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## Establishing Novel Bacteriophage Detection Method Based on Imaging Flow Cytometry

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# Establishing Novel Bacteriophage Detection Method Based on Imaging Flow Cytometry

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**Abstract.** Bacteriophage  $\Phi$ X174 specifically infects *E.coli* cells. It shares the similar biological characteristics with pathogenic virus. Thus, it is widely used as the “model virus” in the evaluation of virus control effect due to its safe operation. In this study, we have established a new bacteriophage detection method based on imaging flow cytometry. Combining the nucleic acid dyeing and capsid protein fluorescence labeling, the method based on imaging flow cytometry shows good linearity and specificity within its detection range. The establishment of this method is of great importance in fast and accurate detection for bacteriophage.

## 1. Introduction

Bacteriophage  $\Phi$ X174 is virus that specifically infects *E.coli* cell. As early as 1977, the researchers have sequenced its whole genome [1]. Bacteriophage  $\Phi$ X174 is a tailless bacteriophage with a diameter of about 300 Å. Bacteriophage  $\Phi$ X174 is similar in size and morphology to some pathogenicity viruses. Hence, as the “model virus”, it is widely used in the evaluation of virus control effect due to its safe operation and easy cultivation [2]. Bacteriophage  $\Phi$ X174 capsid contains a single-stranded genome DNA of 5.3-kb, which encodes four capsid structure proteins: spike protein G; DNA pilot protein H; capsid protein F and DNA-associated protein J [3]. Bacteriophage  $\Phi$ X174 recognizes its host via the interaction between G spike protein and the specific lipopolysaccharides carried on the bacteria surface. Then the G spike protein is destabilized and dissociated, which causing conformational changes of H proteins and F proteins. H and F proteins cooperate and form tubes to facilitate DNA ejection into bacteria cells. H proteins are ejected along with DNA while F proteins are left interacting with the outer membrane [4, 5].

The double-layer technique is widely employed in related research to enumerate and analyze bacteriophage. This method is based on the ability of bacteriophage to lyse its host bacteria, leading to plaque formation within the bacterial lawn [6, 7]. Researchers have developed some approaches to improve the classical double-layer technique, such as: use of dyes to increase the contrast between the plaques and the host lawn, addition of antibiotics to enhance plaque morphology [8, 9]. Although these improvements, the double-layer technique is still time-consuming. Meanwhile, some small and turbid plaques may lead to risk of underestimation, which make the double-layer technique not suitable for rapid and accurate bacteriophage detection. Detection methods based on real time PCR and western



blotting both include complicated sample preparation process, thus they are also hard to accomplish rapid detection [10, 11].

Imaging flow cytometry combines the advantages of single-cell imaging of microscopy with high-throughput analysis of flow cytometry [12]. It greatly increases the spacial resolution of flow cytometry via capturing images of every individual cell, some mistakes generated from flow cytometry can be corrected by observing and analyzing cell images [13, 14]. In our study, we have established a new bacteriophage detection method based on imaging flow cytometry. It not only obviously shorten the detection time but also shows good linearity and specificity within its measure range.

## 2. Materials and methods

### 2.1. Strains and reagents

Bacteriophage ΦX174 (ATCC13706)

*Bacillus subtilis* phage (ATCC80001)

*Escherichia coli* phage (ATCC80002)

*Escherichia coli* 13706

Specific antibody for spike G protein (made in our lab)

SYRB green and secondary antibody Alexa Fluor® 594 (purchased from Abcam)

### 2.2. Methods

Bacteriophage ΦX174 sample preparation

The bacteriophage ΦX174 reference material was dissolved in 2 mL sterile phosphate buffer solution (PBS). Then 10-fold serial dilution of bacteriophage ΦX174 suspension liquid was prepared. The diluted samples were detected by methods based on imaging flow cytometry, as well as double-layer technique.

Double-layer Technique

The double-layer technique was used as previously described [15]. Briefly, 200 μL of a dilution of bacteriophage ΦX174 sample was mixed with 200 μL bacterial suspension cultured overnight at 37°C. This mixture was added into 5 mL half-agar, gently homogenized. Then half-agar was poured into dish previously filled with 15 mL regular agar. The plate were mildly swirled, then incubated at 37°C with up-side down. The plaques could be counted when they appeared to be clear and distinguishable.

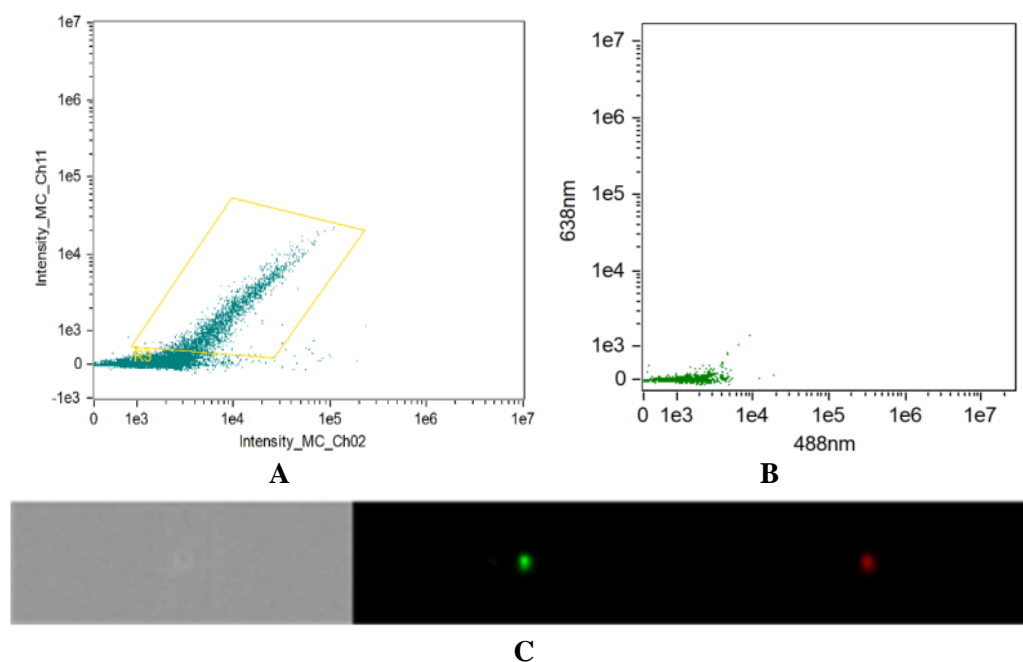
Bacteriophage ΦX174 detection method based on imaging flow cytometry

1 mL bacteriophage ΦX174 ( $1 \times 10^7$  PFU /mL) was incubated with SYRB Green and specific antibody for spike G protein. After the sequential addition of the secondary antibody Alexa Fluor® 594, the mixture was placed at darkness for 30 min. The imaging flow cytometry was applied according to the manufacturer's instructions [16]. Then the analytical software from the manufacturer's website was employed to analyze the data acquired on the imaging flow cytometry.

## 3. Result

### 3.1. Establishment of the bacteriophage detection method based on imaging flow cytometry

After optimization of the reaction condition, SYRB Green was selected as nuclear acid dye. The spike G protein was bond by its specific antibody, then the secondary antibody Alexa Fluor® 594. Based on the labeling of nuclear acid and spike G protein, we can detect the bacteriophage ΦX174 using imaging flow cytometry (Figure 1A C). As a comparison, bacteriophage ΦX174 with no labeling can't be detected (Figure 1B).



**Figure 1.** Detection of bacteriophage based on imaging flow cytometry.

A, B: Bacteriophage  $\Phi$ X174 cells with and without labeling were visualized on dot plot.

C: Representative images of single cell were shown in bright field and two fluorescence detection channels.

### 3.2. The specificity of the imaging flow cytometry based detection method

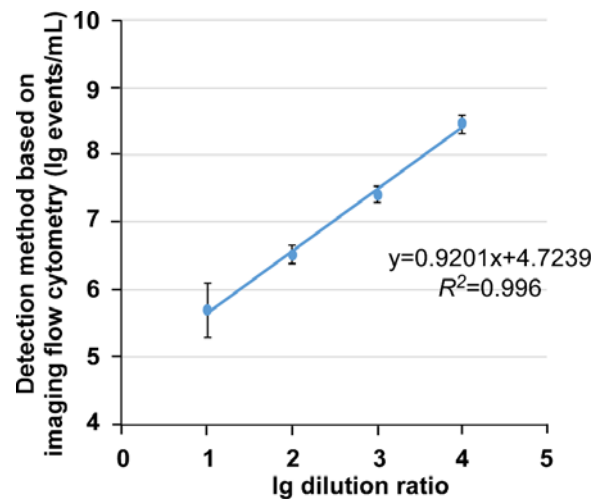
To verify the specificity of the imaging flow cytometry based method for detecting bacteriophage  $\Phi$ X174. We mixed bacteriophage  $\Phi$ X174 with *Bacillus subtilis* phage (ATCC80001) and *Escherichia coli* phage (ATCC80002) at equal ratio and detect bacteriophage  $\Phi$ X174 using the imaging flow cytometry based method. The initial concentration of bacteriophage  $\Phi$ X174 is  $4.5 \times 10^7$  events/mL, and it turned to be  $1.4 \times 10^7$  events/mL detected by imaging flow cytometry after mixture. Then we speculated that the novel method performs specifically for detecting bacteriophage  $\Phi$ X174, which is mainly depended on the specific labeling of spike G protein (Table 1).

**Table 1.** The specificity of detection method based on imaging flow cytometry.

Bacteriophage	Number	Signal detected
Bacteriophage $\Phi$ X174	ATCC 13706	Yes
<i>Bacillus subtilis</i> phage	ATCC 80001	No
<i>Escherichia coli</i> phage	ATCC 80002	No

### 3.3. The linear range of the detection method based on imaging flow cytometry

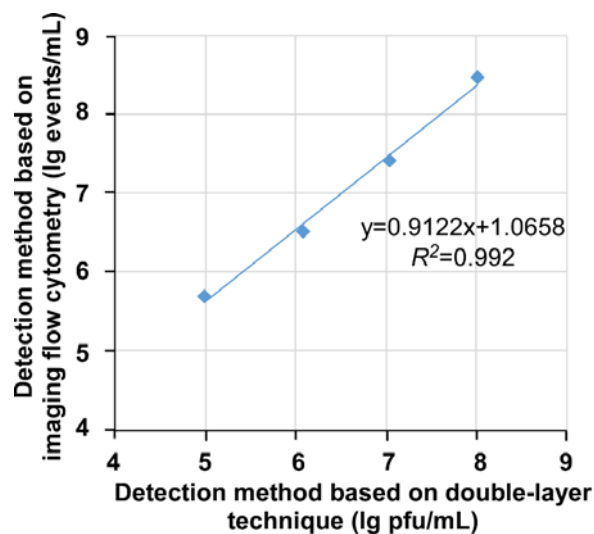
After the gradient dilution of bacteriophage  $\Phi$ X174 with sterile PBS buffer, we employed both imaging flow cytometry and double-layer technique to do the measurement. It turned out that the two methods provide the similar result when the bacteriophage concentration is between  $10^4$  and  $10^7$  PFU/mL. The detection range of the imaging flow cytometry based method is  $1.2 \times 10^4$  to  $2.8 \times 10^7$  PFU/mL. It shows good linearity within its detection range ( $R^2=0.996$ ) (Figure 2).



**Figure 2.** The linearity of detection method based on imaging flow cytometry is good.

### 3.4. The correlation between detection methods separately based on imaging flow cytometry and double-layer technique

Double-layer technique can be applied without expensive equipment. It is still the most widely used detection method by now. Here, we compare the measure result of imaging flow cytometry with that gained from double-layer technique. Their results show significant linear correlation within the detection range ( $R^2=0.992$ ) (Figure 3).



**Figure 3.** The linear relationship between methods based on double-layer technique and imaging flow cytometry.

## 4. Discussion

New infectious diseases caused by highly pathogenic viruses such as SARS, influenza and ebola have posed serious threats to human health and economic development. Protective equipment and facilities are essential to controlling the spread of the disease and protecting the health of medical and scientific personnel [17, 18]. Bacteriophage ΦX174 shares the similar biological characteristics with pathogenic virus. As an ideal "mode virus", it is widely used in the evaluation of virus control effect due to its safe operation and easy cultivation [19]. Considering its importance in the research of virus control, the detection method for bacteriophage ΦX174 also need to further explored.

Double-layer technique is the most widely used detection method for bacteriophage  $\Phi$ X174, which enumerate the plaques to quantify bacteriophage. This method is time-consuming and may cause underestimation because the persence of some small and turbid plaques. Taking its role in virus prevention research in account, it is important to establish novel rapid detection method for bacteriophage  $\Phi$ X174. As we mentioned, imaging flow cytometry enhances the accuracy of conventional flow cytometry via capturing photo of every single cell. Here, combining nuclear acid dyeing and capsid spike G protein labeling, we have developed novel detection method for bacteriophage  $\Phi$ X174 based on imaging flow cytometry. The application of this method shorten the detecion time from over 16 hours to 1 hour. Meanwhile, it shows good linearity and specificity within the detection rage. The exploration of detection method would contribute to improving measure accuracy of bacteriophage  $\Phi$ X174 in all related research.

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