Quantitative Label-Free Cell Proliferation Tracking with a Versatile Electrochemical Impedance Detection Platform

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Abstract. Since the use of impedance measurements for label-free monitoring of cells has become widespread but still the choice of sensing configuration is not unique though crucial for a quantitative interpretation of data, we demonstrate the application of a novel custom multipotentiotstat platform to study optimal detection strategies. Electrochemical Impedance Spectroscopy (EIS) has been used to monitor and compare adhesion of different cell lines. HeLa cells and 3T3 fibroblasts have been cultured for 12 hours on interdigitated electrode arrays integrated into a tailor-made cell culture platform. Both vertical and coplanar interdigitated sensing configuration approaches have been used and compared on the same cell populations.

1. Introduction

Tracking bio-impedance at the microscale is becoming a ubiquitous laboratory tool to monitor in real time and with no need for labels the health and dynamic response of adherent cells to chemical or mechanical triggers, both at single cell level and for large colonies. Automatic and heavily parallel assays can be performed with beneficial impact for instance in diagnostics and drug discovery. As first introduced by Giaever and Keese\textsuperscript{1}, cells are typically grown on planar microelectrodes and their presence and proliferation alters the interfacial impedance that is recorded over time, commonly at a single frequency. Given the amount and importance of the physiological parameters\textsuperscript{2} that can be monitored in comparison with the intrinsically non-specific nature of impedance detection (sensitive to any environmental changes), we study the optimal detection conditions for different model cell lines with an electrochemical platform originally designed for multiparametric investigations of neural stem cells\textsuperscript{3}. In particular, two alternative sensing configurations are experimentally compared by recording impedance spectra for both HeLa cells and mouse fibroblasts using different cell seeding densities. To our best knowledge, this is the first time two sensing configurations are applied simultaneously for the same cell populations.

2. Theory

Avoiding the addition of possibly toxic or otherwise interfering redox molecules to the cell culture medium, no Faradaic phenomena are present and the small-signal impedance equivalent of the electrochemical interface is simply constituted by the double layer capacitance (for standard Phosphate Buffered Saline, PBS, it can be estimated with a specific interfacial capacitance of 0.1
pF/µm²) and the resistance of the ionic solution (PBS conductivity 1.5 S/m). The presence of cells, whose membrane can be considered insulating for frequencies below 1-10 MHz since the membrane capacitance is still not bypassed, obstructs the ionic conductive paths, thus resulting in a monotonic increase in impedance magnitude with the number of cells. Several lumped-parameter equivalent models have been proposed to describe the behaviour of a cell monolayer: typically by an additive intermediate R||C couple (or R||CPE). As more complex models are valid only for a given type of cells (the amount of adhesion and the morphology), we choose to maintain the simple R-C series model also in the presence of cells and to track its “small” variations due to such a growing barrier.

As illustrated in Fig. 1a, in the classic vertical configuration, the sinusoidal voltage is applied to the large and distant counter electrode and the current is sensed at the working electrode. Instead, in the coplanar approach (Fig. 1b), the symmetrical impedance between closely-spaced interdigitated combs is measured. With the proposed platform, it is possible to switch from one configuration to the other in order to perform both measurements on the same comb (green WEa in Fig. 1) covered with the same cell population, approximately at the same time (the time shift between consecutive measurements is a few seconds, i.e. negligible with respect to the time scale of the experiments) thus quantitatively comparing their sensitivity in tracking cell proliferation. It is expected that, at a high enough frequency to enable shorting of the double layer capacitance, the coplanar scheme provides a higher sensitivity due to the higher confinement of the field lines. Unfortunately, the latter requires a higher operating frequency as the solution resistance is smaller (and capacitance is halved). Finally, the impact of parasitics has been carefully analysed and taken into account in the design of the microelectrodes. In fact, as illustrated in Fig. 1c and extensively discussed in [4], besides the connecting wires, the presence of a conductive silicon substrate and large metal leads covered by the Si₃N₄ passivation layer in contact with the solution produces large stray capacitances that might significantly degrade the instrument performance.

3. Materials and Methods

The monitoring platform is composed of two main elements: a cell culture device integrated with a microelectrode chip and a multichannel bipotentiotstat [3]. The cell culture device consists of a micromilled poly(methyl methacrylate) (PMMA) holder characterized by an upper vial, containing up to 600 µL of volume, that allows cell seeding and culture medium exchange. The gold microelectrode chips have been fabricated using a standard lift-off process on a Si/SiO₂ substrate. On each chip, 12 electrode arrays are defined, each consisting of an interdigitated electrode (IDE) (functioning as the working electrode (WE)), a counter electrode (CE) and a reference electrode (RE). The WEs consist of 12 500 µm long fingers having 10 µm width and spacing.

Figure 1. Simultaneous comparison for cell impedance sensing: (a) ECIS vertical configuration vs. (b) interdigitated coplanar configuration and (c) modelling of the parasitic capacitance of the electrodes.
HeLa cells and 3T3 Fibroblasts were grown in 75-cm² cell culture flasks at 37 °C with 5% CO₂ in a humidified atmosphere. Dulbecco’s Modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin was used as culture medium for both cell lines. After reaching 80-90% confluency, the cells were washed with Dulbecco's phosphate buffered saline (PBS) and harvested through trypsinization.

Electrochemical impedance spectroscopic (EIS) tracking was used as a non-invasive biophysical technique to continuously monitor and compare cell adhesion and proliferation of HeLa cells and 3T3 Fibroblasts. Prior to using, in order to clean the gold surface of the microelectrode chip from the ambient contaminants, the chips were treated for 10 minutes with a solution containing 25% H₂O₂/50 mM KOH [5]. The culture chamber was sterilized using 500 mM NaOH. To facilitate cell adhesion, the electrode chip was coated with poly-L-lysine (10 μg/mL). Two different cell densities (about 2.5·10⁵ and 3.5·10⁵ cells) were initially seeded into the chamber and the measurements were performed while the monitoring platform was kept in the incubator. During the first 4 hours after cell seeding, impedance spectra were acquired from each sensor element every 20 minutes, then every hour. In order to limit the current flowing through the cells, a 200 μV sinusoidal signal was applied and 30 points were acquired in the frequency range between 100 Hz and 100kHz, each with an averaging time of 2s. After 24 hours, cells were fixed (for 1 hour with 2% glutaraldehyde solution in PBS, followed by rinsing with PBS and cell culture tested water) and dehydrated with ethanol and imaged using SEM.

All calculations and graphs were obtained using MATLAB software. For each experiment, data acquired from the different sensor elements were averaged and processed to derive a parameter termed Cell Index (CI) [6] that represents the relative impedance change in the system. For each frequency and for each time point, the CI is calculated as CI(t,f) = (|Z(t,f)| / |Z(0,f)|) – 1, where |Z(0,f)| is the magnitude of impedance acquired at the beginning of the experiment, when cells were not yet seeded into the well and |Z(t,f)| is the magnitude of impedance acquired after cell seeding at different time points. Data are represented as mean between experiments ± standard error of mean.

4. Experimental Results

In order to evaluate the behavior of the detection platform and compare the two alternative detection configurations, electrochemical impedance spectroscopic monitoring of two different cell lines was performed. Fig. 2a shows the increase in CI for HeLa cell adhesion and spreading during 12 hours. The spectra were acquired using both the interdigitated coplanar and vertical configuration mode, and the CI was calculated and plotted at the characteristic peak frequency (about 24kHz and 100kHz for the vertical and the coplanar configuration, respectively). For both sensing configurations, the maximum value of CI was reached 10 hours after cell seeding. As can be observed, the coplanar mode provides...
the highest sensitivity reaching the CI of about 1.6. For the vertical configuration, the corresponding value was about 0.45. Fig. 3 shows SEM images of HeLa cells after 12 hours of measurements.

A comparison between the two cell lines, characterized by different adhesion properties, is shown in Fig. 2b. The CI trend over 5 hours of both HeLa cells and fibroblasts confirms that the interdigitated coplanar configuration provides the highest sensitivity for monitoring (CI values 5 hours after cell seeding for HeLa cells and fibroblasts were 0.65 ± 0.02 and 0.9 ± 0.1 using the vertical configuration, as well as 1.4 ± 0.05 and 2 ± 0.15 using coplanar configuration). Moreover, under the same culture conditions and the same cell density, fibroblasts show higher CI values, confirming their stronger adhesion in comparison with HeLa cells. A slightly higher standard error of mean is also observed in the case of fibroblasts, probably due to a larger variability of cell morphology.

5. Conclusions
The presented electrochemical impedance monitoring system allows parallel measurements to study biological phenomena. In order to optimize cell proliferation assays, two alternative configuration modes have been experimentally compared in parallel using different cell lines. It has been clearly demonstrated that for monitoring cell adhesion, the coplanar configuration provides the highest sensitivity and that, as expected, cell lines with higher adhesion to the electrodes provide a higher impedance variation. These experimental results validate the use of the platform and the adopted simple and general equivalent impedance model, representing a quantitative basis for future investigations of more subtle effects such as differentiation of stem cells or discrimination between necrosis and apoptosis.

6. References
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