# Scratching the Surface: An Overview of Scanning Probe Microscopy (SPM)

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The new form of microscopy that was invented in the 1980's moved away from lenses to profilometry, hence the name scanning probe microscopy. This began with the invention of the scanning tunnelling microscope (STM) by Gerd Binnig and Heinrich Rohrer in 1981.

# Introduction

This scanning tunnelling microscope scanned a pointed electrode, referred to as a 'tip' in the context of STM, within a few nanometres of a conducting surface (Binnig & Rohrer, 1982). When a small bias voltage was applied between the sample and the tip, electron tunnelling was initiated. Tunnelling is a guantum mechanical effect that arises due to the particle and wave-like duality of electrons. Solutions of the Schrödinger wave equation reveal that the probability of electron tunnelling decays exponentially with a linearly increasing gap. In the context of STM this manifests itself in the form of exceptional spatial sensitivity toward changes in the tip-sample distance: Binnig and Rohrer's STM utilised this sensitivity to produce an image of a conducting graphite sample surface by raster scanning the electrode tip across it; the resulting image mapped the local density of electronic states at the Fermi level of the graphite. The dependence of tunnelling current upon distance was easily capable of resolving the positions of single atoms in



**Figure 1.** Schematic diagram of the principal components of an AFM. In this representation a tube type piezo scanner is shown which can move in all three directions.

crystalline surfaces. This ground breaking feat won Binnig and Rohrer the Nobel Prize in Physics in 1986, which they shared with the inventor of the electron microscope, Ernst Ruska. Gerd Binnig and colleagues designed a new scanning probe microscope named 'atomic force microscope' (AFM) that could quantify the force interactions between a tip and sample (Binnig, Quate & Gerber, 1986). There are now many

forms of probe microscopes each of which utilises a different physical interaction. They scan the surfaces of materials and the resolvability is linked to the roughness of the surface. In contrast to all other forms of microscopy the flatter the surface, the higher resolution is achieved. Despite this seemingly counterintuitive fact, probe microscopes have several significant advantages over the traditional forms of microscopy. First, because they have no lenses they are not diffraction limited although this has been overcome recently in light microscopy, and second they are not confined to the vacuum environment of the traditional workhorse of ultrahigh resolution - the electron microscope. However, both AFM's and STM's do have the ability to operate in ultra-high vacuum as well but the significant advantage of scanning probe microscopy is its ability to achieve ultra-high resolution in physiologically native environments (air and liquids).

Figure 1 shows a schematic diagram of the principal components of the typical architecture of an AFM. The AFM raster scans the tip across a selected area using the x,y piezo elements of its scanner. Deviations of the cantilever probe as it traverses the surface are detected by reflection of the laser beam onto a photodetector that is split into four quadrants. Each quadrant of the photodetector produces an electrical current proportional to the light intensity falling upon it. The output of the detector as a whole is arranged in such a way that the normal (up and down) motion and lateral (twisting) motion of the AFM cantilever can be recorded in two separate channels: one quantifies the difference between the light intensity falling on the top and bottom pair of quadrants, and a second channel which does the same for the left and right pair of quadrants. AFMs employ a feedback loop to control the tip-sample force. The force that the tip exerts on the sample is given by Hooke's law:

Where, k, is the spring constant of the cantilever and, z, is the distance over which it is deflected away

F=kz

from its undisturbed, relaxed position. So, the key to controlling the force exerted on the sample by the AFM tip is to control the extent of cantilever deflection. The feedback loop uses the varying dc signal from the photodetector 'topography' channel as an input to control the motion of the z piezo element of the scanner, moving it up or down to keep the cantilever at a pre-determined level of deflection as it encounters changes in sample topography. This means that the load exerted by the cantilever is kept constant throughout the scan and this mode is termed 'constant force mode'. It is also variously termed 'dc mode' in reference to the photodetector signal. Control of force is crucially important for non-destructive imaging, but as will be discussed later, in practice there is much more to it than simply quantifying the cantilever displacement.

The AFM image itself is formed by plotting the z piezo excursions that are necessary to maintain constant cantilever deflection against the x,y coordinates of the scanned area. Factors such as the mechanical modulus and surface charge can alter the force interactions as an AFM tip scans over a sample surface (Weisenhorn et al. 1993, Müller & Engel, 1997) and many different modes have been developed over the years which take advantage of the physics of tip-sample interaction. A large area of ongoing research into this aspect of AFM centres on the so-called 'ac mode' of operation (Garcia & Perez, 2002) which is the second principal way of operating AFM. The AFM cantilever is driven into oscillation and feedback control is achieved by using information contained within the ac signal that the cantilever oscillation generates, hence the name.AC mode imaging gives rise to an enormous plethora of measurement possibilities as energy is transferred from the oscillating tip to the sample surface during a scan (Cleveland et al. 1998). For the most common 'ac mode' (Tapping Mode<sup>TM</sup>) the cantilever is driven very close to its natural resonant frequency and feedback control is activated by monitoring the amplitude of the resonating cantilever as it encounters the damping effects caused by tip-

sample interaction. As the name implies, in Tapping mode the tip physically hits the sample surface at the end of each cycle of its oscillation. Tapping is caused by the Pauli exclusion between the atoms on the apex of the AFM tip and the sample surface. Alternatively, at much lower levels of damping, it can involve flying the tip just above the sample surface in a non-contact mode, where the AFM tip moves in and out of the realm of the attractive van der Waals forces without actually touching the sample surface. There are also other special cases of ac mode operation where very low cantilever oscillation amplitudes (in the angstrom range) are advantageous because the cantilever spends more time in the interaction regime rather than hopping in and out of it. In the very low oscillation amplitude regime, a very stiff cantilever is used to avoid jumpto-contact instabilities and control is achieved by feeding back on frequency changes in the cantilever's oscillation, as these are inherently much more sensitive to local perturbations to the tip-sample interaction. The technique is referred to as 'FM-AFM' which stands for frequency modulation AFM. This advanced methodology of AFM has produced dramatic results including true non-contact atomic resolution imaging of crystalline surfaces (Giessibl et al. 2003) and direct measurement of the structuring of water layers occurring at solid-liquid interfaces (Fukuma et al. 2007).

In addition to imaging, a unique and significant aspect of the AFM is its ability to measure forces (Butt et al. 2005). Because the scanner mechanism can move in all three dimensions it is possible to push the tip onto a sample surface to a predetermined loading force and then withdraw it again. As this is done the extent of cantilever bending provides information about the mechanics of the tipsample interaction. Factors such as the mechanical modulus of the sample can be determined through such measurements (Calabri et al. 2008) and, in addition, adhesion between the tip and sample can be quantified by recording the motion of the AFM tip and cantilever upon retraction from the sample

surface (Hinterdorfer et al. 2002). Within these two apparently simple processes lies a surprising range of measurement possibilities and further details about some of these is discussed in the following section.

## **Research** examples

### **Image quantification**

An example that shows the advantage of AFM compared to traditional microscopic techniques is the study of carbohydrates, as they are not possible to image in native states at molecular resolution with electron microscopy due to the low atomic mass of its components. They require either metal coating which limits resolvability (Gunning et al. 1995) or further sample derivatisation techniques such as negative staining. Starch is one of the most common dietary carbohydrate components consumed but there are certain issues with it in terms of its digestibility. Some forms of starch are rapidly digested which produces high glycaemic index (GI) that creates a sugar spike in blood and others are very slow, named resistant starch (Asp et al. 1996). The differences are mainly due to the physical nature of the starch; high GI starch is mainly amorphous and resistant starch has a higher ratio of crystallisation (Buleon et al. 1998). Starch consists of two polysaccharides; amylose a linear polymer of  $\alpha$ -1,4 linked glucose which has secondary structures of single and double helices. The other starch polysaccharide is amylopectin, a similar polymer to amylose as it's composed of a backbone of  $\alpha$ -1,4 linked glucose that contains





complexes



complexes



**Inactive GA** complexes

Figure 3. AFM images of starch polysaccharide amylose, and complexes of amylose-glucoamylase components (scale bars 100nm)



Figure 2. Schematic diagram of Glucoamylase.

highly branched sidechains of  $\alpha$ -1,6 linked glucose. Enzymatic digestion of 'crystalline' starch is important in conveying the health benefits of the resistant starch content of starch-based foods and also in the commercial use of starch as an industrial substrate. There are several enzymes that can digest amorphous starch but crystalline starch is only digestible by a multi-domain enzyme, Glucoamylase I (Figure 2).

The smaller starch-binding domain (SBD) is attached to the larger catalytic domain by a heavily glycosylated linker which separates them. The SBD contains two binding sites. Although it was initially assumed that the starch binding domain simply acted as an anchor for the catalytic domain to digest insoluble crystalline starch, a previous study showed an unexplainable result when SBD was added as an isolated component to a mixture of crystalline starch with a single domain version of Glucoamylase (named Glucoamylase 2) that doesn't have SBD (Williamson et al. 1997). Adding the SBD as an isolated component activated the degradation



Figure 4. Quantification of the contour lengths: (a) native amylose (b) amylose-wild type SBD complex (c) amylose-mutant SBD complex (d) amylose-mutant glucoamylase I complex.

of the crystalline starch by Glucoamylase 2 which pointed out that its role was not just an anchor. So, AFM was used to investigate further detail of SBD's role (Morris *et al.* 2005). The amylose was solubilised by surfactant intercalation to allow molecular visualisation of it in a single helical form (Gunning *et al.* 2003). Subsequent images were then captured of the binding of wild type SBDs, mutated SBDs lacking one of their binding sites, and a catalytically inactivated form of the full enzyme (GA1) to the amylose (Figure 3).

The AFM images revealed an interesting change in the configuration of the extended linear structures of the amylose. SBD binding formed ring-shaped complexes with amylose. The genetically modified mutants of SBD that inactivated one of each of the binding sites produced linear complexes with amylose. The catalytically inactivated full enzyme which maintained its wild type double binding site SBD also formed ring-like complexes with amylose. This result showed that the double binding sites had a secondary effect on the interaction between the enzyme and its substrate. Quantification of the chain lengths (Figure 4) in the images enabled interpretation of how the rings were formed. Although amylose is a polydisperse polymer, the

distribution values of the perimeter of the rings were almost precisely half compared to the distribution of the contour lengths of the linear chains in all of the images which suggested the formation of the rings represented a circular full overlap of the amylose chains. A previously known fact was that the binding sites on the SBD are oriented at 90 degrees relative to each other (Sorimachi *et al.* 1996) and this combination of factors drove the solution shown in Figure 5. The rings are symmetric and closed, the parallel chains cross at 90 degrees and SBDs act as substrates for an expanded double helix. It revealed



Figure 5. Model of the complex formed by SBD binding amylose.



Figure 6. Model of the action of the starch binding domain of Glucoamylase 1.

that the SBD binds the individual amylose chains on either side of its 'face'. This confirmed that SBD can recognise, bind and distort the amylose double helix on the crystal surfaces of resistant starch (Figure 6). Distortion of the amylose helices in a crystalline form is the second role that the starch binding domain plays in Glucoamylase's enzymatic degradation of resistant starch.



Figure 7. (Left) panel; functionalisation of AFM tips by covalent attachment of bio active proteins. (Right) panel; schematic diagram of exploring the spatial distribution of glycans on mucin.

### Force spectroscopy

Force spectroscopy is AFM's unique ability compared to any other form of microscopy. AFM can not only measure the magnitude of forces but also at extremely high spatial resolution which gives it a significant advantage in terms of the data contained within its spectra. A biomolecule that has been very difficult to characterise with the traditional techniques of biochemistry is mucin. It is a glycoprotein which consists of a polypeptide main chain that is heavily substituted with highly branched oligosaccharide sidechains, referred to as 'antennae' (Robbe et al. 2004). The important aspect is that carbohydrates such as sugars can encode dramatically more bio information due to their significantly higher variation in structural arrangement compared to the other bio encoding molecules such as DNA and proteins (Davis 2000).

Sugar molecules have many more linking sites than DNA bases and amino acids, and in addition the orientation of their hydroxyl groups vary and they also have numerous substitution potentials. The effect that the structural variation of carbohydrates has on biomolecular interactions is termed the 'glycocode'. The example of this next section is an AFM force spectroscopy based solution that has revealed new spatial information about the glycan distribution in mucin (Gunning et al. 2013). Figure 7 illustrates the sample preparation protocols for ligand-receptor investigation. The mucin molecules were covalently attached via their N-terminus to glass slides and the AFM tips were functionalised with highly specific carbohydrate binding proteins, named lectins (Iskratch et al. 2009). The covalent attachment of the lectins to the AFM tips includes a heterobifunctional polyethylene glycol (PEG) linker to give it a few nanometres of separation from the apex of the tip as it's less likely to be trapped under the apex of the tip which saves it from being crushed upon contact with the slide. The PEG linker also enables rotational freedom of the lectin so that it can interact specifically with its target epitope (Hinterdorfer et al. 2002).

The functionalised tip is repeatedly lowered until it gently touches the mucin coated glass slide at a controlled level of force (~200 pN) in a physiological buffer (PBS) and then retracted to the appropriate height that slightly exceeds the length of the mucin molecules. It could be considered as a nanoscale form of 'fly fishing'. The motion of the cantilever is recorded over the whole time period to detect when the possible lectin-glycan binding events occur. The raw data output is displayed as force versus distance in Figure 8. The force spectra revealed molecular interaction when the lectin functionalised cantilevers probed mucin coated glass slides. Whilst the approach part of the force distance curves are featureless, multiple unbinding events are seen upon retraction of the AFM cantilever as each lectincarbohydrate bond which forms in-situ is stretched until it ruptures. Each curve contains information



**Figure 8.** Force distance curves of lectin functionalised AFM tips binding mucin glycans. Arbitrary force offsets added to the black and blue curves to separate each data set for clarification.

on binding strength and the distances between the particular carbohydrate recognition events. The three examples shown in Figure

8, taken from the same sample, reflect the heterogeneity of the glycan distribution present within populations of mucin molecules. Ligand-receptor interaction is stochastic hence the 'fly-fishing' process was repeated at least times for each sample 1024 to gather sufficient data for statistical analysis. The experiment was carried out with several different lectins that are specific to the range of the sugar epitopes present in the mucin sidechains to characterise the details of the glycan sidechains at molecular resolution.

Extracting the information in a meaningful way from the force spectra was done by statistical analysis. Initially the measured separations between adhesion events for each lectin were combined to form histograms (Figure 9, left) and then analysed. The distribution of the nearest neighbour site separations can be approximated by a Gamma distribution (Figure 9, right).

The data in Figure 10 shows it also allows comparison of the glycan distribution in mucins from different locations in the GI tract (left, gastric and jejunal mucins) and to track changes induced by enzymatic treatment of the mucin glycans (right). The letters assigned to the curves indicate whether they are

similar (matching letter) or significantly different (changed letter).



Figure 9. Analysis of the binding event data. Left panel: histogram of the distances between the binding events in the force spectra. Right panel: mathematical fitting of the histogram data to a gamma distribution model.



Figure 10. (Left) panel: Density distribution of binding events between 4 different lectins on two mucins from different regions of the GI tract. (Right) panel: enzyme effect on variations in fucose epitope density distribution.

In conclusion, this study showed that force spectroscopy can be used to characterize the distribution of specific carbohydrate species and reveal differences in the highly complex structures of mucins. Fitting the adhesion event distances from the range of the specific binding lectins allows quantitative comparison of the different glycan epitopes present within a given mucin, which reveals detail of the structural composition of the antennae (Figure 11). Despite the inherent heterogeneity



**Figure 11.** Schematic summary of what the binding event distribution analysis conveys.

of the glycan substitution on mucins, the fact that this technique can discriminate between mucins from different regions of the gut and track changes induced by enzymatic attack holds much promise. AFM can not only begin to read the glycocode on

mucin, but also investigate which external factors may re-write it.

# Nano-indentation and Nanomechanics

As already shown, the AFM is capable of highresolution imaging and advanced force spectroscopy. However, having scanned a region of interest, the cantilever/probe assembly can be used to examine the mechanical properties of a wide range of materials and at a range of temperatures. This localised approach of imaging and nano-mechanical properties makes the AFM an indispensable research tool.

As the AFM cantilever obeys simple Hooke's Law (F=kz), the amount of force applied by the cantilever to a surface is based upon the cantilever spring constant. The range of nominal spring constants available from various manufacturers is 0.006N/m to 100N/m. For a modest range of displacement (1nm to 3mm) detection, the minimum/maximum forces using the soft and stiff cantilever spring constants would correspond to 6pN to 300mN. This range of force sensitivity is sufficient to detect an unfolding event of a protein up to making indentations in a stiff polymer.

After calibrating the spring constant and measuring the tip radius (indirectly by scanning a sharper object; or directly by SEM), nanoindentation can be carried out. Figure 12 shows a typical force (F) vs. indentation ( $\delta$ ) plot, where the red curve shows the



Figure 12. (Left) typical force plot showing load, hold and unloading sections (right) modified AFM cantilever with a 20mm diameter glass sphere, scale bar 100mm.

recorded force as the tip approaches the surface – a negative indentation is effectively a separation. For visco-elastic samples, the user is able to hold the maximum load for a period of time; this allows the sample to creep – continuation of deformation under constant stress. Then, the curve in blue shows the recorded force when the cantilever is moving away from the surface; this allows the user to examine adhesive forces.

The dashed black line is a curve fitting to a Hertzian contact mechanics model (inset Figure 12), which returns an elastic modulus for a spherical contact.

Following a nanoindentation, the AFM can readily re-scan the same area, which makes it possible to explore any plastic deformation of the surface (Figure 13). Following this type of experimentation, the residual contact area (A) can be calculated, then the material hardness (H = F/A) can be extracted.

Hertzian contact mechanics allows a straightforward estimation of surface modulus of various samples under any aqueous conditions. However, this type of elastic analysis does have a range of limitations; especially for soft, visco-elastic biomaterials or polymers. Therefore, a number of



Figure 13. (Left) AFM image of single collagen fibril on a silicon substrate with 2 individual force plots, causing plastic deformation. (Right) Closeup on one indentation, estimating the hardness of a collagen fibril ~ 350-600MPa (Grant et al 2008).



Figure 14. (Left) Normalised Indentation creep vs. time curves made on a polymer film at 22, 75 and 100°C. (Right) Short-term and long-term modulus as a function of temperature for a semi crystalline polymer (PET) (Grant et al 2013).

other nanoindentation techniques can be explored, namely time dependent creep of an indenting cone (half angle  $\alpha$ ) using a spring in series with a spring/ dashpot in parallel model (Figure 14). Curve fitting of curves extracts a short-term modulus (E<sub>1</sub>) and a long-term modulus (E<sub>2</sub>), which is restrained by the viscosity of the dashpot ( $\eta$ ) in parallel.

$$\delta^{2}(t) = \frac{\pi}{2} F \cot(\alpha) \left[ \frac{1}{E_{1}} + \frac{1}{E_{2}} \left( 1 - e^{-tE_{2}/\eta} \right) \right]$$

Further visco-elastic analysis can be extracted from creep curves by applying a series of dynamic oscillations of known frequencies on blood vessels (Grant & Twigg 2013) and scar skin tissue (Grant et al 2012). Figure 15 shows the applied force and measured indentation creep against time following



**Figure 15.** Dynamic mechanical analysis using AFM to extract the amount of visco-elastic contact. (Right) Sinusoidal function (black dash line)  $f(t) = A \sin(qt)$  are used to extract the phase lag between the applied force and measured indentation.

oscillations at 8, 4, 2, 1 and 0.5Hz. Simple sinusoidal functions are fitted to the force vs. time and indentation vs. time; extracting the phase difference between the corresponding curves. A zero phase change indicates a purely elastic contact, whereas a phase change of 90° indicates a purely viscous contact.

Recent advances in AFM nano-mechanical technology include the capacity to extract mechanical properties from the sample surface, without any level of indentation - just from scanning alone. AM-FM (amplitude modulation – frequency modulation) is a new technique that allows the user to take advantage of imaging at two resonant frequencies (Proksch & Yablon 2012). For normal AFM imaging, the amplitude of oscillation of the first resonant mode is monitored, with appropriate feedback, to follow the surface (Figure 16). However, a simple equation allows users to utilise the amplitude and phase images from first resonance imaging to obtain a loss tangent image (Figure 17). The loss tangent is the ratio of the amount of dissipative energy to elastic stored energy, which is an indicator of viscoelastic behaviour.

The second resonance is treated slightly differently, in that it is the frequency is monitored and used for feedback, whilst holding the phase at 90°. This change of frequency is directly related to the stiffness of the sample, which can be converted to an elastic modulus with an appropriate conversion factor





Figure 18. Corresponding height and modulus AM-FM images of a PEEK polymer sample, giving a peak distribution moduli of 3.47GPa.

Figure 16. Schematic of AM-FM visco-elastic mapping technique, where the first resonant frequency is used for both topography and loss tangent imaging. The second resonant frequency is used to extract elastic and dissipative characteristics. (Image courtesy of Oxford Instrument Asylum Research, Santa Barbara, CA, USA)



Figure 17. (Left) A 3D representation of a spin-coated blend of polystyrene (PS) and poly-methyl methacrylate (PMMA) (50%:50%) dissolved in chloroform. (Right) Corresponding loss tangent image of the polymer blend, (bottom) distribution of loss tangent data for each of the polymer phases. (Figure 18). This is found by scanning a sample of known moduli (e.g. fused quartz – 69 GPa), before scanning the sample of unknown properties. Using this technique, the range of sample moduli is in the region of 1MPa to 200 GPa. Quantitative nano-mechanical mapping has been used to measure the elastic modulus variations of a polymer surface at 2 and 20 Hz scan rates, yielding values of the Young modulus that are independent of the scan rate (Garcia & Proksch 2013).

In conclusion, atomic force microscopy is an advanced instrument that allows the researcher a multitude of techniques on the widest range of materials. Even though the instrument was only invented 30 years ago, considerable advances have been made in that short space of time. In fact, we have only just scratched the surface (Figure 19).

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Figure 19. A 30mm polycarbonate sample (before/after), where the AFM probe has been used to "scratch" around a superimposed logo.

# **About the Authors**

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first in Britain to use the original form of scanning probe microscopy, the scanning tunnelling microscope (STM) and their research has now moved on to atomic force microscopy (AFM). Patrick is a recognized expert in field of biological AFM and has co-authored an internationally best-selling book on the subject (currently in its second edition). His research areas range from the material science of food ultrastructure to the physiological and biological interactions between food components, the GI tract and the microbiota which inhabit it.

Engineering and a PhD in Biophysics from University of Manchester. Main research interests lie in collagen tissues but fascinated by all aspects of anatomy/physiology in health, disease and aging. Current investigations on a wide range of materials including skin tissue, cornea/sclera, cartilage, blood vessels, bone, cancer cells, drug co-crystals, hydrogels &

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