

Detection of Infective Poliovirus by a Simple, Rapid, and Sensitive Flow Cytometry Method Based on Fluorescence Resonance Energy Transfer Technology[∇]

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The rapid and effective detection of virus infection is critical for clinical management and prevention of disease spread during an outbreak. Several methods have been developed for this purpose, of which classical serological and viral nucleic acid detection are the most common. We describe an alternative approach that utilizes engineered cells expressing fluorescent proteins undergoing fluorescence resonance energy transfer (FRET) upon cleavage by the viral 2A protease (2A^{P^{ro}}) as an indication of infection. Quantification of the infectious-virus titers was resolved by using flow cytometry, and utility was demonstrated for the detection of poliovirus 1 (PV1) infection. Engineered buffalo green monkey kidney (BGMK) cells expressing the cyan fluorescent protein (CFP)-yellow fluorescent protein (YFP) substrate linked by a cleavage recognition site for PV1 2A^{P^{ro}} were infected with different titers of PV1. After incubation at various time points, cells were harvested, washed, and subjected to flow cytometry analysis. The number of infected cells was determined by counting the number of cells with an increased CFP-to-YFP ratio. As early as 5 h postinfection, a significant number of infected cells (3%) was detected by flow cytometry, and cells infected with only 1 PFU were detected after 12 h postinfection. When applied to an environmental water sample spiked with PV1, the flow cytometry-based assay provided a level of sensitivity similar to that of the plaque assay for detecting and quantifying infectious virus particles. This approach, therefore, is more rapid than plaque assays and can be used to detect other viruses that frequently do not form clear plaques on cell cultures.

The rapid and effective detection of infective viruses is critical for clinical disease management and prevention. Current methods to detect and quantify infectious viruses employ mammalian cell cultures that rely on the production of viral cytopathic effects (CPE) or clear plaques. However, cell-based methods are typically slow, and it may take several days or weeks for CPE to appear, depending on the type and concentration of the viruses in the sample. Some viruses such as hepatitis A virus (HAV) do not produce visible CPE without multiple passages in cell culture (3).

A cell-based reporter system using cells expressing a fluorescent protein pair undergoing fluorescence resonance energy transfer (FRET) has been developed for the rapid detection of enteroviral infection (7). FRET is a process wherein energy from an excited donor fluorophore is transferred and excites the acceptor fluorophore, which is located in close proximity (14). In this reporter system, engineered buffalo green monkey kidney (BGMK) cells express a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) linked by a short peptide containing the poliovirus (PV) 2A protease (2A^{P^{ro}}) cleavage site (7). Upon infection, the PV 2A^{P^{ro}} that is produced at an early stage of the virus replication cycle (2) cleaves the linker peptide. A disruption of FRET, as shown by a change in CFP and YFP emission signals (Fig. 1), indicates active viral

replication within the host cells. Even though fluorescent microscopy can be used to monitor the *in vivo* changes in FRET (7), this procedure is tedious, subjective, and not amenable to automation.

Flow cytometry (FC) represents an excellent platform for the rapid, high-throughput detection and quantitation of infected cells from large cell populations while providing statistically significant data. Furthermore, FC can be used to measure FRET signals because of its capacity to detect overlapping signals from individual fluorescent proteins. FC-based methods employing either fluorescent dyes, fluorochrome-labeled antibodies, or fluorescent proteins to analyze virus-infected cells have been used to monitor infection and determine titers of different types and kinds of viruses (1, 4, 6, 8, 10–12, 15, 16). Those methods, however, are time-consuming and labor-intensive, as the procedures included cell fixation, permeabilization, labeling, and washing steps prior to flow cytometry. Here we demonstrated the use of FC for the detection of PV infection using the FRET-based reporter system. Poliovirus (family *Picornaviridae*) was used as the model virus because of the simplicity and ease of its cell culturing (17). Our FC-based detection of virus-infected cells provides fast and quantitative recording of fluorescent signals from individual cells.

MATERIALS AND METHODS

Cell lines and virus. Buffalo green monkey kidney (BGMK) cells were cultured in Eagle's minimal essential medium (MEM) with Earle's salts (Irvine Scientific, Santa Ana, CA) containing 0.075% NaHCO₃, 2 mM L-glutamine (HyClone, Logan, UT), 10 mM nonessential amino acids (NEAA; Gibco BRL, Grand Island, NY), 100 mg/ml penicillin, 100 U/ml streptomycin (HyClone), and 8% (vol/vol) fetal bovine serum (FBS; HyClone) and buffered with 20 mM

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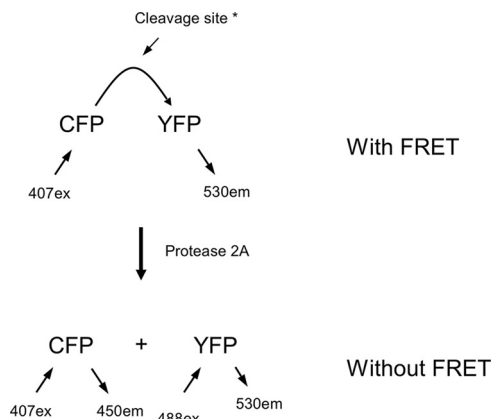


FIG. 1. Principle of FRET. ex, excitation wavelength; em, emission wavelength (in nanometers).

HEPES (pH 7.4). Cells were grown to a confluent monolayer at 37°C with 5% CO₂ in a humidifying incubator. Engineered BGMK cells expressing the fluorescent protein pair (designated BGM-PV) (7) were cultured in the same medium but supplemented with 100 µg/ml G418 (Invitrogen, Carlsbad, CA). Poliovirus 1 (PV1) (strain LSc; ATCC VR-59) was propagated in parental BGMK cells for 3 to 5 days at 37°C. Virus stock was prepared by the freeze-thaw method followed by chloroform extraction (5) and kept in 200-µl aliquots at -20°C until use.

FC and plaque assays. BGM-PV cells were grown in a 6-well plate (Corning, Lowell, MA) until the cells were approximately 90 to 95% confluent. The cell monolayer was washed by using 1× phosphate-buffered saline (PBS) and infected with different concentrations of PV1. After 20 min of adsorption at 37°C, the virus solution was aspirated to remove the unbound virus particles. For FC assays, the virus solution was replaced with 1 ml of maintenance medium (MEM with 2% FBS), and the infected cells were incubated at 37°C for 2 to 24 h. After incubation, the medium was aspirated and replaced with 0.1% trypsin in 0.05% EDTA (Gibco) to detach the cells. A solution containing 10% FBS in 1× Tris-buffered saline (TBS) was added to the detached cells to neutralize trypsin. Cells were harvested by using low-speed centrifugation (1,500 rpm) and resuspended in 1× TBS plus 3 mM EDTA (pH 8.0) to prevent clumping. Cells were sieved by using a 35-µm nylon mesh cell strainer (BD Biosciences, Bedford, MA) prior to flow cytometry analysis. Cells were analyzed by use of a BD FACSAria cell-sorting system using the 407-nm UV laser and the 488-nm argon laser tuned for CFP and YFP excitations, respectively. Emission filters used for detection were 450/40 nm (CFP) and 530/30 nm (YFP). Data acquisition (10⁵ events per sample) and analysis were performed by using BD FACSDiva software.

For the plaque assay, 1 ml of 1% (wt/vol) carboxymethylcellulose (CMC; Sigma-Aldrich) in maintenance medium (with 2% FBS) was overlaid onto the infected-cell monolayer. After 48 h of incubation at 37°C, plaques were visualized by staining with 0.8% (wt/vol) crystal violet (Sigma-Aldrich) in 3.7% (vol/vol) formaldehyde solution.

Analysis of environmental water samples. The performance of the FC-based assay was evaluated by using an urban water runoff sample collected from Sacramento, CA. The water sample was filtered by using a 0.20-µm nylon filter and spiked with PV1 at a concentration of 0, 2, 5, 10, 25, 50, 100, 250, 500, or 1,000 PFU per 1-ml sample and then diluted 1:1 with PBS (pH 7.5). For constructing a calibration curve, PBS buffer was spiked with PV1 using the same viral titer. A 1-ml sample from each virus preparation was used to infect the reporter cell monolayer on each well of a 12-well plate and then subjected to an FC-based assay at 12 h postinfection (hpi) and a plaque assay at 48 hpi. A calibration curve was constructed by plotting the number of infected cells determined by the FC-based assay against the PFU values from the plaque assay and was used to compare the results of the water sample analysis.

The sensitivity of the reporter cells to potential inhibitors present in the water sample was determined by a standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (13).

RESULTS

Detection of PV1 infection by flow cytometry. Experiments were conducted to evaluate if the FC assay is a sensitive

method for the detection of PV1 infection and to determine if the assay was able to detect quantitative differences in the numbers of infected cells in the sample using the FRET-based cellular reporter system for PV 2A^{pro} activity. BGM-PV cells were infected with 10-fold serial dilutions of PV1, with the lowest dilution having a multiplicity of infection (MOI) of 0.4. The quantification of infected cells was accomplished by FC after 8 hpi. Cells were gated based on their size and granularity to include only intact cells. Figure 2 shows representative data plots generated from flow cytometry analysis. After 8 hpi, 8.6% of the cells (counted from 30,000 events) infected with the highest viral titer (MOI = 0.4) (Fig. 2A) showed a decrease in YFP intensity (indicating a disruption of FRET), while less than 0.02% of the uninfected cells were positive (Fig. 2D). Also, the numbers of cells with a disruption in FRET decreased as the amounts of infective viruses were reduced (Fig. 2A to C), suggesting that there is a clear correlation between the infective virus dosages and the number of infected cells. This further suggests that FC can distinguish infected cells from noninfected cells based on the change in the CFP/YFP intensity brought about by the disruption of FRET resulting from viral protease activity.

Kinetics of poliovirus cell infection. The time course of PV1 infection was determined to identify the optimum incubation time for virus detection. BGM-PV cells were infected with PV1 at a lower MOI of 0.2, and cells were collected at 0, 2, 5, 8, 12, 16, and 24 h postinfection (hpi). Infection of cells with a lower MOI provided more cells for the onset of the secondary infection. Infected cells were detected as early as 5 hpi (3% positive) and continued to increase until 8 hpi (4% positive). A second round of infection from matured virions released during cell lysis was observed between 8 and 12 hpi, and the number of infected cells continued to increase over time (Fig. 3). This result demonstrated that PV 2A^{pro} activity was produced and active as early as 5 hpi. Progeny virions, on the other hand, were first released around 8 hpi, and these particles initiated the second round of infection. Thus, 8 hpi is the optimal time that is late enough for the infective virus to produce the viral protease before the release and cell-to-cell spread of the new virus. Assuming highly efficient binding and replication, the number of infected cells at this time point would be a direct measure of the number of infectious virus particles in an inoculum. Increasing numbers of infected cells were detected as the infection progressed, indicating that the FC-based assay may be suitable for monitoring the kinetics of infection.

Comparison of FC-based assay to plaque assay. To determine the sensitivity of the FC-based assay, we directly compared this assay with the plaque assay method for the same dilution or preparation of PV1. Initially, cells were harvested and subjected to flow cytometry analysis after 8 hpi, before the onset of secondary infection. However, at this time point, infected cells were detected only from cells infected with >50 PFU (Fig. 4, inset). A linear relationship ($R^2 = 0.98$) was observed between the FC-based assay and the plaque assay (Fig. 4), indicating that the two assays provide similar measurements of infectious virus in the sample.

The ability of the method to detect a single infectious virus particle is crucial, taking into consideration the low infectious doses of many enteroviruses. To determine if the system could

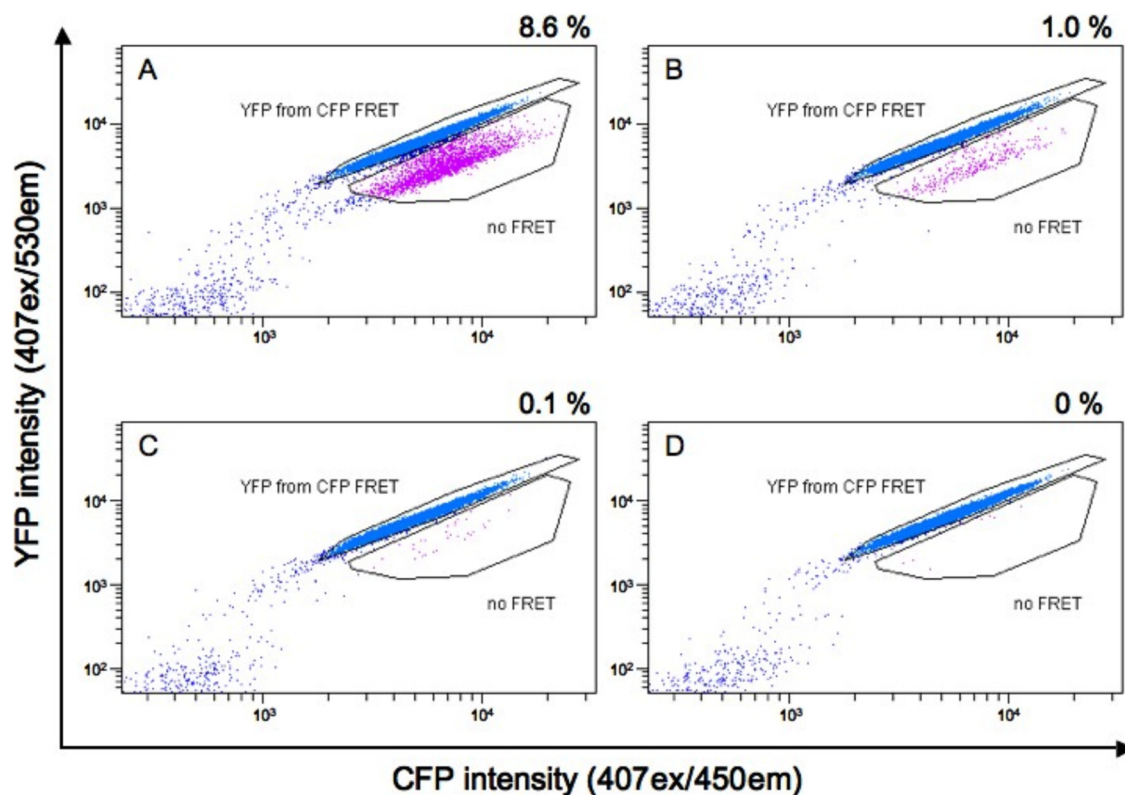


FIG. 2. Poliovirus 1 (PV1) infection of BGM-PV cells. Confluent monolayers of BGM-PV cells (ca. 1.6×10^6 cells) in 6-well plates were infected with 10-fold dilutions of PV1. After 8 h postinfection (hpi), cells were trypsinized, washed with 10% FBS followed by $1 \times$ TBS plus 3 mM EDTA (pH 8.0), resuspended in the latter, and then subjected to flow cytometry. *x* axis, CFP intensity; *y* axis, YFP intensity from CFP excitation. Each dot represents a single event (cell), and dots inside each polygon represent cells that were included in the analysis. The percentage of infected cells shown above each graph was determined by counting the number of cells without a FRET signal divided by the total number of cells counted. (A to C) MOI of 0.4 to 0.004; (D) uninfected cells.

detect as few as 1 PFU, the postinfection time was increased to 12, 16, and 24 h prior to flow cytometry analysis. Prolonging the time allowed the cell-to-cell spread of the infectious virus, thus amplifying the signal for detection. As early as 12 hpi, positive signals from cells challenged with 1 PFU of PV1 were achieved (data not shown). This time frame is much shorter

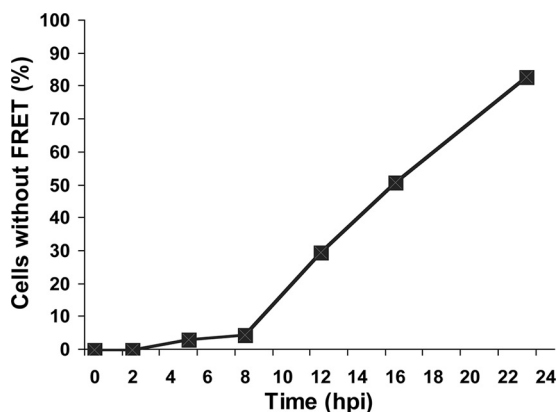


FIG. 3. Time course of PV1 infection of BGM-PV cells at an MOI of 0.2. Cells were grown and infected as described in the text and were harvested at different time points. Those cells that lost FRET due to the uncoupling of the CFP-YFP pair brought about by PV1 infection were counted using FC.

than the 48-h incubation period for the traditional plaque assay and was used in the succeeding experiments. More importantly, these results conclude that the detection of a very low virus titer (i.e., 1 PFU) using a flow cytometer is possible,

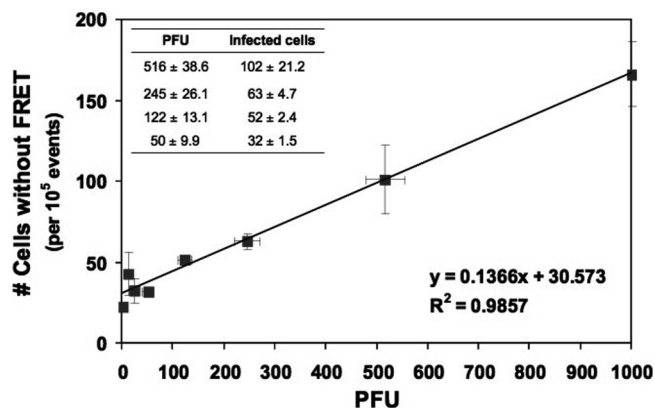


FIG. 4. Direct comparison of FC-based and plaque assays. Confluent monolayers of BGM-PV cells in 12-well plates were infected with PV1 (20 min of adsorption). For the FC-based assay, cells were harvested after 8 h as described in the text and then subjected to flow cytometry. For the plaque assay, infected cells were overlaid with 1% CMC in MEM (plus 2% FBS), incubated for 48 h, and then fixed/stained with 0.8% crystal violet in a 3.7% formaldehyde solution.

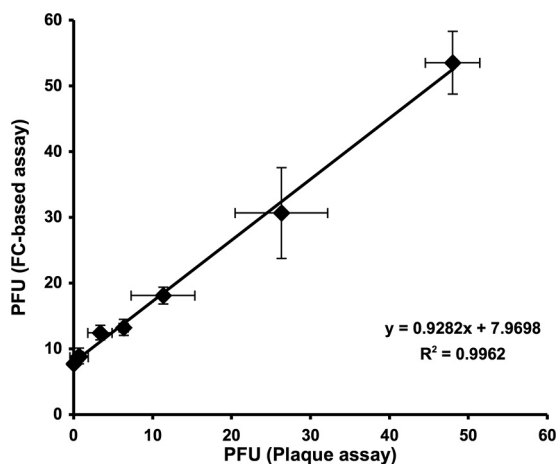


FIG. 5. Comparison of FC-based assay and plaque assay for detecting poliovirus from an urban water runoff sample. The correlation was established by challenging the reporter cells with 2-fold serial dilutions of PV1. The numbers of infected cells were regressed from a calibration curve established in parallel with water analysis and plotted against the numbers of PFU determined by plaque assay. Each data point represents means of 4 replicates.

although detection at this level may no longer be a quantitative measure because the cell-to-cell spread of the virus beyond 8 h decreased the correlation between the initial infective-virus dosage and the number of infective cells.

FC-based assay applied to a surface water sample. To demonstrate the potential of the FC-based assay for environmental applications, a water runoff sample was spiked with known titers of PV1. The sample was diluted with PBS, as the undiluted water runoff sample alone (without PV1) inhibited the growth of the reporter cells as determined by MTT assay and cell toxicity (data not shown). A calibration curve from the FC-based assay and plaque assay was also prepared ($R^2 = 0.979$) by using PBS buffer as a diluent. The average PFU calculated from a calibration curve as determined by the FC-based assay was compared with the results obtained by using the plaque assay. The number of infective PV1 PFU obtained from the two assays exhibited a linear relationship ($R^2 = 0.996$). A Student's *t* test confirmed that there was no significant difference between the two methods tested ($P = 0.12$) at a 95% confidence level. These results suggest that the flow cytometer (FC)-based assay performed as well as the plaque assay (Fig. 5), validating the FC-based assay for quantifying infectious virus particles.

DISCUSSION

In FRET, the excited energy from the donor fluorophore (CFP) is transferred to an acceptor fluorophore (YFP), and that increase in fluorescence intensity of the acceptor is the best indicator of FRET. In our reporter system, a disruption of FRET results in a decrease in the fluorescence intensity of YFP (or increase in the fluorescence intensity of CFP), which indicates active protease activity and viral infection (Fig. 1). By the FRET-based method with flow cytometry, the combined approach provides rapid and automated detection of infectious viruses compared to the conventional plaque assay. Viral titers

determined by FC were comparable to titers obtained by the plaque assay. The FC-based assay using the reporter cells relies on the viral $2A^{pro}$ produced at an early stage (~ 2.5 to 3 hpi) of the viral life cycle (9) and therefore does not require virus assembly or, depending on the virus concentration in the inoculum, cell-to-cell spread. In contrast, to obtain a positive signal with the plaque assay method, virus particles have to enter cells and spread from cell to cell over a period of 2 to 7 days, depending on the specific virus type. The FC-based assay is able to detect virus-infected cells as early as 5 hpi (Fig. 3) and is sensitive enough to detect 1 PFU after 12 hpi. Prolonging the infection time to at least 12 h overcomes the limitation of the FC-based assay compared to the plaque assay in terms of sensitivity.

When applied to an urban water runoff sample spiked with PV1, the FC-based assay provided a level of sensitivity similar to that of the plaque assay for detecting and quantifying infectious-virus particles. Similar to the plaque assay and other cell-based assays, our protease-responsive reporter cells are also susceptible to inhibitors or toxic contaminants present in environmental samples. We observed that dilution prior to analysis reduced the cytotoxic effect of the water sample and ensured that the original composition of the sample was generally preserved. Thus, sample processing prior to analysis must be optimized. Compared to other FC-based methods for virus detection, our FC-based assay method provides a higher speed of detection at a lower cost than serology-based flow cytometry measurements. Since the signal is the direct result of the $2A^{pro}$ activity during viral replication and not from fluorescently labeled antibodies that bind to structural proteins of the virions (10), the reporter system eliminates the need for expensive antibodies, the laborious steps in immunolabeling procedures, and the difficulty in bringing the antibody inside the cells (1, 10).

In the present study, we have shown that FRET combined with flow cytometry can be applied as a fast, sensitive, and efficient detection method for infectious viruses from environmental water samples. A more robust water analysis from several water sources will further validate the applicability of this technology in virus detection in environmental waters. As a single-cell approach, our FC-based assay may also provide a means to rapidly detect or quantify virus isolates that do not form clear plaques on cell monolayers. By altering the protease cleavage site, the infection-based detection method can be tailored to respond either exclusively to a particular viral infection or to all human enteroviruses by using a conserved linker sequence that is recognized by the $2A^{pro}$ of majority of enteroviruses.

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