ABSTRACT

Living cells are crowded with macromolecules and organelles. Yet, it is not fully understood how macromolecular crowding affects the myriad of biochemical reactions, transport and the structural stability of biomolecules that are essential to cellular function and survival. These molecular processes, with or without electrostatic interactions, in living cells are therefore expected to be distinct from those carried out in test tube in dilute solutions where excluded volumes are absent. Thus there is an urgent need to understand the macromolecular crowding effects on cellular and molecular biophysics towards quantitative cell biology. In this report, we investigated how biomimetic crowding affects both the rotational and translation diffusion of a small probe (rhodamine green, RhG). For biomimetic crowding agents, we used Ficoll-70 (synthetic polymer), bovine serum albumin and ovalbumin (proteins) at various concentrations in a buffer at room temperature. As a control, we carried out similar measurements on glycerol-enriched buffer as an environment with homogeneous viscosity as a function of glycerol concentration. The corresponding bulk viscosity was measured independently to test the validity of the Stokes-Einstein model of a diffusing species undergoing a random walk. For rotational diffusion (ps–ns time scale), we used time-resolved anisotropy measurements to examine potential binding of RhG as a function of the crowding agents (surface structure and size). For translational diffusion (µs–s time scale), we used fluorescence correlation spectroscopy for single-molecule fluctuation analysis. Our results allow us to examine the diffusion model of a molecular probe in crowded environments as a function of concentration, length scale, homogeneous versus heterogeneous viscosity, size and surface structures. These biomimetic crowding studies, using non-invasive fluorescence spectroscopy methods, represent an important step towards understanding cellular biophysics and quantitative cell biology.

Keywords: macromolecular crowding, rotational diffusion, translational diffusion, anisotropy, fluorescence correlation spectroscopy, Ficoll, BSA, and ovalbumin.
1. INTRODUCTION

Living cells are crowded with biomolecules and organelles with an estimated concentration of \( \geq 300 \text{ g macromolecules/L} \) (1, 2). This crowded environment differs greatly from buffered solutions typically used in conventional biochemical assays and is likely to have significant impact on biological function of intracellular proteins and how they interact with other biomolecules and organelles inside the living cells. Some of those proteins are not very stable and may sample a range of structural conformations, perhaps by design based on their biological functions in the crowded, dynamic milieu of living cells. The effects of crowded environments on biochemical reactions and transport (e.g., diffusion) have been treated theoretically (3-6). Recent studies have explored the role of crowding in regulating diffusion, protein folding, and protein activities (4, 7), using synthetic polymers (e.g., Ficoll, polyethylene glycol) as crowding agents, and the results were modeled in terms of an excluded volume effect due to hard-sphere repulsions. Recently, it was shown that the excluded volume may also depend on soft (or weak) chemical interactions (e.g., electrostatics, hydrogen bonding) between protein-based crowding agents (e.g., bovine serum albumin, ovalbumin) and the molecule of interest (8-11). Thus, crowding consists of both physical (hard spheres) and chemical (intermolecular) interactions between the crowding agents and the target molecular probe. Such complexity requires the acquisition of single-molecule information along with bulk studies to understand the length- and time-scale dependence associated with crowding effects on protein association kinetics, conformational changes, and biological activities.

To date, the macromolecular crowding effects on intermolecular interactions have been investigated experimentally using a variety of techniques, including aqueous two-phase systems (12, 13). Other experimental techniques used for crowding studies include NMR spectroscopy (14, 15), fluorescence resonance energy transfer (FRET) (16-18), fluorescence correlation spectroscopy (FCS) (19), and small angle x-ray scattering (20). Obstructed diffusion in the crowded milieu of live cells has been reported recently using fluorescence fluctuation analysis of raster scans (21, 22). In addition, NMR spectroscopy has been used to investigate crowding effects in vivo (14) and in vitro (11, 23). Each of these approaches has its own advantages and limitations with respect to sample preparation, spatial and temporal constraints and degree of invasiveness of the technique.

To address the inherent complexity of diffusion in crowded environments, we are using integrated laser-based fluorescence spectroscopy methods for both quantitative and noninvasive studies. Time-resolved fluorescence anisotropy, for example, is used here to assess fast (ps–ns) conformational changes and rotational diffusion of molecular probes (24). In the context of crowding, we can determine whether crowding agents impose intermolecular constraints (e.g., specific or non-specific binding) upon the molecular probe of interest, which would decrease the corresponding rotational rate. For larger molecular tracers, however, crowding agents may induce a compaction of the molecule of interest and may lead to faster rotational correlation times than those measured in the absence of crowding. Using a similar approach, the corresponding fluorescence lifetime is also measured as a means to determine the effects of crowding on the excited-state dynamics of molecular probes. The fluorescence lifetime of a given fluorophore represents the time window during which the rotational diffusion is measured (anisotropy) and variations in the lifetime may also reflect structural changes of the probe within its local microenvironment. For example, Förster resonance energy transfer (FRET) is a powerful
tool to investigate how crowding may influence biomolecular conformations of a donor-acceptor construct using fluorescence lifetime measurements as a function of crowding (16, 25). For translational diffusion (μs–s time scale), we use fluorescence correlation spectroscopy (FCS) to assess the effects of crowding on a molecular probe. FCS allows us to measure the translational diffusion on longer spatial and temporal scales than those measured with time-resolved anisotropy (26). At sufficiently high concentrations of crowding agent, the translational diffusion of the molecular probe will be impeded due to confinement in crowding-induced caging, association reactions (binding), and perhaps cage mobility. These crowding effects are likely to be observed as slower diffusion coefficients as compared with the absence of crowding agents. Importantly, these studies will help test different diffusion models in crowded environments.

2. CROWDING EFFECTS ON MULTISCALE DIFFUSION: A CONCEPT

We hypothesize that the effects of macromolecular crowding on diffusion will depend upon the chemical structure and size of both the molecular tracer and the crowding agents (polymers, proteins or cell extracts). Critically, the spatial and temporal resolution as well as the molecular sensitivity of the experimental techniques used will make a difference in probing different aspects of the diffusion processes in the presence of crowding agents. Figure 1 depicts different scenarios of the proposed model describing macromolecular crowding effects on the diffusion of a molecular probe.

**Figure 1.** A sketch describing possible scenarios for how macromolecular crowding agents (black circles) may influence the diffusion of a molecular tracer (red dot). The dotted circles represent a excluded volume region of weak (or electrostatic) interactions that extends beyond the hard-sphere volume. Binding events (Scenarios II & III) are represented by the changed orange color of the molecular tracer.

To differentiate between homogeneous and heterogeneous (crowding) viscosity, a control experiment is done on glycerol-rich buffer under the same experimental conditions. This control allows us to distinguish among diffusion in viscous solution, confinement in a cage created by the hard-sphere crowding agents, weak interactions, and association reactions (long-lived or transient) that a molecular probe may experience in the crowded milieu of living cells.
Our hypothesis is that the diffusion of spherical molecules in crowded environments may deviate from the Stokes-Einstein model based on (i) the viscosity range in a homogenously viscous environment, (ii) the concentration and the type of crowding agents, and (iii) the spatio-temporal resolution of the experimental technique used. The surface charges and size of the crowding agents are also likely to influence the diffusion processes of a molecular probe. For the excluded volume effect by crowding agents, for example, the diffusion processes will reflect a buffer-like environment as well as caging (or confinement) by the crowding agents. Although molecules diffusing in a crowded environment may encounter buffer-like environments, the apparent diffusion coefficient is likely to be reduced due to confinement (Scenario I) in hard-sphere-induced caging as compared with that in dilute solutions. This confinement may be detected using integrated methods with variable spatial and temporal resolution. It is worth noting that the cage formed by the crowding agent may also be mobile on a much slower time scale.

In Scenario II, there is a potential for binding (specific or non-specific) between the molecular probe and the crowding agents. These binding interactions may be transient or long-lived, based on the chemical structure of both the probe and the crowding agents. For example, this scenario is likely to be observed in a crowded environment of proteins or cell extracts where a plethora of weak interactions may be present. Scenario III describes the presence of intermolecular interactions due to surface charges of both the molecular probe and the crowding agents. These scenarios are likely to be present in complicated crowded environments such as those found in living cells. In controlled environment studies, however, different scenarios may be in play at a given time based on the selection of the crowding agents and/or molecular tracer. Importantly, the length scaling associated with crowding can also be investigated using different experimental methods with diverse spatial, temporal, and molecular sensitivities.

In the work described here, we examined these scenarios in the context of how crowding may influence the rotational and translation diffusion of RhG in homogenous (glycerol-enriched buffer) and heterogeneous (Ficoll-70, bovine serum albumin, BSA, and ovalbumin) environments. We provide a glimpse of this concept-based approach and the full account of our findings will be published elsewhere.

3. MATERIALS AND METHODS

Rhodamine green (RhG; Invitrogen) solutions were prepared using phosphate-buffered saline (PBS; pH 7.4). Synthetic polymers (Ficoll-70 and Ficoll-400) and proteins (BSA and ovalbumin) were used as crowding agents in these studies. As a control for homogeneous viscosity, glycerol-enriched PBS was used under the same experimental conditions. The concentrated glycerol samples were extended up to 900 g/L to cover a wide range of the viscosity that might be found in cellular compartments. In contrast, the protein and polymer samples were prepared up to 300 g/L as reported to match the projected macromolecular crowding in living cells (1, 2). The bulk viscosity of the samples used in these studies was measured using Ubbelohde viscometers, and an Abbe refractometer was used to measure the corresponding refractive indices of the crowded solutions.

For rotational diffusion of RhG, time-resolved fluorescence anisotropy was used and the experimental system was described in details elsewhere (27); see Figure 2A.

Briefly, two-photon infrared (950 nm) laser pulses (120-fs width, 76 MHz repetition rate) were generated using Mira-900 laser system (Coherent). After pulse picking to reduce the pulse repetition rate, the corresponding second harmonic (460 nm, 4.2 MHz) was generated and used for one-photon excitation of RhG solutions at room temperature via a 1.2 NA, 60× water immersion objective (Olympus) on an inverted microscope (IX81, Olympus). The epifluorescence was subsequently filtered, polarization-analyzed using a polarizing beam splitter (Thorlabs) and detected using a two microchannel plate photomultiplier tubes (Hamamatsu). The detected signals were then amplified, routed and registered in a SPC-830 module (Becker & Hickl), whose operation is based on time-correlated single-photon counting principles (31, 32). The SPC module was synchronized using an electronic signal generated by a fast photodiode, which was detecting a small fraction (~5%) of the laser pulses. As a control, pure crowded samples (highest concentration) were measured under the same conditions to assess any background contribution. In addition, the geometrical factor (G factor) in our experimental setup was calibrated using RhG (2–4 µM) and the tail-matching approach (25, 33).
Fluorescence correlation spectroscopy (FCS) was used to characterize the translational diffusion of single RhG molecules through an open observation volume, which was created by the tightly focused laser and the confocal pinhole in front of the detector. The experimental system for FCS is described in detail elsewhere (27); see Figure 2B. Briefly, FCS measurements were carried out using 488-nm excitation via 1.2 NA, 60x water-immersion objective (Olympus) on an inverted microscope (IX81, Olympus). The epifluorescence signal was filtered (HQ525/30M) and steered towards a home-built FCS setup and detected by a single-photon avalanche photodiode (SPAPD, SPCM CD-2969, Perkin-Elmer) through a 50-µm fiber acting as a confocal pinhole. The detected fluorescence fluctuation was detected using external multiple-tau-digital correlator (ALV/6010-160) and analyzed using nonlinear least-square fitting algorithm (OriginPro 8.0). As a control, pure crowded samples (at the highest concentration) were measured under the same conditions to assess any background contribution. In addition, the observation volume of our FCS setup was calibrated using RhG (4 nM) in buffer at room temperature (26, 27).

The concepts and key observables in both time-resolved anisotropy and FCS measurements are highlighted in Figure 3. Following the excitation using a polarized laser pulses, the excited dipole moment of the fluorescent probe depolarizes due to tumbling (rotational) motion. The depolarized emission is then analyzed using a polarizing beam-splitter and the parallel and perpendicularly polarized fluorescence decays are then recorded simultaneously (Figure 3A) using SPC-830 module as described above and used to calculate the corresponding anisotropy decay of a given fluorophore such that (34):

$$r(t) = \frac{I_\parallel(t) - G \cdot I_\perp(t)}{I_\parallel(t) + 2G \cdot I_\perp(t)}$$

The geometrical factor (G) is determined to account for any polarization-based bias in our two MCP detectors. The denominator in Equation (1) corresponds to the fluorescence lifetime, which is measured independently at magic-angle detection. It is worth noting that the factor of “2” in the denominator may be slightly different under epifluorescence excitation using high NA objective microscopy objectives (31). However, in our measurements reported here, we used a 1.2 NA objective with an under-filled back aperture (i.e., the laser beam diameter was smaller than that of the back aperture of the objective). The corresponding anisotropy decays will depend on the chemical structure of the fluorophore, the presence of multiple species, and the surrounding environment. Unlike polymer and proteins crowded environments, the time-resolved anisotropy of RhG in glycerol-enriched buffer was described satisfactorily as a single-exponential decay such that:

$$r(t) = r_0 \exp(-t/\phi)$$

where $r_0$ is the initial anisotropy and $\phi$ is the rotational time of the fluorophore in the a given environment. According to Stokes-Einstein model, the rotational time depends on both the bulk viscosity ($\eta$) and the hydrodynamic volume ($V$) of the rotating moiety at a given temperature ($T$) such that (34):
\[ \phi = \frac{1}{6D_R} = \frac{\eta V}{k_B T} \quad (3) \]

where \( D_R \) is the corresponding rotational diffusion of a spherical fluorophore and \( k_B \) is the Boltzmann constant.

![Diagram](image.png)

**Figure 3.** Basic concepts and observables in time-resolved anisotropy and fluorescence correlation spectroscopy measurements used in these studies. (A) The excited dipole moment of the fluorescent probe depolarizes due to tumbling (rotational) motion. The depolarized emission is then analyzed using a polarizing beam-splitter and the parallel and perpendicularly polarized fluorescence decays are then recorded simultaneously (right). (B) A schematic configuration of the open observation volume in FCS, which is created by both the tightly focused laser beam and the confocal pinhole in front of the detector. In samples of nanomolar concentrations, single molecules diffuse in and out of the observation volume causing time-dependent fluorescence fluctuations (Figure 3B, right). The fluorescence fluctuation is then autocorrelated to yield the average number of molecules that reside in the observation volume, the diffusion time, and the time constant associated with any photophysical processes or chemical reactions.

As shown in Figure 3B, an open observation volume in FCS experiments is created using both a tightly focused laser beam and a confocal pinhole in front of the detector. As single molecules diffuse in and out of the observation volume, time-dependent fluorescence fluctuations or bursts are detected (Figure 3B, right) and then autocorrelated. The corresponding autocorrelation function for three-dimensional diffusion of a given fluorophore can be fitted using the following equation (24, 26, 27, 30):
\[ G(\tau) = N^{-1} \left( 1 + \frac{\tau}{\tau_d} \right)^{-1} \left( 1 + \frac{\tau}{\omega_0^2 \tau_d} \right)^{-\frac{1}{2}} \]

where \( N \) is the average number of molecules that resides in the observation volume at any given time, \( \tau_d \) is the diffusion time, and the structure parameter \( (\omega_0) \) is the ratio of the axial \( (\omega_{xy}) \) to lateral \( (\omega_{xy}) \) extension of the observation volume. In the presence of additional photophysical processes (e.g., intersystem crossing and triplet state population) or chemical reactions, the above mentioned autocorrelation function will be further modified to account for these sources of fluorescence fluctuations (26). It is worth noting that the measured translational diffusion time \( (\tau_d) \) is related to the diffusion coefficient \( (D_T) \), which depends on both the hydrodynamic radius \( (a) \) of the diffusing species in an environment of known viscosity (24, 26, 27, 30):

\[
\tau_d = \frac{\omega_{xy}^2}{4D_T}, \quad \text{and} \quad D_T = \frac{k_B T}{6 \pi \eta a}
\]

Equations 3 and 5 indicate that the rotational and translational diffusion coefficients depend inversely on both the viscosity and the size of the diffusing species. We argue that the separation of these two variables is critical to properly interpret these types of measurements. Within the Stokes-Einstein model, the rotational-to-translational diffusion coefficient ratio depends only on the square of the hydrodynamic radius of the diffusing species in the same environment. As a result, the complementary studies reported here, using time-resolved anisotropy and FCS measurements, enable us to separate the crowding effects on the viscosity and association-related changes of the molecular size.

**4. RESULTS AND DISCUSSION**

**4.1 Crowding agents affect the viscosity and refractive index of the solution**

According to the Stokes-Einstein model, the diffusion coefficient of a spherical molecule depends inversely on the viscosity of the surrounding medium. Here we independently measured the bulk viscosity of crowded solutions using an Ubbelohde viscometer and the results are shown in Figure 4. These results show that the viscosity depends nonlinearly on the concentration of the crowding agents. In contrast with glycerol and protein crowding agents, the polymers (Ficoll-70 and Ficoll-400) exhibit more pronounced effect on the bulk viscosity over the concentration range investigated here. Although extended the concentration range of glycerol up to 900 g/L, the estimated viscosity seems far below what is believed to be the viscosity of the plasma membrane in living cells, which can be up to 100 cP by some estimates (35, 36). In all crowding agents, however, the bulk viscosity goes beyond the reported viscosity of the cytoplasm (~2 cP) in living cells (37, 38). The concentration and viscosity range shown here allow us to use fluorescence spectroscopy methods to determine whether diffusion in crowded environments follows the Stokes-Einstein model.
Figure 4. The bulk viscosity of the solution depends on the concentration of a crowding agent or glycerol. These viscosity measurements were carried out using an Ubbelohde viscometer on glycerol-enriched solutions (270, 340, 410, 480, 550, 620, 690, 760, 830 and 900 g/L). For BSA and ovalbumin concentrations, we used 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 g/L. For Ficoll, we used 40, 80, 120, 160, 200, 240, 280, 320, 360, and 400 g/L concentrations.

According to the Strickler-Berg equation (39), the radiative rate constant of a given fluorophore depends on the squared refractive index of the surrounding medium (Equation 6). The radiative rate constant is directly related to the fluorescence rate constant, or the inverse of the excited state fluorescence lifetime (Equation 7).

Figure 5. The refractive index of crowded solution depends linearly on the concentration of the crowding agents as well as glycerol. These measurements were carried out using an Abbe refractometer and the concentration range is similar to that in Figure 4.

Time-resolved fluorescence anisotropy is used to investigate the rotational diffusion on picosecond to nanosecond timescale, but this approach is limited to the excited state fluorescence lifetime. As a result, we measured the refractive index of the crowded solutions using an Abbe refractometer, and the results are shown in Figure 5. Our results show that the refractive index of
solution depends linearly on the concentration of glycerol, Ficoll, BSA and ovalbumin (Figure 5). It is worth noting that the protein crowding agents used here have a similar effect on the refractive index, which is slightly different from that of both Ficoll and glycerol. We also note that, unlike the viscosity results, the linear dependence of the refractive index was observed over the entire range of concentrations used in these studies.

4.2 The fluorescence decay rate of RhG in crowded solutions can be described satisfactorily by the Strickler-Berg equation

The Strickler-Berg equation (Equation 6) shows that the radiative rate constant for a given fluorophore depends on the squared refractive index of the surrounding medium such that (39):

\[
\tau_{r}^{-1} = k_{r} = 2.88 \times 10^{-9} n^{2} \int F(\tilde{\nu})d\tilde{\nu} \int \varepsilon(\tilde{\nu})d(\ln\tilde{\nu})
\]

This Strickler-Berg equation also shows that the radiative rate constant of a fluorophore can be estimated using steady-state absorption, \( \varepsilon(\tilde{\nu}) \), and emission, \( F(\tilde{\nu}) \), spectroscopy (in the frequency domain) in a given solvent.

![Figure 6](image.png)

**Figure 6.** The fluorescence decay rate of RhG is linearly dependent on the squared refractive index of the crowded solutions using glycerol, BSA, and ovalbumin. These measurements were carried out in a buffer (pH 7.4), as a function of the concentration of crowding agents, at room temperature using magic-angle detection. The concentrations used in these measurements are similar to those shown in Figure 4.

The fluorescence decay rate (\( k_{fl} = \tau_{fl}^{-1} \)) of RhG was measured directly using TCSPC techniques using the same experimental setup described above for time-resolved fluorescence anisotropy (with the exception of using magic-angle detection, \( 57^\circ \) polarization with respect to that of the excitation laser) (25, 33). Importantly, the fluorescence decay rate (\( k_{fl} = \tau_{fl}^{-1} \)) is basically the
The sum of both the radiative \( (k_r) \) and non-radiative rate constants of a \( (k_{nr}) \) fluorophore in a given solvent such that

\[
k_{fl} = \tau_{fl}^{-1} = k_r + k_{nr}
\]  

(7)

It is worth mentioning that the fluorescence quantum yield of a fluorophore in a given solvent is the ratio of the radiative rate constant to the fluorescence rate constant \( (k_f) \). Combining Equations (6) and (7) suggests that the intercept in Figure 6 represents the nonradiative rate constant of a fluorophore in a given crowded solution. As shown in Figure 6, the fluorescence decay rate of RhG is linearly dependent on the squared refractive index of the crowded solutions using glycerol, BSA, and ovalbumin. Importantly, the results also suggest that Strickler-Berg equation remains valid in both homogeneous and heterogeneous environments surrounding RhG.

4.3 The fluorescence anisotropy of RhG decays as a single exponential in glycerol-enriched buffer.

Our rationale for using time-resolved anisotropy measurements is to take advantage of the fast-time restriction imposed on the observed processes associated with the molecular ensemble in a crowded environment (see Figures 2 and 3). Such temporal restrictions will limit slower processes such as translational diffusion, chemical reactions, and confined transport. As a result, time-resolved anisotropy measurements provide a snapshot of existing species such as free molecular probes in buffer-like confined pockets among the population of crowding agents that form the excluded volume, the probe complexes with a crowding agent, and weak interactions between the probe and crowding agents (Figure 1). These molecular snapshots associated with rotational diffusion will serve as a guide for modeling the translational diffusion measured using FCS.

Typical anisotropy decays of RhG in a buffer are shown in Figure 7 as a function of the glycerol concentration (0–900 g/L). These anisotropy decays are described satisfactorily using a single exponential decay within this concentration range as shown on the logarithmic plot (Figure 7, bottom). Under the same experimental conditions, the rotational diffusion of free RhG in a buffer is 130 ± 10 ps at room temperature, which was also used to determine the geometrical factor based of tail-matching approach (40). It is worth noting that the corresponding viscosity range associated with these glycerol concentrations spans the range of 1–42 cP. These results confirm the existence of a single species (monomeric RhG) in glycerol-rich buffers, which is expected because of the homogeneity of such environment.
Currently we are examining whether the corresponding rotational diffusion coefficient of RhG obeys the Stokes-Einstein model over this viscosity range. Previous studies using NMR spectroscopy have shown this to be the case only up to 3.8 cP in glycerol-rich solutions (23, 41). It is conceivable that a molecule undergoing fast rotational diffusion in a very viscous environment may not experience the same bulk viscosity perhaps due to additional factors such as sticking. As a result, it will not be surprising that the measured rotational diffusion coefficient may deviate even slightly from the predicted value using the Stokes-Einstein model based on the bulk viscosity. Such deviations would likely be beyond the uncertainty of time-resolved fluorescence anisotropy because the rotational time at 900 g/L remains close enough to the excited state lifetime of RhG (~3.9 ns). This ratio of rotational time to the fluorescence lifetime seems reasonable for reliable usage of time-resolved anisotropy decays in crowding studies. Critically, these measurements within the stated viscosity range will be useful towards rotational diffusion studies of biomolecules in living cells aimed at quantitative analysis of association reactions and conformational changes using similar techniques.

4.4 The fluorescence fluctuation autocorrelation of RhG indicates a single diffusing species undergoing intersystem crossing in a glycerol-enriched buffer

Translational diffusion of single molecules such as RhG would span a larger spatial and temporal domain as compared with that of rotational diffusion. As a result, the reported complementary
measurements using both time-resolved anisotropy and fluorescence correlation spectroscopy are a valuable means to address the scaling aspect of diffusion in crowded environments. Typical fluorescence fluctuation autocorrelation curves of RhG are shown in Figure 8 as a function of glycerol concentration (0–900 g/L). Under our experimental conditions, both diffusion and, to some extent, intersystem crossing to the triplet state of RhG are responsible for the observed fluctuations. The amplitude ratio of the fluctuation component due to the intersystem crossing is ~15% with an estimated triplet state lifetime of tens of microseconds.

The fitting function under these assumptions was satisfactory in describing the fluctuation autocorrelation of RhG over the viscosity range of 0–45 cP, which corresponds to 0–900 g/L of glycerol. The results also show an apparent increase in the translational diffusion time of RhG throughout the open observation volume as the glycerol concentration (and therefore the corresponding viscosity) increased.

Currently we are calculating the corresponding translational diffusion coefficient of RhG as a function of the viscosity of the glycerol-enriched solutions, which will enable us to examine directly the validity of the Stokes-Einstein model over this viscosity range. Previous studies have shown this to be the case only up to 3.8 cP in glycerol-rich solution using NMR spectroscopy (23, 41). The extended range of viscosity investigated here, along with the sensitivity of fluorescence spectroscopy, will complement those NMR studies. In addition, the comparison between translation and rotational diffusion in a highly viscous environment will help us test the limits of the Stokes-Einstein model using both FCS and time-resolved fluorescence anisotropy methods.

We also observed that the fluorescence fluctuation of RhG in Ficoll and protein-crowded solutions are described satisfactorily using a number of autocorrelation functions such as two diffusion species, anomalous diffusion model, and the three-dimensional diffusion model.
multiplied by an exponential term similar to that of triplet-state population. Our time-resolved anisotropy measurements, however, samples existing species in the crowded environment and therefore provide guidance to which autocorrelation function to use for FCS measurements. A full account of these analyses and other measurements using the Ficoll and protein crowding agents will be described elsewhere.

5. CONCLUSIONS

We demonstrate here that fluorescence correlation spectroscopy and time-resolved fluorescence anisotropy are useful tools to investigate the effects of crowding on a fluorescent tracer molecule. We observe no effects of the crowding agents on refractive index. Taken together, these experimental approaches are promising for use to distinguish between the longer-range and shorter-range effects of crowding on a tracer. These measurements within the stated viscosity range will be useful towards understanding the rotational diffusion of biomolecules in living cells that are aimed at quantitative analysis of association reactions and conformational changes using similar techniques.

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