Atomic force microscopy: applications for molecular biology

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Conventional imaging techniques, such as electron microscopy or X-ray crystallography, have provided important information concerning structures of different proteins, nucleic acids, cellular organelles and virus particles. However, the vacuum and radiation environments of electron microscopy are rather restrictive and destructive for analysis of biomolecular complexes that are unstable under extreme non-physiological conditions. In addition, X-ray crystallography requires at least two-dimensional crystals for analysis, and such crystals do not allow sites of interactions with long, disordered RNA or DNA molecules to be identified. A technique that does allow the interactions between biological molecules to be examined, but does not require crystals of such complexes to be produced, is atomic force microscopy (AFM) (Fig. 1A). AFM was developed initially for physical studies of materials and has produced significant achievements in this field. AFM is fast becoming an important tool for biological research, and allows three-dimensional imaging and measuring of individual complexes at a nanometer scale and under ambient and/or physiological conditions[1,2,3]. This technology has been used to examine the structure of single hairpin ribozymes in solution[4] as well as virus particles[5,6] and polymerase structures and activities[7]. AFM has also allowed the direct visualisation and analysis of living cells[8].

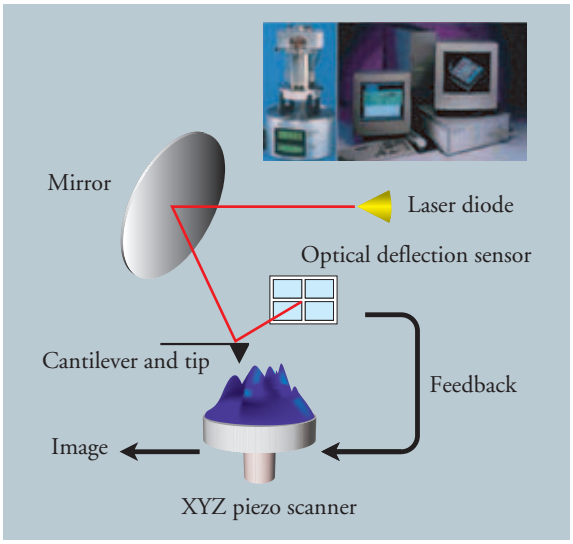

Figure 1 The atomic force microscope and associated monitoring equipment are used to visualise images produced by the deflections of a cantilever holding a tip and a laser diode.

The principle of AFM AFM works by measuring tiny contact forces between the surface and a scanning tip (Fig. 1B). The sharp AFM tip mounted at the end of a flexible cantilever can be used to probe a number of properties of the sample, including its topological features and its mechanical characteristics. Precise lateral and vertical displacement of the sample with respect to the probe is achieved by a piezo scanner holding the sample, or by the cantilever holder. Forces acting between the surface and the tip cause deflection of the cantilever that is registered by a laser beam reflected off the back of the cantilever. The cantilever deflections are used to create a topographic image of the sample when the sample is scanned in the *x-y* (horizontal) direction, or to produce the force curves when the probe is moved in the *z* (vertical) direction. The AFM can operate in different modes. In the contact (constant force) mode the force between the sample surface and the tip is kept constant. The contact mode can be used to image hard and stable samples that are not affected by frictional forces. The tapping (non-contact) mode uses an oscillating tip. When the tip moves towards the surface, it begins to touch or 'tap' the surface. This leads to an energy loss of the oscillating tip, which reduces the tip

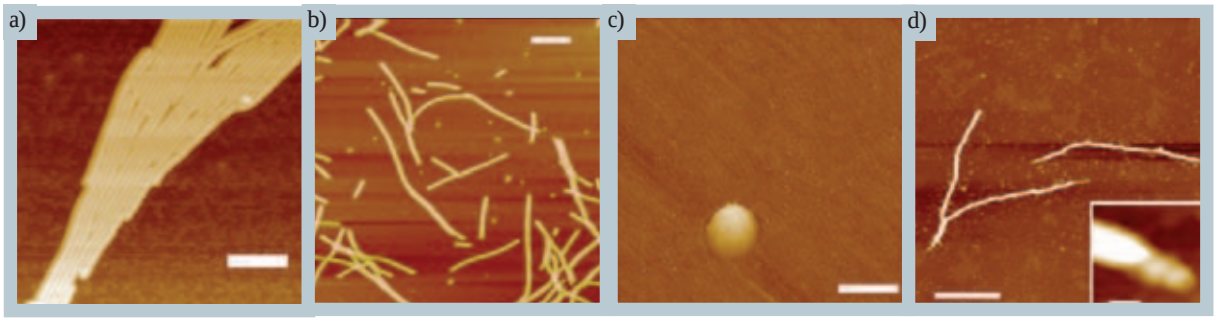

Figure 2 Images generated by atomic force microscopy of different shaped plant virus particles. (a) Aggregated, rigid-rod particles of *Tobacco mosaic virus*; (b) flexuous-rod particles of *Potato virus X;* (c) a single, isometric particle of *Cucumber mosaic virus;* and (d) flexuous-rod particles of *Beet yellows virus.* In (d), note that one end of each virus particle is the same thickness as the rest of the flexuous rod, while the other end is tapered. At higher magnification (see insert), the tapered end is shown to contain morphological features described in the text. The scale bars represent 250 nm (a,b), 40 nm (c), 500 nm (d), or 50 nm.

amplitude. The reduction of the oscillation amplitude is used to identify and measure surface topographic features. The tapping mode operates in air and under liquids.

Applications of AFM Several recent advances in AFM-related technology have enabled the technique to be used under high resolution at the molecular level. Better probes, new imaging techniques, and improved sample preparation methods have all contributed to higher resolution structural imaging. Applications of AFM in molecular and cell biology include the following visualisation and analyses[9]: DNA and RNA, proteins and peptides, protein-nucleic acid complexes, protein-ligand complexes, molecular crystals, and living cells and their organelles. AFM was also employed for studying interactions between different macromolecules, including the interaction between antibody and antigen. Time-lapse, AFM-based techniques for kinetic analysis of protein:nucleic acid and protein:protein interactions in real time also have been developed.

Using AFM, we now have the opportunity to image the surface topography of single cells in aqueous buffer, in real time, as well as at the molecular scale, with spatial and force resolution. In addition, AFM allows us to go beyond topographic imaging by providing information on the physical properties (such as viscoelasticity) of cells and cell organelles, membranes and the cytoskeleton. Specific examples of some of these applications are described below.

AFM studies at the SCRI AFM studies on biological macromolecules were initiated in 2001 in collaboration with Prof. A.G. Fitzgerald at the University of Dundee under a research grant obtained from the Leverhulme Trust. Recently another grant has been awarded to the SCRI from International Association for the Promotion of Co-operation with scientists from New Independent States of the former Soviet Union (INTAS) for the project 'Molecular interactions of a plant virus genome with virus-coded and host cell proteins involved in intercellular virus transport: high-resolution imaging of protein-protein and protein-RNA complexes'. In this project, SCRI will co-ordinate international multidisciplinary collaboration of five teams including the University of Helsinki, Moscow State University, the Advanced Technology Centre (Moscow) and the Centre of Bioengineering (Moscow).

These interdisciplinary studies aim to probe biomolecular 'transport' complexes with AFM. Among the complexes being examined are those formed by viral RNAs and specialised virus-encoded transport proteins involved in processes of cell-to-cell movement through the plasmodesmata (cytoplasmic connections between plant cells), long-distance spread *via* the phloem, and intracellular nuclear transport. AFM is being used for direct imaging and measuring the biomolecular complexes, as well as mapping protein-binding sites on target RNA or DNA molecules; and analysing the oligomerisation state of RNA- or DNA-binding proteins (see below). The binding forces between the biomolecules (protein:protein and protein:nucleic acid) can be measured after immobilisation of the interacting molecules on sensor (tip) and surface (substrate for AFM). This enables the stability and elasticity of the biomolecular complexes to be investigated and factors controlling dissociation of the complexes to be identified.

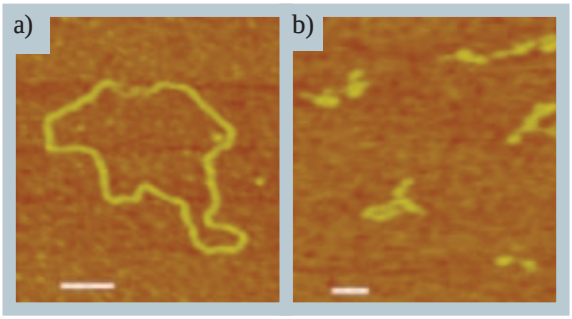

Figure 3 Visualisation of doublestranded DNA (a) and singlestranded RNA (b) by atomic force microscopy. Note that the RNA appears as tangled strands, due to high, internal secondary structure and essential random tertiary structure, while the secondary and tertiary structure of the DNA are more constrained by its doublestranded nature. The scale bars represent 50 nm.

A detailed physical analysis of biomolecular 'transport' complexes and the processes of their formation, trafficking and dissociation is on the cutting edge of plant molecular biology, virology and their interface with physics. Structural and functional characterisation of the 'transport' complexes can provide both novel information on the molecular mechanisms of intracellular and intercellular movement of biological macromolecules, as well as new tools for further studies.

The structure of ordered, biomolecular complexes, such virus particles, can be examined by AFM. For well-characterised virus particles, the structures confirmed those already seen using EM for *Tobacco mosaic virus* (Fig. 2a), *Potato virus X* (Fig. 2b), or *Cucumber mosaic virus* (CMV) (Fig. 2c). We have also studied structure of closteroviruses, filamentous plant viruses with more complex structure (in collaboration with Prof. V.V. Dolja of Oregon State University, Corvallis, OR, USA). For these viruses, such as *Beet yellows virus*, in which additional proteins besides the major capsid protein are present in the particles, morphological differences could be discerned at one end of the virus (Fig. 2d). These gave rise to the 'rattlesnake' structure previously identified by electron microscopy[10,11], but seen at a higher resolution by AFM, due to the masking of the structure by the staining needed to see the virus particles by electron microscopy. AFM allowed more distinct structures to be seen within the 'rattle' at one end of the particle (insert in Fig. 2d). A detailed analysis of the 'rattlesnake' structure together with the biochemical characterisation of virus proteins present in it are very topical, since it has been postulated that this structure is involved in interaction with components of plasmodesmata allowing the virus to move from cell to cell.

Various macromolecules, which have been examined before elsewhere, such as doublestranded (ds) DNA and singlestranded (ss) RNA, were analysed here as a prelude to examining biomolecular complexes containing these macromolecules. These images are shown in Figure 3a and Figure 3b, respectively. Note that ssRNA can be distinguished from dsDNA by the tendency of the former to form tight coils or balls.

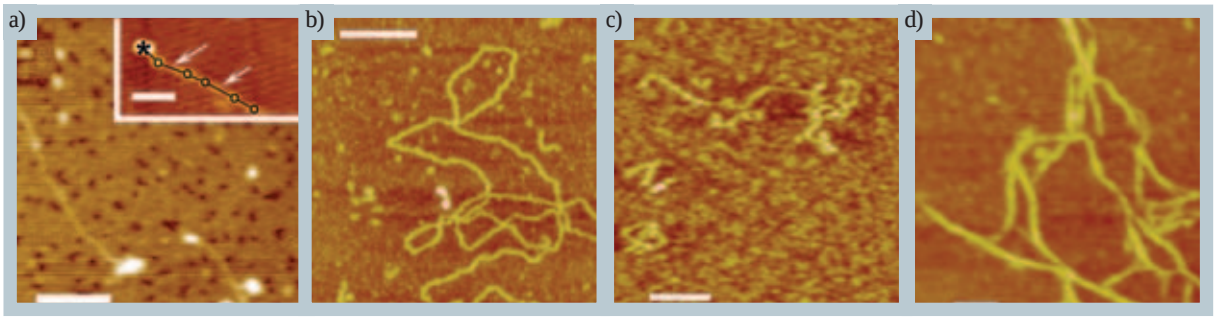

Figure 4 Visualisation of biomolecular RNA:protein complexes by atomic force microscopy. (a) Complexes formed between singlestranded RNA and the ORF4-encoded movement protein of *Groundnut rosette virus* (GRV) show limited binding (white blobs) between the protein and the RNA, with the RNA molecules exposed between the regions covered with protein, shown by arrows and circles, respectively, in the insert in (a). (b) Complexes formed between singlestranded RNA and the 3a movement protein of *Cucumber mosaic virus* (CMV) show the RNA densely packed with movement protein molecules, which are known to bind cooperatively to the RNA, unlike the GRV ORF4-encoded movement protein. (c) Complexes formed between singlestranded RNA and a modified CMV 3a movement protein in which the C-terminal 33 amino acids have been deleted show RNA much less densely packed with protein, with areas of non-coated RNAs being visible as very as small aggregates of protein. (d) Complexes formed between singlestranded RNA and the ORF3-encoded movement protein of GRV show the RNA densely packed into flexuous, rod-like structures by the ORF3-encoded protein, which is known to bind cooperatively to RNA. The scale bars represent 250 nm (a), 50 nm (insert in a), or 100 nm (b, c, d).

A number of viral transport proteins have been shown to be able to bind ssRNA *in vitro*. These RNA:protein complexes could also be visualised by AFM. In the case of the 3a movement protein of CMV, the protein was known to bind to the RNA co-operatively[12]. By contrast, the ORF4-encoded movement protein of *Groundnut rosette virus* (GRV) bound RNA non-co-operatively[13]. Visualisation of these RNA:protein complexes by AFM showed that the GRV ORF4-encoded movement protein bound to RNA incompletely, resulting in various lengths of protein-free segments of RNA (Fig. 4a). By contrast, the CMV 3a movement protein formed highly packed RNA:protein complexes (Fig. 4b). CMV requires its capsid protein in addition to its movement protein for cell-to-cell movement[14]. However, CMV movement protein truncated in its C-terminal 33 amino acids has the ability to mediate viral movement independently of capsid protein[15]. Biochemical assays showed that although CMV C-terminally truncated, movement protein binds viral RNA cooperatively like the wild-type CMV 3a movement protein, this interaction requires fewer protein molecules per molecule of RNA. AFM visualisation of the complexes formed between ssRNA and the CMV C-terminally truncated movement protein showed structures that were less dense (looser?) than the highly dense structures observed for complexes formed between the wildtype CMV 3a protein and ssRNA (Fig. 4c). The densely packed structures of viral RNA and wildtype CMV 3a movement protein, presumably needed for cell-to-cell movement, may prevent RNA molecules from being for expressed (translated) or replicated, unless some other factor, for example the capsid protein, facilitates the release of the viral RNA. However, in the case of the C-terminally truncated 3a movement protein, the complex is not so densely packed and therefore may allow RNA to be released for expression or replication without the need for additional factors such as capsid protein. In support of this is the observation that the GRV ORF4-encoded movement also does not coat viral RNA completely and that may explain why it does not depend on capsid protein for virus movement (for release of viral RNA). This is also true when the GRV ORF4 replaces the CMV 3a gene in infectious CMV RNAs[16].

Finally, we have also visualised a complex formed by viral RNA with the GRV ORF3-encoded protein, which previously was identified as a long-distance RNA movement protein[17] that may be involved in transport of viral RNA through the phloem (Fig. 4d). These latter complexes also exhibit rather dense structures, indicating ordered packaging of the RNA. Further imaging with mutants of the various movement proteins described above will provide a better understanding of the nature of the complexes visualised here, as well as the nature of the forces holding these complexes together and the role of these structures in facilitating virus movement.

While our studies have been limited to those involving viral encoded proteins and their nucleic acids, there is no reason this technique cannot be applied to the analysis of proteins encoded by bacteria, fungi, plants, or animals in various protein or ribonucleoprotein complexes. The applications of AFM to visualising such biomolecular complexes and the effects of alterations in these structures on their functions are just beginning!

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