Fluorescence Correlation Spectroscopy and Förster Resonance Energy Transfer

Thorsten Wohland
Interaction of Light with Matter

incoming light

mirror-like reflection (specular reflection)

diffuse reflection

scattering

internal reflection

transmitted light

luminescence
Internal Conversion

- **Excited Singlet State** $S_1$
- **Ground State** $S_0$
- **Triplet State** $T_1$
- **Absorption**
- **Fluorescence**

**VR**: vibrational relaxation
**IC**: Internal Conversion
Intersystem Crossing

Ground State $S_0$

Excited Singlet State $S_1$

Triplet State $T_1$

Absorption

Fluorescence

Delayed Fluorescence

Photochemical reaction

Phosphorescence

VR: vibrational relaxation

ISC: Intersystem Crossing
Lifetimes, rate constants, and quantum yield

Excitation rate $k_{ex} \sim I$

- Lifetime
  $$\tau = \frac{I}{\Gamma + k_{nr}}$$

- Quantum yield
  $$\phi_f = \frac{\Gamma}{\Gamma + k_{nr}}$$
Fluorescence Properties

- Wavelength (absorption and emission)
- Lifetime (of various states)
- Quantum yield
- Polarization

http://micro.magnet.fsu.edu
F-techniques

Fluorescence Anisotropy

Fluorescence Lifetime

Single Particle Tracking

Fluorescence Recovery after Photobleaching
Förster Resonance Energy Transfer - FRET

A

Intensity [a.u.]

300 400 500 600 700

Wavelength [nm]

B

Efficiency

0 2 4 6 8 10

Distance [nm]

θD, θA, θr, θC2, R, φ

FRET
The actual formula for the FRET rate

\[ k_T(r) = \frac{Q_D \kappa^2}{\tau_D r^6} \left( \frac{9000(\ln 10)}{128\pi^5 N_A n^4} \right) \int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \]

- **Orientation**
- **Distance**
- **Donor lifetime**
- **Spectral overlap**

Donor lifetime

\[ k_T(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6 \]

R_0 is the so-called **Förster radius**. It is the distance at which a FRET pair exhibits 50% FRET. It is a constant for any FRET-pair.

Environmental

\[ E = \frac{k_T}{\tau_D^{-1} + k_T} \]

\[ E = \frac{R_0^6}{R_0^6 + r^6} \]

Förster Distance Calculator
FRET to monitor conformational changes of a virus

- **Donor**: Alexafluor 488 TFP (AF488) labelled protein layer
- **Acceptor**: Dil labelled lipid bilayer

**Near Donor – Acceptor**
- High FRET
- Lower donor fluorescence intensity
- Lower average donor lifetime

**Far Donor – Acceptor**
- Low FRET
- High fluorescence intensity
- High average donor lifetime
Lifetime experiment

\[ \tau_D = \frac{1}{\Gamma + k_{nr} + k_T} \]

**Higher FRET at 25°C**

- **25°C**
  - Intensity (kcts) vs. Time (s)
  - φ²₁ (Low FRET population)
  - τavg (ns)

- **37°C**
  - Intensity (kcts) vs. Time (s)
  - φ²₂ (High FRET population)
  - τavg (ns)

- **Temperature**
  - a₁ (Low FRET population) vs. Temperature
  - a₂ (High FRET population) vs. Temperature
Temperature dependence of lipid bilayer-protein coat distance

Temperature range: 25 to 37°C

DV2 (NGC) transition vs temperature in absence of MgCl$_2$

Temperature (°C): 25 to 37°C (Donor only)

Temperature (°C): 25 to 37°C (Dual labelled)

Temperature (°C): 37°C to 25°C (Dual labelled)

$a_1$ (low FRET population)

$a_2$ (High FRET population)
Single particle spectroscopy

Imaged under TIRF microscope
Single particle spectroscopy

Donor

Acceptor

iSMS

Overlay
Single particle spectroscopy

Molecule 1

- Donor Intensity
  - Background
  - Donor

Molecule 2

- Acceptor Intensity
  - Background
  - Acceptor

Molecule 3

- Overlay
  - Acceptor intensity
  - Donor Intensity

- E_{FRET}
  - Time(s)

*Single particle spectroscopy*
Single particle spectroscopy

(ref: Sean A. McKinney et al. 2002)
Summary 1

• FRET can measure distances in the range of ~10 nm
• It can be measured either by observing the emission wavelength or best by lifetimes
• It can be done in ensembles or on a single molecule level
• Here we demonstrated its application to viral conformations and holiday junction dynamics
Fluorescence Correlation Spectroscopy (FCS)

- What are fluctuations?
- What are correlations?
- How to calculate correlations?
- Fluorescence Correlation Spectroscopy (FCS)
- FRET-FCS
- Fluorescence Cross-Correlation Spectroscopy (FCCS)
- Imaging FCS/FCCS
Fluctuations

$$A + B \iff AB$$

[Diagram showing kinetics and equilibrium with fluctuations represented graphically.]
FCS: General idea

• What is a correlation
• Predicting the future
• Self-similarity
Correlations

\[ \langle a \cdot b \rangle \neq \langle a \rangle \langle b \rangle \]

\[ g = \frac{\langle a \cdot b \rangle}{\langle a \rangle \langle b \rangle} \]

- **Anti-correlation** \( g < 1 \)
- **No correlation** \( g = 1 \)
- **Correlation** \( g > 1 \)

### Example

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Probability</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0.25</td>
<td></td>
<td>&lt;A&gt; = 0.5</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>0.25</td>
<td></td>
<td>&lt;B&gt; = 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25</td>
<td></td>
<td>&lt;AB&gt; = 0.25</td>
</tr>
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<td>0</td>
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<td>0.25</td>
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<td>&lt;AB&gt; = 0.25</td>
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</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0.25</td>
<td></td>
<td>&lt;AB&gt; = 0.25</td>
</tr>
</tbody>
</table>

\[
\langle A \rangle < \langle B \rangle = 1
\]
**Example**

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.2</td>
<td>&lt;A&gt; = 0.5</td>
</tr>
<tr>
<td>0.2</td>
<td>0.2</td>
<td>&lt;B&gt; = 0.5</td>
</tr>
<tr>
<td>0.2</td>
<td>0.2</td>
<td>&lt;AB&gt; = 0.2</td>
</tr>
<tr>
<td>0.3</td>
<td>0.3</td>
<td>&lt;A&gt;&lt;B&gt; = 0.8</td>
</tr>
</tbody>
</table>
### Example

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>Probability</th>
<th>$\langle A \rangle = 0.5$</th>
<th>$\langle B \rangle = 0.5$</th>
<th>$\langle AB \rangle = 0.4$</th>
<th>$\frac{\langle AB \rangle}{\langle A \rangle \langle B \rangle} = 1.6$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="#" alt="Coin A" /></td>
<td><img src="#" alt="Coin B" /></td>
<td>0.1</td>
<td><img src="#" alt="Coin A" /></td>
<td><img src="#" alt="Coin B" /></td>
<td><img src="#" alt="Coin A" /></td>
<td><img src="#" alt="Coin A" /></td>
</tr>
<tr>
<td><img src="#" alt="Coin A" /></td>
<td><img src="#" alt="Coin B" /></td>
<td>0.4</td>
<td><img src="#" alt="Coin A" /></td>
<td><img src="#" alt="Coin B" /></td>
<td><img src="#" alt="Coin A" /></td>
<td><img src="#" alt="Coin A" /></td>
</tr>
<tr>
<td><img src="#" alt="Coin A" /></td>
<td><img src="#" alt="Coin B" /></td>
<td>0.4</td>
<td><img src="#" alt="Coin A" /></td>
<td><img src="#" alt="Coin B" /></td>
<td><img src="#" alt="Coin A" /></td>
<td><img src="#" alt="Coin A" /></td>
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<td>0.1</td>
<td><img src="#" alt="Coin A" /></td>
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<td><img src="#" alt="Coin A" /></td>
<td><img src="#" alt="Coin A" /></td>
</tr>
</tbody>
</table>
## Correlations

### 1. Correlated variables

<table>
<thead>
<tr>
<th></th>
<th>a</th>
<th>b</th>
<th>\langle a \cdot b \rangle \geq \langle a \rangle \langle b \rangle</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1 1 0 1 0 0 1 1 1 0 1 0 1 0 1 1 0 0 0 0</td>
<td>1 1 0 1 0 0 1 1 1 0 1 0 1 0 1 1 0 0 0 0</td>
<td>\langle a \cdot b \rangle = \frac{1}{2}; \langle a \rangle \langle b \rangle = \frac{1}{2} \cdot \frac{1}{2} = \frac{1}{4}</td>
</tr>
</tbody>
</table>

### 2. Anticorrelated variables

<table>
<thead>
<tr>
<th></th>
<th>a</th>
<th>b</th>
<th>\langle a \cdot b \rangle &lt; \langle a \rangle \langle b \rangle</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1 1 0 0 1 0 1 0 1 1 1 0 1 0 0 0 1 0 1 0</td>
<td>0 0 1 1 0 1 0 1 0 0 0 1 0 1 1 1 0 1 0 1</td>
<td>\langle a \cdot b \rangle = 0; \langle a \rangle \langle b \rangle = \frac{1}{2} \cdot \frac{1}{2} = \frac{1}{4}</td>
</tr>
</tbody>
</table>

### 3. Uncorrelated variables

<table>
<thead>
<tr>
<th></th>
<th>a</th>
<th>b</th>
<th>\langle a \cdot b \rangle = \langle a \rangle \langle b \rangle</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1 0 1 0 1 0 1 0 1 0 1 0 1 0 1 0 1 0 1 0</td>
<td>1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1</td>
<td>\langle a \cdot b \rangle = \frac{1}{2}; \langle a \rangle \langle b \rangle = \frac{1}{2} \cdot 1 = \frac{1}{2}</td>
</tr>
</tbody>
</table>

\[ a \cdot b \text{ represents the inner product of } a \text{ and } b. \]
Autocorrelations

\[ \langle a(t) \cdot a(t) \rangle \geq \langle a(t) \rangle \langle a(t) \rangle \]

\[ \langle a(t) \cdot a(t + \tau) \rangle \geq \langle a(t) \rangle \langle a(t + \tau) \rangle \]

\[ G(\tau) = \frac{\langle a(t) \cdot a(t + \tau) \rangle}{\langle a(t) \rangle \langle a(t + \tau) \rangle} \]

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

Stationary Processes
Short time shifts $\tau$

\[ \langle F(t) \cdot F(t + \tau) \rangle \geq \langle F(t) \rangle \langle F(t + \tau) \rangle \]

Blue: $F(t)$
Yellow: $F(t + \tau)$

The intensity peaks always overlap to some extent and thus

\[ \langle F(t) \cdot F(t + \tau_3) \rangle \]
Long time shifts $\tau$

The intensity trace contains a random pattern of intensity peaks. Therefore an overlap of all/many peaks is only achievable at short times.

$$\langle F(t) \cdot F(t + \tau) \rangle \neq \langle F(t) \rangle \langle F(t + \tau) \rangle$$

Blue: $F(t)$
Yellow: $F(t + \tau)$
Periodic signals

\[ \langle F(t) \cdot F(t + \tau) \rangle \approx \langle F(t) \rangle \langle F(t + \tau) \rangle \]

Blue: \( F(t) \)
Yellow: \( F(t + \tau) \)

The intensity trace contains a regular pattern of intensity peaks (i.e. it is repeated). Therefore an overlap of all/many peaks is achievable periodically and the correlation function will show that periodicity.
ACF: Autocorrelation Function (the correlation of a variable with itself)

CCF: Cross-correlation Function (the correlation between two variables)
How is an ACF calculated practically?

Intensity values recorded every nanosecond

To calculate the correlation for the range of seconds you would need 1 billion values ...

If we make the time bins larger then we lose the information at short times.

So best would be to use a varying time scheme.

Correlation Time Schemes

The typical scheme used is called the semi-logarithmic time scale. The first \( n \) channels have a time \( \Delta \tau \). The second group contains \( n/2 \) channels with \( 2 \Delta \tau \). The next group \( n/2 \) channels with \( 4 \Delta \tau \).

1) Each time a new measurement of length \( \Delta \tau \) comes in, calculate all ACF values for lag times 0 to 16\( \Delta \tau \).

2) After 2 measurements of \( \Delta \tau \), correlate the last two newest measurements with all channels in group 2. Then take the last two channels of group 1 and combine them into one channel with width 2\( \Delta \tau \) of group 2 and shift.
Confocal FCS setup

Thompson, *Topics in Fluorescence Spectroscopy Techniques* vol 1 (1991)
Haustein and Schwille, *Biophysics Textbook Online*
FCS: Characteristic Parameters

\[
\tau_D \propto \frac{3}{\sqrt{M}}
\]

\[
G(0) \sim \frac{1}{N}
\]
Correlation Functions

\[ G(\tau) = \frac{1}{\langle C \rangle \pi^{3/2} w_0^2 z_0} \left( 1 + \frac{4D\tau}{w_0^2} \right)^{-1/2} \left( 1 + \frac{4D\tau}{z_0^2} \right)^{-1/2} \]

Number of particles
\[ N = \langle C \rangle V_{eff} = \langle C \rangle \pi^{3/2} w_0^2 z_0 \]

Correlation time
\[ \tau_D = \frac{w_0^2}{4D} \]

Structure factor
\[ K = \frac{z_0}{w_0} \]

\[ G(\tau) = \frac{1}{N} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \left( 1 + \frac{\tau}{K^2 \tau_D} \right)^{-1/2} + G_\infty \]
FCS

Parameters: Width, Amplitude, Shape

Width: characteristic time
Time scales accessible lie between:
1 ns and 1 s

Amplitude: concentration
Accessible range: 50 pM to 1 μM

Shape: Type of process

Live cell measurements

Differences in correlation width
Experimental process

Construction:
EGFR-GFP, EGFR-YFP, EGFR-mRFP

Transfection

Calibration

FCS, FCCS setup

Z-scan

EGFR-mRFP

Free mRFP

Comparison of cytosolic free FPs and membrane fusion EGFR-FPs

EGFR-GFP

GFP

Triplet state at microsecond
Photodynamics at hundred microseconds
Diffusion of membrane-localised EGFR-GFP at tens of milliseconds

Triplet state at microsecond
Photodynamics at hundred microseconds
Diffusion of cytoplasmic GFP 1 milisecond
Lignad-Receptor Binding

How to use amplitude and width of and autocorrelation function
Example: The 5HT$_3$ Receptor

The ligand GR65630 was labeled with Cy5

a) Determination of binding constants
b) And stoichionetry ...
Stoichiometry of ligand binding

$$R + nL \rightleftharpoons RL_n$$
Ligand-Receptor Interactions

Ligands: 0.5 – 1.1 kDa

$C_{12}E_9$ micelle: 60 - 70 kDa

5HT$_3$As-R + micelle: ~320 kDa

---

**Does Binding Occur?**

**Binding Curve**

GR-Cy5: $K_d^{FCS} = 15.7 \pm 8.0 nM$

$K_d^{RBA} = 18.0 \pm 2.0 nM$

Mass: 500±300 kDa

Stoichiometry: 1:1
FRET-FCS

Other fluctuations …
FRET-FCS

Fluorescence acquisition in donor and acceptor channels

Proximity ratio calculated
\[ p = \frac{I_A}{I_A + ID} \]

ACF of Proximity ratio:
\[ G_p(t) = \frac{\langle \delta P(t_0) \delta P(t_0 + t) \rangle}{\langle P(t_0) \rangle^2} \]

Fitting using Stretched Exponential Function

25 degree = 70 µs
50 degree = 37 µs
Dengue Virus 2 envelop dynamics

![Graph showing temperature dependence of DV2 Effective relaxation time and DV2 (NGC) Proximity ratio curves.](image)
Summary 2

- Autocorrelation functions provide a measure for the self-similarity of a signal.
- For a signal in time it provides the capability to predict the future (statistically).
- FCS provides information on dynamics of processes.
- In the case of diffusion it provides diffusion coefficients and concentrations.
- But any fluctuations can be measured and correlated (e.g. FRET-FCS).
Fluorescence Cross-correlation Spectroscopy (FCCS)
Fluorescence Cross-correlation Spectroscopy (FCCS)

Green ($G, GR$) + crosstalk ($R$) + background

Red ($R, GR$) + crosstalk ($G$) + background

GreenRed ($GR$) + crosstalk ($G, R$) + background
SW-FCCS

Fluorophores:
- Quantum dots
- Tandem dyes (energy transfer dyes)
- Organic dyes
- Fluorescent proteins

~2000 counts per second and particle

How to determine the $K_d$

$$[G] + [R] \leftrightarrow [GR]$$

$$K_d = \frac{[G][R]}{[GR]}$$

$$[G][R] = K_d [GR]$$

Line through origin with a slope of $K_d$
Cdc42 is a regulator of membrane trafficking and cytokeletal organization.

IQGAP1 is involved in regulation of cell motility and morphology and is supposed to bind the active GTP bound form of Cdc42.

**Dominant negative** (GDP bound)  
**Constitutively active** (GTP bound)

- GTP: guanosine triphosphate;
- GDP: guanosine diphosphate;
- GEF: guanine nucleotide exchange factors;
- GAP: GTPase-activating proteins;
- GDI: guanine nucleotide exchange inhibitors.
SW-FCCS in zebrafish embryos
Examples of Applications

**EGFR activation and signaling**


**Cytosolic protein interaction (cdc42/IQGAP1)**


**Ligand-receptor binding**

- (Nodal Factor binding to activin receptor II)
  - Wang et al. (eLife, 2016)
IMAGING FCCS
FCS in a confocal system

1) Measurements are not simultaneous
2) Cell damage by long illumination times
Alternative illumination schemes

TIR – Total Internal reflection

VAI – Variable Angle Illumination

SPIM – Single Plane Illumination Microscopy

The z-sectioning of the illumination together with the xy-sectioning provided by the pixels of a camera define multiple observation volumes.


Imaging FCS

G(τ)

Concentration

D

N
Examples

DLPC/DSPC bilayer on glass

GFP-GPI on SH-SY5Y cells

Imaging Fluorescence Cross-Correlation Spectroscopy (TIRF illumination)

Neuroblastoma cell labeled with Dil-C18 (pos. control). 514 nm, 300 μW excitation.
Imaging FCCS on EGFR

Degree of dimerization

\[ q = \frac{G_{GR}(0)}{\text{Min}\{G_G(0), G_R(0)\}} \]
Acknowledgements

Group members
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Wang Xi*
Ng Xue Wen
Anand Paratap Singh*
Nirmalya Bag*
Radek Machan*

Jagadish Sankaran
Kamal Kant Sharma
Huang Shuangru*
Sibel Javas*
Angela Koh
Andreas Karampatzakis
Sarala N. Tantirimudalige
Jonathan Foo
Sapthaswaran Veerapathiran
Anjali Gupta

Collaborators
Vladimir Korzh and Cathleen The (IMCB)
Karuna Sampath (TLL, U of Warwick)
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Timothy Saunders (NUS)