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Analytical methods to determine electrochemical factors in electrotaxis setups and their implications for experimental design

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ABSTRACT

Direct current (DC) stimulation can be used to influence the orientation and migratory behavior of cells and results in cellular electrotaxis. Experimental work on such phenomena commonly relies on electrochemical dissolution of silver:silver–chloride (Ag:AgCl) electrodes to provide the stimulation *via* salt bridges. The strong ionic flow can be expected to influence the cell culture environment. In order to shed more light on which effects that must be considered, and possibly counter balanced, we here characterize a typical DC stimulation system. Silver migration speed was determined by stripping voltammetry. pH variability with stimulation was measured by ratiometric image analysis and conductivity alterations were quantified *via* two electrode impedance spectroscopy. It could be concluded that pH shifts towards more acidic values, in a linear manner with applied charge, after the buffering capability of the culture medium is exceeded. In contrast, the influence on conductivity was of negligible magnitude. Silver ions could enter the culture chamber at low concentrations long before a clear effect on the viability of the cultured cells could be observed. A design rule of 1 cm salt bridge per C of stimulation charge transferred was however sufficient to ensure separation between cells and silver at all times.

1. Introduction

Electrotaxis, or galvanotaxis, can be defined as the directional movement of cells in response to an electric field. Such migration is a known behavior for a variety of cells. Endogenous electric fields occur in the embryo to guide migration and are furthermore important for keratinocyte and fibroblast migration during wound healing [1–6]. It is theorized that electrotactic behavior can be partially responsible for cancer cell migration and metastasis [6–11]. In addition, electric fields can be important for guiding neural regeneration after injury as well as for tissue engineering applications [12,13]. In summary, studies of cellular response to electric fields are of interest for a wide variety of physiological processes. The opportunity to control cellular migration over a surface could potentially also prove useful for a multitude of biotechnological devices in the future.

Most experimental work within this field is performed in cell culture models. The standard experimental setup is based on Ag:AgCl electrodes connected to a cell culture chamber *via* salt bridges. An applied voltage onsets electrochemical dissolution of the Ag:AgCl leading to ionic current in the culture medium. Ionic silver is prevented from entering the culture chamber by ensuring the salt bridges are of sufficient length.

There are various protocols described in literature on how to assemble experimental systems such as the one described above. Common practice was for long time to use a culture dish with two glass slides sealed to the bottom. A narrow slot confined by the two slides defined the culture chamber [14]. More recently, commercial cell culture chambers have been introduced (Ibidi products), which ensures reproducible chamber geometry and facilitates handling. Although the latter is an improvement to the culture dishes, the overall experiment still does not fully meet the requirements on reliability, reproducibility and efficiency that are essential for rapid progress within the field. As a result, the theoretical understanding of why, and under which conditions, cells perform electrotaxis is still incomplete [15]. In a recent critical review Cortese et al. underlines the stressing need for improved experimental methodologies and in particular standardization of devices and protocols [16].

A handful of recent studies describes chip-based designs for cell culture electrotaxis [17–25]. Microsystems for cell culture applications are

Abbreviations: Ag:AgCl, silver:silver–chloride; DC, direct current; PDMS, polydimethylsiloxane; PMMA, poly(methyl methacrylate); RDE, rotating disk electrode; Na_2EDTA, di-sodium-ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; MTT, thiazol-blue-tetrazolin-bromide salt; KNO_3, potassium nitrate.

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already well established within other fields and a wide range of sophisticated chip based tools are described in the literature. Microfluidic systems make it possible to manipulate fractions of cell populations growing on the same chip, exposing them to different electrical fields and/or pharmacological treatments. Using integrated microsensors experimental parameters such as pH and temperature can be monitored. Sophisticated chip-based solutions exploiting the many possibilities offered by the microfluidic environment thus have the power to increase throughput and control without sacrificing precision. Nevertheless, miniaturization also introduces new considerations and constraints on the experiment. This stresses the need for a thorough investigation of the electrochemistry of the standard experiment. Such information is useful both for establishing design rules for microfluidic design and to interpret data from experiments in more traditional setups.

This paper reports on a series of experiments aimed at: 1) quantifying the effect of the ionic exchange on conductivity; 2) monitoring pH over the time course of an experiment; 3) characterizing the flow of ionic silver in salt bridges and 4) studying if there is any negative effect on cell viability and proliferation by medium exposed to the DC field stimulation system. The rationales for studying each of these are outlined in the following sections.

Understanding how stimulation alters conductivity (κ) and pH is essential for reproducibility of experiments. It is common practice to use systems were the current (I) through the chamber is measured as a proxy for the electrical field (E) according to $E = I/\kappa S$. This assumes that cross section (S) can be precisely determined and that conductivity is constant. In contrast to this, the ionic content of the medium is substantially altered since the species actively driven into the channel might differ from the ions leaving, which in turn can be expected to lead to variations also in its conductivity. Furthermore, ionic exchange can be expected to cause alterations in pH which is known to be a highly influential factor on electrotactic behavior. *E.g.* Allen et al. report that a shift from pH 6.2 to 5.8 completely canceled out the directional effect in keratocytes [15].

The use of excessively long agar bridges will limit the possibilities for integration of full systems on chip. It is therefore highly desirable to identify methods allowing for empirical evaluations of the actual migration speed. Furthermore, to investigate to which extent any of the above-mentioned electrochemical side effects might influence cell viability, cytotoxicity assays were performed as a complement.

We here provide data that can serve as basis in the design of future microfluidic electrotaxis systems and facilitate the transition from the previous handmade versions to their microsystems engineered counterparts. Furthermore, for the performance of electrotaxis in general, it is essential that such parameters are considered to ensure a reproducible outcome.

2. Materials and methods

2.1. Electrodes

DC stimulation was provided by a pair of Ag:AgCl disk electrodes (12 mm in diameter, Science Products E-244) connected to a voltage source (TTi PL303QMD MD-P). For voltammetry, requiring a three electrode system, a platinum wire was used as counter electrode and an Ag:AgCl reference electrode was purchased from World Precision Instruments. This particular version has minimal leakage of filling solution and can therefore be assumed not to contribute additional Ag^+ to the sample (Prod no. Flex-Ref, leak rate $< 0.057 * 10^{-8}$ nl/h).

2.2. Agar bridges

All salt bridges in this study were produced using agarose according to the following: a silicone hose, 3 mm in diameter (Versilic® Carl Roth HC63.2), was cut to the desired length. Agarose (Sigma Aldrich A9539) was dissolved in phosphate buffered saline (PBS) at a concentration of 20 mg/ml by microwave heating. The agarose container was carefully weighed before heating and any evaporation was compensated by adding warm deionized water (MilliQ, 18.2 MΩ cm) to the solution. The silicone tubes were filled with the liquid agarose and allowed to cool. Agar bridges were stored in PBS to prevent them from drying. Their impedances were routinely measured and found to be 1.21 kΩ/cm ± 0.05.

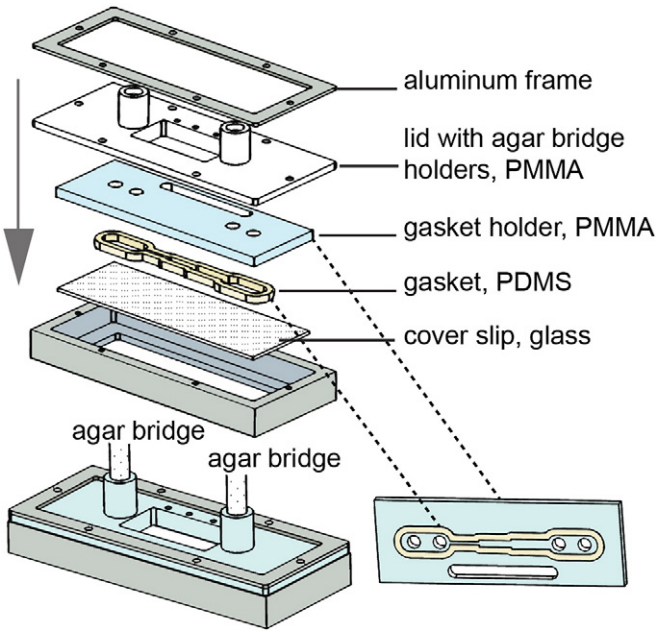
2.3. Electrotaxis chambers

Several types of setups were fabricated in-house to serve as experimental platforms for the measurements. All were based on the general principles described in the paper by Song et al. [14]. The container for the anodic/cathodic Ag:AgCl electrode will be referred to as vial A/C throughout this paper and the cell chamber will be referred to as the DC-chamber.

For conductivity and pH measurements a DC chamber similar to the one presented by Sato et al. was constructed (Schematic 1) [21]. In brief, the chamber was confined by a polydimethylsiloxane (PDMS) gasket sandwiched between a poly(methyl methacrylate) (PMMA) lid and a standard microscopy glass slide. A groove was milled into the lid in order to support the gasket. Estimated height of the compressed chamber is 0.2 mm. Furthermore, a second PMMA lid with holders for the salt bridges was fabricated. The complete system was compressed between an aluminum frame (top), and a base holder, using screws. Agar bridges used for this system were 7.5 cm in length.

For the cytotoxicity studies, a chamber was milled into a solid block of polypropylene (Schematic 2a). The simple system allowed for the exposure of cell culture medium to DC stimulation under sterile conditions. In short, two agar bridges, 5 cm in length, connected vials A and C *via* the DC stimulation chamber containing 1 ml of cell culture medium. Vials A and C contained 2 ml of medium each.

For silver detection, a special container was constructed to allow for rotating disk electrode (RDE) voltammetry within the restricted volume of the DC chamber (Schematic 2b). The RDE was immersed in the DC chamber from top (Schematic 2b) *via* a cylindrical construction enclosing the shaft. This formed a tight seal to the body of the RDE without restricting the free rotation. Vials A and C, both



Schematic 1. The electrotaxis assembly used for pH measurements as well as conductivity measurements. The PDMS gasket is press fit into the PMMA lid and sandwiched on top of a cover slip. The gasket thus comprises the walls of the DC chamber, the PMMA the top and the cover slip the bottom. Estimated chamber height is 200 μm.