Study of the spontaneous activity in neuronal cultures

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Abstract: Action potential occurrences (spikes) were inferred from cellular fluorescence signals acquired through a calcium imaging technique in Dr. Soriano's laboratory. A *peeling* algorithm developed by the Brain Research Institute at the University of Zurich [4] was modified so that, instead of reconstructing simulated spike trains, it could deal with real signals. In this work, we reconstructed spike trains from different cultures and conditions. The reconstruction provided an overview of the collective dynamics of these networks.

I. Introduction

Brain is undeniably the most complex organ in the human body. Both its morphology and functioning are the result of numerous biochemical and biophysical processes interacting in a highly intricate manner across multiple scales in space and time. Neuroscience is thus a field in broad expansion, with many unresolved issues and unexplored approaches. The study of the nervous system dates back to ancient Egypt. A turning point in its evolution was the development of a staining procedure by Camillo Golgi during late 1890s, and the use that Santiago Ramón y Cajal [1] made from this technique, which revealed the morphology of neurons and the structure of the nervous system with astonishing detail.
Since Ramón y Cajal, there has been an astounding development in Neuroscience. However, as stated, there are many issues awaiting to be tackled. One of the major questions that is still not well understood is how the dynamics of individual neurons shape the collective behaviour of the whole network. For this reason, current research focuses both on the brain dynamics and the obtainment of more controllable networks in the form of neuronal cultures. The accessibility of the latter allows the combination of experimental recordings and physical modeling to understand universal mechanisms of neuronal network behaviour. This leads to the actual goal of this work, which is to relate the recordings of neurons with their actual firing patterns. Although this may seem simple, it is actually a very complex problem, and a target of study for many research groups worldwide.

A. Neuronal cultures

Neuronal cultures in Dr. Soriano's lab are prepared as primary cultures from rat embryonic tissues. The tissue is isolated, dissociated and plated in a biocompatible substrate [2]. Primary cultures are very versatile and represent a unique model system for unraveling a wide range of phenomena in Neuroscience and Physics [3].
Here we deal with two types of neuronal cultures: homogeneous cultures (fig. 1A) and aggregated ones (fig. 1B). The former are dissociated neurons affixed in a substrate of adhesive proteins, whereas the latter correspond to neurons platted in absence of such a protein, thus naturally aggregating and shaping a network of clusters connected among them. In this essay, we focused on two homogeneous cultures at DIV=18-19, respectively labeled HOMO_1 and HOMO_2, and one clustered one at DIV=14, labeled CLUS_1.

B. Neuronal activity and calcium imaging

Being able to measure the neuronal activity in these networks is undeniably a big challenge. In this case, we utilized a fluorescence technique called *calcium imaging* which uses either synthetic small-molecules or genetically-encoded fluorescent calcium indicators [4] to acknowledge changes in the intracellular calcium concentration. When a neuron fires, it elicits an action potential and there is an intake of calcium, which binds the fluorescent probe and makes the neuron brighter on the field of view (fig. 1A).

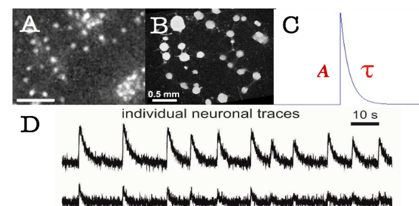

FIG. 1: (A) Homogeneous culture. The figure scale is 100μm (B) Clustered culture. The figure scale is 0.5mm (C) Patron transient, *peeling* algorithm (D) Sample individual neuronal traces.

Spikes are inferred from these calcium influxes (fig.1D), which are converted to fluorescence signals, but due to the noise and sampling rate, the relation is not straightforward. These fluorescence signals are expressed as relative percentage fluorescence signals after background subtraction. The transformation between the intracellular free calcium concentration $[Ca_i^{2+}]$ and the fluorescence signal is given by:

$$\Delta F/F = \Delta F/F_{max} \frac{[Ca^{2+}]_i - [Ca^{2+}]_{rest}}{[Ca^{2+}]_i + K_d} \quad (1)$$

$[Ca^{2+}]_{rest}$ denotes the resting calcium concentration, K_d the dissociation constant of the calcium indicator, and $\Delta F/F_{max}$ the maximal $\Delta F/F$ reached upon saturation. As the spiking is sparse and isolated, this relationship may be presumed linear –providing that fluorescence transients are far from saturation ($[Ca^{2+}]_i << K_d$) – and thus, eq. 1 can be linearized to:

$$\Delta F/F = \Delta F/F_{max}\frac{[Ca^{2+}]_i}{K_d} \tag{2}$$

This linear description is a good approximation in the low firing regime, where APs evoke stereotype, elementary calcium transients that can be approximated with a rapidly rising and exponentially decaying function (fig.1C). However, at higher AP firing rates, it may reach levels sufficiently high to cause substantial saturation of the calcium indicator, the transformation between the calcium concentration and the relative fluorescence becoming thus non-linear [4].

The reason for using calcium imaging in the laboratory is because it enables both *in vitro* and *in vivo* proper network monitoring with relatively simple and cheap optical devices. Despite the major drawback of not being able to discriminate quick or weak spikes because the typical frame rate during acquisition is slower than the cell's firing dynamics, it allows a very high temporal resolution, as well as an exact identification of the neurons involved and the capacity to monitor large populations of neurons, characteristics for which it emerges as a powerful technique [6].

The most important feature of neuronal cultures is their connectivity, which, at the same time, defines their activity. Activity is intimately related to the circuitry of the network, the type of neurons it contains and its dynamics. So to understand how connectivity defines activity, neuronal cultures constitute an excellent model system. At the beginning, the neurons in a culture are isolated, but as the culture grows, neurons start establishing synaptic connections, these networks becoming more and more complex as the culture grows. For sufficiently mature cultures, spontaneous millisecond-lasting firing episodes (bursts) arise, a fraction of the neurons taking part in the collective activity. These bursts are combined with periods of non-firing, thus leading to many and diverse activity patterns, as we are going to observe in further sections (subsec. III C).

C. The *peeling* algorithm

Among all the algorithms developed to infer the spike train underlying a particular observed calcium indicator fluorescence trace, we used the *peeling* algorithm introduced in (Grewe, B.F. et al., 2010) [5], which iteratively subtracts a template elementary calcium transient at event onset times, thus *peeling* away calcium transients until a residual noise trace remains [4]. In simple terms, after having set a carefully parameterized patron transient (fig. 1C), what it does is searching for similar shapes all along the signal fluorescence trace provided. This is why we call it a *matching* algorithm.

Each fluorescence transient detected is typically fitted in a two-step procedure with a model function composed of a rapidly-rising function and a double-exponential decay (the observed decay has two phases: a rapid initial phase followed by a slowly decaying one). In the first step, the onset is fitted in order to determine the start of the event and the onset time constant, and then, the entire calcium transient, so that estimates of amplitudes and time constants for the two decay components can be obtained. In this essay, in order to make the discussion simpler, each AP evoked a stereotype, elementary somatic calcium transient approximated by a single-exponential decay –see sec. III.

The peeled signal is what is called the reconstructed spike train. It may contain false negatives (missed spikes) and false positives (falsely discovered spikes). Theoretically, a comparative approach of the spike time differences (Δt) for all pairs of original and reconstructed spikes is followed, its outcome leading to the creation of two parameters condensing such information: the true positive rate TPR_{AP} (number of correctly detected spikes divided by the original number of spikes, also called sensitivity or recall) and the false discovery rate FDR_{AP} (number of falsely discovered spikes divided by the number of reconstructed spikes, also referred to as precision) [4]. In practice, what we did was play with the model transient amplitude and decay time to graphically maximize the number of real spikes.

Various methods have been explored for inferring spike trains from calcium fluorescence measurements: deconvolution techniques [7], template matching [8], model-based fitting [9] and Monte Carlo methods [10], but what makes this *peeling* algorithm so powerful is its ability to resolve spike times for spikes spaced as close as 40-50ms apart[5].

II. Algorithm modification

As previously stated in the abstract, the goal of this work was to modify the reconstruction algorithm explained in section I C so that it could be applied to experimental data from the Neurophysics laboratory.

The *peeling* algorithm was written in Matlab, and so were all subsequent variations of the code and data analysis carried. In order to modify the original code the minimum possible, a separate program was developed, which called *ModelCalcium*, the main algorithm of the original code, and used it as a function as well. In the developed code, some of the basic experimental parameters were initialized: the duration of the signal and the frame rate at which the images in the experimental setup were taken.

Not only these parameters were duly initialized at the beginning, but also those referred to the reconstruction itself. The most important ones are the following: the portion of the signal that wants to be reconstructed, the time constant for the main exponential decay τ_1 and the time constant τ_2 for the second exponential decay. We set τ_2 to zero, providing that we decided to model the spikes with a single-exponential decay for simplification.

Straightaway, a reading function was needed so that any kind of file in ".txt", ".dat" or ".mat" format (selected by the user) could be read. Afterwards, a function called *fitting* was created, with a triple purpose: firstly, to calculate the average signal over all the neurons in the culture and, secondly, to normalize the stored data while trying to correct the baseline. It is of utmost importance to normalize accurately and correct the possible drift in the baseline in order to rectify two factors that may be affecting the data: the fact that, as time goes by, cells find it more difficult to eliminate the inner calcium when bursting, and the fact that the fluorescent molecule degrades due to the permanent exposure to light excitation (a phenomenon known as *photo-bleaching*).

To properly normalize, the first step taken was getting rid of all those points above the standard deviation. Then, we considered the 5% of those in order to calculate the average noise amplitude F_0. Immediately after, we found the coefficients of a polynomial of degree n fitted using a least squares fit to the data. In this case, $n = 3$ was found optimal enough so that the spikes could be well preserved, i.e. their shape was not altered after the correction. Finally, the fit was subtracted from the original signal to correct for global drifts, and the fluorescence trace normalized to correct for the background brightness level. Mathematically, it is expressed as follows [11]:

$$\tilde{F}(\%) = \frac{(F - F_0) \times 100}{F_0} = \frac{(C - fit) \times 100}{F_0} \qquad (3)$$

with C corresponding to the initial fluorescence data F, fit the polynomial adjusting the data C, and F_0 the average amplitude of the background fluorescence, as previously defined.
The fitting procedure also facilitated another relevant parameter, which is the minimum amplitude of the fluorescence signal to consider an AP (in %), set as twice the width of the signal noise. For a more clear understanding of the signal being treated, we displayed a plot of some representative traces (figs. 2A, B & C). Systematically, the program also provides the signal average over all the neurons. Then, the *ModelCalcium* function was called inside a loop in order to obtain the spike times of all the neurons in the selected file. These time values were saved in a new file and then plotted. This final plot is very clarifying in terms of the activity, as one is able to visually discern whether the firing is simultaneous or scattered.

III. Results and discussion

As previously stated, our goal was to reconstruct different signals after having assessed the parameters needed for a good reconstruction. These parameters were later included in the *ModelCalcium* function for the actual reconstruction. We analysed three files with different characteristics, as summarized in table I. HOMO_1 is an homogeneous culture with very strong bursting, HOMO_2 is also a homogeneous culture, but with very weak bursting (i.e. a bad signal) and CLUS_1 is a clustered culture:

File	# neurons	fps	duration (s)	data size
HOMO_1	25	30	900	29 958
CLUS_1	28	100	1790	179 736
HOMO_2	101	33.33	900	29 987

TABLE I: main characteristics of the files. 'Data size' indicates the number of points for each neuron. 'Fps' refers to frames per second.

In order to have a clearer idea of the signals' behaviour, we plotted the fluorescence traces belonging to the first 12 neurons in each file. Figs. 2A, B & C display the results.

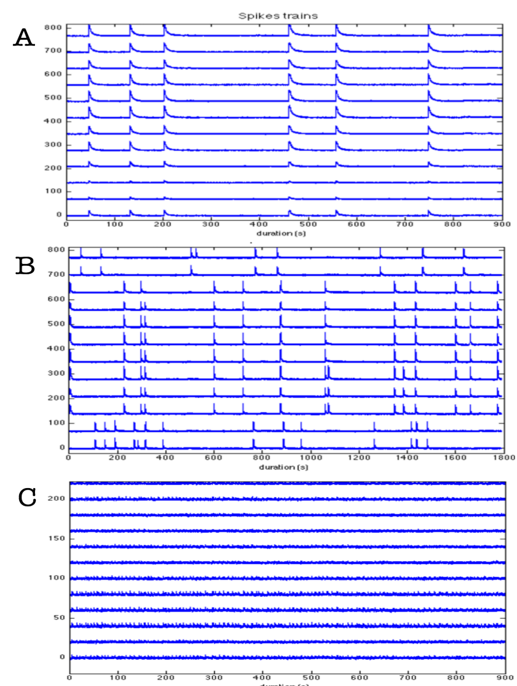

FIG. 2: Fluorescence traces from neurons 1-12 in **(A)** HOMO_1 **(B)** CLUS_1 **(C)** HOMO_2. The vertical axes are $\tilde{F}(\%)$ for each neuron, but vertically shifted for clarity.

As one may observe, especially in the first two culture –see figs. 2A & B–, activity is, regardless of the exhibited activity pattern, characterized by episodes of intense bursts and silent periods between these.

A. Analysis limitations

The *peeling* algorithm used in the *ModelCalcium* function is to be used for accurate experimental signals, that is to say, traces in which the fluorescence levels return to the baseline after the neuron fires. When the noise amplitude is high, the reconstruction process becomes extremely difficult, and, even if one is able to adjust the parameters in a way that it can be finally performed, the false discovery rate FDR_{AP} is so high that the reliability of such a result is really low.

With the implementation made when correcting the baseline -removal of all those points above the standard deviation of the fluorescence values–, reconstruction of noisy signals becomes definitely impossible. In this case, the *std* is so low -there is almost no dispersion from the average– that if one eliminates all those points above this statistical parameter, there are no values left. So, in file HOMO_2, reconstruction was not possible if this procedure was taken. However, if normalization and baseline determination were done taking directly the first 5% of fluorescence values without the *std* treatment, one could get to a reconstruction (of questionable quality, though). Thus, taking into account that, for this file, the *std* treatment had been omitted, we found interesting to include such example in the analysis.

B. Parameters values and limitations

Next, we present another table –tab. II– which summarizes the parameters describing the reconstruction: the portion of data selected for the reconstruction, the decay time constant τ_1 and the amplitude A_1. Notice that, instead of taking the whole signal, we only used a fraction of it in order to reduce the computational time required. The first value was conveniently chosen around 200s, τ_1 was by *trial and error* graphically set after having analysed values in a range [0.5-3], and A_1 was calculated as indicated in the previous section II: as twice the amplitude of the signal noise. In most cases, 1.5 times the value of the background amplitude should be enough so that the program does not miss events or detects false ones, but 2 was estimated a reasonably safer value.
As we took the decision to model the transients with a single-exponential decay function, A_2 was consequently set to zero, but, for some internal issue, the *ModelCalcium* gave errors when setting τ_2 also to zero. For this reason, we initialized τ_2 to a not null value of 0.5.

File	simulation duration (s)	A_1	τ_1 (s)
HOMO_1	200	4.52	1
CLUS_1	200	2.09	1
HOMO_2	200	0.69	1

TABLE II: reconstruction parameters and values

After having initialized the parameters just described, we used the *peeling* algorithm from *ModelCalcium* to exemplify some reconstructions, in which we compared the original fluorescence trace with the extracted spike trains:

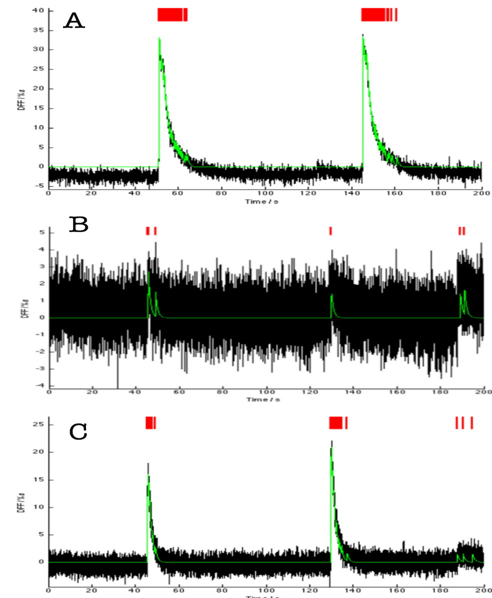

FIG. 3: Reconstructed signal for: (A) Neuron 20 in file HOMO_1 (B) Neuron 14 in file CLUS_1 (C) Neuron 20 in file CLUS_1.

In HOMO_1, as the spikes are clear, a really successful reconstruction was performed. CLUS_1 happens to be a much more interesting focus of discussion. As a cluster, the activity pattern, as we will see in further subsec. III C, is more diverse. By now, we may say that, whereas in neuron 20 reconstruction was satisfactory, in neuron 14, as the level of noise is considerable, and despite not being able to quantify the reconstruction quality, it is visible that one can not take it as a good result. As mentioned before, we include below the reconstruction of the data from 'bad' file HOMO_2:

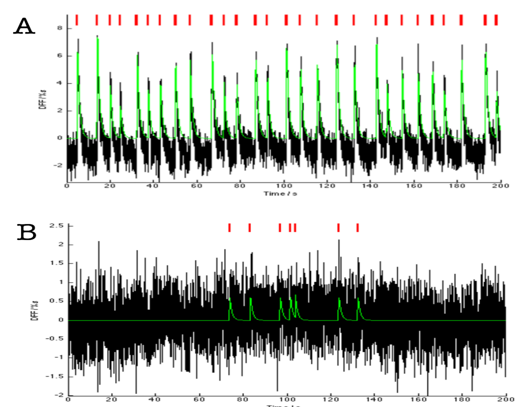

FIG. 4: Reconstructed signal for: (A) Neuron 14 in file HOMO_2 (B) Neuron 20 in file HOMO_2.

In this example, the situation is similar to that of file CLUS_1, despite some differences. A few neurons fire high, others do not. Therefore, there are optimal reconstructions (fig. 4A) and disastrous ones (fig. 4B), in which the program found some spikes that could well be noise.

C. Cultures dynamics and activity patterns

The electrical brain activity has an externally-induced component and an internally-generated spontaneous one. Despite many of the physiological mechanisms that initiate and regulate this spontaneous activity remain unknown, it has been seen that this activity determines important structural and functional features of neuronal circuits. In neuronal cultures, the spontaneous activity patterns depend on the neurons dynamics and the connections among them. The range of patterns one may have is extremely wide. In homogeneous cultures, neurons typically show a coherent activity. As can be seen in the following spike reconstruction raster plots (figs. 5A & B), belonging to homogeneous cultures HOMO_1 and HOMO_2, most of the neurons of the culture fire together in a short time window:

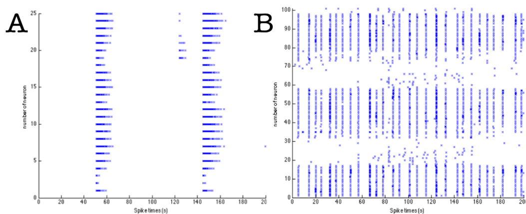

FIG. 5: Spike reconstruction raster plots for networks in homogeneous cultures HOMO_1 and HOMO_2.

This coherent activity is associated with a high synchrony degree among neurons. Besides, in fig. 5B there is another observation worth being made: not all the neurons fire; an important fraction of them remain silent. What is relevant about these two activity behaviours is that they correspond to neurons located in the same spatial regions, a fact that reinforces the above-stated affirmation pointing out the link between neurons' dynamics, activity and connectivity. Note that there are scattered points in the white regions corresponding to neurons that do not fire. They belong to bad reconstructions (i.e. fig. 4B), so these should not be taken into account.

Other interesting dynamical patterns one may have are those of clustered cultures, in which there is a tendency for small groups of aggregates to fire together, rather than the entire network at unison. In the example of fig.6, the 28 neurons in culture CLUS_1 form three clearly distinguishable activity groups:

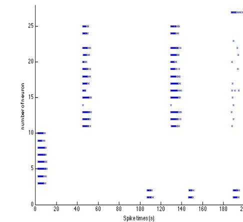

FIG. 6: Spike reconstruction raster plot for clustered network CLUS_1.

IV. Conclusions

Transient adjustment with high parametrization is of extreme complexity. Also of great difficulty is it to correct the signal baseline when normalization is attained. Nevertheless, the *peeling* algorithm was found to be a good tool for extracting the spike trains from experimental calcium data with relatively few parameters. Unluckily, it only works reliably for data with high signal-to-noise ratio and low FDR_{AP}.

Spike reconstruction raster plots allowed to study culture dynamics and spontaneous activity patterns of these cultures: homogeneous cultures showed a synchronous response, whereas clustered networks fired simultaneously by aggregation regions. Inferring culture dynamics after the connectivity networks is relatively easy, but trying to figure out the connectivity patterns from fluorescence traces –this is definitely a daunting task.

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