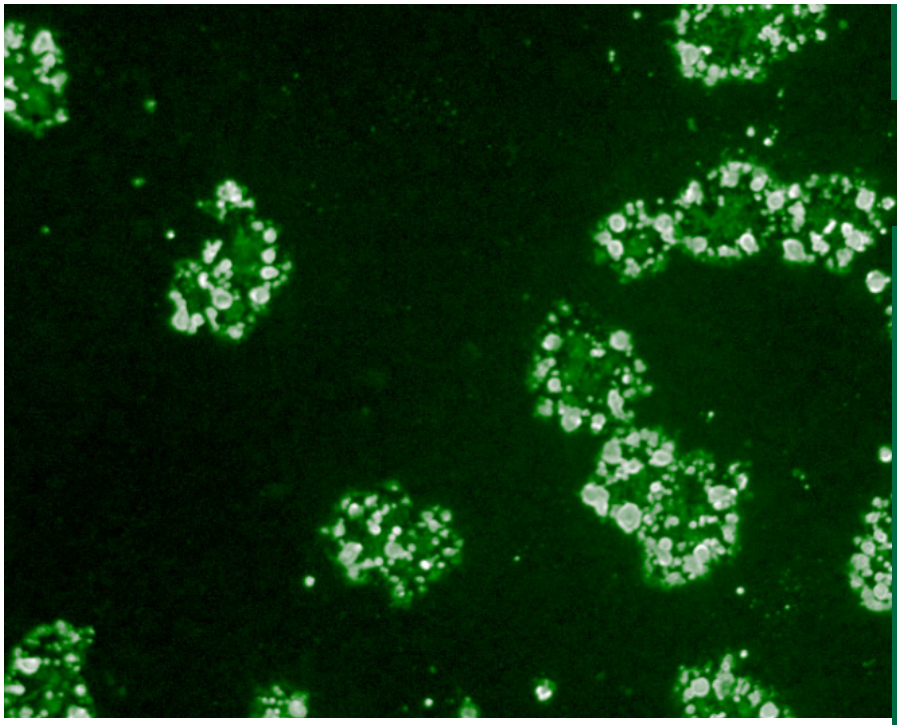

TECHNICAL NOTE

Multimode Detection

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Increased Throughput and Reduced Variability of Virus Plaque Assays with Automated Imaging and Analysis

Introduction

Plaque Assays are a standard method utilized by virologists to determine the concentration of viruses in solution. In contrast to PCR and immunofluorescent methods, which detect only the presence of virus particles, the plaque assay also identifies the amount of infectious virus particles or plaque forming units (PFU).

In plaque assays, a cell monolayer is incubated with a virus solution. If the virus is infectious, the cells usually detach or even lyse so that the monolayer becomes perforated. The infected cells and holes (plaques) are counted and represent the amount of infectious virus particles in solution.

In many laboratories, counting the plaques is performed manually in 6-, 12- or 24-well plates either by visual inspection or using a microscope. This is cumbersome, error prone and very time-consuming. Automated imaging and plaque analysis is rarely used.

In this note we show how the plaque imaging and analysis process can be automated to facilitate virologists in their daily workflow and throughput, using the EnSight™ Multimode Reader and its Kaleido™ Data Acquisition and Analysis Software. Using 96-well microplates further enhances the time saving benefit and qualifies this method for high-throughput screening (HTS) applications. Together with the EnSight's stacker system it is used routinely in compound screening with typically 20-40 plates daily.

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Materials and Methods

Plaque Assay Principle

Adherent cells appropriate for virus growth are seeded in a 96-well microplate (for example: CellCarrier™-96, PerkinElmer) to result in approximately 90% confluency on the day of virus infection. 1:10 serial dilutions of virus are added in culture medium to the wells. The plates are incubated for one hour to allow the virus to infect the cells. After incubation, the virus dilutions are removed from the cell monolayer. 1% agarose is added to the monolayer to prevent further indiscriminate spreading of the virus by diffusion and thus ensures that virus infection is only transmitted between neighboring cells. The cell monolayers are incubated at room temperature until plaques are visible, after 3-10 days.

Plaque Staining

Plaques are stained after removing the agarose cover following standard staining protocols. In this technical note viruses were immuno-stained using specific primary antibodies and Alexa Fluor™ 488 labeled secondary antibodies.

Image Acquisition and Evaluation

For comparison purposes, plaque assays were evaluated manually as well as automatically after three days of incubation, which proved to be optimal for manual counting of this assay. Images were acquired using the EnSight Multimode Plate Reader equipped with imaging module. Manual plaque analysis was performed on these images by counting the plaques by eye. For the automated approach the EnSight system's Data Acquisition and Analysis Software Kaleido was used. The custom assay specific analysis method "Virus Plaque Analysis for Kaleido 2.0" was employed to evaluate the images. With this, the PFUs for each virus dilution were defined, which are utilized to calculate the amount of infectious virus particles in stock solutions.

Assay-specific analysis methods can be easily enabled by selecting a custom analysis file in Kaleido. This file is provided upon request by local PerkinElmer specialists.

Results

Image Evaluation of Lytic and Non-lytic Plaques

After infection, viruses induce structural changes in host cells, also called the cytopathic effect (CPE). These can be morphological alterations such as cell detachment (non-lytic plaques) or even cell lysis leading to holes in the monolayer (lytic plaques) as shown in Figure 1. Both types of plaque can be automatically analyzed by the patent pending approach used in the assay specific analysis method "Virus Plaque Analysis for Kaleido 2.0". This technical note focuses mainly on lytic plaque analysis.

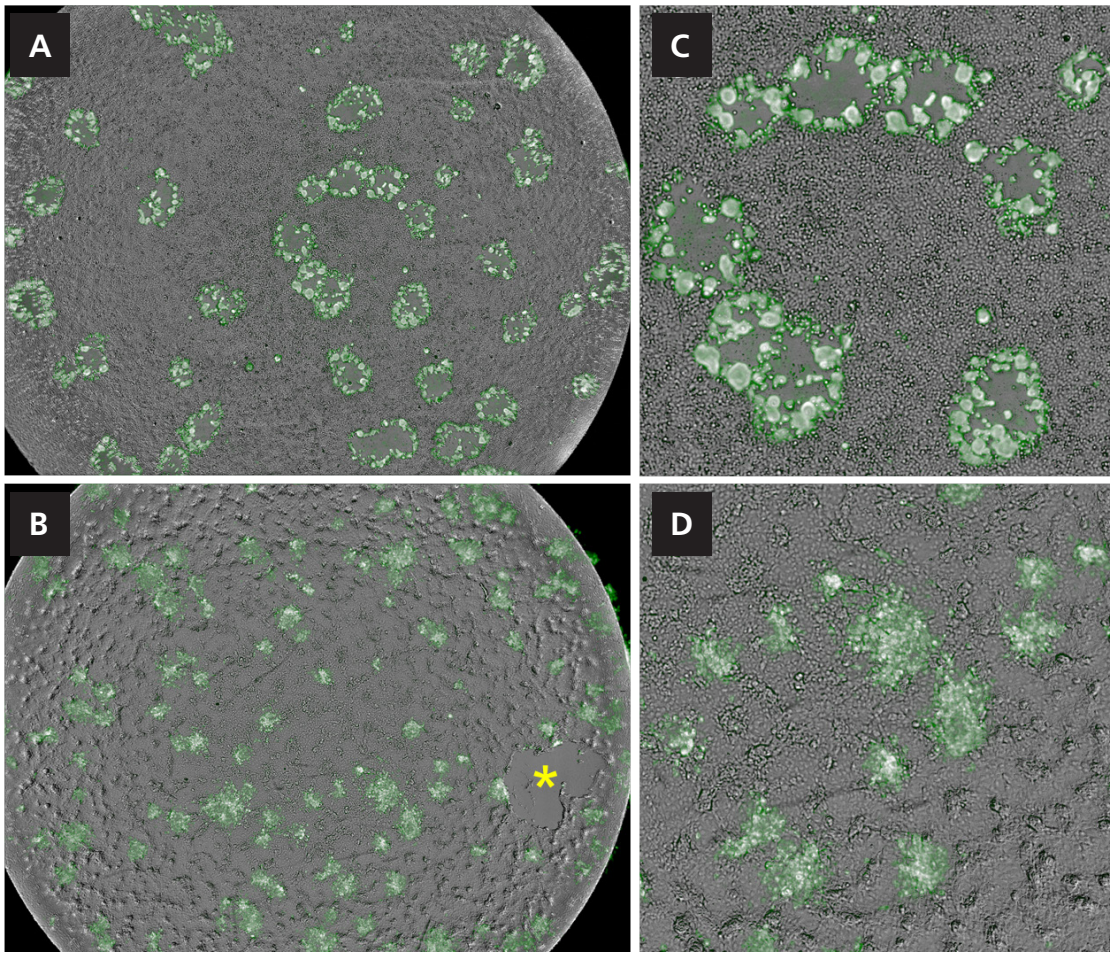


Figure 1. Well overview (left) and detail (right) showing cytopathic effects on the host cell caused by viral infection. The immuno-stained virus is shown by green fluorescence and overlaid with a brightfield image. Lytic plaques (A, C) are formed if the virus causes lysis of the host cell leading to holes in the monolayer. Non-lytic plaques (B, D) are formed if cells are infected and detach from the surface. The yellow asterisk marks a damaged region in the cell monolayer.

For lytic plaque type the image analysis requires a brightfield image, which is used to detect the cell layer. A cell layer in plaque assays can show cell-free centers of lytic plaques, but also damaged regions (see Figure 1B, yellow asterisk). The differentiation between damaged regions and lytic plaques is performed on a virus-specific fluorescence image: infection-specific fluorescence around cell-free regions is used to identify lytic plaques. In addition, non-lytic plaques are detected by searching for fluorescent regions that do not belong to lytic plaques (see Figure 2).

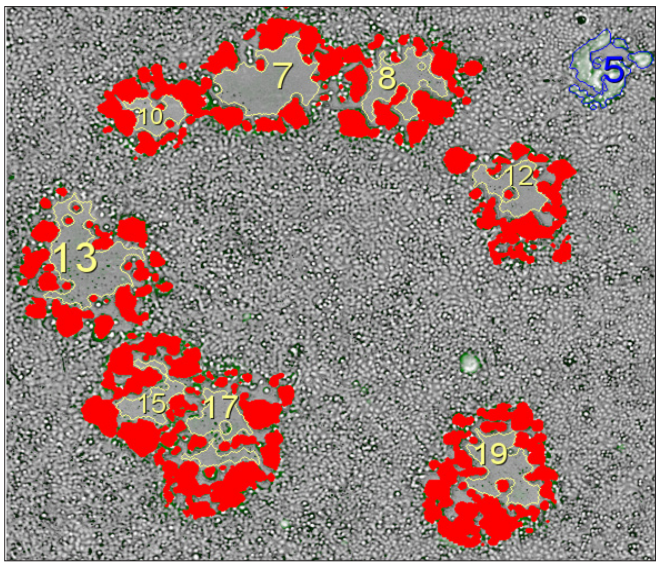


Figure 2. Image analysis mask shown on the brightfield/Alexa Fluor™ 488 overlay image of Figure 1, C. Cell-free regions in the cell layer that are surrounded by virus specific fluorescence (red mask) are defined as lytic-plaques (yellow mask with numbers). Fluorescent regions without any or with a too small cell-free center region are identified as non-lytic plaques (blue mask with number).

The plaque detection analysis method can be fine-tuned by means of several input parameters such as adjustment of texture and signal thresholds or defining minimum and maximum plaque size. This enables precise cell layer and plaque detection under several assay conditions.

The most important information obtained from a plaque assay is the number of plaques. This number is used to determine the concentration of PFUs in stock solution or to verify the impact of compounds on the virulence of viruses. As plaques often fuse at higher concentrations the analysis provides an estimated number of plaques taking also the plaque sizes into account. Of course, the number of actually counted plaques is available as well. Further parameters characterizing the fluorescence signal of infected cells or the background can be used for assay development, quality control or troubleshooting. Cell handling errors are indicated by the parameter "Area Fraction of Invalid Regions [%]" that determines the fraction of damaged regions in the cell layer, which can occur during seeding or pipetting. More parameters can be extracted from the analysis method, but the main output parameters are the ones shown in Table 1.

Table 1. Overview of image requirements and main output parameters of the assay specific analysis method "Virus Plaque Analysis for Kaleido 2.0".

Image Requirements
Brightfield Image for Cell Layer Detection (Optional for non-lytic plaque assays)
Virus Specific Fluorescence Image for Detection of Virus Infected Cell Regions
Main Output Parameters
Estimated Plaque Number (Weighted by Area)
Counted Number of Plaques
Counted Number of Non-lytic Plaques
Median Size of Plaques [µm²]
Fluorescence Signal of Infected Parts of Plaques
Median Fluorescence Signal Outside of the Plaques
Area Fraction of Invalid Regions [%]

Comparison of Automated and Manual Plaque Count

Several experiments were performed to assess the functionality and precision of the image analysis method "Virus Plaque Analysis for Kaleido 2.0" compared to manual plaque counting. The assay plates were imaged on the EnSight multimode reader. The images have been analyzed automatically during the measurement, manual plaque counting has been performed on these images by three persons (Analysts 1-3). A plate overview of Experiment 1 using 1:10 serial virus dilutions is shown in Figure 3.

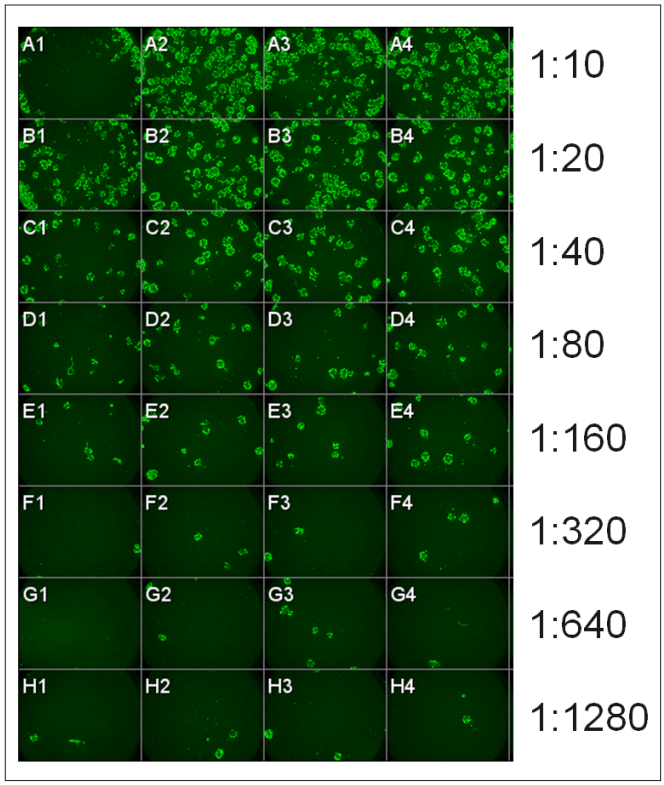
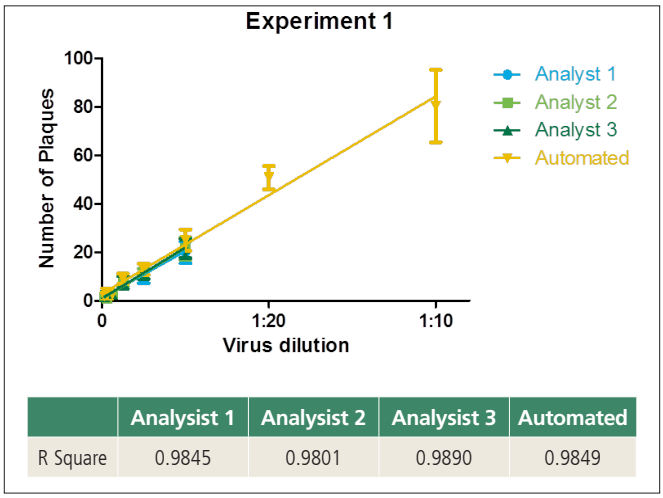


Figure 3. Overview of a plaque assay plate from experiment 1 showing 1:10 serial virus dilutions from top to bottom (A-H) in 4 replicates (1-4). The virus specific Alexa Fluor™ 488 fluorescence is shown in green. Well A1 was excluded as the image analysis reveals a very large "Area Fraction of Invalid Regions", which indicates potential issues in the sample preparation.

The number of counted plaques on the assay plate shown in Figure 3 correlates well with the applied virus dilution (Figure 4). Both automated and manual counting (analyst 1-3) result in very good linear regression and very similar R square values. In contrast, manual counting could not be performed for the two dilution steps 1:10 and 1:20 due to the high plaque density, which leads to merged plaques and makes it difficult to distinguish individual plaques from each other by eye. The automated approach, however, still properly detects individual plaques for both dilutions. The error bar of the lowest, 1:10, dilution is much higher than for other dilutions. Visual inspection of the images and analysis masks revealed that the plaques were counted and estimated correctly, but a varying number of plaques were formed in these wells (see also row A in Figure 3).



	Analysist 1	Analysist 2	Analysist 3	Automated
R Square	0.9845	0.9801	0.9890	0.9849

Figure 4. Linear correlation between virus dilution and number of plaques that were counted either manually by three different analysts or using the automated approach of the EnSight Multimode Reader. For the automated approach the output parameter "Estimated Plaque Number (Weighted by Area)" was used. Error bars indicate the standard deviation of four replicates.

Apart from the two dilutions, 1:10 and 1:20, that were not counted manually, experiment 1 resulted in very consistent plaque counts between all three analysts and the automated approach (Figure 5). However, this was not always the case. As a second experiment in Figure 5 indicates, three different analysts interpret plaques differently: whereas Analyst 1 and Analyst 3 count similar numbers of plaques as the automated approach, Analyst 2 overestimates the number of plaques especially for the 1:160 and 1:620 dilutions. This illustrates that manual counting underlies variations only because each person counts differently and does not define plaques in the same way. In contrast, the automated approach always evaluates plaque characteristics in the same way and is less susceptible to variations from experiment to experiment.

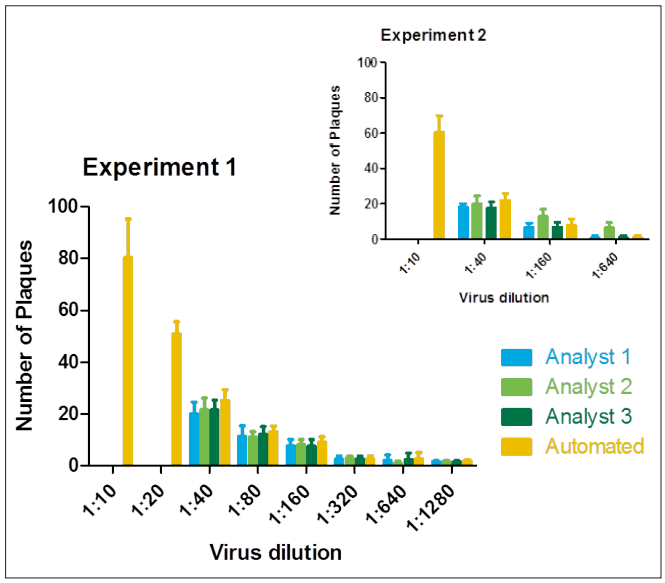


Figure 5. Number of plaques of two independent experiments showing consistent (Experiment 1), but also inconsistent plaque counts between the analysts (Experiment 2, dilution 1:160 and 1:620). For the automated approach the output parameter "Estimated Plaque Number (Weighted by Area)" was used. Error bars indicate the standard deviation of four replicates.

Conclusion

Automated plaque imaging and analysis using the EnSight Multimode Plate Reader enables rapid determination of the concentrations of virus solutions with higher precision and less effort.

- The assay specific analysis method "Virus Plaque Analysis for Kaleido 2.0" detects plaques even at high concentrations, which are difficult to count manually.
- Different CPEs, such as lytic and non-lytic plaques, that are formed by different types of viruses can be detected and analyzed.
- The image analysis method provides many additional output parameters that help with troubleshooting or uncover details of the cell layer that are not usually observed by a person focusing on counting plaques.
- The automated analysis is less error-prone, because it is independent of user specific plaque counts.
- Plaque assays are often performed in six-well plates, which are easier to count manually. However, using 96-well plates and automated analysis increases throughput and saves expensive working time.

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