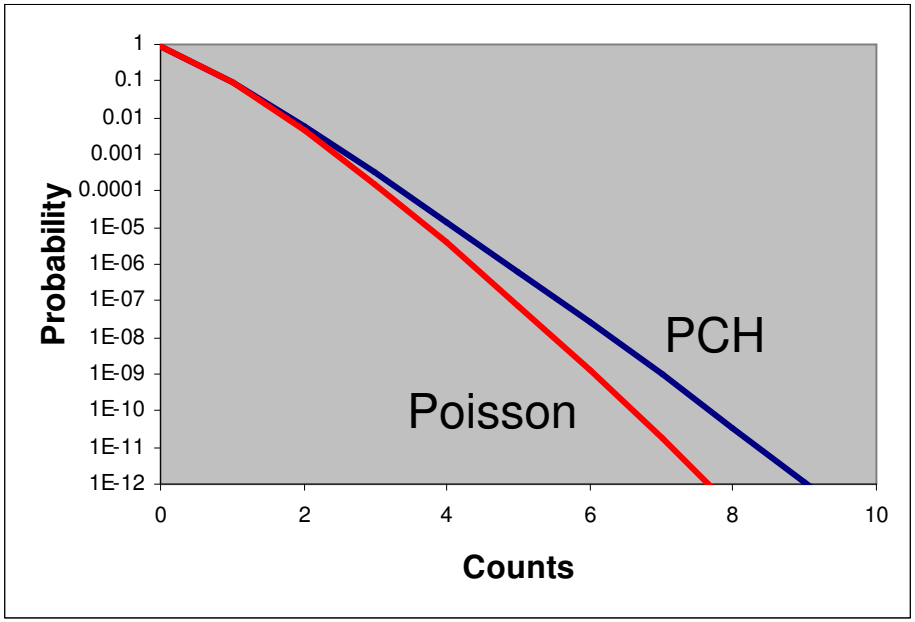
- Definition is same as for FCS
- Can use FCS to determine w_0 (and maybe z_0)

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

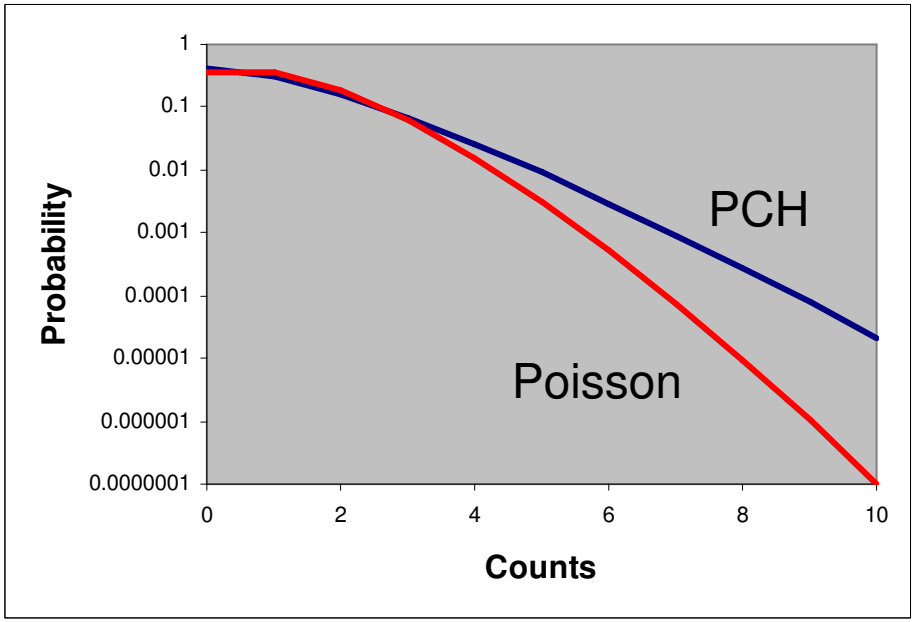
How to Improve Accuracy

- Minimize sources of instrument noise
  - PSF heterogeneity
  - Shot noise
- Maximize particle burst amplitudes

Effect of Brightness



ε = 10,000 cpsm

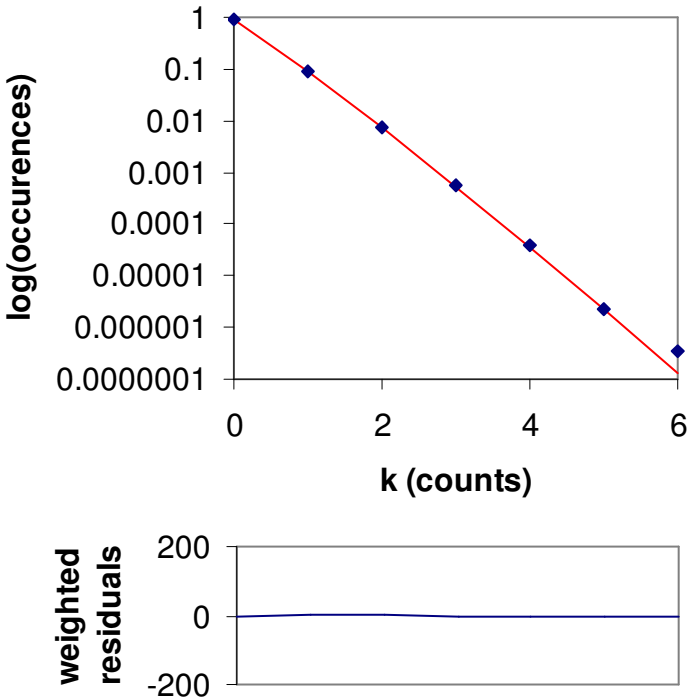


ε = 100,000 cpsm

Saturation Effect

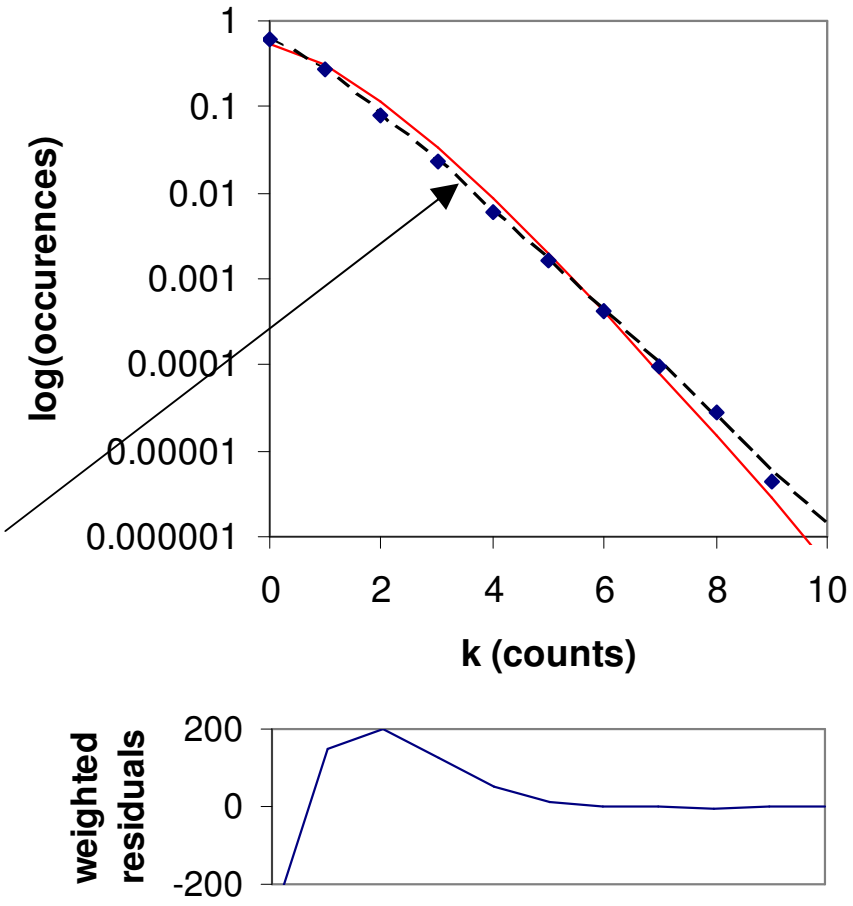
Rhodamine 110 on the Zeiss Confocor 3

10 uW laser



Multi-Species Fit

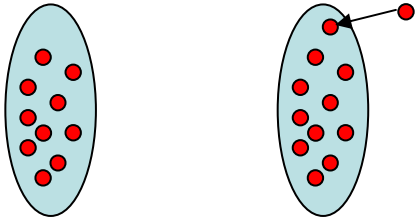
60 uW laser



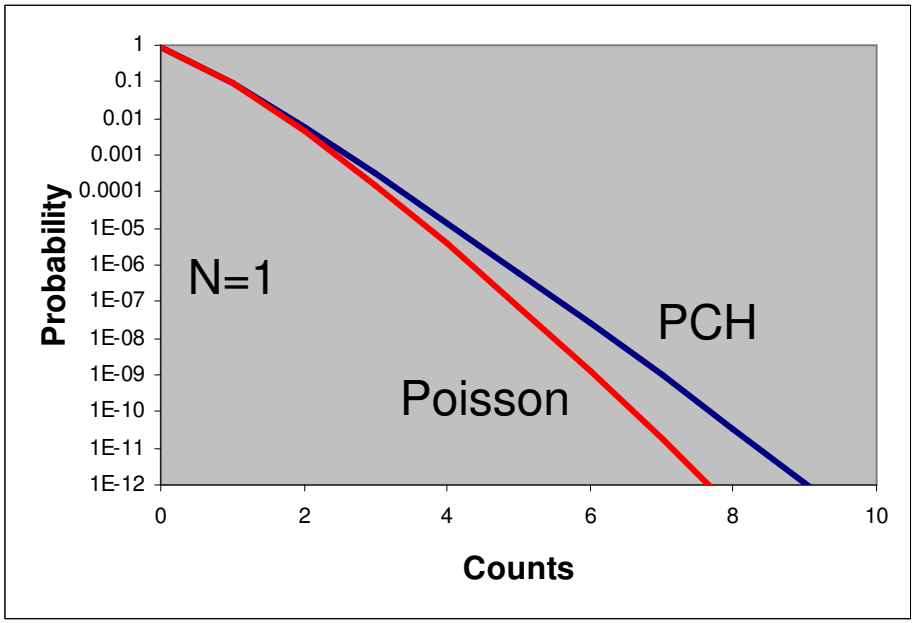
Laser power is not an infinite source of brightness!

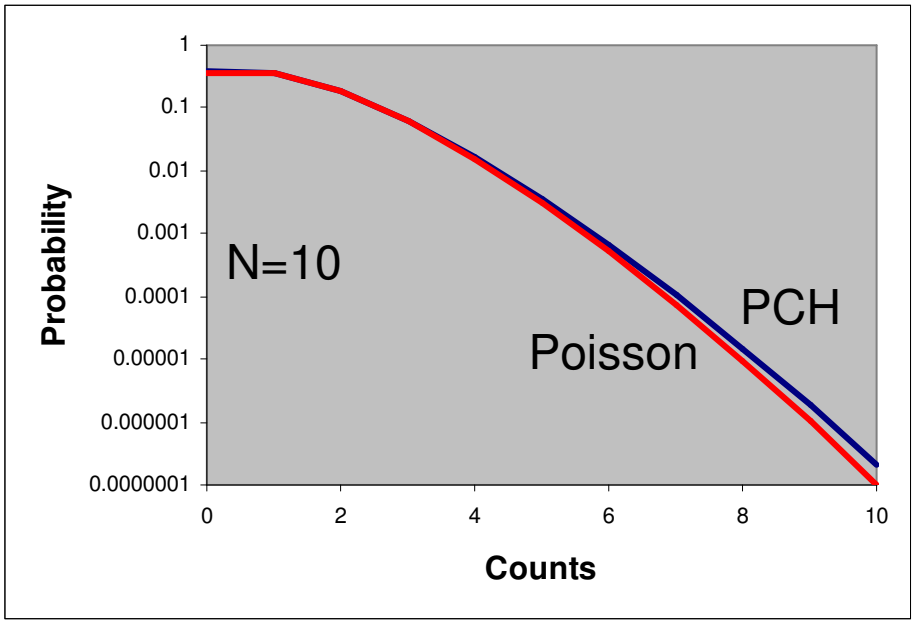
Concentration Effect

Brightness
increases by 100%



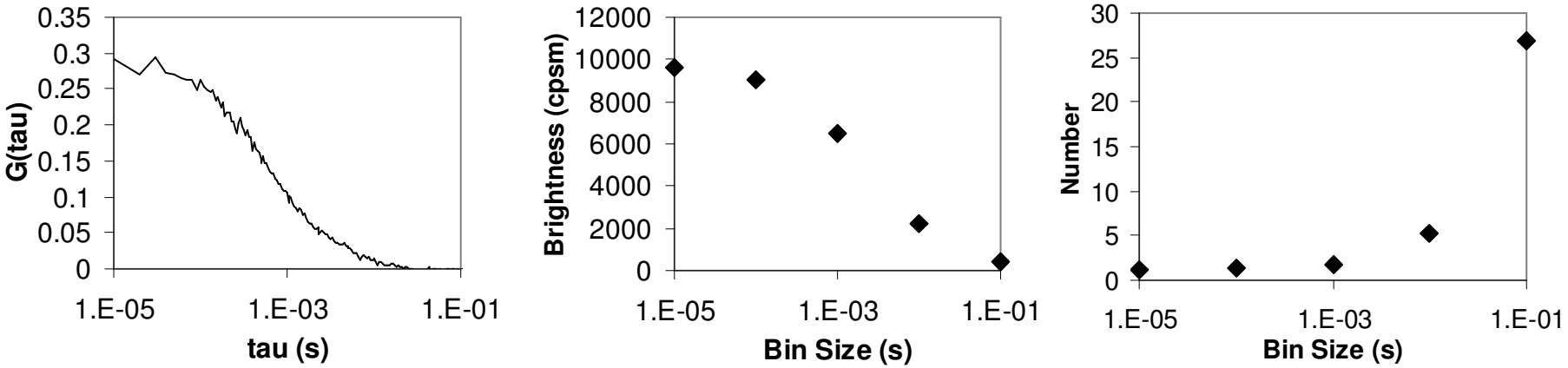
Brightness
increases by 10%





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

Sampling Time Effect

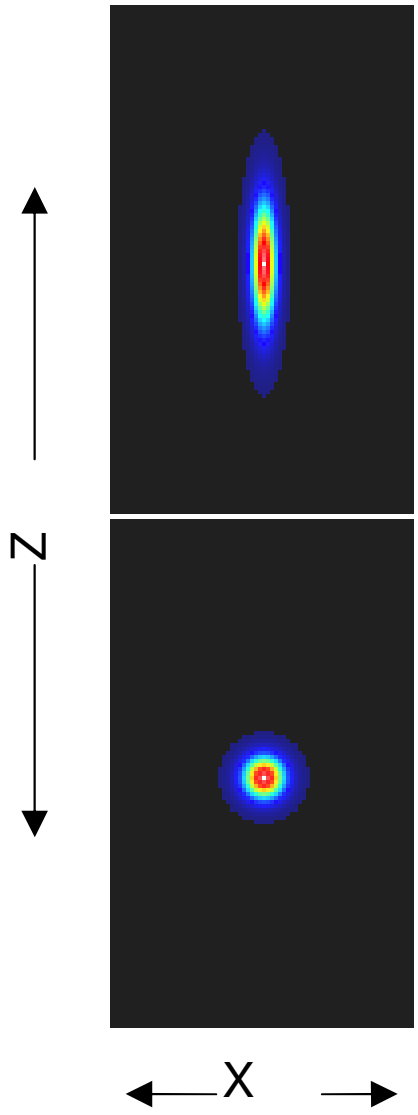


Again, shorter sampling leads to photon limited acquisition

In general sample as long as possible without diffusion averaging

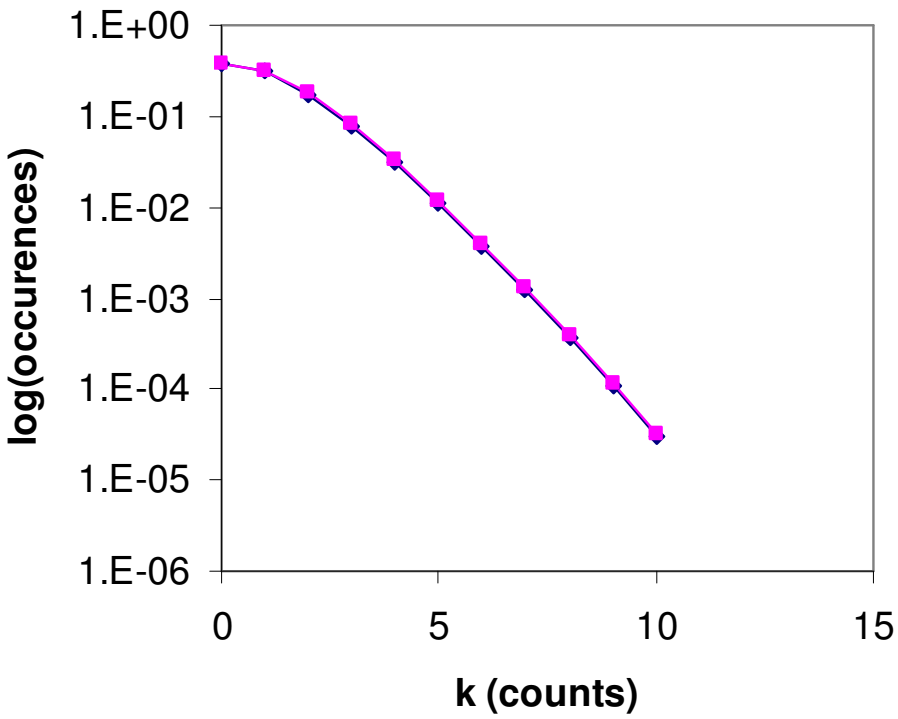
Wu and Mueller, *Biophys. J.*, **2005,** *89,* 2721.

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

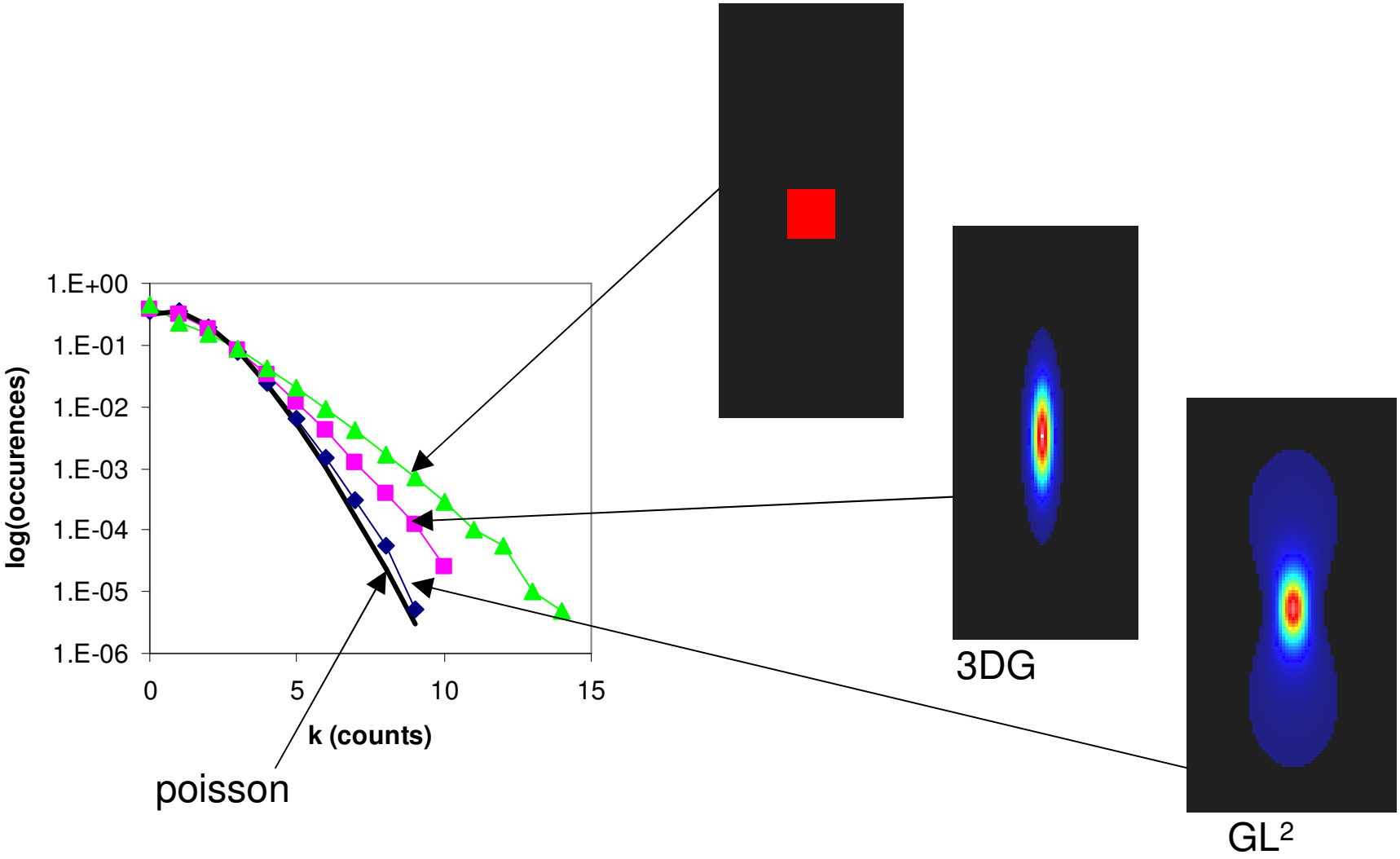


V_{PSF} = 0.08 fL

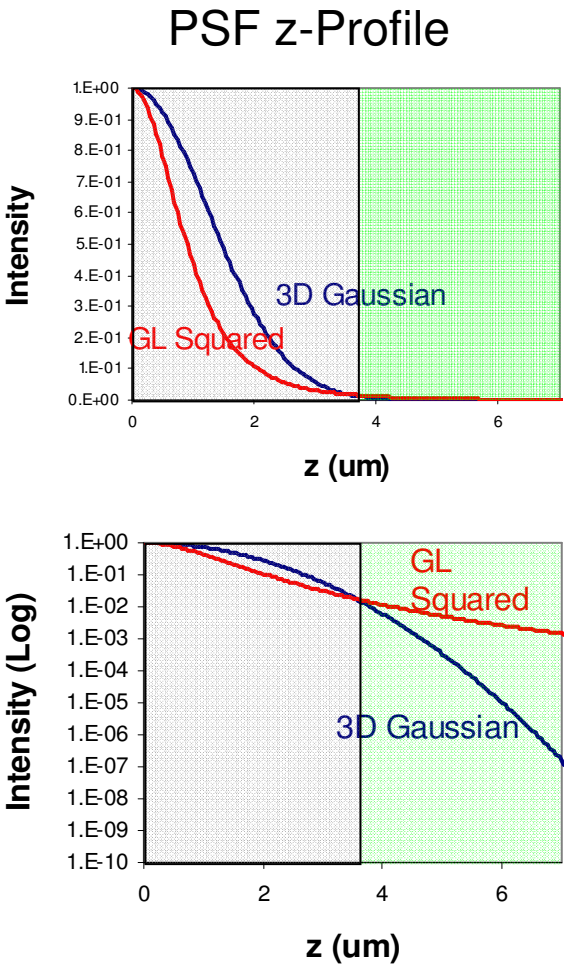
V_{PSF} = 0.08 fL

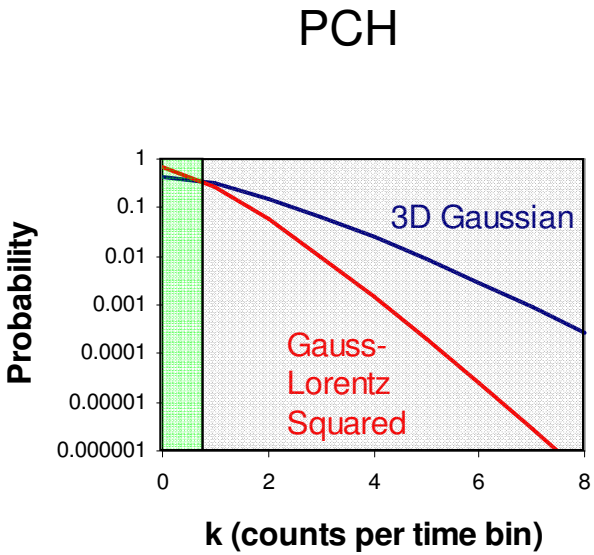


Functional Form DOES Matter



Functional Form Matters for PCH





Point Spread Function Effects

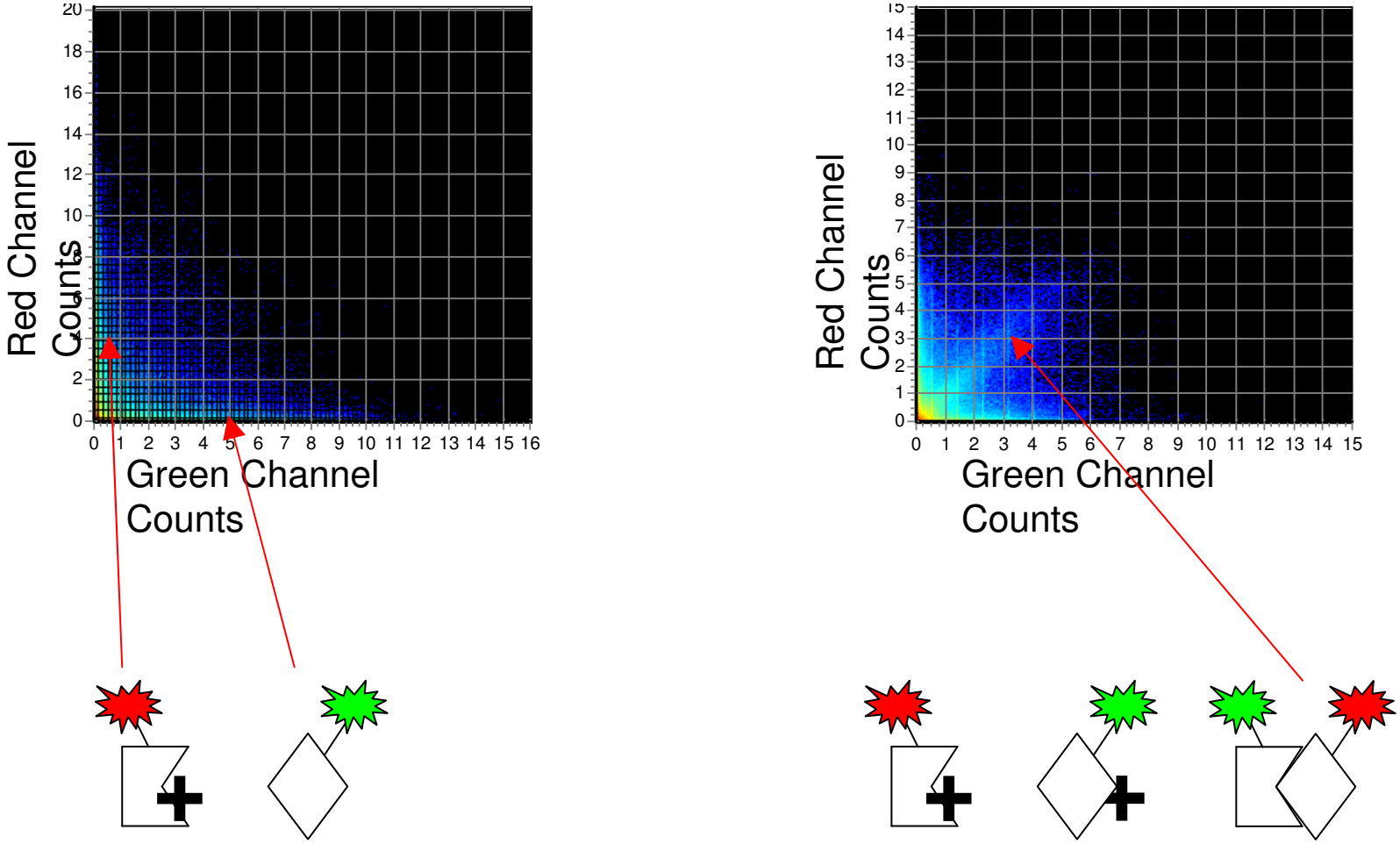
$$p^{(1)}(k) = \frac{1}{V_0} \int_{V_0} Poi\left(k, \varepsilon \overline{PSF}(\vec{r})\right) d\vec{r}$$

This equation will work for ANY PSF shape.

Alternative Methods

- Fluorescence Cumulant Analysis (FCA)
  - Mueller *Biophys. J.* **2004**, *86,* 3981.
  - Similar to method of moments
  - Any distribution can be described by a sum of moments
  - Simple algebraic formulas for cumulants
- Fluorescence Intensity Distribution Analysis (FIDA)
  - Kask et al. *PNAS* **1999,** *96,* 13756.
  - Fits PSF in fourier transformed space
  - Fits to non-physical parameterized PSF

2D PCH


Red Channel Counts
Green Channel Counts
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
0 2 4 6 8 10 12 14 16 18 20
Red Channel Counts
Green Channel Counts
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Calculating the 2D PCH Function

$$PCH(\varepsilon_A, \varepsilon_B, N; k_A, k_B) = \binom{k}{k_A} (\varepsilon_A / \varepsilon)^{k_A} (1 - \varepsilon_A / \varepsilon)^{k - k_A} \cdot PCH(\varepsilon, N; k)$$

the binomial distribution:

$$P(x, k, N) = \binom{N}{k} x^k (1 - x)^{N-k}$$

We can find the 2D PCH function from the single channel PCH function!

Chen et al., *Biophys. J.*, **2005,** *88,* 2177-2192.

Summary

- The photon count histogram can be modeled by integration of component noise sources
- Heterogeneous samples can be resolved through global analysis
- Accuracy is related to magnitude of particle fluctuations relative to instrument fluctuations