# A 3D ENGINEERED PLATFORM FOR FUNCTIONAL MONITORING OF IN VITRO BRAIN MODELS

*Ali Maziz[1], Eduardo Martinez Maria[1], Venkata Suresh Vajrala[1], Asma Eddarir[1], Sophie Pautot[2] and Christian Bergaud[1]*

[1]Laboratory for Analysis and Architecture of Systems (LAAS), CNRS, Toulouse, FRANCE and [2]SYNAXYS neuro engineering systems, Toulouse, FRANCE

## ABSTRACT

This paper reports a three-dimensional (3D) platform for functional monitoring of 3D engineered brain-tissue *in vitro*, which is currently limited by traditional 2D MicroElectrodes Arrays (MEAs) devices. The miniaturized device, based on an integrated flexible probe (z axis) coupled to a MEA glass chip (x and y axis), can capture 3D network electrophysiology of an *in vitro* neural cell assembly. We present recordings of spontaneous electrical activity across the volume of a controlled 3D, millimeter-sized neuronal network for up to four months. Our concept enables a new class of functional mapping arrays for *in vitro* models of three-dimensional electrically active tissue.

## KEYWORDS

3D microelectrodes array, cell culture, brain models *in vitro*, flexible probes, electrophysiology.

## INTRODUCTION

There has been a great deal of interest in developing 3D *in vitro* cell models that accurately replicate the *in vivo* architecture, functionality, and genetic signature of native tissues, which is limited in traditional 2D cell-culture models. 3D brain cell-cultures are examples of these emergent models, in which neural tissues generated from human or rodent primary tissue and/or patient-derived induced pluripotent stem cells (iPSC), have enormous potential for investigating aspects of human brain development, mental and neurological disorders e.g. epilepsy, autism, depression, addiction, etc. [1]. A number of studies in recent years have endeavored to develop 3D *in vitro* models that more accurately represent the function and physiology of the *in vivo* brain, by increasing the complexity of cellular composition and extracellular milieu, bridging the gap between conventional animal research and human clinical studies [2].

Different methodological approaches are employed to characterize 3D brain models *in vitro* using imaging techniques such as a confocal laser scanning system [1] or by monitoring extracellular responses of synaptic connections with a MicroElectrodeArray (MEA) in 2D [3]. Although those electrophysiological recordings through 2D MEAs are a common method of evaluating neuronal function of 3D structures, the main limitation of this approach is that only surface measurements are possible and 3D data are not accessible, leading to inaccurate measurements. A need therefore exists for new technological recordings tools capable of measuring electrophysiological activity from many locations simultaneously throughout a 3D networks of neurons *in vitro*.

Here, we describe a new integrated 3D platform for functional monitoring of 3D engineered brain tissue. This platform consists of a planar MEA coupled with a flexible probe that can capture network-depth electrophysiology of neurons in a 3D *in vitro* matrix (Fig.2). To demonstrate the utility of the 3D device, we monitored the electrophysiological activity of a 3D neuronal cell culture.[4] The 3D cell assembly consisted of hippocampal neurons, dissociated from E18 Sprague Dawley, entrapped in multi-layered silica beads, and seeded through the 3D MEA device, enabling the growth of neural cells in the 3D matrix and the formation and maturation of neural network through the MEA. Detailed device fabrication, 3D culture analysis, as well as electrophysiological recordings from the 3D device, will be discussed.

## ESPERIMENTAL

### 2D MEA fabrication

The design and fabrication process of the MEA is illustrated in Fig. 1A.[5] The planar chip consists of 60 gold microelectrodes (d=20µm) with a 300 µm center-to-center interelectrode distance.

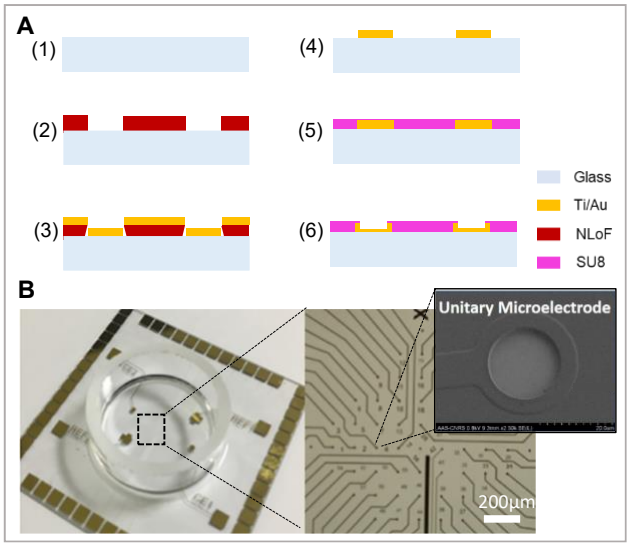


*Figure 1. Planar MEA microfabrication. A) Technological fabrication steps from the starting 4-inch glass wafer to the final SU8-coated wafer. B) Left: picture of a final MEA chip after glass well gluing. Right: optical picture of the microelectrodes array and SEM picture of a single gold microelectrode (20 µm diameter).*

The chips are fabricated using standard microfabrication methods on a 4-inch glass wafer through

the following technological steps (Fig.1A): *1*) wafer cleaning by MW-oxygen plasma (800 W, 5 min, 1000 sccm $O_2$), *2*) spin-coating and photopatterning of a negative photoresist layer (NLoF, 5 µm) for lift-off, *3*) PVD-metallization (Ti/Au, 50 nm/200 nm) and lift-off at room temperature in acetone, *4*) wafer cleaning by piranha cleaning followed by MW-oxygen plasma (200 W, 400 sccm $O_2$, 2 min), *5*) spin-coating and *6*) photopatterning of a 1.5 µm-thick SU8 layer for passivation and openings of the MEA. Finally, a glass well (30 mm inner diameter, 32 mm outer diameter, 10 mm height) was glued onto the MEA to complete it, using PDMS cured at 60 °C for 3 h (Fig.1B).

**Parylene-based flexible probe**

The fabrication process of the flexible parylene C-based probe, with four microdisk microelectrodes (d=40µm) is schematized in Fig. 2A.[6] A 23 µm thick film of Parylene C was deposited using chemical vapor deposition on a 4-inch silicon wafer. Next 50 nm thick Ti and 200 nm thick Au layers were deposited and micropatterned, using a conventional PVD technique followed by a lift-off with AZ-nLof 2035. Subsequently, 1.3 µm thick parylene C was deposited as a passivation layer. Next, the electrode surfaces and the corresponding connection pads were realized by photo-patterning with a 5 µm thick AZ4562 photoresist, followed by the etching of the thin parylene C passivation layer using $O_2$ plasma reactive ion etching. Later, the probes were anistropically etched to establish smooth outlines and vertical sidewalls, using 50 µm thick BPN photoresist and a deep reactive ion etching step. Later, the released implants were stripped off from the leftover photoresist using TechniStrip NF52, thoroughly washed in DI water and stored in a dry place. Finally, the devices were bonded to a customized flexible ribbon cable with golden traces by using epoxy silver and photosensitive glue.[7] All gold microelectrodes were coated with the conducting polymer poly(3,4-ethylenedioxythio-phene):poly(styrene-sulfonate) (PEDOT:PSS) to lower the impedance and obtain a better signal-to-noise ratio for during neural neuron's recordings.[8]

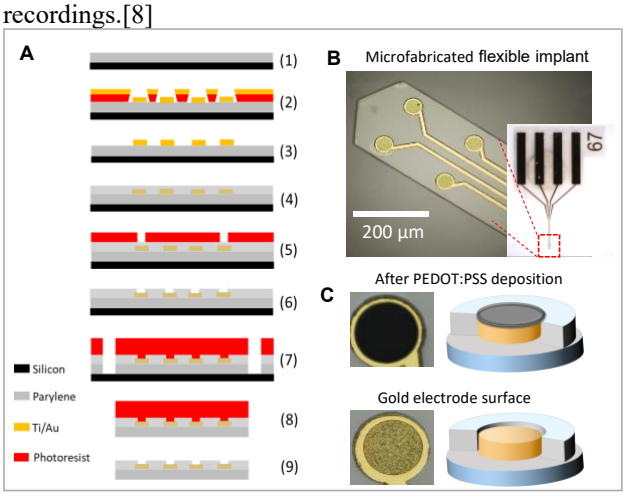


*Figure 2. Parylene-based flexible probe microfabrication. A) Schematic overview of technological steps involved in the microfabrication procedure of the flexible probe with four microdisk microelectrodes (d=40 µm). B) Optical images the parylene-based flexible probe with four microelectrodes (d=40µm).C) Schematic representation and optical images of the PEDOT:PSS-coated gold microelectrodes.*

**Fabrication of cell culture chamber and packaging**

Cell culture chamber and 3D MEA holder (lid and arm) were designed using AutoCAD and printed using a 3D printer 29J + HR (DWS, Italy). The cell culture chamber (d 2.6 cm and h 1 cm) was mounted upon a glass coverslip using PDMS as an adhesive. The holder lid was printed using acrylate resin DS3000 (DWS, Italy) and 405 nm laser at a writing speed of 5.8 m/s. It was designed to hold the flexible probe and align vertically to the 2D glass MEA chip, as shown in Fig.3 and B.

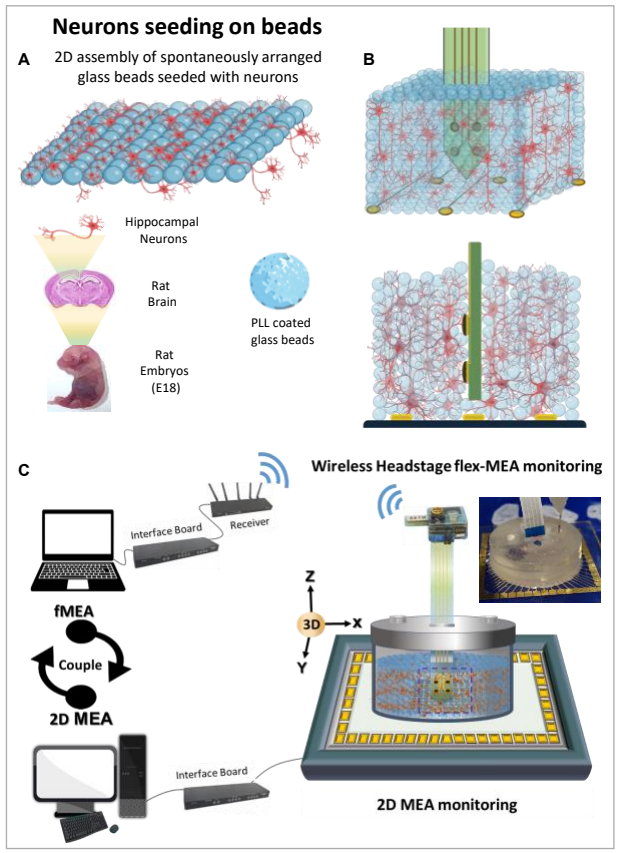


*Figure 3. A) Hippocampal neurons dissociated from E18 Sprague Dawley rat were first entrapped in a multilayered silica beads (d=45 µm). B) The Parylene type microelectrode arrays were assembled vertically (z-direction) within the planar 2D MEA chip (x and y directions) and the cell culture chamber, composed of silica beads seeded with rat embryonic hippocampal neurons. The schematics describe how the microelectrodes interact with the cells within the 3D culture, from a closeup and cross-sectional viewpoints C) The parylene-based probe is connected to a wireless W200-HS4 Multi Channel Systems recorder while the planar MEA is connected to a MEA2100 Multi Channel Systems. Electrophysiological recordings were obtained simultaneously from 4 microelectrodes (z direction) and 60 microelectrodes (x and y directions).*

**3D neuronal culture**

Borosilicate glass spheres (45 µm d, MO-SCI

Specialty Products) and all components of the 3D MEA setups were sterilized overnight in ethanol, followed by an overnight incubation step with poly-lysine solution (PLL). Meanwhile, the hippocampus was removed from rat embryonic day 18 embryos (E18), treated with trypsin and then triturate into a cell suspension (Fig. 3A). The cells were seeded at 100000 cell/ $cm^2$ on PLL-coated glass beads and later they were seeded onto the 3D MEA, enabling the growth of suspended neural cells in the matrix and the formation and maturation of a neural network around the 3D MEA (Fig. 3B).[4, 9] Cells were cultured in neurobasal medium (2 mM L-glutamine, 50 mg/mL Penstrep and 2% B-27) for at 37 ◦C and a third of the total volume was replaced three times a week. The network maturation starts from the 3rd week and the samples were kept in the incubator, following 4 months without further shaking, but with a regular medium exchange.

**Electrophysiological recordings**

A wireless W2100-HS4 electrophysiology system (Multichannel Systems, Reutlingen, Germany) was used to record electrophysiology activity from the flexible parylene-based microelectrodes (z direction). A MEA2100-60 electrophysiology system (Multichannel Systems, Reutlingen, Germany) was used to record electrophysiology activity from the plan 60-microelectrodes array (x and y directions) (Fig. 3C). Recordings sessions were carried out for 30 min at an acquisition frequency of 20 kHz and bandpass filtered between 200 Hz–3000 Hz. A software-supplied spike detector was used to detect spontaneous events that exceeded at least threshold of ±4 μV (an extracellular action potential spike was defined by a lower limit threshold, set at 4.5 × the standard deviation of baseline noise, for each microelectrode.

## RESULTS AND DISCUSSION

**Design and Fabrication of the 3D MEA**

Our 3D electrophysiology platform consists of a planar MEA coupled with a flexible probe that can capture network-depth neural activity in a 3D *in vitro* matrix (Fig.3). We integrated 60 recording gold microelectrodes (d=20 μm) evenly distributed throughout the 2D MEA (x and y directions) (Fig.1), and four parallel microelectrodes (d=40 μm) on the thin parylene shank (z direction) (Fig.2). The integration of the planar MEA, and the probe is designed to allow measurements of neural activities across a large volume within an engineered 3D neural network as well as measurements of the local modulations of the neural networks at a specific site. To maintain the 3D neural network structural fidelity with 3D MEA implanted, we aimed to minimize the probe's dimensions. The shank's length was 3 mm, the width 200 μm, and the thickness 20 μm. All gold microelectrodes were coated with the conducting polymer poly(3,4-ethylenedioxythiophene):poly(styrene-sulfonate) (PEDOT:PSS) to lower the impedance and obtain a better signal-to-noise ratio for during neural neuron's recordings.[8, 10]

**Electrophysiological activity in 3DMEA array**

To demonstrate the ability of the 3D MEA device to monitor the electrophysiological activities of a 3D neuronal biological system, spontaneous electrical recordings were performed on 3D *in vitro* hippocampal neuronal cultures, entrapped in multi-layered silica beads and seeded through the 3D platform (Fig. 3B). We experimentally evaluated the 3D MEA performance by recording extracellular action potentials (spikes). A comparison of single-unit waveforms captured by both 2D and 3D microelectrodes was carried out.

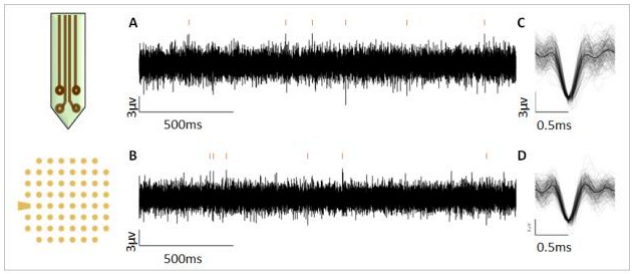


*Figure 4: Electrophysiology recordings from hippocampal neurons at DIV 18. (A) Example of at 300-3000 Hz filtered neuronal spiking data from the 2D MEA (A) and the 3D probe (B), demonstrating the detection of action potential (spikes). (C) and (D) waveform overlay of spikes detected from a the 2D MEA (C) and the 3D parylene-based microelectrodes (D) single microelectrode during a recording experiment.*

The activity of the neuronal network was successfully recorded in all reported experiments using both 2D and 3D microelectrodes. An overlay of spontaneous waveforms captured by both 2D and 3D microelectrodes is shown in Fig. 4. 2D and 3D microelectrodes presented a similar level of spike amplitudes (up to 8 μV), and durations were not significantly different between the two at DIV18.[9] As shown in the literature, they represent the action potentials propagating along axonal branches that are often neglected while measuring with bigger size electrodes [9, 11].

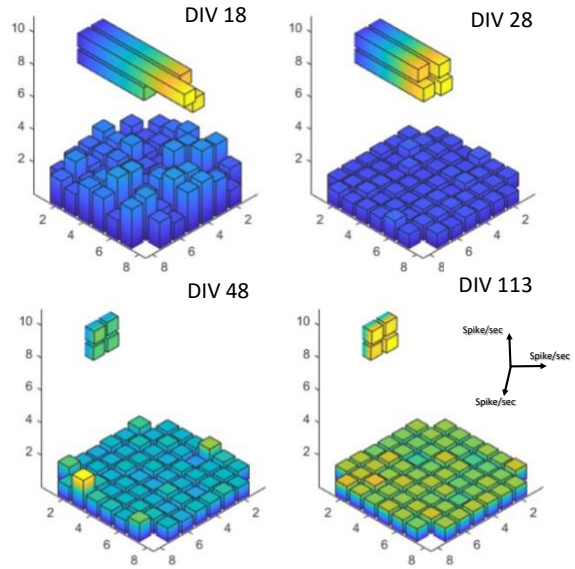


*Figure 5. Map of activities of the 2D MEA (60 microelectrodes) and the 3D probe (4 microelectrodes), shown in selected days of culture: DIV 18, 28, 48 and 113. 3D electrode is shown in horizontal bars and ED MEA is shown as vertical bars.*

**Long-term recordings**

We assessed the ability of the 3D platform to monitor the electrical activity of the 3D neural system on the long-term period (DIV113). The electrophysiological activity was distributed throughout the 3D MEA and we were able to be visually represented in 3D plots based on the location of the electrodes calculated from the probe and the 2D MEA array (Fig.5 and Fig. 6). The electrophysiological activity of the 3D network was analyzed and compared with the 2D recordings (Fig. 6A) From DIV 18 to 34, 3D microelectrodes presented a higher level of spiking rate (3.62 spike/sec) than 2D planar microelectrodes (1.24 spike/sec), while spike amplitudes and durations were not significantly different between the two. Beyond DIV 34 2D and 3D microelectrodes show similar level of spiking activity 5(xxx spike/sec). We observed neuronal activity up to 113 DIV before the culture was terminated for imaging (data not shown). An overlay of the full single unit mapping activity captured by planar 60-microelectrodes (x and y direction) and the four 3D microelectrodes (z direction) is shown in Fig. 6B. Electrophysiological activity was distributed throughout the 3D MEA and we were able to be visually represent the mapping activity of the full 3D neural volume based on the location of the microelectrodes calculated from the probe and the 2D MEA array (Fig.6B).

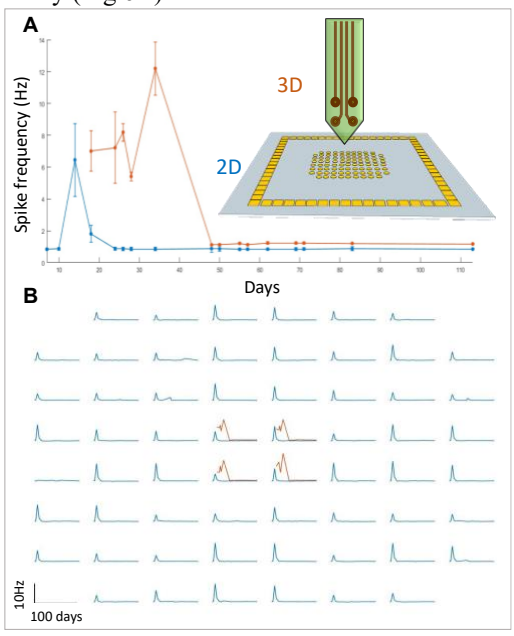


*Figure 6.: Mean activity on each day of culture during 113 days for the 2D MEA (blue) and the 3D probe (red) recordings. We see the peak activity in 3D is higher and later in the culture compared to the peak activity in 2D.*

## CONCLUSIONS

We developed a 3D device that provides a means to electrically monitor 3D-engineered brain-tissue cell cultures. This platform consists of a planar MEA coupled with a flexible probe that can capture network-depth electrophysiology of neurons in a 3D *in vitro* network. We have demonstrated and tested the capability to visualize spatial and temporal mapping of electrophysiological data across a 3D volume of an *in vitro* neural network. These visualizations offer a new technological tool to interrogate 3D-engineered cell models such as neurons, which are currently limited by traditional 2D devices. The 3D platform developed in this study represents one simple arrangement of a 3DMEA, with four electrodes in the z direction. In our future works, the 3D platform can be modified to increase the electrode density to provide greater coverage across the volume of the 3D neural network.[12] We also envision to expand the platform's sensing capabilities towards metabolism activities by sensing levels of dissolved oxygen, pH, glucose, etc. and understanding complex roles of neurotransmitters in cell growth and communications.

## ACKNOWLEDGEMENTS

The authors acknowledge funding from the Agence Nationale de la Recherche (ANR-19-CE19-0002-01 and ANR-19-CE19-0003-01). This work was supported by the French RENATECH network.

## REFERENCES

[1] M. A. Lancaster *et al.*, "Cerebral organoids model human brain development and microcephaly," *Nature,* vol. 501, no. 7467, pp. 373-379, 2013.

[2] M. A. Lancaster and J. A. Knoblich, "Organogenesis in a dish: modeling development and disease using organoid technologies," *Science,* vol. 345, no. 6194, p. 1247125, 2014.

[3] A. P. Passaro and S. L. Stice, "Electrophysiological analysis of brain organoids: Current approaches and advancements," *Frontiers in Neuroscience,* vol. 14, p. 622137, 2021.

[4] S. Pautot, C. Wyart, and E. Y. Isacoff, "Colloid-guided assembly of oriented 3D neuronal networks," *Nature methods,* vol. 5, no. 8, pp. 735-740, 2008.

[5] V. Saunier, E. Flahaut, C. Blatché, C. Bergaud, and A. Maziz, "Carbon nanofiber-PEDOT composite films as novel microelectrode for neural interfaces and biosensing," *Biosensors and Bioelectronics,* p. 112413, 2020.

[6] V. S. Vajrala, V. Saunier, L. G. Nowak, E. Flahaut, C. Bergaud, and A. Maziz, "Nanofibrous PEDOT-Carbon Composite on Flexible Probes for Soft Neural Interfacing," *Frontiers in bioengineering and biotechnology,* vol. 9, 2021.

[7] C. Cointe *et al.*, "Scalable Batch Fabrication of Ultrathin Flexible Neural Probes using Bioresorbable Silk Layer," *Microsystems & Nanoengineering (In Press),* 2022.

[8] V. S. Vajrala, K. Elkhoury, S. Pautot, C. Bergaud, and A. Maziz, "Hollow ring-like flexible electrode architecture enabling subcellular multi-directional neural

interfacing," *Biosensors and Bioelectronics,* p. 115182, 2023.
[9] M. Frega, M. Tedesco, P. Massobrio, M. Pesce, and S. Martinoia, "Network dynamics of 3D engineered neuronal cultures: a new experimental model for in-vitro electrophysiology," *Scientific reports,* vol. 4, no. 1, pp. 1-14, 2014.
[10] A. Maziz, E. Özgür, C. Bergaud, and L. Uzun, "Progress in conducting polymers for biointerfacing and biorecognition applications," *Sensors and Actuators Reports,* vol. 3, p. 100035, 2021.
[11] C. Rossant *et al.*, "Spike sorting for large, dense electrode arrays," *Nature neuroscience,* vol. 19, no. 4, pp. 634-641, 2016.
[12] D. A. Soscia *et al.*, "A flexible 3-dimensional microelectrode array for in vitro brain models," *Lab on a Chip,* vol. 20, no. 5, pp. 901-911, 2020.