JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Virus imaging tools

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Research Article

Volume: 4, Issue: 2
August 2020
Pages: 75-83

ABSTRACT

Viruses are very small physical particles that can not be observed with a normal microscope. Only the largest virus, the poxvirus can be seen in the light microscope. Other tools are required for a detailed examination of viruses in ultrastructural size. Especially for the last 20 years, these advanced and ongoing tools have been used in the investigation of the biological molecules and biological processes of viruses. With the help of virus imaging tools, viruses can be identified in clinical samples, thereby explaining the detailed life cycle of the virus. Thus, these tools help the creation of new and safe vaccines and antiviral medicine. In this review, electron microscopy (EM) tools were given as scanning electron microscopy (SEM), transmission electron microscopy (TEM), cryo-electron microscopy (Cryo-EM) since generally used tools based on EM. Besides EM-based tools, X-ray crystallography which is the basis for cryo-EM and atomic force microscopy (AFM) that is relatively economic and easy to apply compared to other microscopies were also described.

Article History

Received: 10.06.2020
Accepted: 25.08.2020
Available online: 28.08.2020

Keywords: virus, visualisation, equipments

DOI: https://doi.org/10.30704/http-www-jivs-net.750638

To cite this article: Sareyyüpoğlu B.(2020). Virus imaging tools. *Journal of Istanbul Veterinary Sciences, 4*(2), 75-83,

Abbreviated Title: *J Ist Vet Sci*

Introduction

Ultrastructural dimensions about viruses is an important step to get information about morphology, viral entry, replication dynamics, changes in a virus infected-cell. Thus, these tools help the development of new diagnostic and treatment methods against the viruses. The basis of ultrastructural studies was laid in the 1930s by the filtration of viruses through colloid membranes. The first virus imaging method is the electron microscopy (EM) (Risco and Carracosa, 1999; Gelderbloom and Hazelton, 2000; Wagner and Hewlett, 2003; Bartenschaler and Romea Brey, 2015). There are varieties of EM such as SEM, TEM, and Cryo-EM. After EM; other tools were developed as X-ray crystallography, nuclear magnetic resonance, cryo-EM, bioluminescent imaging, and lastly positron emission tomography (Cherry and Gambhir, 2001; Cook et al., 2003). In this review, EM techniques, X-ray crystallography, cryo-EM, and AFM tolls were described.

Electron Microscopy (EM)

Eectron microscopy (EM) has a greater resolution than light microscopy because electrons have a shorter wavelength than light. Therefore, EM allows the image to grow ×10.000 more than a light microscope. It gives information about the virus topography, morphology, composition using the high energy electron beam. Topography

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describes the surface properties of an object, how it looks, its texture, and the direct relationships between these properties and material properties (tightness, reflectability). Morphology, on the other hand, explains the direct relationships between the shape and size of the particles forming the object and the material properties (flexibility, durability) while the composition explains the direct relationships between the elements and building blocks forming the object and the material properties (melting point, reactivity, tightness) (Gelderbloom and Hazelton, 2000; Bartenschaler and Romea Brey, 2015). The first EM developed by Max Knoll and Ernst Ruska in 1930 (Risco and Carracossa, 1999; Goldsmith, 2009; Bartenschaler and Romea Brey, 2015). Then SEM and TEM were developed (Risco and Carracossa, 1999; Goldsmith, 2009; Bartenschaler and Romea Brey, 2015). In general, EM works with a mechanism similar to that of the light microscope, which is its optical equivalent but uses the focused electron beam instead of light to examine the sample. The electron flow, first shaped by the electron source, is accelerated towards the sample using a positive electrical medium. Then, with the help of metal, the finely focused monochromatic light is dropped directly on the sample using magnetic lenses, and then various interactions occur in the sample, affecting the electron beam. These interactions are determined and converted into an image format (Risco and Carracosa, 1999; Gelderbloom and Hazelton, 2000; Wagner and Hewlett, 2003; Bartenschaler and Romea Brey, 2015).

The sizes of viruses are below the visible rays. Therefore, shorter wavelengths are required for viruses to be observed in EM. EM accelerates electrons to give high energy and focus them magnetically. High energy gives electrons a short wavelength, making them smaller in size than viruses (Wagner and Hewlett, 2003; Bartenschaler and Romea Brey, 2015).

The sample preparation steps for EM can be briefly listed as detection, washing, dehydration, embedding, cutting, dying. The purpose of all these processes is to protect the specimen in its current state, to protect the specimen against the procedures to be performed, and to thin the specimen. Thus, sufficient contrast provided so that electrons with limited penetration characteristics can easily pass through the sample (Bartenschaler and Romea Brey, 2015). The electron acceleration and focusing process performed when the sample can be examined and observed under a vacuum. To achieve this, the sample must be completely dry and fixed. During the sample preparation, the image may be lost or altered during the subsequent effects of protein hydration. In other words, works under in vacuum and staining process destroy the sample. This is the main limitation of EM (Wagner and Hewlett, 2003; Bartenschaler and Romea Brey, 2015). EM can be used imaging of many viruses like parapoxviruses, herpesviruses, rotaviruses (Roingeard, 2008). EM was also the first tool used for COVID-19 virus imaging. In that study (Min Kim et al., 2020) after the virus isolated in Vero cells, it was visualized with EM, and confirmed by sequencing.

Scanning electron micrroscopy (SEM)

SEM is another EM type for the visualization of viruses. SEM scans the 3D-high resolution image of virus surfaces. It can achieve sub-nanometer spatial resolution, revealing topological and compositional features. It magnifies an object × 200.000 (Raza, 2012; Haan et al., 2019).

The image of SEM is created by sending electron beams into the sample. Electrons sent on the sample to give charge to the sample. The sample preparation for SEM must have the following properties; the sample must be fixed, dried, have a certain conductivity, and the charge must not be collected regionally on the sample. For conductivity, it is coated with a metal like gold, palladium, or aluminum. For SEM microscopy, it is not necessary to prepare the samples as thinly as in EM. However, in both microscopies, the sample prepared by cold or freezing (Raza, 2012; Haan et al., 2019).

The SEM technique was used to display retroviral gag proteins involved in the cell binding process of retroviruses including HIV, and the result of the study, it was determined that each virus population showed different variations (Parker et al., 2001; Raza, 2012; Haan et al., 2019).

The parts that make up the image in SEM are the electron gun that works as an electron source. The condenser lenses that form a thin electron beam by compressing the electrons coming out of the gun, the beam deflector that allows the electrons to scan the sample inline form, the lenses, the detectors that collect the reflected rays after hitting the sample. The electron beam is better compressed and denser by the photomultiplier tube (PMT) (Haan et al., 2019). A schematic diagram of the SEM are shown in Figure 1 (Raza, 2012).

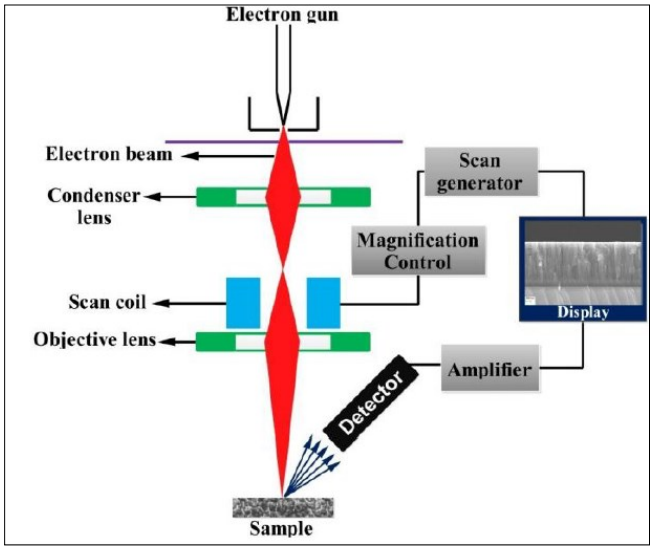

Figure 1. A schematic diagram of SEM (Raza, 2012)

Transmission electron microscopy (TEM)

The first TEM was demonstrated by Max Knoll and Ernst Ruska in 1931, Ruska was awarded the Nobel Prize in physics for the development of TEM contributed to the discovery of many types of viruses and served as a diagnostic tool for interior virus imaging. In the 1990s, the diagnosis of viral infections gained a lot of momentum with the help of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), and via TEM. TEM supported diagnostic methods by performing direct imaging without prior knowledge of the infected agent being investigated. Thus, it is still used today for the detection of infectious agents in situations where the molecular diagnosis is insufficient (Roingeard et al., 2019).

TEM magnifies the examined object ×5000.000. It can obtain information about morphology, crystallographic information, and structure of the virus. Similar to SEM, the electron gun works as an electron source. TEM works just like a slide projector. The projector reflects light through the slide. As the light passes through the slide, it is affected by the structures and objects on the slide. These effects are observed in certain parts of the light beam transmitted by certain parts of the slide. The image is projected on the screen to magnify this transmitted light. The screen made of phosphor (Roingeard et al., 2018; Bartenschaler and Romea Brey, 2015). Samples for TEM should be very thin and it usually stained neural acetate. Then, image transferred a fluorescent screen. Similar to SEM, The lenses of TEM are electromagnetic. This is the main difference from the light microscope. The image is formed around this electromagnetic field. The rest of the microscope suspended in the air except for its anode and cathode. The rays penetrate more easily through the sample when the voltage increased. However, in this condition, the contrast is diminished. The voltage used varies depending on the sample. Variable charge grits control the electron density, in other words, the image brightness. Image brightness can be changed by changing the cathode temperature (Bartenschaler and Romea Brey, 2015; Roingeard, et al., 2018). The layout of optical components in a basic TEM is shown in Figure 2 (Williams and Carter, 2009).

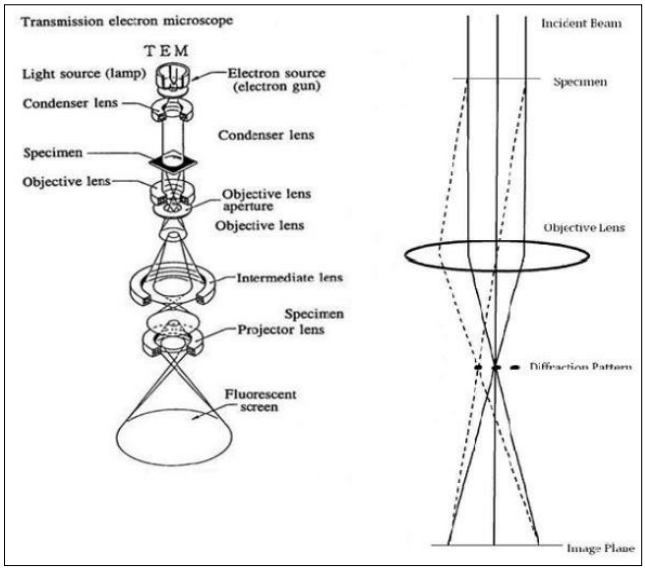

Figure 2. The layout of optical components in a basic TEM (Williams and Carter, 2009).

TEM and SEM are generally used two EM tools for virus imaging. Therefore, the differences between both should be explained in this review. The main difference between the TEM and SEM is the electron scattering feature. SEM uses scattered electrons, but TEM uses transmitted electrons. Therefore, SEM focuses on the sample surface like EM and its composition, whereas TEM provides more details about its internal composition. TEM magnified an object 5000.000x while the SEM 100.000x. In other words, SEM shows only the morphology of samples. TEM can show many characteristics of samples like morphology, crystallization, or even magnetic domains (Bartenschaler and Romea Brey, 2015; Haan et al., 2019). The main limitation in both techniques is that they are both expensive, required trained experts, toxic staining used, and like EM, sample dead during sample preparation because of vacuum and toxic staining.

X-ray crystallography

X-ray crystallography is a basic method to get information about the high resolution of a protein structure. X-ray crystallography provides atomic-resolution structures of proteins and small viruses in order to see the object. Visible rays have a wavelength greater than the distance between atoms. These rays are not enough to see molecules. Therefore, X-rays using electromagnetic radiation have a wavelength that is small enough to see atoms (Carter et al., 1997).

In 1953, Watson and Crick studied the X-ray diffraction model of crystallized DNA. The technique, which records the diffracted X-ray density and spatial distribution emitted from 3-D crystals, makes it possible to investigate many biological specimens. This diffraction model of the structure is measured by a mathematical method called the Fourrier transform. It is important to get the crystals to sufficient size and to be able to form a regular 3D crystal structure and obtain isomorphous heavy-atom derivatives for initial phasing of diffraction data. The main approach in a crystallization trial is to reduce protein solution ability by mixing protein solution with a precipitating solution. If the proteins are protected from amorphous precipitation and/or denaturation, a periodic 3D crystal structure will form (Auer, 1997; Carter et al., 1997; Hewat et al., 1997). X-ray tubes, anode tubes, goniometer, and X-ray microscopes are required with this tool. Moreover, the crystals that can break the X-ray beam are needed, which turns into a diffraction model that can be interpreted by a mathematical operation calculated on the computer. With the computer, the image data is captured when looking at the monitored molecules. X-ray refraction of a unit/ single cell is not evident. Repeating and replicating a unit cell with the help of crystals needs to transform the image. In short, crystals are regular 3-dimensional structures and are important because they are reproducible units of the cell in which they reside. The most difficult and crucial step in X-ray technique is the stage of enlarging crystals. The mostly used technique for enlarging crystals is the dynamic light scattering (DLS) method (Wery et al., 1997). In addition, solution concentration is important to enlarge crystals. In other words, crystals are enlarged changing the saturation degree of the solution (Carter et al., 1997). For example, there is no crystal forming in a non-dense solution. In the low-density solutions, crystals grow, but no new crystals are formed. In the high-density solutions, several crystals begin to grow in a high degree of solution. Then, they grow until when the crystals enclosed in less saturated solutions. The main drawback of X-ray crystallography examined protein must be crystallized. It is a very difficult process besides not all biomolecules suitable for this. Thus, only a very small number of membrane protein structures can be determined by X-ray crystallography. Often a combination of X-ray crystallography and Cryo-EM are used. For this reason, Cryo-EM was developed after X-ray crystallography. Schematic diagram of X-ray crystallography is shown in Figure 3 (Bütner et al., 2015)

Acharya et al. (1990) examined the molecular structure of Foot and Mouth Disease Virus (FMDV) by X-ray crystallography technique. In the study, crystals were formed and diffracted with weekly periods. The X-ray beam produced

by the synchrotron radiation source has helped to unravel the structure of the virus. X-ray crystallography also used for adenovirus imaging (Stewart et al., 1993).

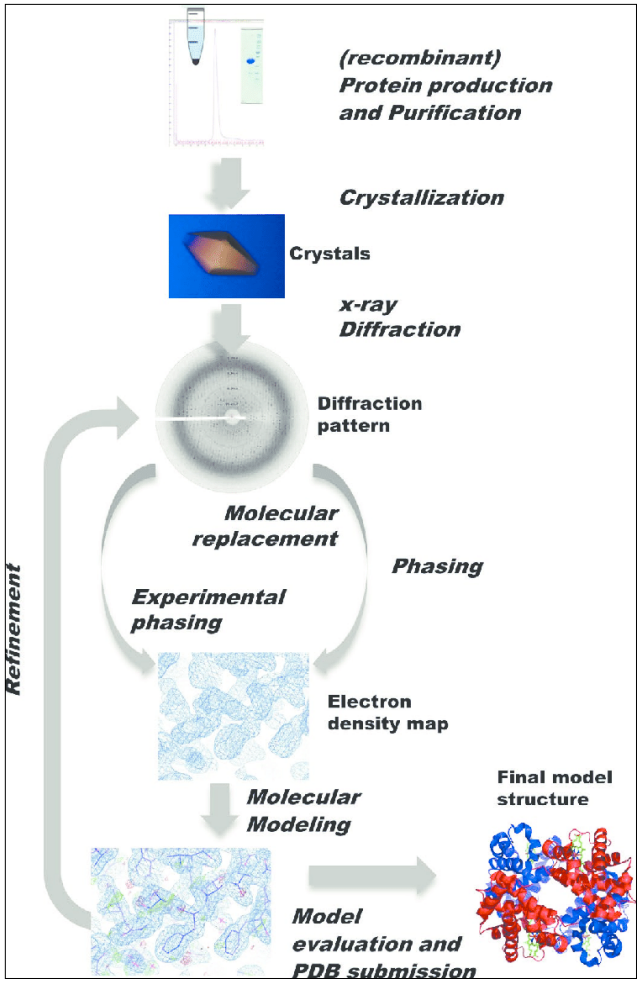

Figure 3. Schematic diagram of X-ray crystallography (Stewart et al., 1993).

Cryo-elektron-mikroskopy (CryoEM)

The Cryo-EM developed to prevent structural deformations that may occur when preparing a sample in a classical EM. Cryo-EM is a powerful tool and it is called a non-crystallography or single-particle reconstruction technique (Yu and Bajaj, 2005). Samples must be cooled by preventing the formation of ice crystals in the Cryo-EM technique. Because ice crystallography damages the ultrastructural structures of proteins (Risco et al., 2002). In 2017, Richard Henderson won Nobel prize with his work in developing detectors for cryo-EM. This technique allows the virus to be observed without loss, as there is no staining, shading, or coating process with heavy metals. In this method, the virus particle is quickly filled into the EM grits. The virus is enclosed in a glassy film (vitrified) layer consisting of thin ice. Since the dye is not used, the particles that receive water are well displayed by taking advantage of the differences between the electron density of the protein or lipid in the structure of the virus and the matrix that is surrounded by a layer of water (Wagner and Hewlett, 2003; Baker et al., 2004).

Vitrification can be achieved by cryosectioning or milling-substitution methods. Cryosectinoning is the removal of sections of vitrified tissue at liquid nitrogen temperatures. However, this approach is technically quite difficult. In the milling substitution, the sample is painted after being physically fixed, dried, and placed well by burying the water at temperatures well below the freezing temperatures. This second approach is quite simple compared to the first one. Nevertheless, due to staining and chemical application in the milling substitution, there are unwanted artifact formations (Auer, 1997).

The main problem with the Cryo-EM is the unexplained displacements of particles floating in the glassy ice environment. Therefore, many of the macromolecules studied with this technique remain dependent on the symmetry of the structures studied (Yu and Bajaj, 2005). In other words, a high concentration of homogeneous virus particles is required to create the image. Therefore, cryo-EM used particularly in the study of icosahedral viruses (Commike and Chiu 2000). The problem is Cryo-EM is that it needs an extraordinary expensive microscope. Similar to TEM and SEM, Cryo-EM also required trained experts. For this aim, in recent years, robots have been used the sample preparation.

In a study (Hewat et al., 1997), X-ray and Cryo-EM techniques were used to investigate the interaction between Foot and Mouth Disease Virus (FMDV) and monoclonal antibody SD6, a strongly neutralizing monoclonal antibody. Accordingly, the structure of the Fab part of the antibody against the virus and the monoclonal antibody was determined using Cryo -EM. In another study (Briggs et al., 2004), a beta

virus and mouse breast carcinoma virus was examined with the Cryo-EM technique, glycoprotein structure, and core formation of the virus was obtained.

Cryo-EM is also used for COVID-19 imaging (Gao et al., 2020). Cryo-EM used for imaging viral RNA polymerases for COVID-19 viruses to develop new coronavirus vaccines and therapeutics. In the study, the viral polymerase NSP12 looks an excellent target for new therapeutics, especially given that lead inhibitor already exists in the form of compounds such as Remdesivir.

Principally, an electron beam sends at a frozen protein solution. Scattered electrons pass through a lens to occur a magnified image on the detector. Therefore, multiple images of protein molecules in different orientations can be collected. A schematic diagram difference between the TEM and Cryo-EM is shown below. As to be seen in the diagram, the main difference to prepare the vitrified sample in Cryo -EM and detector covert the images 3D analysis. A schematic diagram difference between the TEM and Cryo-EM are shown in Figure 4 (Szatanek et al., 2017).

Atomic Force Microscopy (AFM)

AFM is a high-resolution technique used in the imaging of proteins, nucleic acids, and nucleoprotein complexes. Unlike the EM, it is a technique that allows the study of biological structures at a single molecule level and in their natural environment (Kiselyova et al., 2001; Kienberger et al., 2004; Kuznetsov et al., 2001; Kuznetsov and Mcpearson, 2011). AFM developed in 1986 (Binning et al., 1986), which is a better resolution than EM, as well as being in the sample (solid, liquid, gas, etc.) works independently and does not require a long-term sample preparation process. The AFM is a highly suitable tool for surface analysis/topography studies. A probe in the technique is mounted on a retaining arm (spring cantilever) with very precise flexibility. There is also a trestle, optical deflection (installed from a laser diode and photodetector) system where it is placed. The retaining arm is made of silicon or silicon nitride and its dimensions are 100-300 µm in length, 10-30 µm in width, and 0.5-3 µm in thickness. Pressing on the sample with a constant force, this arm moves down and up depending on the topological changes the probe encounters on the sample surface. The x-y-z values recorded during the scan are sent directly to the computer screen and memory using a digital signal processor (DSP). After this stage, image processing and analysis are done with appropriate software (Binning et al. 1986; Colton et al. 1997; Kuznetsov et al. 2001; Kuznetsov and Mcpearson 2011).

The main difference of AFM from other microscopes is that a continuous controlled force is applied to the sample. It provides direct height measurements and surface features without covering, compared to SEM, and provides a topographic contrast easily. When compared to SEM and TEM, it is very practical that a sample preparation process is not as there is no need to cover the sample required in AFM (Colton et al. 1997; Kuznetsov et al. 2001; Kuznetsov and Mcphearson 2011). A schematic diagram of AFM is illustrated in Figure 5 (Balthazar et al., 2013).

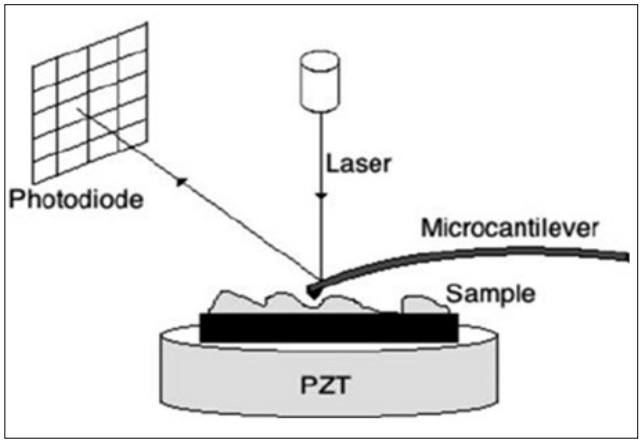

Figure 5. A schematic diagram of AFM (Balthazar et al., 2013).

Kienberger et al. (2004) examined human rhinoviruses within their physiological environment with the help of AFM. Topographic examination of the virus revealed several polygonal areas on the surface of the particle. It has been reported that RNA release occurs at low pH levels and that the length of released RNA is related to capsid length (ranging from 40 to 330 nm), while fork-like extensions may be

related to the characteristic multi-stem loop formation of RNA molecules.

There are several AFM models: constant force, contact, intermittent-contact mode, non-contact, and tapping mode. Topographic imaging can be performed with contact-model AFM. In this model, the tip and sample are in contact. During scanning, the gripper arm continuously touches the sample. However, in this model, imaging is not good if the sample is too weakly attached to the stand. Because during screening, biomolecules are pushed by AFM situluses. Also, the tip should touch the sample with a continuous up-and-down movement. The sideways movement will damage the sample and the tip. Manually controlled styluses can be used to eliminate this problem. Another approach is by tapping-mode AFM or magnetic AC mode models, where the sample is soft and weakly attached to the stand. In this model, the oscillator of the holder arm touches the sample only at intervals in its downward motion. Thus, contact time is reduced and the friction force is minimized. The cryo-AFM technique has also been developed where the sample can be monitored at low temperatures to increase the resolution (Colton et al. 1997; Kienberger et al. 2004; Kuznetsov et al., 2001; Kuznetsov and Mcpearson, 2011).

AFM allows direct imaging of viruses when they are in a watered-down position and can uniquely examine surface tomography. The technique is also used in revealing structural dynamics in vitro. However, it allows the display of surface parts of their internal structures. It is a very successful technique used in the imaging of large viruses, especially in asymmetric structure. In a study conducted (Malkin et al. 2003), an asymmetric and large-structure mature vaccinia virus was investigated by AFM technique.

The interaction of the virus with the cell is one of the fundamental issues of virology. Adsorption of the virus to the cell membrane, penetration, and infection of the cell constitutes the basic steps of virus infection. The AFM technique is a technique that helps to examine the first two steps. However, AFM is used only for the study of simple plant viruses. There is only one study of an animal virus with a cell membrane. In that study (Zaitsev et al. 2002), the interaction of a virus with an erythrocyte blood cell was studied. The erythrocyte membrane has always been a good model for the study of cell-virus interaction. Erythrocytes, which are well defined at the biochemical level, also remain stable during sample preparation in the AFM technique. The physical properties of the erythrocyte membrane were altered as a result of the effects of erythrocyte and virus and these changes were determined by AFM technique. In the study, the AFM technique was reported as an effective technique in the study of cell-virus interaction.

In conclusion, although most commonly used tools are EM, TEM, and Cryo-EM for virus imaging, SEM, X-ray crystallography, PET, bioluminescent imaging, and NMR can be mentioned among the other tools. Sometimes the combination of a few tools is preferred. The study of viruses in ultrastructural dimensions is a significant impact because it provides information about the viral entry, morphology, replication dynamics, changes in a virus infected -cell. Thus, the development of new diagnostic and treatment methods against viruses is only possible by examining them in more detail.

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