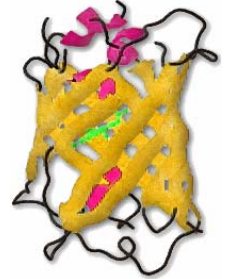


# Report: Fluorescence Correlation Spectroscopy

## 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 molecules (GFP, naturally occurring in jellyfish *Aequorea Victoria*) in aqueous buffer solution.

By temporal analysis of small spontaneous deviations from the average fluorescence, emanating from small illuminated ensembles, the parameters determinable by FCS include: particle concentration, diffusion coefficients, flow rates, aggregate formation, kinetic rate constants of fast reversible or slow irreversible reactions, and triplet lifetimes and populations. Thus the kinetics and thermodynamics of a wide spectrum of processes, that are accompanied by reversible fluorescence changes, are accessible by FCS (Schwille et al., 1999).



The protein is exhibiting pH-dependent intramolecular dynamics, specifically the protonation of chromophore groups. The GFP is in aqueous solution at neutral and higher pH present in an anionic state, with an absorption maximum at ~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<sup>+</sup> laser wavelength) 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.

## Tasks:

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, the protein is less stable and partially denaturizes already short time after the mixing of higher concentrations.
3. *Data Evaluation:* The digital fluorescence signal (pulses corresponding to detected photons) is auto correlated by a hardware correlator, a PC ALV-5000 multiple- $\tau$  correlator card (ALV, Langen, Germany). Evaluation of the curves is carried out by ORIGIN (OriginLab Corporation) 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.

## Equations:

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

- $G(\tau)$  normalized auto correlation of the fluorescence signal  
 $\langle \dots \rangle$  denotes temporal average over the duration of the experiment  
 $F(t)$  measured fluorescence signal at time  $t$   
 $\delta F(t)$  deviation off the temporal average, e.g.  $\delta F(t) := F(t) - \langle F(t) \rangle$

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, which has a

slow time constant  $\tau_{diff}$  compared to  $\tau_{blink}$ .  $\tau_{blink}$  represents another noticeable process in this experiment: the blinking rate, in other words the protonation and deprotonation rate.

Due to the Poissonian nature of the particle number fluctuations at low concentrations  $C(\vec{r}, t)$ , one can conclude that the amplitude  $G(0)$  is inversely proportional to the average number of molecules  $\langle N(t) \rangle$  present in the volume:

$$G(0) = \frac{\langle \delta F(t)^2 \rangle}{\langle F(t) \rangle^2} = \frac{\langle F_0^2 \cdot \delta N(t)^2 \rangle}{\langle F_0 \cdot N(t) \rangle^2} = \frac{F_0^2 \cdot \langle \delta N(t)^2 \rangle}{F_0^2 \cdot \langle N(t) \rangle^2} = \frac{1}{\langle N(t) \rangle} = \frac{1}{\langle C(\vec{r}, t) \rangle V_{eff}} \quad (2)$$

$F_0$  constant fluorescence probability coefficient  
 $V_{eff}$  empirically determined effective volume, see Eq. (4)

In case of a 3-dimensional Gaussian-shaped excitation intensity distribution (see Eq. (5)), the autocorrelation function  $G_{diff}(\tau)$  for freely diffusing molecules of one species with concentration  $C$  is given by

$$G_{diff}(\tau) = \frac{1}{\langle C(\vec{r}, t) \rangle V_{eff}} \frac{1}{1 + \frac{\tau}{\tau_{diff}}} \frac{1}{\sqrt{1 + \frac{r_0^2 \tau}{z_0^2 \tau_{diff}}}} \quad (3)$$

$\tau_{diff}$  diffusion-related time constant, which characterizes the decay of the autocorrelation function  
 $r_0$  equatorial diameter of the prolate spheroid of  $1/e^2$  of the maximal intensity in x- and y-direction  
 $z_0$  polar diameter

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

Measurement profile  $W(\vec{r})$  (defines the excitation and fluorescence intensity distribution in the hanging droplet)

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

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

$D$  diffusion coefficient, which determines how fast the particles move [ $\text{m}^2 \text{s}^{-1}$ ]  
 $\tau_{diff}$  diffusion time

If the molecules, additionally, undergo intramolecular dynamics, which is visible as a blinking of the fluorescence (on/off transitions, where  $\tau_{blink} \ll \tau_{diff}$ ) during their residence time in the focal spot, then  $G(\tau)$  has to be modified as follows:

$$G_{diff+blink}(\tau) = \frac{1 - f_D + f_D e^{-\tau/\tau_{blink}}}{1 - f_D} G_{diff}(\tau) + dc \quad (7)$$

$G_{diff+blink}$  combined normalized auto correlation of the diffusion and blinking processes  
 $f_D$  average fraction of particles in the dark state  
 $\tau_{blink}$  blinking time due to (de)protonation and the related shift of chromophore energy levels  
 $dc$  offset term used to account for effects of a finite data sample

$$\tau_f = \frac{1}{k_{b \rightarrow d} + k_{d \rightarrow b}} \quad (8)$$

$k$  transition rates from the dark to the bright state and vice versa

$$\frac{1}{f} = \frac{1}{b} + \frac{1}{g}, \text{ in case of 1:1-imaging } f = \frac{b}{2} = \frac{g}{2} \quad (9)$$

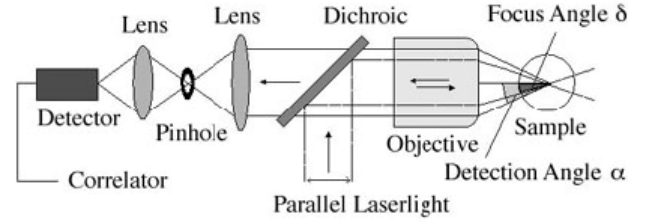
$f$  focal distance  
 $b$  distance between image and lens  
 $g$  distance between image and object

## Experimental Setup:

To minimize the detection volume, to keep the number of simultaneously detected proteins low, confocal optics are used in order to eliminate light from out-of-focus planes. That means a pinhole is placed in the conjugated plane of the hanging droplet sample, which contains the proteins. The data is generated by a highly sensitive avalanche photodiode (APD) which is wired to the hardware correlator. A lens is placed in the middle of the detector and the pinhole, which performs so-called 1:1 imaging. To achieve this, based on Eq. (9) the focal length  $f$  of the lens has to be  $\frac{1}{4}$  of the distance between detector and pinhole.

The  $\text{Ar}^+$  laser used in the experiment has an output power of 14 mW. As both the APD and the proteins could have been damaged by the high intensity, 2 optical filters were used:

1. optical density  $OD$  of 2, reducing the beam power to 1%,  $P_{out} = P_{in} \cdot 10^{-OD} = 140 \mu\text{W}$
2. optical density of 0.1, therefore reducing the beam power to  $P_{out} = 111 \mu\text{W}$



## Experiment:

We received the GFP in a weak buffer stem solution. To find the effect of different pH values, we also received 10 strong buffer solutions with different pH. We then used a computer program to access the autocorrelation data generated by hardware. The program was configured to average 10 runs of 10 seconds each. For each run a frequency time plot was generated which allowed us to find deviations off the usual pattern. We were told that these artifacts (frequency peaks, that were higher than twice the average) were generated by dust particles or proteins stuck to each other. Several times we had to throw away the defective solution and dilute a new part of the stem solution with a new part of the strong buffer.

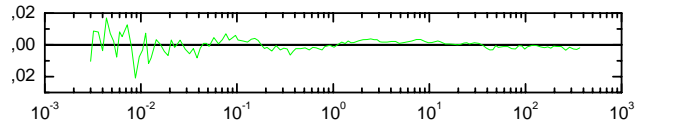
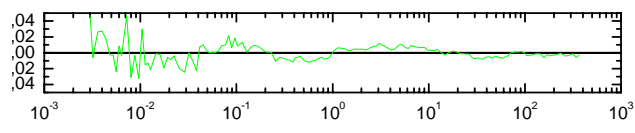
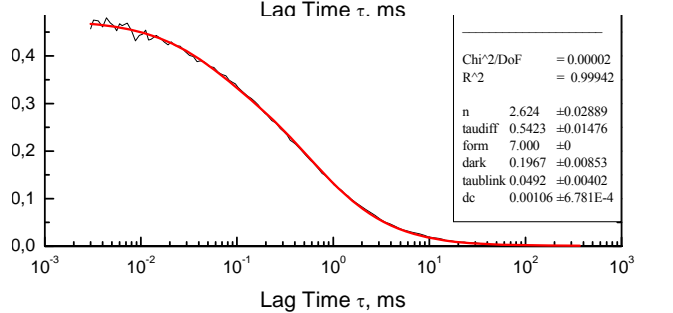
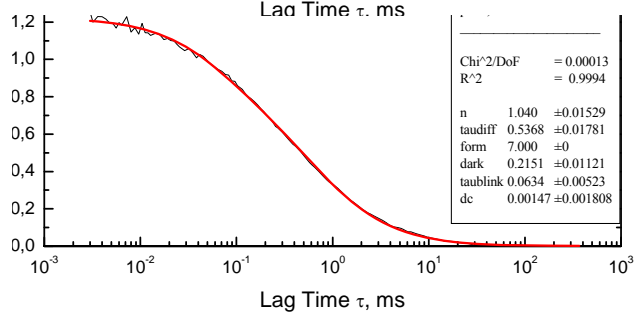
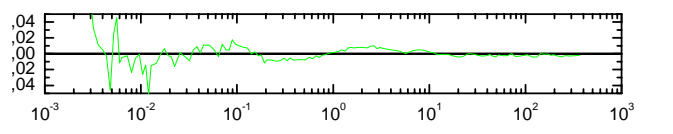
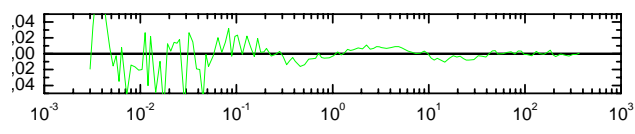
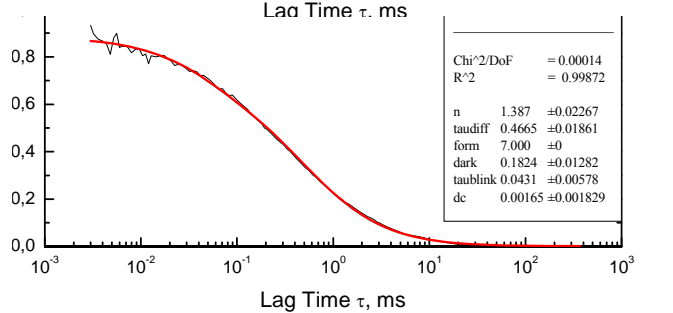
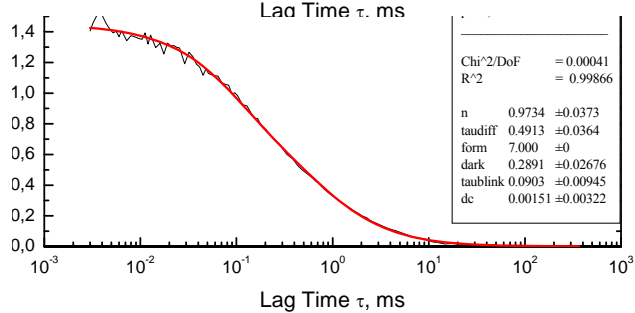
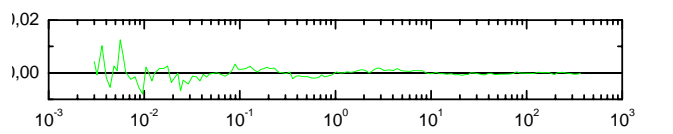
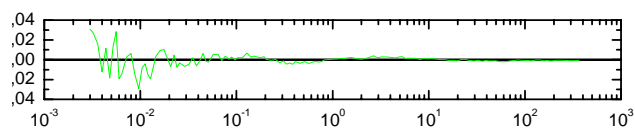
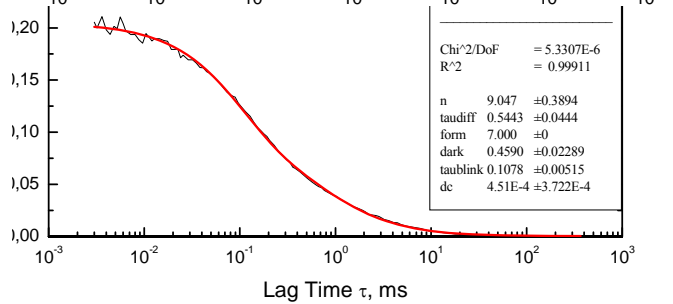
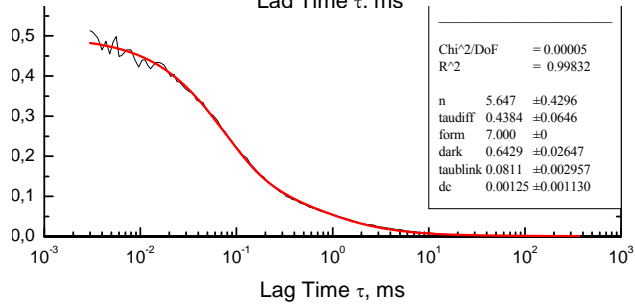
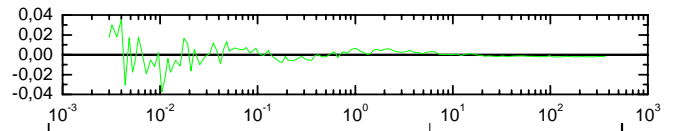
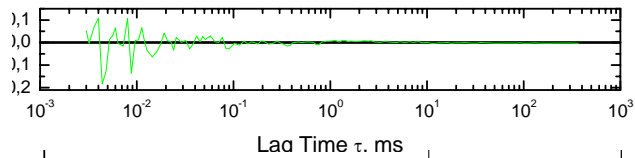
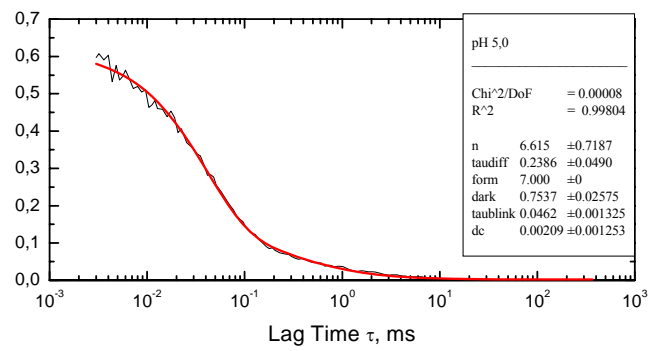
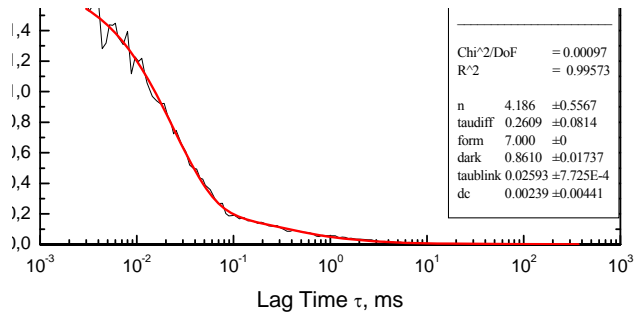
Since we used the confocal setup at very low protein concentrations, the mean number of particles in the focal spot (see Eq. (2)) is of the magnitude of 1, therefore in this sense the used FCS setup can be called a single-molecule method.

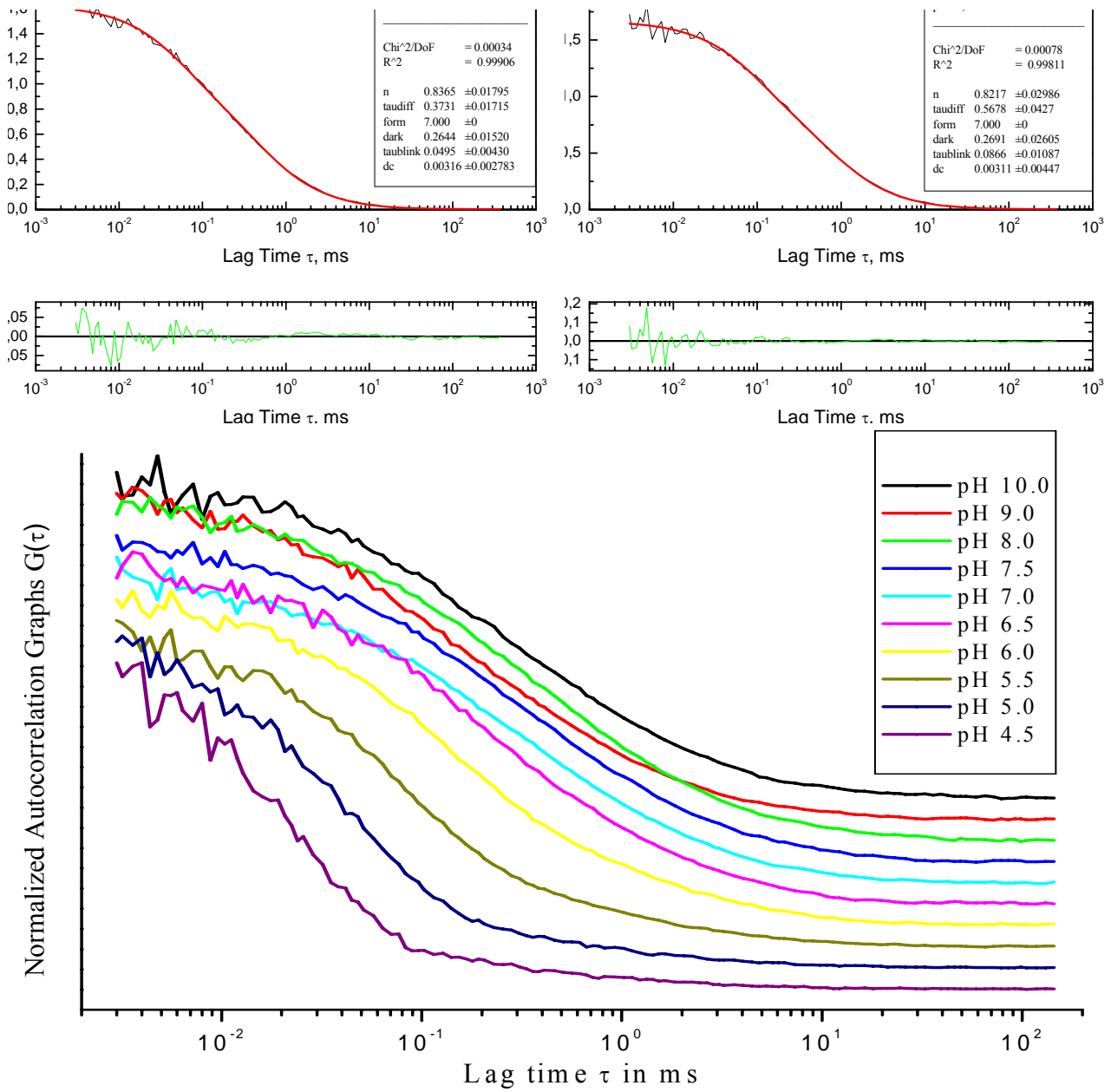
## Results & Calculations:

Data obtained by fitting Eq. (7) to the experimental data in ORIGIN (Marquardt-Levenberg fitting routine):

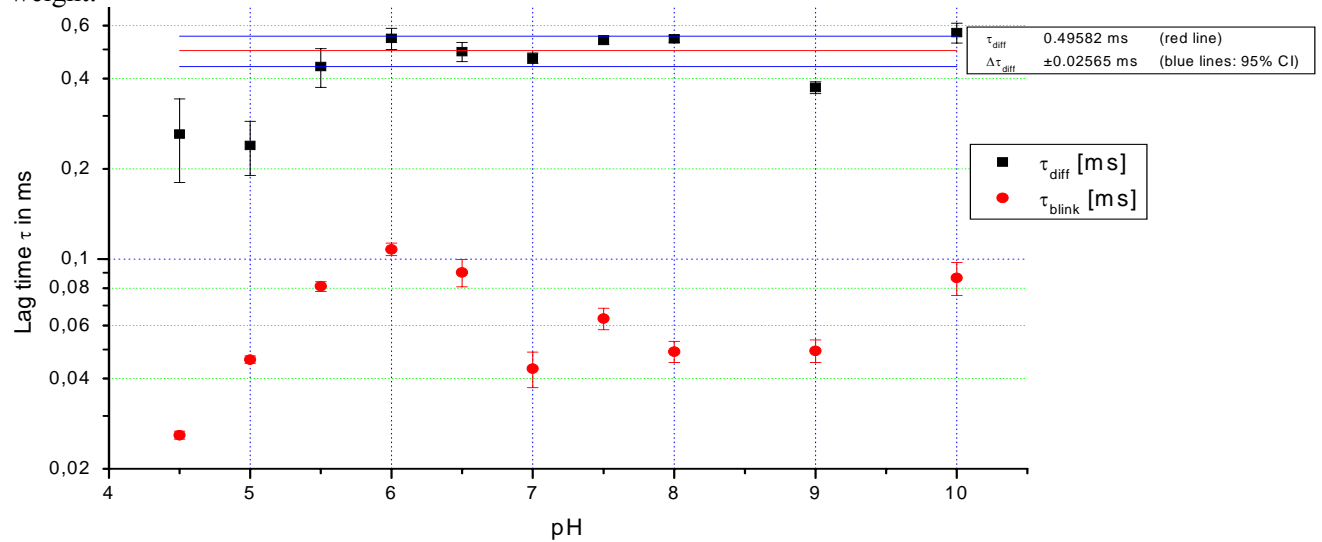
pH	particles $n$	$\Delta n$	$\tau_{diff}$ [ms]	$\Delta\tau_{diff}$ [ms]	dark fraction $f_D$	$\Delta f_D$	$\tau_{blink}$ [ms]	$\Delta\tau_{blink}$ [ms]	$dc$	$\Delta dc$
4.5	4.17	0.56	0.261	0.081	0.861	0.017	0.0259	0.0008	0.0024	0.0044
5.0	6.62	0.72	0.239	0.049	0.754	0.026	0.0462	0.0013	0.0021	0.0013
5.5	5.64	0.43	0.438	0.065	0.643	0.026	0.0811	0.0030	0.0013	0.0011
6.0	9.05	0.39	0.544	0.044	0.459	0.023	0.1078	0.0052	0.0005	0.0004
6.5	0.973	0.037	0.491	0.036	0.289	0.027	0.0903	0.0095	0.0015	0.0032
7.0	1.387	0.023	0.467	0.019	0.182	0.013	0.0431	0.0058	0.0017	0.0018
7.5	1.040	0.015	0.537	0.018	0.215	0.011	0.0634	0.0052	0.0015	0.0018
8.0	2.624	0.029	0.542	0.015	0.197	0.009	0.0492	0.0040	0.0011	0.0007
9.0	0.836	0.018	0.373	0.017	0.264	0.015	0.0495	0.0043	0.0032	0.0028
10.0	0.821	0.030	0.568	0.043	0.269	0.026	0.0866	0.0109	0.0031	0.0045

The following series of diagrams shows the collected fluorescence correlation data (black) and a fit (red):





Although size, shape and mass of the GFPs might change at different pH levels, due to conformation changes and different water structures around the proteins, there should not be a strong dependency of diffusion behavior and pH. Therefore, we fitted a constant through the previously obtained fit data, taking errors as weight:



- Size of measurement volume  $V_{eff}$ : By using Eq. (6) and a diffusion coefficient of  $D = (86.7 \pm 0.6) \cdot 10^{-12} \text{ m}^2/\text{s}$  (R. Swaminathan et al, 1997), we obtain  $r_0 = (415 \pm 12) \text{ nm}$ .  
The ratio  $f = z_0/r_0 = 7$  was given for our setup and therefore  $z_0 = (2.90 \pm 0.08) \mu\text{m}$ .  
Now we can calculate the effective volume by using Eq. (4):

$$V_{eff} = (2.78 \pm 0.24) \cdot 10^{-18} \text{ m}^3 = (2.78 \pm 0.24) \mu\text{m}^3$$

- Absolute concentration of our sample: According to Eq. (2), in theory one can find the concentration by knowing  $G(0)$  of the diffusion process. Unfortunately, the hardware used cannot give accurate results close to  $\tau = 0 \text{ s}$ , so we only measured  $\tau = 0.003 \dots 367 \text{ ms}$ . Additionally, there is the fast protonation process, that adds to the correlation function. By fitting Eq. (7) to the experimental data, as done above, we can get an approximate value of  $n$ .

$$c = \frac{n}{N_A \cdot V_{eff}}, \quad \Delta c = \frac{\Delta n}{N_A \cdot V_{eff}} + \frac{\Delta V_{eff} \cdot n}{N_A \cdot V_{eff}^2}, \text{ where } N_A \text{ is Avogadro's number.}$$

pH	c [n mol/l]	$\Delta c$ [n mol/l]	pH	c [n mol/l]	$\Delta c$ [n mol/l]
4.5	0.35	0.12	7.0	0.68	0.08
5.0	0.97	0.29	7.5	0.49	0.06
5.5	1.20	0.29	8.0	1.26	0.14
6.0	2.92	0.51	9.0	0.37	0.05
6.5	0.41	0.07	10.0	0.36	0.06

- Power density of the excitation laser light within the focal spot: after filtering, the laser power is less than  $P_{out} = 111 \mu\text{W}$ . Therefore, the intensity in the equatorial plane and in the effective volume is less than:

$$w_{plane} = \frac{P_{out}}{\pi r_0^2} = 205 \cdot 10^6 \frac{\text{W}}{\text{m}^2}, \quad w_{volume} = \frac{P_{out}}{V_{eff}} = 39.9 \cdot 10^{12} \frac{\text{W}}{\text{m}^3}$$

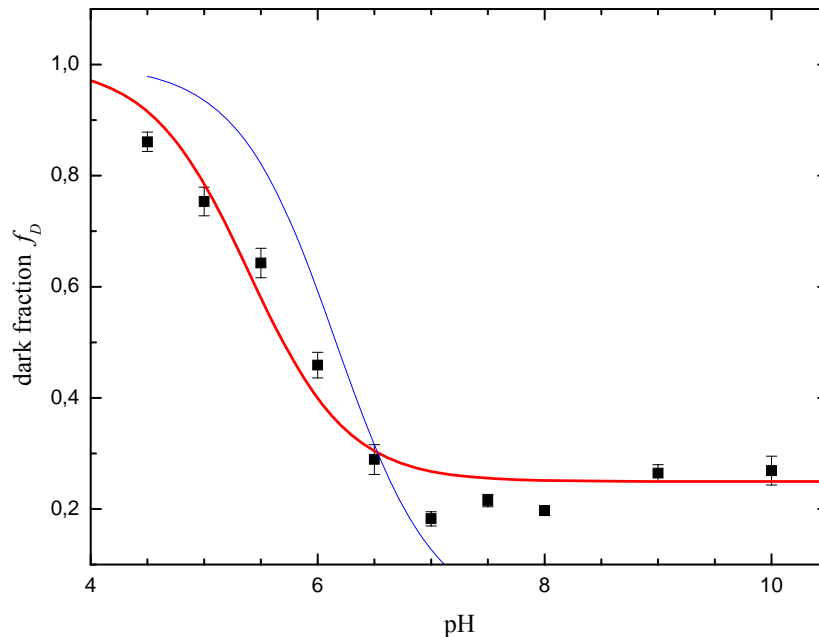
An increase of the power density results in an decrease of the viscosity of water, whereby the diffusion time would decrease. There is also the risk of permanent bleaching of the chromophores.

The particle density is independent of the temperature, but strong lasers can have an optical tweezers effect, which can keep particles in the focal spot.

- variation of autocorrelation curves at low pH: In our measurement the tendency of high peaks in the fluorescence signal rose at lower pH, where higher concentrations were used. These peaks could have been generated by two or more proteins sticking together. Another problem at low pH was the short protein lifetime before bleaching occurred.
- dissociation constant  $K_a$  of protons and the chromophore of eGFP: The theoretical dependence is

$$f_D(pH) = \frac{c_0 + 10^{-pH}}{c_0 + 10^{-pH} + 10^{-pK_a}}, \text{ where } pK_a = -\log_{10}(K_a) \text{ and } c_0 \text{ is a constant.} \quad (9)$$

In the following diagram the above formula 9 has been fitted (red line —) to the  $f_D$  fitting data of the autocorrelation graphs (black squares ■).



In addition to protonation by free protons (see Eq. (10) below, or blue line — in the diagram above) internal protonation as in Eq (9) is assumed.

The obtained values are:

$$\left. \begin{array}{l} c_0 = 10^{-6} \\ pK_a = 5.52 \pm 0.06 \end{array} \right\} \text{ fit of Eq. (9)}$$

$$K_a = (6.88 \pm 0.39) \cdot 10^{-7} \text{ fit of Eq. (10)}$$

- Dependence  $f_D(pH)$  for the case where the internal protonation is neglected. Definitions:

$$pH := -\log[H^+] \Rightarrow [H^+] = 10^{-pH}$$

$$K_a := \frac{[H^+][A^-]}{[HA]} \Rightarrow [HA] = \frac{10^{-pH} \cdot [A^-]}{K_a}$$

$$f_D := \frac{N_D}{N}, \text{ where } N_D \text{ is the number of dark particles, } N \text{ the total number in the focus}$$

For constant concentrations within the focal spot, one can conclude that:

$$f_D = \frac{[HA]}{[HA] + [A^-]} = \frac{\frac{10^{-pH} \cdot \cancel{[A^-]}}{K_a}}{\frac{10^{-pH} \cdot \cancel{[A^-]}}{K_a} + \cancel{[A^-]}} = \frac{\frac{10^{-pH}}{\cancel{K_a}}}{\frac{10^{-pH}}{\cancel{K_a}} + \frac{K_a}{\cancel{K_a}}} = \frac{10^{-pH}}{10^{-pH} + K_a} \quad (10)$$

## Literature

- Swaminathan, R., C. P. Hoang, and A. S. Verkman. 1997. "Photobleaching recovery and anisotropy decay of green fluorescent protein S65T in solution and cells: cytoplasmic viscosity probed by GFP translational and rotational diffusion". *Biophys. J.* 72(4):1900 -1907.
- Schwille P, Haupts U, Maiti S, Webb WW 1999. „Molecular dynamics in living cells observed by fluorescence correlation spectroscopy with one- and two-photon excitation". *Biophys J.* 77(4):2251-65.