Video Article

Interfacing Microfluidics with Microelectrode Arrays for Studying Neuronal Communication and Axonal Signal Propagation

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Abstract

Microelectrode arrays (MEAs) are widely used to study neuronal function *in vitro*. These devices allow concurrent non-invasive recording/stimulation of electrophysiological activity for long periods. However, the property of sensing signals from all sources around every microelectrode can become unfavorable when trying to understand communication and signal propagation in neuronal circuits. In a neuronal network, several neurons can be simultaneously activated and can generate overlapping action potentials, making it difficult to discriminate and track signal propagation. Considering this limitation, we have established an *in vitro* setup focused on assessing electrophysiological communication, which is able to isolate and amplify axonal signals with high spatial and temporal resolution. By interfacing microfluidic devices and MEAs, we are able to compartmentalize neuronal cultures with a well-controlled alignment of the axons and microelectrodes. This setup allows recordings of spike propagation with a high signal-to-noise ratio over the course of several weeks. Combined with specialized data analysis algorithms, it provides detailed quantification of several communication related properties such as propagation velocity, conduction failure, firing rate, anterograde spikes, and coding mechanisms.

This protocol demonstrates how to create a compartmentalized neuronal culture setup over substrate-integrated MEAs, how to culture neurons in this setup, and how to successfully record, analyze and interpret the results from such experiments. Here, we show how the established setup simplifies the understanding of neuronal communication and axonal signal propagation. These platforms pave the way for new *in vitro* models with engineered and controllable neuronal network topographies. They can be used in the context of homogeneous neuronal cultures, or with co-culture configurations where, for example, communication between sensory neurons and other cell types is monitored and assessed. This setup provides very interesting conditions to study, for example, neurodevelopment, neuronal circuits, information coding, neurodegeneration and neuroregeneration approaches.

Video Link

The video component of this article can be found at https://www.jove.com/video/58878/

Introduction

Understanding electrical communication in neuronal circuits is a fundamental step to reveal normal function, and devise therapeutic strategies to address dysfunction. Neurons integrate, compute and relay action potentials (APs) which propagate along their thin axons. Traditional electrophysiological techniques (*e.g.,* patch clamp) are powerful techniques to study neuronal activity but are often limited to the larger cellular structures, such as the soma or the dendrites. Imaging techniques offer an alternative to study axonal signals with high spatial resolution, but they are technically difficult to perform and do not allow long-term measurements[1]. In this context, the combination of microelectrode arrays (MEAs) and microfluidics can make a powerful contribution in disclosing the fundamental properties of neuron´s activity and signal transmission within neuronal networks *in vitro*[2,3].

MEA technology relies on extracellular recordings of neuronal cultures. The main advantages of this electrophysiological methodology are its ability to support long-term, simultaneous stimulation and recording at multiple sites and in a non-invasive way[3]. MEAs are made of biocompatible, high conductive and corrosion resistant microelectrodes embedded in a glass wafer substrate. They are compatible with conventional cell culture bio-coatings, which by promoting cell adhesion significantly increase the sealing resistance between the substrate and cells[3,4]. Moreover, they are versatile in design and may vary in microelectrodes size, geometry and density. Overall, MEAs work as conventional cell culture vessels with the advantage of allowing concurrent live-imaging and electrophysiological recordings/stimulation.

The use of MEA technology has contributed to the study of important features of neural networks[5]. However, there are inherent features that limit the performance of MEAs for studying communication and APs propagation in a neuronal circuit. MEAs enable recordings from single cells

and even subcellular structures like axons, but when compared to somal signals, axonal signals have a very low signal-to-noise ratio (SNR)[6]. Moreover, the characteristic of sensing extracellular field potentials from all sources around every microelectrode hampers the tracking of signal propagation in a neuronal circuit.

Recent studies have demonstrated, however, that better recording conditions can be achieved by having the microelectrodes aligned within narrow microchannels into which axons can grow. This configuration provides a significant increase in the SNR such that propagating axonal signals can be easily detected[7,8,9,10,11,12,13]. The strategy of allying microfluidic devices with MEA technology creates an electrically isolated microenvironment suitable to amplify axonal signals[11]. Moreover, the presence of multiple sensing microelectrodes along a microgroove is fundamental for detection and characterization of axonal signal propagation.

Such *in vitro* platforms with highly controllable neuronal network topographies can be adapted to many research questions[14]. These platforms are suitable to be used in the context of neuronal cultures but can be expanded to engineer co-culture configurations, where the communication between neurons and other cell types can be monitored and assessed. This setup thus provides very interesting conditions to explore a number of neural-related studies such as neurodevelopment, neuronal circuits, information coding, neurodegeneration and neuroregeneration. Furthermore, its combination with emerging models of human induced pluripotent stem cells[15,16] can open new avenues in the development of potential therapies for human diseases that affect the nervous system.

Our lab is using this platform combining microelectrodes with microfluidics (μEF) to understand neuronal processes at the cellular and network level and their implication in the physio- and pathologic nervous system. Given the value of such platform in the field of neuroscience, the purpose of this protocol is to demonstrate how to create a compartmentalized neuronal culture over substrate-integrated MEAs, how to culture neurons in this platform and how to successfully record, analyze and interpret the results from such experiments. This protocol will certainly enrich the experimental toolbox for neuronal cultures in the study of neural communication.

Protocol

All procedures involving animals were performed according to the European Union (EU) Directive 2010/63/EU (transposed to Portuguese legislation by *Decreto-Lei* 113/2013). The experimental protocol (0421/000/000/2017) was approved by the ethics committee of both the Portuguese Official Authority on animal welfare and experimentation (Direção-Geral de Alimentação e Veterinária - DGAV) and of the host Institution.

1. Preparation of Culture Media and Other Solutions

NOTE: Freshly prepare the coating solutions on the day of its use. The unused coating solutions and culture media may be stored at -20 °C or 4 °C as detailed below.

1. Prepare 10 mL of a 0.05% (v/v) Poly(ethylene imine) (PEI) working solution.
    1. Prepare 20 mL of 0.25% (w/v) PEI stock solution by dissolving purified PEI (see **Table of Materials**) in sterile water.
    2. Dilute 2 mL of 0.25% (w/v) PEI stock solution in 8 mL of sterile distilled water. Mix well and use. Unused solution can be stored at 4 °C.

2. Prepare 1 mL of a 5 μg/mL laminin solution.
    1. Dilute 5 μL of 1 mg/mL laminin stock solution in 995 μL of basal medium (see **Table of Materials**). Mix well and use. Unused solution can be stored frozen at -20°C for up to 1-2 weeks.

3. Prepare 1 L of HEPES-buffered Hank's Balanced Salt Solution (H-HBSS).
    1. Prepare 1 M HEPES solution by dissolving 11.9 g of HEPES powder in 50 mL of ultrapure water. Adjust pH to 7.2.
    2. Prepare H-HBSS solution (without calcium and magnesium) by dissolving 5.33 mM potassium chloride, 0.44 mM potassium phosphate monobasic, 138 mM sodium chloride, 0.3 mM sodium phosphate dibasic and 5.6 mM glucose in 800 mL of ultrapure water.
    3. Add 15 mL of 1 M HEPES solution (final concentration of 15 mM).
    4. Adjust pH to 0.2-0.3 units below the desired pH 7.4 using 1 N HCl or 1 N NaOH. Add ultrapure water to make up the final solution volume.
    NOTE pH tends to rise during filtration.
    5. Sterilize by filtration using a 0.22 μm porosity poly(ether sulfone) (PES) membrane.

4. Prepare 10 mL of H-HBSS containing 10% heat-inactivated Fetal Bovine Serum (hiFBS).
    1. Dilute 1 mL of hiFBS in 9 mL of H-HBSS. Mix well and use. Unused solution can be stored at 4 °C for 3-4 weeks.

5. Prepare 5 mL of 1.5 mg/mL trypsin solution.
    1. Immediately before use, dissolve 7.5 mg of trypsin in 5 mL of H-HBSS. Sterilize by filtration using a 0.22 μm porosity PES membrane.

6. Prepare 50 mL of supplemented medium.
    1. In a 50 mL conical tube, mix 48.5 mL of basal medium (see **Table of Materials**), 2% (v/v) B-27 supplement, 0.5 mM L-glutamine and 1% Pen/Strep (10,000 U/mL penicillin and 10,000 μg/mL streptomycin). Supplemented medium can be stored at 4 °C for 3-4 weeks.

2. Microfluidic and MEA Devices Preparation

NOTE: Perform steps 2.1-2.11 on the day before cell seeding.

1. Clean microfluidic devices to remove fabrication and other debris using vinyl tape. Gently press the tape against the device to reach all areas of the device.
2. Air plasma-clean the MEA for 3 min (0.3 mbar) to clean the surface and make it more hydrophilic.
3. Briefly submerge microfluidic devices in 70% ethanol and allow them to air-dry inside a laminar flow hood.
4. Place each MEA in a sterile 90 mm Petri dish and sterilize them by 15 min of ultraviolet (UV) light exposure.
5. Coat the MEA central surface area by incubating with 500 µL of 0.05% (v/v) PEI solution for at least 1 h at 37 °C.
   NOTE: As described by others[17], PEI coating of MEAs results in less cell clustering than using poly(lysine) coating.
6. Aspirate the PEI coating solution from the MEA surface and wash MEAs 4x with 1 mL of sterile distilled water. Be careful to not touch the MEA surface during washing steps as this can damage the electrodes.
7. Guided by a stereomicroscope inside a laminar flow hood, center the MEA chip and add 1 mL of sterile water to the central area of MEA.
8. Place the microfluidic device on top of the MEA surface.
9. Carefully align the microgrooves of microfluidic device with the microelectrode grid of the MEA.
10. When correctly aligned, carefully aspirate the excess water without touching the MEA or the microfluidic device.
11. Incubate the µEFs overnight at 37 °C to dry and allow complete attachment. To facilitate the attachment, gently press the microfluidic device against the MEA.
    NOTE: Perform step 2.12 on the day of cell seeding.
12. Once the µEFs have completely dried, load the microfluidic wells with 150 µL of 5 µg/mL laminin coating solution and incubate at 37 °C for at least 2 h before cell seeding.
    NOTE: When loading µEF with laminin solution, ensure that the solution fills the microgrooves. use a vacuum to remove any air bubble that might be present within the microgrooves.

3. Prefrontal Cortex Dissection

1. Prepare the dissection area for embryo removal.
   1. Disinfect the working surface and the surgical instruments with 70% ethanol.
      NOTE: Though this dissection is not a sterile procedure, disinfection helps to reduce chances of contamination.
   2. Use a clean disposable diaper to confine the dissection area and lay out the surgical instruments for embryo removal.
   3. Prepare 4 90 mm Petri dishes filled with cold H-HBSS and place them on a tray with ice near the dissection area.
2. Euthanize an E-18 time-pregnant Wistar rat by carbon dioxide (CO_2) inhalation.
3. Upon completion of the procedure, remove the animal from the CO_2 chamber and confirm death by a secondary method of euthanasia, such as exsanguination.
4. Place the animal ventral side up on the disposable diaper, spray the lower abdomen with 70% ethanol and, with the help of forceps and scissor, make a U-shape cut through the skin to expose the uterus.
5. Carefully remove the embryos from the uterus by cutting away any connective tissue and place them in one of the Petri dishes with cold H-HBSS.
6. Remove each embryo from its embryonic sac and, with the help of forceps and scissor, decapitate the embryo.
7. Transfer the embryo´s head to a second Petri dish filled with cold H-HBSS.
8. Repeat steps 3.6 - 3.7 for each embryo.
9. Dissect the prefrontal cortex.
   1. Transfer an embryo´s head to a third Petri dish.
   2. Use a pair of forceps to expose the brain.
   3. Remove the brain from its cavity and cover it with cold H-HBSS.
   4. If present, remove and discard the olfactory bulbs. Dissect the prefrontal cortex and remove meninges.
   5. Transfer the prefrontal cortex fragments to a fourth Petri dish filled with cold H-HBSS.
   6. Repeat the steps 3.9.1 - 3.9.5 for each embryo.

4. Cortical Neurons Dissociation and Culture on µEF

NOTE: The following procedures were performed inside a laminar flow hood after a 15 min cycle of UV sterilization. Working surface area as well as all the materials placed inside the hood should be previously disinfected with 70% ethanol.

1. As described in step 1.5, dissolve the previously weighted trypsin in 5 mL of H-HBSS and filter the solution.
2. Collect the cortex pieces previously dissected in a total of 5 mL of H-HBSS. Transfer them to a sterile 15 mL conical tube.
3. Add 2.3 mL of freshly prepared 1.5 mg/mL trypsin solution to the tube containing the cortex fragments.
4. Mix the suspension and incubate at 37 °C for 15 min (briefly agitate every 5 min).
5. Stop tissue digestion and wash out trypsin.
   1. Discard the supernatant containing the trypsin. Add 5 mL of H-HBSS containing 10% hiFBS to inactivate the remaining trypsin; gently agitate.
   2. Let cortex fragments settle down and then discard the supernatant.
   3. Add 5 mL of H-HBSS to remove the hiFBS residues. Let cortex fragments settle down and discard the supernatant.
   4. Wash again the cortex fragments with 5 mL of H-HBSS.
   5. Let cortex fragments settle down and discard the supernatant. Add 5 mL of supplemented neuronal medium.
6. Mechanically dissociate cortex fragments with a 5 mL serologic pipette by slowly pipetting up and down for 10-15 times. If necessary, repeat the procedure using a small caliber pipette until the cell suspension become homogenous.
7. Discard the remaining undissociated tissues by filtering the cell suspension trough a 40 µm cell strainer.
8. Count viable cells and determine the cell density.

1. In a microtube, add 20 µL of 0.4% (w/v) Trypan Blue to a 20 µL cell suspension. Mix well to homogenate the solution and transfer 10 µL to a Neubauer counting chamber.
2. Count viable cells and determine the cell density of viable cells.

9. Adjust cell density to 3.6 x 10^7 cells/mL (if needed, centrifuge cell suspension).
10. Immediately before cell seeding, remove laminin coating from all wells of the µEF. Pipette 50 µL of supplemented medium to each well of the µEF axonal compartment.
11. Seed 5 µL of cell suspension in the µEF somal compartment. Incubate at 37 °C for 1 h, to allow cells attachment to the substrate.
12. Gently fill each well of µEF with warmed supplemented medium. Transfer the µEF to a humidifier incubator at 37 °C supplied with 5% CO_2.
    NOTE: To avoid quick culture medium evaporation, place an opened microtube filled with water inside the Petri dish carrying the µEF.
13. Maintain cultures for the required period by replacing 50% of culture medium every second to third day of culture.
    NOTE: After the experiments, the microfluidics can be detached from MEAs by immersing the µEF in water overnight. If needed, gently peel the microfluidics with the help of forceps. MEAs can be cleaned by overnight incubation with a 1% (v/v) commercial enzymatic detergent (see **Table of Materials**).

5. Recording Spontaneous Neuronal Activity

NOTE: Embryonic cortical neuron cultures on MEAs typically exhibit spontaneous activity as soon as 7 days in vitro (DIV), but take 2-3 weeks (14-21 DIV) to mature and exhibit stable activity. It is up to the experimenter to decide when to start the electrophysiological measurements based on the objectives of the study. In this protocol, the recording of neuronal activity from µEF is demonstrated by using a commercial recording system (see **Table of Materials** for hardware details), with a heating module incorporated. For performing recordings, we used a freely available software (see **Table of Materials** for software details).

1. Turn on the MEA recording system and the temperature controller and allow the heated base plate of the preamplifier to reach 37 °C.
   NOTE: For long-term recordings (>30 min at a time) one should also have a system that maintains a CO_2 atmosphere.
2. Place the µEF on the preamplifier (headstage).
   NOTE: Ensure that the MEA chip is correctly aligned with the reference number in the upper left edge. The MEA chip we use is rotationally symmetrical, but this alignment matches the correct orientation of the pin layout of the electrodes and the hardware IDs.
3. Close and latch the preamplifier lid.
4. To record, open the recording software and define the recording parameters and the path of the recorded data streams (see the software manual for details on how to set-up a recording scheme).
   NOTE: An acquisition with a sampling rate of 20 kHz is generally enough for most applications. A higher sampling frequency is advisable if more precision is required in spike timings. If one intends only to record spikes, set a high-pass filter at 300 Hz, which will attenuate the lower frequency components of the signal. If raw and filtered data are recorded simultaneously, this will significantly increase the data file size. It is always possible to do offline filtering of the raw data. To reduce data file size one can also limit microelectrodes from which to record (e.g., only microelectrodes within microgrooves).
5. Click **Start DAQ** to start data acquisition. Check if the display shows running traces of activity and start recording.
   NOTE: The noise level is usually slightly higher in the microelectrodes corresponding to the microgrooves. If the noise level is too high (peak-to-peak amplitude >50 µV) or unsteady in several microelectrodes, it could be due to dirt hindering a good pin-pad contact. This problem is usually solved by gently cleaning the MEA contact pads and the preamplifier pins using a cotton swab moistened with 70% ethanol.
6. Open the recorded file in the analysis software (see **Table of Materials**) to quickly explore the data.

6. Data Analysis

NOTE: The following steps show how to use the µSpikeHunter software, a computational tool developed at Aguiar's lab (freely available upon email request to pauloaguiar@ineb.up.pt), to analyze data recorded with µEF. A graphical user interface (**Figure 2**) is used to load the data, identify propagating spike waves, determine their direction (anterograde or retrograde) and estimate propagation velocities. µSpikeHunter is compatible with HDF5 files generated from recordings obtained using MEA2100-family systems, which can be used in conjunction with 60-, 120-, and 256-electrode MEAs. The recordings obtained using the multi-channel experimenter can be easily converted to HDF5 files using freely available software (see **Table of Materials**).

1. Click **Browse** to select the recording file. Then, click the File Info button to list the sampling rate, the recording duration as well as a list of the recorded data streams (*e.g.,* raw data, filtered data).
   NOTE: For true propagation velocity estimations, it is recommended to select the raw data stream for analysis.
2. Select microelectrodes (single row or column associated with microgroove) for analysis and set the spike detection threshold value (positive or negative phase) at 6x the standard deviation (SD) of the signal noise. Click **Read Data** to apply the event detector and obtain the identified propagation sequences.
   NOTE: If the criteria are met, a list of detected propagation sequences will populate the analysis panel. The user can then view and interact with several analysis tools for the propagation sequence, including voltage traces, inter-microelectrode cross-correlations, single-sequence propagation velocities, a kymograph, and an audio playback tool. Although the propagation velocity estimation can be obtained for any pair of microelectrodes or as the average of the estimates for all pairs, this estimation is more reliable if the farthest pair is selected.
3. Repeat the previous step for the microelectrode sets of interest. Each time click **Save All Events** to save the time and amplitude of the spikes (on each of the microelectrodes) for all identified propagation sequences to a CSV file.
4. Import the CSV files to data analysis environments for further analysis of the patterns of activity of the culture (*e.g.,* firing rate, inter-spike intervals).

Representative Results

Using the protocol described here, E-18 rat cortical neurons seeded on µEF are able to develop and remain healthy in these culture conditions for over a month. As soon as 3 to 5 days in culture, cortical neurons grow their axons through microgrooves towards the axonal compartment of µEF (**Figure 1**). After 15 days in culture, cortical neurons cultured on µEF are expected to exhibit steady levels of activity and propagation of action potentials along the microgrooves is expected. Older cultures (>14 DIV) tend to be dominated by bursting activity as in conventional MEA recordings[18,19].

Recordings and data analysis

Raw data analysis with µSpikeHunter (**Figure 2**) permitted the detection and extraction of propagating spike waves along sets of 4 microelectrodes (within microgrooves). **Figure 3** displays one of these events. µSpikeHunter allowed for the estimation of propagation velocities, based on the cross-correlations between the voltage waveforms of selected pairs of microelectrodes (providing a time delay) and the associated known inter-electrode distance.

The extracted data was further analyzed using custom designed code in MATLAB. A representative raster plot of the propagating spike waves along 11 (out of 16) microgrooves is shown in **Figure 4a**. The instantaneous firing rate for a high- and a low-activity microgroove is shown in **Figure 4b**.

µEF recordings exhibit varying levels of activity per microelectrode. Frequently, several microelectrodes in the somal compartment are "silent". However, most microelectrodes within microgrooves tend to be active (**Figure 5a**). It is well described that the microgrooves function as signal amplifiers[8]. The amplitude of a recorded signal depends not only on the size of the source currents, but also on the resistivity of the surrounding media. The resistance along a microgroove is particularly high, which greatly increases the amplitude of the measured signals in comparison to those of the microelectrodes in the somal compartment (**Figure 5b**).

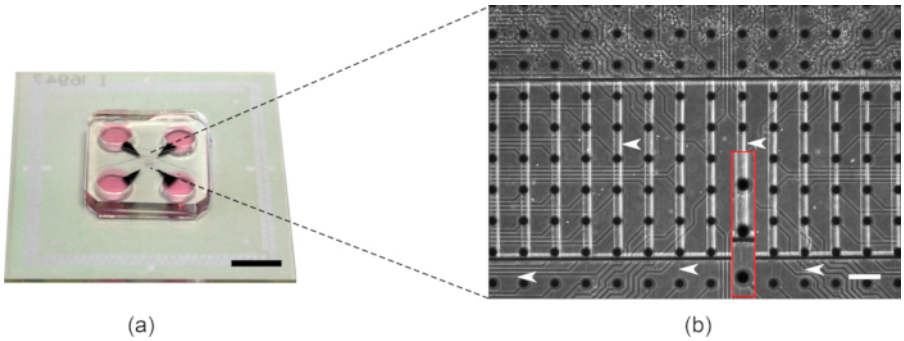

Figure 1. Embryonic rat cortical neurons cultured on µEF. (a) Photograph of µEF. Scale bar: 1 cm. **(b)** Representative image of cortical neurons cultured in the µEF for 5 days, showing several axons crossing the microgrooves and reaching the axonal compartment (arrows). Scale bar: 100 µm. Please click here to view a larger version of this figure.

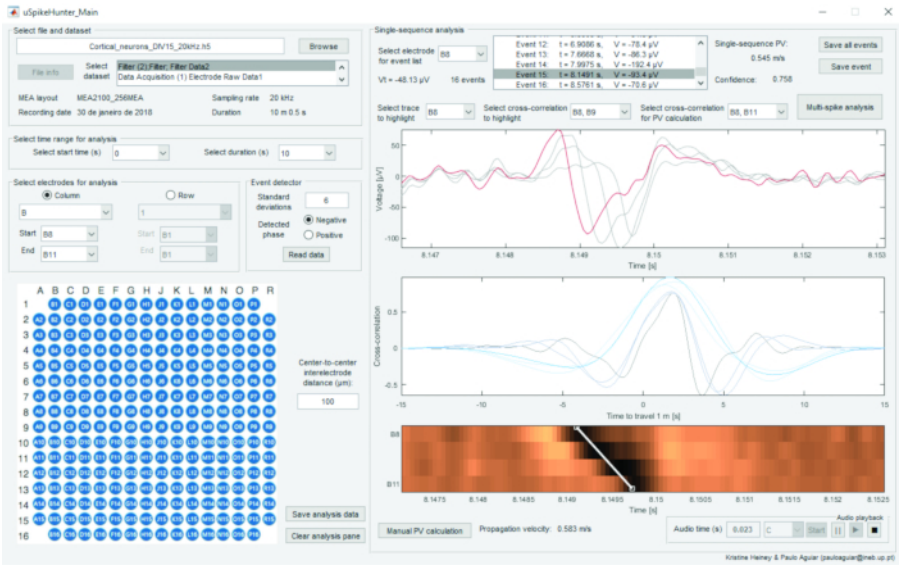

Figure 2. Screen capture of the µSpikeHunter main graphical user interface. The user can load the data recorded with µEF, identify propagating spike waves, determine their direction (anterograde or retrograde) and estimate propagation velocities. A kymograph tool allows the user to manually estimate the propagation velocity based on a line drawn on the kymograph. Please click here to view a larger version of this figure.

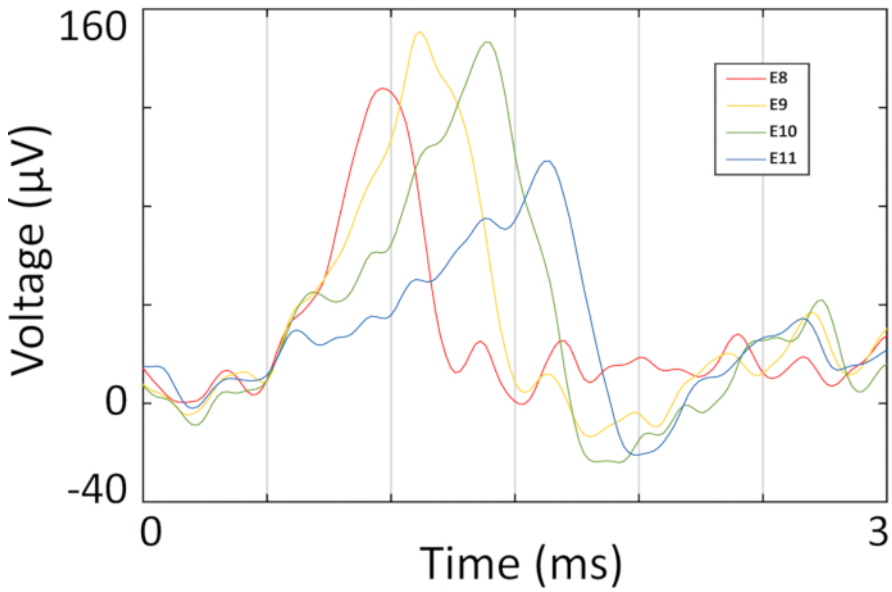

Figure 3. Anterograde propagating spike wave sensed by 4 microelectrodes (E8-E11) within a microgroove. Each trace represents one microelectrode raw recording for 3 ms. After analysis with µSpikeHunter, the cross-correlation between the farthest microelectrodes (E8 and E11) permitted the calculation of a propagation velocity of 0.52 m/s. Please click here to view a larger version of this figure.

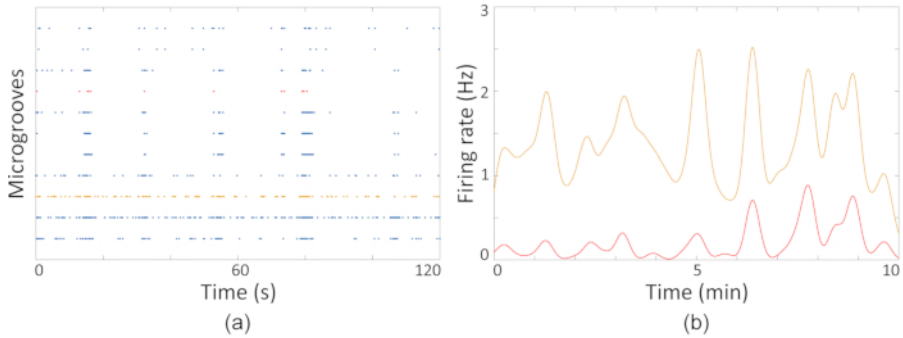

Figure 4. Information barrage through the microgrooves.(a) Representative raster plot of 2 minutes of spontaneous activity recorded along 11 microgrooves. Each dot represents a propagating spike wave (unit) sensed by 4 microelectrodes and identified through the analysis with µSpikeHunter. A high- and a low-activity microgroove are highlighted in yellow and red, respectively. **(b)** Evolution of the instantaneous firing rate (as in rate-coding) for the two highlighted microgrooves along 10 minutes. Only propagating units were considered for the calculation of the firing rate. Note the activity synchronization, despite the different firing rate levels. The instantaneous firing rate was calculated convolving the spike events with a Gaussian kernel with a standard deviation of 100 ms. Please click here to view a larger version of this figure.

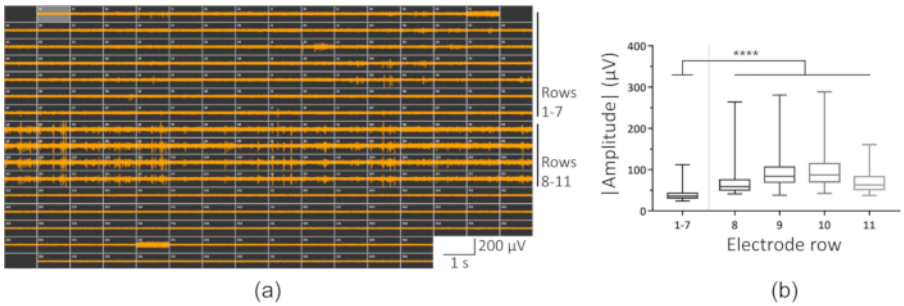

Figure 5. Quality of extracellular recordings in µEF. (a) Time window (1 second) of a µEF recording (rat cortical neurons at 15 days *in vitro*) with a sampling rate of 20 kHz and a high-pass filter at 300 Hz. Electrode rows 1-7 correspond to the somal compartment and rows 8-11 to the microgrooves. (**b)** Box and whisker plot of the full range of spike amplitudes extracted (spikes detected using a threshold method, with negative phase only, set at 6x standard deviation) from the specified rows (total recording time of 10 minutes). The spikes' amplitudes are significantly larger in the microelectrodes within microgrooves (rows 8-11; 16 microgrooves), when compared to the microelectrodes of the somal compartment (rows 1-7; 16 active microelectrodes). One-way ANOVA, Dunn's multiple comparison tests, ****p <0.0001. Please click here to view a larger version of this figure.

Discussion

The protocol presented here shows how to assemble a µEF, comprised of a microfluidic device and a MEA with standard commercially-available designs, and how to analyze the recorded data.

When designing an experiment, researchers must take into account that the *in vitro* model is limited by the MEA fixed grid, which constrains microgroove arrangements. The use of a particular microfluidic or MEA design will depend on the specific experimental needs but, in general, the same procedure steps should apply to different µEF configurations.

A critical decision to be made before µEF assembly is whether the user intends to reuse the assembled µEF in future experiments. Treatment with oxygen plasma on both surfaces can be done to covalently bond microfluidic devices to MEAs[20]. However, this sealing often makes the MEA chips unusable for further experiments, as detaching the microfluidic device irreversibly damages the passivation layer. To circumvent this problem, research groups tend to reuse the mounted µEF, despite the possible drawbacks (*e.g.,* debris from previous experiments). By carefully following the steps outlined in this protocol, from experiment to experiment, one can safely attach and detach microfluidic devices without damaging the MEA.

The use of µEF allows the isolation of a set of axons within the microgrooves. The time required for a reasonable number of axons to cross the microgrooves will greatly depend on the cell seeding procedure, namely seeded cell density. Using the density specified in this protocol, neurons use to cross the whole microgroove within 3 to 5 DIV.

Depending on the culture environment (*i.e.,* incubator and humidity level), it may be required to replace media every 2 to 3 days of culture. When renewing media, remove the media from µEF wells while maintaining media within the main channels. Then, gently add media to the µEF wells allowing it to slowly flow through the main channels before filling up the wells with final media volume. Following these recommendations, we are able to maintain these cultures in healthy conditions for at least a month.

A key advantage of this platform is the ability to isolate, measure and track axonal signals. Although the µEF recording is simple, the great amount of data that can be generated is cumbersome to handle and requires good knowledge of data analysis. The analysis software used here, µSpikeHunter[21], is an advanced yet intuitive computational tool, which allows for the detailed quantification of several related measures (*e.g.,* propagating events, propagation velocity *etc.*) in a few steps. Although data analysis is not the focus of this protocol, the information here provided already allows the extraction of meaningful data from a µEF recording. However, it is important to note that the reliable isolation of a single axon per microgroove remains impracticable. Thus, very complex spike waveforms (due to multiple axons passing through the microgroove) may arise and affect the spike timings and propagation velocity calculations. This limitation can be attenuated by using very narrow microgrooves (below 5 µm)[13] and/or through spike sorting techniques[21], which help distinguish different signal sources.

Even though *in vitro* cultures cannot recapitulate the full *in vivo* complexity, their combination with µEF enables well-controlled bottom-up research approaches. We hope this protocol will help both beginners and proficient MEA users establishing new and reliable models for studying electrophysiological communication in neuronal circuits.

Disclosures

The authors have nothing to disclose.

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