Lecture 3: Introduction to scanning FCS

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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
• Introduction

• The principle of scanning FCS

• Data acquisition, processing and analysis

• Scanning FCS in cells

• Example
When we first applied FCS to cells, a series of problems arose:

- The cell could have moved, so that the volume of observation was not any more the chosen one.
- The average intensity $\langle F(t) \rangle$ 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 effect on the correlation function is given by:

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

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

**Introduction to scanning FCS**
Approaches to FCS in cells

- Manufacturers (Zeiss and ISS) built instruments 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

Single point FCS

Time
0 1 2 4 8
Correlation 1.0 1.0 0.9 0.6 0.3

Correlation function

\[ G(\tau) = \frac{\omega_0^2}{4D} \]

Lag time \( \tau \) (s)

Scanning FCS and RICS

Shift (pixel)
0 1 2 4 8
Correlation 1.00 1.00 0.66 0.14 0.00

\[ \tau_D = \frac{\omega_0}{4D} \]
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 has left the volume of excitation, then we can “multiplex the time” and collect FCS data at several points simultaneously!
The fastest way to scan several points and then 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 acousto-optical beam deflectors.
What is the minimum time required for an orbit so that we will not miss the “fastest” diffusion process in a cell?

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

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(●).
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?
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: $2\pi R/64 \sim 500$nm)

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
D=0.1 µm²/s
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**

![Line plot](image1.png)

![Perform the autocorrelation operation](image2.png)

**Recovered value for** D=0.1 μm²/s ( = to the value input in the simulation! )
Every column should be equivalent for a 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

The G(0) changes from line to line, because the statistics is poor, but the D is pretty constant at the expected value of $D=0.1\mu m^2/s$
Global correlation function: the periodicity is due to the scanning period which is 1 ms.

Global correlation function for a solution experiment

D = 0.1 \mu m^2/s
R = 1 \mu m

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!
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**
Why do scanning FCS in a homogeneous sample?

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 do 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!

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

Data from Pierre Moens (2007)

Detrend? Centering?
Scanning FCS in cells: heterogeneity along adhesions

Single point FCS depicts two-species

<table>
<thead>
<tr>
<th>Paxillin-EGFP</th>
<th>Diffusion (µm²/s)</th>
<th>Fractional Contribution Cytosol (%)</th>
<th>Fractional Contribution Adhesions(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 Monomers</td>
<td>19.6</td>
<td>61</td>
<td>44</td>
</tr>
<tr>
<td>D2 Aggregates?</td>
<td>1.43</td>
<td>39</td>
<td>56</td>
</tr>
</tbody>
</table>

What are these diffusion rates due to?

1. Differences in cell viscosity
2. Paxillin complexed to other proteins
3. Large aggregates of paxillin

Data were sampled at 64kHz (1ms/orbit, 64 points per orbit).

FCS and Scanning FCS results:

- Paxillin moves differently at an adhesion with respect to the cytosol
- Adhesions are heterogeneous
- At the assembling side of the adhesion the fluctuation dynamics is faster and the number of molecules is larger than in stable cellular adhesions

Are the adhesions assembling and disassembling in synchrony? Can we map out protein dynamics in a larger area?

We need a method where we can analyze the entire cell: THE RICS APPROACH
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.

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

- 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!

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

STICS - iMSD  Pair Correlation