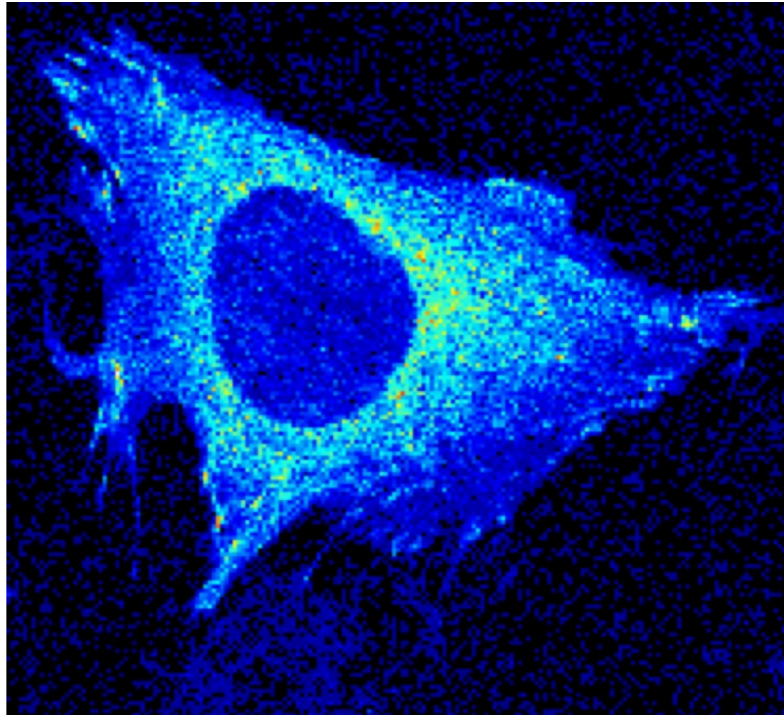# Introduction to FCS

Laboratory for Fluorescence Dynamics
Department of Biomedical Engineering
University of California, Irvine

Outline

**What is diffusion?**
Diffusion of molecules
Diffusion of light
Generalization of the concept of diffusion
Translational diffusion
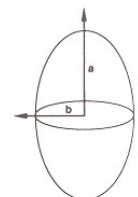Rotational diffusion

Some general rules about diffusion
Diffusion in restricted spaces
Diffusion in presence of flow
Anomalous diffusion

How to measure diffusion?
Macroscopic methods
Molecular level methods
Fluctuation based methods
Single particle tracking methods
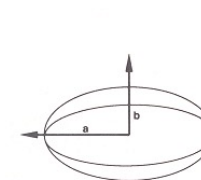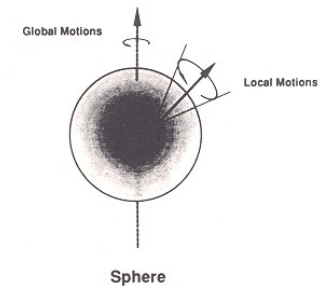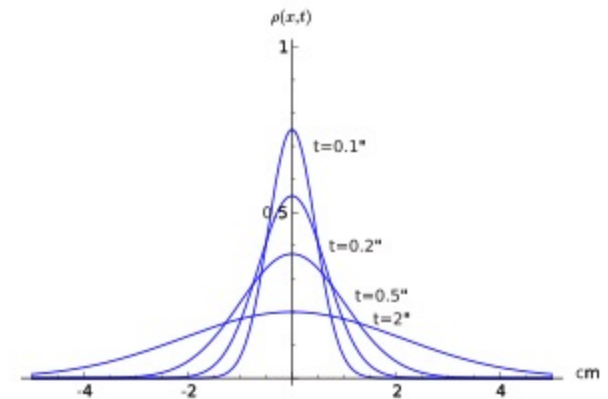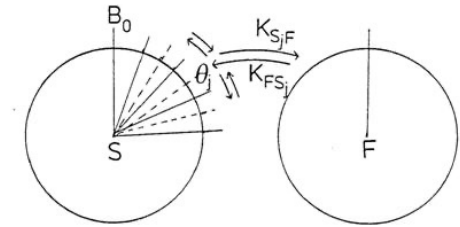
Original observation by Brown: the Brownian motion
(From A Fowler lectures)

In 1827 the English botanist Robert Brown noticed that pollen grains suspended in water jiggled about under the lens of the microscope, following a zigzag path. Even more remarkable was the fact that pollen grains that had been stored for a century moved in the same way.

In 1889 G.L. Gouy found that the "Brownian" movement was more rapid for smaller particles (we do not notice Brownian movement of cars, bricks, or people).

In 1900 F.M. Exner undertook the first quantitative studies, measuring how the motion depended on temperature and particle size.

The first good explanation of Brownian movement was advanced by Desaulx in 1877: "In my way of thinking the phenomenon is a result of thermal molecular motion in the liquid environment (of the particles)." This is indeed the case. A suspended particle is constantly and randomly bombarded from all sides by molecules of the liquid. If the particle is very small, the hits it takes from one side will be stronger than the bumps from other side, causing it to jump. These small random jumps are what make up Brownian motion.

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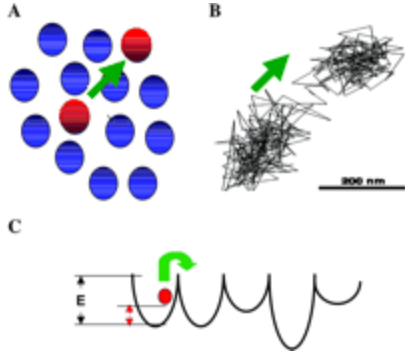
The role of fluctuations
Explanation in terms of fluctuations

Why measure diffusion with optical methods?
        Diffusion in microscopic spaces
        Diffusion in biological materials
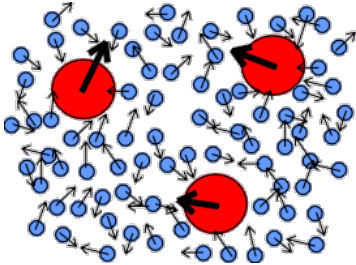
Difference between diffusion, "hopping" and binding

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 {Johnson, J.B., Phys. Rev. 32 pp. 97 (1928), Nyquist, H., Phys. Rev. 32 pp. 110 (1928)}

- 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)

Manfred Eigen

Elliot Elson

Keith Berland

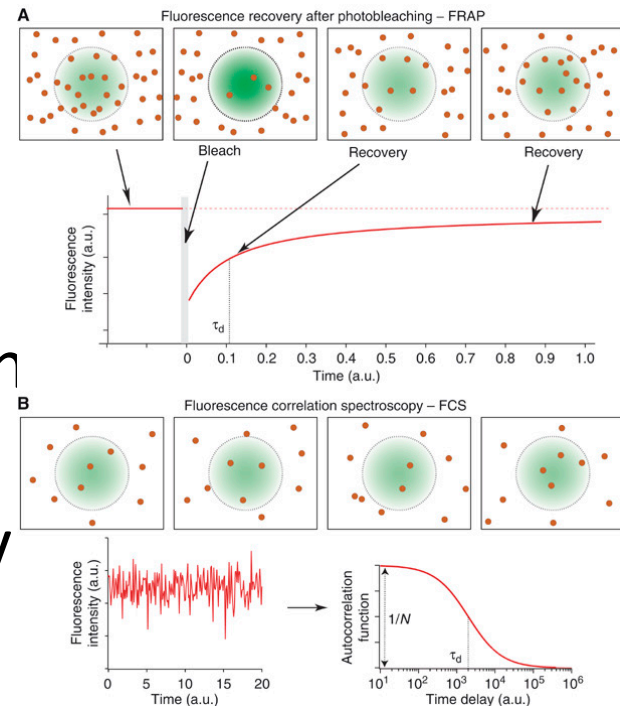# Why we need FCS to measure the internal dynamics in cell??

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.

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
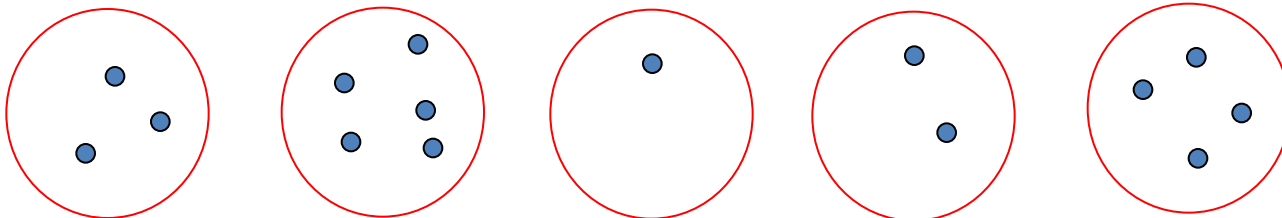
The fluctuation-dissipation principle (systems at equilibrium)

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

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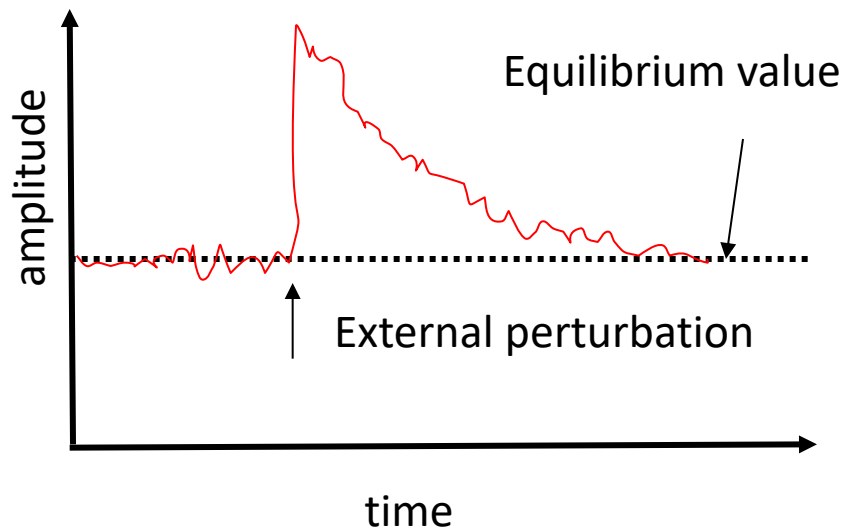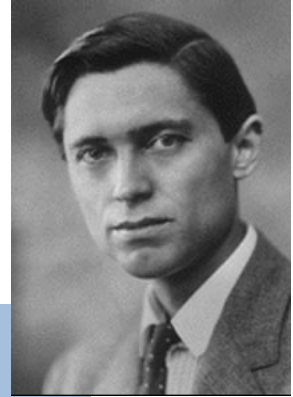

Synchronized
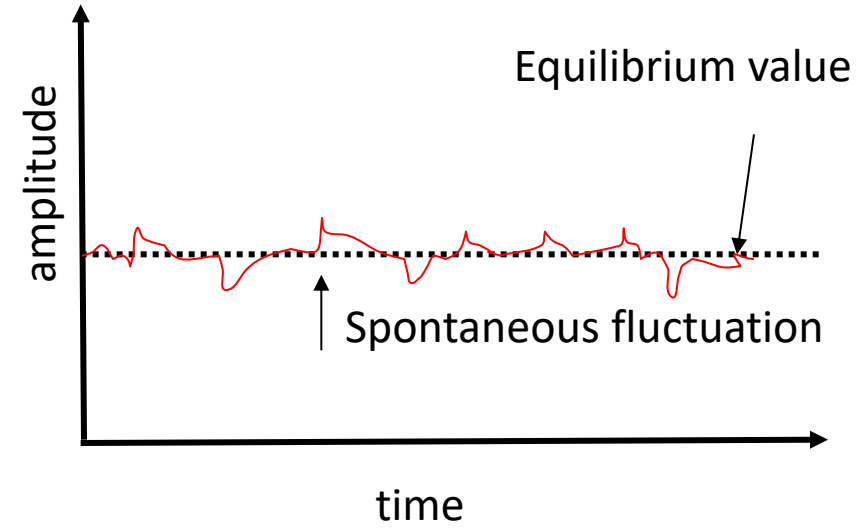
Non-synchronized

First Application of Correlation Spectroscopy
## (Svedberg & Inouye, 1911) *Occupancy Fluctuation*

Experimental data on colloidal gold particles:

```
120002001324123102111311251110233133322111224221226122142345241141311423100100421123123201111000111_21100132000001001100010002322100211000020100 1_3331220002312210240111 02_12221122310001103311102101100101030113121210101211 11211_100032210123020121213211101100233122421100012030101002 217344101010021122114444212114401321233143130112221233101211112224122311133221321100004104320121200113222312 00_2532120332331111002100220130113211131200101314322112211 22323442230321421532200202142123 2320431123120033142234521341104123222 20221
```

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 and spectrum of the fluctuations
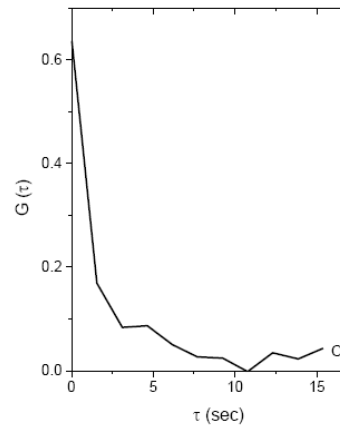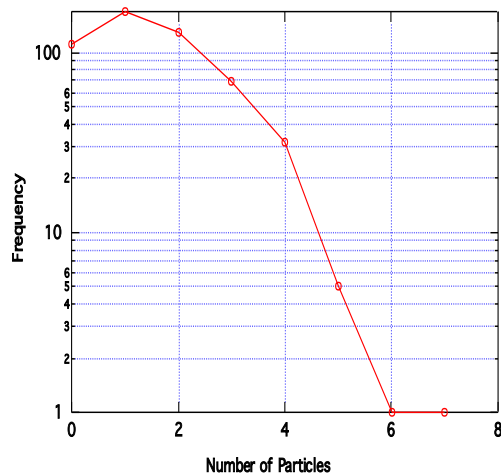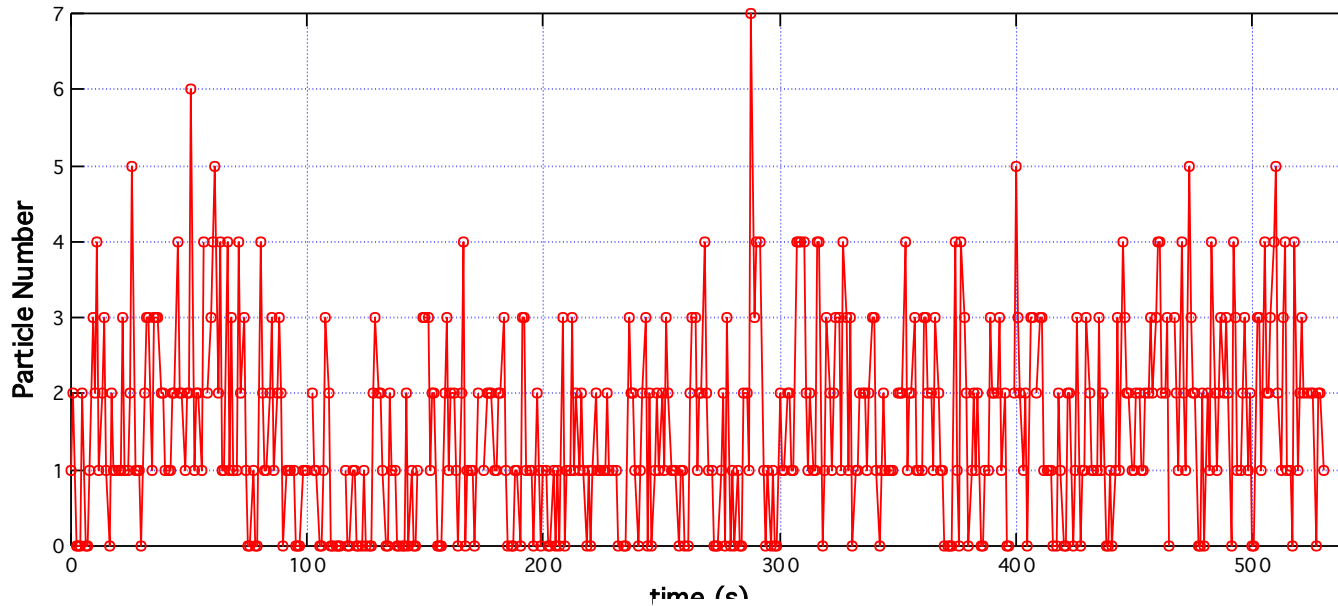
*Histogram of particle counts

*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

We need a small volume!!

Observation
Volume

Sample Space
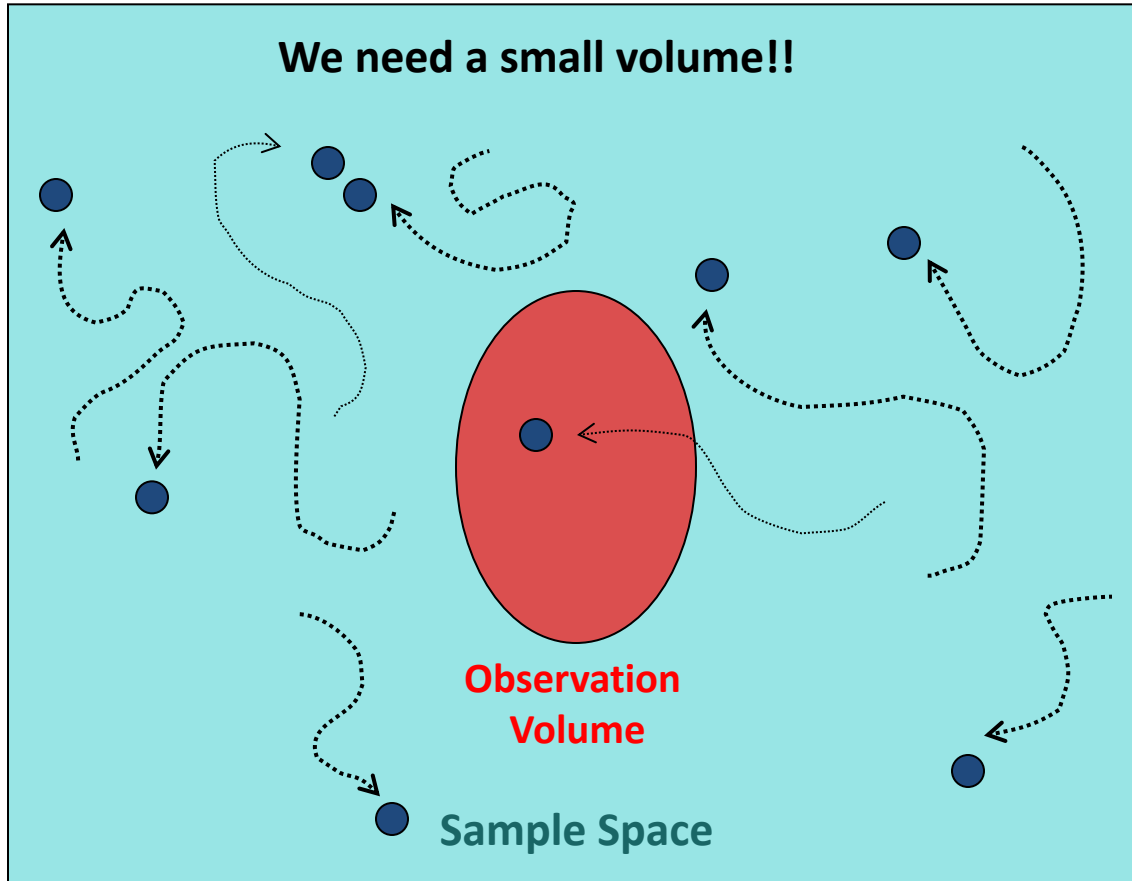
What is Observed?

1. The rate of motion

2. The concentration of particles

3. Changes in the particle fluorescence while under observation, for example conformational transitions
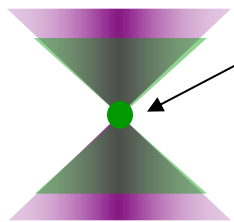
Note that the spectrum of the fluctuations responsible for the motion is "flat" but the spectrum of the fluorescence fluctuations is not

Defining Our Observation Volume under the microscope: One- & Two-Photon Excitation.
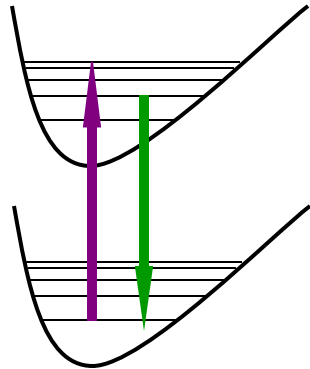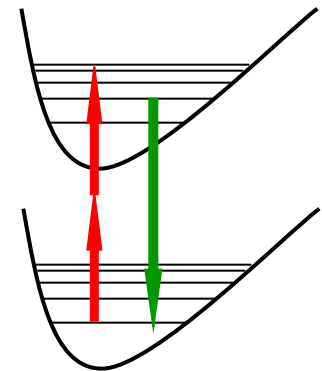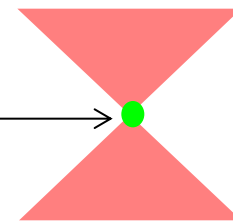
Why confocal detection?
Molecules are small, why to observe a large volume?
- Enhance signal to background ratio
- Define a well-defined and reproducible volume

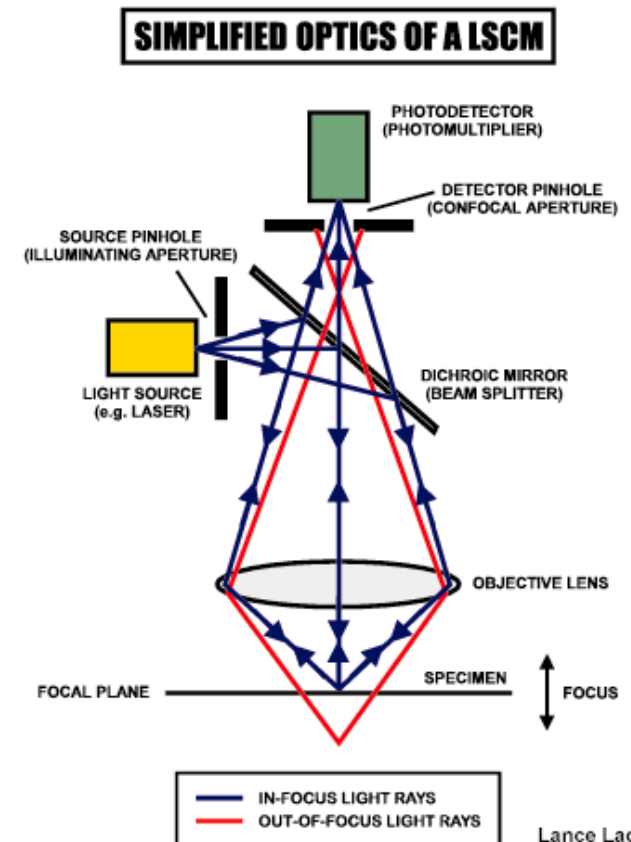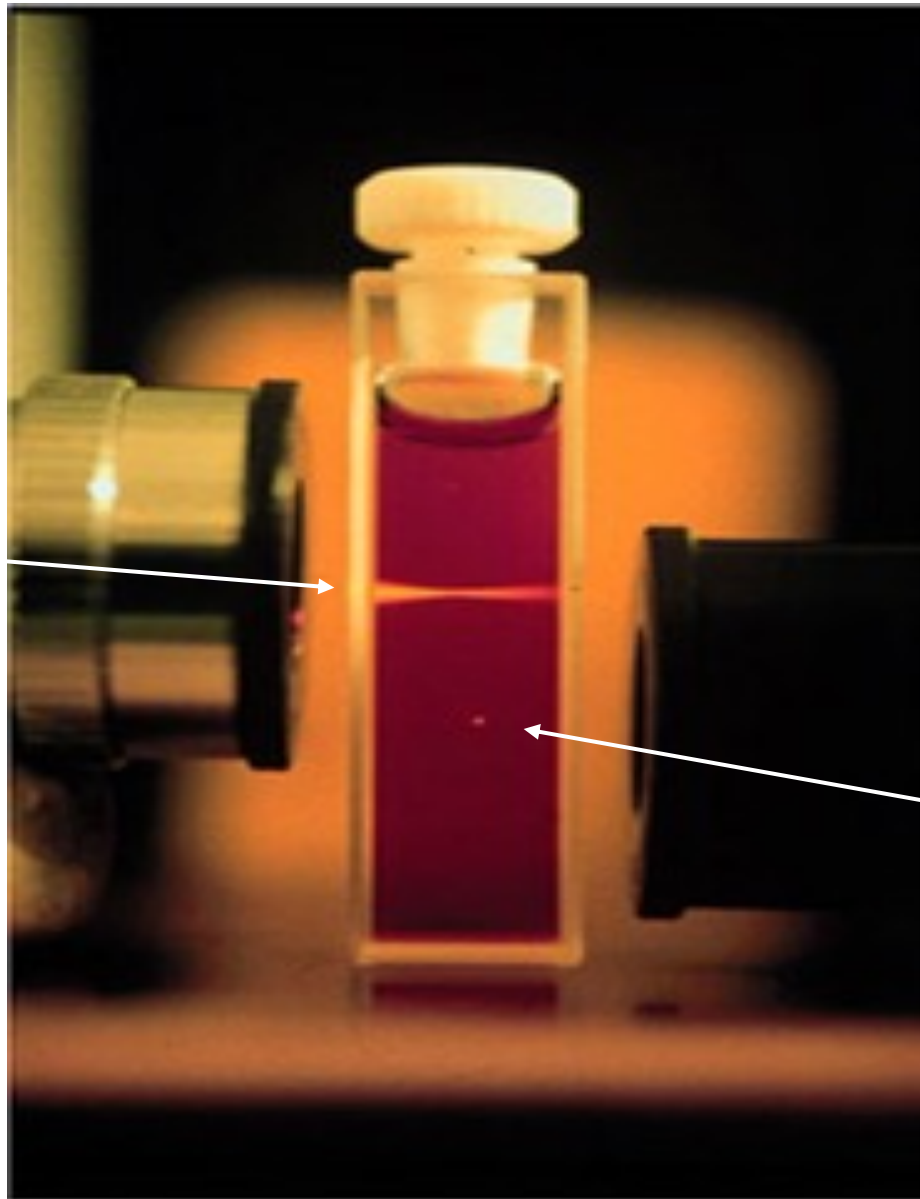## Methods to produce a confocal or small volume

(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

SIMPLIFIED OPTICS OF A LSCM

PHOTODETECTOR
(PHOTOMULTIPLIER)

DETECTOR PINHOLE
(CONFOCAL APERTURE)

SOURCE PINHOLE
(ILLUMINATING APERTURE)

DICHROIC MIRROR
(BEAM SPLITTER)

LIGHT SOURCE
(e.g. LASER)

OBJECTIVE LENS

FOCAL PLANE   SPECIMEN
                              FOCUS

— IN-FOCUS LIGHT RAYS
— OUT-OF-FOCUS LIGHT RAYS

Lance Ladic
ladic@cs.ubc.ca

1-photon

2-photon

Need a pinhole to define a small volume

Brad Amos
MRC, Cambridge, UK

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

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

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

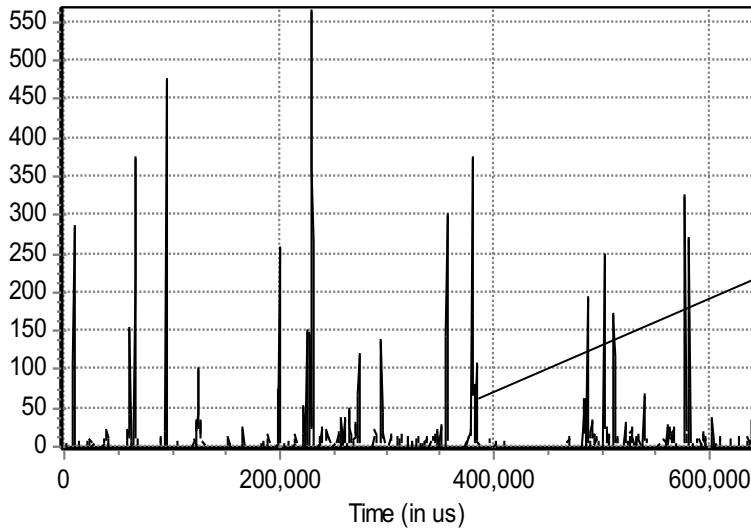$$\delta = 10^{-50} \text{ cm}^4 \text{ sec photon}^{-1} \text{ molecule}^{-1}$$

Enough power to saturate absorption in a diffraction limited spot

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

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

Determination of diffusion by fluctuation spectroscopy

Data presentation and Analysis
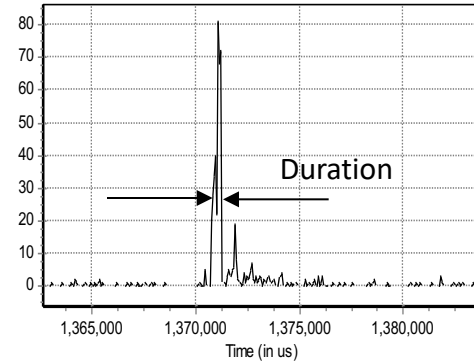
Detail of one time region

The time series

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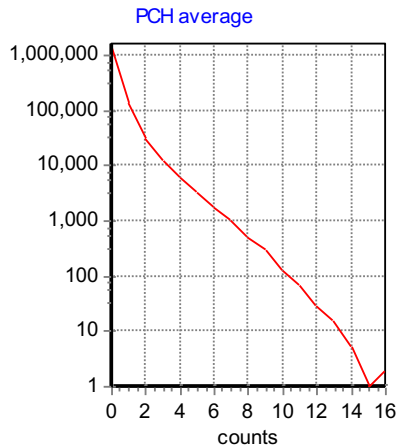
The histogram of the counts in a given time bin (PCH). N and brightness

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

Distribution of the **amplitude** of the fluctuations

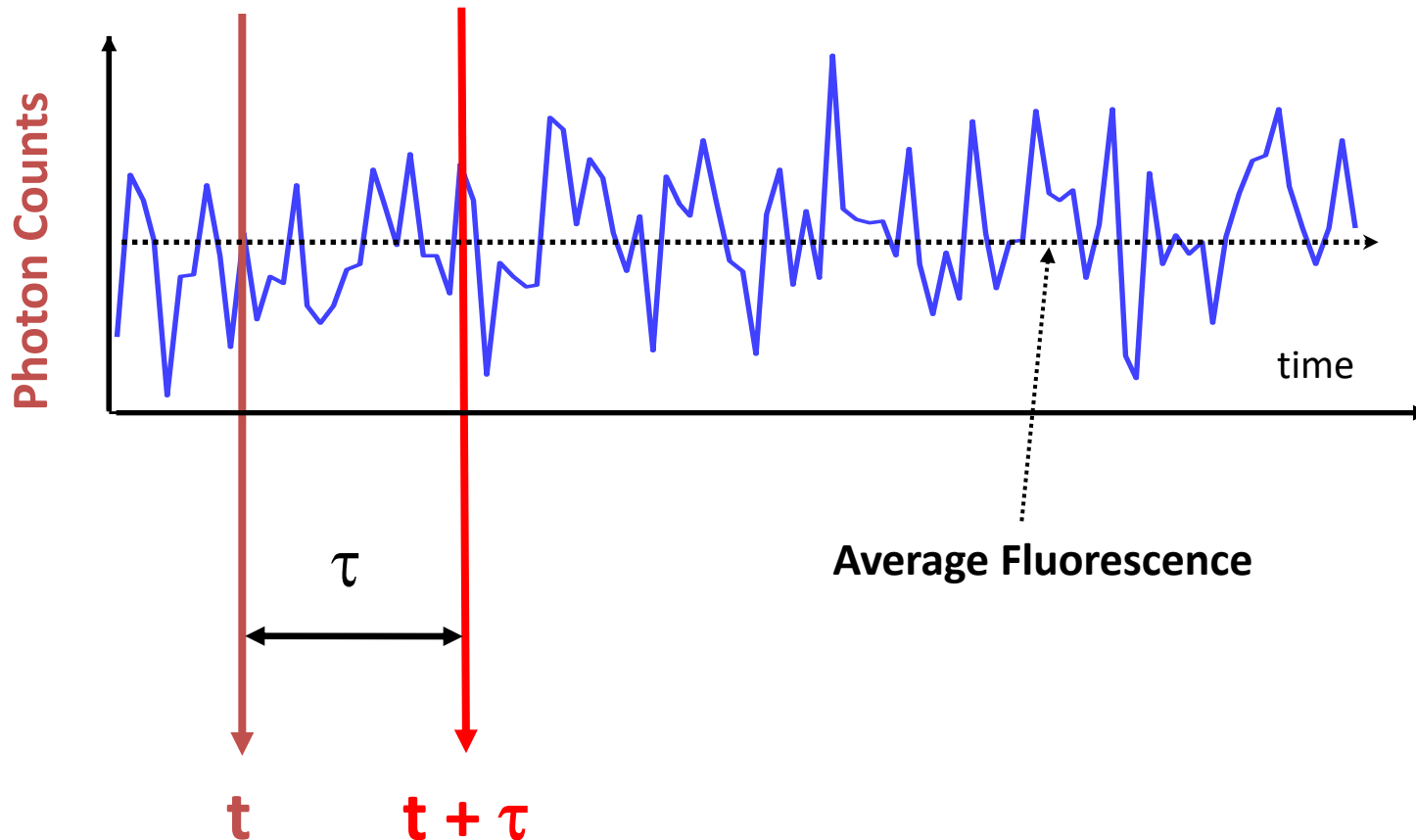Distribution of the **duration** of the fluctuations

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**

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

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

Photon Counts

time

Average Fluorescence

τ

t

t + τ

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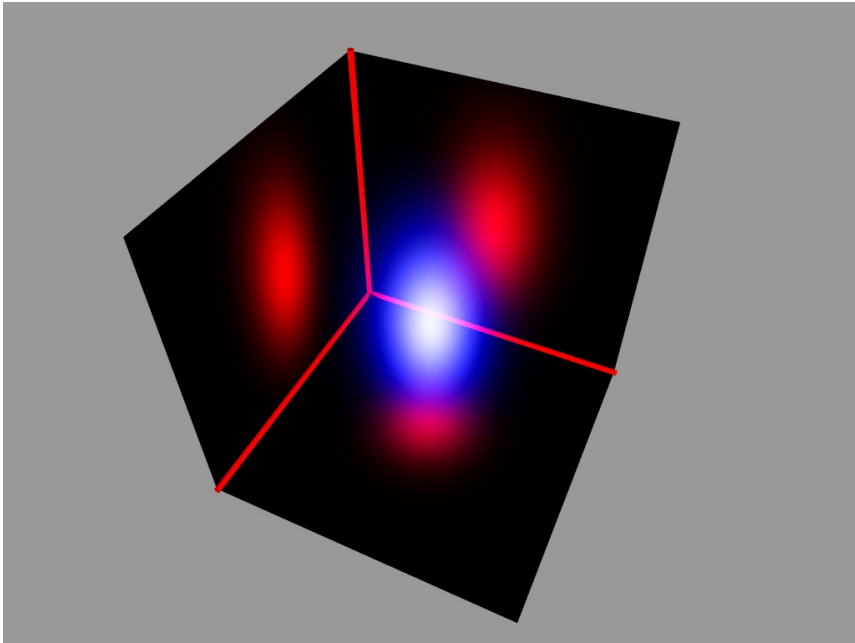
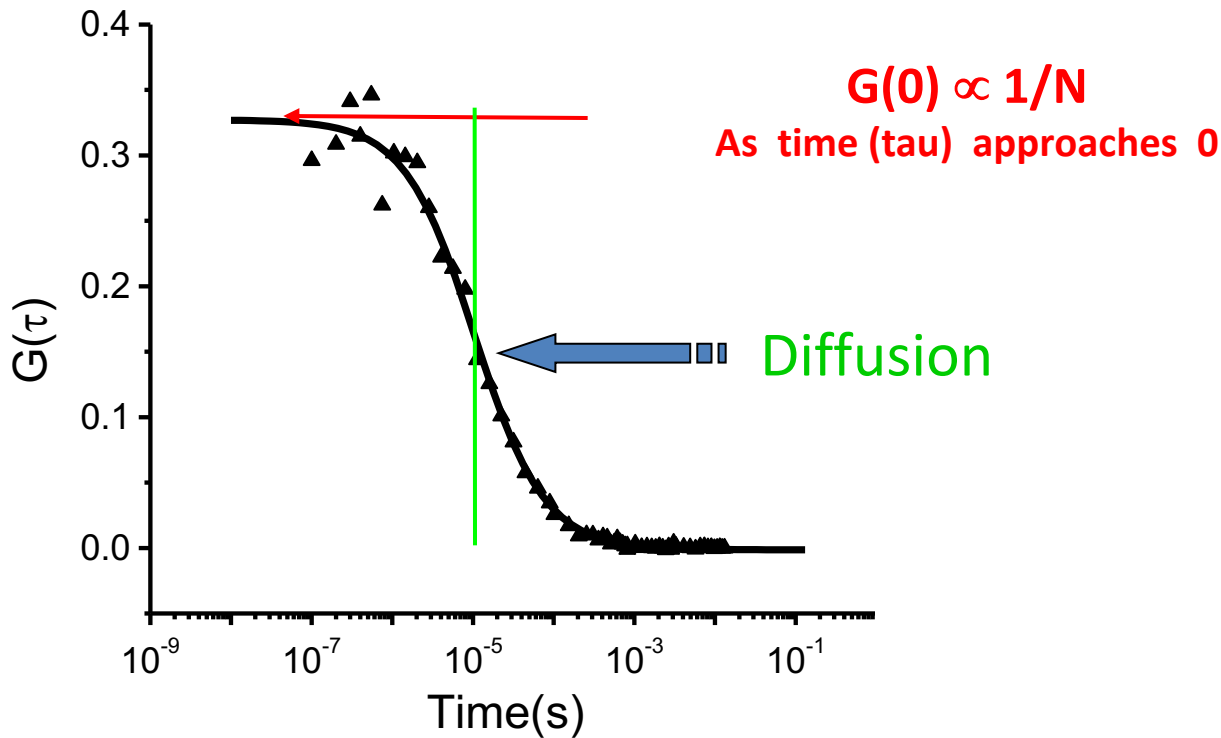$$F(x, y, z) = I_0 I(z) e^{-\frac{2(x^2 + y^2)}{w_0^2}}$$

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

$$I(z) = \frac{1}{1 + \left(\dfrac{z}{w_{oz}}\right)^2} \quad \text{Lorentzian z}$$

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
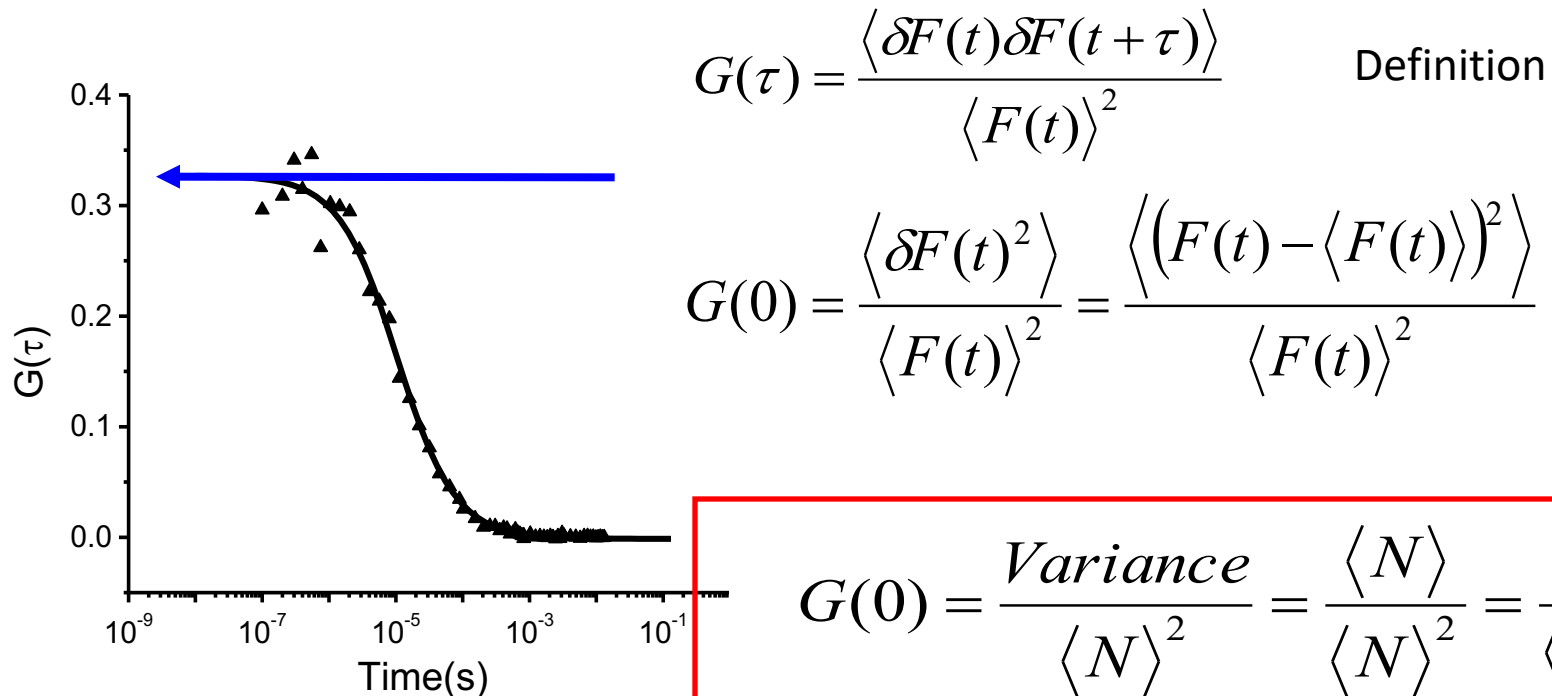
The Effects of Particle Concentration on the Autocorrelation Curve
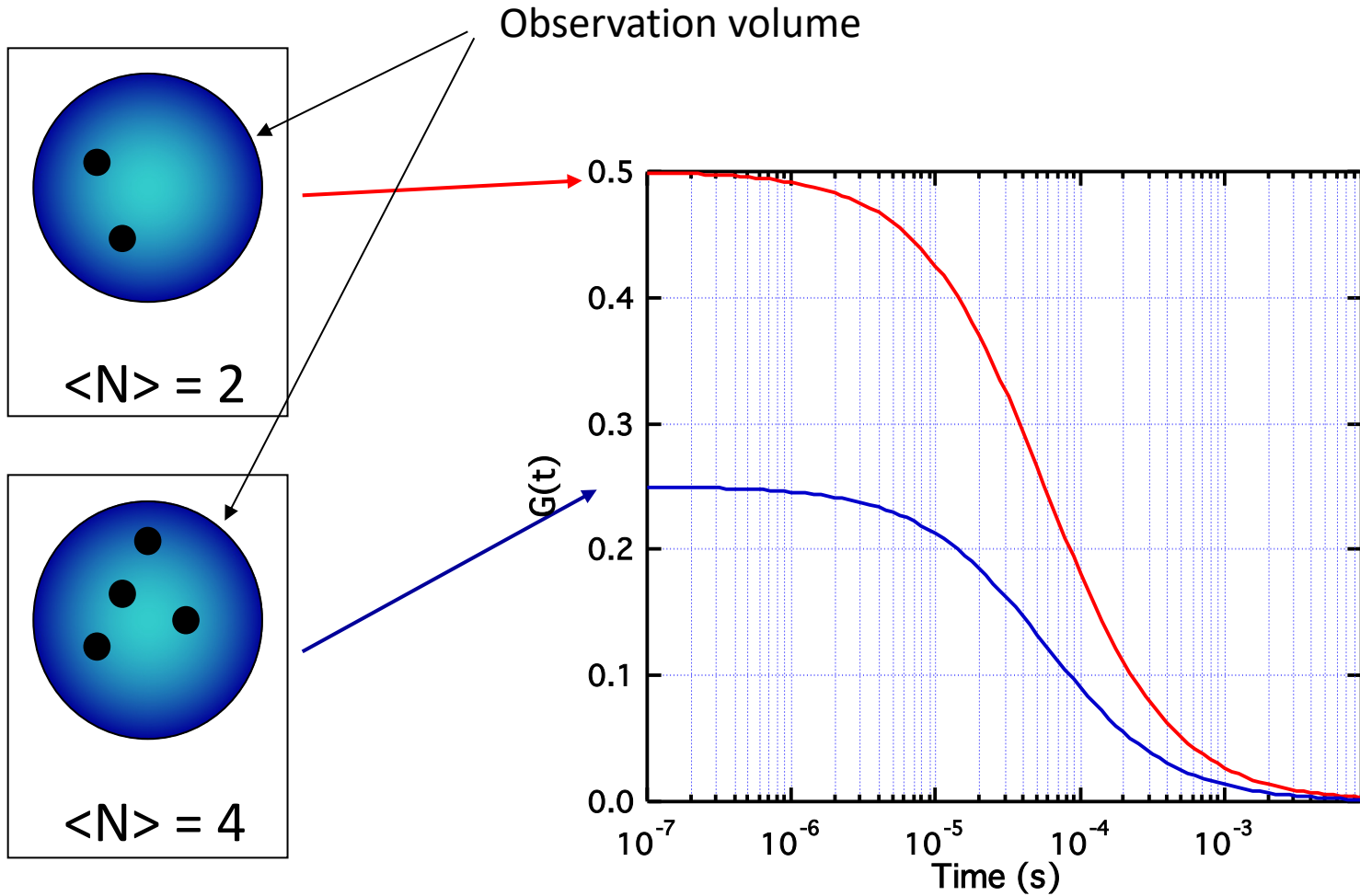
Why Is G(0) Proportional to 1/Particle Number?

A Poisson distribution describes the statistics of particle occupancy fluctuations. For a Poisson distribution the variance is proportional to the average:
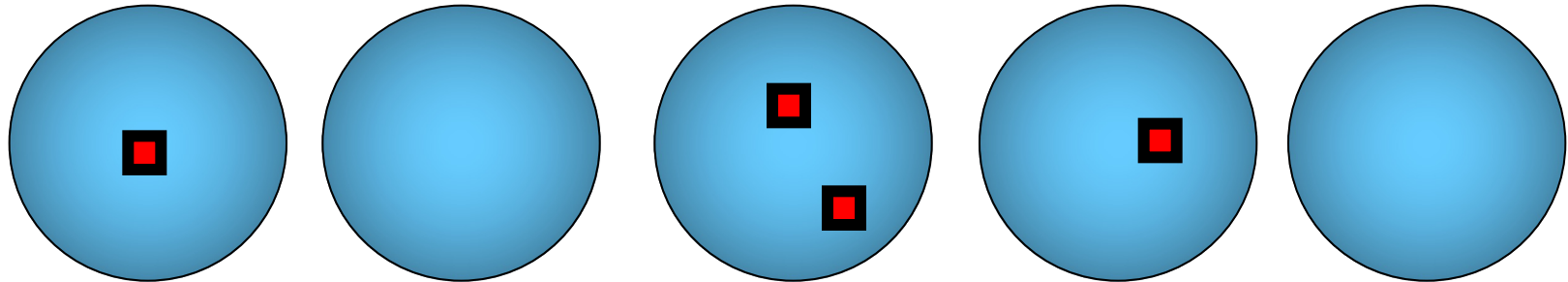
$$< N > = \langle Particle\_Number \rangle = Variance$$

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

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

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

G(0), Particle Brightness and Poisson Statistics

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

$$\langle N \rangle \propto Average^2 \Big/ Variance = \frac{0.275^2}{0.256} = 0.296$$
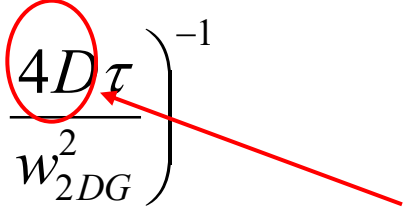
Lets increase the particle brightness by 4x:

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

$$\langle N \rangle \propto \quad \mathbf{0.296}$$

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:

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

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 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)?

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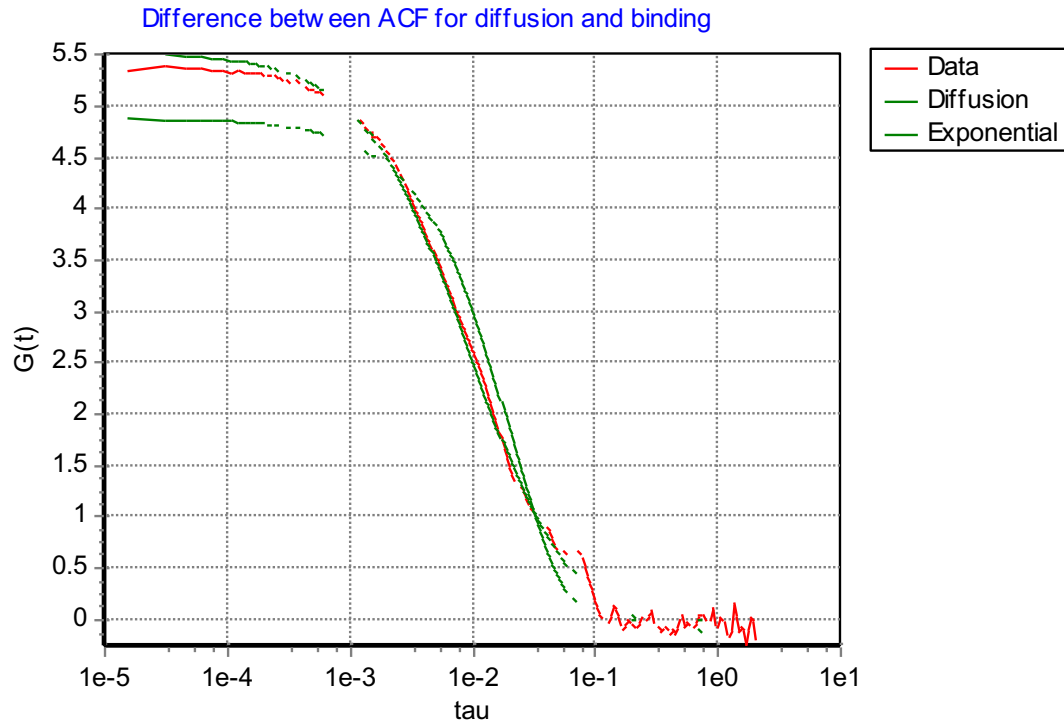
With good S/N it is possible to distinguish between the two processes.
Most of the time diffusion and exponential processes are combined

The Effects of Particle Size on the Autocorrelation Curve

Diffusion Constants

300 um²/s
90 um²/s
71 um²/s

Stokes-Einstein Equation:

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

and

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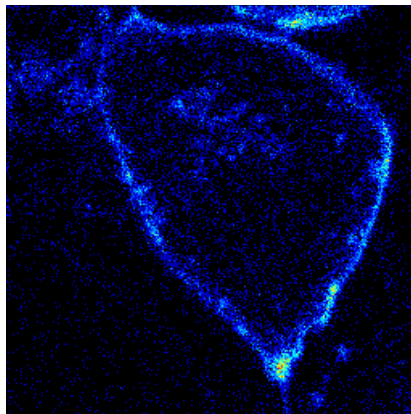

Slow Diffusion

Fast Diffusion

G(t)

Time (s)
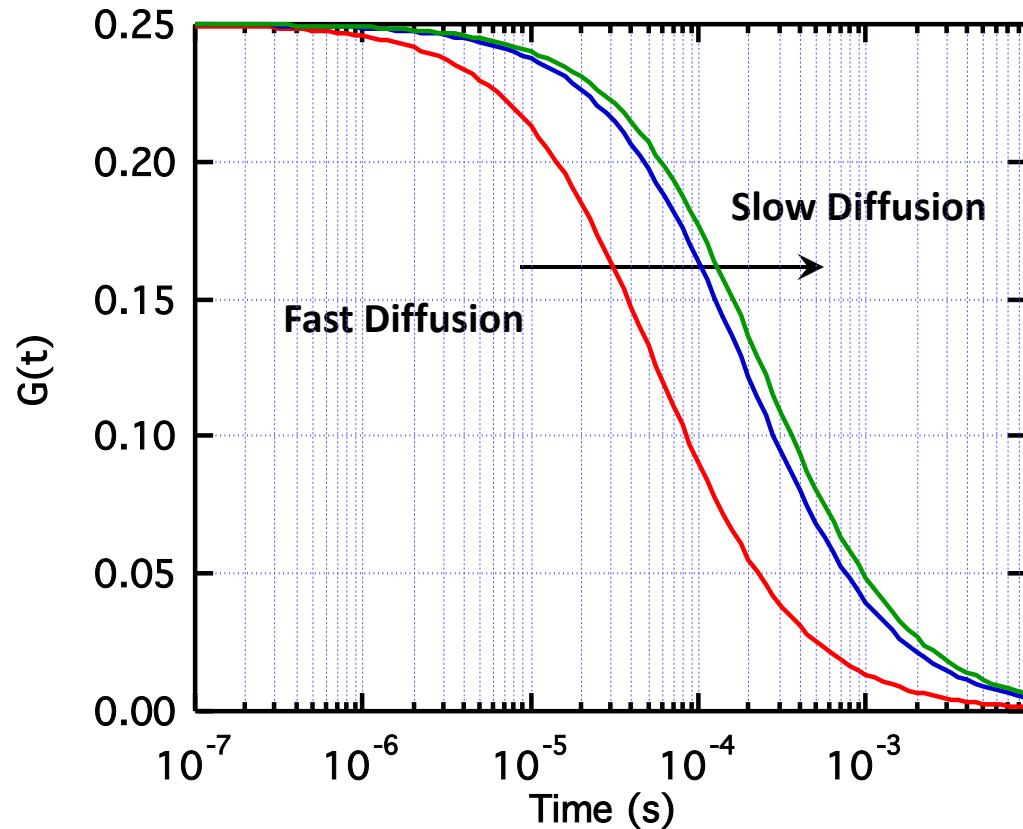
Monomer --> Dimer

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

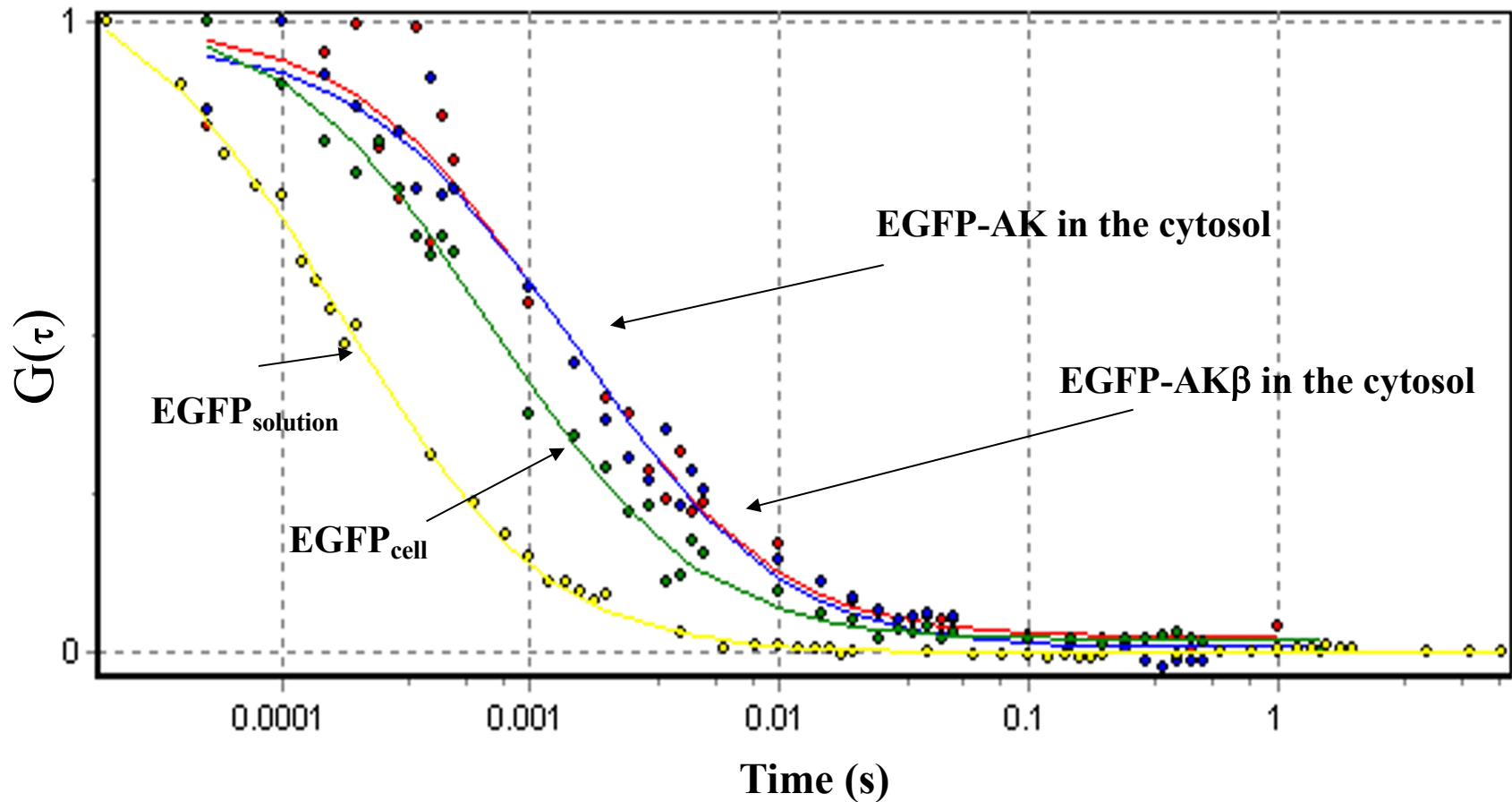# Autocorrelation Adenylate Kinase -EGFP Chimeric Protein in HeLa Cells

Examples of different *Hela* cells transfected with AK1-EGFP
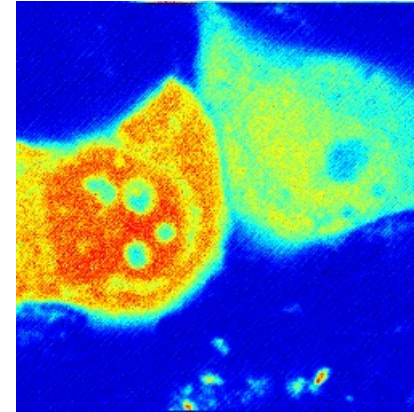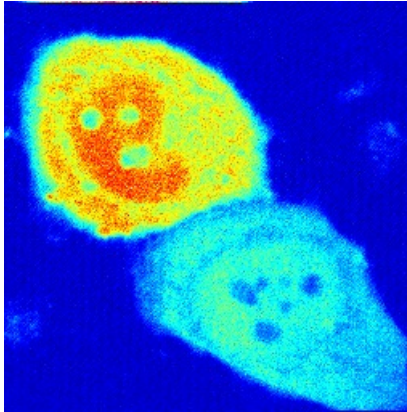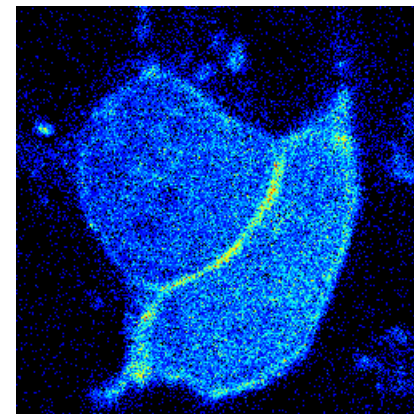
Examples of different *Hela* cells transfected with AK1β -EGFP

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__

Clearly more than one diffusion time
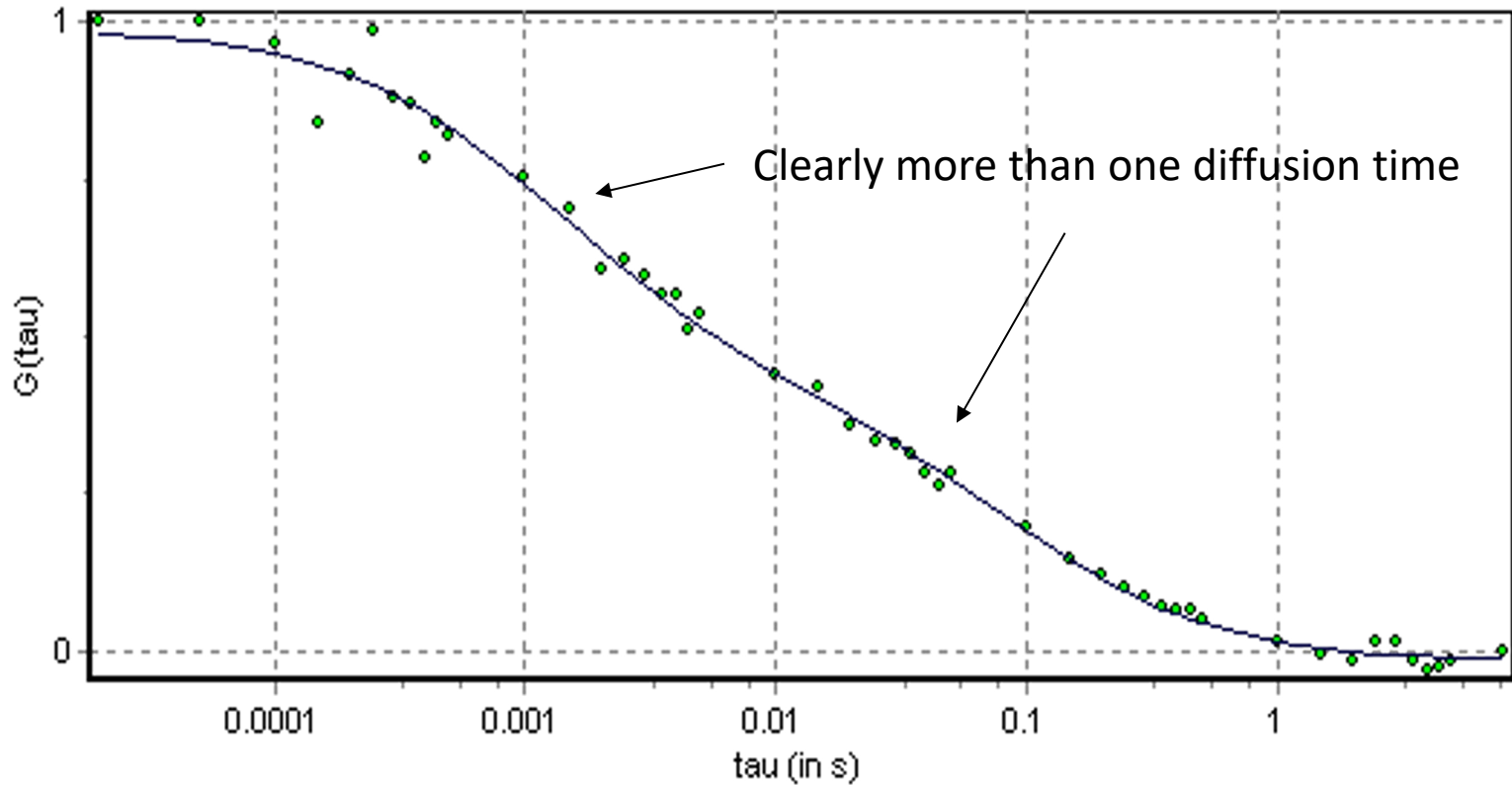
A mixture of AK1b-EGFP in the cytoplasm and membrane of the cell.

Autocorrelation Adenylate Kinaseβ -EGFP
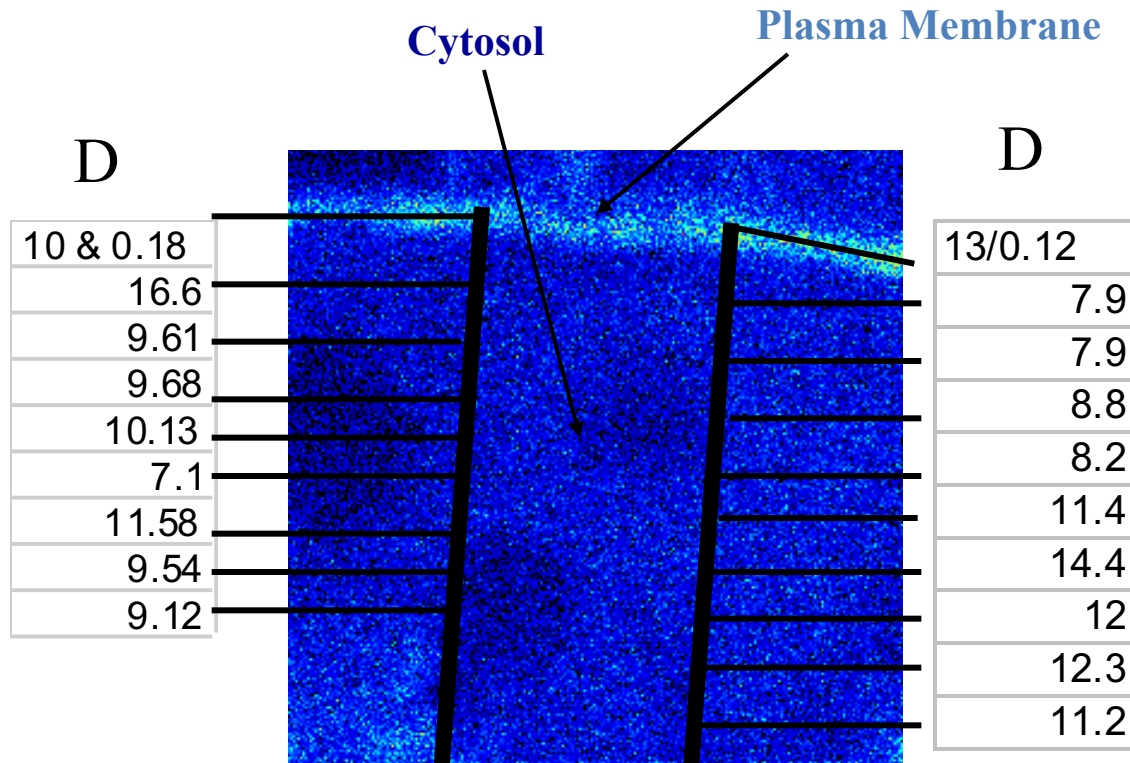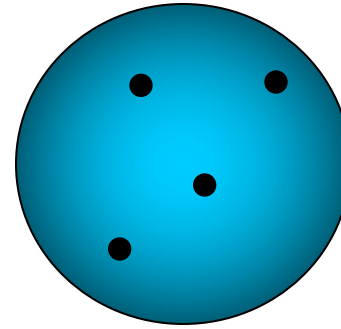

Diffusion constants (um²/s) of AK EGFP-AKβ in the cytosol -EGFP in the cell (HeLa). At the membrane, a dual diffusion rate is calculated from FCS data. Away from the plasma membrane, single diffusion constants are found.
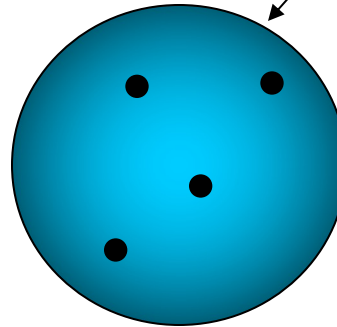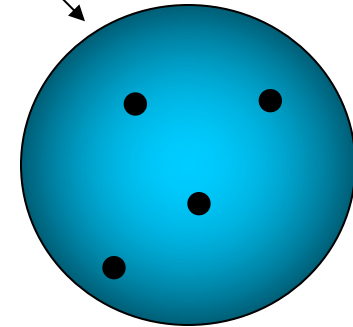
Two Channel Detection:
Cross-correlation
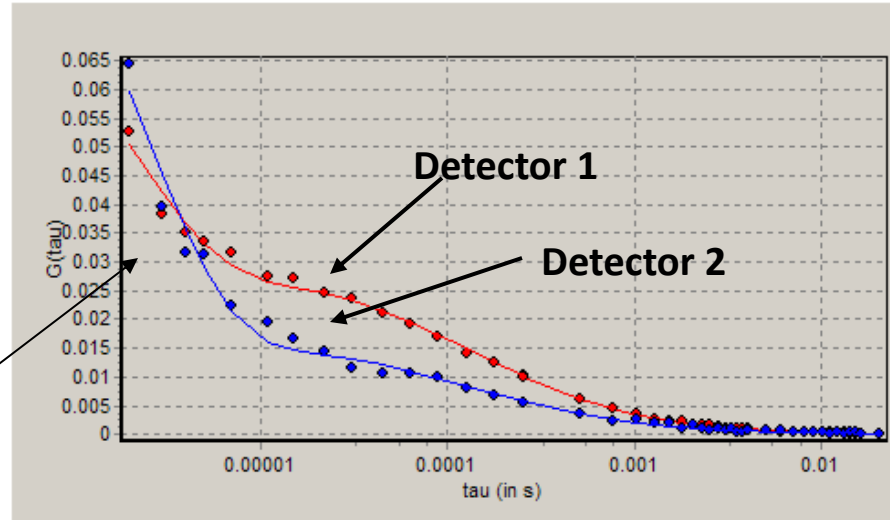
Sample Excitation Volume

Beam Splitter

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

Detector 1   **Detector 2**

Each detector observes the same particles

Removal of Detector Noise by Cross-correlation

11.5 nM Fluorescein

Detector 1

Detector 2
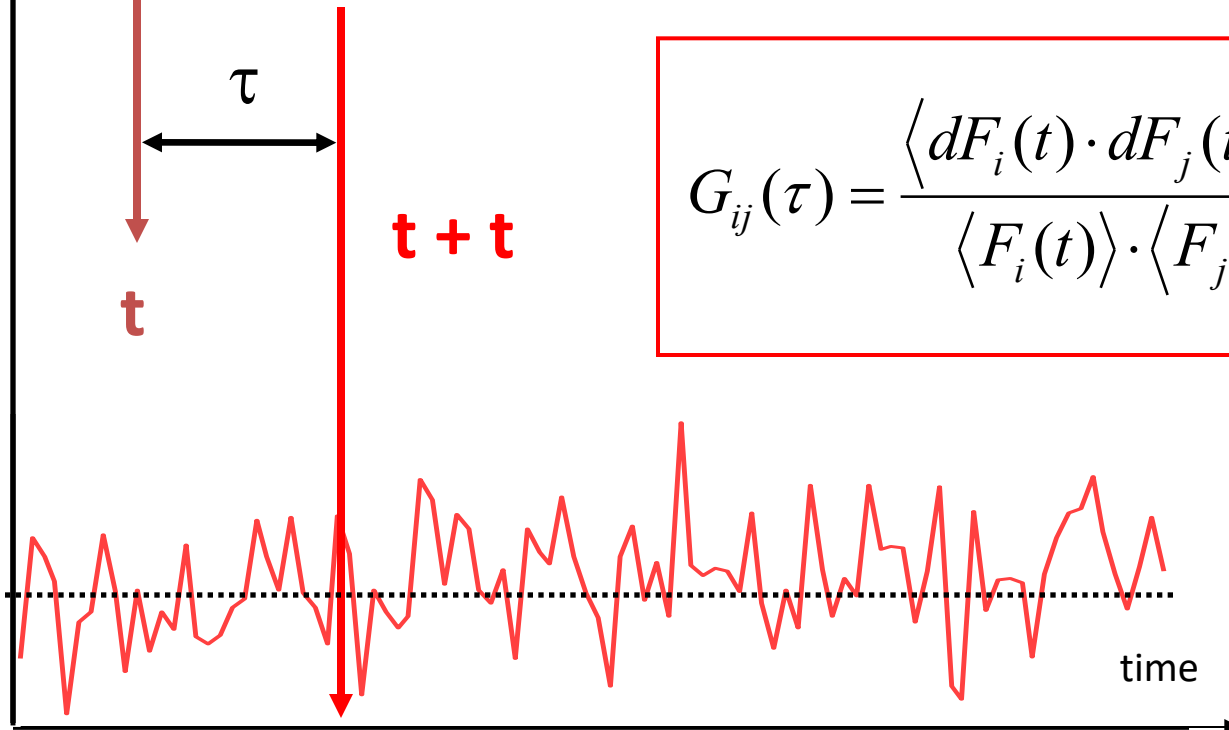
Detector after-pulsing
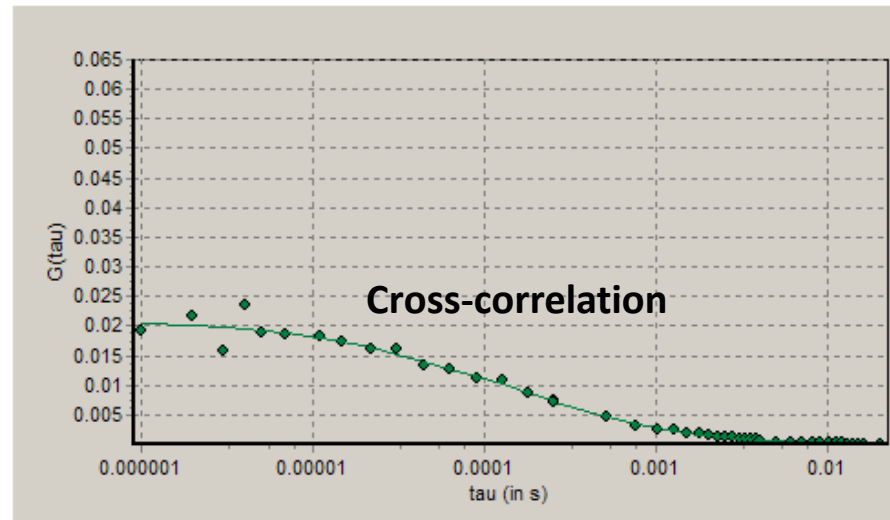
Cross-correlation

Calculating the Cross-correlation Function

Detector 1: F_i

time

τ

t

t + t

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

Detector 2: F_j

time
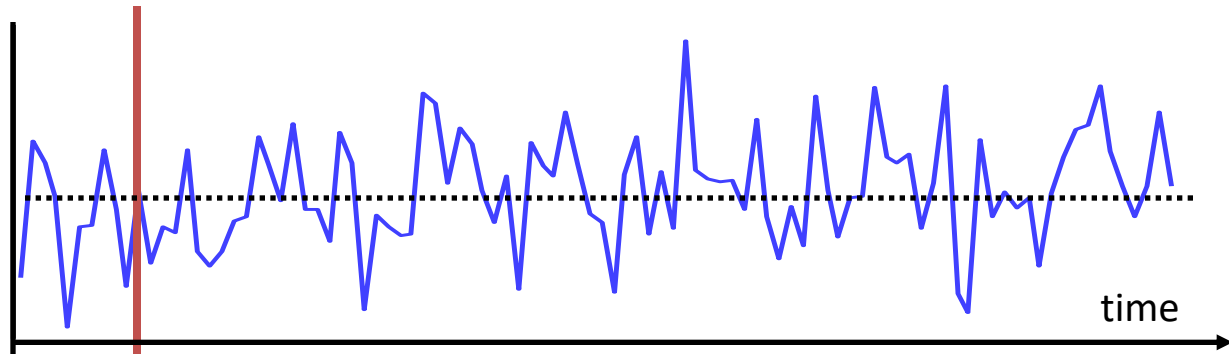
Cross-correlation calculations

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.
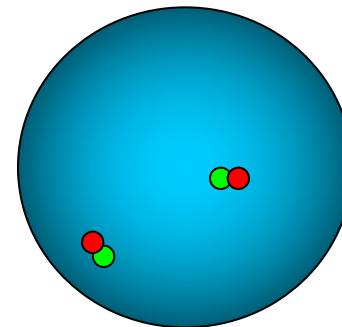
Two-Channel Cross-correlation

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

Sample

Red filter

Green filter

Each detector observes
particles with a particular color
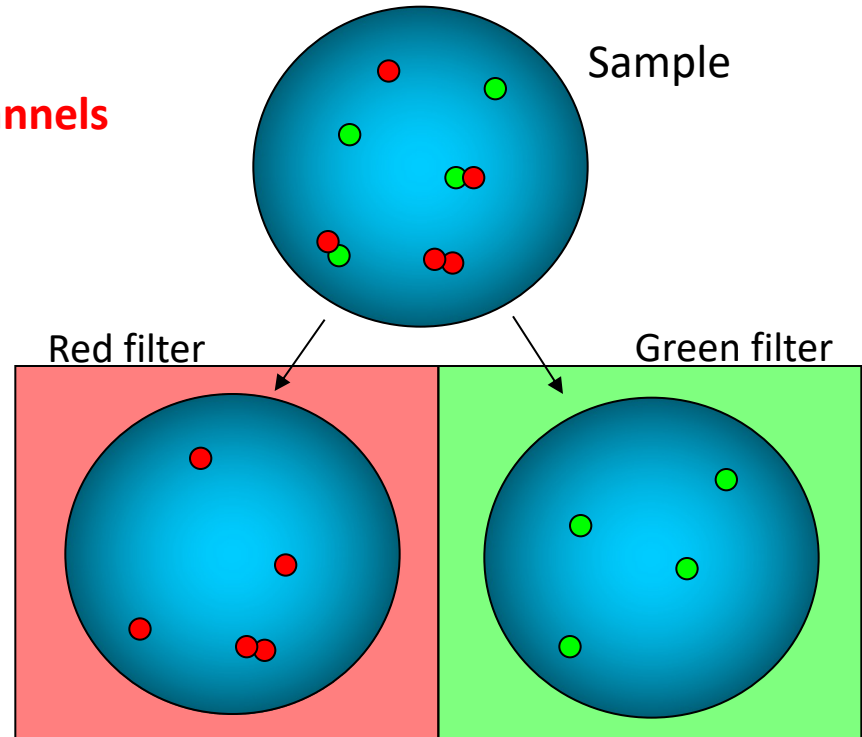
The cross-correlation signal:

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:

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

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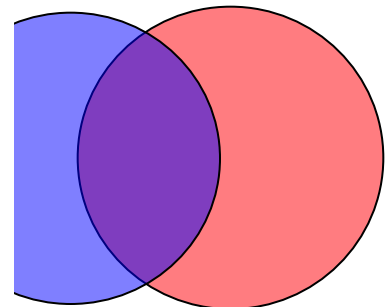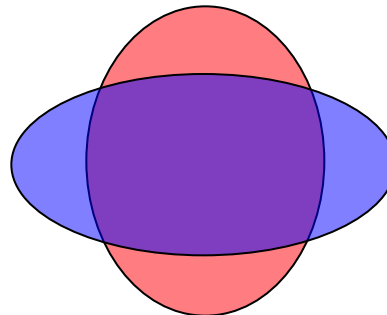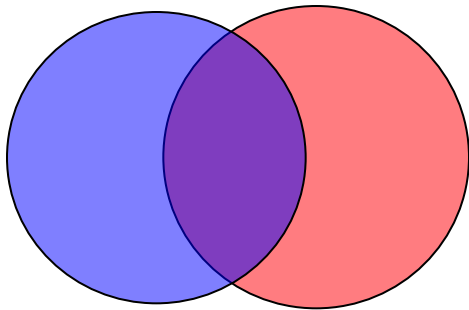
We assume exact match of the observation volumes in our calculations which is difficult to obtain experimentally.

Excitation side:

    (1) Laser alignment

    (2) Chromatic aberration

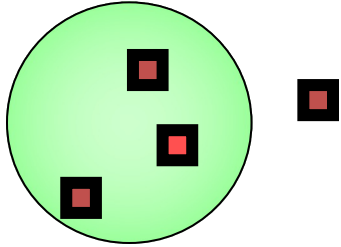   (3) Spherical aberration

Emission side:

    (1) Chromatic aberrations

    (2) Spherical aberrations

    (3) Improper alignment of detectors or pinhole

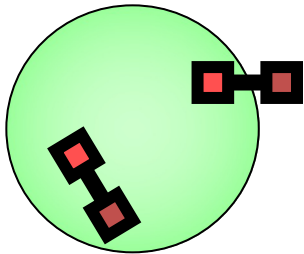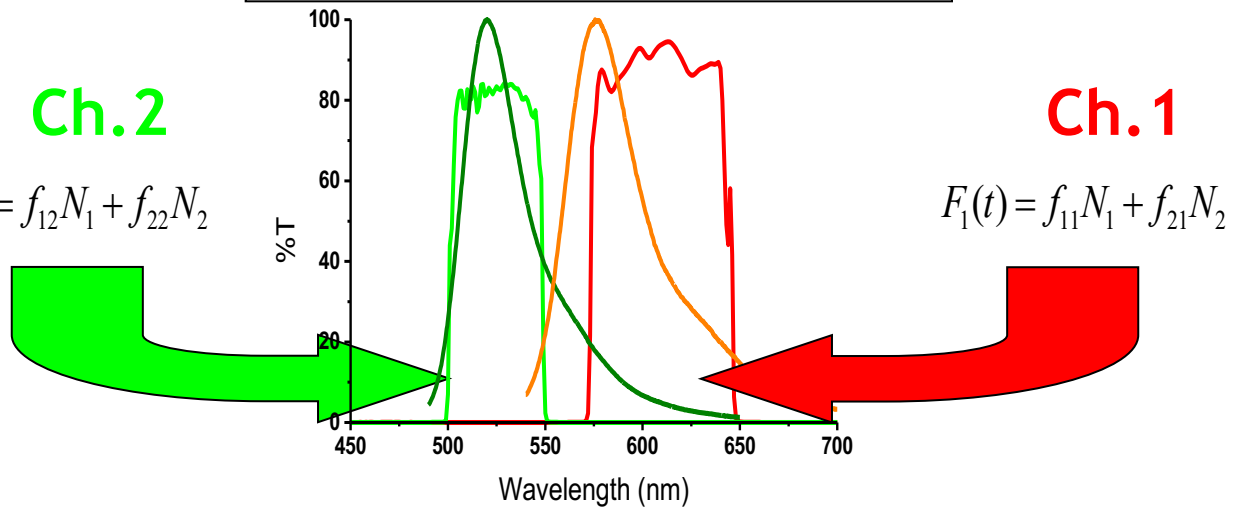   (cropping of the beam and focal point position)

lfd

Uncorrelated

Correlated

Interconverting

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

Ch.2

$$F_2(t) = f_{12}N_1 + f_{22}N_2$$

Ch.1

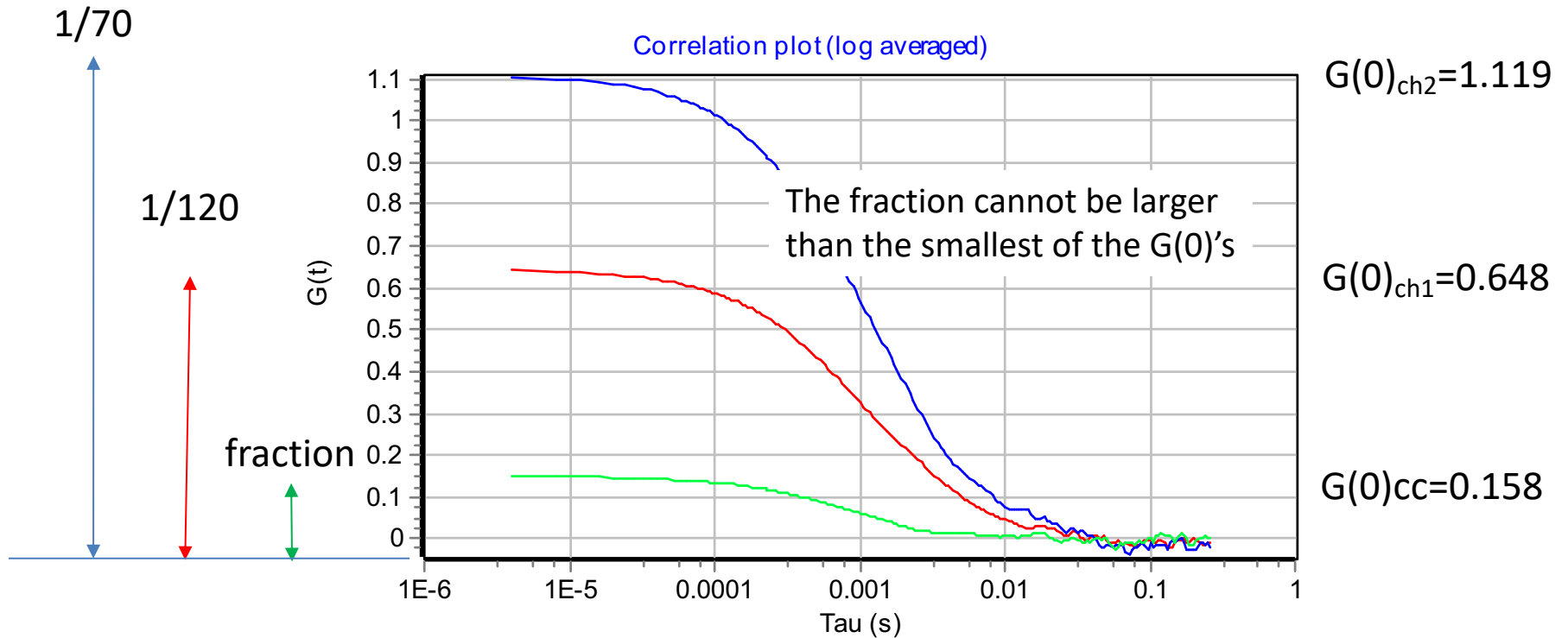$$F_1(t) = f_{11}N_1 + f_{21}N_2$$

Wavelength (nm)
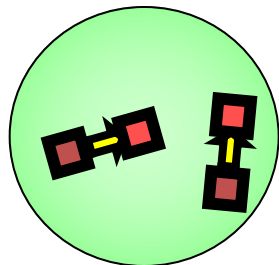
For two _**uncorrelated**_ species, the amplitude of the cross-correlation is proportional to:

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

Simulation of cross-correlation
Same diffusion =10 μm^2/s

	Channel 1	Channel 2		
Molecules	100			
Molecules		50		
Molecules	20	20		

1/70

1/120

fraction

Correlation plot (log averaged)

The fraction cannot be larger than the smallest of the G(0)'s

G(0)$_{ch2}$=1.119

G(0)$_{ch1}$=0.648

G(0)cc=0.158

G(t)

Tau (s)

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 of your questions