A direct micropipette-based calibration method for atomic force microscope cantilevers

Baoyu Liu, Yan Yu, Da-Kang Yao, and Jin-Yu Shao

Department of Biomedical Engineering, Washington University, Saint Louis, Missouri 63130, USA

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In this report, we describe a direct method for calibrating atomic force microscope (AFM) cantilevers with the micropipette aspiration technique (MAT). A closely fitting polystyrene bead inside a micropipette is driven by precisely controlled hydrostatic pressures to apply known loads on the sharp tip of AFM cantilevers, thus providing a calibration at the most functionally relevant position. The new method is capable of calibrating cantilevers with spring constants ranging from 0.01 to hundreds of newtons per meter. Under appropriate loading conditions, this new method yields measurement accuracy and precision both within 10%, with higher performance for softer cantilevers. Furthermore, this method may greatly enhance the accuracy and precision of calibration for colloidal probes. © 2009 American Institute of Physics. [DOI: [10.1063/1.3152220](https://doi.org/10.1063/1.3152220)]

I. INTRODUCTION

Besides being a powerful instrument for high resolution imaging, the atomic force microscope (AFM) has been widely used to apply and measure forces with piconewton (pN) precision in studies such as quantifying cellular mechanical properties,¹ characterizing protein-ligand interactions,² and uncovering protein folding/unfolding mechanisms.³ To ensure reliable quantitative force measurement, accurate knowledge of AFM cantilever stiffness is of great importance.⁴ However, because the stiffness of commercial cantilevers often suffers substantial deviation from their nominal values provided by manufacturers, they have to be individually calibrated in practice.⁵ To this end, many experimental methods have been proposed, which can be categorized into three classes—dimensional, dynamic, and direct.

Dimensional methods calculate the stiffness from geometrical parameters and material properties based on the beam theory in solid mechanics.^{6–8} However, application of these methods is often hindered by practical concerns such as: (i) the cantilever material is not perfectly homogenous (even so, the Young's modulus of a thin layer can be significantly different from the bulk value),⁹ (ii) a cantilever's thickness is difficult to measure and its measurement error is scaled by three because it is cubed in the stiffness formula, and (iii) the thickness of the gold coating on the cantilever's back is usually unknown, which adds uncertainty in the total thickness measurement (moreover, the gold coating might also change the Young's modulus of the cantilever).⁴

Dynamic methods extract the stiffness from thermally driven motion of the cantilever tip.^{10–12} Among them is the widely used added-mass method, which calibrates the stiffness by measuring the resonant frequency before and after attaching a known mass (usually a spherical particle of tungsten or gold) at the cantilever tip.¹¹ Water drops have also been used as the added mass to make this method nondestructive.⁵ A more elegant and perhaps the most popu-

lar one is the thermal noise method, which does not need any mass attachment and derives the stiffness solely from the cantilever thermal noise intensity.¹³ Originally, the cantilever was modeled as a simple one-dimensional harmonic oscillator. At thermal equilibrium, the equipartition theory predicts that the potential energy $k\langle z^2 \rangle / 2$ is equal to $k_B T / 2$, where k , $\langle z^2 \rangle$, k_B , and T are the stiffness, the mean square thermal deflection, the Boltzmann constant, and the absolute temperature. Thus, by computing $\langle z^2 \rangle$ from the cantilever thermal noise spectrum, one can get the stiffness without detailed knowledge of the cantilever mechanical properties. However, as revealed in subsequent publications, the thermal noise calibration is actually cantilever-shape-dependent and material homogeneity also matters.^{14,15} Moreover, the thermal noise calibration can be greatly affected by the laser spot size and position on the cantilever, which are usually not accounted for.¹⁶

Direct methods calibrate the stiffness by loading an AFM cantilever with a known force. The force can be applied with a reference cantilever^{17,18} or an indentation device.^{19,20} In these methods, apart from the requirement of accurate standards, it is very difficult to position the tip of the reference cantilever or the indenter in precise alignment with the tip of the calibrated cantilever. The force can also be applied with a hydrodynamic drag.^{21–23} However, because of the complex nature of the fluid flow problem involved, the hydrodynamic drag method either is semiempirical or requires a microparticle to be attached.

In addition, most available methods are for soft cantilevers with stiffness less than 1 N/m and report calibration uncertainties between 5% and 30%.^{12,24–26} Usually, the calibrated value refers to the stiffness at the cantilever's free end, which is some distance away from the sharp tip where force measurements are performed. Although the position discrepancy can be reconciled by an off-end correction,¹² it needs to assume that the cantilever is homogeneous and has a uniform cross section, which could increase systematic error.

Here, we describe a direct calibration method based on

the micropipette aspiration technique (MAT).²⁷ The cantilever stiffness is calibrated by simultaneously monitoring the applied force on the cantilever and the resulting cantilever deflection. The force is applied directly on the cantilever tip, which offers better positioning accuracy than the reference cantilever or the indentation method. The new method is ideal for calibrating soft cantilevers used in single molecule experiments without *a priori* knowledge of the cantilever geometry or its material properties.

II. MATERIALS AND METHODS

A. The MAT

1. Principle

Like the AFM, the MAT is a force-measurement technique with piconewton precision.²⁸ Its force transducer is a spherical object (e.g., a polystyrene bead or a spherical cell) that fits closely inside a fluid-filled cylindrical tube. Force is applied by controlling the pressure (Δp) inside the tube. As a fluid-mechanics-based technique, the force magnitude in the MAT is linearly dependent on the velocity (U_b) rather than the displacement of the transducer as in the AFM, which is based on solid mechanics. A certain pressure corresponds to a certain free motion velocity (U_f) under which the force is equal to zero. Theoretically, the force (F) exerted by the MAT can be calculated by²⁷

$$F = \frac{\Delta p \cdot \pi D_p^2}{4} \cdot \left[1 - \frac{4}{3} \cdot \frac{D_p - D_b}{D_p} \right] \left(1 - \frac{U_b}{U_f} \right), \quad (1)$$

where D_p and D_b are the tube and transducer diameter, respectively.

2. Experimental setup

The experimental setup of the MAT in this study is the same as described previously.²⁹ It is built around an inverted microscope (Axiovert 200M, Zeiss, Jena, Germany) with standard differential interference contrast (DIC) attachment. A glass micropipette is controlled by a manual mechanical manipulator (Narishige, model MMO-203, East Meadow, NY) and inserted into a water-filled custom-made chamber, which is fixed on the microscope stage with a sample holder. The micropipette is connected to a water reservoir mounted on a motorized nanoscale vertical stage (Physik Instrumente, Model M501.1PD, Germany) controlled with a LABVIEW (National Instruments, Austin, TX) program. The maximal travel distance of the stage is 12.5 mm. Experiments can be recorded either with a CCTV camera (Panasonic, Model WV-BP330, Suzhou, China) or a digital camera (Vision Research, Model Phantom v4.2, Wayne, New Jersey).

3. Force transducer and micropipette

Size-standard polystyrene microparticles (Sigma, St. Louis, MO) were used as the force transducer of the MAT. These uniform particles have a diameter of $20 \pm 0.4 \mu\text{m}$ ($D_b \pm \sigma_{D_b}$, σ represents the standard deviation). Before being added into the chamber, the particles were washed with 1% BSA (in PBS) to prevent nonspecific adhesion. Glass micropipettes were prepared with a vertical pipette puller and a

FIG. 1. A planar view of the AFM chip used in the calibration experiments. The cantilevers are made of silicon nitride with backside gold coating. Their nominal stiffness values are (from left to right) 0.5, 0.1, 0.03, 0.01, and 0.02 N/m, respectively.

microforge.²⁷ The micropipette was also treated with 1% BSA by backfilling with a syringe. The inner diameter was measured with DIC microscopy and modified by a correction factor, which was experimentally determined by measuring the actual micropipette diameters with electron microscopy. Based on the measurements of seven micropipettes with diameters of $\sim 20 \mu\text{m}$, the correction factor (C_f) was found to be 1.122 ± 0.036 ($C_f \pm \sigma_{C_f}$), almost identical to the correction factor we obtained before (1.120 ± 0.016) from micropipettes with diameters of $\sim 8 \mu\text{m}$.²⁹ Both of them agree well with the theoretically predicted value by Engström *et al.*³⁰

B. Calibration

1. Cantilever

The AFM chips used in this study have one cantilever on one side (A) and five cantilevers on the opposite side (B, C, D, E, and F, see Fig. 1 for a planar view, Veeco probes, Model MLCT-EXMT-A-10, Camarillo, CA). The cantilevers are made of silicon nitride and coated with gold on the back. Cantilever B (rectangular), C (triangular), D (triangular), E (triangular), and F (triangular) were calibrated. Their nominal stiffness values are 0.02, 0.01, 0.03, 0.1, and 0.5 N/m, respectively.

2. Geometry

A schematic of the experimental component arrangement inside the chamber is shown in Fig. 2. An AFM chip is glued to and fits snugly in a notch carved out on the vertical wall of a plastic chamber. The gluing is done with vacuum grease so the calibrated cantilever can be intact and usable after being taken off the chamber wall. The micropipette tip is inserted into the chamber and placed at such a position that the cantilever tip is on the same level as the left pole of the force transducer, i.e., the bead. Because both the force transducer and the cantilever tip can be directly viewed under the microscope, we were able to calibrate the cantilever stiffness right at the tip with a high positioning accuracy.

3. Calibration procedure

After the cantilever tip and the force transducer of the MAT were aligned, the pressure inside the micropipette was controlled with the motorized stage to increase linearly from 0 to 50 Pa at a rate of 50 Pa/s (α ; this rate of pressure

FIG. 2. (a) Overview of the experimental component arrangement (not drawn to scale). The experimental chamber has one opening on the right (the two coverslips on the top and bottom are omitted for clarity). The AFM chip is attached in a notch carved on the vertical wall of the experimental chamber. The micropipette is shown as a cylinder. (b) The cantilever tip (only one cantilever is shown) is pressed against by the force-transducer bead of the MAT. The pressing force is controlled by the repulsive pressure inside the micropipette which is in turn controlled by the height of a water reservoir mounted on a nanoscale vertical moving stage (not shown). The inset shows the actual microscopic view of the force-transducer bead and the AFM tip.

increase corresponds to a force loading rate of ~ 15 nN/s), hold at 50 Pa for ~ 1 s and decrease linearly to 0 at the same rate. For each cantilever, this process was repeated approximately ten times and the images such as the inset in Fig. 2(b) were recorded. From the recorded video, the displacement of the bead was tracked with nanometer resolution by a two-dimensional (2D) nanotracking algorithm.³¹ Because the bead was in direct contact with the cantilever tip [Fig. 2(b)], their displacements (L) in the micropipette axial direction (or the normal direction of the cantilever surface) were assumed to be the same. From the displacement data, the stiffness (k) can be calculated as (Appendix A)

$$k = \frac{\pi \alpha D_p (4D_b - D_p)}{12\dot{L}}, \quad (2)$$

where $\dot{L}$ is the time derivative of L and it represents the cantilever-tip velocity.

III. MEASUREMENT ACCURACY

To evaluate the accuracy of our proposed calibration method, here we examine the potential error sources and assess the uncertainties they can cause. According to Eq. (2), the accuracy of measured cantilever stiffness is determined by the accuracy in its components, namely, the pressure load-

ing rate (α), the micropipette and transducer diameter (D_p and D_b), and the cantilever-tip velocity ($\dot{L}$). Among them, due to the high precision of the motorized stage, the parameter α was considered to be accurate and the parameters D_p , D_b , and $\dot{L}$ will be the subjects of the following discussion.

A. Micropipette and bead diameter

The micropipette diameter (D_p) was first measured multiple times with DIC microscopy and expressed as $D_{p\text{DIC}} \pm \sigma_{D_{p\text{DIC}}}$. D_p was then calculated by dividing the DIC measurement with the correction factor ($C_f \pm \sigma_{C_f} = 1.122 \pm 0.036$). The Gaussian error propagation law states that

$$\left(\frac{\sigma_{D_p}}{D_p}\right)^2 = \left(\frac{\sigma_{D_{p\text{DIC}}}}{D_{p\text{DIC}}}\right)^2 + \left(\frac{\sigma_{C_f}}{C_f}\right)^2. \quad (3)$$

Since $\sigma_{D_{p\text{DIC}}}/D_{p\text{DIC}}$ and σ_{C_f}/C_f were 0.8% and 3.2% in our experiments, the uncertainty of the micropipette diameter (σ_{D_p}/D_p) was 3.3%. The bead diameter uncertainty (σ_{D_b}/D_b) was less than 2.0% according to the manufacturer's specification (Sigma), which agrees well with our own measurement (19.9 ± 0.2 μm , $n=18$).²⁹

A closely related parameter is the micropipette-bead gap ($D_p - D_b$), which plays an important role in determining the uncertainty of $\dot{L}$ as discussed in Sec. III B. Based on the uncertainties of D_p and D_b , for a micropipette-bead pair with equal diameter measurements, the standard deviation of the gap is $\sim 3.9\%$ of the micropipette diameter (calculated by $\sqrt{\sigma_{D_p}^2 + \sigma_{D_b}^2}$, assuming the measurements of D_p and D_b are uncorrelated). However, by carefully choosing beads matched closely with individual micropipettes in size (thus creating a correlation between D_p and D_b), the gap can be managed to be less than 3.9% of D_p for most cases.

B. Cantilever-tip velocity ($\dot{L}$)

The measurement accuracy of $\dot{L}$ can be affected by a number of sources. Described below are the major ones—indentation on the bead induced by the loaded cantilever tip, random drift of the cantilever chip, as well as misalignment and thermal fluctuation. To minimize the uncertainty caused by the finite resolution of the 2D nanotracking algorithm (~ 5 nm), the slope of the loading (or unloading) displacements rather than the absolute displacements at the maximal loads was used in our calibration [Eq. (2)].

1. Indentation depth

Because the displacement of the cantilever tip is inferred from the bead displacement, the amount of indentation in the bead induced by the sharp cantilever tip can affect $\dot{L}$ and needs to be evaluated. In an indentation test, loading on the sharp indenter causes not only elastic deformation of the substrate but plastic deformation as well. The plastic deformation leaves a permanent impression on the substrate that conforms to the indenter shape. The indentation depth is determined by the applied load (P), the indenter shape, and the substrate hardness (H).³² H is related to P as $H = P/A$,

FIG. 3. (Color online) (a) The cantilever response to periodic pressure loading and unloading. The pressure first linearly increased from 0 to 50 Pa in 1 s (corresponding to rising displacement), was kept at 50 Pa for ~ 1 s and linearly returned to 0 in 1 s (corresponding to falling displacement). This pressure loading pattern was repeated five times. The black bars represent the average displacement levels at the maximal applied pressure. Note the displacement drift over time. (b) Linear regression of the displacement data, which yields $\dot{L}$, during the second pressure loading in (a).

where A is the effective contact area between the indenter and the substrate, namely, the cross-sectional area of the indenter at the indentation depth. Using a surface hardness of ~ 300 MPa for polystyrene^{33,34} and the maximal loading force of ~ 15 nN in our experiments, we obtained an A value of ~ 50 nm². An AFM cantilever has a four-sided pyramidal tip with a nominal 20 nm radius of curvature at the peak. Assuming a spherical cap at the peak of the cantilever tip, the maximal indentation depth can be estimated to be ~ 0.4 nm.

2. Random drift of the cantilever chip

Mechanical drift of the cantilever chip is unavoidable in actual experiments, which may result from relative movements among the chip, the chamber, and the microscope stage. Because we are dealing with nanometer-level displacements, the otherwise negligible drift could play a significant role in the final cantilever-tip velocity. A manifestation of this drift is the varying magnitude of the maximal displacement of the cantilever tip, as shown in Fig. 3(a). We quantified the drift by measuring the neighboring maximum difference [the level difference between two consecutive black bars in Fig. 3(a)]. As shown in Fig. 4, the drift defined this way seems to be a random process. The average drift magnitude over the loading (or unloading) period (1 s) was 2.7 ± 1.5 nm.

FIG. 4. (Color online) AFM chip drift magnitude over one second measured by the drift of the maximal displacement magnitude during calibration.

3. Misalignment and thermal fluctuation

Ideally, the cantilever tip should be in direct contact with the left pole of a concentric transducer bead inside the micropipette (Fig. 2). In reality, however, a perfect alignment is impossible due to the imperfectness of human vision. First, focal ambiguity introduces uncertainty in finding the true focal plane. Second, in the focal plane, although the positional accuracy in carefully performed experiments can be adjusted to be within 0.1 μm (with a $100\times$ objective and an analog camera, one pixel is equivalent to ~ 0.07 μm in recorded images), a small deviation from symmetry cannot be avoided from time to time. Even for a perfect alignment, thermal motion can cause the bead to deviate from the axisymmetric position and move toward the micropipette wall (Fig. 5). During the pressure loading period, because the bead is pressed against the sharp cantilever tip, the movement of the contact point is restrained but the bead can rotate

FIG. 5. Schematic of the transducer rotation inside the micropipette. Due to imperfect alignment and thermal fluctuation, the transducer can rotate around the cantilever tip during calibration. Because the deflection of the cantilever was measured by tracking the transducer horizontal movement, this rotation might result in undesired error in the cantilever-tip displacement and the calibrated cantilever stiffness. Here the rotational angle (θ) and the micropipette-transducer gap are exaggerated for the purpose of illustration. The transducer rotated from its original position (dashed circle) to a new position (solid circle). At the same time, the transducer center moved a distance of Δx and Δy in the horizontal and vertical direction, respectively, even though the cantilever tip actually did not move.

around the tip. Be it caused by misalignment or thermal fluctuation, under a pushing force, a displaced nonconcentric bead will tend to rotate toward the micropipette wall, leading to fake displacement of the cantilever tip. Fortunately, this rotation is limited by the small micropipette-transducer gap, which is usually less than 3.9% of the micropipette diameter as discussed in Sec. III A. As illustrated in Fig. 5, for a small rotational angle θ , the following relationship applies to the axial and vertical displacement of the transducer center (Δx and Δy),

$$\Delta x = \frac{\Delta y^2}{D_b}. \quad (4)$$

For a 20 μm bead and a maximal Δy value of $\sim 0.78 \mu\text{m}$ ($20 \mu\text{m} \times 3.9\%$), the fake displacement can be as large as 30 nm. One thing needs to be noted is that even when the bead moves away from its symmetric position as it rotates, the force calculation formula [Eq. (1)] remains unchanged,³⁵ which is accurate to the first order of the dimensionless gap $(D_p - D_b)/D_p$ (Appendix B).

The uncertainty from misalignment and thermal fluctuation can overlap with that from the cantilever-chip drift, but it appears that the former plays a dominant role. In the worst case, the total uncertainty in the measurement of the cantilever tip displacement in this study was $0.4 + 2.7 + 30 \approx 33$ nm. Clearly, whether this is significant or not depends on the maximal displacement of the cantilever tip. For the stiffest cantilever we calibrated, the maximal displacement can be estimated to be ~ 30 nm based on the maximal force of ~ 15 nN and the nominal stiffness of 0.5 N/m, which means that the measured cantilever-tip velocity could have up to 100% uncertainty; for the softer cantilevers with a stiffness of 0.03 N/m or less, this uncertainty decreases to within 7%. Therefore, with our current experimental design (i.e., the micropipette diameter, the pressure pattern, and the maximal force were all the same when different cantilevers were calibrated), our new calibration method yields higher accuracy for softer cantilevers. To achieve similar measurement accuracy for stiffer cantilevers, larger beads or higher pressures should be used (see more detailed discussion in Sec. IV). The reason why the transducer rotation causes uncertainty is that the force-transducer displacement of the MAT is assumed to be equal to the cantilever tip displacement. For colloidal probes (cantilevers with large attached spheres as tips), we anticipate that this source of error will be eliminated because the cantilever tip can be directly monitored by tracking the attached sphere.

4. Cumulative uncertainty in stiffness calculation

The uncertainties in all the measurements (D_p , D_b , and $\dot{L}$) propagate according to

$$\begin{aligned} \left(\frac{\sigma_k}{k}\right)^2 &= \left(\frac{\sigma_{D_p}}{D_p}\right)^2 + \left(\frac{\sigma_{(4D_b - D_p)}}{4D_b - D_p}\right)^2 + \left(\frac{\sigma_{\dot{L}}}{\dot{L}}\right)^2 = \frac{10}{9} \left(\frac{\sigma_{D_p}}{D_p}\right)^2 \\ &+ \frac{16}{9} \left(\frac{\sigma_{D_b}}{D_b}\right)^2 + \left(\frac{\sigma_{\dot{L}}}{\dot{L}}\right)^2. \end{aligned} \quad (5)$$

Although σ_{D_p}/D_p and σ_{D_b}/D_b can be determined unambigu-

ously, $\sigma_{\dot{L}}/\dot{L}$ depends on how the pressure loading is applied and what the actual cantilever stiffness is. For the current experimental design, we claim a 7% uncertainty ($\sigma_{\dot{L}}/\dot{L}$) for soft cantilevers (doubling the maximal load will halve this value). On the other hand, for colloidal probes, because the cantilever-chip drift is the only contributing factor for the uncertainty in $\dot{L}$, $\sigma_{\dot{L}}/\dot{L}$ should be easily controlled within 1% for soft cantilevers. Even for stiff cantilevers, 7% uncertainty is within easy reach by using larger transducer beads and micropipettes or larger pressures. If we assume 7% for $\sigma_{\dot{L}}/\dot{L}$, the relative accuracy of the calibrated stiffness (σ_k/k) is 8.2%.

IV. RESULTS AND DISCUSSION

Figure 3(a) shows a typical tracking curve of the cantilever-tip displacement. There is a little vibration at the beginning and the end of each pressure loading (or unloading) period due to the sudden acceleration (or deceleration) of the motorized stage. However, the majority of each period is linear where the pressure loading rate is constant. The cantilever-tip velocity ($\dot{L}$) was extracted by linear regression for each linear segment [Fig. 3(b)] and the stiffness was calculated according to Eq. (2). For comparison, we also calibrated the cantilever stiffness with the thermal noise method by Hutter and Bechhoefer,¹³ which was performed on a Bioscope AFM (Veeco Instruments, Santa Barbara, CA).

Figure 6(a) displays the results for 33 cantilevers calibrated with both the MAT and the thermal noise method. The agreement between the nominal and calibrated stiffness was very poor with relative difference up to 90% (Table I). For the cantilevers with the same nominal stiffness, their calibrated stiffness can vary more than 30%. Two-way repeated measures analysis of variance showed that any of the following three—the nominal values, measured means (of each nominal stiffness group) by the MAT, and measured means by the thermal noise method—was significantly different and within each group, measured means of individual cantilevers were also significantly different ($p < 0.05$). These observations underline the necessity of calibrating individual cantilevers in practice. The maximal relative difference between the two calibration methods was $\sim 30\%$, comparable to previously published results of comparison between the thermal noise and the direct loading method.³⁶ In another paper,¹⁶ it was shown that, if uncorrected, the finite size of the laser spot and its position on the cantilever can result in large errors in the stiffness calibrated by the thermal noise method. The fact that we did not consider this effect might have caused the large discrepancy.

Shown in Fig. 6(c) is the comparison of calibration precision between the MAT method and the thermal noise method. To test the validity of our error analysis, we grouped the cantilevers according to their nominal stiffness and calculated and compared the precision across groups. The precision within each group is defined as the group mean of the relative standard deviation for every individual cantilever (standard deviation divided by individual mean). As expected, the calibration precision by the MAT method de-

FIG. 6. (a) The cumulative results (33 cantilevers in total) for the calibrated stiffness by the MAT method and the thermal noise method. The black line shows the expected values based on the nominal stiffness. (b) Direct comparison of the calibrated stiffness by the two methods for six individual cantilevers with a nominal stiffness of 0.01 N/m. (c) Calibration precision by the MAT method and the thermal noise method. Precision is shown against cantilever nominal stiffness. For each nominal stiffness group, there were at least six cantilevers calibrated. Each cantilever was calibrated multiple times and the percentage standard deviation (standard deviation divided by the mean) was calculated. The precision for each group was defined by the group mean of the percentage standard deviation.

creases as the cantilever stiffness increases (due to the same pressure loading for different stiffness cantilevers), while the precision by the thermal noise method is not sensitive to the cantilever stiffness. To ensure high and consistent precision for cantilevers with various stiffness values, proportionally larger maximal force loads should be employed for stiffer cantilevers, which can be achieved by using larger force-transducer beads or increasing the maximal pressure. For example, with the current experimental setup, using a 150 μm standard bead and a peak pressure of 100 Pa (10 mm water), a peak force of ~ 1700 nN can be applied. Under this con-

dition, even for the stiffest cantilever calibrated here (0.5 N/m), the maximal displacement of the cantilever tip is ~ 3400 nm and the calibration precision (or accuracy) is $\sim 7\%$ (assuming the micropipette-transducer gap to be 3.9% of the micropipette diameter), which are comparable to those of the soft cantilevers calibrated in this study, as shown in Fig. 6(c) (the ones with the nominal stiffness from 0.01 to 0.03 N/m).

Because plastic beads and glass capillaries with standard sizes ranging from several to hundreds of micrometers are commercially available, the only limitation to the usable bead size seems to come from geometric considerations. For instance, the length of cantilever F in Fig. 1 is 85 μm ; therefore, to prevent it from touching the cantilever chip where the cantilever is attached, the standard bead cannot exceed 170 μm in diameter. Another limitation of the current setup is that the maximal pressure that can be applied is restricted by the short travel distance (12.5 mm) of the motorized stage. To circumvent this limitation, one can replace the stage with other manometers such as a U-shaped tube or change the pressure in the micropipette with other means such as air flow.²⁹ With the assistance of a pressure transducer, hundreds of times higher maximal pressures can be applied accurately. This way, much stiffer cantilevers (hundreds of N/m) can be calibrated with high precision.

Our new calibration method directly measures the cantilever stiffness with the MAT. Compared with other direct calibration methods,^{17,23} the MAT method is unique in that it calibrates the stiffness at the most relevant position by directly applying loads at the cantilever tip. This naturally eliminates the need of off-end correction—a common source of systematic error. Another method of direct calibration that is also based on a second force-application technique is the indentation method, where the stiffness is calibrated by applying force on the back of the cantilever with an indenter.^{19,20} The calibration uncertainty was claimed to be within 10%. Due to the stiff indenter transducer (>200 N/m), it produces more accurate measurement for stiffer cantilevers. For the same reason, it cannot calibrate soft cantilevers with stiffness lower than 0.1 N/m—usually the most useful in single molecule experiments. In contrast to the method of indentation, the MAT method proposed here has its intrinsic strength in this aspect. For soft cantilevers, smaller force transducers and micropipettes can be employed and larger maximal cantilever displacements can be managed. Smaller micropipettes afford higher measurement accuracy for the micropipette diameter and reduce the force transducer rotation inside the micropipette. Larger cantilever displacements diminish the relative uncertainty for measurement of the cantilever-tip velocity. Both are beneficial for improving calibration precision and accuracy. Hence, with the MAT method, soft cantilevers with stiffness well below 0.1 N/m can be accurately calibrated.

In calibration of soft cantilevers, the most popular is the thermal noise method. However, there are some concerns for this seemingly perfect way of calibration (convenient, *in situ*, and no requirement for any attached mass). The elegance of the thermal noise method lies in its simplicity. It models the cantilever as a simple harmonic oscillator, following which

TABLE I. Cantilever stiffness comparison among nominal and calibrated values. [The calibrated stiffness is expressed as “mean $\pm$ SD (the number of calibrated cantilevers).” The measured means by MAT and TN for each group are significantly different, as shown by repeated measures ANOVA test ($p < 0.05$). The relative difference was calculated as the group mean of the relative difference for every individual cantilever within the group. MAT and TN represent the MAT calibration method and the thermal noise calibration method, respectively.]

Cantilever stiffness (N/m)			Relative difference		
Nominal	MAT	TN	MAT and nominal (%)	TN and nominal (%)	MAT and TN (%)
0.01	0.018 $\pm$ 0.002(6)	0.019 $\pm$ 0.002(6)	84.21	90.12	6.72
0.02	0.034 $\pm$ 0.006(6)	0.022 $\pm$ 0.005(6)	69.12	19.49	34.53
0.03	0.056 $\pm$ 0.014(9)	0.045 $\pm$ 0.009(9)	87.08	49.28	19.29
0.1	0.146 $\pm$ 0.046(6)	0.113 $\pm$ 0.037(6)	45.02	33.61	17.65
0.5	0.596 $\pm$ 0.285(6)	0.386 $\pm$ 0.119(6)	47.22	25.96	36.17

the stiffness can be calculated according to the equipartition theorem by the simple formula $k = k_B T / \langle z^2 \rangle$ regardless of mechanical properties.¹³ However, an actual cantilever is more complex with multiple vibration modes that are dependent on the cantilever shape and its material property. Therefore, this simple calculation formula has to be modified to include a cantilever-dependent correction factor.^{14,15} Moreover, as mentioned earlier, the calibrated stiffness is sensitive to the laser spot size and position on the cantilever, which needs to be carefully accounted for. These concerns related to the cantilever shape and material properties do not exist in direct calibration methods. Thus, in the absence of a “gold standard,” the MAT method provides a valuable alternative for soft cantilever calibration.

Our new calibration method may also prove very useful especially for the calibration of colloidal probes. Due to well-defined tip curvature and ease of surface chemistry modification, colloidal probes^{37,38} have been widely adopted in numerous studies. For colloidal probes, there are two well-characterized calibration methods that are specifically designed to take advantage of the attached colloidal particle.^{21,22} In both methods, the hydrodynamic drag on the particle due to a flat substrate movement (in the normal direction of the cantilever) was used to determine the stiffness. Because the particle is usually small (~ 10 μm in diameter) compared with the cantilever, it had to be positioned very close to the substrate (the hydrodynamic force on the particle approaches infinity as it approaches the substrate at a finite speed) to ensure that the force on the particle dominates the total drag force on the whole cantilever. At small separation distance, not only is it difficult to obtain the cantilever sensitivity (for converting voltage signals to cantilever tip displacements) due to the asymptotic nature of the hydrodynamic force, but static surface forces will also come into play and complicate data interpretation. Fluid-mechanics-based in nature, our new method, which does not require the sensitivity measurement, can circumvent these problems by confining fluid flow mostly inside the micropipette and generate large enough forces on a separate microparticle to interact with the one attached on the cantilever tip. Moreover, by directly tracking the colloidal particle and thus avoiding the

major source of uncertainty resulting from the MAT transducer rotation, our micropipette-based method should be able to yield much higher precision and accuracy of calibration than that for bare cantilever tips. Consequently, we expect that the new method would work best for either soft or stiff colloidal probes and prove useful for their calibration. However, without a gold standard, how to experimentally examine this theoretically based expectation remains a challenge.

V. SUMMARY

The MAT has been used to apply piconewton-scale forces in many studies of cellular and molecular biomechanics. Here, we explored its nanonewton force capability in the development of a new method for calibrating AFM cantilever stiffness (k). Forces at a constant loading rate were imposed directly on the cantilever tip and the resulting displacements were simultaneously monitored. The stiffness was then calculated with a simple formula [Eq. (2)]. Error analysis showed that the precision and accuracy of calibration for soft cantilevers ($k < 0.05$ N/m) were both within 10%. With little modification of the current experimental design, comparable precision and accuracy for stiffer cantilevers ($k > 1$ N/m) can also be obtained.

There are four major merits of our new calibration method. First, as a direct method, the calibration is independent of the cantilever’s shape or material properties. Second, the calibration yields the stiffness right at the cantilever tip and thus offers exceptional positioning accuracy and eliminates the need for off-end correction. Third, the calibration exhibits higher precision and accuracy toward softer cantilevers, which are widely used in single molecule and single cell studies; meanwhile, it can also be used for stiff cantilevers with stiffness up to hundreds of newtons per meter. Finally, we expect that this method should perform best for either soft or stiff colloidal probes with high precision and accuracy well within 10%.

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APPENDIX A: CALCULATION OF CANTILEVER STIFFNESS

In this appendix, we present a theoretical analysis for calculating the cantilever stiffness (k) from its displacement data (L), the micropipette and transducer diameter (D_p and D_b), and the pressure loading rate (α). This analysis applies to the linear pressure loading period ($\Delta p = \alpha \cdot t$, where $\alpha = 50$ Pa/s in the experiment and t denotes time). For the pressure unloading period, the same conclusion can be drawn with similar analysis.

Assume a quasistatic equilibrium state exists for the cantilever tip and the force transducer of the MAT. The force (F) acting on the force transducer of the MAT is balanced by the elastic force developed in the cantilever and the hydrodynamic force due to the cantilever movement. From Eq. (1), we have

$$F = \frac{\Delta p \cdot \pi D_p^2}{4} \cdot \left(1 - \frac{4 D_p - D_b}{D_p}\right) \left(1 - \frac{U_b}{U_f}\right) = kL + c\dot{L}, \quad (\text{A1})$$

where U_b , U_f , and c are the bead's velocity, its free motion velocity, and the cantilever's hydrodynamic drag coefficient, respectively.

According to low Reynolds number hydrodynamics, the aspiration pressure is proportional to the free motion velocity²⁷

$$\Delta p = \beta \cdot U_f, \quad (\text{A2})$$

where β is a constant dependent on the micropipette/bead radii and the viscosity of the fluid inside the micropipette. The typical value for β in our experiments was ~ 0.1 (pN s/ μm^3). Noting $U_b = \dot{L}$ and rearranging Eq. (A1) yields

$$\dot{L} + \frac{k}{\gamma(\beta + c/\gamma)} \cdot L - \frac{\alpha}{\beta + c/\gamma} \cdot t = 0, \quad (\text{A3})$$

where

$$\gamma = \frac{\pi D_p^2}{4} \cdot \left(1 - \frac{4 D_p - D_b}{D_p}\right).$$

Solving Eq. (A3) with the initial condition of $L=0$ @ $t=0$ allows us to calculate $\dot{L}$ and obtain

$$k = \frac{\alpha\gamma}{\dot{L}} (1 - e^{[-kt/(\beta\gamma+c)]}). \quad (\text{A4})$$

For the softest and longest cantilever ($k=0.01$ N/m = 10^4 pN/ μm), the drag coefficient c is ~ 2 pN s/ μm (Ref. 39) (stiffer and shorter cantilevers have smaller drag coefficients). Considering the typical values of 300 μm^2 and 0.1 pN s/ μm^3 for γ and β , one can estimate the lower bound of $k/(\beta\gamma+c)$ to be ~ 300 /s. Therefore, the exponen-

tial term in Eq. (A4) plays a significant role only at the very short beginning (~ 0.02 s) of the total pressure loading period (1 s). In fact, the initial nonlinear behavior caused by the exponential term is usually indiscernible in our displacement data. Hence, for practical purpose, Eq. (A4) can be simplified to be

$$k = \frac{\alpha\gamma}{\dot{L}} = \frac{\pi\alpha D_p(4D_b - D_p)}{12\dot{L}}. \quad (\text{A5})$$

APPENDIX B: FORCE CALCULATION FOR A NONCONCENTRIC SPHERE IN A CYLINDRICAL TUBE

The problem of creeping flow passing an eccentrically positioned sphere that closely fits inside a tube has been solved with the singular perturbation method by Bungay and Brenner.³⁵ Here, we will only discuss the situation where the sphere is stationary (by virtue of the discussion presented in Appendix A, the sphere velocity can be neglected). We will show that, under this condition, the force calculation formula for a nonconcentric sphere (including the case when the sphere contacts the tube) remains the same as Eq. (1) for $U_b=0$. For a stationary sphere, the force (F) and the pressure (Δp) can be expressed respectively as³⁵

$$F = \mu D_b V_m K_s,$$

$$\Delta p \cdot \pi D_p^2 = \mu D_b V_m M_s, \quad (\text{B1})$$

where μ and V_m are the fluid viscosity and the average flow velocity away from the sphere. K_s and M_s are the dimensionless coefficients that only depend on the sphere-tube geometry and they are defined as

$$K_s = \frac{9\sqrt{2}\pi^2}{8} \eta_0 \varepsilon^{-5/2} \left[1 + \varepsilon \left(\frac{157}{60} + \eta_1 \right) + O(\varepsilon^2) \right],$$

$$M_s = \frac{9\sqrt{2}\pi^2}{2} \eta_0 \varepsilon^{-5/2} \left[1 + \varepsilon \left(\frac{79}{20} + \eta_1 \right) + O(\varepsilon^2) \right], \quad (\text{B2})$$

where

$$\varepsilon = \frac{D_p - D_b}{D_b},$$

$$\eta_0(e) = \frac{1}{2} \pi \left(1 - \frac{1}{2} m \right)^{5/2} \left[\int_0^{\pi/2} \sigma^5 d\theta \right]^{-1},$$

$$\eta_1(e) = \frac{193}{60} - \frac{\eta_0}{30\pi \left(1 - \frac{1}{2} m \right)^{7/2}} \left[90(1-m) \int_0^{\pi/2} \sigma^3 d\theta + 103 \int_0^{\pi/2} \sigma^7 d\theta \right]^{-1}, \quad (\text{B3})$$

in which $\sigma = (1 - m \sin^2 \theta)^{1/2}$ and $m = 2e/(1+e)$. e is a dimensionless parameter to describe the eccentricity of the sphere relative to the tube as defined by

$$e = \frac{2b}{D_b \varepsilon}, \quad 0 \leq e \leq 1, \quad (\text{B4})$$

where b is the perpendicular distance between the sphere center and the tube axis. Both η_0 and η_1 are monotonic functions of e . When the sphere and the tube are in contact ($e=1$), $\eta_0=0.52$ (compared with 1 for $e=0$) and $\eta_1=0.27$ (compared with 0 for $e=0$), which means K_s and M_s will change significantly. However, as shown below, the force applied on a sphere contacting the tube is the same as that on a concentric one for any certain pressure because the decrease in K_s and the increase in V_m [due to the decrease in M_s according to Eq. (B1)] cancel each other out.

We are interested in the functional relationship between F and Δp . Calculating the ratio of their expressions in Eq. (B1) yields

$$\begin{aligned} F &= \frac{\Delta p \cdot \pi D_p^2}{4} \cdot \left[1 - \frac{4}{3} \varepsilon + O(\varepsilon^2) \right] \\ &= \frac{\Delta p \cdot \pi D_p^2}{4} \cdot \left[1 - \frac{4}{3} \bar{\varepsilon} + O(\bar{\varepsilon}^2) \right], \end{aligned} \quad (\text{B5})$$

where $\bar{\varepsilon} = (D_p - D_b)/D_p$, which is essentially the same as Eq. (1) for $U_b=0$ (accurate to the first order of the small dimensionless parameter $\bar{\varepsilon}$). Therefore, in both concentric and non-concentric situations (for e ranging from 0 to 1), the force calculation formula remains the same and Eq. (1) is still valid under nonconcentric conditions (as long as $\bar{\varepsilon} \ll 1$).

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