A New Way to Monitor Virus-Mediated Cytopathogenicity

Introduction

One of the most important procedures in virology is the measurement of viral cytopathic effects (CPE). Providing a direct readout of the number/ concentration of infectious viral particles in a sample, the plaque assay has long been the gold standard for quantifying CPEs. In this technique a confluent monolayer of host cells is infected with varying dilutions of the virus and is then overlaid with a semi-solid material such as dilute agarose gel. When an infected cell lyses, the overlay material prevents the released virions from diffusing through the medium and infecting distal sites. Progeny virions can, however, gain access to neighboring cells in the immediate vicinity. In this manner subsequent rounds of infection and lysis spread laterally in two dimensions, producing a clear cell-free plaque in the midst of an otherwise confluent lawn of cells. Depending on the host cell type and the virus, accurate recognition and counting of plaques may require staining cells with a dye like crystal violet.

By using serial dilutions of virus, a very low multiplicity of infection (MOI) can be achieved. Under this condition each cell that gets infected will be infected by just one virion. After counting the number of plaques in a well, and taking into account the dilution factor used in preparing the virus inoculum, the concentration of virus (titer) in the original sample can be calculated. Titers are usually reported as the number of plaque forming units (PFUs) per unit of volume.

Depending on the virus and host cells being studied, viral plaque formation can take anywhere from days to weeks to be detectable. A single end-point plaque assay provides no information about the onset of CPE or the kinetics of virus-mediated cytotoxicity. Moreover, different cell types and cell densities, as well as viral strains, serotypes, and mutations can cause plaque formation rates and sizes to vary dramatically. Thus, suboptimal selection of a single assay end-point can result in inaccurate calculation of viral titer and lytic activity. In addition, the definition and manual counting of plaques by visual inspection can be highly subjective, giving rise to substantial variability.

Recent peer-reviewed studies of oncolytic viruses (Dyer et al., 2017 and Fajardo et al., 2017) and cancer vaccines (Cross et al., 2015 and Pham et al., 2014) have demonstrated that the xCELLigence® Real-Time Cell Analysis (RTCA) system is a powerful tool for evaluating both virus concentration and cytotoxicity kinetics using a simple workflow with fast read-out and high reproducibility. Using microelectronic biosensors embedded in the bottom of microtiter plate wells, the RTCA assay allows dynamic, real-time, label-free, and non-invasive analysis of cellular events such as virus-mediated cytolysis. Because an agarose overlay is not used in the RTCA procedure, the progeny viruses released from a lysed cell are free to diffuse through the media and infect distant target cells. This unhindered spread of virus throughout the entire well gives rise to the rapid lysis of all cells, providing a quantification of viral titer much more quickly than a plaque assay.

Impedance measurements by the xCELLigence instrument are automatically recorded, at a user-defined frequency, and are plotted by the xCELLigence software using the dimensionless parameter known as Cell Index (CI).

Key Benefits of the xCELLigence RTCA systems for monitoring virus-mediated cytopathogenicity:

- Label Free: No dyes required.
- Fast: Read an entire 96-weel plate in <10 seconds.
- Real-Time: Quantitative monitoring of both fast (hours) and slow (days to weeks) CPE.
- Easy Work Flow: No gel pouring. Requires only the addition of virus to host cells.
- Accurate, Precise and Highly Reproducible.
- Automatic Data Plotting: The Intuitive xCELLigence Software enables facile data display and objective analysis, precluding the subjective data vetting that is common to plaque assays.

This application note describes the experimental setup for assessing vesicular stomatitis virus (VSV)-mediated cytotoxicity of Vero E6 cells and HEK 293 cells using an xCELLigence instrument. The protocol show herein allows the identification of cell proliferation kinetics as well as the optimal time point for viral infection with different cell seeding densities. The assay overcomes many of the limitations of single point plaque assays, and provides direct evidence that RTCA can provide a comprehensive and reliable evaluation of viral cytopathogenicity.

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Materials and Methods

Cells. Cells were cultured in a standard humidified incubator at 37°C with 5% CO_2 saturation. Vero E6, obtained from the ATCC, is an African green monkey kidney-derived cell line with deficiency of the type I interferon genes. HEK 293, obtained from Microbix Biosystems, is a human embryonic kidney cell line with intact interferon system. Both adherent cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, 2 mM glutamine and 1% penicillin/streptomycin.

Virus. The vesicular stomatitis virus (VSV), Serotype Indiana, was grown and titrated on Vero E6 cells at 37°C with 5% CO_2.

Cell Proliferation Assays. For real-time cell analysis, 100 µl of growth media was added to each well of the E-Plate 96 to obtain background readings. For each cell type, a sequential 1:1 dilution series with 7 different cell numbers ranging from 50,000 to 781 cells/well were resuspended in 100 µl of media and then seeded into the E-Plate 96. The E-Plates 96 containing cells were incubated for 30 minutes at room temperature, and placed on the RTCA SP Station located in the cell culture incubator. Cell attachment, spreading, and proliferation were monitored every 30 minutes using the RTCA SP Instrument. Measured impedance recordings from cells in each individual well on the E-Plate 96 were automatically converted to Cell Index (CI) values by the RTCA Software.

Assessment of virus-mediated cytopathogenicity. For viral studies, 25,000 cells/well and 12,500 cells/ well of each cell line were seeded into each well of an E-Plate 96. After 20.5 hours (Vero E6 cells) and 68.5 hours (HEK 293 cells), when the cells had reached either confluency (25,000 cells/well) or were still in the growth phase (12,500 cells/well), cells were infected with the vesicular stomatitis virus (VSV). The E-Plate 96 was removed from the RTCA SP Station, and 800,000 ("high MOI") or 80,000 ("low MOI") PFU VSV, resuspended in 10 µl growth media, were added to the wells. As the control, eight wells were mock-infected by adding 10 µl growth media only. The E-Plate 96 was then placed back immediately into the RTCA SP Station in the incubator, and the CI values were measured every 15 minutes for up to 190 hours.

Results and Discussion

Dymanic Monitoring of Cell Proliferation

To identify the optimal time point for viral infection, a cell proliferation analysis was performed with Vero E6 and HEK 293 cells. Suitable time points for virus infection were defined at 20.5 hours for Vero E6 cells and 68.5 hours for HEK 293 cells **(Figure 1 A & B)**. At these time points, cells were either in the growth phase when 12,500 cells had been used for seeding or in the early stationary phase when 25,000 cells had been used for seeding. Therefore, viral cytopathogenicity was monitored in either the growth phase or the early stationary phase.

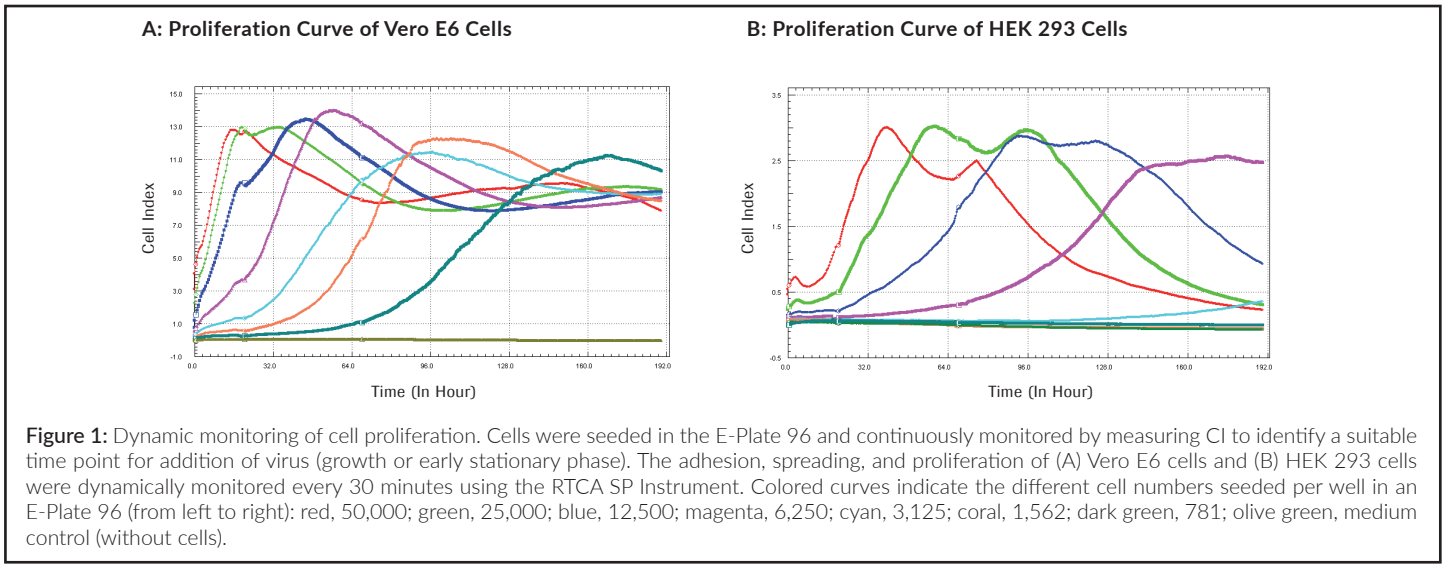

Figure 1: Dynamic monitoring of cell proliferation. Cells were seeded in the E-Plate 96 and continuously monitored by measuring CI to identify a suitable time point for addition of virus (growth or early stationary phase). The adhesion, spreading, and proliferation of (A) Vero E6 cells and (B) HEK 293 cells were dynamically monitored every 30 minutes using the RTCA SP Instrument. Colored curves indicate the different cell numbers seeded per well in an E-Plate 96 (from left to right): red, 50,000; green, 25,000; blue, 12,500; magenta, 6,250; cyan, 3,125; coral, 1,562; dark green, 781; olive green, medium control (without cells).

VSV Cytopathogenicity profile using Vero E6 cells

Based on the dynamic monitoring of cell prolifera- tion, at 20.5 hours after seeding, Vero E6 cells either in growth phase or in early stationary phase were infected with VSV using two different MOIs.

A: VSV Cytopathogenicity profile on Vero E6 Cells (12,500 Cells/well)

B: VSV Cytopathogenicity profile on Vero E6 Cells (25,000 Cells/well)

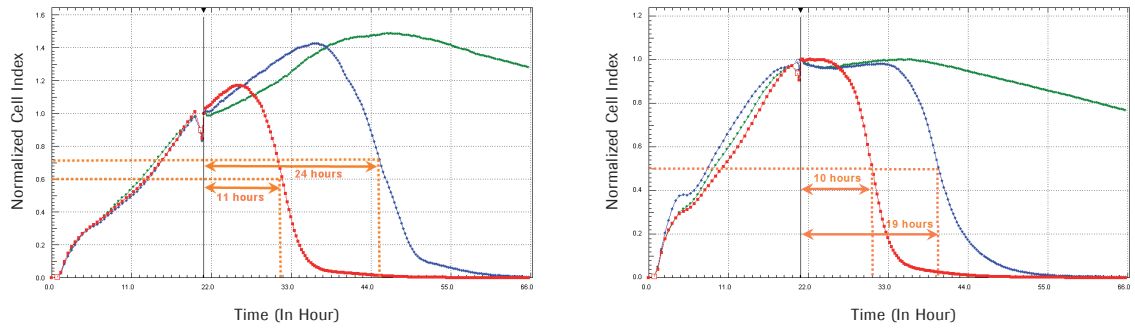

Figure 2: Dynamic monitoring of Vero E6 cells during VSV infection. (A) Normalized Cell Index values of growing cells, (B) Normalized Cell Index values of confluent cells. The virus-mediated effect on adhesion, spreading, and proliferation of the cells was monitored by measuring cell impedance every 15 minutes using the RTCA SP Instrument. Time of addition of virus at 20.5 hours is indicated by the black vertical line. The time point when the Cell Index (CI) value had decreased to 50% of the maximum (CI50) value is indicated by the dotted orange lines. Green curves: control (no viral infection); blue curves: 80,000 PFU VSV; red curves: 800,000 PFU VSV.

When Vero E6 cells were infected with VSV during the growth phase, there was a clear correlation between the amount of virus used for infection and the onset of the virus-mediated cytopathic effect **(Figure 2)**. After infection with a low MOI (80,000 PFU VSV), the cells continued to grow for 15 hours **(Figure 2A, blue curve)** similar to mock-infected cells **(Figure 2A, green curve)**. Thereafter, CI values decreased, indicating that the cells were dying as a consequence of VSV replication. In contrast, mock-infected cells continued to grow. At 24 hours after infection, the CI values had decreased to 50% of the maximum value (CI50) and then continued to decline to zero, indicating complete cell death in the infected culture. In contrast, the CI of Vero E6 cells infected with a high MOI (800,000 PFU VSV) started to decline already at 4 hours post infection **(Figure 2A, red curve)**, and the CI50 was already reached after 11 hours.

Very similar results were obtained when confluent Vero E6 cells were infected **(Figure 2B)**. The CI50 was reached at 10 hours post infection **(high MOI, see Figure 2B, red curve)**, and 19 hours post infection **(low MOI, see Figure 2B, blue curve)** respectively. Again, complete death of the infected cultures was observed, as indicated by the decrease of CI values to zero.

VSV Cytopathogenicity profile using HEK 293 Cells

Based on the dynamic monitoring of cell proliferation, HEK 293 cells in the growth phase and in early stationary phase were infected with VSV 68.5 hours after seeding **(Figure 3 A & B)**.

A: VSV Cytopathogenicity profile on HEK 293 Cells (12,500 Cells/well)

B: VSV Cytopathogenicity profile on HEK 293 Cells (25,000 Cells/well)

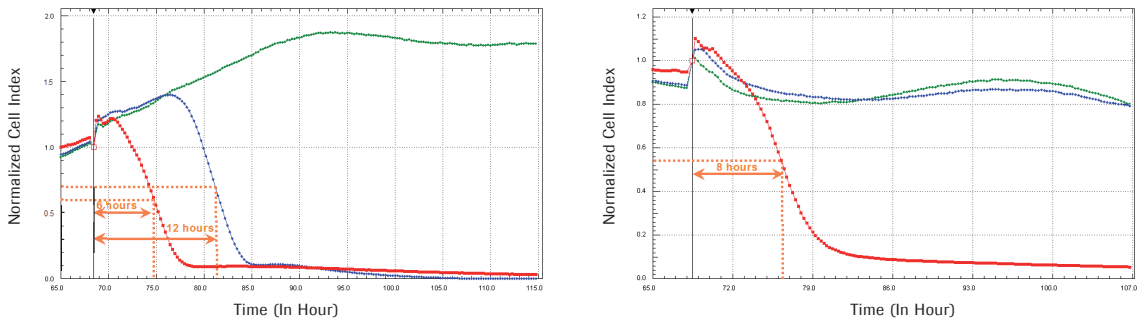

Figure 3: Dynamic monitoring of HEK 293 cells during viral infection. **(A)** Normalized Cell Index values of growing cells, **(B)** Normalized Cell Index values of confluent cells. The virus-mediated effect on adhesion, spreading, and proliferation of the cells was dynamically monitored every 15 minutes using the RTCA SP Instrument. Time of addition of virus at 68.5 hours is indicated by the black vertical line, the time point when the Cell Index had decreased to 50% of the maximum value (CI50) is indicated by the dotted orange lines. Green curves show the control (no viral infection); blue curves: 80,000 PFU VSV; red curves: 800,000 PFU VSV.

HEK 293 cells showed a different response compared to Vero E6 cells when infected with VSV. HEK 293 cells in the growing phase were much more sensitive to VSV infection, as indicated by the drop in CI values to the CI50 value by 6 hours post infection when a high MOI was used **(Figure 3A, red curve)**. Cells infected with a low MOI reached the CI50 by 12 hours post infection **(Figure 3A, blue curve)**. Interestingly, a completely different result was obtained when confluent HEK 293 cells were infected (see Figure 3B). While confluent cells infected with a high MOI exhibited a drop in CI values similar to growing cells **(Figure 3B, red curve)**, cells infected with a low MOI appear to be completely resistant to VSV infection, exhibiting CI values virtually identical to mock-infected cells **(Figure 3B, blue and green curves)**. With respect to the different responses to the VSVinfection, the main difference between Vero E6 and HEK 293 cells is the ability of producing type I interferons. Vero E6 cells are devoid of the interferon genes (Emeny and Morgan 1979).

As a consequence, they cannot upregulate the expression of interferon-induced antiviral active proteins such as MxA and OAS/RNaseL in response to viral infections.

In contrast, HEK 293 cells possess an intact interferon system. Upon viral infection, they produce interferons which activate the JAK/STAT signaling pathway in an autocrine and paracrine manner. As a consequence, the expression of antiviral active proteins is initiated and an antiviral state is established. It is tempting to speculate that the observed resistance of confluent HEK 293 cells to VSV infection with a low MOI is due to the antiviral response mounted by their interferon system.

Additionally, confluent cells may represent a suboptimal environment for VSV replication, because it is well known that confluent cells have reduced metabolic activity compared to growing cells. In line with this hypothesis is the observation that growing HEK 293 cells are much more sensitive to VSV, independent of the MOI used for infection.

On the other hand, it should be noted that the VSV-M protein is known to counteract the interferon system by inhibiting host RNA and protein synthesis which contributes to the shutoff of host-directed gene expression (Ferran and Lucas-Lenard 1997). Therefore, the observed differences in response to VSV infection in growing or confluent Vero E6 and HEK 293 cells and the dependency of the outcome on cell number (and VSV MOI), most likely reflects the interplay of cellular antiviral mechanisms and viral countermeasures.

In contrast to conventional endpoint assays, real-time cell analysis using the xCELLigence System now offers the possibility of continuously monitoring virus-host interactions to better define the responses in which the viral or the cellular activities are more dominant.

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App Note No. 9

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