Advancing Virology Research with High-Resolution AFM Imaging

OXFORD INSTRUMENTS
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Introduction

Infections caused by viruses have been ever-present in human civilization. Each century or decade has brought new epidemics or pandemics, threatening the well-being of millions, especially as people became more sedentary and concentrated in confined cities or communities. Just to name a few, we have seen the emergence of influenza (H1N1) in 1918, polio in the early 1900's, Human Immunodeficiency Virus (HIV) in the 80's, Severe Acute Respiratory Syndrome (SARS-CoV) in the early 2000s, and now, most recently, Covid-19 (SARS-CoV-2) in 2019. These, and other viral infections, require a swift and effective solution, typically in the form of anti-viral drugs or vaccines. In order to develop a treatment or vaccine, a clear understanding of how viruses assemble, interact with their host, elicit immune responses, reproduce, spread, and survive under different conditions is essential. This understanding would then enable researchers to decide which of these steps to target for the most effective treatment or preventative measures.

Historically, overall morphology (helical, icosa-/poly-hedral, spherical or complex) and viral surface structure such as capsomere arrangement (Figure 1) were predominately studied using electron microscopy (SEM, TEM), X-ray crystallography, and optical microscopy. Although SEM and TEM provide high-resolution images of capsids and infected cells, the samples need to be coated and dehydrated and the techniques work best with symmetrical or regular viral structures. Similarly X-ray crystallography provides exceptional resolution but requires symmetrical viral shapes in order to make crystals for analysis. It also does not work well with complex viruses that have tails or external membranes and works best with smaller viruses (<100 nm).[1] Optical microscopy, typically fluorescence, is great for studying the infection process in cells but is limited in resolution (typically ~200 nm). As a result, beginning in the 1990s, researchers started using AFM to image viral capsids in both air and liquid.[2-7]

The Atomic Force Microscope (AFM) has found broad use in the biological sciences largely due to its ability to acquire three-dimensional, high-resolution images of native structures such as biomolecules, cells, tissues, and viruses in fluid under near-physiological conditions. Since AFM is relatively non-destructive and requires minimal sample preparation (i.e. no coating, fixing, labeling, or treatment that is required for optical and electron microscopy), it is frequently used to visualize and monitor dynamic events. As a result, AFM has been instrumental in improving our understanding of the structure, function, and behavior of these biological samples. Another advantage of AFM is that it images individual molecules, viral particles, or cells, which allows observation of individual variability and response. This is in contrast with other techniques that may only provide an average measurement within a population.

Finally, AFM is unique in that it can be used to characterize the mechanical properties of a sample. Researchers can calculate the elastic moduli of individual viral particles (both wildtypes and mutants) to determine capsid strength and measure rupture force to ascertain capsid robustness. Both properties improve our understanding of how viruses can survive in their environments with the capsid protecting their compartmentalized genetic material while also allowing for eventual rupture of the capsid to release their DNA/RNA content, resulting in infection.

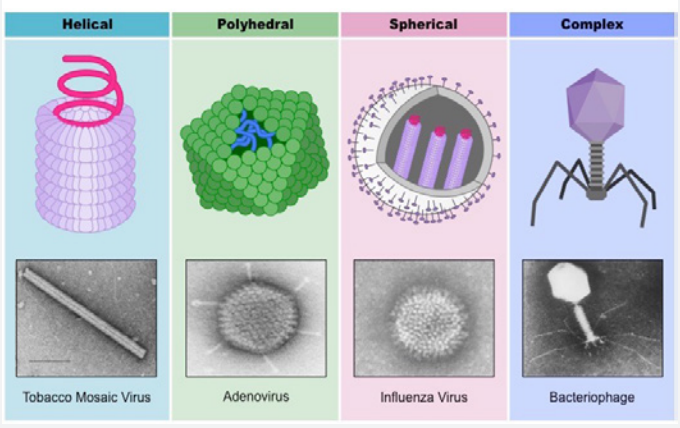

Figure 1: Classification of viruses based on morphology. From Cornell, B. 2016. Virus Classification. Available at: http://ib.bioninja.com.au. [Accessed 13 April 2020]

Fields of Virology Research

AFM research in the field of virology is extensive. We have seen images of fixed and native viruses isolated or attached to their cell host and interacting with a wide range of molecules and material under diverse conditions. We can summarize these studies into the following topics:

1. Easy identification of virus type based on morphology and size.

2. Detailed visualization of structures or capsomere arrangement of isolated viruses/capsids or virus-like particles (VLPs).

3. Direct observation of virus particles on surfaces of plastic, metal, skin, mucous membrane, etc. in native conditions (in air, at relevant temperature and humidity, under normal or UV light regimes).

4. Static and dynamic observation of the interaction of virus particles during infection and particle release of cells.

5. Measurement of how environmental factors such as temperature and pH affect the virus response.

6. Measurement of the effects of antibodies/drugs on viruses to help in the development of effective therapies and vaccines.

7. Characterization of biosensors used in diagnostics (i.e. surface roughness, contamination, etc.).

8. Nanomechanics of the capsids.

Due to the extensive work that has been done with AFM, we will divide the research under two broad topics – topography and dynamics and nanomechanical measurements. This application note will focus on the first topic, summarizing AFM data on high-resolution structure and dynamics of viruses. A second application note will summarize the AFM work completed on the mechanics of viruses.

Virus Structure

Due to the high-resolution imaging capability of the AFM, researchers set out to resolve nanometer sub-structures of capsids. For example, the McPherson Lab was one of the first research groups to publish high-resolution images of over half a dozen viruses, including Brome Mosaic Virus (BMV), Tobacco Mosaic Virus (TMV), and Herpesvirus.[8] Overall shape and capsomere arrangement were easily resolved with the AFM. Since then numerous viruses have been imaged including many plant viruses, influenza, herpes, hepatitis, Murine Leukemia Virus (MLV), Human Immunodeficiency Virus (HIV), and SARS coronavirus. Figure 2 shows a few examples of capsids acquired more recently with the Cypher AFM. Figure 2a shows a Herpes Simplex virus (HSV-1) capsid that was attached to a glass substrate. A "triangle" created by the arrangement of capsomeres, and outlined in black, is clearly visible. An average diameter of 118.96 nm (n=5) was calculated for the HSV-1 capsids; actual diameter is approximately 120 nm. The measured morphology and capsomere arrangement show good agreement with the molecular model of HSV-1, shown in Figure 2b. Figure 2c shows AFM images and the corresponding capsomere schematics of two distinct orientations of the Brome Mosaic Virus (BMV) depending on the arrangement of both pentameric and hexameric capsomeres.[9] Finally Figure 2d shows differences in viral height of Adeno-associated virus (AAV) particles due to differences in substrate binding. Full capsids (last image) have a diameter of 23 nm, very close to the expected value of 25 nm.[10]

Life Cycle

Viruses are technically non-living and require a host for replication. Although viruses are quite diverse in morphology, genetic composition, host and infection, they share a similar life cycle. Figure 3 depicts the life cycle of the influenza virus. AFM measurements have played an important role in many of these stages. Examples of how AFM has been used in some of these stages will be discussed in subsequent sections.

1. Attachment: commonly characterized as a key/lock interaction between viral surface proteins and cell receptors

2. Penetration: via endocytosis

3. Uncoating: release of genetic material

4. Biosynthesis of viral components

5. Assembly (maturation)

6. Release (lysis): viruses are released from the cell membrane

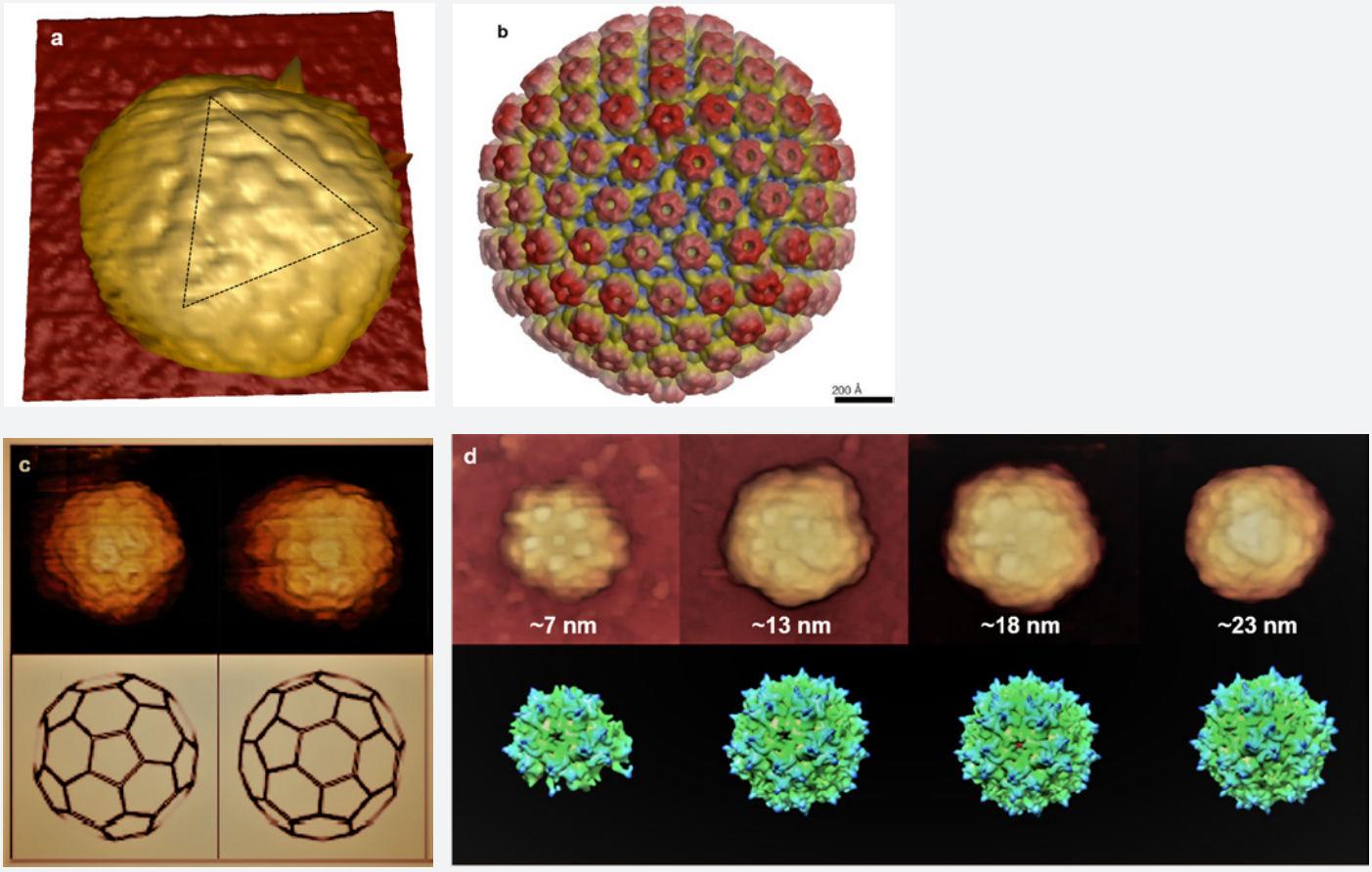

Figure 2: High resolution images of viruses acquired with the Cypher AFM. a) Herpes Simplex 1 (sample courtesy of Alex Evilevitch, University of Lund), b) 3D rendering of HSV-1, c) Brome Mosaic Virus (BMV), d) Adeno-associated virus (AAV).

Monitoring Dynamic Events

One of AFM's main strengths is its ability to monitor dynamic processes in real-time. For viral research, we can improve our understanding of viral assembly, the release of DNA/RNA from virus particles, commonly referred to as "genome uncoating," and viral infection of living cells. Imaging DNA with AFMs has become routine making it straightforward to quickly identify ejected DNA/RNA from the capsid, distinguish between RNA, double, and single stranded DNA based on height differences, and visualize nucleic acid arrangement and packaging, which varies from one virus type to another and is important in the development and release of new viral particles.[8]

Researchers have also studied the budding process in order to better understand the mechanism of release. Initially, viruses were statically observed exiting the cell surface; typically, these cells

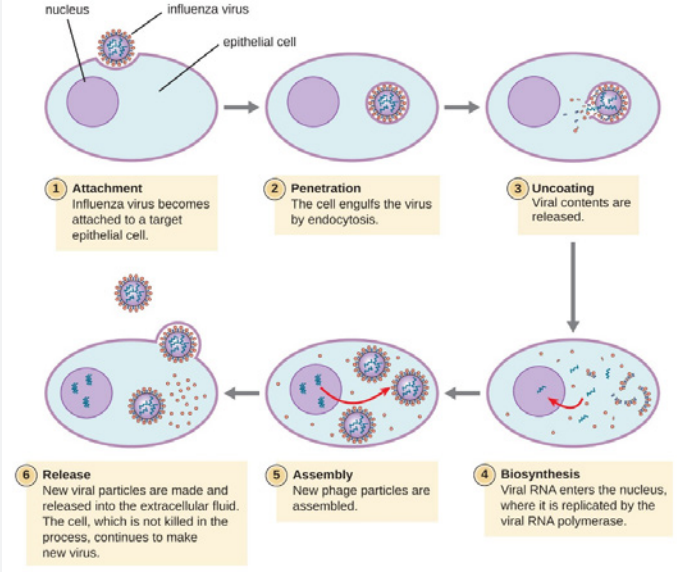

Figure 3: Viral Life Cycle. Courtesy of Lumen Learning: https://courses.lumenlearning.com/microbiology/chapter/the-viral-life-cycle/

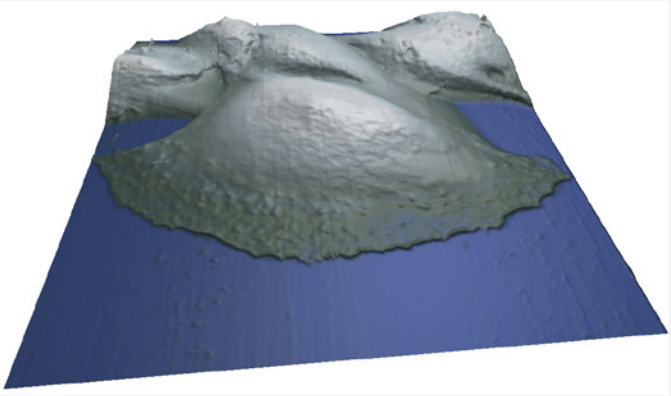

Figure 4: Height image of epithelial lung cell, 90 µm scan.

were fixed.[11] Then research shifted to capturing the dynamic events involved as the viruses are assembled within the cell, bound to the cell membrane, and then released outside the cell. Specifically, for retroviruses such as HIV and MLV, these viruses reorganize the host's actin cytoskeleton to assist in the successful release of viral particles.[12] For other viruses such as the coronavirus (SARS-CoV), a slightly different mechanism was observed by AFM. Infected cells had margins that were thickened and ruffled which appeared to aid in the release of viral particles.[13] Regardless of the mechanism, AFM was successful in resolving emerging viruses and changes in the plasma membrane. And since cells are commonly imaged in contact mode, AFM can not only resolve features on the cell surface but beneath the membrane. Figure 4 is a topographic image of an epithelial lung cell. Both the cell margin and small protrusions beneath the membrane are clearly resolved so any changes to the cell could be followed in real-time.

AFM can be used to determine the effect of environmental factors, such as temperature, humidity, and pH on viruses as these factors influence their functionality and transmission to host cells. Voros et al. (2018) studied heat inactivation of T7 bacteriophage viruses (nonenveloped, short tail), specifically the structural changes from ambient (20°C) to 65°C and 80°C. It is believed that heat inactivation causes a release of genome from within the capsid. At 20°C these nonenveloped viruses are predominately intact with a short conical tail (Figure 5a). There is some DNA release but, as seen in Figure 5a, the majority of capsids, including their tails, are intact. At 65°C there is a significant increase in DNA release from the capsids and a loss of their tails (Figure 5b). The background of the AFM image is covered with DNA strands, the tail complex has opened to release DNA, and the larger spherical structures (marked by the white arrowhead) are thought to be released capsid proteins. At an even higher temperature of 80°C (Figure 5c) there was a disassembly of some capsids even though they became more mechanically stable. DNA concentration in the background is even higher and in addition to capsid proteins, the larger globular structures are believed to be capsid wall fragments. This study confirms that DNA release does occur at temperatures above 65°C for this particular virus.[14]

AFM can also be used to observe the effect of chemicals, drugs, or antibodies on a virus in hopes of developing a successful treatment or vaccine. Hifumi et al. (2015) observed the effect of catalytic antibody (monomeric 23D4) binding to H1N1 influenza A virus surfaces. An H1N1 flu virus is shown both before and after antibody binding in Figure 6a and 6b, respectively. In Figure 6b, several "bumps" can be seen on the virus surface representing bound antibodies, in contrast to the

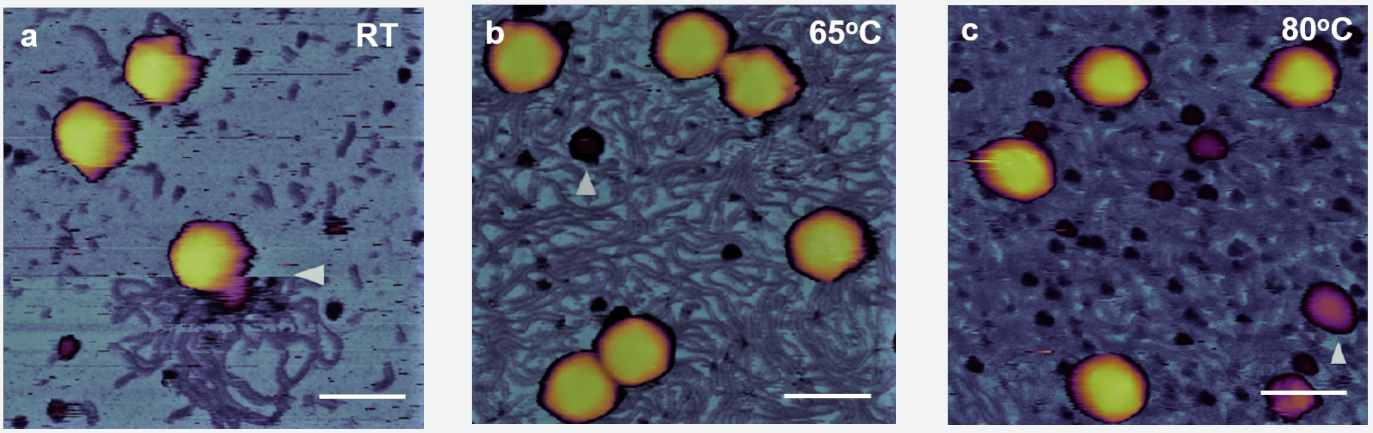

Figure 5: Temperature effects on T7 bacteriophage. a) room temperature (20°C), b) 65°C, c) 80°C, scale bar, 100 nm.

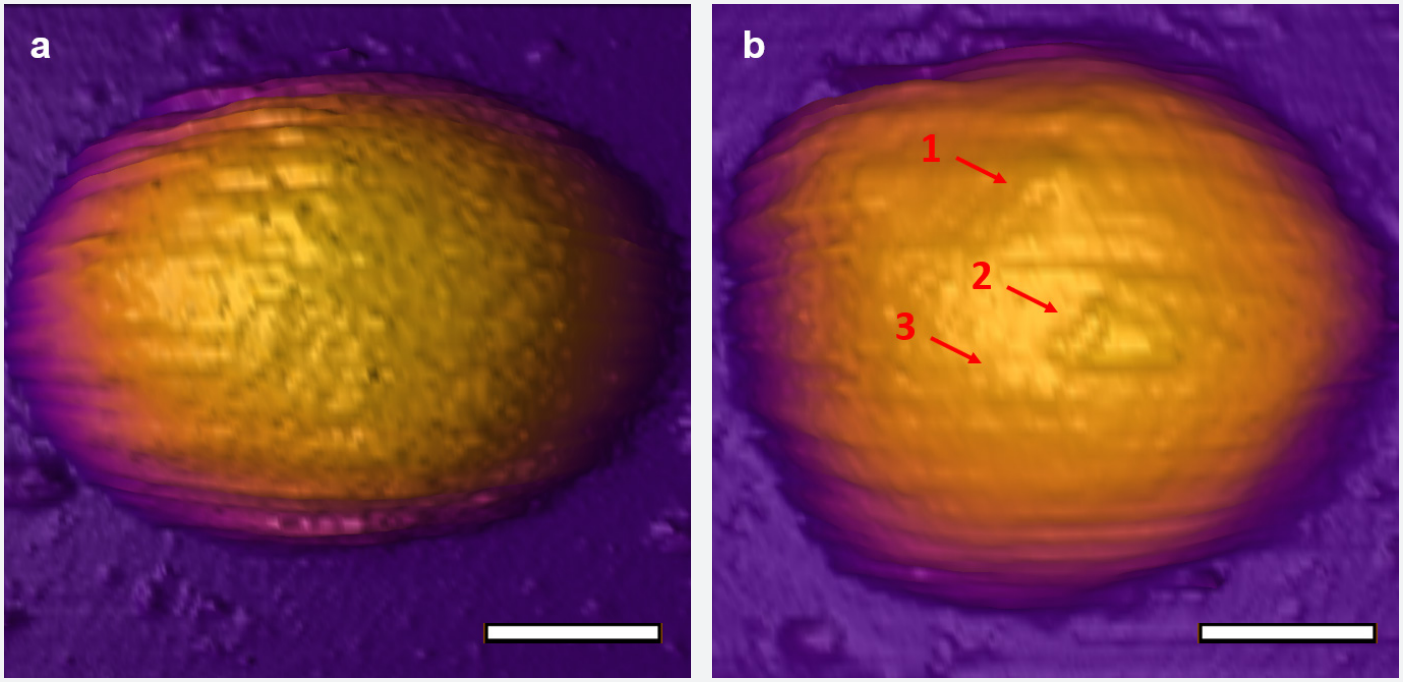

Figure 6: H1N1 influenza virus before (a) and after (b) binding of the catalytic antibody, monomeric 23D4. White scale bar is 40 nm. Red arrows 1, 2 and 3 mark one, three and two antibodies, respectively.

untreated virus (6a) which has a more uniform appearance. This work is important since we can clearly see an interaction between antibody and virus.[15] In theory, viruses could potentially be exposed to several different anti-viral compounds and AFM could be used to determine which ones have the greatest binding/effect on the virus.

Recent infections from the mosquito-borne Zika virus have prompted a renewed interest in understanding how this virus replicates in order to develop an anti-viral drug. One potential target is the RNA-dependent RNA polymerase, NS5, which is involved in replication. AFM was used to observe the macromolecular assembly of monomers and dimers into fiber-like structures of native NS5. With mutants of NS5s, which lacked the ability to form dimers, fibers did not form. Dimer formation is thus a potential target for an anti-viral drug.[16]

Another approach to controlling viral infections and outbreaks is to develop effective vaccines. Collett et al. (2019) used AFM to characterize the structure of Hepatitis C virus-like particles (VLPs). VLPs have proved to be effective vaccines since they cause an immune response and offer protection against future exposure to the virus but are completely non-infectious. AFM results showed differences in size and shape for the four VLP genotypes tested, helping to understand which genotypes would make the most effective vaccine.[17]

Finally, AFM is involved in the development of diagnostic tools. Although AFM is not known to be used in any clinical diagnostic products, it can be used in the development phase to qualify the surfaces of the biosensor devices and measure the deposition of substrate, reactants, and target molecules. To date, Lab-on-graphene-Field Effect Transistor (FET) devices have been developed to detect both influenza and Covid-19.[18,19] AFM can also easily measure surface roughness, so is important in the quality control of these diagnostic devices.

Conclusion

AFM has been successfully used to image capsids with nanometer resolution and observe dynamic events including virus/host cell interactions, DNA/RNA release from capsids, attachment of anti-viral molecules/antibodies to viral surfaces, and environmental effects on structure and functionality (i.e. release of genetic material) of viruses. This research has helped improve our understanding of how viruses behave during infections and where to focus future research in preventing and treating infections.

With the increased use of high-speed AFM imaging we will start to see more data acquired at higher temporal resolution. Figure 7 shows two frames out of a 0.2 frames/second movie of Hepatitis B

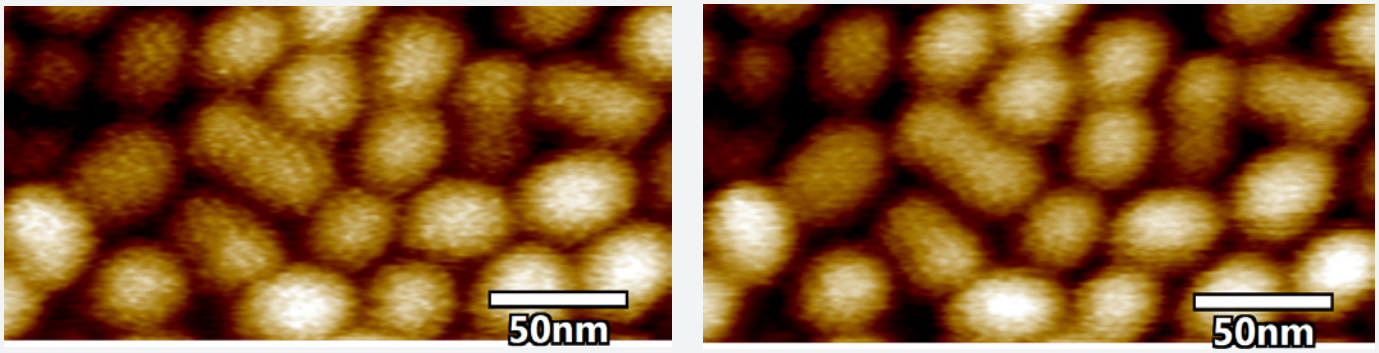

Figure 7: Hepatitis B viruses (HBV) imaged at 0.2 frames/second with Cypher VRS. Frames 12 and 24 are displayed. White scale bar is 50 nm. Sample courtesy of Profs. Shunichi Kuroda and Masumi Iijima, Osaka University, ISIR-SANKEN Lab (https://www.sanken.osaka-u.ac.jp/labs/soc/socmain_english.html).

Viruses (HBV). The spikes on each capsid represent the pre-S1 region of the viral envelope L protein and play an important role in viral infection. When comparing the structures in each frame, we can see some variability in their orientation. Future studies with high speed AFMs could monitor these dynamic changes and look at the interaction of compounds with viral surface structures such as these spikes.

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