Cell-Substrate Lift-off Lithography: Proof of Concept

D D Stupin¹, A A Kornev¹, N A Filatov¹, and S V Koniakhin¹,²

¹St. Petersburg Academic University, Khlopina 8/3, 194021 St. Petersburg, Russia
²Institut Pascal, PHOTON-N2, University Clermont Auvergne, CNRS, 4 avenue Blaise Pascal, 63178 Aubière Cedex, France
E-mail: Stu87@ya.ru, Stupin@spbau.ru

Abstract. In this study, we demonstrate the effectiveness of the lift-off lithography principle for mechanical only and thus chemical-free formation of HeLa cells controlled spatial distribution on the multielectrode array (MEA) surface. It allows creating the cells carrying MEA electrode and cells-free control MEA electrode in close proximity and thus in the same environment (temperature, chemical composition etc.). This scheme provides the additional sensitivity to the single cell and cells population bio-impedance measurements, which was ascertained by detecting the cells reaction on the trypsin-Versene solution addition to the medium.

1. Introduction
Nowadays the bio-electricity and bio-impedance measurements are a hot topic in experimental physics and practical biology [1]. Particularly, impedance-based in vitro studies of the cells population and single cells are widely used in cancer research [2], wound healing investigation [3], in pharmacology [4], and biosensors [5]. The very promising application of impedance measurements requiring high sensitivity is creating the cell-based hazard sensors [6, 7, 8, 9]. In many practical cases, for example in wound healing research and cell-based biosensors, it is necessary to have the possibility to manipulate and control cells spatial distribution on the electrodes. For achieving this goal different approaches were proposed: lethal-dose electroporation [10], microfluidic [11], dielectrophoresis [12] etc. However, despite of the successful realization of the mentioned above methods, they are difficult in implementation and could be aggressive for electrodes, electrodes’ dielectric and cells. Here we propose and describe the implementation of a simple and non-destructive method for preparing arbitrary spatial cells distribution in a multielectrode array dish, which is based on the lift-off lithography idea [13] and resembles the mechanical wounding method [14, 15, 16]. For demonstrating the advantages of our method we have provided the bio-impedance measurement experiment in which we have prepared improved cells-on-electrodes geometry where cells-containing and control empty electrodes were located in close proximity each from other. Such sample processing makes the bio-impedance measurements more sensitive due to the same chemical environment of the electrodes.

2. Motivation
The accurate bio-impedance sensing requires existing of the two identical target electrodes whose immittance spectrum (impedance or admittance, IS) should be measured with respect to common empty reference electrode: the cell-carrying electrode and empty control electrode. The reference electrode is typically equal or larger by its surface than the target electrodes and
could be neglected in impedance interpretation. Such three-electrode experiment design gives the possibility to distinguish cells influence on the electrode impedance from the other purely physical and chemical phenomena (e.g. temperature drift).

In this paper, as example we consider an experiment whose aim is detecting cells detachment from the cell-carrying electrode by it’s electrical admittance measurement before and after replacing cells’ medium with trypsin-Versene solution. The latter is widely used mixture for removing attached cells from Petri dish [17].

According to Giaever-Keese model [18], the electric current through cells carrying electrode pass not only through electrochemical impedance $Z_{e/c}$, as in case of the empty electrode (Fig. 1, left panel), but also through the seal between cells’ membranes and the electrode surface (Fig. 1, middle panel). The latter results in appearance of a serial active resistance of the seal $R_s$ in the impedance of the covered by cells electrode. The total cell/electrolyte/electrode impedance reads as

$$Z_{c/e/e} = Z_{e/c} + R_s.$$ (1)

Obviously, if cells were detached from the electrode (Fig. 1, right panel), it’s IS should be equal to IS of the empty control electrode

$$Z_{c/e/e} = Z_{e/c}.$$ (2)

This criterion could be used for detecting the end of the cell detaching process, which correlates with the cell morphological changes. However the $Z_{e/c}$ depends on cultural media composition homogeneity and temperature homogeneity and also on the distance between the considered electrode and the reference electrode. That’s why it is important to have cells carrying electrode and control empty electrode in close proximity, which is possible to achieve by the proposed technique.

3. Experimental
3.1. Preparing the MEA
The procedure of the MEA preparation with the desired cells spatial distribution consists of three steps. At the first step, a PDMS (polydimethylsiloxane) [19] mask with the desired pattern installed under microscope control by plastic tweezers onto the multielectrode array Petri dish in the preferred orientation [Fig. 2(a)]. In present proof-of-concept study, the mask was prepared from bulk piece of PDMS by cutting using the commercial razor blade (120 µm thickness). Due to good adhesion PDMS strongly attached to the multielectrode array dish, which prevent it’s swimming up in culture media. At the second step, the HeLa cells plating and incubating (37°C, 5% CO$_2$) procedures [Fig. 2(b,c)] were provided. At the third step, since cells were attached to MEA dish and PDMS mask, the latter was mechanically removed by plastic tweezers from the dish bottom without any mechanical damage [Fig. 2(d)] providing the required pattern. It is instructive to notice, that the result of this procedure resembles the result of the semiconductor lift-off lithography [13]. The quality of cut and cell-substrate edge can be seen from Fig. 3(a) and Fig. 3(b) respectively.
For producing PDMS mask the Sylgard 184 (DowCorning, Germany) kit was used. This kit includes a curing agent and a liquid silicone base. The proportion of the agent to the basis of 1:10 was used. Curing occurred during the heat treatment in Carbolite oven (PF30, England) at 65°C during 4 hours.

Figure 2. The proposed cell-substrate lithography scheme. (a) PDMS film installation; (b) plating HeLa cells; (c) cells incubation; (d) removal of the PDMS film.
3.2. Cell-substrate IS measurements

For providing IS measurements of the cells population we have used a multielectrode array 60StimMEA200/30-Ti (MultiChannel systems, Germany) and adhesion HeLa cells, which were obtained from the Bank of Cell Cultures of the Institute of Cytology of the Russian Academy of Sciences. To detect the influence of the cell presence on the contact IS, the MEA IS measurements were conducted before and after the replacing cells’ medium (phosphate saline buffer, Biolot, Russia) with trypsin-Versene solution (Biolot, Russia). The used trypsin-to-Versene ratio was 1:4.

MEA with the desired cell spatial pattern (pair of electrodes in close proximity each from other, one of which is covered by cells, and another is empty) was prepared by the described above PDMS mask-based technique.

The described in Ref. [20] experimental setup and processing protocol were used for conducting the impedance measurements. IS were measured between reference electrode (not presented on photographs) and empty control electrode [Fig. 4(a,b) left electrode], and between reference electrode and covered by cells electrode [Fig. 4(a,b) right electrode]. All three electrodes have the same rectangle shape with 50 µm × 250 µm dimensions. As more illustrative IS representation we have used admittance Nyquist plot for data plotting. Photographs were made by Leica DM4000 microscope (Leica, Germany) under a bright field transmission light.

4. Results and Discussion

The main results are presented in Fig. 3 and Fig. 4. The photographs in Fig. 3(a,b) indicate that PDMS film effectively blocks cells seeding on the MEA dish, and after it’s removing we obtain nearly located covered by cells electrode and the neighboring empty electrode. Such closely-spaced electrodes preparation minimize the influence of cell medium inhomogeneity effects on the IS measurements because the covered by cell electrode and control electrode have the same environment.

In the clockwise rotated and magnified image of the electrodes [Fig. 4(a)] one can see that the left control electrode is cells-free, while the right electrode is fully covered by cells. The corresponding admittance spectra of the electrodes given in Fig. 4(c) indicate that admittance of the cells-free electrode is higher by magnitude than the cells-carrying electrode. This picture is in a good agreement with Giaever-Keese model, see Eq. (1).

After replacing cells’ medium with the trypsin-Versene solution the cells change their morphology and detach from the electrode [Fig. 4(b)]. Due to the fact that the detached cells do not form the seal (additional resistance), they do not affect electrical electrode properties any more. Thus the admittance spectra of the cells-free electrode and electrode with detached cells should be equal to each other [Eq. (2)], which is confirmed by experimental measurements [Fig. 4(d)]. It should be noticed, that after replacing cells’ medium with trypsin-Versene solution the admittance of the control electrode does not change significantly.
Figure 3. Multielectrode array with installed (a) and removed (b) PDMS film. The magnification is 5x. It could be seen that the upper rectangular electrode is covered by cells and the lower rectangular electrode contains no cells.

Figure 4. Experimental results. Clockwise rotated and magnified (20x) image of the prepared electrodes (previously shown in Fig. 3) before (a) and after (b) replacing cell’s medium with trypsin-Versene solution. Admittance spectra of the electrodes before (c) and after (d) replacing cell’s medium.
5. Conclusion

In this paper, we propose and implement a technique for formation the desired spatial distribution of the adhesion HeLa cells in cells population in vitro based on lift-off lithography idea. Using a biocompatible PDMS mask we have prepared the covered by cells and cell-free MEA electrodes in close proximity each from other, i.e. in the same environment, which eliminates the effects of cells media inhomogeneity on the electrical measurements. Finally, using the prepared electrodes we have experimentally confirmed that cells-contained electrode has lower admittance with respect to the empty electrode, however, the admittance of empty and cells-detached electrodes are equal being in agreement with Giaever-Keese model. It is instructively to compare present results with the very recent paper [21] (sent in July 2018, published in August 2018) where the authors use the chemically detaching poly-L-lysine/SU-8/polyvinyl alcohol multilayer structure as a complex-shape mask and fluorescence microscopy for cell detecting. It should be contrasted with present in our study more simple in mask preparation approach followed by the cell IS measurements.

The developed in present study technique is non-destructive, natural, and safe for both the cells and the multielectrode array dish and does not require chemical processing, making it very useful for cells preparation for in vitro bio-impedance and bio-electricity measurements. We believe that results of our study brings biology and physics towards to creation of portable and high-sensitive biosensors.

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