Lecture 7
Introduction to FCS

Enrico Gratton

Laboratory for Fluorescence Dynamics

From cuvette to the microscope

1. Excitation & Emission Spectra
    - Local environment polarity, fluorophore concentration
2. Anisotropy & Polarization
    - Rotational diffusion
3. Quenching
    - Solvent accessibility
    - Character of the local environment
4. Fluorescence Lifetime
    - Dynamic processes (nanosecond timescale)
5. Resonance Energy Transfer
    - Probe-to-probe distance measurements
6. Fluorescence microscopy
    - localization

7. **Fluorescence Correlation Spectroscopy**
    - **Translational & rotational diffusion**
    - **Concentration**
    - **Dynamics**

In the microscope, the spatial location matters: spatial correlations and distributions are a component of the experiment

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 on.

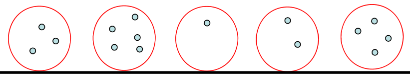
Introduction to “number” fluctuations

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

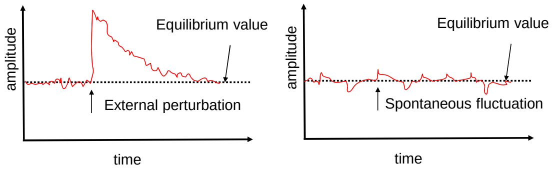
This principle does not tell us anything about the time of the fluctuations



The fluctuation-dissipation principle

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:

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120002001324123102111131125111023313332211122422122612214
23452411413114231001004211231232011110001111_2110013200000
1001100010002322100211000020100 1_333122000231221024011102_
12221122310001103311102101100101030113121210101211111211_10
003221012302012121321110110023312242110001203010100221734
410101002112211444421211440132123314313011222123310121111
2224122311133221321100004104320121200113222312 00_253212033
2331111002100220130113211131200101314322112211223234422 30
3214215322002021421232320431123120033142234521341104123 22
220221
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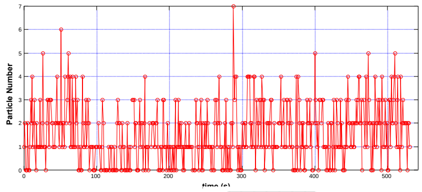
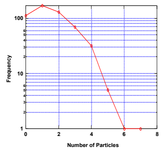
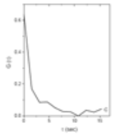
Collected data by counting (by visual inspection) the number of particles in the observation volume as a function of time using a “ultra microscope”

Particle Correlation







*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

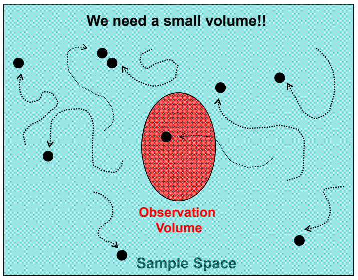
What can cause a fluctuation in the fluorescence signal???

- **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

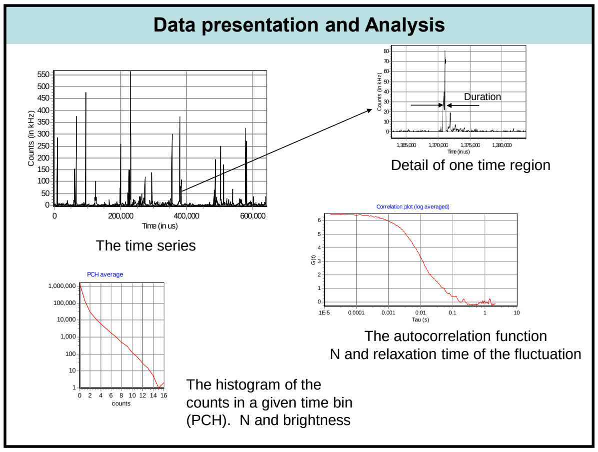
Each of the above processes has its own dynamics. FCS can recover that dynamics

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



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

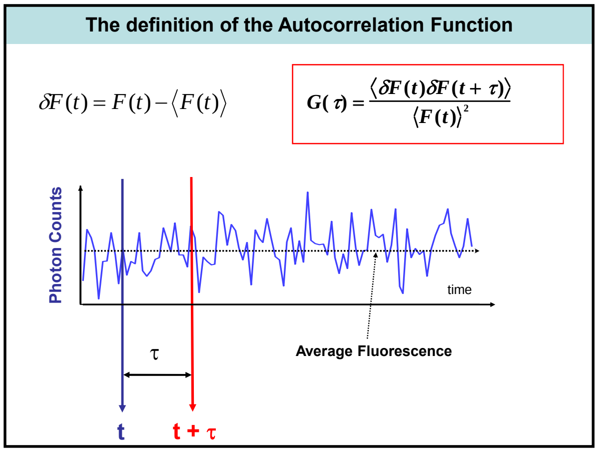
How to extract the information about the fluctuations and their characteristic time?

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**



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

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

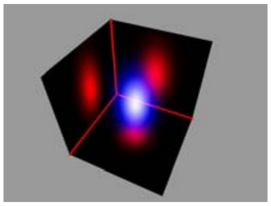
kQ = quantum yield and detector sensitivity (how bright is our probe). This term could contain the fluctuation of the fluorescence intensity due to internal processes

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?



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

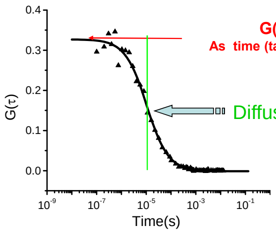
$$I(z) = Exp\left[-\frac{2z^2}{w_{0z}^2}\right]$$ Gaussian z

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

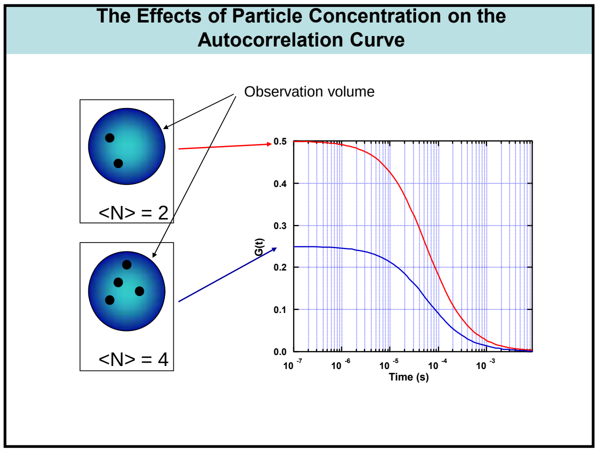
More on the PSF in Jay's lecture

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

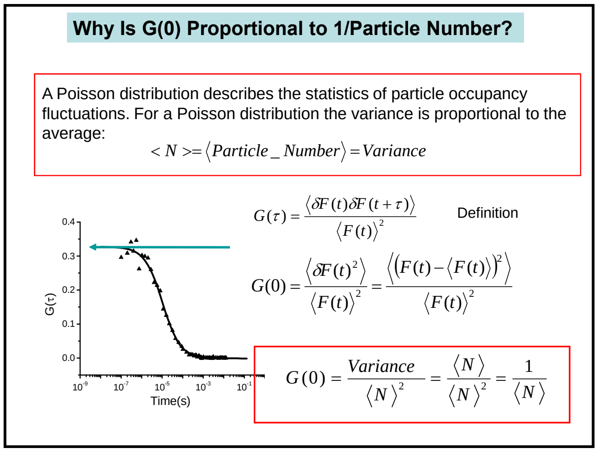
The Autocorrelation Function



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/Particle Number?

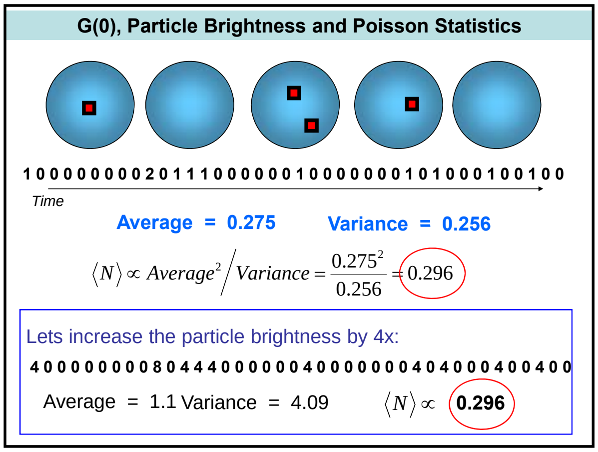
A Poisson distribution describes the statistics of particle occupancy fluctuations. For a Poisson distribution the variance is proportional to the average:

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

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

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

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



G(0), Particle Brightness and Poisson Statistics

1 0 0 0 0 0 0 0 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 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 $S=w/z$

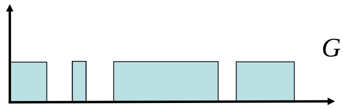
$$G(\tau)=\frac{\gamma}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\cdot\left(1+S^2\cdot\frac{\tau}{\tau_D}\right)^{-\frac{1}{2}}$$

Blinking or other exponential processes:

If the particle blinks during the times it goes through the illumination volume, an additional term appears in the fluctuation amplitude.

How to account for this process??

Reasoning: let us assume that the particle is **not moving** and it is at the center of the PSF.
The intensity will turn **ON** and **OFF**.
The **OFF** time depends on the characteristic blinking time (triplet state lifetime).
The **ON** time depends on the laser intensity. The larger the laser intensity, the lesser is the **ON** time.



$$G(\tau)=\left(1+\frac{T}{1-T}e^{-\frac{\tau}{\tau_T}}\right)$$

Triplet state term:

T is the triplet state amplitude
τ_T is the triplet lifetime.

Blinking and binding processes

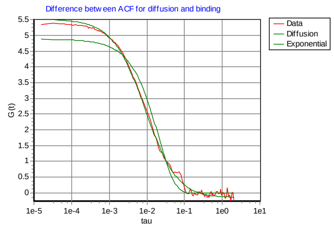
Until now, we assumed that the particle is **not moving**. If we assume that the blinking of the particle is **independent on its movement**, we can use a general principle that states that the correlation function splits in the product of the two independent processes.

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

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

$K = k_f / k_b$ 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(binding) from G(diffusion)?



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

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	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}

The Effects of Particle Size on the Autocorrelation Curve

Diffusion Constants

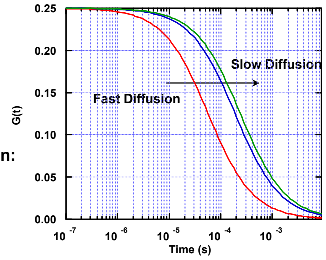
300 $\mathrm{um^2/s}$
90 $\mathrm{um^2/s}$
71 $\mathrm{um^2/s}$

Stokes-Einstein Equation:

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

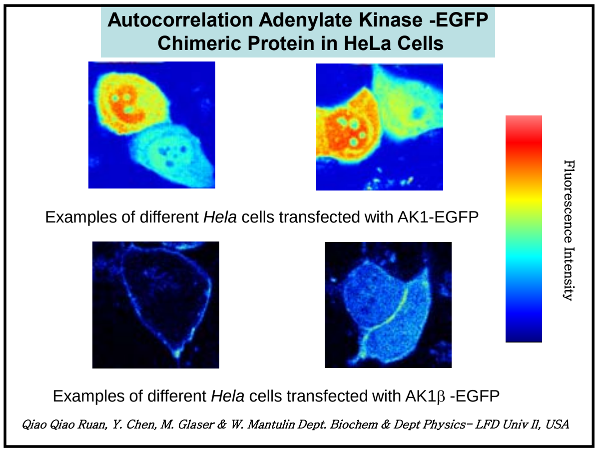
and

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



Monomer --> Dimer

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

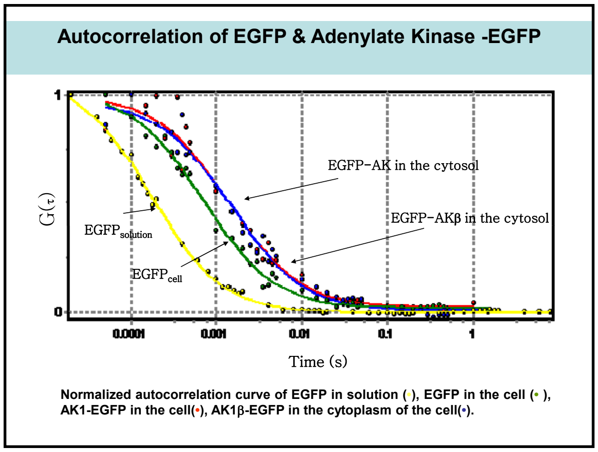


Autocorrelation Adenylate Kinase -EGFP Chimeric Protein in HeLa Cells

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

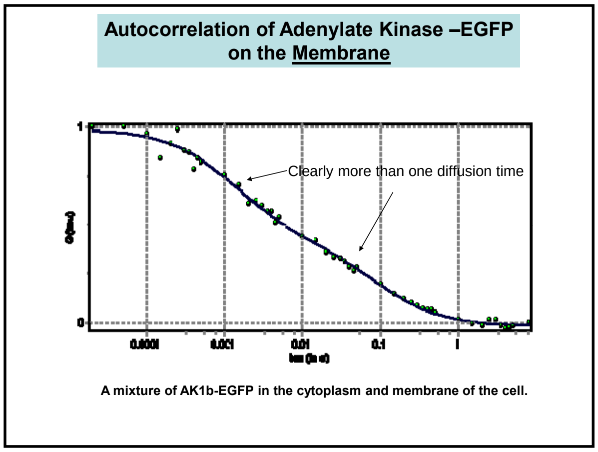
Examples of different *Hela* cells transfected with AK1β -EGFP

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



Autocorrelation of EGFP & Adenylate Kinase -EGFP

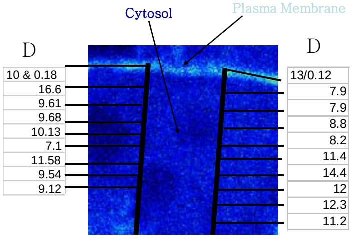
Normalized autocorrelation curve of EGFP in solution (●), EGFP in the cell (●), AK1-EGFP in the cell(●), AK1β-EGFP in the cytoplasm of the cell(●).



Autocorrelation of Adenylate Kinase –EGFP on the Membrane

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

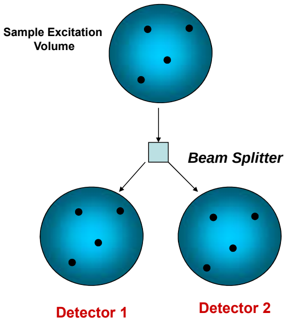
Autocorrelation Adenylate Kinaseβ -EGFP



Diffusion constants (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

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



Sample Excitation Volume

Beam Splitter

Detector 1

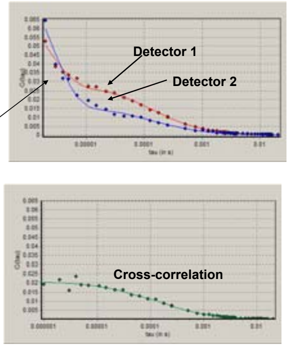
Detector 2

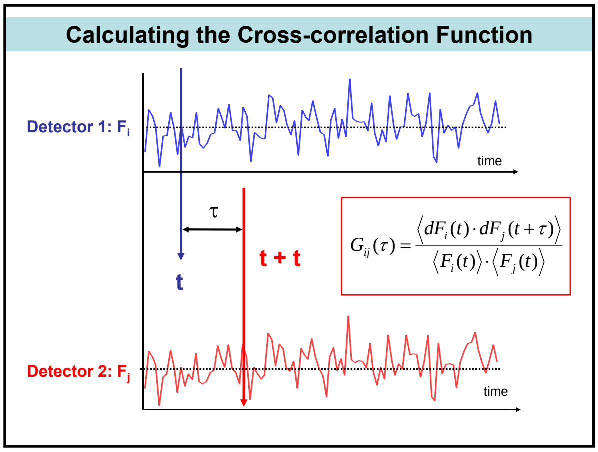
Each detector observes the same particles

Removal of Detector Noise by Cross-correlation

11.5 nM Fluorescein

Detector after-pulsing





Calculating the Cross-correlation Function

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

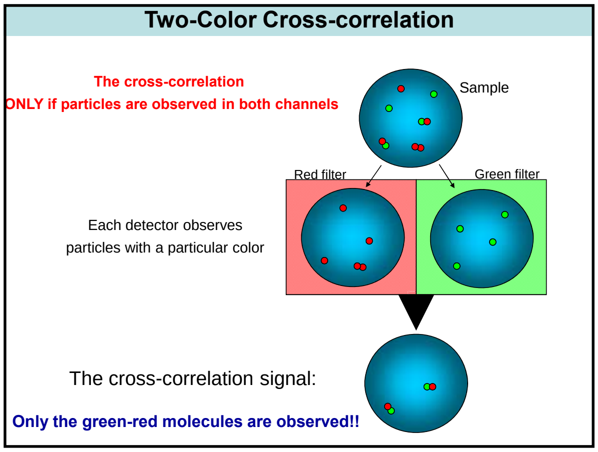
Cross-correlation calculations

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

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

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

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-Color Cross-correlation

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

Each detector observes particles with a particular color

The cross-correlation signal:

Only the green-red molecules are observed!!

Two-color Cross-correlation

Equations are similar to those for the cross correlation using a simple beam splitter:

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

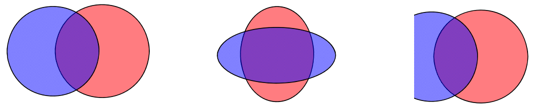
Information Content	**Signal**
Correlated signal from particles having **both colors**.	$G_{12}(\tau)$
Autocorrelation from channel 1 on the **green particles**.	$G_1(\tau)$
Autocorrelation from channel 2 on the **red particles**.	$G_2(\tau)$

Experimental Concerns: Excitation Focusing & Emission Collection

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

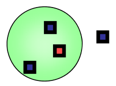
Excitation side:
- (1) Laser alignment
- (2) Chromatic aberration
- (3) Spherical aberration

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



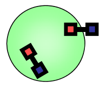
Two-Color Fluctuation Correlation Spectroscopy

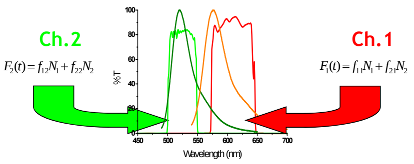
Uncorrelated



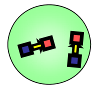
$$G_{ij}(\tau) = \frac{< F_i(t) F_j(t+\tau) >}{< F_i(t) >< F_j(t) >} - 1$$

Correlated



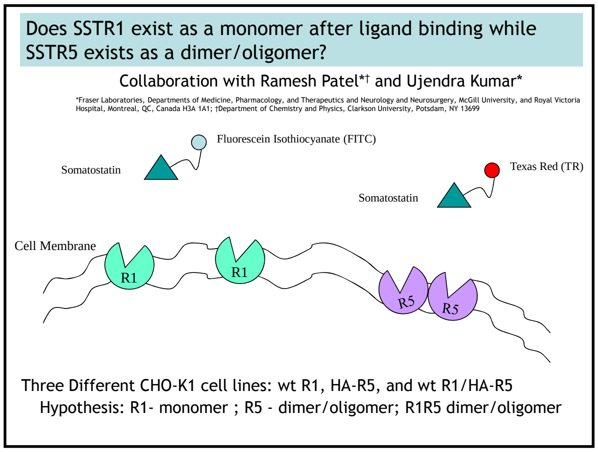


Interconverting



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

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



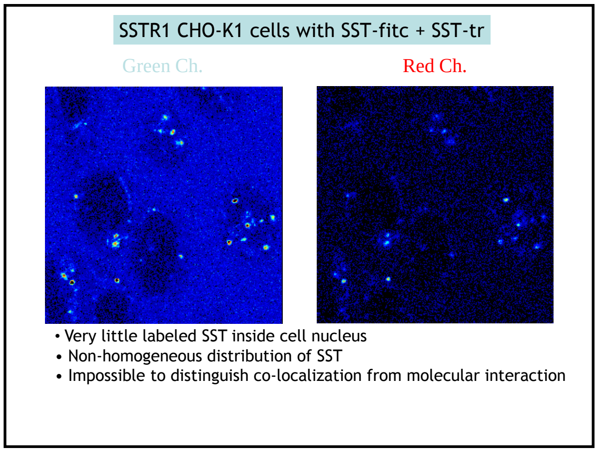
Does SSTR1 exist as a monomer after ligand binding while SSTR5 exists as a dimer/oligomer?

Collaboration with Ramesh Patel*† and Ujendra Kumar*

*Fraser Laboratories, Departments of Medicine, Pharmacology, and Therapeutics and Neurology and Neurosurgery, McGill University, and Royal Victoria Hospital, Montreal, QC, Canada H3A 1A1; †Department of Chemistry and Physics, Clarkson University, Potsdam, NY 13699

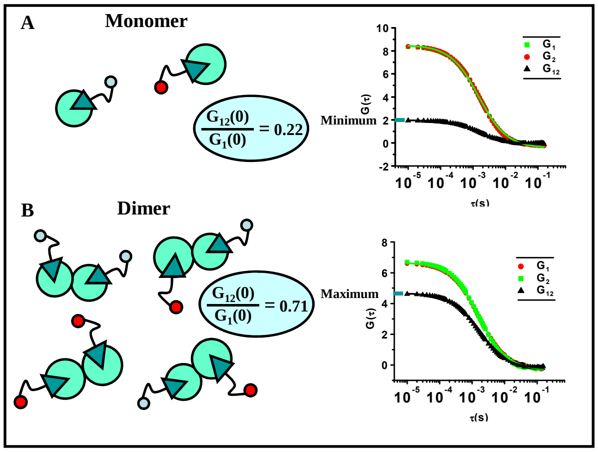
Three Different CHO-K1 cell lines: wt R1, HA-R5, and wt R1/HA-R5

Hypothesis: R1- monomer ; R5 - dimer/oligomer; R1R5 dimer/oligomer

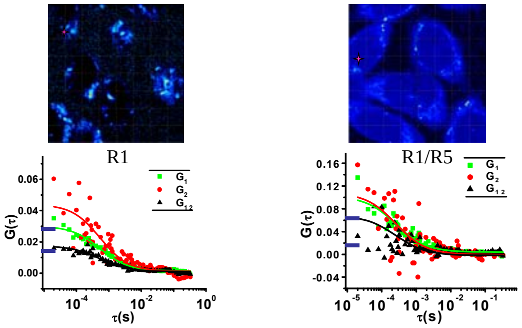


SSTR1 CHO-K1 cells with SST-fitc + SST-tr

- Very little labeled SST inside cell nucleus
- Non-homogeneous distribution of SST
- Impossible to distinguish co-localization from molecular interaction



Experimentally derived auto- and cross-correlation curves from live R1 and R5/R1 expressing CHO-K1 cells using dual-color two-photon FCS.



The R5/R1 expressing cells have a greater cross-correlation relative to the simulated boundaries than the R1 expressing cells, indicating a higher level of dimer/oligomer formation.

Patel, R.C., *et al.*, *Ligand binding to somatostatin receptors induces receptor-specific oligomer formation in live cells.* PNAS, 2002. **99**(5): p. 3294-3299

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?

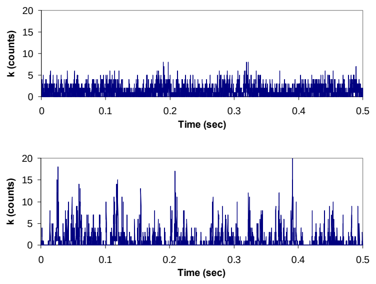
6. ……and many more questions

The Photon Counting Histogram: Statistical Analysis of Single Molecule Populations

Transition from FCS

- The Autocorrelation function only depends on fluctuation duration and fluctuation density (independent of excitation power)
- PCH: distribution of intensities (independent of time)

Fluorescence Trajectories



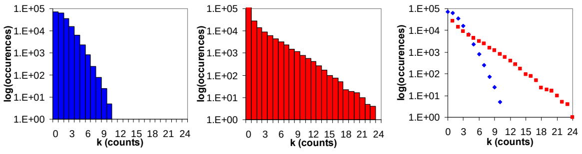
Fluorescent Monomer:

Intensity = 115,000 cps

Aggregate:

Intensity = 111,000 cps

Photon Count Histogram (PCH)

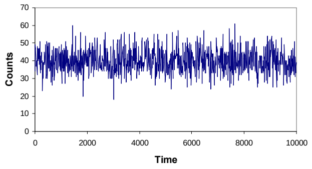


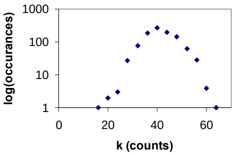
Can we quantitate this?

What contributes to the distribution of intensities?

Contribution from the detector noise

Fixed Particle Noise (Shot Noise)



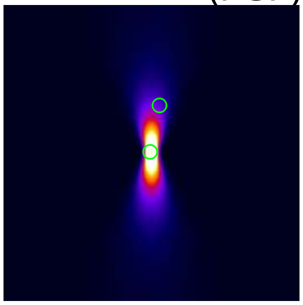


Noise is Poisson

$$Poi(k,\langle k\rangle)=\frac{\langle k\rangle^{k}}{k!}\exp\left(-\langle k\rangle\right)$$

Contribution from the profile of illumination

The Point Spread Function (PSF)



One Photon Confocal:

$$I_{3DG}(r,z)=\exp\left(-\frac{2r^{2}}{\omega_{0}^{2}}-\frac{2z^{2}}{z_{0}^{2}}\right)$$

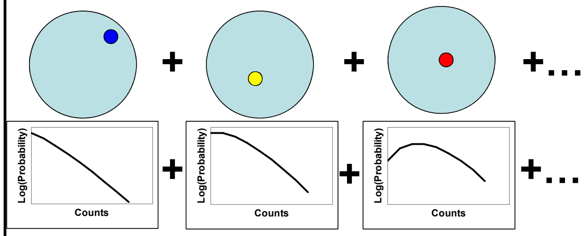
Two Photon:

$$I_{GL^{2}}(r,z)=\frac{4\omega_{0}^{4}}{\pi^{2}\omega^{4}(z)}\exp\left(-\frac{4r^{2}}{\omega^{2}(z)}\right)$$

$$\omega^{2}(z)=\omega_{0}^{2}\left(1+\left(\frac{z}{z_{R}}\right)^{2}\right)$$

$$z_{R}=\frac{\pi\omega_{0}^{2}}{\lambda}$$

Single Particle PCH



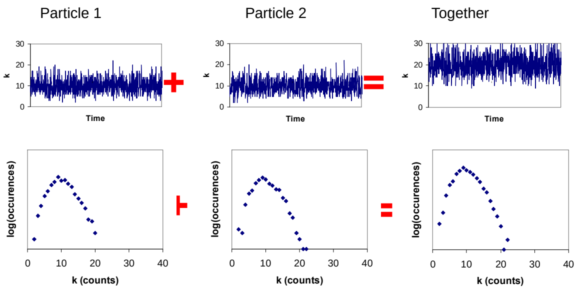
Have to sum up the poissonian distributions for all possible positions of the particle within the PSF

$$p^{(1)}(k)=\frac{1}{V_{0}}\int_{V_{0}}Poi\left(k,\varepsilon\overline{PSF}(\vec{r})\right)d\vec{r}$$

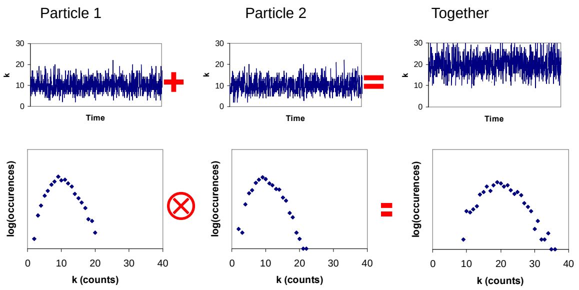
- What if I have two particles in the PSF?
- Have to calculate every possible position of the second particle for each possible position of the first!

Contribution from several particles of same brightn

Combining Distributions

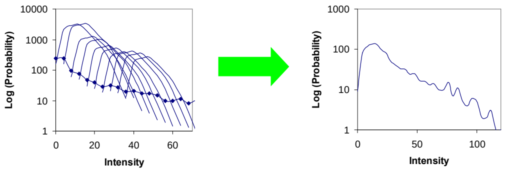


Combining Distributions



Convolution

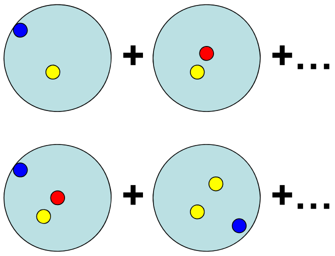
- Sum up all combinations of two probability distributions (joint probability distribution)
- Distributions (particles) must be independent



$$p^{(1+2)}(k) = \sum_{r=0}^{r=k} p^{(1)}(k-r) \cdot p^{(2)}(r)$$

Contribution from particles of different brightn

More Particles



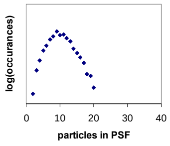
$$p^{(2)}(k) = p^{(1)}(k) \otimes p^{(1)}(k)$$

$$p^{(3)}(k) = p^{(1)}(k) \otimes p^{(2)}(k)$$

$$p^{(n)}(k) = p^{(1)}(k) \otimes p^{(n-1)}(k) = \sum_{r=0}^{r=k} p^{(1)}(k-r) \cdot p^{(n-1)}(r)$$

How Many Particles Do We Have in the PSF?

$$P(n,N) = Poi(n,N)$$



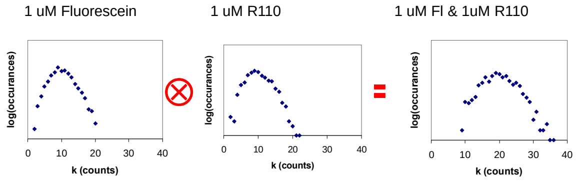
Particle occupation fluctuates around average, N with a poissonian distribution

Calculate poisson weighted average of n particle distributions

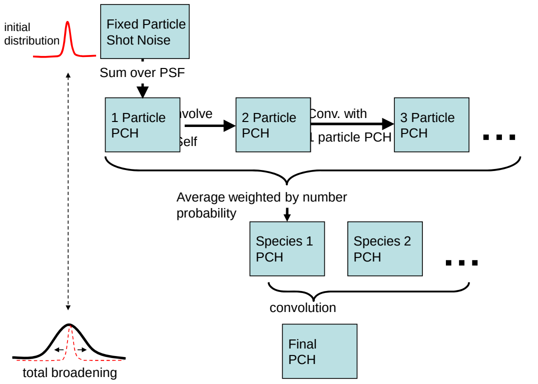
$$PCH(k,N) = \sum_{n} p^{(n)}(k) \cdot P(n,N)$$

Multiple Species

- Species are independent so just convolute!



Recap: Factors that contribute to the final broadening of the PCH



initial distribution

Fixed Particle Shot Noise

Sum over PSF

1 Particle PCH

volve

Self

2 Particle PCH

Conv. with

1 particle PCH

3 Particle PCH

. . .

Average weighted by number probability

Species 1 PCH

Species 2 PCH

. . .

convolution

Final PCH

total broadening

Method

- Sum up Poisson distributions from all possible arrangements and number of fluorophores in excitation volume (PSF)
  - Intensity weighted sum of all possible single particle histograms (Poisson functions)
  - Convolution to get multiple particle histograms
  - Number probability weighted sum of multiple particle histograms
  - Convolution to get multi-species histograms

Chen et al., *Biophys. J.*, **1999,** *77,* 553.

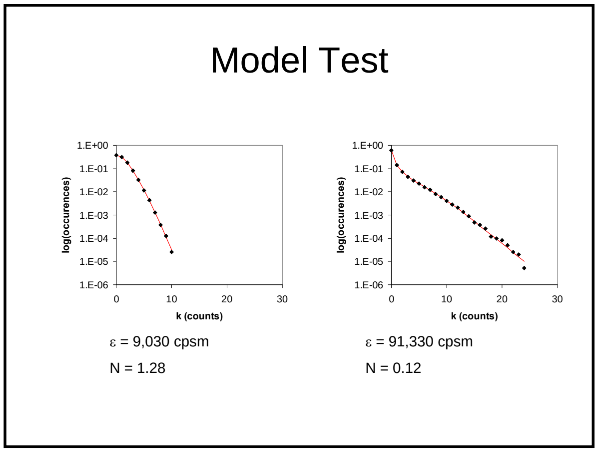
Fitting

$$\chi^2 = \frac{\sum_k \left(M \frac{PCH_{model}(k) - PCH_{observed}(k)}{\sqrt{M \cdot PCH_{observed}(k) \cdot (1 - PCH_{observed}(k))}} \right)^2}{k_{\max} - d}$$

M is number of observations

d is number of fitting parameters

Chen et al., *Biophys. J.*, **1999,** *77,* 553.



Model Test

ε = 9,030 cpsm

N = 1.28

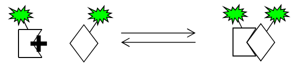
ε = 91,330 cpsm

N = 0.12

Hypothetical situation: Protein Interactions

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

Dimer has double the brightness



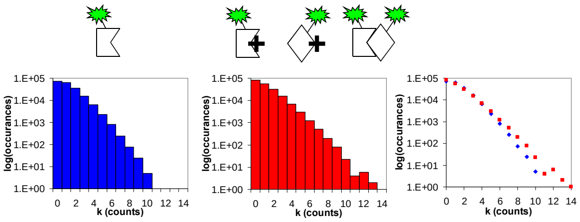
$\varepsilon = \varepsilon_{monomer}$

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

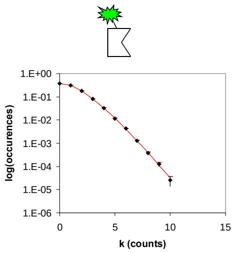
All three species are present in equilibrium mixture

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

Photon Count Histogram (PCH)

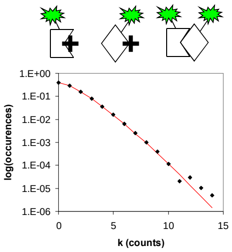


Simulation Solution



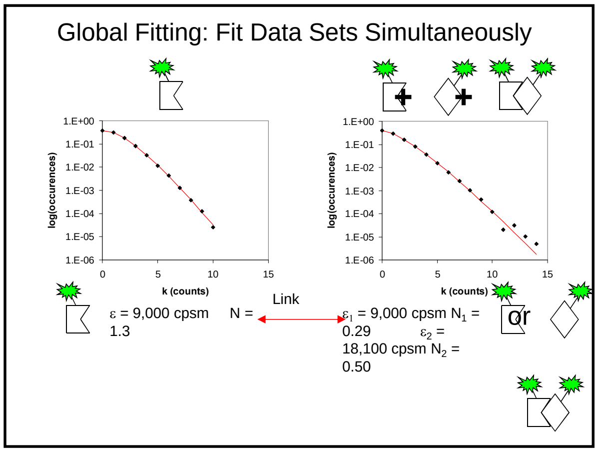
ε = 9,000 cpsm

N = 1.3



ε = 16,000 cpsm

N = 0.73



Global Fitting: Fit Data Sets Simultaneously

ε = 9,000 cpsm N = 1.3

ε_1 = 9,000 cpsm N_1 = 0.29 ε_2 = 18,100 cpsm N_2 = 0.50

What we measure is the number of particles in the PSF. How Do We Get Concentrations?

- N is defined relative to PSF volume $V_{PSF} = \int PSF(\vec{r})d\vec{r}$
- One photon:

$$V_{3DG} = w_0^2 z_0 \left(\pi / 2\right)^{3/2}$$

- Two photon:

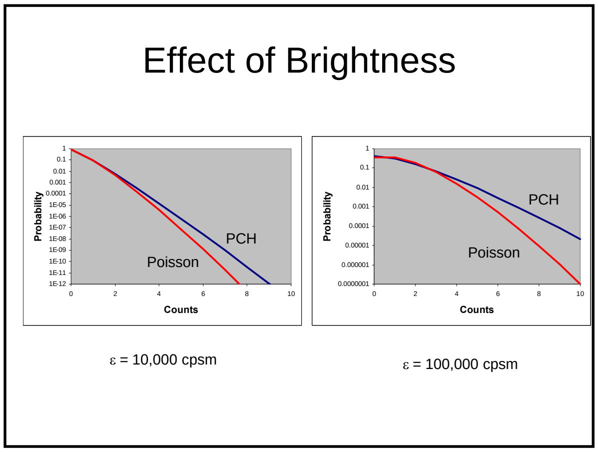
$$V_{GL2} = \frac{\pi w_0^4}{\lambda}$$

- Definition is same as for FCS
- Can use FCS to determine w_0 (and maybe z_0)

w_0 = 0.21 um, z_0 = 1.1 um, V_{PSF} = 0.091 um^3, C = 23 nM

How to Improve Accuracy

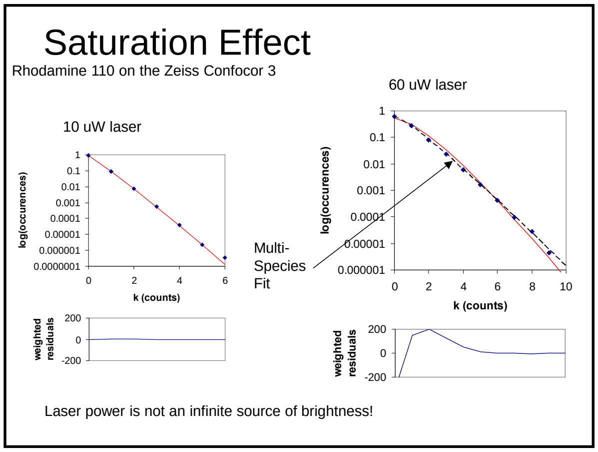
- Minimize sources of instrument noise
  - PSF heterogeneity
  - Shot noise
- Maximize particle burst amplitudes



Effect of Brightness

ε = 10,000 cpsm

ε = 100,000 cpsm



Saturation Effect

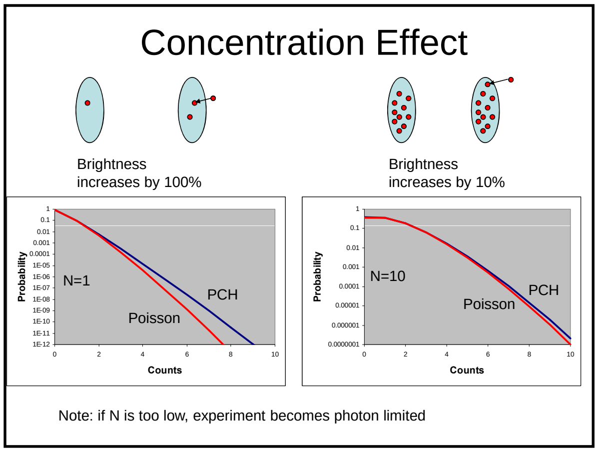
Rhodamine 110 on the Zeiss Confocor 3

10 uW laser

60 uW laser

Multi-Species Fit

Laser power is not an infinite source of brightness!

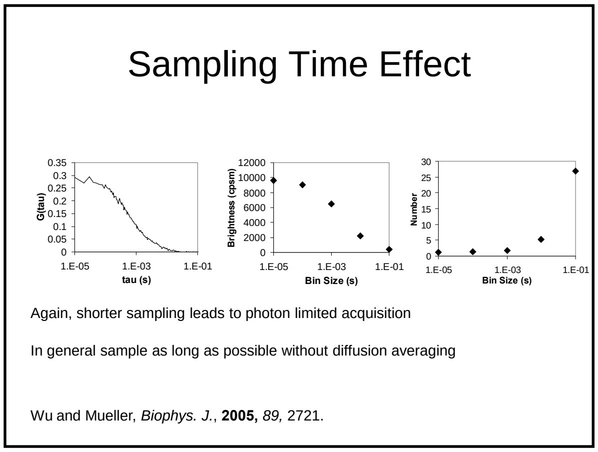


Concentration Effect

Brightness increases by 100%

Brightness increases by 10%

Note: if N is too low, experiment becomes photon limited

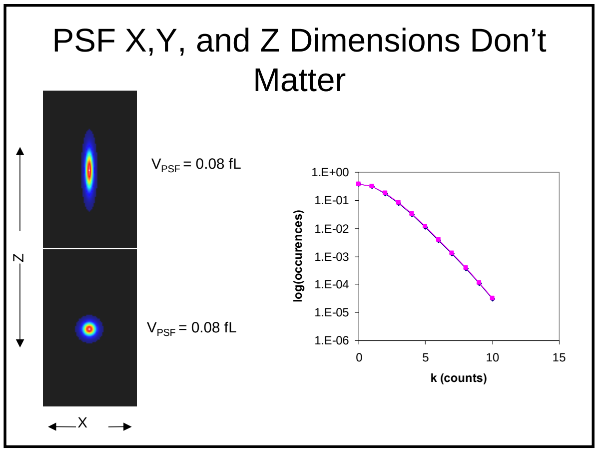


Sampling Time Effect

Again, shorter sampling leads to photon limited acquisition

In general sample as long as possible without diffusion averaging

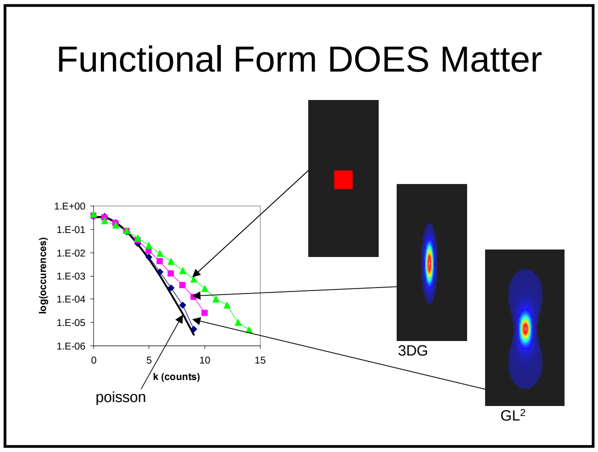
Wu and Mueller, *Biophys. J.*, **2005,** *89,* 2721.



PSF X,Y, and Z Dimensions Don’t Matter

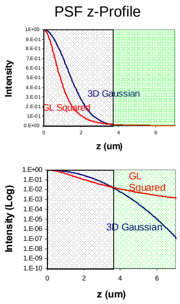
V_{PSF} = 0.08 fL

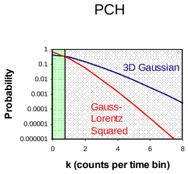
V_{PSF} = 0.08 fL



Functional Form DOES Matter

Functional Form Matters for PCH


PSF z-Profile


PCH

Point Spread Function Effects

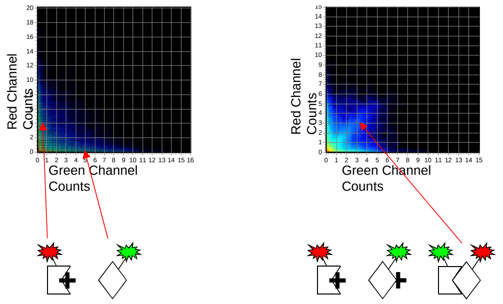
$$p^{(1)}(k) = \frac{1}{V_0}\int_{V_0} Poi\left(k, \varepsilon \overline{PSF}(\vec{r})\right) d\vec{r}$$

This equation will work for ANY PSF shape.

Alternative Methods

- Fluorescence Cumulant Analysis (FCA)
  - Mueller *Biophys. J.* **2004**, *86,* 3981.
  - Similar to method of moments
  - Any distribution can be described by a sum of moments
  - Simple algebraic formulas for cumulants
- Fluorescence Intensity Distribution Analysis (FIDA)
  - Kask et al. *PNAS* **1999,** *96,* 13756.
  - Fits PSF in fourier transformed space
  - Fits to non-physical parameterized PSF

2D PCH



Calculating the 2D PCH Function

$$PCH(\varepsilon_A, \varepsilon_B, N; k_A, k_B) = \binom{k}{k_A} (\varepsilon_A / \varepsilon)^{k_A} (1 - \varepsilon_A / \varepsilon)^{k - k_A} \cdot PCH(\varepsilon, N; k)$$

the binomial distribution:

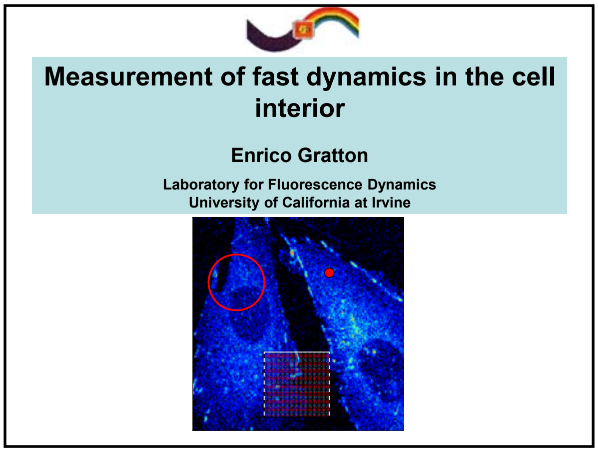
$$P(x, k, N) = \binom{N}{k} x^k (1 - x)^{N-k}$$

We can find the 2D PCH function from the single channel PCH function!

Chen et al., *Biophys. J.*, **2005,** *88,* 2177-2192.

Summary

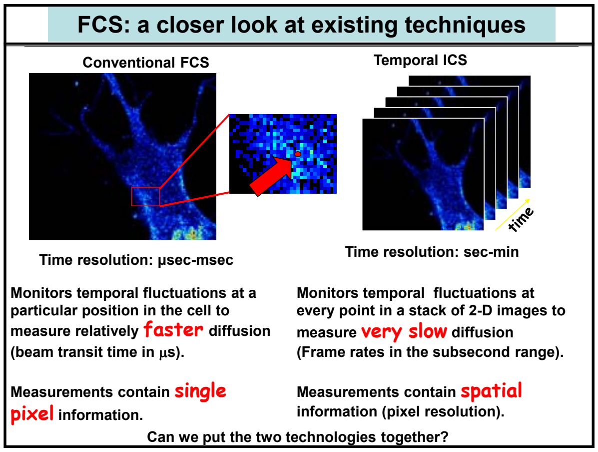
- The photon count histogram can be modeled by integration of component noise sources
- Heterogeneous samples can be resolved through global analysis
- Accuracy is related to magnitude of particle fluctuations relative to instrument fluctuations



Measurement of fast dynamics in the cell interior

Enrico Gratton

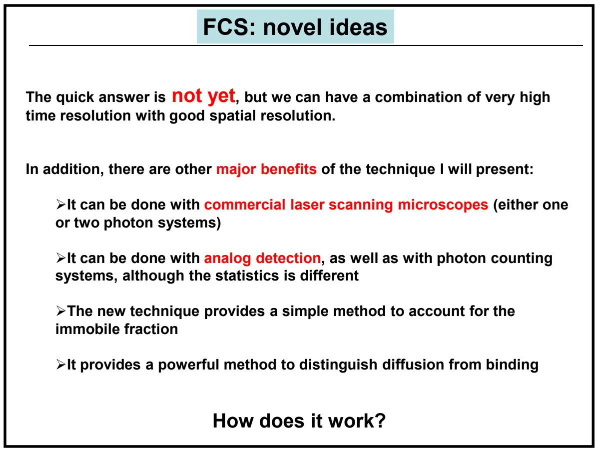
Laboratory for Fluorescence Dynamics
University of California at Irvine



FCS: a closer look at existing techniques

Conventional FCS	Temporal ICS
Time resolution: µsec-msec	Time resolution: sec-min
Monitors temporal fluctuations at a particular position in the cell to measure relatively **faster** diffusion (beam transit time in µs).	Monitors temporal fluctuations at every point in a stack of 2-D images to measure **very slow** diffusion (Frame rates in the subsecond range).
Measurements contain **single pixel** information.	Measurements contain **spatial** information (pixel resolution).

Can we put the two technologies together?



FCS: novel ideas

The quick answer is **not yet**, but we can have a combination of very high time resolution with good spatial resolution.

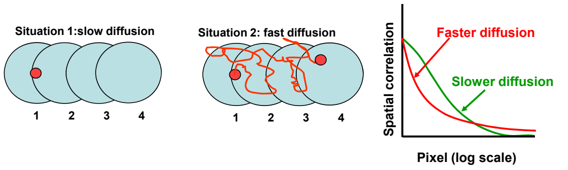
In addition, there are other major benefits of the technique I will present:

- It can be done with commercial laser scanning microscopes (either one or two photon systems)
- It can be done with analog detection, as well as with photon counting systems, although the statistics is different
- The new technique provides a simple method to account for the immobile fraction
- It provides a powerful method to distinguish diffusion from binding

How does it work?

Temporal information hidden in the raster-scan image: the RICS approach

Images obtained with a laser scanning microscope contain temporal information because they are recorded sequentially pixel after pixel as opposed to a camera snap shot.

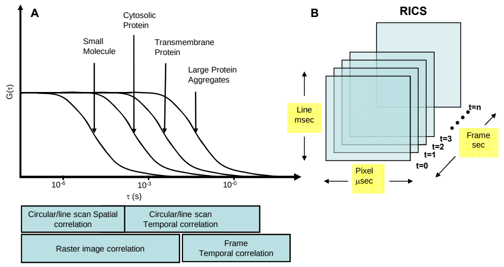


Situation 1: The particle is not moving or slowly moving. A signal is detected at position 1 and 2 but not at 3 and 4. The spatial correlation of the fluorescence at different pixels lasts for the extent of the PSF.

Situation 2: The particle is moving fast. There is a chance to get fluorescence even at location 3 and 4. The spatial extent of the correlation increases depending on the time scale of the diffusion.

RICS= Raster-scan Image Correlation Spectroscopy

Available time scales in RICS



Range of diffusion times accessible by different RICS techniques. Depending of the time scale of the process, pixel (µs), line (ms) or frame (s) correlation methods can be used. Points long a line are microsecond apart. Points in successive lines are millisecond apart and frames are second apart.

What is the new idea? (in few words)

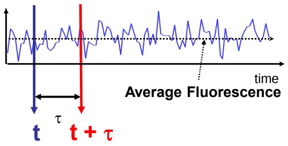
- Because of scanning, spatial and temporal coordinates are related
- By performing spatial correlations, we can obtain temporal (and spatial) information in a very fast time scale as well as in slow time scale
- What we need is to assign a proper time to different spatial locations

The RICS approach: correlation functions

The mathematics and concepts for computation

What is different in RICS is the way the correlation function is calculated. We are familiar with the concept of correlation function of a time series. Definitions:

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



To calculate this function efficiently, the time series must be continuous. Generally, data points are collected every δt. The autocorrelation function is then calculated using either direct numerical algorithms or the FFT method.

If the time series is not continuous, but it has regular gaps the correlation function is modulated (convolution with a periodic square wave).

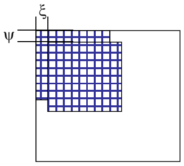
The RICS approach: 2-D spatial correlations

In a raster-scan image, points are measured at different positions and at different times simultaneously

If we consider the time sequence, it is not continuous in time
If we consider the "image", it is contiguous in space

In the RICS approach we calculate the 2-D spatial correlation function (similarly to the ICS method of Petersen and Wiseman)

$$G_{RICS}(\xi,\psi) = \frac{<I(x,y)I(x+\xi,y+\psi)>}{<I(x,y)>^2}$$



The variables ξ and ψ represent spatial increments in the x and y directions, respectively

2-D spatial correlation can be computed very efficiently using FFT methods.

To introduce the "RICS concept" we must account for the relationship between time and position of the scanning laser beam.

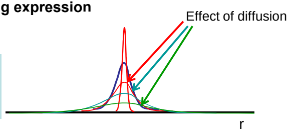
The RICS approach for diffusion

We assume that the correlations due to spatial scanning and the correlations due to the dynamics at a point are "independent", i.e., the dynamics at a point is independent on the scanning motion of the laser beam

$$G_{RICS}(\xi,\psi) = S(\xi,\psi) \mathrm{x} G(\xi,\psi)$$

Consider now the process of diffusion (as one example!). The diffusion kernel can be described by the following expression

$$C(r,t) = \frac{1}{(4\pi Dt)^{3/2}}\exp(-\frac{r^2}{4Dt})$$



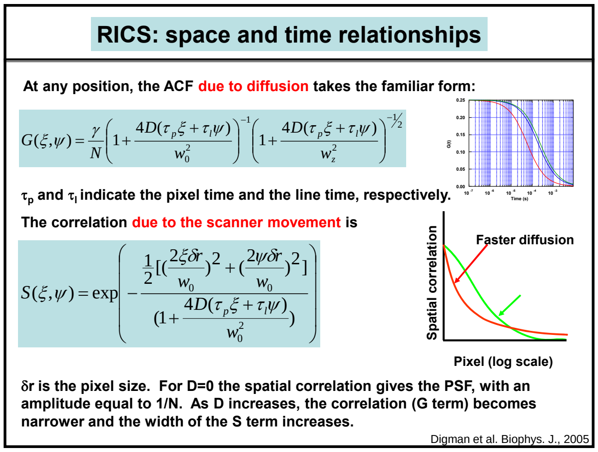
Given a particle at the origin at time zero, it can be found at time t at a distance r with a gaussian probability function with standard deviation that increases as a function of time and amplitude that decreases as a function of time

RICS: space and time relationships

In the usual expressions for FCS we just substitute τ with the term $\tau_p\xi + \tau_{ly}\psi$ where p and l indicate the pixel time and the line time, respectively.

The correlation due to the scanner movement is the expression for the PSF broadened by the molecule diffusion. For D=0 the spatial correlation gives the PSF, with an amplitude equal to 1/N. As D increases, the correlation (G term) becomes narrower and the width of the S term increases.

Digman et al. Biophys. J., 2005



RICS: space and time relationships

At any position, the ACF due to diffusion takes the familiar form:

$$G(\xi,\psi) = \frac{\gamma}{N}\left(1+\frac{4D(\tau_p\xi+\tau_l\psi)}{w_0^2}\right)^{-1}\left(1+\frac{4D(\tau_p\xi+\tau_l\psi)}{w_z^2}\right)^{-1/2}$$

τ_p and τ_l indicate the pixel time and the line time, respectively.

The correlation due to the scanner movement is

$$S(\xi,\psi) = \exp\left(-\frac{\frac{1}{2}\left[\left(\frac{2\xi\delta r}{w_0}\right)^2+\left(\frac{2\psi\delta r}{w_0}\right)^2\right]}{\left(1+\frac{4D(\tau_p\xi+\tau_l\psi)}{w_0^2}\right)}\right)$$

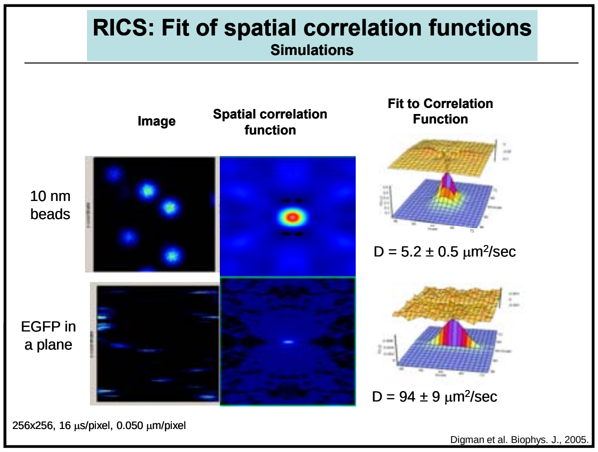
δr is the pixel size. For D=0 the spatial correlation gives the PSF, with an amplitude equal to 1/N. As D increases, the correlation (G term) becomes narrower and the width of the S term increases.

Digman et al. Biophys. J., 2005

Performing a RICS measurement

Setup: any laser confocal microscope

- **Acquire a raster scan image with a pixel time in the microsecond range and a line scan time in the millisecond range**
  - **Example: diffusion of EGFP in solution**
    - **Pixel size=0.05µm**
    - **Pixel time=10µs**
    - **Frame size 256x256**
    - **Acquisition = >10frames**
- **Calculate the 2-D spatial correlation (the RICS analysis).**
- **Fit the 2-D spatial correlation with the previous equations (see Digman et al, BJ 2005).**



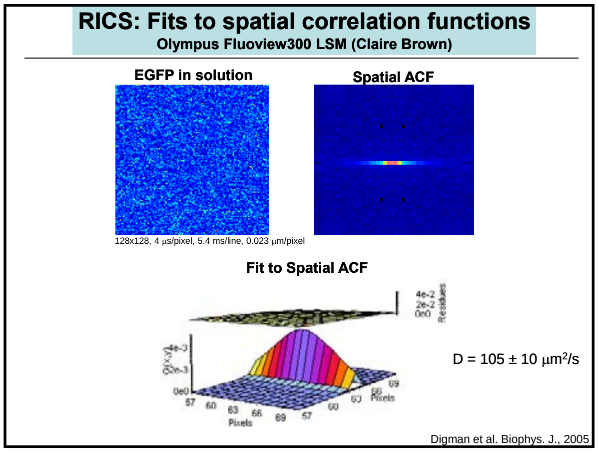
RICS: Fit of spatial correlation functions

Simulations

	Image	Spatial correlation function	Fit to Correlation Function
10 nm beads			D = 5.2 ± 0.5 μm²/sec
EGFP in a plane			D = 94 ± 9 μm²/sec

256x256, 16 μs/pixel, 0.050 μm/pixel

Digman et al. Biophys. J., 2005.



RICS: Fits to spatial correlation functions

Olympus Fluoview300 LSM (Claire Brown)

EGFP in solution

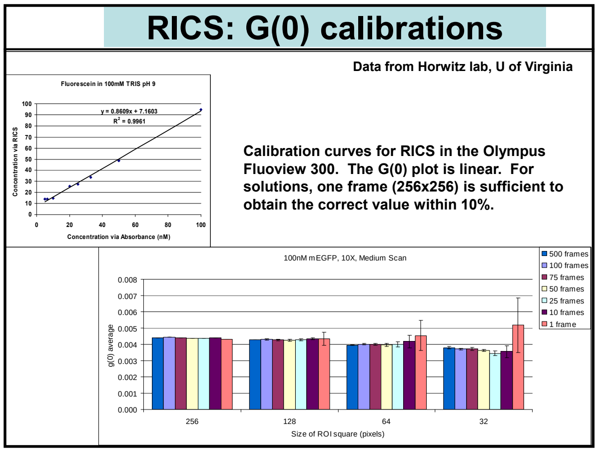
Spatial ACF

128x128, 4 μs/pixel, 5.4 ms/line, 0.023 μm/pixel

Fit to Spatial ACF

D = 105 ± 10 μm²/s

Digman et al. Biophys. J., 2005



RICS: G(0) calibrations

Data from Horwitz lab, U of Virginia

Calibration curves for RICS in the Olympus Fluoview 300. The G(0) plot is linear. For solutions, one frame (256x256) is sufficient to obtain the correct value within 10%.

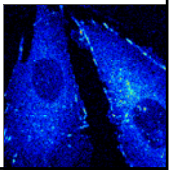
How we go from solutions to cells?

In cells we have an **immobile fraction**: molecules not moving during the course of the experiment.

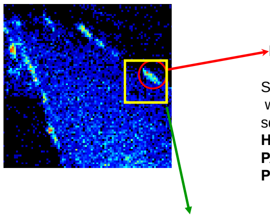
If we perform the 2-D-image correlation operation of an image that contains immobile features, we obtain the transform (power spectrum) of the image. In this transform is impossible to distinguish the moving particles.

We need to separate this immobile fraction from the mobile part before calculating the transform

How is this achieved?



Removal of the immobile fraction



Immobile fraction gives 5000 counts

Since a point with 5,000 counts will fluctuate with an amplitude with the square root of 5,000 (=70)
HOW CAN WE DETECT THE SMALL PARTICLES (molecules) IN THE PRESENCE OF THIS LARGE NOISE?

Small particle give 50 counts

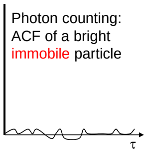
RICS: Removal of slowly varying component

Very often cells (or parts of the cell) move.

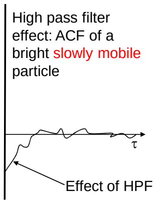
Instead of subtracting the average (over the entire image stack), we could subtract a local moving average. This is equivalent to high-pas filtering of the image sequence: only the fast changing features remain. In our software, it is possible to use different moving average lengths, depending on the speed of motion of the quasi immobile features.

Warning: The principle that the shot noise is time and space uncorrelated is not valid. After high-pass filtering, the intensity of one pixel carries to the next, both in time and in space, introducing correlations that were not there originally. However, the effect of filtering can be predicted and recognized.

Photon counting: ACF of a bright immobile particle

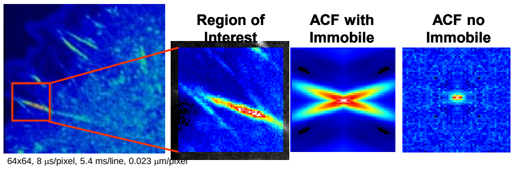


High pass filter effect: ACF of a bright slowly mobile particle



RICS: Immobile Component Removal

Paxillin-EGFP in CHO K1



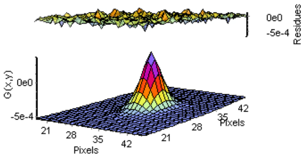
Region of Interest

ACF with Immobile

ACF no Immobile

64x64, 8 μs/pixel, 5.4 ms/line, 0.023 μm/pixel

Fit to ACF with Immobile Component Removed

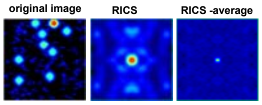


D = 0.49 ± 0.05 μm²/s

Digman et al. Biophys. J., 2005

Subtraction of the immobile fraction

The stack of images contains both mobile and immobile bright particles. The correlation function mostly reflects the shape of the immobile bright particles. After subtraction, the RICS only shows the fast diffusing molecules!

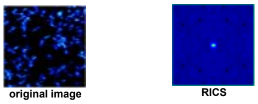


original image

RICS

RICS -average

The stack of images contains only mobile particles. The RICS and the subtracted RICS are identical



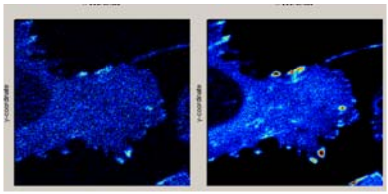
original image

RICS

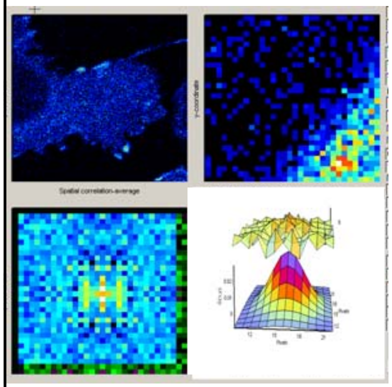
The same method can be used to subtract very slowly moving structure to account for the cell movement

Simultaneous Cell imaging:
ChoK1 cells expressing Paxillin-EGFP

256x256pixel (35.5μm), 32μs/pixel, 10.4ms/line, ω_0=0.42, total run time 5.2min



Procedures for processing images



Pixel size= 0.14200
Pixel time=32
Pixel seq =10.4
Wo(in um)= 0.46000
G(0) = 0.03855
D = 0.21350

Map of paxillin-EGFP diffusion rate