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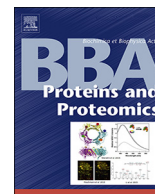
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Review

Single-molecule fluorescence studies of intrinsically disordered proteins and liquid phase separation

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ABSTRACT

Intrinsically disordered proteins (IDPs) are ubiquitous in proteomes and serve in a range of cellular functions including signaling, regulation, transport and enzyme function. IDP misfunction and aggregation are also associated with several diseases including neurodegenerative diseases and cancer. During the past decade, single-molecule methods have become popular for detailed biophysical and structural studies of these complex proteins. This work has included recent applications to cellular liquid-liquid phase separation (LLPS), relevant for functional dynamics of membraneless organelles such as the nucleolus and stress granules. In this concise review, we cover the conceptual motivations for development and application of single-molecule fluorescence methods for such IDP studies. We follow with a few key examples of systems and biophysical problems that have been addressed, and conclude with thoughts for emerging and future directions.

1. Introduction

A long-standing paradigm in biology was that well-defined 3D structures are essential for proteins to perform their functions in the cell. A rapidly expanding body of work over the past couple of decades has challenged this idea [1,2], revealing that a substantial portion of the cellular proteome is comprised of proteins and protein regions that do not strongly encode well defined structures. Nevertheless, these intrinsically disordered proteins (IDPs) and regions (IDRs) are involved in numerous cell functions, with their flexibility and interaction promiscuity often thought to play critical roles in function. Note that in this review (as is often the case in the field), we will use the term IDP to refer to proteins that are predominantly unstructured throughout their sequence as well as proteins containing a mix of IDRs and structured regions. Virtually every class of cellular function is represented by IDPs, including transcription and translation, signaling, transport, and enzyme function. Furthermore, many aspects of cellular misfunction and disease are also associated with protein disorder, including neurodegenerative and other protein aggregation diseases, cancer, viral infection and heart disease.

In recent years, IDPs have also been shown to be important players in cellular liquid-liquid phase separation (LLPS) [3–6]. Phase separation in biology has exploded onto the scene during the past 10 years due to the observation that LLPS underlies the formation and dynamics of

many membrane-less organelles (MLOs) and their roles in essential cellular processes. These macromolecular assemblies generally contain a combination of proteins and nucleic acids, and their formation requires weak multivalent interactions between these component molecules. Of particular interest is that a substantial fraction of the phase-separating proteome contains intrinsically disordered proteins with low-complexity, often repetitive, amino-acid sequences. This is not coincidental, as the disordered nature of these proteins gives them the biochemical flexibility to interact, both specifically and non-specifically, with a wide range of phase-separating molecules. The physical and chemical properties of IDPs, which have been extensively studied over the past few decades, help give membrane-less organelles their diversity in composition, function, and physical properties. This has made the phase separation of IDPs an exciting area of study for biophysicists.

Given the broad importance of IDPs in biology, the physics and chemistry of these proteins are interesting and critical to understanding their function in biology and disease. However, the complexity and flexibility of these proteins makes them hard to study using traditional methods of structural biology and biophysics. Therefore, a major effort in the field has been towards adapting or developing alternative methodologies for studies of these systems.

Along these lines, single-molecule methods have revolutionized studies of complex systems over the past 3 decades and have been

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recently applied to IDPs. These methods can reveal detailed information about protein structure and dynamics that is usually hidden by averaging over an ensemble of molecules [7–9]. In 2006, Crick et al. reported the size scaling behavior in the disordered poly-Q system studied using fluorescence correlation spectroscopy (FCS) [10], a method related to single-molecule fluorescence methods. A year later, Mukhopadhyay et al. reported single-molecule fluorescence studies of the yeast prion protein Sup35, revealing new structural and dynamic features of this system [11]. Since then, other single-molecule studies have probed several such features in a number of IDP systems.

Single-molecule methods can be broadly categorized into fluorescence and manipulation techniques [9]. Single-molecule fluorescence methods include measurements of energy transfer, intensity, fluctuations, and spatial variations which can provide equilibrium or dynamic information about conformation, binding, proximity, size, spatial arrangement, movement and other molecular parameters. For example, one of most commonly used methods is single-molecule Förster resonance energy transfer (smFRET). This method can provide information about conformational distributions and dynamics as well as binding. smFRET has been widely used to study proteins and nucleic acids during the past two decades. The related method of fluorescence correlation spectroscopy (FCS) has used analyses of fluorescence fluctuations to provide measurements of dimensions and rapid molecular fluctuations in IDPs. These and other single-molecule fluorescence methods are discussed in more detail below in the context of specific problems and systems. Single-molecule manipulation methods such as optical and magnetic tweezers and atomic force microscopy (AFM) can also measure several molecular parameters, while simultaneously measuring the molecular responses to exerted forces [12–14]. For folding measurements, all these methods typically attach molecules between two surfaces (beads, AFM tip, quartz, mica, etc.) and forces are generated on the molecule by changing the separation between the surfaces. Different methods are used to measure forces and separation, permitting a range of experiments to be carried out [15].

In this short review, we focus on single-molecule fluorescence studies. The review is not meant to be comprehensive; rather it is meant to provide examples of key types of systems and insights that can be addressed using these methods. A series of examples of IDP studies are provided below, including a brief discussion about techniques used and emerging insights. Several of the systems discussed have been studied with respect to phase separation. However, the application of single-molecule methods to LLPS is still in its infancy. Hence, the complementary examples provided of other IDP studies provide a good overview of the various kinds of experiments that are feasible using these methods. We conclude with our perspectives for emerging and future directions. Several other review articles [16–19] and original papers are cited for broader and more detailed information.

2. A few examples – systems and biophysical problems

2.1. Poly-glutamine: size scaling behavior, water solvent quality and phase separation

Several neurodegenerative diseases are linked with aggregation of expanded poly-glutamine (Q) repeat sequences. The 2006 work by Crick et al. [10] probed polymer scaling behavior of several poly-Q polypeptides differing in glutamine repeat length using fluorescence correlation spectroscopy (FCS). FCS probes fluorescence fluctuations in a small ensemble (few) of molecules using confocal detection and was a precursor to single-molecule fluorescence detection. Several molecular parameters can be extracted by analysis of these fluorescence fluctuations. One of the most basic parameters for diffusing molecules is translational diffusion, which results in signal fluctuations as molecules move in and out of the focal volume. Crick et al. used the fact that translational diffusion times are related to hydrodynamic radii of peptides to study how dimensions scaled as a function of Q repeat length.

The dimensional scaling of long polymers in a solvent is influenced by a balance of polymer-polymer and polymer-solvent interactions. Accordingly, for long polymers in a solvent, the scaling constants assume three different values depending on whether the solvent is a good, neutral or poor solvent. This idea can be translated to the case of IDPs, which can (i) be extended (water is a good solvent) when chain-solvent interactions are favored over chain-chain interactions, (ii) behave like an ideal chain (water is a theta solvent) when chain-chain interactions balance chain-solvent interactions, or (iii) be collapsed (water is a poor solvent) when chain-chain interactions are favored. The measured scaling constant showed that water acts as a poor solvent and poly-Q polypeptides assume an ensemble of collapsed structures. Based on the results, the authors discussed multiple possibilities for poly-Q aggregation, including that it proceeds through formation of disordered oligomeric species, with solvent quality playing a key role in determining which mechanism is dominant. This idea of disordered oligomers is related to droplet formation during LLPS that has subsequently become a topic of intense study [20] as discussed in this review.

2.2. Sup 35-NM – monomer structural and dynamic features, aggregation and phase separation

Amyloidogenic IDPs are involved in several diseases including neurodegenerative and heart diseases. One example where amyloid formation was postulated instead to have a functional cellular role is the yeast prion protein Sup35. This protein is a translational termination factor in yeast. Amyloid formation was previously postulated to act as a regulator of function that could be transmitted over generations [21], and recently phase separation has been linked to cellular fitness [22] (see below).

In 2007, Mukhopadhyay et al. reported the results of a series of single-molecule studies on the amyloid forming (NM) region of Sup35 [11] (Fig. 1). smFRET experiments were first used to probe the overall characteristics of the amyloid forming region of this protein. FRET is the non-radiative transfer of singlet excitation energy from a donor to an acceptor dye. The strong distance dependence of the FRET efficiency has been used to provide a measure of conformation in proteins and other biomolecules. In these experiments, donor and acceptor dyes were attached at positions that spanned this region, and confocal smFRET experiments were used to study the conformational features of freely diffusing single molecules. This experimental mode, which was first developed [23] to test the distance dependence of FRET at single-molecule resolution, allowed studies of Sup35-NM without potential perturbations due to surface tethering, a mode discussed later that is often used in conjunction with imaging for single-molecule experiments. Here, sub-nM concentrations of dual labeled proteins were used in conjunction with the sub-fL detection volume to ensure that almost all signals originated from single molecules. The donor was excited using a laser, and donor and acceptor fluorescence photon counts were recorded as a function of time with sub-ms time-resolution. The single-molecule fluorescence bursts in the resulting time-trajectories were analyzed to produce smFRET histograms, which revealed populations of protein molecules with particular FRET efficiencies.

For Sup35-NM, the smFRET histograms revealed a relatively compact conformational distribution. The low concentrations used in these experiments (~100pM) should highly disfavor aggregation (Fig. 1A). However, to directly study this issue, a complementary single-molecule technique, 2-color coincidence [24], was used to test if the protein still formed small oligomers at these concentrations which might complicate the FRET analysis. Here, two protein samples were separately labeled with blue and red dyes. Once again, confocal detection was used, now with dual excitation. Since diffusion is random, these time traces would only show a significant population of 2-color bursts if the protein oligomerized. The results did not show such a distribution (Fig. 1B), ruling out the existence of a significant fraction of oligomers, and confirming that the above FRET results were indeed from monomers. An smFRET

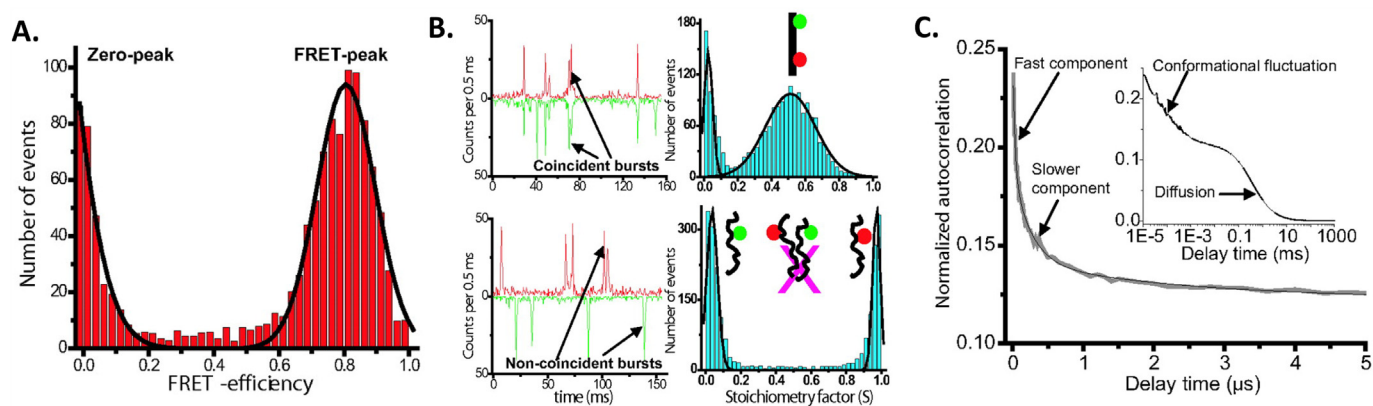


Fig. 1. Single-molecule fluorescence data for Sup35-NM. **A.** smFRET histograms under non-denaturing conditions. **B.** 2-color single-molecule coincidence analysis to rule out oligomerization. **C.** FCS analysis reveal fast fluctuations. Adapted from Mukhopahyay et al. Proc. Natl. Acad. Sci. (2007) 104:2649, Copyright 2007 National Academy of Sciences. See text for additional details.

denaturation analysis was next carried out and showed a single smFRET histogram peak gradually moving to lower FRET efficiency (revealing continuous protein expansion) as a function of denaturant concentration. This was in contrast with results on folded proteins which showed multiple interconverting peaks corresponding to folded and unfolded states [8,25], supporting the idea that Sup35-NM is intrinsically disordered and occupies a range of structures. The relatively narrow FRET peak (Fig. 1A) also indicated that these structures were rapidly interconverting relative to the 0.5 ms data acquisition time. This idea was directly tested using FCS experiments and quenching of attached dyes by nearby tyrosines, showing rapid fluctuations in the ~ 100 ns range. Overall, this set of experiments showed that Sup35-NM is intrinsically disordered and relatively compact (compared to a typical denatured protein) yet is rapidly sampling a range of conformations.

The above work had implications for the aggregation mechanism of the protein, with the authors speculating that aggregation might proceed through the formation of disordered oligomeric species. Initial tests of this idea were made using a combination of single-molecule and ensemble studies [26]. In fluorescence polarization experiments, samples are excited using polarized light and the depolarization of the emitted photons is measured. If the dyes rapidly tumble relative to the excited-state lifetime, the emitted light is depolarized, while increased residual polarization will be observed for slower tumbling dyes. Hence, this method can be used to probe the mobility imparted on the dye and attached IDP by its local environment. For Sup35-NM, the polarization data indicated that initial substantial changes in the environment occurred well before formation of amyloid structure was observed by conventional thioflavin-T fluorescence measurements. These early polarization changes coincided with changes in dye emission intensity due to changes in the environment of the dyes, presumably changing quenching by proximal tyrosine residues as discussed above. Together, these data were consistent with early formation of disordered oligomeric species, that eventually proceeded to form amyloids, one of the models discussed in the previous work by Crick et al.¹⁰

Recent work by Franzmann et al. has shed new light on the functional significance of phase separation of native Sup35 [22]. Using a variety of methods, the authors show that stress-induced pH changes induce liquid-liquid phase separation in Sup35. A liquid-like dense phase is first formed that then changes to a protective gel-like state. This sequence of events is reminiscent of the putative aggregation mechanisms discussed above. The authors also showed that the N-terminal prion domain was a major determinant of phase separation, and that certain charged sequence regions in the M-domain can regulate these phase transitions. Moreover, the C-terminal GTPase domain by itself forms irreversible aggregates in cells under stress and reduced cell fitness. Single-molecule fluorescence methods could be used to

probe various aspects of these interesting transitions, including inter- and intra-domain conformational properties in different phases of the protein system.

2.3. α -synuclein, a Parkinson's disease protein: monomer binding-folding complexity and aggregation

α -synuclein [27] is an IDP whose aggregation has been extensively implicated in Parkinson's disease. It has also been suggested to have functions in synaptic vesicle fusion and vesicle transport. Furthermore, the protein folds upon binding to partners such as membranes and membrane mimics [27]. This coupling of folding and binding is shared by many other IDPs and is often important for IDP function. In 2009, the Rhoades, Subramaniam and Deniz labs separately reported single-molecule FRET studies on this protein [28–30]. These reports showed that smFRET could be used to study details of the coupled folding and binding of this protein. As an example, an ongoing debate in the field raised questions about the precise helical structure the protein assumed on binding to a membrane-like surface - a hairpin-like shape or an extended helix. To test these possibilities directly, Ferreone et al. [30] labeled the protein in locations at the ends of the helical region to create a construct with which well-resolved high and low FRET efficiencies would be observed in the two possible aforementioned helical structures. A series of smFRET experiments encompassing titrations of several concentrations of the lipid-mimic SDS resulted in a 3D histogram that showed a striking series of complex transitions (Fig. 2). Increasing SDS concentration below the critical micelle concentration (CMC) of SDS showed that the system transitioned from an initial disordered state to a hairpin state and then to an extended state. Further titration to above CMC then resulted in a transition back to a hairpin state, and an even higher SDS concentration finally pushed the system again to extended helical states. This work showed that binding to either small amphipathic molecules or membrane-like surfaces of different curvature could result in hairpin and extended helical forms. Follow-up smFRET work using novel microfluidic methods showed that folding to the extended helix occurred following initial binding to SDS and formation of collapsed species, analogous to formation of disordered encounter complexes [31]. Nice examples of such dynamic intermediates have more recently been observed by smFRET in the folding pathways of other systems [32,33]. Several other smFRET studies of this interesting protein have followed. For example, the Rhoades lab has used smFRET to study allosteric effects arising from the floppy C-terminal region of α -synuclein [34]. They also combined smFRET with computational analysis to further characterize in detail the conformational ensembles of this protein [35,36].

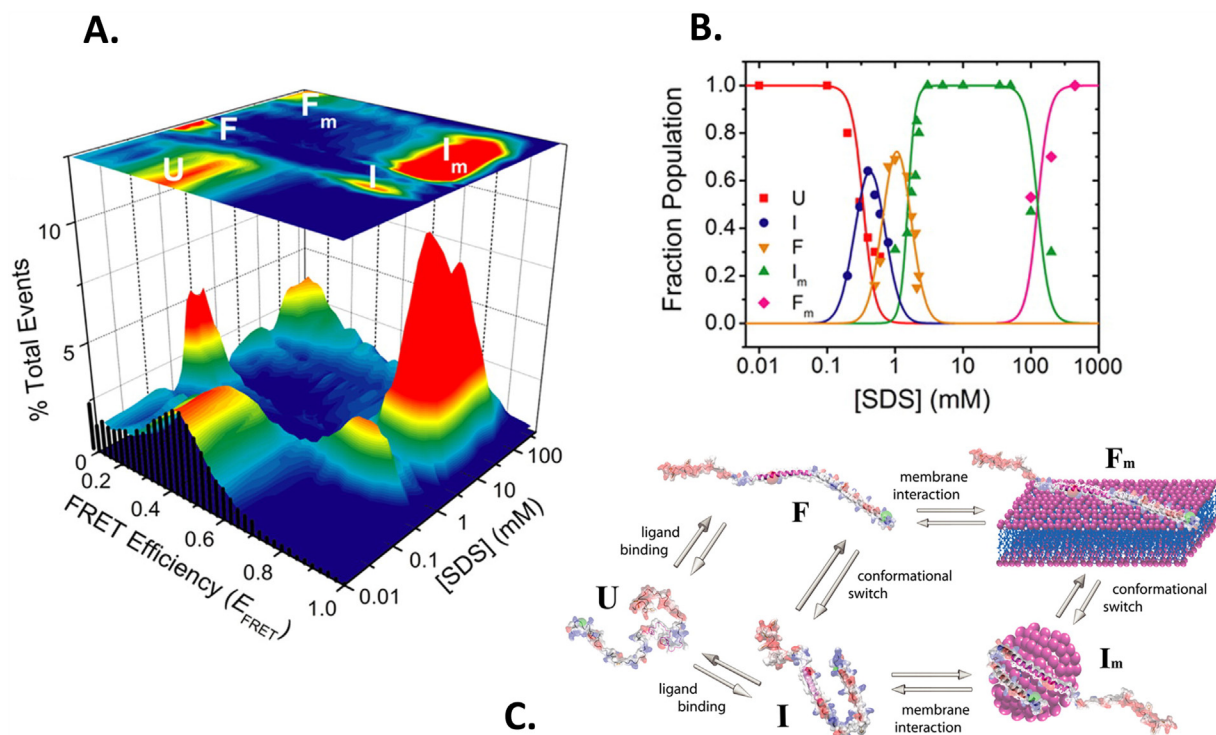


Fig. 2. Single-molecule fluorescence data for α -synuclein. A. 3D smFRET histograms as a function of concentration of the lipid mimic showing a complex folding-binding landscape. B. Populations of different species derived from histograms in A, showing multiple transitions between different species. C. Cartoons of the different states revealed in A. Adapted from Ferreon et al. Proc. Natl. Acad. Sci. (2009) 14:5645. See text for additional details.

2.4. NMDA-sensitive glutamate receptor: effects of post-translational modifications

The effects of post-translational modifications such as phosphorylation on IDPs are an active area of study. These modifications can modulate the charge distribution, disorder-order balance, and corresponding binding and function of IDPs [37], but it is difficult to predict these effects. smFRET is a powerful tool to determine such effects in challenging IDP systems. One example is the study by Choi et al. on the effect of phosphorylation on the C-terminal domain (CTD) of GluN2B, the major Tyr-phosphorylated protein in synapses and essential NMDA-sensitive glutamate receptor (NMDAR) regulator [38]. In this study, the authors used a different single-molecule detection geometry, total internal reflection (TIR). Because TIR can be used to illuminate a thin slice of surface and sample solution, it can eliminate a substantial amount of background signal from other portions of the sample.

GluN2B CTD's function is regulated by Src kinase phosphorylation on the palmitoylation-motif-anchored C-terminal segment of CTD (CTD2). To elucidate the role of CTD2 phosphorylation, eight constructs of CTD2 were designed for donor-acceptor labeling throughout the peptide through site-directed mutagenesis. These peptides were then phosphorylated and encapsulated in biotin-containing liposomes before immobilization on quartz microscope slides. To study the effect of the physiological palmitoylated anchoring of CTD2, N-terminal biotinylated CTD2 constructs were similarly generated and directly immobilized onto the slides [39]. Although a range of different regions of CTD2 were probed by the labeling design, all constructs produced broad distributions around mid-FRET-efficiency values, and a correlation between FRET efficiency and the fluorophore separation in the primary sequence was found in both phosphorylation states, consistent with a lack of structure. However, a small shift to lower median FRET efficiency after phosphorylation regardless of the labeling position, suggested an overall extension of the peptide. This led the authors to refute the induction of structure in CTD2 via phosphorylation, suggesting its allosteric effects on NMDA activity must arise from

interactive or steric outcomes of this expansion.

CTD2 is natively rich in proline, a residue associated with intrinsically disordered proteins and polypeptide expansion [40]. Although a completely Pro-depleted CTD2 mutant was found to lose its solubility, smFRET permitted an assessment of Pro's role in the interactions between CTD2 and the second PDZ domain (PDZ2) of scaffold protein PSD-95, through which the receptor directs synaptic targeting [41]. A FRET-acceptor-labeled Pro-depleted CTD2 construct was used with PDZ2 labeled with donor. The isolation of the Pro-depleted constructs as immobilized single molecules (at low density) prevented aggregation challenges of these aggregation-prone proteins. Using an analysis of the bound and unbound dwell-times, the authors observed that though Pro mutations do not directly affect the PDZ binding site of CTD2, they still reduce the binding affinity via increased dissociation rate constants. Additionally, using intramolecular smFRET, the authors observed that Pro-depletion changes CTD2 conformational dynamics. Overall, they concluded that the dynamics of the cytoplasmic IDR are an important component in allosteric regulation of receptor gating [41].

smFRET studies have also been used to study PTMs in other IDPs, like in alpha-synuclein [42]. Another example is discussed below for the case of nucleophosmin.

2.5. Nucleophosmin: structural changes and complex interplay of interactions - single molecules to phase separation

The nucleolus was one of the first MLOs discovered, with work during the past few years showing their liquid-like nature [43]. It is an essential cellular body, as it is implicated in several important cellular processes including ribosome assembly and stress signaling. Nucleophosmin (NPM1) is one of the many functional IDPs within the nucleolus. It has been shown to interact with a variety of nucleolar biomolecules, including other signaling proteins, ribosomal RNA and DNA. NPM1 is an interesting protein to study in terms of phase separation, because it consists of multiple acidic, basic and low-complexity tracts as

well as folded CTD and N-terminal domain (latter in the pentameric form), giving it variability in interaction modes and multivalency in terms of electrostatic interactions. The versatility of NPM1 is a function of its unique architecture.

Studies by Mitrea et al. showed that *in vitro*, higher salt concentrations were needed to bias the protein towards pentamers, and that phosphorylation could tune this equilibrium [44]. In 2016, Banerjee et al. reported studies of the monomer-pentamer equilibrium of a truncated version of NPM1 that contained the C-terminal oligomerization domain and a part of the adjoining disordered region containing charge tracts [45]. smFRET in conjunction with ensemble experiments provided more detailed information about this equilibrium. The overall reactions occurred in multiple discernible steps, and phosphorylation and partner binding differentially affected these steps. The findings pointed to different points of potential regulation in cells. smFRET studies of this protein were also used to probe aspects of its phase separation, as discussed below.

Work with NPM1 has revealed that it has the ability to phase-separate through several distinct mechanisms [46,47]. It is capable of heterotypic interactions with both positively-charged repeat peptides and negatively-charged ribosomal RNAs. It is also capable of phase separation *via* homotypic interactions between its own component sequences. All of these properties bring up a notable question in the field of IDP phase separation: How does a variety of weak interactions coordinate the phase behavior of an MLO?

smFRET and ensemble turbidity (light scattering by absorbance to report on overall phase separation), SAXS (to understand overall structural features) and mutational analysis (to test molecular components affecting phase separation) were used to study the impact of these weak interactions on the phase separation of NPM1 on its own and in combination with partners [46,47]. smFRET in particular showed changes in conformational features with ionic strength (supporting a model involving a complex interplay of intra- and inter-molecular interactions) and upon moving to phase-separation conditions. These studies revealed the promiscuity of interactions in NPM1, the importance of multivalency among its charged sequences for forming droplets, and how the electrostatic environment dictates the ability of NPM1 to phase separate. Electrostatic mediation of phase separation is important to consider when attempting to understand cellular mechanisms of phase separation. This is because many cellular conditions and stimuli can influence these interactions, whether it be pH, ionic strength, or other specific or non-specific interactions. Phase separation in this sense can also be altered by changes in PTMs and IDP interactions. The variety of molecular mechanisms involved in MLO assembly and dynamics are not yet fully understood, but a better understanding of the biophysical properties of the IDPs involved is essential to generate a complete picture of phase separation in the cell.

2.6. Molecular crowding and osmolytes

As discussed above, single-molecule fluorescence tools have been utilized to help understand the fundamentals of the folding, dynamics and conformational heterogeneity of IDPs and proteins in general. Most experiments were performed in simple conditions *in vitro*, where cellular conditions were probed only in terms of very basic components, such as ionic strength, pH, and binding partners.

Several *in vitro* studies have aimed to probe the influence of key aspects of cellular conditions, while still preserving the tunable and well-defined conditions achievable *in vitro*. For example, as opposed to conditions in most *in vitro* studies, cells have crowded interiors that incorporate high concentrations of a variety of molecules as well as interaction surfaces such as membranes. Careful and extensive studies by Sorzano et al. along with polymer-physics analysis indicated that crowding can cause different degrees of compaction for different IDPs, and this degree is related to the “non-crowded” expanded state of a given IDP [48]. The authors discuss various examples of cellular IDPs

where their results reveal factors that could play key functional roles. In another example, a study by Banerjee et al. showed that 2-dimensional crowding on the surface of model lipid membranes can result in the formation of “hidden” state that is not well-populated in the absence of crowding [49]. Again, given that cellular membranes are very crowded, these results could have interesting implications for the folding, association and function of several membrane-associated IDPs and IDRs. In another related example, Ferreon et al. have shown interesting effects of high concentrations of osmolytes on IDPs. In one case, the osmolytes TMAO and urea could counteract [50] each other's (compaction and expansion) effects on α -synuclein and showed a striking 1:2 counteraction ratio over a broad range of absolute osmolyte concentration [51]. In a different case, TMAO was found to increase the phase separation propensity of the ALS-linked protein TDP-43, but decrease its aggregation propensity [52]. Thus, these two types of phase transitions are related but can be differentially affected by solution conditions.

2.7. Cellular studies

Direct *in cell* and *in vivo* applications of single-molecule fluorescence techniques require several criteria to be fulfilled such as (i) single-molecule emitters must be detectable over cellular autofluorescence; (ii) sample delivery to living cells should be optimized in terms of reproducibility and ideally in high precision in targeting the cellular compartments; (iii) fluorophores must be stable and bright, therefore allowing longer detection times before photobleaching; (iv) data acquisition must be prompt after sample delivery to overcome the depletion of material by degradation; (v) cells must maintain a viable state before and after the delivery of sample, and (vi) ideally, the experimental setup should allow multiparameter data analysis to perform controls and experiment concurrently for the limited number of molecules in question.

Several of these technical issues were first addressed by Sakon and Wengner in a 2010 study, where authors chose microinjection to deliver protein samples into live mammalian cells [53]. With the aid of TIR fluorescence microscopy and single-particle tracking, this technical advance had been leveraged to study the conformational changes associated with SNAP-25, an IDP that folds upon forming a cellular membrane fusion (SNARE) complex. The authors found that SNAP-25, which is specific to neurons, forms promiscuous pairings in different cell lines where it is not endogenously expressed. Using the same method of sample delivery, Schuler and colleagues performed a comprehensive *in vivo* study that recapitulates well-characterized properties of several IDPs [54]. The authors chose to circumvent the cellular autofluorescence problem by using a FRET pair with excitation wavelength above 520 nm. The disordered state of prothymosin- α was shown to be conserved in HeLa cells, by comparison of the radius of gyration in different compartments in the cell to *in vitro* dimensions. Similarly, heat- and cold-denaturation profile of a marginally stable protein, yeast frataxin was studied in cells and found to comply with *in vitro* stability profile. Finally, in combination with recurrence analysis of single-molecule bursts [55], interconversion rates between folded and unfolded forms of protein GB1 domain had been determined. Folding-unfolding relaxation time of protein GB1 domain marginally differed from *in vitro* rates, indicating a small effect of macromolecular crowding takes place within the cells.

While microinjection was proven to be an attractive method for protein delivery into mammalian cells, the large needle diameter and the existence of cell walls make the method unavailable for prokaryotic cells. Kapanidis and colleagues devised a strategy based on electroporation for delivery of folded proteins and DNA into *E.coli* [56,57]. This method can be applicable for delivery into certain types of eukaryotic cells, as the authors have successfully shown that proteins can be delivered into *S. cerevisiae*.

Besides delivery of fluorophore-labeled proteins into cells, fluorophores can be readily expressed as fusion proteins in the form of

fluorescent variants, labeling *via* modified enzymatic activity of native cellular proteins [58]. Due to the generally weaker photophysical properties of fluorophores exclusive to such schemes, the applications are limited. Incorporation of noncanonical amino acids (ncAA) *via* orthogonal tRNA/amino-acyl tRNA synthetase pairs to label proteins using click-chemistry provides high site-specificity. Moreover, many fluorophores that are frequently used for single-molecule fluorescence applications are available in “clickable” moieties. While this approach is widely used for *in vitro* investigations of protein conformation and dynamics with single-molecule fluorescence, low ncAA incorporation efficiency, labeling specificity and efficiency variance in different cellular locations specifically favoring cell surfaces, physical characteristics of ncAAs and fluorophores, together make his method challenging for in cell fluorophore labeling [59]. Lemke and colleagues leveraged this labeling strategy and investigated the conformational dynamics of folded influenza hemagglutinin trimers on viral envelope using smFRET imaging [60]. Particularly because of their subcellular location, IDPs pose a challenge to be labeled *via* click chemistry within cells to study their conformational populations and dynamics using single-molecule fluorescence.

3. Perspectives on emerging and future directions

We anticipate that the application of single-molecule fluorescence studies of IDPs and in particular phase separation will continue to expand in the coming years. In terms of systems, we expect that both a larger array of systems and more in-depth studies of key systems will be carried out. Furthermore, concomitant advances in single-molecule and adjunct methods for such studies are expected to continue and expand.

Such studies in recent years have already begun to reveal new types of biophysical and biologically relevant insights. For example, higher-order complexes with more complex IDPs have begun to reveal interesting information about allostery and cooperativity. For binding of viral oncoprotein E1A to two of its key cellular partners, Ferreon et al. used smFRET studies to show how this IDP can show dramatic shifts in the magnitude and even sign of the binding cooperativity depending on the available E1A regions [61]. The results afforded insights into the complex layers of structural and functional regulation of such IDPs. In other examples, single-molecule fluorescence studies have directly probed the dynamics of disordered or fuzzy [62] complexes, where at least one partner is still unfolded and dynamic. Using a combination of smFRET and other methods, the Lemke lab has shown that nucleoporins, which are key to selective nuclear pore transport, bind nuclear transport receptors in a disordered state and with close to diffusion-limited rates [63]. In a different study, the Schuler lab showed that two IDPs, prothymosin- α and linker histone H1, bind each other with sub-nM affinity and yet remain disordered in the complex [64]. Such charge interactions with dynamic and distributed interfaces are important in phase separation and will also make such binding partners amenable to phase separation as in the case of nucleoporins. In another example, Soranno et al. have studied the question of internal friction in IDPs [65,66]. The authors used multiple methods including smFRET, photoinduced electron transfer mediated contact fluorescence quenching, correlation methods, as well as computation and comparison to theory to quantify friction in IDPs and unfolded proteins. The authors showed that internal friction can have important effects on the dynamics of IDPs and unfolded proteins, with more compact IDPs showing higher friction. This issue is particularly interesting from the point of view of phase separation since the high-density environments of many protein droplets will also likely give rise to friction effects on structural dynamics and potentially function.

Several directions for improving single-molecule methods can be envisioned. For example, further refinement of single-molecule methods such as FRET will be important for moving towards more quantitative measurements of IDP structure and dynamics in more complex droplet environments. Along these lines, multiparameter

fluorescence detection as popularized by the Seidel lab integrates different types of fluorescence photon observables (including color, lifetime, and arrival times) that permit a more global analysis of molecular parameters [67]. Of these, fluorescence lifetime measurements require more complex setups, but can provide important and complementary information, especially when combined with other observables. These types of measurements will be very useful in the context of phase separation. Additionally, studies of molecules in droplets on surfaces would permit long time-trajectories of IDP properties to be studied. Myong and colleagues performed first phase separation studies in this geometry using TIR detection on immobilized RNA molecules within droplets [68,69]. In these studies, smFRET of tethered RNA molecules was used to investigate the conformational changes and dynamics upon incorporation in protein-RNA droplets. Similar studies could also be performed on either immobilized or diffusing IDPs within such surface settled droplets. Since quartz/glass surfaces could influence the properties of proximal IDPs, careful testing of immobilization and comparisons to diffusing molecules would be important. Another direction is related to the fact that many single-molecule measurements need to be done at low (sub-nM) concentrations, which poses a problem for the high concentrations needed for studies of weak interactions and droplets. While studies of intramolecular IDP structure and dynamics in droplets can be performed by doping in a small fraction of dual-labeled protein in a large background of unlabeled protein, this is not the case for studies of intermolecular interactions. One interesting way to overcome this issue is to reduce the detection volume which then allows higher concentrations to be used. For instance, zero-mode waveguides (metallic film with an array of holes smaller than the wavelength of detection light) can reduce the focal volume by orders of magnitude down to the zL level [70,71]. Also, extension of 2-color smFRET to 3 or more colors can provide powerful capabilities for more global analysis of IDPs and their complexes and condensates [72–78]. For example, 3-color smFRET has been used to study the tetramerization domain of p53 [77] as well as α -synuclein [73]. Near-field scanning microscopy can also be used to study IDPs and their aggregates and oligomers with high resolution [79]. Adjunct methods such as chemical biology labeling methods [72,80] and combination with microfluidics will also continue to contribute to improving experimental methods for understanding the molecular underpinnings of phase separation [31,81]. Microfluidic methods that allow quick scanning of a range of interaction, temperature and other conditions [82] might be particularly useful for broadening detailed and controlled studies of IDP phase separation. Other methods could also be further developed or adapted for detection of IDPs and droplets, including single-molecule manipulation and methods that combine single-molecule fluorescence with single-molecule manipulation [83] or other structural methods such as cryo-EM and NMR as well as computational analysis.

We anticipate overall that such advances in single-molecule tools and applications to a larger number of IDP systems both *in vitro* and *in vivo* will continue to reveal and delineate additional biophysical principles related to IDP conformation, molecular-level interactions and phase separation. Novel additional understanding of allosteric [61,84], non-equilibrium and active-matter effects and their influence on function are certain to emerge [85]. These emerging principles are also expected to continue to provide new insight into mechanisms of numerous cellular processes as well as associated diseases.

Conflict of interest

No conflicts of interest to declare.

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