Spontaneous activity in neuronal networks with dictated connectivity

Author: Olga Ortiz Miquel*
Advisor: Jordi Soriano Fradera
Departament de Física de la Matèria Condensada
Facultat de Física, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain

Abstract: We studied spontaneous activity in rat neuronal cultures and compared two configurations, namely *homogeneous* and *patterned*. In the former, neurons grew over a plain glass substrate and therefore could develop isotropically. In the latter, neurons grew on a substrate shaped with topographical obstacles, which dictated the connectivity of the neurons and broke the isotropy of the system. Activity in all networks was recorded using fluorescence calcium imaging. By analyzing and comparing their activity patterns, we have found that activity in patterned cultures is markedly different, with richer spatiotemporal activity fronts and slower propagation speeds.

I. INTRODUCTION

Nowadays, the human brain is yet an unsolved puzzle, despite the years of dedication by the scientists. The brain is a complex system which processes and transmits information through a network of billions of connected neurons. A major open question is the relation between the connectivity of the neuronal network and the dynamics that produces. This question is complicated to solve in the brain given its large size and the impossibility to monitor all neurons at once.

To find alternatives to study the connectivity-activity relationship, neuronal cultures has emerged as a powerful instrument. These *in vitro* neuronal networks are models for the *in vivo* systems such as the brain, in the sense that allow to understand fundamental enigma and universal processes [1], such as spontaneous activity or information coding. Neuronal cultures are accessible, and allow to monitor on the order of 1000 neurons and perturb the characteristics of the network in an easy way.

Neuronal cultures are typically constituted by thousands of neurons grown on a flat glass substrate (Fig 1A, left). A variation precisely designed to understand the link between connectivity and dynamics is the *patterned cultures* [2]. This type of cultures is characterized by the presence of topographical obstacles (Fig. 1A, center and right) to impose a non-flat, non-isotropic substrate. The simplest way to design these obstacles is by using Polydimethylsiloxane (PDMS) molds, and that allow to obtain a dictated connectivity because of the PDMS structure obstacles (Fig. 1A, right).

The aim of this project was to study the initiation and propagation of activity fronts in patterned neuronal cultures, which are explained in detail in the section II. Down below, in section III, we present the main biochemistry employed in these cultures. Then, section IV presents the methods used to analyze the cultures recorded on the lab. Section V shows all the results obtained from the study of a patterned culture, with and without drugs, compared to a homogeneous culture. Finally, a summary of the project is provided in section VI.

II. NEURONAL CULTURES

Neuronal cultures are typically prepared from neuronal tissues extracted from specific regions of rat or mice brains, like the cortex [3]. The extracted tissue is dissociated by pipetting, and neurons settled out in a glass surface, where in the right conditions, they connect again to form a complex neuronal network. The *homogeneous neuronal cultures* that we analyzed were recorded in the UB laboratory of Dr. Soriano using *calcium fluorescence imaging*, a technique that detects the changes in calcium concentration in the neurons upon firing. This technique uses a fluorescent calcium indicator loaded into the cells, leading to fluorescence emission upon neuronal firing that can be recorded with an adequate fluorescence microscope and camera. In these homogeneous culture, all the neurons are distributed in a random way, and they usually establish connections with their nearest neurons. They show spontaneous activity, which is characterized by quasi-periodic activation events in which all neurons in the system fire together in a short time window (*network bursts*).

Patterned cultures are constructed with PDMS molds, which allow to modify the network connectivity (Fig. 1). Connectivity in these cultures can be modified by changing the PDMS molds' shape, giving us different layouts of networks connectivity. Consequently, the spontaneous activity would be different for each structure. To plate the neurons on the PDMS molds, we used adhesive proteins in order to keep them at the same place. By this way, the neurons can grow up only at the top or at the bottom, for example. The rich anisotropy in these cultures can be especially useful to understand the relation between connectivity and activity. The modification of the former through patterning should certainly modify the latter.

The patterned cultures used here were recorded by Soriano's group at the Weizmann Institute of Science (Israel).

* Electronic address: olga.o.m@hotmail.com

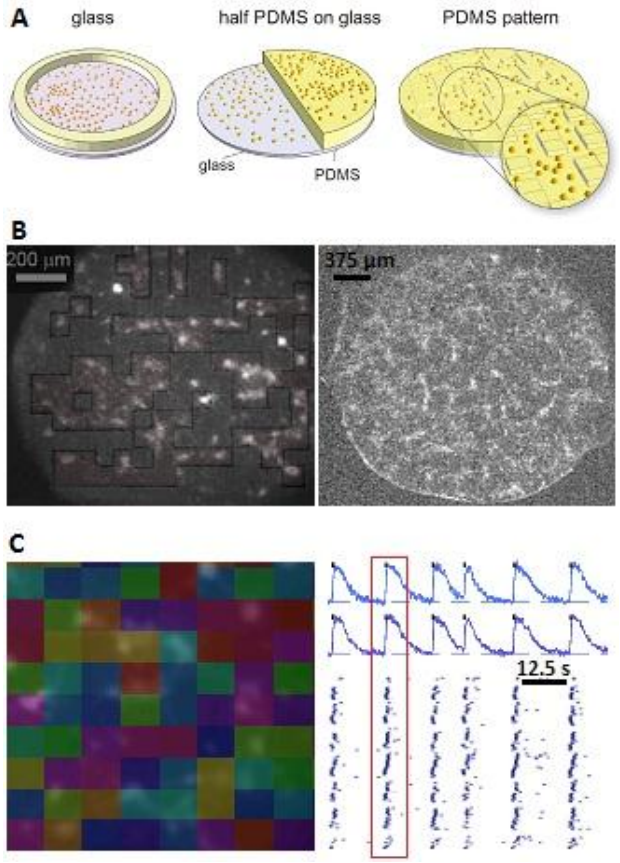

FIG. 1. A. Left: homogeneous culture whose area is limited by the shape of the PDMS mold. Center: half PDMS configuration which divides the culture in two regions. Right: the PDMS patterned produce a dictated connectivity due to the pattern constructed with elevated squares. B. Left: image of the patterned culture firing, with the patterned shape framed. Right: homogeneous culture firing. C. Left: region of the ROIs grid which allows to analyze the traces. Right-top: Fluorescence signal from two traces. Right-bottom: dynamics of the entire network. Each dot is a neuronal activation. X-axis is time, and Y-axis in ROI index.

III. EXPERIMENTS & PHARMACOLOGY

In this project we analyzed a total of 1 homogeneous culture (as reference of standard dynamics) and 4 patterned cultures, two of them with CNQX and the other with bicuculline (BIC). The effect of these drugs is explained below. Fig. 1B shows examples of the studied cultures. The homogeneous culture is young, recorded at day *in vitro* (DIV) 5. The others are more mature, recorded at DIV 14. Recordings in all cases were about 15 min long.

To understand the consequences of the drugs action, it is necessary to know how neurons communicate. Firstly, in a neuronal network, there is the *presynaptic* neuron that emits the signal, and the postsynaptic neuron that receives it. The signal crosses from one to the other through neurotransmitters, which are bound to the corresponding receptors in the dendrites of the postsynaptic neuron.

There are two types of neurotransmitters, those who increase the probability to produce an action potential (*excitatory*), and on the contrary, those who decrease the probability to produce an action potential (*inhibitory*). AMPA is the main excitatory neurotransmitter, and its action can be blocked through CNQX [4]. Correspondingly, GABA is the main inhibitory neurotransmitter, and its corresponding blocker is bicuculine (BIC in the following) [4]. Inhibition plays an important role in regulating activity in the brain, particularly in the hippocampus, a region of the brain which involves the memory formation.

Whereas GABA receptors always receive an inhibitory signal, AMPA glutamate receptors always receive an excitatory one. GABA neurons comprise about the 20% of the network, and the remaining 80% are AMPA excitatory neurons. Excitation is dominant, explaining the easiness for a culture to spontaneously fire.

In this project we analyzed data were CNQX was applied in two different concentrations (50 nM and 100 nM), to study of reduced excitation in the patterned culture. We note the [CNQX]=10 µM is the concentration at which neurons halt activity. Hence, the applied CNQX levels are relatively small.

IV. IMAGE ANALYSIS PIPELINE

We used the Lab software *NETCAL* to study the cultures. Since activity recordings were already provided, in this work we focused on their analysis through the procured software.

The analysis pipeline is as follows. The video of the recording is first loaded into NETCAL, providing first an image of the integrated fluorescence of the entire video (Fig. 1B) and the temporal evolution of the fluorescence frame by frame. This provides a global idea of the culture behavior. The only homogeneous culture analyzed is a young culture, and moreover, it has BIC applied.

Then, we have divided the culture into 440 regions of interest (ROIs) to get the trace of each ROI (Fig. 1C). After that, the traces are smoothed to minimize camera noise effects. Next, they are analyzed to compare different conditions.

All cultures show spontaneous activity in the form of *network bursts*, which represents episodes of intense coherent activity. Fluorescence amplitude increase a lot during this period to slowly return to the resting state (Fig. 1C, right). In NETCAL we used the *Schmitt* method to detect these high amplitude events, converting the fluorescence traces into data points that represent neuronal activations or *spikes*. They are shown in Fig. 1C for a patterned culture, where black dots indicate a neuronal activation.

The above process was repeated for all studied cultures.

V. RESULTS & DATA ANALYSIS

a. Spikes' raster plots

We have analyzed the structure of spontaneous spike trains in the network for each culture. In Fig. 2 we can see examples, reduced to only 100 s for clarity. First of all, in the homogeneous culture (Fig. 2A) there are 4 network bursts

(coherent activation of the neurons) in 100 s. In a homogeneous culture with BIC there is only excitation (E), because all the inhibition has been completely blocked by the drug. The separation between bursts is called *inter-burst interval* (IBI), and it can be seen as quasi-periodic. In general, the periodicity depends on the balance between excitation and inhibition.

In a normal homogeneous culture (E+I), it would be more activity, with a lower IBI, since inhibition prevents neurons from completely depleting their neurotransmitters after an activation, recovering faster and reducing the overall IBI [4]. Comparing with a patterned cultured, we can see that activation is more regular among neurons. We can also identify the burst duration, understood as the repeated activations of a neuron after firing. Neurons in the homogeneous case fire only once, while for the patterned case they fire few times.

For the first patterned cultures, as we can see in Fig. 2B, there are more bursts than in the homogeneous case. This is because of the presence of both excitation and inhibition (E+I). Furthermore, the bursts' duration is higher, resulting in a cloud of dots in the raster plot. This increased burst duration is possibly due to the patterned substrate, which facilitates the neurons to connect much more among themselves and, possibly, reinforces their activity.

With the addition of bicuculine (Fig. 2C) we would have expected a higher IBI, but it is approximately the same. This aspect is discussed in the next section.

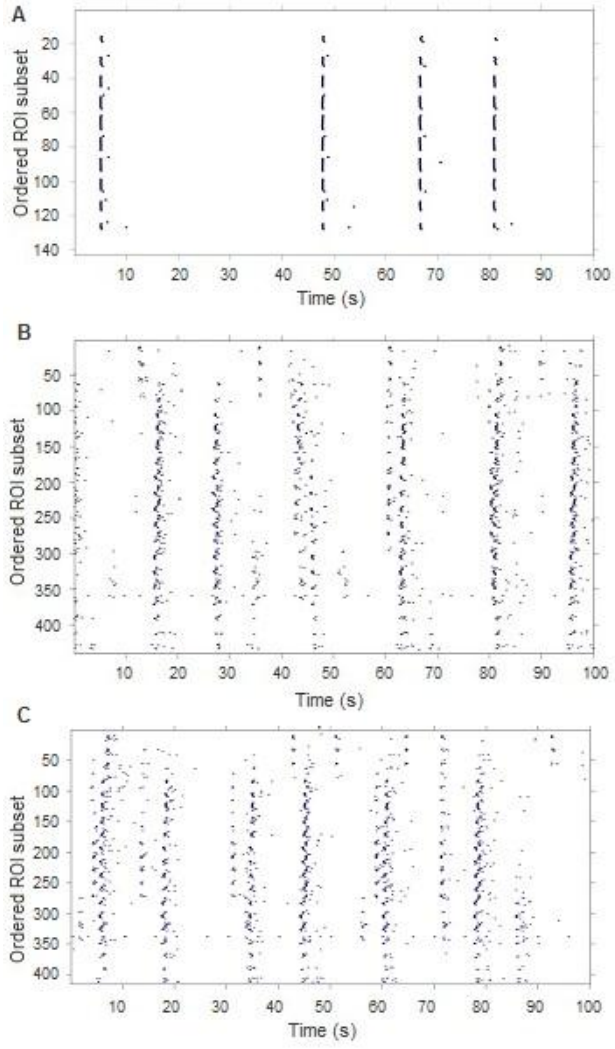

FIG. 2. Raster plots of spiking activity for three different cultures along 100s. A. Homogeneous culture with BIC. B. Normal patterned culture. C. Patterned culture with BIC.

b. Spontaneous activity fronts and statistics

By analyzing the duration of every burst in detail, we can clearly discern the activity patterns of homogeneous and patterned cultures, as we have already deduced from the raster plots. First, we looked at the spatio-temporal structure of the activity fronts. As shown in Fig. 3 (left panels), the burst propagation of the homogeneous culture occurs as a fast circular wave that initiates around the center and propagates towards the edges of the culture in about 0.4-0.8 s. Burst propagation in patterned cultures (Fig. 3, right panels) is about 3-4 times slower, and propagation follows a complex path dictated by the obstacles.

Second, we gathered statistics of the duration of the fronts (Fig. 4A). In general, fronts in homogeneous culture have always a short duration, with a distribution of durations centered at 0.5 ± 0.1 s; whereas the patterned cultures have a longer average duration of 3.1 ± 0.6 s and a broader distribution of values. These results are a proof of the complexity of the patterned culture. While the burst duration of a homogeneous culture has small variability, the values of the patterned culture ranged between 1.9 and 4.2 s.

Third, we looked at the accumulated fraction of active ROIs along time, averaged over different fronts. This means that we counted, at a given time, the number of neurons active at that moment plus those active in previous moments. As we can see in Fig. 4B (thick lines), 80% of the ROIs for homogeneous culture have activated in just 0.2 s, while patterned cultures needed twice that time. Also, the slope is much higher for the homogeneous case, indicating a faster transmission of signal

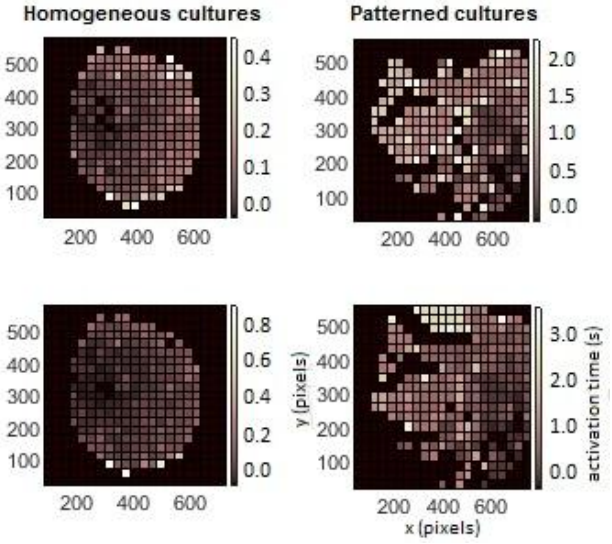

FIG. 3. Burst propagation maps for a homogeneous and a patterned culture, depicting the propagation of the active ROIs throughout the culture. The dark-to-white spots indicate the ROIs temporal sequence of activation (dark: initiation; white: end). Homogeneous cultures propagate as circular waves.

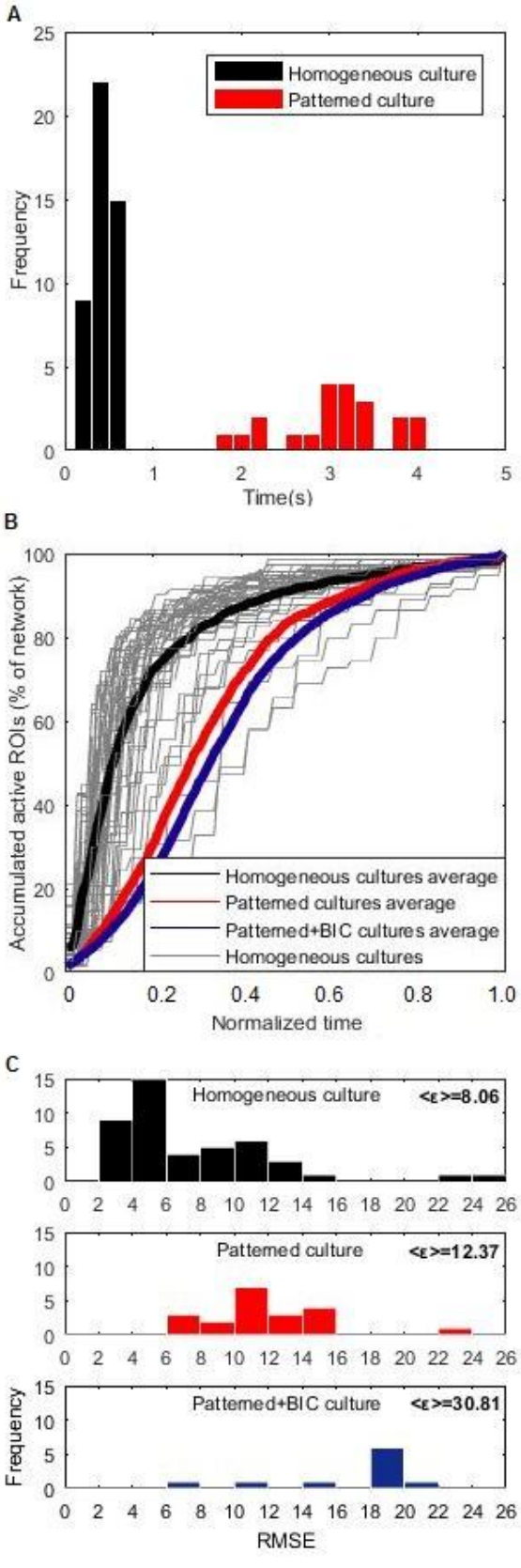

FIG. 4. A. Histogram plot representing the distribution of burst durations. B. Representation of the accumulated active ROIs during a burst along time. Thick black line is the average over n=46 bursts in the homogeneous culture, depicted in grey. Thick red and blue lines are averages in patterned cultures for normal and BIC conditions (n=21 in both). C. Root mean square error (RMSE, ε) for the three cultures. The average RMSE values (top right numbers) and the broadness of the distribution are higher for the patterned culture, particularly with BIC present.

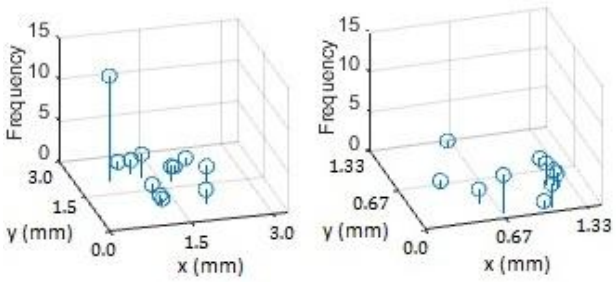

FIG. 5. Frequency map of the initiation point of activity for a homogeneous culture (left), and a patterned culture (right).

among neurons. We must note that these results are calculated using the burst time average of each culture. The addition of BIC slows down the fronts, but not significantly. This contrasts with homogeneous cultures, where the "E-only" culture has a much higher velocity of propagation than the "E+I" culture [1]. We think that the effect of the PDMS pattern is to make connectivity intricate, making communication among distant neurons very difficult, slowing down the fronts, and somehow minimizing the effect of BIC.

In the experiments done with CNQX, the number of bursts decreased drastically regardless for both cases (50 nM and 100 nM). There were only two and three network bursts, respectively, and the statistics too poor to be shown here.

The behavior of homogeneous cultures could be expected theoretically. Indeed, we can first consider a constant neuronal density. Then, we can assume for simplicity that all bursts start at the center, and that advance as circular fronts of equi-spaced rings in Δt intervals, taking as a reference the average difference between a firing neuron and the next one. With this construction, we get a parabola function for the cumulated activity. It can be compared to experimental data only in the short times range, as in the high times range, the experimental function become saturated due to finite-size effects. This behavior seen in the experimental function can be due to the borders of the culture, since initiation does not always occur in the center and some neurons reach the borders sooner than others.

c. Root mean square error among fronts

To analyze the complexity of the patterned cultures, we have determined the root mean square error (RMSE), which measures the overall difference between two functions. In our case we calculated the error between the individual accumulated fronts (for homogeneous, Fig. 4B, grey curves) and the average (for homogeneous Fig. 4B, thick black curve). As shown in Fig. 4C. the homogeneous culture has a low RMSE. This means that the burst' shapes and duration are in general similar, although some particular bursts substantially deviate (Fig. 4B). For patterned cultures, the average RMSE is about 4 times higher, and gets even higher when BIC is applied.

d. Bursts initiation points

To further describe the differences between homogeneous and patterned cultures, we looked at the initiation points of activity, i.e. the regions in the culture where the network burst started. These points were studied in unidimensional cultures (termed burst initiation zones, BIZs) [5] and in two-dimensional cultures as in ours [1].

In all the homogeneous cases we examined, the propagation followed a circular wave, and moreover, only one initiation point dominated. This is shown in Fig. 5, left, where one point at the top-left dominate. This dominant behavior was also observed in detailed explorations [1]. The origin of these points is a complex balance between connectivity, intrinsic neuronal activity and noise [1], but it seems that strong local connectivity plays a major role.

The patterned cultures (Fig. 5, right) had a more dispersed spatial distribution of initiation points. Although the majority of them concentrated on the fourth quadrant, there is no clear dominance of a point. It is possible that the dominant role described in [1] is broken when connectivity is so heterogeneous, but more experiments are required for strong conclusions.

VI. DISCUSSION & CONCLUSIONS

We have focused on the study of patterned cultures, and to give a degree of their complexity we have also used homogeneous cultures for comparison. Overall, we found the following differences interesting. First of all, the raster plot presents important differences in both cases, but neurons in patterned cultures showing higher durations and a clear irregular activity. The burst propagation velocity is approximately ten times higher in patterned cultures than in homogeneous ones. Furthermore, with the addition of bicuculine, the velocity and the IBIs do not seem to change in patterned cultures (contrasting with homogeneous networks, where the IBI increase). This can be due to the dictated conductivity of the pattern, and is a showcase of the complexity of this type of cultures.

In respect to the variability among burst' structure, patterned cultures have a higher standard deviation, as the burst shape is not always the same, in contrast to the homogeneous culture.

Finally, the burst spatio-temporal profile of the patterned culture does not follow a circular wave, but rather it draws a kind of spiral shape. These complex paths may also be related with a rich number of initiation points, possibly as an effect of the intricacy of the connectivity.

In conclusion, the dictated connectivity present in the patterned cultures makes them complex system with distinct differences with respect to homogeneous cultures. The former shows slow, complex activity fronts, while the latter shows fast, regular circular fronts. The study shows that patterned cultures are a valuable system to understand the impact of heterogeneous connectivity on dynamics, and make them an appealing approach to the complexity of the brain.

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