

NANOMECHANICAL METHODS TO STUDY SINGLE CELLS

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Fundamental understanding of the structure and functions of a cell is central to biology. The understanding of many complex biological processes of living organisms would be greatly advanced by analyzing the content of their constituent single cells. The cell, consisting of an enclosing membrane, the cytoplasm, and various organelles (and a nucleus in the case of eukaryotes), has been acknowledged as one of the fundamental building blocks of life. Furthermore, individual cells also have discrete molecular, metabolic, and proteomic identities. Microbial cells such as bacteria, fungal spores, and yeasts, as well as single-cell eukaryotes like protists, have rigid cell walls that play a vital role in protecting the cytoplasm from the outer environment, providing the cell with a robust structure, determining its shape, and controlling its adhesion phenomena. Microbial adhesion processes have major consequences in natural environments (symbiotic interactions, biofouling), medicine (infections), and biotechnology (bioremediation, immobilized cells in bioreactors). Understanding the structure, properties, and functions of cell surfaces is, therefore, of

great significance for both fundamental and applied research. Investigating the functions and properties of a single cell requires developing novel techniques with higher resolution and sensitivity. Efforts to understand the role of a given cell in an organism often entails defining the boundaries of the cell in order to investigate its properties within those boundaries, or isolating a cell from its environment in preparation for further study.

Since the characteristic size of a single cell is typically in the micrometer range, optical imaging is routinely used for low-resolution studies. Optical microscopy has long been used for visualizing microbial cells in their native state. Light microscopy is useful for counting the cells, identifying them, and describing their general morphological details. Cells can be distinguished from each other if the cells display unique physical characteristics. Optical techniques also allow identification and localization of specific cellular components using fluorescent tags where the sample is illuminated with UV light (Rizzuto et al., 1995).

Isolation of single cells from the tissues of an organism and their microscopic analysis using molecular probes such as antibodies can provide details about the structure as well as

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possible functional role of the cells. However, since the resolution of light microscopes is limited by diffraction, it is difficult to obtain structural information at the nanometer level with far-field microscopes.

High-resolution imaging is routinely carried out using electron microscopy techniques that use energetic electron beams for image formation. The use of freeze-fracture and surface replica techniques in transmission electron microscopy makes it possible to visualize cell surface structures at high resolution. However, these approaches are limited by the requirement of vacuum during the analysis, i.e., living cells cannot be directly investigated in their native environments. In addition, electron microscopy cannot provide information on the biophysical properties of the cell surface.

Despite the enormous advances in imaging cells and biomolecules using conventional electron microscopy techniques, high-resolution imaging techniques and high-sensitivity characterization techniques that are compatible with the aqueous environment are highly desired. In recent years, many novel techniques based on scanning probe microscopies have been developed that have myriad applications in studying cells. Here, we introduce scanning probe microscopy and related techniques that can have application in high-resolution imaging of cells and real-time monitoring of multiple cellular components in a multimodal fashion.

SCANNING PROBE MICROSCOPY

Scanning probe microscopes trace their origin back to scanning tunneling microscope (STM) invented by Binnig and Rohrer in the early eighties (Binnig et al., 1982 1986). The working principle of an STM is that the electron tunneling occurs between a conducting sample and a sharp tip positioned a subnanometer distance away from the sample, and the magnitude of the tunneling current varies exponentially with tip-sample distance, such that scanning the tip over an area produces a current image that is directly related to surface morphology. Due to the exponential dependence of the tunneling current with tip-sample separation distance,

surface features of electrically conducting surfaces are greatly amplified. STM is capable of achieving even atomic-scale resolution. However, STM requires electrically conducting samples, and is therefore not well suited for imaging biological samples.

Initially, STM was confined to applications under ultrahigh vacuum conditions. Hansma et al. soon introduced a simpler STM that can operate in air and under solution (Giambattista et al., 1987; Hansma et al., 1988). The ability of STM to operate under these conditions for real-time imaging of surface features with nanometer resolution spurred a sudden and rapid growth in the development of many novel scanning probe microscopes and related techniques. Today, there exist a variety of scanning probe techniques that include STM, atomic force microscopy (AFM), near-field scanning optical microscopy (NSOM), photon scanning tunneling microscopy, scanning electrochemical microscopy, among others. All these techniques are based on scanning a probe over a sample surface at nanometer or subnanometer separation distance, and all offer ways of imaging different physical properties of the sample.

Recent advances in the scanning probe microscopy techniques offer unprecedented opportunities for visualizing cells at nanometer resolution. Since STM requires conducting samples, its application for cellular imaging is very limited. The NSOM and photon scanning tunneling microscope that use near-field optics can be used to obtain images with a resolution of a few nanometers to tenths of nanometers, depending on the size and shape of the probe and the wavelength of the illumination. The AFM, on the other hand, offers subnanometer resolution for topographical imaging. The AFM is more versatile and widely used for imaging biological samples, including surfaces of cells.

ATOMIC FORCE MICROSCOPE

As explained earlier, AFM was a natural outgrowth of ideas catalyzed by the invention of STM. The AFM utilizes the force between a probe and the surface of a sample to map the

topographical details. Unlike the STM, the AFM imaging both conducting and nonconducting samples. The AFM operating principle involves the interaction forces between the AFM probing tip and the sample surface. The probe tip is located at the free end of a cantilever brought in close proximity to the sample by piezoelectric means. The probe tip are microfabricated through photolithography. The force constant of a cantilever is very small, usually a few N/m, which is 2 orders of magnitude smaller than the force constants between



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topographical details of the surface (Fig. 1). Unlike the STM, the AFM can be used for imaging both conductors and insulators since the AFM operating principle is based on the interaction forces between an object and the AFM probing tip. The probing tip, which is located at the free end of a soft cantilever, is brought in close proximity to the surface by piezoelectric means. The cantilevers with a probe tip are microfabricated from silicon through photolithographic techniques. The force constant of a cantilever used in an AFM is very small, usually around 0.1 N/m, which is 2 orders of magnitude smaller than the force constants between atoms in a solid. These

cantilevers have typical dimensions of 100 to 400 μm length, 20 to 40 μm width, and 1 to 2 μm thickness. The deflection of the cantilever is monitored by focusing the beam of a laser diode onto the free end of the cantilever, while monitoring the reflected beam with a position sensitive detector (PSD). A change in the PSD signal denotes bending of the cantilever. Here, it is assumed that the cantilever deflection is proportional to the normal force acting between the tip and the sample. In fact, the signal from PSD really measures the curvature of the cantilever, which is assumed to be proportional to the cantilever deflection. As the cantilever is brought close to the sample, usually by using a

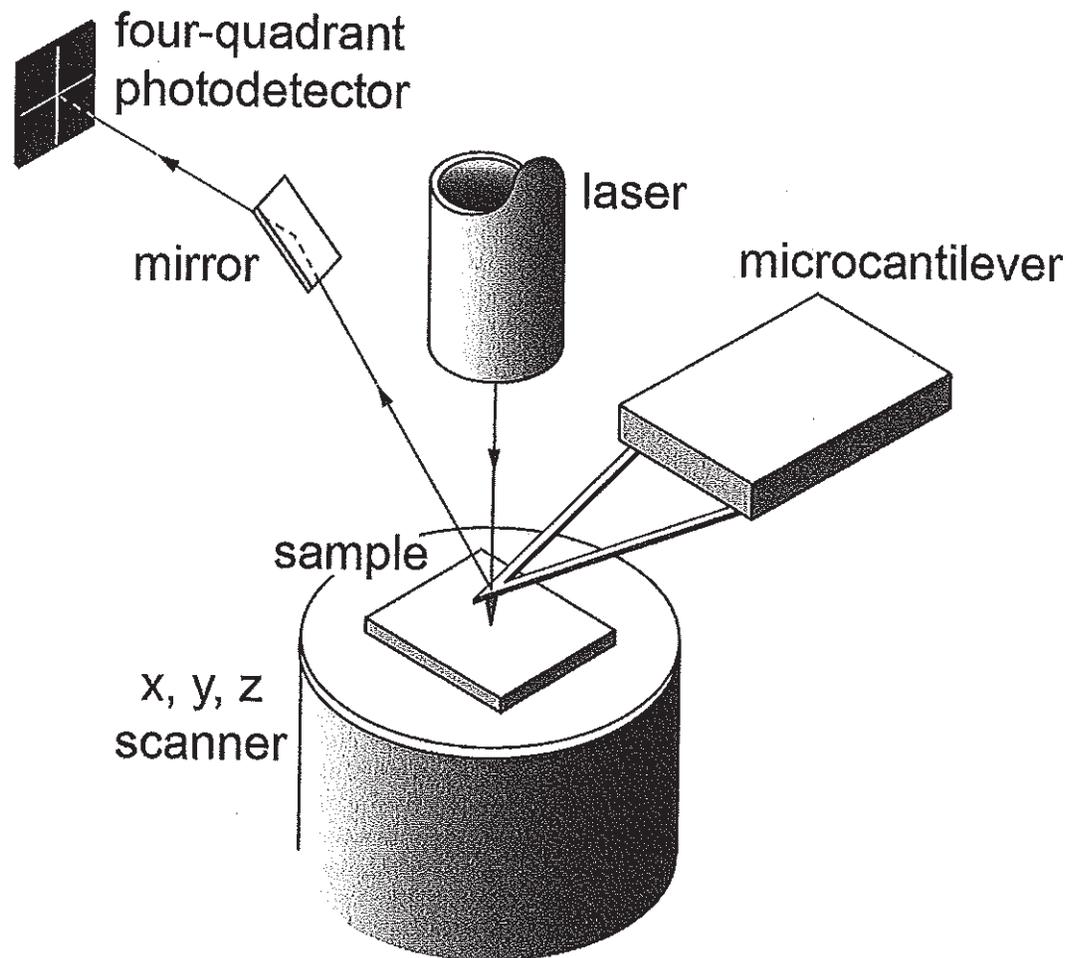


FIGURE 1 Schematic of an atomic force microscope.

piezoelectric element, at a separation distance of a few nanometers, the tip jumps into contact with the surface due to van der Waals force, which is operative at these distances. The interaction force, which is acting between the tip of the cantilever and the sample surface, deflects the cantilever. As the separation distances decrease, the attractive force is replaced with short-range repulsive forces because of the Pauli Exclusion Principle. The strong repulsive force increases rapidly with decreasing separation distance between the tip and the surface. This non-linearity of the repulsion force is responsible for the high resolution observed in AFM images.

Cantilever displacement as small as a fraction of a nanometer can be measured with this method. The force is calculated from knowing the cantilever displacement and the spring constant of the cantilever. The spring constant of the cantilever can be determined by different methods, for example, from the knowledge of its resonance frequency and mass. Typical forces are in the nano-Newton range.

By scanning the probe location in the x - y direction in a systematic fashion, and noting the cantilever deflection as z -coordinate, one acquires high-resolution images of the surface. Since the van der Waals forces in z -direction fall off rapidly with separation distance, the resolution in z -direction is high enough to see atomic steps on a surface. AFM imaging can therefore reveal atomic arrangements on clean surfaces. The x - y imaging size depends on the piezoelectric scanners used for scanning probe (or sample). In general, a single piezoelectric tube is used, whose outer electrode is divided into four quadrants. Electrodes in one set of opposing quadrants are used in push-pull geometry to create deflections for scanning in x -axis (fast scan axis). At the end of the scan, the other two electrodes are used to deflect and hold the tube in y -axis (slow scan axis) for a small distance. This process is repeated until the probe images the entire surface. Piezoelectric scanners with scan ranges varying from a few nanometers to 100 microns are commercially available. The operation of the AFM, therefore, is analogous to the operation of a phonograph, where a sharp

needle is scanned over a grooved surface, although with a different scanning pattern.

Modes of Operation

The two main modes of operation of an AFM are the static (contact) mode and the dynamic mode. In the static mode of operation, the static tip deflection is used as a feedback signal. In the dynamic mode, the change in frequency and amplitude of a resonating cantilever with the tip-sample distance is registered. The often used dynamic mode, also known as the tapping mode, is based on a decrease in the amplitude of the resonating cantilever. The contact mode uses soft, low-frequency cantilevers, while tapping mode relies on rigid, high-frequency cantilevers. The lateral (x - y) resolution is higher for contact mode operation than for tapping mode. Tapping mode, however, overcomes issues associated with friction, electrostatic forces, adhesion, and other difficulties that often plague contact mode AFM. Since the contact mode is based on the measurement of the contact force between the tip and the sample, low-stiffness (low-spring constant) cantilevers are used for detecting displacements. The measurement of static signal is often prone to noise and drift. Also, large capillary forces are present due to condensation of humidity around the tip-sample contact area. Since the contact forces are controlled only in the z -direction, lateral forces can cause problems during scanning. However, the cantilevers are geometrically designed in such a way that lateral forces are minimized. In contact mode, the force between the tip and the surface is maintained constant during scanning by keeping a constant deflection.

In the dynamic mode of operation, the cantilever is externally oscillated at a frequency equal to or close to its resonance frequency. The phase, amplitude, and resonance frequency of oscillation are modified by tip-sample interaction forces. The changes in resonance response due to tip-sample interaction with respect to the external reference oscillation produce information about the topography of the sample. Usually, changes in the amplitude of the cantilever oscillation are used for obtain-

ing topography. The phase variation provides information on the viscoelasticity of the sample and is commonly used to achieve high contrast when imaging biological samples. The ability of AFM to produce images under ambient conditions or in solution makes it an ideal tool for physical studies of biological specimens under physiological conditions. In contact mode, constant-force AFM can show processes including cell infection on live cells and cellular processes that could be imaged with atomic force microscopy. In recent years, high-resolution images of bacterial membranes have also been published (Scheuringer and Hofer et al., 2003). AFM imaging in contact mode of operation is capable of rupturing the cell membrane. Tapping or intermittent-contact mode is generally preferred in studies involving high-resolution imaging of subcellular structures. The main complication that arises in tapping mode of operation is caused by the surrounding liquid environment, making it essential to develop a method to enhance contrast and resolution of images.

Another major application of AFM in cell studies involving real-time imaging of live cells' dynamics such as exocytosis, particle transport, and intercellular interactions and functional cellular response to internal and external perturbations (Butt et al., 1990; Hugel et al., 1991; Kasas et al., 1995; Dufrenoy et al., 2001; Van der Aa et al., 2001). The ability to monitor the dynamic behavior of cells to reduce the cantilever perturbation during scanning and maintaining optimal environmental conditions in terms of temperature and pH changes. Another technical challenge that needs to be overcome relates to the acquisition time for a full scan, which often exceeds the timescale of biological processes being investigated. It is possible to reduce undesirable cellular stimulation by slightly modifying the tapping mode

a grooved surface, scanning pattern.

operation of an AFM mode and the dynamic of operation, the static feedback signal. In the range in frequency and a large cantilever with the large registered. The often-known as the tapping mode in the amplitude of the signal. The contact mode uses soft cantilevers, while tapping mode uses high-frequency cantilevers. The resolution is higher for tapping mode. Tapping mode overcomes issues associated with static forces, adhesion forces that often plague the contact mode is the measurement of a sample, low-stiffness cantilevers are used for the measurement of a sample to noise and drift. Resonance is present due to the damping around the tip-sample interaction. The contact forces are in the lateral direction, lateral forces are minimized during scanning. However, specially designed cantilevers are minimized. In tapping mode, the constant distance between the tip and the sample during scanning is maintained.

In tapping mode of operation, the cantilever is damped at a frequency below the resonance frequency. The resonance frequency is modified by tip-sample interaction changes in resonance frequency. The tip-sample interaction with the reference oscillation is used to measure the topography. Images in the amplitude of the signal are used for obtain-

ing topography. The phase variation contains information on the viscoelastic properties of the sample and is commonly used for obtaining high contrast when imaging soft materials. The ability of AFM to produce images under ambient conditions or in solution makes it an ideal tool for physical studies of biological specimens under physiological conditions. In contact-mode, constant-force AFM imaging, one could show processes induced by viral infection on live cells and cellulose microfibrils that could be imaged with atomic-scale resolution. In recent years, high-resolution AFM images of bacterial membrane proteins have also been published (Scheuring et al., 2002; Horber et al., 2003). AFM imaging of cells by using contact mode of operation often results in rupturing the cell membrane. Tapping mode or intermittent-contact mode is therefore usually preferred in studies involving high-resolution imaging of subcellular structures. The main complication that arises in the tapping mode of operation is the damping caused by the surrounding liquid environment, making it essential to develop an appropriate method to enhance contrast and improve the quality of images.

Another major application of the AFM is cell studies involving real-time monitoring of live cells' dynamics such as exocytosis of viral particles from an infected cell in real-time, intercellular interactions and functions, and the cellular response to internal and external perturbations (Butt et al., 1990; Häberle et al., 1991; Kasas et al., 1995; Duffrène et al., 1999, 2001; Van der Aa et al., 2001). The main issue in monitoring the dynamic behavior of the cell is to reduce the cantilever perturbation while scanning and maintaining optimal environmental conditions in terms of temperature and pH changes. Another technical difficulty that needs to be overcome relates to the requirement to achieve a higher temporal resolution; the acquisition time for a full scan of a living cell often exceeds the timescale of the dynamic processes being investigated. It is possible to reduce undesirable cellular stimulation by slightly modifying the tapping mode of opera-

tion in liquid. This could also be achieved by developing a new technique in which much lower cantilever-loading forces are needed or by designing novel cantilever probes that are biochemically and mechanically compatible with biological samples. One possible solution is to make the temporal resolution higher to speed up the scan rate, though often at the expense of spatial resolution. The current AFM apparatus and techniques not only allow us to monitor certain dynamic cellular processes, such as cell growth, exocytotic and endocytotic events that are fairly slow and do not require high spatial resolution, but also provide the ability to study the cell morphology in real time in the presence of growth factors, hormones, and other biological reagents. With the development of higher-scan-rate AFMs, it could be possible to monitor the processes that occur at the cell membrane during receptor-ligand binding, vesicle transfer, channel blocking or gating, etc., and to obtain information on the delivery of a specific drug with molecular resolution. Information about micromechanical properties is important for cellular systems as it helps to understand the cell architecture and its functions. Local elastic properties of a cell can be quantitatively derived from the force versus distance (F-S) curves obtained at fixed surface points by using AFM.

Figure 2 shows atomic-scale images of a freshly cleaved mica surface acquired with an AFM. The image consists of 512 lines, and each line has 512 pixels. The image shows raw data without any signal processing or pixel averaging. The white areas show a higher force between the atoms at the end of the cantilever tip and the molecular groups on the surface of mica. Hexagonal close packing of molecular arrangements of the mica surface is clearly visible in the image. The image is collected using a tip-surface interaction force of 1 nN.

In addition to atomic and molecular arrangements, nanometer-size objects can be easily visualized with an AFM. Figure 3 shows an image of double-stranded plasmid DNA adsorbed on a mica surface acquired in air (Thundat et al., 1993). Imaging of nanometer-

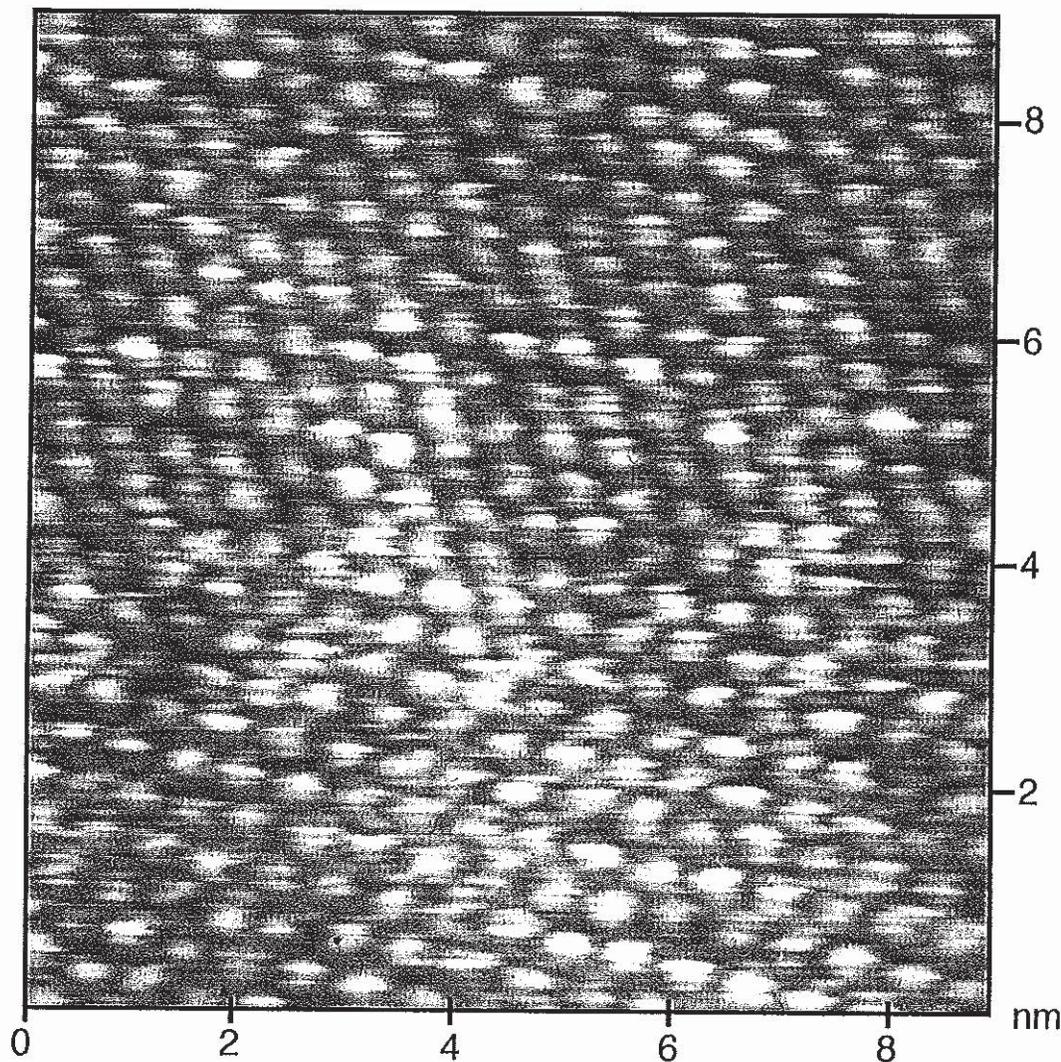


FIGURE 2 Atomic resolution images on a mica surface.

sized objects, such as nanoparticle or a molecule of DNA adsorbed on flat surface, results in geometrical broadening of the image due to finite size of the object (Fig. 3). The apparent width, w , observed in an AFM is approximately, $w = 4\sqrt{Rr}$, where R is the radius of the probe and r is the radius of the object. Typical radius of a micromachined probe tip is around 10 to 15 nm. The z -height, which is related to the van der Waals force between the tip and the surface, does not show any artificial enlargement. In some samples the z -height, however, will be

influenced by the presence of other forces such as electrostatic or magnetic forces.

In ambient conditions when the tip comes in contact with the surface, capillary condensation creates a meniscus force, which has an approximate value of $F_c = 4\pi R\gamma\cos\theta$, where γ is the surface tension of water (72 mN/m) and θ is the contact angle. This produces a force of 9 nN for a tip radius of 10 nm and a contact angle of 0. Therefore, operation of an AFM in high humidity conditions results in loss of resolution due to uncontrolled forces of capillary action.

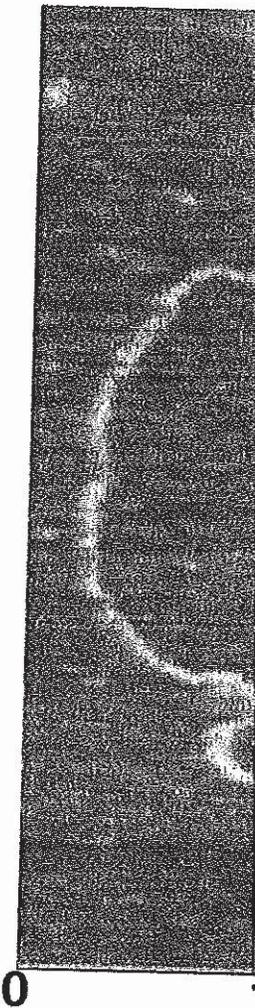


FIGURE 3 Image of double-stranded DNA.

Imaging surfaces under ambient conditions improves the image resolution by reducing capillary forces.

The introduction of dynamic mode AFM in contact mode or the introduction of dynamic mode AFM is an important development. It has significantly reduced the shear forces between the tip and the surface. As a dynamic technique, it has improved the stability of phase imaging. In dynamic mode AFM, the interaction between the probe and the specimen is reduced. The difference between the force exerted on the cantilever and the specimen is reduced. The relationship between the

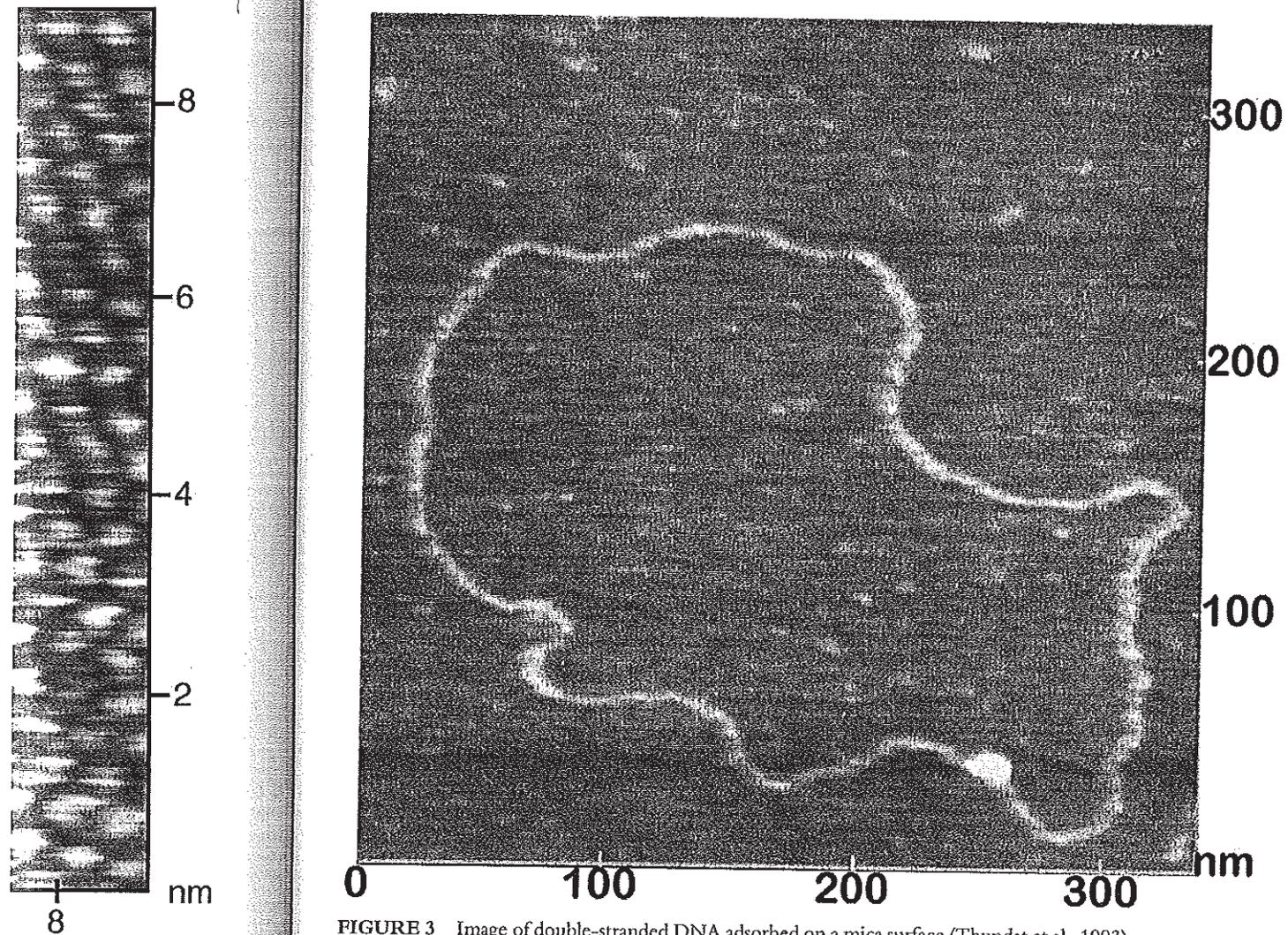


FIGURE 3 Image of double-stranded DNA adsorbed on a mica surface (Thundat et al., 1993).

Imaging surfaces under dry nitrogen or hydrogen improves the image quality due to much reduced capillary forces.

The introduction of the intermittent-contact mode or the tapping mode was an important development because it greatly reduced the shear forces on the specimen and, as a dynamic technique, also offered the possibility of phase imaging. At each point in an image, the interaction between the tip of the probe and the specimen is related to the phase difference between the driving force supplied to the cantilever and the response of the cantilever. The relationship between the nature of

the tip-specimen interaction and phase shift is fairly complicated; however, images constructed from the phase information are not useful in identifying regions of a similar chemical nature. The tapping mode was initially developed for operation in air, and its eventual application in liquid brought the technique in the realm of biological environments. However, oscillation of the cantilever in liquid environments results in damping of the cantilever motion by the liquid. Higher forces are needed to drive the cantilever in liquid, and the damping of the motion results in decreasing the quality factor (Q). To overcome this, the resonant

of other forces such as capillary forces.

When the tip comes into contact, capillary condensation force, which has an energy $E = 4\pi R\gamma\cos\theta$, where γ is the surface energy of water (72 mN/m) and θ is the contact angle, produces a force of 9 nN and a contact angle of 90°. The use of an AFM in high vacuum eliminates the effects of capillary action.

amplitude peak of the cantilever is broadened so that the value of Q ranges from a few hundred in air to around 1 in liquid. This results in greatly reducing the sensitivity to the nature of the tip-specimen interaction, resulting in a phase contrast, which is much weaker. The recent development involving active resonance control when applied to the oscillating cantilever in tapping mode in liquid enables an increase in the effective value of the Q so that tip-specimen forces are drastically reduced. The use of this active-resonance technique significantly reduces the deformation of soft biological and organic specimens, resulting in images that have greater resolution or would otherwise be unattainable; the phase contrast in such images is also tremendously improved. Advantages of AFM include its ability to image insulators, soft samples such as biological materials, and its ability to operate in air, in vacuum, and under solution. Since an AFM image depends on the interaction force between the tip and the sample, specially designed tips can be used for magnetic and electrostatic imaging of certain samples. It is also possible to make the AFM tip similar to a thermocouple for imaging the thermal properties of samples (Wang, 2004; Haeblerle et al., 2006).

In summary, the AFM uses a microfabricated cantilever beam that is an extremely sensitive force sensor and detects the force between the atoms at the end of the cantilever tip and the cell surface. The ability of a cantilever to measure forces as small as subpiconewtons makes it an ideal tool for measuring interaction forces between molecules. The cantilever in an AFM allows application of precise quantifiable forces to single cells in a site-specific manner. Cells also respond to mechanical stress and strain. Understanding the single-cell response to these forces will open a new and exciting area for cell biology. AFM when combined with fluorescence microscopy allows biochemical events in the cell to be correlated with mechanical changes in the cell, making the AFM/fluorescence microscopy a novel tool in the study of mechanotransduction in single cells (Kassies et al., 2005).

The AFM has opened exciting new avenues in microbiology and biophysics for probing microbial cells (Horber et al., 2003). The unprecedented capabilities of AFM include the potential to measure local physical properties such as elasticity and adhesion forces and imaging the surface topography to the nanometer scale under different physiological conditions. With the scope for topographic imaging, one can directly visualize cell surface nanostructures and the changes of cell surface morphology occurring during various physiological processes (such as germination, division) (Hoh et al., 1992; Nagao and Dvorak, 1998). More improvements in the current sample preparation techniques, instrumentation, and experimental conditions could bring subnanometer-level resolution to these living cells for monitoring molecular conformational changes, as is already the case with reconstituted microbial surface layers (Fotiadis et al., 2002; Bahatyrova et al., 2004).

AFM produces not only high-resolution imaging of cellular structures below the optical limit, which is quite "natural" for this method, but also has the potential to study the micromechanical properties of the cell and the ability to monitor cell dynamics and intracytoplasmic processes in real time (Radmacher et al., 1996; Ricci et al., 1997). With AFM, cells can be imaged with practically little or no sample pretreatment, which is noteworthy in most native physiological media such as aqueous solutions. In addition to several advantages over conventional microscopic techniques, AFM can also be combined with other methods such as electron microscopy, scanning near-field optical microscopy, and others for further improvement (Vesenska et al., 1995; Haydon et al., 1996; Proksch et al., 1996; Langer et al., 1997). Direct imaging of fixed or living cells and subcellular structures gives us important information on the structure and features of the membrane, organelles, and cytoskeleton of cells. The AFM also has the potential to image, localize, and identify integral membrane proteins at the surface of living cells (Lal and John, 1994; Bao and Suresh, 2003).

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Photonic Force Microscopy

AFM and related techniques are based on sensing normal forces by using a cantilever beam that is not capable of obtaining information from sidewalls. Since living cells exist in three dimensions, cantilever-based AFM cannot be used as a tool for obtaining three-dimensional images. The AFM is a surface tool with performance that is directly coupled to the flatness of the underlying surface examined. Imaging sidewalls or inside cells is impossible because of the instrument's mechanical connection to the imaging tip. Hence, a scanning probe microscope without a mechanical connection to the tip, working with extremely minute loading forces, would be an ideal complementary technique for the study of live cells with the AFM. Photonic force microscope (PFM) is such an imaging tool recently developed at the European Molecular Biology Laboratory in Heidelberg that can be used for three-dimensional imaging (Pralle et al., 2000).

The PFM utilizes a micron- or submicron-sized bead in an optical trap as the imaging probe. Unlike in the AFM, where the tip is attached to a cantilever anchored on a rigid mass, the bead is essentially free floating and can probe sidewalls. The bead is trapped in the three-dimensional trapping potential of a focused laser beam. Trapping and manipulating micrometer-sized beads with a laser beam focused in a fluid were originally described by Ashkin et al. (1986). The depth and shape of the trapping potential can be determined from the difference in the refractive index between the bead and the fluid medium, the bead diameter, and the laser intensity and the beam profile. The trapped bead executes Brownian motion inside the trap as if the bead is attached to an invisible spring with a spring constant 3 to 4 orders of magnitude smaller than the cantilever used in an AFM. The effective spring constant of the trapped bead can be tuned by varying the intensity of the laser beam. The beads commonly used have a radius of 50 nm. However, it is also possible to use even smaller beads (~10 nm) provided the refractive index

of the bead material is sufficiently high, for example, metals.

PFM uses a three-dimensional detection system for determining bead position with respect to trapping potential. This allows measurement of the force (magnitude and direction) acting on the bead with subpiconewton precision on a timescale of microseconds. The Brownian envelope of the bead motion, which is much larger than the bead diameter, is used for probing the three-dimensional shape of the object. Beads used as scanning probe tips can be moved along a surface to make interaction force measurements between the tip and the surface in the pico- and subpiconewton force range. If latex or glass beads are used as tips, many standard chemical surface modifications are commercially available with a bead size of 200 to 400 nm, for which the objective used to focus the laser provides a good optical control for the readings.

The image resolution obtained with these beads by scanning them across a surface while measuring the interaction forces is limited by the interaction region between bead and sample and is also limited by thermal fluctuations, which can be up to 100 nm, depending on the trapping potential. With a detection system giving a spatial resolution of better than 1 nm and a time resolution of 1 μ s, this limitation can be easily overcome by using the thermal fluctuations as a random scan generator for the exploration (within milliseconds) of a small three-dimensional volume of several tenths of nanometers. Such a method opens up many new applications because the position probability measured for a certain volume reflects the presence of other objects in this volume, the interaction potential with these objects, and the interaction with the surrounding medium. By applying such a technique, three-dimensional polymer networks can be imaged, the mechanical properties of single molecules binding the bead to a surface can be measured, and the viscosity of the membrane of living cells in areas smaller than 100 nm in diameter can be determined if beads are linked to single-membrane components.

Scanning Acoustic Holography

AFM can image surface features with sub-nanometer resolution in the z -direction since the force used as a signal is nonlinear. The nonlinear relation results in amplification of signal in z -direction. The signals in the x - y directions have no amplification. One of the disadvantages of AFM is that it cannot obtain subsurface information. All images obtained with an AFM are surface features. In 2005 Shekhawat and Dravid developed a scanning probe technique called scanning near-field ultrasonic holography (SNFUH) (Shekhawat and Dravid, 2005). SNFUH is a modification of contact-mode AFM. In this modification the sample is vibrated at megahertz frequencies by attaching a piezoelectric crystal to the sample holder. The acoustic waves traveling through the sample vibrate the cantilever at the same frequency. Since this high-frequency vibration of the cantilever cannot be detected by optical beam deflection, a beat frequency technique is utilized to detect cantilever motion. The contact-mode cantilever of the AFM is excited at a frequency close to the frequency of sample vibration. This superposition of two frequencies makes the cantilever vibrate at a beat frequency, which is the difference between the two excitation frequencies. The excitation frequencies can be adjusted in such a way that the beat frequency lies below 800 kHz, which can be detected with electronics used for optical beam deflection. The DC deflection of the cantilever can be used as the topographic image of the sample. The phase of the vibration at the beat frequency as a function of x - y scan results in phase image of the sample. For properly tuned frequencies the phase image shows subsurface features that cannot be seen in topographs.

SNFUH could be an ideal technique for imaging cells where subsurface information is highly desired, for example, investigating the nucleus of a cell or imaging microtubules. SNFUH, however, is so new that it has not been applied to many exciting problems. Shekhawat and Dravid have employed SNFUH

to image malarial parasites within red blood cells (Shekhawat and Dravid, 2005). Figure 4 shows AFM topography of red blood cells on a mica surface. Figure 5 shows an image of a macrophage from a mouse lung.

Near-Field Scanning Optical Microscopy

For a quantum particle, simultaneous measurement or knowledge of its position and momentum is impossible (Heisenberg, 1927). When trying to resolve small details of a sample by light microscopy, one experiences a similar measurement limitation. Abbe observed in 1873 that the smallest distance that can be resolved between two lines (spatial resolution) by optical microscopy is limited (Abbe, 1873). Therefore, objects that are closer than about one-third of the wavelength of the illuminating light cannot be distinguished. Breaking this diffraction limit has been at the center of many efforts (Stelzer, 2002). Based on the original idea of Syngé in 1928, subwavelength resolution optical microscopy was demonstrated in 1986 by Pohl and Betzig, who used an optical fiber in the near-field (Pohl et al., 1984; Betzig et al., 1986). Since then, many efforts have been made to use NSOM for the study of biological samples and investigation of single molecules (Eddin, 2001; Kulzer and Orrit, 2004). Fluorescence microscopy provides a noninvasive method for cell biology (Stephens and Allan, 2003), and NSOM beats the diffraction limit; thus the combination of the two provides a powerful platform to obtain structural information. Room temperature detection of single-molecule fluorescence by NSOM was reported in 1993 (Heinz and Hoh, 1999). In recent years, another fusion of features from two powerful microscopies has emerged. Replacing its probe with a microcantilever that has an aperture through which light would be conducted may augment an NSOM. This complex probe allows force measurements on the same point on the sample from where an optical signal is also collected (Wissenschaftliche Instrumente und Technologie GmbH, Ulm, Germany).



FIGURE 4 / diluted in phosphate buffer and fixed with paraformaldehyde.

CANTILEVER-BASED SPECTROSCOPY

Force Spectroscopy
Mechanical forces and vibrations play vital roles in biological organisms. Force spectroscopy is a dynamic analytical technique for the study of the mechanical properties of molecules and the physical properties of the bonds. In general, AFMs operate at the nanoscale, but they have several applications in nanotechnology, such as force sensors for detecting DNA strands and interactions between antibodies and antigens, as cited in the literature (Le Schaumann et al., 2000).

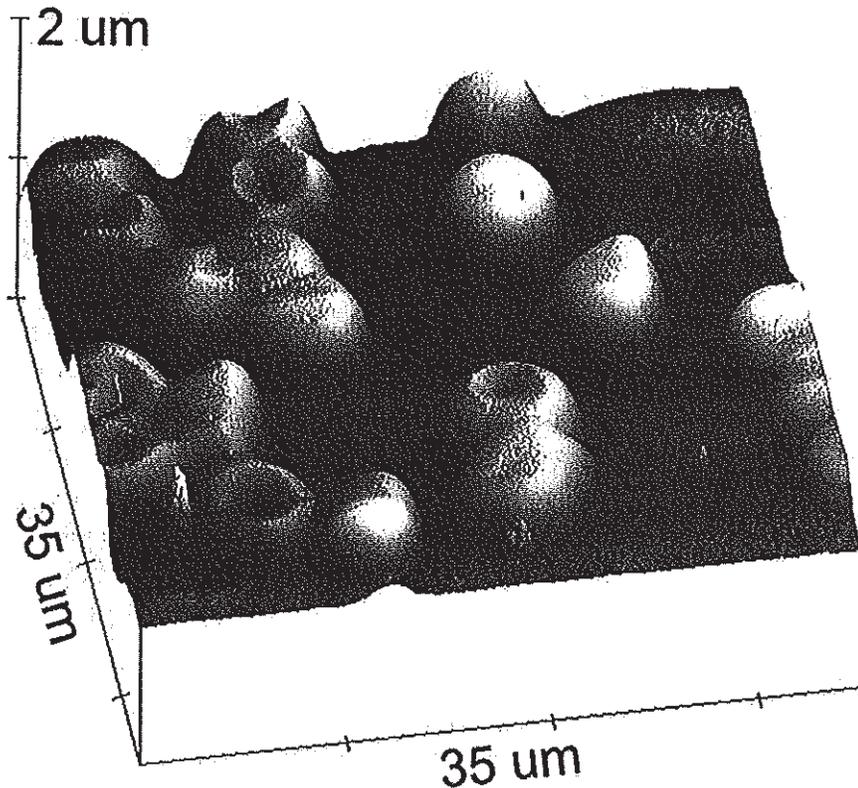


FIGURE 4 AFM (contact-mode) images of erythrocytes from mice. Blood samples were diluted in phosphate-buffered saline, centrifuged onto freshly cleaved mica by using a cytospin, and fixed with methanol.

CANTILEVER-BASED SPECTROSCOPY

Force Spectroscopy

Mechanical forces and molecular conformations play vital roles in the function of biological organisms. Force spectroscopy is a powerful dynamic analytical technique that allows the study of the mechanical properties of large molecules and the properties of chemical bonds. In general, AFMs are used for imaging at the nanoscale, but they also have found potential applications in nanomanipulation and as force sensors for detecting forces between DNA strands and interacting forces between antibodies and antigens; other examples are cited in the literature (Lee et al., 1994; Clausen-Schaumann et al., 2000). This single-molecule

analytic technique allows much finer control of the molecule under study. Single molecular force spectroscopy offers a new way to measure directly the strength of single covalent bonds. Force-distance curves (Fig. 6) provide complementary information on surface forces, interatomic forces, adhesion, and nanomechanics, yielding new insight into the mechanisms of biological events such as cell adhesion and aggregation.

There are several ways to manipulate single molecules accurately. The two most common methods are the optical or magnetic tweezers and the AFM cantilevers. The force sensor is usually a micrometer-sized bead or a cantilever with displacements that can be measured to determine the force. In all of these techniques, a

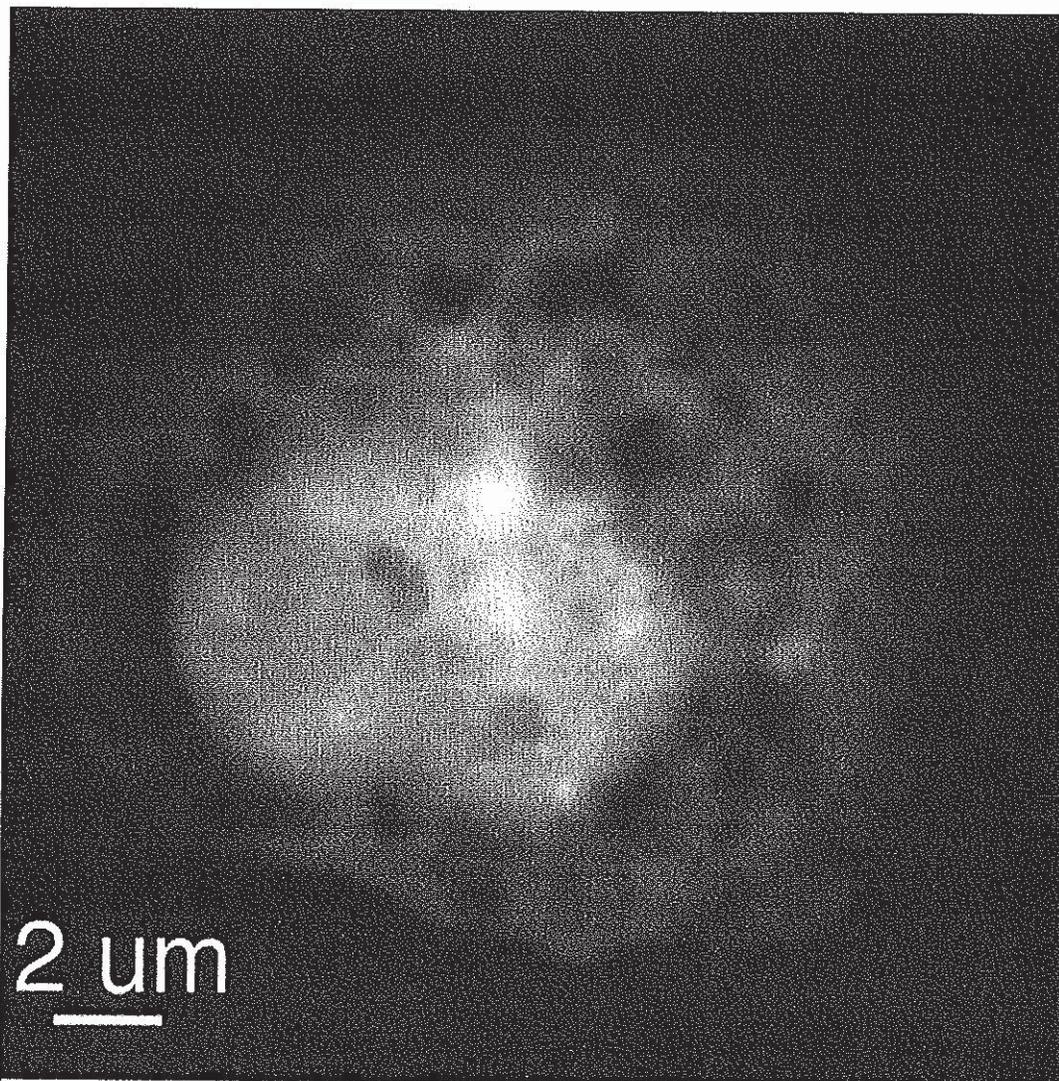


FIGURE 5 AFM image of a macrophage from mouse lungs. Cells were centrifuged onto freshly cleaved mica by using a cytospin and fixed with methanol.

biomolecule, such as protein or DNA, has one end adhered to a surface and the other to a force sensor. Force spectroscopy is mainly used to make measurements of elasticity, especially biopolymers such as RNA and DNA. Force spectroscopy is also used to unravel details on protein unfolding by making the proteins adsorb onto a gold surface and then by stretching it. The unfolding is carefully observed, and the characteristic pattern is plotted in a force versus elongation graph. Valuable information

about protein elasticity and the unfolding pattern can be obtained with this technique. Force spectroscopy is also applied in the study of mechanical resistance of chemical bonds.

Photothermal Spectroscopy

Microcantilevers may be used to obtain unique spectroscopic information. A silicon cantilever interacts with an incident beam of photons through a number of channels. The net effect is a mechanical response that can be measured, for

Tip Vertical



Vertical

example, by using localized surface plasmon resonance. Understanding the involved mechanism of the observed response is of interest, as it goes well beyond the scope of this review. In the fundamental physics of photothermal scattering of photons in a medium, one may coat a cantilever with a material that contains an analyte of interest. The spectrum may be acquired by scanning the coated cantilever with a laser beam. The analyte may also be detected by scanning the cantilever without a need for a laser. In such explicit sensing methods, the photothermal spectra of nanogram quantities of *Bacillus anthracis* and *Bacillus cereus* were obtained (Wig et al., 2006). In comparison with those of *B. cereus*, the relative differences observed in the photothermal deflection spectra were included in the system for chemical identification. The data obtained with the cantilever sensor were compared with those of spectroscopic techniques, suggesting possible uses of cantilevers as sensitive detectors of bioagents. Clear benefits

Tip Vertical

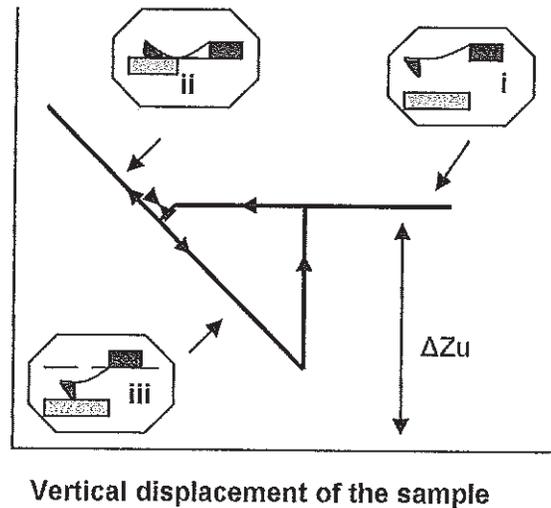


FIGURE 6 Cantilever deflection in an AFM due to contact with a surface. (i) The system is in equilibrium; (ii) the tip is in contact with the sample and the cantilever is compressed; and (iii) the force due to the extended cantilever equals the adhesive force, and it snaps back into the equilibrium position. ΔZ_u is the vertical displacement.

example, by using lock-in detection. Understanding the involved mechanisms for the observed response is ongoing research and is well beyond the scope of this chapter. Bypassing the fundamental physics associated with the scattering of photons in sensing applications, one may coat a cantilever with a solution that contains an analyte of interest whereby a spectrum may be acquired by illuminating the coated cantilever with a broadband light source that can be scanned with a monochromator. The analyte may also be adsorbed on the cantilever without a need for coating. Through such explicit sensing methodology to implement photothermal spectroscopy, first infrared spectra of nanogram quantities of *Bacillus anthracis* and *Bacillus cereus* were reported in 2006 (Wig et al.). In comparing the *B. anthracis* results with those of *B. cereus* by monitoring the relative differences observed in the photothermal deflections spectra (Fig. 7), it was concluded that the system could be used for chemical identification. Furthermore, when the data obtained with the cantilever-based sensor were compared with traditional spectroscopic techniques, similar results were obtained, suggesting possible uses of the microcantilevers as sensitive detectors of minute quantities of bioagents. Clear benefits of a faster, simpler

sample preparation that requires a much lower concentration of spores and the low-cost platform highlight the advantage of nanomechanical sensing, where molecules undergoing transitions modify the dynamic or static state of the cantilever (Wig et al., 2006). We note that a spectrum from the mechanical response of the cantilever is also observed in the absence of any molecules, and thus any analyte-based spectral data acquired by such a sensing platform have to account for the “natural” spectrum of the cantilever (Wig et al., 2004).

CANTILEVER-BASED SENSING

Adsorption-Induced Response

The AFM microcantilever can be used as a physical, chemical, or biological sensor. The cantilevers are extremely sensitive force sensors. They are usually microfabricated from silicon by using conventional photolithographic masking and etching techniques. Typical dimensions of a cantilever are 100 μm in length, 40 μm in width, and 1 μm in thickness. Silicon and silicon nitride cantilevers and cantilever arrays that utilize optical beam deflection for signal transduction are commercially available. Piezoresistive cantilever arrays are also commercially available. The deflection of a piezoresistive can-

onto freshly cleaved mica

and the unfolding path this technique. Force applied in the study of chemical bonds.

Spectroscopy

used to obtain unique information. A silicon cantilever is illuminated by a collimated beam of photons through optical channels. The net effect is that the force that can be measured, for

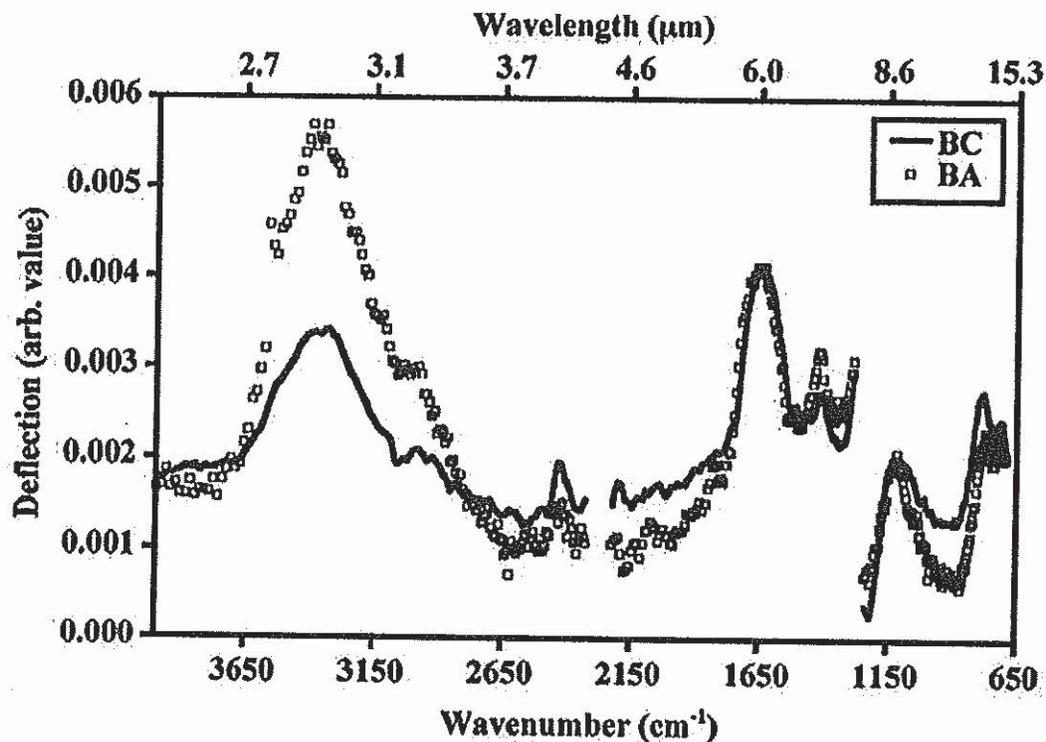


FIGURE 7 Deflections of two cantilevers coated with *B. anthracis* (BA) and *B. cereus* (BC) when exposed to infrared light. The deflection response as a function of infrared wavelength resembles infrared absorption spectra of the adsorbed material (Wig et al., 2006).

tilver is detected as a change in its resistivity without need for optical components.

Microcantilever sensors can be operated in dynamic or static modes. In the dynamic mode, mass loading due to molecular adsorption is detected by monitoring the variation in resonance frequency of the cantilever. In the static mode of operation, molecular adsorption on one side of the cantilever results in a differential surface stress that can be detected by monitoring the bending of the cantilever. The dynamic mode, where resonance frequency is monitored, is very similar to the operation of other gravimetric sensors, such as quartz crystal microbalance and surface acoustic wave transducers (Ballantine et al., 1996; Liu et al., 2003). The sensitivity of the dynamic mode of operation is directly related to the frequency of the cantilever; the higher the frequency, the higher the sensitivity. In the static mode with cantilevers with low frequency and spring

constant, large deflections are observed due to adsorption-induced forces. Figure 6 shows a schematic diagram of cantilever bending due to differential molecular adsorption. Microcantilever-based sensing satisfies many requirements of an ideal biosensor in the sense that the microcantilevers can be operated under solution, are capable of simultaneously detecting many analytes, and the overall sensor can have a small footprint, hence potentially portable. Microcantilever-based sensors often utilize receptors that are immobilized on the cantilever surface for molecular recognition.

The microcantilever is an ideal displacement sensor. The ability to detect motion of a cantilever beam with nanometer precision makes the cantilever ideal for measuring bending. Cantilever bending can be related to adsorption/desorption of molecules through adsorption forces. As molecular reactions on a surface

are ultimately driven of the surface, the free a change in surface st produce no observabl the surface of a bul induced surface stress cantilever if the adsor surface. Adsorption-i should not be confus dimensional changes s polymer films on cant adsorption-induced st of magnitude higher t variation mass sensors cies in the range of ten

Microcantilever d function of adsorbate tion is confined to a si (or when there is dif opposite sides of the c not know the absolute face stress, we can only relation can be deriv bending and changes. Stoney's formula and cantilever bending (Sto relation can be derived curvature of the cantilev ential surface stress:

$$\frac{1}{R} = \frac{\delta\sigma}{E(1-\nu)}$$

where R is the cantilever and E are Poisson's ratio of the cantilever, and $\delta\sigma$ differential surface stress. Surface free energy, γ , can Shuttleworth equation ($\sigma = \gamma + \epsilon\gamma$)

$$\sigma = \gamma + \epsilon\gamma$$

where σ is the surface stress, ϵ is defined as the ratio of surface area, $\partial A = \frac{dA}{A}$. Since the cantilever is very small compared to the substrate, the strain

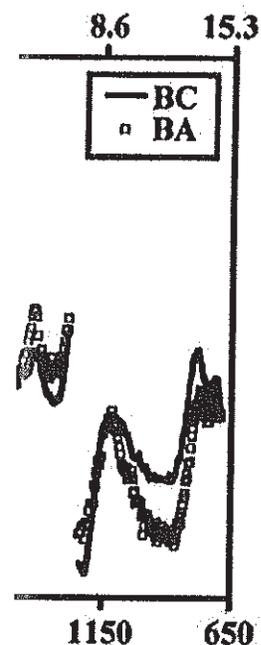


Figure 6 shows the infrared absorption spectra of cantilever bending (BC) when exposed to

adsorption of molecules. Figure 6 shows the infrared absorption spectra of cantilever bending (BC) and molecular adsorption (BA). The BC trace shows a prominent peak at 8.6 μm, while the BA trace shows a peak at 15.3 μm. Microcantilever-based sensors that are immobilized on a surface

are an ideal displacement sensor to detect motion of a cantilever. The precision makes it possible to measure bending. This can be related to adsorption of molecules through adsorption reactions on a surface

are ultimately driven by free energy reduction of the surface, the free energy reduction leads to a change in surface stress. Although they would produce no observable macroscopic change on the surface of a bulk solid, the adsorption-induced surface stresses are sufficient to bend a cantilever if the adsorption is confined to one surface. Adsorption-induced forces, however, should not be confused with bending due to dimensional changes such as swelling of thicker polymer films on cantilevers. The sensitivity of adsorption-induced stress sensors can be orders of magnitude higher than those of frequency-variation mass sensors (for resonance frequencies in the range of tenths of kilohertz).

Microcantilever deflection changes as a function of adsorbate coverage when adsorption is confined to a single side of a cantilever (or when there is differential adsorption on opposite sides of the cantilever). Since we do not know the absolute value of the initial surface stress, we can only measure its variation. A relation can be derived between cantilever bending and changes in surface stress from Stoney's formula and equations that describe cantilever bending (Stoney, 1909). Specifically, a relation can be derived between the radius of curvature of the cantilever beam and the differential surface stress:

$$\frac{1}{R} = \frac{6(1-\nu)}{Et^2} \delta\sigma$$

where R is the cantilever's radius of curvature, ν and E are Poisson's ratio and Young's modulus for the substrate, respectively, t is the thickness of the cantilever, and $\delta\sigma = \Delta\sigma_1 - \Delta\sigma_2$ is the differential surface stress. Surface stress, σ , and surface free energy, γ , can be related using the Shuttleworth equation (Shuttleworth, 1950):

$$\sigma = \gamma + \left(\frac{\partial\gamma}{\partial\epsilon} \right)$$

where σ is the surface stress. The surface strain, $\partial\epsilon$, is defined as the ratio of change in surface area, $\partial\epsilon = \frac{dA}{A}$. Since the bending of the cantilever is very small compared to the length of the cantilever, the strain contribution is only

in the part-per-million (10^{-6}) range. Therefore, one can possibly neglect the contribution from surface strain effects and equate the free energy change to surface stress variation (Butt, 1996). By using equation (2), a relationship between the cantilever deflection, h , and the differential surface stress, $\delta\sigma$, is obtained as:

$$h = \frac{3L^2(1-\nu)}{Et^2} \delta\sigma$$

where L is the cantilever length. Therefore, the deflection of the cantilever (Fig. 8) is directly proportional to the adsorption-induced differential surface stress. Surface stress has units of N/m or J/m². Equation (3) shows a linear relation between cantilever bending and differential surface stress. Adsorption-induced forces are applicable only for monolayer films and, as mentioned above, should not be confused with bending due to dimensional changes such as swelling of thicker polymer films. It should also not be confused with deflection due to weight of the adsorbed molecules. The deflection due to weight is extremely small, for example, for a cantilever with a spring constant of 0.1 N/m; the bending due to weight of 1 ng of adsorbed material will be 0.1 nm.

The minimum detectable signal for cantilever bending depends on the geometry and the material properties of the cantilever. For a silicon nitride cantilever that is 200 microns long and 0.5 micron thick, with the Young's modulus $E = 8.5 \times 10^{10}$ N/m² and the Poisson's ratio, $\nu = 0.27$, a surface stress of 0.2 mJ/m² will result in a deflection of 1 nm at the end. Because a cantilever's deflection strongly depends on geometry, the surface stress change, which is directly related to molecular adsorption on the cantilever surface, is a more convenient quantity of the reactions for comparison of various measurements. Changes in free energy density in biomolecular reactions are usually in the range of 1 to 50 mJ/m² but can be as high as 900 mJ/m².

Microcantilever-Based Physical Sensors
Microcantilever can be used as a physical sensor for measuring changes in temperature, flow

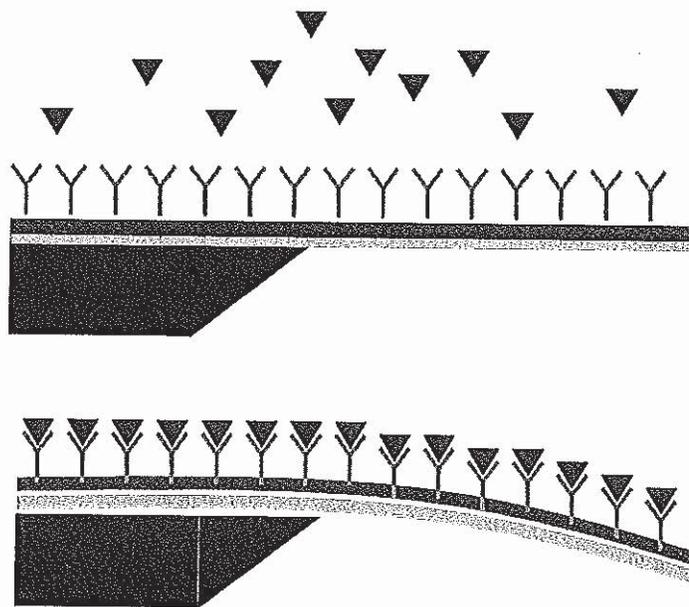


FIGURE 8 A cartoon of cantilever bending caused by binding of target molecules to immobilized probe molecules present on the surface of the cantilever. The probes are immobilized only on one side, and target binding induces a differential stress on the cantilever.

rate, pH, etc. (Datskos et al., 1998; Ji et al., 2001; Muralidharan et al., 2001; Mehta et al., 2001; Cherian and Thundat, 2002). The cantilever needs to be tuned for particular applications. For example, using cantilever as a temperature sensor requires making the cantilever bimetallic by depositing a thin layer of metal on the silicon cantilever. Change in temperature makes the cantilever bend due to a differential thermal expansion of metal and silicon. Developing a microcantilever pH sensor requires coating one side of the cantilever with a material that accumulates surface charge proportional to the pH of the surrounding solution. For example, silicon nitride has many groups that ionize, depending on the pH of the solution. It is also possible to immobilize self-assembled monolayers on one side of the cantilever, which can respond to variation in pH. A properly fashioned cantilever can be an ideal flow rate sensor where cantilever bending varies as a function of flow rate.

Microcantilever-Based Biosensors

Being a very simple structure, the microcantilever beam has the potential of being a highly effective sensing element offering numerous

transduction applications. Micromachined cantilevers, in addition to their ability in characterizing surface features in AFM, also lend themselves well to numerous chemical and biological sensing applications wherein the presence of an analyte is manifested mechanically in cantilever deflection and/or a resonance frequency change. The selectivity, sensitivity, compactness, cost, low power consumption, and versatility of microcantilever sensors make them highly suitable for biological sensing. Biosensors are sensors in which biomolecular interactions are used as sensing reactions. Biomolecular interactions, when combined with a microcantilever platform, can produce an extremely powerful biosensing design (Thundat et al., 1997; Raiteri et al., 2001; Wu et al., 2001a, 2001b; Hansen et al., 2001; Ming et al., 2003). The resonance frequency of a microcantilever shifts sensitively due to mass loading. Since the resonance response is very broad under solution, mass detection sensitivity is lower for operation under solution. However, adsorption-induced bending is not affected by presence of solution. Therefore, cantilever bending is usually used as a sensor signal for sensing under solution. The specificity of the

cantilever sensor originates from the specificity of probe-target interaction. Adsorption of target molecules on the cantilever causes it to bend. In a control experiment, a cantilever with antibodies immobilized on its surface when exposed to a solution containing 1×10^6 cells/ml of bacteria in a buffered saline solution does not show any appreciable deflection. Injection of sample solution containing target bacteria causes bending observed with the cantilever. This is most probably due to the adsorption of the solution.

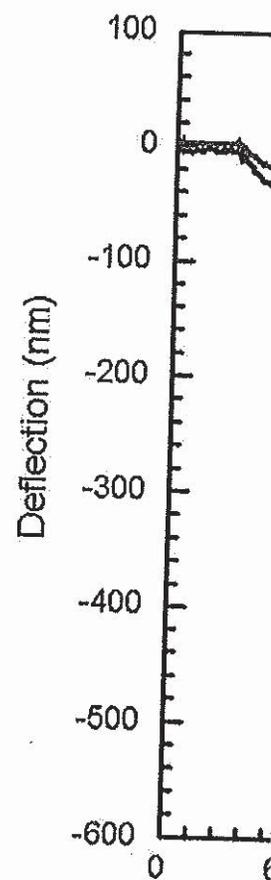


FIGURE 9 Cantilever deflection of antibodies shows bending due to adsorption of target bacteria. The specificity of cantilever is due to changes in ion

cantilever sensor originates from the specificity of probe-target interactions. Nonspecific adsorption of target molecules does not cause the cantilever to bend. Figure 9 shows bending of a cantilever with immobilized antibodies when exposed to a solution of *Francisella tularensis* (1×10^6 cells/ml in 0.1M phosphate-buffered saline solution) (Ji et al., 2004). The cantilever without any immobilized antibodies does not show any appreciable bending from injection of sample solution. The relatively small bending observed with the reference cantilever is most probably due to change in ionic concentration of the solution. This result was observed

with a setup where cantilever bending was monitored with an optical beam. The optical beam deflection technique is influenced by concentration of ionic species in the solution. The adsorption of organisms on the antibody-covered cantilevers causes a large deflection compared to that of a bare cantilever. The continued bending of the cantilever observed in Fig. 9 is probably due to movement of the organisms on the cantilever surface.

Figure 10 shows cantilever bending due to adsorption of thiolated single-stranded DNA (ssDNA) on a cantilever with a thin layer of gold coating on one side. In the case of ssDNA

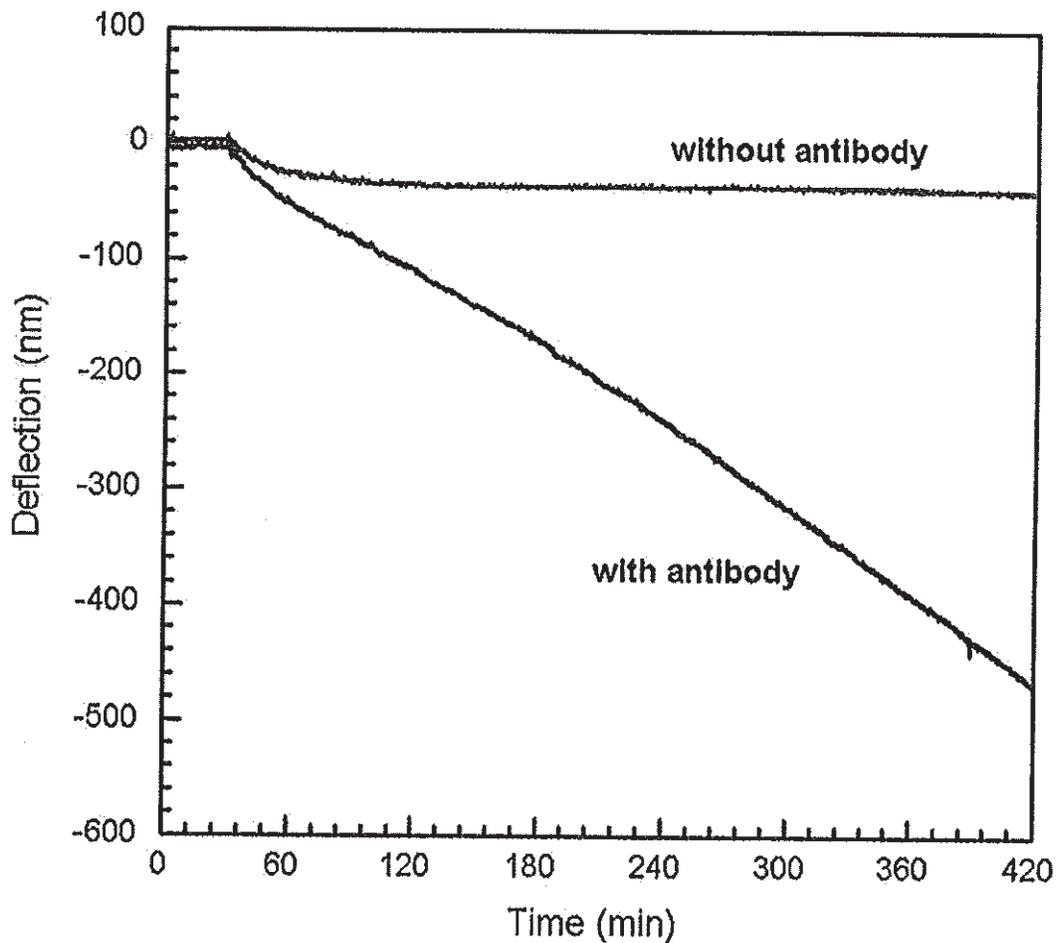
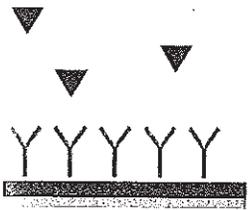


FIGURE 9 Cantilever deflection as a function of exposure to tularemia in solution. Cantilever with immobilized antibodies shows bending due to antibody-antigen interaction. The small deflection observed with uncoated cantilever is due to changes in ionic concentration of the solution.

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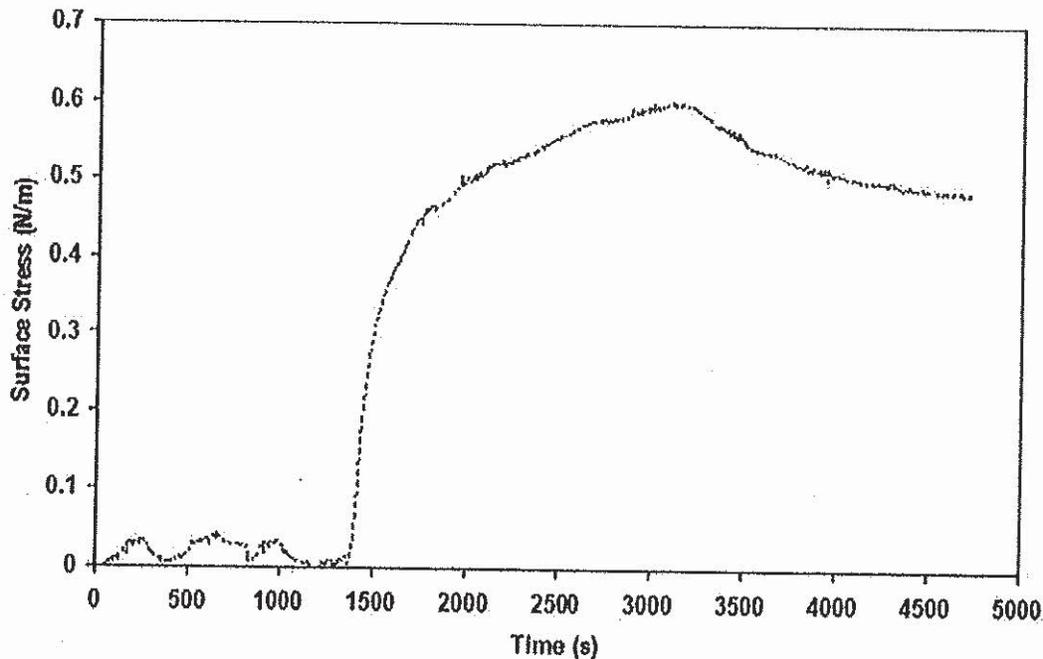


FIGURE 10 Surface stress (proportional to cantilever bending) observed on a cantilever as a function of time during thiolated ssDNA immobilization on the cantilever surface.

adsorption, the cantilever reaches a steady state due to saturation of available adsorption sites on the cantilever surface. The cantilevers used in the ssDNA experiments were piezoresistive cantilevers where the cantilever resistance changes as a function of cantilever bending.

The effect of interferents such as changes in ionic concentrations, pH, temperature, and flow rate can be overcome by using reference cantilevers (Boisen et al., 2000; Thaysen et al., 2001; Shekhawat et al., 2006). Also, it is possible to fabricate multiple cantilevers in an array for detection of multiple targets. Recently there have been many advances in fabrication of cantilevers with electronic signal transduction. Cantilevers with electronic readout have the inherent advantages over the optical beam deflection approach. In addition, electronic readout is compatible with array arrangement and packaging into a small integrated system.

CONCLUSIONS

We have described a variety of techniques based on scanning probe microscopy that have

become available in recent years and can be effectively extended to sensing, physiological studies, and diagnostics in biological and microbiological analyses. These truly interdisciplinary developments have immense potential to transcend academic and industrial barriers and are expected to allow significant advancements in nanoscale studies in biological systems. Certainly, as the probes of the discussed microscopes, that is, the microcantilever, the bead, the optical fibers, etc., improve, thanks to recent developments in material research and processing, manufacturing, and the necessary understanding of the involved physics, important knowledge is expected to transpire with regard to biological functions. In particular, reduction in size of the probes and alteration of the mechanical, electronic, and optical properties have been witnessed to provide higher resolution, operation on a wider range of sample material in more realistic environments, and richer interpretation of the collected data. Although the presented material provides the basic framework of the current cellular and bio-

molecular imaging probes may be envisioned multifunctional such robust data may be collected of biological samples.

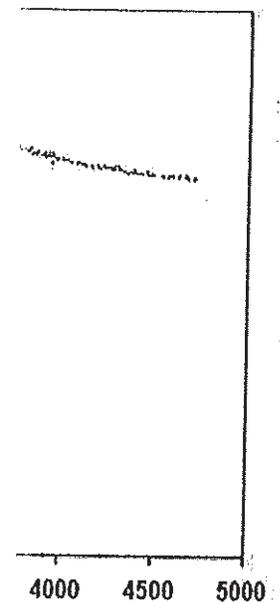
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molecular imaging techniques, the future probes may be envisioned to be hybrid and multifunctional such that reproducible and robust data may be collected on a large number of biological samples.

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cent years and can be sensing, physiological in biological and micro- se truly interdisciplinary mense potential to trans- industrial barriers and are ificant advancements in biological systems. Cer- of the discussed micro- cantilever, the bead, the prove, thanks to recent ial research and process- d the necessary under- ved physics, important to transpire with regard In particular, reduction and alteration of the and optical properties provide higher resolu- wider range of sample stic environments, and of the collected data. d material provides the current cellular and bio-

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