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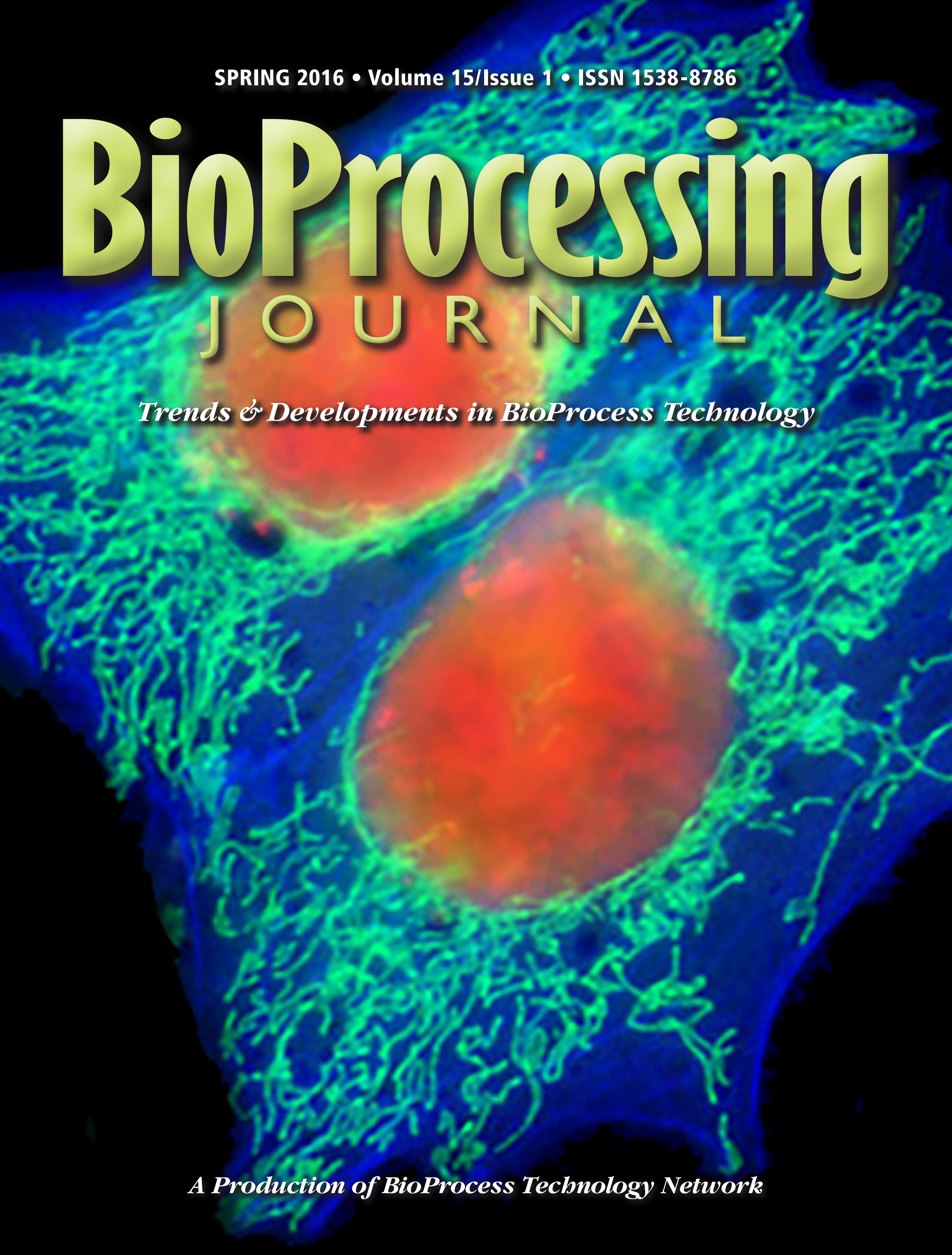

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TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

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Development of a Novel Flow Cytometry-Based Titration Assay to Quantify Herpes Simplex Virus Type 1 (HSV-1)

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Abstract

Plaque assays have traditionally been a reliable way to determine the titer of a lytic virus. However, this method has several shortcomings in that it is time-consuming, labor intensive, and suffers from limited sensitivity. In this article, we describe a novel flow cytometry-based titration assay to quantify green fluorescent protein-labeled herpes simplex virus type 1 (HSV-1-GFP). Using this assay, we were able to directly quantify ten-fold dilutions of the virus in which every GFP-positive cell could be counted. In a head-to-head comparison with a traditional plaque assay, the flow cytometry assay showed a greater linear range and was accomplished in less than half the time of the plaque assay. Additionally, the cells prepared for flow cytometry could also be directly visualized by fluorescence microscopy. These results with HSV-1-GFP show proof of concept and are of practical use to herpesvirus researchers. Additionally, this technique could be easily modified to study other lytic or non-lytic viruses using antibodies against viral antigens.

Introduction

Plaque assays are one of the most commonly used techniques in the field of virology. They provide a reliable approach to quantitatively determine the specific titer of a lytic virus through the generation of plaque-forming units (pfu) on susceptible cells. Plaque assays currently constitute the "gold standard" for quantifying virus, thus allowing precise calculation of multiplicity of infection (MOI) for viral infectivity studies *in vitro* and *in vivo*. For certain viruses, such as HSV-1, a well-studied human double-stranded DNA virus[1], the plaque assay is a time- and labor-intensive process. One day is spent seeding the cells, another for viral infection, and then three more for incubation, followed by detection through manual counting of the plaques. Thus, from cell seeding to plaque staining, the entire process takes five days. There is also the potential for human error when counting plaques as identification of individual plaques is subjective. Here, we report a flow cytometry method using a GFP-labeled strain of HSV-1 that can be performed in less than half the time, and yields more sensitive and accurate results than the traditional plaque assay.

Materials and Methods

Cell Culture and Virus Preparation

CV-1 (African green monkey kidney fibroblast) cells were obtained from American Type Culture Collection (ATCC® CCL-70™) and maintained in 1× Dulbecco's Modification of Eagle's Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. HSV-1 virus (17+ strain) containing a GFP tag (HSV-1-GFP) located on viral protein 22 (VP22)[2] was amplified to create a stock virus. The virus stocks were generated and titered on CV-1 cells. Cell-released virus was amplified by the following method: CV-1 cells in ten T-150 flasks were grown to 70% confluency in culture media (1× DMEM, 10% FBS, 2mM L-glutamine). The culture media was removed and stock HSV-1-GFP cell-released virus was added in 10 mL of virus media (1× DMEM, 5% FBS, 2 mM L-glutamine, 100 units pen/strep, 0.1 mM gentamicin, 10 mM HEPES) at an MOI of 0.01 to the flasks. The flasks were incubated for one hour with rocking in a humidified incubator at 37 °C with 5% CO_2. The virus inoculum was removed and then 35 mL of fresh culture media added. The cells were incubated for 3–5 days in a humidified incubator at 37 °C with 5% CO_2 until >90% cytopathic effect (CPE) was observed. After incubation, the media/cell mixture was combined by pipetting, placed into 50 mL conical tubes, and centrifuged at 1,000 × *g* for ten minutes. The supernatant was transferred into fresh conical tubes and centrifuged again at 1,000 × *g* for six minutes. The supernatant was then transferred into Thinwall Ultra Clear™ tubes (Beckman Coulter), loaded into an SW28 rotor (Beckman Coulter) and ultracentrifuged at 100,000 × *g* for 80 minutes in an Optima™ L-90K ultracentrifuge (Beckman Coulter). The supernatant was aspirated and the remaining pellet resuspended in 1 mL Hank's Balanced Salt Solution (HBSS) with 2% FBS. The virus was stored in 20 µL aliquots in HBSS with 2% FBS at −80 °C.

Plaque Assay

CV-1 cells (8.5×10^5) were plated and grown in a single monolayer to 70–90% confluency in a six-well plate. A ten-fold dilution series of HSV-1-GFP was prepared and plated in 1 mL of HBSS plus 2% FBS. After initial inoculation, the virus was incubated with rocking for one hour in a humidified incubator at 37°C with 5% CO_2. The viral inoculum was removed and 2 mL plaque media (1× DMEM, 2 mM L-glutamine, 1% human serum, and 1% FBS) was added. Plates were incubated for three days at 37°C in 5% CO_2. After three days, the plaque media was removed and 1 mL of a methylene blue solution (H_2O, 95% methanol, and 0.25% methylene blue) was added to each well for five minutes and then gently washed with H_2O. Plaques were subsequently visualized and manually counted. The plaque assays were done in duplicate and the number of plaques from each dilution were averaged. Results shown are representative of four experiments.

Flow Cytometry

CV-1 cells (8.5×10^5) were plated as a single monolayer and infected with ten-fold dilutions of HSV-1-GFP in a manner identical to the process used for plaque assaying. After initial inoculation, the virus was incubated with rocking for one hour in a humidified incubator at 37°C with 5% CO_2. Virus solution was removed, 2 mL culture media was added to the plates, and cells were further incubated for 16 hours. On the following day, the cells were processed for flow cytometry as follows: The culture media was aspirated and the cells were washed twice with 1× phosphate buffered solution (PBS). Trypsin (1 mL) was added and incubated at 37°C with 5% CO_2 for five minutes. Next, 1 mL of HBSS plus 2% FBS was added and the entire mixture was transferred to a flow cytometry tube. The samples were centrifuged at 500 × *g* for six minutes at 4°C, the supernatant was removed, and then 300 µL 4% paraformaldehyde and 300 µL PBS were added. The samples were analyzed on a FACSCalibur™ flow cytometer (Becton Dickinson) with DxP 8-color 488/637/407 upgrade (Cytek Development). A total of 20,000 events were collected using a BluFL1 488 nm laser and then analyzed on the Cytek FlowJo Collector's Edition 7.5.110.6. The cells were gated to exclude cellular debris and multiple cell aggregates. Results are representative of three experiments.

Fluorescence Microscopy

Approximately 1.4×10^4 of the cells prepared for flow cytometry were plated on Premium Superfrost™ microscope slides (Fisher). Ten µL of Vectashield mounting medium with DAPI (Vector Laboratories) was added directly on top of the cells and then coverslips were mounted. Prepared slides were incubated in the dark for one hour and then visualized on an Olympus microscope (model BX50). The pictures were taken with an Olympus microscope-mounted camera (model DP70). Cells were quantified with ImageJ software (National Institutes of Health).

Results

Flow Cytometry-Based Titration Assay Quantifies HSV-1 Infected Cells

CV-1 cells were infected with HSV-1-GFP at an MOI of 10, 1, 0.1, 0.01, 0.001, and 0.0001. Sixteen hours post-infection, the cells were processed for flow cytometry and analyzed. Flow cytometry plots demonstrated a dose-dependent reduction of GFP-positive cells as the MOI decreased (**Figure 1A–F**). Quantification of the virus dilutions produced a linear ten-fold dilution for which every GFP-positive cell could be accounted for over a five log range (**Figure 1G**). As each GFP-positive cell represents an HSV-1 infected cell, this assay is superior to the traditional plaque assay in that single infected cells may be directly quantified.

Flow Cytometry-Based Titration Assay Has a Broader Dynamic Range Than a Traditional HSV-1 Plaque Assay

To determine the relative sensitivity of the flow cytometry-based titration assay versus the plaque assay, CV-1 cells were inoculated with serial dilutions of HSV-1-GFP (10^{-4} to 10^{-10}) and subjected to both assays simultaneously. As shown in **Table 1**, at 10^{-4} to 10^{-7} dilutions, the plaque

TABLE 1. Comparison of the traditional plaque assay to the flow cytometry-based titration assay.

Dilution Factor	Number of Plaques	Titer From Plaque Assay (PFU/mL)	% of GFP+ Cells From Flow Cytometry	Number of GFP+ Cells From Flow Cytometry	Titer From Flow Cytometry (GFP+ cells/mL)
10^{-4}	TNTC	—	99.6	8943	8.9×10^7
10^{-5}	TNTC	—	64.8	9576	9.5×10^8
10^{-6}	TNTC	—	10.0	1630	1.6×10^9
10^{-7}	TNTC	—	1.2	208	2.0×10^9
10^{-8}	88	8.8×10^9	0.18	29	2.9×10^9
10^{-9}	7	7.0×10^9	0.025	4	4.0×10^9
10^{-10}	0	—	0.0062	1	1.0×10^{10}

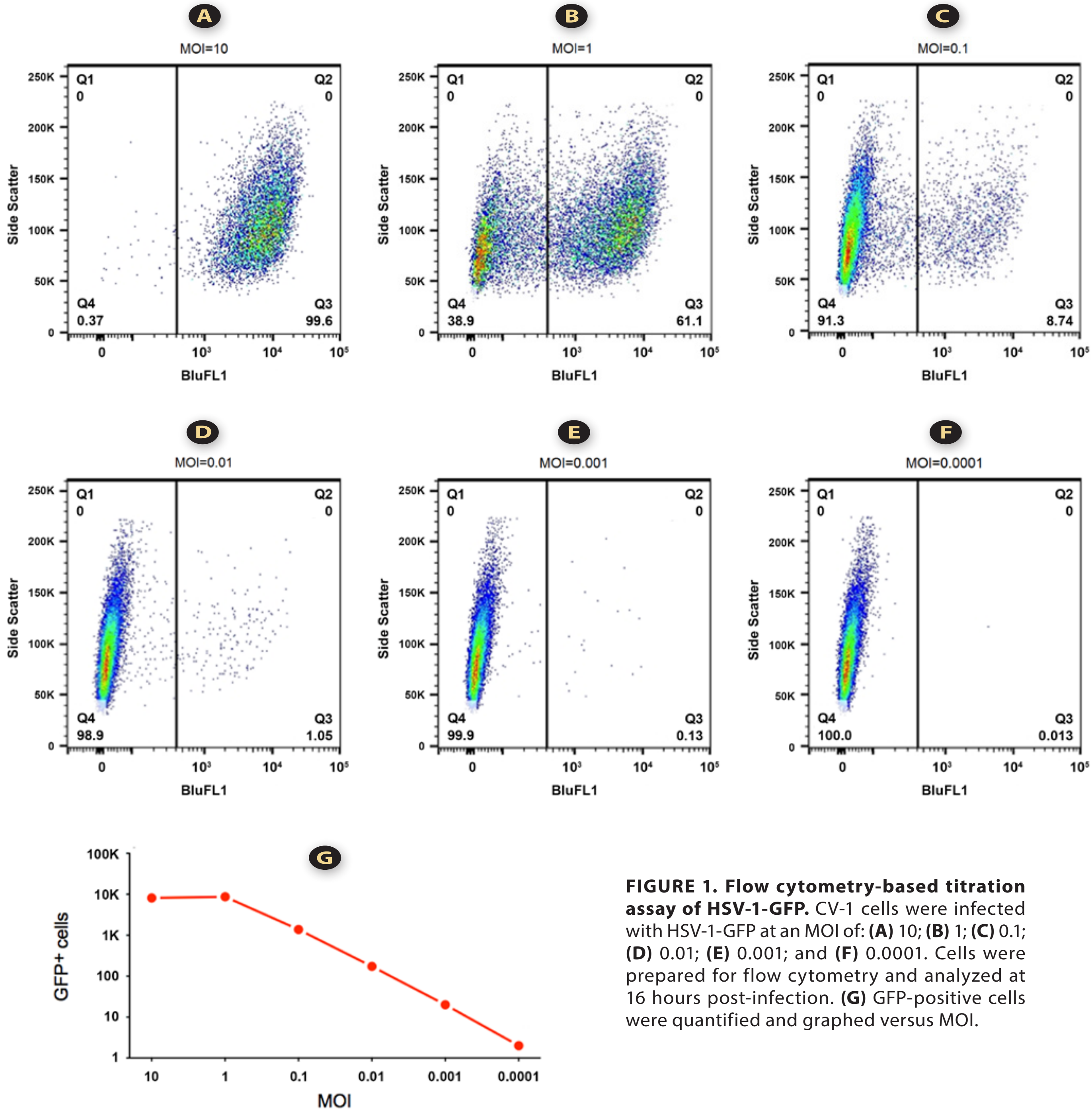
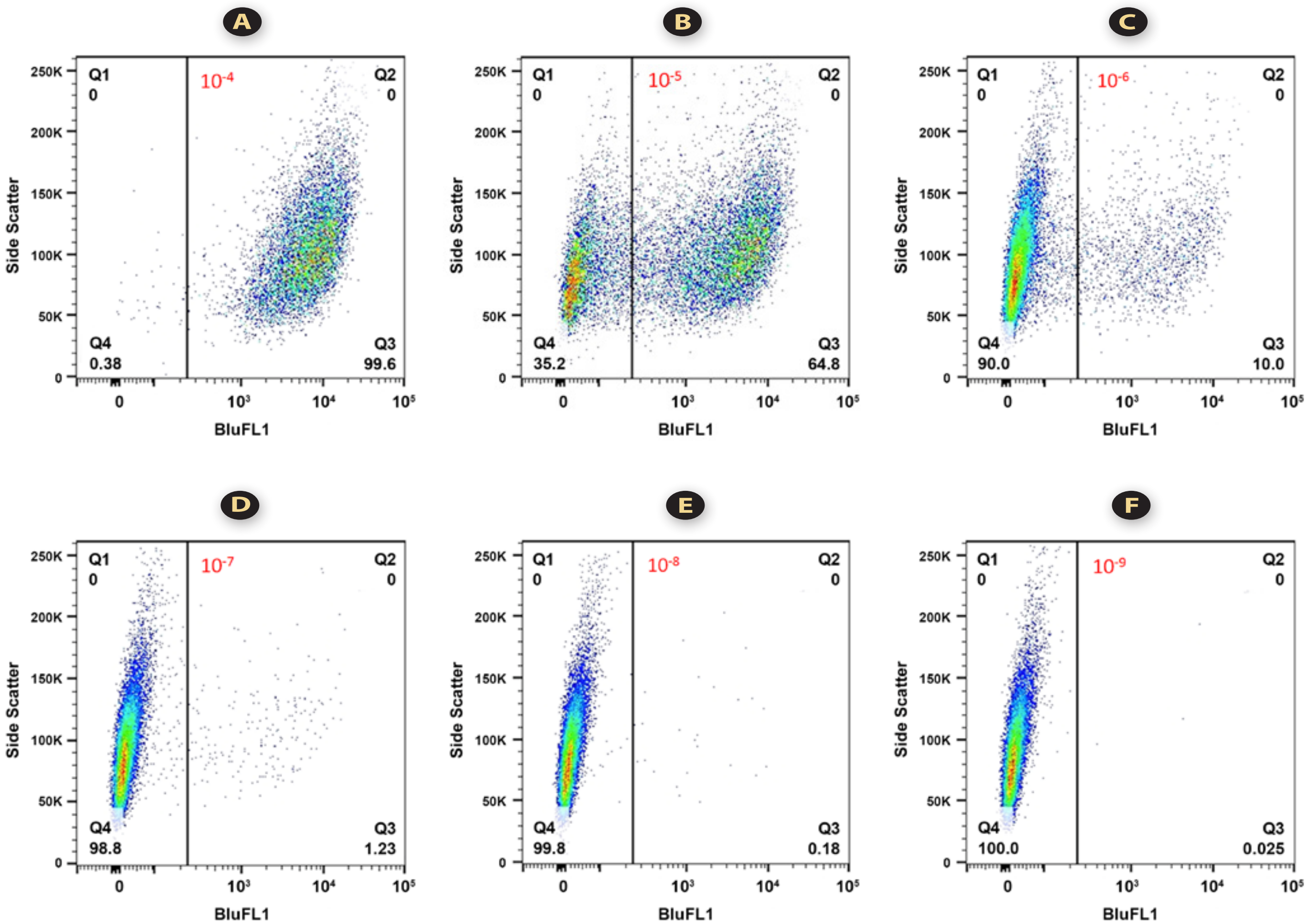


FIGURE 1. Flow cytometry-based titration assay of HSV-1-GFP. CV-1 cells were infected with HSV-1-GFP at an MOI of: **(A)** 10; **(B)** 1; **(C)** 0.1; **(D)** 0.01; **(E)** 0.001; and **(F)** 0.0001. Cells were prepared for flow cytometry and analyzed at 16 hours post-infection. **(G)** GFP-positive cells were quantified and graphed versus MOI.

assays were too numerous to count (TNTC). In contrast, with flow cytometry, each infected cell had the ability to be visualized (**Figure 2**) and thus could be quantified (**Table 1**). At 10^{-8} to 10^{-9} dilutions, the number of viral plaques roughly correlated with the number of GFP-positive cells determined by flow cytometry (**Table 1**). We should note that the flow cytometric gating for single healthy cells may give titer estimates slightly different than a conventional end-point plaque assay (typically lower, as can be seen in **Table 1**, when comparing the one dilution that can be used in the conventional assay determination), but choices for gating can be addressed by any individual user of this approach to suit the characteristics of the virus used. Lastly, the linear range of the flow cytometry-based titration assay was much broader than the plaque assay allowing for determination of viral titer at each viral dilution tested (**Table 1**).



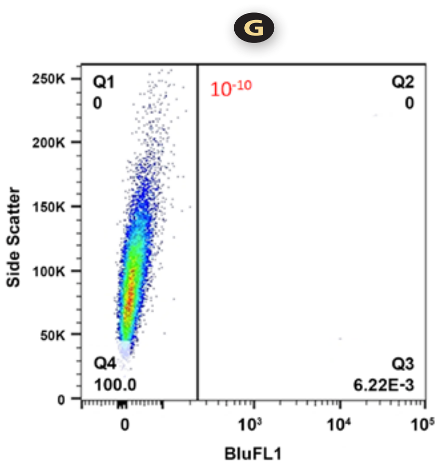


FIGURE 2. The flow cytometry based titration assay has a broader dynamic range than the plaque assay. CV-1 cells were grown in DMEM to 70–90% confluency. The cells were infected with HSV-1-GFP at 10^{-4} to 10^{-10}, washed, incubated for 16 hours, and then analyzed by flow cytometry. The viral dilutions are indicated on the flow charts.

Flow Cytometry-Based Titration Assay Provides Visual Fluorescent Images

An additional advantage to using the HSV-1-GFP labeled virus was that it was possible to directly visualize the cells prepared for flow cytometry by fluorescent microscopy. Cells infected with HSV-1-GFP at the indicated dilutions stained with DAPI were visualized by fluorescence microscopy and showed a similar number of cells in each field (**Figure 3A–D**). Cells infected with HSV-1-GFP could be directly visualized by fluorescence microscopy and demonstrated a dose-dependent GFP signal (**Figure 3E–H**).

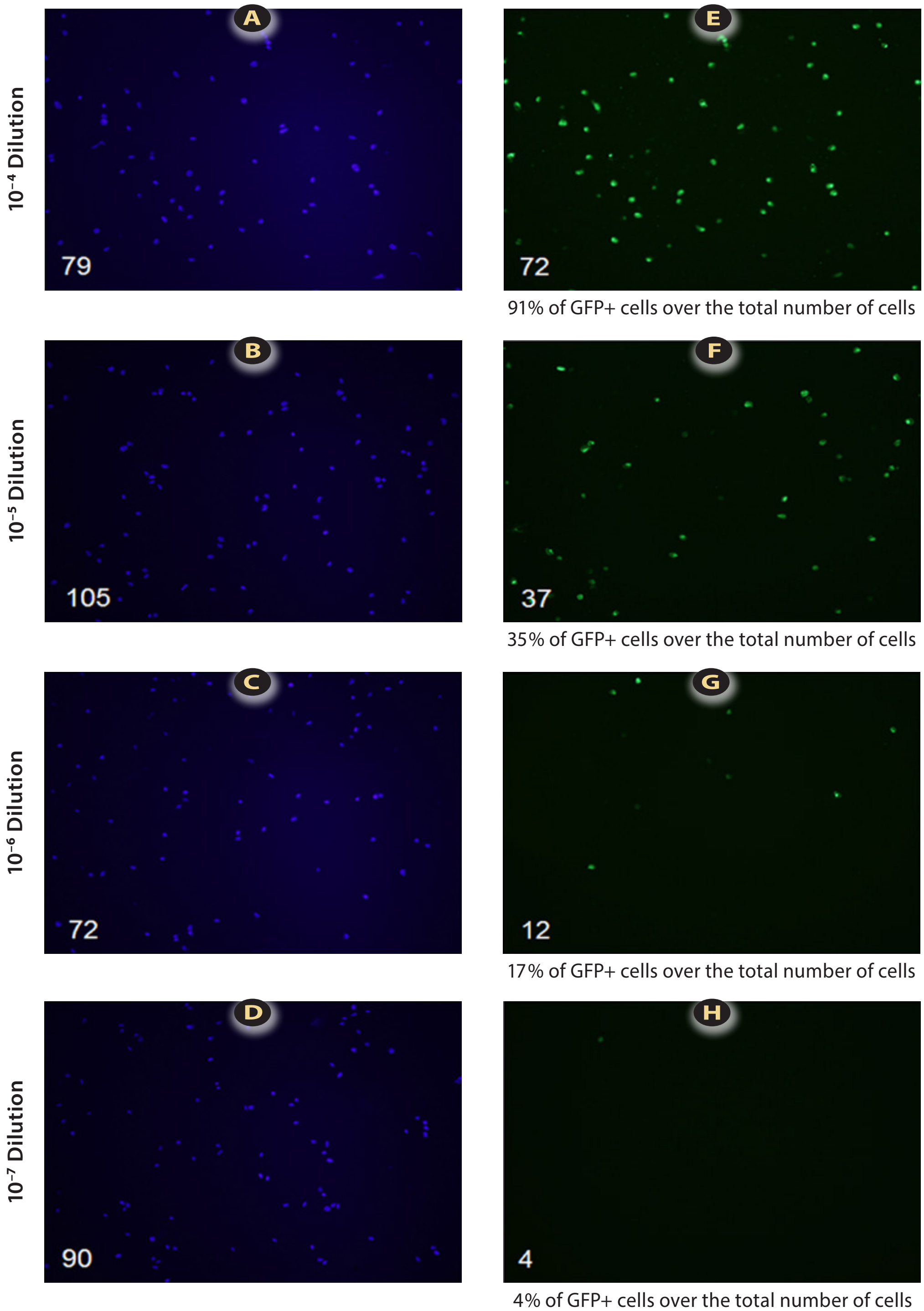


FIGURE 3. The flow cytometry-based titration assay allows for visualization of fluorescent images of HSV-1 infected cells. Approximately 1.4×10^4 cells infected with HSV-1-GFP at 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions prepared for flow cytometry were placed on slides and visualized by fluorescent microscopy. **(A–D)** DAPI stained cells and their amounts; and **(E–H)** GFP-positive cells and their amounts. (Images were photographed at 100× magnification.)

Discussion

Traditional plaque assays for lytic viruses are a time-honored virological technique for quantifying the infectivity of a viral preparation and efficacy of anti-viral interventions. In the case of HSV-1, a widely studied human pathogen, serial dilutions of the virus stock are added to a near confluent monolayer of CV-1 cells and allowed to incubate for an hour. After the incubation period, the viral inoculum is washed off and the cells are further incubated for three more days in the presence of a neutralizing antibody derived from normal human serum. During this time, the virus infects individual cells and replicates to produce progeny virions which spread to adjacent cells by cell-to-cell contact. After three days, the medium is removed and a solution of methylene blue is added. This solution allows visualization of the cells, and where the cells have been lysed by the virus, the visible plaques can be counted to determine the infectivity of the virus as expressed in PFU/mL. While the plaque assay is a standard technique in the field, it is time-consuming and limited in its sensitivity. In this paper, we have described a flow cytometry-based titration assay that we believe is superior to the traditional plaque assay in that it is: (1) rapid; (2) has a broad dynamic range; and (3) allows for direct quantitation of virally infected cells. Along with direct visualization of the virally infected cells, it is important to note that the flow cytometry assay can be performed 16 hours after infection. Therefore, it is far more rapid than the traditional plaque assay. Assaying at 16 hours post-infection also provides a more precise indication of the initially infected cells as opposed to the plaque assay, which takes 3–4 days to visualize plaques produced by lysis of the neighboring cells. In addition to the technical advantages of this methodology, the ability to track individually infected viral cells may also allow researchers to unravel the basic mechanisms of the early infectious process of herpesviruses. It would also be possible to use this methodology to quantify other viruses including non-lytic viruses that are engineered to carry a GFP, a similar fluorescent tag, or alternatively stained for viral antigens.

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