Fluorescence Correlation and Cross-Correlation Spectroscopy for the measurement of molecular dynamics and interactions

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Outline

- Fluorescence Correlation Spectroscopy (FCS)
  - Introduction to basics of FCS
  - How to use amplitude, width and shape to obtain quantitative information
- Fluorescence Cross-Correlation Spectroscopy (FCCS)
  - Measurement of interactions and affinity constants ($K_d$s)
- FCS limitations and workarounds
- Imaging FCS
  - Motivation and principles
  - Example: Organization of Wnt3 in zebrafish membranes
Fluctuations

A + B ⇄ AB

[AB]

Time

equilibrium

kinetics

fluctuations

fluctuations

3
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: \( g > 1 \)

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 $\tau$

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

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

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

\[
\langle F(t) \cdot F(t + \tau) \rangle = \langle F(t) \rangle \langle F(t + \tau) \rangle
\]
Periodic signals

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

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

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)
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 $4 \Delta \tau$. 

$n=16$

$\Delta \tau$

$n=8$

$2\Delta \tau$

$n=8$

$4\Delta \tau$

...
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
FCS: Characteristic Parameters

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

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

\[ G(\tau) = \frac{\langle F(t+\tau)F(t) \rangle}{\langle F(t) \rangle^2} = \frac{\langle \delta F(t)\delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} + 1 \]
Correlation Functions

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

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

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

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

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

Afterpulsing (APD)

$\Delta \tau$

$T_{\text{max}}$
Removal of afterpulsing by cross-correlation
Data Fitting: Raw data
Weighted data fits

<table>
<thead>
<tr>
<th></th>
<th>non-weighted fit</th>
<th>weighted fit</th>
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<tbody>
<tr>
<td>$N$</td>
<td>2.601</td>
<td>2.591</td>
</tr>
<tr>
<td>$\tau_D$</td>
<td>4.781e-05</td>
<td>4.739e-05</td>
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<tr>
<td>$\tau_{Trip}$</td>
<td>4.992e-06</td>
<td>4.406e-06</td>
</tr>
<tr>
<td>$F_{Trip}$</td>
<td>1.010e-01</td>
<td>8.187e-02</td>
</tr>
<tr>
<td>$K$</td>
<td>6.089</td>
<td>5.843</td>
</tr>
<tr>
<td>$G_\infty$</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Standard deviation

<table>
<thead>
<tr>
<th></th>
<th>non-weighted fit</th>
<th>weighted fit</th>
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</thead>
<tbody>
<tr>
<td>$N$</td>
<td>9.377e-02 (3.6 %)</td>
<td>6.294e-02 (2.4 %)</td>
</tr>
<tr>
<td>$\tau_D$</td>
<td>4.254e-06 (8.9 %)</td>
<td>2.699e-06 (5.7 %)</td>
</tr>
<tr>
<td>$\tau_{Trip}$</td>
<td>4.128e-06 (83 %)</td>
<td>2.439e-06 (55 %)</td>
</tr>
<tr>
<td>$F_{Trip}$</td>
<td>3.780e-02 (37 %)</td>
<td>1.853e-02 (23 %)</td>
</tr>
<tr>
<td>$K$</td>
<td>1.940 (32 %)</td>
<td>1.219 (21 %)</td>
</tr>
<tr>
<td>$G_\infty$</td>
<td>8.698e-05</td>
<td>5.586e-05</td>
</tr>
</tbody>
</table>

Measured over 10 experiments
How to use the FCS amplitude

Aggregation numbers of detergent/lipid micelles
Determination of the aggregation of detergent and LPS

Micelle Formation

R18 non-flu oligomers -> Micelle formation->dissolution of R18 oligomers and incorporation into micelles with fluorescence increase
Determination of the aggregation of detergent and LPS

Yu et al., Analytica Chimica Acta 556 (2006) 216–225
Determination of the aggregation of detergent and LPS

\[ C_{pr} < C_{mic} \]

\[ C_{pr} = C_{mic} \]

\[ C_{pr} > C_{mic} \]

\[ C_{12E9} \]

\[ c_{cmc} = 93-105 \, \mu M; \, N_{agg} = 112-132 \]

\[ (c_{cmc} = 80 \, \mu M; \, N_{agg} = 120) \]

\[ c_{cmc} = 1.3-1.6 \, \mu M; \, N_{agg} = 43-49 \]
How to use the FCS width

Ligand affinities for the 5HT$_3$ receptor
Measurements in Solution

A: Ligand in Buffer solution

B: Ligand + Detergent

C: Ligand+Detergent +Receptor

D: Ligand+Detergent +Competitor

Parameters: correlation times $(\tau_1, \tau_2, \tau_3)$, fraction of particles $(Y_1, Y_2, Y_3)$
Ligand-Receptor Interactions

Ligands: 0.5 – 1.1 kDa
C\textsubscript{12}E\textsubscript{9} micelle: 60 - 70 kDa
5HT\textsubscript{3As} - R + micelle: ~320 kDa

Binding Curve

\[ K_d^{FCS} = 15.7 \pm 8.0 \text{nM} \]
\[ K_d^{RBA} = 18.0 \pm 2.0 \text{nM} \]
How to use the FCS shape

Determination of morphogen secretion in live zebrafish
Wnt Signaling

Canonical Wnt Pathway
Wnt/β-catenin signaling

Non-Canonical Wnt Pathway
β-catenin independent Wnt signaling

1. Is Wnt3 secreted?
2. Where in the membrane does Wnt3 reside?

Wnt3EGFP Expression in the Cerebellum

Wnt3EGFP Secretion to the Brain Ventricle

ce: cerebellum; BV: brain ventricle
Bayesian Model Selection

- Bayes’ Theorem

\[
P(M_k|y) = \frac{P(y|M_k)P(M_k)}{P(y)}
\]

\[
P(y|M_k) = \int_{\beta} P(y|\beta, M_k)P(\beta|M_k)\,d\beta
\]

\[
P(y|\beta) = \frac{1}{(2\pi)^{n/2}\sqrt{\det(C)}}\exp\left\{-\frac{1}{2}\left[y - f(x, \beta)\right]^T C^{-1}\left[y - f(x, \beta)\right]\right\}
\]

\[
\times [y - f(x, \beta)]
\]
Zebrafish FCS measurements

1% low melting agarose
glass-bottom culture dish
60X dorsal view

Detection Volume
Protein
Membrane

τ₁ = 775 μs
τ₂ = 29 ms

ce: cerebellum; BV: brain ventricle
Fluorescence Cross-Correlation Spectroscopy
Fluorescence Cross-correlation Spectroscopy (FCCS)
SW-FCCS

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


~2000 counts per second and particle
How to determine the $K_d$

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

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

Line through origin with a slope of $K_d$

Normalized Frequency

$\ln K_D$ (ln nM)

Normalized Frequency

$\ln K_D$ (ln nM)
Nodal/Acvr2b interactions in live zebrafish

Wang et al., eLife 2016, 5:e13879
Examples of Applications

Membrane proteins: EGFR dimerization
Yavas et al., *Biophys. J.* 111(10) - 2241-2254 (2016)

Membrane and cytosolic proteins: EGFR activation

Cytosolic protein (cdc42 and effectors: IQGAP1, N-WASP etc.)

Protein - DNA: Sox2/Oct4 DNA motif binding and cooperativity
EMBO Reports 2015 16(9)p1177
bioarxiv 052530

Nuclear proteins: K_d's of kinetochore protein interactions (CENPs)
Some FCS limitations and solutions
Scanning FCS: The problem of immobile particles

- Moving particles
- Immobile particles
- Scanning Beam

ACF curve for pure diffusion
No correlation
ACF curve for scanning

\[ G(\tau) \]
Scanning FCS: The problem of moving membranes
Bleach Correction

Wavelet Shrinking

Fitting of a multi-exponential decay

Imaging FCS
FCS in a confocal system

1) Measurements are not simultaneous
2) Cell damage by long illumination times
Imaging FCS
Examples

DLPC/DSPC bilayer on glass

GFP-GPI on SH-SY5Y cells

FCS videos

- hIAPP treated Dil-C18 labelled SH-SY5Y cell
- RhoPE labelled DOPC bilayer
- GFP-GPI transfected SH-SY5Y cell at different temperature
Imaging FCCS on EGFR

Degree of dimerization

\[ q = \frac{G_{GR}(0)}{\text{Min}\{G_{G}(0), G_{R}(0)\}} \]
Single Plane Illumination Microscopy (SPIM)
SPIM-FCS in 3D

Giant Unilamellar Vesicles (GUV)

RhoPE-labelled DOPC:DOPG (10:1)

Dil-C<sub>18</sub> labelled live SH-SY5Y cell

Ng et al., Biophys. J., (2016) accepted
Imaging FCS diffusion law

Free Diffusion
$D_{\text{micro}} = D_{\text{macro}}$

Raft Partitioning
$D_{\text{micro}} < D_{\text{macro}}$

Meshwork
$D_{\text{micro}} > D_{\text{macro}}$

Pixel binning

EMCCD chip

$\tau_D(A_{\text{eff}}) = \tau_0 + \frac{A_{\text{eff}}}{D}$

Wawrezinieck et al. Biophysical Journal, 2005
Huang and Pralle, arXiv:1101.5087
Imaging FCS diffusion law

\[ \tau_D(A_{eff}) = \tau_0 + \frac{A_{eff}}{D_{eff}} \]

- Bag et al., *Biophysical Journal*, 2015

<table>
<thead>
<tr>
<th>Diffusion mode</th>
<th>( \tau_0 ) [s]</th>
<th>( \frac{D_{eff}}{D_{ACF}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Domain confined</td>
<td>&gt; 0</td>
<td>&gt; 1</td>
</tr>
</tbody>
</table>
SH-SY5Y membrane organization

\[ \tau_b [s] \]

\[ A_{\text{eff}} \left[ \mu m^2 \right] \]

\[ D \left[ \mu m^2/s \right] \]

Frequency

- Dil-C_18
- GFP-GPI
- GFP-GPI mβCD
- GFP-GPI CO
Wnt3 membrane localization in zebrafish
Inhibition of palmitoylation

![Graphs showing inhibition of palmitoylation with different treatments.](image)
ImageJ Plugin for Imaging FCS

http://dbs.nus.edu.sg/lab/BFL

Krieger et al. Nat. Prot. 2015, 10 (12) 1948-1972
Summary

• FCS provides measures for concentrations and diffusion coefficients
• These parameters can be quantified and can be used to derive secondary parameters (affinity, stoichiometry etc.)
• (SW-) FCCS provides an easy readout for interactions via ACF and CCF amplitudes
• Imaging FCS multiplexes FCS and FCCS measurements and can be used to make time lapse FCS videos
• TIRF and SPIM modes provide high S/N 2D and 3D measurements, respectively
• The spatiotemporal information in imaging FCS provides information beyond the diffraction limit via the diffusion laws
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