# FCS, fluorescence correlation spectroscopy



→ current biological research: aims to characterize elementary processes on the level of individual proteins and nucleic acids

→ high-resolution spatial and temporal analysis of extremely low concentrated biomolecules

→ parameter of interest: not fluorescence intensity but spontaneous intensity fluctuations,

determines: local concentrations, mobility coefficients, rate constants of in/intra-molecular interactions of labeled biomolecules in nanomolar concentrations.

→ developed: early 1970's, relaxation analysis method (W. Webb et al.)

classical relaxation methods: ① external perturbation (p, T jumps)
② gain information about kinetic parameters

pictures

FCS: takes advantage of minute fluctuations

→ temporally autocorrelated →

information about processes governing molecular dynamics

- early measurements: poor signal/noise ratios

- Task: find out if a friend entered a shopping cluster → not possible due to high concentration of people

  Better: watch if the friend enters/leaves your own living room

⇒ Concept of FCS: using low concentrations of molecules that each of them contributes substantially to the measured signal! → analyses of spontaneous fluctuations possible

→ 1) use of lasers

2) ultrasensitive detectors (single photon sensitivity)

3) confocal detection (Rigler, Stockholm)

(+ 2-photon excitation)

picture set up

⇒ confocal volume < 1 fL, approx. volume of E. coli cell

→ standard tool for high-throughput screening

# Autocorrelation analysis

- focus on one particular species of fluorescent particles
- fluctuations: quantified by temporally auto-correlating the recorded intensity signal
- Number of molecules in the confocal volume at a given time governed by a Poissonian distribution.

root mean square fluctuation of the particle number N given by:

$$\frac{\sqrt{\langle (\delta N)^2 \rangle}}{\langle N \rangle} = \frac{\sqrt{\langle (N - \langle N \rangle)^2 \rangle}}{\langle N \rangle} = \frac{1}{\sqrt{\langle N \rangle}}$$

- increasing number of particles → decreased relative fluctuations → minimize number of molecules in the confocal volume (higher than background)

temporal average should be between 0.1 and $10^3$

$V_{confocal} \approx$ one fL ⇒ concentrations between $< 10^{-9}$ M to $10^{-6}$ M

- Fluctuations of the fluorescence signal: deviations from the average, defined as

$$\delta F(t) = F(t) - \langle F(t) \rangle \quad , \langle F(t) \rangle = \frac{1}{T} \int_0^T F(t)\, dt$$

Fluctuation due to changes in the local concentration $\delta c$ in effective volume $V_{eff}$ of the focal spot

$$\Rightarrow \quad \delta F(t) = \kappa \int_V I_{ex}(\vec{r}) \cdot S(\vec{r}) \cdot \delta(\sigma \cdot q \cdot C(\vec{r},t)\, dV$$

$\underline{\kappa}$: overall detection efficiency $\underline{I_{exp}(\vec{r})}$: spatial distribution of the excitation energy, maximum $= E_0$ (amplitude)

$\underline{S}(\vec{r})$ : optical transfer function of the pinhole-objective combination (spatial collection efficiency, dimensionless)

$\underline{\delta(\sigma \cdot q \cdot C(\vec{r},t)}$ : dynamics of the fluorophore on the single particle level,

$\delta\sigma$ fluctuation of the molecular absorption cross-section

$\delta q$ fluctuations in the quantum yield

$\delta C(\vec{r},t)$ local particle concentration fluctuation at time $t$ because of Brownian motion

Simplification:

$I_{exp}(\vec{r}) / I_0 \cdot S(\vec{r}) \rightarrow$ combined into $W(\vec{r})$ : describes the spatial distribution of the emitted light, approx. by a 3d-Gaussian

$\kappa, \alpha, q$ combined with the amplitude $I_0$

to $\eta_0 = I_0 \cdot \kappa \cdot \alpha \cdot q$ photon count rate (per (molecule and second))

$\Rightarrow$ as a measure of the signal to noise ratio used of different setups

$$\Rightarrow \delta F(t) = \int_V W(r)\, \delta(\eta C(\vec{r},t))\, dV$$

normalized autocorrelation function, defined:

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

$\Rightarrow$ show picture

Signal is analyzed with respect to its self-similarity after the lag time $\tau$,

$G(0)$: autocorrelation amplitude, normalized of the fluctuating fluorescence signal $\delta F(t)$

Assumptions: - $\delta \eta = 0$ (fluorescent dye does not change)

- only particles are freely diffusing with the diffusion coefficient D $\rightarrow$ number density autocorrelation

$$\langle \delta C(\vec{r},0) \cdot \delta C(\vec{r}',t) \rangle = \langle C \rangle \frac{1}{(4\pi D \tau)^{-3/2}} \cdot e^{-\left(\frac{(r-r')^2}{4D\tau}\right)}$$

- $\tau_D$: lateral diffusion time that a particle stays in the focal volume

$$\tau_D = \frac{r_0^2}{4D} \Leftrightarrow \text{diffusion coefficient obtained!!}$$

- effective focal volume $V_{eff} = \pi^{3/2} \cdot r_0^2 \cdot z_0$

$\Rightarrow$ auto correlation function for a freely diffusing species of molecules:

$$G(\tau) = \frac{1}{V_{eff}\langle C\rangle} \cdot \frac{1}{\left(1+\frac{\tau}{\tau_D}\right)} \cdot \frac{1}{\sqrt{1+\left(\frac{r_0}{z_0}\right)^2 \cdot \frac{\tau}{\tau_D}}}$$

$r_0, z_0$: known from calibration experiments

local concentration of fluorophores: from the amplitude $G(0)$

$$G(0) = \frac{1}{\langle N\rangle} = \frac{1}{V_{eff}\cdot\langle C\rangle} \Rightarrow \langle C\rangle = \frac{1}{V_{eff}\cdot G(0)}$$

$\Rightarrow$ Diffusion coefficient: derived from a characteristic decay time $\tau_D$

Complications:
- "flickering" due to transition in triplett state $\rightarrow$ dark factor introduced $\left(1 - T + T\cdot e^{-\tau/\tau_{Triplett}}\right)$
- reactions influencing the mobility:
  Mobility term $M_i(\tau)$

$$\Rightarrow \underset{\text{motion}}{G(\tau)} = \frac{1}{V_{eff}} \cdot \frac{\sum_i \eta_i \langle C_i \rangle M_i(\tau)}{\left(\sum_i \eta_i \langle C_i \rangle\right)^2}$$

$i$ = all different species

generalized, accounting for all possible motion, weighted with the relative emission rate

free 3D motion: $M_i(\tau) = \frac{1}{(1 + \tau/\tau_{D,i})\sqrt{1 + \left(\frac{r_0}{z_0}\right)^2 \cdot \tau/\tau_{D,i}}}$

2D (membranes) $M_i(\tau) = \frac{1}{(1 + \tau/\tau_{D,i})}$

active transport, velocity $v_i$ $M_i(\tau) = e^{-\left(\frac{\tau \cdot v_i}{r_0}\right)^2}$

anomalous diffusion: $\tau/\tau_{D,i}$ replaced by $\left(\frac{\tau}{\tau_{anom,i}}\right)^{\alpha}$

(diffusion eq. $\langle r^2 \rangle = 4 D t^{\alpha}$ for 2d) $\alpha < 1$

- Rotational Brownian diffusion → addition of factor ($\exp(C \Theta \tau)$)

→ Cross-correlation:

1) Correlation between measurements in two volumes ⇒ yields flow velocity

2) dual ~~(brother)~~ color:
two dyes in the same detection element
⇒ interaction between two (labeled) molecular species

Applications

- concentration and aggregation measurements

- diffusion analysis example → change of mobility as seen by D

* molecular interactions,

- ligand binding can be monitored
- pure ligands/~~proteins~~ show different behavior in ~~the~~ terms of the autocorrelation curves
- ligands are labeled, mean ratio should be 8 and more, at the end/start only one species (unbound/bound) is present

→ DNA binding to short DNA fragments

→ membrane adsorption: proinsulin C-peptide

- Conformational changes,
example: denaturated albumin (unfolded)

Einstein - Relation $D = \frac{kT}{6\pi\eta R}$ ← R changed

= Diffusion coefficient:
relation
between $\tau_D$ (diffusion time) $\tau = \frac{\omega^2}{4D}$
and D

$\omega$ = radius of Gaussian beam profile

( extracted from : biophysics textbook online,
http://www.biophysics.org/education/schwille.pdf )