Dear Editor,

For virologists, it is crucial to confidently determine the concentration of infectious particles that are utilized and produced in experiments (Dulbecco, 1952; Bushar and Sagripanti, 1990; LaBarre and Lowy, 2001; Gueret et al., 2002; Gao et al., 2009; Kutner et al., 2009; Grigorov et al., 2011; Knipe and Howley, 2013). With respect to Herpes Simplex Virus-1 (HSV-1), this is generally accomplished with plaque assays involving an adherent cell line such as Vero cells, which are plated at appropriate confluency on a multi-well plate and allowed to attach overnight. The cells are infected with an unknown concentration of viral inoculum in a serial dilution (often 4 empirically derived dilutions) with statistical replicates (often 3). The inoculum is allowed to bind to the cells for 1 hour and is then removed. The infection is allowed to progress until localized points of cell death or plaques can be observed by the naked eye or with light magnification to determine a macroscopic cytopathic effect. This often takes 24–48 hours for most HSV-1 strains (Hsia et al., 2013; Bedadala et al., 2014; Figliozzi et al., 2014). The entire process generally takes 72 hours and relies on the accuracy of the subjective count. Furthermore, the extensive labor involved in the staining and counting for this multi-well plate format limits the total number of samples that can be reasonably processed to about 8 samples on 4 plates per assay without a dedicated technician or some automation. In addition, the multiple staining and washing steps can cause the monolayer of cells to become damaged, thus rendering the assay a failure.

Here, we introduce an additional method that we believe has merits with respect to reducing labor, decreasing wait times, improving objectivity, and decreasing long-term costs. This new method, called fluorescently labeled infected cell inoculum titration (FLICIT), utilizes high-throughput fluorescent microscopy instrumentation such as Cytation 3 from Biotek, and is therefore only applicable to transgenic viruses that cause host cells to express fluorescent proteins. For the purpose of developing this method, we used the recombinant virus HSV-1 strain 17-Syn+, which expresses green fluorescent protein (GFP) (Figure 1A) and exhibits the same replication pattern as the wild-type counterpart (Foster et al., 1998). This new method relies on titrating the inoculum so that dilution ensures that the rate of infection is less than 50%, with cells infected by nearly one viral particle each; thus, each fluorescent signal can be attributed to a single viral particle.

Early attempts at testing this approach revealed that this one-to-one ratio was difficult to achieve. Upon further testing and observation, it became clear that this phenomenon was best described by a distribution that is very familiar to virologists, the Poisson distribution (PD). The PD models discrete events that occur over a fixed period of time or space, such as the number of infected cells inoculated with a fixed number of viral particles for a fixed time or well size (Knipe and Howley, 2013). We assumed that our observed rate of infection would also follow the PD; accordingly, use of an equation that models the PD could then be used to estimate the number of infectious viral particles in an unknown concentration of inoculum.

Since FLICIT quantifies the proportion of cells that are infected by the inoculum, and the PD in its standard form solves for the proportion, the PD must be rearranged to solve for its variable $m$, in order to utilize PD to determine the multiplicity of infection (MOI) and the viral titer. The MOI is the ratio of the number of infectious particles relative to the number of host cells exposed to the inoculum (Knipe and Howley, 2013). For example, an experiment using an MOI of 1 should, in theory, use 1 viral particle per cell. However, the infection process is stochastic, which means that the number of virus particles absorbed by a host cell will vary (Knipe and Howley, 2013). Thus, the efficiency of infection is dependent on a statistical probability of host cells absorbing different numbers of viral particles, thereby preventing every cell from receiving an infectious viral particle. In fact, for many viruses, the probability that an individual cell will absorb a given number of viral particles can be modeled using the PD, where the prob-
Ability is a function of the number of outcomes and the mean expected outcome, as follows:

$$P\{n\} = \frac{m^n e^{-m}}{n!}$$

Where $P\{n\}$ is the probability that any cell is infected with $n$ particles, $m$ is the MOI, and $n$ is the number of particles in a given cell (Knipe and Howley, 2013). The probability that a cell does not become infected, $n = 0$, simplifies the equation as follows, since $0!$ and $m^0$ both equal 1:

$$P\{0\} = e^{-m}$$

If all possible outcomes equals 1, then $P\{0\}$ and the probability of a cell receiving more than 0 viral particles, $P\{n > 0\}$, is also equal to 1.

$$P\{0\} + P\{n > 0\} = 1$$

Solving for $P\{0\}$ and rearranging the equations gives:

$$e^{-m} = 1 - P\{n > 0\}$$

Taking the natural log of both sides and multiplying by -1 gives iPd

$$m = - \ln (1 - P\{n > 0\})$$

Thus, we can approximate that:
We can achieve the above enumerations by counting the number of green fluorescent cells, due to infection by the recombinant GFP-HSV-1, and the number of blue fluorescent cells stained by DAPI (Held, 2015).

In order to properly assess the statistical power of this method, we must properly carry and manipulate the inherent propagation of error that the equation and method yield. Since iPD is in the form of:

\[ f = \alpha \ln(hA) \]

we can calculate the standard deviation as explained in Harris’s Quantitative Chemical Analysis, Sixth Edition page 56 (Harris, 2003):

\[ \sigma_f \approx \frac{\sigma_x}{A} \]

The Cytation 3 system from BioTek is an automated fluorescent microscopic imaging station and plate reader. It is capable of automatically capturing fluorescent images in several fluorescent channels and enables optical magnification in multiple locations of every well on multiwall plates. After capturing the images, the accompanied Gen 5 software can analyze each image using a cell analysis feature (Figure 1A). The cell analysis feature uses an algorithm to simulate a “rolling ball” over the image. As the “ball rolls over” an area of pixels that meets the fluorescent intensity and size parameters set by the user, the software counts the area as a cell. The software then counts the number of fluorescent cells in each well and in each fluorescent channel.

Data from the assays of the lab standard stock HSV-1 inoculum were performed and compared to test our hypothesis and the validity of the mathematical calculations. Without the use of the iPD, the FLICIT assay failed to correctly quantify the viral titer (Figure 1B). Upon implementing the iPD, the FLICIT assay measured the titer of the stock virus to within 4% of the measurement obtained by the plaque assay and to within 0.1% of the expected value (Figure 1B). Furthermore, FLICIT was used to measure the viral concentration of a dilution series of the lab stock virus. The triplicate results were graphed (Figure 1C) as a scatter plot, and yielded a best fit trend line with a slope of 0.9907 and an \( R^2 \) of 0.9996.

The FLICIT assay exhibits several advantages over traditional plaque assays. For example, many chimeric viruses contain transgenes that can modify viral replication (Bedadala et al., 2014). In that regard, it is difficult to correctly measure the titer of this virus and compare it to its wild-type counterpart using the plaque assay since the replication was altered by the transgene and thus the plaque assay result might not accurately reflect the true titer of the recombinant virus. This issue can be easily resolved using FLICIT with the replication inhibitor acyclovir, since it calculates the green fluorescence from the infected cells without relying on completion of replication.

In summary, the plaque assay required over 3 hours of labor and a total of 4 days to complete in order to observe a macroscopic cytopathic effect. The assay requires use of a 24-well plate with \( 5 \times 10^6 \) Vero cells to test two inoculums in triplicate. FLICIT relies on the cellular expression of GFP, which can be observed and quantified in our system at 8 hours post-infection. Therefore, FLICIT is able to complete the analysis in under 24 hours. FLICIT can simultaneously titer up to 24 samples per plate, which required under 3 hours of labor to prepare and initiate in the automated quantification. Based on comparison of the time required, labor hours, and costs between the two methods, it is our opinion that FLICIT is superior over plaque assays with respect to all three parameters. Specifically, FLICIT is approximately 12-fold cheaper to run, requires one-twelfth of the labor hours per sample tested, and can yield results in a quarter of the time required for the plaque assay. These combined advantages make FLICIT exponentially more cost-effective overall than the plaque assay.

FOOTNOTES

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