An Experimental Platform Aimed at Long Lasting Electrophysiological Multichannel Recordings of Neuronal Cultures

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Keywords: Microelectrode arrays, Neuronal cultures, Long-term recordings

Abstract: The elucidation of physio-pathological mechanisms expressed by a neuronal network over extended time scales (i.e., from hours to weeks) is the goal of many neurophysiological and neuropharmacological *in vitro* studies. In this context, a challenging technological requirement is the establishment of an experimental setup able to collect long-term neuronal signals. In this work we report the development of a compact environmental chamber designed to perform prolonged recordings of the bioelectrical activity exhibited by neuronal networks grown on MicroElectrode Arrays (MEAs). To reproduce an environment suitable for cells growth (temperature, pH and humidity) the chamber was coupled with a temperature control system and an air humidifying module. Validation tests demonstrated that the environment inside the portable chamber is comparable to standard cell incubators environment. To collect neuronal extracellular signals, custom multichannel pre-processing boards have been developed and integrated with the chamber. With this equipment, we were able to reliably record spontaneous neuronal electrical activity from hippocampal cultures grown inside the chamber for several hours, which is not possible with the standard MEA recording setup due to environmental fluctuations. This system can collect multichannel data from neuronal cultures over long periods, providing an effective solution for long-term studies of neural activity.

1 INTRODUCTION

A central goal of the modern neuroscience is to understand the relationship between the functional connectivity of neuronal circuits and their physiological or pathological features. The dissociated culture of primary central neurons provides a convenient test system to reach this aim. Indeed, *in vitro* cultures retain many characteristics of their *in vivo* counterparts, but they are simpler and more accessible for investigations and manipulations (Eckmann et al., 2007). In this context, neuronal cultures grown on MicroElectrode Arrays (MEAs) represent a powerful tool thanks to the non invasive and multisite approach (Johnstone et al., 2010, Rossi et al., 2011). However, *in vitro* neuronal ensembles are extremely sensitive to changes in the surrounding environment (temperature, pH, humidity) (Biffi et al., 2012). Thus, the establishment of an experimental setup able to maintain stable conditions is an absolute requirement in order to design truly significant experiments and collect reliable data with MEAs.

Nowadays, standard MEA-based experimental platforms are well-established setups for several neurobiological applications where short recordings (i.e., from 10 minutes to a couple of hours) are adequate to gather the information of interest. Nevertheless, the possibility to perform longer investigations of neuronal activity is a challenge to throw light on physiological or pathological mechanisms evolving over longer time windows (e.g. degeneration of functional connectivity).

Some effort has been already directed at improving standard setups in order to reach this aim (Potter and DeMarse, 2001). Among the proposed solutions, an effective solution is represented by

Regalia G., Biffi E., Lucchini A., Capriata M., Achilli S., Menegon A., Ferrigno G., Colombo L. and Pedrocchi A..
An Experimental Platform Aimed at Long Lasting Electrophysiological Multichannel Recordings of Neuronal Cultures.
DOI: 10.5220/0004659005510557
In *Proceedings of the 5th International Joint Conference on Computational Intelligence* (SSCN-2013), pages 551-557
ISBN: 978-989-8565-77-8

compact commercial top stage incubators for microscopy analysis (e.g., Ibidi GmbH, Okolab s.r.l.) which provide an effective environment control but they are hardly modifiable to be coupled to MEA technology. On the other hand, custom setups built up for MEA-based experiments, do not include the control of all environmental parameters and they usually provide temporary solutions (Novellino et al., 2011). Recently, it was devised a novel system which merges an effective environmental control and multisite recordings capability. This system is a pilot environmental chamber for a single neuronal culture grown on a MEA coupled to external commercial electronics (Biffi et al., 2012). However, more effort has to be done to improve the osmolarity control, to advance the quality of recorded neuronal signals and to increase the throughput of the system.

To tackle the challenge of environmental stability during *in vitro* electrophysiological experiments and to fulfil the requirements of environmental stability, good quality recordings and multi-MEA format, we designed and validated a stand-alone platform for multi site experiments with neuronal networks. In particular, we present here: (i) the design of the environmental chamber and a preliminary quantitative characterization of the environmental control (ii) the design and validation of a custom modular multichannel front-end and (iii) preliminary results regarding prolonged recordings with this experimental platform.

2 MATERIALS AND METHODS

2.1 Multi-MEA Environmental Chamber Realization

Figure 1 reports a schematic representation of the environmental chamber. The chamber has been realized by assembling two PMMA boxes: an external one (220x220x45 mm) and an internal one (180x180x30 mm), the latter being surrounded by a water jacket. Both boxes are sealed with an airtight top plate, through which we drilled openings for air inlet and outlet, for the insertion of a temperature probe and for medium exchange from outside. In the internal box, we placed a reference well in the centre and four 50x50 mm housings for 60 channel MEA chips in the corners. The recording electronics (paragraph 2.2) contacts the 60 pads of each MEA chip by means of vertical gold spring probes. Signals are carried outside by means of a 68-pin connector inserted and sealed through the top plate. Openings

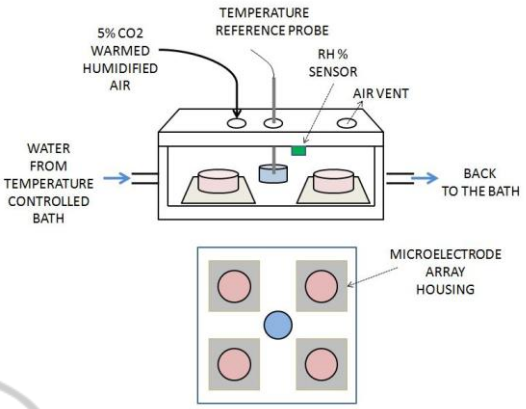

Figure 1: Schematic representation of the environmental chamber (top) and top view of MEAs housings (bottom).

for microscope objective insertion were drilled below the four MEA housings.

2.1.1 Temperature Control

The chamber heating is obtained by means of a circulating bath (E306, Ecoline, Lauda GmbH) and a feedback Proportional-Integral control, as previously described (Biffi et al., 2012). Preliminary simulations of the temperature distribution in the MEA housings were performed with a FEM code (Comsol). During experiments, the temperature of culturing medium contained in Petri dishes located in the four MEA housings was monitored by thermo-couples to verify its maintenance in a physiological range (37 ± 0.5 °C).

2.1.2 PH Stabilization

To maintain the pH of the medium in a physiological range (7.2-7.4), an air flow enriched with CO_2 is injected in the chamber from a gas cylinder. Experimental tests have been conducted to link the air flow rate to the CO_2 content inside the chamber. 20% O_2, 75% N_2, and 5% CO_2 air flow rates spanning from 70 to 500 ml/min were set by means of a flow meter (NG series, Platon SaS). For each flow rate, the gaseous CO_2 percentage in the chamber was measured by a CO_2 tester (Heraeus, Thermo Scientific).

2.1.2 Humidity Regulation

To slow medium evaporation, the flow is warmed and humidified by an independent bubbling module placed onto the lab bench. This unit is formed by a glass bottle containing a glass micro-filter candle to improve the creation of bubbles, and thus the air

humidification. A Nickel-Chrome heating wire (10 W), insulated by a silicon sheath, is placed into the bottle to worm up the gas flow. This element is powered on when a miniaturized humidity sensor (SHT15, Sensirion AG), integrated into the chamber, measures values of relative humidity (RH) lower than 85% and it is switched off when RH reaches 95%. In its first version, the control is implemented by software (USB6009 and Labview, National Instruments). An heating resistive wire is bound to the tube which connects the bubbling module to the chamber, to prevent air cooling. To have a reference for the miniaturized sensor, RH measurements after the bubbling column and inside the environmental chamber have been performed with a commercial probe (HMP233, Vaisala Inc.).

A high RH level could induce condensation in the chamber, thus raising the probability of water droplets to drain on the cells and decreasing visibility from outside. Condensation of the water vapour over the inner face of the top plate would arise if its temperature lowers under the dew point. To avoid this drawback, heating elements could be placed on the top plate. To suitably design the heaters, a FEM model of the steady-state heat transfer through the whole environmental chamber has been implemented (Comsol).

2.2 MEA Recording Boards Design

To perform electrical recordings from cultures grown on four 60-channel MEA chips, custom pre-processing boards have been designed and coupled to a multichannel commercial data acquisition system (USB-ME240, 50 kHz, 16 bit, Multi Channel Systems, MCS, GmbH).

The whole processing chain was designed to fulfil the main following requirements: (1) high gain (~ 60 dB), suitable for the amplification of neuronal signals *in vitro* (20-400 µV peak to-peak) (2) neuronal spike bandwidth (300Hz - 5 kHz), (3) low noise (< 20 µV peak-to-peak, i.e., typical thermal and background biological noise at the microelectrodes). The defined circuitry (Figure 2, top) consists of a pre-amplification stage (gain 100, high pass cut frequency at 300 Hz), a Butterworth high-pass filter (gain 3, cut frequency at 300 Hz) and a Butterworth low pass filter followed by a RC low pass (gain 3, cut frequency at 5 kHz). The performance of the circuit was simulated with Spice software (LTSpice, Linear Technology).

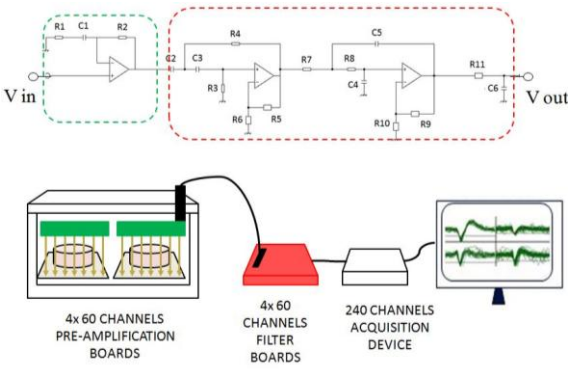

Figure 2: Schematic of the custom front-end circuitry (top) and assembly of the boards (bottom).

For each 60 channel MEA, the pre-amplification stage was designed to be implemented on two 30-channel boards (65x65 mm) placed inside the culture chamber (Figure 2, in green), in order to avoid the degradation of the signal-to-noise ratio (SNR) of recordings. A squared hole has been drilled in the centre of each board in order to allow visibility of the cultures and to insert tubes from medium exchange from the top. The following filter stage was implemented on two 100x100 mm 30-channel external boards (Figure 2, in red). Surface mounted components and low noise, precision opamps were chosen. Four copies of pre-amplifiers and filter modules were realized in order to record simultaneously from four MEAs (i.e., 240 channels).

The assessment of the real frequency response gain of each board was obtained by providing sine waves (peak-to-peak amplitude equal to 100 µV for the pre-amplification stage and 10 mV for the filter stage) by means of a sinusoidal wave generator with frequency varying between 1 Hz and 10 kHz, for each channel. The input-referred noise (300 Hz - 5 kHz) of each board was tested measuring the channel output and dividing it by the overall nominal gain, with inputs connected to ground. The channel crosstalk was measured by sending a 1 kHz controlled sine wave to one channel, and recording from directly adjacent ones (with inputs grounded). After testing each board independently, pre-amplification and filter boards were connected and tested together.

2.3 Electrophysiological Recordings From Neuronal Cultures

Figure 3: (A) Picture of the realized environmental chamber. (B) Assembled electronic boards for the internal pre-amplifier stage (left) and external filter stage (right). (C) Inverted microscope image of a neuronal culture grown on a MEA taken from the chamber (bar scale 10 μm).

Neuronal cultures on MEAs were obtained with a standard protocol, described in (Ghezzi et al., 2008, Biffi et al., 2012 b). Short recordings (< 30 min) from hippocampal neurons (CD1 mice, E17.5, 200.000 cells/MEA) grown on standard MEA chips (60 electrodes, MCS GmbH) were performed during the 2nd and 3rd week of maturation, with the aim of evaluating signal quality and spikes morphology. Moreover, to evaluate the feasibility of prolonged experiments with the system, we performed recordings lasting several hours (>2 h). Spikes were detected by a comparison with a threshold based on noise level (as described in Biffi et al., 2010). Then, time stamps were analyzed by means of standard signal processing (Matlab®) (Biffi et al., 2011). The SNR for a single channel has been defined averaging the spikes amplitudes over the recording window and dividing by the noise over the first 500 ms. The mean firing rate was chosen as a global descriptor of network activity (Biffi et al., 2011), computed as the ratio between the total number of spikes and the number of active channels, reported in Hz.

3 RESULTS

3.1 Multi-MEA Environmental Chamber

Figure 3A reports the chamber assembled. Figure 3B shows the custom pre-amplifier board (left) and filter board (right). Figure 3C shows a neuronal network on microelectrodes of a MEA chip. The image was taken by using an inverted microscope (Axiovert 135 TV, Zeiss). The 5x differential interference contrast objective was inserted beneath the MEA housing through the opening in the bottom wall. The quality of the picture attests the possibility of duly monitoring cells inside the chamber during network maturation.

3.1.1 Temperature Control

The FEM simulations confirm that in all the possible operative conditions, the temperature difference among the MEA allocations is negligible. To maintain the desired temperature in the MEA housings the set-point has been set to 36.8 °C. After the initial heating phase (almost 60 minutes), temperature measurements compare well among the 4 housings and show small oscillations around 37°C (i.e., <0.5°C peak-to-peak) (Figure 4).

3.1.2 PH Stability

As a result of the air flow rate characterization, flow rates ranging from 140 ml/min to 500 ml/min allow to balance CO_2 losses occurring along the tubing system and in the chamber and to reach 5% CO_2, i.e., the value needed to maintain cell culture pH at 7.4 (Biffi et al., 2012). After these experiments we chose a flow rate equal to 200 ml/min for further experiments as a trade-off between a physiological pH value, a good humidification (better for higher flow rates) and the gas cylinder consumption.

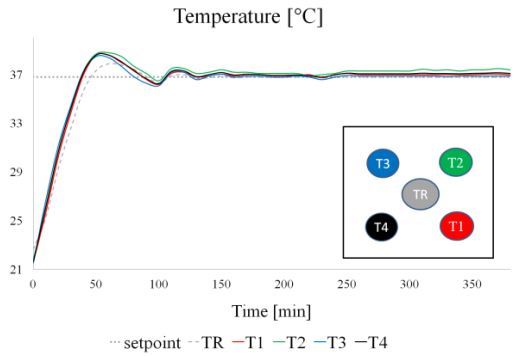

Figure 4: Temperature time course measured at the reference well (dotted line) and at the four MEAs allocations (T1-T4). The insert is a top view of the temperature measurement points.

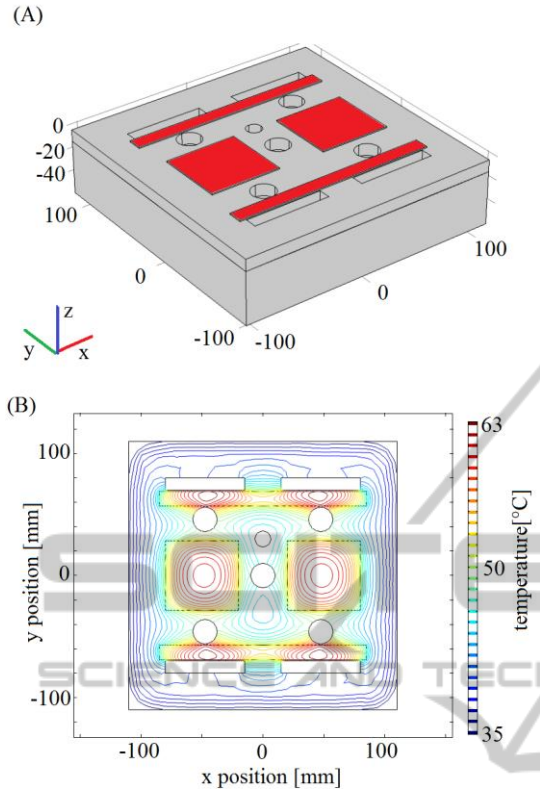

Figure 5: (A) 3D chamber geometry with heating elements on the top plate (red). (B) Temperature contour in the lower side of the top plate.

3.1.3 Humidity Maintenance

With the selected flow rate of 200 ml/min, the control system maintains almost 95% RH in the bubbling column (water temperature 65 °C, air temperature 27 °C), which corresponds to almost 85% RH in the chamber (air temperature 35°C). The simplest solution to overcome condensation on the inner side of the top plate turns out to be the use of electrical strip heaters on the outer surface of the cover. A suitable heater configuration consists of two couples of heaters (170×12 mm, 0.75 W and 60×57 mm, 1 W, respectively), shown in Figure 5A. They keep the top plate inner surface temperature above the dew point (i.e., 35 °C, 85% RH) inducing a maximum temperature of almost 60°C, which is lower than PMMA melting point (Figure 5B). Moreover, their allocation preserve visibility from the outside of the chamber. To limit the power consumption and to avoid accidental contact with hot surfaces, an insulating, removable layer of rubber foam, 10 mm thick, has been simulated above the heaters. Preliminary experiments with real heaters seem to confirm the simulations (maximum temperature of almost 58 °C) but further tests have to be performed to verify that the heaters do not interfere with cells viability and quality of the recordings.

3.2 Boards Performances

The defined front-end circuit is suitable for the analog processing of *in vitro* neuronal signals. The measured whole gain of pre-amplifiers and filter boards is in agreement with calculations and Spice simulations (absolute error equal to ~1.6 dB in the bandwidth, Figure 6). Also, noise performances of the whole chain in terms of input-noise (4 μV RMS, mean of the 60 channels) and cross-talk gain (-36 dB, mean of the 60 channels) are comparable to noise as measured from the commercial equipment and other custom setups (Bottino et al., 2009, Rolston et al., 2009).

3.3 Long Lasting Recordings of Neuronal Networks

The environmental chamber and the custom electronic described above, were used to perform electrophysiological multichannel recordings from hippocampal neuronal cultures. We observed a mean SNR equal to 5.4 dB, which is comparable to recordings performed with standard equipment in our lab. Furthermore, the recorded biphasic spike waves compare well to those described in the literature (Rolston et al., 2009) (Figure 7).

Moreover, preliminary results show that neuronal cells inside the controlled environment do not undergo the activity decline, that typically occurs with the standard setup when the recording time windows is longer than 2 hours. Several cultures were recorded for time windows lasting from 3 to 12 hours. As an example, Figure 8 reports the spike rate of a neuronal culture briefly recorded in the standard setup (with only temperature control) and immediately after in the environmental and recording chamber for almost 4 hours (temperature, RH% and gaseous CO_2 control). Apart from the initial adaptation due to the repositioning, neuronal activity inside the chamber is stable over the time window. Furthermore, it is characterized by a mean value (2.6 Hz) and fluctuations (± 0.78 Hz) comparable to the activity recorded by the standard setup over a shorter time window (2.3 Hz ± 0.68 Hz), which demonstrates the reliability of long-term data (i.e., the culture kept staying in a physiological state throughout the 4 hours).

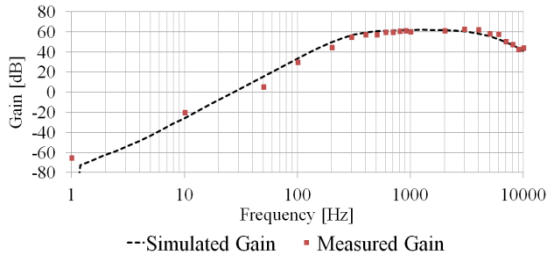

Figure 6: Comparison between the simulated gain and the measured one (pre-amplifiers + filter boards).

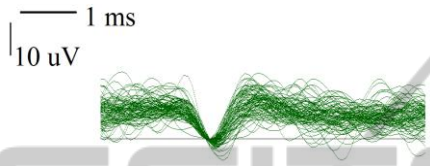

Figure 7: Overlapped neuronal spikes recorded from a MEA channel inside the environmental chamber.

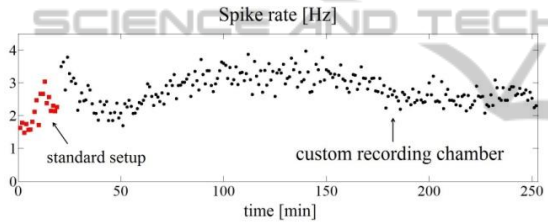

Figure 8: Spike rate of a neuronal culture recorded in the standard setup and in the environmental recording chamber. Mean spike rates over 1minute bins are reported.

4 DISCUSSION

To tackle the challenge of environmental stability during *in vitro* electrophysiological experiments, we designed and validated a stand-alone platform aimed at maintaining a controlled environment while growing and recording from neuronal networks on MEAs.

To realize a controlled environment, we connected the chamber to a temperature controller and a system to inject air enriched with CO_2 and water vapour. We demonstrated that the chamber maintains a stable physiological temperature in each of the four MEA housings. Moreover this preliminary measurements suggest that a flow rate of 200 ml/min is optimal to obtain (i) a CO_2 percentage almost equal to the quantity contained in the air delivered by the gas source (ii) a quite high level of RH in the chamber, i.e. 85% RH, which is comparable to other commercial top stage incubators (Ibidi GmbH).

Regarding the custom front-end, it is suitable to be coupled with the chamber, both in terms of sizes, environmental compatibility and recording performances. Moreover, the realized boards are cheaper and more easily replicable than commercial recording front-end devices or custom CMOS-based systems (Rolston et al., 2009). Preliminary results assessed the feasibility of performing experiments with MEAs longer than standard ones (i.e., 2 hours) thanks to the stable, physiological environment. Regarding the throughput of the system, the actual prototype houses four 60-channel MEA chips, which means up to 24 cultures if 6-well MEAs (9 electrodes per well) are used (MCS GmbH).

Future work will include an improvement of the humidification system, the integration of gas sensors in the chamber and repeated experimental tests to assess the reproducibility of the system.

5 CONCLUSIONS

In this work we presented the design and preliminary validation of a challenging stand-alone platform for parallel prolonged experiments from neuronal cells grown on MEAs. Our final aim is to provide a compact technological tool for an electrophysiological laboratory, independent from both bulky incubators and expensive front-end equipments, and easy to handle for experimenters. This system provides new perspectives for *in vitro* long-term, high-throughput electrophysiological studies on neuronal cultures on MEAs.

ACKNOWLEDGEMENTS

Authors would like to thank people from the Alembic facility for their support and Dr. De Ceglia for the dissection of hippocampi.

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