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. 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.
E Sight 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, 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. 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 from a single simple assay.

Materials and methods
Cell maintenance and assays were conducted at 37 °C/5% CO₂ 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 (MOI) 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. All eSight assays employed Agilent’s E-Plate VIEW microplates (part number 00300601030). The antibodies and proteins used in this study are listed in the more detailed full length application note 5994-3258EN (from which this technical overview is derived).

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 10k 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 MOI =1) 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 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
Proof of concept
Figure 1 displays images for uninfected cells, and cells infected with adenovirus-GFP that had or had not been pre-incubated with the soluble domain of coxsackie adenovirus receptor (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.

In this particular assay neutralizing activity can be detected in three different ways. A neutralizing antibody can 1) prevent red target cells from lysing, 2) block emergence of the virus-encoded GFP signal, or 3) prevent the virus-induced drop in the impedance signal. Using any one of these readouts, if an antibody suppressed the CPE by >50% at the 120-hour time point, it was regarded as being an effective neutralizer.* Using this criterion, two of the antibodies were determined to be positive hits.

*This definition need not be used by others. The criterion for being an effective neutralizer can be adjusted by the user.
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 (Figure 2). After uninfected cells reach confluence at ~50 hours the impedance signal gradually decreases 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).

Figure 1. 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.

Figure 2. Impedance-based quantification of neutralization efficacy. 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.
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 2).

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 2 is legitimate and not simply an artifact of antibody interacting with host cells directly.

Figure 3 shows two different types of image-based analyses of antibody 9C12’s neutralization capabilities. While Figure 3A tracks adenovirus-mediated destruction of red host cells, Figure 3B tracks production of the adenovirus-encoded GFP using green total integrated intensity.** By plotting the area under these curves as a function of the antibody concentration, dose response curves were generated (data not shown). The associated IC_{50} values are displayed in Figure 3C alongside the IC_{50} value generated in a similar manner from the impedance data in Figure 2. Considering the fact that this is a cell-based assay, the correlation between the IC_{50} values from the different readouts is excellent. This is at least partially a consequence of the impedance and imaging data being acquired at the same time and from the same population of cells (i.e. in the same well).

** 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.

### Considerations for assay format

Although using eSight to monitor a virally encoded fluorophore is a very efficient means of studying virus neutralizing antibodies (Figure 3B), this approach is not compatible with wild type viruses and is only relevant to the small percentage of viruses that have been appropriately engineered. Using host cells that express a fluorescent protein is a generally more accessible alternative. As a third option cells can be labeled with exogenous fluorescent dyes, but these do not work in all cell types and care should be taken to ensure that the dyes are not functionally disruptive.

<table>
<thead>
<tr>
<th>IC_{50} of Antibody 9C12 (10k Cells/Well + MOI = 1)</th>
<th>IC_{50}</th>
<th>R^2</th>
</tr>
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<tbody>
<tr>
<td>Impedance</td>
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<td>0.9927</td>
</tr>
<tr>
<td>No. Red Nuclei</td>
<td>19 ng/mL</td>
<td>0.9515</td>
</tr>
<tr>
<td>Green Total Integrated Intensity</td>
<td>30 ng/mL</td>
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</tbody>
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**Figure 3.** Image-based quantification of neutralization efficacy for antibody 9C12 under the 10k cells/well + MOI = 1 assay condition. (A) Number of red nuclei as a function of time. (B) Green total integrated intensity as a function of time. (C) 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.
With the above in mind, in some situations label-free methods for tracking viral CPEs are particularly attractive. Towards this end, eSight’s percent brightfield confluence (not shown here) and impedance (Figure 2) readouts can be used.

When designing an eSight assay it is important to recognize that the time at which a viral CPE becomes detectable varies depending on which readout is being used, as well as the conditions under which the assay is being run. For example, when using a low concentration of adenovirus-GFP (MOI less than 1) expression of the viral GFP precedes any virus-induced changes in impedance. In contrast, medium to high concentrations of virus cause an immediate drop in impedance that precedes viral GFP expression by ~5 hours (data not shown). At MOIs above 25 the virus-induced drop in the impedance signal is so rapid that the neutralizing capability of an antibody can be evaluated in as little as 1 hour (this more extensive data set is included in the full application note from which this technical overview is derived). If accelerating the time to results is critical, and if high virus concentrations are accessible, impedance provides a bona fide functional assay that identifies neutralizing activity with unprecedented speed.

**Conclusion**

The data presented here clearly demonstrate the eSight’s ability to screen antibodies for virus neutralizing activity, and to subsequently quantify the efficacy of hits. Using the protocol shown in this Tech Note, impedance has successfully been employed to study neutralization of 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 transferring 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.

While generation of a primary result and a confirmatory result using an 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.

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 just a few of the arenas where this assay is being adopted globally.

**References**