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ORIGINAL PAPER

Determining if a System is Heterogeneous: The Analysis of Single Molecule Rotational Correlation Functions and Their Limitations

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Abstract Single molecule spectroscopy can be utilized to measure distributions of individual molecular properties that may be averaged out in the ensemble measurement. For example, complex dynamics in disordered systems can be investigated by observing single molecule rotations via fluorescence spectroscopy. The rotational time of a single transient can be calculated from the correlation function of the reduced linear dichroism signal which fluctuates over time as the molecule reorients in its surroundings. Distributions of rotational time constants can be used to characterize the heterogeneity of molecular environments in the material. This paper reviews some theoretical studies on (1) the high numerical aperture effects on the final correlation function, and how it can be related to optical anisotropy decays in a bulk measurement; (2) the statistical errors resulting from the finite observation length that will propagate into distributions of rotational times. These lead to the discussions on how to interpret correctly the distribution of properties measured from a set of single molecule data, and to determine if in fact the system is heterogeneous.

Keywords Single molecule · Heterogeneous dynamics · Rotation · Polymer dynamics · Glass transition

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Introduction

Molecular interactions and chemical reactions in chemistry texts are generally described on a single molecule basis. However, our knowledge of molecular interactions and chemical dynamics has come almost exclusively from experiments on ensembles of molecules. In order to extract molecular information from ensemble data, most experiments examined homogenous systems such as molecules in the gas phase, single crystals, or neat liquids or solution. In these homogeneous media, all the molecules experience a similar environment enabling the ensemble average to effectively report homogeneous molecular information. In inhomogeneous systems, a variety of spectroscopic techniques have been developed to measure their homogeneous optical properties [1]. In general, these methods focused on extracting homogeneous information rather than characterizing the heterogeneity of the system. In many biological and material systems one would like to directly study these underlying heterogeneous distributions. Such measurements have become possible through the development of single molecule spectroscopy (SMS) which allows for the direct measurement of individual molecular environments [2–4]. SMS depends on repeated measurements of the same molecule over time yielding a trajectory of intensity, emission frequency, polarization, or other properties. The great advantage of SMS is its ability to probe directly distributions of properties that might otherwise not be revealed in a bulk measurement. While not ensemble averaged, the single molecule measurement is inherently time averaged as the data for a single molecule are collected over a period of repeated excitation. Since signals from a single molecule are small, longer measurements will greatly improve the signal-to-noise, but with the loss of information about fluctuations in the system. Short measurements

can provide information on faster time scales, but as the observation time gets shorter the statistical uncertainty grows [5, 6]. This leads to an inherent difficulty with SM measurements, in such that it is possible to make very short observations of a system in which all the molecules are in fact identical, and yet obtain distinct properties for each of the molecules as a result of the large uncertainties associated with the short measurement time. Ideally, besides measuring a distribution of properties, one would also like to examine how this distribution changes with time. Therefore, it is very important to understand how observation time in SM experiments affects the inherent distribution of properties measured. Two important comparisons should be made when characterizing a distribution measured from single molecules. First, the ensemble of single molecules measured individually should yield the bulk average result. This requires a clear understanding of how to directly compare the single molecule and ensemble measurements. Second, the distribution should be compared to the distribution expected to arise merely as a result of the statistics associated with the measurement.

This paper will explore these two criteria in the context of single molecule rotation [7–11]. Single molecule rotations can be tracked by analyzing the polarization of fluorescence signal, and the rotational time scale can be determined by calculating a correlation function for these polarization transients. However, the period of time that a fluorescent dye can be observed in a SM experiment is finite because of photochemical bleaching. Therefore the ability to accurately estimate the rotational correlation function and the resulting rotational time constant is affected by this limited observation time.

Relating single molecule correlation functions to ensemble measurements

SMS has been widely applied to all areas of science interested in studying individual molecular behaviors in complex systems rather than looking at the ensemble average. A very useful property to explore is the rotational dynamics, which is often achieved optically by probing the transition dipole orientations of fluorescent probes embedded in the system. The rotation of the probe molecule can be used to measure the relaxation of the local environment of the probe [7, 8, 10–16]. A critical question in all of these studies is whether the rotational motion is best described as homogeneous or heterogeneous. Is the rotational motion governed by one timescale (homogeneous) or a distribution of time scales (heterogeneous)? If there is a distribution, is that distribution static or dynamic?

In the experimental scheme discussed here, emission from a single fluorescent probe molecule is collected by an objective and split into two orthogonal polarizations on the plane perpendicular to the objective axis [17]. Signals collected on these two directions are designated I_s and I_p, and they are proportional to the projections of the transition dipole moment onto these two polarization directions. One common approach to measure the orientation of the transition dipole is to calculate the reduced linear dichroism:

$$A(t) = \frac{I_s(t) - I_p(t)}{I_s(t) + I_p(t)}. \tag{1}$$

A time constant associated with the transition dipole rotation can be determined by calculating the autocorrelation function of the dichroism signal:

$$C(t) = \frac{\sum_{\tau=0}^{T-t} A(\tau)A(\tau + t)}{\sum_{\tau=0}^{T} A(\tau)A(\tau)}. \tag{2}$$

Drawing an analogy of this correlation function with that measured in an ensemble fluorescence anisotropy experiment, one would expect a single exponential decay from a system in which there was homogeneous rotational diffusion. A multi-exponential decay could imply multiple time scales of rotations experienced by the molecule during its observation. However, to make such a comparison quantitative we need to understand how to relate the measured quantity $A(t)$ to the molecular rotational correlation function. It has been shown that for an isotropic diffusion case, $C(t)$ can be expressed in terms of spherical harmonic functions [18, 19]:

$$C(t) = \sum_l a_l C_l(t), \tag{3}$$

where each $C(t)$ is given by

$$C_l(t) = e^{-l(l+1)Dt}, \tag{4}$$

l labels a Legendre polynomial P_l and its corresponding coefficient, a_l, and D is the rotational diffusion constant. Ensemble anisotropy experiments measure a decay that results from only the l=2 correlation function. For the single molecule case, one must expand the measured dichroism into the spherical harmonics to examine all the terms that will constitute the correlation function. The coefficients a_l can be calculated by projecting the dichroism into spherical harmonic coordinates:

$$a_l = \frac{1}{4\pi} \sum_m \left| \int_0^{2\pi} d\Phi \int_0^{\pi} d\Theta \sin\Theta A(\Theta, \Phi) Y_{l,m}(\Theta, \Phi) \right|^2. \tag{5}$$

In an ideal case where no optics are considered, $A(t)$=cos (2Φ), Φ is the azimuthal angle of the transition dipole.

Therefore, $A(t)$ is simply representative of the projected in-plane orientation of the emission dipole, and has most probable values at ±1. The correlation function for $A(t)$=cos (2Φ) can be computed by expanding $A(t)$ into the spherical harmonics (Eq. 5) and summing the correlation functions for each l weighted by their coefficients (Eqs. 3, 4). Hinze et al. [19] had demonstrated that for $A(t)$ in the limit of zero numerical aperture (NA), terms from l=2 up to l=20 were required. The resulting decay would then be multi-exponential, but can be fit by a stretched exponential function : $f(t) = \exp(-t/\tau_c)^{\beta}$. The resulting fit yielded τ_c=0.87/6D and β=0.87. For homogeneous diffusion the ensemble anisotropy decay would yield a single exponential giving τ_c= 1/6D and β=1. This result suggested an inherent nonexponential factor for an isotropic decay and therefore only decays with β<0.84 could be regarded as indications for intrinsically nonexponential behaviors. It was further suggested that a better signature of heterogeneity would be the differences between correlation functions for different molecules rather than the non-exponentiality of a single molecule decay.

While the results from Hinze hold in the limit of zero NA, single molecule experiments generally require high NA objectives in order to efficiently collect the most photons from the single emitter. The high NA has the effect of altering the polarization of the light that is collected so that the dichroism signal is no longer simply related to the in-plane projection of the dipole [20]. Such effects from high NA optics should be considered for any analysis of single molecule polarizations. The fluorescent signals that are detected on two directions are functions of the dipole orientation angles Φ and Θ, NA, and the index of refraction of the medium, n. Putting together all these parameters would give a more complicated reduced linear dichroism:

$$A(t)_{\mathrm{NA}} = \frac{C\sin^2\Theta\cos 2\Phi}{A + B\sin^2\Theta}, \tag{6}$$

where A, B, C are constants defined by NA and n. When NA=0, Eq. 6 reduces to cos(2Φ). Following the previous analysis, the correlation function of $A(t)_{\mathrm{NA}}$ can be readily calculated by expanding the measured quantity into spherical harmonics using Eq. 5. In the high NA case (e.g. NA=1.2), the coefficients a_l decrease dramatically with l (Fig. 1), and the resulting decay is nearly entirely described by the l=2 component, $C_2(t) \approx \exp(-6Dt)$, which is the same single exponential decay measured in the ensemble anisotropy experiments. When fitting the correlation function that includes the terms from l=2 to l= 20 with a stretched exponential, it yields a decay whose τ_c= 1/6D and β=1. The end result is that the polarization effects cause the measured correlation function to return to an essentially single exponential decay. Another consequence of the polarization is that the distribution of $A(t)_{\mathrm{NA}}$ values is not peaked at ±1 as the $A(t)$=cos(2Φ) would be. Instead the distribution is peaked at 0, and trails off at the extremes. While homogeneous diffusion will yield single exponential correlation functions for the single molecule transients, this does not lead to the conclusion that a nonexponential decay is a proof for heterogeneous environments. Most importantly, full analysis of the polarization allows us to quantitatively compare the single molecule results to the ensemble results. The average of all SM data can be compared to the ensemble average to ensure similar environments are probed in both experiments. To test for heterogeneity would still require a comparison of the results obtained from different molecules. A more extensive statistical analysis is required to determine if the distribution of SM results arises from heterogeneity or is merely due to limited data sampling. The same is true for judging the importance of a non-exponential correlation function. Non-exponential decays could indicate that the dynamics of the system are not simply diffusive or they could arise from poor statistics.

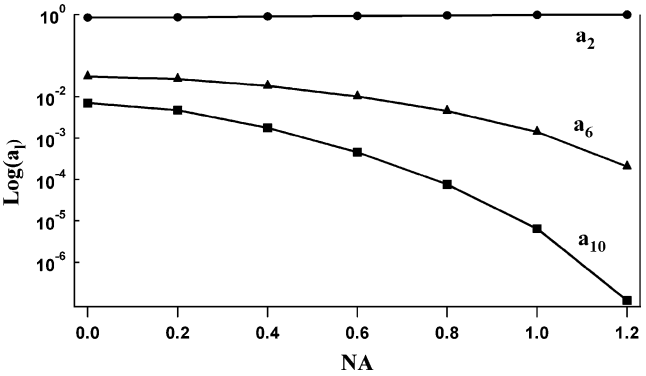

Fig. 1 The effects of high NA on the correlation function coefficients, a_l. Numerical values of a_2, a_6, and a_{10} are shown here for NA=0 to NA=1.2. Note the logarithmic scale. Except for a_2, all higher-order a_l terms decrease rapidly as NA increases

Statistical analysis of single molecule distributions

To better understand the decay of the single molecule correlation functions and the distributions that can arise in single molecule observations, a careful analysis must be made of the statistics of the measurement. Single molecule data exhibit fluctuations which might not necessarily converge to a specific value during a finite observation. In characterizing the system, measurements are conducted to determine a particular quantity, M. This could be any number of quantities such as emission wavelength or orientation. In the case discussed this would be the dichroism, $A(t)$. In the sense of ergodicity [21], the bulk measurement, which corresponds to the limit of spatial

average $\langle M \rangle_s$, is assumed a priori to be the same as the limit of time average $\langle M \rangle_t$. However, in a SM experiment, if we track only a few molecular trajectories during a finite period of time, they might not be able to represent the entire space of possible trajectories. Unless we can show that the data converge to a specific distribution, these trajectories can only be treated as single events, which belong to a larger data set. For the SM rotational experiment, we measure the fluorescence signal from a single molecular probe embedded in the matrix. The signal carries information about the local environment as a function of time. Suppose the environment is isotropic and time invariant, the properties characterized from the observed trajectory will approach the ensemble value if the observation is long enough. However, in the case of anisotropic system, the probe might not be able to sample enough configurations to give a sufficient statistical result. Even worse, if the properties of the system are non-stationary due to time dependent environmental changes, each trajectory must be treated independently.

Commonly, correlation functions are used in analyzing the rotational trajectories as mentioned above. Since the correlation function $C(t)$ calculates the joint probability of two points on the trajectory separated by time lag t, the number of pairs used in the estimation will affect the statistics of this correlation. It can be shown that for a stationary process, the variance of correlation function is inversely proportional to the trajectory length [22]. Thus, even if the system is stationary, the correlation function calculated from a trajectory of finite length exhibits fluctuations. As a consequence, if one characterizes the correlation function by fitting it to a parameterized function, a stretched exponential for example, the variances in the correlation function will propagate to the other parameters. Another way to state this is that if one wants to characterize the time scale for molecular rotation, one needs to observe the molecule long enough for it to reorient. But the question is: how long? The answer depends on both the rate of reorientation and the precision with which one would like to characterize the time constant.

In our previous work [5], we determined the "natural distribution" in τ_F and β_F calculated from isotropic rotational diffusion trajectories of given length T. In each case the distributions of both τ_F and β_F broaden as T becomes short. Not only do the distributions grow but the average values begin to deviate from the true values for the rotational constant and β=1. Given these distributions, it is now possible to directly compare a distribution measured experimentally and determine if it varies significantly from what would be expected based on the statistics and normal diffusion. Figure 2 shows the simulated distributions in τ_F and β_F for given trajectories of length T. Note that the simulation is based on the isotropic rotational diffusion

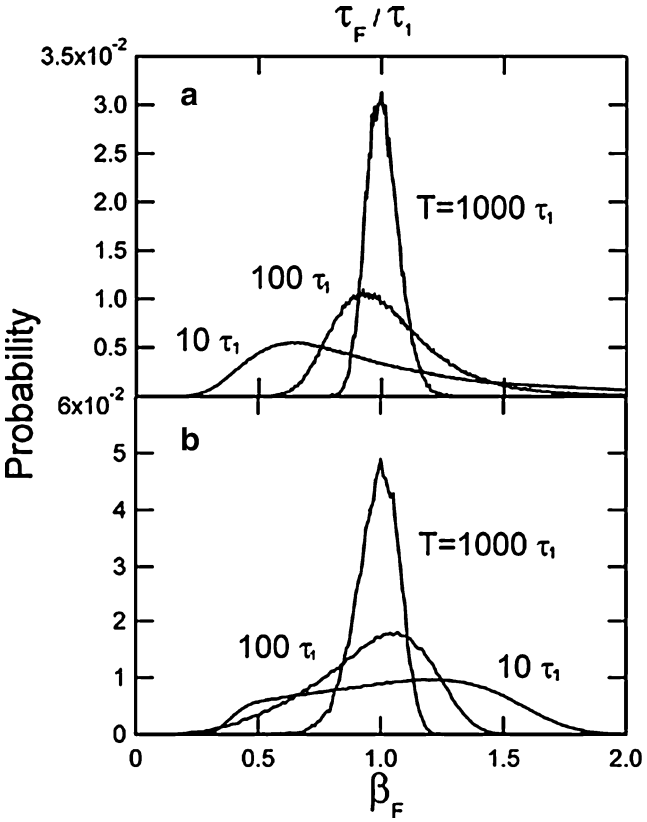

Fig. 2 Distributions of **a** τ_F and **b** β_F with respect to different sample sizes T ($10\tau_1$, $100\tau_1$, and 1,000 τ_1). Each curve is calculated from 1,000 independent trajectories l =1, τ_1=200, length 10^7 broken into pieces of desired length T

with a single diffusion constant. The broadenings in τ_F and β_F are purely due to finite sampling, and theoretically the distributions can be applied to correlation functions of any rank l. For a traditional dichroism measurement cooperated with high NA objective, the correlation function mainly corresponds to second rank spherical component. In the following discussion, we'll see how the methods mentioned above are applied to analyze the single molecule data of rotational motion in a polymer film just above its glass transition. With considerations of high NA and finite sampling effects, we will examine the data to evaluate the ability of single molecule orientational measurements to characterize the polymer heterogeneity.

Examination of experimental SM data

We will now analyze some of our own experimental data of single Rhodamine 6G molecules embedded in poly (cyclohexyl acrylate) at room temperature. The concentration of R6G molecules is as dilute as 1 nM to ensure that they are sparsely spread out in the polymer. First, an image of the spin-cast sample is collected to locate single R6G molecules. Then the target single molecule is moved into the center of the excitation

laser spot for polarization measurement. Fluorescent signals are collected through the same objective (NA=1.25) and split into two orthogonal polarizations for detection. Figure 3 displays the raw signals I_s (black line) and I_p (grey line) recorded at time intervals of 0.2 s. The two signals have some anti-correlation relation at some periods, which makes sense as the transition dipole moment rotates around the axes and will result in a larger intensity on one polarization if it is well aligned on that direction. However, as evident from Fig. 4a, the reduced linear dichroism values do not reach ±1 because the polarizing effects from the high NA objective do not give zero intensity on one polarization at any given dipole orientation. Instead, the most probable value of measured dichroism $A(t)_{\text{exp}}$ is zero when the dipole is aligned nearly parallel to the objective axis, in which case the projections of the emission polarization become almost equal onto the two detection axes. Histogram of dichroism values for this transient is shown in Fig. 4b and it is centered about zero as would be expected when considering the NA. Figure 5 is the autocorrelation of $A(t)_{\text{exp}}$ in Fig. 4a, and the dashed line is the fit to a stretched exponential function. Interestingly, fitting results vary slightly depending on the constraints on the fitting parameters. When amplitude is held at 1 and $0<\beta<1$, the fitted result gives β_F=1.00, τ_F=22.187 s. Statistically speaking, there is no need to constrain β within 0 and 1, although it is a common practice when fitting to the stretched exponential as the values greater than one are non-physical (but could occur as a result of the statistical fluctuations). Rather than fitting all the points in the decay, the fit can be restricted to a certain time lag q, beyond which the standard errors will be greater than the estimated autocorrelation [6]. For the decay in Fig. 5 if we restrict the fitting range to lag q (58 s for this autocorrelation function), hold amplitude at 1, and let β fit freely, the result is β_F=0.906, τ_F= 25.139 s. In addition to fitting range, the relative length of the transient to the rotation time is important. For the entire set of 58 single molecule data

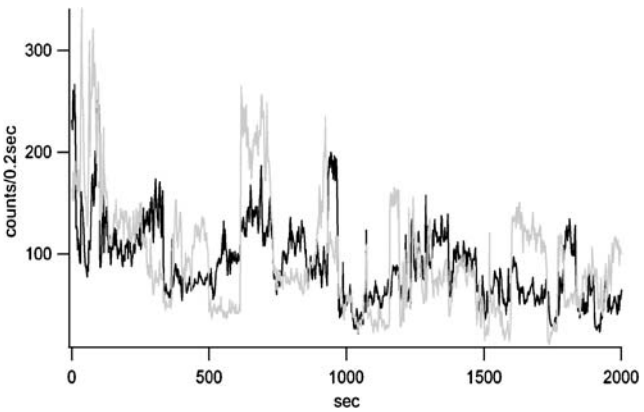

Fig. 3 Fluorescent signals of a single R6G molecule embedded in poly(cyclohexyl arylate) at room temperature. I_s (*black*) and I_p (*grey*) denote two orthogonal fluorescent polarizations being measured. Data recorded at 0.2-s intervals

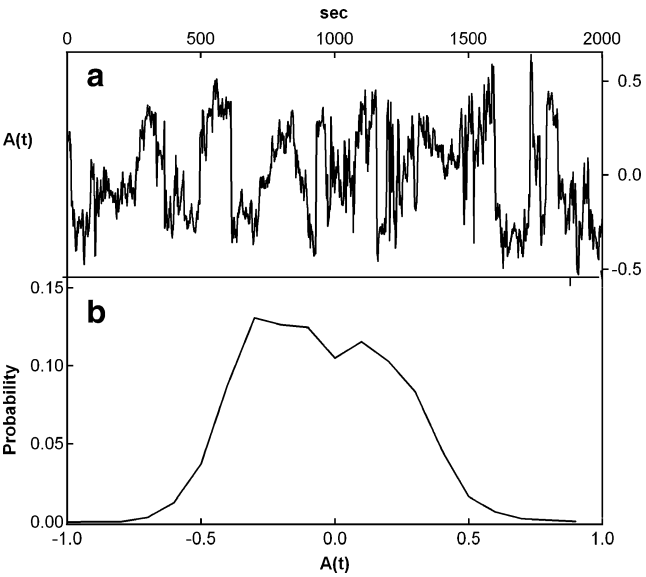

Fig. 4 **a** is the reduced linear dichroism $A(t)_{\text{exp}}$ calculated from I_s and I_p in Fig. 3. Note that the values of dichroism do not reach ±1 and as shown in **b**, the distribution of $A(t)_{\text{exp}}$ is centered around 0 and tails off at both ends

collected, the average correlation time τ_{avg}=130.791 s. This means that all the transient lengths are on the order of 1–60 times the average rotational constant τ_{avg}. For the transient in Fig. 3, the length of observation is 2,000 s, which is approximately 15 times of τ_{avg}. The average ratio T/τ_{avg} of all transient molecules is 14.5. While this observation time might seem to be sufficient, it is short enough that the measured τ_{avg} could deviate significantly from the true value of the rotational constant.

Figure 6 shows the probability distributions of τ_F (Fig. 6a) and β_F (Fig. 6b) for 58 single molecule transients, to which the stretched exponential function is fit freely with no constraints. Note that τ_F is normalized to τ_{avg}. The distribution appears to be quite broad with the longest times more than an order of magnitude longer than the shortest. The stretching exponents also show a broad distribution ranging from single exponential decays to highly non-exponential correlation functions. However, even if the single molecule measurements yield a distribution of values, we should not simply jump to the conclusion that the molecules have different rotational times because they are experiencing a distribution of environments. To better understand the origin of the distributions we simulated trajectories based upon isotropic diffusion for a homogeneous system. The simulation has only one rotational correlation time, τ_l, and therefore the true correlation function should be a perfect single exponential for an infinitely long trajectory. When broken into pieces of finite transients we can see how short trajectory lengths affect the estimation of the correlation function and how these errors are propagated into distributions of τ_F and β_F (Fig. 2).

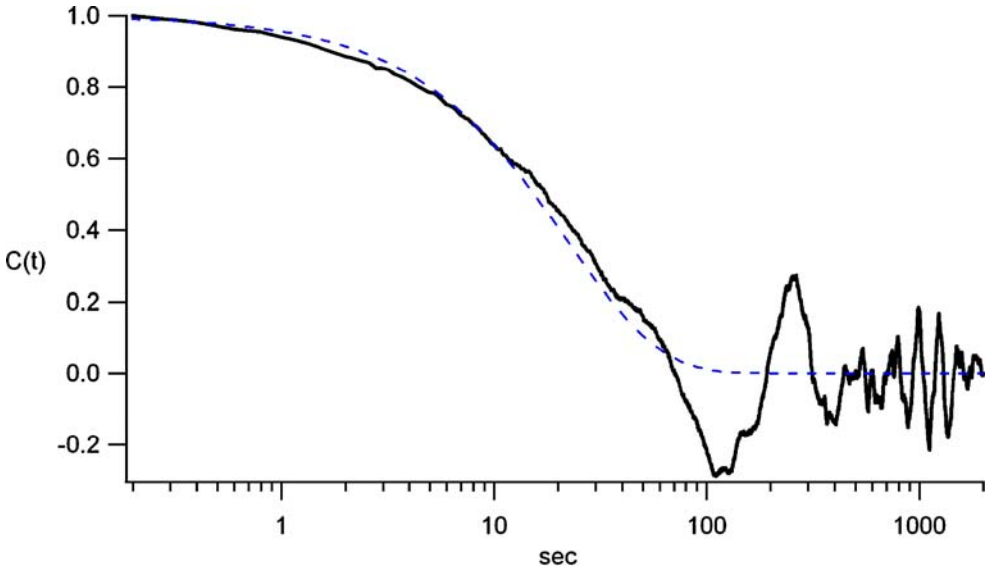

Fig. 5 Correlation function of the linear dichroism in Fig. 4a fit to a stretched exponential function (*dashed line*)

Comparing the experimental data to the simulation, there is a clear resemblance between these distributions. The distribution of τ_F (Fig. 6a) is peaked at a shorter time, giving possibly an underestimation of the true rotational time as the result of insufficient transient lengths. Since the average T/τ_{avg} ratio for this data set is 14.5, we will use the simulated $T=15\tau_l$ transients for comparison [5]. It is a challenge to try to compare these distributions statistically because they are not Gaussian distributions, but we can make a rough comparison by performing the chi-square test. The chi-square test is used to see if the standard deviation of experimental data is equal to a specific value,

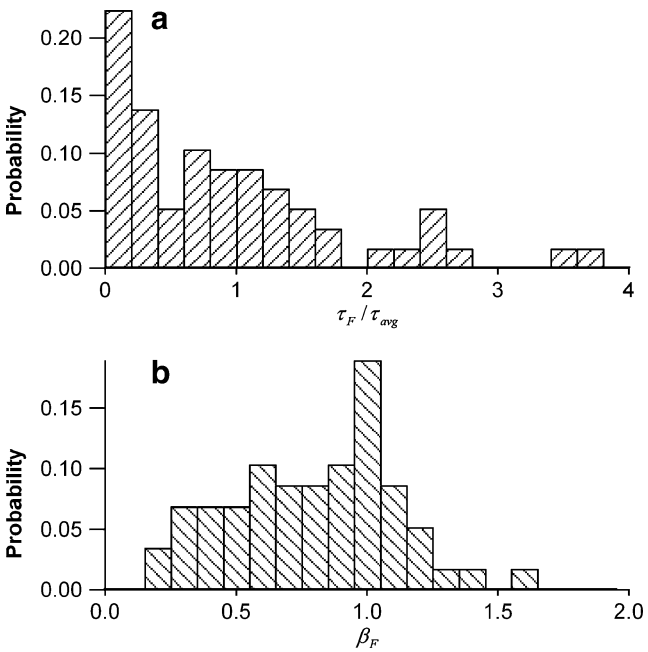

Fig. 6 Distributions of **a** τ_F, normalized to τ_{avg}; and **b** β_F, measured from 58 single molecule transients. Average value for τ_F is 130.791 s, β_F is 0.846

in this case it would be the standard deviation of the simulated transients (length=$15\tau_l$) [23]. If the test value is greater than the upper critical value χ^2_α or smaller than the lower critical value $\chi^2_{1-\alpha}$ at significance level α, we can reject the null hypothesis that the standard deviation of the experiment is equal to the simulation. If the test value falls inside the upper and lower critical values, then the two standard deviations are not significantly different from each other. The standard deviation $S(\tau_F)_{exp}$ for experimental τ_F is 0.954, and for the simulated data $S(\tau_F)_{sim}=0.813$. The calculated one-way chi-square test statistic value is: $P(\tau_F) = (N-1)\left(\frac{S(\tau_F)_{exp}}{S(\tau_F)_{sim}}\right)^2 = 78.552$, which is in between the upper critical value $\chi^2_{0.05}$= 75.624 at 5% significance level and $\chi^2_{0.01}$= 84.733 at 1% significance level. Because this chi-square test value is at the upper cut-off region between two significance levels, we can not explicitly accept or reject the null hypothesis. Therefore we are hesitant to conclude that the SM experiment results demonstrate that the system is heterogeneous since the distribution of rotational constant is very similar to the inherent distribution expected from the statistics. It can possibly be a pure diffusion system but due to other factors such as background signals that the measurement results are not truthful of the real SM properties. Same comparison can be made for the histogram of β_F (Fig. 6b), which shows a wide distribution of values ranging from 0 to 1.6, and has an average of 0.846, standard deviation of 0.317. Simulation yields a distribution of β_F that is sharply peaked at 1 for long trajectories ($T=1{,}000\tau_l$) but broadened for short trajectories ($T=10\tau_l$; Fig. 2b). The standard deviation of β_F for $T=15\tau_l$ transients is 0.339. Performing a chi-square test on β_F for the experimental and simulated data, we obtain $P(\beta_F)$=49.689, which falls inside the lower critical value of $\chi^2_{0.90}$=43.816 at 10% significance level. Therefore the distribution of experimental β_F is likely to be the same as

the simulation. It's worth mentioning that the simulated $T=15\tau_l$ transients have average $\beta_F=1.012$, but the experiment yields average $\beta_F=0.846$, which is not expected for a pure diffusion. These comparisons imply that extra consideration is required to check if the experimental distributions are significantly greater than the inherent statistical widths when analyzing correlation functions, and that we can not simply regard a distribution of rotational constants τ_F and β_F from a set of single transients as evidence of dynamical heterogeneity because insufficient transient lengths will bias the fitting results from the true value. Although we used a chi-square test here, it is not an ideal method, because the distributions of τ_F and β_F from both the experiment and simulation are not normal distributions, and we did not truncate each single transient to the same length as in the simulated data. Further exploration of non-single diffusion models should be able to provide more insights for determining system heterogeneity.

Unfortunately, one of the biggest challenges in SMS is the observable "lifetime" of probe molecules, which are often shortened by irreversible photochemical reactions (photobleaching). To obtain a better estimation of the true rotation time one can extend the transient length, for example, by using very low probing power [24], reducing exposure time, removing triplet quencher such as oxygen, etc. [25]. Experiments that measure self-reproducible properties, such as the turnover events in single molecule enzymology [26], can record extremely long transients that provide excellent estimation of the true correlation functions.

Conclusions

We showed in this paper how to analyze SMS correlation function data to compare to the ensemble measurements, and to realize there are inherent statistical errors in SM experiments. In the first part of discussion, it was demonstrated that by taking into account the effects of high NA objective, autocorrelation function of the measured dichroism signal from a single fluorophore undergoing pure rotation still manifests a single exponential decay, which suggests that a nonexponential decay can be interpreted as an indication for complex dynamics. This is, however, valid only for an infinite transient molecule. In real experiments, one has to evaluate carefully the quality of the observed single molecule data that is affected by several factors such as trajectory length and signal-to-noise ratio (which we did not discuss here). Even a pure rotation could yield a distribution of values as a result of statistical errors. Comparison of SMS average value to bulk average is also crucial in ensuring that the same properties are being measured in both cases. From the SM experimental results for probe rotation in the acrylate polymer that are discussed, it is not clear if the broad distribution in both time constants and stretching exponents are the result of heterogeneity or statistics. However, the similarity in the standard deviations of the experimental distributions and those from the diffusion simulation suggest that any heterogeneity would be significantly smaller than the measured distributions. A more conclusive comparison could be made with longer trajectories, more molecules, and a decisive statistical test. It should be noted that the length of trajectories in these studies is as long or longer than any single molecule rotational trajectory present in the literature. Finally, while the analysis presented here is for rotational dynamics, general conclusion should apply to all other SM measurements. One should not jump to the conclusion of heterogeneity based upon observing a distribution of individually measured properties. The distribution should be compared to the natural width from statistical fluctuations in order to determine if the underlying dynamics are homogeneous or heterogeneous.

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