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Force spectroscopy of single protein molecules using an atomic force microscope

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Corresponding Author:	Zack Scholl Duke University Durham, North Carolina UNITED STATES
Corresponding Author's Institution:	Duke University
Corresponding Author E-Mail:	zns@duke.edu;zack.scholl@gmail.com
Order of Authors:	Zack Scholl Qing Li Eric Josephs Dimitra Apostolidou Piotr E Marszalek
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Dr. Nandita Singh
Senior Science Editor
JoVE

Dear Dr. Singh,

Thank you for reviewing our invited manuscript entitled “Force spectroscopy of single protein molecules using an atomic force microscope.” We note that the authors are now listed as Zackary N. Scholl, Qing Li, Eric Josephs, Dmitra Apostolidou and Piotr E. Marszalek.

We have closely followed the editor’s comments to adapt our manuscript to the JoVE style - including adding abbreviations, more references, and increased usage of the imperative tense. Also, as suggested, we have clarified the language in the cases where our manuscript had significant overlap with our previous work (these areas are marked in red). Additionally, as suggested by both the editor and reviewers, we have also significantly expanded the Protein purification steps, namely the transformation and purification steps. Finally, as specified by the editor, we have added three new paragraphs to address the critical steps, limitations, applications and significance of this method.

While one reviewer said that our account of AFM was well written, we received several points of consideration to address from all reviewers. Reviewers 1 and 2 suggested making the protocols more general in terms of proteins and methods used. To this end, we have adopted more general terminology where appropriate and we have included references about the construction of our particular AFM setup to make our setup easier to replicate in other laboratories. We have also followed the reviewers concerns to include more information about the DNA sequences used and have introduced a new figure showing a plasmid map for a plasmid we have made available through AddGene that is readily used with our method. Following Reviewer 3’s suggestions we have also included more information on the analysis of AFM data.

We hope that these changes make our manuscript suitable for publication in *JoVE*.

Sincerely,

Zackary Scholl, Ph.D.

TITLE:

Force Spectroscopy of Single Protein Molecules Using an Atomic Force Microscope

AUTHORS AND AFFILIATIONS:

Zackary N. Scholl, Qing Li, Eric Josephs, Dimitra Apostolidou, Piotr E. Marszalek

Mechanical Engineering and Materials Science, Duke University, Durham, NC, USA

Corresponding Author:

Piotr E. Marszalek

pemar@duke.edu

Email Addresses of Co-authors:

Zackary N. Scholl (zns@duke.edu)

Qing Li (ql29@duke.edu)

Eric Josephs (ej20@duke.edu)

Dimitra Apostolidou (da150@duke.edu)

KEYWORDS:

Atomic force microscope, polyprotein, protein folding, single-molecule, force spectroscopy, protein purification

SUMMARY:

We describe the detailed procedures and strategies to measure the mechanical properties and mechanical unfolding pathways of single protein molecules using an atomic force microscope. We also show representative results as a reference for selection and justification of good single protein molecule recordings.

ABSTRACT:

The determination of the folding process of proteins from their amino acid sequence to their native 3D structure is an important problem in biology. Atomic force microscopy (AFM) can address this problem by enabling stretching and relaxation of single protein molecules, which gives direct evidence of specific unfolding and refolding characteristics. AFM-based single-molecule force-spectroscopy (AFM-SMFS) provides a means to consistently measure high-energy conformations in proteins that are not possible in traditional bulk (biochemical) measurements. Although numerous papers were published to show principles of AFM-SMFS, it is not easy to conduct SMFS experiments due to a lack of an exhaustively complete protocol. In this study, we briefly illustrate the principles of AFM and extensively detail the protocols, procedures, and data analysis as a guideline to achieve good results from SMFS experiments. We demonstrate representative SMFS results of single protein mechanical unfolding measurements and we provide troubleshooting strategies for some commonly encountered problems.

INTRODUCTION:

Advances in single molecule force spectroscopy (SMFS) by AFM have enabled mechanical manipulation and precise characterization of single protein molecules. This characterization has produced novel insights about protein mechanics^{1,2}, protein folding³, protein–ligand interactions⁴, protein-protein interactions⁵, and protein-based engineered materials^{6–8}. SMFS is especially useful for studying protein unfolding, as stretching by AFM allows the chemical and physical bonds within the protein molecule to gradually extend according to their stiffness, which gives rise to a continually increasing contour length. This overstretching of a protein molecule can produce an abrupt transition in the force-extension curve resulting in a rupture event (or force peak). The force peak gives direct information on the unfolding force and structural change of the protein during the mechanical unfolding process. One of the first studies using AFM measured titin¹ and found novel aspects of protein unfolding and refolding under physiological conditions without the use of unnatural denaturants like concentrated chemicals or extreme temperatures.

SMFS experiments are conducted on a variety of instruments, though here we consider only the AFM. The AFM is composed of four main elements: the probe, the detector, the sample holder, and the piezoelectric scanner. The probe is a sharp tip on the free-swinging end of a cantilever. After calibration, bending of the cantilever during stretching of an attached molecule is measured using a laser beam that is reflected off the back of the cantilever to precisely determine forces using Hooke's law. The reflected laser beam projects into a quadrant photodiode detector which produces a voltage in proportion to the displacement of the laser beam from the diode center. The substrate with the protein sample in fluid is mounted onto a 3D piezoelectric stage that can be controlled with sub-nanometer precision. A computer reads the voltage from the photodiode detectors and controls the 3D stage through a computer-controlled voltage supply. These piezo actuator stages are usually equipped with capacitive or strain-gauge position sensors to precisely measure piezo displacement and to correct hysteresis through feed-back control system. The sensor signal output from the piezo controller is converted into distance using the voltage constant of the piezo that is factory-calibrated. An example force-extension curve from a pulling experiment is shown in **Figure 2**.

There are two types of AFM-SMFS experiments: constant velocity and constant force pulling measurements. Constant force SMFS measurements are described in Oberhauser *et al.*⁹, while here we focus on constant velocity measurements. A typical AFM constant-velocity pulling experiment is done by providing voltage to a piezo to gently move a substrate relative to a cantilever tip. A typical experiment has the tip initially pressing against the surface. The pulling measurement is begun by moving the substrate away from the tip to bring out of contact. If a protein comes into contact with the tip initially, it will be pulled and the unfolding trace of force against displacement will be measured. The substrate is then brought back into contact with the tip and a relaxing trace is measured where protein folding can be determined from the force displacement.

PROTOCOL:

1. Protein Preparation

1.1. DNA cloning.

1.1.1. Synthesize a DNA sequence of interest, for example, the DNA sequence of NI₁₀C¹⁰, or isolate *via* PCR from the host organism using standard molecular biology techniques¹¹. Flank the gene of interest with restriction sites during synthesis or by placing sites in the 5'-end of the PCR primers to correspond to a module in the plasmid pEMI91 (Addgene #74888)¹².

1.1.2. Separately digest both the plasmid pEMI91¹² and the DNA sequence of interest with a pair of restriction sites so that the sequence of interest will be flanked by the tandem I91 repeats (see **Figure 1**). Follow the standard protocol for the restriction sites.

1.1.3. Purify the digested product using gel electrophoresis and then ligate the products using T4 DNA ligase, following a standard protocol. After ligation, transform the plasmid into *E. coli* cells for plasmid purification and purify the plasmid using standard protocols. Sequence using T7 primers or internal pEMI91 primers to verify that the sequence was successfully transformed.

1.2. Transformation.

1.2.1. Remove protein expression cells, such as C41 (DE3) pLysS cells, from a -80 °C freezer and thaw completely on ice.

1.2.2. Add 1 µL of plasmid DNA to the cells and stir briefly; pipetting up and down is not advisable as it will introduce air bubbles and warm the cells.

1.2.3. Incubate the culture tube containing the cells and the plasmid on ice for 30 min.

1.2.4. Heat shock the cells in a water bath at 42 °C for 45 s.

1.2.5. Return the tube to ice for 2 min.

1.2.6. Place 950 µL of LB broth in the cells and shake at 250 rpm for 1 h at 37 °C.

1.2.7. Place about 200 µL of the transformation onto LB plates containing 100 µg/mL Ampicillin. If using a plasmid other than pEMI91, then use the appropriate antibiotic for that plasmid. Place plate overnight in an incubator at 37 °C. The next day, remove plate from incubator, wrap with parafilm, and store refrigerated up to 1 month.

1.3. Protein expression.

1.3.1. Inoculate 15 mL of LB broth medium with 100 µg/mL ampicillin in a 50 mL tube at 37 °C for overnight growth by touching a sterile pipette tip to a single bacterial colony on the plate and pipetting up and down in the LB.

1.3.2. Transfer the 15 mL culture into 1 L of LB broth medium with 100 µg/mL ampicillin and shake for 4-12 h at 37 °C (until OD₆₀₀ > 0.8).

1.3.3. Collect 10 mL of the culture for plasmid preparation.

1.3.4. Add 0.2-1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and lower the temperature to room temperature for overnight expression.

1.3.5. Harvest cells the next day by splitting into four tubes and centrifuging at 4,000 × g for 40 min, and then freeze the pellet at -80 °C for several hours.

1.3.6. Send the plasmids extracted from the cells for sequencing with the primers corresponding to the inserted cassette of the protein¹² and verify the fidelity of the inserted DNA.

1.4. Protein purification.

1.4.1. Thaw one tube of frozen cells for 30 min at room temperature.

1.4.2. Suspend thawed cells in lysis buffer (38 mL of water, 2 mL of glycerol, 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM CaCl₂, 10 µg/mL DNase, 1 mM PMSF, 1 mM TCEP, 500 µg/mL lysozyme) and shake on ice for 1 h.

1.4.3. Freeze the lysed cells at -80 °C for several hours and then re-thaw at room temperature.

1.4.4. Spin the lysate down at 13,100 × g for 30 min at 4 °C.

1.4.5. Run the supernatant through a gravity flow column for the specific tag (*e.g.*, Strep-tag or His-tag).

1.4.6. Perform a buffer exchange using a centrifugal filter using the appropriate buffer for Strep-tag or His-tag and store at 4 °C before use.

2. Slides Preparation for Sample Preparation

2.1. Prepare glass slides using Piranha solution.

2.1.1. Place 10-30 pieces of round glass cover slips (radius of 7.5 mm) into a 40 mL glass beaker.

Note: Perform this step and following steps in a hood. Follow standard procedures for dealing with dangerous chemicals during this step.

2.1.2. Add 30 mL of concentrated (18.4 M) sulfuric acid.

2.1.3. Add 10 mL of 30% hydrogen peroxide.

2.1.4. Heat the mixture for 10-30 min to 95 °C.

2.1.5. Carefully decant the Piranha solution into a separate waste container (to be reused or discarded).

2.1.6. Rinse the slides with deionized water to remove Piranha solution.

2.1.7. Suspend the slides in 40 mL of acetone.

2.1.8. Decant acetone into a waste container and re-suspend slides in 40 mL of ethanol.

2.1.9. Use clean forceps to carefully extract one slide at a time and dry with argon or purified air.

2.1.10. Place clean and dried slides under vacuum until the gold evaporation, or under argon for long-term storage.

2.2. Prepare gold coated slides (optional).

Note: This step requires the use of a vacuum (e-beam) evaporator, which is available in many clean room facilities at major universities.

2.2.1. Cut five microscope slides (25 mm x 75 mm rectangular shape) in half using a glasscutter.

2.2.2. Apply double-sided adhesive tape across the middle of each of the 10 half microscope slides.

2.2.3. Cut the sticky side of a sticky note and apply to the double-sided tape so that the sticky side of the note is face-up. The sticky note is generally less adhesive, but still firmly attaches to slides to prevent future breaking. Then those half microscope slides can now work as glass slides holder.

2.2.4. Carefully press four clean glass slides (from section 2.1.10) to each corner of the sticky side of the holder.

2.2.5. Move the glass slides to an e-beam evaporator. Follow the specifications of the evaporator to apply 70 nm of chromium and 300 nm of gold to the surface.

2.2.6. Store the gold-coated slides under argon.

3. Sample Preparation

3.1. Prepare slide.

3.1.1. Select a clean iron disk (15 mm diameter) and attach an adhesive tab to it.

3.1.2. Unattach the cover piece of the adhesive tab.

3.1.3. Select a piece of clean glass (from section 2.1.10) or gold (from section 2.2.7) and firmly place it onto the sticky side of the iron disk, this will be the sample slide.

3.2. Deposit protein onto slide.

3.2.1. Dialyze the polyprotein into the buffer for the experiment (25 mM Tris-HCl, pH 7.6 with 150 mM NaCl) using a buffer exchange column such as a 0.5-mL centrifugal filter. Spin the protein in the column for 10 min at 13,000 rpm and then reverse the column and elute the protein into a new buffer by spinning at 1,000 rpm for 2 min.

3.2.2. Determine the approximate protein concentration by measuring the absorbance at 280 nm, using a spectrophotometer.

3.2.3. Dilute the protein to 10-100 $\mu\text{g}/\text{mL}$ in a final volume of 100 μL .

3.2.4. Apply the 100 μL of protein solution onto the center of the slide. Be careful at this stage not to let any liquid enter beneath into the gap between the glass and the iron slide, as this can cause swelling of the adhesive during the experiment and uncontrolled sample movements.

Note: Gold slides are hydrophobic, and a spherical droplet will form, while glass slides are more hydrophilic and the protein solution may spread.

3.2.5. Let the sample sit at room temperature for 10-60 min. During this time, proceed to the next step to begin setting up the atomic force microscope.

4. Atomic Force Microscope (AFM) Setup

Note: The following is a general description for setting up the AFM, and some specific details may differ depending on the specific instrumentation used. The instrumentation used is partially home-built and described in detail in Scholl¹³.

255
256 4.1. Mount the cantilever onto an AFM cell.
257

258 4.1.1. Choose the cantilever with appropriate properties for the application. Use cantilevers
259 with spring constants 4-10 pN/nm for low unfolding forces (~10-50 pN), while use cantilevers
260 with spring constants 15-100 pN/nm for high unfolding forces.
261

262 4.1.2. Carefully pick up the cantilever from the end and place it into the probe holding cell.
263

264 4.1.3. Make sure the holding cell firmly holds the cantilever in place before continuing.
265

266 4.1.4. Place the holding cell into the AFM head for alignment of the laser.
267

268 4.2. Align the laser in the AFM head.
269

270 4.2.1. Place the head onto an inverted microscope and connect a battery pack to the AFM head
271 for powering the laser in the head.
272

273 4.2.2. Attach a camera to the microscope detector so that the laser light can be visualized onto
274 a TV or monitor.
275

276 4.2.3. Position the laser so that it is located onto the tip of the cantilever
277

278 4.3. Mount head with sample.
279

280 4.3.1. Flush 10 μ L of buffer into each of the ports of the probe holding cell.
281

282 4.3.2. Take the sample slide which has been incubating and decant 60 μ L of fluid from the
283 incubated solution. Place the sample slide onto the magnet above the piezo.
284

285 4.3.3. Make sure that the AFM stage is in the elevated position. Then place the AFM head onto
286 the stage so that the AFM cantilever is above the sample droplet.
287

288 4.4. Center the reflected laser beam onto the photodiode.
289

290 4.4.1. Cut a small piece of paper and place it in front of the AFM photodiode.
291

292 4.4.2. Reposition the AFM laser using the knobs so that the laser spot on the paper becomes
293 focused and bright.
294

295 4.4.3. Adjust the AFM head mirror so that the laser hits the photodiode to maximize the total
296 signal in all quadrants ($A+B+C+D$) and causes the difference signal between top two and bottom
297 two quadrants to be zero ($A+B-C-D$).

5. Atomic Force Microscope Calibration

5.1. Measure the power spectrum.

5.1.1. On the filter connected to the AFM, turn the filter setting for the AFM head signal to full bandwidth.

5.1.2. On the AFM itself, make sure that the piezo is off as this will add noise to the signal.

5.1.3. Use the AFM software to measure the average of 512 calculations of the power spectrum from 1024 data points per calculation.

5.1.4. Use the AFM software to integrate the power spectral density across the first peak, which corresponds to the main mode of vibration for the cantilever.

5.2. Calculate photodiode sensitivity.

5.2.1. On the AFM itself, turn the piezo controller on and change the filter setting to 500 Hz (low-pass filter).

5.2.2. Monitor the difference signal which should be fluctuating around zero in the AFM software. Swiftly move the AFM head down a few hundred micrometers using micropositioners. Repeat moving the head down and monitor the difference signal for consistent jumps. The surface is very nearby when the signal jumps start increasing in height.

5.2.3. As soon as the deflection signal saturates upon contact with the surface, move the head away from the surface slightly.

5.2.4. In the AFM software, use the input controls to regulate the piezo voltage to find the surface, by moving up or down 100-5000 nm. If the surface is still not in reach, continue to reduce the distance between the head and the sample manually.

5.2.5. In the AFM software, conduct a pulling experiment with a scan size of about 500 nm so that the tip comes in contact for about 100 nm of piezo travel.

5.2.6. Measure the slope of the linear region of the photodiode signal *versus* piezo displacement curve where the AFM tip remains in contact with the substrate surface.

5.2.7. Calculate the spring constant and sensitivity using the slope and the integrated power

spectrum ($k_c = \frac{k_B T}{\langle \delta V^2 \rangle * slope^2}$).

6. Data Acquisition

6.1. Preparation.

6.1.1. On the filters connected to the AFM, set the filter setting so that the sampling frequency is at least twice the bandwidth (Nyquist criterion), which will give an upper bound for the low-pass cutoff.

6.2. Measurements.

6.2.1. Set the scan size to the total theoretical size of the unfolding polypeptide (number of amino acids \times 0.365) plus about 40% to allow for pressing against the substrate. For example, pulling a 9x I91 construct will have a theoretical unfolded length of about 300 nm, so the scan size should be about 420 nm.

6.2.2. In the AFM software, position the cantilever so that it is 80% of the scan-size away from the surface (in this case, about 340 nm away from the surface).

6.2.3. In the AFM software, set the scan speed to 300 nm/s initially.

Note: This speed can be modified to be slower, or faster, depending on the application.

6.2.4. Perform a pulling experiment and measure the resulting position of the piezo and the photodiode signal. Use the AFM software to initiate the scan.

6.2.5. In the AFM software, continue to perform measurements until there are about 10,000 recordings.

Note: Generally, the pickup rate of a positive-controlled molecule is around 0.5%¹⁴, so 10,000 are necessary to be able to collect enough data to analyze.

6.3. Automation.

6.3.1. Determine a threshold force level so the process can be automated by adjusting the Z piezo position so that it sits within a threshold force level¹⁴. After each pull, the X piezo position should move, until it reaches the end at which it should move in Y once and then reverse position in X (raster scan).

6.3.2. In the software, save recordings that have a highest registered force above the baseline of at least 50 pN.

Note: Smaller force thresholds are needed if the entire polypeptide is expected to be lower force or it is unknown.

7. Data Analysis

7.1. Normalize data.

7.1.1. For each recording, compute the extension using $\text{extension} = \text{displacement} - F/kc$ (kc is from 5.2.7).

7.1.2. Determine the baseline force level by taking the average of either the beginning or end of the force-extension trace, where no force peaks are present and where the cantilever is not pressing against the surface. The entire force-extension curve can then be shifted by this mean value.

7.1.3. Translate the data so that the extension is aligned along the zero y-axis. This is because the molecule should be set to zero extension at the beginning of a trace.

7.2. Identifying protein of interest.

7.2.1. Identify recordings with at least four I91 events that provide a single-molecule fingerprint. These recordings capture true “single-molecule measurements”.

7.2.2. Save events that are denoted single-molecule events for further analysis.

7.3. Determine contour-length increment.

7.3.1. For each recording, fit a worm-like chain model to unfolding event, $F = \frac{k_b T}{p} \left(\frac{1}{4(1-x/L)^2} - \frac{1}{4} + \frac{x}{L} \right)$ where $k_b T = 4.1 \text{ pN nm}$ at room temperature, p is the persistence length (typically 0.4 to 1 nm), x is the extension in nanometers, and L is the contour length in nanometers.

Note: Form of the worm-like chain is an interpolated solution to the exact, a more exact numerical solution is found in Bouchiat *et al.*¹⁵. Alternate fitting models can be found in Su *et al.*¹⁶.

7.3.2. Calculate the difference between the values for L determined for two consecutive unfolding events, and this difference is coined the “contour-length increment”.

7.4. Determine rupture force.

7.4.1. Calculate the rupture force for a given unfolding event by taking the highest point before the biggest drop in the unfolding curve.

REPRESENTATIVE RESULTS:

Representative results from this protocol are shown in **Figure 2**. Both panels show representative force-extension curves from proteins. The top shows results from a I91 polyprotein, while the bottom shows the I91 protein flanking a protein-of-interest, the NI₁₀C molecule. These recordings show the characteristic force of I91 (200 pN) and contour length increment (28 nm) which indicates that the alignment and calibration of the AFM was successful. These force-extension curves can then be analyzed by worm-like chains (dashed line) which help to determine the force-independent length of the molecule and determine the number of unfolded residues. Once analyzed, the contour-length increments (difference between subsequent contour-lengths) and the unfolding force can be used to determine the protein stability, unfolding rate, and unfolding pathway²⁰.

FIGURE LEGENDS:

Figure 1: Plasmid map of polyprotein. This polyprotein plasmid map sequence and physical DNA is available through the Addgene repository (www.addgene.org/74888). It shows the prototypical polyprotein design for AFM, where a polyprotein is composed of 8 identical repeats of I91 protein (gray boxes) which flank a protein of interest (red). Each module contains a unique restriction site which allows customization.

Figure 2: Representative SMFS results. A. Representative force-extension curve for poly I91 protein. Mean contour length increment between peaks is ~28 nm, and unfolding forces are between 100-200 pN. **B.** Representative force-extension curve for (I91)₃-NI₁₀C-(I91)₃ protein. Mean contour length increment for ankyrin repeat is ~10.5 nm, and unfolding forces are between 8-25 pN. The regular saw-tooth pattern for ankyrin repeats is followed by I91 unfolding peaks. The pulling speed for both (A) and (B) are 0.02 nm/ms.

DISCUSSION:

A critical step in the protocol is the use of a polyprotein, described in step 1.1.2, which serves as a positive control to “fingerprint” single-molecule events. Generally, there must be unfolding events of the polyprotein proteins (for I91, this means an unfolding force of about 200 pN and contour length increment of about 28 nm) to unambiguously conclude that the protein of interest has been unfolded. For example, when the protein of interest is flanked by three I91 domains from either side, then there must be at least four I91 events to conclude that it is positively a single-molecule event. If there is not a positive control through a polyprotein, or other means, then any data is liable to misinterpretation.

The cloning strategy for the plasmid depends on the gene and plasmid of interest. We have made available a polyprotein plasmid which can be used in conjunction with this protocol¹². There are plasmids available for this step that contain unique restriction sites for simple cloning^{17,18}. These plasmids only require a unique set of restriction sites to easily clone in the protein of interest into the plasmid. There are also methods available to create this plasmid from scratch using Gibson assembly¹⁹ which has the advantage of easily modifying the flanking proteins while inserting the

protein of interest. There are also plasmids with modular polyproteins with codon shuffled domains that provide a simple way to efficiently sequence the entire protein of interest, aiding in ensuring the fidelity¹⁸.

The choice of gold or glass depends on whether the protein has specific attachment available. Gold substrates allow for forming Au-Cys bonds between the polyprotein and the surface. If a Cys is appended to the end of the polyprotein, then this can serve as a way to specifically attach the protein to the surface. Glass substrates can be used by themselves, which are useful for some proteins which do not adsorb well to gold and are also simpler to generate. Glass slides can also be used as a precursor to using specific attachment with specially made polyproteins²¹. Mica substrates can also be used, which may be useful for some proteins which have specific charge sites that can bind to the negatively charged surface.

AFM spectroscopy on proteins has several important limitations. Sample preparation may fail if the protein cannot be spliced into a polyprotein. Proteins that are too small to produce measurable contour-length increments or too weak to resist force will also not be able to be resolved by AFM. Typical resolution in AFM experiments only allows resolving forces as low as 10-15 pN due to the typical RMS noise in AFM cantilevers, although recent advances have made available better resolutions with advanced instrumentation²². The speed for constant velocity experiments is also limited to about 4 nm/s²³, and the best resolution achieved can typically detect states shorter than 10 μ s²². If proteins are less mechanically stable, they are better characterized using optical tweezers which has a lower force range and typically a higher temporal resolution. There are still improvements in the future available to AFM, like combining with FRET²⁴, using AFM imaging to determine protein positions²⁵ and increasing resolution with specially designed cantilevers²⁶.

The AFM technique is significant with respect to existing methods because it allows extracting kinetic parameters, like unfolding rate and transition state distance on a single molecule level²⁷. While other traditional biochemical methods (NMR, chemical denaturation, stopped-flow fluorescence) can also obtain information on kinetic parameters, the results of these methods are functions of a protein ensemble and not a single molecule. This makes an important difference in cases where there are different and distinct ensembles of a single protein. AFM has shown to be able to distinguish the subpopulations, instead of averaging them together²⁸.

The detailed explanation of the SMFS experiment on protein molecules above still does not anticipate possible problems that can be solved with experience. In the following we discuss common problems and troubleshooting associated with operating an AFM for constant-velocity experiments.

Sinusoidal-shaped force baseline. The sinusoidal shape is detrimental to determine correct force peak value since it is difficult to calculate the baseline. The problem is caused by interference of the laser reflected from the cantilever. To alleviate this problem, the position of the laser spot on the cantilever can be slightly shifted as long as the new position still retains a good sum signal

from the photodiode. If the problem still persists, consider repositioning the cantilever in the probe holding cell.

Photodiode signal does not saturate in one-step when touching the surface the first time. When touching the surface, the photodiode signal jumps in several steps until it reaches the saturated value. This means that the tip is not the lowest point in the AFM head and some other place on the probe chip may touch the sample surface prior to the tip of the cantilever. This problem has to be fixed in order to continue the SMFS measurements and get correct force-extension curve. To fix the problem, try moving the position of the cantilever in the probe holding cell back and forth and adjust the tightness of the hook which grasps the cantilever in place. If this does not help, try switching to a different cantilever on the same chip or even change to a new cantilever chip.

Many events upon retraction, or no events at all. This is related to finding the correct protein concentration for the experiment. If there's always a single big force peak (> 500 pN) appearing on the stretching trace and no other peaks are displayed while conducting experiments on gold coated surface, the cantilever is actually measuring gold-gold interaction since the sample molecules on the surface are too sparse. In this case, the concentration of the protein sample deposited on the gold surface should be increased. However, if there are multiple irregularly shaped peaks appearing on every stretching trace of each pulling cycle, it indicates that there are too many molecules absorbed to the surface that formed a layer or a complicated network on the surface. In this case, the sample needs to be washed by the buffer used to remove excess protein molecules. Sometimes preparing a new sample with lower concentration of protein is necessary to get rid of this problem. If the new sample also cannot solve the problem, check the frosted annular groove on the AFM holding cell to see if any liquid penetrated into it. The groove should stay dry during the experiment since any liquid in the groove would couple the vibration of the sample into the holding cell and cause the photodiode signal to change abnormally when the cantilever and the surface are in touch.

Spurious modulation of photodiode signal, between ramps. This is indicative of flow that can push the cantilever back and forth, which is usually caused by a small bubble that is transiting through the fluid channels. This type of problem will disrupt measurements with huge signal increases or decreases. To solve this problem, raise the head from the substrate, so that the fluid around the cantilever no longer touches the fluid around the substrate. Then bring them back together, and move the head back down to the surface. The reformation of the fluid column in the AFM cell is usually enough to disrupt the bubble from the fluid channels, but if not, repeating is necessary.

Systematic increase or decrease in photodiode signal. Drifting is a phenomenon that is present throughout each experiment, where the cantilevers and the tip will always drift very fast upon touching the liquid drop on the sample surface. Drift can be caused by temperature equilibration, in which the temperature-induced expansion or contraction of the cantilever causes vibration in the signal. Waiting for more than 10 min before conducting the first experiment will significantly

decrease drifting speed. When conducting pulling experiments, if the stretching and relaxing trace do not coincide with each other, then there's a hysteresis between the two halves of the pulling cycle, which corresponds to a fast drifting rate and requires additional time to reach equilibrium. If the force baseline tilts upwards with a small angle, the drifting speed is faster than the pulling speed and it's better to suspend the experiment to wait for less vibration of the tip.

Force-extension curve initial region is not vertical. It is useful to look at the force-extension plot of recordings after they have been acquired from a newly calibrated setup. If these recordings do not show a vertical region when the cantilever presses against the surface, then the calibration may be an issue and it should be repeated from step 5.

Photodiode signal disappears after some time. This could be due to the evaporation of the buffer in the fluid cell. It is important to rehydrate the cell by applying more buffer every hour or so, in order to keep the sample from drying out and making it unusable for further experiments.

Intrinsic noise is high (> 30 pN). A large amount of noise (measured as the RMS of the calibrated photodiode fluctuations) is indicative of external noise. AFM experiments should be done on an air table to reduce the amount of coupling to the building, but external noise can also be caused by insecure stage, auditory noise nearby, or coupling of the air table to nearby static objects (*e.g.*, a chair). The noise may also come from the electronic controlling system of the AFM (*e.g.*, unstable connection in the electronic system).

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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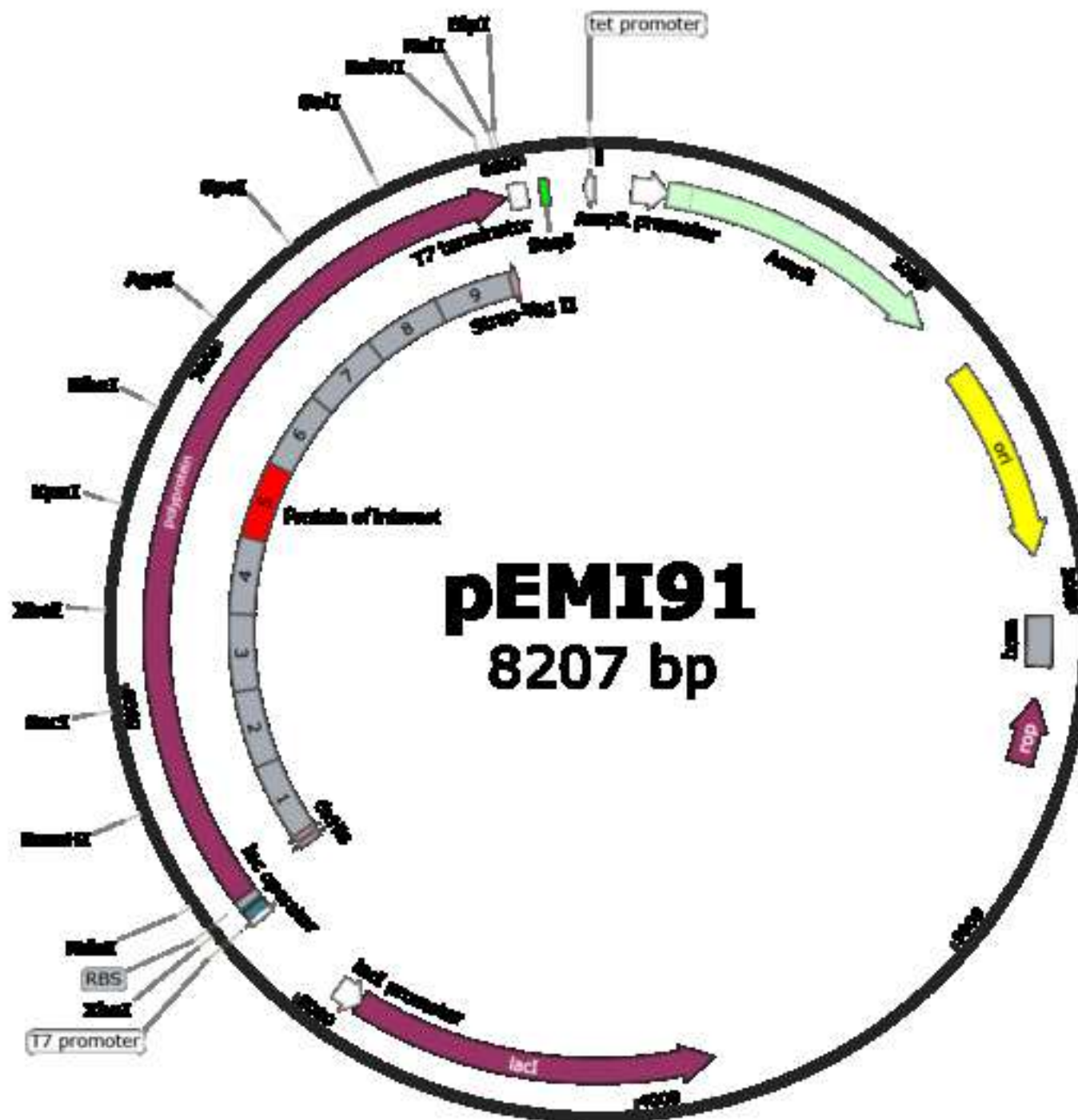
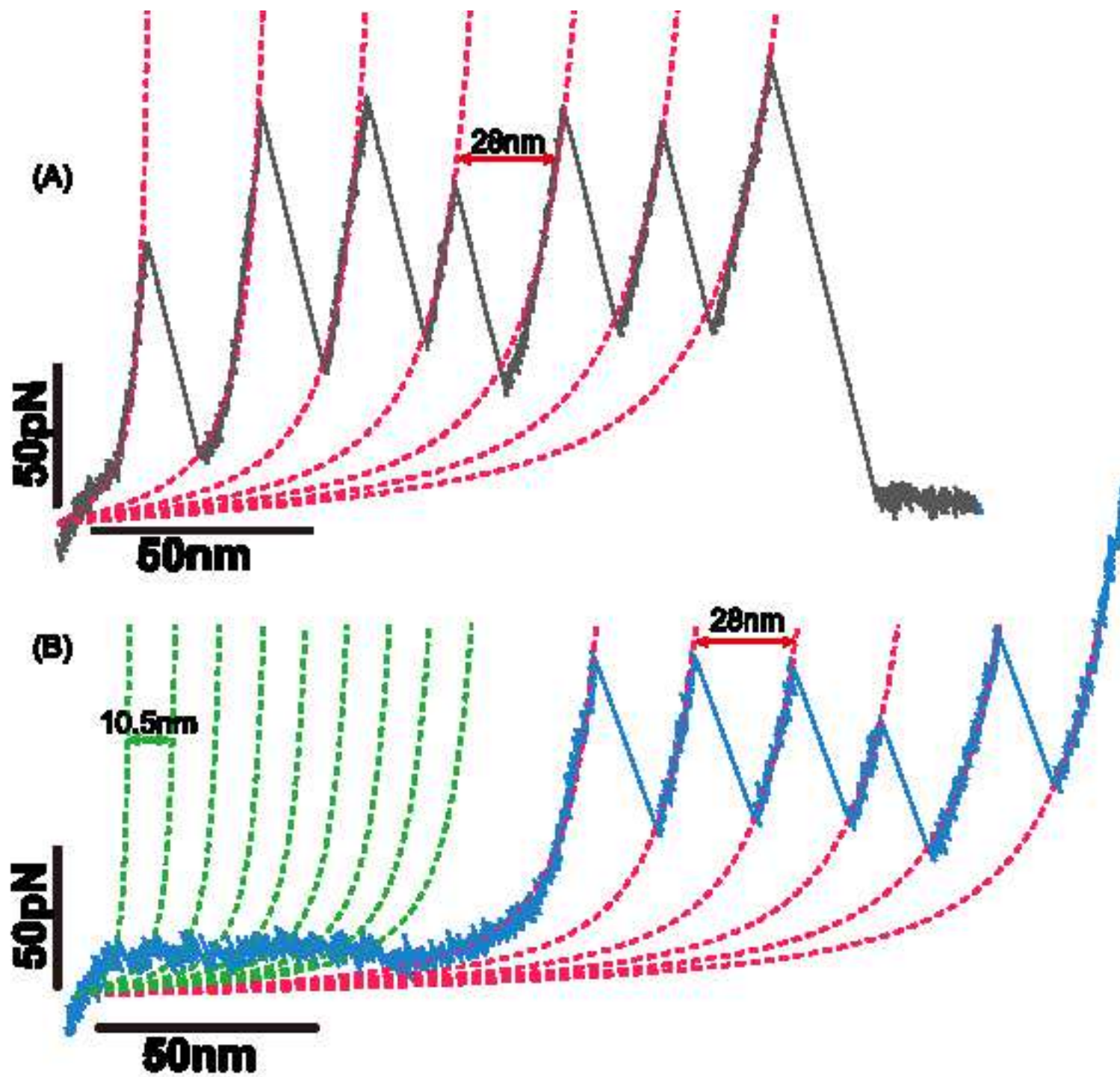


Figure 2



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
AFM Specimen Discs, 15mm diameter	Ted Pella, Inc.	16218	Serve as base for glass substrate
Round Glass Coverslips, 15mm diameter No.1 Thick	Ted Pella, Inc.	26024	serve as glass substrate and base for gold coating
Adhesive Tabs	Ted Pella, Inc.	16079	Paste on AFM Specimen Discs to provide a sticky face for al
STD Multimode head assembly	Bruker Nano Inc.	1B75C	AFM head
Glass probe holder	Bruker Nano Inc.	MTFML-V2	Glass probe holder for scanning in fluid with the MultiMod
Microlever AFM probes	Bruker Nano Inc.	MLCT	Silicon Nitride cantilevers with Silicon Nitride tips, ideal for
AFM probes with Au coated tips	Bruker Nano Inc.	OBL-10	Cantilevers for pulling on proteins with low unfolding force
Multifunction Data Acquisition (DAQ) Card,16-Bit, 1 MS/s (Multichannel), 1.25 MS/s (1-Channel), 32 Analog Inputs	National Instruments	PCI-6259	Data Acquisition for signals from AFM head and Piezo Actu
LISA Linear Piezo Stage Actuators	LP Physik Instrumente	P-753.11C	Piezo Actuator to control the position of substrate and peri
XY Piezo Stage	LP	P-541.2CD	Piezo Actuator to control the position of substrate and scar

Attaching glass coverslips

AFM.

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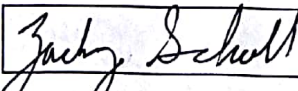
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Name:	Zackary Scholl	
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We thank the editor for considering our manuscript and have appropriately addressed all points raised.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have thoroughly proofread the manuscript.

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We have thoroughly defined all abbreviations.

6. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Please rewrite the text to avoid this overlap. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. These sections are highlighted with red text in the attached revised manuscript.

We have modified these sections to use original language.

7. Please include a space between all numbers and their corresponding figures: Figure 1, etc.

We have modified all the corresponding text.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have clarified the text.

9. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

We have followed this suggestion.

10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have expanded respective sections and added references where appropriate.

11. What is the DNA sequence of interest? We need specific examples in order to film.

We have specified “NI₁₀C” as a example DNA sequence of interest. This matches the example shown in Figure 1.

12. [1.1.1.](#): How? Citations?

Isolating the DNA sequence is done using standard molecular biology techniques. They are described many places, so we have included a reference to the most authoritative source for this technique.

13. 1.2.1: How is the transformation done?

We have thoroughly expanded this section to include information on how the transformation is done.

14. 1.4.1: What is the composition of the lysis buffer? Please provide all concentrations and volumes used throughout.

We added the concentrations and volumes for the entire composition of the lysis buffer.

15. 2.1.3: What concentration of sulfuric acid?

The sulfuric acid is concentrated. We added this clarification.

16. 2.14: Heat to what temperature?

It should be heated to 95°C, we have updated this in the manuscript.

17. 3.2.1: Dialyze how? What solution is used? What membrane is used and for how long?

We have clarified the dialysis method and the solution used.

18. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted about 2.25 pages of the Protocol which are most important and specific to our method.

19. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We have checked this.

20. For the AFM, please specify what is done manually and what is done in software. If done in software, please provide all user input commands: File | Save | etc.

We have clarified which parts of the protocol pertain to the AFM and which parts pertain to the AFM software.

21. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

We have added a paragraph into the Discussion to explain the representative results.

22. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol**
- b) Any modifications and troubleshooting of the technique**
- c) Any limitations of the technique**
- d) The significance with respect to existing methods**
- e) Any future applications of the technique**

Currently, there is mainly just troubleshooting steps of the protocol

We apologize for the gap in our manuscript. We have added three new paragraphs that address the critical steps, the limitations of the technique, future applications, and significance of this method.

Reviewer #1

While reviewer says that our account of force spectroscopy is well written they have some concerned about the usefulness and information.

The reviewer is unclear on how useful this account is, because they find that some parts are very detailed, but other parts are treated very superficially so that it would be impossible to understand without reference to other protocols / reviews.

We understand the reviewer's concerns. Though not specified by the reviewer, we believe we identified that the protein preparation section was the least thorough, so we have added more steps regarding this section.

The reviewer requests more information about the cloning strategies and polyprotein design.

We have added a new paragraph in the discussion which discusses the different cloning mechanisms and has references to their specific implementation and design.

The reviewer notes that the detail given for protein expression (including the selective marker) would result in this protocol not working for other constructs.

We have modified the manuscript to be more general when describing the protein construct and only allude to the selective marker we use (Ampicillin) as a specific example.

The reviewer notes it would be good to discuss attachment strategies and the rationale for using mica/glass or gold.

In the discussion we have added a new paragraph which describes attachment strategies and the rationale for using glass, gold, or mica.

The reviewer specifies that it should be made clear that the instructions given for the AFM are again instrument specific.

We added a note to Section 4 specifying that our instructions, though general, may differ for specific implementations of the AFM equipment.

The reviewer suggests that a figure showing the constructs at the DNA and protein level from which the data in Figure 1 would be highly instructive.

We thank the reviewer for this suggestion and have included this figure to accompany our representative results.

Reviewer #2

The reviewer states that as written the methods are only understandable if investigating the protein of interest using the particular atomic force microscope used by the authors.

We agree with the reviewer, that we describe a method for use with a specific AFM. However, most of the methods we propose are quite general to all AFMs, including the experimental setup and the general calibration and setup of the AFM because almost all modern AFMs are now composed of the same set of components (AFM head housing photodiode with laser, 3D piezo electric scanner device moving the AFM cantilever or the sample, coarse stage movement mechanical stage for initial approach and sample slide and the chamber that houses it). We refrain from describing specific actions, like interacting with specific software components which would be specific to a particular AFM setup.

We also note that the AFM we describe is mostly constructed in-house and the components are described in publication by Scholl (PhD thesis), 2016, which makes our setup feasible for development in other laboratories without being prohibitively expensive like other commercial instruments.

The reviewer requests more information about sample preparation and a map of the DNA sequence because it is unclear how the sequence is placed between tandem repeats and how tags are included.

We have added more information into the protein preparation section and also included a plasmid map of a plasmid that we have made available through AddGene for use with this protocol.

It is unclear what types of sequences should be placed between tandem repeats of your protein of interest and how tags are included for purification.

The new figure showing a plasmid map of a plasmid we have prepared for this protocol shows the tags used for purification and the sequences of the tandem repeats.

The authors indicate in 1.3.1 that ampicillin is used, however, depending on the DNA vector used, different types of antibiotics are necessary.

We agree that different DNA vectors will need different antibiotics, and we have added this as a note in the protocol.

More information should be provided about the suppliers of the different reagents used in the sample preparation outlined in Section 1. If commercial protein purification kits are used, this should be indicated or more details provided for the purification protocol.

We have added more information, including concentrations and volumes, for all the buffers we use in Section 1.

More information should be provided about the basis for adsorbing samples on treated glass slides.

We have added a paragraph in the discussion which discusses the pros and cons for different slides and their adsorption.

The reviewer asks whether this treatment work on all types of proteins?

Proteins that are too small to give contour length increments or too weak to resist force will also not be able to be resolved by AFM. We have added this point into the discussion.

The reviewer notes that a reader would benefit also from trouble-shooting on sample preparation, including optimizing protein concentration on samples, and pitfalls in expressing and purifying.

We have added into the discussion some points about pitfalls in expression, namely if the protein of interest fails to behave well inside a polyprotein.

In the troubleshooting for AFM we also include information for optimizing protein concentrations on samples, as we suggest using positive feedback – when no events are found or too many events are found we suggested changing the protein concentration. As proteins are quite heterogeneous it is difficult to provide a more quantitative metric for the protein concentration.

The reviewer notes that we refer to an "Up Mode" and "Down Mode and that a more general terminology is needed.

We have removed those phrases and replaced with a more general terminology.

The author requests that the worm like chain model should be included and alternate models should be indicated.

We have added in the worm-like chain formula and added a reference to alternative models that can be used.

Reviewer #3:

The reviewer observes that an AFM image of the single protein is not provided and asks how it is possible to verify that experiments are performed on a single molecule?

The presence of a single molecule is controlled by the flanking tandem proteins of a known domain. In the protocol we describe using I91 domains which have a characteristic unfolding force and contour length increment. Therefore, if the protein of interest is flanked by four I91 domains on either side, we would expect a single molecule to have the unfolding of at least five I91 domains (to ensure that either side has been unfolded). As long as the protein of interest unfolds at a lower force than I91 (which is often the case), then you can expect that a single molecule of the protein of interest has been unfolded. This is in the discussion of our protocols.

The protocol we describe here is not geared towards membrane proteins, which benefit from added AFM imaging. In our case, however, the proteins are often too small to be seen by AFM imaging so there is no benefit to those experiments. We have added this into the discussion.

The reviewer asks how does one deduct force vibration data from the background.

We have noted in the discussion that a typical RMS noise of AFM is about 5-15 pN and for that reason the minimum resolving force must be above that level. Typically, and that applies also to our AFM setup, the instrument itself is mounted on a vibration isolation platform, such as an air table, which greatly reduces the ambient mechanical noise. In addition, the acquired photodiode

signal that measures cantilever bending due to molecular elasticity and ambient noise (thermal noise), is passed through a low-pass filter that further decreases the measurement force noise and improves the signal to noise ratio.

“The force normalization process experimental data is detrimental to method paper, I would love to see authors explaining how the force curve changes when AFM tip touches the protein surface and what are the differences between normal molecules and proteins. If authors do not mention all these critical factors, no one can follow the methods.”

This reviewer’s comment is unclear to us and we would appreciate further clarification as to what exactly the reviewer means by “changes when AFM tip touches the protein surface” and what he/she means by “normal molecules”.

The reviewer did not find the Figure captions.

We do not believe the Figure captions were cut off, they were shown before the Discussion and after the Protocol.