Cell culture chamber with gas supply for prolonged recording of human neuronal cells on microelectrode array

Cell Culture Chamber with Gas Supply for Prolonged Recording of Human Neuronal Cells on Microelectrode Array

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Background: **Typically, live cell analyses are performed outside an incubator in an ambient air, where the lack of sufficient CO_2 supply results in a fast change of pH and the high evaporation causes concentration drifts in the culture medium. That limits the experiment time for tens of minutes. In many applications, e.g. in neurotoxicity studies, a prolonged measurement of extracellular activity is, however, essential.**

New Method: **We demonstrate a simple cell culture chamber that enables stable culture conditions during prolonged extracellular recordings on a microelectrode array (MEA) outside an incubator. The proposed chamber consists of a gas permeable silicone structure that enables gas transfer into the chamber.**

Results: **We show that the culture chamber supports the growth of the human embryonic stem cell (hESC)-derived neurons both inside and outside an incubator. The structure provides very low evaporation, stable pH and osmolarity, and maintains strong signaling of hESC-derived neuronal networks over three-day MEA experiments.**

Comparison with Existing Methods: **Existing systems are typically complex including continuous perfusion of medium or relatively large amount of gas to supply. The proposed chamber requires only a supply of very low flow rate (1.5 ml/min) of non-humidified 5% CO_2 gas. Utilizing dry gas supply makes the proposed chamber simple to use.**

Conclusion: **Using the proposed culture structure on top of MEA, we can maintain hESC-derived neural networks over three days outside an incubator. Technically, the structure requires very low flow rate of dry gas supporting, however, low evaporation and maintaining the pH of the culture.**

***Index Terms*—Cell culture environment, Human embryonic stem cells, Long-term culture, MEA, Neuronal cells, Osmolarity, PDMS, pH**

1. Introduction

Live cell analyses are typically performed outside an incubator in an ambient air with low CO_2 concentration and low humidity. The lack of sufficient CO_2 supply results in a fast change of pH of the cell culture medium and the high evaporation causes concentration drifts. These facts limit the experiment time to tens of minutes particularly in applications which use bicarbonate buffer in culture medium. In many applications, e.g. in neurotoxicity studies, prolonged measurement of extracellular activity is, however, essential.

Extracellular activity of cells can be recorded using microelectrode arrays (MEA). MEA technology has become an important tool for electrophysiological studies of cells *in vitro* since its invention in early 1970's (Thomas et al. 1972). Since then, the technology has significantly improved and the number of application areas has increased substantially. Still, there is an unmet need to create a stable and controllable environment in MEA systems, particularly for prolonged measurements. That need is especially recognized in the fields of neuronal and cardiac drug screening, toxicity analysis, network learning and detection of chronic responses in cultures (Kehat et al., 2002; Johnstone et al., 2010; Ylä-Outinen et al., 2010). Those studies would benefit long-term (from hours to days) continuous measurement periods to gain detailed information about dynamics of the signaling (Ylä-Outinen et al., 2010; Odawara et al., 2016). Also, the fast development in the stem cell field, especially in the field of human embryonic stem cells (hESC) has broaden up new areas such as studies of human brain development and disease modelling that will surely benefit from prolonged MEA measurements (Narkilahti, 2014; Nat, 2011).

Usually, cells on a MEA-plate are cultured in a humidified incubator with 37 °C and 5% CO_2. However, to record cellular activities, the MEA-plate is placed on a signal amplifier, which is not typically compatible with humid environments. Thus, MEA recordings are performed outside the incubator in ambient air in lower temperature, humidity, and CO_2 concentration, which drastically change the culture conditions. This limits the experiment time typically to tens of minutes.

Changes in the culture conditions affect the cell behavior. For example, evaporation of the culture medium is often significant due to the low ambient humidity. Evaporation increases the osmolarity of the culture medium, and particularly in small volume systems (tens of µl), osmolarity shifts can significantly influence the cell growth as reported by Heo et al. (2007). The evaporation can be reduced by sealing the culture chamber, for example, using a fluorinated ethylene-propylene (FEP) (Potter and Demarse, 2001) or a polydimethylsiloxane (PDMS) (Blau et al., 2009) membrane. These materials have low water vapor permeability but are gas permeable, which is an advantage when supplying CO_2 and other gases through the material. Without additional CO_2 supply, pH of the medium start to increase immediately and pH cannot be maintained in physiological level more than few tens of minutes (Potter and Demarse, 2001; Blau et al., 2009). Therefore, sufficient supply

of CO_2 is the most critical parameter for maintaining pH in medium supplemented with sodium bicarbonate, such as DMEM/F12/Neurobasal, a usual medium base for neuronal cultures (Lappalainen et al., 2010; Salimi et al., 2014; Ylä-Outinen et al., 2014). Typically, 5% concentration of CO_2 maintains the pH of the culture medium in at a physiologically relevant level. Sufficient CO_2 supply maintains the pH of a healthy culture between 7.2 - 7.4, while insufficient CO_2 supply increases the pH of the culture medium to a level harmful for the cells cells (Potter and Demarse, 2001). On the other hand, other media compounds, such as HEPES buffered medium solutions may not require CO_2 to maintain the pH but are reported to be slightly phototoxic (Lepe-Zuniga et al., 1987). Furthermore, HEPES buffered medium may not support the culture of some type of cells.

To overcome challenges to maintain the cultures during long-term MEA recordings, few research groups have developed several types of bioreactors and environment chambers (Mukai et al., 2003; Blau and Ziegler, 2001; Li et al., 2011; Saalfank et al., 2015; Biffi et al., 2012). In the study of Mukai et al., (2003) a MEA system was implemented inside a humidified incubator with continuous perfusion of medium. However, MEA systems are typically not recommended to be placed inside the humid environment because of a high risk of corrosion. In other studies (Blau and Ziegler, 2001; Li et al., 2011), continuous or periodic perfusion of medium was required to maintain the small volume of culture medium in a closed system. Periodic and/or continuous perfusion was also required in the study of Saalfank *et al.*, (2015) in which they were able to perform the continuous MEA recordings up to 70 days. However, they maintained the pH of the culture medium in ambient CO_2 using HEPES buffered medium. An environment chamber introduced by Biffi et al. (2012) required the use of a high flow (70 ml/min) rate of humidified gas with high concentration (10 %) of CO_2 to maintain the culture inside a large environment chamber. Overall, settings described above include several limitations, such as a requirement for continuous medium perfusion during the long pre-cultivation period that typically takes 3-8 weeks for hESC-derived neuronal cell cultures before actual electrophysiological measurements. Also, increased complexity and incompatibility with electronics because of the humidified gas, and a substantial supply or lack of CO_2 limit the use of these systems.

In this paper, we introduce a compact cell culture chamber for prolonged MEA measurements outside an incubator. The compact structure is placed reversibly on a MEA-plate and it includes a large medium reservoir. Therefore, cells can be cultured in the structure in an incubator prior to actual MEA experiments and normal medium exchange periods can be maintained. During the MEA recordings, the proposed structure requires only a supply of very low flow rate (1.5 ml/min) of non-humidified (dry) 5% CO_2 gas. CO_2 is transferred through a gas permeable but water vapor-tight silicone structure to the culture medium.

The proposed gas supply structure is compared with three other implementations designed for similar use. Computational simulations are utilized to analyze the distribution of CO_2 concentrations. Evaporation, pH, and osmolarity of the culture medium are experimentally evaluated. Finally, the functionality of the proposed structure is demonstrated in a continuous three-day MEA measurement. We show that the proposed structure with a continuous low flow rate of non-humidified CO_2 supply provides a stable cell culture environment for continuous and successful MEA recordings of hESC-derived neuronal networks while retaining physiologically relevant pH and osmolarity levels.

2. Materials and methods

2.1 Culture chamber implementation

In this study, we fabricated and compared four different culture chamber implementations designed for MEA measurements outside an incubator. Three of the implementations include CO_2 supply and are presented in Fig. 1 as: (a) gas flow between two PDMS rings in a cell culture chamber (later called Structure 1), (b) gas flow above a thin PDMS membrane on top of a culture chamber filled with medium (later called Structure 2), and (c) gas flow over the culture medium (without a gas permeable membrane; later called Structure 3). In addition, the most commonly used implementation for short term MEA recordings is a sealed structure surrounded with ambient CO_2 concentration, as described by Potter and Demarse (2001). Similar implementation without gas supply was used as a fourth structure (later called Structure 4, Fig. 1d). All four structures were compared computationally and experimentally.

All structures include a culture chamber and a connection cap. The culture chambers are placed reversibly on top of standard MEA plates (Multi Channel Systems GmbH, Germany) or a glass plate. The culture chambers provide a restricted area for cells to grow and a large volume of medium enabling a standard culture protocol (medium exchange three times per week). There are two different culture chamber constructions used: one for Structure 1 and another for Structures 2-4. Each structure has its own connection cap depending on the gas supply method used in the particular culture chamber. The connection cap enables a tubing connection for gas supply and seals the culture chamber when experiments are performed outside an incubator.

The culture chamber with the gas ring (Structure 1) consists of four main parts, as shown in Fig. 1a: a 500 µm thick PDMS base (1); an outer PDMS ring (2); an inner PDMS ring (3); and a rigid glass plate (4). The PDMS base (similar in all four Structures, Fig. 1 a-d) contains a restricted cell area in the middle of the chamber, which was found to be advantageous for the formation of functional *in vitro* neuronal networks (Kreutzer et al., 2012). The PDMS base also enables bonding of the inner and outer rings, and thus, forming a small volume, sealed gas zone around the culture chamber between the rings. The glass plate on top of the culture chamber, together with the connection cap, prevents the gas exchange through the top of the structure. The culture chambers in Structures 2-4 consist of two parts: the PDMS base (500 µm) and a PDMS ring (as shown in Fig. 1b, c, and d).

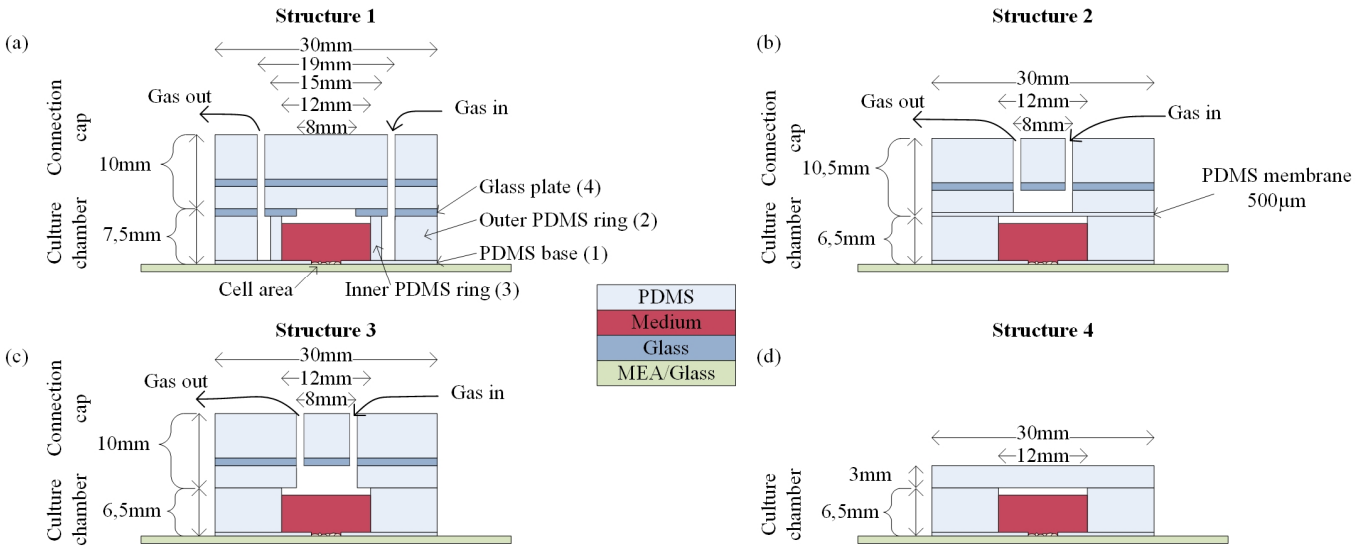

Fig. 1. Four fabricated structures for experiments. a) Structure 1: gas flow between two PDMS rings in a cell culture chamber and diffuses through the inner PDMS ring to the cell culture area. This structure consist of four main parts a 500 µm thick PDMS base with opening for cell area (1); an outer PDMS ring (2); an inner PDMS ring (3); and a rigid glass plate (4). b) Structure 2: gas flow over the thin PDMS membrane on top of medium. c) Structure 3: gas flow over the culture medium. d) Structure 4: no gas supply. Structures presented here are in scale.

The gas connection caps consist of a glass plate and two PDMS sheets (Structures 1-3, Fig. 1 a-c). The lower PDMS sheet (3 mm thick) provides an adhesion surface to the culture chambers. The glass plate keeps the connection cap stiff which helps the top PDMS sheet (6 mm thick) to provide tight connections for tubing.

The connection cap for Structure 2 includes also a 500 µm thick gas permeable membrane to provide a sealed gas zone on top of the culture chamber. In the experiments, we used the 500 µm thick membrane, whereas in computational simulations we also used 100 µm and 1000 µm thick membranes.

PDMS (Sylgard 184, Dow Corning, USA) was prepared using a standard protocol. First, a silicone elastomer pre-polymer (base) and a cross-linker (curing agent) were thoroughly combined at a mixing ratio of 10:1 (weight ratio). Second, air bubbles formed during mixing were removed in a vacuum desiccator. Third, the mixture was casted to a mold. Finally, the mixture was cured in an oven (Binder ED 53, Binder GmbH, Germany) at 65°C for ten hours.

The PDMS rings were fabricated by punching a 6 mm thick bulk PDMS sheet using custom-made punching tools. For Structure 1, the inner ring was punched using 12-mm and 15-mm punching tools and the outer ring was made with 19-mm and 30-mm punching tools. Similarly, PDMS rings for Structures 2-4 were punched using 12-mm and 30-mm punching tools. The PDMS base, for all structures, was prepared by casting 8 g of liquid PDMS on polystyrene (PS) plate (Ø 140 mm) and curing in a levelled rack in the oven to achieve the wanted thickness (~500 µm).

1 mm thick glass plates (Ø 30 mm) were purchased from Akilasi Oy (Tampere, Finland). For Structure 1, an 8 mm hole was drilled in the middle of the glass plate for seeding cells and supplying medium. 1 mm holes were drilled at 9 mm from the center for the gas connections.

The glass and the PDMS parts were bonded together using oxygen plasma (Vision 320 Mk II, Advanced Vacuum Scandinavia AB, Sweden) with the following parameters: O_2 flow rate of 30 sccm, pressure of 30 mTorr, power of 30 W, and time of 15 s.

2.2 Numerical simulations

Diffusion and distribution of CO_2 inside the structures were simulated. More specifically, we modelled the dynamics of CO_2 distribution and concentration in the cell area of each gas supply structure (Structures 1-3). Structure 2 was modelled with three different gas permeable membrane thicknesses: 100 µm, 500 µm, and 1000 µm to compare the effect of the membrane thickness to the dynamics of CO_2 distribution in the culture chamber. Two-dimensional axial-symmetric models were created and solved using commercial finite-element software COMSOL Multiphysics® Version 4.4 (COMSOL, Inc., Burlington, MA, USA). The simulation parameters were according to previous work, where a CO_2 transport model was validated (Mäki et al. 2015). Briefly, "Transport of diluted species equations" using Fick's law was used as the governing equation to calculate time-dependent CO_2 concentrations. As an initial condition, it was considered that each phase (gas, liquid, PDMS) were exposed to air (i.e. CO_2 level ~0.033%). A fixed input CO_2 concentration was set to the boundaries, where the gas was applied, and no-flux condition was set on the glass boundaries. In the model, a validity of ideal gas behavior and Henry's Law was assumed. Furthermore, constant temperature and pressure conditions were implemented and any liquid evaporation, transportation of other molecules than CO_2, and reactions between CO_2 and liquid were not considered.

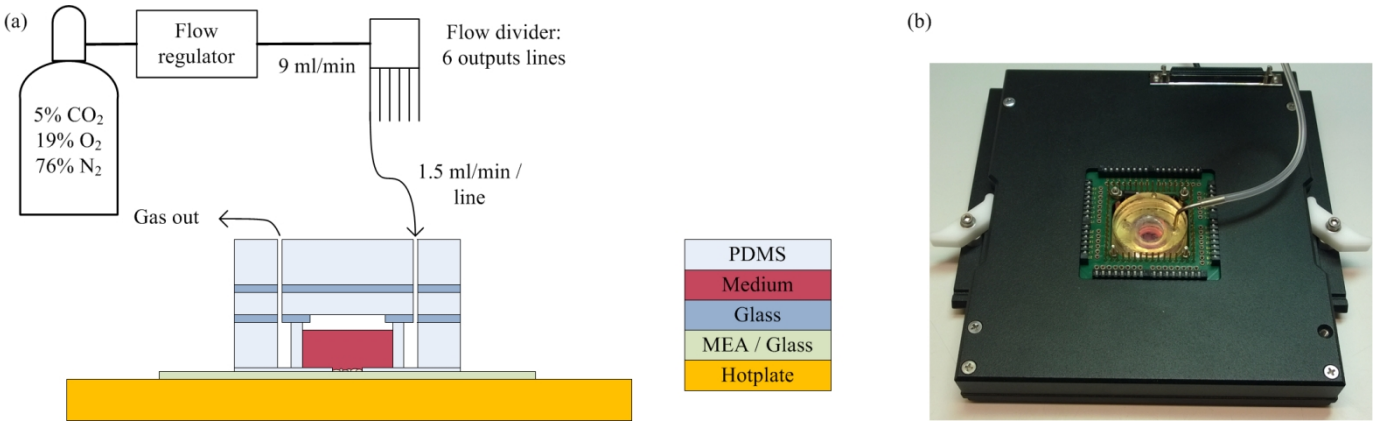

Fig. 2. Gas supply setup include gas tank of gas mixture, flow regulator, and flow divider to divide flow into six equal flow lines. One flow line is connected to one connection cap of gas supply structure. Color coding show the materials and components used in order. a) Example of test setup (here with Structure 1) for the pH, evaporation, and osmolarity measurements with and without cells on hotplate. b) Real test setup for long-term MEA recordings.

2.3 Cell culture and assessing neuronal network development

Human embryonic stem cells (cell line Regea 08/023) were differentiated towards neuronal cells, as described by Lappalainen et al. (2010). Briefly, differentiation was implemented as neurospheres for eight weeks in a suspension culture in differentiation medium consisting of 1:1 DMEM/F-12 and Neurobasal medium (both Life technologies) with fibroblast growth factor (FGF, R&D Systems) and additional supplements. BioMediTech has Pirkanmaa Hospital District's ethical approval to derivate, culture, and differentiate hESCs (Skottman, R05116) and permission from the National Authority for Medicolegal Affairs (FIMEA 1426/32/300/05) to conduct human stem cell research.

After this, cells were plated either on glass coverslips or standard MEA plates (standard 8 ´ 8 electrode layout, 200/30iR-Ti, without the glass ring; Multi Channels Systems GmbH (MCS), Germany) as described earlier (Heikkilä et al., 2009). Experiments with cells were performed with Structures 1 and 4. During the cell maturation process of three to five weeks in an incubator (+37°C, 5% CO_2), the electrical activity of the developing neuronal networks was measured once a week using the amplifier from MCS (MEA2100-2´60-2-system). Only functionally maturated cultures were selected for the long-term MEA experiments. Criteria for a functional culture were as follows: at least 15 active electrodes/MEA (active electrode = at least 5 spikes/min detected) and total number of spikes/active electrode is at least 50 spikes/5min.

2.4 Experimental setups

We used premixed gas (5% CO_2, 19% O_2, and 76% N_2) in a pressurized cylinder purchased from AGA (Finland). Gas flow was regulated using a flow regulator (red-y GSC-A9KA-FF22, Vögtlin, Switzerland) and divided into six equal flow lines (equal flow resistances in the flow divider). This provided 1.5 ml/min flow rate for each output line. Each output flow line can be connected to the individual structure or left open (See Fig. 2). The low flow resistance through the structures enables unplugging a structure without affecting the flow-through rate of the other structures.

2.5 Evaporation, pH, and osmolarity of cell culture medium

Evaporation and pH of medium were first evaluated for all four structures on a hotplate outside the incubator without cells, (see Fig. 2a). In these experiments, the culture chambers were filled with medium (DMEM/F-12 + 1% penicillin/streptomycin). This mimics the measurement conditions during the MEA recordings and provides clarifying information of evaporation and pH without cells.

Evaporation was determined using a precision scale (Presica 610-MC-FR SCS, Presica Instruments AG, Switzerland) by weighting the structure implementation (the culture chamber, the connection cap, and the glass/MEA plate) several times during the three-day experiment. Evaporation experiments were repeated three times for each structure (n=3/structure). Evaporation was estimated by calculating the mass change during the experiment.

The pH analysis was performed as an end-point measurement and pH was measured using a microFET pH meter (SI600with microFET sensor, Sentron, The Netherlands). pH was determined by taking a 200 µl sample, with a 1 ml pipette, pipetting close to the cell area and therefore, it can be considered to provide a good estimation on the pH at the cell area. Furthermore, we used the pipette tip directly as a container for the sensor probe for pH reading. This technique enabled us to avoid drifting of the sample because of atmospheric measurement conditions (low CO_2). We determined the pH for all four structures at four selected time points (4, 14, 19, and 72h) and this was repeated three times for each structure (n=3/structure/time point).

Osmolarity was also analyzed as an end-point measurement and it was determined by taking a 50 µl sample of medium after the experiment using OsmoMat 030 osmometer (GonoTec GmbH, Germany). Osmolarity was measured after the experiments on the hotplate with cells on Structure 1 (n=3) and after one MEA recording (n=1).

Reference measurements were conducted in two different incubators. pH and osmolarity were measured from the cultures with cells (n=3) in HERAcell 150i (Thermo Scientific™, Thermo Fisher Scientific, MA, USA) and evaporation without

cells (n=15) in Binder CB 150 (Binder GmbH, Germany). In both reference experiments, structures were inside Petri dishes without the connection cap. Reference measurements were performed after 69h and 72h, respectively.

2.6 Immunocytochemistry

To characterize hESC-derived neuronal cultures after the hotplate experiments, cells were fixed at the end of the experiment with 4% paraformaldehyde. Immunocytochemical analysis with rabbit anti-MAP-2 antibody (Chemicon) was performed as previously described [11]. Cells were examined using an Olympus microscope (IX51, Olympus) equipped with a fluorescence unit and a camera (DP30BW, Olympus).

2.7 Long-term MEA recordings

HESC-derived neuronal cells cultured on MEA plates with Structures 1 and 4 were used for long-term MEA recordings. During the experiment, the activity of cells was recorded for ten minutes every hour for three days. Extracellular spikes were detected using MC_Rack software (settings: 200 Hz high-pass filter and the spikes threshold set to STD ´ 5 from the baseline). Also, the number of active electrodes (= at least 5 spikes/min) and average spiking per active electrode (= sum of all spikes from all active electrodes during ten minutes recording divided by the number of active electrodes) was calculated at each measurement point. Long-term MEA recordings were performed three times with Structure 1 (n=3, one recording for 72h and two recordings for 24h) and once with Structure 4 (n=1).

3. Results

3.1 Simulation results

Computational simulations clearly demonstrate the differences in CO_2 concentrations inside the culture chambers between the structures (Fig. 3). When comparing the CO_2 concentrations, it is clearly seen that the supplied gas is uniformly distributed in Structure 1. The gas is supplied around the culture well, and thus, in equilibrium it is equally distributed throughout the culture chamber. Therefore, the exact set point value (5% CO_2) can be reached in the cell area (Fig. 4a). In Structures 2 and 3 where the gas is supplied from the top of the chamber, the concentration of CO_2 in the cell area never reaches the 5% set point value.

The thickness of the membrane between the gas supply and the culture chamber affects the distribution of CO_2 as studied with Structure 2. The thicker the membrane the lower the CO_2 concentration is in the cell area in equilibrium, which is 2.7%, 2.4%, and 2.1% with 100 µm, 500 µm, and 1000 µm membranes, respectively (as shown in Fig. 4a). Surprisingly, the set point value is not reached either in Structure 3. The gas is supplied directly on top of the culture medium but ambient air diffuses through thick PDMS wall affecting the CO_2 concentration in the cell area where the concentration reaches the value of 4.4%.

3.2 Evaporation, pH, and osmolarity of cell culture medium

Evaporation was studied for all structures on the hotplate as shown in Fig 4b. Evaporation of the culture medium from

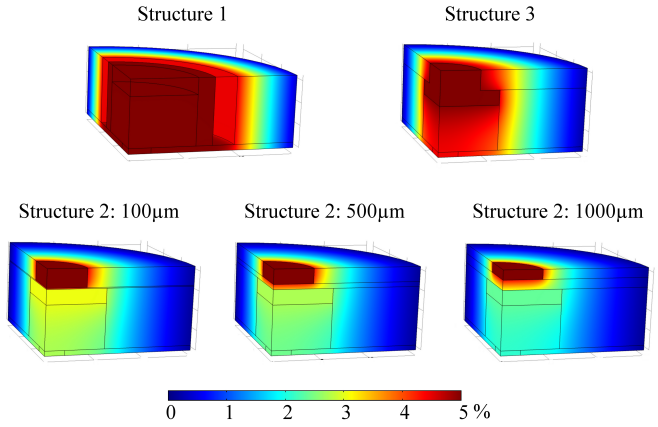

Fig. 3. Distribution of CO_2 concentration inside the culture chambers after 10h. Structure 1 provides gas supply surround the culture chamber. For Structure 3 the gas is supplied on top of the cell culture medium without the sealing membrane. Distribution of CO_2 concentration inside the Structure 2 was simulated with three different membrane thicknesses; 100 µm, 500 µm, and 1000 µm membrane between the culture chamber and gas supply.

Structure 1 was measured to be 44 ± 5 µl /72h (n=3). This corresponds to 7.4% ± 0.8% of the initial volume of the medium (600 µl) and provides the evaporation rate as low as 0.62 ± 0.07 µl/h, as concluded in Table 1. Correspondingly, the evaporation from all closed chamber Structures (1, 2, and 4, n=3 for each structure) was approximately the same, as shown in Fig 4b.

Cell experiments on hotplate were also conducted with the Structure 1 achieving low evaporation values as well (80 ± 14 µl /69h, n=3, corresponding to 13.4% ± 2.4% total evaporation and 1.16 ± 0.20 µl/h evaporation rate). Evaporation from the structures used outside an incubator is two to four times smaller than evaporation of the culture medium from the structures placed inside the incubator which is 171 ± 58 µl /72h (n=15), corresponding to 28.5% ± 9.7% of total evaporation and evaporation rate of 2.48 ± 0.84 µl/h.

By contrast, the medium in Structure 3 (the structure without the membrane) dried completely when the dry gas was supplied through the culture chamber. This was due to absorption and hold of moisture by the non-humidified gas while flowing through the culture chamber. Further, the condensate liquid on the connection cap often blocked the gas supply during the experiments and the pressure in the gas supply system was not sufficiently high to break the blockage (push the liquid through the 1mm hole in PDMS). Thus, the blockage prevented the efficient CO_2 supply to the chamber, and pH started to rise. To summarize, in Structure 3 the medium either evaporates completely or the pH rises too much to maintain a healthy cell culture. Therefore, this structure was not used in further studies.

The pH level of properly gasified fresh medium in 37 °C (DMEM/F12) is 7.2 to 7.4. A similar pH level was maintained in Structure 1 during the three-day experiments on the hotplate without cells (7.43 ± 0.03, n=3) and with the hESC-derived neurons (7.33 ± 0.01, n=3), as shown in Fig. 4c. These results are consistent with the reference measurements inside the incubator where the pH was 7.39 ± 0.07 (n=3). In contrast, higher pH levels were measured in other structures after 72h experiments. In Structure 4, pH increased rapidly at the beginning of the experiments (4 h, pH >8). The rapid change was expected, because there was no CO_2 for buffering pH in the

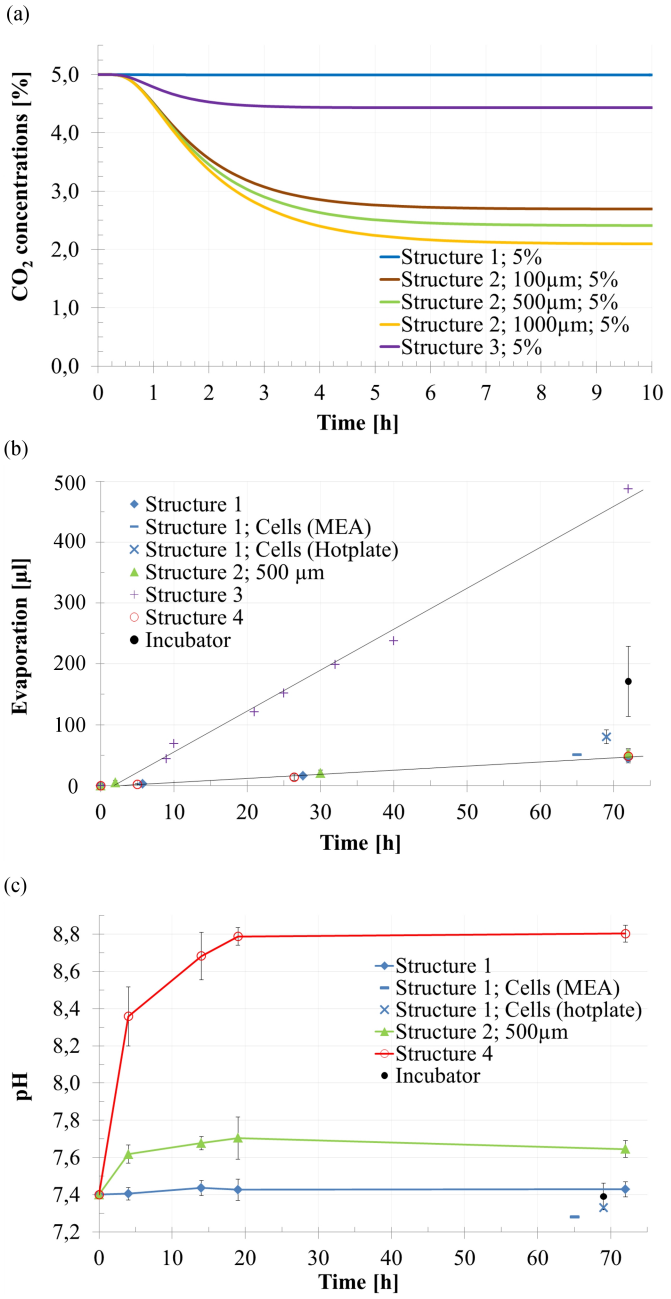

Fig. 4. a) Simulation results for CO_2 concentration on cell area in different structures. Comparison of Structure 1 (blue line), Structure 2 (green line), and Structure 3 (purple line) with supply of 5% CO_2 concentration. Graphics also shows the comparison of the CO_2 concentration inside the Structure 2 with 100 µm (brown line), 500 µm (green line), and 1000 µm (yellow line) gas permeable membranes. b) Evaporation of the medium measured from the Structure 1 (blue diamond), Structure 2 (green triangle), Structure 3 (purple plus), and Structure 4 (red circle) during the three days experiments on hotplate. Graphics also present evaporation during the cell experiments on MEA recordings (blue dash) and on hotplate (blue cross) and comparison to evaporation inside the incubator (black dot). c) pH measurements from the cell area of the structures during the three days experiments.

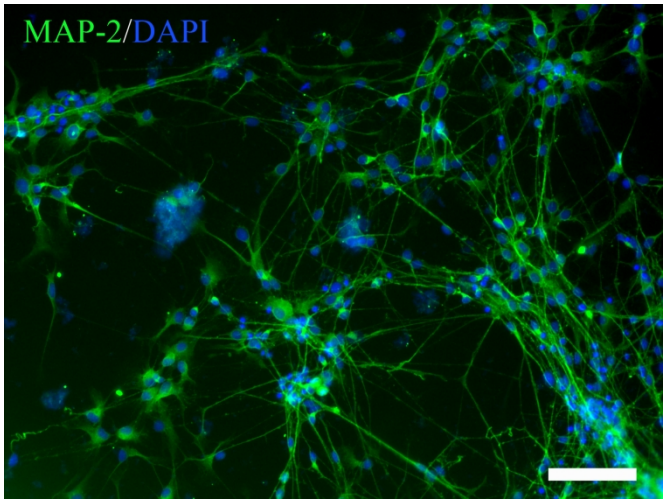

Fig. 5. Characterization of hESC-derived neuronal cells cultured on glass coverslip with Structure 1 on a hotplate experiment. Neuronal culture stained positively for neuronal marker MAP-2. DAPI was included for nuclei identification. Scale bar 100µm.

medium. In Structure 2, pH reached 7.62 ± 0.05 (n=3) after four hours and it was maintained until the end of the experiment (7.65 ± 0.06, n=3, 3 days). These results are consistent with the simulation results which revealed insufficient CO_2 supply to the culture chamber. Both, experimental and simulation results verify that pH cannot be maintained at a relevant level if CO_2 concentration is less than 5% in the cell area. In this study, Structure 1 was the only structure capable for maintaining the physiologically relevant pH with the 5% CO_2 supply.

Osmolarity of fresh DMEM/F12/Neurobasal medium was 267 mOsm/l. Osmolarity in cell experiments with Structure 1 on the hotplate was 314 ± 2 mOsm/l (n=3, 3 days). Cultures in the incubator provided the osmolarity value of 376 ± 7 mOsm/l (n=3, 3 days).

3.3 Immunocytochemistry

Immunocytochemical analysis was done for all three neuronal cultures on glass plate (n=3) after culturing them on a hotplate for three days using the Structure 1. Before the experiment, cells were cultured inside the incubator for two weeks. The analysis showed that cells stained positively with neuronal marker MAP-2 providing also typical neuronal and neuronal network morphology. This confirms that cultures were vital and kept in proper conditions during the experiment (Fig. 5).

3.4 Long-term MEA recordings

Long-term MEA recordings with hESC-derived neuronal cells demonstrated that Structure 1 provided a very good and stable culture environment outside an incubator. Cells cultured on MEA plates in Structure 1 and Structure 4 were monitored using a MEA amplifier for three days continuously (setup example shown in Fig. 2b). Neuronal cell cultures showed spontaneous activity during the entire measurement period (See Fig. 6). During a long-term experiment, the number of active electrodes and general spiking activity remained the same level with some natural fluctuation. This means that the cell cultures are as functional and healthy at the end of the recording period

as at the beginning. That also proofs that our culture chamber with gas supply keeps the cells healthy and vital outside the incubator conditions for at least 3 days. Comparing the activity patterns in Structure 1 and Structure 4, it is obvious that constant CO_2 supply is required for successful long-term measurements. At the beginning, the activity is strong in both test structures. However, in Structure 4, the activity begins to decrease drastically after eight hours without the supply of CO_2 and the entire culture is completely silent after 14 hours and eventually dies.

In Structure 1, the pH, evaporation, and osmolarity of the cell culture medium remained at preferred levels as measured after three-day MEA recordings. Evaporation was in the same range as measured on the hotplate being 51 µl (8.5% and 0.78 µl/h), as shown in Fig. 4b. Also the pH remained below 7.4 (7.28, as shown in Fig. 4c) and osmolarity was maintained as low as 347 mOsm/l. Overall, neuronal cultures in Structure 1 remained highly active and vital during the three-day continuous MEA recordings outside the incubator.

4. Discussion

Live cell analyses outside an incubator suffer from the lack of stability of cell culture conditions, particularly using bicarbonate buffer solutions as the culture medium. The lack of sufficient CO_2 supply results in a fast change of pH of the cell culture medium and the evaporation causes concentration drifts. That limits the experiment time typically to tens of minutes without additional systems to maintain the culture conditions.

There are only few reported studies to demonstrate long-term MEA recordings outside a standard incubator. However, these systems include some limitations. For example, using a small volume of the culture medium in a closed system requires continuous or periodical medium perfusion (Blau and Ziegler, 2001; Li et al., 2011) that is impractical for cultures requiring long pre-cultivation period. Typical pre-cultivation period for hESC-derived neuronal cell cultures before actual electrophysiological measurements is 3-8 weeks. Furthermore, in a busy laboratory, there might be tens of cultures at the same time in the pre-cultivation phase, and thus, it is not practical to keep such many pumps running parallel. Biffi et al. (2012) introduced an environment chamber without medium perfusion where they were able to recording and maintain the cultures up to 21 days. However, that "mini-incubator" required a relatively large flow rate (70 ml/min) of 10% CO_2 to maintain the cultures. Moreover, thick chamber walls limit high resolution

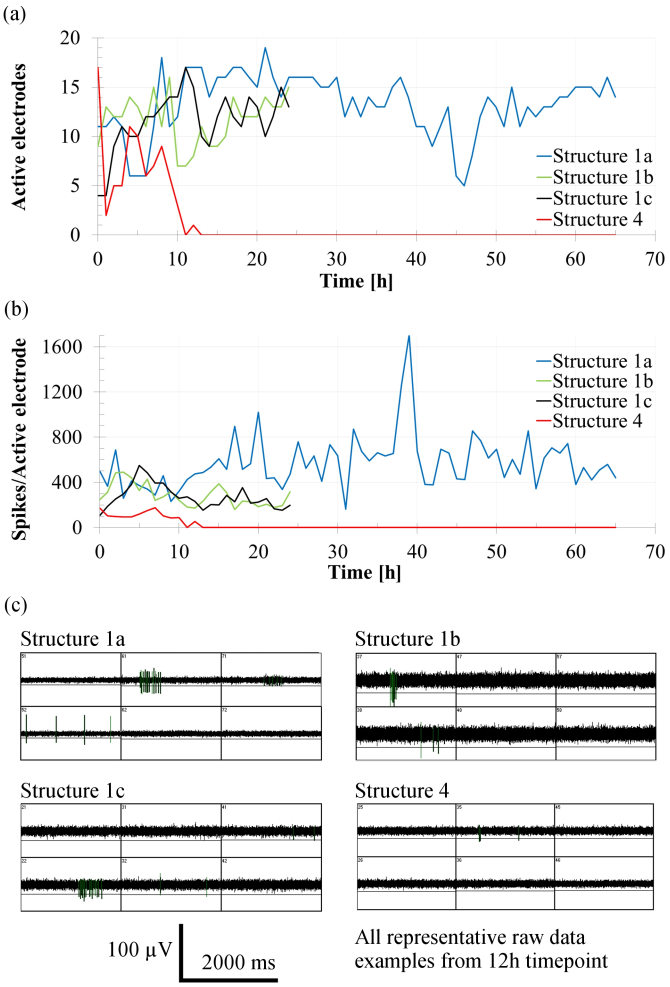

Fig. 6. Four continuous long-term MEA measurement were performed for hPSC-derived neuronal cell cultures to demonstrate the functionality of the gas supply structure. Neuronal network show strong and stable activity over three days (blue lines) when using Structure 1 to supply 5% CO_2. During the long-term MEA recordings (blue, green and black lines), there is all the time a large number of a) active electrodes and b) spikes per active electrode (in average). This demonstrate that the neuronal network is as functional and healthy at the end of the recording period as at the beginning and therefore verify the functionality of our gas supply structure for long-term MEA recordings outside controlled incubator conditions. In comparison, using Structure 4, without the gas supply, neural network activity diminishes and no activity is seen after 14h (red lines). c) Examples of representative raw data plots from each MEA measurement (1a-c, and 4). The plots show MEA data for each MEA plate recorded in the area containing 6 electrodes at time point 12 h. Spike detection threshold is set to -5 ´ SD of background noise for each electrode. The vertical scale bar is 100 µV and the horizontal scale bar is 2000 ms.

TABLE I
RESULTS OF EVAPORATION, PH, AND OSMOLARITY MEASUREMENTS OUTSIDE AN INCUBATOR ON HOTPLATE AND ON MEA HEAD STAGE COMPARED TO REFERENCE MEASUREMENTS INSIDE THE INCUBATOR.

	Hotplate without cells [a]	Cell experiments		Incubator (reference)
		On hotplate [a]	On MEA	
Evaporation [%]	7.4% ± 0.8%	13.4% ± 2.4%	8.5%	28.5% ± 9.7% [b]
Evaporation [µl/h]	0.62 ± 0.07	1.16 ± 0.20	0.78	2.48 ± 0.84 [b]
pH	7.43 ± 0.03	7.33 ± 0.01	7.28	7.39 ± 0.07 [c]
Osmolarity [mOsm/l]		314 ± 2	347	376 ± 7 [c]

[a]Structure 1 on glass plate (n=3)
[b]Average from all structures (1, 2, 3, and 4) without cells in incubator. Structures on glass plates, without connection cap, and inside Petri dishes (n=15).
[c]Structure 1 with cells on MEA plate and inside Petri dishes (n=3).

imaging with short distance objectives and modified electrical connections affect slightly to signals. Saalfrank et al. (2015) introduced a practical gravity driven system with large medium reservoir for continuous long-term MEA recordings. However, they used HEPES buffered medium suitable for use in ambient air. HEPES is not suitable for some type of cells and it also is reported to be slightly phototoxic (Lepe-Zuniga et al., 1987).

In this paper, we compare four different culture structures made of gas permeable PDMS and glass. One of the typical approaches to supply gas to a culture is from the top of the culture well through a gas permeable membrane (Oppegard et al., 2009). Supplying gas from atop works when the culture chamber itself is impermeable to gas and the gas supply layer is placed very close the cells. However, placing the gas supply layer close the cells reduce the volume of the medium in the cell area and thus, medium needs to be changed more frequently (daily) (Oppegard et al., 2009) or, alternatively, continuous medium perfusion is required in long-lasting experiments (Blau and Ziegler, 2001; Li et al., 2011). By placing the gas supply layer further away from the cell layer, a proper CO_2 concentration cannot be maintained in PDMS-based culture wells, as we demonstrated in this paper. Our results show that CO_2 concentration in the cell area remains as low as 2.4% and the pH increases (7.62) using the structure where gas supply layer is 6mm from the cell layer and separated with 500 µm thick membrane (Structure 2). Even though the wall thickness of the culture chamber is 9mm, which is almost twice the height of the medium on top of the cells, it is not sufficient to maintain the supplied CO_2 concentration in the cell area. In the proposed structure, instead, the medium chamber is large, thus containing enough nutrients for long-term experiments without a need for additional medium perfusion. The culture chamber is closed with a connection cap and separated from the CO_2 supply with a gas permeable silicone ring that enables efficient gas supply to maintain the pH at a correct level. Closed structure also guarantees low evaporation, even if supplying non-humidified gas.

A general association is that humidified gas would be required to avoid evaporation. However, we demonstrate that even though a non-humidified (dry) gas mixture is used, the evaporation rate remains very low (7.4%; 0.62 µl/h) in our gas supply structure used outside an incubator. The evaporation rate of this closed gas supply structure is four times smaller than the evaporation rate of medium in the same structure without the connection cap but placed in a normal ventilated Petri dish inside the incubator (28.5%; 2.48 µl/h). Potter and DeMarse (2001) and Blau et al. (2009) also demonstrated that using a gas permeable lid to seal a culture chamber, the evaporation rate can be remarkably reduced compared to a standard Petri dish inside an incubator. However, these lids do not maintain the culture conditions during prolonged experiments outside an incubator. Without CO_2 gas supply, pH will increase fast changing culture conditions, as we also demonstrated using Structure 4.

In our setup, dry gas is supplied directly from a premixed tank. Gas flow is regulated and divided into six equal flow paths that have the same flow resistance (See Fig. 2). Each output is set to a very small flow rate (1.5 ml/min). The flow rate is very small compared to other reported systems. For example, commercial microscope well-plate incubators (e.g. ibidi) are used with humidified air with high flow rate (>100 ml/min). Regalia et al. (2014) used humidified gas with flow rates up to 100 ml/min but could not reach the set value (10% CO_2 in that case) inside their home-made environment chamber housing four MEA plates. It might be possible to achieve the target concentration by using a higher flow rate, but that empties even large gas tanks relatively fast.

Humidified air also causes problems in small air channels, as we demonstrated by using the structure where gas is supplied on top of the culture medium in the culture chamber (Structure 3). Condensate water drops (from the culture medium) constantly blocked the flow path in that structure. If the flow path of CO_2 is blocked, the pH cannot be maintained anymore. In our setup, the blockage of the flow path happens because pressure remains low even if the line was blocked (blocked line ~0.3 kPa). This kind of low pressure system is, however, beneficial to use since even small unwanted pressure variations can affect the cell fate (Maul et al., 2007; Reinwald et al., 2015). To summarize this part, the humidified gas supply systems are unreliable in structures having small scale air channels because of a high risk to block the flow path by condensate water drops. Furthermore, additional parts, such as water bottles, connectors, and pipes, in series with a gas supply line make the entire system much more complex. Therefore, it is advantageous to use dry gas, but a gas permeable structure needs to be used between the dry gas and the culture medium to avoid evaporation.

We have demonstrated that using the compact and simple gas supply structure on top of a MEA plate we can culture and maintain strongly signaling hESC-derived neuronal networks both outside and inside an incubator without bulky and complex medium perfusion or humidifier systems. In addition, a restricted cell area makes cell plating easy on the MEA electrodes, saves cell and coating expenses, and enhances the growth and development of neural networks, as reported earlier Kreutzer et al. (2012). Therefore, the proposed gas supply structure provides easy to use and well-maintaining environment for prolonged MEA recordings.

5. Conclusion

Long-term MEA recordings are useful, for example, in drug screening and toxicological experiments wherein it is advantageous to follow the extracellular activity continuously for a long time period. This results in more detailed information about dynamic changes in network signaling revealing more information about treatment effects to the network. Moreover, *in vitro* learning experiments require stable long term environment. These kinds of experiments are typically performed outside an incubator exposing the culture to an ambient air. The lack of sufficient CO_2 supply results in a fast change of pH of the bicarbonate-based cell culture medium and the evaporation causes concentration drifts. That limits the experiment time typically to tens of minutes without additional systems to maintain the culture conditions. In this paper, we demonstrated the functionality of a gas supply structure in long-term MEA recordings outside controlled incubator conditions. The gas supply structure provides stable culture conditions on a MEA-plate outside an incubator, only connecting the supply of very low flow rate (1.5 ml/min) of non-humidified (dry) 5%

CO_2 gas. That is only 2% from the flow rates reported in other studies. The gas is provided surround the culture chamber, through a gas permeable and watertight silicone structure to the culture medium. We demonstrate that even without continuous perfusion of medium and/or a complex humidified gas supply system, the gas supply structure provides stable and physiologically correct pH and osmolarity levels, with low evaporation on the MEA-plate outside the incubator facilitating over three days continuous recordings. Furthermore, the structure enables not only long-term measurements outside an incubator but also enhanced culture of hESC-derived neurons inside an incubator between the MEA measurements.

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