Reconstructing the intrinsic potential energy landscape of interfacial interactions with thermally modulated force spectroscopy

Alan Y. Liu and Todd A. Sulchek*

*George W. Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, Georgia 30332, USA
and Parker H. Petit Institute for Bioengineering and Biosciences, Georgia Institute of Technology, Atlanta, Georgia 30332, USA*

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Force probes are powerful experimental tools to measure the strength and physical extent of interfacial and intermolecular interactions at nanometer scales. However, because of the stochastic nature of force measurements, most force spectroscopy models require a massive quantity of data in order to obtain meaningful energetic information of the interaction. We developed a force spectroscopy framework based on thermally modulated atomic force microscopy (AFM) force measurements capable of reconstructing energy landscapes of interfacial interactions over 100 nm distances from a handful of force curves. To address the challenge of insufficient sampling at the key points of the interaction, we defined exact equilibrium forces to serve as fiduciary markers that can be used to reliably overlay repeated force curves. The equilibrium force markers create a major advantage in that multiple undersampled force measurements can be compiled as one fully sampled measurement of key regions of two-state binding. The Boltzmann method was directly applied to reconstruct the underlying energy landscape in undersampled regions and combined with the direct integration of the force profile in sufficiently sampled regions to fully determine the energy landscape. We experimentally demonstrate the application of the method to find the intrinsic, continuous force and energy landscape reconstructed along an unprecedented 100 nm distance from merely 15 AFM force curves.

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I. INTRODUCTION

As chemical, physical, and biological interactions are all governed by their interaction energy landscapes [1,2], experimental methods to probe the underlying energy landscape are essential to understand fundamental interactions. Optical tweezer (OT) based single-molecule force spectroscopy pioneered the experimental reconstruction of the energy landscape of an interaction field using the Boltzmann based method to define DNA and RNA folding interactions [3–5]. However, OT based reconstruction methods are primarily applied to folding interactions. Biomolecule unfolding measurements have an inherent advantage to reconstruction as the molecule contour length can serve as a fiduciary marker by which energy curves can be overlaid. In contrast to folding energy landscapes, interfacial interactions are typically stronger, therefore the well gradients are larger, which requires higher sampling in these regions and a stiffer force probe. Also, in the case of adhesive and interfacial interactions, there do not currently exist natural fiduciary markers by which multiple force curves can be combined. Therefore applying Boltzmann methods require highly precise sampling of all points of the

energy landscape and they are in general more challenging to perform [6–9]. To study interfacial and adhesive interactions, a variety of reconstruction methods have been developed, primarily implemented on atomic force microscopy and surface force apparatus. These methods either use high sampling within a limited range of reconstruction [6–8] or result in poor sampling in high gradient regions of the landscape [9]. To increase sampling of key regions of the energy landscape, Haider *et al.* applied the weighted histogram analysis method (WHAM) to atomic force microscopy (AFM) force data to directly reconstruct the underlying energy landscapes of the tip-sample interactions [10]. The method required sampling of at least a quarter million data points per nanometer of the reaction coordinate and needed an extremely slow probe velocity (below 1 nm/sec) over the entire force curve. Therefore, the method was only used to reconstruct a 5 nm portion of the underlying energy landscape. In another approach, Walder *et al.* significantly improved the force stability of the AFM measurement with customized AFM cantilevers to reconstruct a one-dimensional (1D) trajectory of energy landscape of RNA unfolding [6]. However, the reconstruction range was limited by the thermal fluctuation of the customized probes, which is typically a few nanometers.

An intuitive way to estimate the underlying energy landscape is to simply integrate the interaction force of probes over the approach or retraction path in a force-distance measurement. However, sensitive force probes, such as AFM, OT, and biomembrane force probe (BFP), undergo an unstable “snap” in the force-distance experiment, therefore the full force landscape cannot be accurately measured in the region of the

*Corresponding author: todd.sulchek@me.gatech.edu

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FIG. 1. (a) Schematic of AFM force measurements. (b) The heat map represents a 2D projection of the 3D surface of energy level for the simulated probe in the force-distance coordinate and the green curve is the interaction force landscape on AFM coordinate. (c) The energetic view of the cross section at the dashed position of (b). The combined potential energy landscape E is the sum of probe's harmonic potential V and interaction potential landscape U . The inner plot highlights the shape and key parameter of the wells of combined landscape E .

snap [11]. In an AFM schema, we can illustrate this phenomenon by observing the deflection (d) of the probe, defined as the difference between the controlled actuator position (Z_s) and the interactant position (Z_{tip} , tip position, also referred to as tip sample separation), as shown in Fig. 1(a). For a high probe stiffness, the measured force-distance profile is identical to the intrinsic interaction force profile. But, if the probe stiffness is lower than the maximum gradient of the interactive force profile, the cantilever becomes bistable and the ideal measured force-distance profile twists into an inverse-S shape curve, shown as the green curve in Fig. 1(b). A detailed discussion on force landscape transforming from interaction coordinate into AFM coordinate is shown in Fig. S1 of the Supplemental Material [12]. A soft probe therefore exhibits a rapid snap from one probe position to another on the S curve during both the approach and retraction. Consequently, a critical portion of the interaction force well is overlooked, which results in an inevitable reconstruction error by directly integrating the force during both the approach (underestimate) and retraction (overestimate), as illustrated in Fig. S2 of the Supplemental Material [12]. Since the information loss in the region of the snap significantly contributes to the energy well, the integration method is inaccurate even if the probe faithfully measures the force profile before and after the snap.

In this Letter, we developed a framework to define characteristic forces to serve as reliable fiduciary markers of regions of the force curve that are typically undersampled in adhe-

sive interactions. These characteristic forces can be recorded at various probe equivalent temperatures with respect to the intrinsic force landscape and collectively compiled to improve the sampling of adhesive force curves. Our method was used to reconstruct the full energy and force landscape of an interaction over a long reaction coordinate range. Finally, with the sufficiently sampled force curves, we demonstrate experimentally that the full force and energy landscape of an unprecedented 100 nm long distance of the interaction can be reconstructed collectively from only 15 force-distance measurements.

A. Defining equilibrium characteristic forces from AFM force-distance measurements

To take advantage of the spatial resolution of the force-distance measurement to reconstruct the intrinsic energy landscape, we first introduce a framework to project an energetic perspective of the probe onto the AFM force measurement coordinate. In the energetic view, the cantilever force transducer is modeled as a thermally activated particle oscillating over a constrained harmonic potential [13,14]. During a force measurement, the cantilever spring can map the local potential energy constraint (E) that consists of a combination of cantilever harmonic potential (V) and the underlying interaction potential energy landscape (U), as shown in Fig. 1(c) [13,15]. A plot of a three-dimensional energy surface projected as a two-dimensional heat map is shown

in Fig. 1(b). The heat map represents the potential energy at all the probe positions along the AFM coordinate in which the red color represents high energy level, the blue color represents low energy level, and the contour lines connect locations with equal energy. The green trace is the transformed force landscape (see Sec. S1 for a discussion [12]), which defines the static equilibrium positions of the probe. The probe can snap at the transitional region where the two wells of $E(Z_S, d)$ provide two stable states, and the unbound (d_u) and bound (d_b) deflection positions are defined at Z_S at the minimum of the two energy wells, as shown in Fig. 1(c) inset. Because the energy barrier to escape from the unbound state (ΔE_u) and the bound state (ΔE_b) are different at the same Z_S , the state transition typically occurs at different Z_S positions during approach and retraction (see Sec. S3 for a discussion [12]). Consequently, a full approach and retraction cycle of force-distance measurement samples two different pathways along the force profile. Two characteristic adhesion forces are thus observed from bound and unbound states: $f_b = kd_b$ and $f_u = kd_u$, respectively, where k is the stiffness of the probe [16]. These two characteristic forces can serve as landmarks to determine the inaccessible portion within the S-curved region under a quasistatic condition as well as points that identify inaccurate regions of direct integration of the force curve.

From a thermodynamic point of view, the probability distribution of the probe fluctuation follows the Boltzmann relation under the energy landscape $E(Z_S, d)$ with a quasistatic assumption: $P(d, Z_S, T) = \exp[-\Delta E(d, Z_S)/k_B T]$, where k_B is the Boltzmann constant and T is the effective probe temperature. For the two-well energy landscape, such as demonstrated in Fig. 1(c), we note that characteristic forces change depending on loading rate and probe temperature. We can therefore define equilibrium forces at which the probe probability of binding and unbinding are equal, $P_b = P_u$. Thus the probe obtains thermodynamic equilibrium between bound and unbound states [Fig. 2(a)]. In principle, a point of equilibrium can be found at any probe temperature. However, in the finite measurement times of experimental practice we observe metastable transitional states at which binding and unbinding are observed only above a critical temperature for a given sampling rate and probe velocity (see the discussion in Sec. S4 [12]). For probe temperatures above this critical value, the equilibrium forces and position can be obtained experimentally by performing a force curve and finding the probe location with a probability distribution function (PDF) of equal area for the bound and unbound states as described in the Methods section.

B. Thermally modulated force spectroscopy to obtain equilibrium forces

While the Boltzmann equation predicts the probe to have a bimodal distribution at all Z_S of which $E(d, Z_S)$ exhibit a two-well shape, empirically we typically observe a single-snap transition between bound and unbound positions in a force-distance measurement. The single-snap transition results from the time span being insufficient to sample the rare,

FIG. 2. (a) Illustration of probability and representative deflection of bound and unbound states. (b) Simulated AFM approach force-distance curves at different temperatures. The fluctuation amplitude of the probe is seen to increase as the temperature increases. (c) The probability density function of three approaching temperatures over the potential landscape at dashed position in (b). The probability is presented as the distance between the dashed probability curves and the solid energy curve.

high energy state of two energy wells Z_S for a probe moving at a constant rate. We can address the insufficient sampling of rare states by increasing the thermal fluctuations of the sampling probe to produce a probability density function $P(d)$ more evenly distributed across the energy landscape [10,17]. To visualize the impact of probe temperature on the sampling of the interaction, in Fig. 2(b) we simulated force-distance approach measurements at low (red), moderate (blue), and high temperature (yellow). The higher temperature probe is able to overcome a higher energy barrier ΔE_b leading to an earlier Z_S of binding during the approach. Considering the dashed-boxed Z_S position of the energy landscape $E(d, Z_S)$ of Fig. 2(b) for three different temperatures, Fig. 2(c) provides the probability distribution $P(d, Z_S)$ (dotted line) for three different temperatures. At a low probe temperature of 100 K (red), the probe does not sample the interaction at all and remains entirely constrained by the harmonic potential of the probe. At moderate probe temperature of 400 K (blue), the thermal energy is sufficient to overcome ΔE_b but not enough for ΔE_u , thus we see a single-snap transition at this location. At a high temperature of 1000 K (yellow), we observe a probe behaving with transitional snaps and a PDF that samples the entire energy landscape, as neither ΔE_u nor ΔE_b are sufficient to constrain the probe.

The thermally modulated force spectroscopy (TM-FS) method in which the effective probe temperature is increased can thus be used to determine equilibrium forces for adhesive interactions. The method is equivalent to that observed in dynamic force spectroscopy (DFS) analysis in which lowering the loading rate lowers the force needed to observe unbinding, until a plateaued equilibrium rupture force is reached at which binding and unbinding occurs [13,18]. Using conventional DFS measurements, one can possibly observe similar transitional interactions at approach or retraction by slowing the loading rate to quasiequilibrium [14,18–20]. However, there is a practical limit to low probe velocities due to instrumental drift or long lifetime biological interactions [21–23]. In such cases, modulating the probe temperature can be an effective alternative approach to measure equilibrium conditions for a practical velocity range (see discussion in Sec. S5 [12]) [24].

C. Reconstructing the intrinsic potential energy landscape using inverse Boltzmann relation

The TM-FS method was used to define a point of equilibrium at a high probe temperature at which bound and unbound states have equal probability [Fig. 2(a)]. We then used this method collect sufficient sampling data and minimize the computational error of the inverse Boltzmann method [3–5,10,12]. The combined potential landscape (green) in Fig. 3(a) shows the reconstruction result of inverse Boltzmann relation at the point of equilibrium of a simulated force-distance measurement at $T = 1200$ K. The intrinsic potential energy $U(Z_{tip})$ was obtained by subtracting the harmonic potential $V(d)$ of the probe from the measured energy landscape $E(d, Z_S)$.

Since the Boltzmann method is typically only used to probe a small portion of potential landscape defined by the range

FIG. 3. (a) The combined energy landscape $[E(Z_S, d)]$ calculated from simulated AFM force data. The corresponding potential energy $[U(Z_{Tip})]$ can be obtained by subtracting the harmonic potential $[V(d)]$ from $E(Z_S, d)$. (b) The equilibrium force path (green) can be determined by comparing deflection at bound and unbound position to approach (blue) and retraction (purple) measurements. (c) The equivalent single-snap force-distance measurement (upper) and the corresponding reconstructed potential energy landscape (lower). The shaded area indicates the integrated energy from the force-distance curve.

FIG. 4. (a) An unfiltered experimental force-distance data of tip-sample interaction. The point of equilibrium is determined at the point where probability of bound and unbound states is the same. The inner plot demonstrates the histogram at the point of equilibrium. (b) The inverse Boltzmann reconstructed potential landscape at $T_{eq} = 1753$ K. The dotted line marks the bound (orange) and unbound (blue) position on the deflection coordinate of the probe. $\Delta U_{s,eq}$ and $\Delta x_{s,eq}$ are the potential difference and distance at transition states, respectively. (c) The equivalent snap path at $T_{eq} = 1753$ K can be determined from approach and retraction curve at room temperature. (d) Comparison of the Boltzmann method (black) and direct integration of approach (blue, highlight at inner figure) and retraction (orange) force curves to reconstruct the potential energy landscape. ΔU_0 represents the potential well depth and Δx_β is the barrier distance of the interaction.

of probe fluctuation at a particular Z_S , it can nevertheless be applied to complement the direct integration method at the key unsampled portion (illustrated in Fig. S2 [12]). Therefore, the full potential landscape can be obtained by stitching Boltzmann reconstruction results together with direct integration of force-distance curve before and after the snap. The simple approach to implement is to define an equivalent snap path on the force-distance measurement cycle utilizing two deflections ($d_{u,eq}$ and $d_{b,eq}$) obtained from the measured combined landscape ($E(d, Z_{S,eq})$), as shown in Fig. 3(b). Then the integrated energy landscape can be stitched to the Boltzmann reconstructed energy landscape at two ends of the snap path with low error, as shown in Fig. 3(c) (see stitching detail in the Method section). When applied to reconstruct the potential well depth from simulated interactions, these methods showed a small error of 0.26% comparing to the analytical value [black dashed curve in Fig. 3(c) compared to Fig. S2D [12]].

D. Experimentally determining the intrinsic potential energy landscape

We implement TM-FS on an AFM to measure the electrostatic landscape between a gold-coated silicon nitride sharp AFM tip and a mica sample. Thermal modulation of the probe was accomplished with a white noise excitation method to

increase the stochastic fluctuation amplitude of the probe to mimic higher equivalent temperatures [10]. We only used data from the approach measurements in this work to avoid possible uncertainty on contact during retraction measurements [16,25]. As the excitation increased, we observed transitional snaps at excitation values above 5V ($T_{eq} = 1111$ K, as shown in Fig. S6 [12]). An evaluation of sampling of bound and unbound positions at eight different temperatures ($T_{eq} = 1111$ K to $T_{eq} = 1861$ K) showed similar results (Fig. S7 [12]). We show the full potential energy landscape reconstruction here at an equivalent temperature of $T_{eq} = 1753$ K in Fig. 4. The point of equilibrium can be identified from unfiltered deflection data at which bound and unbound states possess equal probability, as shown in Fig. 4(a). An important consideration is that the point of equilibrium for an interaction at a given temperature is uniquely identified and indicates an identical physical location of the interaction. As a result, we can acquire multiple force curves to average the reconstruction results within the range of equilibrium forces. A representative reconstructed potential landscape of 15 force-distance measurements is shown in Fig. 4(b), with the data analysis routine shown in Fig. S7 [12]. Next, we use $d_{eq,b}$ and $d_{eq,u}$ at 1753 K obtained from the window between the points of equilibrium [Fig. 4(a) inset] to define the equivalent snap path from approach and retract force-distance curves obtained at

room temperature (25 °C), as shown in Fig. 4(c). Using the TM-FS method, the potential landscape can be reconstructed by stitching together the approach energy integration, the retraction energy integration, and Boltzmann reconstructed potential landscape, as the black curve shown in Fig. 4(d) (see the stitching detail in the Methods section). The reconstruction result showed that the interaction potential landscape of a gold-coated silicon nitride tip and mica surface is composed of a long-range repulsive interaction and a short-range attractive interaction.

A comparison of the Boltzmann reconstruction to direct integration of the entire approach force-distance curve reveals a more pronounced energy well [Fig. 4(d) inset], as expected from Sec. S2 [12]. However, we observed an unexpected large deviation between retraction integrated energy and Boltzmann reconstructed landscape. We evaluate this deviation by deriving the underlying force landscape from Boltzmann reconstructed potential landscape, and found that retraction adhesion is outside the S-shaped region of the intrinsic force landscape, as shown in Fig. S8 [12]. This may be a result of the remodeling of the adhesive interaction upon retraction after the firm probe contact [16,26]. A potential energy difference at transition states can also be estimated using DFS model proposed by Friddle *et al.*: $F_{eq} = \sqrt{2k\Delta G}$, where F_{eq} in Friddle's model is $f_{eq,b} = 178.8$ pN, the calibrated $k = 111.63$ pN/nm, and $\Delta G = U_0 - T\Delta S$ is a free energy term offset by an entropic component contributed by the force probe [13]. The result of the Friddle model, $\Delta G = 1.437 \times 10^{-19}$ J, shows a 10% difference from our result, $\Delta U_{s,eq} = 1.291 \times 10^{-19}$ J. A possible source of difference between the two methods includes the assumptions of the shape of the energy landscape in the Friddle model in which no interactions exist far from binding [13]. The intrinsic potential well depth (ΔU_0) and distance (Δx_β) of the energy barrier can be easily recognized in the full potential landscape in Fig. 4(d). Taken together, the combination of the Boltzmann method and the TM-FS method can directly measure the detailed potential energy landscape with only a handful of force-distance measurements.

II. DISCUSSION

An essential condition for accurate energy landscape reconstruction is to sufficiently sample the interaction at regions of high force gradient. Prior studies often fulfilled such condition with slow probe velocity, which requires excellent force stability that can best be achieved by custom instrumentation [22,23] or extremely long equilibrium time [10]. The TM-FS method provided an alternative approach, by varying the equivalent temperature, to enhance the sampling of higher energy positions that can significantly reduce the requirement of instrumentational stability. Furthermore, by defining equilibrium forces, we established an interaction coordinate that does not change with repeated force measurements. Correspondingly, we were able to quantitatively include multiple force curves to provide sufficient sampling of the interaction. With these methods, we reconstructed the underlying energy landscape of tip-sample interaction from merely 15 AFM force measurements. Another advantage of TM-FS framework is that no assumptions of the shape of interaction landscape are needed. Although our demonstration shows a single-well

nonspecific interaction, it can be easily extended to predict probe reaction over a more complex energy landscape [17] or applied to biomolecular experiments. Additionally, the reconstruction method can possibly be applied without excitation. As a proof of concept, Fig. S9 [12] shows how the energy and force reconstruction can be performed on the biotin-avidin interaction [12]. However, we note that with no thermal modulation, it is likely the energy well was undersampled, underestimating the well depth.

The equivalent temperature should not be confused with the environmental temperature of the interaction during the measurements. Rather, it is a physical quantity to describe the 1D fluctuation brought by external excitation to the force probe which is not expected to change the temperature of the molecules of the surrounding or attached on the probe, yet provide effective sampling of strong interactions.

Most prior studies based on Boltzmann relation only obtained a reconstruction of a small portion, within a few nanometers, of the interaction, since the method is optimal at the strongest interactive position where two distinctive states are formed. Nonetheless, long distance interactions, such as electrostatic force, are not well probed (e.g., interfacial interactions in Fig. 4). Our method enables a quantitative way to directly combine direct integration and Boltzmann reconstructed energy landscapes, and therefore we were able to reconstruct an unprecedented 100 nm range of underlying energy landscape in addition to the 2 nm highly varying region reconstructed from the Boltzmann method. Thus observing the energy landscape of both long-range repulsive interactions and short-range attractive interactions is now possible.

III. CONCLUSION

We have developed a framework from the energetic perspective of the force probe to define characteristic forces of equilibrium under various equivalent temperatures on the intrinsic force landscape. Under this framework, we demonstrated that the intrinsic potential energy landscape can be measured by tuning the equivalent temperature of the AFM probe computationally and experimentally. At high equivalent probe temperature, the probe equilibrium position $Z_{s,eq}(T)$ can be found at which transitional bound and unbound states are of equal probability. The inverse Boltzmann relation can then be applied at the point of equilibrium because both bound and unbound states are maximally sampled. Furthermore, the Boltzmann reconstructed potential landscape can be stitched together with integrated potential energy profile using characteristic forces from approach and retract force curves to obtain a full range intrinsic potential energy landscape. We experimentally reconstructed the full intrinsic potential energy landscape between a silicon nitride AFM tip and mica surface over a 100 nm force distance from a mere 15 force-distance measurement. We anticipate that this method can be applied to single-molecule experiments (as demonstrated in Sec. S9 [12]) to obtain the complete energy landscape of adhesive interactions of interest.

IV. METHODS

AFM measurements. The AFM probe-mica experiments were performed with an MFP-3D AFM (Asylum research).

The equivalent temperature of the probe was modulated with direct white-noise excitation through iDrive™ (Asylum research) cantilevers. The white noise excitation signal was generated by a DS 345 function generator (Stanford research) and directly sent to the AFM controller. Silicon nitride BL-TR400PB cantilevers, coated with gold on both sides, compatible with iDrive (nominal $k = 0.09$ N/m) were used as received from the manufacturer. All the cantilevers were calibrated using the thermal Sader method [27] before the measurements. Mica disks (Ted Pella), 10 mm in diameter and of the highest grade, V1, were glued to the glass bottom of a FluoroDish™ then cleaved no longer than 5 min prior to measurements. All the experiments were carried out in deionized water (milli-Q) and with the entire mica disk and cantilever immersed in water. All the measurements shown were operated at 10 nm/s velocity both approaching and retracting under 50 kHz sampling rate with a 25 kHz low-pass filtering.

Simulation method. The simulation of TM-FS method was implemented in PYTHON using a damped simple harmonic oscillator cantilever model approaching/retracting to/from the tip-sample interaction following a sixth order chemical force profile that has a single well potential energy characteristic, as described previously [10]. The thermal fluctuations are modeled as Gaussian noise with a variance of $4k_B T b B$, where k_B , T , b , and B are the Boltzmann constant, temperature in kelvin, damping coefficient, and frequency bandwidth of 200 kHz respectively. The spring constant $k = 0.5$ N/m, the quality factor $Q = 3$, and the resonance frequency $f_0 = 25$ kHz were used for all the simulations shown in this work. The damping coefficient b was determined using equivalent mass $m = k/(2\pi f_0)^2$, k , and Q through $b = \sqrt{km}/Q$. The velocity of simulated measurements was set to be 1 nm/s unless further specified because this velocity is slow enough to ensure that all the force measurements are in the quasistatic region while still having reasonable computational time. A higher probe velocity will provide similar results with sufficiently high equivalent temperature; further details are discussed in Sec. S11 [12].

Calculating the point of equilibrium from force-distance measurements. The first step to obtain equilibrium force from force-distance data is to create a small window scanning through the deflection data. In each location, a KDE (kernel density estimation) smoothened histogram [28] was created and used to find the minimum normalized probability at

which the accumulated probability is equal to 0.5, or 50%. This algorithm can find windows with a bimodal distribution where two modes have the same accumulated probability (see Sec. S10 for details [12]). In the context of AFM force measurements, these windows containing 50-50 bimodal distributions correspond to the point of equilibrium for measurements with bistable states, or the location where the snap occurs for single-snap measurements. The optimization of window size is discussed in Sec. S12 [12]. The deflections of two peak values (d_u and d_b) in the bimodal probability density distribution correspond to represented forces at bound and unbound states [(Fig. 2(a)). All the analysis routine was implemented with custom PYTHON script. We have shared the source code for simulation and data analysis routine via GitHub [29].

Stitch the Boltzmann reconstructed energy curve to integration of force-distance curves. The full-length scale energy landscape reconstruction is constructed from three individual energy curves: (1) integration of approach force-distance curve, (2) Boltzmann reconstruction, and (3) integration of retract force-distance curve. The approach and retract force curves are collected from no-excitation measurements because lower equivalent temperature probe would capture a longer distance of the intrinsic force landscape before the snaps occur. The Boltzmann results were reconstructed from high equivalent probe temperature because a better PDF is obtained in such condition. The first step to stitch the curves together is to truncate the approach integration curve at d_u found at the point of equilibrium at specified equivalent temperature used in the Boltzmann method. Then the Boltzmann reconstructed energy curve can be smoothly stitched to the approach integrated energy curve at $d_{u, eq}$. The second step is to apply the same method to the retraction integrated curve at d_b but stitch the energy curve after $d_{b, eq}$, and the full-length energy landscapes can be obtained.

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