The pair correlation approach

Laboratory for Fluorescence Dynamics
Outline of the presentation

• 2 minutes history/review of FCS (relevant to this talk)
• The pair-correlation concept
• Detection and localization of barriers
• Application to nucleocytoplasmic shuttling
• Summary
2 minutes history of (FCS) Fluorescence Correlation Spectroscopy

- FCS was first described by Magde, Elson and Webb “Thermodynamic fluctuations in a reacting system. Measurement by fluorescence correlation spectroscopy”. Physical Review Letters (1972). In this paper the description of the chemical reactions was based on “changes in concentration” using differential equations describing the system as a “continuum”

- The “particle” interpretation of FCS experiments appeared. It took some time to realize that fluctuations were evidence of single molecules (Rudolf Rigler and Manfred Eigen (1974))

- The question of the observation of fluctuations from the same molecule is even more recent (Zeno Földes-Papp, 2001)

- ...and the use of spatial correlations for FCS was developed few years ago (Digman and Gratton, 2005)
FCS and spatial correlations

• The idea of using spatial correlations to measure diffusion and flow is very recent: RICS (Digman and Gratton, 2005)

• Spatial (and temporal) correlations have the potential to provide a more complete description of transport of single molecules in non-homogeneous media

• This is currently done by Single-Particle Tracking (SPT)

Can we join the detailed description of SPT but using “many molecules”? 
Fluctuation Spectroscopy for Flow

Many important papers in the FCS field. For example flow was measured with the dual foci method (Ries, J., and P. Schwille. 2006. Biophys. J.)

In our approach we use spatial and temporal correlations to measure local diffusion and transport of molecules.
Our approach provides a map of diffusion coefficients and of barriers to diffusion.

\[ G(\tau) = \frac{\langle F(t) \cdot F(t+\tau) \rangle}{\langle F(t) \rangle \langle F(t) \rangle} - 1 \]
The pair-correlation concept

We perform a single-molecule measurement in a sea of many molecules.

Correlate in time the fluctuations at many points in a grid. Measure the time for a particle at a given point to appear at another point.

The pair-correlation concept

A particle observed at time t=0 at the origin can be found at a distance ‘r’ with a probability proportional to the fluorescence intensity at a given distance.

The pair-correlation concept

Only the same particle will produce an average positive cross-correlation with a given time delay at two different points. Why?

\[
G(\tau, \delta r) = \frac{\langle F(t, 0) \cdot F(t + \tau, \delta r) \rangle}{\langle F(t, 0) \rangle \langle F(t, \delta r) \rangle} - 1
\]
• When the distance between the two points is small, points are in within the PSF, which produces correlation of the intensity fluctuations at very short time.

• As the distance increases, the time cross-correlation curve starts at very low amplitude and then increases at a later time.

• The characteristics anticorrelation at short time is the signature that we are detecting the “same” molecule at a later time.

• Dilution experiments show that the shape of the pCF(n) is the same irrespective of concentration.

The pair-correlation concept

• The pCF(n) function has a maximum at a given time which depends on the diffusion coefficient. The faster is the diffusion, the shorter is the time for the maximum.

• The pCF(n) function has also a “width” which depends on the dimension of the motion (2D or 3D or fractal). In a τ-log scale representation the width is constant.

• The amplitude of the pCF(n) depends on $1/n$ in 2D and $1/n^2$ in 3D.
Beads in solution

Detection and localization of barriers

Simulation of particles diffusing in restricted zones. The particles cannot cross the boundaries of the zone. Barriers to flow appears as “dark” (no diffusion) vertical lines in the pCF(n) carpet.
Application to nucleocytoplasmic shuttling

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
Application to nucleocytoplasmic shuttling
Nucleocytoplasmic shuttling: state of the art

1. **FRAP experiments: the nuclear envelope is a barrier to free molecular diffusion:** For 10, 27, and 56 kDa proteins, passive diffusion is slowed 100-fold at the NE boundary compared to diffusion within the nucleus or the cytoplasm, as expected for the reduced cross-sectional area of the NPCs. (Wei et al. Biophys J (2003), 84: 1317–1327)
   - ensemble averaging measurement (no single molecule information)
   - passive and active fluxes cannot be discriminated

2. **Single molecule experiment:** Active transport substrates can cross the NPC very quickly. The mean residence time at the NPC is of approximately **10 ms** (Yang et al. PNAS (2004), 101: 12887–12892)
   - requires the observation of isolated particles for long time
   - requires complex experimental procedures (purification, labeling, permeabilization)
Application to nucleocytoplasmic shuttling

PSF 

6.3 µs 

100 nm 

1 2 3 4 .... 31 32 

100 nm 

1 2 3 4 .... 31 32 

0.47 ms 

1 2 3 4 .... 31 32 

N C NE 

N C NE 

time 

1 32
ACF analysis

"main cytoplasmic events" (e.g. slowly moving organelles, structures, cytoplasmic heterogeneity)

D1 = 8.2 μm²/s
D2 = 0.0009 μm²/s

D = 11.6 ± 1.5/3.4 ± 0.6 μm²/s

Intracompartment diffusion

- the maximum of correlation <10 ms

Nucleus-to-cytoplasm passive diffusion

- The pCF algorithm detects a considerable lengthening of the time of the maximum when two positions across NE are correlated.
- The maximum of correlation is in the 100-500 ms range.

This huge delay is in keeping with the presence of a barrier to free diffusion across NE.

Nucleus-to-cytoplasm passive diffusion

NE is located at position 15 along the line

Cytoplasm-to-nucleus active import

Localization of import carriers

Control experiments-1

- Under energy depletion passive diffusion restores a homogenous NLS-GFP distribution across NE, as it is the only route available for protein shuttling.
- By cross-correlating the two compartments, we observe the typical transit delays of molecules undergoing passive diffusion through NPCs in both directions.

Control experiments-2

Diffusion at the border of two neighbor cells: as expected, no flow of proteins from one cell to the other was observed, even after long times.

• The PC method measures molecular flow at the ~100 nm scale
• Experiments with beads reproduce the PC(n) predictions (maximum at a give time, negative correlation at shorter times)
• Simulations show that we should be able to detect diffusion boundaries that are ~200 nm apart (or farther)
• Experiments with molecules in cells show obvious barriers to diffusion at the junction between cells
• Experiments with nls-EGFP show passive and active transport through nuclear pore complexes
• Diffusion in the nucleus shows disconnected regions of flow
• Connectivity depends on the cell cycle