



Review Article

Atomic force microscopy in biology and biomedicine

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ARTICLE INFO

Article history:

Received 11 June 2012

Received in revised form

3 July 2012

Accepted 17 July 2012

Keywords:

Atomic force microscopy

Bacteriology

Cell imaging

Electrostatic measurements

Virology

ABSTRACT

In order to gain better understanding of the behavior of complex biological systems, it is sometimes necessary to monitor biological samples in their native state and in their physiological environments. However, until recently, bioscientists have been unable to obtain real-space images of biological and biochemical structures in their physiological aqueous environments with a resolution better than the diffraction limit of conventional optical microscopy, which is approximately 350 nm. The invention of the atomic force microscope by Binnig, Quate and Gerber in 1986 brought new hope in this area. Modifications and improvements to the atomic force microscope in the past two decades have enabled the observation of biological samples from large structures, such as hair and whole cells, down to individual molecules of nucleic acids and proteins with submolecular resolution. This review introduces the basic principles of atomic force microscopy and recent developments in its applications in biological and biochemical research, including those in the fields of virology, bacteriology, cell biology and nucleic acid, protein and peptide studies, as well as electrostatic measurements in biological samples.

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1. Introduction

Since its invention in 1986 [1], the atomic force microscope has become one of the most important tools for imaging the surfaces of objects at nanometer scale resolutions. It is considered by many to be a strong competitor to conventional methods for the investigation of structures, such as in electron microscopy and X-ray scattering. In the beginning, atomic force microscopy (AFM) was applied almost exclusively to characterize the surfaces of non-biological materials [2–5], and even today its major applications are still in the visualization of microcircuits, material sciences and nanotechnology. The application of the AFM to biological and biomedical research has increased exponentially [4]. The atomic force microscope enjoys many advantages over conventional optical microscopes and electron microscopes. It has several important advantages in studies of biological samples. First, when preparing samples for AFM experiments, no freezing, metal coating, vacuum or dye is needed. Therefore, there is little

disruption of samples in preparation for imaging, and the functions of the biological samples can be maintained. Second, the atomic force microscope is capable of operating in air as well as in liquid, so samples can be scanned in their physiological buffer solutions. The resolution of AFM scanning is extremely high, and atomic resolution on hard surfaces and molecular resolution on soft samples are frequently achieved. At this level of resolution, images of single biomolecules at work, which cannot be seen by other imaging techniques, can be obtained. This article will introduce the basic principle and operation modes of the atomic force microscope and review a range of its applications in biological and biochemical studies.

2. The principle of AFM

The key step for the popularization and commercialization of the atomic force microscope was the introduction of the optical lever for the detection of cantilever movement [6]. The basic configuration of this design is shown in Fig. 1A. In this approach, the AFM consists of four major parts: a cantilever with a sharp tip (Fig. 1B), normally made of silicon or silicon nitride, mounted underneath it; a piezo-scanner that drives the cantilever; a laser diode; and a position sensitive detector. As the tip scans over the

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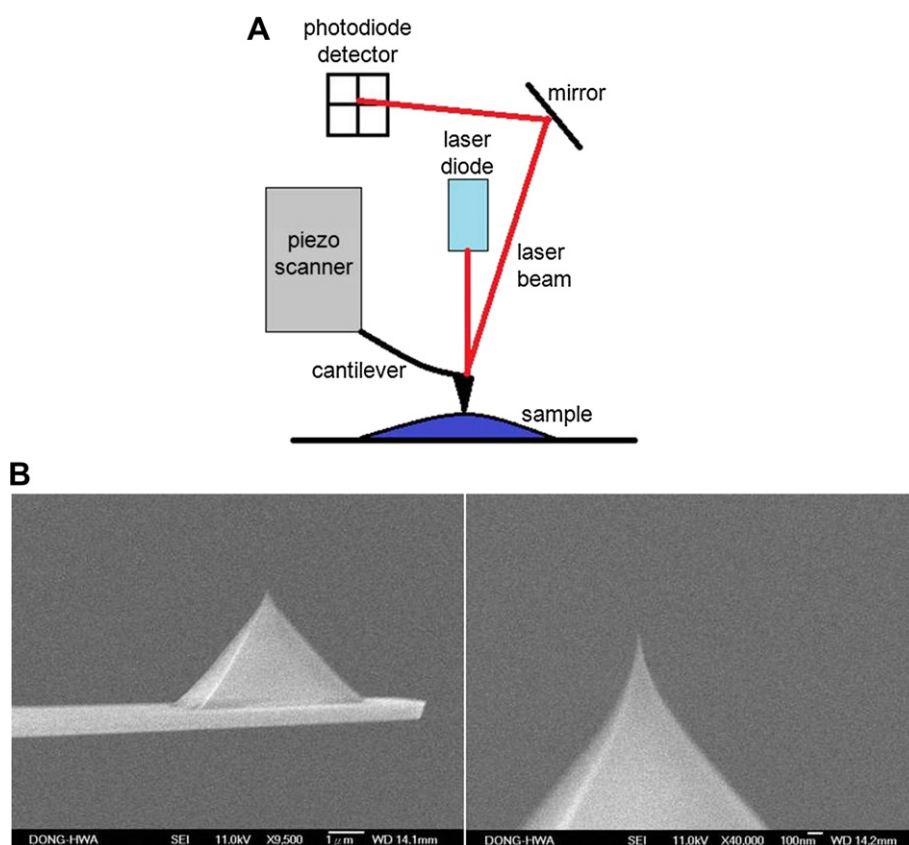


Fig. 1. A typical atomic force microscopy detection scheme (A) and electron micrographs of the AFM tip (B). The cantilever is bent by a given force, which is controlled by the piezo-scanner and the topography of the sample surface when scanning. This gives a deflection of the laser beam, which is generated by the laser diode and reflects off the cantilever. The amount of laser deflection can then be detected by the photodiode detector. The AFM creates topographic images of the surface by plotting the laser beam deflection as the AFM tip scans over the surface. The AFM probe is normally made of silicon or a silicon nitride pyramid with a sharp tip. The terminal radius of the tip ranges from 5 to 40 nm, as shown in (B).

surface, the interactions between the AFM tip and the features on the surface cause displacement of the cantilever. This displacement is measured by detecting the deflection of a weak laser beam, generated by the laser diode, reflecting off the back of the cantilever with the photodiode detector. The atomic force microscope creates topographic images of the surface by plotting the laser beam deflection as its tip scans over the surface. This design greatly improved the sensitivity of the design of the microscope as cantilever displacement can easily be amplified by the light path. Atomic resolution images of a variety of surfaces have been achieved with this design. However, the most important point of this design for biomedical purposes is that it makes operation of the atomic force microscope possible in ambient environments or in aqueous solutions at room temperature or at 37°C. These conditions are required for imaging native biological samples in their functional and physiological environments.

3. Modes of operation

The interatomic interactions between point-like objects or atoms can be repulsive or attractive [7]. This gives options for the atomic force microscope to operate in different modes. Among these modes, the most popular are the contact mode and non-contact mode.

In the contact mode, a force is applied by the AFM on the sample, and therefore the interaction between the AFM probe and the sample is repulsive. As the scanner gently traces the tip across the sample, the contact force causes the cantilever to flex to accommodate changes in the sample's topography. The normal

force applied creates a substantial frictional force when the probe scans over the surfaces. Both the normal and the frictional force can damage vulnerable biological samples. To image these kinds of samples, the force applied must be carefully controlled.

In the non-contact mode, the cantilever is oscillated at a distance (normally 5–15 nm) above the sample surface. The attractive force between the tip and the surface, which is largely due to van der Waals forces [8] or Coulomb and dipole interactions [7], changes depending on distance. These force changes induce alterations in the resonant behavior of the oscillating cantilever. Frequency shift or phase and amplitude changes can be used to generate images. Real non-contact imaging is extremely difficult to achieve. In order to achieve the highest resolution, the probe has to be brought close enough to the surface to effectively detect the attractive force gradient. Thus, the oscillating probe very often slightly touches the sample surface, and becomes intermittent-contact (or tapping) mode AFM.

Despite the fact that non-contact mode AFM has the advantage of reducing the contact and shear forces between the tip and the samples, therefore reducing the possibility of damaging soft biological samples, it still has many disadvantages that make it less popular than the contact mode. First, the operating force of non-contact mode AFM is located in the attractive regions of the force–distance curve, which includes many different forces; therefore the force behavior between the probe and the sample is not easy to determine and calibrate [9]. Second, the resolution and contrast of non-contact mode AFM in liquid is not as good as that in air, as fluid damping greatly reduces the sensitivity of the oscillating probe [10]. Third, in some cases it has been found that soft

structures on the surfaces may still be deformed by the oscillating atomic force microscope tip [11].

4. AFM sample preparation and imaging in aqueous environments

As the atomic force microscope uses a physical scanning technique to obtain images of samples, sample preparation for AFM imaging is relatively simple, and no freezing, metal coating, vacuum or dye is required. For protein and DNA, or even bacteria samples, the sample solutions or suspensions can simply be dropped onto extremely flat surfaces, such as mica, graphite and silicon/silicon nitride discs, and the sample can be adsorbed onto the surfaces by weak forces, such as van der Waals interactions. For cell samples, it is possible to image living cells grown on or attached to glass coverslips or modified surfaces, such as polylysine-coated surfaces, without fixation treatments. However, mammalian cells are sometimes very sensitive to the environment and physical contact. In order to avoid the deformation of cells by contact with an atomic force microscope tip during scanning, the non-contact mode of operation should be used. Scientists sometimes prefer to fix their samples using standard cell fixation techniques with formaldehyde or glutaraldehyde before imaging. However, if these treatments are used, the cells are no longer 'alive'.

Most commercial atomic force microscopes sold today have the ability to image samples in liquids, as they all adopt the same method of optical detection to monitor the displacement of the probe. The samples can simply be covered with water or buffer solution, and the probe can then be placed straight into the liquid to scan the samples in it. The laser beam can still penetrate the liquid to detect changes in the atomic force microscope probe. Manufacturers have also developed a variety of condition-controlled containers for biological sample imaging in liquid. Some of them allow buffer changes with syringes or pumps (for example, the liquid cell of Nanoscope IV, Veeco, New York, USA) and some of them have precise temperature-control functions, which is considered very important for living cell imaging (for example, JPK BioCell, Berlin, Germany).

5. AFM in virology

Viruses are small agents that infect all types of organisms, from animals to plants to bacteria. Viruses are also among the most serious pathogens in human history. Understanding the molecular basis of viruses has the potential to yield new and exciting strategies for therapeutic treatments. However, the methods currently available to treat virus infections are not always helpful. Part of the reason for this is that the development of affective strategies for virus treatments is limited by a lack of knowledge of viruses. In order to have a real understanding of viruses, direct visualization of virus particles is sometimes required. The sizes of viruses range from 20 to 250–400 nm, which is beyond the resolution range of conventional optical microscopy, so visualization of virus particles was carried out by electron microscopy. Its high resolution and relatively simple sample preparation mean that the atomic force microscope has become a new option for direct virus imaging. The atomic force microscope has been applied to image virus morphology on tobacco mosaic virus and other large plant viruses in their crystalline form [12–14], and the Moloney murine leukemia virus and HIV on cell surfaces [15,16]. Images of single virus particles of the herpes virus, vaccinia virus, and minivirus have also been achieved using AFM [14,17–20]. AFM has also been applied to measure the triangulation number, T , in the icosahedral capsid of Ty3 protoretrovirus [21]. These studies have given great

insight into how viruses are assembled and formed, and how virus components are organized in intact virus particles.

Although the morphologies of virus particles have been imaged using AFM, the pathogenicity of viruses is related to many other parameters that should be addressed. For this, the AFM has been applied to investigate the auto-assembly process of the core proteins of the HIV and Mason-Pfizer monkey viruses [22]. In this study, the importance of the deletion of a proline residue at the N-terminus in a mature virus capsid protein on the formation of sphere-shaped virus capsids was observed. The authors also suggested the roles played by capsid protein–RNA associations on maintenance of the regular assembly of HIV core-like particles based on AFM observations. The self-assembled particles of the recombinant hepatitis B virus (HBV) core antigen and its mutants expressed in the yeast *Saccharomyces cerevisiae* system were also analyzed by AFM [23,24]. The authors found that there were two populations of HBV core particles of different sizes formed in the yeast expression system they applied, and these were also found in the native particles isolated from human liver. The size distributions of the particles isolated from the yeast expression system and human liver were nearly identical. They also found that the sequence of the HBV core protein between amino acids 81 and 116, which is a recognition site for human T-cells, was not important for virus core assembly. This finding could imply that the mutations within this region could help the virus escape attack from the human immune system without affecting virus assembly. AFM imaging of hepatitis C virus core particles is also possible. Fig. 2A shows a three-dimensional image of three hepatitis C virus core-like particles assembled by C-terminus truncated core protein 1–116. AFM can also be applied to visualize virus-related events, such as size-dependent virus particle lysis by the N-terminal region of the hepatitis virus non-structural protein NS5A [25], and the formation of lipid raft microdomains in cells induced by the hepatitis C virus replication complex [26].

6. Application in DNA and chromosome studies

Transcription is one of the central biochemical processes in gene expression, but it is still not fully understood. Scientists have applied AFM to investigate the mechanism by which transcription is initiated in *Escherichia coli* by imaging the real-time interactions between DNA and RNA polymerase, providing new insights into the common mechanisms of all DNA–RNA transcriptions [27,28]. AFM can also be used to directly measure the folding force between RNA strands at pico-Newton levels with modified tips and a force spectroscopy technique [29]. The atomic force microscope has also been used to study chromatin and metaphase chromosome structures [30–33]. The results of these studies revealed that the chromatid arm has ridges and grooves along its length that are related to the G/Q-positive and G/Q-negative bands, respectively. The chromatid could also be produced by compaction of highly twisted chromatin fiber loops, and its compaction tended to be stronger in the ridged regions than the grooved regions of the chromosomes. In addition, structural models for the chromatin fiber were proposed based on AFM results [34]. These studies proved the usefulness of AFM in obtaining three-dimensional surface topography in both ambient and physiological liquid conditions. Fig. 3 shows the results of AFM imaging of human chromosomes treated with the standard cytogenetic Giemsa-trypsin-Giemsa method. The atomic force microscope is able to obtain much greater resolution images of G-banded chromosomes than conventional optical microscopic methods. With this high resolution, small defects on chromosomes may now be resolved by AFM. The atomic force microscope may therefore have the potential to become a new method in karyotyping.

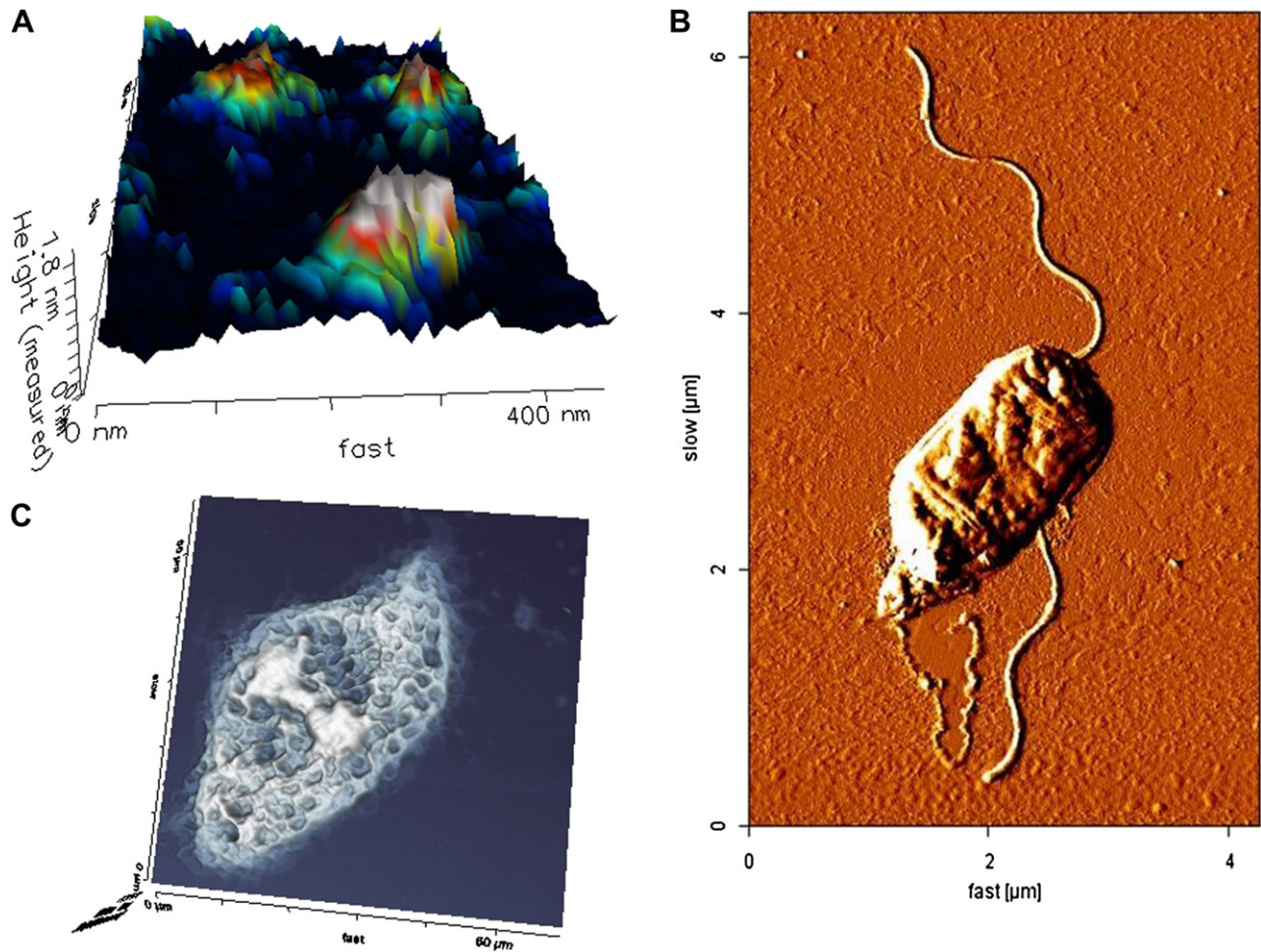


Fig. 2. (A) Atomic force microscope (AFM) three-dimensional image of three core-like particles assembled by hepatitis C virus core protein 1–116. The particles were assembled by dialysis against phosphate buffered saline (PBS), pH7.0, and imaged in PBS pH7.0 on a mica surface. (B) AFM image of a flagellated *E. coli* cell. The *E. coli* cell is in its late log phase, and was twice washed with PBS to remove the culture medium and air-dried on mica surfaces for imaging. (C) Three-dimensional view of the cytoskeleton network of an A549 cancer cell imaged by the AFM. In addition to the complex cytoskeleton network, tiny protrusions around the cell edge are also visible. This cell was fixed with a standard glutaraldehyde method on a glass cover-slide.

7. Imaging bacteria

Events happening on bacteria surfaces are smaller than micrometer scales and are sometimes at the scale of a few nanometers. AFM is therefore especially suitable for studying bacterial surfaces in detail. The atomic force microscope has been applied to characterize the changes on the cell surface during the life cycle of *Streptomyces coelicolor* [35]. AFM was also used to visualize bacterial pili and flagella [36–38]. Fig. 2B shows an example of AFM imaging of a flagellated bacterial cell. The flagella and features on the bacterial cell are clearly seen. The most common application of AFM in bacteriology is in studies of changes on bacterial surfaces induced by alteration in condition or treatment with antimicrobial agents. The influence of culture conditions on *E. coli* O157:H7 biofilm formation was investigated using this technique [39]. Studies of the antimicrobial action of antibiotics [40] and antimicrobial peptides [41–45] have also been carried out. We also applied AFM to investigate the antibacterial mechanism of visible light-responsive nano-photocatalysts [46]. Our results revealed preferential damage at the apical terminus on rod-like bacterial cells by photocatalysis, which had not been observed by other techniques such as electron microscopy.

AFM is able to yield images of bacteria in great detail, so valuable information on the mode of action of antimicrobial agents can be

obtained. This might lead to the better design of antimicrobial drugs to combat the problems created by the increasing resistance of bacteria to antibiotics.

8. Mammalian cell imaging

Mammalian cell imaging is another AFM application in biomedicine. The advantage here lies in its capability to measure living cells at a single cell level and in liquid conditions mimicking the natural environment. It is capable of revealing specific cellular structures such as cytoskeletons, filopodia, lamellipodia and microvilli. Fig. 2C shows an example of a cancer cell imaged by AFM. The cell nucleus and complex cytoskeleton structure as well as small features protruding from the cell are visible. AFM can also be applied to visualize submembranous cytoplasmic structures [47] and identify differentiated cells from stem cells [48]. A large number of AFM applications in mammalian cell studies are related to cancer cells. While the mechanical properties of cancer cells have been measured by AFM and their use has been proposed for cancer cell identification [49], the majority of applications in studies have been the observation and analysis of cell morphology. Morphological changes in cancer cells after treatment with anticancer agents such as paclitaxel, colchicine or cytarabine have been observed [50–52]. The inhibition of cytoskeleton function and cell

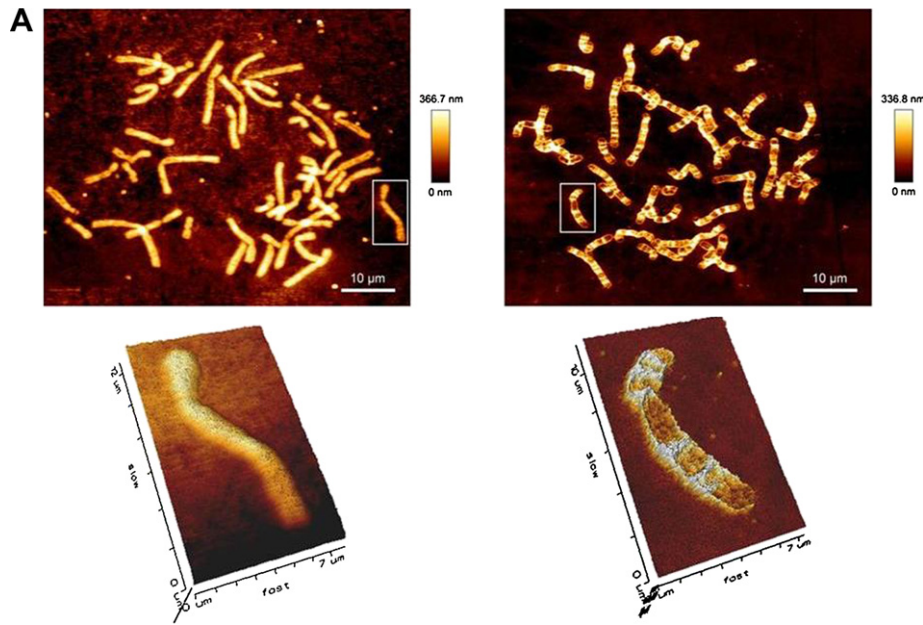


Fig. 3. Visualization of human chromosomes using the atomic force microscope. (A) The AFM images of human chromosomes before (left) and after (right) cytogenetic Giemsa-trypsin-Giemsa treatment.

migration of lung carcinoma cells by the drug 7-chloro-6-piperidin-1-yl-quinoline-5,8-dione was also investigated [53]. Scientists have also used AFM to study the role played by cellular proteins in the migration and invasion of breast cancer cells [54]. In our previous study, we applied AFM to visualize and confirm cancer cell apoptosis induced by antroquinol, a derivative of *Antrodia camphorata* [55]. Despite its inability to monitor events inside cells, AFM has already proven to be a powerful tool in cell biology research because of its ability to resolve nanoscale features on the surfaces and edges of cells.

9. Protein and peptide analysis and biological membrane studies

The atomic force microscope has already yielded important information on proteins that cannot be provided by other techniques. For example, AFM experiments revealed the differences in length between phosphorylated and non-phosphorylated myosin monomers that were not detected using electron microscopy [56]. AFM has been applied to visualize misfolding and follow the interaction between protein molecules, including α -synuclein, β -amyloid peptide and lysozyme, as a function of pH [57]. The β -amyloid peptides are responsible for the amyloid plaques associated with Alzheimer's disease. The toxicity of β -amyloid peptides on brain cells is thought to be induced during aggregation of the peptides [58,59]. As a result, knowledge of the aggregation process in β -amyloid peptides is central for understanding this disease. In 2006, Mastrangelo and co-workers characterized the structure and formation of β -amyloid peptide assemblies using AFM, and captured images from the smallest molecular weight soluble oligomers to β -amyloid fibrils [60]. This study provided a possible mechanism for how peptides are aggregated into the fibrils found in amyloid plaques in the brains of patients with Alzheimer's disease.

For membrane protein research, as early as 1998, Muller and Engel's group in Switzerland achieved sub-nanometer resolution

imaging of the two-dimensional crystals of bacteriorhodopsin from *Halobacterium salinarum*, OmpF porin from *Escherichia coli*, the head-tail connector from phage $\phi 29$, and the hexagonally packed intermediate layer from *Deinococcus radiodurans*. Time-dependent and force-induced structural changes in native (or nearly native) membrane proteins were also monitored with AFM at this level of resolution [61,62]. Furthermore, even the voltage and pH-induced conformational changes of membrane proteins in reconstructed protein crystals were able to be monitored [62]. Apart from imaging, AFM can also be applied to force measurements where scanning is not always required. The atomic force microscope is able to apply forces on the sample surfaces at a level as low as 10^{-11} N; its force sensitivity is high. Some long-range forces between atomic force microscope tips and samples within tens of nanometers above the surface, such as electrostatic interactions, have been measured using the atomic force microscope tip approach curves at particular points on the surfaces in aqueous environments. AFM has also been used to measure the unfolding force of membrane proteins using modified tips to pull membrane proteins out of the membranes [63]. AFM force measurements have great potential in biological membrane studies, as many biochemical reactions, such as protein or lipid phosphorylation that result in a charge density changes on the surface of biological samples, can be detected *in situ*.

10. Electrostatic imaging

Many biochemical reactions result in changes in the electric charges of biological materials. For example, lipid and protein phosphorylations, which are important events frequently linked to the functions of proteins and signal transductions of cells, increase negative charges on biological samples. It would be desirable to be able to directly visualize these biochemical events *in situ*. As the atomic force microscope can apply a force of as little as 0.01 nN, electrostatic interactions are detectable and the biochemical reactions thus become measurable. In colloid science, it is most

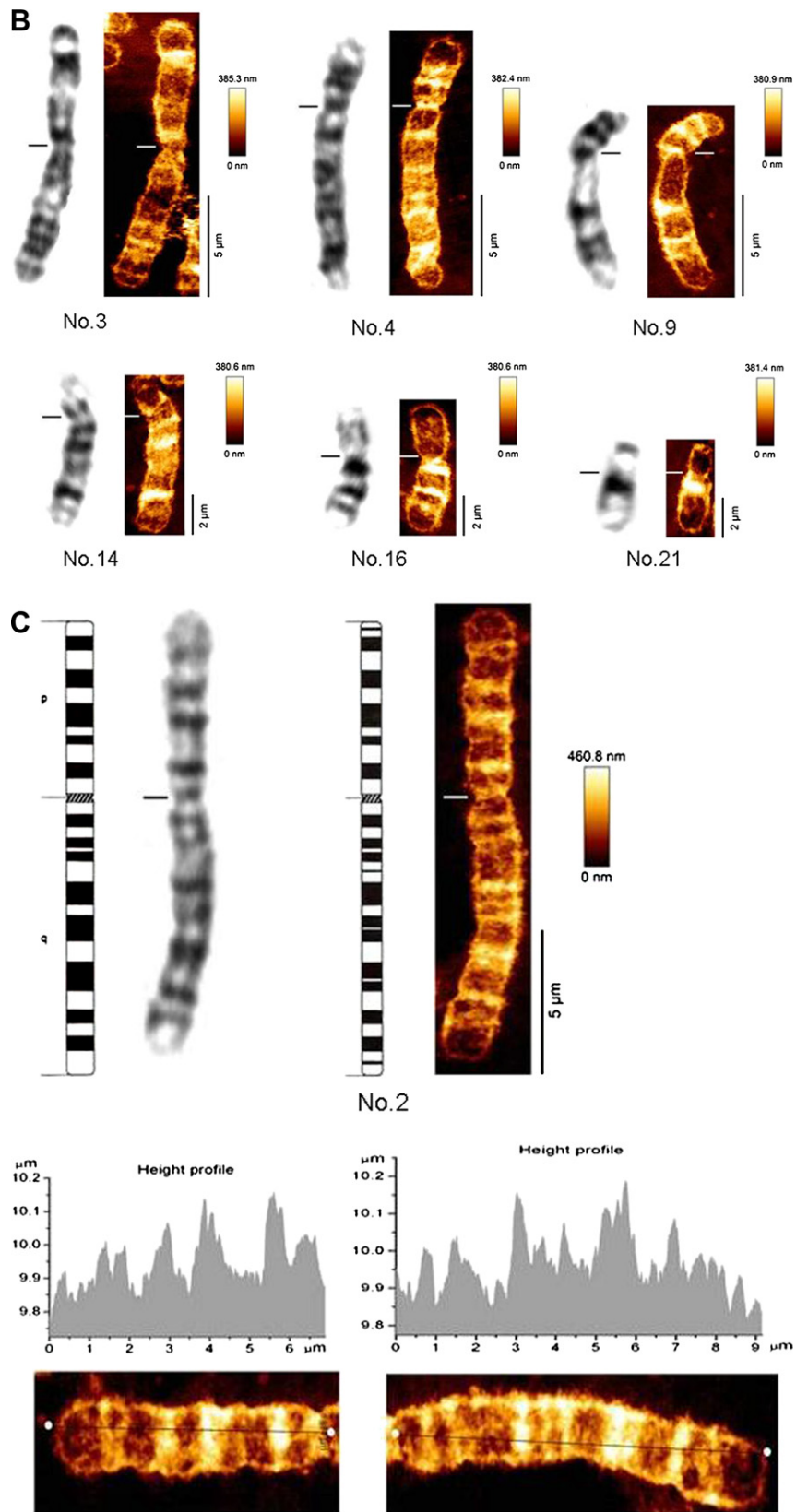


Fig. 3. (Continued) (B) The identity and comparison between classic optical microscopic imaging and AFM imaging of Giemsa-trypsin-Giemsa treated chromosomes. (C) AFM height analysis of the No. 2 human chromosome showing that the AFM can visualize more G-bands than the traditional optical method. Some of the single bands observed with the classic method actually consist of multiple thinner bands, as revealed by the AFM.

important to understand the forces between charged objects in an aqueous electrolyte solution [64]. In AFM experiments, the major force consideration is explained by the Derjaguin, Landau, Verwey, Overbeek (DLVO) theory [8]. The DLVO force (F_{DLVO}) considers only two major forces, the electrostatic interaction (F_{el}) and the van der Waals interaction (F_{vdW}), and neglects other minor forces such as hydrogen forces, steric forces and ion radii effects [65]. The DLVO force can be written as follows [8, 65]: $F_{DLVO}(Z) = F_{el}(Z) + F_{vdW}(Z)$.

For a sphere and a flat surface, which represent the atomic force microscope tip and the sample surface, the electrostatic and van der Waals interactions are $F_{el}(Z) = 4\pi\sigma_s\sigma_t R\lambda_D/\epsilon_e\epsilon_0 e^{-Z/\lambda_D}$ and $F_{vdW}(Z) = H_a R/6Z^2$, respectively [8], where H_a is the Hamaker constant, Z is the distance between a sphere (atomic force microscope tip) and a surface, R is the radius of a sphere. ϵ_e and ϵ_0 are the dielectric constant and permittivity of the free space. σ_s , σ_p and σ_t are the surface charge densities of the sample, support and tip, respectively. λ_D is the Debye length, which indicates the exponential decrease in the potential resulting from screening the surface charges with electrolytes. As a result, the Debye length is highly dependent on the concentration (c_c) and the valence of the electrolyte in the aqueous solution [8]. At room temperature, the Debye length is measured as follows: $\lambda_D = 0.304/\sqrt{c_c}$ for monovalent (1:1) electrolytes, $\lambda_D = 0.174/\sqrt{c_c}$ for divalent (1:2 or 2:1) electrolytes, and $\lambda_D = 0.152/\sqrt{c_c}$ for divalent (2:2) electrolytes [65,66].

DLVO forces also affect the AFM-measured height of biological samples. In AFM experiments, the tip can easily be pushed up by the repulsive electrostatic interactions between it and the samples. This effect is particularly obvious in low electrolyte concentrations. In these conditions, the sample height measured by the atomic force microscope will be greater than the actual height of the sample. In high electrolyte concentrations, almost all repulsive electrostatic interactions are screened out. As a result, the measured height of the AFM sample should be very close to the sample's actual height. If the sample heights measured in high and low electrolyte concentrations are compared, the electrostatic contribution of the measured sample height is obtained, and this electrostatic height contribution can be converted into the surface charge density of the sample based on the DLVO theory. The charge distribution of membrane proteins were identified by Philippsen et al. [67], and the measurements and mapping of the phosphorylation on biological membranes were demonstrated by Liou et al. [68].

11. Conclusions

The major disadvantage of the atomic force microscope is that it can only obtain surface information from samples. As a result, AFM cannot replace the valuable functions of optical microscopy, scanning fluorescence microscopy or transmission electron microscopy. However, AFM has a nanometer-scale resolution and the ability to operate in liquid environments, which are key requirements in biological imaging. The operation range of the atomic force microscope is suitable for characterizing structures from the molecular to the cellular scale. In addition, AFM has the unique ability to measure molecular forces with high sensitivity. These applications have been exploited to reveal structural details and define the molecular forces involved in a variety of biological systems. Measurements of electrostatic characteristics are also among the emerging advances that can facilitate the analysis of biological and biomedical samples. The need for detailed imaging at the molecular level and for monitoring dynamic biological processes will continue. AFM is therefore likely to play an important and enduring role in biological and biomedical research.

Acknowledgments

This work was supported by the National Science Council of Taiwan R.O.C. under grant No. 96-2120-M-259-001 and Tzu Chi University under grant No. TCIRP96004-01.

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