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Short communication

Use of electric cell-substrate impedance sensing as a tool for quantifying cytopathic effect in influenza A virus infected MDCK cells in real-time

Morgan H. McCoy [1], Eugenia Wang *

University of Louisville, Department of Biochemistry and Molecular Biology, Gheens Center on Aging, Delia Baxter Building II, rm 102, 580 South Preston Street, Louisville, KY 40202, USA

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Abstract

Resulting from its subjective nature, cytopathic effect (CPE) due to virus infection in cell culture has long been difficult to quantify. This report illustrates the use of electric cell-substrate impedance sensing (ECIS) for monitoring the progression of CPE due to influenza A virus infection. ECIS monitors the impedance of a non-invasive ac current flowing through cell culture medium by gold film electrodes placed on the surface of the culture dish. As cultured cells attach and spread onto the electrodes, the current is impeded proportional to the number of attached cells, the number of tight junctions between cells and the shortness in distance between the cells and the substratum. In the case at hand, a healthy monolayer of cells was insulted with influenza A virus infection and exhibited a characteristic rounded cell morphology and cell detachment. These effects resulted in reduced impedance, which was monitored with ECIS. Since data obtained through ECIS are both quantitative and in real-time, it was possible to monitor continuously cell behavior during infection. This, in turn, allowed for a more detailed and comprehensive data set to analyze. More importantly, through ammonium chloride treatment of cells, it was also shown that ECIS may be exploited to examine a treatment's effect on the reduction of resistance because of its antiviral activity. Thus, ECIS may be a powerful approach for screening antiviral compounds quantitatively in a real-time fashion.

Influenza A virus contains a segmented, negative sense, single stranded RNA genome and is thought to be the cause of upwards of 500,000 deaths globally each year (World Health Organization, 2004). The introduction of a particularly virulent avian influenza virus into the human population of Hong Kong in 1997 (Claas et al., 1998; Subbarao et al., 1998), compounded with the continued circulation of similar viruses in the area lead many to believe that another pandemic influenza will occur in the not too distant future (Kaye and Pringle, 2005; Palese, 2004). With conditions as they are, research leading to new antiviral drugs is needed urgently. Efficient methods for studying drugs' antiviral potential are often extremely time consuming and are limited by the techniques available for studying the effects of virus replication in cell culture systems.

Resulting from their ability to produce quantitative data, assays for cell viability or apoptosis are used often in lieu of microscopy. Smee et al. (2002) compare many of the "... colorimetric, fluorometric and visual methods ..." currently available for analyzing the anti-influenza and cell toxicity characteristics of compounds. In the methods described, measurement of cell viability is the common denominator. Cell viability or apoptosis assays, such as the MTT or TUNEL staining methods, respectively, typically require harvesting cells at multiple times post-infection and are, therefore, limited by the number of replicates feasible for a given experiment. Arbitrary harvest intervals post-infection are often used based on previous experience or simple practicality. This creates a situation where key events taking place between harvest intervals may be ignored. Alternatively, time lapse microscopy can be used to observe the effects of viral infection in cell culture. This method permits observation of cells

* Corresponding author. Tel.: +1 502 852 2556; fax: +1 502 852 2555.
E-mail addresses: morgan.mccoy@uky.edu (M.H. McCoy), eugenia.wang@louisville.edu (E. Wang).
[1] Present address: University of Kentucky, Livestock Disease Diagnostic Center, P.O. Box 14125, Lexington, KY 40512, USA.

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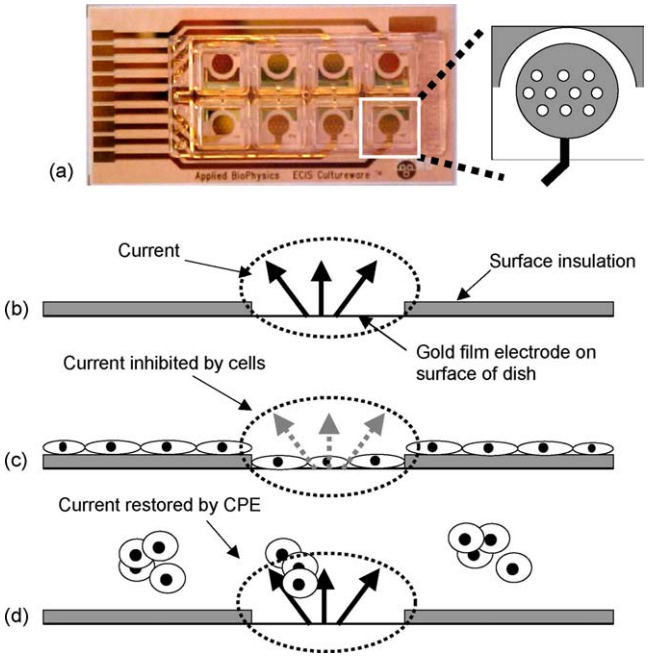

Fig. 1. Electric cell-substrate impedance sensing (ECIS) measured viral cytopathic effect (CPE). (a) In ECIS cells are grown on the surface of culture dishes lined with gold film electrodes. The depicted 8W10E 8 well culture dish has 10 non-insulated circular areas per well for current flow, which are illustrated in the expanded drawing of a culture well. (b) A devise measures a non-invasive ac current as it flows through the culture medium. (c) As cells attach and spread on the surface of the electrodes the current is impeded. (d) The CPE resulting from viral infection releases cells from the surface of the dishes, which restores the current.

in real-time, which the MTT and TUNEL staining methods do not. Unfortunately, what time lapse microscopy gains in the way of real-time observation it loses in the power of quantitation. Electric cell-substrate impedance sensing (ECIS) (Applied BioPhysics, Inc.) is a technology which is not only capable of producing quantitative data, but is also able to monitor experiments in real-time (Giaever and Keese, 1984).

ECIS measures the interaction between cells and the substrate to which they are attached via gold-film electrodes placed on the surface of culture dishes (Fig. 1). Through these electrodes a device measures the ability of the cell monolayer to impede a non-invasive ac signal. The 8W10E (Applied BioPhysics Inc.) eight well culture dish contains an identical circular electrode in each well, which in turn has 10 smaller identical circular un-insulated openings to allow for current flow (Fig. 1). As the number of cells attaching and spreading on the electrodes increase, so does the impedance of current flowing through the culture medium (Fig. 1). This signal impedance is accomplished not only through the insulation of the electrodes by the cell membranes, but also through the tight junctions formed between neighboring cells and the distance the cells are from the substrate to which they are attached. This technology has been exploited in such studies as cell morphology, cell-substrate interactions, cell layer barrier function, cell motility and wound healing (Giaever and Keese, 1993, 1991, 1986; Keese et al., 2004; Lo et al., 1993; Mitra et al., 1991; Tiruppathi et al., 1992; Wegener et al., 2000). Since cytopathic effect (CPE) due to viral infection is typically characterized by a rounded cell morphology and detachment from the surface of the culture dish, it was hypothesised that ECIS would be useful in quantifying the level of CPE due to viral infection (Fig. 1). To test this hypothesis, a working model of influenza A virus infection of MDCK cells was employed.

This report shows that as the CPE caused by influenza A virus infection became more severe, the signal impedance from the cell monolayer was reduced in a dose-dependent manner. Additionally, upon pretreatment with ammonium chloride (NH_4Cl), which inhibits virus entry into the cell (Jakeman et al., 1991), the reduction in signal impedance due to influenza infection was abolished. Since data were collected from a single group of cells throughout the course of the experiment, rather than from multiple time-point replicates often necessary for cell viability or apoptosis assays, ECIS eliminated effectively the possible introduction of variability between replicates. Furthermore, since ECIS was in real-time, up-to-the-minute data were obtained, allowing for greater accuracy in plotting the timeline of CPE. Collectively these findings illustrate that this technology could be exploited in the investigation of processes affecting the rate and severity of CPE in cell culture including, but not limited to, antiviral drugs and signal transduction pathways affecting virus replication.

Prior to cell seeding the 8W10E test array was equilibrated with 200 μL of growth medium (EMEM containing 10% fetal bovine serum (FBS) and antibiotics) per well for at least 30 min in a humidified, 37 °C, 5% CO_2 incubator. ECIS data collection began upon seeding of MDCK cells at a concentration of 1×10^5 cells per well in growth medium. Once the resistance had reached a plateau, generally between 24 and 36 h post-seeding, the cells were washed twice with serum free EMEM containing antibiotics and subsequently infected in duplicate with influenza/A/PR/8/34 virus. Virus was diluted in serum free EMEM containing 1 μg/mL TPCK treated trypsin, antibiotics and included MOIs of 1, 5 or 10 in addition to mock controls. Following a 1 h inoculation period the virus was removed and replaced with maintenance medium (EMEM containing 0.125% bovine serum albumin (BSA) and antibiotics). ECIS data were collected continuously at 400, 4000 and 40,000 Hz frequencies until approximately 48 h post-infection (PI). The data collected at 4000 Hz are depicted in Fig. 2a. Initially all cells, including mock controls, exhibited a spike in resistance before beginning to descend. These measurements corresponded to the time directly after addition of the inoculum to the cells and therefore have been attributed to the physical manipulation the cells underwent during inoculation. Approximately 3–4 h PI, cells which received an MOI of 5 or 10 exhibited an initial rise in resistance peaking 7–8 h PI before falling. Although lagging slightly behind, cells which received an MOI of 1 exhibited similar readings. By 48 h PI, the resistance of the cells infected at an MOI of 5 and 10 had dropped to levels

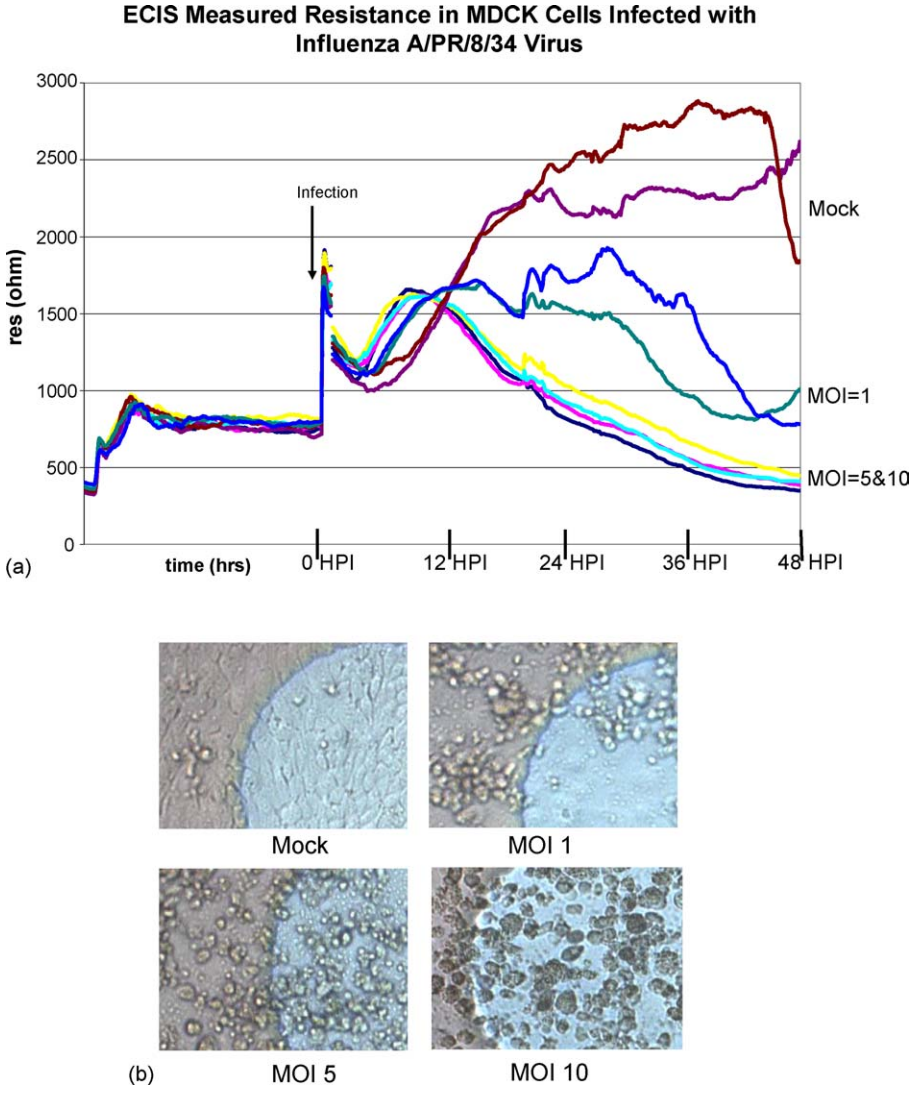

Fig. 2. ECIS measurements from influenza/A/PR/8 virus infected MDCK Cells. MDCK cells undergoing ECIS measurement were inoculated in duplicate with influenza A virus at MOIs of 1, 5 and 10 in addition to mock controls. (a) Approximately 48 h post infection (HPI) the experiment was terminated and the resistance at 4000 Hz was plotted against time. (b) To confirm virus infection the presence of cytopathic effect (CPE) was visualized and photographed 48 HPI. The blue area in the photographs is a non-insulated area of an electrode on the surface of the culture dish. This experiment was performed three times with similar results.

approximating those prior to cell seeding. Cells infected at an MOI of 1, although not as severe, also exhibited lower resistance compared to mock controls. Photographs taken of the cells 48 h PI illustrated the varying degree of CPE in the virus infected cells compared to the mock controls. The ECIS data agreed with the observed CPE, which was indistinguishable in cells infected at an MOI of 5 or 10 compared to the reduced CPE seen in cells infected at an MOI of 1 (Fig. 2b).

To confirm ECIS could be used for the analysis of compounds or treatment affecting virus replication, MDCK cells were seeded as before in an 8W10E test array. Once the resistance had stabilized, the cells were either pretreated with 20 mM NH_4Cl or left untreated in maintenance medium for 1 h. Cells were then either inoculated with the PR8 influenza A virus at an MOI of 5 or with a mock inoculum in serum free EMEM containing 1 μg/mL TPCK treated trypsin and antibiotics. Cells pretreated with 20 mM NH_4Cl remained under NH_4Cl treatment throughout the experiment, including the inoculation period. Data were collected by ECIS as before for approximately 48 h PI and are depicted in Fig. 3a. Pretreatment of MDCK cells with 20 mM NH_4Cl, a known virus entry inhibitor (Jakeman et al., 1991), resulted in similar resistance curves for both mock and influenza inoculated cells. The only cells exhibiting the characteristic rise and subsequent fall in resistance following inoculation were the un-NH_4Cl treated, PR8 influenza A virus inoculated positive controls.

The cause for the brief rise in resistance of the cell monolayer following influenza A virus inoculation is unknown. However, since pretreatment with NH_4Cl appeared to abolish this phenomenon, the cause is not likely to be due to virus attachment to the cell since virus attachment was likely not

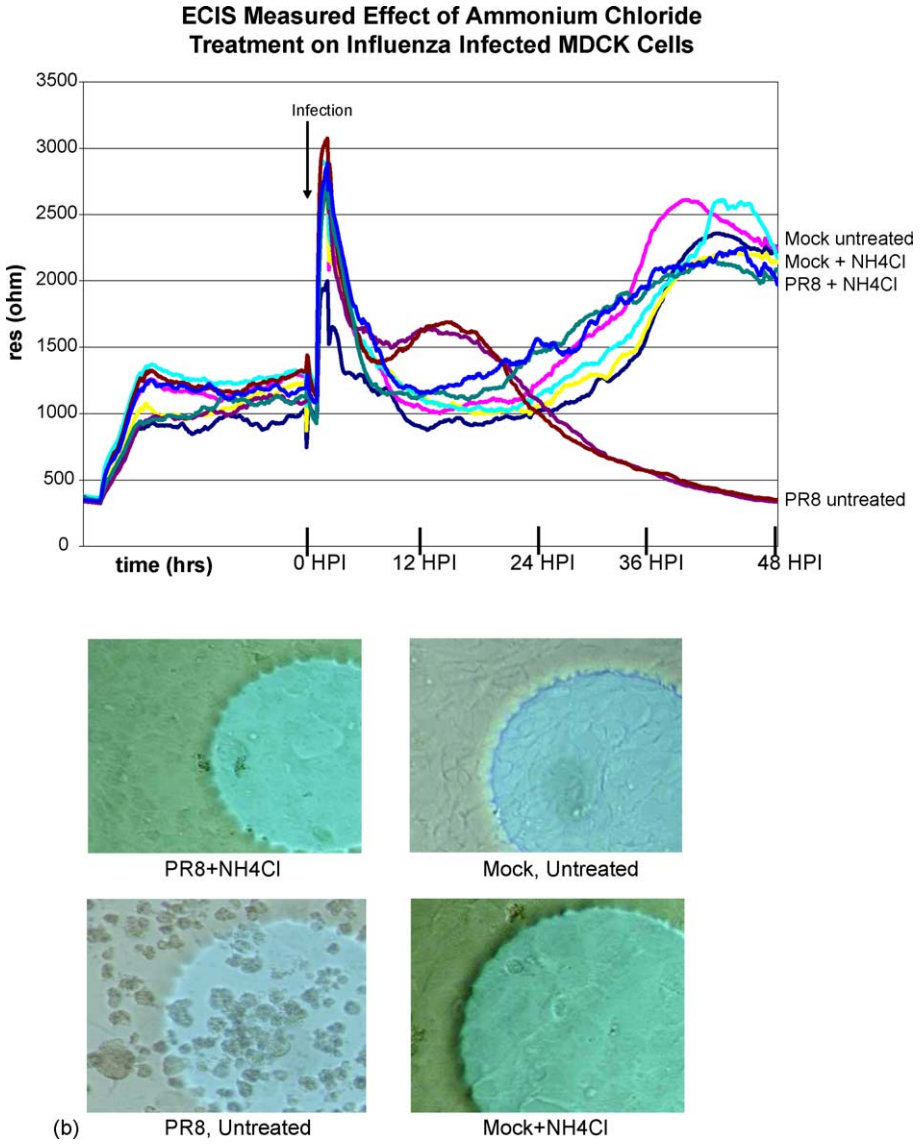

Fig. 3. ECIS measured effect of ammonium chloride treatment on influenza infected MDCK cells. MDCK cells undergoing ECIS measurement were either treated with 20 mM NH_4Cl or left untreated for 1 h prior to inoculation with either influenza/A/PR/8/34 at an MOI of 5 or a mock inoculum. (a) Approximately 48 h post infection (HPI) the experiment was terminated and the resistance at 4000 Hz was plotted against time. (b) To confirm virus infection the presence of cytopathic effect (CPE) was visualized and photographed 48 HPI. The blue area in the photographs is a non-insulated area of an electrode on the surface of the culture dish. This experiment was performed twice with similar results.

affected by this treatment. Accordingly, virus entry into the cell could have caused a series of inter- and intra-cellular remodeling events, which in turn could have resulted in an increased number of tight junctions between cells in the monolayer and/or a decreased distance between the cells and the surface of the culture dish. Both of which would result in an increase in the observed resistance (Giaever and Keese, 1991). Ultimately, once the CPE were to overcome the resistance increase due to cellular remodeling, the trend would reverse leading to a reduction in the measured resistance similar to the current observations (Figs. 2a and 3a).

Although the ECIS instrument collected data at three different frequencies: 400, 4000 and 40,000 Hz; the data collected at 4000 Hz appeared to provide more meaningful results. The general trend was the same in all three instances; however, a greater separation in the resistances measured between the variables was observed at 4000 Hz (data not shown). Since all viral infections vary with regard to their specific kinetics of pathogenesis, it is doubtful that 4000 Hz is optimal for all. Therefore, it would be recommended that data be collected at three frequencies to ensure a comprehensive sampling.

Owing to the massive cytopathology in cell culture, influenza A virus infection of MDCK cells was an obvious test model for the use of ECIS in measuring CPE. Inhibition of CPE in influenza infected cells through pretreatment of NH_4Cl was also observable with ECIS, illustrating its potential in screening antiviral compounds. In fact, any viral

infection resulting in CPE together with that inhibition of CPE in cell culture could theoretically be analyzed using ECIS. Additionally, in light of the observed signal impedance spike directly following manipulation of the cells at the time of inoculation and the characteristic rise and fall of resistance following influenza A virus inoculation, ECIS appears to have great sensitivity for detecting changes in cells that may not necessarily be observable under conventional microscopy. Since the number of tight junctions and the distance between the cells and the substrate to which they are attached affect the flow of current through the system (Giaever and Keese, 1991), it is possible to measure changes in these two characteristics that might otherwise go unnoticed. Therefore, it may be worthwhile to use ECIS for monitoring non-cytopathic virus infections where, although no gross pathology is observed, small or subtle changes in the cell monolayer may be detected.

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