Chapter 19

Single-Molecule and Ensemble Methods to Probe Initial Stages of RNP Granule Assembly

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Abstract

Ribonucleoprotein (RNP) granules are membraneless organelles, consisting of high local concentrations of RNA and proteins bearing intrinsically disordered regions (IDRs). They are formed by liquid-liquid phase separation (LLPS). In neurodegenerative diseases such as ALS, mutations in granule proteins such as FUS and TDP-43 accelerate abnormal liquid to solid transition of RNP granules, leading to formation of fiber-like structures. Methods to study granules must be carefully selected based on the stage of granule's life. Here we describe a strategic combination of single-molecule biophysical and ensemble biochemical techniques that may be employed to extract insightful information about early stages of RNP granule formation. Protein-RNA interaction and stoichiometry of the complex in the early soluble stage of RNP assembly can be probed by *single-molecule FRET (smFRET) assay* and *electrophoretic mobility shift assay (EMSA)*, respectively. RNP-RNP interaction that likely contributes to RNP nucleation can be reported on by a smFRET-based RNA *annealing assay*. The next stage in the assembly pathway, that is, phase separation from diffused to liquid-like droplets, may be monitored by a *phase separation assay*. Finally, RNP granules isolated from mammalian cells can be investigated using a unique *single-molecule pull-down (SiMPull) assay*.

Key words RNP granules, Single-molecule FRET (smFRET), Phase separation assay, Electrophoretic mobility shift assay (EMSA), Single-molecule pull-down (SiMPull), LAF-1

1 Introduction

Stress granules (SGs) are a subclass of RNP granules that assemble in eukaryotic cells under stress. They are composed of RNAs and RNA-binding proteins (RBPs) [1]. Many of the SG proteins such as FUS and TDP-43 contain long stretches of intrinsically disordered regions (IDRs) and RNA recognition motifs (RRMs). The low complexity and the nature of the amino acid composition of the IDRs render the SG proteins highly interactive. Multivalent RNA-protein and protein-protein interactions drive liquid-liquid phase separation (LLPS), forming liquid droplet-like SGs [2]. However, nature of SGs can change, potentially losing their fluidity and maturing to hydrogels and eventually to more solid-like fibrillar structures that are a hallmark of neurodegenerative diseases [3].

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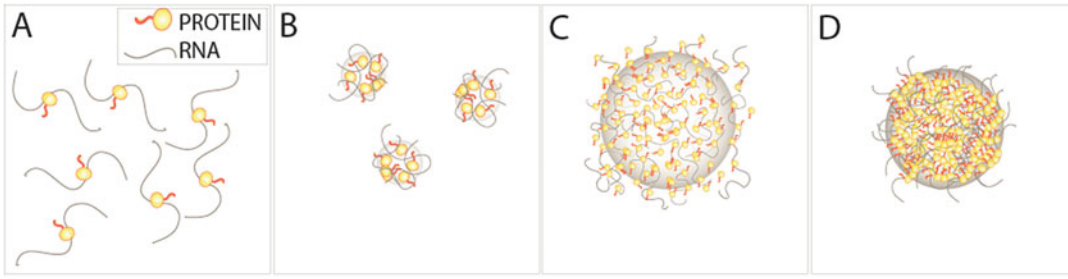

Fig. 1 Stages of granule development. (**a**) Soluble, (**b**) nucleation, (**c**) LLPS, (**d**) gel or solid-like

Indeed, FUS and TDP-43 inclusions are associated with disease pathology in ALS patients. Aberrant liquid-solid transition is accelerated by ALS-linked mutations, numerous of which have been identified in these proteins [4]. Example of RNP granules in other organisms include P granules in *C. elegans* [5]. Similar to SGs, P granules are also composed of IDR proteins such as LAF-1 (a DEAD-box helicase). Understanding the molecular mechanisms that contribute to disease onset and progression requires identifying the molecular differences between the normal wild-type SG proteins and the disease mutants, starting from the very early stages of granule assembly.

In this chapter, we describe single-molecule biophysical and ensemble biochemical techniques, an appropriate blend of which may be employed to probe early-stage molecular events of RNP granule formation. Furthermore, this combinatory approach enables connecting molecular mechanisms with biophysical properties of in vitro droplets and cellular granules. We envision the early stages to entail (a) *the soluble phase*, where proteins and RNA are soluble and remain diffused; (b) *the nucleation phase*, when RNA-protein, protein-protein, and RNP-RNP interactions tune RNP assembly; (c) *the growth phase*, in which nucleated granules continue to grow; and (d) *the maturation phase*, in which granules start losing their liquid nature and convert to solid-like aggregates, a process likely accelerated by aberrant interactions when IDR granule proteins bear disease mutations (Fig. 1). Accordingly, we categorize our methods as per their utility in probing each of these stages. In addition, we also describe a unique single-molecule method that can probe granules isolated from cells at the early stages of nucleation.

1.1 Phase Separation

This assay clearly defines the phase boundary, moving from the soluble to the phase-separated space. It allows probing biophysical properties of RNP droplets, shedding light into both the *growth and maturation phase* of the droplets. Recombinant protein expression constructs must be carefully designed for this assay. Since IDR proteins are inherently prone to aggregation, in some cases, a

protein solubility-enhancing tag such as MBP or GST may need to be fused to the gene encoding the IDR protein, via a linker that encodes a protease cleavage site. This will enable purification of the soluble protein. In these cases, when conducting phase separation assays [6], the solubility tag can be cleaved off by using an appropriate protease. Once the protein is liberated from its tag, it is expected to start phase separating and forming demixed liquid droplets, under the correct buffer conditions. These droplets can then be microscopically imaged. Scoring positive or negative for droplet formation can generate a phase diagram [7]. More detailed analyses such as assessment of droplet number, size, and growth rate may also be conducted. Furthermore, FRAP (fluorescence recovery after photobleaching) assay may be employed to determine droplet fluidity, a change of which over time will provide insight into the process of droplet "aging." Hence, the phase separation assay may be used to test droplet tuning parameters including salt and protein concentration, RNA, ATP, and temperature.

1.2 smFRET

Interaction of RNA with the IDR proteins has been demonstrated to be a critical tuning parameter for both in vitro droplets and cellular granules [7–11]. In order to understand such interactions, it is important to first probe the nature of the RNA-protein interaction in the *soluble phase*. For this, we employ a smFRET binding assay based on total internal reflection fluorescence (TIRF) microscopy (Fig. 2) [12]. An RNA substrate that is dual labeled with a pair of FRET fluorophores is immobilized on a PEG-passivated quartz slide (that is used on the TIRF microscope) via biotin-NeutrAvidin linkage. Changes in FRET signal, as indicated by distance changes between the fluorophores, can report not only on protein binding to the RNA but also on the RNA conformational changes that are being induced by protein binding. IDR protein-induced

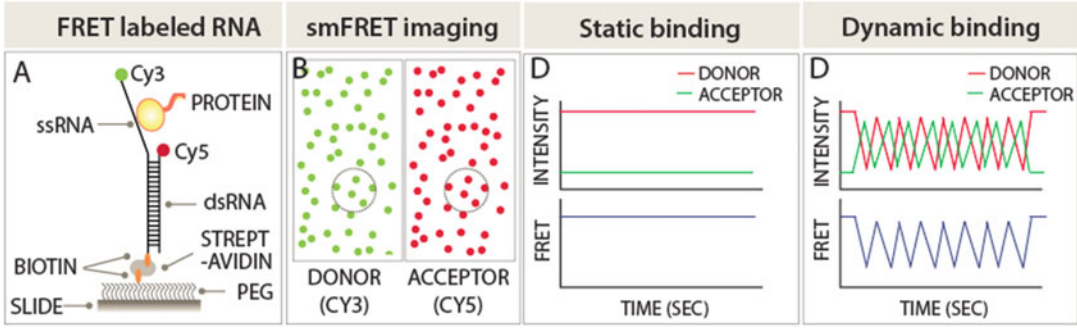

Fig. 2 smFRET detection. (**a**) FRET-RNA or DNA substrate immobilized to PEG surface. (**b**) Cy3 and Cy5 signal from same set of molecules (circle). (**c**, **d**) Individual smFRET traces reporting static (**c**) versus dynamic protein binding to single strand RNA

conformational dynamics on the RNA have been liked to granule fluidity [7, 8]. Thus, this assay can report on differences in protein-induced RNA dynamics between a wild type and a disease mutant variant of an SG protein, thereby identifying early-stage molecular differences that may impact eventual disease onset. These early differences can be potential target for drug design.

1.3 EMSA

EMSA has conventionally been used to characterize protein-nucleic acid interactions [13]. We utilize this assay to probe the stoichiometry of the protein-RNA complexes that form in the *soluble phase* preceding LLPS. By mixing a fluorophore-labeled RNA (same as the smFRET assay) and the IDR protein in an appropriate reaction buffer, we can monitor the different species of varying sizes formed, based on their differential migration on native gel electrophoresis (Fig. 3). Stoichiometric information obtained from EMSA can be co-related to RNA conformational dynamics information from smFRET assay, thereby providing insights into the stoichiometry and nature of soluble phase interactions that may ultimately govern properties of the phase-separated space.

1.4 RNA Annealing

RNP-RNP interaction is a critical factor regulating granule properties both pre and post granule *nucleation phase*. We describe here an smFRET-based annealing assay [8] that can report on

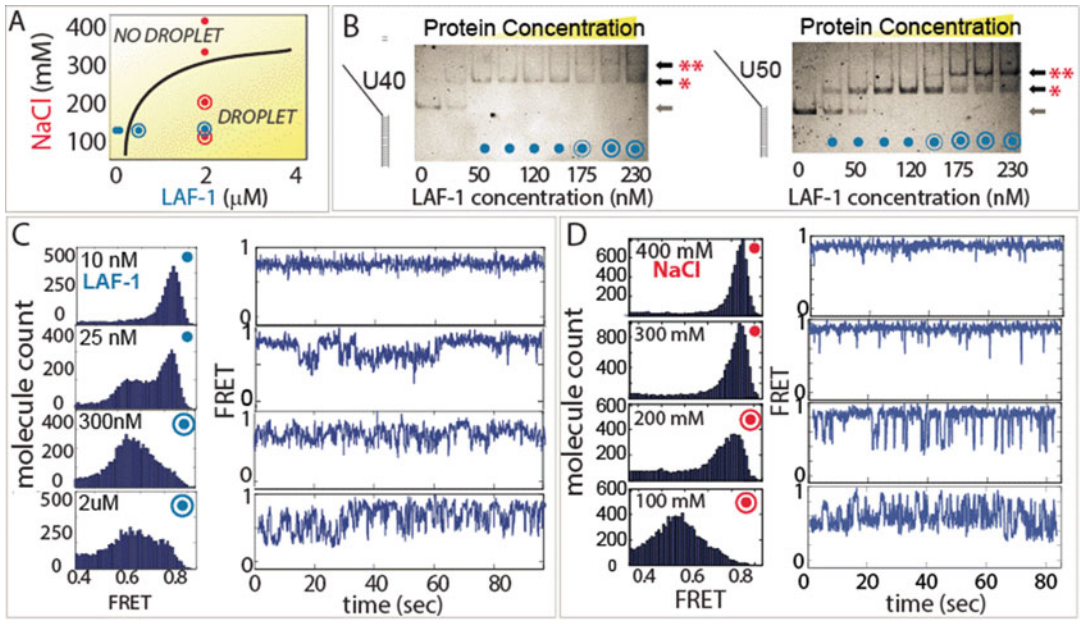

Fig. 3 LAF-1 induces dynamics on ssRNA in droplet forming condition. (**a**) Experimental conditions cutting across phase boundary in [LAF-1] and [NACl]. (**b**) Droplet forming conditions coincides with dimerization of LAF-1 denoted by double red asterix. (**c**, **d**) In droplet forming condition of high [LAF-1] LAF-1 induces dynamic mobility on ssRNA, evidenced by FRET fluctuation

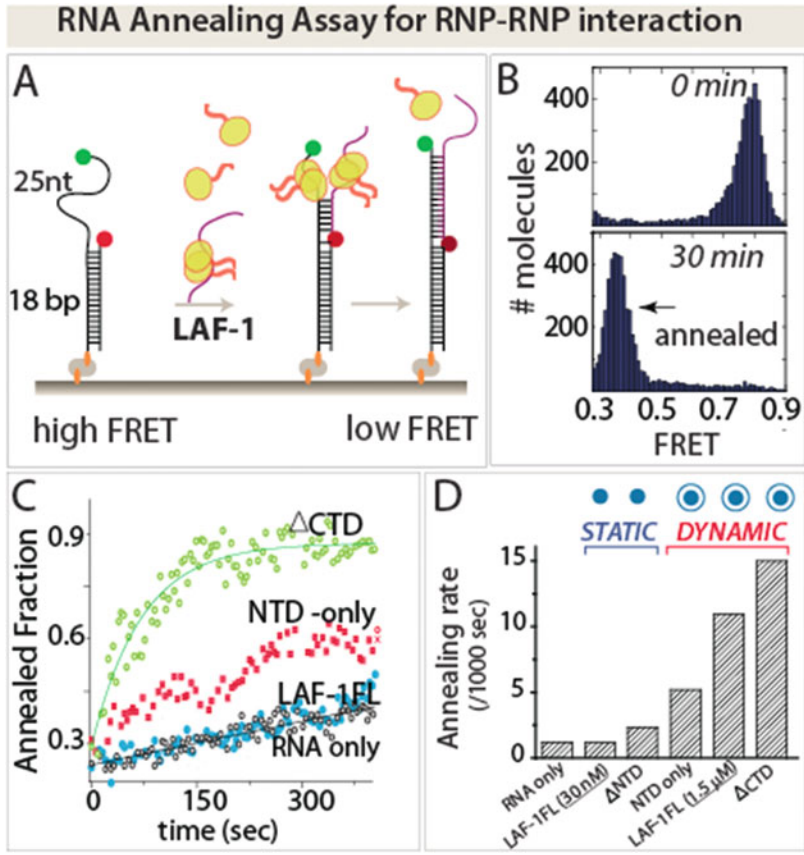

Fig. 4 LAF-1-RNA dynamics promote RNA annealing. (**a**) High FRET converts to low FRET upon RNA annealing. (**b**) FRET histogram before (top) and after (bottom) annealing. (**c**) Kinetic analysis of RNA annealing reaction. (**d**) Annealing rate for various mutants that represent static vs. dynamic LAF-1-RNA interaction

hybridization of two complementary RNA strands, an act that likely requires association between two sets of RNA-protein complexes, i.e., RNP-RNP contact. A FRET-pair fluorophore-labeled RNA substrate is immobilized on the TIRF microscope PEG-passivated slide (similar to the aforementioned smFRET assay). A preincubated mixture of a complementary RNA strand and IDR protein is applied on the slide (Fig. 4). We then monitor changes in FRET signal produced by annealing of the two complementary strands, thus reporting on RNP-RNP interaction.

1.5 SiMPull

Size and/or oligomeric state of cellular granules at the very early stage of the *nucleation phase* can be reliably measured using a uniquely customized pull-down assay, called single-molecule pull-down (SiMPull) assay [14, 15] (Fig. 5). This technique combines traditional pull-down assay principles with TIRF-based single-molecule fluorescence microscopy. It allows scope for probing cellular granules in a non-perturbing fashion. Fluorescently tagged SG protein(s) may be expressed in mammalian cells, followed by

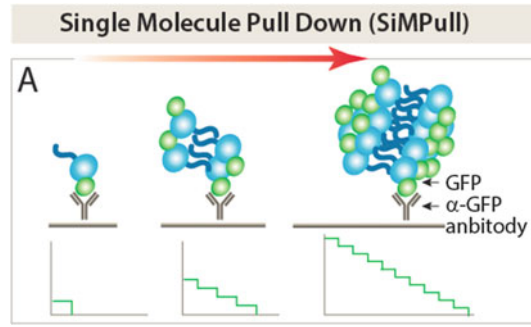

Fig. 5 Probing molecular assembly of granules. (**a**) Single-molecule pull-down assay can reveal the multimeric state of target proteins in cellular granules by quantifying photobleaching steps

application of stress, and cell lysis. The cell lysate is then applied on a PEG-passivated TIRF microscope slide that is coated with an antibody against the expressed SG protein. This allows for the fluorescently tagged SG protein molecules to be surface immobilized. Higher protein oligomers will be manifested as bright spots in fluorescence TIRF imaging, as opposed to low intensity spots exhibited to lower oligomers or monomers. Counting the number of photobleaching steps provides insight into the oligomeric state of the cellular granules.

2 Materials

2.1 Instrument and Common Reagents

1. For TIRF-based measurements, we use a home-built *TIRF-FRET microscope* [12].
2. Reagents for *quartz slides (passivated with a mixture of biotin-PEG and m-PEG)* to be used on the TIRF microscope are described elsewhere [16, 17] and will not be discussed here.
3. *Single-molecule imaging buffer*: 1 mg/mL glucose oxidase, 0.2% glucose, 2 mM 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic (Trolox), and 0.01 mg/mL catalase.
4. *Single-molecule wash buffer*: T50 buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl).
5. NeutrAvidin (Thermo Fisher). Reconstitute to a stock of 5 mg/mL in T50 buffer.
6. *Purified IDR proteins* (*see* **Note 1**). We will use LAF-1 as an example here.

2.2 Phase Separation Assay

1. Inverted microscope with 60× or 100× objective (Nikon).
2. Chambered cover glass (Lab-Tek or Grace Biolabs).
3. *Phase separation buffer*: 100 mM NaCl, 50 mM Tris-HCl, pH 7.5 (*see* **Notes 2** and **3**).

2.3 Electrophoretic Mobility Shift Assay (EMSA)

1. *EMSA reaction buffer*: 50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 100 mM β-mercaptoethanol, 0.1 mg/mL BSA.
2. Precast 6% DNA retardation (polyacrylamide) gels (Invitrogen).
3. *Electrophoresis buffer*: 0.5× TBE.
4. Typhoon scanner (GE) (fluorescence mode).

2.4 smFRET Binding Assay & Annealing Assay

1. *RNA substrate*: Partial duplex RNA with single strand (ss) overhang, labeled with a pair of FRET-suitable fluorophores such as Cy3 (donor) and Cy5 (acceptor) (Fig. 2) (*see* **Notes 4–6**). For the smFRET binding assay, the ss overhang of the RNA substrate is a poly(U) sequence to prevent secondary structure formation. We refer to this substrate as "binding substrate." However, for the annealing assay, the ss overhang may be a mixed sequence. We refer to this substrate as the "annealing substrate."
2. *ssRNA* that is complementary to the ss overhang of the partial duplex annealing RNA substrate. This will be used in the annealing assay.
3. *Single-molecule reaction buffer*: 50 mM Tris-HCl, pH 7.5, 125 mM NaCl.

2.5 Single-Molecule Pull-Down Assay (SiMPull)

1. HEK293 or HeLa cells (ATCC) and appropriate growth media.
2. 6-well cell culture plates (Corning).
3. Plasmids expressing fluorescently tagged SG protein of interest.
4. Transfection reagent. We use Lipofectamine 2000 (Thermo Fisher).
5. *SiMPull cell lysis buffer*: RIPA buffer (Thermo Fisher) + protease inhibitor cocktail tablet (Roche).
6. Antibody against the SG protein of interest that has been overexpressed in cells.

3 Methods

Perform all assays at room temperature, unless otherwise stated.

3.1 Phase Separation Assay

1. Prepare 50–100 μL reactions by mixing IDR protein and phase separation buffer (*see* **Notes 2** and **3**) in chambered cover glass imaging chambers.
2. Image droplets using DIC imaging with a 100× objective. Time points for imaging will depend on droplet nucleation and growth rate of the IDR protein of interest.

3. Construct the phase diagram, based on positive/negative score for droplet formation.
4. Several parameters may be systematically varied in this assay to test for its impact on LLPS of IDR protein and hence on the phase diagram. These include salt and protein concentration, presence/absence of RNA, temperature, crowding agents, etc.
5. When using fluorescently labeled IDR protein or RNA, droplets may be imaged by fluorescence imaging, apart from DIC.

3.2 Single-Molecule FRET (smFRET) Binding Assay

1. Assemble PEG-passivated slide and coverslip into sample chambers, as described in [16]. Let slide and coverslip thaw to reach room temperature before assembling.
2. Dilute stock NeutrAvidin solution 1:100, and then apply (~30 μL) on the biotin-PEG-slide chambers. Wait for ~2 min, ensuring biotin-NeutrAvidin attachment, and then wash each chamber with T50 buffer (~100 μL).
3. Flow in 30–100 pM of the binding RNA substrate (Fig. 2) in the chambers. Wait for ~2 min for the RNA molecules to be immobilized on the slide via biotin (on the RNA substrate)-NeutrAvidin linkage. Wash away excess RNA substrate with T50 buffer (*see* **Notes 7** and **8**).
4. Flow in different concentrations of IDR protein, for example, LAF-1 (one concentration per slide chamber) (*see* **Note 9**) in single-molecule reaction buffer supplemented with single-molecule imaging buffer.
5. Record movies (~20 short movies each of 20 frames; and ~5 long movies each of 1200 frames) at different time points, using the TIRF microscope. As a control, always perform these measurements for RNA substrate alone.
6. *Data analysis*: We analyze the RNA-protein smFRET binding data in two ways—(i) For each condition, a representative smFRET histogram is generated by averaging the initial ten frames from thousands of single RNA molecules recorded over 20 movies. This shows the FRET signal distribution of all collected molecules. In Fig. 3c, the U50 RNA binding substrate alone shows a low FRET peak. Upon addition of low (~10 nM) LAF-1 concentration, the peak shifts to high FRET, whereas addition of intermediate (~25 nM) LAF-1 generates both a mid and high FRET peak. This mixed behavior shifts to a complete high FRET peak when high (>300 nM) LAF-1 concentrations are applied to the U50 RNA substrate. (ii) For more in-depth analysis, we then look at the time traces of individual RNA molecules. These show how the donor (Cy3) and acceptor (Cy5) intensities are changing and the resulting FRET signal changes. In Fig. 3c, d, individual time traces show the following behavior, under different conditions—time

traces of U50 RNA alone show static low FRET signal (corresponding histogram showed low FRET peak); low concentration LAF-1 binding to U50 shows time traces with static high FRET signal (corresponding histogram showed high FRET peak); and increasing concentrations of LAF-1 binding to U50 show emerging dynamics in FRET signal, fluctuating between high and mid FRET (corresponding histogram showed a broad mid FRET peak). *Thus, the smFRET data (derived from histograms and individual time traces), shed light into unprecendented details of RNA conformational dynamics induced by LAF-1 concentrations varying across the soluble to the phase-separated droplet phase.*

7. *Combined data interpretation from EMSA and smFRET binding assays*: However, it is the combination of these two ensemble and single-molecule methods that paints the emerging picture of molecular events occurring as an IDR protein like LAF-1 transitions across its phase boundary. In presence of low concentrations of LAF-1, when EMSA showed a single shifted band, smFRET traces exhibited static FRET signal. This indicates tight compaction of the RNA (the two dyes are now close to each other, *see* Fig. 3b–d). When LAF-1 concentrations correspond to within the phase boundary (high concentrations), EMSA primarily showed double shift and smFRET traces exhibited FRET fluctuations. This suggests that multimerization of LAF-1 induces conformational dynamics on the RNA (the distance between the two dyes changing). *Thus, overall, this combinatory approach helps us understand the molecular details of how monomer LAF-1-bound tightly wrapped RNA converts to multimer LAF-1-bound dynamic RNA, a stage likely primed for nucleation of droplets.*

3.3 Electrophoretic Mobility Shift Assay (EMSA)

1. Fluorescently labeled RNA binding substrate (the same as used for smFRET binding assay) and varying concentrations of IDR protein (e.g., LAF-1) (*see* **Note 7**) were mixed in the EMSA reaction buffer and incubated for 20 min at room temperature.
2. The reaction mixtures were mixed with loading dye, followed by electrophoresis on 6% DNA retardation polyacrylamide gels at 150 V for 50 min.
3. The gel was then scanned using fluorescence mode on a Typhoon scanner.
4. *Data analysis*: RNA binding substrate with 30 poly(U) ss overhang (U30) showed one shifted band (relative to the unbound RNA band), across low to high LAF-1 concentrations, indicating monomer LAF-1 binding to the RNA. RNA binding substrate with 40 and 50 poly(U) ss overhang (U40 and U50, respectively) showed a second super shifted band, indicative of

multimer LAF-1 binding to these longer RNA substrates. *Thus, this data informs us about the oligomeric state of LAF-1-RNA complexes across the phase boundary.*

3.4 Annealing Assay

1. Perform **steps 1** and **2** of smFRET binding assay, described above.
2. Flow in 30–100 pM of the *annealing RNA substrate* (Fig. 4) in the chambers. Wait for ~2 min for the RNA molecules to be immobilized on the slide via biotin (on the RNA substrate)-NeutrAvidin linkage. Wash away excess RNA substrate with T50 buffer (*see* **Notes 7** and **8**).
3. Flow into the chambers a preincubated (for 5 min) mixture of LAF-1 and 1 nM ssRNA (complementary to ss overhang of the annealing RNA substrate) in single-molecule reaction buffer supplemented with single-molecule imaging buffer (*see* **Note 10**).
4. Record movies (~20 short movies each of 20 frames) at different time points, using the TIRF microscope. As a control, always perform these measurements for RNA substrate alone.
5. *Data interpretation*: The smFRET histograms show that the RNA annealing substrate alone shows high FRET (since due to the mixed sequence in the ss overhang of this substrate, the dyes are close) (Fig. 4a). The preincubated mixture of LAF-1 with ss complementary RNA (that was applied in **step 3**) is expected to contain LAF-1 in complex with ssRNA as well as some free LAF-1 that can interact with the immobilized RNA substrate on surface (**step 2**). Annealing between the immobilized RNA and the ssRNA is expected to decrease FRET since the two dyes (on the immobilized RNA) will now be far apart separated by the annealed duplex RNA region (Fig. 4a). We find that RNA annealing (indicated by FRET peak shift from high to low in the smFRET histogram) is enhanced by LAF-1 concentrations within LLPS conditions, i.e., conditions that promote dynamic LAF-1-RNA interaction in the smFRET binding assay (Fig. 4c). Thus, LAF-1, in granule forming conditions, can promote RNP-RNP interaction, which likely contributes to the nucleation stage of granules.

3.5 Single-Molecule Pull-Down Assay (SiMPull)

1. Seed and growth HEK293 cells to ~70% confluency. We usually use 6-well dishes.
2. Transfect cells with fluorescently tagged granule forming protein of interest. We use Lipofectamine transfection reagent, as per manufacturer's protocol. Let protein express for 24 h.

3. Apply desired stress reagent to cells, for the recommended time. As a control, keep a sample without stress.
4. Check for protein expression (fluorescence) by cellular imaging under a fluorescent microscope.
5. Lyse cells using SiMPull cell lysis buffer on ice for 30 min (*see* **Note 11**). Keep lysates on ice till ready to be used.
6. To prepare TIRF microscope slide: Perform **steps 1–2** of smFRET binding assay.
7. Apply the biotin-conjugated antibody (*see* **Note 12**) against the granule protein of interest (that has been overexpressed in the cells). This coats the slide surface with the antibody, via biotin-NeutrAvidin linkage. Wait ~2 min. Wash away excess antibody with T50 buffer.
8. Apply diluted cell lysate on the slide (*see* **Note 13**). Wait ~2 min. Fluorescently tagged overexpressed granule protein of interest will be pulled down to the surface via its antibody (that was surface tethered in **step 6**). The goal is to achieve single-molecule density of fluorescently tagged protein on surface.
9. Using the appropriate laser excitation (depending on the fluorescent tag on the granule protein of interest), record ~5–10 movies each of ~900 frames, for each sample condition.
10. *Data analysis and interpretation*: The fluorescently tagged target granule protein of interest will be captured on the slide and manifested as a single fluorescent spot in the acquired image (Fig. 5). Analyzing the time traces of each of these spots will show the photobleaching events of the fluorescent tag. Combined information from the total intensity of each spot and counting the number of photobleaching steps individual traces provide us insight into the oligomeric state of the granule protein of interest that has been pulled down. For example, in absence of stress, these spots are expected to be low intensity, with time traces showing single-step photobleaching, indicative of monomeric proteins being pulled down. However, in presence of stress, the spots may become high intensity, and corresponding time traces will show multiple photobleaching steps (proportional to the number of fluorescent protein units present in each spot), suggesting multimeric protein complexes. Thus, analysis of SiMPull data provides unique information on oligomeric state of cellular protein clusters in the early stages of RNP formation, when they are potentially on their way to granule formation.

4 Notes

1. IDR proteins are inherently aggregation-prone and hence can be challenging to purify. Yet, high yield and purity are critical factors that must be met for conducting the assays described here. To prevent aggregation during purification and afterward during storage, these proteins are often purified in high salt (~1 M) buffer also containing low concentrations of urea (~1 M). Strategies to purify these proteins have been described by several groups [6, 7, 10, 18].
2. If IDR protein contains a solubility tag that needs to be cleaved off, add the appropriate protease to the abovementioned phase separation buffer and the requisite protease buffer (as per manufacturer's protocol).
3. Specific salt and protein concentrations at which IDR proteins will phase separate into droplets will depend on the protein. This concentration needs to be optimized from the phase diagram of the corresponding IDR protein. *For LAF-1 the phase diagram has been defined* [7].
4. We order RNA strands from IDT (Coralville, IA). For labeling the RNA strands, we use Cy3 or Cy5-NHS ester with 3′- or 5′-amine modified RNA strands, also ordered from IDT.
5. One strand of the RNA substrate must be conjugated to a biotin at one end so that the RNA substrate can be immobilized on the PEG-slide via biotin-NeutrAvidin linkage.
6. Typical position of the Cy3 and Cy5 labels is at either end of the ss overhang, which generally ranges from 40 to 70 nucleotides to allow multimerization of IDR proteins. The duplex region is typically 18-mer.
7. Meaningful concentrations to be used for IDR proteins range from low to high, corresponding to the transition from the soluble to LLPS-dependent droplet formation, deduced from the phase diagram of the protein. For example, for LAF-1, based on its phase diagram, we varied protein concentration from 10 nM to 2 μM.
8. RNA substrates are usually prepared and stored as 10 μM stocks at −80 °C. This is then serially diluted to one-time use 10 nM aliquots that are stored at −20 °C.
9. The exact concentration of RNA substrate to be applied on the slide chambers will need to be optimized. The number of RNA substrate molecules immobilized on the slide will depend on the density of biotin-PEG on the slide. *The goal is to achieve single-molecule density of RNA substrate on slide surface.*

10. Varying LAF-1 concentrations may be applied. We suggest using a range that corresponds to the static (low LAF-1) and dynamic (high LAF-1) LAF-1-RNA interaction behavior observed in the smFRET binding assay.
11. To ensure preservation of structure and oligomeric state of cellular granules, we prefer using mild lysis conditions. These include mild detergent (NP-40 in RIPA buffer), gently scraping attached cells off plate, and no centrifugation after lysis.
12. The concentration of antibody to be applied on surface will have to be empirically determined. For example, for a biotin-conjugated GFP antibody (Rockland), 10 nM is a good starting point for application on surface.
13. Dilution factor of cell lysate will have to be empirically determined. As an example, for cells harvested from one well of a 6-well plate (~0.8×10^6 cells), exhibiting ~50% transfection efficiency, we use 1:10,000 as a starting dilution.

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