

Electronic Supplementary Information for

Probe Dependence of Spatially Heterogeneous Dynamics in Supercooled Glycerol as Revealed by Single Molecule Microscopy

Stephan A. Mackowiak, Lindsay M. Leone, and Laura J. Kaufman^{a)}

Department of Chemistry, Columbia University, New York, NY 10027

^{a)} corresponding author

Heating Correction

Sample stage temperature is measured using a 100 Ω platinum resistive temperature device (Pt RTD) mounted directly on the sample stage with the vacuum grease Apiezon N. The temperature of the sample stage is controlled using a temperature controller (Lakeshore 331S). Sample stage temperature is stable within 50 mK of the set-point, with an absolute accuracy set by the Pt RTD calibration (estimated to be better than 200 mK). Sample temperature can be somewhat higher than Pt sensor temperature due to absorption of laser light by the Silicon wafer, which is $\approx 50\%$ absorptive in the visible range¹. To test for power dependence and potentially correct for it in the samples measured, power series are performed. Data is taken at multiple laser powers at multiple temperatures since only a small range of powers allows for sufficiently long trajectories and sufficient signal to noise for accurate analysis at a given temperature. Such power series must be performed on each sample, as thermal contact and thus heat dissipation varies from sample to sample because of differences in the thickness of the Apiezon N used to affix the sample to the cryostat.

Because the absorption of laser light changes the temperature of the sample, we first describe correcting the set temperature to the actual temperature while not adjusting the measured rotational relaxation times of the probes, as these values are accurate for the corrected temperature. To make the heating correction in the manner described below, one must assume the temperature dependence of the rotational relaxation time of the fluorophore follows the temperature dependence of the viscosity of

glycerol. The temperature dependence of glycerol's viscosity is known to vary with temperature according to the Vogel-Fulcher-Tammann-Hesse (VFTH) law,

$$\log \eta = \log \eta_0 + \frac{B}{T - T_0}, \quad (\text{S1})$$

with η the viscosity, T the temperature in Kelvin, and B , η_0 , and T_0 fit parameters. The VFTH parameters for glycerol in the temperature range investigated here as determined by rheology are $B = 1260$, $\eta_0 = 7.9 \times 10^{-8}$, and $T_0 = 118^2$.

Calculation of actual temperature from set temperature and measured rotational correlation times of the probes requires use of the DSE equation (Eqn. 5 in the text). However, neither T nor V_h are known *a priori*; thus, the actual temperature and V_h are obtained simultaneously using a self-consistent approach. As an initial guess for V_h , the low temperature rotational correlation times values are fit using the DSE equation and the temperature of the sample stage as reported by the Pt sensor. At low temperatures, very low laser powers are typically used and little heating is expected. The obtained hydrodynamic volume and the DSE equation are then used to numerically calculate corrected temperatures of the sample, which should be higher than the sample stage temperatures due to the laser heating. Based on the obtained temperatures for $\langle \tau_c \rangle$ values (where $\langle \tau_c \rangle$ refers to the median relaxation time obtained from all single molecules in a given movie) at each of the set temperatures, an average heating effect in terms of K/mW is calculated. This heating effect is then used to calculate a new actual temperature for each $\langle \tau_c \rangle$ value. Using this new temperature and the experimental $\langle \tau_c \rangle$ values, a new best-fit V_h can be obtained using the DSE equation. Comparing the experimental $\langle \tau_c \rangle$ values at the corrected temperatures with the $\langle \tau_c \rangle$ values predicted from the DSE equation and best-fit hydrodynamic volume provides a measure with which to evaluate the correction. If the difference in the resulting best-fit V_h between iterations is greater than 1%, the temperature correction is repeated using the last obtained V_h . In each iteration, a new V_h and new corrected temperatures are obtained by fitting the $\langle \tau_c \rangle$ values and their temperature dependence to

the DSE equation. The calculation is repeated until the difference between calculated V_h in two subsequent iterations is $< 1\%$.

Actual temperature at which data is collected can be ascertained as described above. However, it is often desirable to combine data from different movies and/or different samples. While each of these data sets may be collected at the same set temperature, they may not be collected at identical actual temperature. To attain sufficient data at a given temperature, correcting the values for the relaxation times of the probes to the set temperature rather than correcting the set temperature to an actual temperature is necessary. Doing this assumes that time-temperature superposition holds over the temperature range between the set and actual temperatures of the combined data. Given that this range is less than 2K at the highest temperatures investigated and less than 0.2K at the lowest temperatures considered, this is expected to be a good assumption.

Combining Equation S1 with the DSE equation gives the following relationship:

$$\tau_1 = \tau_2 * \frac{T_2}{T_1} * 10^{\left(\frac{B}{T_1-T_0}\right) - \left(\frac{B}{T_2-T_0}\right)} \quad (\text{S2})$$

where τ_2 is the measured rotational relaxation time, T_2 is the actual temperature taking the heating effect into account, B and T_0 are the VFTH parameters, T_1 is the set temperature of the sample stage, and τ_1 is the corrected relaxation time for the sample stage temperature T_1 . Thus, when the actual temperature T_2 is known, the τ_2 values can be corrected to the sample stage temperature T_1 by multiplying these values by the ratio τ_1/τ_2 .

Fig. S1 shows (a) raw data, (b) temperature corrected data, and (c) relaxation time corrected data on the dpPDI data set also shown (after relaxation time correction) in Figs. 3 and 4 in the manuscript. Fig. S1a shows the experimental data and the eventual best-fit DSE line, corresponding to a hydrodynamic volume of 2.02 nm^3 . The best-fit V_h for the uncorrected data, the starting point of the iterative process, is 1.94 nm^3 . At low temperature, where low laser powers are employed, the points are close to the DSE line. At higher temperature, a deviation is apparent, which is due to more substantial heating at the higher laser

powers used at these temperatures. As described above, the actual temperatures corresponding to the measured $\langle\tau_c\rangle$ values are first found (Fig. S1b), and then the $\langle\tau_c\rangle$ values are corrected to those expected at the sample stage temperatures (Fig. S1c). As expected, following the correction, correlation between $\langle\tau_c\rangle$ values and laser power is no longer present and all points are near the best-fit DSE line, which yields a final hydrodynamic volume of 2.02 nm^3 .

References

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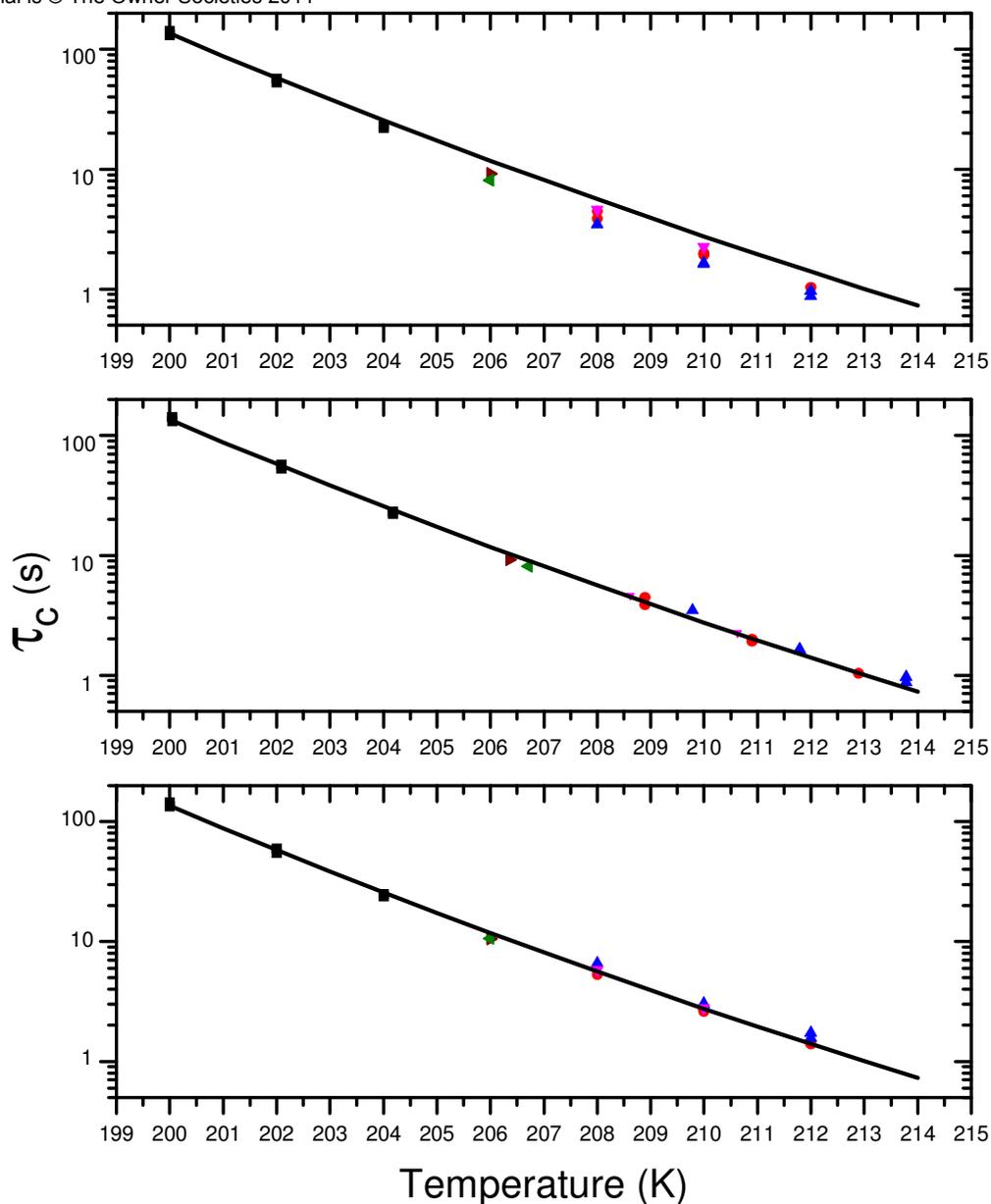


Figure S1. Median τ_c values for each of 23 movies of dpPDI before and after heating correction with eventual best-fit DSE line corresponding to $V_h = 2.02 \text{ nm}^3$. The different symbols represent different laser powers - blue upright triangle: 20mW, red circle: 10mW, magenta downright triangle: 7mW, olive left triangle: 8mW, wine right triangle: 4mW, black square: 0.5mW - 2mW. (a) Uncorrected experimental data. (b) Uncorrected median τ_c values with corresponding actual temperature. (c) Corrected median τ_c values for sample stage set temperature, as also shown in Fig. 3 in the manuscript.