

# EFS

## ***Organisation:***

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## ***Time and place:***

Friday, 8:00

room E023 (ground floor)

Biotechnologisches Zentrum, Tatzberg 47-51

## ***Experiment:***

### **Investigating intramolecular protein dynamics on a single molecule scale by confocal fluorescence correlation spectroscopy (FCS)**

**Goal:** To learn about the theoretical concept and the experimental implementation of confocal Fluorescence Correlation Spectroscopy (FCS) on a system of freely diffusing Green Fluorescent Protein (GFP) molecules in aqueous buffer solution exhibiting pH-dependent intramolecular dynamics. The basic measurement parameters of FCS such as concentration, molecular diffusion coefficient and kinetic rate constants are introduced and measured on small molecular ensembles consisting of several molecules.

## **Theoretical Background:**

### *Fluorescence correlation spectroscopy (FCS)*

In contrast to conventional applications of fluorescence spectroscopy, FCS is a technique that derives thermodynamic and kinetic information not from an ensemble average but from small spontaneous deviations from equilibrium that give rise to fluctuations in fluorescence emission. In order to resolve such spontaneous fluctuations, the systems under observation have to be kept extremely small. Ideally, ensembles consisting of several or even single molecules at any time are probed. This condition is achieved by combining ultrasmall measurement volumes with extremely low sample concentrations. In confocal FCS geometries which are at present the most popular, the reduction of the measurement volume to approx. 1 fl is achieved by epi-illumination of a high numerical aperture ( $NA > 0.9$ ) objectives with parallel laser beam and imaging the focal spot onto a diaphragm or pinhole in the image plane.

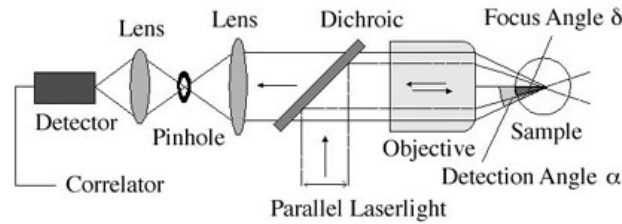


Figure 1: Confocal setup for FCS detection. The laser light is focused to a diffraction-limited volume, which is imaged onto a pinhole or optical fiber of  $\sim 100 \mu\text{m}$  diameter. This results in an open volume element of approx.  $10^{-15} \text{ l}$ .

The temporal fluctuations of the fluorescence signal  $F(t)$  recorded with a very sensitive detector (avalanche photodiode, APD) are analyzed by means of normalized fluorescence autocorrelation function  $G(\tau)$  with lag time  $\tau$ :

$$G(\tau) \equiv \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \quad (1)$$

where  $\delta F(t) \equiv F(t) - \langle F(t) \rangle$  and  $\langle \cdot \rangle$  is the temporal average over the duration of the experiment. The autocorrelation function reflects the decay of spontaneous fluctuations of  $F(t)$ . The amplitude and the shape of  $G(\tau)$  are determined by the underlying processes. The most common source of fluctuations in  $F(t)$  is the fluctuation of the number of molecules in the measurement volume due to the random 3D diffusion. In this case, the characteristic decay time of  $G(\tau)$  reflects the average residence time of the molecules in the focal spot. Due to the Poissonian nature of the particle number fluctuations at low concentrations, the amplitude  $G(0)$  is inversely proportional to the average number of molecules present in the volume:

$$G(0) = \frac{1}{N} = \frac{1}{CV_{\text{eff}}} \quad (2)$$

In case of a 3-dimensional Gaussian-shaped excitation intensity distribution, the autocorrelation function  $G_{\text{diff}}(\tau)$  for freely diffusing molecules of one species with concentration  $C$  is given by:

$$G_{\text{diff}}(\tau) = \frac{1}{CV_{\text{eff}}} \frac{1}{1 + \tau/\tau_d} \frac{1}{\sqrt{1 + r_0^2 \tau / z_0^2 \tau_d}} \quad (3)$$

where  $V_{\text{eff}}$  is the size of the effective volume element:

$$V_{\text{eff}} = \pi^{3/2} r_0^2 z_0 \quad (4)$$

and  $\tau_d$  the characteristic residence time of a molecule within  $V_{\text{eff}}$ . The parameters  $r_0$  and  $z_0$  are the  $1/e^2$  half axes of the measurement profile  $W(\mathbf{r})$  in the sample space which is considered to be Gaussian in all three dimensions:

$$W(\vec{r}) = e^{-2\frac{x^2+y^2}{r_0^2}} e^{-2\frac{z^2}{z_0^2}} \quad (5)$$

The relationship between  $\tau_d$  and the diffusion coefficient  $D$  is given by

$$\tau_d = \frac{r_0^2}{4D} \quad (6)$$

The diffusion time  $\tau_d$  which characterizes the decay of the autocorrelation function is then determined by the diffusion coefficient of the molecules  $D$  (how fast the molecules move) and the parameter  $r_0$  (the size of the volume over which the molecules move).

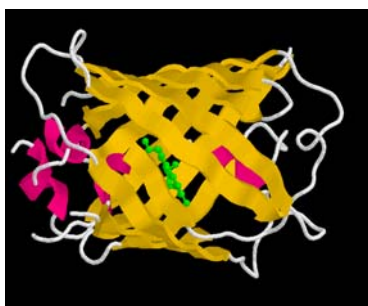
If the molecules additionally undergo intramolecular dynamics reflected as blinking of the fluorescence (on/off transitions) on a fast time scale during their residence time in the focal spot,  $G(\tau)$  has to be modified as follows (in case where  $\tau_f \ll \tau_d$ ):

$$G_{diff+fast}(\tau) = \frac{(1 - f_D + f_D \cdot e^{-\tau/\tau_f})}{(1 - f_D)} G_{diff}(\tau) \quad (7)$$

where  $f_D$  is the average fraction of particles in the dark state, and  $\tau_f$  is the inverse of the characteristic blinking rate:  $\tau_f = 1/(k_b + k_d)$  with  $k_b$  and  $k_d$  being the respective transition rates from the dark to the bright state and vice versa.

### *The investigated system*

The green fluorescent protein is in aqueous solution at neutral and higher pH present in an anionic state with absorption maximum at  $\sim 490$  nm. It has been shown that at low pH the chromophore gets protonated by external protons leading to the shift of its absorption maximum towards a shorter wavelength of around 400 nm. The protein is no longer excitable at 488 nm (the  $Ar^+$  laser used in Praktikum) and appears dark (non-fluorescent) during the lifetime of the protonated form. Thus, if the protonation/deprotonation-induced blinking of fluorescence emission is measured on a single molecule scale as accomplished by FCS, protonation and deprotonation rate constants can be determined.



*Fig. 2: Green fluorescent protein (GFP), a small protein (26 kDa, 1Da = 1g/mol), naturally occurring in jellyfish Aequorea victoria, has become a remarkable tool in fluorescence microscopy. Upon excitation in the visible spectral range (e.g. at 488 nm), the protein itself exhibits a strong native fluorescence which arises from a chromophoric unit inside the protein's barrel-like tertiary structure (chromophore in green).*

The protonation/deprotonation reaction:

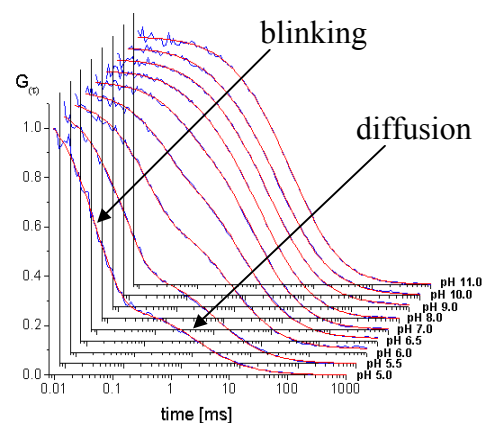
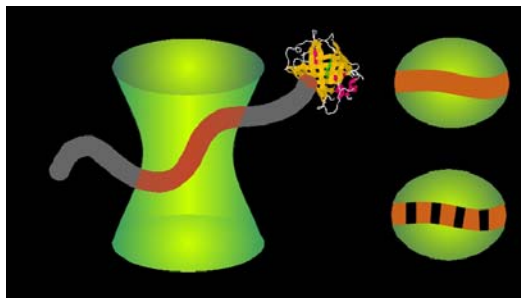
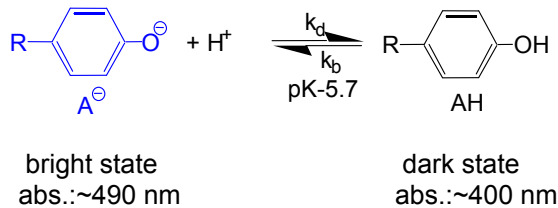


Fig. 3: The above figures show the protonation scheme with dark intervals given by the protonated form of the chromophore (left picture), as well as the correlation curves that are recorded from solutions of GFP in buffers of different pH. It can be seen that the dark fraction, given by the amplitude of the fast exponential process, increases with decreasing pH, while the fast time constant  $\tau_f = 1/(k_b + k_d) = 1/(k_b + k_d[H^+])$  decreases.

## Practical work:

### 1. Setup

The FCS-measurements are carried out in sample droplets of an aqueous buffer solution with a custom-made microscope setup using excitation wavelength of 488 nm. The optics (mirrors, filters, objective, lenses, pinhole) are installed in the microscope module. The laser is coupled in externally. The excitation intensity needs to be adapted to the experimental conditions.

### 2. Materials

The GFP is studied in buffer solutions of different pH values. The GFP mutant under investigation is eGFP (enhanced GFP, Clontech), exhibiting stronger fluorescence and higher stability compared to the wild-type protein. The phosphate/citrate buffer at pH values ranging from 4.5 to 10.0 is used to prepare the solutions. The protein concentration should be less than 100 nM. At low pH values where the protein is less stable and partially denaturates already short time after mixing higher concentrations are used.

### 3. Data Evaluation

The digital fluorescence signal (pulses corresponding to detected photons) is autocorrelated by a hardware correlator, a PC ALV-5000 multiple- $\tau$  correlator card (ALV, Langen, Germany). Evaluation of the curves is carried out by ORIGIN (MicroCal Software, Northampton, MA) by using the Marquardt-Levenberg fitting routine. The measured curves are fitted to the respective theoretical model (diffusion, blinking dynamics etc.) based on Eq. 7.

### Questions and Tasks:

- In what sense can FCS be called a single-molecule method (even though the number of molecules probed at any time may be larger than one)? Compare, for example, with a standard absorption measurement of concentrated solution in a spectrophotometer. ✓
- What conditions a system has to fulfill to be investigated by FCS?
- What are the main quantities measurable with FCS?
- Calculate the size of the experimentally defined measurement volume element  $V_{\text{eff}}$ . Use the value of the diffusion coefficient of eGFP reported in literature:  $D = 8.7 \cdot 10^{-7} \text{ cm}^2/\text{s}$  (R. Swaminathan, Biophys. J., **72**, 1900, 1997). ✓
- Calculate the absolute concentration of your sample (in mol/l, exemplary for one measurement of choice) ✓
- Calculate the power density of the excitation laser light within the focal spot (pay attention to the correct units). How would much larger or much smaller excitation power affect the results of the measurement (diffusion time, particle number, ...)? ✓
- The autocorrelation curves at very low pH often vary between the different runs (10-20 s periods of measurement). Why? ✓
- The equilibrium between the anionic and the protonated form of GFP at any pH is characterized by the dissociation constant  $K_a$  (the amino acid Tyrosine in the case of the here studied eGFP) or its negative decadic logarithm  $\text{p}K_a \equiv -\log_{10} K_a$ . The theoretical value of  $\text{p}K_a$  is about 5.5. It can be determined experimentally from the measured dependence of the dark fraction (fraction of the molecules in the dark state)  $f_D$  on pH. The theoretical dependence  $f_D(\text{pH})$  is described by the following equation:

$$f_D(\text{pH}) = \frac{c_0 + 10^{-\text{pH}}}{c_0 + 10^{-\text{pH}} + 10^{-\text{p}K_a}} \quad (9)$$

where  $c_0$  is a constant. In addition to protonation by free protons (see eq. 8) internal protonation is assumed. Plot the experimentally determined  $f_D$  vs. pH, fit this dependence to eq. 9, and determine  $\text{p}K_a$ .

- Derive the dependence  $f_D(\text{pH})$  for the case where the internal protonation is neglected by using the definition of  $f_D$ , the definition of pH, and the definition of  $K_a$ :  $K_a = ([\text{H}^+][\text{A}^-])/[\text{HA}]$ . Show that this function does not fit the experimental data.
- (Optional) Derive the equation 9. The possibility of internal protonation can be included in the derivation of eq. 9 in two ways. Either the total proton concentration is assumed to be that determined by pH (bulk) plus the protons

available for internal protonation  $c_0$ , or the internal protonation can be described by the reaction scheme  $A^- \leftrightarrow AH'$  with a dissociation constant  $K_a' = [A^-]/[HA']$ , and the total concentration of molecules in the protonated (dark) state is then  $[HA] + [HA']$ . Both ways lead to the same result with a fixed relationship between the constants  $c_0$ ,  $K_a'$  and  $K_a$ .

## Literature

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*Who would like to read more...*

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