Lecture 3: Introduction to scanning FCS (sFCS)

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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

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

- Bleaching of the immobile fraction occurred, causing a large deviation of the apparent correlation curve
• 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. Many state-of-the-art commercial microscope now come with modules for FCS measurements.

• Although these technological implementation represent a step forward to a more widespread use of correlation spectroscopy, here at LFD we developed a different approach:

**the scanning FCS principle**


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FCS and sFCS

**Single point FCS**

<table>
<thead>
<tr>
<th>Time</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
</tr>
<tr>
<td>8</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Scanning FCS**

<table>
<thead>
<tr>
<th>Time</th>
<th>Shift (pixel)</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
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<td>0.3</td>
</tr>
</tbody>
</table>

**Correlation function**

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

**Lag time**

\[ \tau_D = \frac{\omega_0^2}{4D} \]
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 less than 1 ms using conventional galvo drivers and in microseconds using acoustooptical beam deflectors.
What is the maximum 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 $20 \, \mu m^2/s$. The transit across the laser beam (assuming a $w_0$ of 350 nm) is about 1.5 ms!

Therefore **0.5 to 1 ms** per orbit should catch the GFP diffusing in a cell. Faster diffusing molecules will be partially missed.
Normalized autocorrelation curve of EGFP in solution (•), EGFP in the cell (●), AK1-EGFP in the cell (○), AK1β-EGFP in the cytoplasm of the cell (●).
Acquiring sFCS data

Diffusing particles

Light is collected along the orbit, generally at 64 or 128 points. If the orbit period is 1 ms, 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 is the distance between points 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: $2\pi R/64 \approx 500$nm)

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

Data processing in scanning FCS

6x6 μm image size, 1 μm orbit radius, D=0.1 μm²/s
How do 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 are we obtaining?
- What is the sampling time along one of these columns?
- What is the dwell time along one of these columns?
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.

The $G(0)$ changes from line to line, because the concentration is low, but the $D$ is pretty constant at the expected value of $D=0.1\,\mu m^2/s$.

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

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

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

\[ D = 10 \mu m^2/s \]

We are not scanning fast enough!

No spatial correlations!

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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.
Welcome to the real world!

Scanning a moving target: GUV

De-trend?
Centering?

Data from Pierre Moens (2007)

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sFCS in cells

64 points sampled along the orbit
Period of scanning is 1 ms,
Radius of scanning is 2 μm
Distance between pixels 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?
sFCS in cells: Cellular adhesions

CHO-k1 cells expressing Paxillin-EGFP

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

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


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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**
Summary

- Circular and 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)
- 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.

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