

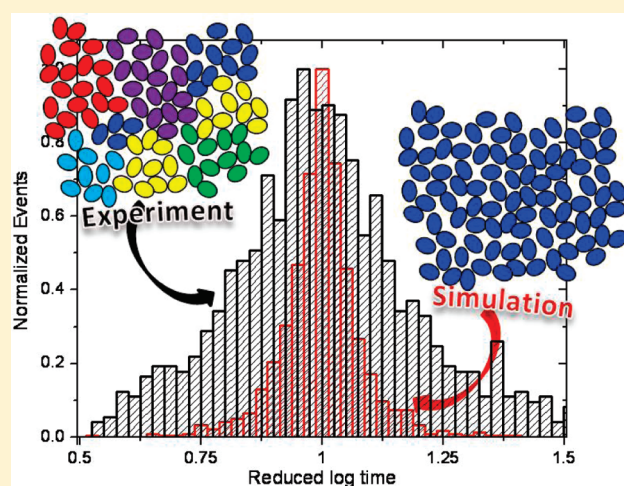
When the Heterogeneous Appears Homogeneous: Discrepant Measures of Heterogeneity in Single-Molecule Observables

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ABSTRACT: Supercooled liquids demonstrate stretched exponential relaxations consistent with the presence of spatially heterogeneous dynamics. Many experimental results are consistent with this picture, but differences in experimental approach may lead to different conclusions about the degree of heterogeneity in a given system. Here we investigate whether observables accessible with single-molecule (SM) approaches are consistent with each other and with ensemble measurements. In particular, the distribution of rotational relaxation times, τ_c , obtained from SM measurements is compared with the stretching exponent determined from a quasi-ensemble treatment of the same data. It is shown that the time-limited trajectories typical of SM experiments can lead to a stretching exponent that suggests homogeneous dynamics even in the presence of heterogeneous dynamics. After correction for the time-limited trajectories, additional discrepancy remains between stretching exponents measured via SM experiments and ensemble techniques. The remaining difference is attributed to the limited dynamic range of the SM experiments.

SECTION: Macromolecules, Soft Matter



Supercooled liquids and glasses have provoked substantial interest, in large measure because of the interesting dynamics these systems display. On the basis of experiments, simulations, and theoretical work, a picture of the supercooled liquid as a mosaic of local and interchanging dynamic environments has emerged.¹ Such heterogeneous dynamics are not limited to molecular supercooled liquids but indeed are found in a variety of crowded systems.^{2–4} One of the key findings of ensemble experiments on supercooled liquids that supports the view of the system as a mosaic of local environments is the presence of stretched exponential relaxations. In particular, autocorrelation functions (ACFs) of various observables have been found to be well fit by the form $C(t) = Ae^{-(t/\tau_{\text{fit}})^\beta}$ with $0 \leq \beta \leq 1$ and τ_{fit} related to a mean relaxation time, $\tau_c = (\tau_{\text{fit}}/\beta)\Gamma(1/\beta)$. In such experiments, the deviation of β from 1 is typically associated with the degree of heterogeneity in the system.¹ The stretched exponential relaxations in supercooled liquids are most commonly attributed to spatial and temporal averaging over a distribution of single exponential relaxations.

The existence of heterogeneous dynamics in supercooled liquids encourages the application of subensemble approaches to their study. In particular, in recent years, single-molecule (SM) fluorescence microscopy following the rotational relaxation of fluorescent probes in supercooled liquids has been used to establish the presence of dynamic heterogeneity in these systems as well as to investigate the length scales over which regions of particular

dynamics exist and the time scales over which they persist.^{5–11} Whereas SM approaches yield information concealed in ensemble experiments, when sufficient data on sufficient single molecules are collected, these experiments are also expected to return results obtained in bulk experiments. However, for a variety of reasons, this is not always found to be the case.^{12–14} Discrepancies may emerge due to the fact that SM fluorescence microscopy generally yields much shorter trajectories relative to the time scales of interest as a result of single-molecule photobleaching events than do ensemble experiments. Indeed, it has previously been shown that time-limited SM trajectories can suggest the presence of heterogeneity even in the presence of homogeneous dynamics.¹³ In this simulation-based study motivated by recent experimental results,⁷ we consider the inverse phenomenon, that time-limited trajectories may cause systems displaying heterogeneous dynamics to appear homogeneous in some SM evaluations.

SM experiments in supercooled liquids are often carried out such that they are analogues of bulk fluorescence depolarization measurements. Fluorescence from probe molecules is used to construct SM linear dichroism (LD) trajectories, and the ACF of each LD trajectory is fit to either single or stretched exponential decays. Whereas the magnitude of the deviation of β from 1.0 of

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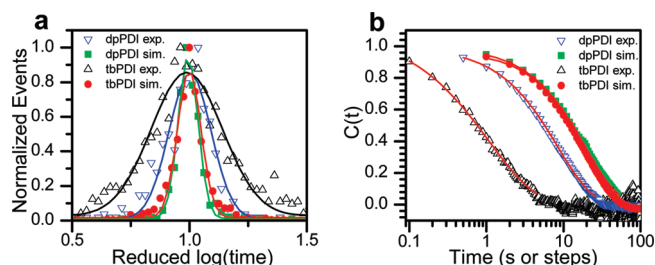


Figure 1. (a) τ_c distributions obtained from experiments (open symbols) and simulations (filled symbols). Experimental τ_c distributions are obtained from stretched exponential fits of 438 dpPDI (blue open inverted triangles) and 1071 tbPDI (black open triangles) molecules, respectively. Distributions are fit to the best fit Gaussian function shown as a solid line. Median τ_c values are 10.7 and 3.0 s, and full width at half-maximum (fwhm) are 0.42 and 0.71 for dpPDI and tbPDI, respectively. The distributions are shifted to peak at the same position for straightforward comparison of widths. Simulations of homogeneous rotational diffusion with median τ_c set to 20 steps are performed, and the trajectories are truncated to match the length of the dpPDI (green filled squares) and tbPDI (red filled circles) trajectories relative to the extracted experimental τ_c values. fwhm values are 0.20 and 0.23 for the simulations matching the dpPDI and tbPDI trajectory lengths, respectively. (b) Quasi-ensemble ACFs and fits to stretched exponential decays for experimental dpPDI (blue open inverted triangles) and tbPDI (black open triangles) data. Representative ACF_{QE}s from a given movie are shown; particular β_{QE} values for the data shown are 0.95 and 0.71, and mean β_{QE} values over the 3 dpPDI and 11 tbPDI movies collected are 0.95 and 0.78, respectively. Quasi-ensemble ACFs from simulations with trajectory lengths set by to match those in dpPDI (green filled squares) and tbPDI (red filled circles) experiments are also shown and $\beta_{QE} = 1.00$ and 1.12, respectively.

an ACF measured in an ensemble experiment is generally interpreted as correlating with the degree of heterogeneity in the system, for SM LD ACFs, this is not generally assumed.^{1,6,13} This is because typical SM trajectories are 10–200 τ_c , short enough such that trajectories associated with homogeneous dynamics may be best fit by stretched exponential decays with β values significantly different than 1.0.¹³ Whereas the β values from SM LD ACFs thus do not give access to particular molecules' extent of heterogeneous dynamics, fits of many SM LD ACFs do suggest the breadth of heterogeneity of the system via the extracted τ_c distribution. Whereas individual τ_c values are also affected by the time-limited nature of the trajectories, for typical trajectory lengths, deviations of extracted τ_c values from actual τ_c values are rather small and symmetric.^{7,13} Such deviations are therefore not expected to significantly affect either median τ_c or breadth of measured τ_c values.

In recent experiments, probes were embedded in supercooled glycerol near its glass-transition temperature (190 K), and wide ranges of rotational relaxation times were found.^{5–7} Figure 1a shows the distribution of τ_c values measured at 206 K for two different perylene dicarboximide probes, dpPDI ($M = 598.65$ g/mol) and tbPDI ($M = 799.96$ g/mol). In contrast with initial expectation based on the molecular mass of these two probes, dpPDI was found to exhibit slower rotational relaxation than tbPDI, presumably as a result of hydrogen bonding present between dpPDI and glycerol that is sterically hindered between tbPDI and glycerol.⁷ The distributions of both probes' rotational relaxation times are approximately log-normal, and the $\log(\tau_c)$ distributions are well-fit by Gaussian functions. Whereas the distributions appear broad, some spread may be attributable to the time-limited nature of the trajectories. To investigate this, we

simulated particles exhibiting homogeneous rotational diffusion with a given τ_c . If such trajectories were infinitely long, the distribution would be a Dirac-delta function at τ_c . Actual trajectory length distribution in the experiments can be approximated by taking each single molecule trajectory length and dividing it by the extracted τ_c for that molecule. Upon establishing this distribution of trajectory lengths, the simulated LD trajectories are truncated consistent with the experimental distribution. ACFs are calculated from those LD trajectories and are fit to stretched exponential decays from which τ_c values are extracted. The two simulated τ_c distributions are clearly narrower than the experimentally measured distributions (Figure 1a), providing evidence that the distributions measured in the experiments are inconsistent with those expected for homogeneous rotational diffusion even given the time-limited nature of the measured SM trajectories. Discrepancy between the measured and simulated distributions is apparent for both probes but is especially striking for tbPDI, where the relatively fast probe rotation may allow the probe to report a more substantial proportion of the heterogeneous dynamics in supercooled glycerol than does the slower dpPDI probe.⁷

To attempt to connect the SM results to those attained in ensemble experiments, the SM ACFs can be averaged to obtain a quasi-ensemble ACF (ACF_{QE}). The ACF_{QE} is then fit to a stretched exponential function. The best-fit stretched exponentials associated with the four distributions in Figure 1a are shown in Figure 1b. The quasi-ensemble stretching exponents (β_{QE}) for the two distributions associated with homogeneous rotational diffusion are 1.00 and 1.12 for dpPDI- and tbPDI-based simulations, respectively. This result is not necessarily expected given the spread in τ_c that emerges from the time-limited trajectories, but it does indicate that in the presence of homogeneous dynamics the β_{QE} value will not erroneously suggest heterogeneous dynamics in the presence of SM trajectories of typical length. The average β_{QE} values obtained for the experimental dpPDI and tbPDI τ_c distributions data are 0.95 and 0.78, respectively. Whereas these values are certainly not equal to 1.0, the value expected for homogeneous rotational diffusion, they are substantially higher than those measured from a variety of ensemble experiments on supercooled glycerol, none of which employ large probes as do the SM experiments. These ensemble experiments generally yield β values of 0.4 to 0.6. (See ref 15 for a summary.) While the presence of the probe is a possible source of discrepancy, another possibility is that time-limited trajectories suppress deviations of β_{QE} from 1.0. Therefore, whereas previous studies have assessed the influence of limited trajectory length on the appearance of homogeneous dynamics, here we assess its influence on heterogeneous dynamics modeled as a distribution of single exponential decays.

Because experimentally we and others have found that single molecule rotational relaxation times of probes in supercooled glycerol are log-normally distributed,^{5,7} we model the heterogeneous system as such and vary the full width half-maximum (fwhm) of the $\log(\tau_c)$ distribution from 0.0 (homogeneous dynamics) to 1.2, with the median τ_c ($\tau_{c,med}$) set to 100 steps for each distribution of 1000 particles. In the simulations, each trajectory itself is homogeneous, including no changes of dynamics (temporal heterogeneity). Whereas there remains disagreement about whether single molecule experiments in supercooled glycerol show evidence of such temporal heterogeneity,^{5–7} here we focus only on systems displaying spatial heterogeneity, in part because temporal heterogeneity is very difficult to model. For the

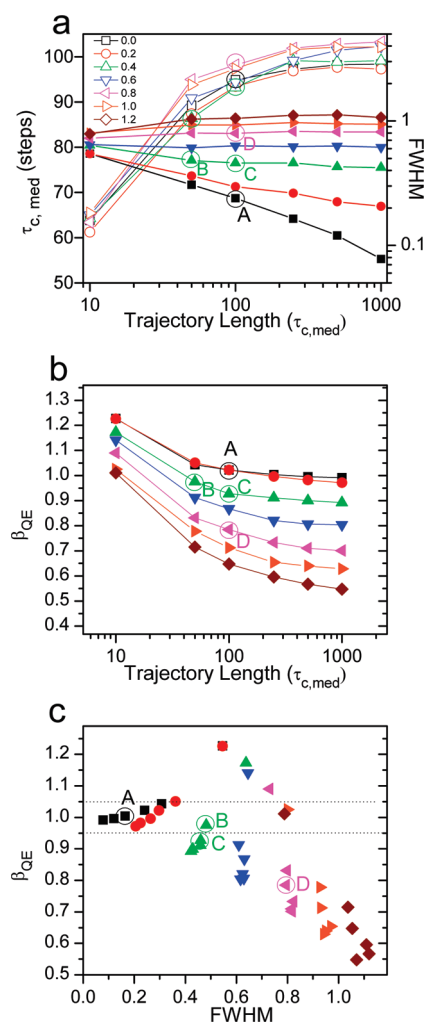


Figure 2. (a) Median τ_c (left, open symbols) and fwhm (right, closed symbols) values from Gaussian fits to the obtained τ_c distributions as a function of trajectory length. Data are shown for homogeneous rotational diffusion (fwhm = 0.0, black squares) as well as distributions of particles undergoing homogeneous diffusion with $fwhm_{input}$ of 0.2 (red circles), 0.4 (green triangles), 0.6 (blue inverted triangles), 0.8 (pink left facing triangles), 1.0 (orange right facing triangles), and 1.2 (maroon diamonds). Points A–D are described in the text. (b) β_{QE} of ACF_{QE} s constructed from the data associated with panel a versus trajectory length. Symbols represent the same $fwhm_{input}$ values as in panel a. (c) β_{QE} values as a function of measured fwhm. Lines are drawn at 0.95 and 1.05 as guides to the eye. Symbols represent the same $fwhm_{input}$ values as in panel a.

input distributions, fwhm is determined as 2.35σ , with σ the standard deviation of the $\log(\tau_c)$ distribution, and we term this quantity $fwhm_{input}$. Instead of employing a distribution of trajectory lengths, as for Figure 1, here all trajectories are truncated to a given length between 10 and $1000 \tau_{c,med}$ and are fit to obtain output τ_c values. We note that whereas our SM experiments are collected on average for $100 \tau_{c,med}$, other reports use trajectories as short as $10 \tau_{c,med}$.^{7,11} The effect of time-limited trajectories on the obtained fwhm and median τ_c of a distribution of rotational relaxation times is assessed by fitting the obtained $\log(\tau_c)$ distribution to a Gaussian (Figure 2a). Here, as expected, with increasing trajectory length, $\tau_{c,med}$ of the distribution approaches the true value of 100 steps, and the fwhm approaches $fwhm_{input}$. In the case of homogeneous dynamics ($fwhm_{input} = 0.0$), the

obtained fwhm is 0.55 for the shortest trajectory length of $10 \tau_{c,med}$ and decreases quickly with increasing trajectory length. Similarly, it is shown that the median τ_c is underestimated for short trajectories. These results are consistent with previous studies on the effects of time-limited trajectories on homogeneous dynamics.^{6,13} For heterogeneous systems with $fwhm_{input}$ of <0.6 , the measured fwhm of the shortest trajectory measurements is overestimated, as in the homogeneous case. However, the degree of overestimation even at trajectories of just $10 \tau_{c,med}$ decreases quickly with increasing $fwhm_{input}$. Additionally, for $fwhm_{input}$ of ≥ 0.6 , there is relatively little change in the obtained fwhm with changing trajectory length over the range investigated. For $fwhm_{input}$ of 1.0 and 1.2, the measured fwhm is somewhat underestimated at all trajectory lengths; this occurs because for broad distributions, 1000 particles do not reproduce a smooth distribution, leading to a fit that is somewhat too narrow. We note, however, that the fwhm of the obtained distribution calculated as 2.35σ does return 1.0 and 1.2 for $1000 \tau_{c,med}$ trajectories, indicating that the distributions returned for long trajectory lengths do approximate the input distributions. We report the fwhm from fit in Figure 2 rather than as $fwhm = 2.35\sigma$, by analogy to our past and current treatment of experimental results.

Calculating and fitting the ACF_{QE} s from the distributions described in Figure 2a gives β_{QE} values shown in Figure 2b. For all distributions studied, β_{QE} decreases with increasing trajectory length, reaching the expected value of 1.0 for a homogeneous system and a value of ~ 0.58 for the most heterogeneous system studied here at trajectory lengths of $1000 \tau_{c,med}$. We note that β_{QE} values in the vicinity of 1.0 ($1.05 \geq \beta \geq 0.95$) are found for a variety of distributions (Figure 2c). For example, a homogeneous distribution ($fwhm_{input} = 0.0$) that is broadened to display a fwhm of 0.24 by time-limited trajectories of $100 \tau_{c,med}$ (point A) and a heterogeneous distribution with a $fwhm_{input}$ of 0.4 broadened to 0.48 by time-limited trajectories of $50 \tau_{c,med}$ (point B) have β_{QE} values in this range, differing by only 0.04. Indeed, Figure 2 shows that even for a log-normal distribution with $fwhm_{input}$ up to 1.2, a fit of the ACF_{QE} can suggest homogeneous dynamics by returning a β_{QE} value near 1.0. This behavior emerges in part because obtained β_{QE} values depend more strongly on details of the individual ACFs than does the obtained fwhm. Figure 3 shows a distribution of extracted τ_c values for a given distribution with $fwhm_{input} = 0.6$ for trajectory lengths of $100 \tau_{c,med}$ and $500 \tau_{c,med}$. As expected, the τ_c values for particular molecules are not identical for the two distributions. However, both distributions are well-fit by a Gaussian on a log scale and return identical fwhm values. Despite this, the β_{QE} value is different for the two distributions, with $\beta_{QE} = 0.87$ and 0.81 for trajectory lengths of $100 \tau_{c,med}$ and $500 \tau_{c,med}$, respectively. The experimentally measured τ_c distributions for dpPDI and tbPDI shown in Figure 1 (fwhm of 0.42 and 0.71, respectively) and the median trajectory length in these experiments (116 and $91 \tau_{c,med}$, respectively) are most closely represented by points C and D in Figure 2. The β_{QE} values measured (0.95 and 0.78, Figure 1b) are nearly identical to those that would be expected for an input fwhm distribution of single-exponential decays of that magnitude ($\beta_{QE} = 0.93$ for point C and 0.78 for point D). Extrapolating to the $1000 \tau_{c,med}$ result, we then could expect that in the long-time trajectory limit the β_{QE} values would fall to 0.89 and 0.70, respectively, for dpPDI and tbPDI measurements.

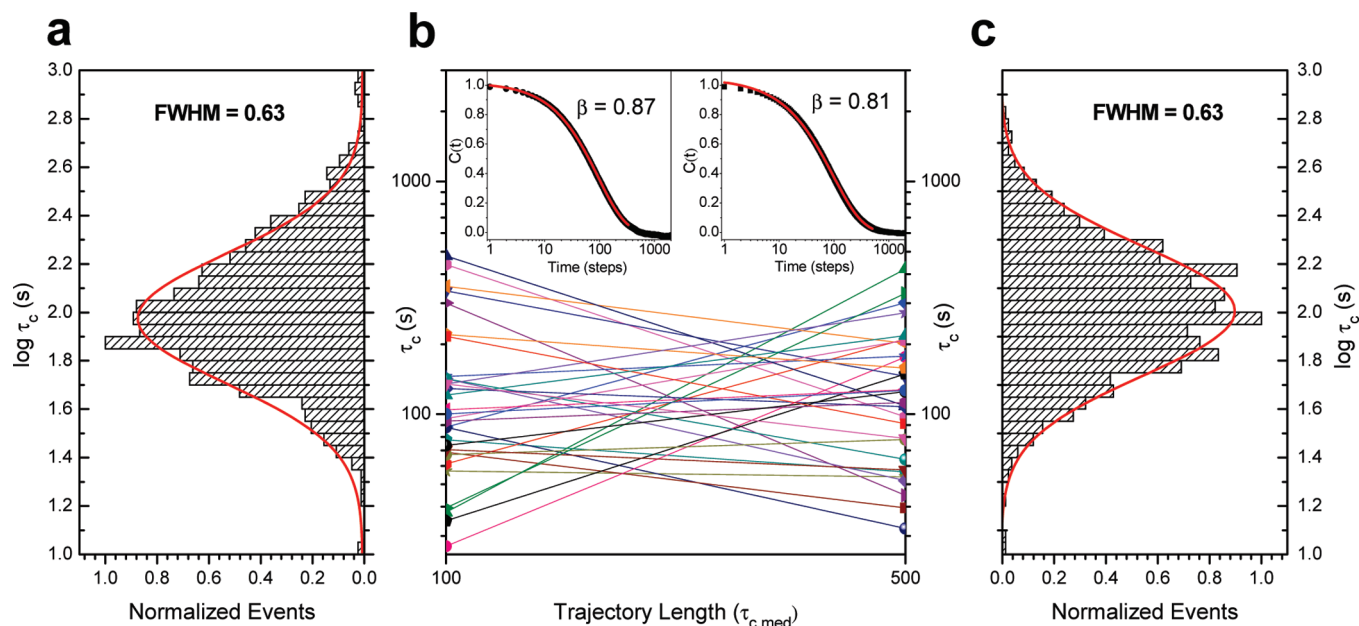


Figure 3. (a) τ_c distribution for 100 $\tau_{c,med}$ trajectory length and $fwhm_{input} = 0.60$. Obtained $fwhm$ is 0.63. (b) Obtained τ_c for 30 (of 1000) randomly selected particles with 100 $\tau_{c,med}$ (left axis) and 500 $\tau_{c,med}$ (right axis) trajectory length and $fwhm_{input} = 0.60$. Insets show that the ACF_{QES} return different β_{QE} values of 0.87 and 0.81, respectively, in accordance with Figure 2. (c) τ_c distribution for 500 $\tau_{c,med}$ trajectory length and $fwhm_{input} = 0.60$. As in panel a, obtained $fwhm$ is 0.63.

We have shown that time-limited trajectories alone may give rise to β_{QE} values that are larger than they would be in the limit of infinitely long trajectories and may imply dynamical homogeneity even in the presence of heterogeneity. We also note, however, that the long time trajectory length limit results return β_{QE} values that are greater than those measured for glycerol via ensemble experiments. The remaining discrepancy between the SM β_{QE} values and the ensemble measured β values may be related to the limited dynamic range of the SM studies. First, we note that analytically a stretched exponential is associated with a log-normal distribution of relaxation times only in the limit of small β (~ 0.25 , smaller than that measured in either ensemble or SM experiments in supercooled glycerol).^{16–18} For larger β values, the distribution peaks at longer times relative to τ_c but has a tail that develops toward short times. Whereas the log-normal distribution is not the analytical solution for the Laplace transform of the stretched exponential, in practice, log-normal distributions of single exponential decays do return forms that are very well fit by stretched exponentials. To investigate whether distributions of similar breadth but bearing short-time rather than long-time tails yield similar β_{QE} values, a short-time bearing tail distribution mirroring the log-normal distribution of $fwhm_{input} = 1.0$ was prepared and investigated for trajectory length of 1000 $\tau_{c,med}$. The output distribution has an $fwhm = 0.11$, with this quantity measured directly as the width of the obtained distribution at half-maximum, and $\beta_{QE} = 0.98$. Comparison to Figure 2 shows that this β_{QE} is very similar to that which would be expected from a log-normal distribution of this width. Thus, β_{QE} appears to depend more strongly on the overall width of the distribution than on its particular shape. As such, the remaining discrepancy between extrapolated long-time limit β_{QE} values and β values measured in ensemble experiments cannot immediately be attributed to loss of either short time or long time relaxations. Straightforward experiments increasing and decreasing frame rate do not show evidence of additional relaxations outside the

range picked up in experiments where frame rate is tuned to match expected $\tau_{c,med}$ (unpublished data). As such, the likely constriction of dynamic range in SM experiments is due to the presence of the relatively large and slow probes that average over heterogeneous environments in space, time, or both.

In summary, we have shown that β_{QE} values obtained from SM measurements may be artificially increased in the presence of typical length SM trajectories, allowing for dynamics that are heterogeneous to appear homogeneous by this measure even in the presence of a τ_c distribution inconsistent with homogeneous rotational diffusion. Whereas this study has been directed at understanding spatially heterogeneous dynamics in supercooled liquids, this finding applies to any time-limited SM trajectories from which relaxation times are extracted. With respect to supercooled glycerol, we additionally show that for measured log-normal SM distributions of rotational relaxation times, the expected long-time limit β_{QE} is still smaller than that measured in many ensemble experiments. One possible explanation for the remaining discrepancy is the limited dynamic range of the SM experiments, most likely due to probe averaging over small and fast regions in the system.

EXPERIMENTAL SECTION

The probes *N,N'*-bis(2,6-dimethylphenyl)-3,4,9,10-perylene dicarboximide (dpPDI, $M = 598.65$ g/mol) and *N,N'*-bis(2,5-*tert*-butylphenyl)-3,4,9,10-perylene dicarboximide (tbPDI, $M = 799.96$ g/mol) were added to spectrophotometric grade glycerol at ~ 1 nM. The PDI/glycerol solutions were spin-coated onto a silicon wafer to produce a film of several hundred nanometers thickness. The sample was placed in a microscopy cryostat and cooled at ~ 5 K/min to 206 K. A standard wide-field epifluorescence microscope with an air objective ($NA = 0.75$) was employed. For such a configuration, the LD ACF is dominated by the second spherical harmonic and will therefore yield a single exponential decay for isotropic rotational diffusion.^{6,12} A Wollaston

prism in the detection arm was used to collect fluorescence in two orthogonal polarizations from the probes. We recorded 14-bit movies onto an EMCCD camera with frame rate adjusted to ~ 20 frames per $\tau_{c,med}$. More details on sample preparation, microscope configuration, and data collection parameters are reported in ref 7. Background-subtracted fluorescence intensities, $I_{||}$ and $I_{\perp}$, were recorded for each molecule in each frame of the movie. LD was then constructed as $LD = (I_{||} - I_{\perp}) / (I_{||} + I_{\perp})$. The quantity $a(t) = LD(t) - \langle LD(t) \rangle$ was used to construct the ACF: $C(t) = (\sum_t a(t')a(t'+t)) / (\sum_t a(t')a(t'))$, and the ACFs were fit to stretched exponential functions, $C(t) = Ae^{-(t/\tau_{fit})^\beta}$, using least-squares fitting. The functions were fit until they decayed to 0.05. τ_{fit} and A values were unconstrained and β values were constrained to $0.3 \leq \beta \leq 2.0$. τ_{fit} values were translated to τ_c values using $\tau_c = (\tau_{fit}/\beta)\Gamma(1/\beta)$, and τ_c values were heating corrected.⁷ Data were obtained for 438 dpPDI molecules over 1 sample and 3 movies and 1071 tbPDI molecules over 2 samples and 11 movies, respectively. Reported fwhm values presented here differ slightly from those presented in ref 7 because here all ACFs were fit to stretched exponentials for simplicity, whereas in ref 7 trajectory-dependent fitting was performed.^{6,7} To construct the quasi-ensemble ACFs, dpPDI and tbPDI ACFs from all individual trajectories in a given movie were normalized, averaged, and fit to stretched exponential functions. ACFs between movies were not averaged because the procedure used to heat-correct τ_c values is not straightforwardly extensible to heat-correcting ACFs.

Simulations of 3D rotational diffusion of a unit vector representing the transition dipole of a fluorophore were performed.^{6,7} The rotational diffusion constant D_r and thus $\tau_c = 1/6D_r$ was set by choosing the average angle by which the unit vector would rotate per step. From the simulated trajectories, x -, y -, and z -components of the dipole orientation were used to calculate the parallel and orthogonal intensities. For this calculation, wide-field excitation and detection with an NA = 0.75 objective, analogous to our experimental microscope configuration, were assumed, and thus LD ACFs are again expected to be single exponential in the presence of homogeneous rotational diffusion. As in the SM experiments, LD was calculated from the two orthogonal intensities. We added 30% Gaussian noise relative to the mean signal for consistency with experimental data, although the presence of noise does not affect conclusions of this study. Simulations used in Figure 1 were constructed with median τ_c of 20 steps and were varied in length, as described in the text. Simulations associated with Figures 2 and 3 were performed on log-normal distributions of τ_c values (with median $\tau_c = 100$ steps) with $fwhm = 2.35\sigma$ of the $\log(\tau_c)$ distribution varied from 0.0 to 1.2 in increments of 0.2. For all simulations, LD ACFs were constructed and fit to stretched exponential decays. ACFs were fit and ACF_{QES} were constructed, as described for experimental results.

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