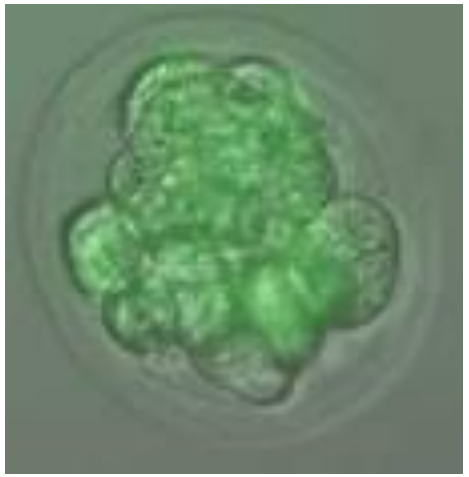
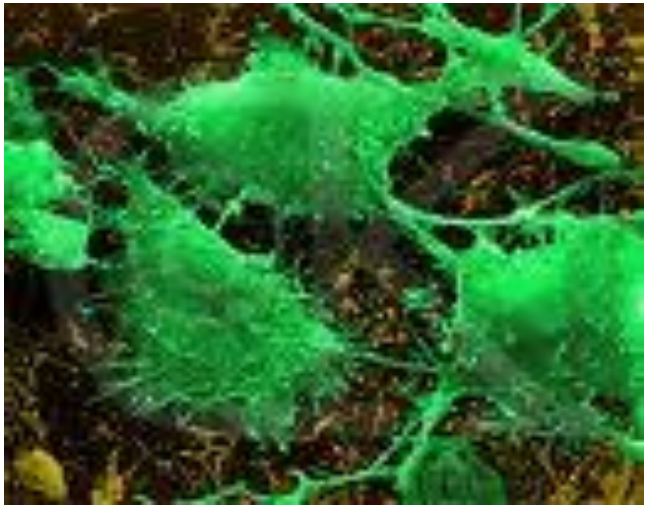
Quantitative Fluorescence Microscopy for the Study of Protein Interactions, Signaling, and Molecular Microenvironment

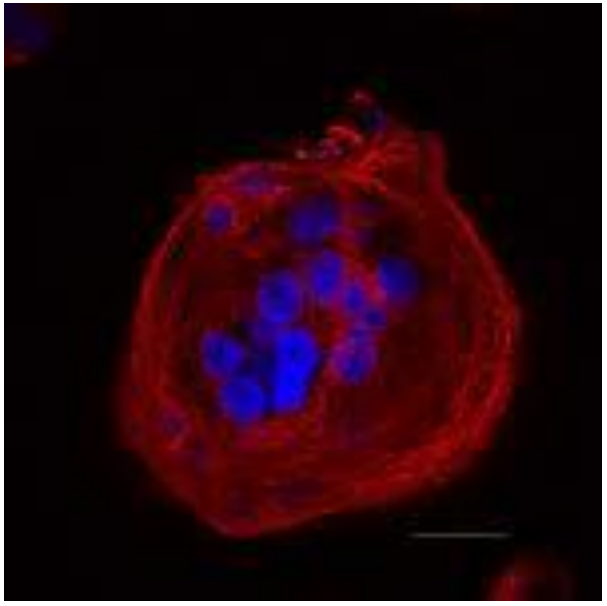
Advanced Fluorescence Microscopy Workshop, Urbana-Champaign Aug. 17-20 2015

Beniamino Barbieri

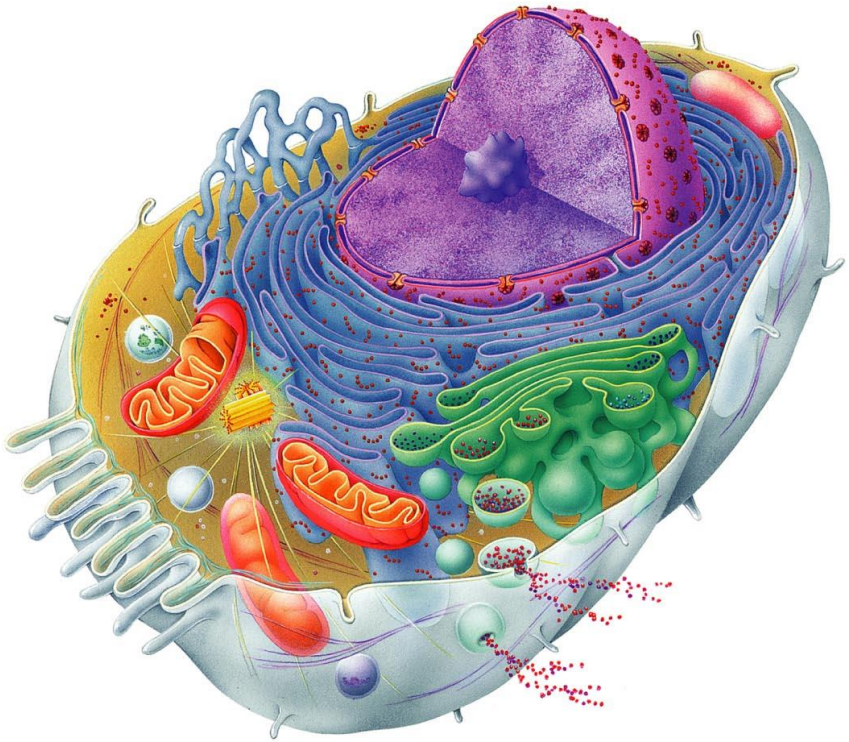
Cell Images





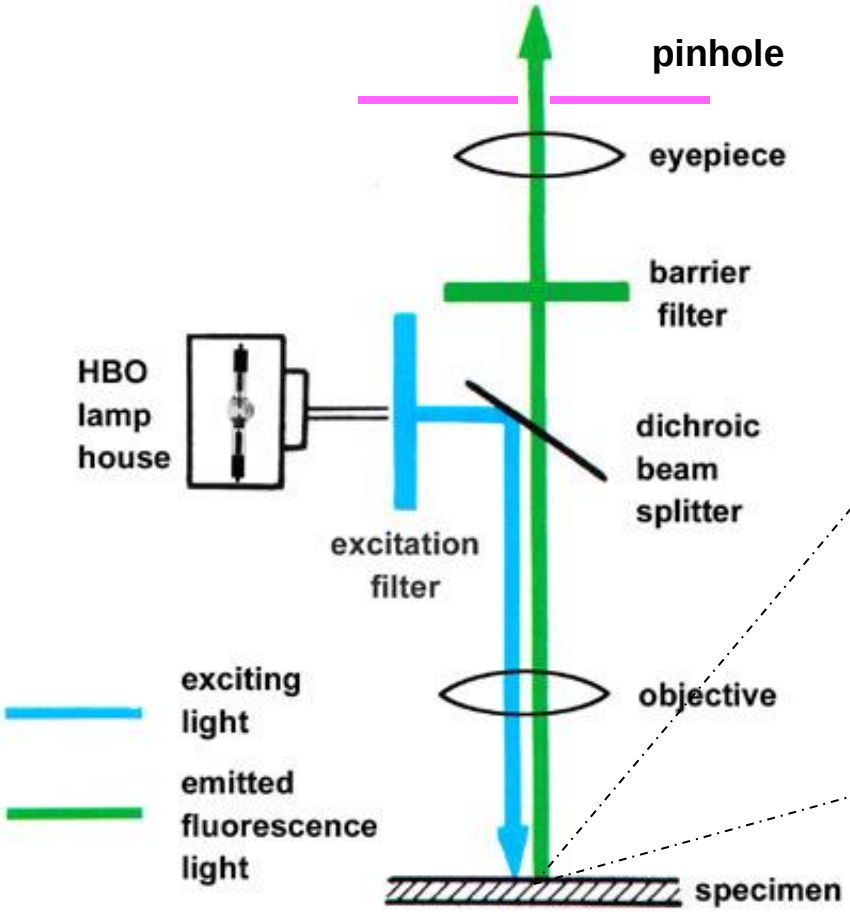


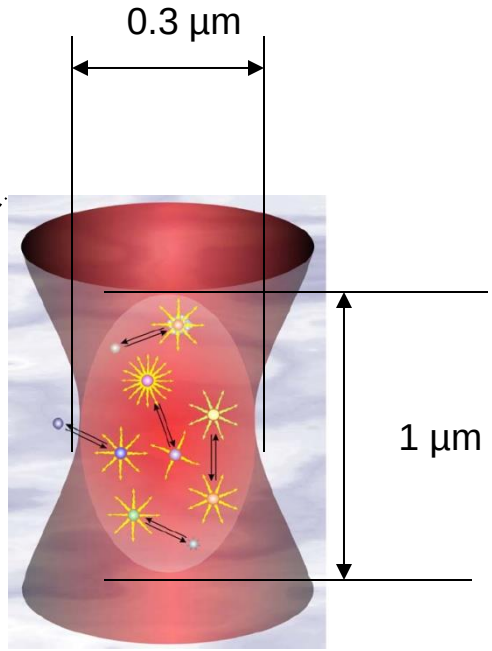
Cell Structure



- In a cell we may want to locate the areas where we have clusters of molecules versus single molecules.
- In cells, both the concentration and the clustering of proteins can differ in various locations and change during biological processes.

Fluorescence microscope





Volume: 10^{-15} L
Size (µm): 0.3 x 1
No. molecules: ≈ 1

What is Fluorescence?

FLUORESCENCE is the light emitted by an atom or molecule after a finite duration subsequent to the absorption of photons.

Specifically, the emitted light arises from the transition of the excited species from its first excited electronic singlet level to its ground electronic level.

The development of highly sophisticated fluorescent probe chemistries, new lasers and microscopy approaches and site-directed mutagenesis has led to many novel applications of fluorescence in the chemical, physical and life sciences. Fluorescence methodologies are now widely used in the biochemical and biophysical areas, in clinical chemistry and diagnostics and in cell biology and molecular biology.

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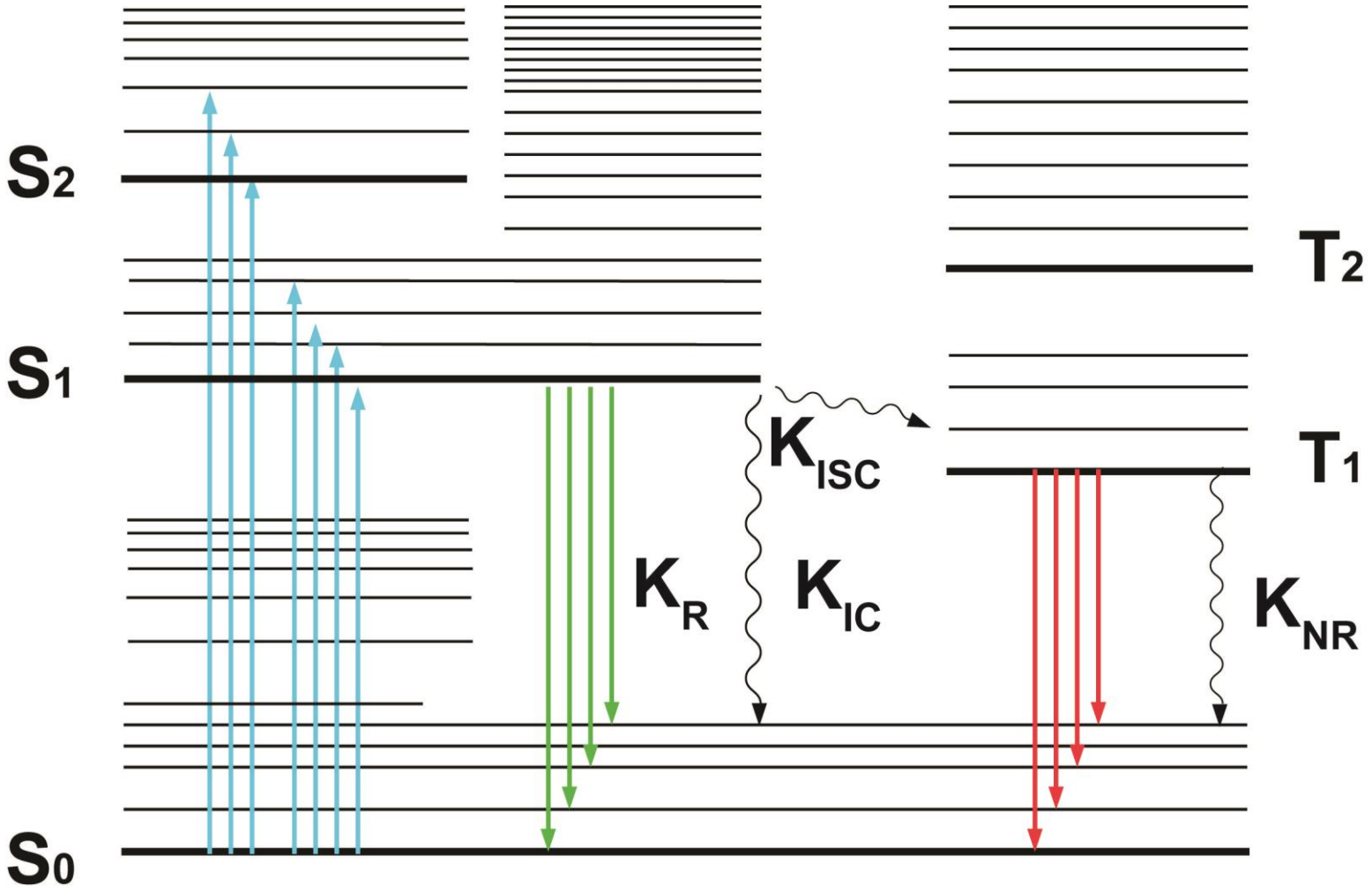
Perrin-Jabłoński diagram



Fluorescence Tools

- Fluorescence Fluctuation Spectroscopy (FFS)
- Fluorescence Lifetime Imaging (FLIM)
- Particle Tracking and nanoimaging

What is Fluorescence Fluctuations Spectroscopy (FFS)

Single point

- FCS (Magde, Elson and Webb, 1972)
- PCH (Chen et al., 1999)
- FIDA (Kask et al., 1999)
- FCA (Mueller, 2004)

Imaging

- ICS (Peterson et al. 1993)
- RICS (Digman et al. 2005)
- N & B (Digman et al., 2008)

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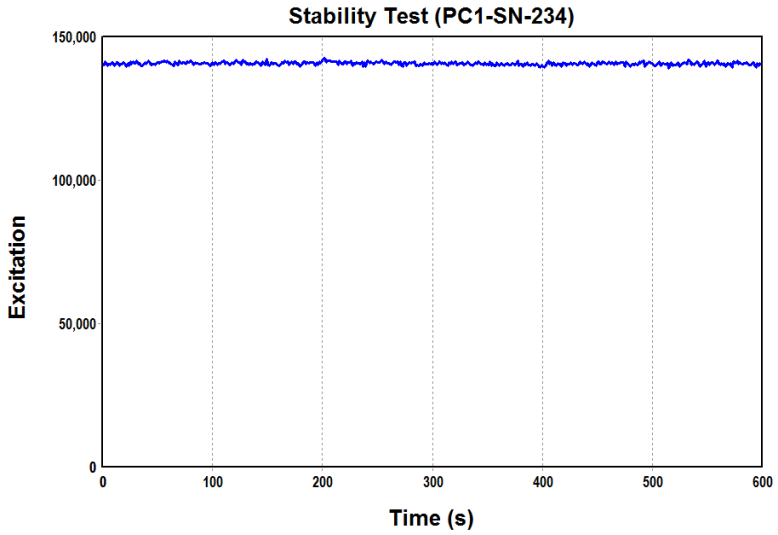
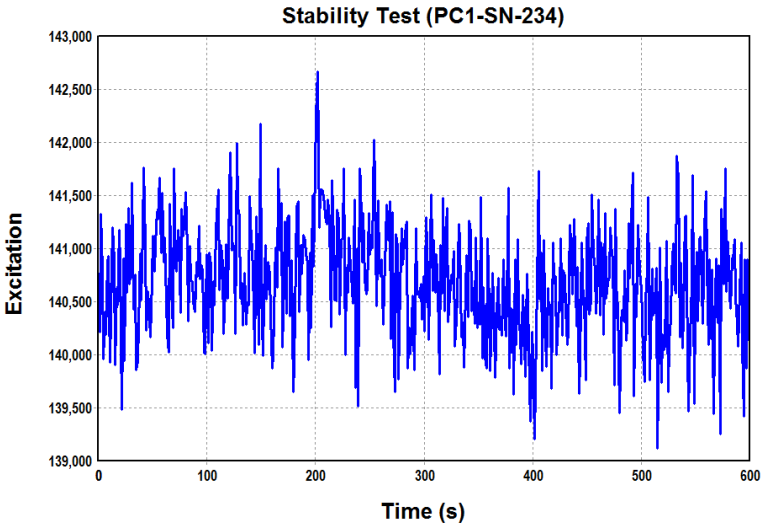
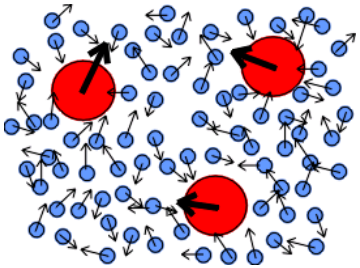
Fluorescence: is it noise?

Acquisition of a steady fluorescence signal over time





Brownian motion



Why do we need FFS to measure the internal dynamics in cells?

Methods based on perturbation

- Typically FRAP (fluorescence recovery after photobleaching)

Methods based on fluctuations

- Typically FCS and dynamic ICS methods

There is a fundamental difference between the two approaches, although they are related as to the physical phenomena they report on.

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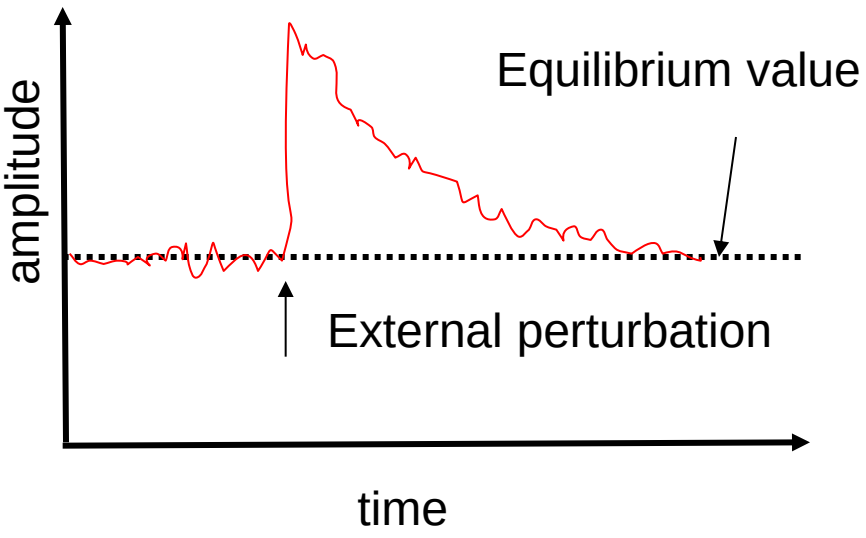
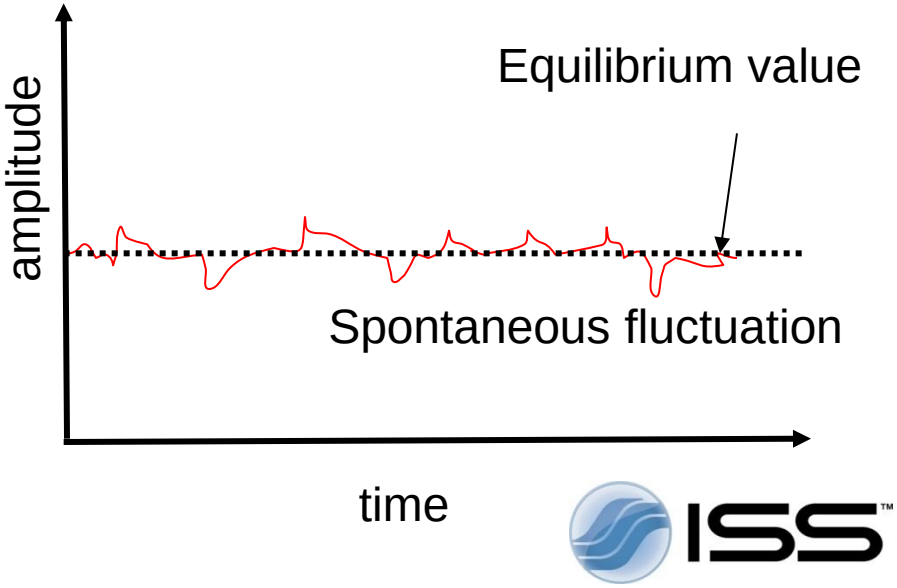
The fluctuation-dissipation principle

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

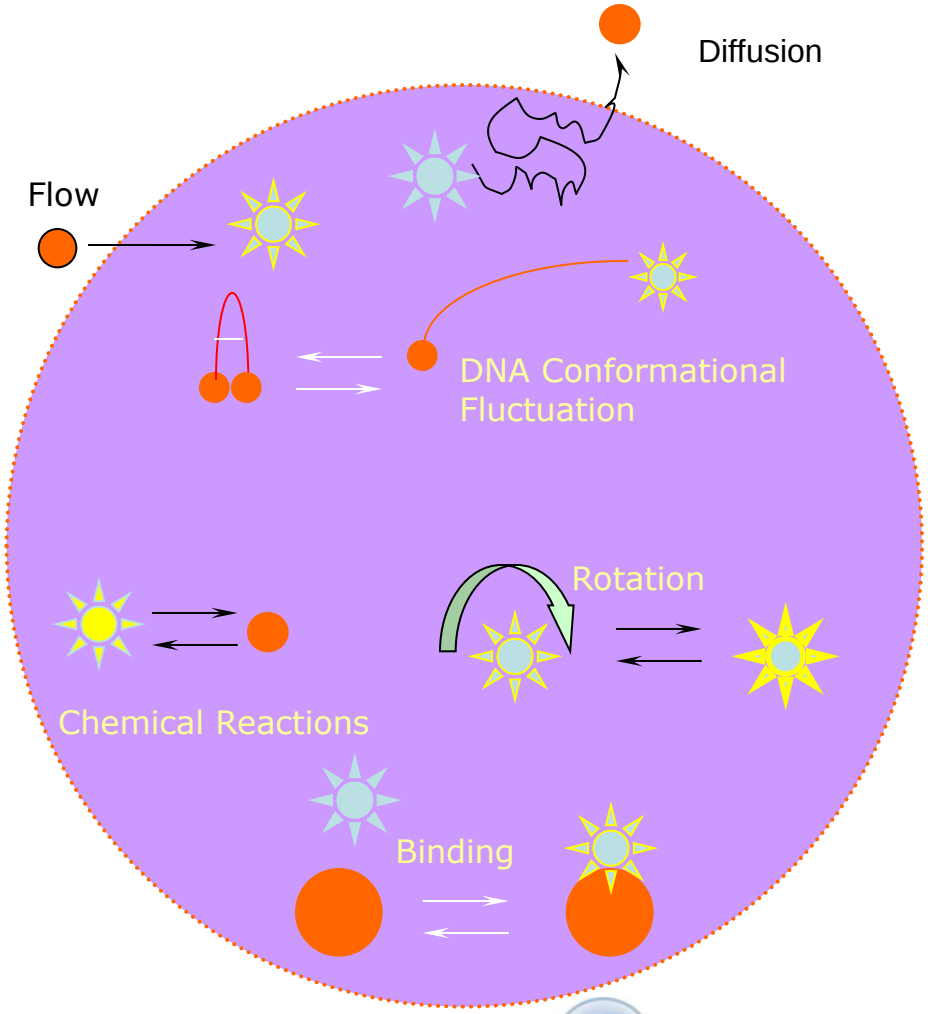
Spontaneous energy fluctuations in a part of the system, can cause the system to locally go out of equilibrium. These spontaneous fluctuations **dissipate** with the same time constant as if we had externally perturbed the equilibrium of the system.





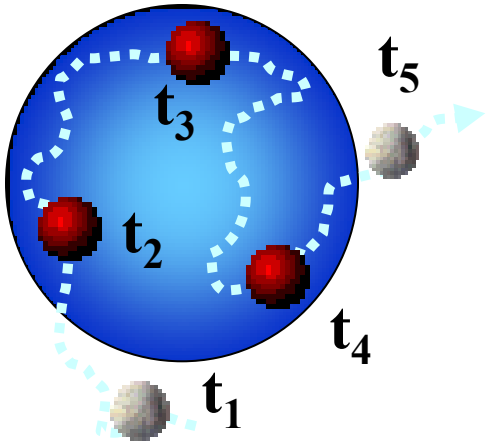
What can cause a fluctuation in the fluorescence?

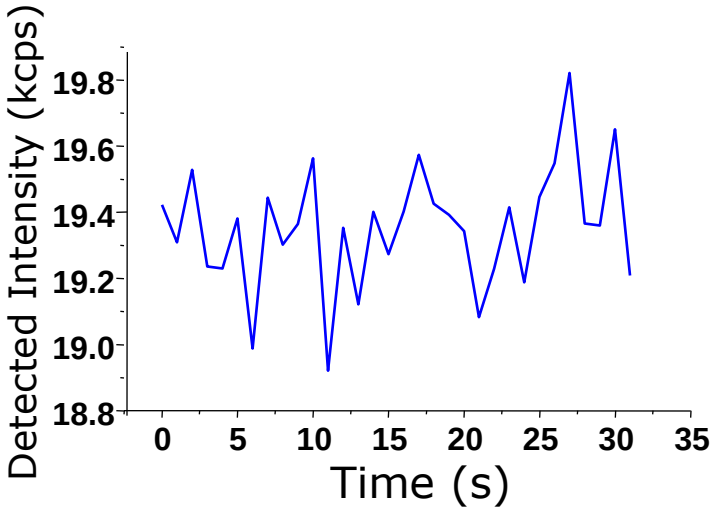
- Number of fluorescent molecules in the volume of observation
- Diffusion or binding
- Conformational Dynamics
- Rotational Motion
- Protein Folding
- Blinking
- And many more



The Signal

Role of the confocal volume





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

Distribution of the **duration** of the fluctuations

Distribution of the **amplitude** of the fluctuations

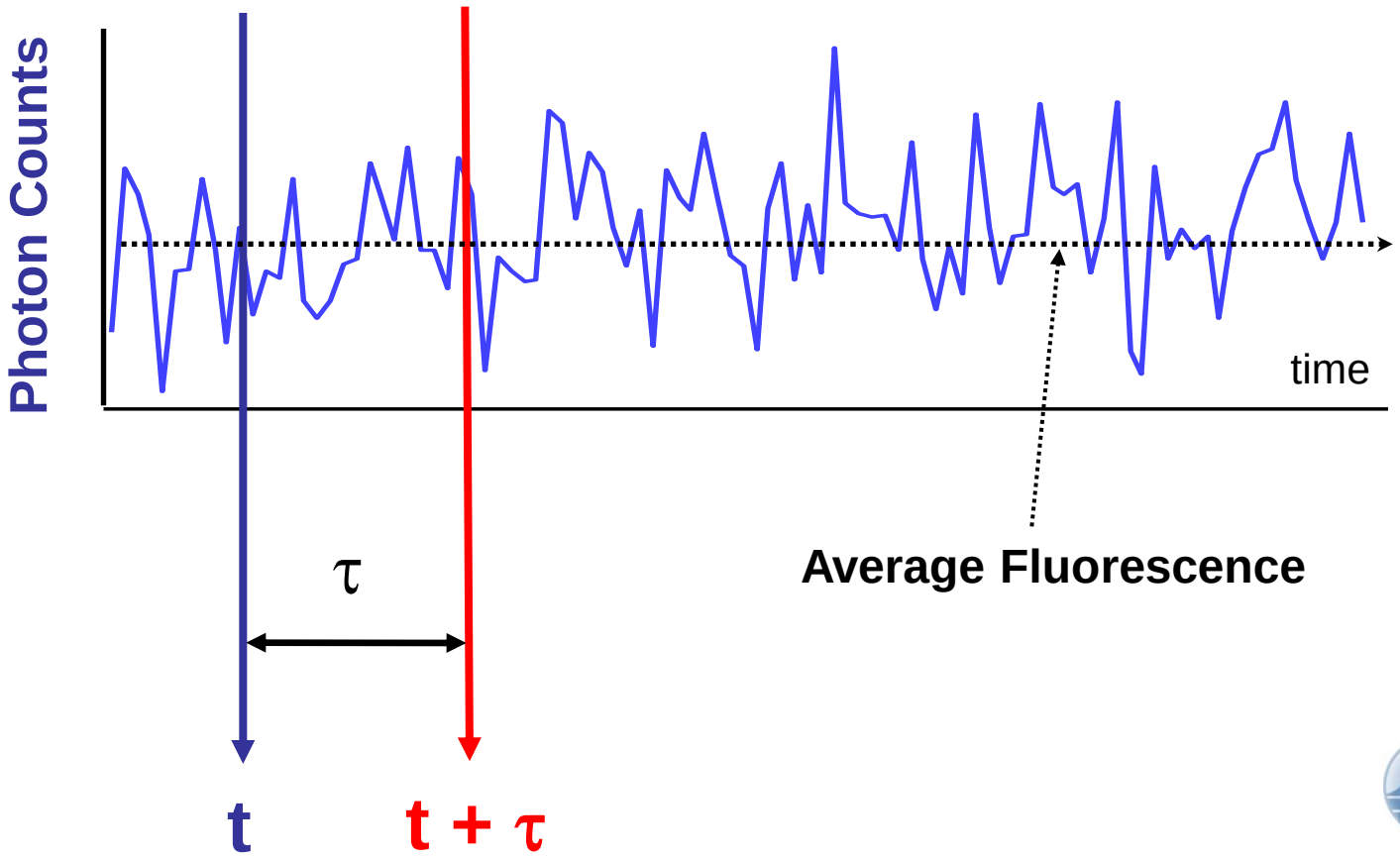
To extract the distribution of the duration of the fluctuations we use a math based on calculation of the **correlation function**

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

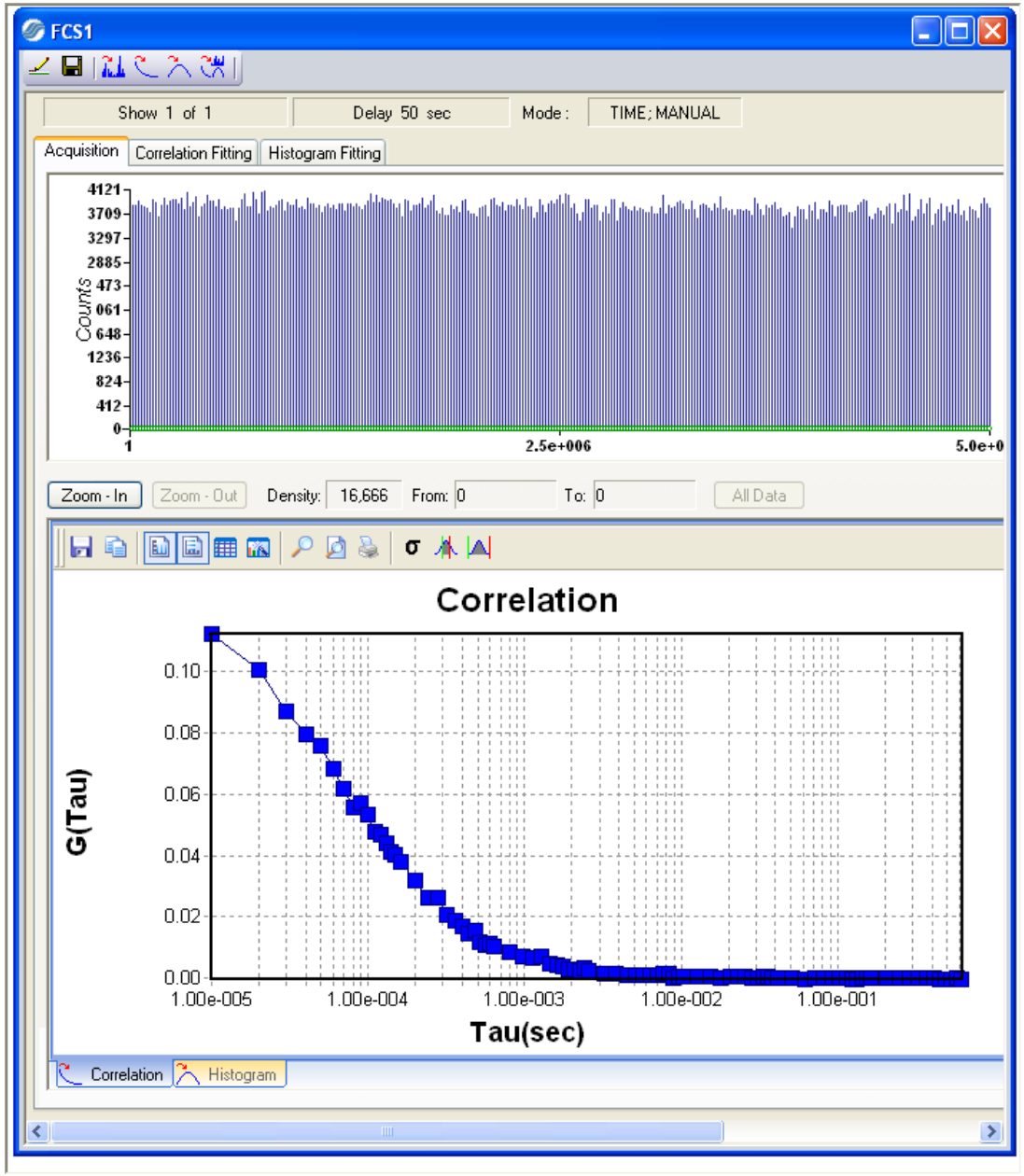
The definition of the Autocorrelation Function

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

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



The autocorrelation function



What determines the intensity of the fluorescence signal?

This is the fundamental equation in FCS

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

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

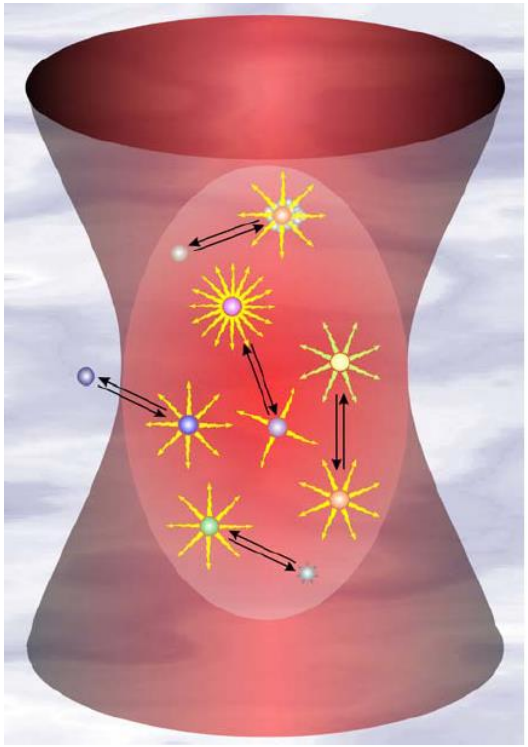
The value of F(t) depends on the profile of illumination!

The role of the observation volume

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

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

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

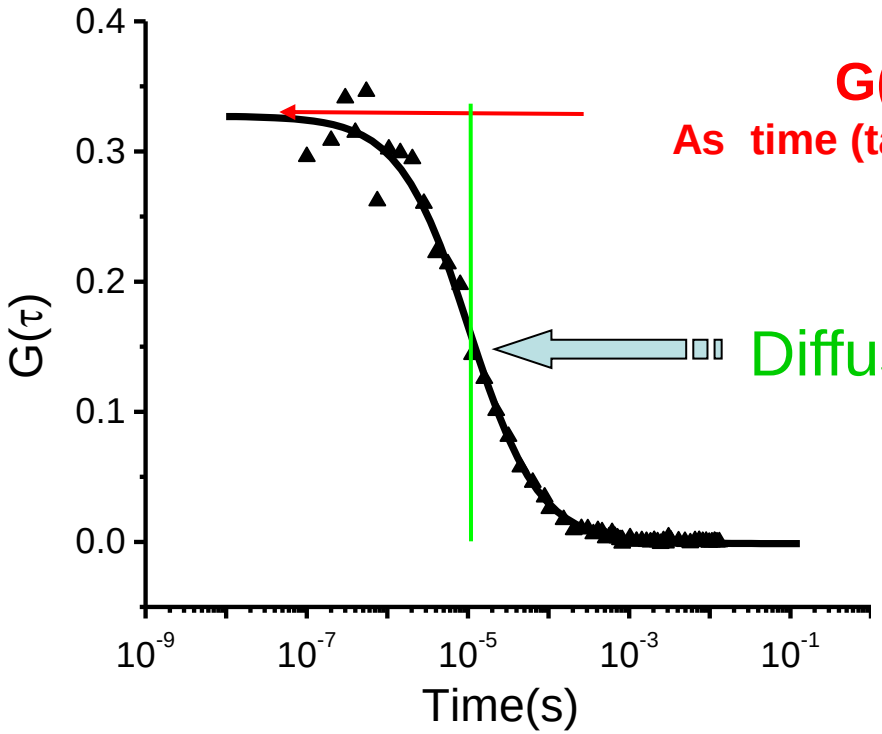


Volume: 10^{-15} L

Size (μm): 0.3 x 1

No. of molecules: ≈ 1

The Autocorrelation Function



G(0) ∝ 1/N

As time (tau) approaches 0

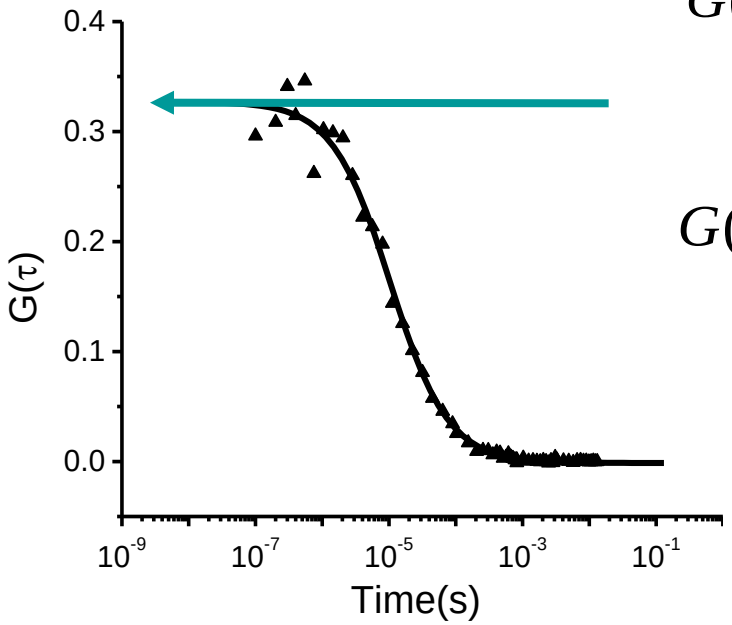
Diffusion

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

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

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

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

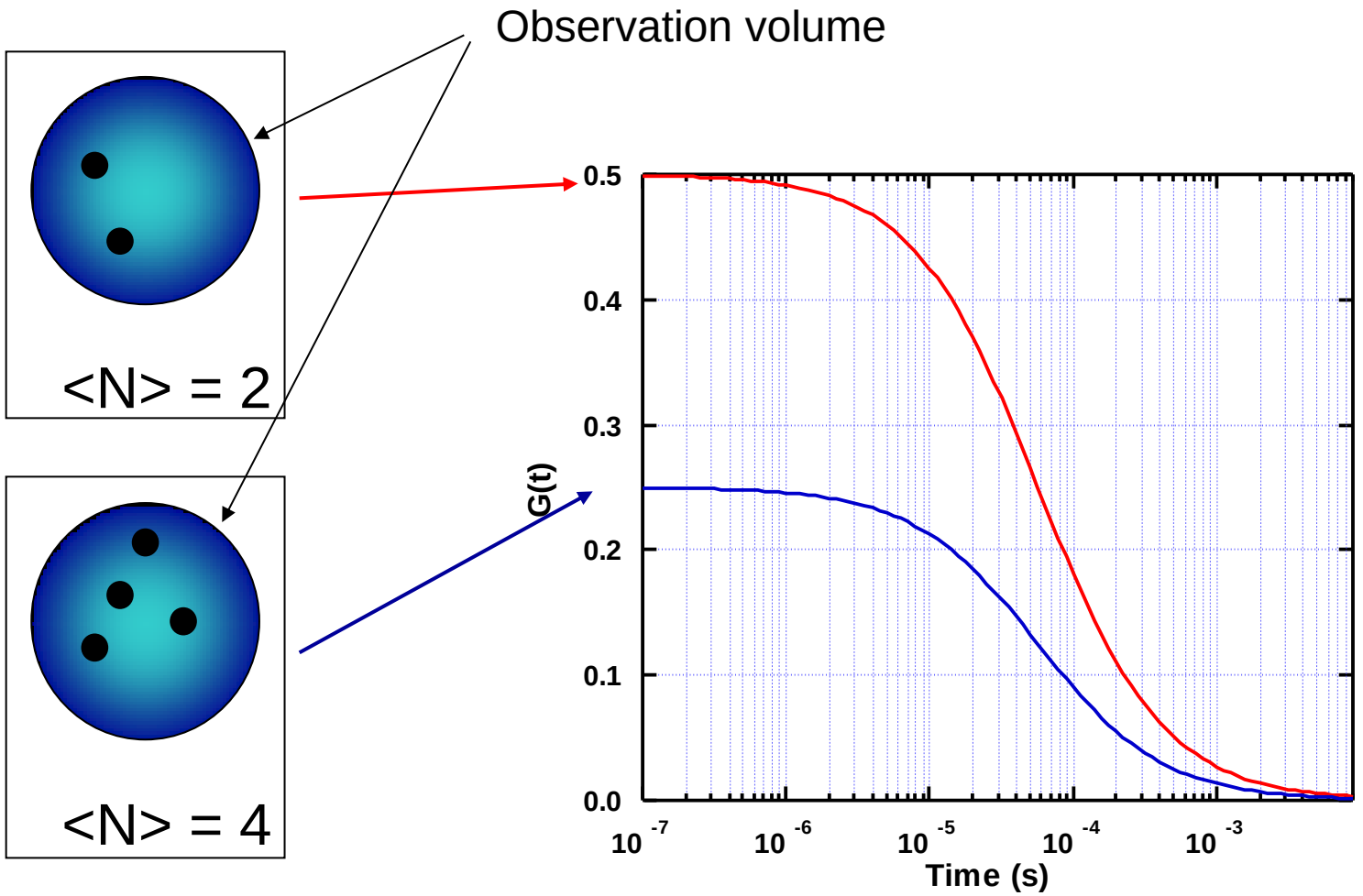


$$G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$ Definition

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

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

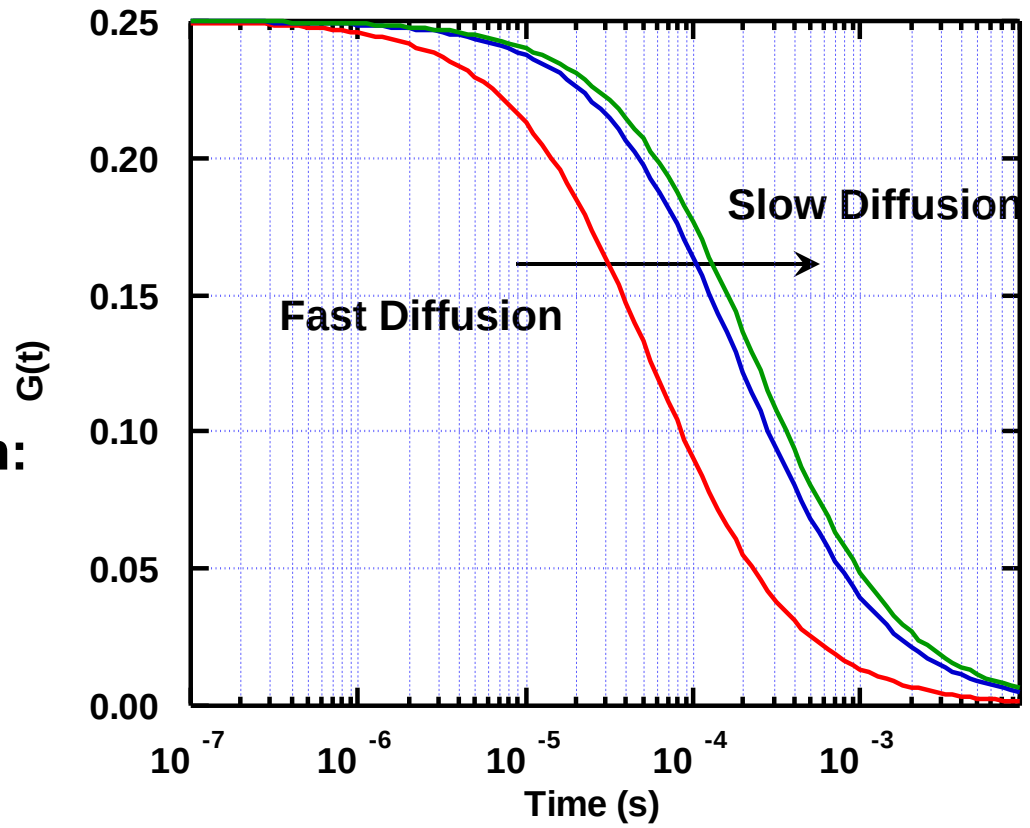
The Effects of Particle Concentration on the Autocorrelation Curve



The Effects of Particle Size on the Autocorrelation Curve

Diffusion Constants

300 μm²/s
90 μm²/s
71 μm²/s



Stokes-Einstein Equation:

$$D = \frac{kT}{6\pi\eta r}$$

$$\langle x \rangle = \sqrt{6D}\,\sqrt{t}$$

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

Monomer --> Dimer

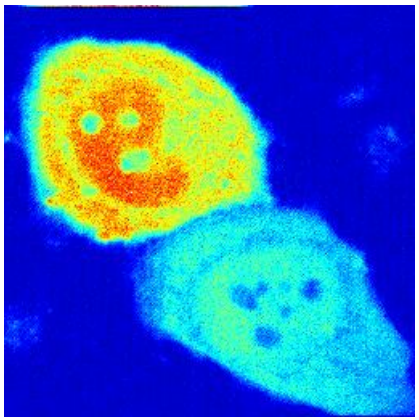
Only a change in D by a factor of $2^{1/3}$, or 1.26

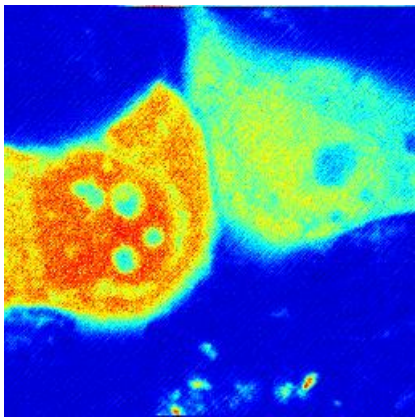
Table of characteristic times for diffusion

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

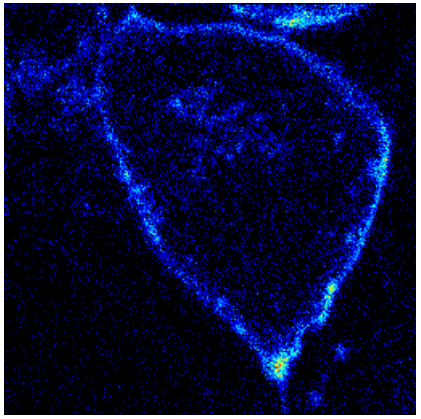
Volume	**Device**	**Size(μm)**	**Molecules**	**Time**
milliliter	cuvette	10000	$6x10^{14}$	10^{4}
microliter	plate well	1000	$6x10^{11}$	10^{2}
nanoliter	microfabrication	100	$6x10^{8}$	1
picoliter	typical cell	10	$6x10^{5}$	10^{-2}
femtoliter	confocal volume	1	$6x10^{2}$	10^{-4}
attoliter	nanofabrication	0.1	$6x10^{-1}$	10^{-6}

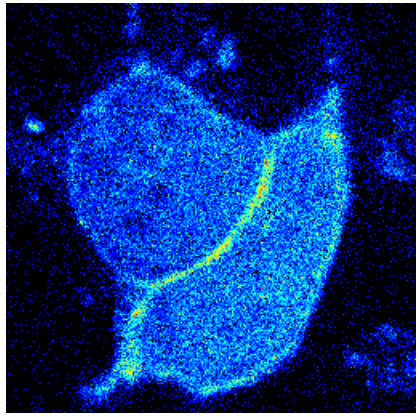
Autocorrelation Adenylate Kinase -EGFP Chimeric Protein in HeLa Cells





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



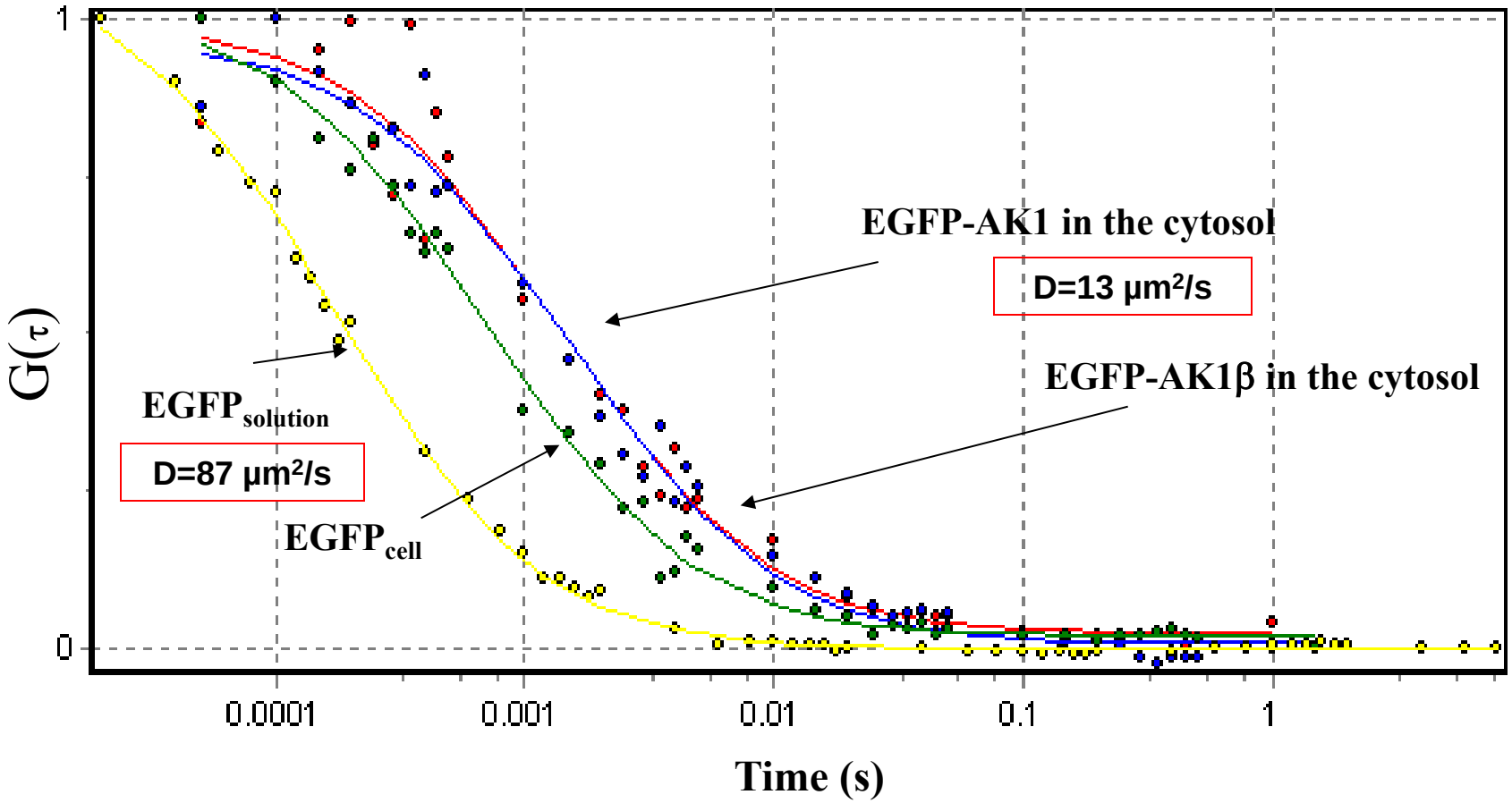


Examples of different *HeLa* cells transfected with AK1β -EGFP

Fluorescence Intensity

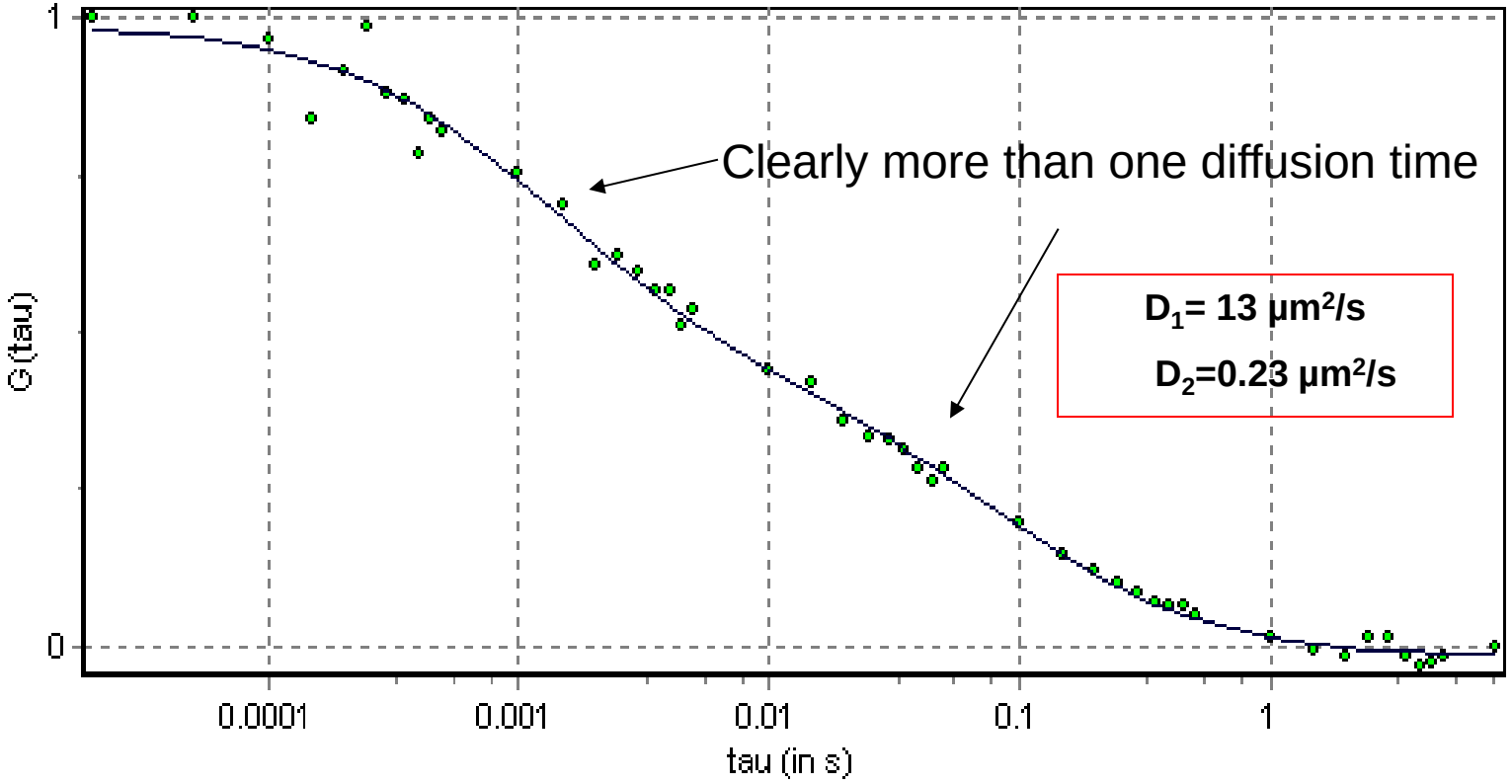
Qiao Qiao Ruan, Y. Chen, E. Gratton, M. Glaser & W. Mantulin; Biophys. J. 83 (2002)

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(•).

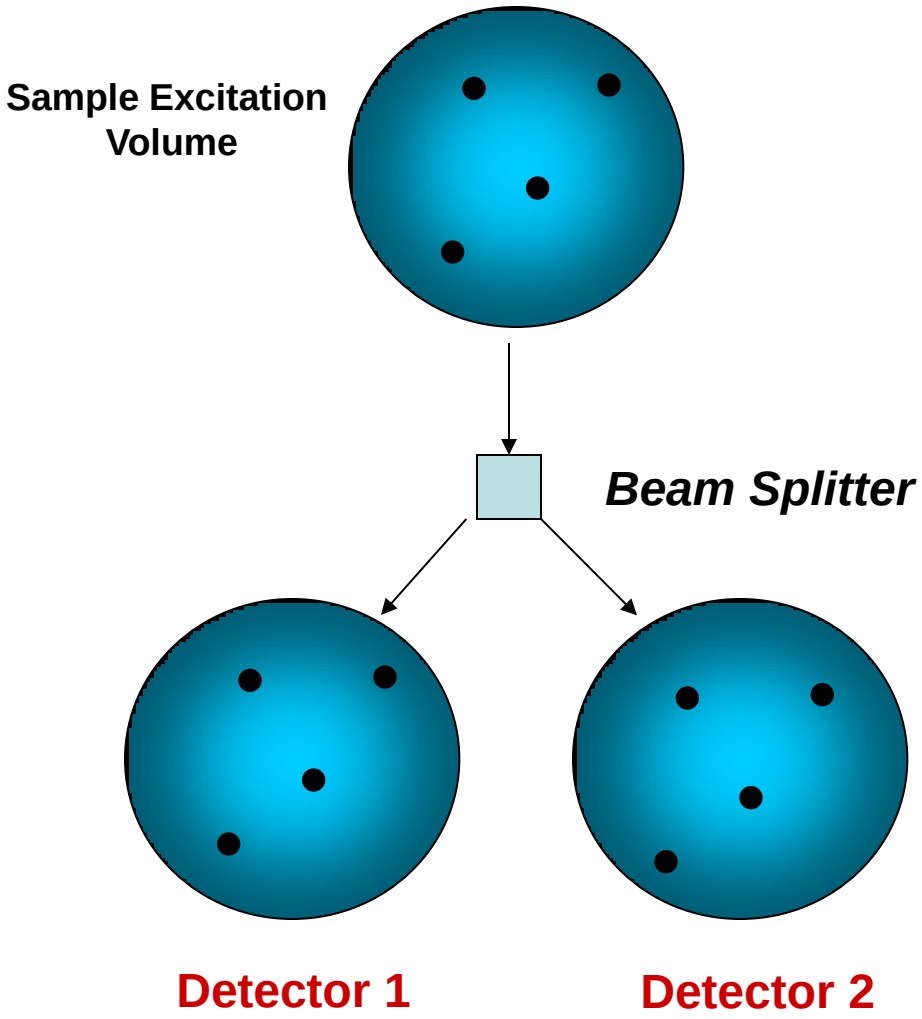
Autocorrelation of Adenylate Kinase –EGFP on the Membrane



A mixture of AK1ß-EGFP in the plasma membrane of the cell.

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

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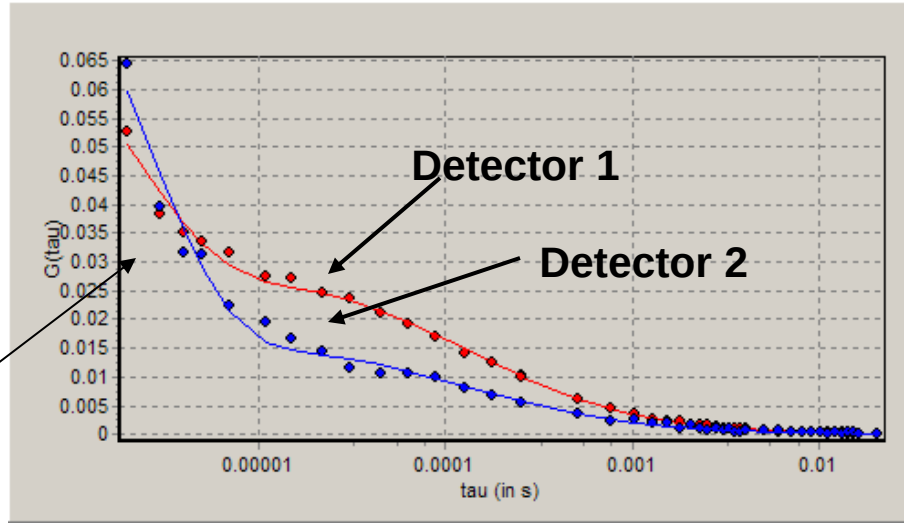
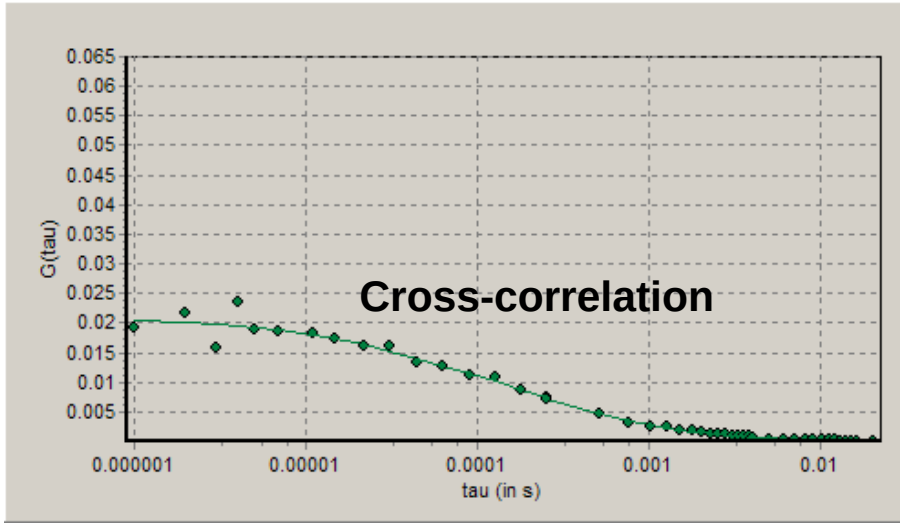
Removal of Detector Noise by Cross-correlation

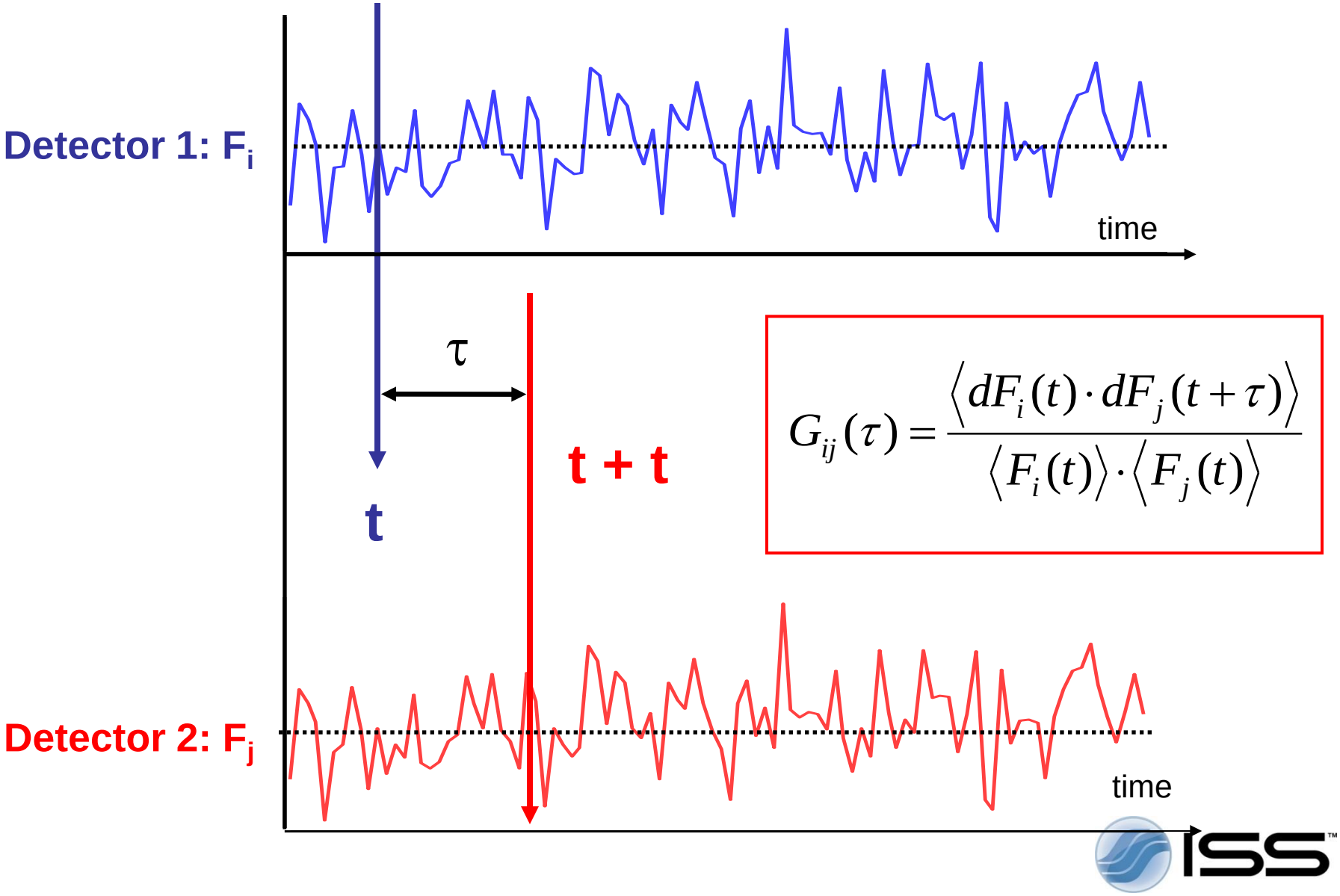
11.5 nM Fluorescein



Detector after-pulsing



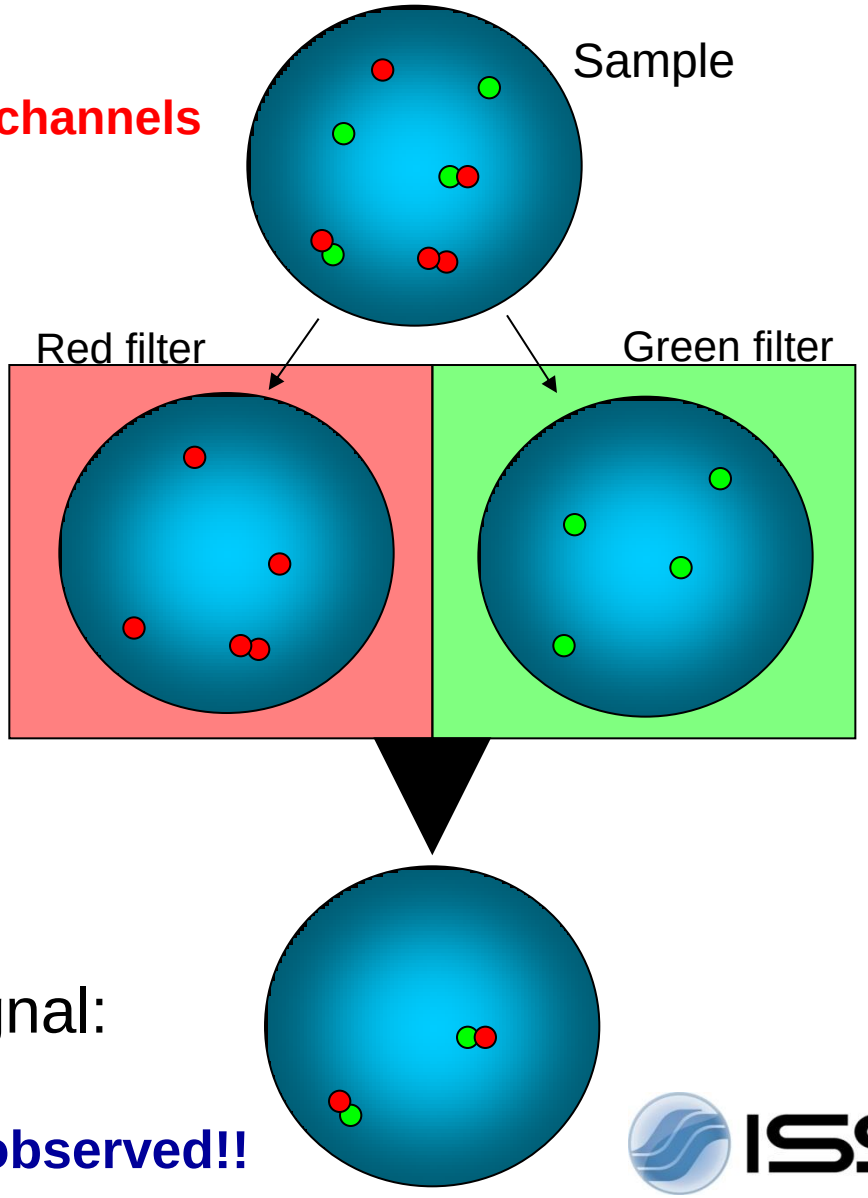
Calculating the Cross-correlation Function



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

Two-Color Cross-correlation

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

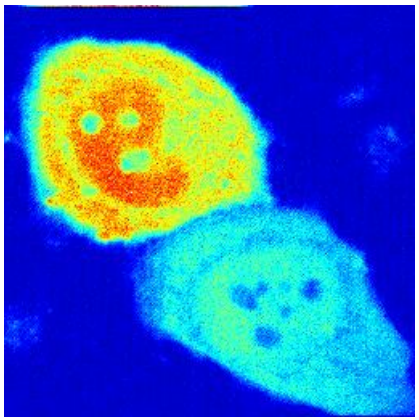


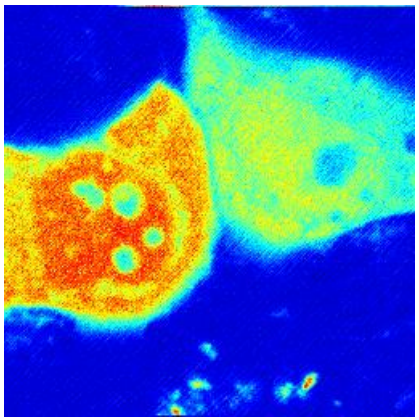
Each detector observes particles with a particular color

The cross-correlation signal:

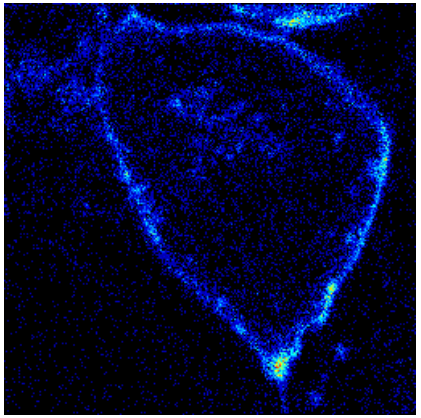
Only the green-red molecules are observed!!

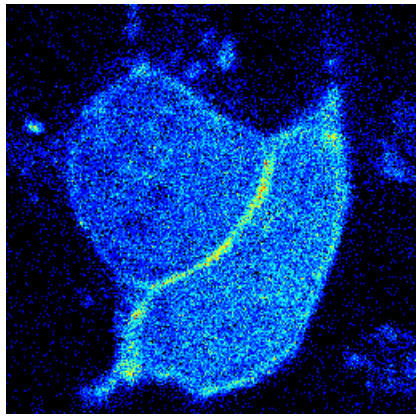
Autocorrelation Adenylate Kinase -EGFP Chimeric Protein in HeLa Cells





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





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

Fluorescence Intensity

Q. Ruan, Y. Chen, E. Gratton, M. Glaser and W. Mantulin; Biophys. J. 83 (2002)

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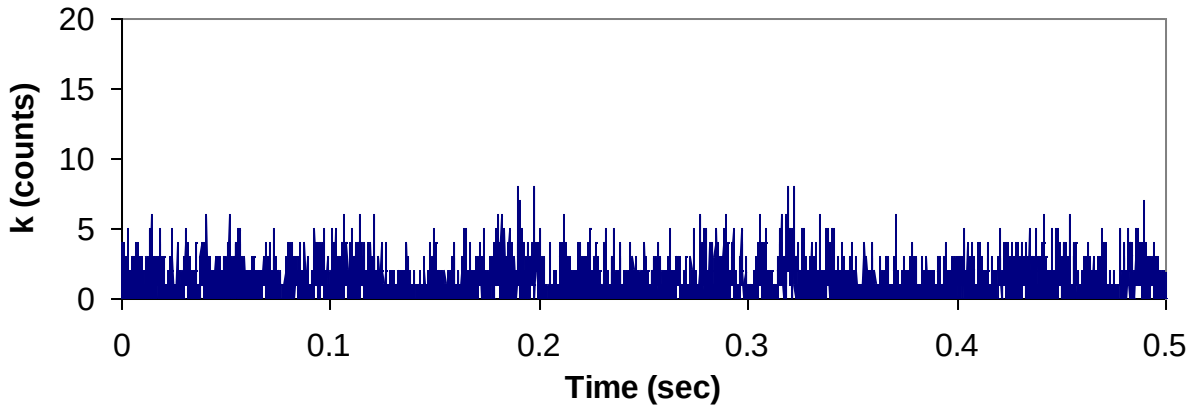
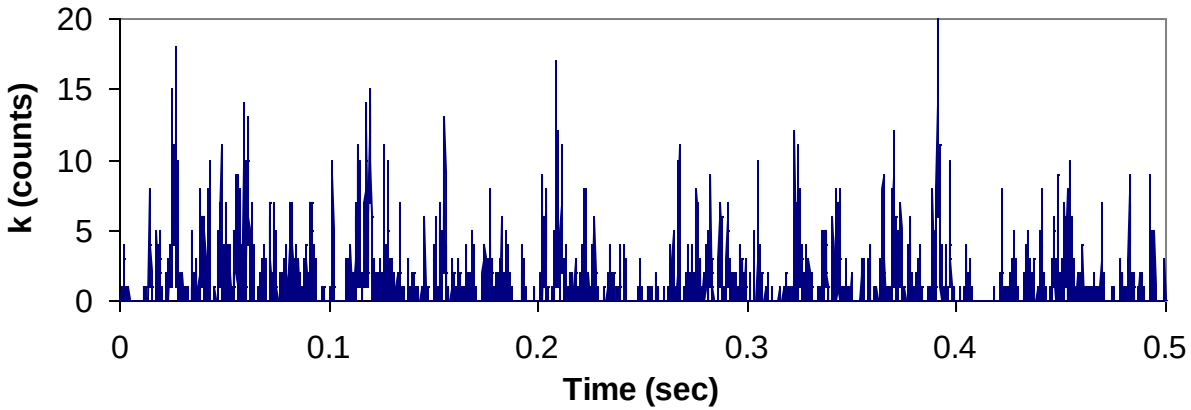
Fluorescence Trajectories



Fluorescent Monomer:

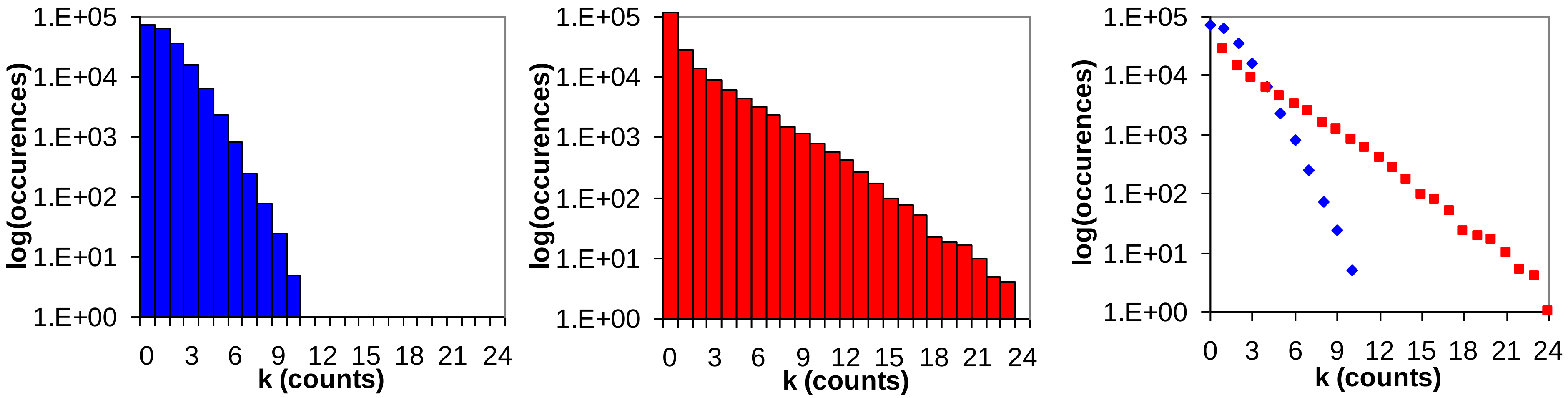
Intensity = 115,000 cps



Aggregate:

Intensity = 111,000 cps

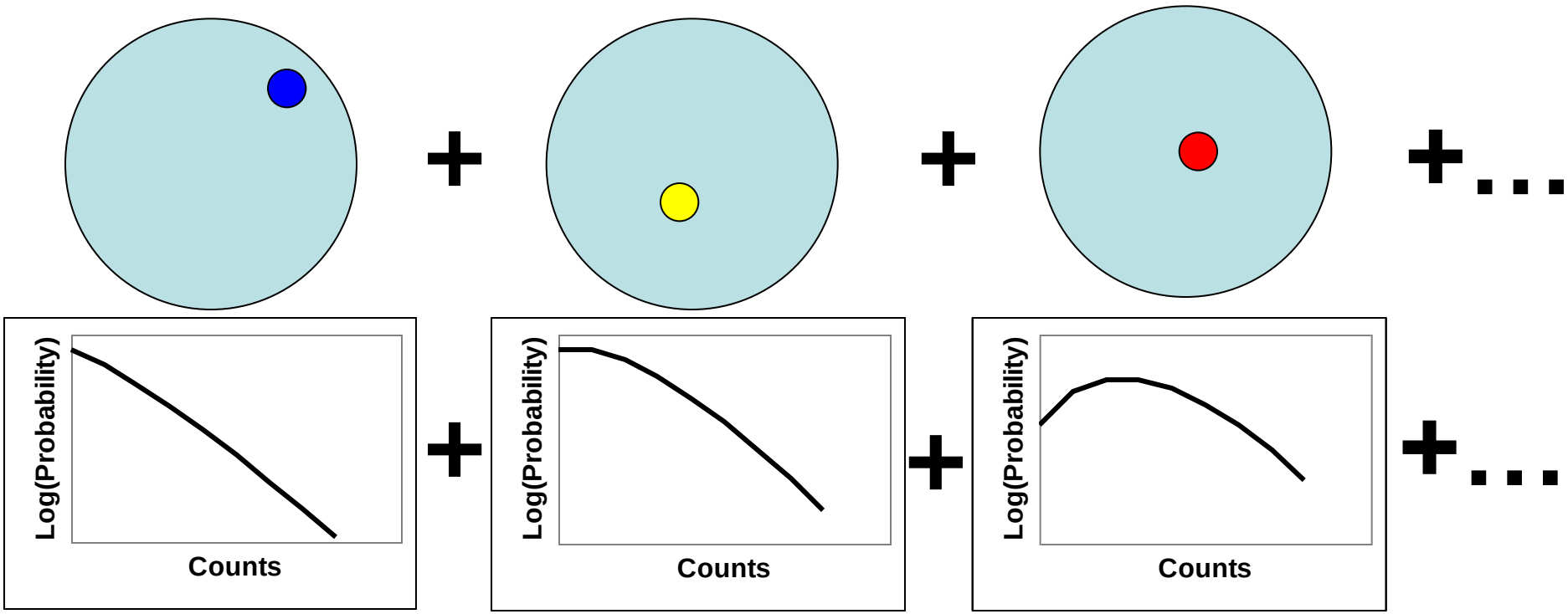
Photon Count Histogram (PCH)



Can we quantitate this?

What contributes to the distribution of intensities?

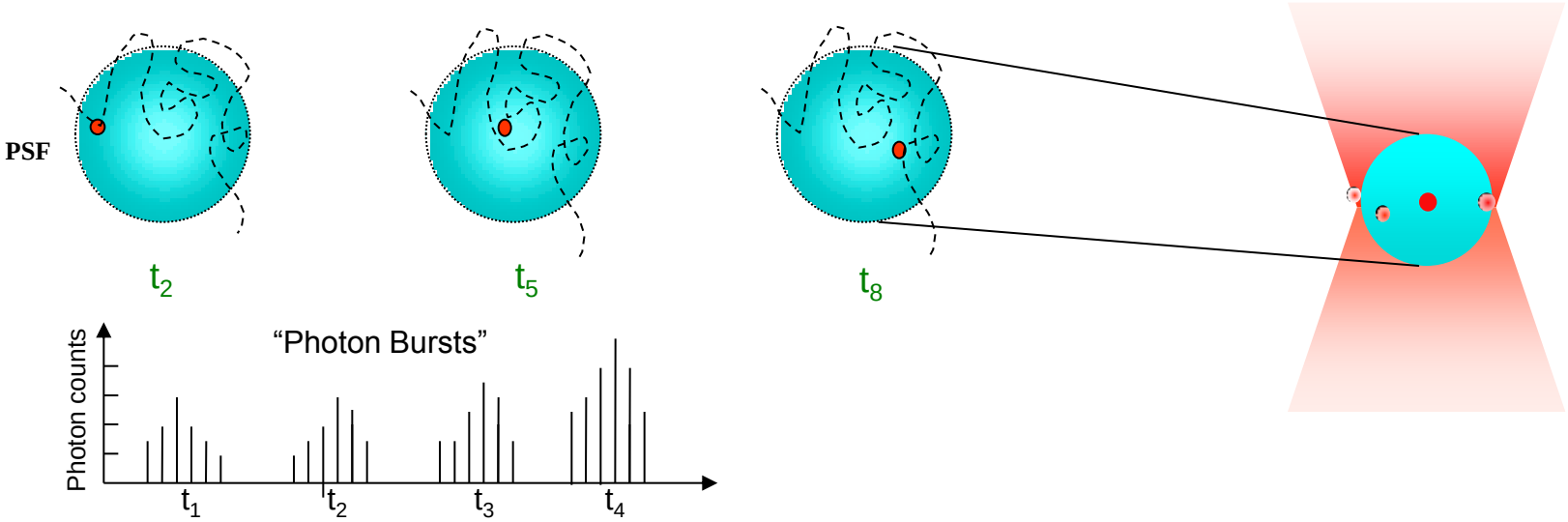
Single Particle PCH



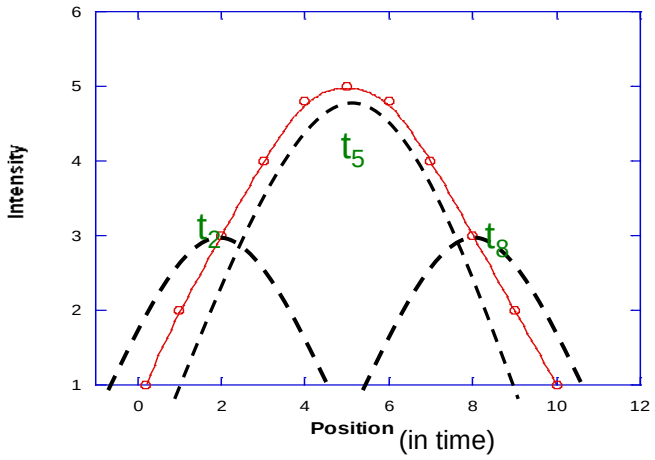
Have to sum up the poissonian distributions for all possible positions of the particle within the PSF

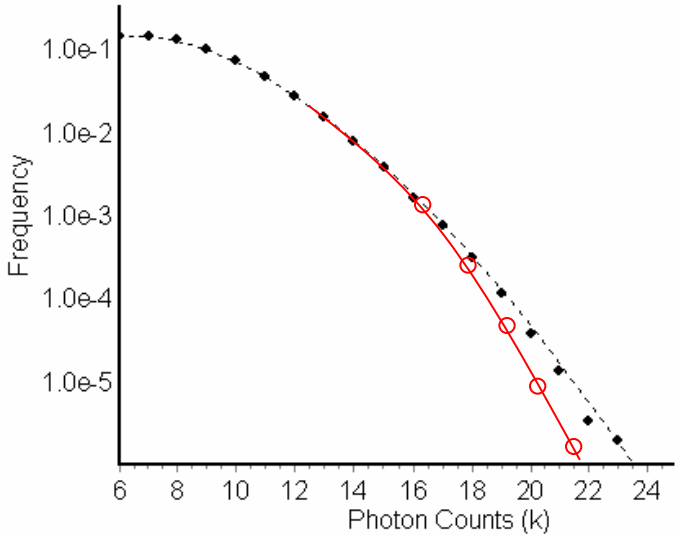
$$p^{(1)}(k) = \frac{1}{V_0} \int_{V_0} Poi\left(k, \varepsilon \overline{PSF}(\vec{r})\right) d\vec{r}$$

The average photon count rate of bursts determines the molecular brightness of the labeled protein



The intensity distribution accounts for the fluctuations of photons from the molecule freely diffusing through the exitation profile. Thus, the overall photon counting count distrubution is the weighted superposition of individual Poissonian distributions for each intensity values with a scaling amplitude. The fluctuations light intensity results in a broadeing of photon count distribution with respect to a pure poisson distribution. As the fluctuations increases, the photon count distribution broadens.





Multiple-Particle PCH

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

The Photon Counting Histogram: Statistical Analysis of Single Molecule Populations

- The Autocorrelation function only depends on fluctuation duration and fluctuation density (independent of excitation power)

- PCH: distribution of intensities (independent of time)

Hypothetical situation: Protein Interactions

- 2 proteins are labeled with a fluorophore
- Proteins are soluble
- How do we assess interactions between these proteins?

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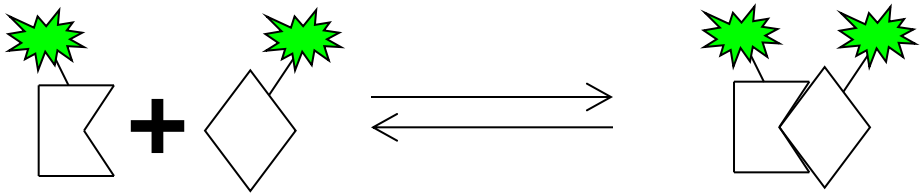
Dimer has double the brightness

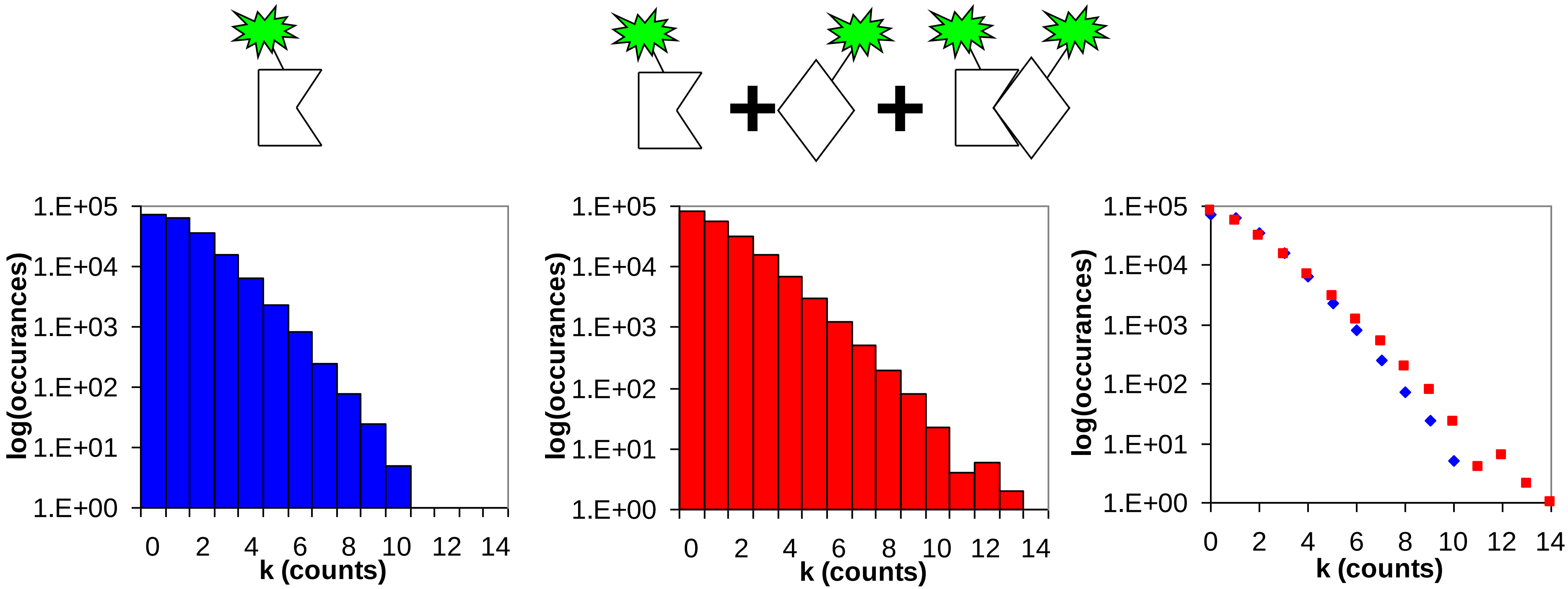


$\varepsilon = \varepsilon_{monomer}$ $\varepsilon = 2 \times \varepsilon_{monomer}$

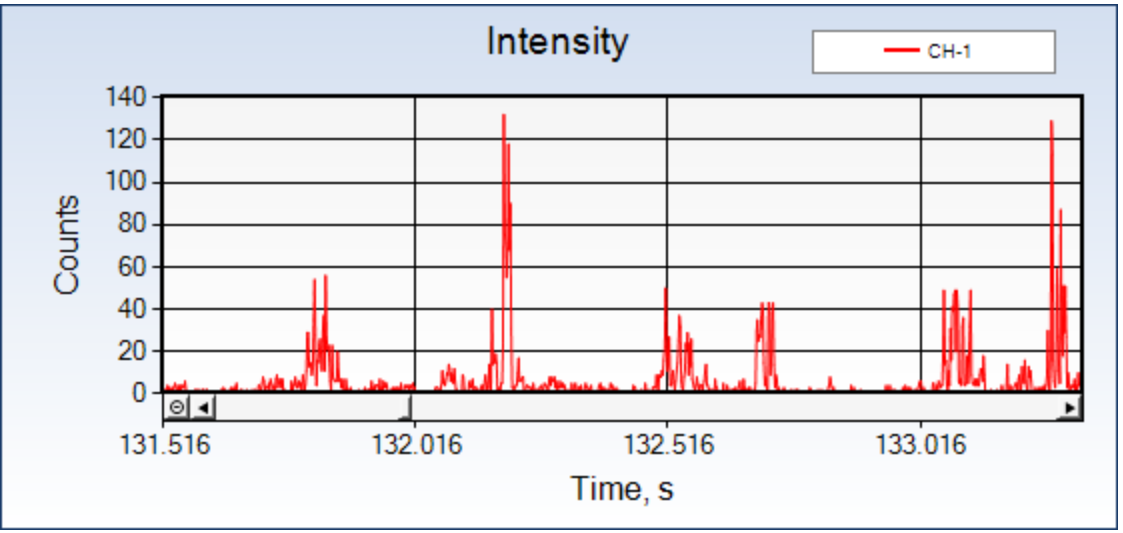
All three species are present in equilibrium mixture

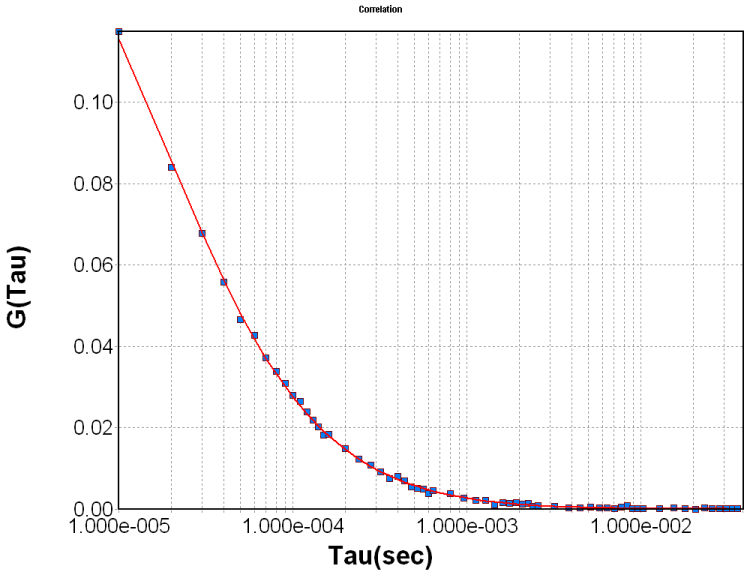
Typical one photon $\varepsilon_{monomer}$ = 10,000 cpsm

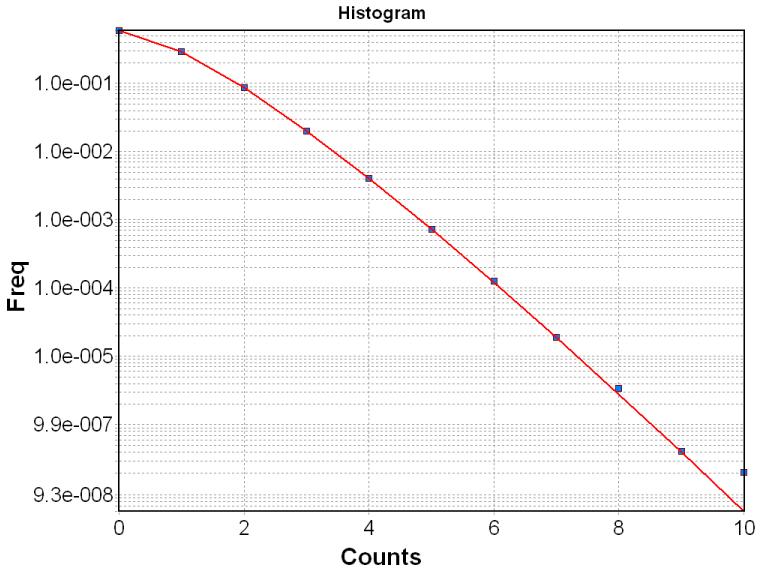
Photon Count Histogram (PCH)



Data Presentation and Analysis







FCS parameters: <N> and **D**

PCH parameters: <N> and ε

<N>: no. of particles in the observation volume

D: diffusion coefficient

ε: brightness

Complementary information obtained with the same measurement

using Autocorrelation function (FCS):

- ✓ Diffusion Coefficient (diffusion times)
- ✓ No. of particles in observation volume

using Photon Counting Histogram (PCH) analysis:

- ✓ Brightness
- ✓ No. of particles in observation volume

- The Autocorrelation function only depends on fluctuation duration and fluctuation density (independent of excitation power)

- PCH: distribution of intensities (independent of time)

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What about … ?

- In a cell we may want to locate the areas where we have clusters of molecules versus single molecules.
- In cells, both the concentration and the clustering of proteins can differ in various locations and change during biological processes.
- Number & Brightness (N&B) measures the average number of molecules and brightness in each pixel of an image.

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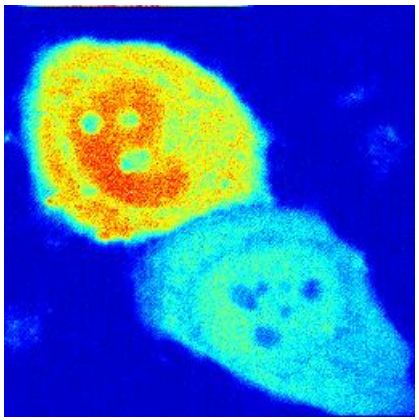
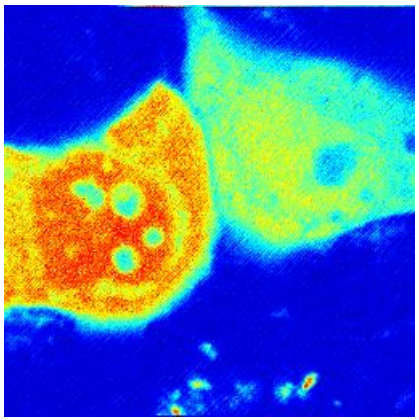
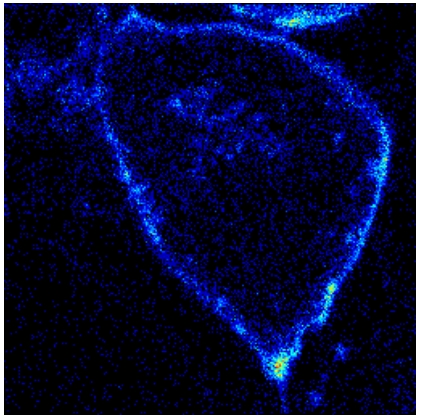
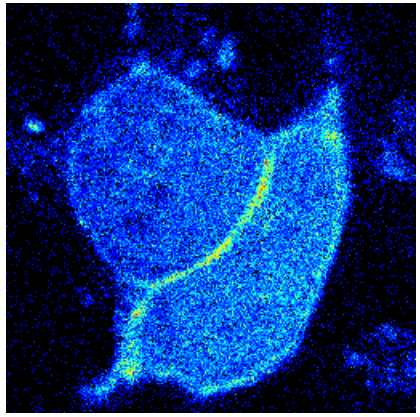
Autocorrelation Adenylate Kinase -EGFP Chimeric Protein in HeLa Cells





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





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

Fluorescence Intensity

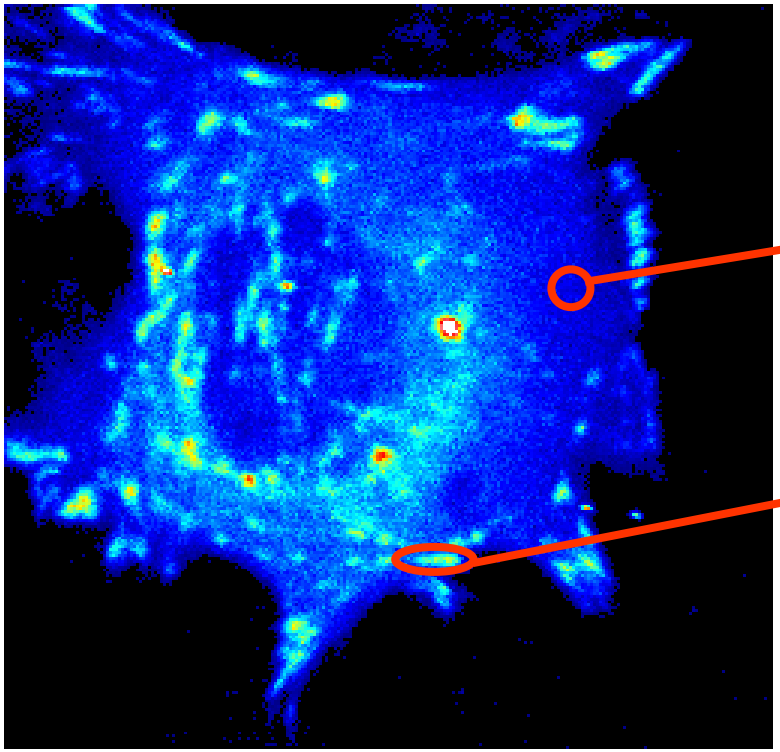
Q. Ruan, Y. Chen, E. Gratton, M. Glaser and W. Mantulin; Biophys. J. 83 (2002)

Existing Methods to determine protein concentration and aggregation of proteins in cells

- Calibrate the fluorescence intensity
- FRET
- ICS, Image Correlation Spectroscopy
- PCH

Intensity

1. Calibration of the free fluorophore based on intensity



Average intensity of MEF cells expressing Paxillin-EGFP

A **INTENSITY**

31,250counts/sec

B

93,750 counts/sec

If "free" EGFP at 10nM gave 30,000 counts/sec then the conclusion would be that :

A **=10nM**

B **= 30nM**

However, it doesn't give you the size distribution
Only concentration is given

The Number and Brightness (N&B) analysis

Purpose: Provide a pixel resolution map of molecular number and aggregation in cells

Method: First and second moment of the fluorescence intensity distribution at each pixel

Source: Raster scanned image obtained with laser scanning microscopes
TIRF with fast cameras
Spinning disk confocal microscope

Output: The N and B maps, B vs intensity 2D histogram

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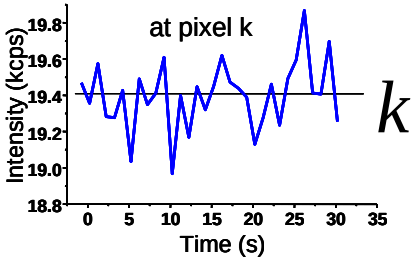
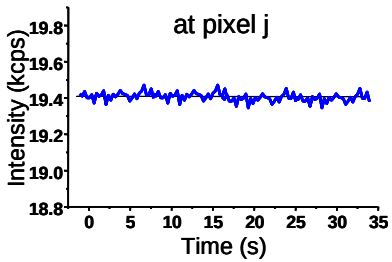
How to distinguish pixels with many dim molecules from pixels with few bright molecules?

Average (first moment)

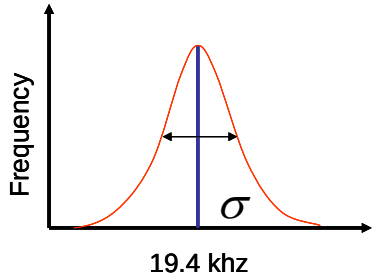
$$< k >= \frac{\sum_i k_i}{K}$$

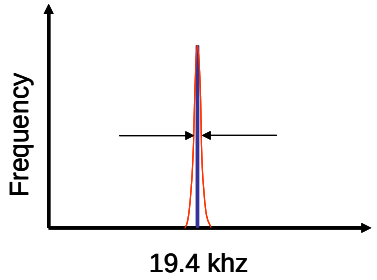


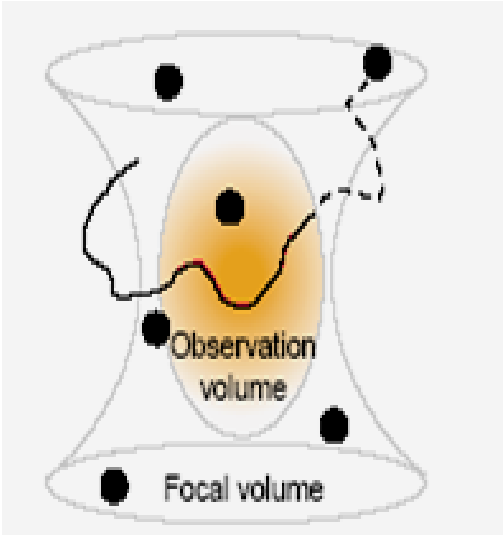


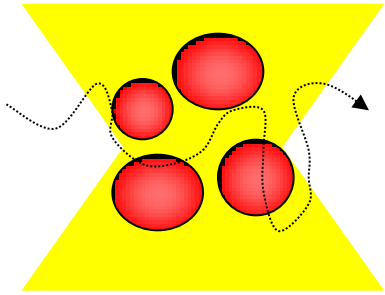
Variance (second moment)

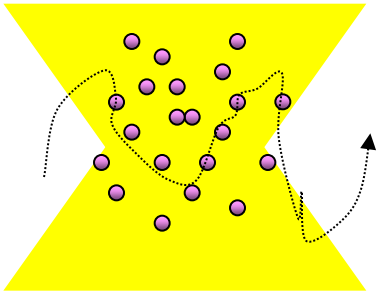
$$\sigma^2 = \frac{\sum_i (k_i - < k >)^2}{K}$$











- **Given two series of equal average, the larger is the variance, the less molecules contribute to the average. The ratio of the square of the average intensity ($<k>^2$) to the variance (σ^2) is proportional to the average number of particles <N>.**

$$G(0) = \sigma^2/<k>^2 = 1/N$$

* Originally developed by Qian and Elson (1990) for solution measurements.

Calculating protein aggregates from images

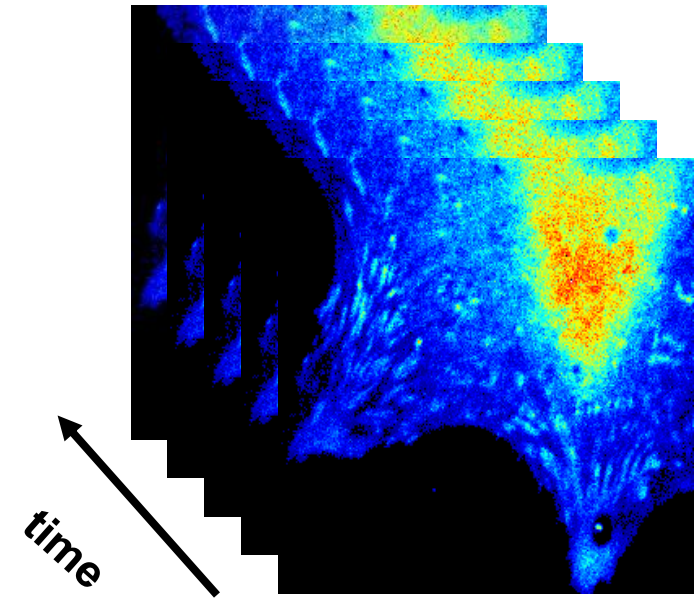
This analysis provides a map of <N> and brightness (B) for every pixel in the image. The units of brightness are related to the pixel dwell time and they are “counts/dwell time/molecule”.

$$< k >= \frac{\sum_i k_i}{K} \qquad \sigma^2 = \frac{\sum_i (k_i - < k >)^2}{K}$$

$$< N >= \frac{< k >^2}{\sigma^2}$$

$$B = \frac{< k >}{< N >} = \frac{\sigma^2}{< k >}$$

σ^2 = Variance
<k> = Average counts
N = Apparent number of molecules
B = Apparent molecular brightness
K = # of frames analyzed

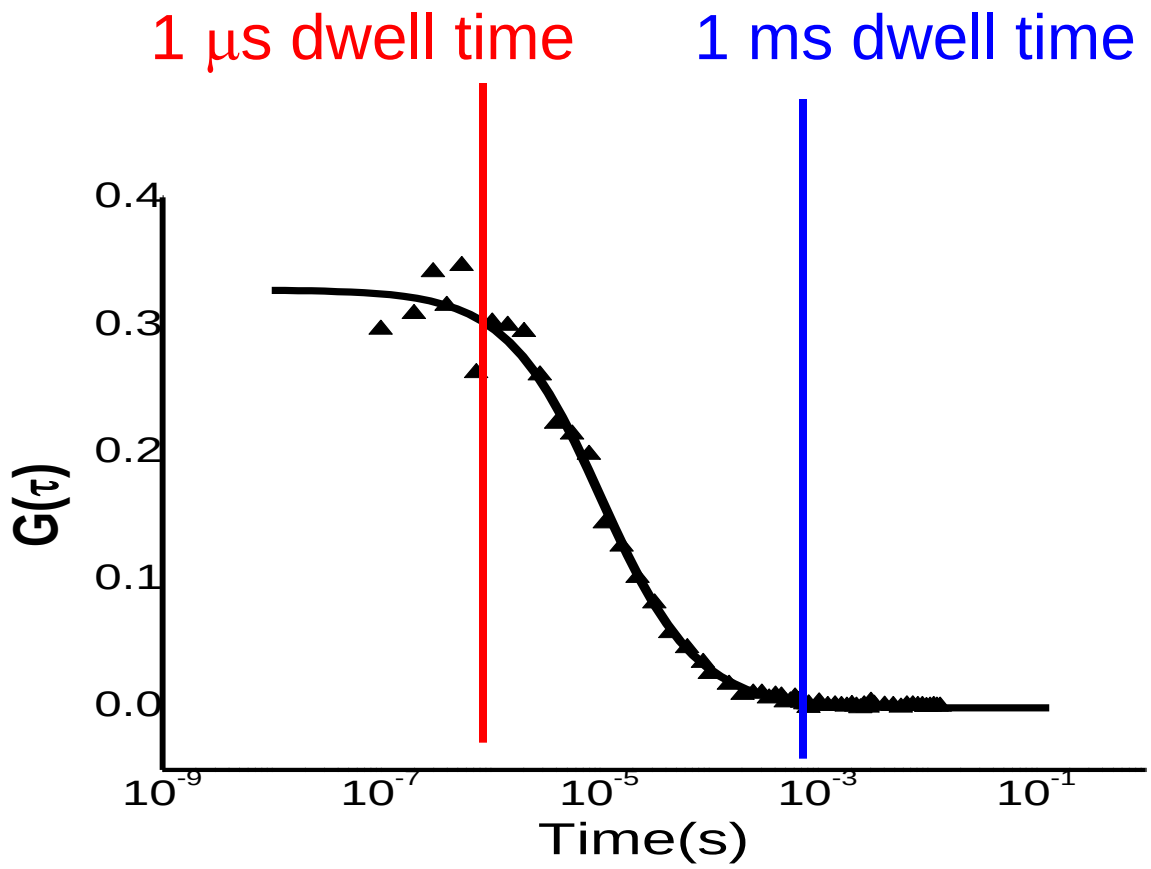


ISS

Selecting the dwell time

To increase the apparent brightness we could increase the dwell time, since the brightness is measured in counts/dwell time/molecule.

Increasing the dwell time decreases the amplitude of the fluctuation.



What contributes to the variance?

The measured variance contains two terms, the variance due to the particle number fluctuation and the variance due to the detector count statistics noise

$$\sigma^2 = \sigma_n^2 + \sigma_d^2$$

Variance due to particle number fluctuations $\sigma_n^2 = \varepsilon^2 n$

Variance due to detector shot noise $\sigma_d^2 = \varepsilon n$ (for the photon counting detector)

Both depend on the intrinsic brightness and the number of molecules.
We can invert the equations and obtain n and ε

n is the true number of molecules
ε is the true molecular brightness

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How to Calculate n and ε

$$B = \frac{\sigma^2}{<k>} = \frac{\sigma_n^{\,2}}{<k>} + \frac{\sigma_d^{\,2}}{<k>} = \frac{\varepsilon^2 n}{\varepsilon n} + \frac{\sigma_d^{\,2}}{<k>} = \varepsilon + 1$$

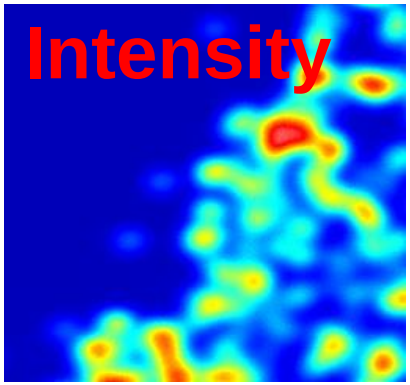
This ratio identifies pixels of different brightness due to mobile particles.

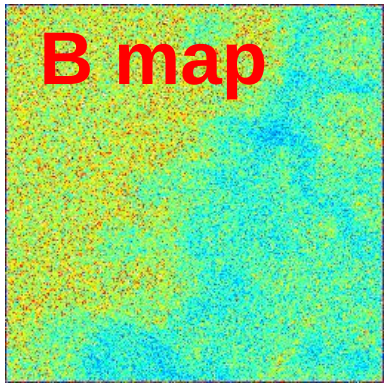
The “true” number of molecules n and the “true” molecular brightness for mobile particles can be obtained from

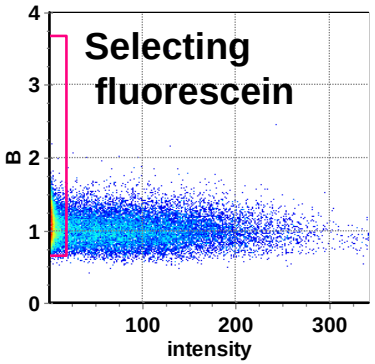
$$n = \frac{<k>^2}{\sigma^2 - <k>} \qquad \varepsilon = \frac{\sigma^2 - <k>}{<k>}$$

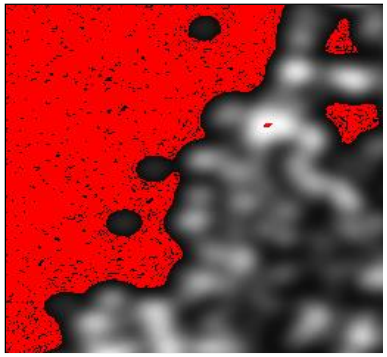
If there are regions of immobile particles, n cannot be calculated because for the immobile fraction the variance is = <k>. For this reason, several plots are offered to help the operator to identify regions of mobile and immobile particles. Particularly useful is the plot of N*vs*B.

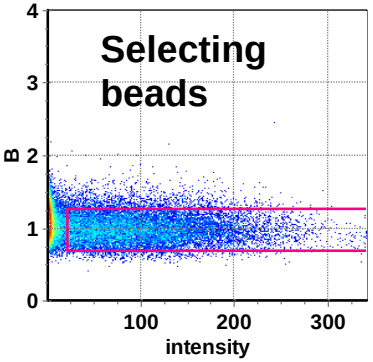
The effect of the immobile part: with photon counting detectors (Fluorescent beads in a sea of 100nM Fluorescein).

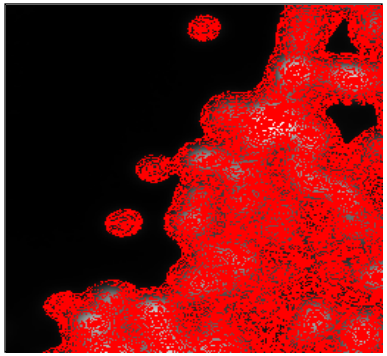


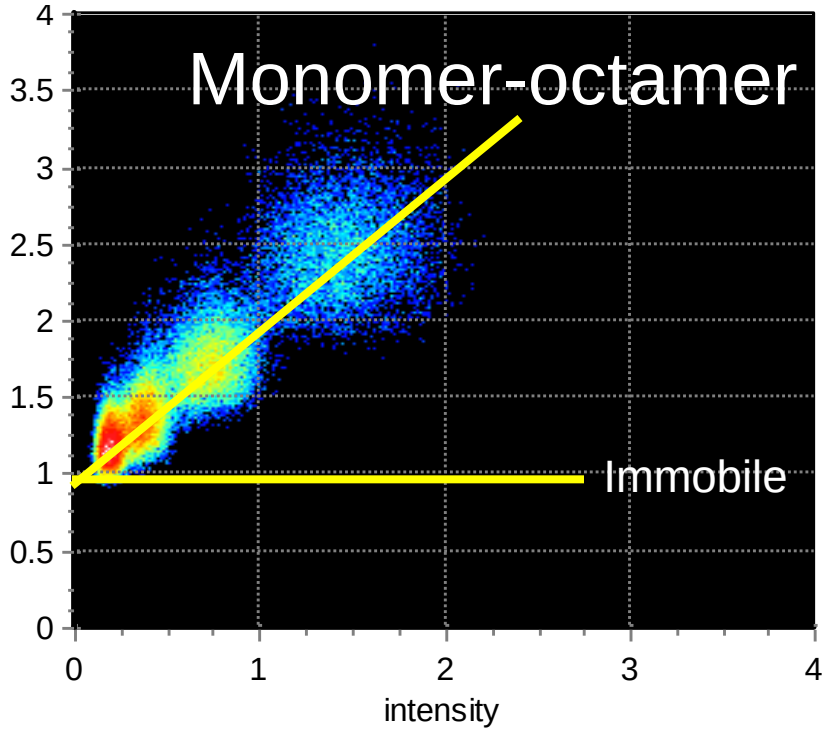






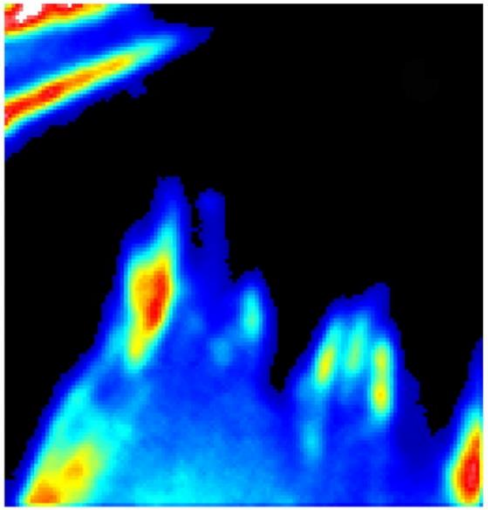




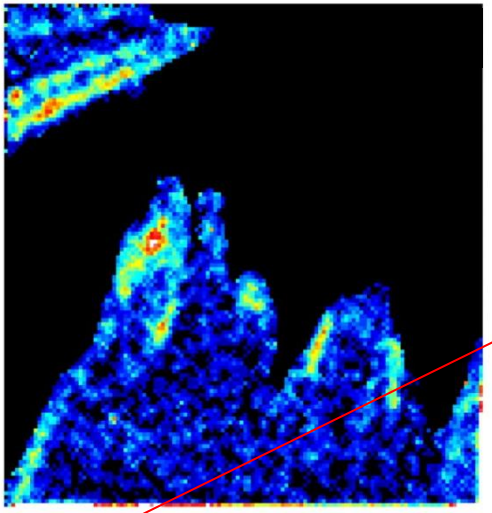


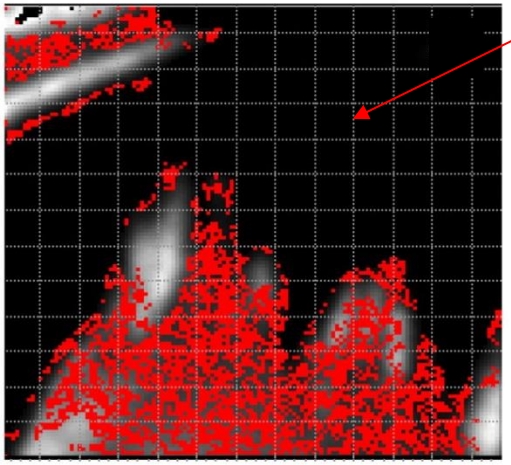
Paxillin assembles as monomers and disassembles as aggregates as large as 8-12

Average intensity

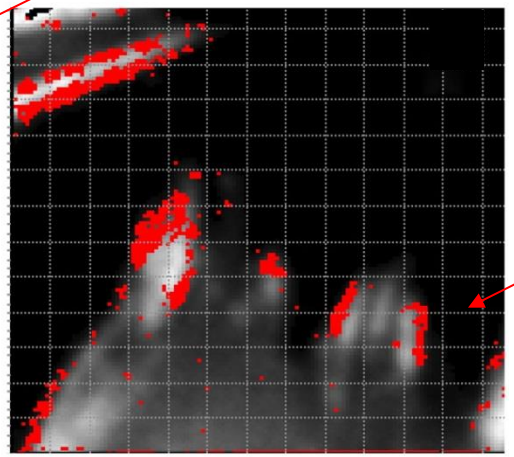


Molecular Brightness

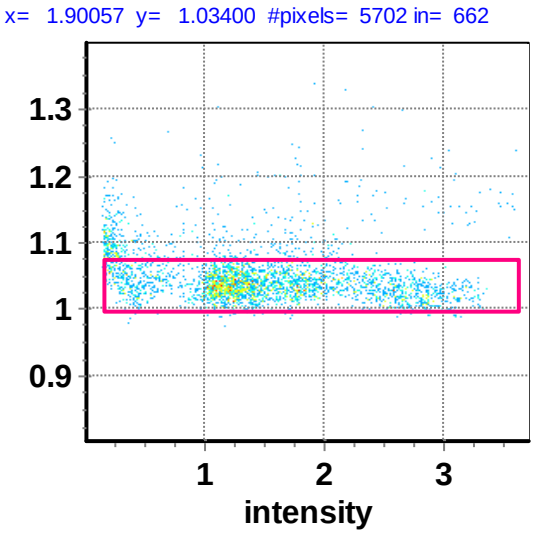


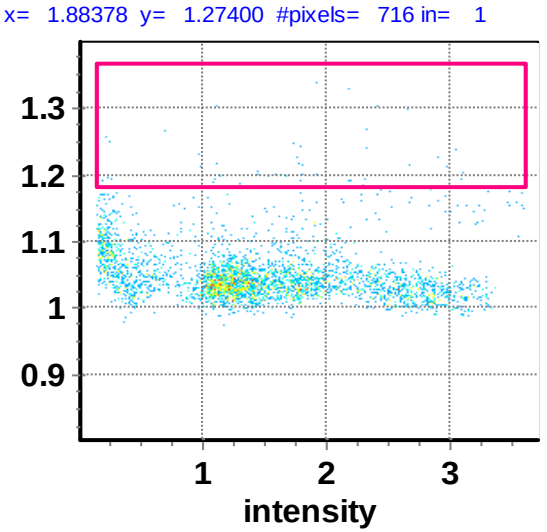


Selected Monomers



Selected >5mers



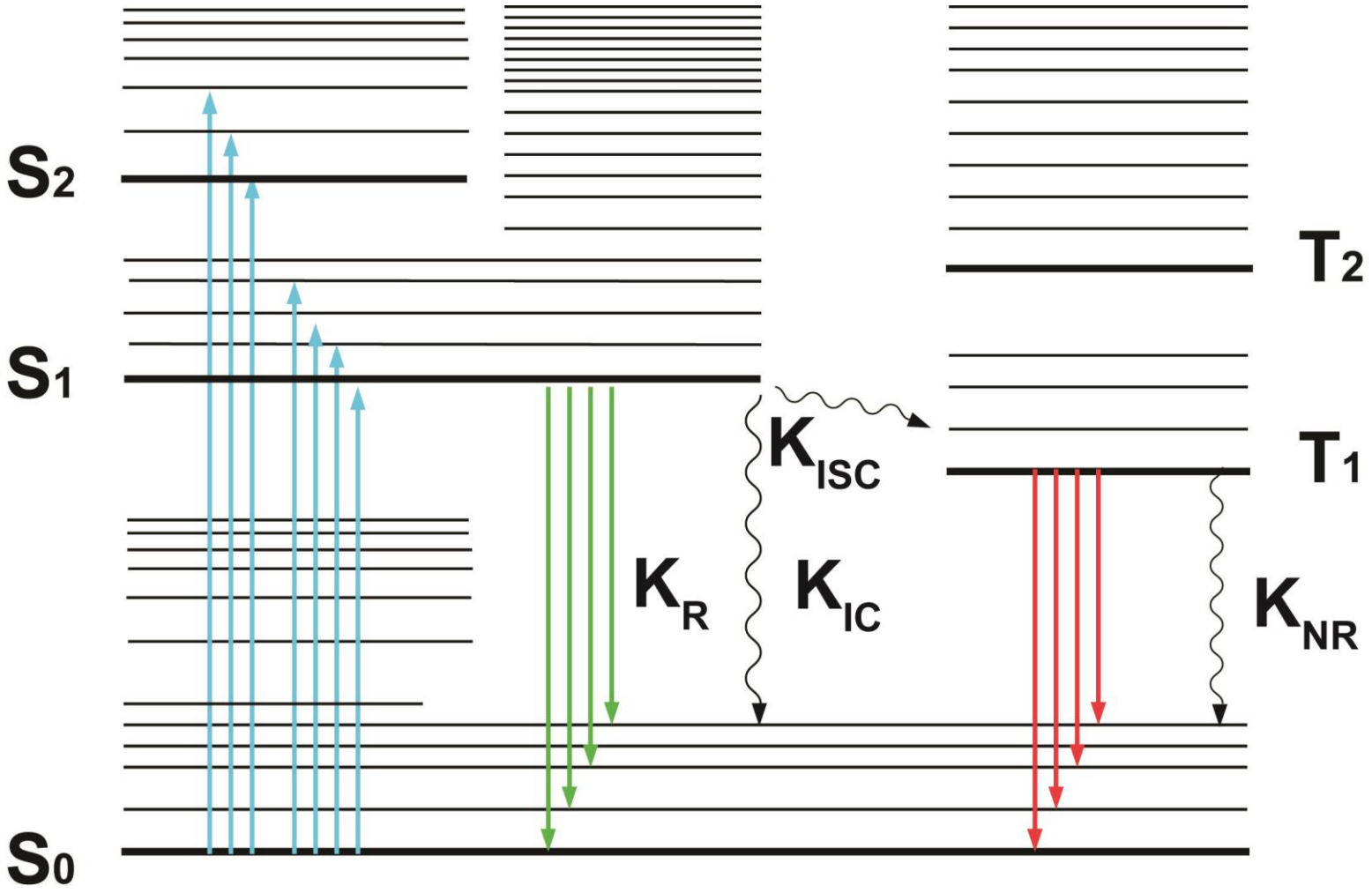


Digman, M.A., et al, Biophys J. 2008 Mar 15;94(6):2320-32

Summary

- FCS provides the dynamics and the <N> in the observation volume.
- PCH provides the brightness and the <N> in the observation volume.
- N&B distinguishes between number of molecules and molecular brightness in the same pixel. The immobile fraction can be separated since it has a brightness value =1.

Perrin-Jabłoński diagram



What is the Lifetime?

Absorption and emission processes are concepts that involve a population of molecules. In general, if N_1 is the population of the excited level S_1, the population is described by the relation:

$$\frac{dN_1}{dt} = -(k_R + k_{NR})N_1 + f(t)$$

$$N_1 = N_1(0)e^{-t/\tau_S}$$

$$\tau_S = \frac{1}{k_R + k_{NR}}$$

τ_S is the lifetime of excited state S_1

If a population of fluorophores is excited at time t=0, after a time the number of molecules in τ is decreased to 1/e or to about 36.8%

Application to Förster Resonance Energy Transfer (FRET)

FRET strongly depends on the distance between the donor and acceptor:

Förster calculated the rate of transfer to be

$$k_T = \frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6$$

τ_D is the lifetime of the donor D in the absence of the acceptor A.
R is the distance between the two groups
R_o is called the Förster distance
E is transfer efficiency (fraction of photons absorbed by the D and transferred to A)

$$E = 1 - \frac{\tau_D}{\tau_A}$$

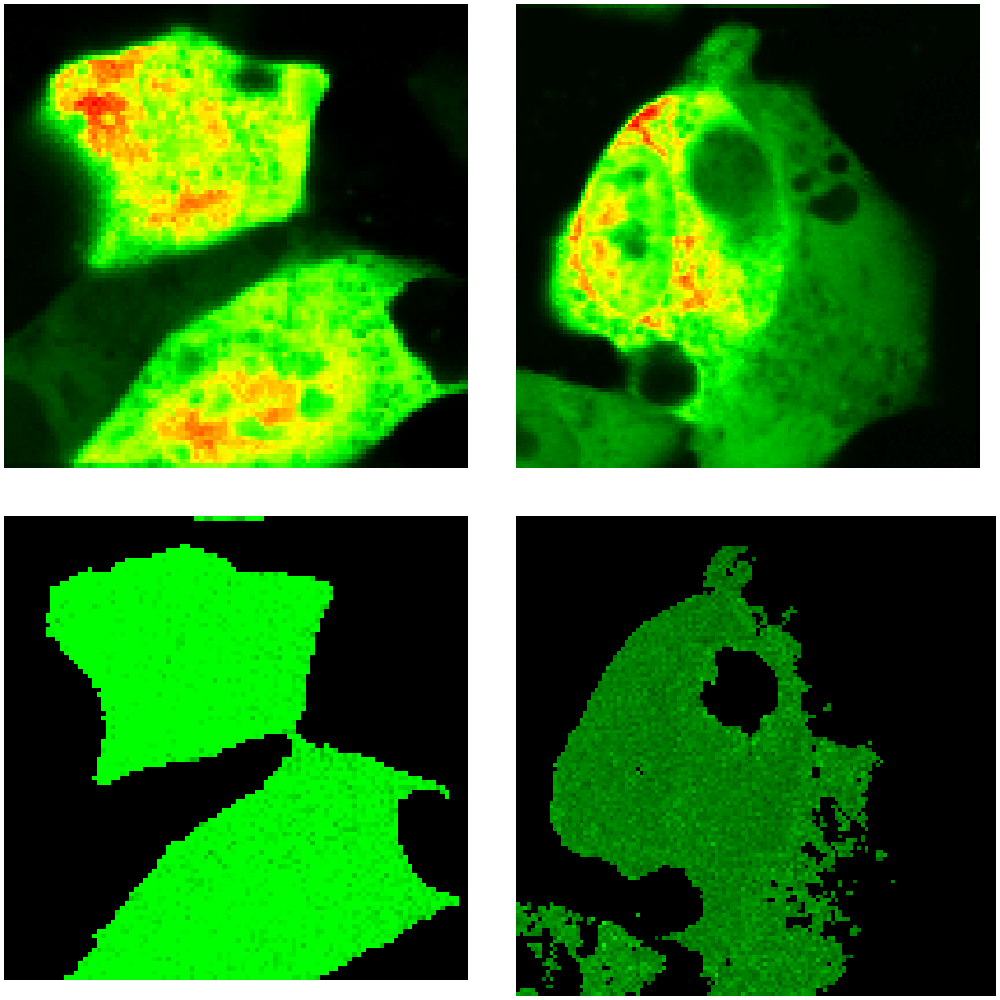
FRET

Images of opossum kidney cells expressing a CFP (left column) and CFP/YFP tandem protein (right column).

Top: intensity

Bottom: phase lifetime

Left: 2.1 ns; Right: 1.5 ns



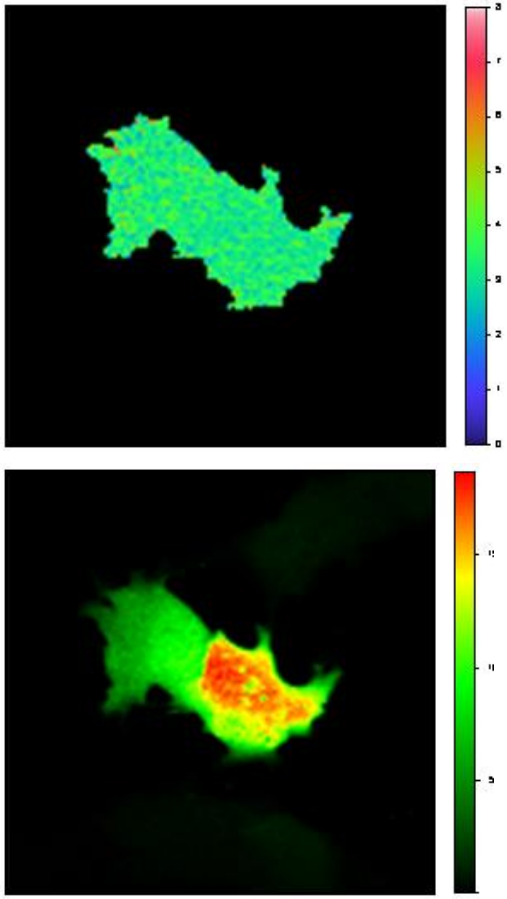
Images obtained in **frequency domain: Ti-Sapphire** – 2-photon exc. at 850 nm

The images in channel 1 were collected through a 520 – 560 nm YFP filter and in channel 2 through a 460 – 500 nm CFP filter.

Courtesy of Dr. Moshe Levi

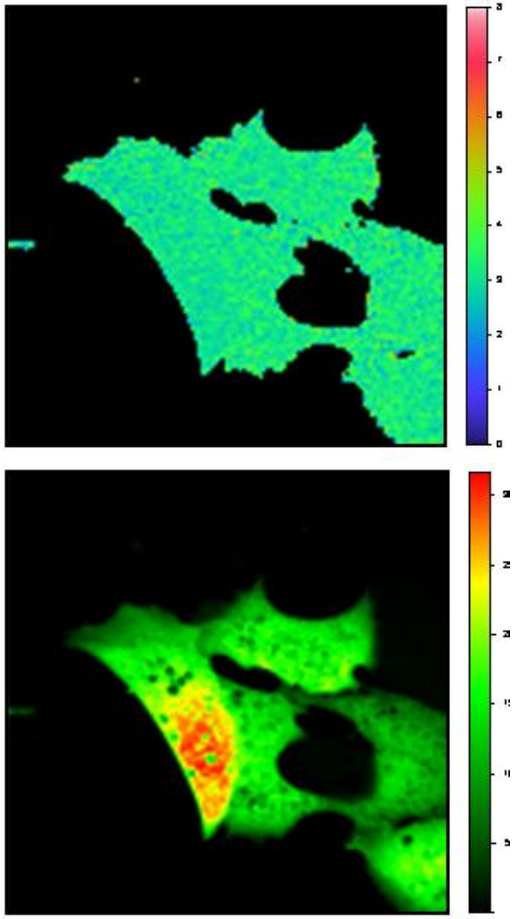
Applications to Cerulean-Venus pairs FRET

Cerulean



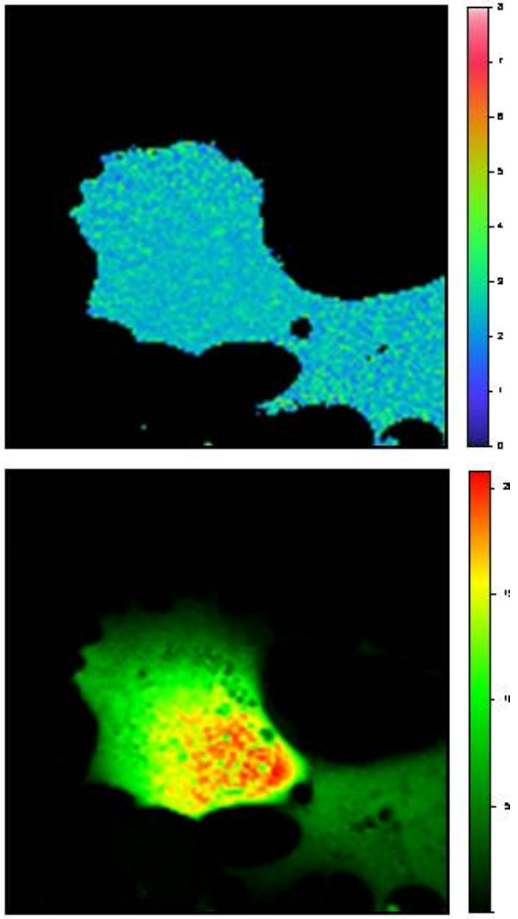
τ = 3.06 ns

Cerulean 32-venus



τ = 2.63 ns
E = 14%

Cerulean 17-venus



τ = 2.2 ns
E = 27%

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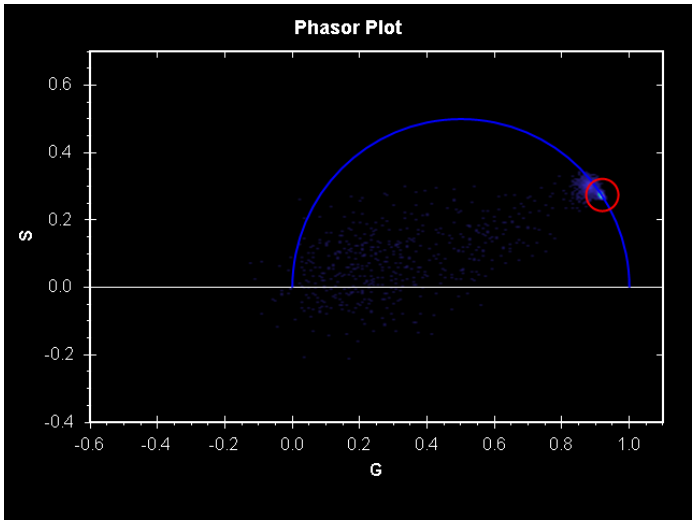
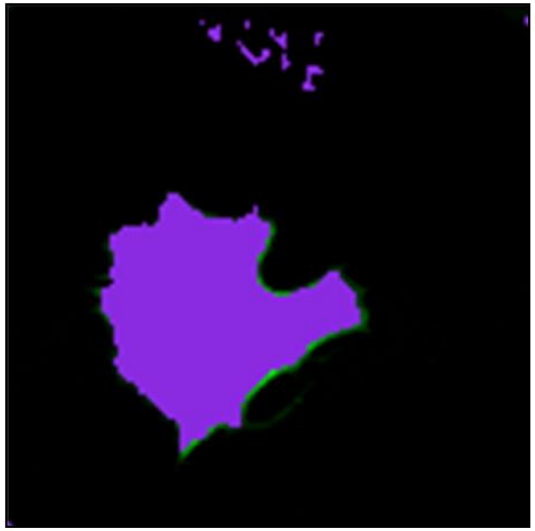
Courtesy of Dr. A. Periasamy

Polar Plots

C17v

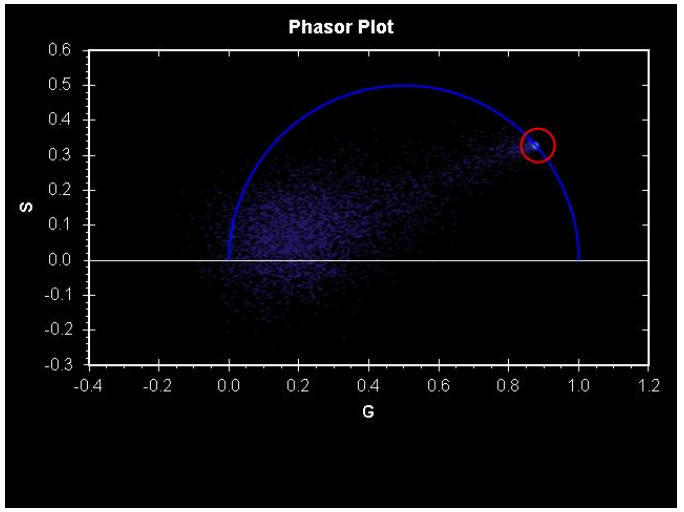
2.2 ns





C32v

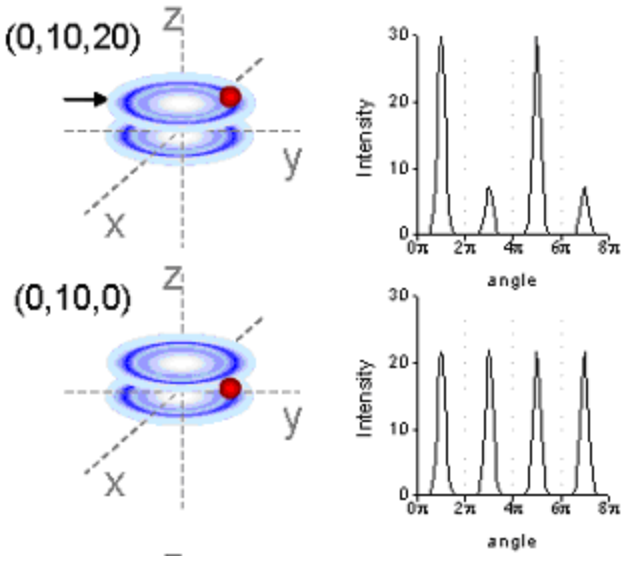
2.6 ns

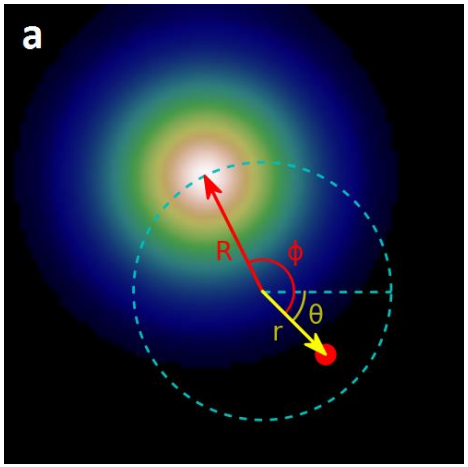


Summary FLIM

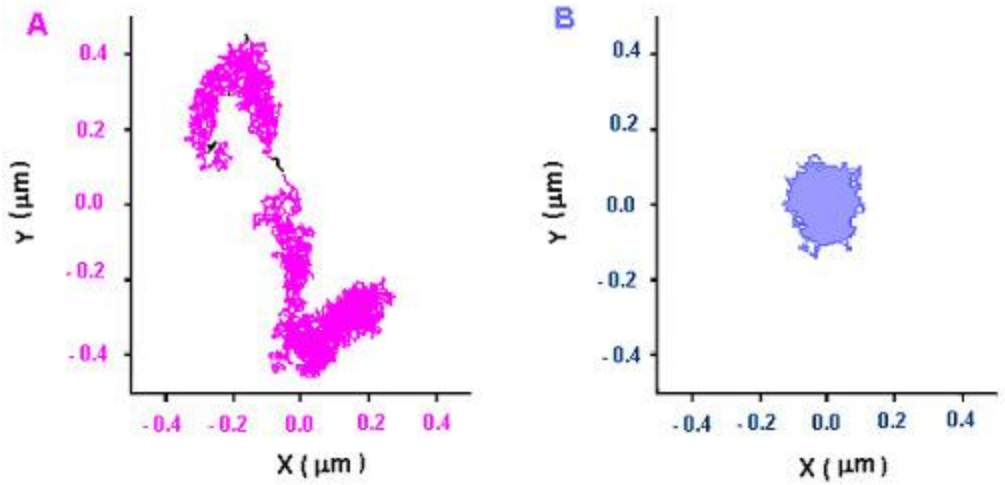
- Lifetime measurements provide a powerful tool for the characterization of several processes in materials and life sciences
- The new DFD approach makes FLIM in cells faster and more sensitive
- The Polar Plot approach greatly simplifies the data analysis
- FLIM can be added to most commercial LSM

Particle Tracking and Nanoimaging



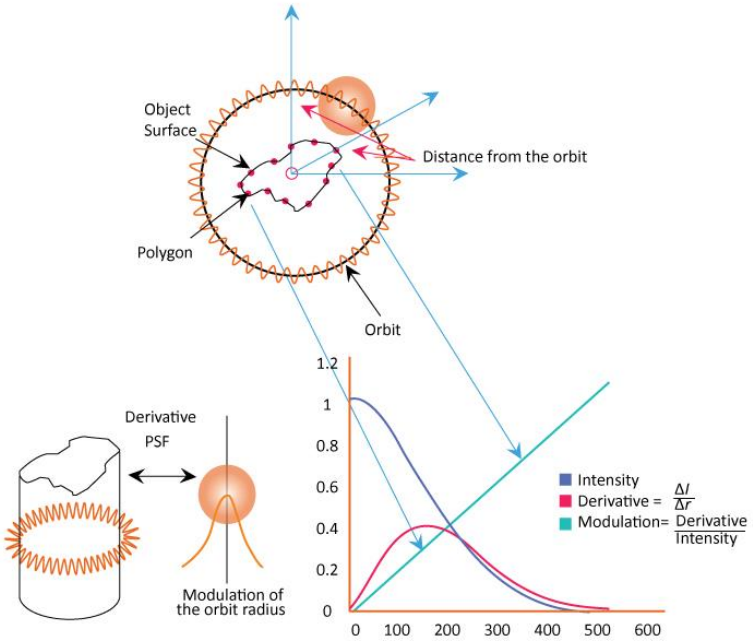


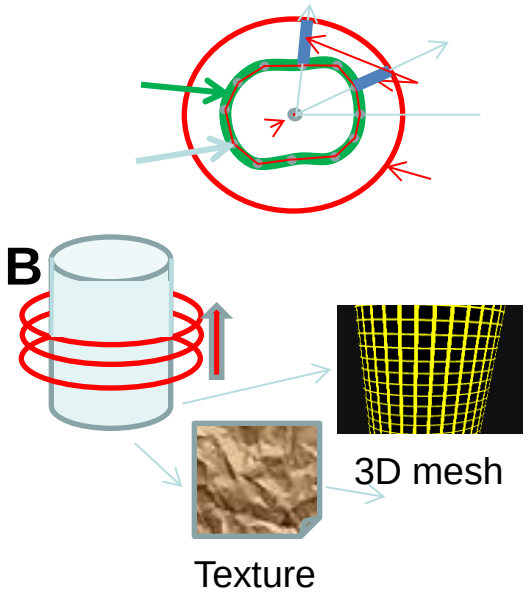
the radius of the orbit should be half of the width (ω_0) of PSF

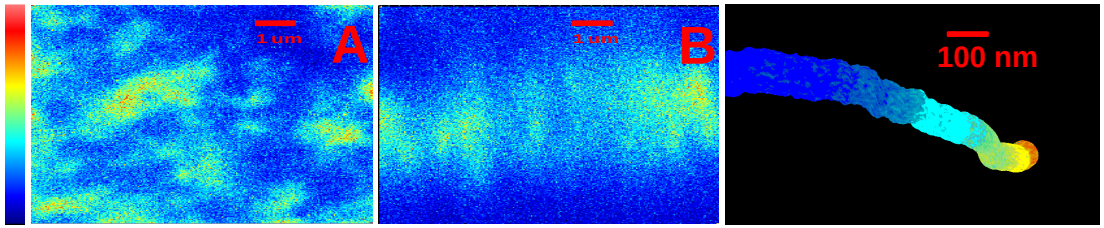


Dynamics of chromatin in living C6-14 cells. Trajectories recovered in a control (A) and an azidetreated (B) cell.

Particle Tracking and Nanoimaging







What are the requirements on the instrumentation?

- Optics and microscopes
  - High-speed galvo-mirrors
- Light Sources
- Light detectors
  - Avalanche photodiodes
  - GaAs PMTs
  - Hybrid PMTs

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