Introduction to Fluorescence Correlation Spectroscopy (FCS)

Fluctuation analysis goes back to the beginning of the century

- Brownian motion (1827). Einstein explanation of Brownian motion (1905)
- Noise in resistors (Johnson, Nyquist), white noise
- Noise in telephone-telegraph lines. Mathematics of the noise. Spectral analysis
- I/f noise
- Noise in lasers (1960)
- Dynamic light scattering
- Noise in chemical reactions (Eigen)
- FCS (fluorescence correlation spectroscopy, 1972) Elson, Magde, Webb
- FCS in cells, 2-photon FCS (Berland et al, 1994)
- First commercial instrument (Zeiss 1998)

Fluorescence Parameters & Methods

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

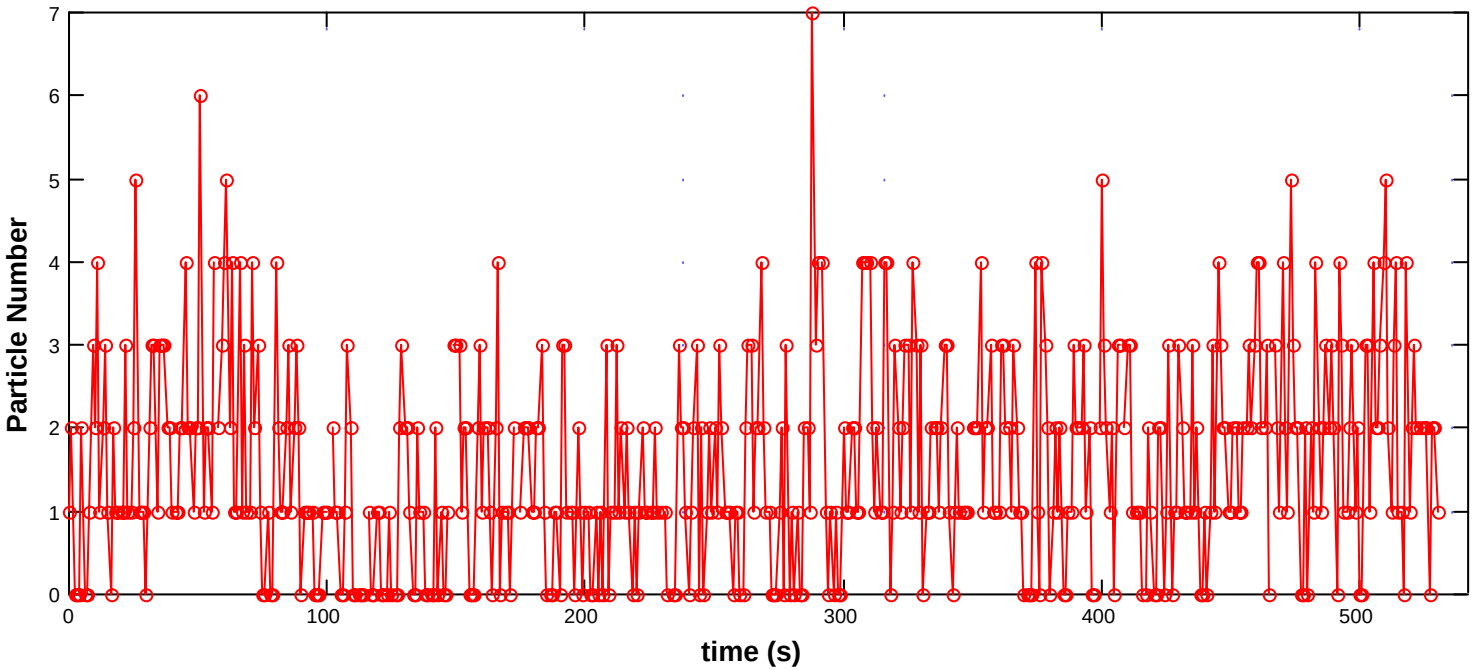
First Application of Correlation Spectroscopy

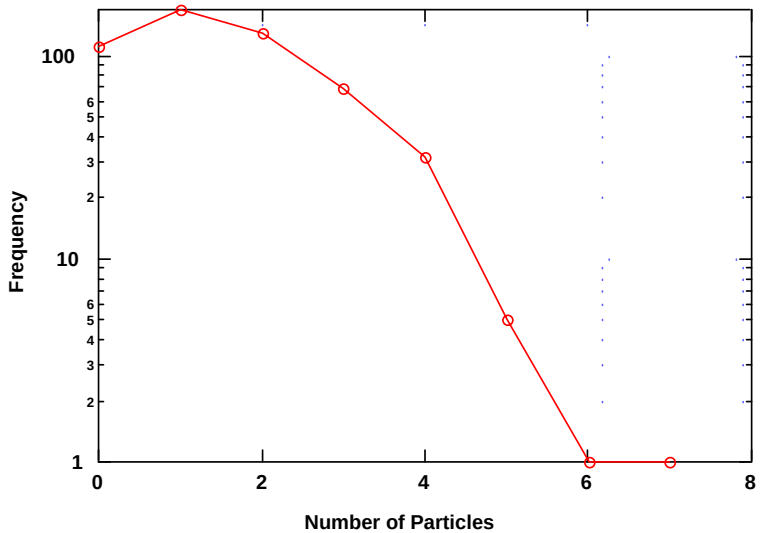
(Svedberg & Inouye, 1911) *Occupancy Fluctuation*

Experimental data on colloidal gold particles:

120002001324123102111131125111023313332211122422122612214
234524114131142310010042112312320111100011 1_2110013200000
100110001000232210021100002010 01_33312200023122102401110 2_
122211223100011033111021011001010301131212101012111121 1_10
003221012302012121321110110023312242110001203010100221734
410101002112211444421211440132123314313011222123310121111
222412231113322132110000410432012120011322231200_253212033
233111100210022013011321113120010131432211221122323442230
321421532200202142123232043112312003314223452134110412322
220221

Particle Correlation



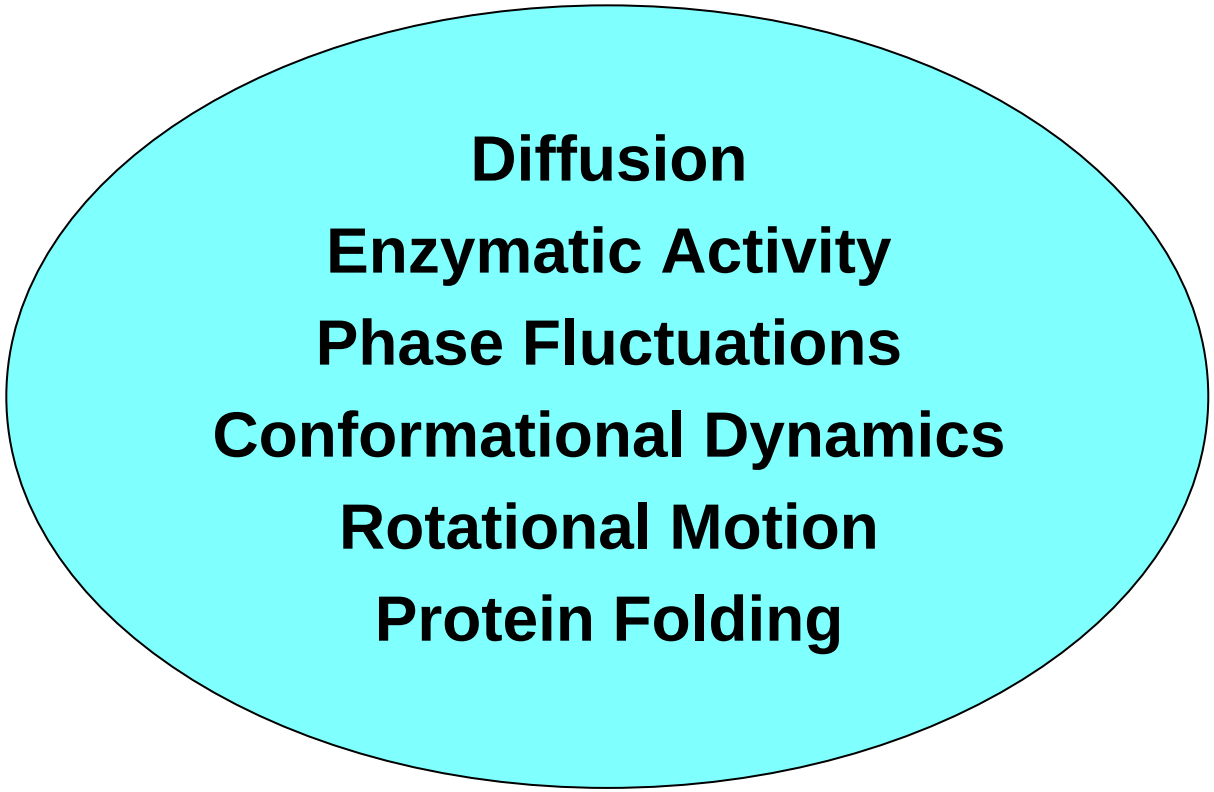


***Histogram of particle counts**

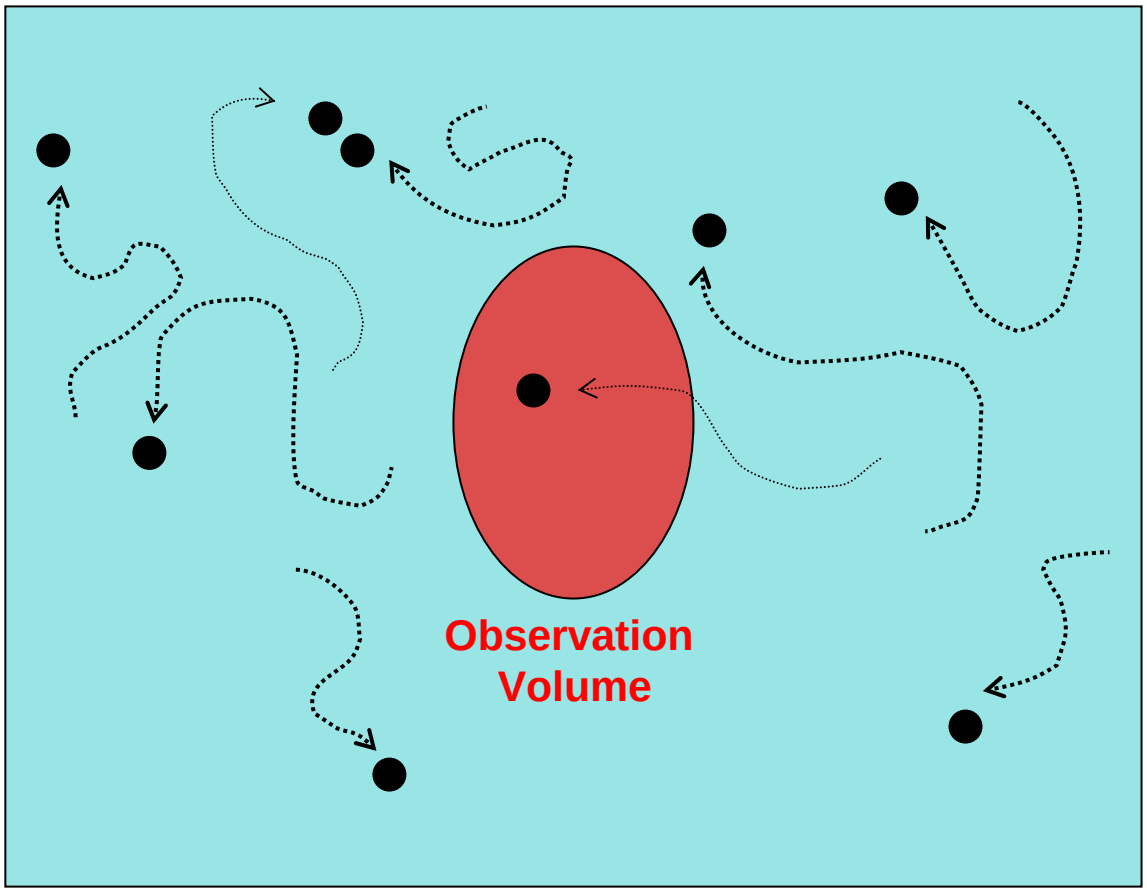
***Poisson behavior**

***Autocorrelation not available**

In FCS
Fluctuations are in the Fluorescence Signal



Generating Fluctuations By Motion



Sample Space

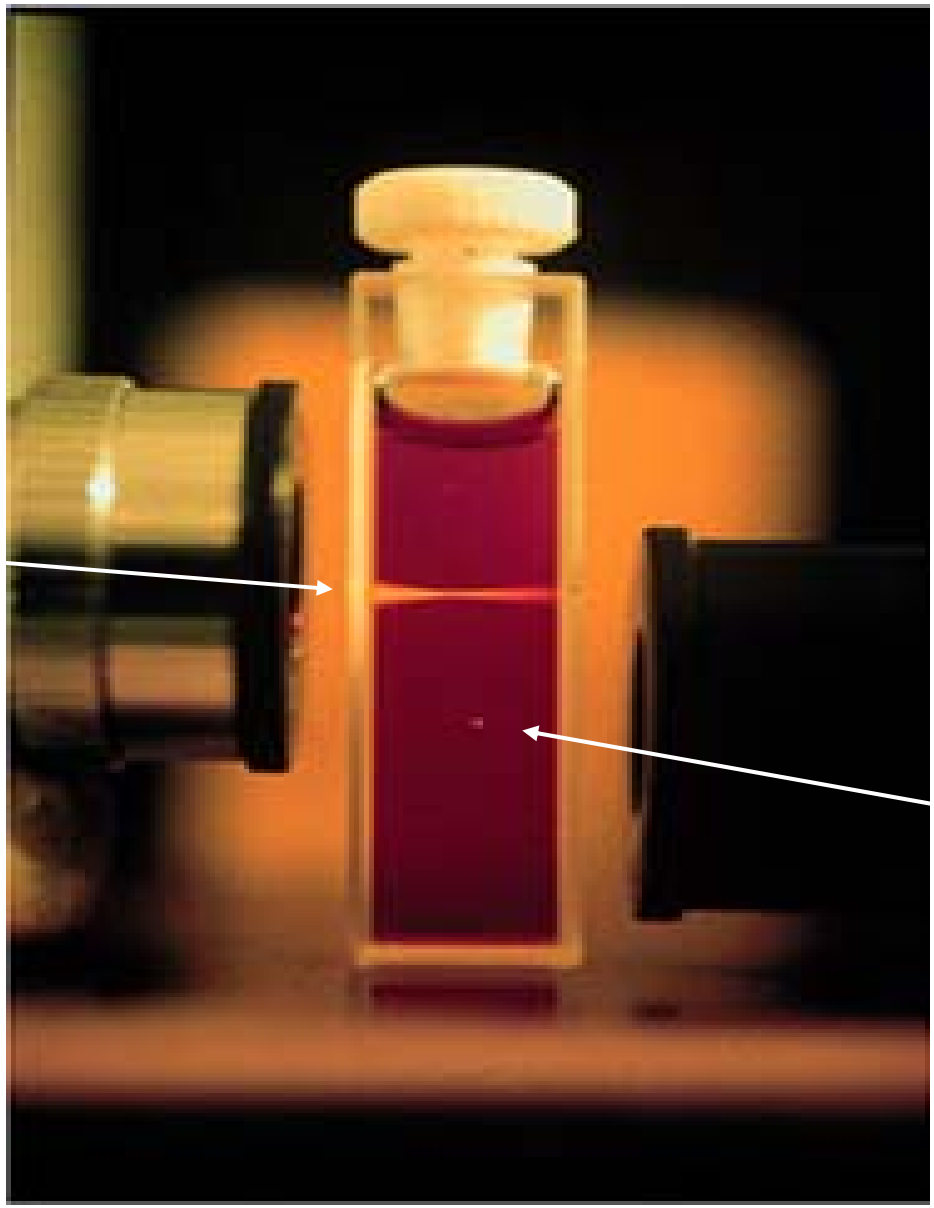
What is Observed?

1. The Rate of Motion

2. The Concentration of Particles

3. Changes in the Particle Fluorescence while under Observation

1-photon



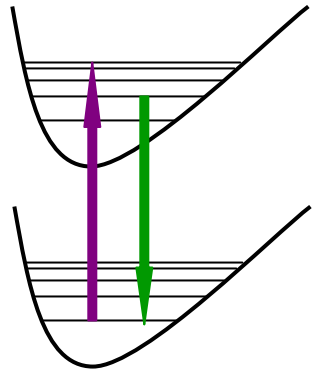
2-photon

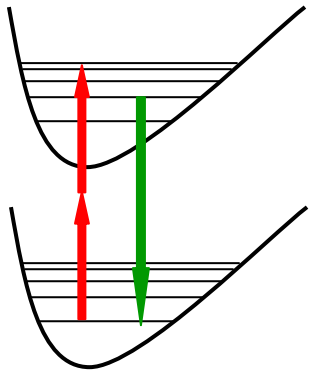
Brad Amos
MRC, Cambridge, UK

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

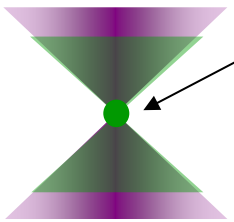
1 - Photon

2 - Photon

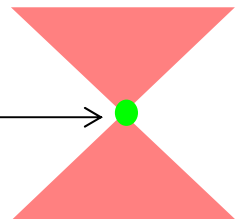




Defined by the pinhole size, wavelength, magnification and numerical aperture of the objective



Approximately 1 μm^3



Defined by the wavelength and numerical aperture of the objective

$$n_a \approx \frac{d}{\tau}\left(\frac{p\pi\ A^2}{fhc\lambda}\right)^2$$

n_a Photon pairs absorbed per laser pulse
p Average power
τ pulse duration
f laser repetition frequency
A Numerical aperture
λ Laser wavelength
d cross-section

From Webb, Denk and Strickler, 1990

Why confocal detection?

Molecules are small, why to observe a large volume?

- Enhance signal to background ratio
- Define a well-defined and reproducible volume

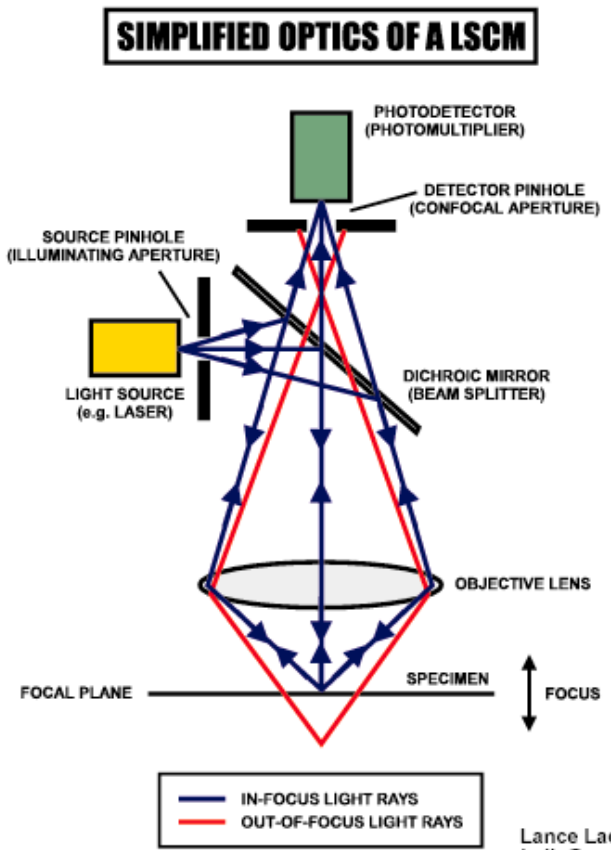
Methods to produce a confocal or small volume

(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 light, not applicable to cells)

- Nanofabrication
- Local field enhancement
- Near-field effects



Lance Ladic
ladic@cs.ubc.ca

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}

Laser technology needed for two-photon excitation

Ti:Sapphire lasers have pulse duration of about 100 fs

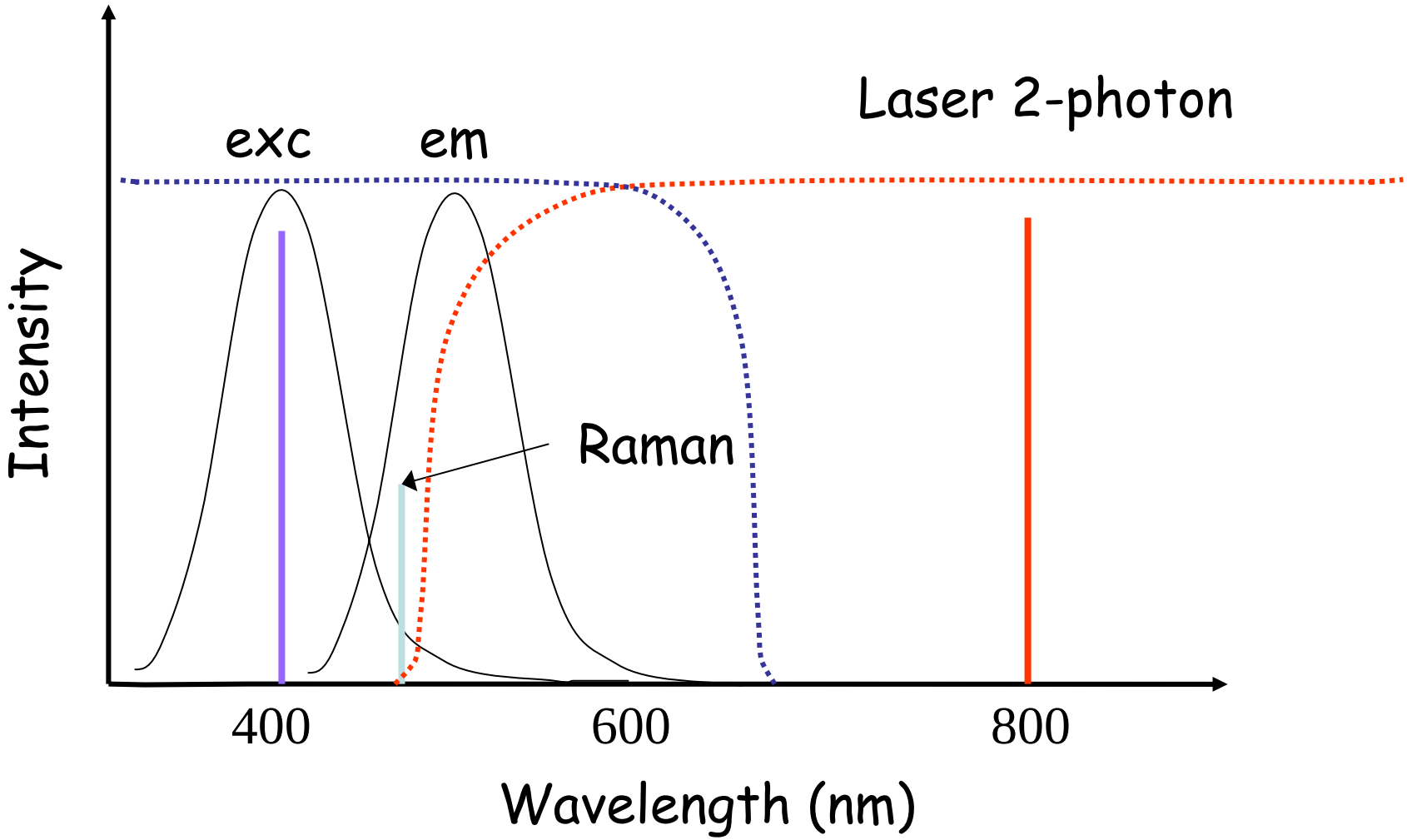
Average power is about 1 W at 80 MHz repetition rate

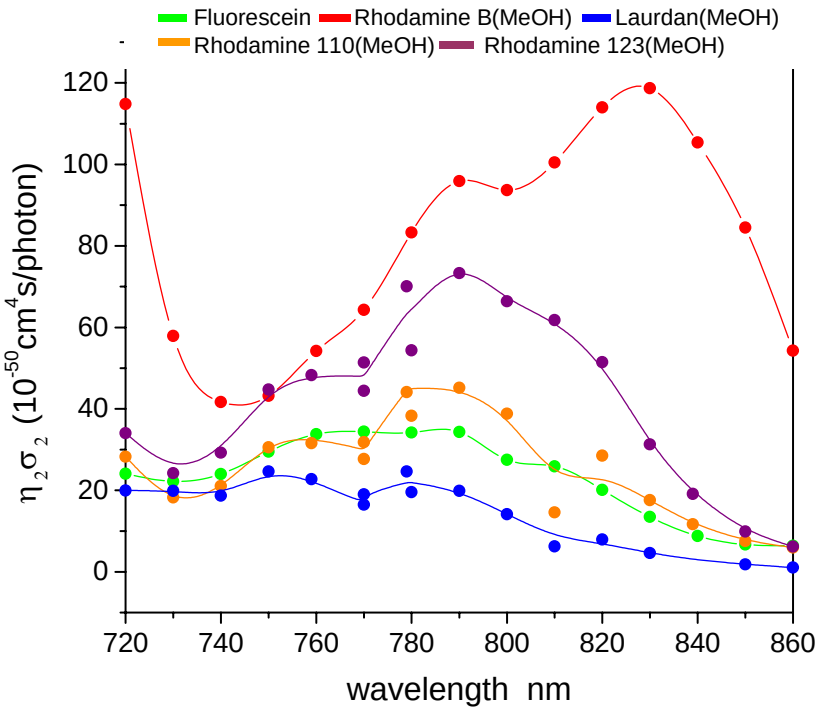
About 12.5 nJ per pulse (about 125 kW peak-power)

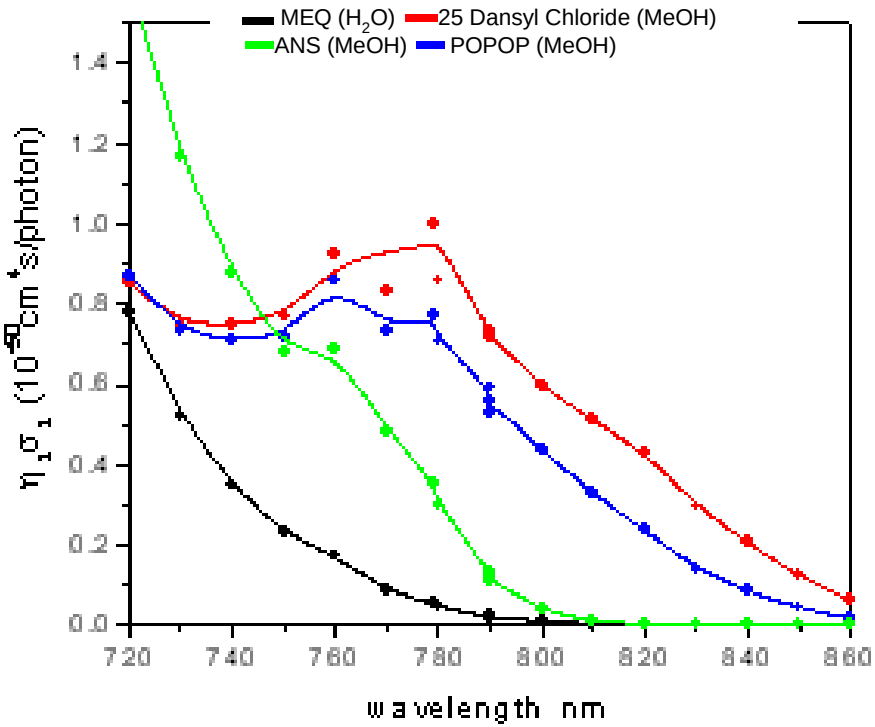
Two-photon cross sections are typically about

$$\delta = 10^{-50}\ \mathrm{cm}^4\ \mathrm{sec\ photon}^{-1}\ \mathrm{molecule}^{-1}$$

Enough power to saturate absorption in a diffraction limited spot

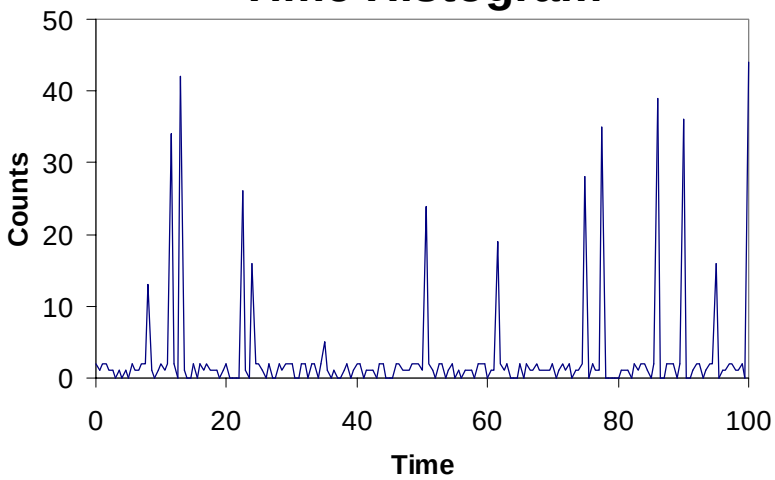

Laser 2-photon
exc
em
Intensity
Raman
400
600
800
Wavelength (nm)


Fluorescein
Rhodamine B(MeOH)
Laurdan(MeOH)
Rhodamine 110(MeOH)
Rhodamine 123(MeOH)
$\eta_2\sigma_2$ (10^{-50}cm^{4}s/photon)
wavelength nm

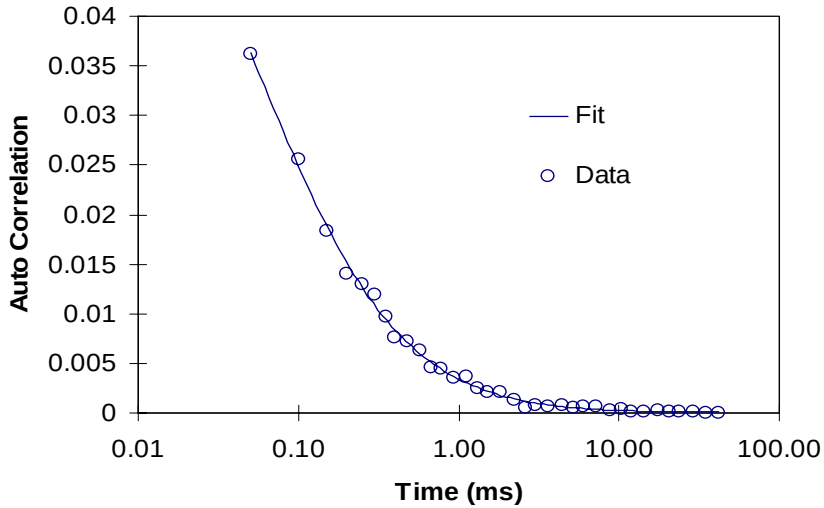

MEQ (H_2O)
25 Dansyl Chloride (MeOH)
ANS (MeOH)
POPOP (MeOH)
$\eta_1\sigma_1$ (10^{-50}cm^{4}s/photon)
wavelength nm

FCS Data Treatment & Analysis

Time Histogram

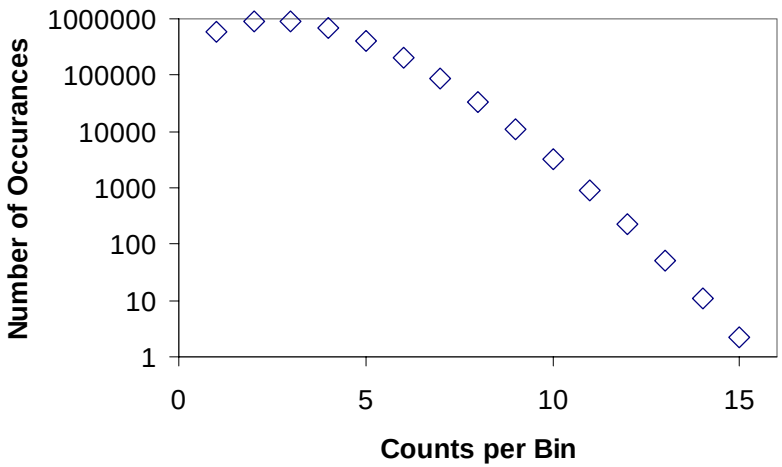


Autocorrelation



Autocorrelation Parameters: G(0) & $k_{reaction}$

Photon Counting Histogram (PCH)



PCH Parameters: <N> & ϵ

Autocorrelation Function

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

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

Factors influencing the fluorescence signal:

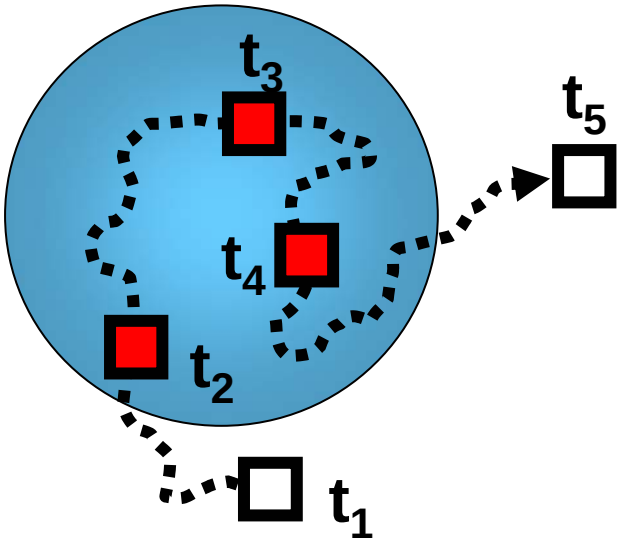
$$F(t) = \kappa Q \int d\mathbf{r}\, W(\mathbf{r}) C(\mathbf{r}, t)$$

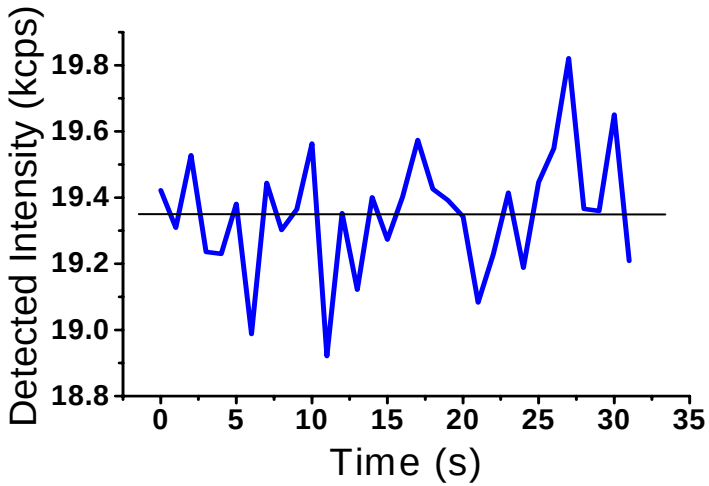
kQ = quantum yield and detector sensitivity (how bright is our probe)

W(r) describes our observation volume

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

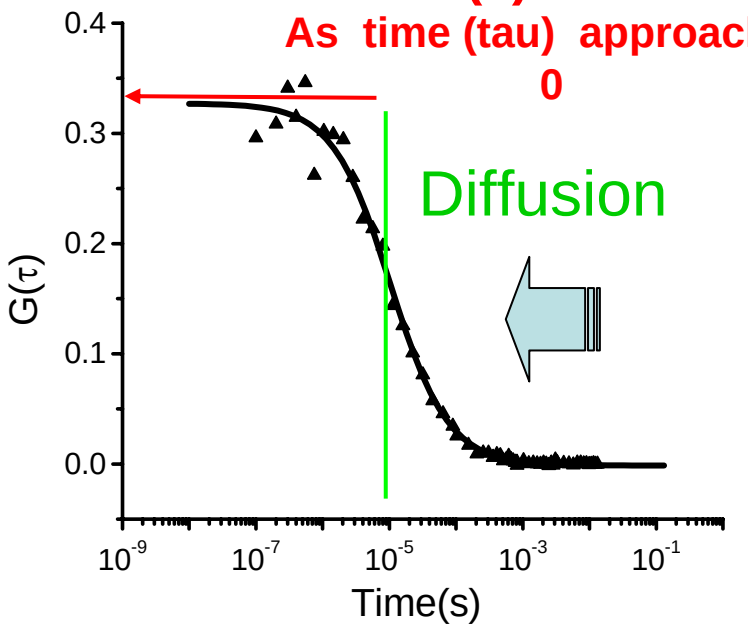
The Autocorrelation Function





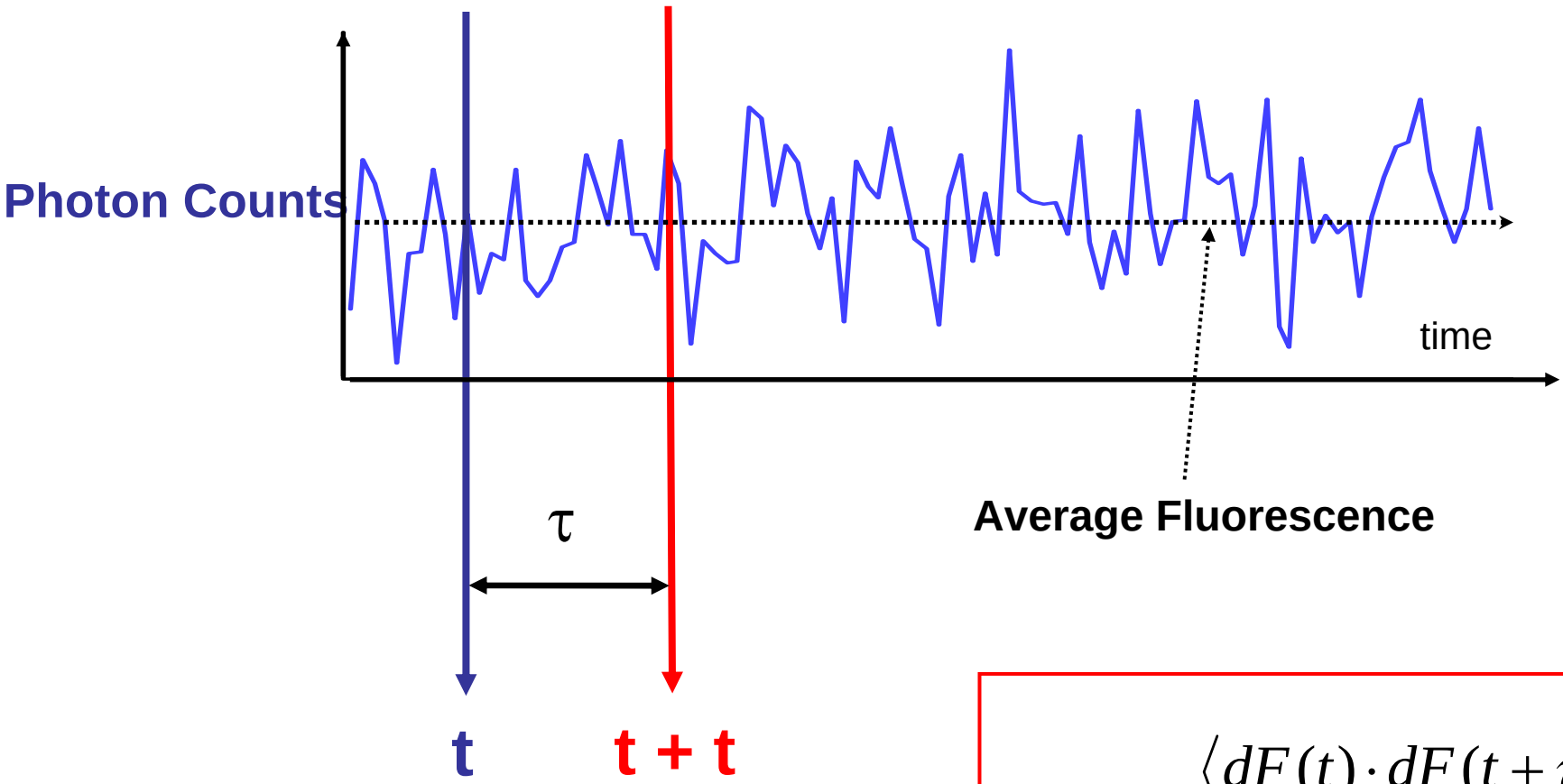
G(0) ∝ 1/N

As time (tau) approaches 0



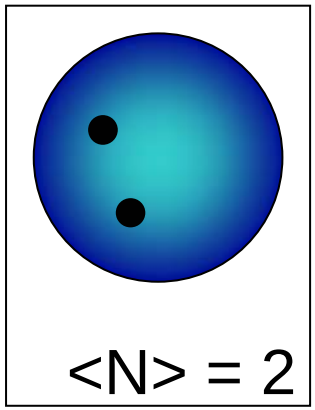
$$G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

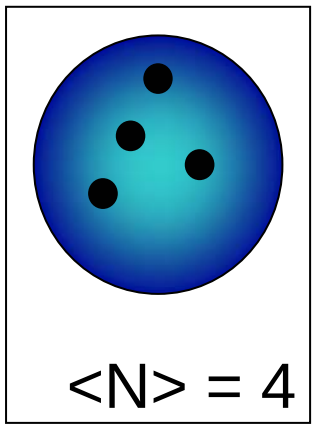
Calculating the Autocorrelation Function

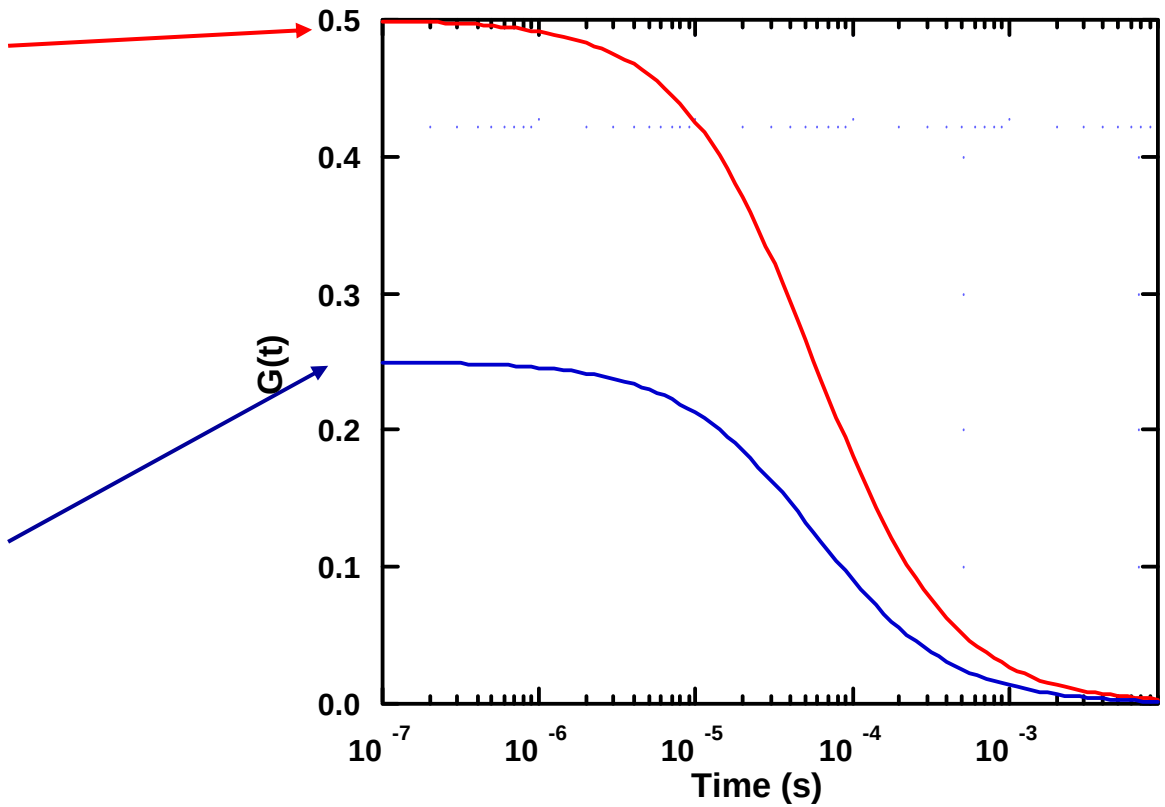


$$G(\tau) = \frac{\langle dF(t) \cdot dF(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

The Effects of Particle Concentration on the Autocorrelation Curve



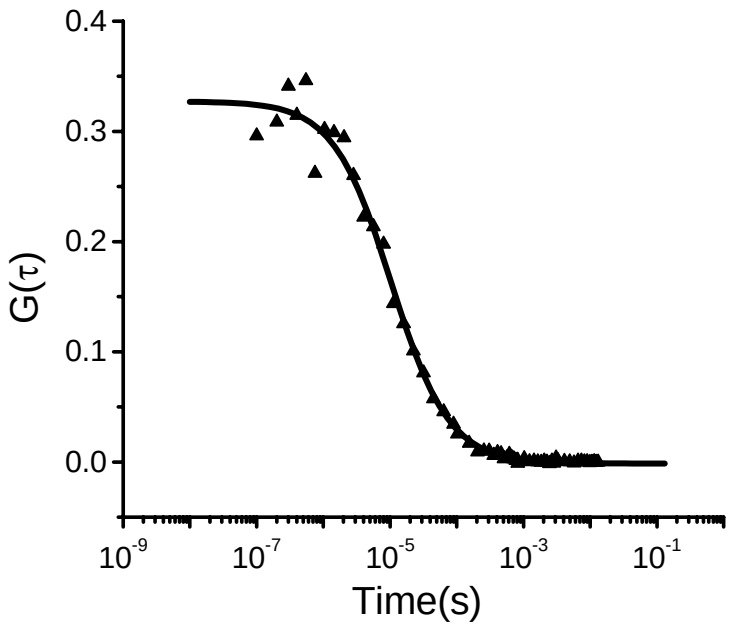




Why Is G(0) Proportional to 1/Particle Number?

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

$$\langle Particle \quad Number \rangle = Variance$$

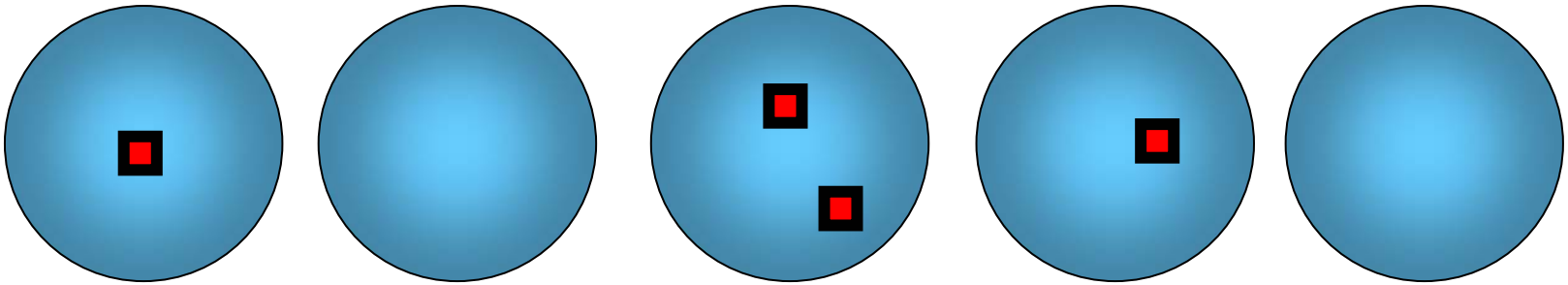


$$G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

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

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

G(0), Particle Brightness and Poisson Statistics



1 0 0 0 0 0 0 0 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**

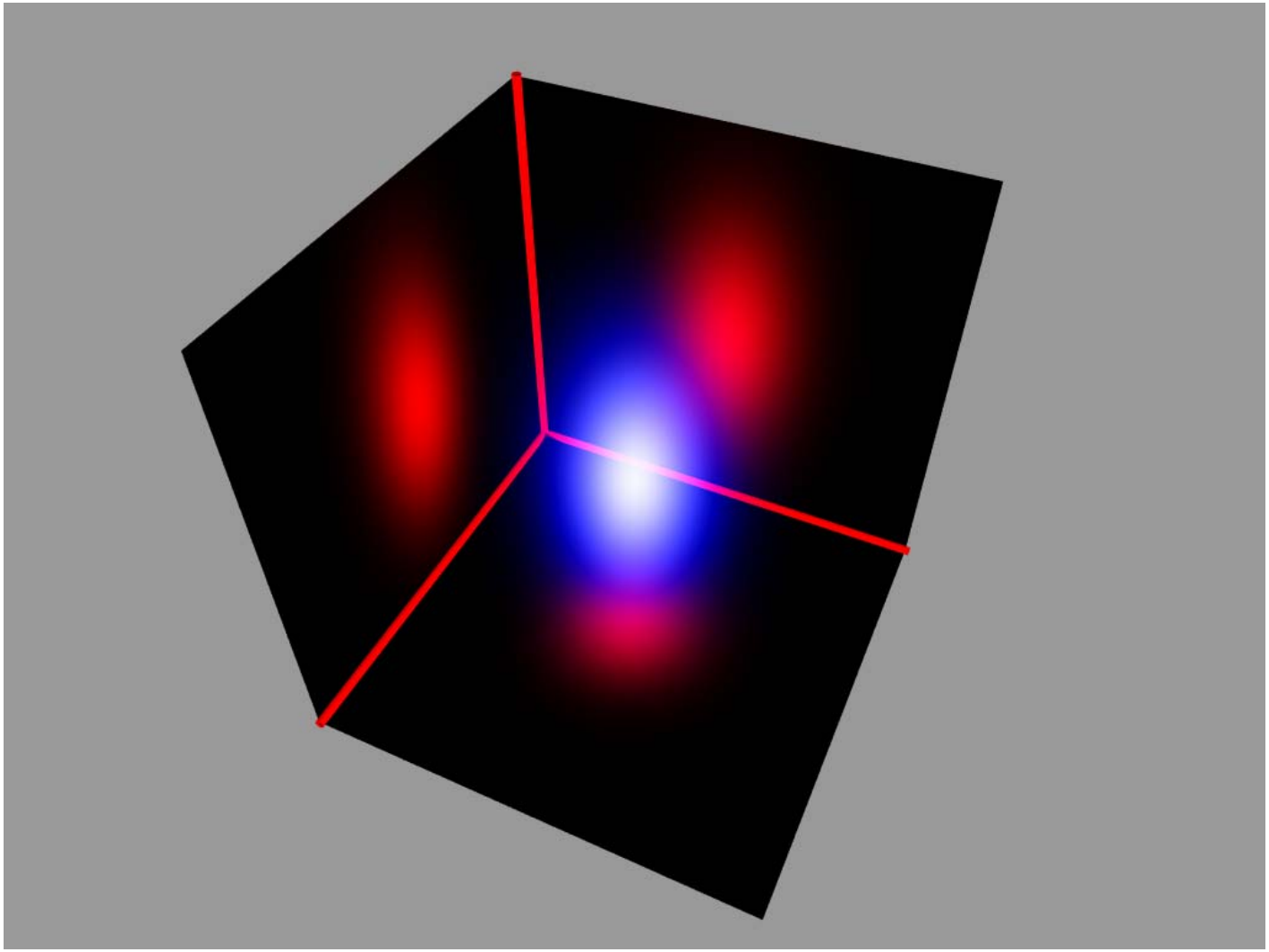
$$\langle N \rangle \propto Average^2 \Big/ Variance = \frac{0.275^2}{0.256} = 0.296$$

Lets increase the particle brightness by 4x:

4 0 0 0 0 0 0 0 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**

What about the excitation (or observation) volume shape?



Effect of Shape on the (Two-Photon) Autocorrelation Functions:

For a 2-dimensional Gaussian excitation volume:

$$G(\tau) = \frac{\gamma}{N}\left(1 + \frac{8D\tau}{w_{2\,DG}^2}\right)^{-1}$$

1-photon equation contains a 4, instead of 8

For a 3-dimensional Gaussian excitation volume:

$$G(\tau) = \frac{\gamma}{N}\left(1 + \frac{8D\tau}{w_{3\,DG}^2}\right)^{-1}\left(1 + \frac{8D\tau}{z_{3\,DG}^2}\right)^{-1/2}$$

Additional Equations:

3D Gaussian 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.

Reaction during transit (Triplet state term):

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

..where T is the triplet state equilibrium population and τ_T is the triplet lifetime.

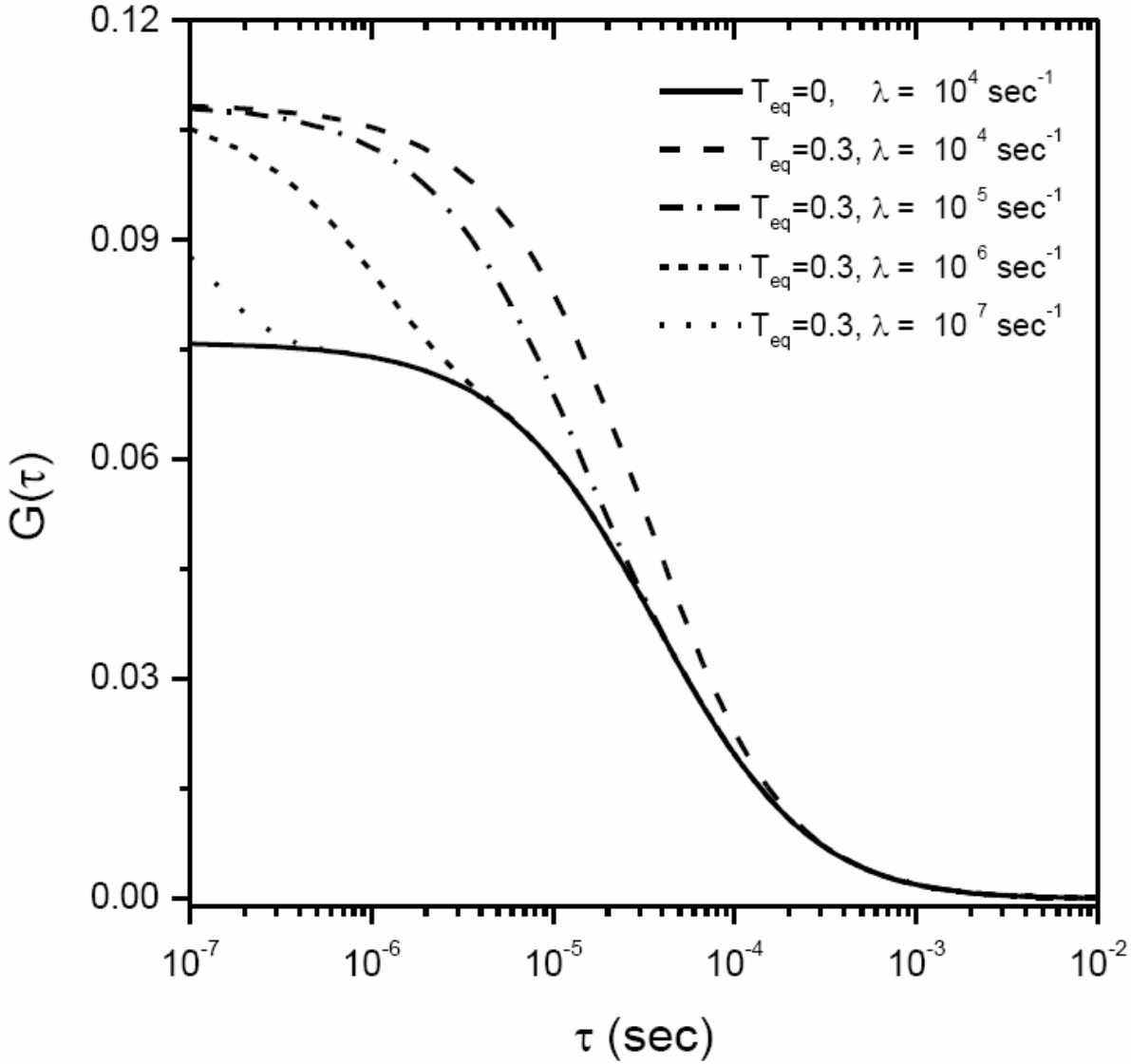
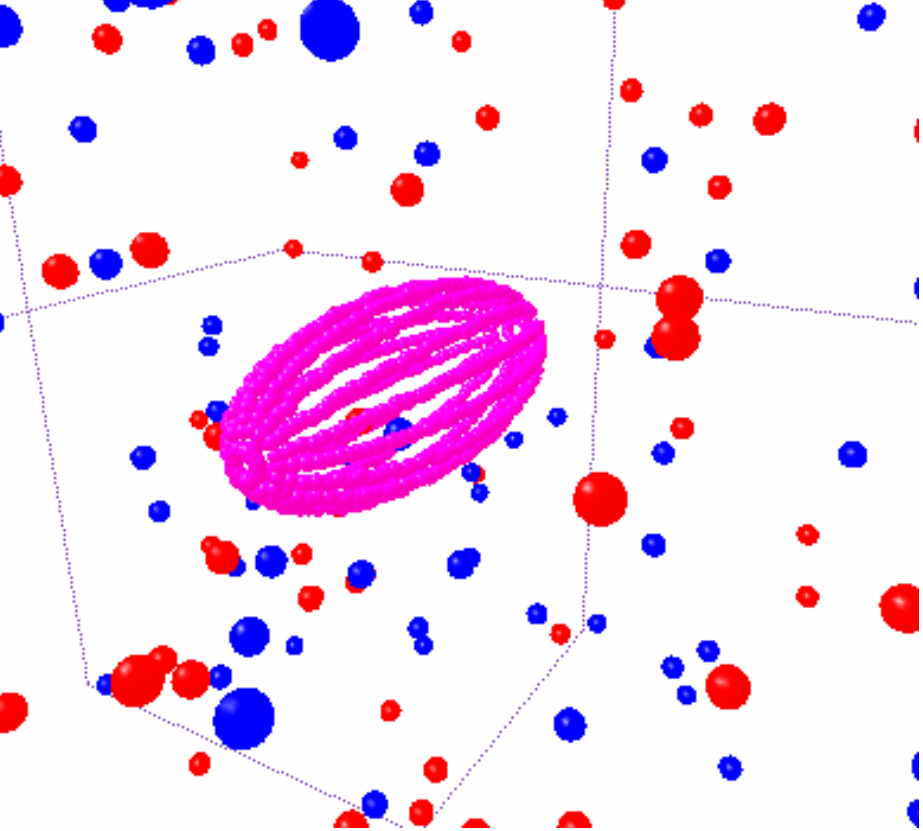


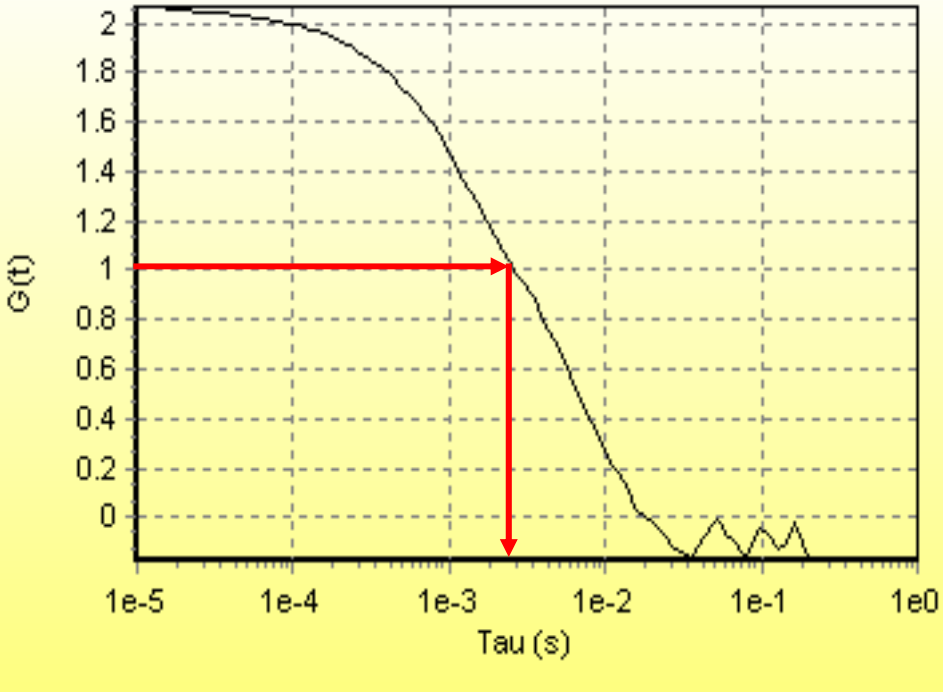
Figure 4.2 Simulation of autocorrelation functions using equation (4.12). The diffusion coefficient used is $300\,\mu m^2/\sec$, $w_{3DG} = 0.3\mu m$, $z_{3DG} = 1.5\mu m$.



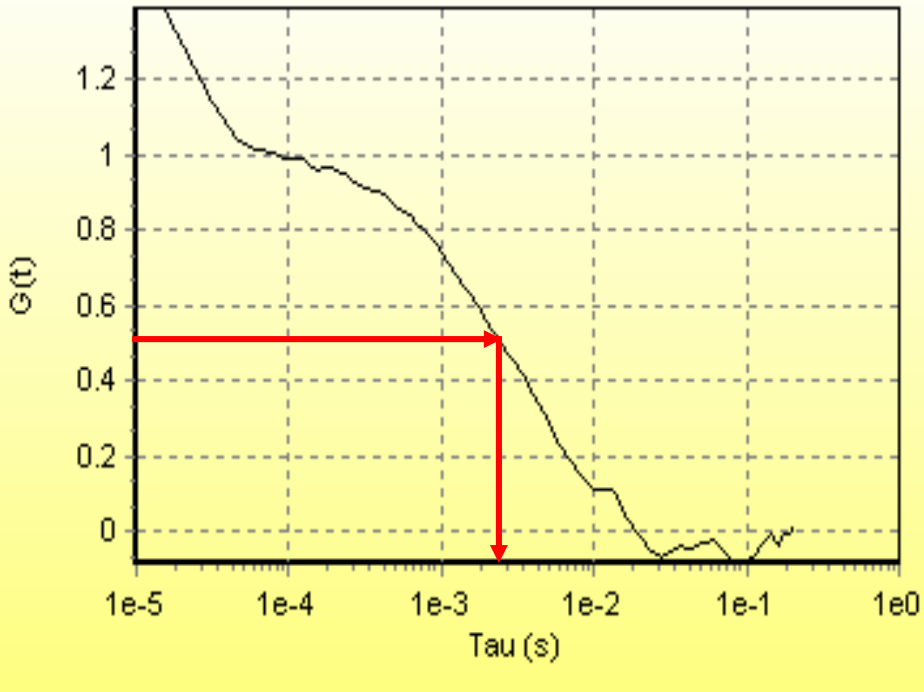
Box size=6.4 μm
Diffusion coefficient D=23 μm^2/s
Periodic boundary conditions

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

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



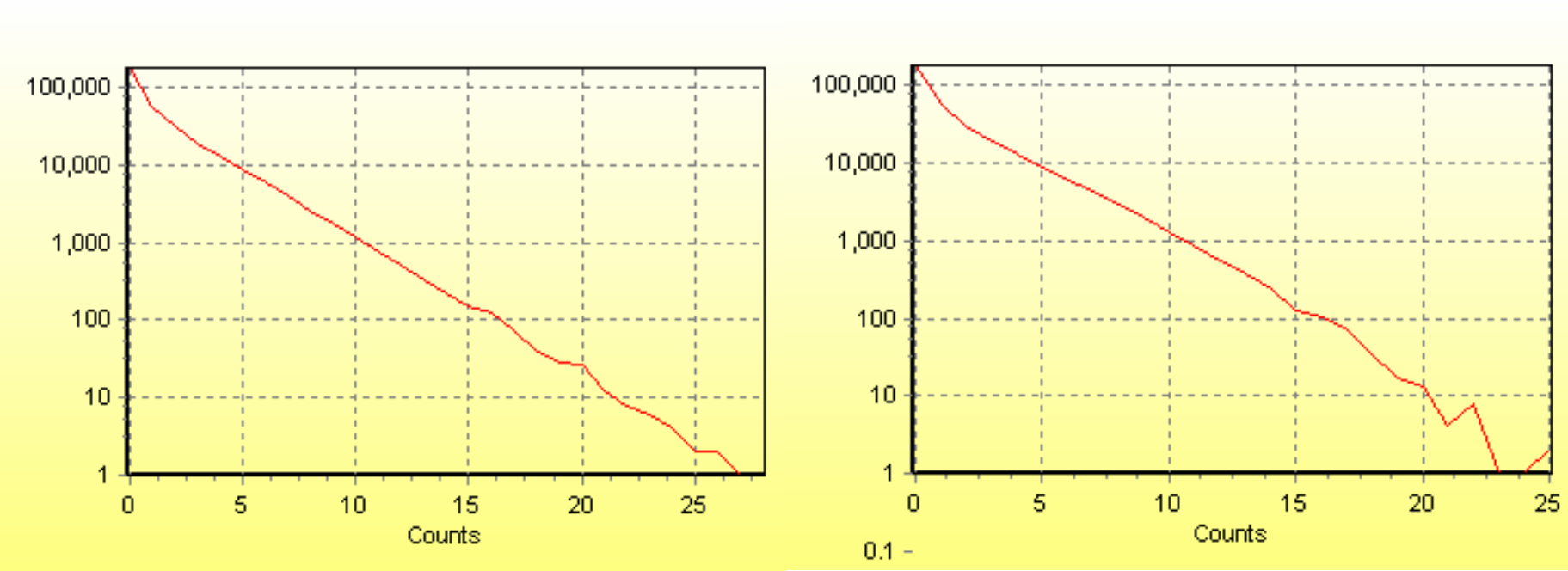
Correlation function for **pure diffusion**



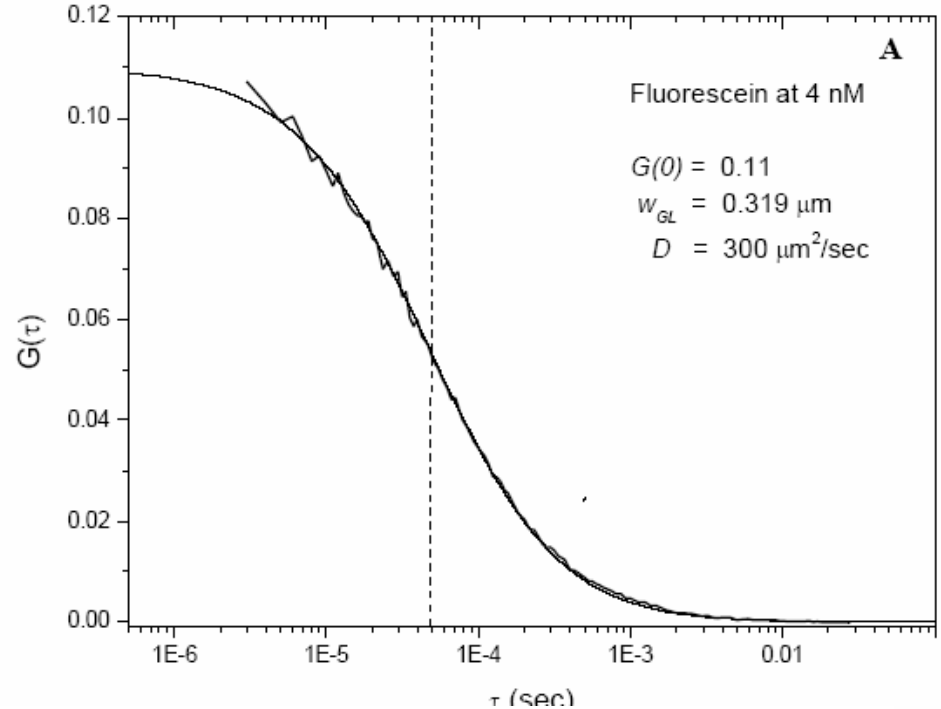
Correlation function for **diffusion and excited-state reaction** (triplet state)

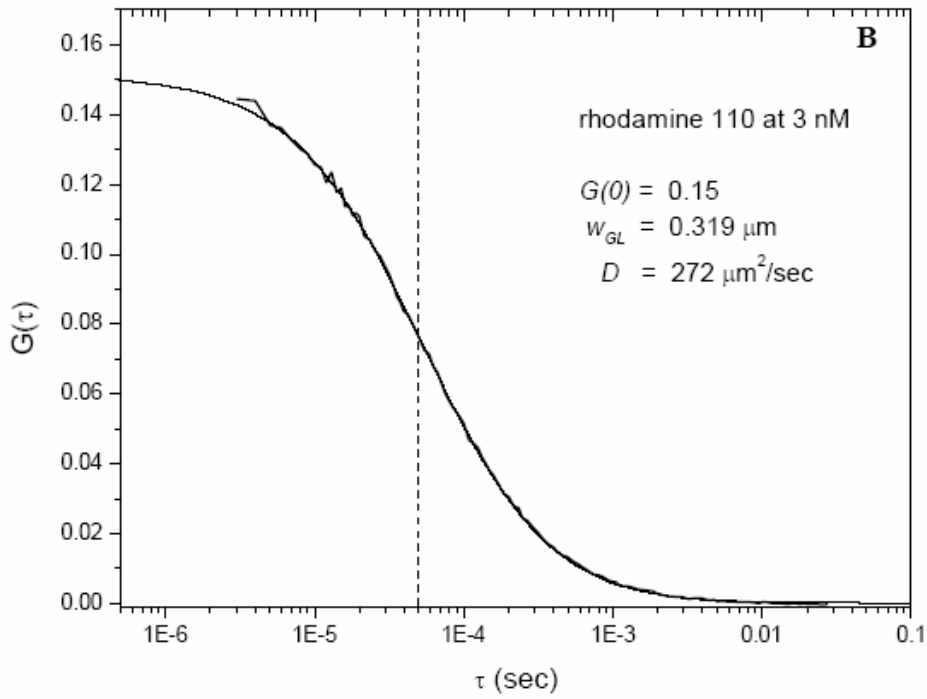
Panel 1: 100 particles in a box of approximately 6.4 μm side and a PSF of 0.5 μm waist and 1.5 μm axial waist.

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)?

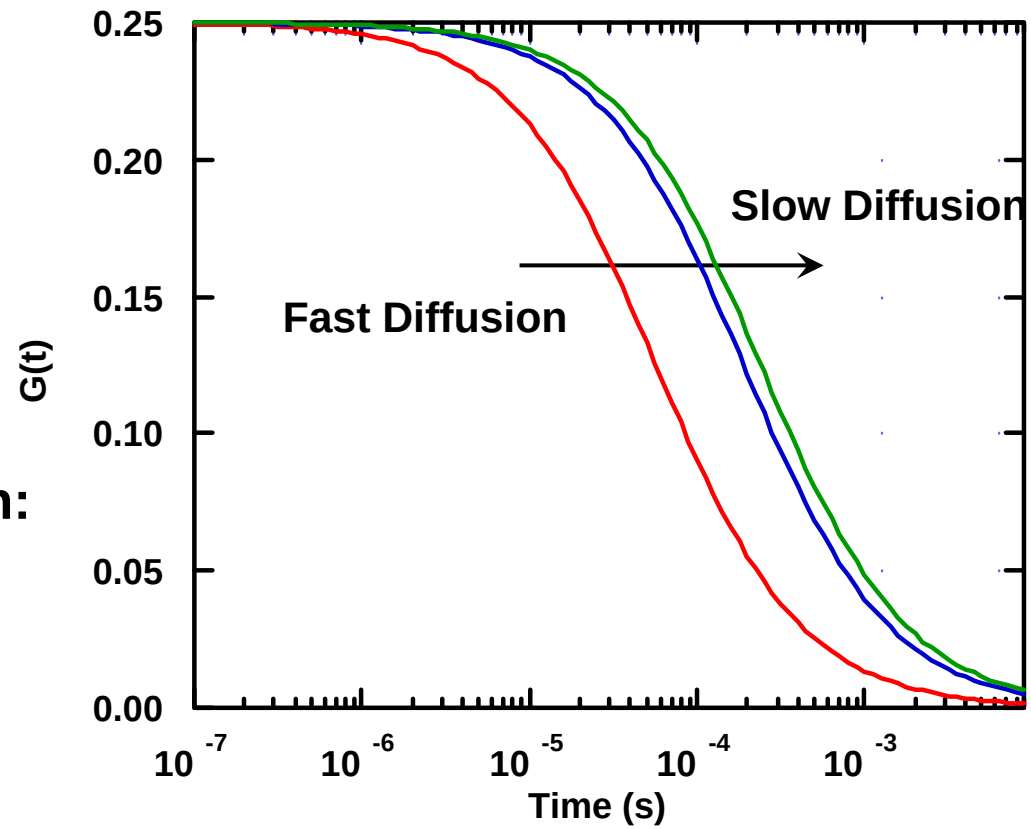

A
Fluorescein at 4 nM
G(0) = 0.11
w_{GL} = 0.319 μm
D = 300 μm²/sec
G(τ)
τ (sec)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1E-6
1E-5
1E-4
1E-3
0.01


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

The Effects of Particle Size on the Autocorrelation Curve

Diffusion Constants

300 μm²/s
90 μm²/s
71 μm²/s



Stokes-Einstein Equation:

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

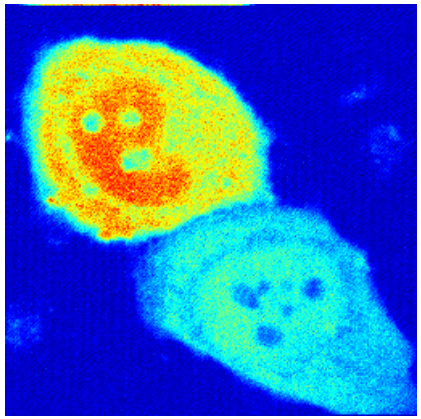
and

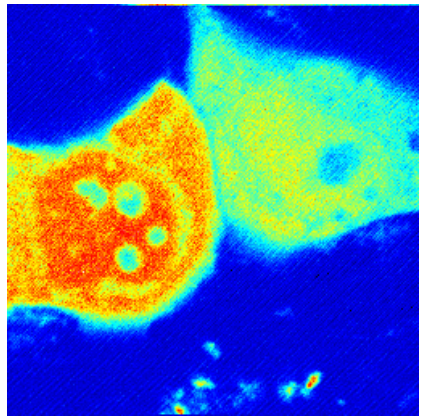
$$MW \propto Volume \propto r^3$$

Monomer --> Dimer

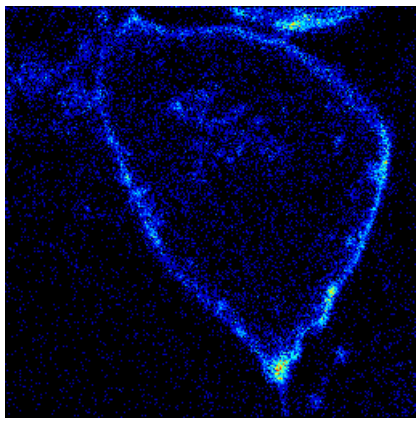
Only a change in D by a factor of $2^{1/3}$, or 1.26

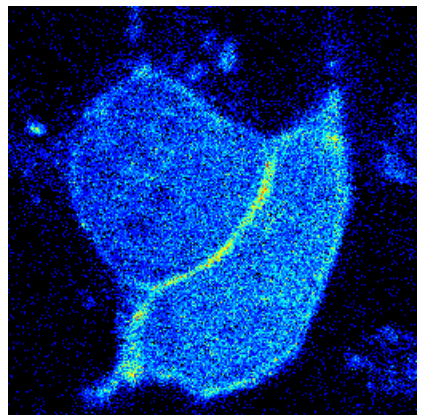
Autocorrelation Adenylate Kinase -EGFP Chimeric Protein in HeLa Cells





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



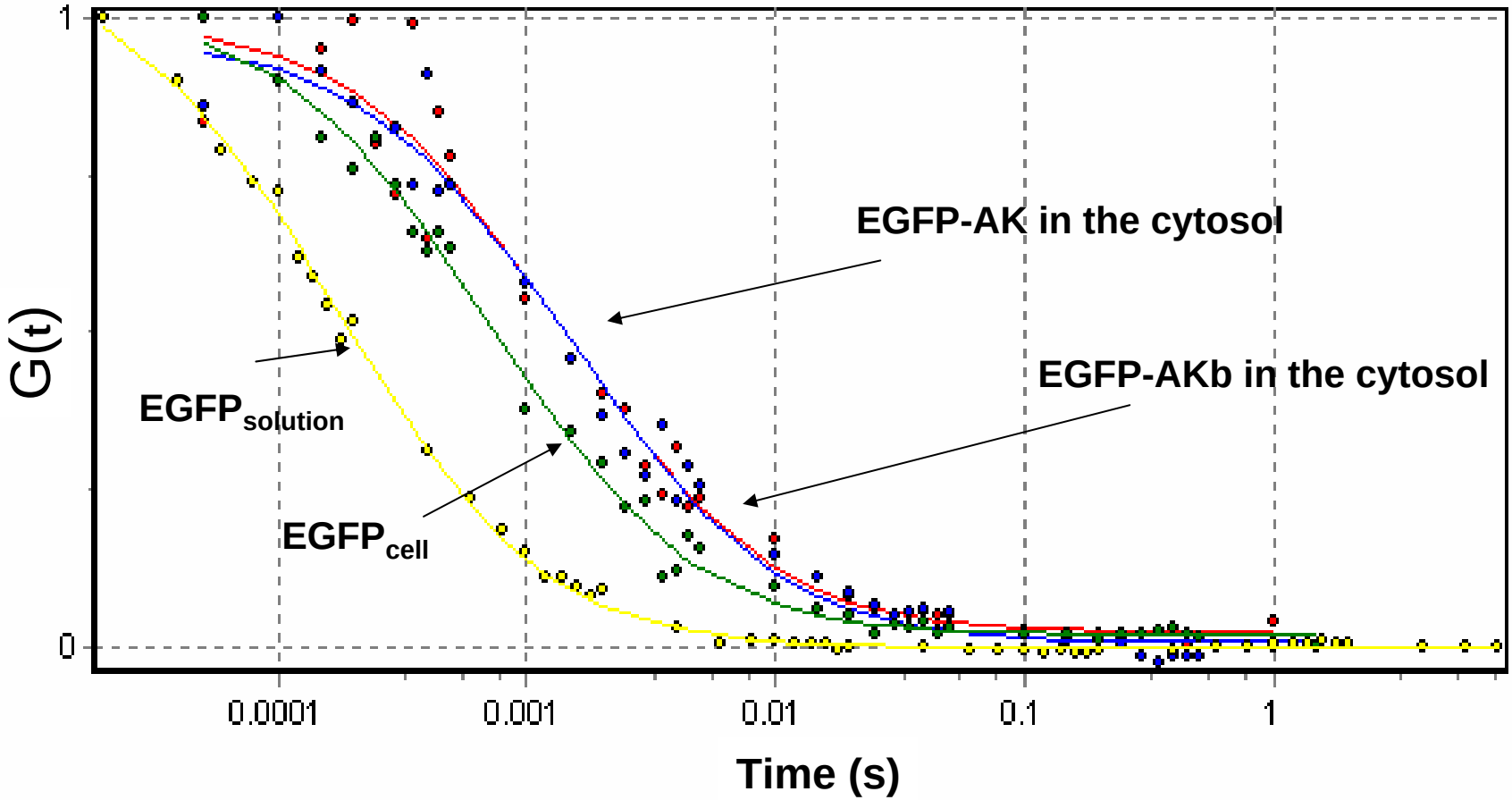


Examples of different *Hela* cells transfected with AK1b -EGFP

Fluorescence Intensity

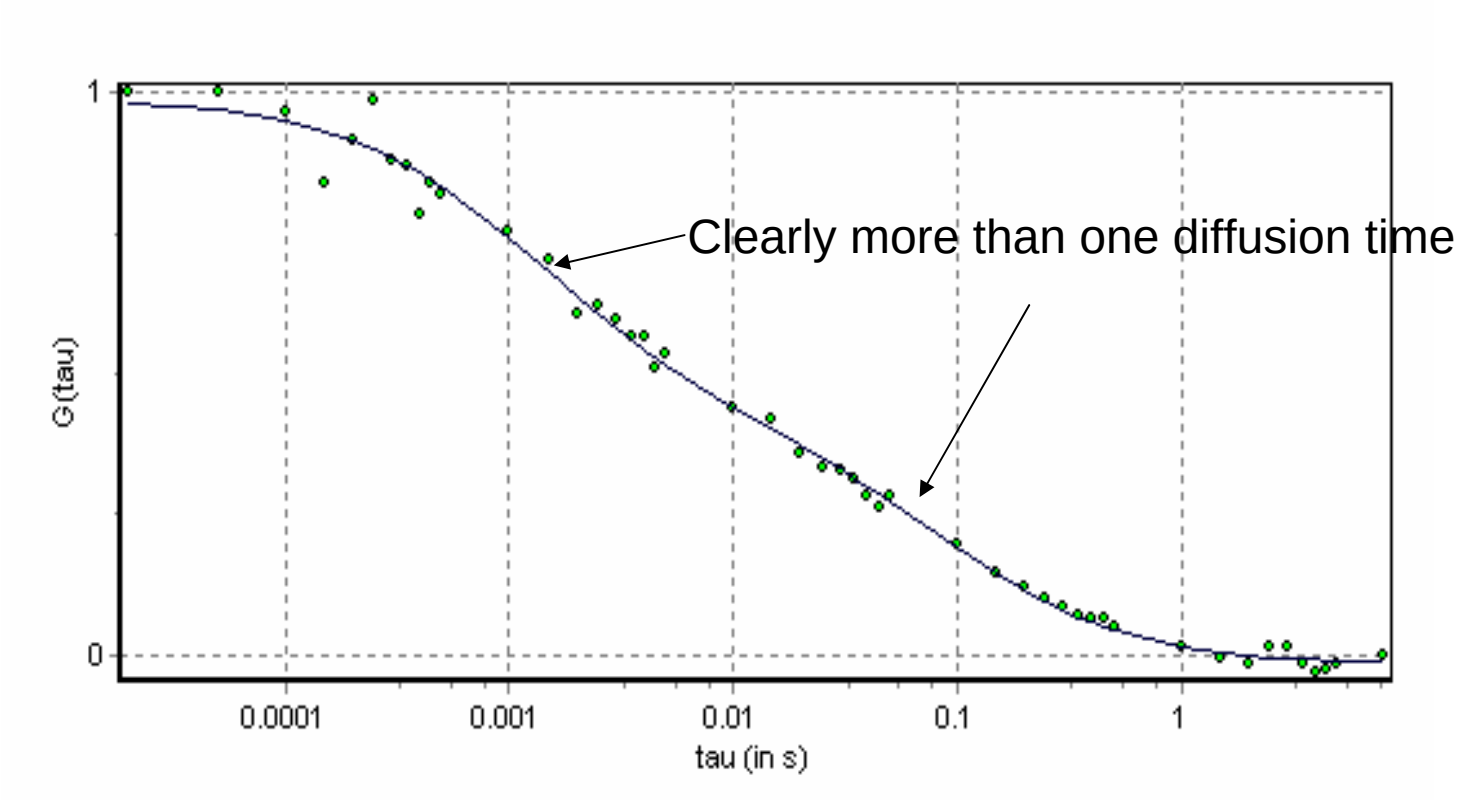
Qiao Qiao Ruan, Y. Chen, M. Glaser & W. Mantulin Dept. Biochem & Dept Physics- LFD Univ Il, USA

Autocorrelation of EGFP & Adenylate Kinase -EGFP



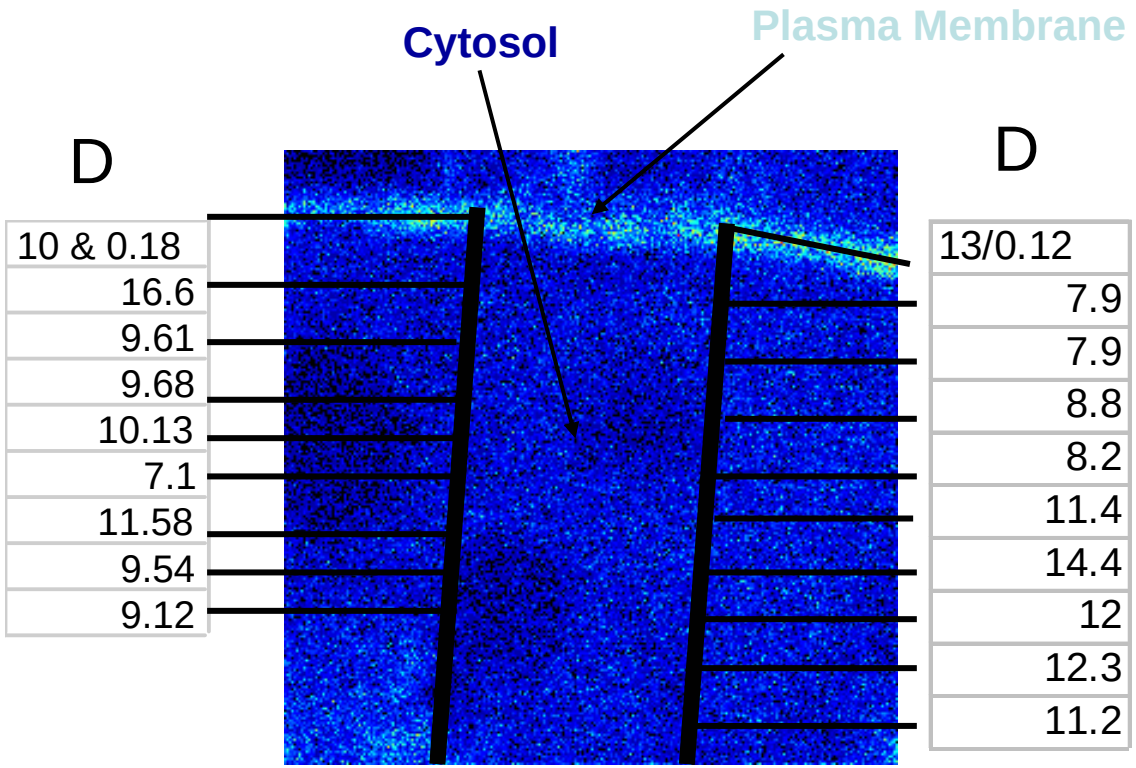
Normalized autocorrelation curve of EGFP in solution (•), EGFP in the cell (•), AK1-EGFP in the cell(•), AK1b-EGFP in the cytoplasm of the cell(•).

Autocorrelation of Adenylate Kinase –EGFP on the Membrane



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

Autocorrelation Adenylate Kinaseb -EGFP



Diffusion constants (µm^2/s) of AK EGFP-AKb 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.

Multiple Species

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

Autocorrelation function can be used:

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

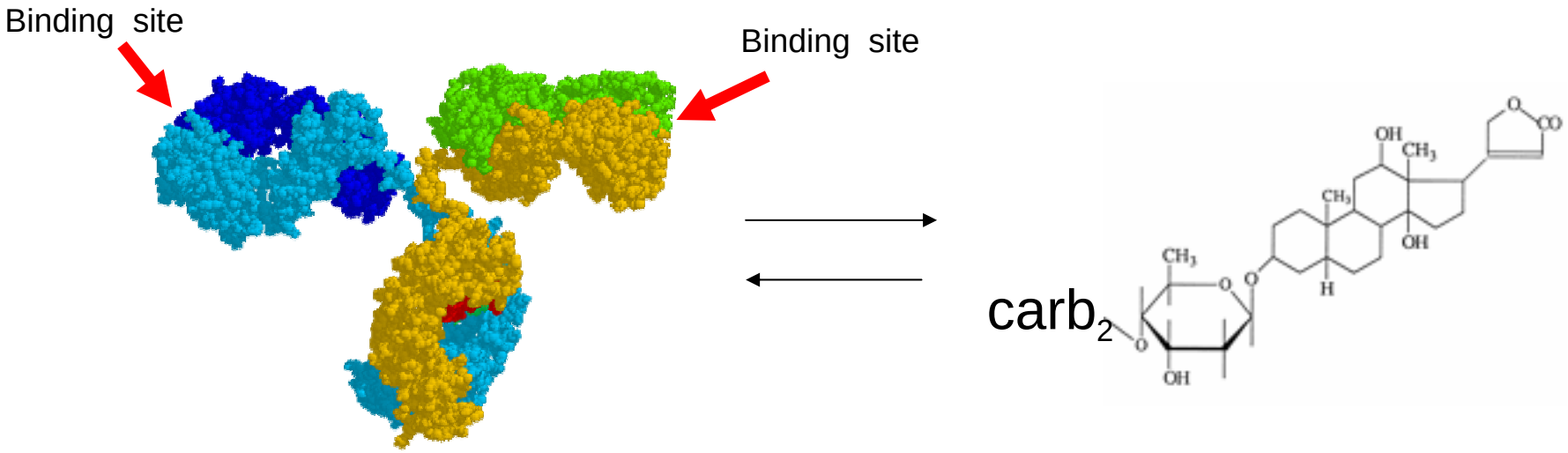
(2D-Gaussian Shape)

!

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

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

Antibody - Hapten Interactions

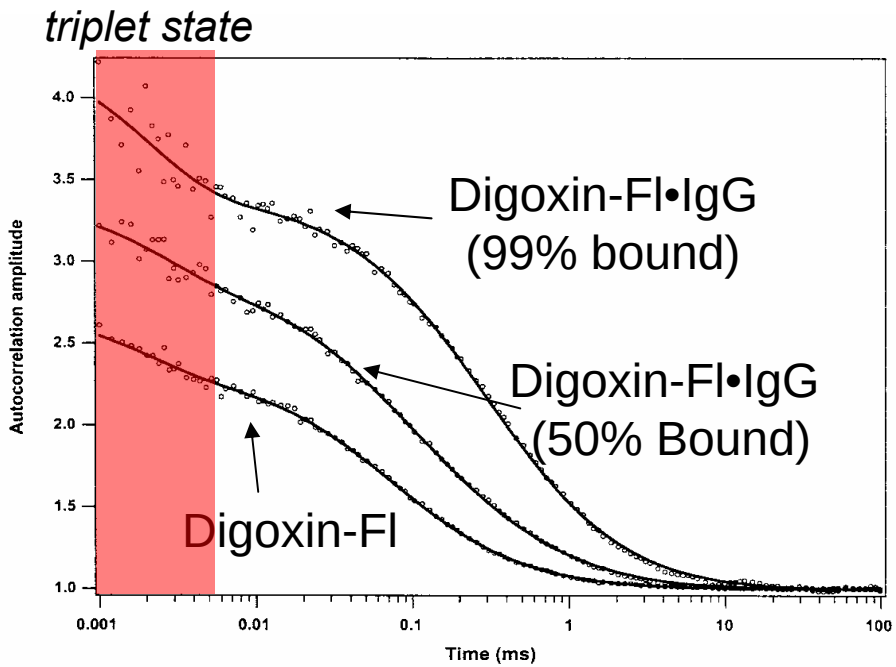


Mouse IgG: The two heavy chains are shown in yellow and light blue. The two light chains are shown in green and dark blue..*J.Harris, S.B.Larson, K.W.Hasel, A.McPherson, "Refined structure of an intact IgG2a monoclonal antibody", Biochemistry 36: 1581, (1997)*.

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.

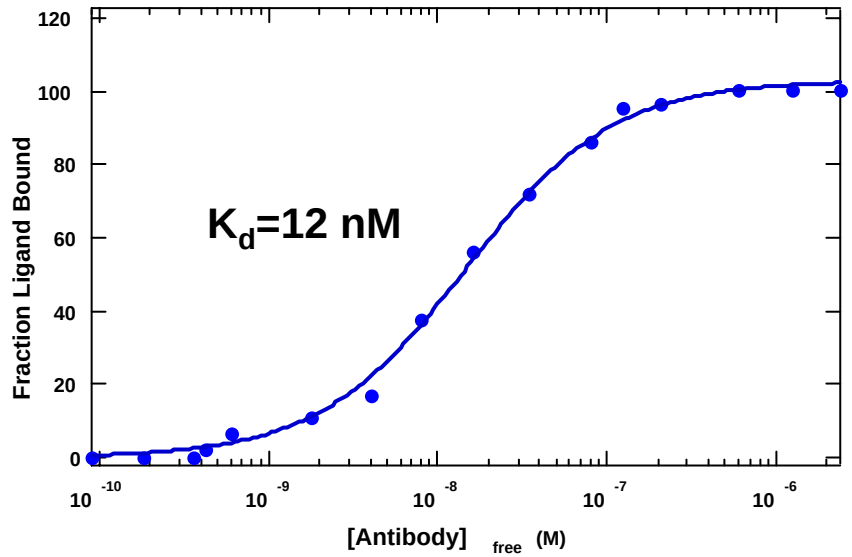
Anti-Digoxin Antibody (IgG) Binding to Digoxin-Fluorescein

Autocorrelation curves:



Binding titration from the autocorrelation analyses:

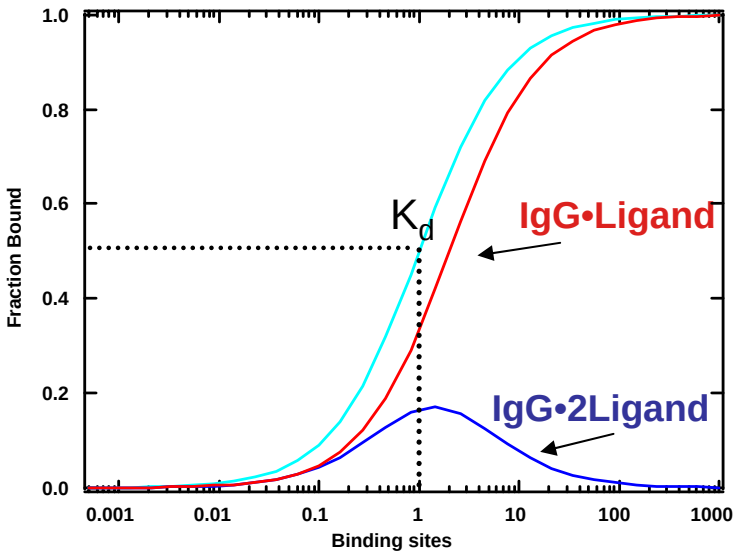
$$F_b = \frac{m \cdot S_{free}}{K_d + S_{free}} + c$$

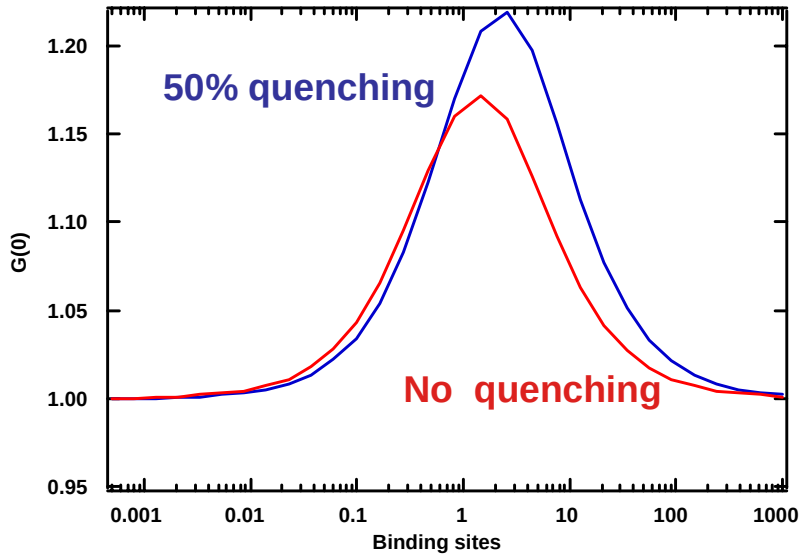


S. Tetin, K. Swift, & , E, Matayoshi , 2003

Two Binding Site Model

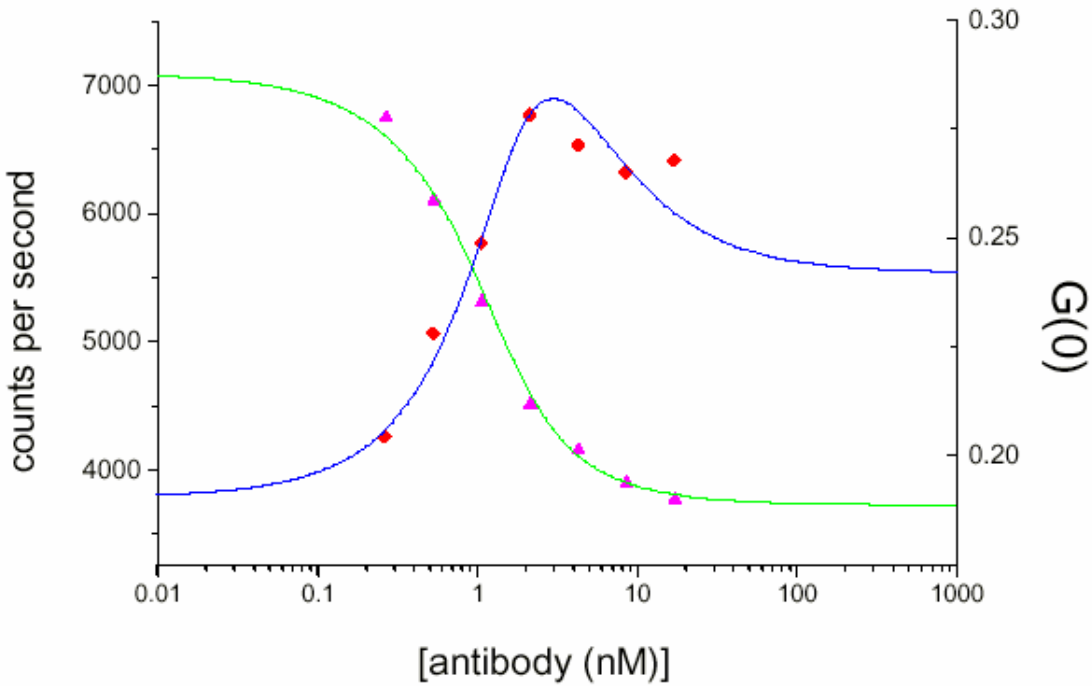
IgG + 2 Ligand-Fl ⟷ IgG•Ligand-Fl + Ligand-Fl ⟷ IgG•2Ligand-Fl





[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