Chapter 5

MORPHOLOGY, MECHANICAL PROPERTIES
AND MANIPULATION OF LIVING CELLS:
ATOMIC FORCE MICROSCOPY

Gregory S. Watson*, Jolanta A. Watsona and Sverre Myhrab

aNanoscale Science and Technology Centre, School of Science, Griffith University;
Nathan, Australia
bOxford University, Begbroke Science Park, Sandy Lane, Yarnton, UK

ABSTRACT

Atomic force microscopy (AFM) is becoming an increasingly important tool-of-the-trade in the life sciences. In the study of morphology and dynamics of cells it has the particular merit of being able to interact non-destructively with live cells in vitro. Thus it occupies a unique niche in the suite of techniques that has so far been dominated by the photon- and electron-optical microscopies and spectroscopies. In addition, AFM in the lateral force and force-versus-distance operational modes can gain access to information that cannot be obtained by other means. Recent results obtained by AFM analysis of human fibroblasts and cancer cells (MDA) are described and discussed.

Keywords: Atomic Force Microscopy, fibroblasts, cancer cells, mechanical properties, F-d curves, morphology.

* Corresponding Author: Email: G.Watson@griffith.edu.au; Phone: +617 3875 7531; Fax: +617 3875 7656
1. **INTRODUCTION**

The force-sensing members of the large family of scanning probe microscopies (SPM), of which the atomic force microscope (AFM) is the most widely used technique, have become important tools during the past decade for visualizing, characterizing and manipulating objects and processes on the meso- and nano-scale. The AFM, in particular, has had impact in the life sciences. In cell science the pioneering work with AFM was carried out in the early 90’s [Gould et al. 1990, Henderson et al. 1992, Hoh and Hansma 1992]. The methodologies have now reached a stage of relative maturity [Hong and Lei 1999 and Czajkowski et al. 2000]. The principal merit of the AFM is as a non-intrusive local probe of live cells and their dynamics in the biofluid environment.

2. **ELEMENTS OF ATOMIC FORCE MICROSCOPY**

Detailed accounts of the technicalities of AFM instrumentation can be found in the literature, e.g., Myhra [1998] and Sarid [1991]. The basic/typical elements of a generic current-generation AFM are shown in figure 1.

![Figure 1. Schematic of a generic AFM. The segments of the photodetector are labelled A through D. The respective signals can be manipulated so as to detect either simple bending/buckling or torsional deformation of the lever. The piezo-electric driver can be activated in order to engage the tapping mode. The specimen is translated in x-y-z space by the piezo-electric scanner.](image)
The probe is at the heart of the system. It consists of a force-sensing/imposing lever and an integral tip at the free end of the lever. The interaction between tip and surface causes deformation of the lever. That deformation is ‘sensed’ by an optical lever system where a collimated beam from a laser diode is incident on the top surface of the lever at the location of the tip. The optical beam is reflected and then detected by a quad-segment position-sensitive photo-detector (PSPD). If the deformation is that of simple deflection, analogous to that of a diving board, arising from out-of-plane force components, then the deflected light beam will be detected by the top-bottom segments. If, on the other hand, the lever undergoes torsional deformation in response to in-plane force components acting at the apex of the tip, then the deflected light beam will be detected by left-right segments of the PSPD. The angular sensitivity of the detection system is in the range $10^{-6}$-$10^{-7}$ radians. Since the deflection of the cantilever, in terms of $z$-displacement at its free end, is proportional to the angular change, then the top-bottom PSPD signal is a measure of the $z$-excursion of the tip from an arbitrary set point. Because the tip is rigidly attached to the lever, the $z$-excursion of the tip will translate to an equal bending of the lever. The system is quasi-static; consequently the net force acting between tip and surface is balanced by the force imposed by the lever.

Most AFM instruments have adopted the scheme whereby the specimen is translated while the probe is held stationary. Localization and translation in $x$-$y$-$z$ space is effected by a scanner consisting of piezo-electric elements. (A piezo-electric material has the property that there is direct correspondence between an applied electric field and dimensional change. The field arises from application of a voltage. Typical figures of merit for a piezoelectric scanner range from a few to several hundred nm of dimensional change per volt applied to the scanner element). A high-resolution stage may have a maximum $x$-$y$ field of view of less than $1\times1$ $\mu$m$^2$, while other scanners may have fields of view of more than $100\times100$ $\mu$m$^2$; the latter capability is useful when a large area needs to be surveyed, but extreme resolution is not required (as in the case of analysis of living cells). A raster is generated by application of ramp- or sawtooth-signals to the $x$-$y$ elements of the scanning scanner. The resolution for a particular scanner is generally of order $10^{-5}$ times the field of view. Thus the smallest raster increment ranges from less than 0.1 to more than 10 nm, depending on the scanner and on the number of pixels in the image. The $z$-range of a scanner is generally about a tenth of the maximum field of view, and defines the ‘depth of focus’, while the resolution along the $z$-direction inherent in the scanner is correspondingly better (typically 0.01 nm) than in the $x$-$y$ plane.

The signals from the quad segments are conditioned (i.e., buffered, amplified and filtered). The top-bottom difference signal is compared against a set-point, when the instrument is operated in the constant force mode (in actuality the mode is maintaining constant lever deflection). Any deviation from the set-point, arising from a change in $z$-height at a particular location on the surface will then generate a feed-back signal. That signal is managed by an electronic feed-back loop, the output from which is used to control the height of the $z$-scanner so as to take the deviation back to zero. The change in $z$-scanner height required to maintain constant force constitutes the information that is used to generate a contour map of the surface (in this case a map where the contours correspond to the $z$-scanner extension required to maintain constant lever force). The usual implicit assumption for the interpretation of the resultant topographic image is that the surface is much stiffer than the lever; (i.e., the lever is the only compliant element). In the case of ‘soft’ surfaces that assumption must be treated with some scepticism. If the surface is relatively flat, such as in
the case of a cleaved crystalline face, then the feed-back loop can be turned off, and the z-height information is obtained directly from the difference signal from the top-bottom segments (the image is then obtained in the constant height mode).

2.1. THE PROBE

The tip is the primary sensor of interactions with the surface, and defines the location of the measurement, while the lever is the force transducer. The quality of the probe is defined by a number of parameters including:

- Radius of curvature at the tip apex, \( R_{\text{tip}} \), (rarely less than 2 nm, and up to 50 nm for a ‘standard’ probe).
- The aspect ratio of the tip, \( A_r \), usually taken as the ratio of tip height to half width, defines the steepest gradient of surface features that can be traced (specially ‘sharpened’ tips may have an aspect ratio of 10, while routine tips may have a figure of near unity).
- Tip height, \( h \), will affect the range of height variations that can be probed reliably, it will also affect the sensitivity to torsional forces (tip height may range from < 3 to > 25 \( \mu \)m).
- Lever stiffness arises from its geometry and the elastic modulus of the material which will determine its response to forces being sensed and will affect the range of forces being imposed by the tip on the surface (the normal force constant, \( k_N \), ranges from 0.001 to 100 N/m, while the torsional force constant, \( k_T \), tends to be higher by factors of 10-100). The actual spring constant for a particular lever can be calibrated in anticipation of F-d analysis in accord with one of several methods described in the literature [Cleveland et al. 1993 and Gibson et al. 1997a].

A lever with a force constant of 1 N/m will impose/sense a force of 1 nN, if there is a z-deflection of 1 nm. One might think that a soft lever in combination with a z-resolution of 0.01 nm would allow force sensitivity in the sub-pN range. Unfortunately thermal fluctuations impose a limit of 1-10 pN on resolution. The probe is typically microfabricated from doped Si or from nominally stoichiometric \( \text{Si}_3\text{N}_4 \). A V-shaped two-beam geometry is generally adopted when torsional rigidity is required, while a single-beam ‘diving board’ configuration is preferable when quantitative lateral force measurements are to be obtained. In many cases, especially when biosystems are probed, the surface chemistry of the tip will be an important factor. Clean Si or \( \text{Si}_3\text{N}_4 \) will be coated by a thin native oxide and will therefore present a hydrophilic surface (thus the tip can be functionalized by biochemical agents that will couple to an oxide). However, probes stored in air are generally hydrophobic due to adsorbed hydrocarbon contamination. Special purpose probes are now available for particular applications; e.g., Au-coatings are preferred for thiol coupling, a nano-tube or graphite spike can be grown on the tip apex in cases where extreme tip sharpness and high aspect ratio are required.

The choice of probe depends on the type of measurement (e.g., contact mode imaging requires a soft lever, while intermittent contact mode imaging is commonly carried out with a
stiffer lever), the roughness and hardness of the sample, and the desired resolution (hard and flat specimens are suitable for short and sharp tips and stiff levers). Biomaterials are ‘soft’ objects in the context of AFM analysis. Accordingly it is necessary to work with a lever with $k_N$ in the range 0.001-0.1 N/m in order to avoid excessive tip indentation. New probes will come with the manufacturer’s nominal figures of merit. In many cases it is necessary to determine the characteristics of particular probes. The actual parameters for a particular probe can be calibrated/measured by one of several methods described in the literature (e.g., Gibson et al. [1996]). The probe is a consumable item, due to wear, contamination or accidental damage. The cost ranges from a few dollars/probe for the ‘standard’ varieties to more than $100 for a special purpose probe.

2.2. OPERATIONAL MODES

Contact Mode Imaging

Short-range interatomic interactions at the point of tip-to-surface contact are balanced by the quasi-static bending of the lever. Since the force constant of a lattice potential is in the range $10^2$-$10^3$ N/m, while that of the lever is typically 0.01-1 N/m, the compliance of the system is principally confined to that of the lever. However, biomaterials are ‘soft’ with effective force constants comparable to that of the lever. Thus the surface will become deformed by forces imposed by the tip, leading to an extended area of contact, and a corresponding degradation of lateral resolution.

Intermittent Contact Mode Imaging

The lever is now stimulated by excitation of a piezoelectric actuator at the anchor point to oscillate at, or near, its free-running resonance frequency (from <10 to ca. 500 kHz depending on the stiffness of the lever). The tip is then located in the z-direction with respect to the surface so that the tip enters the short-range force field of the surface at the point of closest approach. The effect is to turn the probe into a damped and driven anharmonic oscillator. The damping causes a decrement in amplitude of oscillation and a change in phase with respect to the phase of the driving signal and the free-running amplitude of the probe. Those changes depend on the strength of the short-range interactions at ‘contact’; the respective decrement and shift constitute variables that can be compared against a set-point. In many cases the intermittent-contact AC mode (also known as ‘tapping mode™’) will result in better image quality for ‘soft’ specimens. The mode has two distinct advantages. The effect of lateral forces is substantially eliminated (important because biomaterials tend to have low resistance to shear stress). Also, since the interaction now arises from an impulse action at one extreme of the oscillatory motion of the tip, then the inertia of the sample will resist deformation. However, the resolution of AC mode imaging depends on the stiffness of the lever (related to the free-running frequency) and the width of the resonance envelope. The latter is severely degraded in water, and soft levers are preferred for analysis of live cells. While AC mode

**Lateral force Imaging/Analysis**

In-plane as well as out-of-plane force components will act on the tip at the point of contact with the surface. The former will exert a friction force on the tip in the direction of travel. If the fast-scan raster direction is perpendicular to the long axis of the lever, then the lateral friction force will cause a torsional deformation of the lever that can be sensed by the signal on the left-right PSPD segments. To a first approximation the effect is similar to that of macroscopic friction between two objects in sliding contact. The relationship between lateral force, $F_L$, being sensed, and normal force, $F_N$, being imposed by the lever is given by equation 1,

$$F_L = \mu (F_N + F_A)$$

where $\mu$ is the coefficient of friction and $F_A$ is the force of adhesion between tip and surface ($F_N + F_A$ is the total loading force) [Gibson \textit{et al.} 1997b]. In the single asperity regime, when a sharp tip is sliding across a hard surface, the relationship is more complex [Carpick and Salmeron 1997]. A surface may be laterally differentiated by virtue of variation in surface chemistry (the differentiation may not manifest itself in the topographical contrast). A chemical contrast will result from chemical differentiation that manifests itself as a change in adhesive force. Hence the mode is often called chemical force microscopy (or friction force microscopy (FFM)) [Noy \textit{et al.} 1995]. It is difficult to describe fully and simply the frictional force dependence over a large dynamic range of force loading for all AFM experiments. However equation 2 offers a phenomenological trend describing ‘hard’ materials in contact for different contact regimes.

$$F_L = A F_{Total}^n + \mu F_{Total}^{2/3} + F_{L-Contact}$$

where $A$ and $\mu$ are constants. At low total loading forces in the range (a$\rightarrow$b) a change in contact area with increasing load will yield the $n \neq 1$ exponent. The area of contact between a spherically shaped tip (at the tip apex), and a flat surface (for purely elastic deformations) will typically be proportional to $F_{Total}^{2/3}$. The second term in the equation describes the multi asperity case for higher loading forces (c$\rightarrow$d). The last term in the equation ($F_{L-Contact}$) takes account of the condition when there is a finite frictional force at zero total load (i.e., a finite contact area may be present at zero load). Thus the lateral dependence at low loads is typically non-linear while a linear dependence is often seen at high loads.
Force versus Distance (F-d) Analysis

Quasi-static F-d analysis can be undertaken by holding the tip at a particular x-y location above the surface. The sample is then driven towards the tip at a rate that is slow in comparison with the mechanical response of the system. The net force is sensed during the approach, contact and retraction parts of the cycle. Two idealized response curves are shown in figure 2, with stage travel and lever deflection plotted on the horizontal and vertical axes, respectively. The vertical units can be converted into forces sensed/applied by the lever by the simple expediency of multiplying the deflection by \( k \). The curve in figure 2 (a) represents the case when both tip and a surface are incompressible, and when the surface is covered with a thin adsorbed aqueous film. The various segments represent:

- **Approach half-cycle:**
  - AB - tip and surface are well-separated, no interaction;
  - BC - the tip senses the attractive interaction from the meniscus layer, the force constant of interaction exceeds \( k \), and the tip snaps into contact with the ‘hard’ surface. There is a regime of instability due to the force constant of interaction being greater than that of the lever;
  - CD - tip and surface are incompressible, and the stage travel distance must therefore be equal to the lever deflection.

- **Retract half-cycle:**
  - DE - the system retraces itself since all deformations/deflections are elastic;
  - EF - the meniscus interaction has increased due to capillary action, and there will be a greater lift-off instability/discontinuity (than for the snap-on);
  - FA - return to large separation and no interaction.

![Diagram](image)

Figure 2. Generic outcomes of F-d analysis for: (a) an incompressible tip and a surface, with the surface being covered by an adsorbed aqueous film, and (b) a compliant surface so that the tip indents the surface. The surface undergoes deformation in response to the force being applied by the lever, in combination with partial elastic recovery.

This kind of curve is representative of events for an air-ambient instrument due to adsorbed moisture. The meniscus interaction is generally a nuisance feature in that it will mask other surface mechanical effects. The meniscus can be eliminated by carrying out F-d
analysis under water (or some other fluid ambient). The ‘hard’ surface F-d curve is used to calibrate the detection system so that a measurable detector response can be related accurately to the lever deflection. Applications of F-d analysis in cellular biology, biomolecular interactions and protein folding are now well-established. Several reports have described current state of the art [Burnham and Colton 1989, Zlatanova et al. 2000, Best and Clarke 2002].

The schematic curve in figure 2 (b) shows lever deflection as a function of tip indentation of the specimen surface, or separation from the surface, (i.e., the difference between stage travel and lever deflection), and thus illustrates other surface mechanical aspects of the system which are accessible to F-d analysis. The shape of the curve assumes that at no stage is the constant force of interaction greater than \( k_N \) (hence instabilities at snap-on and lift-off are suppressed). The information content of the generic F-d curve in figure 2 (b) may be summarized as follows:

- The force constant of interaction, \( k_i \), is simply the slope of the curve, \( k_N z_L / \left| z_d - z_L \right| \), where \( z_d \) and \( z_L \) refer to stage travel and lever deflection, respectively.
- The forces at ‘contact’ on approach and retract, \( F_A \) and \( F_R \), may be defined at the inflection points where the force constants of interaction are the greatest.
- The snap-on and lift-off forces, \( F_{SO} \) and \( F_{LO} \), are measured at the points of greatest net attraction. The latter is generally taken to be the force of adhesion.
- The distances \( z_{RO} \) and \( z_{LO} \) are measures of the elastic recovery and the plastic indentation, both at zero lever loading.
- The extent of hysteresis in the system is given by the area enclosed by the approach and retract curves.

The parameters defined above cannot readily be related to the familiar macroscopic definitions of mechanical properties, e.g., hardness, adhesion, Young’s modulus, tensile strength, flexural strength, etc, unless the system can be specified further (e.g., tip shape, contact area, surface free energy of tip, etc) and unless additional assumptions are made (homogeneity, isotropy, surface topography, etc). Typical examples of what insight can be gained by F-d analysis can be found in the literature, e.g., Blach et al. [2001a] and Blach et al. [2001b].

A consequence of the discussion above is the need to match the force constant of the lever to that of the interaction being investigated in order to extract maximum information. If there is miss-match, then either the lever will be the only compliant element, and no information is obtained about the surface, or the surface will be the only compliant element, and the deflection of the lever is not measurable.

### 2.3. Utility of AFM in the Context of Cell Biology

Periods of high rates of progress in visualization and analysis of bio-systems at the level of the single cell have substantially coincided with the emergence of new techniques. The
invention and subsequent maturing of photon optical microscopies and spectroscopies were the initial drivers of rapid progress. These techniques have the merits of being able to interact non-destructively with viable cells in a biocompatible fluid. However, the effects of diffraction places a lower limit on spatial resolution of ca. 1 μm, as well as imposing a similar limit on the linear size of the volume over which analytical information will be averaged.

Electron-optical microscopies and associated analytical techniques have provided the means to improve the spatial resolution by more than a factor of $10^3$, while the information volume is now approaching that of a single atom, in the case of the latest-generation high resolution TEM instruments using high luminosity and fine-focus beams in combination with scanning/convergent beam operational modes. However, the impressive advances in resolution have come at the expense of the techniques being profoundly destructive for viable cells (due to requirement for specimen preparation, and exposure to ionising radiation and a vacuum environment). More recently fine-focus ion beam analytical techniques with spatial resolution down to ca. 100 nm (dynamic imaging secondary ion mass spectrometry (SIMS)) are beginning to have an impact in cell biology.

This is the context in which atomic force microscopy is carving out its niche as an important complementary tool-of-the-trade for cell biologists. Areas that have had the greatest impact to date, and some that are likely to have considerable scope for further development and exploitation include:

- **Surface topography/morphology** – AFM generated great initial excitement due to its ability to map surface topography in three dimensions with nm spatial resolution. That performance is obtainable for minimal specimen preparation, and for specimens that were conventionally thought to be ‘difficult’ (e.g., insulators). However, biomaterials in general, and living cells in particular, are ‘soft’ objects (Young’s modulus is typically of order 10 kPa). When the relatively minute forces, in the nN and pN range, are imposed on the tip, there will be considerable indentation and deformation of the sample being probed, thus leading to a greater than expected tip-to-sample contact area, and correspondingly degraded spatial resolution. On the other hand, unlike other imaging techniques the depth of focus for AFM is equal to the z-range of the scanning stage (usually 5-10 μm), thus offering true 3-dimensional mapping at sub-nm resolution in the z-direction.

- **Internal structure** – While the plasma membrane is extremely soft and relatively fragile a cell maintains its shape due to its internal cytoskeletal structure. Likewise, other components of the internal cellular machinery can be ‘hard’ in comparison with the plasma membrane. Thus the force-sensing/imposing probe can map internal structure, in manner similar to ‘palpating’, by deforming the membrane. The palpation effect is shown in figure 3.
- **Mechanical properties** – AFM has the unique attribute among the high resolution imaging techniques of being a local probe of nano-mechanical properties. In the force versus distance, (F-d), operational mode the force-sensing/imposing lever responds to interactions between tip and surface as the tip is being driven towards, or away from the surface, at a defined point in the x-y plane. Thus it is possible to measure the local stiffness directly, and Young’s modulus indirectly, local tip-to-surface adhesion and plastic versus elastic deformation and recovery. The tip may be functionalised, see figure 4, in order to carry out local molecular sensing, and to generate chemical image contrast in a laterally differentiated surface.
Figure 4. Schematic illustration of AFM-based measurement of biomolecular interactions. Tip and substrate are functionalised via silane coupling (other couplings may be used). A particular biomolecule is then attached to the tip, while a counter-molecule is located in the specimen surface, or attached to the substrate.

− *Dynamics* – The ability to interact with live cells in vitro in combination with either sequential image acquisition or continuous force sensing provide the means for tracking the dynamics of intra- or inter-cell processes. The former operational mode is relatively slow and is most suitable for events where the temporal evolution takes place on a time scale of several minutes to hours. For instance, aspects of cytoskeletal dynamics, cell motility or conformational response to external stimuli fall into this category. Continuous monitoring of local dimensional change in the z-direction, such as in the case of spontaneously beating cardiomyocytes, can be carried out at a temporal resolution that is essentially limited by the resonance frequency of the force-sensing lever (typically 10 kHz).

− *Probe-induced manipulation* – While most AFM contact interactions will, to varying extents, perturb cellular systems, the operation of the AFM can be configured as a device for micro- and nano-manipulation. The emphasis then is on deliberate and purposeful application of force so as to alter the bio-system. In the case of the bio-system being a cell, alteration might entail a translation in the x-y plane, with the intention of gaining insight into the interaction of a particular cell with a substrate. More intrusive, and terminal, alteration can be carried out with a stiffer probe and/or higher force loadings, and thus greater applied force, in order to disassemble the cell. The AFM is now essentially acting as a nano-version of a microtome, with the advantage that the cell can be imaged in situ before and after alteration, as demonstrated in figure 5 (a) and (b), respectively, showing a human fibroblast cell.
Figure 5. (a) Topographical images showing a living human fibroblast cell prior to (a) and post (b) microtoming using the AFM tip at higher force loadings.

3. TECHNICAL ISSUES

The technical details described below show typical cell culture preparations for AFM imaging. The procedures are representative of general procedures used for other cell lines.

3.1. Cell Culture - Handling and Preparation for In vitro Analysis by AFM

Primary human skin fibroblasts (HSF), human breast cancer cells (MDA-MB-231) are generally maintained as monolayer cultures in RPMI-1640 containing 15 mM NaHCO$_3$, and supplemented with 10% heat-inactivated fetal calf serum (FCS). The cultures are grown in a humidified environment with a 5% CO$_2$ atmosphere at 37°C. There are two reliable methods for preparation of live cells for AFM analysis.

3.1.1. Live Cells (i)

In preparation for AFM analysis cells are harvested by trypsin/versene treatment and seeded onto 35 mm tissue culture treated dishes (Nunc 153066) containing 12 mm glass coverslips. After overnight incubation at 37°C these coverslips were removed and washed five times with serum free media (RPMI) containing 15 mM NaHCO$_3$. The coverslips can then be attached to standard AFM mounting plates, before being covered with a droplet of fluid, in anticipation of in vitro analysis.

3.1.2. Live Cells (ii)

The cells were harvested and grown overnight in 35 mm sterile tissue culture dishes. The preferred dishes have been pre-treated by the manufacturer so as to present a negatively charged and hydrophilic surface, thus ensuring better cell adherence. The cells are then washed, as above, and covered to a depth of 2-3 mm with serum free media.
3.1.3. Dehydrated Cells
The preparatory sequence is identical to that described above. However, instead of placing the cultures in a biocompatible liquid for analysis, they are now rinsed in order to remove excess salts, and then allowed to dry in air for 30 min or more, and then investigated by AFM over durations up to 3 h. In some cases it may be possible to continue analysis for durations up to 48 h.

3.2. Fluid Cell
The examples of AFM analyses described below were carried out with a ThermoMicroscope TMX-2000 multitechnique SPM. Some of the results were obtained with a Discoverer using an open fluid cell defined by a droplet of water trapped between the glass window on the detector stage and the coverslip substrate (similar to that described in the literature [Hoh and Hansma, 1992]). On other occasions the Explorer provided increased flexibility and convenience. Again, an open cell could be defined by a trapped droplet as in the case of the Discoverer. It is preferable and more convenient, however, to grow the cell culture directly in a culture dish that will then constitute the fluid cell for the Explorer. The preferred scanner for the latter procedure had a maximum field of view of 130×130 µm$^2$ and a z-range of 10 µm.

3.3. Fluid Ambient Environment
3.3.1. Maintenance of Static Conditions
The principal variables requiring control are those of temperature and fluid volume. If the fluid cell is defined by a trapped droplet, then frequent replenishment of the reservoir is required. Thus imaging conditions will need to be reestablished at regular intervals (typically 30 mins), but there is then opportunity also to reestablish optimum temperature. A larger fluid cell, such as a culture dish, with a volume of some 5 ml or more, will have a longer life-time with respect to evaporative losses, and the greater thermal inertia will promote temperature stability. Long-term stability over some hours will require replenishment, however. The optimum temperature can then be reestablished by total replacement of the media. Flow-through replacement from an external reservoir is another option. Another alternative is that of continuous heating of the fluid cell by a hot stage; the disadvantages then being associated with thermal contraction/expansion and with thermal convection currents in the fluid.

3.3.2. Injection of Reactants for Dynamic Studies
In the case of slow dynamics being investigated, reactants may be introduced when there is replenishment or replacement of media, or directly via an access port. A practised operator can usually reestablish imaging/analysis conditions within a few minutes. More rapid injection and mixing is required if fast dynamics is being investigated. Access to the fluid cell is generally restricted by the compact design of most instruments. Accordingly a flow-through cell arrangement may be the better choice when interruption to the imaging
4. Illustrative Case Studies

4.1. Dehydrated Cell

Actual spatial resolution of AFM analysis will always depend on the ‘contact’ area, for any given operational mode and set of probe parameters. Thus an image of a hard surface will reveal more detailed information than that for a soft surface. The point is illustrated by the image in figure 6 (a) of a section of a dehydrated cell (cf. subsequent images of living cells). The sketch shown in figure 6 (b) below illustrates the effect of ‘tip convolution’, which is the most common artefact. In essence, the finite width of the tip causes broadening in the x-y plane of the image of any real object. If the apex of the tip is taken to be a spherical section, then apparent width of a cytoskeletal fibre is given by equation 3.

\[
W = 2\sqrt{\left(\left(R_{\text{Fib}} + T_{\text{Mem}}\right) + R_{\text{Tip}}\right)^2 - \left(R_{\text{Tip}} - \left(R_{\text{Fib}} + T_{\text{Mem}}\right)\right)^2}
\]  

The finite thickness of the collapsed plasma membrane, \(T_{\text{mem}}\), has been taken into account. While the apparent width in the image, \(W\), of a fibre is some 100 nm, the deconvoluted width, \(R_{\text{Fib}}\), is more likely to be 30-45 nm. It should be noted that the height of a feature, such as a cytoskeletal fibre in the plane of the substrate will be a ‘true’ height generally unaffected by tip shape.

Figure 6. (a) AFM contact mode image (shown in reverse contrast) of a human fibroblast cell. A high resolution contact mode image shows the nucleus and surrounding structure. (b) The schematic shows the geometry of convolution between tip shape, fibre and plasma membrane.
4.2. Live Cells

Methodological aspects of a number of AFM-based studies of living fibroblasts are summarized in Table 1. The choice of substrate, and subsequent coating, figured prominently in most of the early studies in order to ensure reliable and stable attachment of the cells. It is also evident that most of the imaging was carried out in the contact mode, as opposed to the tapping mode. It is significant that ‘soft’ levers ($k_N < 0.1 \text{ N/m}$) were found to produce the best results.

**Table 1. Summary of AFM-based studies**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Substrate</th>
<th>Substrate Coating</th>
<th>AFM mode</th>
<th>Probe(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts etc.</td>
<td>Glass cover slip</td>
<td>None</td>
<td>Contact</td>
<td>$Si_3N_4$, $k_N = 0.01, 0.03$</td>
<td>Kuznetsov <em>et al.</em> 1997</td>
</tr>
<tr>
<td>L929</td>
<td>‘Culture dish’</td>
<td>None</td>
<td>Contact, F-d</td>
<td>$Si_3N_4$, $k_N = 0.03, 0.06$</td>
<td>Wu <em>et al.</em> 1998</td>
</tr>
<tr>
<td>Fibroblasts etc.</td>
<td>‘Plastic’ dish</td>
<td>None</td>
<td>Contact</td>
<td>$Si_3N_4$, $k_N = 0.03$</td>
<td>Braet <em>et al.</em> 1998</td>
</tr>
<tr>
<td>3T3</td>
<td>‘Plastic’ dish</td>
<td>None</td>
<td>Contact, F-d</td>
<td>$Si_3N_4$, $k_N = 0.08$</td>
<td>Rotsch <em>et al.</em> 1999</td>
</tr>
<tr>
<td>3T6</td>
<td>Glass cover slip</td>
<td>None</td>
<td>Contact, F-d</td>
<td>$Si_3N_4$, $k_N = 0.01$</td>
<td>Ricci <em>et al.</em> 1997</td>
</tr>
<tr>
<td>3T3, NRK</td>
<td>‘Plastic’ dish</td>
<td>None</td>
<td>Contact, F-d</td>
<td>$Si_3N_4$, $k_N = 0.08$</td>
<td>Rotsch and Radmacher 2000</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>Glass dish</td>
<td>Fibronectin</td>
<td>Contact, F-d</td>
<td>$Si_3N_4$, $k_N = 0.018$</td>
<td>Haga <em>et al.</em> 2000a</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>Glass dish</td>
<td>Fibronectin</td>
<td>Contact, F-d(m)*</td>
<td>$Si_3N_4$, $k_N = 0.03$</td>
<td>Haga <em>et al.</em> 2000b</td>
</tr>
</tbody>
</table>

* A viscoelastic modulation method was adopted for the particular study.

Typical outcomes of contact mode imaging of live human fibroblasts are shown in figures 7 and 8. Figure 7 (a) shows a near-confluent layer. An image of an isolated cell is also shown in figure 7 (b), with a corresponding contour line shown in (c). The image in figure 8 (a) clearly shows the internal cytoskeletal structure. Figure 8 (b) shows a corresponding line profile.
Figure 7. (a) AFM images of living fibroblast cells in a near confluent state. (b) An isolated cell with a contour line illustrating 3-D information content in the AFM images.

Figure 8. (a) An AFM contact mode image of a living fibroblast demonstrating that the more rigid cytoskeletal structure can be mapped due to deformation of the soft plasma membrane. (b) shows a corresponding line profile.
4.3. Cellular Dynamics

As mentioned above the AFM is uniquely capable of tracking the temporal evolution of systems consisting of living cells in a biocompatible fluid. There are distinctly different methodologies for ‘slow’ (> 10 min) and ‘fast’ (< 10 min) dynamics. These are described in greater detail in sections 4.3.1 and 4.3.2 below.

4.3.1. Slow Dynamics

It is possible to establish good imaging conditions within a few minutes. The acquisition of an image takes typically 1-10 mins. Thus sequential imaging over a particular field of view can track cell dynamics *in vitro* on the time scale of some minutes. A soft lever \( k_N < 0.01 \) N/m in combination with a low applied force (< 1 nN) will enhance information arising from the ‘softer’ elements of the cell, while a stiffer lever and greater applied force will deform the plasma membrane and enhance visualization of the less compressible cytoskeletal and intracellular structures. The more informative studies have exploited the latter strategy in order to gain insight into cytoskeletal dynamics, (e.g., Bushell *et al.* [1999], Schoenenberger and Hoh [1994], Braet *et al.* [1998a, b], Rotsch and Radmacher [2000]). An example of investigations of slow intracellular dynamics is shown in a sequence of images in figure 9 (A) to (D), where the intracellular nucleation and growth over a period of 3 h of formazan crystals is apparent. The crystals arise from enzymatic conversion of a tetrazolium salt during the MTT assay of viable cells [Bushell *et al.* 1999]. Another example of slow cellular dynamics being revealed by AFM imaging is shown in figure 10. A live fibroblast was subjected to exposure to cytochalasin (a known cytoskeletal inhibitor).
Figure 9. The sequence of images show the intra-cellular nucleation and growth of formazan crystals (arising from the MTT test for viable cells).

Figure 10. Successive images showing the effect on a live fibroblast of exposure to cytochalasin for 0 (a) and 20 (b) minutes.

Figure 11 shows the effects of MDA cells and fibroblasts upon exposure to glutaraldehyde which crosslinks the plasma membrane. The fibroblasts and MDA cells were initially imaged in order to obtain a suitable location. The cells were then exposed to glutaraldehyde by injection via a micro syringe through a thin tube attached to the AFM head.
leading to the culture dish. This procedure ensured that the position of the tip in the x-y plane was positioned precisely above the desired location. The acquisition of F-d curves was undertaken just prior to the treatment, and immediately after the exposure to the cells.

Figure 11. Topographical images of an MDA cell and human fibroblasts with corresponding F-d curves showing the extent of cellular stiffening over time as a result of the addition of glutaraldehyde. Curve 1 (shown in blue for both cells) was obtained on the hard incompressible culture dish substrate.

The mechanical response of the cells was found to change with time following the addition of gluteraldehyde. The changes were monitored in real time and show progressive mechanical stiffening of the cell membrane. Even at relatively low concentrations (0.002%) both of the cell lines show significant hardening in less than 15 minutes. The MDA cell showed an indentation of ca. 200 nm and ca. 3000 nm at time 844 sec and 0 sec, respectively. F-d data was obtained on two sections of the human skin cell indicated on the topographical image in figure 11 at points B and C. The data show the cell region ‘B’ to be far more compliant than region ‘C’, as indicated by F-d curve 2 and 4, respectively. After the injection of glutaraldehyde, region ‘B’ becomes stiffer after 455 seconds (indentation decreases from ca. 1900 nm to ca. 1000 nm) and region ‘C’ significantly hardens after 723 seconds (indentation decreases from ca. 500 nm to ca. 40 nm). An earlier study, using a higher concentration of glutaraldehyde on Madin-Darby Canine Kidney (MDCK) cells, demonstrated significant cell stiffening within 4 min after exposure (Hoh and Schoenenberger 1994).
4.3.2. Fast Dynamics

Biological activity on the sub-second time scale can be observed and analyzed by AFM methodologies by monitoring the deflection of a lever stationary in the x-y plane, and sensed effectively in the constant height mode (i.e., where the time-constant of the feed-back loop is longer than that of the biological response mechanism). The tip is simply landed at an appropriate location predetermined from an image. The x-y scan function is deactivated, and the dynamic response is monitored through the z-deflection of the lever. A soft lever is most appropriate, since the probe ideally should be a passive participant in the temporal evolution. The method has been deployed with considerable success in the case of cardiomyocytes [Shroff et al. 1995, Domke et al. 1999], as shown in figure 12 (a) and (b). There is clearly considerable scope for applications of similar methodologies for investigations of other manifestations of cellular dynamics.

Figure 12. The trace in (a) illustrates an irregular beat of a live cardiomyocyte. The image and traces in (b) demonstrate that both amplitude and frequency depend on the location within a single cell (reproduced with permission from Springer Verlag).
4.4. Nano-mechanical (F-d) Analysis

The cytoskeleton is a complex 3-dimensional network composed of actin filaments, intermediate filaments and microtubules. Each component serves a specific function. For instance, the actin filaments, when subjected to stress, will coalesce to form stress fibers, that then play a major role in forming attachment of a cell to a substrate.

A lever-induced force will cause deformation at the tip-to-surface interface. If the tip is taken to be incompressible, then the deformation is confined to the sample. The depth of indentation as a function of applied force will depend on the shape of the tip and on the mechanical properties of the surface. The Bilodeau model [Bilodeau 1992] describes the indentation of an elastic half-space by a regular $n$-sided pyramidal probe; a standard Si$_3$N$_4$ probe tip, to a very high degree of accuracy. When $n = 4$, the Bilodeau model predicts the following relationship (equation 4) between force, $F$, and depth of indentation, $\Delta z$, from which an equivalent Young’s modulus, $E$, can be obtained.

$$F = \frac{3E \tan \alpha}{4(1-\nu^2)} \Delta z^2$$  \hspace{1cm} (4)

where $\alpha$ is the opening half-angle of the pyramid (55° for a standard Si$_3$N$_4$ tip), and $\nu$ is Poisson’s ratio (in the range 0.3-0.5). Other expressions are relevant to tips of different shape (e.g., cylindrical, spherical or parabolic). The salient results of studies in the literature are summarized in Table 2.

Table 2. Summary of studies of mechanical properties of living cells

<table>
<thead>
<tr>
<th>Young’s Modulus (kPa)</th>
<th>Cell line</th>
<th>Quoted tip shape</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C)-Cell; (N)-Nucleus; (E)-Cell Edge</td>
<td></td>
<td>Approximation</td>
<td></td>
</tr>
<tr>
<td>7.22±0.46 (N); 2.97±0.79 (C); Near Nucleus 1.27±0.36 (E)</td>
<td>Human umbilical, Vein, endothelial cells (HUVECs)</td>
<td>Conical</td>
<td>Mathur et al. 2000</td>
</tr>
<tr>
<td>2</td>
<td>Liver, endothelial cells</td>
<td></td>
<td>Braet et al. 1998</td>
</tr>
<tr>
<td>3-5 (E); leading edge of cell 12 (E) stable edge cell</td>
<td>Fibroblasts</td>
<td></td>
<td>Rotsch et al. 1997</td>
</tr>
<tr>
<td>5 (C)</td>
<td>3T3 Fibroblasts</td>
<td>Sneddon</td>
<td>Rotsch and Radmacher et al. 2000</td>
</tr>
<tr>
<td>1.5-4 (N); 100 (E)</td>
<td>Human platelets</td>
<td></td>
<td>Rrotsch and Radmacher et al. 1996</td>
</tr>
<tr>
<td>2-17 (C)</td>
<td>Human fibroblasts</td>
<td>Sneddon conical</td>
<td>Bushell et al. 1999</td>
</tr>
<tr>
<td>1-10 (C)</td>
<td>Human fibroblasts</td>
<td>Sneddon conical</td>
<td>Bushell et al. 2003</td>
</tr>
<tr>
<td>4-100; 4 (N)</td>
<td>Mouse fibroblasts (NIH3T3)</td>
<td>Conical (50-200nm)</td>
<td>Haga et al. 2000a,b</td>
</tr>
<tr>
<td>(N) 10 \times softer than surrounding regions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.57±0.37 (C)</td>
<td>Human lung epithelial cells</td>
<td>Pyramidal</td>
<td>Alcaraz et al. 2003</td>
</tr>
<tr>
<td>-</td>
<td>Madine-Darby, canine kidney (MDCK)</td>
<td>Spherical</td>
<td>A-Hassan et al. 1998</td>
</tr>
<tr>
<td>5 (C)</td>
<td>3T3 Fibroblasts</td>
<td>Sneddon</td>
<td>Rotsch and Radmacher et al. 2000</td>
</tr>
<tr>
<td>5-8 (C)</td>
<td>Smooth muscle cell</td>
<td>Hertz conical and spherical</td>
<td>Engler et al. 2004</td>
</tr>
<tr>
<td>0.1-1.4 (C)</td>
<td>NIH 3T3 Fibroblasts</td>
<td>Parabolic</td>
<td>Mahaffy et al. 2000</td>
</tr>
</tbody>
</table>
4.4.1. Human Fibroblasts

F-d analysis of a cell will show a variety of outcomes because of the complex and heterogeneous content of the cytoplasm which contains organelles, cytoskeletal components, and water substrate effects. Prior to F-d analysis, a topographical image is acquired in order to locate a suitable area for study. F-d analysis is undertaken immediately after the image acquisition in order to minimise the effect of any cell motion [Rotsch et al. 1999]. A topographical image is then acquired post F-d analysis to ensure the location of the cell did not change.

Two topographical images of human fibroblast cells acquired at scan areas of 105×105 and 20×20 µm$^2$ are shown in figure 13 (a) and (b), respectively. A range of F-d curves were acquired on the various cellular regions which are shown in figure 13 (labelled 1 to 8). Curves 1 to 4 correspond to figure 13 (a) and (b) with the locations indicated by the labels A, B and C. Curves 5 to 8 show interaction at different imaging locations/conditions. Curve 1 shows a calibration curve (shown in blue) obtained on a hard surface (the bottom of the culture dish in this case) and on the cell membrane (shown in black) located on region ‘A’ in figure 13 (a). This alignment of contact points of the two curves allows for a direct estimation of the extent of indentation at a particular applied force. By processing this F-d curve and utilising a pyramidal tip approximation, Young’s modulus was determined to be ca. 9 kPa. This value is in good agreement with those in the literature (e.g., Bushell et al. [1999] and Wu et al. [1998]). However errors in the calculations (e.g., indentation depth, Young’s modulus, etc) will arise in the event of any uncertainty in the contact point location of the F-d curve obtained at a soft region on the cell surface.
Figure 13. Some of the F-d curves in this figure have been shifted along the x-axis in order to emphasize specific features. (a) and (b) Topographical images of human fibroblast cells obtained at a scan area of 105×105 and 20×20 μm², respectively. F-d curves 1 and 2 correspond to regions shown as points A and B on the topographical images in (a). Curves 3 and 4 correspond to the topographical image in (b). Curves 5 and 6 show distinct features marked as ‘X’ and curves 7 and 8 show adhesive components (red curves).
Curves 2 show F-d data obtained on the base of the culture dish (blue curve), and for regions ‘A’ and ‘B’ on the cell membrane shown in figure 13 (a). The curves clearly show that region ‘A’ is significantly more compliant than region ‘B’ (ca. 600 nm difference in indentation between curve ‘A’ and ‘B’). The topographical image of region B shows a higher concentration of cytoskeletal fibres. Haga et al. [2000] carried out a study on mouse fibroblasts of the influence of the actin filaments, intermediate filaments and microtubules by correlating AFM cell elastic responses with confocal laser scanning microscopy (CLSM). They found that regions with high density of actin filaments correlated with higher cell stiffness, regions with the highest concentration of microtubules showed low Young’s modulus values whereas the density of intermediate filaments seemed to show a correlation with cell stiffness. Other work [Bushell et al. 1999, 2000] has also shown that higher concentrations of cytoskeletal fibers contribute to greater cell stiffness. Tsai et al. [1998] have also concluded that microtubules have little influence on the mechanical properties on a neutrophils cell line and concluded that actin filaments are the primary structural determinants of neutrophil mechanical properties.

Curves 3 and 4 correspond to F-d measurements obtained on the cell regions shown in figure 13 (b). Curve 3 demonstrates full indentation whereby the tip apex compresses the cell membrane resulting in the tip coming into close contact with the underlying culture dish surface (see also figure 3). This is also demonstrated on region ‘B’ in curve 4, however in this case the full indentation is obtained at a higher force loading (ca. 11 nN as compared to ca. 5 nN for curve 3); a consequence of the cell thickness at the two locations. F-d curves exhibiting contact or near contact with the cell surface and the underlying substrate as shown in curves 3 and 4 can provide a reasonably accurate measurement of the cell height in the unloaded state.

Curves 5 and 6 show distinct structure along the approach curve (at locations marked “X”). These F-d curves were obtained at a location on the cell surface where little apparent structure was evident from the topographical image. The F-d response of the tip in these cases may be the result of membrane penetration or movement of internal cell components on a time scale comparable with the speed of approach of the tip during data acquisition.

Adhesive effects (shown in red) of the F-d data are shown in curves 7 and 8. When adhesion is measured in air, the interaction is predominantly a result of a meniscus layer. This is eliminated when the experiments are carried out in a fluid environment. The adhesion shown here is a result of the interaction between the tip and the cell membrane (which includes van der Waals forces and any electrostatic interactions). Curve 7 shows the result of carrying out F-d analysis using a contaminated tip. In F-d curve number 8, however, the retract curve is characterised by a series of prominent jumps. Afrin et al [2004] have observed a similar effect on 3T3 fibroblast cells with functionalised tips. They concluded that the effect is probably the result of stretching and removal of the cell membrane proteins.

4.4.2. MDA Cells (Breast cancer Cells)

Figure 14 shows topographical images of an MDA cell with the various locations of F-d analysis indicated on the two images by labels A (representing the hard incompressible culture dish substrate) to G. F-d curves 1 to 3 reveal the extent of indentation at varying locations on the cell membrane. In curve 1, the extent of indentation at a force of ca. 3 nN was found to be ca 1300 nm. In curve 2, the values at points C and D, for a force of ca. 7 nN, were found to be ca. 1200 and 1500 nm, respectively. At points E, F and G, the indentations
at a force of ca. 12 nN were found to be 800, 900 and 1300 nm, respectively. The Young’s modulus inferred from locations A and D (curve 2) was found to be ca. 2.5 kPa. This value is generally lower than values obtained on fibroblast cells (see also table 2).

![Topographical image showing a breast cancer cell (MDA) with points A to G denoting the locations of F-d curve acquisition. F-d curves 1 to 3 reveal the extent of cellular deformation/indentation.](image)

Figure 14. Topographical image showing a breast cancer cell (MDA) with points A to G denoting the locations of F-d curve acquisition. F-d curves 1 to 3 reveal the extent of cellular deformation/indentation.

5. **FUTURE PROSPECTS**

The emphasis in this chapter is to describe those applications in the context of analysis of whole cells that are uniquely suited to the technique. While it is rather more hazardous to speculate about the future, than describing the past, one might guess that the future developments for AFM will build on those attributes. For instance, greater insight into cell
dynamics through continuous monitoring of live cells would seem to be an area of great promise. Indeed one might see the system emerging as a future test bed for evaluating the response of live cells to new drugs, or in response to other physico-chemical variables in the local biocompatible fluid environment. Likewise, AFM-based nano-scale manipulation of cells has the great merit of being possible in combination with simultaneous imaging.

Current trends in instrumentation are likely to promote and enhance new applications in AFM analysis of live cells. For instance, while past generations of instruments tended to be general purpose multi-technique platforms, the next generations are likely to be optimised for particular requirements. Thus more user-friendly dedicated bio-AFM systems will become available in the near future. Similarly next-generation instruments will have much faster electronics, in combination with high-resonance probes, thus allowing imaging at TV scan rates, and much improved F-d data acquisition rates. While new operational modes were appearing once a month in the early days, the pace of invention has now slackened somewhat. Nevertheless new and clever ideas will emerge from time to time, and some of those will have a significant impact in the field of cellular biology.

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