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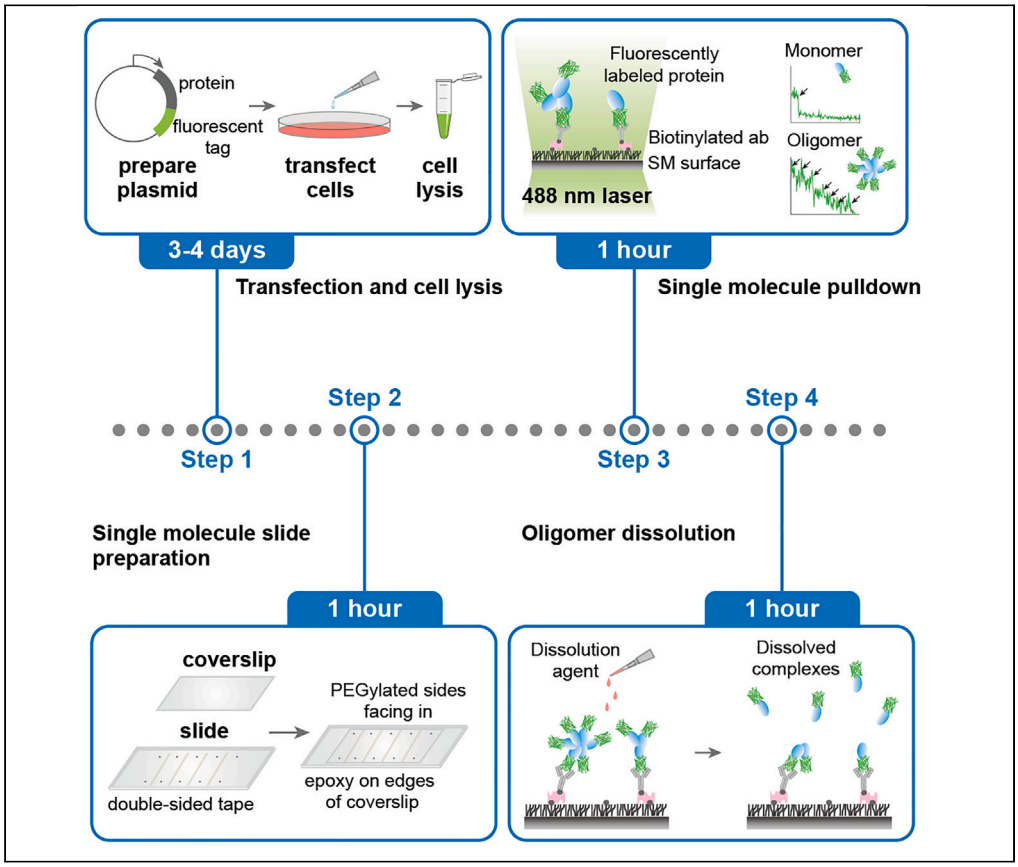
Protocol for single-molecule pull-down of fluorescently tagged oligomers from cell lysates



Mutations in intrinsically disordered proteins drive the irreversible formation of pathological aggregates, a hallmark of neurodegenerative diseases. Here, we present a protocol to pull down fluorescently tagged proteins to characterize their basal oligomeric states. We describe steps for transfection and cell lysis, single-molecule slide preparation and pull-down, and oligomer dissolution. This protocol enables visualization of protein oligomers with single-molecule resolution. In addition, differences in oligomerization may provide insight on condensation or aggregation propensity in differing mutated or cell stress conditions.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Isolation and pull-down of basal protein oligomers from cell lysates

Dissolution of protein oligomers to provide insight on oligomer formation

Detailed steps for slide preparation and assembly for single-molecule pull-down

Basal protein oligomerization to provide insight on protein condensation

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Protocol for single-molecule pull-down of fluorescently tagged oligomers from cell lysates

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SUMMARY

Mutations in intrinsically disordered proteins drive the irreversible formation of pathological aggregates, a hallmark of neurodegenerative diseases. Here, we present a protocol to pull down fluorescently tagged proteins to characterize their basal oligomeric states. We describe steps for transfection and cell lysis, single-molecule slide preparation and pull-down, and oligomer dissolution. This protocol enables visualization of protein oligomers with single-molecule resolution. In addition, differences in oligomerization may provide insight on condensation or aggregation propensity in differing mutated or cell stress conditions. For complete details on the use and execution of this protocol, please refer to Djaja et al.[1]

BEFORE YOU BEGIN

These protocols are designed to pull down fluorescently-tagged proteins of interest from cellular lysates. The protocol below describes specific steps for using SH-SY5Y cells. However, we have used this protocol in HEK293 cells. The protocol also uses calcium phosphate transfection, but lipofectamine, nucleofection, or other forms of transfection can be used.

Ensure compliance 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. Carefully consider fluorescent tags for proteins of interest. Certain tags (e.g., GFP, YFP) enable optimal detection of photobleaching steps, while other tags (e.g., mCherry, RFP) result in less optimal detection of photobleaching steps. Experiments illustrated throughout the paper used eGFP fluorescent tags. mCherry fluorescent tags were also tested but resulted in suboptimal photobleaching detection. Fluorescent tags should exhibit photobleaching properties while enabling detection via total internal reflection fluorescence microscope.

It is advisable that you perform the following steps before beginning the protocols.

Plasmid preparation

Timing: 3 days

1. Transform Bacteria using DH5α competent cells.

a. Remove tube(s) of DH5α from –80°C to thaw on ice for 5 min.
b. Label a microcentrifuge tube noting plasmid of interest and leave tube on ice.
c. Add desired amount of plasmid DNA to tube.

Note: 10 pg–100 ng is sufficient for transformation.

d. Then add 25 μL DH5α cells to tube and mix by pipetting slowly.
e. Incubate DH5α cells with DNA on ice for 30 min.
f. Heat shock for 40 s at 42°C.
g. Incubate on ice for 5 min.
h. Add entire contents of tube containing cells and DNA to 1 mL LB.
i. Allow cells to recover in LB for 1 h while shaking at 200–250 rpm at 37°C.
j. Prepare a 1:10 or 1:100 dilution of cells and plate 100 μL of dilution on LB agar plate with appropriate antibiotic.
k. Spread the bacteria with sterile beads or a cell spreader.
l. Store agar plate in 37°C incubator for 12 h.

2. Inoculate bacterial colonies.

Note: Amount of culture can be adjusted to prepare desired amount of plasmid DNA.

a. Label a 14 mL bacterial culture tube.
b. Add 5 mL LB to tube.
c. Add appropriate antibiotic to tube.
d. Select a colony for inoculation and place in tube.
e. Incubate tube for 12–18 h while shaking at 200–250 rpm at 37°C.

3. Plasmid DNA extraction
a. Place contents of bacterial culture tube into 5 mL centrifuge tube.
b. Spin down at 10,000 × *g* for 1 min at 20°C–25°C.
c. Decant or aspirate and discard culture media.
d. Proceed with any DNA extraction protocol. We used the E.Z.N.A. Plasmid DNA Mini Kit I (V-spin).
e. Following DNA extraction, measure concentration and dilute accordingly.

Note: We prepared DNA plasmid dilutions such that concentration was between 0.1–0.5 μg/μL.

f. Store DNA at −20°C.

SH-SY5Y cell culturing and transient transfection

Timing: 4 days

4. Culture SH-SY5Y cells.
a. Culture SH-SY5Y cells on a 100 × 15 mm Petri dish using 8 mL fresh DMEM and 300–400 μL cells or approximately 2 × 10^6 cells.
b. Cells should be plated such that they have 20%–30% confluency the following day. Cells may be counted to ensure the appropriate density.

5. Calcium phosphate transfection.

Note: Other transfection protocols may be used if preferred. For our experiments, we performed calcium phosphate transfection in SH-SY5Y cells as this yielded high transfection efficiency.

a. Prepare 2.5 M $CaCl_2$.
b. Prepare 2X HBS buffer.
c. Warm $CaCl_2$ and ddH_2O to 37°C 15 min prior to transfection.
d. Let 2X HBS sit at 20°C–25°C prior to transfection.
e. Prepare microcentrifuge tubes.
f. Add warm 66 μL $CaCl_2$ to each tube.
g. Add warm ddH_2O to tubes.

Note: ddH_2O and DNA volume should total 574 μL. Therefore, if 1 μL of DNA is added, 573 μL of ddH_2O should be added.

h. Add DNA to tube at desired concentration.

Note: 0.1–1 μg of DNA is sufficient for transfection. We used low plasmid concentrations to minimize over-expression.

i. Pipet solution several times to mix.
j. Slowly add 640 μL 2X HBS to tube.
k. Introduce bubbles into the solution by pipetting air into the mixture 10 times with a P200 pipet tip (transfection efficiency is increased when solution is bubbled vs. pipet mixing only).
l. Let solution in tubes incubate at 20°C–25°C for 5–10 min.
m. Add transfection mixture dropwise into Petri dishes.
n. Gently shake Petri dishes to mix solution.
o. The following day or 12 h after transfection, remove media from Petri dishes and replace with new media.

Slide preparation

Timing: 2 days

Slide preparation ensures that new quartz slides and glass coverslips are properly biotinylated to enable capture of biotin-conjugated molecules on the single molecule surface. Many slide and coverslip pairs can be prepared in advance and left in long-term storage at −80°C. We refer the reader to further instructions and a video on slide preparation.[2,3]

6. Drill five pairs of holes (using a 0.75 mm diamond drill bit) along the longer edges of the quartz slide for flow inlet and outlet.
7. Prepare slides and coverslips 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 may be prepared since 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 dH_2O to the slide holders.
   c. Prepare empty slide holders and a 500 mL Erlenmeyer flask and add dH_2O to slide holders and flask. (Erlenmeyer flask is used to later prepare aminosilane mixture and should be properly cleaned following the instructions below.)
   d. Sonicate empty and filled slide holders and flask for 10 min using an ultrasonic cleaner.
   e. Remove dH_2O from slide holders and flask.
   f. Add methanol to the slide holders and flask.
   g. Sonicate for 15 min.
   h. Remove methanol from slide holders and flask.

i. Fill slide holders and flask with acetone.
j. Sonicate again for 15 min.
k. Remove acetone from slide holders and flask.
l. Rinse slide holders and flask with dH_2O.
m. Equilibrate aminosilane to 20°C–25°C.

8. KOH cleaning.
   a. Prepare 1 M KOH.
   b. Fill slide holders and flask with 1 M KOH.
   c. Sonicate for 30 min.
   d. Rinse slide holders and flask with dH_2O three times.
   e. Remove dH_2O and add methanol to slide holders and flask.
   f. Sonicate for 30 min.
   g. Remove methanol from empty slide holders and dry.
   h. Rinse slides and coverslips in slide holders with dH_2O.
   i. Carefully burn slides and coverslips to dry completely.

   Note: Slides and coverslips are extremely brittle especially after the KOH wash. Apply minimal pressure to slides and coverslips to prevent them from breaking.

   j. Place dried slides and coverslips in dry, empty slide holders.
9. Aminosilane preparation.
   a. Fill flask with 196 mL methanol and 2 mL glacial acetic acid.
   b. Mix by swirling the flask.
   c. Add 2 mL N-(2-aminoethyl)-3-aminopropyltrimethoxysilane and mix by swirling the flask.
   d. Add aminosilane/methanol/acetic acid solution into the slide holders with slides and coverslips.
   e. Incubate slide holders in the dark for 10 min.
   f. Sonicate for 1 min.
   g. Incubate again in the dark for 10 min.
10. PEGylation of slides.
    a. Prepare PEGylation chambers for humid incubation using slide boxes with dH_2O in the boxes.
    b. Rinse slides and coverslips with methanol twice.
    c. Rinse slides and coverslips with dH_2O twice.
    d. Air dry the slides and coverslips.

    Note: Slides and coverslips can be gently dried with a nitrogen gas stream for quicker drying.

    e. Prepare 10 mL of 100 mM $NaHCO_3$.
    f. Weigh out 160 mg mPEG-SVA and 2–4 mg Biotin-PEG-SVA per 10 slides and coverslip pairs.
    g. Add mPEG-SVA and Biotin-PEG-SVA to 800 μL 100 mM $NaHCO_3$.
    h. Centrifuge the PEG/Biotin-PEG solution at 10,000 × *g* for 2 min.
    i. Transfer the supernatant fraction to a new tube.
    j. Pipet 80 μL PEG/Biotin-PEG solution onto the surface of each slide.
    k. Carefully lay the coverslips on the slides and try to avoid air bubbles.
    l. Incubate in the dark for 4–12 h.
11. Final rinsing and storage.
    a. Carefully separate the slide/coverslip pairs.
    b. Wash the slides and coverslips separately with dH_2O.
    c. Air dry the slides and coverslips or use the nitrogen gas stream.
    d. Carefully transfer each slide and coverslip pair to a 50 mL conical tube.
    e. Place the PEGylated surfaces facing away from each other.
    f. Place conical tubes in vacuum-sealable bags for storage at −20°C for up to 1 year.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-GFP antibody biotin conjugated (1:200 dilution)	Rockland	600-406-215; RRID: AB_828168
Bacterial and virus strains		
NEB Turbo competent *E. coli* (high efficiency)	New England Biolabs	C2984H
Chemicals, peptides, and recombinant proteins		
Gibco 1X DPBS, no calcium, no magnesium	Fisher Scientific	14-190-144
AcTEV protease	Fisher Scientific	12-575-015
Alconox powdered precision cleaner	Fisher Scientific	16-000-104
Biotin-PEG-SVA, MW 5,000	Laysan Bio, Inc.	Biotin-PEG-SVA-5000-100 mg
mPEG-succinimidyl valerate, MW 5,000	Laysan Bio, Inc.	MPEG-SVA-5000-1 *g*
N-(2-aminoethyl)-3-aminopropyltrimethoxysilane	United Chemical Technologies	1760-24-3
NeutrAvidin protein	Thermo Fisher Scientific	31000
cOmplete protease inhibitor cocktail	Roche	11697498001
Sodium (meta)arsenite (>90%)	Millipore Sigma	S7400-100G
D-sorbitol	Millipore Sigma	S1876-100G
Ribonuclease A from bovine pancreas	Millipore Sigma	R6513-50MG
1,6-Hexanediol, 99%	Millipore Sigma	240117-50G
Guanidine hydrochloride	Millipore Sigma	G3272-100G
NP40 cell lysis buffer	Thermo Fisher Scientific	FNN0021
Karyopherin β2	Guo et al., 2018	N/A
DMEM, high glucose, no glutamine	Thermo Fisher Scientific	11960-044
Fetal bovine serum, certified, United States	Thermo Fisher Scientific	16000-044
Sodium bicarbonate 7.5% solution	Thermo Fisher Scientific	250-80-094
Sodium pyruvate (100 mM)	Thermo Fisher Scientific	113-60-070
L-glutamine (200 mM)	Thermo Fisher Scientific	250-30-081
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	15-140-122
Experimental models: Cell lines		
Human: SH-SY5Y	ATCC	Cat# CRL-2266; RRID: CVCL_0019
Recombinant DNA		
pcDNA3.1(+)-N-eGFP	GenScript	N/A
pFUS-WT-GFP_pcDNA3.1(+)-N-eGFP	GenScript	N/A
Software and algorithms		
SmCamera	Roy et al., 2008	https://cplc.illinois.edu/research/tools
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
IDL	Harris Geospatial	https://www.l3harrisgeospatial.com/Software-Technology/IDL
Prism 7	GraphPad (https://www.graphpad.com/ scientific-software/prism/)	N/A
Other		
Fisherbrand Petri dishes with clear lid	Fisher Scientific	FB0875713
E.Z.N.A. Plasmid DNA Mini Kit I (V-spin)	Omega Bio-tek	D6943-01
5 Minute rapid-curing, general purpose adhesive epoxy, 25 mL tube	Devcon	14250
Fisherbrand razor blades	Fisher Scientific	12-640
Diamond drill 0.75 mm (100 pack)	Crystalite Corporation	C5250510
Glass Coplin slide staining jar w/cover, for 75 × 25 mm slides	Cole-Parmer	UX-48585-20
Quartz microscope slides (1″ × 3″ × 1 mm)	G Finkenbeiner, Inc.	N/A
VSR coverglass (24 mm × 40 mm)	VWR	16004-306
Scotch 665 permanent double-sided tape, 1/2″ 3 250″, clear, pack of 3 rolls	Office Depot	391775
Olympus low auto fluorescence immersion oil	Edmund Optics	86-834

MATERIALS AND EQUIPMENT

A prism-type total internal reflection fluorescence (TIRF) microscope is required for single molecule measurements. Many of these scopes are homebuilt, and there are papers with detailed instructions for constructing and optimizing TIRF microscopes.[4] Because of the complexity of building TIRF microscopes, we refer the reader to these publications for further instructions. Traditional TIRF microscopes are built with 532 and 647 nm lasers required for FRET (Fluorescence Resonance Energy Transfer) imaging; ensure that the TIRF microscope is equipped with a 488 nm laser to obtain GFP photobleaching steps. Commercially available objective-type TIRF microscopes may also be used, but the signal-to-noise ratio can be lower and the access to the excitation and emission beams is often limited.

Some of the reagents listed in the key resources table can be swapped for equivalent products. This list was constructed for GFP-tagged FUS (fused in sarcoma) plasmid constructs with a GFP-only plasmid control. GFP tags can be swapped for alternative fluorescent tags and protein of interest can be exchanged. Biotinylated antibodies used for pull-down of GFP are commercially available, but can be swapped for pull-down of other fluorescent molecules of interest.

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**

Filter sterilize with a 0.22 µm filter. T50 Buffer may be stored at 20°C–25°C indefinitely.

SH-SY5Y Cell Culture Media (500 mL)

Reagent	Final concentration	Amount
DMEM, high glucose, no glutamine	N/A	425 mL
FBS	10%	50 mL
200 mM glutamine	2 mM	5 mL
10 mg/mL pen-strep	100 µg/mL	5 mL
7.5% sodium bicarbonate	0.15%	10 mL
100 mM sodium pyruvate	1 mM	5 mL
Total	**N/A**	**500 mL**

Filter sterilize with a 0.22 µm vacuum filter. Cell culture media can be stored at 4°C for 3–4 weeks.

2X HBS Buffer

Reagent	Final concentration	Amount
NaCl	274 mM	800 mg
KCl	10 mM	37.275 mg
$Na_2HPO_4\ 7H_2O$	1.4 mM	19 mg
Dextrose (D-glucose)	15 mM	148.6 mg
HEPES	42 mM	500 mg
ddH_2O	N/A	To 50 mL
Total	**N/A**	**50 mL**

pH solution to 7.00–7.05. Filter sterilize with a 0.22 µm filter. 2X HBS can be stored at 4°C for 12 months, but is generally used before this point. For long-term storage, 2X HBS can be stored at −20°C for 2 years.

Other solutions

- 2.5 M $CaCl_2$: Add 13.87 *g* $CaCl_2 \cdot 2H_2O$ in 50 mL ddH_2O, filter to sterilize.

Filter sterilize with a 0.22 µm filter. Store $CaCl_2$ solution at 4°C indefinitely.

- Cell lysis buffer: 10 mL NP-40 cell lysis buffer supplemented with 1 EDTA-free Protease Inhibitor Cocktail.

Cell lysis buffer should be kept on ice or at 4°C during the experiment and protease inhibitor cocktail should be added right before every experiment.

Alternatives: Other cell lysis buffer solutions can be used. Alternative cell transfection protocols may be followed instead. Media for cell culture should be modified for the cell line of interest.

STEP-BY-STEP METHOD DETAILS

Slide assembly

Timing: ~1 h

For this step, the single-molecule slide is assembled using the previously prepared slide and coverslip. Chambers are created within the slide by attaching the coverslip to the slide using double-sided tape and sealing the edges with epoxy. Each pair of pre-drilled holes will serve as an inlet and an outlet for each chamber within the slide.

1. Warm the slide to 20°C–25°C for 15 min.
2. Tear off a 10 cm strip of double-sided tape and place on a glass plate or other sterile surface.
3. Use a clean razor blade to cut the tape into 6–8 10 cm-long strips.
4. Carefully remove the slide from the conical tube and place the PEGylated side face-up on a glass plate.
5. Mark the coverslip with a sharpie to denote the PEGylated or non-PEGylated side of the coverslip.

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 coverslip can then be dried with a nitrogen gas stream or air dried before continuing to assemble the slide.

6. Lift a tape strip and apply the strip to the slide between each pair of drilled holes and along the outer edge of the first and final pair of holes.
7. Use a pipet tip to gently secure the tape onto the slide.
8. Remove the coverslip and place it on the taped slide.

Note: The PEGylated side of the coverslip and the slide should be facing each other.

9. Seal the slide and coverslip by gently pressing with a pipet tip.
10. Cut the excess tape on each end of the slide using a razor blade.
11. Seal the outer edges of the slide and coverslip by applying quick-drying epoxy with a pipet tip.

⚠ CRITICAL: Be careful not to apply too much epoxy or it will flow too far into the lane and will block that channel.

12. Allow the epoxy to dry for 20 min before use.

Pause point: Single-molecule slides can be assembled in advance. Store assembled slides in vacuum-sealed bags at −20°C.

Single molecule pull-down

Timing: ~1 h

This method uses cells expressing the fluorescently-tagged protein of interest for pull-down onto the single-molecule slide. Photobleaching the singly-tagged proteins will then report on the oligomerization status of the complex. Additional reagents can be applied to the isolated complexes as desired. If cell stress conditions are desired, treat cells with cell stressor or stress condition prior to the pull-down experiment.

13. Set up the TIRF microscope for the single molecule pull-down experiment.
    a. Turn the camera on.
    b. Ensure that appropriate laser(s) are on.
14. Open IDL for single-molecule trace processing.

Note: Single-molecule slide and cell lysis will be prepared simultaneously.

Optional: Treat cells with stress condition prior to cell lysis if this is applicable to the experiment.

15. Take Petri dish from incubator.
16. Decant or aspirate media from Petri dish and discard.
17. Rinse Petri dish with 1 mL ice cold 1X dPBS and discard.
18. Add 1 mL ice cold cell lysis buffer to Petri dish and incubate on ice.
19. While cells are lysing, prepare single-molecule slide for pull-down.
20. Apply 30 μL neutravidin diluted in T50 buffer at a 20 μg/μL concentration to one channel within the slide and incubate for ~2 min.
21. Apply 50 μL T50 buffer to rinse off unbound neutravidin.
22. Apply 30 μL biotin-conjugated GFP antibody at a 5 μg/mL concentration after diluting 1 mg/mL stock antibody in T50 buffer to the slide and incubate for ~2 min.
23. Apply 50 μL T50 buffer to rinse off unbound antibody.

Note: By this point, cells will have been lysed for ~5 min. Cell lysis time can be adjusted accordingly depending on type of cells used.

24. Place lysed cells in Petri dish into microcentrifuge tube and pipet several times to further lyse.
25. Prepare a dilution of cell lysis in T50 buffer.

Note: Cell lysates can be diluted to 1:100-1:1000 and should be optimized accordingly such that a surface density of ~100–300 molecules is obtained on the single-molecule surface (2,500 μm^2 imaging area). Gentle cell lysis has been confirmed using western blot to ensure effectiveness of cell lysis and extraction of nuclear and cytosolic proteins. Cell lysates are not centrifuged and are pipetted gently to ensure minimal condensate disruption.

26. Apply 50 μL diluted cell lysate onto the slide and incubate for ~2 min.
27. Apply 50 μL T50 buffer to rinse off unbound complexes.
28. Set up slide on TIRF microscope.
    a. Apply water to the objective on the TIRF microscope.
    b. Place the single-molecule slide on the objective with the coverslip facing down.
    c. Clean the prism with 100% ethanol.
    d. Apply 1 drop of immersion oil to the prism and attach the prism to the microscope so that the oil surface is touching the slide.
    e. Adjust the beam alignment for TIRF imaging.

29. Optimize the gain, exposure time, laser intensity, and z-plane focus for imaging the single-molecule surface. Settings are optimal when single molecule spots are detectable on the surface with minimal background, and with proper cell density to prevent under- or over-saturation.
30. Acquire 6–10+ videos capturing 600 frames at 100 ms/frame acquisition speed in different fields of view at maximum laser intensity in the excitation channel corresponding to the tagged protein.

⚠ CRITICAL: Perform a photobleaching control with the fluorescent tag alone.

Oligomer dissolution

Timing: ~1 h

Once fluorescently-tagged proteins are effectively pulled down using the method described above, additional reagents can be added to promote complex disassembly. Robust dissolution agents will result in an increase in monomer population of complexes and a decrease in higher-order oligomers. Dissolution experiments require obtaining photobleaching steps before and after treatment with dissolving agent. Once fluorescently-tagged proteins have been successfully pulled down and photobleaching steps acquired as described in the steps above, the following steps can be taken using the same channel.

31. Carefully pipet 50 μL of dilution reagent at desired concentration into the channel and incubate for ~2–10 min.

Note: Concentration and incubation time of dilution reagent should be optimized for the experimental condition desired.

32. Perform two washes with 50 μL T50 buffer to remove dissolving reagent and unbound complexes from the slide.

Optional: Slide can be removed from the TIRF microscope to add dissolving agent and perform T50 washes. In this case, clean slide and prism with a Kimwipe and 100% ethanol to remove excess oil. Gently wipe microscope objective with lens paper to remove excess water. Then proceed with adding dilution reagents and performing T50 buffer washes. Set up the slide on the TIRF microscope as previously described in Step 28.

33. Obtain photobleaching steps of fluorescently-tagged proteins.
34. Acquire 6–10+ videos capturing 600 frames at 100 ms/frame acquisition speed in different fields of view at maximum laser intensity in the excitation channel corresponding to the tagged protein.

⚠ CRITICAL: Maintain the same gain, exposure time, laser intensity, and z-plane focus for imaging the single-molecule surface and obtaining photobleaching steps.

EXPECTED OUTCOMES

If fluorescently-tagged proteins are isolated from cell lysates and pulled down (Figure 1A), the fluorescence intensity of the complexes can be plotted as a function of time, resulting in single-molecule "traces" (Figure 1B). Successful photobleaching of complexes will result in photobleaching steps as depicted. Some single-molecule traces will be generated from background signal or from stochastic noise. Such traces (Figure 3A.) should be discarded and excluded from analysis of photobleaching steps. Poor slide preparation, excessive light in the imaging room, and contaminants in buffer may

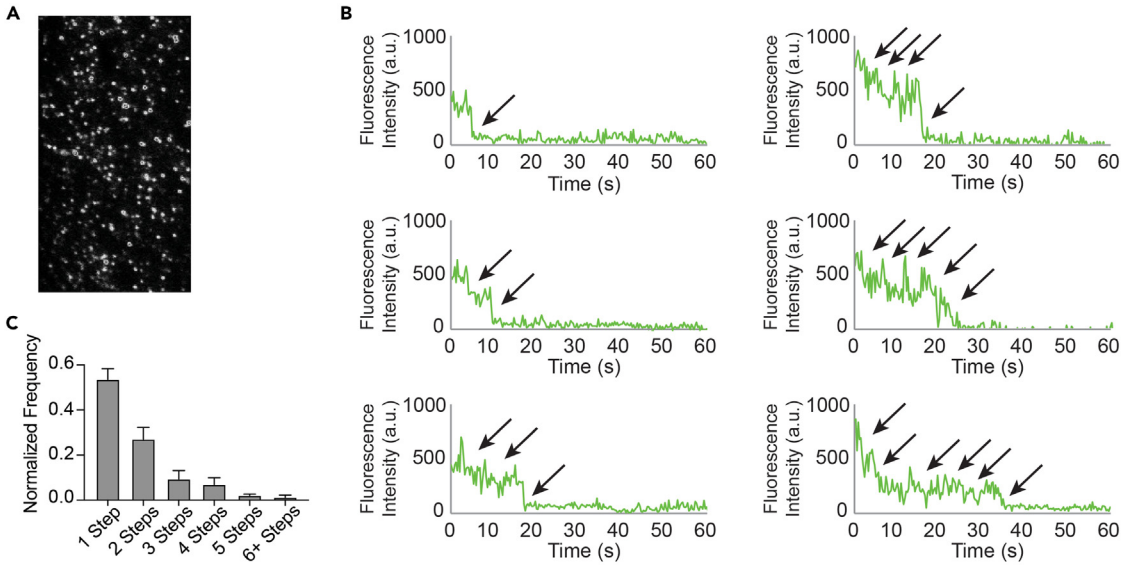

Figure 1. SiMPull can effectively isolate fluorescently-tagged proteins from cell lysates

(A) FUS-GFP complexes isolated from cells are captured and visualized on the single molecule surface and shown as individual dots.

(B) Representative photobleaching steps of individual FUS-GFP complexes.

(C) Photobleaching step quantification and distribution.

also cause increased background noise and should be avoided as much as possible. Only traces where photobleaching steps are resolvable should be included in analysis.

Below are some expected outcomes for the single molecule pull-down experiment where single molecules can be visualized on the imaging surface at a density of approximately 100–300 molecules per 2,500 μm^2 imaging area (Figure 1A).

Fluorescence intensity should decrease in a stepwise manner resulting in photobleaching steps as depicted in Figure 1B. The photobleaching steps can be counted to inform on the oligomerization status of fluorescently-tagged proteins. Photobleaching step distribution can be plotted to highlight changes in oligomerization (Figure 1C).

Dissolution experiments that are performed to dissolve protein complexes or oligomers should result in increased monomer formation or an overall decrease in higher-order oligomerization. Proper controls should be performed using a protein denaturant such as guanidine hydrochloride, urea, or sodium dodecyl sulfate. Strong protein denaturants should result in increased monomer formation, with the possibility of some loss of fluorescence due to complete protein denaturation. Milder protein denaturants result in increased monomer formation with less loss of fluorescence. Single molecule traces should be obtained prior to and following treatment with dissolving agent. Successful dissolution will result in decreased photobleaching steps as shown in Figure 2A. Some oligomers are resistant to certain dissolving agents; therefore, performing a positive control with a strong protein denaturant is critical for each dissolution experiment. Application of dissolving agent and extra T50 buffer washes may result in unbinding of protein complexes from antibody or from protein-antibody complexes on the single molecule surface (Figure 2B). Therefore, additional videos may need to be obtained to ensure enough single molecule traces are generated to capture a representative number of traces before and after dissolving agent addition.

QUANTIFICATION AND STATISTICAL ANALYSIS

Photobleaching steps generated from single molecule traces can be manually counted. Blinded analysis should be performed to prevent bias. Photobleaching steps can then be plotted

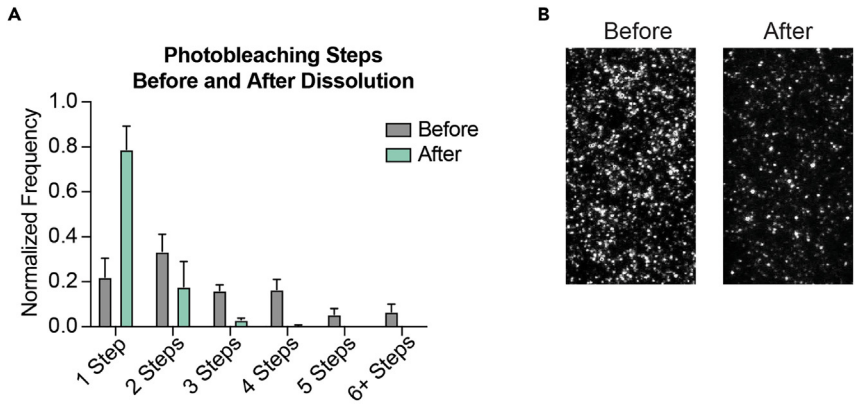

Figure 2. Dissolution experiments can be performed to dissolve isolated complexes

(A) Photobleaching step quantification before and after dissolution experiment.

(B) Strong dissolving agents may result in unbinding and loss of complexes on the single molecule surface.

as a histogram to show distribution of steps and can also be summarized as an average photobleaching step number.

LIMITATIONS

Cell lysis conditions may slightly perturb the oligomerization of fluorescently-tagged proteins. To this end, we prevent harsh lysis conditions by using a mild detergent and prevent excessively harsh conditions by excluding centrifugation or manual disruption of cell lysates with a homogenizer or sonicator. Mild lysis of cells should enable preservation of protein oligomerization at a basal state. We previously confirmed through western blot that mild lysis does properly solubilize the cell membrane and nuclear membrane for pull-down of cytosolic and nuclear proteins.

Single molecule traces obtained may result in excessive noise and background signal that makes quantification of photobleaching steps difficult. Therefore, single molecule traces that appear noisy or do not show clear stepwise decreases in fluorescent intensity should be excluded from analysis, with the understanding that this may result in exclusion of data from much higher oligomeric states. The lack of commercially available prism-type TIRF microscopes can make this protocol inaccessible to many labs.

TROUBLESHOOTING

Problem 1

Obtaining the appropriate density of molecules on the single molecule surface is critical (Figure 3). If too many molecules are present on the surface, an oversaturation of fluorescence intensity may occur, resulting in unreliable single molecule traces (Figure 3B, right panel). Conversely, if too few molecules are present on the single molecule surface (Figure 3B left panel), it may be difficult to distinguish molecules from background noise (Figure 3A), and will result in the need to obtain many more videos of single molecule traces to gain a representative sample of reliable traces (Related to step 25).

Potential solution

To circumvent this issue, one should perform serial dilutions of cell lysate to obtain an appropriate concentration that results in ~100–300 molecules on the imaging surface. Obtaining roughly the same number of molecules among different conditions will help maintain uniformity when gathering single molecule traces.

Problem 2

Fluorescently-tagged proteins may not photobleach (Related to steps 29–30).

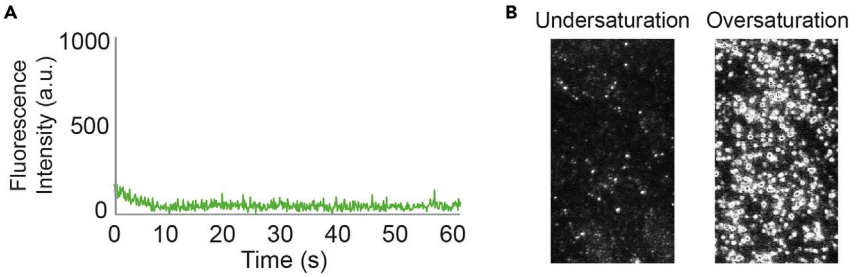

Figure 3. Optimization should be performed to obtain the appropriate density of molecules on the single molecule surface

(A) Representative single molecule trace of background signal.

(B) FUS-GFP complexes on single molecule surface are shown as individual dots in undersaturated and oversaturated conditions.

Potential solution

To prevent this issue, a fluorescent tag such as GFP should be used to enable optimal photobleaching steps. Fluorescent tags with weaker signal may not yield distinct photobleaching steps. The laser intensity can also be increased to enhance photobleaching.

Problem 3

Fluorescent tag may induce dimerization or higher order oligomerization (Related to steps 29–30).

Potential solution

A control with the fluorescent tag only should be performed to determine oligomerization due to the tag alone. Any additional dimerization or oligomerization due to the fluorescent tag should be subtracted from all other conditions to minimize an inaccurate increase in oligomerization.

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://zenodo.org/doi/10.5281/zenodo.10045465). 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.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.D. and S.M.; methodology, N.D.; writing, N.D. and S.M.; supervision, S.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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