Rapid determination of purity, integrity and titer of viruses by Fluorescence Nanoparticle Tracking Analysis (F-NTA) using the bacteriophage Phi6

Abstract

Determination of the titer of viruses and bacteriophages is an indispensable key technology in virological research and for diagnostic purposes. Depending on the method used, the measurements are either qualitative or quantitative, very time-consuming, and do not provide information about integrity or aggregation behaviour of the virus particles. Nanoparticle Tracking Analysis with the Particle Metrix ZetaView® instrument allows the user to perform a rapid concentration determination of virus particles. Using the bacteriophage Phi6 as an example, we show how titer, purity and integrity of phage particles can be measured quickly and reliably using the fluorescence detection capability of the Particle Metrix ZetaView® instrument.

Keywords: Bacteriophage Phi6, dsRNA, lipid layer, Sybr™Gold, Cellbrite® Fix 640, F-NTA, titer, integrity, detergent

Introduction

The level of infectious or non-infectious virus particles can be determined by several methods, all of which have shortcomings:

A: The tissue culture infectious dose 50 (TCID50) provides only a qualitative measurement and is defined as the dilution of a virus required to infect 50% of a given cell culture. It detects the presence or absence of cytopathic effects after cells are infected with serial dilutions of a virus or bacteriophage sample and is therefore very time consuming. There are several methods available to calculate the TCID50 (1-3).

B: Detection of viral nucleic acids by qPCR quantifies the viral load (copies/mL) to be calculated from the cycle threshold (Ct) value, for example in clinical samples or in virological research (4-6). However, viral load comparisons from lab to lab are often problematic without strict adherence to recognized standards and assay controls. Additionally, this method does not provide any information about the structural integrity or aggregation of the virus particles.

C: The plaque assay provides a quantitative but very time-consuming (24-72 hours) determination of infectious virus particles by quantifying plaques, i.e., areas of lysed cells in a cell culture, which are counted and determined as plaque forming units (pfu) (7-9).

All mentioned techniques are very time-consuming and require highly trained lab personnel. Particularly, in virological research and during production of virus-based therapeutics, one must find out in a quick, simple, and precise measurement the total number of virus particles as well as the structural integrity and presumed aggregation, which is considered to be an indication of a limited shelf life.

Due to the topicality of the corona pandemic, we performed our study with the enveloped spherical bacteriophage Phi6. Recent research showed that Phi6 can be used perfectly as a surrogate for SARS-CoV-2 (10) because this virion is very similar to SARS-CoV-2 in terms of geometry, spikes, structure, size, and genetic material, but is non-infectious to humans. Using a Phi6 preparation, we can show that Nanoparticle Tracking Analysis (NTA), performed by the Particle Metrix ZetaView® instrument, is a powerful method

for the characterization of nanoparticles such as viruses or bacteriophages in suspension in the size range between 25 and 1000 nm. A microscopic setup enables real-time visualization of the phage particles below the diffraction limit of conventional microscopes and captures the Brownian motion of each individual particle in a video.

Based on the Stokes-Einstein relationship, NTA measurement calculates the diffusion coefficient and the hydrodynamic diameter for each individual particle. The direct real-time visualization makes NTA a rapid and versatile method for size and concentration determination in both scattered light and fluorescence modes.

Furthermore, by using Sybr™Gold nucleic acid stain and Cellbrite® Fix 640 membrane dye, we obtained additional information such as the purity of the preparation and the structural integrity of the phage particles by fluorescence detection in a much shorter time compared to conventional methods.

Material & Methods

Bacteriophage Phi6 preparation

CsCl-purified bacteriophages Phi6 were obtained from the Leibniz Institute DSMZ in Braunschweig (German Collection of Microorganisms and Cell Cultures GmbH). According to the supplier, the phage titer was measured at $4x10^{10}$ pfu/ml.

Fluorescence labelling

For dsRNA labelling, 1 µl of a 1:50 dilution of Sybr™Gold nucleic acid stain (ThermoFisher Scientific Waltham, MA, USA) was mixed with 1 µl of the CsCl-purified bacteriophage Phi6 and incubated for 1 hour in the dark at room temperature. After incubation, the preparation was filled to 2000 µl with PBS buffer (Gibco, Fisher Scientific Paisley, UK) and subjected to NTA measurement in scatter and fluorescence mode. When using PBS, care was taken to ensure that the buffer was free of particles.

For staining the phages' lipid layer, 1 µl of a 1:100 dilution of Cellbrite® Fix 640 (Biotium Inc., Freemont, CA, USA) was added to 1 µl of the CsCl-purified phage Phi6 preparation and incubated for 15 minutes in the dark at room temperature. The staining mixture was subsequently filled to 2000 µl with PBS buffer before measurement in scatter and fluorescence mode.

For lysis controls, NP-40 alternative (in the following just named NP-40) (Calbiochem, Merck, Darmstadt, Germany), commercially available Sterillium® hand disinfectant (Bode Chemie, Hamburg, Germany), and SDS (Carl Roth, Karlsruhe, Germany) solution were used. SDS and Sterillium® were filtered using an 0.02 µm syringe filter. Sterillium® was subsequently diluted to 50% with PBS buffer, whereas the SDS solution was brought to a dilution of 0.25% and NP-40 to 0.5% with PBS buffer, respectively. After incubation, 2 µl of 50% Sterillium® disinfectant or 2 µl of 0.25% SDS or 0.5% NP-40 solution were added to the labelling preparation and incubated 30 additional minutes at room temperature in the dark. Afterwards, the preparation was filled with PBS buffer to 2000 µl and subjected to NTA measurement in scatter and fluorescence mode.

Nanoparticle Tracking Analysis

NTA measurements were carried out using a ZetaView® PMX-420 QUATT instrument equipped with the software version 8.05.14 SP7. All measurement parameters used are listed in the table below.

Table 1: Measurement parameters used for scatter and fluorescence mode.

Measurement Parameters	Scatter Mode (λ=488 nm)	Fluorescence Mode (λ=488F500 nm)	Fluorescence Mode (λ=640F660 nm)
Positions	11	11	11
#Cycles	2	2	2
Number of Frames	30	30	30
Sensitivity	80	95	94
Shutter	100	100	200
Min. Brightness	30	30	30
Max. Area	1000	1000	1000
Min. Area	10	10	10
Tracelength	15	15	15
Frame Rate (fps)	30	30	30
Tracking Radius	100	100	100

The purity of the phage preparation was calculated using the formula shown below:

$$purity = \frac{conc.\ fluorescence\ mode}{conc.\ scatter\ mode}$$

Results & Conclusions

Total particle titer

For downstream experiments, analysis of the total particle concentration of the CsCl-purified bacteriophage Phi6 preparation is of great interest. Therefore, the bacteriophage preparation was first measured in scatter mode of the ZetaView® instrument and compared with the pfu value provided by the supplier. This result is very important as it gives information about the ratio of the total number of particles and the number of infectious phages in the preparation. In addition, the shape of the size distribution provides information on whether aggregates of phage particles are present or not. A representative size histogram derived from a scatter measurement is shown in Figure 1.

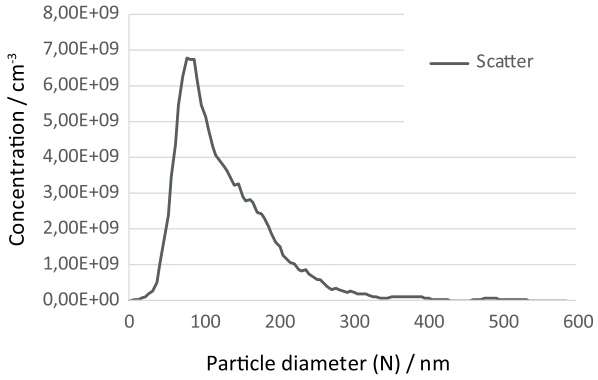

Figure 1: Size distribution of total particle count of bacteriophage Phi6 preparation measured by NTA in scatter mode.

NTA detection in scatter mode

Scatter-based NTA measurements of the phage Phi6 preparation resulted in a peak diameter of 87.4 nm which is in very good agreement with published data showing the intact complete phage with a diameter between 60 and 100 nm (11). The median (X50) is calculated to 114.3 nm and therefore slightly larger than in published data which is most likely due to the presence of aggregated phages or, to a lesser extent, other nanoparticles such as salt precipitates or protein aggregates. The measured concentration is $7.08x10^{10}$ particles per ml. This is about 1.8 times higher than the value of $4x10^{10}$ pfu/ml given by the supplier for the preparation. However, this finding is not surprising since the pfu value relates only to the number of infectious phage particles but not to the total particle count where non-infective fragments are included.

Particularly during production of virus-based assays or therapeutics, the total count of intact virion particles, infective or non-infective, is of great importance.

In the following experiments we show that fluorescence based NTA (F-NTA) allows detection of the phages' nucleic acid and lipid layer while collecting size and concentration information. Thus F-NTA provides a rapid, simple, and precise tool for assessing the integrity of phage particles. As mentioned previously, dsRNA and the phages' lipid layer were labelled with the nucleic acid stain Sybr™Gold and membrane stain Cellbrite® Fix 640, respectively, leading to much more specific F-NTA detections. Furthermore, by knowing the concentration of phage particles in scatter and fluorescence mode, the purity of the phage preparation can be calculated. Representative histograms derived from F-NTA measurements are shown in Figure 2.

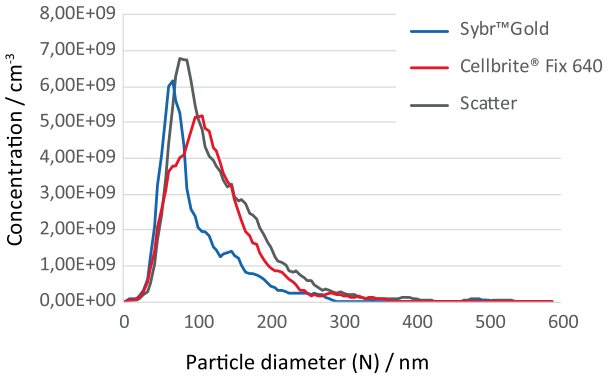

Figure 2: Example of an F-NTA measurement of a bacteriophage Phi6 preparation labelled with Sybr™Gold nucleic acid stain (blue curve) and Cellbrite® Fix 640 lipid layer stain (red curve) compared to scatter-based NTA (grey curve). Purity was calculated to be 82% for dsRNA and 85% for lipid layer containing phage particles.

F-NTA measurements of Sybr™Gold and Cellbrite® Fix 640 led to much more specific results, since the focus of the measurements was now on phage particles possessing dsRNA and a lipid layer and can therefore be considered intact. All F-NTA measurements with Sybr™Gold and Cellbrite® Fix 640 were performed in at least 5 replicates.

F-NTA detection of Sybr™Gold

Fluorescence-based NTA measurement with Sybr™Gold labelled bacteriophages resulted in a diameter (X50) of 86.2 nm which is in very good agreement to published data (11). The measured particle concentration is $5.75x10^{10}$ particles per ml which is much closer to the value of $4x10^{10}$ pfu/ml given by the supplier for this preparation. The fact that the number of particles measured via F-NTA is still somewhat higher than the number provided by the supplier is most likely due to the fact that non-infectious bacteriophages that carry dsRNA are also included in the F-NTA measurement result. With regard to dsRNA-containing particles detected by F-NTA, we calculated a purity of around 82% of intact bacteriophages in the preparation.

F-NTA detection of Cellbrite® Fix 640

F-NTA size measurement of Cellbrite® Fix 640 labelled phage particles resulted in 120.4 nm (X50). This result differs significantly from published data (11). The effect that the diameter of membrane-containing biological nanoparticles after labelling their lipid layer is larger than expected, suggests that the integration of the membrane dye causes the diameter of the particles to swell slightly. However, it is not known to what extent the interaction of the used membrane dye influences the spatial expansion of the phages' lipid layer and thus makes the phage particles appear larger during the measurement. The measured particle concentration is $6.05x10^{10}$ particles per ml which meets the pfu value of $4x10^{10}$ pfu/ml provided by the supplier quite well.

Similar to the dsRNA labelling, the difference of particle count between the number of membranous phage particles measured by F-NTA and the number derived from the plaque assay is likely an indication that intact but non-infectious bacteriophages are included in the F-NTA results. However, it cannot be ruled out that individual membrane-containing particles may be detected in the phage preparation that may have been detached during the purification procedure and are no longer associated with phage particles.

To ensure that the detected particles are membrane-coated phage particles, lysis controls were carried out. Depending on their strength and chemical content, suitable detergents should only destroy the lipid layer or lyse the entire phage particles.

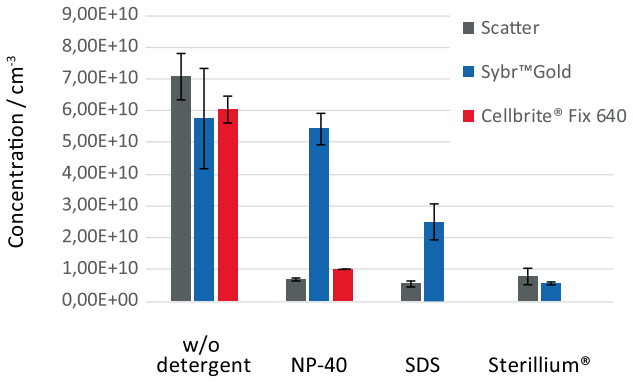

Figure 3: Comparison of lysis controls using different detergents with regard to the total particle concentration of unlabelled particles (scatter) and those labelled with Sybr™Gold (dsRNA) or Cellbrite® Fix 640 (lipid layer).

Figure 3 shows a summary of results of untreated phage preparations and phage particles treated with detergent. Treatment with NP-40 resulted in a 90% reduction of phage particles detected in scatter mode and 84% reduction detected in the red fluorescence channel. The significant decrease in the number of bacteriophages indicates that treatment with NP-40 causes almost complete lysis of the lipid layer, resulting in only 16% of membrane coated particles detected in the red fluorescence channel. However, the phages' dsRNA could still be detected by F-NTA in the green fluorescence channel after NP-40 treatment. Most likely, these are either intact protein capsids containing phage RNA, or individual RNA genomes released into solution after NP-40 lysis.

Treatment of Phi6 with SDS or Sterillium® disinfectant resulted in a complete absence of detectable lipid layers in the red fluorescence channel. In addition, further reduction of the total particle count was observed in scatter mode (SDS 93% reduction, Sterillium® 90% reduction) as well as in the green fluorescence channel for Sybr™Gold labelled dsRNA containing phage particles (SDS 57% reduction, Sterillium® 90% reduction).

Negative controls without Phi6 bacteriophages but with all other components show no or only a very small number of particles compared to Phi6 positive samples (see Figure 4).

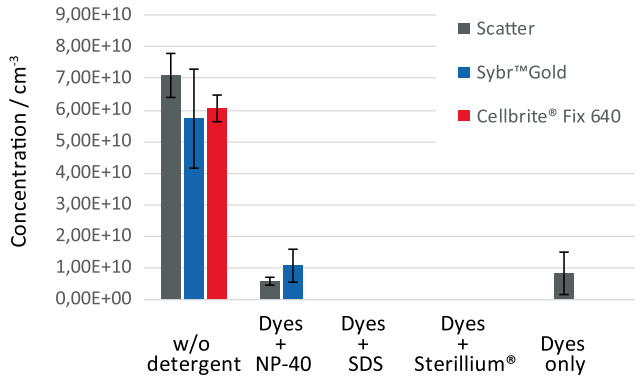

Figure 4: Negative controls without Phi6 bacteriophages but with all other components labelled with Sybr™Gold (dsRNA) or Cellbrite® Fix 640 (lipid layer) and analyzed in scatter and fluorescence mode. The first data set serves as comparison and represents labelled Phi6 without detergent (ref. figure 3).

The combination of both dyes, Sybr™Gold and Cellbrite Fix® 640 with NP-40 showed a small number of particles, both in the scatter and in the green fluorescence channel, which is not the case in the samples treated with SDS and Sterillium®. Most likely, this can be explained by the formation of micelles by NP-40 and incorporation of Sybr™Gold into these micelles. However, it cannot be ruled out that the dyes are not completely particle-free or that interaction of both dyes in the solution may form particles as partly seen in the last data set.

Summary

Using the example of the bacteriophage Phi6, the data clearly show that labelling with the nucleic acid stain Sybr™Gold and the lipophilic membrane dye Cellbrite® Fix 640 can identify and discriminate bacteriophages from other, non-vesicular nanoparticles in the preparation by using F-NTA. Furthermore, with F-NTA, both the titer and the integrity of the virus particles could be determined much more specifically than is possible with scatter-based NTA. Titer measurements using only scatter-based NTA may lead to an overestimated number of phages and therefore to incorrect results, since those analyses include non-phage particles in the solution due to the lack of specific fluorescent markers. Hence, F-NTA makes the method significantly superior to scatter-based NTA and thus provides greater accuracy when determining integrity and titer of viruses.

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Head Office

Particle Metrix GmbH
Wildmoos 4
D-82266 Inning / Germany

+49-8143-99172-0
info@particle-metrix.de

US Office

Particle Metrix Inc.
Mebane, NC 27302 / USA

+1-919-667-6960
usa@particle-metrix.com

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