1 / fluorescence correlation spectroscopy

- sensitive technique : high-resolution spatial and temporal analysis of extremely low concentrated biomolecules
- not intensity but intensity fluctuations are parameter of interest !
- all parameters giving rise to fluctuations in the fluorescence signal are principally accessible
- first introduced by Madge, Elson, Webb 1972 (DNA – drug intercalation dynamics)

Principle :

- observation volume must be small enough that each molecule contributes substantially to the signal measured
- → fluorescent labeled biomolecules diffusing in aqueous buffers in very small volumes (femtoliter – range ↔ E. coli cell)

experimental procedure

- exciting laser beam (Ar, Ar-Kr, He-Ne), focused on a sample (water immersion objectives, NA > 0.9) over a dichroic mirror
- fluorescence light → objective → dichroic and emission filter, pinhole at the image plane: blocks all light not originating from the focal region
- Computer: performs autocorrelation of the fluorescence signal
- dyes: rhodamines, cyanines, GFP.

Theory

Autocorrelation analysis

- self-similarity of a time signal measured
- → yields characteristic time constants of the underlying process

Molecules in focal volume at any time: governed by Poissonian distribution
root mean square fluctuation of particle number N:

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

- N should be between 0.1 and 10^3 (otherwise too much noise : $C \ll 10^{-9}$ M to 10^{-6} M

- fluorescence in the focal spot: recorded photon by photon

definition of the fluorescence fluctuations:

$$\delta F(t) = F(t) - \langle F(t) \rangle$$ deviation from temporal average

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

All fluctuations arise only from changes in local concentration δC in the effective volume of the focal spot

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

κ = overall detection efficiency

$I_{ex}(r)$ = Spatial distribution of the excitation energy with the maximum I_0 (amplitude)

$S(r)$ optical transfer function (efficiency of set-up)

$\delta\sigma$: fluctuations in the molecular absorption cross-sections

δq : fluctuations in the quantum yield

$\delta C(r,t)$: fluctuations in the local particle concentration (brownian motion)

Simplification

$I_{ex}(r) / I_0 \times S(r)$: combined into a spatial distribution function of emitted light (Gaussian-3D)

$$\left(W(\vec{r}) = \exp\left(-2\frac{x^2+y^2}{r_0^2}\right) \exp\left(-2\frac{z^2}{z_0^2}\right) \right)$$

→ remaining parameters: combined to η_0, giving the photon count-rate per detected molecule per second

$$\eta_0 = I_0 \cdot \kappa \cdot \sigma \cdot q$$

⇒ now we get:

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

Def.: normalized auto-correlation function

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

Signal analyzed with respect to its self-similarity after lag time τ, amplitude G_0:

Substitution yields

$$G(\tau) = \frac{\iint W(r)\, W(r') \left\langle \delta(\eta \cdot C(r,t))\, \delta(\eta \cdot C(r',t+\tau)) \right\rangle dV dV'}{\left(\int W(r) \left\langle \delta(\eta \cdot C(r,t)) \right\rangle dV \right)^2}$$

Simplifications if η and c are constant;
if the fluorophore's properties do not change $\Rightarrow \delta\eta = 0$

$$\Rightarrow G(\tau) = \frac{\iint w(r)\, w(r') \langle \delta C(r,0)\, \delta C(r',\tau) \rangle \, dV\, dV'}{\left(\langle C \rangle \int w(r)\, dV \right)^2}$$

Considering only particles freely diffusing in three dimensions with the diffusion coefficient D:

$$\langle \delta C(r,0)\, \delta C(r',\tau) \rangle = \langle C \rangle \frac{1}{(4\pi D \tau)^{-\frac{3}{2}}} \cdot \exp\left(\frac{(r-r')^2}{4D\tau}\right)$$

number-density autocorrelation term can be calculated

$$\Rightarrow G(\tau) = \frac{1}{\langle C \rangle (4\pi D \tau)^{-3/2}} \cdot \frac{\iint w(r)\, w(r') \cdot \exp\left(\frac{(r-r')^2}{4D\tau}\right) dV\, dV'}{\left(\int w(r)\, dV \right)^2}$$

Furthermore: $\tau_D = \frac{r_0^2}{4D}$ (lateral diffusion time, which the particle stays in the focal vol.)

and (trick)
$$V_{eff} = \frac{\left(\int w(r)\, dV \right)^2}{\int w^2(r)\, dV} = \pi^{3/2} \cdot r_0^2 \cdot z_0$$

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

$\frac{1}{V_{eff}\langle C\rangle}$ = inverse of the particle number in the focal volume

r_0, t_0 : known from calibration experiments

↓ local concentration can be determined from the amplitude of $G(0)$

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

diffusion coefficient: can be derived from τ_D!

— Complications: - fluorescents properties can change (blinking, triplet states need a long time to decay)

- reactions: influence mobility

⇒ Can be taken into account

— anomalous diffusion in cell membranes:

$\langle r^2\rangle = 4Dt^{\alpha}$ ↓ $\frac{\tau}{\tau_D}$ becomes $\left(\frac{\tau}{\tau_{anomal.}}\right)^{\alpha}$

with $\alpha < 1$

Transmission electron microscopy

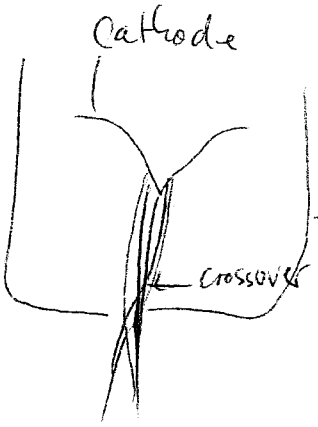
- Solid samples radiated (through) by electrons
- up to a resolution to 0,1 nm crystals of atoms / molecules can be mapped
- biological samples: 2–3 nm
- developed in the 1930's (Ruska, von Borries, Knoll)

General: – microscopic tube (vacuum)

- electron beam led to the sample by a lense system (magnetic lenses)
- objective generates the sample picture
- electron source: tungsten wire)

$U_0 = -60 - 100\,kV$

$\Rightarrow v = \sqrt{\frac{2eU_0}{m}} \approx 55\,\%$ of c



lense errors

- spherical aberration $d_S = \frac{1}{2} C_S \cdot \alpha^3$ (α = aperture angle)
- scattering error (resolution due to scattering effects:

$$d_B = \frac{0.6 \cdot \lambda}{\sin\alpha}$$

– further errors

chromatic error : $d_c = C_c \cdot \frac{\Delta E}{E} \cdot \alpha$ (C_c = chromatic error constant; ΔE = energy width of electrons)

Sum of errors

$$d = d_s + d_B = \frac{1}{2} C_s \cdot \alpha^3 + 0.6 \cdot \frac{\lambda}{\alpha}$$

minimum of $d(\alpha)$ $\Rightarrow$ $d \approx 0.75 \cdot (C_s \cdot \lambda^3)^{1/4}$

at an optical aperture angle $\alpha_{opt} \approx \left\{ \frac{0.4 \cdot \lambda}{C_s} \right\}^{1/4}$

with $C_s \cong 1$ mm (typical)

$\Rightarrow$ 100 kV – e^- ; $\lambda = 3{,}7$ pm $\Rightarrow$ $\alpha_{opt} \approx 6 \cdot 10^{-3}$

$d \approx 0.4$ mm (diffraction neglected!)

in the electron microscope the resolution is determined by the spherical aberration!

Interaction with the sample

possibilities

– no scattering

– scattering at a nucleus (coulomb), elastically as reflected by the scattering length (elastic)

$$\sigma_{el} \sim \frac{\lambda^2 \cdot Z^{4/3}}{\pi}$$

stronger increase with high Ordnungszahlen

– inelastic electron scattering

$\sigma_{inel.} \sim Z^{1/3}$

$\Rightarrow \sigma_{el}/\sigma_{inel} = Z/26$

⟶ resulting in inelastic scattering for light elements, while elastic scattering dominates for heavy elements (high angles)

↓

contrast enhancement by heavy elements

⟶ energy of inelastic scattering is converted

to a) vibrations of atomic electrons: continuum radiation

b) ionization: ~~free~~ off outer shell electrons

→ fluorescence (due to replacement)

inner shell electrons:

→ X-ray generation; used for element identification

→ breaking of covalent bonds: sample degradation

c) heat (thick samples which mainly absorb)

Recording of the picture

– fluorescence screen, – photographic film, – photo-multiplyer, CCD