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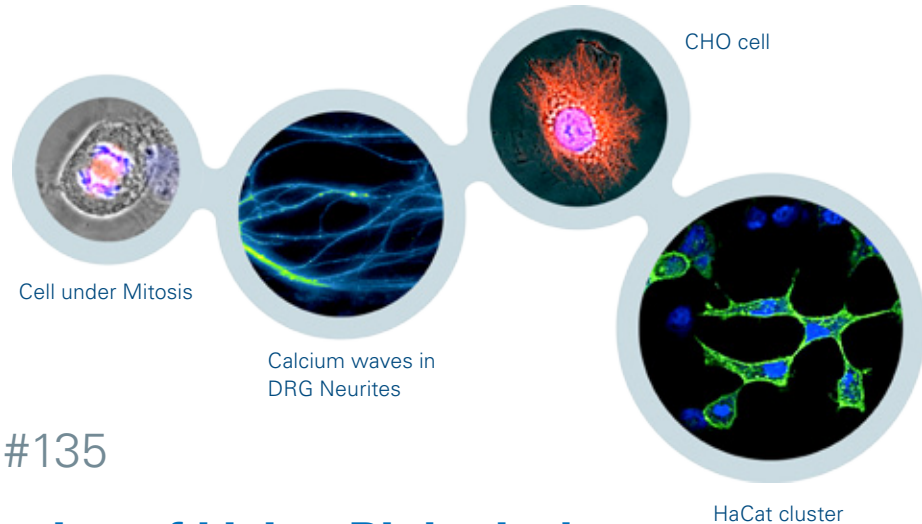
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Application Note #135

Quantitative Imaging of Living Biological Samples by PeakForce QNM Atomic Force Microscopy

It is now well established that measuring the mechanical properties of living cells *ex vivo* can be a good indicator of the health of the organism from which they were extracted. Atomic force microscopy (AFM) is a powerful investigation and diagnostic tool, especially in force mode. Nevertheless, force spectroscopy suffers from several limitations, including the speed of acquisition, a relative lack of resolution, and the fact that it doesn't directly provide quantitative information. In order to fill this gap, Bruker has developed a new imaging technique, PeakForce QNM[®], which provides much more informative data at a high resolution and with remarkable ease-of-use. Up to now, this very promising technique has been tested successfully on a wide range of samples but not on living organisms. This application note gives an overview of the biological samples that can be imaged using this technique and the information that can be extracted from the atomic force microscopy data.

AFM and Cell Mechanics

Since its development,¹ AFM has proven itself to be a tool of choice to image super soft biological samples, especially with the emergence of TappingMode^{™2} and force spectroscopy³ and the fact that it is one of the few microscopy techniques that allows observation of cells under near-physiological conditions.⁴ It is now well documented that the mechanical properties of living cells

can be quantified by AFM and used to estimate the effect of drug treatment or different types of pathologies, as well as such natural processes as aging.⁵⁻⁷ For instance, it is becoming more and more evident that cancer cells and their normal homologues express clearly different mechanical properties.⁸⁻¹¹ Moreover, AFM is often used to correlate elastic behavior and cell migration or division.¹²⁻¹⁵ The vast majority of these studies are based on TappingMode, single-force curves, or force-volume measurements.

TappingMode offers the advantage of applying negligible nominal, friction and shear forces, and phase imaging reflects the energy dissipated between the tip and the sample during each tap on the surface. Nevertheless, the nature of the phase signal remains always unclear since it is a mixed contribution of several surface and volume parameters, including adhesion forces, contact area, elasticity, viscosity and dissipation. Even if working at specific ratio setpoints allows more sensitivity to surface or volume properties, a quantitative extraction is impossible.

Force volume is another powerful technique based on force measurements achieved on a matrix of points defined by the user. Stiffness (and by extrapolation the sample's Young's modulus) and the adhesion between the tip and the sample can be extracted from each force curve. If the tip is functionalized with a molecule of interest, specific unbinding events can also be detected on the retraction

curve. Although it is very commonly used in the AFM world to probe cell mechanics, force volume suffers from two main drawbacks. First, acquiring high-resolution data is highly time consuming. This is why in the majority of force volume studies, the stiffness and adhesion images have a much lower resolution than the height channel. Secondly, the elastic properties cannot be directly quantified. The data have to be post-processed by using external software so that the Young's modulus can be extracted.

To overcome these bottlenecks, Bruker has developed PeakForce QNM. This revolutionary new mode is based on Bruker's new Peak Force Tapping™ technology, with which the probe is oscillated at a low frequency while force curves are generated each time the AFM tip taps on the sample's surface. Many parameters can be extracted quantitatively in real time from each of these force curves, which makes PeakForce QNM the most powerful AFM technique for nanoscale material property characterization available today.

Easy, High-Resolution Quantification of Sample Mechanical Properties

PeakForce QNM enables direct extraction of quantitative nanomechanical information from biological samples without sample damage. It's based on Peak Force Tapping technology, during which the probe is oscillated in a similar fashion as it is in TappingMode, but at far below the resonance frequency (1 or 2 kHz depending on the tool). Each time the tip and the sample are brought together, a force curve is captured. However, where the feedback loop maintains the tapping amplitude constant in TappingMode, Peak Force Tapping controls the maximum peak force on the probe. These forces can be controlled at levels much lower than contact mode and even lower than

TappingMode, allowing operation on even the most delicate biological samples.

Figure 1 illustrates the different force fields experienced by the probe during an approach-retract cycle, as well as all the information that can be extracted from the generated force curves. When the probe approaches the sample (figure 1a), it's pulled down toward the surface by attractive forces, which are mainly capillary, Van der Waals and electrostatic forces. At point B, those negative forces become higher than the cantilever's stiffness, which causes the tip to pull to the surface and then start indenting into the sample until the Z-position of the modulation reaches its maximum (point C). This position represents the maximum peak force value, which is used for the feedback control. After this point, the probe starts withdrawing until it reaches the pull-off point (the maximum adhesion point, which also corresponds to the minimum force). Then the tip continues retracting and reaches back to its original position (E) where (as in A) no more force field affects its motion.

By eliminating the time variable, one can plot the force versus the tip-sample distance (figure 1b), from which much information can be obtained. As in regular force mode, the maximum adhesion force between the tip and the sample can be extracted as the step height between the base line and the pull-off point. The peak force is defined as the vertical distance between the base line and the turn-away point. The deformation corresponds to the horizontal distance between the contact point and the turn-away point. By extrapolating the retraction curve close to the contact area, the software determines the Young's modulus using a DMT¹⁶ fit. This mechanics model assumes that the contact principle remains the same as in the Hertzian model

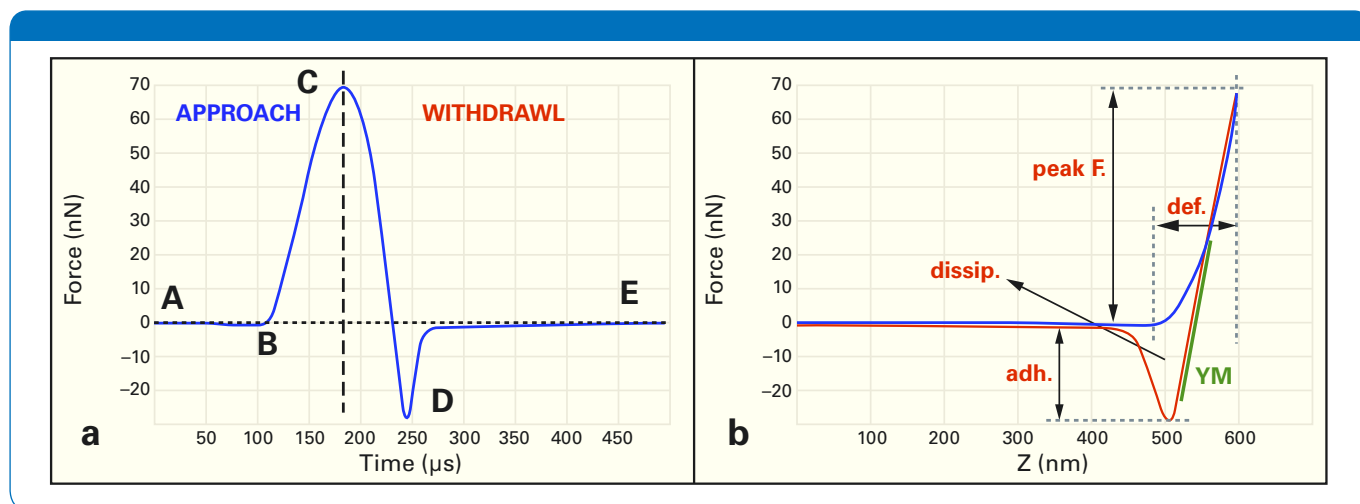


Figure 1: Working principle of PeakForce QNM. While the probe is oscillated, a force curve is recorded for each pixel of the image. To distinguish between the different portions of the tip trajectory, this example was recorded by using a TAP150A probe, which is typically used to image rather stiff and poorly compliant samples. On biological samples, the typical peak force can be up to a thousand times lower.

but considers additional attractive interactions focused inside an annulus located outside of the contact area (figure 2a). In that case, and considering the contact between a sphere and an elastic half-space, the force is related to the deformation by:

$$F = \frac{4}{3} E^* R^{1/2} d^{3/2} \quad \text{and} \quad \alpha = \sqrt{Rd}$$

where E^* represents the reduced Young's modulus, R the tip radius and d the deformation depth.

Eventually, the energy dissipated by the tip and the sample during each tap on the surface is obtained by integrating the area between the approach and the retraction curves.

Direct Quantification of AFM Signals on Biological Samples

When the probe is calibrated prior to the experiment, all the signals mentioned above will be directly quantitative. This calibration can be done as follows:

- 1) Engage on a stiff part of the sample (like glass) and record a force curve from which the deflection sensitivity can be calculated.
- 2) Withdraw and calculate the spring constant using "Thermal Tune".
- 3) Record a topography image of the Tipcheck sample to get a value of the tip radius R .
- 4) Having entered the estimated R value, adjust the deformation on a sample of choice. The sample to be scanned should have similar mechanical properties as the biological sample that will be investigated during the experiment. For non-biological samples, a list of reference samples, as well as a list of suitable AFM probes, can be found in Bruker Application Note #128.¹⁷ For purely biological samples, the tip can be calibrated on commercial gelatin gels, if the Young's modulus of the sample of choice is around 100 kPa. For softer samples, home-made calibration samples based on gelatin¹⁸ or agarose¹⁹ can enable measurements down to 1 kPa.

As the exploration of the mechanical properties of living cells is a key requirement today, and also a real technical challenge, the present document will review a list of typical applications that can be addressed using Bruker's PeakForce QNM technique, from the simplest to the most complex examples. Although this newly released technique has been successfully tested on polymers,²⁰ this application note marks the first time that it has been applied to living and super soft samples.

In most of the existing publications, the Hertz theory is used to estimate the contact mechanics because it's by far the

simplest since it considers that there are no adhesion forces between the tip and the sample. This is not true, although they can be greatly reduced in liquid. The DMT assumption is based on a Hertzian geometry but considers that the adhesion forces exist and are located outside the contact area and tends to underestimate it. It is mainly applicable to poorly compliant samples, having low adhesive properties and low contact radius.

On most of the tested samples, a Sneddon fit²¹ was used to extract the Young's modulus by capturing a HSDC (High Speed Data Capture) file on a scan line at a very high resolution (a few thousand of force curves). By comparing the force and height profiles, the non-desired parts (force curves captured on a portion of the sample that is not of interest, such as glass) can be excluded manually. The remaining force curves can be exported as a single file, post-processed by an external program,²² and the average Young's modulus can be calculated by considering different contact theories, such as the Sneddon model.

This mechanical theory considers the contact between an elastic half-space deformed by a rigid conical indenter (figure 2b), determining that the load is proportional to the square of the penetration depth. It is not applicable to ceramics or polymers but is commonly considered as most adapted for biological samples. The indentation depth and the tip radius are related by:

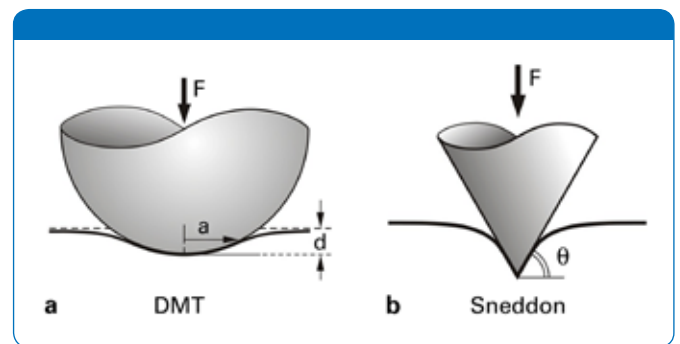


Figure 2: Contact mechanics in AFM. In a, the DMT fit is based on a Hertzian assumption but states that the adhesion forces are focused outside the contact area. This is well adapted to high-density polymers and poorly deformable samples. In b, the Sneddon fit considers the tip as an infinite conical indenter, which is well adapted to soft (biological) and deformable samples.

On such samples, a wide range of AFM probes have been tested and the recommendation is given in figure 3. The softest existing probes on the market are OBL-B, which have a nominal spring constant of 0.006 N/m and are thus appropriate to investigate super soft living cells, such as neurons.

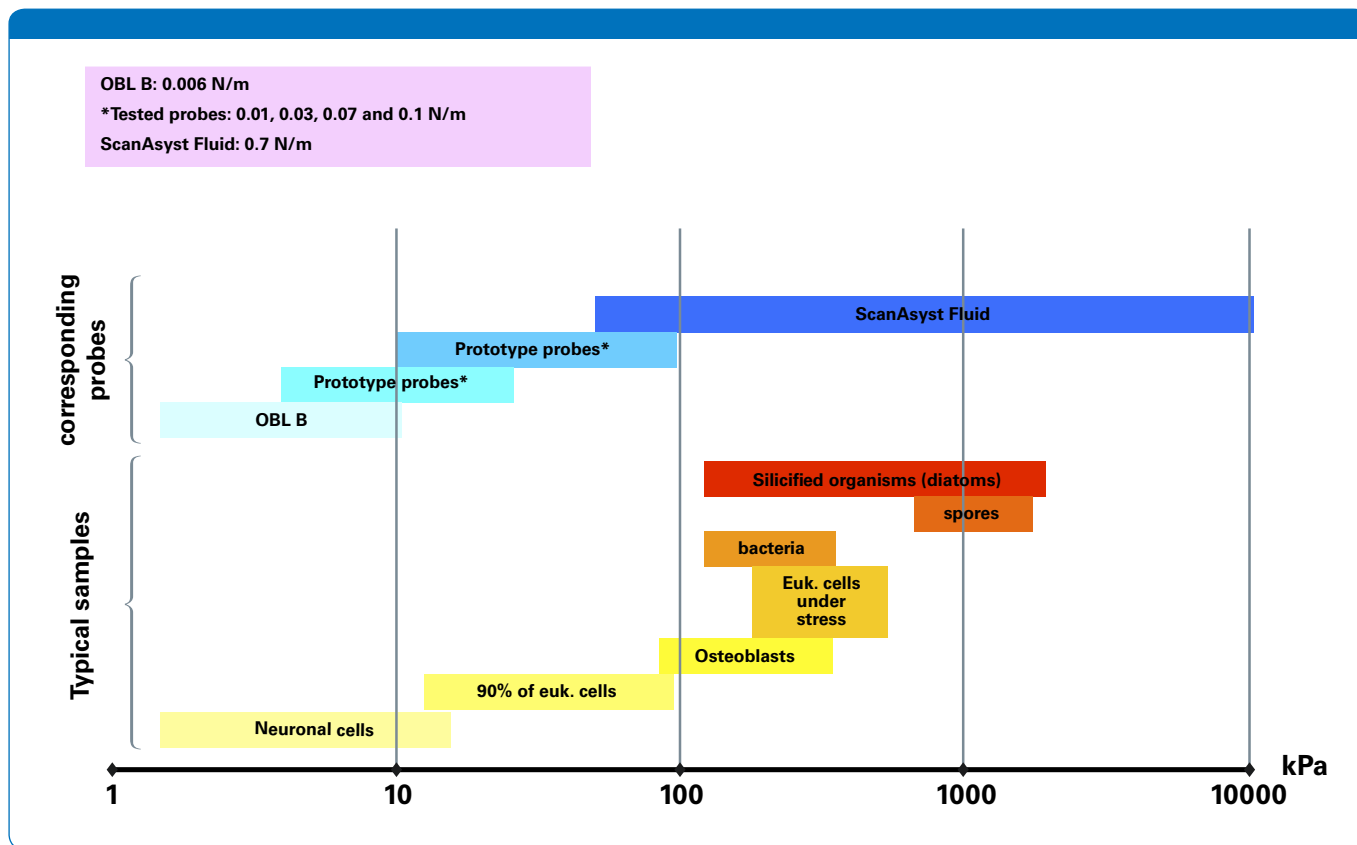


Figure 3: Range of compliance of various biological samples and corresponding AFM probes recommended for Peak Force Tapping. Depending on their type, eukaryotic cells can exhibit very different mechanical properties. Neurons can be extremely soft (down to 1kPa) whereas bone cell can be as robust as bacteria. In order to properly probe the cell properties, selecting the right spring constant and thus sensitivity is mandatory.

Imaging Biological Samples with PeakForce QNM

Marine biological samples are often composed of a mixture of soft and rigid components. As an example, diatoms, unicellular algae that appeared 180 million years ago, have a cell wall called frustule that is made of pure silica coated with organic molecules, such as pectin and other types of polysaccharides.²³ Those barriers are riddled with holes that act as filters and pores, enabling communication between the inner part of the diatom and the external environment.

A sample of water taken from the Adriatic Sea was put on a glass slide and investigated by PeakForce QNM. Other than very relevant observations on living diatoms,²⁴ some cell wall remnants were also found in the suspension. Figure 4 gives an example how those structures look. The 3D-topography profile reveals a characteristic waffle-like structure with pores 100 nm in size and an average height of 20 nm. The adhesion channel shows a marked contrast between the bottom of the pores (about 50 pN in average) and the rest of the cell wall (less than 20 pN). However, the most informative channels are the elasticity and the deformation data. On both channels the three

portions of the frustule are distinguished, each exhibiting clearly different mechanical properties: the center of the pore (average Young's modulus of ~300 kPa and average deformation of ~7 nm), the ring around the pore (~75 kPa and ~25 nm) and the core part of the cell wall, which seem to have intermediate mechanical properties (~200 kPa and ~10 nm). Those differences might be explained by the presence of components of different nature. As expected, the deformation is low for stiffer portions of the sample and vice-versa.

Additional experiments were carried out on *Escherichia coli* K12 bacteria. *E. coli* are gram-negative, aerobic/anaerobic and non-sporulating bacteria of about 2 μm in length and 500 nm in width and height. They are rather robust bacteria that can live on a wide variety of substrates. Some non-pathogenic strains are of high interest because they can be used as therapeutic, so-called probiotic, agents to treat various diseases.^{25,26} Unlike most of *E. coli* species, K12 strains are able to multiply in the intestine and are particularly resistant to antibodies. One of their other characteristics is that they possess pili (see figure 5a) that

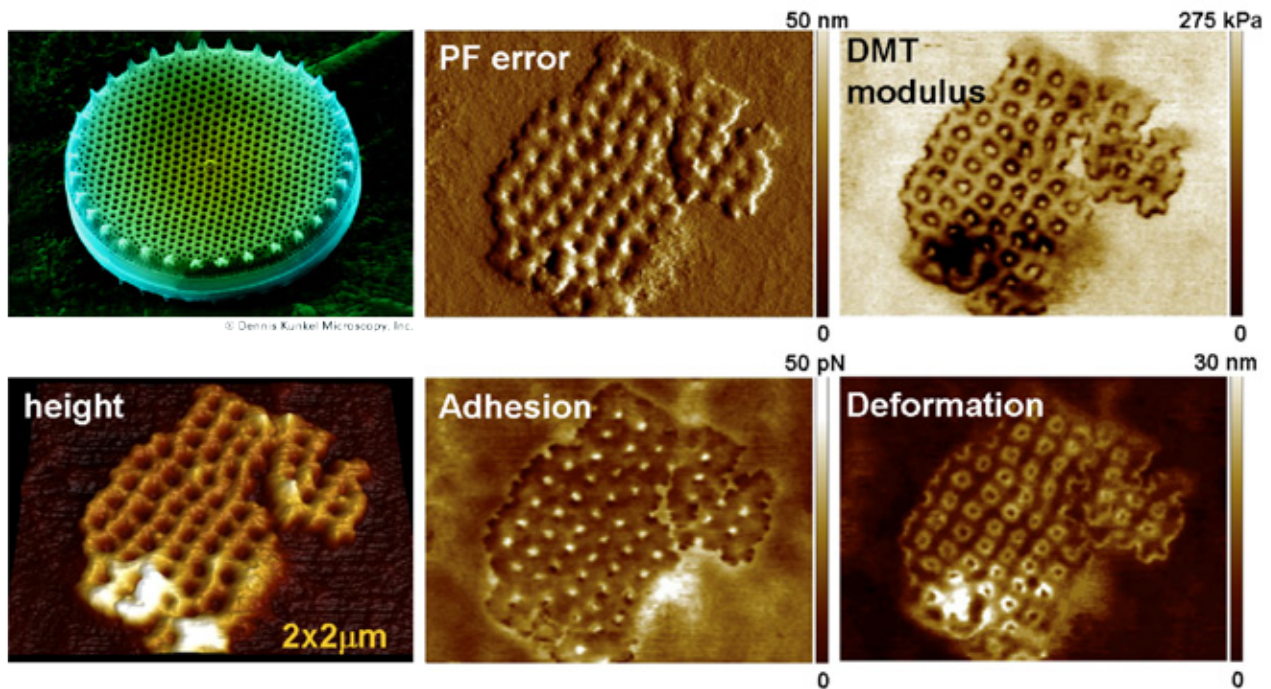


Figure 4: Imaging of phytoplankton cell wall with a BioScope Catalyst AFM. Top left: electron microscopy image of a diatom, sample courtesy of Dennis Kunkel, Astrographics. Most of the PeakForce QNM channels provide a remarkable contrast and high-resolution features.

typically retract under depletion conditions or any stressing environment. Until now, imaging those bacteria alive with the AFM, in any mode, has been a considerable challenge and a historically elusive result.

Figure 5 shows high-resolution images of such living bacteria, easily obtained in less than one hour. As can be seen on the 3D-representation of the height channel (figure 5b), the pili are no longer visible, which can be explained by the fact that extracting from their suspension medium and spreading them on a dish induces a stress that causes those pili to retract. Prior to the experiment, the AFM probe was calibrated on a gelatin sample and thus the elastic modulus could be calculated. Figure 5c shows the DMT modulus channel. By using a Sneddon fit, the average Young's modulus was determined to be 183 kPa, which perfectly matches previous observations.²⁷

Monitoring Cell Dynamics in Real Time

All living cells are dynamic, changing shape due to rearrangement of their cytoskeleton scaffold and spreading and migrating on the cell culture substrate. These processes and the mechanical changes that accompany them can be monitored with PeakForce QNM. Moreover, cells can be kept alive for long periods of time by using Bruker's Perfusing Stage Incubator (PSI) accessory, which consists of an enclosed incubation chamber that fits with cell culture

dishes.²⁸ The inlets and outlets for gas and liquids, as well as a heating plate enable introduction of the appropriate serum, gas and temperature to bacteria or eukaryotic cells for their growth and maintenance.

In another set of experiments, PeakForce QNM was used to investigate glioblastoma cells. Glioblastoma are by far the most common and malignant form of brain cancer. The most common factors underlying the cause of the disease are exposure to cytomegalovirus,²⁹ smoking³⁰ and electric fields.³¹ Moreover, this affection is not easy to cure, due to the fact that most of therapeutic agents cannot cross the hemato-encephalic barrier and because conventional therapy is very likely to damage the brain. For these reasons, most existing treatments have a more palliative than healing effect. This is why there is an increasing interest in trying to achieve better insight in how the tumor is mediated.

Though the signaling pathways are particularly complex, it is well known that the capacity of a cell to be invasive is directly connected to its intrinsic mechanical properties. Living glioblastoma cells have been imaged by PeakForce QNM on the BioScope Catalyst™ and maintained alive for the duration of the experiment by the use of the PSI. This technology allows the user to apply a very gentle to moderate force on the sample, depending on the

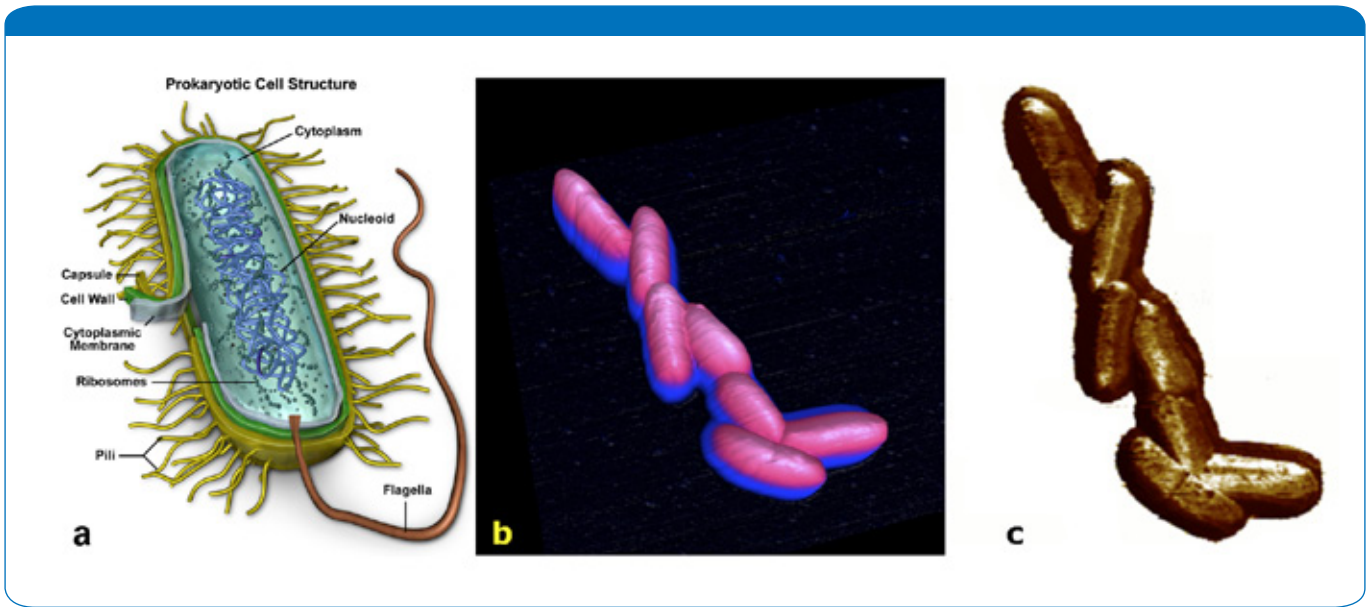


Figure 5: E. Coli K12 bacteria imaged by PeakForce QNM on a BioScope Catalyst AFM. In a, the structure of the strain is drawn. In b, an AFM 10x10µm 3D-height representation of a cluster of bacteria is shown. In c, Young's modulus channel (z-scale: 0-4GPa) is depicted. This is the first time that such bacteria has been imaged alive by AFM.

information needed. When applying a very light force on the sample, the topmost features of the cell (glycocalyx, protrusions) can be probed. On the other hand, applying a slightly higher force is required to sense the organelles and the cytoskeleton located underneath the plasma membrane.

Probing the real mechanical properties of the sample also requires indentation of the sample (and thus flex in the cantilever) by at least a hundred nanometers. Figure 6a shows a typical high-resolution image obtained on living glioblastoma while applying a moderate force (~300 pN).

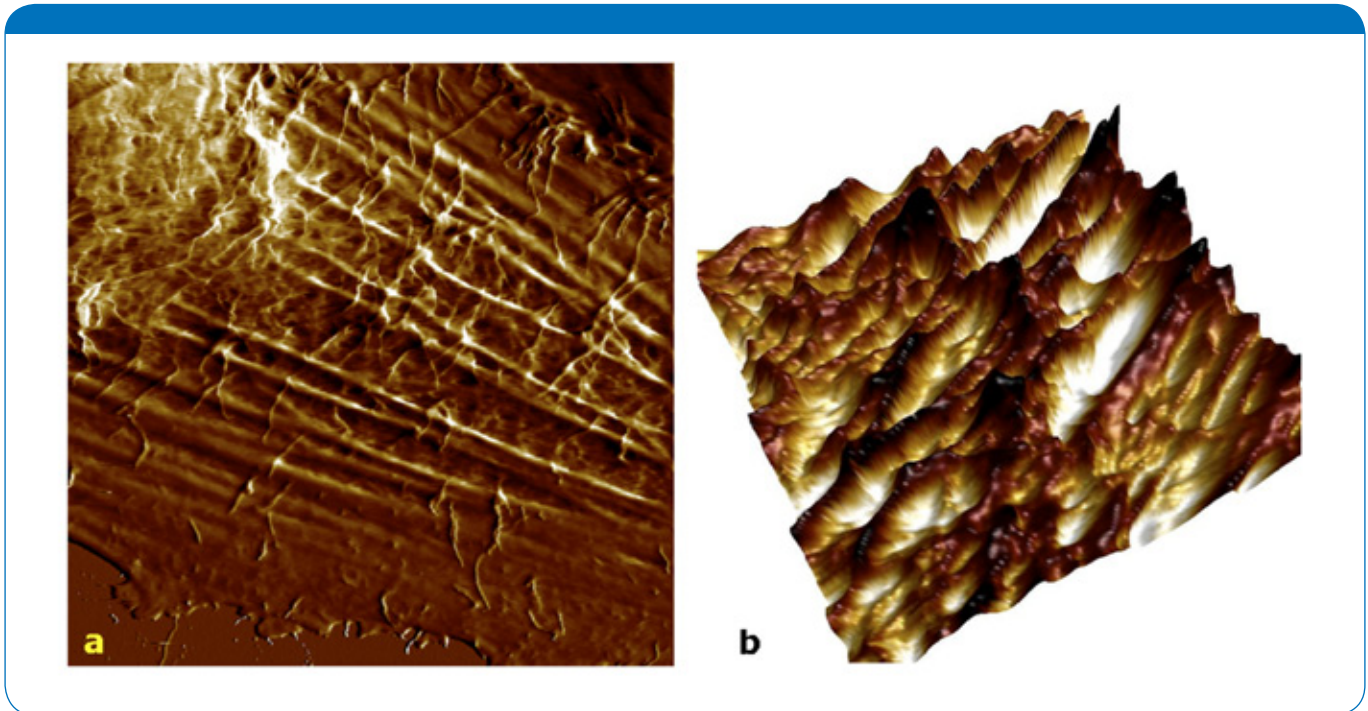


Figure 6: Images of living glioblastoma cells by PeakForce QNM and the BioScope Catalyst AFM. In a, 40x40µm height image recorded at a moderate force shows both topmost and internal structures. In b, 15x15µm 3D overlay of topography and deformation channels is shown. Bruker's BioScope Catalyst with Perfusing Stage Incubator offers the best balance of living cell imaging for long-term experiments.

At such a scanning force, both topmost and more inner features (most likely the cell cytoskeleton) can be detected. Figure 6b is a 3D representation of the cell topography with a "deformation skin."

This technology and setup can also be used to monitor changes in cell properties in response to external stimuli. Keratinocytes are the major components of the outermost layer of the human skin. They establish tight junctions with other types of cells, such as neurons, and represent the main barrier against UV radiations for the human body. Studying such cells by AFM helps researchers understand the process of skin cancer or other impairments. It has also been shown that an excess of keratin produced by those cells can undergo programmed cell death, though the full mechanism is still unknown.³²

HaCat is an immortal cell line of human keratinocytes that is widely investigated in cytology and also represents a good candidate to explore the potential of PeakForce QNM. The cells were exposed to an oxidative agent capable of inducing a stress. In response to this chemical aggression, the cells tend to transform and synthesize so-called actin stress fibers. A typical medium-resolution image is shown in figure 7. On the peak force error channel, structural changes, such as the morphology of the cells themselves are observed. These tend to adopt a cocoon-like structure. Stress fibrils are also clearly visible around the center of the cell. The changes in mechanical properties are also obvious, the Young's modulus increases and deformation decreases in response to the drug treatment. The adhesion channel also provides some interesting information, though

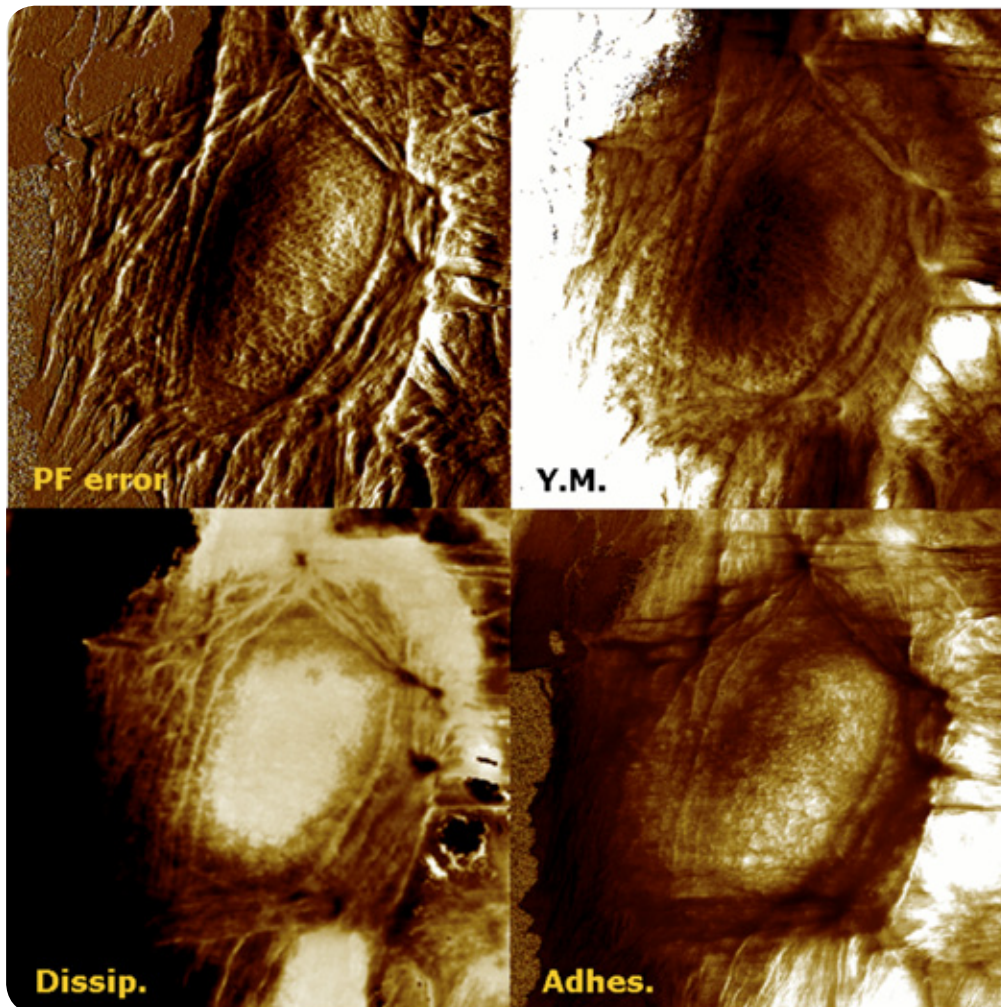


Figure 7: 75x75µm BioScope Catalyst and PeakForce QNM image of living HaCat cells under oxidative stress. The cells react by rapidly synthesizing stress fibrils to establish contacts with adjacent cells. Such dynamic processes can also be tracked by using this technique.

the adhesion contrast between the cell and the glass is rather homogeneous (which could be expected as non-specific adhesion forces tend to be more uniform in liquid environment). There is a dramatic decrease in the average adhesion between neighbor cells. This might indicate the presence of cellular adhesion molecules (CAMs). Such an assumption could be verified by the use of AFM probes functionalized with specific antibodies directed

against different types of CAMs. In PeakForce QNM, a force curve is made for each pixel of the image, thus the resolution is the same on all the channels. This example illustrates how easy and fast (384x384 pixel resolution images can be captured in 6 to 9 minutes) it is to directly and in a quantitative manner probe changes in topography and mechanical properties of living cells in response to drug treatments.

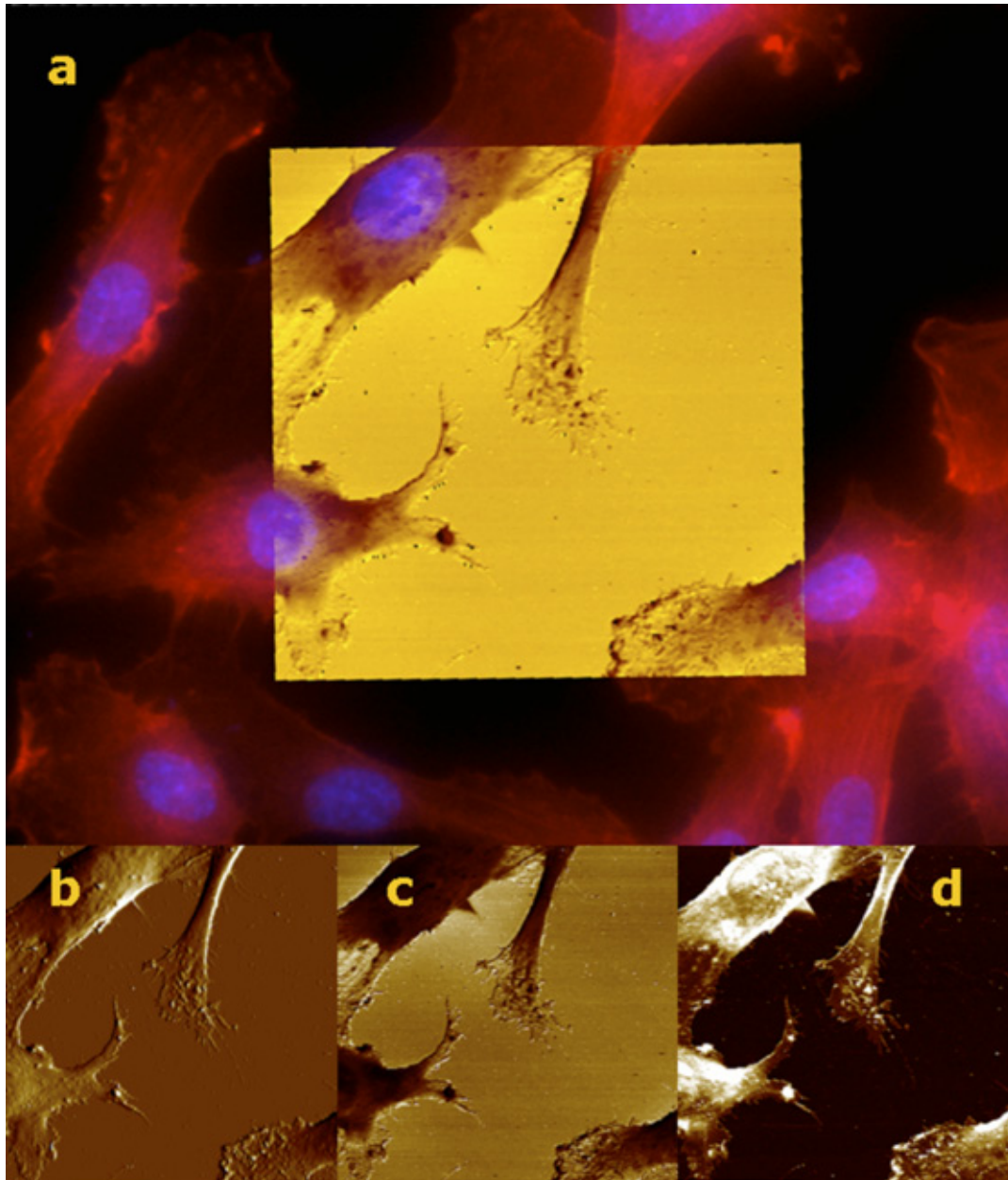


Figure 8: Overlay of fluorescence and AFM images of living HUVEC cells created with MIRO on a BioScope Catalyst. The main benefit of MIRO is to enable the display of optical and AFM information simultaneously. When operating with functionalized probes, a “Point & Shoot” option can also be used to accurately trigger force measurement at desired locations without losing the ligand.

Overlaying AFM and Optical Channels

Another of the current key challenges for biological applications is to be able to get optical and AFM information simultaneously. Bruker's exclusive Microscope Image Registration and Overlay (MIRO™) feature can be used to easily import optical/fluorescence images into NanoScope® software and overlay them with AFM images. After a short calibration, the user can select the location to make the AFM scan. Thus the sample automatically can be moved to the desired position and the AFM image can be captured pixel by pixel, and fully integrated into the optical image.

Figure 8 shows an overlay achieved on living endothelial cells. The fluorescence image (double-staining DAPI for nucleus and α -phalloidin for actin filaments) is set as the background and overlapped with an AFM image made of a mix of two channels: peak force error and Young's modulus. The transparency was set at 50% so that a direct correlation can be made between the different parts of the cells (visible by AFM topography and fluorescence) and their corresponding mechanical properties (Young's modulus AFM channel). In b, c and d the individual peak force error, Young's modulus and deformation AFM images are represented. It can clearly be seen in the elasticity and deformation channels that on the edges of cells where the thickness is too low, the influence of the scaffold (glass) on the mechanical properties of the sample is non negligible, whereas on the core part of the cells, the average Young's modulus is much more reliable (45.3 kPa). For a matter of clarity, only three AFM channels are shown here, but eight different signals can be displayed simultaneously.

Conclusion

The applications shown above demonstrate that Peak Force Tapping is by far the most powerful and quantitative high-resolution AFM technique available today to probe quantitative chemical and mechanical properties of living biological samples with an acquisition speed comparable to TappingMode. The number of different mechanical properties that can be characterized exceeds that of other commonly used AFM modes. Its potential paves the way for many exciting new applications in the field of biology, especially in cancer research and cardiovascular diseases.

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