

Probing Diffusion and Viscosity in Multiphase Coacervates and Cellular Condensates with Raster Image Correlation Spectroscopy

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(Received 10 November 2025; accepted 16 June 2026; published 27 July 2026)

Raster image correlation spectroscopy (RICS) is a nondestructive, fluorescence-based method to probe molecular diffusion and effective viscosity in biomolecular condensates. By measuring fluorescence intensity autocorrelations of fluorescent probes in a scanning confocal microscope image, RICS enables the calculation of effective, local condensate viscosities both in reconstituted condensates and in living cells. Here we demonstrate the use of RICS as a powerful tool to probe local diffusion and length-scale-dependent viscosities in multiphase coacervates and stress granules without disrupting them. We quantify the local viscosity in core and shell regions of multiphase coacervates, and inside stress granules and the surrounding cytoplasm in HEK293T cells without relying on labeled proteins. By comparing different probe types, we further show that globular probes such as folded proteins and quantum dots experience a length-scale-dependent viscosity in condensates, whereas dextran-based polymeric probes exhibit an apparent size-independent diffusivity over three orders of magnitude in molecular weight. We attribute these findings to a progressive collapse with increasing size of the polymer chains inside condensates, combined with a weakening of sticky probe-condensate interactions. These findings emphasize the need for due care when using probes to characterize condensate properties. Altogether, our results show that RICS is a useful tool for studying condensate properties *in situ* that can be accessed on conventional confocal microscopes.

DOI: [10.1103/msq9-61tz](https://doi.org/10.1103/msq9-61tz)

I. INTRODUCTION

Liquid-liquid phase separation of biomolecules into condensates has emerged as a key mechanism in cellular organization, capable of concentrating proteins and nucleic acids, modulating biochemical reactions, and regulating signal transduction pathways [1,2]. Recent findings suggest that dysregulation of biomolecular condensate formation and alterations in the physical properties of condensates may be linked to various types of diseases [3]. However, a detailed characterization of microscopic condensate properties is often lacking, obscuring the mechanistic understanding of this link [4–8]. Furthermore, knowledge of condensate viscosity is often crucial for determining other condensate properties, such as interfacial tension in optical tweezers [9,10], and zeta-potential determination via microelectrophoresis [11].

The viscosity of condensates is a property that depends on the length scale at which it is probed, owing to their supramolecular structural organization. Nanoviscosity is the viscosity experienced by nanometer-sized probes [12,13], as opposed to (macro)viscosity or bulk viscosity, which is the viscosity experienced by probes with sizes on the micron scale or larger. Both microrheology using microparticles and

rheometry applied to condensates measure the latter, bulk viscosity. However, nanoviscosity is a key condensate parameter because most client molecules that partition into and out of condensates, including small proteins, peptides, RNAs, and metabolites, have hydrodynamic radii of only a few nanometers. Recent single-molecule FRET and MD simulations by Galvanetto *et al.* show that the apparent viscosity indeed decreases as the probe size decreases [14]. The diffusion of the smallest probes is thus largely dictated by the nanoviscosity and can have direct effects on reaction rates that are diffusion limited, such as some enzymatic reactions.

However, techniques to probe condensate viscosity in small and often multiphasic bodies that also take into account the dependence on length scale are currently lacking. Here we demonstrate that Raster Image Correlation Spectroscopy (RICS) can be used as an accessible, fluorescence-based approach for measuring spatially resolved molecular diffusion within biomolecular condensates, both in reconstituted condensates and in condensates in living cells [15,16].

The most commonly used technique to study diffusion in condensates is Fluorescence Recovery After Photobleaching (FRAP). FRAP is frequently used to quantify probe diffusion coefficients in condensates and to distinguish between mobile and immobile molecular fractions [17,18]. However, FRAP has limitations, including noncontinuous diffusion measurements, potential cytotoxicity from high-intensity laser exposure, and sensitivity to the chosen analysis model [18]. Fluorescence Correlation Spectroscopy (FCS) is another technique that can measure diffusion coefficients, but typically requires nanomolar concentrations and specific instruments, and does not easily allow for areas of interest [14,19].

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In contrast to these techniques, RICS enables continuous measurements at the same site over time without requiring photobleaching, making it less invasive and potentially suited for studying dynamic changes. Additionally, it has a wider range of compatible concentrations, and its ability to resolve spatial differences in diffusion coefficients provides further advantages.

RICS is a versatile fluorescence microscopy technique developed by Digman and coworkers in 2005 [15,16,20] that can be used to measure diffusion of fluorophores in a large variety of contexts [15,21]. For instance, Hendrix has shown that the technique can be used for measuring diffusion coefficients of GFP in cytoplasm [22]. It is based on analyzing spatiotemporal correlations in fluorescence intensity fluctuations across a series of images. The intensity autocorrelation function provides information on both the average number of molecules within the observation volume and their diffusion coefficients. Unlike FCS, which relies on a static focal volume, RICS scans the sample using a known pixel dwell time (τ_p) and line scan time (τ_l), allowing for the measurement of a broad range of diffusion coefficients [16,20]. Furthermore, Arbitrary-Region RICS (ARICS) extends this approach by enabling spatially resolved diffusion measurements within arbitrary regions of interest [23].

ARICS is particularly suited for measurements of local probe concentrations and viscosities in condensates with complex architectures or in complex environments that are difficult to probe with other techniques. For instance, multiphase condensates can be analyzed by splitting the frame into multiple regions of interest to analyze one phase at a time. ARICS is also well suited for analyzing biomolecular condensates in cells, to separate the condensate area from the rest of the cell. In addition, probing the local viscosity of biomolecular condensates, such as stress granules, inside living cells is challenging with FRAP and FCS.

In this study, we demonstrate that ARICS can be used to quantify the local viscosity of condensates with up to four orders of magnitude variation in viscosity. We use a tunable arginine-rich peptide/polyU model system and find that the local viscosity in these systems is insensitive to additives such as ATP and client proteins such as NPM1. We then use ARICS to analyze the viscosity of multiphase condensates and stress granules in HEK293T cells. Finally, we investigate the effect of probe type and size on the observed diffusion in condensates. Interestingly, we find distinctly different size-dependent diffusion for globular and polymeric probes. Our results underscore the versatility of ARICS for probing condensate properties, while highlighting the need for careful selection of probes and analysis of their dynamics.

II. METHODS

A. Chemicals

All reagents were purchased from Sigma-Aldrich unless mentioned otherwise. [RGXGG]₅ variants were custom ordered from GenScript as TFA salts with $\geq 95.0\%$ purity. Poly-D,L-lysine(100) and poly-D,L-glutamic acid(100) were obtained from Alamanda Polymers. Grafted poly-L-lysine PEG, PLL(20)-g[3.5]-PEG(2), was purchased from SuSoS

AG (Switzerland). Fluorescently labeled dextrans (FITC-dextran: 10 k, 40 k; TRITC-dextran: 4.4 k, 70 k, 155 k, 500 k, 2000 k; purchased from Fisher Scientific) were used for size-dependent diffusion studies. Branching is mostly unknown for the probes used; however, lower MW dextrans are known to have less branching. Globular probes used for length-scale-dependent viscosity, in order of molecular weight, were aprotinin-AF488 (AF488-NHS ester from Lumiprobe GmbH, Aprotinin from Sigma-Aldrich), ubiquitin-AF488 (AF488-NHS ester from Lumiprobe GmbH, ubiquitin from Sigma-Aldrich), NPM1 conjugated with Alexa Fluor 555 (from Yewdall *et al.* [24]), NPM1-EGFP (from Yewdall *et al.* [24]), Albumin-TRITC (Sigma-Aldrich), and CdSe/ZnS core-shell type quantum dots (Sigma-Aldrich). Molecular weight of CdSe/ZnS quantum dots was estimated to be ~ 1300 kDa, based on a 1 nm ZnS shell and a density of 4.10 g/cm^3 for ZnS and 5.81 g/cm^3 for CdSe.

B. RICS setup and data acquisition

The RICS analysis was performed using a Leica SP8 confocal microscope equipped with a single-photon detector and 18-well chambered polymer coverslips (Ibidi GmbH, Germany). Samples were left to equilibrate and settle for approximately 20 min prior to measuring. All measurements were taken with a $63\times$ oil immersion objective, at 10 and/or 100 Hz line scanning speed depending on the viscosity of the sample, with the latter being used for low viscosity measurements. The frames were digitally zoomed in until the pixel size reached 20 nm, and images were taken at 256×256 pixels. Measurements were taken with 15 frames per data point. RICS analysis was performed using MIA and MIAfit from the PAM software [25]. One of two corrections was performed on the data to prevent insoluble aggregates from affecting the analysis. Depending on the movement of the droplets during measurement time, the correction was either done by subtracting the average pixel value and adding the mean frame value, or by subtracting a moving average of 15 pixels over four frames. The autocorrelation function (ACF) data were fitted using the RICS 3D diffusion model.

The temperature of our samples was determined using a temperature probe in the sample solution as the laser was active and found to be approximately 24°C . Determination of the lateral (w_r) and axial (w_z) radii was done by analyzing the PSF of 200 nm fluorescent beads (Fluoresbrite Multifluorescent Microspheres, $0.20 \mu\text{m}$ diameter) in PSFj, taking the geometric mean, and converting the FWHM to $1/e^2$ [26]. We find $w_r = 0.216 \pm 0.004 \mu\text{m}$ and $w_z = 0.713 \pm 0.025 \mu\text{m}$ for our setup.

Concentrations calculated from RICS analysis were done using the average number of molecules in the excitation volume N , according to $c = \frac{N}{N_A \cdot V}$. The excitation volume V was calculated with $V_{\text{eff}} = \pi^{\frac{3}{2}} w_r^2 w_z$ [27] to be $0.19 \mu\text{m}^3$. Length-scale-dependent viscosity was analyzed using a two-layer depletion model, implemented with custom Python scripts.

C. Slide modification

The 18-well chambered glass coverslips (Ibidi GmbH, Germany) were modified using PLL-g-PEG. Slides were cleaned

in a plasma cleaner prior to overnight incubation with PLL-g-PEG at 0.01 mg/ml. Wells were washed with Milli-Q water after removal of PLL-g-PEG and left to dry before use.

D. Condensates preparation

[RGXGG]₅/polyU condensates were formed under the following conditions unless mentioned otherwise: 5 mM peptide and equal charge ratio polyU, 50 mM HEPES (pH 7.4), 150 mM NaCl, and 10 nM Alexa 488, or 0.01 mg/ml labeled dextran. pLys/pGlu condensates were prepared at 0.49 mg/ml and 0.36 mg/ml respectively, in the same buffer conditions.

E. Labeling of proteins

Aprotinin and ubiquitin were labeled with AF488-NHS ester by mixing the protein with the dye dissolved in anhydrous DMSO at 1:10 ratio in pH 8.0 HEPES. Mixtures were incubated overnight at 4 °C in the dark. Excess dye was removed by using centrifugal filters with 3 kDa MWCO.

F. Multiphase condensates

Two distinct condensates types (pLys/UTP and R₁₀/UTP) were individually prepared and adjusted to the same salt concentration before being combined in a separate tube. Multiphase condensates were formed at final concentrations of 2.5 mM pLys, 5 mM UTP, 50 mM HEPES (pH 7.4), 5 mM MgCl₂, and 30 mM NaCl. Gentle pipetting was done (3×) before each measurement to ensure mixing of the components.

G. Preparation of stress granule samples

Cells from a HEK293T line with stable expression of G3BP1-APEX-GFP fusion protein were seeded in a 0.4 single-channel Ibidi slide two days before microscopy. A 100 µl of 5000 cells/ml suspension in DMEM was used for seeding. Formation of stress granules was induced by replacing medium with DMEM containing 400 µM sodium arsenite, and microscopy was performed immediately after.

III. RESULTS AND DISCUSSION

A. Quantification of *in vitro* condensate viscosity using RICS

RICS operates by scanning a sample and computing spatial correlations in fluorescence intensity, thereby generating an autocorrelation function (ACF) that reflects both the diffusion coefficient (D) and the average number of molecules (N) within the excitation volume [Figs. 1(a) and 1(b)]. Spatial lags in x and y directions (ξ , ψ , respectively) have a characteristic lag time (τ_p and τ_l) based on imaging settings. The diffusion coefficient can be calculated as follows [Eq. (1)] [15,20]:

$$G(\xi, \psi) = \frac{\gamma}{N} \left(1 + \frac{4D(\tau_p \xi + \tau_l \psi)}{w_r^2} \right)^{-1} \times \left(1 + \frac{4D(\tau_p \xi + \tau_l \psi)}{w_z^2} \right)^{-1/2} \times \exp \left(-\frac{\delta r^2(\xi^2 + \psi^2)}{w_r^2 + 4D(\tau_p \xi + \tau_l \psi)} \right), \quad (1)$$

where w_r is the waist of the point-spread function (PSF) in the radial direction and w_z in the axial direction, which is assumed to be $3w_r$. The illumination correction factor γ is set to $\gamma = 2^{-\frac{3}{2}}$ for 3D Gaussian. Diffusion coefficient values can be converted to viscosity as experienced by the probe using the Stokes-Einstein relation $D = \frac{k_B T}{6\pi \eta r_p}$, where r_p is the hydrodynamic radius of the probe. Conversion of diffusion coefficient to viscosity through the Stokes-Einstein relation assumes free diffusion, which is a simplification. In condensates, probe motion can deviate from these assumptions due to transient interactions with the condensate scaffold, spatial heterogeneity, and viscoelastic network dynamics, all of which can make the measured diffusivity probe-, length-, and timescale-dependent. For this reason, we describe our converted diffusion coefficients not as nanoviscosity, but as effective viscosity (η_{eff}), as they are probe-dependent.

To test the use of RICS for measurements of viscosity in condensates we determined the effective viscosity of [RGXGG]₅/polyU model condensates, developed by Banerjee and co-workers [28], using Alexa Fluor 488 (AF488) as a small-molecule probe [Fig. 1(c)]. We use the term "effective viscosity" to describe the viscosity experienced by the probe, which is a result of the probe size-linked viscosity and (transient) chemical interactions with the condensate material. We tested sticker amino acids (X) with varying interaction strengths ($S < R < F < Y < W$), and hypothesized that stronger attractive interactions (electrostatic, cation- π , π - π) would increase effective viscosity. We indeed observed that stronger interactions result in increased viscosity, with measured values spanning ~ 0.1 to 60 Pa s (example fits are shown in Supplemental Material 1 [29]). The trends and calculated effective viscosities for the different stickers agree with literature where optical tweezers were used to determine condensate viscosity [28]. Apart from [RGSGG]₅/polyU, we find approximately four times lower effective viscosity within the condensates than their bulk viscosity. Differences in absolute measured viscosity are mostly due to the difference in measuring effective viscosity or macroviscosity; while we probe the movement of guest molecules on nanoscopic scale, optical tweezers with microscopic beads probe the bulk properties of the material, leading to similar trends but shifts in magnitude.

Additionally, mixing [RGYGG]₅ with [RGSGG]₅ yielded condensates with intermediate and tunable effective viscosities that follow a quadratic scaling law [Fig. 1(d)]. Using this model system, we also measured how adding relevant biological molecules, such as ATP and a protein (nucleophosmin-1, NPM1) influenced condensate effective viscosity. Figures 1(e) and 1(f) show that the addition of ATP had no effect on the condensate effective viscosity, in spite of its reported role as a hydrotrope in condensates consisting of hydrophobic proteins [30]. NPM1 only slightly reduced the condensate effective viscosity. We hypothesize that ATP is too small to displace polyU for interaction with the peptides, so it leaves the effective viscosity unchanged. By contrast, NPM1 may be able to bind to the polyU through its disordered regions, lowering the peptide-RNA interactions, leading to a decrease in effective viscosity.

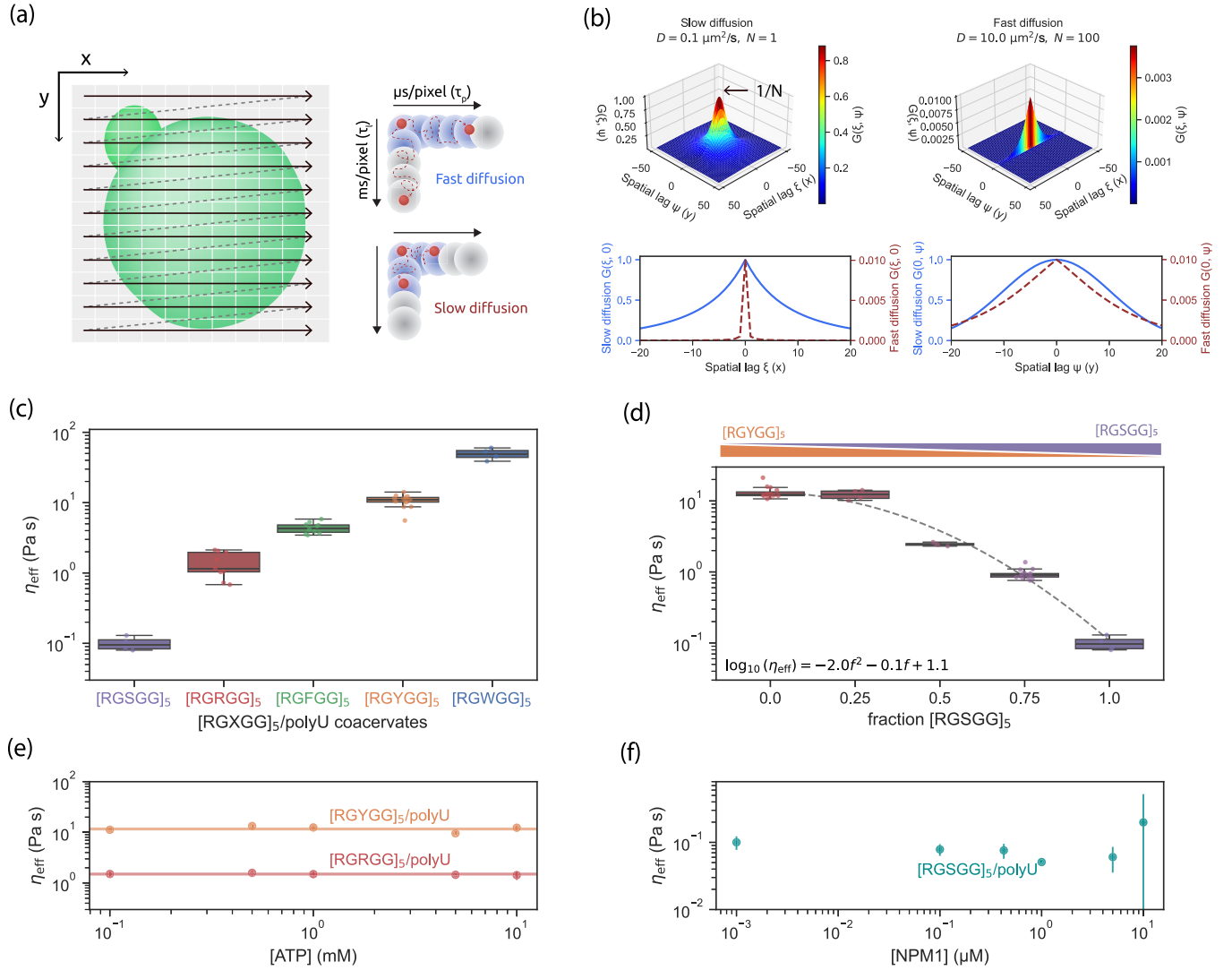


FIG. 1. Effective viscosity of [RGXGG]₅/polyU condensates as measured by RICS. (a) A schematic illustration of RICS. A confocal laser scanning microscope scans over an image with a fixed time between pixels (τ_p) and between lines (τ_l). Depending on the diffusion speed, molecules are more likely to be seen in adjacent pixels (fast diffusion) or in subsequent lines (slow diffusion). (b) An autocorrelation curve G is calculated for spatial lag in the y direction (ψ) and x direction (ξ). Slow and fast diffusing molecules lead to different autocorrelation curves, which can be fit to determine the diffusion coefficient. The peak of the autocorrelation curve is inversely correlated with the number of molecules in the observation volume. (c) Measured effective viscosity for [RGXGG]₅/polyU condensates using freely diffusing Alexa Fluor 488 partitioned into the droplets. (d) Mixing of two distinct peptides, [RGSGG]₅ and [RGYGG]₅, leads to intermediate effective viscosities that follow a quadratic scaling law; fit line and equation are shown. (e) Addition of ATP to the condensates leads to no changes in the effective viscosity. Error bars represent the standard deviation; horizontal lines are drawn to guide the eye. (f) Nucleophosmin-1 (NPM1) can slightly alter the effective viscosity of [RGSGG]₅/polyU condensates. Error bars represent the standard deviation.

B. Spatially resolved viscosity measurement of multiphase condensates and stress granules

Having demonstrated RICS-derived viscosities against established peptide-RNA models, we next turned to more complex contexts. Characterizing viscosity in multiphase condensates is challenging, particularly within inner phases [31]. ARICS, however, provides a direct and nondestructive method for the determination of diffusion, and thus effective viscosity, for fluorophores within both the inner and outer phases of multiphase condensates [Fig. 2(a)]. We measured the diffusion, and, consequently, the effective viscosity of both phases of pLys/UTP with R₁₀/UTP multiphase condensates, using

free AF488 as a probe [Figs. 2(b) and 2(c)]. Furthermore, we determined the probe concentration in both phases using the average number of fluorophores in the observation volume (N) and a calculated excitation volume [Fig. 2(d)]. Our data show that the inner phase exhibits over an order of magnitude higher effective viscosity than the outer phase, highlighting the distinct microenvironments within multiphase condensates. To demonstrate the utility of ARICS in complex cellular environments, we applied it to HEK293T cells expressing G3BP1-GFP [Fig. 2(e)]. G3BP1 is a key protein involved in stress granule formation [32]. Stress granule formation upon arsenite treatment is widely used as a model system for cellular condensate analysis [33]. Our RICS

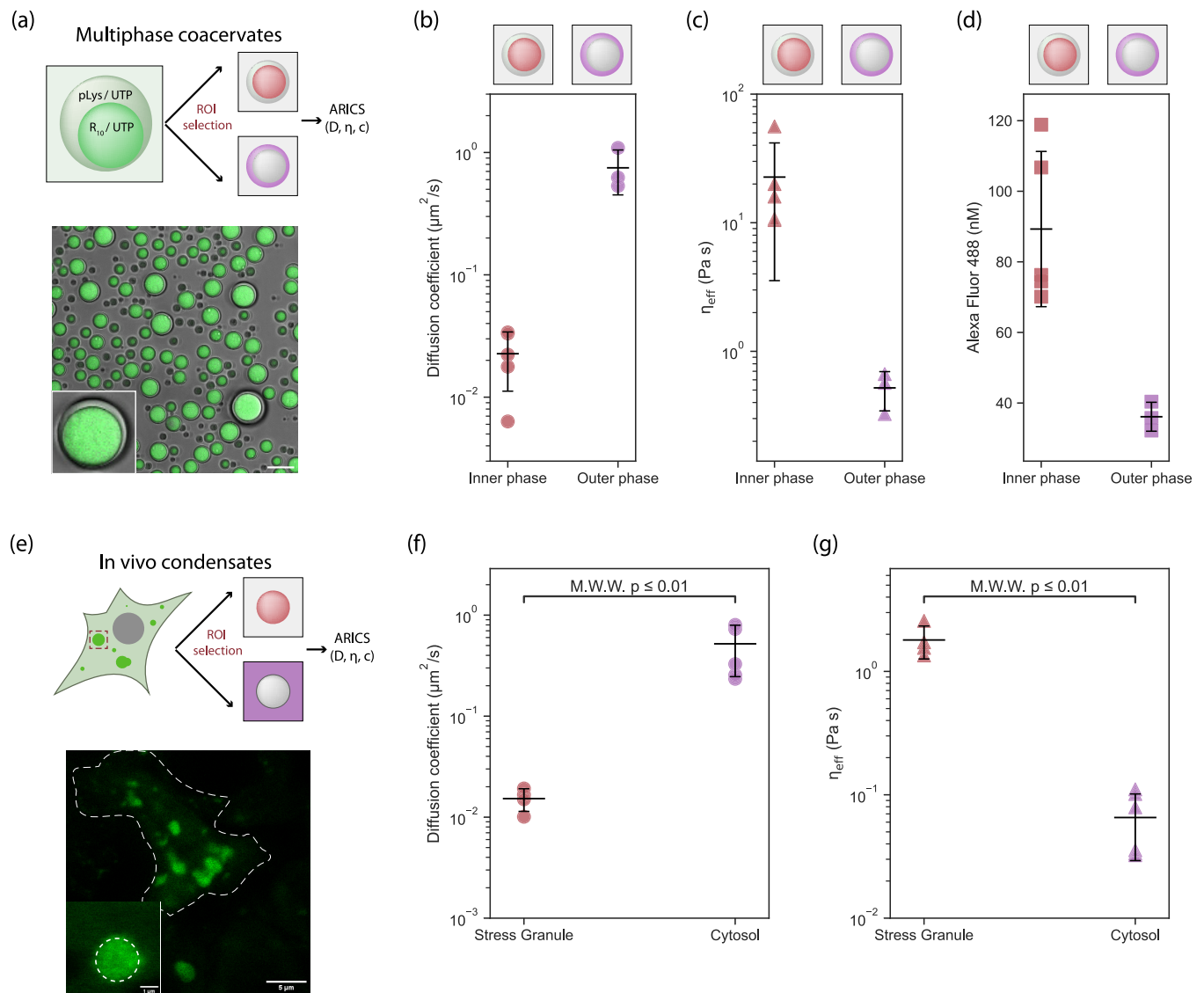


FIG. 2. ARICS analysis of multiphase coacervates and cellular G3BP1 stress granules. (a) ARICS enables the determination of the diffusion coefficient, effective viscosity, and AF488 concentration in both phases of multiphase condensates. Confocal microscopy of multiphase pLys/UTP R₁₀/UTP condensates with AF488 as free label (green). Scale bar = 20 μm. (b) Comparison of the diffusion coefficients found for the inner phase and outer phase, revealing over an order of magnitude difference ($n = 5$ for inner phase, $n = 3$ for outer phase). (c) Effective viscosity within multiphase pLys/UTP R₁₀/UTP condensates. (d) Quantification of AF488 concentration in each phase, derived from the average number of fluorophores in the excitation volume. (e) Representative image of G3BP1-GFP condensates inside HEK293T cells after stressing with arsenite. Inset: Example frame used in RICS analysis, with the white circle indicating the boundary between the condensed and dilute phases in the analysis. (f) Comparison of diffusion coefficients of G3BP1-GFP between the granules and the surrounding cytosol. (g) Calculated effective viscosities demonstrating a significant increase within stress granules. Statistical significance was tested using a two-sided Mann-Whitney U test. Black lines indicate mean and standard deviation.

measurements revealed that G3BP1-GFP diffuses at $\sim 0.02 \pm 0.01 \mu\text{m}^2/\text{s}$ within stress granules and $\sim 0.52 \pm 0.27 \mu\text{m}^2/\text{s}$ in the surrounding cytosol, roughly $25\times$ slower [Fig. 2(f)]. Assuming a hydrodynamic radius of G3BP1-GFP of 8.4 nm [34], we calculated the effective viscosity to be 1.80 ± 0.54 Pa s within granules in contrast to 0.07 ± 0.04 Pa s in the cytosol [Fig. 2(g)]. This is in line with single-molecule diffusion measurements of G3BP1 in stress granules ($\sim 0.04 \mu\text{m}^2/\text{s}$) [35]. While these results show that RICS can be used for measuring properties of condensates in cells, there is a practical limitation caused by movement of stress granules in

cells. Selection of time windows where droplet movement and deformation are minimal is important for obtaining unbiased autocorrelation functions.

C. Probe-size-dependent diffusion in condensates

Using diffusion measurements within condensates can help understand more properties of condensates, such as mesh size. Mesh size is a fundamental physical characteristic of biomolecular condensates arising from the interior entangled network that influences their selective permeability, diffusion

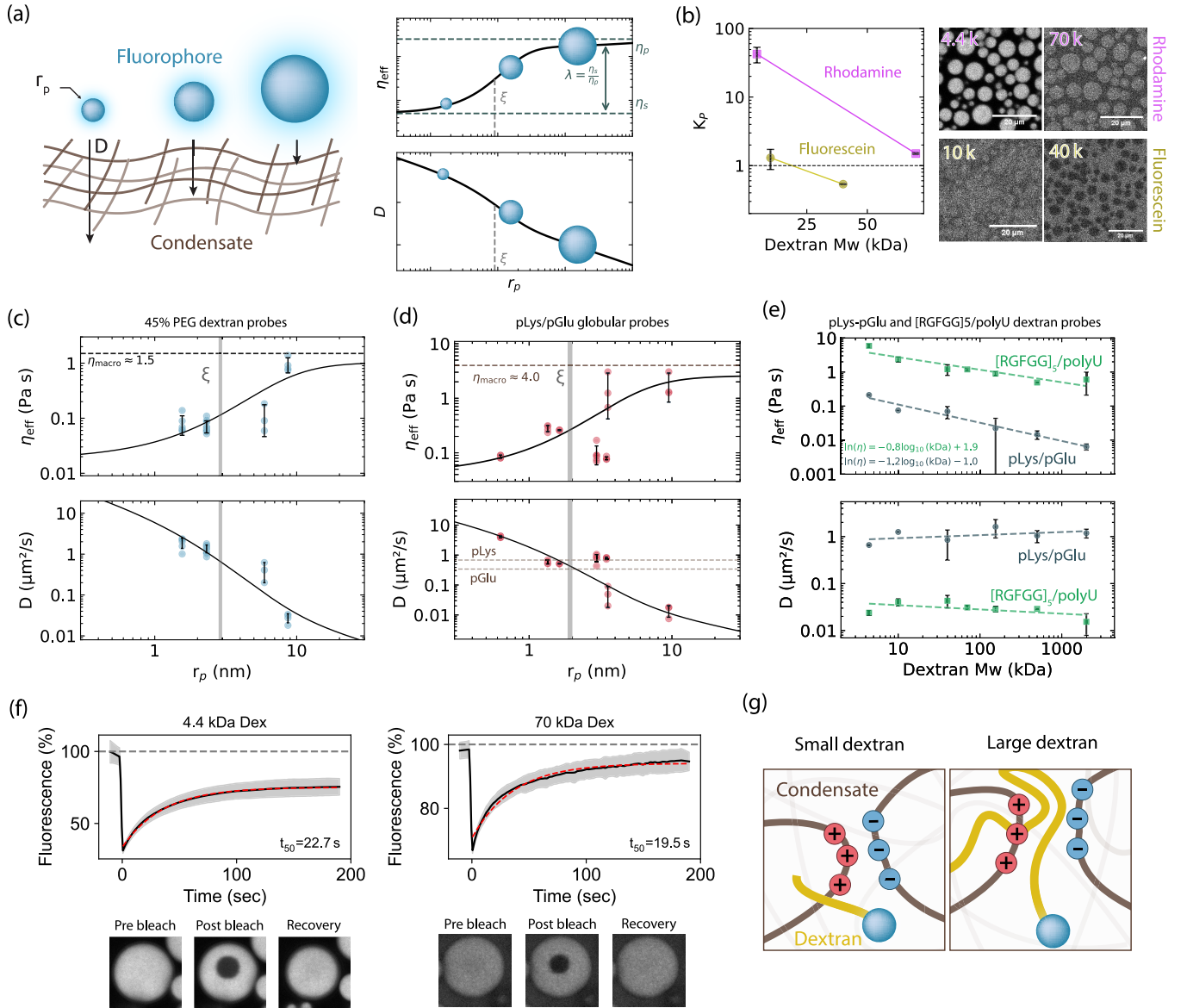


FIG. 3. Probe size-dependent viscosity in condensates. (a) Illustration of length-scale dependent viscosity. Probe size determines viscosity as movement through the condensate mesh becomes more limited, whereas probes smaller than the mesh size are able to diffuse better. (b) Partition coefficients of FITC and RITC labeled dextrans into pLys/pGlu condensates. Fluorescein labeled dextrans show lower partitioning than rhodamine labeled dextrans. (c) Length-scale dependent viscosity of fluorescently labeled dextran probes in 45% PEG (Mw = 20 k). Fit parameters: $\xi = 2.9$ nm, $\lambda = 0.01$. Macroscopic viscosity estimated from Ref. [44]. (d) Length-scale-dependent viscosity for Alexa Fluor 488 and globular probes in pLys/pGlu condensates. Globular probes in order of size: AF488, Aprotinin-AF488, Ubiquitin-AF488, NPM1-AF555, BSA-TRITC, NPM1-EGFP, CdSe/ZnS quantum dots. Fit parameters: $\xi = 1.93$ nm, $\lambda = 0.01$. Macroscopic viscosity is obtained from Ref. [45]. Lower: Dotted lines labeled pLys and pGlu show the diffusion coefficient of AF488-labeled pLys and pGlu. (e) Diffusion coefficients and viscosities of dextran probes in pLys/pGlu and [RGFGG]₅/polyU condensates. Power fits are shown ($R^2 = 0.96$ and 0.86 for the plot of the effective viscosity as a function of molecular weight of [RGFGG]₅/polyU and pLys/pGlu, respectively). (f) FRAP recovery curves of 4.4 k and 70 k dextran-TRITC in [RGFGG]₅/polyU condensates show similar recovery times ($n = 3$). Line shown is the average and area drawn indicates the standard deviation. (g) Schematic illustration of dextran probe in the condensate environment. Dextrans may remain mobile throughout the material by moving through transient gaps, lowering local effective viscosity, and clustering together. Error bars indicate the standard deviation.

dynamics, and biochemical activity [14,36,37]. The mesh size can provide functionality in condensates by selectively allowing small reactants that can form larger products, which are then released from the condensates [38]. The concept of length-scale-dependent viscosity (LSDV) predicts that

effective viscosity increases with probe size (r_p) when probes approach the mesh size (ξ) of the surrounding entangled network [36,39,40]. In this framework, guests inside the mesh are described as (solid) spheres [Fig. 3(a)] surrounded by depleted mesh polymers. We describe LSDV using the two-layer

depletion model of Tuinier and coworkers [41,42], which relates the probe diffusion coefficient to a depleted shell of thickness ξ around the probe

$$D = \frac{k_B T}{6\pi\eta_s\alpha g'(\lambda, \frac{\xi}{\alpha})}, \quad (2)$$

where α is the probe radius, $\lambda = \eta_s/\eta_p$, η_s is the local viscosity in the depleted shell, η_p the macroscopic viscosity, ξ the depletion thickness (taken as the mesh size), and g' the hydrodynamic correction for translational drag, evaluated using the closed-form Eq. (2) of Tuinier *et al.* [41]. The model assumes a simplified two-layer geometry and is most accurate when the probe is large relative to the mesh ($\xi/\alpha \leq 0.3$). Many guests, such as (partially) unfolded proteins or peptides, could be better described as chain-like molecules, which sphere-based LSDV models do not account for.

RICS is a suitable technique for measuring diffusion of probe shape and size inside condensates to determine the apparent mesh size. Previous studies have quantified condensate mesh sizes based on partitioning of probes of different sizes [36]. The crossover from inclusion to exclusion is then taken as an estimate of the mesh size, which is justified by the rationale that guests larger than the mesh size are excluded from the condensate. While this may be true for probes that do not interact with and can deform the mesh, it has already been shown that even small fluorescent labels can severely impact the partitioning behavior of guest molecules [43]. In addition, large proteins and nanoparticles have been found to be included in condensates, apparently counteracting this simple estimate of the mesh size [43]. Indeed, we observed that changing the fluorescent label used on identical guest molecules dramatically impacts the partitioning of these probes: rhodamine-labeled dextrans exhibit one to two orders of magnitude stronger partitioning than fluorescein-labeled dextrans [Fig. 3(b)]. The crossover from inclusion to exclusion is thus not an accurate estimate of the mesh size. Therefore, we set out to show that RICS can be used to measure length-scale dependent viscosities and estimate condensate mesh sizes.

We first provide a proof of concept that RICS can be used to measure length-scale dependent viscosities in a well-characterized model system of 45% (w/v) PEG (Mw = 20 k) and linear rhodamine-labeled dextrans as probes, for which LSDV has previously been observed [44]. As expected, we find an increase in effective viscosity with increasing dextran probe size [Fig. 3(c)] and find an apparent mesh size of ~ 2.9 nm using Eq. (2), which is close to literature values [44].

Next, we examined the diffusion of globular probes (labeled proteins and quantum dots; details in Table I) inside pLys/pGlu condensates. Similar to the 45% PEG solution, we observe a pronounced increase in effective viscosity and a drop in diffusion for larger probe sizes [Fig. 3(d)]. Using Eq. (2), we find a mesh size of the condensate of $\xi \approx 1.9$ nm, similar to previous reports for condensates in literature and our scaling estimate (Appendix 1) [36,46]. To further support our analysis, we also analyzed the diffusion of the same globular probes in a different model condensate, consisting of NPM1 and polyU RNA. In this system,

TABLE I. Probes used for length-scale dependent viscosity measurements.

Probe	r_p (nm)	Reference
Alexa Fluor 488	0.63	Ref. [47]
Aprotinin-AF488	1.35	Ref. [48]
Ubiquitin-AF488	1.63	Ref. [49]
NPM1-AF555	2.97	Estimated from M_w
BSA-TRITC	3.48	Ref. [50]
NPM1-EGFP	3.56	Estimated from M_w
CdSe/ZnS	9.5	Estimated from M_w
4.4 k Dex ^a	1.56	
10 k Dex	2.32	
40 k Dex	4.52	
70 k Dex	5.92	
155 k Dex	8.68	
500 k Dex	15.26	
2000 k Dex	29.74	

^aDextran sizes were estimated using supplier data and fitting to a power law to calculate unlisted sizes.

we also see a pronounced increase in effective viscosity for larger probe sizes (Appendix 2, Fig. 4) and obtain $\xi \approx 1.0$ nm. These results show that LSDV analysis can be used for both peptide-based (pLys/pGlu) model condensates and protein/RNA (NPM1/polyU) condensate models.

Surprisingly, when we used labeled dextrans instead of globular probes, we observed that the diffusion coefficients remained largely constant with increasing probe size [Fig. 3(e)], showing that sphere-based LSDV does not fully capture diffusion dynamics of polymeric probes in condensates, contrary to the measurements in 45% PEG. We also find this in [RGFGG]₅/polyU condensates and confirm our findings using FRAP, which showed similar recovery times for both 4.4 k and 70 k dextran [Fig. 3(f)].

The diffusion of the dextran probes was faster than the diffusion of AF488 labeled pLys and pGlu themselves, being 0.33 ± 0.07 and $0.49 \pm 0.50 \mu\text{m}^2/\text{s}$, respectively. We hypothesize that this is due to the dextrans having fewer and weaker attractive interactions with the condensate mesh than the scaffold peptides themselves. As a result, small dextrans (up to $R_G \sim \xi$ or $M_w \approx 26.4$ kDa) can move relatively unhindered through the holes in the condensate mesh. Nevertheless, we would expect much larger dextrans to diffuse more slowly and even become partly entangled in the network if they remain in a swollen or ideal chain conformation. The paradoxical fact that we see a much faster diffusion of large dextrans than expected leads us to hypothesize that the dextrans increasingly collapse into globules as their size increases, because the effective dextran-condensate interaction is moderately unfavorable [i.e., $0 < \chi_{\text{dex-cond}} < 0.5$; see also Fig. 3(b)]. This progressive collapse can be seen as a local phase separation, and makes the effective probe size smaller than our estimates of R_G [which we use in Eq. (2) for r_p] based on an ideal chain. It also leads to fewer sticky interactions between the dextran and condensate mesh per unit length, and a local reduction in the density of ion pairs between the condensate scaffold molecules. Thus, the nearly constant or slightly

decreasing diffusion coefficient with increasing dextran size for two different condensate model systems can be explained by a combination of decreased effective probe size and decreased local effective viscosity [Fig. 3(g)].

Our findings highlight that the probe used for determining viscosity in condensates significantly impacts discoveries. On top of probe-size effects, probe conformation also has to be taken into account. This has implications for reactions taking place in biological condensates involving different guest molecules. For example, guests that have little structure, such as DNA/RNA and globular probes, where mesh size is a critical parameter to determine diffusion coefficients, will experience different length-dependent viscosity, which can be limiting in diffusion-limited reactions.

IV. CONCLUSIONS

We have shown that Raster Image Correlation Spectroscopy (RICS) is a powerful and flexible method for measuring diffusion and effective viscosity in a range of biomolecular condensates. By applying RICS to both *in vitro* and live-cell systems, we were able to capture differences in molecular mobility across environments that vary in composition, structure, and complexity. Using these results, we gain new insights into the physicochemical properties of condensates.

Using peptide-RNA condensates, multiphase condensates, and stress granules in cells, we demonstrated that RICS can resolve both subtle and large changes in viscosity with spatial selectivity. We also found that diffusion of guest probes depends not only on the properties of the condensate, but also on the conformation of the diffusing molecule. We find that spherical, globular probes follow length-scale-dependent viscosity dynamics, whereas unfolded probes remain largely constant in their diffusion, likely due to weak interactions with the matrix and reduction in the density of interactions between the condensate mesh. This has implications for biological condensates, as different guests will experience condensate viscosity differently, and will be affected differently by changes (for instance in a liquid-solid-transition). For instance, this can affect the kinetics of diffusion-limited reactions, where small globular probes and large polymer-like reactants are less affected by their size, but larger globular reactants are limited by the mesh size.

These results establish RICS as a reliable, minimally invasive tool for studying the physical properties of condensates in a variety of contexts. It offers a widely accessible way to explore how environmental factors influence dynamics of guests within condensates, and to study physico-chemical properties of condensates themselves.

ACKNOWLEDGMENTS

This work was supported financially by a Vidi grant from the Dutch Research Council (NWO). HEK293T cells expressing G3BP1-APEX-GFP were obtained as a gift from Dr. Joep Joosten. Point spread functions were determined with the help of Dr. Jelle Postma.

B.S.V. and E.S. conceived the project and designed experiments. B.S.V. performed and analyzed experiments. I.B.A.S. and B.S.V. performed calibration experiments. W.P.L. prepared cells for stress granule experiments. B.S.V. and E.S. wrote the manuscript, with input and revisions from all authors.

DATA AVAILABILITY

The source data generated in this study have been deposited in the Radboud Data Repository [51].

APPENDIX

1. pLys-pGlu mesh size estimation

We estimate the mesh size using $\xi = R_G(c/c^*)^{-3/4}$, where R_G is the radius of gyration of the polymers, c the total concentration, and c^* the overlap concentration ($c^* = 1/V_{\text{coil}}$) [52]. For ideal chains, R_G is given by $\sqrt{\frac{L_c b}{6}}$, and $V_{\text{coil}} = \frac{4}{3}\pi R_G^3$. Using a contour length (L_c) of 36.5 nm and a Kuhn length (b) of 1.2 nm [53], results in a mesh size of ~ 2.1 nm for pLys/pGlu condensates.

2. NPM1/polyU length-scale-dependent viscosity

Effective viscosity for probes of various sizes in NPM1/polyU droplets. Droplets were prepared as described in Ref. [11]. Fit parameters are $\xi = 1.0$ nm, $\lambda = 0.0001$.

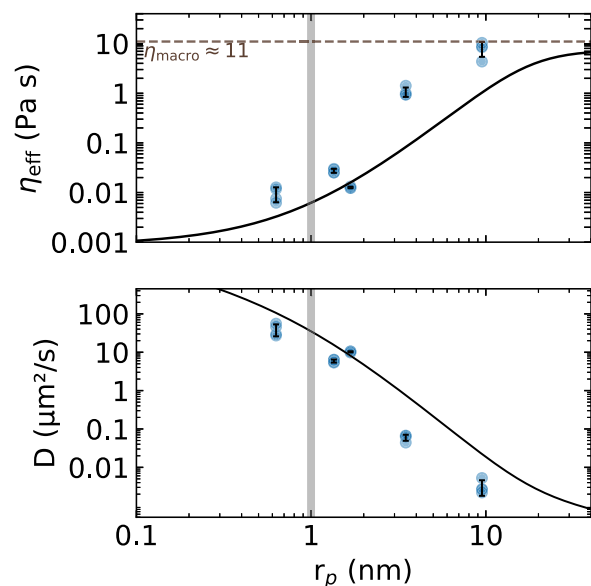


FIG. 4. LSDV of globular probes in NPM1/polyU condensates. Effective viscosity (top) and diffusion coefficient (bottom) as a function of probe hydrodynamic radius for AF488 and the globular probes listed in Table I. The solid line is a two-layer depletion model fit; the vertical band denotes the fitted mesh size (ξ). Error bars indicate the standard deviation.

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