Electric Cell-substrate Impedance Sensing: A Label-free Method to Continuously Monitor Cell Behaviors In Vitro

Electric Cell-substrate Impedance Sensing (ECIS[®]) is a method used to measure cell behaviors continuously in real-time without the use of intrusive labeling techniques. By using a non-invasive alternating current and a multifrequency approach, ECIS[®] can quantify barrier function, cell proliferation rates, cytotoxicity, migration and more. In this application note, we will discuss the use of simple and complex impedance and how using a spectrum of frequencies can identify and monitor cellular behaviors in vitro.

Introduction

Ohms law (V = IR) states that the voltage across a resistor is equal to the product of the direct current (DC) (amperes) and the resistance (ohms). The same law holds for alternating current (AC) circuits where the current oscillates in a sinusoidal fashion at a given frequency, but instead of resistance, AC deals with impedance (Z), which is also measured in ohms. This impedance can come from different sources. Part of it can be pure resistance, but capacitors and coils (inductors) have reactance (measured in ohms) that can contribute to the total impedance.

Electric Cell-substrate Impedance Sensing (ECIS®) takes advantage of the properties of alternating currents to quantify cellular behaviors. ECIS® was first described by the founders of Applied BioPhysics in a PNAS paper (Giaever & Keese, 1984). The early work at General Electric's R&D Center demonstrated that one could

monitor some aspects of the behavior of cultured cells by growing them upon electrodes that served as substrates, applying very weak alternating currents, and monitoring the impedance of the electrodes. In the ECIS[®] setup, there is no *inductive* reactance to consider, but the cell membranes, as well as the gold electrodes, have *capacitive* reactance that can alter the total measured impedance.

In this application note, we will attempt to explain 1) the concept of using multiple AC frequencies do determine cell behaviors, 2) the concepts of simple and complex impedance, and 3) what we can learn about cells in culture from these impedance data. As a first example, consider the model in Figure 1, where the impedance is plotted as a function of time. As the cells attach and spread upon the electrode, the current is impeded by the plasma membranes resulting in the impedance increase and subsequent decrease from a treatment that results in disruption of the cell monolayer.



Figure 1: Schematic representation of ECIS data with impedance vs time. As cells grow and cover electrodes, impedance data rises proportional to cell coverage of the gold electrodes.



The Importance of AC Frequency

Cellular impedance using ECIS[®] involves applying a known AC current and measuring the voltage across two ECIS[®] electrodes. The ratio of measured voltage to the applied current is the impedance, but remember, there is another variable to consider – the AC frequency. In ECIS[®] measurements, the frequency can be varied from a few Hertz to a hundred thousand Hertz, and the choice of frequency can determine what aspect of the cell monolayer is measured.

When a monolayer of cells forms on the ECIS[®] electrodesubstrate, current can flow in two different paths:

1. Through the culture medium surrounding the cells:



2. Capacitively coupling through the cell membranes:



Current, carried by ions in the culture medium, flows from the electrode-substrate into the narrow spaces between the substrate and basal membrane, around the focal adhesions, and up through the paracellular space between adjacent cells. This path is purely resistive, and its resistance does not change with the AC frequency.

As the alternating current oscillates, there is a transfer of electrical charge between the conducting culture medium and the surface of the insulating cell membrane—this oscillating redistribution of charge results in a capacitive current. No ions pass through the membrane, neverthe-less by alternately charging and discharging the membrane, there is a resulting current flow in the circuit.

The important difference in this current path is that the capacitive reactance (X_c) that contributes to the overall impedance, varies inversely with the AC frequency. At relatively low frequencies, the reactance is high, and there is little capacitive flow of current. Since current will always follow the path of least resistance, most of the current at this frequency flows through the solution under and between the cells (red arrows) (Figure 2a). Alternatively, the membrane reactance is low at high frequencies, and most of the current now passes capacitively through the plasma membranes (green arrows) (Figure 2b).



Figure 2: Sources of impedance of alternating current paths through cell monolayers. (a) At low AC frequency, the majority of impedance is from resistance due to the current flowing around the paracellular space (red arrows), whereas with (b) high AC frequency, the majority of impedance is due to the current capacitively coupling through the cell membranes (green arrows).



Simple Impedance

Simple impedance takes advantage of Ohm's law (V = IZ) by applying a fixed alternating current (I) and measuring the subsequent voltage (V) changes allowing for the calculation of the impedance (Z) caused by the cells. The following figure is an example of simple impedance using different AC frequencies applied to electrodes freshly inoculated with MDCK cells at sufficient numbers to achieve confluence upon attachment and spreading. The time course of impedance is measured both at a high frequency (40 kHz) and a low frequency (400 Hz). Notice the very different time courses observed depending only upon the AC frequency being used.

At high frequency, the impedance data plateau approximately 3 hours post inoculation (the first bluedotted line) (Figure 3a). The same cell samples, however, monitored at low frequency, have barely started to increase, and it is not until ~10 hours postinoculation that we see the data reaching a plateau (the second blue-dotted line) (Figure 3b).

To aid in the interpretation of these data, similar inoculations of MDCK cells were stained for the tight

junction proteins zonula occludens-1 (ZO-1) and E-cadherin at the two different times denoted by the blue dotted lines. It is clear that at the 3-hour mark, the cells have formed a confluent monolayer, but the stains remain diffuse (Figure 3c). By the 10-hour mark, however, the stains are localized at the tight junctions between the cells (Figure 3d).

Complex Impedance

Unlike the simple impedance, where we use Ohm's law to determine Z in ohms, the complex impedance is a measure of the pure resistance (R) and the capacitive reactance (X_c) (both measured in ohms) that together account for the overall impedance*.

*The two quantities add as the square root of the sum of the resistance squared and the reactance squared.

 $Z = \sqrt{(R^2 + X_2^2)}$



Figure 3: Comparison of confluence and barrier formation of MDCK II cells. (a) ECIS[®] readings at high AC frequency (40,000 Hz) reveals plateauing impedance at ~3 hours suggesting confluence of the monolayer versus (b) low AC frequency showing plateauing at ~10 hours suggesting maturation of cell-cell junctional formation. (c) Fluorescence images of the same seeding parameters as the ECIS[®] measurements for zona occludens, E-cadherin, and F-actin 3 hours after inoculation and (d) 10 hours after inoculation. Graphs and figures courtesy of Joachim Wegener, University of Regensburg.



The breakdown of the impedance into these two sources is possible because *capacitance* in the system results in a phase difference between the current and the voltage. This is shown diagrammatically in the figure below, where we apply a known alternating current (I; dotted trace) and measure the voltage (V; solid trace) as a function of time. Notice that the two sinusoidal curves have the same frequency but are shifted such that the voltage lags the current, with the curves peaking at different times. This shift is the phase difference (ϕ) and is expressed in degrees where one complete sinusoidal cycle is 360 degrees.



Based on both the voltage amplitude and the phase difference, the software determines what value of **resistance** and **capacitance** in a series RC circuit would give this result. So we end up with a time course measurement of series resistance, measured in ohms, and of series capacitance, measured in nanoFarads (nF).

As previously mentioned, the reactance varies inversely with the AC frequency (f). The equation below gives the exact relationship between capacitance and capacitive reactance (ohms):

$$X_{c} = 1/(2\pi fC)$$

One can see changes related to cell behavior when measuring simple impedance with multiple frequencies, but these are qualitative in nature. With complex impedance, one can quantitatively describe specific morphological properties of cultured cells.

Cell Behaviors Measured with ECIS

Barrier Function

As previously mentioned, the resistive portion of the total impedance results from the path of the alternating current flowing beneath and around the paracellular space, making low frequency resistance ideal for barrier function measurements. In the following figure, MDCK



I cells were seeded at time 0 and monitored at low AC frequency (250 Hz) until a plateau in the resistance data indicating a mature barrier formation (Figure 4). Once the barrier formation was confirmed, the cells were treated with varying concentrations of known barrier disrupter cytochalasin D (Stevenson & Begg, 1994), and were then continued to be monitored with ECIS[®].

Proliferation

When current is applied at high AC frequency (>32,000 Hz), capacitance is easily measured as most current capacitively couples through the cell membranes. The open gold-film electrode (without cells) has a relatively high specific capacitance of ~10 microFarad/cm². As the cells attach and spread upon the gold electrode, their membrane capacitance of ~1 microFarad/cm² is in series with the electrode capacitance. This results in a *drop* in the measured capacitance, reaching a minimum once the monolayer of cells is confluent.

It has been shown that the capacitance measured at high AC frequency decreases linearly with the fractional area covered by the cells (Wegener, Keese, & Giaever, 2000). An example of this is shown in Figure 5 where MDCK I cells were seeded at decreasing quantities, and the capacitance was monitored as the cells attached and proliferated over the ECIS[®] electrodes until plateauing at the bottom indicating confluence.

Attachment & Spreading

Traditional "counting attached cell assays" can only quantify the number of cells attached to any ECM coating. ECIS assays give feedback of the behavior of the cells on the varying ECM models. When cells are seeded at a high enough density eliminating the need for mitosis to achieve confluence, the quick drop in capacitance represents the attachment and spreading in morphology of the cells (Hung et al., 2022). In the following graph, MDCK II cells were seeded at a confluent density and then attachment and spreading were subsequently monitored with ECIS® with either fibronectin, laminin, vitronectin, or albumin (BSA) as the substrate protein (Wegener, Keese, & Giaever, 2000). t₁₀ represents the time necessary to reach half of the maximum capacitance from the cell-free electrode to the confluent layer as displayed on the laminin curve.

Cytotoxicity

Not only can ECIS measure the attachment and proliferation of cells in culture as they cover the electrodes, it can also measure the cytotoxicity of cells (Xiao, Lachance, Sunahara, & Luong, 2002). Similar to reading high frequency (> 32,000 Hz) for proliferation in terms of cell coverage, cytotoxicity can also be quantified in the same way. As cells go through necrosis in response to toxic treatments, they will lift off of the gold electrode, hence reducing impedance (or increasing capacitance). In the following example, BSC-1 cells were grown to confluence and then treated with varying concentrations of dimethyl sulfoxide (DMSO) and subsequently monitored with ECIS[®] using high frequency capacitance (Figure 7).



Figure 4: Barrier disruption of MDCKI cells. Low frequency resistance monitoring of MDCK 1 cells treated with varying concentrations of Cytochalasin D.



Figure 6: Attachment rates of MDCK II cells. High frequency capacitance monitoring of MDCK II cells with fibronectin, laminin, vitronectin, or BSA as the substrate protein.



Automated Wound-Healing

ECIS instruments include an elevated field mode (EFM) that allows users to wound the cells in tissue culture by dramatically increasing the electrical current from the standard, noninvasive measuring current. For wounding purposes, the EFM severely electroporates the cells resulting in cell necrosis (Keese, Wegener, Walker, & Giaever, 2004). The wound is restricted to only those cells on the electrode allowing for a precise wounding area. Once the wounding mode is completed, the instrument returns to the normal measurement mode and monitors the migration of the surrounding cells back to a confluent monolayer (Figure 8).







Figure 7: Cytotoxicty of BSC-1 cells. High frequency capacitance monitoring of BSC-1 cells treated with varying concentrations of dimethyl sulfoxide.



Figure 8: ECIS[®] automated electrical wounding of MDCK II cells while monitoring with high frequency capacitance for migration rates.

Conclusion

Electric Cell-substrate Impedance Sensing allows researchers to measure in vitro cell behaviors like barrier function, cell proliferation, cytotoxicity, and more continuously and label-free while the cells remain under incubated conditions. By taking advantage of ohm's

Giaever, I., & Keese, C. R. (1984). Monitoring fibroblast behavior in tissue culture with an applied electric field. Proceedings of the National Academy of Sciences of the United States of America, 81(12), 3761–3764. Retrieved from http://www.pubmedcentral. nih.gov/articlerender.fcgi?artid=345299&tool=pmcentrez&rendertype=abstract

Hung, Y., Chiu, W., Fuh, S., Lai, Y., Tung, T., Huang, C., & Lo, C.-M. (2022). ECIS Based Electric Fence Method for Measurement of Human Keratinocyte Migration on Different Substrates. Biosensors, 12(5), 1–15. https://doi.org/10.3390/bios12050293

Keese, C. R., Wegener, J., Walker, S. R., & Giaever, I. (2004). Electrical wound-healing assay for cells in vitro. Proceedings of the National Academy of Sciences of the United States of America, 101(6), 1554–1559. https://doi.org/10.1073/ pnas.0307588100

Stevenson, B. R., & Begg, D. A. (1994). Concentration-dependent effects of cytochalasin D on tight junctions and actin filaments in MDCK epithelial cells. Journal of Cell Science, 107(3). https://doi.org/10.1242/jcs.107.3.367

Wegener, J., Keese, C. R., & Giaever, I. (2000). Electric cell-substrate impedance sensing (ECIS) as a noninvasive means to monitor the kinetics of cell spreading to artificial surfaces. Experimental Cell Research, 259(1), 158–166. https://doi. org/10.1006/excr.2000.4919

Xiao, C., Lachance, B., Sunahara, G., & Luong, J. H. T. (2002). Assessment of cytotoxicity using electric cell-substrate impedance sensing: concentration and time response function approach. Analytical Chemistry, 74(22), 5748–5753. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12463358



law and the properties of alternating currents, ECIS[®] can identify and quantify these cell behaviors by using a multifrequency approach and can isolate the sources of cellular impedance to the resistive and capacitive pathways using complex impedance.