Differences in activity and functional connectivity between bi-dimensional and three-dimensional neuronal cultures

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Abstract: Three-dimensional (3D) neuronal cultures can provide a more desirable *in vitro* approach to emulate brain-like conditions. To prove it, 2D and 3D neuronal cultures were compared. During different *days in vitro* (DIV) after formation, spontaneous neuronal activity was recorded through calcium fluorescence imaging for data extraction. We show that activity in 3D cultures is higher and more varied. These cultures are functionally organized in modules that are spatially localized. In comparison, 2D cultures show less variability in activity, higher connectivity between neurons and a poor organization in modules. We ascribe the differences to the capacity of 3D cultures to form connections of short distance, favouring small microcircuits that coexist, while the 2D cultures have long–range connections that shape a strongly coupled and rigid system.

I. INTRODUCTION

Living neuronal networks, from those present in small worms up to the brain, are one of the most interesting complex systems in nature. Such complexity makes difficult to model and understand how they work, since a great wealth of dynamical and biochemical processes occur at the same time. Thus, *in vitro* networks in the form of neuronal cultures have been used to simplify the networks and take better control on them, gradually adding richer characteristics to grasp key aspects of the brain and its functioning. Additionally, understanding how neuronal networks are structurally organized and how they generate complex dynamics is the first step towards a comprehensive knowledge of other networks (e.g. social networks or economics). Many complex systems exhibit similar macroscopic behavior despite their differences in microscopic composition or mechanisms of interaction. Graph theory, which represents a network as a set of nodes and describes the interrelations among them as edges, is a key tool in studying and analysing the properties of these types of systems [1].

Neuronal cultures are a powerful tool to develop models of brain connectivity *in vitro*, especially three–dimensional (3D) cultures. These type of cultures provide a more realistic environment than bi–dimensional (2D) ones, which are generally grown on flat substrates. Indeed, including a third dimension provides a high surface area for growth and migration, where some cellular behaviours can occur, such as differentiation or maturation [2]. Furthermore, innovations in bioengineering have brought to light new biomaterials that imitate the brain environment, thus allowing for more specific studies (such as specific cell types or circuits) and avoiding the use of animals in research. These studies have recognised advantages in establishing models of neurodegenerative diseases or drugs effects, which lately could be applied to clinical therapies [3].

Here, we used these innovations in bioengineering to generate neuronal cultures in small, ∼15 mm diameter wells. 3D cultures were investigated during several days and compared to 2D cultures. For both, we quantified some measures related to culture activity and some other measures that describe network functional connectivity, the latter obtained using graph theory concepts.

II. EXPERIMENTAL PROCEDURE

A. Neuronal cultures & calcium imaging

Neuronal culturing was performed in accordance with the Ethical Committee for Animal Experimentation of the University of Barcelona. We used primary neurons from Sprague–Dawley rat brain cortices. As shown in Fig. 1A, 2D cultures corresponded to dissociated neurons that were placed on 4 mm diameter glass coverslips [4], while 3D neuronal cultures were prepared in wells filled with PEGylated Fibrinogen, a semi–synthetic hydrogel, to provide physiological conditions closer to the brain. The wells were 15 mm in diameter and 10 mm height. These hydrogels are elastic and gelatinous biomaterials made from cross–linked polymer chains which imitate the native extracellular matrix of the brain. Hydrogels culturing maintains the advantage of *in vitro* cultures (accessibility & easy manipulation) while advancing towards more realistic, brain–like *in vivo* models [3]. In both preparations, neurons randomly connect to one another and form a spontaneously active circuit within 5 days.

Calcium fluorescence imaging is a microscopy technique that allows us to measure neuronal activity changes by tracking variations in Ca^{2+} concentration, an ion that plays an important role in neuronal activity. For both cultures, the fluorescence indicator GCaMP6s was introduced into neurons through an adeno-associated virus (AAV), which 'infects' the neurons with the indicator, and therefore allows to track neuronal activity through several days [3]. When cultures are irradiated with blue

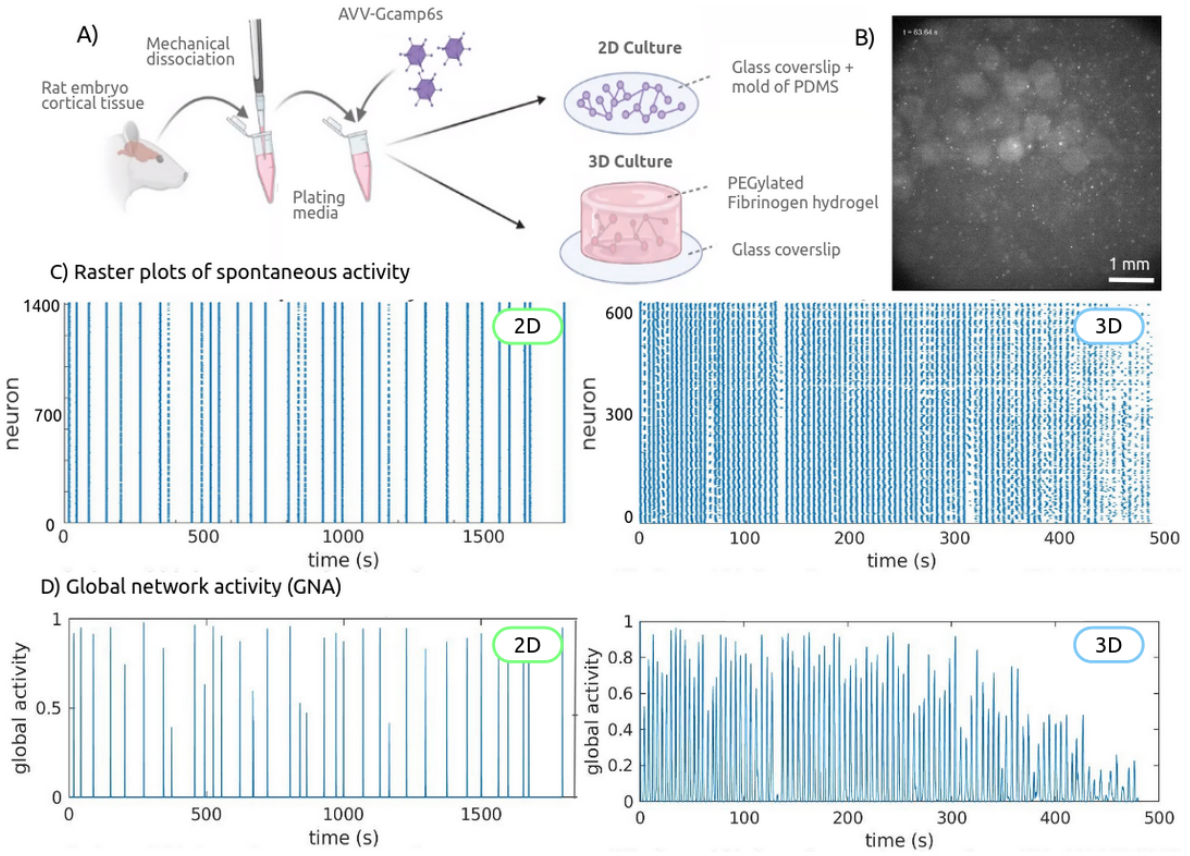

FIG. 1: **A:** Sketch of the culture preparation, from rat embryo cortical tissue to the different neuronal cultures. **B:** Fluorescence image of the 3D culture. Bright spots are active neurons, with some of them out of focus. **C:** Raster plot in which we represent the spontaneous activity (spike events) along time for each neuron in the network. Left: 2D culture with 1414 neurons. Right: 3D culture with 625 neurons. **D:** Corresponding Global Network Activity (GNA) of the above raster plots. Significant network bursts are those where at least 20% of the culture fires in a time window of 1s.

light ($\lambda = 490$ nm), fluorescence proteins bound to calcium emit green light ($\lambda = 514$ nm) that can be detected. Thus, network activity was actually monitored as changes in Ca^{2+} levels at the moment of neuronal firing, when calcium concentration (≈ 100 nM) rises by two orders of magnitude.

Fluorescence imaging was performed for 30 min in 2D and 10 min in 3D networks, and at 50 frames/s at 25ºC. In both case, cultures were investigated at different days *in vitro* (DIV), i.e. different maturation stages. We note that, in both cultures, wide–field fluorescence microscopy was used, with the camera focusing on a single plane. This was a problem in the 3D cultures since it was not possible to focus all neurons, but only a fraction of them. Many neurons actually appeared as out–of–focus objects (Fig. 1B). To precisely monitor 3D cultures one needs light–sheet microscopy, a highly expensive technology that is presently under development in Dr. Soriano's lab in collaboration with the Institute of Photonic Sciences (ICFO).

B. Image & data analysis

The recordings were analyzed using the NETCAL software [5] run on MatLab. First, an average fluorescence image of the recording was created and the background was removed. Then, Regions Of Interest (ROIs) were created as a grid in the field of view, and filling a circle (for 2D, 625 ROIs) or a square (for 3D, 1444 ROIs). The average fluorescence along time was then extracted for each ROI i and normalised by their relative fluorescence intensity $f_i(t) = (F_i(t) - F_0)/F_0$ where F_i and F_0 are the fluorescence intensity and the background fluorescence, respectively [6]. Each ROI was ascribed as a neuron for simplicity, although a ROI could contain more than a neuron, particularly in 3D. The timing of a neuronal activation (*spike*) was computed using the Schmitt trigger method where two thresholds are used to infer the timing of a spike. This method scans the fluorescence traces for events that first pass a high threshold for at least 100 ms until they decay below a second lower threshold. Thus, the train of activity for a neuron is expressed as ''1' for the existence of an event or '0' otherwise. Spike events are plotted as raster plots (Fig. 1C).

Once the raster plot was obtained, we exported the data to analyse it with software packages from Dr. Soriano's Lab. A first software computes the global network activity (GNA, Fig. 1D). Sharp peaks where at least 20% of the neurons fired simultaneously in a 1 s window reveal strong collective activity (*network bursts*). The selected bursts were then analyzed to compute their velocity of propagation, the inter–burst interval, and the probability distribution of burst initiation points. A second software computes the network functional connectivity and two network measures, namely the *global efficiency* G_{eff} and the *modularity* Q. The representation of the networks was performed in Gephi [7].

III. THEORETICAL METHODS

A. Activity Analysis

The first approach for comparing activity in cultures is using the *global network activity* (GNA). The 20% threshold is used to remove sporadic activity and small bursts. GNA is useful since, conceptually, a network is 'dynamically poor' when GNA values are always high, i.e. all neurons fire simultaneously, and hence only one dynamic state is present (all firing or nothing). 2D dynamics correspond to this situation (Fig. 1D, left), and contrasts with the much richer 3D case (Fig. 1D, right). The *inter–burst interval* measures the time between bursts. Therefore, the 'network activity' (bursts/s) is its inverse. Consequently, cultures more dynamically active show lower burst intervals, while cultures with less activity show higher intervals. On the other hand, the origin of coactivations is established as the center of mass of the first four active neurons. Thereby, *bursts velocity* is estimated through a linear fit $d(t)$, where d is the distance from a neuron to the origin, and t its activation time. This approach particularly works well when burst propagation is circular. This model is not accurate when looking at the actual front waves of bursts, however approximations that go beyond this one are outside the scope of this work.

B. Functional connectivity

Graph theory is used to describe networks as a set of nodes connected through edges. From the raster plots, we determine if two neurons are coupled or not by computing *Pearson's correlation coefficient* [6]:

$$r_{ij} = \frac{\sum_t [x_i(t) - \bar{x}_i][x_j(t) - \bar{x}_j]}{\sqrt{\sum_t [x_i(t) - \bar{x}_i]^2}\sqrt{\sum_t [x_j(t) - \bar{x}_j]^2}}, \qquad (1)$$

where $x_i(t)$ and $x_j(t)$ are the binary signals representing '1' for the presence of a spike at time t and '0' otherwise, and $\bar{x}_i(t)$ and $\bar{x}_j(t)$ their average values. When two neurons fire synchronously over time their correlation coefficient $r_{ij} \approx 1$; otherwise two uncorrelated neurons will provide $r_{ij} \approx 0$. Thus, for all neuronal pairs, r_{ij} values vary between 0 and 1. To establish significant functional connections, the software of Dr. Soriano calculates the Pearson values for randomized versions of the raster plots, r^R_{ij}. Thus, those $r_{ij} < r^R_{ij}$ can occur by chance, and are automatically set to 0. For simplicity, all significant connections are set to '1'.

To quantify the capacity of the network to exchange and propagate information among all neurons, the *global efficiency* G_{eff} is used, which is given by [8]:

$$G_{\text{eff}} = \frac{1}{N(N-1)} \sum_{j \neq i} d_{ij}^{-1}, \qquad (2)$$

where N refers to the total number of neurons and d_{ij} denotes the minimum topological distance between neurons i and j i.e. the shortest path length. The global efficiency varies between 0 (neurons completely isolated) and 1 (fully connected network).

Additionally, we introduce *modularity* Q, related to the structure of the network, which quantifies the strength of division into communities (like groups of friends in a social network) [9]. Q is obtained through complex mathematical algorithms, but conceptually it is 0 when the network itself is the only community, and 1 when there are as many communities as neurons. Typically, a modularity around $Q \simeq 0.3$ means a strong capacity of organization into communities.

IV. RESULTS & DISCUSSION

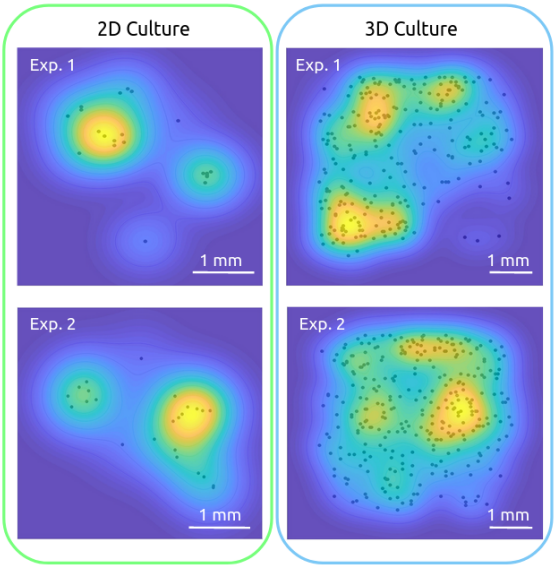

FIG. 2: **Initiation points distribution.** Left 2 columns are 2D cultures; right 2 columns are 3D cultures. Each column refers to different experiments at DIV7. Black dots represent burst's origin, while the probability distribution function is indicated as a colour contour. The darker the colour, the lower the probability for activity to start in that point.

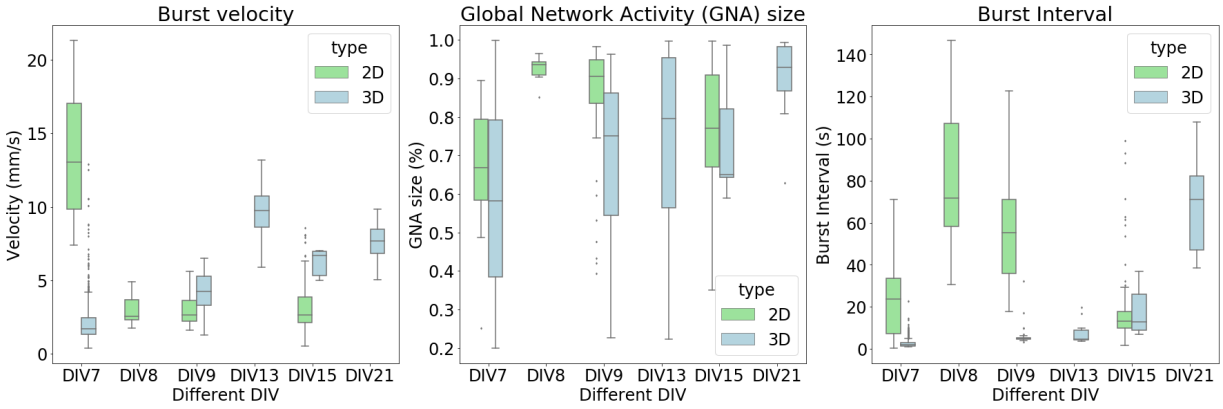

FIG. 3: **Statistical final results**. Green and blue bars correspond to 2D and 3D neuronal cultures, respectively. From left to right: evolution of burst velocity (mm/s), GNA size (% of network) and inter–burst interval (s) box plots.

We analysed a data set of 4 recordings for each type of culture along different DIVs. In 2D cultures we had a time duration of 2000 s and an average of 1400 neurons per culture, while in 3D cultures, recordings lasted 500 s and there were about 600 neurons per culture. From each recording, we obtained raster plots, showed in Fig. 1C, and computed the different measures introduced above.

In Fig. 2, we represented burst initiation points and its probability distribution for both type of culturing at DIV7. Both cultures showed different patterns of coactivation origins and number of bursts. Interestingly, the initiation points for 2D cultures were more localized in certain region, i.e. activity preferentially started in the same location, while in 3D cultures initiation was broadly distributed. This result indicates that 3D cultures facilitate a richer dynamical variability.

Fig. 3 shows all the statistics that we could obtain from the analysis of activity, including the burst velocity, GNA size and inter–burst interval. First, 2D cultures velocity values are (except for the first day) quite similar throughout days *in vitro*, whereas in 3D case we see lower initial velocity values that sightly increase over time. In 2D cultures at DIV7, it is noteworthy a high velocity values with strong variability as compared to other DIVs. We conjecture that this contrasting change in velocity occurs due to the circular propagation assumption, that may fail at cultures that are so young, probably because neurons are not sufficiently connected for a propagating front to take shape, and activity occurs in a erratic manner.

We also computed the distribution of GNA data. Cultures with high GNA values are considered to be 'dynamically poor' as we can see in 2D. Here, the majority of GNA values are over 50% meaning that most of neurons fire simultaneously. The distribution of GNA values is also narrower, indicating that coactivation sizes are very similar among themselves. By contrast, a widespread 3D GNA values represent an enhancement in dynamical variability. We hypothesize that the 3D environment facilitates a rich variability in connectivity throughout the volume that helps alternating groups of neurons to activate, and not always the same ones. We also note that hydrogels deteriorate (possibly by the neurons themselves), and that data at DIV15 and DIV21 contained less than 10% of the bursts observed in other days.

To complete the activity analysis, we observed that 2D cultures showed higher inter–burst intervals than 3D cultures. Thus, 3D cultures were in general much more active than the 2D ones. This failed at DIV15 and DIV21, when 3D cultures start to die.

From a functional connectivity point of view, by looking at the results of Fig. 4, we observed that both cultures are organised in communities or modules. Communities in 2D are more mixed up than in 3D, which are assembled into more compact patterns. In order to quantify functional connectivity, we have used global efficiency G_{eff} and modularity Q. We only show results obtained at DIV9 because of their consistency with other DIVs. G_{eff} is higher in 2D cultures, whereas Q is higher in 3D. Therefore, neurons in 2D cultures are more connected between them, possibly because neurons can establish long–range connections. This also explains that modules are mixed up in space. In contrast, 3D neuronal cultures have lower connectivity and are more organized in modules, which are physically compact. This indicates that neurons in 3D connect preferentially to their neighbours, and that the hydrogel itself may difficult the formation of long–range connections.

The limitations of 3D culturing experiments are basically because the large variability of networks since the connections are set randomly and, therefore, can not be repeated exactly. Moreover, the rigidity of hydrogels affects to these connections. New methods are being found to fabricate scaffolds with lasers in order to control density and position of neurons, making experiments more reproducible and with less variability [10]. In addition, as mentioned above, the problem of out–of–focus neurons must be solved using light–sheet microscopy because we cannot see out–of–focus neurons at first place and they add noise to the signal of others.

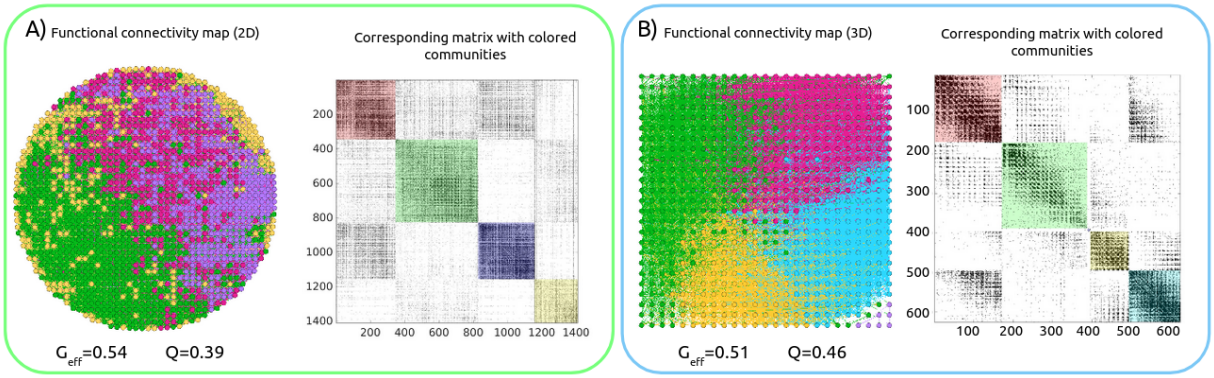

FIG. 4: **Functional connectivity. A:** 2D culture at DIV9. **B:** 3D culture at DIV 9. For each panel, the left plot shows the functional connectivity map done using Gephi with G_{eff} and Q values. Round objects are neurons, coloured according to their community. The right plot shows the corresponding connectivity matrix of binarized Pearson's values between neurons (i,j). Black dots are functional connections. Matrices are organized and coloured according to the obtained functional communities.

V. CONCLUSIONS

- 3D cultures are dynamically richer because they show widespread GNA values and lower inter–burst intervals. Velocity was difficult to measure in general, both in 2D and 3D.
- The probability distribution of initiation points allowed us to observe different patterns of coactivation origins and number of bursts between 2D and 3D neuronal cultures. Whereas in 2D culture origins are focused in a certain region, there are a large amount of bursts and they are more distributed in 3D cultures. Consequently, this higher spatial variability for the latter is in agreement with the results obtained with the activity analysis.
- Functional connectivity reveals that both cultures are organised in communities. A high G_{eff} is observed in 2D cultures, and a high Q in 3D ones. We conclude that neurons in 2D cultures are highly connected and less organised in modules because of long-range connections. In contrast, 3D neurons are highly organised in modules even though they are less connected, indicating that local and varied directions of connections were established.
- Data from DIV 15 and 21 in 3D neuronal cultures show different results compared with early DIVs. This could be due to hydrogel degradation. Moreover, variability in 3D cultures is high, so we are not able to reproduce experiments exactly. Thus, laser–fabricated 3D scaffolds might be a solution [10], as well as using light–sheet microscopy to solve issues with out–of–focus neurons.

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