Chapter 12

Mechanically Watching the ClpXP Proteolytic Machinery

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Abstract

Energy-dependent protein degradation is studied through the dual bead ClpXP motility assay. Processing of folded proteins involves recognition, unfolding, translocation, and degradation stages. A dual optical trap, in a passive force-clamp geometry, exhibits bead-to-bead displacements that directly follow subprocesses underlying protein degradation. Discrete nanometer-scale displacements of the bead position reveal steps, dwells and pauses during the unfolding and translocation substeps. With a few structural modifications to the protease machinery and an engineered substrate, the assay represents a "chassis" for the measurement of a wide range of substrates and related machinery. The methods described faithfully record our assay as implemented, including substrate design, wet assay preparation, and the motility assay experiment protocol. The strategies herein permit adaptation of the ClpXP mechanical assay to a wide range of protein degradation systems.

Key words ClpXP, Optical tweezers, Proteasome, Dual trap, Unfolding, Translocation, Single molecule degradation, Passive force clamp, ATPase, AAA+ protease

1 Introduction

ClpXP is a member of the AAA+ (ATPase associated with various cellular activities) protease family in *Escherichia coli* and model for understanding how energy-dependent protein degradation machinery, such as the eukaryotic proteasome, work in all cells. The ClpXP system processes proteins tagged for destruction by performing many tasks including recognition, unfolding, translocation and degradation. ClpXP consists of the ClpX and ClpP multimeric rings stacked in a barrel like geometry with rings encompassing a central axis of symmetry. The system also contains mirror like symmetry with two back-to-back seven member ClpP rings, forming a central degradation chamber, capped by two 6 member ClpX rings where proteins enter. The ATP processing ClpX rings perform recognition, unfolding and translocation tasks that control access to ClpP by feeding an unfolded polypeptide through a small opening to the inner chamber of ClpP where catalytic degradation of the peptide backbone occurs [1].

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The broader AAA+ superfamily of molecular machines, which function in diverse cellular processes, contains a massive number of members, over 30,000, found in all forms of life [2–5]. Some of the best-known examples used for single molecule studies are cytoplasmic dynein [6, 7], which is a processive microtubule minus end-directed motor, and the Φ29 DNA-packaging motor, which transports and packages the phage genome [8–10]. AAA+ member activities are diverse [11], and include circadian clock oscillators [12], microtubule-severing enzymes such as Katanin and Spastin [13], and Hsp104 and ClpB, which disassemble protein aggregates [14].

From a geometrical and design perspective, the diverse family of AAA+ motors retains ring hierarchy where sub-tasks are organized within or adjacent to ring elements [15]. From a protein sequence perspective these systems may assemble from separate AAA+ ATPase subunits (as in ClpX and ClpP), be concatenated into a single large polypeptide (as in dynein) or linked sequentially vertically from one ring function to the next such as in FtsH [16].

The assay detailed in this chapter overcame a number of challenges. Fortunately, a substantial foundation of work detailing the biochemical requirements for the initiation of ClpXP's processing of a wide range of proteins helped us to establish a reliable single-molecule assay. The assay includes inherently fluorescent substrates such as GFP and CFP, recognition sequences, and details relating to the nucleotide identity and/or concentration and underlying requirements for executing recognition, unfolding and translocation tasks [17]. Effectively, ClpXP travels on a protein-based track, navigating along both folded and unfolded elements of the polypeptide chain. While a foundation of single molecule literature/work detailed methods for motility experiments using actin, microtubules, DNA, and RNA as substrates, adaptation to a polypeptide track was required. Unlike these other tracks, the only regular periodicity of the ClpXP track is the peptide bond. ClpXP must therefore denature diverse structures of folded proteins as well as process strands of varied amino acid sequences.

Motility is initiated through a peptide based signal (the ssrA tag) that when covalently linked to a protein, facilitates recognition by ClpXP. Still a number of challenges were present when adapting these well-established bulk assay protocols to the single molecule arena. Disassembly of the ClpXP system at very low concentrations utilized in single-molecule studies proved to be fatal as the ClpX ring equilibrium would shift to monomers. A single chain hexamer was developed to link the ring together using a variant of ClpX lacking it's N-terminal domain [18]. A biotin molecule was also engineered within the ring to permit physical immobilization to a coverslip surface or bead.

The first robust single-molecule assay for ClpXP immobilized ClpXP to a streptavidin-coated surface and utilized GFP as well as Cy3-tagged substrates [19]. Here, many ClpXP motors could be

watched simultaneously in a single field of view by following degradation of the fluorescent substrates. A strategy was developed to stall and synchronize ClpXP activity. To do this, a substrate was engineered with a recognition tag followed by a weakened titin domain followed by the more mechanically stable GFP and a terminal handle that was functionalized with a Cy3 fluorescent dye. The titin domain contains the V15P single point mutation, which can be degraded by ClpXP using ATPγS as a fuel substitute for ATP. Unlike V15P, GFP degradation stalls in the presence of ATPγS and requires ATP for successful processing [20]. Synchronization of the motors through exchange of ATP for ATPγS utilized a strategy to load ATP into the ClpX nucleotide binding pockets but prevent hydrolysis by chelating and starving the system of magnesium, which is required for hydrolysis. The fluorescence-based assay permitted direct observation of the degradation kinetics of ClpXP and featured many of the assay elements needed for adapting to a mechanical based optical tweezers measurement.

Adapting the single molecule fluorescence assay to optical tweezers was first attempted by attaching ClpXP to the coverslip surface followed by introducing substrates tethered to beads, but given the nanometer-scale displacements and the low motor velocity (~3 nm/s), motility records suffered from large sample drift. Furthermore, all pre-assembled tethers in the flow cell initiate motility at the start of the experiment, creating a problem in experimental throughput for optical trapping measurements, which typically observe a single molecule at a time. These problems could be partially solved using the dual-bead system (Fig. 1), which

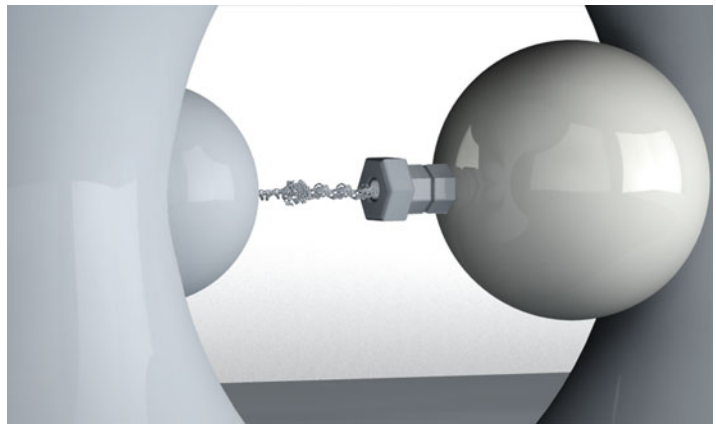

Fig. 1 Rendering of the dual bead optical trap assay for mechanically visualizing ClpXP machinery. ClpXP recognizes, unfolds, translocates, and degrades protein substrates tagged for destruction. Two beads of slightly different sizes are held using two optical traps originating from the same laser source. A protein multimer containing a recognition sequence, four titin^{I27} domains and a HaloTag is linked to the smaller bead using a DNA tether as in Cordova et al. [24]. The slightly larger bead is functionalized with ClpXP. The hexameric ClpX recognizes, unfolds, and translocates the polypeptide into ClpP, which catalytically degrades the polypeptide. By measuring precise bead-to-bead separation, displacements due to unfolding and translocation steps along the polypeptide can be recorded

offers a number of advantages to the tethered-bead assay. First, due to the use of a common mode differential strategy for the detection of the distance between two beads held by a dual-beam optical trap but synthesized by the same laser source, it is not subject to stage drift and therefore exhibits lower noise. Second, each substrate-motor tether can be assembled individually, in real time, permitting multiple experiments per slide. Third, much of the physical linkages could be more easily controlled on bead surfaces rather than cover glass surfaces, which can be finicky.

In addition to the dual-bead geometry, use of a multi-domain substrate was advantageous for two reasons. The presence of several folded domains in a single substrate allows for multiple unfolding and translocation events to be measured from a single tether thereby achieving "more bang for your buck" during an experiment. Additionally, this substrate design ensures that we catch unfolding and translocation events that might be missed in a single domain substrate due to the time it takes to begin data collection once an active tether is formed in the presence of ATP.

Early work establishing the dual-bead assay to investigate ClpXP motility used a filamin polyprotein [21], and single or double GFP substrates [22]. These assays revealed sequential unfolding and translocation as well as the force velocity relation and direct observation of stepping. More recent studies include the work by Sen et al. [23], who investigated translocation and unfolding of GFP and unfolded substrates under varied nucleotide concentrations, and the work by Cordova et al. [24], which demonstrated unfolding and stepping through a series of weakened titin domains and ClpX mutants that lacked the ability to hydrolyze ATP. Olivares et al. [25] adapted the assay to investigate ClpAP motility, which features a double ring ATPase structure and a biotin labeled ClpP construct for bead attachment. More recently, Iosefson et al. [26] probed motility of ClpX constructs bearing pore-loop mutations. Throughout these studies a foundation of solution-based biochemical work has been integral to the development of assays and the interpretation of motor function. The ability to directly watch mechanical sub-processes and manipulate motor performance using these single-molecule assays has revealed much about ClpXP and proteasome-related machinery.

2 Materials

2.1 Motor System

Two strategies are used to link the AAA+ protease to a polystyrene trapping bead. One involves biotinylation of a single chain variant of ClpX missing is N-terminal domain (^{SC}ClpXΔN), which is dispensable for the degradation of ssrA-tagged substrates [18]. Complexes of ClpXP are assembled on bead surfaces by adding excess ClpP in the flow chamber during an experiment. A second strategy

uses a biotinylated ClpP variant that can bind a AAA+ unfoldase on one face of the heptameric ring ($ClpP^{platform}$) [25]. The advantage of this method of attaching a AAA+ protease to a bead is that single chain variants of the ATPase ring are not required and use of full-length enzymes is possible. $ClpP^{platform}$ is bound to a streptavidin-coated bead and the ATPase ClpX or ClpA are added in solution. Free ATPase can optionally be removed by centrifugation of the resulting ClpXP or ClpAP beads followed by washing the beads with a solution containing ATP before use in an experiment. Components of the motor system include:

1. ClpX: In its native state, ClpX forms a hexamer by association of individual ClpX ATPase domains. The dilute enzyme concentrations typical in single-molecule studies (~pM) did not permit stable ring formation from ClpX monomers and required using a ClpX variant where the individual domains (residues 61–423) were linked in a "single chain hexamer", ^{SC}ClpXΔN. This construct is produced using an *E. coli* expression system and also includes a biotin tag on the C terminus. In order to investigate the roles of individual ATPase subunits, a ClpX system has been generated linking two trimers through sortase-based coupling [27].
2. ClpP: The ClpP-His_6 unit is a seven membered ring. Two rings associate symmetrically to form a 14-member chamber where catalytic degradation occurs. This double ring system naturally binds to the ClpX hexamer. The ring system is stable at single-molecule concentrations.
3. Biotin linker: A biotin molecule is used to tether ClpXP to a bead. Typically, the biotin is located on the ClpX ring as part of the single-chain expression. Biotin molecules can also be incorporated within ClpP.

2.2 Buffers for Protein Expression, Purification, Modification, Filtration, and Storage

1. Lysis Buffer (LB1): 20 mM Hepes pH 7.6, 10 mM imidazole, 400 mM NaCl, 100 mM KCl, 10 % glycerol, 10 mM β-mercaptoethanol.
2. S-Buffer: 50 mM sodium phosphate pH 8.0, 1 M NaCl, 10 % glycerol, 5 mM imidazole.
3. Elution Buffer (EB1): 20 mM Hepes pH 7.6, 250 mM imidazole, 400 mM NaCl, 100 mM KCl, 10 % glycerol, 10 mM β-mercaptoethanol.
4. W20 Buffer: 50 mM sodium phosphate pH 8.0, 1 M NaCl, 10 % glycerol, 20 mM imidazole.
5. W500 Buffer: 50 mM sodium phosphate pH 8.0, 1 M NaCl, 10 % glycerol, 500 mM imidazole.
6. ResBuffer: 50 mM Hepes pH 7.6, 150 mM KCl, 0.5 mM EDTA, 10 % glycerol.

7. Biotinylation Buffer (BB1): 20 mM Hepes pH 7.6, 300 mM KCl, 10 % glycerol, 0.5 mM TCEP.
8. S300 Buffer (SB1): 20 mM Hepes pH 7.6, 300 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 % glycerol, 0.5 mM TCEP.
9. Ring Separation Buffer: 50 mM Hepes pH 7.6, 0.7 M KCl.
10. W-20B Buffer: 50 mM Hepes pH 7.6, 150 mM KCl, 0.5 mM EDTA, 20 mM imidazole, 10 % glycerol.
11. W-500B Buffer: 50 mM Hepes pH 7.6, 150 mM KCl, 0.5 mM EDTA, 500 mM imidazole, 10 % glycerol.
12. ClpP Storage Buffer: 50 mM Hepes pH 7.6, 150 mM KCl, 0.5 mM EDTA, 10 % glycerol.

2.3 Multidomain Substrate System

The multidomain substrate contains an N-terminal HaloTag® domain fused to four repeats of the ~100 amino acid I27 domain of the human muscle protein titin ($titin^{I27}$) and ends with the *E. coli* ssrA degradation tag, which allows for efficient targeting to the ClpXP and ClpAP ATP-dependent proteases. HaloTag is a modified haloalkane dehalogenase that is used to covalently link our substrate to a 3500 bp DNA linker [24, 25]. This long ~1 μm linker allows for sufficient separation of the two beads used in the experiment, ensuring that beads remain trapped by the appropriate laser. Similar strategies have been used to covalently link substrate to DNA using a SNAP-tag®, which is the modified DNA repair protein O^6-alkylguanine-DNA alkyltransferase [22, 23]. $Titin^{I27}$ possesses a β-sheet-rich, Ig-like fold and is used here as a model substrate for examining how protein stability affects the kinetics of unfolding. The mutation of valines to prolines near the C-terminus of $titin^{I27}$ (V13P and V15P) affect the thermodynamic, kinetic and mechanical stabilities such that the wild-type protein (WT) is the most resistant to unfolding with increasing weakening of the structure as one disrupts the C-terminal strand where the AAA+ protease is pulling on (i.e., WT > V15P > V13P) [28]. Additionally, carboxymethylation of buried cysteines within $titin^{I27}$ yields an unfolded protein for the study of polypeptide translocation [28]. The very C-terminus of the substrate contains a hexahistidine (His_6) tag for purification and the ssrA tag, which is a small peptide sequence that is cotranslationally appended to incomplete proteins that arise due to ribosomal stalling and efficiently targets these proteins to ATP-dependent proteases like ClpXP in bacteria [29]. Substrates used in published work are found in Table 1.

2.3.1 Specific Components of the Multidomain Substrate

1. Recognition domain: a C-terminal AANDENYALAA ssrA tag.
2. His_6 tag.
3. Multi-domain protein substrate: These typically consist of sequential polyprotein elements that form the motility substrate of ClpXP. Elements include immunoglobulin repeats 1–8 of filamin A (accession CAI43198; residues 279–1066),

Table 1
lists various substrate elements, residue length, typical unfolding distance and unfolding time from dual bead ClpXP mechanical degradation assays

Substrate	Residue length (aa)	Unfolding distance (nm)	Unfolding time (s)	References
Filamin A (Ig 1–8)	98	15	5–25	[21]
Titin I27 (wt)	89	12	>55	[24]
Titin I27 (V13P)	89	12	5.9	[24–26]
Titin I27 (V15P)	89	12	17	[24]
GFP	238[a]	50	9.1	[22, 23, 25, 26]
Halo Tag	294[b]	25	8.7	[21, 24–26]

[a]Truncated version [20]
[b]150–170 amino acids are degraded by ClpXP

residues 5303–5341 of human titin (accession CAA62188; corresponding to the C-terminal half of the I27 domain), mutant I27 domains and GFP domains.

4. Linker: a linker of 47 residues containing two TEV protease sites.
5. HaloTag: haloalkane dehalogenase, a protein used as a covalent linker to 3500 bp DNA.

2.3.2 DNA Tethers for Substrate and Aptamer

1. Forward primer (25 μL, 20 μM): 5′-Biotin-AAT CCG CTT TGC TTC TGA CT-3′ diluted from 100 μM with ddH_2O (IDT custom oligo).
2. Reverse primer (25 μL, 20 μM): 5′-Amino-TTG AAA TAC CGA CCG TGT GA-3′ diluted from 100 μM with ddH_2O (IDT custom oligo).
3. dNTPs (8 μL, 25 μM) (New England Biolabs cat#N04475).
4. M13mp18 plasmid (5 μL, 50 ng/μL) (Bayou Biolabs cat#P-105).
5. TE buffer (Ambion cat#AM9849).
6. UltraPure water (727 μL) (Invitrogen cat#10977-015).
7. Phusion DNA Polymerase (10 μL) (New England Biolabs cat#M0530S).
8. QIAquick PCR purification kit (Qiagen cat#28106).
9. UV–Vis spectrophotometer (Thermo Scientific NanoDrop 2000).

2.3.3 DNA-Amine to Thiol-HaloTag Ligand Conjugation

1. NH_2-DNA (200 ng/μL in PBS).
2. UltraPure distilled water (Invitrogen cat#10977-015).

3. Sulfo-SMCC (Pierce cat#22622 in 2 mg sealed tubes).
4. PBS buffer at pH 7.4.
5. For HaloTag conjugation: HaloTag Thiol Ligand (Promega cat#P6761).
6. For aptamer conjugation: glass binding peptide with cysteine and two glycine residues added at the N-terminus (CGGRSGRRRSHHHRL).
7. Bio-Rad Micro Bio-Spin 30 columns (MBS30) (BioRad cat#732-6223).
8. Bio-Rad Micro Bio-Spin 6 columns (MBS6) (BioRad cat#732-6221).
9. UV–Vis spectrophotometer (Thermo Scientific NanoDrop 2000).

2.4 Protein Degradation Buffer (PD buffer)

1. 0.59575 g HEPES (25 mM).
2. 0.7455 g KCl (100 mM).
3. 0.2033 g $MgCl_2$ (10 mM).
4. 90 mL ddH_2O.
5. 10 mL glycerol (10 %).
6. 100 μL Tween 20 (0.1 %).
7. 0.2 μm filter.
8. Mix everything. Adjust pH to 7.6 with KOH. Pass through a 0.2 μm filter.

2.5 ATP Regenerating System (Prepared at 20×)

1. Creatine phosphokinase (Calbiochem 238395).
2. Creatine phosphate (Calbiochem 2380).
3. Adenosine triphosphate (Sigma A7699).
4. Potassium hydroxide (Sigma 221473).
5. PD buffer.

2.6 Oxygen Scavenging System (Prepared at 100×)

1. 16,500 U/mL glucose oxidase (Sigma, G2133).
2. 217,000 U/mL catalase (Sigma, C100) in PD.
3. 500 mg/mL D-Glucose (Sigma, G8270) inPD.

2.7 Flow Chambers

1. Scotch Permanent Double Sided Tape (3 M cat#137DM-2).
2. Potassium hydroxide (Sigma 221473).
3. Glass coverslips (24 × 40, thickness 1.5) (Fisher cat#12-544-C).
4. Glass microscopy slides (VWR cat# 48312-068) chemically etched in ethanolic base as below.

2.8 ClpXP Dumbbell Assay

1. ClpX (biotinylated) 3 μL, ~5–6 μM.
2. ClpP (with his tag) 11 μL, 20 μM.
3. ATP, 20× with regeneration system.
4. HaloTag terminated substrate, 3 μL, 20 μM.
5. HaloTag–3500 bp DNA–biotin in PBS, 15 μL, ~150 ng/μL.
6. A08–3500 bp DNA–biotin, 50 μL of 0.5 ng/μL.
7. 1 μm streptavidin coated polystyrene beads (Polysciences Inc 24162-1).
8. 1.26 μm streptavidin coated polystyrene beads (Spherotech SVP-10-5).
9. Oxygen scavenging system at 100×.
10. Dithiothreitol (100 mM, Thermo 20290).
11. Casein (Sigma C7078).
12. Bovine serum albumin (Calbiochem 2905-OP).
13. PD buffer.
14. PBS.
15. 0.2 μM syringe filters (Fisher SLMP025SS).
16. Microcentrifuge tubes (1.5 and 0.7 mL, Fisher 02-682-556 and 05-408-120).
17. Flow cells.
18. Vacuum grease (VWR 59323-011).

2.9 Dual-Beam Trapping Instrument

The optical trapping microscope is a high-end custom-built instrument featuring separate trap and position sensing systems and a high degree of automation. The design contains many of the features described by Lang et al. [30]. The dual trap is achieved by incorporating a pair of polarizers as detailed by Visscher et al. [31]. When designing a system one must consider stability throughout the system ranging from choosing high quality components to housing the instrument in an acoustically quiet, tight temperature controlled environment. The system is only as stable as the noisiest component. Herein we list some of the specific choices in components as a guide.

1. Microscope, Nikon TE 2000-U, mechanically stabilized through direct bolting to the optical table and custom machined sample stage, objective holder, trap injecting mirror holder and condenser side detector branch. Our objective is a Nikon Oil IR, 1.40 NA and the condenser is a wide lens Oil 1.4 NA.
2. Trapping laser: IPG Photonics model YLD-10-1064. This laser is fiber coupled, reasonably stable with a reasonable mode. Fiber coupling permits locating the laser outside of the

trapping instrument room such that heat dissipation and acoustical noise do not interfere with the microscope. Fiber coupling also helps to reduce pointing instabilities and other noise sources related to laser cavities.

3. Detection laser and subsystem: Avanex A1998 PLM, a 14-pin butterfly laser mounted in a Thor labs housing and driven by a Thor Labs Controller (ITC 510). The laser outputs 975 nm light through a single-mode fiber that can be output-coupled to produce a beam of the desired width. The optical fiber launch helps to reduce noise associated with pointing. The laser also features a fiber Bragg grating which stabilizes the lasing mode. The laser is isolated along the detection branch with an Andover 970FS10-50 band pass filter. A number of wavelengths and laser sources are appropriate for the detection laser. The primary requirement should be stability and wavelength cross-section with the position sensing detectors. Our instrument utilizes dual lateral position sensing devices, PSD's, from Pacific Silicon Sensor (model 10-018) with an approximately 1 cm^2 active area for Silicon based detection. Signals from the PSD's are amplified approximately 10× using low noise voltage-regulated amplifier chips and fed into an "anti-alias filter" (Kron-Hite Model 3384, 8 pole LP/HP Butterworth Bessel).
4. Some of the automated features include: computerized ability to move the optical trap through acoustic optic deflectors or AOD's (IntraAction DTD-276HD6); movement of the sample through a piezo stage (Physik Instrumente P-527.3CD stage and E-710.3CD controller); shutters for turning on and off beams (Uniblitz); and picomotor actuators for fine positioning of trap and detection lasers (NewFocus).

3 Methods

3.1 Expression of Proteins in E. coli

1. Use freshly transformed *E. coli* expression strains harboring plasmids for expression of multidomain ssrA-tagged substrate, ^{SC}ClpXΔN, ClpP-His_6, or $ClpP^{platform}$ variants ClpP-TEV-His_6 and $ClpP^{M5A}$-bioAP-His_6 described below.
2. Grow overnight at 37 °C on LB-agar with the appropriate antibiotic.
3. Multidomain substrate and ^{SC}ClpXΔN are overexpressed in an *E. coli* ER2566 lysis strain described in Shin et al. [19], which is available upon request or the commercially available BLR (DE3), *recA*$^-$, which prevents the unwanted recombination of repeat sequences. ClpP variants are expressed in JK10 cells (clpP::cat, Δlon, slyD::kan, λDE3) described in Kenniston et al. [32].

4. Pick colony from overnight plate and grow 50 mL overnight culture in LB antibiotic at 37 °C.
5. Transfer 10 mL overnight culture into 1 L LB-antibiotic and grow, shaking at 37 °C. When OD_{600} reaches ~0.6, cool at 4 °C for 15 min and lower shaker temperature to 25 °C. Induce cultures with 0.5 mM IPTG and grow for 3–4 h. Harvest cells at 4000 × *g* for 15 min at 4 °C. Resuspend cells in 10 mL Lysis Buffer per each gram of cell paste. For ClpP variants, resuspend cells in 20 mL S-buffer per 1 L cells.
6. Flash-freeze in liquid nitrogen and store pellets at −80 °C.

3.2 Ni-NTA Purification of Multidomain Substrate and ClpX Proteins

The following steps are performed at 4 °C unless stated otherwise.

1. Thaw cells in ice water bath for 15–20 min. Make sure that cells are well suspended and then pass through a French press at 25 kpsi. If you do not have a French press, sonication or freeze–thaw cycles with lysozyme can be done to break open the cells. Add 1 mM PMSF to lysate. If lysate is viscous, incubate with 500 U benzonase and 2 mM $MgCl_2$ for 20 min. Clarify lysate at 30,000 × *g* for 30 min.
2. Equilibrate NiNTA resin (Qiagen) with LB1 (use 1 mL resin per 2 L culture). Add supernatant to resin and bind in batch for 1 h. Collect resin at 1000 × *g*, discard supernatant and wash in batch with 30 mL LB1 per mL resin. Transfer resin to empty column and wash with more LB1 (40 mL per mL resin). Elute with 10–15 mL Elution Buffer, taking 1 mL fractions and check purification by SDS-PAGE.
3. Pool fractions and concentrate using a centrifugal concentration device (such as Millipore Centriprep Ultracel YM-30 Cat No 4306) according to the manufacturer's instructions.

3.3 Ni-NTA Purification of ClpP Variant Proteins

The following steps are performed at 4 °C unless stated otherwise.

1. Thaw cells in ice water bath for 15–20 min. Make sure that cells are well suspended and then pass through a French press at 25 kpsi. If you do not have a French press, sonication or freeze–thaw cycles with lysozyme can be done to break open the cells. If lysate is viscous, incubate with 500 U benzonase and 2 mM $MgCl_2$ for 20 min. Clarify lysate at 30,000 × *g* for 30 min.
2. Equilibrate NiNTA resin (Qiagen) with S-buffer (use 1 mL resin per 1 L culture). Add supernatant to resin and bind in batch for 1 h. Collect resin at 1000 × *g* and discard supernatant. Transfer resin to empty column and wash with 50 mL S-buffer per mL resin. Wash with W20 buffer. Elute with 15 mL W500 buffer per mL resin, taking 1.5 mL fractions and check purification by SDS-PAGE.

3. Pool fractions, concentrate and buffer exchange into ResBuffer using a centrifugal concentration device according to the manufacturer's instructions. Briefly, for buffer exchange, concentrated protein (1–2 mL for a 15 mL concentration device) is diluted with ResBuffer to the maximum volume of the concentration device and centrifuged following the manufacturer's instructions. Concentration and dilution is repeated for a total of three times.

3.4 Exogenous Biotinylation of Proteins with Biotin Ligase, BirA

For ^{SC}ClpXΔN or ClpP-biotin acceptor peptide-His_6, concentrated proteins purified by Ni-NTA are buffer exchanged into Biotinylation Buffer by dialysis or using PD-10 Desalting Columns (GE Healthcare Life Sciences).

Add 50 mM bicine, pH 8.3, 4 mM ATP, 5 mM Mg-acetate, 1 mM D-biotin, and 1 μM BirA to the buffer exchanged protein. A BirA-His_6 plasmid is available through Addgene (#20857) with subsequent purification described by Howarth et al. [33]. Incubate reaction for 1–1.5 h at room temperature.

3.5 Gel Filtration of Proteins

Multidomain substrate and ^{SC}ClpXΔN are further purified on a Sephacryl S-300 HR size exclusion column in S300 Buffer. No more than 5 % of the total column volume is loaded on the column to ensure proper separation of proteins from the contaminants. Fractions are collected, checked by SDS-PAGE and pooled. If required, pooled fractions are concentrated using a centrifugal concentration device, apportioned into small aliquots, flash-frozen in liquid nitrogen and stored at −80 °C.

3.6 $ClpP^{platform}$ Ring Assembly

$ClpP^{platform}$ consists of a ClpP variant with abrogated AAA+ unfoldase binding activity (M5A; Bewley et al. [34]) containing a biotin acceptor peptide (sequence GLNDIFEAQKIEWHE), C-terminal hexahistidine tag ($ClpP^{M5A}$-bioAP-His_6) and WT ClpP (expressed as ClpP-TEV-His_6). ClpP tetradecamers are first disassembled into heptameric rings at high salt and no glycerol. Variants are then mixed and reassembled into 14-mers by lowering salt concentration and adding glycerol.

1. For M5A variant, exchange into BB1 and follow protocol for exogenous biotinylation of protein described above.
2. Add TEV (20 μM) to ClpP-TEV-His_6 and rock overnight at 4 °C. A His_6-TEV-polyArg plasmid is available through Addgene (#8827) with subsequent purification described by Kapust et al. [35].
3. Pass overnight ClpP/TEV reaction over Ni-NTA column and wash with ResBuffer. Collect flow-through.
4. Mix excess of tagless WT ClpP variant with biotinylated M5A variant (5:1 tagless:M5A) and dialyze against Ring Separation Buffer for 3 h at room temperature.

5. Dilute ring separated mixture in 50 mM Hepes pH 7.6 and glycerol to give a final concentration of 0.3 M KCl and 15 % glycerol. Incubate at 4 °C overnight.
6. Pass $ClpP^{platform}$ mix over Ni-NTA column. Wash with 40 mL W-20B buffer per 1 mL Ni-NTA resin. Elute with W-500B buffer. Concentrate on centrifugal concentration device. Exchange into ClpP storage buffer.
7. Check by SDS-PAGE, apportion into small aliquots, flash-freeze in liquid nitrogen and store at −80 °C.

3.7 PCR Amplification of 3500 bp DNA Tether Formation for Substrate and Aptamer

1. PCR reaction mixture. Mix the following items: 715 μL Ultra-Pure water, 25 μL of 20 μM biotin primer in TE buffer, 25 μL of 20 μM amino primer in TE buffer, 20 μL of 10 mM dNTPs mixture, 5 μL of 50 ng/μL m13mp18 plasmid in TE buffer, 200 μL of 5× GC buffer, and 10 μL Phusion polymerase.
2. Mix thoroughly by gently pipetting the solution up and down.
3. Add 100 μL of PCR reaction mixture to 10 flat-cap PCR tubes.
4. Immediately transfer tubes to PCR machine and run the following program/cycles: (1) 98 °C during 30 s, (2) 98 °C during 10 s, (3) 49 °C during 30 s, (4) 72 °C during 90 s, (5) repeat **steps 2–4** for a total of 35×, (6) 72 °C during 10 min, (7) keep at 4 °C.
5. Purify DNA fragments using the Qiagen PCR purification kit. In the last step, resuspend DNA using 30 μL of 1× PBS instead of the elution buffer included in the kit.
6. Measure nucleic acid concentration of the purified DNA using the NanoDrop. Normal yields are ~200 ng/μL (100 nM for 3500 bp dsDNA) (*see* **Note 1**).

3.8 SMCC Based Coupling of 3500 bp DNA-Amine to Thiol-HaloTag or Thiol-Aptamer Ligand Conjugation

1. Remove a 2 mg Sulfo-SMCC tube from the freezer and equilibrate to room temperature for 30 min.
2. Dissolve 2 mg Sulfo-SMCC in 200 μL UltraPure water by perforating the tube with a pipette tip only delivering the water. Mix for ~15 min.
3. Immediately combine 60 μL of Sulfo-SMCC solution with 60 μL NH_2-DNA-Biotin at ~200 ng/μL.
4. Incubate for 2 h at room temperature on a rotator.
5. Remove unreacted Sulfo-SMCC with gel chromatography columns: Exchange buffer in six MBS6 tubes (*see* **Note 2**). Place 60 μL of DNA-SMCC solution in two columns. Spin column at 100 × *g* for 4 min and collect flow-through. Clean a total of three times.
6. Combine cleaned DNA-Maleimide with 2 μL of 100 mM Thiol-HaloTag Ligand (or 2 μL of Thiol-aptamer ligand), wrap in aluminum foil and rotate overnight at 4 °C.

7. Remove unreacted HaloTag Ligand using gel chromatography columns: Prepare 10 MBS6 columns by exchanging the buffer to PBS as described above in **step 5**. Place a maximum of 70 μL DNA-HaloTag Ligand (or DNA-aptamer) on each column. Spin at 1000 × *g* for 4 min and retain flow-through. Clean a total of three times.
8. Measure nucleic acid concentration of the biotin-DNA-HaloTag Ligand (or biotin-DNA-aptamer) using the NanoDrop. The NanoDrop nucleic acid setting estimates the DNA concentration by measuring the ratio of absorption by nucleic acids at 260/280 nm. Normal yields produce ~100 ng/μL.

3.9 HaloTag-Based Coupling of 3500 bp DNA to Multi-domain Substrate

Mix 3 μL of 20 μM substrate with 15 μL of ~150 ng/μL (~0.1 μM) HaloTag–3500 bp DNA–biotin in PBS (*see* **Note 3**). Wrap in aluminum foil to maintain in the dark. Incubate rotating overnight in a cold room or cold environment.

3.10 ATP Regeneration Solution at 20×

1. Prepare the following stock solutions in PD buffer and keep on ice: 300 mM ATP (pH to 7.6 with KOH), 3 mg/mL creatine phosphokinase, 1 M creatine phosphate.
2. Mix the following on ice: 633.4 μL PD, 200 μL creatine phosphokinase stock, 100 μL creatine phosphate stock, and 66.6 μL ATP stock.
3. Vortex and aliquot 40 μL volumes per tube.
4. Flash-freeze with liquid nitrogen and store at −80 °C. Aliquots are used within 3 months.

3.11 Chemical Etching of Glass Coverslips

This procedure was adapted from a protocol originally used in the Block Lab.

1. Dissolve ~100 g of KOH pellets in ~300 mL ethanol in a large 1 L beaker. This takes about 30 min.
2. Load teflon coverslip racks with glass coverslips.
3. Fill 2 or more beakers with ~300 mL ddH_2O and degas in a bath sonicator for 5 min.
4. Fill a beaker with ~300 mL ethanol and degas for 5 min.
5. Submerge one coverslip rack in the KOH/ethanol solution and sonicate for 5 min.
6. Wash the coverslips by dipping the rack up and down and spinning it in the ethanol beaker.
7. Wash the coverslips similarly in the ddH_2O beaker.
8. Submerge the coverslip rack in the second ddH_2O beaker and sonicate for 5 min.
9. Remove the coverslip rack and spritz each coverslip with a ddH_2O spray bottle, use lots of water.

10. Spritz the coverslips with an ethanol filled spray bottle. Spritz until the ethanol smoothly flows off the coverslip surface.
11. Repeat the steps with other racks of coverslips.
12. Place coverslips in an oven for ~15 min.
13. Store the racks in sealed plastic Nalgene containers.

3.12 Flow Cell Construction

1. Place two double sticky tape across the short axis of the glass slides with an approximately 5 mm space between them. This gap creates a volume between 10 and 15 μL.
2. Place an etched coverslip on top of the two pieces of tape. Align so that the long axis of the coverslip is perpendicular to the long axis of the slide.
3. Use a wooden Q-tip, the bottom of an Eppendorf tube, or a large pipette tip to gently press the cover slip and slide together to seal the tape. Start at one edge of the tape and work systematically in one direction to push bubbles out of the tape glass interface. The cloudy interface will become clearer when done properly.

3.13 Motility Assay and Wet Preparation

1. Prepare surface blocking buffer: make a 1 mg/mL casein solution in PBS, filter with 0.2 μM syringe filter and keep in a microtube on ice (*see* **Note 4**).
2. Prepare anti-clumping buffer: make a 5 mg/mL BSA solution in PD (typically 3-6 mL is made at this stage by weighing 15–30 mg of BSA), vortex to dissolve, filter with 0.2 μM syringe filter, transfer 1.5 mL of buffer to a 1.5 mL microtube and add 15 μL of DDT at 100 mM and keep on ice (*see* **Note 5**).
3. Introduce 0.5 ng/μL A08–3500 bp DNA–biotin into flow chamber and incubate for 30 min in a humidity chamber at room temperature (*see* **Note 6**). When adding solution to the flow cell for the first time, introduce adjacent to the cover glass centered on the opening of the channel and allow capillary action to fill the flow cell (*see* **Note 7**) (see also illustrations in Fig. 2).
4. Casein incubation: wash out unbound A08–DNA–biotin and coat the coverslip surface by flowing 100 μL of casein solution through the chamber. Incubate for 20 min at room temperature (*see* **Note 8**).
5. Bead solution preparation: bead solutions are prepared during the casein incubation. In two separate tubes, mix 180 μL PD with 3 μL of 1 μm and 1.26 μm streptavidin bead stock solution, respectively. Wash beads by spinning down at 8000 × *g* for 3 min. Remove supernatant and resuspend

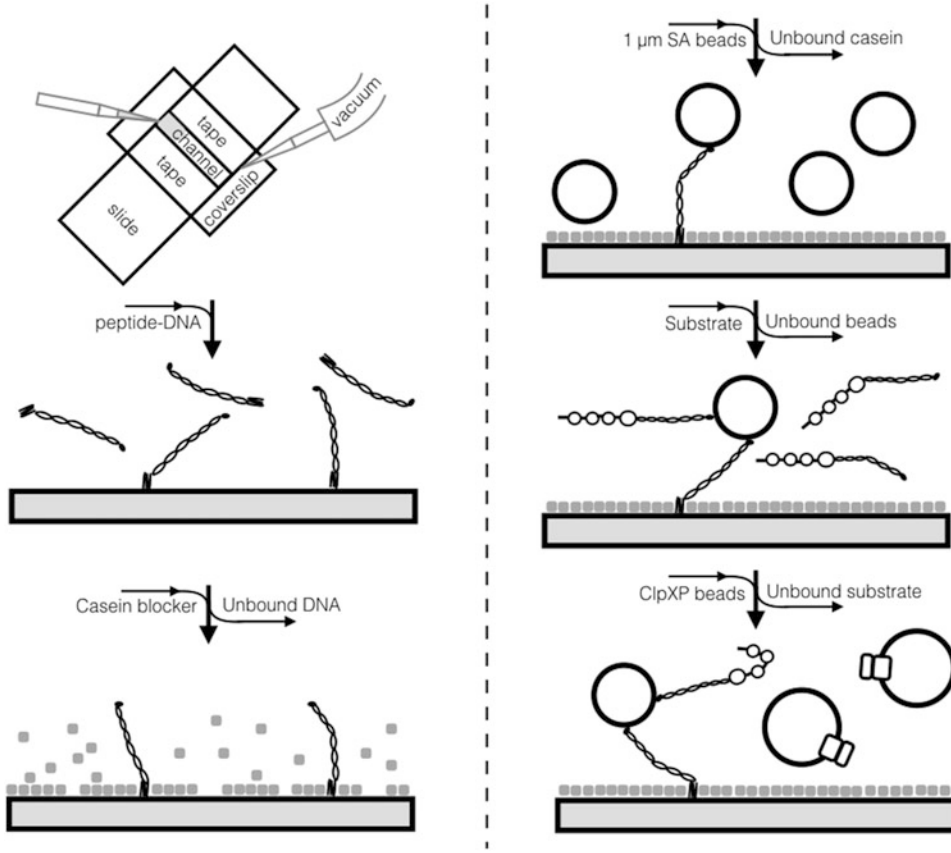

Fig. 2 Cartoon outlining ClpXP flow cell preparation. A solution (~10 μL) containing A08 peptide-DNA is added into a flow cell (*panel 1*) directly at the channel entry. The peptide-DNA is allowed to bind the glass coverslip (*panel 2*) by incubating in a humidity chamber. Casein blocking buffer is then introduced to coat the coverslip surface around the pre-bound peptide-DNA tethers (*panel 3*) to prevent nonspecific binding of assay components during upcoming steps. This step also removes unbound peptide-DNA. Streptavidin-coated beads (1 μm in diameter) are then flowed in and allowed to bind the biotinylated surface-tethered DNA strands (*panel 4*). Unbound beads are removed using a buffer wash, after which substrate–DNA–biotin constructs are flowed into the slide chamber (*panel 5*) to bind the surface tethered beads. Excess substrate-DNA is then washed out, and a final reaction mixture that includes ClpXP beads (1.26 μm in diameter) and ATP is flowed in (*panel 6*). At this point, the slide is sealed with vacuum grease and loaded onto the optical tweezers instrument for dumbbell assembly and measurement

thoroughly in 180 μL PD. After centrifugation a white pellet of beads will be clearly visible near the bottom of the microtube. Use a pipette to remove the supernatant without disturbing the pellet. Repeat for a total of three washes. The final resuspension is in 60 μL of BSA solution. Sonicate both bead solutions for 2 min at 40 % in a cup sonicator. Fill the cup sonicator with cold water, but not ice. After sonication, keep beads on ice.

6. Flow 20 μL of cleaned 1 μm streptavidin beads into flow chamber and incubate for 10 min. This chamber is cloudy like slightly diluted milk.
7. During the bead incubation, functionalize 1.26 μm beads with ClpXP. Mix 14 μL of 1.26 μm streptavidin beads solution, 5 μL of ~5–6 μM ClpX, 2.3 μL of 44.4 μM ClpP, and 6 μL of 10× ATP with regeneration system and 60 μL BSA solution. Allow protein to bind beads by incubating for 30 min at room temperature. To remove unbound ClpXP, spin down at 6000 × *g* for 1 min. Carefully remove supernatant as the pellet is very small/almost invisible. Look closely to make sure that pellet is not removed along with the supernatant and resuspend gently in 51 μL BSA solution, 6 μL of 20× ATP regeneration system, and 3 μL of 44.4 μM ClpP. Keep on ice.
8. Wash the flow cell with 100 μL of BSA solution to remove unbound beads. After this wash the flow cell is clear, not milky.
9. Mix 6 μL of substrate–DNA–biotin with 12 μL of BSA solution, flow in channel and incubate for 20 min at room temperature.
10. Wash channel with 100 μL BSA solution to remove unbound substrate-DNA-biotin.
11. Mix the following immediately before flowing into flow cell: 15 μL of 1.26 μm ClpXP functionalized beads, 8.1 μL of BSA solution, 6 μL of 20× ATP with regeneration system, 0.3 μL of 100× catalase, 0.3 μL of 100× glucose oxidase, and 0.3 μL of 100× glucose.
12. Flow into channel and seal chamber with vacuum grease. Start a timer for 1 h after which ATP regeneration system is no longer effective.

3.14 Motility Assay Measurement

1. Warm up instrument by turning lasers and other equipment on prior to the wet assay preparation. Load a dummy flow cell filled with water and adjust the power of the lasers to achieve the desired trap stiffnesses. Trap 2 is typically 3–5× stiffer than trap 1. Typically, powers are measured prior to the laser being reflected into the objective. For a force clamp of ~10 pN, our instrument requires ~220 mW for Trap 2 and 75 mW for Trap 1 measured before the microscope objective.
2. Load slide on microscope. Typically only the fine focus is adjusted as the day-to-day height is very consistent given the tight tolerances on coverslip and tape dimensions. A single drop of DF oil (Cargille) is used between the objective and cover glass. Between the microscope slide and condenser, the laser beams can be quite wide and thus generous ~3–4 drops of Type A (Nikon) oil is recommended.

3. Start LabVIEW programs including Trap 1 and Trap 2 calibration routines, voltage recording routines and a program to move the AOD controlled trap. Voltages are typically recorded at 3000 scans/s. Filtering is typically set to 1.5 kHz. Have these programs ready to go as open windows on the recording computer so that routines can be easily initiated.
4. Focus near the surface and survey fields of view to identify suitable locations with 1 μm substrate- and A08–DNA-tethered beads where performing the assay would be unimpeded by neighboring beads (see illustrations in Fig. 3).
5. Turn on Trap 1 and capture a 1.26 μm ClpXP coated bead and bring near the surface bound substrate beads.
6. Fish along the diagonal to achieve a connection by mechanically moving or tapping the stage position micrometers to see if there is inter-bead tension. When you see inter-bead tension, turn on Trap 2 to capture the 1 μm substrate-bound bead and displace the stage horizontally away from Trap 1 to break the connection between the 1 μm bead and surface. Defocus to drop the cover glass slightly below the suspended dumbbell so that the beads are clear of interacting with the surface.
7. Check the voltage recording for Trap 1 to verify that a single tether is connected between the beads. Single tethers will provide consistent voltages at these stages. Start recording Trap 1 and Trap 2 position voltages.
8. Adjust the MHz values of Trap 1 to move to the passive force clamp location along the diagonal. This is typically done in two or more stages so that the bead is not lost and kicked out of the trap by too drastic of a move. The passive force clamp region MHz values are 25.93/25.93 for our instrument, with conversions of approximately 3400 nm/MHz on each axis. Both vertical and horizontal AOD's are moved to maintain the dumbbell oriented along the diagonal.
9. Record the experiment as illustrated in Figs. 4 and 5. Be as quiet as possible and keep the room dark as light signals may be detected by the position sensing subsystem. By zooming the voltage window one may be able to directly watch small displacements, typically unfolding events, although usually data processing after the experiment is required to ensure the presence or lack of successful degradation activity for a specific dumbbell. Tether connections can break spontaneously or at the end of the motility record indicating the experiment is finished.
10. Calibrate beads at the end of each dumbbell. First move Trap 1 back to the 26.00/26.00 MHz location and calibrate Trap 1 by running the fifth order calibration routine for the

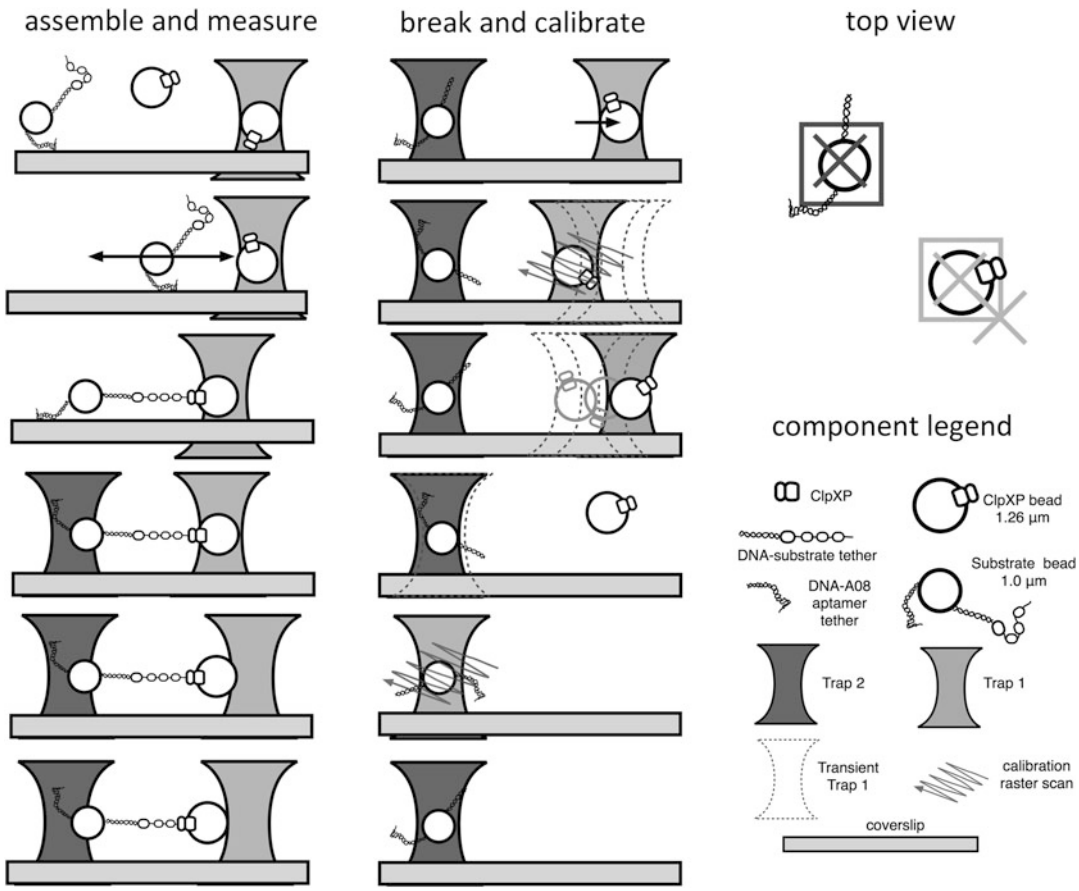

Fig. 3 Scenes depicting a 12-step process for active assembly, measurement and calibration of the ClpXP mechanical assay. Scenes are read from *top* to *bottom. Assemble and measure. Scene 1*: substrate tethered beads are attached to the cover glass surface through a DNA-aptamer tether. Slightly larger ClpXP labeled beads are free in solution, trapped with Trap 1 and brought in the vicinity of the substrate bead. *Scenes 2* and *3*: a fishing procedure is initiated by gently moving the ClpXP bead near the substrate bead until correlated motion is observed indicating recognition and attachment of the substrate. *Scene 4*: Trap 2 is then turned on and the sample translated away, diagonally in the *XY* plane, from the ClpXP bead as well as dropped in *Z* so that the aptamer-surface interaction breaks and a suspended dual bead assay is achieved. *Scene 5*: Trap 1 is then moved away from Trap 2 to achieve the passive force clamp location. This motion is performed in two or three moves so that the bead can be maintained stably in Trap 1 during the sudden trap displacements. *Scene 6*: the system is held in the passive force clamp geometry while recording bead position to observe motility until break. *Break and calibrate*. Upon break in *Scene 7*, Trap 1 is moved back to the measurement zone (*Scene 8*) and calibrated through a raster scan displacement to map position to voltage signals. Again, Trap 1 is moved in stages so that the bead can be held without release. *Scene 9*: Trap 1 moves the ClpXP bead away, near the passive force clamp location, so that it can be released at a location where Trap 2 will not capture it. *Scene 10*: Trap 1 is then moved, while off, to overlap with the Trap 2 location. *Scene 11* Trap 1 is turned on and Trap 2 is turned off. Trap 1 then raster scans the substrate bead to map the position calibration for the Trap 2 location. *Scene 12*: at the end of the raster scan, Trap 2 is then turned on and Trap 1 is turned off, so that the stiffness of Trap 2 with the substrate bead can be determined

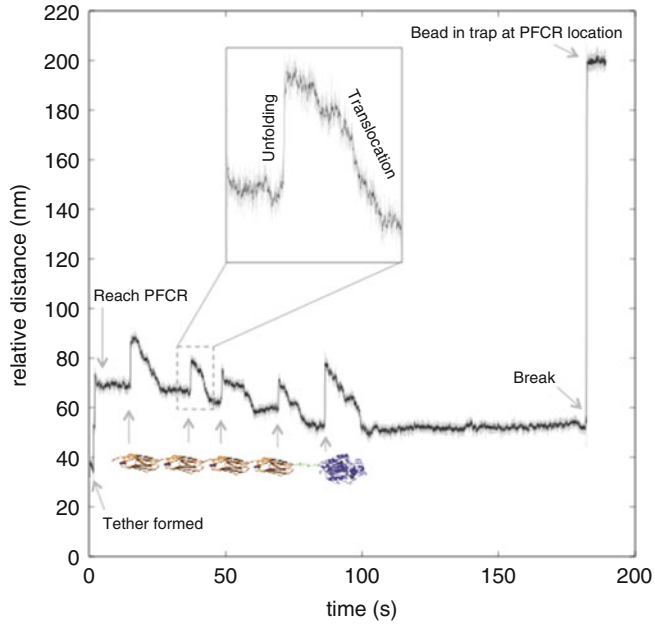

Fig. 4 Representative trace showing global bead position and motility record. Initially, position recoding is started within ~5 s of dumbbell formation. Then, the position of Trap 1 is moved away to place the bead in Trap 1 at the passive force clamp region (PFCR). Usually, 2–3 AOD steps are used to reach this trap location. Upon reaching the PFCR, position data is recorded until dumbbell rupture/break, which is evident by a large change in bead position as both beads return to the center of their respective trap positions. Before tether rupture, successful unfolding and translocation events (*inset*) results in small bead movements, which are often difficult to observe during acquisition, but are evident after data processing. Upon dumbbell rupture, continuous position recording is stopped and saved, and position/force calibration procedures are carried out

"Trap 1 location." After the Trap 1 calibration, move Trap 1 away from Trap 2 (such as by stepping past the force clamp region) and release the 1.26 μm bead about 1 μm farther away so that it does not fall into Trap 2. Turn off Trap 1 and move to overlap with Trap 2 (at 26.50/26.50 MHz). Turn on Trap 1 and turn off Trap 2, which should be holding the 1.0 μm substrate bead at the same location. Run the fifth order calibration routine for the "Trap 2 location." After the position scanning portion of the program, but before the stiffness determination, turn on Trap 2 and turn off Trap 1. This trick allows for mapping the position space for the "Trap 2 location" using the AOD controlled trap, while recording the stiffness of Trap 2 used in the experiment.

11. Two file sets of fifth order parameters, one for the Trap 1 location and one for the Trap 2 location are recorded along with two stiffness files and data streams for the position voltages for each trap.

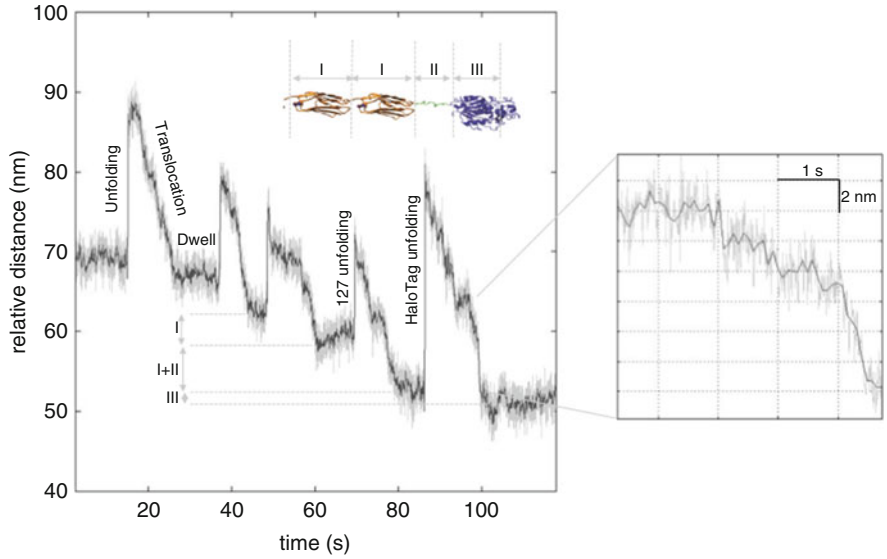

Fig. 5 Signature features of a single molecule degradation trace. For ClpXP mediated degradation of multidomain substrates, such as the Halo-V13P($\times$4)-ssrA substrate in this trace, tracking changes in inter-bead distances with time shows abrupt displacements in bead position directly followed by continuous slower decreases in distance. The abrupt increases in relative distance correspond to the unfolding of single domains. Depending on the domain being unfolded the magnitude of the distance change varies. For example, the first four unfolding events (~15 nm) in this trace correspond to V13P domains, while the fifth larger unfolding event (~25 nm) corresponds to the terminal HaloTag domain. Translocation of each domain directly follows unfolding and is evident by a stepwise decrease in distance. The steps during translocation range in size from ~1–4 nm (*inset*). Following translocation of a domain, there are periods of constant inter-bead distance, which correspond to unfolding dwells (the time necessary for ClpXP to achieve successful unfolding of the abutting domain). Consecutive dwell periods are spaced by the distance of a folded domain (I), the folded domain plus a linker (I + II), or the portion of HaloTag successfully unfolded (III)

3.15 Passive Force Clamp Determination

The passive force clamp makes use of the nonlinear stiffness at the extreme edge of an optical trap. Typically, linear ranges where "Hookean" type behavior is observed lie within ~100–150 nm of the trap center. At the edge of the trap, the force becomes weaker and the transition region represents a "constant force zone" as described by Greenleaf et al. [36]. Determining the passive force clamp location can be performed using a DNA tether or with a ClpX tether as shown in Fig. 6.

4 Notes

1. DNA gel electrophoresis is performed to ensure the PCR amplification reaction and purification yields the desired length and quality of DNA. Here, standard DNA gel electrophoresis methods are employed (Owl EasyCast B1 Mini Gel

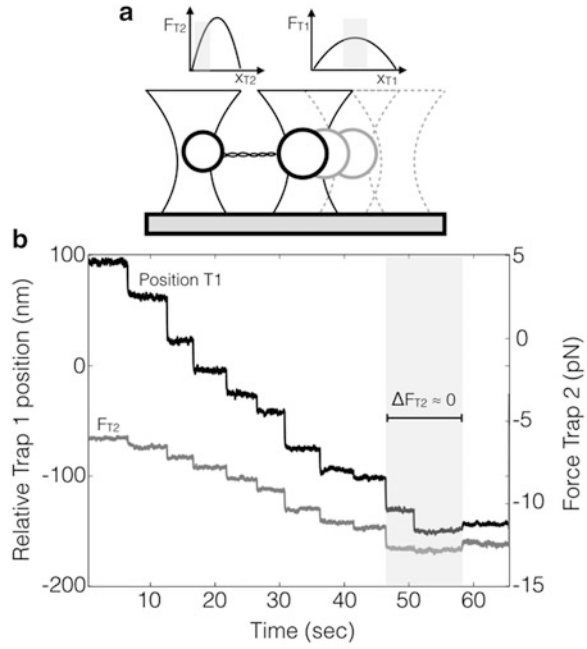

Fig. 6 Passive force clamp (PFC) tutorial. To determine the location of the PFCR, we use the same procedure described in this chapter to form dumbbells, except the Biotin–DNA–HaloTag–Substrate construct is replaced with a strand of Biotin–DNA–Digoxigenin and an Anti-digoxigenin-coated bead is used instead of the ClpXP-coated bead. Upon formation of the DNA dumbbell, Trap 1 is stepped away from Trap 2 (Panel **a**) using AOD movements. Since Trap 2 is three times stiffer than Trap 1, as Trap 1 is moved away, the bead in Trap 1 is in turn pulled out from the center of the trap. The Trap 1 position is continually stepped until the dumbbell connection breaks, or the bead in Trap 1 pulls out far enough so that Trap 2 swallows it. The position of the PFC is evident when viewing the position and forces plot for the test. The trace in Panel (**b**) shows that the force in Trap 2 changes with every AOD step taken in Trap 1, except at ~50 s where the force in Trap 2 remains nearly constant when an AOD step is taken in Trap 1. We note the number of AOD steps required to place the bead in Trap 1 in this region, and use this position during the ClpXP experiments

Electrophoresis System). An agarose gel is used (VWR EM-2120) with a dsDNA ladder mixture (Bayou biolabs L-201) and a SybrGreen at 10,000× (Molecular Probes S7563) to indicate the DNA bands. If the band is not nicely resolved, for example squiggly or smeared, it is possible the concentration of DNA in the well is too high. Titrate the concentration for better band resolution.

2. During the SMCC conjugation, primary amines must not be present. Samples in Tris buffer (TE) must undergo a buffer exchange: Invert/mix the MBS6 or MBS30 column, snap tip, and drain for 2 min in a 2 mL tube. Empty flow-through, spin at 1000 × *g* for 2 min, and discard flow-through. Load 500 μL PBS, spin 1000 × *g* for 1 min and discard flow-through. Repeat the PBS load and spin a total of three times.

3. Excess substrate is needed for sufficient labeling of the long DNA spacer. Excess substrate is removed during the dumbbell assembly process steps.
4. This solution is made fresh every day and used within 6 h.
5. This solution is made fresh everyday and used within 6 h, and BSA must always be stored at 4 °C.
6. Humidity chambers are formed using an empty pipette tip box. The bottom is filled with water. Flow cells are stored on the lid of the empty pipette tip holding manifold with the lid closed. The humidity chamber is vital in preventing evaporation of the flow cell contents during incubation periods in this assay. All incubations are at room temperature.
7. Normally 3–5 tethers per field of view are desired, so the A08–DNA concentration can be tuned to achieve the appropriate density.
8. A vacuum linked pipette tip is used to establish flow through the channel. Flow rates can be controlled more readily through such application of vacuum on the opposite side of a channel. Although liquid is initially introduced into an empty channel directly near the cover glass slide inlet, subsequent washes are introduced by dripping solution farther away from the inlet to help control flow rates. Flow rates are slow (typically 100 μL over 3 min) to avoid unbinding and shearing of surface bound flow cell contents such as A08-tethered beads.

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