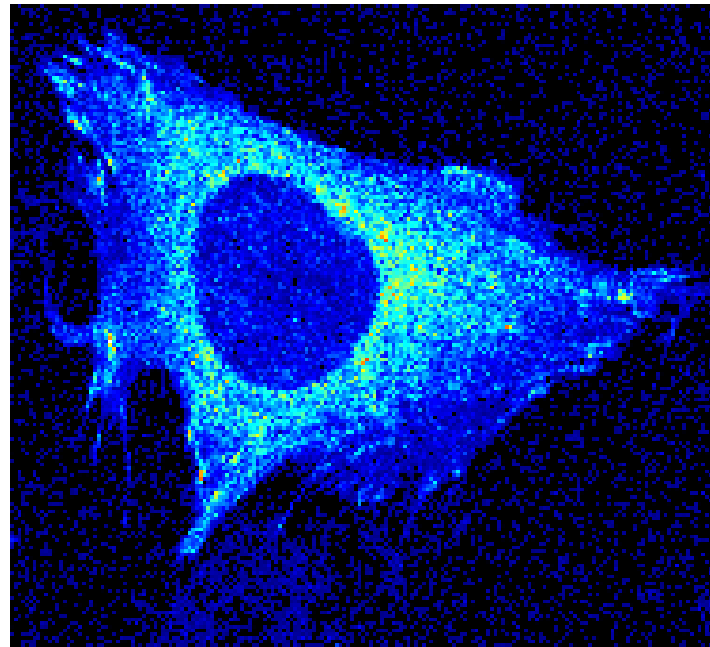


Introduction to FCS

Enrico Gratton

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



Outline

What is diffusion?

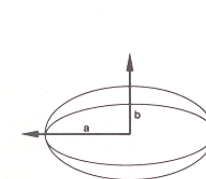
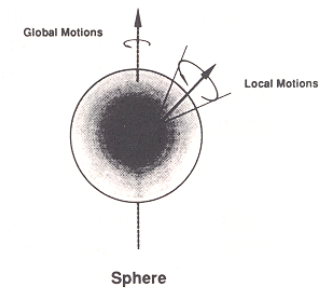
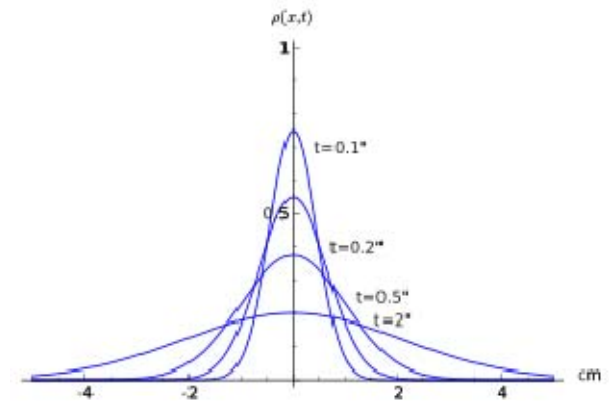
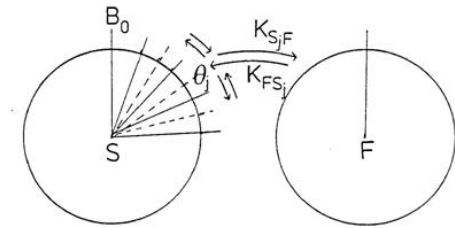
- Diffusion of molecules
- Diffusion of light
- Generalization of the concept of diffusion
 - Translational diffusion
 - Rotational diffusion

Some general rules about diffusion

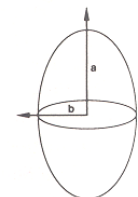
- Diffusion in restricted spaces
- Diffusion in presence of flow
- Anomalous diffusion

How to measure diffusion?

- Macroscopic methods
- Molecular level methods
 - Fluctuation based methods
 - Single particle tracking methods



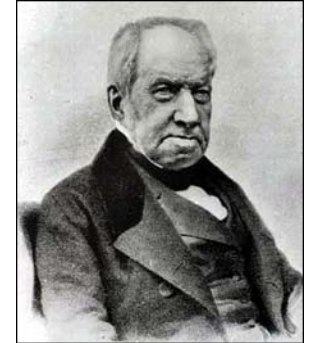
Oblate Ellipsoid



Prolate Ellipsoid

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.

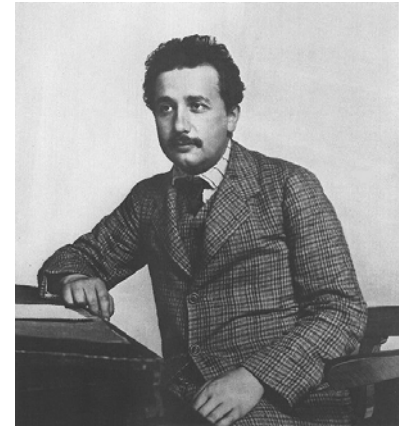


In 1889 G.L. Gouy found that the "Brownian" movement was more rapid for smaller particles (we do not notice Brownian movement of cars, bricks, or people).

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

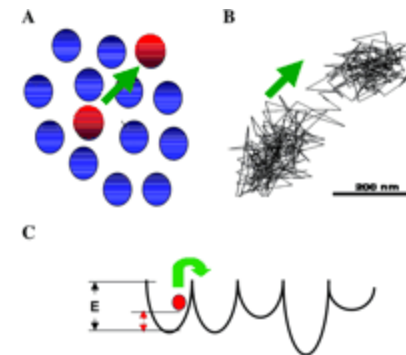
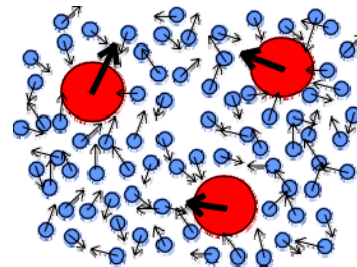
Explanation in terms of fluctuations

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
- $1/f$ 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

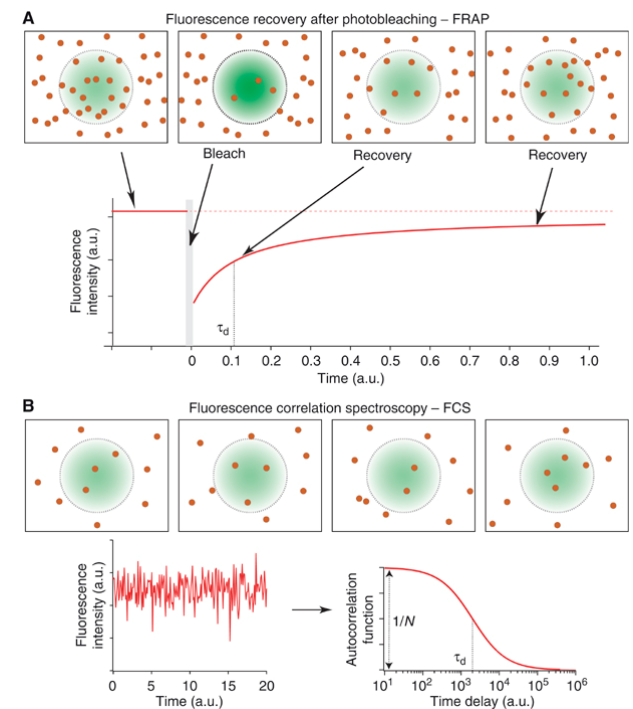
Typically FRAP (fluorescence recovery after photobleaching)



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.



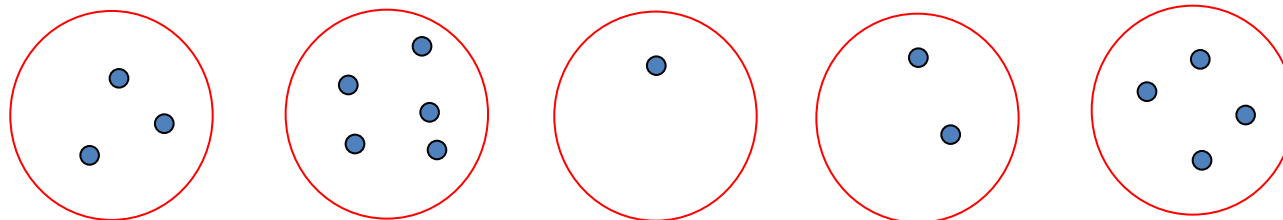
Introduction to “number” fluctuations

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

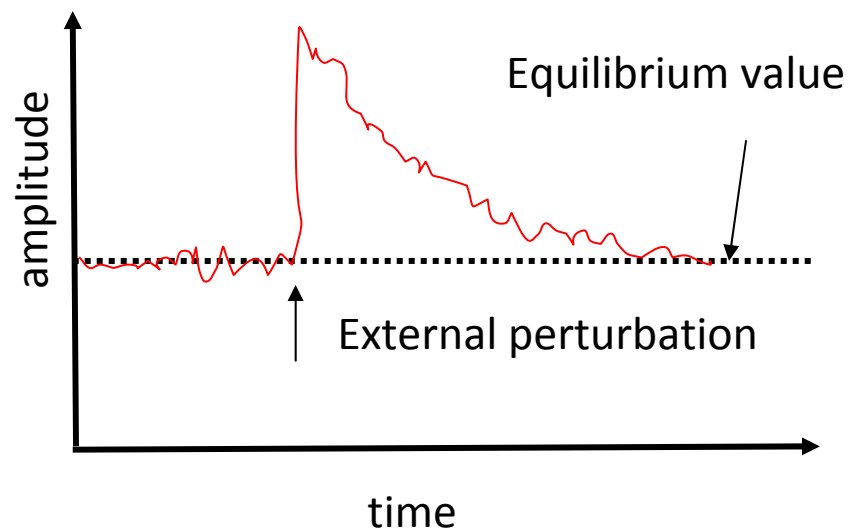
This principle does not tell us anything about the time of the fluctuations



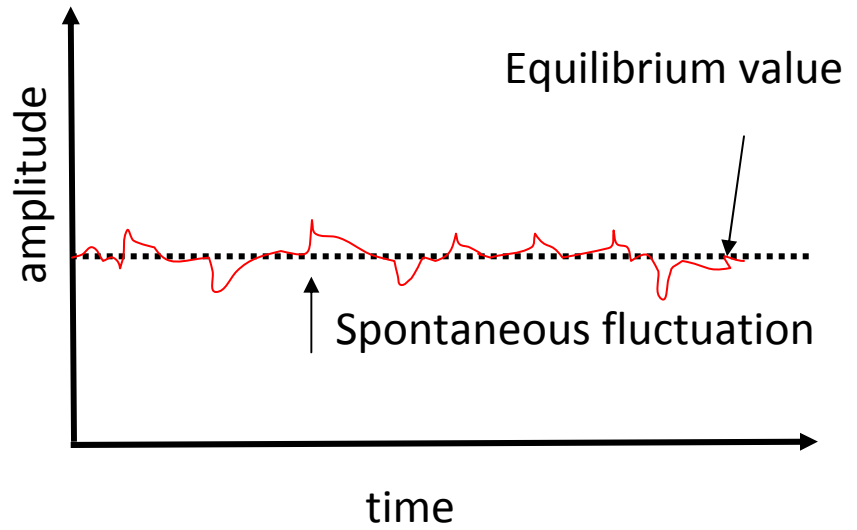
The fluctuation-dissipation principle (systems at equilibrium)

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*

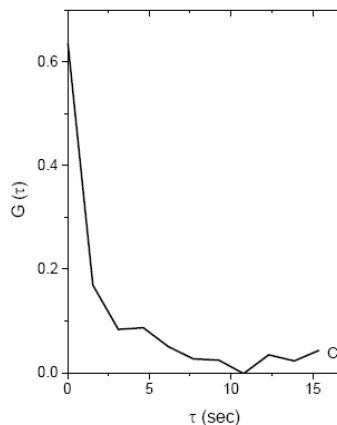
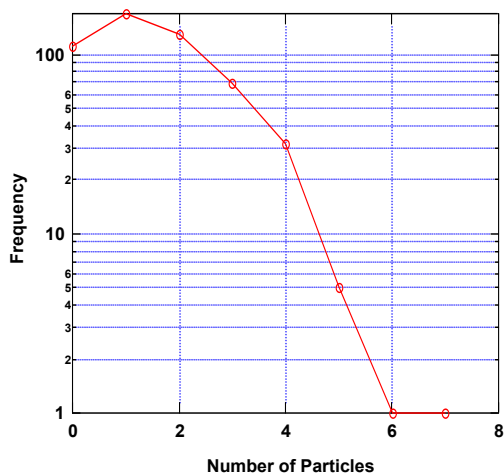
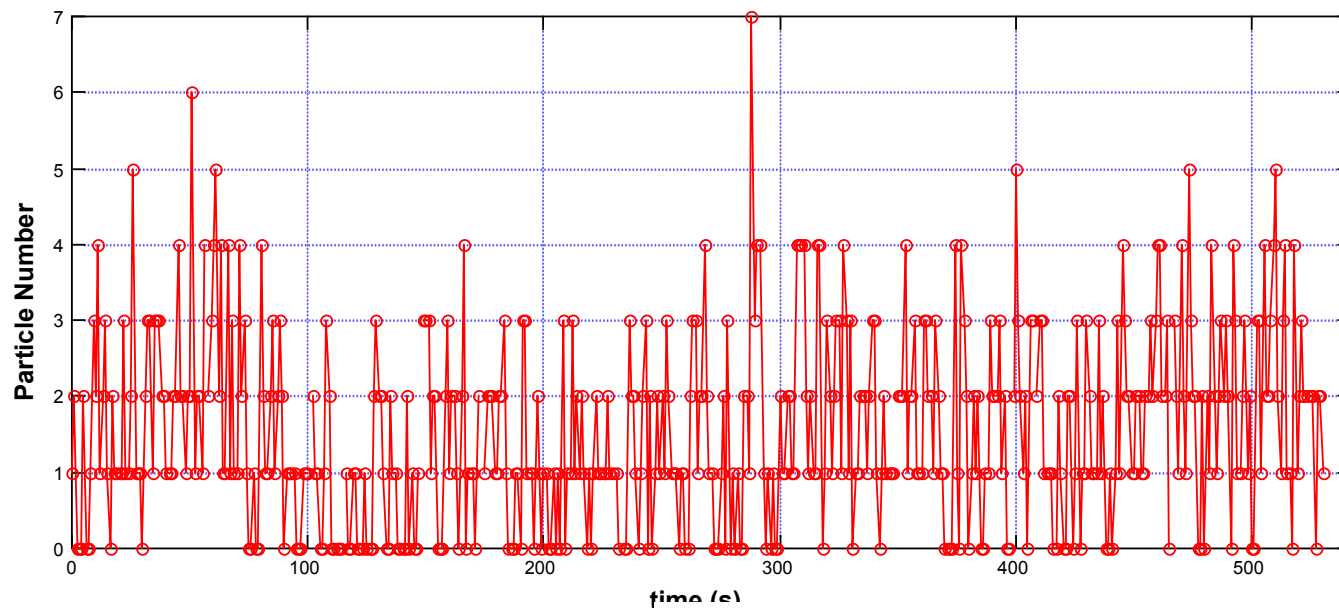
Experimental data on colloidal gold particles:

```
120002001324123102111131125111023313332211122422122612214
2345241141311423100100421123123201111000111_2110013200000
10011000100023221002110000201001_333122000231221024011102_
1222112231000110331110210110010103011312121010121111211_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 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

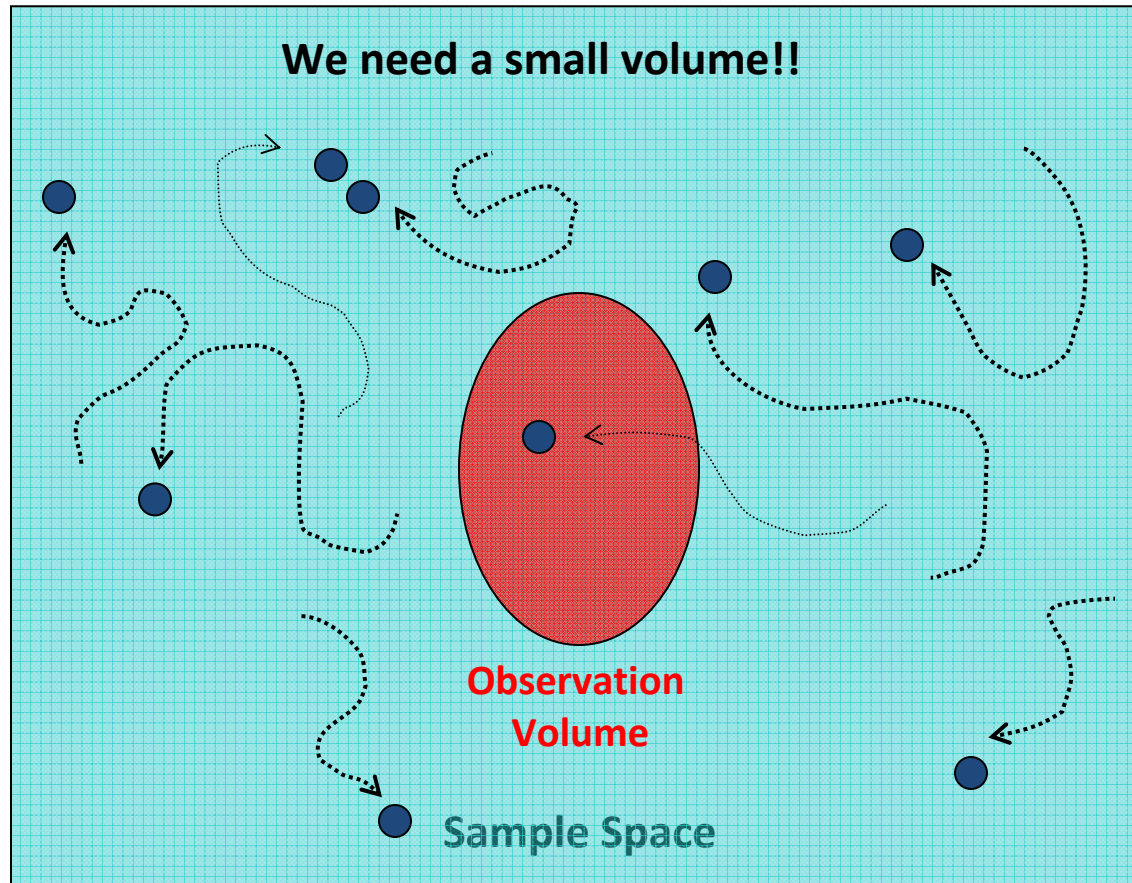
What can cause a fluctuation in the fluorescence signal???

- **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



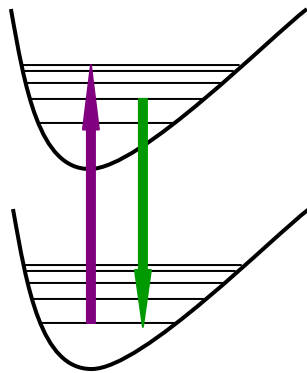
What is Observed?

1. The rate of motion
2. The concentration of particles
3. Changes in the particle fluorescence while under observation, for example conformational transitions

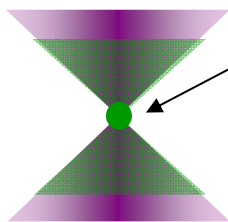
Note that the spectrum of the fluctuations responsible for the motion is “flat” but the spectrum of the fluorescence fluctuations is not

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

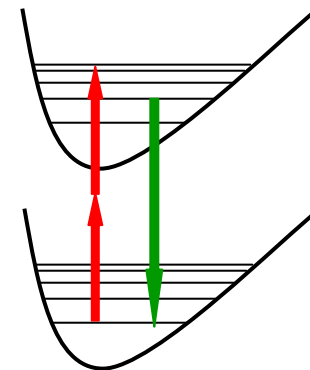
1 - Photon



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

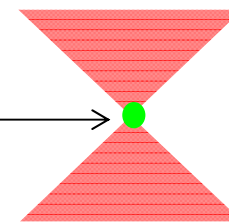


2 - Photon



Approximately $1 \text{ } \mu\text{m}^3$

Defined by the wavelength
and numerical aperture of the
objective



Why confocal detection?

Molecules are small, why to observe a large volume?

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

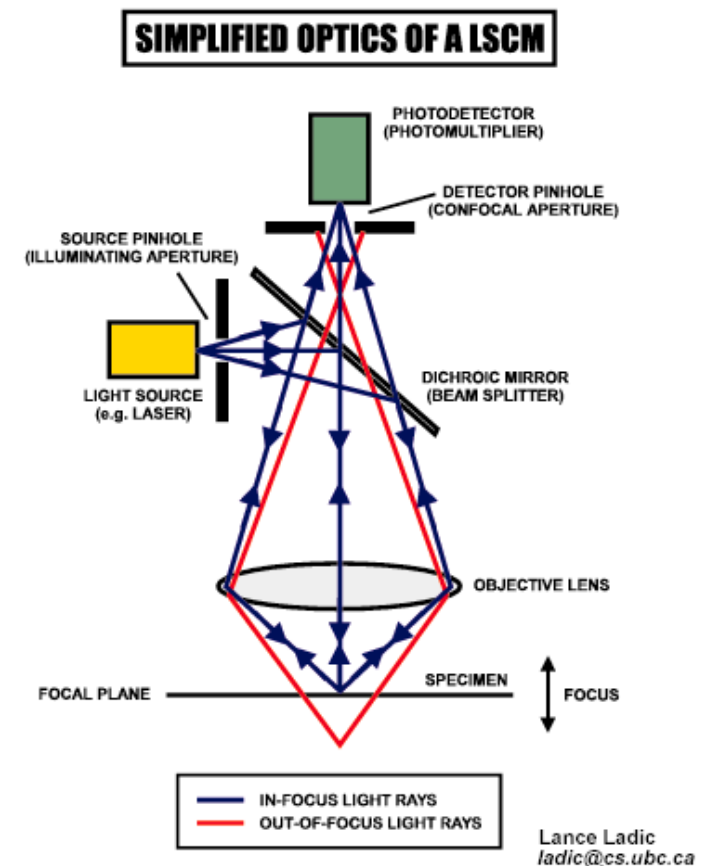
Methods to produce a confocal or small volume

(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)

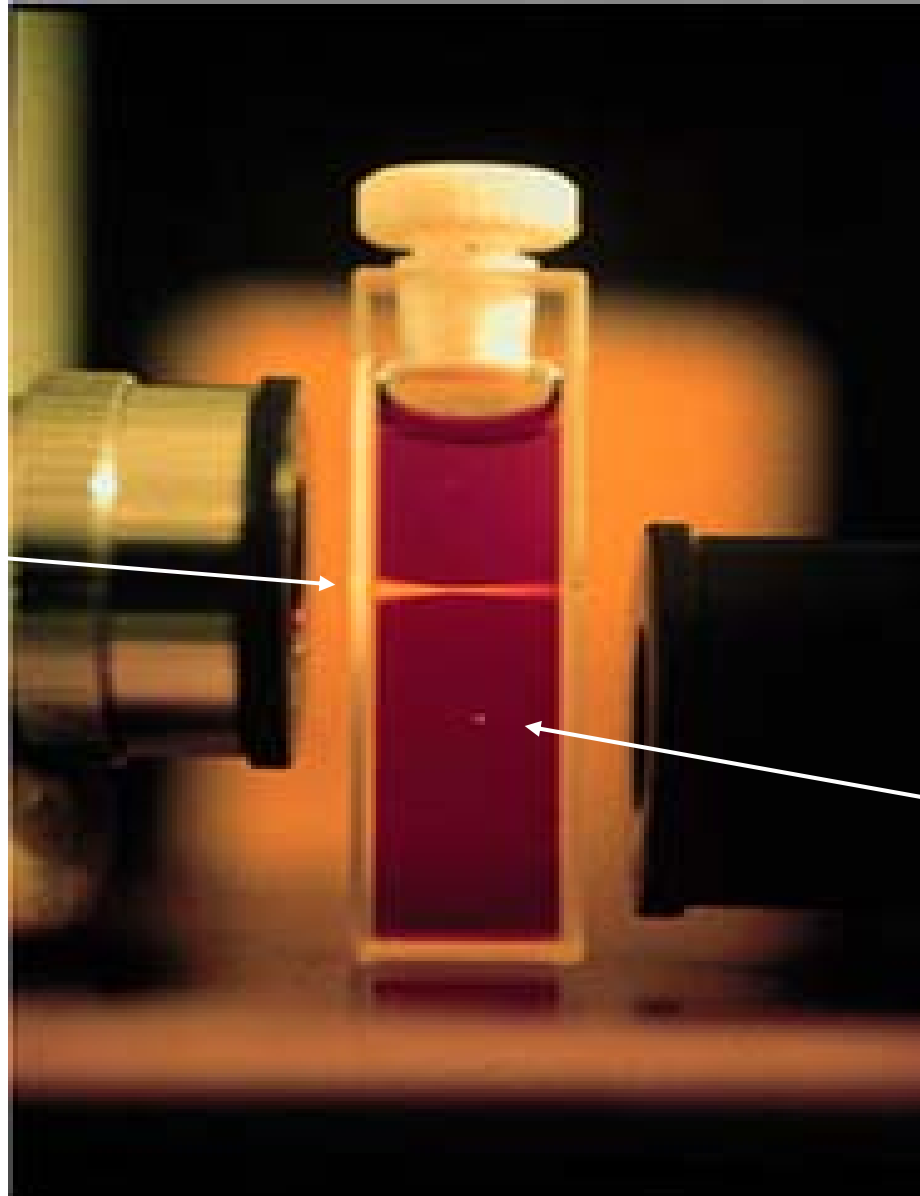
(not limited by light, not applicable to cells)

- Nanofabrication
- Local field enhancement
- Near-field effects



1-photon

Need a pinhole to
define a small volume



2-photon

Brad Amos
MRC, Cambridge, UK

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

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

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

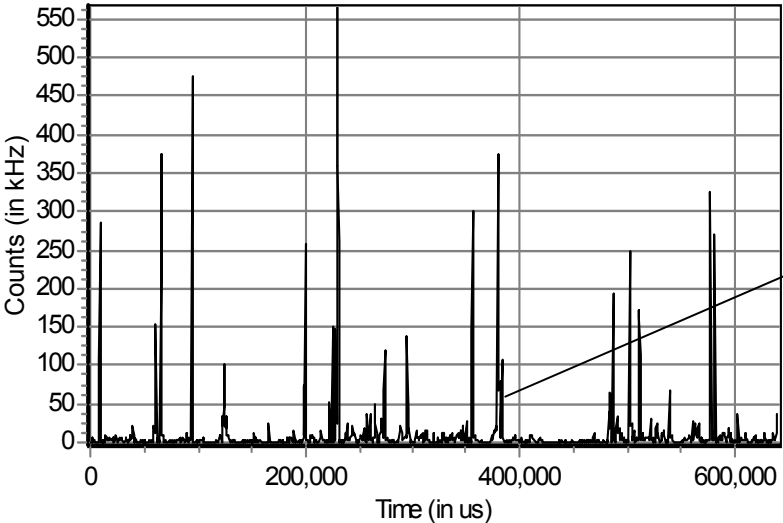
Enough power to saturate absorption in a diffraction limited spot

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

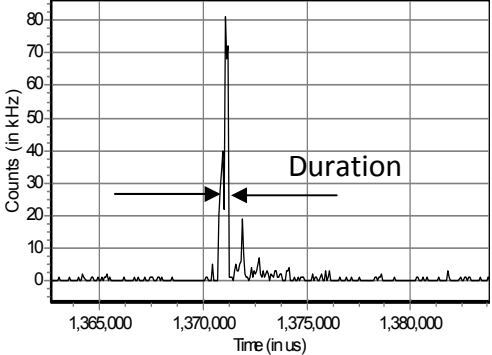
Volume	Device	Size(μm)	Molecules	Time
milliliter	cuvette	10000	6×10^{14}	10^4
microliter	plate well	1000	6×10^{11}	10^2
nanoliter	microfabrication	100	6×10^8	1
picoliter	typical cell	10	6×10^5	10^{-2}
femtoliter	confocal volume	1	6×10^2	10^{-4}
attoliter	nanofabrication	0.1	6×10^{-1}	10^{-6}

Determination of diffusion by fluctuation spectroscopy

Data presentation and Analysis



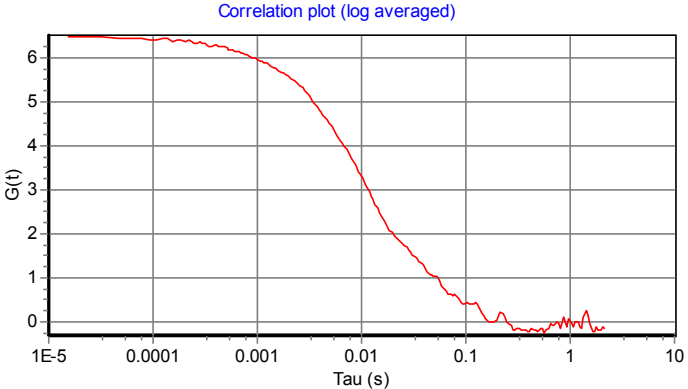
The time series



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

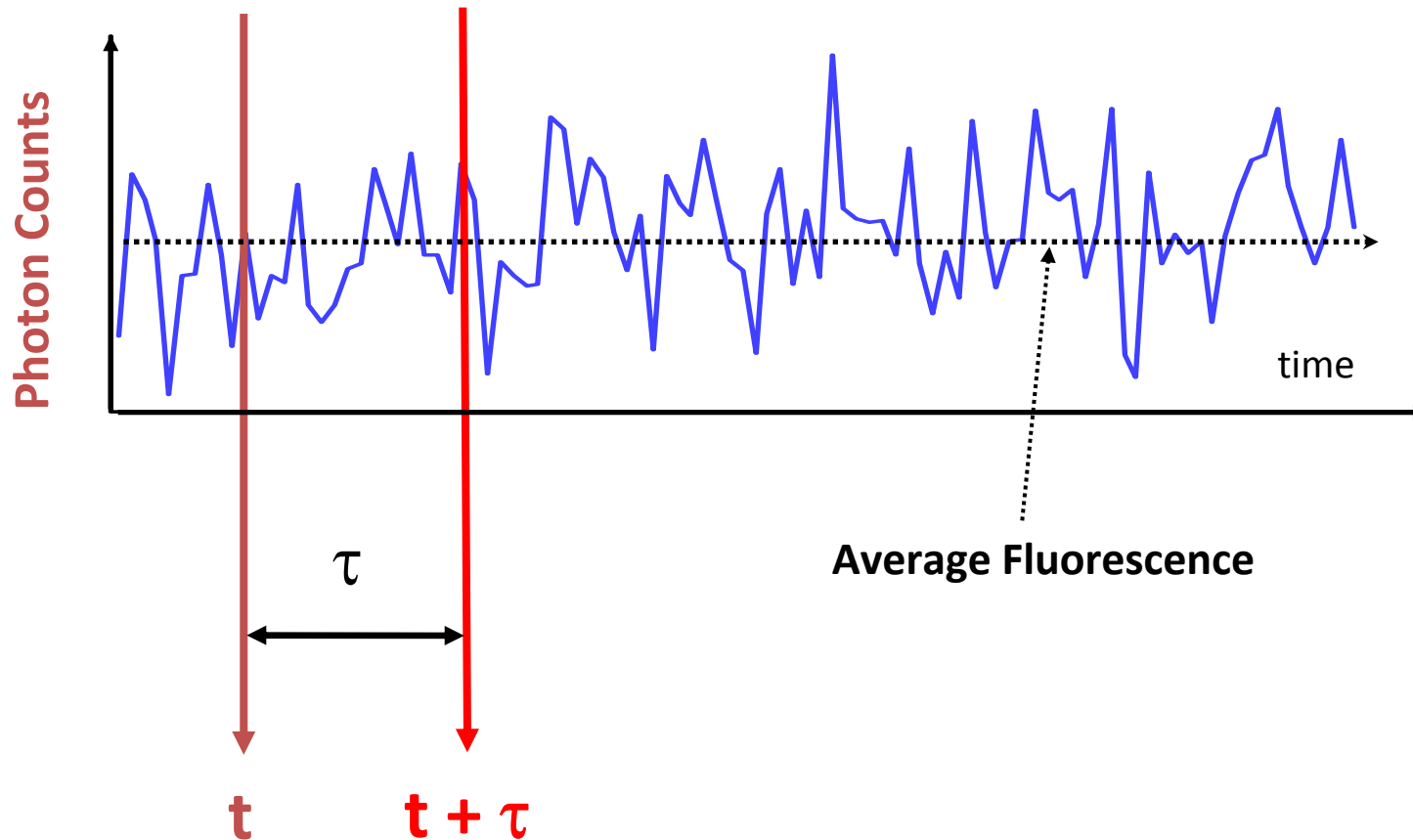
To extract the distribution of the duration of the fluctuations we use a math based on calculation of the **correlation function**

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

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

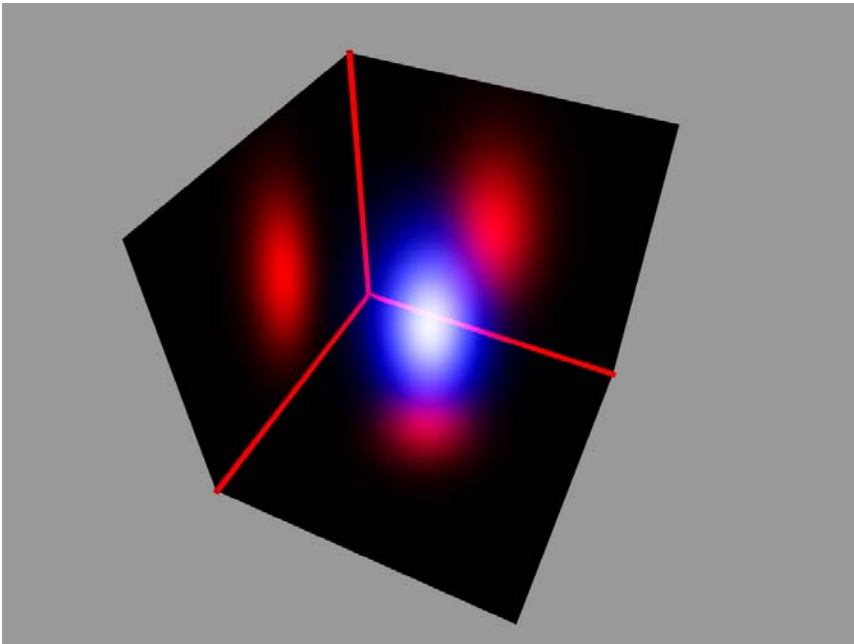
κQ = quantum yield and detector sensitivity (how bright is our probe). This term could contain the fluctuation of the fluorescence intensity due to internal processes

$W(\mathbf{r})$ describes the profile of illumination

$C(\mathbf{r}, t)$ 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?



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

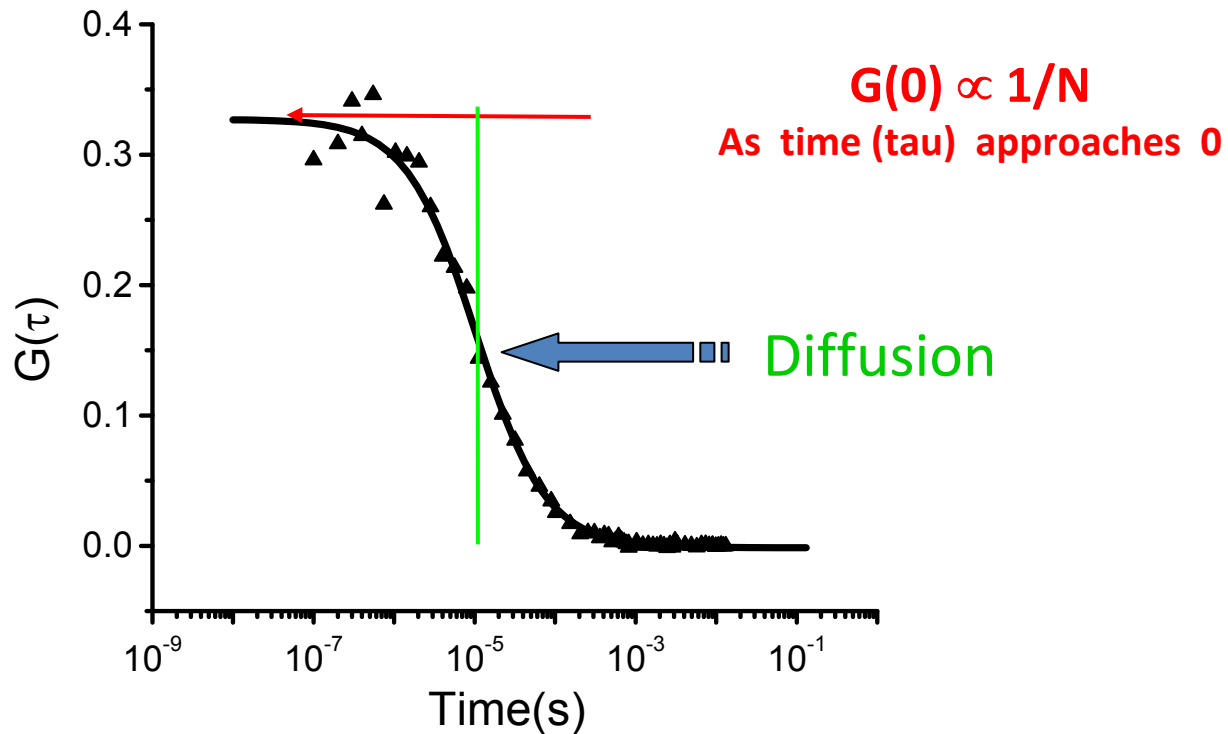
$$I(z) = \text{Exp}\left[-\frac{2z^2}{w_{0z}^2}\right] \quad \text{Gaussian } z$$

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

More on the PSF in Jay's lecture

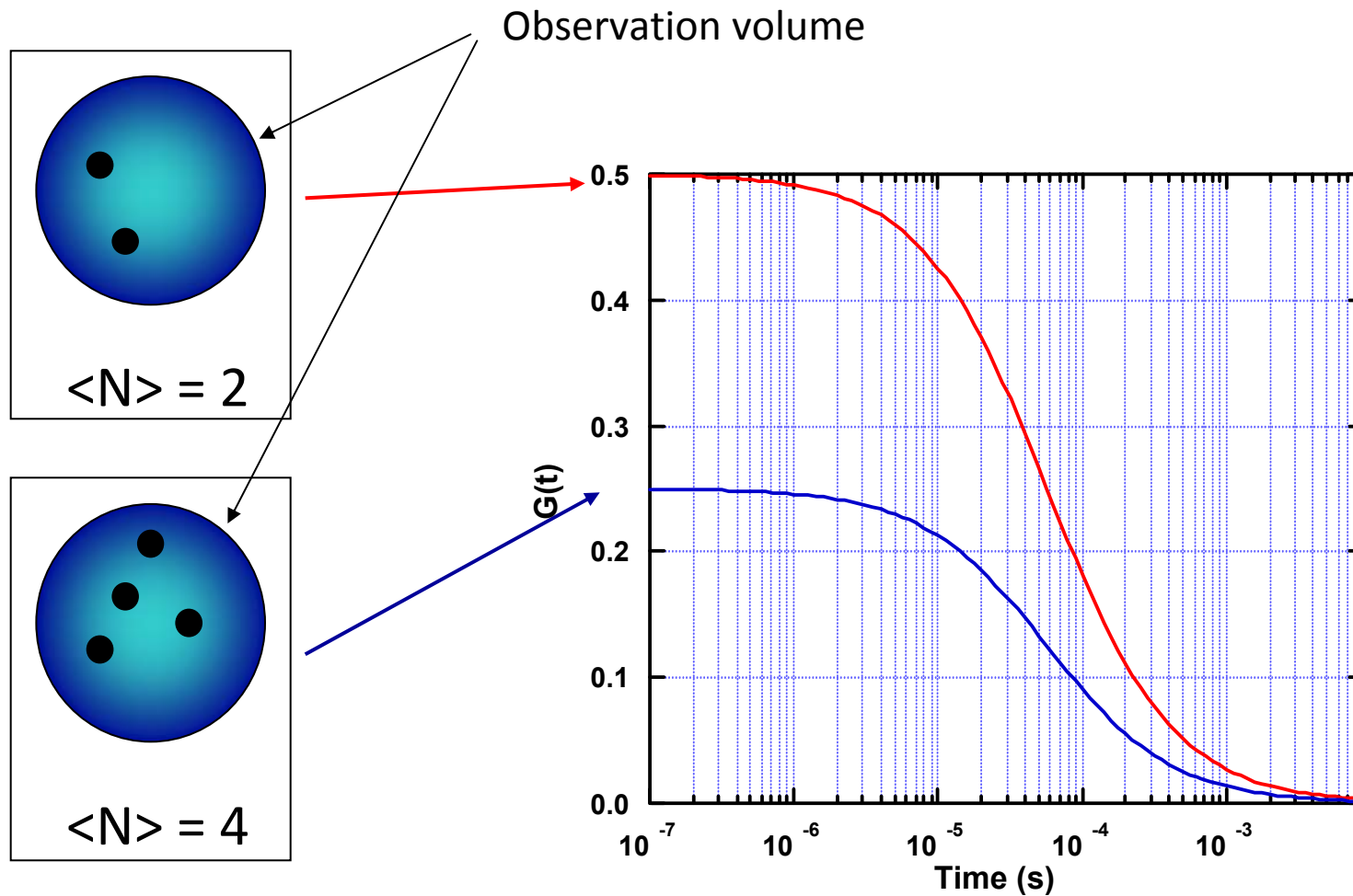
For the 2-photon case, these expression must be squared

The Autocorrelation Function



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

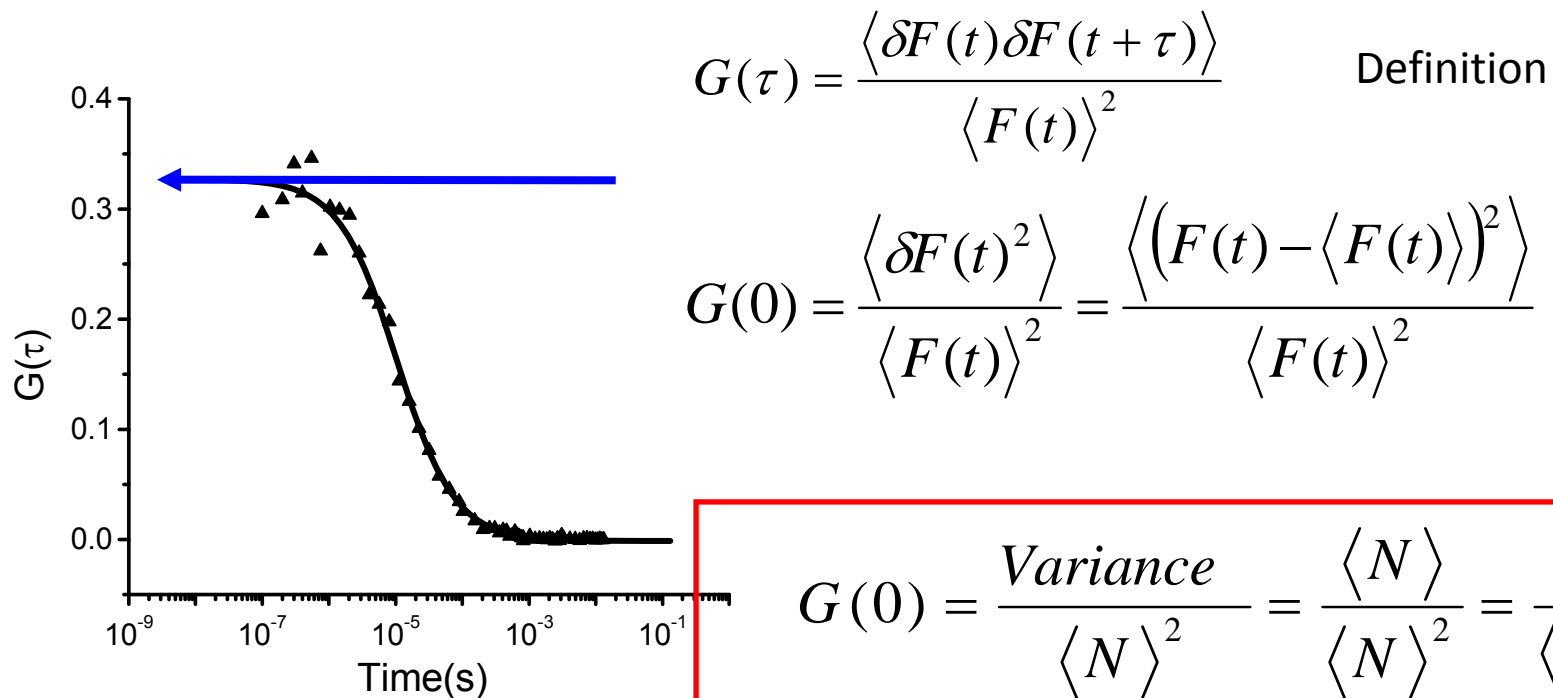
The Effects of Particle Concentration on the Autocorrelation Curve



Why Is $G(0)$ Proportional to $1/\text{Particle Number}$?

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

$$\langle N \rangle = \langle \text{Particle_Number} \rangle = \text{Variance}$$

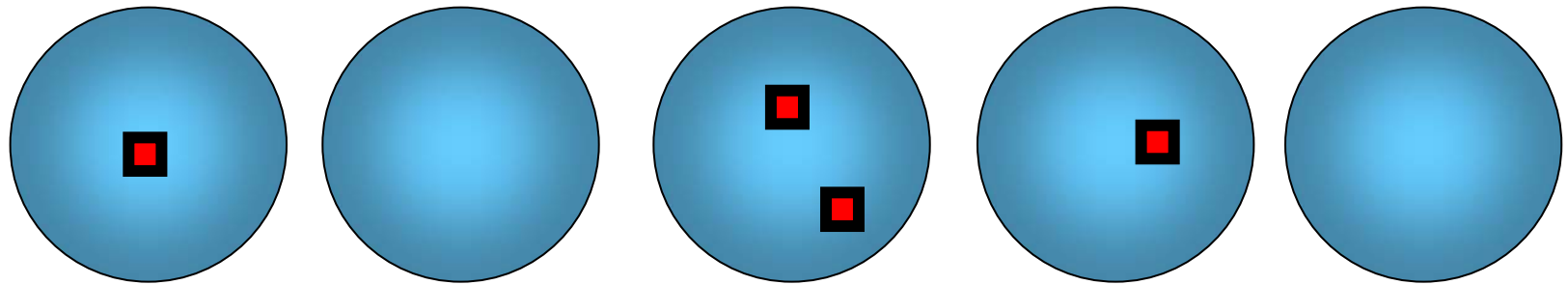


$$G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \quad \text{Definition}$$

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

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

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 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

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

Lets increase the particle brightness by 4x:

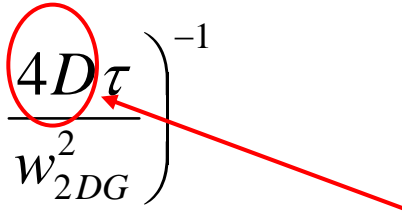
4 0 0 0 0 0 0 0 0 8 0 4 4 4 0 0 0 0 0 0 4 0 0 0 0 0 0 0 4 0 4 0 0 0 4 0 0 4 0 0

Average = 1.1 Variance = 4.09

$$\langle N \rangle \propto 0.296$$

Effect of Shape on the (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 $S = w/z$

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

Blinking and binding processes

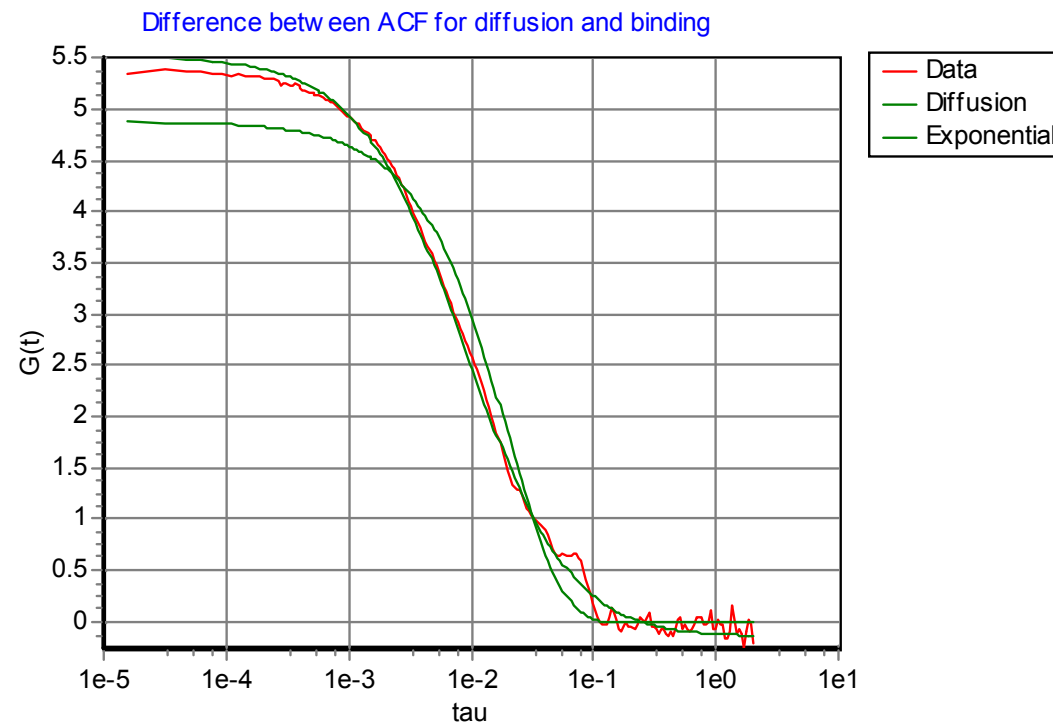
Until now, we assumed that the particle is **not moving**. If we assume that the blinking of the particle is **independent on its movement**, we can use a general principle that states that the correlation function splits in the product of the two independent processes.

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

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

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

How different is $G(\text{binding})$ from $G(\text{diffusion})$?



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 $\mu\text{m}^2/\text{s}$

90 $\mu\text{m}^2/\text{s}$

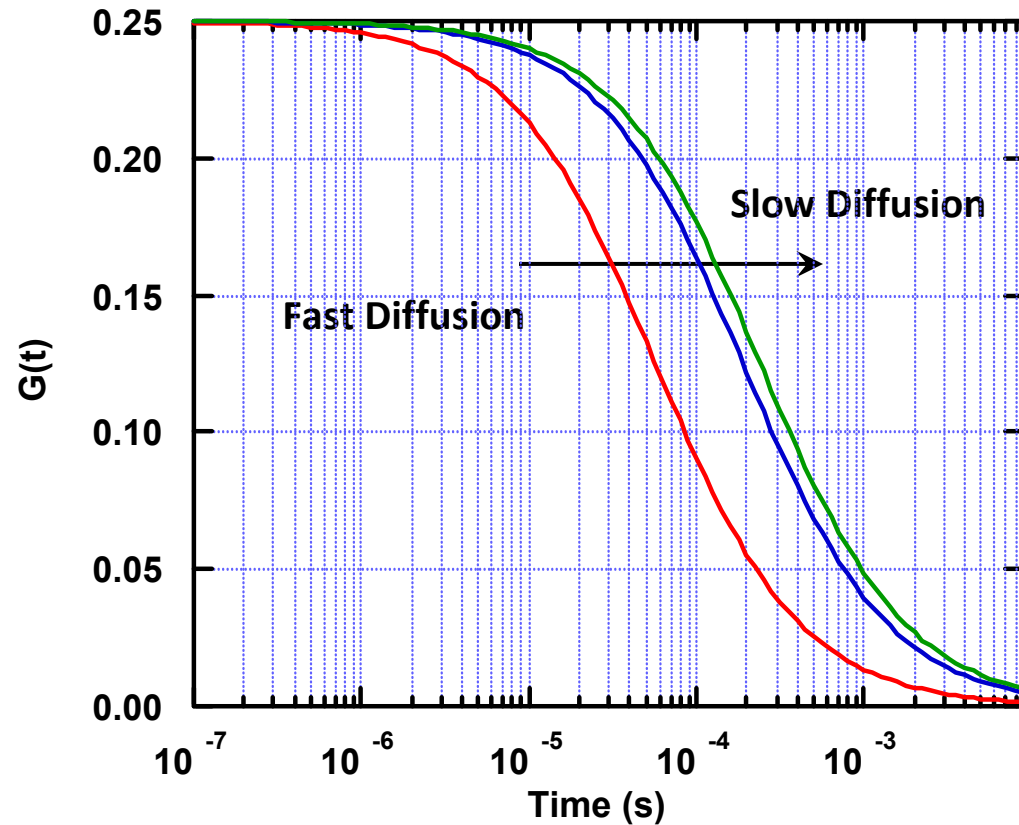
71 $\mu\text{m}^2/\text{s}$

Stokes-Einstein Equation:

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

and

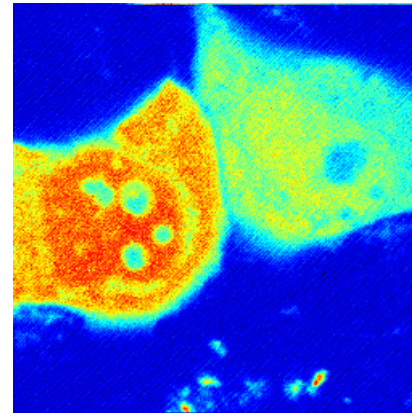
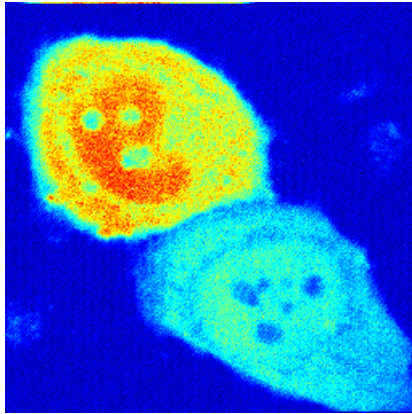
$$MW \propto \text{Volume} \propto r^3$$



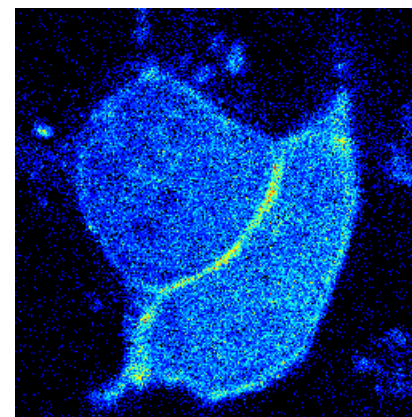
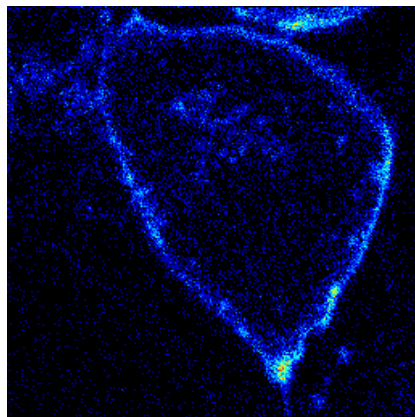
Monomer --> Dimer

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

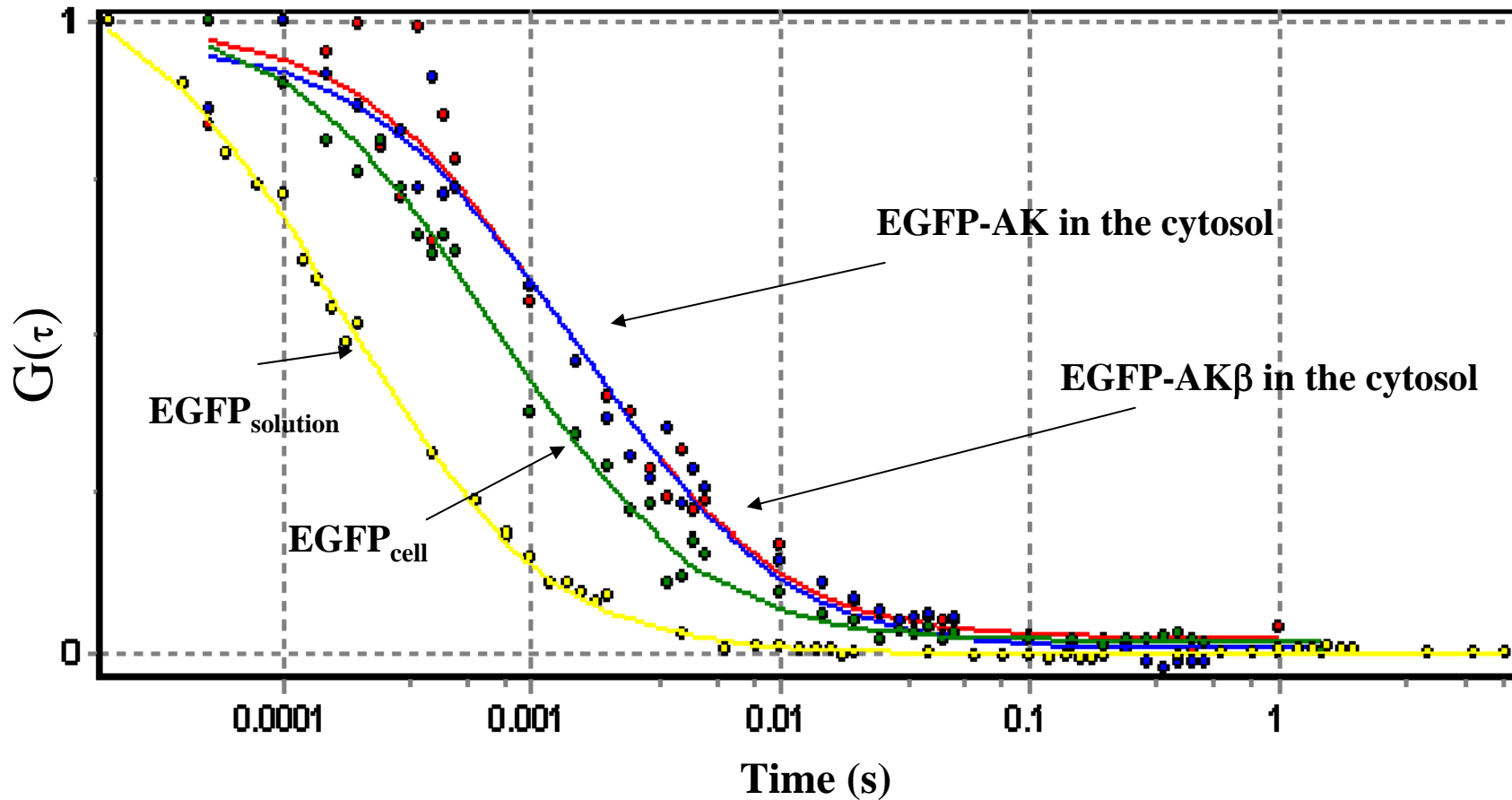


Fluorescence Intensity

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

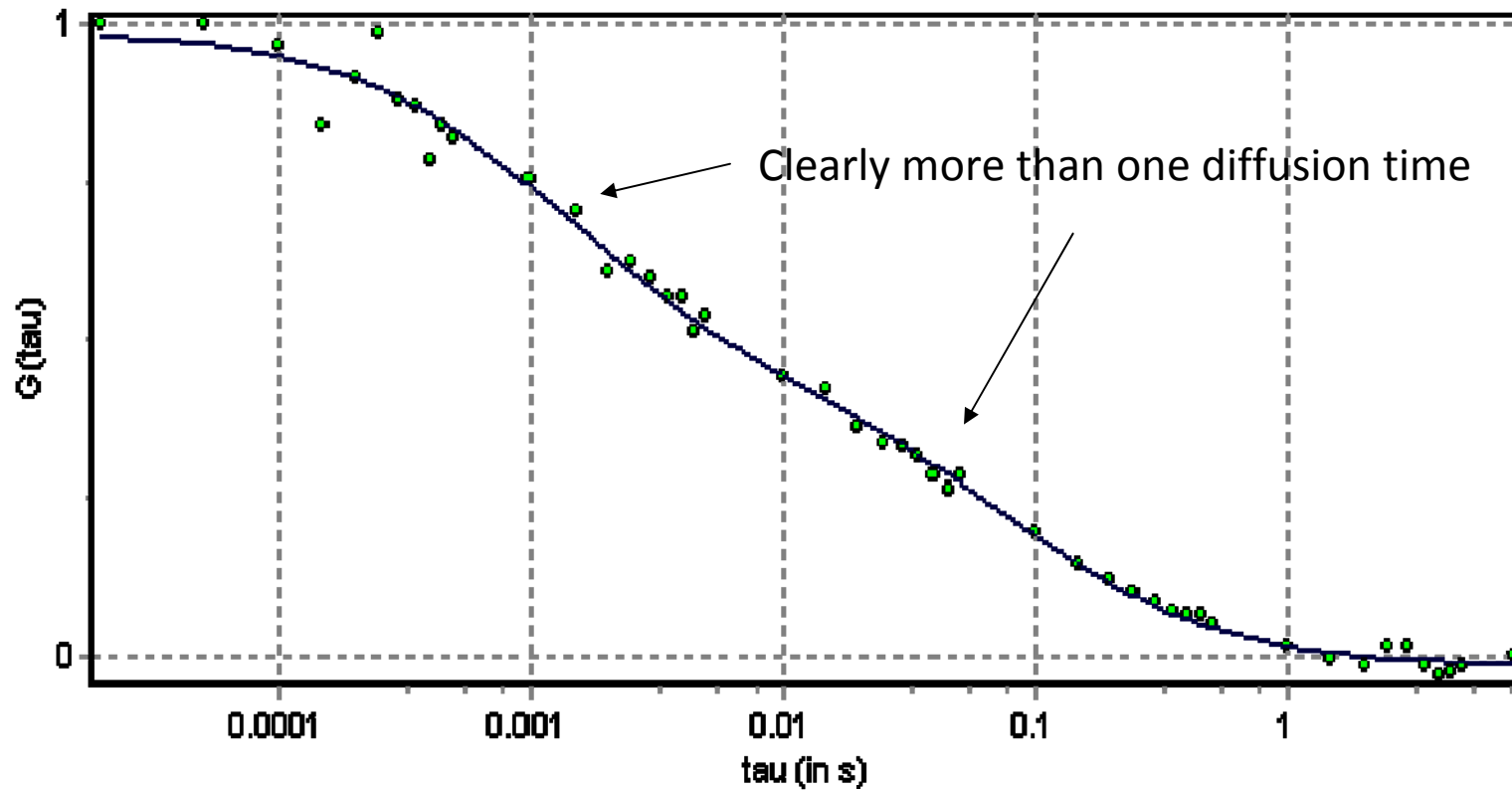
Qiao Qiao Ruan, Y. Chen, M. Glaser & W. Mantulin Dept. Biochem & Dept Physics- LFD Univ Il, USA

Autocorrelation of EGFP & Adenylate Kinase -EGFP



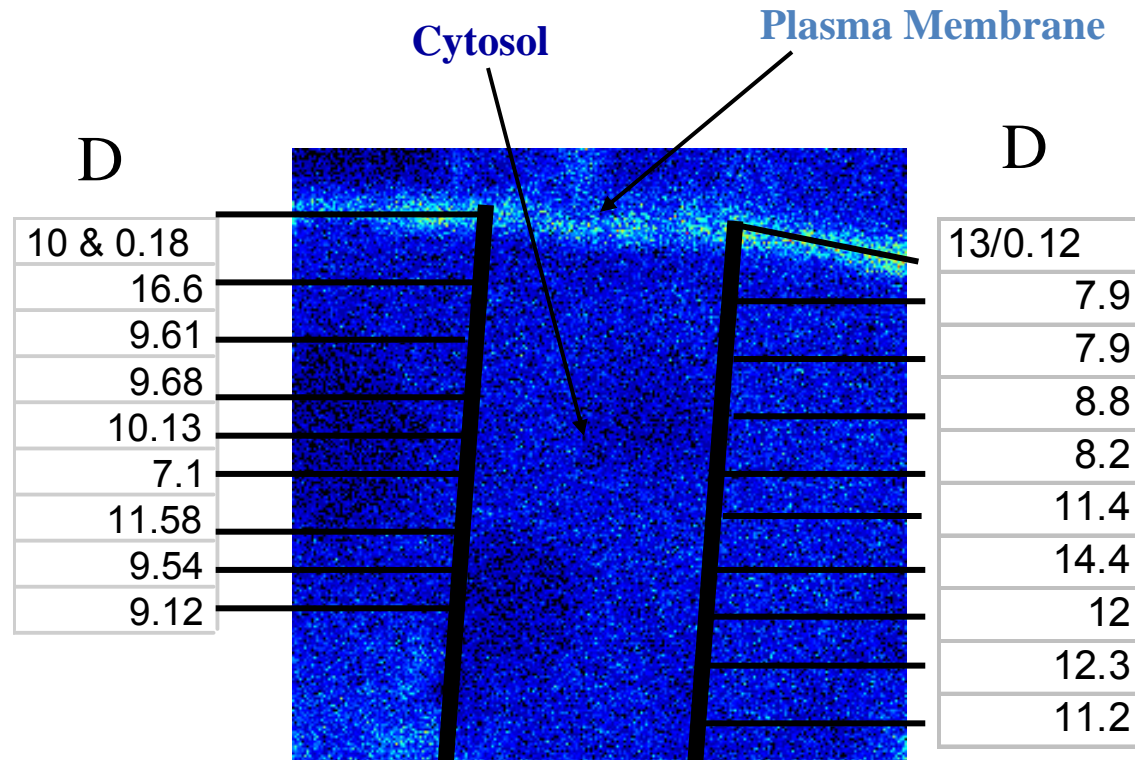
Normalized autocorrelation curve of EGFP in solution (\bullet), EGFP in the cell (\bullet), AK1-EGFP in the cell(\bullet), AK1 β -EGFP in the cytoplasm of the cell(\bullet).

Autocorrelation of Adenylate Kinase –EGFP on the Membrane



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

Autocorrelation Adenylate Kinase β -EGFP

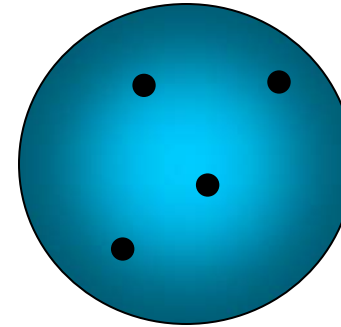


Diffusion constants ($\mu\text{m}^2/\text{s}$) of AK EGFP-AK β in the cytosol -EGFP in the cell (HeLa). At the membrane, a dual diffusion rate is calculated from FCS data. Away from the plasma membrane, single diffusion constants are found.

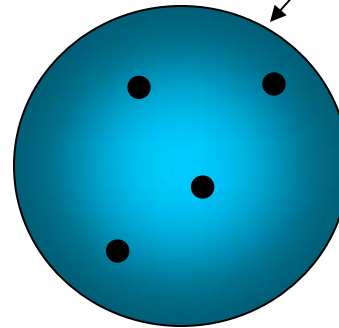
Two Channel Detection: Cross-correlation

— lfd

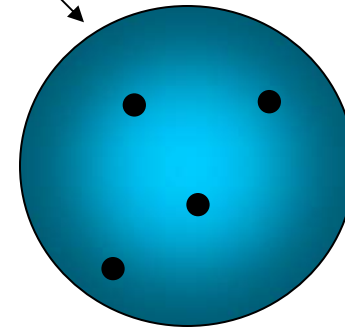
Sample Excitation
Volume



Beam Splitter



Detector 1



Detector 2

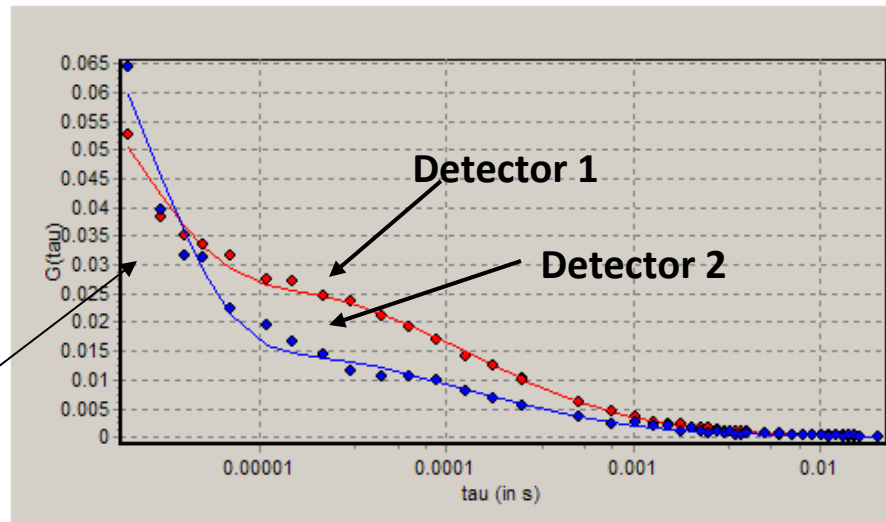
1. Increases signal to noise by isolating correlated signals.
2. Corrects for PMT noise

Each detector observes
the same particles

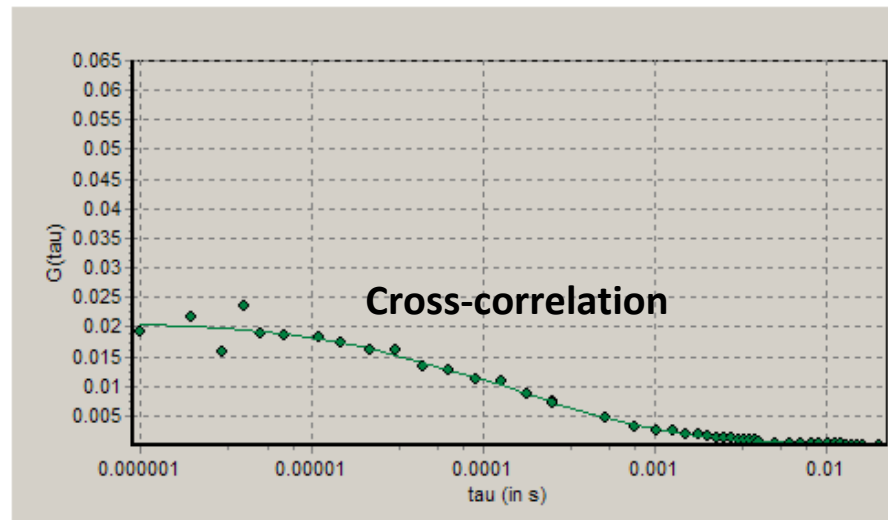
Removal of Detector Noise by Cross-correlation

— lfd

11.5 nM Fluorescein

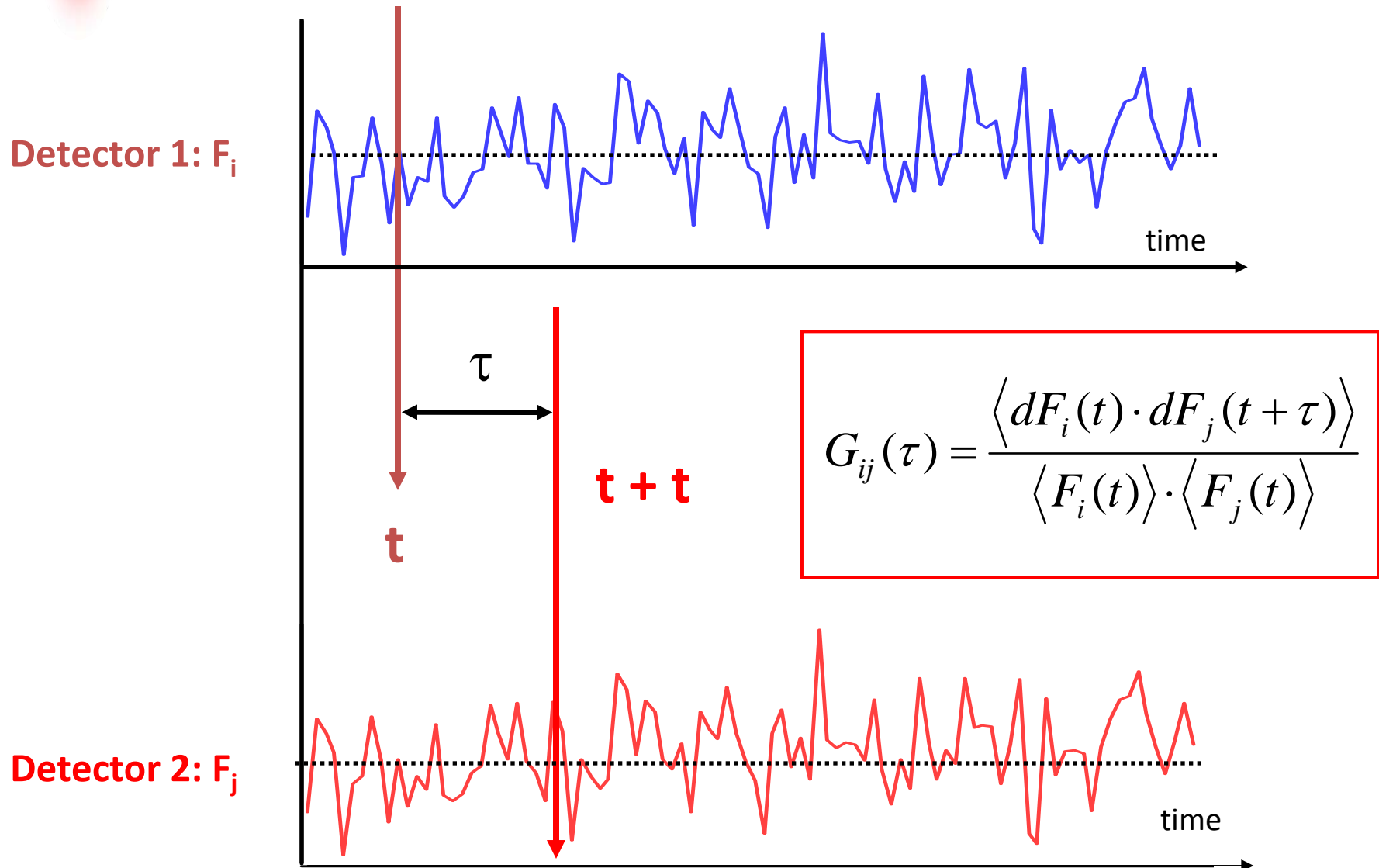


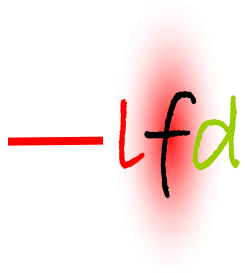
Detector after-pulsing



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Calculating the Cross-correlation Function





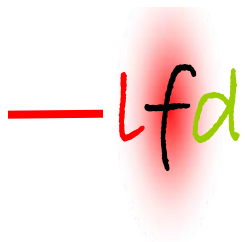
Cross-correlation calculations

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

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

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

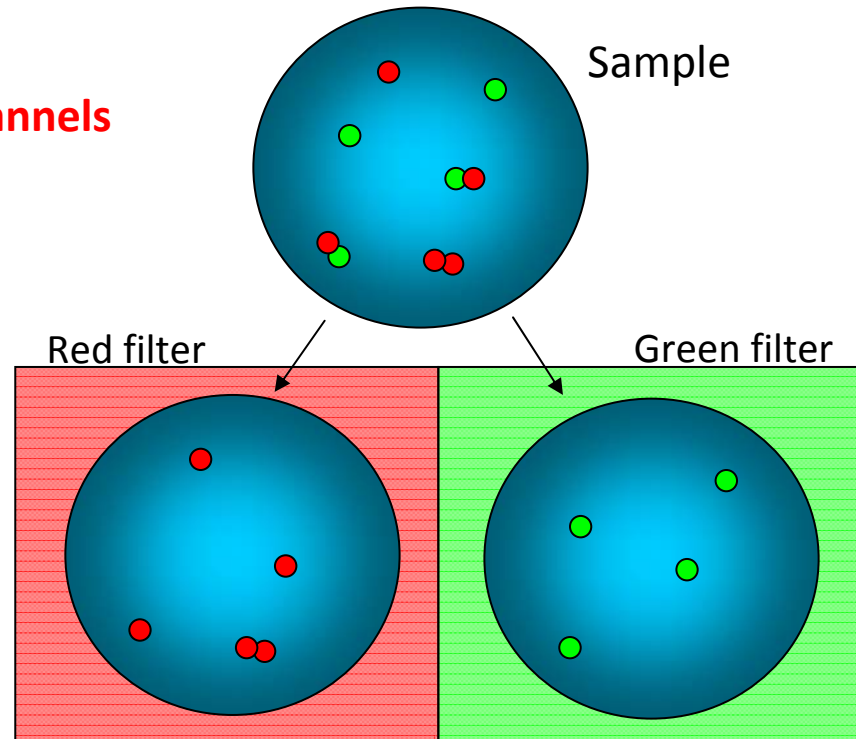
G_{12} is commonly used to denote the cross-correlation and G_1 and G_2 for the autocorrelation of the individual detectors. Sometimes you will see $G_x(0)$ or $C(0)$ used for the cross-correlation.



Two-Channel Cross-correlation

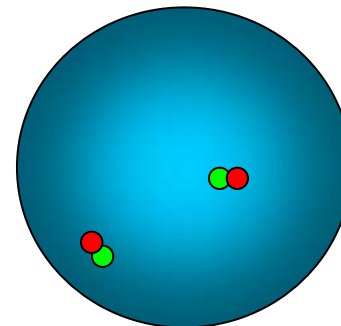
**The cross-correlation
ONLY if particles are observed in both channels**

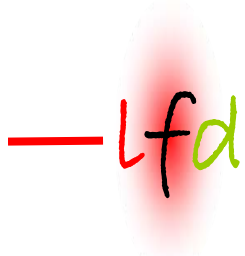
Each detector observes
particles with a particular color



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

— lfd

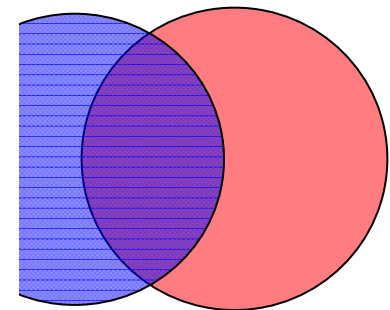
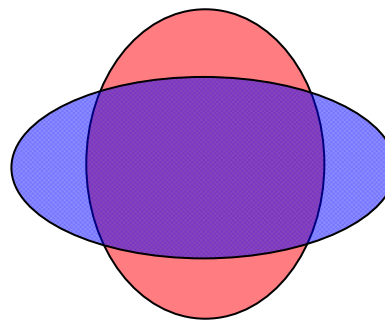
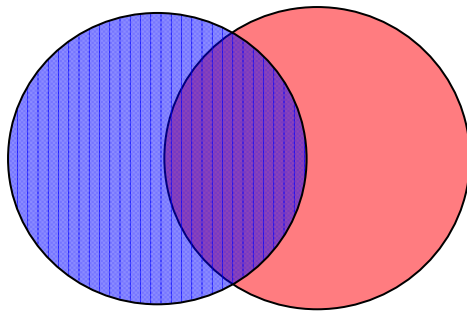
*assume exact match of the observation volumes in our calculations
which is difficult to obtain experimentally.*

Excitation side:

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

Emission side:

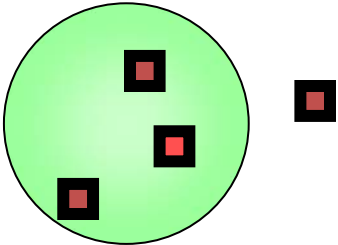
- (1) Chromatic aberrations
- (2) Spherical aberrations
- (3) Improper alignment of detectors or pinhole
(cropping of the beam and focal point position)



— *lfd*

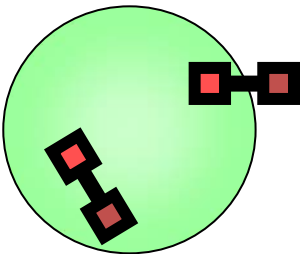
Two-Color FCS

Uncorrelated



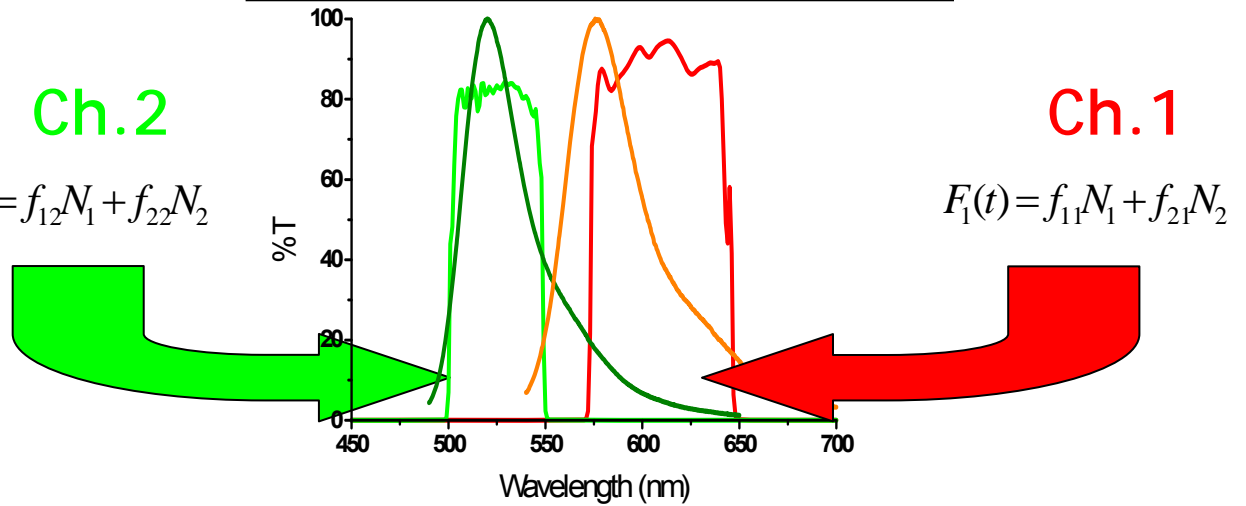
$$G_{ij}(\tau) = \frac{\langle F_i(t)F_j(t+\tau) \rangle}{\langle F_i(t) \rangle \langle F_j(t) \rangle} - 1$$

Correlated

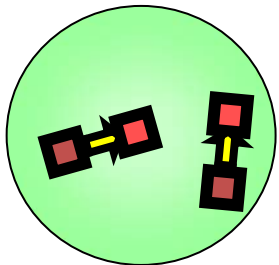


$$F_2(t) = f_{12}N_1 + f_{22}N_2$$

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



Interconverting



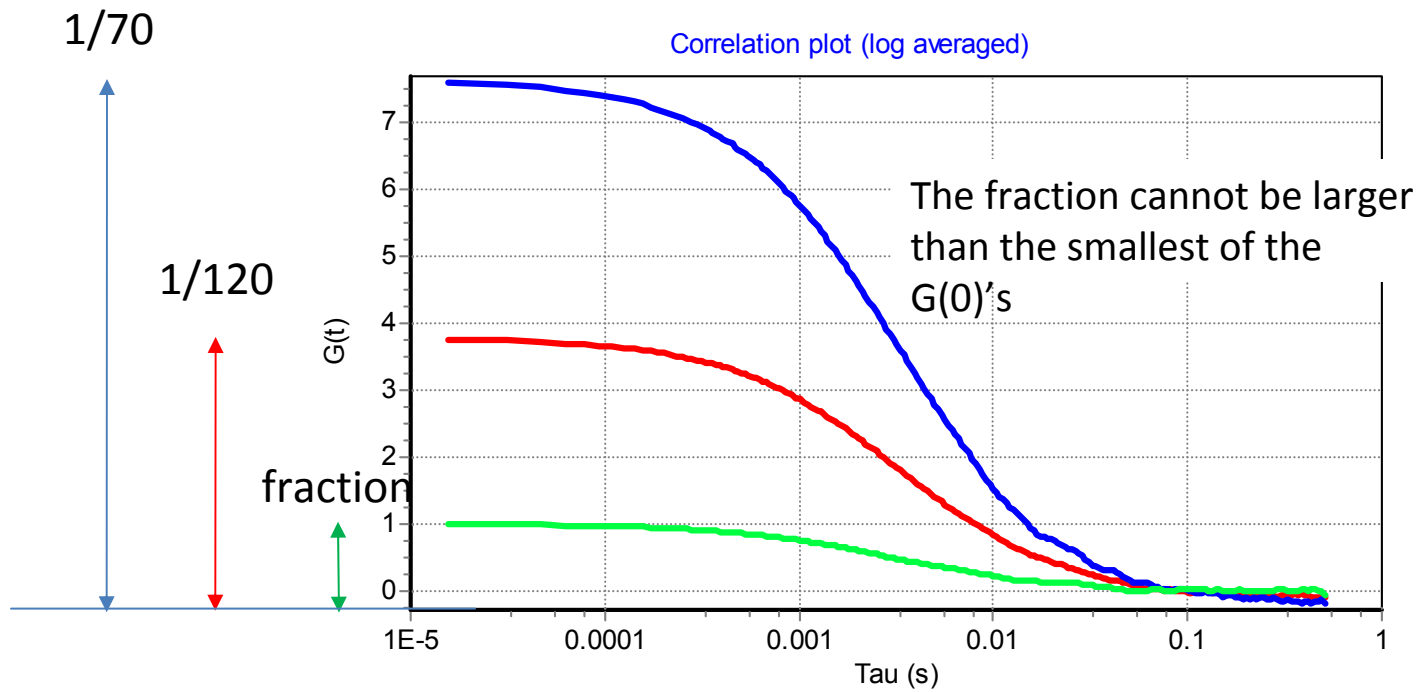
For two uncorrelated species, the amplitude of the cross-correlation is proportional to:

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

Simulation of cross-correlation

Same diffusion = $10 \mu\text{m}^2/\text{s}$

	Channel 1	Channel 2		
Molecules	100			
Molecules		50		
Molecules	20	20		



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