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Single-molecule fluorescence spectroscopy of biomolecular condensates

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Single-molecule spectroscopy is emerging as a powerful approach for elucidating the molecular structure and dynamics of biomolecular condensates. Unlike ensemble methods, it can resolve heterogeneous conformations, dynamics, interactions, concentrations, and transport properties across a broad range of length- and timescales, even for minute amounts of biomolecules. Recent applications, especially fluorescence-based methods such as single-molecule Förster resonance energy transfer, fluorescence correlation spectroscopy (FCS), fluorescence anisotropy, and nanosecond FCS, have revealed how proteins and nucleic acids behave within dense phases, and how molecular-scale dynamics relate to mesoscopic properties and biological function. Combined with molecular simulations, these measurements yield mechanistic insight into condensate organization, dynamics, and aging. We highlight recent advances, key applications, and promising directions for probing the properties of condensates with single-molecule spectroscopy.

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Introduction

The investigation of biomolecular condensates poses new conceptual and experimental challenges compared to the classical view of biomolecular assemblies. Condensates lack the simple stoichiometries of biomolecular complexes; they often contain heterogeneous mixtures of proteins, nucleic acids and other solutes; the proteins involved can be folded or disordered; the relevant length scales of condensates extend from the Ångström scale of individual amino acid side chains to the micrometer scale of droplets; both the structural and the dynamic properties of condensates are essential for developing a coherent picture; and while we can elucidate many physical principles of condensate mechanisms *in vitro*, understanding their cellular role requires *in-vivo* experiments. Some aspects are readily accessible and have been studied in great depth based on fluorescence imaging, such as the formation of droplets under a wide range of conditions, and their morphology and composition through fluorescence labeling of selected components, but an increasing number of experimental methods are being employed to study the many fascinating facets of biomolecular phase separation [1]. In view of the formidable challenge of understanding the molecular-scale behavior of condensates, single-molecule spectroscopy has become a particularly promising approach, with its core strengths of resolving structural and dynamic heterogeneity [2,3] across a broad range of timescales [4,5] as well as structural detail in folded and disordered biomolecules [6,7]. Owing to its extreme sensitivity, it enables even minute amounts of a particular biomolecule to be probed within condensates. We summarize here the recent developments and applications of single-molecule spectroscopy to biomolecular condensates, with a focus on fluorescence spectroscopy, and outline where we expect the most important advances.

The potential of single-molecule fluorescence spectroscopy (Figure 1) for investigating biomolecular condensates was recognized early on [8–12], but the number of applications remained limited, at least in part for technical reasons. Single-molecule spectroscopy requires the detection of individual molecules,

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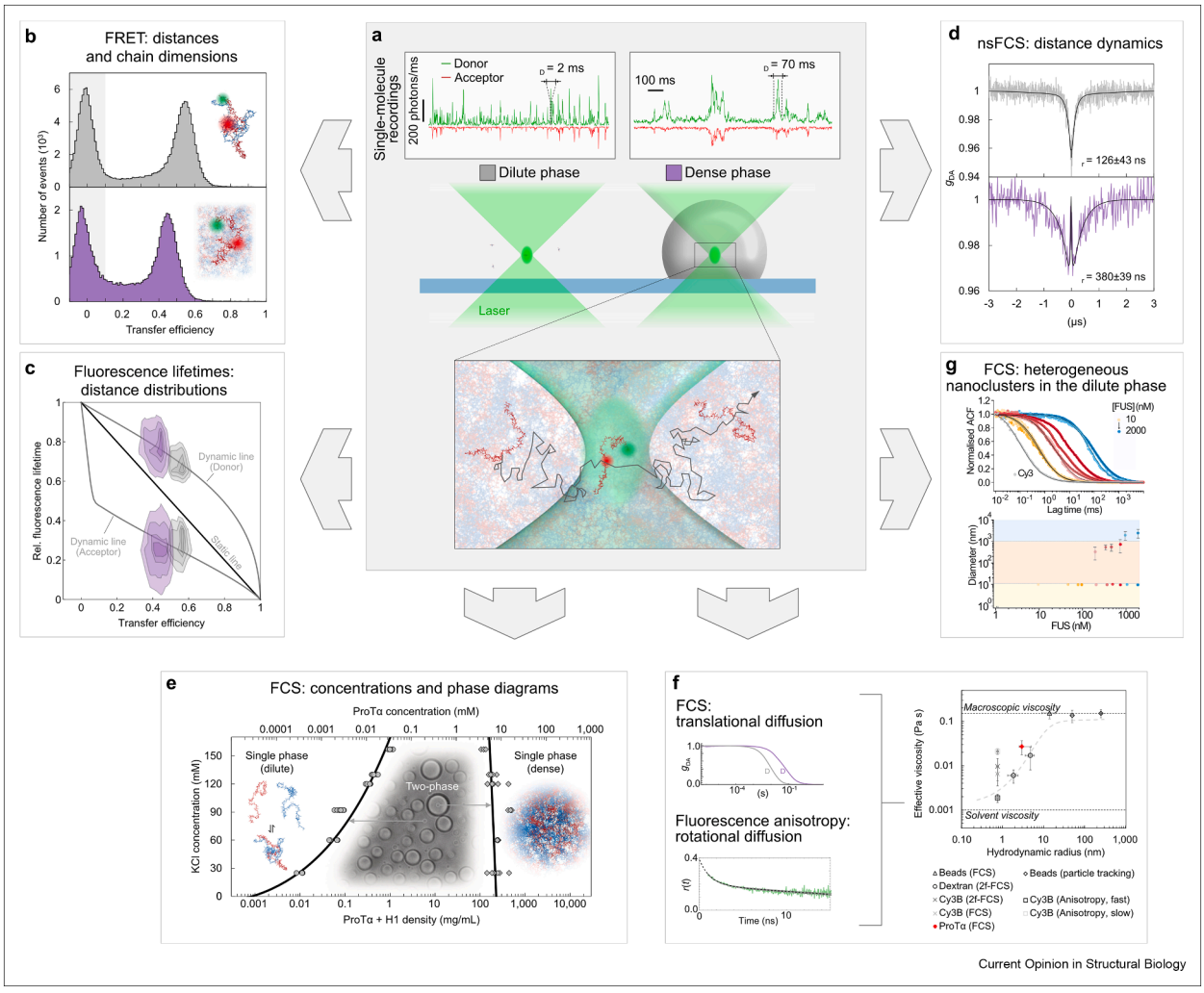
Figure 1



Overview of information on biomolecular condensates available from confocal single-molecule fluorescence spectroscopy. (**a**) Illustration of the confocal volume positioned either in the dilute phase or inside a condensate droplet. The top row shows examples of single-molecule fluorescence time traces recorded in each phase for the donor (green) and acceptor (red) channels of a double-labeled molecule undergoing FRET [22]. A hallmark signature confirming the single-molecule regime is the combination of a constant low background with well-separated fluorescence bursts, each corresponding to an individual double-labeled molecule crossing the confocal volume. The longer burst duration in the dense phase compared to the dilute phase reflects the lower diffusion coefficient inside the droplet. (**b**) Single-molecule FRET efficiency histograms reporting on intramolecular distances of a doubly-labeled molecule in the dilute (gray) and dense (purple) phases, revealing chain expansion upon condensation as evidenced by a shift toward lower FRET efficiency [22] (shaded region: donor-only signal). (**c**) Fluorescence donor and acceptor lifetimes vs. transfer efficiency analysis. Displacement from the static FRET line [29] toward the dynamic line indicates rapid conformational dynamics of IDPs both in the dense and dilute phases [22]. (**d**) Nanosecond FCS (nsFCS) donor–acceptor cross-correlation curves [22]. The relaxation on the nano- to microsecond timescale reports on end-to-end distance reconfiguration dynamics of the molecules in the dense and dilute phases. These experimental data show slowed chain dynamics within the condensates. (**e**) Phase diagram of the ProTα/H1 system as a function of KCl concentration, determined by FCS [22]. The binodal (solid line) separates the homogeneous dilute- and dense-phase regions from the two-phase coexistence region, within which both phases can be probed independently by positioning the confocal volume accordingly. (**f**) Diffusion coefficients and effective viscosities quantified with fluorescence spectroscopy. Illustrations of FCS curves (top left) reporting on translational diffusion [23], and fluorescence anisotropy decays reporting on rotational diffusion (bottom left) [22]. The resulting effective viscosity as a function of the hydrodynamic radius of the probe particle (right) reveals a steep length scale dependence in the dense phase. (**g**) FCS shows the concentration-dependent formation of heterogeneous nanoclusters in the dilute phase of FUS [44]. Normalized autocorrelation curves (top) shift to longer lag times with increasing FUS concentration, and hydrodynamic diameters from a two-component FCS model (bottom) indicate the presence of larger assemblies well below the threshold concentration for macroscopic phase separation. The experimental data in panels (a–f) are from Galvanetto et al. [22]; FCS inset of panel (c) is from Galvanetto et al. [23]; panel (g) is from Ge et al. [44].

typically at picomolar concentrations, against the background from fluorescence impurities and scattered light. Scattered light can be eliminated efficiently by combining the spatial selection of a small observation volume with the spectral separation between scattering and fluorescence [13,14], and fluorescent impurities can usually be kept sufficiently low in dilute solutions. In the dense phase of condensates, however, with

biomolecule concentrations in the range of hundreds of milligrams per milliliter, even small contaminations carried over from sample preparation can dominate the fluorescence signal and prevent single-molecule detection. It is thus no coincidence that methods such as fluorescence correlation spectroscopy [15] (FCS), which also exploit fluorescence-intensity fluctuations but can be applied at nanomolar (and even low micromolar) concentrations of the fluorescent species, soon became an important technique for monitoring translational diffusion and quantifying biomolecule concentrations in condensates, including the phase boundaries as well as the transition from the dilute to the dense phase [16–18]. However, single-molecule spectroscopy can provide substantially more molecular information [19–21], including molecular-scale distances from Förster resonance energy transfer (FRET), distance distributions based on fluorescence lifetimes, rotational motion from fluorescence anisotropy, and rapid distance dynamics from nanosecond FCS (nsFCS).

Multiparameter single-molecule spectroscopy in biomolecular condensates

We illustrate the versatility of such multiparameter single-molecule fluorescence spectroscopy [19] based on recent work on complex coacervates formed by two highly charged intrinsically disordered proteins (IDPs), histone H1 and prothymosin α (ProTα) [22,23] (Figure 1). Taking full advantage of multiparameter spectroscopy requires confocal fluorescence detection in combination with multichannel time-correlated single-photon counting, where photons are sorted by polarization and wavelength [19–21] (Figure 1). The sample typically consists almost exclusively of unlabeled biomolecules; the fluorescently labeled molecules are added at picomolar concentrations to ensure that the probability of more than one fluorescent molecule in the detection volume at any time is negligible. One benefit of the confocal detection scheme is that the femtoliter confocal volume can be positioned selectively in either the dilute phase or the interior of a dense-phase droplet, which allows the signal from the different phases to be clearly distinguished (Figure 1a). Individual fluorescent molecules diffusing through the laser focus will then result in bursts of photons that are well separated if their concentration is sufficiently low. It is critical to assess how clearly these bursts can be distinguished from the background fluorescence. Control experiments should thus be performed in the absence of labeled molecules, and the resulting background fluorescence compared with that measured in the presence of labeled molecules; the two background levels should be approximately the same. In our experience, reliable single-molecule analysis generally requires a substantial number of bursts that are clearly distinguishable from the background, with peak intensities typically at least three times the background level. The signal from these bursts can then be analyzed to obtain information regarding many different aspects of the system.

For a protein or nucleic acid of interest labeled with a donor and acceptor dye for FRET, a key piece of information is the transfer efficiency quantified from the number of donor and acceptor photons, from which the inter-dye distance distribution can be inferred [24] to characterize the conformational ensemble at the molecular scale (Figure 1b). In terms of quality control, the FRET efficiency histogram is another important indicator of whether single-molecule conditions have been achieved. If the background signal or the concentration of labeled molecules is too high, the donor-only and FRET populations merge into a single unresolved peak, precluding a quantitative analysis of molecular conformations. An interesting question in the context of biomolecular condensates is whether the molecules expand or compact upon transfer from the dilute to the dense phase [8–10,22,23,25–28]. In the case of H1-ProTα, for instance, an expansion is observed in the droplets compared to the dimer that dominates the dilute phase [22,23]. Additionally, the donor and acceptor fluorescence lifetimes provide information on the width of the underlying distance distribution [29], which is particularly useful for IDPs and single-stranded nucleic acids [22,24,30] (Figure 1c). For these kinds of disordered molecules, an exciting piece of information is how the chain dynamics in the droplets differ from those in the dilute phase. Such distance dynamics can be measured with nsFCS based on the fluorescence intensity fluctuations of donor and acceptor as the distance between them fluctuates [24,31] (Figure 1d). A surprising finding for H1-ProTα was that the chain dynamics slow by only about a factor of three, although the bulk viscosity of the droplets is two orders of magnitude greater than that of the dilute phase [22].

Confocal single-molecule fluorescence measurements can provide not only information on the conformations, interactions, and dynamics of the phase-separating molecules, but also on the properties of the dilute and dense phases on different length- and timescales. For instance, from the fluorescence intensities and the amplitudes of FCS curves, the concentrations of molecules in the dilute and dense phases can be inferred, corresponding to the dilute- and dense-phase branches of the binodal in the phase diagram [16,17,22,23] (Figure 1e). From FCS and fluorescence anisotropy analysis, the mobility of molecules of different sizes can be determined (Figures 1f and 1g). Another interesting property of biomolecular condensates is their viscoelasticity [32,33], which can be quantified with methods such as particle tracking-based microrheology [34]. However, condensates are complex network fluids, and their material properties are thus length-scale-dependent [35]. Correspondingly, the viscosity

inferred via the Stokes–Einstein relation from measurements of diffusivity depends on the size of the particles used. The diffusion of beads can be tracked by standard microscopy, but the diffusion of smaller particles, such as proteins or smaller tracers, is too fast for this approach. In this case, FCS is an ideal approach for quantifying translational diffusion coefficients [15], ideally complemented with two-focus FCS, which is robust to differences in refractive index [22,36] (Figure 1f). The shortest length scales, where the local viscosity is dominated by the solvent, can be accessed by measuring the rotational diffusion of free fluorophores based on time-resolved fluorescence anisotropy. For H1-ProTα condensates, the combination of bead tracking, FCS, and fluorescence anisotropy thus enabled virtually the entire range of viscosities to be probed, from the solvent to the bulk viscosity, and the length scale dependence is described remarkably well by the theory of depletion interactions [22,37].

Examples of single-molecule spectroscopy of condensates

Given its versatility, single-molecule spectroscopy has been used to address a variety of interesting questions in condensate research, and new methods are being developed and adapted continuously to extend its scope. We briefly summarize some recent examples that illustrate such developments and the resulting insights.

Single-molecule experiments can help to advance our understanding of the interactions that are present within condensates. Trussina et al. [38] investigated reconstituted ribonucleoprotein granules, which store mRNA and repress its translation, and whose formation is driven by the protein G3BP1. Using single-molecule FRET, the authors found that a marginally stable RNA hairpin that is predominantly folded in the dilute phase is unfolded and conformationally dynamic in the dense phase (Figure 2a). G3BP1 has higher affinity for the unfolded than the folded hairpin, suggesting that it unfolds RNA in the condensates and may thus facilitate intermolecular RNA–RNA interactions. The complex balance of interactions between proteins and RNA in such ribonucleoprotein condensates affects the stability of the condensates, their internal dynamics, and the partitioning of molecules between dense and dilute phases.

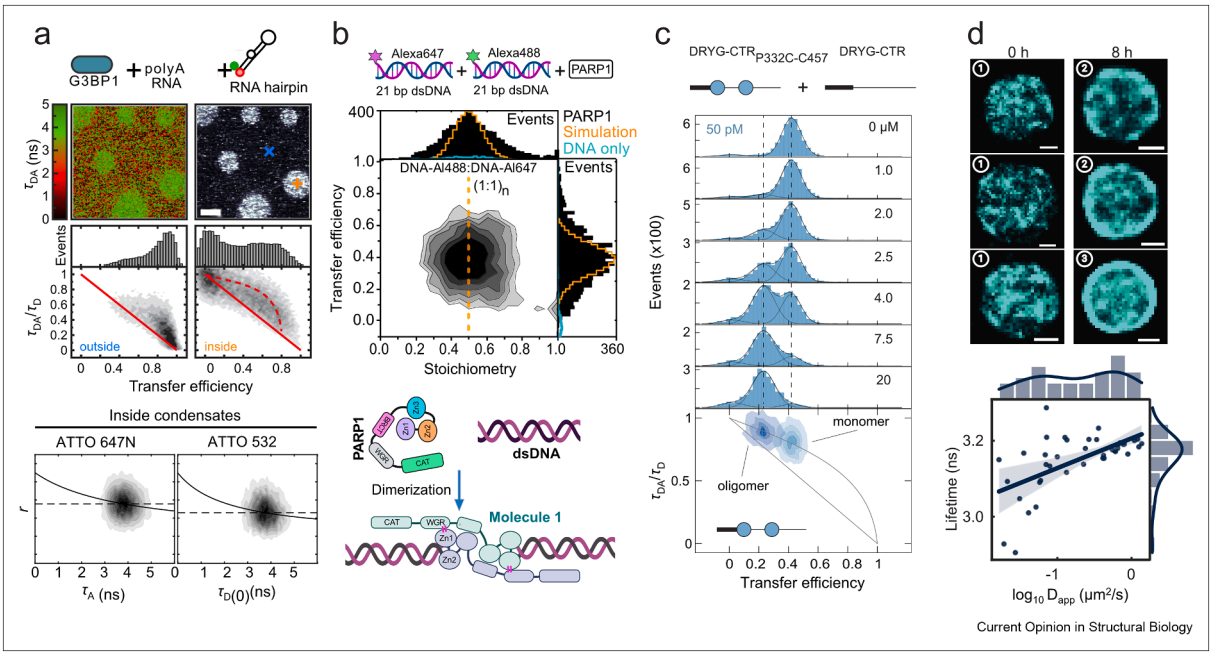
Another important question in the field of biomolecular condensates is the functional role of such assemblies. In a remarkable study, Chappidi et al. [39] found that poly(ADP-ribose) (PAR) polymerase 1 (PARP1) and DNA co-condense at the site of DNA breaks to hold the broken DNA ends together and facilitate the assembly of repair proteins. They employed optical tweezers to demonstrate that the condensates indeed exert a force that keeps the DNA ends together. With intermolecular single-molecule FRET experiments between donor- and acceptor-labeled DNA and unlabeled PARP1, they further showed that the protein–protein interactions between two PARP1 molecules can bridge two DNA molecules (Figure 2b). The resulting complex then promotes the formation of larger condensates that recruit other proteins and become enzymatically active for DNA repair. Cases such as this one illustrate the power of single-molecule experiments in revealing surprising functions of biomolecular condensates.

An emerging topic is the presence of protein oligomers or larger clusters in the dilute phase [40–44]. Nucleation theory in its simplest form assumes an equilibrium between the dense phase and a homogeneous dilute phase, so the role of such clusters is an intriguing question. A particularly elegant single-molecule study is the work of Swain et al. [45], who used an integrative approach to investigate the transition of the intrinsically disordered region (IDR) of the translation initiation factor eIF4B from monomers to condensates via oligomers containing up to approximately 15 protein molecules. With single-molecule FRET, the authors were able to map the conformational ensembles of eIF4B-IDR in the monomeric and oligomeric states. They observed an expansion of the chains upon oligomerization and showed that the proteins remain disordered within the oligomers (Figure 2c). Interestingly, the expansion was more pronounced in the DRYG-rich C-terminal region than in other parts of the protein. The authors were further able to quantify the rapid exchange of individual molecules between monomeric and oligomeric states on the low-millisecond timescale [45] by recurrence analysis [46]. In combination with coarse-grained molecular simulations, they were thus able to provide detailed mechanistic insight into such unconventional oligomerization processes. An important open question is whether and how such clusters are mechanistically linked to the formation of mesoscopic dense phases, or whether they result from independent processes.

Integration with other methods and promising method development

Besides the existing single-molecule spectroscopy methods and their application to biomolecular condensates, there are many promising developments that are likely to further enhance the insights to be gained from these techniques. One key development is the increasing synergy between single-molecule experiments and molecular simulations across different length- and timescales [47,48]. Distances or distance distributions from single-molecule FRET, biopolymer dynamics from nsFCS, or translational diffusion measurements by FCS can be directly compared with simulations. The experimental data constitute stringent benchmarks of the simulations and can thus increase the confidence in the simulation results, which, in turn,

Figure 2



Recent highlights of single-molecule spectroscopy applied to biomolecular condensates. (**a**) Folding and conformational ensemble of a client RNA hairpin in polyA RNA and G3BP1 condensates [38]. Fluorescence lifetime imaging (FLIM) and fluorescence intensity micrographs of condensates with a donor/acceptor-labeled RNA hairpin (top). Fluorescence-lifetime vs. transfer-efficiency histograms of the RNA hairpin outside and inside the condensates (middle) from regions indicated by blue and orange symbols, respectively, indicate that the hairpin is predominantly folded outside and unfolded inside the condensate, with a broad ensemble of dynamic conformers, as evidenced by the positions on the dynamic FRET line. Anisotropy measurements of the donor- and acceptor-labeled hairpin demonstrate slower rotational motion inside compared to outside the condensate (bottom). (**b**) Investigation of PARP1-mediated dsDNA oligomerization [39]. The transfer efficiency of a mixture of donor- and acceptor-labeled dsDNA fragments in the presence of PARP1 (top) demonstrates that PARP1 induces dimerization of dsDNA fragments. The stoichiometry distribution is centered around 0.5, indicating predominantly 1:1 donor–acceptor species, but the stoichiometry distribution is broader than expected for purely dimeric species (orange histogram), suggesting the presence of higher-order species. PARP1-induced DNA dimerization is an early step for PARP1-DNA condensate formation (bottom). (**c**) Formation of disordered oligomers by eIF4B [45]. Transfer efficiency histograms of C-terminally labeled eIF4B with increasing concentrations of unlabeled protein show the emergence of a low-transfer efficiency population and concomitant decrease of the monomeric population (vertical dashed lines). The lifetime vs. transfer efficiency histogram shows that disorder is retained in the oligomers, as both species fall on the dynamic line. (**d**) Single-molecule localization microscopy and confocal spectroscopy facilitate the characterization of nanoscale heterogeneity and aging of FUS condensates [56]. Single-molecule localization maps of labeled FUS in FUS condensates at different times (top), showing nanodomains in FUS condensates that localize at the periphery. FLIM-FCS in the condensates (bottom) supports the heterogeneity in the condensates, showing a bimodal distribution of diffusion coefficients and a correlation between fluorescence lifetimes and diffusion coefficients. The experimental data in panel (a) are from Trussina et al. [38], (b) from Chappidi et al. [39], (c) from Swain et al. [45], and (d) from Gao et al. [56].

offer a mechanistic interpretation of the measurements that is difficult to achieve in experiments alone. An example is the use of coarse-grained simulations of eIF4B-IDR oligomers to develop a molecular model of the nature of these assemblies and the conformational distributions of the constituent proteins [45].

Obtaining information on the absolute timescale of dynamics from simulations typically requires all-atom explicit-solvent models [49], and the integration with single-molecule experiments benefits on the one hand from the improvements in time resolution of the measurements, and on the other from the increasing system sizes and longer timescales accessible with high-performance computing [4]. A case in point is the comparison between experiments and simulations for the H1-ProTα condensates [22,23], where the all-atom MD simulations reproduced experimental observables including protein concentrations, translational diffusion coefficients, chain dimensions, and chain reconfiguration times. Besides a wealth of information on the conformational properties and interactions of the molecules in the dense phase, the simulations revealed the rapid dynamic shuffling of the interactions between charged side chains within a few nanoseconds, which is likely to be an important factor for the extreme dynamics on the molecular scale despite the large bulk viscosity of the condensates [22,23]. Many further opportunities arise from the rich theoretical framework for phase separation, polymer interactions, and polymer dynamics that can aid

the interpretation of experimental results [50—55], such as the link between molecular and mesoscopic properties provided by the Rouse model with entanglement [23].

Finally, we would like to mention some of the most promising developments from the experimental side that are likely to impact the investigation of biomolecular condensates and help solve important open questions in the field. Some of the most exciting and challenging topics, such as the structural organization and heterogeneity within condensates, the formation of nanodomains, and how these processes regulate transport, aging, and function, are starting to be addressed by fluorescence methods, including tracking-based techniques and super-resolution fluorescence microscopy [56—61]. Single-molecule microscopy provides an ideal complement to the spectroscopic techniques we focus on here, as recently reviewed in detail by Sumrall et al. [62], and the approaches often go hand in hand, as illustrated by the combination of single-molecule tracking and corresponding localization density maps with fluorescence lifetime imaging (FLIM) for identifying nanodomains linked to the aging of condensates (Figure 2d). Another important single-molecule method complementary to fluorescence is force spectroscopy based on optical or magnetic tweezers. These techniques provide an ideal platform for interrogating processes such as condensation on DNA and the resulting forces [39,53,63]. For multiplexing the investigation of protein-DNA interactions, DNA curtains [64] provide a powerful platform, and processes such as the nucleation of condensates on nucleic acids can be monitored with surface-based experiments and total internal reflection fluorescence (TIRF) [65,66]. Another important challenge is the investigation of biomolecular condensates with realistic compositions, ideally directly within their cellular environment. Key advances in this area have already been made with in-cell single-molecule tracking, for instance to elucidate the role of RNA in stress granules and processing bodies [67,68], or for detecting heterogeneous diffusion in chromatin [69]. Further important contributions are likely to come from the identification of the components based on single-molecule pulldown experiments [66] and the application of advanced single-molecule methods directly in live cells [58,70,71]. A particularly important development will be to further advance the incorporation of noncanonical amino acids to enable the transition from in-cell ensemble [72] to single-molecule FRET measurements. In the years to come, we thus expect many important contributions to our understanding of biomolecular condensates to result from single-molecule spectroscopy.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Benjamin Schuler reports financial support provided by the Swiss National Science Foundation, the Novo Nordisk Foundation, and the European Union. Nicola Galvanetto reports financial support provided by the Ernst Hadorn Foundation. Aritra Chowdhury reports financial support provided by the European Union. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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* of special interest
** of outstanding interest

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