



Quantitative FCS: Determination of the Confocal Volume by FCS and Bead Scanning with the MicroTime 200

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Introduction

Fluorescence Correlation Spectroscopy (FCS) is used to determine concentrations and diffusion constants in the pico- to nanomolar concentration region with broad applications in Biology and Chemistry. However, the method is correlated to a broad range of measurement parameters and other factors as background contributions which make quantitative results very often difficult to obtain. Quantitative results rely on the size of the confocal volume which has to be determined experimentally. The confocal volume is difficult to measure in situ and is sensitive to saturation and bleaching of the dye molecules, optical aberrations and variations of the index of refraction as observed in biological specimen.

The spatial resolution of a confocal fluorescence microscope is usually described by the confocal volume, that is the excitation volume folded with the detection volume. It depends on the excitation intensity distribution (EID) as well as on the collection efficiency function (CEF) of the excited fluorescence. Furthermore it depends on sample properties like the refractive index of the sample containing medium, the photophysics of the fluorophore, cover slide thickness and other experimental conditions.

In this application note we present three methods for the determination of the confocal parameters for quantitative FCS measurements:

1. Measuring a dilution series of a sample, which has the advantage to be applicable to any dye with known concentration
2. Measuring FCS of a sample with a known diffusion coefficient to determine the confocal volume in the FCS curve fitting. This method has the benefit that the sample concentration does not need to be determined exactly.
3. The third method measures directly the confocal volume via raster scanning of a sub-resolution fluorescent bead with high precision.

The results of the measurements are compared to each other in respect of their application for quantitative FCS.

Experimental details

The measurements were performed on a MicroTime 200 confocal microscope (PicoQuant) [1]. A modified Olympus IX71, equipped with a Olympus UPlanAPO NA 1.2 water immersion objective, served as a microscope body. Laser in-coupling and fluorescence collection was performed through the main optical unit, which was connected to the microscope body via the right sideport. As excitation source, a 635 nm pulsed laser diode with a repetition rate of 40 MHz was used (LDH-P-635). The laser beam passed an adjustable attenuator and was coupled via a polarization maintaining single mode fiber into the main optical unit. There, the beam was directed via a dichroic mirror (z467/638pc) into the Olympus IX71. Using an additional beamsplitter, a part of the excitation light was directed to a photo diode. The photo diode was calibrated with a laser power meter and was used to determine the excitation light intensities. Simultaneously, a back reflection from the sample was directed onto a CCD camera. Monitoring of the back reflection allows exact repositioning of the z-position of the cover slide surface. As scanning was performed with a xyz-piezo scanner, the FCS measurements can be performed at a defined distance to the glass surface.

The fluorescence emission passed the dichroic, an emission filter (HQ687/70) and was focused on a pinhole. The pinhole size was set to 63 μm . After the pinhole, the fluorescence light was divided via a 50/50 beam splitter cube and focused on 2 SPCM-AQR SPAD detectors (Perkin Elmer). All measurements and data analysis was performed using the SymPhoTime Software (PicoQuant).

Bead scanning was performed on 100 nm fluorescent beads (TetraSpeck, Invitrogen) that were immobilized on a glass surface. An area with a small particle density was selected and the beads with the weakest fluorescence were chosen for scanning. The scanning speed was set to 20 nm/pixel and stacks were recorded with 100 nm resolution in z - direction.

FCS measurements were performed on solutions of ATTO 655-COOH (carboxy group - AD 655-2, Atto-Tec). The dye has the advantage to have negligible triplet state contributions. The objective col-

lar of the water immersion objective was optimized in order to achieve the highest molecular brightness i.e. the mean fluorescence count rate per dye molecule.

The stock solution was further diluted to the desired concentration by subsequently pipetting into triple distilled water with 0.05 % Tween 20 added to prevent aggregation and surface adhesion. The initial concentration was measured spectroscopically with a UV-160A UV-VIS absorption spectrometer (Shimadzu) and found to be $(3.8 \pm 0.2) \mu\text{M}$. This stock solution was diluted to $1 \mu\text{M}$ for the sake of even numbers. To further minimize surface adsorption, pipetting volumes were kept relatively large (typically above $100 \mu\text{l}$) and polypropylene tubes (Plastibrand, Brand) were used as sample containers. Uncertainties of the samples increase with the number of dilution steps involved. The uncertainties due to the concentration measurement of the stock solution and the pipetting steps were calculated according to the information given by the manufacturers and plotted as error bars in Fig. 2 and Fig. 3. For the measurements the sample was pipetted into the cap of a polypropylene tube (approximately $80 \mu\text{l}$) and put onto a cover slide.

The FCS measurements (5 min) were performed $20 \mu\text{m}$ above the cover slide surface at a temperature of $(23 \pm 2)^\circ\text{C}$. The fluorescence was split on two detectors and the cross correlation function was calculated to remove the influence of detector afterpulsing in the calculated correlation curves.

Theory

Measuring the confocal volume with FCS

If the measured signal fluctuations are only due to diffusion, the amplitude of the auto- or crosscorrelation equals the inverse number of fluorescent molecules present in the effective volume V_{eff} on average. Please note, that the term effective volume V_{eff} will be used here, which is not identical to the confocal volume V_{conf} . If the volumes is approximated with a 3-dimensional Gaussian shape function, the effective volume is larger than the confocal volume V_{conf} by a factor of $2^{3/2}$:

$$V_{\text{conf}} = \left(\frac{\pi}{2}\right)^{3/2} w_0^2 z_0 = \left(\frac{1}{2}\right)^{3/2} V_{\text{eff}} \quad [\text{Eq. 1}]$$

w_0 is the lateral and z_0 the axial $1/e^2$ -radius of the confocal volume. z_0 is usually expressed in terms of $z_0 = k * w_0$ with k being the eccentricity of the confocal volume.

The first approach of determining the effective volume (and therefore the confocal volume) is to measure the correlation amplitude of a sample with known concentration [2]. The advantage of this procedure is that the measurement can be performed under similar conditions as in the experiment

of interest. This method allows to calculate the effective volume according to:

$$V_{\text{eff}} = \frac{1}{G_0 N_A c} \quad [\text{Eq. 2}]$$

G_0 is the correlation amplitude, c the sample concentration in molar units and $N_A = 6.022 \times 10^{23}$ is the number of molecules in one mol (Avogadro number).

A second advantage of this approach is that no special FCS model and hence no assumption about the shape of the confocal volume need to be applied, as G_0 can be extracted without any fitting procedure. However, the method also assumes that photophysics (like triplet states) can be neglected.

The second approach relies on the assumption, that the confocal volume can be approximated by a 3-dimensional Gaussian shape. In this case the autocorrelation function can be calculated analytically:

$$G(t) = G_0 \cdot \left(1 + \frac{t}{\tau}\right)^{-1} \cdot \left(1 + \frac{t}{k^2 \tau}\right)^{-1/2} \quad [\text{Eq. 3}]$$

τ is the lag-time for which the correlation has dropped to half of its maximum. It can be interpreted as the average time a molecule needs to transverse the confocal volume by diffusion. It is connected with the lateral extension of the confocal volume by the diffusion coefficient D :

$$w_0^2 = 4D\tau \quad [\text{Eq. 4}]$$

If the diffusion coefficient is known, the size of the confocal volume can be extracted using [Eq. 5] which follows from [Eq.1] and [Eq. 4]. τ and k are determined by fitting the correlated data with [Eq. 3]. Knowledge about the concentration of the sample is not necessary, as the concentration can be directly obtained from the FCS analysis.

For this method it is essential to use only adequate

$$V_{\text{eff}} = \pi^{3/2} k (4D\tau)^{3/2} \quad [\text{Eq. 5}]$$

laser power for the FCS measurements since the confocal volume depends on the laser power in a way that saturation of the dye enlarges the confocal volume. A good way to determine the maximum allowed laser power for a measurement is to monitor the fluorescence intensity as a function of laser power for a sample with approx. 100 nM concentration diluted in the solution of interest. The FCS measurement should be done with the highest laser power displaying a power dependency still in the linear regime [Fig. 1]. In our case this corresponds to a laser power of $89 \mu\text{W}$. The laser power used for the calibration measurement should then also be utilized for the final FCS experiment. If the diffusion time of

interest in the final experiment is much longer than during the calibration measurements possible bleaching of the dye can occur, demanding even lower excitation power.

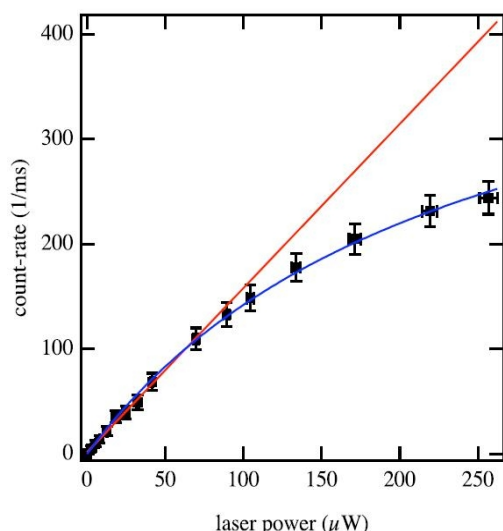


Fig. 1: Saturation curve of the dye ATTO655 measured at 100 nM concentration

Results

a) Determination of the confocal volume using samples with known concentration from a dilution series

At first we will discuss the determination of the confocal volume by analyzing the fluorescence correlation of a sample with known concentration. Instead of measuring the number of particles for a single concentration we analyzed G_0 for a Atto-655 dilution series covering 6 orders of magnitude, ranging from 1 μ M to 1 pM. This approach gives a far better accuracy and reveals the suitable concentration range.

Fig. 2 shows the particle numbers $\langle N \rangle = 1/G_0$ on average present in the effective detection volume extracted from the correlation amplitudes for the different sample concentrations measured. Note that both axis have logarithmic scales to cover the large concentration range measured.

The black squares are the apparent numbers of particles calculated as $\langle N \rangle = 1/G_0$, while the red squares are the numbers of particles calculated after correction for the influence of the uncorrelated background signal.

A linear dependence between the average number of particles and the concentration is expected and the resulting slope can be interpreted as the effective volume. While $\langle N_{app} \rangle$ (black squares) decreases with decreasing sample concentration for larger concentrations, this trend is inverted for concentrations below approx. 1 nM. This increase at low sample concentrations is caused by the

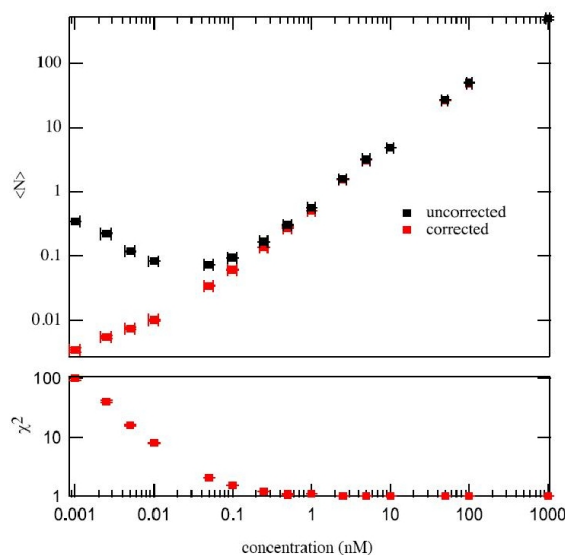


Fig. 2: Dilution series of Atto655 in H₂O. Above: $\langle N \rangle$, average number of particles found in the effective volume; below: background correction factor χ^2

increasing contribution of the uncorrelated background signal, which becomes more prominent at low sample concentration and damps the correlation amplitude. As a consequence, the reduction of the correlation amplitude leads to an apparently higher particle concentration in the effective volume and has therefore to be taken into account for a correct analysis of the results.

The influence of the uncorrelated background signal on the correlation amplitude can be taken into account through a correctional factor χ^2 [3,4] :

$$\frac{1}{\chi^2} = \frac{1}{(1 + \langle b \rangle / \langle f \rangle)^2} \quad [\text{Eq. 6}]$$

$\langle b \rangle$ is the average background count rate measured on a sample containing only solvent, $\langle f \rangle$ is the (virtual) count rate of the actual sample without any background contribution, which is calculated from the measured count rate $\langle F \rangle$ reduced by the background count rate ($\langle f \rangle = \langle F \rangle - \langle b \rangle$). As can be seen in Fig. 2, χ^2 increases with decreasing concentration.

Taken the influence of the uncorrelated background into account, the number of particles present on average in the effective volume can be calculated according to the following equation:

$$\langle N \rangle = \frac{1}{\chi^2 G_0} \quad \text{Eq. 7}$$

This equation holds only for molecules with negligible contribution of fluctuations between a non-fluorescent and a fluorescent state (like triplet) on a microsecond time scale, which is the case for the measured ATTO 655 dye. The background corrected particle numbers, $\langle N \rangle$, are shown as red

squares in Fig. 2. A more or less linear dependence on the sample concentration down to a concentration of 50 pM (3×10^{13} particles per liter) is obtained. To get a measure of the linearity of the dilution series the logarithm of $\langle N \rangle$ was calculated and plotted versus the logarithm of the pipetted sample concentration (Fig. 3).

When trying to fit the whole concentration range the fit tends to strongly weight the higher concentration samples because the concentrations were varied logarithmically. The complete concentration range was therefore fitted applying a logarithmic weighting of the data (red curve in Fig. 3). The offset of $(3.7 \pm 0.6) \times 10^{-3}$ can be explained originating from impurities from the solvent, most probably stemming from the Tween 20. If the offset would correspond to impurities with the same molecular brightness as ATTO 655, it would correspond to a concentration of 6.3×10^{-13} M. $\langle N \rangle$ is reproduced well over the whole concentration range (6 orders of magnitude) as can be seen in the residues plotted in Fig. 3 (top). From these results V_{eff} was found to be (1.0 ± 0.1) fl.

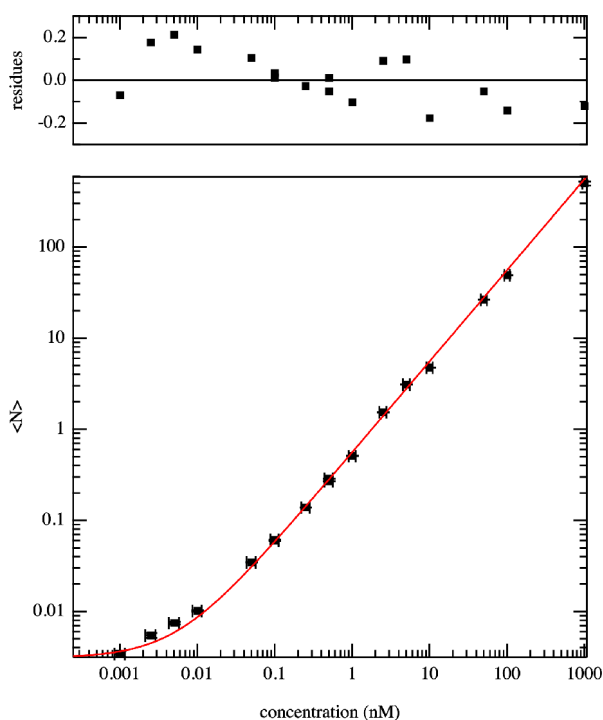


Fig. 3: Dilution series of ATTO655 in H₂O. Linear fit and its relative residuals (top)

b) Determination of the confocal volume from the FCS fit of a sample with known diffusion coefficient

The lateral and axial dimensions of the confocal volume can be extracted from the fit of the correlated data if the diffusion coefficient of the fluorescent sample is known. Assuming that the confocal volume can be approximated by a three dimensional Gaussian function, the dimensions of the

confocal volume can be derived from the fit parameters using [Eq. 8]:

$$G(t) = G_0 \cdot \left(1 + \frac{4Dt}{w_0^2}\right)^{-1} \cdot \left(1 + \frac{4Dt}{k^2 w_0^2}\right)^{-1/2} \quad [\text{Eq. 8}]$$

While w_0 and k were determined by fitting [Eq. 8] to the experimental data, the effective volume V_{eff} was calculated from w_0 and k as

$$V_{\text{eff}} = \pi^{3/2} w_0^3 k.$$

As a reference the diffusion coefficient of ATTO 655 was measured with two focus FCS in a low and with NMR in a high concentration regime by Thomas Dertinger et al. [6]. In these measurements, the diffusion coefficient D was determined to be $(426 \pm 8) \mu\text{m}^2/\text{s}$ in pure water at 25°C. In our sample chamber the temperature could, however, not be determined as accurately and a sample temperature of $(23 \pm 2)^\circ\text{C}$ was assumed. The temperature dependence of D can be linearly approximated for the temperature differences in question with 2.6% increase per 1°C [6], which leads to a diffusion coefficient of $D = (404 \pm 10) \mu\text{m}^2/\text{s}$ for 23°C. Changes of the diffusion coefficient due the addition of Tween 20 was not taken into account in the following analysis.

Since the correlation of the data was only calculated for lag times smaller than 100 ms, the correlation may not drop to zero completely. We therefore introduced an additional parameter C_0 in the analysis. C_0 was always found to be about 1000 times smaller than the correlation amplitudes along with more than 100% uncertainty. The introduction of C_0 is therefore only a means of stabilizing the fit process.

The fits reproduce the correlated data quite well for all three concentrations. However, the resulting parameter of the eccentricity of the confocal volume $k = z_0/w_0$ for the 1 pM sample has been found to be 21 ± 447 (expected values fall between 2 and 6), indicating that the fit is unable to yield reasonable values for the shape of the confocal volume. For 100 nM the uncertainty of the eccentricity is as well high. For a concentration of 0.25 nM all parameters of the fit could be acquired with reasonable uncertainties. The examples shown in Fig. 4 indicate that the quality of the parameters that can be extracted by fitting the correlated data vary depending on the sample concentration. Fig. 5 shows the lateral $1/e^2$ radius w_0 , the eccentricity k and the resulting effective volume for the sample concentrations between 1 pM and 1 μM . In order to calculate the effective volume from a known diffusion coefficient, [Eq. 5] equation can be applied. All three parameters are expected to be constant since they only depend on the experimental setup but not on the sample concentration.

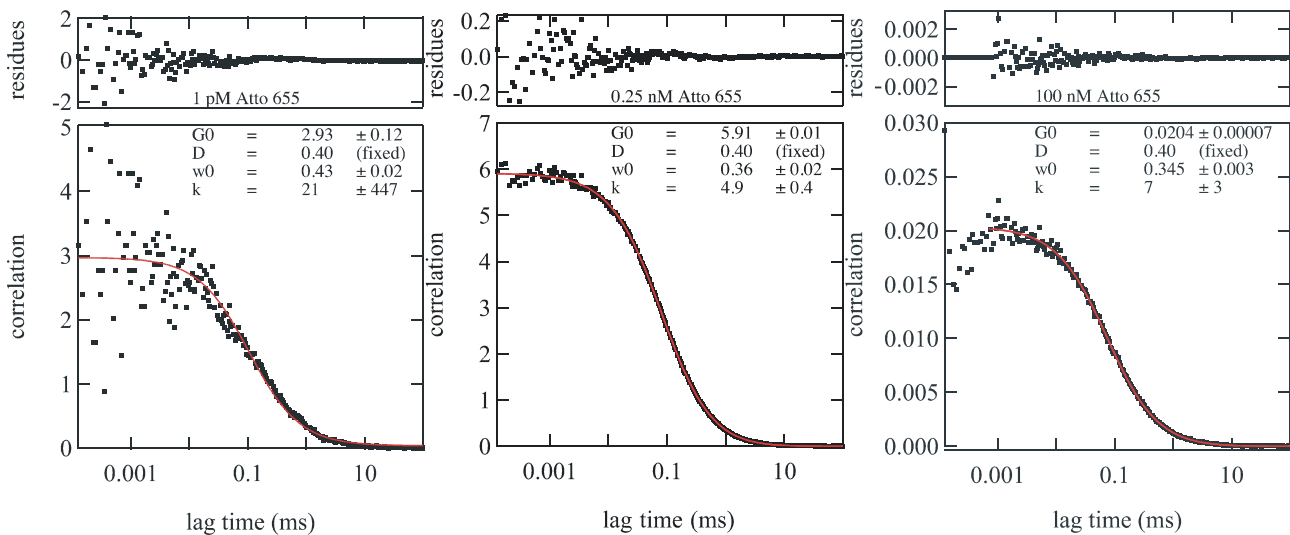


Fig. 4: Fluorescence correlation curves of 3 different dilutions for ATTO 655 and their respective fits. The best result is obtained with a concentration of 0.25 nM.

From [Eq. 8] it becomes obvious that k can only be extracted with a relative high uncertainty since its contribution to the shape of the correlation function is rather small. k is also strongly correlated to ω_0 , making it difficult to find both parameters with accuracy especially if the quality of the correlated data becomes poorer. As can be seen in Fig. 5 the uncertainty of k increases strongly for low and high concentrations. For low concentrations this is due to low statistics and for high concentration due to

the low correlation amplitude. For the 100 nM sample the correlation amplitude is only 0.02. The correlation drops from this value to zero on a time scale of about 4 orders of magnitude. The dependence of the correlation on the lag time is very weak and the fit parameters therefore difficult to extract. Only in the range of 0.1 nM to 5 nM the fit yields reasonable values with acceptable uncertainties for the eccentricity. This region is indicated by the vertical lines in Fig. 5. The achieved average values in this region are $w_0 = (0.36 \pm 0.02) \mu\text{m}$, $k = (4.6 \pm 0.5)$, and the average confocal volume in this region is calculated to be $V_{\text{eff}} = (1.2 \pm 0.15) \text{fl}$ as indicated by the horizontal line in Fig. 5c. For this calculation the diffusion coefficient D of $(404 \pm 10) \mu\text{m}^2/\text{s}$ for 23°C was taken into account [6].

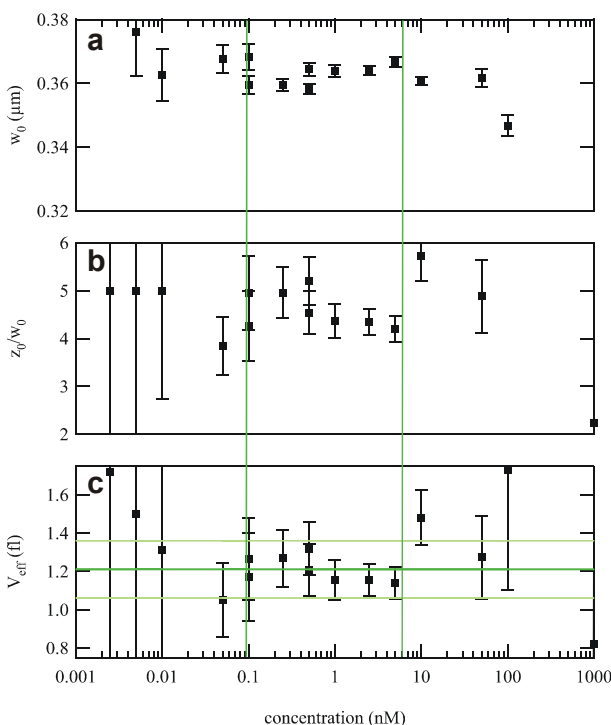


Fig. 5: Dilution series of ATTO655 in H_2O :

- Beam waist radius ($1/e^2$)
- eccentricity $k_0 = z_0 / \omega_0$
- resulting effective volume found by fitting the correlated data with the autocorrelation function for pure diffusion and a three dimensional confocal Gaussian volume

c) Determination of the confocal volume by bead scanning

A third method to determine the confocal volume is a measurement based on the imaging of subresolution fluorescent beads (in this case of 100 nm diameter). Besides the laser excitation power, the measurement conditions were identical as for the FCS measurements mentioned above. However, it is important to correct for eventually different thicknesses of the microscope cover slide with the objective correction collar. Subresolution beads can be treated like a point source and be used in order to scan the confocal volume.

For confocal volume determination, the ability of the MicroTime 200 system equipped with an axial piezo positioner was used to scan not only in lateral xy -direction but also in axial direction (see Fig. 6). The sections in xy , xz and yz directions were fitted with a 2-dimensional Gaussian distribution.

These measurements yielded a lateral radius ω_0 of

$(0.38 \pm 0.02) \mu\text{m}$ and an eccentricity k of 3.2 ± 0.2 ; resulting in an effective volume of (1.0 ± 0.1) fl. The scanner accuracy is ± 3 nm and can be disregarded as a source of uncertainty.

$$I = I_0 + I_{max} \cdot e^{-2 \left[\left(\frac{x-x_0}{w_x} \right)^2 + \left(\frac{y-y_0}{w_y} \right)^2 \right]} \quad [\text{Eq. 9}]$$

d) Determination of the diffusion coefficient

In order to check whether confocal volumes determined by bead-scanning can help determining diffusion coefficients in FCS measurements, the beam waist and the eccentricity found by bead scanning were used as parameters in FCS fitting. Instead of [Eq. 8], [Eq. 10] is used to fit the correlated data:

$$G(t) = G_0 \cdot \left(1 + \frac{t}{\tau} \right)^{-1} \cdot \left(1 + \frac{t}{k^2 \tau} \right)^{-1/2} + C_0 \quad [\text{Eq. 10}]$$

For k , the value of 3.2 ± 0.2 obtained in bead scanning measurements was chosen. The fit yields the diffusion time τ , which is plotted in Fig. 7 for the different concentrations of the dilution series.

The diffusion constant is then calculated according to equation [Eq. 11] with $w_0 = 0.38 \mu\text{m}$ derived from the bead measurements.

$$D = \frac{w_0^2}{4\tau} \quad [\text{Eq. 11}]$$

Fig. 7 shows the resulting diffusion times τ . Since k is kept constant, the trustworthy concentration range is larger compared to Fig. 5 and reaches from 50 pM to 50 nM.

The average diffusion time for the samples in this concentrations range is 0.0849 ± 0.001 ms.

According to [Eq. 11] this corresponds to a diffusion coefficient of $(0.425 \pm 0.40 \mu\text{m}^2/\text{s})$. Considering the uncertainties, the value is in accordance with the diffusion coefficient of ATTO 655 measured by Dertinger et al. Of $(0.404 \pm 0.10) \mu\text{m}^2/\text{s}$ at 23°C [6].

Conclusion

We have investigated two methods of obtaining the effective volume from FCS measurements and compared the results with the effective volume resulting from imaging of fluorescent microspheres (beads).

For the FCS calibration measurements care has to be taken in order to choose the right concentration range and in addition an adequate illumination power since saturation influences the size and shape of the confocal detection volume.

Obtaining the effective volume directly from FCS measurements has the advantage of a measurement in similar environmental conditions e.g. in an aqueous solution.

Applying a dilution series the absolute concentration has to be known leading to uncertainties arising from dilution steps and from the measurement of the absorption coefficient.

From dilution series, including samples with concentrations of 250 pM to 1 μM , the effective volume could be determined with an uncertainty of 10%. We assume that in this concentration range sample loss due to surface adsorption is not of concern. The positive side of the method is the independence of additional parameters. It can be applied to any dyes with known absorption coefficient.

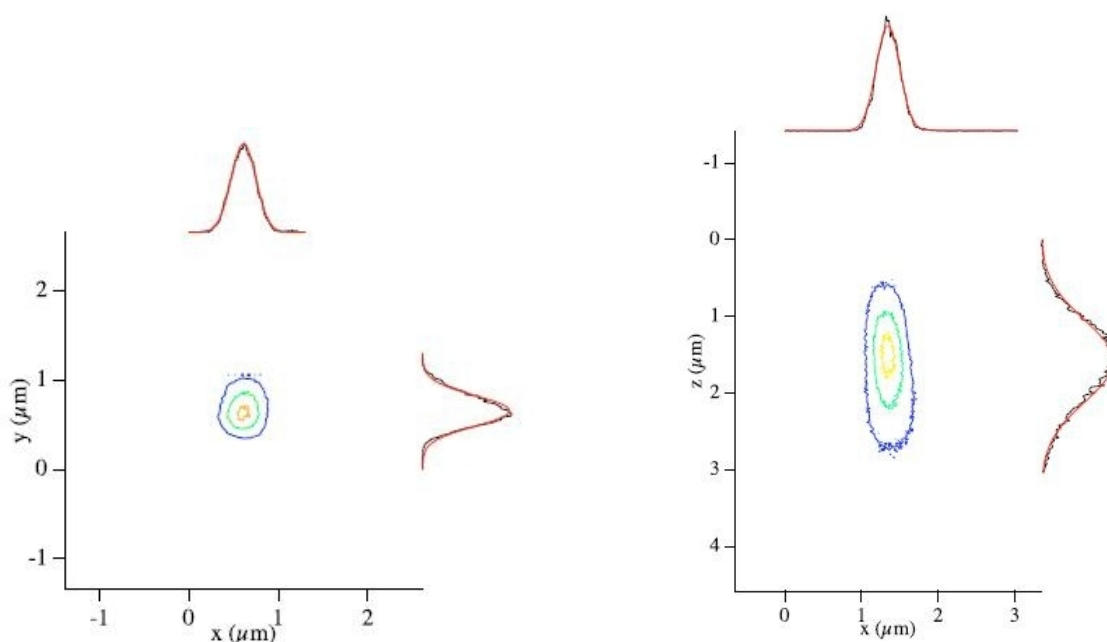


Fig. 6: Isosurface representations of a TetraSpeck bead (left: xy-scan; right: xz-scan). The contour lines indicate the intensity profiles $(1/e^2)I_{max}$, $0.5I_{max}$ and $0.9I_{max}$. Line profiles and Gaussian fits through the lines are shown at the borders.

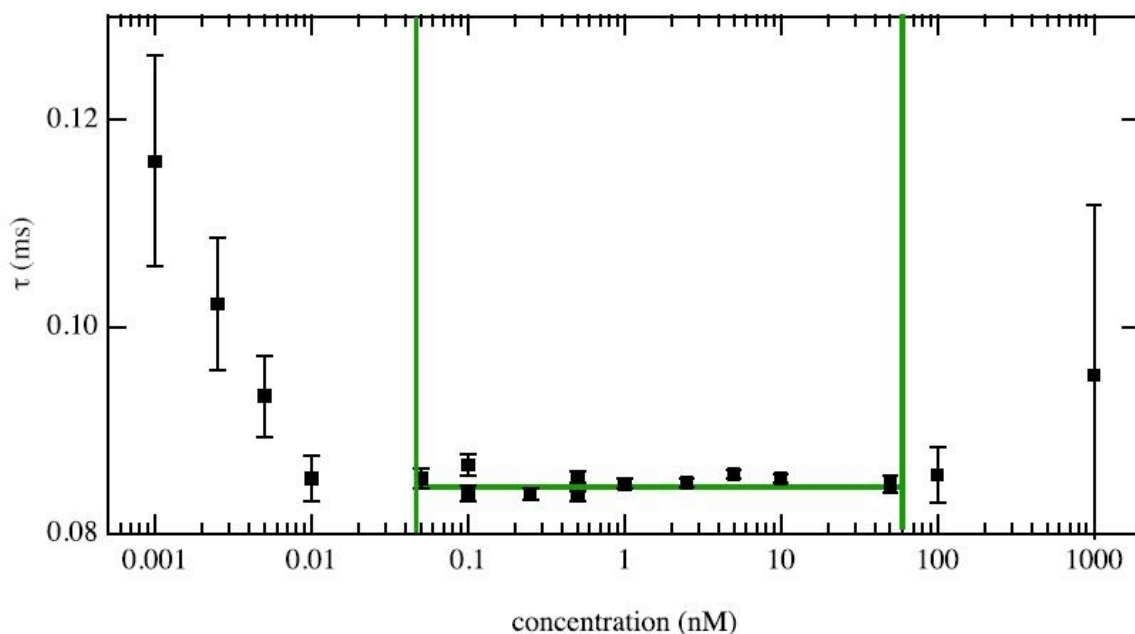


Fig. 7: fitted correlation time τ for different concentrations

The second method based on a known diffusion coefficient applies FCS curve fitting. Using the same theoretical model to describe the correlation curve for the volume calibration as well as for the FCS measurement can help to reduce the error arising from an incomplete description of the correlation curve. We approximated the confocal detection volume with a 3-dimensional Gaussian function. Since this approach involves fitting of the model function to the experimental data the quality of the correlated data also influences the precision of the findings. We found that the determination of the confocal volume was only possible for samples with concentrations between 100 pM and 5 nM. The advantage of this method is that there is no need to know the exact sample concentration and therefore sample loss due to adsorption does not influence the result. However, the diffusion coefficient must have been determined before with high accuracy, which is only the case for few dyes.

The last method detects the detection volume through raster scanning of a subresolution fluorescent bead with high precision. It therefore relies on the accuracy of the scanner. As the bead is normally immobilized to the surface of a microscope cover slide, the detection volume is not measured in solution. Possible optical aberrations through the aqueous environment are not taken into account. Furthermore a bead with a dye with spectral properties corresponding to the dye used in the FCS measurements should be selected. However, the method is beyond its limitations fast and reliable.

The results of the three methods are listed in the following table:

Method	Effective detection volume
Dilution series	(1.0 ± 0.1) fl ($\pm 10\%$)
Known diffusion coefficient	(1.2 ± 0.15) fl ($\pm 13\%$)
Bead scanning	(1.0 ± 0.1) fl ($\pm 10\%$)

Taking the uncertainties into account all methods lead to comparable results. The effective confocal volume could be determined with all methods with an uncertainty of $\sim 10\%$. The results show that it is possible to perform quantitative FCS measurements with the MicroTime 200 confocal microscope. All displayed methods for the determination of the confocal volume can be applied. In case of the bead scanning method it was shown that the diffusion coefficient could be determined with a precision of 10%. The method of choice should be selected according to the properties of the sample.

Further reading

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