Functional connectivity analysis of clustered neuronal cultures

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Abstract: The brain is an extremely complex structure and neuroscience is just a relatively new-born field. In this work we take a look on the process of how rat cortical neurons *in vitro* shape neuronal assemblies (clusters) with rich activity patterns. We use fluorescence imaging, trace each cluster individually and extract the exact timing of their neuronal bursts. We monitor cultures from their formation to their death, and use a genetically encoded calcium indicator (GECI) to monitor the same culture along different days. Using tools from network theory, for each day *in vitro* and culture we characterize their activity, connectivity traits and efficiency. We finally investigate possible correlations between these network properties and the maturation of the cultures.

I. INTRODUCTION

The neuron is the element unit of animal brain. By itself it is a cell that by voltage–gated ion channels can generate and propagate electrical signals. But there is not much that a neuron by itself can do. It is not until we have thousands of them carefully coupled between each other that they can serve any purpose. An interconnected system of neurons forms a *network*. A network is any system with sub–units that are linked onto a whole. It is the neuronal network itself and how its nodes (neurons) are connected what makes a functional brain. We still cannot fully understand how does the translation between the mesoscale and the microscale in a brain works, but in the lasts twenty years several techniques have been developed to investigate living brain cells, procuring important advances concerning brain mapping and functional interactions [1], [2].

In vitro neuronal networks are specially interesting since they have a manageable number of nodes or neurons, present spontaneous activity and can be precisely monitored. When cortical tissue is dissociated into individual cells, and despite the loss of the native connectivity, they will self–reorganize, form a new network by themselves and start exhibiting activity again [3]. The motivation behind our work is to gain insight into this fascinating process and its continuity. There is indeed a wide difference between the activity in a living brain and a neuronal culture, but the latter is a key tool to understanding the former. Indeed, the more we understand these cultures, the more useful they will be to understanding universal mechanisms in the brain.

When neurons in culture are free to move, they assemble onto neuronal *clusters*, which measure from 20 to 200 μm and contain hundreds of neurons. These clusters will connect to one another, shaping a neuronal network where nodes are the clusters and the links are the dendrites connecting the clusters. Clustered networks and the homogeneous ones present different properties; for example the cluster–like network presents more stability and a modular behavior [3].

It is also known [3] that when a neuronal network is formed, it will start to communicate with itself via *network bursts*, i.e. the co-activation of different clusters in a short time window. The timing and pattern of these bursts will provide us with the data to investigate these cultures, and will inform us of major network properties.

In this project we will work with *in vitro* neuronal cultures derived from rat cortical tissue, with the objective of studying how these neuronal networks and their properties evolve along days. The special component of this project is that, for first time in Soriano's laboratory, we count with a viral–infection protocol that allows us to incorporate specific genetic material onto the neurons, namely a a genetically encoded calcium indicator (GECI). The protein expressed by the neurons becomes fluorescence when binds calcium, and therefore we can monitor neuronal activity through fluorescence calcium imaging. GECI is completely innocuous to the cells, allowing us to trace the same culture from its formation to its death, without the need of having to change culture every measure and, thus, biasing the results.

II. EXPERIMENTAL SETUP AND PROCEDURE

Our neuronal cultures were prepared with the assistance of Sorianos's research group. Cortical tissue was extracted from deceased rat embryos and dissociated through pipetting. Neurons were plated on sterile 13 mm glasses in the presence a thin layer of polydimethylsiloxane (PDMS), which was pierced with four holes 3 mm diameter (Fig. 1).

At this stage we left the culture inside an incubator at 37°C and 95% humidity. The next day *in vitro* (DIV), we treated infected the neurons with the GECI, and waited for the neurons to assembly themselves into clusters, shaping the physical network. We started to monitor them in the microscope at DIV 5, when they started to show activity. Once the clusters are formed, their physical appearance and position do not substan-

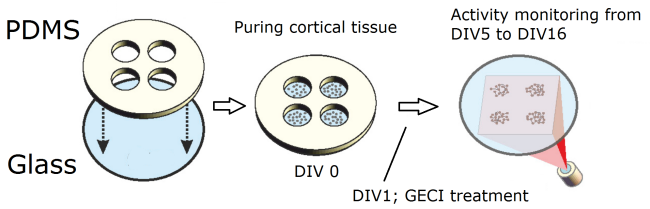

FIG. 1: Culture preparation. We prevented the neurons to anchor on the glass, so they could move freely and self-organize into neuronal clusters. The PDMS kept each culture separated, avoiding them to interconnect.

tially change along the next days [3].

For the image and data acquisition, we placed the cultures on an inverted microscope prepared for fluorescence. Firing clusters were observed as bright objects on the images (Fig. 2). Images were recorded at sufficient spatial resolution to see individual clusters well, and at high speed (100 frames/s) to characterize well network dynamics. Each measurement consisted of 20 minutes of spontaneous activity, every day along two weeks.

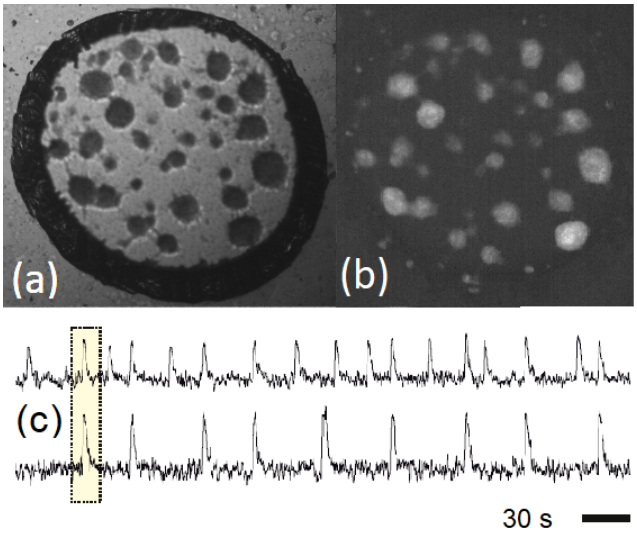

FIG. 2: (a) Bright field image of culture C32 (see table) at DIV9. (b) Corresponding fluorescence, integrated over a 100 frames. All the clusters can be seen bright, revealing bursting. (c) Characteristic activity traces of 2 clusters. Sharp peaks are activations. The yellow box marks two clusters that fire together in a short time window (network burst).

The result from a measurement was a high resolution video of the entire culture where we could precisely discern its clusters and firing patterns. In order to analyze the data we had to mark every single cluster as *region of interest* (ROI), finally obtaining a matrix of each ROI brightness level as a function of time [Fig. 2(c)].

The acquired data was processed with the software ClustDyn, developed in MATLAB by Sara Teller in Soriano's group. With it we essentially took every ROI and identified the firing events. In order to do that, a script was made to detected whenever the *fluorescence signal* had a sharp rise with an amplitude much higher than the surrounding noise. A co–activations of clusters in a given time shaped a network burst [Fig. 2(c)].

The final data was analyzed to extract, for each network burst, what we call a *sequence vector*, i.e. the set of clusters and their activation times that fired together. This information allowed us to see how the clusters were *functionally* connected to one another. We used time–delays to quantify such functional links. The idea is that the shorter the time–delay in the co–activation of any two clusters, the stronger their coupling (*weight*). By extending this analysis to all clusters and sequences, we could finally build the functional network of the culture. We note this network will be *directed* and *weighted*.

III. RESULTS

A. General culture properties

A total of 5 different cultures were recorded, labeled as **C1**, **C2**, **C31**, **C32** and **C33**, where the three last ones came from the same dissection. We also took data from an extra experiment labeled **CM**, which was monitored by a former lab member. The measurements took place from DIV5 to the death of the neurons, which occurred between DIV10 and DIV16, depending on the culture.

The table in Fig. 3 shows the number of clusters active each day and the days investigated. We can see how drastically different is every culture from each other, due to biological variability, which is already forewarning us that the results from network analysis may strongly vary from culture to culture. We note that the number of physical clusters (observed on the images) was approximately constant, but the number of active clusters varied along development. This observation also illustrates the biological complexity of such a neuronal network.

n.º Clusters	C1	C2	C31	C32	C33	CM
DIV5	21		26	30	9	68
DIV6			30	30	12	
DIV7	35	25	30	29	10	67
DIV8			21	21	9	68
DIV9	35		24	23	11	68
DIV10						68
DIV11						68
DIV12	33	12		14		68
DIV13	35			11		68
DIV14	35	10				68
DIV15		10				68
DIV16	32					68

FIG. 3: Number of *active* clusters per culture along development. The table also informs us of how many days the culture lasted, and in which days we could not measure.

In the firsts days (DIV $5-6$) the cultures showed weak activity and the frequency of bursts was irregular. This

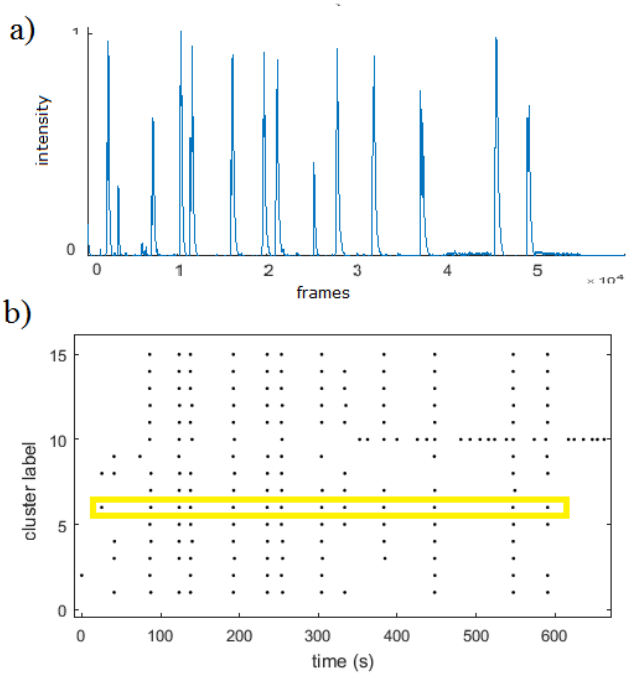

FIG. 4: (a) Bursts of a healthy cluster, for culture 31, during an entire measurement at DIV9. (b) Raster plot showing all cluster activations in the culture. The cluster marked in yellow is the same as in (a).

was probably due to important changes during early maturation, in which the culture creates connections and adjusts its firing patterns. As days passed, cultures showed higher activity and the frequency of firing stabilized.

The decreasing number of *active* clusters as cultures matured was intriguing. From DIV 10 onwards, some clusters began to burst less and with less intensity, until they could not be set as functional nodes. When a substantial number of clusters showed that state, we labeled the culture as dead and stopped the measurement. The small number of active nodes could lead to biased data.

An example of the problems associated with data analysis is provided in Fig. 4(a). When a cluster activates, its fluorescence ramps up and returns to the baseline as an exponential decay. This slow decay could cause some problems when it came to measuring young clusters. For example, when cluster activity was very high, a second burst could occur during the fluorescence decay, causing a merge of the two activity peaks onto one, making the identification of activation times very difficult.

Fig. 4(b) shows how the activation of a cluster often led to other activations in the same time window. If we consider a cluster (for instance the one highlighted in yellow) we can see that other clusters systematically fired with this one. Each concurrent activation is a *network burst*. Those clusters should be connected to systematically fire together. The analysis of the data in terms of network theory provides information about this connectivity.

B. Network properties

With the aid of the ClustDyn software, we extracted the different network properties of the cultures along their development. We calculated, at both a local and a global scale, the period between bursts, the network strength and efficiency, the density of links and the tendency of the networks to exhibit *small world* properties.

1. *Density of links and strength*

By defining the *degree* of a node as its number of links, then the *density of links* is the mean degree of the entire network. Since the networks are weighted, we can also define the *strength* of a node as the sum of all total weights [3], [4], $S_i = \sum_j (w_{ij} + w_{ji})$. The average over all nodes provides the mean strength of the network.

We must note that w is the normalized weight of a link, between 0 and 1, and informs us on the relative coupling strength between nodes. The strength can be also viewed as link density but taking into account the weights.

Fig. 5 provides an example of obtained functional networks, and the evolution of the link density and strength. Connectivity is weak at early days, DIV 6, and swiftly increase during maturation. The culture reaches a state of high connectivity by DIV9. When the degradation begins by DIV 12, clusters become silent and several links are lost, decreasing the network functionality.

2. *Global Efficiency*

It quantifies the exchange of information across the whole network, and is given by [3], [4]

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

where d_{ij} is the shortest path between nodes i and j, i.e. the minimum number of steps to reach each other.

The evolution of the *global efficiency*, averaged over cultures, is provided in Fig. 6. First, we can see a general increasing trend despite the fact that cultures died in different days, indicating that the network cohesiveness increases during development, probably due to the formation of connections. Second, G_{Eff} from DIV 5 to 7 shows strong oscillations. They may be a result of some sort of regulation effort, in which the culture quickly creates and modifies connections to reach activity and an efficient network as soon as possible. Once activity is regular, network interconnectivity gradually increases.

We observed that the evolution of link density and strength was very similar to the evolution of the *global efficiency*. That is to be expected as these three properties provide a measure of the interconnection of the network.

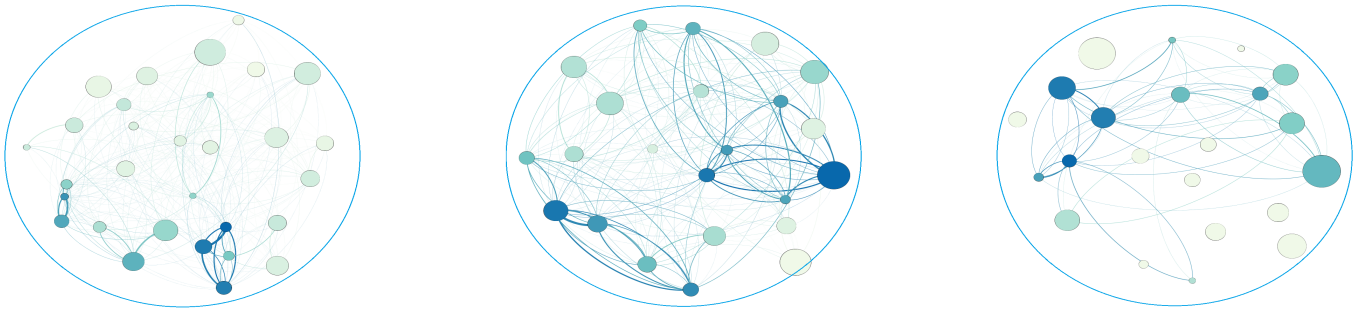

FIG. 5: From left to right, graphs of functional networks for culture C32 at DIV 6, 9 and 12. Only active clusters are depicted. Cluster size is the same as in the images. Clustes color is coded according to the strength; the darker the color, the higher the strength. Links' width and color are coded according to their strength; the darker the color, the higher the property.

3. *Mean Sequence Firing Rate*

It measures how much the clusters fire [4]. The comparison between *firing rate* and *global efficiency* along development for the 4 cultures is provided in Fig. 7. Here we found an interesting observation. The firing rate shows peaks, and coincide with the times at which the *global efficiency* starts to increase. Afterwards, the firing rate decreases. This decrease may be caused by self–regulation mechanisms of activity at early developmental times, and the degradation of the culture at later times.

In figure 7 we showed the network Strength instead the *global efficiency* since both grafics look almost identical.

4. *Small World properties*

Not only biological systems, but also in any real-world network, there is a kind of network organization that we constantly find, that is the *small world* property. A small world network characterizes itself for having high overall clustering and a relatively short path-length between any of their nodes. This means that every node is connected

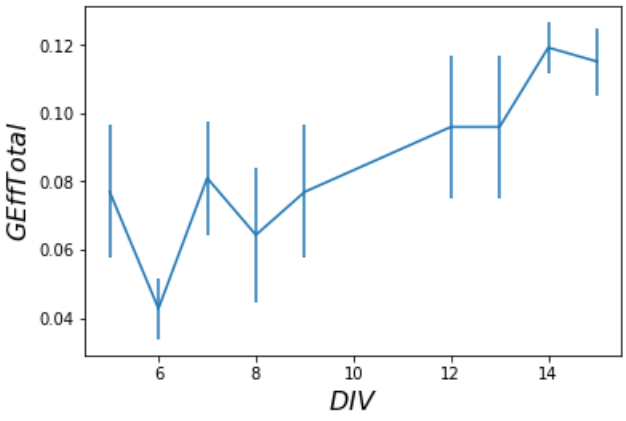

FIG. 6: Evolution of the global efficiency, averaged over 3 cultures. Error bars show standard deviation.

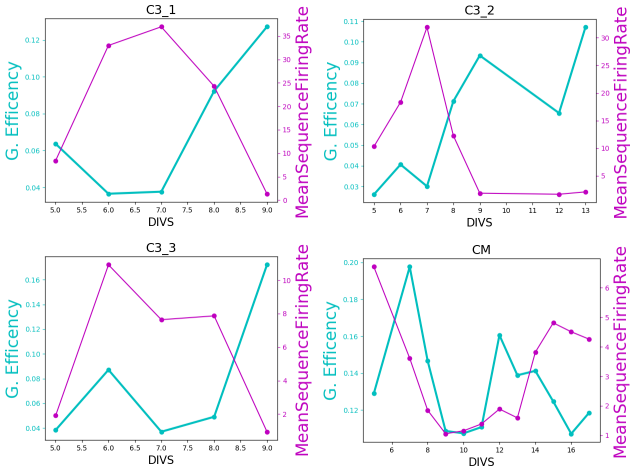

FIG. 7: Simultaneous graphic representation of the the network Strength and the Mean Sequence Firing Rate for the cultures C31, C32, C33 and CM.

to their neighbors, with the existence of some long links. Hence, it is possible to reach a given node from any other in a small number of steps (short path-length).

We calculated how our networks adjusted to a small world scenario as [4]

$$SW = \left(\frac{CC}{CC^r}\right)\left(\frac{G_{Eff}}{G^r_{Eff}}\right), \tag{2}$$

where CC is the *Clustering Coefficient*, the degree to which nodes in a graph tend to cluster together. It is calculated by dividing the number of closed triplets inside the network between the sum of closed triplets and open triplets (three nodes and two connections). The super-script r indicates the random-graph equivalent of the given property.

We made this analysis for each culture. The results fro 3 cultures are shown in Fig. 8. We found $SW \simeq 0$ at DIV 5, to rapidly increase to almost reach a clear small world trait ($SW \simeq 1$) around DIV9. Unfortunately, cultures died afterwards, so a complete evolution is lacking. Maybe they continued growing in DIV10 and DIV11, but

we cannot know since we could not measure those days. Only the CM culture neatly reached the small world condition, but it is obviously a too poor statistics. We could say that neuronal cultures tend to form small world networks, but we can not make a rigorous conclusion since not all our cultures reached this state.

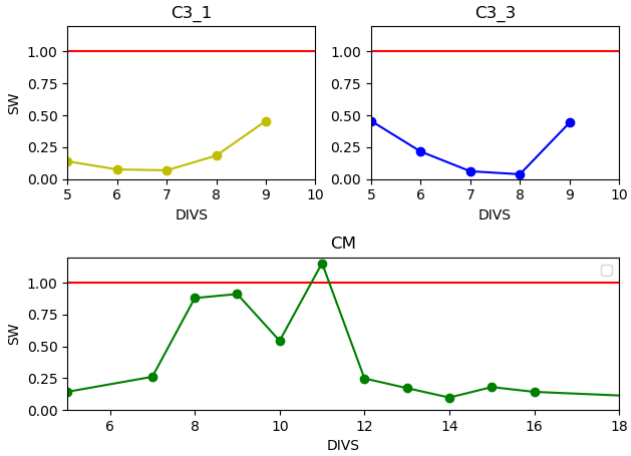

FIG. 8: 'Small world' evolution of cultures C31, C33 and CM.

IV. DISCUSSION AND CONCLUSIONS

This project offered the opportunity to work with *in vitro* neuronal cultures, which allowed us to carry out first-hand work in the fascinating field of neuroscience. We still have a lot of questions about the brain, and despite that, just working with the approximation of rat neuronal cultures was a enlightening. Cultures are not brain–like systems, but give valuable statistical data.

For first time the Soriano's lab had the chance to work with GECI, a calcium indicator that does not poison neurons and thus allows for the observation of the same neuronal culture along days. This approach alone was a major motivation for the present study.

In this study we observed that rat neurons *in vitro* start to present activity between DIVs 4 and 5, after the physical culture has established. Just after they begin showing activity, they start a process of maturation, which goes from DIV5 to DIC $7-9$. That maturation translates in an improvement of the culture strength, link density and global efficiency. The placement of the clusters is initially random, but, fascinating enough, the culture rapidly self–organizes itself into a fully functional network. We say that the maturation ends when the culture *global efficiency* no longer increases. After that point, G_{Eff} can experience some of variations around the lasts days of the culture, which is when it starts to degrade.

Another interesting observation was that the cultures' firing rate peaked just when the derivative of the global efficiency was maximum. This indicates that the biggest network modifications in terms of maturation and global efficiency occur when activity boosts. It is like if the cultures reach a growth spurt, rapidly bursting and testing themselves. This phenomenon was found in cultures C31, C32, C33 and CM. The most interesting changes occurred by DIV 9, when the cultures approached the small world state. Two other cultures, however, showed a frequency bursting peak but they did not adjust to the timing criteria we just mentioned.

We recommend to perform future experimentation in neuronal cultures at DIVs just after this firing frequency peak is observed, which does not need a lot of data treatment, since it is when the culture has reached full maturation and has not yet begun to degrade. This conclusion would have been possible to reach without the use of GECI, since the firing rate and global efficiency peaks happened at different DIVs, and without GECI we can only see a single DIV per culture.

That said, it would be really interesting to continue investigating these *in vitro* neuronal cultures since biological variability still plays a big factor in the results; the more experiments are made, the more conclusions we will be able to reach.

Acknowledgments

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[1] Bullmore Ed., Sporns O., (2009), Nature Reviews, Neuroscience.
[2] Bassett D., Sports O., (2017), Nature America.
[3] S. Teller Amado, (2015), PhD thesis, Universitat de Barcelona.
[4] S. Boccalettia, V. Latorab, Y. Morenod, M. Chavezf , D. Hwanga, (2005), Elsevier, Physics Reports
[5] Teller, S. et al., (2015) Sci. Rep. **5**, 17261.
[6] Teller, S., Granell, C., De Domenico, M., Soriano, J., Gómez, S., A., et al. (2014), PLoS Comput Biol 10, e1003796.
[7] Zamora-López, G., Zhou, C., and Kurths, J.,(2011), Front Neur Circ **5**, 83.