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Independent impedimetric analysis of two cell populations co-cultured on opposite sides of a porous support

Kathrin Hajek, Joachim Wegener*

Institut für Analytische Chemie, Chemo- und Biosensorik, Universität Regensburg, Universitätsstr. 31, 93053 Regensburg, Germany

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ABSTRACT

The transepithelial or -endothelial electrical resistance (TEER) is a very common and routinely recorded parameter describing the expression of barrier-forming cell-cell contacts (tight junctions) in quantitative terms. To determine TEER, barrier-forming cell monolayers are cultured on porous filter supports that separate two fluid compartments. The frequency-dependent impedance of the cell layer is then recorded and analyzed by means of equivalent circuit modelling providing TEER and the cell layer capacitance. The latter serves as a quantitative indicator for membrane topography. When cells are co-cultured on opposite sides of such a porous support to model more complex biological barriers, TEER readings will integrate over both cell layers and the individual contributions are not assessable. This study describes the modification of commonly used porous filter inserts by coating their backside with a thin gold-film. When this gold-film is used as an additional electrode, both cell layers can be studied separately by impedance analysis. The electrical parameters of either cell layer are assessable independently by switching between different electrode combinations. The performance of this new approach is illustrated and documented by experiments that (i) follow the *de novo* formation of cell junctions between initially suspended cells and (ii) the manipulation of mature cell-cell junctions by cytoskeleton-active drugs. Both assays confirm that both cell layers are monitored entirely independently.

1. Introduction

Measuring the transepithelial or transendothelial electrical resistance (TEER) is a well-established non-invasive approach to characterize the tightness of barrier-forming cell layers *in vitro* [1]. Besides assessment of permeability coefficients (P_E) for molecular tracers and hydraulic conductivity (L_P), reading TEER is considered as the most convenient and fastest *in situ* technique capable of reporting on changes in barrier function with a time resolution of a few seconds if needed [2]. In the regular setup to record permeability coefficients for molecular or ionic tracers the cells of interest are grown to monolayers on permeable filter supports that separate two fluid compartments. The filter supports themselves are ideally very permeable so that ion or more general probe transfer from one compartment to the other is determined by the barrier properties of the cells. The transfer rate of hydrophilic marker compounds or ions, both of which are not membrane permeable, is considered as a measure for the tightness of intercellular junctions, most notably the tight junctions [3]. The entire setup is considered an *in vitro* model of an interfacial tissue like the blood vessels, the skin or the lining of gut or bladder [4].

For TEER readings two electrodes are introduced in either one of the two fluid compartments. One electrode from either side is used to apply a well-defined constant current I (DC) across the cell layer. The two remaining electrodes measure the associated voltage drop U. Ohm's law allows calculating the integral resistance of cell layer and filter support as $R_{total} = U/I$. Subtracting the resistance of the cell-free filter provides the TEER of the cell layer in units of $\Omega \cdot cm^2$. Despite of some experimental problems, the so-called *chop-stick* setup with point-like electrodes fixed to a fork-like structure that allows introducing the electrodes in both fluid compartments at the same time from the side, are still used heavily [5]. Most recently, setups have been introduced with only one electrode per compartment that is used for current injection and voltage reading at the same time [6]. Based on this blueprint commercial devices have been designed and are marketed worldwide. These electrodes are no longer point-like but circular plates with roughly the same area as the filter support providing a very homogeneous electric field that prevents an overestimation of TEER values. However, these two electrode arrangements are no longer compatible with DC measurements but require AC. Under these conditions Ohm's law returns the system's impedance and not the resistance. But when the impedance is measured at several discrete

* Correspondence to: Institut fuer Analytische Chemie, Chemo, & Biosensorik, Universitaet Regensburg, Universitaetstr. 31, 93053 Regensburg, Germany.
E-mail address: Joachim.Wegener@ur.de (J. Wegener).

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frequencies over an extended frequency range (impedance spectroscopy) and the data is subsequently analyzed by equivalent circuit modelling, the experiment returns not only TEER but also the cell layer capacitance C_{cl} that reports on the topography of the plasma membrane. The latter is an important but often underestimated experimental parameter when transporting epithelial cell layers are studied. The interested reader is referred to a recent review for more details [2].

Impedance spectroscopy of cell-covered filter inserts has become a standard technique to investigate all kinds of barrier-forming cell types in the last decades [7,8]. Epithelial or endothelial cell layers are probed with respect to tight junctions formation during cell attachment and spreading [6] or changes in their barrier function in response to a certain stimulus [9–12]. The majority of these studies is performed with cell monolayers. However, in some cases epithelial or endothelial barrier function is only properly expressed when the barrier forming cells are co-cultured with other cells that reside in their direct neighborhood *in vivo* so that cell-cell communication is possible. One of the most well characterized examples is the *blood-brain barrier* that is responsible for separating the circulating blood flow from the interstitial fluid of the CNS. *In vitro* models of this barrier revealed that blood-brain barrier endothelial cells show a remarkably higher TEER when they are cultured in presence of astrocytes and or pericytes [13–15]. Both of which are in contact to the brain capillary endothelial cells *in vivo*. These co-cultures are technically established by growing endothelial cells on one side of the filter and astrocytes/perciytes on the other. The filter support is thin enough to allow for physical or chemical communication between these two cell types. When TEER values were recorded for these co-cultures, only the integral TEER of the entire co-culture was assessable. So far these measurements cannot return the individual contributions of either cell species. This may be negligible when endothelial-astrocyte co-cultures are studied since astrocytes do not form a significant barrier. Data interpretation may, however, become difficult for those biological barriers that are synergistically established by endothelial and epithelial cells as in the choroid plexus [16] or the renal glomeruli [17]. Effects of drugs or toxins cannot be attributed individually to one of the two co-cultured cell types.

Facing this limitation, we designed and fabricated a permeable gold electrode based on commercially available Transwell® filter inserts, that enables measuring the complex impedance of each of the two co-cultured cell layers separately. Thus, the individual responses of the cells on opposite sides of the porous electrode to any kind of stimulus are assessable. The integrated impedance over both cell types is additionally available in this experimental setup.

2. Material and methods

2.1. Modification of Transwell® filter inserts: Preparation of porous gold electrodes

Corning Transwell® permeable supports (polycarbonate membrane, growth area: 1.12 cm^2, pore diameter: 400 nm, pore density: $10^8/cm^2$) were purchased from LMS Consult (Germany). A 100 nm thick gold film was deposited upon the backside side of the porous membrane using a Bal-Tec sputter coater SCD 050 (Bal-Tec AG, Balzers, Lie) equipped with a real-time thickness monitor. The gold-covered membrane was electrically connected to a copper wire by a silver conductive adhesive (Conrad, Germany). The copper wire and the contact area were insulated against the electrolyte solution by the use of non-cytotoxic silicone glue (Master Fix Aquarium Silikon, Warenimport und Handels GmbH, Austria). The setup is illustrated in Fig. 1.

2.2. Cell culture

High resistant (hr) and low resistant (lr) strains of Mardin-Darby canine kidney cells also referred to as strain I and II (MDCK-I/MDCK-II), respectively, were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) and used between passage 47–58 (MDCK-lr) and 24–27 (MDCK-hr), respectively. Both MDCK strains were kept in a humidified atmosphere at 37 °C with 5 % (v/v) CO_2. The epithelial cell lines were grown to confluence in standard culture flasks (Sarstedt, Germany) using MEM-Earle medium (Sigma Aldrich, Germany) containing 1 g/L D-glucose, 4 mM L-glutamine, 100 µg/mL penicillin/streptomycin and 5 % (v/v) fetal calf serum (Biochrom AG, Germany). The culture medium was routinely exchanged every three days. Subcultivation was performed once a week using standard trypsinization procedures: cell suspensions were prepared using 0.25 % (w/v) trypsin-EDTA solution (Sigma Aldrich) for MDCK-I cells and 0.025 % (w/v) for MDCK-II cells.

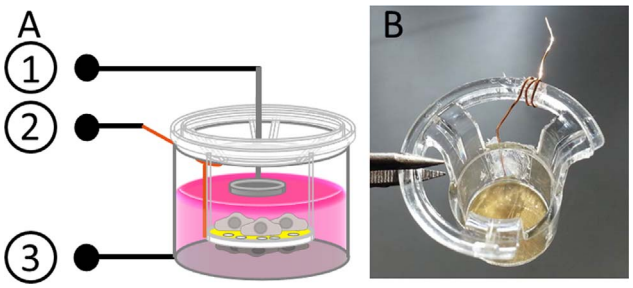

Fig. 1. (**A**) Experimental setup to individually measure the frequency-dependent impedance of two cell populations grown on opposite sides of a permeable filter support (Transwell®). The stainless steel electrode (1) dips into the upper compartment of the device. Electrode 2 represents the porous gold film electrode established on the backside of a standard Transwell® filter insert. The stainless steel plate underneath the membrane serves as electrode 3 as well as bottom of the measurement chamber. A glass ring is mounted on top of electrode 3 to mechanically fix the filter insert and to form the lower fluid compartment. (**B**) Photograph of the modified filter insert with an insulated copper wire to connect the gold-film electrode.

2.3. Impedance measurements

All measurements were carried out in a standard cell culture incubator at 37 °C and 5 % (v/v) CO_2. The experimental setup for impedance analysis of filter inserts consists of a Lexan®-slide as the base plate. A 2 mm thick plate made from stainless steel was glued to this base plate forming the bottom electrode of the chamber (electrode 3, cp. Fig. 1). A glass ring (Ø = 24 mm, h = 18 mm) was mounted on top of the steel plate electrode by using non-toxic silicone glue. The glass ring forms a fluid compartment and mechanically fixes the filter inserts above the bottom electrode. A second, stamp-shaped stainless steel electrode (electrode 1, cp. Fig. 1) dips into the electrolyte solution from above the filter membrane [6]. The two steel electrodes as well as the porous gold electrode (electrode 2; cp. Fig. 1) were electrically connected to an impedance analyzer (Solatron Instruments, SI-1260, UK) *via* a home-made relay that allows addressing of the different electrode pairs (1–2, 1–3, 2–3) individually as indicated in Fig. 1. Relay and impedance analyzer were controlled by a regular PC using data acquisition software written in LabView. Impedance data were recorded in a frequency range of 1 – 10^5 Hz by applying a non-invasive AC voltage of 10 mV (rms) to a given electrode pair with the DC potential being clamped to zero. The latter is necessary to avoid any battery effect between the two different metals used as electrodes. The overall impedance of the co-culture was measured in the common '*transfilter mode*' (electrode 1 *vs.* electrode 3, ***mode 1–3***). The impedance of the cells growing on the upper side of the filter support was recorded by using electrodes 1 and 2 (***mode 1–2***) whereas the impedance of the cells grown on the backside of the filter was recorded by readings between electrode 2 and 3 (***mode 2–3***).