

Heterogeneity in Single-Molecule Observables in the Study of Supercooled Liquids

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Abstract

Bulk approaches to studying heterogeneous systems obscure important details, as they report average behavior rather than the distribution of behaviors in such environments. Small-molecule and polymeric supercooled liquids, which display heterogeneity in their dynamics without an underlying structural heterogeneity that sets those dynamics, are important constituents of this category of condensed matter systems. A variety of approaches have been devised to unravel ensemble averaging in supercooled liquids. This review focuses on the ultimate subensemble approach, single-molecule measurements, as they have been applied to the study of supercooled liquids. We detail how three key experimental observables (single-molecule probe rotation, translation, and fluorescence lifetime) have been employed to provide detail on dynamic heterogeneity in supercooled liquids. Special attention is given to the potential for, but also the challenges in, discriminating spatial and temporal heterogeneity and detailing the length scales and timescales of heterogeneity in these systems.

SM: single molecule

Supercooled liquid:

a system lacking long-range order that exists between its melting temperature and the glass transition temperature

1. INTRODUCTION

Optical single-molecule (SM) techniques emerged more than 20 years ago with experiments monitoring single probe molecule behavior in solids at cryogenic temperatures (1, 2). With a variety of advances, particularly in detection, optical SM measurements were extended to much higher temperatures, and both near-field and far-field detection was demonstrated in the subsequent decade (3–8). Reviews of early SM work can be found in References 9–11. Since that time, the most commonly employed optical SM approaches have monitored properties of individual fluorescent probe molecules using far-field microscopy. These techniques have been most widely used to investigate the properties and behavior of biological molecules *in vitro*, with a drive toward moving such measurements into live cells (9, 12–14). The appeal of SM experiments for the study of biological systems stems largely from the significant inhomogeneity expected in such systems: It is only through the determination of individual molecules' behaviors and elucidation of the distribution, rather than the average, of a given quantity that a full picture of the inhomogeneous system can be attained. Of course, heterogeneous condensed matter systems exist outside the biological domain as well, and optical SM approaches are increasingly being used in other condensed matter systems either known or suspected to bear heterogeneity (15).

Among these systems, there are several classes of materials. The most straightforward to describe and assess are those with structural heterogeneities, systems with static, distinct structural motifs on nano-, micro-, or mesoscopic length scales. The systems in this class most thoroughly studied with optical SM approaches are mesoporous silica materials (16–37). In addition, room-temperature optical SM microscopy has been applied to the study of defects in crystals (38), systems undergoing crystallization (39), gels (40–43), and liquid crystals (44–48). In such systems, SM probe behavior tends to map onto local structures of the host: For example, in a mesoporous material, probe molecules between or within large hydrated pores may be mobile, whereas those trapped within small pores or on pore surfaces may be immobile (25, 30).

Systems lacking obvious structural heterogeneity may also display heterogeneity in dynamics. Supercooled liquids (see the sidebar) comprising either small molecules or polymers are the most prominent constituent of this category. In supercooled liquids, no signs of structural inhomogeneity are present, but a variety of experiments suggest the presence of regions of distinct and

SUPERCOOLED LIQUIDS

Liquids between their melting and glass transition temperatures that have not undergone a first-order phase transition to a crystalline solid are known as supercooled liquids. Supercooled liquids are metastable, lack long-range order, and display interesting properties. One such property is a strong, typically non-Arrhenius, increase in viscosity, η , with a decrease in temperature. Additionally, relaxations in supercooled liquids differ from those seen in typical liquids, in which exponential decays indicate a single underlying timescale governing the relaxation process; in contrast, relaxations in supercooled liquids tend to decay with a stretched exponential form, indicating a wide array of underlying timescales. Another interesting feature of the dynamics of supercooled liquids is the phenomenon of rotational-translational decoupling, whereby rotational dynamics track bulk viscosity, but translational dynamics are enhanced relative to those properties. The non-Arrhenius decay of viscosity, nonexponential relaxations, and rotational-translational decoupling are all consistent with the idea that instantaneous dynamics in different nanoscopic regions of the supercooled liquid differ from each other and fluctuate in time. This is known as dynamic heterogeneity or spatially heterogeneous dynamics, the underlying cause of which remains a central question in condensed matter science.

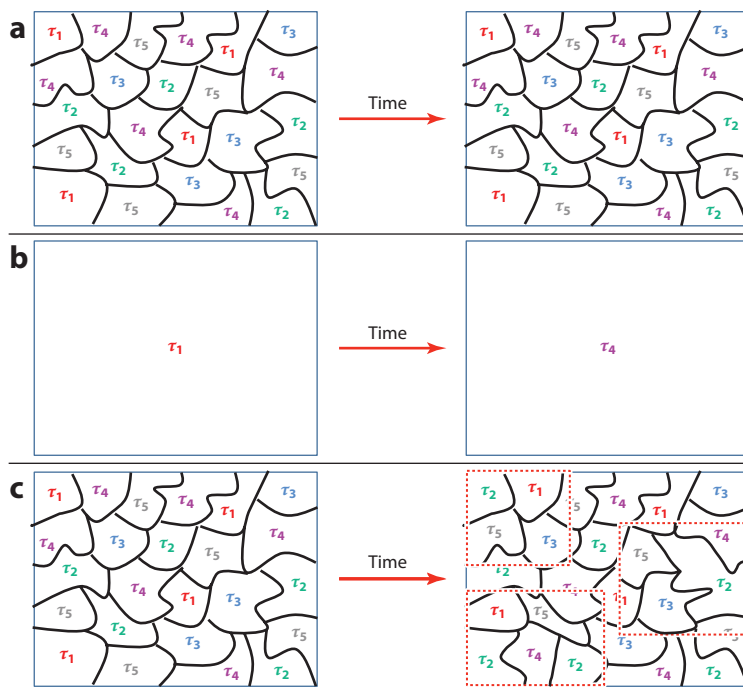


Figure 1

A supercooled liquid lacks structural heterogeneity but displays dynamic heterogeneity: The τ values represent the dynamics of host molecules. (a) A system with spatial heterogeneity, in which different regions of the sample exhibit different and unchanging dynamics as a function of position. (b) A system with temporal heterogeneity, in which the entire sample exhibits a single dynamical behavior that changes on the timescale of the experiment. (c) A system with spatial and temporal heterogeneity. Areas in red highlight possible changes over time: At the upper left, the dynamics change although regions bearing particular dynamics remain the same shape and size; at the right, regions change shape but the regional dynamics remain fixed; and at the lower left, both the regional shape and dynamics change.

interchanging dynamics (49, 50). We refer to these systems as displaying spatially heterogeneous dynamics or dynamic heterogeneity. In supercooled liquids, ensemble measurements probing relaxations have shown nonexponential and, in particular, stretched exponential decays. Whereas an exponential relaxation points to a single timescale underlying the monitored process, a stretched exponential suggests a broad array of underlying timescales. These timescales may represent (a) sets of molecules in the system displaying different, but fixed, relaxations (spatial heterogeneity); (b) molecules undergoing identical, but shifting, relaxations (temporal heterogeneity); or (c) a combination of both (**Figure 1**). The development and implementation of subensemble techniques have been extremely valuable in demonstrating the presence of dynamic heterogeneity and in beginning to detail the length scales and timescales over which it exists (49, 50). In supercooled poly(vinylacetate) (PVAc), multidimensional nuclear magnetic resonance (NMR) was used to selectively follow the dynamics of a slow subensemble of the system (51). It was found that such a subset existed and that it evolved toward the average relaxation time over long times. This experiment demonstrated the existence of temporal heterogeneity and identified a characteristic timescale, an exchange time, over which slow dynamics become average in this system. Probe-bearing subensemble techniques have also been used: The fluorescent probe tetracene in the small-molecule glass former *ortho*-terphenyl (OTP) was exposed to a deep photobleach that

Dynamic heterogeneity:

dynamics that differ as a function of location and/or time in a sample

Ensemble measurement:

technique that averages over large numbers of molecules, potentially obscuring heterogeneity in a sample

PVAc:

poly(vinylacetate)

Exchange time: time between changes in dynamics of a system or region thereof exhibiting temporal heterogeneity

OTP: *ortho*-terphenyl

Glass transition temperature, T_g : empirically defined temperature at which a supercooled liquid becomes a glass; often defined as the temperature at which $\tau_\alpha = 100$ s

FL: fluorescence lifetime

LD: linear dichroism

spared slowly rotating probe molecules—the rotational dynamics of this slow subset of molecules were then followed (52). As in the NMR experiment, at long times the slow molecules evolved toward average dynamics, demonstrating the presence of temporal heterogeneity, this time in a small-molecule glass former and using an optical technique. Although both experiments demonstrated the presence of temporal heterogeneity, the measured timescales for exchange were quite different. This has since been rationalized by considering the potential temperature dependence of the exchange time (49, 52, 53). Other experiments have focused on elucidating length scales of heterogeneity in supercooled liquids. Tracht et al. (54, 55) employed a multidimensional NMR technique to infer a length scale of ~ 3 nm in PVAc near the glass transition temperature (T_g). Additional experiments have suggested similar length scales in both polymeric and small-molecule glass formers (56–60).

Although these and other experiments have provided convincing evidence that dynamic heterogeneity exists in supercooled liquids, difficulties in clearly differentiating spatial and temporal heterogeneity suggest a role for SM experiments. SM approaches represent the ultimate subensemble experiment: They access molecular length scales and timescales and hold promise for directly identifying spatial and temporal heterogeneity and the length scales and timescales over which they persist (**Figure 2**). This review describes the most common optical SM approaches applied to the study of supercooled liquids, measurement of probe rotation, translation, and fluorescence lifetime (FL). We describe how such experiments have been used to identify and quantify spatial and temporal heterogeneity as well as the significant practical challenges to detailing spatial and temporal heterogeneity with these approaches. We conclude by suggesting approaches to overcoming some of these challenges.

2. SINGLE-MOLECULE APPROACHES FOR IDENTIFYING HETEROGENEITY IN SUPERCOOLED LIQUIDS

2.1. Measurements of Probe Rotation

The SM technique most commonly employed to demonstrate the presence of dynamic heterogeneity in molecular and polymeric supercooled liquids involves monitoring SM probe orientation (61–76).

2.1.1. General approach. In experiments that monitor SM probe orientation, probe molecule fluorescence is excited in either a confocal or wide-field configuration, and polarization of the emitted fluorescence is monitored (62, 63, 65, 66, 68–72, 74–76). A polarizing beam splitter is placed before the detector to collect two orthogonal polarization components of the fluorescence ($I_{||}$ and I_{\perp}) simultaneously. Because the polarization of the emitted light depends on the orientation of the probe transition dipole with respect to the detection system, when the two signal intensities change in an anticorrelated manner, this reflects probe rotation. From the measured intensities, linear dichroism (LD) is obtained via $LD = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$. This observable, related to the in-plane orientation of the molecule, has been used to track the evolution of probe orientation in supercooled liquids. We note also that techniques that elucidate both in-plane and out-of-plane angles have been suggested and implemented (62, 66, 73, 77–79). These studies and complementary simulations suggest that both in-plane and full three-dimensional rotational measurements allow faithful SM reporting of host dynamics. Here we restrict our discussion to the measurement and analysis of LD.

2.1.2. Assessing heterogeneity: visual inspection. To move from LD trajectories recorded for SM probes to probe rotational correlation times (τ_c) as depicted by the τ values in **Figure 2**

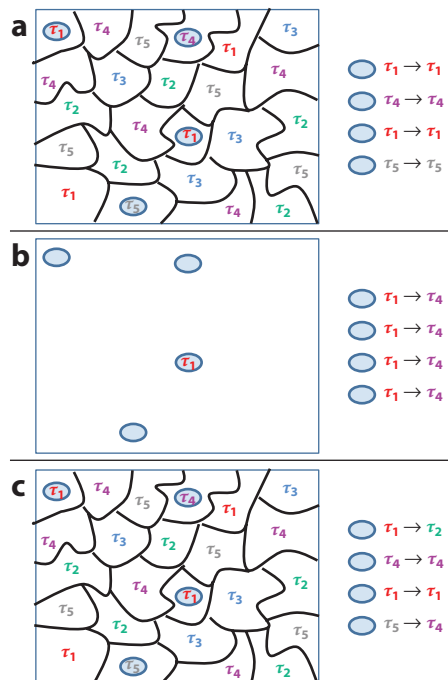


Figure 2

A supercooled liquid with (a) spatial, (b) temporal, or (c) spatial and temporal heterogeneity as depicted in **Figure 1** with the same time evolution (not shown) as illustrated there. Four single-molecule probes are shown at the same positions in each sample. For ideal probes that mirror the dynamics of the surrounding host, probe reports of host dynamics over time are shown on the right. The τ values represent probe rotational correlation times, probe translational diffusion constants, or probe fluorescence lifetimes, the observables discussed in detail in this review. (a) For the system with spatial heterogeneity, each probe demonstrates different τ values that do not change over time. (b) For the system with temporal heterogeneity, each probe displays the same τ value, and it evolves in time. (c) When both spatial and temporal heterogeneity are present, different probes display different τ values, some of which change on the experimental timescale.

requires additional analysis, as described in Section 2.1.3. Host heterogeneity, however, may be identified without such analysis, through visual inspection of LD trajectories. **Figure 3** shows LD trajectories of three rubrene probe molecules embedded in glycerol at 204 K ($1.07T_g$). **Figure 3a** shows a typical LD trace of a probe molecule in a supercooled liquid: The full range of possible LD values ($-1.0 \leq LD \leq 1.0$) is explored, and LD varies in time. Although relatively few SM experiments have been performed in molecular supercooled liquids, LD trajectories in these systems (68, 70, 72) and in polymeric ones (62, 63, 65, 66, 69, 71, 74–76) show qualitatively similar behavior, with perhaps more abrupt changes in LD seen in polymeric supercooled liquids. We note that a priori it is not known whether probes (or host molecules) in supercooled liquids will experience rotational relaxation through free diffusion consisting of small angular motions or through a more complex process, perhaps including small librations interspersed with large angular jumps. The latter may be expected if supercooled liquids relax through cooperative rearrangements, as proposed by Adam & Gibbs (80). Zooming in on a short time window of the LD trajectory of rubrene in glycerol does reveal some relatively large LD jumps (**Figure 3a**, inset). Similar jumps are seen in polymeric supercooled liquids, even when LD data are collected at a higher rate (20 Hz) than that shown here (5 Hz) (e.g., see 74).

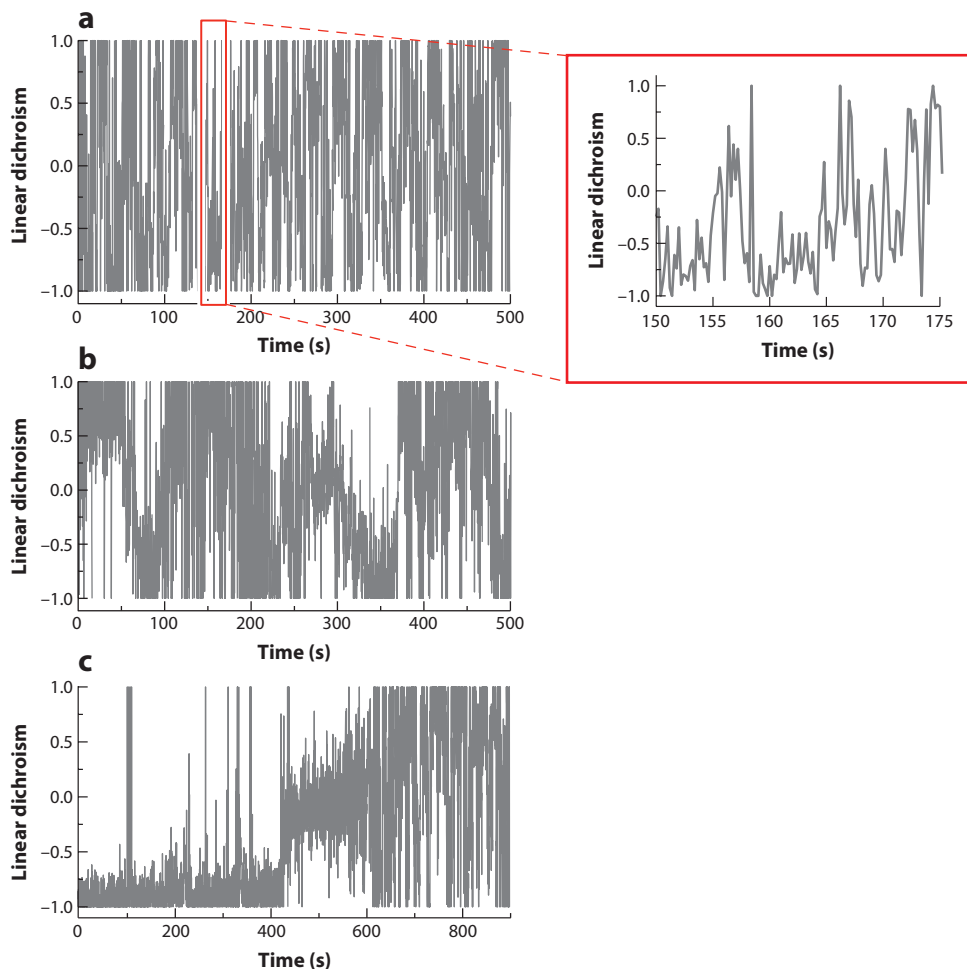


Figure 3

Linear-dichroism trajectory of a (*a*) fast, (*b*) slow, and (*c*) heterogeneous rubrene probe in glycerol at 204 K ($1.07T_g$). Figure adapted with permission from Reference 70. Copyright American Institute of Physics.

The trajectory shown in **Figure 3a** differs from that shown in **Figure 3b** in the average rate of change of LD and thus the average rate of probe rotation. The presence of two such SM probes at different locations in the same sample at the same time suggests the presence of spatial heterogeneity in the system. **Figure 3c** shows the LD trajectory of a molecule that appears to exhibit different rotational dynamics over the course of the experiment, with little change in LD apparent for most of the trajectory followed by fast oscillations of LD toward the end of the trajectory, providing evidence of temporal heterogeneity. Trajectories demonstrating obvious changes in probe dynamics were seen but were relatively rare in this and other SM LD measurements in supercooled liquids (62, 63, 65, 66, 68–72, 74–76). In cases in which alterations in the rate of LD change can be identified by eye, exchange times (times between changes in dynamics) can be quantified (62). When evaluating exchange times and comparing them across different experiments, one must consider the time window that can be interrogated with a particular experiment. For visual inspection of an SM LD trace, that window spans from approximately tens

of frames to the length of the trajectory, with evaluated timescales relative to the host structural relaxation, or α -relaxation, timescale (τ_α) differing with the particular probe. In cases in which no obvious changes in LD are seen, such changes may exist outside of the interrogated window and/or be too subtle to discriminate by eye.

2.1.3. Assessing heterogeneity: autocorrelation functions. Going beyond visual inspection of LD trajectories for investigation of dynamic heterogeneity encourages the analysis of autocorrelation functions (ACFs). For SM LD trajectories, an ACF is constructed, usually via $C(t) = \frac{\sum_{t'} a(t')a(t'+t)}{\sum_{t'} a(t')a(t')}$ with $a(t) = LD(t) - \langle LD(t) \rangle$. In supercooled liquids, measured ACFs are generally fit to stretched exponential functions, $C(t) = Ke^{-(\frac{t}{\tau_{fit}})^\beta}$ with the rotational correlation time of the probe, τ_c , given by $\tau_c = \int_{t=0}^{\infty} C(t) dt = \frac{\tau_{fit}}{\beta} \Gamma(\frac{1}{\beta})$. Because LD is a quantity similar to fluorescence polarization as measured in bulk experiments, it was initially assumed that LD ACFs would mirror the decay measured in fluorescence depolarization experiments (81, 82). As described in Section 1, deviations of such decays from exponential behavior ($\beta < 1$) have been assumed to indicate the presence of heterogeneity. Although in bulk experiments such deviations could arise from either spatial or temporal heterogeneity, for SM LD ACFs, deviation from exponentiality was expected to report solely on temporal heterogeneity, as no averaging over molecules in different environments would occur. For the probe molecules schematically depicted in **Figure 2c**, two probes would undergo no changes in dynamics over the time course of the experiment; these molecules would be expected to yield LD ACFs that decay exponentially, with $\beta = 1$ and $\tau_c = \tau_{fit}$. The two other probes would experience a change in dynamics during the experiment: Assuming multiple changes in dynamics over the course of the experiment, the SM ACF would be best fit by a stretched exponential with $\beta < 1$ and τ_c an average of the instantaneous rotational correlation times. The degree of deviation of β from 1 would provide a measure of the degree of temporal heterogeneity that the particular probe, and its local environment, experienced. Following this line of reasoning, another expectation was that in the presence of temporal heterogeneity, constructing an ACF from an early portion of an LD trajectory would yield an exponential decay (for the probe had not yet experienced alterations in the surrounding host dynamics), whereas fitting the ACF from the full LD trajectory would return a stretched exponential decay. Several challenges to these ideas quickly emerged.

Challenge: mixed-rank rotational correlation function. Bulk measurements of rotational relaxation including fluorescence anisotropy, dielectric spectroscopy, and NMR yield rotational correlation functions of specific rank, l (C_l). For homogeneous rotational diffusion, these are directly related to the rotational diffusion constant, D , through $C_l(t) = e^{-Dl(l+1)t}$. However, it was pointed out that LD ACFs have contributions from all even-rank correlation functions (81, 83). The takeaway is that SM LD ACFs can yield nonexponential decays, even for molecules exhibiting homogeneous isotropic rotational diffusion. The problem was found to be more pronounced for anisotropic rotational diffusion and less pronounced for rotation that occurs through large angular jumps (81). Following this work, several papers carefully considered how this would manifest in typical SM microscopy configurations, which employ high-numerical aperture objective lenses. These papers concluded that as typically implemented, C_2 would dominate the LD ACF and thus an exponential decay would be expected for a probe undergoing isotropic or anisotropic rotational diffusion (70, 74, 82, 84).

Challenge: time-limited trajectories. A second challenge to the idea that the nonexponentiality of individual SM LD ACFs indicates temporal heterogeneity emerged. Here the time-limited nature

α -relaxation time, τ_α : structural relaxation of a supercooled liquid related to the escape of a molecule from a cage of neighboring molecules

ACF: autocorrelation function

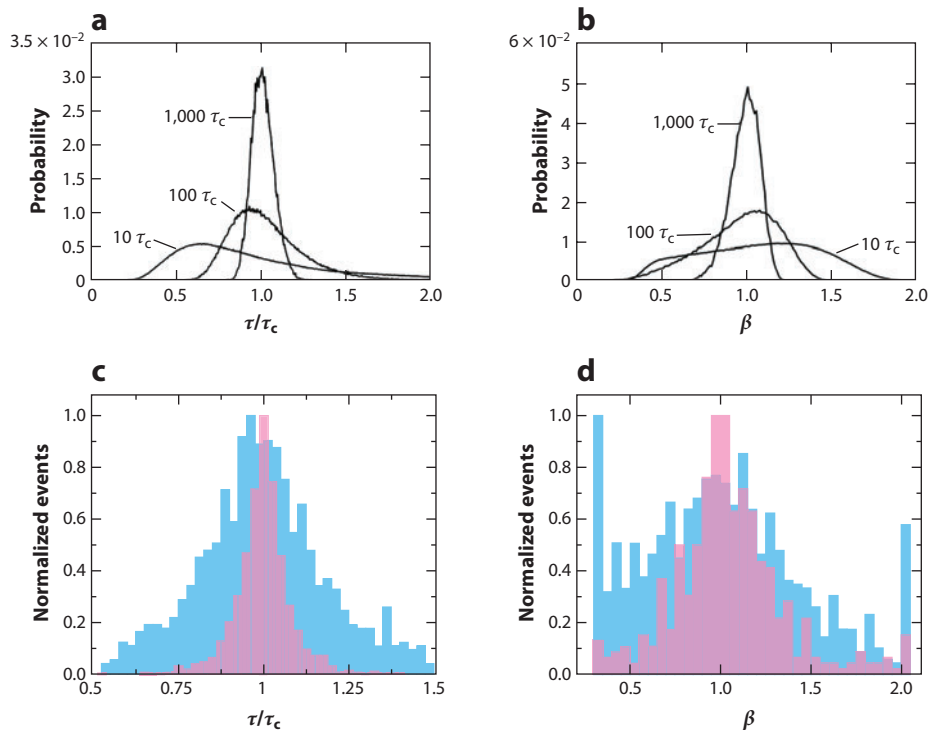


Figure 4

(a) Extracted τ values relative to known τ_c and (b) β values as a function of trajectory length for trajectories of 10, 100, and $1,000\tau_c$ from fitting autocorrelation functions to stretched exponential functions. Panels *a* and *b* adapted with permission from Reference 86. Copyright American Institute of Physics. (c) τ_c and (d) β distributions obtained from experiments (blue bars) and simulations (magenta bars) for a tbPDI probe in glycerol at $1.08T_g$. τ values are plotted relative to the mean τ_c value obtained. Simulated fits are constrained to have $0.3 < \beta < 2.0$. Simulated distributions are attained from 1,000 particles undergoing homogeneous rotational diffusion with trajectories truncated to match experimental trajectory lengths relative to the extracted τ_c values. Measured τ_c and β distributions are broader and differently shaped than those expected of molecules undergoing homogeneous rotational diffusion. Panel *c* adapted in part with permission from Reference 89. Copyright 2011 American Chemical Society. Panel *d* is constructed from the same data shown in panel *c* and is described in Reference 89.

of LD trajectories imposed by photobleaching of the SM probes, as well as particular ACF fitting procedures, was recognized to affect both the extracted timescales and exponentiality of the ACFs (70, 84–88). Simulations of isotropic homogeneous rotational diffusion yielding LD trajectories of a variety of lengths were performed. Fitting the individual LD ACFs revealed a broad distribution of extracted τ values relative to the known τ_c as well as a broad spread of β values (Figure 4*a,b*) (86). SM experiments in supercooled liquids have typically been $\sim 100\tau_c$, not sufficiently long to avoid a significant spread in β values emerging simply from the time-limited nature of the trajectories. Given this finding, the deviation from exponentiality of typical individual SM LD ACFs should not be used to demonstrate the presence of temporal heterogeneity.

Although the nonexponentiality of typical SM LD ACFs cannot straightforwardly demonstrate temporal heterogeneity, fitting these decays and examining the distribution of best-fit β and τ_c values can be useful. Indeed, fitting each SM LD ACF and building distributions of extracted τ_c

values has been the most common approach to demonstrating spatial heterogeneity in both small-molecule and polymeric glass formers (62, 63, 65, 68, 71, 72, 74, 75), although in the presence of both spatial and temporal heterogeneity this approach cannot cleanly isolate spatial heterogeneity. Wide distributions of τ_c and β values are typically attained, and these distributions are broader than those expected for homogeneous systems, even given the limited experimental trajectory lengths (**Figure 4c,d**) (89). These broad measured distributions thus do provide some evidence for the presence of spatial and temporal heterogeneity in these systems.

2.1.4. Assessing heterogeneity: sliding-window autocorrelations. Although SM LD ACFs cannot be used to demonstrate temporal heterogeneity for the reasons discussed above, developing a method to detect temporal heterogeneity and extract exchange times that is more rigorous than visual inspection is desirable. Schob et al. (65) proposed using a sliding-window ACF, an approach also adopted by Mackowiak et al. (70, 72). In this approach, a window is placed over a portion of the LD trajectory, and the data within that window are used to construct an ACF from which a τ_c value is obtained. The window is moved along the trajectory, and τ_c values are obtained for each window position. Schob et al. (65) extracted a single exchange time from these τ_c trajectories by plotting their width as a function of the length of the trajectory, a function that increases and then plateaus with trajectory length. Mackowiak et al. (70, 72) performed simulations of homogeneous rotational diffusion to make statistical judgments about whether any obtained τ_c trajectory was inconsistent with homogeneous rotational diffusion. For four types of probe molecules in glycerol, 15–35% of the assessed probes were found to display τ_c trajectories inconsistent with homogeneous rotational diffusion. Even with such careful identification of trajectories suggestive of temporal heterogeneity, establishing exchange times from these τ_c trajectories was performed via visual inspection. This yielded exchange times of $\sim 25\tau_c$. Although this finding is consistent with other SM findings arrived at through different approaches (62, 65, 67, 75), this approach to quantifying exchange time suffers from many of the same limitations as does identifying exchange purely via visual inspection.

2.1.5. Assessing heterogeneity: beyond the standard window. As described in Section 2.1.2, visual observation may allow for the detection of temporal heterogeneity; however, it may only do so in a distinct window. For the approaches described above, on the long end this window is set by the trajectory length, which itself is typically set by probe photobleaching. On the short end, the window is set by a combination of the probe τ_c and sensitivity of the chosen analysis to the change in τ_c .

It is relatively straightforward to detect changes in dynamics that may happen at times longer than the typical trajectory length. Zondervan et al. (68) found no evidence of temporal heterogeneity in typical LD trajectories of *N,N'*-bis(2,5-di-*tert*-butylphenyl)-3,4,9,10-perylenedicarboximide (tbPDI) probes in supercooled glycerol. To search for potential exchange on longer timescales, relatively short LD trajectories from a set of molecules were collected serially with waiting times of 10^5 – $10^6\tau_\alpha$ between data-collection periods. The authors did not find clear changes in single probe τ_c values, even over these long periods. The conclusion was that supercooled glycerol did not display evidence of temporal heterogeneity in this experiment at times up to $10^6\tau_\alpha$. However, across all probe molecules tracked, a broad spread of τ_c values was observed. Thus this experiment suggested that supercooled glycerol displayed spatial heterogeneity but not temporal heterogeneity (as depicted in **Figure 1a**). A similar long-time experiment on another perylene diimide (PDI) dye in glycerol was performed by Mackowiak et al. (72). Simulations of homogeneous rotational diffusion similar to those described in Section 2.1.4 were performed to provide a statistical measure by which to judge whether a measured change in a probe molecule's τ_c was inconsistent with homogeneous rotational diffusion. It was concluded that there was evidence for temporal

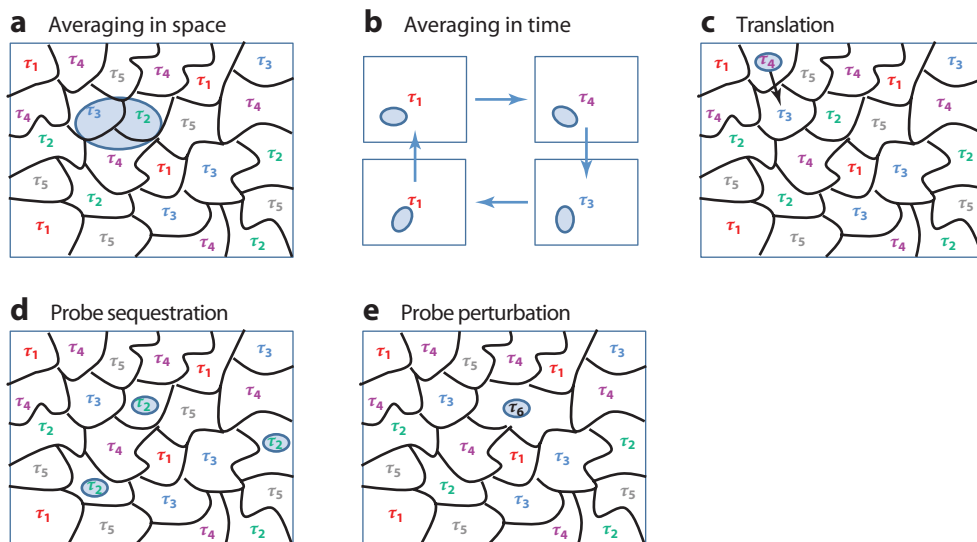


Figure 5

Schematic depiction of a variety of potential complications in probe measurement and reporting in systems bearing dynamic heterogeneity. (a) The probe may average in space over regions of particular dynamics (discussed in Section 2.1.5). (b) The probe may average in time over fluctuating dynamics (Section 2.1.5). (c) Probe reports of changing dynamics may reflect probe translation rather than dynamic exchange (Section 2.1.6). (d) The probe may sequester into regions of the sample demonstrating particular dynamics (Section 3.1). (e) The probe may alter dynamics in its immediate environment (Section 3.2).

heterogeneity, but that differences arising from limited trajectory lengths alone could account for many of the measured changes in τ_c values. Differences in the results of these two studies—both found evidence for spatial heterogeneity in supercooled glycerol but only one found evidence for temporal heterogeneity in the system—may be related to differences in sample preparation (as described in Section 3.1 below).

It is more challenging to assess temporal heterogeneity with SM probe rotation measurements on timescales faster than the typical lower bound imposed by probe τ_c . In the initial probe-bearing subensemble experiment that demonstrated the presence of dynamic heterogeneity in OTP, the probe was tetracene, carefully chosen not only to resemble OTP in molecular structure, but also to have a τ_c value similar to τ_α of OTP (49, 52). Such careful choice of probes is not possible in SM work, for which only fluorophores with very high quantum yield and photostability can be employed. As such, SM studies are typically performed with rather bulky, conjugated probes that have τ_c values much larger than the τ_α of the host. This is true not only in the case of small-molecule glass formers but also for polymers. Examples can be seen in recent experiments: tbPDI in glycerol displays $\tau_c/\tau_\alpha \approx 20$, whereas Rhodamine 6G (R6G) in PVAc has a $\tau_c/\tau_\alpha \approx 50$ (72, 76). These are among the smallest such ratios accessed to date.

Probe-dependent SM studies offer one way to infer temporal heterogeneity on faster timescales than τ_c . This approach is inspired by bulk studies (90–93). In these bulk experiments, some probes (those with the smallest molecular weights, space filling volumes, and rotational correlation times) yield relaxations that are fit to stretched exponential decays with small β values. Larger, slower probes in the same hosts yield single exponential decays, suggesting that these probes average over dynamic heterogeneity in the sample, as depicted in **Figure 5a,b**. With smaller and faster probes, less averaging occurs, and in this way probes may act as molecular rulers (or clocks) for measuring spatial (or temporal) heterogeneity.

PDI: perylene diimide

R6G: Rhodamine 6G

Although the choice of probe in SM experiments is restricted, probe-dependent studies can be performed. SM probes in supercooled systems do display a range of τ_c values inconsistent with homogeneous rotation (as shown in **Figure 4c**), thus demonstrating the presence of dynamic heterogeneity in these systems. It is not immediately apparent, however, that the probes report the full breadth of heterogeneity in the samples. This is highlighted by differences between the width of dielectric spectra in the α -relaxation regime and that of SM τ_c distributions. In the case of SM measurements, if the distribution of τ_c values increases with decreasing probe size and probe τ_c , it may be concluded that the smaller, faster probe is averaging less in space and/or time and is reporting a greater proportion of the inherent host heterogeneity. In the limit of small-enough and fast-enough probes, a plateau in width of the τ_c distribution is expected. Although probe size and probe τ_c typically track together, this is occasionally not the case. Mackowiak et al. (72) monitored three PDI probes in glycerol, two of which could hydrogen bond with glycerol. This slowed the two probes relative to the third (tbPDI), which happened to be the largest; thus the largest probe had the smallest τ_c . This probe demonstrated the widest distribution of τ_c values, suggesting that the probe size differential did not set the difference in the τ_c distribution breadth but the probe speed differential did. Here the probes appear to be acting as molecular clocks, with the faster ones reporting more heterogeneity in the system. These results suggest bounds on spatial and temporal heterogeneity in supercooled glycerol: The three PDI probes are all sufficiently small to report (at least a proportion of) host spatial heterogeneity, and the size differential between them (≈ 0.2 nm along their transition dipole) does not lead to a noticeable difference in τ_c distribution. This result is consistent with the measured length scale of heterogeneity in supercooled glycerol as obtained from multidimensional NMR measurements of ≈ 1.5 nm (56, 58). For temporal heterogeneity, the results suggest that dynamic exchange occurs on timescales faster than or similar to the fastest probe rotational correlation time ($\approx 20\tau_\alpha$), consistent with another recent SM study that quantifies exchange in a manner that avoids dependence on long LD trajectories (76).

2.1.6. Additional consideration: translation. In describing how SM probe rotation may report on spatial and temporal heterogeneity above, we assume that the probe remains within a region of particular dynamics during a typical experiment. This, however, may not be a good assumption. For example, in glycerol a typical SM probe of $V \approx 1$ nm³ would have a translational diffusion constant of $D_T \approx 8.2 \times 10^{-22}$ m² s⁻¹ at $1.05T_g$ and $D_T \approx 8.1 \times 10^{-19}$ m² s⁻¹ at $1.15T_g$ (94, 95). For the more fragile OTP, the range would be $D_T \approx 3.3 \times 10^{-20}$ to 9.6×10^{-16} m² s⁻¹ for the same temperature range (95, 96). These estimates assume no rotational-translational decoupling, which would increase the translational diffusion constants. Given these diffusion constants, a typical probe in glycerol (OTP) at $1.05T_g$ would traverse a distance of 5 nm (31) over a typical (5,000-s) experiment. An experiment constructed to interrogate long times, spanning $10^6\tau_\alpha$, for example, may result in the probe displacement of hundreds of nanometers. Given the estimates of spatial heterogeneity of 1–4 nm (54–60), probes may certainly traverse regions of different dynamics over the course of a typical experiment. Thus when SM probes are found to alter their dynamics over the course of an experiment, this should be evaluated not only as possible evidence of temporal heterogeneity but also as possible evidence of spatial heterogeneity coupled with probe translation (**Figure 5c**) (75). To the best of our knowledge, no work has attempted to discriminate these possibilities. One possible approach to do so would employ a probe tethered to a coverslip such that it could rotate and report local host dynamic exchanges but could not translate. To a different end, such an approach was used by Biju et al. (97) in supercooled PVAc.

2.2. Measurements of Probe Translation

Whereas probe rotation measurements have been widely used to interrogate spatially heterogeneous dynamics in supercooled liquids, probe translation can also be monitored for this purpose.

PMA:

polymethylacrylate

FIONA: fluorescence imaging with one nanometer accuracy**MSD:** mean-square displacement

Indeed, one way in which to discriminate between a change in probe dynamics due to dynamic exchange versus that due to translation into a region bearing different dynamics is tracking SM position while monitoring SM rotational dynamics. Measuring the translational behavior of molecules at the SM level in supercooled liquids is also of interest for other reasons. Several experiments have shown evidence for rotational-translational decoupling, the phenomenon whereby translational diffusion is enhanced relative to viscosity and rotational diffusion (49, 50). Given the presence of dynamic heterogeneity, one viable explanation for rotational-translational decoupling is that measurements of rotational diffusion preferentially report on a different subset of molecules than those probed in measurements of translational diffusion (49). Measuring both types of mobility simultaneously on an SM level could clarify whether rotational-translational decoupling can be an SM phenomenon in which a given molecule translates with a diffusion constant faster than would be expected from its rotational diffusion constant.

2.2.1. General approach. Although fluorescence correlation spectroscopy can be used to extract translational diffusion constants through measurement of many SMs, it does not allow explicit SM tracking and cannot reveal a distribution of translational diffusion constants. Thus we comment here only on SM tracking experiments that are the direct complement to the rotational studies described in Section 2.1. Although relatively few such experiments have been performed, SM particle tracking has been demonstrated in both structurally heterogeneous and homogeneous condensed phase systems (30, 31, 65, 98). As for probe rotational measurements, in systems of homogeneous structure, a continuity of diffusion constants is expected. In the first such SM measurement in a supercooled liquid, Schob et al. (65) investigated R6G in polymethylacrylate (PMA) at $1.2\text{--}1.4T_g$. At these temperatures, the probe molecules had diffusion constants of $\sim 10^{-13} \text{ m}^2 \text{ s}^{-1}$ and thus were expected to move hundreds of nanometers—beyond the diffraction-limited fluorescent spot—over the course of the experiment. Performing single-particle tracking in supercooled liquids in the temperature regime in which dynamic heterogeneity is expected ($1\text{--}1.2T_g$) requires a technique that allows localization of the molecule within the imaged diffraction limited spot. This can be achieved with localization microscopies such as fluorescence imaging with one nanometer accuracy (FIONA) (99). This approach has recently been applied to track a large PDI derivative in a 25-nm polystyrene film near T_g (98).

2.2.2. Assessing heterogeneity: mean-square displacement and beyond. In References 65 and 98, diffusion constants were extracted from SM tracks using mean-square displacements (MSDs), $\langle r^2(t) \rangle = \langle [r(t + \tau) - r(\tau)]^2 \rangle$. In the diffusive regime, this quantity increases linearly with time. The diffusion constant can be extracted by fitting the linear portion of the function. For in-plane imaging, the slope of the line yields D_T via $\text{slope} = 4D_T t$. Plotting MSDs from various SMs and acquiring a distribution of D_T values is similar in spirit to fitting LD ACFs to assemble a distribution of τ_c values as a report of spatial heterogeneity. It is unsurprising that extracting D_T values from MSDs must be done with care for reasons similar to those described for extracting τ_c (and β) values from LD ACFs. First, the trajectory length is crucial: In supercooled liquids, caged behavior precedes the escape from the cage that ultimately allows for diffusion. Trajectories must be sufficiently long to reach the diffusive regime; moreover, trajectories should be at least 100 times longer than the relaxation time to give accurate D_T values (100). Additional factors contributing to broadened distributions of extracted relative to actual D_T values, including the particular choice of regime over which to fit the MSD, have been described by Saxton (100). Flier et al. (98) used a variant of this approach that assesses step-length distributions as a function of lag times (101). This approach has been tested with simulations and has been shown to allow more accurate extraction of D_T values for relatively short trajectories (101). In their studies, both Schob

et al. (65) and Flier et al. (98) found a distribution of diffusion constants. Flier et al. performed Monte Carlo simulations to compare measured D_T distributions with simulated ones given experimental trajectory lengths. At temperatures below $\sim 1.1T_g$, measured D_T distributions (which monitor diffusion down to $D_T \approx 10^{-17} \text{ m}^2 \text{ s}^{-1}$) are substantially different from simulated ones, suggesting the presence of spatial heterogeneity in much the way that reported τ_c distributions do (68, 72).

Given that a single diffusion constant is extracted from an MSD, this quantity is particularly ill-suited for the identification of temporal heterogeneity. Again in analogy with measurements of rotational diffusion, monitoring changes in the diffusion constant for a given molecule over the course of an experiment is challenging, although the analysis of single-particle tracks in colloidal supercooled liquids and simulated glassy systems provides guidance (102–106). Monitoring step-size distributions and their deviations from Gaussian functions is one approach. Using slightly different techniques, both Schob et al. and Flier et al. did consider step-size distributions: Neither found evidence for changes in (most) probes' velocities over the course of the experiments. Additional experiments are necessary to establish whether translational SM measurements will prove useful in detailing spatial and temporal heterogeneities in supercooled liquids given the challenges of tracking probe molecules on relevant length scales for sufficiently long times.

2.3. Measurements of Fluorescence Lifetime

Another class of SM techniques monitors the behavior of environmentally sensitive probes. SM observables in this class that have been followed to study polymers both above and below T_g include blinking (39, 107–110) and emission spectra (111, 112), both of which may change as a function of environmental rigidity. The most commonly tracked observable in this class is FL, and FL measurements have been applied broadly to study polymeric systems both below T_g (97, 113–120) and above T_g (69, 74, 97, 116, 121, 122), but to date they have not been applied to the study of small-molecule supercooled liquids.

2.3.1. General approach and assessing heterogeneity. In SM FL experiments, probe molecules are typically excited with pulsed lasers, and fluorescence is collected using avalanche photodiodes equipped with time-correlated single-photon-counting electronics. The decay of the photon count as a function of time lag (nanoseconds) binned over some longer time period is measured and fit. In polymeric systems both below and above T_g , fluctuations in the FL are seen over time. These fluctuations are more obvious above T_g , where “the lifetime trajectory exhibits a strong hopping character on the measurement timescale of several hundred seconds” (121). Because emitted fluorescence depends on an effective transition dipole of the probe and the induced dipoles in its local environment, FL fluctuations emerge from changes in the positions and polarizabilities of the probe and/or the molecules (or monomers and holes, for a polymeric melt) around the probe (116, 121, 123, 124).

FL measurements typically have not been analyzed in a manner that focuses on dynamic heterogeneity; however, some analyses of this type have been performed. In some instances, FL measurements have been used to characterize spatial heterogeneity in polymers above and below T_g (69, 115, 122). More regularly, such measurements have been used to identify temporal heterogeneity, as is natural given the observed FL fluctuations. Minimal binning and bin-free techniques have been applied to assess possible changes in FL over the shortest accessible timescales (69, 74, 115, 122, 125, 126). Such minimal binning is critical in observing fluctuations in the FL over the course of a given SM trajectory. Careful binning analysis, controls on homogeneous systems, and complementary simulations all support that the measured fluctuations can emerge

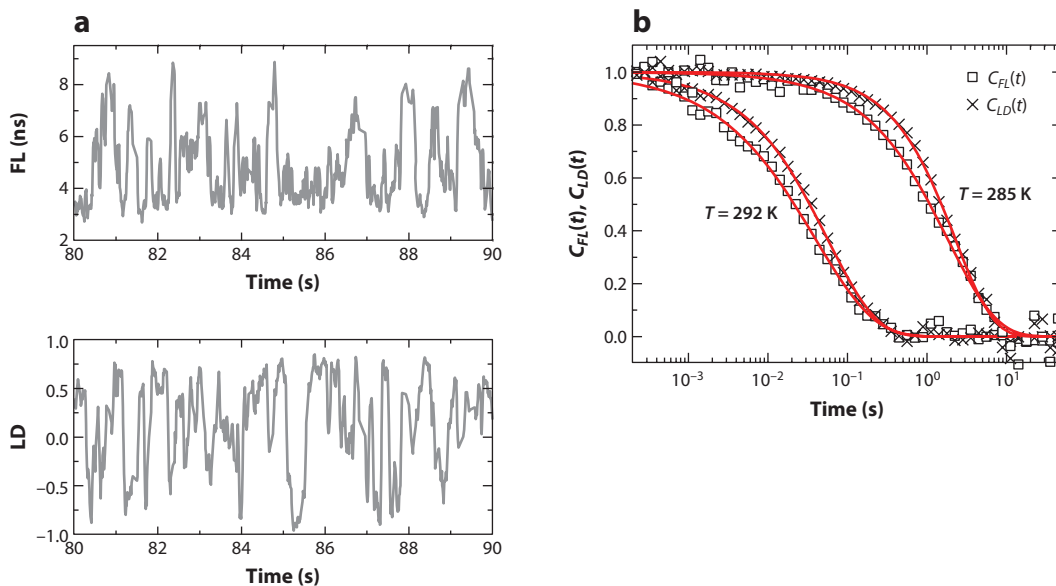


Figure 6

(a) Representative fluorescence lifetime (FL) and linear dichroism (LD) trajectories collected simultaneously (with 50-ms binning) from a single BODIPY molecule in PMA at $1.04T_g$. Fluctuations in both observables, some of which are coincident, are evident. (b) FL (squares) and LD (crosses) autocorrelation functions constructed via bin-free analysis and fits at 1.01 and $1.04T_g$. The fits yield $\tau_{c,FL} = 0.040$ s, $\beta_{FL} = 0.59$, $\tau_{c,LD} = 0.056$, and $\beta_{LD} = 0.71$ at $1.04T_g$ and $\tau_{c,FL} = 1.82$ s, $\beta_{FL} = 0.71$, $\tau_{c,LD} = 2.27$ s, and $\beta_{LD} = 0.91$ at $1.01T_g$. Figure reproduced with permission of the Royal Society of Chemistry from Reference 74.

from changes in the local environment around the probe, i.e., temporal heterogeneity (69, 88, 121, 122). Although temporal heterogeneity has thus been established by this technique, quantities such as the exchange time have not been assessed in these studies.

2.3.2. Comparison to measurements of probe rotation. New experiments and simulations that simultaneously investigate FL and LD hold promise for revealing details of dynamic heterogeneity in supercooled liquids (69, 74). Whereas LD changes only if the probe reorients, FL changes either if the probe rotates or if the surrounding host dynamics change. Braeken et al. (69) studied a terylene diimide probe in PMA at $1.03T_g$. In this study, most molecules exhibited changes in both observables, but only a subset of lifetime fluctuations coincided with abrupt changes in LD. Fits to FL and LD ACFs revealed similar but not identical timescales. The finding was interpreted as the two measurements probing relaxation on different spatial scales (69), although it may also be related to the fact that LD ACFs track $C_2(t)$, whereas FL ACFs track $C_4(t)$ (88). In the case of homogeneous rotational diffusion, the extracted relaxation times will differ by a factor of $10/3$, although for relaxation through large angular reorientations, the values will be identical (88, 127, 128). Both simulations and experiments suggest that a mix of small and large angular jumps is at play in probe reorientation in polymer melts (74, 79, 88). Another dual LD and FL study was performed with BODIPY in PMA at 1.01 and $1.04T_g$. Here more similar timescales were found for the decay of FL and LD ACFs, and both were fit with stretched exponential functions, with the deviation from exponentiality larger in FL ACFs than in LD ACFs (Figure 6). This has been interpreted as the slower LD relaxations allowing for more substantial averaging over dynamic exchange, although caveats related to the short trajectory length described in Section 2.1.3 are

also at play here. We note that in both the dual FL and LD studies described above, as usual, the measured probe relaxation was slower than the α -relaxation of the host in the absence of the probe. Indeed, the finding of similar decay times for both LD and FL studies reveals that FL studies do not avoid the challenges associated with spatial and/or temporal probe averaging that exist in rotational and translational SM measurements (**Figure 5a,b**).

Because FL studies generally have not focused on using the technique to detail heterogeneity, additional analysis of SM FL data—including the extraction of exchange times from fluctuating FL traces—may be useful. In addition, probe-dependent studies may help clarify the probe averaging that occurs in FL measurements. Finally, performing dual LD and FL studies in small-molecule glass formers may yield interesting information on the mechanisms of probe and host relaxation in those systems.

3. ADDITIONAL CONSIDERATIONS ON SINGLE-MOLECULE PROBE REPORTS OF HETEROGENEITY

In the experiments described above, aside from potential averaging in space and/or time (Section 2.1.5 and **Figure 5a,b**), it has been assumed that probes directly reflect the dynamics of the host as those dynamics would exist in the absence of the probes. It is also possible, however, that this is not the case. There are two ways in which a probe may not faithfully report on the host: if the probe sequesters into certain regions of the system or if the probe alters its local environment (**Figure 5d,e**). Experimentally, many SM probe rotation experiments have demonstrated that probe dynamics slow with decreasing temperature in parallel with host dynamics as investigated in the absence of probes (62, 67, 68, 72, 75). This is a necessary but insufficient condition to demonstrate that probes report faithfully on the dynamic heterogeneity of the host. Because it is quite difficult to ascertain whether a probe is mirroring the system in which it is embedded when only the probe can be monitored, both probe-dependent studies and simulations are useful here. Simulations that compare the host in the presence and absence of the probe and monitor both the probe and the (dynamically heterogeneous) system around the probe can be particularly powerful in ascertaining how probes report on spatial and temporal heterogeneity.

3.1. Probe Sequestration

Because SM probes are doped at such low concentrations in their host, it is not necessary for them to be strongly soluble in the host. For example, nonpolar rubrene was used as an SM probe of supercooled glycerol (70). In such a case, it is possible that the probes will preferentially be solvated by host molecules in a particular configuration, possibly inducing the host molecules to adopt configurations that would be absent in the neat system. Similarly, the hydrogen-bonding PDI probes employed by Mackowiak et al. (72) in glycerol may sequester in regions that maximize (or encourage the rearrangement of glycerol molecules to accommodate) those interactions. If a probe segregates in a particular type of environment (**Figure 5d**), it may experience and report a less heterogeneous environment than is present in the host in the absence of such probes.

The possibility of probe sequestration was invoked by Zondervan et al. (68) to help explain their finding of significant spatial heterogeneity without evidence of temporal heterogeneity in supercooled glycerol as assessed via LD experiments. They suggest that supercooled glycerol may exist as a set of liquid lakes separated by a nearly static network. This static network may involve few glycerol molecules but may span the sample, separating it into distinct regions. Each region may have slightly different properties such as density, and SM probes in each liquid lake would reflect this via different τ_c values, consistent with the presence of spatial heterogeneity. If the SM probes exist only in the liquid lakes and cannot insert into or cross the tenuous, perhaps

crystalline, network segregating them, the probes will not report (and the overall system will not have) temporal heterogeneity. Later work supported the idea that supercooled glycerol prepared in the manner described in this work (with a particular temperature history including slow cooling) did have such a network and that probes may exclude themselves from that network and/or other regions of the sample (129–132). Although there have been simulations of glassy systems in which probes have been embedded, they have not given much insight into the question of probe segregation as they are typically coarse grained and do not include the chemical detail likely necessary to lead to probe sequestration.

3.2. Probe Perturbation

Just as probes may sequester into a particular region that is not representative of the full set of environments present in the host, a probe could also locally alter a sample (**Figure 5e**) and then report preferentially on that altered environment, preventing the probe from experiencing and reporting the same degree of heterogeneity as the host in the absence of the probe and suppressing dynamic exchange. Simulations in which the host and probe are followed simultaneously are of utmost utility to clarify these issues. Simulated systems that have been shown to exhibit the slow and heterogeneous dynamics displayed by molecular and polymeric supercooled liquids have been developed (102, 133–136). Several groups have introduced probes into these systems to examine whether these probes mirror the heterogeneous dynamics of their surroundings. Studies considering the dynamics of an ellipsoid or needle in arrays of spheres or point obstacles reveal interesting behaviors that may be relevant to understanding SM studies in supercooled liquids (137–145). The most immediately relevant studies for understanding how SM probes report heterogeneity in structurally homogeneous, dynamically heterogeneous systems have been performed by groups also performing SM experiments.

Vallee and coworkers (79, 88, 121, 146) have performed extensive studies of dumbbell probes embedded in systems of bead-spring chains representing short polymers (from 5 to 25 monomers) as model polymeric melts. For a dumbbell probe (dimer) in a sea of oligomers, they demonstrated that the dynamics of the probe molecule, as reflected in the intermediate scattering function, mirror matrix dynamics as they exist both in the presence and absence of the probe (79, 121). This simulation also confirmed that probe FL fluctuations are sensitive to matrix α -relaxation. These findings, however, may depend sensitively on matrix and probe details (147, 148). More recent simulations focus on the sensitivity of probe reports of host dynamics to probe mass, size, and host chain length (146). Although subtle differences were seen as a function of probe size and probe presence, the overall finding was that the probe reflects the dynamics of the system. Mackowiak and colleagues (149, 150) studied probe and host behavior in a system of spheres exhibiting glassy behavior with embedded spherical probes of up to six times the host radius. For these probes, most of which are much larger relative to the host particles than those studied by Vallee and coworkers, the presence of the probe did affect the average translational mobility of particles in the surroundings, particularly near the probe (149). A later study investigated the mobility of the probe in this altered environment and found that the translational motion of the probe was decreased, even though the probe's presence increased the translational mobility of the surrounding host particles. This unintuitive result was caused by a preferential directionality of the translations of particles surrounding the probe, which enhanced probe caging (150). Although there are no published explicit analyses of the heterogeneity of host environments as a function of probe size and shape, this information is attainable from typical simulations and can further elucidate how SM experiments may reflect the details of spatial and temporal heterogeneity in supercooled liquids.

4. CONCLUSIONS AND OUTLOOK

The introduction of SM techniques to the study of supercooled systems a decade ago brought the promise of clearly identifying spatial and/or temporal heterogeneity in these systems as well as delineating the length scales and timescales over which this heterogeneity exists. In the intervening years, SM experiments have provided a clear demonstration of dynamic heterogeneity in supercooled liquids. However, careful consideration of the experimental techniques and data analyses employed has pointed to difficulties in straightforwardly distinguishing spatial and temporal heterogeneity and assessing length scales and timescales of heterogeneity with these measurements. With a more nuanced understanding of the challenges in hand, new experiments and analysis techniques supplemented with complementary, realistic simulations are now being deployed to tackle these questions. Among the approaches and SM studies that can add to the existing understanding of spatially heterogeneous dynamics in supercooled liquids are the following: (a) a continued drive toward careful choice of probe molecules picked so as to match host size and chemical functionality as closely as possible; (b) further probe-dependent studies, including ones in which the probe size and probe dynamics do not track each other to help discriminate spatial and temporal averaging by the probe and delineate length scales and timescales of host heterogeneity; (c) the adoption of analyses that simultaneously develop best fits for both spatial and temporal heterogeneity; (d) additional attempts to study multiple SM observables simultaneously, including dual translational and rotational measurements as made possible through localization microscopy; (e) the extension of techniques traditionally used only in polymeric systems to small-molecule glass formers; and (f) simulations including probes of realistic size and chemical functionality, with a focus on how probes report on dynamic heterogeneity. With these tools, additional details of dynamic heterogeneity in supercooled liquids can be elucidated, holding promise for distinguishing between and testing predictions of various theories of the glass transition.

SUMMARY POINTS

1. SM studies have been used to characterize both structurally heterogeneous and homogeneous systems suspected of exhibiting heterogeneous dynamics.
2. In supercooled liquids, which are structurally homogeneous and dynamically heterogeneous, optical SM experiments hold the promise of elucidating the length scales and timescales of heterogeneity.
3. The most commonly employed SM approaches applied to the study of supercooled liquids have been measurements of probe LD and FL.
4. Although it was initially believed that stretched exponential decays of SM ACFs indicated temporal heterogeneity of the host, it was later appreciated that experimental details, chiefly time-limited trajectories, preclude this conclusion.
5. Evidence for temporal heterogeneity in SM studies of supercooled liquids has emerged from visual inspection of LD and FL trajectories, probe-dependent experiments, and a comparison of the distribution of probe behavior with simulation.
6. Additional challenges to identifying temporal heterogeneity and quantifying the exchange time from SM optical experiments include the possibility of probe averaging in space and/or time, probe translation through regions of distinct dynamics, probe sequestration in specific host regions, and probe perturbation of the heterogeneous dynamics of the host.

7. Future SM experiments that use probes well matched to the host, track multiple observables, and are compared carefully to simulations hold promise for revealing more detail on the length scales and timescales of heterogeneity in supercooled liquids.

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62. Presents the first published SM study of a small-molecule glass former.

65. Presents the first attempt to measure translation in a supercooled liquid at the SM level.

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