Chapter 15

Measuring the Elastic Properties of Living Cells with Atomic Force Microscopy Indentation

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Abstract

Atomic force microscopy (AFM) is a powerful and versatile tool for probing the mechanical properties of biological samples. This chapter describes the procedures for using AFM indentation to measure the elastic moduli of living cells. We include step-by-step instructions for cantilever calibration and data acquisition using a combined AFM/optical microscope system, as well as a detailed protocol for data analysis. Our protocol is written specifically for the BioScope™ Catalyst™ AFM system (Bruker AXS Inc.); however, most of the general concepts can be readily translated to other commercial systems.

Key words: Scanning probe microscope, Nanoindentation, Force spectroscopy, Mechanobiology, Cell mechanics, Young's modulus, Stiffness, Elasticity

1. Introduction

The mechanical properties of cells influence their ability to change shape, exert force (1), migrate (2, 3), and sense physical stimuli in their microenvironment (4–6), such as matrix stiffness and shear stress. Studies have also shown that changes in the mechanical properties of cells can serve as a biomarker for various diseases, including anemia (7), muscular dystrophy (8), pulmonary and cardiac diseases (9), and several types of cancer (8, 10–13). A number of tools have therefore been developed to measure the mechanical properties of living cells, including atomic force microscopy (AFM) indentation (14), micropipette aspiration (15, 16), particle tracking microrheology (17), and magnetic twisting cytometry (18). Of these, AFM is arguably the most widely used and versatile technique. In addition to measuring cellular mechanical properties, AFM can also be used to image cells through a variety of contrast mechanisms (19, 20), apply tensile forces to cells (21), and measure cellular contractile forces (22).

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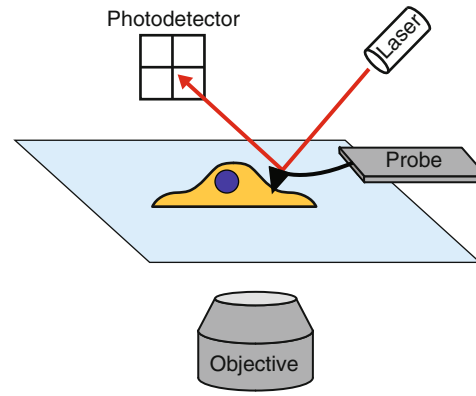

Fig. 1. Schematic of an AFM indentation experiment depicting a probe with a pyramidal tip indenting a cell.

In AFM, piezoelectric actuators are typically used to control the x, y, and z position of a probe, which consists of a flexible cantilever with a micron-sized tip attached to a rectangular substrate (chip). When the probe is near a surface, attractive and repulsive forces between the tip and the sample cause deflection of the cantilever, which is tracked by a laser reflected off the back of the cantilever onto a position-sensitive photodiode detector. To measure the elastic modulus of a sample, the sample is indented by the probe, and the cantilever deflection is measured as a function of the probe's z position (Fig. 1). The cantilever deflection is used to calculate the indentation force of the probe based on Hooke's law, $F = k \times d$ where d is the cantilever deflection and k is the cantilever spring constant. The resulting force-vs.-indentation curve is then typically fit to the Hertz model, which was originally developed to describe the indentation of two elastic spheres (23) and has since been modified to describe the indentation of an elastic infinite half-space by a small, rigid tip of defined geometry (24–26). From this fitted curve, the elastic modulus of the sample can be extracted. These force curves can be conducted in liquid and at ambient temperatures, which permits direct measurement of living cells and tissues. To locate cells and position the probe over specific subcellular locations, the AFM system is often integrated with an optical microscope, which can also be used to monitor cellular structure and function during the experiment.

In this chapter, we provide a detailed protocol for measuring the elastic moduli of living cells, which includes calibration of the cantilever spring constant, acquisition of force curves on cells, and data analysis. Throughout the chapter, we include advice and troubleshooting tips based on our own experiences with these measurements. In Chapter 17 of the first edition of *Cell Imaging Techniques: Methods and Protocols* (27), Costa outlined methods for performing force measurements on cells using the original

BioScope™ AFM system from Veeco Instruments (now known as Bruker AXS Inc.). Here, we focus on the more recently developed BioScope™ Catalyst™ AFM system (Bruker AXS Inc.) and provide more specific, step-by-step instructions for acquiring single force curves on cells with the goal of calculating the average elastic modulus of a population of cells. Note that throughout this chapter we have indicated software commands by quotation marks (e.g., "initialize the stage").

2. Materials

2.1. AFM System

The BioScope™ Catalyst™ AFM system is fully integrated with an inverted optical microscope to enable simultaneous force measurements and optical imaging. The open design of the AFM head permits a direct light path from the condenser to the optical objective for optimal phase contrast and differential interference contrast (DIC) imaging. The open design also facilitates the use of cell culture dishes and easy access to the sample. The following components are supplied with the AFM system:

1. BioScope™ Catalyst™ head.
2. Nanoscope V controller.
3. BioScope™ Catalyst™ Electronics Interface Box ("E-box").
4. BioScope™ Catalyst™ baseplate with large NA sample holder plate.
5. EasyAlign™ for infrared laser alignment.
6. Joystick for controlling *x*, *y*, *z* motors.
7. Nanoscope software (version 8.10).
8. Probe holder for submersion in liquid.
9. Probe stand for securing probe holder while loading and unloading probes.
10. Magnetic sample substrate clamps.
11. Heater stage and controller (optional, see Note 1).
12. Petri dish perfusion cell (optional, see Note 1).

2.2. Additional Microscope Components

1. Nikon Eclipse Ti-E inverted light microscope (Nikon Instruments Inc., Melville, NY) with ×20 or ×40 Ph2 objective for phase imaging (see Note 2).
2. Controller for microscope shutters (e.g., Lambda 10-3, Sutter Instrument Company, Novato, CA).
3. Vibration isolation table.

4. Two computers: one with the Nanoscope software and AFM drivers installed and the other with the optical microscope software and drivers installed.
5. Digital camera (optional).
6. Fluorescence lamp (optional).

2.3. Additional Supplies

1. AFM probes appropriate for contact mode in fluid with spring constants around 0.01–0.2 N/m and a reflective coating on the back side of the cantilever (see Note 3).
2. Tweezers with non-scratching synthetic tips (e.g., Carbofib tweezers, Aven, Inc., Ann Arbor, MI) to avoid damaging the glass window on the liquid probe holder.
3. Standard 25 mm × 75 mm microscope slides.
4. Hydrophobic solution (OMS Opto Chemicals) to treat microscope slides (see Note 4).
5. Standard cell culture reagents.
6. Standard cell culture dishes (35 mm, 60 mm) or glass coverslips (see Note 5).
7. 50 mm glass-bottom Petri dishes (WillCo Wells).
8. Pipette.
9. Kimwipes® for cleanup.

3. Methods

3.1. Sample Preparation

1. Seed cells in cell culture dishes or on glass coverslips (see Note 5). We recommend seeding at a cell density that is sub-confluent but greater than 5,000 cells/cm^2 to make locating cells easier and to reduce travel time between cells.
2. Allow cells to adhere and spread (2–24 h) prior to force measurements.

3.2. Cantilever Spring Constant Calibration

The actual spring constant of a cantilever can vary significantly from the nominal spring constant estimated by the manufacturer, and it is important to measure this value before each experiment. Various methods for measuring cantilever spring constants have been reviewed elsewhere (28, 29). We use the Thermal Tune method built into the Nanoscope software, which measures the thermal fluctuations of the cantilever as a function of time, calculates the power spectrum of the fluctuations, fits the chosen peak to a Lorentzian curve, and integrates under the curve to calculate the cantilever spring constant. This measurement should be conducted in air with a clean, dry cantilever, and it first requires

measurement of the "deflection sensitivity" (i.e., the conversion factor between cantilever deflection and photodetector voltage) on a material that is very stiff compared to the cantilever, such as glass or tissue culture plastic.

1. First turn on the computers and then turn on the Nanoscope V controller and the E-box. The optical microscope components should also be turned on.
2. Open the Nanoscope software, and under experiment type choose "contact mode in fluid." This will turn the infrared laser on. Do not initialize the stage yet because the laser first needs to be aligned on the AFM probe.
3. Slide the probe holder for submersion in liquid onto the probe stand. Using tweezers with non-scratching synthetic tips (to protect the glass window on the probe holder), carefully pick up an AFM probe substrate by the sides (see Note 6) and place it on the probe holder with the tip facing up and with the cantilever of interest above the glass window. Push down on the probe stand to raise the spring-loaded clamp and slide the probe under the clamp so that the cantilever is in the center of the glass window. If there are additional cantilevers on the other end of the probe (under the clamp), they will likely be damaged and should not be used.
4. Stand the AFM head up vertically on the EasyAlign and slide the probe holder onto the *z* scanner. Lower the AFM head horizontally onto the EasyAlign (without liquid in the dish holder) so that the three legs on the head fit into the three dents in the EasyAlign. Turn the EasyAlign on and adjust the AFM head so that the cantilever is in view, adjusting the focus and brightness knobs as necessary.
5. Use the beam positioning knobs on the AFM head to move the laser spot onto the end of the cantilever (where the tip is located). Use the horizontal detector positioning knob (–H+) on the AFM head to set the horizontal deflection to 0 and the vertical detector positioning knob (–V+) to set the vertical deflection to a negative number (between –2 and –6 V). The laser sum should be a positive number greater than 2 and should be greatest when the laser is positioned on the cantilever (see Note 7).
6. With the AFM head still resting on the EasyAlign, follow the prompts in the Nanoscope software to "initialize the stage" and "wake up" the scanners.
7. Place a clean, dry microscope slide onto the sample holder plate on the microscope stage and secure the slide with the appropriate sample clamp.
8. Use the Navigation Menu in the Nanoscope software to ensure that the AFM head is near its highest position and then place

the head on the stage so that the three legs on the head fit into the three dents on the baseplate.

9. Use the optical microscope to focus on the top surface of the microscope slide. Using either the joystick or the Navigation Menu in the software, carefully lower the AFM head closer to the glass slide but still far enough away to be out of focus. If the *z* position of the AFM head is unclear, it is best to stay far away from the glass surface to avoid crashing the probe and *z* scanner; note that the greater the initial probe-sample separation distance, the more time will be needed to engage.
10. In the Scan Menu, set the scan size to zero and the deflection setpoint to around 1.5 V. Then in the Engage Menu, use the "slow engage" function to automatically bring the AFM head down to the surface. During this process, the scanner is set to scan mode and the AFM head is lowered to the surface until the vertical cantilever deflection reaches the specified deflection setpoint (see Note 8). Once the deflection setpoint is reached, the tip will start scanning the surface. The default engage settings can be used ("SPM engage step size" = 1 μm, "SPM withdraw step size" = 100 μm), or alternatively, we prefer to use an "SPM engage step size" of 5 μm to speed up the engagement process.
11. Once the probe is engaged at the surface, switch to the Ramp Menu and raise the AFM head 10 μm by going to "Microscope" in the menu bar and choosing "step motor" or by clicking the "step motor" icon in the RealTime Status Window. Be sure to check the box: "allow stage motion while engaged".
12. Look through the optical microscope and check that the cantilever is in view. If necessary, temporarily raise the AFM head another 10–20 μm and adjust the stage using the baseplate positioning knobs (see Note 9).
13. In the Ramp Menu under the expanded view, choose the following settings: "ramp output" = Z, "ramp size" = 12 μm, "forward and reverse velocities" = 10 μm/s (which corresponds to 0.42 Hz), "number of samples" = 2,048, "Z-closed loop" = ON (see Note 10), "trigger mode" = relative, "data type" = deflection error, "trigger threshold" = 1–2 V (or about 50–100 nm), "start mode" = motor step, and "end mode" = retracted. Adjust the vertical deflection to be around −2 V (see Note 11), and then click the "single ramp" button in the toolbar to acquire a force curve on the glass slide (see Note 12).
14. In the graph, set channel 1 to "deflection error," which plots the data as vertical deflection vs. *z* position. The extension part of the curve should have a flat baseline followed by a sharp, positive slope denoting deflection of the cantilever by the glass surface. The slope of this line gives the "deflection sensitivity"

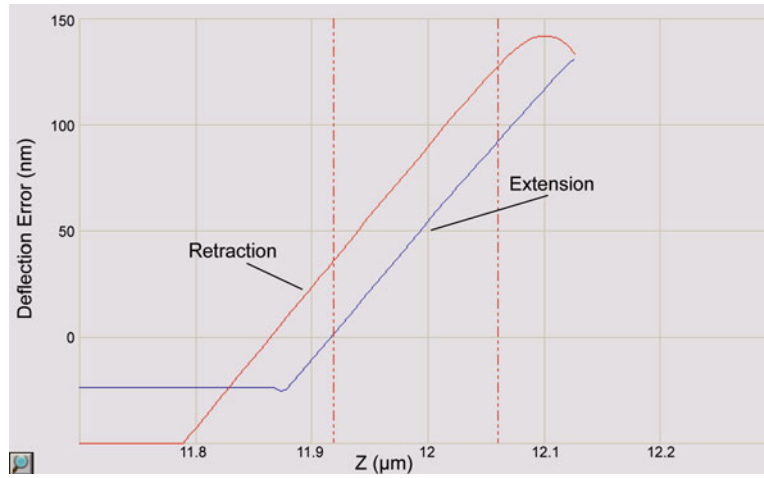

Fig. 2. Screenshot from the Nanoscope software showing a magnified view of a force curve taken on a glass slide. The extension portion of the curve between the *vertical dashed lines* is used to calculate the "deflection sensitivity." For clarity, the extension and retraction curves are labeled, and the font size for the axes has been enlarged.

in nm/V, which enables the vertical deflection value to be converted from volts to nanometers. Zoom into the sloped region of the curve by holding "Ctrl" and using the mouse to draw a box around the curve. Then click to the left or right of the graph to drag two red, dashed lines onto the graph and use these lines to mark the boundaries of the sloped region to be fit with a straight line (Fig. 2). Click the "update sensitivity" button in the toolbar to calculate the slope of the line and save this value. Click the magnifying glass button in the bottom left corner of the graph to zoom back out.

15. Bring the AFM head up at least 200 μm by clicking the "withdraw" button several times. Set both the vertical and horizontal deflections to 0 by adjusting the detector positioning knobs on the AFM head and click the "Thermal Tune" button in the toolbar. Do not change the laser beam position (see Note 13).
16. In the Thermal Tune Menu, check the box for "Lorentzian (Air)," choose the "thermal tune range" of 5–2,000 kHz, enter 1.144 for "deflection sensitivity correction" for v-shaped cantilevers or 1.106 for rectangular cantilevers, and then click the "acquire data" button. Zoom into the largest peak (which should be within the frequency range estimated by the probe manufacturer) and drag red lines onto either side of it to define the boundaries for fitting. Click "fit data" and "calculate spring constant" and then save this value.

3.3. Deflection Sensitivity Calibration in Water

Since the force measurements on cells will be conducted in liquid, the deflection sensitivity must first be recalibrated on a glass slide in water.

1. Place a 50 mm Petri dish in the dish holder of the EasyAlign and fill it with about 2–3 mL of deionized water.
2. Stand the AFM head up vertically on the EasyAlign and use a pipette to carefully place one or two drops of deionized water onto the probe. Carefully lay the AFM head down horizontally so that the tip holder is in contact with the water, but only partially submerged. Do not allow liquid to reach the electrical connections between the probe holder and the *z* scanner.
3. Turn the EasyAlign on and realign the laser onto the end of the cantilever if necessary. Set the horizontal deflection to 0 and the vertical deflection to around −6 V.
4. Add a few drops of water to the microscope slide (see Note 4) and place the AFM head onto the stage so that the probe contacts the water and forms a meniscus.
5. Wait a few minutes for the vertical deflection to stabilize (see Note 14), reset it to −6 V, and then perform a "slow engage" as in step 10 of Subheading 3.2. Switch to the Ramp Menu and raise the AFM head 10 μm using the step motor.
6. Follow steps 13 and 14 of Subheading 3.2 to update the "deflection sensitivity" in water and save this value.
7. Withdraw the AFM head at least 300 μm and place it horizontally on the EasyAlign so that the probe is in contact with the water in the dish (see Note 15).

3.4. Force Measurements on Cells

Since physically indenting a cell can stimulate rearrangements of the cytoskeleton and possibly alter cellular mechanical properties (30, 31), we recommend indenting each cell only once and assaying a large number of cells to account for both spatial heterogeneity within a cell as well as cell to cell heterogeneity across the population.

1. Place the cell culture sample on the microscope stage and secure it with the appropriate magnetic sample clamp. If the sample is on a coverslip, remove the coverslip from the cell culture medium, wick excess medium from the bottom of the coverslip with a Kimwipe®, place it on a microscope slide (preferably hydrophobic), and add a few drops of medium to the top of the coverslip. Do not add too much medium or it will run underneath the coverslip and cause it to float.
2. Carefully place the AFM head on the microscope stage so that the probe contacts the cell culture medium and forms a meniscus. If the sample is thick, first raise the AFM head further using the joystick or the Navigation Menu to avoid crashing the probe.
3. Repeat step 5 of Subheading 3.3 to engage on the surface and then use the joystick to position a cell under the AFM cantilever.

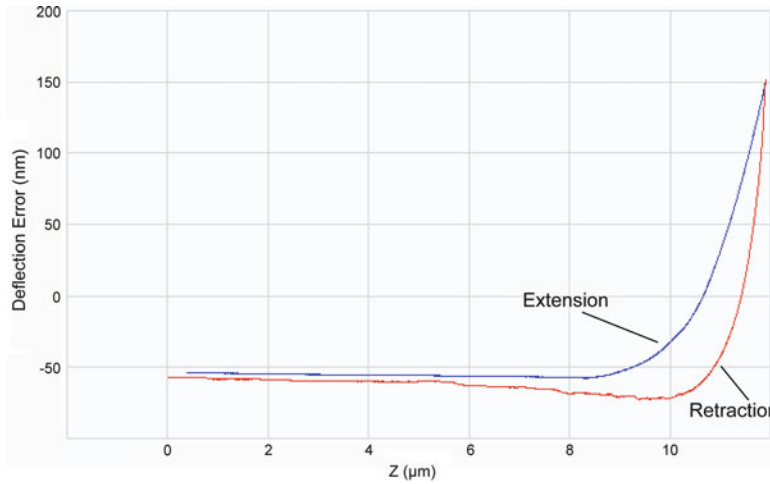

Fig. 3. Screenshot from the Nanoscope software showing a force curve taken on a living cell plotted as the vertical deflection (nm) vs. the *z* position of the probe (μm). The initial position of the probe is near Z = 0, and Z increases as the probe is lowered down to the cell during extension. Once the cantilever deflection reaches the deflection setpoint, the cantilever is retracted back to the initial *z* position. For clarity, the extension and retraction curves are labeled, and the font size for the axes has been enlarged.

When measuring the elastic modulus of a cell, the tip should be near the center of the cell but not above the nucleus.

4. In the Ramp Menu, change the "forward and reverse velocities" to 5 μm/s (which corresponds to 0.21 Hz), make sure the "Z-closed loop" is on, set the "trigger threshold" to about 100 nm, and click the "continuous save" button to save the data in a designated folder.
5. Click the "single approach" button to acquire a force curve (see Note 12). The curve should have a flat baseline with a smooth indentation (Fig. 3), and in the RealTime Status Window, the colored bar indicating how far the tip was extended should be green (see Note 16).
6. Use the joystick to move another cell under the AFM tip and continue taking force curves (see Note 17). Throughout the experiment, use the step motor to move the AFM head up and down as appropriate and adjust the vertical deflection to always be between −2 and −6 V. Be sure to raise the AFM head before moving to a different part of the sample in case the sample surface is not flat. If the cells are rounded or vary significantly in height, raise the AFM head between measurements to avoid scraping cells off the substrate and onto the AFM cantilever (see Note 18). If the probe has multiple cantilevers and one of the unused cantilevers hangs lower than the cantilever being used, that unused tip will drag along the surface. In this case, move along the sample in a zig-zag pattern to avoid indenting

cells that have already been touched by the low hanging tip. Alternatively, the longer cantilevers can be carefully sheared off with a tweezer prior to mounting the probe.

7. When ready to switch samples, withdraw the AFM head at least 300 μm and place it horizontally on the EasyAlign so that the probe is in contact with the water in the dish and repeat step 1 through step 6.
8. When finished for the day, stand the AFM head up vertically on the EasyAlign box and wick away excess liquid from the bottom of the probe holder with a Kimwipe®. Slide the probe holder off of the scanner and place it on the probe stand.
9. Use the tweezers with synthetic tips to carefully remove the probe from the probe holder and dip it in deionized water. Wick away excess water from the probe by touching the bottom of it to a Kimwipe® and then place it back in the probe container. We reuse probes several times until they appear dirty or their spring constants change significantly.
10. Clean the probe holder with water, dry it with a Kimwipe®, and place it back in its container. To decontaminate the probe holder, a 15% bleach solution can be used.
11. After exporting data (see Subheading 3.5), close the Nanoscope software and turn off the Nanoscope controller. The E-box can stay on. Turn off the microscope and its accessories, including the camera, light source, and shutter controller.

3.5. Data Analysis

We now outline the strategy for analyzing a single force curve in which we first plot the data, define the baseline and normalize the curve, calculate the contact point, and fit the data to a Hertz model for a pyramidal tip to calculate the elastic modulus (see Note 19). We use MATLAB® to analyze our data, but other programs capable of nonlinear least squares fitting should also work.

1. To export the data from the Nanoscope software, right-click on the files and choose to export as ASCII files. Choose "deflection error" for data type, "native" for units, and check the boxes for "extend" and "ramp" under the force curve options. The software will export each force curve as a separate text file with the first column containing the z position of the probe in nanometers (which starts at 0 and increases as the probe is lowered to the sample) and the second column containing the vertical deflection of the cantilever during probe extension in volts (see Note 20).
2. Import the data into MATLAB® as a 2 column matrix and multiply the second column by the "deflection sensitivity" (nm/V) measured in water to convert the vertical deflection from volts to nanometers.

3. Plot the z position data on the x-axis and the vertical deflection data on the y-axis. Define the baseline of the curve by choosing two points along the flat part of the curve, at least 2 μm apart, and calculate the slope of the line between these two points. Multiply the slope by the z position data and subtract these values from the vertical deflection data to straighten the curve so that the baseline is perfectly horizontal.
4. Calculate the average deflection along the baseline and subtract this value from the vertical deflection data to normalize the curve so that it starts at zero deflection.
5. Several methods have been developed to calculate the contact point, which is the z position of the probe when it first indents the cell (32). We use a formula from Domke and Radmacher (33, 34) in which two points of deflection are used to fit the indentation portion of the curve and to extrapolate back to where the deflection is near zero:

$$\mathrm{cp} = \frac{(z_2 - d_2) - (z_1 - d_1)(d_2 / d_1)^n}{1 - (d_2 / d_1)^n}$$

where cp is the z position at the contact point, d_1 and z_1 refer to the vertical deflection and z position of the first data point to be fit, d_2 and z_2 refer to the vertical deflection and z position of the second data point to be fit, and $n = 0.5$ for a pyramidal/conical tip (or $n = 2/3$ for a spherical tip). We have found that choosing vertical deflections around 5–15 nm for the first point and 100 nm for the second point (or the maximum deflection) gives good results. Overlay the contact point on a plot of the vertical deflection vs. the z position data to confirm that the point lies just before where the curve starts to slope upwards.
6. For each data point after the contact point, calculate the indentation force, $F = k \times d$, and the indentation depth, $\delta = z - d$, where k is the cantilever spring constant (N/m), d is the vertical deflection (nm), and z is the z position of the probe (nm).
7. Calculate the elastic modulus (E) of the cell by using a nonlinear least squares fitting method (e.g., lsqcurvefit) to fit the indentation force (F) to the Hertz model for a pyramidal tip (25):

$$F = \frac{3}{4} \times \frac{E \tan(\alpha)\delta^2}{(1 - \nu^2)}$$

where δ is the indentation depth (nm), α is the tip half angle in radians (estimated by the probe manufacturer), and ν is the Poisson's ratio of the cell, which is frequently assumed to be 0.5 but has been measured for some cell types to be around 0.37 (35, 36). Fit the data up to 1–2 μm of indentation into the cell and plot the fit against the experimental data to confirm that the fit is appropriate (see Note 21).

4. Notes

1. The BioScope Catalyst system has an optional heated stage that replaces the sample holder plate in the baseplate and a perfusion cell that enables circulation of both liquid and gas through the cell culture sample. While these features permit long-term culturing of cells during AFM experiments, both the heating and fluid circulation can introduce noise in the cantilever deflection. Therefore, for short experiments, we typically do not use these features and instead take measurements for no more than 30 min after the cells are removed from the incubator. For longer experiments, CO_2-independent media can also be used.
2. When the Bioscope Catalyst head is placed on the microscope stage, the optical light path is partially blocked by the scanner. This can result in poor phase contrast, especially for the Ph1 phase rings that are typically used in ×10 and ×20 objectives. We have found that Ph2 phase ring objectives give good phase contrast, and we prefer to use a ×20 Ph2 objective (CFI Plan Apochromat DM20x, Nikon Instruments Inc., Melville, NY). Note that the phase ring should be aligned while the AFM head is engaged on a sample in liquid.
3. When choosing an AFM probe, it is important to consider the geometry of the tip because sharp tips can puncture the cell and thereby underestimate the elastic modulus. (On a force curve, a puncture event appears as an abrupt, sawtooth-like decrease in force during indentation.) Spherical tips, usually colloidal probes, are ideal for indenting cells (*37*), but are expensive if purchased commercially and can be difficult to assemble in the lab with consistent geometric and mechanical properties. We prefer to use the TR400PSA (OTR4) silicon nitride probes (Olympus), which have cantilevers with spring constants of 0.02 and 0.08 N/m and tetrahedral pyramidal tips with a 35° half angle. These tips are sharpened for imaging, but we do not usually experience cell puncture events. In the past, we preferred the DNP and MLCT silicon nitride probes (Veeco Instruments, now known as Bruker AXS Inc.), which were unsharpened with the same pyramidal tip shape, but the recent versions of these probes have sharper aspect ratios with pyramid half angles of 15–25°.
4. We have found that treating microscope slides with hydrophobic solution makes cantilever calibration in water easier by preventing the water from wetting the surface. Also, when cells are cultured on a coverslip that is then placed on top of a microscope slide, making the slide hydrophobic prevents liquid from running underneath the coverslip.

5. The Bioscope Catalyst has sample clamps that hold 35 and 60 mm cell culture dishes, 50 mm Petri dishes, and standard microscope slides. We do not recommend using 35 mm culture dishes, however, because the probe holder can collide with the walls of a small dish. For most experiments, we prefer to seed cells on 18 or 25 mm circular glass coverslips, which are placed on top of a microscope slide with a few droplets of medium before force measurements. For cells that are not well adhered to the substrate, we prefer to use 50 mm glass-bottom Petri dishes since transferring the coverslips and adding medium can dislodge the cells. Note that the surface area available for probing is limited by the range of XY stage movement to a square of 14 mm × 14 mm.
6. AFM cantilevers are fragile and will break if handled roughly. Grasp the probe substrate from the sides near the center and be careful not to flip or drop it. Visually inspect the probe before use to ensure that the cantilevers are clean and intact.
7. If the laser sum is low despite the laser being correctly positioned on the end of the cantilever, the mirrors that direct the laser onto the photodiode detector may be completely misaligned. Try turning both the horizontal and vertical detector positioning knobs from one extreme to the other until the laser sum increases and the vertical deflection can be set to a negative number between –2 and –6.
8. If the vertical deflection equals the deflection setpoint before the probe reaches the surface (which can happen due to noise fluctuations or drift), the software will assume that the surface has been reached and false engage. If this happens, set the vertical deflection to a more negative value and initiate "slow engage" again.
9. If the cantilever is still not in the field of view, raise the AFM head by clicking the "withdraw" button and place the head on the EasyAlign. Check to see that the baseplate is centered on the microscope and/or try repositioning the probe in the center of the glass window on the probe holder.
10. The "Z-closed loop" corrects for drift in the movement of the *z* piezo and should be on for all calibration steps and force measurements. While most of the ramp settings are saved after withdrawing and reengaging, the "Z-closed loop setting" is not and needs to be reset each time the probe is engaged. To check whether the "Z-closed loop" was on for previous measurements, check the "height sensor" data. If the "Z-closed loop" was on, the extension and retraction "height sensor" curves will be straight lines and overlapping.
11. The vertical deflection values range from –12 to +12 V, and it is important to stay within these boundaries for accurate results.

Since the vertical deflection becomes increasingly positive during a force measurement, the vertical deflection should be set to a negative number between –2 and –6 V. When the "deflection sensitivity" is first calculated in air, however, the vertical deflection should be set to –1 or –2 V to be near the value used when calculating the cantilever spring constant.

12. During a "single ramp," the probe is lowered to the surface, raised up by the "ramp size," and then lowered again until the "trigger threshold" is reached. This double tapping ensures that the probe always reaches the surface. In contrast, for a "single approach" the probe is lowered only once until the "trigger threshold" is reached or until the *z* scanner is fully extended. We use "single ramp" for cantilever calibration because it always produces an ideal force curve, but we prefer to use "single approach" for cell measurements to avoid contacting the cell more than once.
13. Each time the laser position is moved during the experiment, the "deflection sensitivity" must be recalibrated because the laser will be reflected onto the photodiode detector at a different angle.
14. When the probe is lowered onto a sample in liquid, the cantilever may maximally deflect and then rapidly relax back down. The vertical deflection often levels out after a few minutes, but it can continue to drift for 10–20 min. We usually wait only a few minutes before acquiring data and adjust the vertical deflection between measurements to account for drift if necessary.
15. Once the AFM probe is wet, always hold the AFM head horizontally to keep the probe wet and to prevent water from dripping near the electrical connections between the probe holder and the AFM head. Do not let the probe dry while sitting in the probe holder. When the water starts to evaporate, the surface tension generated between the probe and the probe holder may permanently bend the cantilevers.
16. The colored bar in the RealTime Status Window indicates how far the *z* scanner was extended when the tip contacted the surface. Green is ideal and means that the *z* scanner was in the middle of its range of extension when the tip contacted the surface, red means that the tip was either too close or too far from the surface (and these data should not be used), and yellow is in between. The positions of the two black horizontal lines on the colored bar indicate whether the AFM head should be moved up or down before the next measurement. If the lines are near the top of the bar, the tip is too close to the

surface and the AFM head should be moved up using the step motor. If the lines are near the bottom of the bar, the tip is too far from the surface and the AFM head should be moved down. If the colored bar is red and raising/lowering the AFM head does not help, check that the vertical deflection is between –2 and –6 V.

17. To work quickly, we view the sample through the microscope eyepiece (because this viewing window is larger than through the camera port) and control the Nanoscope software from that position using a wireless mouse. By doing this and by moving the sample to the next cell as early as possible (while the probe is still retracting from the previous force curve), we are able to attain 100–150 force curves in 30 min.
18. When cell debris accumulates on the AFM cantilever, it can interfere with the force measurements. When cell debris is visible, perform a force curve on glass to determine if it interferes with the measurement. To remove cell debris, gently lift the AFM head out of the liquid and then place it back down, repeating if necessary.
19. The Hertz model includes assumptions that the material being indented is isotropic, linearly elastic, and infinitely deep, none of which are strictly valid for a cell. Several modifications to the Hertz model have been developed to try to account for these limitations (38–41), but most researchers still use variations of the Hertz equations ((25, 26) for a pyramidal tip, (24) for a conical tip, (24) for a spherical tip). In addition, since calculating the contact point for soft samples can be difficult, at least one group has developed a strategy for analyzing force curves without needing to define a contact point (42).
20. While the data can be exported in "display units" to show the vertical deflection in nanometers instead of volts, we prefer to export the data as "native units" and to use the "deflection sensitivity" to convert the values ourselves. This is particularly important if the probe was changed during the experiment or if the "deflection sensitivity" was recalibrated.
21. We fit the data multiple times using a series of increasing indentation depths (e.g., 0.5, 1, 1.5, 2, 2.5 μm, etc.) and confirm that the elastic modulus for each of the fits is similar. Occasionally, the elastic modulus increases significantly with increasing indentation depth, which can indicate that the cell was too thin in that region and that the mechanical properties of the underlying substrate were detected. Alternatively, this has also been proposed to reflect nonlinear elastic behavior of the cell (27).

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