Evolution of functional connectivity in neuronal cultures

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Abstract: We study the temporal evolution of three–dimensional rat cortical neurons *in vitro*. Data is obtained through calcium fluorescence imaging, allowing us to record the spontaneous activity of the culture. We monitor these cultures during their second and third week after formation and use graph theory to characterize some relevant network parameters, allowing us to pinpoint the functionality and complexity of such cultures. We show that cultures are more synchronous as they mature, with a higher global efficiency and connectivity. We also discuss about the reliability of current existing methods to estimate the richness of the network in terms of activity and the impact of spatial constraints on the functional traits of the cultures, particularly at early days.

I. INTRODUCTION

For many years, scientists from a wide variety of fields have been trying to understand how such a complex and unknown structure as the brain works. The impressive complex structural network that is formed by the neuronal elements of the brain has been known since the nineteenth century [1]. Networks are an object of study in a wide range of disciplines as a phenomena of the natural, social and technological world. Network science uses graph theory as a common toolset to analyze the properties of networks. This branch of mathematics represents a system as a number of nodes and describes the interrelations among them as edges. Research has been carried out in multiple species, using graph theory to obtain descriptive measures that report on local and global features of network topology. The data acquired shows nonrandom behavior, such as big clustering and short path length, and network communities (modules) linked by highly connected hub nodes [2].

The vast majority of connectivity studies in neuronal networks has been carried out in small living systems called neuronal cultures, which are dissociated neurons grown *in vitro*. This kind of cultures allows for a wide variety of preparations, from simple homogeneous assemblies to complex bioengineered designs, as they are accessible and easy to manipulate [3]. *In vitro* preparations of animal neurons have been broadly used for making noteworthy scientific advance in the understanding of neurodegenerative diseases and neuronal functional development. Specifically, rodents' brain cells are the most usual resort for cells *in vitro* due to their affordability, easy manipulation and also the fast formation and maturation of the neuronal networks [4].

The essential purpose of these cultures is to offer a controlled, scaled–down and reproducible stage in which the analysis of intricate brain functions can be done. Generally, efforts aimed at grasping the complex functioning of neuronal networks are based mostly on two–dimensional studies. However, the natural environment of neurons is a complex three–dimensional (3D) extracellular matrix where they can project connections in all directions. Consequently, 3D cultures furnish physiological conditions much closer to the brain and enable the reproduction of much more complex architectures. Furthermore, such cultures enhance cell growth and differentiation, improve cell-to-cell and cell-to-matrix interactions, and eventually neurons exhibit richer functional dynamics and an extensive repertoire of activity patterns [4].

II. EXPERIMENTAL SETUP AND PROCEDURE

A. Neuronal cultures in hydrogels

Our 3D neuronal cultures were prepared in a semi-synthetic hydrogel named *PEGylated Fibrinogen* (Fig. 1). Hydrogels are elastic, gelatinous structures made from cross–linked polymer chains. These biomaterials imitate the native extracellular matrix of the brain and that maintains the structural integrity of the neurons. This type of culturing in hydrogels maintains the advantages of *in vitro* preparations (accessibility, easy manipulation) while advancing towards more realistic, brain–like *in vivo* models [4].

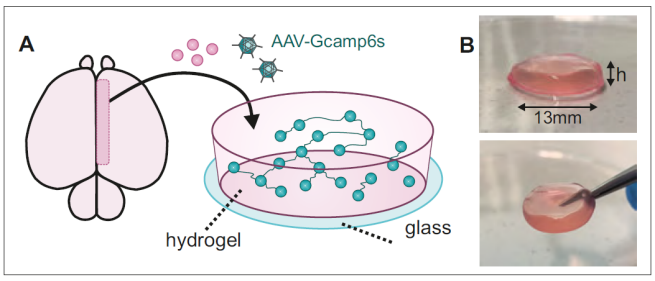

FIG. 1: 'PEGylated Fibrinogen' hydrogel culture of cortical neurons from rat embryos. (A) Sketch of culture preparation, combining neurons from rat brains, the GCamp6s fluorescence indicator and the hydrogel. (B) Photo of a typical hydrogel culture. Adapted with permission from Ref. [4].

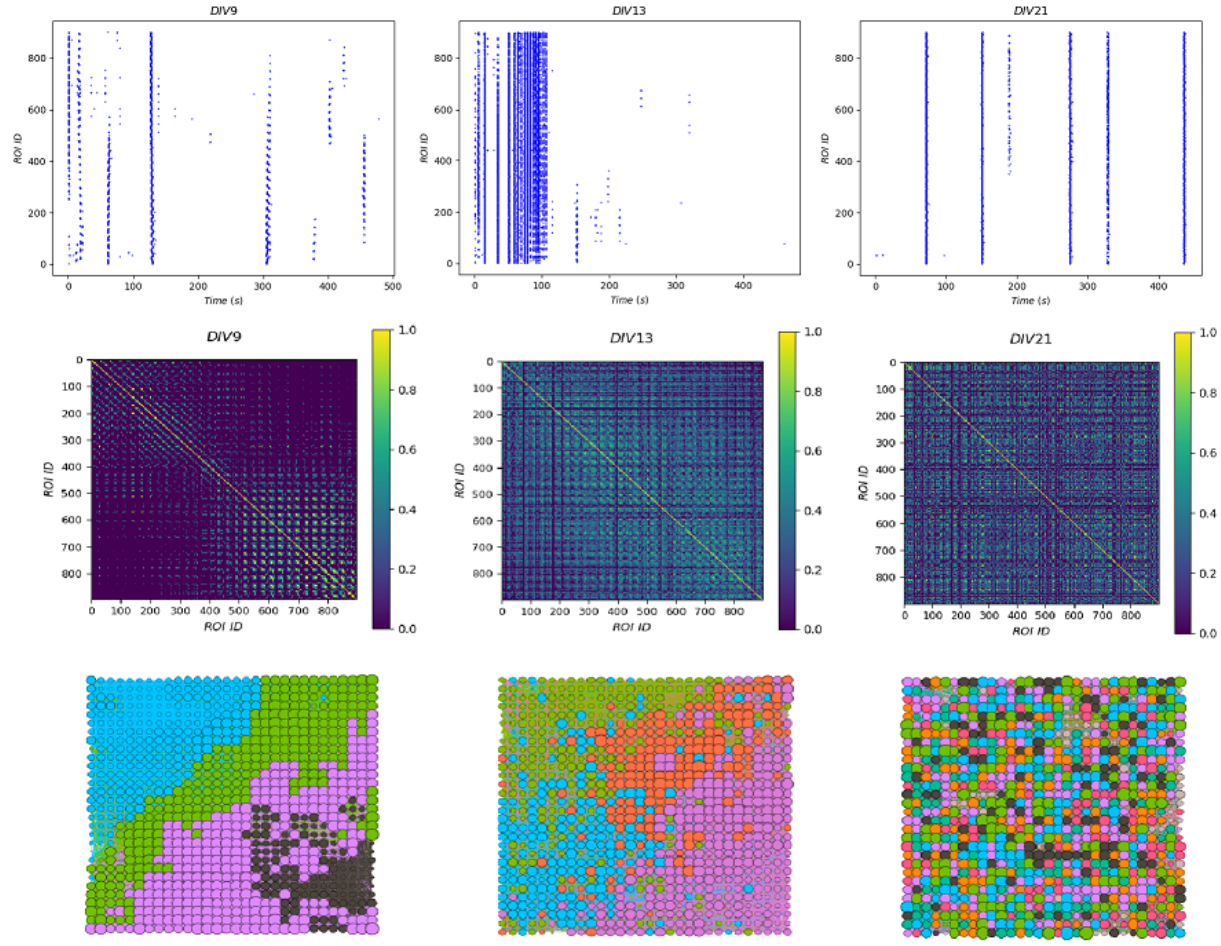

FIG. 2: Analyzed data. Columns correspond to DIV 9, 13 and 21, respectively. Top: raster plots of neuronal activations in the culture. Center: cross-correlation matrix. The color bar is the Pearson correlation coefficient. Bottom: representation of the network using Gephi. Colors indicate modules of preferentially connected neurons.

B. Calcium fluorescence imaging

Calcium imaging was used in this study to analyze 3D neuronal cultures for the purpose of describing their interactions as a network. The functioning of fluorescence microscopy is that a fluorophore excited by a certain energy (through radiation of a specific wavelength) emits a light that can be detected. Normally, green fluorescence proteins that emit green light ($\lambda = 514\,nm$) when irradiated by blue light ($\lambda = 490\,nm$) are employed.

Neurons are highly polarized cells, with such polarization being the basis of the flow of information in the nervous system. Consequently, neurons display a low intracellular calcium concentration (≈ 100 nM) that will rise around to orders of magnitudes at the moment of firing. Therefore, we can track calcium transients when neurons activate by using calcium-binding fluorescent proteins. Our optical system will detect those changes and will register a increase in the fluorescent signal at the moment that a neuron fires.To record these activations, cells in the neuronal culture were infected with adeno-associated viruses (AAV) encoding for the fluorescence indicator GCaMP6 at the moment of preparation [4, 5]. Neurons will genetically encode the indicator and express it continuously. Thus, we can track the same culture again and again for several days.

In the present study 3D homogeneous primary cultures in which neurons are distributed uniformly were used. Given the COVID-19 pandemic, it was not possible to carry out full experiments in the laboratory from the beginning. Therefore, Dr. Soriano provided three previously recorded videos, corresponding to neuronal cultures in *day in vitro* (DIV) 9, 13 and 21, in which firing neurons were observed as bright objects. All the recordings had approximately the same duration, around 500 seconds. Once the videos were available they were processed using NETCAL, Dr. Soriano's Lab software, through which data about the position of each neuron or *region of interest* (ROI) and the times of firing for each ROI were acquired (Fig. 2). With the obtained data a statistical analysis was conducted with Python, except the representation of the network, which was done in Gephi.

III. RESULTS AND DISCUSSION

A. General network properties

An important remark is that cultures evolve and therefore they change behavior constantly, with neurons creating new connections among them, or even degrading and dying due to changes in the environment or the deterioration of the hydrogel. However, despite these changes, we observed a general trend in which the culture gained synchrony over the days. This is shown in the raster plots of Fig. 2, with neurons starting to fire in small groups to gradually fire neatly together by DIV 22.

The analysis of synchrony leads to the study of the correlation among neurons. In order to do so, the cross–correlation (CC) amongst ROIs was computed using Python library SciPy, which includes a function that evaluates Pearson Correlation between two vectors, i.e., our ROIs. Following this method the adjacency matrix was obtained (Fig. 2), which indicates which neuron connects more strongly with others based on the Pearson value. Such figure provides a graphic representation of the modularity of the network (box–like groups along the diagonal of the matrix) and the connections existing among modules. The network displays a modular behavior particularly in the first day, as 4 modules can be seen in DIV 9 adjacency matrix. This modules can be represented over the space (Fig. 2, bottom), and indicate that they are spatially compact, suggesting that neurons preferentially connect to their neighbors. In the following days the modularity of the culture seems to decrease as the neurons become more interconnected. This observation suggests the creation of well–interconnected communities. The spatial maps show a complete mixture, indicating that many connections are long range.

A convenient method to estimate the modularity of the network in each DIV is using the Louvain algorithm for community detection, which provides a parameter that describes the proportion of within-group connections in relation to between-group connections [4], being $Q = 1$ the value corresponding to completely isolated neurons and $Q = 0$ an all-to-all connectivity. As shown in Fig. 3, Q shows a noticeable decrease in the first days, whilst in the later days it displays a light increase that suggests that the culture is reinforcing local connectivity.

B. Global efficiency and degree evolution

The analyses of the network in the previous section did not provide any details on how information is exchanged globally across the culture. For the purpose of quantifying the transport and communication within the network we use the Global Efficiency, given by [4, 6]

$$E = \frac{1}{N(N-1)} \sum_{i,j \in N, i \neq j} \frac{1}{d_{ij}}, \qquad (1)$$

where d_{ij} is the shortest path between nodes i and j, i.e. the minimum number of edges to be crossed to reach one another.

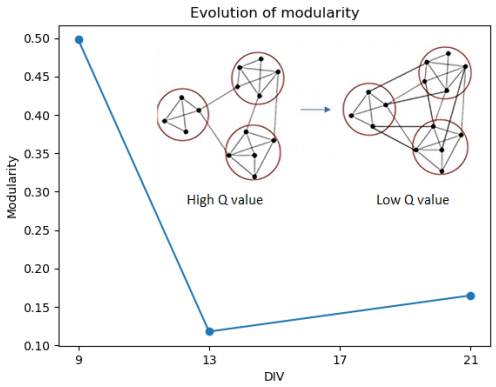

FIG. 3: Modularity decreases in the first 4 days, then it stabilizes seeking balance between activity and energy consumption. If data from subsequent DIV was available the figure would show a stability plateau. The inset shows a sketch of the decrease in modularity in a model network.

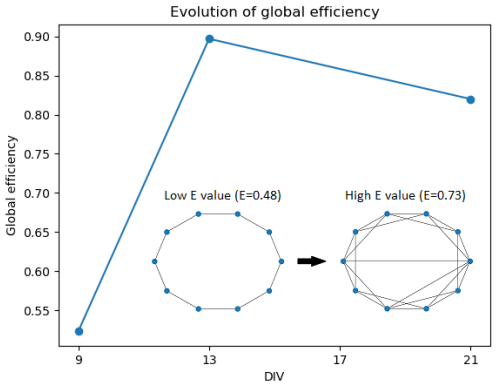

FIG. 4: Global efficiency rapidly grows in 4 days indicating that the neurons are becoming more connected, then it stabilizes.The inset shows an example of a model network in which the global efficiency increases as more edges are created.

Figure 4 shows that E abruptly rises between DIV 9 and 13, which concurs with the creation of well-interconnected communities of neurons mentioned in the previous section. Afterwards, between DIV 13 and 21, as local connectivity is enhanced and the network becomes slightly more modular in correspondence with Fig. 3, global efficiency experiments a slight decrease.

Another interesting parameter to monitor throughout the days is the degree distribution, which measures the probability $p_k(k)$ that a node has k connections to other nodes [6]. These distributions are shown in Fig. 5 as histograms. It is noticeable that DIV 13 and 21 present a much higher number of important nodes or hubs, i.e.

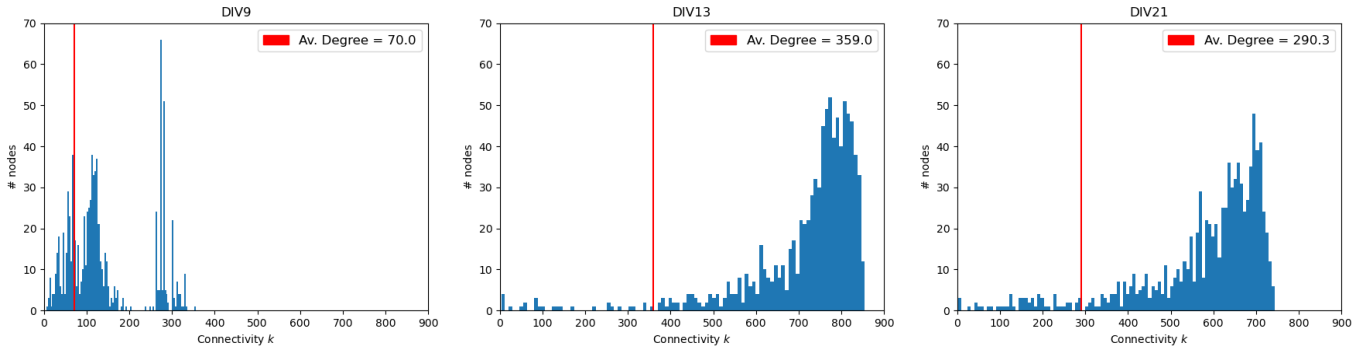

FIG. 5: Histograms of degree distribution of the network and average degree (red line) for each DIV, displaying the creation of densely linked nodes with time, i.e. neurons with very high degree. This is observed by the shift of the distributions towards the right, high k values.

nodes that exhibit a large number of links to other nodes, as compared to DIV 9, again indicating an increase in connectivity along time.

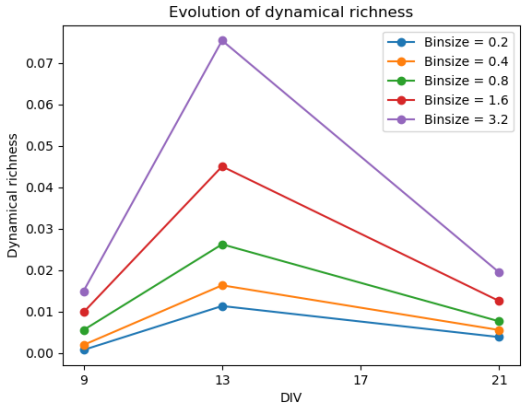

FIG. 6: Evolution of the dynamical richness of the culture throughout the DIV measuring the global network activations using different bin sizes and dynamical richness as a function of bin size for each DIV.

It is also noteworthy that the average degree of the cultures is maximum in DIV 13 instead of DIV 21 (Fig. 5), as would be expected if the neurons kept creating connections. We conjecture that this occurs because of neuronal homeostatic plasticity, in which the culture prunes connections to balance activity and energy consumption, effectually reaching an optimal 'set point'. Neuronal plasticity can be classified in two groups: Hebbian plasticity and homeostatic plasticity. Hebbian plasticity is a positive feedback that increases synaptic strength as a result repeated activations between neurons that facilitate subsequent activations, whereas homeostatic plasticity is a negative feedback that attempts to reach an optimal basal 'set point' of firing rates by either increasing or decreasing synaptic strength [4].

Thus, we conjecture that between DIV 9 and DIV 13 neurons create a large number of connections among them in order to maximize activity so that the neuronal culture becomes active as soon as possible. Then, between DIV 13 and DIV 21, homeostatic mechanisms rapidly eliminate edges of the network so as to find a balance between activity and energy consumption.

C. Functional complexity and dynamical richness

As a way to provide a direct measure for spatiotemporal variability let us introduce dynamical richness (Θ), following Yamamoto *et al.* [7] and Zamora-López *et al.* [8], in order to quantify the ability of the network to exhibit a vast variety of dynamical states. The higher the value, the richer the neuronal culture is. Consequently, a network will present $\Theta = 0$ for either completely random activity or persistent whole–network synchronization, and $\Theta = 1$ for a full range of coactivation patterns. Dynamical richness is computed as

$$\Theta = \Theta_{CC} \cdot \Theta_{GNA} = \left(1 - \frac{m}{2(m-1)} \sum_{\mu=1}^{m} \left| p_\mu(r_{ij}) - \frac{1}{m} \right| \right) \cdot \left(1 - \frac{m}{2(m-1)} \sum_{\mu=1}^{m} \left| p_\mu(\Gamma_t) - \frac{1}{m} \right| \right), \quad (2)$$

where the first term refers to the variability among pairwise cross–correlations (CC), defined as functional complexity by Zamora-López *et al.* [8], and the second one describes the variability of global network activations (GNA). Θ is then computed by using the distributions $p(r_{ij})$ and $p(\Gamma_t)$ for CC and GNA, respectively, and $m = 20$ as the number of bins used for evaluating the distributions.

A parameter that plays an essential role when estimating the distribution for the GNA is the size of the time bin that is considered to contain neurons that are firing simultaneously. To illustrate the importance of the bin

size, dynamical richness was computed through different bin sizes using the data of the three DIV (Fig. 6).

As the analyzed culture is homogeneous, it does not exhibit a high value for Θ in any of the DIVs (Figs. 6 and 7). Nevertheless, it is possible to notice that all the bin sizes studied display the same behavior throughout the days as Θ grows between DIV 9 and 13. This is because neurons keep creating connections and Θ slightly decreases between DIV 13 and 21 due to the emergence of synchronization. It is also observable that these changes are more significant when using a large bin size (Fig. 6).

Fig. 7 shows the changes in dynamical richness within the same DIV using different bin sizes. It is noteworthy that increasing the size of the bin does not have the same effect for all three days, since DIV 13, which displays the higher value of Θ, is much more sensitive to the modification of this parameter. Therefore, it may be sensible to suggest that above a certain threshold of richness and complexity of the neuronal culture, the Θ value of the network heavily depends on the chosen binning.

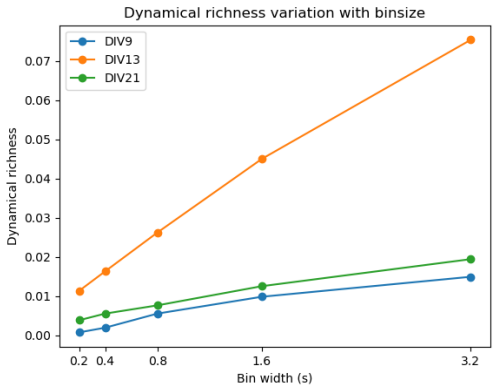

FIG. 7: Dynamical richness of the network as a function of bin size for each DIV.

IV. CONCLUSIONS

The previously exposed results and figures lead us to two meaningful results of great importance in order to comprehend the evolution of neuronal cultures in terms of their functional connectivity and activity.

Firstly, the representation of the network in Fig. 2 clearly shows that connections among neurons in the early days are of short range, whereas in the last days of observation correlation among neurons can be exhibited in a much larger range. This shows that neuronal cultures, specially during the early days, are an example of networks in which spatial embedding plays a key role during the formation and development of the network, which confers an additional feature to the strictly mathematical graph theory.

Furthermore, it is also shown that we still lack of a solid method to quantify the richness of a network in terms of its activity. All existing techniques require the definition of a bin width that will contain global network activations, which affects the analysis considerably. With a view to elect the optimal bin size to evaluate the complexity of the network, one can try to extract from the raster plot the approximate duration of a single activation. Thus, we could avoid both splitting such activation in two different bins and considering different firings to have the same origin.

In summary, despite the great progress made in neuroscience towards the understanding of neuronal cultures, a new procedure to estimate the complexity of the network that is not unbiased by the bin size needs to be found. In addition, the metrical dependence when creating connections among nodes needs to be taken into account if we wish to extrapolate the results obtained from the study of our 3D culture to such an intricate system as the brain.

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