Application Note

Microbiology

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Monitoring Viral Infection of Mammalian Cells Using Digital Fluorescence Microscopy

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Abstract

Virus quantification involves the determination of the number of viruses in a specific volume. One method to determine a viral titer is the endpoint dilution assay, which determines a 50% tissue culture infective dose ($TCID_{50}$). This method involves infecting tissue culture cells and is useful for viruses that do not form plaques. This application note demonstrates the ability of the Agilent BioTek Cytation 5 cell imaging multimode reader and Agilent BioTek Gen5 microplate reader and imager software to determine infection levels of human immunodeficiency virus (HIV) with HeLa cells in culture.

Introduction

Viral quantitation determines the number of viruses in a specific volume of fluid. Its utility is most apparent in the production of viral vaccines or recombinant proteins that use viral vectors as a means for cellular entry or propagation. There are several different methods of virus quantification; the most notable is the plaque assay, where viral infection causes cell lysis. In this assay, a confluent monolayer of cells is infected with virus, causing the infected cells to lyse. Surrounding cells will then become infected and eventually a larger area of the cell monolayer will have been denuded. These areas, referred to as plaques, can be counted to calculate a plaque forming unit, which is one measure of virus quantity. The focus forming assay (FAA) is related to the plaque assay except that it uses fluorescent immunostaining with labeled antibodies to determine viral infection. This method is useful for viruses that do not lyse cell membranes as part of their life cycle. A modification of the two previously described methods is the endpoint dilution assay, which calculates a 50% tissue culture infective dose ($TCID_{50}$). This quantitates the amount of virus required to kill or infect 50% of the inoculated host cells, and is akin to an LD_{50} or an IC_{50} commonly determined during drug development.

HIV is a retrovirus that causes HIV infection and acquired immunodeficiency syndrome (AIDS).[1] HIV is a lentivirus that infects a number of different cell types and integrates into the cellular genome as part of its growth cycle (Figure 1).[2]

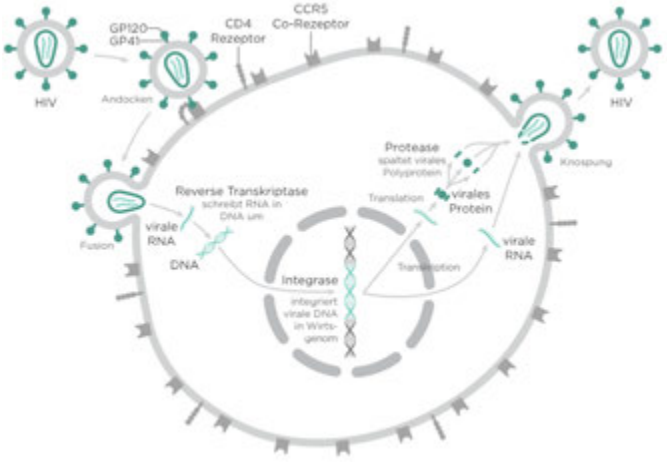

Figure 1. Schematic diagram of HIV lentivirus infection into mammalian cells. Image supplied courtesy of Thomas Splettstoesser.

Materials and methods

Cell culture

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO_2. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency.

Virus infection

For experiments, cells were plated into Corning 3904 black-sided clear-bottom 96-well microplates at 5,000 cells per well. Cells were allowed to grow overnight, with HIV infection the following day. Different wells were infected with 1:2 titrations of either HIV-GFP or HIV-mCherry expressing strains of virus. After 48 hours, the media was aspirated and the cells were fixed with 2% paraformaldehyde (100 µL) overnight.

Staining

After fixation, paraformaldehyde solution was removed by aspiration and wells washed once with PBS. DAPI solution (0.25 µg/mL) was added and cells were stained for 15 minutes at room temperature. DAPI solution was then removed with aspiration and the cells were washed once with PBS. PBS (100 µL) was then added to the wells to maintain hydration during imaging.

Imaging

Experiments were imaged using a Cytation 5 cell imaging multimode reader configured with DAPI, GFP, and Texas Red light cubes. The imager uses LED light sources in conjunction with band pass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cube uses a 337/50 excitation filter and a 447/60 emission filter, the GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter, while the Texas Red light cube uses a 585/29 excitation filter and a 624/40 emission filter.

Image analysis

Typically, samples were imaged by capturing a montage of images and creating a stitched composite image of a wider field of view. Montage (5 × 4) tiles of three-color overlaid 4x objective images were digitally stitched using Gen5 software. Object mask counting of the DAPI channel was used to identify cell nuclei. Subpopulation analysis was used to determine the mean fluorescence intensity of the Texas Red and GFP channels as a means to assess mCherry- and GFP-positive cells, respectively. Cell nuclei object masks were delineated using a threshold of 12,000 with a 5 µm minimum and 50 µm maximum size exclusion. Subpopulation analysis defined GFP- and mCherry-positive wells with mean GFP and TR fluorescence thresholds of 11,000 and 7,000, respectively.

Flow cytometry

HeLa cells infected with the same titrations of GFP-tagged HIV as those used for imaging were fixed and stained then harvested for flow cytometry. The negative control was used to set GFP positive gating. The same gate was then used for all subsequent flow cytometry analysis.

Results and discussion

Viral infection can be demonstrated with fluorescence imaging. As shown in Figure 2, infection of HeLa cells with GFP-tagged virus leads to significant green fluorescence. Likewise, infection of cells with mCherry-tagged virus can also be detected relative to the uninfected negative control (Figure 3).

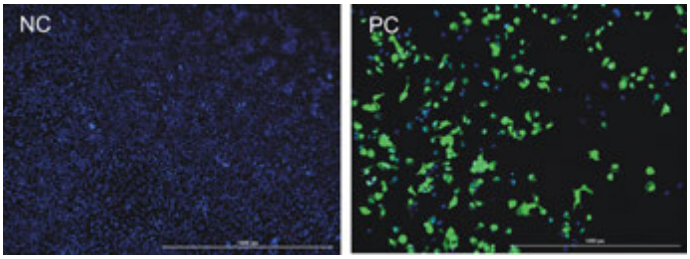

Figure 2. Comparison of HIV-GFP infected and noninfected HeLa cells. Digital 4x in the DAPI, Texas Red, and GFP channels were captured and images overlaid.

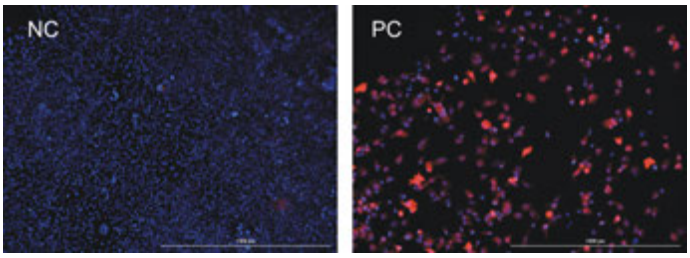

Figure 3. Comparison of HIV-mCherry infected and noninfected HeLa cells. Digital 4x in the DAPI, Texas Red, and GFP channels were captured and images overlaid.

These data also demonstrate the fidelity of signal as both experiments involved three-color imagining. Only cells infected with the GFP-tagged virus exhibited green fluorescence (Figure 2), and only those cells infected with mCherry-tagged virus had signal in the Texas Red channel (Figure 3). The uninfected negative controls for either experiment only had DAPI staining.

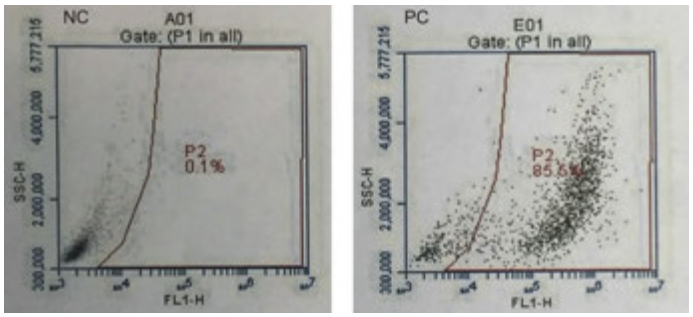

Figure 4. Comparison of flow cytometer scatterplot analysis of HIV-GFP infected and noninfected HeLa cells. The output from flow cytometry of DAPI-stained infected cells were plotted such green fluorescence intensity is plotted on the axis, while blue fluorescence plotted on the abscissa.

The presence of HIV virus has a significant effect on total HeLa cell numbers present at the time of fixation (Figure 5). Cell counts of stained nuclei made at different virus titers show a decrease in the number of stained HeLa cell nuclei with increasing viral load. Image-based analysis of either HIV-GFP or HIV-mCherry infected cells show a decrease from 41,000 cells in the uninfected control to 6,000 cells in the wells with the highest virus concentration. Likewise, flow cytometry cell counts show a similar pattern with the exception of the negative control, which reports a 50% higher cell count. The difference between the two systems is most likely attributed to the close packing of confluent cells, making it difficult for image analysis to discern separate nuclei at very high densities.

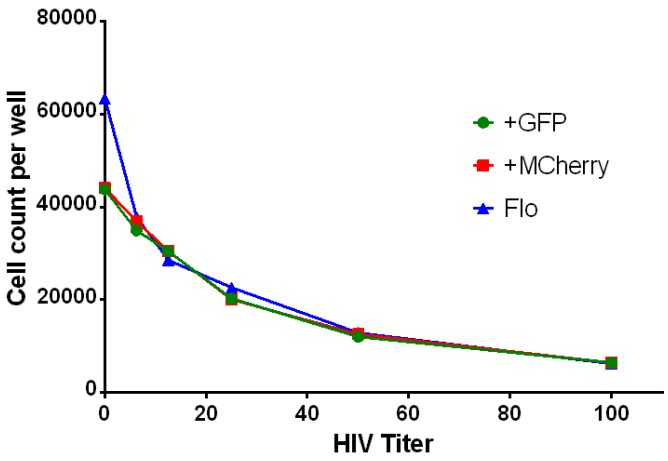

Figure 5. Whole-well cell counts of HIV-infected HeLa cells. DAPI-stained nuclei object masks were counted from HeLa cells infected with either HIV-GFP or HIV-mCherry virus. In each case, full well analysis was performed on montage images that were subsequently stitched using Agilent BioTek Gen5 microplate reader and imager software. Comparison was also made using cell counts from a flow cytometer of trypsinized HIV-GFP infected cells run in parallel.

Using DAPI staining to identify a nuclear mask for image analysis, the extent of infection can be tracked using subpopulation analysis. Objects defined by the primary threshold mask as nuclei can then be subjected to subpopulation analysis based on other fluorescent colors. When the percentages of GFP-positive cells are plotted, an increase in the positive cell count was observed that corresponds with the viral load of HIV-GFP (Figure 6). Cells infected with mCherry-tagged HIV do not show any increase when selected for GFP.

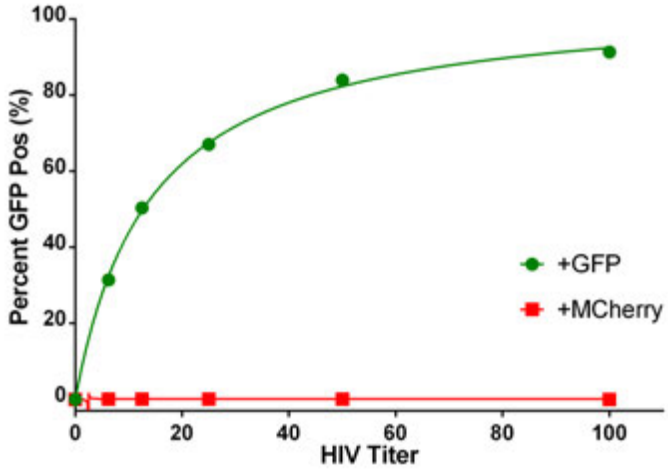

Figure 6. Percent of GFP positive HIV infected HeLa cells. HeLa cells were infected with a range of viral titers with both HIV-GFP and HIV-mCherry virus. Total cell number was determined using a nuclear mask of DAPI-stained nuclei and subpopulation analysis of GFP expressing cells. Data were reported as a percentage of cells positive for GFP.

When the same wells are examined for mCherry, an increase in the positive percentage was seen, which correlates with the virus levels of HIV-mCherry present in the well (Figure 7). Cells exposed only to HIV-GFP do not show any increase in mCherry signal.

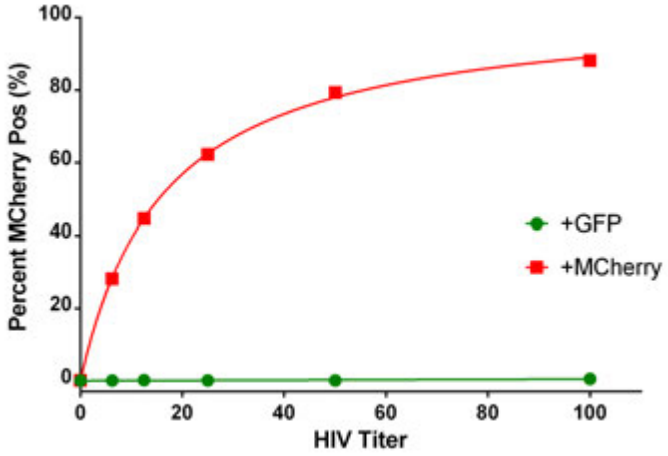

Figure 7. Percent of mCherry-positive HIV infected HeLa cells. HeLa cells were infected with a range of viral titers with both HIV-GFP and HIV-mCherry virus. Total cell number was determined using a nuclear mask of DAPI-stained nuclei and subpopulation analysis of mCherry expressing cells. Data were reported as a percentage of cells positive for mCherry.

A comparison between the calculated percentage of HIV-GFP positive cells using either image analysis or flow cytometry shows very good agreement between the two methods (Figure 8). The $TCID_{50}$ for the virus titration using flow cytometry was 11.95 while image analysis data have a $TCID_{50}$ of 12.68. Similar results would be expected with the mCherry (data not shown).

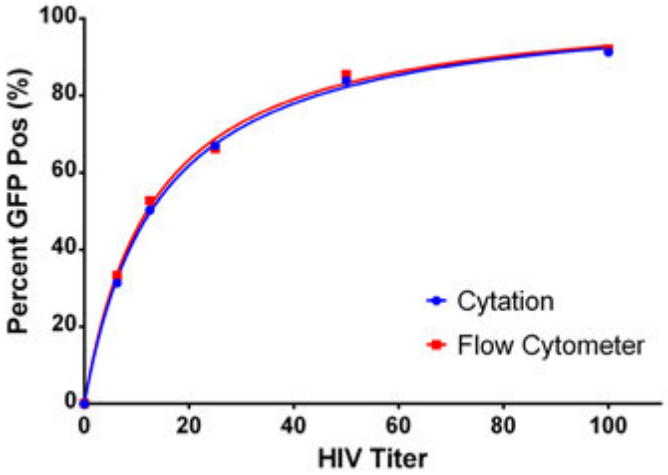

Figure 8. Comparison of flow cytometry and image-based analysis. HeLa cells infected with various titers of HIV-GFP were analyzed for the expression of GFP using either flow cytometry or image-based analysis of digital microscopy. Data were expressed as the percent positive at each viral titer.

Conclusion

These data demonstrate the ability of the Agilent BioTek Cytation 5 cell imaging multimode reader and Agilent BioTek Gen5 microplate reader and imager software to track increases in virus infection when using fluorescently tagged viral particles. Using three-color analyses, it was shown that the signal increase as a result of virus infection is specific to the tag attached to the virus.

In these experiments, whole-well montage images were obtained and the separate tiles stitched into a single data file. This practice eliminates duplicate counting of the same cell in adjacent images. In addition, using stitched montage images of the entire well for analysis provides reliable whole well measurements without the need to extrapolate based on the surface area of the well examined with a single image.

Total cell counting and the percentage of HIV-positive cells were used as a means to compare image-based analysis with flow cytometry. With very high cell densities, the close proximity of cell nuclei makes accurate cell counts difficult, with the result being an undercounting of the true number. However, when the cell density is such that cell are discrete image analysis returns values similar to that seen with flow cytometry. Using subpopulation analysis, the percentage of HIV positive cells was found to be identical with either flow cytometry or image analysis.

References

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