

A practical guide to AFM force spectroscopy and data analysis

"Fishing" for single molecules

The atomic force microscope (AFM) is best known for its high-resolution imaging capabilities, but it is also a powerful tool for sensitive force measurements. The AFM can be used to "fish" for single molecules and investigate their structure and biological function. The study of interaction forces with the AFM has led to deeper understanding of many biological and physical processes down to the single molecule level.

The AFM tip is able to probe an extremely small interaction area (using a tip radius in the range of 5-50 nanometers), and this gives it a high sensitivity to small forces. To make these forces accessible, the tip is suspended on an very soft spring, the AFM cantilever (images in Figure 1). These cantilevers are usually silicon or silicon nitride beams, 100-200 microns long, and allow forces in the pico-Newton range to be measured. This corresponds to the order of magnitude of forces that are required to separate receptors from ligands or to break a single hydrogen bond.

In AFM imaging modes, the cantilever is usually scanned over the surface to produce a three dimensional image of the surface. In AFM "force spectroscopy" experiments, the cantilever and tip are moved directly towards the sample until they are in contact with it, and then retracted again, while the interaction between the tip and sample is measured. This may then be repeated at different locations to build up a map of the tip-surface interaction, or can be repeated at the same point to give a full statistical understanding of the interaction.

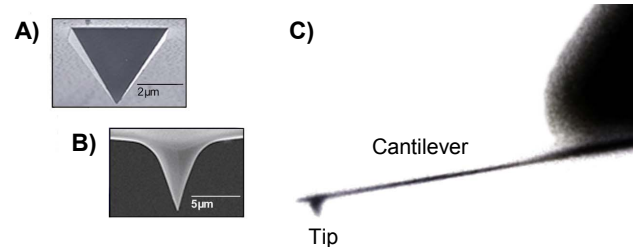


Fig 1. Scanning electron microscopy images of typical pyramidal (A) and cone-shaped (B) AFM tips and an optical microscope image of an AFM cantilever and tip (C).

The applications of these experiments range from nano-mechanical investigations of elastic properties to protein unfolding and investigations of single chemical bonds [1]. This technical report introduces the data collected in force curves and the standard steps that are used in converting the data for a quantitative force analysis.

Overview

The schematic diagram in Figure 2 shows the movement of the cantilever and tip during the force spectroscopy experiment – towards the sample (the approach part) and then away again (the retract part). The image series reflects different points in the approach and retract cycle, the lateral position of the cantilever is usually constant. A flexible software interface allows for waiting times at different points of the movement – for example at the sample surface to give time for molecules to adsorb to the cantilever tip or at the end of the retract movement, to give the surface time to recover.

Table 1 gives a summary of interactions that have been measured using the atomic force microscope, to give an idea of the experiments that are possible and the parts of the force curves where particular interactions are seen.

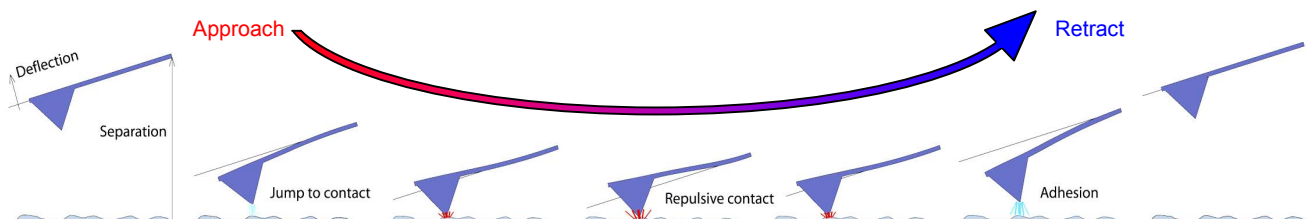


Fig. 2 Schematic diagram of the vertical tip movement during the approach and retract parts of a force spectroscopy experiment.

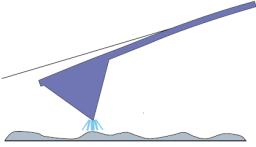
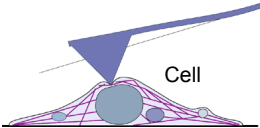
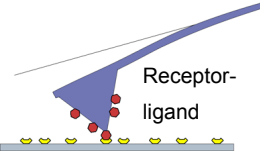
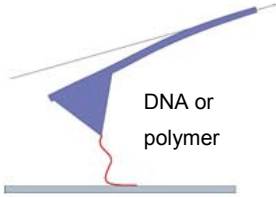
Approach	
Tip far away (10 - 100 microns)	No interaction
Tip approaching (few microns)	<ul style="list-style-type: none"> • Electrostatic forces • Long-range interactions from adsorbed molecules, e.g. polymer brush
Tip close to surface (nanometers to atomic distances)	<ul style="list-style-type: none"> • Van der Waals • Capillary forces (in air) • DLVO/screened electrostatics (in aqueous solutions) • Chemical potential • Magnetic • Solvation forces (water layering) 
Contact	
Tip indenting sample	<ul style="list-style-type: none"> • Stiffness (Young's modulus, elastic response) • Viscoelastic response (variable rates or indentation depth) • Measurement of active forces (e.g. generated by cells) 
Retract	
Tip lifting off surface (few atomic distances to nanometers)	<p>Adhesion:</p> <ul style="list-style-type: none"> • Non-specific (including chemical affinity, surface coatings) • Ligand-receptor (e.g. antibody-antigen) • DNA hybridisation (e.g. matched or mismatched pairs) • Cell surface interactions 
Tip further away (nanometers to hundreds of nanometers)	<p>Stretched molecules between tip and surface:</p> <ul style="list-style-type: none"> • Protein unfolding, pulling out of membranes • Entropic elasticity • DNA stiffness, structural transitions and "melting" • Other conformational changes in stretched molecules, e.g. chair-to-boat transition in sugar rings <p>Other stretched attachments e.g. membrane tethers formed on cells</p> 
Tip far from surface (1-5 microns)	<p>Connections broken between the tip and surface, no further interaction.</p> <p>Adhesion strength can be measured between attached molecules and the surface when the attachments break.</p>

Table 1. Overview of some of the interactions measured at different points during a force spectroscopy cycle.

The forces experienced by the cantilever as it approaches from several microns above the surface can give information about long-range interactions, such as electrostatic effects. As the cantilever comes close to the surface (nanometers or atomic distances), shorter-range forces, such as Van der Waals or capillary forces can be measured. When the sum of these forces is attractive, the tip may jump into contact with the surface, an effect referred to as “snap-in”.

Once the cantilever has made contact with the surface, it may be pushed into the surface with some force, in which case the viscoelastic properties of the sample can be investigated, to give the Young's modulus, or stiffness for different strain rates or maximum applied forces. When the cantilever is pulled away from the surface, adhesion forces can be measured, and single molecules attached between the tip and surface can be stretched.

Understanding simple force curves

The data from an experiment is often displayed as a simple x-y plot. The height positions for the approach or retract of the cantilever are usually chosen as the x-axis, and the cantilever property that is being measured is the y-axis. This is usually the deflection of the cantilever, which can give a direct measure of the interaction force. Sometimes other signals are used – for example current or fluorescence, and particularly in dynamic AFM spectroscopy modes the amplitude or phase of the cantilever oscillation. These “force-distance” plots are often referred to as force curves. Unfortunately, there are many different conventions for how the data is displayed, so data from different sources may look quite different.

The most direct way to plot the data shows the movement of the piezo during the force curve (as a distance) against the deflection of the cantilever. The deflection is measured by an optical beam deflection setup which delivers an electrical signal (in Volts, as the signal from the photodiode) that is proportional to the cantilever deflection. The example shown in Figure 3 reflects a typical interaction for an uncoated cantilever in air approaching a hard, incompressible hydrophilic surface such as glass or

mica. Approach (red) and retract (blue) curves are both plotted on the same axes.

As the cantilever approaches the surface, initially the forces are too small to give a measurable deflection of the cantilever, and the cantilever remains in its undisturbed position. At some tip-sample distance, the attractive forces (usually Van der Waals, and capillary forces in air) overcome the cantilever spring constant and the tip jumps into contact with the surface. Once the tip is in contact with the sample, it remains on the surface as the separation between the base of the cantilever and the sample decreases further, causing a deflection of the cantilever and an increase in the repulsive contact force.

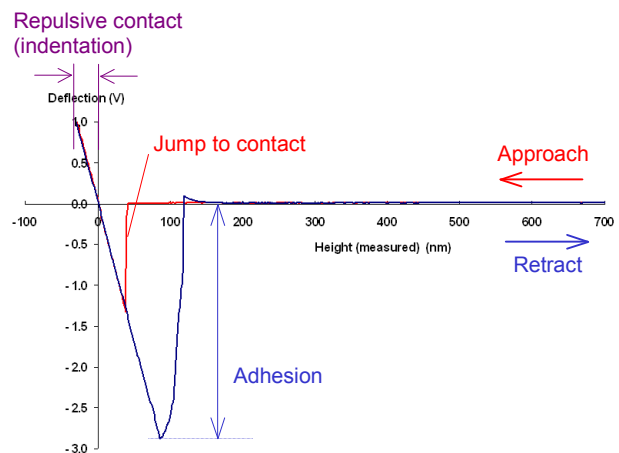


Fig. 3 Plot of approach (red) and retract (blue) curves for a contact mode cantilever and clean mica in air.

As the cantilever is retracted from the surface, often the tip remains in contact with the surface due to some adhesion and the cantilever is deflected downwards. For measurements in air, such as the one shown in Figure 3, there is usually adhesion from capillary forces between the tip and sample.

Hydrophilic surfaces are covered with a thin (few nanometers) water layer in ambient conditions, and often the tip and sample are both hydrophilic. In this case, the water layers can join when the tip and sample are close together, forming a capillary neck between them. When the cantilever is retracted from the sample this combined water layer causes a strong adhesion. At some tip-sample distance, the force from the cantilever will be enough to

overcome the adhesion, and the tip will break free. Often the adhesion between the tip and the sample in water is actually reduced, since there is no longer this special water surface layer interaction, as can be seen from the reduced adhesion in the curves in Figure 4.

Calibration of the cantilever deflection

The deflection of the cantilever spring is directly proportional to the tip-sample interaction force, but there are two measurements required to convert the photodetector signal into a quantitative value of force. The first stage is to calibrate the distance that the cantilever actually deflects for a certain measured change in photodetector voltage. This value depends on type of cantilever, but also on the optical path of the AFM detection laser, and will be slightly different each time the cantilever is mounted in the instrument. Once the deflection of the cantilever is known as a distance, *x*, the spring constant, *k*, is needed to convert this value into a force *F*, using the well-known Hooke's law:

$$F = kx$$

A force curve between a plain cantilever tip and a bare hard substrate is used to determine the sensitivity of the experimental setup. This is a measurement of the deflection of the tip in nanometers for a given movement of the detection laser on the photodetector. The repulsive contact region, where the deflection rises steeply upwards, is linear for a hard surface and tip. Therefore the software can easily determine the factor for converting Volts into nanometers. This measurement can then be used for calibrating the applied forces when the samples of interest are investigated. The sensitivity can then also be used to set the oscillation amplitude in intermittent contact mode as actual nanometers of oscillation.

Since the hard repulsive interaction regime is used for the sensitivity measurement, it should usually be performed at the end of an experiment, to avoid damaging the tip. Figure 4 shows part of a force curve from a plain contact mode cantilever on mica under aqueous buffer solution (PBS). The data here is the "raw" information, as cantilever deflection is displayed in Volts. The gradient of the

repulsive contact region is marked on the figure, and the calculation in this case gave a sensitivity of 22 nm/V. This value can then be used to convert the deflection data along the whole curve into nanometers.

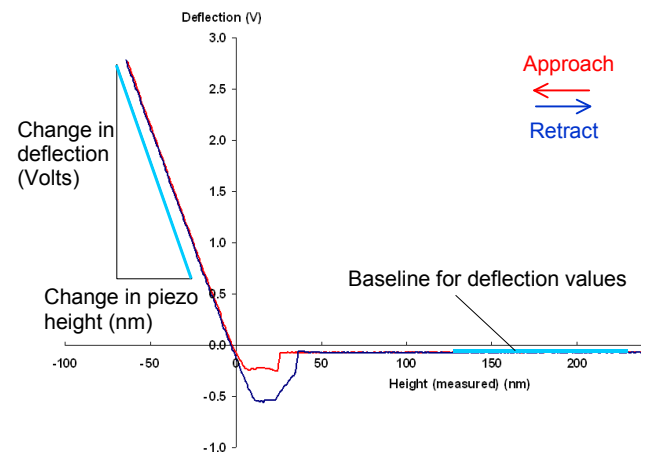


Fig. 4 Plot of approach (red) and retract (blue) curves in water. The gradient chosen for sensitivity measurements and the baseline offset for the deflection are both marked on this plot.

When the cantilever is far from the surface, the interaction forces are virtually zero (the flat part of the curve on the right hand side in Figure 4). This offset (which may be due to the initial settings of the equipment, or to thermal drift) should be subtracted from all the deflection data in order to calculate the true interaction force. The baseline value is also marked in Figure 4. Note that the very flat baseline is due to the suppression of optical interference effects by the state-of-the-art detection system employed in the NanoWizard® AFM.

For older systems or homebuilt equipment it is important to take account of the piezo hysteresis in these measurements, in order to get an accurate value for the sensitivity. When a piezo moves over a relatively large range, nonlinearity and hysteresis are problems that can lead to false data for the cantilever position. These problems can be overcome by using a separate system to simultaneously measure the z-piezo position during the movement, such as the precision sensors used in the JPK NanoWizard® AFM. This measured z value is independent of the piezo nonlinearity and hysteresis.

The benefits of piezo linearization can be seen in Figure 5, where the same force measurement is shown with linearized (A) and unlinearized (B) height scales. In the upper image (A), where the sensor height values are used, the approach and retract curves exactly overlay, as they should for a hard clean sample surface. The natural piezo hysteresis can be seen in the curves in the lower panel (B).

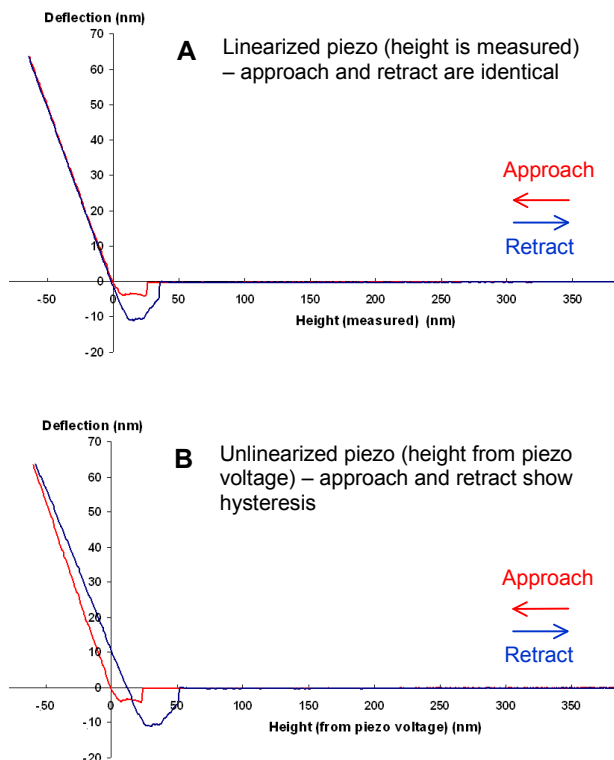


Fig. 5 Comparison of results obtained with piezo linearization (A) and without piezo linearization (B). Plots of approach (red) and retract (blue) curves using a contact mode cantilever and clean mica in water.

The curves in Figure 5 show the converted data from the experiment in Figure 4. The baseline has been shifted to take account of the deflection offset far from the surface, and the sensitivity value has been used to convert the deflection into nanometers. The deflection values in Figure 5A are now ready to be converted to units of force.

Cantilever spring constant calibration

For quantitative force measurement, the spring constant of the cantilever must be calibrated, so that the nanometers

deflection of the cantilever can be converted into actual force values. There are various different ways of calibrating spring constants of cantilevers, depending on the equipment that is available, and only the three most common methods are described here. Each method is subject to some limitations, and if the force measurement is particularly important, the spring constant should be calibrated using more than one method to verify the results.

Calculation from the cantilever geometry

When cantilevers are purchased, they are usually supplied with a data sheet, which includes the spring constant. The values are generally given as a range, and may have been calculated from the average cantilever geometry, rather than having been experimentally measured for each cantilever. It is possible to calculate the spring constant, if the exact shape of the cantilever, and the Young's modulus of the material is known.

A problem with this method is that the spring constant depends on the thickness of the cantilever cubed. This value is also the smallest dimension of the cantilever. Therefore the spring constant is extremely sensitive to slight differences in cantilever thickness between batches of cantilevers. The quoted spring constant values from manufacturers are useful for choosing a cantilever for a particular application, but are usually not reliable for quantitative force measurements.

Measurement using a reference cantilever

When a reference cantilever with a known spring constant is available, other cantilever spring constants can be calculated by comparing the deflection of the two cantilevers when they are pushed together [2]. The unknown cantilever can be mounted as normal in the AFM, and the reference cantilever is used as the "sample". The cantilevers are approached to each other so that they just overlap at the end, and a force curve is performed.

The slope of the repulsive contact part of a force-distance curve on a hard surface and on the reference cantilever must be compared. The deflection of the unknown cantilever compared with the reference cantilever gives a

conversion for the spring constant. This calculation is most accurate if the two cantilevers have similar values. The method is relatively straightforward if the AFM is equipped with a good optical imaging system, so that the cantilevers can be well aligned, but properly calibrated reference cantilevers are expensive.

Measurement using the thermal noise method

Fluctuations in the environment (in air or fluid) constantly provide small force impulses, as can be seen for example in the diffusion of small particles (Brownian motion). Soft cantilevers are susceptible to thermal fluctuations, and the AFM can be used to measure and analyse the movements. The thermal noise spectrum is a plot of the cantilever fluctuations as a function of frequency; on average the greatest amplitude will be seen around the cantilever resonance frequency. An example is shown in Figure 6. Longer integration times of the cantilever motion give better signal to noise in the spectrum.

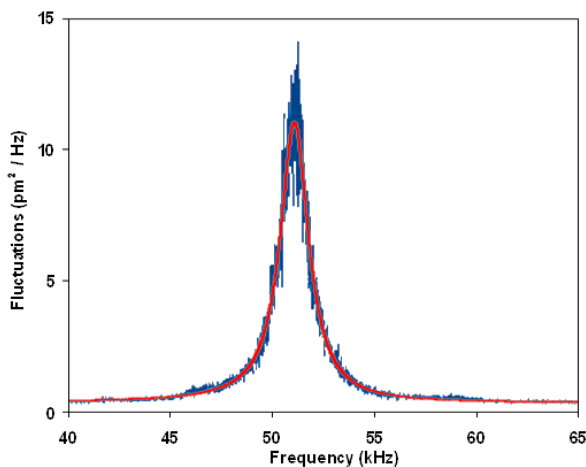


Fig. 6 Thermal noise measurement using the JPK software of a contact mode cantilever in air, showing the measured cantilever resonance peak (blue) and the Lorentz fit (red). The fit in this case gave a spring constant of 0.147N/m.

The amplitude of the fluctuations for a given temperature depends only on the spring constant of the cantilever [3]. The thermal resonance curve can therefore be fitted to a Lorentz function, which allows calculation of the spring constant. This method has the advantage that it can be done in-situ with software analysis. Thermal noise

measurement and automatic spring constant calculation is offered as standard in the JPK SPM software, so this can be performed without any extra equipment.

Force conversion and resolution

When the deflection of the cantilever is known in units of length, as in Figure 5A, then it is straightforward to convert to force units. The deflection (in meters) is multiplied by the spring constant of the cantilever (in N/m) to give a force in Newtons. For molecular interactions, forces are usually seen in the range of picoNewtons (1 pN = 10⁻¹² N) to nanoNewtons (1nN = 10⁻⁹ N).

So far, all the conversion has been in the y-axis, or deflection/force values. There is also one conversion needed for the height, or x-axis values, however. The change in piezo height has been used for the distance between the tip and the sample, but in fact this must be corrected for the deflection of the cantilever. If the cantilever deflects towards the sample, for example, then the separation between the tip and the surface will be less than expected from the piezo position alone. Since the cantilever deflection and position are available in the same units, this can be easily corrected by subtracting the cantilever deflection from the piezo height.

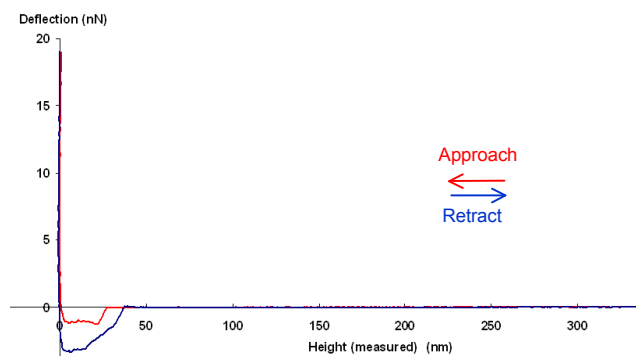


Fig 7. Force-separation (corrected height) plot for the data shown in Figures 4-5.

This conversion leads to the gradient of the steep upwards part of the curve becoming vertical. Once the tip is in contact with the surface, the tip-sample separation remains at zero while the force is increased by the cantilever pushing towards the surface. An example of the final

converted curve is shown in Figure 7. Here the x-axis is plotted as the actual tip-sample separation, rather than the piezo movement and the deflection values are now displayed in units of force. The data here would now be ready for analysis.

One important consideration for force measurements is the smallest detectable force. This is affected by many different factors, and the properties of the cantilever itself play an important role in determining the force resolution. In the AFM instrument, the deflection of the cantilever is measured by the optical detection system and there will be a limit on the smallest detectable deflection. For softer cantilevers, this limit on the detectable deflection corresponds to a lower force value. Therefore it might seem that the force resolution should always be improved by using a softer cantilever.

When the forces and distances involved correspond to the thermal energy, however, the thermal fluctuations of the cantilever become significant. The scale for thermal energy at room temperature can be calculated by using the Boltzmann constant, so for example at 25°C, $k_B T = 4.1 \times 10^{-20}$ J. The units of energy correspond to units of force times units of distance, so 1 Joule = 1m x 1N. It can be helpful to express the thermal energy in units that are more appropriate to single molecules, for example as $k_B T = 41$ pN nm. The spring constants for the cantilevers can also be expressed in more helpful size ranges; for example, a soft cantilever for force spectroscopy with a spring constant value of 0.01 N/m is equivalent to 10 pN/nm. A force of 10pN would therefore cause a deflection of 1 nm for this cantilever.

Softer cantilevers have larger thermal fluctuations, and the soft cantilevers used for many single-molecule force spectroscopy experiments have significant thermal fluctuations. In this case, the force resolution is usually limited by the thermal noise of the cantilever. The effective limit on the force resolution for a particular experiment can be seen from the noise on the deflection baseline.

An example curve for a soft cantilever (10 pN/nm spring constant) is shown in Figure 8, for parameters closer to the

normal force spectroscopy experiments. Here, for example, the maximum applied force is much lower than in the measurements for sensitivity calibration in Figures 4-7. To show the force resolution, a part of the baseline in Figure 8 is shown with a magnified height scale in the inset curve.

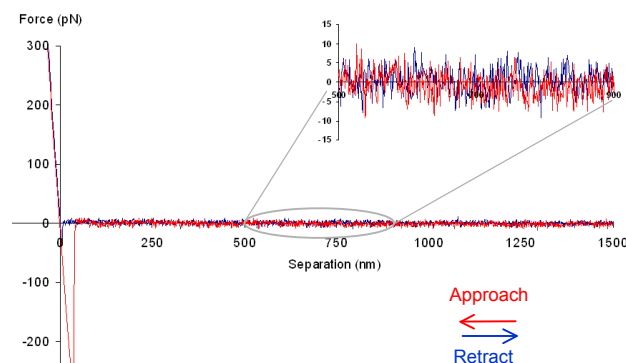


Fig 8. Force-separation plot for a 10pN/nm cantilever in buffer. A part of the baseline is displayed in a magnified view in the inset curve, to show the resolution limit of the force data due to thermal fluctuations.

Advanced force measurement applications

Over a soft, compressible sample in liquid, such as a gel or a living cell, the overall shape of the force curves is often quite different from the hard-surface examples shown here. The curves are more likely to show a gradual increase in force, without the sharp onset of the interactions seen in air. The gradient of the repulsive contact region may not be constant, as the sample stiffness (change in indentation depth for a given change in force) may change as the structure is compressed. For thin samples on a hard surface, the linear repulsive contact regime may be seen at large deflections, as the tip may indent the sample enough to feel the supporting surface below.

The tip-sample contact area will change as the tip indents a soft surface, so sometimes a larger object with a well-known shape is attached to the tip. This may be a micron-sized sphere or bead, such as the fluorescent microspheres used as calibration standards. This gives a more defined and reproducible contact area for a given indentation depth. This can be useful for indentation

experiments, and also for adhesion measurements if single molecule experiments are not the aim.

When the tip is retracted from the surface, there is often a hysteresis observable, if the sample is not perfectly elastic, since on the retract movement the surface has already been compressed. If the piezo hysteresis has been eliminated (as, for example, in Figure 5A), then this sample hysteresis can be measured accurately for different approach and retract rates.

Different types of adhesion events can be seen, depending on what links the tip and the sample. If long molecules connect the tip and sample, then the stretching of these tethers produces characteristic curved sections on the retract deflection curves. When the molecule is pulled free from either the tip or sample, or unfolds or even breaks somewhere between, the deflection jumps sharply as the tip is freed. Multiple events may be seen as connections of different lengths are stretched and broken as the tip retracts.

Other physical properties can also be measured, as well as the actual interaction force [4]. Electrical signals, such as the conductivity through the tip and sample, or external properties such as fluorescence can be monitored during the tip movement. In dynamic force spectroscopy experiments the cantilever is oscillated during the movement, and the oscillation amplitude and phase signals can be measured. This can give information about the viscoelastic properties (elastic and dissipative response) of the sample. If this is combined with a magnetized tip or an

external electrical potential, information about magnetic and electric properties of the sample can be obtained.

Varying the speed of the tip movement can give further information about the sample responses. The approach and retract rates can be varied independently, and waiting times added at the surface or at the retracted position. This flexibility allows exploration of the time-dependent response of the sample, for example adsorption times or on-rates for binding, relaxation times for mechanical properties, or re-folding rates for proteins.

A detailed review of force spectroscopy applications is outside the scope of this technical note. The steps outlined in this report to convert the cantilever deflection into quantitative force measurements are the starting point for any more complex analysis. The NanoWizard® hardware and software provides the perfect combination to study forces on the nano scale.

Literature

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