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Cellular impedance measurement as a new tool for poxvirus titration, antibody neutralization testing and evaluation of antiviral substances

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ABSTRACT

Impedance-based biosensing known as real-time cell electronic sensing (RT-CES) belongs to an emerging technology for analyzing the status of cells *in vitro*. In the present study protocols were developed for an RT-CES-based system (xCELLigence™, Roche Applied Science, ACEA Biosciences Inc.) to supplement conventional techniques in pox virology. First, proliferation of cells susceptible to orthopoxviruses was monitored. For virus titration cells were infected with vaccinia virus and cell status, represented by the dimensionless impedance-based cell index (CI), was monitored. A virus-dose dependent decrease in electrical impedance could be shown. Calculation of calibration curves at a suitable CI covering a dynamic range of 4 log enabled the quantification of virus titers in unknown samples. Similarly, antiviral effects could be determined as shown for anti-poxviral agents ST-246 and Cidofovir. Published values for the *in vitro* concentration that inhibited virus replication by 50% (IC_{50}) could be confirmed while cytotoxicity in effective concentrations was excluded in long-term incubation experiments. Finally, an RT-CES-based virus neutralization test was established. Various poxvirus-specific antibodies were examined for their neutralizing activity and a calculation mode for the neutralizing antibody titer was introduced. In summary, the presented RT-CES-based methods outmatch end-point assays by observing the cell population throughout the entire experiment while workload and time to result are reduced.

1. Introduction

Virological standard procedures include virus titration for estimating the number of infectious particles [1], plaque reduction neutralization tests (PRNT) for determining neutralizing antibody (Ab) titers [2] and characterization of antiviral agents by means of cytotoxicity and antiviral effects [3,4]. Although these techniques are laborious, they are irreplaceable for (orthopox) virus research. Hence, simplified and time-saving approaches might be beneficial.

Orthopoxvirus (OPV) titration is usually performed on a cell layer within microtiter vessels by infection of cells with serial dilutions of a viral stock solution. After a 3–5 day incubation period followed by washing and staining procedures, virally induced plaques are quantified to calculate the number of infectious particles (or plaque-forming units [PFU]) within the viral stock solution. Based on this quantification, antiviral substances and virus neutralizing Abs can be evaluated. For the evaluation of a compounds' antiviral activity determination of its cytotoxicity is a necessity and commonly performed by colorimetric assays using WST-1 or MTT [5]. Ab neutralizing activities are conventionally determined by preincubation of virus particles and Abs before being used in a quantitative plaque titration assay. These simple and potent techniques are laborious and, as end-point assays, allow assessment only after a defined time span, but not during the time course of the experiment.

In order to simplify these techniques by reducing work load and hands-on time the xCELLigence™ RT-CES system was employed for routine virological standard methods.

The utilized RT-CES system is equipped with 96-cavity plates that contain microelectronic impedance sensor arrays capable of measuring distinct changes in interactions between cells and vessel ground (see [6] for a detailed description). Changes in cell number, morphology and interplay with the cavity ground lead to measurable impedance shifts. These were recorded over time and represented by a dimensionless parameter called cell index (CI).

Abbreviations: CI, cell index; IC_{50}, 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OPV, orthopoxvirus; RT-CES, real-time cell electronic sensing; WST, water soluble tetrazolium salt.

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Thus, the system offers the possibility to non-invasively quantify cell status in real-time.

The general benefit of using RT-CES in cell culture experiments has been shown for live monitoring of cell growth and quality control [7–9], cytotoxicity assays [6,10,11], cell adhesion and spreading [12] immune cell activation [13], receptor tyrosine kinase activation [14], G protein-coupled receptor activation [15] as well as cell motion [16]. Preliminary tests presented in an application note by Roche Applied Science showed that impedance-based methods allow the monitoring of cell infection with vesicular stomatitis virus [17].

In this study, we systematically evaluated the RT-CES xCELLigence™ system for accurate poxvirus titration, antiviral compound testing including drug-related cytotoxicity and as a screening tool for OPV neutralizing Abs.

2. Materials and methods

2.1. Viruses and cell culture

HEK293 (ATCC ID: CRL-1573) and HEp-2 (ATCC ID: CCL-23) cells were both cultured in standard D-MEM medium containing 5% FCS and 2 mM L-glutamine, while VERO E6 (ATCC ID: CRL-1586) cells required an FCS concentration of 10%.

The following OPV were used: vaccinia virus (VACV) IHD-W (ATCC ID: VR-1441), VACV Western Reserve (WR, ATCC ID: VR-1354), VACV New York City Board of Health (NYCBOH, ATCC ID: VR-1536), VACV Copenhagen (COP, kindly provided by Dr. Albert Zimmermann), VACV Lister Elstree (LE, kindly provided by Bavarian Nordic), cowpox virus (CPXV) Brighton Red (BR, ATCC ID: VR-302), camelpox virus (CMLV) CP-19 (kindly provided by Dr. Hermann Meyer). High-titer viral stocks were produced on HEp-2 cells according to standard procedures [18].

2.2. Deployment of RT-CES in cell culture

The xCELLigence™ system (RTCA-SP; Roche Applied Science, Mannheim, Germany) used in this work consists of single-use E-plates inserted into an RTCA single-plate (SP) station which is located within the incubator. Moreover, an analyzer unit is placed outside the incubator and links the SP station with a computer. The RT-CES monitors the impedance of each distinct cavity of the E-plate and delivers CI values at time points specified by the user. The CI is calculated by the software according to Eq. (1):

$$\mathrm{CI} = \max_{i=1,\ldots,N}\left[\frac{R_{\mathrm{cell}}(f_i)}{R_{\mathrm{b}}(f_i)} - 1\right] \quad (1)$$

where R_{cell} stands for resistance of the electrode with attached cells and R_{b} stands for resistance of the electrode without attached cells.

The equation defines the CI as a dimensionless parameter which increases with higher cell numbers (or larger cells or stronger cell adhesion) and decreases with lower cell numbers (or smaller cells or weaker cell adhesion).

For evaluation of CI background, which is the CI signal without cells, E-plates were filled with 50 μl of pre-warmed culture medium. Thereafter cells were seeded into the E-plate cavities in pre-warmed culture medium and E-plates were placed within the plate station for monitoring of CI. To visualize the cell line-specific unique proliferation characteristics, growth curves of different cell numbers of each cell line used were recorded initially and cell numbers were optimized.

2.3. Virus titration

The conventional plaque titration test was performed on VERO E6 cells in 24-cavity plates by using tenfold serial sample dilutions from 10^{-1} to 10^{-6} according to standard procedures [18].

In order to perform virus titration with the xCELLigence™ system HEK293 cells were seeded at 7500 cells per cavity (as determined with a Neubauer chamber) into the E-plates as described above. After initial growth data collection for up to 16 h, cells were infected with VACV IHD-W (50 μl per cavity in pre-warmed culture medium) in concentrations ranging from 12.5 to 125,000 PFU/cavity. Finally, four VACV samples (LE, WR, NYCBOH, COP) of unknown titer were subjected to the adhered cells in three tenfold dilutions ($n = 3$). The CI value for all samples was measured for up to 100 h post infection (p.i.) in comparison to uninfected cells. The same samples were quantified by plaque titration in parallel.

2.4. Determination of antiviral effects and cytotoxicity of ST-246 and Cidofovir

ST-246 [19,20] and Cidofovir (CDV, Vistide® [21]) are known to display anti-OPV activity and were used for the evaluation of the RT-CES system's potential in characterizing antiviral compounds. Therefore, cytotoxicity and the *in vitro* concentration that inhibited virus replication by 50% (IC_{50}) of ST-246 and CDV were determined by RT-CES. Once background CI was recorded, 7500 HEp-2 cells/cavity were seeded into the E-Plate in a volume of 90 μl. After allowing the cells to adhere for 24 h, cytotoxicity of both compounds was determined by addition of ST-246 at final concentrations of 1–500 μM and CDV at final concentrations of 100–1000 μM in a volume of 10 μl and in the absence of virus. CI was recorded for 170 h in triplicate. For determination of dose-dependent antiviral effects, infection of adhered cells with VACV NYCBOH (multiplicity of infection (MOI) 0.15; 1125 PFU/cavity) and simultaneous addition of antiviral compounds were performed in a volume of 10 μl. ST-246 and CDV were added in triplicate at final concentrations of 10–1000 nM and 50–1000 μM, respectively. CI values were recorded and after 90 h the IC_{50} was calculated either by the spreadsheet software Graph Pad Prism version 5.01 (Graph Pad Software, San Diego, California, USA), using the log (agonist) vs. response equation, or by xCELLigence™ software version 1.2, selecting the dose–response curve (CI at time point vs. concentration) and the sigmoidal dose–response formula. For comparison, cytotoxicity of ST-246 and CDV was conventionally determined by a colorimetric assay using the cell proliferation agent WST-1 according to the manufacturer's instructions (Roche Applied Science). Calculated values for IC_{50} were compared to the literature.

2.5. NT of OPV neutralizing Abs

The conventional virus PRNT was based on the plaque titration test (see Section 2.3). A selection of sera and Ab preparations from different species was used for PRNT (Table 2). Abs and sera were treated at 56 °C for 1 h in order to inactivate complement factors and centrifuged shortly to remove any possible precipitates.

Formation of virus–Ab complexes was allowed by pre-incubating VACV IHD-W (MOI 0.1; 2.5×10^4 PFU) with serial Ab dilutions for 1 h at 37 °C. The Ab-virus mixture was applied to 2.5×10^5 VERO E6 cells. The neutralizing titer (which is defined as the dilution of Ab that induces a 50% plaque reduction compared to the non-inhibited control sample, NT_{50}) was calculated after 5 days for each Ab.

For the RT-CES based NT test of Abs from different species, 50 μl of medium were used for initial background measurement followed by seeding of 5000 HEK293 cells in a volume of 50 μl and monitoring of CI change for 4 h. A virus dilution of VACV IHD-W (MOI 0.1; 500 PFU) was prepared in 50 μl of medium per cavity. Abs were diluted serially twofold from 1:10 to 1:2560 in 50 μl aliquots of medium. Abs and virus were combined to the final dilution of 1:20 to 1:5120 in a volume of 100 μl and incubated for 1 h at 37 °C in a separate 96-well plate. Finally the 100 μl of

virus–Ab mixture were given into the E-plate containing cultured cells for a total volume of 200 µl. As reference samples, cells were incubated with the highest serum concentration and with virus alone, representing cell growth without virus and the maximal cell growth inhibition by VACV, respectively. CI was monitored and after 30 h p.i. the neutralizing titer was calculated by spreadsheet software.

3. Results

3.1. RT-CES-based virus titration

Infection experiments were performed with several OPV strains (VACV IHD-W, WR, NYCBOH, COP, LE, CPXV BR, CMLV CP-19, see Section 2.1) using HEK293, HEp-2 or VERO E6 cells. Representative titration data is shown for VACV IHD-W on HEK293 cells (Fig. 1A), displaying the CI progression dependent on experiment time and virus amount. After 16 h of cell growth, infection with different PFU was performed and was visible as a slight peak within the CI curve. The characteristics of each curve were a typical cell growth phase (CI increase) followed by a maximum and a final CI decrease reflecting virus-mediated cytopathic effects of different intensity depending on the PFU and nutrient depletion as seen in the uninfected control. Time to CI maximum was clearly PFU dependent. In addition, there was a strong linear correlation between the log virus concentration and the time after infection to reach a certain CI value. This CI value was selected to cover the broadest range of virus concentrations which was a value of CI = 1 for the calibration run shown in Fig. 1A. By plotting time to reach CI = 1 over the log virus concentration a calibration curve was constructed (Fig. 1B). Finally, the titer of four unknown samples was determined. To obtain CI values in the range of the calibration curve, three tenfold dilutions of virus stocks were applied. Time to reach CI = 1 was determined and virus titer was calculated according to the equation.

$$\mathrm{PFU/ml} = 20 \times 10^{\frac{t(\mathrm{CI}=1)-107.4}{-13.9}} \quad (2)$$

(see colored symbols in Fig. 1B). A comparison to the results obtained from conventional plaque testing is given in Table 1 and shows a strong correlation for both techniques.

Taken together, the CI progression after OPV infection is clearly PFU dependent and permits determination of OPV titers in unknown samples.

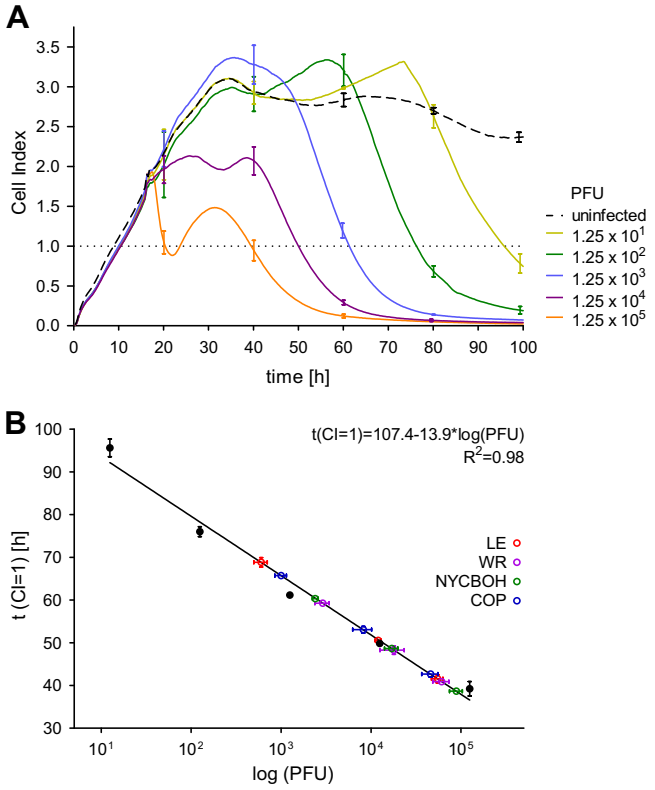

Fig. 1. VACV titration by RT-CES. 7500 HEK293 cells were seeded into each well of the E-Plate. After 16 h cells were infected with serial dilutions of VACV IHD-W and CI development was monitored. (A) CI values recorded over time. (B) Calibration curve for VACV IHD-W to quantify samples with unknown titer, as presented as colored symbols. Time to reach CI = 1 is plotted over log virus concentration/cavity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Cytotoxicity and antiviral effect testing

For the evaluation of cytotoxicity, uninfected cells were incubated with ST-246 or CDV at different concentrations and CI was recorded for 170 h (data not shown). For both compounds cytotoxicity became evident after 100 h p.i. at >500 µM, but not at 100 µM. Anti-OPV effects were analyzed by incubation of OPV-infected cells with antiviral agents at different concentrations. The antiviral efficacy was indicated by an increased CI in samples with ST-246 or CDV compared to infected cells without compound. Although viral replication could not be inhibited completely by ST-246 or CDV, growth curves of compound-treated samples approached the curve of the uninfected control sample. CDV mediated antiviral effects at concentrations of >100 µM (Fig. 2). The resulting IC_{50} was calculated to be 183 ± 2 µM. For ST-246 anti-OPV effects were observed for concentrations of >50 nM, and the resulting IC_{50} was 25 ± 2 nM (data not shown). IC_{50} values calculated by spreadsheet software or the xCELLigence™ software were identical.

In conclusion, dose-dependent anti-OPV effects of two substances could be determined by RT-CES while cytotoxicity in effective antiviral concentrations was ruled out.

3.3. Neutralizing activity of anti-OPV Abs

Similar to virus titration, the data for NT activity was collected in terms of CI values. Fig. 3 shows the CI progression after application of anti-OPV IgY or rat-Abs and Table 2 shows the data for sera from distinct species (chicken, human, rat and elephant). Serum treated (1:20) HEK293 cells showed a typical growth curve without reaching the maximum before 30 h. Infected cells showed a lower maximum CI value (0.60 ± 0.01) after approximately 22 h followed by a CI decrease (Fig. 3). Dilution of neutralizing Abs was reflected by the corresponding CI progression. Abs displaying high neutralizing activity allowed growth of cells similar to that of untreated cells, while a decreasing Ab concentration was accompanied by a CI decrease.

Table 1
Comparison of OPV titers obtained from RT-CES and conventional plaque testing for four different VACV.

	Titer by RT-CES		Titer by plaque-test	
	PFU/ml	SD[a]	PFU/ml	SD[a]
LE	1.6×107	±07.7%	8.9×10^6	±13.9%
WR	3.6×10^7	±20.1%	5.0×10^7	±12.9%
NYCBOH	3.3×10^7	±11.2%	6.3×10^7	±10.3%
COP	1.5×10^7	±14.0%	5.0×10^7	±19.6%

[a] SD = standard deviation.

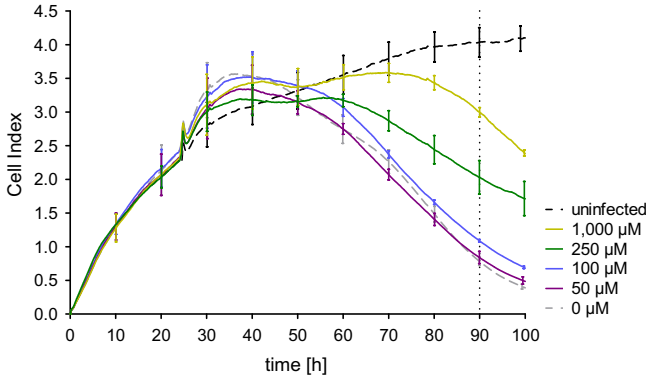

Fig. 2. Determination of anti-OPV effects of Cidofovir by RT-CES. 7500 HEp-2 cells were seeded into each well of the E-Plate. After 24 h cells were infected with VACV NYCBOH (MOI 0.15; 1125 PFU) and 50–1000 µM Cidofovir was added in triplicate. Growth curves were recorded by the xCELLigence™ system.

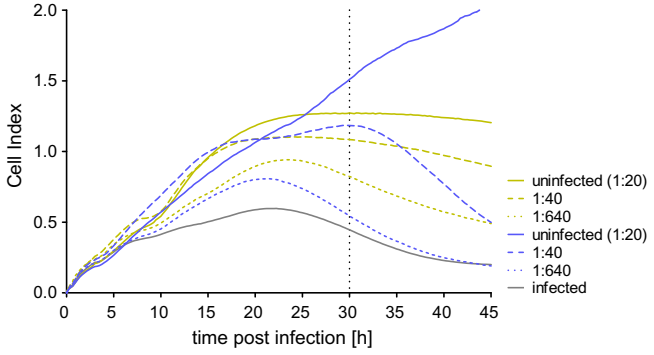

Fig. 3. VACV neutralization by anti-OPV rat-Abs or IgY by using RT-CES. 5000 HEK293 cells were infected with 500 PFU of VACV IHD-W after pre-incubation with serial dilutions of anti-OPV rat-Abs (shown in green) or IgY (shown in blue). CI was monitored for 45 h. The *x*-axis shows experiment time and the *y*-axis the appending CI values. The infected control without sera is displayed in gray. Neutralization experiments were performed in duplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Quantification of neutralizing Ab titer by RT-CES

Table 2 summarizes the neutralizing titers of the sera tested ranging from 1:320 for Omri Gam™ and anti-OPV IgY Abs to 1:80 for elephant sera. For determination of NT_{50} by RT-CES the CI of uninfected serum treated cells (CI_U) and infected (CI_I) control cells was compared 30 h p.i. A CI value exactly in between CI_U and CI_I at the same time point was calculated to reflect 50% neutralization as in the classical PRNT:

$$CI_{NT50} = \frac{1}{2}(CI_U - CI_I) + CI_I \quad (3)$$

The Ab dilution with a CI nearest to the calculated CI_{NT50} ($CI_0 \leqslant CI_{NT50}$) represented the minimal neutralizing titer of the Ab. The CI_{NT50} values determined by this method are shown in Table 2 as $RTNT_{50}$ (real-time neutralization titer 50).

A further goal of this study was to establish an accurate calculation method for quantification of virus neutralization. To this end, determined CI values after 30 h p.i. and corresponding Ab dilutions were plotted in a logarithmic manner. The equation for the linear slope of the graph was utilized to calculate the Ab dilution exactly corresponding to CI_{NT50}. The quantitative $RTNT_{50}$ ($qRTNT_{50}$) could be calculated according to (4).

$$CI = \text{slope} \times \ln(\text{Ab titer}) + \text{axis intercept} \quad (4)$$

After transformation and insertion of CI_{NT50} for CI and $qRTNT_{50}$ for Ab titer, the following Eq. (5) was formed:

$$qRTNT_{50} = e^{\left(\frac{CI_{NT50} - \text{axis intercept}}{\text{slope}}\right)} \quad (5)$$

The calculation of $qRTNT_{50}$ is shown exemplarily in the case of rat anti-OPV Ab. The $qRTNT_{50}$ was calculated as shown in Eqs. (6) and (7):

$$CI_{NT50} = \frac{1}{2}(1.27 - 0.33) + 0.33 = 0.80 \quad (6)$$

$$qRTNT_{50(rat)} = e^{\left(\frac{0.80 - 1.43}{-0.12}\right)} = \underline{\underline{191}} \quad (7)$$

All calculated $RTNT_{50}$ values can be found in Table 2. In summary, RTNT values can be determined by comparison to uninfected and infected cells.

4. Discussion

In the present study RT-CES was evaluated for its benefits in complementing standard virological techniques. To this end, the CI value as a measure of the cell number, morphology and strength of adhesion of cells, was used to quantify virally induced cytopathic effects. Since the OPV used in this study induce the formation of plaques in infected cells and are capable to inflict substantial damage on the entire cell culture [2,22,23], RT-CES should be applicable to monitor the status of poxvirus infected cells.

After OPV infection a PFU-dependent decrease in electrical impedance could be detected, indicating an option for poxvirus titration. After an initial growth phase, the CI values in cultures of higher virus load decreased earlier than in those of lower virus load. Samples could be quantified in a linear dynamic range from 12.5 to 125000 PFU per cavity, reflecting 250–2.5×10^6 PFU/ml. Since the xCELLigence™ system provides real-time data, the selection of a qualified CI value enabling the quantification of high and low virus concentrations can be done without additional operative effort (see Fig. 1B). Each virus–cell combination needs its own calibration experiment with virus dilutions of known titer to choose the appropriate CI due to different growth characteristics of cells and viruses. However, on each plate duplicates of 45 viral samples can be analyzed and compared to the calibration curve measured in parallel, emphasizing the applicability of the RT-CES system for high-throughput applications.

Based on virus quantification, RT-CES was used to characterize antiviral agents. For already established anti-OPV compounds

Table 2
Neutralization titers of several anti-OPV Abs and sera determined by classical PRNT and RTNT 30 h p.i.

Ab/serum	Description	$PRNT_{50}$	$RTNT_{50}$	$qRTNT_{50}$
OmriGam™	Serum, VACV-vaccinated human	1:320	1:160	1:170
Rat Ab	Serum, OPV-infected rat	1:160	1:160	1:191
Elephant Ab	Serum, OPV-infected elephant	1:80	1:40	1:82
Anti-OPV IgY	IgY [24]	1:320	1:160	1:93

(ST-246 and CDV) the range of IC_{50} values could be reproduced. The online analysis of growth curves allowed the selection of an individually determined optimal time point for calculation of IC_{50} (90 h) on the one hand, while cytotoxic effects could be shown to occur only later in the time course of the experiment (data not shown). Moreover, temporary effects on cell viability would have been identified by the real-time monitoring of the cell status, an advantage in contrast to end-point assays.

For evaluation of neutralizing Abs both, conventional PRNT and the here presented RTNT, use virus titration as their basic principle. Virally induced plaques are counted and plaque reduction due to the presence of Abs with neutralizing activity is determined. Using RT-CES, neutralizing activity of Abs could be monitored by increased CI compared to the infected control without Abs treatment. Interestingly, in samples with a low Ab dilution cell growth was slightly increased in comparison to uninfected cells, which makes a serum treated cell sample the most reliable positive control to be compared to infected cells.

A further advantage of RT-CES for the screening of neutralization activity of Abs was the option to perform quantifications when compared to calibration curves generated by analyzing dilutions of neutralizing Abs. For one species, serial dilutions can serve as reference for several samples of the same species. This approach introduced here allowed quantitative real-time NT with the neutralization titer described as quantitative $RTNT_{50}$ or $qRTNT_{50}$.

Further advantages of RT-CES for applications in the virological lab were the speed (RTNT was performed within 40 h [including infection] compared to 3–5 days in the conventional way) and options for automation and workload reduction. The possibility to use multiple samples in parallel as well as the application of the CI as parameter led to a higher objectivity as compared to the case of plaque counting.

However, in initial trials it could be shown that in some cases cavities at the edge of the E-plate provided poor cell growth conditions when monitored over several days. This fact, possibly due to medium evaporation, led to a reduced number of analyzable cavities of a 96-well plate that could be alleviated by application of phosphate buffered saline to the inter-cavity space according to the manufacturer's specifications. Further constraints of the xCELLigence™ system were the exclusive analysis of adherent cell lines and difficulties in performing microscopic analyses with E-plates. Finally, the interplay of the three parameters cell number, morphology and adherence, that had an impact on the impedance and therefore also on the CI value, hampers the interpretation of the CI values gained.

In summary, the RT-CES was assessed as a useful tool in virology which could provide additional data when compared to classical methods. The system allowed dense real-time data collection over several days, combined with low operative effort, and avoided the danger of potentially missing significant events as may happen in end-point assays. The usability of RT-CES could be shown for OPV titration, characterization of potentially new antiviral drugs and neutralizing Abs. Although the RT-CES can not replace conventional quantitative methods in virology, it will help in high throughput screenings to reduce workload and time to result.

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