Biotechnol Lett (2019) 41:1105–1110
https://doi.org/10.1007/s10529-019-02720-3

ORIGINAL RESEARCH PAPER

Imaging aquatic animal cells and associated pathogens by atomic force microscopy in air

Chen Li · Yan Liang · Menglin Xu · Hailiang Wang · Chongming Wang · Jie Huang

Received: 30 May 2019 / Accepted: 7 August 2019 / Published online: 13 August 2019

Abstract Atomic force microscopy (AFM) is a sophisticated imaging tool with nanoscale resolution that is widely used in structural biology, cell biology, and material science, among other fields. However, to date it has rarely been applied to the study of aquatic animals, especially on one of the main cultured species, shrimp. One reason for this is that no shrimp cell line established until now, primary cell is fragile and difficult to be studied under AFM. In this study, we used AFM to image three different types of biological material from shrimp (*Litopenaeus vannamei*) in air, including hemocytes and two associated pathogens. Without obvious deformations when the cells were imaged in air and in the case for the haemocytes and the cells were fixed as well. The result suggests hydrophobic glass coverslips are a suitable substrate for adhesion of these samples. The method described here can be applied to the preparation of other fragile biological samples from aquatic animals for high-resolution analyses of host–pathogen interactions and other basic physiological processes.

Keywords Atomic force microscopy · Imaging · Shrimp · Cells · Viruses · Bacteria

C. Li · Y. Liang · M. Xu · H. Wang · C. Wang · J. Huang (✉)
Key Laboratory of Maricultural Organism Disease Control, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, No. 106 Nanjing Road, Qingdao 266071, China
e-mail: huangjie@ysfri.ac.cn

C. Li · Y. Liang · H. Wang · C. Wang · J. Huang
Function Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266071, China

C. Li · Y. Liang · H. Wang · C. Wang · J. Huang
Qingdao Key Laboratory of Mariculture Epidemiology and Biosecurity, Qingdao 266071, China

Introduction

Atomic force microscopy (AFM) has nanoscale resolution and can be used for three-dimensional (3D) characterization and dynamic measurement of research objects. AFM has been used in previous studies to characterize chemically induced chromosomal damage in animal cells such as Chinese hamster ovary cells, to image human chromosomes (Ratanavalachai and William 1996; Tamayo 2003; Tamiya et al. 2003), also commonly used to image molecular assemblies (Mayer et al. 2016; Marchante et al. 2017; Galloway et al. 2017); thus, AFM has been applied to structural, and cell biology, material science, and medical research (Fotiadis et al. 2002; Alonso and Goldmann 2003; Francis et al. 2010). However, it has rarely been applied to research on aquatic animals. There are no aquatic invertebrate cell lines that have

been successfully established to date and only primary cells are available for investigations at the cellular level due to the difficulty of sample preparation.

In this study we developed a simple method for imaging hemocytes, white spot syndrome virus (WSSV), and *Bacillus firmus* (*B. firmus*) isolated from shrimp (*L. vannamei*), which are representative aquatic animal cells and pathogens (Yang et al. 1997; Lightner et al. 1998; Sun et al. 2012; Liu et al. 2012). The samples were mounted on glass coverslips that were hydrophobized in order to improve sample adsorption and reduce the risk of cellular damage. This method can be used to investigate the topography of any type of fragile sample including cells, viruses, and bacteria by AFM.

Materials and methods

Preparation of hydrophobic glass coverslips

The glass coverslips were cleaned in a solution of 10% KOH and 90% ethanol, dried with a gentle stream of nitrogen, and exposed to a hexamethyldisilazane (HMDS) vapor (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Roos 2011).

WSSV preparation for AFM imaging

Intact WSSV virus particles from infected crayfish tissue were purified by differential centrifugation (Xie et al. 2005), and a 20-µl drop of the solution was placed in the middle of hydrophobic glass coverslips followed by incubation at 4 °C for 30 min. After two thorough rinses with TN buffer composed of 20 mmol/l Tris–HCl (pH 7.4) and 400 mmol/l NaCl, the sample were dried under a stream of nitrogen for AFM imaging.

Bacterial cell preparation for AFM imaging

Bacillus firmus was isolated from the intestines of shrimp and identified in our laboratory, and was stored at − 80 °C. Bacterial cells were cultured overnight at 37 °C in 2216E liquid medium and collected by low-speed centrifugation, washed twice, and diluted in sterilized water. A drop of the sample was placed on a hydrophobic glass coverslip and dried with clean nitrogen for AFM imaging.

Animal cell preparation for AFM imaging

Hemocytes cells of healthy *L. vannamei* (body length: 8–10 cm) were isolated and centrifuged at 1000×*g* for 7 min. The flocculent precipitate was resuspended in 2 × L15 (Gibco, Grand Island, NY, USA) containing 19% (v/v) fetal calf serum and 1% (v/v) penicillin and streptomycin and incubated in a cell culture dish (Φ35 mm; Corning Inc., Corning, NY, USA) at 28 °C for 2 h. The medium was removed and the cells were washed twice for 3 min each with 2 × phosphate-buffered saline composed of 16 g/l NaCl, 5.8 g/l $Na_2HPO_4{\cdot}12\ H_2O$, 0.4 g/l KCl, and 0.4 g/l KH_2PO_4 (pH 7.4); the cells were then fixed in 2.5% glutaraldehyde (Solarbio, Beijing, China) for 10 min and thoroughly rinsed twice with distilled water before drying under clean nitrogen for AFM imaging.

Imaging by AFM

The samples were imaged by AFM (Model 5500 microscope; Agilent Technologies, Santa Clara, CA, USA) in the tapping mode in air. Rectangular Si cantilever type II probes were used to image the virus and bacteria in air at a resonant frequency of 75 kHz and elastic constant of 2.8 N/m (N9812B; Agilent Technologies). Type VII probes were used to image shrimp cells in air at a resonant frequency of 43 kHz and elastic constant of 0.14 N/m (N9866A; Agilent Technologies). All of the probes were also used to image the polymer in liquid; stable images were obtained at a scanning rate of 1.0–3.5 Hz.

Results

Analysis of WSSV topography by AFM imaging

Intact WSSV viral particles at an appropriate concentration were immobilized on hydrophobic glass coverslips to reduce the risk of damaging the fragile viral envelope. The 3D topography analysis by AFM revealed that although the envelope was incomplete, the WSSV had a length and width of 343.3 ± 85.2 and 135.9 ± 29.0 nm (N = 20), respectively (Fig. 1), which is consistent with results obtained by transmission electron microscopy (Xie et al. 2005).

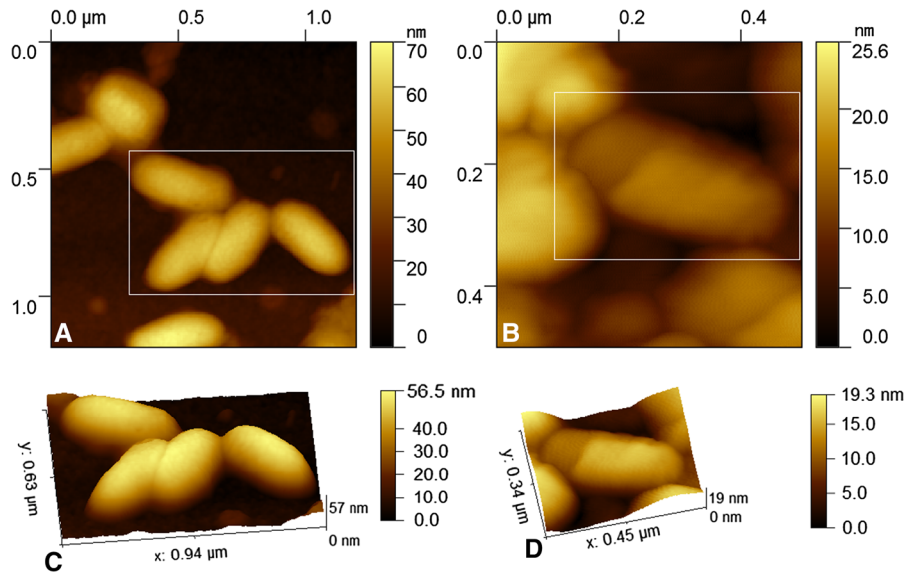

Fig. 1 Surface topography of WSSV particles imaged by AFM. **a** WSSV particles with intact envelopes. **b** WSSV particles with incomplete envelopes. **c**, **d** Analysis of the 3D topography of WSSV particles in the area enclosed by a rectangle in (**a**, **b**) using the open source software Gwyddion

Analysis of *B. firmus* topography by AFM imaging

Two flagella were observed on *B. firmus* cells (Fig. 2a), each with a diameter of 9.4 ± 0.7 nm (N = 20) (Fig. 2b). However, AFM imaging also revealed many aflagellate bacteria, suggesting that the flagellum is easily lost in the culture environment or during sample preparation.

Analysis of shrimp cell topography by AFM imaging

The surface characteristics of primary cultured hemocytes of *L. vannamei* were examined from AFM images (Fig. 3). The byssus and inclusions of cells growing on the substrate were clearly visible, and the position of inclusions was higher than byssus in many hemocytes cells imaged by AFM in air. In addition, the cell membrane appeared fragile and sticky.

Discussion

Sample preparation is a key step for imaging by AFM. Modified substrates can improve the adsorption of sample surface proteins and thereby reduce the risk of damage to fragile samples. Many types of substrate have been used for AFM including mica, glass, and

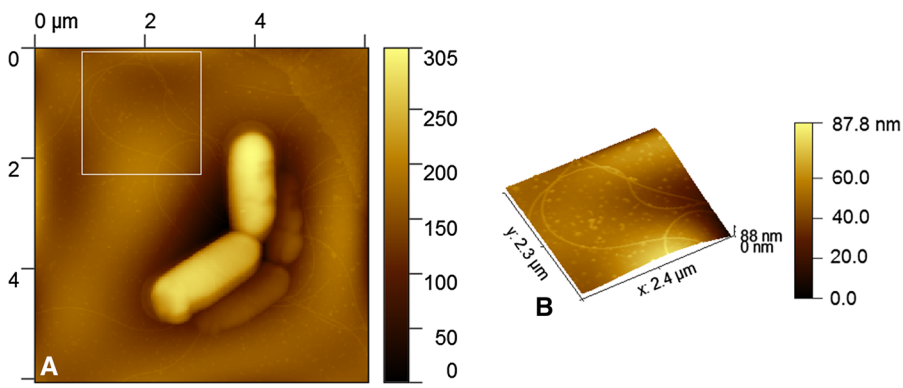

Fig. 2 Surface topography of *B. firmus* cells imaged by AFM. **a** In this image, two cells are superposed on four cells, with scattered flagella also visible. **b** Analysis of the 3D topography of flagella in the area enclosed by a square in (**a**) using the open source software Gwyddion

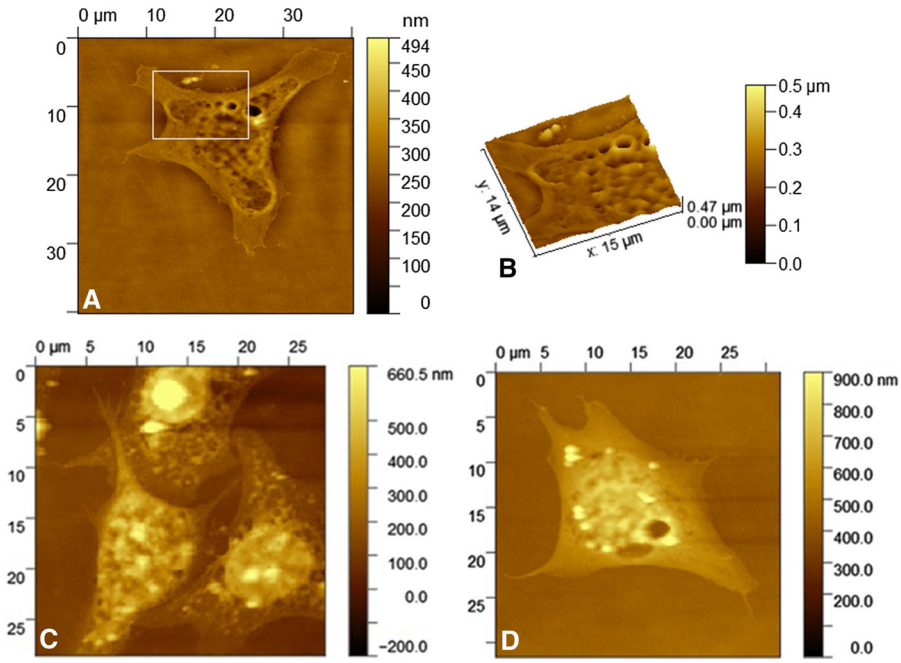

Fig. 3 Surface topography of primary cultured hemocytes cells of *L. vannamei* imaged by AFM. **a** The byssus and cell inclusions of hemocytes were clearly visible. **b** Analysis of the 3D topography of cell inclusions in the area enclosed by the square in panel A using the open source software Gwyddion. **c**, **d** The position of inclusions was higher than byssus in many hemocytes cells imaged by AFM in air

graphite, among others. Tobacco mosaic virus samples have been prepared on mica and graphite for AFM imaging (Dubrovin et al. 2004). Chemical modification of a mica surface was shown to enhance the adhesion of viral particles, which allowed the acquisition of highly reproducible images (Dubrovin et al. 2007). Glass coated with poly-L-lysine, graphite processed with poly-L-lysine, and highly oriented pyrolytic graphite have been employed to image herpes simplex virus by AFM, with the latter two substrates showing superior capacity for promoting viral particle adsorption (Liashkovich et al. 2008). Lei et al. described the rapid and label-free detection of WSSV using a surface plasmon resonance (SPR) device based on gold films prepared by electroless plating (Lei et al. 2008). Liu et al. described a method to image WSSV which suspended in PBS buffer were deposited onto freshly cleaved mica surface (Liu et al. 2010). In this study we used glass coverslips that were rendered hydrophobic with HMDS to image shrimp hemocytes and two associated pathogens in air, the image of all three particles showed in an improved resolution and quality without obvious deformations. And these can be further processed with other reagents to broaden their applicability to the research in liquid.

In order to preserve sample integrity, a low centrifugal force was applied during virus purification and bacterial enrichment to avoid damaging the capsule membrane and flagella, respectively. Hemocytes were obtained from healthy *L. vannamei* and fixed in glutaraldehyde, which helped to prevent cell degradation, autolysis, and deformation. Since the cell membrane was fragile and sticky, we used probes with lower resonance frequency and elastic constants and a lower scanning rate, since the drag force between the probe and edge can reduce image resolution.

The morphology *L. vannamei* hemocytes, WSSV, and *B. firmus* were described detailed by ATM in this study. The measured length of the WSSV nucleocapsid increased when the envelope was disrupted, and the structures of the virion envelope and nucleocapsid were not clearly visible due to the low resolution of imaging in air and the limitations of the sample preparation method. Interaction of some WSSV capsule membrane proteins with the host cell has been observed by immunofluorescence imaging—e.g., vp37 with BP53 (Liu et al. 2009; Liang et al. 2015; Li et al. 2016) and vp28 with PmRab7 (Sritunyaluksana et al. 2006). However, the mechanism of infection has not been fully described. Sample visualization at the single-particle level by AFM could be used to highlight mechanism of infection.

AFM is widely used in nanoscale imaging and related dynamic measurement. Marchante et al. used

quantitative AFM and single-particle image analysis to characterize the morphology and suprastructure of the synthetic Sup35NM prion particles, and resolve their size distribution and particle concentration to a high quantitative detail (Marchante et al. 2017). In many of the molecular structure of study, to get more comprehensive detailed data, need to combine a variety of ways to get data for analysis. Galloway et al. revealed a network of hexagonal and irregular features on the SiO_2-SAGE surface using various methods, such as PDMPO fluorescence spectra, TEM images, SEM images, AFM plots, CLEM images, bright field and fluorescence LM images and so on (Galloway et al. 2017). These researches are mostly leading the research field of aquatic animals to some extent. As we know, aquaculture is a growing big industrial in the world, and shrimp is one of mainly cultured species, also facing threaten by major diseases and new emerging diseases. Researches on diseases prevention and control are an urgent need in aquaculture. Due to unsuccessful establishment of shrimp cell lines, some challenging technologies and methods are limited applied in research on diseases prevention and control measurement in aquaculture.

We used laser confocal microscopy to research the co-localization between WSSV membrane protein with binding sites on shrimp hemocytes to reveal the virus infection mechanisms (Ma et al. 2014, 2016; Liang et al. 2015; Liu et al. 2015; Li et al. 2016). However, the limitation of optical microscope magnification and the resolution confined to subcellular localization relied on the accumulation of fluorescent signals, which lacking accurate analysis the ultrastructure on cell surface. With nanoscale resolution, AFM is a promising tool to study the mechanism of virus infection in ultrastructure scale. The method described in this study can be employed to examine the topography of fragile samples such as the primary cells of aquatic animals and their associated pathogens, would laid a foundation for the high-resolution analyses of host–pathogen interactions and other basic physiological processes in aquatic animals.

Conclusions

The results of this study provide the description of the 3D morphology of hemocytes of *L. vannamei* and WSSV particles and *B. firmus* associated with this species, which was resolved by AFM imaging in air. The method of sample preparation and imaging by AFM described in this study would lay a foundation for the high-resolution analyses of host–pathogen interactions and other basic physiological processes in aquatic animals.

Acknowledgements This study was supported by the National Natural Science Foundation of China (Grant No. 31302233); Special Scientific Research Funds for Central Non-Profit Institutes, Yellow Sea Fisheries Research Institute (Grant No. 20603022018014); the projects under Central Public-interest Scientific Institution Basal Research Fund, CAFS (Grant No: 2017HY-ZD1002) and China Agriculture Research System (Grant No. CARS-47).

References

Alonso JL, Goldmann WH (2003) Feeling the forces atomic microscopy in cell biology. Life Sci 72:2553–2560

Dubrovin EV, Kirikova MN, Novikov VK, Sgalari G, MacKintosh FC, Carrascosa JL, Schmidt CF, Wuite GJ (2004) Study of the peculiarities of adhesion of tobacco mosaic virus by atomic force microscopy. Colloid J 66(6):673–678

Dubrovin EV, Drygin YF, Novikov VK, Yaminsky IV (2007) Atomic force microscopy as a tool of inspection of viral infection. Nanomedicine 3(2):128–131

Fotiadis D, Scheuring S, Müller SA, Engel A, Müller DJ (2002) Imaging and manipulation of biological structures with the AFM. Micron 33:385–397

Francis LW, Lewis PD, Wright CJ, Conlan RS (2010) Atomic force microscopy comes of age. Biol Cell 102:133–143

Galloway JM, Senior L, Fletcher JM, Beesley JL, Hodgson LR, Harniman RL, Mantell JM, Coombs J, Rhys GG, Xue WF, Mosayebi M, Linden N, Liverpool TB, Curnow P, Verkade P, Woolfson DN (2017) Bioinspired silicification reveals structural detail in self-assembled peptide cages. ACS Nano 12(2):1420–1432

Lei Y, Chen HY, Dai HP, Zeng ZR, Lin Y, Zhou FM, Pang DW (2008) Electroless-plated gold films for sensitive surface plasmon resonance detection of white spot syndrome virus. Biosens Bioelectron 23(7):1200–1207

Li C, Gao XX, Huang J, Liang Y (2016) Studies of the viral binding proteins of shrimp BP53, a receptor of white spot syndrome virus. J Invertebr Pathol 134:48–53

Liang Y, Xu ML, Wang XW, Gao XX, Cheng JJ, Li C, Huang J (2015) ATP synthesis is active on the cell surface of the shrimp *Litopenaeus vannamei* and is suppressed by WSSV infection. Virol J 12:49

Liashkovich I, Hafezi W, Kühn JE, Oberleithner H, Kramer A, Shahin V (2008) Exceptional mechanical and structural stability of HSV-1 unveiled with fluid atomic force microscopy. J Cell Sci 121(14):2287–2292

Lightner DV, Hasson KW, White BL, Redman RM (1998) Experimental infection of western hemisphere penaeid

shrimp with Asian white spot syndrome virus and Asian yellow head virus. J Aquat Anim Health 10:271–281

Liu QH, Zhang XL, Ma CY, Liang Y, Huang J (2009) VP37 of white spot syndrome virus interact with shrimp cells. Lett Appl Microbiol 48:44–50

Liu YJ, Wu JL, Chen H, Hew CL, Yan J (2010) DNA condensates organized by the capsid protein VP15 in White Spot Syndrome Virus. Virology 408(2):197–203

Liu J, Song XL, Liu L, Chai PC, Huang J (2012) Effects of digestive tract probiotics on immune enzyme activity and anti-WSSV ability of *Litopenaeus vannamei*. J Fish China 36(3):444–450

Liu QH, Ma FF, Guan GK, Wang XF, Li C, Huang J (2015) White spot syndrome virus VP51 interact with ribosomal protein L7 of *Litopenaeus vannamei*. Fish Shellfish Immunol 44:382–388

Ma FF, Liu QH, Guan GK, Li C, Huang J (2014) Arginine kinase of *Litopenaeus vanname* involved in white spot syndrome virus infection. Gene 539:99–106

Ma CY, Gao Q, Liang Y, Li C, Liu C, Huang J (2016) Shrimp arginine kinase being a binding protein of WSSV envelope protein VP31. Chin J Oceanol Limnol 34(6):1287–1296

Marchante R, Beal DM, Koloteva-Levine N, Purton TJ, Tuite MF, Xue WF (2017) The physical dimensions of amyloid aggregates control their infective potential as prion particles. eLife 6:27109

Mayer MJ, Juodeikis R, Brown IR, Frank S, Palmer DJ, Deery E, Beal DM, Xue WF, Warren MJ (2016) Effect of bioengineering on size, shape, composition and rigidity of bacterial microcompartments. Sci Rep 6:36899

Ratanavalachai TC, William WA (1996) Effects of reactive oxygen species (ROS) modulators, TEMPOL and catalase, on methoxyacetaldehyde (MALD)-induced chromosome aberrations in Chinese hamster ovary (CHO)-AS52 cells. Mutat Res 357:25–33

Roos WH (2011) How to perform a nanoindentation experiment on a virus. Methods Mol Biol 783:251–264

Sritunyalucksana K, Wannapapho W, Lo CF, Flegel TW (2006) PmRab7 is a VP28- binding protein involved in white spot syndrome virus infection in shrimp. J Virol 80:10734–10742

Sun Y, Liu F, Song XL, Mai KS, Li YH, Huang J (2012) Effects of adding probiotics in the feed on non-specific immune gene expression and disease resistance of *Litopenaeus Vannamei*. Oceanol Limnol Sin 43(4):845–851

Tamayo J (2003) Structure of human chromosomes studied by atomic force microscopy. J Struct Biol 141:198–207

Tamiya E, Saito M, Iwabuchi S, Morita Y (2003) Nanoscopic imaging of human chromosomes via a scanning near-field optical/atomic-force microscopy (SNOAM). Sci Technol Adv Mater 4:61–67

Xie XX, Li HY, Xu LM, Yang F (2005) A simple and efficient method for purification of intact white spot syndrome virus (WSSV) viral particles. Virus Res 108:63–67

Yang F, Wang W, Chen RZ, Xu X (1997) A simple and efficient method for purification of prawn baculovirus DNA. J Virol Methods 67:1–4