Measuring and Understanding Force Distance Curves

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Measuring and Understanding Force-Distance Curves

An AFM is an incredibly flexible and powerful instrument. A large part of this versatility comes from the ability of the AFM to make non-topographic measurements. That is, to do more than simply measure images of a sample surface. AFM has been used to develop a wide range of different experiments, all based on the ability of AFM to probe a sample on the nanoscale. Because of this, AFM has been referred to as a "nanotechnology toolbox". Many of these measurements are based on measuring force-distance curves (sometimes known as F-D curves). Unlike normal AFM imaging, when measuring force-distance curves, the probe of the AFM is moved in a vertical direction (perpendicular to the sample surface), instead of scanning along the surface horizontally. While this is carried out, the AFM measures the force between the probe and the sample as a function of the distance the probe moves; thus a force-distance curve can be plotted.

There are a large number of possible applications of force-distance curves. The most common ones will be described here. One of the most frequently used applications of measuring force-distance curves is what's known as a nanoindentation measurement. In this mode, the stiffness or other viscoelastic properties of a material can be measured. Since the probe is small, and the AFM works as a highly accurate nanopositioner, it's possible to measure these properties in very small samples, or alternatively form a "map" of sample stiffness with nanoscale resolution. Force-distance curves can also be used to determine the force of adhesion between a colloid probe and a surface. This can be useful in modelling colloidal interactions. In biology, force-distance curves are often used to measure the force of interaction between different molecules, such as antibodies and antigens. This method is sometimes known as "force spectroscopy", as a range of interaction forces are usually measured. This idea can be further extended to measure the interactions between molecules immobilized on the probe and a cell surface, or even to measure forces between two cells. AFM force-distance curves can also probe protein unfolding by measuring the force on the probe while denaturing a protein by physically pulling it.

There are three different stages to review when considering force-distance experiments:

A. Preparing for the experiment

   This can mean planning the type of experiment, and in some cases preparing the probe needed, or performing special sample preparation.

B. Measuring the force-distance curves

   In general this is quite simple, although the technique is quite different to that used in imaging experiments.

C. Processing and analyzing force curves

   Unlike AFM images, force curves generally need considerable processing and analysis to obtain meaningful information from them.

The following sections of the application note will explain each of these three methods.

Preparing to Measure Force-Distance Curves

The first step is planning the experiment. It is useful to consider what kind of experiment will be performed. For example, to determine sample stiffness, nanoindentation experiments are performed. These use the data recorded with the probe moving towards the sample. On the other hand, for adhesion measurements, the data recorded while the probe is moving away from the sample are used. The difference between these data sets is illustrated in figure 1 which shows a schematic force-distance curve.

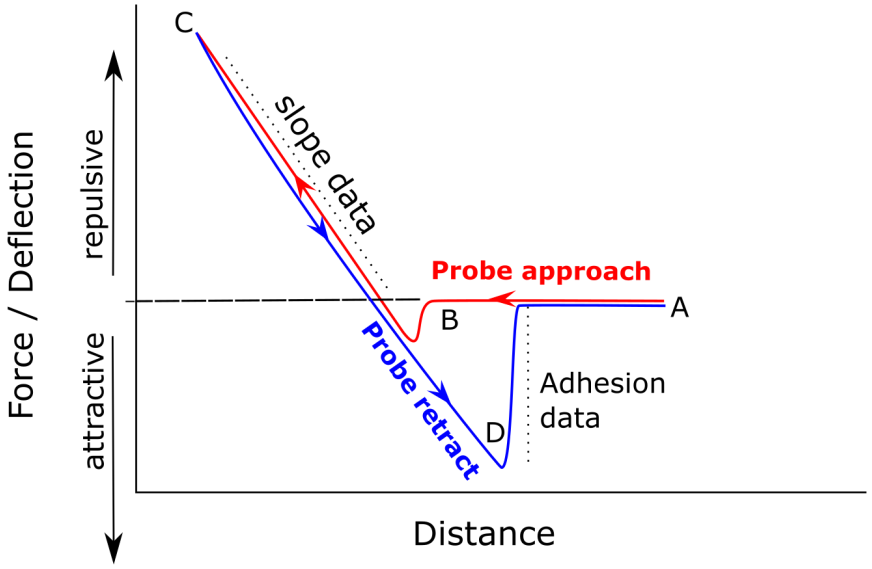


Figure 1: schematic force-distance curve

As shown in figure 1, different parts of the curve are used for different experiments. Fortunately, the AFMWorkshop advanced force-distance software measures and records both parts of the curve by default. For nanoindentation, it's worth considering where the "reverse trigger" (point C in figure 1), will be set, as this is important for nanoindentation, but less so for adhesion measurements.

One of the most important considerations in planning force-distance experiments is deciding which probe to use. For most experiments, the considerations that need to be taken into account are two-fold: firstly, the spring constant of the cantilever is important, and secondly, so is the tip shape. For nanoindentation experiments, the spring constant of the probe used must be similar to that of the sample. For example, a very stiff probe will push through very soft materials without deflecting at all, but could be appropriate for a much stiffer sample. Usually for force spectroscopy, highly flexible levers are used, as this maximizes the sensitivity of the experiment. For nanoindentation, the shape of the probe tip is extremely important. The most accurate results will be obtained by using a probe with a well-known shape, such as a colloid probe[1]. Whichever probe is used, the geometry used must be input as a parameter in the fitting process (see below). The size of the probe tip is also important, if very small features are to be probed, or high resolution in the case of mapping experiment is important, a sharp tip, similar to those used for imaging may be used. However, for very soft or sensitive materials, or heterogeneous samples, where averaging is important (for example, biological cells), a large, micron-scale probe could be used. It's worth mentioning that

colloid probes for these experiments can be bought ready-made from some vendors [2], or prepared in-house[3]. For force spectroscopy experiments, normal probes are typically used, although some preparation of the probe is usually carried out.

Preparation of Probes

Making adhesion measurements only makes sense if the experimenter understands what the nature of the probe is. In some cases, interaction with a normal AFM probe (i.e. a pyramid-shaped tip made of silicon or silicon nitride) might be useful. Even in these cases, it is important to thoroughly clean the probe before making measurements, since probes as delivered from the manufacturer can be contaminated with any number of materials. Several methods for cleaning AFM probes have been described [4–6]. A very common and simple method is the use of uv-induced air plasma. In most cases, the interaction between specific materials is to be probed, meaning the nature of the probe surface must be altered. In this case, there are several methods used to modify the probe so that the interaction between two materials of interest can be probed. For intermolecular interactions, normal probes are typically chemically modified, in order to present a molecule of interest at the probe tip. Many different methods of carrying out this process have been discussed, some popular ones including physisorption and the use of silane chemistry[7]. For other types of interactions, such as particle-particle, particle surface, or cell-cell probing, typically the probe is modified so as to adhere to a cell or particle, then the particle is picked up using the AFM from a surface, and used as a probe[8]. Note that for any of these probe-modification strategies to be successfully used, it's best to begin with a probe cleaning step as described above.

Another consideration is the environment used for probing. This should match that most relevant for the application under study, since AFM can be applied in almost any environment. For example, many biological interactions should be measured under physiological conditions, and often at a controlled temperature also. On the other hand, for some measurements, interactions under ambient conditions can be probed. It is important to remember that under these conditions, capillary forces from water on the probe and sample tend to dominate. Some researchers control the humidity in the probing environment to overcome this issue. For nanoindentation measurements, the environment may be less important, unless the sample needs to be kept hydrated. So for nanoindentation of cells, a physiological environment would normally be used (i.e. buffer or growth medium), while for polymers or nanostructures, an ambient environment works fine.

Sample preparation for measuring force-distance curves needs no special steps, other than those typically used for imaging, and can even be less stringent, since the interaction in force-distance measurement is perpendicular to the surface no lateral forces are generated. In general, the sample must be clean, and well fixed-down onto a substrate.

Measuring Force-Distance Curves

In general, measuring force-distance curves is a simple procedure. Force-distance curves are measured in contact mode, so the instrument is set-up in a way similar to that used for contact mode measurements.

Usually, the first step is to make an approach in contact mode. However, there are some circumstances in which it might be advantageous to begin measuring curves without moving into contact first. For instance, with a specially modified probe, the modification of the probe might be so delicate that the user worries about the approach mechanism damaging the probe coating. In

general, however, the probe approach routine in the software should be set up so that no damage occurs on approach. If the user wishes to measure force-distance curves before approach, the advanced-force distance module in the AFM Workshop software allows the user to try this by selecting “non-feedback mode” in the advanced force-distance tab.

Once the system is in feedback with the sample surface, generally the first step is to perform a force curve for calibration purposes. Calibration is discussed in the next section.

Calibrating Cantilevers

There are two aspects of the system that need to be calibrated for each experiment. These are light lever sensitivity, and cantilever spring constant. The light lever sensitivity is measured in nm/V, and is a measure of how the photodetector in the AFM reacts to bending of the cantilever. This factor is sometimes also known as inverse optical lever sensitivity or invOLS. This is a property of the optical alignment as well as instrument design, and changes depending on exactly how the optical alignment is made. Thus, if the laser is moved on the cantilever, the sensitivity must be determined again. The simplest way to determine this factor is to measure a force curve on an incompressible surface. In this way, the slope of the force curve in the contact region represents cantilever bending only, and so gives a value for the light lever sensitivity as shown in the figure below.

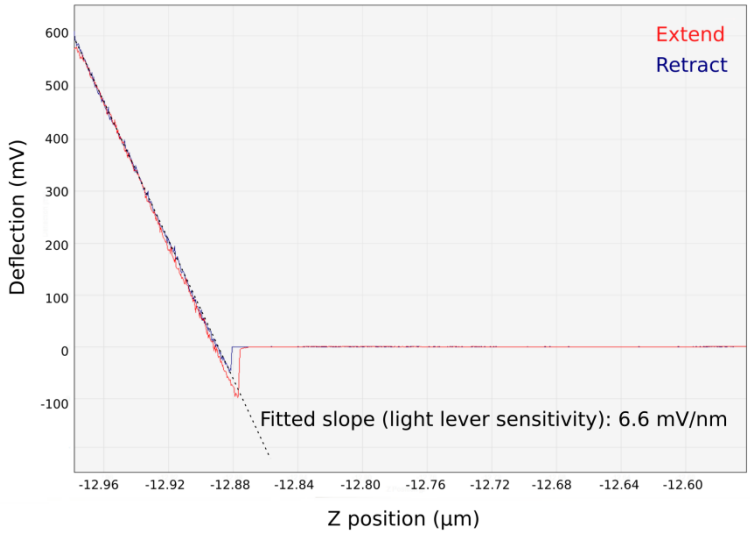


Figure 2: light lever sensitivity determination. A straight line is fitted to the slope of a force curve measured on an incompressible sample to determine the light lever sensitivity

Once the light lever sensitivity is obtained, deflections in volts can be easily converted to nanometers by multiplying by this factor. The second calibration factor required is the cantilever spring constant. Although commercial cantilevers are sold with an estimated spring constant, the average value supplied with the cantilever can be wildly inaccurate [9]. There are several methods described to calibrate cantilever spring constant. Of these, possibly the most accurate is the use of laser vibrometer[10], however this a rather expensive instrument. A commonly-used method is based on determining the thermal noise-induced vibration[11, 12]. Finally, a very popular method that is simple to carry out in the so-called Sader method which is based on having known cantilever geometry and measuring the quality factor of the oscillation resonance [13, 14]. AFMWorkshop supplies an applet that can perform this calibration. Having determined this value which is expressed

in newtons per meter, the deflection is transformed into a force, typically in the 100s of piconewtons range. In this way, quantitative data can be extracted from force-distance curves, whether in adhesion or nanoindentation modes.

As illustrated below for the AFMWorkshop control software, most AFM control software offers a wide variety of settings that can be applied to the collection of force curves. For many experiments most of these settings will not need to be altered. To collect a single for curve in the AFMWorkshop Software, carry out the following procedure.

1. Choose a location within the scan range by clicking within the box marked Image (bottom left). If you have already acquired an image, you can choose a clean region, or a place that is otherwise of interest.
2. Perform a Tip Approach if not already in feedback by pressing the Tip Approach “Start” button, and waiting until approach is complete.
3. Choose the length of the force curve. For a 1 micron force-distance curve, select 500 nm in Z retract and 500 nm in Z extend.
4. Check the T-B Reverse Trigger box, and use as T-B Value, a value about 200 mV above the Current T-B Value. To apply less force, decrease this difference, to apply more force, increase it.
5. To measure the force curve, click ”Start”. The curve will be recorded and displayed. The file-saving behavior can be set using the “Files” control.

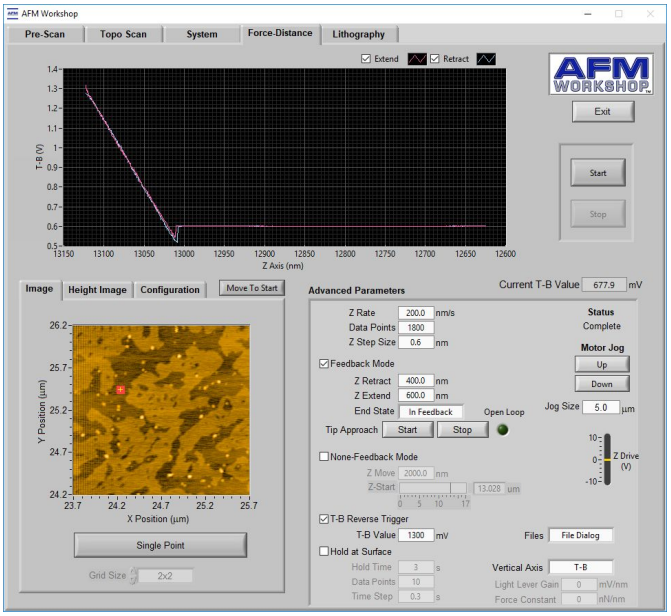


Figure 3: The advanced force-distance tab in use.

Force-distance curve maps, also sometimes known as layered imaging or force volume, can be extremely useful in a range of scenarios. At its most basic, a force map can be used to quickly obtain a large number of FD curves on a sample, such as is usually required for statistically significant results. In addition, force mapping can be used to determine the spatial distribution of different

materials. For example, in material science, it can be used to determine the distribution of different components in a composite material. In biology, it can be used to determine the distribution of receptors on a cell surface. In this mode, the software sets up a grid of points across a predetermined area. At each point a force curve is measured in turn. This method can even be used as an imaging method, since lateral forces are completely eliminated. This can be useful for imaging of extremely sensitive or delicate samples. The image below shows the AFMWorkshop software in the force map mode.

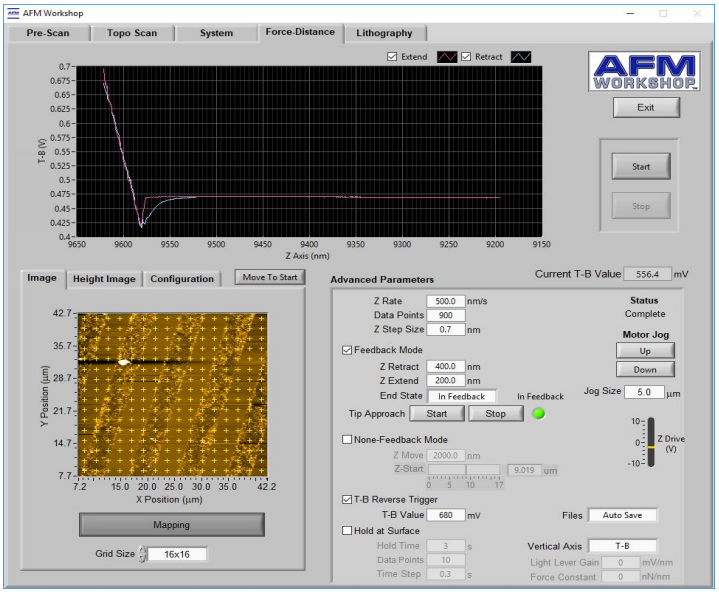


Figure 4: AFMWorkshop software in Force mapping mode. The yellow crosses in the image at bottom left show where the force curves will be measured.

For force mapping, it's generally advised to obtain one or more single force curves before measuring the map, to optimize F-D curve conditions. After this, it's a simple matter of choosing the relevant area, the number of points required, and clicking start. One word of caution: force mapping can be extremely time-consuming. In the example above, 16×16 curves, with 800 nm ramps at 500 nm/s would take approximately ten minutes to run. But if the resolution was increased to 128 × 128 F-D curves, the map would take about 10 hours to acquire.

Understanding and Processing Force-Distance Curves

Force-distance curves can have a variety of different features depending on the sample, the probe, and the probing environment. Some example of different curves are shown in the figure below.

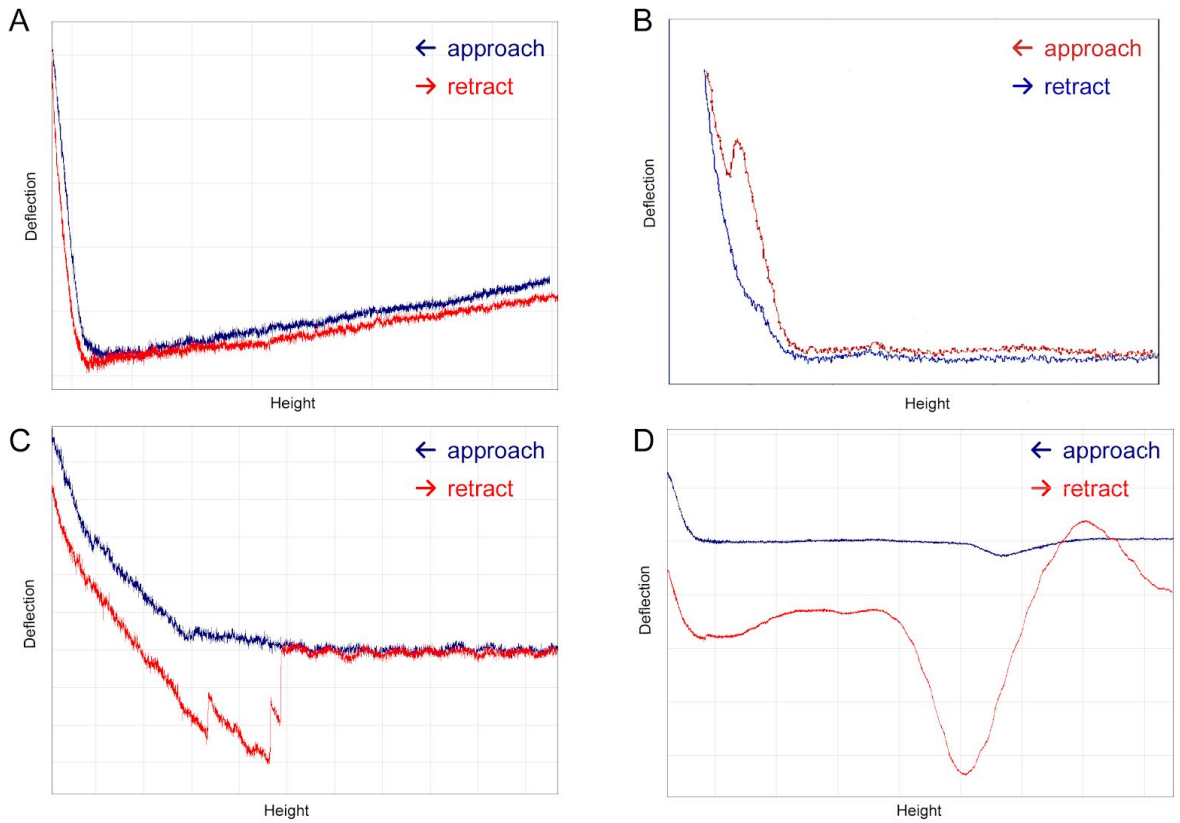


Figure 5: A number of different "non-standard" force curves. Some of these curves represent problems in the experimental setup, while others show unusual sample behavior

The first thing to note is that all these curves look quite different than the schematic curve in figure 1, and also different than the (real) curve in figure 2. Figure 2 shows a typical curve obtained on a hard surface in air. However, even under such conditions, the result obtained can vary quite a lot.

In figure 5A, the slope of the rightmost part of the extend curve is not horizontal. This can happen due to a variety of reasons, including laser interference, poor sample grounding, thermal drift, or other long range probe-sample forces. As mentioned below, one of the most important processing steps for force-distance curves is fitting the slope of this approach part of the curve, which can overcome such effects.

In figure 5B, there is a discontinuity on the upwards slope section of the curve, where the probe is indenting into the sample (the approach curve). This is a fairly common feature, and indicates a "breakthrough" of some material on the sample, or possibly force-induced movement of a feature of the sample. For example, when studying supported lipid bilayers, this feature can be used to confirm the presence of a bilayer, or even to measure properties of the bilayer [15].

On the other hand, in figure 5C, the retract curve is unusual. Compared to a "simple" curve such as that shown in figure 2, there appear to be multiple pulloff events of various sizes. In fact, this curve was measured in an experiment measuring cell-cell adhesion, and reflects the multiple receptor pairs that the two cells made, and are subsequently broken during retraction. It's also worth noting that the retract curve is separated from the approach curve. This is probably due to viscous behavior of the cell. Biological materials rarely show ideal elastic behavior.

Finally, in figure D, we see a transient event in the retract curve, possibly caused by something passing through the laser path during measurement. This curve was measured in liquid, and it could have been a floating cell or other piece of debris passing through the laser beam. In a case like this, the force-distance curve would often be discarded if the effect altered the analysis of the curve.

In processing force-distance curves, there are several steps that are commonly applied to all curves, while some others are used only for certain kinds of experiments. The common steps will be discussed first.

Application of Calibration Factors

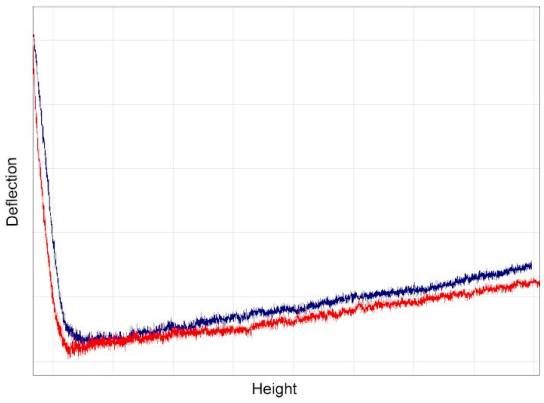
The first step in processing of force-distance files is commonly calibration of the raw data using the measured values of the light lever sensitivity and the cantilever spring constant. These are measured for each experiment, as determined above. The application of these factors is simple, in the AFMWorkshop Advanced Force-Distance tab, simply select "Vertical axis". Setting this to "Force" allows the user to type in the values of "Light Lever Gain" and "Force Constant" (cantilever spring constant). Once this is done, the F-D curves will be displayed and saved in force rather than deflection automatically. This procedure converts the file from deflection-distance curve to a force-distance curve.

Correction for Cantilever Bending

The second step is to take account of cantilever bending, and convert piezo extension to distance. Again, this is a simple operation, which can be done with a simple click. In the analysis software SPIP, this is carried out automatically, if the Sensitivity (V/nm) value is entered, the result appearing in the force/sensitivity window. SPIP can also calculate the sensitivity for you if you do not have a measured value, to do this check the "Auto Calculate sensitivity" checkbox. After this step, the curve is a true force-distance curve.

Baseline Fitting

As mentioned above, many force-distance curves have tilted baselines (the normally flat, linear part of the curve where the probe is far from the surface). This operation simply fits a straight line to remove this tilt. Typically, the resulting horizontal line is set to be at zero force (0 nN). This operation is shown below.



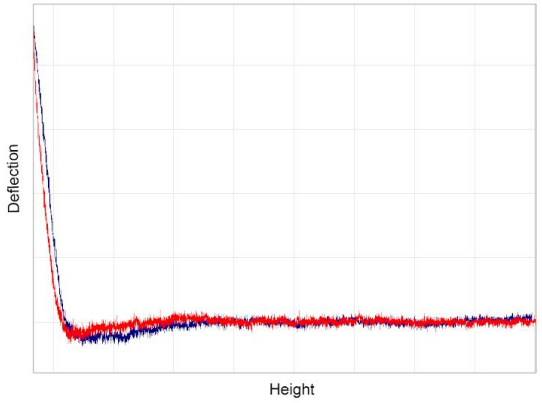


Figure 6: Baseline removal. The left figure shows the raw data, and on the right, the same data after baseline fitting. This step is important for all subsequent analysis.

In general, the most common types of measurements can be grouped into two categories: nanoindentation measurements, and adhesion (or force spectroscopy) measurements. Within each category there are further types of experiments. In data processing, analysis of nanoindentation measurements requires processing of the extension curve; while adhesion measurements are made using the retract curve.

Procedures for Analysis of Nanoindentation Data

The first step in analysis of data for nanoindentation is determination of the contact point. This is the point at which contact with the surface is first made. For stiff surfaces, this is easy to determine, while for soft or composite materials, such as cells, it can be trickier. This procedure can be manual, or commonly dedicated software will have an automated routine to determine this, and sometimes an option for the user to override the automatically –determined result if necessary. To perform this operation in SPIP, using the automated routine, open the Force Curve Analysis panel, switch to the Indentation panel, and select “auto” under “Point of zero indentation”. The force (separation) window will now reflect the new contact point as calculated by the automated routine.

Modelling of Nanoindentation

In order to determine the Young’s modulus of a sample, the curve is modelled using an extension of the Hertz model which is appropriate to a rounded probe tip (e.g. a colloid probe). On the other hand, for sharp pyramidal probes, a cone model (Sneddon’s law) can be more appropriate [1]. Several variables must be known in order to make this modelling. This includes the Poisson’s ratio of the material under study. This is generally taken to be 0.5 for biological samples, and estimates of the value can be found for other materials in the literature or on-line. Finally, the geometry of the probe used is needed. This can be determined via a number of methods [16], but can also be estimated from manufacturer’s guidelines. Once these factors are inserted into the procedure, fitting is done automatically by the routine. A visual representation is usually presented to the user, to determine the quality of the fit some examples of good and bad fit are shown below. It’s also possible to use the residual of the non-linear regression to determine quality of fit. Typically, in a run of 100 nanoindentation experiments, 5 to 25% might be rejected by the user. These poor fits can occur due to a variety of reasons, but are mostly due to heterogeneities in the sample composition, probe alterations, or transient fluctuations in the probing environment (see figure 5). To perform this procedure using your data in SPIP, under fitting model, select either cone indentation (Sneddon) or sphere indentation (Hertz), depending on the shape of the probe used. On the Indentation tab, the approach curve should be selected under “curve to fit”. The Poisson’s ratio and probe shape should be altered based on the probe and sample, respectively. It’s recommended to activate the Inspection Box, and manually select the “force fit range”. Upon clicking “Calculate”, a new window will pop up with the calculated parameters. It might be necessary to click “Field Chooser” in this window and check Young’s Modulus to ensure the correct results are shown.

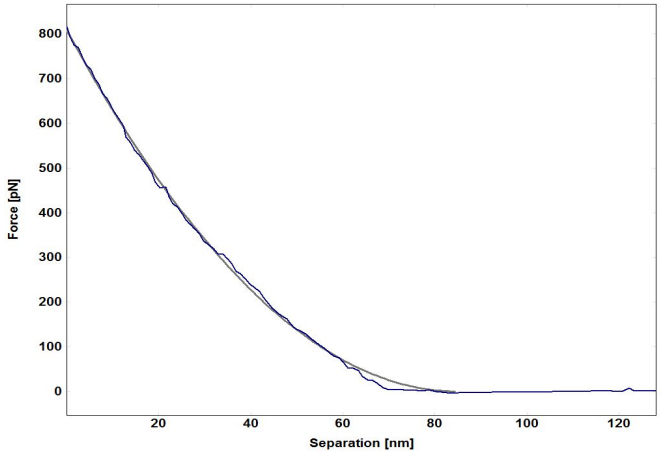


Figure 7. Example of fitting to nanoindentation data. The data is plotted in blue, and the fitting in gray. This result gave a value of 61 kPa for the Young's modulus of a 5 percent agarose gel, matching literature values.

Procedures for Analysis of Adhesion Data

In adhesion measurements, a variety of values can be obtained, depending on the complexity of the force curves obtained and the system probed, but the most common factors measured are total adhesion, and work done. These are illustrated in the figure below. Adhesion is very simple to obtain, and can be measured manually, by just measuring the height (in newtons) of the pulloff feature in the curve. The work done is measured as the area of the adhesion portion of the curve below the 0nN line. Either curve can be determined simply by the software as illustrated in the figure below.

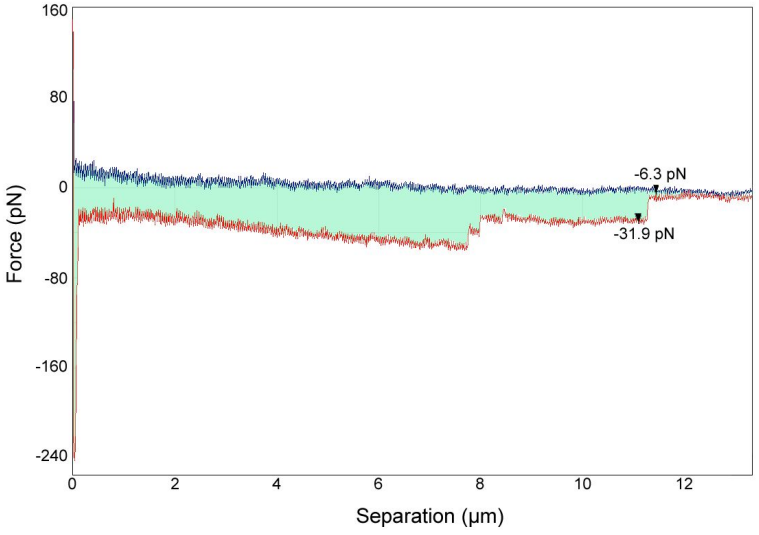


Figure 8: Analysis of adhesion data. The green area shows the total energy in the interaction (in this case 0.562 fJ), while the difference between the two markers show the adhesion force for an individual step, which is 25.6 pN.

More complex analyses can be required in certain cases, for example, to measure protein unfolding, fitting to a work-like-chain model is usually used, while for cell-cell adhesion determination of multiple pull-off forces can be carried out.

Overall, measurement of force-distance curves add a wealth of possibilities to AAFM measurements, in a hugely diverse number of scientific and technical fields. Care must be taken in experimental planning, and considering the correct probe, and probe-modification for the experiment. Also, data analysis is important, and can be time-consuming. However, these methods greatly increase the power of atomic force microscopy.

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