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Atomic Force Microscopy of Biofilms—Imaging, Interactions, and Mechanics

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Abstract

Atomic force microscopy (AFM) has proven itself to be a powerful and diverse tool for the study of microbial systems on both single and multicellular scales including complex biofilms. This chapter will review how AFM and its derivatives have been used to unravel the nanoscale forces governing the structure and behavior of biofilms, thus providing unique insight into the control of microbial populations within clinical and industrial environments. Diversification of AFM-based technologies has allowed for the creation of a truly multiparametric platform, enabling the interrogation of all aspects of microbial systems. Advances in traditional AFM operation have allowed, for the first time, insight into the topographical landscape of both microbial cells and spores, which, when combined with high-speed AFM's ability to resolve the structure of surface macromolecules, have provided, with unparalleled detail, visualization of this complex environmental interface. The application of AFM force spectroscopies has enabled the analysis of many microbial nanomechanical properties including macromolecule folding pathways, receptor ligand binding events, microbial adhesion forces, biofilm mechanical properties, and antimicrobial/antibiofilm affectivities. Thus, AFM has offered an outstanding glimpse into the biofilm, how its inhabitants create and use this complex adaptive interface, and perhaps most importantly what can be done to control this.

Keywords: atomic force microscopy, imaging, force measurement, nanomechanical properties, adhesion

1. Introduction

Biofilms remain a primary concern in industrial and clinical fields. The tendency of planktonic cells to form these structures in moist environments and the resulting increase in resistance

to antimicrobials, in combination with an increasing frequency of innate antimicrobial resistance, demonstrates the continued need for novel biofilm control strategies and innovative methods to unravel the fundamental properties of biofilms. Atomic force microscopy (AFM) has proven to be a useful addition to the microscopy family providing imaging and force measurement capabilities that can interrogate the nanoscale properties of surfaces. Indeed, AFM has been used with great success to provide novel insight into the structure of biofilms and the interplay of interaction forces and mechanical properties that govern the behavior of biofilms and their response to chemical and physical attack as part of control strategies. AFM can be used to study whole biofilms or the influence of their component parts, from bacterial surface proteins to extracellular polysaccharides (EPSs) and individual cells. This chapter will first introduce the reader to the basic operation of the instrument relevant to the study of biofilms. The different capabilities of the instrument and their application to biofilm will be then reviewed with examples from the authors' laboratory.

2. AFM basic principles

AFM was first developed as part of the family of scanning probe microscopies in 1986 [1]. It was very quickly applied to the imaging of biological materials, including DNA, bacteria, viruses, and mammalian cells [2]. The components of atomic force microscope is shown in **Figure 1**. A very small, sharp tip held at the free end of a cantilever systematically scans a surface of interest to generate a topographical image. The tip is held in intimate contact with the surface, and its apex has a radius of curvature in the range of nanometers, which sets the image resolution. As the tip is systematically scanned across the surface, it encounters surface forces that cause the cantilever to be deflected. The deflection of the cantilever is monitored

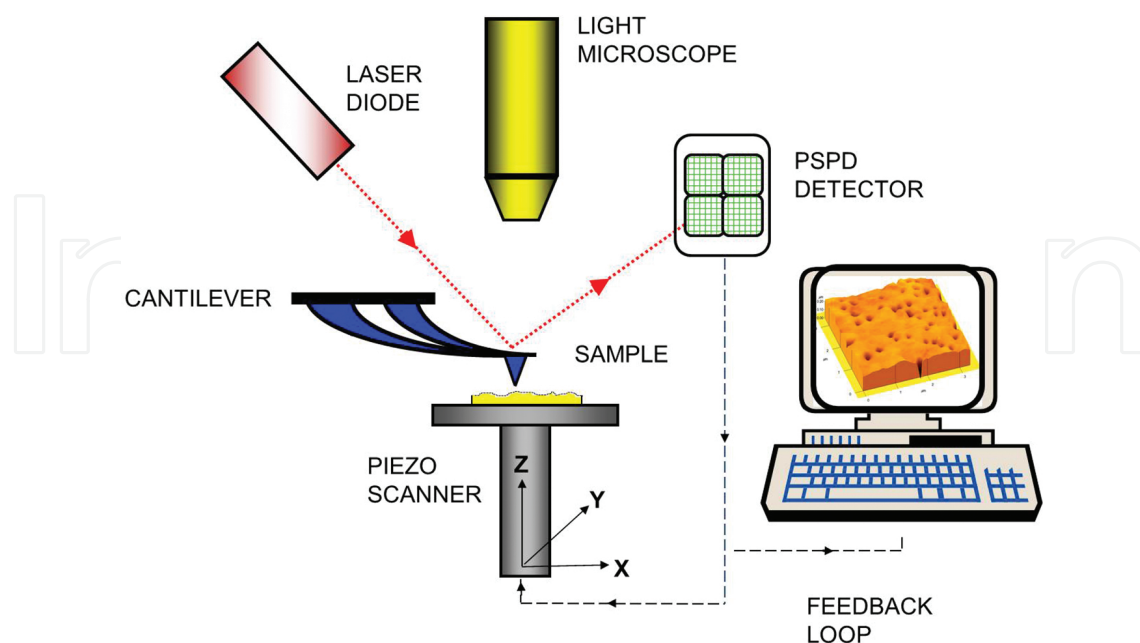


Figure 1. A schematic representation of the AFM instrument.

by the displacement of a reflected laser beam and used to create a topographical image. In contact mode, the forces of the bent cantilever keep the tip in intimate contact with the surface.

When imaging a soft sample such as a bacterial cell surface or biofilm, the tapping mode or intermittent contact mode is used. The intermittent contact of this imaging mode reduces the degree of friction or drag on a sample compared with imaging in contact mode. To achieve the intermittent contact, a vibrating cantilever technique is used, and the changes in the vibrational parameters are monitored as the cantilever scans the surface. In response to changes in topography, the piezo-scanner moves up and down to maintain a constant vibration of the cantilever, and the feedback signal is used to produce the image data set. A further advantage of this imaging mode is that measurement of the phase angle between the free oscillation at the end of the cantilever and the imposed driving vibration provides a map of phase angle across a surface; this data, referred to as phase imaging, is captured simultaneously as the standard topographical data. This phase angle is often used to qualitatively distinguish between materials on the surfaces of heterogeneous samples as the phase angle change is a function of the mechanical properties of the surface and the area of contact between the AFM tip and the surface.

The advantages of tapping mode have meant that this is the most frequently used method when imaging soft biological samples. The authors have found tapping mode in combination with phase imaging extremely useful in identifying structures on the cells and within biofilm.

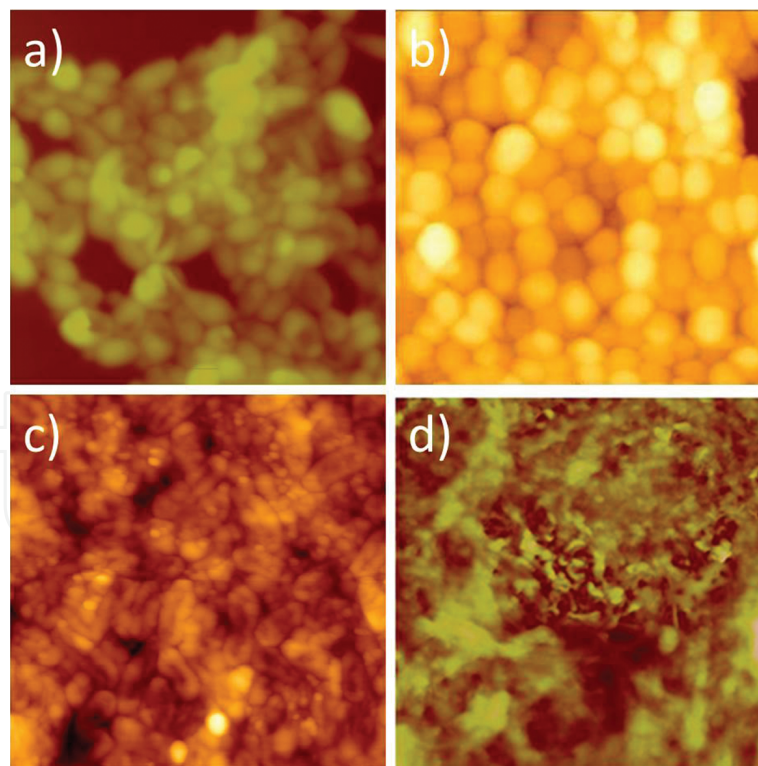


Figure 2. AFM tapping mode images of microbial biofilms: (a) *Candida tropicalis* (50 μm^2), (b) *Staphylococcus aureus* (10 μm^2), (c) *Pseudomonas aeruginosa* (10 μm^2), (d) mixed species biofilm at an industrially fouled reverse osmosis membrane (10 μm^2).

Figure 2 presents AFM tapping mode images of a range of microbial biofilms. When imaging biofilms, the mechanical robustness of a biofilm should be considered; it is simpler to image model biofilms with minimum components, which have been grown on adhesion-promoting substrates, compared to biofilms that have been sampled from natural or industrial settings that consist of multiple components (**Figure 2d**). As AFM imaging is a technique that relies on surface contact, the imaging of a hydrated diffuse biofilm is very difficult without fixation methods.

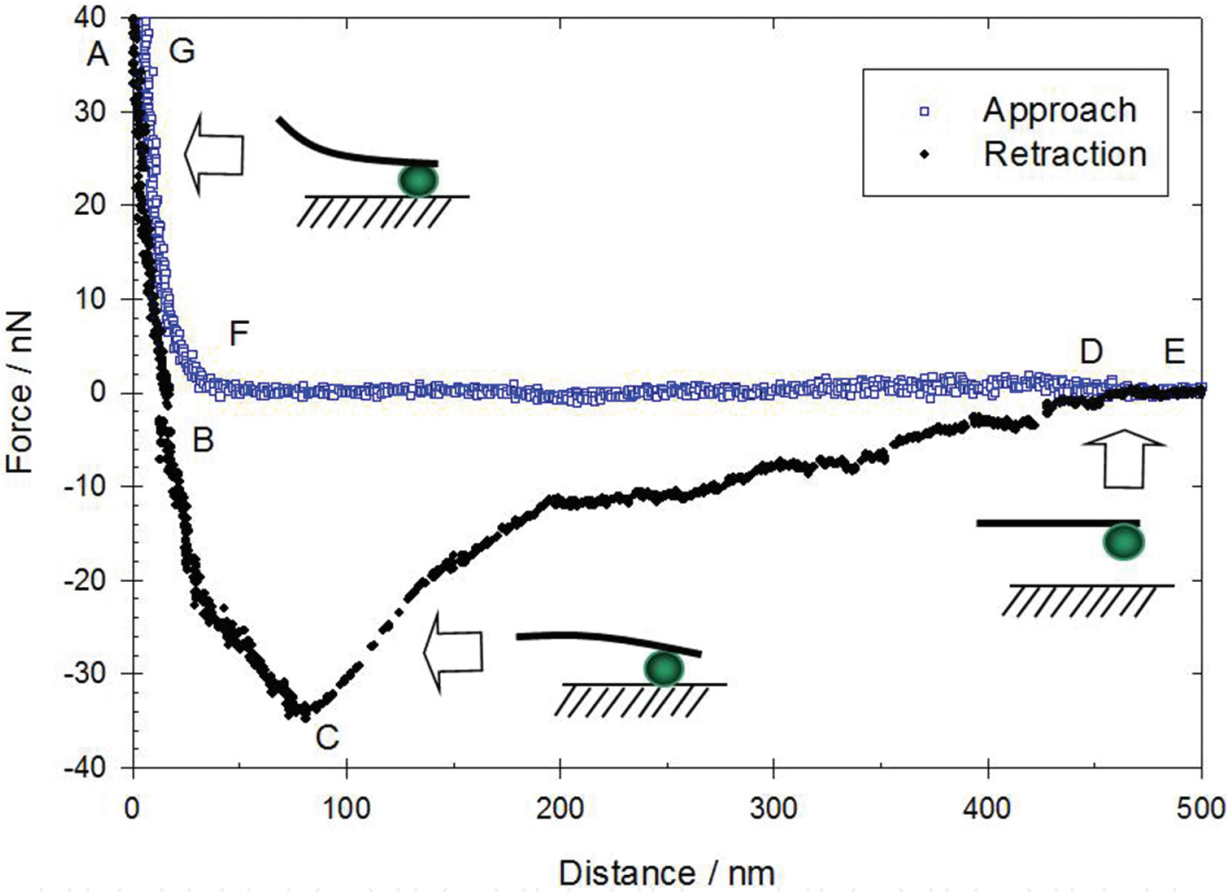


Figure 3. A typical force measurement between an AFM cell probe (*Saccharomyces cerevisiae*) and a surface in a process-relevant environment (10^{-2} M NaCl).

The AFM can measure the forces of interactions between surfaces, which have obvious implications in the study of biofilms. AFM has been added to the group of instruments that can be used to study microbial interactions involved in biofilm formation. Such instruments include flow chambers, micropipette aspiration, and centrifugation devices. However, AFM has the advantage of allowing the imaging and identification of points of interest on a surface prior to the measurement of the forces of interaction. AFM also allows the direct measurement of forces as opposed to techniques that estimate force from the application of shear to a cell

population. In addition, surface forces are measured over very small contact areas, minimizing contamination problems. To generate a force–distance curve, the deflection of the cantilever is recorded as a function of tip-to-sample separation, as the piezo-scanner of the AFM brings the sample and tip together. The deflection of the cantilever is converted to a value of force using Hooke's law. Force–distance curves are characteristic of the system under study. For biofilms, they have features that reflect the chemical and physical properties of the surfaces that are interacting, including the substrate, the cells, EPS, and the AFM probe. **Figure 3** shows a typical force measurement between an AFM cell probe (*Saccharomyces cerevisiae*) and a surface in a process-relevant environment (10^{-2} M NaCl) [3]. The force is plotted as a function of separation distance and shows some key features for the characterization of the surfaces involved. At position D (referring to **Figure 3**), the cantilever and probe are moving independently of the surface, as the probe is brought into contact with the surface, until at position F it encounters physiochemical forces, which in this case are repulsive and likely to be dominated by electrostatic forces. The extension of the scanner continues to push the cell into contact (F–G) until a predefined loading force is reached, whereupon the movement is reversed and the probe is retracted away from the surface by the retraction of the piezo-scanner. At position C, the bending of the cantilever is inflected and the forces in the bent cantilever begin to rupture the adhesion between the cell and the surface. If this was an inorganic hard particle, a sudden break in contact would be observed. However, with the yeast cell with macromolecular tethers (and any deformable surface), a sequential breaking of contact is observed as the forces in the bent cantilever peel the cell from the surface, until at position E the cell probe is moving independently of the surface. The adhesion measurement is determined from the difference in force between positions C and D. Integration between the approach and diffraction curves gives an estimate of the energy of adhesion. The mechanical properties of the system can be determined from the contact region (F–G and A–C) and the adhesion component of the curve (C–D).

Operating the AFM as a nanoindenter allows the measurement of microbial cell and biofilm mechanical properties, which include elastic moduli and turgor pressure [2]. **Figure 4** shows how the indentation depth is measured by comparison between force curves measured at a reference hard surface and at the softer sample surface. The indentation depth can then be plotted as a function of applied force and compared with a theoretical framework to quantify sample mechanical properties. The most commonly used theoretical framework is based on the Hertz model, which describes the elastic deformation of two perfectly homogeneous smooth bodies touching under load. The geometry of the system is assumed to consist of an indenter with a parabolic shape and a sample that is of much greater thickness than the indentation depth. The Hertz model that describes force on the cantilever $F(\delta)$ as a function of indentation depth is:

$$F(\delta)_{\text{parabolic}} = \frac{4E\sqrt{R}}{3(1-\nu^2)} \delta^{3/2}$$

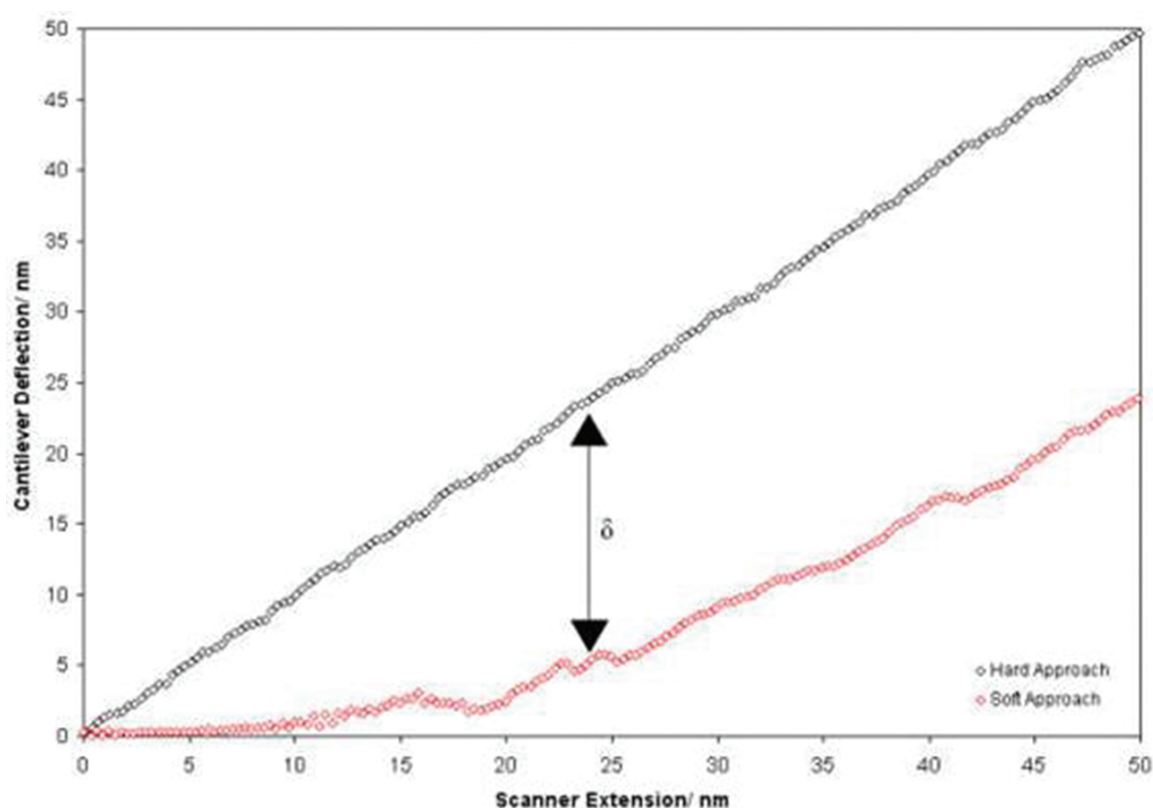


Figure 4. The measurement of indentation depth (δ) by comparison of the slope of the contact region of force curves at hard and soft surfaces.

where the tip is approximated with the radius R , the depth of indentation is denoted by δ , E is the Young's modulus of the sample surface, and ν is the Poisson ratio for the sample material (assumed to be a value of 0.5 for biological samples). Other theoretical frameworks have been used to interrogate AFM nanoindentation curves such as the JKR (Johnson, Kendall, Roberts) model. When choosing which model to use and interpreting the data, a number of considerations should be taken into account. The mechanical properties of microbial cells and biofilms will not be homogeneous across their surface and will be a convolution of whole cell compression as well as material close to the tip. In addition, nanoindentation is an invasive technique which applies a disruptive force to the surface. Repeated indentation at the same location on the cell or biofilm will disrupt the structure and its mechanical robustness rendering subsequent measurements invalid.

3. Imaging

Examination of microbial systems in native, aqueous environments is central to the validity of the data collected. However, AFM imaging in such environments is often difficult due to a number of factors. For instance, microbial cells are often attached to the surface via weak Lifshitz-Van der Waals forces, and as a result are easily disrupted by the scanning of an AFM

cantilever, resulting in the destruction of the sample [4, 5]. Additionally, microbial cells are often motile with some recent papers suggesting that motility may even be the largest governing factor in the physiological imaging of microbes [6]. Consequently, immobilization of microbial cells prior to analysis has become imperative to the application of AFM in the imaging of microbial systems.

3.1. Cell immobilization for single-cell analysis

Immobilization of microbial cells has often proven to be the most problematic step in the imaging of microbial samples under aqueous conditions. The immobilization must be secure enough to withstand the lateral forces exerted by the tip during scanning, but benign enough to not force physiochemical, physiological, or nanomechanical changes in the sample. As a result, a number of different techniques have arisen; these protocols can be broadly divided into two categories: mechanical, whereby microbial cells are physically trapped within a porous media, and chemical, whereby chemical treatment of the substrate is used to facilitate binding.

Initial studies into the use of mechanical protocols to immobilize microbes utilized agar or membranes with pore diameters similar to the cell diameter of the organism to be captured [4, 7–9] (**Figure 5**). Later work expanded upon this through the use of more complex or functionalized surfaces such as lithographically patterned silica [5, 10–12]. Though, while mechanical entrapment offers immobilization secure enough to alleviate the destructive scanning of the cantilever, the immobilization is sporadic and unpredictable, reducing the reproducibility of

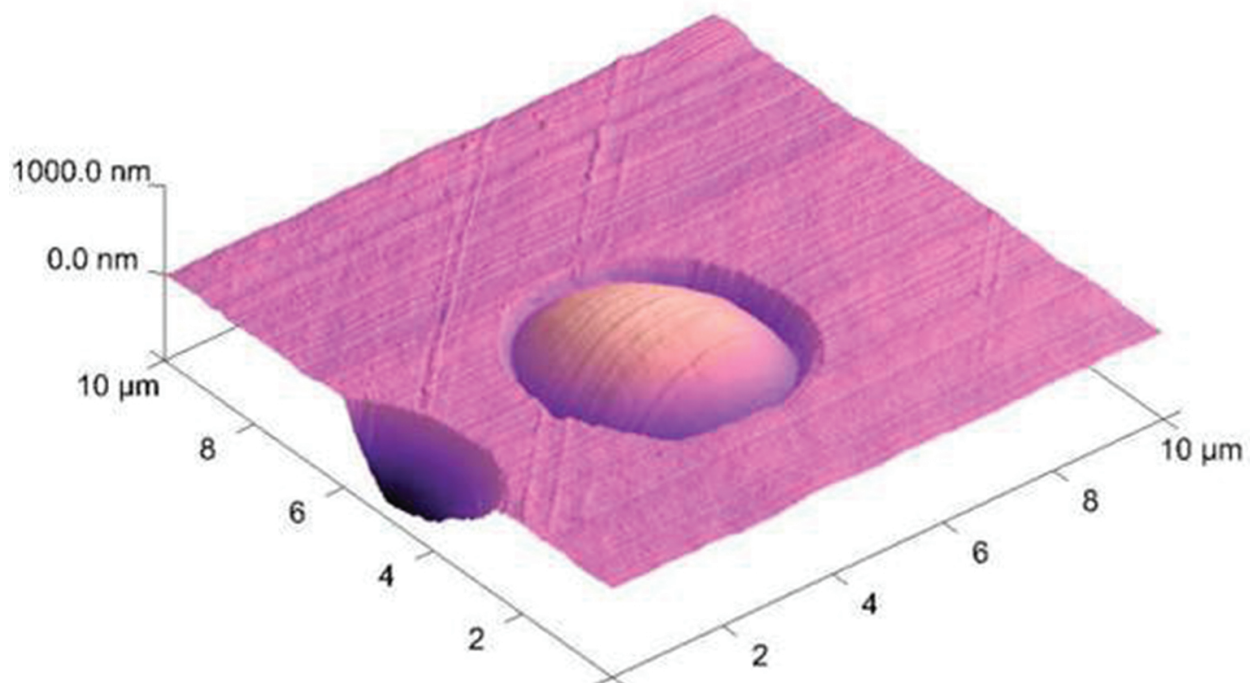


Figure 5. A yeast cell (*Saccharomyces cerevisiae*) trapped in a microfiltration membrane prior to AFM study.

the results. Recent work by Formosa et al. [13] developed a protocol in which selective tuning of polydimethylsiloxane (PDMS) stamps were used to immobilize spherical microorganisms of various sizes. The protocol requires the creation of a glass and chromium blank that holds the microstructure, from which the pattern is transferred to a silicon wafer by deep reactive ion etching. The dimensions of the silicon master can be varied with the group reporting dimensions of 1.5–6 μm wide, a pitch of 0.5 μm , and a depth of 1–4 μm , accommodating a variety of target cell sizes. A PDMS stamp is then cast from the silicon wafer master and cells deposited through the use of convective and capillary forces. Further work by the group has shown this immobilization technique to be an effective way to immobilize spherical cells, in this case *S. cerevisiae* and *Candida albicans*, and, spore of *Aspergillus fumigatus* with no effect on viability [14–17]. Additionally, the technique allows for the rectification of one of AFMs greatest flaws, analysis of multiple cells to achieve statistical significance. Previously, this has not been feasible using other immobilization techniques due to the relatively low rate and sporadic nature of deposition; thus, the development of a platform capable of producing arrays of uniform cells for multiparametric analysis will increase the reliability of AFM analysis. However, this technique is limited due to its inability to immobilize nonspherical organisms.

A number of chemical fixation methods for the immobilization of microbial cells have been used, including, poly-L-lysine, trimethoxysilyl-propyl-diethylenetriamine, mica, and carboxyl group cross-linking [18–22]. While these techniques offer a high level of immobilization, some cross-linking agents have been shown to negatively impact the nanocharacteristics and viability of the immobilized cells [23]. Despite this, some techniques, such as the use of photocatalytically active silicon, also offer a high level of cell orientation and organized immobilization not offered by conventional mechanical techniques, which, depending on application may be favorable over the associated reduction in viability [24]. Other recent advances also indicate that the addition of divalent cations, such as Mg^{2+} and Ca^{2+} , and glucose may provide optimal attachment without the associated reduction in viability. In one such study, Lonergan et al. [25] reported that *Escherichia coli* cells immobilized on poly-L-lysine in $0.01\times$ PBS-S, with a rehabilitation period in minimal media were sufficiently immobilized to perform AFM analysis while maintaining membrane integrity.

3.2. Cell topography

Analysis of the topography of single cells has proven to be a powerful addition in the real-time visualization of cellular surface structures. However, the structural landscape of metabolically active cells exists in a constant state of flux; thus, the ability to image surface morphologies under physiological conditions is vital for characterization. Previous studies have utilized AFM to image a number of key microbial features. In 2010, negative mutants of cell wall polysaccharide (WPS-) of *Lactococcus lactis* were shown by AFM imaging to exhibit a 25 nm corded like structure perpendicular to the long axis of the cell; further mutagenesis studies confirmed that these structures were not due to hydrolysis, and AFM chemical spectroscopy (imaging with a functionalized tip) using LysM confirmed that the bands consisted of peptidoglycan [26]. In a more recent study, an in-depth analysis of *Streptococcus agalactiae* (Group B *Streptococci*) peptidoglycan confirmed the presence of approximately 25 nm corded

structure running perpendicular to the long axis of the cell [27]. However, during this study the bands were found to periodically interlink to form a net-like structure. Imaging of other Group B *Streptococci* showed that this net-like structure, while exhibiting some variation in pore dimensions, remained constant. The group then imaged a number of cell wall deficient mutants in an attempt to identify structural abnormalities associated with other surface macromolecules; however, no significant alterations in the peptidoglycan net to suggest macromolecular anchoring were observed. Significant alterations in the solute concentration were found to alter the net-like structure with the group observing a near doubling (~25 to ~47 nm) of the peptidoglycan bands, suggesting that the net-like structure may influence adaptation of the cell to changes in turgor pressure. Similarly, the growth phase of the organism was found to have a significant effect on peptidoglycan structure; topographical images of a high proportion of Group B *Streptococci* grown to stationary were shown to exhibit a tendency to express a rough peptidoglycan layer as opposed to the previously described net-like structure. Upon further investigation, this roughness was shown to consist of highly ordered strands aligned in parallel with the divisional plane having a periodicity of approximately 4.5 nm; the group suggests that these may in fact be glycan strands; however, the structure and density of the strands prevented the researchers from coming to a clear conclusion.

As outlined above, in vitro AFM has been used to map the topography on cellular structures at a number of cell life stages, as in the work of Abscali et al. [28] who examined changes in the macromolecular structure of the cell wall of *Streptomyces coelicolor* during its life cycle from vegetative hyphae to spores. Yet, such studies merely offer a snapshot of cellular processes. Thus, several studies have aimed to image the dynamics of cellular processes. Germination of *Bacillus atrophaeus* has been successfully imaged; post exposure to a germination solution, the rodlets comprising the spore coat were shown to disassemble and form 2–3 nm etched pits [29]. The pits were subsequently shown to mature into highly orientated fissures perpendicular to the rodlet orientation, beneath which a highly ordered hexagonal structure was observed. The study continued to image the germinating spore through to the emergence of the germling cell, and the spore fissures were observed to form apertures of approximately 70 nm that dilated with germination. In vitro analysis of the germling confirmed the presence of vegetative cell wall structures prior to emergence, which were similar to those of mature vegetative cells.

3.3. Microbial cellular surface layers

Microbial membranes consist of a number of surface layers, the outermost of which, the S-layer, consists of a monomolecular layer composed of self-assembling single proteins, or glycoprotein monomers exhibiting oblique, square, or hexagonal symmetry. Due to its self-assembling nature and its role in many innate immunities associated with microbes, S-layers have become the focus of many AFM studies. Initial studies into S-layers successfully imaged PS2 monomers of *Corynebacterium glutamicum* and in the process highlighted the presence of a bilayer of hexagonally arranged monomers and a nanogrooved substrate; further work suggests that this substrate may be involved in the creation of the monolayer [30, 31].

In a recent study, the nanomechanical and structural properties of *Propionibacterium freudenreichii* surface layer protein A (SlpA) was characterized [32]. SlpA was found to consist of a hexagonal *p1* monomer with a high level of disorder; upon heating to 45°C, SlpA was found to maintain structural integrity post recrystallization. However, a marked reduction in the elasticity of the SlpA layer from 4.2 ± 0.9 MPa at 25°C to 1.8 ± 0.3 and 0.9 ± 0.1 MPa for 35 and 45°C, respectively, demonstrate that while topographically comparable, the nanomechanical properties of SlpA had altered. Additional work conducted by the group showed the SlpA exhibited the same, albeit less pronounced, behavior in response to alteration in pH. The topographical characteristics of SlpA were maintained to pH 3; however, a corresponding reduction in the elastic properties was observed: 5.7 ± 1.4 MPa and 5.5 ± 1.6 MPa at pH 6.7 and 5, respectively, followed by a reduction to 2.2 ± 0.3 MPa at pH 3. The group attributes this reduction in the elastic properties to be a result of a number of physiochemical interactions such as the reduction in pH below that of the theoretical pI of SlpA and protonation of the disordered regions.

3.4. High-speed AFM

While spatial resolution using AFM has remained high, the lack of high temporal resolution has limited the application of topographical studies of microbial systems. Optimal scan speed varies; however, the minimum is restricted to the order of approximately 30 s for an AFM image. This level of temporal resolution is sufficient for the imaging of relatively low fluctuating structures and processes, such as S-layers and cell division. The high-resolution imaging of surface macromolecules has remained elusive due to the limited speed of standard AFM imaging. However, the recent development of high-speed AFM (HS-AFM) has enabled the resolution of such structures primarily due to HS-AFMs to show exceptional temporal resolution (>100 ms) and significantly reduced scanning forces [33–35].

In one such series of studies, the dynamics of conformational changes of bacteriorhodopsin (bR) was successfully imaged in response to electrochemical radiation stimulation [33, 36, 37]. During initial studies, the group observed conformational changes in the form of a 0.69 ± 0.15 nm displacement of the center mass of the trimer structure when exposed to green light. Furthermore, the group was able to ascertain that these changes in the center mass were actually the result of the displacement of trimer monomers into close proximity with monomers of neighboring trimers via displacement of the E-F loop. Through combination of selective mutagenesis and HS-AFM, Yamashita et al. [33] were able to characterize the monomer association of bR trimers. During the study, five bR mutants were created: W10I, Y131I, W12I, F135I, and W12F, and HS-AFM used to image the structure of each trimer within the membrane. The study showed that W12I and F135I mutants were unable to form membrane-stable trimers, with only a small number of trimers assembling and quickly dissipating. Conversely, W10I, Y131I, and W12F were able to form a stable trimer structure, suggesting the presence of an aromatic residue at positions 12 and 135, which is essential to the formation of a stable trimer.

Further HS-AFM studies have been able to track the motion of membrane-bound macromolecules through three-dimensional space. In one such study, the rotational and translational

membrane dynamics of outer-membrane protein F (OmpF) were imaged to an optical resolution of approximately 750 Å [38].

While initial studies using HS-AFM revolved around its ability to resolve surface macromolecules, some focus has shifted to topographical analysis. In the first such study, the surface of *Magnetospirillum magneticum* was found in contradiction to initial models to consist of a very highly ordered series of nanometer-sized pores consistent with that of porin molecules [34]. Further work set out to ascertain if this was in fact a characteristic of all *Proteobacteria*, wherein Oestreicher et al. [39] imaged the surfaces of the *E. coli* and *Rhodobacter sphaeroides*. This was shown to be the case, and nanometer-sized pores of 8 and 6.6 nm were observed for *E. coli* and *R. sphaeroides*, respectively. Oestreicher et al. [39] concluded that due to the similarities in distribution and size when compared to *M. magneticum* (7 nm), and with the crystal structure size estimation of the outer membrane proteins of *E. coli* (OmpF and OmpC)—7.5 and 7.38 nm, respectively—that they must also be porins.

4. Force spectroscopy

AFM force measurement has been used extensively to study biological systems. In the past, AFM was limited to physics laboratories, and microbiologists focused on the benefits of AFM to imaging of single bacteria; bacterial studies were restricted to model surfaces, and the heterogeneity inherent to natural systems compromised quantification and discouraged the use of AFM force measurement. However, AFM technology has been disseminated to microbial laboratories that have the advantage of prior knowledge to guide AFM research strategies. In addition, the advent of improved data capture rates has permitted statistically viable AFM measurements to quantitatively characterize biological systems including biofilms. Modern AFM studies of biofilm orchestrate AFM imaging of microbial surfaces with force spectroscopy to unravel structure function relationships. The force-curves measured at surfaces have a number of components which can be used to characterize the mechanical and interaction properties of biofilms that are now discussed.

4.1. Microbial surface proteins

Surface macromolecules play an essential role in a number of physiological processes essential to the success of microbes including adhesion and existence within a biofilm; the activity of these molecules has been shown to be dependent on a number of environmental conditions [40–45]. Consequently, research into the nanomechanical and physiological properties of surface macromolecules has expanded over the last decade with the fundamentals of AFM tip-molecule binding forces in vitro having become well documented [46–48].

Several models have been described to interpret the nanomechanical properties of long-chain surface macromolecules. Typically, these models revolve around the use of the Worm-Like Chain (WLC) and Freely Jointed Chain (FJC) models, as these allow for the description of force-curve profiles and the definition of tether and binding partner interaction entropy, thus leading to contour length (L_0) definition [49, 50]. Defining L_0 offers a number of advantages, such as

the filtering of noise and predictions in the unfolding pathways of uncharacterized protein complexes [51, 52]. If the structure is unknown, L_0 allows collaboration of experimentally derived data to a theoretical value defined from the estimation of the sum of individual components fitted to a normal (Gaussian) distribution, therefore acting as a confirmation that the interaction is the one of interest, while offering a level of insight into the unbinding pathway. Studies conducted by Farrance et al. [53] expanded on traditional models, whereby a physical basis for the prediction of L_0 was described. The model, through the use of theoretically idealized tethering surfaces and the probability of two such chains meeting, is able to predict the distributions expected from experimentally derived data with a high level of agreement to existing studies.

4.2. Functional proteins at microbial surfaces

Microbial adhesion to biotic and abiotic surfaces is reliant on a number of macromolecular interaction including binding of small microbial peptides (SMPs), capsules, recognition proteins, fimbriae, and flagella. Single-molecule force spectroscopy (SMFS) has been used to characterize a number of microbial surface-bound receptors including antibiotic receptor ligand sites, fimbriae, flagella, and adhesins [54–56]. In an interesting example of the use of SMFS, the holdfast proteins of *Caulobacter crescentus* were characterized for adhesion to surfaces of varying polarities [57]. Holdfasts were allowed to adhere to each surface for an extended period of time greater than 16 h and imaged via AFM to determine the height and diameter; it was found that the holdfast height varied independently of the surface polarity; from 5 to 100 nm, however, the average height varied between 30.6 ± 2.4 nm and 21.5 ± 0.9 nm for mica and graphite, respectively. Holdfast foot diameter was also found to vary on both surfaces: 90.2 ± 2.7 nm for mica and 119.2 ± 4.1 nm for graphite; however, both showed large distributions in the data—30–280 nm and 45–450 nm, respectively. The group then proceeded to access the binding strength on holdfast-coated cantilevers to mica, graphite, clean glass, and 3-TMSM-treated glass, and the maximum adhesion force was measured—0.05, 0.08, 0.13, and 0.66 nN, respectively. Adhesion was concluded to be primarily a result of residence time and surface polarity.

4.3. Microbial mechanical properties

One of the distinct features of AFM over other SPMs is its ability to quantifiably resolve physiochemical properties of materials at the nanoscale. To date, AFM has been used to resolve the nanomechanical behaviours of a bacteria in a number of ways, from single-cell indentation studies to the characterization of molecular appendages such as pili and flagellum [58]. A number of techniques can be employed dependent on the type of nanomechanical measurement that is required, with most alterations involving functionalization of the cantilever. All nanomechanical studies revolve around the use of the force-curve analysis as detailed earlier in this review.

5. Adhesion studies

Biofilm adhesion qualities have been measured through AFM in a number of ways. EPS has been confirmed as a major mechanism controlling biofilm adhesion [59–62]. As a result, a number of studies have been undertaken to assess the effect of growth conditions, chemical treatments, and novel antimicrobials on the production of EPS and the reduction in adhesion. Oh et al. [61] used AFM force spectroscopy to study the influence of nutrient concentrations on *E. coli* biofilm maturation. The adhesion of an AFM tip at the surface of the biofilm increased as biofilms matured, indicating a release and accumulation of extracellular polymeric substances over the cell surface after primary colonization. Nunez et al. [63] used AFM imaging and force measurement to study the action of *Bdellovibrio bacteriovorus* on *E. coli* biofilms. AFM characterized the change in *E. coli* cells, as they were attacked by the predatory bacterium with cells changing from rod-shaped to a round shape, with a shrunken texture and the visible coil of *B. bacteriovorus* growing inside. *Bdellovibrio bacteriovorus* was shown to prevent biofilm formation and destroy established biofilms. This work was extended by Volle et al. [64] who used force spectroscopy to observe that the spring constant of predated *E. coli* cells was three times softer than that of normal cells and that there was change in cell wall morphology on predation, as there was much larger adhesion forces between an AFM tip and predated cells. This important work demonstrates that dynamic events in living unfixed cells can be characterized and investigated using AFM. Rodriguez et al. [65] used AFM force measurements to study the formation of *Listeria monocytogenes* biofilms at stainless steel surfaces. They found that the adhesiveness of biofilms was not influenced by contact time, loading force, or relative humidity, but surface chemistry is important; force measurements using SiO₂ and polyethylene colloid probes showed that *L. monocytogenes* cells within a biofilm adhered more strongly to hydrophobic surfaces. The mechanical properties of the surface that biofilms form at are important determinants on the properties of the biofilm.

Oh et al. [66] studied the formation of *Pseudomonas aeruginosa* biofilms at a range of surfaces including steel, rubber, and polypropylene. Biofilms were treated with hot water, and all surfaces with and without biofilms were characterized using AFM. Force spectroscopy revealed that adhesion was greatest at the untreated biofilm surfaces and that the reduction of adhesion after hot water treatment indicated the removal of extracellular matrix from the biofilm.

6. Indentation studies

AFM has been implemented to analyze several mechanical properties of microbial cells, such as elasticity and hardness [56, 67, 68]. Typically, this is done using the Hertz model, wherein the indentation of a material by a nonadherent probe can be used to calculate the elastic modulus of the substrate. Volle et al. [69] measured cell spring constants and AFM tip adhesion on cells within the biofilms of *E. coli*, *Pseudomonas putida*, *Bacillus subtilis*, and *Micrococcus luteus*. Gram-positive bacteria were observed to have largest spring constants with all cells having values in the range 0.16 ± 0.01 to 0.41 ± 0.01 N/m. These workers also demonstrated that the mechanical properties of chemically fixed cells are significantly different. Fang et al. [60] also

used AFM force spectroscopy to quantify tip-cell adhesion and surface elasticity of sulfate-reducing bacteria (SRB) biofilms. To achieve this, they used a force volume technique to map forces across the biofilm surface. Greater adhesion was measured at the cell-cell and cell-substratum interfaces; this was compared to a smaller and constant force at the bacterial cell surfaces and argued to be due to the accumulation of EPS at the interfaces. Another interesting study conducted by Longo et al. [70] demonstrated that AFM can be used to characterize the variations in nanomechanical properties across a single cell membrane. In the study, nanoindentation was performed across the surface of an immobilized *E. coli* cell, and it was found that there was a variation in the Young's modulus of the cell membrane. Upon further analysis, this heterogeneity was attributed to the presence of submembranous structures, hinting at the possibility that AFM may be capable of resolving the organization of such structures.

As confidence in the technique grew, focus of nanoindentation studies shifted from single cells to biofilms. However, use of the classic Hertz model to interpret the viscoelastic properties of biofilms, until recently, remained problematic [71, 72]. In a recent example of one such study, the elastic moduli of *P. aeruginosa* was found to be heterogeneous in nature, varying between approximately 40 and 45 kPa [73]. SEM and AFM topographical studies of the same sample showed variations in packing density of the cells throughout the biofilm, offering possible insight into the cause of the variation in mechanical properties. However, these variations may also be the result of underlying physiological structures such as nutrient channels. Finite element analysis performed by the group showed that the variation may be a result of the combined effect of the EPS and cell orientation.

There have been further studies into the nanomechanical properties of biofilms that have focused on the effect of growth conditions and novel antimicrobials on the nanostructure of biofilms. One such study showed that increasing concentrations of CaCl_2 resulted in not only an increase in EPS production but also alterations in EPS structure of *Pseudomonas fluorescens* biofilms [74]. Consequently, a reduction in stiffness and increase in both viscosity and adhesive forces were observed. In another study, AFM was used to assess the changes in the nanomechanical properties of *P. aeruginosa* and *Acinetobacter baumannii* biofilms after treatment with OligoG. During the study, OligoG was found to significantly lower Young's moduli and increase the surface roughness (R_a) when compared to untreated biofilms [75]. However, this study highlights one of the main challenges facing the characterization of biofilms via AFM: continuity of sample preparation. In the aforementioned study, the biofilms were dried prior to analysis, while others made use of hydrated samples. While both techniques remain valid, interstudy comparisons will remain difficult until a level of interstudy continuity is achieved.

7. Single-cell force spectroscopy

Single-cell force spectroscopy (SCFS) has become an essential tool in unravelling the forces involved in intermicrobial, host-microbe, and substrate-microbe binding. This is of particu-

lar importance in the field of biofilm formation as the forces governing such interactions are pertinent in the initiation of a biofilm. The research was pioneered by Bowen et al. [76] who first constructed a cell probe to measure the adhesion of *S. cerevisiae* cells at surfaces (**Figure 6**). The author then moved this on to look at the adhesion of fungal and bacterial spores [77, 78]. Protocols for the construction of cell probes have varied in the method of cantilevers functionalization: electrostatic compounds; poly(ethyleneimine) (PEI), poly-L-lysine, or hydrophobic substances, and the use of glue, chemical fixation, and bio-inspired wet adhesives have all been used, and in the type of probe that was created: single versus multicellular [3, 79–85]. While all methods succeeded in the creation of a cellular functionalized tip and the acquisition of adhesive force-curve data, the results and validity of the techniques varied.

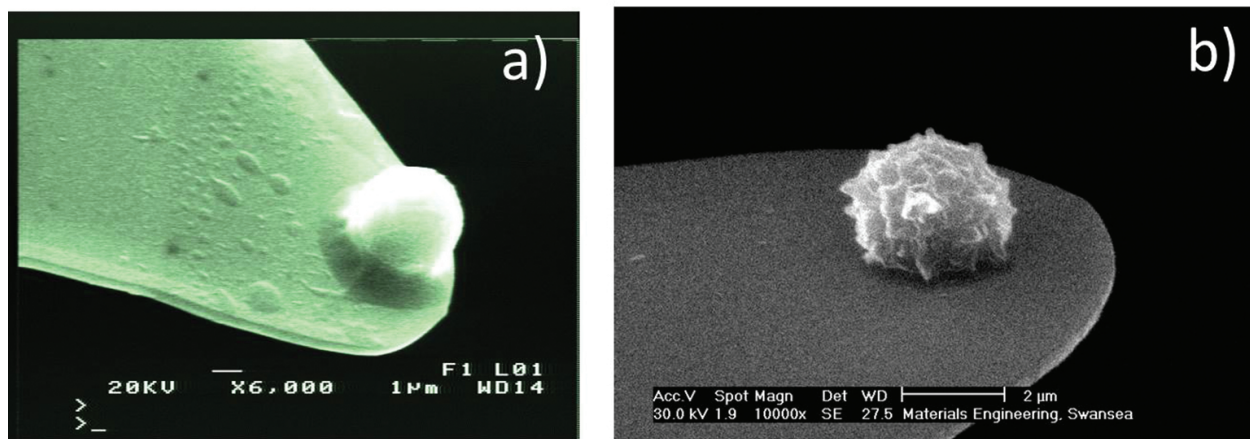


Figure 6. Scanning electron images of AFM probes used in single cell force spectroscopy (SCFS). (a) *Saccharomyces cerevisiae* and (b) *Aspergillus niger*.

Recently, a method for the direct immobilization of single microbial cells was developed [86]. A colloidal probe was attached to the tip of a cantilever and coated in polydopamine, and a single microbial cell was then attached to the colloid particle. Fluorescence microscopy validated the viability and orientation of the microbial cell, and force-curve analysis was performed across a number of surfaces and a number of probes to ensure reproducibility of results. The technique was shown to offer a high level of cell orientation; thus, a high level of control of the surface area, ensuring reproducibility of results and enabling statistical analysis of force curves. The group went on to create cellular probes functionalized with *Lactococcus plantarum*, *C. albicans*, and *Staphylococcus epidermidis* to prove the versatility of the technique [84].

Studies using SCFS have characterized a number of microbial binding structures, such as bacterial pili, to show how these structures influence microbial adhesion. During one such study, the nanomechanical binding of *P. aeruginosa* type IV pili to a hydrophobic substrate was examined. During the study, type IV pili were shown to have the same constant force plateaus associated with a nanospring-like mechanism; this may be explained by the fracturing of internal amino acid bonds and the unravelling of the three-dimensional structure to resist the increase in mechanical force. This model is consistent with the previous interpretations of

Gram-negative pili structure [87–89]. In a similar study, strains of *Lactococcus lactis* were immobilized onto polyethyleneimine (PEI)-coated cantilever, and adhesion to a pig gastric mucin-coated substrate was characterized [90]. In the study, long-range adhesion was found to be predominantly the result of pili-mediated binding, while surface adhesion was primarily mediated by mucus-binding adhesins.

The implementation of SCFS has not been limited to the characterization of microbial binding to surfaces. While uncommon, the use of SCFS to characterize microbial aggregation and the formation of heterogeneous biofilms has grown as a field in recent years. One such interaction to be studied is the common co-colonization of *S. epidermidis* and *C. albicans*; a recent study attempting to characterize such an interaction showed that despite the complex nature, SCFM is able to offer a window of insight into the adhesion forces at work [91]. During the study, the group was able to establish that *S. epidermidis* adhesion was strongly influenced by the life stage of *C. albicans* and primarily mediated by the binding of long-range macromolecules.

SCFS techniques have been used to study the mechanisms of biofilm control agents. Chaw et al. [92] measured the adhesion between *S. epidermidis*-coated AFM tips and a substrate before and after addition of silver ions (50 ppb) to the liquid medium. For both *S. epidermis* strains studied, the adhesion decreased and was argued to demonstrate how the biofilm matrix is destabilized in the presence of silver ions.

8. Conclusion

AFM has provided researchers with the tools necessary to unravel the intimate, complex, and traditionally illusive processes governing the formation and resilience of biofilms. AFM has provided the platform necessary for the application of classical engineering techniques, such as indentation analysis in the exploration of microbial nanomechanics with unprecedented resolution. Nanoindentation studies have elucidated the heterogeneity of the microbial membrane landscape. Studies utilizing nanoindentation have provided evidence of the variation in Young's moduli of both single cells and biofilms, while also hinting at the possible application of the technique in the visualization of the assembly of submembranous structures. AFM studies have also demonstrated the importance of such measurements in the evaluation of novel antimicrobial and other therapeutics.

Through the use of functionalized cantilevers, SMFS has revolutionized our understanding of microbial cell surface topography and nanomechanical properties. Tips functionalized with ligands or with alterations in hydrophobicity have been used to not only map the receptor landscape at the macroscale, but also to visualize the structure of individual membrane-bound protein complexes. AFM quantification of the nanoscale forces of adhesion has offered unparalleled insight into the forces governing microbial adhesion, a crucial event in biofilm formation, and how these individual forces may be manipulated to promote dissolution. The formation of cellular probes has been a mainstay of microbial-based AFM, and this continues with the recent development of protocols for the immobilization of a singular, highly orientated bacterial cells.

In conclusion, microbiology and the study of biofilms is no longer a microscience. The elucidation of microbial behavior at the nanoscale has now become an essential avenue of research in the understanding of the complex interplay of the microbial world, and AFM has proved itself to be an essential tool in this endeavor. The increase in sensitivity and analytical power, as well as ingenuity shown by researchers in the creation of more imaginative probes will ensure that unique insights into biofilms through AFM will continue.

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