Low frequency impedance spectroscopy of cell monolayers using the four-electrode method

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Abstract. We have developed an impedance spectroscopy system for monitoring changes in extracellular conductivity and cell-to-cell coupling in cells cultured on microelectrode arrays (MEAs). Impedance spectra were measured at low frequencies (0.5-10kHz) using the four-electrode technique and standard electrophysiological instruments. A reduction in extracellular conductivity (using isotonic sucrose solution) was observed as an elevation of impedance at all measured frequencies. A reduction in cell coupling (using heptanol) was observed as an elevation of impedance at low frequencies (<100Hz) and small reductions or no change in impedance at higher frequencies. Monolayer confluency and cell adhesion to electrodes were major factors in the measurements. The effect of disrupting cell adhesion (using trypsin) was most apparent at frequencies below 1kHz. Impedance was dependent upon the spacing between stimulating electrodes. Impedance reduced with increasing spacing. Overall, using commercially available MEAs and the four-electrode method provides a fast and reliable way to measure cell monolayer impedances.

1. Background
Electrical impedance spectroscopy (EIS) is emerging as a tool for fast non-invasive monitoring of cell cultures. EIS measurements can be made using two main techniques. One is to use two electrodes, where one electrode is used for sensing and the other is a reference electrode. This is typically used for monitoring cell adhesion, motility, proliferation, and cell toxicity [1]. Electrode surface areas in the two electrodes technique are typically large to reduce electrode impedance, however this hampers spatial sampling and the ability to accurately measure local cell densities and cell migration velocities.

Another technique for EIS is to use four electrodes, where current flows between two outer electrodes (stimulating electrodes) and potential differences are measured between two inner electrodes (measuring electrodes). This reduces effects of electrode impedance and electrode polarization. This is important when using grids of small electrodes (i.e. microelectrode arrays (MEAs) with 10µm dia electrodes are now available) to achieve high spatial resolutions, especially for measurements at low frequencies where electrode-electrolyte impedance effects are significant. Recently the four-electrode technique has been used in multi-electrode systems to provide increased accuracy compared to the two electrode technique [2, 3].

From a theoretical basis, changes in intracellular and extracellular conductivities can be measured using the four-electrode technique with either multi-site stimulation or frequency modulation.
Adequately small spacing between stimulating electrodes is required for this to work using frequency modulation [4-6]. At low frequencies, relatively little current penetrates the cell; it is shunted through the extracellular space. At high frequencies, the capacitive impedance of the membrane is low, so a significant amount of current flows through the cell. Accordingly, frequency dependent impedance changes may indicate relative differences between intra- and extracellular conductivities. Our goal has been to use this concept for assessment of extracellular conductivity and cell-to-cell coupling in cell culture monolayers.

Our objectives were to 1) show the effect of stimulation electrode spacing on four-electrode impedance measurements of fibroblast cell monolayers and 2) measure changes in four-electrode impedance spectra caused by alterations of extracellular conductivity and cell-to-cell coupling. To accomplish these objectives, cell monolayers were grown on commercially available MEAs. Impedance spectra were measured at low frequencies using standard electrophysiological instruments.

2. Materials and Methods

MEAs (Multichannel Systems, Reutlingen, Germany) having either gold (100µm dia) or titanium nitride (10µm dia) contact electrodes with inter-electrode spacings of either 700µm or 30µm (figure 1A) were mounted within an adapter (Multichannel Systems, MEAadapt) that provided pin-outs for each electrode. Current ($I_{stim}$) was injected between stimulating electrodes (the two outermost pairs, $A_1$:$D_1$ and $A_4$:$D_4$, figure 1B) using an AC/DC current source (Keithley 6220) at frequencies between 0.5Hz and 10kHz (3 points per decade). Extracellular potentials ($\Delta \Phi$) were measured from the two center electrodes (B:C, figure 1B) using a differential amplifier (Dagan EX4-400). Signals were recorded using a laptop PC, DAQ hardware (NI DAQCard-6062E), and custom LabVIEW software. Sampling frequencies ranged from 1000sps to 90Ksps and were set at least 10 times higher than the frequency of current injection. The amplitude of extracellular potentials ($|\Delta \Phi|$) was measured using custom Matlab routines by fitting potentials at each frequency to a sine wave at the frequency of the stimulating current. Impedance magnitude at each frequency was then calculated as $|Z| = |\Delta \Phi| / |I_{stim}|$.

First, impedance was measured for clean MEAs containing 750 µL of either 10% FBS or Tyrode’s solution. Current limits were set to prevent solution ionization, usually a max of 100nA. MEAs were then plated with fibroblasts (PT67). Cell concentrations varied between 0.5 and 2 million cells per mL. Cultures were incubated overnight and measurements were acquired the next day at room temperature after replacing the FBS solution with a buffered Tyrode’s solution. Cell-to-cell coupling and extracellular conductivities were then sequentially altered. Heptanol (a gap junction uncoupler) was applied (1mM) and impedance spectra were measured after 10 minutes. Cultures were carefully rinsed three times to remove heptanol and 750 µL of isotonic sucrose solution was added to the culture. Impedance spectra were measured after 10 minutes. Trypsin was then added to cultures, cells were washed from the MEA, and 750 µL of Tyrode’s solution was added to the cultures.
MEA. Impedance measurements were repeated. MEAs were then treated with an enzymatic pH neutral detergent (Tergzyme, Alconox Inc.) and impedance measurements were repeated.

3. Results and Discussion

Impedance at 0.5Hz for clean MEAs (100μm electrode dia) having 750 μL of dionized water, isotonic sucrose solution, or FBS solution was approximately 2000Ω, 1000Ω, 200Ω, respectively. Confluent monolayers were obtained a day after cell plating (figure 2). To ensure confluency and electrode coverage, monolayers were inspected using a confocal microscope (Zeiss LSM510) after staining the cells with CellTracker™ (Invitrogen) (figure 2).

Impedance was dependent upon the spacing between stimulating electrodes (figures 3&4). Typically, impedance measured at larger stimulation electrode spacing was lower than impedance measured at smaller stimulation electrode spacing. This effect might be described by simple volume conductor theory if the supplied current always remained outside of the cells. However, the validity of our approach relies upon the redistribution of current, in part, to the intracellular volume. In this situation a description that accounts for the separate contributions of the intracellular and extracellular domains becomes necessary. Presently, there is no analytic description of this redistribution, unless an

![Figure 3](image1.png)

**Figure 3.** Impedance spectra before and after applying trypsin and after cleaning the MEA (100 μm electrode dia). **A:** Spectra using a stimulation electrode spacing of 4.9mm. **B:** Spectra using a stimulation electrode spacing of 2.1mm.

![Figure 4](image2.png)

**Figure 4.** Alterations of extracellular conductivity and cell-to-cell coupling. Average impedance change (left) and typical impedance spectra (right) are shown. Error bars represent MSE. **A:** Replacement of cell culture media with isotonic sucrose solution. **B:** Application of heptanol (1mM).

![Figure 5](image3.png)

**Figure 5.** Top: Confluent monolayer on a High-Dense MEA (10 μm dia electrodes). **Bottom:** Impedance spectra (90 μm stim electrode spacing).
assumption of uniform, isotropic conductivities is valid. However, there are compelling reasons to believe that this assumption does not apply to our experimental conditions. A goal of future studies is to develop an analytic framework for describing the redistribution of current in our cell monolayers.

Impedance was drastically reduced after the application of trypsin (figure 3), indicating that cells were adhered to MEA electrodes and that adhesion was disrupted after applying trypsin. The effect of trypsin was most apparent at frequencies below 1kHz. Impedance was further reduced after cleaning the electrodes with an enzymatic detergent, indicating the presence of residual proteins after the detachment of cells (figure 3). Impedance spectra measured from non-confluent monolayers showed little difference before and after the application of trypsin, indicating that measurements are dependent upon adequate coverage of the electrodes with cells.

Reducing extracellular conductivity with isotonic sucrose solution increased impedance at all frequencies below 10kHz (figure 4A). Specific changes were 125%±44% at 0.5Hz, 75%±17% at 5Hz, and 300%±19% at 5kHz. The magnitude of impedance changes after reducing gap junction conductance were less and appeared to be frequency dependent (figure 4B). Impedance increased at lower frequencies (27%±13% at 0.5Hz and 16%±12% at 5Hz) while impedance decreased at higher frequencies (-8.4%±20% at 5kHz), but was not significant.

Cells were also plated on MEAs having titanium nitride electrodes (10µm dia, High-Dense MEAs). We found that although the effect of electrode impedance is less using the four-electrode technique, impedance measured using the 10µm dia electrodes was at least an order of magnitude higher than impedance measured using 100µm dia electrodes (figure 5). In addition, impedance differences measured before and after applying trypsin to cells were usually smaller for the 10µm dia electrodes.

Impedance measurements required low levels of stimulation currents and measurements of small potential differences, therefore minimization of noise was important. This was done by shielding the equipment and placing the MEA adapter within a Faraday cage. Sine wave fitting to measured potential differences minimized the effect residual noise levels. Raw signal to noise ratios (SNRs) were lowest at low frequencies. SNR at 1Hz was 60dB while SNR at 5kHz was 5dB.

Since changes in phase due to monolayer perturbations might be more sensitive than changes in the impedance magnitude [7], future work will involve the development of techniques for measuring impedance magnitude and phase and for conducting studies within an incubator.

4. Summary and Conclusions
We have shown how MEAs and the four-electrode method can be used for non-invasive measurements of cell monolayer conductivities, with emphasis at low frequencies (0.5Hz to 10kHz). Impedance was dependent upon the spacing between stimulating electrodes. Impedance reduced with increasing spacing. Reducing extracellular conductivity (replacing FBS with isotonic sucrose solution) was observed as an elevation of impedance at all measured frequencies. Reduced cell coupling (using heptanol) was observed as an elevation of impedance at low frequencies (<100Hz) and small reductions or no change in impedance at higher frequencies. Accordingly, monolayer confluency and cell adhesion to electrodes were major factors in the measurements. The use of commercially available MEAs provided a fast and reliable way to measure monolayer impedances.

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