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Frequency variation analysis in neuronal cultures for stimulus response characterization

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	computational capabilities so it can be useful for robotic applications.
Response to Reviewers:	Following the kind suggestions of the editor, we have rewritten the former last paragraph in Introduction to clarify the novelty of the manuscript and also we have introduced a new separate paragraph at the end of Introduction to explain the structure of rest of the paper. The subsections of the former section 2 have been renumbered and reorganized as sections and their titles have been revised to better reflect the structure and sequence of the work. We have made a new proofreading of the whole MS to correct any minor English errors. Response to minor issues indicated by Reviewer #1: 1. "in-vitro" changed to "in vitro" and with italics formatting. 2. Inserted "of" in "has been the use of the post-stimulus". 3. "an MEA" was changed to "a MEA", considering MEA as an acronym pronounced as a word and not letter by letter. 4. "an hibrot" corrected to "a hybrot" and also corrected "Hybrots" in the rest of MS.

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Abstract *In vitro* neuronal cultures embodied in a closed-loop control system have been used recently to study neuronal dynamics. This allows the development of neurons in a controlled environment with the purpose of exploring the computational capabilities of such biological neural networks. Due to the intrinsic properties of *in vitro* neuronal cultures and how the neuronal tissue grows in them, the ways in which signals are transmitted and generated within and throughout the culture can be difficult to characterize. The neural code is formed by patterns of spikes whose properties are in essence non-linear and non-stationary. The usual approach for this characterization has been the use of the post-stimulus time histogram (PSTH). PSTH is calculated by counting the spikes detected in each neuronal culture electrode during some time windows after a stimulus in one of the electrodes. The objective is to find pairs of electrodes where stimulation in one of the pair produces a response in the other but not in the rest of the electrodes in other pairs. The aim of this work is to explore possible ways of extracting relevant information from the global response to culture stimulus by studying the patterns of variation over time for the firing rate, estimated from inverse inter-spike intervals, in each electrode. Machine-learning methods can then be applied to distinguish the electrode being stimulated from the whole culture response, in order to obtain a better characterization of the culture and its computational capabilities so it can be useful for robotic applications.

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Keywords MEA · Dissociated neurons · Neuronal stimulation · *Hybrots* · Machine-Learning

1 Introduction

Our brain is continuously processing all our sensory inputs to produce behavioural responses. In order to deal with these processes, neuron circuits generate multidimensional dynamics that encode multiple internal and external features. During the last decades, many researchers have approached the study of these processes by working with cell cultures of dissociated neurons, where it was expected that spontaneous circuits would emerge. The main idea behind that approach is such that the combination of controlled external electrical stimulation with the recording of the electrical output of the neuron culture would allow for the dynamics of the implicit circuits to be understood.

In neuron cell cultures, the early development of neuron circuits is controlled both by the spontaneous activity of the circuit [1] and by the interaction with the environment [2,3]. Despite the fact that cell cultures are grown in isolation, without any kind of input/output feedback, self-organized neuron circuits present spontaneous activity in the form of global oscillations, which are recorded in the whole multi-electrode array (MEA) [1,2,3]. Such spontaneous activity must be taken into account when neuron cultures receive any kind of stimulation, as the internal dynamics of the circuit may affect the respective response to stimulation.

According to Xydas *et al.* [4], the use of artificial sensory inputs and effector outputs seems to be a logical solution to overcome the re-embodiment problem. This has been one of the main motivations to study neuron cultures grown over a substrate-embedded MEA, merged with robots or simulated animals. As is the case with Braitenberg's vehicles, robots with simple hardware architectures that directly couple sensors and motors. Valentino Braitenberg [5] showed, by using different types of sensor to motor connections, how transfer functions were formed producing different observable behaviours of the robot in response to its environment. Several authors have used Braitenberg's vehicles as an approach to create and make sense of closed-loop systems [6,7,8].

Many attempts have been made to merge biological computation with robotics, where the principal aim was to explore the computational capabilities and learning capacities of such *in vitro* neuron cultures. In the future, this approach could allow us further understanding of how neuronal networks manage information in order to create high order behaviours. DeMarse *et al.* [9] made a neuronally controlled *animat* (artificial animal), to study "how information is processed and encoded in living cultured neuron networks by interfacing them to a computer-generated animal". The main objective was to study the interaction between the sensory inputs of these *animats* and the dissociated neuron culture in a MEA. Later, Bakkum *et al.* [10] delved into the study with such *animats* by researching about "how learning, memory and information processing in real time" is performed. They studied a promising approach by merging a disembodied neuron culture with a real robot, called a *hybrot* (hybrid robot), where they hypothesized that "if a given neuronal reaction is repeatable with low variance, then the response may be used to control a robot to handle a specific task". Applying pairs of electrical stimuli to different electrodes they found a way to produce stable non-linear responses that they characterized in order to control the robot's behaviour.

Also, Warwick *et al.* [8] proposed a "hybrid system incorporating closed-loop control of a mobile robot by a dissociated culture of neurons". They developed a methodological approach to characterize the instantaneous firing rate of the neuron culture in several electrodes in order to map the inputs and outputs of the overall system. Finally, Tessadori *et al.* [6,11] improved the previously mentioned approaches by adding one more step in the experiment, where after each collision due to a bad response of the cell culture, they stimulated the circuit to enhance the plasticity over the selected input-output electrodes.

The main requirement in any neuron culture embodiment is the characterization of neuronal dynamics after stimulation to identify which channels of the MEA can be used as sensory inputs (i.e. connected to robot's sensors) and which channels are to be used as motor outputs (i.e. connected to robot's wheels). The usual mathematical tool used for this characterization has been the post-stimulus time histogram (PSTH) [7,8]. The PSTH is calculated by counting the spikes detected in each neuronal culture electrode during some time windows after a stimulus in one of the electrodes. The objective is to find pairs of electrodes where stimulation in one of the pair produces a response in the other but not in the rest of the electrodes in other pairs. This electrode selection method cannot be applied to neuronal cultures with high connectivity, because in that case, it is difficult to find pairs of electrodes connected and isolated from the rest of the stimulus.

In this paper, we tried to overcome the difficulties involved in the PSTH method for characterizing pairs of connected electrodes in a neuron culture. To this end, a new method was developed and applied that uses the patterns in variation over time (immediately after a stimulus) of the firing rate at each electrode, estimated from inverse inter-spike intervals (IISI), to distinguish the electrode being stimulated. Jimbo *et al.* [12] reported that stimulation on different electrode positions can produce immediate differences in the firing rate properties on the recording electrodes, as a result of the interaction between excitatory and inhibitory synaptic pathways towards the recorded electrode. Following that, the main hypothesis is that the neuron culture has a global activation topology with a distinctive response depending on the stimulus localization, that can be used to obtain probably more than two simple pairs of inputs and outputs and thus allow its application to other more complex Braitenberg's vehicles.

The rest of the paper is organized as follows. Section 2 describes the experimental set-up including the process to select the electrodes to stimulate. In section 3 the preprocessing of data is explained. The pattern analysis of the data obtained is explained in section 4. Section 5 describes the machine-learning method applied to detect the stimulated electrode. In section 6 the robustness throughout the lifetime of neuronal culture is discussed. Finally, some concluding remarks and future perspectives are given in section 7.

2 Experimental set-up

Our set-up consists of a set of MultiChannel Systems (MCS) devices. A standard multi-electrode array (MEA) is composed of 60 Ti/Au/TiN electrodes of 30 µm in diameter, arranged in an 8×8 square grid and spaced 200 µm from each other that allow simultaneous recording and stimulation. A commercial 60-channel amplifier (MEA1060-BC) with limited frequency in the range of $10\,\text{Hz} - 3000\,\text{Hz}$ was used.

The stimulation was performed using the stimulator (STG1002) capable of multiplexing the signals for the stimulation of 60 electrodes in a nearly parallel process. The data were digitized through data acquisition cards with an analogue-digital interface. Each channel was sampled at a frequency of 25 kHz where the signal was filtered by a 2nd-order Butterworth bandpass filter in the range $300\,\text{Hz} \leq \omega \leq 1300\,\text{Hz}$, acquired with the MC-Rack software, also provided by MCS. Spike shapes and timestamps were extracted using MC-Rack software.

Embryonic rat brains (E16) were dissociated and the obtained cells were seeded over a substrate-embedded MEA from MCS. Cell cultures were maintained using a standard culture medium and were kept in an incubator with constant 37 °C temperature and 5 % CO_2. Experiments started 20 days after seeding. Spike shapes and time stamps were extracted using MC-Rack software from MCS.

The stimulus consisted of a train of 20 biphasic pulses of $\pm 2\,\text{V}$ amplitude and 1 ms period applied to a small subset of previously selected electrodes, with an inter-stimulus interval of 4 s. A series of 200 stimulations were applied to each of the selected electrodes, and the evoked response in the rest of the MEA was recorded.

In order to select the electrodes to stimulate, a first experiment was recorded at day 20 after the seeding of the experiment. We stimulated 10 times each electrode of the MEA and selected the two electrodes which evoked the greatest response in the whole array.

3 Data preprocessing

The recorded data were preprocessed to filter out the noise. In the first step, the root mean square (RMS) of each spike raw data was computed. The distribution of RMS values for all spikes is different from those events that are more similar to noise or to artefacts generated from stimulation. The events with an RMS value that were two standard deviations above or below the mean value were discarded.

As a second step to remove any existing noise that was wrongly classified as a spike, a 5th-degree polynomial was adjusted to each of the detected events using a 2 ms window centred in the minimum value. The obtained coefficients of each event were analysed using a density-based spatial clustering of applications with noise (DBSCAN) algorithm [13] that can find outliers respect to the main cluster of spike models. The events detected as outliers by the DBSCAN algorithm were also discarded.

Any stimulation trial that did not produce a significant increase in global activity, probably because a high burst of spontaneous activity had just occurred, was discarded and not considered for further analysis. In addition, only those electrodes that showed a significant firing rate after some stimulus were selected for analysis. To do so, we compared the firing rate of each electrode in a 150 ms window before and after stimulation (Wilcoxon test, $p < 0.05$). Only 26 electrodes, showing a significant response to stimulation, were selected. Figure 1 shows some examples of the evolution over time of the IISI immediately after a stimulus.

4 Pattern analysis

A total of 200 valid trials of a stimulus was performed on each of the two stimulation electrodes. The inverse inter-spike interval (IISI) of the activity in each one of the

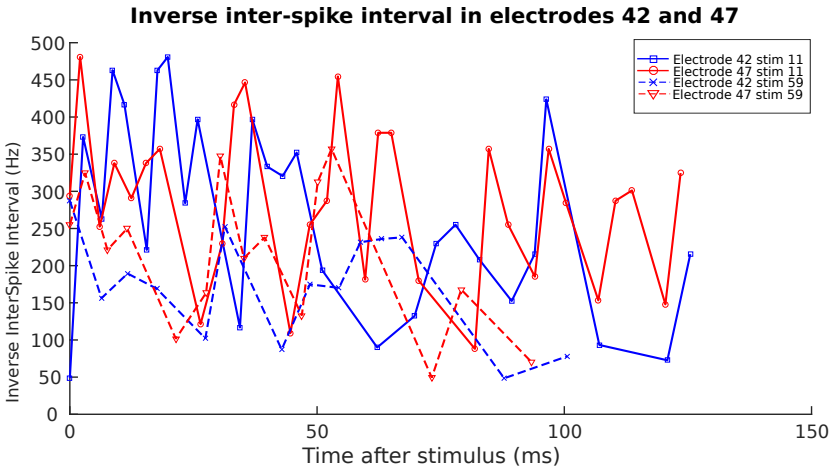


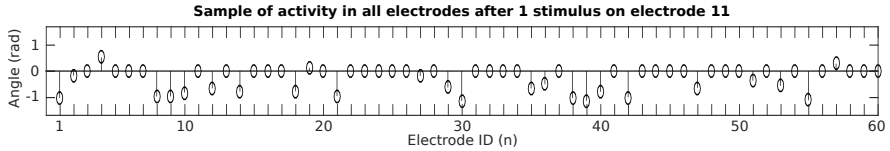
Figure 1: Samples of the evolution of sequences of inverse inter-spike intervals from two electrodes (indexes 42 and 47) immediately after a stimulation in other two electrodes (11 and 59).

26 selected responsive electrodes was computed along the 150 ms after each stimulus. Therefore, 200 vectors for each active electrode after a stimulation in each of the selected electrodes were obtained. Once these vectors were stored, we computed a linear regression to the IISI of each electrode and trial, in order to obtain a meaningful feature to predict the used stimulation electrode of a given trial. Thus, the corresponding slope (variation in time) of the IISI was used as a representation of the individual response to that stimulus on each electrode. The sequences with a very low number of spikes were eliminated from the analysis, replacing their slope with a zero value, as a regression on them could produce spurious values.

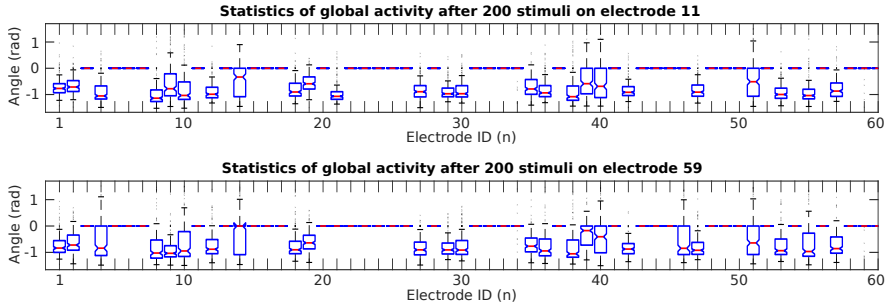
After the representation of each sequence by the slope, the pattern of responses on each electrode was used as a fingerprint to characterize the whole culture firing dynamics. The slopes from the linear regression were mainly negative (indicating a decrease) but slopes are not bounded and they could have large and isolated values. Therefore, slopes were normalised using the arctan() function, converting them to angles in radians that only vary from $-\pi/2$ to $+\pi/2$, in order to normalise all the information in a limited range of values. That limited range favours the use of the data in machine learning methods. Figure 2a shows an example of the global activity fingerprint after one stimulus through the representation of the angles of IISI variations for all the electrodes. Figure 2b shows the distribution statistics of the angles of IISI for responses of each electrode after stimulation in two selected electrodes.

5 Stimulated electrode detection by machine-learning

Finally, the fingerprints of the electrodes in culture response to each stimulation in two selected electrodes were used to train a supervised artificial neural network (ANN) [14] in order to classify which of the electrodes were stimulated. The 31 electrodes that showed no activity at all in any stimulation, the stimulated electrodes, and the



(a) Example of global activity fingerprint after one stimulus on electrode 11.



(b) Statistical distributions (box and whiskers diagrams) of angles of IISI responses for all electrodes after 200 stimulations in electrodes 11 and 59.

Figure 2: Global activity in all electrodes measured by the angle (in radians) of the IISI response after each stimulus. The electrode ID number is the order of each electrode as read by software, not the label (column-row position) in MEA.

ground electrode were discarded. The angles of firing rate variation of the remaining 26 electrodes were used to build the dataset.

The ANN was a standard multilayer perceptron with 26 inputs, a hidden layer of seven neurons with a hyperbolic tangent sigmoid transfer function, and one output with the standard sigmoid function to classify the stimulated neuron for the input pattern. The ANN was trained with the Levenberg-Marquardt backpropagation algorithm in MATLAB software using default values for the parameters.

The dataset had 400 patterns after pre-processing and filtering, 200 from stimulation in electrode number 11 and 200 patterns in electrode 59. The training was repeated ten times using different random weight initialization and pattern presentation order. After training, the average correct classification was $(97.9 \pm 1.7)\,\%$.

By visual inspection, comparing the two diagrams in figure 2b, it can be noted that there are two electrodes that have a distinctive response depending on the stimulated electrode. These are the recording electrodes 21 and 46. These electrodes have a high impact on the training because they make it "easy" for the ANN to distinguish between both classes. In order to test if the information was restricted to a very small subset of electrodes, or if the stimulation electrode could be also inferred from the rest of the circuit, we trained a new ANN excluding those electrodes with the biggest differences among stimulation conditions. Despite we could appreciate a decrease in the performance of the classifier, it was still far beyond chance classification $(69 \pm 10)\,\%$. This confirms that additional information about the stimulation electrodes can be extracted from the response on other electrodes different from those more evident ones.

Even though the analysis of the experiments was done off-line, all the computations are simple and could be optimized and adapted to run on-board a robot. The training

of the ANN can be done previously off-board, and then the resulting weights could be frozen and translated into the robot to identify neuronal culture response online.

6 Robustness throughout the lifetime of neuronal culture

It was necessary to check if the used strategy was robust during the lifetime of a neuronal culture, so the experiment was repeated up to nine times on different days. In figure 3 the variation in the percentage of correct classification by using the IISI strategy is shown. It can be seen that taking into account all electrodes with significant activity (blue line), the correct classification never goes below 80 %. On the other hand, the classification without taking into account the distinctive electrodes for each training set it does not even fall below 60 %.

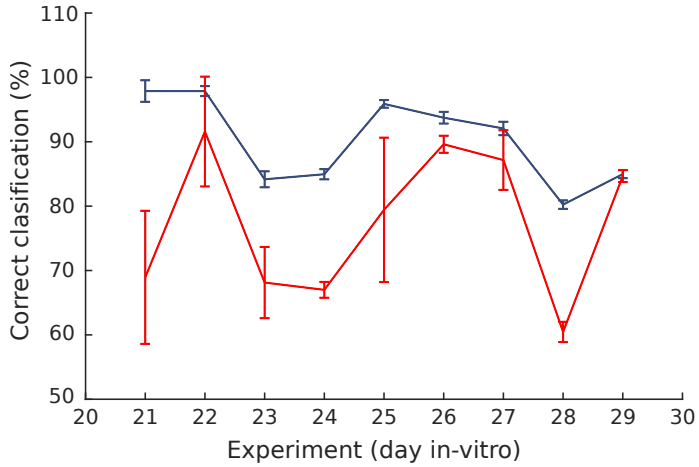


Figure 3: Evolution of the culture characterization over 9 days starting after 20 days *in vitro*. The classification results using all electrodes with significant activity, excluding stimulated electrodes, is shown in blue. The same results but excluding also the distinctive electrodes (21 and 46) for each case is shown in red.

There was a clear correlation between the classification results in both cases (including or not the distinctive electrodes). It can be supposed that the effect of stimulation in the electrodes produces different flows of neural activity in each experiment, but the data provided by the IISI method for all the electrodes can still give valuable information about that process.

7 Conclusions and future perspectives

The available evidence about neurons firing properties indicates that pulse trains are both, analogical (duration of the train of pulses) and digital ("all or nothing" behaviour) [15], as well as they manage information changes depending on the density of the train of pulses. These properties suggest a strong dependency of the neural code in the

frequency domain and its variation over time. The approximation presented here can take advantage of these properties to obtain a better characterization of the neuronal culture response, thus making it more useful for culture embedding in robots. Also, this method allows exploring and extending the possible forms to use the global response in a neuronal culture, because it does not depend on the response at specific electrodes after stimulation but on parallel computational processes of the neurons connected in the neuronal culture. Moreover, this approach allows us to analyse which electrodes are more relevant for the classification of a given stimulus based on the learned weights of the artificial neurons to study their possible role in the initiation of spontaneous activity or the study of neural circuit connectivity. The results show that the neural code can be modified by the local modulating activities when the correct electrode is stimulated, and the resulting neural flow can be used for characterization. It can be observed through comparison and correlation of data, with and without taking into account the distinctive electrodes, that IISI method can provide valuable information about the effects that electrode stimulation can produce on neural activity over time in the culture. In addition, the fact that the classification results increase and decrease over a few days may be due to cell migration and the generation of new connections formed along the growth of the culture. These changes can alter the neural flow produced after stimulation. The cell migration can occur in the reading electrodes as in the stimulated electrodes, that is, if one or more axon migrated slightly in the stimulation electrodes, it can result in that the stimulus has a different effect in the network with less important propagation.

The experiments were based on stimulation in only one electrode each time, making more difficult to affect the local pathways, so we expect that the results can be improved by having into account different stimulus properties, such as, the stimulus amplitude, frequency, duration and position, and by using a bigger subset of electrodes for stimulation in each experiment. Also, the number of stimuli performed could be increased in order to obtain enough data for the process of characterization through the ANN.

Considering the excitability of the self-organized circuit, any change in the Excitatory/Inhibitory balance, by means of using glutamate or GABA blockers, will cause major changes both in the spontaneous and evoked activity of the network. The addition of Mg^{2+} to the extracellular medium suppresses the bursting activity after stimulation and, in contrast, the addition of bicuculline increases it. These changes could drive the neuronal network dynamics into two different dynamical states: a non-chaotic deterministic behaviour system and a chaotic system where the system exhibits large spatial-temporal fluctuations [16]. Nevertheless, this exceeds the aim of this paper, as we intend to characterise the evoked response of self-organized, unaltered, neuronal circuits and to reproduce the experimental conditions described by DeMarse *et al.* [9] and Novellino *et al.* [6], where no blockers were used.

In summary, this study opens a new insight in the exploration of the computational capabilities of neuronal cultures, in terms of frequency domain and global activity response, to obtain a more complex mapping of stimulus and response in order to create more complex behaviours in a robotic context.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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