Scanning FCS

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4th LFD Workshop in Advanced Fluorescence Imaging and Dynamics
October 26–30, 2009, Irvine, CA.
Introduction

Intensity trace

Photon Counting Histogram

Autocorrelation

- the amplitude of the fluctuation $G(0)$
- relaxation time of the fluctuation $D_{coef}$

-molecular brightness ($\varepsilon$)
-number of particles
FCS inside the cells

AK EGFP-AKβ in the cytosol and membrane of HeLa cell.

At the membrane, a dual diffusion rate is calculated from FCS data.

Away from the plasma membrane, single diffusion constants are found.

We need to be able to scan several points as rapidly as possible.
What do we need?

*We want to collect FCS data at several points simultaneously.*

- Move the point at which we acquire FCS to other points.

- Return to the original point before the particle had left the volume of excitation.

Thus, collect FCS data at several points simultaneously.

**How fast we can return to the original point.**

**How fast the particle moves.**
How fast can we return to the original point?

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

An orbit could be performed in times of less than 1 ms, using conventional galvo drivers.
How fast particles move inside the cell?

EGFP diffuses with an apparent \( D_{\text{coef}} \) of approximately 20 \( \text{us}^2/\text{s} \). The transit across the laser beam in about 1.5 ms.

The transit across the laser beam in about 1.5 ms

Therefore 0.5 to 1 ms per orbit should catch the GFP diffusing in the cell.

Faster diffusing molecules will be partially missed.
How fast particles move inside the cell?

- **EGFP cytosol (20 um²)/s**  
  $t = 1.5$ ms

- **EGFP-Adenylate Kinase-1 cytosol (8 um²/s)**  
  $t = 3.8$ s

- **EGFP (34KDa)**  
  Solution (94 um²/s)  
  $t = 0.3$ ms

- **EGFP-Adenylate Kinase-1b cytosol**

**Legend**
- Fast scanners  
  0.1 ms
- Our scanner  
  1 ms
- Line FCS  
  3-4 ms

**Time (seconds)**
Therefore 1 ms per orbit should be sufficient to catch the fastest diffusion process inside the cell.

Faster diffusion processes will be partially missed.
How do we take the data?

Orbit = 1ms

Let’s take data from 6 points

1 2 3 4 5 6
How many points we want to sample?

Non-overlapped PSF

Overlapped PSF

If PSF overlap and we have now an extra information..... spatial correlation information (RICS lecture)
Data representation

Orbit = 1 ms
Radius of the orbit = 1.52 μm
Perimeter of the orbit = 9.52 μm
Points per orbit = 64
Distance between points = 9.52/64 = 0.148 μm
Dwell time = 1 ms/64 = 16 μs
Carpet analysis

Every column is the equivalent of single point FCS

Spatial information

Temporal information

Autocorrelation

PCH
Carpet analysis

Every column is equivalent to an homogeneous sample, so that we can calculate the AFC 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 $D=0.1\,\mu\text{m}^2/\text{s}$.
What do we see in real cellular world?

Clearly, if we calculate the autocorrelation function along columns, there will be a common component due to the slow variation of the average intensity. Instead, we are interested in the fast fluctuations!!
Dealing with slow-changing signals

How to properly detrend the data without changing the statistics at short times?

One common approach is to calculate the ACF in small pieces only

**ACF, all data in a column**
256K segment length

**ACF, all data in a column**
32K segment length
Addition of random uncorrelated numbers

Every column will be divided in segments.

For each segment the average will be calculated.

The largest average will be stored.

For each segment, random “uncorrelated” numbers will be added in a quantity that will make the average counts in each segment to be equal to the largest average.

After this operation the trends should have disappear. However, since different columns have different average, the difference in intensity in the columns will be preserved.
Addition of random uncorrelated numbers

Divide it in segments

Get the average of each piece

Largest average

random “uncorrelated” numbers will be added in a quantity that will make the average counts in each segment to be equal to the largest average

After this operation the trends should have disappear.
However, since different columns have different average, the difference in intensity in the columns will be preserved.
After detrending

Detrended at 10000 lines

Carpet

Average. Vertical Intensity

Autocorrelation
After detrending

Detrended at 10000 lines

Carpet

Average Vertical Intensity

Autocorrelation
After detrending

Detrended at 6000 lines

Carpet

Average Vertical Intensity

Autocorrelation
More dramatic detrending
Yeast ribosomal stalk heterogeneity in vivo shown by two-photon FCS and molecular brightness analysis.

Garcia-Marcos, A.
S.A. Sánchez
P. Parada
J.S. Eid
D.M. Jameson
M. Remacha
E. Gratton
J.P.G. Ballesta

Biophys J. 94(7):2884-2890.2008
The biological question

The reconstruction of the 80S eEF2 sordarin

S. cerevisiae ribosome

Simplified diagram
The biological question

What is the organization of the acidic proteins on the stalk in vivo?

Homogeneous distribution

Heterogeneous distribution

The various ribosome types may translate mRNAs with different efficiencies, and specific messengers might even be translated by some of the ribosome subpopulations. In this way the pattern of proteins will be related to the equilibrium between the different ribosomes.
Molecular biology approach

EGFP-mutants single and double labeled

P0

1α, 2α

1β, 2β

P0
<table>
<thead>
<tr>
<th>Name of the strain</th>
<th>Lacks wt protein</th>
<th>Expresses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- KanGP0/P0GFP</td>
<td>P0</td>
<td>P0-GFP</td>
</tr>
<tr>
<td>2- KanGP0-D4/P0GFP/2αGFP</td>
<td>P0, P2α</td>
<td>P0-GFP, P2α-GFP</td>
</tr>
<tr>
<td>3- KanGP0-D6/P0GFP/1βGFP</td>
<td>P0, P1β</td>
<td>P0-GFP, P1β-GFP</td>
</tr>
<tr>
<td>4- D4/2αGFP</td>
<td>P2α</td>
<td>P2α-GFP</td>
</tr>
<tr>
<td>5- D5/2βGFP</td>
<td>P2β</td>
<td>P2β-GFP</td>
</tr>
<tr>
<td>6- D6/1βGFP</td>
<td>P1β</td>
<td>P1β-GFP</td>
</tr>
<tr>
<td>7- D7/1αGFP</td>
<td>P1α</td>
<td>P1α-GFP</td>
</tr>
<tr>
<td>8- D45/2αGFP</td>
<td>P2α, P2β</td>
<td>P2α-GFP</td>
</tr>
<tr>
<td>9- D45/2βGFP</td>
<td>P2α, P2β</td>
<td>P2β-GFP</td>
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<td>P2α-GFP</td>
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<td>P2α-GFP</td>
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<td>P2α-GFP, P2α-GFP</td>
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<td>P1α-GFP</td>
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<td>P2β-GFP</td>
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<td>P2β, P1β</td>
<td>P1β-GFP</td>
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<td>19- D56/2βGFP/1βGFP</td>
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<td>P2β-GFP, P1β-GFP</td>
</tr>
<tr>
<td>20- D57/2βGFP</td>
<td>P2β, P1α</td>
<td>P2β-GFP</td>
</tr>
<tr>
<td>21- D57/1αGFP</td>
<td>P2β, P1α</td>
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<td>22- D57/2αGFP/1αGFP</td>
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<tr>
<td>25- D67/1αGFP/1αGFP</td>
<td>P1β, P1α</td>
<td>P1β-GFP, P1α-GFP</td>
</tr>
</tbody>
</table>
Experimental question

Can we distinguish between particles having 1 or 2 labeled proteins \textit{in vivo}?

![Diagram showing two states: one with one labeled protein and brightness = 1, and another with two labeled proteins and brightness = 2.]

Experimental approach

Data Acquisition: scanning FCS
Data analysis: Molecular brightness and PCH analysis \textit{in vivo}
Microscopy approach

sFCS and PCH analysis

- Frequency = 64000 Hz
- Period = 1000 ms
- Radius = 1.52 um
- Perimeter = 9.52 um
- Size of pixel in the orbit = 9.52/64 = 0.148 um

PCH analysis
Experimental setup

Cells $\text{Abs}_{600\text{nm}} = 0.5$

25°C
Results representation

Data box chart

- Maximum value
- 95th percentile
- 75th percentile
- Mean
- Median
- 25th percentile
- 5th percentile
- Minimum value
- Interquartile range
Results

![Box plot showing normalized brightness for different conditions.](image-url)

- Native protein missing
  - EGFP protein expressed
  - EGFP P0, 2α, 1β, 1α, 1β, 2α, 1β, P0, 2α, P0, 1β
Interpretation of the results

What is the origin of the fluctuations?

AUTOCORRELATION ANALYSIS
Average $D_{\text{coef}}$ for all the strains
0.02-0.06 $\mu$m$^2$/s

![Graph showing autocorrelation analysis with G(0) = 0.003 and sFCS]
Average $D_{\text{coef}}$ for all the strains 0.02-0.06 µm$^2$/s

FREE PROTEINS at high concentration?

- Acidic Proteins-GFP ≈ 39-60 kD
- Theoretical $\text{coef} ≈ 90 \text{ µm}^2/\text{s}$
- EGFP inside cells ≈ 20 µm$^2$/s
- We did not see fast component.

POLYSOMES?

From the Biology
At 25°C and low O$_2$, they are not present

FREE RI BOSOMES?

- Theoretical $D_{\text{coef}} (5 \text{ MD}) = 12 \text{ µm}^2/\text{s}$
- Measured $D_{\text{coef}}$ purified Rib = 15 +/- 2 µm$^2$/s
- Predicted inside cell ≈ 1-3 µm$^2$/s**

We are looking at the ribosome but:

Fluctuations are really slow
Origin of the GP fluctuations

Diffusion process

Apparent $D_{\text{coef}}$ will decrease

Binding process

Apparent $D_{\text{coef}}$ will not change

Point spread function scaling analysis

*Biophys J. 94(7):2884-2890.*
Point spread function scaling analysis

*Biophys J.* 94(7):2884-2890.

We are looking at a Diffusion Process
Conclusions

We are measuring the Diffusion of ribosomes.

PHC analysis *in vivo* can distinguish between species having 1 or 2 EGFP labeled proteins.