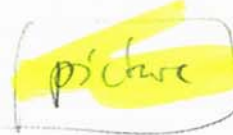


FCS, fluorescence correlation spectroscopy - A-

- 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 inter/intra-molecular interactions of labeled biomolecules in nanomolar concentrations.

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

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

FCS: takes advantage of minute fluctuations

→ temporally autocorrelated →

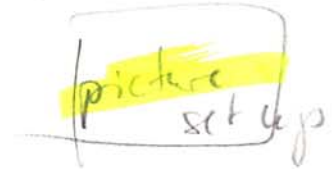
information about processes governing molecular dynamics

- early measurements: poor signal/noise ratios⁻⁸ -
- Task: find out if a friend entered a shopping center → not possible due to high concentration of people

Better: wait 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)



⇒ Confocal volume $< 1 \mu\text{L}$, approx. volume of *E. coli* cell

→ Standard tool for high-throughput screening

Autocorrelation analysis

- 1 -

- 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:

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

- increasing number of particles \rightarrow decreased relative fluctuations \rightarrow minimize number of molecules in the confocal volume (higher than background)
temporal average should be between 0.1 and 10^3
 $V_{\text{confocal}} \approx \text{one fL} \Rightarrow$ concentrations between $< 10^{-9} \text{ M}$
to 10^{-6} M

- Fluctuations of the fluorescence signal: defined as deviation from the average

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

-2-

Fluctuation due to changes in the local concentration δc in effective volume V_{eff} of the focal spot

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

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

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

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

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

δq fluctuations in the quantum yield

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

Simplification:

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

by a 3d-Gaussian

k, σ, q combined with the amplitude I_0 -3-

$$I_0 \eta_0 = I_0 \cdot k \cdot \sigma \cdot q \quad \begin{array}{l} \text{photon count rate} \\ (\text{ps (molecule and second)}) \end{array}$$

→ as a measure of the signal to noise ratio used of different setups

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

normalized autocorrelation function, defined:

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} \Rightarrow \text{show picture}$$

Signal is analyzed with respect to its self-similarity after the lag time τ ,

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

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

- only particles are freely diffusing with the diffusion coefficient D → number density autocorrelation

$$\langle \delta C(\vec{r}_1, 0) \cdot \delta C(\vec{r}_2, \tau) \rangle = \langle C \rangle \cdot \frac{1}{(4\pi D \tau)^{3/2}} \cdot e^{-\frac{(\vec{r}_1 - \vec{r}_2)^2}{4D\tau}}$$

- τ_D : lateral diffusion time that a particle stays in the focal volume. -4-

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

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

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

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

r_0, z_0 : known from calibration experiments

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

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

\Rightarrow Diffusion coefficient: derived from a characteristic decay time τ_D

Complications:

- "bleaching" due to transition in triplet state \rightarrow dark factor introduced $(1 - T + T \cdot e^{-\frac{\tau}{\tau_{\text{Triplet}}}})$

- reactions influencing the mobility:
Mobility term $M_i(\tau)$

$$\Rightarrow G_{\text{motion}}(\gamma) = \frac{1}{v_{\text{eff}}} \frac{\sum_i \gamma_i \langle c_i \rangle \Psi_i(\gamma)}{\left(\sum_i \gamma_i \langle c_i \rangle \right)^2} \quad i = \text{all different species}$$

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

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

2D (membranes) $\Psi_i(\gamma) = \frac{1}{\left(1 + \gamma/\gamma_{D,i}\right)}$

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

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

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

- Rotational Brownian diffusion \rightarrow additional factor $(\exp(-c\theta\gamma))$

→ Cross-correlation:

-6-

1) Correlation between measurements in two volumes yields flow velocity

2) Dual ~~channel~~ color:

two dyes in the same detection element
⇒ interactions 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, mass ratio should be 8 and more, at the end/start only one species (unbound/bound) is present
- DNA binding to short DNA fragment
- membrane adsorption: proinsulin
C-peptide

- Conformational changes,

-2-

example: denaturated albumin (unfolding)

Einstein - Relation

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

- Diffusion coefficient:
relation

between τ_D (diffusion time)
and D $\tau = \frac{\omega^2}{4D}$

$\omega =$ radius of Gaussian beam profile

(extracted from: biophysics textbook outline,
<http://www.biophysics.org/education/schwilke.pdf>)