Structured PDMS chambers for enhanced human neuronal cell activity on MEA platforms

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Abstract Structured poly(dimethylsiloxane) (PDMS) chambers were designed and fabricated to enhance the signaling of human embryonic stem cell (hESC) - derived neuronal networks on microelectrode array (MEA) platforms. The structured PDMS chambers enable cell seeding on restricted areas and thus, reduce the amount of needed coating materials and cells. In addition, the neuronal cells formed spontaneously active networks faster in the structured PDMS chambers than in control chambers. In the PDMS chambers, the neuronal networks were more active and able to develop their signaling into organized signal trains faster than control cultures. The PDMS chamber design enables much more repeatable analysis and rapid growth of functional neuronal network *in vitro*. Moreover, due to its easy and cheap fabrication process, new configurations can be easily fabricated based on investigator requirements.

Keywords

Cell culturing

Electrical activity

Human embryonic stem cells

Microelectrode array

Poly(dimethylsiloxane)

1 Introduction

Neurodegenerative diseases are a major economic burden to 21st century societies. Stroke alone is the third largest cause of death and the leading cause of long-term disability in Western industrialized countries. Neuronal cells derived from stem cells provide several potential means for reducing this burden. They can be used for understanding the disease mechanisms, developing new drugs, and treating the diseases through transplantation therapies[1-3]. However, more information and knowledge is needed to thoroughly understand the functional properties of stem cell derived neurons and neuronal networks so that these cells can be fully utilized for research and therapeutic purposes. Microelectrode array (MEA) measurements are a widely used method to characterize the electrical activity of human-origin neuronal cells[2, 4, 5].

MEA platforms are commercially available in different configurations from several companies (e.g., Multi Channel Systems [MCS] GmbH, Germany; Ayanda Biosystems SA, Switzerland; Alpha Med Scientific, Inc, Japan; Axion Biosystems, NW, USA). There are, however, limitations in the cultivation chambers that impede the efficient and high-throughput analysis of stem cell derived neuronal cells on MEA platforms. Human embryonic stem cells (hESC) and their neuronal derivates are especially demanding, laborious, and expensive to culture and therefore, the limitations of the cultivation chambers are more severe in studies with these cells. For example, formation of neuronal networks on top of the electrode area is not controlled and thus, the reproducible rate of similar networks is low. In addition, the growth of neuronal cells on large surfaces presents challenges by lowering the signaling rates and network formation. The large surface areas also increase the required amount of coating substances and cells to be seeded, and lead to more costly experiments. Thus, it is reasonable to

optimize the cultivation methods on the MEA platforms to improve the attachment and maturation process of the neuronal cells and possibly reduce the costs of the experiments.

In this study we report the design of structured poly(dimethylsiloxane) (PDMS) chambers, their use on various commercially available MEA platforms, and the subsequent neuronal cell recordings. Our aim is to improve the neuronal cell attachment and maturation process, and allow more controlled growth over the electrode area without affecting the development and maturation of functional neuronal networks. The paper will discuss three chamber configurations (a one-well structure and two six-well structures) and compare their performance to a commercial chamber.

2 Materials and methods

2.1 MEA platforms

Microelectrode array (MEA) technology has been used for cell network studies for decades[6-8]. MEAs are widely used in different fields of cell research and tissue engineering, from single cell recordings using Si_3N_4 cages[9] to slice cultures[10]. MEAs are used for monitoring spontaneous activity of neuronal networks[11], response to electrical and pharmaceutical stimuli[4, 12], plasticity[13], and toxicological screening[5, 14].

In this study, we used MEA platforms from Multi Channel Systems (MCS) GmbH (Germany). For the one-well chamber studies, we used MEA platforms with a standard electrode (200/30iR-Ti, layout 8×8) layout without a glass ring on the substrate. The same electrode layout with a glass ring served as the platform for the control study. Six-well MEA platforms (6wellMEA200/30iR-Ti-w/o, layout 6×(3×3)) were used in the six-well PDMS chambers studies.

2.2 Design and fabrication of one-well PDMS chambers

Previously, many research groups have reported cell and tissue studies utilizing PDMS microstructures[10, 15-27]. Recently, PDMS has been used to fabricate microfluidic structures for hESC studies[28-37], but they have not been used with MEA platforms. PDMS is a very good material for a laboratory use and is advantageous for inexpensive prototyping applications. PDMS is resistant to most chemicals[38], gas permeable[39], optically transparent down to wavelengths of 290 nm[40], and it can be used to replicate features just a few nanometers in size[41].

A PDMS chamber including one well, from now on referred to as the one-well chamber, was designed for the MEA platforms having all 60 electrodes in the middle of the platform. Similar to the commercial glass ring chamber produced by MCS, the one-well PDMS chamber included a reservoir to store up to 1000 µl of cell culture medium. The structured PDMS chamber featured a PDMS bottom with an open cell attachment area (4 mm in diameter, ~13 mm^2) around the electrode area (1.4mm × 1.4mm, ~2 mm^2) to guide the cells to grow and attach onto the electrodes, and another opening (16 mm^2) for the reference electrode as shown in Fig. 1a.

To fabricate the one-well chambers, a mold for casting the PDMS was designed. The mold comprised of two lathe-machined parts as shown in Fig. 1b: a support made of polytetrafluoroethylene (PTFE) and an insert made of polyoxymethylene (POM). The support provides a place for the insert, which is tightly attached with a screw from the bottom. As a result, the insert can be easily exchanged to vary the shape and size of the medium chamber. In addition, the two-part approach was used to facilitate the removal of the casting from the mold. In the present study, the insert was 19 mm in diameter and 7.0 mm high. The

insert includes bosses 0.5 mm in height, as shown in Fig. 1b. These raised patterns leave openings in the PDMS membrane for the cell attachment area and the reference electrode.

The fabrication procedure for the one-well chamber included the following steps: preparing a mixture of PDMS, casting the PDMS mixture, degassing the mixture, placing a glass plate on top of the mold, curing the PDMS mixture, and removing the culture chamber from the mold.

The mold was used for replicating the culture chambers from PDMS. We used a two-component PDMS elastomer (Sylgard® 184, Dow Corning, purchased from Ellsworth Adhesives AB, Sweden). The base (liquid silicone rubber) and the curing agent were thoroughly mixed with a weight ratio of 10:1. After mixing, the mixture was cast to a clean mold. The mold was completely filled, covering the patterns of the insert. The mold containing the PDMS mixture was then placed into a vacuum chamber (~100 mbar absolute pressure) for 20 min to remove air bubbles that were created during the mixing and trapped in the uncured liquid silicone.

After the bubbles were removed, a clean glass plate was pressed tightly on top of the mold to contact only the bosses. Hence, these patterned areas were open after removing the PDMS structure from the mold. The glass plate also flattens the PDMS surface and thus increases the efficiency of reversible bonding to the MEA platform or other smooth surfaces. A small weight of 500 g was placed on top of the glass plate during oven curing (2 h at 70°C).

Removal of the PDMS chamber from the mold is performed carefully so as to not break the thin bottom layer, which is only 0.5 mm thick. The PDMS chambers were sterilized in an autoclave before bonding to the MEA platforms. A completed PDMS chamber on a MEA platform is illustrated in Fig. 1c.

2.3 Parallel fabrication of multiple one-well chambers

A mold used for the parallel fabrication of nine one-well PDMS chambers is illustrated in Fig. 2a. The mold facilitates the production of nine preforms of one-well culturing chambers as shown in Fig. 2b. The fabrication procedure of preforms was the same as used for a single chamber as described previously. The cured PDMS preforms were finalized by punching two holes (each 4 mm in diameter) with a biopsy punch for the cell attachment area and the reference electrode located in the middle and at the edge of the chamber, respectively. Fig. 2c illustrates a one-well PDMS culture chamber used in the experiments of this study.

2.4 Design and fabrication of six-well PDMS chambers

Six-well PDMS chambers were designed for MEA platforms, which included six electrode areas that each contained nine electrodes. Two chamber configurations were fabricated to demonstrate the versatility of a single MEA platform with different six-well PDMS chambers. In Configuration I, shown in Fig. 3a, each electrode area has its own medium reservoir and cell attachment area. In Configuration II, shown in Fig. 3b, all electrode areas share a larger common medium reservoir, but have their own structures to guide the cell attachment and growth. In addition to the cell guidance openings for the electrode areas, the bottom plate includes a large opening for the reference electrode in the middle. Both chamber designs are used with the same six-well MEA platform.

Configuration I with six separate medium reservoirs was designed to increase the number of parallel experiments on one MEA platform. Two different medium reservoir sizes, for volumes of 150 µl and 250 µl, were fabricated to

determine the appropriate volume of medium required for a practical medium change interval of three times per week.

Configuration I (Fig. 3a) was fabricated from two parts. The top part, providing the walls of the containers, was punched out from a bulk (thickness 6.5 mm) PDMS sheet using a 32 mm diameter custom-made punch. Thereafter, three 6 mm ∅ holes and three 8 mm ∅ holes were punched through the disk for the medium reservoirs using custom-made punches. The bottom part was punched out from a bulk (thickness 0.5 mm) PDMS sheet with the 32 mm (in diameter) punch. The two parts were bonded irreversibly using an oxygen plasma treatment (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. Finally, the six openings for the cell attachment areas (2 mm ∅, ~3 mm^2) were punched through the membrane using the biopsy punch.

Configuration II (Fig. 3b) utilized a common medium reservoir to increase the number of replicates in the same culture conditions. This design also comprised of two parts. The top part (32 mm ∅) was punched out from the bulk (thick 6.5 mm) PDMS sheet, and a 22 mm ∅ hole was punched in the middle. A thin bottom membrane was fabricated and bonded as in Configuration I. Six 2 mm ∅ openings for the cell attachment areas and one 8 mm ∅ opening for the common reference electrode in the middle were also punched.

2.5 Assembly and cleaning

A smooth PDMS surface forms a reversible bond with a flat surface due to Van der Waals interaction that can seal structures water tightly[42]**.** In this study, PDMS chambers were reversibly bonded on MEA platforms. The bond strength was sufficient for long-term cell culture, and could also sustain the mechanical forces

that were encountered during the installation of covers before measurements under a microscope.

The PDMS chambers and the MEA platforms were cleaned with detergent solution (1% (w/v) Terg-A-Zyme, Sigma-Aldrich, St. Louis, MO, US), after every experiment. After an overnight bath in detergent solution, components were washed carefully with de-ionized water and then sterilized using 70% ethanol, or an autoclave. Thereafter, components were ready for the next experiment.

2.6 Cell source and culturing

The cells used in the experiment were hESC-derived neuronal cells from lineages Regea 08/023 and Regea 06/015. Regea has ethical approval to derivate, culture, and differentiate the hESCs (Skottman, R05116) as well as permission for human stem cell research from Valvira (1426/32/300/05). First, the hESCs were mechanically cut into small pieces and the cell aggregates were placed into neural differentiation medium containing Dulbecco's Modified Eagle's Medium/F12 (Gibco, Invitrogen, Finland) and Neurobasal medium (Gibco) 1:1, supplemented with 2 mM GlutaMax (Gibco, USA), 1 × B27 (Gibco) and 1 × N2 (Gibco), 20 ng/ml basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN), and penicillin/streptomycin (25 U/ml, Cambrex, Belgium). In this medium, the hESCs formed neurospheres in a suspension culture, as previously described [43]. The medium was changed three times per week and spheres were mechanically dissected on a weekly basis. After ~8 weeks of differentiation in the suspension culture, cells turned into neuronal cells[43] and were ready to be seeded onto MEAs.

To assess the quality of the cells to be seeded, mycoplasma tests, hESC karyotyping, and gene and protein expression analyses of neuronal cells were

routinely performed. Cells used in this study had a normal karyotype, were mycoplasma free, and had a normal neuronal phenotype.

2.7 Surface coating and cell seeding

The PDMS chambers were first bonded reversibly onto the MEA platforms, as described above. The cell attachment areas were then coated using a two-step coating procedure as previously described[4]. Briefly, polyethyleneimide (PEI, 0.05% w/v, Sigma-Aldrich) solution was pipetted onto the electrode area and incubated overnight at +4°C. The MEA platforms were rinsed three times with sterile water and allowed to dry. Thereafter, a laminin solution (40 µl, 20 µg/ml human laminin, Sigma-Aldrich) was added to the cell attachment area and incubated overnight at +4 °C. In control experiments including the glass ring chambers, the same two-step coating procedure was used, but 1000 µl of PEI and laminin solutions were added to uniformly cover the entire surface.

Cells were seeded onto the MEA platform as very small aggregates (~50-100 µm ∅). In total, approximately ten aggregates per one-well chamber (PDMS chambers and glass ring chambers) were seeded onto the electrode area. In six-well designs, cells were plated both as aggregates and as single cell suspensions to evaluate the suitability of commonly used seeding methods for PDMS chambers. In the aggregate plating, 1-2 small aggregates were placed to each well. In the suspension plating, cell aggregates were entzymically dissociated into single cell suspensions with TrypLE Select (Invitrogen) for 15 min. Cells were washed with cell culture medium and 20 000 cells were plated to each well. Neural differentiation medium without the basic fibroblast growth factor (bFGF) was used in the beginning of the seeding. After three days, the brain-derived neurotrophic factor (BDNF, 5 ng/ml, Gibco) and bFGF (4 ng/ml) were added into

the cell culture medium to enhance the neuronal maturation. MEA platforms were stored in Petri dishes inside an incubator (+37°C, 5% CO_2) between the MEA recordings, and the medium was changed three times per week.

2.8 Covers

Before the cell recordings, the MEA chambers were sealed inside a laminar flow hood. The one-well chambers were sealed with a semipermeable membrane[44] (ALA MEA-MEM, ALA Scientific Instruments Inc., Westbury, NY) installed over a PTFE cap (MCS). The six-well PDMS chambers were sealed with a 3 mm thick PDMS membrane.

2.9 Data acquisition and analysis

A MEA amplifier (MEA-1060 Amplifier, MCS) was used for signal filtering and amplification. The amplifier was placed on top of a phase-contrast microscope (XI51, Olympus, Finland) for simultaneous imaging. The temperature on the MEA was maintained with a specific controller (TC02, MCS) and a heating element (set to 38.5°C, He-Inv25, MCS) was placed under the MEA platform. Data was recorded using the MC_Rack program. Post-recording analyses were performed with MC_Rack, MC_DataTool (both from MCS), MATLAB (MathWorks, Natick, MA), and NeuroExplorer (Nex Technologies, Littleton, MA). Spikes in the recorded signals were detected with MC_Rack. The spike detection threshold was set to a level of -5× standard deviation of the noise in each channel.

2.10. Statistical analyses

All statistical analyses were performed with IBM SPSS-software (version 19, IBM, Armonc, NY, USA). Statistical analysis was performed using nonparametric Kruskall-Wallis test followed by Mann-Whitney U test. The p-value ≤ 0.05 is considered significant.

3 Experiments and results

To quantify and demonstrate the performance of the cell signaling in the different cultivation chambers, two performance measurements were defined. The influence of the cultivation chamber on the cells was evaluated by investigating the attachment of the cells and the development of functional neuronal networks.

The cell attachment was studied by evaluating whether the cells attach and start to form networks within two days after seeding. The development of functional networks was measured by recording the electrical activity using the MEAs for 15 days and counting the number of active electrode channels and the number of spikes in the active channels. A channel was defined as active when at least five neuronal spikes were recorded per 250-s recording period. The number of active channels describes how many cells are electrically active in the population and the number of spikes describes the quality of the activity.

In the experiments, the performance in the one-well PDMS chambers and in the two types of six-well PDMS chambers was compared to the control - the commercial one-well glass chamber.

3.1 Cell attachment

A successful cell attachment was counted if a seeded cell aggregate attached, started to grow, and formed a network within two days after seeding.

The results indicate that the cell aggregates attach equally well in the one-well PDMS chambers (success rate 32/36 = 89%) as in the glass ring chambers (success rate 35/41 = 85%). In the six-well designs, the attachment rate was similar (success rate 35/40 = 88% for Configuration I, and 18/20 = 90%, for configuration II).

3.2 Development of functional networks in one-well designs

Typically, hESC-derived neuronal signaling develops in three stages: 1) initially some individual single spikes occur sporadically, 2) after two weeks, more organized signal trains appear, and 3) finally signaling develops to synchronous bursting activity[4]. In the experiments of this study, the same neuronal network maturation patterns were detected in the cultures in the PDMS chambers (Fig. 4).

Figure 4 shows representative phase-contrast images of networks, raster plots, and raw data of neuronal networks at 8, 11, 13, and 15 days in dish (DID). The raster plots show the network activities of all 60 MEA channels over the 250 s recording time, and the raw data plots show detailed signal development from single spiking (DID 8) to prominent training activity (DID 15).

As described earlier, in this study the activity of a neuronal network is evaluated by measuring the active number of MEA channels and the number of spikes in the neuronal signals. The number of active channels in a MEA and the average number of spikes in an active channel/MEA are shown in Fig. 5. The development of the signaling is shown at time points of 8, 11, 13, and 15 (DID). Typically, neuronal networks in the PDMS chambers were more active compared to networks in the control chambers. This was evaluated by counting the number of the active channels at all time points. Thus, at 8 and 11 DID there was a significantly larger number of active channels to neuronal networks growing in

the PDMS chambers ($p = 0.047$ and 0.014, respectively) compared to the controls. In addition, the number of spikes was higher in the PDMS chambers than in the glass chambers at same time points ($p = 0.028$ and $p = 0.009$, respectively) compared to controls.

3.3 Development of functional networks in six-well designs

MEA platforms with Configuration I were used to compare single cell and aggregate seeding methods and measure activities up to 49 DID. Both seeding methods resulted in active networks, although the maturation time varied. The maturation of the single cell plated networks was significantly slower compared to the aggregate cultures at 7 to 35 DID follow up time. The comparison of maturation speed of neuronal networks between aggregate and single cell suspension method is shown in Fig. 6. Both network types eventually developed into training-like activity, in 9 or 49 DID, for aggregates and single cell culture, respectively.

When compared to one-well chambers, the networks in the six-well designs developed similarly, as shown in Fig. 7. The results also showed that the use of the separate small medium chambers (150µl and 250µl) does not affect the signal quality and provides easy and targeted cell seeding. Even the smaller medium reservoir (150 µl) in the six-well design was large enough for sufficient nutrient exchange in static cultivation in an incubator, and the standard medium exchange cycle (every three days) was sufficient.

4 Discussion

The results presented in the previous section demonstrate the benefits of the structured PDMS chambers in MEA recordings of neuronal networks derived

from human embryonic stem cells. In the PDMS chambers, strong signaling and maturing networks formed in a shorter period of time than on the glass ring chambers. The networks in the PDMS chambers became active earlier and developed significantly more prominent training activities when compared to the glass ring chambers. This shows that neuronal networks can mature faster, and consequently cultures can be prepared for experimental measurements sooner. Similar amounts of aggregates were adequate for network formation in both chambers. However, with the six-well PDMS designs, an extremely small number of cells (1-2 aggregates or 20 000 cells in suspension) was sufficient to support growth of viable neuronal networks. Importantly by using either culturing method, functional neuronal networks are formed although it takes significantly more time for the single cell cultures to maturate compared to the aggregate cultures. Depending on the application, both types of seeding methods/cultures can be grown in six-well PDMS designs.

Preparing the mature cultures faster is not the only advantage of using the structured PDMS chambers. With the PDMS chambers, the volume of the coating solution (human laminin in neuronal studies) can be reduced by 25, which results in significant cost savings in high-throughput experiments.

In the medium change (every three days), a manual aspirating pipette is used with fast suction. In the PDMS chambers, the cells do not experience fluid-induced shear stress since the structured PDMS chamber protects them. Therefore, the requirement for precisely positioning the pipette tip is not as important in the PDMS chambers providing an easier and faster medium change. The smaller cell attachment area also offers more repeatable and efficient cell seeding and thus, increases the number of successful experiments.

The versatile use of the relatively expensive MEA platforms is also one of the advantages we propose in this study. For example, the six-well chamber with separate medium reservoirs is useful for pharmacological and toxicological experiments in the MEA platform[2]. The six-well chamber with a common medium reservoir, in turn, increases the number of replicates for experiments in which the same environment is essential. The same MEA platform can be utilized for both purposes simply by changing the PDMS structure on top. This is not possible with fixed chambers.

The PDMS chamber is easy to position accurately and attach reversibly to commercial MEA platforms. The reversible bond is watertight and stable for long-term cell culturing. The bond strength is sufficient to keep the PDMS chamber on a MEA platform during all steps in the measurement procedure, including multiple reattachments of the sealing cap. However, the reversibly bonded PDMS chambers can be simply detached by peeling them from the MEA platforms. Moreover, the separate parts are easy to clean because both parts (the PDMS chamber and the MEA platform) can be sterilized separately in an autoclave or with ethanol and/or UV-light.

5 Conclusions

In this study, we developed structured PDMS cultivation chambers that enhance the maturation process and spontaneous activity of hESC-derived neuronal cells. These cells formed strongly signaling, dense, and functional networks faster in our structured PDMS chambers than in control MEA platforms with a fixed glass ring.

Using the PDMS structures with the limited cell attachment area, the amount of coating materials, the initial number of cells, and the amount of

culturing medium can be reduced significantly in MEA studies. Reversible bonding to a MEA platform makes the PDMS chamber easy to detach, clean, sterilize, and replace. Therefore, single PDMS chamber can be used with different MEA configurations or single MEA platform with different PDMS chamber configurations to enhance the versatility of MEA platforms.

Acknowledgement

The study was part of the project STEMFUNC funded by the Academy of Finland. This study is also financially supported by the Competitive Research Funding of the Tampere University Hospital (Grant 9L064). We wish to thank the personnel of IBT for their support of stem cell culturing.

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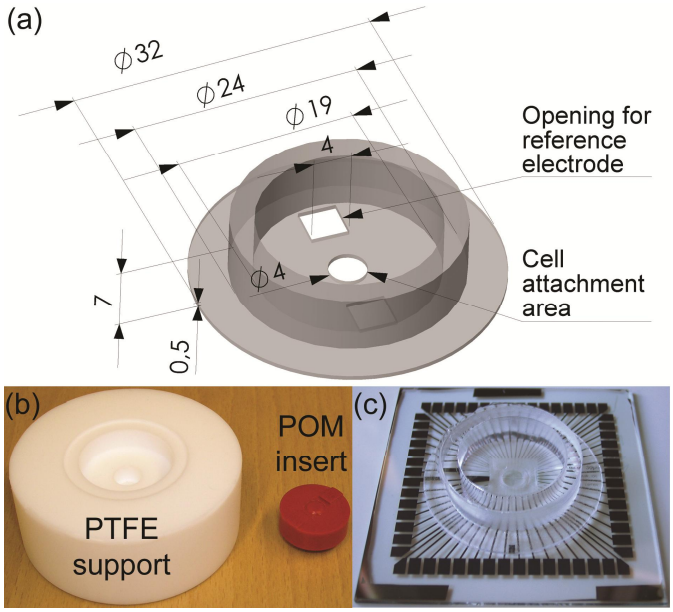

Fig. 1 (a) A one-well PDMS chamber design. Dimensions are in [mm]. (b) Mold for one-well PDMS chamber. (c) One-well PDMS chamber on a MEA platform

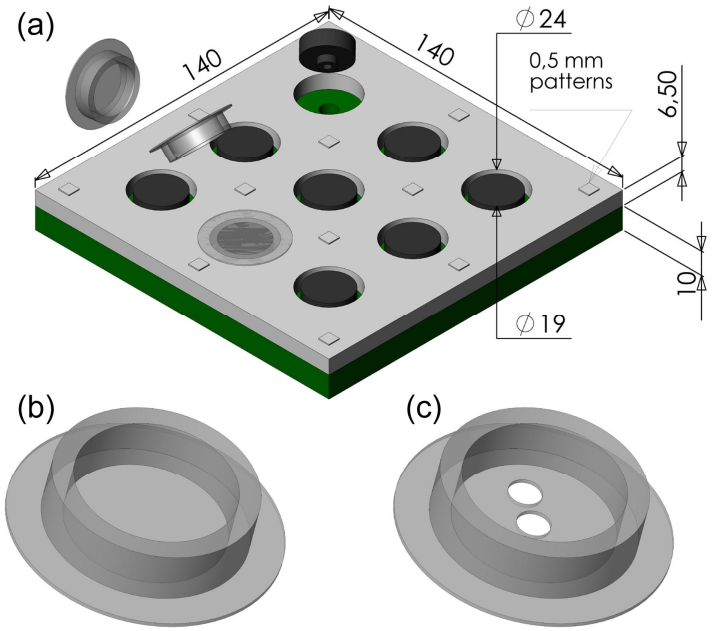

Fig. 2 (a) Mold for nine preforms of a one-well PDMS chamber. Dimensions are in [mm] (b) Preform for a one-well PDMS chamber. (c) Example of a custom-made PDMS chamber

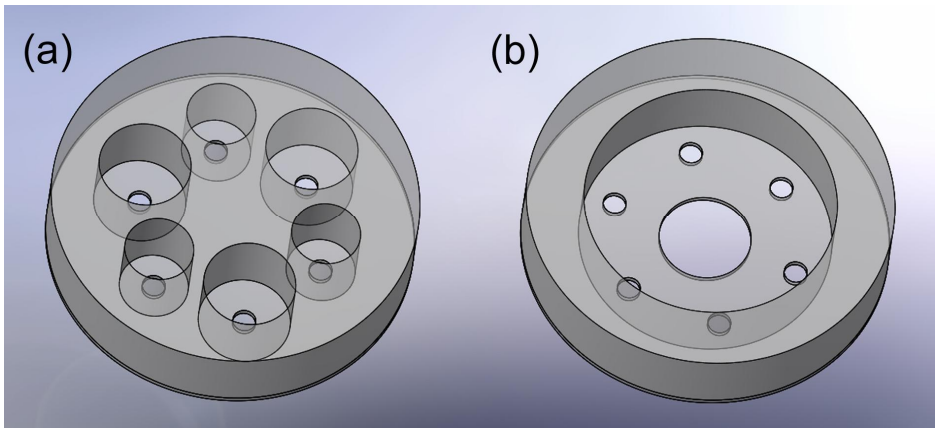

Fig. 3 (a) A six-well PDMS chamber with separate medium reservoirs called as Configuration I. (b) A six-well PDMS chamber with common medium reservoir called as Configuration II

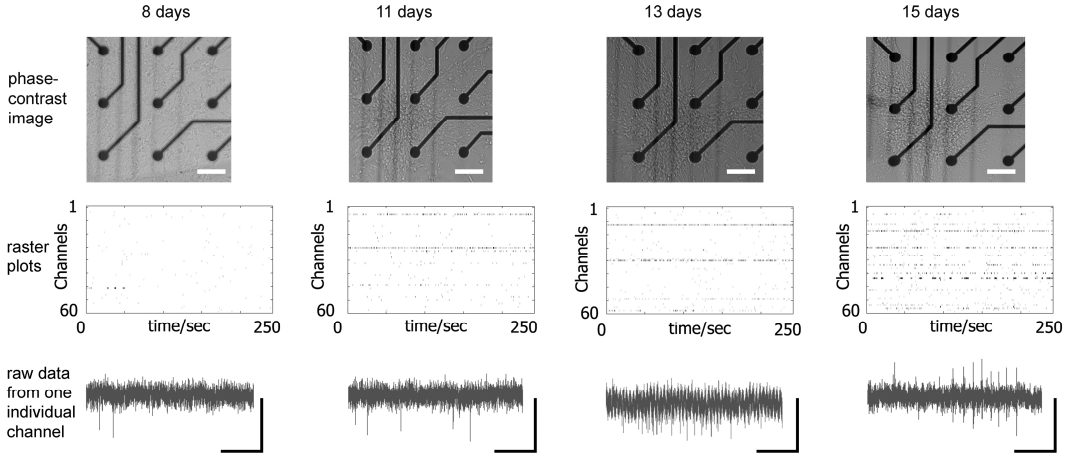

Fig. 4 Spontaneous network activities in MEAs were measured at 8, 11, 13, and 15 DID. The phase-contrast images show the growth of neuronal networks. Phase-contrast images were taken from the same PDMS-MEA platform and same location on different days. The scalebar is 100 µm. In raster plots all 60 channels are presented over a 250 s measuring period and the raster is presented if spike/spikes are detected at 1 s bin. The raw data plots were taken from one channel for 1 s. Scalebars: for x-axis= 250 µs, for y-axis=20 µV. Representative phase-contrast microscopy images shows the growth of the cells in MEA platform, and raster-plots of the activity and raw data plots show maturation of

neuronal networks at both networks and the one channel levels for each time-point.

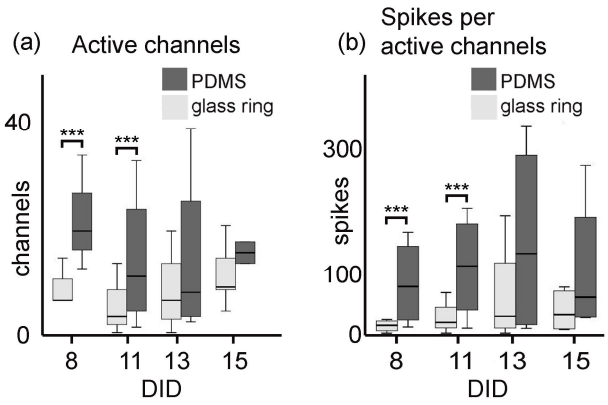

Fig. 5 (a) Number of active channels from aggregate cultures of neuronal cells in glass-ring MEAs and PDMS chamber MEAs at time-points 8, 11, 13, and 15 DID. (b) Number of detected spikes per active channel from aggregate cultures and in glass-ring MEAs and PDMS chamber MEAs at time-points 8, 11, 13, and 15 DID. For graphs, spikes were detected from 250 s measurement period and the results from at least 5 MEAs per group (n=8) were plotted. Data is plotted as box plot were median, quartiles and minimum and maximum values were shown. *** = $p < 0.05$.

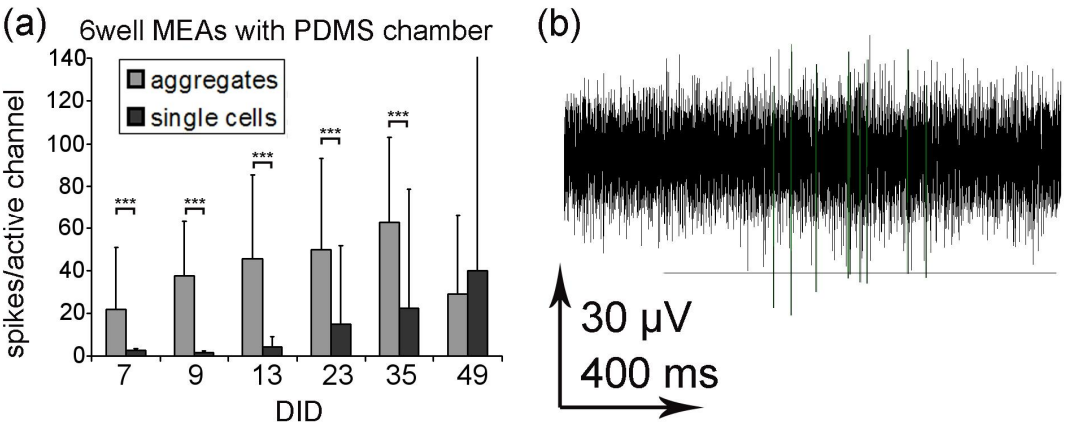

Fig. 6 (a) Cells either plated as single cell suspension or as aggregates into six-well Configuration I. Spikes per active channel were plotted as mean, and error

bars shows standard deviation. (b) Typical training activity was developed in both single cell and aggregate plated networks. Faster development was detected from aggregate plated networks. *** = $p < 0.05$.

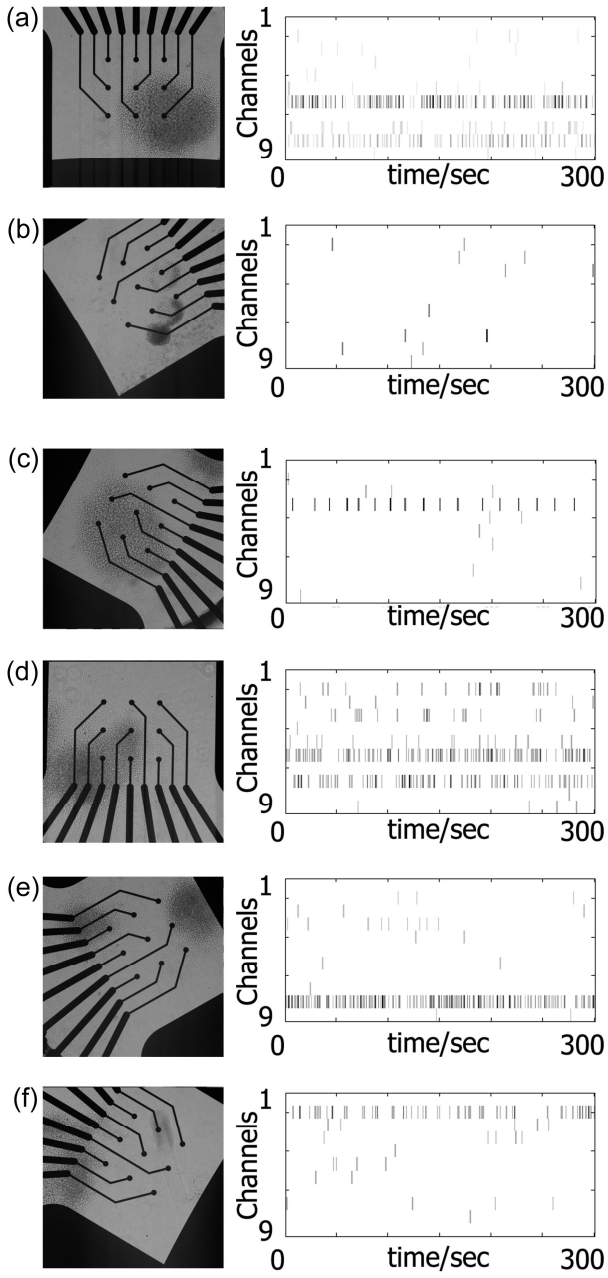

Fig. 7 Representative phase-contrast microscopy images and raster plots from cells on six-well MEA with Configuration I PDMS chamber. They formed good networks and expressed spontaneous signaling at 16 DID in all separate wells. All six wells presented (a)-(f) from one MEA