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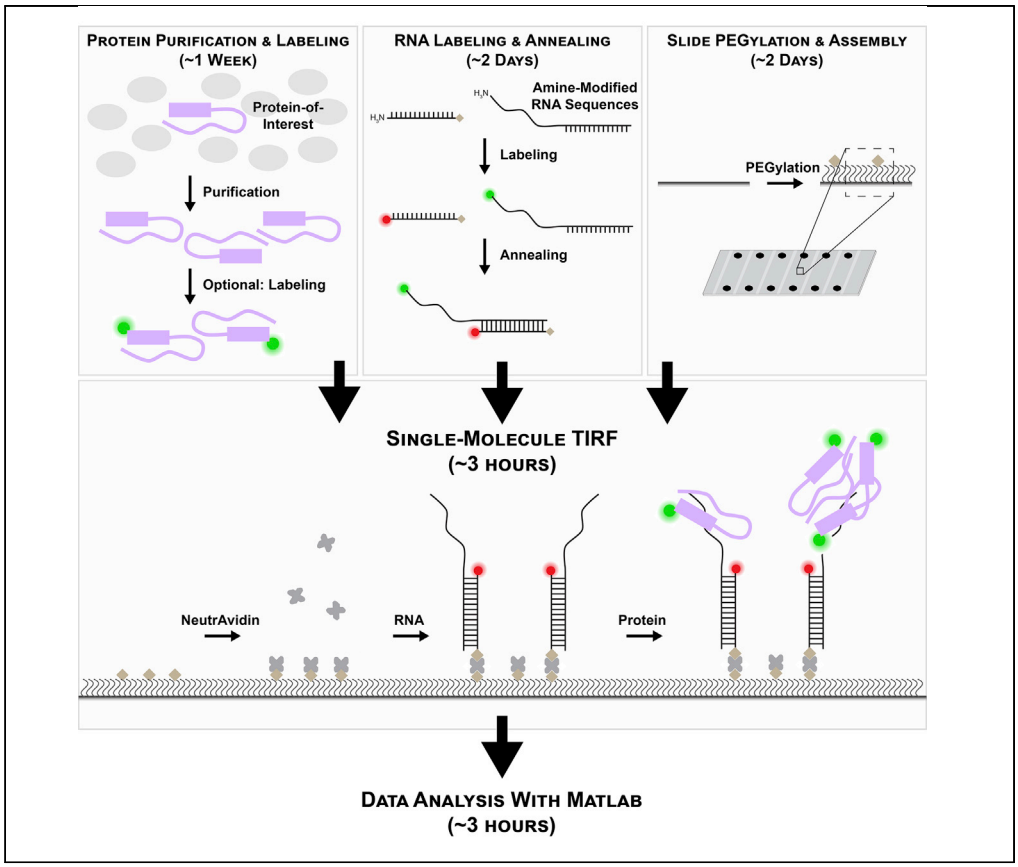
Single molecule probing of disordered RNA binding proteins



Liquid-liquid phase separation of intrinsically disordered proteins is known to underlie diverse pathologies such as neurodegeneration, cancer, and aging. The nucleation step of condensate formation is of critical importance for understanding how healthy and disease-associated condensates differ. Here, we describe four orthogonal single-molecule techniques that enable molecular tracking of the RNA-protein interaction, RNA-induced oligomerization, and kinetics of nucleation. These approaches allow researchers to directly interrogate the initial steps of liquid-liquid phase separation.

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Highlights

Single-molecule FRET experiments report on RNA conformation changes during binding

Oligomerization of disordered proteins is determined by photobleaching tagged proteins

Real-time nucleation generates kinetics data of oligomerization onto RNA

Colocalization of 2 disordered proteins is identified by alternating excitation

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Single molecule probing of disordered RNA binding proteins

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SUMMARY

Liquid-liquid phase separation of intrinsically disordered proteins is known to underlie diverse pathologies such as neurodegeneration, cancer, and aging. The nucleation step of condensate formation is of critical importance for understanding how healthy and disease-associated condensates differ. Here, we describe four orthogonal single-molecule techniques that enable molecular tracking of the RNA-protein interaction, RNA-induced oligomerization, and kinetics of nucleation. These approaches allow researchers to directly interrogate the initial steps of liquid-liquid phase separation.

For complete details on the use and execution of this profile, please refer to Niaki et al. (2020), Rhine et al. (2020), and Rhine et al. (2022).

BEFORE YOU BEGIN

These protocols are designed to quantify the nucleation of phase separation-prone proteins, especially in the context of RNA or DNA binding. It is advisable that you perform the following steps before beginning the protocols:

First, comply with university or institutional requirements for the safe use of lasers. Many prism-type TIRF microscopes are home-built. Class 3 and Class 4 lasers often have strict safety protocols, and the use of eye protection is recommended.

Second, test the RNA or DNA binding of your protein. Using RNA or DNA as a single-molecule tether simplifies the following protocols, and RNA or DNA with biotinylation and/or fluorescent labels can be purchased from suppliers such as Integrated DNA Technologies. In this paper, we generally refer to RNA, but modified DNAs can easily be substituted if that is the preferred binding partner of the protein-of-interest.

Third, carefully consider the tags used for protein purification. Certain tags (e.g., GFP) can disrupt the conformation of disordered proteins, depending on their proximity to functional or oligomerizing domains. Moreover, cleavage of some purification tags may not be desirable if one is using that tag to tether the protein to the single-molecule surface via antibody immobilization.

Purifying disordered proteins

Timing: ~1 week

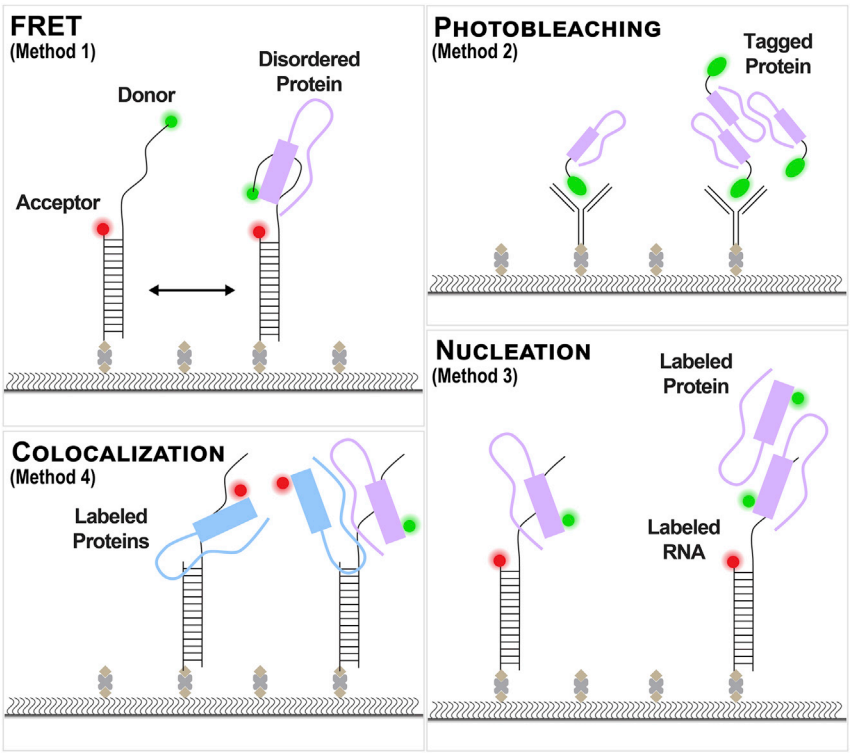

Figure 1. Labeling scheme for TIRF experiments with disordered proteins

An overview of the protein and RNA labeling for each of the 4 methods in this protocol. *Top Left*, FRET uses a FRET dye pair, which can both be attached to the RNA. Alternatively, one dye can be attached to the RNA and one can be on the protein. *Top Right*, Photobleaching experiments are optimized for fluorescent tags such as GFP or mCherry, which are cloned into the protein's coding sequence. *Bottom Right*, Nucleation assays require a labeled protein, and the RNA duplex can be labeled for easier visualization on the surface. *Bottom Left*, Colocalization assays require an unlabeled RNA and two labeled proteins.

Note: This section is generalized because protein purification protocols are highly dependent on the identity of the protein-of-interest.

1. Clone the protein coding sequence into the expression vector of choice
   a. Placement of tags should be cognizant of the functional protein domains and any perturbations should be experimentally verified before use in single-molecule assays. Figure 1 describes the protein and RNA labeling/tag schemes for each of the 4 single-molecule methods described in this paper.
   b. Solubility tags such as maltose binding protein (MBP) or glutathione S-transferase (GST) may be needed to prevent condensation during the purification process. Site-specific proteases can be used to cleave this tag before experiments.
2. Express proteins for purification
   a. Bacterial expression of disordered proteins may need to be tuned to prevent aggregation of the protein prior to purification. Induction temperature, duration, and optical density may need to be adjusted to slow bacterial growth.
   b. Pellets may usually be stored at −80°C until one is ready to proceed to the purification steps.
3. Purify the protein using column chromatography
   a. DNase and RNase should be added to lysis buffer to prevent co-purification of native DNA or RNAs (and other proteins bound to these DNA or RNAs) that will reduce the purity of proteins.
   b. High salt/denaturing (e.g., urea, guanidinium hydrochloride) conditions can prevent premature aggregation of proteins during the purification process.

c. An initial affinity column may be sufficient to purify certain proteins. The protein purity can be assessed by Coomassie staining of SDS-PAGE.
d. If contaminating proteins are present after the initial column, size-exclusion chromatography or another method (e.g., ion-exchange or a Heparin column) can be used to further purify the protein-of-interest.
e. Concentrating disordered proteins should be avoided as this can lead to premature oligomerization or aggregation.

Fluorescent labeling of proteins

Timing: ~2 h

4. If applicable, transfer the purified protein to labeling-appropriate buffer (e.g., Tris should be removed for NHS-ester labeling) by using desalting columns.
5. Incubate the purified protein with the appropriate fluorophore.
   a. Examples of fluorophores commonly used for TIRF microscopy include Alexa dyes (Alexa-488, etc.) and Cyanine dyes (Cy3, Cy5, etc.).
   b. Maleimide (for cysteine labeling) or NHS-ester conjugation (for lysine labeling) chemistries are commonly used for protein labeling. For NHS-ester reactions, the incubation time and conditions will need to be optimized to prevent excessive labeling of lysines (i.e., >100% labeling efficiency).
6. Remove excess dye using desalting columns. Quantify the protein and dye concentrations using a Nanodrop spectrophotometer. A fluorometer may also be used, but larger volumes are usually required for these instruments.

RNA labeling and annealing

Timing: ~2 days

7. RNA labeling reaction
   a. Prepare the RNA labeling reaction by combining the 5 μL 1 mM amine-modified probe, 0.1–0.2 mg NHS-ester conjugated dye, and 5 μL freshly-made 100 mM $NaHCO_3$ in 50 μL dH_2O.

   Note: Dyes are often provided as 1 or 5 mg powders. The powder can be resuspended in a small volume of DMSO and aliquoted into 0.1 or 0.2 mg aliquots; the DMSO is then evaporated via vacuum centrifugation or lyophilization for a long-term storage at −80°C.

   b. Incubate the reaction for 12–16 h in the dark with rotation.

8. Ethanol precipitation and washing
   a. Add 3 μL 5 M NaCl and 125 μL ice-cold 100% ethanol.
   b. Incubate at −20°C for 30 min.
   c. Centrifuge at 21000×*g* for 30 min at 4°C.
   d. Aspirate the supernatant fraction; be sure not to disturb the RNA-containing pellet.
   e. Carefully wash with 200 μL ice-cold 70% (v/v) ethanol.
   f. Dissolve the pellet in 50 μL dH_2O.
   g. Repeat steps a–e.
   h. Air-dry the pellet in the dark for 10–20 min.
9. Resuspend the pellet in dH_2O and quantify the RNA concentration and dye concentration using a Nanodrop spectrophotometer. A fluorometer may also be used, but larger volumes are usually required for these instruments.
10. Annealing

a. Combine the two 18-mer strands with each other in a 1.2:1 (non-biotin:biotinylated) molar mixture.
b. Anneal by incubating the solution at 85°C for 5 min, followed by a 2°C/min cooldown to 4°C. A thermocycler may be used for this cooldown, or a heat block can be heated to 85°C and turned off for a slow temperature decrease to 20°C followed by refrigeration/freezing.
c. Dispense the annealed RNA into single-use 10 nM aliquots (~5–10 μL). The higher-concentration stock may be stored at −20°C or −80°C for up to 1 year.

11. The quality of the annealed RNA can be assessed using a non-denaturing acrylamide gel.

Slide preparation

Timing: ~2 days

12. Drill pairs of holes (using a 0.75 mm diamond drill bit) along the longer edges of the slide for flow inlet and outlet (refer to Methods video S1 to see what a slide looks like prior to assembly). Five or six pairs is feasible on one quartz slide.

13. Pre-clean slides for PEGylation
    a. Place slide/coverslip pairs in a slide holder diagonally. The diagonal placement in the slide holder helps prevent the slides or coverslips from sticking to each other.
        i. Extra coverslips should be prepared because they tend to become brittle and break after KOH treatment.
        ii. Slides and coverslips should be handled with a clean pair of forceps for this entire protocol.
    b. Add 2.5% (w/v) alconox to the slide holders.
    c. Sonicate for 10 min using an ultrasonic cleaner.
    d. Rinse slides in the slide holder five times with dH_2O.
    e. Add acetone to the slide holders.
    f. Sonicate for 15 min.
    g. Fill the slide holders with methanol.
    h. Sonicate again for 15 min.
    i. Rinse slide holders with dH_2O again.

14. KOH cleaning
    a. Equilibrate the aminosilane to 20°C–25°C.
    b. Fill the slide holders with 1 M KOH.
    c. Sonicate for 20 min.
    d. Rinse the slides with dH_2O and then fill with fresh dH_2O.
    e. Sonicate again for 20 min.
    f. Burn the slides and coverslips with a Bunsen burner or flamethrower – each slide should be burned for ~2 min.

Note: The coverslips become especially brittle following the KOH wash, so they often break during this burning step. Try to apply the minimum amount of pressure to hold the slides.

Note: Burning the slides helps reduce the amount of dirt and other contaminants that might be visible during TIRF microscopy, especially for RNA substrates. However, one can attempt this protocol without burning the slides to see if there is a noticeable difference in slide cleanliness.

    g. Rinse the slide holders with methanol and then fill with methanol.
    h. Sonicate for 10 min.

15. Aminosilane preparation
    a. Fill a 500 mL flask with 200 mL methanol and 10 mL glacial acetic acid. Mix by pipetting.
    b. Sonicate the flask for 1 min.
    c. Add 2 mL N-(2-Aminoethyl)-3-Aminopropyltrimethoxysilane. This reaction occurs rapidly.
    d. Split the aminosilane/methanol/acetic acid solution into the slide holders.
    e. Sonicate for 30 s, then incubate in the dark for 10 min. Repeat this step once.

16. PEGylation of slides
    a. Fill water into the PEGylation chamber for humid incubation.
    b. Rinse slides and coverslips with methanol.
    c. Manually pipet dH_2O into the drilled slide holes and the regions between each pair of holes for cleaning.
    d. Dry the slides with a nitrogen gas stream.

    Optional: The slides can instead be air-dried for a few hours if a nitrogen gas stream is unavailable.

    e. Weigh out 100 mg mPEG-SVA and ~3 mg Biotin-PEG-SVA per 10 slides and add to 650 μL 100 mM $NaHCO_3$. Vortex as needed.
    f. Centrifuge the PEG/Biotin-PEG solution at 8000×*g* for 1 min.
    g. Transfer the supernatant fraction to a new tube.
    h. Pipet 65 μL PEG/Biotin-PEG solution onto the surface of each slide. Carefully lay the coverslips on the slides and try to avoid air bubbles.
    i. Incubate in the dark for 4 h.

    Pause Point: Slides and coverslips can be incubated for 12–16 h instead.

17. Final rinsing and storage
    a. Carefully separate the slide/coverslip pairs.
    b. Wash the slides and coverslips separately with dH_2O.
    c. Dry the slides and coverslips with the nitrogen gas stream and carefully transfer each slide/coverslip pair to a 50 mL conical vial. The PEGylated surfaces should face away from each other.
    d. Slides can be combined in groups of three and placed in vacuum-sealable bags for storage at −20°C for up to 1 year.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GFP Antibody Biotin Conjugated (Rabbit)	Rockland Antibodies & Assays	CAT#600-406-215
Chemicals, peptides, and recombinant proteins		
AcTEV Protease	Fisher Scientific	CAT#12-575-015
Alconox™ Powdered Precision Cleaner	Fisher Scientific	CAT#16-000-104
Biotin-PEG-SVA, MW 5,000	Laysan Bio Inc.	CAT#Biotin-PEG-SVA-5000-100mg
Catalase from bovine liver	Sigma-Aldrich	CAT#C3155
Cy3 NHS Ester	Cytiva	CAT#PA13101
Cy5 NHS Ester	Cytiva	CAT#PA15100
Glucose Oxidase from Aspergillus niger	Sigma-Aldrich	CAT#G2133
mPEG-Succinimidyl Valerate, MW 5,000	Laysan Bio Inc.	CAT#MPEG-SVA-5000-1g
N-(2-Aminoethyl)-3-Aminopropyltrimethoxysilane	United Chemical Technologies	CAT#1760-24-3

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NeutrAvidin Protein	Thermo Scientific	CAT#31000
Rnase Inhibitor, Murine	New England Biolabs	CAT#M0314L
Trolox(R), 97%, ACROS Organics™	Fisher Scientific	CAT#AC218940010
Oligonucleotides		
Biotin-18mer: 5′- /biotin/rUrGrG rCrGrA rCrGrG rCrArG rCrGrA rGrGrC/3AmMO/-3′	Integrated DNA Technologies	N/A
U50-18mer: 5′- /5AmMC6/rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrGrC rCrCrU rCrGrC rUrGrC rCrGrU rCrGrC rCrA-3′	Integrated DNA Technologies	N/A
Software and algorithms		
IDL	Harris Geospatial	https://www.l3harrisgeospatial.com/Software-Technology/IDL
ImageJ (Fiji)	National Institutes of Health	https://imagej.nih.gov/ij/
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
smCamera	(Roy et al., 2008)	https://cplc.illinois.edu/research/tools
Other		
5 Minute® Rapid-Curing, General Purpose Adhesive Epoxy, 25 mL Tube	All-Spec	CAT#14250
Chemyx Inc fusion 200 Touch Syringe Pump	Fisher Scientific	CAT#NC0670590
Diamond Drill 0.75 mm (100 pack)	Crystalite Corporation	CAT#C5250510
ETT PTFE Tubing Size 26, 100 ft, Natural Color	Weico Wire	CAT#ETT-26
Fisherbrand™ Glass Staining Dishes for 16 Slides	Dynalon	CAT#235505
Pellin Broca Prisms	Eksma Optics	CAT#325-1206
Quartz Microscope Slides (1″ × 3″ × 1 mm)	G Finkenbeiner Inc.	N/A
Scotch® 665 Permanent Double-Sided Tape, 1/2" × 250", Clear, Pack Of 3 Rolls	Office Depot	CAT#391775
TetraSpeck™ Microspheres, 0.1 µm, fluorescent blue/green/orange/dark red	Thermo Fisher Scientific	CAT#T7279
VMM-D3 Three-Channel Shutter Driver	Vincent Associates	CAT#VMM-D3
VSR Coverglass (24 mm × 40 mm)	VWR	CAT#16004-306
Zeba™ Spin Desalting Columns, 7K MWCO, 0.5 mL	Thermo Fisher Scientific	CAT#89883

MATERIALS AND EQUIPMENT

A prism-type total internal reflection fluorescence (TIRF) microscope is required for these single-molecule measurements. Many of these scopes are homebuilt, and there are papers with detailed instructions for constructing and optimizing TIRF microscopes (Joo and Ha, 2012). Because of the complexity of building TIRF microscopes, we refer the reader to these publications for further instructions. Commercially-available objective-type TIRF microscopes may be used, but the signal-to-noise ratio is generally poor and the alignment of the excitation and emission beams is difficult. A shutter manager for alternating excitation is required for colocalization experiments, and it should be used to image acceptor molecules for FRET data analysis (see below) (Kapanidis et al., 2015). Flow experiments (i.e., nucleation, colocalization, and some FRET assays below) require a syringe pump for withdrawing buffers from a buffer reservoir into a syringe (Yang and Ha, 2018).

Some of the reagents listed in the Key Resources Table can be swapped for equivalent products. This list was constructed for an MBP-6xHis-FUS (maltose-binding protein; fused in sarcoma) construct with a TEV cleavage site located between the 6xHis tag and the FUS coding sequence; if a different protease recognition site is used, the TEV protease can be swapped to the corresponding site-specific protease. The dyes used for labeling can also be changed to the preferred labeling scheme (e.g., maleimide) or excitation wavelength (e.g., 488 nm or 750 nm). The RNA sequence can also be changed as needed; sequences with hairpin or stem-loop structures may bind certain disordered proteins more effectively. Finally, other biotinylated antibodies can be used for tethering the purified protein to the surface.

T50 buffer (50 mL)		
Reagent	Final concentration	Amount
1 M Tris, pH 7.4	10 mM	500 μL
5 M NaCl	50 mM	500 μL
dH_2O	n/a	49 mL
Total	**n/a**	**50 mL**
T50 Buffer may be stored at 25°C indefinitely.		

100× gloxy (100 μL)		
Reagent	Final concentration	Amount
Glucose Oxidase	n/a	10 mg
~1000 U/μL Catalase	20 U/μL	2 μL
T50 Buffer	n/a	98 μL
Total	**n/a**	**100 μL**
Gloxy can be stored at 4°C for up to 8 weeks.		

Imaging buffer (500 μL)		
Reagent	Final concentration	Amount
3.2 M KCl	100 mM	15 μL
1 M Tris, pH 7.4	20 mM	10 μL
20% (w/v) glucose	0.4% (w/v)	10 μL
100× Gloxy	1×	5 μL
10000 U/mL TEV Protease	40 U/mL	2 μL
40000 U/mL RNase Inhibitor	160 U/mL	2 μL
10 mM Trolox	~10 mM	456 μL
Total	**n/a**	**500 μL**
Imaging Buffer may be stored at 25°C for up to 8 h, and it should be made fresh for each experiment.		

Note: Trolox (10 mM) can be stored at −20°C for up to 6 months and at 4°C for up to 8 weeks.

Note: Salt and pH of the Imaging Buffer may be adjusted depending on the protein being tested. TEV (or equivalent protease) may also be pre-incubated with the protein-of-interest before the experiment. Because excess TEV is added to the Imaging Buffer, cleavage of the protease recognition site is usually rapid and pre-incubation is unnecessary. TEV may also be omitted depending on the experiment.

Alternatives: Other oxygen scavenging systems may be used in lieu of glucose oxidase and glucose.

STEP-BY-STEP METHOD DETAILS

Slide assembly

Timing: ~1 h

In this step, the single-molecule slide is assembled by attaching the coverslip and slide with epoxy. Lanes for each reaction are delineated with double-sided tape. For flow experiments, epoxy molds and buffer reservoirs are added for automated flow of solutions. Please see Methods video S1 for a visual walkthrough of how to assemble TIRF slides.

1. Assemble the slide
   a. Warm the slide to 20°C–25°C for ~10 min.
   b. Carefully remove the slide from the conical vial and place the PEGylated side face-up on a glass plate or other sterile surface.
   c. Remove the coverslip and use a fine-tip sharpie to mark the non-PEGylated side of the coverslip. Place the coverslip back in the 50 mL conical vial for safekeeping.

   Note: If it is unclear which side of the slide is PEGylated, one can gently pipet distilled water onto each side of the slide. The PEGylated side will have less surface tension and the water will move around more easily when the slide is tilted. The water can then be dried with a nitrogen gas stream.

   d. Tear off a ~10-cm-long piece of double-sided tape. Use a clean razor blade to slice the tape into ~1 mm × 10 cm-long strips. The strips can then be cut down the middle for ~10–12 1 mm × 5 cm strips.
   e. Lift the tape strip from the sterile surface and gently apply one strip to the slide surface between each pair of drilled holes and along the outer edge of the first and final pair of holes.
   f. Remove the coverslip and lower it onto the taped slide.
      i. The PEGylated side of the coverslip and the slide should be facing each other.
      ii. Seal the slide and coverslip by gently pressing with a 1000-μL pipet tip and/or the blunt edge of a razor blade.
   g. Cut the excess tape on each end of the slide using a razor blade.
   h. Seal the outer edges of the slide and coverslip by applying quick-drying epoxy with a 200-μL pipet tip.

   ⚠ CRITICAL: Be careful not to apply too much epoxy or it will flow too far into the lane via capillary action and "plug" a lane.

   i. Allow the epoxy to dry for 5–10 min, then return the slide to the 50 mL conical vial until use in the experiment.

2. Flow slide preparation (optional for methods 1 and 2, required for methods 3 and 4)
   a. For each lane, cut and save the upper 1 cm of a 200-μL pipet tip to make a buffer reservoir that will be attached to the top of the slide.
   b. Use epoxy to attach the buffer reservoir around each of the drilled holes at the top of the slide.

   Note: Pipet tips (10 μL) can be inserted into each of the holes to prevent epoxy from sealing the lane.

   c. Insert 10-μL pipet tips into the other drilled holes and seal with epoxy to create a mold for the syringe mold.
   d. Wait ~30 min until the epoxy has completely dried.
   e. Carefully remove the 10-μL pipet tips.
   f. Prepare the syringe lead by cutting and discarding the upper ~2 cm of a 10 μL pipet tip and attaching the remaining portion of the tip to 2 mm tubing with epoxy.
   g. Attach the tubing to a 10-mL syringe with a needle luer-lock attachment. Fasten the syringe into the syringe pump and set the pump to withdraw 40 μL with a ~5 s delay.

Method 1: FRET of RNA & disordered proteins

Timing: ~3 h

This step uses a FRET pair of dyes to report on the distance between two different regions in a protein-RNA interaction. The FRET dyes can be attached to each end of the annealed 18-mer RNA strand, or one dye can be replaced with a site-specific label on the protein. The FRET pair reports conformational data on the protein and RNA. Salt, protein concentration, pH, dye location, and other parameters can be modified. Flow experiments are optional here, but the initial binding of the protein to the RNA can induce unique shifts in the FRET efficiency that report on temporary conformational changes of the protein and/or RNA.

3. Map the donor/acceptor channel xy-offset using a bead slide map
   a. Make a low-density bead mixture by diluting 3 μL tetraspeck beads in 77 μL T50.
   b. Pipet the bead solution onto a non-PEGylated/untreated quartz slide.
   c. Gently sandwich the slide with an untreated coverslip. Dab the edges with a tissue to collect excess buffer.
   d. Seal the outside edges of the slide and coverslip with fast-drying epoxy.
   e. Allow the epoxy to solidify for ~10 min and transfer to a 50 mL conical vial for safekeeping.
   f. Apply water to the objective on the TIRF microscope.
   g. Place the slide on the objective; the coverslip should be facing down.
   h. Clean the prism with ethanol. Apply ~1 drop of immersion oil and attach the prism to the microscope so that the oil surface is touching the slide.
   i. Turn on the fluorescent laser to illuminate the beads. Adjust the bead alignment as needed. Troubleshooting 1
   j. Optimize the gain, exposure time, laser intensity, and z-plane focus.
   k. Acquire several short (1–2 s) movies for mapping the offset (see quantification and statistical analysis for further instructions).

   Note: Bead slides can be stored in the dark at 20°C–25°C for up to 1–2 months.

4. Flow reagents onto the single-molecule slide for FRET measurements. Each lane has a pair of holes, and a 200-μL tip is inserted into one hole while the flow-through is collected from the other hole.
   a. Replace the bead slide on the TIRF microscope with the assembled single-molecule slide from steps 1–2. The water and oil immersion surfaces will need to be prepared again as described in steps 3.f-3.j.
   b. Flow 50 μL 100 μg/mL NeutrAvidin onto the single-molecule surface and collect the flow-through with a folded tissue.
   c. Incubate for ~5 min.
   d. Wash the lane with 50 μL T50 buffer.
   e. Flow 50 μL 50 pM annealed RNA onto the single-molecule surface.

   ⚠ CRITICAL: The RNA density on the single-molecule surface should be concentrated enough to measure many binding events at once, but not too dense so as to saturate the surface; approximately 200–300 molecules per field-of-view is usually optimal. When using a new RNA construct, one lane should be used as a test to determine the labeling efficiency. If the RNA is too dense, move to the next lane and use a two- or three-fold lower concentration of RNA. If the RNA is too sparse, flow a more highly-concentrated RNA stock (e.g. 100 pM or 200 pM) into the same lane and evaluate the labeling density. This process can be repeated and fine-tuned until the optimal labeling density is achieved. It is also possible to adjust the biotin-PEG concentration on the slides, but slides are usually made in batches so adjusting the RNA concentration is more straightforward and efficient.

   Note: Photobleaching will be severe until Imaging Buffer is added to the channel.

f. Incubate for ~5 min.
g. Wash the lane with 50 μL T50 buffer again. Troubleshooting 2
h. Flow 50 μL Imaging Buffer.
i. Acquire 1–3 long (~60–80 s) videos and 8–10 short (~1–2 s) videos with the donor excitation laser; longer videos are used to evaluate the FRET efficiency of individual molecules over extended time ranges whereas short movies are used to gather the FRET efficiencies of many different molecules to construct a histogram of FRET efficiency for this condition. If a shutter manager is being used and the RNA is FRET labeled, a quick 1-s excitation with the acceptor laser can be used to help filter for RNA molecules with both the donor and acceptor dyes. Troubleshooting 3
j. Flow 50 μL Imaging Buffer with your protein-of-interest at the desired concentration.

Note: If the protein is labeled, the maximum concentration is ~10 nM to avoid saturating the camera's detector.

Note: If the flow setup is being used, acquire a long video (60+ s) while flowing the protein onto the single-molecule surface with the syringe pump.

k. Acquire 3–5 long videos and 10–12 short videos in different fields of view with the donor excitation laser. Troubleshooting 4
l. More videos can be acquired after incubating the protein with the RNA for an extended period of time (e.g., 30+ min).

Note: Lanes can be reused by either regenerating the slide as described previously (Paul et al., 2021), or by flowing additional Imaging Buffer conditions (e.g. increased protein or salt concentrations) in the previously-used lane.

m. The movies are processed as described below (see quantification and statistical analysis).

5. Use the other lanes to perform the appropriate controls, e.g., flowing protein without RNA immobilization, or flowing a control protein like the solubility tag or protease alone. Slides may also be regenerated as described previously (Paul et al., 2021).

Method 2: photobleaching of fluorescently tagged disordered proteins

Timing: ~3 h

In this method, a fluorescently tagged (e.g., GFP, mCherry, etc.) disordered protein is tethered to the surface with an antibody raised against the fluorescent protein, and the tag is photobleached with strong laser exposure. The photobleaching steps are counted during the trace analysis (see below) to report on the oligomerization status of the protein, which varies depending on the protein concentration and other parameters. These experiments can be performed with a flow slide, but it is not necessary.

6. Set up the TIRF microscope for the photobleaching experiment.
   a. Apply water to the objective on the TIRF microscope.
   b. Place the assembled single-molecule slide from steps 1–2 on the objective; the coverslip should be facing down.
   c. Clean the prism with ethanol. Apply ~1 drop of immersion oil and attach the prism to the microscope so that the oil surface is touching the slide.
   d. Adjust the beam alignment for TIRF imaging.
   e. Optimize the gain, exposure time, laser intensity, and z-plane focus for imaging the single-molecule surface.

7. Photobleaching of fluorescently-tagged proteins.
   a. Flow 50 μL 100 μg/mL NeutrAvidin onto the single-molecule surface and collect the flow-through with a folded tissue.
   b. Incubate for ~5 min.
   c. Wash the lane with 50 μL T50 buffer.
   d. Flow 50 μL 10 nM biotinylated anti-tag (e.g., anti-GFP) antibody.

   Note: As with RNA immobilization, this concentration may need to be optimized depending on the labeling density of the fluorescently tagged protein.

   e. Incubate for ~5 min.
   f. Wash again with 50 μL T50 buffer.
   g. Flow 50 μL Imaging Buffer containing the fluorescently tagged protein. The concentration of protein can be reliably increased up to 50 nM without saturating the imaging surface.
   h. Acquire 5+ long (60+ s) videos in different fields of view at maximum laser intensity in the excitation channel corresponding to the tagged protein. Troubleshooting 5

8. Perform a photobleaching control with the fluorescent tag alone (e.g., GFP without the conjugated RNA binding protein).

Method 3: nucleation of fluorescently labeled disordered proteins

Timing: ~2 h

Here, a fluorescently labeled (e.g., Cy3, Cy5, etc.) protein is flowed onto the single-molecule surface, which is coated with unlabeled RNA. The RNA may also be labeled with a dye that does not interfere with the protein. The association kinetics between the RNA and protein and the oligomerization status of the protein can be determined from the single-molecule traces (see below). This method requires the automated flow of a syringe pump to accurately measure the fluorescence intensity of the protein as it is first interacting with the RNA substrate.

9. Set up the TIRF microscope for a flow experiment.
   a. Apply water to the objective on the TIRF microscope.
   b. Place the assembled single-molecule slide from steps 1–2 on the objective; the coverslip should be facing down.
   c. Attach a 5–10 mL syringe to the syringe pump and connect it to a needle attachment. Carefully insert the vacuum tubing with a 10-μL tip attached into the needle. Insert the tip into the epoxy mold on the slide.

   Note: When using the syringe pump, less volume should be withdrawn than is added to the buffer reservoir. For instance, if 50 μL of solution is added to the reservoir, 40 μL should be withdrawn by the pump. A slower flow rate of ~1 mL/min is recommended.

   d. Clean the prism with ethanol. Apply ~1 drop of immersion oil and attach the prism to the microscope so that the oil surface is touching the slide.
   e. Adjust the beam alignment for TIRF imaging.
   f. Optimize the gain, exposure time, laser intensity, and z-plane focus for imaging the single-molecule surface.

10. Nucleation of fluorescently labeled proteins on the single-molecule surface.
    a. Flow 40 μL 100 μg/mL NeutrAvidin by adding 50 μL to the buffer reservoir and withdrawing the solution through the syringe pump.
    b. Incubate for ~5 min.

c. Wash the lane with 40 μL T50 buffer using the syringe pump to withdraw the T50 through the lane.
d. Flow 40 μL 50 pM unlabeled RNA with the syringe pump.

Note: The RNA can also be labeled for easier visualization of the single-molecule surface. However, the label should not interfere with the nucleation imaging. For instance, if the protein is Cy3-labeled, the RNA should be in a different channel such as Cy5. The RNA can be imaged during the flow video before the protein is added to the lane.

e. Incubate for ~5 min.
f. Wash using the syringe pump again with 40 μL T50 buffer.
g. Flow 40 μL Imaging Buffer without the protein.

⚠ CRITICAL: Although the surface will not usually be imaged here, it is important to flow the Imaging Buffer before the flow experiment. This is because a dramatic change in buffer (e.g. T50 buffer to Imaging Buffer) may cause premature capillary flow of the protein-containing Imaging Buffer into the lane, which can compromise the nucleation imaging.

h. Add 40 μL Imaging Buffer with the labeled protein to the buffer reservoir. The protein concentration should not exceed 10 nM, or the surface may become saturated.
i. Prepare the syringe pump with a 10-s delay (or manually delay the flow for 10 s) and find an ideal region for acquiring the video.
j. Start acquisition of a long (60+ s) video and begin the delayed flow simultaneously.

11. Controls should be performed with single-molecule surfaces lacking RNA and with labeled control proteins.

Method 4: colocalization of 2+ fluorescently labeled disordered proteins

Timing: ~2 h

Finally, this protocol uses a similar approach as method 3, but with two or more labeled proteins. Alternating excitation of multiple lasers is used to evaluate whether multiple disordered proteins are co-oligomerizing on single RNA molecules. This method requires the automated flow of a syringe pump to accurately measure the fluorescence intensity of the proteins as they are first interacting with the RNA substrate.

12. Repeat Step 9.

13. Nucleation of two or more proteins on a single-molecule slide.
    a. Set up an automatic shutter manager for alternating excitation. The laser should switch every ~1 s to capture co-binding events.
    b. Flow 40 μL 100 μg/mL NeutrAvidin by adding 50 μL to the buffer reservoir and withdrawing the solution through the syringe pump.
    c. Incubate for ~5 min.
    d. Wash the lane with 40 μL T50 buffer using the syringe pump to withdraw the T50 through the lane.
    e. Flow 40 μL 50 pM unlabeled RNA with the syringe pump.

Note: It is more difficult to focus the z-plane for unlabeled RNA samples. It may be helpful to use one lane as a control for finding the focal plane, or background fluorescence can be used for preparing the TIRF microscope.

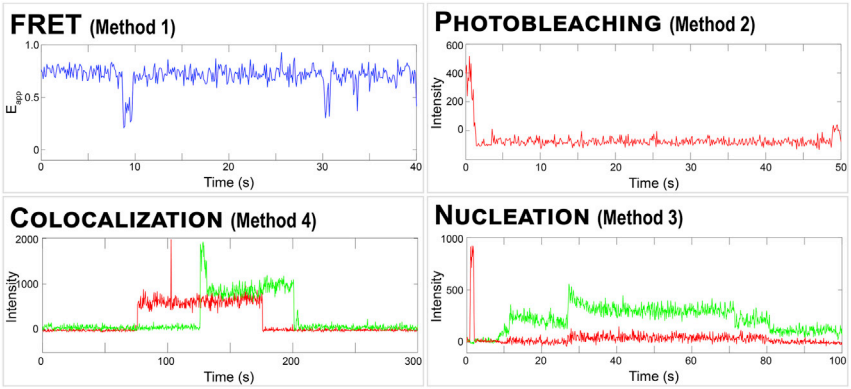

Figure 2. Sample TIRF experiment traces

Raw data from single-molecule experiments performed with each of the described techniques. *Top Left*, FRET over time of 500 nM G156E-FUS in 20 mM Tris and 100 mM KCl with the glucose oxidase scavenging system. *Top Right*, 5 nM GFP-FUS in 20 mM Tris and 100 mM KCl. *Bottom Right*, 5 nM Cy3-FUS in 20 mM Tris and 100 mM KCl with the oxygen scavenging system and TEV; the initial Cy5 intensity is the Cy5-RNA exposed for 1 s. *Bottom Left*, Two-color nucleation of 2.5 nM Cy3-FUS and Cy5-FUS in 20 mM Tris and 100 mM KCl with the scavenging system and TEV protease.

f. Add 40 μL Imaging Buffer with both labeled proteins to the buffer reservoir. The protein concentration in each fluorescence channel should not exceed 10 nM, or the surface may become saturated.
g. Prepare the syringe pump with a 10-s delay (or manually delay the flow for 10 s) and find an ideal region for acquiring the video.
h. Start acquisition of a long (60+ s) video and begin the delayed flow simultaneously.

EXPECTED OUTCOMES

If the individual molecules are resolvable, the fluorescence intensity of each RNA or protein can be plotted as a function of time – these are single-molecule "traces." Due to the stochasticity of single-molecule binding, some traces will be unusable or have poor quality data; these traces should be excluded from formal analysis. The proportion of unusable traces can be as high as 50% of all detected molecules depending on the complexity of the experiment; these unusable traces may arise from (1) incomplete labeling of the RNA, (2) poor slide preparation, and/or (3) rapid photobleaching. Tips for reducing the number of unusable traces are provided in the troubleshooting section below. A typical high-quality trace for each assay is shown in Figure 2. Below are the expected outcomes for each of the four methods discussed above:

Method 1 (FRET): The FRET efficiency should deviate from the native RNA FRET efficiency, which is dependent on the sequence folding and length, when the protein-of-interest binds. Some proteins may also cause dynamic FRET efficiency fluctuations or cyclical FRET efficiency changes upon binding.

Method 2 (Photobleaching): The fluorescence intensity should decrease in a stepwise manner as the individual fluorescent proteins are photobleached by the high laser power. The distinct photobleaching steps should be countable for quantification.

Method 3 (Nucleation): The fluorescence intensity of the labeled protein channel should increase in a stepwise manner as proteins bind to the tethered RNA. The proteins will likely photobleach due to the constant laser exposure, though additional controls (i.e., FRET experiments) need to be performed to determine whether the decrease is due to unbinding or photobleaching.

Method 4 (Multicolor nucleation): The fluorescence intensity of either or both fluorescence channels should increase in a stepwise manner, like the outcome for method 3.

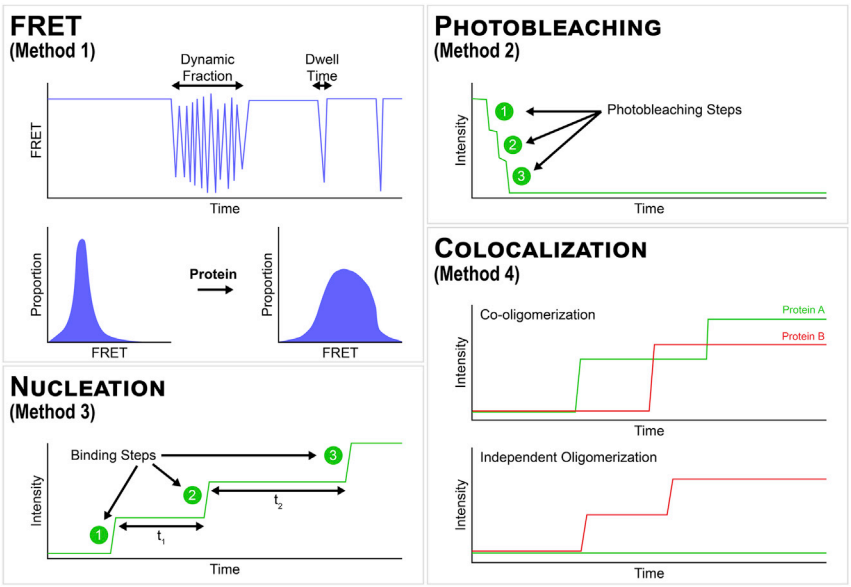

Figure 3. Quantification of TIRF experiments

Examples of data that can be quantified from each type of trace obtained in this protocol. *Top Left*, FRET values can report on the conformational dynamics of protein-RNA binding through individual traces (top) or histograms of all molecules (bottom). FRET fluctuations will vary depending on the conditions, but repetitive fluctuations may be correlated with dynamic RNA interactions in condensates. *Top Right*, Each photobleaching step can be counted and quantified to report on the oligomerization of the protein captured by the biotinylated antibody. *Bottom Right*, Example traces of colocalized or independent oligomerization on a single RNA. *Bottom Left*, Nucleation traces report on the overall oligomerization status per unit time and the time elapsed between each oligomerization event, which can be used to determine association rates for each protein oligomer.

QUANTIFICATION AND STATISTICAL ANALYSIS

The analysis pipeline for single-molecule movies follows the same initial flow. First, individual molecules are mapped; the offset between multiple channels is corrected using a "map" that is generated from the bead slide movies (see method 1: FRET of RNA & disordered proteins). Second, the fluorescence intensity over time is extracted using custom programs or scripts written in IDL (see data and code availability). Third, the traces are quantified and analyzed in another analysis program such as Matlab. Figure 3 shows some of the values that can be determined from traces for each experiment, and the analysis is discussed in greater detail below. Methods videos S2 and S3 show the raw pre-processed videos for a nucleation and colocalization experiment, respectively.

After calculating the fluorescence intensity of each molecule over time, the traces can be plotted using Matlab (see data and code availability) or an equivalent data analysis program. For FRET data, the apparent FRET efficiency (E_{app}) is calculated using the following equation:

$$E_{app} = \frac{I_A}{I_D + I_A},$$

where I_A is the intensity of the FRET acceptor and I_D is the intensity of the FRET donor. The background dye fluorescence is subtracted from each intensity; we perform this correction by subtracting the intensity of the surrounding pixels. The detector efficiency γ may impact the apparent FRET efficiency, and we refer the reader to another publication discussing this factor in more detail (Hellenkamp et al., 2018). A further correction for fluorescence leakage between the donor and acceptor emission channels may be needed depending on the instrument and fluorophores; previous publications cover this correction in more detail (Roy et al., 2008). This leakage, α, usually ranges between 0.1 to 0.2 for commonly used FRET dye pairs.

$$E_{app} = \frac{I_A^0 - \alpha \times I_D^0}{I_D^0 + I_A^0 - \alpha \times I_D^0}$$

The FRET efficiency for all molecules can be visualized as a histogram, and the FRET efficiency for each molecule is plotted individually for each trace. Fluctuations in the FRET efficiency value can be interpreted for more detailed information about the conformational dynamics of the protein-RNA complex, though the exact implications of the data vary depending on the research question.

Photobleaching and nucleation data is processed similarly, but the mapping and FRET calculations are usually not performed. The photobleaching steps can be manually counted, or a standard curve corresponding to the initial fluorescence and the number of steps can be constructed if the illumination on the surface was even. Nucleation is quantified in a similar manner but with fluorescence increases corresponding to binding events. The kinetics of each binding event can also be determined from the nucleation data.

Colocalization between two or more proteins requires deinterleaving the alternating excitation of the two channels (Kapanidis et al., 2015). The colocalization between the two proteins can then be manually quantified by overlapping fluorescence intensities in the deinterleaved traces.

Finally, the movie files from each experiment can be exported and processed using ImageJ for use in presentations or manuscripts.

LIMITATIONS

Single-molecule assays are a powerful tool to interrogate the behavior of individual disordered proteins as they interact with RNA and oligomerize into higher-order structures. However, disordered proteins may not tolerate certain labeling chemistries or modifications to their structure to accommodate site-specific maleimide labeling, limiting the effectiveness of structural FRET-based assays. Nucleation and photobleaching assays also may saturate the camera detector at high concentrations (~tens of nM), which is still far below the concentrations used to form droplets *in vitro* and reported concentrations *in vivo* (~μM). The lack of commercially available prism-type TIRF microscopes also makes these experiments inaccessible for many labs.

TROUBLESHOOTING

Problem 1

The single-molecule surface is not visible and/or the microscope cannot be aligned, which may occur in methods 1, 2, 3, or 4.

Potential solution

Contact between the oil and the slide/quartz prism surfaces needs to be clean and devoid of air bubbles. The evanescent field (i.e., single-molecule region) will not be visible if the oil surface is deformed. To solve this issue, try simply cleaning the prism and slide, reapplying the oil, and attempting to align the excitation laser beam again. The bead slide should be extremely bright and easy to focus/align, and it can also be used to help realign the laser if it is knocked out of focus during a single-molecule experiment.

Problem 2

RNA spots disappear after flowing T50 or Imaging Buffer, which may occur in methods 1 or 3.

Potential solution

Due to the low concentration of RNA on the surface of the slide, even a small amount of RNase contamination can lead to a loss of RNA foci. RNAse contamination can occur with either T50 or Imaging Buffer. For T50 contamination, one should simply remake the buffer. For Imaging Buffer,

RNase Inhibitor should be added because the likely source of contamination is the catalase, which cannot be omitted unless an alternative oxygen scavenging system is used.

Problem 3

There are many aggregates/large spots on the surface, which may occur in methods 1, 2, 3, or 4.

Potential solution

Poor quality of the TIRF slides can lead to pieces of dirt or dust fluorescing on the surface during movie acquisitions. The slide preparation protocol is designed to decrease the background as much as possible, and one should avoid handling the slides with their gloves as much as possible.

Problem 4

The fluorescence signal rapidly decreases during video acquisition, which may be a problem in method 1.

Potential solution

The most likely cause of this issue is intense laser exposure during the video acquisition. One can lower the laser intensity or expand the excitation spot; the key is balancing the laser exposure so that it is low enough to avoid photobleaching but high enough to have good fluorescence signal. The oxygen scavenging system may have expired, so a new batch should be prepared. If the issue persists after trying these two solutions, there may be RNAse contamination from the protein-of-interest. One should avoid adding RNase to the protein after purification; instead add it to the lysis buffer, which should still remove most RNA prior to purification.

Problem 5

The fluorescently tagged protein is not photobleaching, which may be an issue during method 2.

Potential solution

Here, the oxygen scavenging system may need to be omitted from the Imaging Buffer for effective photobleaching of GFP, mCherry, etc. The laser intensity can also be increased or the size of the excitation spot can be reduced to increase the effective laser power across the single-molecule surface.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sua Myong (smyong@jhu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Single-molecule data acquisition and analysis package can all be obtained freely from CPLC's website (https://cplc.illinois.edu/research/tools). MATLAB code from this manuscript can be downloaded from Github: (https://github.com/Myong-Lab). IDL (http://www.exelisvis.co.uk/ProductsServices/IDL.aspx), and MATLAB (https://www.mathworks.com/) can be downloaded with academic or individual licenses from their respective distributors. ImageJ is an open-source program available from the NIH (https://imagej.nih.gov/ij/).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101131.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.R. and S.M.; methodology, K.R.; writing, K.R. and S.M.; supervision, S.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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