Impedance Sensing for Monitoring Neuronal Coverage and Comparison With Microscopy

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Abstract—We investigated the applicability of electric impedance sensing (IS) to monitor the coverage of adhered dissociated neuronal cells on glass substrates with embedded electrodes. IS is a sensitive method for the quantification of changes in cell morphology and cell mobility, making it suitable to study aggregation kinetics. Various sizes of electrodes were compared for the real-time recording of the impedance of adhering cells, at eight frequencies (range: 5 Hz–20 kHz). The real part of the impedance showed to be most sensitive at frequencies of 10 and 20 kHz for the two largest electrodes (7850 and 125 600 μm^2). Compared to simultaneous microscopic evaluation of cell coverage and cell spreading, IS shows more detail.

Index Terms—Aggregation, electric impedance sensing (IS), neuronal cell coverage, neuronal cultures.

I. INTRODUCTION

TO ASSAY the process of attachment of cells to artificial surfaces several techniques have been used. Most quantitative studies on adhesion involve employing forces like centrifugal acceleration and laminar shear flow [1], [2]. These techniques are laborious and noncontinuous. Another technique is microscopy, often in combination with immunocytochemical staining, direct cell counting, or time lapse cinematography [3]–[5]. Electric impedance sensing (IS) is a continuous method, providing quantitative data on several cultures simultaneously with a relatively high time resolution [6]–[10].

In IS cells are cultured on microelectrodes and submitted to an ac current. Current can flow through the cell membranes and via the openings between tightly adhered, but not totally confluent cells.

The impedance measured depends on a number of variables, such as adhesion tightness, cell type, surface area of the electrode, frequency, and confluency of cells. Depending on the application an optimal set of variables has to be chosen. In the methods section this will be further elaborated on.

Assuming that cells are firmly adhered (sealed) to the substrates, and stay adhered when openings between the cells grow

or shrink, changes in cell confluency will affect mainly the intercellular resistance (spreading resistance, or constriction resistance). Due to the low resistivity of the culturing fluid, compared to the membrane impedance and sealing resistance, even slight changes in the openings have very large effects on the impedance, as given in [9] and [10].

IS has proven valuable for study of the cell or tissue interface and the monitoring of changes in mammalian cell-culture morphology [7]–[10]. Several electric models of electrode-fluid-cell/tissue have been developed [7]–[10]. Changes in cell shape caused by various biochemicals like α -thrombin [11] and prostaglandin E [12] have been monitored as well as changes caused by cytotoxic agents [13], virus infections [14], or even very small changes in morphology caused by periodic injection of CO_2 in cell culture incubators [15].

So far, no research has been reported in which IS was applied on dissociated primary neuronal cell cultures. IS has been reported in studies involving mammalian cell types with tight intracellular clefts, whereas neurons have far less-defined cell-cell contacts and do not divide. In a study by Bieberich and Guiseppi-Elie [16], the neuronal differentiated cancer cell line PC-12 showed an almost 3% higher impedance compared to nondifferentiated PC-12 cells. Another study demonstrated a significant increase in impedance during the attachment of neuroblastoma cells on an electrode [17].

In this study, neuronal cultures were investigated during normal development in two ways: IS and microscopy. Directly after plating, the neurons start to spread and make contact with surrounding cells, leading to a rather confluent monolayer of neurons. Both methods were compared to test whether IS shows more details than standard microscopy.

II. MATERIALS AND METHODS

A. Planar Electrodes

Fig. 1 shows an overview of the four electrode sizes used for IS. Dimensions of the electrodes are 78, 1962, 7850, and 125 600 μm^2 . Electrodes were patterned on 1 cm^2 glass plates. Each glass plate contained 19 electrodes of one size. Glass was used as a substrate in order to have transparency between electrodes. Gold electrode structures were created by photolithography and reactive ion etching. An insulation sandwiched layer combination of SiO_2 – Si_3N_4 – SiO_2 (144–396–144 nm thick) was deposited by plasma-enhanced chemical vapor deposition. The insulation was etched away above the electrodes. After fabrication, the electrode plates were cleaned ultrasonically in an acetone bath.

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Fig. 1. Cell-covered gold electrodes (78, 1962, 7850, and 125 600 μm^2).

Fig. 2. Schematic view of an electrode covered with neurons. The total current I_{total} splits up into three pathways. Current I_{spread} finding its way “easily” through the small spaces between the cells, I_{seal} , the leakage current through the gaps between the substrate and the cells, and I_{cell} , the current through the cells.

Neurons were plated and cultured on and around the electrode areas (see Fig. 1). The electrode glass plates were precoated with 50 $\mu\text{g}/\text{mL}$ polyethyleneimine (PEI; Fluka, Buchs, Switzerland). For the positioning of a neuronal culture, a glass ring was placed on the substrate during cell plating.

B. Impedance Model

Fig. 2 shows a schematic view of an electrode covered by neurons. The impedance spectrum of this system can be analyzed using an equivalent RC circuit (see Fig. 3). Assuming that cells are firmly adhered to the substrates (so R_{seal} is very high, typical value 5 $\text{M}\Omega$; see [9]), and that they stay adhered when openings between the cells grow or shrink, changes in cell confluency will affect mainly the intercellular resistance (spreading resistance R_{spread}). Due to the low resistivity of the culturing fluid, compared to the membrane impedance and R_{seal} , even slight changes in the openings have very large effects on the impedance, as given in [9] and [10] (for proof, see last two paragraphs of this section). Therefore, this model can be simplified

Fig. 3. Equivalent circuit. Z_{elec} is the impedance of the electrode–electrolyte interface (Helmholtz double layer), R_{spread} is the resistance of the intercellular open spaces and bulk fluid, and R_{seal} , the sealing resistance between the cells and substrate. The $R_{\text{mem}} C_{\text{mem}}$ part accounts for the neuronal cell membranes.

Fig. 4. Simplified equivalent circuit for an electrode.

to only Z_{elec} in series with R_{spread} (see Fig. 4)

$$Z = Z_{\text{elec}} + R_{\text{spread}} = \frac{K}{(i\omega)^m} + R_{\text{spread}}. \quad (1)$$

The first term represents the equivalent impedance of the electrode–electrolyte interface that is frequency dependent, and K is a size-dependent constant [8], [19]. Power m usually takes values around 0.6–0.7, indicating the nontruly capacitive nature of the Helmholtz layer.

R_{spread} can be modeled as the resistance of a fluid conductor, seen by a small source with “electrode” radius r_e ,

$$R_{\text{spread}} = \frac{\sqrt{2}}{2\pi\sigma r_e} \approx \frac{1}{4.44\sigma r_e} \quad (2)$$

with σ the conductivity of the culturing medium ($\sigma = 1.65 \text{ S/m}$). Therefore, the radius r_e is the equivalent radius of the electrode surface that is not covered by cells and depends on the cell coverage. Electrode coverage with neurons will have a main effect on the second term, as corridors will shrink/vanish when cells attach more firmly to each other, until complete confluence is reached. The neurons now impede the passage of current, thereby increasing the total impedance.

The model approach chosen is certainly not the only possible one. A number of other, but usually more complex models exist in literature [6], [8]. However, given the purpose of analysis of development of confluency of cells, the proposed model has the strength of simplicity, with only three parameters.

We still have to check numerically whether our simplification of the electrical circuit model is justified, and so whether the ratio of R_{spread} and membrane impedance is sufficiently low. For parameters C_m and σ_m , we have cellular membrane capacitance $C_m = 1 \mu\text{F}/\text{cm}^2$, membrane conductance $\sigma_m = 0.3 \text{ mS}/\text{cm}^2$ [9].

Assuming full confluency and regarding now all cells as one giant cell, with surface dimensions the same as the four electrodes areas (78, 1962, 7850, and 125 600 μm^2), one calculates $C_{m \text{ all cells}} = 0.39, 9.81, 39.25$, and 628 pF, respectively, and $R_{m \text{ all cells}} = 4270, 170, 42.5$, and 2.65 $\text{M}\Omega$, respectively. Together, they determine the membrane real impedance for

each frequency. For example, for the $7850 \mu\text{m}^2$ surface, and at 10 kHz, one calculates $R_m = 42.5 \text{ M}\Omega$, in parallel with Z_C (real part) = $406 \text{ k}\Omega$. Therefore, the membranes form together a real impedance of about $400 \text{ k}\Omega$, or less (when less cells present, i.e., not completely confluent). On the other hand, voids between the cells of 0.1%, 1%, or 10% yield R_{spread} values of 123, 40, and $12.2 \text{ k}\Omega$, respectively (2). Combining these values, one may conclude that at voids of 1% and 10% the circuit simplification is justified, as R_{spread} is 10–32 times smaller than the membrane real impedance, respectively. At 0.1% open space, the simplification is a bit too strong for the $7850 \mu\text{m}^2$, 10 kHz combination, but will get better justified for lower frequencies or smaller electrodes. Obviously, the R_{seal} value of $5 \text{ M}\Omega$ is large enough to be neglected, compared to the above values.

Cortical cells have a typical somatic diameter of $20 \mu\text{m}$. This implies that the smallest electrode, area $78 \mu\text{m}^2$, will be probably covered by a few cells only, or even one cell. In case of one cell, the interface lacks the spread current component. This one-cell coverage case has been analyzed previously in detail by Buitenweg *et al.* [9], [10].

C. Cell Culturing

Cerebral cortical neurons from newborn rats (P2) were used for all experiments in this study. Brains were taken out after decapitation, the meninges of the cortices were removed, and the basal ganglia as well as the hippocampus was prepared free. The remaining cortices were collected in a tube with chemically defined R12 culture medium [20] and trypsin for chemical dissociation. After removal of trypsin, $150 \mu\text{L}$ of soybean trypsin inhibitor and $125 \mu\text{L}$ of DNase I (20 000 units, Life Technology, Carlsbad) are added. A solution of single neurons was obtained by mechanical dissociation of the cortical tissue. The neuron solution was centrifuged at 1200 r/min for 5 min. The supernatant was removed and the pellet of neurons resuspended. Neurons were plated and cultured on the described electrodes precoated with $50 \mu\text{g/mL}$ PEI (Fluka). PEI is a cell–substrate adhesive enabling the neurons to adhere to the nonadhesive glass, it is routinely used in neuronal cell culturing. Cells were kept in serum-free R12 medium under standard conditions of 37°C and 5% CO_2 in air. A cell concentration of approximately 10^6 cells/cm^2 was used in all experiments. During measurements, the neuron cultures were placed into a small incubator keeping the temperature at 37°C . In total, five platings were done from five different rats ($N = 5$).

D. Measurement Setup

All impedance measurements were carried out using a programmable signal source (HP 4194A), a home-built impedance measuring circuit, and a data acquisition system in a Labview environment [9], [10]. This setup was used in combination with cell culturing chambers containing the electrodes. The cultures were kept at 37°C under sterile conditions during measurements on a NIKON DIAPHOT inverted microscope. Applied frequencies were 5, 10, 50, 100, 500, 1000, 10 000, and 20 000 Hz. The measurements were controlled by the same computer that recorded and saved the real and imaginary part of the impedance.

Cultures were monitored during their development, starting shortly before cell plating of the electrodes, until cultures formed compact monolayers of neurons and aggregation was just starting. In the first 12 h, the electrodes were monitored by a set of measurements done every 3 h in which six impedance spectra were obtained with an interval of 2 min on all four devices. After 12 h, the cultures were measured every 24 h ending the experiment after 144 h.

Measurement sessions ended on day 6 by the addition of trypsin while monitoring its effect on the impedance of the cell-covered electrode. During trypsin digestion, the time interval between measurements was 5 min until electrodes appeared to be free of neurons.

E. Imaging Technique

The gold electrode lacked the transparency for direct optical monitoring of the electrode surface. We used the visible area directly surrounding the electrodes to indirectly determine the neuronal coverage of the electrode surface. Percentage of coverage was determined by converting digital color photographs into an 8-bit grayscale photograph using CorelDraw software. The histogram of the grayscale photograph was used for segmentation of the picture into a black-and-white photo. The ratio of the number of black-to-white pixels is the percentage of the electrode area covered by cells. Pictures of the electrode areas were made after every impedance measurement. On each photograph, an area of $200 \mu\text{m}^2$ was taken at four different positions. The average percentage of coverage at these four areas was calculated.

III. RESULTS

A. Electrodes

In the first experiment, the optimum electrode size of planar electrodes for neuronal coverage was investigated at frequencies of 5, 10, 100, 500, 1000, 10 000, and 20 000 Hz. The sensitivity of these electrodes for cell coverage was calculated as a percentage of the increase in impedance after maximum coverage of the electrodes with neuronal cells. Maximum electrode coverage was accomplished by culturing at a cell density of $1 \times 10^6 \text{ cells/cm}^2$, during six days, before the period that at some parts of the culture aggregates were developing. The development of a neuronal culture during these six days is represented in Figs. 5 and 6. In these figures, we plotted the real and imaginary parts of the impedance together with the change in cell coverage in the electrode area. Directly after cell seeding, a clear rise in the real part of the impedance at a frequency of 10 kHz is seen due to the attachment and spreading of the neuronal cells. After 6 h, the increase flattens, but progresses slowly. This effect on the real impedance is not seen at a frequency of 100 Hz (see Fig. 5). Fig. 7(a)–(d) shows the impedance loci for all electrode sizes for both noncovered electrodes and electrodes covered with a 6-day-old neuron culture. The highest frequencies show the lowest real and imaginary impedance. As expected, larger electrodes demonstrate lower impedances. The presence of a 6-day-old neuronal culture on top of the electrodes

Fig. 5. Real impedance (left vertical scale) during the development of a neuronal cell culture after cell seeding at frequencies of 100 Hz ($\blacktriangle$) and 10 kHz ($\bullet$) (electrode size $7850 \mu\text{m}^2$). Microscopy: Percentage cell coverage ($\circ$) is indicated on the right vertical axis. Inserts: Enlarged version of initial impedance development. $N = 5$.

Fig. 6. Imaginary impedance (left vertical scale) during the development of a neuronal cell culture after seeding at frequencies of 100 Hz ($\blacktriangle$) and 10 kHz ($\bullet$) (electrode size $7850 \mu\text{m}^2$). Microscopy: Percentage cell coverage ($\circ$) is indicated on the right vertical axis. Inserts: Enlarged version of initial impedance development. $N = 5$.

causes an increase of the real impedance at higher frequencies (horizontal shift to the right).

As can be seen in Fig. 8, the impedance of the applied electrodes at low frequencies show a small rise of impedances at frequencies below 500 Hz. Standard deviations are relatively high.

Strongest effects were obtained using the $7850 \mu\text{m}^2$ electrodes at frequencies of 10 and 20 kHz. At these frequencies, the cell coverage of electrodes alters the real impedance with more than 250% (see Fig. 8). In contrast, effects on the imaginary part of the impedance were low at all frequencies, with a maximum change of 14% at 10 Hz ($1962 \mu\text{m}^2$ electrode, data not shown). This makes the imaginary part of the impedance less attractive for future use in electric cell sensing. Therefore, for further monitoring of neuronal development in culture, the

(a)

(b)

(c)

(d)

Fig. 7. (a)–(d) Impedance locus of electrodes with full cell coverage and without cells ($\circ$ and $\bullet$, respectively) at 5, 10, 50, 100, 500, 1000, 10 000, and 20 000 Hz going (from upper right to lower left). Electrodes sizes: (a) $78 \mu\text{m}^2$. (b) $1962 \mu\text{m}^2$. (c) $850 \mu\text{m}^2$. (d) $125\,600 \mu\text{m}^2$. Bars indicate standard deviation. $N = 5$.

Fig. 8. Percentage of change in real impedance between bare and neuron-covered electrodes of various sizes ($78 \mu\text{m}^2$ = gray, $1962 \mu\text{m}^2$ = white, $7850 \mu\text{m}^2$ = black, and $125\,600 \mu\text{m}^2$ = hatched). $N = 5$.

Fig. 9. Real impedance during trypsin digestion of a neuronal cell culture (electrode size $7850 \mu\text{m}^2$, frequency 10 kHz). $N = 5$.

$7850 \mu\text{m}^2$ electrode was used to record the real impedance at a frequency of 10 000 Hz.

After six days, the experiments were finalized by the addition of trypsin, serving as a control to see if the impedance was effected by anything else than culture development. Impedances decreased to the noncovered value in about 40 min (see Fig. 9).

B. Model Fit

Impedance loci of electrodes have been simulated by fitting (1) to the measured impedance loci. Fig. 10 represents the measured and fitted loci of both 7850 and $125\,600 \mu\text{m}^2$ electrodes, before and during cell coverage at frequencies of 5, 10, 100, 500, 1000, 10 000, and 20 000 Hz. The highest frequencies are plotted in the lower left corner of the graph. As frequency decreases, both real Z and imaginary Z increase. The two leftmost curves are those of the $125\,600 \mu\text{m}^2$ electrodes (most left curve is the uncovered case). The loci of the noncovered and cell covered $7850 \mu\text{m}^2$ electrodes represent much higher real and imaginary impedances as expected. Like in Fig. 7, the presence of a 6-day-old neuronal culture on top of the electrodes causes an increase of the real impedance at higher frequencies for both electrode

Fig. 10. Example of two impedance loci ($7850 \mu\text{m}^2$ and $125\,600 \mu\text{m}^2$ electrodes) before and after neuron coverage with the modeled loci fitted to the measured data. —●— = $7850 \mu\text{m}^2$ noncovered measured, —○— = $7850 \mu\text{m}^2$ noncovered fit; —◆— = $7850 \mu\text{m}^2$ covered measured, —◇— = $7850 \mu\text{m}^2$ covered fit; —▲— = $125\,600 \mu\text{m}^2$ noncovered measured, —△— = $125\,600 \mu\text{m}^2$ noncovered fit; —■— = $125\,600 \mu\text{m}^2$ covered measured, —□— = $125\,600 \mu\text{m}^2$ covered fit.

TABLE I
MODEL PARAMETERS FITTED TO THE EXPERIMENTAL IMPEDANCE SPECTRA OF NONCOVERED (BARE) AND COVERED ELECTRODES

Electrode size μm^2	R_{spread} [Ohm]	m	K
78 bare	0.00	0.76	$2.60 \cdot 10^9$
78 covered	$3.04 \cdot 10^5$	0.72	$2.24 \cdot 10^9$
1962 bare	$1.19 \cdot 10^3$	0.74	$3.86 \cdot 10^8$
1962 covered	$7.92 \cdot 10^4$	0.73	$3.98 \cdot 10^8$
7850 bare	$8.46 \cdot 10^3$	0.81	$1.24 \cdot 10^8$
7850 covered	$4.13 \cdot 10^4$	0.83	$1.51 \cdot 10^8$
125600 bare	$1.34 \cdot 10^3$	0.89	$3.23 \cdot 10^7$
125600 covered	$4.36 \cdot 10^3$	0.88	$3.34 \cdot 10^7$

For each electrode size, parameters m and k do not change much after neuron coverage. The spreading resistance, however, increases drastically after coverage.

sizes. This increase is represented by the horizontal shift of the impedance locus in the lower left corner of the loci.

The impedance loci could be fitted by a multivariable least-square-fit selection procedure of values for the parameters K , m , and R_{spread} . The values are listed in Table I (noncovered electrodes and neuron-covered electrodes). The tables and plotted impedance loci show that neuron coverage mainly affects the real-valued R_{spread} .

C. Calculation of Change in Impedance Based on Microscopy

In Fig. 11, the increase of the real impedance $\Delta\text{Re}Z$ (equal to R_{spread} , filled circles) of the $7850 \mu\text{m}^2$ electrode during culture development is plotted, together with the percentage of electrode coverage, as determined from microscopy and image analysis (open-circle symbols). The experimental $\Delta\text{Re}Z$ data (filled circles) in Fig. 11 are derived from Fig. 5 by subtracting the real impedance of the uncovered electrode, thereby obtaining the change in real impedance during culture development. (In contrast to the real impedance, there is nearly no change in imaginary impedance at high frequency between uncovered and fully covered electrode condition.) On the other hand, the change in real impedance can be derived from the optical

Fig. 11. Measured change ΔReZ during the development of a neuronal cell culture after seeding at 10 kHz ($\bullet$) on the left y-axis. Change ΔReZ calculated from the cell coverage, determined by image analysis ($\blacktriangle$) on the left axis. Percentage cell coverage obtained by microscopy, right y-axis ($\circ$). $N = 5$. Electrode size = $7850 \mu m^2$.

coverage data using the following equation:

$$\Delta ReZ = R_{spread} = \frac{\sqrt{2}}{2\pi\sigma r_e} \approx \frac{1}{4.44\sigma r_e}.$$

As conductivity, we used 1.65 S/m. Radius r_e is the equivalent radius of the noncovered electrode surface and can be calculated from the optically determined electrode coverage A_e

$$r_e = \sqrt{\left(\frac{A_e}{\pi}\right)}.$$

This “optically inferred” change in impedance R_{spread} is also plotted in Fig. 11 (triangle symbols).

The difference between the two curves (measured versus optically inferred one) is striking. The absolute values differ considerably, but also the detailed course over time, the IS-measured curve showing the most detail.

IV. DISCUSSION

IS of cellular systems has shown to be effective in monitoring cell spreading and adhesion. Change in impedance is mainly caused by the progressive “insulating” properties of cells. So far IS has been applied on cell types proliferating in 2-D monolayers with tight intracellular spaces, like epithelial and endothelial cells. However, neurons do not proliferate and cell junctions are far less tight. Electrodes were applied in neuronal cell sensing to study the applicability of IS in the monitoring of neuronal cell cultures. Four sizes of electrodes were compared. For all electrodes, a clear effect of neuronal cell covering on the electrode impedance has been demonstrated; the maximal effect was seen for an electrode with size 7850. Increase of real impedance after cell coverage was 254% at 10 kHz. Wegener *et al.* [7] indicated a frequency of 40 kHz as measured optimum for cell sensing (but for epithelial cells, electrode surface $50\,000 \mu m^2$).

Neuronal cultures that are kept longer than six days *in vitro* have a denser morphology compared to the final state of the cultures measured in this study. Aggregation of neurons in such

cultures, however, causes nonhomogeneous covering of electrodes, and is therefore, less interesting for this study.

We also tested interdigitated electrodes (results not shown). They were reported as more applicable for IS [18] because of better sensitivity and reproducibility. The results obtained in this study do not support this conclusion for neuronal cultures. Possible reason is the larger intercellular space in neuronal cultures, resulting in a lower R_{spread} , outshining the capacitive effect. After completely removing the neuronal cultures by trypsin digestion, electrode impedance turned back to the initial impedance of the empty electrodes.

The monitoring of neuronal cultures in development is presented in Fig. 5. At 10 kHz, over 50% of the increase in real impedance is caused by the attachment and spreading of neurons in the first 3 h. The percentage of electrode coverage (optical) demonstrates a similar increasing trend as the real impedance during the first 24 h. No further increase in neuronal coverage of the electrode is seen after 24 h. The real impedance, however, increases further after 24 h. This indicates that IS can detect changes in neuronal cultures that are undetectable using normal microscopy.

The impedance inferred from the optically determined cell coverage is plotted in Fig. 11 (triangles). This calculated impedance is considerably less than the measured impedance (closed circles) and shows less detail. This implies that microscopy reveals too much open space (maximum coverage in Fig. 11 is 93%, so 7% open space). There may be several reasons why microscopy gives less accurate results. Only an on-top view of a culture can be achieved, making it difficult to obtain data from the cell–substrate area. Also, at high cell densities, neurons are at close proximity. At these small distances, the halo effect caused by phase-contrast microscopy [21] obscures much of the clear vision on the soma’s distal regions (which consist of very thin lamellae) and cellular processes. The halo effect makes it also hard to distinguish somas from axonal outgrowth, cell debris, and noncovered substrate. In Fig. 11, at a maximum cell coverage of 93%, the extent of cell–cell contacts seems to be poor. However, when we detached the tissue from the substrate, we observed a floating “monopiece” sheet of cells, indicating a much better than poor extent of cell–cell contact in dense neuronal cultures. The conclusion can be drawn that IS shows more details and is more accurate about coverage. It is also a relatively simple technique, yet yielding quantitative data on culture development.

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