Dear Dr. Nguyen,

thank you for the helpful comments on our manuscript "Covalent Attachment of Single Molecules for AFM based Force Spectroscopy". We have rewritten, clarified and streamlined several parts of the manuscript in response to the editorial and reviewers' comments. In addition, we have optimized some representation of the data for better visualization, in particular Figure 5. We believe that we could address every single concern as detailed in the point by point response (see below).

None of the figures has been previously used for publication. The manuscript has been proofread by a native speaker.

We hope that this revised manuscript is acceptable for publication in JoVE and we are looking forward to your response.

Kind regards,

B.N. Balzer

Point by point response to **reviewer comments**

Reviewer #1

The manuscript by Kolberg et al presents a progress in sample preparing for single-molecule AFM. They use a short PEG linker for further attachment of long polymers. The manuscript can be accepted after revision.

We thank the reviewer for acknowledging our manuscript.

1. What is the exact structure of the silane end group for maleimide-polyethylene glycol-silane?

The term "silane" has been changed to "triethoxysilane" in the manuscript and in the material list for a better description of the end group. It reads now:
"The polymers used for this protocol are: maleimide-polyethylene glycol-triethoxysilane (silane-PEG-mal, 5 kDa), […]" (cf. lines 104ff.).
The exact chemical structure of the silane-based linkers to a hydroxy-activated substrate could be represented as mixture of different alkoxy- and siloxane moieties, as previously investigated by using different spectroscopic methods (see the literature: Gutmann et al., Dynamic Nuclear Polarization Signal Amplification as a Sensitive Probe for Specific Functionalization of Complex Paper Substrates, The Journal of Physical Chemistry C, 121, 3896-3903, 2017). In brief, both anchoring to the substrate via siloxane-chemistry and the presence of intermolecular –O-Si-O- bonds between the linkers are possible. Accordingly, the detailed structure of maleimide-polyethylene glycol-triethoxysilane is given in ***Figure 1****.*

2. The authors used oxygen for the plasma treatment. It will be better if the performance of plasma treatment using air is provided.

Oxygen plasma treatment is used to introduce hydroxyl groups to the AFM cantilever tips surface, which consists of Si_3N_4, with a high degree of coverage to increase the efficiency of further chemical reactions. The oxidation of silicon nitride surfaces in this way is described in the literature as a reliable procedure (cf. C. Jimenez et al. Surface and Coatings Technology ***25****, 147-154, 1991). Furthermore, our experience in the past 15 years has shown that a high oxygen content for the plasma process leads to a more successful functionalization process. Thus, we added the following to note to step 3.1.:*
"The efficiency of the plasma treatment for further functionalization steps scales with the content of oxygen in the plasma chamber." (cf. lines 150f.).

3. From Fig 3A, one can see that many force signals appear before 150 nm, which can be assigned to multiple chains. Thus, it is important to control the surface density of the attached

polymers. What can be done for this? This is very important since the authors claim that in other studies "cooperative effects might dominate the results because interactions to neighboring polymers cannot be excluded".

The surface density of the attached polymers can be controlled by mixing functional and non-functional linker molecules. Additionally, the concentration of the target polymer (e.g., PS, PEG or PNiPAM) can be varied. The following notes to step 3.2.1. and step 3.3.1.2. account for these aspects:
"If binding of multiple probe polymers is observed in the SMFS experiment, mixing silane-PEG-mal with non-functional silane-PEG can reduce the number of anchoring points." (cf. lines 173ff.) and "If binding of multiple probe polymers is observed in the SMFS experiment, the concentration of the polymer should be reduced" (cf. lines 223f.).

Reviewer #2:

Manuscript Summary:

In their MS entitled "Covalent Attachment of Single Molecules for AFM based Force Spectroscopy", Adrianna Kolberg and coauthors describe a profound and detailed chemistry for specific surface functionalization for single molecule force AFM experiments on polymers. Additionally they give convincing examples of their results obtained from those substrates and strategies to intrinsically monitor the quality of the experiment and the functionalization.

We thank the reviewer for acknowledging our manuscript.

Major Concerns: None

Minor Concerns:

1. The MS is basically written well, but please check for miss-spellings:

P6 L171 "question" -> acquisition?, P8 L248 "you" -> your? L285 "are" -> a and carefully check the chosen words: P13 L432ff "This reduces ... reproducibility" ???

The mentioned miss-spellings have been corrected and the manuscript was proofread by a native speaker.

A few points might be worth to be revised as well:

2. P8 §6: In case of tidy polymers the calibration of the cantilever possibly might be better after the experiment in order to keep the functionalization of the apex in good shape?

We thank the reviewer for the very valuable comment and added the following note to step 5.10.: "For strong adhesion between polymer and surface, the calibration can be done after the actual experiment to preserve the functionalization." (cf. lines 348f.)

3. P13 L429 to what refers the term "the procedure" to?

For clarification, the term "the procedure" has been changed to "functionalization procedure".

4. L438 what exactly are "control AFM cantilevers"? can you by those? are they treated in a specific way...?

The term "control AFM cantilevers" refers to cantilevers that went through all steps of functionalization (including the linker system) except the binding of the respective target polymer (e.g., PS, PEG, PNiPAM). This has been clarified by adding a note to step 3.2.5.: "Control AFM cantilever chips have gone through all functionalization steps except the polymer attachment (step 3.3). They serve to prove the cleanliness of the functionalization process, the

AFM cantilever chip holder system, the surfaces and the solvents used for the SMFS experiment." (cf. lines 196ff.)

5. L439 ff This sentence is rather misleading: do the authors evaluate only from a total of 2% of force curves all their experiments and discard from those even further the nonspecific ones? Or should this describe a control experiment without any functionalization of the cantilever? Please clarify.

This sentence refers to control experiments. All force-extension curves of the respective experiment with the target polymer have been evaluated. The criterion for cleanliness is the following: if less than 2% of the evaluated curves from the control cantilever show events (such as plateaus or stretching events) the control AFM cantilevers are considered as clean. For clarification "in the control experiment" was added:
"The functionalization process and the fluid cell for performing the experiments are only considered to be clean, if a small number of force-extension curves show stretches or plateaus in the control experiment (in the presented examples less than 2%)." (cf. lines 549ff.).

6. P14 L447 please specify or quantify "simple"

The word "simple" has been deleted for clarification.

7. Fig. 3D and 4A and P13 427 fff: how could a master curve as the blue one show such a pronounced undulation on top of the vertical displacement due to the temperature? This must be either a highly reproducible and interesting feature of the polymer itself -since it results from overlaying several curves- or is this just an artifact of the substraction of a "representative" goldlayer-interference pattern curve?

In the latter case I strongly suggest to either substract "master" goldlayer-interference pattern curves or strongly smoothed or a fitted pattern that represents only the low frequency pattern of the interference rather than the full noise and speckle patterns of a single "representative" force curve. Please re-think the deconvolution of the gold surface in particular for experiments with "weak" force patterns or steps close to the noise level...

*We did not apply the interference correction for the curves given in **Figures 3** and **4**. Thus, the undulation on top of the blue master curve does not result from any type of interference pattern or the respective correction algorithm. Nevertheless, thank you very much for your suggestion, because at the same time, introducing a smoothing step for the representative force-extension curve has proven to be very valuable. Thus, we added a note to step 6.4. revised step 6.7.:*
"Smooth the representative force-extension curve in order to obtain the low frequency pattern of the interference." (cf. lines 380f.). and "Subtract the (smoothed) representative force-extension […]" (cf. lines 388f.).

Reviewer #3:

Manuscript Summary:

The authors describe a methodology to link polymers to AFM tips and describe their approach to executing single molecule pulling experiments and data analysis. Their linking chemistry is based on common moieties used for decades now, albeit they emphasize that their approach saves a step. I don't see any issue with the chemistry method as is. However, there are a number of issues that arise when reading the single-molecule pulling portions of the paper. I suggest major revisions.

We thank the reviewer for the comments and rewrote parts of the manuscript according to the reviewers' suggestions. In addition, we like to emphasize that our approach does not only save a step, but is also more robust than previously published approaches. Although some changes might look very minor, altogether these improvements help a lot for stably linking polymers to AFM tips.

Major Concerns:

1. Page 7-8: The steps used to perform a single molecule AFM experiment have been described in far greater detail in several methods/review papers in the literature. Someone reading this manuscript would not be able to carry out the experiments unless, for example, they were also using a machine that automatically calculates the invOLS and spring constant (many systems do not, particularly older models). The authors should either elaborate on these topics, or clearly point to some of the more complete SMFS methodology papers which provide the reader with deeper context and step-by-step explanations accompanied with figures.

We have revised the description for the determination of the InvOLS and spring constant. It does now give a deeper theoretical justification and cites literature that describes both processes in full detail. Steps 5.6. and 5.7. read now:

"Approach the surface and determine the InvOLS (inverse optical lever sensitivity) by taking force-extension curves on a hard surface (such as silicon oxide). For this, take the deflection signal of the photodetector (in V) vs piezo distance and determine the slope of the part representing the indentation of the AFM cantilever tip into the underlying surface (repulsive regime) using a linear function. In order to reduce errors, take the average of at least five values as final InvOLS value. For further details, see the literature [4,39].

NOTE: The InvOLS can only be reliably determined on hard surfaces. In the case of experiments on soft surfaces or interfaces make sure you place a hard surface close to your soft surfaces. Then, the InvOLS calibration can be done before or after your soft surface experiments without the need of disassembling the AFM setup." (cf. lines 306ff.)

and

"For spring constant determination, move the AFM cantilever to a height with neither attractive nor repulsive interactions between AFM cantilever tip and surface (several µm). Then, take a thermal noise spectrum where the power spectral density (PSD) vs frequency is plotted. The following steps are usually performed by automated built-in functions in

commercial AFM software: first, the acquired thermal noise spectrum is analyzed by fitting a function to the PSD, e.g., a simple harmonic oscillator (SHO). The fit is done up to the minimum between the first and second resonance. Second, the area under the fitted part of the PSD vs frequency plot is determined representing the mean square displacement of the AFM cantilever in vertical direction. Finally, the equipartition theorem is used to obtain the AFM cantilever force constant[28,50].
NOTE: An appropriate frequency range should be used comprising the first resonance peak of the AFM cantilever. To get a satisfactory signal-to-noise ratio, at least 10 PSDs should be accumulated with the highest possible frequency resolution." (cf. lines 318ff.)

2. Page 9, line 296-298: Again, a reader new to SMFS would not understand how to perform the tip-surface transformation of the data from this brief mention of a vital part of the processing. To make this method paper valuable to a novice reader, it should be stated more clearly how to find information on performing this operation.

For clarification, we have changed the order of the steps and rewritten step 6.2. (including a reference for further details) which now reads:
"Subtract the deflection of the AFM cantilever (after multiplication of the raw deflection signal with the InvOLS) from the distance driven by the piezo elements in vertical direction in order to obtain the true extension (tip-surface distance)[4]." (cf. lines 358ff.).

3. Page 9-10: The authors claim that seeing a single rupture event is evidence of a single-polymer. However, it very likely that when two polymers of similar length share a load, the rupture of the first bond can lead to a catastrophic failure of the remaining bond. This is because the second suddenly see a spike in their load force upon the breakage of the first. Given that a second bond could fail instantaneously with the first bond, it is not enough to simply state that seeing one rupture event indicates a single-polymer. Rather, a true signature of a single polymer is found from the stiffness of the force-extension trace [See Sulchek et al, Biophys J. 2006]. I suggest the authors clarify their definition of what constitutes a single bond in light of the past work.

Thank you for pointing out that we did not discuss the possibility of two polymers rupturing at the same length. This is, because we consider this scenario as very unlikely and the likelihood is further decreased with the very high resolution in extension (time) current AFMs provide. Nevertheless, we now discuss this possibility in our revised manuscript and therefore complete our definition of a true signature of a single polymer:
"A clear stretching pattern with no further drops or maxima is essential for having proper single molecule stretching events. Additionally, the dependence of rupture force on the force loading rate at rupture or the complete elastic response of the stretching curve should be analyzed in order to exclude simultaneous desorption of multiple polymer[59,60]." (cf. lines 553ff.)

4. Page 10, line 332: A note on nomenclature here. The "contour length" of a polymer defines the maximum length of the polymer at its maximum extension. This is not the same as the extension at rupture. For example, a polymer with a contour length of Lc = 500nm will rupture at a distribution of extensions before 500nm is reached. This should be revised in the text.

Thank you for pointing this out. We try to clarify what we take as the contour length in our revised manuscript. The principal problem is that usually a certain model is used to determine the contour length, i.e., the contour length values depend on the model that is used. One would obtain different contour length when using, e.g., the FJC, WLC or FRJ model to fit the force-extension curves. This becomes even more evident, once extensible versions of these models are used. Therefore, we here decided to be "model free" and therefore use the extension at 500 pN as reference length value for rescaling. In our opinion, this is the best way to compare different measurements in the presented cases. 500 pN are chosen, because then conformational fluctuations and solvent effects are negligible, cf. Hugel et al., Highly Stretched Single Polymers: Atomic-Force-Microscope Experiments versus Ab-Initio Theory. Phys. Rev. Lett.,94, 048301, 2005), see also response to point 5. To avoid confusion, we omitted the sentence:
"[…] of a polymer with a contour length of about 350 nm." (cf. line 332 of the original manuscript).

5. Page 10, line 339-347: The rescaling here is problematic. Naturally, a polymer's extension is scaled by its contour length, Lc, as defined above. All theories of polymer extension follow a dependence on the ratio x/Lc where x is the extension. Hence it is common in the literature to find that authors rescale polymer force-extension profiles to Lc, not an arbitrary force-extension value. Why do the authors not use any of the myriad of polymer models on their data to get the true Lc? They state that the WLC and FJC do not fit the data, but for decades it has been known that these two simple models only fit the low-force entropic regime. The extensible versions of these models fit many polymers very well. In particular, the PEG force-extension curve is well-described by the two-state model of Oesterhelt, Rief and Gaub (New J. Phys, 1999, Equation 2). This equation has also been used by Sulchek et al (PNAS 2005, Biophys J. 2006) to characterize bond number using PEG linkers.

We appreciate the work of Oesterhelt et al. and Sulchek et al. . However, as explained in response to point 4, there is no "true" contour length. In addition, the models mentioned by the referee assume a completely entropic chain (extended with diverse features). Such a description fails for several polymers in solvents, especially for PEG (see literature: Liese et al., Hydration Effects Turn a Highly Stretched Polymer from an Entropic into an Energetic Spring. ACS Nano, 11(1), 702–712, 2017). This can for example be seen if the FJC model is investigated in detail: The Kuhn length one obtains for most (if not all) polymers in aqueous environment has no physical meaning, because it does not match the actual chemical bond length, i.e., the basis of the model. Taking a well-defined extension value corresponding to a certain force

value seems more reliable than taking a polymer model-based contour length Lc. Therefore, we decided to use the respective extension values at a force of 500 pN for rescaling, see response to point 4. We have described the above-mentioned discrepancies by the following paragraph:
"While a polymer model such as the wormlike chain (WLC) or the freely jointed chain (FJC) model cannot reproduce the respective force-extension behavior for PEG or PNiPAM properly over the entire extension range[18,29,41,65,66]*, such a polymer model might be valuable for other polymeric and protein systems* [10,15,67,68]*." (cf. lines 579ff.)*

6. Page 10, line 362-363: It is not clear why the plateau force and extension are important. What is learned from this analysis? In particular, does the force and extension of the plateau relate to the intermolecular interactions between the polymer and surface?

Plateau-like force-extension curves are observed when the polymer is desorbed from a surface in liquid environment in equilibrium on the timescale of the experiment (see literature: Hugel, T., & Seitz, M. The study of molecular interactions by AFM force spectroscopy. Macromolecular Rapid Communications, 22(13), 989–1016, 2001; Horinek et al. PNAS, 105 (8), 2842, 2008; Krysiak et al., Peptide Desorption Kinetics from Single Molecule Force Spectroscopy Studies. Journal of the American Chemical Society, 136(2), 688–697, 2014). These force-extension curves yield free energies of adsorption, equilibrium adsorbed polymer lengths and some information on friction properties. Yes, plateau forces are closely related to the intermolecular interactions between the polymer and the surface (via the adsorption free energy).
Plateau curves have been the topic of many AFM based single molecule publications. In order to give a short introduction instead of an in-depth review we added the following paragraph to the Example 2 section including literature for further understanding of plateau-like force-extension curves:
"Plateau-like desorption is observed when the dynamics of the probed bonds are much faster than the pulling rate of the AFM cantilever tip (quasi-equilibrium). Desorption forces of plateau-like force-extension curves directly provide adhesion free energies by integrating the force-extension trace[54]*. They have been used to determine electrostatic, dispersive and hydrophobic interactions as well as friction properties of single polymers on surfaces in liquid environment*[2,4,23,51,54,55]*." (cf. lines 443ff.)*

7. Page 11, line 366-367: The authors also state that this plateau can serve as a fingerprint. While this may seem likely, do the authors have any data from other polymer/substrate systems that support the idea of using this plateau as a polymer fingerprint?

This is indeed a crucial point, which we tried to explain with Example 2 and Figure 5. We rewrote this part and modified Figure 5 to further clarify that both, the plateau height and the plateau length are single polymer fingerprints. For a single plateau this will be difficult, but if several plateaus are measured, then the force distribution will show distinct values (to first approximation multiples of the lowest force plateau). The lowest force plateau corresponds

then to a single polymer event. See for example the revised Figure 5D or the following literature: Scherer et al., Intermolecular Interactions of Polymer Molecules Determined by Single-Molecule Force Spectroscopy. Macromolecules, 38(23), 9821–9825, 2005; Friedsam et al., Adsorption energies of single charged polymers, Europhys. Lett., 72(5), 844, 2005. In general, the distribution of force values can pinpoint the number of polymers desorbed at the same time. Corresponding force-extension curves show step-like force-extension curves.

The length of the plateau is another very good fingerprint because it correlates with the contour length of the polymer. This has been thoroughly discussed by the following literature: Krysiak et al., Peptide Desorption Kinetics from Single Molecule Force Spectroscopy Studies. JACS, 136(2), 688–697, 2014. For clarification, we rewrote the Example 2 section and revised ***Figure 5*** *taking comparing data sets with one (****Figure 5(A)*** *and* ***(B)****) and two different polymers bound to the AFM cantilever tip (****Figure 5(C)*** *and* ***(D****)):*

"Example 2: Desorption of PS from a SAM surface in water.

The desorption of PS from a SAM surface in water can be used to determine the desorption force and length and thereby quantify the hydrophobic interaction. After calibration, at least two force maps were recorded at two different spots of the surface. When the polymer attachment is successful, the force-extension curves show plateaus of constant force, as characteristic feature, see ***Figures 5****(****A****) and* ***5(C)****. Plateau-like desorption is observed when the dynamics of the probed bonds are much faster than the pulling rate of the AFM cantilever tip (quasi-equilibrium). Desorption forces of plateau-like force-extension curves directly provide adhesion free energies by integrating the force-extension trace[54]. They have been used to determine electrostatic, dispersive and hydrophobic interactions as well as friction properties of single polymers on surfaces in liquid environment[2,4,23,51,54,55].*

Each plateau of constant force was fitted with a sigmoidal curve to determine the desorption force and desorption length, which were then plotted in histograms. The histograms were fitted with a Gaussian to extract the maximum value and standard deviation. For a better overview, the desorption force and length values were displayed together in a scatter plot, as given in ***Figures 5****(****B****) and (****D****).*

For polystyrene on SAM in water, the determined desorption forces correspond to previously obtained values[19,23]. As the desorption length correlates with the polymer contour length[51], the desorption length distribution can be used as a proof of the covalent binding of the respective polymer to the AFM cantilever tip. Thus, the desorption length serves as a fingerprint.

For more than one polymer attached to the AFM cantilever tip, cascades of plateaus (discrete steps) are observed in the force-extension curves[56]. Each plateau represents the desorption of a polymer at a different extension. The experiment given in ***Figures 5****(****C****) and (****D****) shows a typical case of two polymers attached to the cantilever tip at the same time. By fitting the final rupture, a bimodal distribution can be found for the desorption length, while the desorption force shows a narrow distribution. In this case, the smaller desorption length could be found in 90% of the force-extension curves, either as a single plateau or as an additional plateau on the longer plateau, as shown in* ***Figure 5****(****C****). The higher desorption length was found in 37% of the obtained force-extension curves. So, the desorption length distribution can be used to determine the number of different polymers attached to the AFM cantilever tip. Furthermore,*

a narrow distribution of the desorption length values is a good indication that one and the same single polymer was probed in the obtained force-extension curves. At the same, time a superposition of the respective forces-extension can be used to decide whether one and the same single polymer was measured.
After this experiment, further experiments with the PS polymer can be performed varying, e.g., substrate (solid surface as well as polymer films), solvent conditions, temperature, velocity or dwell time." (cf. lines 438ff.).

8. Page 11, line 367-368: The authors suggest the plateau can be used as a diagnostic to prove that a single polymer is formed. This isn't clear and should be explained.

See answer to the previous comment.

Minor Concerns:

9. Figure 4 caption: The stretching "free energy" is mentioned here and in many places within the paper. However, it is not actually measured or shown anywhere. Perhaps the use of the term should be reduced, or some demonstration/discussion of measuring free energy should be performed.

Thank you for pointing this out. We have revised the manuscript by underlining the role of free energy for further analysis:
"Additionally, the stretching free energy can be obtained from the force-extension master curves by determining the area under the curve for any given force value. This can be used for extracting energetic and entropic contributions of the stretching free energy with the help of molecular dynamics (MD) simulations[18]." (cf. lines 433ff.) and "Desorption forces of plateau-like force-extension curves directly provide adhesion free energies by integrating the force-extension trace[54]." (cf. lines 445f.)