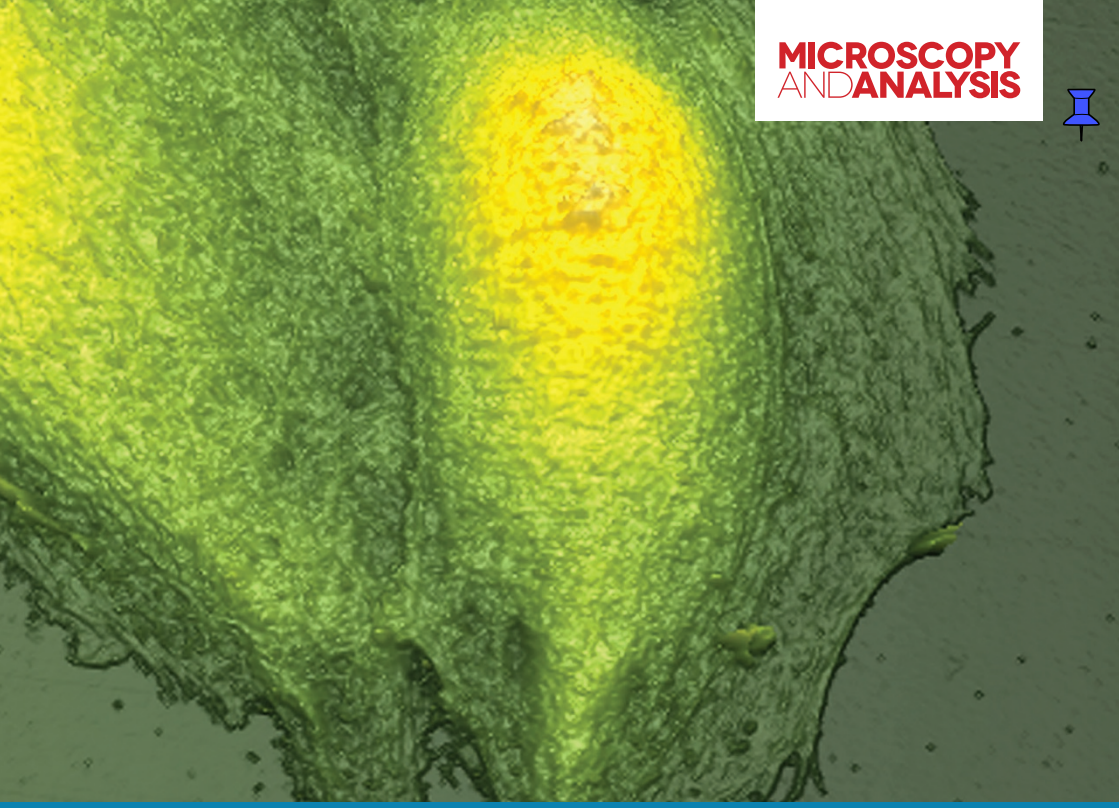

MICROSCOPY AND ANALYSIS

Atomic Force Microscopy for Life Sciences

Essential Knowledge Briefings

First Edition, 2017

BRUKER

Front cover image: PeakForce Tapping atomic force microscopy topography image of live Madin-Darby canine kidney (MDCK) cells (95μm image).

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About Essential Knowledge Briefings

Essential Knowledge Briefings, published by John Wiley & Sons, comprise a series of short guides to the latest techniques, applications and equipment used in analytical science. Revised and updated annually, EKBs are an essential resource for scientists working in both academia and industry looking to update their understanding of key developments within each specialty. Free to download in a range of electronic formats, the EKB range is available at www.essentialknowledgebriefings.com

INTRODUCTION

Atomic force microscopy (AFM) is becoming an increasingly important tool in biological and biomedical studies. This is due to its very high resolution, and also because, unlike other types of microscopy, it is not just an imaging technique. AFM can also provide nanometer-resolution surface mapping for many mechanical and electrical properties, such as elasticity, stiffness, conductivity and surface potential.

Life science researchers are increasingly aware that these properties can have a big impact on a range of cellular functions, including communication, signaling, cell division and differentiation, and even tumor metastasis and infection. In fact, understanding how these physical properties affect cells could be the key to differentiating between healthy and diseased states. They could also become a vital parameter in the study of pathophysiology, as well as in the development of new therapeutic approaches.

AFM uses a cantilever with a sharp tip attached to the free end to detect varying forces as the tip is scanned over a sample surface. It has proved ideal for observing, exploring and manipulating the surface of a variety of samples, ranging from individual biomolecules such as DNA or proteins to individual cells and even whole tissue samples.

There are many variations of this basic approach, which are usually referred to as AFM modes. These include several 'primary' imaging modes, which are used nearly universally and differ mainly in the way the tip interacts with the sample surface, as well as numerous 'secondary' or specialty modes, which often involve specialized tips and cantilevers. These secondary modes, which can usually be collected at the same time as primary mode topographic

information, are being used to reveal novel information about cell properties.

One of the big attractions of AFM for life scientists is the possibility of conducting experiments with live cells under physiologically relevant conditions. Again, unlike many other microscopy techniques, AFM can operate under ambient conditions, even in liquid, and offers some benefits in sample preparation protocols as well.

This Essential Knowledge Briefing (EKB) introduces AFM and its life science research capabilities; it is one of a pair of EKBs on AFM, with its sister publication looking at materials research. Beginning with a detailed explanation of the operation of a typical AFM instrument, including the role of the tip, cantilever and photodetector, the EKB also outlines how AFM has evolved from primarily being a materials characterization technique to becoming an important life sciences research tool.

It describes the primary imaging modes and gives a brief introduction to some significant secondary modes, before moving on to explain some of the unique challenges involved in studying biological samples with AFM, including the always present concern of preparing biological samples and selecting the right probe. Finally, it looks at how newer AFM instruments are providing faster imaging and a larger selection of modes, and how AFM is being combined with advanced forms of light microscopy to specifically target biological applications. In addition, the EKB also includes several case studies detailing how life science researchers are using AFM in their work.

HISTORY AND BACKGROUND

AFM is a powerful and versatile method for studying sample surfaces at the atomic scale. Rather than producing a magnified optical image of a sample, it involves scanning a cantilever with a sharp tip across the surface of a material, and measuring the forces created by the interaction between the tip and the surface. Because the tip closely tracks the surface, AFM can create detailed maps of surface topography.

It is one of several related techniques for creating topographical maps at the atomic level, collectively termed scanning probe microscopy (SPM). The first type of SPM to be developed was scanning tunneling microscopy (STM), which uses quantum effects to create an electrical current that flows between the tip and surface. The size of this current is a function of the distance from the tip to the surface.

STM was invented in 1981 by two researchers at IBM, Gerd Binnig and Heinrich Rohrer, and was one of the first microscopy techniques to allow scientists to 'see' the world at the level of individual atoms and molecules. Though STM revolutionized microscopy, it has its disadvantages. Because it relies on measuring a tunneling current, it can only be used on conducting materials such as metals. In 1986, the same year that Binnig shared a Nobel Prize for inventing STM, he and colleagues replaced the fixed tunneling tip with a flexible cantilever to produce the first atomic force microscope.

Rather than measure changes in the tunneling current as the tip is scanned across the surface, AFM measures changes in the forces between the tip of the cantilever and the surface of the sample. These forces affect the position or movement of the cantilever, with

the degree of deflection depending on the size of the forces, which increase as the tip gets closer to the sample surface. Because the deflection of the cantilever does not depend on an electrical current flowing between the tip and the sample, AFM can be used on non-conductive samples, and can even produce maps of the surface of biological samples.

In the first atomic force microscope, the tip comprised a tiny diamond glued onto the end of a cantilever made from a thin gold strip, while the deflection was measured using the tunneling current generated between the strip and a wire hanging above. Today, AFM cantilevers are usually made from silicon, with the cantilever and tip typically manufactured as a single unit. The tip, which can have a variety of coatings to allow additional surface properties to be measured, is typically less than 5μm in height and 10nm in diameter at the apex, while the cantilever is 100–500μm in length. For imaging large structures like whole cells, however, larger tips are generally more suited (around 10μm in size).

The horizontal and vertical position of the tip over the sample surface is controlled by a scanner made from a piezoelectric ceramic that expands and contracts in a controlled way when a voltage is applied. This scanner is attached either to the cantilever, allowing it to control the position of the tip directly (tip scanning), or to the base the sample is placed on, moving the base under a fixed tip (sample scanning).

In either case, the tip is scanned over the surface of the sample in a raster pattern, a set of zig-zagged lines that cover a square or rectangle. Surface measurements are taken at equally spaced intervals along the scan lines to build up an image made of pixels, similar to how an image is constructed on a television screen. The

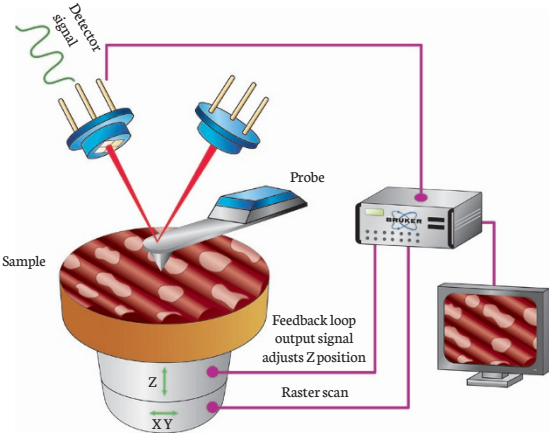


Figure 1. Schematic of basic SPM operation

zig-zag pattern means that data are collected as the scanner moves both left-to-right (trace) and right-to-left (retrace). Collecting data in both directions helps to remove artifacts that don't accurately reflect the sample surface.

To begin a scan, the tip is brought close to the surface. As the tip is then scanned across the surface, forces between the tip and sample surface cause the cantilever to bend. When the tip begins to interact with the surface, these are attractive van der Waals forces; once the tip gets very close to the surface, they become repulsive electrostatic forces.

The deflection of the cantilever caused by these forces is recorded by bouncing a laser off the back of the cantilever onto a position-sensitive photodetector, with any deflection altering the position of the laser beam. Because the distance between the cantilever and the photodetector is much greater than the length of the cantilever, this set-up produces amplification, allowing the photodetector to detect tiny deflections at the sub-Ångstrom scale (Figure 1).

The map of surface topography can be built up directly from these recorded deflections, by comparing the deflected signal with a reference signal known as the setpoint. Alternatively, it can be produced from the voltage that needs to be applied to the scanner to correct these deflections by moving the tip such that the signal returns to the setpoint, with a higher voltage required for larger deflections. Building the map from the applied voltage, known as Z feedback, is slower, as the scanner needs to be moved up and down, but it is better at imaging irregular surfaces.

In addition to producing a visual representation of topography, AFM can be used to reveal many chemical and mechanical features of the surface, including variations in elasticity, stiffness, *etc.* It can also be used as a spectroscopy tool to probe the forces at specific points on the surface. This produces a typical force–distance curve, plotting the force on the tip as a function of its distance from the surface, which can reveal information about such things as the adhesion of surface contaminants and sample elastic properties.

This ability to probe various different surface features has proven to be of great use for the study of biological samples, from DNA (Figure 2) and proteins to individual cells and whole tissue samples. Indeed, AFM was quickly adopted by life scientists as a way to probe intact cells at unprecedented resolution, with red blood cells and bacteria being some of the first cells to be studied in the late 1980s.

Producing topographical images of the cellular surface was the first focus, but as AFM techniques became more advanced, scientists found they could perform a range of other studies. In the 1990s, researchers began using AFM to physically manipulate cells

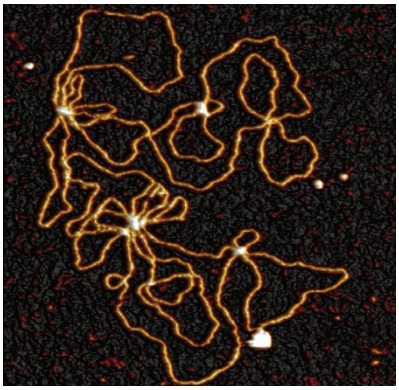


Figure 2. PeakForce Tapping resolution of plasmid DNA (419nm image)

and study their mechanical response to stimuli, such as changes in volume and elasticity, and showed that AFM was fast enough to follow dynamic physiological processes. They also started using AFM to probe the activity of specific biomolecules, such as the binding of ligands to protein receptors.

Today, AFM is regularly used to assess the structure, behavior and localization of specific membrane proteins on cells, such as transporters and receptors, as well as cell-cell adhesion and cell communication. It can also be used to study diffusion properties, signal transduction cascades and the generation of transmembrane potentials.

The ability to study individual cells represents one of the most powerful life science applications of AFM. Bacterial cells are a popular type of sample, as they can tolerate longer AFM sessions without detrimental effects. Mammalian cells, on the other hand, are more sensitive to environmental conditions, but are also increasingly popular objects of study since they can provide researchers with a greater insight into how diseases develop and help in the development of new therapeutic drugs. Many of the

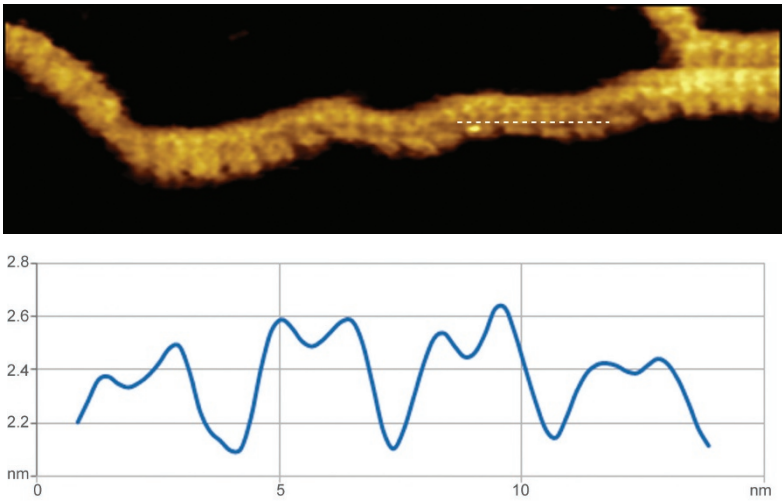


Figure 3. Highest submolecular resolution of the DNA double helix, showing both the major and minor grooves. The graph shows the PeakForce Tapping force curve from the dotted line in the image above

more recent advances in AFM for biology have been to more successfully study these more sensitive cell types.

Over the last several decades many different types of cells have been studied with AFM, including fibroblasts, breast cancer lines, stem cells, muscle cells, epithelial cells and bone cells. Further, due to its special capabilities, AFM is often used to probe mechanical properties rather than image tissue samples such as brain, bone, cartilage and ocular tissues.

AFM has thus helped to further understanding about a wide range of biological processes, including embryonic development, immune response, cell migration and tissue differentiation. Biomedical researchers are also regularly using AFM to study medical conditions such as cancer, malaria, sickle cell anemia, hepatic fibrosis, cardiovascular disease, muscular dystrophy and bone disorders.

IN PRACTICE

AFM can be operated in several main or primary imaging modes, which typically differ in the way the tip physically interacts with the sample surface. There are also numerous secondary or specialty modes, which are often specific applications of the main modes and are proprietary to specific developers of AFM systems.

The simplest mode is known as contact mode, in which the probe is in permanent physical contact with the sample surface as it performs a raster scan, with changes in topography causing the cantilever to bend up and down. Although simple, fast and sensitive, contact mode has a major drawback: the lateral forces exerted on the sample as the tip is scanned across can be very high. For delicate biological samples such as live cells, these unwanted lateral forces can not only damage the sample, but can also blur the image and lower the resolution.

In 1992, engineers at Digital Instruments (now Bruker) developed tapping mode. In this mode, the cantilever oscillates at or near its resonance frequency, but with a large enough amplitude that the tip touches the surface intermittently with a tapping action. Tapping mode is ideal for many biological applications because, although the force applied can actually be much higher than in contact mode, the act of tapping considerably reduces the damage inflicted to samples. It also generates a much higher signal, as it is affected by both attractive and repulsive forces.

Another benefit of the tapping motion is that it prevents the tip from being trapped by the thin layer of water when studying samples in fluid, which can be a major problem in non-contact

mode. Because of all these advantages, tapping mode is now the most widely used AFM primary mode for biological applications.

In 2009, Bruker introduced an enhanced tapping mode called PeakForce Tapping. In PeakForce Tapping, the cantilever is oscillated in a sine wave pattern at a frequency that can be an order of magnitude less than the resonant frequency; typical frequencies are 2kHz as compared to the typical 60–500kHz for tapping mode.

As the tip approaches the surface, the attractive van der Waals forces increase until the tip jumps into contact. At this point, the tip experiences repulsive forces, which eventually dominate the attractive ones. PeakForce Tapping uses an intelligent algorithm to extract the peak force experienced by the tip, which occurs at the point where the tip begins to withdraw from the surface. This means that PeakForce Tapping can produce an instantaneous quantifiable measurement of the peak force during each oscillatory cycle. In contrast, tapping mode measures the average oscillatory amplitude while the tip is in contact with the surface.

While contact and tapping modes focus primarily on producing three-dimensional (3D) images of surface topography, measuring surface roughness or determining height profiles, PeakForce Tapping and other secondary modes are able to probe various chemical and mechanical features of the surface of biological samples (for example, Figure 5).

For life science applications, a very popular secondary mode is PeakForce Quantitative Nanomechanical Mapping (QNM). In essence, this method combines PeakForce Tapping for imaging the sample with force spectroscopy for obtaining mechanical data. The resultant data can be analyzed to reveal simultaneous

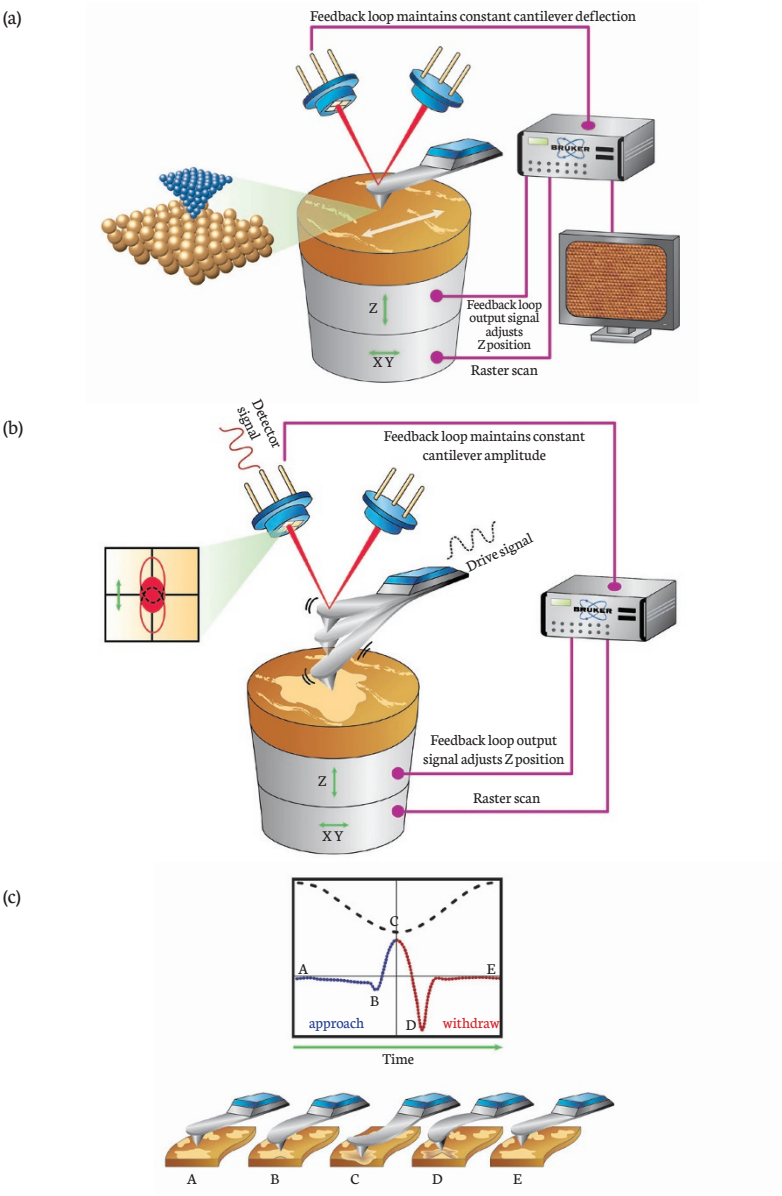


Figure 4. Schematics of primary AFM modes: (a) contact mode, (b) tapping mode, (c) PeakForce Tapping mode

information about various surface characteristics, including adhesion and deformation. As such, it has been used to study many biological processes, including the regulation of cell–cell adhesion by membrane proteins and the changes in stiffness that occur when healthy cells become cancerous.

These and other AFM modes can be used for studying both fixed cells and live cells. Studying chemically fixed cells is fairly straightforward: they can be imaged for long periods under ambient conditions, without requiring specific temperatures or pHs. This relative ease of analysis comes at the expense of not being able to study any mechanical properties or dynamic events. In contrast, studying live cells requires specific buffers and maintaining set pHs, temperatures and salinity levels, which differ for different types of cells.

Live-cell imaging also often employs functionalized tips, allowing AFM to be used for actively manipulating cellular samples rather than just imaging or analyzing them. By attaching a specific ligand to the tip, for example, AFM can be used for studying the binding between this ligand and specific proteins on the sample surface. This can help to reveal the presence and distribution of particular receptors on cellular membranes, which is useful for

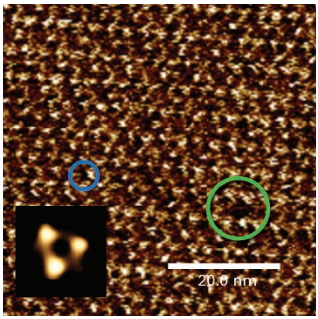


Figure 5. PeakForce Tapping AFM image of the bacteriorhodopsin membrane protein lattice structure taken on an inverted optical microscope. The inset shows a single particle averaging of the bacteriorhodopsin trimer. The green circle shows a single lattice defect. The blue circle shows the lattice substructures (Z scale = 0.6nm)

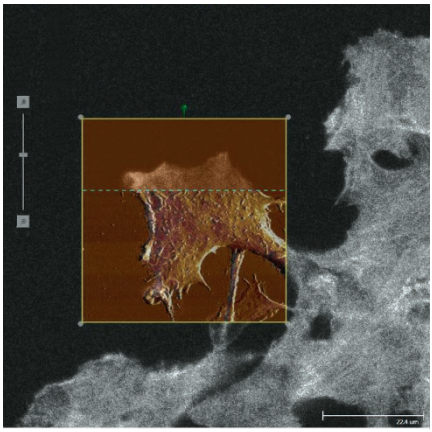


Figure 6. Line-by-line synchronization of atomic force and confocal microscopy for true correlation of AFM and optical data

understanding how malfunctioning of cellular recognition systems can lead to disease.

In addition to analyzing cellular organelles and individual cells, AFM is also used to analyze multiple cells in an aggregate environment. The most common approach involves freezing biological tissue and then removing thin slices, which are glued to a glass slide for analysis. In this case, AFM is not usually used for imaging, but for obtaining mechanical data about the tissue.

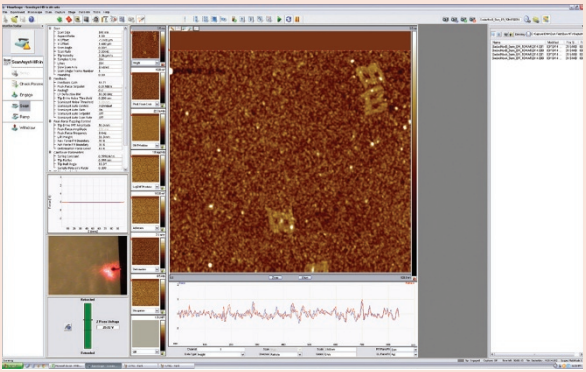
When studying biological material, AFM is often combined with some form of light microscopy. This can be useful for directing the AFM analysis, with researchers identifying regions of interest with a light microscope for more detailed study with AFM, and for comparing the topological map with a visual image. In addition, by its very nature, AFM is a purely surface technique, whereas forms of light microscopy such as confocal microscopy can image inside cells to identify cellular components. This can provide a deeper understanding of dynamic cellular events that start at the membrane.

CASE STUDY 1. DNA origami

For researchers at Boise State University in Idaho, USA, AFM comes in very handy when fabricating novel structures made from DNA. Using a technique known as 'DNA origami', the team recently created the university's 'B' logo out of DNA strands as part of a summer project for an undergraduate student.

Invented by Paul Rothemund at Caltech, DNA origami involves binding many short strands of single-stranded DNA to a long scaffold strand. The short strands possess complementary sequences and by binding with each other they fold the long strand into a desired shape, just like in paper origami. In this study, the researchers made the 'B' shape using 170 separate short DNA strands.

'The "B" project was started as a way to train a student in design of DNA origami nanostructures,' says Elton Graugnard, Assistant Professor in the Department of Materials Science and Engineering. 'The "B" was chosen as a fun design to use in the process of learning the DNA origami technique.'



Formation of Boise 'B' DNA displayed on screen of the MultiMode 8 AFM (Bruker Nano Surfaces). Photo by Kelly Schutt

To check if the process worked, they used PeakForce Tapping, imaging the DNA structure under fluid to achieve the highest resolution. 'Since [these DNA structures] are negatively charged, they are created in a solution with positive ions. To image the structures, a drop of the solution is placed onto a mica surface. Mica is atomically flat, so it makes the structures easy to image with AFM,' explains Graugnard. Using this approach, Graugnard and his team successfully detected a 'B' shape made from DNA, around 100x80nm in size and 2nm thick; they even managed to resolve the little holes inside the Bs.

Origami 'B' 3D AFM image

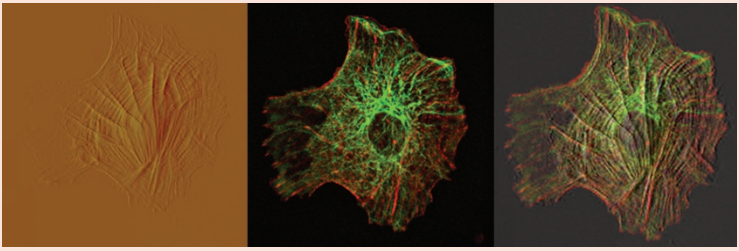
'Our group uses AFM extensively,' says Graugnard. 'It is very easy to obtain high-quality AFM images of DNA origami nanostructures on mica.' Their main goal is to develop future electronic computer circuits made from DNA, and AFM is one of the main weapons in their research arsenal.

CASE STUDY 2. Live cell response to mechanical stimulation

Andreea Trache at Texas A&M University Health Science Center in the USA is using AFM to help her understand cellular adaptations to mechanical stresses from a biophysical perspective. 'AFM is the only technique available that directly provides structural, mechanical and functional information at high resolution in living specimens and also in real time,' she says.

Keen to take AFM to the next level, Trache and her team developed a new system that integrates AFM with total internal reflection fluorescence (TIRF) microscopy and spinning-disk confocal microscopy for imaging live cells. This allows them to combine mechanical stimulation using matrix-functionalized AFM tips with real-time fluorescence microscopy.

The system has now become standard in their research and has, for example, allowed the team to show that cytoskeletal tension affects the actomyosin apparatus, which in turn coordinates the cell's ability to adapt to external forces. 'The AFM-optical imaging integrated system is broadly applicable across a wide range of



Live smooth muscle cell expressing tubulin-EGFP (green) and actin-mRFP (red) imaged by an atomic force microscope (left) and spinning-disk confocal microscope (center). The image on the right shows the overlay of the AFM and confocal images. Images courtesy of Soon Mi Lim and Andreea Trache

molecular dynamic studies in any adherent live cells, allowing direct optical imaging of cell responses to mechanical stimulation in real time,' says Trache.

For Trache and her team, integration is the future. New approaches for combining AFM with classical light microscopy can take researchers towards a deeper understanding of the dynamics of biological processes and allow a wide range of different studies, from discerning single molecule interactions to monitoring live cell responses. 'The newer instrument developments bring advances to the challenging aspects of this technology,' says Trache. 'Most importantly, the development of instrumentation for increasing acquisition speed to allow for real-time imaging of fast biological processes.'

Lim SM, Trzeciakowski JP, Sreenivasappa H, *et al.* RhoA-induced cytoskeletal tension controls adaptive cellular remodeling to mechanical signalling. *Integr Biol* 2012;4:615–27. (http://dx.doi.org/10.1039/C2IB20008B)

Sreenivasappa H, Chaki SP, Lim SM, *et al.* Selective regulation of cytoskeletal tension and cell-matrix adhesion by RhoA and Src. *Integr Biol (Camb)* 2014;6:743–54. (http://dx.doi.org/10.1039/C4IB00019F)

Trache A, Lim SM. Live cell response to mechanical stimulation studied by integrated optical and atomic force microscopy. *J Vis Exp* 2010;44:2072. (http://dx.doi.org/10.3791/2072)

Trache A, Lim SM. Integrated microscopy for real-time imaging of mechanotransduction studies in live cells. *J Biomed Opt* 2009;14:1–13. (http://dx.doi.org/10.1117/1.3155517)

CASE STUDY 3. Virus binding to living cells

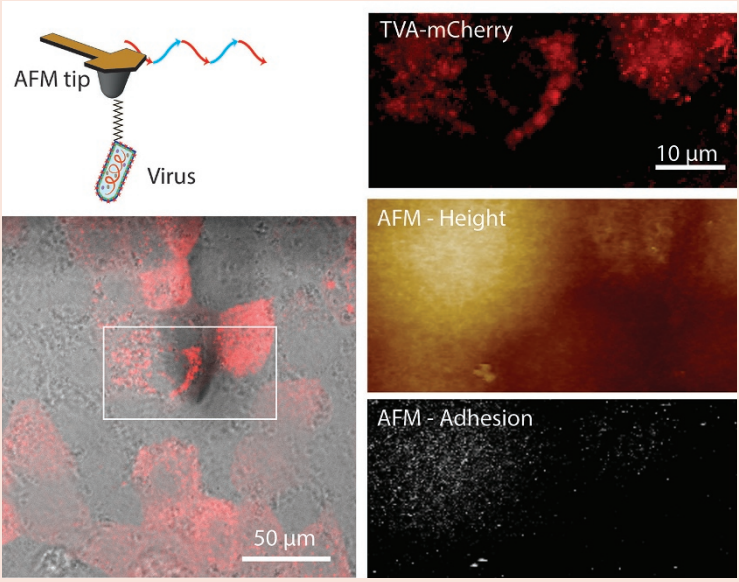
Developing and testing new drugs that can stop viruses entering cells is a possible use of an AFM technique developed by a team led by David Alsteens at the Université Catholique de Louvain, Belgium. Alsteens and his team studied how an engineered rabies virus binds to the surface of animal cells – the first and key step in the process of entering and infecting a cell.

How easily a virus binds to the surface of a living cell depends on temperature, pH and the composition of the cell membrane, and varies across the membrane and over time. Previously, however, there was no technique for imaging live cells, identifying individual receptors on their surface, and measuring the strength to which a virus binds to them in real time.

To develop such a technique, the team combined force–distance curve-based AFM (FD-based AFM) and confocal microscopy with a special culture chamber able to keep mammal cells alive for days. This new technique allowed them to study – for the first time – how a virus binds to individual receptors on the surface of live cells at nanoscale resolution (<50nm).

The team used FD-based AFM with a single virus tethered to the AFM tip to image the cell surface and identify virus binding sites. By combining this with confocal microscopy, they could image individual receptors and map the interaction between the virus and receptors as it occurred. By looking at the force–distance curves, they deduced that the virus detached multiple times from the surface, suggesting that it bound to several different receptors in the first millisecond of contact. Each successive bond strengthened the attachment between the virus and the host cell, until they were tightly

bound. The virus then moved to the next stage of infection – entering the cell.



Results from combined FD-based AFM and confocal microscopy. First column: schematic showing virus tethered to the AFM tip and differential interference contrast and fluorescence microscopy images showing two populations of canine kidney cells – cells non-susceptible to the engineered rabies virus (non-fluorescent cells) and susceptible cells (cells expressing the TVA receptor). Second column: fluorescent image, FD-based AFM height image of the same cells and corresponding adhesion image where only susceptible cells show binding to the virus-coated AFM tip

Alsteens D, Newton R, Schubert R, *et al.* Nanomechanical mapping of first binding steps of a virus to animal cells. *Nat Nanotechnol* 2016;12:177–83. (http://dx.doi.org/10.1038/NNANO.2016.228)

PROBLEMS AND SOLUTIONS

As with any other imaging technique, AFM has certain limitations. For example, the technique is prone to certain artifacts produced by unsuitable tips or poor operating environments. Although it's impossible to completely eliminate these artifacts, their occurrence has been reduced drastically by recent innovations in AFM systems designed specifically for biological research. The remaining common artifacts can be minimized with good laboratory practices.

The main tip-related issue experienced is known as 'double tip'. This is caused when a dirty and contaminated tip results in two (or more) points on the tip touching the sample at the same time, generating duplicate images. In this case, the only solution is a new tip, which can cost from a few tens of dollars up to hundreds of dollars for the more specialized cantilever/probe combinations. 'Double tip' can be particularly problematic when imaging live cells, where tips are more likely to become covered in cellular debris, metabolic products or cellular secretions.

For cantilevers, it's important to choose the correct size. For example, for a cell that is 5µm long, such as a yeast cell, a 1µm tip would not be the best option. In this case, as the tip moves across the surface, the cell would touch the back of the cantilever and reverse the images. This would lead to the tip producing a triangular-shaped artifact in the image of the cell.

When preparing biological samples for AFM, one of the most important considerations is finding a safe and effective way to immobilize the sample on a substrate, with typical substrate materials including glass, mica and silicon. Ultimately,

the selection of the substrate depends on the sample type and target molecules being studied.

For example, DNA is commonly attached to mica for AFM studies, as its planar structure can easily be cleaned using sticky tape to obtain an atomically flat surface. One disadvantage with mica, however, is that it has a slight negative charge, preventing adsorption of negatively charged DNA. To overcome this negative charge, the DNA can be attached with nickel or magnesium cations, or the mica can be modified with 3-aminopropyltriethoxysilane (APTES) or 1-3-aminopropylsilatrane (APS).

Lipid bilayers and whole cells need a different approach. Some cells can be immobilized directly onto glass with epoxy glue, with force measurements performed in a liquid environment. Microbial cells are an exception, as they cannot easily be glued to glass. Even in culture, these organisms tend to retain their shape and, unlike mammalian cells, don't spread in a Petri dish. This means the point of contact between the substrate and the cell is minimal, increasing the risk of the cell being dragged along by the tip during scanning. It's better to immobilize microbial cells to porous polycarbonate membranes, or to bind them to a positively charged surface such as gelatin or poly-l-lysine, or to amino- or carboxyl-functionalized surfaces.

Some AFM systems include an incubation chamber for live-cell imaging. Conditions inside these chambers are tightly controlled to mimic normal physiological conditions as closely as possible and to keep cells alive during the experiments.

One further challenge is relating the nanoscale information obtained by AFM to biological information at larger scales. AFM can produce a great deal of data about the mechanical properties of

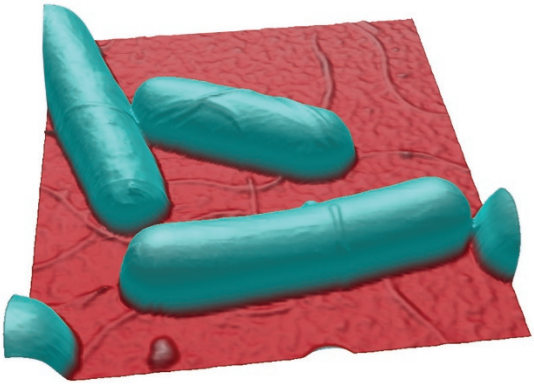


Figure 7. PeakForce Tapping AFM image of live *E. coli* cells

biological samples, but it can be difficult to understand how this relates to chemical and biological properties, such as protein morphology and distribution across the cellular membrane.

Several studies may be required to determine which AFM parameters contain meaningful information about specific cellular functions. For example, researchers are starting to explore the idea that stiffness and elasticity data acquired by AFM can be a potential biomarker for identifying stages of disease. This is because elasticity reflects the condition of a cell's cytoskeleton, which is important for cell migration and development.

WHAT'S NEXT?

One of the great advantages of AFM is that it is much more than merely an imaging technique. By measuring the interaction forces between biomolecules, it can also determine important mechanical properties, which may be crucial for understanding biological and pathological processes and also lead to the development of new therapeutic approaches. However, there are several areas that AFM developers are still actively pursuing to enable an even broader use of the technique in the life sciences. For example, atomic force microscope manufacturers have been developing and commercializing tips that are softer (lower stiffness) but sharper (smaller tip radius) than conventional AFM tips, which will allow the detection of smaller interaction forces and smaller recognition sites on cell surfaces. New specialty tips like these are entering the market every year.

Functionalized tips are also expanding the range of information that AFM can reveal about biological samples. Nevertheless, researchers still don't have a full understanding of how functionalized tips may affect the sample being analyzed. For example, when using ligands attached to the end of a tip, it can be difficult to distinguish specific interactions between ligands and target receptors from non-specific interactions with other biomolecules. The development and subsequent research around functionalized tips is a very active and exciting area in bio-AFM, with an ever increasing number of technical articles on the subject being published by top journals.

Despite better tips and scanning techniques, there is always some risk in AFM-based life science research that the target molecules will be pulled or ripped from the membrane by the tip,

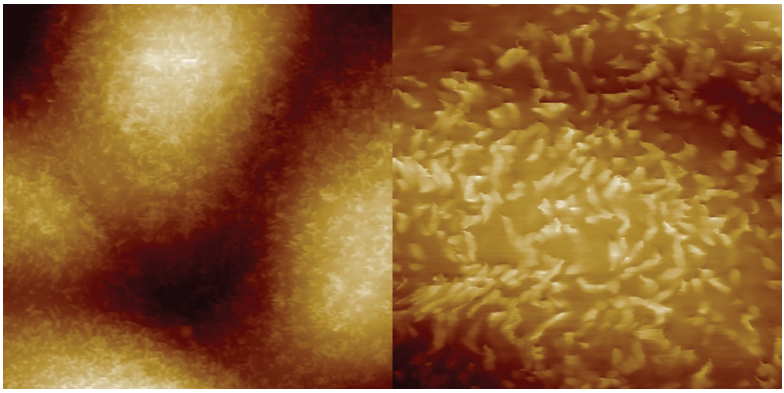


Figure 8. PeakForce Tapping enables the first AFM imaging of individual microvilli on living MDCK cells. 25µm (left), 10µm (right) images captured at 1kHz PeakForce QNM. Images obtained by Dr Hermann Schillers, University of Münster

potentially causing irreversible conformational changes and influencing cellular activities. And even with the much smaller tips being developed, it can remain challenging to determine exactly where the individual molecules are in relation to the cell surface, which may influence the results. One way to solve this problem is with fluorescence microscopy, which is very familiar to biologists. In AFM studies, this involves tagging the protein of interest with a fluorescent label capable of detecting conformational changes that can then reveal any damage caused by the functionalized tips, or that can help in determining molecule-to-cell surface data.

AFM is already often combined with light microscopy, including fluorescence microscopy, to compare the topological map with a visual image and also to direct the AFM analysis. The main difficulty with combining these two techniques has been that they work at such widely different magnifications, making it difficult to match up the same features on an image produced by a light microscope and a topographical map produced by AFM.

Much work has been done over the last several years to make this combination of techniques more effective.

One solution involves a version of light microscopy that employs AFM technology to overcome the diffraction limit that restricts the resolution of conventional light microscopy to 250nm. Known as near-field scanning optical microscopy (NSOM), it involves focusing light through a narrow optical fiber onto a sample and collecting the scattered light as the beam is scanned across. Because the interaction between the light and the sample surface is extremely localized, NSOM can have a spatial resolution of as little as 10nm. Combining these two scanning techniques allows optical and topographical images of a sample to be obtained simultaneously.

What NSOM lacks, however, is the wide field of view offered by traditional light microscopy and the ability to image beneath the surface. Both of these abilities can now be provided by several recently developed super-resolution microscopy techniques, which were collectively awarded the Nobel prize for chemistry in 2014. These are advanced versions of fluorescence microscopy that, by controlling or analyzing the fluorescence signal, can also bypass the diffraction limit, bringing the resolution down to around 10nm while keeping the wide field of view and sub-surface imaging. Already, scientists have successfully used combinations of AFM and super-resolution microscopy to study protein aggregates, DNA and actin filaments.

The two techniques can also produce complementary information. One research group used super-resolution microscopy to visualize the microtubule cytoskeleton of healthy and cancerous mammalian cells and AFM to analyze the cells' stiffness and elasticity. This allowed them to correlate the location and structure of

the microtubules with the measured stiffness and elasticity. (See Case Study 2 for another example of the advantages of combining AFM with light microscopy.)

Ultimately, life science researchers would like the ability to scan live cells in their natural environments to study fast-paced cellular events, such as the working of membrane receptors and transporters, and cell-to-cell interactions. Recent advances in AFM technology, including ultra-small cantilevers, more sensitive optics and photodetectors, and high-speed electronics, have made significant progress toward this goal by increasing the speed of AFM.

There's no doubt that as AFM continues to improve in terms of speed, ease of use and range of modes, it will continue to be an increasingly indispensable technique for life science researchers, who are only now beginning to 'scratch the surface' of its vast potential.

FURTHER INFORMATION

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