Introduction to scanning FCS

Enrico Gratton

University of California Irvine

Laboratory for Fluorescence Dynamics

The principle of FCS and scanning FCS

- ✓ Introduction to number fluctuations
- ✓ Measuring single molecules passing through the volume of illumination

Scanning FCS provides spatiotemporal correlations

Outline

- Introduction
- The principle of scanning FCS
- Data acquisition, processing and analysis
- Scanning FCS in cells
- Example

Introduction to scanning FCS

When we first applied FCS to cells, a series of problem arose

- The average intensity suddenly changed, perhaps due to the passage of a vesicle at the point of observation
- Bleaching of the immobile fraction occurred, causing a large deviation of the apparent correlation curve
- The cell could have moved, so that the volume of observation was not any more the chosen one

Approaches to FCS in cells

- Manufacturers (Zeiss and ISS) built instrument for solution experiments. They were asked by many researchers to be able to directly perform FCS measurements in cells

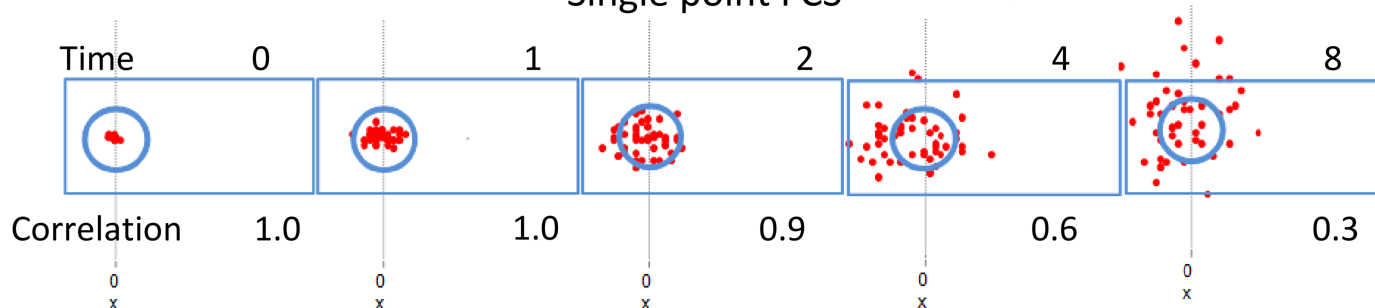
- Zeiss produced the Confocor 2 and Confocor 3, in which it was possible to alternate the capability of performing FCS at one point with the confocal unit

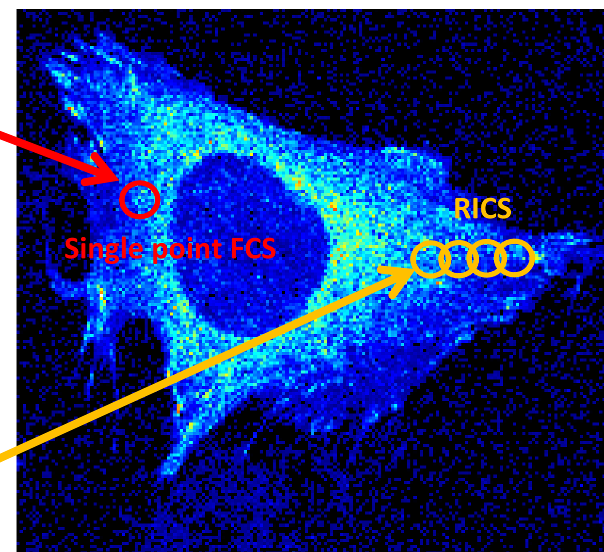
- ISS produced an instrument to raster scan the sample in a "conventional FCS unit", thereby joining imaging with FCS, but always at two separate times

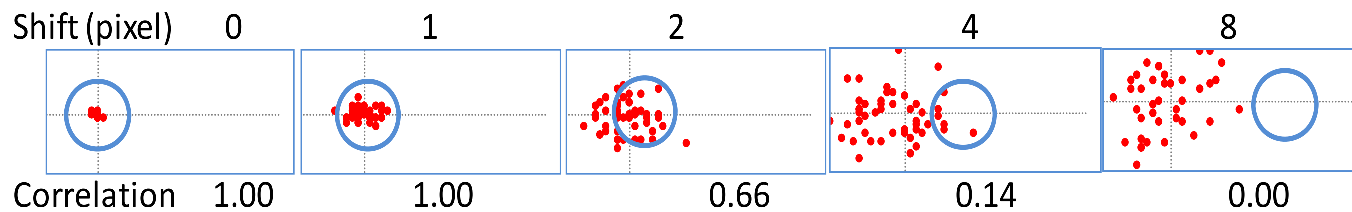
At the LFD we took a radically different approach:

the scanning FCS principle

Fluctuation analysis: single point and scanning

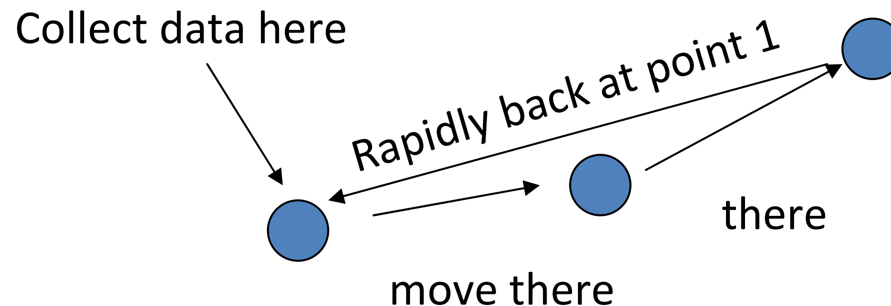






The principle of scanning FCS

If we can move the point at which we acquire FCS data fast enough to other points and then **return** to the original point "before" the particle had left the volume of excitation, then we can "multiplex the time" and collect FCS data at several points simultaneously!

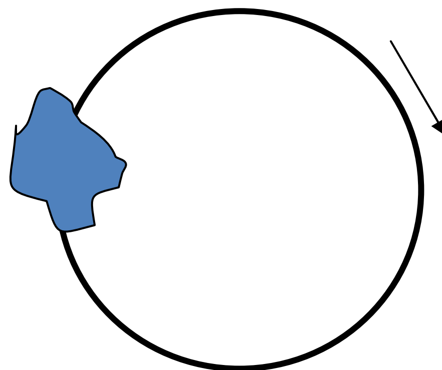


Why circular scanning? Circular scanning is faster!

The fastest way to scan several points and the return to the original point is to perform a circular orbit using the scanner galvo.

The x- and y-galvos are driven by 2 sine waves shifted by 90 degrees, thereby obtaining a projected orbit on the sample.

One orbit could be performed in times of less than 1 ms, using conventional galvo drivers and in microseconds using AOD



Timing in scanning FCS

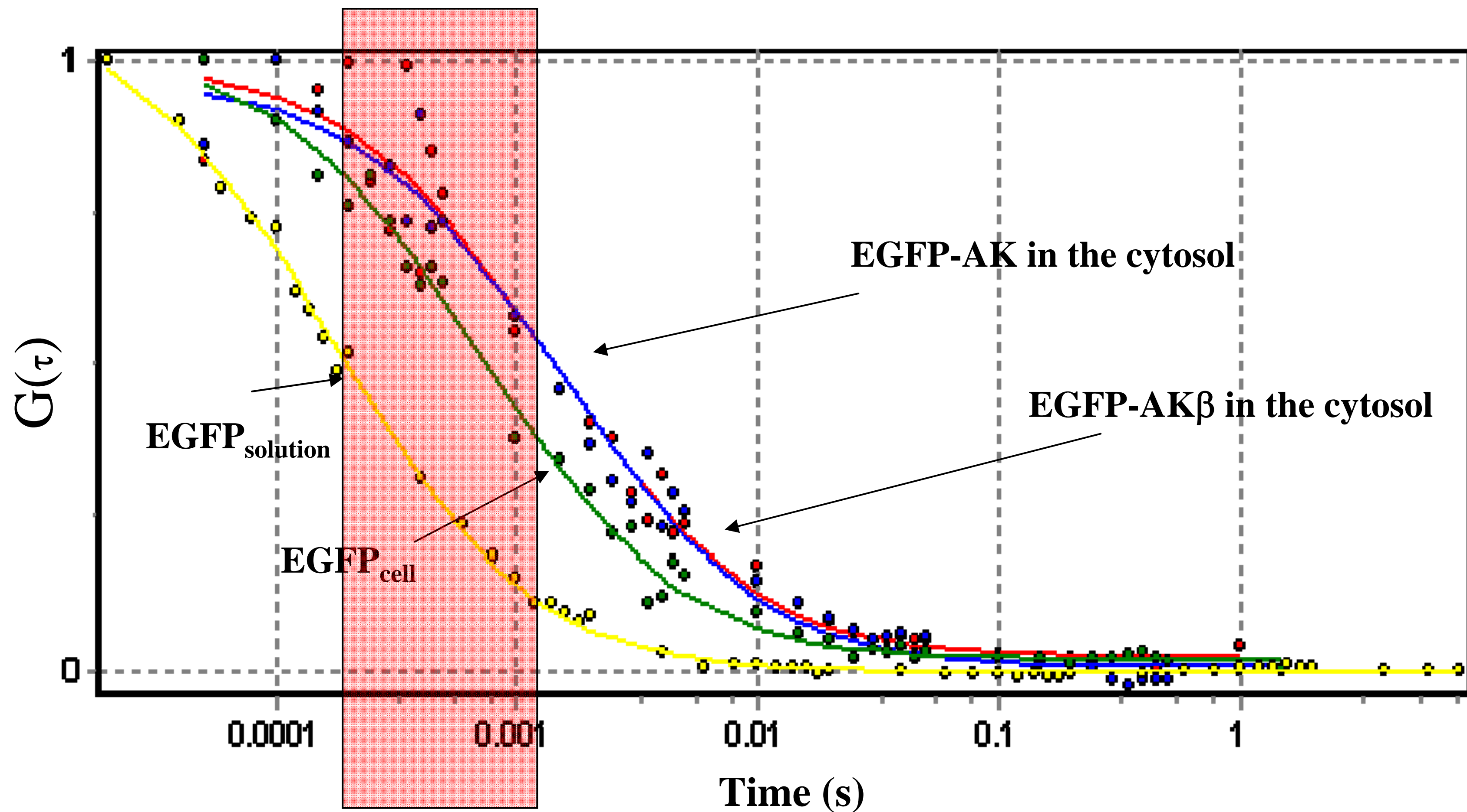
What is the minimum time required for an orbit so that we will not miss the "fastest" diffusion process in a cell?

EGFP diffuses with an apparent diffusion of approximately **20 $\mu m^2/s$**. The transit across the laser beam (assuming a w_0 of 0.35 μm) is about 1.5 ms! (formula used: time=$w_o^2/4D$)

Therefore **0.5 to 1 ms** per orbit should catch the GFP diffusing in a cell. Faster diffusing molecules will be partially missed.

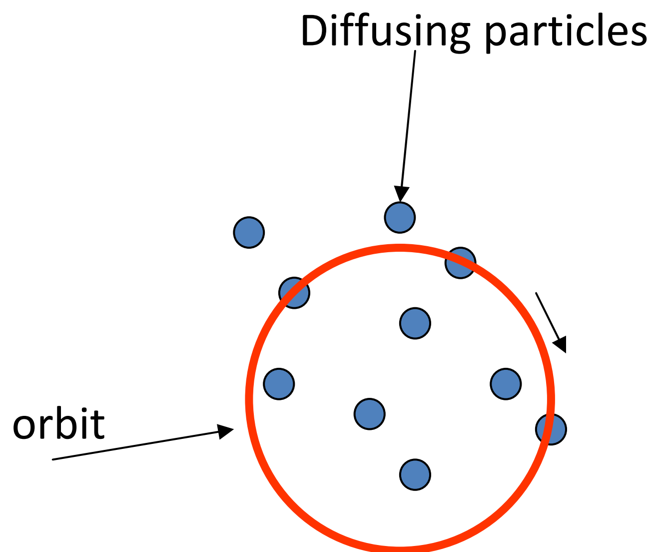
Instead, faster blinking and other fast intramolecular processes will not be missed!! (why?)

Autocorrelation of EGFP & Adenylate Kinase -EGFP



Normalized autocorrelation curve of EGFP in solution (•), EGFP in the cell (•), AK1-EGFP in the cell(•), AK1b-EGFP in the cytoplasm of the cell(•).

Acquiring scanning-FCS data



Light is collected along the orbit, generally at 64 or 128 points. If the orbit period is 1ms, the dwell time at each point is about 16 μs (64 points) or 8 μs (128 points).

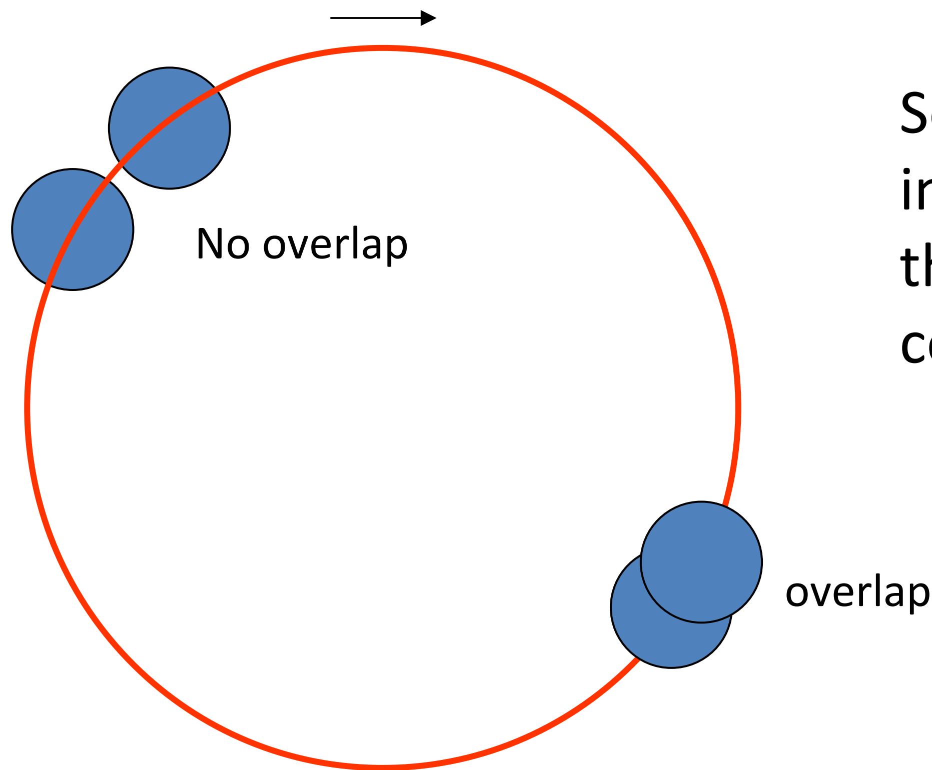
The separation between the points depends on the orbit radius.

For an orbit radius of 5 μm, the length of the orbit is about 32 μm. At 64 points per orbit the average distance is about 0.5 μm (0.25 μm at 128 points).

Why the distance between points is important?

Overlapping volumes in scanning FCS

If the orbit radius is larger than 5 μm, the points are separated by more than the width of the PSF
(assuming 64 points per orbit: 2πR/64~500nm)

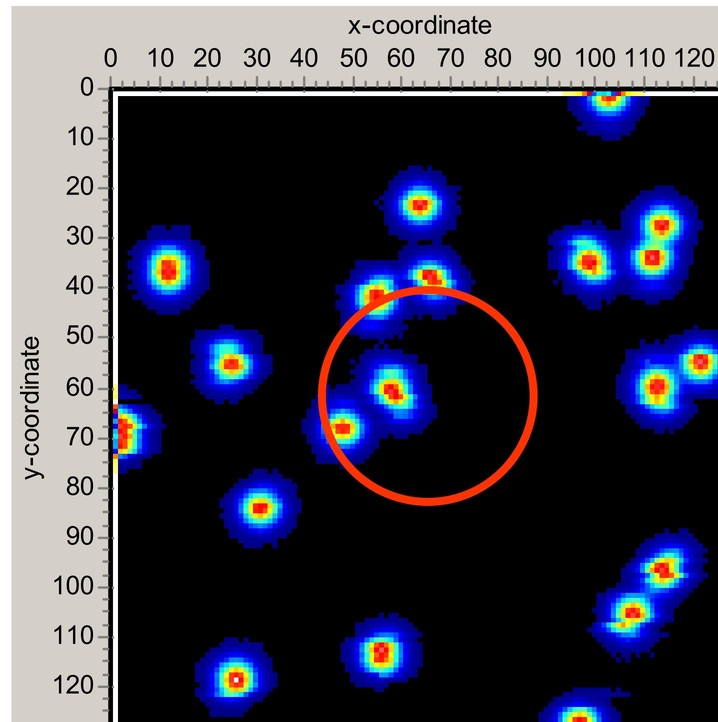


Setting the conditions of the instrument for **no-overlap** limits the capability of obtaining spatial correlations along the orbit

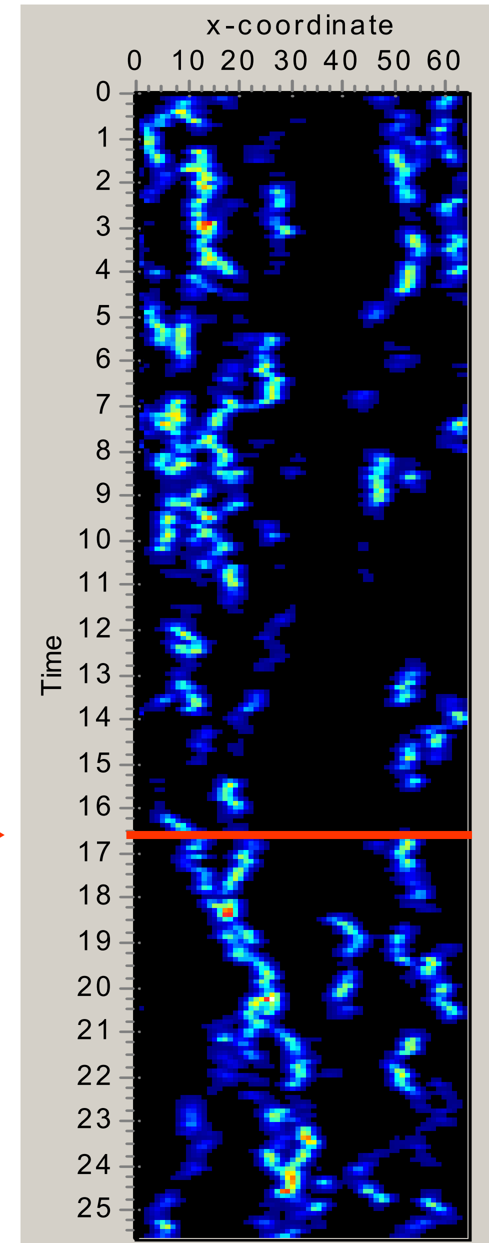
Data processing in scanning FCS

The data stream is presented as a **"carpet"** in which the **horizontal coordinate** represents data along the orbit and the **vertical coordinate** represents data at successive orbits (Hyperspace).

6 μm image
1 μm radius orbit



"carpet"

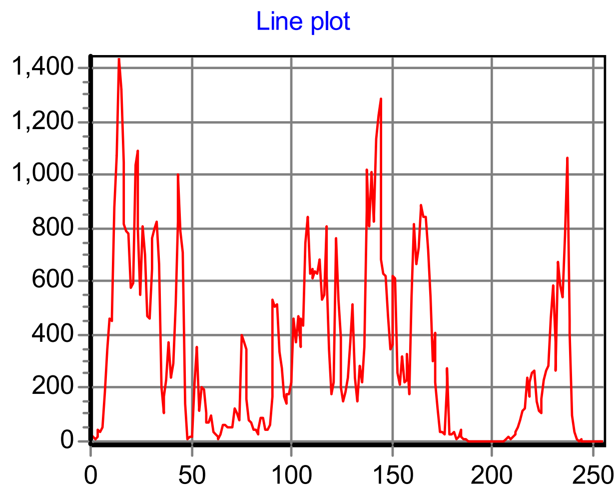


Analyzing data in scanning FCS

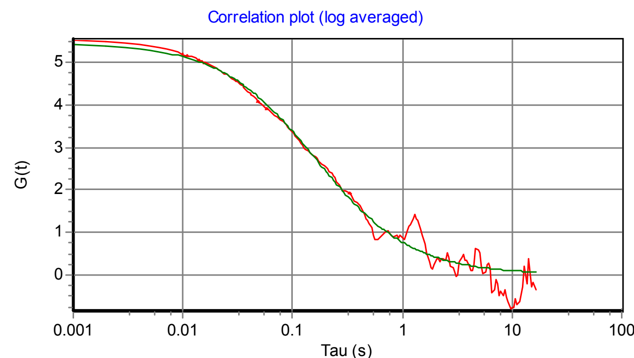
How we proceed to determine the **diffusion** of particles, the **number** of particles and their **brightness**??

- Select a column of the carpet. It is a time sequence at a specific point of the orbit!
- Perform autocorrelation operation along a column
- What we obtain?
- What is the sampling time along one of these column?
- What is the dwell time along one of these columns?

Intensity along a column



Perform the autocorrelation operation

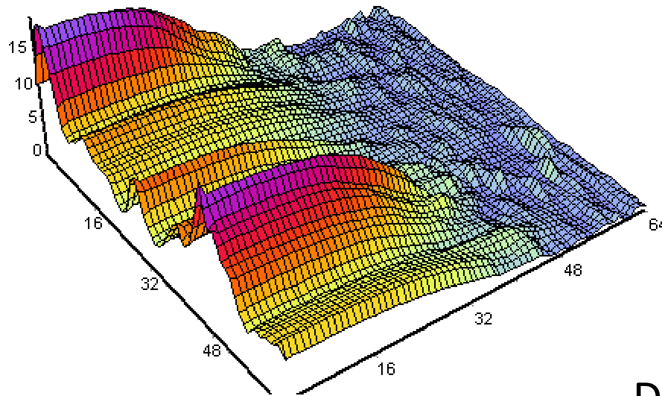


Recovered value for D=0.1 $\mu m^2/s$(= to the value input in the simulation!)

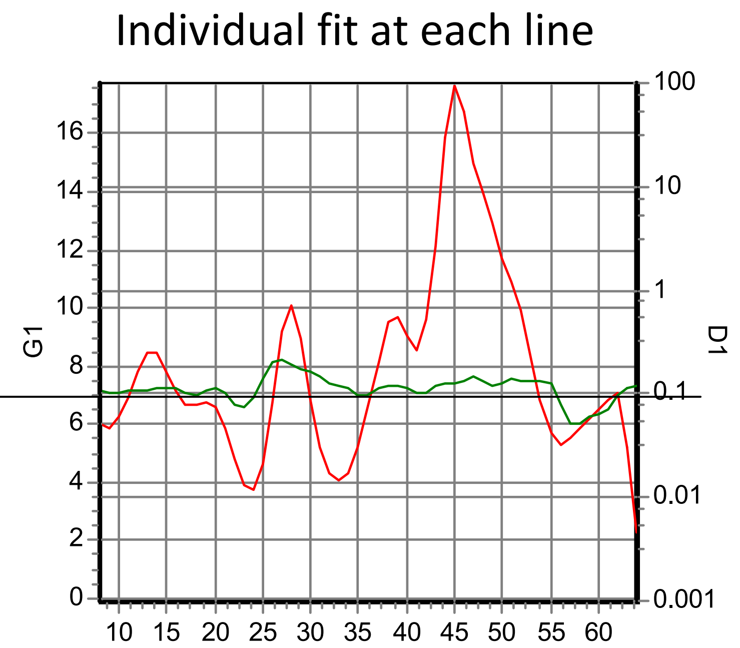
Carpet analysis

Every column should be equivalent for an homogeneous sample, so that we can calculate the ACF for every column and then fit all the columns either globally or individually.

ACF along each column
The calculation takes few seconds

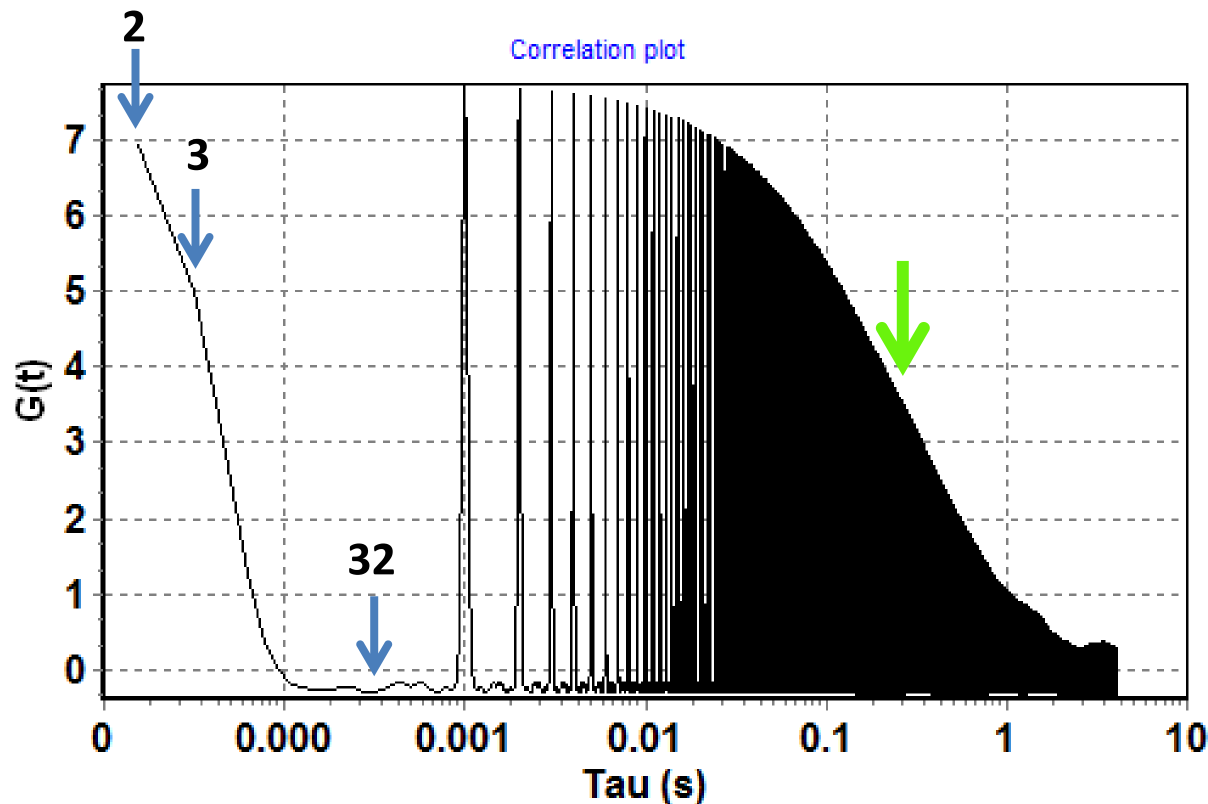


D=0.1μm²/s



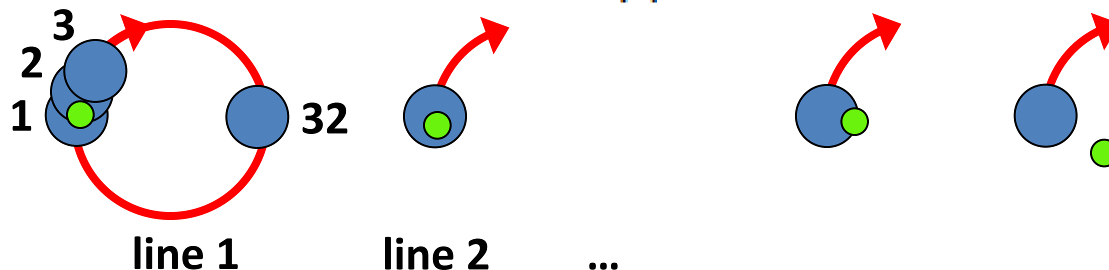
The G(0) changes from line to line, because the statistics is poor, but the D is pretty constant at the expected value of D=0.1um²/s

Global correlation function for a solution experiment



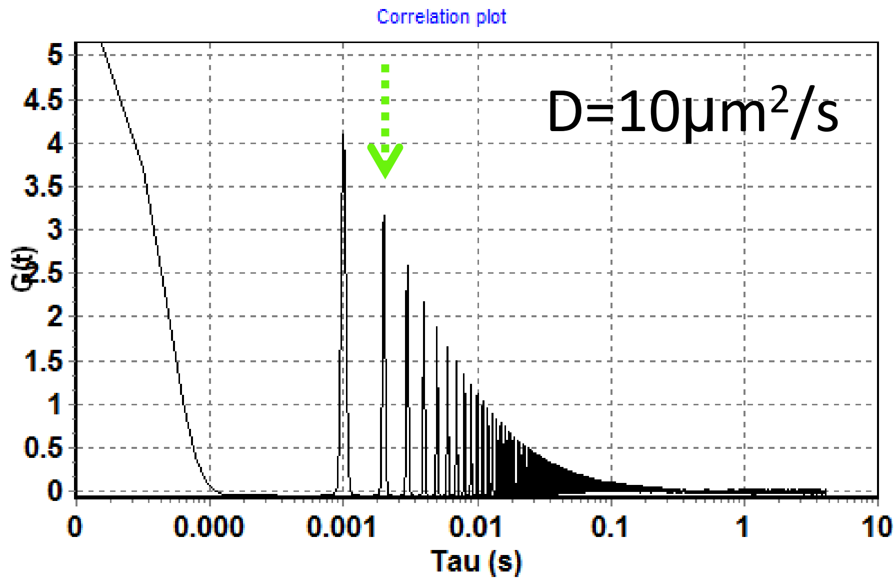
Global correlation function
The periodicity is due to the scanning period which is 1 ms

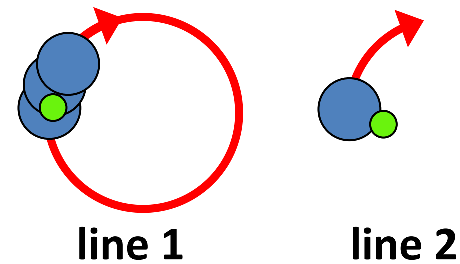
D=0.1μm²/s
R=1μm



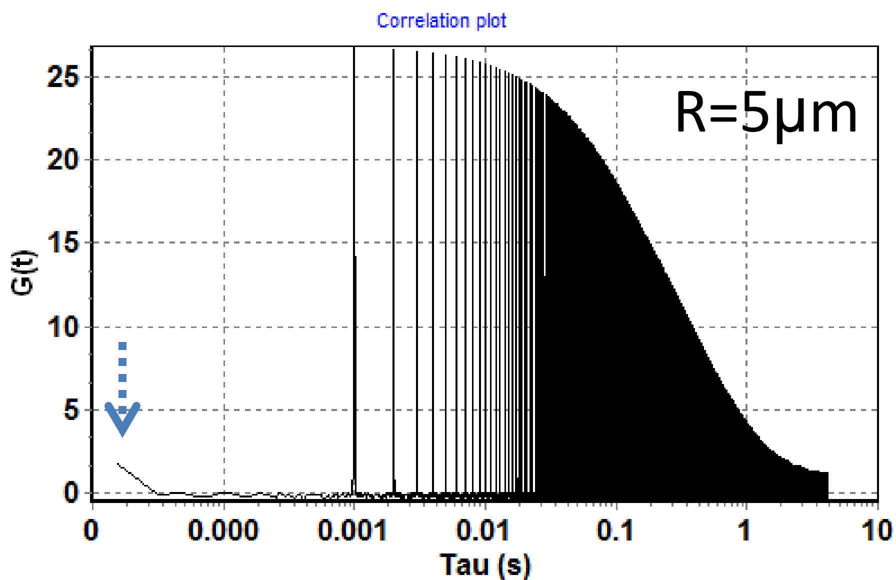
Clearly, we are sampling fast with respect to the relaxation due to diffusion. (How can we see that this is the case?)

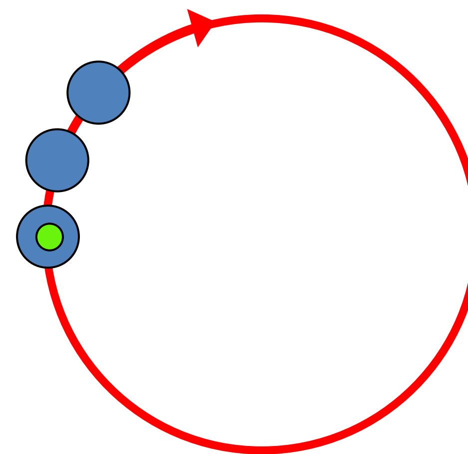
Global correlation function for a solution experiment





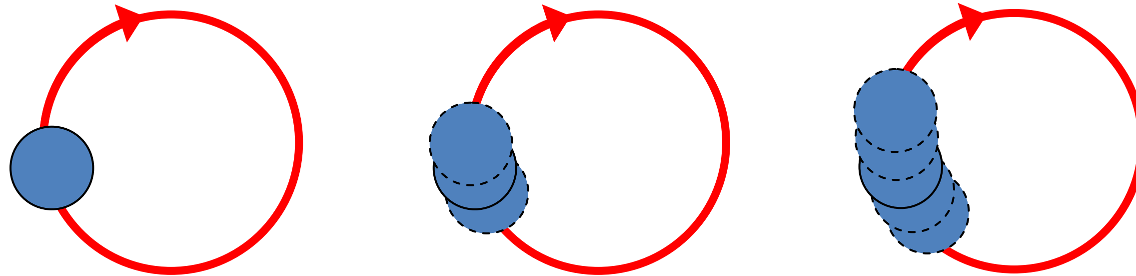
We are not scanning fast enough!



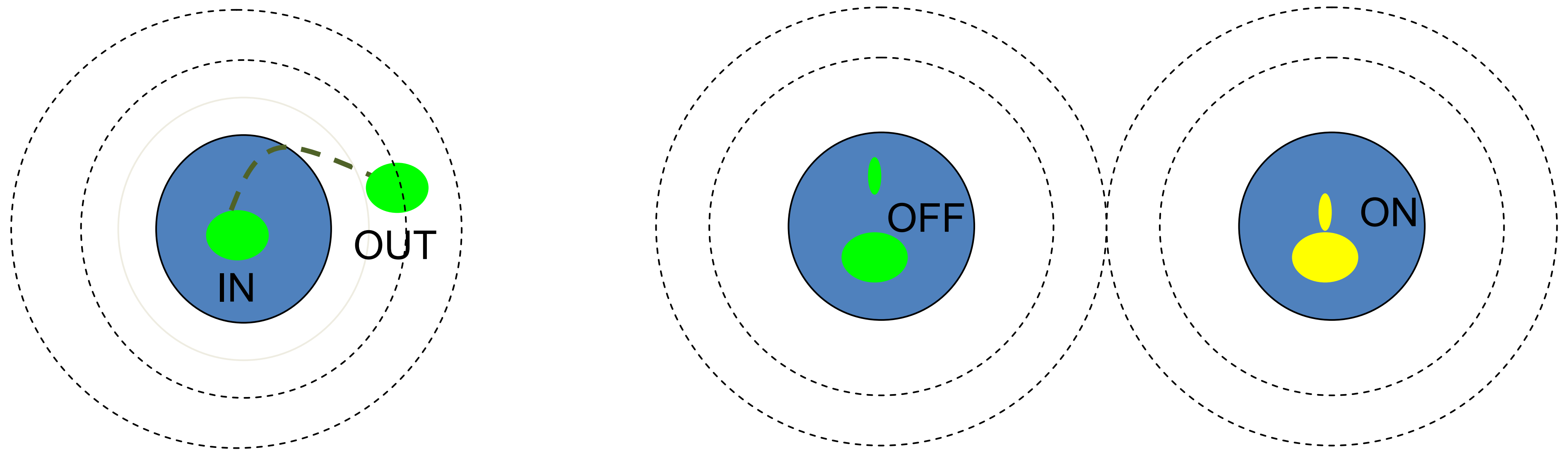


No spatial correlations!

How to distinguish Diffusion from Binding?



PSF scaling analysis: we can average adjacent columns to increase the apparent size of the PSF

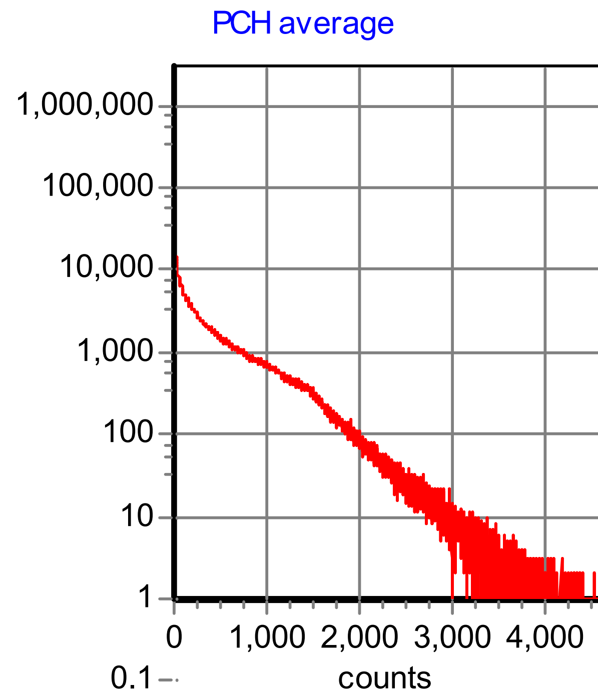


Diffusion: Fluctuations come from particle IN and OUT the focal volume
→ Apparent D_{coef} will decrease

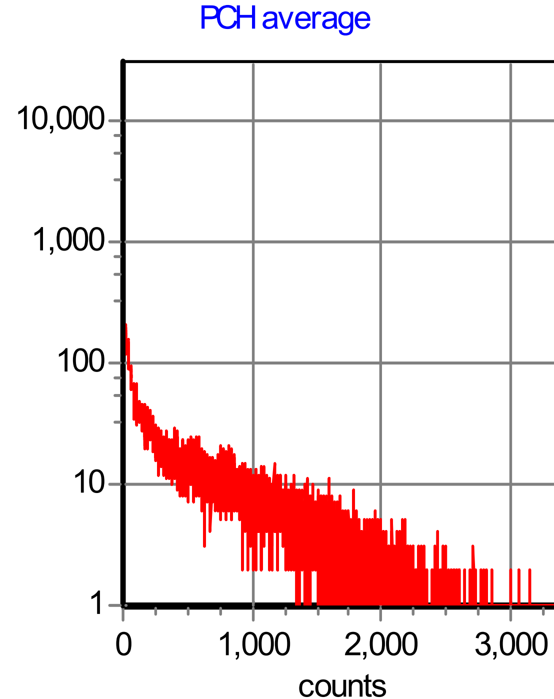
Binding: Protein ON and OFF from an immobile structure
→ Apparent D_{coef} will not change

PCH analysis at each column

What about the PCH analysis, can that be done?
Since we have a sequence, we can plot the histogram first globally and then individually for each column



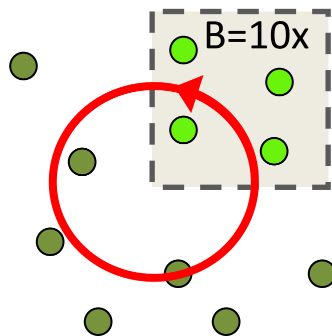
Global histogram
(more statistics!)

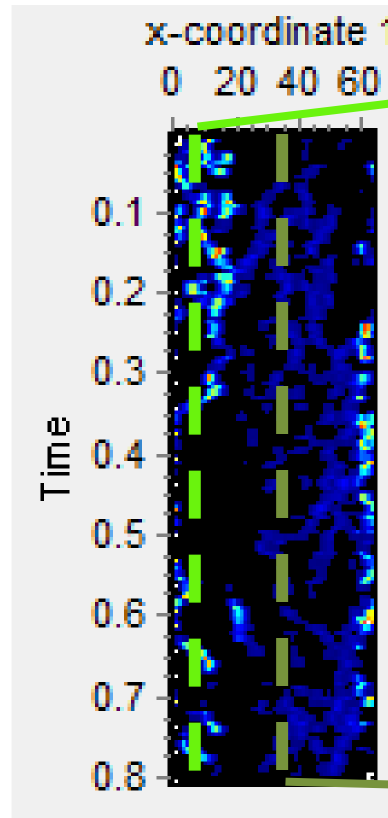


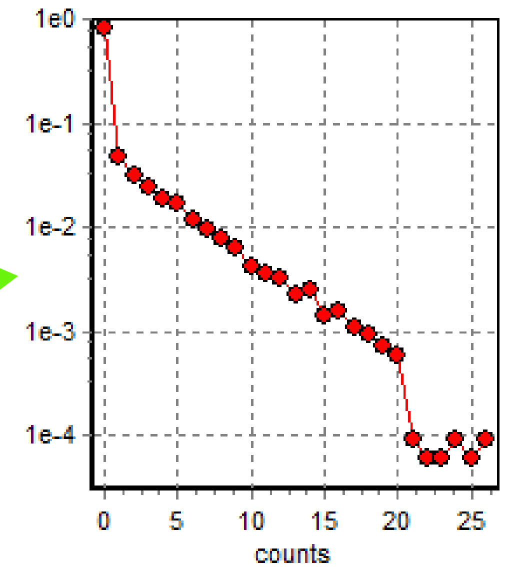
Single histogram at one column

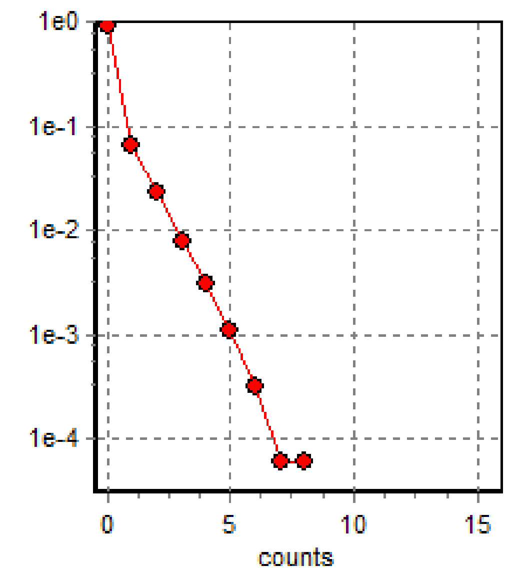
PCH analysis at each column

Simulation: scanning FCS through zones of different brightness









Why scanning FCS in homogeneous samples?

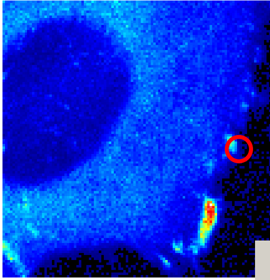
Is there any advantage to perform scanning FCS instead of single point FCS for a solution sample?

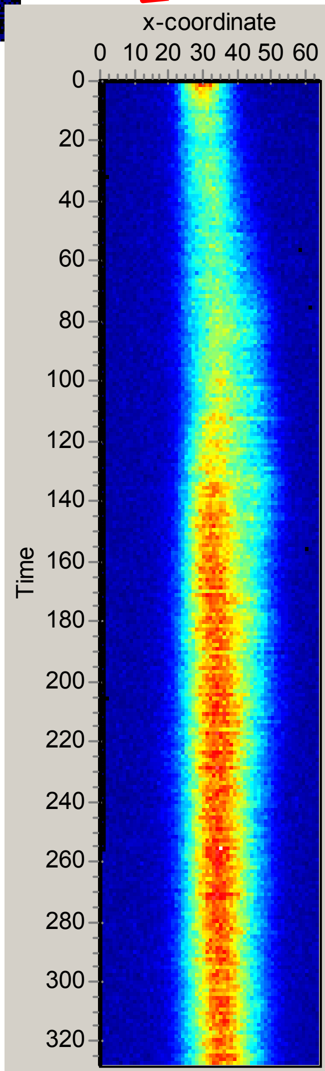
A major issue in FCS is that we need the volume of the PSF to calculate the diffusion coefficient

In scanning FCS we know the distance between points along the orbit. We can calculate the time for a molecule to diffuse between the two volumes

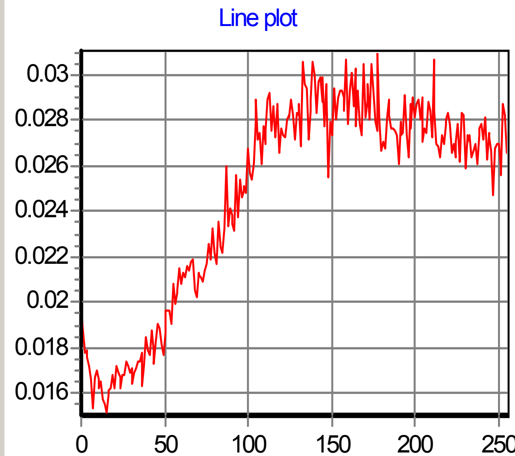
What about cross-correlation between columns?

Scanning FCS in cells (some surprises!)





Example of scanning at an adhesion



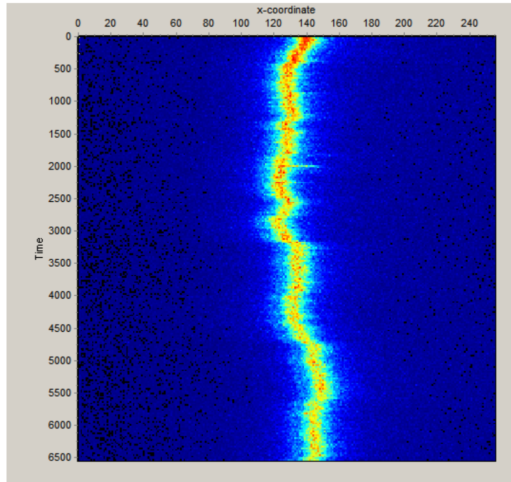
64 points sampled along the orbit
Period of scanning is 1 ms,
Radius of scanning is 2 μm
Distance between pixel is about 0.2 μm

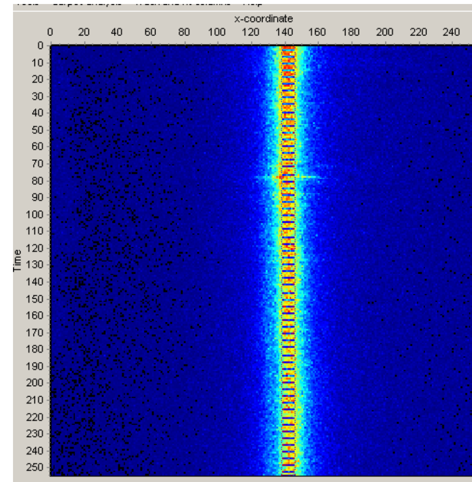
The "real world"
What we do with the 'changes in intensity"?
There is some fast initial bleaching followed up by a slow increase in intensity

What are the questions?

- What is the apparent "diffusion" coefficient of paxillin ?
- Is the diffusion coefficient homogeneous?
- Is paxillin monomeric (i.e., what is the brightness)?
- What is the number of particles in the different parts of the adhesion?

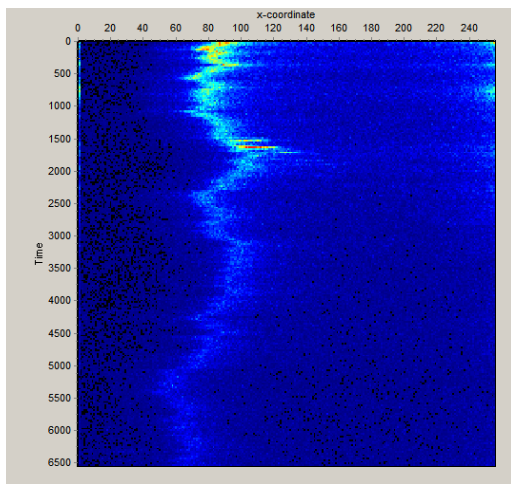
Welcome to the real world!

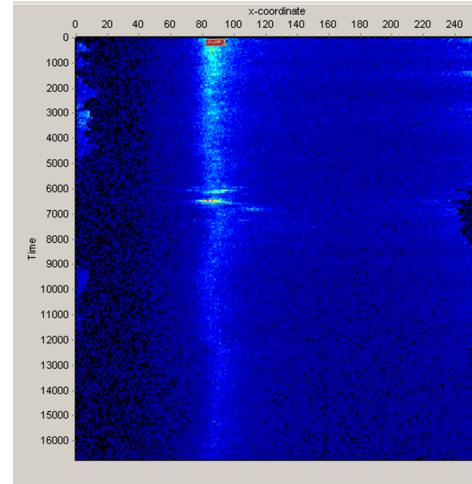




Detrend?

Centering?

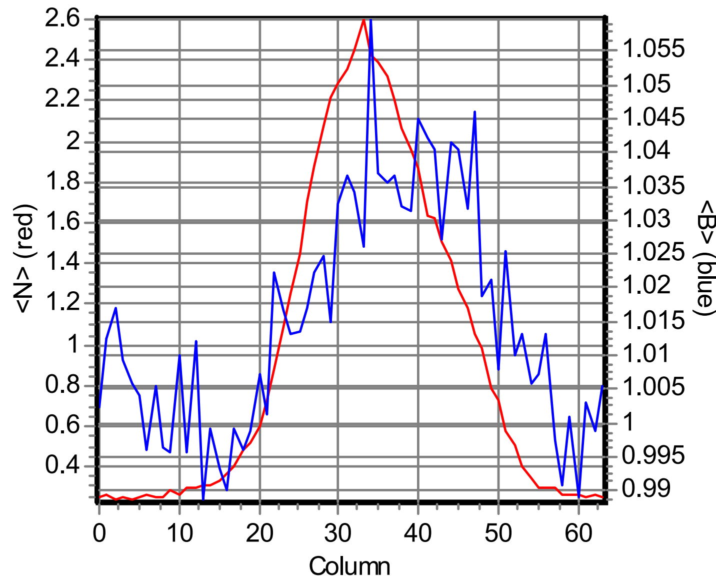




Data from Pierre Moens (2007)

Scanning a moving target: GUV. How to determine the diffusion in the membrane?

Carpet Brightness and Number analysis



Bin by 8 (what is this?)

Now the right part of the adhesion shows larger brightness. Also the number of molecules and the brightness curve are displaced one with respect to the other.

This analysis shows the map of the brightness across the adhesion

Was the amplitude statistics modified by filtering the slow varying component??

Described so far

Circular versus line-scanning

Line scanning can be performed with any confocal microscope

Line scanning is not as fast as circular scanning (few ms versus a fraction of a ms)

For homogeneous samples, is there any advantage in performing scanning-FCS (either circular or line) with respect to single point FCS??

Filtering operations on the data and integrity of the original statistics

Observations

Even in the "simplest" implementation, FCS in cells requires precautions in data analysis and interpretation

Maps of diffusion coefficients, number of particles and brightness can be obtained if we can deal with slowly varying fluctuations

The software for data analysis must offer a series of tools to the user for data filtering, analysis and presentation. It is not enough to collect line scanning data!

The user must set up the instrument parameters (line period, dwell time, etc) for the particular experiment

What is next?

This was an “introduction” to scanning FCS

We discussed the analysis of the carpet columns as individual time traces at separate points

We have not considered the correlation between adjacent columns or between distant columns

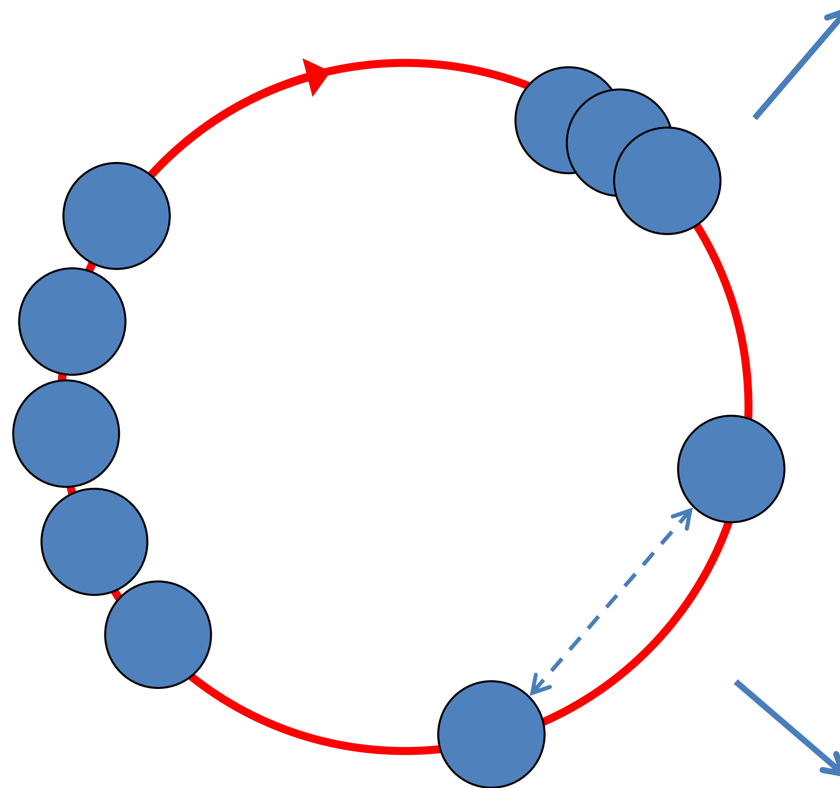
We need to develop new concepts and mathematical tools to account for these spatial correlations

As we understand the scanning experiment we discover a new world about fluctuation methods that was not possible to explore with single point FCS

What is next?

Spatial Resolution

RICS

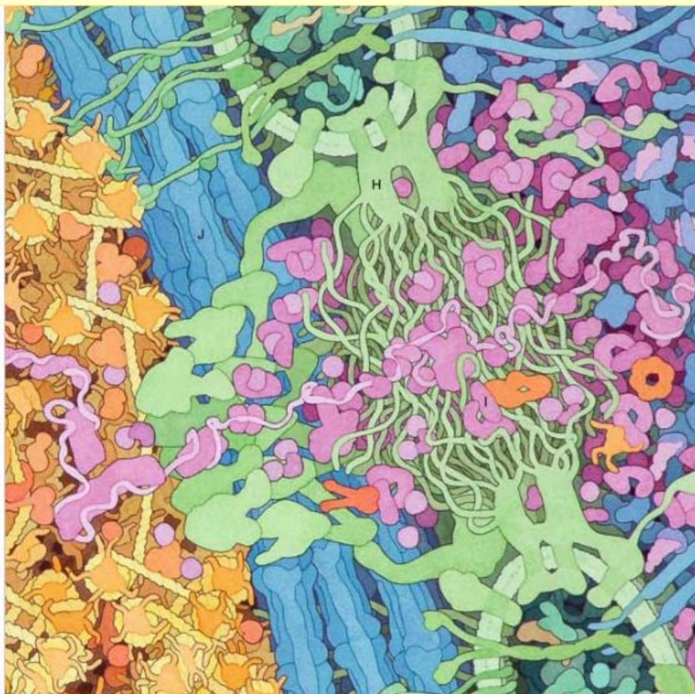


Orbital Tracking

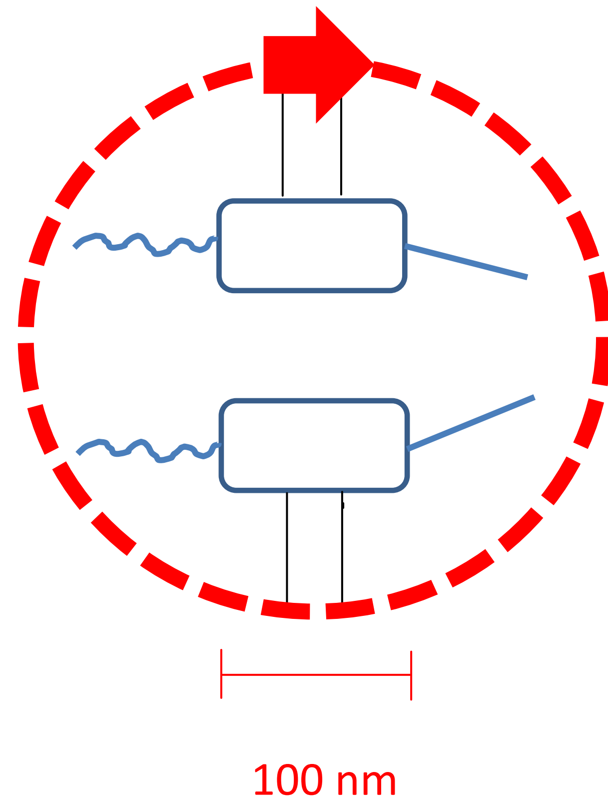
Pair Correlation

Example

Scanning FCS on single Nuclear Pore Complexes (NPCs)



David Goodsell, *The machinery of life*



In collaboration with: Francesco Cardarelli, NEST, Scuola Normale Superiore, Pisa, Italy

Example

The NPC regulates nucleocytoplasmic transport through:

Passive diffusion

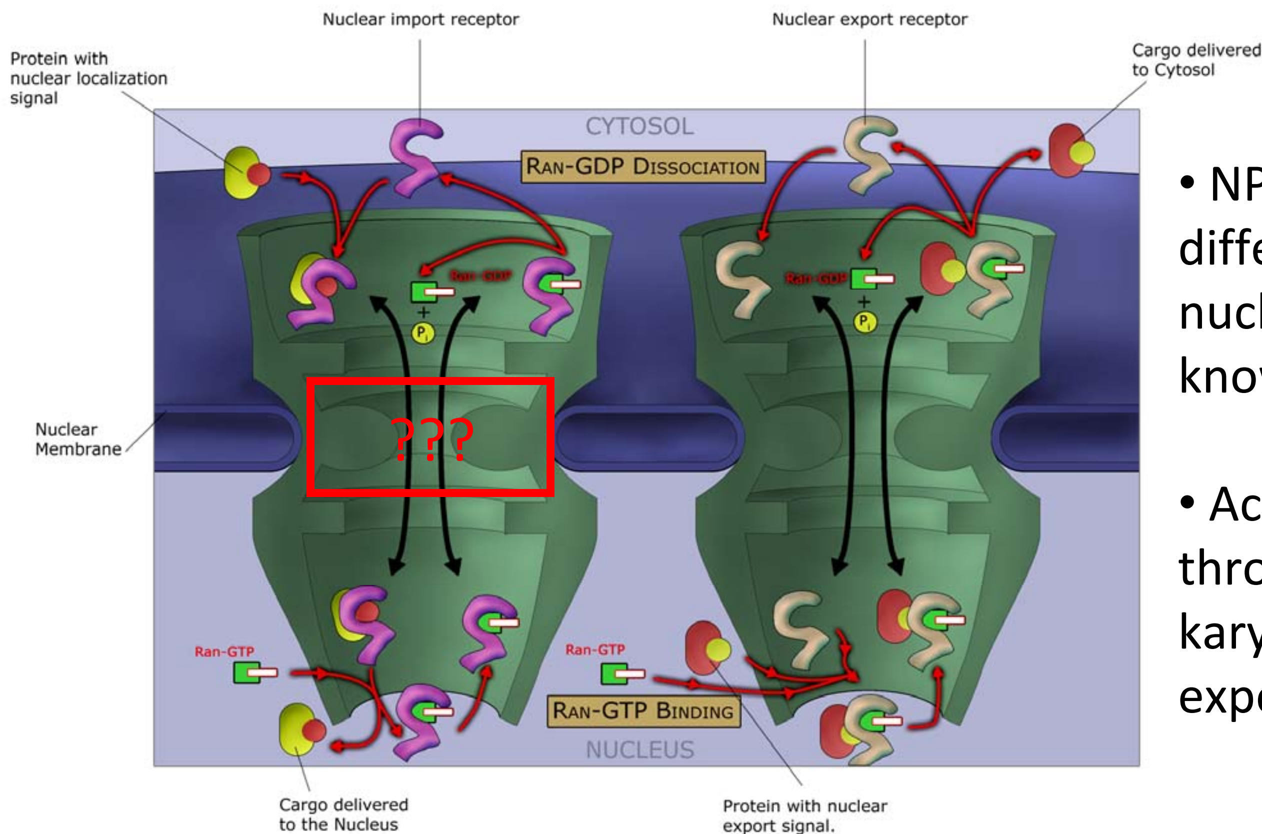
1. Bidirectional through the NPC
2. Regulated by molecular size (limit: 60-70 kDa)
3. Energy-independent

Active import

1. Unidirectional through the nuclear pore complex (NPC)
2. Driven by specific aminoacidic sequences (NLS/NES)
3. Not affected by molecular size
4. Energy-dependent

Example

Molecular transport across the NPC

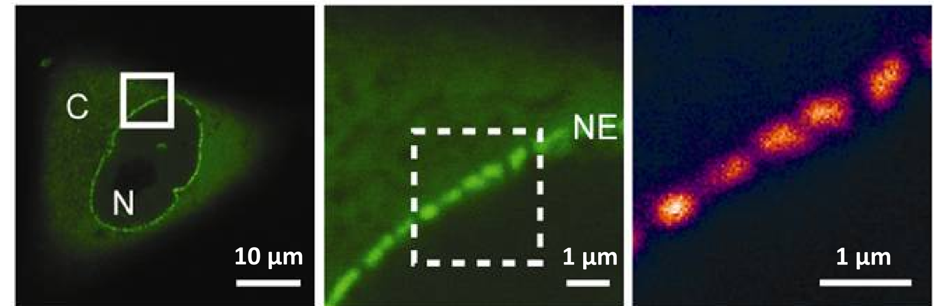


- NPC consists of about 30 different polypeptides called nucleoporins (Nups), but little is known about their organization
- Active transport is mediated through receptors called karyopherins (importins and exportins)

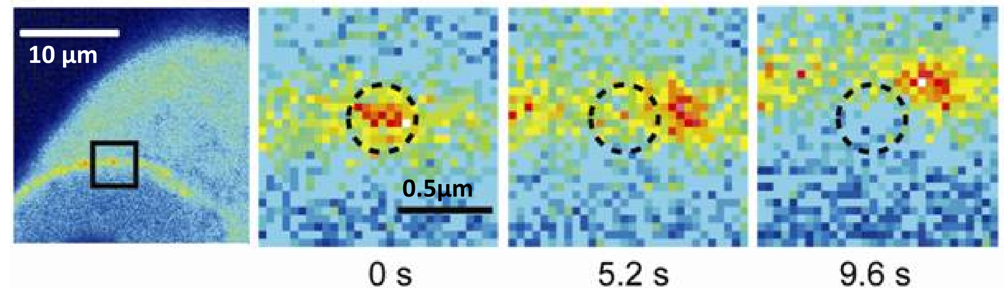
Can we apply scanning FCS to study dynamics through the pore?

Example

• Kapβ1-GFP is able to bind nucleoporins and we use it as a dynamic marker of NPCs.



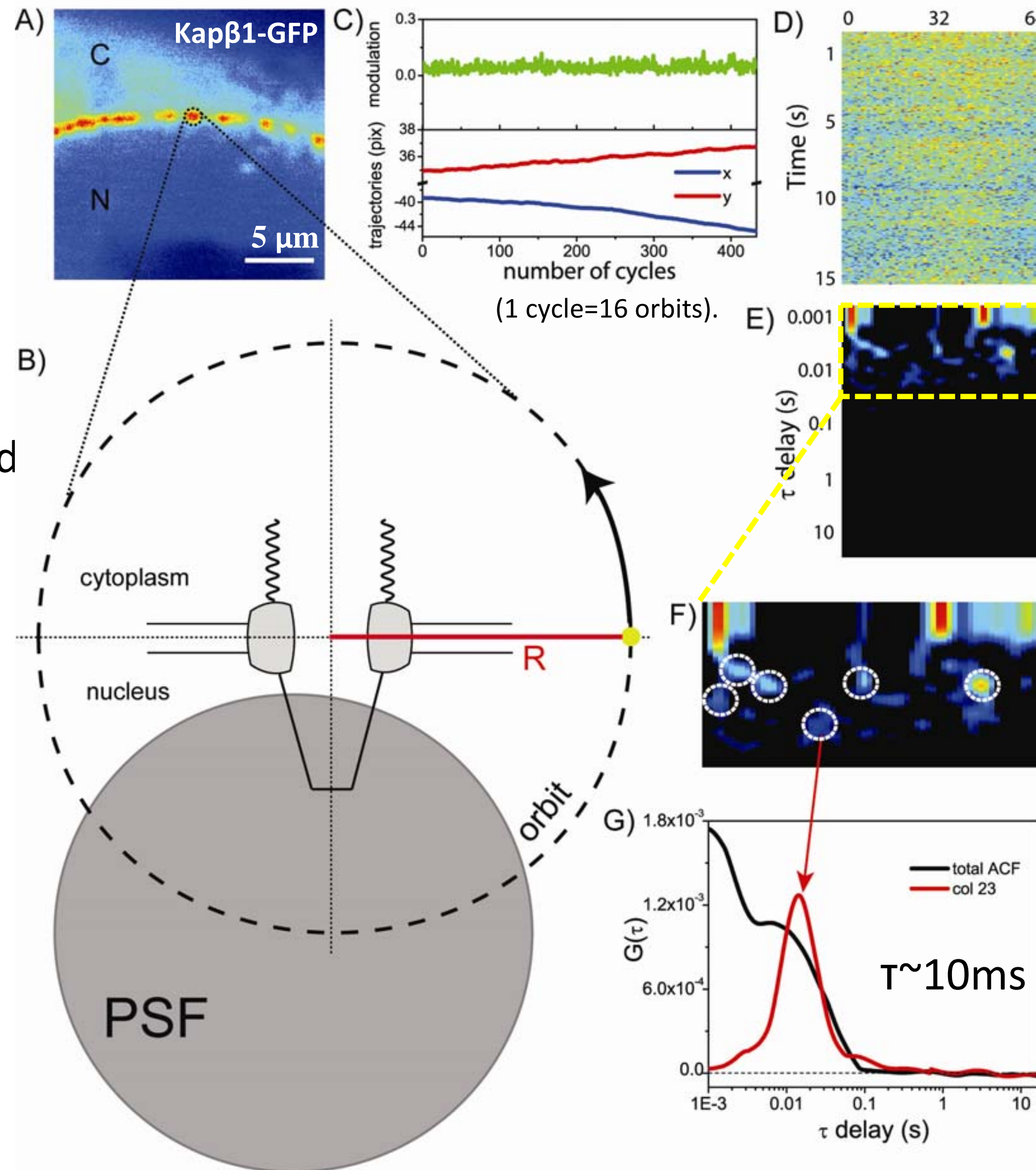
• The entire NPC can perform local nanometer diffusive motion within the nuclear envelope or follow global rearrangements of the cell. It is crucial that we subtract this motion if we want to distinguish between the diffusion of the molecules from the overall thermal motion of the NPC.



→Scanning FCS + Orbital tracking of the NPC

Example

The PSF is scanned along a 64-points orbit of 180nm in radius (R) around the pore



Fluorescence intensity along the orbit over time.

total ACF carpet

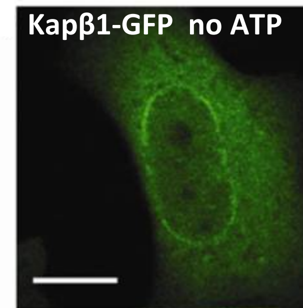
Average ACF plot (black) and ACF of column 23 (red).

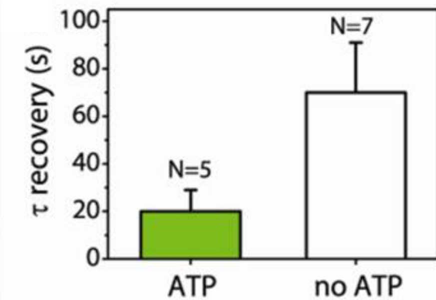
Example

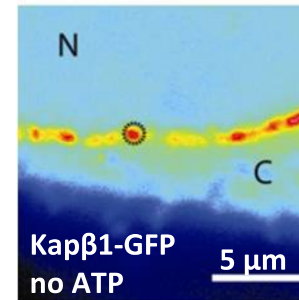
- Localization of Kapβ1-GFP in energy-depleting conditions. Cumulative FRAP results show the energy dependence of Kapβ1 shuttling.

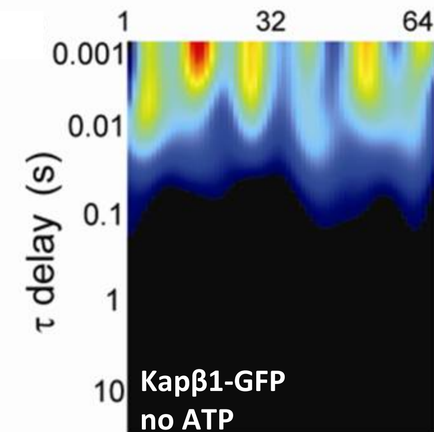
- A single NPC in energy-depleting conditions is analyzed by the scanning FCS + Tracking. The obtained ACF carpet and the average ACF curve show absence of detectable humps along the orbit.

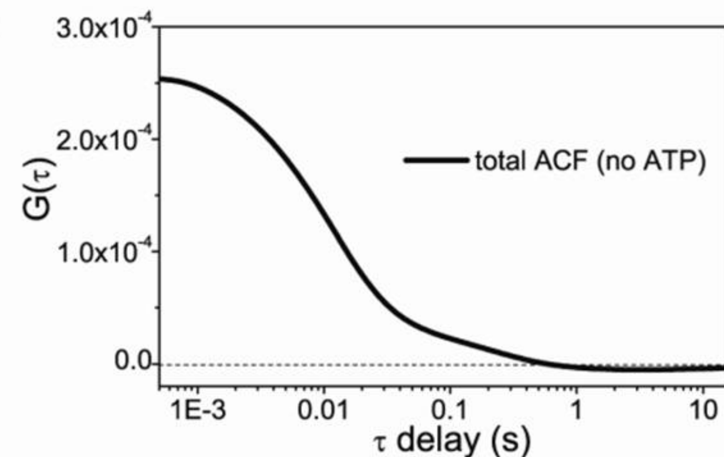
The hump is dependent on energy





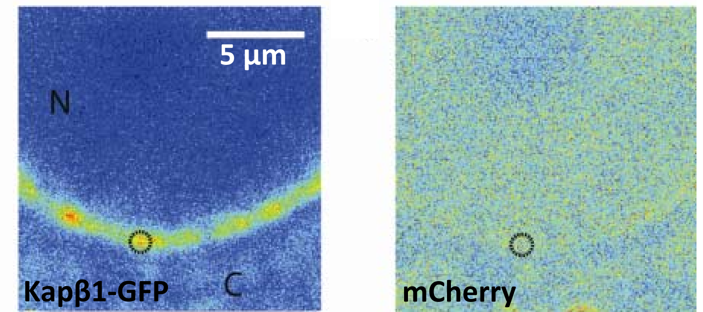




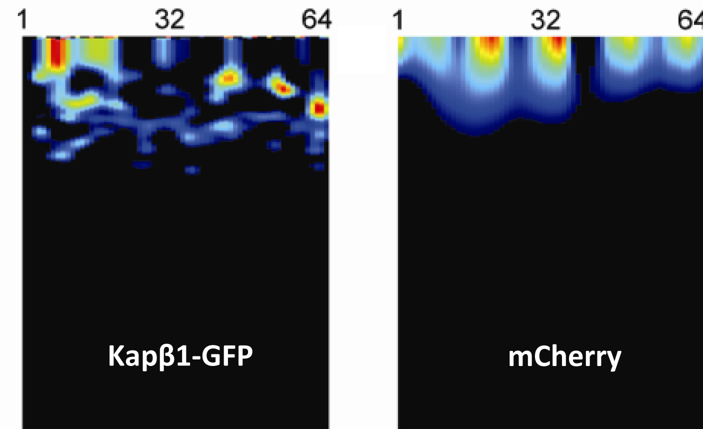


Example

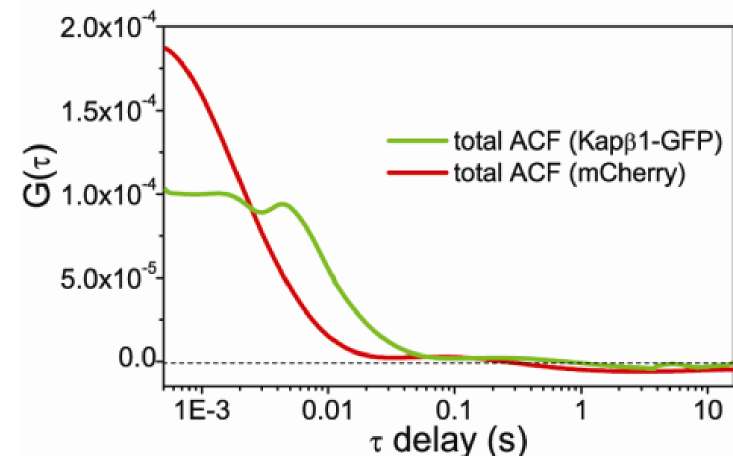
- We performed the experiment on cells co-expressing Kapβ1-GFP and mCherry to check if the effect was specific to Kapβ1 properties



- ACF carpets obtained in the two channels are different: the humps are visible only in the Kapβ1-GFP channel. The mCherry channel shows passive diffusion.



- The average ACF curves show the different behavior of Kapβ1-GFP and mCherry at the pore.



The hump is dependent on Kapβ1 properties

Conclusions

- Scanning FCS can be applied in combination with a tracking algorithm to study molecular transport across single NPCs in live cells
- The ACF shows a characteristic time distribution corresponding to the shuttling of Kapβ1-GFP through the NPC
- The pair correlation analysis (not shown) can also be applied to discriminate between diffusive motion and directed transport across the NPC channel