Two-photon FCS Tutorial

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What is FCS?

FCS : Fluorescence Correlation Spectroscopy

FCS is a technique for acquiring dynamical information from spontaneous fluorescence fluctuations at thermal equilibrium. It's a very useful tool in Molecular Biophysics applications both in vivo and in vitro.

The power in FCS lies in its capability to extract dynamics over a wide range of time scales (microseconds to seconds) from sample concentrations ranging from subnamolar to micromolar.
FCS has been used to measure:

- Translational diffusion coefficients, rate of active transport and other mobility-related parameters

- Concentrations and degree of aggregation of biomolecules

- Chemical kinetic rate constants

- Molecular interactions, including the rate of conformational dynamics, binding kinetics and dissociation constants
How does it work?

First: Observe spontaneous fluctuations in fluorescence intensity and record fluorescence intensity versus time.

Fluctuations in fluorescence intensity can be due to motion of fluorescent molecules in and out of the observation volume (because of Brownian motion, active transport, etc.) or due to physical or chemical dynamics that facilitates conversion from bright to dark states.
Autocorrelation Function

Second: Calculate autocorrelation function from fluorescence fluctuations and compare with known models

Autocorrelation Function:

\[ G(\tau) = \frac{\langle \delta F(r,t) \delta F(r',t + \tau) \rangle}{\langle F \rangle^2} \]

Fluorescence fluctuations: \( \delta F(t) = F(t) - \langle F \rangle \)
where \( \langle F \rangle \equiv \text{time-averaged fluorescence intensity} \)

The autocorrelation function measures the average duration of a fluorescence fluctuation. From the shape and decay rate of the autocorrelation function, we can determine the underlying physical dynamics that lead to the fluorescence fluctuation.
Shape and decay rate of $G(\tau)$ provide information on the mechanisms that lead to fluorescence fluctuations.

Different fluorophores under different conditions have different rates of decay of the autocorrelation curve. Compare freely diffusing fluorescent dye ($\text{Diffusion coefficient} = 0.3 \, \mu\text{m}^2/\text{ms}, \text{MW} = 500 \, \text{g/mol}$) to a small protein molecule ($D = 0.07 \, \mu\text{m}^2/\text{ms}, \text{MW} = 30000 \, \text{g/mol}$) in water. The same protein would diffuse more slowly inside the cell cytoplasm and in the cell membrane. Note also that the characteristic shapes of the FCS curves for random diffusion is different when there is active transport and anomalous diffusion.
Resolution of fluorescence fluctuations

It is easier to detect fluctuations in fluorescence when looking at a small number of molecules. This is achieved by two-photon excitation in a very small observation volume (~0.1 femtoliter) containing a few molecules at a time.

Assuming the laser excitation has a three-dimensional-Gaussian profile: 

\[ S(r) = S_{3DG}(\rho, z) = \exp\left(-\frac{2\rho^2}{\omega_0^2}\right) \exp\left(-\frac{2z^2}{z_0^2}\right) \]

The beam waists, \( \omega_0 \) along the radial direction and \( z_0 \) along the axial direction, are defined by the laser intensity decay length of \( 1/e^2 \) from the origin.

Observation volume:

\[ V_{3DG} = \frac{\pi^{\frac{3}{2}}}{8} \omega_0^2 z_0 \]
Aside: Derivation of $G(\tau)$ for freely diffusing molecules

$$G(\tau) = \frac{\langle \delta F(\mathbf{r}, t) \delta F(\mathbf{r}', t + \tau) \rangle}{\langle F \rangle^2} \quad \delta F(t) = F(t) - \langle F \rangle$$

For random diffusion, fluorescence fluctuations are due to changes in local concentration $\delta C(\mathbf{r}, t)$.

So, $\delta F(t) = \kappa \sigma_2 \eta I^2 \int \delta C(\mathbf{r}, t) S(\mathbf{r})^2 d^3r$

where $\kappa$ = constant, $\sigma_2$ = two-photon cross section of fluorescent dye, $\eta$ = quantum yield of fluorescent dye, $I^2$ = laser intensity, $S(\mathbf{r})$ is the laser excitation profile.

Therefore,

$$G(\tau) = \frac{\iint S(\mathbf{r})^2 S(\mathbf{r}')^2 \langle \delta C(\mathbf{r}, t + \tau) \delta C(\mathbf{r}', t) \rangle d^3r d^3r'}{\langle C \rangle \int S(\mathbf{r})^2 d^3r}$$

$$\langle \delta C(\mathbf{r}, t + \tau) \delta C(\mathbf{r}', t) \rangle = \langle \delta C(\mathbf{r}, \tau) \delta C(\mathbf{r}', 0) \rangle = \frac{\langle C \rangle}{(4\pi D\tau)^2} \exp \left( -\frac{||\mathbf{r} - \mathbf{r}'||^2}{4D\tau} \right) \quad \langle C \rangle \text{ is average of concentration and } D \text{ is diffusion coefficient}$$

$$G(\tau) = \frac{\gamma_{3 DG}}{\langle C \rangle V_{3 DG}} \frac{1}{\left(1 + 8D\tau/\omega_0^2\right)\left(1 + 8D\tau/z_0^2\right)^{1/2}}$$

$$\gamma_{3 DG} = \frac{1}{2\sqrt{2}} \quad V_{3 DG} = \frac{\pi^2}{8} \omega_0^2 z_0$$
The amplitude of the correlation function can be used to determine the sample concentration.
FCS Instrumentation

Components:

• Femtosecond laser for two-photon excitation

• High Numerical Aperture (NA) objective lenses for single-molecule detection

• Dichroic mirror for separation of excited and emitted light

• Efficient photon-collecting detectors

• Data acquisition device
Aside: Two-photon Excitation

In two-photon excitation, 2 lower energy photons are absorbed simultaneously to reach the excited state.

**Advantages of two-photon excitation:**

- Inherent axial sectioning (no need for confocal pinholes)
- Lesser photodamage effects on live cells
- Better penetration depth
- Large spectral difference between excitation and emission wavelengths
- Allows simultaneous excitation of spectral-emission separable dyes for dual-color applications

**One-photon excitation:**  
\[ F \sim I \]

**Two-photon excitation:**  
\[ F \sim I^2 \]
Applications of Two-Photon FCS

Mobility measurements in *in vivo*

Importin receptor human importin α fused with EGFP transfected in HEK cells

The mobility of the importin α is 2~4 times slower than EGFP *in vivo*. This difference cannot be explained by molecule weight change alone. EGFP-tagged importin α is three times the size of EGFP, which will only result in a diffusion coefficient decrease by 30%. Note also that FCS data for importin α fits better to a two-component or anomalous diffusion model.
Applications of Two-Photon FCS

Measuring molecular interactions by change in diffusion coefficients upon binding to a larger molecule

Diffusion coefficients decrease upon binding of a small fluorescent to a large molecule to a larger non-fluorescent target molecule. Mixtures of bound and unbound species result in two-component diffusion. Concentrations determined from the correlation curves upon titration of the non-fluorescent molecule can be used to determine dissociation constants and binding energies.
Applications of Two-Photon FCS

Measuring molecular interactions by change in amplitudes

Two samples (one made of monomers, the other made of dimers) can have the same fluorescence intensity but different correlation amplitudes. The dimers have twice the G(0) as the monomers, corresponding to twice the molecular brightness.
Applications of Two-Photon FCS

Measuring molecular interactions by cross-correlation

Interacting molecules are labeled with two spectrally distinct fluorophores that are resolved in two separate detectors. Cross-correlation between the two channels are calculated. Fluorescence fluctuations between the two detectors are only correlated if the two molecules are interacting.
Applications of Two-Photon FCS

Conformational dynamics and binding kinetics can be measured by FCS if reactions change the fluorescent properties of the fluorophore.

There is conversion from dark to bright states as molecule traverses the observation volume.

Resulting FCS curves:
Other Fluctuation Spectroscopy techniques

- **Distribution analysis**
  - Uses the same instrumentation as FCS
  - Different ways to analyze fluctuation data that complements FCS
  - PCH/FIDA, cumulant analysis, etc.

- **Image correlation spectroscopy**
  - Also monitors spatial fluctuations
  - Very useful in studying slowly diffusing species (membrane bound molecules, etc.)