Application Note

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# Detecting and Characterizing Virus Neutralizing Antibodies in Real Time Using Cellular Impedance and Live-Cell Imaging


**Authors**

Nancy Li, Yama Abassi, Xiaobo Wang, and Brandon J. Lamarche
Agilent Technologies, Inc.


## Introduction

By binding to specific proteins[1], carbohydrates[2], and/or lipids[3] on the surface of viruses, antibodies can sterically block interactions with cognate host cell receptors and thereby prevent infection. Although numerous biophysical assays can interrogate antibody-antigen interactions, it is well established that an antibody's ability to bind a purified antigen does not always correlate with its ability to "neutralize" the virus (i.e. block infection) within the complex milieu of the host cell membrane. For this reason, functional assays that monitor the bona fide infection of host cells are regarded as the gold standard for identifying and characterizing virus neutralizing antibodies. Chief among these assays is the plaque reduction neutralization test (PRNT), which evaluates changes in the number of plaques that are produced when purified virus is preincubated with antibody (or serum) prior to exposure to host cells. Although the PRNT has been the gold standard for decades, the extensive hands on time required to run and quantify a PRNT make it unsuitable for many studies, particularly when higher throughput is needed. As a highly efficient replacement of the PRNT, this study uses the real-time impedance monitoring and live-cell imaging capabilities of the Agilent xCELLigence RTCA eSight instrument to both screen for neutralizing antibodies and to subsequently quantify the efficacy of hits. After adding the virus + antibody mixture to host cells, no further hands-on time is required. Data are acquired continuously and, depending on the multiplicity of infection that is used, neutralizing activity is detectable in as little as 1 hour. Because of its extreme sensitivity, the impedance readout provides information about the mechanism of neutralization, making it possible to differentiate between antibodies that prevent cellular uptake of virus versus antibodies that noncanonically inhibit the virus after it has already gained access to the cell's interior. The overall flexibility of this eSight assay makes it amenable to the study of neutralizing antibodies in diverse contexts including, but not limited to, vaccine development, convalescent plasma testing, and antibody engineering.

### eSight assay principle

The eSight is currently the only instrument in the world that interrogates cell health and behavior using cellular impedance and live cell imaging simultaneously. When using eSight's specialized microplates, which contain gold biosensor arrays integrated into the bottom of all 96 wells (Figure 1), real-time impedance measurements track changes in cell number, cell size, cell-substrate attachment strength, and cell-cell interactions (i.e. barrier function). Because each of these parameters changes during a typical viral cytopathic effect (CPE), impedance provides a very sensitive readout of host cell health throughout the full continuum of a viral infection. Antibody-mediated suppression of the CPE is readily detected as changes in both the kinetics and magnitude of the impedance signal. Positioned in between the gold biosensors, a microscopy viewing window enables eSight to concurrently collect live cell images that track the CPE, and an antibody's mitigation of it, using brightfield as well as red, green, and blue fluorescence channels (Figure 1). This ability to monitor neutralizing activity in real time from two orthogonal perspectives, using the same population of cells, provides both a primary and confirmatory result all from a single simple assay.

## Materials and methods

Cell maintenance and assays were conducted at 37 °C/5% $CO_2$ in DMEM media (ATCC; part number 30-2002) containing 10% heat inactivated FBS (Corning, part number 35016CV). The HEK293A-Red cell line, which stably expresses nuclear-localized red fluorescent protein (RFP), was produced by transducing HEK293A cells (Life Technologies; part number R70507) with Agilent eLenti Red (part number 8711011) at a multiplicity of infection of 1. From day 2 to day 11 postinfection, 1 µg/mL puromycin was included in the growth medium to select for transductants. Adenovirus-GFP (Vector Biolabs; part number 1060) is an adenovirus 5 that encodes cytoplasmic eGFP, which is expressed behind a CMV promoter. This virus also has the E1 and E3 regions deleted, making it replication incompetent. Because the HEK293A cell line has the adenovirus E1 and E3 regions stably integrated into its genome, it supports replication of the mutant adenovirus being used here. The antibodies and proteins used in this study are listed in Table 1. Agilent E-Plate VIEW microplates were used (part number 00300601030).

For a typical assay, 50 µL of media was added to E-Plate wells followed by recording the background impedance signal. Wells were subsequently seeded with 50 µL of media containing either 10k or 40k host cells. After allowing the cells to settle for 30 minutes at room temperature, the plate was transferred to the eSight instrument to monitor cell adhesion and proliferation overnight. 20 hours post cell seeding, each well was treated with 100 µL of media containing adenovirus-GFP (at different concentrations) that either had or had not been pre-incubated for 45 minutes at 25 °C with neutralizing antibody (more details are provided in the Results section). Data acquisition was then continued out to 120 hours. For the initial viral CPE assays shown in Figures 2 and 3, HEK293A cells were used and the growth medium contained Agilent eLive Red dye (part number 8711040) diluted 500-fold and 100 µM verapamil (an efflux pump inhibitor). For all subsequent assays HEK293A-Red cells were used without dye or verapamil.

For all assays impedance was measured every 15 minutes, while images were acquired once per hour. In each well, four fields of view were captured for each channel (brightfield, red, and green). Exposure times were as follows: brightfield (automatically adjusted by the eSight software), red (300 ms), green (300 ms).

## Results and discussion

### Monitoring viral CPEs using impedance

Before running virus neutralization assays, it is important to become familiar with the unmitigated CPE of the particular virus and host cells being used. Towards this end, HEK293A cells were seeded in an E-Plate at a density of 10k/well, and 20 hours later were infected with adenovirus-GFP that had been 5x serially diluted. Using the uninfected control to establish a frame of reference, the black impedance trace in Figure 2A shows a rapid increase over the first 5 hours post cell seeding, which corresponds to cell adhesion. This is followed by a slower proliferation phase that extends out to ~55 hours, after which it plateaus – indicating that the cells have become confluent and are covering the entire surface area of the gold biosensors. Note that this proliferation phase displays a transient "blip" at the 20 hour time point when the plate was removed from the eSight and 100 µL of virus-free media was added to these duplicate wells.

At very low virus concentrations the impedance traces are essentially identical to that of the negative control. Upon increasing the virus concentration to $1.1 \times 10^3$ pfu/mL a modest CPE is observable, with the impedance trace begining to drop below that of the negative control at ~100 hours (Figure 2A; turquoise trace). Using the photos collected by eSight to determine the actual number of red host cells that were present at the 20-hour time point when virus was added, this $1.1 \times 10^3$ pfu/mL sample is calculated to have a multiplicity of infection (MOI) of 0.0082. While this MOI produces a

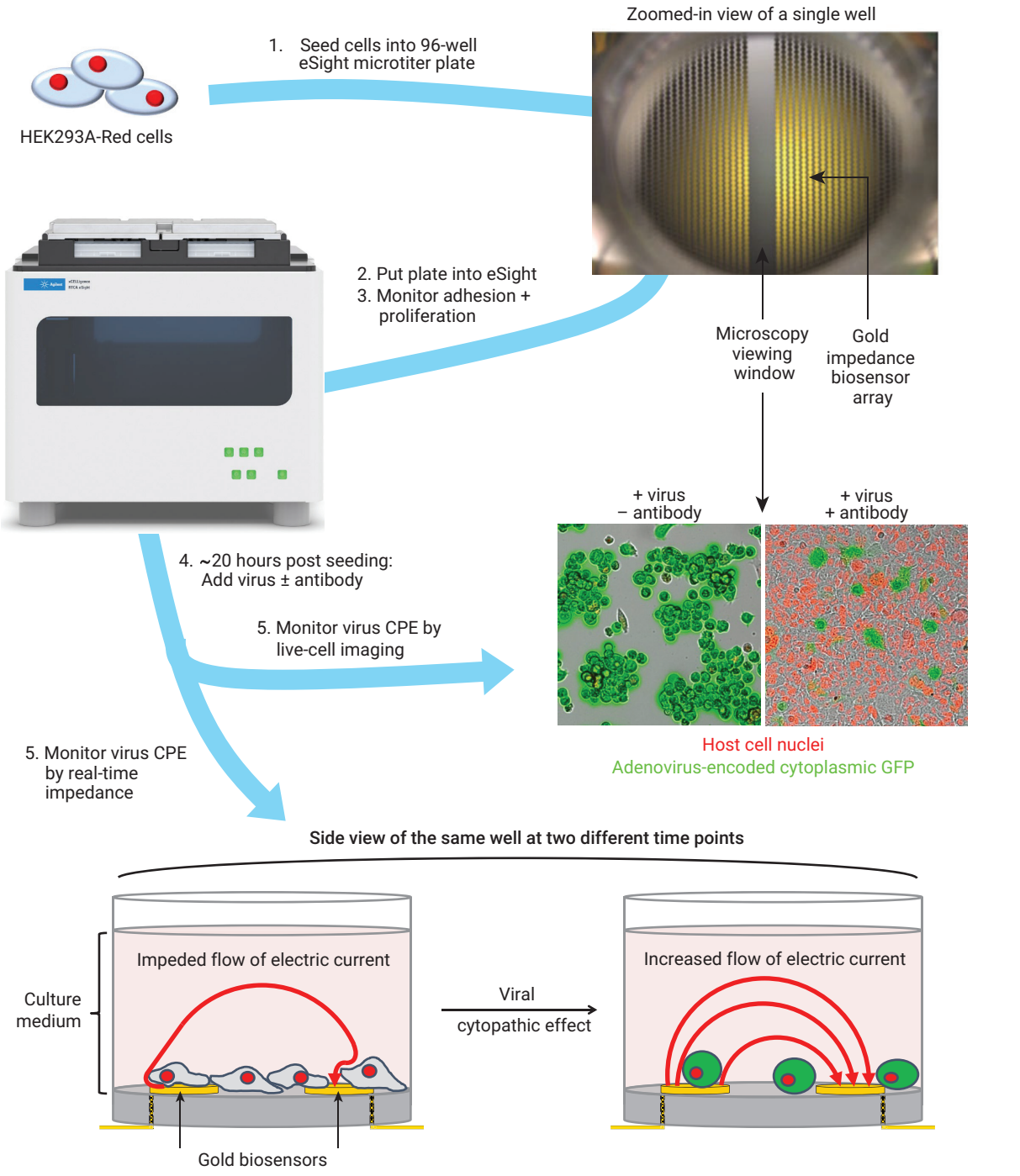
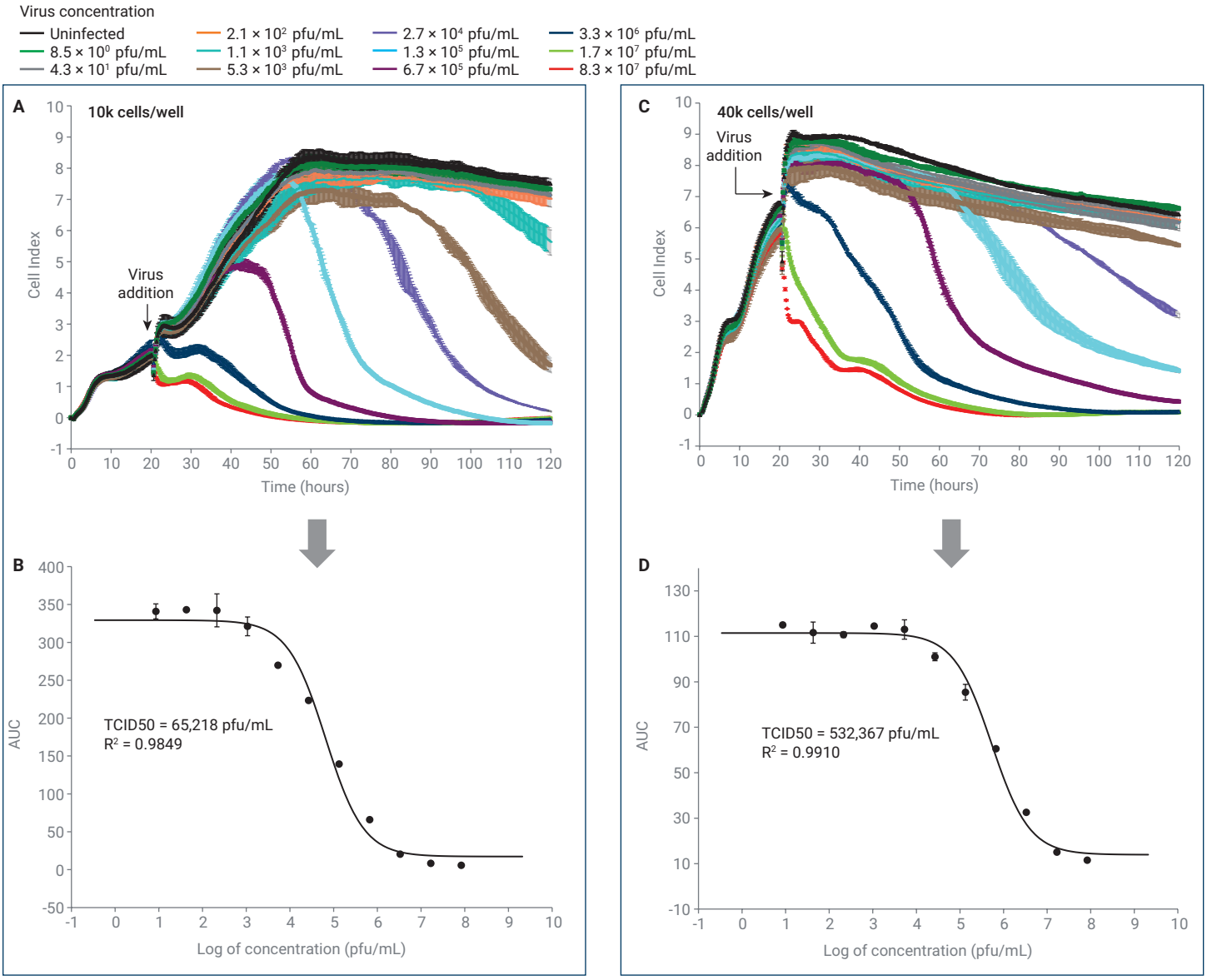



**Figure 1.** eSight workflow for identifying and characterizing virus neutralizing antibodies in real-time. See text for details.




**E**

| Conditions Selected For Use in All Subsequent Assays | | | |
|---|---|---|---|
| **Cell Seeding Density** | **Virus Concentration (pfu/mL)** | **Virus Concentration (TCID50)** | **Virus Concentration (MOI*)** |
| 10k Cells/Well | $1.3 \times 10^5$ | 2 | 1 |
| | $8.3 \times 10^7$ | 1,280 | 641 |
| 40k Cells/Well | $1.3 \times 10^5$ | 0.25 | 0.4 |
| | $8.3 \times 10^7$ | 156 | 222 |

*MOIs were calculated based on the known virus concentration and the actual number of cells present at the moment of virus addition (as counted by eSight).

**Figure 2.** Impedance-based monitoring and quantification of the cytopathic effect induced by adenovirus-GFP. (A) Impedance as a function of time when different virus concentrations are used to infect HEK293A cells that were seeded at a density of 10k cells/well. Error bars = standard deviation for two replicate wells; because of the high temporal resolution, error bars are packed together tightly – so the thickness of a given impedance trace reflects reproducibility. (C-D) Similar to panels "A" and "B", but for a cell seeding density of 40k cells/well. (E) Summary of the four infection conditions selected for use in the neutralizing antibody assays shown below. Virus concentration in units of TCID50 = (virus concentration in pfu/mL) / (TCID50 value from panel "B" or panel "D"). Virus concentration in units of MOI = (virus concentration in pfu/mL)x(0.1 mL) / (actual number of cells present at the time of infection‡). ‡Rather than using the number of cells seeded 20 hours prior to infection, this number is based on cell counts taken directly from eSight immediately before virus addition.

CPE that is right at the limit of what is detectable by impedance for this virus + host cell combination, it is important to recognize that simply extending the duration of the assay beyond 120 hours enables CPEs to manifest more fully and therefore makes it possible to detect even lower concentrations of virus (data not shown). As long as a well receives a single active virion, a CPE will be detectable by impedance.

Progressing through the 5x dilution series clearly shows the strong correlation between the amount of virus added and the rate at which the impedance signal decreases. At a virus concentration of $1.3 \times 10^5$ pfu/mL (Figure 2A; light blue trace), the impedance signal drops all the way to zero by the 120 hour time point, indicating that the entire population of cells in these duplicate wells has been killed. Note that at the three highest concentrations of virus the impedance curves (red, lime green, and dark blue) take on a biphasic shape where an extremely rapid decrease over the first few hours is followed by a slight "recovery" and then a more gradual decrease. The physical relevance of these biphasic curves will be discussed in more detail later in the Results section.

As one way to evaluate the host cell killing efficacy of adenovirus-GFP, the area under the impedance curves in Figure 2A was plotted as a function of virus concentration. The corresponding dose-response curve in Figure 2B has a classical sigmoid shape with excellent fitting. The inflection point of this curve is here defined as the "TCID50" (tissue culture infectious dose required to achieve a 50% killing response). This result indicates that for the conditions being used here, an adenovirus-GFP concentration of 65,218 pfu/mL is required in order to kill 50% of the cells in the well by the 120-hour time point.

When the same adenovirus dilution series is used to infect wells that were seeded with four times as many cells (40k/well), the overall trends in impedance are largely similar (Figure 2C). One important difference is that for a given virus concentration, the CPE is more robust for 10k cells/well than it is for 40k cells/well. This is especially evident when comparing the impedance traces (Figures 2A and 2C) for a low virus concentration, and also when comparing the TCID50 values calculated for each cell seeding density: 65,218 versus 532,367 pfu/mL for 10k and 40k cells/well, respectively (Figures 2B and 2D). That adenovirus-GFP is a more efficient killer at 10k cells/well than at 40k cells/well is at least partially a consequence of MOI: for a fixed virus dilution, the MOI is higher for the 10k cells/well condition than it is for the 40k cells/well condition. The differential killing efficacy at the two different cell seeding densities may also reflect the replicative status of the host cells during the early stages of infection. When cells are seeded at a density of 10k/well they have the capacity to continue proliferating out to the ~55-hour time point. In contrast, when cells are seeded at a density of 40k/well they have the capacity to proliferate only to the ~25-hour time point (compare the time at which the black impedance trace plateaus in Figures 2A and 2C). Because adenovirus is a more efficient killer of cells that are actively dividing[4], less virus should be required for eliciting a robust CPE at 10k cells/well than at 40k cells/well.

The above data demonstrate how easily impedance can be used to continuously and quantitatively monitor viral CPEs, and provides a frame of reference for how the kinetics and magnitude of the impedance signal vary as a function of cell density and virus concentration. Although the specifics will vary for different virus + host cell systems, the general trends observed here are common across diverse families of viruses.

Using this assay to detect/characterize neutralizing antibodies simply involves preincubating virus with antibody before adding the mixture to host cells. Under conditions where a virus is capable of inducing a CPE, an effective neutralizing antibody will mitigate the virus-induced decline in the impedance signal. The extent of neutralization, and the time at which neutralization becomes detectable, will vary some depending on assay conditions. In order to demonstrate the efficacy of this impedance-based approach over a broad range of conditions, the neutralization assays conducted in below sections were run at both 10k and 40k cells/well, and at both low ($1.3 \times 10^5$ pfu/mL) and high ($8.3 \times 10^7$ pfu/mL) virus concentrations. These four conditions are summarized in Figure 2E, which also shows the virus concentrations in terms of MOI and TCID50 (see the legend of Figure 2 for a description of how these concentrations were calculated).

## Corroborating impedance with imaging

Importantly, the live-cell images collected by eSight confirm that the impedance signal is an accurate reflection of the physical status of the host cells as they progress through the full continuum of the CPE (Figures 3A and 3B). While brightfield images alone are sufficient for tracking the progression of the CPE, inclusion of the red channel (host cell nuclei) and green channel (virus-encoded GFP) both improves the aesthetics and increases the information richness. As one example of this, Figure 3C displays the inverse correlation between host cell death observed by impedance and the expression of viral GFP. As would be expected, these data demonstrate that viral gene expression precedes the death cascade phenotype of the host cells. It is worth noting that even though cells are still present at the 120-hour time point, the impedance signal has dropped to zero (Figures 3A and 3B). Because cellular impedance results from intimate interaction between the gold biosensors and the phospholipid bilayer of the host cells, this data indicates that those cells which are still present at late time points are completely detached from the well bottom.

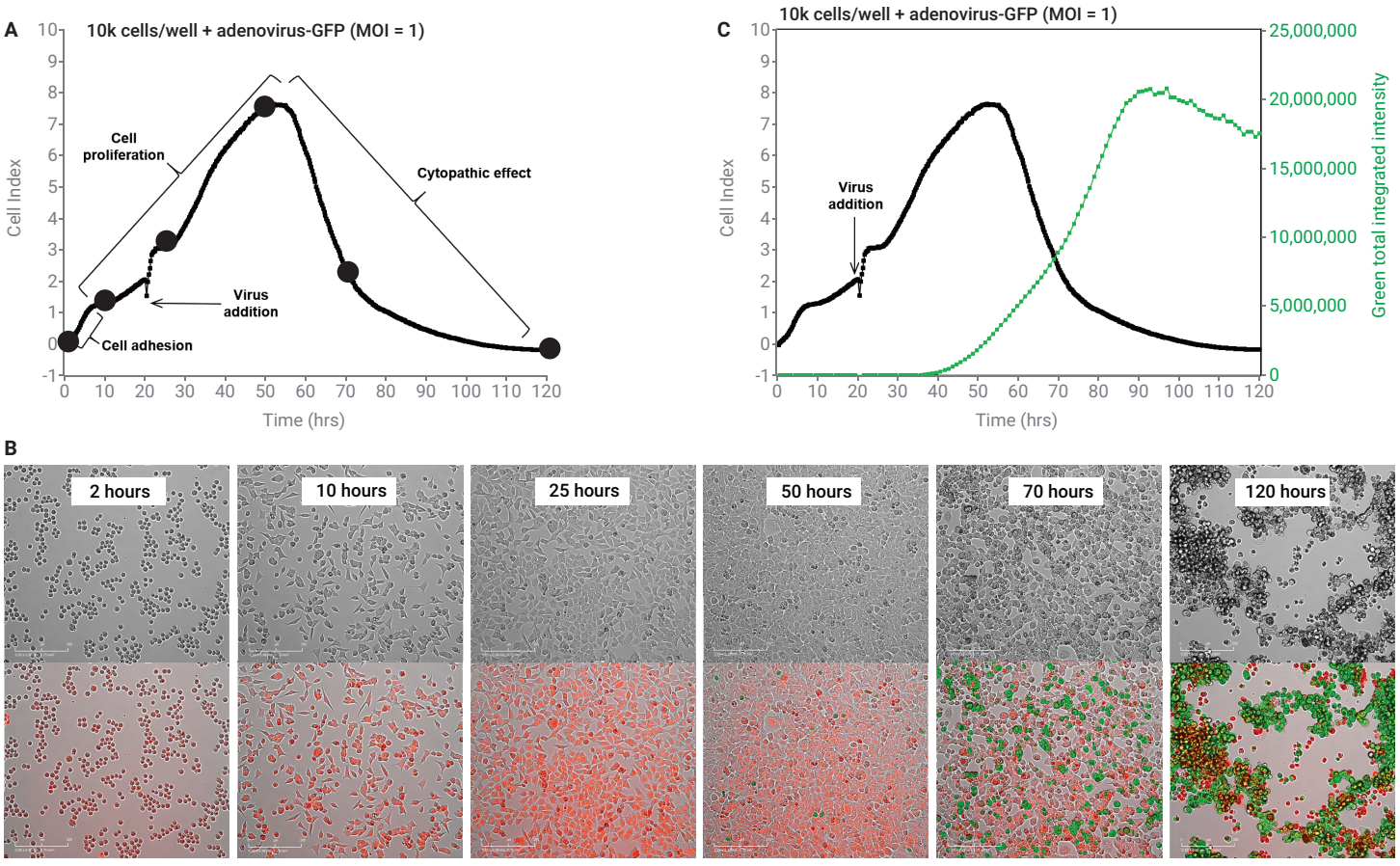



**Figure 3.** Combining eSight's impedance- and image-based readouts to track the adenovirus-GFP cytopathic effect. (A) A single impedance trace for 10k cells/well infected with adenovirus-GFP at MOI = 1. Distinct phases of the curve, and the cellular phenomena that underlie them, are denoted. The black dots correspond to the time points shown in panel B. (B) Photos for key time points in the impedance trace of panel A. While the upper panels are just brightfield, the lower panels include the red channel (host cell nuclei) and the green channel (virus-encoded GFP). Scale bars = 200 µm. (C) Juxtaposing impedance-based detection of cell killing (black trace) with image-based detection of viral GFP expression (green trace).

If fluorescent labeling of target cells is deemed to be advantageous, the most straightforward approach is to simply include Agilent eLive Red dye in the growth medium. This dye is nonfluorescent until it diffuses through the plasma membrane and binds to dsDNA. Although useful for most cell types, this dye labels some cells (including HEK293A) with poor efficiency (Figure 4; left panel). To remedy this, the drug verapamil can be included in the growth medium to block the efflux pumps that actively secrete dye molecules from the cell. While this is effective (Figure 4; middle panel), it is always important to verify that inclusion of verapamil is not perturbing the biology one wishes to study. In this case, it was found that inclusion of verapamil makes the HEK293A cells more sensitive to adenovirus-mediated killing (data not shown). This is consistent with recent findings for oncolytic adenoviruses[5] and is not surprising considering the fact that verapamil inhibits organic anion trasnsporter 2[6], a protein that adenovirus upregulates.[7] In studies such as this, where the CPE is merely being used to detect whether an antibody has neutralizing activity, a systemic reduction in killing efficiency will not detract from the assay's objective. However, as a more conservative alternative to the use of verapamil, HEK293A cells were instead engineered to express nuclear-localized red fluorescent protein (Figure 4; right panel). These cells, hereafter refered to as HEK293A-Red, were used for all subsequent assays.

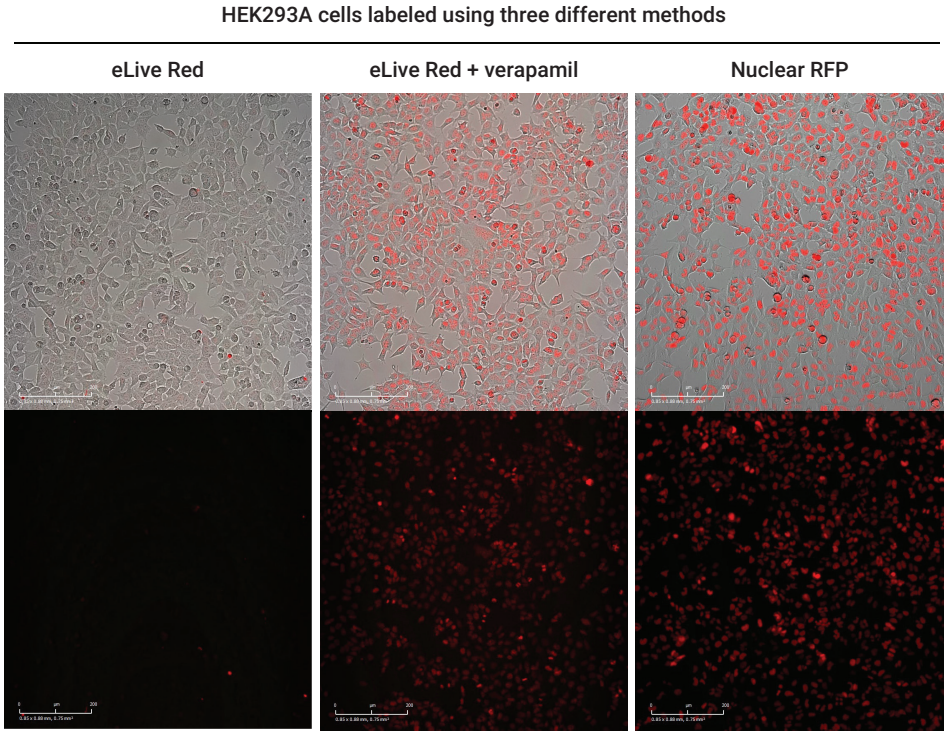



**Figure 4.** Labeling HEK293A cells with eLive Red, eLive Red + verapamil, or by stably expressing nuclear RFP (via lentiviral transduction).

### Detecting neutralizing activity: Positive and negative controls

Antibody clone 27F11 was employed as a negative control. Although this antibody binds to the adenovirus hexon protein, it specifically recognizes an epitope that is only exposed to the interior of the capsid (Figure 5A). For this reason, antibody 27F11 should be incapable of mitigating the adenovirus-GFP-induced cytopathic effect. As expected, preincubating adenovirus-GFP with 27F11 (3 µg/mL) for 45 minutes at 25 °C has little to no impact on the extent or rate of host cell killing at all four of the cell density and virus concentration combinations that were examined (Figures 5B through 5E).

Adenovirus gains access to host cells primarily by interaction of its fiber protein with the coxsackie-adenovirus receptor (CAR) in the host cell membrane (Figure 6A). As a positive control for neutralizing activity, 30 µg/mL of the soluble domain of CAR was preincubated with adenovirus-GFP for 45 minutes at 25 °C before adding the mixture to host cells. When using 40k cells/well and the high virus concentration of MOI = 222, the soluble CAR has minimal impact on the killing efficacy of the virus (Figure 6B). In stark contrast, when using 40k cells/well with the low virus concentration of MOI = 0.4, the soluble CAR completely blocks the ability of adenovirus-GFP to kill the host cells (Figure 6C). This highlights the importance of using appropriate assay conditions where the quantity of virus is sufficient for inducing an observable CPE, but not so high that it completely overwhelms the neutralizing agent. In similar fashion, when working with 10k cells/well the soluble CAR displays minimal neutralizing activity when the virus concentration is high (MOI = 641) but robust neutralizing activity when the virus concentration is low (MOI = 1) (Figures 6D and 6E). Finally, note that the soluble CAR neutralizes more efficiently when using 40k cells/well than it does when using 10k cells/well. This reiterates the fact that even when neutralizing activity is detectable, the magnitude/kinetics of neutralization will vary depending on the exact conditions under which the assay is being run.

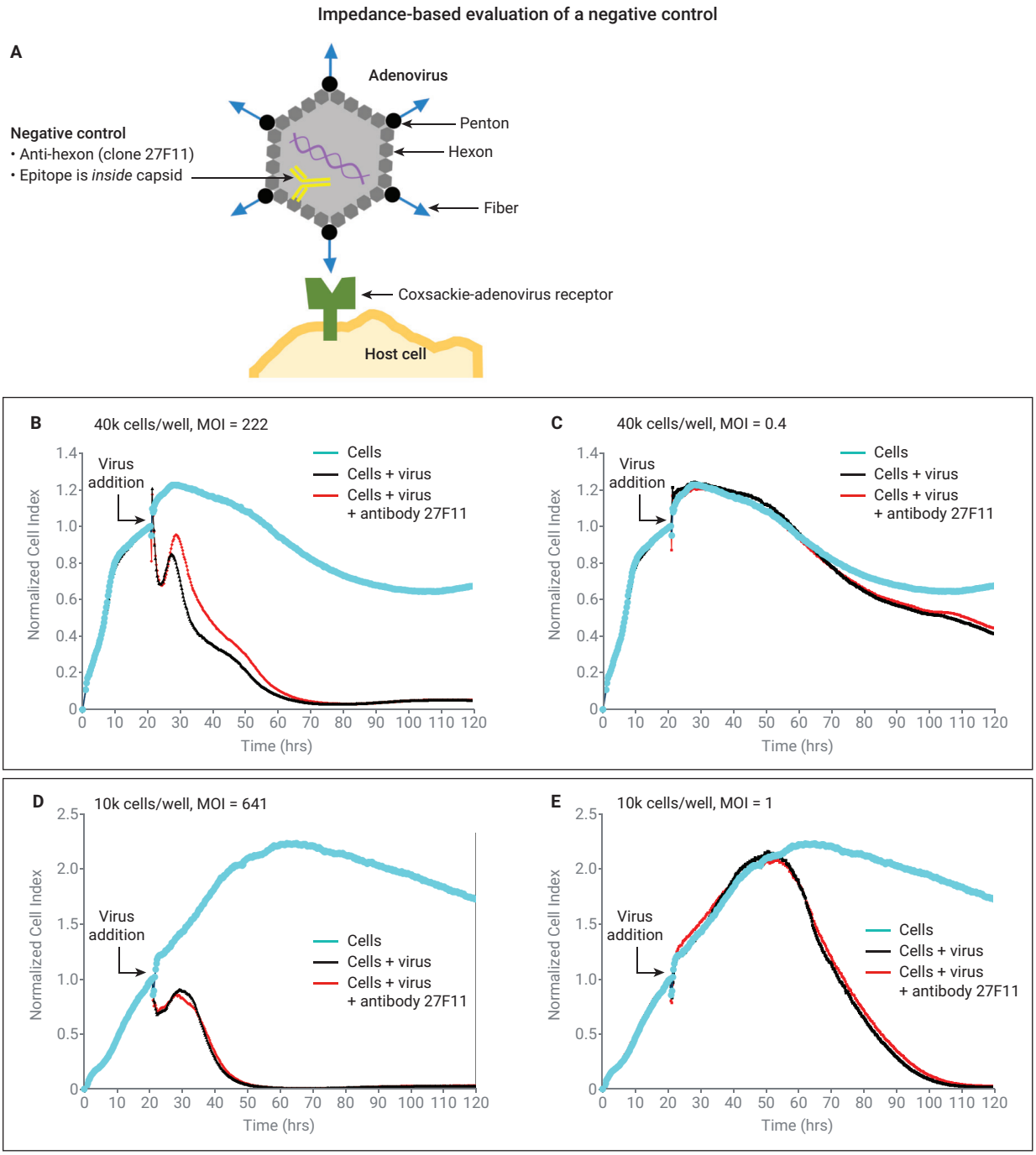



**Figure 5.** Impedance-based evaluation of a negative control. (A) Schematic showing key adenovirus proteins, the adenovirus receptor in the host cell membrane, and the binding site of the negative control antibody (yellow; this binds to an epitope that is only exposed to the interior of the adenovirus capsid). (B,C) Impedance response for 40k cells/well infected at MOI = 222 or 0.4 after preincubation with or without antibody 27F11. (D,E) Impedance response for 10k cells/well infected at MOI = 641 or 1 after preincubation with or without antibody 27F11. Antibody 27F11 was used at a concentration of 3 µg/mL. See the legend of Figure 2 for a description of how exact MOIs were determined.

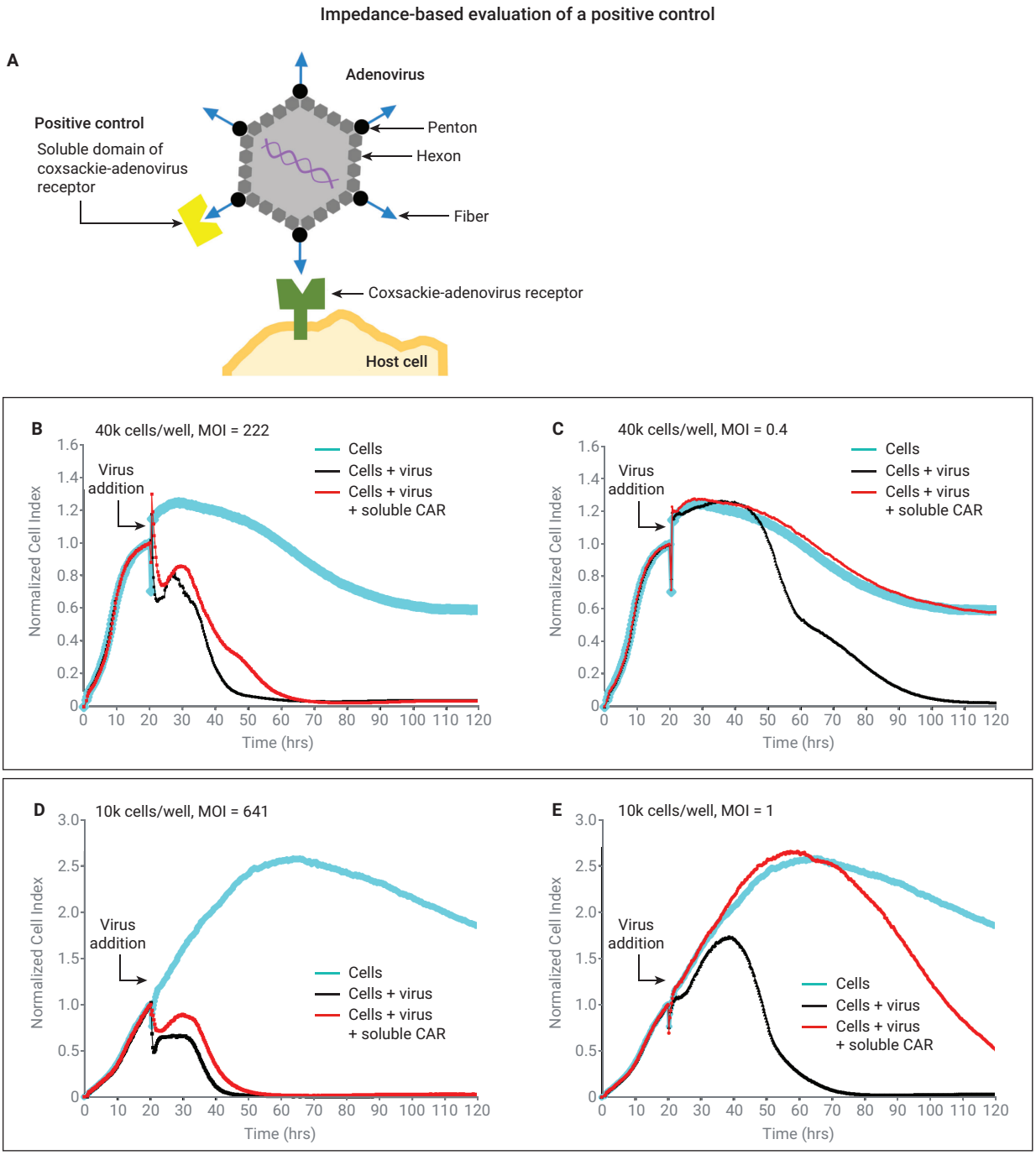



**Figure 6.** Impedance-based evaluation of a positive control. (A) Schematic showing key adenovirus proteins, the adenovirus receptor in the host cell membrane, and binding of the soluble domain of the CAR receptor (yellow) to the adenovirus fiber protein. (B,C) Impedance response for 40k cells/well infected at MOI = 222 or 0.4 after preincubation with or without soluble CAR. (D,E) Impedance response for 10k cells/well infected at MOI = 641 or 1 after preincubation with or without soluble CAR. The soluble domain of CAR was used at a concentration of 30 µg/mL. See the legend of Figure 2 for a description of how exact MOIs were determined.

As corroboration of the above impedance results, Figure 7 displays images for uninfected cells, and cells infected with adenovirus-GFP that had or had not been pre-incubated with the soluble domain of CAR. Whereas the untreated virus causes a massive CPE where 100% of the cells are positive for the virus-encoded GFP, virus pretreated with CAR is unable to elicit a CPE and only a fraction of the cells are GFP-positive.

## Screening for neutralizing antibodies

Next, seven different antibodies were screened for their ability to mitigate the CPE of adenovirus-GFP. These included a polyclonal antibody raised against intact adenovirus 5 capsids, as well as monoclonal antibodies with specificity for the adenovirus hexon and fiber proteins, and a monoclonal antibody against CAR. The assay format was similar to that described above, with the preincubation step being 45 minutes at 25 °C and the antibody concentrations being 3 µg/mL. The one exception was for antibody ab6982, which was used at a 25-fold dilution (the actual concentration of this polyclonal serum was not provided by the manufacturer). If an antibody suppressed the CPE by >50% at the 120-hour time point, when using the low virus concentration at both 10k and 40k cells/well, it was regarded

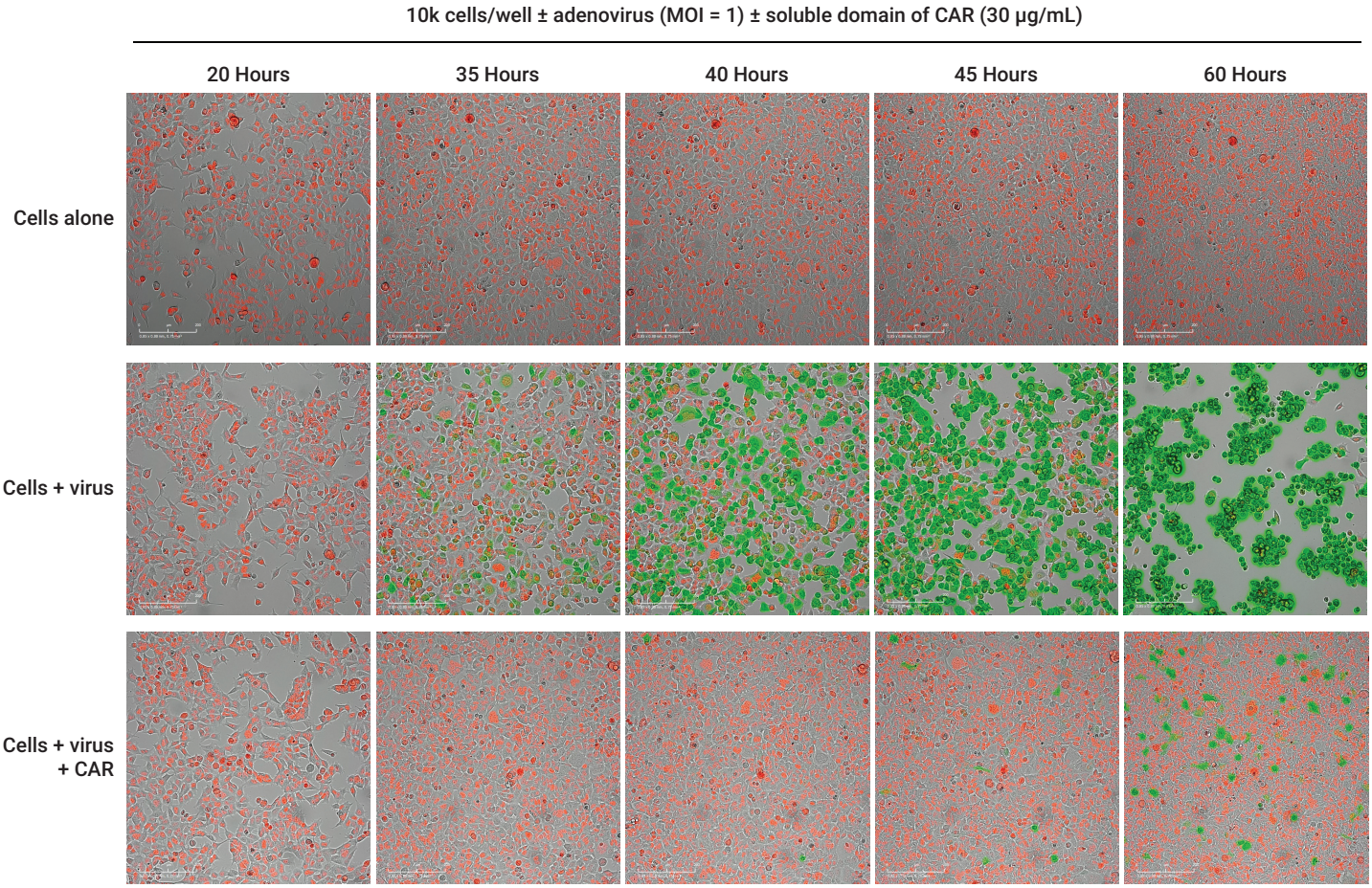



**Figure 7.** Image-based tracking of the ability of the soluble domain of CAR (30 µg/mL) to neutralize adenovirus-GFP (MOI = 1) infection of HEK293A-Red cells (10k/well). See text for details. Scale bars = 200 µm.

as being an effective neutralizer.* Using this criterion, two of the antibodies were determined to be positive hits (Table 1).

**Table 1.** Summary of neutralizing antibody screening results.

| Target Protein That Antibody Was Raised Against | Antibody Clone | Neutralizing Activity |
|---|---|---|
| Adenovirus 5 Intact Capsids | ab6982 (abcam) | Yes |
| Adenovirus Hexon | TC31-9C12.C9 (U. Iowa Hybridoma Bank) | Yes |
| Adenovirus Hexon | TC31-27F11.C2 (U. Iowa Hybridoma Bank) | No |
| Adenovirus Hexon | 8C4 (abcam) | No |
| Adenovirus Hexon | 1E11 (abcam) | No |
| Adenovirus Fiber | 4D2 (abcam) | No |
| Adenovirus Fiber | 3F13 (abcam) | No |
| Adenovirus Fiber | ab168893 (abcam)<br>This is not an antibody. It is the soluble domain of the coxsackie-adenovirus receptor. | Yes |
| Coxsackie-Adenovirus Receptor | EPR23305-44 (abcam) | No |

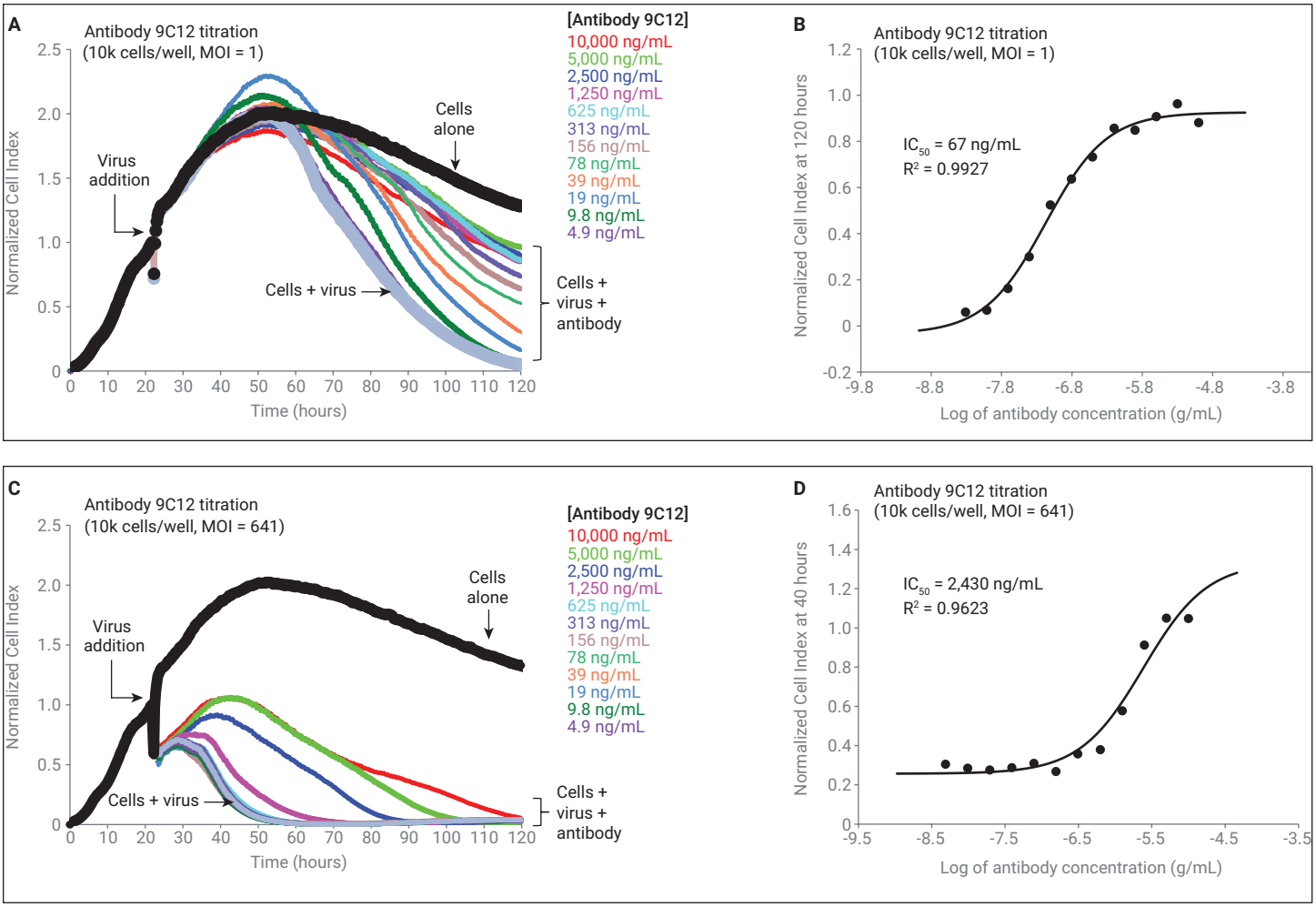
## Characterizing the efficacy of neutralizing antibodies

The neutralizing efficacy of one of the screening hits, anti-hexon monoclonal antibody 9C12, was evaluated by repeating the assay with adenovirus-GFP that had been preincubated with different concentrations of the antibody spanning from 4.9 to 10,000 ng/mL. Although the assay was run under all four of the cell density and virus concentration combinations, Figure 8A focuses on just the 10k cells/well + MOI = 1 condition. Under this condition, after uninfected cells reach confluence at ~50 hours the impedance signal gradually declines out to 120 hours (thick black data trace). This indicates that the uninfected cells are becoming unhealthy in the late time regime. Importantly, the presence of virus effects a much more rapid decline in the impedance signal (thick grey data trace). Preincubation with antibody 9C12 at 4.9 ng/mL has minimal impact on the rate of host cell killing (thin purple data trace). However, 9C12 concentrations ranging from 9.8 to 10,000 ng/mL cause a progressive reduction in the killing capacity of the virus (Figure 8A). Plotting the value of the impedance signal at the 120-hour time point as a function of antibody concentration produces the dose response curve in Figure 8B, which indicates that antibody 9C12 has an $IC_{50}$ of 67 ng/mL.

When the antibody 9C12 titration is repeated using the same cell seeding density (10k/well) but the virus concentration is increased from MOI = 1 to MOI = 641 the killing kinetics increase dramatically (Figure 8C), and producing a dose response curve based on the impedance values at the 120-hour time point is no longer appropriate. Instead, under these conditions the best fitting is obtained by analyzing the impedance values at the 40-hour time point – which yields an $IC_{50}$ of 2,430 ng/mL (Figure 8D). This increase in $IC_{50}$ as the virus concentration is increased is also observed at the higher (40k/well) cell seeding density (Figure 8E), and is expected: when more virus is being used in the assay, more antibody is needed to effect neutralization. It is interesting to note that more antibody is needed to neutralize virus when using 10k cells/well than when using 40k cells/well which, again, highlights the condition-dependence of the results and emphasizes the need for standardization when attempting to compare the efficacy of different antibodies.

Finally, it is worth noting that antibodies have the potential to interact directly with cells, independent of their interaction with virus. For this reason, it is always worthwhile to include a cell + antibody control (i.e. in the absence of virus). Treating HEK293A-Red cells with antibody 9C12, across the entire concentration range, was found to have minimal impact on the impedance signal of the cells (data not shown). This helps to confirm that the neutralization seen in Figure 8 is legitimate and not simply an artifact of antibody interacting with host cells directly.

*This definition need not be used by others. The criterion for being an effective neutralizer can be adjusted by the user.




**E**

| $IC_{50}$ of Antibody 9C12 for Neutralizing Adenovirus-GFP Under Four Different Infection Conditions | | |
|---|---|---|
| | High Virus Concentration | Low Virus Concentration |
| 40k Cells/Well | 1,150 ng/mL | 24 ng/mL |
| 10k Cells/Well | 2,430 ng/mL | 67 ng/mL |

**Figure 8.** Impedance-based quantification of neutralization efficacy. (A) Infection of HEK293A-Red cells (10k/well) by adenovirus-GFP (MOI = 1) that was preincubated with monoclonal antibody 9C12 at concentrations ranging from 4.9 to 10,000 ng/mL. (B) Dose-response curve based on plotting the value of the impedance traces from panel A at the 120-hour time point as a function of antibody concentration. (C,D) Similar to panels A and B, but for the higher virus concentration of MOI = 641. (E) Summary of the $IC_{50}$ values for antibody 9C12 when assayed under all four combinations of cell density and virus concentration.

## Rapid detection of neutralization

For standard plaque reduction neutralization tests (PRNTs) it is necessary to wait ~3 to 12 days before plaques can accurately be evaluated. The key here is giving the virus sufficient time to spread outwards from the initially infected cell into neighboring cells, allowing it to form a robust plaque that is detectable optically. In contrast, when using impedance even the very early stages of infection are detectable. When 10k cells/well are infected with adenovirus-GFP at MOI = 1, it takes only 15 hours for the cytopathic effect to become apparent (Figure 9A). For the anti-capsid antibody ab6982, which was the second "hit" from the screening library, 15 hours post virus addition is also the time point where neutralizing activity becomes detectable (i.e. this is where the red and black impedance traces begin to separate from one another in Figure 9A). Importantly, the time at which this neutralization becomes detectable is highly dependent upon the concentration of virus that is being used. When increasing the virus concentration from MOI = 1 up to MOI = 641 the CPE becomes apparent in only 1 hour (Figure 9B). While this rapid drop in impedance is associated with virus-induced cell shrinkage and occurs many hours before expression of the viral GFP (data not shown), more work is needed to characterize this mechanistically. Because this phenomenon has been observed across diverse virus families including both enveloped and nonenveloped viruses, the use of high virus concentration appears to be a general tool for reducing even further the time required to identify neutralizing activity by impedance.

## Identifying different mechanisms of action

Perhaps the most common perception of how a neutralizing antibody functions is that it binds to the surface of a virion and sterically blocks its ability to interact with its cognate receptor, thereby preventing uptake into a cell. Antibody ab6982 appears to function in this manner: whereas a high concentration (MOI = 641) of untreated adenovirus-GFP effects an immediate drop in impedance, ab6982 blocks both this drop in impedance (Figure 10A) and the downstream expression of viral GFP (not shown). In contrast, antibodies with alternative mechanisms of neutralization have also been identified. As one example, antibody 9C12 has been shown to bind to the adenovirus hexon protein and remain attached to the virion all the way through the endocytic pathway. Upon virion escape from the endosome, the constant region of antibody 9C12 is recognized by the cytoplasmic antibody receptor TRIM21, leading to degradation of the virion by the proteasome.[8-10] This atypical mechanism of action is captured in the impedance signal: 9C12 is incapable of blocking the initial rapid drop in impedance that is associated with virus uptake, but greatly reduces the kinetics of subsequent cell killing (Figure 10B). This ability to differentiate between different mechanisms of inhibition, which results from the continuous nature of the eSight readout, simply is not possible with traditional virus neutralization assays.

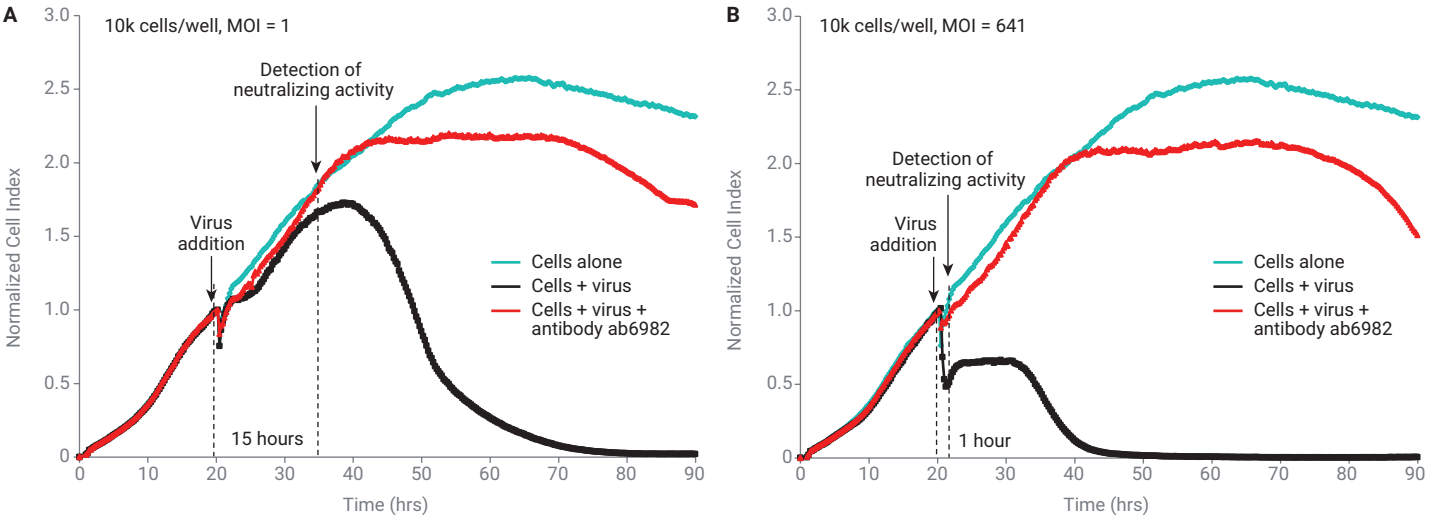



**Figure 9.** The time required for detecting virus neutralizing activity by impedance is dependent upon virus MOI. (A) When 10k cells/well are infected at an MOI of 1, it takes ~15 hours for the neutralizing activity of antibody ab6982 to be detectable. (B) When 10k cells/well are infected at an MOI of 641, it takes only ~1 hour for the neutralizing activity of antibody ab6982 to be detectable. During the virus + antibody preincubation step, ab6982 was used at a 25-fold dilution.

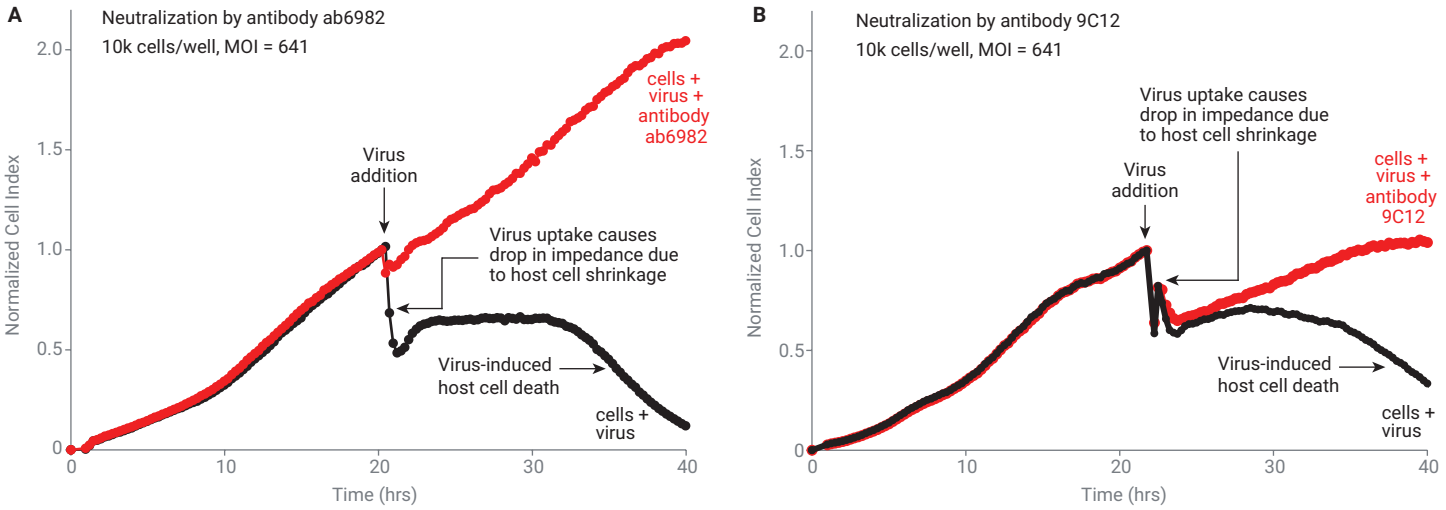



**Figure 10.** Identifying different mechanisms of antibody-mediated virus neutralization. (A) Polyclonal antibody ab6982 blocks both the virus-mediated rapid drop in impedance and the subsequent slower cell killing phase. Collectively, these data are consistent with 9C12 blocking virus uptake into the cell. (B) Monoclonal antibody 9C12 fails to block the virus-mediated rapid drop in impedance, but subsequently inhibits killing of the host cells. Collectively, this is consistent with 9C12's established ability to pass through the endocytic pathway with adenovirus, and then cause proteasomal degradation of virions in the cytoplasm.

## Conclusion

The data presented here clearly demonstrate the eSight's ability to screen antibodies for virus neutralizing acitivity, and to subsequently quantify the efficacy of hits. Using the protocol shown in this app note, impedance has successfully been employed with diverse viruses, including those which are enveloped and nonenveloped, as well as viruses that effect host cell lysis or fusion. A distinguishing feature of the assay is its simplicity, requiring very little hands-on time. In a typical assay format, host cells are seeded and then allowed to adhere and proliferate overnight, followed by addition of the virus + antibody solution. No additional handling or processing steps are needed. Importantly though, the assay is highly amenable to variations in this protocol – including infecting cells while in suspension and then transfering the cell + virus mixture into the E-Plate. By using tethering antibodies to first capture the cells on the well bottom, impedance can also be used to study neutralizing antibody mitigation of CPEs in host cell types that are not naturally adherent.

The manner in which the data was presented here reflects the approach of typical eSight users – where impedance is the primary/quantitative readout and images are employed for qualitative confirmation. However, image-based quantification is also possible. Figure 11 shows three different types of image-based analyses of antibody 9C12's impact on the 10k cells/well + MOI = 1 assay condition. In panel A, it is clear that analyzing % brightfield confluence isn't useful for this particular condition: the CPE simply is not severe enough to translate into substantial changes in confluence (because many dead/detatched cells remain resting on the well bottom; see photo insert in Figure 11A). Contrast this with Figure 8A where impedance readily detected the impact of antibody 9C12 under the same assay condition. This difference highlights the sensitivity of impedance – which is a consequence of it being a composite readout that reflects changes in cell number, size, attachment strength, and barrier function. In Figure 11B, tracking the number of red nuclei over time effectively elucidates 9C12's neutralizing activity. The reason for this is that even though many dead cells remain in the well out through 120 hours they tend to lose the red fluorescence of their nuclei as they die (see photo insert in Figure 11A). Finally, monitoring the total integrated intensity (TII*) of the virus-encoded GFP is a very effective means of quantifying 9C12's neutralizing activity (Figure 11C). Importantly, when the area under the red nuclei curves and the area under the green TII curves are plotted as a function of antibody 9C12 concentration they yield excellent dose response curves (Figure 11D), with $IC_{50}$ values that differ only 2 to 3-fold compared to that determined using impedance (19, 30, and 67 ng/mL, respectively) (Figure 11D).

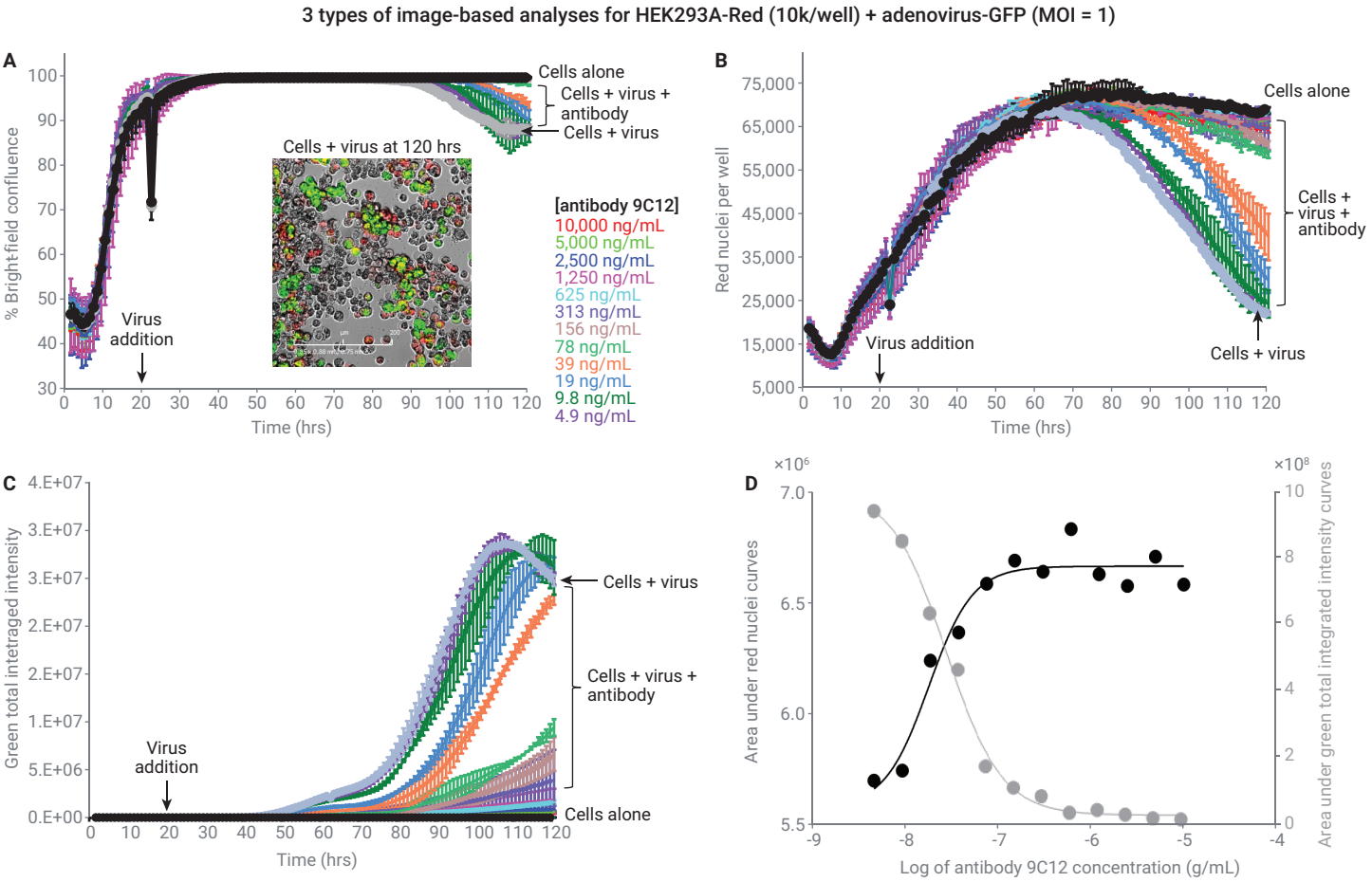
Considering the fact that this is a cell-based assay, the correlation between the different readouts is excellent, and is at least partially a consequence of the impedance and imaging data being acquired from the same poplation of cells. While generation of a primary result and a confirmatory result using an

* Total integrated intensity is the summation of all green light collected from the well bottom. As such, it reflects both the number of green cells and the intensity of the green signal in each of those cells.

orthogonal method is considered best practice, this is rarely done due to the time and cost required. With eSight, both primary and confirmatory results are provided from a single simple assay.

Although using eSight to monitor a virally encoded fluorophore is a very efficient means of studying virus neutralizing antibodies (Figure 11C), this approach is only relevant to the small percentage of viruses that have been appropriately engineered. Using fluorescently labeled host cells is a useful, and generally more accessible, alternative. The third option, exogenous fluorescent dyes, aren't universally useful for all host cell types and care should be taken to ensure that they are not functionally disruptive (Figure 4 and the associated discussion).

When using a low concentration of adenovirus-GFP, expression of the viral GFP precedes any virus-induced changes in impedance (Figure 3C). In contrast, medium to high concentrations of virus cause an immediate drop in impedance (Figures 2A, 2C, and 9B) that precedes viral GFP expression by ~5 hours (data not shown). If accelerating the time to results is critical,

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| $IC_{50}$ of Antibody 9C12 (10k Cells/Well + MOI = 1) | | |
|---|---|---|
| | $IC_{50}$ | $R^2$ |
| Impedance | 67 ng/mL | 0.9927 |
| % Brightfield Confluence | ----- | ----- |
| No. Red Nuclei | 19 ng/mL | 0.9515 |
| Green Total Integrated Intensity | 30 ng/mL | 0.9956 |

**Figure 11.** Image-based quantification of neutralization efficacy for antibody 9C12 under the 10k cells/well + MOI = 1 assay condition. (A) % brightfield confluence as a function of time. Photo insert is for cells infected with virus in the absence of antibody, at the 120-hour time point. Scale bar = 200 µm. (B) Number of red nuclei as a function of time. (C) Green total integrated intensity as a function of time. (D) Dose-response curves based on the area under the red nuclei curves from panel B or the green total integrated intensity curves from panel C. (E) Comparison of $IC_{50}$ values for antibody 9C12, when assayed under the 10k cells/well + MOI = 1 assay condition, as determined using impedance versus image-based analyses.

and if high virus concentrations are accessible, impedance provides a bona fide functional assay that identifies neutralizing activity with unprecedented speed. Moreover, the information that impedance provides regarding the antibody's mechanism of neutralization (Figure 10) simply is not accessible using traditional methods.

Whether using real-time impedance, live cell imaging, or both, the eSight assay described here provides a much simpler, faster, and more information-rich alternative to traditional virus neutralizing antibody assays. In addition to basic research and development, convalescent plasma/therapeutic antibody testing and vaccine development are only a few of the arenas where this assay is being adopted globally.

## References


1. Forthal, D. N. Functions of Antibodies. *Microbiol Spectr.* **2014**, *2(4)*, 1.
2. Pejchal, R. *et al.* A Potent and Broad Neutralizing Antibody Recognizes And Penetrates the HIV Glycan Shield. *Science* **2011**, *334(6059)*, 1097.
3. Chen, J. *et al.* Mechanism of HIV-1 Neutralization by Antibodies Targeting a Membrane-Proximal Region of gp41. *J. Virol*. **2014**, *88(2)*, 1249.
4. Vaillancourt, M. T. *et al.* Inefficient Killing of Quiescent Human Epithelial Cells by Replicating Adenoviruses: Potential Implications for Their Use as Oncolytic Agents. *Cancer Gene Ther.* **2005**, *12(8)*, 691.
5. Gros, A. *et al.* Verapamil Enhances the Antitumoral Efficacy of Oncolytic Adenoviruses. *Mol. Ther*. **2010**, *18(5)*, 903.
6. Zolk, O. *et al.* Structural Determinants of Inhibitor Interaction with the Human Organic Cation Transporter OCT2 (SLC22A2). *Naunyn Schmiedebergs Arch. Pharmacol*. **2009**, *379(4)*, 337.
7. Miakotina, O. L. *et al.* Adenovirus Stimulates Choline Efflux by Increasing Expression of Organic Cation Transporter-2. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2005**, *288(1)*, L93.
8. Varghese, R. *et al.* Postentry Neutralization of Adenovirus Type 5 by an Antihexon Antibody. *J. Virol*. **2004**, *78*, 12320.
9. Smith, J. G. *et al.* Neutralizing Antibody Blocks Adenovirus Infection by Arresting Microtubule-Dependent Cytoplasmic Transport. *J. Virol.* **2008**, *82*, 6492.
10. Bottermann, M. *et al.* TRIM21 Mediates Antibody Inhibition of Adenovirus-Based Gene Delivery and Vaccination. *PNAS* **2018**, *115*, 10440.


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