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Long-term brain-on-chip: Multielectrode array recordings in 3D neural cell cultures

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ABSTRACT
This study presents sensing of network bursts in a three-dimensional (3D) cell culture system consisting of a microbioreactor and a multielectrode array (MEA), i.e., brain-on-chip, to interpret neural network dynamics in a label-free manner. While our initial results reported an increased single spiking activity already over the course of days 7, 14, and 21 in vitro, the advanced long-term analysis of the data set (including a last timepoint at day 79) here demonstrates a proof-of-principle for following bursting patterns upon maturation of the network in the microbioreactor as an add-on device for a commercial MEA recording system. These quantitative electrophysiological findings, including mean bursting rate, mean burst duration, and network burst dynamics, confirm a 2D to 3D transition in coherence with the literature.

I. INTRODUCTION
Human three-dimensional (3D) cell cultures are exquisitely positioned to imitate the complex in vivo cell-cell and cell-tissue behavior. Compared to two-dimensional (2D) cultures, the 3D approach improves the expression of differentiated functions and tissue organization. 3D cell culture models can, therefore, fill the gap between animal and 2D cell culture studies and harness the potential to unlock complex content-rich experiments based on human cell sources and reduce the extensive use of animals in (bio)medical studies.1 Furthermore, by means of 3D cell cultures and their analysis, these systems can be enabled to decipher biological functions, including disease mechanisms, of the human body which are not detectable in 2D culture formats or animal models.2–4

Despite all these benefits, quantitative data collection of 3D cell cultures is challenging. They often need advanced confocal (fluorescence) microscopy with enhanced spatial resolution in all directions,5 while potentially content-rich optical techniques can be very time-consuming and expert-labor intensive,6 and additionally, fluorescence-based characterization techniques are mostly performed as an endpoint method, i.e., being toxic and static.7 Therefore, there is a need for label-free in situ assays. This requirement makes electrical measurement techniques a method of choice for readouts in 3D cell cultures.8 The development of impedance-based sensors8 and advances in multielectrode arrays (MEAs)9 for electrophysiological recording techniques in human induced pluripotent stem cell (hiPSC)-derived 3D cultures of cortical neurons are the foundation for an effective brain-on-chip (BoC) technology.7 Generally, MEAs have planar electrodes on glass surfaces and require very close vicinity of the neurons for high signal-to-noise ratio data collection. Nevertheless, this well-known arrangement of electrodes can also act as a transducer for the signals generated in the spatially extended 3D network to retrieve biologically
TABLE I. Long-term culturing studies of neural cell culture in 2D and 3D and monitored electrically via MEA, emphasizing cell culture type and method, cell type, and culture duration (days in vitro).

| Reference | Culture type          | Culture method                                      | Cell type                                      | Days in vitro |
|-----------|-----------------------|-----------------------------------------------------|-----------------------------------------------|---------------|
| 12        | 2D                    | Laminin-521 substrate                               | hiPSCs-derived neurons                         | 130           |
| 13        | Poly-d, l-ornithine and laminin coating on MEA | hiPSCs-derived neurons                             |                                               | 90            |
| 14        | Poly-d-lysine and laminin coating on MEA        | hiPSCs-derived neurons                             |                                               | 95            |
| 15        | 3D                    | 3D construction around electrodes of 3D MEA        | Rats’ primary cortical neurons                 | 14            |
| 16        | 3D                    | 3D construction by using glass microbeads          | Hippocampal neurons dissociated from E18 Sprague Dawley rats | 28            |
| 11        | Matrigel*             | SH-SY5Y and hiPSCs-derived cortical neurons        |                                               | 21            |
| 17        | Encapsulated in extracellular matrix-collagen hydrogel | hiPSC-derived neurons and astrocytes               |                                               | 38            |
| This study| Matrigel*             | hiPSCs-derived cortical neurons                     |                                               | 79            |

meaningful results. Previously in our group, we introduced such a concept of signal collection using a MEMS add-on, i.e., a micro-bioreactor, which can be reversibly assembled on a MEA plate to maintain a 3D cell culture. This 3D culture system consists of a circular cell culture chamber, surrounded by a ring-shaped microfluidic channel.\cite{10,11} Cell culture chamber and microfluidic channel are separated by a porous barrier. As a preliminary result, we reported that hiPSC-derived cortical neurons can be cultured up to day 21 by using this system. It was also shown that spontaneous spikes, having 100\textmu V amplitudes, were present already at day 7. Moreover, burst formation, consisting of higher amplitude (400\textmu V) spikes, was observed at days 14 and 21.\cite{11}

In this research paper, we completed the analysis of the data set, received from this unique experiment with cultured hiPSC-derived cortical neurons on MEA, in a quantitative manner up to 79 days-in vitro (DIV) as an example of illustrating the value of MEMS technology in this emerging application field of BoC technology. By means of the encircling microfluidic channel and the porous cell culture wall from polyethersulfone (PES), neurons did not lose their contact with 2D electrodes of MEA since nutrients were provided in 3D from the top and the side walls. There are a few studies which present long-term culturing of neuronal cell culture in 2D (Table I). However, for 3D, the culture durations are shorter than 2 months (Table I). To the best of our knowledge, this analysis of data, received by brain-on-chip-based sensing of network bursts in a 3D neuronal cell culture system, gave us an opportunity to obtain the widest electrophysiological data repertoire belonging to hiPSCs-derived cortical neurons in 3D cell culture established in the literature so far.

II. MATERIALS AND METHODS

A. Integration of micro-bioreactor and MEA

Our micro-bioreactor was fabricated by using soft lithography of polydimethylsiloxane (PDMS) in a mechanically machined poly(methyl methacrylate) mold and porous PES cylindrical membranes. Details of this fabrication are presented in Ref. 11. Figure 1 depicts our brain-on-chip 3D cell culture system as a schematic in parts (a) and an assembled fashion (b), which had been previously introduced in Ref. 11. In brief, the operational procedure of the system assembly steps can be described as follows: a 2D cell layer is formed first. Next, cells inside a hydrogel are seeded into the porous PES tube, i.e., the culture chamber [Fig. 1(c)]. Cell medium is loaded into the microfluidic channel (volume = 120\textmu l), encircling the PES tube (wall thickness <500\textmu m), to provide nutrients to cells and wash removal via the side wall [Fig. 1(c)] and subsequently cell culture medium is topped up in the cell culture chamber from the top, too. MEA electrodes acquire in situ and label-free response of the 3D cultured networks. Two different experimental groups were prepared in the cell experiments [Fig. 1(c)] by applying the same procedures for surface coating and cell seeding. One group was a 3D cell culture in a micro-bioreactor on MEA and the other was a 3D cell culture, prepared in a confined area on MEA, provided only by a plain PDMS ring without microfluidic exchange. The latter serves as a culture control to assess the effect of applying a surrounding microfluidic channel in such 3D cultures.

B. hiPSCs-derived cortical neuron cultures

hiPSC-derived cortical neurons (Axol Bioscience, Cambridge, UK) were differentiated and cultured based on the description of the manufacturer,\cite{18} which should result in a pure population of cortical neurons. In brief, neural progenitor cells were thawed and subsequently seeded into a micro-bioreactor integrated with a MEA, coated with ReadySet and SureBond (Axol Bioscience, UK), consecutively, before cell seeding. After 30 min, cells mixed with a hydrogel, i.e., Corning Matrigel matrix (Life Sciences, USA), were added on top of them to obtain 3D cell culture and culture medium was topped up. Details of this procedure are described already in Ref. 11.

C. Spike, burst, and network burst (NB) detection and analysis

Measurements were carried out via a 2100MEA system (Multi Channel Systems GmbH, Germany) by using 120 electrodes MEA plate, having a plastic ring with an inner diameter of 26.5 mm on it (120MEA200/30 iR-Ti-pr). Cells were kept within neural differentiation medium (Axol Bioscience, UK) for 9 days and subsequently in neural maintenance XF medium (Axol Bioscience, UK) for the
remaining days in culture. The temperature of MEA plate was set to 37 °C through measurements to minimize disturbances on cells. More than four different measurements were taken from the microbioreactor culture at consecutive 1 min durations between different DIV, ranging from day 6 to 79. **MULTICHANNEL ANALYZER** software (Multi Channel Systems GmbH, Germany) was utilized to examine these measurements. Experimental settings in this software are presented in Sec. I in the supplementary material.29 Electrode data were sampled at 20 kHz. After acquisition, they were high pass filtered by second order Butterworth filter with 200 Hz cut off frequency. A Notch filter at 50 Hz was applied in series with it. Examples of filtered data for both groups 1 and 2 are presented in Sec. II A in the supplementary material.29

The first parameter to understand firing behavior of a neuronal cell culture is spikes. Spontaneous spikes are the action potential discharges without applying stimulation.19 For spike detection, voltage threshold was chosen as five times \( V_{\text{noise-rms}} \).20,21 Bursts occur when spikes become more organized.22 The level of organization in spikes to define a burst is determined with respect to features of spikes, including interspike intervals. Network bursts

| Maximum interval to start burst | 10 ms |
|-------------------------------|-------|
| Maximum interval to end burst  | 50 ms |
| Minimum interval between bursts| 100 ms|
| Minimum duration of bursts     | 300 ms|
| Minimum number of spikes in bursts | 10 |
| Minimum active channels        | 2     |
| Minimum spontaneous channels   | 50% of BC |

**FIG. 1.** Schematic of microbioreactor in detail (a) and MEA-assembled brain-on-chip 3D culture system (b). (c) Cell seeding procedure and experimental groups.
FIG. 2. Electrophysiological recordings of hiPSC-derived cortical neurons in microbioreactor (group 1), presenting the quantity of spikes (a), average burst number (b), in-burst spike ratio (c), mean burst duration (d), and NB rate (e) and duration (f). Data are presented as avg ± SD.
(NBs) are composed of synchronized bursts all over the network.\textsuperscript{22} There should be a threshold like minimum spontaneous channels. In this study, we defined it as the half of total number of bursting channels. For burst and NB recognition, the parameters are presented in Table II.\textsuperscript{23}

**D. Statistical analysis**

More than four measurements at each DIV were taken consecutively for 1 min. Data were presented as average (avg) ± standard deviation (SD). Statistical correlation between the averages of these measurements at different DIVs was investigated by one-way ANOVA technique using Fisher’s Least Significant Difference test. p values of <0.05 were considered statistically different. Origin Pro 2019 (OriginLab) was used for these analyses.

**III. RESULTS AND DISCUSSION**

3D cell culture recordings were performed up to day 79 in the microbioreactor/MEA (group 1) and day 29 in PDMS ring/MEA (group 2). In supplementary video S1 in the supplementary material,\textsuperscript{29} an example measurement with microbioreactor at day 35 is presented. After 29 DIV, the PDMS ring culture rapidly degraded or lost connection with the electrodes and was discarded. The microbioreactor culture was still producing signals after day 79 but was discarded shortly thereafter for practical experimental reasons. Spike and burst numbers, average burst duration, NB occurrence and duration, and in-burst spike percentages were investigated for these two cell cultures to make neuronal network analysis, quantitatively. Spikes, bursts, and NBs were detected at 6 DIV for both groups although burst amount (4 ± 4 bursts/min) was significantly low and stochastic in group 1 and relatively higher (14 ± 9 bursts/min) but randomly distributed in 5 min for group 2. All three types of negative spikes (mono-, bi-, and tri-phasic) were observed as early as day 6, indicating good coupling between the different parts of neurons, such as soma and axon, and MEA electrodes.\textsuperscript{24} Measurements were taken as 1 min durations day-by-day. Minute-to-minute fluctuations occurred in neuron dynamics of both groups (for whole sets of data, please see Secs. II A and II C in the supplementary material).\textsuperscript{29} Fluctuating behavior was expected for mature neuronal networks after 3-week in vitro.\textsuperscript{22} Therefore, comparisons between recordings at different DIVs were carried out by ANOVA one-way analysis at p = 0.05 level in which average values per minute were considered.

For group 1 (microbioreactor), the average of spike numbers increased up to day 19 with significant differences (at p = 0.05 level) between day 6, 7, 8, 9, 10, 12, 14, and 19 [Fig. 2(a)]. It started to decrease at day 21. There was no meaningful variation in spike number up to day 27. At day 27, an abrupt spike number reduction occurred although there was no significant difference in the active electrode (AE) and bursting electrode (BE) numbers for day 25, 26, and 27 (10 ± 1, 10 ± 1, and 9 ± 2 for AEs and 7 ± 2, 5 ± 1, and 4 ± 2 for BE, respectively). This oscillating behavior, i.e., increase and decrease in spike number, was observed between days 35 and 51 and between days 51 and 72. These tendencies were followed by burst numbers, also [Fig. 2(b)]. Although there was no specific variation in active and bursting electrode numbers (AE and BE number graphs with respect to DIVs are presented in Sec. II D in Fig. 3. Fixed cell images showing MAP2 (red) and DAPI (blue) stained neurons in 3D. Yellow, white, and blue arrows indicate appearing and disappearing parts of neurons obtained by z-stack setting from bottom (a) to top (d) of 3D culture.
FIG. 4. Electrophysiological recordings of hiPSC-derived cortical neurons in PDMS ring (group 2), presenting the quantity of spikes (a), average burst number (b), in-burst spike ratio (c), mean burst duration (d), and NB rate (e) and duration (f). Data are presented as avg ± SD.
the supplementary material), this behavior of cells can be interpreted as neurons started to remodel their environment and changed their position by migration into the third dimension within the Matrigel. Overall, the increase in spike number and high order patterns of bursts indicated neuronal maturity in 3D cell culture.

Based on Frega et al., the number of spikes included in bursts (i.e., in-burst spikes) were divided by total number of spikes and the obtained value was named in-burst spike ratio. Up to the end of week 3, there was a significant increase to 85% in the mean value of in-burst spike ratio (at p = 0.05 level, n = 1) [Fig. 2(c)]. This is coherent with the maturation level of 2D cell culture. According to Frega et al., spikes form clusters other than being in random format after 3 weeks in 2D cell culture. These clusters are grouped as bursts, showing maturation in neurons. However, at day 22, there was a sharp decrease in in-burst spike ratio of cells up to 59% in microbioreactor and after this point no statistically significant increase occurred in in-burst spike ratio up to day 33 [Fig. 2(c)]. This in-burst spike ratio (60% as average) showed a transition to 3D cell culture. The largest minute-to-minute fluctuation occurred in spike number at day 22. This can also be a clue for 3D transition of cells. Additionally, supporting random spike ratio tendency as evidence for 3D transition, average burst number for 1 min increased up to 86 ± 8 bursts/min at 19 DIV and significantly (p < 0.05) lower number of bursts (40 ± 30 bursts/min) were observed at day 22 [Fig. 2(b)]. There is no conclusive evidence in the literature about a significant increase in mean burst duration within the Matrigel. Overall, the increase in spike number and high order patterns of bursts indicated neuronal maturity in 3D cell culture. Maturity of 2D cell culture can be reached up to 59% in microbioreactor and after this point no statistically significant increase occurred in in-burst spike ratio up to day 33 [Fig. 2(c)]. This in-burst spike ratio (60% as average) showed a transition to 3D cell culture. The largest minute-to-minute fluctuation occurred in spike number at day 22. This can also be a clue for 3D transition of cells. Additionally, supporting random spike ratio tendency as evidence for 3D transition, average burst number for 1 min increased up to 86 ± 8 bursts/min at 19 DIV and significantly (p < 0.05) lower number of bursts (40 ± 30 bursts/min) were observed at day 22 [Fig. 2(b)]. There is no conclusive evidence in the literature about a significant increase in mean burst duration within the Matrigel. According to Frega et al., spikes form clusters other than being in random format after 3 weeks in 2D cell culture. These clusters are grouped as bursts, showing maturation in neurons. However, at day 22, there was a sharp decrease in in-burst spike ratio of cells up to 59% in microbioreactor and after this point no statistically significant increase occurred in in-burst spike ratio up to day 33 [Fig. 2(c)]. This in-burst spike ratio (60% as average) showed a transition to 3D cell culture. The largest minute-to-minute fluctuation occurred in spike number at day 22. This can also be a clue for 3D transition of cells. Additionally, supporting random spike ratio tendency as evidence for 3D transition, average burst number for 1 min increased up to 86 ± 8 bursts/min at 19 DIV and significantly (p < 0.05) lower number of bursts (40 ± 30 bursts/min) were observed at day 22 [Fig. 2(b)]. There is no conclusive evidence in the literature about a significant increase in mean burst duration within the Matrigel. Overall, the increase in spike number and high order patterns of bursts indicated neuronal maturity in 3D cell culture.

It might not be interpreted as the death of cells since the occurrence of spikes, bursts, and NBs up to the end of day 79 provides sufficient evidence that the cell culture was healthy throughout the entire analysis time. Variable NB duration promotes the evidences in the literature at which it was claimed that the duration of NB in the 3D cell culture can be either close to the ones in 2D or longer than that of 2D [Fig. 2(f)].

As evidence for 3D cell culture, fluorescent cell images from different heights of cell culture inside microbioreactor are presented in Fig. 3 from bottom (a) to top (d) of cell culture. The z-stack (obtained in confocal microscope, Leica TCS, SP5X) video from which these images are taken is presented as supplementary video in the supplementary material.

For group 2 (PDMS ring), significant decrease in spike number [Fig. 4(a)] and burst number [Fig. 4(b)] occurred 1 week before compared to the microbioreactor experiment. There was no encircling microfluidic channel in PDMS ring. Nutrition of cells was achieved from top of Matrigel only. Therefore, cells might want to immediately reach the top and 3D transition may have occurred earlier. Average in-burst spike ratio was under 60% around day 16 as expected for 3D transition in Matrigel although there was no significant decrease between days 12, 14, 16, and 19 [Fig. 4(c)]. Figure 4(d) presents average burst durations, which were similar with the durations obtained in Ref. 12 for longer period in 2D hiPSCs culture and were also longer than indicated in Ref. 16 for the same period in 3D cell culture of hippocampal neurons, dissociated from E18 Sprague Dawley rats. Figures 4(e) and 4(f) show the NB number for 1 min and average NB durations, respectively. No values were presented for days 22 and 29 since no NB formation was observed in these days. There was no significant day-to-day variation between NB numbers and durations for group 2. If group 1 and group 2 are compared for 4 weeks in vitro, NB numbers are higher and NB durations are longer in group 1 than those of group 2. This observation offers us an argument for the importance of the microbioreactor design, which had an encircling microfluidic channel providing shear-free exchange of nutrients and waste in and out of the 3D cell culture chamber. Hence, we hypothesis that by means of this property, cells did not lose their contact with 2D MEA electrodes and data collection could be achieved for longer times in 3D cell culture as ever reported before in the literature for such type of culture arrangements.

IV. SUMMARY AND CONCLUSIONS

The collection of network activities by electrophysiological recordings provides us a strong clue for arranging proper microenvironments and maintenance of hiPSC-derived cortical neuron cultures utilizing, for example, an encircling microfluidic channel in such cultures. While cells demonstrated their transition to a 3D configuration after two to three weeks in vitro in both, the simple PDMS ring confined as well as the microbioreactor culture on MEA plates, our brain-on-chip 3D cell culture system applying a porous side wall in the culture chamber with an encircling flow channel can be successfully used for applications studying healthy or pathophysiological stem cell-derived neural microtissues aiming for a long-term study in a label-free and noninvasive manner. For this purpose, the efforts in proposing new research with a focus on
repeatability and reliability analysis of this system with technical
and biological replicas for such long culture durations in vitro are
deemed justified.

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DATA AVAILABILITY

The data that support the findings of this study are available
from the corresponding author upon reasonable request.

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