Synthetic and Systems Biotechnology 7 (2022) 1066–1072

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Original Research Article

A new method for quantitative analysis of M13 bacteriophage by atomic force microscopy

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ARTICLE INFO

Keywords:
AFM
M13 bacteriophage titer
Mathematical deviation
Visualization
Fail to form plaque

ABSTRACT

Quantitative analysis is essential for virus research, especially in determining the virus titer. The classical method plaque assay is time-consuming, complex, and difficult for the phages that cannot form apparent plaque on the solid medium. In order to realize rapid and effective detection, a new method combining atomic force microscopy (AFM) observation and mathematical calculation is established. In this research, M13 phages with an appropriate dilution ratio were observed and counted by AFM. Based on the counting results, the titer of M13 phages can be calculated simply through mathematical substitution. Instead of cultivating overnight in plaque assay, this new method can be implemented within a few hours. Moreover, it is a method that can achieve visualization for titer determination and have the potential to determine the phages that fail to form apparent plaque, which is significant in virus quantitative assessment.

1. Introduction

Quantitative assessment is a relatively primary subject in virology research, which is of vital importance in biochemistry, especially under the severe situation of the COVID-19 pandemic currently. Different methods have been developed for quantitative analysis and the determination of titer based on the properties of all kinds of viruses.

Bacteriophage (also known as phage) is a general type of virus that can infect and replicate with bacterial cells [1]. Phages usually use bacteria as their hosts exclusively and can cause them to lysis when infected. They do not have a complete cell structure and often use materials and substances of the infected bacteria to grow and propagate. Therefore, it is difficult for phages to survive alone once they leave the hosts. The classical method plaque assay is widely used in phage research to enumerate phages and titer determination. Many phages can form large and well-defined plaques that are easy to observe and detect [2]. Therefore, titer determination can be achieved by plaque assay, and the plaque-forming unit represents the total number of viruses with infectivity. This method is usually performed by using phages to infect *E. coli*. After cultivating on a solid medium after infection, the titer can be calculated by counting the number of plaques grown on the medium. However, this method tends to be less reproducible and time-consuming, requiring 18–24 h to determine the titer [3]. Also, because of the non-infectious or prematurely degraded phages, the number of phages counted from the plaque assay is often less than the actual titer. In addition, phages that may form small and turbid plaques or even fail to form plaques on the plates are difficult to detect and count. Therefore, the results from the classical plaque assay might be unreliable [2]. Progress has been made based on the plaque assay to overcome this problem. Some studies have applied antibiotics and glycerol supplementing to increase plaque size [2,4]. However, these methods may reduce the phage titer to 70% and restrain their application in phage biology research [4]. The drop cast method has been reported for phage quantification without applying two agar layers and other supplements on the plate [4]. However, compared with the plaque assay, the results from drop cast have a lower standard deviation in phage count [4]. And it can only provide first-approximation phage titer and still require further validation for more accuracy [4]. Therefore, it is difficult to realize efficient quantitative assessment, especially for the phages that fail to form prominent plaques.

Other methods have been developed for the detection and titer measurement of different viruses these years, including the enzyme-

Peer review under responsibility of KeAi Communications Co., Ltd.

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https://doi.org/10.1016/j.synbio.2022.07.001
Received 17 April 2022; Received in revised form 29 June 2022; Accepted 7 July 2022
Available online 13 July 2022

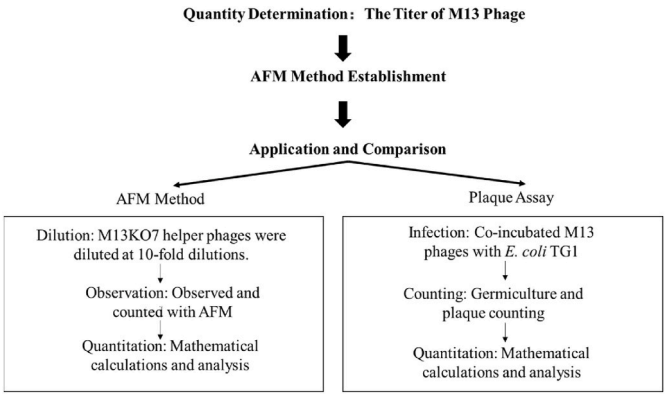

Fig. 1. The scheme of the AFM method for the determination of M13 phages and comparison with plaque assay.

linked immunosorbent assay (ELISA) and the quantitative polymerase chain reaction (qPCR) [3,5–8]. Furthermore, based on qPCR, new methods have also been studied using TaqMan and SYBR Green quantitative PCR [9]. These methods have been researched with M13 and T7 phages from phage display experiments by quantifying the genomic copy number of the two phages through TaqMan or SYBR Green qPCR and referencing against the standard curves with known concentrations [9]. These methods showed comparable accuracy to classical plaque assay, and the sensitivity of the measurement was approximately 100–1000 times higher than the traditional direct and sandwich ELISAs to detect M13 phage [9]. However, the ELISA method requires a standard sample with known standard curves as references. Besides, the qPCR analysis is less rigorous and cannot distinguish between proteins derived from complete viral particles and empty capsids. Researchers have also presented a new method by constructing a nano-flow cytometer (nFCM) [10]. They combined fluorescence analysis to detect individual silica nanoparticles and viruses, which whereas small as 24 nm and 27 nm in diameter, using light scattering [10]. However, this method is complicated to operate and requires an advanced experimental environment. Therefore, despite a few new methods to determine virus titers, it is still challenging to realize a rapid, convenient, and efficient virus quantitative assessment method.

With the advances and technological development, atomic force microscopy (AFM) gradually plays an essential role in biochemistry study. This instrument was introduced by Gerd Binnig, Calvin Quate, and Christoph Gerber in 1986 [11]. It can provide nanometer resolution imaging and has been used to study nano-scale phenomena [12]. And it has been widely applied in different research areas, including physics, chemistry, biology, and engineering [12]. Moreover, AFM has shown great potential in viral research because this instrument can work in different conditions, including in liquids [13], which is significant for virus study. Under an environment close to physiological conditions for biological samples [13], single biomolecules or biological objects can be studied using AFM. Therefore, it has been used as a powerful tool to help provide a deeper understanding of single molecules in structural biology, molecular biology, and cell biology research.

In this research, we established a new method for viral titer measurement combining AFM observation and mathematical derivation. M13 phage was selected as an example in this research. This method could achieve rapid detection and quantitative visualization, which might provide a novel idea to quantify phages and titer assessment. More importantly, it can be used to determine the titer of the phages that cannot form plaque on a plate. Also, this method could help expand the application of AFM in biochemistry. The scheme of this research is shown in Fig. 1.

2. Materials and methods

2.1. Materials

M13 phages were prepared by our laboratory and observed by AFM (NT-MDT Solver Next SKLSWJC-09). 10 × PBS buffer (pH 7.2–7.4) was bought from Solarbio (Cat. #P1022-500). Ethanol absolute was bought from RHAWN (CAS # 64-17-5). *E. coli* TG1 was bought from Biomed (Cat. # BC120-01). The monocrystalline silicon wafer was bought from Saichi (Ningbo) Electronic Technology Co., Ltd. The contact angle was measured by Contact Angle Meter (Model: JC2000D1, Shanghai Zhongchen)

2.2. Methods

2.2.1. AFM method

The prepared M13 phages were diluted at 10-fold dilutions with PBS buffer. The AFM monocrystalline silicon wafer surface was carefully washed by ethanol absolute three times and ultrapure water (18.2 kΩ) twice to remove impurities. 50 μL of each diluted sample was dropped on the freshly cleaned monocrystalline silicon wafer. After adsorption for hours, the droplets were removed. Then, the monocrystalline silicon surface was gently washed with ultrapure water five times and dried with a dryer. All the samples were observed and photographed under AFM.

2.2.2. Plaque assay

The prepared M13 phages were serially diluted with PBS buffer at 10-fold dilutions. 50 μL of each diluted sample was taken and added to 50 μL E. *coli* TG1 from the logarithmic growth phase, respectively. All the mixtures were placed on a shaker at 150 rpm for 30 min to realize full infection. Then, each mixture was evenly spread on a Luria-Bertani solid culture medium (LB medium) incubated at 37 °C overnight. The plaques were counted, and the titer was calculated.

2.3. Statistical analysis

The results from the AFM method and plaque assay were compared and analyzed statistically.

3. Results

3.1. Derivation equations for phage adsorption

a) A general equation for adsorption

The adsorption of phages on the silicon wafer surface plays an important role when establishing the AFM calculation method. Therefore, the degree of adsorption was studied through mathematical derivation with specific conditions such as temperature and pressure.

Four assumptions were made when deriving the formula: (1) The adsorption between phages and the silicon wafer surface is an irreversible system in general. (2) The whole process conforms to monomolecular layer adsorption. (3) With the contact area between the droplet and silicon wafer surface expressed by S, the adsorption rate v is proportional to S with a coefficient k_1. (4) The concentration of the droplet c, which can also be used to represent the titer of phages, has a relationship with the adsorption rate v as well. And v is proportional to c with a coefficient k_2. Thus, the adsorption rate v can be described as

$$v = k_1 S + k_2 c \tag{3.1.1}$$

Then, during the adsorption time t, the total amount of phage adsorbed on the silicon wafer surface $n(t)$ is

$$\mathrm{n}(t) = v \cdot t \tag{3.1.2}$$

While in the process of phages being adsorbed to the silicon wafer

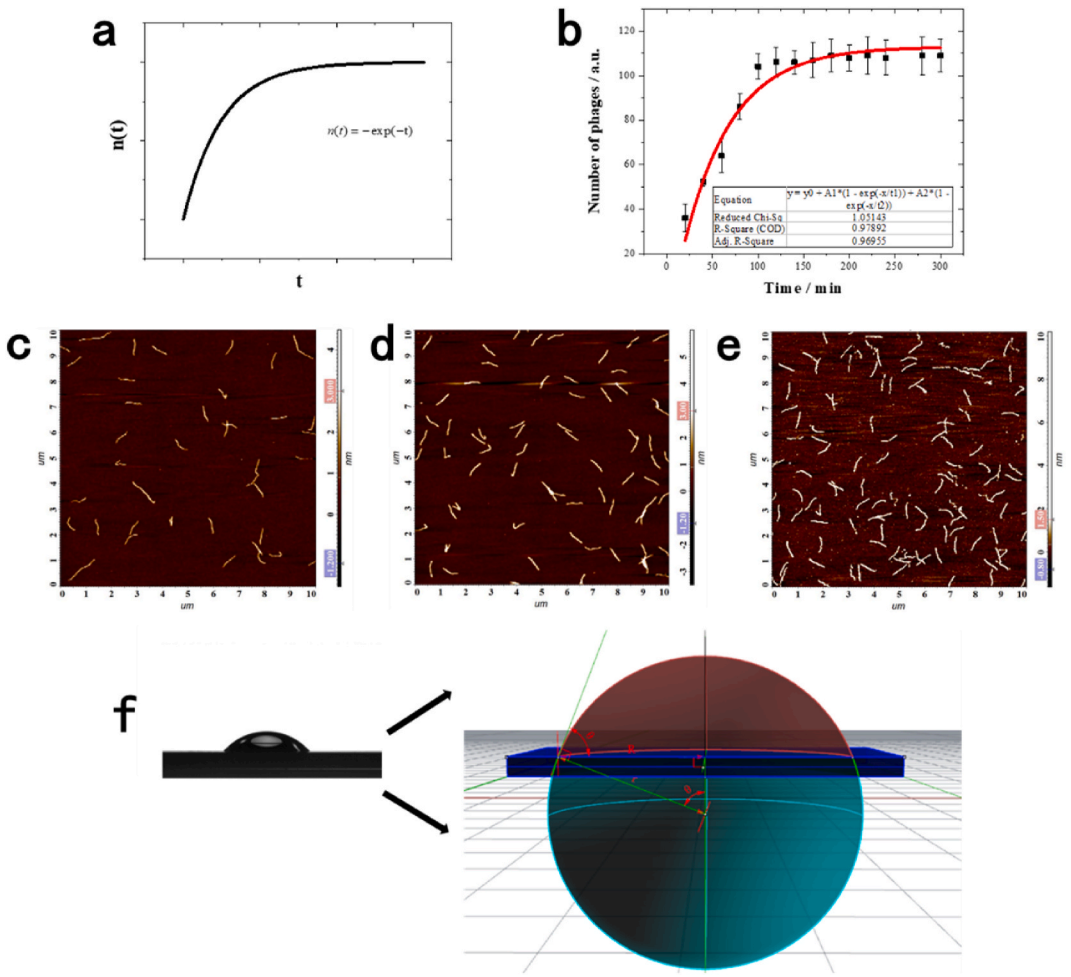

Fig. 2. a) Fitting curve based on the exponential function, b) Fitting curve based on experimental results. c) M13 phage image for 20 min adsorption, d) M13 phage image for 60 min adsorption, e) M13 phage image for 120 min adsorption, f) Calculation of contact area by solid geometry.

surface, S and c will be changed. Suppose $S^{'}$ and $c^{'}$ are the area occupied by every single phage and the concentration change in droplet when each phage falls onto the surface, respectively. Therefore, the adsorption rate v can be transformed to

$$v = k_1[S - S^{'} \cdot n(t)] + k_2[c - c^{'} \cdot n(t)] \tag{3.1.3}$$

And after substitution and integral calculation, $n(t)$ can be expressed as

$$n(t) = \frac{k_1 S + k_2 c}{k_1 S^{'} + k_2 c^{'}}\{1 - \exp[-(t + B_1)(k_1 S^{'} + k_2 c^{'})]\}$$

$$(B_1 = \text{constant}) \tag{3.1.4}$$

b) The equation for M13 phage adsorption

The M13 phage has a cylindrical shape with a length of 880 nm and a diameter of 6.6 nm [14]. Every M13 phage can be regarded as a line segment based on its unique aspect ratio. In addition, the contact area S is fixed for a 50 μL-droplet placed on the silicon wafer surface because of gravity and the nature of the contact surface. Thus, the entire adsorption process can be studied under a one-dimensional coordinate, and the adsorption rate v can be simplified to

$$v = k_2[c - c^{'} \cdot n(t)] \tag{3.1.5}$$

Accordingly, the total amount of phage adsorbed on the silicon wafer surface $n(t)$ can be indicated as

$$n(t) = \frac{c}{c^{'}}\{1 - \exp[-(t + B_2) \cdot (k_2 c^{'})]\}$$

$$(B_2 = \text{constant}) \tag{3.1.6}$$

c) Study of adsorption time

As derived above, it is evident that the total amount of phage absorbed on the silicon wafer surface $n(t)$ and the adsorption time t follow an exponential function based on e when other variables or constant terms in the equation are confirmed and fixed. Fig. 2(a) is an approximate fitting curve based on the exponential relationship between the two variables. As t becomes larger, $n(t)$ follows an upward trend. And when t reaching a particular value, almost remains the same. Accordingly, a hypothesis can be proposed that the total amount of phage absorbed on the silicon wafer surface should be nearly the same during adequate adsorption time. Also, the whole process achieves complete adsorption. Therefore, to obtain the optimum adsorption time, the identical M13 phage samples at different adsorption times were observed by AFM and counted within the same scanning area as Fig. 2 (c), (d), and (e) show. The relationship between the numb $n(t)$ er of phages and adsorption time was investigated via a fitting curve, as shown in Fig. 2(b). The figure obtained from the experiment is in high accordance with the fitting curve derived from the adsorption process, which may verify the reliability of the deduced adsorption equations. As the images demonstrate, the process almost realizes saturation when the

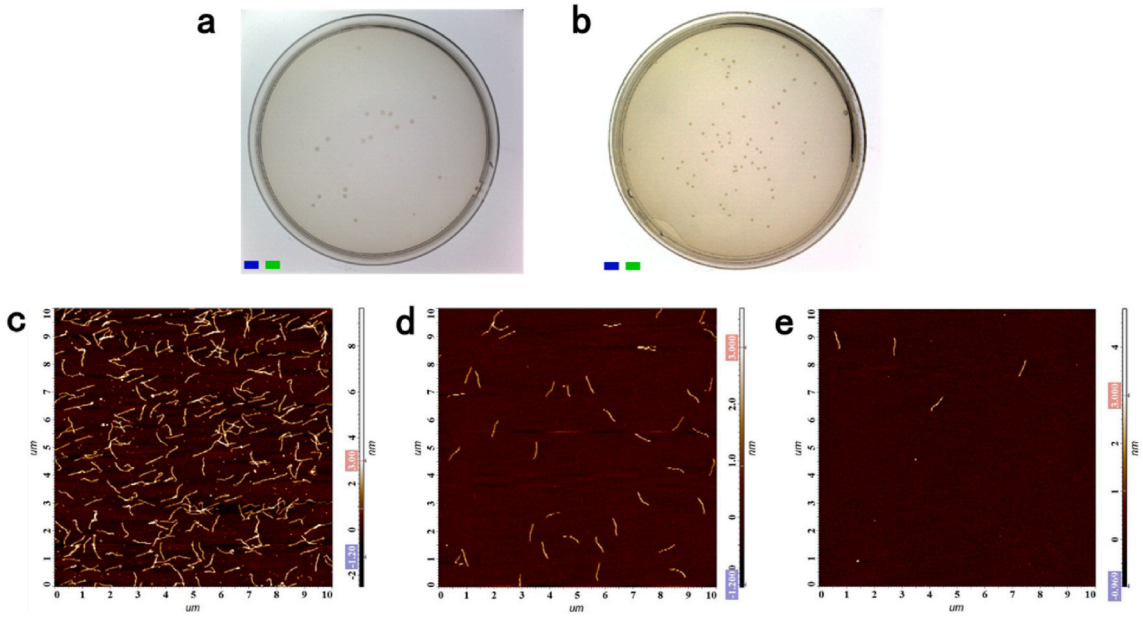

Fig. 3. Plaque assay for titer determination. a) dilution factor: 10^{11}, number of observed plaques: 18, b) Dilution factor: 10^{10}, number of observed plaques: 64. Comparison of AFM images of the different dilution factors. c) dilution factor: 10^{10}, d) dilution factor: 10^{11}, e) dilution factor: 10^{12}.

adsorption time reaches 2 h. And with the adsorption time continuing to increase, the amount of adsorbed phages changes little.

3.2. Determination of M13 phage titer

If there are n_{obs} phages that can be observed under AFM within the scanning area L^2, there will be a relationship between n_{obs}, $n(t)$, L^2 and contact area S.

$$\frac{n_{obs}}{L^2}=\frac{n(t)}{S} \tag{3.2.1}$$

As analyzed above, almost all the phages N in the droplet are adsorbed on the silicon wafer surface with adequate adsorption time, and the whole process reaches saturation. The absorbency $n(t)$ can be used to approximate N.

$$N \simeq n(t) \tag{3.2.2}$$

Thus, with the volume of the droplet V, its concentration can be represented as follows.

$$c=\frac{N}{V} \tag{3.2.3}$$

In addition, with dilution multiple A, the titer of phages, which may also be expressed by the concentration of the stock solution of phages $c_{stock solution}$, can be calculated using the formula below.

$$Titer=c_{stock solution}=c\cdot A \tag{3.2.4}$$

Therefore, based on the above derivation, the equation for titer determination by AFM can be summarized.

$$Titer=c_{stock solution}=\frac{n_{obs}\cdot S}{V\cdot L^2}\cdot A \tag{3.2.5}$$

3.3. Calculation of contact area

The contact area between the droplet and silicon wafer surface can be considered as a regular circle. In order to have a more accurate result, solid geometry and calculus can be used to calculate the contact area. Fig. 2(f) shows that the droplet with volume V has the following relationship with the radius of the contact circle R, the radius of the fitting sphere r, and the contact angle θ of the droplet.

$$R=r\cdot\sin\theta \tag{3.3.1}$$

$$V=2\pi\int_0^{r\sin\theta} x\cdot\left(\sqrt{r^2-x^2}-r\cdot\cos\theta\right)dx \tag{3.3.2}$$

After calculus calculation and substitution, the area of the contact surface circle can be indicated as

$$S=\pi R^2=\pi\cdot\sin^2\theta\left[\frac{3V}{2\pi\left(1-\cos\theta-\frac{1}{2}\cos\theta\cdot\sin^2\theta\right)}\right]^{\frac{2}{3}} \tag{3.3.3}$$

Therefore, by measuring the contact angle of the droplet using a contact angle meter, the area of the contact surface can be obtained.

3.4. Establishment of the method for detecting M13 phage titer by AFM

The formula derivation and mathematical calculations mentioned in the previous section provide a foundation for constructing the new method. In addition, two more conditions are required. (a) Firstly, the stock solution of phages is supposed to be diluted with an appropriate ratio, guaranteeing the phages in the AFM scanning area can be clearly observed and counted. (b) Secondly, the phages in the droplet are almost adsorbed on the silicon wafer surface within sufficient time, reaching a saturation state.

Therefore, the method can be finally summarized. The unknown phage sample should be diluted with PBS and placed a drop of a specific volume on a monocrystalline silicon wafer. The phages are expected to be observed and counted through AFM after adsorption for at least 2 h. Then, formula (3.2.5) can be used to calculate the titer of phages. Usually, the adsorption time is supposed to be 2-h, and the droplet volume can be 50 μL.

3.5. Method validation

a) plaque assay

The solid medium plates were shown in Fig. 3(a) and (b) after

Table 1
Measurement of contact angle and contact area.

	Contact Angle ($\theta/^{\circ}$)
(1)	49.61
(2)	48.01
(3)	46.96
(4)	53.12
(5)	51.59
Average	49.86

$$S = \pi R^2 = \pi \cdot \sin^2\theta \left[\frac{3V}{2\pi\left(1-\cos\theta-\frac{1}{2}\cos\theta\cdot\sin^2\theta\right)}\right]^{\frac{2}{3}} = 50.20\ \mu m^2$$

infection for a phage solution with an unknown titer. Since 50 μL investigated phages were placed on each solid culture medium, the average titer was calculated as 2.6×10^{13} PFU mL^{-1}.

b) AFM method

AFM observation was performed with different dilution factors for the same M13 phages, as Fig. 3(c), (d), and (e) show. By comparison, the phages were clear and countable in the image obtained from the dilution ratio of 10^{11} and 10^{12}. However, the counting result was more reliable and statistically significant for 10^{11}. Thus, the phage titer in the stock solution was calculated from the dilution factor of 10^{11}. The measurement results for contact angle and calculation for contact surface are shown in Table 1.

Also, the samples prepared with dilution ratio 10^{11} were investigated and filmed at different spots by AFM repeatedly, as Fig. 4 shows, and the results were counted and analyzed in Table 2.

c) Statistical analysis

For plaque assay, the average titer for M13 phage stock solution was 2.6×10^{13} PFU mL^{-1}. In contrast, the titer determined by the new AFM method was 5.12×10^{13} VP mL^{-1} after replicating. The results were correlated between the two methods with a relatively strong coefficient R square. Also, the difference between the two is statistically significant, as shown in Fig. 5 (a) and (b)

4. Discussion

4.1. Sufficient adsorption and saturation

In this research, complete adsorption means that the phages in droplets are absorbed entirely on the silicon wafer surface. At the same time, saturation represents that the silicon wafer surface attains the maximum adsorption capacity. This method is based on the condition of sufficient adsorption. However, the controversial point is to determine whether the saturation state achieved by adequate adsorption time can represent sufficient adsorption. Almost all the phages in the droplet can be adsorbed to the surface.

This question might be demonstrated by the AFM figures of phages from different dilution ratios. The arrangement of phages in the figures is relatively uniform, and the distribution density is comparatively low. Thus, there is still some space to accommodate extra phages. Also, with the assumption of "irreversible adsorption," it may be shown that when the adsorption reaches saturation, most of the phages in the droplet have been adsorbed to the surface, realizing sufficient adsorption. Therefore,

Table 2
Detecting titer of M13 phage by AFM method.

Numbering	a	b	c	d	e	f
Dilution ratio (A)	10^{11}	10^{11}	10^{11}	10^{11}	10^{11}	10^{11}
Volume of droplet (V/μL)	50	50	50	50	50	50
Scanning area (L^2/μm^2)	10×10	10×10	10×10	10×10	10×10	10×10
The counted number of phages (n_{obs})	55	56	60	40	45	50
Contract area (S/μm^2)	50.20	50.20	50.20	50.20	50.20	50.20
$Titer = c_{stock\,solution} = \frac{n_{obs}\cdot S}{V\cdot L^2}A$						
Titer (virus particles/VP mL^{-1})	5.52×10^{13}	5.62×10^{13}	6.02×10^{13}	4.02×10^{13}	4.52×10^{13}	5.02×10^{13}
Average Titer (virus particles/VP mL^{-1})	5.12×10^{13}					

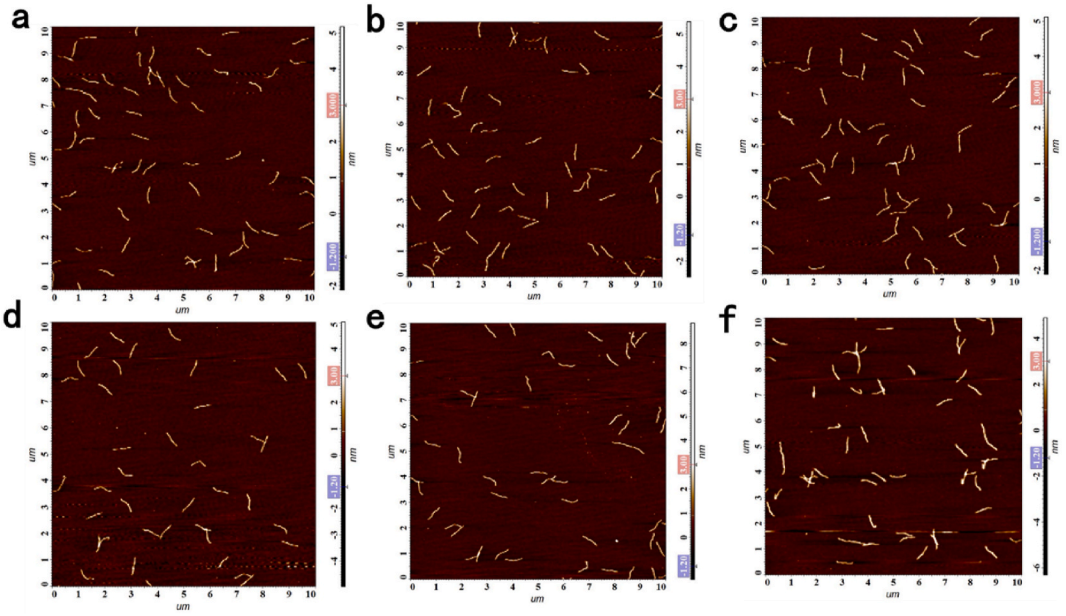

Fig. 4. Repeated observation for phages from 10^{11} dilution factor by AFM.

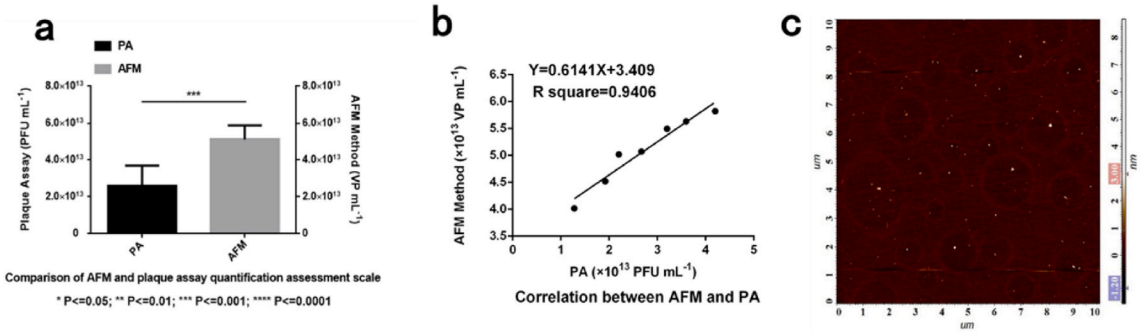

Fig. 5. Statistical analysis and comparison for the two methods. a) comparison of AFM and plaque assay quantification assessment, b) correlation between AFM and plaque assay. Discussion for the time selection: c) AFM image for 12-h adsorption.

the calculated results of the titer are efficient and reliable. In addition, there is another suppose in the deviation: the phages are arranged following the monomolecular layer adsorption. This assumption can be proved by AFM figures as well.

4.2. Selection of time

In the previous experiments and analysis, 2-h has been selected as the shortest time to achieve saturation. As Fig. 2(b) indicates, with time increasing, the number of phages adsorbed will increase gradually, but the growth rate puts the brakes on. However, the situation did not follow the trend strictly due to the volatilization of the droplet and the precipitation of PBS solution. Fig. 5(c) shows the AFM results after 12-h adsorption. It is difficult to distinguish and count the phages directly. In order to have sufficient adsorption and ensure stability of the droplets, the selection of adsorption time is set to 2-h, which could satisfy these requirements, shorten the measurement time and improve efficiency.

4.3. Comparison between plaque assay and AFM method

Plaque assay is based on counting the plaque formed by infected bacteria and is treated as the gold standard for titer detection. Effective and infectious phages can be quantified through this method. However, the operation of plaque assay is complicated, which may lead to fairly uncertainty and error. In addition, the results can be significantly affected by the health of the host bacteria, the characteristics of the solid medium culture, and other factors. Apart from that, it is a highly time-consuming and laborious process to perform.

The advantages of the AFM method are, first of all, that the quantitative visualization of virus titer detection can be realized. Phage particles can be observed and calculated directly through this approach. Secondly, this method can determine the titer of phages that fail to form apparent plaque on a solid medium, which is of vital importance in quantitative assessment. Besides, the AFM method is easy to perform and time-saving, and the results of the AFM method sometimes are reliable compared with the classic plaque assay. For example, in some immunological research, the preparation of vaccines based on inactivated viruses, regardless of the activity of the virus, is the intact virus particles that stimulate antigen production [10]. It is impossible to distinguish whether the phages are active through the AFM method, and all visible phages can be counted instead of active ones. Therefore, the AFM method can be helpful and significant in this area.

5. Conclusion

In summary, a new method to determine the titer of M13 phages using AFM has been established and validated. Based on AFM observation and mathematical calculation, this method can be visible and rapid for regular use in quantity assessment for virus research. Also, compared with the gold standard plaque assay, it is more time-efficient and suitable for virus-based study and immunological development. In this paper, the M13 phage is focused on. It also presents the potential of titer determination for phages that fail to form obvious plaques on the solid medium. In the future, this derivation idea can be employed for other types of viruses, and the prospect of this AFM method may be broadened. Meanwhile, this application of AFM has been expanded in cell and molecular biology research as well.

CRediT authorship contribution statement

Yuting Wu: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. **Shuai Liu:** Conceptualization, Investigation. **Zhiwei Liu:** Funding acquisition. **Bing Liu:** Supervision. **Bin Du:** Resources, Methodology. **Zhaoyang Tong:** Project administration, Writing – review & editing. **Jianjie Xu:** Supervision.

Declaration of competing interest

The authors declare that they do not have any competing financial or personal interests which can influence the work reported in this paper.

Acknowledgment

We thank the Foundation of State Key Laboratory of NBC Protection for Civilian SKLNBC2018-03 and SKLNBC2020-02 for financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.07.001.

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