Lecture 1
Introduction to FCS

Enrico Gratton

Laboratory for Fluorescence Dynamics
From cuvette to the microscope

1. Excitation & Emission Spectra
   • Local environment polarity, fluorophore concentration
2. Anisotropy & Polarization
   • Rotational diffusion
3. Quenching
   • Solvent accessibility
   • Character of the local environment
4. Fluorescence Lifetime
   • Dynamic processes (nanosecond timescale)
5. Resonance Energy Transfer
   • Probe-to-probe distance measurements
6. Fluorescence microscopy
   • Localization

7. Fluorescence Correlation Spectroscopy
   • Translational & rotational diffusion
   • Concentration
   • Dynamics

In the microscope, the spatial location matters: spatial correlations and distributions are a component of the experiment
Why we need FCS to measure the internal dynamics in cell?

Methods based on perturbation
   Typically FRAP (fluorescence recovery after photobleaching)

Methods based on fluctuations
   Typically FCS and dynamic ICS methods

There is a fundamental difference between the two approaches, although they are related as to the physical phenomena they report on.
In any open volume, the number of molecules or particles fluctuate according to a Poisson statistics (if the particles are not-interacting)

The average number depends on the concentration of the particles and the size of the volume

The variance is equal to the number of particles in the volume

This principle does not tell us anything about the time of the fluctuations
The fluctuation-dissipation principle

If we perturb a system from equilibrium, it returns to the average value with a characteristic time that depends on the process responsible for returning the system to equilibrium.

**Spontaneous** energy fluctuations in a part of the system, can cause the system to locally go out of equilibrium. These spontaneous fluctuations **dissipate** with the same time constant as if we had externally perturbed the equilibrium of the system.
Experimental data on colloidal gold particles:

```
120002001324123102111131125111023313332211122422122612214
2345241141311423100100421123123201111000111_2110013200000
10011000100023221002110000201001_333122000231221024011102_
1222112231000110331110210110010103011312121010121111211_10
00322101230201212321110110023312242110001203010100221734
41010100211221144442121144013212331431301122123310121111
222412231113322132110000410432012120011322231200_253212033
2331111002100220130113211113120010131432211221122323442230
321421532200202142123232043112312003314223452134110412322
220221
```

Collected data by counting (by visual inspection) the number of particles in the observation volume as a function of time using a “ultra microscope”
Particle Correlation

- Histogram of particle counts
- Poisson behavior
- Autocorrelation not available in the original paper. It can be easily calculated today.

Comments to this paper conclude that scattering will not be suitable to observe single molecules, but fluorescence could...
What can cause a fluctuation in the fluorescence signal???

- Number of fluorescent molecules in the volume of observation, diffusion or binding
- Conformational Dynamics
- Rotational Motion if polarizers are used either in emission or excitation
- Protein Folding
- Blinking
- And many more

Example of processes that could generate fluctuations

Each of the above processes has its own dynamics. FCS can recover that dynamics
Generating Fluctuations By Motion

What is Observed?

1. The Rate of Motion
2. The Concentration of Particles
3. Changes in the Particle Fluorescence while under Observation, for example conformational transitions
Defining Our Observation Volume under the microscope: One- & Two-Photon Excitation.

1 - Photon

- Defined by the pinhole size, wavelength, magnification, and numerical aperture of the objective
- Approximately 1 μm³

2 - Photon

- Defined by the wavelength and numerical aperture of the objective
1-photon

Need a pinhole to define a small volume

2-photon

Brad Amos
MRC, Cambridge, UK
Data presentation and Analysis

- The time series
- The autocorrelation function
- The histogram of the counts in a given time bin (PCH). N and brightness
- Detail of one time region
- Correlation plot (log averaged)
- Tau (s) values: $1 \times 10^{-5}$, 0.0001, 0.001, 0.01, 0.1, 1, 10
How to extract the information about the fluctuations and their characteristic time?

Distribution of the **amplitude** of the fluctuations

Distribution of the **duration** of the fluctuations

To extract the distribution of the duration of the fluctuations we use a math based on calculation of the **correlation function**.

To extract the distribution of the amplitude of the fluctuations, we use a math based on the **PCH distribution**.
The definition of the Autocorrelation Function

\[ \delta F(t) = F(t) - \langle F(t) \rangle \]

\[ G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \]
What determines the intensity of the fluorescence signal?

This is the fundamental equation in FCS

\[ F(t) = \kappa Q \int d\mathbf{r} \ W(\mathbf{r})C(\mathbf{r}, t) \]

- \( \kappa Q \) = quantum yield and detector sensitivity (how bright is our probe). This term could contain the fluctuation of the fluorescence intensity due to internal processes.
- \( W(\mathbf{r}) \) describes the profile of illumination.
- \( C(\mathbf{r}, t) \) is a function of the fluorophore concentration over time. This is the term that contains the “physics” of the diffusion processes.

The value of \( F(t) \) depends on the profile of illumination!
What about the excitation (or observation) volume shape?

\[
F(x, y, z) = I_0 I(z) e^{-\frac{2(x^2+y^2)}{w_0^2}}
\]

\[
I(z) = \text{Exp}
\begin{bmatrix}
-2z^2 \\
\frac{w_0}{w_0 z}
\end{bmatrix}
\]

Gaussian z

\[
I(z) = \frac{1}{1 + \left(\frac{z}{w_0 z}\right)^2}
\]

Lorentzian z

More on the PSF in Jay’s lecture

For the 2-photon case, these expression must be squared
In the simplest case, two parameters define the autocorrelation function: the amplitude of the fluctuation \( G(0) \) and the characteristic relaxation time of the fluctuation. 

\[
G(0) \propto \frac{1}{N}
\]

As time (tau) approaches 0.

**Diffusion**
The Effects of Particle Concentration on the Autocorrelation Curve

\[ <N> = 2 \]

\[ <N> = 4 \]

Graph showing the autocorrelation function \( G(t) \) over time with two observation volumes, one for \( <N> = 2 \) and another for \( <N> = 4 \). The graph indicates a decrease in \( G(t) \) as a function of time, illustrating the impact of particle concentration on the autocorrelation curve.
Why Is $G(0)$ Proportional to $1$/Particle Number?

A Poisson distribution describes the statistics of particle occupancy fluctuations. For a Poisson distribution the variance is proportional to the average:

$$< N > = \langle \text{Particle Number} \rangle = \text{Variance}$$

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

Definition

$$G(0) = \frac{\langle \delta F(t)^2 \rangle}{\langle F(t) \rangle^2} = \frac{\langle (F(t) - \langle F(t) \rangle)^2 \rangle}{\langle F(t) \rangle^2}$$

$$G(0) = \frac{\text{Variance}}{\langle N \rangle^2} = \frac{\langle N \rangle}{\langle N \rangle^2} = \frac{1}{\langle N \rangle}$$
G(0), Particle Brightness and Poisson Statistics

\[
\begin{align*}
\langle N \rangle & \propto \frac{\text{Average}^2}{\text{Variance}} = \frac{0.275^2}{0.256} = 0.296 \\
\text{Average} &= 0.275 \quad \text{Variance} = 0.256
\end{align*}
\]

Lets increase the particle brightness by 4x:

\[
\begin{align*}
\langle N \rangle & \propto \frac{\text{Average}^2}{\text{Variance}} = \frac{1.1^2}{4.09} = 0.296 \\
\text{Average} &= 1.1 \quad \text{Variance} = 4.09
\end{align*}
\]
Effect of Shape on the (Two-Photon) Autocorrelation Functions:

For a 2-dimensional Gaussian excitation volume:

\[
G(\tau) = \frac{\gamma}{N} \left( 1 + \frac{4D\, \tau}{w_{2DG}^2} \right)^{-1}
\]

For a 3-dimensional Gaussian excitation volume:

\[
G(\tau) = \frac{\gamma}{N} \left( 1 + \frac{4D\, \tau}{w_{3DG}^2} \right)^{-1} \left( 1 + \frac{4D\, \tau}{z_{3DG}^2} \right)^{-1/2}
\]

3D Gaussian “time” analysis: with \( \tau_D = w^2/4D \) and \( S = w/z \)

\[
G(\tau) = \frac{\gamma}{N} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \left( 1 + S^2 \cdot \frac{\tau}{\tau_D} \right)^{-1/2}
\]

2-photon equation contains a 8, instead of 4
Blinking or other exponential processes:

If the particle blinks during the times it goes through the illumination volume, an additional term appears in the fluctuation amplitude.

**How to account for this process?**

**Reasoning:** let us assume that the particle is **not moving** and it is at the center of the PSF.
The intensity will turn **ON** and **OFF**.
The **OFF** time depends on the characteristic blinking time (triplet state lifetime).
The **ON** time depends on the laser intensity. The larger the laser intensity, the lesser is the **ON** time.

\[
G(\tau) = (1 + \frac{T}{1-T} e^{-\frac{\tau}{\tau_T}})
\]

**Triplet state term:**  
*\(T\) is the triplet state amplitude \(\tau_T\) is the triplet lifetime.*
Until now, we assumed that the particle is not moving. If we assume that the blinking of the particle is independent on its movement, we can use a general principle that states that the correlation function splits in the product of the two independent processes.

\[ G_{Total}(\tau) = G_{Blinking}(\tau) \cdot G_{Diffusion}(\tau) \]

\[ G_{Binding}(\tau) = \left[ 1 + K \left( f_A - \frac{f_B}{K} \right)^2 e^{-\lambda \tau} \right] \]

\( K = k_f / k_b \) is the equilibrium coefficient; \( \lambda = k_f + k_b \) is the apparent reaction rate coefficient; and \( f_j \) is the fractional intensity contribution of species \( j \).
How different is $G$(binding) from $G$(diffusion)?

With good S/N it is possible to distinguish between the two processes. Most of the time diffusion and exponential processes are combined.
# Table of characteristic times for diffusion

**Orders of magnitude (for 1 μM solution, small molecule, water)**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Device</th>
<th>Size(μm)</th>
<th>Molecules</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>milliliter</td>
<td>cuvette</td>
<td>10000</td>
<td>6x10^{14}</td>
<td>10^4</td>
</tr>
<tr>
<td>microliter</td>
<td>plate well</td>
<td>1000</td>
<td>6x10^{11}</td>
<td>10^2</td>
</tr>
<tr>
<td>nanoliter</td>
<td>microfabrication</td>
<td>100</td>
<td>6x10^8</td>
<td>1</td>
</tr>
<tr>
<td>picoliter</td>
<td>typical cell</td>
<td>10</td>
<td>6x10^5</td>
<td>10^{-2}</td>
</tr>
<tr>
<td>femtoliter</td>
<td>confocal volume</td>
<td>1</td>
<td>6x10^2</td>
<td>10^{-4}</td>
</tr>
<tr>
<td>attoliter</td>
<td>nanofabrication</td>
<td>0.1</td>
<td>6x10^{-1}</td>
<td>10^{-6}</td>
</tr>
</tbody>
</table>
The Effects of Particle Size on the Autocorrelation Curve

Diffusion Constants

- 300 \( \text{um}^2/\text{s} \)
- 90 \( \text{um}^2/\text{s} \)
- 71 \( \text{um}^2/\text{s} \)

Stokes-Einstein Equation:

\[
D = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot r}
\]

and

\[
MW \propto Volume \propto r^3
\]

Monomer --> Dimer

Only a change in D by a factor of \(2^{1/3}\), or 1.26
Examples of different *HeLa* cells transfected with AK1-EGFP

Examples of different *HeLa* cells transfected with AK1β-EGFP

*Qiao Qiao Ruan, Y. Chen, M. Glaser & W. Mantulin Dept. Biochem & Dept Physics– LFD Univ II, USA*
Normalized autocorrelation curve of EGFP in solution (•), EGFP in the cell (•), AK1-EGFP in the cell(•), AK1β-EGFP in the cytoplasm of the cell(•).
Autocorrelation of Adenylate Kinase –EGFP on the Membrane

Clearly more than one diffusion time

A mixture of AK1b-EGFP in the cytoplasm and membrane of the cell.
**Autocorrelation Adenylate Kinaseβ -EGFP**

Diffusion constants (um²/s) of AK EGFP–AKβ in the cytosol -EGFP in the cell (HeLa). At the membrane, a dual diffusion rate is calculated from FCS data. Away from the plasma membrane, single diffusion constants are found.
Two Channel Detection: Cross-correlation

1. Increases signal to noise by isolating correlated signals.
2. Corrects for PMT noise

Sample Excitation Volume

Beam Splitter

Detector 1

Detector 2

Each detector observes the same particles
Removal of Detector Noise by Cross-correlation

11.5 nM Fluorescein

Detector after-pulsing

Cross-correlation
Calculating the Cross-correlation Function

Detector 1: $F_i$

Detector 2: $F_j$

$G_{ij}(\tau) = \frac{\langle dF_i(t) \cdot dF_j(t + \tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle}$
Cross-correlation calculations

One uses the same fitting functions you would use for the standard autocorrelation curves.

Thus, for a 3-dimensional Gaussian excitation volume one uses:

\[ G_{12}(\tau) = \frac{\gamma}{N_{12}} \left( 1 + \frac{4D_{12}\tau}{w^2} \right)^{-1} \left( 1 + \frac{4D_{12}\tau}{z^2} \right)^{-1/2} \]

\( G_{12} \) is commonly used to denote the cross-correlation and \( G_1 \) and \( G_2 \) for the autocorrelation of the individual detectors. Sometimes you will see \( G_x(0) \) or \( C(0) \) used for the cross-correlation.
Two-Color Cross-correlation

The cross-correlation ONLY if particles are observed in both channels.

Each detector observes particles with a particular color.

The cross-correlation signal:

Only the green-red molecules are observed!!
Two-color Cross-correlation

Equations are similar to those for the cross correlation using a simple beam splitter:

$$G_{ij}(\tau) = \frac{\langle dF_i(t) \cdot dF_j(t + \tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle}$$

### Information Content

<table>
<thead>
<tr>
<th>Correlated signal from particles having both colors.</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{12}(\tau)$</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Autocorrelation from channel 1 on the green particles.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_1(\tau)$</td>
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</table>

<table>
<thead>
<tr>
<th>Autocorrelation from channel 2 on the red particles.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_2(\tau)$</td>
<td></td>
</tr>
</tbody>
</table>
Experimental Concerns: Excitation Focusing & Emission Collection

We assume exact match of the observation volumes in our calculations which is difficult to obtain experimentally.

Excitation side:
(1) Laser alignment
(2) Chromatic aberration
(3) Spherical aberration

Emission side:
(1) Chromatic aberrations
(2) Spherical aberrations
(3) Improper alignment of detectors or pinhole
  (cropping of the beam and focal point position)
Two-Color Fluctuation Correlation Spectroscopy

Uncorrelated

\[
G_{ij}(\tau) = \frac{\langle F_i(t) F_j(t + \tau) \rangle}{\langle F_i(t) \rangle \langle F_j(t) \rangle} - 1
\]

Correlated

For two uncorrelated species, the amplitude of the cross-correlation is proportional to:

\[
G_{12}(0) \propto \left[ \frac{f_{11}f_{12}\langle N_1 \rangle + f_{21}f_{22}\langle N_2 \rangle}{f_{11}f_{12}\langle N_1 \rangle^2 + (f_{11}f_{22} + f_{21}f_{12})\langle N_1 \rangle\langle N_2 \rangle + f_{21}f_{22}\langle N_2 \rangle^2} \right]
\]

Interconverting
Does SSTR1 exist as a monomer after ligand binding while SSTR5 exists as a dimer/oligomer?

Collaboration with Ramesh Patel*† and Ujendra Kumar*

*Fraser Laboratories, Departments of Medicine, Pharmacology, and Therapeutics and Neurology and Neurosurgery, McGill University, and Royal Victoria Hospital, Montreal, QC, Canada H3A 1A1; †Department of Chemistry and Physics, Clarkson University, Potsdam, NY 13699

Three Different CHO-K1 cell lines: wt R1, HA-R5, and wt R1/HA-R5

Hypothesis: R1 - monomer; R5 - dimer/oligomer; R1R5 dimer/oligomer
SSTR1 CHO-K1 cells with SST-fitc + SST-tr

Green Ch.

- Very little labeled SST inside cell nucleus
- Non-homogeneous distribution of SST
- Impossible to distinguish co-localization from molecular interaction

Red Ch.
A  
Monomer  

\[
\frac{G_{12}(0)}{G_1(0)} = 0.22
\]

B  
Dimer  

\[
\frac{G_{12}(0)}{G_1(0)} = 0.71
\]
Experimentally derived auto- and cross-correlation curves from live R1 and R5/R1 expressing CHO-K1 cells using dual-color two-photon FCS.

The R5/R1 expressing cells have a greater cross-correlation relative to the simulated boundaries than the R1 expressing cells, indicating a higher level of dimer/oligomer formation.

Discussion

1. The PSF: how much it affects our estimation of the processes?
2. Models for diffusion, anomalous?
3. Binding?
4. FRET (dynamic FRET)?
5. Bleaching?

6. ……and many more questions