Investigation of Viruses Using Atomic Force Microscopy

Introduction

The recent outbreak of Covid-19 and the resulting global pandemic affecting billions of people worldwide has shown how a nanoscopic particle can change the world around us. An organic entity, a hundred nanometers (nms) in size, composed of proteins, lipids and RNA, and hardly considered to be a living organism, is responsible for more than 1 million deaths [1], millions of job losses, and billions of $/€ in economic losses within just 9 months of its emergence.

For a long time, researchers debated on the existence of small, microscopically invisible pathogens suspected to be responsible for multiple diseases in plants and animals. Only the discovery of the electron microscope in 1933 made it possible to visualize them for the first time. Since then, thousands of viruses have been imaged and classified.

The advance of atomic force microscopy (AFM) in the early 1990s led to the imaging of biological samples in liquid with nanometer resolution and removed the electron microscope's dominance as the sole tool for the investigation of viruses.

In this application note, we would like to present some prominent examples of AFM's potential for investigating various types of viruses. Not only AFM's imaging capabilities will be highlighted here, but also its ability to measure the mechanical properties of viruses, their interactions with and adhesion to cells, and electrical properties.

High-resolution imaging of viruses

AFM's ability to visualize individual virions in a liquid environment makes it a very attractive tool. The biggest consideration (relevant for any AFM imaging technique) is the proper immobilization of the sample.

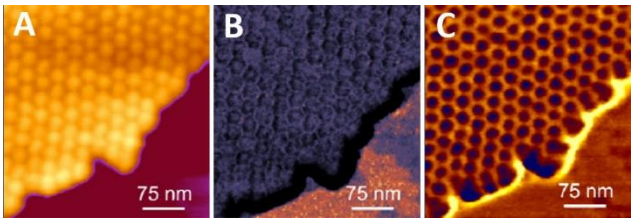

Fig. 1: High resolution imaging of genetically modified tomato bushy stunt virus capsids. *Virus capsids support each other enabling stable and higher resolution imaging. Imaging was done in buffer solution using the QI mode. (**A**) Height image (range: 30 nm), (**B**) Young's modulus map (range: 3 GPa), (**C**) Tip-sample adhesion map (range: 2 Nn).*

The majority of known viruses are spherical or cylindrical in shape and are several tens to several hundreds of nms in height. Weak immobilization, high imaging forces and/or scanning rates could easily lead to virus detachment from the surface. Clusters or 2D crystals of viruses (Fig. 1), where individual virions support each other and lateral motion is restricted, can be easier to image and higher resolution achieved. Due to their glyco/lipo-protein structure, virions could be isolated on a flat surface using standard protocols for molecule immobilization [2, 3], or by coating the surfaces with adhesion molecules to facilitate attachment (e.g. poly-L-lysine). Hydrophobic surfaces, like highly oriented pyrolytic graphite (HOPG) or silanized glass have been reported to immobilize herpes simplex virus (HSV)-1 [4]. As most viruses are >20-50 nm in size, an atomically flat substrate is not needed for imaging and regular glass could be used. Some reports, however, indicate that glass, as opposed to mica and HOPG, can induce capsids disassembly and DNA release [4]. The ability to operate at different temperatures allows AFM users study temperature induced degradation of the viruses. One of the recent works by Sharma *et al.* demonstrated that a mildly elevated temperature (34 °C) can rapidly cause dramatic degradation of SARS-CoV-2 virus-like particles both in wet and dry conditions [5].

AFM imaging modes for virus visualization

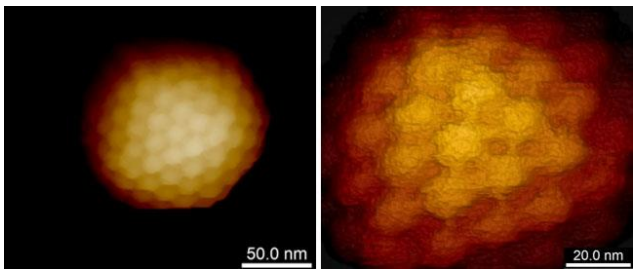

Fig. 2: High resolution imaging of the herpes simplex virus capsid. *Single virus capsid immobilized on silanized glass and imaged in buffer solution using PeakForce Tapping mode and Bruker PEAKFORCE-HIRS-F-B probe. The height scale is 110 nm (left image) and 50 nm (right image). Individual capsomers are clearly seen. Sample courtesy of Prof. A. Evilevitch, Univ. of Illinois at Urbana-Champaign, USA.*

Despite their robustness and ability to withstand severe environmental conditions, viruses appear to be sensitive to *in vitro* AFM imaging, especially when high-resolution is aimed for. To utilize the full imaging potential of AFM, extremely low imaging forces are required to resolve fine structural details like proteins or capsomers on the virus surface (Fig. 2).

Bruker is proud to be the inventor of the modes that have revolutionized high-resolution imaging in liquid: **Tapping mode** (1994), **PeakForce Tapping (PFT) mode** (2010), and **Quantitative Imaging (QI) mode** (2011). They have not only made high-resolution imaging in liquid possible but have also made it easy to use, regardless of the AFM experience. The first high resolution images of native infectious SARS-CoV-2 virions were acquired using the QI mode [6]. For more information about these modes, please refer to the following application notes [7–15].

Imaging virus uptake and release

Viruses cannot function and multiply by themselves, they need a host organism, which can be either a bacterium, yeast, plant, or mammalian cell. Modern atomic force microscopes can easily visualize all of the above in native physiological conditions, with a resolution down to 50-100 nm on living samples and 20-50 nm on fixed specimens. AFM enables the visualization of the remarkable process of virus uptake and release *in vitro.* By making small adjustments to the forces when imaging soft mammalian cells with AFM, it is possible to visualize either structures on the surface of the cell, or, by applying a stronger force, to see the cytoskeleton and other structures beneath the plasma membrane (Fig. 3 A,E).

The Bruker PeakForce Tapping (PFT) and QI modes can record force-distance curves at every pixel of an image, making it possible to reconstruct the so-called topography at zero force, regardless of what imaging force was used (Fig. 3 C,E). It is, therefore, possible to visualize: (i) viruses attached to the cell membrane, (ii) partially internalized viruses, or (iii) viruses inside cells which have just been internalized or are about to be released (Fig. 4).

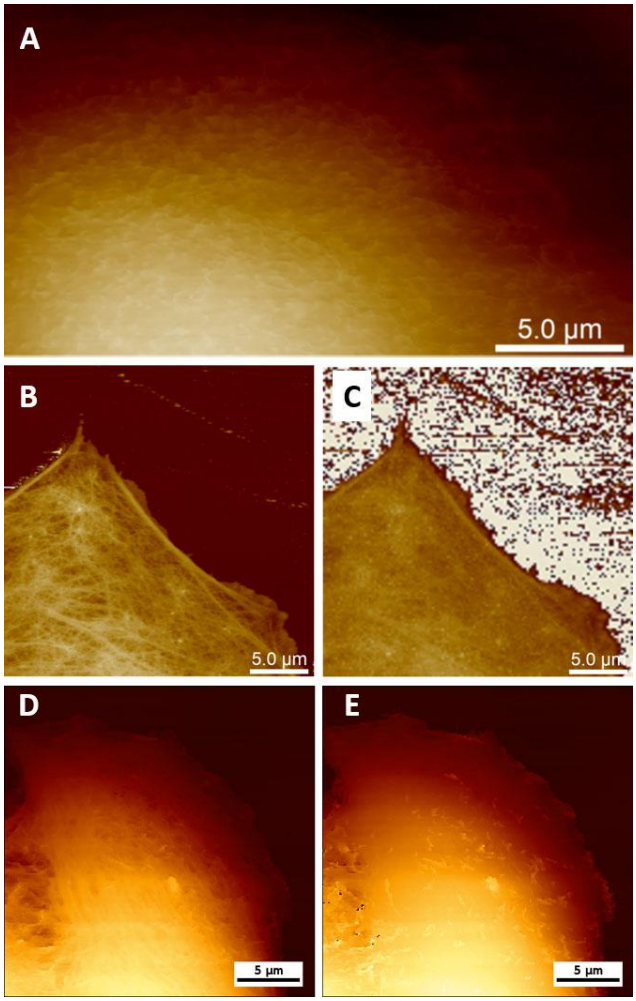

Fig. 3: Live cell imaging using AFM. *(**A**) Low force PFT imaging enables visualisation of microvilli on the surface of MDCK cells. (**B**) High force PFT imaging enables the visualisation of structures beneath the cell membrane (e.g. cytoskeleton) on MC3T3 cells (sample courtesy of Simone Weigel, IBG KIT, Karlsruhe, Germany). (**C**) Real Height (image at zero force) of the image (B) calculated in NanoScope Analysis. (**D, E**) Topography of the Vero cell imaged using QI mode at 300 pN (D) and reconstructed at 0 pN (E).*

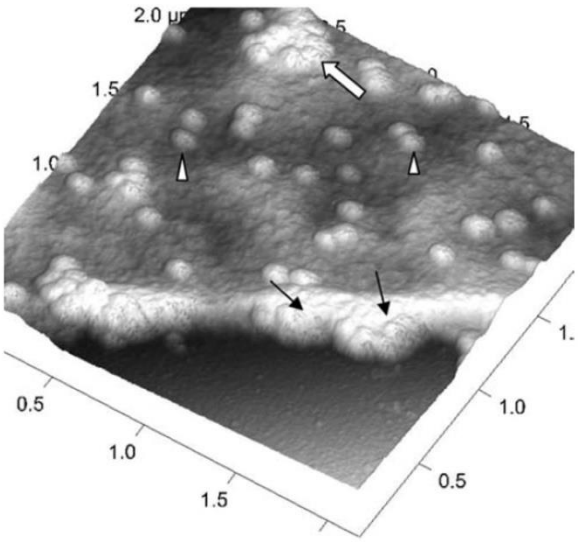

Fig. 4: A three-dimensional AFM image of a Vero cell infected with severe acute respiratory syndrome (SARS)–associated coronavirus. *Many intracellular viruses are visible just under the plasma membrane (arrows). Extruded virus particles are present on other areas of the cell surface (arrowheads). The thick white arrow shows a large clump of virus particles just beneath the plasma membrane. Adopted from* [16] *under open access usage.*

The latest developments in high-speed imaging and the introduction of the Bruker NanoWizard ULTRA Speed 2 AFM with scanning capabilities of up to 10 frames per second enable the study of virus uptake and release at high temporal resolution, well above the speed it occurs at *in vivo* [17].

Probing the mechanical properties of viruses

As mentioned above, virus particles can be affected and even damaged if high imaging forces are used. It has also been reported that retroviruses like human immuno-deficiency virus (HIV) and moloney murine leukemia virus (MLV) undergo both structural and mechanical changes during their maturation process. Matured virus particles were shown to be two-fold (for MLV) to ten times (for HIV) softer than their immature counterparts [18, 19]. As there is a strong correlation between virus stiffness and its ability to enter target cells [19], probing the mechanical properties of these nanoobjects is of crucial importance.

The ability of an atomic force microscope to apply a precise load in the range of pN to µN with nanometre spatial resolution, makes it a perfect tool for probing the mechanical properties of viruses. Modes such as single-point ramping or spectroscopy on a single viral capsid can be complemented with the simultaneous imaging and mapping of mechanical properties with the PeakForce Quantitative Nanomechanical Mapping (PF-QNM) and QI modes. Figure 5 shows an example of how single-force spectroscopy can be used to probe the stiffness of HSV-1 capsids. After acquiring the AFM image of the virus particles, the AFM probe can be positioned precisely on top of the individual capsid for indentation measurements. Subsequent treatment of the capsids with genome releasing chemicals (e.g. guanidine hydrochloride, GuHCl) allows *in situ* measurement of chemically induced stiffness change (Fig. 5 bottom).

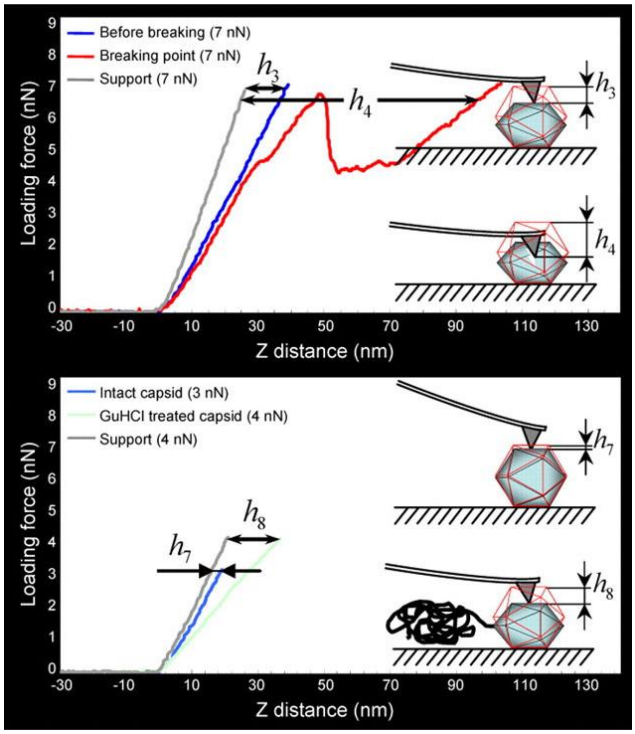

Fig. 5: Mechanical probing of HSV-1 capsids. *(**Top**) The threshold loading force of 7 nN causes mechanical failure of the capsids. The linear curve (dark blue) was taken when the capsid was still intact, the next round of probing (red curve) caused mechanical failure of the capsid. (**Bottom**) Application of GuHCl induces genome release and consequently leads to decreased stiffness of the capsids (light blue curve is taken from intact capsid, that in light green from GuHCl-treated capsid). h_3 to h_8 represent the indentation values. Images are adopted with permission from [4], J. Cell Sci.*

Measuring virus adhesion to host cells

For most viruses, the beginning of the life cycle starts with absorption to the host cell. There are multiple ways in which this occurs, however, the main issue common to all is that there must be some kind of structural complementarity. There must be a structure on the virus that recognizes a

structure on the host cell to which it can bind. For example, HIV binds to a receptor on T-cells, SARS-CoV-2 binds through its S like glycoprotein spike to the angiotensin-converting enzyme 2 (ACE2) receptor on the cell surface [20]. AFM is ideal for characterizing individual virus binding events to host cells both at high temporal and spatial resolutions.

The example below shows that PF-QNM in combination with a virus-functionalized AFM probe not only enabled imaging of the virus binding associated receptors on the surface of living cells but also the quantification of the kinetic and thermodynamic parameters of virus-cell surface receptor interactions [21]. Individual enveloped rabies virus (RABV) particles were covalently attached to the AFM probe through the flexible polyethylene glycol (PEG) linker (Fig. 6).

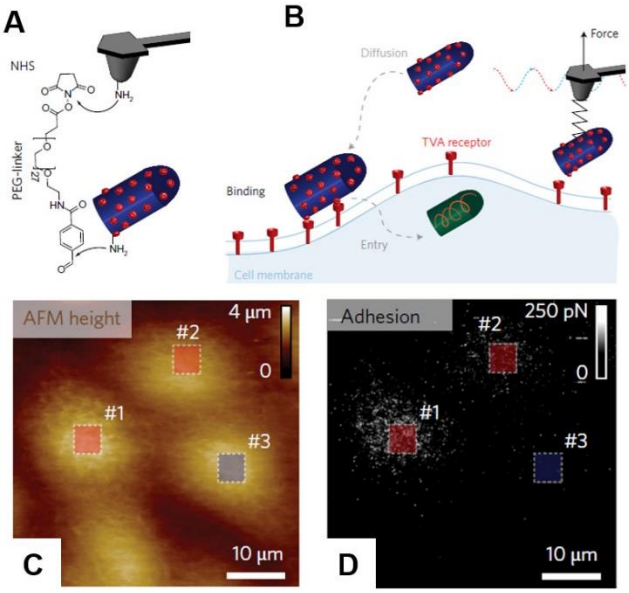

Fig. 6: PF-QNM AFM imaging and simultaneous detection of virus binding. *(**A**) AFM tip was functionalized with PEG_{27} spacer grafted to a free amino group of the EnvA-RABV glycoprotein. (**B**) Scheme of the experiment. Before cell entry, the virus must diffuse and attach to the cell surface. This initial attachment of the virus to the cell involves specific binding of EnvA glycoproteins to complementary TVA receptors. The specific binding of the virus activates EnvA, after which the virus enters the cell via membrane fusion. Height (**C**) and Adhesion/Recognition (**D**) images of mapping EnvA–RABV virus binding to MDCK–TVA cells. Images are adopted with permission from [21], Nature Nano.*

The virus was modified to carry the Avian Sarcoma Leukosis virus subgroup A (EnvA) glycoprotein on its surface instead of the typical glycoprotein G. Such a modification allows the virus to bind to MDCK cells expressing an avian tumour virus receptor (TVA). As discussed above, the PF-QNM mode, executing force-distance curves, can probe mechanical properties by fitting the approach or retraction section of the curve with a corresponding contact mechanical model. In this example, the retraction section of the curve was used to detect and measure the specific unbinding forces between the EnvA on the virus attached to the AFM probe and the TVA expressed on the cell's surface. Coupling AFM with a confocal microscope enables verification of the presence of TVA receptors on the surface of MDCK cells using fluorescent labelling [21]. The free-energy landscape of the virus-receptor bond was analysed by plotting detected rupture forces over the loading rates ($LR=\Delta F/\Delta t$). To obtain information on the kinetics of the ligand-receptor bond, the force curve acquisition (or dynamic force spectroscopy) must be executed at various loading rates. Technically this means repeating the measurements at different pulling rates. The advantage of the PF-QNM approach used here lies in the sinusoidal nature of the probe movement, meaning that the tip velocity changes during the oscillation cycle. In the work presented here, it allows researchers to achieve a loading rate spanning three orders of magnitude without the need to repeat the measurements. Multiple virus-receptor bonds were detected, the first one forming for a relatively short duration and low free energy, which, however, it is suggested increases quickly (≤1 ms) as additional bonds rapidly form [21].

Several critical elements need to be considered for such measurements. Performing experiments under physiological conditions (temperature and pH) is very important, as these parameters can affect both the cell's physiology and virus binding. The applied forces should not exceed several hundreds of pN (500 pN was used in this work), in order to maintain a gentle contact between the attached cargo and the soft, dynamic cell surface. The spring constant of the AFM probe needs to be low enough to enable gentle imaging while being sensitive enough to detect weak unbinding forces. The probe does not need to be sharp, as high contact pressure will be applied to the cells, even when using soft cantilevers. One considerable problem when doing force-curve type mapping in a viscous liquid environment is hydrodynamic drag. During measurements, the AFM probe travels a distance of hundreds of nms to the cells and back at a velocity of several hundreds of micrometres per second. The cantilever, therefore, acts like a paddle going through the liquid. To minimize the hydrodynamic drag, it is better to employ as small cantilever as possible, with the tip being as tall as possible, which is, of course, not trivial from a probe engineering point of view.

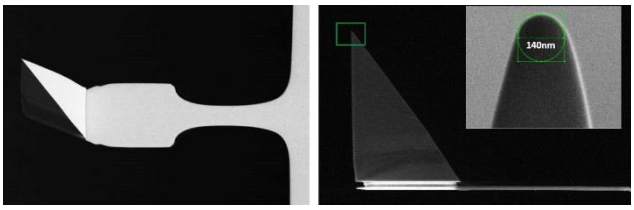

Fig. 7: Scanning electron microscopy image of a PFQNM-LC probe. *The 19 µm tip is mounted on a paddle-shaped 54 µm long cantilever. The insert shows the sharpened end of a tip made up of a 0.8-1 µm long protrusion on top of the pyramidal structure with an opening angle of 15 degrees and an end radius of 70 nm. The "paddle"-like cantilever shape helps minimize hydrodynamic drag and keep the spring constant low (0.1 N/m nominal, every probe comes pre-calibrated).*

The probe used in this work was the Bruker PFQNM-LC (live cell) probe specifically designed for imaging and mapping the mechanical properties of living cells (Fig. 7).

Can we do both? Simultaneous adhesion and mechanical mapping of the virus-host complex

We have demonstrated that AFM can be used to effectively map the mechanical properties of viruses and measure the binding kinetics to the host cell. Correlating this information would be very useful, and therefore the question arises – can we do both simultaneously? Both PF-QNM and QI modes generate force curve-based images from which both tip-sample adhesion and the mechanical properties of the sample can be calculated.

One of the first examples of where structural, adhesion and elasticity images of complex biological samples were acquired simultaneously at high temporal and spatial resolutions and with biochemical specificity was demonstrated using PF-QNM together with a chemically modified probe to detect the extrusion of individual filamentous bacteriophages from the surface of living *Escherichia coli* bacteria (Fig. 8). The elasticity of the bacteria was measured simultaneously, showing that bacteriophage extrusion localizes at soft domains. These soft nanodomains might appear as a result of a disrupted peptidoglycan assembly as the bacteriophage building machinery is localized near the bacterial septum. Deep analysis of the force-distance curves recorded for bacteria infected with bacteriophage particles showed linear force behaviour very different from the forces observed on bacteriophage particles absorbed on solid surfaces. This characteristic force signature could indicate a true extrusion of bacteriophages from bacteria and not a non-specific adsorbance to the cell surface [22].

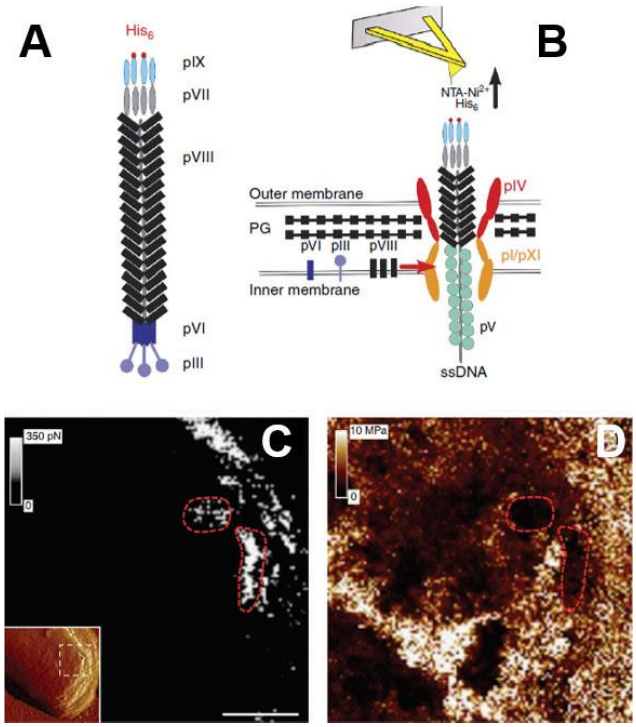

Fig. 8: Simultaneous imaging of the adhesion and elasticity of infected bacteria. (A) *Filamentous bacteriophages were genetically engineered to display His-tag on their pIX tail. (**B**) Single phages assembled in and escaping from the bacterial cell wall were detected via their His-pIX tail using an AFM tip modified with Ni^{2+} - NTA groups. The main components of the assembly machinery are shown in the image. Adhesion map (**C**) is directly correlated with elasticity map (**D**) recorded by the PF-QNM AFM. The inset in (C) is the high-resolution structural image of the bacteria (error signal), the white dash square area corresponds to (C) and (D). The red dashed lines emphasize the organization of the bacteriophages into soft nanodomains surrounded by stiff material. Scale bars are 125 nm (C, D). Images are adopted with permission from [22],* Nature Comm.

Electrical measurements of viruses

The ability to measure the electrical properties of materials at high spatial resolution makes AFM an attractive technique for many researchers. Bruker developments in combining its low force, high-resolution imaging PFT mode with multiple electrical characterization techniques have been widely accepted by the community, leading to numerous publications in this field. Modes such as **PeakForce TUNA** [23], **PeakForce KPFM** [24], **PeakForce sMIM** [25], **PeakForce SECM** [26] and **DataCube** [27] have made the simultaneous acquisition of mechanical and electrical properties possible on samples that, before, had been considered challenging: soft polymers and organic photo-voltaic materials.

Biological samples had rarely been investigated using electrical measurements, mainly due to the difficulties such measurements present. Furthermore, studying biological samples in native physiological conditions usually means performing the measurements in a buffer solution or even in growth media, or, explicitly in a conductive liquid. This, from both an AFM and AFM probe perspective, makes electrical measurements very challenging, if not impossible.

Here we would like to present two examples of the electrical study of viruses using AFM: (i) virus isoelectric point determination using chemical force microscopy, and (ii) electrochemical-AFM imaging of redox-immunomarked proteins on native potyviruses.

Virus isoelectric point determination using chemical force microscopy

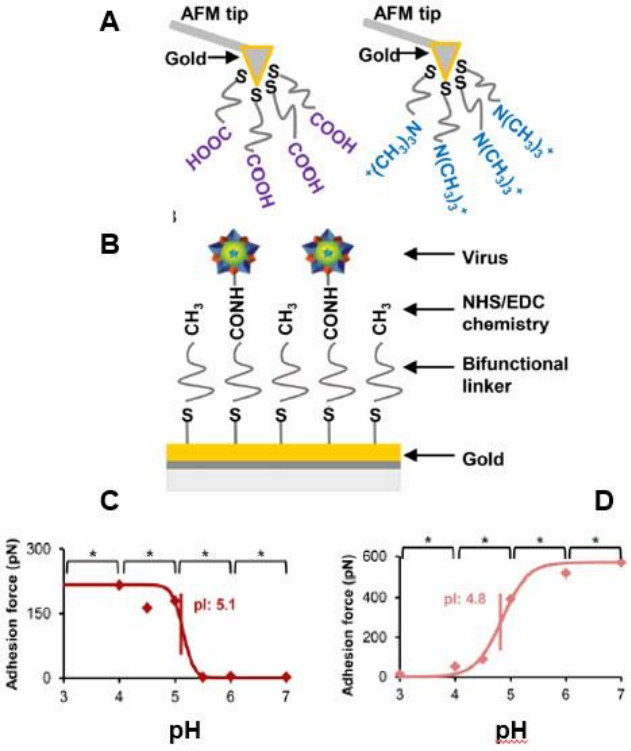

Fig. 9: Virus isoelectric point determination using chemical force microscopy. (A) *AFM probes were functionalized with either a negatively charged carboxyl acid group or a positively charged quaternary amine group. (**B**) Virus particles were immobilized with NHS/EDC chemistry. Porcine parvovirus isoelectric point determination using CFM with COO- probe (**C**) and CFM with NR4+ probe (**D**). Adapted with permission from* [32]*. American Chemical Society.*

Understanding single virus surface charge can be very useful and could serve multiple purposes: (i) estimate its adhesion to a charged surface [28], (ii) design filters that could purify viruses based on electrostatic adsorption [29, 30], and (iii) separate viral capsids that lack nucleic acids from full virions using anion exchange chromatography [31]. One way to characterize virus surface charge is to measure the isoelectric point (pI). Traditionally, a bulk viral solution is used to determine virus pI. However, due to heterogeneity between viral capsids, a single-particle method could provide more information. The AFM-based technique chemical force microscopy (CFM) enables the detection of the surface charge of viral capsids at the single-particle level and allows a comparison of the surface charge of different virus types [32]. Figure 9 depicts an example of CFM, where an AFM probe was functionalized either with a positive or a negative charge to determine the pI of non-enveloped porcine parvovirus (PPV) and enveloped bovine diarrhea virus. The adhesion forces between the functionalized tip and virus were measured using a force-spectroscopy approach in 20 mM citrate or phosphate buffers of varying pH (3-6) [32].

Electrochemical-AFM imaging of redox-immuno-marked proteins on native potyviruses

The above example deals with the charge of a single virion. However, the surface of a virus is decorated with various molecules, the detection and visualization of which is also of great importance. Such structures are either too small or too soft to be studied by AFM alone. AFM techniques such as AFM-tip-enhanced Raman spectroscopy [33] and AFM-infrared spectroscopy [34] could be of great help here. However, at present their implementation in liquid remains challenging. The AFM-based scanning electrochemical microscopy (SECM) using a nanoelectrode as a local probe to monitor electrochemical processes occurring at the surface could be a better solution here.

The Bruker BioAFM SECM module allows the investigation of electrochemical currents on the nanometre scale simultaneously to AFM topographical imaging [36]. In addition, with this module these experiments can be performed on inverted optical microscopes (IOMs) and the temperature can be adjusted as required by the specimen of interest. Figure 10, shows an example of individual lettuce mosaic virus (LMV) and potato virus A (PVA) particles decorated with redox antibodies being investigated by the Tapping mode based SECM technique [35]. Specific immunomarking using antibodies decorated with redox (ferrocenylated)-PEG chains, enables the *in situ* mapping and distribution of proteins on individual virus particles and makes the localization of individual proteins possible.

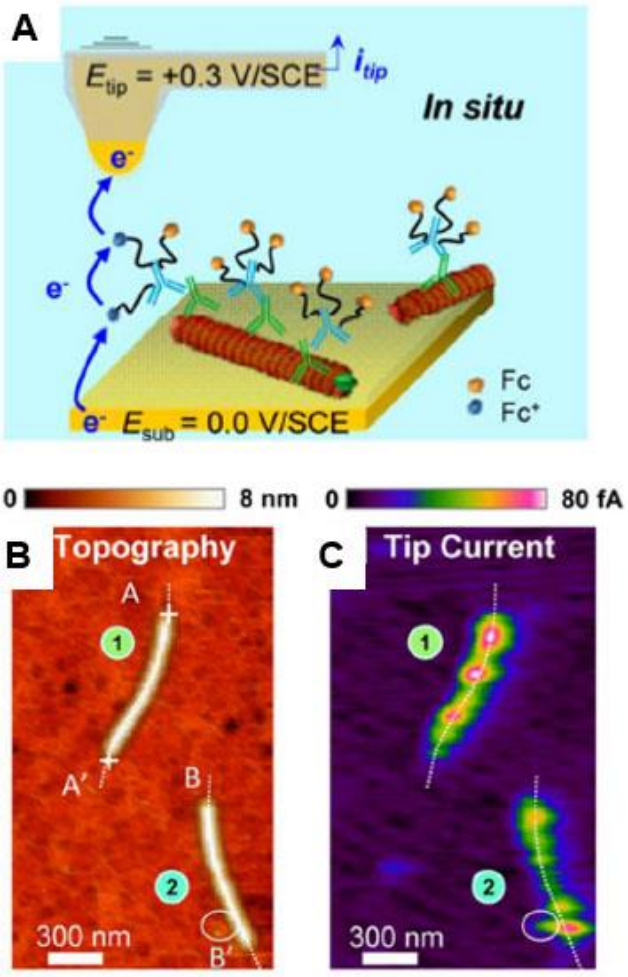

Fig. 10: In situ AFM-SECM tapping mode imaging of coat proteins-marked lettuce mosaic virus immobilized on a gold substrate. (A) *Scheme of redox cycling of the ferrocene (Fc) heads borne by the Fc-PEGylated antibodies generating the tip current. Simultaneously acquired images of topography* ***(B)*** *and raw tip current* ***(C)****. Imaging medium: 10 mM pH 7.4 phosphate buffer. Adapted with permission from [35]. American Chemical Society.*

Conclusion

This application note has only just touched on a few prominent examples of the potential of AFM to study viruses. It clearly shows that the modern AFM technology can not only perform high-resolution topography imaging but also the advanced mechanical and electrical characterization of virus particles and their life cycle.

Bruker continuously strives to expand the capabilities of the Atomic Force Microscopy technology by developing new techniques that support scientists in their Life Science research and which, we hope, will enable new and exciting discoveries in the field of virology.

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8. Pittenger B, Slade A, Berquand A Application Note #141 Toward Quantitative Nanomechanical Measurements on Live Cells with PeakForce QNM

9. Application Note - Investigation of living cells using JPK's QI mode

10. Application Note - QI mode - Quantitative Imaging with NanoWizard 3 AFM

11. Application Note - Quantitative and fast AFM co-localized with STED microscopy in living cell experiments

12. Pittenger B, Erina N, Su C Application Note #128 Quantitative Mechanical Property Mapping at the Nanoscale with PeakForce QNM

13. Slade A, Berquand A, DeWolf P Application Note #131 BioScope Catalyst: Accessing All Biological Size Scales with High Resolution AFM Imaging

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