Single-Molecule FRET-Resolved Protein Dynamics – from Plasmid to Data in Six Steps

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Chapter 13

Single-Molecule FRET-Resolved Protein Dynamics – from Plasmid to Data in Six Steps

Benjamin Vermeer, Jannick van Ossenbruggen, and Sonja Schmid

Abstract

Single-molecule Förster resonance energy transfer (smFRET) is a powerful technique for the detection of conformational dynamics of biomolecules. While many smFRET experiments are performed using dye-labeled DNA, here we describe a comprehensive protocol to resolve the conformational dynamics of a protein system – notably from plasmid to data. Using the example of the heat-shock protein Hsp90, we describe the protein production and threefold site-specific bioconjugation, the smFRET measurement using total internal reflection fluorescence microscopy (TIRFM), and raw data processing to reveal time-resolved protein dynamics. The described smFRET approach is readily transferrable to the study of many more all-protein systems and their conformational energy landscape.

Key words Single-molecule FRET, Protein dynamics, Protein purification, Bioconjugation, Surface functionalization, Chaperone protein Hsp90

1 Introduction

1.1 smFRET Enables Monitoring of Protein Dynamics

Dynamic processes are critical for the functioning of proteins. Protein dynamics occur both within single proteins (intramolecular) and between proteins or other biomolecules (intermolecular). In general ensemble experiments, protein dynamics are usually averaged out, while single-molecule techniques allow one to watch them in real time. Single-molecule Förster resonance energy transfer (smFRET), based on the FRET mechanism described by Förster in 1948, is a powerful fluorescence-based approach that allows researchers to reveal nanoscale dynamics over a vast time scale (picoseconds to minutes [1–3]), enabling insights into protein translation [4], DNA synthesis [5], membrane transport [3], DNA maintenance [6], and many more processes that are critical to life.

Herein, we describe a single-molecule FRET protocol to measure protein dynamics of surface-tethered proteins using total internal reflection fluorescence microscopy (TIRFM). This approach is

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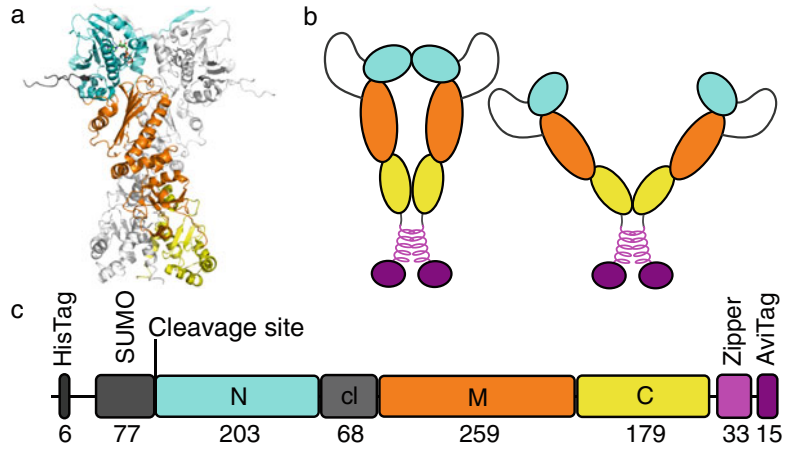

Fig. 1 Heat-shock protein 90 (Hsp90) structure and expressed construct. (**a**) Crystal structure of a closed Hsp90 dimer (PDB:2CG9). (**b**) Schematic representation of the Hsp90 dimer in the closed (l) and open "v-shaped" (r) conformation. (**c**) Schematic representation of the Hsp90 protein construct described in this protocol. The N-terminal HisTag and the SUMO domain are cleaved off during protein purification

capable of probing dynamic processes from milliseconds to minutes. The protein of interest in this protocol is the non-constitutively expressed heat-shock protein 90 (Hsp90) from *Saccharomyces cerevisiae* (yHsp82). This chaperone protein is a slow ATPase and forms homodimers (Fig. 1a). Hsp90 is known to exist in a range of v-shaped open and compact closed conformations (Fig. 1b), the dynamics of which can be probed by smFRET. Our protocol is organized in six concise steps (Fig. 2): (i) plasmid transformation into competent *E. coli*; (ii) protein expression with in vivo biotinylation; (iii) protein purification; (iv) site-specific fluorescent labeling; (v) surface functionalization and smFRET recording; and (vi) data processing. This six-step protocol is largely transferrable to other cytosolic all-protein systems.

1.2 Transformation of Plasmid into Competent Escherichia coli

The starting material is a pET-28b(+) vector with the DNA sequence encoding the Hsp90 in the open reading frame downstream of the T7 promotor. From 5′ to 3′, the protein-coding DNA sequence includes N- to C-terminus in the protein (Fig. 1c): a 6x histidine tag for purification, a SUMO domain for traceless removal of the His-Tag after purification, the yHsp82 sequence with an N298C point mutation introducing a single cysteine for specific thiol-reactive dye labeling, an AviTag for site-specific in vivo biotinylation, and a *Drosophila melanogaster* kinesin neck linker coiled-coil zipper domain (DmKHC [7]) for high dimerization affinity of Hsp90 homodimers (*see* **Note 1**). The plasmids are transformed by heat shock into chemically competent bacterial cells [8] (*see* **Note 2**) and selected with kanamycin.

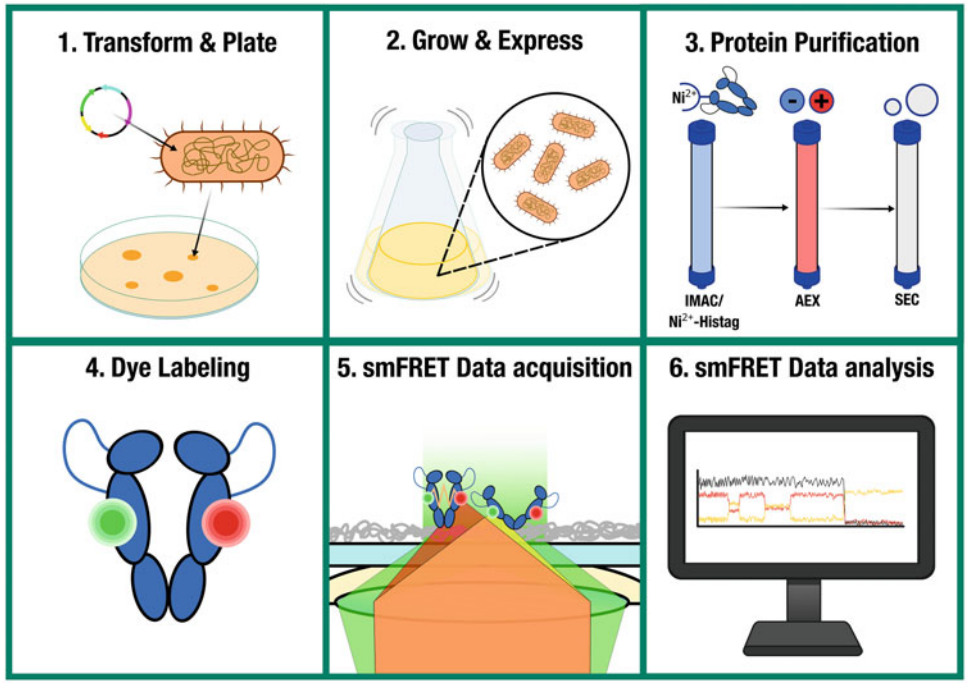

Fig. 2 Overview of the six steps from plasmid to smFRET dynamics data. Schematic representation of the necessary procedures: (1) Plasmid transformation into *E.coli*, (2) growing transformed *E.coli* and protein expression, (3) purifying Hsp90 from the *E.coli* lysate using a nickel, AEX and SEC column, (4) site-specific dye labeling of Hsp90, (5) single-molecule FRET data acquisition using a total internal reflection (TIR) setup, and (6) processing the smFRET data to study protein dynamics

1.3 Expression of the Protein of Interest

Transformed cells are grown to exponential growth phase in nutrient-rich Terrific Broth (TB) medium. Once an optical density (OD) between 0.8 and 1.0 is reached, isopropyl β-D-1 thiogalactopyranoside (IPTG) is added to induce protein expression. Proteins are biotinylated in vivo by adding biotin upon IPTG addition in the exponential growth phase. Native BirA ligase in *E. coli* then specifically biotinylates the expressed protein construct on the lysine residue of the AviTag [9, 10]. Four hours after induction, the cells are harvested from the medium by centrifugation at 5000 rotations per minute (rpm), at 4° Celsius. Next, the cells in the pellet are resuspended and lysed by sonication, after which the cell debris is pelleted by centrifugation at 50.000G at 4 °C and the supernatant is collected as a crude protein sample.

1.4 Purification of the Protein of Interest

Three purification steps are performed. First, the cell lysate is subjected to a His-Tag purification step using nickel-affinity chromatography [11]. An artificial six-residue histidine repeat (6x His-Tag) is included in the protein construct to ensure high-affinity binding to the immobilized nickel. The construct is eluted

from the column using a buffer high in imidazole which competes for nickel binding with the 6x His-Tag. Next, the eluate is dialyzed into a low-salt buffer required for anion exchange chromatography. During dialysis, the 6x His-Tag is cleaved from the protein construct using ubiquitin-like protease 1 (Ulp1) SUMO protease from *Saccharomyces cerevisiae*, which specifically recognizes the tertiary structure of the SUMO domain and cuts it off in a traceless way. Ulp1 cleaves the peptide bond after the double glycine at the C-terminal end of the SUMO domain, which directly precedes the Hsp90 start methionine, thus removing the entire SUMO domain plus 6x His-Tag, yielding the native Hsp90 N-terminus. Next, anion exchange chromatography is performed to purify the protein from nucleic acids as well as (differently) charged protein species. In short, under low-salt conditions, the overall negatively charged Hsp90 will bind to a cationic resin, followed by elution with a higher-salt gradient. Lastly, size-exclusion chromatography is used to remove smaller and larger proteins and to elute purified Hsp90.

1.5 Dye Labeling of the Protein of Interest

To observe the Hsp90 dimers with smFRET, one monomer is labeled with a donor dye and the other monomer with an acceptor dye. The purified Hsp90 proteins are engineered to contain a single cysteine residue at position 298 in the protein sequence. Such a cysteine substitution for thiol-specific labeling should be placed in an unstructured region of the protein to prevent interference with protein folding and function. The thiol group of reduced cysteines couples to maleimide groups, and thus dyes with maleimide functionalization can be used to site-specifically label proteins on cysteine residues [12]. Alternative site-specific labeling methods include amine-reactive labeling at the N-terminus or at lysine residues (which occur frequently in proteins) [12], and many other bio-orthogonal (click) reactions, e.g., using unnatural amino acids incorporated by genetic code expansion [13].

1.6 Detection of Protein Dynamics Using smFRET

The application of smFRET to detect nanoscale distance changes has been carefully described and reviewed [14–16]. In short, Förster resonance energy transfer (FRET) is caused by the dipole-dipole interaction between the transition dipoles of two fluorophores which leads to a non-radiative energy transfer from an electronically excited "donor" fluorophore to a ground-state "acceptor" fluorophore – if certain conditions are fulfilled: (i) spectral overlap between donor emission and acceptor excitation, (ii) non-perpendicular orientation of the transition dipoles of the donor and acceptor fluorophore, and (iii) a spatial separation of donor and acceptor of generally less than 10 nm [14–16]. The latter is due to the R^{-6}-dependence of the dipole-dipole interaction, which in turn allows one to infer the inter-dye distance based on the energy transfer efficiency between the donor and acceptor.

For this purpose, a FRET efficiency (FRET E) is defined as the ratio of acceptor fluorescence to total fluorescence, after a range of corrections detailed previously [14]:

$$E = \frac{I_{DA}}{I_{DA} + I_{DD}} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$$

where E is the energy transfer efficiency, R is the inter-dye distance, and R_0 is the Förster radius defined as the inter-dye distance at which the FRET efficiency equals 50% [14].

Single-molecule FRET is frequently measured using total internal reflection fluorescence microscopy (TIRFM) or confocal detection. Both techniques can detect single molecules in dilute samples, thanks to the low fluorescent background involved [16]. Here, we focus on TIRFM which allows one to monitor the behavior of single biomolecules over time in a parallelized manner, meaning hundreds or even thousands of molecules are observed simultaneously. The molecules of interest are tethered to a passivated glass surface. Using alternating laser excitation (ALEX, often with a green and red laser), photophysical effects can be revealed to prevent their misinterpretation as conformational dynamics [17].

1.7 Data Processing

From the acquired raw smFRET data, i.e., TIF image stacks, intensity trajectories are extracted for each single molecule in the field of view. Several selection criteria distinguish an smFRET trajectory from noise and other artifacts: (i) single-step photobleaching of the donor and acceptor; (ii) stable intensity plateaus; and (iii) few blinking events. Out of these selected trajectories, only the relevant range (before either the donor or the acceptor photobleaches) is further analyzed and corrected for several experimental cross-talks as previously described [14]. Using smFRET, one can directly observe sequential conformational changes and the order in which these occur. From the corrected smFRET data, a global conformational state distribution can be extracted. For a deeper quantitative understanding of the protein kinetics, e.g., Hidden-Markov Model (HMM), algorithms can be used to infer all the rate constants involved. The use of HMMs and other approaches to extract single-molecule kinetics from smFRET data has been detailed extensively elsewhere [18, 19]. Hence, we focus here on the basics of smFRET data processing that lead up to such kinetic analyses.

Altogether, this protocol describes all six necessary steps to get from a protein-encoding plasmid to time-resolved protein dynamics. Using the example of Hsp90, we provide representative examples of chromatography results, time-resolved fluorescence trajectories of single molecules, and global conformational state distributions in FRET efficiency histograms that are readily transferrable to other all-protein systems. Our smFRET data reveals that

the Hsp90 ATPase undergoes thermally driven conformational changes even in the absence of a chemical energy source, such as ATP, which illustrates the unique insight that smFRET can give into protein behavior.

2 Materials

Unless specified otherwise, all solutions are prepared using ultrapure water (18 MΩ-cm at 25 °C) and analytical grade reagents, and all reagents are stored at room temperature. All chemicals are purchased from Sigma-Aldrich (Saint-Louis, USA), unless specified otherwise. Adhere to local waste disposal regulations.

2.1 Transformation of Plasmid into Competent E. coli

1. pET-28b-Hsp90 plasmid.
2. Rosetta DE3 BL21 derived competent *Escherichia coli* cell stock (*see* **Note 3**).
3. 30 mg/mL kanamycin in distilled water. Filtered (0.2 μm) under sterile conditions (store at −20 °C).
4. 35 mg/mL ultrapure chloramphenicol in 70% ethanol. Filtered (0.2 μm) under sterile conditions (store at −20 °C).
5. Lysogeny broth (LB) medium in distilled water.
6. LB medium +1.5 g agar-agar powder per 100 mL.
7. Plastic petri dishes (Ø10 cm).
8. Cell spreader (glass or metal).
9. Water bath/stove (able to reach 42 °C).
10. Shaking incubator (able to reach 37 °C and shake horizontally at 200 rpm).
11. Syringe (50 mL) and attachable 0.2 μm filters.

2.2 Expression of the Protein of Interest

1. Terrific broth (TB) in distilled water.
2. Kanamycin stock (see Subheading 2.1).
3. Chloramphenicol stock (see Subheading 2.1).
4. 20% glucose solution (*see* **Note 4**).
5. Biotin 5 mM solution: add 12 mg of D-biotin to 10 ml of warm (microwaved) 10 mM bicine buffer (pH 8.3). Filter (0.2 μm) under sterile conditions (*see* **Note 4**).
6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) 1 M in distilled water. Filter (0.2 μm nylon syringe filter) under sterile conditions.
7. cOmplete^{TM} protease inhibitor tablets, EDTA-free (Roche, Basel, Switzerland).

8. Resuspension/wash buffer, 40 mM HEPES, 150 mM NaCl, 20 mM imidazole, pH 7.5.
9. Autoclave (able to heat up to 134 °C).
10. Shaking incubator (able to reach 100 rpm and 37 °C).
11. Lab Vortex Mixer.
12. Optical density reader.
13. Cooled centrifuge (able to reach 50.000G and 4 °C).
14. 20 kHz sonicator probe capable of cell lysis in 50 mL falcon tube (or another machine capable of lysing cells, e.g., a French press).
15. Syringe (50 mL) and attachable 0.2 μm filters.

2.3 Purification of the Protein of Interest

1. HisTag wash buffer, 40 mM HEPES, 150 mM NaCl, 20 mM imidazole, pH 7.5.
2. HisTag elution buffer, 40 mM HEPES, 150 mM NaCl, 1 M imidazole, pH 7.5
3. 20% ethanol.
4. Ulp1 SUMO protease (Sigma-Aldrich, Saint-Louis, USA).
5. AEX wash buffer, 40 mM HEPES, 30 mM NaCl, pH 7.5.
6. AEX elution buffer, 40 mM HEPES, 1 M NaCl, pH 7.5.
7. SEC buffer. 40 mM HEPES, 150 mM KCl, pH 7.5.
8. NanoDrop 2000/2000c spectrophotometer.
9. Vacuum pump and buffer filtration equipment.
10. Syringe (50 mL) and 0.45 μm nylon syringe filters.
11. Protein purification system (e.g., ÄKTA start or pure and associated UNICORN program).
12. Ni Sepharose High Performance column, e.g., HisTrap HP 5 mL nickel column (Cytiva, Marlborough, USA).
13. SDS-PAGE gel, e.g., precast 4–15% mini-PROTEAN TGX gel (Bio-rad, Hercules, USA).
14. Snakeskin dialysis tubing 10 K MWCO 22 mm × 10.5 m (Thermofisher, Waltham, USA).
15. Quaternary ammonium anion exchange column, e.g., HiTrap Q HP 5 mL Sepharose AEX column (Cytiva, Marlborough, USA).
16. Centrifugal filter tube (30 kDa), e.g., Amicon Pro Affinity Concentrator (Millipore, Burlington, USA).
17. Superdex 200 prep-grade 26/600 size-exclusion column (Cytiva, Marlborough, USA).

2.4 Fluorescent Labeling of the Protein of Interest

1. Phosphate-buffered saline (PBS) solution (pH 6.7).
2. Maleimide-functionalized ATTO550 (Attotec, Siegen, Germany).
3. Maleimide-functionalized ATTO647N (Attotec, Siegen, Germany).
4. Dimethyl sulfoxide (DMSO), ACS-grade.
5. 100 mM TCEP solution in ultrapure water. Adjust pH to 7 using sodium hydroxide. Store in aliquots at −20 °C.
6. HKM Hsp90 buffer, 40 mM HEPES, 150 mM KCl, 10 mM $MgCl_2$, pH 7.5.
7. NanoDrop 2000/2000c spectrophotometer.
8. PD MiniTrap™ G-25 columns (Cytiva, Marlborough, USA).
9. Water bath/tube incubator (able to keep constant temperature at 47 °C).

2.5 smFRET of Protein Dynamics

1. mPEG SVA MW 5000 (mPEG-Succinimidyl Valerate, MW 5000 – 1 g) (Laysan Bio, Arab, US).
2. Biotin-PEG-SC MW 5000 (Laysan Bio, Arab, US).
3. Vectabond (Vectorlabs, Newark, US).
4. Acetone for spectroscopy.
5. MOPS buffer, 50 mM, pH 7.5. Filtered through 0.2 μm nylon syringe filter.
6. 0.1 M sodium bicarbonate buffer, pH 8.5. Filter (0.2 μm) the solution. Store in aliquots at −20 °C.
7. Methyl-PEG-NHS-Ester (ThermoFisher, Waltham, USA) in DMSO to a final concentration of 250 mM. Store aliquots under nitrogen gas at −20 °C.
8. Nitrogen gas (for gentle blow-drying).
9. HKM wash buffer, 40 mM HEPES, 150 mM KCl, 10 mM $MgCl_2$., pH 7.5.
10. HKM Hsp90 measurement buffer 40 mM HEPES, 150 mM KCl, 10 mM $MgCl_2$, pH 7.5, with 2 mM AMP-PNP (Sigma-Aldrich, Saint-Louis, USA).
11. High-precision No. 1.5H (tol ± 5 μm) glass coverslips (Paul Marienfeld, Lauda-Königshofen, Germany).
12. Aluminum foil.
13. Chamber furnace (able to reach 500 °C, e.g.,Vecstar with a Eurotherm controller).
14. Coplin glass slide staining jar for coverslips and glass slides (e.g., Epredia RA lamb glass coplin jars, FisherScientific, Waltham, USA).

15. Tweezers.
16. Silicone gaskets (Grace BioLabs, Bend, USA).
17. Ø10 mm round glass cover slides (Paul Marienfeld, Lauda-Königshofen, Germany).
18. 1.5 mL microtubes.
19. Tabletop vortex apparatus.
20. Tabletop centrifuge for microtubes (capable of spinning at 13000 rpm).
21. TIRFM setup with an electron-multiplying charge-coupled device (EMCCD) or complementary metal oxide semiconductor (CMOS) camera capable of detecting single molecules.
22. Laser excitation control hardware and software (e.g., [20]).
23. Microscope acquisition software (e.g., Andor SOLIS).

2.6 smFRET Data Processing

1. ImageJ or Fiji.
2. smFRET analysis software. Our group utilizes a code package written for Igor Pro developed in the laboratory of Thorsten Hugel.

3 Methods

All procedures are carried out at room temperature unless specified otherwise. Sterile conditions refer to either under the flame of a Bunsen burner or a sterile fume hood.

3.1 Transformation of Plasmid into Competent E. coli

1. Autoclave 100 mL of LB + agar at 120 °C for 20 min. Once it has cooled enough to hold it in your hands, but not enough to solidify, add 100 μL of kanamycin stock (final concentration 30 μg/mL) and 20 μL of chloramphenicol stock (final concentration 7 μg/mL) to the medium, and swirl gently to disperse the antibiotics without forming bubbles.
2. Under sterile conditions, pour ~14 mL of medium into a sterile 10 cm diameter petri dish. Gently swirl to evenly distribute, and let it cool down and solidify (*see* **Notes 5** and **6**).
3. Thaw competent cells and pDNA on ice. Pipette 1 μL pDNA stock to 50 μL competent cell stock, and gently resuspend the mixture. Keep the cells on ice for 15 min.
4. Heat-shock the cells at 42 °C for 2 min and then keep them on ice for 15 min.
5. Add 1 mL of warm (~37 °C) LB without antibiotics to the cells. Incubate at 37 °C, shaking at 200 rpm, for 1 h.
6. Plate the cells and incubate overnight (12–16 h) at 37 °C.

3.2 Expression of the Protein of Interest

1. Make 2 L of TB and 300 mL of LB medium. Autoclave media and growth Erlenmeyers at 120 °C for 20 min. Allow media to cool before adding antibiotics.
2. Under sterile conditions, add kanamycin (final 30 μg/mL) and chloramphenicol (final 7 μg/mL) to the LB, and add kanamycin stock (final 30 μg/mL) and filter-sterilized 20% glucose solution (final concentration 0.5%) to the TB (*see* **Note 7**).
3. Divide the LB evenly between two 200 mL Erlenmeyers. Place a pipette tip with a colony from the plate in both Erlenmeyers, and put an airable cap on them.
4. Place both Erlenmeyers in the shaker overnight, or around 16–18 h, at 37 °C, and 200 rpm.
5. Autoclave four 2 L Erlenmeyer flasks at 134 °C for 3 min.
6. Pour 500 mL TB in each of the 2 L flasks under sterile conditions. Take 1 mL as a blank for the optical density (OD) measurements. Pre-warm the flasks to 37 °C overnight.
7. Add 12.5 mL preculture to each of the 2 L Erlenmeyer flasks, and place them in a shaking incubator (37 °C, 100 rpm shaking) (*see* **Note 8**).
8. Every 30–60 min, measure the OD at 600 nm (OD_{600}) of the TB. Once the OD_{600} is between 0.8 and 1.0, add 5 mL of 5 mM biotin solution per 500 mL culture (final concentration 50 μM) (*see* **Note 9**).
9. Induce protein expression for 4 h with 0.5 mL filter-sterilized IPTG per 500 mL culture (final concentration 1 mM) in each flask (*see* **Note 10**).
10. Centrifuge post-induction cells at 4000G at 4 °C for 15 min.
11. Add 4 cOmplete protease inhibitor tablets to 40 mL of resuspension volume.
12. Decant the supernatant from the centrifuge bottles, and resuspend the *E. coli* containing pellets in the 40 mL resuspension buffer in a cold room (*see* **Note 11**).
13. Lyse cells on ice using a sonicator at amplitude 60%. Sonicate six times for 10 s with a 50 s interval (*see* **Note 12**).
14. Centrifuge the obtained *E. coli* lysate at 50.000G at 4 °C for 1 h. Carefully decant or pipette the supernatant into a falcon tube. Store the supernatant at −20 °C.

3.3 Purification of the Protein of Interest

The protein purification step consists of three consecutive column separation steps: a nickel affinity column, an anion exchange (AEX) column, and a size-exclusion (SEC) column.

3.3.1 His-Tag Purification

1. Filter (0.45 μm) ultrapure water, resuspension buffer, elution buffer, and the 20% ethanol using a vacuum pump, and the lysate with a syringe and attachable filter. Using the filtered

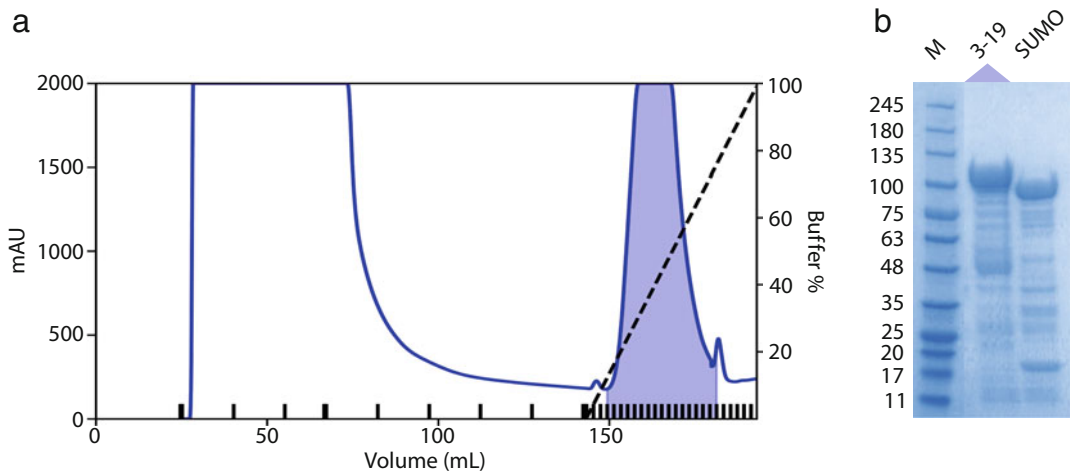

Fig. 3 Hsp90 expression and His-Tag purification results. (**a**) Chromatogram for His-Tag purification of Hsp90 from *E.coli* lysate. The blue line displays UV absorbance at 280 nm, indicating the level of protein. The first peak (between 20 mL and 100 mL) is the column flowthrough. The second peak (between 140 mL and 190 mL) is the column eluent, mostly consisting of His-Tagged Hsp90. (**b**) SDS-PAGE of fractions collected from the Nickel column eluate and the pooled fractions after SUMO-cleavage

solutions, clean the protein purification system tubing first with 10 mL ultrapure water, then 10 mL elution buffer, and then 10 mL ultrapure water again.

2. Attach the His-Tag Column into the flow path of the protein purification system in a drop-to-drop fashion with ultrapure water, and wash the column with 5CV of ultrapure water (*see* **Note 13**).

Automate or manually perform the following steps (the chromatogram should look like Fig. 3):

3. Equilibrate column with wash buffer (5 CV, 5 mL/min).
4. Apply filtered lysate to column (2 mL/min, *see* **Note 14**).
5. Wash out unbound protein with wash buffer (15 CV, 5 mL/min, *see* **Note 15**).
6. Elute bound protein through gradient elution (10 CV, 5 mL/min, from 0% to 100% elution buffer). The Hsp90 elution peak is at ~33% elution buffer (i.e., 300 mM imidazole), at conductivity 17–20 mS/cm (*see* **Note 16**).
7. Elute remaining protein and equilibrate with elution buffer (5 CV, 5 mL/min).
8. Clean the tubing according to the protein purification systems manual.
9. Perform an SDS-PAGE gel run on the eluate fractions collected during gradient elution to determine which fractions contain your protein.

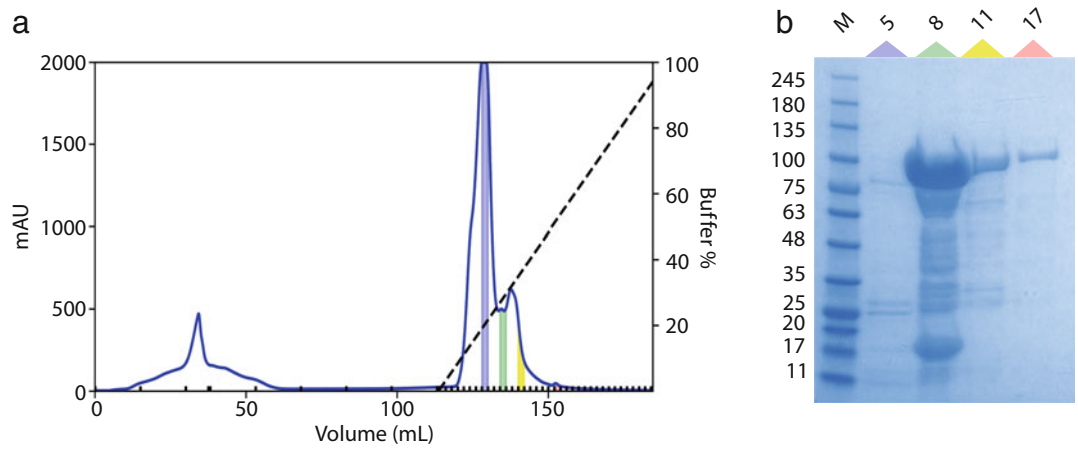

Fig. 4 Hsp90 expression and AEX purification results. (**a**) Chromatogram for AEX purification of Hsp90. The blue line displays UV absorbance at 280 nm, a proxy for the protein concentration. The first peak (between 10 and 60 mL) is the column flowthrough. The second peak (between 120 and 140 mL) is the column eluent, mostly consisting of Hsp90. (**b**) SDS-PAGE of fractions collected from the AEX column eluate

10. Pool the protein-containing fractions. Treat with SUMO protease (~20 U/mg protein, or perform an assay to determine protease activity at different protein:protease ratios) overnight at 4 °C.
11. Dialyze sample against Q-A buffer with a Snakeskin membrane at 4 °C overnight (can be combined with SUMO protease treatment in previous step).

3.3.2 Anion Exchange Chromatography (AEX)

The AEX purification uses the same protocol as described for the HisTag purification, with two exceptions: the column used and the binding and elution buffer (volume) used. Elution is performed by a linear gradient over 15 CV, going from 30 mM NaCl to 1 M NaCl. The Hsp90 elution peak can be expected at ~26% elution buffer and at a conductivity of ~43 mS/cm. The chromatogram should look like Fig. 4.

1. After purification, concentrate the pooled eluate using a 30 kDa centrifugal filter tube (check the concentration with a Nanodrop until the desired concentration is reached) (*see* **Note 17**). Ensure that the final sample volume is less than the maximum volume allowed on the SEC column (see manufacturer instructions).

3.3.3 Size-Exclusion Chromatography (SEC)

1. Filter (0.45 μm) the ultrapure water and the SEC buffer using the vacuum pump and the sample with a syringe and an attachable filter (0.45 um). Using the filtered ultrapure water, purge any air in the ÄKTA machine tubing between buffers and pump.

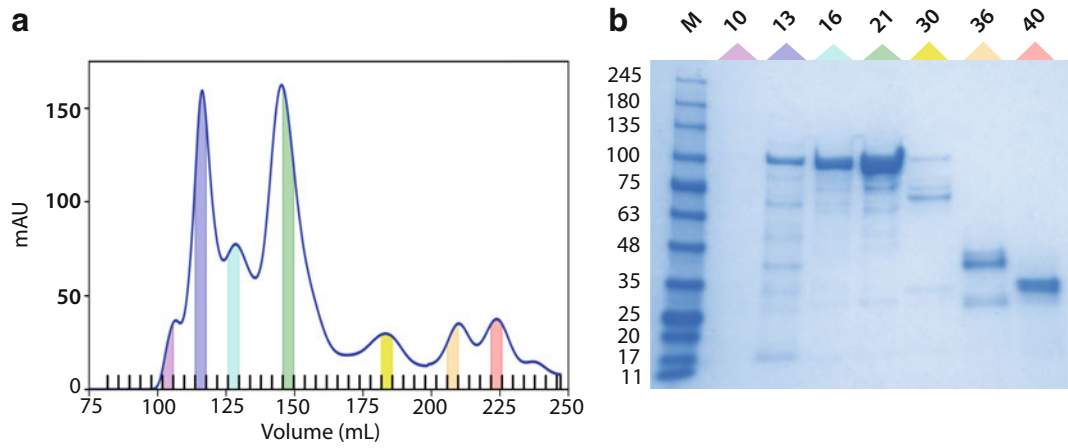

Fig. 5 Hsp90 expression and SEC purification results. (**a**) Chromatogram for SEC purification of Hsp90. The blue line displays UV absorbance at 280 nm, indicating the protein concentration. (**b**) SDS-PAGE of fractions collected from the SEC column eluate

2. Attach the SEC column into the flow path of the protein purification system in a drop-to-drop fashion with ultrapure water, and remove the transport device (filled with 20% EtOH) from the bottom of the column and connect it to the correct tubing.

Automate or manually perform the following steps (the chromatogram should look like Fig. 5):

3. Remove storage solution by washing with 1 CV ultrapure water (2.6 mL/min) and then with 2 CV SEC buffer (4.3 mL/min).
4. Apply sample to the column (2.6 mL/min).
5. Run 1.1 CV of SEC buffer through the column (2.6 mL/min) until sample elutes. In our column, Hsp90 elutes after around 0.5 CV in several peaks due to its elution in various forms (dimeric, tetrameric) (see Fig. 5). Reducing conditions can be used to prevent thiol bridge formation.
6. Clean the column and chromatography system according to the manufacturer's instructions.
7. Shock-freeze the purified protein sample in liquid nitrogen, and store at −20 °C.

3.4 Dye Labeling of the Protein

Prior to dye labeling, determine the Hsp90 concentration of the SEC eluate using the Nanodrop. The monomeric yHsp82-N298C construct (plus Avitag and zipper) has a calculated extinction coefficient of 57300 (in water) and a molecular weight of 81.37 kDa (calculated using Expasy's ProtParam tool [21]). Proper labeling is achieved with an initial protein sample concentration of 60–70 μM.

1. With gloves on, use a pipette tip to gently transfer a few grains of lyophilized dyes (≪1 mg) into a fresh microtube. Dissolve the dye grains in 20 μL DMSO. Store the main stock under inert gas at −20 °C.
2. Dilute DMSO-dissolved stock solution in 1X PBS (pH 6.7) to two times the concentration of the protein sample. Prepare blanking solutions for the prepared dye dilutions. Determine dye concentrations diluted in buffer using the nanodrop.
3. Reduce cysteine bonds in the protein sample by addition of 10 mM TCEP in a 9:1 ratio of protein:100 mM TCEP, and incubate in the dark for 30 min at room temperature (preparation volume should be between 0.2 and 0.5 mL).
4. Prepare a MiniTrap G-25 column for centrifugation by eluting 1CV of ultrapure water, and 1CV of 1X PBS buffer (pH 6.7) twice to equilibrate the column with the PBS buffer.
5. Remove TCEP and change buffer to PBS pH 6.7 with the MiniTrap G-25 column.
6. Use the Nanodrop to determine the protein concentration after TCEP removal to ensure a concentration higher than 30 μM.
7. Split the Hsp90 protein sample into two subsets (*see* **Note 18**). Add a twofold molar excess of maleimide dye (to account for maleimide hydrolysis in the buffer during dye incubation) to both subsets, and incubate in the dark for 1 h at room temperature (*see* **Note 19**).
8. Repeat **step 4** with measurement buffer on a new MiniTrap G-25 column before removing dye excess and exchanging buffer to measurement buffer.
9. Determine the degree of labeling (DOL) to verify the efficiency of covalent dye labeling of your protein (see AttoTec instructions [22]). The yeast Hsp82-N298C construct yielded a DOL of 83% for ATTO550 and 92% for ATTO647N (without an initial centrifugation step to remove potential protein aggregates).
10. Exchange monomers from the current donor-only and acceptor-only dimers by incubating the mixed sample for 20 min at 47 °C in order to accelerate the opening of the coiled coil zipper. Then, remove possible aggregates by extensive centrifugation at 14000 × *g* for 1 h at 4 °C (room temperature may also be possible).
11. Measure the approximate protein concentration of your samples by UV-VIS (i.e., based on the covalently coupled dye concentrations) in a Nanodrop spectrometer. The final concentration of the double-labeled yHsp82 sample is approximately 10 μM.

12. Store the dye-labeled protein samples in small aliquots (10–50 μL) in protein lo-bind tubes at 4 °C (*see* **Note 20**).

This dye labeling protocol is based on a protocol described in the supplemental information of Hellenkamp et al. [23].

3.5 smFRET of Protein Dynamics

3.5.1 Coverslip Cleaning

1. Burn the high-precision 1.5H coverslips in a furnace to remove organic impurities. Set the heating rate at 90 °C/hr, heat the slides at 500 °C for 1 h, and cool the furnace (overnight) at 150 °C/hr. Wear gloves when handling the coverslips to prevent contamination.
2. Wrap the coverslips in clean aluminum foil for storage.

3.5.2 Vectabond Coating

1. Transfer the burned coverslips into a glass coverslip staining jar.
2. Immerse the coverslips completely in spectroscopy-grade acetone for 5 min.
3. Add Vectabond solution into the staining jar to a final 2% (v/v) Vectabond solution in acetone with gloves and in a fume hood, as Vectabond is toxic. Pipette up and down ten times all over the jar surface to mix the Vectabond and the acetone.
4. Leave the coverslips in the solution for 5 min.
5. Pour the Vectabond-acetone solution in a toxic waste collection container. The glass coverslips will not easily slip out during the pouring.
6. Wash (i.e., 1 min incubation) the coverslips once with 50 mL acetone (discard in toxic waste collection container). Then, outside the fume hood, wash the coverslips three times with 50 mL ultrapure water (discard in sink).
7. Dry the coverslips under a gentle nitrogen gas flow, and place them in a clean, empty pipette tip box.

3.5.3 First PEG Coating

1. Prepare chambers by placing gaskets on the coverslips. Press gaskets to the glass with tweezers. Check that there are no air bubbles between the silicone and the glass.

   Prepare polyethylene glycol (PEG) solution, consisting of MOPS buffer, 50 mM pH 7.5 (filtered, stored at 4 °C), NHS-PEG, and biotin-PEG-NHS. For 20 gasket wells:
2. Weigh off 3 mg biotin-PEG-NHS without using a spatula, as the powder will stick, by gently tapping powder into a fresh 1.5 mL microtube.
3. Repeat the same procedure for 80 mg NHS-PEG in a second 1.5 mL microtube. (Note: PEG powders must be stored under nitrogen gas; *very* gently flow nitrogen gas into storage tubes before resealing them with parafilm and storing at −20 °C).

   Dissolving the PEG solution must be done quickly. The hydrolytic half-life is ~20.4 min at pH 8 according to the

manufacturer [24]. Furthermore, it is very important that all of the biotin-PEG is completely dissolved to prevent patches of surface-localized molecules in the experiment, whereas a homogenous surface coverage is desired.

4. Pipet the MOPS buffer to the NHS-PEG microtube. Vortex rapidly (10 s) to suspend the NHS-PEG powder in the buffer.
5. Transfer the NHS-PEG solution by pipetting it to the biotin-NHS-PEG microtube. Vortex (30 s) to dissolve the PEG in the buffer completely.
6. Centrifuge for 1 min at 13,000 g to remove all bubbles and to spin down any dirt or aggregates.
7. Pipet 20 μL PEG solution into each well. Be careful not to touch any potential pellet (even if invisible to the eye), and prevent bubble formation.
8. Incubate the solution in the gasket wells for 3 h at room temperature. Ensure that the solution does not dry up by placing the coverslips in a closed box with saturating humidity. Crystallization of the salt may result in undesirable autofluorescent signals.
9. Rinse the gasket wells by pouring ultrapure water over the wells.
10. Dry the coverslips under gentle nitrogen gas flow.

3.5.4 Second PEG Coating

1. Prepare the second PEGylation solution. For each gasket well, prepare 20 μL of second PEGylation solution. Mix in a 1:9 ratio (2:18 μL for 20 μL) 250 mM MS4-PEG:sodium bicarbonate buffer.
2. With the slides in a clean, empty pipette tip box, add 20 μL of second PEGylation solution to each gasket well. Incubate in the dark for at least 30 minutes under saturating humidity, preferably overnight. After incubation, rinse the gasket wells with ultrapure water and dry the wells with a gentle nitrogen gas flow. Store the slides in nitrogen gas in 50 mL falcon tubes, at −20 °C.
3. The silicone gaskets can be cleaned by successive rinsing with methanol, ultrapure water, and acetone, followed by overnight washing in acetone.

This protocol was adapted from protocols from the Kapanidis [5] *and Joo* [25] *labs.*

3.5.5 smFRET Data Acquisition

For data acquisition, an objective-type total internal reflection fluorescence (TIRF) microscope is used (Fig. 6, as described previously [26]). In this setup, green (520 nm) and red (638 nm) diode lasers (Lasertack, Fuldabrück, Germany) are passed through spectral

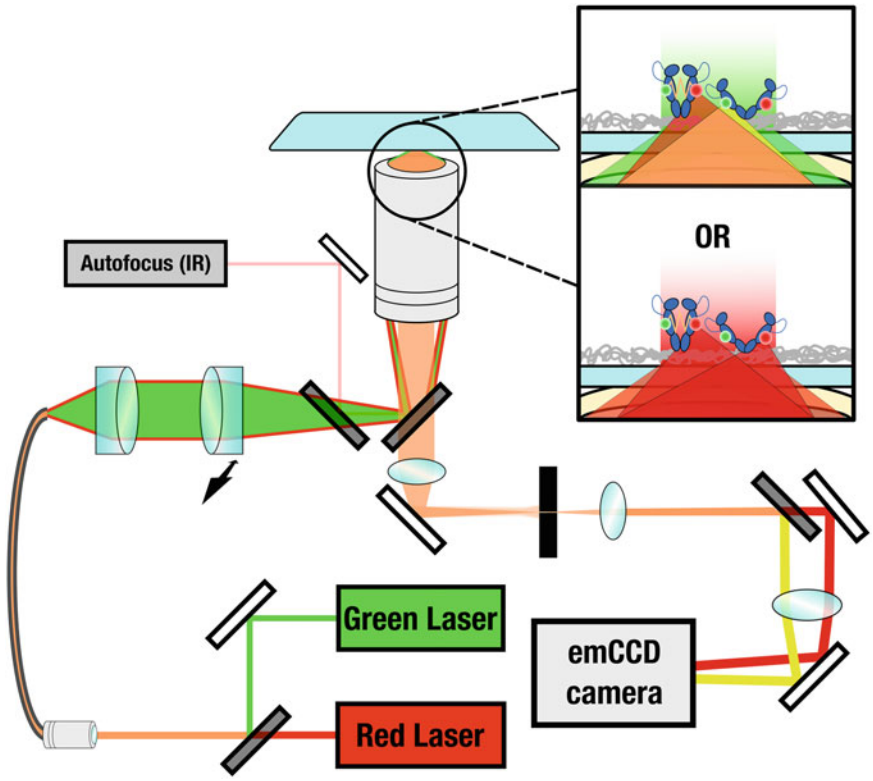

Fig. 6 Schematic representation of the smFRET TIRF setup. 520-nm (green) and 638-nm (red) diode lasers are coupled into an optical fiber. After beam expansion, the excitation light is focused onto the back-focal plane of the objective. The collimated excitation beam undergoes total internal reflection (TIR) at the coverslip-buffer interface, creating an evanescent field that excites the surface tethered fluorophores. Fluorescence emission is collected through the same objective, spectrally split, and guided to the detector (e.g. an Electron Multiplying Charge-Coupled Device, EMCCD). Open rectangles: regular mirrors. Grey-shaded rectangles: dichroic mirrors. The lens focusing the lasers onto the objective back-focal plane is mounted on a horizontal translation stage to adjust the TIR angle. The inset depicts the situation under green (top) and red excitation (bottom). More details are provided in Subheading 3.5.2

filters (resp. ZET520/20x and ZET63/20x, Chroma Technology, Bellow Falls, USA) aligned using a silver-coated mirror and a dichroic mirror and coupled into a single-mode fiber (Thorlabs, Germany) using a translation stage. After the lasers exit the fiber, they pass a collimating lens. An achromatic lens on a translatable stage (to set the TIR angle) focuses the lasers onto the back-focal plane of the objective via a dichroic mirror (ZT532/640rpc, Chroma Technology), which is reflective for the excitation photons and transmits the emission photons. The fluorescence emission collected through the objective is passed through a slit to remove off-axis beams. Before reaching the detector, a ZT640rdc dichroic mirror (Chroma Technology) separates the donor and acceptor emission. The emitted photons are detected using an EMCCD (or s-CMOS) camera (e.g., Andor iXon Ultra 897, Andor, Oxford,

UK). The objective is mounted on an XYZ translation stage (e.g., MS-2000, ASI, Eugene, USA).

1. Switch on the lasers for at least 10 minutes before measurement for the laser source to warm up, yielding a stable laser intensity. Using a power meter, determine the laser power delivered to the sample (*see* **Note 21**).
2. Carefully place a small amount of immersion oil on the TIR objective. The immersion oil refractive index should closely match the refractive index of the TIR objective (e.g., refractive index of 1.518).
3. Place the glass slide (gasket wells up) above the TIR objective. Add 20 μL of wash buffer in the gasket well. Use the TIR objective translation stage to carefully move the TIR objective in the Z-direction until the glass-buffer plane is in focus (*see* **Note 22**).
4. Empty the gasket well. Add 20 μL Neutravidin (0.25 mg/mL) solution. Incubate for 5 min to promote interaction with the biotin-PEG on the slide. Then wash the gasket well three times with 200 μL wash buffer per rinse, using a two-pipette approach: simultaneous discharge from one side of the gasket well and aspiration from the opposite side to create a manual flow inside the well.
5. Centrifuge labeled Hsp90 sample (~10 μM) for 3 min at 13.000 × *g* to spin down potential aggregates. Dilute Hsp90 25x into Hsp90 measurement buffer with 2 mM AMP-PNP. Incubate in the dark for 45 min. Prepare picomolar Hsp90 dilutions in Hsp90 measurement buffer with 2 mM AMP-PNP (*see* **Note 23**).
6. To obtain a desired coverage (100–400 molecules per field of view of 60 × 30 μm), iteratively add Hsp90 at higher concentration to the gasket well. Start at a Hsp90 concentration of 10 pM.
7. Incubate the protein sample in the gasket well for 2 min to allow for the biotin-neutravidin interaction. Keep checking the surface coverage during this time: single-molecule positions should be distinguishable (*see* Fig. 7).
8. At a desired coverage, wash out Hsp90 with wash buffer (3x200μL) to prevent further immobilizations during measurement.
9. Add 20 μL Hsp90 measurement buffer with 2 mM AMP-PNP to the gasket well. Cover the gasket well with a round glass coverslip to prevent oxygen entry into the solution during the measurements. Allow the measurement buffer to equilibrate in the gasket well for 2 min before starting a measurement.

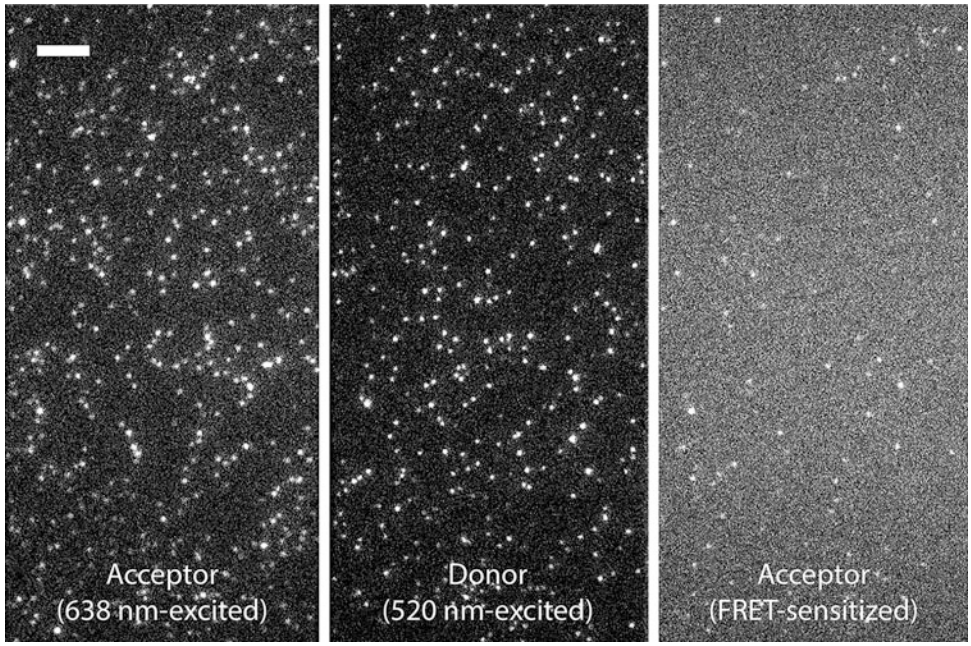

Fig. 7 Molecular coverage on the field-of-view, resulting in highly parallel detection of individual molecules. Shown are the three detection channels in an smFRET experiment using alternating laser excitation, as indicated. Signals too close in space are excluded from analysis. The scale bar is 5 μm

10. Set time resolution to 100 ms per frame (i.e., an ALEX time resolution of 200 ms). Set the amount of frames to measure. Remeasure laser powers (520 nm: 8.3 mW, 638 nm: 1.4 mW) to verify the stability of lasers.
11. Acquire ten (or more) movies at different fields of view.

3.6 smFRET Data Processing

Procedures of single-molecule FRET data processing have been described in multiple publications [14, 16, 27]. Here, data analysis is performed in Igor Pro, using code developed in the laboratory of Thorsten Hugel. A comprehensive list of alternative smFRET analysis software can be found in [16].

1. If required, correct for horizontal drift in your acquired data (e.g., using the NanoJ Core ImageJ plugin [28]).
2. Geometrically calibrate the two detection channels using a movie acquired with static markers (fiducial markers or fluorescent beads) on the microscope slide.

   Manually or automatically select trajectories, according to the following criteria:
3. Donor and acceptor bleach in a single step.
4. Trajectories display no or few blinking events.
5. The total fluorescence intensity remains constant over time.

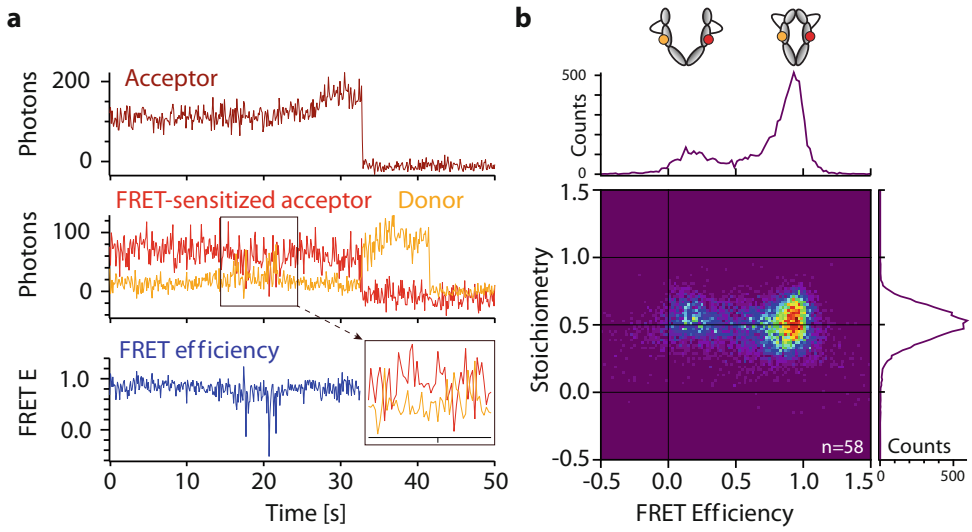

Fig. 8 Single-molecule FRET data of the Hsp90 chaperone protein in the presence of non-hydrolysable AMP-PNP. (**a**) Time-resolved trajectory of a single Hsp90 protein residing mainly in the closed conformation (high FRET E) with three conformational opening transitions leading to short dwells in the open conformation (low FRET E). Top: directly excited acceptor signal as a control for photophysics. Middle: donor and FRET-sensitized acceptor signal: their anticorrelated spikes indicate conformational transitions (inset: zoom-in of anticorrelated spikes), followed by acceptor bleaching leading to a sharp increase in donor intensity (no more FRET). Bottom: calculated FRET efficiency. (**b**) The stoichiometry versus FRET efficiency histogram shows the relative population of Hsp90's open and closed conformations. In the presence of AMP-PNP, a large high-FRET population and a small low-FRET population is observed, indicating that Hsp90 occurs mainly in the closed conformation with only short excursions to open conformations. The data of $n = 58$ Hsp90 molecules was corrected according to Ref. [14]

An exemplary trajectory displaying Hsp90 dynamics, notably in the presence of AMP-PNP, is shown in Fig. 8a.

6. Prepare the selected trajectories for further data analysis: select the relevant trajectory range (i.e., with simultaneous donor and acceptor emission) for each single-molecule trajectory.

   Plot the selected trajectories in a stoichiometry versus efficiency (ES) histogram.

7. Uncorrected apparent efficiency (E_{app}):

$$E_{app} = \frac{DA}{DD + DA}$$

   where DA is the FRET-sensitized acceptor signal and DD is the donor signal after donor excitation (i.e., 520 nm here).

8. Uncorrected apparent stoichiometry (S_{app}):

$$S_{app} = \frac{DD + DA}{DD + DA + AA}$$

   where AA is the acceptor signal after acceptor excitation (i.e., 638 nm here).

9. Correct for background intensity, donor leakage (α factor), direct acceptor excitation (δ factor), fluorophore photon absorption probability and laser intensity correction factor (β factor), and a fluorophore quantum yield and detection correction factor (γ factor), as detailed in [14]. The corrected SE histogram of Hsp90 dynamics in the presence of AMP-PNP is shown in Fig. 8b. The donor leakage and direct acceptor excitation correction factors were determined to be 0.06, the β factor 1.55, and the γ factor 0.80 for the custom-built TIRF setup used.

The presence of AMP-PNP is known to lock Hsp90 mainly in its closed conformation, as shown by X-ray crystallography [29]. The smFRET data shows that indeed, in the presence of the non-hydrolyzable ATP analog AMP-PNP, Hsp90 is predominantly observed in its closed state. Interestingly, however, smFRET reveals that even when bound to AMP-PNP, single Hsp90 proteins can exhibit conformational dynamics (Fig. 8a). As there was no ATP present that could provide energy through hydrolysis, these dynamics can only arise from thermal fluctuations. This underlines the power of single-molecule FRET to elucidate the nuanced behavior of individual proteins in the presence and absence of a chemical energy source.

4 Notes

1. yHsp82 contains no cysteines naturally, allowing for the insertion of a single cysteine for specific fluorescent labeling. When designing labeling positions in your protein, ensure that there are no other cysteines accessible to the dye.
2. Alternatively, transformation can be performed by electroporation using electrocompetent cells.
3. Rosetta is a BL21-derived *E. coli* strain with an additional plasmid encoding tRNAs for codons uncommon in bacteria, several of which Hsp82 contains. However, when isolating a different (bacterial) protein without these uncommon codons, it would be best to simply use BL21, as Rosetta limits you to colE1-based plasmids (such as the pET plasmid used here) and has additional metabolic burden due to the extra plasmid.
4. The 20% glucose and biotin solution are only necessary if in vivo biotinylation is performed.
5. Additional plates with concentrated as well as diluted transformed cells should be made for a first experiment when transformation efficiency is still unknown.
6. Agar plates can be stored for a maximum of 2 weeks in a plastic bag or parafilm wrap at 4 °C.

7. Glucose is added for optimal suppression of pET plasmid DNA expression (which happens under control of the lac operon) during bacterial cell growth. Omittance of glucose may also result in good protein yields.
8. While aeration by shaking can improve growth, shaking too fast may result in undesirable foam formation. Prevent foaming in the culture flasks by properly adjusting the shaking speed. Our method includes baffled flasks, which improve aeration but may also quickly cause foaming at higher shaking speeds.
9. The amount of time it takes for the culture to reach an OD_{600} of 0.8–1.0 is highly dependent on the protein being expressed. In our experience with two different plasmids, the initial growth phase took 3 versus 8 h. Keep this in mind when planning your experiment with a new plasmid. Pre-warming the culture medium before adding pre-culture helps accelerating this step.
10. For a first-time protein production, it is strongly recommended to take an expression control, i.e., transformed cell culture before and after induction with IPTG, and verify specific expression of protein of the correct size by SDS-PAGE.
11. The resuspension can be stored at −20 °C until resuming with the procedure. After thawing, gently invert the resuspension several times.
12. Ensure that the sonic probe does not touch the falcon tube or the tube will absorb the vibrations. The resuspension should turn more transparent. Repeat the sonication step when necessary. Sample viscosity may be reduced by addition of DNase to degrade genomic DNA.
13. It is key to prevent air from entering the columns. This may damage the resin which binds your proteins. Therefore, take care to prevent air bubbles in the tubing. If air bubbles do enter the tubing at any step, purge them out before letting (more) fluid run over the columns.
14. Load filtrated lysate from a falcon tube or beaker. Make sure not to introduce any air bubbles. When almost all sample is applied, slowly add binding buffer to the lysate until all lysate is applied to the column to prevent air aspiration into the tubing.
15. The column may become saturated during lysate application, contain some unbound protein of interest during wash out, or contain not yet eluted protein of interest during remaining protein elution. It is therefore recommended to collect the eluate during these phases and analyze these fractions on an SDS-PAGE gel.
16. For a first protein purification round, an increasing elution buffer gradient should be used, instead of a fixed

concentration, to determine at which imidazole (HisTag) or salt (AEX) concentration the protein elutes.

17. The membranes of the centrifugal filter contain trace amounts of glycerin. It is recommended to rinse and centrifugate with ultrapure water before use. The filters can be reused for concentrating the same protein, as long as the membranes are stored in ultrapure water.
18. Depending on the conformational dynamics that the experimenter wants to probe, multiple unique cysteine mutant proteins may need to be produced.
19. Shorter incubation than 1 h (e.g., 10–30 min) may already be sufficient for efficient labeling of the protein.
20. In our experience, *dye-labeled* protein stored at −20 °C for extended periods of time (>1 month) yields poor results in smFRET measurements. Therefore, we recommend to only perform smFRET measurements with freshly dye-labeled protein, and keep dye-labeled samples at 4 °C for up to a week. Storing purified protein at −20 °C for longer periods works well.
21. Ideally, the experimenter reports a value for laser power delivered per area of glass slide (W/cm^2). At our setup, we measure the power (in mW) directly after the fiber outlet. For a frame rate of 10 Hz, suitable laser powers (determined in this way) are 8.3 mW for the green (520 nm) laser and 1.4 mW for the red (638 nm) laser using the ATTO550 and ATTO647N dyes.
22. A useful starting position to find the glass-buffer focus plane is to move the objective toward the silica coverslip until the flat part of the objective is exactly covered with immersion oil. From this point, to prevent coverslip breaking, first bring the oil-glass interface into focus by moving the objective away from the coverslip. The oil-glass interface should be directly recognizable by many signals in the field of view. Then, slowly move the objective toward the coverslip until the glass-buffer plane is in focus, recognized primarily in the green channel by sparse signals from autofluorescent particles.
23. Photobleaching and photoblinking can severely shorten or introduce artifacts to smFRET experiments. Enzymatic systems that scavenge oxygen combined with antioxidants can be employed to reduce such effects [30]. However, with such systems, additional proteins are introduced into the measurement, which is undesirable for protein studies. Alternatively, oxygen can be replaced by an inert gas such as nitrogen or argon to extend single-molecule observations – the broadest bandwidth (observation time divided by time resolution) currently measured with smFRET was achieved with argon-saturated buffers; see [31]. In addition, dye replenishment

schemes (DyeCycling [32] and REFRESH-FRET [33]) are being developed that will vastly extend the covered time range and the total information gain per single molecule.

5 Data Availability

The single-molecule trajectories recorded in this work are deposited on Zenodo (doi: https://doi.org/10.5281/zenodo.7273266).

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