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A flow cytometric granularity assay for the quantification of infectious virus

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ABSTRACT

A flow cytometry-based assay was developed to assess the infective titer of two recombinant viruses: a recombinant herpes simplex type 2 (rHSV-2) and a recombinant canary pox (rALVAC.gfp). This method uses granularity of infected Vero and QT-35 cells, respectively, and correlates this to the infectious titer of virus samples. The percent of the cell populations with a high level of granularity could accurately be correlated to viral titers obtained through a traditional plaque assay, with R^2 values greater than 0.8 using a semi-logarithmic scale. This approach offers a rapid, high-throughput method for infectious virus titration with similar accuracy to a traditional plaque assay.

1. Introduction

To support research and development of new virus-based vaccines and therapeutics, rapid infectious virus enumeration is critical in a variety of different manufacturing, analytical and clinical activities. Traditional methods employed to determine the infectious virus titer in samples include the plaque assay and the cell culture infectious dose ($CCID_{50}$) assay; both methods are typically labor intensive, have low throughput because of long incubation times (3–7 days), and suffer from high inter-assay variability [1]. Furthermore, rapid titer approximations that target total virus particles, e.g., staining surface proteins or genetic material, or by examining particle diffusion patterns, are unable to differentiate between infectious and defective virus.

Flow cytometry has also been used as a method to determine infectious virus titer, capitalizing on its ability to amass large datasets from the rapid analysis of thousands of single cells [2–5]. Traditional flow cytometers use the scattering or emission of light to discriminate cells with different physical and biological characteristics within a large population. Forward scattered light (FSC) (0.5–5° from the incident beam) and side scattered light (SSC) (15–150° from the incident beam) give the relative size and granularity of the cells, respectively [6]. Laser light can also be used to excite fluorophores from which emitted light can be quantified and used to detect specific biological features of individual cells. As a result, flow cytometry has been used to quantify infectious viruses using the expression of genes encoding for fluorescent proteins in infected cells [3,7–10]; the labelling of viral proteins using fluorescent antibody conjugates [11]; or the morphological changes in the cells [12]. However, the use of fluorescent reporter proteins requires a specifically engineered cell line or virus, which can be time-consuming to develop, while either antibody-detection of viral proteins or the use of intercalating dyes targeting the viral genomes often requires customized reagents that are not readily available. Capturing early cellular physical changes caused by viral infection offers a simpler method of detecting infectious virions. Specifically, changes in granularity have been observed during the course of infection of insect cells by baculovirus, human embryonic kidney cells (HEK293) by adenovirus, foot and mouth disease virus in baby hamster kidney cells (BHK-21), and mouse neuroblastoma cells (MNA) by rabies virus [11–15].

Abbreviations: AV529-19, a Vero cell line designed specifically to express the U_L5 and U_L29 viral genes; BHK-21, baby hamster kidney cells; $CCID_{50}$, cell culture infectious dose; %CV, coefficient of variation; DI, deionized water; DMEM/F12, Dulbecco's Modified Eagle with Ham's F12 50:50 Mix Medium; D-PBS, Dulbecco's Phosphate Buffered Saline; EDTA, ethylenediaminetetraacetic acid; FC, flow cytometry-based assay; HEK293, human embryonic kidney cells; HI-FBS, heat inactivated fetal bovine serum; hpi, hours post infection; FSC, forward scattered light; LOD, limit of detection; MNA, mouse neuroblastoma cells; MOI, multiplicity of infection; PFU, plaque forming units; QT-35, quail muscle fibroblast cells; rALVAC.gfp, recombinant attenuated canarypox virus; rHSV-2, recombinant herpes simplex virus serotype 2, also known as ACAM529; SSC, side scattered light.

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Here, we present a method to quantify the infectious titer of two recombinant viruses, a recombinant herpes simplex virus serotype 2 (rHSV-2) and a recombinant attenuated canarypox virus (rALVAC.gfp), based on post-infection increases in the granularity of Vero and QT-35 cells, respectively, as measured by commercial flow cytometers. Additionally, we show that changes in the granularity of the population are indeed due to replication of the virus, that the method can be applied to two different host-virus pairs (rHSV-2/Vero and canarypox/QT-35), and that the incubation time for detection is dependent on the replication cycle of the virus-host pair. We also show that the method can be extended to different flow cytometers including one with a 96-well auto-sampler, realizing the potential as a rapid, high-throughput approach.

2. Materials and methods

2.1. Cell lines and viral strains used in single tube assay development

The rHSV-2 virus used in this study is a monoclonal mutant strain that is replication-deficient in non-complementary cells due to the absence of the U_L5 and U_L29 viral genes (also known as ACAM529 [16]). The rHSV-2 was stored at −80 °C, thawed in a 37C water bath immediately before use, and diluted with cold (4 °C) cell culture media prior to infection of AV529-19 cells (Sanofi Pasteur, Toronto, Canada) or Vero cells (ATCC, Manassas, VA, USA). AV529-19 is a research working cell bank of a complementing Vero cell line designed specifically to express the U_L5 and U_L29 viral genes. Both AV529-19 and Vero cells were maintained in Dulbecco's Modified Eagle Medium/Ham's F12 50:50 mix (DMEM/F12) (Corning Cellgro, Manassas, VA, USA) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) (Gibco Life Technologies, Burlington, Canada), and 4 mM L-glutamine (Sigma-Aldrich, Oakville, Canada), and incubated at 37 °C in 5% CO_2.

rALVAC.gfp (Sanofi Pasteur, Toronto, Canada) is a recombinant attenuated canarypox virus engineered to express green fluorescent protein (GFP) under control of the vaccinia virus H6 early-late promoter. rALVAC.gfp was stored at −80 °C, thawed in a 37 °C water bath immediately before use, and then diluted with cold (4 °C) cell culture media prior to infection of QT-35 cells (ECCAC, Salisbury, United Kingdom). QT-35 cells were maintained in Eagle's Minimum Essential Media (Sigma-Aldrich, Oakville, Canada), supplemented with 10% HI-FBS (Gibco Life Technologies, Burlington, Canada), 1% Non-Essential Amino Acids (Sigma-Aldrich, Oakville, Canada), and 4 mM L-glutamine (Sigma-Aldrich, Oakville, Canada), and incubated at 37 °C in 5% CO_2.

All cultures were grown in surface tissue culture flasks with vented caps (Thermo Scientific, Waltham, MA, USA).

2.2. Cell line and virus strains used in Semi-Automated flow cytometry assay

AV529-19 cells were maintained in a proprietary serum-free medium (Sanofi Pasteur, Toronto, Canada) in T75, T175 or T225 tissue culture flasks with vent caps and incubated at 37 °C in 5% CO_2. Cell passaging was conducted using recombinant trypsin (Roche Custom Biotech, Indianapolis, IN, USA) and trypsin inhibitor (Sigma Aldrich, Saint Louis, MO, USA) prepared in cell culture media. Cell counts were conducted using an NC-100 NucleoCounter (Chemo-Metec A/C, Allerod, Denmark).

Virus samples were obtained either from bioreactor cultivations or spinner flask experiments, and were of different levels of purity. The calibrator stock had an established titer of 4.6×10^6 plaque forming units per mL (PFU/mL) from repeated plaque assays conducted at Sanofi Pasteur.

2.3. Plaque assay to determine infectious virus titer

AV529-19 cells were seeded on 12-well tissue culture plates at a concentration of 3×10^5 cells per well and incubated overnight. The next day, the cells were washed once with Dulbecco's Phosphate Buffered Saline (D-PBS) (Life Technologies, Burlington, Canada) and incubated with 200 μL of serially diluted virus samples. The cells and virus were incubated at 37 °C for 1 h, with gentle rocking every 15 min. After 1 h, 1 mL of overlay media (DMEM/F12), 4 mM L-glutamine, 1% HI-FBS, 1% penicillin/streptomycin, and 0.75% methyl cellulose) was added and the cells were incubated for an additional 48 h. After incubation, the overlay media was removed and the plates were stained with 300 μL of 1% crystal violet (Sigma-Aldrich, Oakville, Canada) in 70% methanol solution for 30 min. The crystal violet solution was removed, the plates were rinsed with deionized (DI) water and then manually counted (between 50 and 200 plaques) based on the number of visible plaques to determine the titer.

2.4. $CCID_{50}$ assay to determine infectious virus titer

Since rALVAC.gfp does not form well-defined plaques, $CCID_{50}$ assays were performed. QT-35 cells were seeded onto a 96-well plate at a concentration of 3×10^4 cells per well and infected with serially diluted rALVAC.gfp virus with 10 replicates. After 5–8 days of incubation at 37 °C in 5% CO_2, individual wells were examined for evidence of cytopathic effect due to the presence of infectious virus. The titer was calculated as $CCID_{50}$/mL and then converted to PFU/mL by multiplying the value by 0.7, based on the Poisson ratio [17].

2.5. Sample preparation for single tube assay measurements

100 μL of AV529-19, Vero or QT-35 cells were seeded into 96-well plates at a concentration of 3.0×10^5 cells/mL and incubated overnight at 37 °C in 5% CO_2. The next day, the spent media was aspirated and the cells were washed once with D-PBS. Virus samples were serially diluted in cold (4 °C) media just prior to infection. The cells were subsequently incubated with 50 μL of diluted virus for a set amount of time at 37 °C in 5% CO_2 in order for the infection to proceed (see Fig. 1A for typical plate layout used to establish the method; Supplementary Fig. S1 shows alternative layouts to maximize the number of samples per plate). Each plate included a virus calibrator (titered using plaque assays) in order to create a standard curve to correlate the percentage of cells with increased granularity to infectious titer. Test samples included up to 8 serial dilutions in triplicate. After incubation, the spent media was aspirated and the cells were treated with 100 μL of TrypLE Select™ (Gibco, Thermo Fisher Scientific, Burlington, Canada) for 10 min to dissociate the cells. The cells were then fixed in 4% formaldehyde (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at 4 °C. Each sample was transferred from the 96-well plate into 5 mL round bottom culture tubes (VWR, Mississauga, Canada) with a final volume of 200 μL for individual flow cytometric measurements.

A FACSCalibur flow cytometer from BD Biosciences (San Jose, CA) equipped with a 15 mW air-cooled argon-ion laser with an excitation frequency of 488 nm, required single sample input from 5 mL tubes. Samples were run for 2000 events at the high flow setting (60 μL/min).

2.6. Flow cytometry sample preparation for Semi-Automated flow cytometry assay

50 μL of AV529-19 cells were seeded into a 96-well plate at a concentration of 8×10^5 cells/mL. Cells were allowed to attach

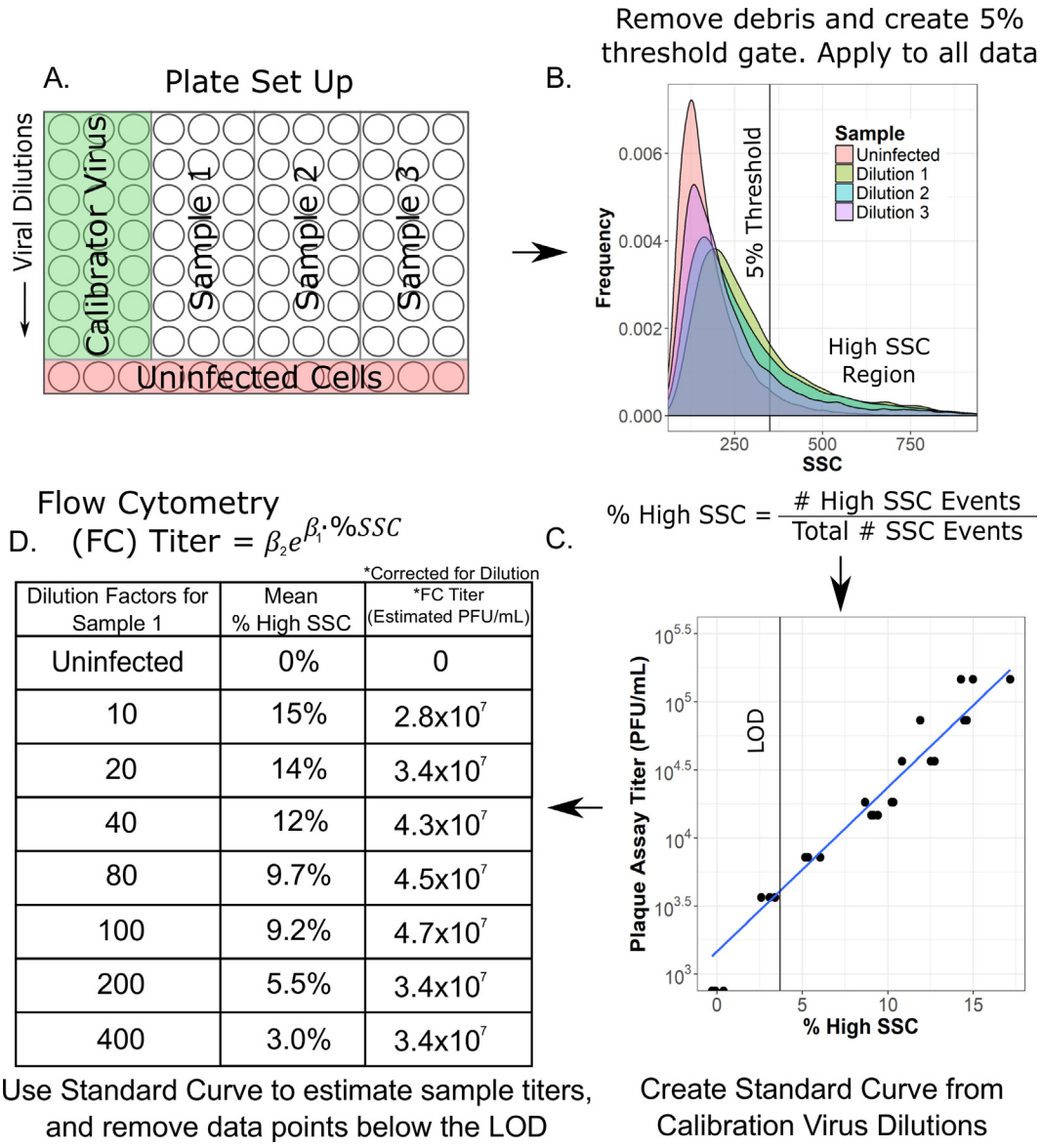

Fig. 1. Schematic displaying key steps for the flow cytometry-based assay for quantifying the infectious virus by individually loaded sample measurements. A. Sample of a 96-well plate set up for calibrator and samples to be measured in triplicate at 7 different viral dilutions. B. A typical SSC histogram plot of the data obtained from a flow cytometer with a 5% threshold gate (black vertical line) separating the low and high SSC regions. C. A standard curve using the calibrator virus to calculate the β values for the standard curve. D. A sample table showing the final estimates for the infectious titer of one virus sample. The data points that appear below the LOD are not to be included in the final infectious titer estimate.

for 2 h before adding 50 μL of serially diluted virus samples. Each plate included a virus calibrator in order to create a standard curve. The plates were incubated for 20 h, treated with 50 μL of trypsin solution containing 5 mM EDTA (ethylenediaminetetraacetic acid) (Corning, Manassas, VA, USA) per well for 5–7 min until cells rounded and detached from the surface, followed by the addition of trypsin inhibitor to stop the trypsinization reaction. Each well was triturated prior to the addition of a 50 μL formaldehyde (2% final concentration) and Pluronic™ F-68 solution (Sigma Aldrich, Irvine, UK) (0.2% final concentration) in order to prevent clumping. The cells were allowed to fix for 1 h at room temperature. Each well was triturated for a second time and 180 μL from each well was transferred to a low binding round-bottom polypropylene 96-well plate (Thermo Scientific, Waltham, MA, USA) to be analyzed by flow cytometry.

A BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA) was used to process cells directly from a 96-well plate. 5000 events from each well were recorded with a maximum run time of 1 min. A threshold of 500,000 for FSC and 50,000 for SSC was used. The plates were agitated for 15 s between each well reading using the BD CSampler plate holder accessory.

2.7. Data visualization and analysis

Data from the FACSCalibur and Accuri C6 flow cytometers were analyzed using R statistical software (www.r-project.org) and the data was imported using the 'flowCore' package. FlowJo X 10.0.6 (FlowJo, LLC) software was also used for analysis.

To remove debris and dead cells, events with FSC less than 300 and 3×10^6 for each cytometer were removed from the data, respectively. SSC histograms were made for each sample and a gated threshold that encompassed 95% of uninfected cells was set (Fig. 1B). For each infected sample measured, the percent of

cells with an SSC greater than this threshold were determined (% High SSC).

A standard curve was created by plotting the % High SSC versus the virus titer (PFU/mL) for a serially diluted calibrator virus (Fig. 1C). The data was first linearized by taking the natural logarithm of the titer and correlating it to the mean value of the triplicate % High SSC values. Linear least squares was chosen as the regression method to fit the data and to calculate the constants. The final semi logarithmic equation is shown below:

$$Flow\ Cytometry\ (FC)\ Titer = \beta_2 e^{\beta_1 \cdot \%SSC} \quad (1)$$

This equation was used to estimate the titer of infectious virus from the % High SSC for each sample, then multiplied by its dilution factor and averaged to obtain a final titer (Fig. 1D). Data points that were below the limit of detection (LOD, calculated by using 3 times the variance of the blanks samples) were not used to predict the estimated titer [18].

3. Results

3.1. SSC as an indicator of infection

AV529-19 cells were infected with rHSV-2 at a multiplicity of infection (MOI) of 0.1 and monitored for 36 h using flow cytometry. Fig. 2A depicts the physical changes that occurred in the cells post-infection. Over time, the cells became rounded and formed

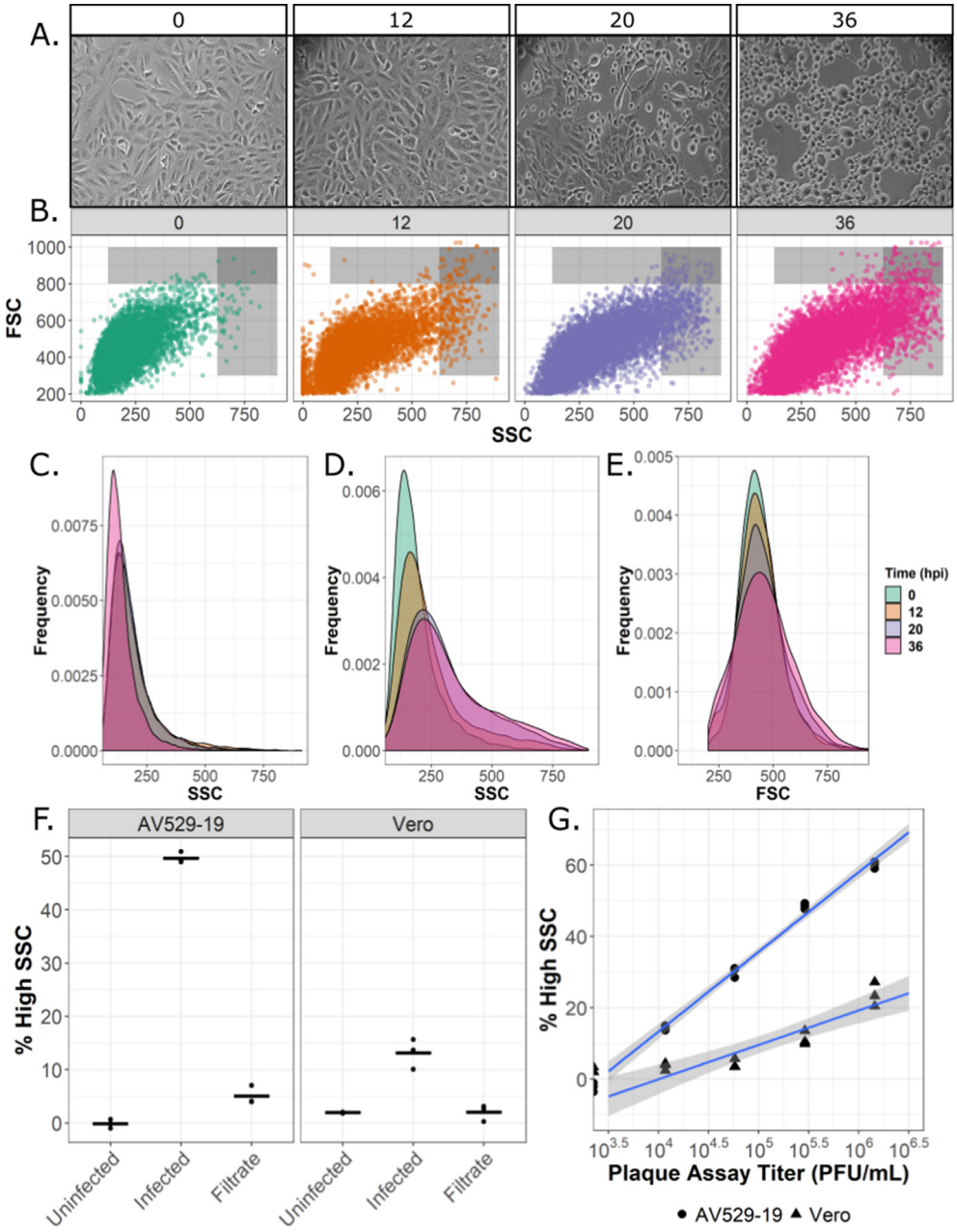

Fig. 2. A. AV529-19 cells infected with rHSV-2 at an MOI of 0.1 over 36 h. B. Forward (FSC) versus side scatter (SSC) plots that show the shift of the data points further into the greyed regions which represent high forwards scatter and side scatter values. C. A histogram of SSC for uninfected cells over 36 h D. A histogram of SSC of AV529-19 cells infected at an MOI of 0.1 over 36 h E. The histogram of FSC for the infected cell population. F. The percent of cells that had high side scatter (% High SSC) values after being incubated for 20 h with fresh media (Uninfected), virus (Infected; final viral titer of 3.6×10^5 PFU/mL), or virus filtrate (Filtrate) (n = 3). G. AV529-19 and Vero cells infected with rHSV-2 virus using 5 dilutions and their granularity was measured after 20 hpi. The titer of rHSV-2 was determined by a plaque assay (n = 3).

large syncytial cells at 36 h post infection (hpi). Accompanying these physical observations, flow cytometry revealed that there was also an increase in the overall granularity of the cells, which can be seen by the shift of the population to higher SSC values (Fig. 2B). The histograms of the SSC values of the population also show this shift (Fig. 2D), while for uninfected cells there was no shifting peak (Fig. 2C). In comparison to SSC, the peaks of the FSC histograms did not shift in the first 20 hpi, with only a slight increase seen by 36 hpi. This indicates that the relative size of the majority of cells remained comparatively constant throughout the infection (Fig. 2E). Based on these observations, SSC was chosen as the parameter to monitor infected cell populations.

To account for natural increases in granularity in uninfected AV529-19 cultures over time, the SSC of uninfected cells was tracked for 36 h (Fig. 2C). The granularity of uninfected AV529-19 cells decreased after 20 h (Fig. 2C). Additionally, to ensure that no soluble factors in the virus sample could alter the host cells' SSC profile, such as the presence of cytokines or other cell-signalling molecules, AV529-19 cells were exposed to virus samples filtered through a 0.1 μm filter to remove the virus (HSV-2 has a diameter of 0.185–0.225 μm [19]). In this mock infection, AV529-19 cells were exposed to the viral filtrate and only showed a small increase in the number of cells with high SSC compared with uninfected cells. This value was 10 times less than the response from the virus-containing sample (Fig. 2F).

To study whether the increase in cell granularity was linked to viral entry and replication, AV529-19 and Vero cells were infected with rHSV-2. Although rHSV-2 can complete part of its life cycle in Vero cells, it cannot replicate its DNA and form infectious progeny without the presence of the U_L5 and U_L29 gene products [20]. Fig. 2G shows that over a range of MOIs (calculated based on plaque assay results), the % High SSC values in infected AV529-19 cells were far greater than for wild-type Vero cells. This demonstrated that increases in granularity depended on the ability of the virus to not only enter, but also replicate inside the host cell.

To determine whether similar granularity changes occurred in other virus-cell systems, the granularity of QT-35 cells was tracked using flow cytometry for 72 h following infection with rALVAC.gfp. Infected QT-35 cells showed visible signs of infection over 72 h (Fig. 3A). A scatterplot of the SSC versus GFP Intensity at 48 hpi

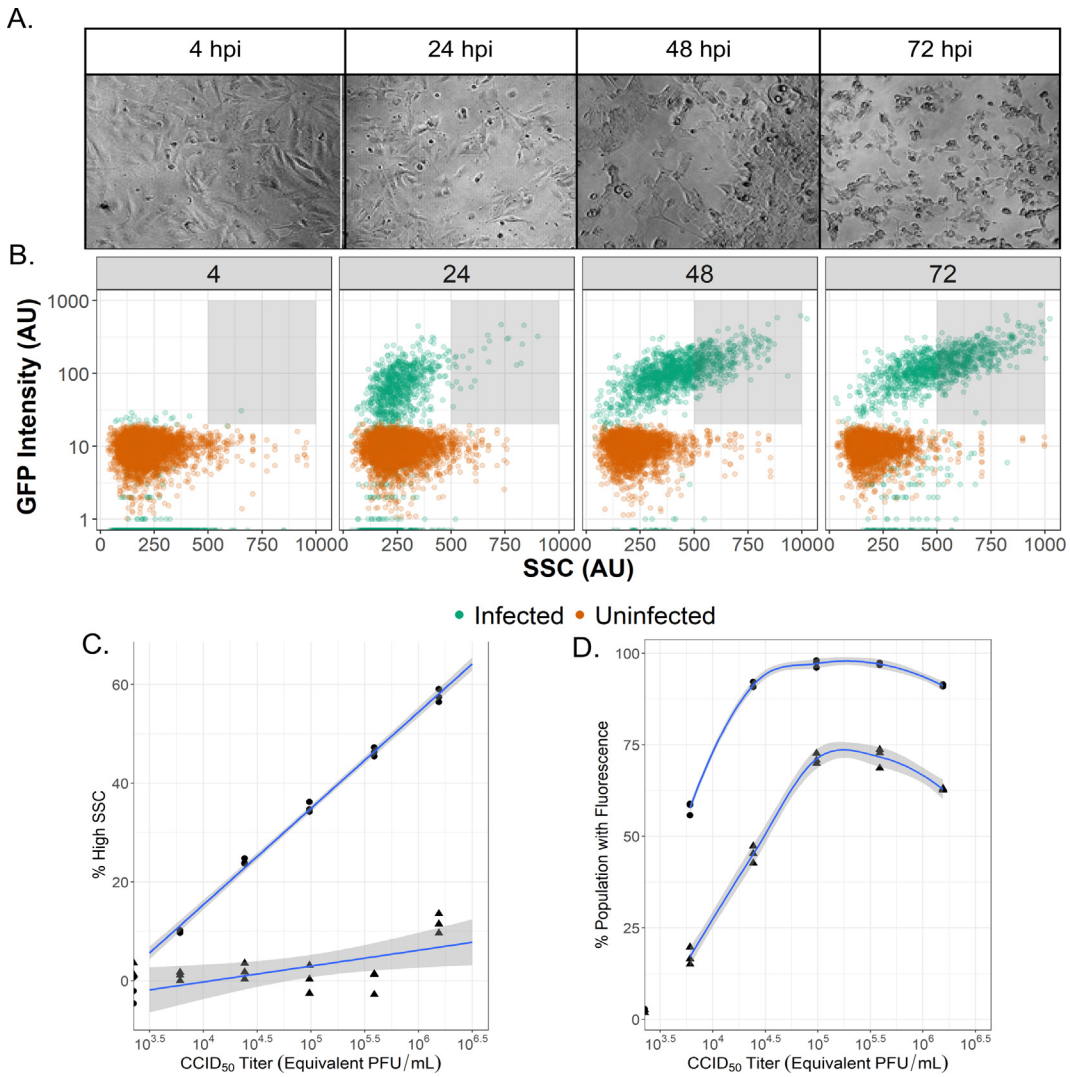

Fig. 3. A. The morphological changes of QT-35 cells infected with rALVAC.gfp at an MOI of 0.1 over 72 h under 10x magnification. B. Scatter plots of QT-35 cells infected with rALVAC.gfp with an MOI of 0.1. The cells expressing GFP and have increased granularity over 72 h. C. QT-35 and Vero cells were infected with rALVAV.gfp using several different viral dilutions and incubated for 48 h. The percent of the population with high granularity post infection is plotted versus the infectious titer of the virus. The titer of rALVAC.gfp was determined using a $CCID_{50}$ assay and converted to PFU/mL units. D. The percent of the cell population producing GFP after 48 hpi versus the infectious titer of the virus. For panels C and D: filled circles (●) are experiments performed with QT-35 cells; filled triangles (▲) are experiments performed with Vero cells.

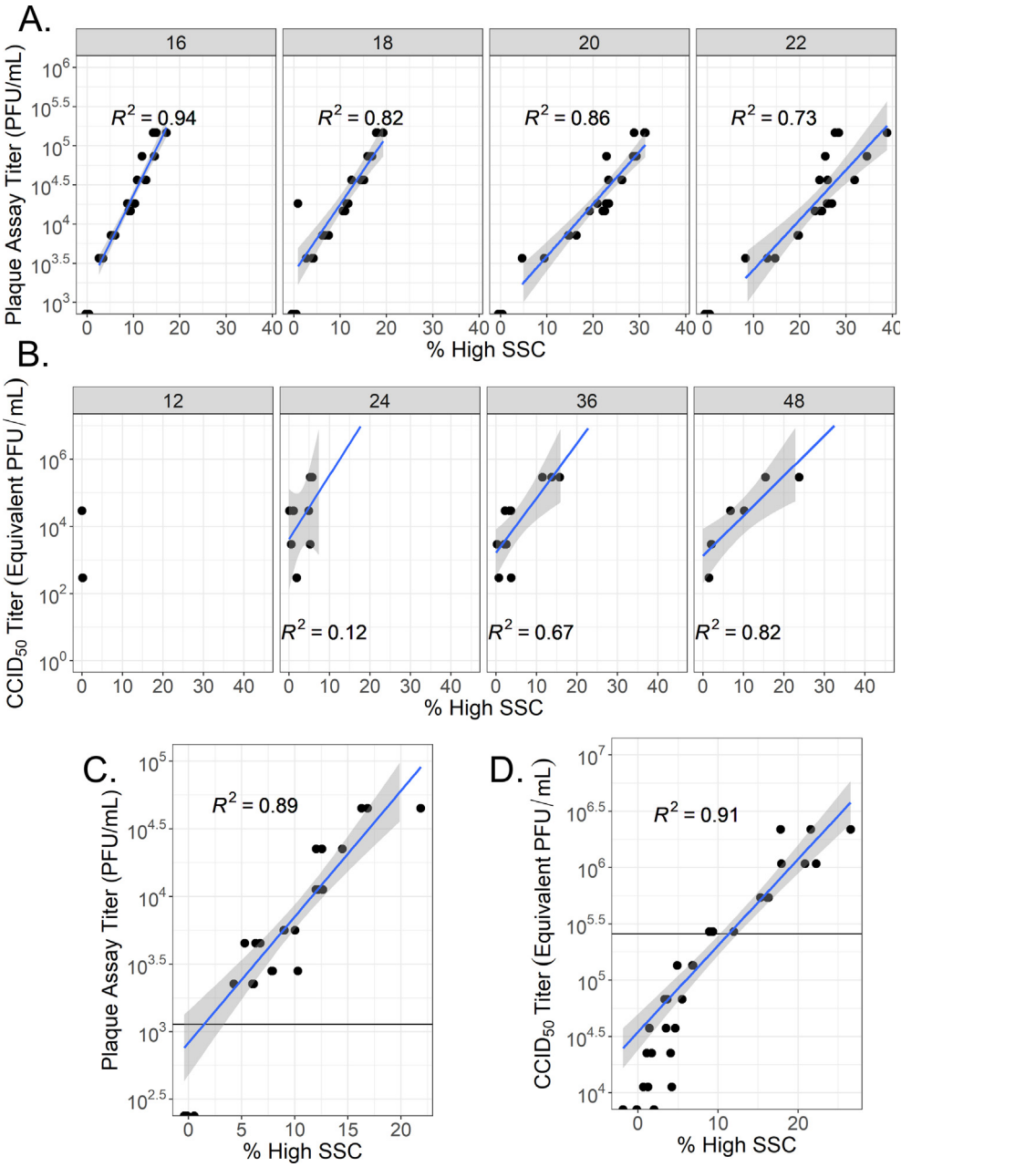

Fig. 4. A. AV529-19 cells were infected with 6 different dilutions of rHSV-2 and tracked over time. The 4 chosen times coincide with when HSV-2 late phase genes are typically expressed (16 hpi) and the end of the first viral replication cycle (20 hpi) [18]. B. QT-35 cells were infected with 3 different dilutions of rALVAC.gfp and the percent of the population with high granularity was tracked over 2 days. Poxvirus late genes can be expressed from 140 min post infection to 48 hpi [21,22]. The gray shaded region represents a 95% confidence interval and the solid blue line is the line of best fit based on Eq. (1). C. 7 different dilutions (n = 3) of rHSV-2 were used to infect the AV529-19 cell line and plotted to demonstrate the relationship between the % of cells with high SSC histogram and the concentration of infectious virus. The black horizontal line represents the LOD. D. 9 different dilutions of rALVAC.gfp (n = 3) were used to infect QT-35 cells and the % high SSC is plotted against the titer of the virus which was calculated using a $CCID_{50}$. The line of best fit (blue) was created using the data points that are above the LOD (black horizontal line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Validation parameters that were used to evaluate the best time post infection to estimate the infectious titer of rHSV-2. The coefficient of variation was calculated from a single plaque assay using 3 different dilutions, each with 3 independent replicates.

Hours post infection	Range	Linear fit (R^2)	Estimated PFU/mL ($1\,0^6$)	Coefficient of variation $\left(\frac{s}{x}\right)$
16	0–17%	0.94	3.8	18.1%
18	0–19.3%	0.82	4.1	25.8%
20	0–31.3%	0.86	3.9	34.0%
22	0–34.5%	0.73	4.2	32.7%
Plaque Assay	N/A	N/A	3.9	18.4%

Table 2
Validation parameters that were used to evaluate the best time post infection to estimate the infectious titer of rALVAC.gfp. Five separate $CCID_{50}$ were conducted to obtain an estimate of the virus titer and coefficient of variation.

Hours post infection	Range	Linear fit (R^2)	Estimated $CCID_{50}$/mL ($1\,0^{7}$)	Coefficient of variation $\left(\frac{s}{x}\right)$
12	N/A	N/A	N/A	N/A
24	11.4%	0.12	2.7	116%
36	15.7%	0.67	8.9	181%
48	23.7%	0.82	3.0	165%
$CCID_{50}$	N/A	N/A	4.0	107.8%

Table 3
Comparison of the results obtained from the plaque assays (n = 3) and the flow cytometry-based (FC) assay (n = 3). The difference between the mean values of each assay was calculated ($abs\left(\frac{FC\ \ mean-plaque\ \ mean}{plaque\ \ mean}\right)x100\%$).

Sample	Plaque assay ($1\,0^{6}$) PFU/mL ± SD	FC Assay ($1\,0^{6}$) Estimated PFU/mL ± SD	% Difference
A	6.0 ± 1.8	7.3 ± 3.9	21%
B	6.3 ± 4.1	6.1 ± 2.3	3.1%
C	6.3 ± 2.2	2.3 ± 0.93	63%
D	6.3 ± 1.0	3.1 ± 1.0	51%
E	62 ± 7.7	66 ± 27	6.4%

shows that for the cell population analysed, individual cells that were infected with poxvirus i.e. cells that were expressing GFP, also had higher SSC. This increased further at 72 hpi (Fig. 3B).

To further confirm that the progression of viral replication led to an increase in SSC in infected cells, QT-35 and Vero cells were infected with rALVAC.gfp. Since rALVAC.gfp is a canarypox virus, it has the ability to enter into mammalian cells and produce some early-phase viral proteins (including GFP), but is unable to complete its replications cycle [21]. No major increase in granularity was observed in Vero cells, even though over 50% of the cells were expressing GFP. This indicates that the rALVAC.gfp virus was able to successfully enter and express some genes in the Vero cells (Fig. 3C & D). Fig. 3C demonstrates that there is an increase in the SSC for QT-35 cells compared with what is observed for Vero cells. Together with the above observations for rHSV-2 and non-complementary Vero cells, a substantial progression through the virus life cycle in infected cells is most likely required to produce an appreciable increase in cell granularity.

3.2. Development of assay parameters

To evaluate the relationship between the increased numbers of cells with high levels of granularity, an empirical correlation was developed using a semi-logarithmic linear model (Eq. (1)). Purified virus samples containing rHSV-2 or rALVAC.gfp were serially diluted and used to infect AV529-19 or QT-35 cells, respectively. The infected cells were analyzed using flow cytometry at 4 different time points that were chosen based on the replication cycle of the wild-type virus. Correlation plots comparing the virus titer to the percent of cells with increased granularity (high SSC) at different times post-infection are presented in Fig. 4A and B, and in Tables 1 and 2. The data was fit to Eq. (1) and the optimal duration of the infection period was chosen based on the ability to discriminate within the selected range of virus concentrations (i.e. greatest range of data). Using the coefficient of determination (R^2), it was determined that although 16 hpi created the best fit with the least unexplained variability for rHSV-2, any time between 16 hpi and 20 hpi could be used ($R^2 > 0.8$; Fig. 4A and Table 1). For rALVAC.gfp, 48 hpi was deemed the optimal incubation time with QT-35 cells (Fig. 4B and Table 2).

To determine the minimum levels of virus that are detectable using the individually loaded sample method, the lower LOD was calculated using the variation of the blank (uninfected cells, n = 12). The LOD represents the lowest signal that can be reliably distinguished from the blank and can be detected [18]. It was estimated by taking the average of the blank and then adding 3 times the standard deviation. The LOD for rHSV-2 with AV529-19 cells was found to be approximately 840 PFU/mL (Fig. 4C). For rALVAC.gfp with QT35 cells, the LOD was found to be 140,000 PFU/mL (Fig. 4D).

3.3. Qualification of the flow cytometry-based assay for individually loaded samples

To assess the accuracy of the assay, 5 different rHSV-2 samples were quantified using the flow cytometry-based assay and a plaque assay (Table 3). Based on the standard curve obtained using the serially diluted calibrator, the constants (β values) for Eq. (1) were determined and the equation was used to estimate the titer of the virus for the samples A-E. The plaque assay and flow cytometry-based assay results are displayed in Table 3 for comparison.

The precision of the assay was determined by quantifying a purified homogenous sample of rHSV-2 over 3 different days using 2 different operators. Precision was considered at two levels: as agreement within a single run between assays and operators, as well as inter-run agreement over 3 days. The results from all 3 runs are summarized in Table 4. It was found that the data obtained by both operators spanned approximately the same range (12% variance between runs) and the linearity of the data obtained by each operator verified that the standard curve had a good fit ($R^2 > 0.9$). The inter-assay variability from each operator was estimated to be 9–12%, and the operator variability was estimated to be 3–7%. The overall assay precision was estimated to be $\pm 4.4 \times 10^5$ estimated PFU/mL.

Table 4
The average titers for each run and the coefficient of variation (%CV) were calculated for the flow cytometry-based (FC) assay and plaque assay. The percent difference between the FC assay and the traditional plaque assay was calculated for both operators.

	FC assay (Estimated PFUx10^6/mL)			Difference between operators	
	Operator 1	Operator 2	Plaque assay (PFUx10^6/mL)	Operator 1	Operator 2
Run 1	4.8	5.3	5.2	−7.8%	2.3%
Run 2	4.1	4.3	4.6	−12%	−6.7%
Run 3	4.8	4.4	4.8	1.5%	−8.4%
Inter assay (%CV)	9.2%	12%	5.9%		

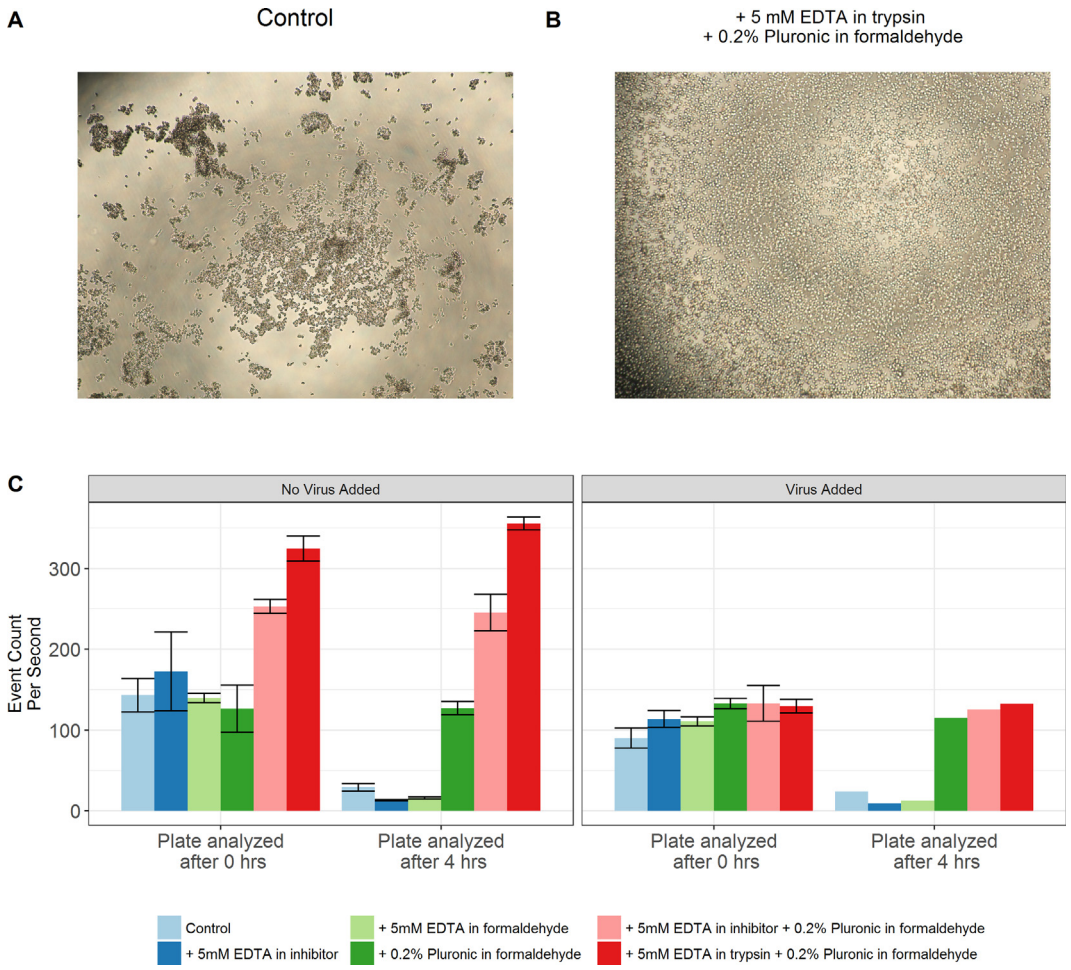

Fig. 5. Adaptation to Semi-Automated format. A. Control: a sample image of a well post trypsinization and left resting for 4 h. B. Sample image after the addition of EDTA, and Pluronic™ F-68 to create a single cell suspension after 4 h. C. Ability of the cytometer to aspirate cells for analysis. Addition of EDTA and Pluronic™ F-68 at different stages during cell preparation for the flow cytometer was investigated to reduce the ability of the cells to settle and clump, while the samples awaited aspiration. "No Virus Added" is the analysis of cells that have not been incubated with virus solutions (n = 2). "Virus Added" is the analysis of cells that have been incubated with the virus for 24 h (n = 2 for 0 hrs, n = 1 for 4 hrs).

The inter-assay variation for both the flow cytometer assay and the plaque assay was compared over 3 separate runs. The flow cytometry-based assay did demonstrate a larger standard deviation in the inter-assay variability than the plaque assay, with the difference between the mean values of the assays over the 3 runs being less than 12%. As the titer of the calibrator used for the flow cytometry assay is determined from plaque assays, the variation in the flow cytometer assay not only contains variances from the assay itself, but also from the variability of the plaque assay when calculating a final estimated titer. Based on these results, the robustness of the individual sample tube format flow cytometer assay was considered to be acceptable.

3.4. Adaption of the flow cytometer assay to a semi-automated format

To enable higher throughput than the single tube format rHSV-2 assay, the assay was adapted to a semi-automated format compatible with a flow cytometer with an automated plate sampler. One of the challenges using this format was the time required to complete the 96 flow cytometry measurements, which was approximately 4 h. Using the protocol developed for individually loaded single tube assay, the cells had a tendency to form aggregates in the wells prior to analysis, despite the agitation feature of the robotic arm (Fig. 5A), which led to a reduction in the number of cells that could be sampled by the flow cytometer over time (Fig. 5C). To prevent cell aggregation following the trypsinization, trypsin inhibition and fixation steps, the addition of EDTA and Pluronic™ F-68 to the cells at different steps in the assay was investigated. The most consistent reduction in aggregation was found to be when 5 mM EDTA was present in the trypsinization solution and 0.2% Pluronic™ F-68 was present in the fixation solution. Fig. 5B shows that the cells remained as single cells over 4 h, while Fig. 5C shows an increase in the event rate with the addition of EDTA and Pluronic™ F-68 to the 96-well plate.

3.5. Comparison of the semi-automated flow cytometry assay with the plaque assay

To verify the utility of the semi-automated flow cytometry-based assay for use in process development, where a high-throughput and less labor-intensive assay is desirable, 23 viral samples were measured in both a plaque assay and a flow cytometry assay for rHSV-2. Fig. 6 illustrates how the estimated titers from the flow cytometry-based assay compares to the results obtained from the plaque assay. Titers ranging from 10^5 PFU/mL up to 10^7 PFU/mL show similar results, but for titers above 10^7 PFU/mL, the flow cytometry-based assay tends to overestimate the viral titer.

4. Discussion

Infectious virus quantification is a key parameter in manufacturing and formulation development for early-stage viral vaccine candidates. Faster turnaround time and increased throughput of the flow cytometry assay are expected to contribute to reducing overall vaccine development timelines. The method presented here provides a simple, rapid, and high-throughput flow cytometry-based assay for the enumeration of infectious virus to support continuous process monitoring and process development of viral vaccines. Flow cytometry was used to measure changes in cells that were infected with rHSV-2 or rALVAC.gfp. In contrast to previous reports, which use fluorescence to detect infection, this method uses the changes in cell granularity to estimate the infectious titer of the virus. It was found that the increase in cell granularity could be reliably correlated with the infectious viral titer after 16 hpi for AV529-19 cells infected with rHSV-2 and 36 hpi for QT-35 cells infected with rALVAC.gfp. Although the reason for the change in cell granularity was not determined, this report demonstrates that the virus must be able to successfully enter, and replicate in the host cell.

The guidelines issued by the International Council for Harmonisation (ICH) and USP 1033 Biological Assay Validation were used to assess the assay in terms of specificity, linearity, limits of detection, accuracy, and precision [22,23]. The flow cytometry-based assay proved to have high linearity between the natural logarithm of the virus titer and the increase in the granularity of the cells, which was assessed by calculating the percent of the population with high side scatter. The linearity occurred above the limit of detection and spanned the range of 10^3–10^5 PFU/mL for rHSV-2 and $10^{5.5}$–$10^{6.5}$ equivalent PFU/mL for rALVAC.gfp ($R^2 \approx 0.9$). The flow cytometry-based assay requires at least 10^3 PFU/mL of virus (approximately the limit of detection), making it less suitable for applications that require high sensitivity. For applications with higher virus titers, such as biomanufacturing, this linearity can be exploited to rapidly quantify virus samples. This relationship was used to quantify 5 different rHSV-2 viral samples in triplicate using the flow cytometry-based assay and was compared to the results obtained using a plaque assay. There was a difference that ranged between 3 and 63%, and all titers were in the same order of magnitude between both assays. The flow cytometry-based assay was adapted to a semi-automated format and was used to titer 23 separate viral batches. The assay had to be modified to prevent the AV529-19 cells from aggregating after trypsinization and settling to the bottom of the 96-well plate during the automated plate reading step. The addition of Pluronic™ F-68 and EDTA, coupled with a mechanical agitation step and use of low binding polypropylene 96-well plates, were able to prevent clumping and settling while the automated plate reader was taking measurements.

The use of an automated plate reader reduced the amount of labour and time required to obtain granularity measurements of the cell populations. Since this assay depends on the increase in granularity of infected cells compared with uninfected cells, it is always necessary to have an uninfected control to confirm that the granularity of the uninfected population is sufficiently low. It is also important to have a standard curve with each assay to correlate the granularity of the cells with the known titer of a

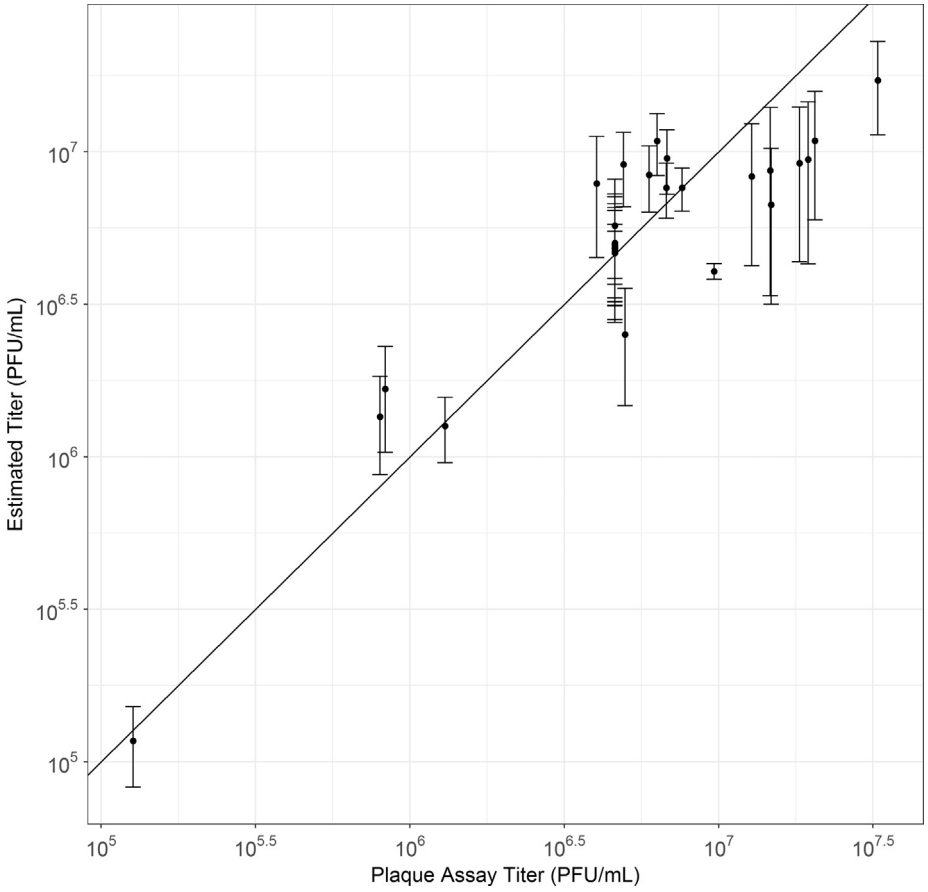

Fig. 6. 23 different rHSV-2 virus preparations were titered using AV529-19 cells and the semi-automated flow cytometry assay or the plaque assay. The black solid line represents a perfect correlation between plaque assay and flow cytometry titers.

calibrator virus. Many factors can affect cell granularity such as media composition, cell health, quality of the virus sample, and cell age. The media and virus storage buffer composition are important for cell health since it is part of the environment that surrounds the cells in tissue culture. Batch to batch variability in the formulation of the media or virus storage buffer may contribute to inter-assay variation. Additionally, it was noted during these experiments that when the AV529-19 cells were passaged over 20 times, their growth slowed and uninfected cells appeared to have a higher granularity (Supplementary Fig. S2). This made it difficult to distinguish uninfected cells from infected cells based on their granularity. A standard curve, along with uninfected cells as a control, are necessary to capture these random affects in the parameter values (β) of the model (Eq. (1)).

The flow cytometry method described here is attractive for three main reasons: it is a simple detection method, it reduces assay time, and it has higher throughput as compared to traditional methods such as the plaque assay or $CCID_{50}$. The flow cytometry-based assay relies on changes in cell granularity and does not require the use of fluorescent reporter proteins or stains, which greatly simplifies the titration process. The amount of work conducted by one operator is reduced by coupling the use of electronic and manual multichannel pipettes with 96-well plates that can accommodate more samples. Due to this, hands-on time per plate, which can be used to measure up to 11 different viral samples (Supplementary Fig. S1), is approximately 2 h. In addition, assay results are available after 28 h using the flow cytometry-based assay for rHSV-2 and rALVAC.gfp titers can be obtained within 48 h. Due to this, up to 44 samples could theoretically be run per week by a single operator without much difficulty, with results available in 5 days, with total hands-on time being ~8 h. This assay will help improve the development of viral bioprocesses by facilitating process monitoring and decision making and offering researchers a rapid and high-throughput titration assay.

5. Conclusion

This work demonstrates that the level of cell granularity, as measured by flow cytometry, can be correlated to the natural logarithm of the infectious viral titer. The results show that non-fluorescent detection of viral infection through flow cytometry is a rapid and reliable method for quantifying concentrated viral titers, which can facilitate the improvement and optimization of virus production.

Declaration of interest

The author declares that there is no conflict of interest.

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Conflict of Interest statements

M.L., J.M., and M.A. have no conflicts of interest. C.N., C.K., S.G., P.F., A.Z. are employees at Sanofi Pasteur.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.02.059.

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