Benjamin K. Naggay[1], Tobias B. Schmidt[1], Karen Ende, and Ralf Kemkemer[1]*

Development of a multi-well-chip for studying 2D and 3D tumor cell migration and spheroid growth in electrical fields

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Abstract: Endogenous electrical fields play an important role in various physiological and pathological events. Yet the effects of electrical cues on processes such as wound healing, tumor development or metastasis are still rarely investigated, though it is known that direct current electrical fields can alter cell migration or proliferation *in vitro*. Several 2D experimental models for studying cell responses to direct current electrical fields have been presented and characterized but suitable experimental models for electrotaxis studies in 3D are rare. Here we present a novel, easy-to-produce, multi-well-based galvanotactic-chamber for the use in 2D and 3D cell experiments for investigations on the influence of electrical fields on tumor cell migration and tumor spheroid growth. Our presented system allows the simultaneous application of electrical field to cells in four chambers, either cultured on the bottom of the culture-plate (2D) or embedded in hydrogel filled channels (3D). The set-up is also suitable for, live-cell-imaging. Validation tests show stable electrical fields and high cell viabilities inside the channel. Tumor spheroids of various diameters can be exposed to direct current electrical fields up to one week.

Keywords: electrical field, galvanotaxis, electrotaxis, cell motility, 2D & 3D cell culture, tumor cells

1 Introduction

Cancer development, tumor growth, and metastasis are complex processes that are based on complex signaling events [1]. A multitude of cellular interactions and biochemical signals but also factors like pH, O_2-concentrations or the mechanical properties of the extracellular matrix (ECM) affect the progression of a tumor [1, 2]. One of the physical cues exerted by the extracellular surrounding are endogenously (*in vivo*) occurring electrical fields (EFs) [1, 3]. Such electrical fields

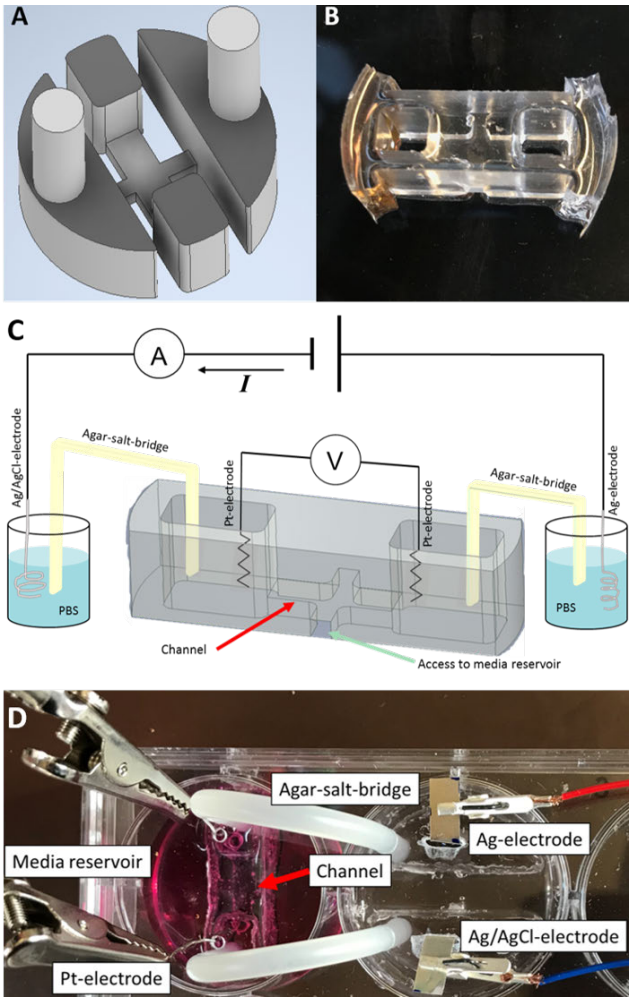

Fig. 1: A) Image of the 3D model of the self-designed 6-well-insert enabling the production of PDMS-molds for the galvanotactic-chamber. B) Image of the PDMS-mold produced via the 6-well-insert shown in (A). C) Scheme of one galvanotactic-chamber showing agar-salt-bridges (light yellow), channel for cell migration and spheroid growth experiments (red arrow), electrolyte reservoirs (blue), access to extra media reservoir (green arrow) and connections for the measurements of current and voltage. D) Image of one completely assembled chamber with Ag/AgCl-electrodes, two easy exchangeable agar-bridges, Pt-electrodes as measuring electrodes, and the channel for the hydrogel (red arrow). Four such inserts are assembled in one standard 6-well plate.

***Corresponding author: Ralf Kemkemer**[1], Reutlingen University, Alteburgstraße 150, Reutlingen, Germany and Max Planck Institute for Medical Research, Heisenbergstraße 3, Stuttgart, Germany, e-mail: ralf.kemkemer@reutlingen-university.de
Benjamin K. Naggay[1], **Tobias B. Schmidt**[1], **Karen Ende,** Reutlingen University, Alteburgstraße 150, Reutlingen, Germany,
[1]contributed equally

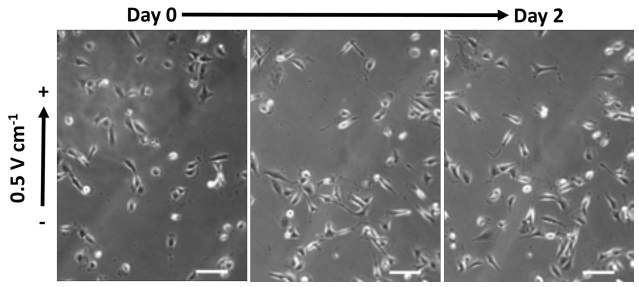

Fig. 2: Representative time-lapse images of the 2D galvanotactic experiments, at 24 h intervals. Scale bar 100 μm.

occur due to gradients in ion concentrations present in most tissues, often as a result of a locally different regulated transmembrane resting potential of cells [3].

While in developmental biology several studies have reported about the impact of bioelectrical signals on cell behavior and tissue patterning, there is limited knowledge about how endogenous EFs affect tumor development [3, 4]. On the cellular level, 2D *in vitro* studies revealed that proliferation or the active migration of many cell types is affected by small electrical fields, typical in the range of a few hundred mV mm^{-1}. Active migration due to an electrical field is coined as galvano- or electrotaxis and has been investigated in different tumor cells such as breast cancer, prostate cancer or lung cancer cells, suggesting a possible involvement of galvanotaxis in cancer metastasis [5, 6]. For example, the cell line MDA-MB-231, human derived breast cancer cells migrate towards the anode in direct current EFs (dcEFs) unlike lung cancer or prostate cancer cells which migrate to the cathode [7]. These observations suggest that galvanotaxis of different tumor cells can be different in specific physiological environments and disease models. Despite these advances and the development of 2D experimental models for studying cell responses to dcEF, suitable experimental models for electrotaxis studies in 3D are still rare.

Here we present an innovative and easy-to-use, multi-well-based system for studying the response of cells and tumor models (spheroids) to electrical fields in 2D or 3D experiments (see Figure 1A to 1D). The system allows the application of EFs to four experimental chambers laden with cells, either cultured on the bottom of the culture-plate (2D) or embedded in hydrogel filled channels (3D), and simultaneous live-cell-imaging of the samples. Inserts for the multi-well plates are fabricated with polydimethylsiloxane (PDMS) via mold replication of a 3D printed model (see Figure 1A and 1B). For EF applications, agar-salt-bridges are used to connect the electrode reservoirs to the chambers containing the spheroid-laden hydrogel in 3D or cell media for 2D experiments (see Figure 1C and 1D). Multicellular tumors spheroids embedded in hydrogels are used as a model for 3D tumor environments and exposed to different dcEFs. Validation tests show stable electrical fields and high cell viabilities inside the channel. Furthermore, the system allows easy handling and imaging, of the tumor spheroids. In addition, results show that tumor spheroids of various diameters can be exposed to dcEFs over several days. Replaceable agar-salt-bridges and electrodes allow dcEF experiments for more than 3 days, not interrupting the live-cell-imaging.

2 Materials and Methods

2.1 Cell culture

The breast cancer cell line MDA-MB-231 was used for all experiments. The cells were cultivated in DMEM (ThermoFisher Scientific) supplemented with 10 % fetal calf serum (ThermoFisher Scientific), and 1 % penicillin/streptomycin (ThermoFisher Scientific). The cells were cultured inside an incubator at 37 °C, and 5 % CO_2 and passaged at confluence of 80 %.

2.2 Spheroid production

A 125 mL glass spinner flask (Magna Flex, Wheaton) was silanized with Sigmacote® (Merck) according to the manufacturer protocol and conditioned with 105 mL DMEM cell media (ThermoFisher Scientific) inside the incubator for 30 min. Then 20 mL of a cell suspension (cell density: 3.5 x 10^5 cells mL^{-1}) was added into the spinner flask. The spinner flask was put onto a magnetic stirrer (MagStir Genie, Scientific Industries) inside the incubator and kept spinning at 30 - 90 rpm for up to seven days. For media exchange, the stirrer was turned off and the aggregates were allowed to settle down. The media was partially removed and the same amount of new media was added subsequently. To harvest the cell spheroids the stirrer plate was turned off and parts of suspension was removed carefully without allowing the aggregates to sink.

2.3 Hydrogel fabrication and cell encapsulation - 3D experiments

For the hydrogel, collagen type I (Rat tail, Merck) with a stock concentration of 6.9 mg mL^{-1} was mixed with sterile PBS, agarose stock solution and cell spheroids. The final concentration of agarose was 0.5 %, and 0.25 mg mL^{-1} for collagen type I. The preheated agarose stock solution (at a temperature

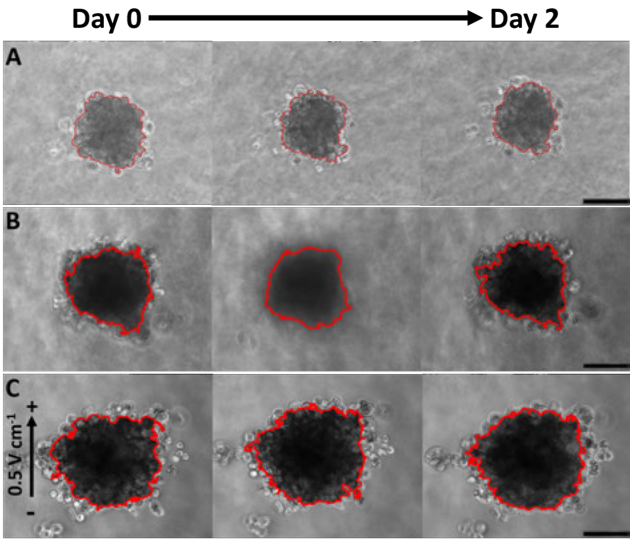

Fig. 3: Representative time-lapse images of the 3D galvanotactic experiments at 24 h intervals. A) Image of a cell spheroid in a transwell. B) Image of a cell spheroid in our presented set-up as a control without an EF. C) Image of a cell spheroid in our presented set-up with an electrical field of 0.5 V cm^{-1}. Scale bar 100 μm.

between 38 °C and 40 °C) was added to the prepared Eppendorf tube. Subsequently, the spheroid enriched cell media was pipetted into the liquid hydrogel solution, and gently mixed by pipetting up and down, and the solution was pipetted into the channel or transwell insert, avoiding air bubbles. Then the hydrogel was allowed to solidify for 30 min at room temperature and another hour inside the incubator. Excess of hydrogel inside the channel reservoirs was removed and discarded. Prewarmed cell media was added to each reservoir and the channel was checked for spheroids inside.

2.4 Preparing the electrotaxis chamber for cell seeding - 2D experiments

The electrotaxis chamber was sterilized under the workbench by UV-light radiation for 20 min and washed directly with 400 μL ethanol (70 %). Following, the chamber was washed twice with sterile milli-q water to remove all the residing ethanol, and then 200 μL collagen solution (collagen type I, rat tail, Merck, end concentration: 100 μg L^{-1} in PBS) was pipetted into the channel. Two sterile wet paper towels were added into the gaps of the 6-well-plate, to minimize evaporation of the PBS. The whole plate was sealed with parafilm and stored inside a refrigerator (4 °C) overnight.

2.5 Microscopy and image analysis

Time-lapse phase-contrast images were collected every 10 min using an inverted microscope (Zeiss Axiovert 200M; Zeiss, Germany) with a 10x objective (CP-Achromat; Zeiss, Germany). A customized live-cell chamber for the microscope was used to maintain cell culture conditions at 37 °C, 5 % CO_2 and relative humidity of 30 %. Time-lapse images were stacked and edited with ImageJ (Version 1.51; NIH, USA). For later analysis of spheroid we used an ImageJ macro from Ivanov et. al. [8] and customized it to our requirements.

3 Results

Construction of the set-up: As basis for our novel set-up, we used conventionally available 6-well-plates (Greiner Bio One) and developed fitting well-inserts with appropriate dimensions. The inserts were produced by replicating a 3D master mold with PDMS (Dow Corning, Sylgard 184). The PDMS-inserts separate four different cell culture media reservoirs and have a cell chamber in the center of the insert (see Figure 1A to 1D). Two opposite reservoirs are connected to the current-applying electrodes via agar-salt-bridges (2 % agarose in PBS). The electrical field is applied between these two reservoirs via the cell chamber. The other two reservoirs are connected to the cell chamber by small channels and serve as cell culture media reservoirs for improving the cell culture conditions (see Figure 1C). 3D construction sketches of the replica mold were made with the software Autodesk Inventor Professional 2020.

Prototypes of the replica master mold for the inserts were produced by 3D printing with a 3D stereolithography printer (Fuse 2 SLA, formlabs). The replica master mold was printed with Clear V4 resin (formlabs) and then washed twice with isopropyl alcohol for 5 min in the "Form Wash" (formlabs). The resin was then cured for 10 min at 60 °C under UV-light radiation with the "Form Cure" (formlabs). Support structures were cut off and the surface was smoothed for later reduction of light scattering of the PDMS inserts. For final production of the inserts, replica master molds were fabricated from aluminum with a professional CNC milling machine.

Assembly of the set-up: PDMS was used for the producing the well-inserts. To reduce adhesion between PDMS and the master molds, the PMMA replica mold prints and a 6-well-plate were treated with 3-Aminopropyltriethoxysilane (APTES) (98 %, abcr GmbH, lot: AB 110815). APTES (200 μL) was pipetted into a watch glass inside a desiccator. The objects were placed next to the watch glass and were treated for 2 h at 0.3 mbar, and room temperature inside the va-

por phase. The aluminum or the treated PMMA models were placed inside the wells of an unused 6-well-plate. Then liquid PDMS (1:10 crosslinker:base) was poured into the wells until it reached a height of approximately 1 cm. The PDMS inside the well-plate was cured in an oven at 80 °C for 3 h. After removal, the solid PDMS forms were washed with ethanol (70 %) and dried with nitrogen gas subsequently. Dust particles on the bottom were removed via sticky tape prior to the bonding.

To bind the PDMS mold inside a new untreated 6-well-plate, a thin layer of liquid PDMS (1:20 crosslinker:base) was spread out onto a glass slide (76 x 26 x 1 mm, Labsolute). The PDMS mold was put onto the liquid PDMS and removed subsequently so that a thin layer of liquid PDMS was stuck on the bottom. Then the PDMS mold was put inside the well and cured in the oven at 80 °C for 2 h. In preliminary tests, we could show, that "gluing" the PDMS mold via liquid PDMS is an easy and sufficient way for getting a tight bonding into the well.

Electrode productions: Silver sheets (70 x 6 x 0.1 mm) were rolled up for an increased surface area. Then one sheet (anode) was placed in a 1 M HCl solution together with a Pt-wire (cathode) for chlorination. Electrodes were chlorinated for 5 min at 5 V and afterwards stored in milli-q water till usage.

Validation experiments: As validation tests, we seeded MDA-MB-231 cells into our self-developed set-up and applied an electrical field. We could show that our system is capable to maintain the necessary culture conditions for long-term 2D galvanotactic experiments (>3days). Due to the depletion of the Ag/AgCl-electrode, while performing an experiment, we developed a system that allows an easy exchange of the Ag/AgCl-electrodes and agar-salt-bridges without interrupting the live-cell imaging. As a further proof-of-concept of the device capabilities, we dispersed the tumor cell spheroids in collagen gel and put the solution in the self-developed system and into transwell inserts as a reference. The first 3D experiments showed, that the spheroids could be held viable (also for several days) and grew in all conditions.

4 Conclusion and Outlook

Detailed mechanisms of the cell response to the extracellular matrix in combination with electrical fields are not jet understood but 3D galvanotactic-chambers are a promising tool to get further insights. We described the implementation of a new and easy to use galvanotactic-chamber to systematically investigate the impact of electrical fields on the growth behavior of cell spheroids and the migration behavior of cells in 2D and 3D experiments. Our set-up allows to study the response of cell spheroids to EFs and the presented ECM in a high-throughput fashion, and therefore providing a useful tool for basic cancer and metastasis research.

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