Time-averaged fluorescence intensity analysis in fluorescence fluctuation polarization sensitive experiments

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Abstract: In fluorescence fluctuation polarization sensitive experiments, the limitations associated with detecting the rotational timescale are usually eliminated by applying fluorescence correlation spectroscopy analysis. In this paper, the variance of the time-averaged fluorescence intensity extracted from the second moment of the measured fluorescence intensity is analyzed in the short time limit, before fluctuations resulting from rotational diffusion average out. Since rotational correlation times of fluorescence molecules are typically much lower than the temporal resolution of the system, independently of the time bins used, averaging over an ensemble of time-averaged trajectories was performed in order to construct the time-averaged intensity distribution, thus improving the signal-to-noise ratio. Rotational correlation times of fluorescein molecules in different viscosities of the medium within the range of the anti-bunching time (1-10 ns) were then extracted using this method.

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1. Introduction

Fluorescence spectroscopy and microscopy techniques use the sensitivity of the fluorescence signal to the environment of the fluorescence probe in order to gain important information about the investigated system [1]. When studying samples with low concentrations of fluorescent species or even individual molecules, as well as when operating the fluorescence detection system at high temporal resolution, the measured signal is typically weak and fluctuating. These fluctuations are used in fluctuation-sensitive techniques to gain better insight into the underlying physical evolution of the system, and are therefore of great interest [2]. Various fluorescence fluctuation techniques have been extensively applied in chemistry, biophysics, biology and related fields, in studies of photochemistry, diffusion properties, chemical kinetics, aggregation, conformational fluctuations [3–7], both in vitro [8] and in living cells [9–11], on small molecular ensembles as well as on single molecules [12,13]. The main advantage of these methods is that they report on the stochastic fluctuations that are the key to biological mechanisms and if sufficiently fast, reveal macromolecular dynamics directly, without the need to synchronize large ensembles and interpret relaxation decays in kinetic terms [14].

Observed fluorescence fluctuations usually have several independent sources. First, signal fluctuations originate from a varying number of fluorescent molecules in the probed volume, reorientation of the molecule or its internal dynamics, or from variations in the emission properties of the molecules [12,15–17]. A second source of fluctuations that is particularly

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relevant at low light levels or short integration times, is the variation of the photon counts due to the stochastic nature of light emission and detection [18–20]. Other fluctuation sources can be fluorescence background, impurities or scattered light [21–23].

In fluorescence fluctuation polarization sensitive experiments, if the sample is excited with linearly polarized light at a low intensity, or if detection is polarization-dependent (or both), rotation of the molecule leads to observable fluctuations in the fluorescence signal [24,25]. Typically, these fluctuations contribute a term that has the characteristic time scale of the rotational correlation time to the fluorescence correlation function [3,26]. However, in most applications it is difficult to detect the rotational correlation time by fluorescence correlation spectroscopy (FCS), as it is short in the order of the anti-bunching time (at 1–10 ns), the dead time of the detector (at 10–100 ns), or the triplet relaxation time (at 0.1–1 μs). In these cases measurement of the fluorescence rotational correlation time is usually obtained by correlating the reduced linear dichroism signal measured by detecting any two orthogonal polarizations in the in-plane of the sample, and taking their difference divided by their sum. These signals fluctuate in the course of time due to molecular rotational dynamics [27–29]. Normalization with the measured fluorescence intensity ensures that the dichroism signal depends solely on the orientation of the emission dipole and not on the absorption efficiency. The analogy of the dichroism signal to what is often called polarization could lead to some confusion. In fluorescence polarization methods applied on an ensembles of molecules polarization and anisotropy can be interchanged easily [30]. However, in the case of single molecule fluorescence, one would have to know the polarization components in all three directions to calculate the anisotropy. Subsequently, the autocorrelation function of the dichroism signal is calculated. Although this autocorrelation function gives valuable information about the time scale and the geometric aspects of molecular reorientations [28], these measurements also require a very good signal-to-noise ratio (SNR) for subsequent data analysis and are limited also with regard to the time resolution of the system. Several approaches have been presented aiming to increase temporal resolution of these measurements [28,31,32]. Recently it has been suggested to improve the temporal resolution of these measurements by analyzing higher moments of the polarization dependent fluorescence signal [33]. The concept that there is more information in higher moments of fluorescence fluctuations has already been applied by several authors [34–37].

In this paper, the time-integrated measured intensity of the fluctuating polarization sensitive fluorescence signal is used to extract rotational dynamics information. This procedure is conceptually different from that performed in fluorescence correlation methods. In correlation analysis, information is extracted by explicitly analyzing the time course of the fluctuating signal. Therefore, the shortest correlation time that can be probed is determined by how rapidly the signal can be continuously recorded. Since it is explicitly assumed that the sampling time is much shorter than the characteristic timescale of fluctuations in the fluorescence signal, a few photons are collected during a short sampling period and as a result the SNR of the data is poor. Thus, longer sampling times are needed to improve the signal. In contrast, with a time integrated intensity analysis, the actual time course of the signal is not analyzed. Rather, the sample is probed by measuring how the mean and variance of the time-integrated measured signal vary with different integration times. However, as opposed to standard cumulant analysis (FCA) [36] in which higher moments of the intensity that is measured by one detection channel are analyzed, in this paper the intensity that is analyzed has an out-of-plane dipole orientation dependency and is measured by two polarization channels. Moreover, as opposed to FCA in which the brightness and the number of molecules in the optical observation volume are extracted from higher moments of the intensity for arbitrary sampling times [37], in this paper the rotational diffusion time of the diffusing molecule is extracted from the short time behavior of variance of the time averaged intensity. In fluorescence fluctuation polarization sensitive experiments, fluctuations due to the rotational dynamics of the fluorescence molecule that occur during the integration time will be
manifested by increasing the variance of the time-averaged fluorescence signal. In this approach, the timescale of the fluctuations that are probed is given by the timescale of the integration time used. By analyzing the mean and the variance of the time-averaged fluorescence signals in the time limit where fluctuations as a result of rotational diffusion are not averaged out, rotational correlation times of the fluorescent molecule can then be extracted. The time-averaged fluorescence signal itself is extracted from the first and second moments of the measured intensity. Since rotational correlation times are typically much shorter than the temporal resolution of the system independently of the time bins used, one must acquire ensemble measurements for each sample in order to obtain a good SNR. Here, molecule brightness is not an issue to be considered, since only the polarization sensitivity of the measured signal is quantified and not the total emitted photon counts [12,33]. This method is applied here for the simple case of fluorescein, which is considered a spherical rotor characterized with a single-exponential diffusion time [38]. Various mixtures of fluorescein in glycerol/buffer provide a convenient framework to examine the ability of this method to separate nano-seconds rotational correlation times.

The paper is constructed as follows: Section 2 gives a brief theoretical discussion of the methods used. The experimental setup, samples, simulation and the data analysis are described in Section 3. In Section 4, the fluorescence polarization sensitive signal of fluorescein in glycerol/buffer mixtures is analyzed. Finally, a brief discussion on the limitations and future prospects of the method are given in Section 5.

2. Theory

2.1 Time-averaged fluorescence intensity

Fluorophores preferentially absorb photons whose electric field vectors are aligned parallel to the transition dipoles of the molecule [30]. These dipoles have a well-defined orientation with respect to the molecular axes, with an azimuthal angle \( \theta \) and a polar angle \( \phi \). Similarly, emission also occurs with light polarized along a fixed axis. The experimental situation analyzed in the remainder of this paper is depicted in Fig. 1 together with the definition of the axis system and the angles used. The system considers a plane wave emission polarized along the molecular transition dipole moment. In this case, the fluorescence intensity signals, \( I_\parallel \) and \( I_\perp \), are proportional to the projections of the transition moment onto the two polarization directions being measured. The cone of light collected by a lens is given by the numerical aperture (NA) of the collecting objective, such that the collection angle \( \alpha \) is given by:

\[
\alpha = \sin^{-1}(\text{NA}/n)
\]

(1)

where \( n \) is the refraction index of the immersion medium. Following Fourkas [39], the detected signals \( I_\parallel \) and \( I_\perp \) are related to the transition dipole orientation according to:

\[
I_\parallel = I_e(A + B \sin^2 \varphi + C \sin^2 \varphi \cos 2\theta)
\]

(2)

\[
I_\perp = I_e(A + B \sin^2 \varphi - C \sin^2 \varphi \cos 2\theta)
\]

(3)

where \( A, B, \) and \( C \) are constants evaluated in terms of \( \alpha \), and \( I_e \) is the total emitted fluorescence intensity determined by the molecular brightness (which is proportional to the product of the quantum yield, the excitation of the fluorophore and the instrument efficiency). According to Eqs. (2) and (3) the measured intensity, is always \( \sin^2 \varphi \) dependent. That is:

\[
I(t) = I_\parallel(t) + I_\perp(t) = 2I_e(A + B \sin^2 \varphi \cos 2\theta)(t)
\]

(4)

whereas the total emission intensity measured from one channel, i.e. \( 2I_\parallel + 4I_\perp \) or more simply \( I_\parallel + 2I_\perp \) [30, 40, 41] is also \( \theta \) dependent. However, in single fluorophore experiments where a
large numerical aperture (NA) must be used, the total emission intensity is not always
equivalent to \( I_0 + 2I_\perp \), as it is for narrow beam angles [42,43]. That is, \( I_\perp \) is in many cases not
a single-exponent, i.e. not entirely free of the anisotropy decay. The impact of a large NA on
the rotational correlation time estimation from the rotational correlation function has been
treated in different ways [27,28,44].

Fig. 1. A schematic representation of the experimental system. The sample is excited by a laser
diode (LD) in 470 nm with a repetition rate of 50 MHz. The polarization of light emitted by the
fluorophore depends on the orientation of the transition dipole with respect to the detection
system. The orientation of the transition dipole is represented by the angles \( \phi \) and \( \theta \). \( I_\parallel \) and \( I_\perp \)
are orthogonal polarization components of the fluorescence signal, separated by a polarizing
beam splitter (BS). The fluorescence signal is detected at the same time by two separate
photomultipliers (HPM hybrid detectors) and delivered to the TCSPC card for processing.

For a spherical isotropic rotor, the diffusion of \( \phi \) is described by the one-dimensional
rotational diffusion equation with a single-exponential solution and rotational correlation time
\( \tau_r \) [45]. In this case \( I(t) \) fluctuates in the time scale of \( \phi \) [33,46]. The time limit in which
these fluctuations are not averaged out can be quantitatively described by the photon counting
distribution. For a stationary dipole, Poisson distribution fully describes the statistics of
photon counting detection. However, a change in dipole orientation will also cause a change
in the mean Poisson quantity. If this change is random, then the arrival of photons to the
detector, and hence their subsequent detection, is a doubly stochastic process [46]. The
relation between the continuous fluorescence intensity distribution and the discrete
photoelectron distribution is given by Mandel’s formula [47]:

\[
\langle P(n,T) \rangle = \frac{\Omega^n}{n!} e^{-\Omega} P(\Omega) d\Omega
\]

where the time integrated intensity \( \Omega(t) = \alpha \int_0^t I(t') dt' \) is expressed in units of photon counts
and the measured intensity, \( I(t) \), is in units of photon counts per second. \( \alpha \) is the quantum
efficiency of the detector which is assumed here for simplicity to be 1. The mean of the distribution is given by:

\[ \langle \Omega(t) \rangle = \frac{1}{\Omega} \int_0^\infty \Omega(t) P(\Omega) d\Omega \]  

(6)

where \( \langle \Omega(t) \rangle = \langle \Omega(t) \rangle / t \) is the time average of the measured intensity. The variance is given by:

\[ \langle \Omega^2(t) \rangle - \langle \Omega(t) \rangle^2 = \int_0^\infty \Omega(t)^2 P(\Omega) d\Omega - \left( \int_0^\infty \Omega(t) P(\Omega) d\Omega \right)^2 \]

(7)

Fluctuations in \( \langle \Omega(t) \rangle \) are described by the distribution:

\[ P(T, t) = \delta \left( T - \frac{1}{T} \int_0^T I(t) dt \right) \]

(8)

where \( T \) is the integration time used in the experiment. In the time limit \( T >> \tau_r \), the distribution approaches a delta function centered around the mean number of photon counts \( P(T, T) = \delta (T - \langle I(t) \rangle) \). For \( T \) that is short in comparison to the characteristic translational diffusion correlation time, and long in comparison to the rotational correlation time, \( P(T, T) \) describes the average number of photons that fluctuate due to rotational diffusion. In this time limit where fluctuations resulting from rotational diffusion are not averaged out, the molecule is assumed to be stationary (frozen) as regards to translational diffusion, but still rotating. The variance of the distribution for this case is given by [48]:

\[ \langle T(T)^2 \rangle - \langle T(T) \rangle^2 = \frac{\langle \Omega^2(t) \rangle}{T^2} - \left( \frac{\langle \Omega(t) \rangle}{T} \right)^2 = \frac{2}{T^2} \left[ \int_0^T dt(T - t) \langle \delta I(t) \delta I(0) \rangle \right] \]

(9)

where \( \delta I = I - \langle I \rangle \) and the integrand \( \langle \delta I(t) \delta I(0) \rangle \) is the autocorrelation function of \( I(t) \). For most cases of molecular rotational symmetry and transition dipole geometry relevant to fluctuation polarization sensitive experiments, the autocorrelation function is approximated by a stretched-exponential rise term [28,49]. That is

\[ \langle \delta I(t) \delta I(0) \rangle = Fe^{-\beta \tau_r \alpha} \]

(10)

where \( F \) is an empirical pre-exponential fitting constant corresponding to the magnitude of the dipole rotation effect, and \( \beta \) describes the system’s heterogeneity. That is, the variance of the time averaged intensity is a direct measure of the self-similarity of a time series intensity described by \( \tau_r \). The benefit of using Eq. (9) can be demonstrated by the simple case of a spherical molecule with a transition dipole that exhibits isotropic rotation in time. In this case the autocorrelation function is a single exponent where \( \beta = 1 \). Substitution of Eq. (10) in Eq. (9) yields:

\[ \langle T(T)^2 \rangle - \langle T(T) \rangle^2 = \frac{2}{T^2} \left[ \int_0^T (T - t)Fe^{-\beta \tau_r \alpha} dt \right] = \frac{2F\tau_r}{T^2} + \frac{2F\tau_r^2}{T^2} (-1 + e^{-\beta \tau_r \alpha}) \]

(11)

Clearly, in the time limit \( T >> \tau_r \), \( \langle T(T)^2 \rangle - \langle T(T) \rangle^2 \) is linearly decreased in time according to
As an example, consider $\tau_r = 10$ ns and $F = 1$. In Fig. 2(a) the variance of the time-averaged signal is compared to the autocorrelation function. Clearly, the decrease rate of the variance of the time-averaged intensity (blue line) is slower than that of the autocorrelation function (red line). In probing fast rotational diffusion processes where the temporal resolution of the system is rather limited, this slow convergence rate could be advantageous.

In Fig. 2(b) the variance of the time-averaged intensity is plotted for different $\tau_r$ values. Generally, as $\tau_r$ values increase, the variance of the time-averaged intensity exhibits a slower decrease in time.

In conclusion, the variance of the time-averaged intensity extracted from the variance of the time-integrated measured intensity, is analyzed in the short time limit where fluctuations resulting from rotational diffusion are not averaged out. The slow convergence rate of the variance and its strong dependency on $\tau_r$ allow the use of larger time bins for tracking rotational diffusion fluctuations.

2.2 The influence of dead time on the time-averaged intensity

Since detectors are never ideal, it is essential to understand the influence of detectors on signal statistics to correctly interpret the experimental data. Most of the theories of fluorescence fluctuation spectroscopy have considered the case of ideal photo-detection [50]. Unfortunately, photo-detectors are never ideal and typically suffer both dead-time and afterpulse effects. Dead-time is a fixed period of time after registration of a photon during which the detector cannot detect another photon [51]. Afterpulse generation depends on detector type [52]. Typically, in the context of fluorescence fluctuation experiments, it is safe to ignore afterpulsing [50]. Since dead-time takes up a larger proportion of the time interval at high sampling frequencies, it should affect data taken at those frequencies more strongly than data taken at lower frequencies. For an experiment with a pulsed excitation source, the relative time in which the system is active is:

$$\delta = \frac{n \lambda}{T}$$  \hspace{1cm} (13)
where \( \lambda \) is the excitation period and \( n \) is the number of pulses in which the system is active during integration time \( T \). The effect of dead time is described accordingly by

\[
\frac{t_d}{T} = 1 - \delta,
\]

where \( t_d \) is the dead-time. Based on these assumptions Eq. (11) is rewritten as:

\[
\langle T(T)^2 \rangle - \langle T(T) \rangle^2 = \delta \frac{2}{T^2} \int_0^T (T - t\delta) e^{-t\delta} dt = \frac{2F_T}{T^2} \left[ T - \tau_r + e^{-T\delta} \left( \tau_r + (\delta - 1) \right) \right]
\]

Equation (14)

\[
(14)
\]

According to Eq. (14), dead time yields slower rate of decrease of the variance of the time-averaged intensity. As a consequence, the SNR of the data is decreased for the given integration time. Clearly, for \( \delta = 1 \), Eq. (11) is recovered.

![Graph showing the variance of the time-averaged intensity](image)

**Fig. 3.** The variance of the time-averaged intensity for \( \tau_r = 10 \text{ns} \) (solid black line - ideal measurement (\( \delta = 1 \)), dashed black line - \( \frac{t_d}{T} = 1/100 \)) and for \( \tau_r = 500 \text{ns} \) (solid red line - ideal measurement (\( \delta = 1 \))).

In Fig. 3 comparison of the variance of the time-averaged intensity for \( \tau_r = 10 \text{ ns} \) between an ideal measurement (\( \delta = 1 \)) (solid black line) and a non-ideal measurement, where \( \frac{t_d}{T} = 1/100 \) (dashed black line). Clearly, dead time yields higher measured \( \tau_r \) values, as demonstrated by comparing it to an ideal measurement where \( \tau_r = 500 \text{ ns} \) (solid red line).

**3. Materials and methods**

**3.1 Experimental set-up**

Experiments were carried out on an OLYMPUS IX-81 inverted microscope with a 60x, NA = 1.2 oil immersion objective (Olympus, Japan) as described in Fig. 1. Fluorescence excitation was provided by a linearly polarized 470 nm pulsed laser diode (Becker & Hickl, BDL-470-SMC, Berlin, Germany) with a repetition rate of 50MHz. The laser beam is coupled into a polarization-maintaining single-mode fiber and delivered to a commercial confocal scan head (DCS-120, Becker & Hickl, Berlin, Germany), collimated, reflected by a dichroic mirror and brought to its diffraction-limited focus inside the sample. The laser power per illumination spot was typically 10 \( \mu \text{W} \). Fluorescence signals were collected by the same objective through the dichroic beam splitter and focused to a confocal pinhole, which serves to reject the out-of-focus light. After the pinhole, the light was collimated, split by a polarizing beam splitter cube and focused onto two single-photon hybrid detectors (HPD), (HPM-100-40, Becker & Hickl, Berlin, Germany). The two detectors were synchronized and operated with high gain in order.
to avoid afterpulsing. The dark count rate for these detectors was typically 500–700 counts per second at the room temperature. Single photon counting electronics (SPC-150, Becker & Hickl, Berlin, Germany) independently recorded the detected photons of both detectors with an absolute temporal resolution of two picoseconds on a common time frame. The single photon counting electronic channels store each individual photon with its time from the last laser pulse (“micro time”) and its time from the start of the experiment (“macro time”). Fluorescein solutions (1 nM for the fluorescence fluctuation experiments and 1 μM for the time-resolved anisotropy decay measurements), were prepared by dissolving fluorescein (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) solutions having a pH of 7.4 and different viscosities (different glycerol in PBS concentrations: 0, 20%, 60%, and 80% glycerol by weight). The PBS buffer was used in order to maintain a constant pH in all measurements. Glycerol-concentration is known to change the fluorescein characterizations like FLT and quantum yield [40] but not change the absorbance and emission spectrum [30,53]. Viscosities of these solutions at 20°C were estimated at 1, 4, 10.8, 59.6 cP respectively, by a four-parameter correlation function [54].

3.2 Simulations

Monte Carlo simulations of polarized fluorescence fluctuation experiment for 1D linear transition dipoles of single molecules in solution were carried out by diffusion of Eulerian angles on the surface of a sphere, following a typical stochastic Wiener process. Briefly, each Euler angle is incremented by a Gaussian step-size distribution with a mean of zero and standard deviation inversely proportional to the rotational diffusion time constant for the corresponding molecular axis. The resulting orientation and NA-dependent signal collected by the orthogonal polarization channels of a confocal microscope were then calculated using Eqs. (1)- (3) for the corresponding time window to generate the simulated data set. Following completion, the time orientation and signal time course data were processed to yield the mean and the variance of the time-averaged intensity. Typical TCSPC counting losses due to pile-up effect were neglected in our simulation [31,51,55]. Losses efficiency of optical components and finite detection area were all considered with the relative weight of each factor.

3.3 Data analysis

An individual discrete-time series of \( n \) fluorescence intensity values measured by a single optical detection channel \( A \) at regular time intervals \( \{\delta t, t_0 + \delta t, t_0 + 2\delta t, \ldots, t_{\text{end}}\} \) (satisfying the condition \( t_{\text{end}} - t_0 = (n-1)\delta t \)) was considered as an \( n \)-element vector \( \{A_0, A_1, \ldots, A_{n-1}\} \). The difference vector is given by the difference between each element \( A_i \) and the mean \( \langle A \rangle \), resulting in the vector \( I_{\delta = \tau} = \{A_0 - \langle A \rangle, A_1 - \langle A \rangle, \ldots, A_{n-1} - \langle A \rangle\} \) that contains both positive and negative values. The autocorrelation of \( A \) corresponding to positive time delay \( \tau \) where \( \tau = m\delta t \) (\( m \in \mathbb{N} \)), has created a \( \tau \)-shifted difference vector of length \( n-m \), \( I_{\delta = \tau} = \{A_{(n-m)} - \langle A \rangle, A_{(2n-m)} - \langle A \rangle, \ldots, A_n - \langle A \rangle\} \). For a given two polarization sensitive channels \( A \) and \( B \) (|| and \( \perp \)), the normalized autocorrelation for each channel is a vector dot product of \( I_{A:B} \) and the first \( (n-m) \) elements of \( I_{A:B} \), scaled by \( \frac{1}{n-m} \), divided by the squared norm of \( I_{A:B} \) scaled by \( \frac{1}{n} \):

\[
G(\tau) = \frac{1}{n-m} \left[ I_{A:B} \cdot I_{A:B}^{(n-m)} \right] \cdot \frac{1}{n} \left\| I_{A:B} \right\|^2
\]

(15)
The cross-correlation is then calculated by:

\[ G_{a,b}(\tau) = \frac{1}{n-m} \left[ I_B \cdot I_A^{(n-m)} \right] \]

where normalization is achieved by using a dot product of the two non-shifted difference vectors \( I_A, I_B \). The time-averaged intensity was calculated by:

\[ \langle T \rangle = \left\{ \frac{1}{n} \left[ I_A + I_B \right] \right\} \]

where \( \langle ... \rangle \) denotes ensemble averaging over \( l \) measurements. The time-averaged variance was then calculated by:

\[ \text{Var}(\langle T \rangle) = \frac{1}{\langle \langle T^2 \rangle \rangle - \langle \langle T \rangle \rangle^2} \]

The steady state polarization was calculated by:

\[ P = \frac{I_A - I_B}{I_A + I_B} \]

All simulations and data analysis programs were written in MATLAB.

4. Results

4.1 Autocorrelation and cross-correlation analysis

All fluorescence data was collected using the microscopy setup described in Section 3. Excitation power was kept low (typically 50 \( \mu \)W) to avoid bleaching during acquisition. For each experiment ~20\( \mu \)l of the sample solution was placed on a coverslip separating the sample from the oil immersion objective. All samples were measured separately for 100sec. An autocorrelation analysis was applied for each one of the two orthogonal polarization detection channels and cross-correlation analysis of both channels, for the macro-time data of photon arrival for the different samples. As an example, Fig. 4 demonstrates the autocorrelation (solid blue – parallel polarization, solid red – perpendicular polarization) and cross-correlation data (solid green) data for the fluorescein in 0\% glycerol solution Fig. 4(a) and fluorescein in 80\% glycerol solution Fig. 4(b). Clearly, both the autocorrelation and cross-correlation traces exhibit the same decrease in time at the same timescale for both samples. The same indications were found for the other samples (20\% and 60\% glycerol, data not shown). This low sensitivity of the autocorrelation traces to nano-seconds rapid changes in the fluorescence signal as result of rotational diffusion is already well known and discussed in literature [56].
Fig. 4. Autocorrelation (solid blue- parallel polarization, solid red- perpendicular polarization) and cross-correlation (solid green) data for the fluorescein in 0% glycerol solution (a) and fluorescein in 80% glycerol solution (b). The rotational correlation component of the autocorrelation and cross-correlation traces is completely absent for both solutions.

4.2 Photon counts distribution of the measured intensity

In this section we consider fluctuations in the measured signal $I = I_\parallel + I_\perp$. In order to determine the smallest timescale that allows the probing of rotational diffusion, the time integrated intensity $\Omega_i$ was analyzed in various time scales (Eq. (6)). As shown in Fig. 5(a), both $\Omega_\parallel$ and $\Omega_\perp$ linearly increase in time. The slower increase of $\Omega_i$ for the higher glycerol solutions in the longer time limit yields slower convergence of $\langle T \rangle$ [33]. However, by zooming to lower time limits in which fluctuations are shown not to be averaged out [0:100µs], alterations between the different concentrations become unnoticeable. Within this time range, photons of the measured signal were sorted into different sizes of time bins. Since the time bins used are shorter than fluorescein translational diffusion time (~0.1ms) [30], all dynamic information due to translational diffusion contained in the correlations between different bins is lost [57]. On the other hand, since the rotational correlation time of fluorescein is much shorter (1-10’s ns [38]), rotational diffusion information is preserved. Although the Poissonian photon counts distribution for Nano-molar concentrations of fluorescein within this time range is expected to become broader [12], fluctuations as a result of rotational diffusion yield additional broadening that is $\tau_r$ dependent [46]. Comparison of the photon counting distribution for the various samples with the Poisson distribution for a mean equal to the corresponding average photon counts of the experimental histogram, for time bins of $1 \times 10^{-5}$ sec, is shown in Fig. 5(b). Clearly, differences between various samples are significant in the photon counts distribution. That is, although the photon counts distribution is Super-Poissonian for all samples as expected, it is broader for the lower Glycerol solutions. This deviation of the experimental data from the Poisson distribution is much more pronounced in the logarithmic representation as compared to the linear scale (shown in the inset in Fig. 5(b)). The molecular brightness of the sample (mean number of photons detected from one molecule per unit time) typically quantified in photon counting histogram (PCH) experiment is not discussed, since only fluctuations in the measured signal are considered ($I_\parallel + I_\perp$) [12,33]. Thus $\tau_r$ can be extracted for time bins that are lower or at least equal to those that yield significant deviations between the histogram to the Poisson distribution (i.e $1 \times 10^{-5}$).
4.3 Time averaged measured intensity

Extraction of molecular diffusion information from the photon counting distribution is possible by analyzing higher moments of the distribution with arbitrary bin widths and long integration times [37]. In the present study the variance of the time averaged intensity extracted from the variance of the time integrated measured intensity, $I = I_\parallel + I_\perp$, is analyzed with arbitrary bin widths in the time limit where fluctuations are not averaged out. Since the rotational correlation times measured in this work are much shorter than the temporal resolution of the system, independently of the time bins that are used, repeating the experiment many times over ensembles of time-averaged trajectories was done in order to construct the time averaged intensity distribution, thus obtaining the best possible SNR. Typical photon counts trace for the fluorescein in 0% glycerol solution (a) and fluorescein in 80% glycerol solution (b) are shown in Fig. 6 for the parallel polarization (solid blue) and perpendicular polarization (solid red), for time bins of $1 \times 10^{-4}$ sec, which is the longer time limit where the molecule can be considered as “frozen” regarding translational diffusion. The higher fluorescence intensity obtained in the parallel polarization component compared to the perpendicular polarization component, for both solutions; indicate rotational diffusion of the fluorescein. However, the time average $\bar{I}(t)$ (green line) decreases faster for the fluorescein in 0% glycerol solution than for the fluorescein in 80% glycerol solution, since photon count rate is higher for the 0% glycerol solution than for the 80% glycerol solution.
As shorter time bins are used, the averaged polarization for all samples decreases. For time bins of $1 \times 10^{-4}$ sec the mean polarization (Eq. (19)) is clearly high for all glycerol concentrations ($p_{\text{mean}} = 0.35$ while $p_{\text{mean}}$ is the average steady-state polarization of all samples). However, for shorter time bins the averaged polarization significantly decreases ($p_{\text{mean}} = 0.06$ for time bins of $1 \times 10^{-5}$ sec and $p_{\text{mean}} = 0.005$ for time bins of $1 \times 10^{-6}$ sec). On the other hand, as the time averaged variance $\left< \left< I(t)^2 \right> - \left< I(t) \right>^2 \right>$ is analyzed for time bins of $1 \times 10^{-6}$, differences between the various samples become significant (Fig. 7). As shown in Fig. 7(a) for the normalized variance, fluctuations are averaged out faster for the less viscous samples. As the solution becomes more viscous, fluorescein rotates slower and therefore it takes longer for $\left< \left< I(t)^2 \right> - \left< I(t) \right>^2 \right>$ to reach equilibrium. A close look indicates that for the 80% glycerol solution in the range of 15-20 $\mu$sec, deviations of the experimental curve from the theoretical curve are smaller than the simulation curve. However, for the same solution in the range of 20-27 $\mu$sec, deviations of the simulation curve from the theoretical curve are smaller.

Thus the quantitative significance of the time-averaged variance for the more viscous solutions in this short time limit is poor, as fluctuations for the more viscous samples are averaged out much slower than for the less viscous samples. Furthermore, the less viscous samples have lower maximum values at longer times as found in theory (Fig. 2(b)). This maximum value is more distinguished by applying additional time averaging over the variance (Fig. 7(b)). Clearly, for all solutions dead time of the TCSPC system yields slower decrease of the variance as found in theory (Fig. 3) at the cost of lower SNR. Various $\tau_r$ values are then extracted by fitting the experimental results with simulation and theory. Generally, $\tau_r$ is remarkably higher when increasing solution viscosity for the 1 nM fluorescein solution. The 0% glycerol solution is characterized by $\tau_r$ of $0.3 \pm 0.054$ ns, the 20% glycerol solution with $\tau_r$ of $1 \pm 0.22$ ns, the 60% glycerol solution with $\tau_r$ of about $3 \pm 0.285$ ns and the 80% glycerol solution with $\tau_r$ of about $10 \pm 0.318$ ns. These values, derived from the non-imaging single-point measurement, are in good agreement with values derived from other time-resolved bulk imaging measurements, and demonstrate the quantitative accuracy that can be achieved with this method for relatively short integration time [58]. Typically, for higher $\tau_r$ values the SNR is decreased as shown in Fig. 7. By increasing the time bin size and averaging over a larger ensemble of time averaged trajectories the SNR can be improved (data not shown). In the time-resolved anisotropy decay measurements for the 1 $\mu$M fluorescein solution, the 0% glycerol solution was characterized by $\tau_r$ of $0.3 \pm 0.05$ ns, the 20% glycerol solution with $\tau_r$ of $0.7 \pm 0.07$ ns, the 60% glycerol solution with $\tau_r$ of about $2 \pm 0.09$ ns and the 80% glycerol solution with $\tau_r$ of about $10 \pm 0.102$ ns (Fig. 8). This slight sensitivity of $\tau_r$ to the fluorescein concentration is somewhat not surprising and already discussed in literature [40,59,60].

![Fig. 6](image1.png)

Fig. 6. Typical photon count trajectory for fluorescein in 0% glycerol solution (a) and fluorescein in 80% glycerol solution (b), in the parallel polarization (solid blue) and perpendicular polarization (solid red). The non-normalized time average of $I_{\text{measured}}$ for each solution is represented by the solid green line.
4.4 Hydrodynamic radius calculation of fluorescein

For a spherical rotor in an isotropic medium, the rotational diffusion constant $D_r$ is directly proportional to the viscosity of the solvent ($\eta$) and to the hydrodynamic radius ($R$) of the molecule according to Stokes-Einstein-Debye Equation [45]:

$$D_r = \frac{K_BT}{8\pi\eta R}$$

(20)

Where $D_r = 1/6\tau_r$, $K_B$ is the Boltzmann constant and $T$ is the absolute temperature. A plot of the rotational correlation time $\tau_r$ versus the viscosity is shown in Fig. 8. The slope of the solid straight line fits the fluorescein radius of $0.53 \pm 0.05$ nm according to Eq. (20). This result is in agreement with previous reports of bulk measurement [58].
5. Discussion

Variation of the dipole orientation during a finite measurement time can alter the statistical properties of the measured signal in fluorescence fluctuation polarization sensitive experiments [33]. Since fast fluctuations may be obscured during the integration time, the temporal resolution of the system must be short enough to allow tracking them. In most photon counting systems this restriction impedes resolving processes that involve sub microsecond macromolecular dynamics such as rotational diffusion. In this work the time-averaged measured polarization sensitive fluorescence is analyzed. This kind of analysis is conceptually different from that performed in fluorescence correlation polarization sensitive methods in which information is extracted by explicitly analyzing the time course of the fluctuating polarization signal. Therefore, the shortest correlation time that can be probed is determined by how rapidly the polarization signal can be continuously recorded. In this paper, the actual time course of the signal is not analyzed. Rather, the sample is probed by how the mean and variance of the time averaged measured intensity ($\overline{I_\parallel} + \overline{I_\perp}$) vary with different time bins for very short integration times. The time averaged measured signal is extracted from the second moment of the time integrated measured intensity. The efficiency of using time averaged intensities is known from statistical optics. If the process is ergodic and if the measurement time is infinite, then the time average of the process is equal to the corresponding ensemble average. For an ergodic process such as Brownian diffusion we expect the width of the probability distribution of the fluorescence intensity to vanish in the long-time limit, and the distribution to reduce asymptotically to a $\delta$ function centered on the ensemble averaged intensity, i.e. $P(\overline{T}(t), t) \rightarrow \delta(\overline{T}(t) - \langle I(t) \rangle)$. However, in real experiment, the measurement time might be long, but it is always finite. Hence, it is natural to ask what the fluctuations of this process are. Such an analysis sheds light on deviations from the equilibrium average due to finite time measurement. By analyzing these deviations in...
polarization sensitive experiments for short integration times, rotational correlation times of the molecules can be estimated.

In this paper various fluorescein in glycerol/PBS mixtures, having different rotational correlation times ($\tau_r$) are examined. After determination of the time limit in which fluctuations in the fluorescence signal detected in the two orthogonal polarizations are not averaged out, the measured fluorescence signals, $I_\parallel + I_\perp$, is analyzed. It is experimentally validated that for short integration times, the time averaging rate of the measured intensity is rotational diffusion dependent, as found in the theory [33,46]. Rotational correlation times of the various samples are then extracted, by fitting the experimental results to theory and simulation. In the time limit where fluctuations are already averaged out, the time integrated measured intensity linearly increases with the integration time, indicating steady state behavior (Fig. 5(a)). Since the SNR for slower diffusion rates is poor, larger ensembles for larger time bins are needed. In certain cases, other methods that use correlation analysis might be preferred. In this work arbitrary bin widths are considered, a complete theoretical model that describes the effect of time binning on the amplitude of higher moments is not given. Since the suggested method is currently limited to isotropic Brownian diffusion described by single rotational correlation time, it is considered at this stage as a complementary approach to the standard autocorrelation methods. For discrete molecule reorientation process, a generalized model must be developed.

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