Rapid quantification of vesicular stomatitis virus in Vero cells using Laser Force Cytology

Colin G. Hebert a, Nicole DiNardo b, Zachary L. Evans a, Sean J. Hart a, Anna-Barbara Hachmann b,*

a LumaCyte, LLC, 1145 River Road, Suite 16, Charlottesville, VA 22901, USA
b Thermo Fisher Scientific, Inc., 3175 Stable Road, Grand Island, NY 14072, USA

ARTICLE INFO

Abstract

The ability to rapidly and accurately determine viral infectivity can help improve the speed of vaccine product development and manufacturing. Current methods to determine infectious viral titers, such as the end-point dilution (50% tissue culture infective dose, TCID50) and plaque assays are slow, labor intensive, and often subjective. In order to accelerate virus quantification, Laser Force Cytology (LFC) was used to monitor vesicular stomatitis virus (VSV) infection in Vero (African green monkey kidney) cells. LFC uses a combination of optical and fluidic forces to interrogate single cells without the use of labels or antibodies. Using a combination of variables measured by the Radiance LFC instrument (LumaCyte), an infection metric was developed that correlates well with the viral titer as measured by TCID50 and shortens the timeframe from infection to titer determination from 3 days to 16 h (a 4.5 fold reduction). A correlation was also developed between in-process cellular measurements and the viral titer of collected supernatant, demonstrating the potential for real-time infectivity measurements. Overall, these results demonstrate the utility of LFC as a tool for rapid infectivity measurements throughout the vaccine development process.

© 2018 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Many processes across the biopharmaceutical industry require quantification of viruses. Applications range from vaccine manufacturing, to serum neutralization assays for clinical efficacy, to viral clearance measurements of raw materials or finished goods. The most commonly accepted methods include the plaque and endpoint dilution (TCID50) assays, followed by immunological methods [1–8]. Plaque and TCID50 assays are labor intensive and can take up to two weeks (Table 1), depending on the pathogen [9,10]. Since the assays and their interpretation can be subjective and highly variable, a sufficient number of replicates must be performed to obtain statistically significant results [11,12]. This extends the timeline of pharmaceutical process development and can cause considerable delays to the release of finished goods. For vaccines this rate-limiting step is of particular concern, as it can be challenging to detect peak titer and delay the time between harvest and final product release, which requires accurate infectivity measurements [13–15].

Novel vaccine development process can take decades, with reports of the average development time ranging from 18 to 30 years [16–18]. A number of process parameters can be modified to accelerate time to launch. Recent advances in cell culture media development have improved the manufacturing process to the point where an optimized serum-free medium formulation can increase growth performance and vaccine titers, product quality, and product purification requirements [19–21]. High throughput screening methods are available to improve development of new cell culture media for growth and protein production. Along those lines, a rapid screening method for virus titers would greatly facilitate media and process optimization for vaccine production.

Several virus quantification techniques use instruments to accelerate virus quantification and analysis (Table 2), including qPCR, ELISA, immunofluorescence foci assay, and flow cytometry. Methods based on antibody or fluorescent labeling face several limitations. They are often limited by the type of virus that can be quantified, may not distinguish between non-infectious and infectious virus particles, and can have a reduced limit of detection as a result of background from other substances in the sample matrix [22]. In this study, we have evaluated the use of Laser Force Cytology (LFC) to determine the infectivity of vesicular stomatitis virus (VSV) produced in Vero cells. The Radiance LFC instrument uses a unique combination of advanced optics and microfluidics to analyze suspended cells based upon their intrinsic properties, while simultaneously taking high resolution video of each cell. LFC does not require fluorescent antibody or dye labeling to differentiate cell phenotypes, but instead uses optical force or pressure to impact cellular structures and transfer momentum. The
combination of optical and fluidic forces has been used to characterize a number of features, including cell differentiation, viral infection, and cell deformability [23–27]. Specifically, subtle cellular changes in membranes, cytoplasmic organelles, and nuclear features manifest themselves as changes in the velocity, size, shape, and orientation of cells as they pass through the region of laser photon pressure. These biochemical and biophysical interactions can be quantified to characterize cells in a label-free manner.

Vero cells were isolated from African green monkey (Ceropithecus aethiops) kidney cells in 1962 and are commonly used for the production of human vaccines, including polio, EV-71, rabies, influenza, and rotavirus [28–30]. They are adherent cells that can be cultured in flasks, cell factories, and in bioreactors supported by microcarriers. VSV belongs to the Rhabdoviridae family and is characterized by a rapid lytic growth cycle [31]. It is zoonotic, can cause flu-like symptoms in humans, and foot and mouth disease in cattle, horses, and pigs. Because of its broad host range, the envelope glycoprotein (VSV G) is commonly used as a coat protein for lentiviral vectors [32]. VSV was chosen as an analog for rabies virus as both viruses share many structural and functional similarities.

2. Materials and methods

2.1. Cell culture

Vero cells (ATCC CCL-81) were cultured in MEM α with 10% FBS or VP-SFM (Gibco), supplemented with 6 mM glutamine, in a humidified incubator at 37 °C with 5% CO2. Cultures were passaged with Trypsin-EDTA (0.05%) or TrypLE™ (Gibco). Trypsin activity was quenched with Defined Trypsin Inhibitor (Gibco) and cells were washed with medium before determination of viable cell densities with a Vi-CELL XR Cell Counter (Beckman Coulter).

2.2. Virus propagation and infection

Vero cells were grown in T-75 flasks or 6-well plates in MEM α with 10% FBS or VP-SFM (Gibco). For infection, the growth medium was removed, the cell monolayer washed with DPBS (Gibco), and the medium replaced with Advanced MEM or VP-SFM (Gibco). All media were supplemented with 6 mM glutamine. Cultures were infected with vesicular stomatitis virus (ATCC VR-1415) at the MOIs indicated. Cultures were observed daily for signs of cytopathic effect (CPE). The culture supernatant was used for virus quantification with a TCID₅₀ assay, and the cells were detached for analysis with the Radiance (LumaCyte).

2.3. Sample preparation

Infected cells were harvested using Trypsin-EDTA and Defined Trypsin Inhibitor and washed before resuspension in Sample Dilution Buffer (LumaCyte). Cells were diluted to a concentration of approximately 500,000 cells/mL. 200 μL of the cell suspension was measured on a Radiance instrument (LumaCyte). Radiance employs optical force (laser photon pressure) and image capture together with microfluidics to image and analyze single cells. The instrument processes and analyzes images to generate a multiparameter description of each cell. These data are stored in a database describing the analyzed cell population.

2.4. Virus titer determination (TCID₅₀)

Virus production in the infected supernatant was determined by measuring the TCID₅₀ using the endpoint dilution assay. In short, Vero cells were seeded in MEM α with 10% FBS in 96-well plates at densities that reached near 100% confluence in 24 h. The cells were then washed with DPBS and medium was
plates were incubated at 37°C with 5% CO2 and observed daily for CPE. Once a clear CPE was visible, positive wells were identified and titers were calculated based on Spearman & Kärber algorithm [33,34].

2.5. Radiance instrument and Laser Force Cytology data analysis

The Radiance instrument was operated at a laser power of 7 W and a channel flow rate of 3000 nL/min. Sample injection flow rate was between 200 and 600 nL/min depending upon cell concentration. The system was tested with LumaCyte calibration beads (LFA-A-3004, LumaCyte, Charlottesville, VA) daily to ensure optimum system operation and repeatability. In order to demonstrate system stability, uninfected Vero cells and Vero cells infected with VSV were fixed using 0.5% paraformaldehyde and a cell stabilization fluid (SF1600, LumaCyte, Charlottesville, VA), stored at 4°C, and analyzed in Radiance over a 19 day period. The mean uninfected velocity was 1730 μm/s ± 13 μm/s (0.8% CV), and the mean infected velocity was 1542 μm/s ± 14 μm/s (0.9% CV), indicating excellent instrumental reproducibility over an extended period (Fig. S1). Such instrumental stability ensures that variations seen during infectivity or other biological experiments are due to biological differences, not instrumental drift.

LFC data was initially analyzed and explored using LumaCyte’s Analyzer software. For each replicate of every biological condition, single cell data was collected on a population of cells for a number of measured variables including velocity, size, and eccentricity. Scatter plots and histograms for each of these variables were used to visualize population trends for a given infection condition. Comparative analysis between uninfected cells and cells with varying levels of infection and/or time revealed key metrics that could be used, both individually and collectively, to summarize an entire population’s behavior using census (population average) data. These census data were then plotted against calculated TCID50 values to produce univariable titer calibration curves.

Using Solo Model Exporter software (Eigenvector Research Inc.), LFC data was also input into the PLS Toolbox (partial least squares) to develop a multivariate titer calibration curve. Specifically, the LFC data included velocity, size, size normalized velocity, and eccentricity measurements along with all respective standard deviations as X input variable data, while the Y input variable data were the TCID50 values. The output of the PLS Toolbox were latent variables, which were then used as an infection metric variable (x) plotted against the calculated TCID50 values (y) to produce a multivariate titer calibration curve.

3. Results and discussion

Initial experiments focused on determining the optimal parameters to facilitate the rapid measurement of viral infectivity using LFC in VSV infected Vero cells. Parameters included MOI, incubation time post infection and the dynamic range of initial virus concentrations. As a first step, the changes in LFC measurements that occur upon infection were identified and quantified. This laid the groundwork for the development of an infection metric that can describe the changes occurring upon infection and be used to correlate LFC measurements with traditional cell-based infectivity measurements such as the plaque assay and TCID50.

A selection of these changes is illustrated in Fig. 1. Panels A-D represent Size vs. Velocity scatter plots comparing Vero cells infected with VSV at MOI 0.1 to uninfected cells at 8, 12, 16, and 20 h post-infection, respectively. Image analysis reveals information about the size as the two-dimensional area of the cell, and the velocity, as the speed of the cell moving through the zone of laser interaction. Velocity is inversely proportional to the optical force acting on a cell, so as the optical force acting on a cell increases, its velocity decreases. Thus, cells with a higher optical force have a lower velocity and vice versa. As the infection progresses, cells exposed to the virus decrease in both size and velocity, starting with averages of 370 μm² and 1731 μm/s at 8 h and ending with 258 μm² and 1670 μm/s at 20 h. In comparison, uninfected cells remain relatively consistent, starting at 387 μm² and 1714 μm/s at 8 h and ending at 389 μm² and 1735 μm/s at 20 h. This decrease in velocity indicates an increase in optical force upon infection, which has been observed previously in other cell/virus systems [23]. This increase in force is likely a result of the combination of the physical presence of virions within the cell as well as the changes to the host cell that occur upon infection or exposure to a pathogen [25].

It is important to note that in addition to an overall decrease in the average velocity of VSV infected cells, there is also an increase in the distribution of velocity values, as evidenced by an increase in the velocity standard deviation from 75 μm/s at 8 h to 165 μm/s at 20 h, compared to 76 μm/s and 73 μm/s for uninfected cells at the same time points. These trends are illustrated in representative scatter plots showing the time course of infection (Fig. 1). At 8 h the uninfected and VSV infected populations are nearly identical, but as time progresses, cells in the infected population decrease in both size and velocity while at the same time increasing in velocity standard deviation. At the 20 h time point (Fig. 1D), the separation between the two conditions can be clearly seen [35–37]. This increase in the velocity distribution of infected cells can be attributed to the heterogeneous nature of viral infection. Initially, not all cells will be infected, resulting in a mixed population of uninfected and infected cells. As the infection progresses, additional cells will become infected with varying progress, further increasing the velocity distribution.

In addition to the numerical data collected on each cell, multiple images were collected that can be used as an additional source of information between different sub-populations of cells. Representative images from both the uninfected (top three) and VSV infected (bottom six) populations are shown in Fig. 1E along with the size and velocity for each cell. The infected cells represent a broad range of size and velocity within that population, the first three cells from the infected population are low velocity and the last three cells from the infected population are high in velocity and smaller. In general, uninfected cells are uniform in contrast and appearance despite variation in size and velocity within the population, while higher velocity cells from the infected population are darker and have greater variation in shape and appearance. In addition, the smaller and high velocity cells are markedly different in appearance than the other cells shown but are similar in appearance to each other. The infected cells are round, indistinct, have low contrast relative to normal cells, and few internal details or structures can be discerned. Combined with the fact that the percentage of cells in the sub-population increases as the infection progresses (shown in Fig. 1 A–D), this indicates that these cells are compromised in some way. An additional reason that the velocity standard deviation increases with infection is due to the increase in the population of high velocity and low size cells as the infection progresses. All of the images for each cell are saved and associated with the numerical data, allowing for its use in future quantitative studies if desired, including image analysis and classification techniques.

The optical force of each cell is dependent upon its size and refractive index [38]. Cell size and velocity change with infection, and velocity is a function of both the optical force and the fluidic drag force [39]. A parameter was developed in order to normalize by the size; thus, helping describe the effects on velocity independent of cell size. This parameter is termed size normalized velocity (SNV) and is described by the following equation:
Size Normalized Velocity $[s^{-1}] = \frac{\text{Velocity}_{\text{fluid}} - \text{Velocity}_{\text{cell}}}{\text{Radius}_{\text{cell}}} [\mu m/s]$

Because the velocity component of the SNV calculation is the difference between the fluid velocity and the cell velocity, SNV increases as the optical force on a cell increases and vice versa. Size normalized velocity histograms are shown in Fig. 2 for the sample populations of both VSV infected and uninfected Vero cells that were shown in Fig. 1. As the infection progresses, the SNV for the infected population increases consistently from a value of 23.2 at 8 h to 33.1 at 20 h. In addition, the percentage of cells above a certain threshold value can be defined, similar to gating in flow

Fig. 1. Size and velocity time course data. Scatter plots comparing the size and velocity of Vero cells infected with VSV at an MOI of 0.1 to uninfected control cells. Representative plots are shown at 8, 12, 16, and 20 h post infection (A–D, respectively). Each point on the graph represents one cell. Representative cell images (E) are also shown, with their corresponding velocity and size values. The top row of three images are uninfected cells and the bottom six images are from the infected population.
cytometry [40]. In this case, an empirically determined threshold value of 30 was selected, and the percentage of cells above that threshold value is shown for both cell populations on the histograms. The percentage of high SNV cells for the uninfected population remains low and consistent throughout the time course, while it increases significantly for the VSV infected cells, rising from 6.3% at 8 h to 48% at 20 h. Even at 12 h, the percentage of high SNV cells for the infected cells is 13.8%, which is more than 4 standard deviations above the mean percentage for uninfected cells.

Once the changes that occur during infection were identified, a wider range of time points and MOIs were tested. These were used to develop the infection metric. Based on the changes seen in size and velocity in initial experiments, the infection metric selected was the product of the velocity standard deviation and the percentage of cells with SNV greater than 30. This infection metric correlates well with the MOI and the initial virus concentration.

Once the optimal time of 16 h had been established, the next step was to further quantify the correlation between LFC measurements and traditional cell-based infectivity measurements, in this case TCID50. The flexibility of LFC allows for two potential correlations. In the first, measurements are correlated to the initial concentration of virus, which demonstrate that LFC can be used in a similar fashion to TCID50 or plaque assay (shown in Fig. 3A). Specifically, a virus sample at multiple dilutions is added to cells, which are incubated, harvested, and then analyzed using a Radiance instrument. In the case of the VSV infected Vero cells, the incubation time for LFC is only 16 h compared to 72 h for plate-based assays. In general, the incubation time is 3-7 shorter for LFC than with traditional methods. In the second method (see Fig. 3C), LFC measurements are correlated with infectivity results from the supernatant collected at the same time as the samples for LFC. Once either of the correlations has been established, Radiance measurements can be used to calculate the corresponding TCID50 value of a sample.

Once the changes that occur during infection were identified, a wider range of time points and MOIs were tested. These were used to develop the infection metric. Based on the changes seen in size and velocity in initial experiments, the infection metric selected was the product of the velocity standard deviation and the percentage of cells with SNV greater than 30. This infection metric correlates well with the MOI and the initial virus concentration in the experiments (see also Fig. 3).

\[
\text{Infection Metric} = \text{Standard Deviation}_{\text{Velocity}} \times (\% \text{ High SNV Cells}_{\text{SNV>30}})
\]

Fig. S2 shows a time course of the infection metric over a range of MOIs and time points. No significant difference in the infection metric is seen between uninfected and VSV infected cells until 12 h, at which point there are minimal differences between MOIs 0.1 and below. At 16 h post infection, there is a much larger dynamic range of values for the infection metric; a trend between MOI and infection metric can be seen. At the 20 and 24 h time points, the infection has progressed to a point where the majority of the cells at the highest MOI values have been lysed, making it difficult to collect and analyze these samples. Thus, the 16 h time point was chosen for all additional studies. Notably, this is far less than the standard incubation time of 72 h until a CPE is observed [41].

Once the optimal time of 16 h had been established, the next step was to further quantify the correlation between LFC measurements and traditional cell-based infectivity measurements, in this case TCID50. The flexibility of LFC allows for two potential correlations. In the first, measurements are correlated to the initial concentration of virus, which demonstrate that LFC can be used in a similar fashion to TCID50 or plaque assay (shown in Fig. 3A). Specifically, a virus sample at multiple dilutions is added to cells, which are incubated, harvested, and then analyzed using a Radiance instrument. In the case of the VSV infected Vero cells, the incubation time for LFC is only 16 h compared to 72 h for plate-based assays. In general, the incubation time is 3-7 shorter with LFC than with traditional methods. In the second method (see Fig. 3C), LFC measurements are correlated with infectivity results from the supernatant collected at the same time as the samples for LFC. Once either of the correlations has been established, Radiance measurements can be used to calculate the corresponding TCID50 value of a sample.

Fig. 3A and B show the correlation between the infection metric taken at the 16 h time point and the initial virus concentration, which was varied by changing the MOI from 1.0 to 0.0001 (4 logs). The infection metric increases with MOI, ranging from 10.5 ± 2.5 for MOI 0.0001 (corresponding to 50 PFU in a 6-well plate) to 91.4 ± 18.8 for MOI 1, compared to 5.1 ± 1.3 for uninfected cells. Therefore, the infection metric at the lowest MOI is more than 4 standard deviations higher than uninfected cells, demonstrating the sensitivity of the technique. Fig. 3B shows the regression analysis correlating the average infection metric values to the MOI. A good fit is obtained using a power curve, resulting in \( R^2 = 0.97 \). In addition, the average difference between the actual MOI and the predicted MOI was 0.22 ± 0.15 log_{10} (shown in Table S1), which compares favorably with other studies using traditional assays [42]. The infection metric is consistent in cells grown in both classical medium containing serum as well as serum-free medium.
Fig. S3 shows statistically similar infection metric values for cells infected at the same MOI grown in MEM and VP-SFM, aligning with the fact that TCID50 values are similar in both media (data not shown). Overall, this demonstrates the ability of LFC measurements to correlate with traditional TCID50 measurements with a shorter incubation time and without having to manually check and score wells, potentially reducing subjectivity.

In Fig. 3C and D, cells were analyzed using LFC and compared to subsequent TCID50 values on supernatants taken at the same time points. The resulting correlation allows for the near real-time measurement of infectivity, eliminating the need for lengthy titer determination assays. For instance, this can be useful when comparing various media conditions or monitoring a production process. TCID50 values for three MOIs taken at different time points are shown in Fig. 3C; the values range from 2.8e6 to 2.8e8 TCID50/mL. A multivariate infection metric was developed using partial least squares (PLS) analysis on the LFC data including velocity, size, size normalized velocity, and eccentricity measurements along with their respective standard deviations. Although this approach requires additional computational effort, it can be more robust and eliminate the need for the user to optimize metrics. Shown in Fig. 3D, is the fit between the multivariate infection metric developed with the PLS analysis and the experimental TCID50 values, resulting in an average difference of 0.16 ± 0.22 log10 with all but one of the predicted values within 0.18 log10 (Table S2).

In summary, Fig. 4A compares LFC to the most commonly used virus quantification methods, TCID50 and plaque assays. The methods differ in their workflow to determine the virus concentration of a given sample. For a TCID50 assay, a serially diluted virus sample is added to a monolayer of cells and incubated until CPE is visible. Depending on the virus, it can take from 3 to 15 days until the plates can be analyzed. To determine this time, plates initially need to be monitored daily utilizing a microscope. The titer is calculated from the concentration where half of the wells show CPE. Like the TCID50, the plaque assay also uses infection of a monolayer of cells with a serially diluted virus sample. After a brief incubation time, the plates are overlaid with a plaquing medium. If historical data is not available, the incubation time needs to be optimized before plates can be washed and stained; it can also range from 3 to 15 days. The long incubation time, along with the inherent variability of the counting of plaques and scoring of wells of a TCID50, prolong vaccine development processes. To reduce the assay duration, the use of LFC can shorten the post-infection incubation time to 0.5–3 days. In addition, the instrument run time is less than 10 min per sample. An optional autosampler can run up to 96 samples in succession, significantly reducing hands-on time.

A specific example is shown in Fig. 4B, where Vero cells are infected with VSV under multiple experimental conditions, for instance different cell culture media. Using conventional assays, each sample has to be monitored (3+ days) until CPE is observed. Collected samples can be analyzed using a TCID50 or plaque assay, which can add another 3+ days until results are available. Using the Radiance, in turn, the samples from the experimental conditions...
can be harvested before CPE is visible (often less than 24 h) and analyzed on the instrument. Harvested samples can be tested immediately, fixed, or frozen for later analysis. Results can be obtained within minutes.

An example timeline illustrating the potential time saving for a 12-arm experiment is outlined in Table 1. The total time estimate to analyze 12 samples ranges from 8 to 21 days for conventional assays, while the LFC based process is estimated to take only 2–4 days as a result of measurements made throughout the process. A comparison between different methods of commonly used viral quantification techniques is shown in Table 2, which vary in reproducibility, time, cost, and labor intensity. Overall, the timeframe extends from days to less than an hour, as is the case for the Radiance.

4. Conclusions

The ability to rapidly measure viral titers could greatly accelerate multiple aspects of vaccine development and other viral-based applications. For vaccine process development, the iterative optimization of conditions, including media development, would benefit from the reduction of time to obtain titer data before initiating next experiments. Results presented here demonstrate the ability for LFC to function in at least two complementary scenarios. First,
a Radiance based analysis is compared to TCID50 and plaque-based assays with the starting point being a crude or purified viral stock. Due to its large dynamic range, once the calibration curve is developed, the LFC based analysis uses fewer dilutions and requires a 3–7× shorter incubation time, which depends upon the cell line and virus used. For VSV, the 72 h incubation was shortened to 16 h (a 4.5× reduction). LFC also eliminates subjectivity related to TCID50 or plaque-based assay analysis. Due to its ability to measure cells directly, LFC can be used to drastically improve the throughput of comparative experiments. In the second scenario, separate populations of cells are infected under different conditions, such as various media, to evaluate performance. Using conventional infectivity assays, samples must be collected and stored throughout the process before a separate TCID50 or plaque measurement can be made on each sample. In contrast, LFC provides results in less than 10 min after the cell sample is drawn allowing for real-time in-process modifications. While LFC has been demonstrated with a particular cell line and virus in this work, numerous viruses and cell lines (more than a dozen of each to date) have been tested successfully using Radiance. The ability to make rapid, cell-based infectivity measurements has the potential to impact the entire vaccine development life cycle from R&D to clinical trials and manufacturing, reducing the cost and time associated with developing and producing life-saving vaccines.

Contributors

CH, ND, ZE, and AH designed and performed the experiments for this publication. All authors contributed to the interpretation and analysis of the data; they all approved the final manuscript for submission.

Conflicts of interest

ND and AH are employees of Thermo Fisher Scientific, and CH, ZE, and SH are employees of LumaCyte. Thermo Fisher Scientific and LumaCyte funded this study in part. CH, ZE, and SH, have an ownership interest in LumaCyte, which is a privately held company. CH, ZE, and SH are authors on patents or patent applications developed or licensed by LumaCyte.

Acknowledgements

The authors would like to thank Keya Rodrigues and Margaret McCoy for their assistance with data collected on VSV fixed cell stability.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.09.002.

References


