Atomic Force Microscopy

A Tool to Unveil the Mystery of Biological Systems

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Real-time AFM, On-Substrate lysis, p53 DNA binding, bioscope, tapping mode, nucleus.

This article focuses on one of the promising and emerging nanolevel imaging techniques: Atomic Force Microscopy (AFM). In recent studies, AFM has been extensively used to understand intricate biological phenomena like prokaryotic and eukaryotic genome organization, different DNA transaction activities, protein chaperoning and also protein-nucleic acid organization in viruses.

Introduction

Biological systems are complex and are full of mysteries. Understanding function in relation to structure is crucial in many biological systems. A microscope (*Box* 1), which is used to magnify, resolve and visualize a substance that is impossible to see by naked eyes, plays a vital role in various biological studies. We can visualize macroscopic organisms like animals, plants, and insects by the unaided eye but not bacteria, viruses or individual proteins. Biological structures have sizes variable over such a wide range that it is not possible for a single microscopic technique to analyze all of them. Therefore, different types of microscopes with varying magnification and resolving abilities were developed to unveil the structural complexities of biomolecules (*Figure* 1). New technologies such as live-cell imaging and Fast Scanning AFM are proving to be invaluable in understanding function at the molecular level.

Over the last 100 years, several types of microscopes have been designed and used in various fields. The microscopes work on different principles, and have a broad range of applications. This article focuses on the usage of Atomic Force Microscope in the field of biology.

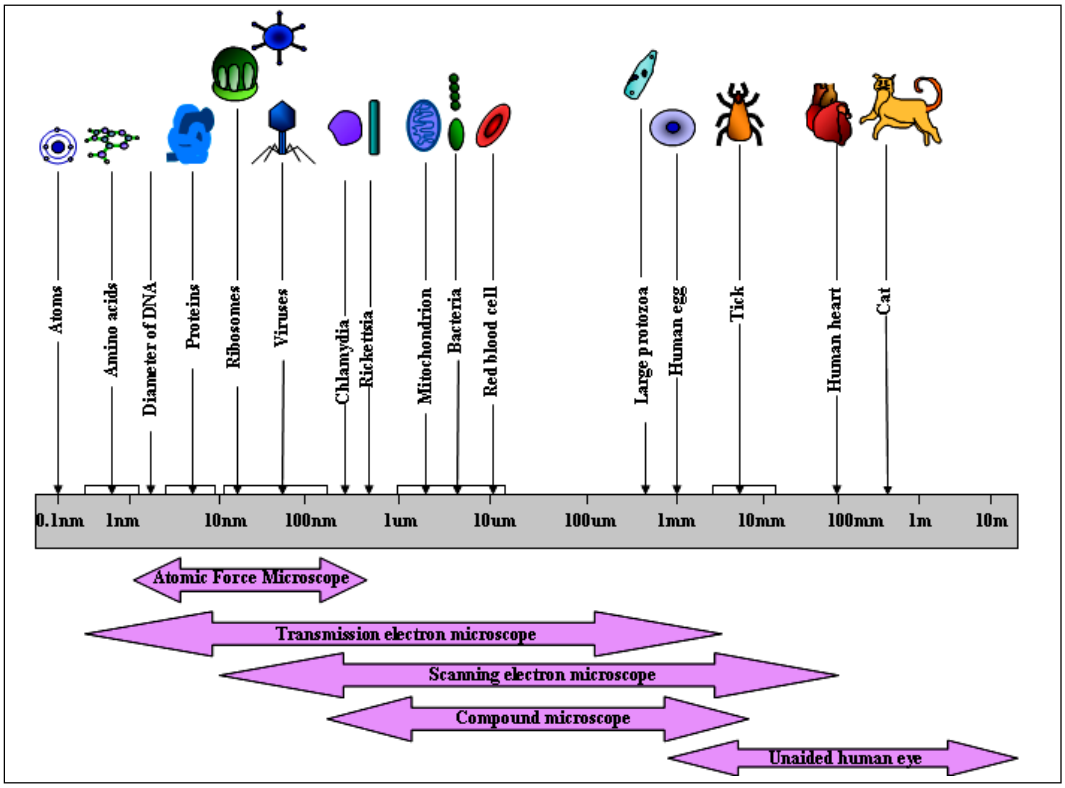
Box 1. Microscopy

Microscopy is the observation and examination of minute objects using a microscope, which will provide a magnified image of an object not visible to the naked eye. It can be divided into three main branches:

- Optical Microscopy
- Electron Microscopy
- Scanning Probe Microscopy (SPM)

Here, we will revise our concept on the principles of light microscopy. A light microscope is the most simplified form of a microscope which uses visible light and a combination of lenses to magnify tiny objects. It uses a very simple principle, where visible light transmitted or reflected from a sample through a lens gives a magnified view of the sample. It is of mainly two types, viz., Bright Field Microscope and Dark Field Microscopes. An optical microscope has a broad range of applications ranging from the field of biotechnology, nanophysics, pharmaceutical research, microelectronics and microbiology. It is also extensively used in medical diagnosis or histopathology analysis.

Figure 1. Magnifying range of different microscopes: The size of biological samples varies from several meters to few nanometers (right to left). Complex or large structures ranging from meters to millimeters can be easily visualized by normal human eye. Compound microscope can be used to see substances of lengths ranging from several millimeters to around 100 nanometers, while transmission electron microscope is used to magnify substances ranging in length between several millimeters and a few nanometers.



Inventors	Type of Microscope	Year
Zacharias Jansen	First Microscope	1590
Antoni van Leenwenhoek	Simple Microscope	1674
Richard Zsigmondy	Ultramicroscope	1903
Frits Zernike	Phase-contrast microscope	1932
Max Knoll and Ernst Ruska	Electron Microscope	1931
Gerd Binnig and Heinrich Rohrer	Scanning Tunneling Microscope	1981
Gerd Binnig and Quate	Atomic Force Microscope	1986

Table 1. A brief history of microscopes.

The history of the development of ultra high-resolution microscopes began with the invention of electron microscope in the 1930s (*Table* 1). It offered a new tool for biologists to study various biological substances at a resolution and magnification which was not achievable by other microscopic techniques at that time. Though the resolution achieved by electron microscope can be at the atomic level, the intrinsic contrast of biological materials was so low that either contrast-enhancing agents (i.e., negative stains or metal shadowing) or image averaging (cryo-EM) was often required. These drawbacks were overcome when scanning probe microscopy (SPM) was invented. SPM includes a large group of instruments used to image and measure properties of materials, chemicals and biological surfaces. The images are obtained by scanning across the surface with a sharp probe that monitors and assembles the tip–sample interactions to generate an image. There are two forms of SPM: Scanning Tunneling Microscope (STM) and Atomic Force Microscope (AFM) (*Figure* 2 and *Box* 2). STM was invented by Gerd Binnig and Heinrich Rohrer, in the early eighties for which they received the Nobel Prize in 1986. But the biggest limitation of STM is that it can only image materials that can conduct a tunneling current. Atomic Force Microscope (AFM) was invented by Binnig and Quate in 1986 to overcome the limitations of STM. AFM generates images, almost at the level of atomic resolution, by measuring the contour of the sample. The image is created by quantifying the forces between the probe (cantilever tip) and the sample

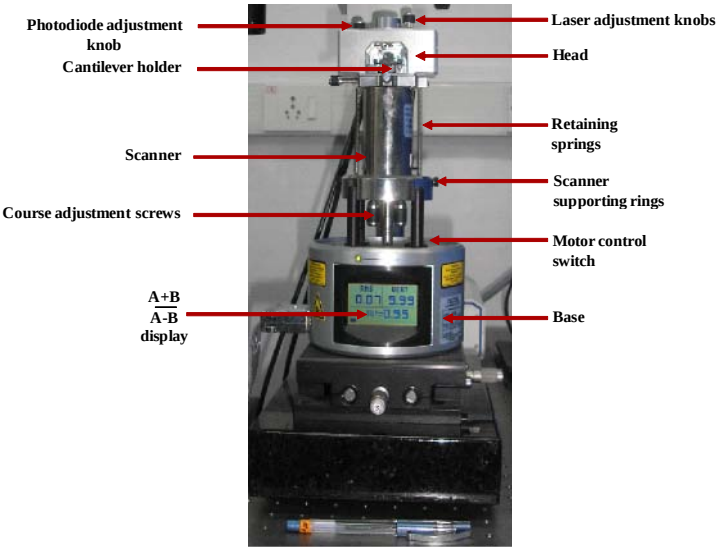


Figure 2. A modern AFM.

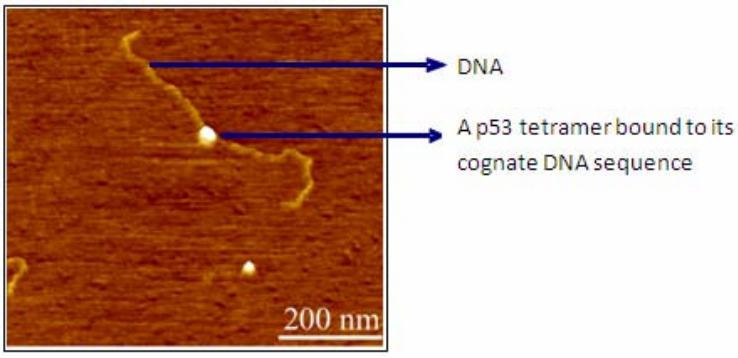
Box 2. Why named Atomic Force Microscope?

Atomic: AFM can achieve atomic resolution by using a sharp cantilever tip. Images obtained by AFM can distinguish two closely spaced atoms (but in reality it may not be always achievable).

Force: The image is created by quantifying the forces between the probe (cantilever tip) and the sample surface

Microscope: AFM amplifies the image of a sample.

For example:
Individual protein molecules are very small, and are not visible through naked eyes or even by using other light microscopic techniques. But the structural contour of the protein molecule can be imaged by using AFM. As we see here, the tumor suppressor protein p53, a 393KDa protein is impossible to be imaged by light microscopes, but AFM can be used to image four molecules of p53 bound to a small fragment of DNA. Here we can also comfortably see the DNA molecule.



Glossary

Adhesive force: Forces of attraction between a liquid and another surface.

Artifacts: Any visible result of a procedure which is caused by the procedure itself and not by the sample. It is a substance or structure not naturally present in the sample being observed but formed by artificial means, as during preparation of a microscope slide.

Cantilever is a small beam supported on only one end used especially made from silicon or silicon nitride. It has a tiny tip attached to the free end of the cantilever which contacts the sample. The cantilever scans over the sample surface and the image is obtained.

Chromatin is the nucleoprotein complex consisting of Histones, DNA, RNA and non-histone proteins present in eukaryotic cell nucleus.

Chromatin dynamics is the constant folding and unfolding of chromatin.

Cognate site is a specific binding site in the DNA sequence for a particular molecule.

Conglomerates are a combination or aggregation of several parts into one mass.

Dipole–dipole interactions are caused by permanent dipoles in molecules. When one atom is covalently bonded to another with different **electronegativity**, the electronegative atom draws the electrons in the bond nearer to itself, becoming slightly negative. Conversely, the other atom becomes slightly positive. Electrostatic forces are generated between the opposing charges and the molecules align themselves to increase the attraction.

Electron Microscope (EM): Electron microscope uses an electron beam to magnify very small objects, such as DNA and other cellular structures. The common types of electron microscope are Transmission Electron Microscope (TEM), Scanning Electron Microscope (SEM), Reflection Electron Microscope (REM), and Scanning Transmission Electron Microscope (STEM).

HeLa cells are immortal cell lines used in scientific research. It was derived from cervical cancer cells.

Newton is the SI unit of force. 1 Newton (N) is defined as the amount of force required to give a mass of 1Kg an acceleration of $1ms^{-2}$; 1 Newton (N) = $1Kg.m/s^2$.

Scanning Probe Microscopy (SPM) is a branch of microscopy that forms images of surfaces using a small cantilever that scans over the specimen.

Scanning Tunneling Microscope (STM) is a powerful instrument for imaging surfaces at the atomic level. Gerd Binnig and Heinrich Rohrer (at IBM Zürich) earned the Nobel Prize in Physics in 1986 for the development of STM. STM can image and manipulate individual atoms within materials. But, STM can only image materials that can conduct a tunneling current.

Spring constant is the extent of elasticity of a material. It is calculated by using the formula:

$$k=F/x,$$

where k= spring constant, F = force applied to the spring in N (Newtons), x = extension or compression of the spring in m (meters).

van der Waals force (or **van der Waals interaction**) is the attractive or repulsive force between molecules (or between parts of the same molecule) other than those due to covalent bonds or to the electrostatic interaction of ions with one another or with neutral molecules.

Virion is the single infective viral particle.

surface. AFM can provide extremely high-resolution images by using an ultra-small probe tip at the end of a cantilever. AFM was originally devised to study material surfaces; later it was modified for biological samples first by Marti and colleagues.

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Due to several advantages, AFM is being widely used as an imaging tool in biological studies:

i) High-resolution three-dimensional x, y and z (normal to the surface) images can be obtained. The resolution in the x–y plane ranges from 0.1 to 1.0 nm and in the z direction it is 0.001 nm (atomic resolution).
ii) AFM images are free of any artifacts while images obtained by EM consist of a large amount of artifacts which in several cases result in misleading conclusions.
iii) AFM requires neither a vacuum environment nor any special sample preparation. Instead, imaging can also be performed in liquid medium, which permits the samples to be analyzed in a near native condition.
iv) Sample preparation of AFM is comparatively simple and less time consuming.

Components of a Modern AFM (Nanoscope Multimode)

A modern nanoscope multimode consists of four instruments assembled together: nanoscope controller, a computer with a control and display, multimode, and two monitors.

The multimode is the most vital component of the AFM. It contains an optical head, a removable scanner and a multimode base. The head contains the probe, the laser and photodiode array, and the adjustment knobs that are used to align the system. Generally, the sample is placed on top of the scanner containing the piezotube which controls the movement of the sample. The base has controls for raising and lowering the probe, a mode selector switch and a digital display.

Principle of AFM

Both AFM and light microscope amplify the image of the sample

Both AFM and light microscope amplify the image of the sample but the major difference between them is that the former does not use visible light; instead it uses a cantilever made from silicon or silicon nitride having a very low spring constant to image a sample.

AFM records a three-dimensional image of the surface topography of the sample under a constant applied force which provides a maximum resolution image.

but the major difference between them is that the former does not use visible light; instead it uses a cantilever made from silicon or silicon nitride having a very low spring constant to image a sample. At one end of the cantilever, a very sharp tip (around 100–200μm long and 20–60 nm radius of curvature) is fabricated using semiconductor processing techniques. The cantilever scans above the surface of the sample by progressively moving backward and forward across the surface. A piezo-electric crystal raises or lowers the cantilever to maintain a constant bending of the cantilever. The force exerted on the tip varies with the difference in the surface height and thus leads to the bending of the cantilever. A laser beam gets constantly reflected from the top of the cantilever towards a position-sensitive photodetector consisting of four side-by-side photodiodes (*Figure* 3). This laser beam detects the bend occurring in the cantilever and calculates the actual position of the cantilever. Thus, AFM records a three-dimensional image of the surface topography of the sample under a constant applied force (as low as nano Newton range), which provides a maximum resolution image without causing any damage to the sample surface.

As the tip scans the surface of the sample, the force between the tip and the sample varies. This change in force is sensed by the tip

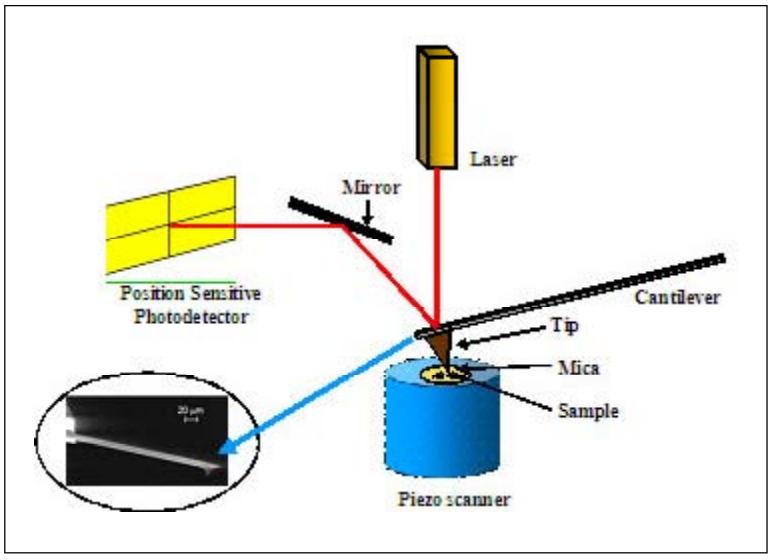

Figure 3. Mode of action of an AFM: AFM consists of an extremely sharp tip (brown color) mounted or integrated at the end of a tiny cantilever spring which is moved by a mechanical scanner/Piezo scanner (blue color) over the surface to be observed/ scanned. A magnified image of a real cantilever is also shown.

attached to the flexible cantilever. The amount of force between the probe and the sample is dependent on the spring constant of the cantilever and the distance between the probe and the sample surface. According to Hooke's Law, this force can be described as $F = -k\,\Delta x$, where F is the force, k is the spring constant and x is the cantilever deflection.

The interactions between the tip of the cantilever and the sample surfaces are regulated by different types of forces, viz., van der Waals forces, capillary and adhesive forces, and double layer forces. Among these, van der Waals force is most commonly associated with AFM. In the contact region, the distance between the tip of the cantilever and the sample surface is less than a few angstroms (Å). Thus the probe experiences repulsive van der Waals force. In the non-contact region, the tip is several tens to hundreds of angstroms away from the sample surface and hence experiences an attractive van der Waals force. Various scanning modes operate in different regions of the van der Waals force vs distance curve as represented in *Figure* 4. The contact mode operates in the repulsive region and the non-contact mode operates in the non-repulsive region of the curve, while the intermittent or tapping mode fluctuates between the two.

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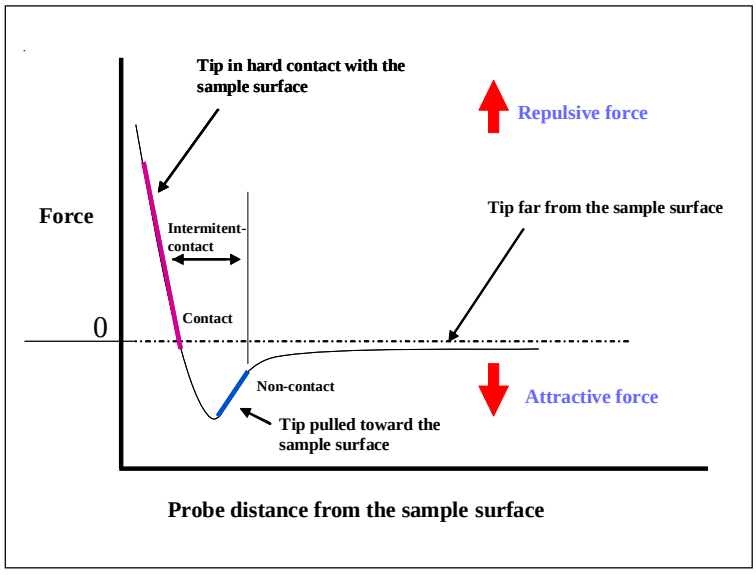
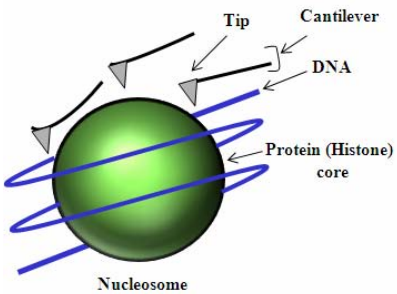


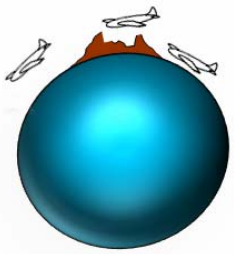
Figure 4. Graphical representation of force–distance relationship: The Y-axis of the curve represents the interatomic force acting between the cantilever and the sample surface, while the X-axis represents the distance between the cantilever and the sample surface. In the contact zone of the curve, a repulsive interatomic force mainly prevails between the cantilever and the sample where the cantilever is held a few angstroms from the sample surface. Here the AFM tip makes soft 'physical contact' with the sample. In the non-contact zone, the cantilever is held away from the sample surface and an attractive interatomic force prevails.

Box 3. Simple Understanding of the Principle of AFM Cantilever Tip Scan over a Sample

The cantilever scans the beads on a string structure (chromatin). It moves over the sample surface. The van der Waals forces between the cantilever and sample surface generates electrical signals which in turn produce the topographic image. The mobility of the cantilever could be compared with the long distance flight travel over the earth, where it moves over the contour of the earth just as the cantilever scans over the contour of the sample, in this case nucleosome.



Scanning of chromatin unit, nucleosome (complex of DNA and histone protein unit present in the eukaryotic cell nuclei)



A flight flying over the earth

Operating Modes of AFM

***Contact Mode*:** Contact mode or repulsive mode is the simplest mode of operation for AFM. This mode of operation involves sideways scanning by the cantilever tip over the sample surface. The tip makes soft physical contact with the sample and the scanner gently traces the tip across the sample. However, this mode is also associated with disadvantages, viz., general integrity of the delicate biological samples can be lost. This drawback prevents the use of contact mode in studies pertaining to biological substances. In addition, contact mode yields very low resolution with large soft samples. As biological substances are very soft and delicate, the vertical and shear forces exerted by the tip can damage the sample. Therefore, contact mode is not ideal for biological systems. This lacuna can be overcome by applying tapping mode in fluid that reduces the shear forces and minimizes the damage to biological samples.

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***Tapping Mode*:** Tapping mode or the intermittent contact AFM is the most preferred operating mode for high-resolution topographic imaging of subcellular structures, and soft and delicate biological samples.

Box 4. Applications of AFM in Various Fields

- **Compound semiconductor:** Imaging compound semiconductor surfaces on atomic scale.
- **Electronic materials:** AFM tip is used to write and erase nanoscale **electronic** structures.
- **Data storage:** AFM is nowadays used for data storage due to space and cost constraints.
- **Life science:** AFM measures surface structures of various biological substances, and is capable of visualizing biological objects from living cells down to single molecule levels.
- **Pharmaceuticals:** In pharmaceutical industries, AFM is used for drug crystallization study, particle characterization and tablet coatings.
- **Semiconductor:** AFM is used in characterizing semiconductors.
- **Optics:** AFM is used in optics for the metrological measurement, since the optical profilers are unable to image transparent specimens.
- **Polymers:** Polymers are generally insulators. High resolution images of the polymers are very difficult to obtain using SEM/TEM, as the samples need to be coated with a conductive layer. AFM however can generate images of polymers without sample preparation.

Tapping mode in air: In this operating mode, a small piezoelectric crystal mounted in the multimode AFM tip holder makes the cantilever oscillate up and down at or slightly below its resonance frequency. The tip oscillates vertically, alternately contacts the surface and lifts off. The amplitude of this oscillation typically ranges from 20nm to 100nm. The oscillating tip lightly touches or 'taps' on the sample surface during scanning. When the tip comes close to the sample surface, forces like van der Waals force, dipole-dipole interactions, electrostatic forces, etc., act on the cantilever and lead to a decrease in the amplitude of oscillation. Thus, the image is obtained by imaging the force of the oscillating contacts of the cantilever tip with the sample surface.

Tapping mode in fluids: Tapping mode operation in aqueous medium is a very useful tool for biologists because the samples are in a state that closely resembles the *in vivo* environment as compared to dehydrated samples.

In biological studies tapping mode and non-contact modes are most extensively used.

***Non-Contact Mode*:** This mode is preferentially used in the study of chromatin dynamics. The Non-Contact AFM (or NC-AFM) operates with increased tip–sample separation without being in

AFM has emerged as an excellent tool for biological studies, as the magnification and resolution achieved is quite satisfactory and with limited artifacts.

contact with the samples under normal imaging conditions. The cantilever oscillates above the sample surface with small amplitude at a frequency larger than its resonance frequency. This results in increased sensitivity in comparison with tapping mode AFM. Outstanding spatial resolution can be achieved by performing NC-AFM in ultra high vacuum (UVH). In biological studies tapping mode and non-contact modes are most extensively used.

Need of AFM in Biological Sciences

Biological samples have been studied by a variety of microscopic techniques, starting from light microscope, compound microscope and electron microscope, but each technique has its own limitations. Magnification achieved by compound microscope is not satisfactory, while electron microscope provides a lot of artifacts which could in turn convey wrong and misleading information. Sample preparation for electron microscope is also very complex and laborious. AFM has emerged as an excellent tool for biological studies, as the magnification and resolution achieved is quite satisfactory and with limited artifacts. Proper sample preparation could lead to a resolution as low as 2–3 nm. Nowadays, the combination of AFM and confocal microscopy has introduced another important technique called *Bioscope*, which has added another useful feature to the AFM systems. If the cells are stained with a fluorescent marker, then simultaneous images obtained by AFM and fluorescent microscope could be compared (as in *Figure* 7b on p.639), which could provide valuable information about the sample.

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The AFM has been used to visualize different biologically important structures, ranging from DNA–protein complexes, viruses to subcellular components of eukaryotic cell. It has also enabled us to examine the dynamic structures arising from changes in different physiological conditions (e.g., change in the shapes of bacteria upon treatment with various drugs). In the following section we will describe a few biological studies in which AFM has been used.

Study of Viruses with AFM

Viruses are sub-microscopic particles that are considered as entities between living and non-living because they cannot replicate outside the host cell. They are composed of an outer protein coat surrounding the genetic material that consists of either DNA or RNA. AFM was first used for virology in 1992 by Kolbe and colleagues for the study of T4 bacteriophages. AFM can be used to study intact virion, without causing any damage by fixation or staining and has the ability to analyze host–viral interaction in real-time.

AFM observation revealed that the width of TMV is in the range of 25–35nm while the height is in the range of 17–23nm in tapping mode and 19–23nm in the case of contact mode.

Tobacco Mosaic Virus (TMV)

Tobacco mosaic virus (TMV) is a rod-shaped virus containing single-stranded RNA as its genetic material (*Figure* 5a). TMV infects plants especially tobacco and other specific members of the family *Solanaceae*. It causes the mosaic disease in tobacco. AFM observations revealed that the width of TMV is in the range of 25–35nm while the height is in the range of 17–23nm in tapping mode and 19–23nm in the case of contact mode (*Figure* 5b). The structure of TMV is very interesting and artistic. The protein subunits are arranged in a helical fashion with three turns of viral helix consisting of 49 subunits. The single-stranded RNA follows the viral helix where each protein is attached to three nucleotides. One of the most intensively studied TMV proteins is the Coat Protein (CP). It forms the virus particle and in turn protects the viral RNA. CP assembles into the virus particle and this ability helps in the long distance movement of the virus. CP is essentially made up of two layers of α-helices oriented perpendicular to the axis of the virus particle, while the RNA remains deeply embedded in the CP.

An interesting finding using AFM suggests that clusters of gp120 do not form spikes on the HIV surface.

HIV

The shape of HIV virion (*Fig*ure 5c) is roughly spherical with reasonable uniformity and a diameter of about 120nm. Tufts-like proteins called gp120 are found on the surface of the HIV particle. Around 100 gp120 molecules are present per particle

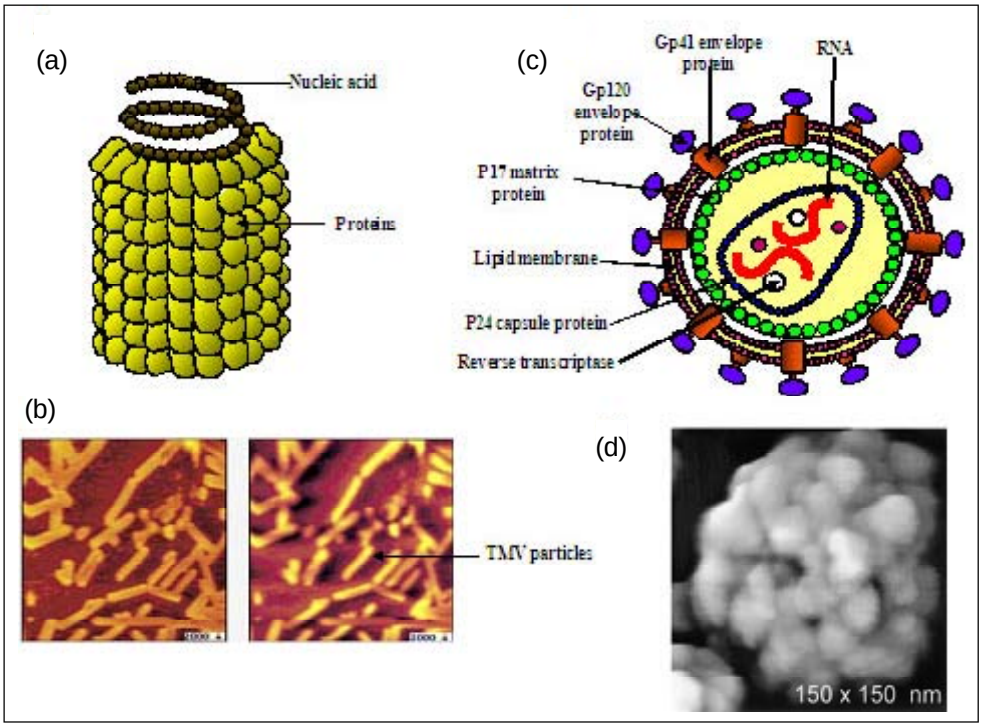


Figure 5. Visualization of viruses by AFM:
(a) Artistic representation of the Tobacco Mosaic Virus (TMV)
(b) AFM image of TMV
(From Internet).
(c) Schematic representation of Human Immunodeficiency Virus (HIV)
(d) AFM image of HIV
(Courtesy: Alex McPherson).

with a diameter of 200Å. The gp120 is found to be non-covalently connected to the lipid membrane through another protein called gp41. gp41 is a glycoprotein which associates with gp120 to form the secondary basis for entry of HIV into the cell. The structure of gp41 is known from X-ray crystallography which shows that it is a three-fold symmetrical coiled-coil trimer consisting of six long α helices. An interesting finding using AFM suggests that clusters of gp120 do not form spikes on the HIV surface. The appearance of gp120 as spikes on the HIV surface by negative staining microscope could just be an artifact of the heavy metal stain penetrating between enveloped proteins. Later, this paradox was solved by using AFM, which revealed that gp120 does not form spikes on the HIV surface (*Figure* 5d). Combined gp41 and gp120 is found to be as mushroom shaped, with large, exposed, exterior surfaces. A conformational change in gp41 protein is found to be involved in membrane fusion between HIV and the target cell plasma membrane. The conformational change includes an early exposed hairpin conformation and a late low

energy six-helix bundle (SHB) conformation. The reason for the folding of gp41 into SHB conformation is to slow down membrane disruption effects induced by early gp41. The presence of SHBs in viral fusion proteins clearly demonstrates that this structure is critical in the viral fusion process prior to integration of viral genome into the host genome. Thus AFM provided the most clear, artifact-free image of HIV.

Vaccinia is found to be about 350nm long and is more ellipsoidal (most of the viruses are classical brick-shaped objects) and height measured under AFM is found to be mainly in the range of 240–290 nm, which is more than the range previously obtained by cryo SEM and EM.

Vaccinia Virus (Pox Virus)

Vaccinia virus is a member of *Poxviridae* family containing double-stranded DNA. It causes small pox. For proper knowledge about the infection, the understanding of the arrangement of the viral surface protein is extremely essential. *Vaccinia* is found to be about 350nm long and is more ellipsoidal (most of the viruses are classical brick-shaped objects) and height measured under AFM is found to be mainly in the range of 240–290 nm, which is more than the range previously obtained by cryo SEM and EM**.** The outer surface is relatively rough with a high-density array of punctuate protrusions of around 25–30 nm diameter. Two membrane proteins, a trimeric 14 KDa fusion protein and a 32 KDa adsorption protein, play crucial roles in viral infectivity. These two proteins are essential in the early phase of virus–host interactions. 'Surface tubules' formed by a 54–58 KDa protein is also found on the surface which is thought to be responsible for the adhesion of the virion to the cell.

Efficiency of nucleoid condensation in bacteria can be effectively evaluated by the principle that, un-condensed nucleoid extends fibrous structures around the lysed cell while the DNA/DNA-protein fiber remains in the condensed nucleoid structure upon lysis.

Study of Signal-Dependent Organization of Bacterial Genome

The nucleoprotein organization of bacterial genome has been extensively studied by employing AFM. The organization details would help in extracting extremely valuable information for future drugs against some of the pathogenic bacteria. One of the important protocols which is followed to study organizational structure of bacterial genome by AFM is 'On-Substrate Lysis'. This procedure is extremely effective in removing the cellular membrane and visualizing the bacterial nucleoid under AFM. By

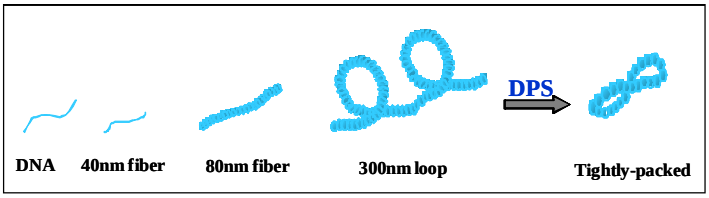
this method, efficiency of nucleoid condensation in bacteria can be effectively evaluated by the principle that, un-condensed nucleoid extends fibrous structures around the lysed cell while the DNA/DNA-protein fiber remains in the condensed nucleoid structure upon lysis. This method has been widely implemented to gather information about the size of the compacted nucleoid of both *Staphylococcus aureus* and *Escherichia coli*.

Furthermore, the studies conducted by AFM strengthen the earlier findings that bacteria do not contain a well-defined nucleus and the whole genomic DNA is packed in the cell in the form of a nucleoid. In eukaryotes, histones, non-histone proteins and RNA are involved in the organization of the genome into tiny confines of the nucleus. However, in bacteria Hu proteins play a major role in organizing the nucleiod by utilizing the physical/chemical properties of DNA–protein interactions. In addition to Hu proteins, different proteins like Histone-like Nucleoid Structuring (H–NS) proteins, Structural Maintenance of Chromosomes (SMC) proteins and DNA topoisomerases also play a pivotal role in the construction and maintenance of the higher order structure[1] of bacterial genome.

[1]The higher order structure protects the genomic DNA against various environmental stresses.

It has been reported that, DPS (DNA binding protein from starved cells) induces the transformation of the bacterial genomic DNA into a condensed state (*Figure* 6). DPS is a 19 KDa protein, which is a member of Fe-binding protein family. It is expressed in bacteria at the time of stress and protects the genomic DNA

***Figure 6. DNA-binding protein from starved cells (DPS) induces transformation of the bacterial genomic DNA into a condensed state: DNA in association with other nucleoid proteins forms a 40nm fiber. The 40nm fiber structure then condenses and super solenoids into an 80nm fiber (from left panel to right). A looped structure then forms from the 80nm fiber. After several series of changes of structure, the nucleoid forms a tight compact structure (right panel). The tight compaction of the bacterial nucleoid is largely influenced by DPS, a non-specific DNA-binding protein in* Escherichia coli.** (For AFM image of these structures, see, Joongbaek Kim *et al*, *Nucleic Acids Res.*, Vol. 32, No. 6, 2004.)



against oxidative stress, UV light, nuclease cleavage, thermal shock and acid. DPS can also restrict the production of hydroxyl radicals by reducing the intracellular level of Fe^{2+} when the cell is exposed to oxidative stress.

Atomic force microscopy studies suggest that, in *E. coli*, DPS induces nucleoid condensation in oxidative stress, while Fis (a nucleoid-associated protein) inhibits DPS activity in the log phase. In *S. aureus* and other gram positive bacteria (such as *Bacillus subtilis*), the homolog of DPS, i.e., MrgA (Metallo regulated genes A) is present. But, unlike DPS in *E. coli*, MrgA can condense the nucleoid of *S. aureus* in the log phase, where the analog of Fis is missing.

Nuclear Architecture as Revealed under AFM

Nucleus is the largest organelle in a eukaryotic cell (*Figure* 7a). It is called the brain of the cell as it contains the whole genetic material packed inside it. The diameter of a mammalian cell ranges from 10–20 μm. The nuclear envelope separates the eukaryotic genome from the cytoplasm and acts as a protective shell. Recent findings suggest that the nuclear architecture plays a significant role in the dynamic regulation of the genome function: AFM has been used extensively to understand the structure–function organization of different sub-nuclear organelle. Nucleus

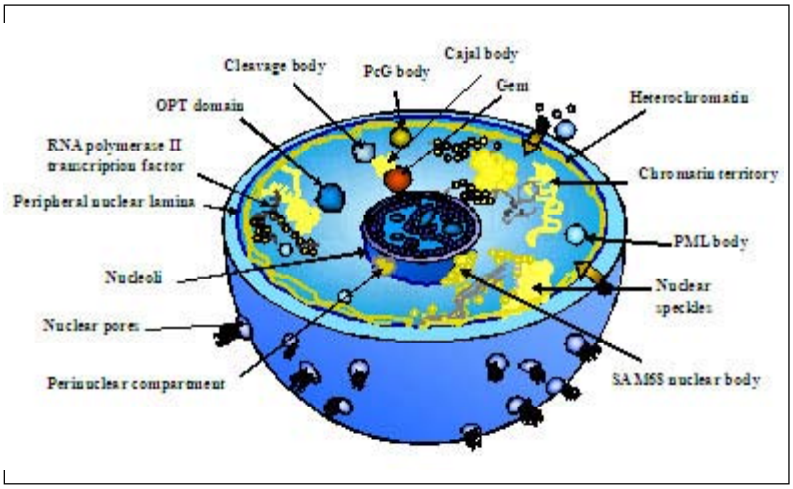


Figure 7a. Eukaryotic nucleus: Pictorial representation of the subnuclear particles of a eukaryotic nucleus.

The nucleolus is the most extensively studied intranuclear domain.

can be compartmentalized into two quarters: Chromosome territory and the inter-chromatin compartment. The chromosome territory consists of the chromatin, heterochromatin (located below the nuclear envelope surrounding the nucleolus) and the euchromatin (present between the boundary of chromatin territory and the interchromatin compartment). On the other hand, the interchromatin compartment comprises of nuclear matrix, nucleolus, nuclear speckle, cajal body and promyelocytic leukemia (PML) body.

Nuclear matrix was first identified under electron microscope and biochemically it was defined as a remnant structure after treatment of a eukaryotic cell with detergent, high salt buffer and DNase I. Later nuclear matrix was viewed under AFM as fibrous and dotted structures inside the nucleus by gently treating HeLa cells with Triton X-100, ammonium sulfate, and DNase I. Biochemical techniques have enabled identification of various proteins present in the nuclear matrix. The study with a specialized AFM technique called Pico-TREC^{TM} revealed that the MAR/SAR-binding proteins bind to the MAR/SAR of the chromosomal DNA and play a vital role in attaching the nuclear matrix inside the nucleus. SP 120, a major MAR-binding protein has also been identified. This technique involves recognition of a particular protein by using an antibody attached with the cantilever which in turn will provide the topographic image with that specific probe. The nucleolus is the most extensively studied intranuclear domain. It can be easily distinguished from other sub-nuclear structures, as it has no membrane. It is a reservoir of rRNA genes (rDNA), rRNAs, and more than 700 proteins containing RNA transcription factors, processing enzymes, and ribosomal protein subunits. Nucleolus is found to be a rigid structure and it has been confirmed by AFM (*Fig*ure 7b).

Mitochondria visualized by AFM appear ellipsoidal with an average diameter of approximately 600 nm.

Apart from nucleus, AFM is also used in the study of other cellular organelles such as mitochondria and chloroplasts. Mitochondria visualized by AFM appear ellipsoidal with an average diameter of approximately 600nm. The majority of the mitochondria are found as single particles, while a very small portion of the

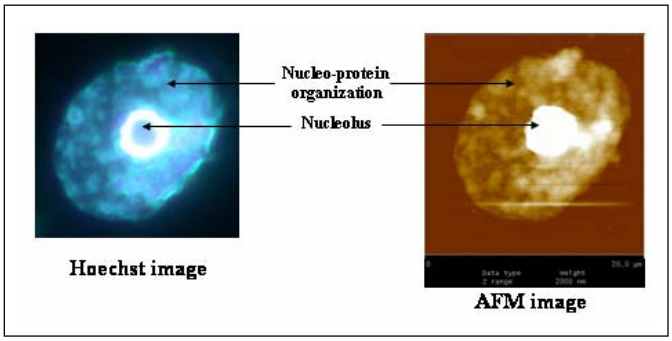


Figure 7(b). Eukaryotic nucleus: AFM image of HeLa cell nucleus in tapping mode under air using BIOSCOPE AFM. The cells were observed either by fluorescence microscopy after staining with Hoechst or by AFM without staining.

Courtesy: Laboratory of Plasma Membrane and Nuclear Signaling, Graduate School of Biostudies, Kyoto University, Japan.

population exists in aggregates with fewer than eight mitochondria per aggregate. Chloroplasts isolated from cucumber plants appear as rough and rugged surface under tapping mode AFM. The average size of a chloroplast has been reported to be approximately 10μm and 3μm in height.

Visualizing DNA Dynamics under AFM

Visualization of DNA and RNA is routinely done under AFM and they appear as thread-like structures. Small features like helix turn of B-DNA have also been imaged. Bare uncoated double-stranded DNA and complexes with proteins are also studied routinely, mainly with the tapping mode. Recently, DNA damage under AFM has also been extensively studied by Wenfang and colleagues. Cellular DNA exposed to formaldehyde and hydrogen peroxide undergoes breakage and crosslink when viewed under AFM. It has also been reported that, with high concentrations of formaldehyde, DNA is found to form conglomerates. Supercoiling of DNA is brought about by winding of DNA around several DNA-binding proteins and also due to some topological limitations imposed on closed circular plasmids. Supercoiling of DNA is essential for transcription, replication, recombination and also in maintaining the genomic stability. Supercoiled DNA and relaxed DNA can be easily distinguished with help of AFM (*Figure* 8a).

Supercoiling of DNA is brought about by winding of DNA around several DNA-binding proteins and also due to some topological limitations imposed on closed circular plasmids.

AFM has a broad level of applications in the quantitative as well as qualitative analysis of the molecular mechanisms of DNA-protein interaction. For example, the binding of tumor suppressor

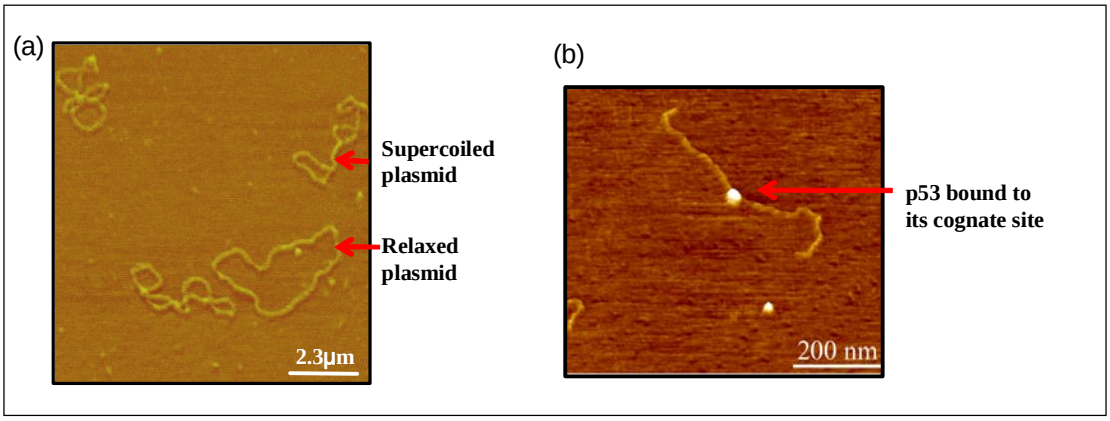


Figure 8. AFM images of DNA and DNA-protein complexes: (a) AFM image of circular plasmid DNA scanned using tapping mode under air with 5nm height. (b) AFM image of p53 binding on its cognate site located in a 1Kb DNA fragment.

Courtesy: H A Kishore, K Hizume, K Takeyasu and T K Kundu: Unpublished

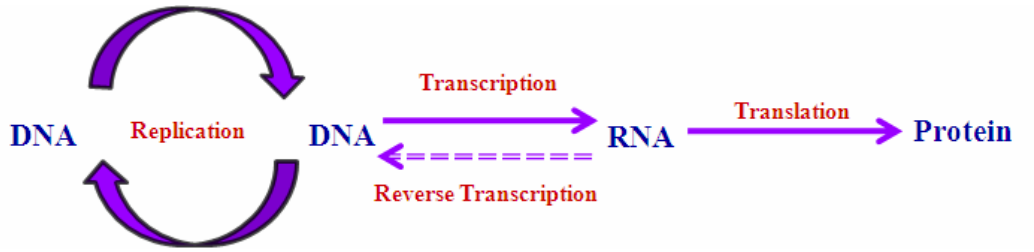
protein, p53 to its cognate DNA binding site(s) has been investigated by AFM. As depicted in *Figure* 8b, p53 (tetramer: four molecules of p53 together) forms a specific DNA-protein complex at its cognate site (DNA-binding sites), present in the 1Kb promoter DNA fragment. The structure of chromatin has also been broadly studied under AFM by various research groups in recent past. AFM has been also been effectively used to study the effects of various non-histone proteins on chromatin structure.

Conclusion

With the advancement of technology, new generation Atomic Force Microscope has been developed by which the real time images of different biochemical reactions can be imaged. These techniques together are called Fast-Atomic Force Microscope. Furthermore, live imaging of living cells have also been developed in combination with other spectroscopic techniques, such as Raman spectroscopy. These technologies will also be useful in chemical sciences, nanotechnology and single molecule experiments.

Box 6. A Real Experiment, where AFM has been Used to understand some Biological Phenomena.

Introduction: DNA is the genetic material for most of the living organisms except in some viruses. The whole phenomenon of transfer of information from DNA to protein through RNA is called the Central Dogma of molecular biology.



Proteins formed by translation regulate the phenotypic expressions of the organism. Thus, transcription is an extremely important phenomenon which regulates the rate of protein expression in a living cell. Transcription in turn is regulated by several DNA-binding proteins. tumor suppressor protein p53 is one such DNA binding protein which acts as a transcription factor. Mutation of p53 in the DNA binding domain can reduce the DNA binding ability of p53, which leads to various cancers. p53 binding to its cognate DNA sequence can be directly monitored by using AFM.

Experiment:

- Wild type p53 and mutant p53 (mutation in the DNA binding domain) were taken in separate vials and were mixed with the required amount of the DNA containing p53 binding sequence in buffer containing 10mM Tris-Cl and 10mM $MgCl_2$.
- Reaction mixture was incubated at 30^o C for 30 minutes.
- 5μ of the solution was dropped on a freshly cleaved mica surface and incubated for around 10 minutes and was then washed with water.
- Mica surface was then dried with nitrogen gas and was directly used for AFM analysis.

Observation: In the reaction where wild type p53 was used, quite a few DNA-Protein complexes could be observed where p53 is bound in the relatively same position, whereas in the case of the mutant protein no such structure could be observed (*Figure* 8 b).

Conclusion: Wild type p53 binds with its cognate DNA sequence, while mutant p53 is unable to bind to its cognate DNA sequence.

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