# Introduction to Fluorescence Correlation Spectroscopy (FCS)

***Theodore Hazlett***

***Principles of Fluorescence Techniques***
***Genova, Italy***
***June 14-16, 2004***

# 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

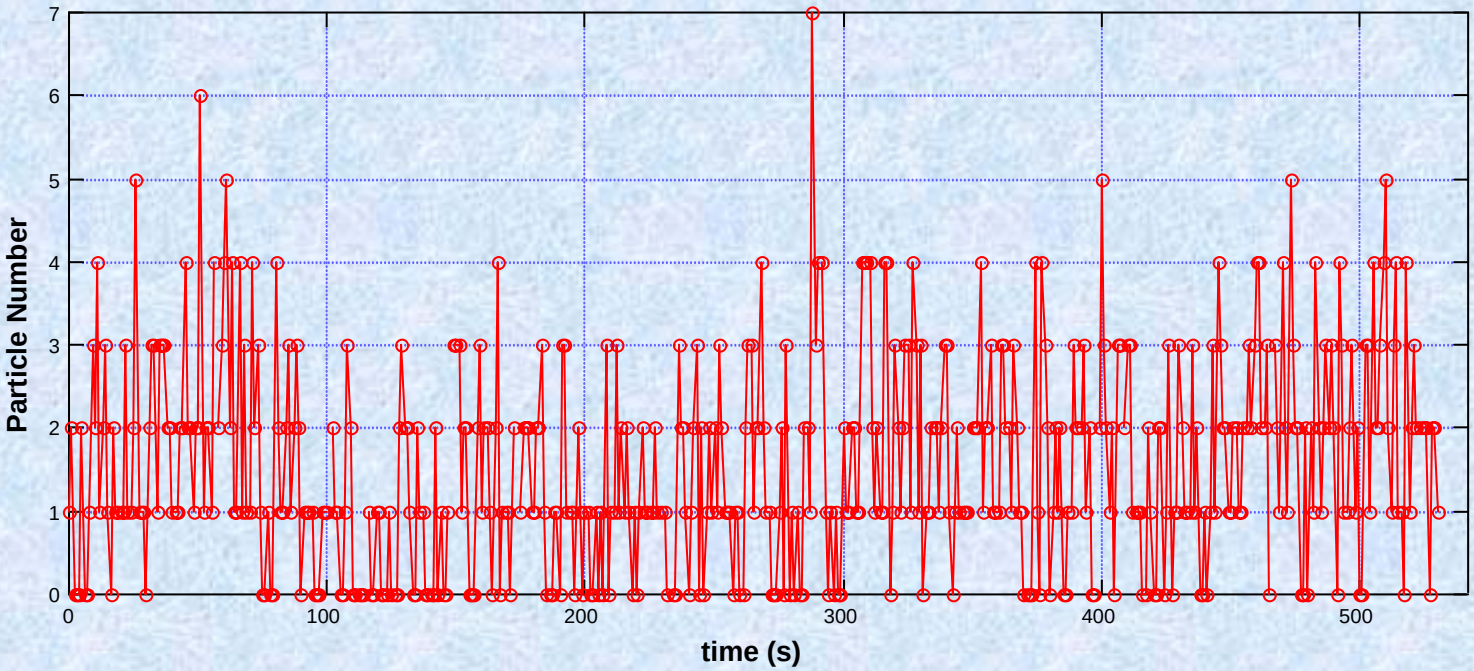
(Svedberg & Inouye, 1911) *Occupancy Fluctuation*

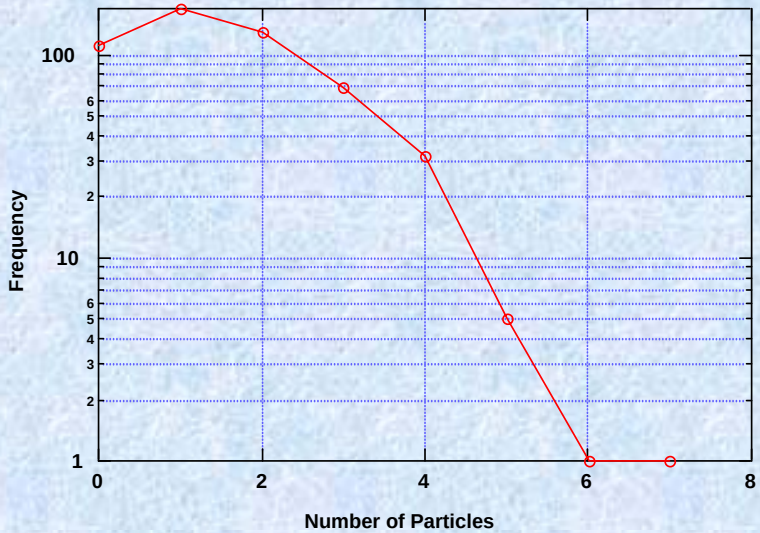
**Experimental data on colloidal gold particles:**

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

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

# Particle Correlation



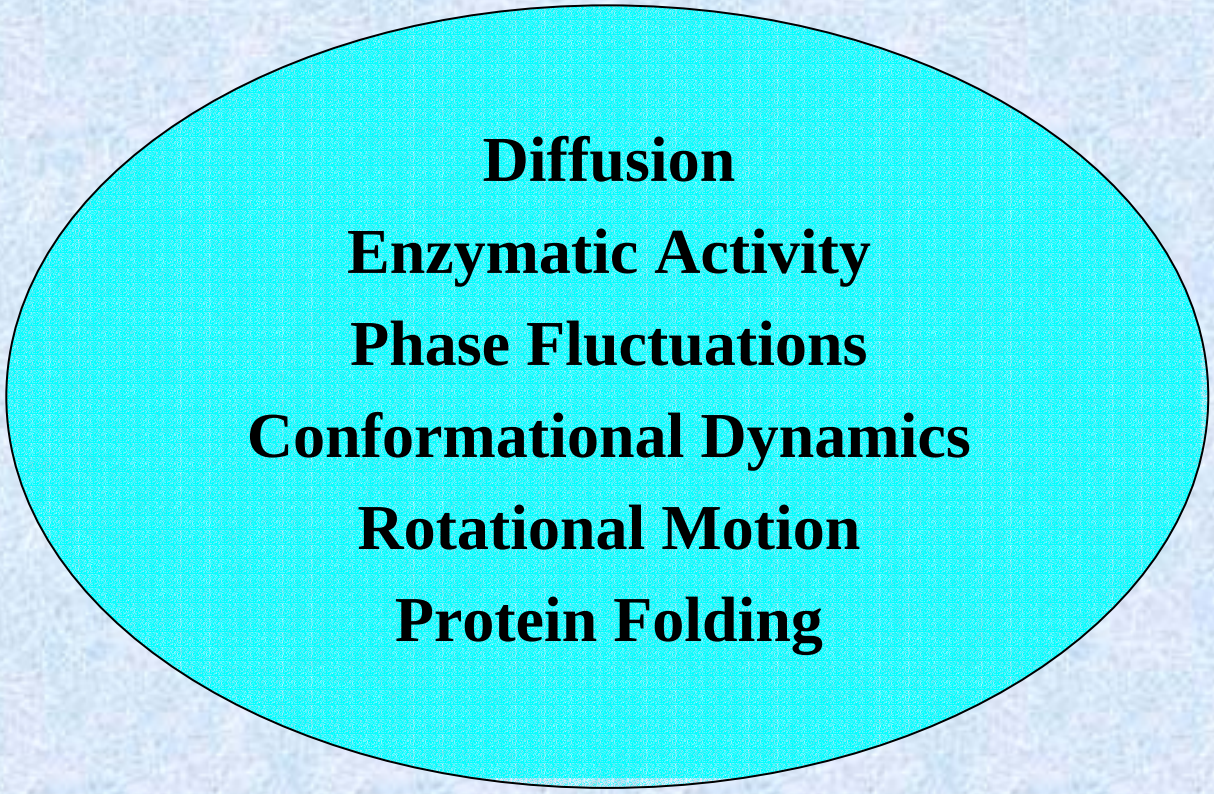




*Histogram of particle counts

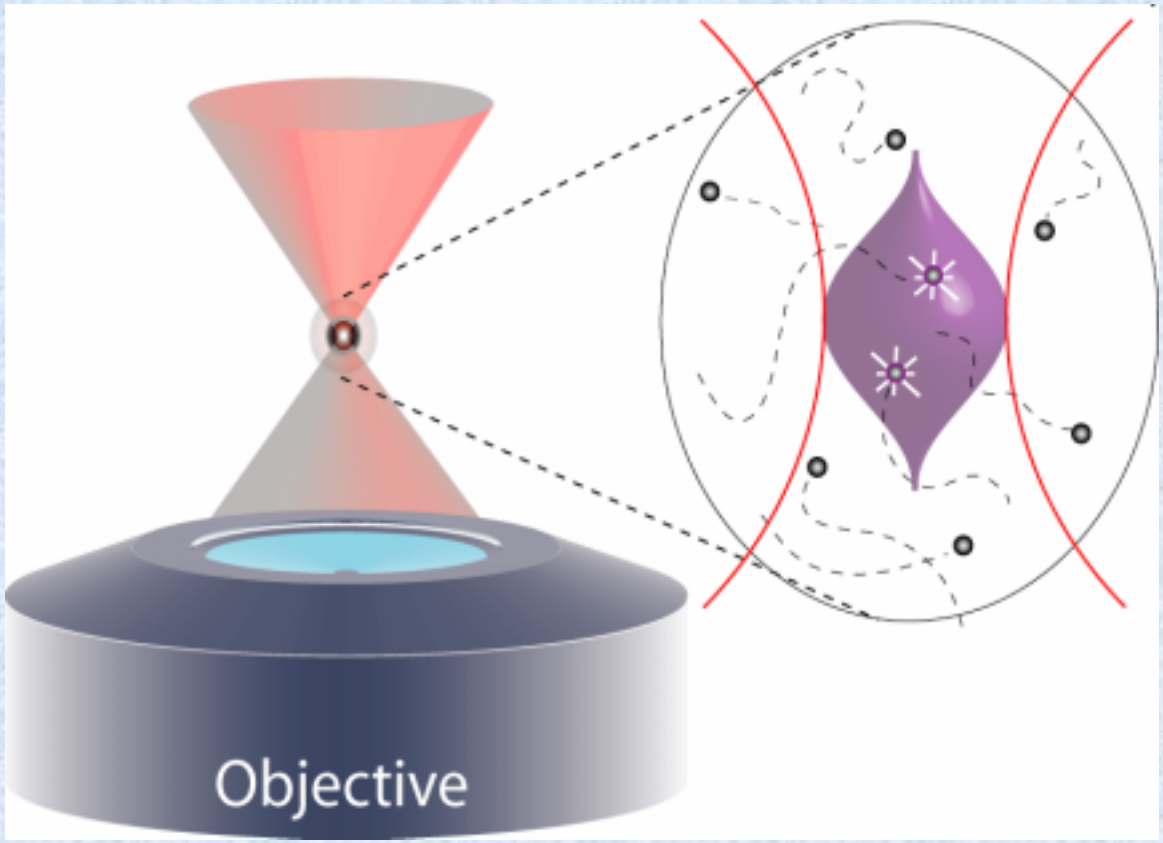
*Poisson behavior

*Autocorrelation not available

# In FCS
# Fluctuations are in the Fluorescence Signal



# Generating Fluctuations By Motion




## What is Observed?

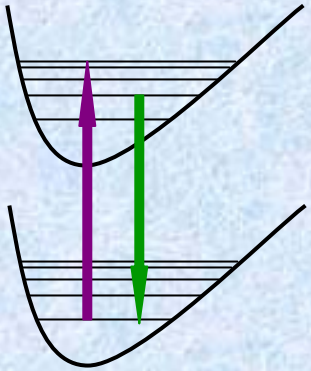
1. The rate of motion.

2. The concentration of particles.

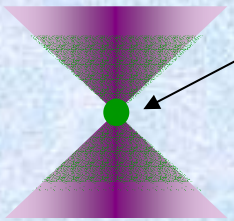
3. Changes in the particle fluorescence while under observation.

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

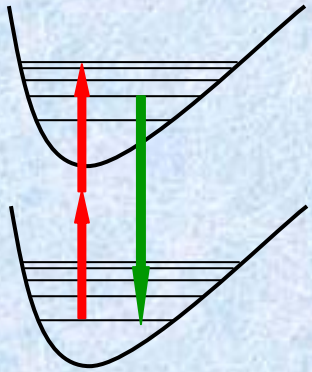
1 - Photon



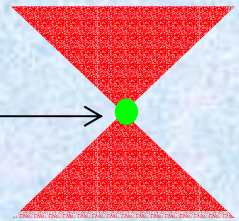
Defined by the pinhole size, wavelength, magnification and numerical aperture of the objective



2 - Photon

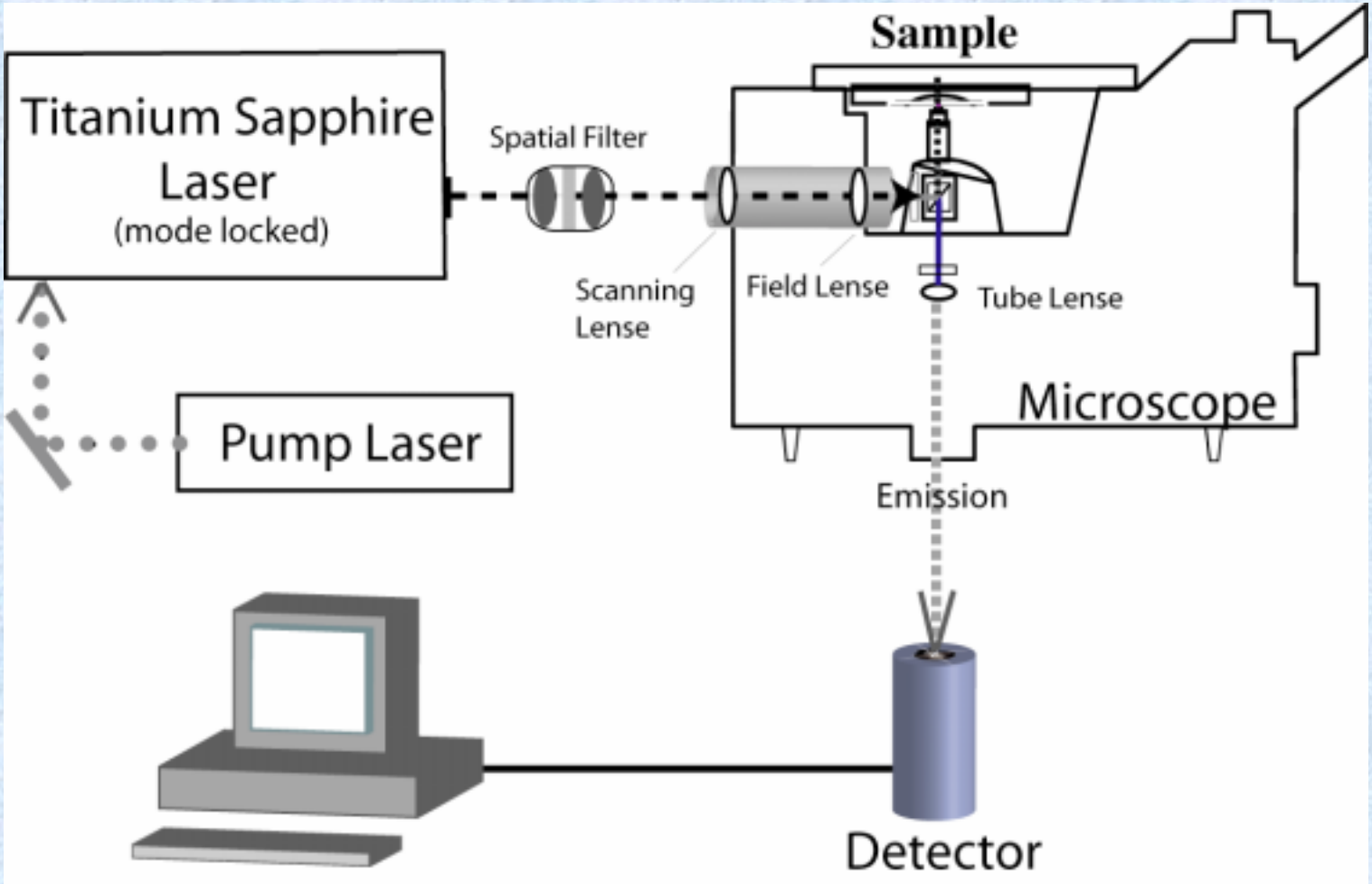


Approximately 1 $um^3$



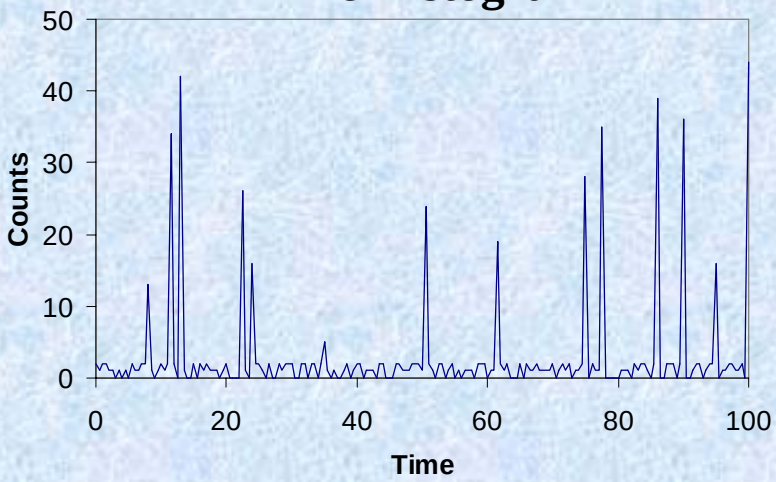
Defined by the wavelength and numerical aperture of the objective


Brad Amos
MRC, Cambridge, UK

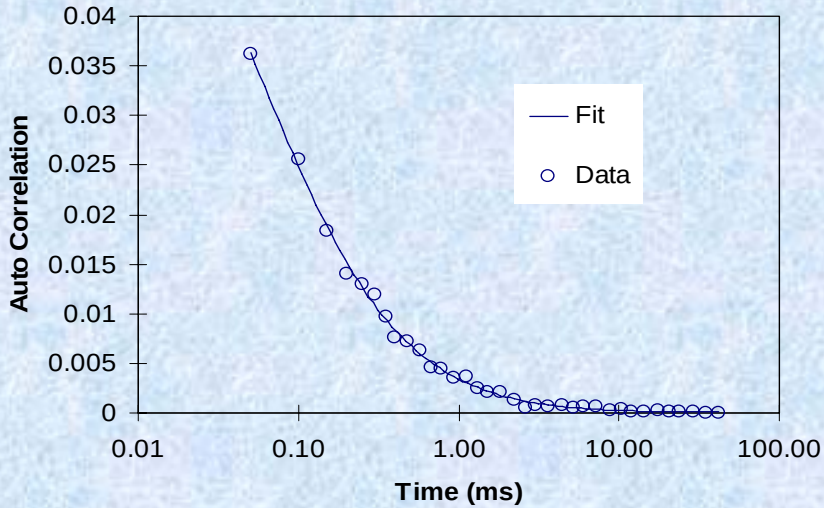

Sample
Titanium Sapphire Laser
(mode locked)
Spatial Filter
Scanning Lense
Field Lense
Tube Lense
Microscope
Pump Laser
Emission
Detector

# Data Treatment & Analysis

## Time Histogram

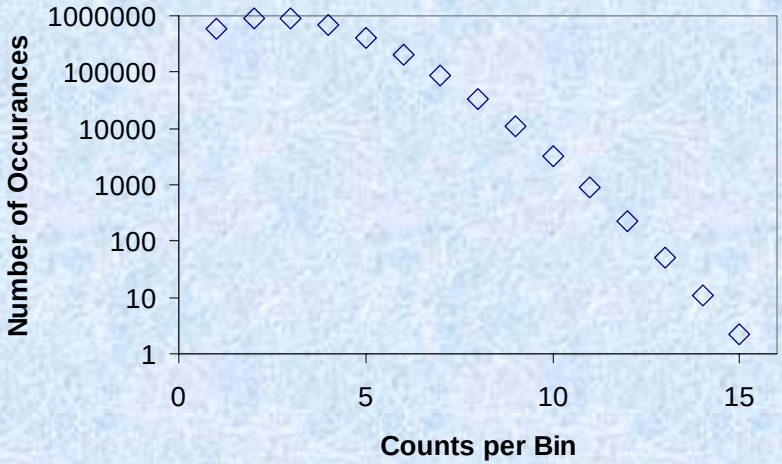



## Autocorrelation




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

## Photon Counting Histogram (PCH)




**PCH Parameters: <N> & $\varepsilon$**

# 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:

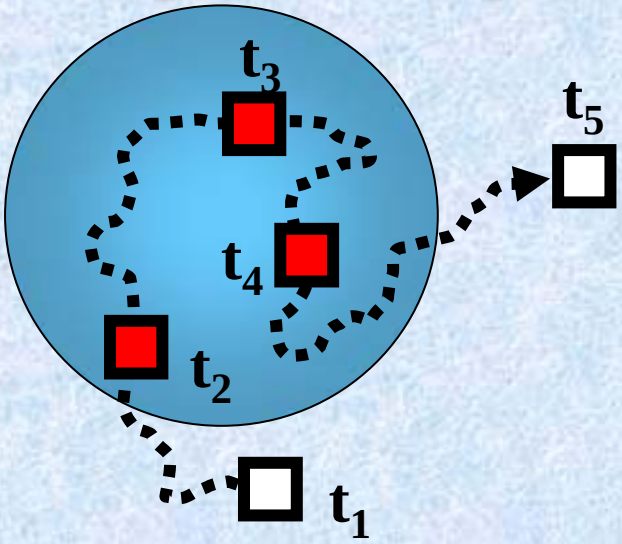
$$F(t) = \kappa Q \int d\mathbf{r}\, W(\mathbf{r}) C(\mathbf{r}, t)$$

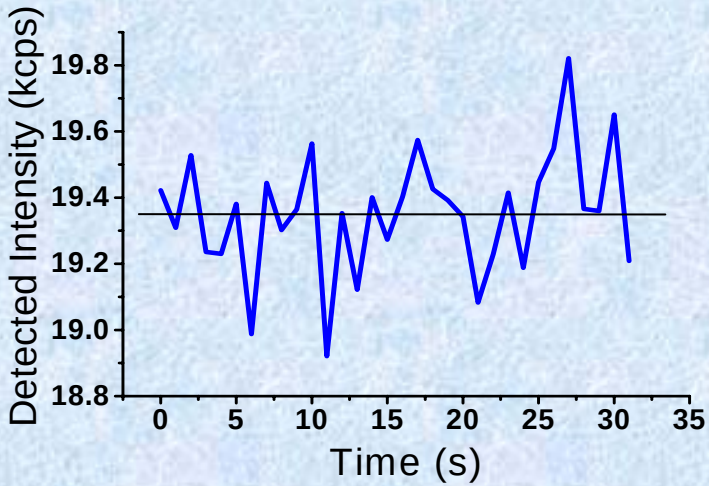
κQ = quantum yield and detector sensitivity (how bright is our probe)

W(r) describes our observation volume

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

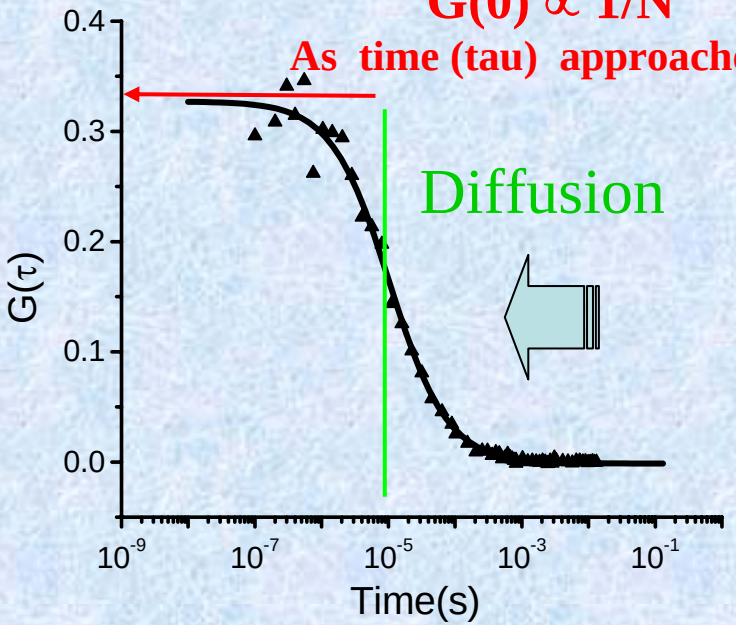
# The Autocorrelation Function




**G(0) ∝ 1/N**

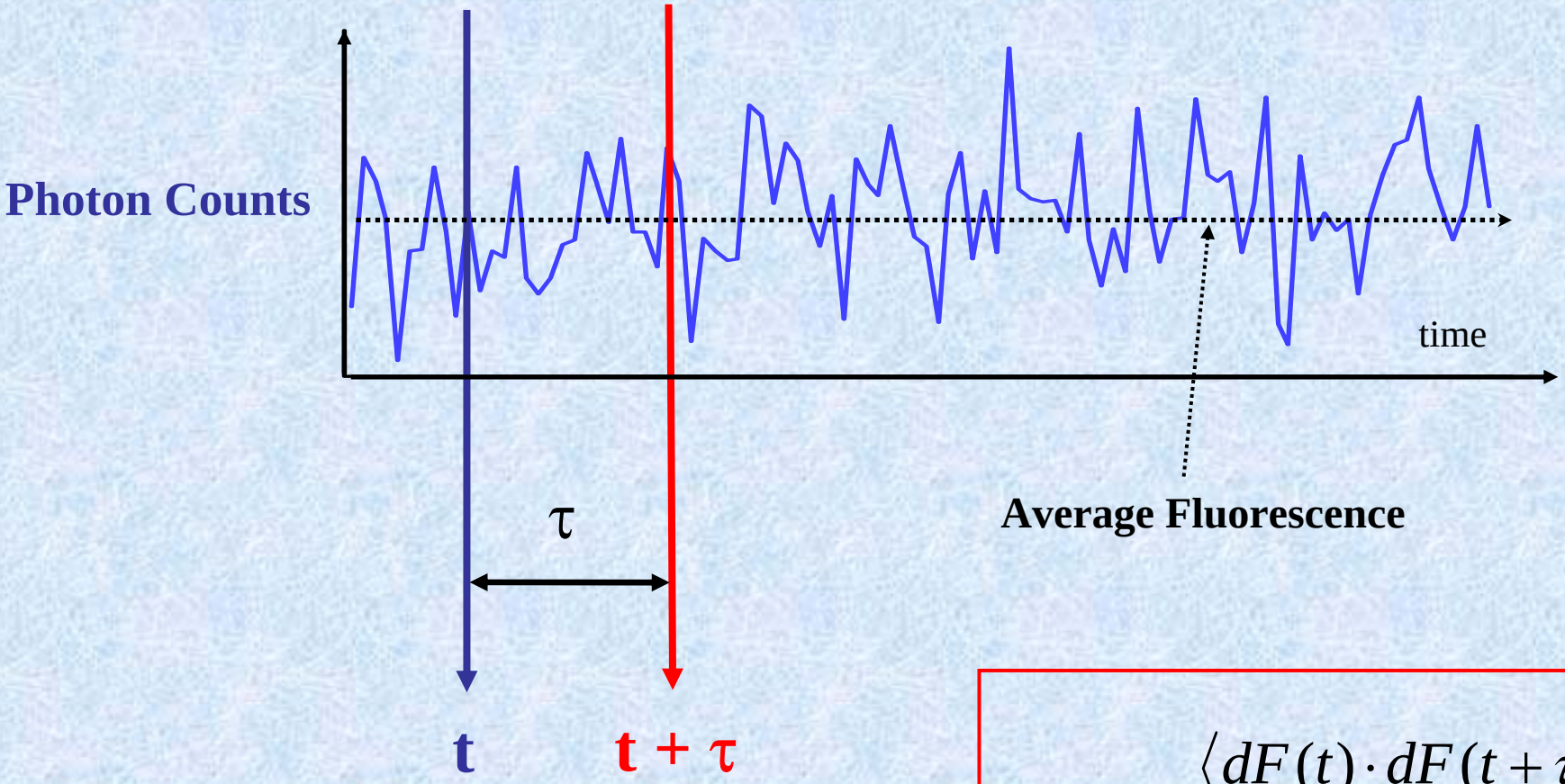
**As time (tau) approaches 0**




Diffusion

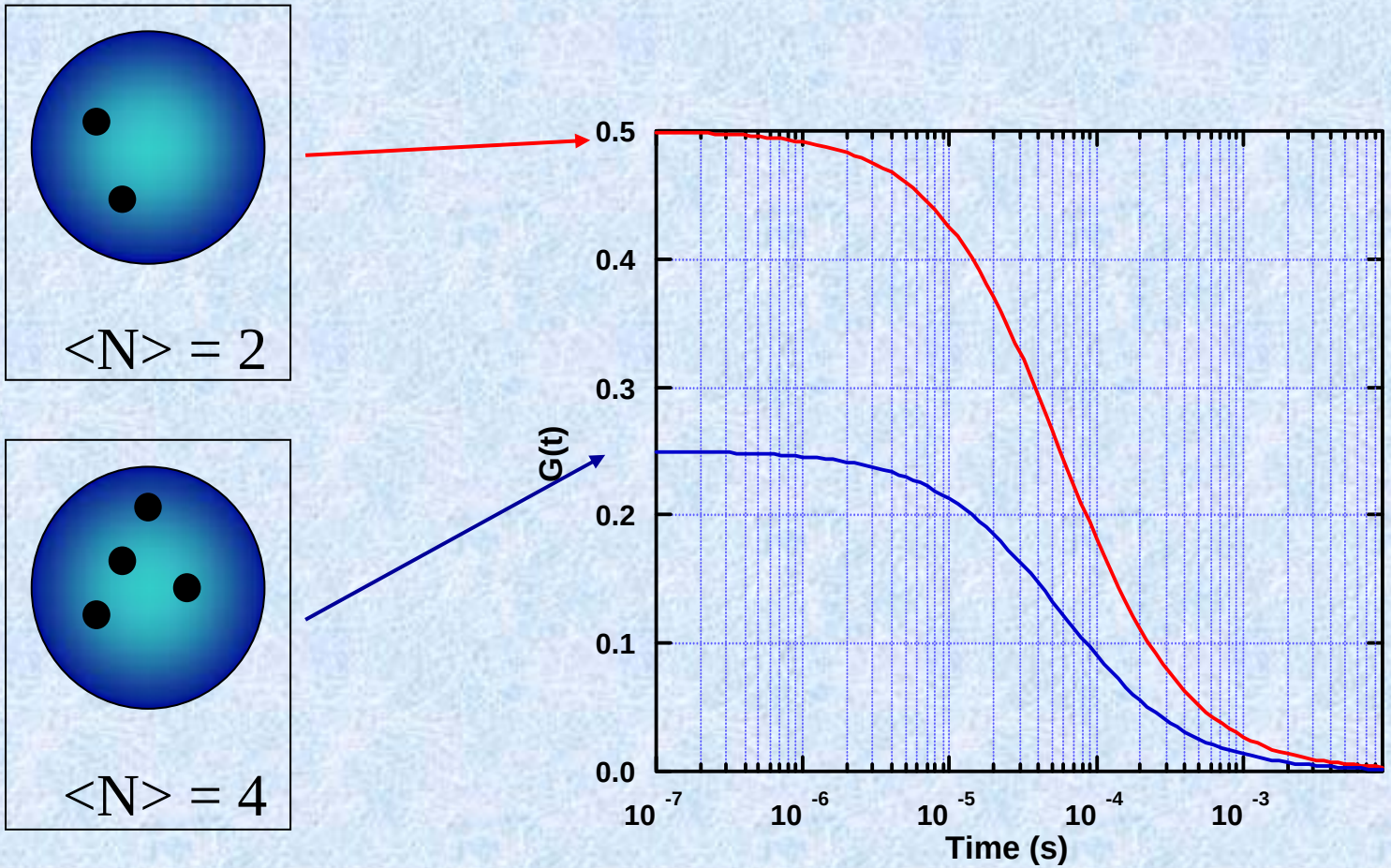
$$G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

# Calculating the Autocorrelation Function




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

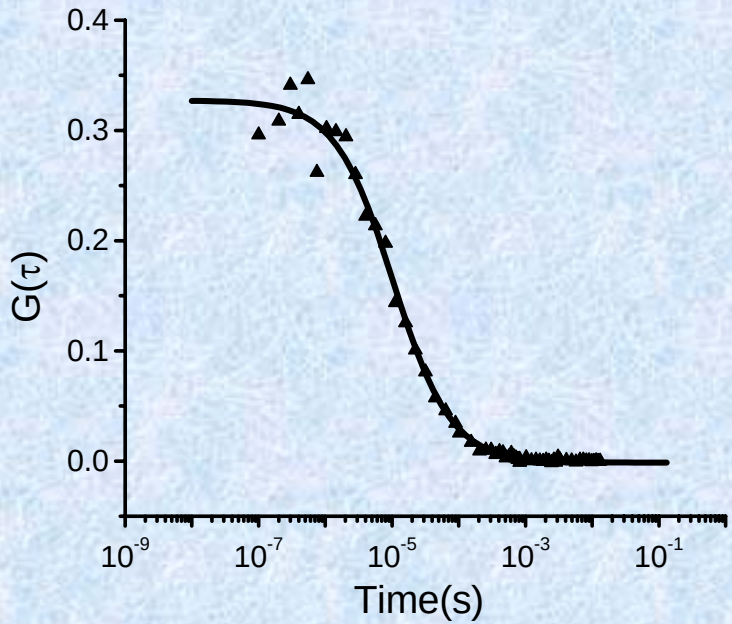
# The Effects of Particle Concentration on the Autocorrelation Curve



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

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

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

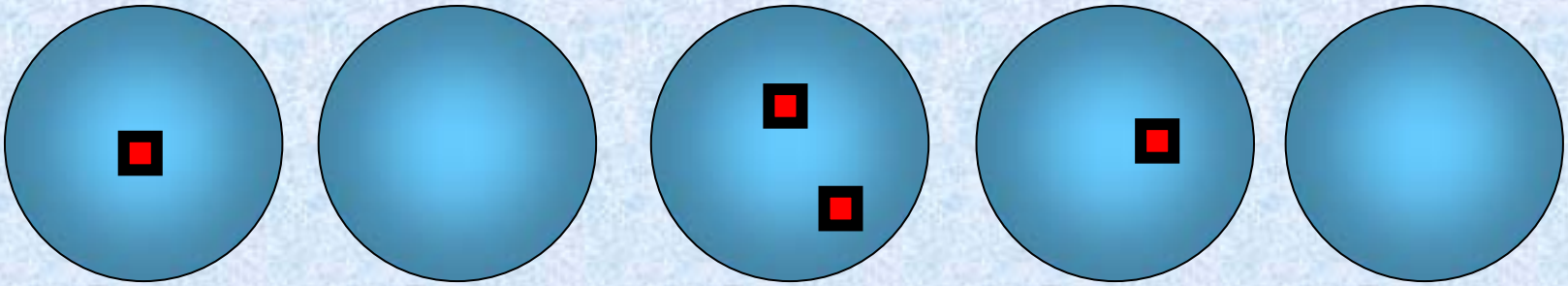



$$G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

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

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

# G(0), Particle Brightness and Poisson Statistics



**1 0 0 0 0 0 0 0 2 0 1 1 1 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 1 0 0 1 0 0 1 0 0**

*Time*

**Average = 0.275** **Variance = 0.256**

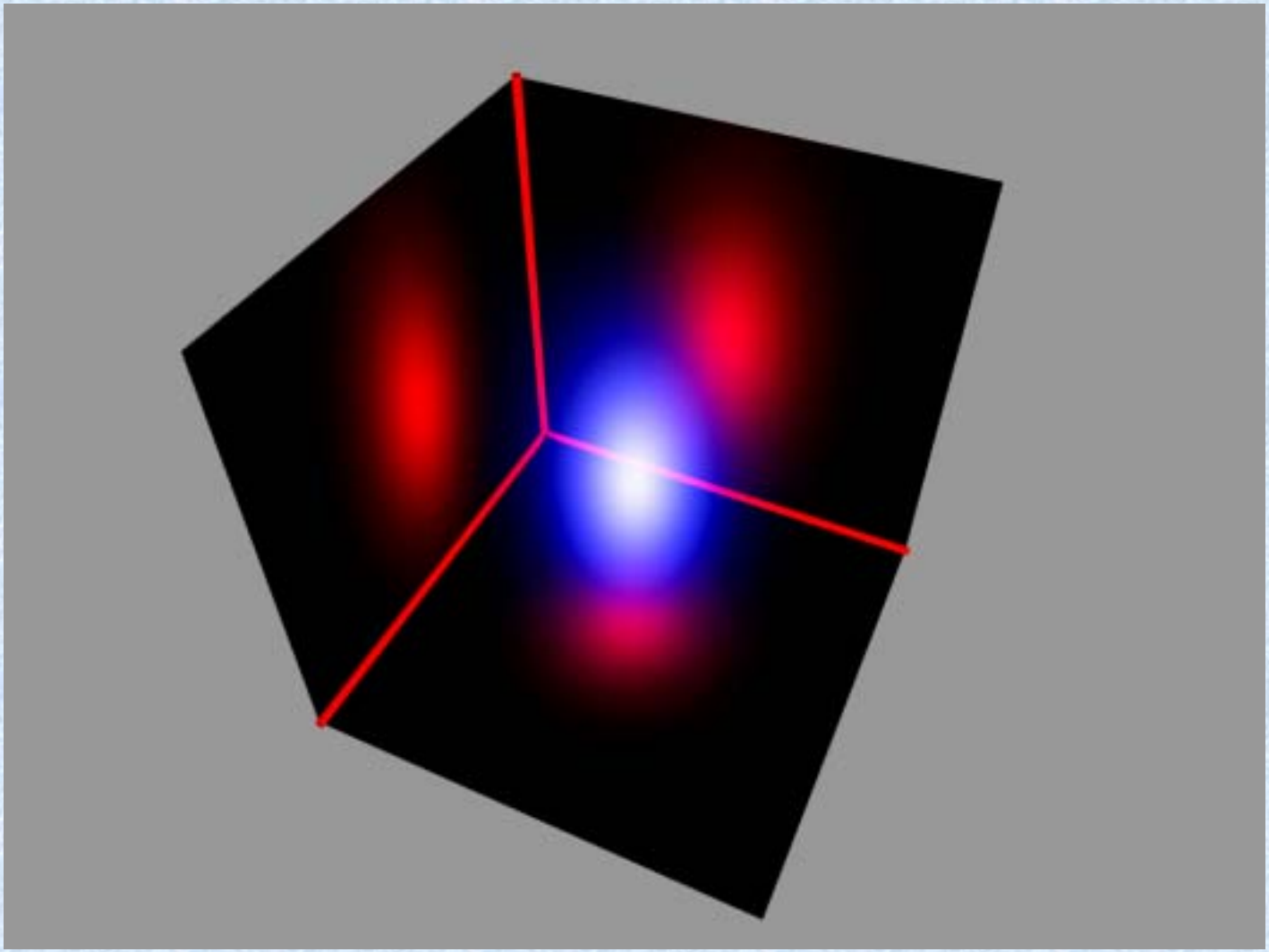
$$\langle N \rangle \propto Average^2 \Big/ Variance = \frac{0.275^2}{0.256} = 0.296$$

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 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 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, $\tau_D$ 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.*

**Triplet state term:**

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

*..where T is the triplet state amplitude and $\tau_T$ is the triplet lifetime.*

# The Effects of Particle Size on the Autocorrelation Curve

**Diffusion Constants**

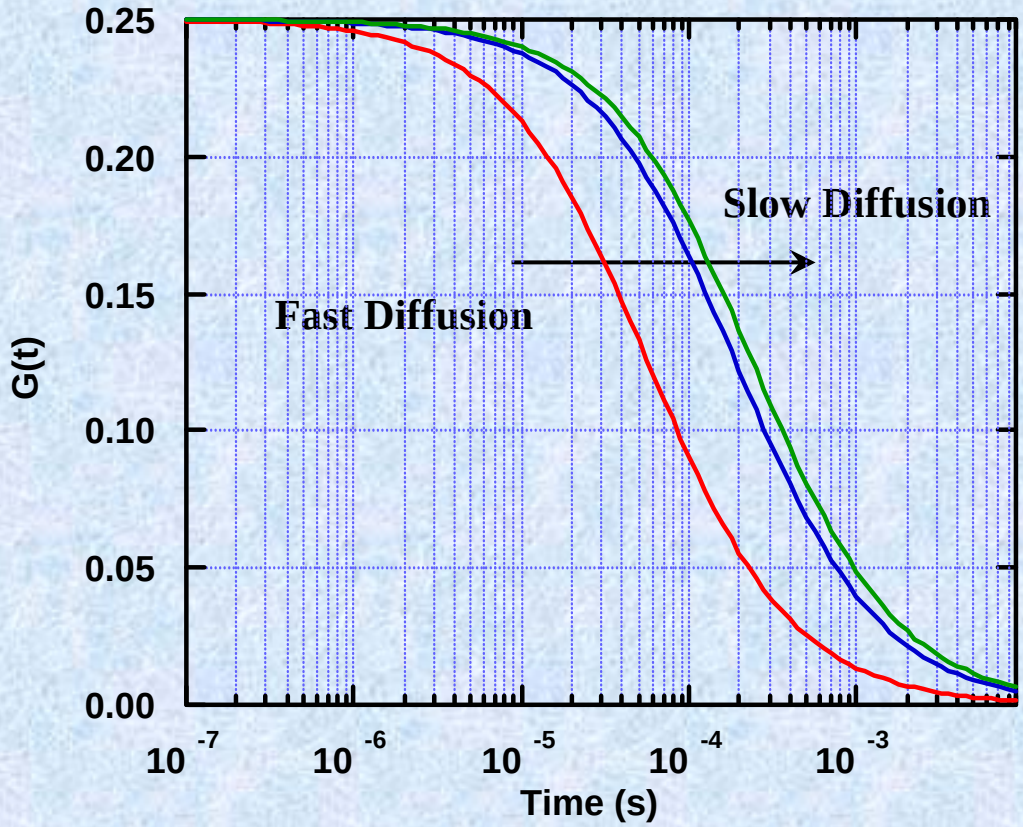
300 $um^2/s$
90 $um^2/s$
71 $um^2/s$

**Stokes-Einstein Equation:**

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

*and*

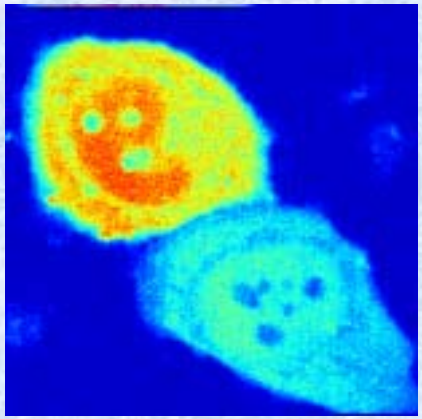
$$MW \propto Volume \propto r^3$$

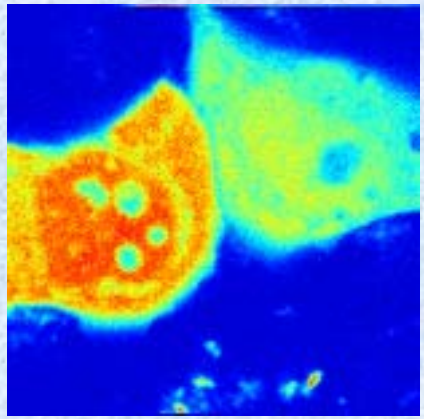



Monomer --> Dimer

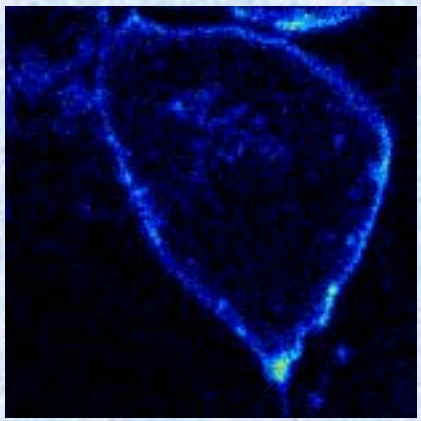
**Only a change in D by a factor of $2^{1/3}$, or 1.26**

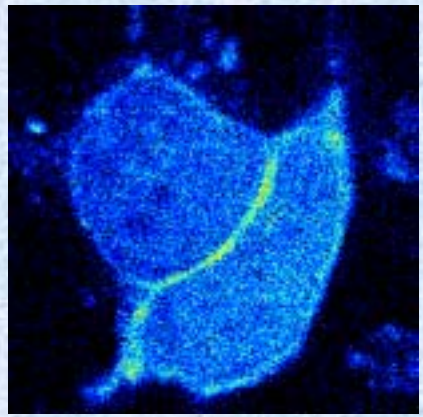
# Autocorrelation Adenylate Kinase -EGFP Chimeric Protein in HeLa Cells





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



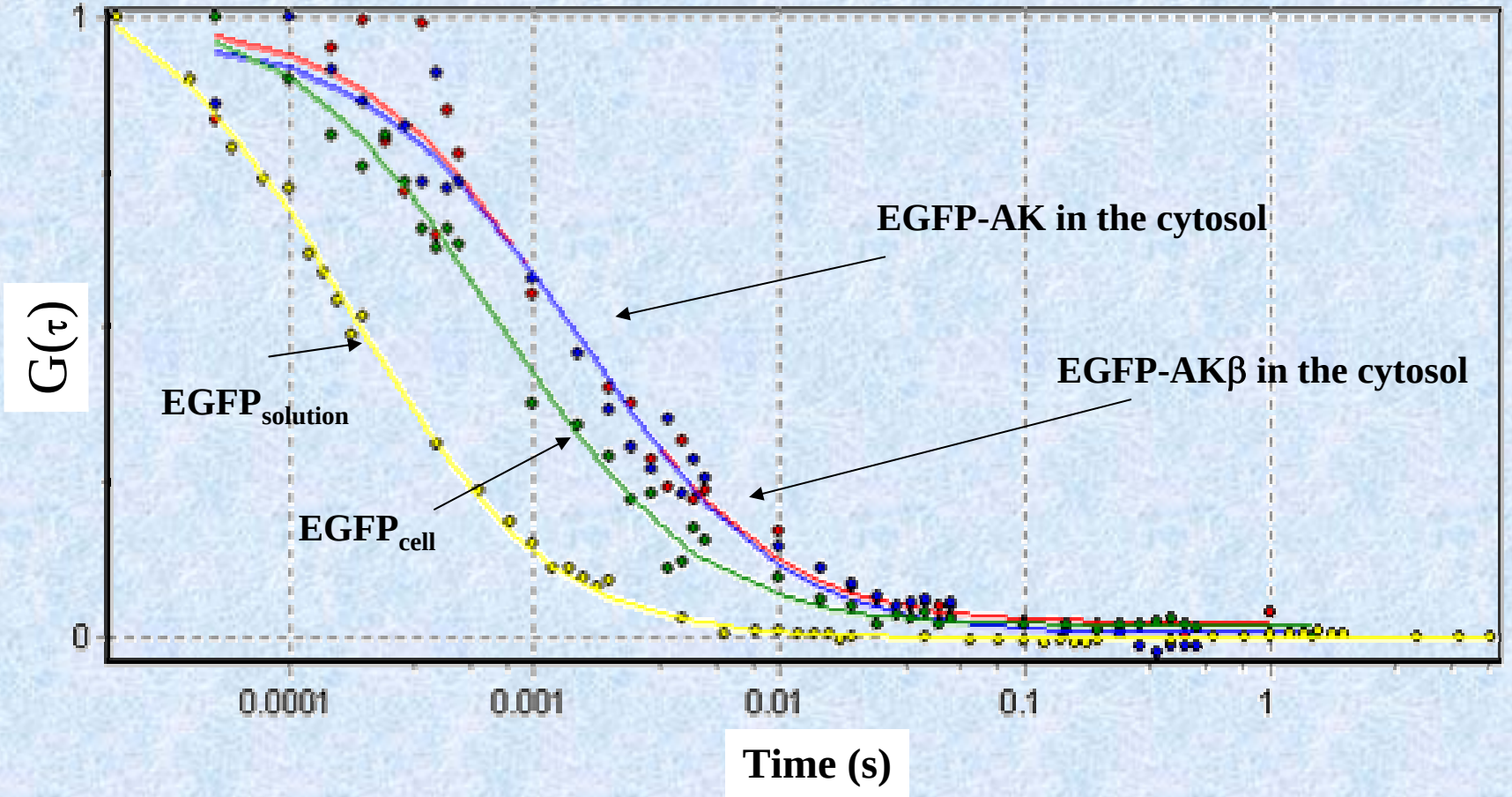


Examples of different *Hela* cells transfected with AK1β -EGFP

Fluorescence Intensity

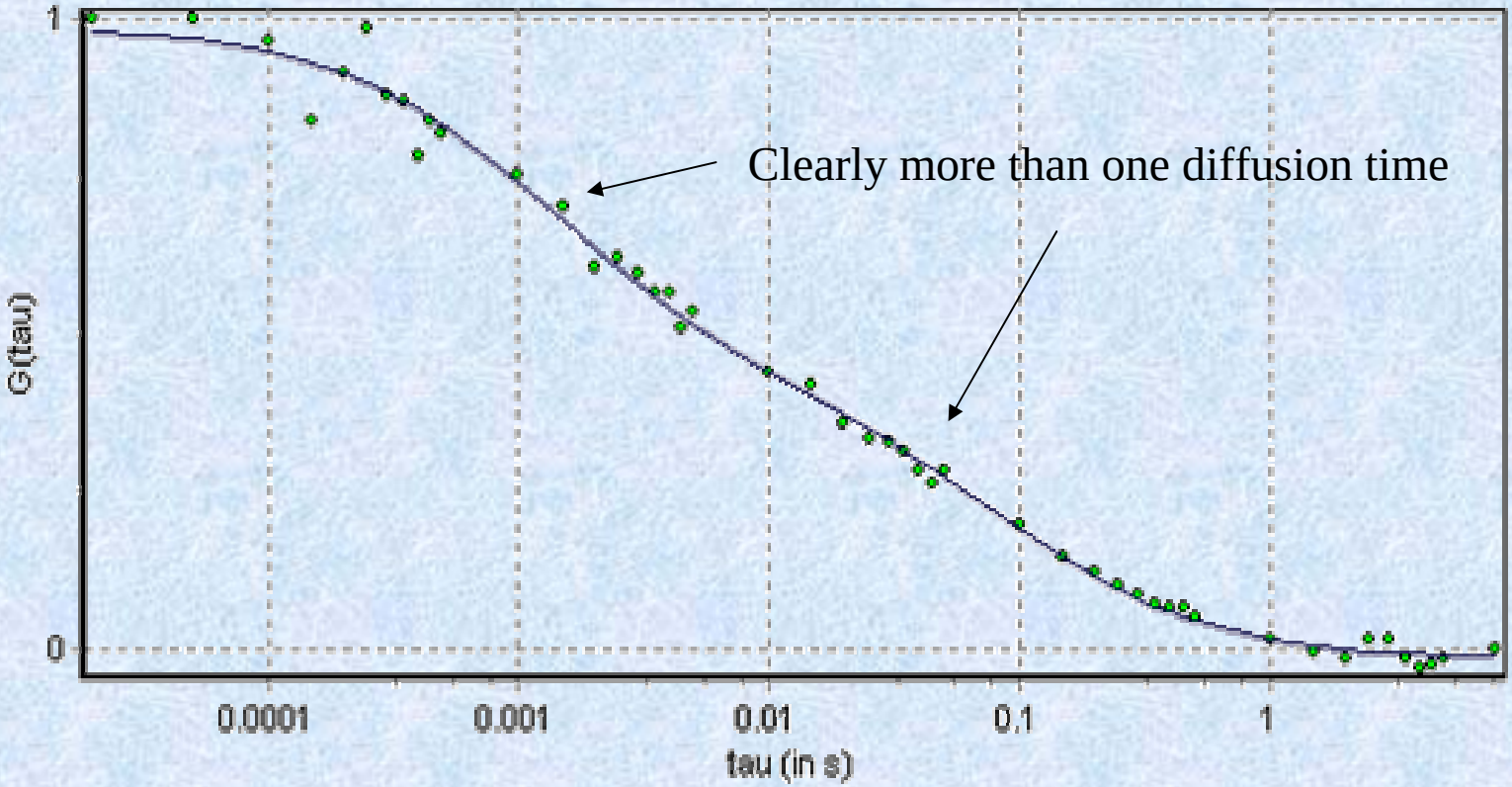
***Qiao Qiao Ruan, Y. Chen, M. Glaser & W. Mantulin Dept. Biochem & Dept Physics- LFD Univ Il, USA***

# Autocorrelation of EGFP & Adenylate Kinase -EGFP



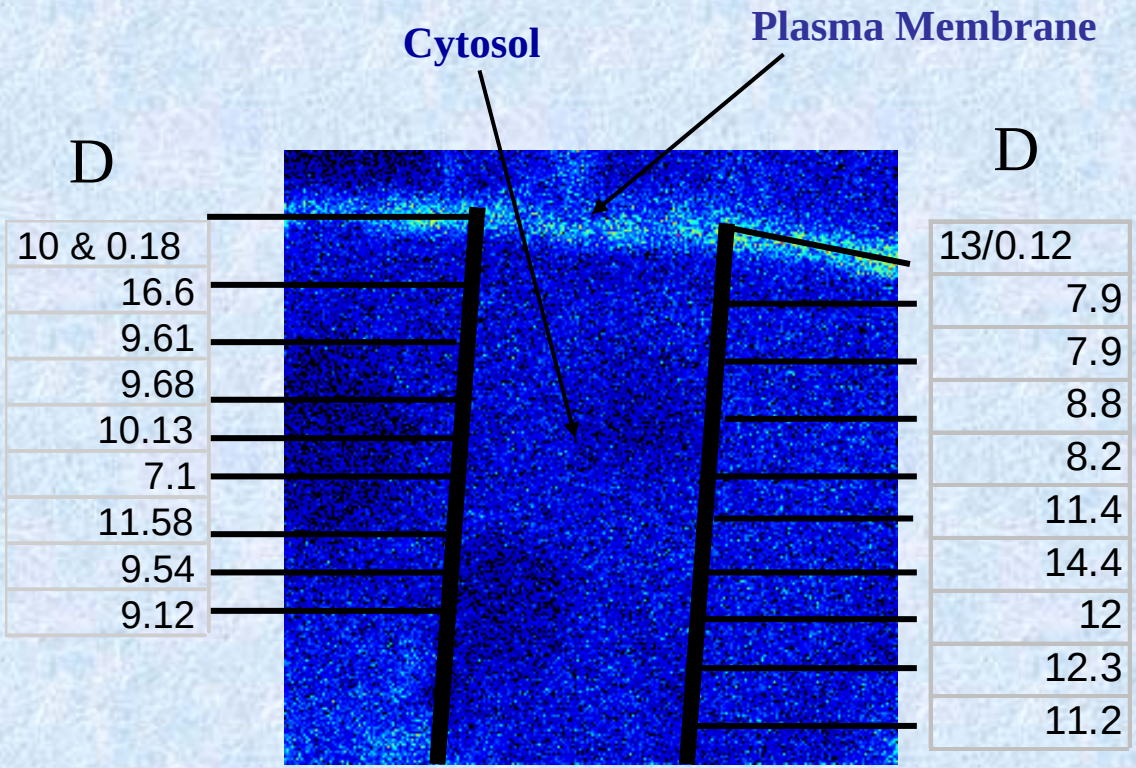

Normalized autocorrelation curve of EGFP in solution (•), EGFP in the cell (•), AK1-EGFP in the cell(•), AK1β-EGFP in the cytoplasm of the cell(•).

# 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 costants are found.**

# Multiple Species

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

*Autocorrelation function can be used:*

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

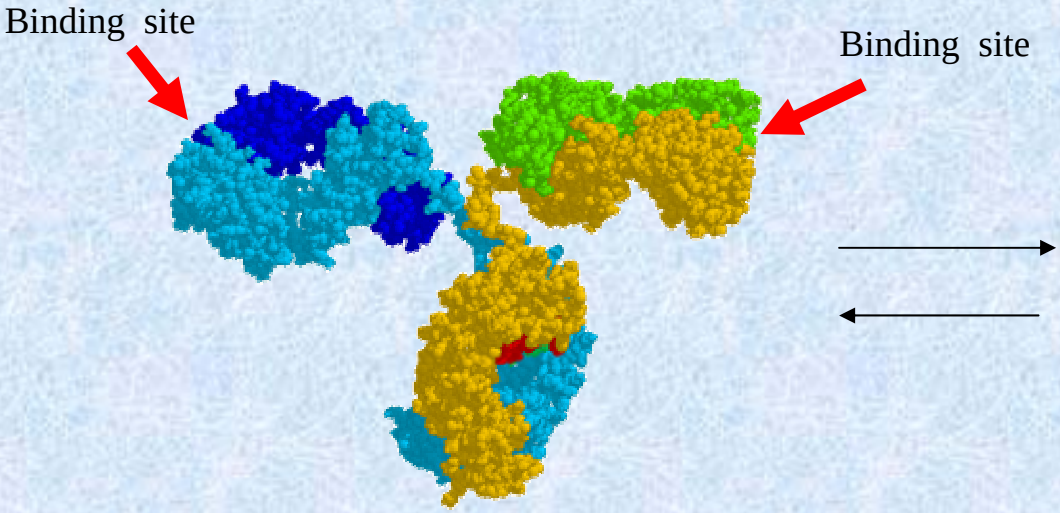
(2D-Gaussian Shape)

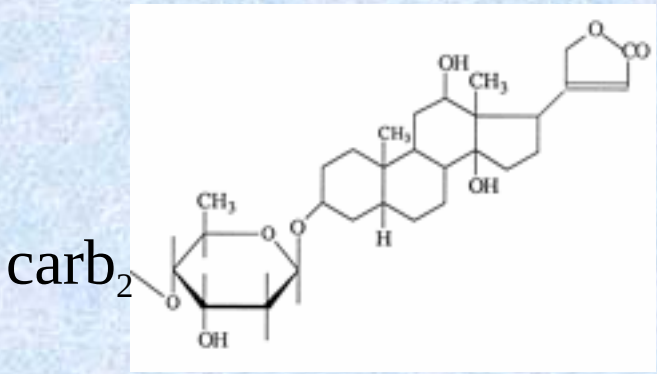
!

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

*$G(0)_{sample}$ is no longer $\gamma/N$ !*

# Antibody - Hapten Interactions



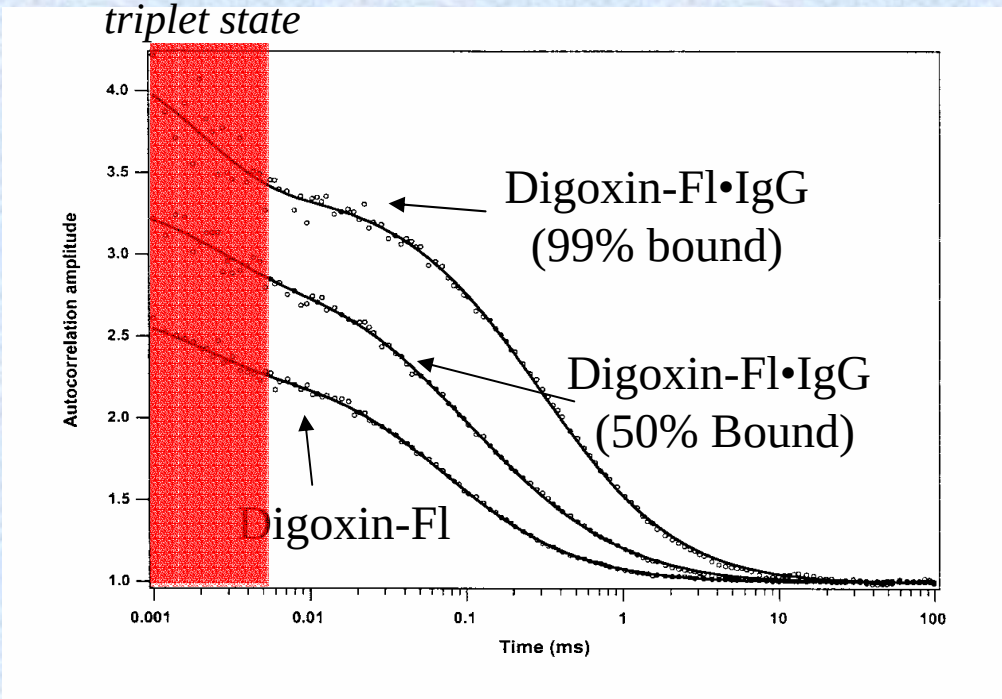




**Mouse IgG**: The two heavy chains are shown in yellow and light blue. The two light chains are shown in green and dark blue..*J.Harris, S.B.Larson, K.W.Hasel, A.McPherson, "Refined structure of an intact IgG2a monoclonal antibody", Biochemistry 36: 1581, (1997).*

**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.

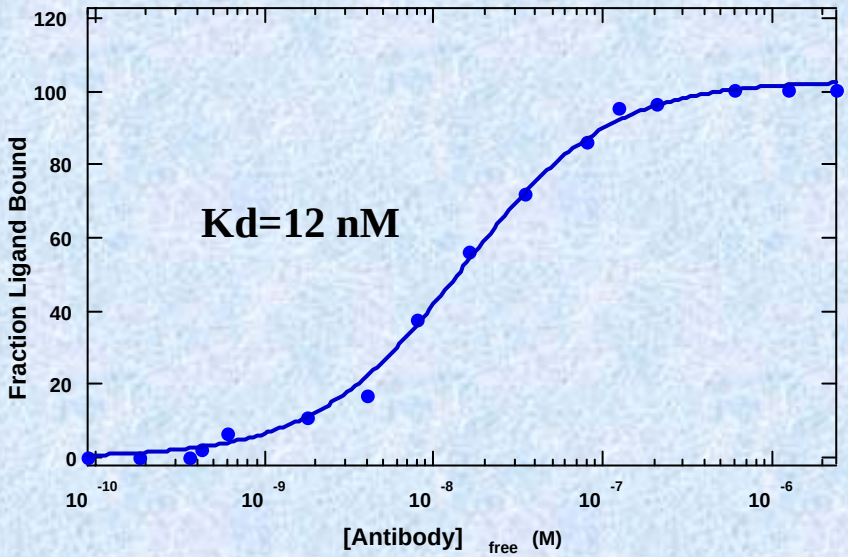
# Anti-Digoxin Antibody (IgG) Binding to Digoxin-Fluorescein

**Autocorrelation curves**:




**Binding titration from the autocorrelation analyses:**

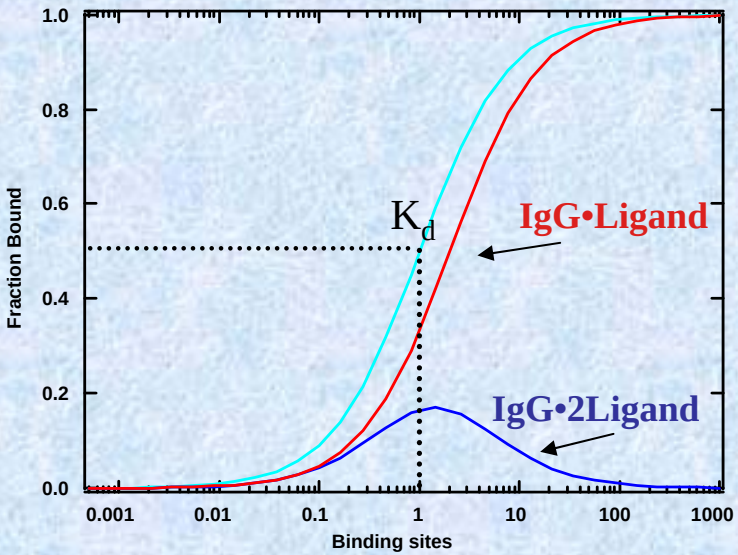
$$F_b = \frac{m \cdot S_{free}}{K_d + S_{free}} + c$$

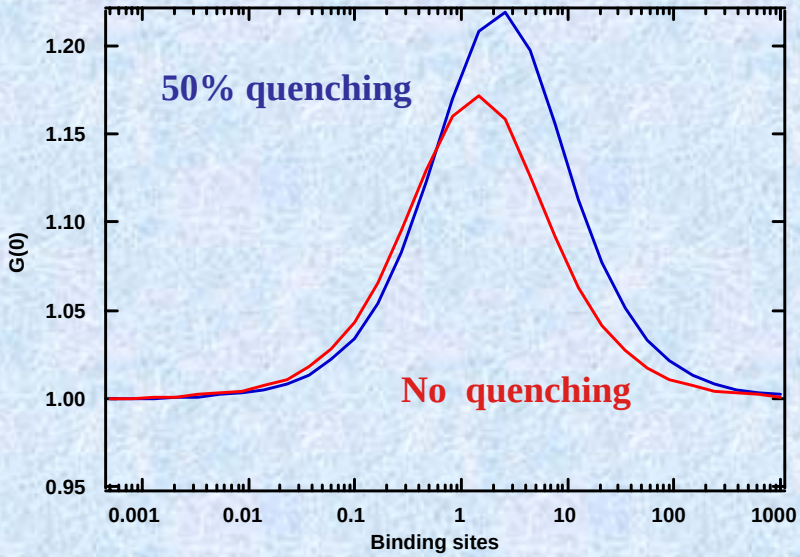



S. Tetin, K. Swift, & , E, Matayoshi , (in press)

# Two Binding Site Model

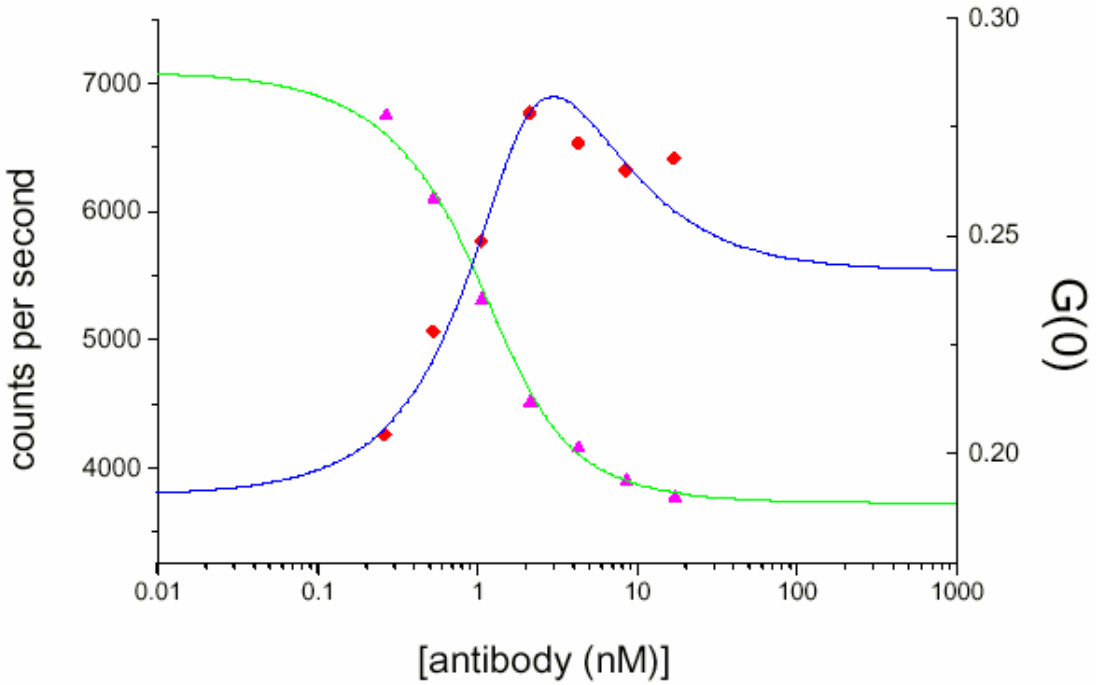
IgG + 2 Ligand-Fl ⟷ IgG•Ligand-Fl + Ligand-Fl ⟷ IgG•2Ligand-Fl




[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 Go becomes the only parameter to distinguish species, but we know that:

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

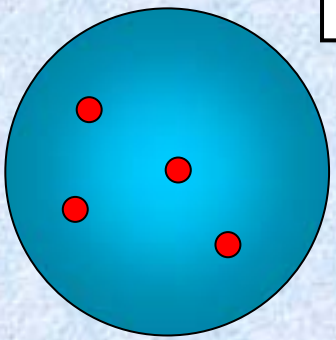
**The autocorrelation function is not suitable for analysis of this kind of data without additional information.**

We need a different type of analysis

# Photon Counting Histogram (PCH)

**Aim:** **To resolve species from differences in their molecular brightnesses**

**Poisson Distribution:** $$p(N) = \frac{\langle N \rangle^N \cdot e^{-\langle N \rangle}}{N!}$$



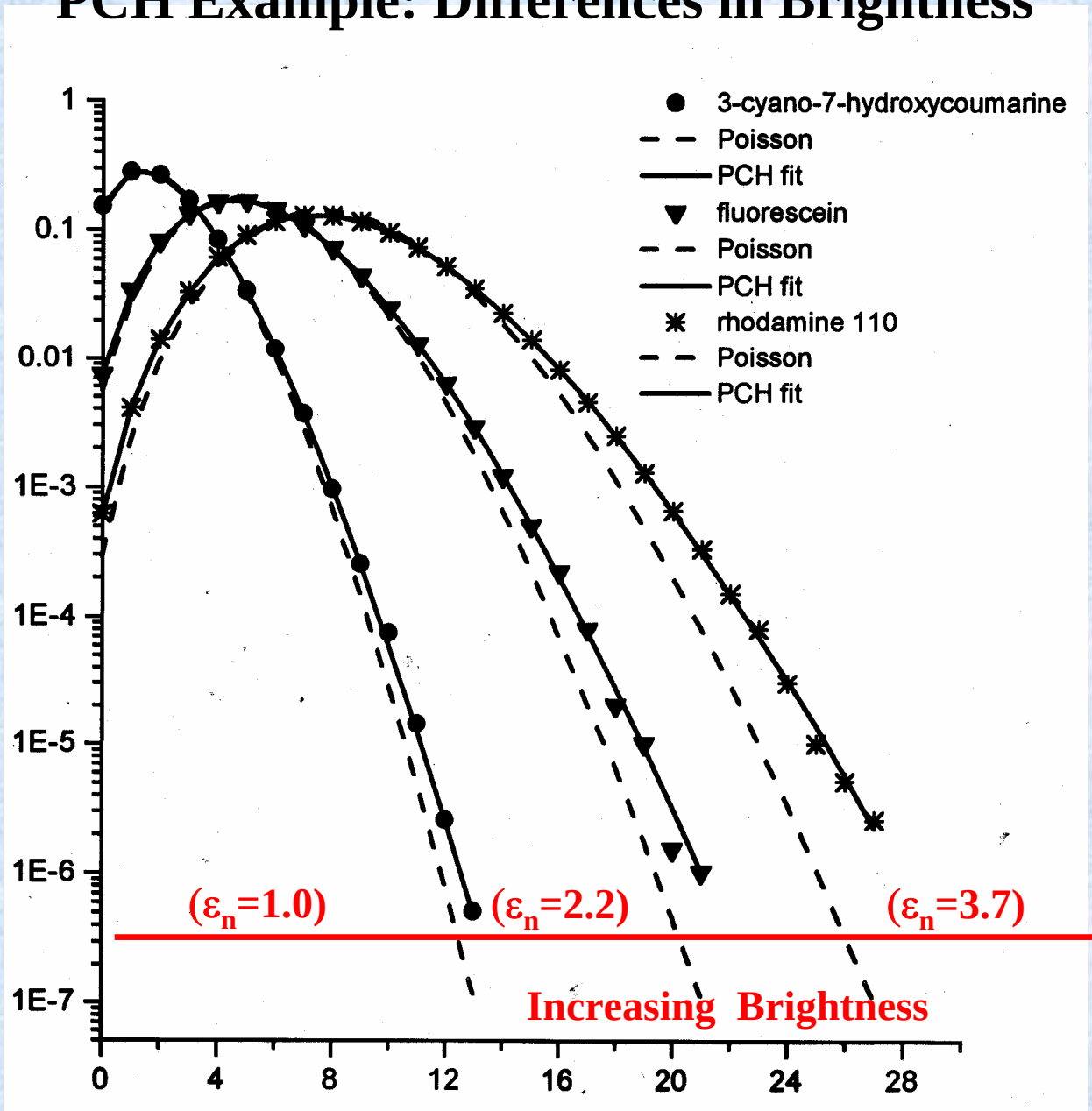
**Sources of Non-Poissonian Noise**

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

**Single Species:** $$p(k) = PCH(\varepsilon, \langle N \rangle)$$

Where p(k) is the probability of observing **k** photon counts

# PCH Example: Differences in Brightness



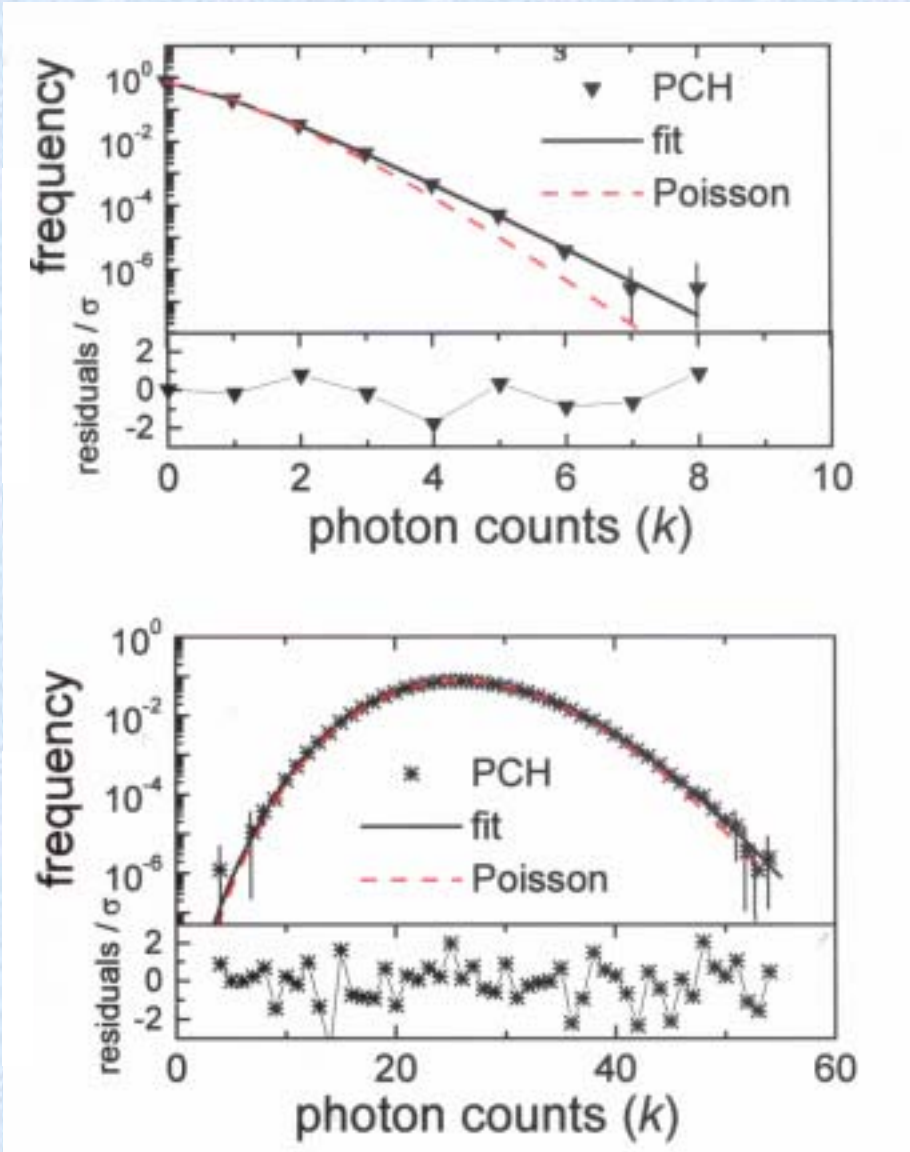
# Single Species PCH: Concentration

5.5 nM Fluorescein

Fit:
ε = 16,000 cpsm
N = 0.3

550 nM Fluorescein

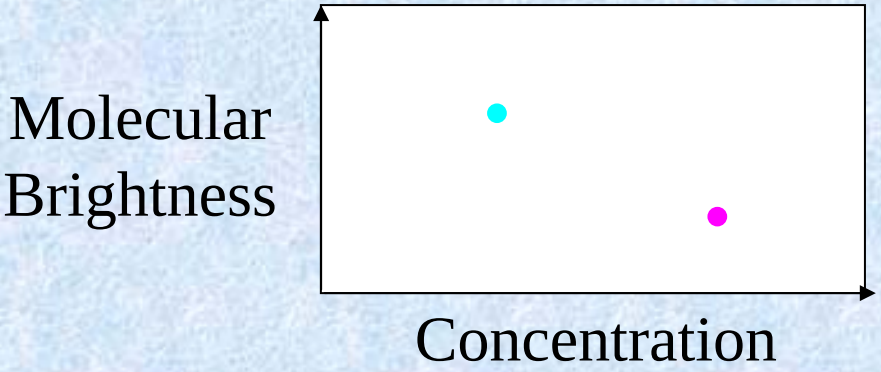
Fit:
ε = 16,000 cpsm
N = 33

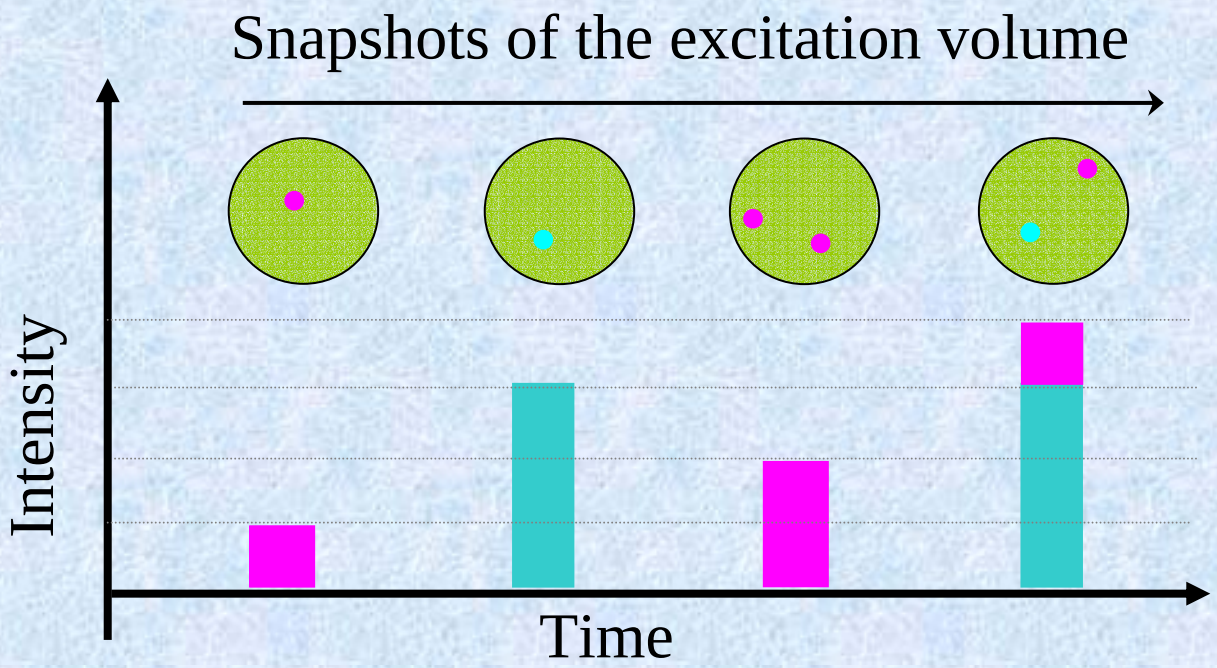



**As particle concentration increases the PCH approaches a Poisson distribution**

# Photon Counting Histogram: Multispecies

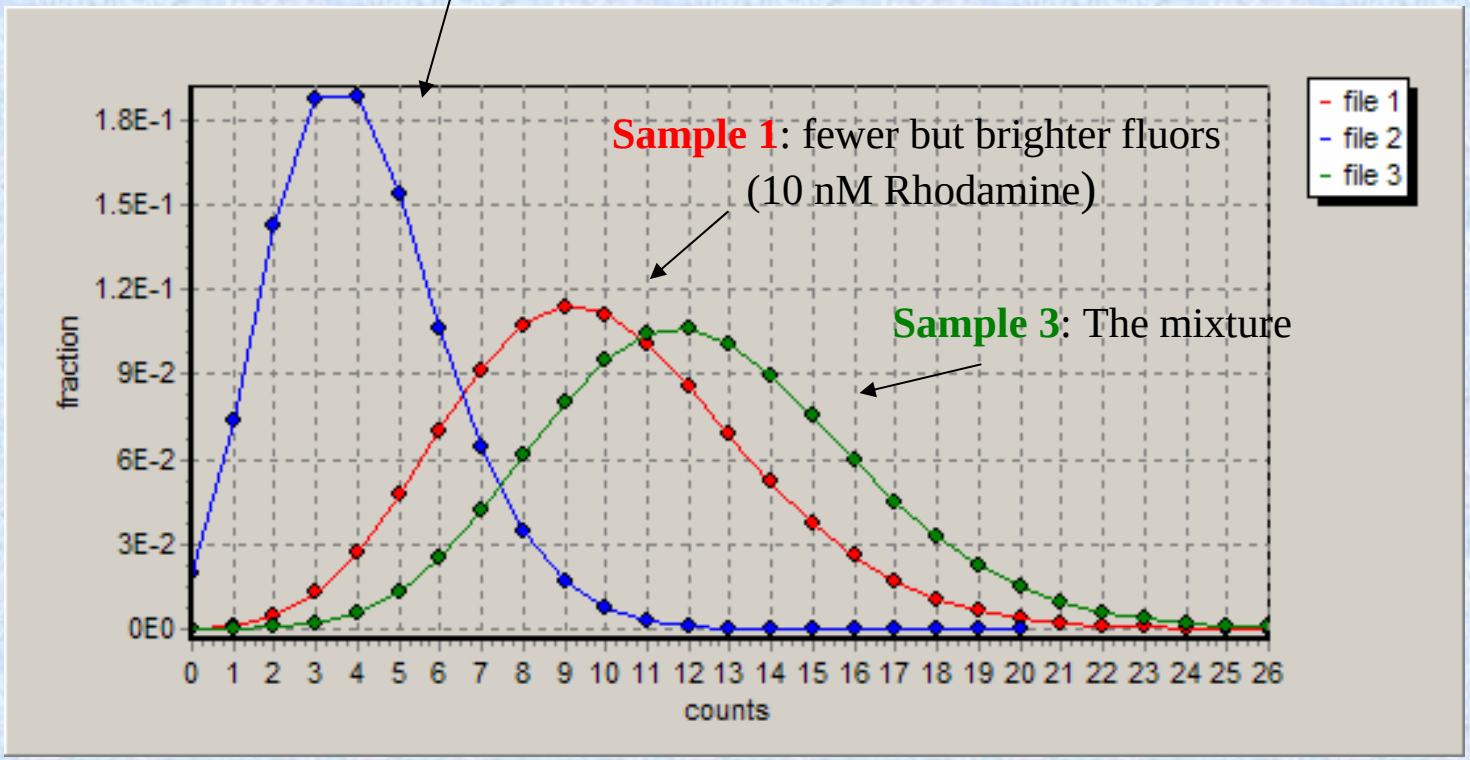
Binary Mixture: $p(k) = PCH(\varepsilon_1, \langle N_1 \rangle) \otimes PCH(\varepsilon_2, \langle N_2 \rangle)$



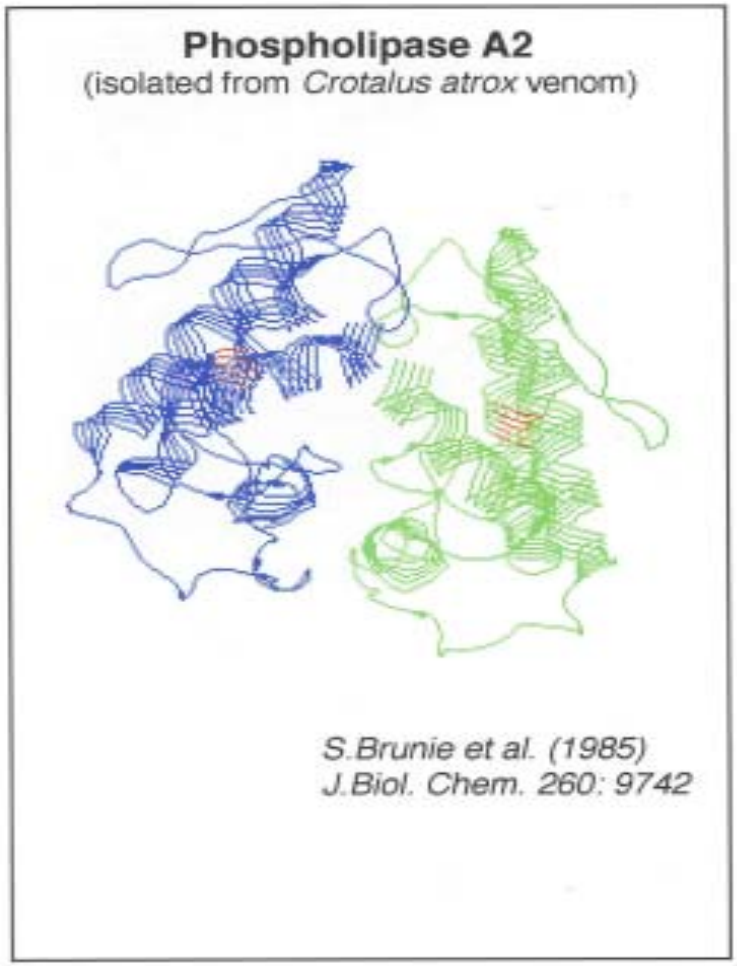
# Photon Counting Histogram: Multispecies

**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 $A_2$



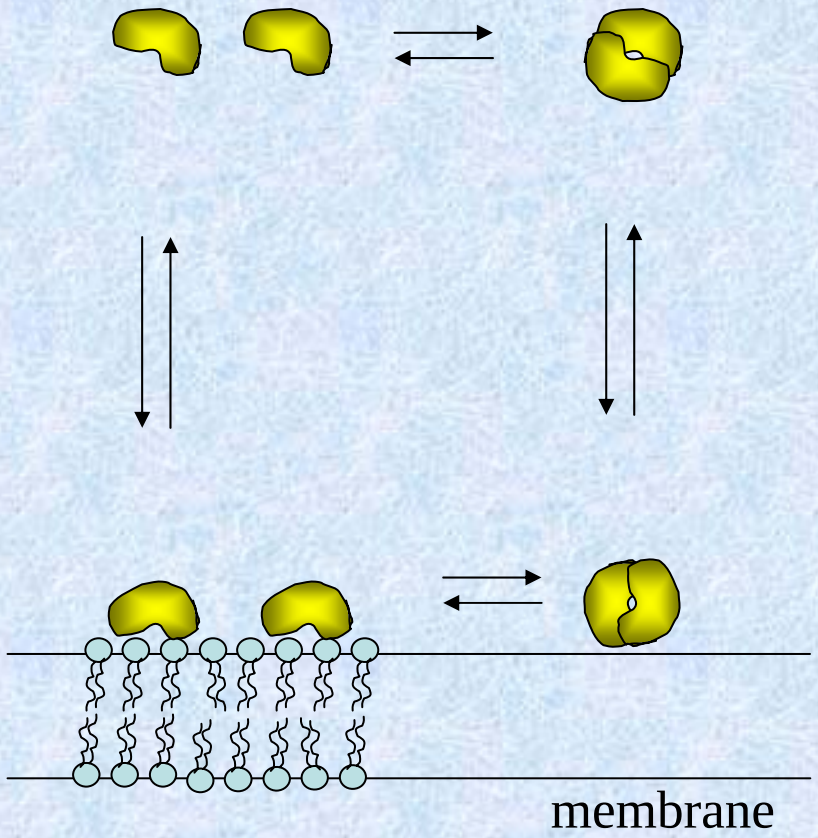

***Sanchez, S. A., Y. Chen, J. D. Mueller, E. Gratton, T. L. Hazlett. (2001) Biochemistry, 40, 6903-6911.***

# $sPLA_2$ Interfacial Binding

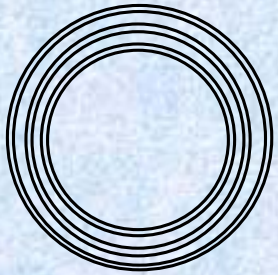
**$sPLA_2$ Self-Association**

**$sPLA_2$ Membrane Binding**

**Interfacial $sPLA_2$Self-Association**

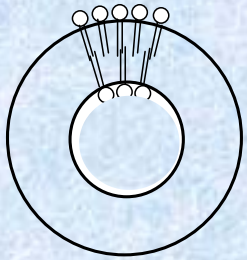


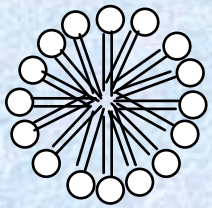
# Lipid Interfaces



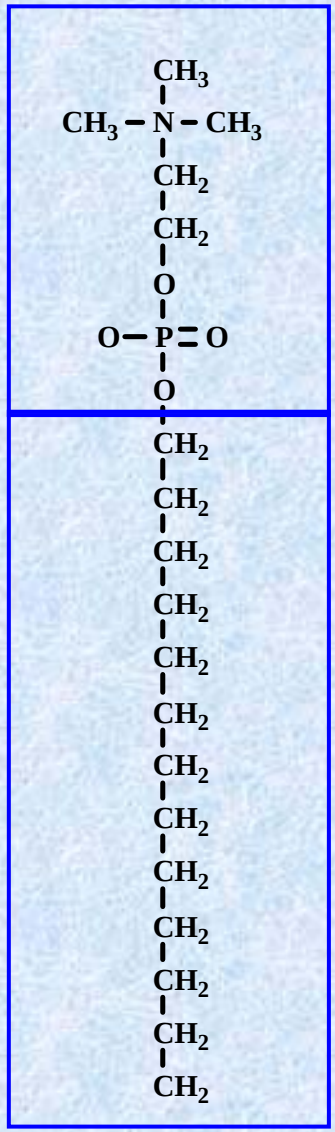
Multibilayers
(MLVs)

Vesicles
(SUVs, LUVs
& GUVs)





Micelles




**Choline Group**

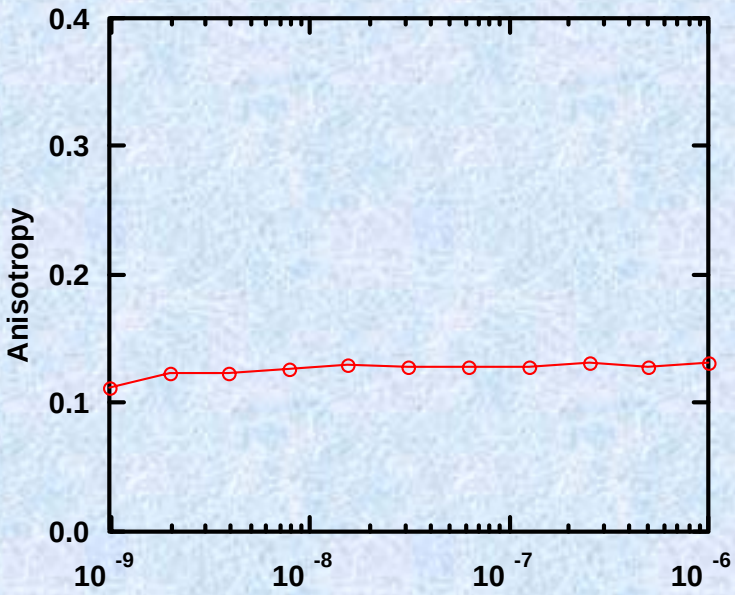
**12 Carbon Tail**

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

# In Solution: *a Tight Dimer*

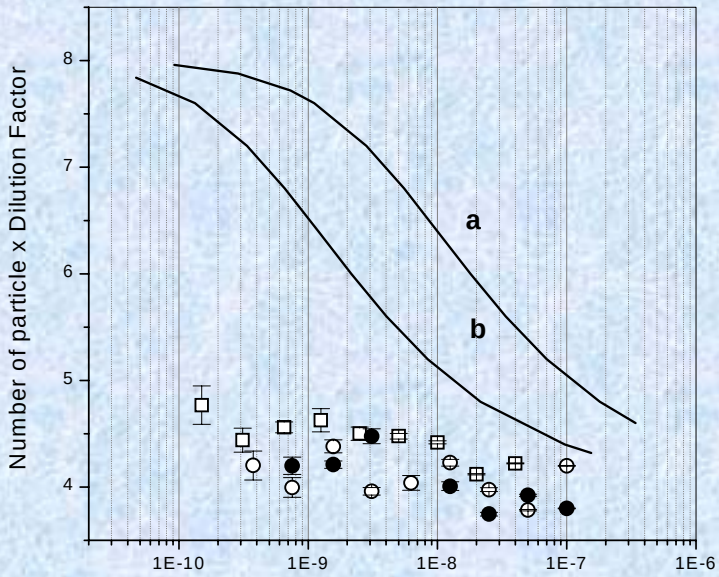
# Fluorescein-sPLA$_2$

Steady-State Anisotropy




**[Fl-sPLA$_2$]**

Fluorescence Correlation Spectroscopy




**[Fl-sPLA$_2$]**

***Time-Resolved Anisotropy***:

**Phi1 = 12.8 ns (0.43)**

**Phi2 = 0.50 ns (0.57)**

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

## 1. Autocorrelation

**sPLA$_2$**

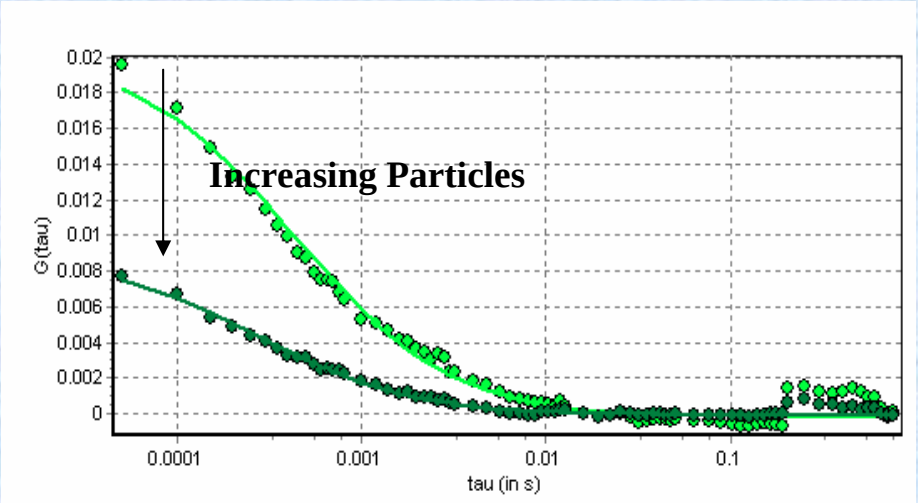
G(0)=0.021

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

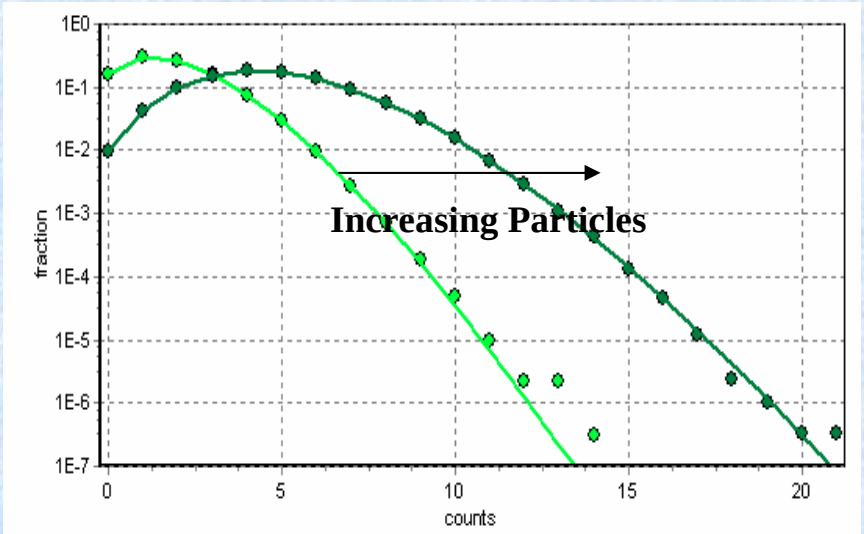
N = 3.29

**sPLA$_2$ + 3M Urea**

ε = 0.6

N = 8.48

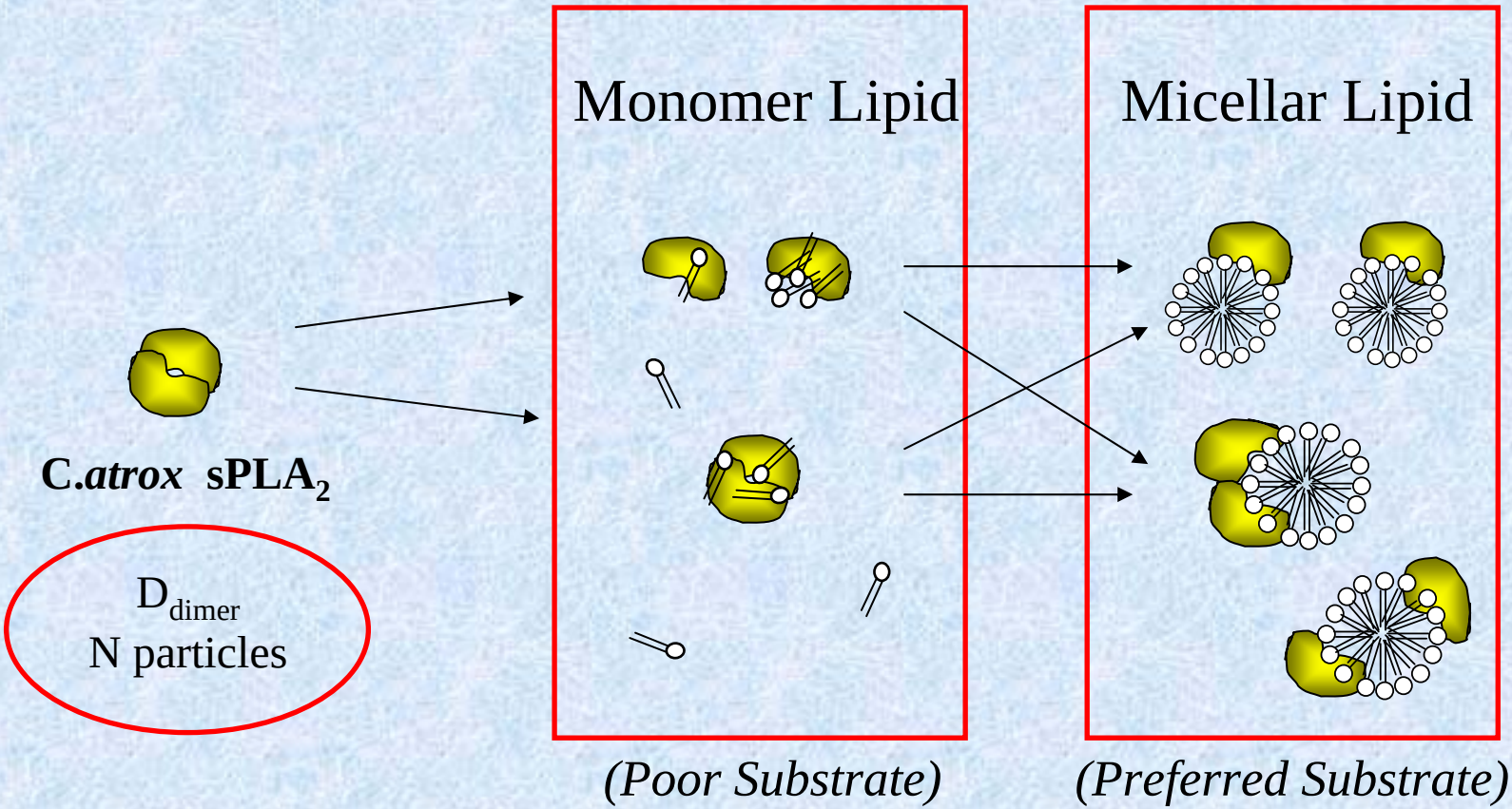
*Adjusted for viscosity differences*




Change in number of particles, little change in brightness

# The Critical Question:
# Is $sPLA_2$ a Dimer in the Presence of Interfacial Lipid?

*What Could We Expect to Find in the FCS Data?*




Observing Fluorescein-labeled $sPLA_2$

# FCS on Fluorescein - $sPLA_2$ in Buffer (RED) and with DPC Micelles ( BLUE )

## 1. Autocorrelation Analysis

$sPLA_2$

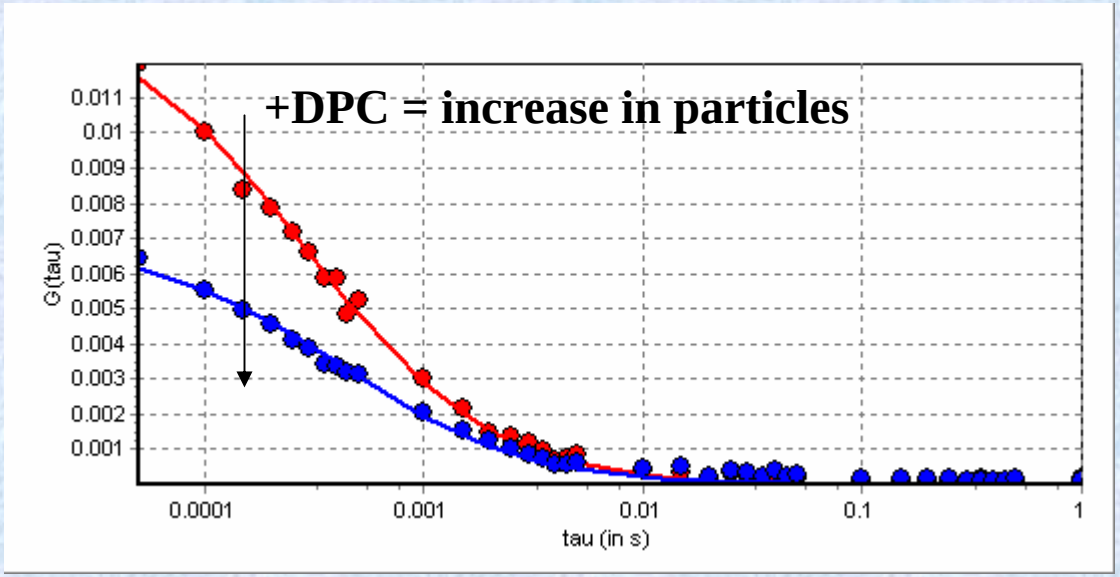
$G_0 = 0.0137$

$D = 75\ um^2/s$

$sPLA_2$ + 20 mM DPC

$G_0 = 0.0069$

$D = 55\ um^2/s$




## 2. PCH Analysis

$sPLA_2$

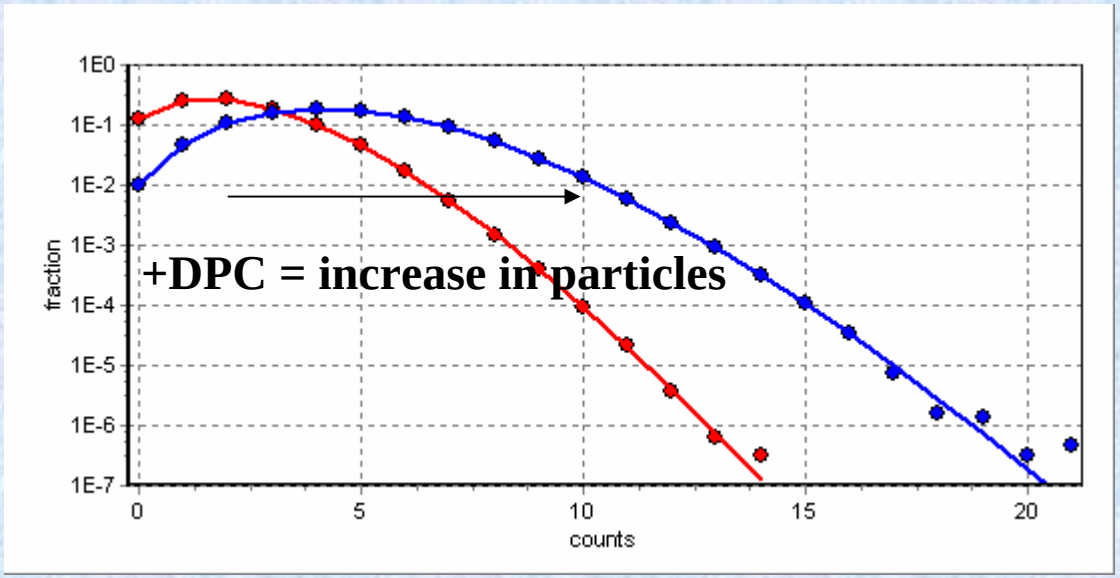
$\varepsilon = 0.41$

$N = 6.5$

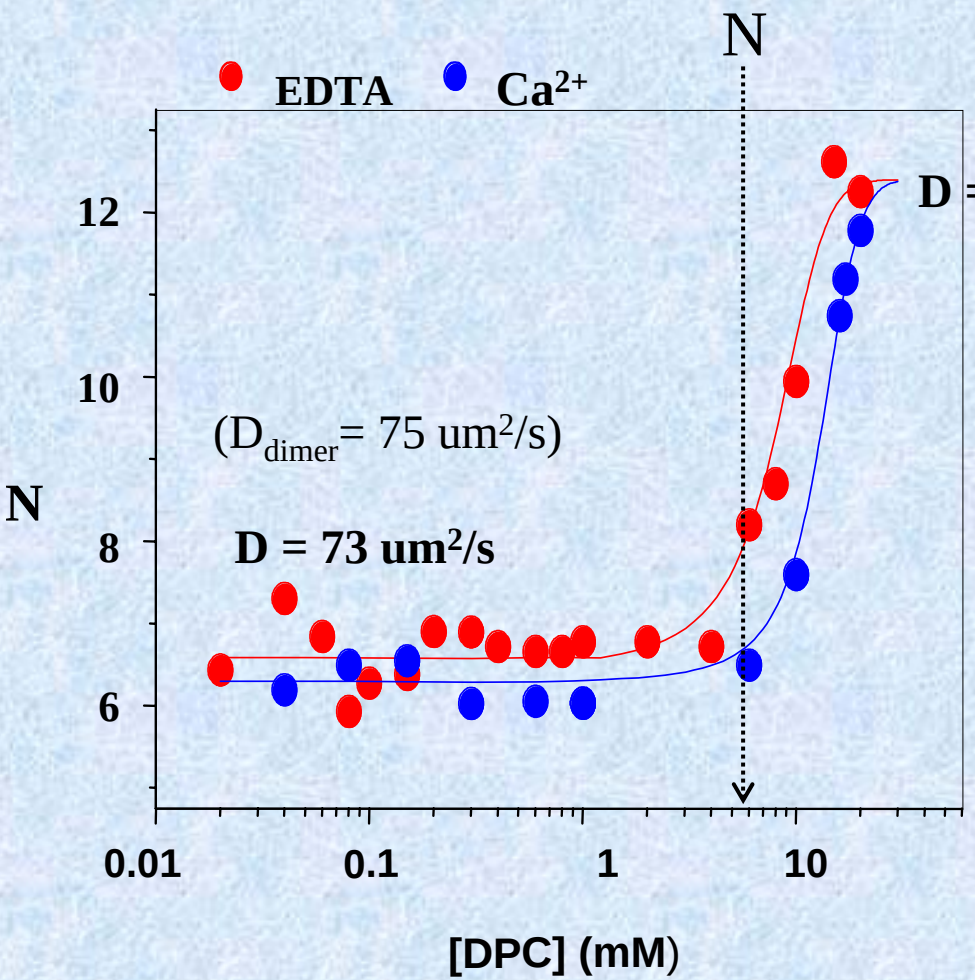
$sPLA_2$ + 20 mM DPC

$\varepsilon = 0.45$

$N = 12.2$



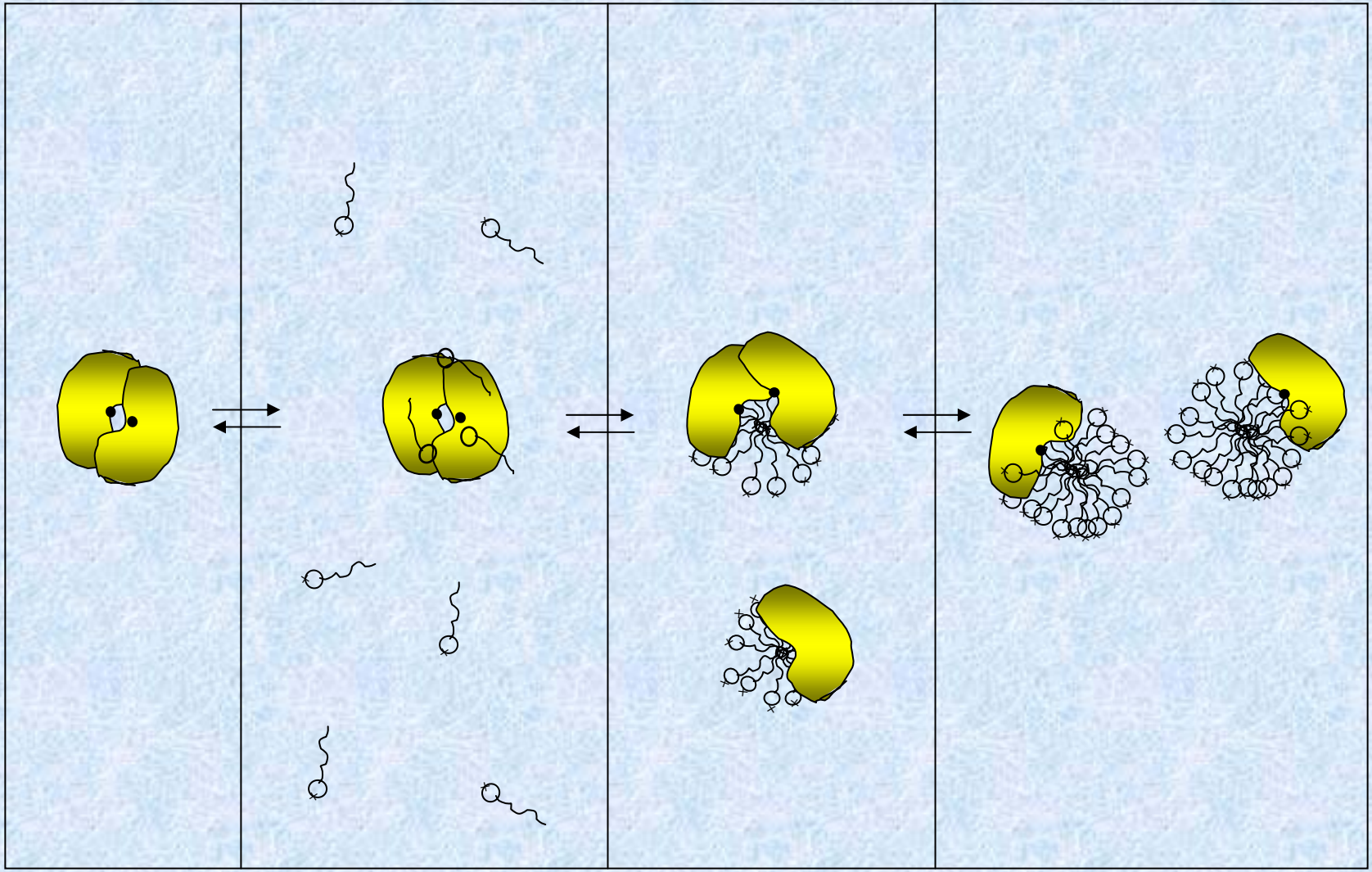
# Fluorescein-sPLA$_2$ Interaction with DPC




**D = 55-60 um$^2$/s** ($D_{micelle}$=57 um$^2$/s)

- The PLA$_2$ dimer dissociates in the presence of micelles.
- Active enzyme form in a micellar system is monomeric.

# Schematic of $sPLA_2$ - Dodecylphosphocholine Interactions



$sPLA_2$

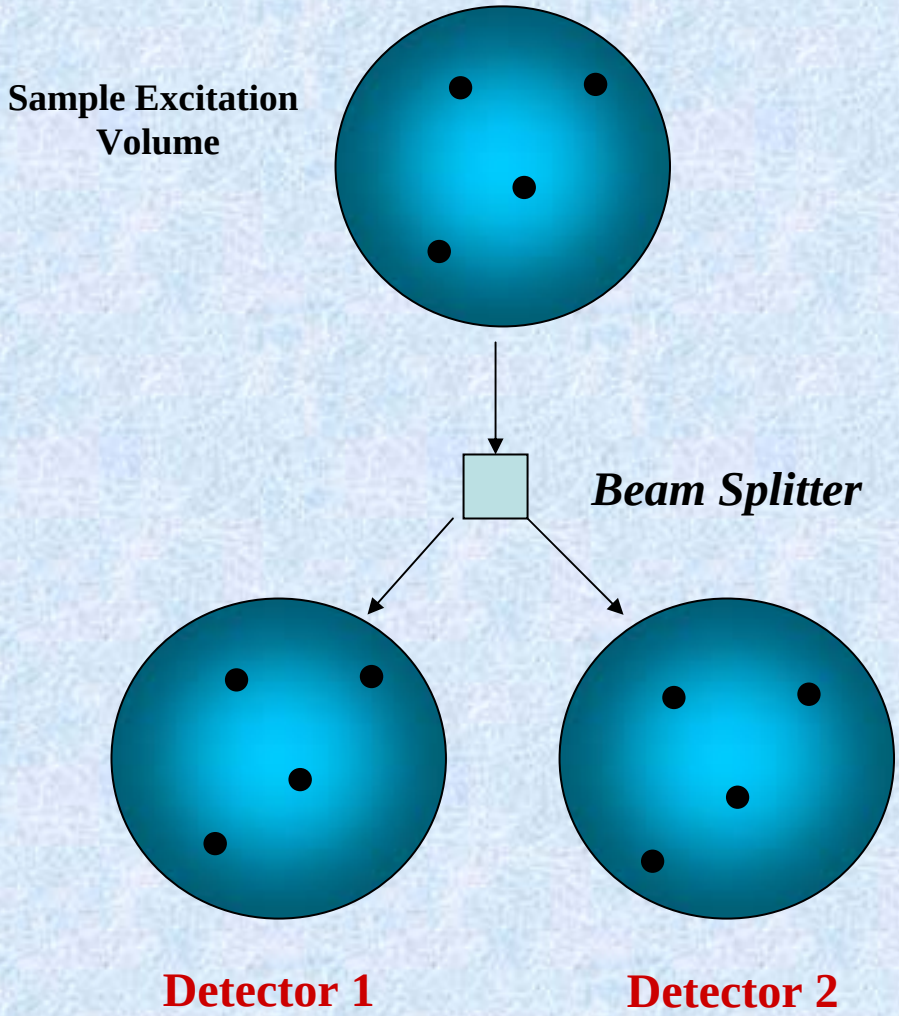
Monomer-Lipid Association

Co-Micelle

$sPLA_2$-Micelle

# Two Channel Detection: Cross-correlation

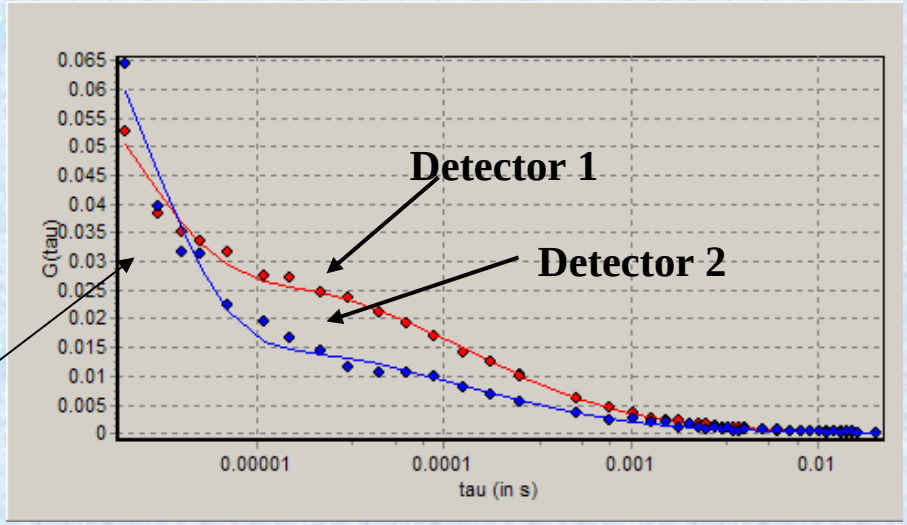
1. Increases signal to noise by isolating correlated signals.
2. Corrects for PMT noise



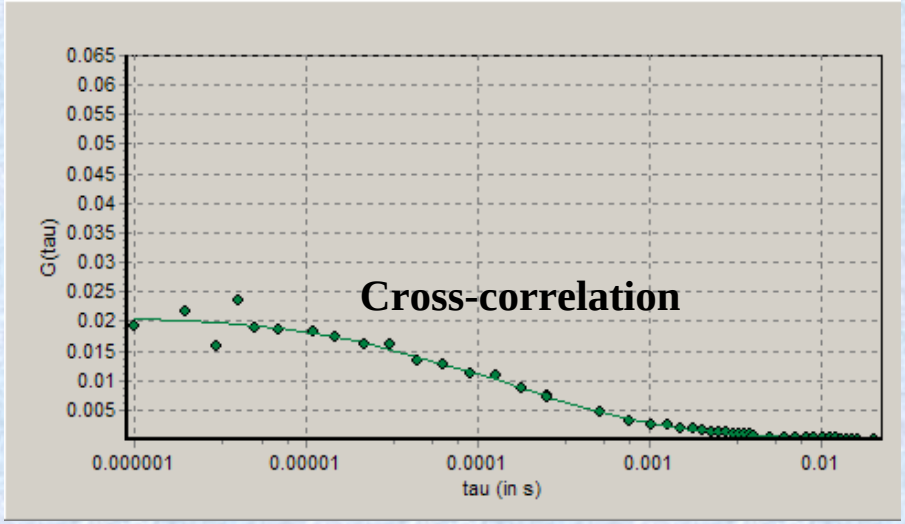

Each detector observes the same particles

# Removal of Detector Noise by Cross-correlation

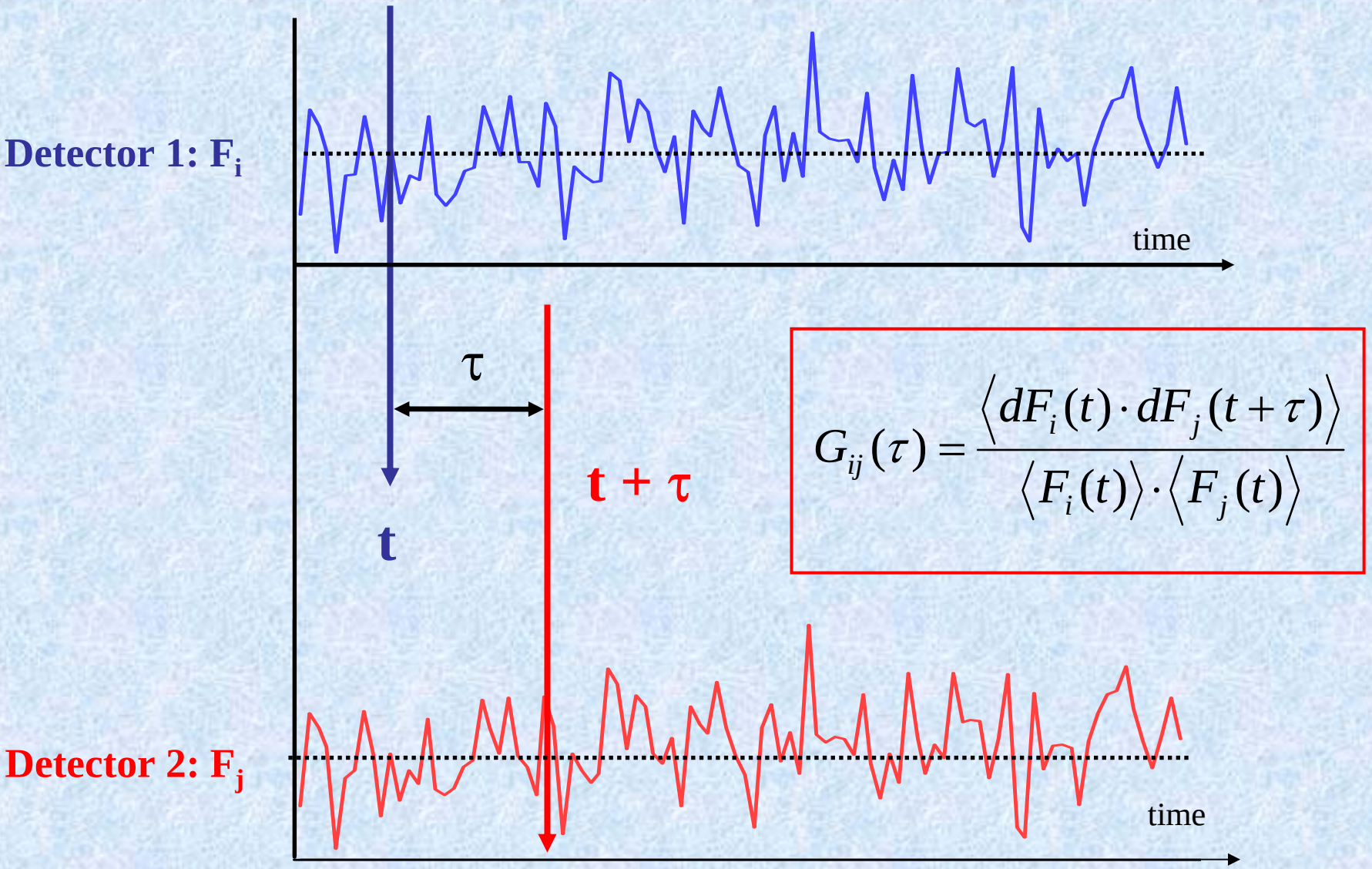
**11.5 nM Fluorescein**




**Detector after-pulsing**



# Calculating the Cross-correlation Function




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

# Cross-Correlation Calculations

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

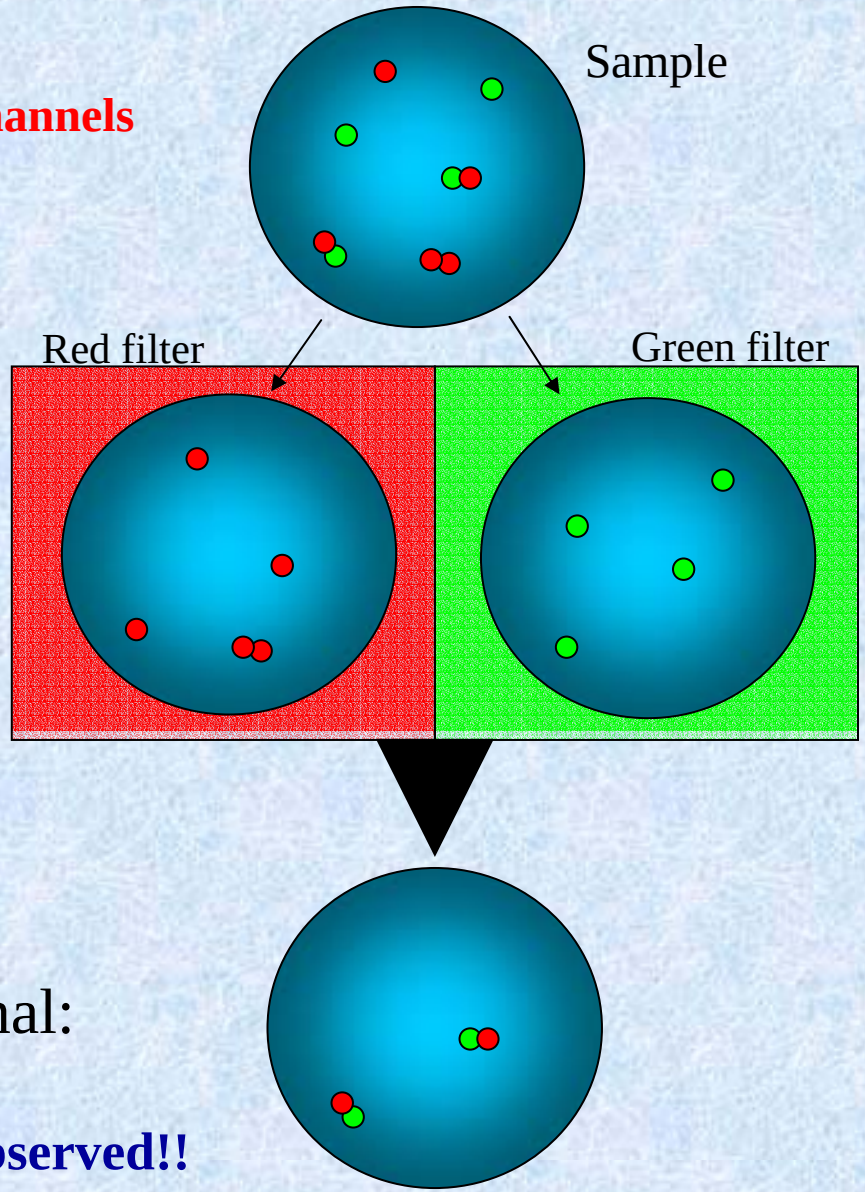
**Thus, for a 3-dimensional Gaussian excitation volume one uses:**

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

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

# 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

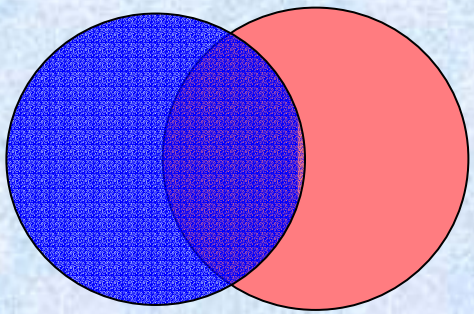
*We assume exact match of the observation volumes in our calculations which is difficult to obtain experimentally.*

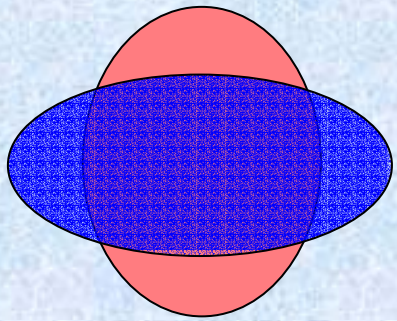
**Excitation side:**

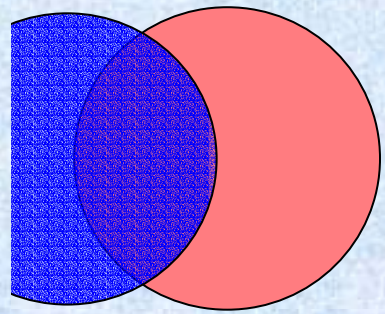
(1) Laser alignment
(2) Chromatic aberration
(3) Spherical aberration

**Emission side:**

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

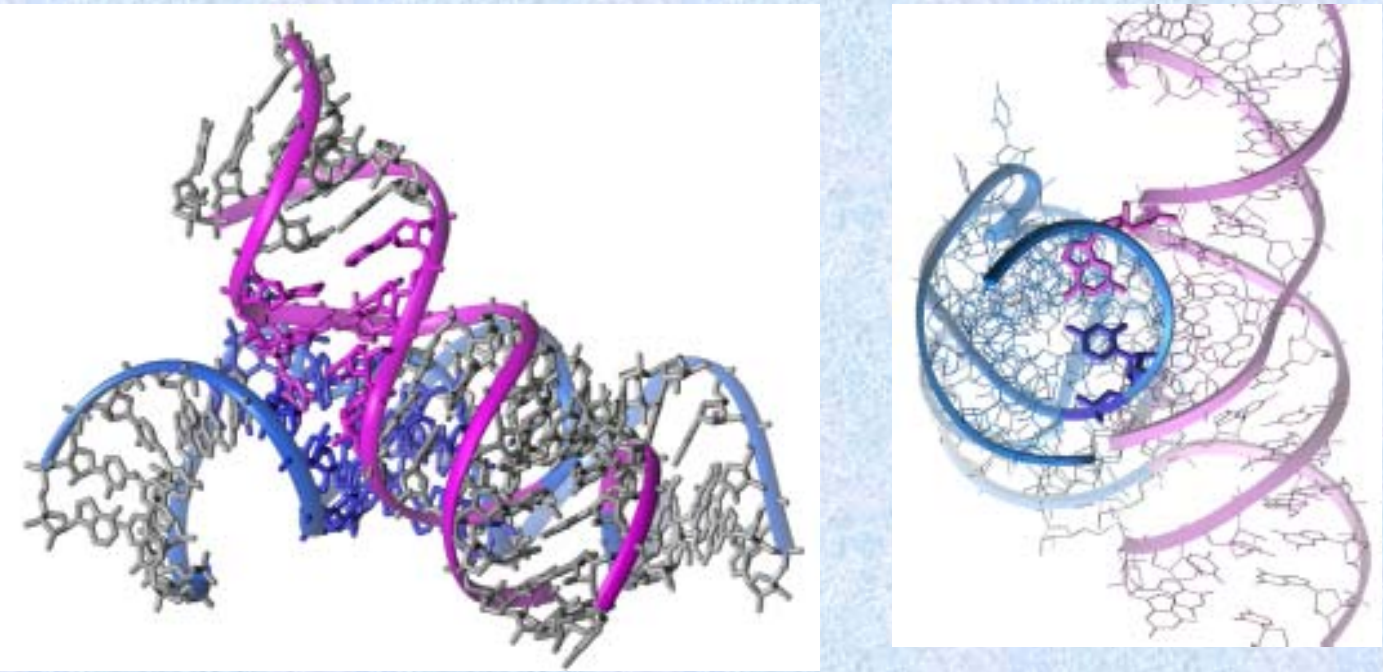






# Single Molecule Application of FCS

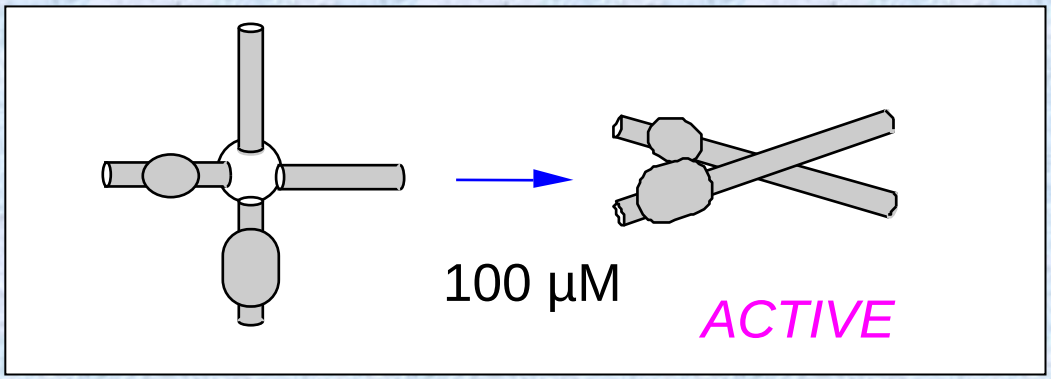
***E. Tan, T. Ha & R. Clegg (UIUC, LFD)***



Crystal Structure of Hairpin Ribozyme

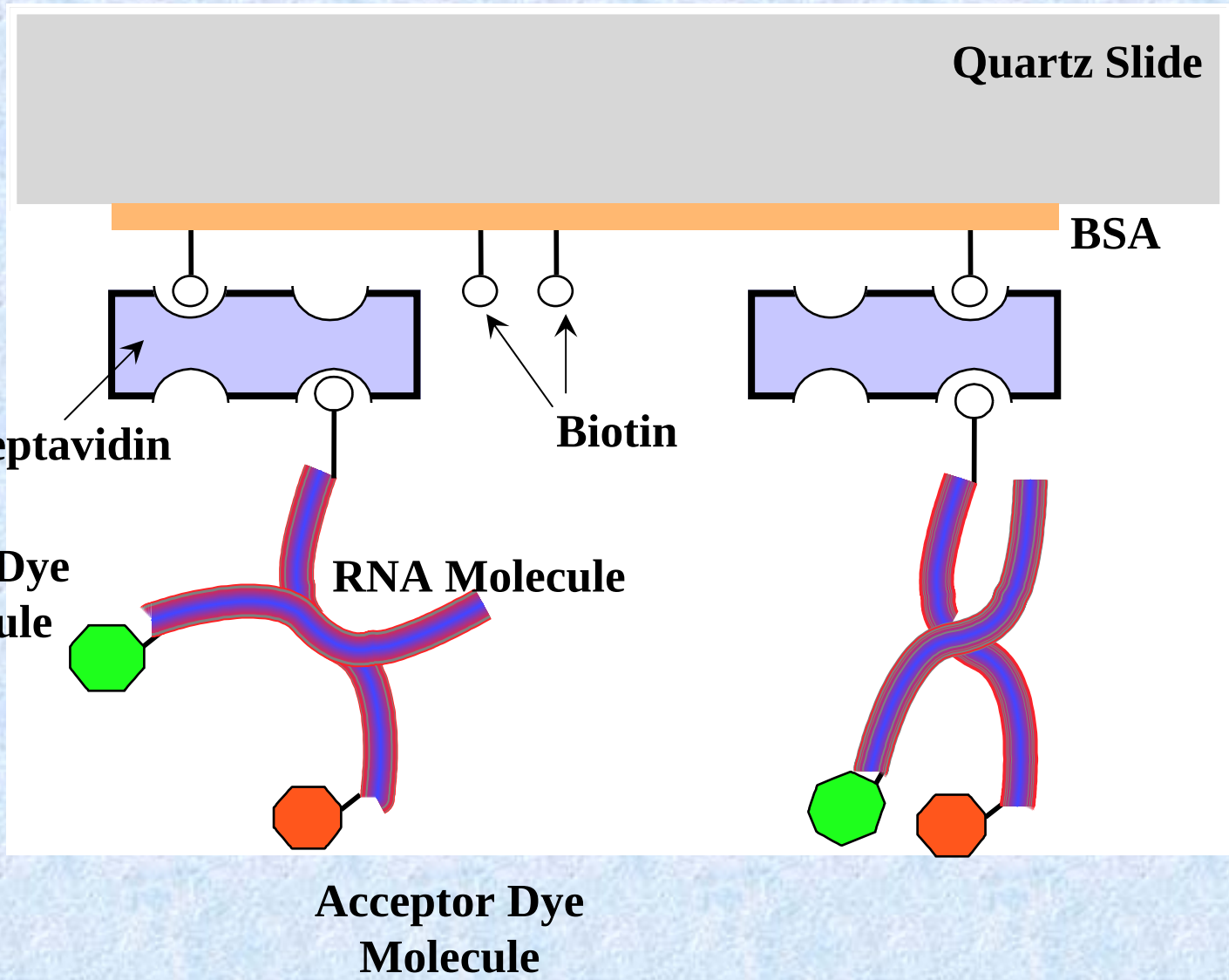
PB Rupert & A Ferré d'Amaré *Nature* **410**, 780-786 (2001).

# HAIRPIN RIBOZYME : FOLDING OF THE JUNCTION

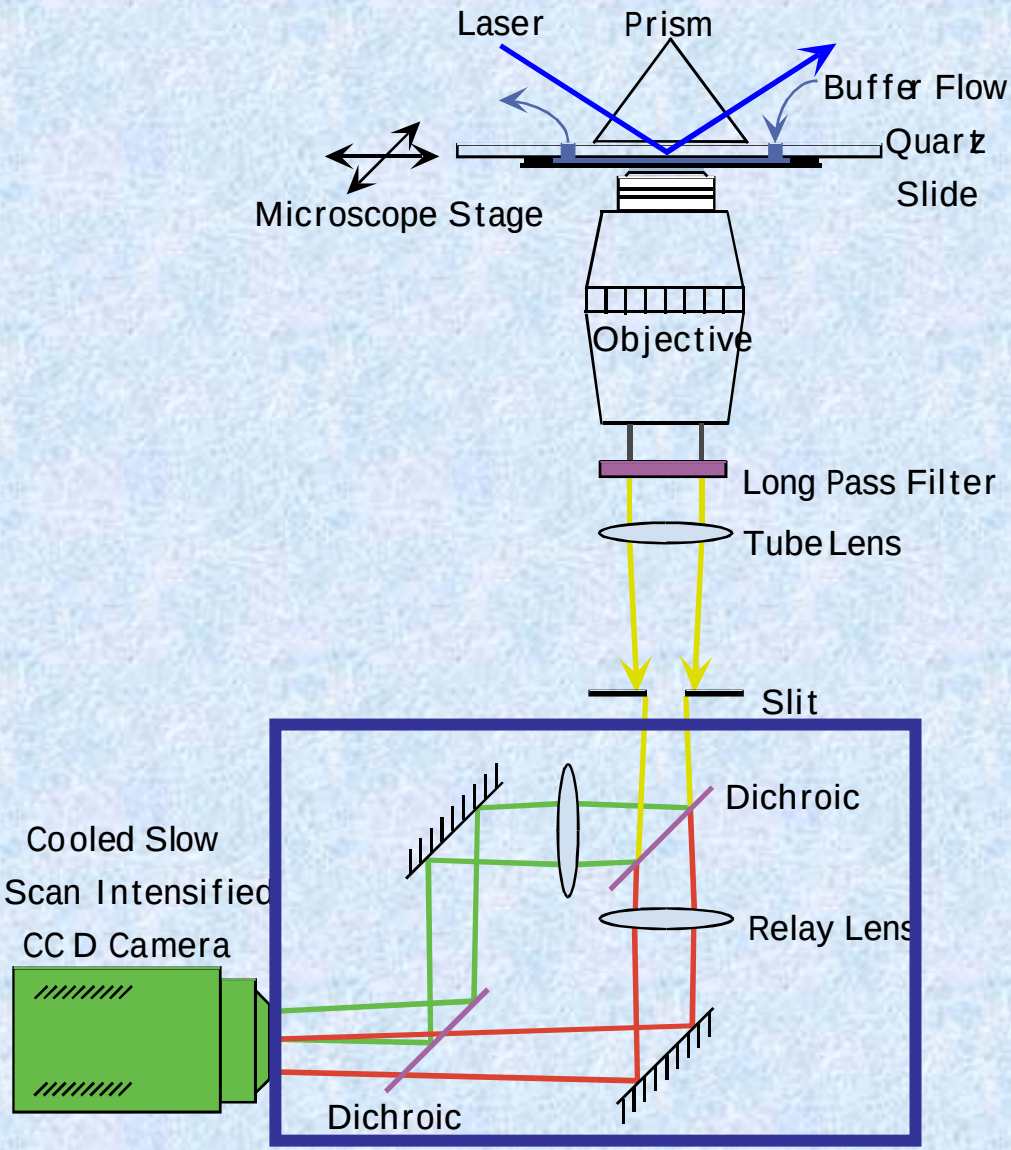



Z Zhao, TJ Wilson, K Maxwell & DMJ Lilley *RNA* 6, 1833-1846 (2000)

# Immobilization Strategy



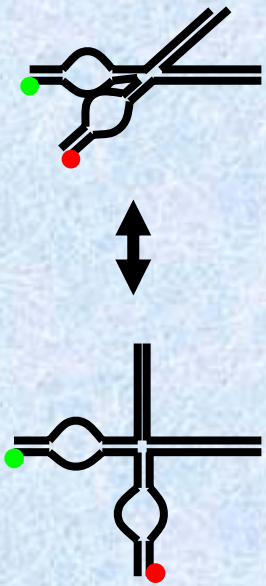
# Dual Channel TIR Imaging

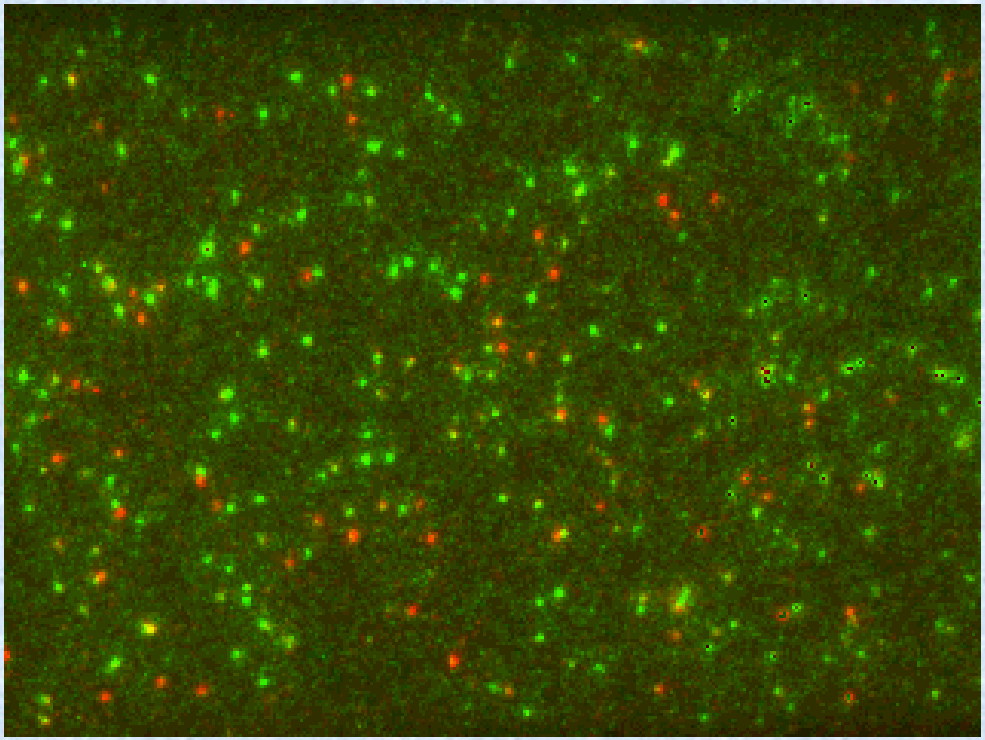


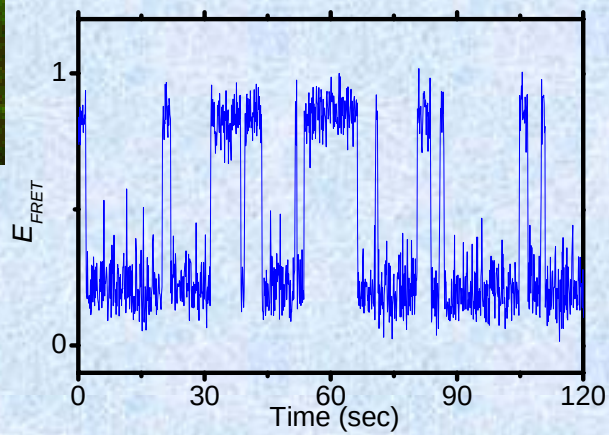

- 100s of molecules
- ~30 ms time resolution
- Flow delivery system
- Dual channel imaging

*Courtesy of T.Ha, Dept. Physics, UIUC,*

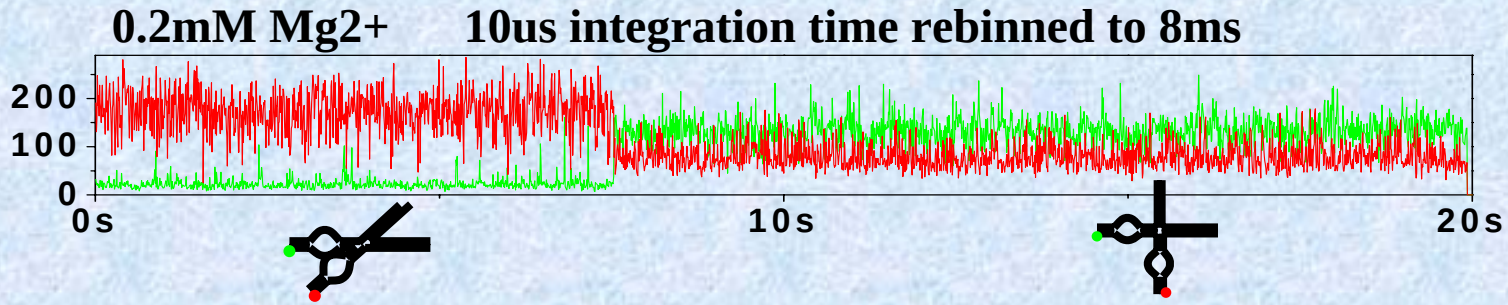
# Hairpin Ribozyme Movie
# Real Time (10 Frames/Sec)

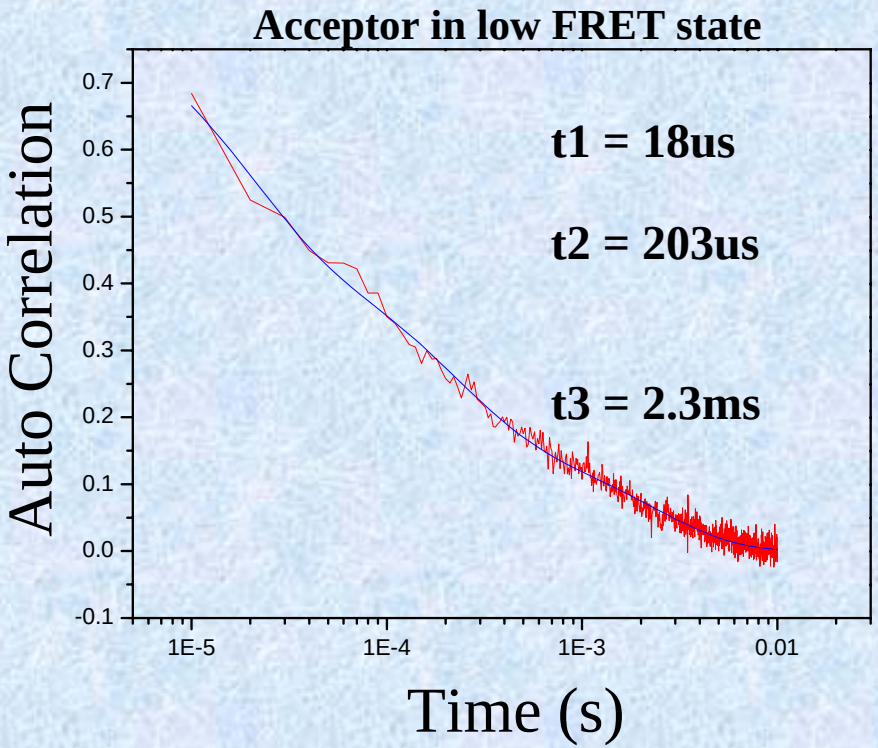


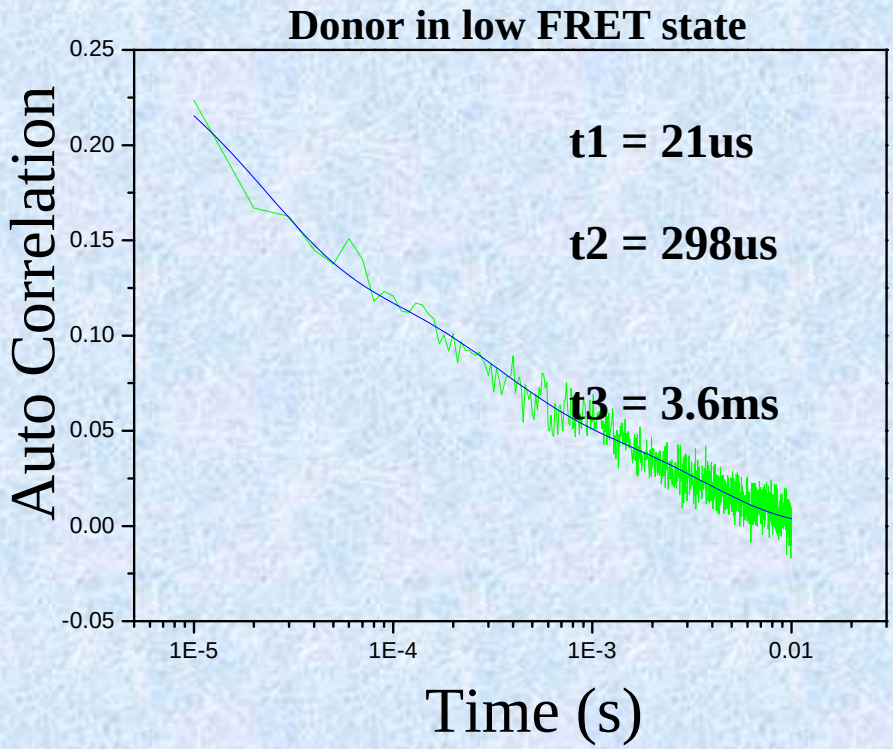




# Dynamics of Hairpin Ribozyme at Low FRET State

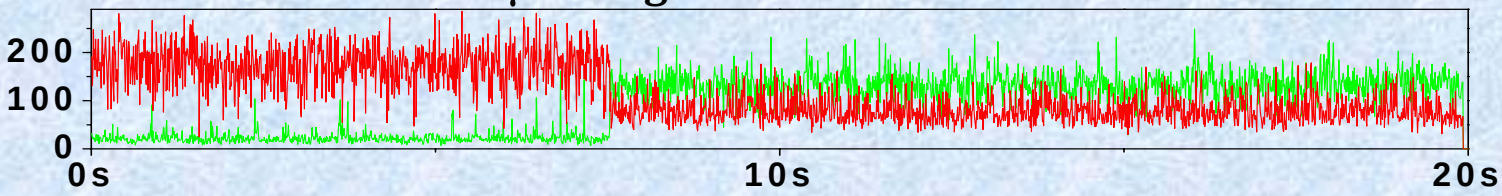


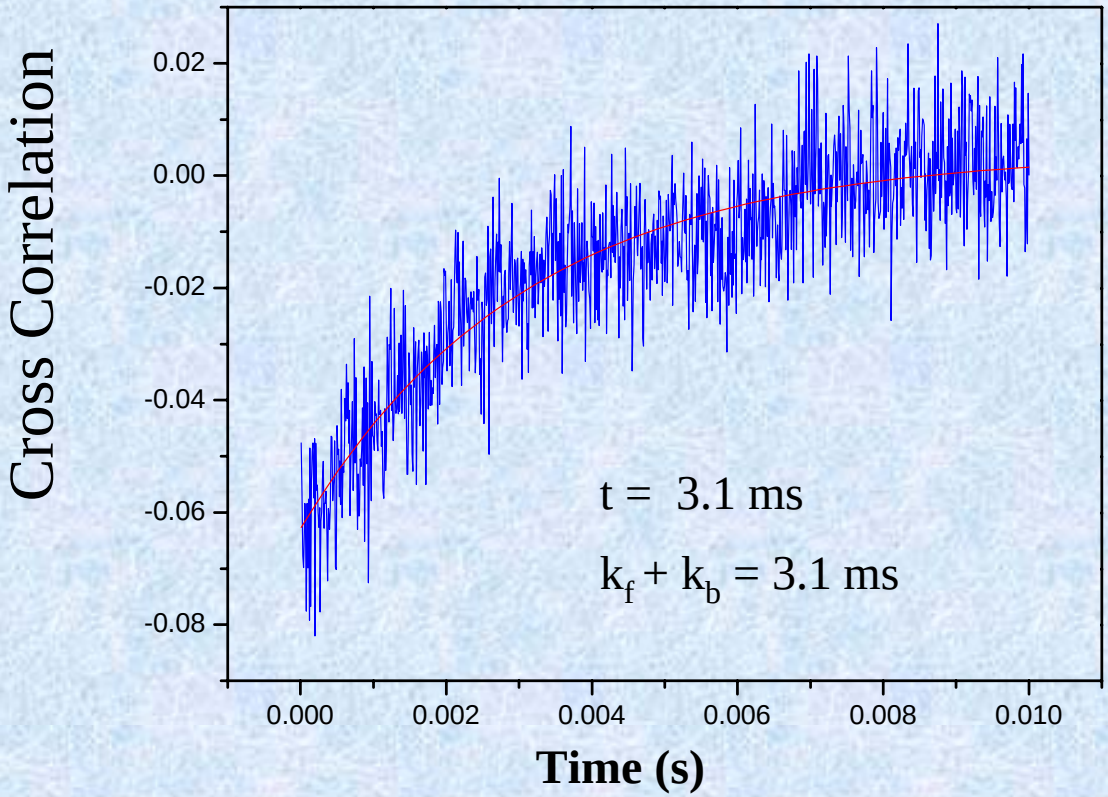



# Dynamics of Hairpin Ribozyme at Low FRET State

**10μs integration time rebinned to 8ms**




**Cross -correlation of acceptor and donor signal from 10 s to 20 s using 10μs data**



# FRET Efficiency Distribution in Low $Mg^{++}$