Impedance Based Biosensor Array for Monitoring Mammalian Cell Behavior

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Abstract
Functional genomic studies and drug candidate testing require tracking of cell activity with high throughput. Electrical impedance measurements on multiple electrodes are highly attractive in this application because of the potential for direct computer control. Impedance measurements over a wide range of frequency were performed on four sensing electrodes to study the mouse fibroblast cell behavior. We report the experimental observation of electrode impedance changes caused by cell growth and also the cell response to Protein Kinase Inhibitor (H-7) which affects cell attachments. The most notable signal is the peak magnitude of the normalized impedance change. The peak magnitude is related to the effective cell blockage which depends on the cell coverage, cell-substrate contacts and spaces. During cell growth we observed the peak magnitude increased over the time and saturated. Furthermore, by characterizing the peak magnitude we analyzed the effect of drug H-7 on the cell.

Keywords
Electrode impedance, impedance spectroscopy, fibroblast

INTRODUCTION
Impedance based microelectrode sensor arrays have emerged as a promising means for screening cells. Giaever and his coworkers developed an electrical cell-substrate impedance sensor in 1986 to monitor cell proliferation, morphology, and motility [1]. The impedance sensor consists of two metal electrodes: one large common reference electrode and one small working electrode. The cells were cultured on the electrodes. The AC impedance at an appropriate frequency between the two electrodes was measured, recorded and correlated with cell activity. Subsequently, Ehret et al. monitored the impedance change during cell growth and observed the toxic effect of heavy metal ions (cadmium) on the signal with an interdigitated electrode structure [2]. Due to the size of the electrodes, the measured impedance changes in all these studies represent the average effect of a large number of cells.

In our work, we are aiming to develop an array of cell-sized sensors with active addressing devices for electronic detection of cell behavior *in vitro*, that is more effective and efficient than existing technology using microscopic observation [3].

In this paper we present the experimentally observed cell effect on the intermediate-sized sensing electrode impedance and the impedance change due to the cell response to the drugs. The purpose is to attain understanding of how the impedance change is related to the cell activity and analyze the cell responses to the drug with the impedance sensors. In addition, these studies provide information necessary for the design of cell-sized sensor arrays.

EXPERIMENTAL PROTOCOL

Electrode Fabrication
Electrode array fabrication was performed by using a lift-off process. Photoresist was spun onto clean fused silica wafers and patterned using standard photolithographic techniques. Then a 35 nm adhesion layer of Cr and 150 nm Au were deposited onto the patterned photoresist by DC sputtering. The electrode pattern was created by soaking the wafer in the acetone to lift off the metal not adhering to the substrate. Four sites ranging in diameter from 100 μm to 800 μm were fabricated on a 2.5 cm × 4 cm substrate as depicted in Figure 1. Different electrode areas were used, in part, to establish if known area-related behaviors were manifested by bare electrodes before interpreting signals from cells on electrodes. The four sites were separated by grounded metal lines. Parasitic capacitance between the interconnect lines and the liquid medium was reduced by painting the interconnect about 150 μm away from the electrodes with silicone EP30HT from Masterbond Inc. (Hackensack, NJ). The exact electrode areas were calculated from microscope images. A bottomless plastic well cut from a 48-well culture dish was bonded onto the substrate, enclosing the sensors and forming a chamber for cell culture. During the measurements, the well was placed in a temperature-controlled chamber mounted on an electronic probe station. The chamber temperature was controlled at 37 ± 1 °C.

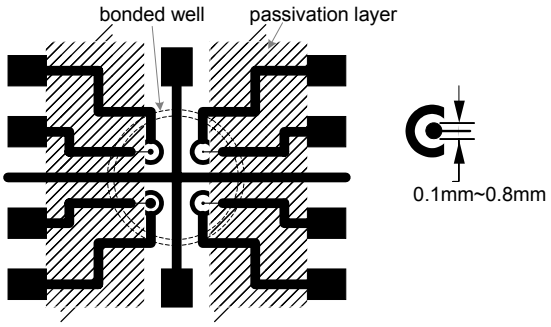

Figure 1. Sensor configuration.

Impedance Measurement Set-up

Admittance magnitude and phase was measured with a HP 4192A admittance meter (Hewlett Packard, Palo Alto, CA). The AC probe signal used during these measurements was 50 mV peak-to-peak and 50-80 measurements were taken over the frequency range from 100 to 106 Hz. Collection of a complete set of measurements over this frequency range took 60-90 sec. The admittance meter was switched between sensor sites using a National Instruments ER-16 relay board (National Instruments, Austin, TX), as shown in Figure 2. Data was acquired under computer control using Labview™ (National Instruments, Austin, TX) and stored for later analysis.

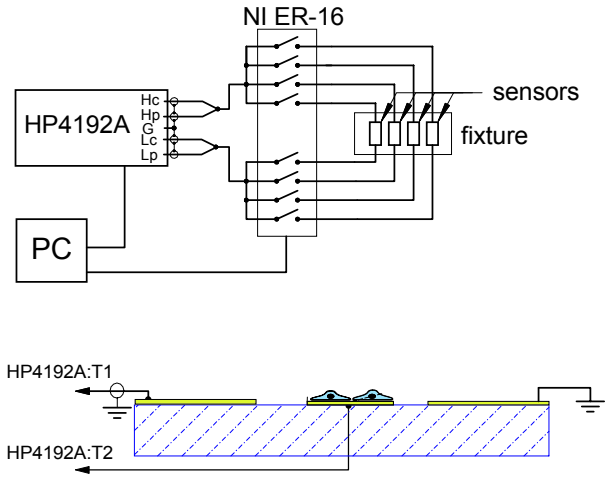

Figure 2. Impedance measurement set up.

Cell Culture

3T3 fibroblast cells were grown on standard 10 cm diameter polystyrene culture dishes (Corning Costar, Acton, MA) and maintained in Dulbecco's Modified Eagle medium (Gibco Laboratories, Grand Island, NY) supplemented with calf serum (10%), 500 unit/ml penicillin, 500 µg/ml streptomycin, 2 mM L-Glutamine, and 5 mM HEPES buffer, at 37 ^{o}C, and 5% CO_2 in air. The same medium was used to sustain cells during the impedance monitoring experiments.

Drug H-7 Experiment

Protein kinase inhibitor, 1-(5-iso-quinolinylsulfonyl)-2-methylpiperazine (H-7), was obtained from Sigma Chemical Co. (St Louis, MO). H-7 was added to the growth medium to achieve a concentration equal to 120 µM. H-7 treatment reduces the abundance of large fibers in the cell cytoskeletal organization. To reverse the effects of H-7, the medium containing H-7 was replaced with fresh medium lacking H-7.

ELECTRODE CHARACTERIZATION

Impedance Without Cells

When an electrode with area A is immersed into the liquid, an electrified double layer forms at the interface due to the chemical reactions[4]. When a small-signal current passes through the electrified interface, the current will encounter the faradic impedance due to electron exchange at the interface, diffusive ion transport and capacitive current flow through the double layer. The electrode interface impedanceis inversely proportional to the electrode area and frequency dependent, which can be represented by $Z(\omega)/A$. When the impedance between a small electrode and a large counter electrode is measured, the measured impedance will be dominated by the small electrode impedance. The total impedance of the electrochemical system consists of the faradic impedance $Z(\omega)/A$ at the electrode-solution interfaces in series with the solution resistance R_S , as shown in Figure 3(a). Spreading resistance is proportional to the inverse square root of electrode area and is frequency independent. At low frequencies, the measured impedance is mainly due to the faradic interface impedance. At high frequencies, the spreading resistance R_S dominates over $Z(\omega)/A$. In the measurement with cell culture medium as the conductive solution, the electrode impedance (Z) had the frequency (f) dependence $f^{-0.92}$ for low frequencies and flattened out at high frequencies. The measured impedance magnitude is shown in Fig. 4 for four electrodes of different area.

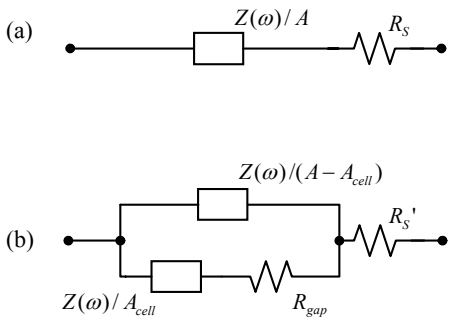

Figure 3. Equivalent circuits at electrode-solution interface (a) without cells and (b) with cells.

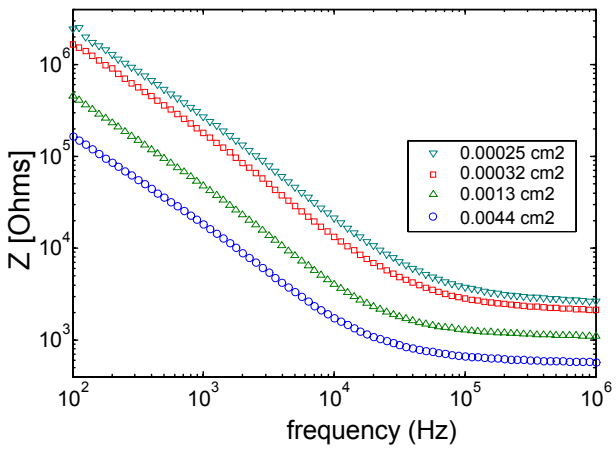

Figure 4. Plot of impedance magnitude over a frequency range of 100 Hz-10^6 Hz in logarithm scale for four electrodes with different area.

Impedance With Cells

Growth of cells causes changes of electrode impedance of order 100-200% which are difficult to see when $Z(f)$ is plotted on a logarithmic scale. To more clearly reveal the impedance change due to cell growth, we plot the normalized impedance change which is defined as

$$r = \frac{Z_{cell} - Z_{no\ cell}}{Z_{no\ cell}}$$

When a cell grows on the electrode, the cell forms adhesion contacts to the electrode, which accounts for 15-20% of the cell surface area. Apart from the contact areas, the average gap between the cell and the electrode surface is 50 nm-150 nm (Figure. 5). Because the cell membrane is highly insulating, the current flowing from the cell-covered portion A_{cell} must flow laterally through the resistive gap region. This effect can be approximately modeled by a resistance R_{gap}. So with cell presence there are two branches for the current flow: the exposed electrode portion with impedance of $Z(\omega)/(A\text{-}A_{cell})$ and the cell-covered portion with impedance of $Z(\omega)/A_{cell}$ in series with R_{gap}, shown in Figure. 3(b).

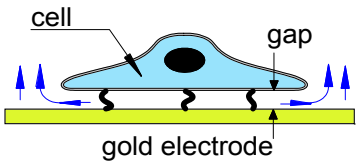

Figure 5. Cell-electrode junction showing cell focal adhesion contacts and current flowing(blue arrows) laterally through the cell-electrode gap.

At low frequencies, the electrode impedance $Z(\omega)/A_{cell}$ is large compared to the effective gap resistance, and the impedance magnitude is essentially unaffected by the cell (Figure 6). The electrode impedance decreases as the frequency increases. At a critical frequency the electrode impedance under the cell $Z(\omega)/A_{cell}$ becomes less than R_{gap} and the measured impedance is approximately equal to the gap resistance R_{gap} until the impedance of the uncovered electrode $Z(\omega)/(A\text{-}A_{cell})$ becomes smaller than R_{gap} and the measured impedance follows $Z(\omega)/(A\text{-}A_{cell})$. So the impedance is substantially increased by the cell presence at moderate frequencies. At high frequencies, spreading resistance determines the impedance. Since a portion of the electrode area is blocked by the cell at high frequencies, the spreading resistance is increased slightly and this is modeled by $R_S' > R_S$, giving a moderate impedance increase at high frequencies. As a result, plots of the normalized impedance change will show a peak with an amplitude which depends on cell coverage and cell-electrode gap, as shown in Figure 6. The position of the peak frequency is related to the size of contiguous cell colonies.

The impedance change caused by cells has been calculated analytically using a simplified model [5] and we have performed finite element simulations in order to obtain further insight [6]. Qualitatively, the predicted changes in impedance due to cell growth are consistent with the simplified equivalent circuit model discussed here.

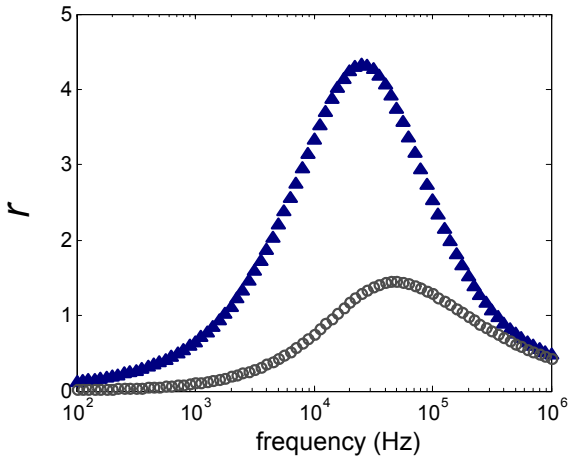

Figure 6. Normalized impedance change with cell presence (○) 0.25 μm cell-electrode gap; and (▲) 0.025 μm cell-electrode gap, calculated using the equivalent circuit of Fig. 3(b) (30 μm diameter cells with 90% coverage on 0.13 mm^2 electrode)

RESULTS AND DISCUSSION

Cell Growth Experiment

The electrodes were rinsed with deionized water and the well was then filled with fresh culture medium. Impedance for four electrodes with different surface area was measured over a wide frequency range from 100 Hz to 1 MHz. Then cells were prepared as described above. The suspended cells were inoculated into the well containing the sensors and cultured at 37 °C. The impedance was measured every half hour.

The normalized impedance change is plotted in Figure 7(a). As cells settled down, attached and spread on the electrodes over the time, the impedance decreased slightly at low frequencies and increased strongly at high frequencies, with a peak at intermediate frequency. The height of the peak provides an indication of the cell coverage on the electrodes and how close and extensively the cells spread on the surface. Note that the magnitude of the peak in Figure 7(a) increases with time (Figure 7(b)) and shifts slightly to lower frequency, which suggests that cell-substrate contacts increase monotonically with time. In addition, the peak shift to the left in the first two hours also reflect that the cell-surface separation distance decreases with elapsed time.

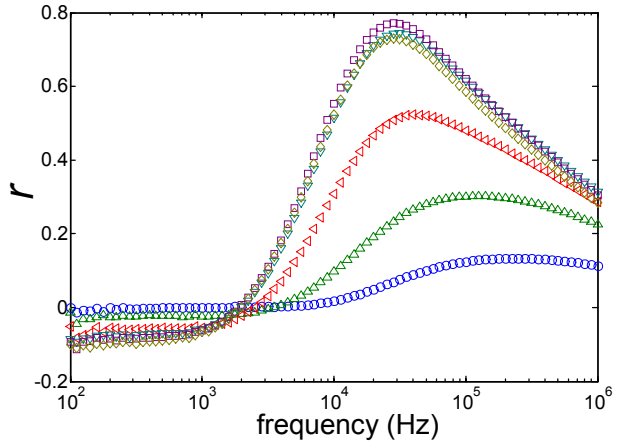

Figure 7 (a). Plot of normalized impedance change(r) over 5 hours after 3T3 fibroblasts were deposited: (○) 15 min, (△) 0.5 hr, (◁) 2 hr, (▽) 3 hr, (□) 4 hr, and (◇) 5 hr after deposition. 90% to 95% cell coverage on substrate was observed by optical microscope. The electrode area was 0.13 mm^2.

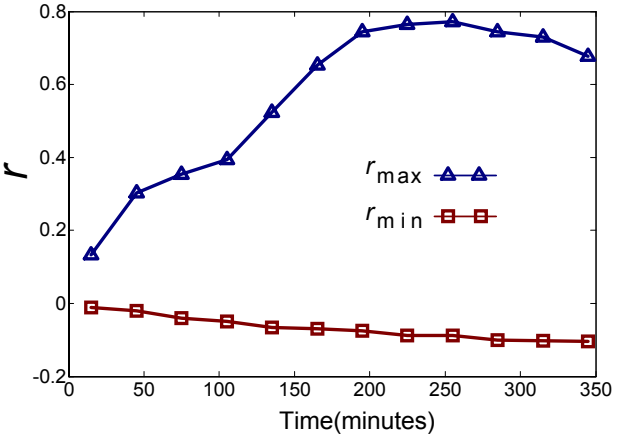

Figure 7(b). Normalized impedance peak magnitude (r_{max}) and magnitude at 300 Hz (r_{min}) as a function of time after cell deposition.

Furthermore, we have observed that the normalized impedance ratio r decreases monotonically with cell growth at low frequencies (Figure 7(b)). This is unexpected as simulations and theory shows that the either increase the impedance by blocking current flow or leave the impedance unchanged. By a series of experiments, we have determined that the increase in impedance at low frequency cannot be attributed to exposure to the cell growth medium itself for hours. Consequently we attribute the observed decrease in impedance at low frequencies to the adsorption of materials produced during cell growth. When fibroblast cells attach to the substrate, extracellular protein matrix (e.g. fibronection or collagen) are created to form the focal contacts to the substrate. We believe that adsorbed proteins or similar groups on the surface shift the isoelectric point of the electrode surface so that the local ion concentration increases near the surface. Higher local ion concentrations will decrease the value of the diffusive impedance and increase the interfacial capacitance, yielding the behavior observed here. Thus, the changes in low frequency impedance may yield additional information about cell metabolism.

Drug H-7 Effect on Sensor Impedance

The cell response to the drug H-7, a protein kinase inhibitor, was first studied in culture dishes by optical observations. To visualize stress fibers of the cytoskeletal structure, the cells were fixed and labelled, and the stained cells were visualized using epifluorescence microscopy. Figure 8 shows the change of cell cytoskeletal organization after exposure to H-7. In the absence of H-7, typical cell spreading and cytoskeletal organization were observed, shown in Figure 8 (a). H-7 treatment reduced the abundance of large fibers (bright lines in the image) over the course of 4 hours, as shown in Figure 8(b). The loss of the fibers resulted in less cell-substrate contacts without dramatic change of cell morphology [7].

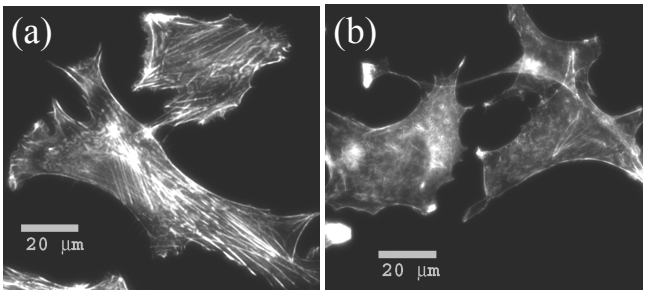

Figure 8. Experimentally observed cell cytoskeletal organization before (a) and after (b) exposure to H-7.

Impedance measurement was then performed to study the cell response to H-7. In the experiment, the cells were cultured on the electrodes for 16 hours to reach an essentially confluent monolayer. Then the impedance measurement was started. Two hours later, the H-7 was added into the medium to attain a final concentration of 120 μM. The cells were cultured in the medium with H-7 for four hours. Then H-7 was removed by replacing the medium. The impedance was continuously monitored. Figure 9(a) shows how the

normalized impedance change *r* was altered in this experiment.

Over the two hours before exposure to H-7, the peak increased slightly by 13%. The addition of H-7 caused the measured impedance to decline over time. The peak decreased to 68% and 10% of the original value after 15 minutes and 4 hours of exposure to H-7, respectively. After eliminating H-7 via medium replacement the impedance recovered to approximately 70% of the initial value within 2 hours. The change of the peak magnitude over the time is illustrated in Figure 9(b). The time scale over which the impedance decreased after exposure to H-7 is consistent with the time scale that H-7 was optically observed to alter the abundance of large stress fibers. Because cell loss or death was not observed after H-7 exposure, the decreased impedance indicates that cell-substrate contacts diminished, allowing more electrical current to pass through the gap between the cell and the electrode. The change of cell physiology and the consequent alteration of current flow are schematically illustrated in Figure 10. Since the period of 2 hours is too short for cells to proliferate, the impedance recovery after H-7 removal indicates the reestablishment of more and/or more extensive cell-substrate contacts that block electrode area as well as reduced cell-electrode separation distance. Recovery of some types of cell-substrate contacts is consistent with prior reports of submembrane protein plagues restoration after H-7 removal [8].

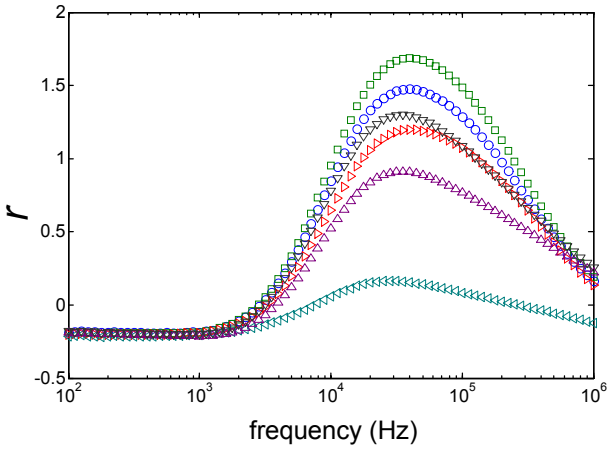

Figure 9(a). Normalized impedance change in the H-7 experiment: (○) 0 hr and (□) 2 hr after 16 hours of cell incubation. H-7 was added into the medium and the impedance decreased (▷) 15 minutes and (◁) 4 hr later. After replacing the medium with medium without H-7, the impedance was measured (△) 1 hr (▽) 2 hr later and recovered partly to the value prior to H-7 treatment. Electrode area was 0.13 mm^2.

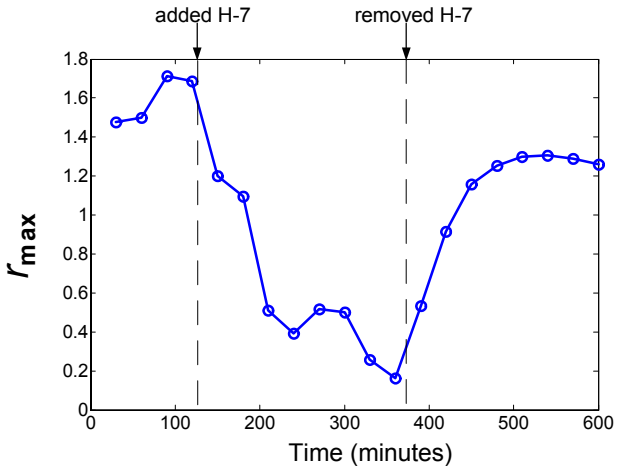

Figure 9(b). Normalized impedance peak magnitude change over the time when adding in H-7 and removing H-7

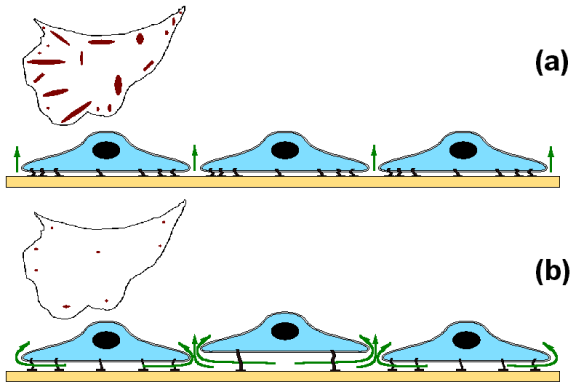

Figure 10. Cell response to H-7 deduced from impedance measurements: (a) before exposure to H-7(b) after exposure to H-7.

CONCLUSIONS

We have shown that manipulation of the cell physiology caused large, easily observable changes in the electrical impedance. The peak magnitude and the position of the normalized impedance change were found to be a function of cell parameters: cell attachment, cell coverage, etc. The H-7 experiment shows that impedance sensor is an effective tool to screen the cell response to drugs. We envision the peak magnitude and the frequency dependence of the peak may be useful as a signature of the cell type. In addition, the impedance decrease caused by the protein products can be used to characterize the cell metabolism

Furthermore, we observed that in the same experiment, the smaller the electrode, the higher the peak magnitude, and the higher the frequency at which the peak is located. These results suggest that cell-sized sensor array will give larger peak magnitude and will be more effective for studying the cell response to drugs.

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