Response to Reviewers

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 JoVEeditor 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.

2. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi: DOI (YEAR).] For more than 6 authors, list only the first author then et al.

We used the latest version of JoVE's Endnote style to format references.

3. Please abbreviate all journal titles.

As specified, we are using the latest JoVE Endnote style.

4. Please include volume, issue numbers, and DOIs for all references.

As specified, we are using the latest JoVE Endnote style.

5. Please define all abbreviations before use.

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 "$NI_{10}C$" 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 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 procs 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.