Video Article

Interfacing 3D Engineered Neuronal Cultures to Micro-Electrode Arrays: An Innovative *In Vitro* Experimental Model

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Abstract

Currently, large-scale networks derived from dissociated neurons growing and developing *in vitro* on extracellular micro-transducer devices are the gold-standard experimental model to study basic neurophysiological mechanisms involved in the formation and maintenance of neuronal cell assemblies. However, *in vitro* studies have been limited to the recording of the electrophysiological activity generated by bi-dimensional (2D) neural networks. Nonetheless, given the intricate relationship between structure and dynamics, a significant improvement is necessary to investigate the formation and the developing dynamics of three-dimensional (3D) networks. In this work, a novel experimental platform in which 3D hippocampal or cortical networks are coupled to planar Micro-Electrode Arrays (MEAs) is presented. 3D networks are realized by seeding neurons in a scaffold constituted of glass microbeads (30-40 µm in diameter) on which neurons are able to grow and form complex interconnected 3D assemblies. In this way, it is possible to design engineered 3D networks made up of 5-8 layers with an expected final cell density. The increasing complexity in the morphological organization of the 3D assembly induces an enhancement of the electrophysiological patterns displayed by this type of networks. Compared with the standard 2D networks, where highly stereotyped bursting activity emerges, the 3D structure alters the bursting activity in terms of duration and frequency, as well as it allows observation of more random spiking activity. In this sense, the developed 3D model more closely resembles *in vivo* neural networks.

Video Link

The video component of this article can be found at http://www.jove.com/video/53080/

Introduction

In vitro two-dimensional (2D) neural networks coupled to Micro-Electrode Arrays (MEAs)arethe gold-standard experimental model adopted to study the interplay between neuronal dynamics and the underlying connectivity. During the development, neurons recreate complex networks which display well definedspatio-temporalpatternsof activity[1,2] (*i.e.*, bursts, network bursts, random spiking activity). MEAs record the electrophysiological activity from many sites (from tens to thousands of microelectrodes), allowing a detailed investigation of the expressed dynamics at the network level. In addition, the use of dissociated cultures makes possibile to design engineered-networks. It is easier to understand in this way the functional relationships between the recorded electrophysiological activity and the parameters of the network organization like cell density[3], degree of modularity[4,5], presence of heterogeneous neuronal populations[6], etc. However, all *in vitro* studies on dissociated cultured cells are based on 2D neuronal networks. This approach leads to oversimplifications with respect to the *in vivo* (intrinsically 3-dimensional, 3D) system: (i) in a 2D model, somata and growth cones are flattened and the axons-dendrites outgrowth cannot spread in all directions[7]. (ii) 2D *in vitro* networks exhibit stereotyped electrophysiological dynamics dominated by bursting activity involving most of the neurons of the network[8].

Recently, different solutions have been developed to allow the construction of *in vitro* 3D dissociated neuronal networks. The common idea consists in creating a scaffold where neurons can grow in a 3D fashion. Such a scaffold can be realized with polymer gels and solid porous matrices[9-13]. By exploiting the mechanical properties of the polymers, it is possible to embed cells inside these structures by defining a uniform block of 3D cultures of neurospheres[11]. The main feature of this approach is the rigid mechanical property of the neurospheres[9,12]. However, these materials have limited porosity, and they do not guarantee cell migration inside the matrix. To overcome this drawback, a possible solution consists in slicing the matrix into 'unit' modules. Unfortunately, the size and shape diversity of the particles could hamper the packing into regular layered structures. In[7], Cullen and coworkers designed a 3D neuronal construct made up of neurons and/or astrocytes within a bioactive extracellular matrix-based scaffold. Such an engineered neural tissue allowed *in vitro* investigations to study and manipulate neurobiological responses within 3D micro-environments. This model consisted of neurons and glia distributed throughout the extracellular matrix (ECM) and/or hydrogel scaffolds (500-600 µm thick). In this condition, an optimum cell viability (greater than 90%) was found by plating cells at a final density of about 3,750 - 5,000 cells/mm^3. It must be noted that such a density value is far lower than the one in the *in vivo* condition, where the cell density of the mouse brain cortex is about 90,000 cells/mm^3[14]. To overcome this limitation Pautot and coworkers[15] realized a 3D *in vitro*

system where cell density and network connectivity are controlled to resemble *in vivo* conditions while enabling real-time imaging of the network. Practically, this method is based on the concept that dissociated cultured neurons are able to grow on *silica* microbeads. These beads provide a growth surface large enough for neuronal cell bodies to adhere and for their arborizations to grow, mature, extend, and define synaptic contacts to other neurons. This method exploits the spontaneous assembly properties of mono-dispersed beads to form 3D layered hexagonal arrays containing distinct subsets of neurons on different layers with constrained connectivity among neurons on different beads. The achieved cell density with this method was about 75,000 cells/mm^3.

Recently, we have adapted Pautot's method to MEAs[16]: the obtained results show that the 3D electrophysiological activity presents a wider repertoire of activities than the one expressed by 2D networks. 3D mature cultures exhibit an enhanced dynamic in which both network burst and random spike activity coexist. Similarly, Tang-Schomer and coworkers[17] realized a silk protein-based porous scaffold which maintains a primary cortical culture *in vitro* for some months, and recorded the electrophysiological activity by means of a tungsten electrode.

In this work, the experimental procedures to build 3D neuronal networks coupled to MEAs will be described.

Protocol

The experimental protocol was approved by the European Animal Care Legislation (2010/63/EU), by the Italian Ministry of Health in accordance with the D.L. 116/1992 and by the guidelines of the University of Genova (Prot. N. 13130, May 2011). All efforts were made to reduce the number of animals for the project and to minimize their suffering.

1. Preparation of Materials and Supports

1. Construct the mold to build the PDMS (Poly-Dimethyl-Siloxane) constraint by means of a CNC (Computer Numerical Control) milling machine. Such a mold is realized in polycarbonate with the central cylinder in polytetrafluoroethylene (PTFE). This material allows an easier extraction of the mask once the PDMS has hardened.
   NOTE: The design of the mold has been performed by Computer-Aided-Design (CAD) and then delivered to the CNC milling machine.
2. Prepare the PDMS elastomer. PDMS is an organic polymer. It is composed of two elements, a curing agent and a polymer. Mix them by a volume ratio of 1:10, one part of curing agent and nine parts of polymer. Put the two components in a Petri dish, shake and insert the mix in the vacuum chamber for 10 min to eliminate air bubbles. 3 g of PDMS are sufficient for one constraint.
3. Construct the PDMS constraint. Insert the PDMS material into the molder and put it into the oven for 30 min at 120 °C. The PDMS constraint has the shape of an ideal cylinder with an external and internal diameter of 22.0 and 3.0 mm, respectively and a height of 650 µm.
4. The day before the plating, couple the mask to the active area of the Micro-Electrode Arrays (MEAs) by means of a tweezer under a stereomicroscope and sterilize the PDMS mask in the oven at 120 °C.
5. Sterilize the glass microbeads (nominal diameter of 40±2 µm; certified mean diameter of 42.3±1.1 µm) in 70% ethanol for 2 hr in a conical vial and rotate every 30 min to expose all microbeads.
6. Remove the ethanol solution from the vial and rinse the microbeads two times with sterilized water.
7. Condition the central part of the MEA area delimited by the PDMS mask (diameter equals to 3.0 mm) with 24 µl of mixed solution of Poly-D-Lysine and Laminin at 0.05 µg/ml.
8. Coat the microbeads with adhesion proteins, Laminin and Poly-D-Lysine at 0.05 µg/ml and leave them overnight in the incubator at 37 °C, to obtain a stable and long-lasting neuronal network.
9. Remove the adhesion factors both from the glass surface of the MEA and from the glass microbeads. Aspirate approximately 95% of the coating solution with a pipette and apply a small volume- 24µl- of sterile water on the MEA surface. Aspirate again more than 95% of the water and let the MEA dry under the laminar hood for 1 hr before plating the cells.
   NOTE: In the case of microbeads instead, aspirate the coating solution and make a first wash with sterilized water and a second one with the basal medium and its supplement such as B27, in order to condition the glass surface where neurons will be plated. Do not let the microbeads dry. Leave them in suspension in the culture medium inside the vial.
10. Distribute, by means of a pipette -200 µl-, the suspension of treated microbeads (about 32,000) onto a multiwell plate with membrane insert (pore diameter 0.4 µm) where they will self-assemble forming a uniform layer.
11. Fill each well of the membrane with 0.5 ml of medium and put it in the incubator (T = 37.0 ^{o}C, CO_2 = 5%) until the neurons are ready to be plated (3.2).

2. Dissection of Embryos and Dissociation of Tissue

1. House adult female rats (200-250 g) at a constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light–dark schedule (light-on 7 AM–7 PM) in the animal facility. Ensure that food and water are freely available.
2. Anesthetize a pregnant female rat after 18 days of development (E18) using 3% isoflurane. Then, sacrifice the animal by cervical dislocation.
3. Remove hippocampi from each rat embryo and place them into ice cold Hank's balanced salt solution without Ca^{2+} and Mg^{2+}. At day 18 of development hippocampus and cortex tissues are very soft and do not need to be cut into small pieces. Further details can be found[18,19].
4. Dissociate the tissue in 0.125% of Trypsin/Hank's solution containing 0.05% of DNAse for 18-20 min at 37 °C.
5. Remove the supernatant solution with a Pasteur pipette and stop the enzymatic digestion by adding medium with 10% fetal bovine serum (FBS) for 5 min.
6. Remove the medium with FBS and wash once with the medium with its supplement, 1% L-glutamine, gentamicin 10 µg/ml. Remove again the medium with a Pasteur pipette and refill it again with a small amount (500 µl) of growth medium with its supplement, 1% L-glutamine, gentamicin 10 µg/ml.
7. Dissociate the tissue pellet mechanically with a narrow Pasteur pipette until a milky suspension of cells is apparent. It is not necessary to centrifuge the cell suspension.

8. Dilute the small volume of cell suspension with the growth medium to obtain a final volume of 2.0 ml. Count the obtained cellular concentration with a hemocytometer chamber. Dilute this concentration at 1:5 in order to obtain the desired cell concentration of 600-700 cells/µl.

3. Cell Plating

1. Plate cells at a density of about 2,000 cells/mm^2 onto the active area of the MEA, defined by the PDMS constraint to create a 2D neuronal network.
   NOTE: Each hippocampus contains about 5 x 10^5 cells, (1 x 10^6 for a single embryo). Dissect 6 embryos to get a total amount of 6 x 10^6 cells in 2 ml, and an estimated concentration of 3,000 cells/µl. Dilute this concentration at 1:5 in order to obtain the desired cell concentration and plate about 600-700 cells/µl. If the MEA area delimited by the PDMS constraint is about 7.065 mm^2, and the total number of plated cells 600 cells/µl x 24 µl = 14,400 cells, the final density of 2,038 cells/mm^2.
2. Place the MEA devices into the incubator with humidified CO_2 atmosphere (5%) at 37 °C.
3. Distribute 160 µl of the suspension with a cell concentration of 600-700 cells/µl (about 100,000 cells) onto the surface of the microbeads monolayer positioned inside the multiwell plates to complete the preliminary step for the construction of three-dimensional culture. Put the multiwell plates in the incubator with humidified CO_2 atmosphere (5%) at 37 °C.

4. 3D Neuronal Network Construction

1. 6-8 hr after the plating, transfer the suspension (microbeads with neurons) from the multiwell plates very carefully inside the area delimited by the PDMS constraint by using a pipette set for a volume of about 30-40 µl. After each transfer, wait for about half a minute, to allow the microbeads to self-assemble in a hexagonal compact structure.
2. Once all the layers are deposited and spontaneously assembled, refill with a large drop of about 300 µl of medium the top of the area delimited by the PDMS constraint.
3. Put the 3D structure coupled to the MEA in incubator (T = 37.0 ^oC, CO_2 = 5%) for 48 hr before adding a final volume (about 1 ml) of growth medium culture with its supplement.
   NOTE: Consider the total number of beads on the multiwell plates with membrane insert (30,000) and the number of beads on a single layer onto the MEA device (6,000). The resulting 3D structure is composed of 5 *layers* of microbeads and cells. Taking into account that the 3D neuronal network is not geometrically perfect, the resulting 3D structure could be composed of 5-8 layers.
4. The day after plating, carefully add the final volume of the medium (about 900 µl) inside the MEA ring. Maintain the 3D cultures in a humidified CO_2 atmosphere (5%) at 37 °C for 4-5 weeks. Replace half of the medium once a week.

5. Confocal Microscopy Acquisition

1. Fix the biological samples to the stage of the microscope in a holder. Set the laser (Argon, 496-555 nm), the scan speed (400 Hz) at which to acquire the image, the gain and offset (-0.3%) of PMT for each image to capture the correct bandwidth emission filter and in order to avoid saturation and to increase the signal to noise ratio. The Results Section reports the values used to acquire the images of **Figure 4**.
2. For the acquisition of the *z*-stack sequence, first select, the value of *z* position of the top and the bottom layer of the sample, and then select the step size to make the acquisition.

Representative Results

In this experimental procedure, a relevant role is played by the microbeads that define the mechanical scaffold for the growth of the 3D neuronal network. **Figures 1A and B** display the arrangement of the microbeads (nominal diameter of 40±2 µm; certified mean diameter of 42.3± 1.1 µm) in the plane. The peculiarity of such structures is that microbeads spontaneously self-assemble defining a compact hexagonal geometry (**Figure 1B and C**). The so generated scaffold guarantees a consistent growth surface for neurites and a large enough interstitial space for the metabolic exchange of the cell bodies. This microbead dimension proves to be a reasonable compromise for interstitial spacing and final neuronal density. Considering that the PDMS structure recreates an “ideal” cylinder, and since the compact hexagonal structure impact factor (HPF) is equal to 0.74, the maximum number of beads (diameter of 40 µm) contained within the PDMS constraint is about 100,000, which corresponds to about 6,000 microbeads per each layer. As explained in the Protocol Section, these microbeads are pre-conditioned with the adhesion factors (*i.e.*, Laminin and Poly-D-Lysine). This procedure guarantees that neurons adhere to the microbeads. **Figure 1D** shows an example where three neurons are coupled to a microbead with a diameter of 40 µm.

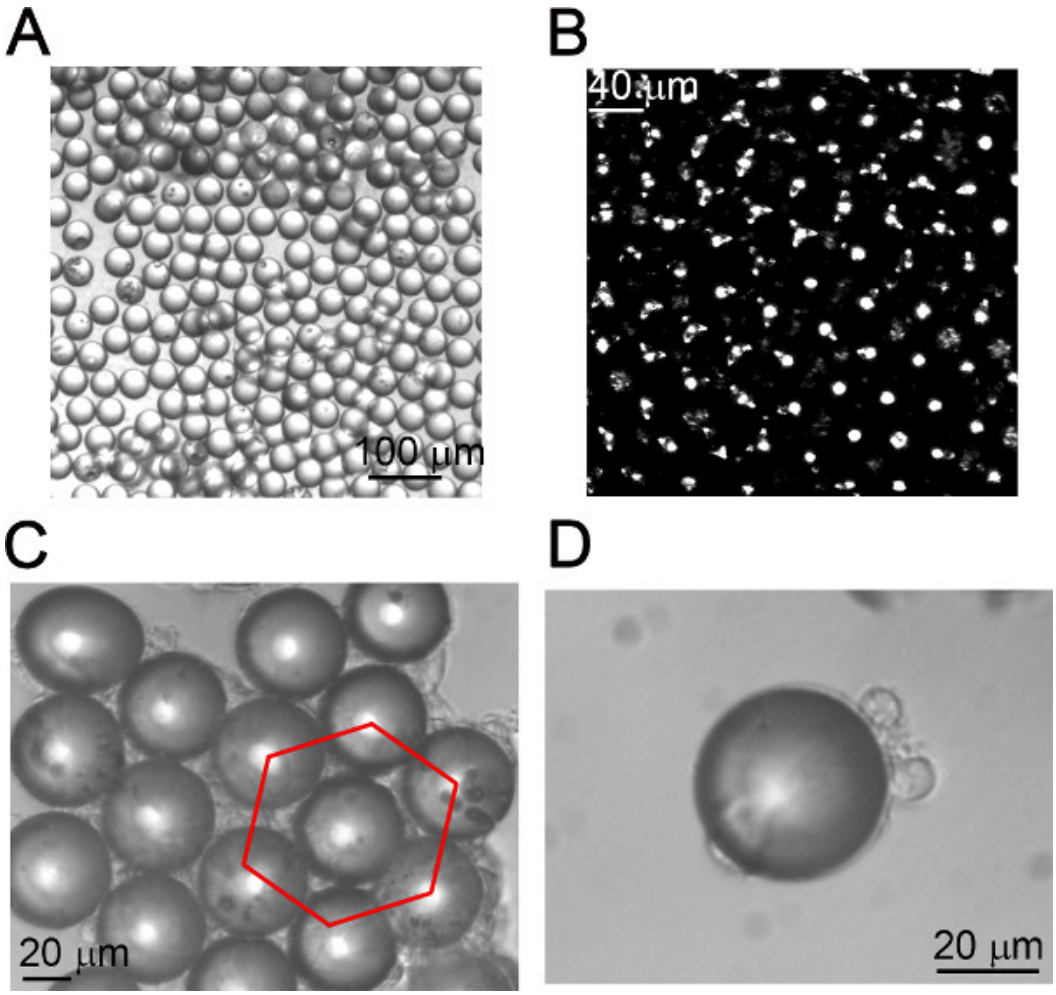

Figure 1. Images of the microbeads used to create the scaffold for the neuronal network. (A, B) Two examples of monolayers of microbeads on the surface of a multiwell plate with membrane insert. **(C)** Microbeads settled under gravitational force and spontaneously assembled into 2D hexagonal arrays. Once the first layer is fully packed, other beads are added, building a second ordered layer having the same hexagonal symmetry. Further addition of microbeads resulted in the construction of a packed 3D assembly. The free space between the microbeads is filled by neuronal processes and cell bodies. **(D)** Three neurons are anchored on the surface of a single microbead, previously conditioned with adhesion factors (Laminin and Poly-D-Lysine). Please click here to view a larger version of this figure.

The interfacing of the microbeads and neurons with the active area of a MEA is due to an elastomeric constraint (**Figure 2C**), realized with the mold depicted in **Figures 2A and B**. **Figure 2A** shows the layout and the selected dimensions. The active area of the MEA is delimited by coupling the PDMS structure of **Figure 2C** to the MEA substrate (**Figure 2D)**. In this way, neurons are forced to adhere to the active electrode area of the MEA. Subsequently, the glass microbead stacks with adherent neurons will be accommodated on this first layer. **Figure 2D** shows a cross-section of the final configuration, where the role of the PDMS structure can be clearly appreciated. The space delimited by the PDMS structure contains the mixture of microbeads and neurons (white powder in the bottom panel of **Figure 2D**). The PDMS structure has the shape of a cylinder built with the mold depicted in **Figure 2B** with the following dimensions: external and internal diameter of 22.0 and 3.0 mm respectively and height of 650 µm. The mold was built using polycarbonate and PTFE which make an ideal smooth and resistant material to facilitate the extraction of the elastomeric mask.The PDMS constraints display a shorter lifetime, since the sticky properties of the PDMS decrease after each usage. Typically, each PDMS structure can be successfully used three times.

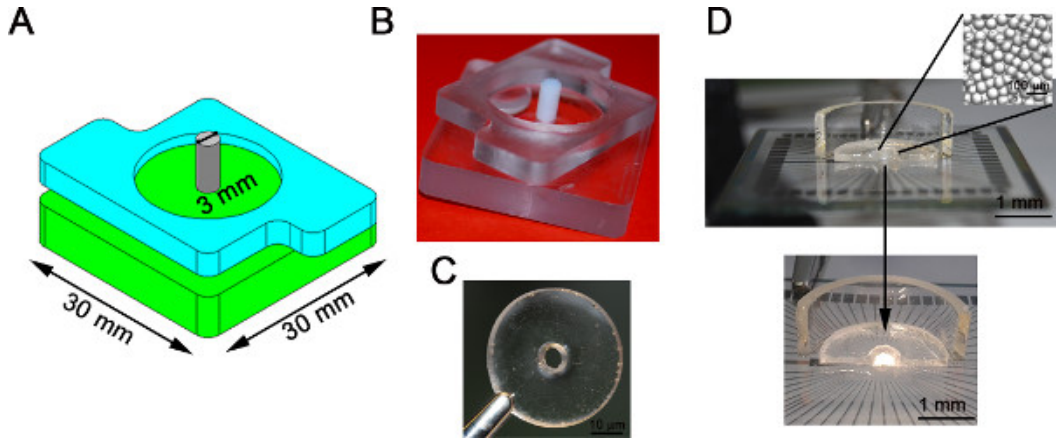

Figure 2. Materials for building the 3D neuronal network. (A) Design and **(B)** image of the mold used to create the physical constraint. The grey 3 mm diameter cylinder corresponds to the hole of the constraint depicted in **(C)**. **(C)** PDMS constraint placed on the active area of a Micro-Electrode Array (MEA). The use of such a physical barrier allows to contain the microbeads within a target area (about 7 mm^2) and limit the development of the 3D network. **(D)** Cross-section of the MEA system and constraint. Microbeads (depicted by the microscope in the top right corner) are filled into the space delimited by the PDMS constraint (bottom panel). Please click here to view a larger version of this figure.

After the confinement of the active area of the MEA by means of the PDMS mask, the protocol continues as depicted in the panels of **Figure 3**, describing the main steps to realize the assembly of neurons and microbeads.

The first preliminary step consists in plating dissociated neurons onto the MEA surface which was previously pre-coated with the adhesion molecules Laminin and Poly-D-Lysine (**Figure 3A**). It is essential that the 2D neuronal network adheres directly to the active area of the MEA: only under these conditions, the recording of the electrophysiological signals (*i.e.*, spikes and bursts) is feasible. This first part of the protocol corresponds to the standard procedure used to plate 2D neurons. The actual construction of the 3D system is depicted in **Figures 3B, C, and D**. Firstly, microbeads are positioned into the multiwell plates with membrane insert supports (**Figure 3B**); afterwards, a 160 µl suspension with a cell concentration of 600-700 cells/µl (about 100,000 cells) is distributed onto the microbeads monolayer (**Figure 3C**). Considering that cells can occupy half of the bead area, the total free surface is 75 mm^2. The cell density on the microbeads monolayer is about 1,500 cells/mm^2. The utilized multiwell plates with membrane insert present a porous membrane with a surface of 33.6 mm^2. By considering a virtual cylindrical shape with a base area equal to the porous membrane surface and a height of 40 µm (bead diameter), a uniform layer composed of 30,000 microbeads is coupled to the multiwell plates with membrane insert. The so realized suspension of microbeads and neurons stays for about 6-8 hr inside the multiwell plates with membrane insert. Once the aforementioned steps are completed, the construction of the 3D network can start. At this time, the mixture of neurons and microbeads is removed from the multiwell plates with membrane insert and placed on the 2D neuronal network previously plated onto the area defined by the PDMS constraint (**Figure 3D**). During this procedure, a loss of about 20-30% of neurons occurs due to (i) mechanical manipulations and (ii) to the transfer outside the membrane. By considering the base area of the constraint (7.06 mm^2) and a height of 178.56 µm (5 layers of beads), and dividing the number of cells by the volume of the ideal cylinder formed by the 5 layers of beads, the resulting 3D neuronal network cell density is about 80,000 cells/mm^3.

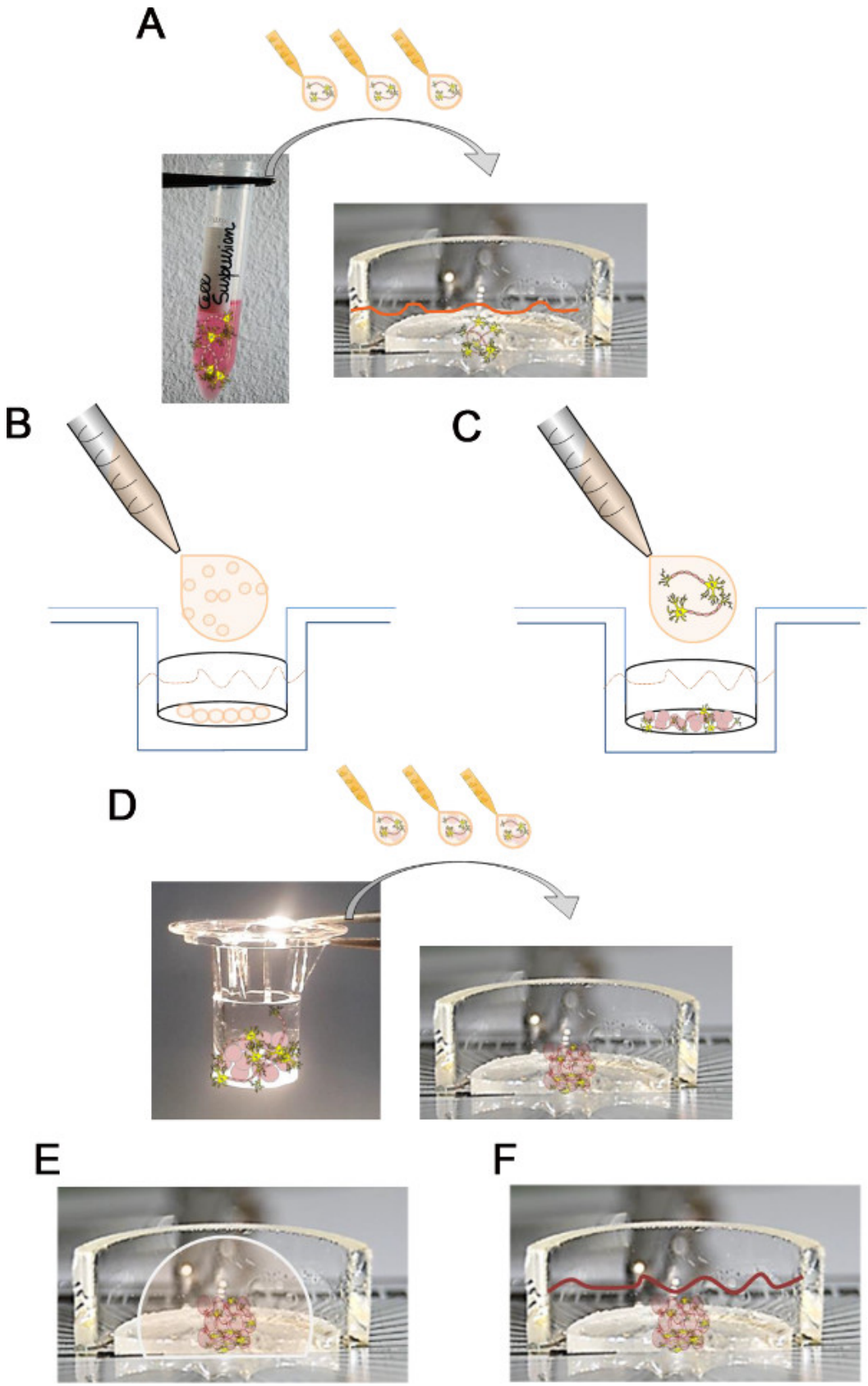

Figure 3. Sketch of the fundamental steps to create 2D and 3D neuronal networks. (A) Plating of neurons on the active area of a MEA. The suspension of neurons is plated onto the MEA surface, previously conditioned with adhesion factors and delimited by the PDMS constraint. This step allows to realize: i) a classical 2D neuronal network coupled to MEA; ii) the first layer of a 3D network from which the electrophysiological activity will be recorded. **(B)** Microbeads and **(C)** neurons are delivered to the surface of the multiwell plates with membrane insert after 6-8 hr.

(D) The suspension of neurons and microbeads is moved from the multiwell plates with membrane insert to the 2D network. The repetition of this step several times allows to define a dense multi-layer 3D structure. **(E)** When the 3D structure is completed, a drop of medium is added, and the resulting structure is stored in the incubator for 48 hr. **(F)** After 48 hr, the 3D culture is completely refilled with the final volume of the medium. Please click here to view a larger version of this figure.

Once the first layer has been positioned, the same operation is repeated to obtain a packed 3D ensemble. The resulting 3D structure reorganizes itself defining a colloidal crystal that self-assembles in a compact structure made up of about 5-8 layers of microbeads and neurons (**Figure 4**). Once all the layers are formed, the 3D network has to be refilled with a drop of 300 µl of medium (**Figure 3E**). At this stage, 3D networks coupled to MEAs can be stored in the incubator for 48 hr. After that, 1 ml of medium is added to fill the ring of the MEA (**Figure 3F**).

Once all the layers have been assembled, neurites grow over the microbead scaffold reaching a final cell density of about 80,000 cells/mm^3. It is worth noticing that the obtained value is not far from 92,000 cells/mm^3, the average neuronal density in the mouse brain cortex[14].

Figure 4 displays three examples of 3D networks coupled to MEAs captured by means of confocal microscopy upright. In **Figure 4A**, the DIC (differential interference contrast) image shows a single layer of beads coupled to the MEA surface; the microscope was coupled with 20.0 N.A. 0.50 water objective and the images were acquired with Transmitted PMT included to the microscope and a Laser Argon (488nm). The pictures show very clearly the spatial layout of the electrodes (black dots) and the regular spatial organization of the microbeads.

In **Figure 4B**, an immunofluorescence staining for Map-2 (red signal) displays the distribution of dendritic arborizations and somata of the neurons around microbeads directly coupled to the electrode plane of MEA (the presence of the black contacts can be observed). **Figure 4B** is a maximum projection of a *z*-stack sequence (108 µm) with the step size equals to 1.53 µm. Images were acquired with 40.0 N.A. 0.80 water objective; the excitation is Argon Laser (488 nm), the emission bandwidth 496nm-655nm.

Finally, **Figure 4C** displays the maximum projection of a *z*-stack sequence (187.79 µm) of a 3D hippocampal culture (step size is 2.27 µm). The *z*-stack sequence was acquired with 25.0 N.A. 0.95 water objective. For green channel, an Argon laser 488nm and an emission filter bandwidth 499nm-551nm were used. For red channel, a 543 nm laser and an emission filter bandwidth 555nm-645nm were used. The channels were acquired in sequence mode. A highly interconnected network emerges where neurons are labelled for NeuN expressed in mature post-mitotic neurons (red signal) and for Gaba (green signal, yellow in merge). Gaba antibody revealed a positivity for inhibitory neurons both in the somata and neurites.

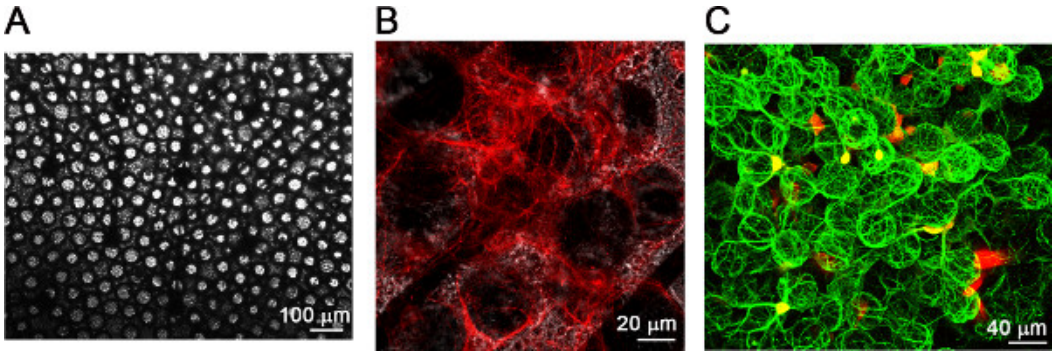

Figure 4. Three examples of 3D networks captured by confocal microscopy. (A) DIC (differential interference contrast) image showing a single layer of beads coupled to the MEA surface; the spatial layout of the electrodes (black dots) is observable. **(B)** Immunofluorescence staining for Map-2 (red signal) displays the distribution of dendritic arborizations and somata of the neurons around microbeads directly coupled to the electrode plane of the MEA. **(C)** Maximum projection of a *z*-stack sequence (187.79 µm) of a 3D culture displaying a highly interconnected network where neurons are labelled for NeuN expressed in mature post-mitotic neurons (red signal) and for Gaba (green signal, yellow in merge). Gaba antibody revealed a positivity for inhibitory neurons both in the somata and neurites. Please click here to view a larger version of this figure.

To understand whether the presence of a 3D structure modulates the network dynamics, the so generated electrophysiological activity was compared to conventional 2D neuronal networks grown over MEAs, which represent the reference model.

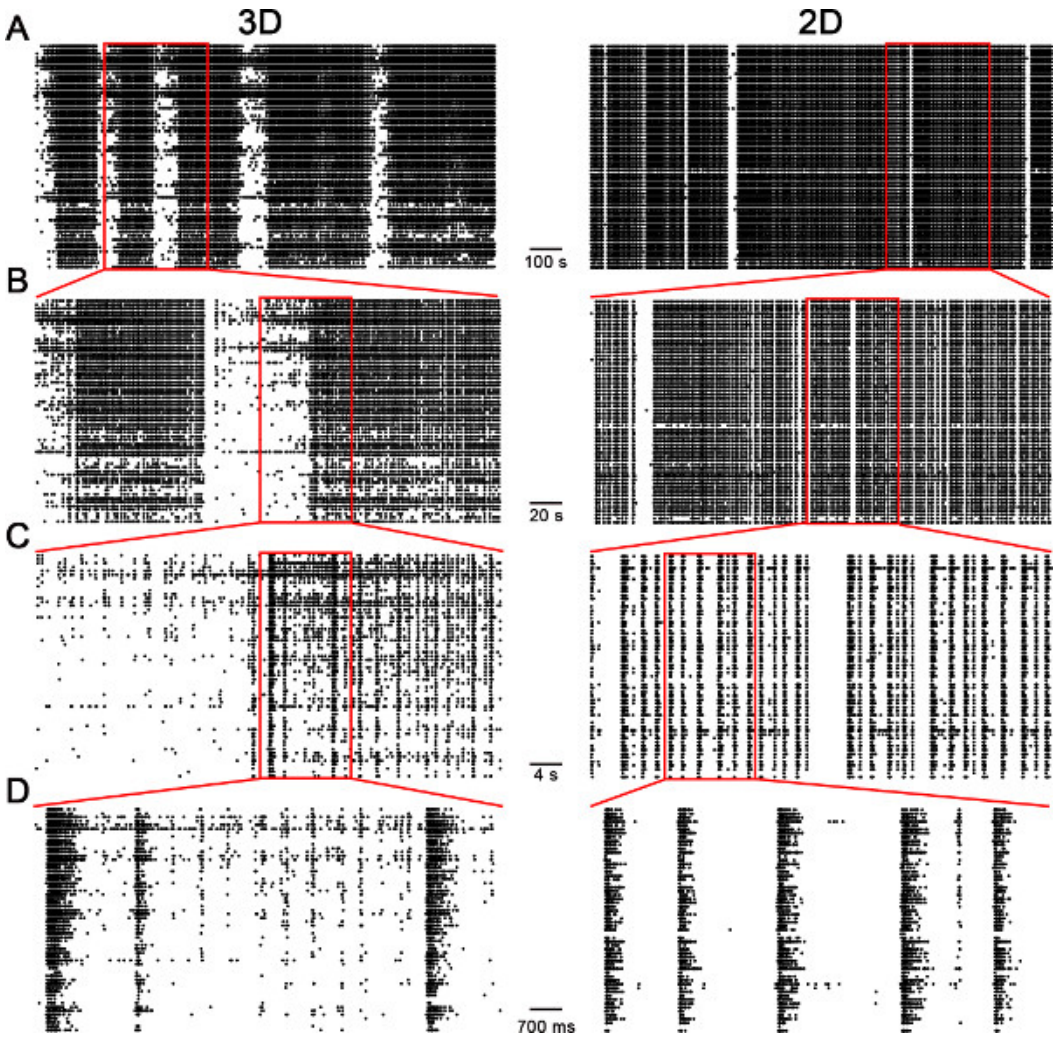

Figure 5. Comparison between the network dynamics exhibited by 3D (left column) and 2D (right column) hippocampal cultures. At each time scale (**A**: 1,200 sec, **B**: 300 sec; **C**: 60 sec; **D**: 10 sec), 3D assemblies display a variety of signatures of activity (*i.e.*, short and long network bursts, random spiking activity, synchronized bursts), while 2D ones display a more pronounced stereotyped activity with only high synchronized bursts. Please click here to view a larger version of this figure.

Figure 5 shows two raster plots of two representative experiments performed on a 3D (left panels) and 2D (right panels) hippocampal culture during the fourth week *in vitro*. These plots show the electrical activity of the network recorded on all active electrodes (*i.e.*, electrodes with a firing rate greater than 0.1 spikes/s).

The dynamics of 2D networks display a quasi-synchronous activity composed mainly of network bursts. The signature of this dynamic is the presence of synchronized events which involve most of the recording channels interspersed by period in which almost no activity is recorded (see the right panel of **Figure 5D**). This behavior can be appreciated at different time scales, as the different levels of magnification underline.

In the case of a 3D network, the signature of the network dynamics consists of a wider repertoire of activities characterized by: (i) less global synchrony, (ii) more random spiking activity, (iii) periods in which sub-networks (*i.e.*, a subset of microelectrodes) present a more synchronous activity with the occurrence of network bursts. Furthermore, the duration of the network bursts is more variable as well: there is the presence of network bursts with a duration similar to the one observed in the 2D networks, but there are also longer network bursts. A complete and quantitative characterization of the emergent 3D dynamics obtained by using such a methods can be found in[16]. As anticipated, the 3D neuronal network exhibits also a significant 'random spiking' and non-synchronous bursting activity. The plausibility of the dynamics generated by these 3D structures have been proofed with some control-experiments (*e.g.*, presence of a 2D network coupled to a MEA with a stack of bare microbeads and 3D networks assembled onto the microbeads with a bare MEA), as reported in[16].

Discussion

In this work, a novel experimental *in vitro* platform made up of 3D engineered neuronal cultures coupled to MEAs for network electrophysiology has been presented. The use of microbeads as scaffold to allow the neuritic outgrowth along the *z*-axis has been tailored to be integrated with the planar MEA. In this way, the obtained micro-system results in a valid and reliable *in vitro* 3D model to study the emergent electrophysiological dynamics[16].

MEA recording set-up

MEA devices consist of a culture chamber with an integrated array of substrate-embedded micro-electrodes capable of measuring extracellular signals from electro-active tissues. The typical recorded signals consist of spikes (*i.e.,* single supra-threshold voltage variation representing the electrical activity of one or more neurons) and bursts (*i.e.,* sequence of highly packed spikes often occurring simultaneously on several channels). The recordings of neuronal networks activity with MEA-based systems produce huge amount of data (*e.g.,* a 4 hr experiment with a 60 channels MEA produces a record of about 15 GB). This implies the need to have efficient tools to process and analyze the data, especially in the case of studies that require long recordings. In general, the recording of the electrophysiological signals is obtained by a direct extracellular transduction with the neuronal cultures, and the acquisition of a voltage signal. After an amplification and filtering stage, signals are sampled 10-50 kHz and stored. The raw signals are then peak-detected by a custom developed software tool. The recording system consisted of (1) MEA amplifier, (2) personal computer equipped with A/D acquisition board, (3) recording software, (d) heating system and temperature controller, (4) CO2 atmosphere-maintaining and evaporation-preventing systems.

The building of the 3D neuronal network goes through two critical steps: the first one is the distribution of the correct amount of beads on the multiwell plates with membrane insert surface, the second one is the transfer of the suspension of microbeads with adherent neurons from the multiwell plates with membrane insert to: (i) the 2D neuronal network plated onto the active area of the MEA (first layer); (ii) the already settled layers. This operation should be repeated as many times as the number of required layers. During the first week of culture, a rapid growth of neuritic extensions takes place around the microbeads belonging at the different layers. The close connection between neurons and microbeads stabilizes the biological samples and allow to move it from the incubator to change/replace the medium and to record with the MEA amplifier the electrical activity without worrying about damaging it.

Since the electrophysiological activity of the entire network is recorded only from the bottom layer (*i.e.*, the one directly coupled to the MEA), the active area must be covered by a conventional 2D network. The other layers will be added step by step over the 2D one, and long-range connections will be spread among the layers. Practically, the other layers are added 6-8 hr later than the 2D one by deposing the mixture of neurons and microbeads. The choice of this temporal window allows for the formation of a few synaptic connections in the 2D layer, and guarantees the possibility to establish connections to the upper layers too.

Two factors make the use of multiwell plates with membrane insert necessary to realize the 3D networks: (i) since neurons and microbeads present different sedimentation speeds, the use of multiwell plates with membrane insert guarantees a uniform distribution of neurons on the top of the microbeads: on average, each microbead may be surrounded by the same number of neurons. (ii) By exploiting the elastic properties of the multiwell plates with membrane insert, the backwash of the solution of microbeads coupled to neurons is easier than using solid surfaces like polycarbonate multi-wells.

The presented work follows the approach proposed by Pautot and coworkers[15]: in that study the use of *silica* beads provided a growth surface large enough for neuronal cell bodies to adhere and for their processes to grow, mature and produce pre- and post-synaptic specializations. A valid alternative to the use of beads scaffold comes from hydrogels matrices or bioactive and biodegradable beads made up of natural biopolymer like chitosan. Chitin and its deacetylated derivative, chitosan, are non-toxic, antibacterial, biocompatible and biodegradable biopolymers. The time constant of such biodegradation process is compatible with the network outgrow[20,21].

This study may constitute a reference work for the future development of advanced 3D neuronal model systems to systematically study the interplay between network dynamics and structure. In a short time, we hope to be able to transfer the 3D network construct on a new generation of devices[22]. The fabrication technique involves the production of electrodes in the form of micro-pillars in a variable heights between 50 and 350 µm.

Disclosures

The authors declare that they have no competing financial interests.

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