Quantitative Measurement of Varicella-Zoster Virus Infection by Semiautomated Flow Cytometry[▽]

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Varicella-zoster virus (VZV; human herpesvirus 3) is the etiological cause of chickenpox and, upon reactivation from latency, zoster. Currently, vaccines are available to prevent both diseases effectively. A critical requirement for the manufacturing of safe and potent vaccines is the measurement of the biological activity to ensure proper dosing and efficacy, while minimizing potentially harmful secondary effects induced by immunization. In the case of live virus-containing vaccines, such as VZV-containing vaccines, biological activity is determined using an infectivity assay in a susceptible cellular host in vitro. Infectivity measurements generally rely on the enumeration of plaques by visual inspection of an infected cell monolayer. These plaque assays are generally very tedious and labor intensive and have modest throughput and high associated variability. In this study, we have developed a flow cytometry assay to measure the infectivity of the attenuated vaccine strain (vOka/Merck) of VZV in MRC-5 cells with improved throughput. The assay is performed in 96-well tissue culture microtiter plates and is based on the detection and quantification of infected cells expressing VZV glycoproteins on their surfaces. Multiple assay parameters have been investigated, including specificity, limit of detection, limit of quantification, range of linear response, signal-to-noise ratio, and precision. This novel assay appears to be in good concordance with the classical plaque assay results and therefore provides a viable, higher-throughput alternative to the plaque assay.

Varicella-zoster virus (VZV; human herpesvirus 3) is a member of the *Alphaherpesvirinae* family. It is the etiological cause of two distinct and common diseases in humans: chickenpox and zoster. Exposure of immunologically naïve individuals to VZV results in chickenpox, a condition typically occurring during the first two decades of life. Chickenpox is usually a mild disease, although severe complications have been reported, especially in immune-compromised individuals or patients suffering from hematopoietic malignancies (29, 31). Resolution of the primary infection does not result in complete elimination of the virus, which subsists in a latent stage in sensory neural ganglia, despite sustained cellular and humoral immunity (1). This latent stage can be maintained for the remainder of the individual's life span. VZV reactivation from latency causes the symptoms of zoster which can be associated with severe and debilitating pain. A significant fraction of patients (up to 20%) will eventually suffer from long-term chronic neuralgia (postherpetic neuralgia) due to permanent nerve damage. The causes of reactivation are not fully understood, but a combination of fatigue, stress, and a declining level of cell-mediated immunity seems to be implicated. Indeed, there

is a strong link between the rate of clinical reactivation and the increase in age of the affected patients (8).

Several pediatric live attenuated vaccine formulations, which have proven very efficacious at preventing chickenpox in children, while being well tolerated and safe, are commercially available. More recently, a high-dose formulation of the vOka/Merck strain has been approved by the U.S. Food and Drug Administration (FDA) for the prevention of shingles in adults 60 years of age and older (20). In both age groups, clinical efficacy, as measured by the induction of a protective cellular and humoral immune response, has been tentatively correlated with the level of infectivity of the vaccine (6). As a consequence, all aspects of vaccine production, formulation, and clinical dosage are based on the precise and accurate measurement of the concentration of VZV infectious units in relevant test articles (crude manufacturing process intermediates, final vaccine containers). Measurement of infectivity is paramount to ensure that a safe and efficacious vaccine is administered to each patient.

A commonly accepted definition of infectious units is the PFU, which is determined by plaque assays. Plaque assays have been previously described for a wide variety of viruses and rely on the appearance of localized foci of infection, characterized by damage, or cytopathic effect (CPE), in a monolayer of susceptible cells. They are normally sensitive, but are time consuming, labor intensive, and subject to counting errors. In the particular case of the attenuated vOka/Merck strain, the appearance of detectable CPE in cell culture takes several days at the multiplicities of infection used to enable manual counting, further compromising turnaround time and assay throughput.

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In this study, we describe an alternate infectivity assay for the attenuated VZV (vOka/Merck) strain, based on the enumeration of infected cells 24 to 72 h postinfection by semi-automated capillary flow cytometry. The discrimination of infected cells from noninfected cells is performed by indirect immunofluorescence to detect the expression of viral glycoproteins on the surface of infected cells. The new assay provides a rapid, higher-throughput alternative to the classical plaque assay. Critical analytical parameters, such as specificity, dynamic range, limits of quantitation, and variance components, are evaluated and discussed.

MATERIALS AND METHODS

Cells, cell maintenance, and viruses. MRC-5 human diploid lung fibroblast cells (ATCC CCL-171; Manassas, VA) were maintained in 150-cm² vented T-flasks (BD Falcon, Bedford, MA) in William's modified Eagle's medium with 4 mM MgSO₄ (WEM; HyClone, Logan, UT) supplemented with 10% γ -irradiated, iron-fortified bovine calf serum (BCS; HyClone, Logan, UT), 2 mM L-glutamine (Mediatech, Herndon, VA), and 50 μ g/ml neomycin sulfate (Sigma, St. Louis, MO). Cells were passaged twice a week, every 72 or 96 h, by harvesting cells with 0.05% trypsin-EDTA (Gibco, Invitrogen, Carlsbad, CA) for 5 min at 37°C and by seeding vented 150-cm² T-flasks at 20,000 cells/cm² in 50 ml culture medium. This culture routine resulted in cells gaining on average 3.0 (standard error of the mean, ± 0.10) population-doubling level (PDL) units per passage. PDL was calculated using the following formula: $PDL_{\text{harvest}} = \log(X_V \cdot \text{harvest} / X_V \cdot \text{plant}) / \log 2 + PDL_{\text{plant}}$, where X_V is the concentration of viable cells per ml. Viable cell counts were obtained manually by enumerating cells excluding trypan blue, using a hemocytometer. Cell trains were maintained below a PDL of 60 throughout the study; senescence and growth arrest were observed at a PDL of >80 (data not shown). The VZV-containing samples used in this study consisted of the attenuated vaccine vOka/Merck strain in various formulations and matrices originating from discrete processing steps of the Varivax and/or Zostavax vaccine production processes. Sample matrices were either cell associated (CA) or cell free (CF) when samples were collected prior to or after cell homogenization, respectively. Virus inactivation was performed in the CF matrix by incubating lyophilized vaccine for 56 days at 56°C (23) or by treatment in the liquid form for 15 min under a UV lamp in a biosafety cabinet.

Reagents and antibodies. Accumax and Accutase (Innovative Cell Technologies, San Diego, CA) were used to detach and disaggregate cells as recommended by the manufacturer. Mouse monoclonal anti-VZV glycoprotein E (gE) {clone 9C8, immunoglobulin G1(κ) [IgG1(κ)] (19), fully glycosylated anti-VZV gE [clone 13B1, IgG1(κ)] (9), anti-VZV gB [clone 10G6, IgG1(κ)] (17), anti-VZV gH [clone 6A6, IgG1(κ)] (5), anti-VZV gI [clone 8C4, IgG1(κ)] (12), anti-VZV major coat protein (MCP) [clone 3H2, IgG1(κ)], and anti-VZV IE62 [clone 8B11, IgG1(κ)] (11) antibodies, used for the original antigen expressing screening experiments, were obtained from Virusys Corporation (Sykesville, MO), and used and stored as recommended by the supplier. The monoclonal anti-VZV gH (clone A1, IgG2a) antibody used for the final assay was prepared as described previously (11).

Culture dish VZV infection procedure. The VZV vOka/Merck strain was propagated in 50-mm-diameter cell culture dishes (Corning Life Sciences, Corning, NY) by coculture of VZV-infected MRC-5 cells, with MRC-5 target cells previously grown to full confluence ($\geq 1.5 \times 10^5$ cells/cm²) at a cell-to-cell ratio ranging from 1 infected cell to 30 to 125 target cells for up to 5 days. Cells were harvested either by trypsinization or treatment with Accumax and then stained as described below.

Microtiter plate VZV infection procedure. The flow cytometry-based VZV infectivity assay described in this study is comprised of six discrete steps: cell planting, infection, harvest, staining, acquisition, and data analysis. Cells were planted for the infectivity assay by harvesting vented 150-cm² T-flasks grown for 3 or 4 days by treatment for 10 min with 5 ml of 0.05% trypsin-EDTA at 37°C in a humidified incubator with 5% CO₂. The trypsin digestion was quenched by addition of 15 ml of prewarmed WEM supplemented with 10% γ -irradiated BCS, and the cells were triturated using a serological pipette to ensure the generation of a single-cell suspension. Cells were diluted to 5×10^5 cell/ml, and 96-well plates were seeded with 50,000 cell/well in a 100- μ l final volume. The plates were then incubated for 24 h in a humidified incubator at 37°C with 5% CO₂. The day of infection, VZV-containing test articles were rapidly thawed at 37°C and immediately transferred to ice to minimize infectivity losses. Dilution curves were generated in 96-well dilution blocks on ice by targeting the first

dilution to $\sim 5,000$ PFU/well followed by 10 twofold serial dilutions in WEM supplemented with 2% BCA, 2 mM L-glutamine, and 50 μ g/ml neomycin sulfate. The 96-well plates were infected by transferring 200 μ l of the virus dilutions, using a multichannel pipette. The plates were centrifuged for 5 min at $900 \times g$ at 4°C and returned at 35.5°C for 48 to 72 h in a humidified incubator with 5% CO₂.

VZV glycoprotein staining and flow cytometry acquisition. Infected plates were harvested by removing the spent supernatant, washing the monolayer once with 200 μ l/well of Dulbecco's phosphate-buffered saline (PBS; Gibco, Invitrogen, Carlsbad, CA), and treating with 40 μ l/well of Accumax reagent. The plates were incubated for 5 min at 37°C in a humidified incubator gassed with 5% CO₂, and the reactions were stopped by the addition of 60 μ l/well of WEM supplemented with 10% BCS. One hundred microliters of wash-and-stain buffer (WSB; 5% fetal calf serum in Dulbecco's PBS) was added to each well, and cells were resuspended using a tabletop Vortex Genie (Scientific Industries, Bohemia, NY) and transferred to a U-bottom non-tissue culture 96-well plate (Corning Life Sciences, Corning, NY). The cells were pelleted for 5 min at $900 \times g$ at 4°C, washed in 200 μ l of WSB, and pelleted again. After aspiration of the supernatant, cells were resuspended in 50 μ l/well of diluted anti-gH mouse monoclonal antibody (clone A1, IgG2a; 4 μ g/ml) in WSB and left to stain for 20 min at room temperature (22°C). The staining solution was diluted by addition of 150 μ l/well of cold WSB, and cells were pelleted and washed as described above. The cells were stained in 50 μ l/well of a mixture of 1:10 7-amino actinomycin D (7-AAD) and 1:5 diluted goat anti-mouse F(ab')₂ IgG-R-phycoerythrin (RPE) conjugate (Guava Technologies, Hayward, CA) in WSB for 20 min at 22°C. The cells were recovered and washed once in WSB as described above for the primary stain and transferred to a flat-bottom, ultralow-cluster, 96-well plate (Corning Life Sciences, Corning, NY) for acquisition. Plates were acquired as soon as possible or stored for up to 4 h, protected from light at 4°C without noticeable loss of signal intensity or cellular viability. Viability measurements were performed as recommended by the manufacturer, using the VIAcount Flex kit (Guava Technologies, Hayward, CA).

Crystal violet staining. MRC-5 cells were grown as described above. The spent culture media were removed by aspiration, followed by treatment with a panel of detachment reagents. After incubation in the detachment reagents (5 to 15 min at 37°C, with 5% CO₂), the cells were resuspended with a multichannel micropipette and transferred to a fresh plate for viability and counting assays using the Guava VIAcount Flex kit (see above). The original plate was washed twice with 250 μ l/well of PBS and then incubated for 30 min at 37°C with 5% CO₂ in 100 μ l/well of 0.5% crystal violet in methanol. After incubation, the crystal violet solution was discarded and the plate washed three times with deionized H₂O and dried by inverted blotting on a piece of absorbent paper. One hundred microliters of 100% methanol was added to each well, and the plate was left to agitate on an orbital shaker for 10 min at room temperature. The amount of released crystal violet was measured by spectrophotometry at 570 nm.

Data acquisition and analysis. Flow cytometric data were acquired on calibrated tabletop semiautomated Guava EasyCyte flow cytometers (Guava Technologies, Hayward, CA), using the dedicated acquisition and analysis software module Guava ExpressPlus, part of the CytoSoft v3.6.1 software. Five thousand events were collected for each well/condition. The well mixing setting was put on high for 10 s/well. Acquisition parameters, such as forward scatter (FSC) threshold, FSC gain, photomultiplier tube voltage gain, and compensation parameters, were set using the negative control and the most infected well. As depicted in Fig. 1, the collection of events of each well was reduced to live cell-sized events by successive logical gating on 7-AAD-negative and cell-sized events. Discrimination between live negative and live infected cells was achieved using a marker tool set to encompass less than 1% of the negative-control, mock-infected cells. The resulting Microsoft Excel file (comma separated values, or .CSV) was fitted using four-parameter logistical functions. Fifty-percent infective concentration values were reported and relative potencies calculated by parallel line analysis, using an externally calibrated standard of known infectivity (PFU/ml).

Precision analysis (variance component analysis). To assess the precision of the flow cytometry assay, a variance component analysis (25) was performed on the natural log transformation of individual relative potencies. The assay format was one plate for each run, and each plate has two series of the same sample. Thus, the total variability was decomposed into the following two components: variability between runs ($\sigma_{b-\text{run}}^2$) and variability within a run (or plate), including variability between two series of the same sample ($\sigma_{b-\text{series}}^2$) and variability related to estimated relative potencies ($\sigma_{\text{parallel-line-approach}}^2$). The variability (σ^2) of the natural log of a reportable value based on n runs and k replicated series per run (plate) was calculated as $\frac{\sigma_{b-\text{run}}^2}{n} + \frac{\sigma_{\text{parallel-line-approach}}^2 + \sigma_{b-\text{series}}^2}{n \times k}$. The precision of the reportable value was expressed by the percent relative standard

FIG. 1. Measurement of VZV infection using flow cytometry. (a) FSC (x axis) and red fluorescence intensity channel (RED; y axis) correlation dot plot showing selective gating (gray events) on 7-AAD-negative events (live cells and nonnucleated debris). (b) RPE-conjugated gH staining intensity (yellow fluorescence intensity channel; x axis) and FSC (y axis) correlation dot plot. Selective gating on cellular-sized events is indicated in gray, while subcellular debris (black) is excluded from the analysis. This plot is logically gated on the plot in panel a to include only live events. (c) The RPE-conjugated gH staining intensity histogram, logically gated on plots a and b, displays a characteristic biphasic frequency distribution corresponding to uninfected cells (gray) and infected, high-fluorescence, gH-positive cells (black). The marker bars 1 and 2 are used to calculate the fraction of infected cells in each sample or well.

deviation (%RSD) (28), calculated as $\%RSD = \sqrt{e^{n^2} - 1} \times 100\%$, based upon a log-normal distribution.

Concordance analysis. The concordance in relative potencies between the results of the flow cytometry assay and the plaque assay was statistically assessed by errors-in-variables regression (4, 27), as follows: $X_i = \xi_i + \delta_i$, $Y_i = \eta_i + \epsilon_i$, and $\eta_i = \alpha + \beta\xi_i$, where X_i and Y_i are natural log-transformed relative potencies from flow cytometry and plaque assays on the i th sample ($i = 1, 2, \dots, n$). ξ_i and η_i are the unobservable true values of X_i and Y_i , δ_i and ϵ_i are measurement errors, and α and β are the intercept and concordance slope, respectively. The VSV plaque assay was performed by standard methods, as described in reference 13.

RESULTS

The use of flow cytometry to measure the number of infected cells has been demonstrated previously for other types of viruses (18). These methods were aimed at the measurement of the fraction of infected cells in a sample previously infected with a virus suspension and relied on the assumption that only one round of infection had occurred and that virus adhesion is quantitative and synchronous. At early time points, therefore, the presence of virus antigen on a single cell can be considered to result from infection by an individual infectious particle in the original inoculum.

We first compared commercially available and house-produced monoclonal antibodies for their ability to stain VZV-infected human diploid fibroblast cells (MRC-5) in a standard indirect immunofluorescence approach, using RPE secondary conjugates as fluorophores. The monoclonal antibodies tested were directed at both immediate early/early genes (IE62) and late, structural proteins (VZV gE, gI, gB, and gH and MCP) and were titrated to measure mean fluorescence intensities at saturation. It appeared that staining of glycoproteins, with the notable exception of gB, consistently resulted in high staining intensity (data not shown). A particular monoclonal antibody directed against gH (described by Keller et al. [11]) was selected for further assay development. The high fluorescence and saturating signals achieved using this antibody are consistent with the notion that structural proteins of viruses are generally produced in abundance. Besides offering the poten-

tial to develop a more sensitive assay, staining for gH on the surface of infected cells also circumvents the need for cell fixation and permeabilization that would be required to stain for cytoplasmic and/or nuclear VZV antigens (IE62, MCP). In addition, the choice of gH as a reporter antigen also allowed us to counterstain the cells, using the semipermeable vitality dye 7-AAD (Fig. 1) to identify and quantify live-infected cells, in which, presumably, productive infections are occurring. The final two-color, two-step, logical gating procedure (Fig. 1) allowed for the precise discrimination of live-infected cells from a significant portion of dead cells (resulting from the cytopathic nature of VZV) and a large portion of debris. The proportion of debris is variable and can originate both from the cells themselves, through biologic processes such as apoptosis or mechanical stresses induced during recovery and/or acquisition (e.g., mixing). The analysis strategy effectively excludes debris and hence provides a safeguard against this source of variability.

To maximize the sensitivity of the assay, we examined the kinetics of VZV expression in an MRC-5 monolayer to identify the point at which the signal is maximal for harvest. This study was performed in 60-mm petri dishes, using a cryo-preserved VZV-infected sample as the inoculum, diluted to a cell-to-cell ratio of $\sim 1:100$. As seen in Fig. 2a, signs of VZV infection are noticeable as early as 12 h postinfection, with infection being quantitative by 48 h. When the data are compared to the appearance of CPE, it is noteworthy that morphological signs of infection, often used to gauge the success of VZV infection, are trailing viral markers by about 30 h (Fig. 2b). This is corroborated by the measurement of apoptosis in infected cells, which shows that significant caspase activity only becomes detectable after 72 h of infection (data not shown). In contrast, inactivated virus (either by heat or UV irradiation) does not induce a similar wave of infection in the target monolayer, indicating that live virus is required to induce the infection event (Fig. 2c). In addition, these data also demonstrate that the multiplicity-of-infection range is well chosen and that

the background due to noninfectious material (resulting from heat or UV inactivation of the inoculum) is well controlled. Also noteworthy is the fact that the infection kinetics are very dependent on the nature of the inoculum. As seen in Fig. 2c, kinetics of infection using a CA (trypsin digestion) inoculum are much faster than kinetics observed using a CF (sonication) inoculum, suggesting that the mechanisms of infection are different. This is consistent with recent data on the entry of CA VZV compared to free virions (7).

One of the critical requirements of flow cytometry analysis is the generation of a suitable cell suspension, preferably consisting of single cells and devoid of aggregates and particulates. In the case of the MRC-5 cell line, which is a highly adherent human diploid lung fibroblast cell line, this requires not only that the cells to be recovered from the cell culture substrate while preserving their surface expression characteristics, but also that they be maintained in suspension during the time required for analysis. An additional complication is the fact that VZV glycoproteins, such as gE, are highly sensitive to trypsin degradation (15). To ensure that the cells were recovered with minimal impact on their fluorescence characteristics, multiple cell detachment reagents were compared. Initial experiments with various formulations of EDTA alone were unsuccessful at detaching the cell monolayer and were not pursued further (data not shown). Comparisons of various formulations of trypsin with trypsin-free enzymatic reagents have revealed that the Accumax reagent was a viable alternative to trypsin-based reagents. Accutase and Accumax are enzymatic cell detachment reagents consisting of a mixture of proteases of crustacean origin, containing collagenolytic and DNase activities, and have been used successfully in a range of delicate applications (2, 21, 24, 30, 33). Indeed, cell recovery from tissue culture-treated 96-well plates using Accumax was indistinguishable from that using standard trypsin-EDTA, and viability of the cells was preserved to similar levels (Fig. 3a). A control experiment to quantify the recovery of cells from 96-well plates by using crystal violet staining confirmed that both Accumax and trypsin-EDTA yield comparable, quantitative (>95%) recovery compared to untreated monolayers (Fig. 3b). Comparison of recovered cells for staining for VZV gH indicates that Accumax offers an extended window of treatment during which the fraction of infected cells is stable (Fig. 3c) and also offers less staining variability than does trypsin-EDTA. Finally, the examination of fluorescence intensity histograms reveals that there is a significant drop in fluorescence after 6 min of treatment with trypsin-EDTA compared to Accumax (Fig. 3d). Accumax is clearly a superior reagent for preserving cell surface staining and was used in all subsequent experiments.

During acquisition of flow cytometric data, cells need to

remain in suspension for the entire duration of the acquisition of a plate (typically over 30 min). In the case of MRC-5, a significant fraction of the cells were actually readhering to the plate during acquisition, as evidenced by a precipitous drop in acquisition rate relative to that of the first well acquired on the plate (Fig. 4a). This resulted in a significant increase in the acquisition time and selective bias for analysis of cell debris, as only cells readhered. To correct for this, cells were transferred to low-cluster, hydrogel-coated flat-bottom plates, and two mixing settings were compared. The improvement in acquisition rate afforded by the transfer of cells to low-cluster plates was evident even at the lower mixing setting, but the variability of acquisition rates remained high, possibly because of settling issues (Fig. 4a). Mixing the sample more extensively resulted in a well-maintained acquisition rate in all 96 wells of the plate, with overall lower variability. Under these acquisition conditions, the stability of the signal across a plate was evaluated by performing an infection with a VZV sample at a single multiplicity of infection. The signal intensity and discrimination of infected cells appear to be well maintained across the plate, with no evidence of systematic trends across the plate (Fig. 4b).

As noted previously, one of the hallmarks of VZV preparations grown in cell culture is the presence of a large excess of deficient, noninfectious particles. Because the ratio of physical particles to infectious particles of VZV in cell culture-produced virus is so high, the background noise of any assay aimed at measuring the infectivity of VZV must be evaluated. We have carefully examined the background component of the flow cytometry assay by performing an extended series of two-fold dilutions and comparing the signal recovered at 2 h postinfection, when replication has not yet occurred, to the signal obtained 48 h into the assay. These data (Fig. 5a) suggest a useful working dynamic range of a little more than 2 orders of magnitude (range, $256\times$) between saturation and the limit of detection (LOD; in this range the signal-to-noise ratio is larger than 10). The maximal signal-to-noise ratio is attained roughly in the center of the dynamic range at 10^{-3} PFU/cell (signal-to-noise ratio of 79.9). The LOD of the assay can be estimated by measuring the standard deviation of the lowest nonzero dilution and multiplying by 3.29 (99% confidence interval) and comparing the signal to that of the infection harvested at 2 h. The minimal fraction of infected cells that can be reliably detected (with 99% confidence) is 4.3%, which corresponds to an infectious-particle-to-cell ratio of 10^{-4} PFU/cell. The theoretical limit of quantitation (LOQ) of the assay ($5\times$ the LOD) is therefore estimated to be 5×10^{-4} PFU/cell. Taking into consideration the format of the assay, the LOD and LOQ of this assay enable the detection of 1,000 PFU/ml and the quantification of 5,000 PFU/ml in

FIG. 2. Progression of VZV infection in MRC-5 cells measured by flow cytometry. (a) Characteristic RPE-conjugated gH-staining intensity (yellow fluorescence intensity channel; x axis) and FSC (y axis) correlation contour plots illustrating the course of an infection with a CA VZV inoculum in MRC-5 cells. (b) Fraction of gH-positive cells (open circles) as a function of hours postinfection compared to the appearance of signs of CPE (closed circles). Mock-infected (closed inverted triangles) and isotype-staining controls (open triangles) are included to demonstrate the specificity of the staining. Error bars represent 1 standard deviation for cytometric measurements ($n = 3$ experiments), while the CPE curve is representative of a single experiment. (c) Infection kinetics of CA (closed circles) and CF (closed squares) samples in MRC-5 cells, using the flow cytometry assay. Control infections with heat-inactivated (CF/ Δ ; open squares) and UV-inactivated (CF/UV; open triangles) CF samples demonstrating that the signal observed is dependent on the presence of live attenuated virus.

FIG. 3. Detachment of MRC-5 cells and preservation of surface VZV glycoproteins. (a) Recovery and viability of MRC-5 cells from tissue culture-treated 96-well plates, using a panel of different detachment reagents. Measurements for cell concentration (gray bars) and cell viability (closed circles) are performed using the Guava VIACount Flex kit. Cell recovery data are plotted as averages over 24 wells with associated 95% confidence intervals. Guava CDR (cell dispersal reagent) is an accessory reagent used to disaggregate cells for viability measurements. (b) Measurement of the amount of cells remaining on the substrate after cell detachment using crystal violet staining. Yield of detachment is indicated as a percentage in each bar (control is set at 0.0%). Data are plotted as average optical densities at 570 nm (OD 570 nm) with associated 95% confidence intervals (over 16 wells). (c) Stability of the measured fraction of gH-positive cells as a function of exposure time to trypsin-EDTA (closed circles) or Accumax (open circles) cell detachment reagents. Data are plotted as averages of four wells with associated 95% confidence intervals. (d) Overlay of fluorescence intensity histograms for RPE-conjugated VZV gH after 6 min treatment with trypsin-EDTA (dark gray) and Accumax (light gray). The black histogram represents staining for mock-infected cells.

VZV test articles, respectively. To evaluate the ability of this assay to discriminate small differences in infectivity, a series of closely related samples was prepared by successive predilution of the same sample at slightly different levels. It appears that the assay is able to discriminate at least 25% differences in samples with similar matrices, an important fact to consider when measuring samples produced from related arms of a single experiment (Fig. 5b).

To assess the precision of this assay, a variance component analysis was performed. The analysis was based on data collected from 12 identical runs of VZV CA samples and nine identical runs of VZV CF samples. The precision of the assay is summarized in Table 1. It appears that the root variabilities for CA and CF samples are very similar (40%), which should allow for discriminating samples that are about 1.5-fold different in infectivity with good statistical confidence. This confirms

FIG. 4. Cell readherence and plate effect. (a) Observed acquisition rates normalized to the first well acquired (well A1) in a 96-well format assay stained and acquired in a U-bottom tissue culture plate at low mixing setting (closed circles) and a U-bottom non-tissue culture plate and acquired in a hydrogel low-cluster flat-bottom plate at low mixing setting (open squares) or at high mixing setting (open diamonds). Data are presented as an average over an entire dilution curve (12 wells) with associated 95% confidence intervals. (b) Mean fluorescence intensity (M.F.I.; closed circles) of gH staining and fraction of positive cells (open circles) for a 96-well plate infected with a single dilution of the same VZV test article.

the results obtained on serially diluted samples (see above), where discrimination between the 1.25 $\times$ and 1 $\times$ samples is marginal, whereas the discrimination between the 1.5 $\times$ sample and 1 $\times$ sample is convincing.

Finally, in order to evaluate the performance of the new assay, we compared the infectivity results obtained by the flow

cytometry assay to those generated by the plaque assay. This concordance analysis was performed using flow and plaque assay infectivity results from 60 independent samples. Infectivity results for the flow assay were estimated by the parallel-logistic (four-parameter fit) method against an infectivity standard calibrated by the plaque assay. The concordance plot is

FIG. 5. Assay dynamic range, background, and discrimination. (a) Assay dynamic response over 17 twofold dilutions of a VZV test article, spanning 5 orders of magnitude of infected-cell-to-target-cell ratio (PFU/cell). Signal is measured at 48 h postinfection (closed circles), and nonspecific background is estimated at 2 h postinfection (open circles). Data are presented as averages over 12 dilution curves with associated 95% confidence intervals. (b) Discrimination illustrated by infection with the same test article at various predilution factors (closed circles, 1 $\times$; open circles, 1.25 $\times$; closed triangles, 1.5 $\times$; open triangles, 2 $\times$; and closed squares, 4 $\times$) to demonstrate recovery of small differences in infectivity. Data are presented as averages of three representative curves at each predilution level with associated 95% confidence interval. The data are fitted using a four-parameter logistic function.

TABLE 1. Variance component analysis summary^a

Matrix type	Replicate curve (intrarun variability)	% CV for indicated replicate run (interrun variability)				
		1	3	6	9	12
CA	1	41.7	23.4	16.5	13.4	11.6
	2	32.3	18.3	12.9	10.5	9.1
	3	28.6	16.3	11.5	9.4	8.1
	4	26.6	15.2	10.7	8.7	7.6
	5	25.3	14.5	10.2	8.3	7.2
	6	24.5	14.0	9.9	8.1	7.0
CF	1	41.3	23.2	16.3	13.3	11.5
	2	31.1	17.7	12.5	10.2	8.8
	3	27.1	15.5	10.9	8.9	7.7
	4	24.9	14.2	10.0	8.2	7.1
	5	23.5	13.4	9.5	7.7	6.7
	6	22.5	12.9	9.1	7.4	6.4

^a Percent coefficient of variation (% CV), reported as a function of assay replication level (intrarun and interrun) for CA and CF matrices. The root variability (one replicate curve, one replicate run) is shown in boldface type for both matrix types.

shown in Fig. 6. It appears that the two assays are indeed correlated but that the slope is quite different from the unit (0.68). This may indicate that the two assays, while scoring infectious units, do not score the same subpopulation of infectious units. Heterogeneity in plaque morphology has been described for a large number of viruses, including VZV, and could be the result of genotypic heterogeneity. The fact that the slope is less than 1 indicates that the flow assay is registering slightly more infectious units than is the plaque assay. This might be due to the fact that the flow-based assay relies on centrifugation to synchronize the infection while the plaque assay does not. Hence, a higher proportion of infectious units may interact with the monolayer, resulting in a higher number of hits.

DISCUSSION

The application of flow cytometry to quantify virus infectivity has been described previously for various enveloped and nonenveloped viruses (reviewed in reference 18). These studies are based on direct enumeration of infected cells in the flow cytometer and discrimination from noninfected cells by immunostaining using monoclonal antibodies specific for viral antigens. Three conditions have to be satisfied to use the number of antigen-positive cells as a surrogate measurement of infectious units in the original sample: (i) the assay has to be performed under conditions where the multiplicity of infection is less than 1; (ii) the infection is not limited by Brownian motion (e.g., infection is synchronized and quantitative); and (iii) the number of cumulative replicative cycles is less than or equal to 1. Generally, the results of these experiments have been correlated with plaque assay results quite successfully for a number of viruses (3, 10, 14, 16, 22, 32).

We took a similar approach to design a flow cytometric infectivity assay for the attenuated vOka/Merck VZV strain with improved throughput characteristics and potential for high-throughput operation. However, two distinctive properties of this virus in cell culture had to be taken into account during assay development. First, the yield of replication and

assembly of VZV production in cell culture are extremely low. Indeed, the ratio of physical particles to infectious particles can range from 10^5 to 10^6 (26). The consequence is that the large excess of debris, including viral protein and DNA not associated with infectivity potentially introduced in the assay, can generate significant background and decrease its overall dynamic range. Therefore, the assay needs to be operated at a multiplicity-of-infection range that could severely limit sensitivity. Second, VZV grows with slow kinetics in vitro. Therefore, the generation of sufficient concentrations of viral antigens for detection requires longer incubation times. Given the propensity of VZV to propagate by cell-to-cell interaction without completing its full replicative cycle (34), the premise that only a single replicative cycle is measured cannot be verified. Therefore, we opted for a design including an internal infectivity control, calibrated externally to the plaque assay, in order to account for multiple asynchronous replicative cycles. In addition, infection experiments in this study were performed using particulate virus or infected cells and were not limited by Brownian motion to ensure quantitative and synchronous virus adsorption. Critical assay parameters for each of the six steps of the procedure were systematically investigated, and a summary of the final optimized procedure is provided in a tabulated format in Table 2.

The assay reported in this study presents many desirable attributes. Notably, the high signal-to-noise ratio permits the measurement of samples with low levels of infectivity. This high sensitivity is a key benefit of flow cytometry for this application. Since size/morphology discrimination of cellular events from debris is possible, the burden of noninfectious, yet immunoreactive, material is effectively reduced in the analysis. The assay has good precision characteristics, comparable to those of most plaque assays, suggesting that this variability is

FIG. 6. Assay concordance plot. Bivariate scatter correlation plot between the infectivity of VZV-containing test articles measured using the flow-based infectivity assay (x axis) and the plaque assay (y axis). The solid line is the ideal linear curve with slope of 1. The dashed line is the observed concordance curve ($\text{LnPlaque} = 0.68 \times \text{LnFlow} + 3.62$).

TABLE 2. Optimized critical assay parameters^a

Step	Cell or VZV type	Cell density or MOI	Microtiter plate type	Medium and/or reagent (concn)	Time and temp	Comment(s)
Cell planting	MRC-5 cells	5×10^4 cells/well, 0.1 ml/well	FB TC	WMEM + 10% BCS	24 h at 37°C	
Infection	CA or CF VZV	10^2 to 10^5 PFU/cell	FB TC	WMEM + 2% BCS	48 h (CA) or 72 h (CF) at 35.5°C	Centrifugation required
Harvest	Infected cells		FB TC	Accumax (40 μ l/well)	5 min at 37°C	
Staining	Infected cells		RB NTC	Anti-gH (4 μ g/ml) GAM-RPE (1:5) 7-AAD (1:10)	40 min at RT (total)	
Acquisition	Infected cells		FB LC		~90 min at RT	5,000 events/well, high mixing setting for 10 s/well
Analysis						Two-color, two step gating, four-parameter logistical regression

^a Step-by-step description of critical assay parameters and their values in the final optimized VZV infectivity assay. All microtiter plates were sourced from Corning Life Sciences (Corning, NY). FB, flat-bottom; RB, round-bottom; TC, tissue culture; NTC, non-tissue culture; LC, low cluster; GAM, goat anti-mouse antibody; MOI, multiplicity of infection; RT, room temperature.

inherent to the adsorption and infection events. However, the higher density and potential for high-throughput operation of this assay make it a promising alternative to the classical plaque assay.

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