NanoBiotechnology

ISSN 1551-1286/05/01:337–346/$30.00 (Online) 1551-1294
DOI: 10.1385/Nano:1:4:337

Original Article

Delocalization of Vaccinia Virus Components Observed by Atomic Force and Fluorescence Microscopy

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Abstract

Better understanding of viral genomes is emerging as an urgent need as these genomes evolve and pandemic fears surface and for better understanding of viral infection processes. To address this need, we report a method to visualize intact, viral DNA and its interaction with viral proteins with the use of the atomic force microscope (AFM) in conjunction with fluorescence microscopy. Through a series of multifaceted experiments, we were able to visualize time-dependent progressive stages of proteolytic digestion and disassembly of extracellular enveloped vaccinia virus particles. After a 1-h treatment, the viral particles were partially digested and the viral cores showed slight disassociation in the AFM as evidenced by height analysis of individual virions. Most of the components of the virions were still intact. Further verification with florescence microscopy with nucleophilic and lipophilic stains demonstrated that viral DNA was, indeed still, co-localized within the viral core. However, with prolonged treatment with proteinase K and sodium dodecylsulfate, the AFM revealed that the viral core completely collapsed onto the substrate and had delocalized from the enclosed DNA. This process was again verified using fluorescence microscopy, the viral DNA was observed to be completely released from the viral core, in globular condensed form. These studies suggest that AFM imaging and fluorescence microscopy verification with stains specific for different constituents of viral particles is a valuable method to study the structural and mechano elastic properties of virus morphology and interactions of viral nucleoproteins with its DNA core.

(Nanobiotechnology DOI: 10.1385/Nano:1:4:337)

Key Words: AFM; SPM; vaccinia; poxvirus; nanoscopy; delocalization.

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Humana Press

Imaging of virus particles is customarily done by scanning or transmission electron microscopy *(1)* however, these methods require the samples to be fixed, dried, coated, and laboriously prepared for imaging. These methods are incompatible with processing and analysis of virus particles in real time. Furthermore, sample integrity may be compromised during preparation and their use in imaging of dynamic changes can be very limited. The atomic force microscope provides atomic sized resolutions of surfaces by measuring force generated by height or other physical properties of the material as the microscope probe tip scans over them *(2)*. There are three basic modes of imaging in atomic force microscopy. In the AFM's attractive or "non contact" mode the tip does not touch the sample, but the AFM derives three-dimensional topographical images based on measurements of attractive forces. Similarly, in "contact mode" the tip is in close proximity with the sample during scanning and any vertical deflection of the cantilever induced by repulsive forces between the tip and sample is monitored by the AFM's detection apparatus. In the third mode of imaging, tapping

mode, the cantilever is driven into resonance frequency. Thus, any changes in the resonant frequency of the cantilever during a scan can be measured and used to generate an image.

In our study, we generated two types of images during each scan: height and phase images. Height images are constructed by using a laser and position-sensitive detector to monitor the cantilever's deflection from or attraction to the sample during the scan. They essentially depict all the topographical features of a sample. Likewise, in phase imaging, the mechanoelastic properties of a sample during a scan can be probed by driving the cantilever at resonant frequency in the tapping mode and mapping the phase lag between the periodic signal and the oscillations of the cantilever.

Atomic force microscope (AFM) can have vast applications in structural biology due to its powerful imaging capabilities. Surfaces of biological particles, such as viruses can be characterized at the nanoscale dimensions using an AFM. Bacteriophage T4 was initially imaged by Kolbe et al. *(3)*. They were able to differentiate between the head and tail of the phage and later Ikai et al. were also able to refine the imaging and obtain the dimensions of the head, tail, and tail fiber of the phage within a few nanometers of resolution *(4,5)*. Other viruses have been studied in great structural detail including the tobacco mosaic virus by Imai et al. *(6)*. Vaccinia virus belongs to the Poxviridae virus family and forms the basis of the smallpox vaccine. Attenuated strains of vaccinia virus have been used extensively as gene therapy vector. The members of Poxviridae have characteristically large DNA genomes. Vaccinia virions are generally brick-shaped with a size of 220–450 nm length, 140–260 nm width, and 140–260 nm thickness. These particles are assembled in two infectious forms: the intracellular mature virus (IMV) and the extracellular enveloped virus (EMV) *(7)*. The IMV form is a precursor to the EMV forms and the two forms are differentiated by EMV having an extra viral membrane, which is acquired through viral membrane wrapping in the trans-Golgi network of a host cell's secretory pathway *(8)*. Both particles possess a genome-containing coat, composed of an array of highly cross-linked individual protein subunits *(9)*. Enclosed within the viral coat is linear, double-stranded DNA of approx 196 kb *(9)*. The complete sequencing of the viral genome has revealed 273 potential gene sites with approx 100 proteins identified to play some key structural roles *(10,11)*. In addition, proteins and transcription factors encapsidated within the virion mediates DNA replication and early transcription in the host cells' cytoplasm *(12)*.

Western-reserve strain of vaccinia virus (obtained from S. Broyles, Purdue University) was propagated in HeLa S3 cells and stored at –80°C until further processing as described previously *(13)*. All cells and viruses were originally obtained from American Type Culture Collection (ATCC, Manassas, VA). Cell-culture-grown viruses were purified by either sucrose gradient centrifugation (SGC) or after the gradient purification filtered through 1 µm pore size nitrocellulose membrane filters. For the SGC, the cell lysates containing virus particles were layered on top of a sucrose gradient, 5–15%, and centrifuged at 20,000*g* for 60 min after which the viral band was recovered and stored at –80°C. Prior to each experiment, the virus samples were treated with short-wave ultraviolet light to neutralize the infectivity of the viruses by cross-linking viral DNA.

The protocol for digestion of Western reserve strain of vaccinia virus involved two incubations of vaccinia virions in 20 m*M* EDTA, 0.5% SDS, 0.5–1 mg/mL proteinase K, and 20 m*M* Tris-HCl (pH 7.5) at 37°C. A stop buffer consisting of 2% PBS and glycine was added to both samples after 1 and at 2 h to inhibit further digestion. After each digestion and stopping the reactions, the samples were stored at –20°C until imaging.

Digital Instruments, Dimension 3100 atomic force microscope with Nanoscope IV controller (Veeco, Santa Barbara, CA) and etched silicon tips with tip radius of 5–10 nm and resonant frequency of 75 kHz were used for the image acquisition of the virus samples in air-dried conditions using tapping mode. Height and phase images were collected by DI program and analyzed by using an image analysis program (WS × M, Nanotec Electronica, Madrid, Spain). The approximate particle density of vaccinia virus was 10^7 particles/mL and these were deposited on a 1 × 1 cm silicon chip and imaged *exsitu.*

Images of the digested vaccinia virus particles were acquired by a fluorescent microscope (Nikon Eclipse 600FN) equipped with a cooled-CCD camera (Pixera 600CL) and appropriate filters for FITC, TexasRed, and DAPI. Images were analyzed by ImageJ software (W. Rasband. Available from NIH website http://rsb.info.nih.gov/ij/). Spectrally separated red and green images were processed by an ImageJ plugin (J. Walter. Spectral Unmixing plugins).

Undigested vaccinia virus particles were imaged *exsitu* using tapping mode AFM. The samples were deposited on a silicon chip and allowed to air-dry for approx 1 h. Visual analysis of the phase images show intact individual virions, as seen in Fig. 1. The AFM scanning was performed with the following parameters: *Z*-amplitude: 118.07 nm; *X/Y*- amplitude: 4.2 µm; X-frequency: 0.528 Hz. The particles were brick to ovoid shaped and the dimensions of the particles in AFM image was about 582 ± 13 nm long, 465 ± 28 nm wide, and 94 ± 15 nm as shown Figs. 1A, B, and C. The size is slightly larger than expected, possibily due to collapse of the particle during drying or imaging. Further imaging using scanning electron micrography (SEM) confirms that the vaccinia virions indeed possess the intact morphology (Fig. 1D).

In probing the vaccinia genome and visually capturing its delocalization from the viral capsid, we initially began with a combined treatment consisting of a strong metal-complexing agent, a disulfide reducing agent, and a protease on virus sample for approx 1 h. EDTA was used to promote the disintegration of the vaccinia virus membrane due to its strong metal-binding effects as observed on retroviral membranes previously *(14)*. EDTA was also used in conjunction with a protein disulfide reducing agent SDS and proteolytic enzyme proteinase K. Digestive treatments include 20 m*M* EDTA, 0.5% SDS, 0.5–1 mg/mL proteinase K, and 20 m*M*

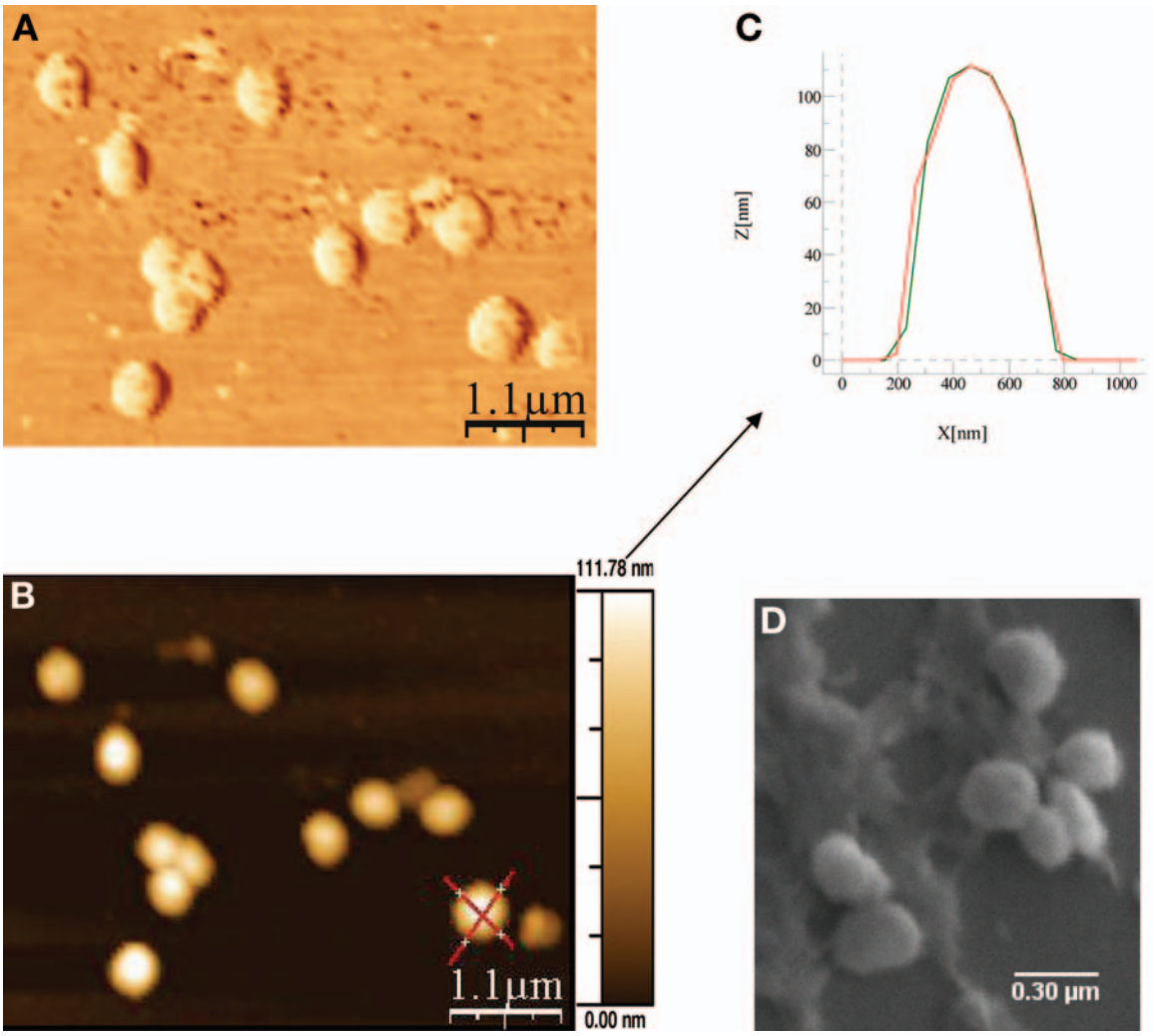

Fig. 1. Intact vaccinia virus particles imaged by tapping mode AFM **(A)**, phase image **(B)**, height image **(C)** extracted dimensions of the viral particles with lateral dimension of 592 nm and 109 nm in height, **(D)** SEM image of vaccinia virus on the silicon surface demonstrate an intact morphology of the individual virions confirming the AFM results. Vaccinia virions were air-dried on 1 cm x 1 cm silicon substrate and imaged *ex situ*. Particle density was 10^7 particles/mL. AFM scanning parameters: *Z*-amplitude: 118.07 nm; *X*/*Y*-amplitude: 4.2 µm; *X*-frequency: 0.528 Hz.

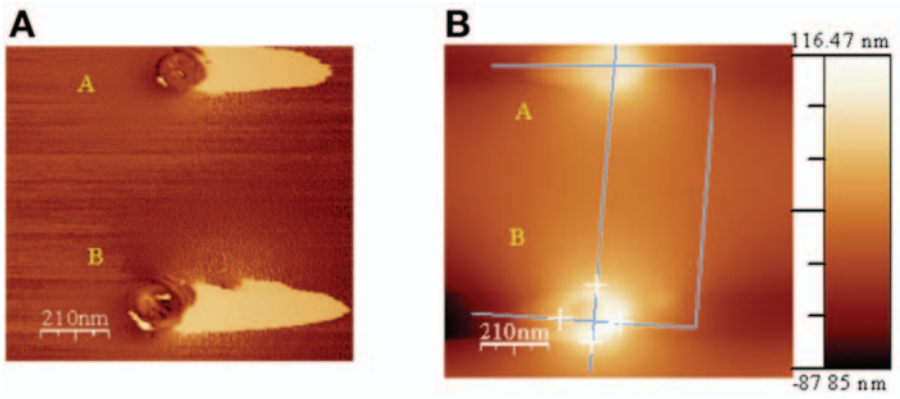

Fig. 2. *(Continued)*

Tris-HCl (pH 7.5) at 37°C. Stop buffer (2% PBS and glycine) was added approx 1 h after digestion to terminate the digestive treatment. The results obtained from the AFM indicated only a slight digestion of individual vaccinia virions upon 1 h digestive treatment, as shown in Fig. 2A–D. The slight depressions, clearly visible in Fig. 2C are most likely the initial

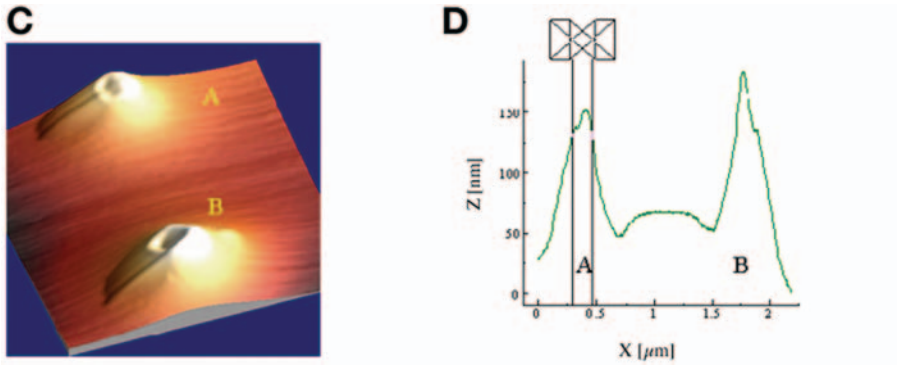

Fig. 2. AFM images of the vaccinia virions after they were air-dried on a silicon substrate and subjected to 1 h of digestion with proteinase K in presence of SDS and EDTA. **(A)** phase image, **(B)** height image of vaccinia virions A and B, **(C)** 3-D reconstruction of 1-h digested virions A and B, **(D)** A profile plot of the virions A and B. AFM Scanning Parameters: Z-amplitude: 194.33 nm; X/Y-amplitude: 1.1 μm; X-frequency: 2.188 Hz.

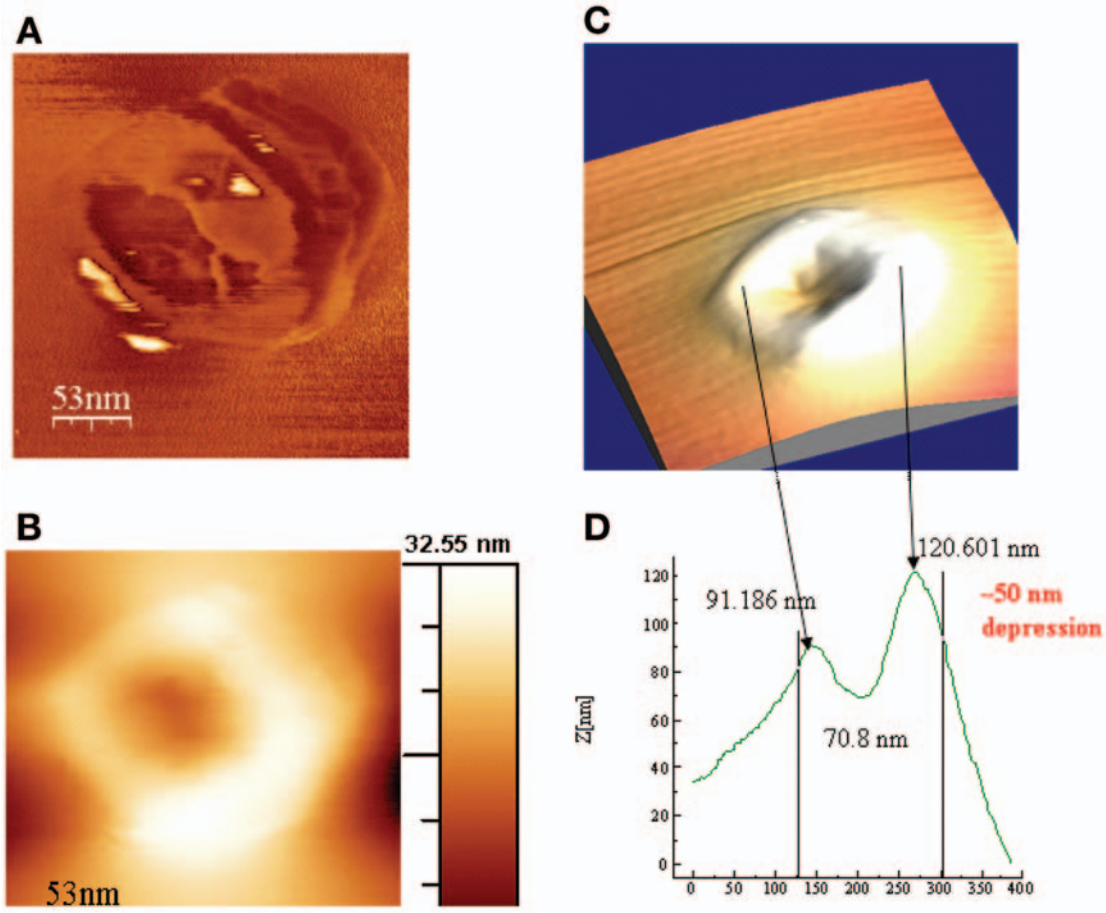

Fig. 3. High-resolution AFM images of 1-h digested vaccinia virion. **(A)** phase image, **(B)** height images. Approximately 90 nm diameter and 50 nm depression due to 1-h proteolysis and disassembly of the viral capsid **(C)**, size analysis performed on the three-dimensional reconstruction of the 1-h digested samples show lateral dimensions of 206 nm by 176 nm for virion B **(D)**.

stages of the digestion. It can be seen that virion A possesses lateral dimensions of about 465 nm × 300 nm and a height of 150 nm, while virion B shows lateral dimensions of 467 nm × 303 nm and a height of 181 nm. These measurements are consistent with the notion that there is only a slight digestion of the 1-h treated sample due to increased lateral values than compared to lateral dimensions obtained by Malkin et al. on intact, air-dried vaccinia virions approx 300–360 by 240–280 nm *(9)*. Higher magnification images of the particles digested 1 h, as shown more clearly in Fig. 3A–D,

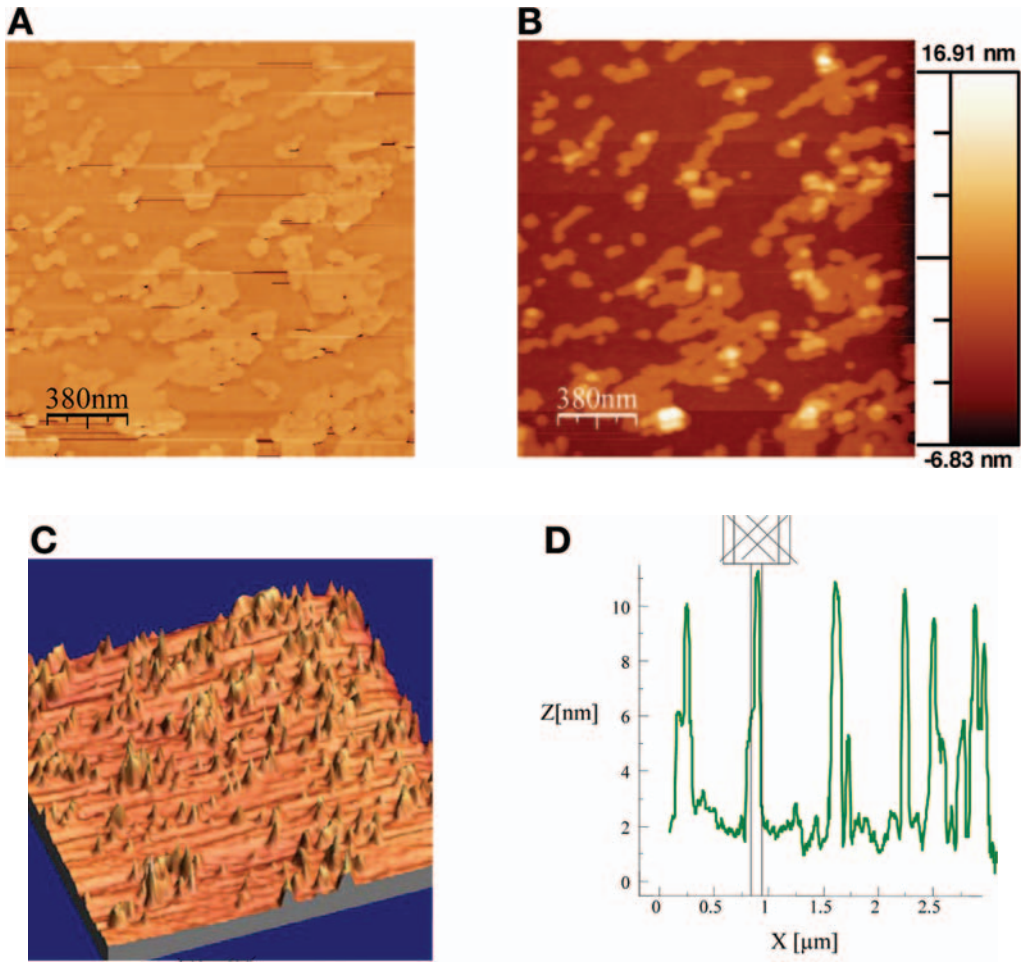

Fig. 4. AFM images of vaccinina virions after 2 h digestions and lysing, **(A)** phase image, **(B)** height image of lysed vaccinia virions, **(C)** 3-D reconstruction of the 2-h digested virions using WS × M image analysis program reveal the peaks of the exposed DNA, **(D)** size analysis of the DNA. Stop buffer (2% PBS and glycine) was added approx 2 h upon digestion. Particle density was 10^7 particles/mL. AFM scanning parameters: *Z*-amplitude: 23.74 nm; *X*/*Y*-amplitude: 1.9 μm; *X*-frequency: 3.283 Hz.

revealed the partial digestion of the particles. Height analysis indicates a slight depression of the viral core of virion B, approx 50 nm in depth, (Fig. 3D). These images clearly show that the vaccinia virions are partially digested on a 1-h digestive treatment.

More extended treatment with 20 m*M* EDTA, 0.5% SDS, proteinase K, and 20 m*M* of Tris-HCl (pH 7.5) at 37°C for up to 2-h resulted in the exposure of intact DNA as shown in Fig. 4A–D and Fig. 5A–D. Figure 4B shows many digested particles within the same image and a height analysis of 10 particles in the same scan shows an average height of 9.8 nm with standard deviation of 0.36 nm, indicating some uniformity and consistency in the characteristics of the digested particles. Figure 5 shows that the viral remains are about 4.7 nm high and the viral core is about 64 nm in diameter and 9 nm high. Upon higher-resolution AFM imaging in Fig. 6A–D, it is quite evident that proteinaceous capsid that encapsulates the genomes has collapsed, seen as plateaus in Fig. 6C, and that the individual compacted-genomic DNA's are exposed, also seen as peaks in Fig. 6C. These images reveal that the viral capsid has indeed delocalized from the intact DNA and the capsid can be seen as disintegrated and suspended upon the substrate. A final size analysis performed on the DNA from the 2-h digested samples, as shown in Fig. 7, reveals average lateral dimensions of 64 nm by 30 nm and a height of approx 10 nm with respect to the substrate.

Unlike Malkin et al., we provide a method to confirm the identity of the DNA observed in the AFM using fluorescence microscopy *(9)*. Both virus samples utilized in the AFM-based study were observed under fluorescence microscopy using stains that have affinity for DNA or lipids *(15)*. Virions from both samples were labeled with Yo-Yo-1, a green fluorescent nucleophilic dye, and DiI, a red fluorescent lipophilic dye. In the sample exposed to digestion enzymes for 1 h, a light yellow–orange hue was observed, as shown in Fig. 8. The inset of this color due the overlap of the red and green

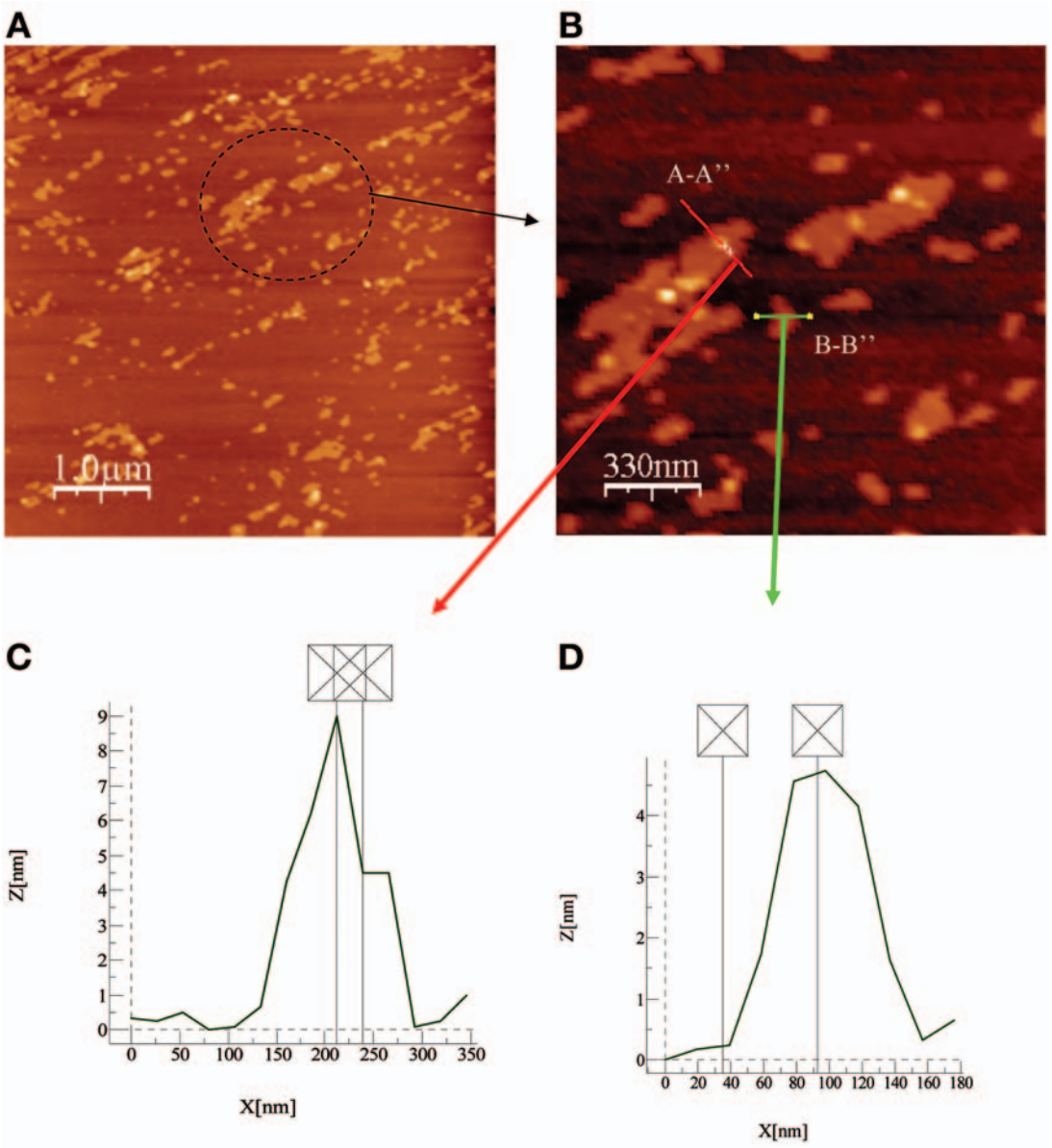

Fig. 5. Completely digested (2 h) and collapsed vaccinia virus virion. **(A)** height images of vaccinia DNA fully exposed and delocalized from the surrounding viral capsid upon digestive treatments, **(B)** zoom in image of the region indicated, **(C)** scan across region *A-A′* showing viral remains are about 4.7 nm in height and the dimensions of the viral core is about 64 nm in diameter and 9 nm in height, **(D)** scan across region *B-B′* showing the viral remains of about 4.7 nm. Scanning parameters: *Z*-amplitude: 18.24 nm; *X*/*Y*-amplitude: 1.1 nm; *X*-frequency: 1.127 Hz

dyes clearly demonstrates that the viral DNA and lipid membrane are colocalized, as shown in Fig. 8B. Conversely, in the 2-h samples, distinct red and green fluorescence were observed, as shown in Fig. 8C. The red dye has essentially delocalized and surrounded the green dye. These observations are very consistent with the results obtained from the AFM with the 2-h sample, as seen in Fig. 8C. This strong correlation between the fluorescent microscopy and AFM images helps confirm the notion that the viral capsid has collapsed upon progression with the digestion from 1 h to 2 h and has delocalized from the intact globular DNA lying underneath. The progressive emergence of the viral DNA from the viral capsid within the 2-h time frame can be seen in Fig. 8D.

Vaccinia virions are composed of proteins (90%), lipids (5%), and viral DNA (3%). The viral genome is approx 196 bp long and it is a linear double-stranded DNA within an approx 260 nm wide, 330 nm long, and 125 nm thickness capsid as observed in SEM images *(1,7)*. Because the capsid is shaped

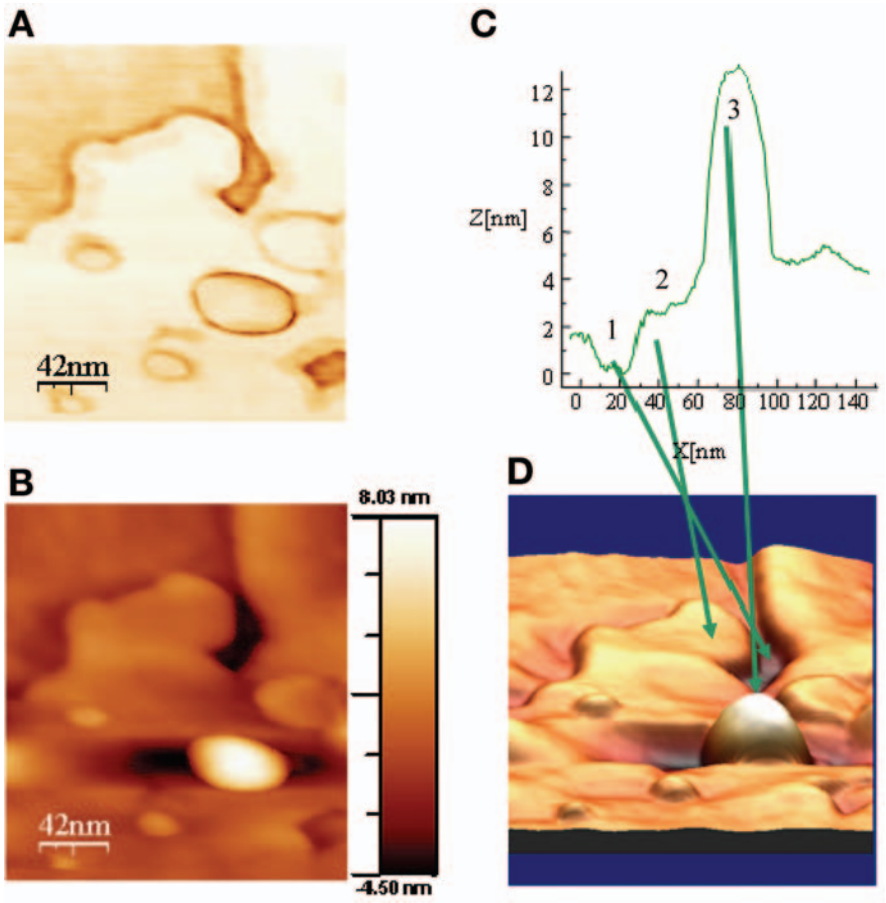

Fig. 6. High-resolution AFM images of collapsed viral capsids after 2 h digestions. The enclosed vaccinia DNA is exposed upon the collapse of the surrounding viral capsids. **(A)** Phase image, **(B)** height image of vaccinia virion, **(C)** size analysis of the DNA peaks. 1: Viral membrane (2 nm) consistent with the expected size of membranes. 2: Substrate layer. 3: Globular viral DNA 12 nm height from the substrate. **(D)** Three-dimensional reconstruction of the images in **(A)** and **(B)**. AFM scanning parameters: *Z*-amplitude: 18.24 nm; *X*/*Y*-amplitude: 1.1 nm; *X*-frequency: 1.127 Hz.

like a brick, it would have a volume of approx $1 \times 10^7 \text{nm}^3$. In the relaxed form, genomic DNA can be considered as cylindrical and the volume *(V)* occupied by the viral DNA would be $V=\pi r^2 l$, l being the genome length and r being the diameter of a base pair (approx 2 nm). Because every 10-bp per turn occurs in the DNA double helix at every 0.34 nm, this would correspond to a genome length of 66,640 nm and volume of $8.37 \times 10^5 \text{ nm}^3$. If this volume is condensed in a sphere with 100% efficiency, then the diameter of the sphere would be approx 117 nm. We measure the DNA diameter as approx 65 nm from our AFM images, which is in the same order of magnitude and confirms our AFM analysis. It is also evident that the compacted DNA does not fully occupy the internal space. It is possible that this space is occupied by the viral proteins and other material. In the collapsed form, the virus particle height seems to be only 4 nm without the DNA core; these low profile features that are clearly visible could correspond to the viral envelope and or partially digested proteins from other virions. During our studies, we did not observe the tubular structures that have been pointed out by other groups *(9)*. Fluorescence images clearly show the enzymatic digestion of the viral particle and increased access of the DNA-selective stain to the viral DNA. This picture was also corroborated by the 2-h digestion and AFM imaging.

Acknowledgments

Azam Ghafoor was funded by Howard Hughes Medical Institute Summer Undergraduate Research Fellowship in the Department of Biological Sciences at Purdue University and by the Summer Undergraduate Research Fellowship (SURF) in the College of Engineering at Purdue University. We would like to acknowledge Chia-Ping Huang for performing the SEM imaging. We also thank NIH (NIBIB grant number R21/R33EB00778-01) for supporting the work and for supporting Demir Akin.

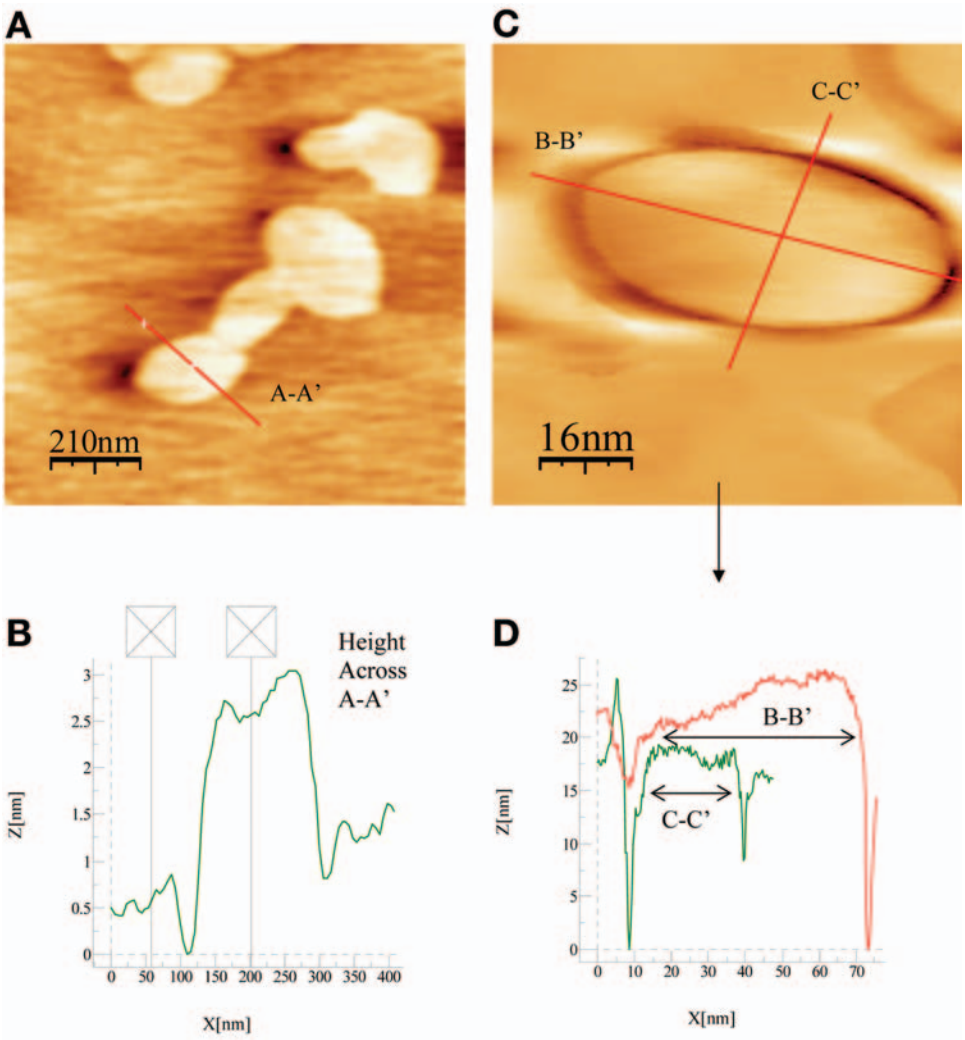

Fig. 7. AFM analysis of completely released vaccinia genome. **(A)** 1 μm x 1 μm scan of the released but still partially compacted vaccinia viral DNA, **(B)** line scan across *A-A′* across the regions *B* showing that vaccinia virus DNA remains are about 2.5 nm high, **(C)** high-resolution scan of the released highly compacted-ovoid DNA, **(D)** line scans across *B-B′* and *C-C′* showing large diameter of 64 nm and small diameter of 30 nm. The height of viral DNA is 10 nm.

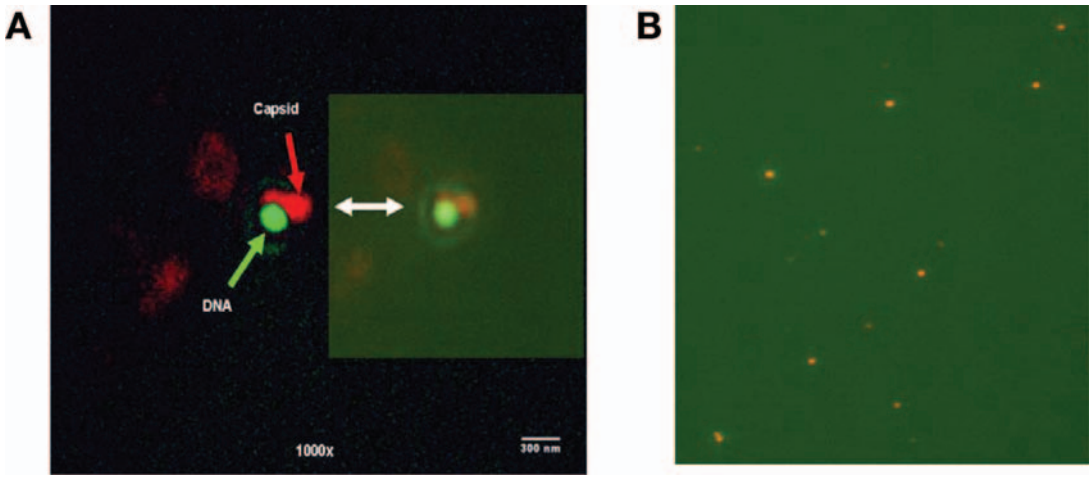

Fig. 8. *(Continued)*

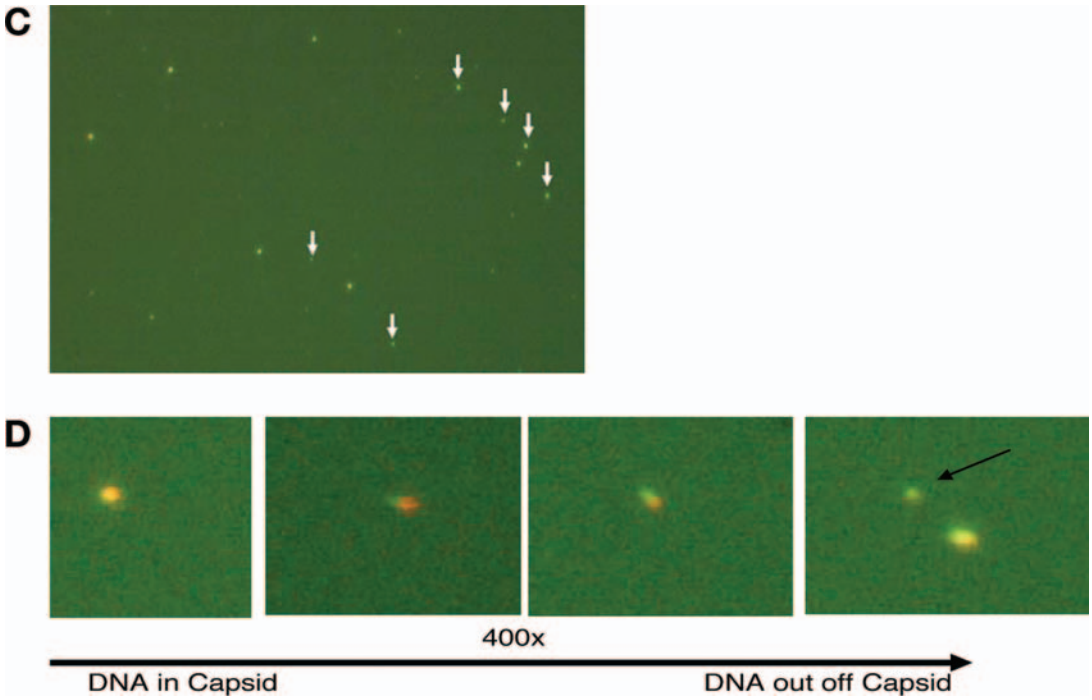

Fig. 8. Fluorescence-based verification of release of viral DNA. The vaccinia virion was lysed by proteinase K for and 2 h and the virion was labeled fluorescent red with a *lipophilic* tracer stain (DiI) and the viral DNA was stained fluorescent green with a *nucleophilic* dye (YOYO-1). Images of the digested vaccinia viral particles were acquired by a cooled-CCD camera and images were analyzed by ImageJ software. **(A)** Spectrally unmixed images of vaccinia virus virions after 2 h digestion by proteinase K. Colocalization of the viral DNA and the capsid is clearly evident in the inset as the yellow color development occurs at the point of colocalization, **(B)** fluorescence images of the virions after 1 h enzymatic digestion. **(C)** Fluorescence images of the virions after 2 h enzymatic digestion. Emerging viral DNA is shown by the arrows, **(D)** progression of the emergence of the viral DNA from the viral capsid within the 2 h time frame. Emerged viral DNA is shown by the black arrow.

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