Imaging of Cells, Viruses, and Virus - Infected Cells by Atomic Force Microscopy

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Abstract: Atomic force microscopy (AFM), by virtue of its ability to obtain images at the nanometer scale of soft samples, in fluids, and through non-perturbing forces, has proven a valuable technology for the investigation of biological specimens. We show that it can provide precise images of viruses, even including the distribution of capsomeres on their surfaces, and images of living cells. Combining the two, we studied the infection of cells, microbial and mammalian, by a range of different viruses that include retroviruses, bacteriophages, and large cytoplasmic viruses such as mimivirus. The method holds great promise for further development and applications in molecular biology, cell science, and medical pathology.

Keywords: probe microscopy; fibroblasts; retroviruses; mimivirus; phages; imaging; mimivirus

Unlike most kinds of microscopy, AFM does not rely on the passage through or reflection from the sample by particles (electrons) or waves (light), but depends on mechanical forces between a physical probe and the surface of the sample. Hence it falls in the category of what is termed probe microscopy. The principle of AFM, illustrated in Figure 1, along with the instrument, could hardly be simpler, being little more than that of a blind man with a cane or stick feeling his way through the physical world of hard objects, but at the nanometer scale (for extensive treatments and other biological applications see: [1-5]).

An AFM, however, applies the principle in a very systematic way and with unusual sensitivity. A stylus with a very sharp tip (~20 nm radius) is drawn back and forth in a raster manner, one line at a time, over a flat substrate (glass, plastic, mica) of about 20 mm diameter on which a sample has been displayed. At close intervals along the line the vertical deflection of a low spring constant cantilever, from which the stylus protrudes at one end, is recorded. When the traces from all of the successive lines are assembled, then one has a topographical map of the entire plane containing the sample. Vertical deflections occur because of physical encounters between the tip and whatever object might occupy a specific point at a particular time. The tip simply rides up and over and down the other side of structural features as they appear before it. The topographical map then is an image of the objects spread across the substrate plane.

The tip of the cantilever does not really touch, in the common physical sense, the object beneath it, but passes over and interacts with it by what are termed “aggregate atomic forces,” whose exact nature remain somewhat obscure, particularly for soft, deformable samples. A consequence of this soft touch interaction is that the sample is not damaged, changed, or even physically perturbed by the scanning motion of the tip so long as sufficient care is exercised. The scanning may be carried out in the dry state, in air, or in fluids. This feature is of great advantage for biological samples, including living cells and tissues, whose structures and processes are generally sensitive to dehydration and must be maintained in buffer or culture media.

There are two principal modes in which an AFM may be operated, and these are “contact mode” and “tapping mode.” In contact mode the stylus is simply “dragged” across the sample plane without losing contact with the surface below. This, however, creates lateral friction, of little consequence if the sample being scanned is materially “hard,” but damaging if the sample is “soft.” Biological samples are characteristically “soft.” In tapping mode, the AFM blind man taps his stick, the cantilever tip, as he moves ahead, lifting it slightly between each tap. This eliminates most lateral friction.

Fig. 1 In (a) is a schematic drawing illustrating the principles of an atomic force microscope. The vertical deflection that the cantilever tip experiences upon encountering some topographical feature on a specimen is amplified through a reflected laser beam, which is tracked and reported by a split diode photodiode detector. Scanning takes place in a fluid filled (or dry if preferred) cell of about 75 μl volume. The sample is translated in a raster manner by piezoelectric positioners upon which the fluid cell is mounted. In (b) is an AFM instrument consisting of the scanning apparatus, about the size of a kitchen coffee maker, two monitors, and the electronic controllers that drive the scanner.
Tapping mode is used almost exclusively with biological samples such as tissues, cells and microbes. The tip is not really tapped in this mode, but constantly vibrates in the vertical plane with frequencies in the range of 200 kHz in air to 10 kHz in fluid, with a scan frequency of about 1 Hz. With soft biological samples where scanning must be relatively slow in order to capture detail, the acquisition time for a single image is usually from 1 to 4 minutes. Because cellular processes, division, fusion, virus budding, etc. are rather slow, a two minute acquisition time is generally more than adequate to record dynamic processes. More detailed procedures for scanning with AFM are provided in references [1, 3-6].

**Fig. 2** Schematic illustration of the convolution of the shape of the AFM tip with the shape of the feature, or particle being scanned. The side of the cantilever tip contacts the object and begins to produce a deflection of the cantilever before the tip apex actually reaches the object. Similarly the opposite side of the tip is still in contact with the object even after the apex itself has passed. Thus the total deflection implies a virtual lateral dimension for the object greater than its actual dimension. The difference between the virtual and actual dimension is a function of the width of the cantilever tip. The sharper the tip, the more accurate the observed dimensions, and the greater the resolution attainable.

In defining the resolution of the method, it is necessary to discriminate between lateral resolution, points in the x, y plane, and vertical resolution, the heights of features above the xy plane. Because the image is a convolution of the tip shape and the shape of the object beneath it (see Fig 2), the lateral resolution is modest, usually in the range of 10 to 20 nm at best. Resolution is essentially limited by the acuity of the tip. Vertical resolution, however, is basically independent of tip shape or sharpness and is very good, on the order of several Angstroms. Thus in making quantitative measures of dimensions, vertical values are used whenever possible. Spherically and cylindrically symmetrical objects are, therefore, straightforward to size very accurately. The same is true of center to center distances in arrays, periodic and otherwise, such as the mimivirus in Figure 3.

**Fig. 3** Arrays of mimivirus, the largest virus known in nature, obtained by bursting infected cells. The diameters of the particles can be readily defined by measuring their heights above the substrate plane, or their center to center distances in the arrays. Note that the particles assume an ordered pattern that resembles, but is not the same as a crystalline array. Scan areas are (a) 12 x 12 um, (b) 25 x 25 um, and (c) 6 x 6 um.

Finally, how can the AFM achieve such extraordinary precision using such a crude principle as a tapping stick? The answer lies in two features of the AFM instrument, its ability to position an object, or determine its position, with extreme precision, and its ability to amplify the tiny deflections experienced by the cantilever tip as it moves over the substrate. The first of these is based on the application of piezoelectric positioning technology, which in practice provides “stepping motor” action at the sub nanometer scale. The detection of small vertical fluctuations in tip position is equally clever. A laser beam is reflected from the top surface of the cantilever as it moves over the substrate surface. Small deflections in the tip position are translated into large deflections, some distance away, of the reflected laser beam. These are tracked and recorded by a split diode, photomultiplier device. AFM software then translates the beam deflections back into $z$ values for the tip.

A few words are necessary with regard sample choice and preparation for AFM analysis. If one is investigating phenomena involving structural features, or changes in these features, in the range of a few hundred nanometers to tens of microns resolution, then AFM applications are rather straightforward. Tip pressure in this range is so slight that no treatment or modification of the sample is necessary. Thus the surface features of normal or infected microbial, animal or plant cells can be observed without consequence while they remain in their culture or physiological medium in a fully hydrated and live state, as are some of those in Figures 4 through 7. They can be observed to move, divide, fuse [7] and carry out other physiological processes such as phagocytosis.
layers of protein, polysaccharide, or nucleic acid. Visualization of the results are then effected by AFM. Such membranes and lipids, and a wide variety of proteases, glycosidases, and nucleases of diverse specificities that degrade specimen are chemicals, such as dithiothreitol that breaks disulfide linkages, detergents such as NP40 that removes membranes, also information about what lies just beneath the surface, such as the cellular cytoskeleton. It does not, however, mean that AFM cannot be used to explore the interior of biological specimens.

On the other hand, when scan areas become smaller than about 10µ², tip pressure becomes significant with soft biological samples. In particular, cell membranes deform under the tip, damage may occur to the specimen, and images eventually become impossible to obtain. To record images at high magnification (small scan areas), it is usually necessary to fix, or treat biological samples with glutaraldehyde or some other crosslinking agent to stiffen them. Osmium tetroxide fixation may also be used for membranes. Living samples are no longer possible if fixation is carried out, but static biological detail is largely preserved and precisely recorded.

When dealing with any AFM sample, it is essential that it be made immobile on the substrate. If you touch an object and it moves or deforms, you cannot specify its position or shape. Thus it is necessary that the object under study, a cell, a virus, be strongly fixed to the substrate, unless you are using the AFM to track and image dynamic processes. With most biological samples such as cells and viruses or large cellular organelles, if the substrate is first coated with poly-L-lysine, then the specimen will adhere tenaciously to the substrate. For some other types of specimens, such as nucleic acids, pretreatment of the substrate with the salts of divalent metal ions such as MgCl₂ suffices to hold RNA or DNA in place [6, 8].

It should be noted that scanning in fluids is technically more difficult for several reasons, as one might well imagine. For living specimens and for inanimate specimens as well, including viruses, structure is certainly better preserved when it is fully hydrated. Some resolution, however, is sacrificed by scanning in fluids. Scanning in air is usually carried out with cantilever tips made of silicon, and these can be sharpened more efficiently than the tips made of silicon nitride that are generally used in fluids. Scanning of air dried samples is common, considerably easier, faster, and yields higher resolution images in many cases. In most studies, scanning in air is routinely employed and scanning in fluid carried out to accurately record detail of particular biological interest.

The topographical information obtained from a two dimensional scan over the substrate surface can be presented in a number of visual formats, and indeed, several of these are common in the literature. The authors usually use, and recommend presenting the topography as a pattern of light and dark intensity representing height above substrate. In such images, dark regions represent features that rise only slightly above the substrate plane, while lighter areas imply a greater height above substrate. The virtue of this representation is that the image presents the sample topography in a familiar way, it looks to the observer as if it were recorded with a common camera using reflected or transmitted light. Thus cells and viruses look just as you might expect them to look if only you had a powerful enough light microscope. There is really no mystery in interpreting such AFM images; they are what they look like.

More complete reviews of the imaging of viruses by AFM are available in references [9-11]. It should be pointed out that in addition to imaging, AFM is also an extremely useful tool for measuring forces, including the responses of cell membranes and virus shells, and for manipulating small particles, including individual virions. A fine review of these latter applications is presented in reference [12].

**DISSECTION OF BIOLOGICAL SPECIMENS:** AFM, as should be evident from the above description, provides information about the surfaces of objects and, because there may be some slight deformation of soft samples and elastic membranes, also information about what lies just beneath the surface, such as the cellular cytoskeleton. It does not otherwise allow visualization of what is internal to the specimen as does EM or confocal microscopy. This does not, however, mean that AFM cannot be used to explore the interior of biological specimens.

Dissection has a long and distinguished history of application in biological investigations, though generally carried out with picks and scalpels on organisms, tissues, and organs. What medical doctor has not dissected a cadaver during his studies, what plant pathologist a flower? In those cases hand tools were employed to strip away successive layers of structure and a microscope, or the eye alone, employed to visualize the newly revealed material. The same approach can be applied to individual cells, viruses, and virus infected cells.

In the cases of biological samples dissection is at the micron and nanometer level and the tools for reducing the specimen are chemicals, such as dithiothreitol that breaks disulphide linkages, detergents such as NP40 that removes membranes and lipids, and a wide variety of proteases, glycosidases, and nucleases of diverse specificities that degrade layers of protein, polysaccharide, or nucleic acid. Visualization of the results are then effected by AFM. Such approaches have now been widely applied in the determination of the detailed structures and architectures of cells and viruses [9, 10].
Fig. 5 At the top, in (a) through (d), is a 3T3, fibroblast derived cell undergoing division while being visualized by AFM. Most striking in the series is the swelling and protrusion of the cell nucleus which splits into the nuclei of the two daughter cells. The time intervals are (a) time 0, (b) 189 min, (c) 231 min, (d) 254 min. At bottom, in (e) through (f), several 3T3 cells migrate to a common area and begin fusing into an aggregate. The series illustrates how AFM may be used to monitor the progress of living cells and visually record dynamic cellular processes, such as division, without significantly perturbing the system. These images were recorded in cell culture using contact mode AFM. Scan areas are (a) – (d) 100 x 100 um, (e) 132 x 132 um, (f) 88 x 88 um, (g) 150 x 150 um, and (h) 150 x 150 um.

CELLS: Mammalian cells, from an AFM perspective, are quite large, often exceeding 50 um in dimensions and they can be readily visualized in their culture media, or some other suitable fluid without the need of fixation. Thus they can be observed for extended periods, hours or even days if sterility is maintained. Various events of their life cycles can be recorded such as cell division (Fig 5a-d), motility and fusion (Fig 5e-h) and cell death (Fig 6), to name just a few examples. None of these observations require very high magnification, disturbance of the cells by the scanning probe is minimal, and many specimens or particles in a preparation can be visualized, recorded, and compared [7].

Fig. 6 In (a) is a healthy osteosarcoma cell in culture moving across the AFM substrate. The arrow indicates its direction of migration by means of its extending pseudopodia. In (b), after 12 hours in the fluid cell of the AFM, nutrients and oxygen begin to be exhausted and the cells exhibits the characteristics of cell death, the appearance of blebs and the broad extended pseudopodia. Scan areas are 45 x 45 um.

As noted above, because of the slight pressure exerted on the exterior of a cell, the membrane deforms inward to some extent and what the probe senses is not what is necessarily on the cell surface but what lies just beneath. It is appropriate to think of running your index finger over your rib cage. Your finger senses principally the rib bones below the skin, not the skin itself.

Fig. 7 Two fibroblasts in culture have been imaged using contact mode AFM during which there is significant cantilever tip pressure that reveals the structure below the surface. The cell images are presented in two different visual modes. In both cases, the network of cytoskeletal and stress fibers undergirding the plasma membrane are apparent. Scan areas are (a) 130 x 130 um, and (b) 100 x 100 um.

In Fig 7, for example, the cytoskeletal and stress fibers lying below the external membrane of a 3T3 cell are exhibited following a scan over the cell surface. The depth sampled by the probe can be regulated to some extent by adjusting tip pressure. The images in Fig 7 were recorded in contact mode where tip pressure and lateral resistance were useful forces for the purpose of the study. Just as the stress fibers appeared in Fig 7, in Fig 5a-d, where a cell was undergoing division.
division, the increased volume and counter pressure of the dividing nucleus served to make that a prominent feature of the series of images of a living fibroblast.

**VIRUSES:** Viruses that infect plants, animals and microorganisms range in size from about 16 nm diameter in the smallest case to about 750 nm in the largest. Icosahedral plant viruses seldom exceed 50 nm diameter and their satellite viruses 20 nm. Phage heads tend to be about 100 nm in length and width, animal retroviruses about 150 nm, animal pox viruses and large isometric algal and insect viruses about 200 to 400 nm in dimensions, and the very largest viruses known, such as mimivirus, may reach sizes of 750 nm diameter, the size of mycoplasms and very small living cells. All of these fall within the range of investigation by AFM.

**Fig. 8** In (a) is an AFM image of the surface of a satellite tobacco mosaic virus (STMV) crystal, and in (b) an image, not of a crystal, but a mass of closely packed brome mosaic virus (BMV) on the AFM substrate. In (b) the capsomeres of the T = 3 virus begin to emerge. In (c) and (d) are increasingly higher magnification images of turnip yellow mosaic virus (TYMV) in a hexagonal crystal. In all of the images the samples were in their mother liquors and the scans were acquired in tapping mode AFM. The scan areas are (a) 200 x 200 nm, (b) 150 x 150 nm, (c) 140 x 140 nm, and (d) 100 x 100 nm.

**Fig. 9** In (a) and (b) are images of cyanophages, myoviruses in this case, that infect cyanobacteria. They are similar to the terrestrial T4 phage that infects E. coli bacteria. The extended tail apparatus and firm heads indicates that the phage have not yet extruded their DNA. In (c) and (d) are two cyanophage that have expelled their DNA. This is indicated by the contracted tail sheaths and the collapsed heads. These images were obtained from air dried samples where full heads maintain their shapes while empty heads are readily recognized by their collapse. Tail fibers, in all cases, can be seen extending from the base plates. The scan areas are (a) 400 x 400 nm, (b) 400 x 400 nm, (c) 340 x 340 nm, and (d) 300 x 300 nm.

Details of virus particles less than 30 nm diameter are difficult to resolve unambiguously by AFM, but when they form vast arrays or arrange themselves periodically in crystals, as in Fig 8, the eye tends to average structure and often capsomeres can be discriminated. In addition, images of crystals can be subjected to digital filtering of different types, as, for example, by Fourier filtering [7, 13]. Bacteriophages, because of their distinctive architectures of icosahedrally derived heads, helical tails, and attachment fibers are easily recognized and imaged, as in Fig 9, and even capsomeres on the heads can be resolved as seen in Fig 10 [14, 15]. Retroviruses, such as MuLV and HIV are roughly spherical particles, but their exteriors do not exhibit any symmetrical arrangement of proteins. The retroviruses display surfaces having a tufted appearance due to clusters of receptor proteins poised to engage host cells [16, 17]. Examples are seen in Fig 11. Pox viruses too lack any external symmetrical distribution of proteins, and in fact like many animal viruses, are coated by one or more lipid membranes, or bilayers. *Vaccinia*, a close relative of smallpox virus, is an example and is shown in fig 12 as imaged by AFM [18].

**Fig. 10** In (a) and (b) are increasingly higher magnifications of a mutant of T4 bacteriophage that fails to make the external capsid proteins hoc and soc that normally mask the capsids. In both AFM images, obtained in the culture medium, the hexameric and pentameric capsomeres of the icosahedral heads are clearly visible, allowing the investigator to directly determine the triangulation number of the icosahedron. Scan areas are (a) 90 x 90 nm and (b) 50 x 50 nm.
Fig. 11 In (a) through (d) are four high magnification, high resolution images of virions of the retrovirus Moloney Mouse Leukemic Virus, which has a diameter of about 120 nm. The tufted appearance of the protein clusters on the particle surfaces, the target cell receptors, are clearly evident. These AFM images were obtained in fluid where biological structure is best preserved.

Fig. 12 An aggregate of three vaccinia virus on the AFM substrate after air drying. The more or less rectangular shape of the virions only appears after drying and is not evident for samples investigated in fluids.

A large iridovirus known as PBCV-1 that infects algae is shown in Fig 13b. Such viruses are bounded by 20 large equilateral triangular plates tiled with coat proteins of three fold symmetry. The final architecture is in fact icosahedral and PBCV-1 has a remarkable triangulation number of 169 [19]. The facets of these “large cytoplasmic viruses,” so named because they replicate entirely in the host cell’s cytoplasm, can be scanned by AFM with sufficient acuity that the individual coat proteins, and proteins which form the fivefold vertexes can be resolved [20].

Fig. 13 In (a) is a single Ty3 retrotransposon particle, a pseudo retrovirus that infects yeast. The Ty3 particle is icosahedral and the pattern of hexameric and pentameric capsomeres on its surface allowed direct determination of its T = 7 triangulation number. Its diameter is about 60 nm. In (b) is an AFM image of a virion of the algal iridovirus PBCV-1. The icosahedral virion has a very high triangulation number of 169 and is composed of vast arrays of a triangular capsid protein that forms a honeycomb network on its surface.

Fig. 14 This abbreviated series of AFM images illustrates the process of enzymatic and chemical dissection of a large virus, in this case the pox virus vaccinia. In (a) is an intact virion. In (b) the virion after treatment with detergents and a reducing agent which reveals the presence of the lateral body and the texture of the inner core of the particle. In (c) are the empty sacs, presumably of lipid membrane and protein, that encaseidate the viral genome. In (d) is the massive amount of DNA spilled by vaccinia particles when the nucleic acid is induced to burst from the sacs seen in (c).

Figures 14 and 15 illustrate the process of nanoscale dissection described above as applied to two large viruses. The largest virus currently known in nature is mimivirus, a 750 nm diameter, large cytoplasmic virus that infects amoeba; [21, 22]. In successive shells, starting from the exterior is a forest of heavily glycosylated surface fibers tipped by special proteins. Upon treatment first with glycosidases and then by a combination of proteinases, the fibers are removed to reveal a layer of matrix protein below. Further proteolytic degradation removes the matrix protein and exposes an icosahedral capsid and an unusual “stargate” apparatus that is peculiar to mimivirus. It functions in DNA release once the virus enters the amoeba. Finally, the capsid can be induced to regurgitate an interior membrane sac containing nearly 1000 genes of double stranded, highly compacted DNA that is thoroughly complexed with associated
proteins [21]. Similar procedures have been applied to dissect vaccinia virus shown in Figure 14 [18], and to explore the assembly of various viruses inside their host cells.

Fig. 15 An abbreviated series of AFM images illustrating the dissection of another large virus, the amoeba virus known as mimivirus. In (a) are intact virions coated with hundreds of surface fibers, but nonetheless exhibiting the unusual star shape apparatus that, aside from size, is its principle characteristic. In (b) after treatment with lysozyme and several proteases, the “stargate”, an accessory apparatus responsible for the emission of the viral DNA inside the host cell is revealed in full. In (d) we see the icosahedral capsid of the mimivirus and the honeycomb arrangement of the capsid proteins with depressions separated by 14 nm. In (d) DNA from a burst virion is displayed on the AFM substrate showing it to be heavily complexed with accessory proteins.

Fig. 16 In (a) is a 3T3 fibroblast cell in culture before infection with Moloney Mouse Leukemic Virus (MuLV). The very prominent bulge in the cell at lower left is the cell nucleus. In (b) is a low magnification image of the surface of a MuLV infected 3T3 cell where viral particle (bright spots) can be seen budding from the cell. In (c) and (d) are AFM images at higher magnification of viral particles emerging from the cell surfaces. Scan areas are (a) 40 x 40 um, (b) 5 x 5 um, (c) 300 x 300 nm, and (d) 430 x 430 nm.

Fig. 17 If 3T3 fibroblasts in culture are infected with a mutant MuLV virus deficient in the capability to properly bud from the host cells, then AFM images like those in (a) and at higher magnification in (b) are recorded. The virus, unable to properly separate from the host cells, produce the comet shaped protuberances on the cell surfaces. Scan areas are (a) 30 x 30 um, and (b) 10 x 10 um.

VIRUS INFECTED CELLS: AFM imaging of cells and viruses is combined in investigations of virus infected cells. Such studies may explore not only the natural process of infection, but also infection with mutant viruses, or infection in the presence of drugs or other bioactive agents [17, 23, 24]. In Fig 16 are cell surfaces of 3T3 fibroblasts infected with MuLV and at the highest magnification, the budding of the viral particles through the plasma membrane can be clearly visualized. If a mutant MuLV is used in place of the native virus for infection, the virus release tends to fail or be incomplete and a very different picture of virus emergence is obtained in Fig 17 [23]. In Fig 18 are human lymphocytes in culture, and in Fig 19 one such lymphocyte massively infected with HIV. Again, the budding viruses are well resolved. Figure 20 shows a healthy, dried amoeba cell, compared with a corresponding amoeba heavily infected with mimivirus.
**Fig. 18** In the four frames (a) through (d) are AFM images of four human lymphocyte cells in culture that illustrate the enormous variability in their external appearances, and the complex arrangements of membranes, microvilli, and other complicated structures on their surfaces.

**Fig. 19** A human lymphocyte in culture is massively infected with HIV seen budding through the surface of the cell.

**Fig. 20** In (a) is a normal, uninfected amoeba cell that has been air dried on the AFM substrate, has split open, and has subsequently been scanned. The plasma membrane is evident and the nucleus, flattened, has moved to the right side. In (b) is another amoeba in a late stage of infection with mimivirus. Thousands of the virus can be seen gathered around the periphery of the cell. The remnant of a viral “factory” is the light mass within the cell in the lower left. Scan areas are 60 x 60 um.

The value of AFM for studying virus host interactions has been largely neglected to this point. It should be clear, even from the few examples presented here, however, that AFM offers an alternative approach to EM and other microscopies that is not competitive, but is complementary. The images and the information they present are fundamentally different than those produced by other techniques, they contain different kinds of information, and they add significantly to our conception and understanding of biological structure and phenomena. In particular, AFM presents the direct imaging of cellular dynamics at the nanometer scale. It also allows the visualization of pathological states and processes as illustrated here by viral infection.

**References**


