Fluorescence Correlation Spectroscopy \textit{in vivo}

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FCS & FCCS

Fluorescence correlation spectroscopy  
Fluorescence cross-correlation spectroscopy

What can we measure in cells?

FCS:
- diffusion coefficient $D$; comparison of mobilities
- concentration; relative changes in concentration

dual color FCCS:
- dynamic co-localization: are the molecules diffusing together?
- kinetics of binding / dissociation
diffusion coefficient $D$ describes how fast molecules diffuse

$$D = \frac{r_0^2}{\tau_D}$$

- $r_0^2$: measurement volume size; determined by calibration
- $\tau_D$: ‘diffusion time’, characteristic transit time through the volume
diffusion coefficient $D$

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Principles

Practicalities

Application examples

diffusion coefficient $D$

$g(\tau) = \frac{\langle F(t)F(t+\tau) \rangle}{\langle F(t) \rangle^2} - 1$

$g(\tau) = \frac{1}{N\left(1+\frac{\tau}{\tau_D}\right)}\sqrt{1+\frac{\tau}{\tau_D w^2}}$
diffusion coefficient $D$

range of diffusion coefficients that can be measured:
molecules diffuse independently of each other  
⇒ Poisson distribution of the number $n$ of molecules in focus

$\langle n \rangle$ — mean number of molecules in focus  
$\sigma^2_n = \langle n \rangle$ — variance of $n$

$F = \eta n$ — fluorescence = molecular brightness $\cdot n$

$$g(0) = \left. \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \right|_{\tau \to 0} = \frac{\langle (F - \langle F \rangle)^2 \rangle}{\langle F \rangle^2} = \frac{\sigma_F^2}{\langle F \rangle^2} =$$

$$= \frac{\sigma_n^2}{\langle n \rangle^2} = \frac{1}{\langle n \rangle} = \frac{1}{cV_{\text{eff}}}$$
Are the molecules diffusing together?

- two molecules labeled with two different dyes: green and red
- two lasers to excite both dyes
- two overlapping measurement volumes
- two detectors, one for each dye

**calculate cross-correlation:**

\[
g_X(\tau) = \frac{\langle (F_1(t) - \langle F_1 \rangle)(F_2(t + \tau) - \langle F_2 \rangle) \rangle}{\langle F_1 \rangle \langle F_2 \rangle}
\]
the meaning of cross-correlation

\[ g_1(0) = \frac{1}{n_1 + n_{12}} \]
\[ g_2(0) = \frac{1}{n_2 + n_{12}} \]
\[ g_\times(0) = \frac{n_{12}}{(n_1 + n_{12})(n_2 + n_{12})} \]

\[ \frac{g_\times(0)}{g_2(0)} = \frac{n_{12}}{n_1 + n_{12}} \rightarrow \text{fraction of green in complex with red} \]
\[ \frac{g_\times(0)}{g_1(0)} = \frac{n_{12}}{n_2 + n_{12}} \rightarrow \text{fraction of red in complex in complex with green} \]
How to choose the right fluorescent label?

properties to focus on:

- spectral characteristics
- photostability
- molecular brightness
- photophysics
dye spectra and filters

- available laser lines
- available filters and beam splitters
- minimum spectral overlap!
dye spectra and filters

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molecular brightness $\eta$

$\eta = \text{number of photons detected per molecule per second (kHz)}$

$\eta$ depends on many factors:
- excitation intensity
- dye extinction coefficient
- dye fluorescence quantum yield
- overall detection efficiency (objective, filters, detector)

The higher $\eta$ the better S/N ratio, **but avoid photobleaching and saturation!**
molecular brightness $\eta$

$\eta = \text{number of photons detected per molecule per second (kHz)}$

The higher $\eta$ the better S/N ratio, but avoid photobleaching and saturation!

in cells: typically $\eta=1-5\ \text{kHz}$
many dyes exhibit additional kinetics at $\mu$s – sub-ms time range:

- triplet transitions
- protonation equilibria, isomerisation, . . .

The less fast kinetics the better!

GFP: protonation, stronger at low pH
red FPs: stronger and at longer $\tau$
Cy–5: strong blinking
Atto 655: practically none
potential problems

- background signal
- photobleaching
- measurement volume size and shape
- spectral cross-talk
- model
background signal

detected signal other than the fluorescence of the used dye(s)

temporal behaviour:
- uncorrelated (constant)
- correlated $\Rightarrow$ from diffusing molecules
background signal

detected signal other than the fluorescence of the used dye(s)

temporal behaviour:
- uncorrelated (constant)
- correlated ⇒ from diffusing molecules

constant background lowers the amplitude $g(0)$:

affected autocorrelation $g'(\tau) = g(\tau) \left( \frac{\langle F \rangle}{\langle F \rangle + B} \right)^2$

→ determine the background $B$ and correct the amplitude $g(0)$
background signal

detected signal other than the fluorescence of the used dye(s)

temporal behaviour:
- uncorrelated (constant)
- correlated ⇒ from diffusing molecules

correlated background should be included in the analysis as an additional component
**photobleaching**

photobleaching = loss of fluorescence due to a photochemical reaction

can lead to two effects:

- ‘static’ (long-term): depletion of the fluorescent molecules
- ‘dynamic’: apparent lowering of the diffusion time $\tau_D$
photobleaching = loss of fluorescence due to a photochemical reaction

can lead to two effects:

- ‘static’ (long-term): depletion of the fluorescent molecules
- ‘dynamic’: apparent lowering of the diffusion time $\tau_D$

**Diagram:**
- **Static** effect shows a gradual decay of fluorescence intensity.
- **Dynamic** effect indicates a faster decay due to diffusion and bleaching.

**Graph:**
- The $g(\tau)/g(0)$ vs. $\tau$ plot shows the decay over time for different samples.

**Legend:**
- **Alexa 546**
- **Dynamic** and **Static** behaviors are highlighted.

**Equation:**
- $\tau_D$ represents the diffusion time in the dynamic effect.
photobleaching

photobleaching = loss of fluorescence due to a photochemical reaction

can lead to two effects:

- ‘static’ (long-term): depletion of the fluorescent molecules
- ‘dynamic’: apparent lowering of the diffusion time $\tau_D$

FCCS: reduction of cross-correlation amplitude by bleaching one of the labels in a double-labeled complex
FCS: distortions of the volume size can affect:
- measured diffusion coefficient $D$ via $r_0$ in $D = r_0^2 / \tau_D$
- measured concentration via changed $V_{\text{eff}}$: $g(0) = 1 / cV_{\text{eff}}$

FCCS: in addition to the above, the volume overlap affects $g_X(\tau)$

— use objective correction collar for coverslip thickness correction
spectral crosstalk

the green dye is partially detected in the red channel

→ this leads to false-positive cross-correlation!

- alternating excitation → avoid crosstalk artefacts
- triplet kinetics in $g_X(\tau) \Rightarrow$ indication of crosstalk
spectral crosstalk - correction

label fluorescence: $f_1, f_2$
detected fluorescence: $F_1 = f_1 + \gamma f_2, F_2 = f_2 + \beta f_1$

determine the crosstalk factors $\beta$ and $\gamma$

measured (channel correlation): $g_{F_1}(\tau) = \langle \delta F_1(t) \delta F_1(t + \tau) \rangle$
wanted (label correlation): $g_{f_1}(\tau) = \langle \delta f_1(t) \delta f_1(t + \tau) \rangle$

$$
\begin{pmatrix}
g_{f_1} \\
g_{f_2} \\
g_{f_12}
\end{pmatrix} = \frac{1}{(1 - \gamma \beta)^2} \begin{pmatrix}
1 & \gamma^2 & -2\gamma \\
\beta^2 & 1 & -2\beta \\
-\beta & -\gamma & 1 + \gamma \beta
\end{pmatrix} \begin{pmatrix}
g_{F_1} \\
g_{F_2} \\
g_{F_{12}}
\end{pmatrix}
$$
the fitting model typically includes:
- triplet or fast kinetics exponential term
- one or two diffusion terms

\[ g(\tau) = g(0) \frac{1 - T + Te^{-\tau/\tau_f}}{1 - T} \times \]
\[ \times \left( \frac{p}{\sqrt{1 + \tau / (\tau_{D1} w^2) } (1 + \tau / \tau_{D1})} + \frac{1 - p}{\sqrt{1 + \tau / (\tau_{D2} w^2) } (1 + \tau / \tau_{D2})} \right) \]
the fitting model typically includes:

- triplet or fast kinetics exponential term
- one or two diffusion terms

\[
g(\tau) = \frac{1}{t} e^{-t/\tau} + \frac{1}{t} e^{-2t/\tau}
\]
controls

- biological — no dye interference with function
controls

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- setup alignment: test on a known standard
  - determine volume size $r_0$
  - adjust the correction collar
controls

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- measure the dyes in conditions as close as possible to the cellular environment
  - the same buffer, pH, ...
  - determine optimal excitation intensity
  - quantify fast kinetics
controls

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- **FCCS**: positive and negative controls
  - positive: determine maximum measurable cross-correlation
  - negative: exclude cross-talk effects
two-photon excitation

excitation by absorption of two photons, $\lambda_{\text{ex}} = 750–950$ nm

positive features:
- lower overall photobleaching
- lower phototoxicity
- lower background
- deeper penetration (tissues)
- one wavelength to excite two dyes

negative features:
- stronger in-focus photobleaching
- lower S/N ratio
- expensive
RNA interference = mechanism of inhibition of gene expression

- point of inhibition: mRNA → degradation or repression of translation
- specific; specificity provided by a short dsRNA: siRNA ‘short interfering RNA’; based on complementarity of nucleotide sequences between siRNA and mRNA
RISC — ‘RNA-induced silencing complex’
siRNA: guide strand and passenger strand
goal

in vivo study of:

- loading of siRNA to RISC
- relationship of the nuclear and cytoplasmatic RISC
**labeling**

**EGFP-Ago2**
- create a stable EGFP-Ago2 human cell line
- controls to check that everything fully functional

**Cy–5-siRNA**
- label either guide strand or passenger strand
- microinject
**Experimental Setup**

Microscope: Zeiss LSM 510 with Confocor 3

λ<sub>ex</sub> = 488, 633 nm; bs1: 488/633

bs2: LP635; em: 505–610 + LP655
volume size:
- Alexa 488: $r_0 = 0.19 \mu m$
- Atto 655: $r_0 = 0.24 \mu m$

maximum cross-correlation: 80%, dsRNA with Rh6G and Cy–5

fast kinetics (triplet, protonation):
- EGFP: $\tau_f = 130 \mu s$
- Cy–5: $\tau_f = 70 \mu s$
- position the focus away from membranes and cellular structures
- duration: $8 \times 30$ s; bad runs removed
- molecular brightness: $\sim 4$ kHz in each channel
- fitting model: 2 components + fast kinetics
- apply correction for autofluorescence background (uncorrelated, measured in cells without EGFP)
results: size of EGFP-Ago2

measure diffusion of EGFP-Ago2 in cytoplasm and in nucleus

\[
D \, \mu m^2 s^{-1} \\
\begin{array}{l|cc}
\text{control (EGFP)} & \text{EGFP-Ago2} \\
\hline
\text{cytoplasm} & 25.5 \pm 0.9 & 5.4 \pm 0.2 \\
\text{nucleus} & 24.5 \pm 0.5 & 13.7 \pm 0.5 \\
\end{array}
\]
results: size of EGFP-Ago2

measure diffusion of EGFP-Ago2 in cytoplasm and in nucleus

estimate the size from $D$:

$$D = \frac{kT}{6\pi\eta R}$$

cytoplasm $3 \pm 0.6$ MDa

nucleus $158 \pm 26$ kDa

→ determined molecular weight of RISC in vivo
results: size of EGFP-Ago2

measure diffusion of EGFP-Ago2 in cytoplasm and in nucleus

particle numbers: $4.7 \pm 0.5 \times$ higher abundance in cytoplasm; agreement with intensity from imaging
loading of siRNA to RISC — FCCS

- label either guide or passenger strand of siRNA with Cy–5
- measure cross-correlation between siRNA and EGFP-Ago2

3 hours after microinjection

cross-correlation: guide strand 32%
passenger strand 2%
Principles  
Practicalities  
Application examples  

RNA interference pathway  
Cholera toxin cellular uptake  

loading of siRNA to RISC — FCCS  

dependence on the time after microinjection  

passenger strand:  
- max. 5% cross-corr.  
- all gone after 12 h  

guide strand:  
- max. 20% cross-corr.  
- still present after 48 h  

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Fluorescence Correlation Spectroscopy in vivo
inhibition of nuclear import/export
RNA interference: conclusion

- **FCS**: determined the size of RISC in cytoplasm and nucleus (from diffusion coefficients of EGFP-Ago2)
- **FCCS**: monitored the kinetics of incorporation of siRNA into RISC, both strands, both in cytoplasm and nucleus
- all measurements in vivo, at expression levels similar to endogeneous
Cholera toxin uptake

cholera toxin AB₅ — two subunits: A, B

- A: enzymatic reaction in the cytosol (28.5 kDa)
- B: needed for membrane binding and cellular uptake (11 kDa)

interaction with the cell:
- binds to the membrane
- taken up by the cell via endocytosis
- internalized in vesicles
- later accumulates in Golgi

goal: use FCCS to follow the A and B subunits during and after endocytosis
labeling

A: Cy–2 488 nm
B: Cy–5 633 nm

- labeling not complete → many unlabeled subunits
- cholera toxin added to cells in a buffer, washed after few minutes
observe cross-correlation of native and denatured (high temperature) cholera toxin in solution

loss of cross-correlation at high temperature → separation of the subunits
position focus on the membrane:

- initially: photobleaching of immobile or slowly moving molecules
- later: signal from molecules in vesicles near the membrane
on the cell membrane

position focus on the membrane:

- initially: photobleaching of immobile or slowly moving molecules
- later: signal from molecules in vesicles near the membrane

![Fluorescence Correlation Spectroscopy in vivo](image)
in cytoplasm

high cross-correlation $\Rightarrow$ A and B subunits diffuse together:

- either bound to each other
- or confined to the same vesicle
\[ \tau_{\text{diff}} \sim 20 \text{ ms} \quad \rightarrow \quad D = 0.6 \mu m^2 s^{-1} \quad \rightarrow \quad 2R = 150 \text{ nm} \]

\[ \rightarrow \quad \text{in agreement with the expected intracellular vesicle size} \]
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in cytoplasm

large spikes → exclude from the analysis
after initial bleaching period:

- strongly reduced cross-correlation
- different diffusion times of the two labels

⇒ the two subunits are separated and diffuse independently
single-labeled toxins

⇒ differently labeled toxins become confined to the same vesicles
different toxins

non-zero cross-correlation of two different toxins $\Rightarrow$ the two toxins have the same or overlapping intracellular pathway
in cytoplasm the subunits diffuse together, most likely within vesicles
separation of subunits does not occur before the cargo reaches the Golgi body
cholera and shiga toxin are transported in the same vesicles
incomplete labeling prevents more quantitative analysis
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J. Mütze, T. Ohrt, P. Schwille, Fluorescence correlation spectroscopy *in vivo*, *Laser & Photonics Reviews* (September 2009)


