Review

Atomic force microscopy for university students: applications in biomaterials

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Abstract

Atomic force microscopy (AFM) is a powerful tool used in the investigation of the structural and mechanical properties of a wide range of materials including biomaterials. It provides the ability to acquire high resolution images of biomaterials at the nanoscale. It also provides information about the response of specific areas under controlled applied force, which leads to the mechanical characterization of the sample at the nanoscale. The wide range of information provided by AFM has established it as a powerful research tool. In this paper, we present a general overview of the basic operation and functions of AFM applications in biomaterials. The basic operation of AFM is explained in detail with a focus on the real interactions that take place at the nanoscale level during imaging. AFM’s ability to provide the mechanical characterization (force curves) of specific areas at the nanoscale is also explained. The basic models of applied mechanics that are used for processing the data obtained by the force curves are presented. The aim of this paper is to provide university students and young scientists in the fields of biophysics and nanotechnology with a better understanding of AFM.

Keywords: atomic force microscopy (AFM), biophysics, nanoscale imaging, force spectroscopy, nanoscale mechanics

(Some figures may appear in colour only in the online journal)
1. Introduction

Atomic force microscopy (AFM) is part of the scanning probe microscopy branch of microscopy [1], and its operation relies on the interaction of a small tip located on the edge of a sensitive cantilever with a sample surface at the nanoscale level. AFM was developed to image nonconducting samples [2], with the basic goal of improving imaging resolution analysis over that obtained with optical microscopy. However, AFM has proven to be a powerful instrument that is able to provide a wide range of information apart from imaging. In particular, force spectroscopy measurements combined with high resolution imaging techniques provide the ability to completely characterize a sample surface at the nanoscale. In addition, AFM has a major advantage compared with other microscopy techniques (i.e. scanning electron microscopy (SEM) and transmission electron microscopy (TEM)), which is its ability to acquire high resolution images of native biological samples in environmental or liquid conditions (i.e. avoiding sample preparation which is often responsible for artifacts) [2].

Today, there are numerous applications of AFM, ranging from the imaging of the smallest biomolecules to the mechanical characterization of materials and biomaterials [2–14]. In this paper, the basic operation of AFM for the imaging and mechanical measurement of biological samples is presented. The aim of this paper is to provide a wide range of information regarding the physical principles of the operation of AFM suited to university students and young scientists in the fields of biophysics and nanotechnology. Firstly, the basic parts of an AFM apparatus are explained. Then, the imaging modes are explained in detail with a focus on the physical principles of the interactions that occur at the nanoscale during the imaging procedure. AFM images from real AFM experiments are presented. In addition, AFM’s ability to provide mechanical characterization of specific areas at the nanoscale is presented (force spectroscopy), and the basic contact mechanics models used to analyze AFM data are discussed. Furthermore, typical force curves and Young’s modulus maps obtained on biological samples are shown. The wide range of information obtained using AFM in biophysics and explained in this paper will contribute to the literature available for university students and young scientists who want to understand basic AFM theory and techniques.

1.1. Basic operation

The basic operating principle of AFM is based on a sharp tip that is mounted on the edge of a flexible cantilever in order to scan a sample surface [3, 15]. The tip comes in contact with the sample and ‘grazes’ the sample, like the old fashion record-player needles in gramophones. The motion of the sample relative to the tip is controlled using a piezoelectric actuator with sub-Angstrom accuracy. During the scanning procedure, the cantilever deflects and this motion which is based on the tip–sample interaction is monitored by a laser beam pointed at the back of the cantilever (figure 1). The laser beam is reflected towards a photodetector in order to record the motion of the cantilever towards and away from the sample surface during scanning. In addition, a feedback circuit which is connected to the deflection of the cantilever sensor is responsible for maintaining the tip–sample surface interaction at a constant level by controlling the tip–sample surface’s relative distance [16]. The amount of the feedback signal at each scanning point is measured in order to construct a 3D image that represents the sample’s surface topography. Consequently, AFM systems generate images by ‘feeling’ the surface, rather than ‘looking’ at the samples like other types of microscopes do (e.g., optical microscope, SEM) [17, 18].
1.2. Force versus distance

Forces arise during the interaction between the tip and sample surface. At large distances the resultant force is attractive (van der Waals force) [19]. Conversely at small distances the resultant force is repulsive (overlapping of electron orbitals between the tip and sample) [19]. These forces can be approximated using the Lennard–Jones potential (figure 2) [20]:

\[ V(d) = 4\varepsilon \left[ \left( \frac{d_e}{d} \right)^{12} - \left( \frac{d_e}{d} \right)^6 \right] \]  

(1)

Figure 1. The basic operation of AFM. A sample surface is being scanned by a tip mounted on the edge of a cantilever. The deflection of the cantilever is being monitored using a laser beam.

Figure 2. The Lennard–Jones potential. When \( d > d_{eq} \) the resultant force is attractive and when \( d < d_{eq} \) the resultant force is repulsive. When \( d = d_{eq} \) the total force is zero, \( \Sigma F = 0 \).
where, $V$, $d$ is the intermolecular potential and the distance between the two atoms or molecules, $\varepsilon$ is the well depth (a measure of how strongly the two particles attract each other) and $d_{eq}$ is the distance at which $V = 0$.

When the separation distance is $d = d_{eq}$ (where $d_{eq}$ is the equilibrium distance) the total force is zero (figure 3).

The forces that are applied between the tip and the sample surface are presented in figure 3. When $d > d_{eq}$ the resultant force is attractive and when $d < d_{eq}$ the resultant force is repulsive. The diagram in figure 3(a) approximates the interaction forces between the tip and the sample surface. In figure 3(b) the forces applied on the AFM tip in three different regions are presented.
If, \( d > d_{eq} \), \( F_T = F_R - F_A < 0 \)

If, \( d = d_{eq} \), \( F_T = F_R - F_A = 0 \)

If, \( d < d_{eq} \), \( F_T = F_R - F_A > 0 \)

where, \( F_R \) is the repulsive force and \( F_A \) the attractive force and \( F_T \) the total force on the tip.

### 2. AFM imaging modes

Depending on the experiment and the sample that is being scanned, AFM can work in three basic imaging modes: contact, tapping and noncontact. The force between the tip and the sample surface defines the operation mode. Generally, the force between the AFM tip and the sample surface will be repulsive (if the tip–sample distance is less than 1 nm) or attractive (if the tip–sample distance >100 nm). Hence, when the force between the tip and the sample surface is continuously repulsive, and the tip is always in contact with the sample, the operation mode is contact mode. Conversely, if the force between the tip and sample is continuously attractive, and the tip never touches the sample surface, the operation mode is the noncontact mode. Furthermore, there is an additional mode, the intermittent mode or tapping mode in which the cantilever is oscillated at a frequency near its resonance and the net force is attractive or repulsive depending on the tip’s position.

#### 2.1. Contact mode

The first imaging mode that was developed is the contact mode [21], in which the tip is always in contact with the sample surface (figure 4(a)). In contact mode, the cantilever’s deflection remains constant (hence, the applied force on the sample surface is always constant) during the scanning procedure. The resultant force on the sample is repulsive [22], despite the attractive forces on the tip due to the presence of a thin water layer on the sample. In order to minimize the attractive forces due to the water layer, the contact mode can be applied in an aqueous environment.
The contact mode is the best choice for imaging flat and rigid surfaces. However, image artifacts cannot always be avoided (e.g., surface tilt) and can dominate over the characteristics of the surface topography. The possibility of the domination of imaging artifacts increases when large areas (e.g., 100 × 100 μm) are being scanned or when the imaging rate is too slow (e.g., 0.5 Hz). However, low-frequency scanning artifacts can be avoided using the error mode, in which the cantilever deflection signal is recorded in order to maintain the fast response of the feedback \[2, 23\]. The error mode provides information about how well the desired applied force (i.e., the desired cantilever deflection) is maintained during imaging. In the contact mode the user can obtain height images, error or deflection images and lateral force images (see figure 4(b)). The deflection images are formed by the error signal, which is the difference, measured in volts by the photodetector, between the setpoint and actual cantilever deflection. On the other hand, the lateral force images (some manufacturers refer to this type of image as a friction image) are formed by the cantilever’s twisting motion when the tip passes over regions of varying friction. The contrast in lateral force images may be due to changes in frictional properties across the sample or by variations in topography.

2.2. Tapping mode

The aim of the tapping mode is to minimize sample surface damage on soft compliant samples \[10\]. Its operation is based on the monitoring of the oscillation amplitude. Firstly, the cantilever is oscillated near its resonance frequency with an initial value of amplitude that is damped when the tip touches the sample surface. In addition, the resonance frequency is modified by the tip–surface interaction (the increase in resonance frequency).

The recording of the feedback signal allows for maintaining a constant value to the tip’s oscillation amplitude in order to obtain the topography of the sample. In the tapping mode, the tip is not continuously in contact with the sample surface (figure 5), thus the possibility of damaging the sample is significantly reduced.

The tapping mode is the most appropriate mode for imaging soft biological samples with high resolution.

In the tapping mode topography and phase images are simultaneously acquired to obtain information on the various properties of the sample \[17\]. In phase imaging the detector signal is in phase \(\varphi\) of the cantilever oscillations relative to the phase of the drive signal \[10\]. AFM phase imaging goes beyond topographical features and it is the appropriate method for detecting variations in the composition, friction and viscoelasticity enhancing contrast of heterogeneous samples \[10, 24\].

In figure 6, the same area of a collagen gel is shown imaged using the contact mode (a) and the tapping mode (b).
2.3. Noncontact mode

In noncontact mode the applied force on the tip is attractive because the tip is not in contact with the sample (figure 7). The cantilever is oscillated near its resonance frequency under the influence of an alternating voltage due to a piezoelectric element. During the relative movement of the sample surface toward the tip the oscillation amplitude is reduced and the oscillation frequency of the cantilever changes due to the interaction forces between the tip and sample.

Figure 6. Contact mode imaging (a) versus tapping mode imaging (b). The two images are of the same collagen gel imaged using the contact mode (a) compared with it imaged using the tapping mode (b). The images were taken with a Cypher ES, Asylum, Oxford Instruments microscope with CSG10m NT-MDT probes.

Figure 7. Noncontact mode. (a) The cantilever is oscillated near its resonance frequency. (b) During the relative movement of the sample surface toward the tip the oscillation amplitude is reduced and the oscillation frequency of the cantilever changes due to the interaction forces between the tip and sample.
movement of the sample surface toward the tip, the oscillation amplitude is reduced and the oscillation frequency of the cantilever changes due to the interaction forces between the tip and the sample (decrease of the resonance frequency). However, during the imaging procedure the oscillation frequency remains constant. The interaction forces between the tip and the sample are smaller than those in the contact mode. The noncontact mode finds application in very soft samples (e.g. biological samples) in order to avoid scratching the sample surface. In addition, it must be noted that the noncontact mode is mostly applied in a high vacuum environment \[25\]. Despite the fact that the noncontact mode can provide valuable results \[26\], it is not used in air because the noncontact conditions can barely be maintained due to the presence of a surface contamination layer.

2.4. Applied forces in the AFM imaging modes

The choice of AFM imaging mode is made depending on the sample being tested. For a nonbiological sample, the contact mode can be used which is easily applied and there in no possibility of harming the sample. For very soft biological samples the noncontact mode is the preferred choice. However, the limitations (as mentioned in section 2.3) of the noncontact mode have led to the use of the tapping mode for biological samples. In the contact mode, the tip is always in contact with the sample surface and the resultant force is repulsive. In the noncontact mode, the tip is never in contact with the sample during the imaging procedure. Hence, the interaction force between the tip and the sample is attractive. In the tapping mode, the tip alternately moves toward the sample, making contact with it, and then moves away from the sample; thus depending on the tip–sample distance the net force can be attractive or repulsive. In figure 8, the net force in relation to distance is presented. In the diagram the blue box shows the range of the interaction forces in the contact mode and the pink and green boxes the range of the interaction forces in the noncontact and the tapping modes, respectively.

\[\text{Figure 8. Force–distance diagram. In the contact mode, the interaction force between the tip and the sample surface is repulsive. In the noncontact mode the interaction force is attractive and in the tapping mode the interaction can be attractive or repulsive depending on the distance between the tip and the sample surface.}\]
3. Force spectroscopy

3.1. Force–displacement curves

AFM provides the ability to determine the mechanical properties of a sample using force spectroscopy [12, 14, 27]. Force spectroscopy is performed by moving the sample up and down towards the tip in order to measure the cantilever’s corresponding deflection. As a result a cantilever deflection–displacement curve can be created at a single location on the tested sample surface. The curve is created by measuring the bending of the cantilever during the up and down movement of the sample. The deflection of the cantilever leads to the calculation of the applied force on the sample surface, using the simple equation \( F = kd \), where \( F \) is the applied force, \( k \) is the spring constant of the cantilever and \( d \) is the deflection of the cantilever. The force–displacement curve (figure 9) has two curves, the loading curve (the movement of the tip towards the sample) and the unloading curve (the movement of the tip away from the sample).

The right side of the curve (in which \( F = 0 \), the cantilever is undeflected) represents the area in which the AFM tip is far away from the sample surface (i.e. the scanner tube is fully retracted). As the sample moves towards the tip, the tip ‘jumps into’ the sample due to a thin layer of humidity on the sample surface which is responsible for a negative (attractive) net force on the tip (snap-in point in figure 9). The sample moves towards the tip and continues until the cantilever is bent away from the tip (repulsive net force) and the deflection reaches the selected value (maximum applied force). After the full extension of the scanner, it begins to move in the opposite motion (i.e. to retract). The separation between the sample surface and the tip occurs at the snap-out point in which the net force is negative (the thin layer of humidity on the sample surface applies a strong attractive force on the AFM tip). It must be noted that the horizontal offset between the loading and the unloading curves is a result of

![Figure 9. A force–displacement curve obtained on a hard sample in air. The loading part of the curve represents the movement of the sample towards the tip. The unloading part represents the opposite movement of the sample (away from the tip). The snap-in point represents the first contact of the tip with the sample surface (loading curve). The snap-out point represents the point in which the tip separates from the sample (unloading curve).](image-url)
scanner hysteresis. In addition, the shift between the loading and the unloading curves can also be the inverse of that depicted in figure 9. It must also be noted that the approach and retraction curves may not coincide due to mechanical behaviors of the sample, i.e. plasticity, viscoelasticity, viscoplasticity etc.

In addition, it must be noted that the graph in figure 9 represents a force–displacement curve obtained on a hard sample in which no indentation occurs. However, in many samples (e.g. biological samples) the motion towards the tip (z) is larger than the deflection of the cantilever (d), due to the indentation. The relationship between the piezo movement of the sample and the indentation (h) is \( z = h + d \) (figure 10). Hence the difference between the piezo movement of the sample and the cantilever deflection is equal to the indentation depth.

In practice, in order to calculate the indentation depth for a specific applied force, a hard reference sample must firstly be used in which no indentation occurs. The reference sample must be several orders of magnitude stiffer than the tip, in order not to become indented. Using a reference sample the piezo movement is equal to the deflection of the cantilever since the indentation depth is zero (\( h = 0 \)). In addition, the force–displacement curve on a stiff sample is linear (the applied force is proportional to the piezo movement of the sample, since \( z = d \)).

After the acquisition of the linear curve on the hard reference sample, a force–displacement curve must be obtained on the soft sample of interest. The indentation depth for every applied force value can be obtained by creating a force–indentation curve. The difference between the piezo-displacement for the hard and the soft samples in order to succeed the same deflection of the cantilever results in the indentation depth (figure 11) [12, 27].
The comparison of the force–displacement curves on the soft and the stiff sample is generally carried out using the unloading curves \[5, 6, 27\]. Deformation during loading can potentially be both elastic and plastic. However, during unloading, only the elastic displacements are recovered (the elastic nature of the unloading curve facilitates the analysis) \[6\].

A hard sample (AFM grating), a biological sample (collagen fibril) and the unloading force–displacement curves (obtained on the hard sample and on collagen, black and red curves, respectively) are presented in figure 12.

### 3.2. Application of the contact mechanism theory in AFM experiments

As was presented in section 3.1 the AFM tip can be used as an indenter in order to apply controlled vertical force to a sample surface \[4, 12, 27\]. Depending on the tip’s shape and the indentation depth, the AFM tip can be approximated as a solid sphere, cone or paraboloid of revolution \[12, 28, 29\]. Hence, the classic theory of the Hertz model \[9, 13, 30–33\] can be approximately applied and provide valuable results. The Hertz model (which is the most widely applied model) was firstly established to describe the contact between two spherical elastic bodies. However, several modifications have been made in order to include other basic
The most basic modification was the consideration of the sample as an elastic half space. In addition, in the case of a rigid cone, indenting a soft flat surface (elastic half space), the Sneddon model can be applied. In most AFM experiments, the AFM tip radius is several orders of magnitude smaller than the dimensions of the sample. Hence, the interaction between the tip and the sample can be considered as being the interaction between the indenter and an elastic half space.

In addition, the application of the Hertz model is valid under the following assumptions:

1. The sample is isotropic and homogeneous.
2. The contact between the tip and the sample is adhesionless and frictionless.
3. The contact geometry is assumed to be axisymmetric, smooth and continuous.

3.2.1. Assumption of AFM tip as a sphere. Spherical AFM tips (figure 13), like borosilicate glass spheres, can be used for indentation experiments, mostly on biological samples (e.g., cells, articular cartilage). In addition, if the AFM tip has a pyramidal geometry but the indentation depth is small compared with the tip radius, the AFM tip can be approximated as a sphere.

The relationship between the applied force and the indentation depth is provided by the equation:

\[ F = \frac{4}{3} E^* R^{1/2} h^{3/2} \]  

where, \( F \) is the applied force, \( E^* \) is the combined elastic modulus of the contacting bodies, \( R \) is the tip radius and \( h \) the indentation depth. In addition,

\[ \frac{1}{E^*} = \frac{1 - v_i^2}{E_i} + \frac{1 - v^2}{E} \]  

where, \( E_i \) and \( v_i \), and \( E \) and \( v \), represent the elastic modulus and Poisson ratio of the indenter and the specimen, respectively.
However, providing that the tip is several orders of magnitude stiffer than the sample, equation (3) can be expressed in the form:

\[
\frac{1}{E^*} = \frac{1 - \nu^2}{E}.
\]  

(4)

Hence, equation (2) is transformed as follows:

\[
F = \frac{4ER^{1/2}h^{3/2}}{3(1 - \nu^2)}.
\]  

(5)

Young’s modulus can be easily calculated using the linearized Hertz model [8, 34, 35]. The slope $S$ of the linear curve $F^{2/3} - h$ leads to the calculation of the Young modulus value (figure 14). The linearized force–indentation curves are expressed by the equation:

\[
F^{2/3} = \left[ \frac{4ER^{1/2}}{3(1 - \nu^2)} \right]^{2/3} h \Rightarrow F^{2/3} = Sh
\]  

(6)

where $S = \left[ \frac{4ER^{1/2}}{3(1 - \nu^2)} \right]^{2/3}$ is the slope of the linear curve $F^{2/3} - h$. In conclusion, Young’s modulus is calculated by the equation:

\[
E = \left[ \frac{3(1 - \nu^2)}{4R^{1/2}} \right]S^{3/2}.
\]  

(7)

A similar analysis can be done when considering the tip as a paraboloid of revolution (figure 15). If the indentation depth is much smaller than the tip radius, Young’s modulus can be calculated using the same equations [27, 29, 36].

3.2.2. Assumption of AFM tip as a cone (Sneddon model). Most of the tips used in AFM experiments have pyramidal geometry [5, 6, 10, 12, 27–29, 34, 35, 39]. If the indentation depth is high, the pyramidal tip can be approximated to a cone (figure 16), which results in the same projected area to the pyramidal tip.
The relationship between the applied force and the indentation depth (when the tip is several orders of magnitude stiffer than the sample) is provided by the equation [37, 38]:

\[
F = \frac{2E (\tan \theta) h^2}{\pi (1 - \nu^2)}
\]  

(8)

where, \( F \) is the applied force, \( E \), \( \nu \) are the elastic modulus and Poisson ratio of the sample, respectively, \( \theta \) is the cone half-angle and \( h \) is the indentation depth. Using equation (8) Young’s modulus of the sample can be easily determined.
3.2.3. Calculation of Young’s modulus using a general equation. Young’s modulus can be also calculated using the basic formula [5, 6, 12, 27, 31]:

\[ E = \frac{\sqrt{\pi}}{2} \frac{S}{\sqrt{A_c}} \]  

(9)

where, \( E \) is Young’s modulus, \( \nu \) the sample’s Poisson ratio, \( S \) the contact stiffness and \( A_c \), an area function related to the effective cross-sectional or projected area of the indenter. The contact stiffness can be determined by the slope of the upper unloading part of the force–indentation curve, thus \( S = \frac{dF}{dh} \bigg|_{h_{\text{max}}} \) [5, 6, 12, 27, 31]. Equation (9) can be applied in every case of a rigid smooth frictionless axisymmetric indenter [31] (regardless of the indenter’s shape). However, it must be noted that the projected area depends on the tip’s shape.

For spherical indenters [29]:

\[ A_c = \pi R^2 (2R - h_c). \]  

(10)

For conical indenters [39]:

\[ A_c = \pi h_c^2 \tan^2 \theta. \]  

(11)

The depth at which contact is made between the indenter and the sample during indentation is defined as contact depth \( h_c \) [5, 6, 12, 27, 31].

\[ h_c = h_{\text{max}} - \varepsilon \frac{P_{\text{max}}}{S}, \]  

(12)

where \( h_{\text{max}} \) is the maximum indentation depth and \( \varepsilon \) is a constant that depends on the indenter geometry. For conical geometry \( \varepsilon = 0.72 \) and for a paraboloid of revolution geometry \( \varepsilon = 0.75 \) [31]. The contact depth is illustrated in figures 13, 15, 16.

In addition, it must be noted that the typical Poisson ratio \( \nu \) of a solid material is in the range of \( 0 < \nu < 0.5 \). However, the uncertainty of the exact value of the Poisson ratio provides uncertainty in the Young modulus calculation. Hence, in order to avoid the uncertainty of the exact value of Poisson’s ratio, the reduced modulus, \( E^* = \frac{E}{1 - \nu^2} \), can be calculated.

4. Young’s modulus maps

4.1. Force volume mode

Force spectroscopy can also be extended to the so-called force volume mode. In this mode the force–displacement curves are obtained from a large number of points in the region of interest. After acquiring the force–distance curves the Young’s modulus for each point can be calculated and Young’s modulus values for every point can be presented in a Young modulus map (figure 17) [40].

4.2. Force scanning method

A Young modulus map can alternatively be created using the force scanning method [8, 34, 35]. In the force scanning method, a specific area is scanned several times with different setpoint forces. Each image obtained is characterized by \( z \)-values (which represent
the height of each point). The indentation values are calculated by subtracting the $z$-values from an arbitrary contact–point height $z_0$ [8]. Hence, the creation of arrays that consist of indentation values at every point of the scanned area leads to the calculation of Young’s modulus values at each point of the image (i.e. the creation of the Young modulus map). The Young modulus value for each point is calculated using the linearized Hertz model as was presented in section 3.2.1.

5. Advanced techniques

5.1. Peak force tapping mode

The operation of the peak force tapping mode is similar to the tapping mode due to the fact that the lateral forces are avoided by intermittent contact with the sample. However, it must be noted that its operation is in the nonresonant mode. Its major advantage is that it combines the benefits of tapping and contact mode imaging (i.e. control of direct force and avoidance of lateral forces) [44]. The oscillation in the peak force tapping mode is performed at all frequencies well below the cantilever resonance. A typical frequency for the probe oscillation is 2 kHz (which is far below the resonant frequency of the cantilever). Typical peak to peak amplitudes in air are $\sim$300 nm [45]. The probe is periodically in contact with the sample surface for a short time period ($<100 \mu s$) [45]. Hence, a periodic force is applied onto the sample surface, and individual force–separation curves can be created and collected for each ‘tap’ of the tip onto the sample surface [46]. The innovation of the peak force tapping mode is based on the control of the peak force (maximum normal force) applied on the sample surface at each point. Using the feedback system, the maximum applied force is kept constant during scanning. The data on adhesion forces, surface deformation and topography are separated from the individual force–separation curves at each point. The principles of the force–separation curves [46] are comparable to the force–displacement curves for the Young modulus calculation in the classic nanoindentation procedure. For the estimation of the

![Figure 17. Topography and Young’s modulus map of cancer-associated fibroblasts. The images were acquired with a PicoPlus, Molecular Imaging/Agilent microscope, in the contact mode under phosphate-buffered saline (PBS) with SICON, Applied NanoStructures. The data processing was accomplished by the open source software called AtomicJ, an open source software for the analysis of force curves [41].](image-url)
mechanical properties of the sample using the peak force tapping mode, the Derjaguin–Muller–Toporov (DMT) model [45] can be used and leads to the estimation of the sample’s reduced modulus which is described by the following equation:

$$F_{\text{INT}} = \frac{4}{3} E^* R (d - d_0)^3 + F_{\text{ADH}}$$

where $F_{\text{INT}}$ is the tip–sample force, $E^*$ is the combined reduced modulus (of the tip and the sample), $R$ is the tip radius, $d_0$ is the resting position of the surface, $d - d_0$ the deformation of the sample and $F_{\text{ADH}}$ the adhesion force during contact.

5.2. Amplitude modulation-frequency modulation (AM-FM) viscoelastic mapping

AM-FM viscoelastic mapping (hereinafter referred to as AM-FM) is a new and advanced AFM technique. This technique is used for the nanomechanical characterization of samples and it can be applied to soft surfaces such as biomaterials. AM-FM is a dynamic force-based method that has its roots in research on multifrequency and bimodal AFM [47, 48]. In this technique the AFM probe is excited by two sinusoidal signals at the resonant flexural frequencies of the first and the second mode. Each mode can be operated in either AM or FM [49]. Consequently, this technique operates at two cantilever resonances at once and can simultaneously map the topography and the nanomechanical properties of soft-matter surfaces [50]. As a result it provides a wide range of mechanical information, such as Young’s modulus and contact stiffness. AM-FM works like the tapping mode and as a result it is characterized by the benefits and advantages of the tapping mode including fast scanning, high spatial resolution and gentle forces. Although AM-FM is only provided by a few AFM manufacturers (i.e. Asylum Research) and is very new, it has already demonstrated that it can be a very powerful tool for mechanical characterization at the nanoscale [51, 52]. Figure 18 illustrates the topography and the Young modulus map of a collagen gel imaged in air using the AM-FM technique. Different Young’s modulus values on different nanoregions of the collagen gel are clearly observed.

![Figure 18. AM-FM imaging. Topography (left) and Young’s modulus map (right) of collagen gel, respectively (the images were taken with a Cypher ES, Asylum, Oxford Instruments microscope with AC160TS-R3, Olympus probes).](image-url)
6. Force spectroscopy of biological samples and biomaterials

AFM has been extensively used for the mechanical characterization of biomaterials at the nanoscale. The determination of the Young modulus of biomaterials and biological samples like collagen, cells or articular cartilage under various conditions is of great importance to biophysics research. Hence, typical results from the literature are provided in the next sections. In addition, typical Young’s modulus values of collagen, cells and articular cartilage under various conditions are presented in table 1.

6.1. Collagen

The importance of collagen in biophysics and biomaterials research is great due to the amount of collagen in mammals. Specifically, most parts of the mammalian body (e.g., tissue, skin, bone, cartilage, or tendons) contain collagen in the form of collagen fibrils. Hence, the mechanical characterization of collagen at the nanoscale is crucial. Many researchers have calculated Young’s modulus of collagen fibrils. In particular, Jolandan et al [42] investigated the mechanical heterogeneity of type I collagen fibrils due to D-band periodicity. The average values were ∼2.2 GPa and ∼1.2 GPa for the overlapping and gap regions, respectively. Furthermore, the resulting alteration of the Young modulus values between the gap and overlapping regions was confirmed by Kontomaris et al [27] who estimated that Young’s modulus of the overlapping and gap regions of type I collagen fibrils are in the range of 0.74–1.43 GPa due to the mechanical heterogeneity of the collagen fibrils. Moreover, Strasser et al [13] calculated the average values of Young’s modulus of type I collagen fibrils at approximately 1.2 ± 1 GPa, while Yadavalli et al estimated Young’s modulus at 1.03 ± 0.31 GPa [43]. Furthermore, the mechanical properties of collagen fibrils have been studied by Heim et al who found average values of 1–2 GPa [14] and Wenger et al whose results were in the range of 5–11.5 GPa [29]. Due to the stiffness of collagen (small indentation depths) the most common approximation of the tip for the Young modulus calculation is the spherical approximation.

6.2. Cells

The determination of the mechanical properties of various types of cells is a critical issue in the area of biophysics. Many researchers have calculated Young’s modulus of various types of cells. In particular, Mathur et al determined the Young modulus variations of human umbilical vein endothelial cells in the range of 1–7 kPa [53]. Murakoshi et al calculated Young’s modulus of outer hair cells from the cochlea of mice at ∼2 kPa [54]. Furthermore, the age-dependent apparent elastic modulus of cardiac myocytes was estimated by Lieber et al in the range of 35–43 kPa [55]. Moreover, the values of human aortic endothelial cells were calculated as ∼1.5 kPa and ∼5.6 kPa for two distinct populations [56].

In addition, the determination of the mechanical properties of the cells under various conditions is a critical issue because it leads to the detection of pathological conditions like cancer. In particular a change in a cell’s Young’s modulus is a characteristic of cancer cells [57]. The alteration in Young’s modulus is of major importance because it affects the way that the cells spread [58]. The use of AFM has provided scientists with the ability to calculate Young’s modulus of metastatic cancer cells from patients with suspected breast, lung and pancreatic cancers [58, 59]. In particular, the cell stiffness of cancer cells can be ∼70% softer than that of benign cells, e.g., the Young modulus of cancer cells obtained from a woman detected with breast ductal adenocarcinoma was in the range of 0.50 ± 0.08 kPa. In contrast
Table 1. Typical measurements of Young’s modulus on collagen, cells and articular cartilage using AFM.

| Collagen | Young’s modulus | Cells | Young’s modulus | Articular Cartilage | Young’s modulus |
|----------|----------------|-------|----------------|---------------------|-----------------|
| Kontomaris et al \cite{27} (2015) | 0.74–1.43 GPa | Reference | Young’s modulus of fibroblasts with different levels of cancer transformation were found to be 40%–80% softer than that of the normal fibroblasts. | Loparic et al \cite{39} (2010) | Microscale 1.3 ± 0.4 MPa |
| Yadavalli et al \cite{43} (2010) | 1.03 ± 0.31 GPa | Li at al \cite{61} (2008) | Young’s modulus of cancer cells was found to be significantly lower (1.4–1.8 times) than that of the normal epithelial cells | | Nanoscale 22.3 ± 1.5 kPa (PG) 184 ± 50 kPa (Collagen) |
| Jolandan and Yu \cite{42} (2009) | ~1.2–2.2 GPa | Cross et al \cite{58} (2007) | Normal cells 1.93 ± 0.50 kPa Breast ductal adenocarcinoma 0.50 ± 0.08 kPa | Stolz et al \cite{64} (2009) | Microscale 2.3 ± 0.4 MPa |
| Wenger et al \cite{29} (2007) | 3.75–11.5 GPa | Costa et al \cite{56} (2006) | The values of human aortic endothelial cells were ~1.5 kPa and ~5.6 kPa for two distinct populations. | Patel et al 2003 \cite{62} | Intercellular matrix of articular cartilage |
| Murakoshi et al \cite{54} (2006) | | Outer hair cells from the cochlea of mice 2 kPa | | Tomkoria et al 2004 \cite{63} | Superficial zone: ~0.52 MPa, calcifying deep zone: ~1.69 MPa |
| Strasser et al \cite{13} (2007) | 1.2 ± 1 GPa | Lieber et al \cite{55} (2004) | The age-dependent apparent elastic moduli of cardiac myocytes were in the range of 35–43 kPa. | Stolz et al \cite{28} (2004) | Nanoscale 0.021 MPa |
| Mathur et al \cite{53} (2000) | | Human umbilical vein endothelial cells (HUVECs) with regional variations 1–7 kPa | | | Microscale 2.6 MPa |
to the above results Young’s modulus of the normal cells was calculated to be in the range of 1.93 ± 0.50 kPa [58]. Hence, mechanical analysis can distinguish normal cells from cancerous cells, even in cases where the normal and the cancerous cells have similar shapes. Moreover, Efremov et al studied the mechanical properties of cells with different levels of cancer transformation [60]. The transformed cells were found to be 40%–80% softer than the normal cells. Li et al investigated the Young modulus differences of breast epithelial cells and malignant breast epithelial cells using AFM. Young’s modulus of the cancer cells was found to significantly lower (1.4–1.8 times) than that of the normal epithelial cells at physiological temperature (37 °C) [61].

The most common approximation of the tip for the Young modulus calculation for cells is the spherical approximation; however, the conical approximation can be also used [58, 60].

6.3. Articular cartilage

Young’s modulus of articular cartilage can be also calculated using AFM. For example, it has been reported that the elastic modulus of the intercellular matrix of articular cartilage exhibits a depth-dependent increase from the articular surface (superficial zone: ∼0.52 MPa, calcifying deep zone: ∼1.69 MPa) [62, 63]. In addition, AFM is a powerful tool that can provide information regarding the functionality of articular cartilage and detect diseases such as osteoarthritis at the initial stages. The investigation of the mechanical properties of articular cartilage as a method of the diagnosis of diseases at early stages has been reported by many researchers [28, 39, 64]. Stolz et al [28] calculated Young’s modulus of normal articular cartilage at the microscale and nanoscale at 2.6 MPa and 0.021 MPa, respectively. Loparic et al obtained results in the same order of magnitude, in a case in which they calculated the microstiffness of articular cartilage at 1.3 ± 0.4 MPa and the nanostiffness at 22.3 ± 1.5 kPa (cartilage’s soft proteoglycan (PG) gel) and 184 ± 50 kPa (collagen meshwork) [39]. In addition, Stolz et al proved that in the early stages of osteoarthritis the microstiffness of articular cartilage remains constant. However, the early stages of osteoarthritis could only be detected at the nanometer scale. According to the Stolz research, nanostiffness reduces from 83 kPa (normal cartilage) to 5.6 kPa (grade 3 of osteoarthritis) [64]. Hence, the ability to detect early changes in the mechanical properties of cartilage using AFM at the nanometer scale has opened new prospects for the use of AFM as a clinical tool.

7. Conclusion

In this paper an overview of the basic operation and applications of AFM in biomaterials was presented. The physical principles of nanoscale interactions between the AFM tip and the sample surface were clearly explained. In addition, the ability of AFM for the mechanical characterization of a sample surface was presented. This paper will contribute to the literature as a tool that provides a coherent presentation of the basic operation and applications of AFM to help biophysics and nanotechnology students in understanding AFM.

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