Enhancing Hebbian Learning in Biological Neural Cultures Through Electrical Stimulation

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Abstract— **Electric stimulation has been widely used to induce changes in neuronal cultures coupled to microelectrode arrays (MEAs). In this paper, we used low-frequency current stimulation on dissociated cultures of hippocampal cells to study how neuronal cultures could be trained with this kind of stimulation. We show that persistent and synchronous stimulation of adjacent electrodes may be used for creating adjacent physical or logical connections in the connectivity graph following Hebb's Law.**

Keywords- Cultured neural networks; Hebbian Law; Induced plasticity; Learning.

I. INTRODUCTION

Using biological nervous systems as conventional computer elements is a fascinating problem that permits the hybridization between Neuroscience and Computer Science. This synergic approach can provide a deeper understanding of natural perception and may be used for the design of new computing devices based on natural computational paradigms. Classical computational paradigms consist in serial and supervised processing computations with high-frequency clocks silicon processors, with moderate power consumption, and fixed circuits structure. However the brain uses millions of biological processors, with dynamic structure, slow commutations compared with silicon circuits, low power consumption and unsupervised learning. This kind of computation is more related to perceptual recognition [1,2], due to the natural variance of the perceptive patterns and the a priori lack of knowledge about the perceptual domain.

A real biological processor with millions of biological neurons and a huge number of interconnections would provide much more computational power instead of their low transition rates due to high number of computing elements and the extraordinary network capability of adaptation and reconfiguration to unknown environments. This extraordinary capability is related with natural unsupervised learning.

Microelectrode Arrays (MEAs) have been designed for direct culturing neural cells over silicon or glass substrates, providing the capability to stimulate and record simultaneously populations of neural cells. The use of dissociated cortical neurons cultured onto MEAs represents a useful experimental model to characterize both the spontaneous behaviour of neuronal populations and their activity in response to electrical and pharmacological changes.

Learning is a natural process that needs the creation and modulation of sets of associations between stimuli and responses. Many different stimulation protocols have been used to induced changes in the electrophysiological activity of neural cultures looking for achieve learning [3-13] and low-frequency stimulation has brought good results to researchers enhancing bursting activity in cortical cultures [10,11].

Hebbian learning describes a basic mechanism for synaptic plasticity wherein an increase in synaptic efficacy arises from the presynaptic cell's repeated and persistent stimulation of the postsynaptic cell. The theory is commonly evoked to explain some types of associative learning in which simultaneous activation of cells leads to pronounced increases in synaptic strength. Basically the efficiency of a synaptic connection is increased when presynaptic activity is synchronous with post-synaptic activity. In this work, we use this kind of stimulation to create adjacent physical or logical connections in the connectivity graphs using Hebb's Law.

In previous papers, we used a specific low-frequency current stimulation on dissociated cultures of hippocampal cells to study how neuronal cultures could be trained with this kind of stimulation [14, 15]. We showed that persistent and synchronous stimulation of adjacent electrodes may be used for creating adjacent physical or logical connections in the connectivity graph following Hebb's Law. In later experiments, we have used different parameters for this stimulation to check if those connections can be created stimulating with different configurations. The results provided in this paper show that low-frequency stimulation can create adjacent connections with different amplitude values. In addition, we present new results explaining when such connections have been established in a dissociated culture of hippocampal neurons grown onto a MEA.

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The outline of the paper is as follows. We first introduce the evolution of the related work on this topic. Next, we present the methods for addressing Hebbian Learning throw electrical stimulation. The following section shows the results obtained using a specific stimulation with our experimental setup on hippocampal cultures to train them. We conclude by discussing some crucial aspects of the research and the remaining challenges.

II. RELATED WORK

The first studies demonstrating functional plasticity in cultured networks began 1990s. The research group of Akio Kawana at NTT in Japan reported that tetanic stimulation through one or several electrodes resulted in plasticity [3]. They observed a change in the probability of evoking bursts by test pulses, as well as a change in the rate of spontaneous bursting, as a result of repeatedly evoking bursts using strong tetanic stimulation. Jimbo et al. reported similar results with a different tetanic stimulation and used voltage clamp to observe inward currents associated with evoked bursts [4]. The following year, Jimbo et al. reported that tetanizing a single electrode resulted in changes in the responses to test pulses to other electrodes [5]. In another paper, Jimbo et al. used simultaneous tetanization through a pair of electrodes to induce more precise forms of plasticity, expressed in detailed spike patterns evoked by electrical (probe) pulses [6]. Since then, a few other groups have reported on other forms of plasticity in MEA neural cultures.

Typically, these later papers have focused on more abstract plasticity results, more related to the network level than to the synaptic level. For instance, Shahaf and Marom reported that networks could be learn to respond in specific ways to test pulses, by repeatedly stimulating until the desired response was obtained [7], while Ruaro et al. reported that cultured networks could learn to extract a specific pattern from a complex image that had been presented repeatedly as spatial patterns of multielectrode stimulation [8].

In the following years, researchers have tried using more complex stimulation patterns in order to induce plasticity in neural cultures. Wagenaar et al. [9] looked for plasticity expressed in changes in spontaneous burst patterns, and in array-wide response patterns to electrical stimuli, following several induction protocols related to the previous ones, as well as some novel ones. Madhavan et al. [10] investigated patterns of spontaneous multi-single-unit activity to study the potential role of bursts of action potentials in memory mechanisms. Their analysis revealed spatiotemporally diverse bursts occurring in well-defined patterns, which remained stable for several hours. Chao et al. [11] compared five established statistical methods to one of their own design, called center of activity trajectory (CAT), to quantify functional plasticity at the network level. Stegenga studied the possibility of changing the spatio-temporal structure of spontaneous bursts using different configurations of tetanic stimulation. They obtained a profile of the array-wide spiking rate, a *burst profile* (BP) and also calculated the per-electrode spiking rate profile, the *phase profiles* (PPs). None of their stimulation methods had a measurable effect on of the specific burst statistics (peak firing rate, rise and fall times). However, they found many PP changes in their experiments, which can be seen as a confirmation that the analysis is sensitive to changes in the network.

Other researchers have focused in changing some stimulation parameters (voltage vs. current, frequency, amplitude…) to achieve learning. Brewer et al. [12] used chronic stimulation for getting an increase in evoked spike counts per stimulus and in spiking rate. The results obtained suggested that plastic network changes induced by chronic stimulation enhance the reliability of information transmission and the efficiency of multi-synaptic network communication. In turn, Martinoia et al. [13] applied low-frequency stimulation constantly applied over weeks. They found that the stimulation had a delayed effect modulating responsiveness capability of the network without directly affecting its intrinsic *in vitro* development.

III. METHODS

A. Cell Culture Preparation

Dissociated cultures of hippocampal CA1-CA3 neurons were prepared from E17.5 sibling embryos (Figure 1). During the extraction of the hippocampus a small amount of cortical tissue will have inevitably also been included. Tissue was kept in 2ml of HBSS. 10mg/ml of trypsin was added to the medium and placed in a 37° C water bath for 13 min for subsequent dissociation. The tissue was then transferred to a 15 ml falcon containing 4ml of NB/FBS and triturated using combination of fine pore fire polished Pasteur pipettes (Volac). Cells were then transferred onto 12 well plates (Corning Incorporated) containing glass coverslips (Thermo Scientific).

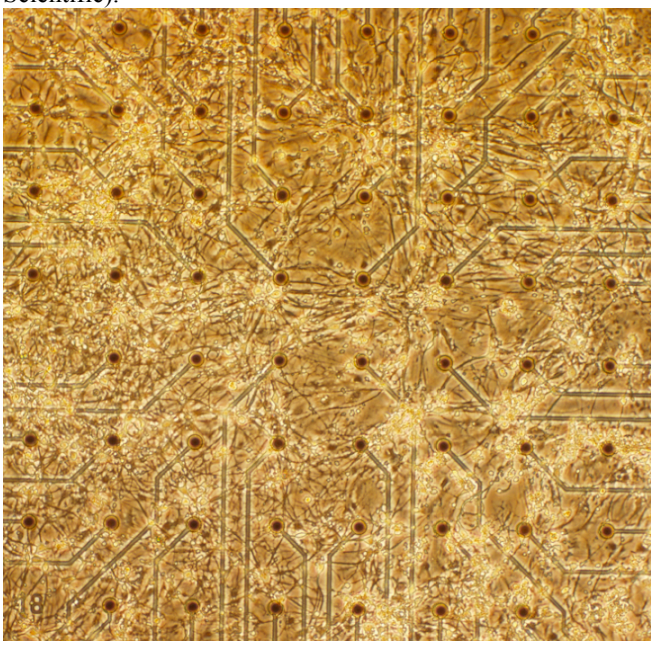

Figure 1. Hippocampal CA1-CA3 culture (21 DIV) on a microelectrodes array.

The coverslips were pre-treated overnight with PDL (50mg/ml), a synthetic molecule used as a coating to enhance cell attachment. The PDL was then aspirated away and the coverslips washed twice with PBS. This was then followed by a final coating of laminin (50μg/ml), a protein found in the extracellular matrix, to further help anchor the dissociated hippocampal cells. The cells were maintained in a mixture of 500ml NB/B27 (promotes neural growth) and 500ml NB/FBS (promotes glial growth), each supplemented with Glutamax and Pen/Strep (dilution 1/100). Glutamax improves cells viability and growth while preventing build up of ammonia and Pen/Strep helps to prevent any infections. Cell density for each coverslip was roughly 200000 cells. Cells were kept in an incubator at 37˚ C in 6% CO2.

B. *Expeerimental Setup*

Microelectrode arrays (Multichannel systems, MCS) consisted of 60 TiN/SiN planar round electrodes (200 μm electrode spacing, 30 μm electrode diameter) arrange in a 8x8 grid were used. Two pairs of electrodes were selected for creating functional connections between them. The activity of all cultures was recorded using a MEA60 System (MCS). After 1200X amplification, signals were sampled at 10kHz and acquired through the data acquisition card and MCRack software (MCS). Electrical stimuli were delivered through a two-channel stimulator (MCS STG1002) to each pair of electrodes.

C. *Expeerimental Protocol*

Three experiments were carried out using a total of 15 cultures during 2-3 weeks. Five cultures were stimulated in each experiment with a low-frequency electrical stimulation, which differs only on a few parameters. Each experiment started when cultures had 14DIV. The following summarizes the experiments and stimulation applied to the cultures:

1) *Experiment1 (E1): Cultures ID48-52*

a) *Days of experiment:* 16.

b) *Stimulation1:* train of 5 biphasic pulses cathodic-first (50 μA peak, 100 μs phase, 50ms ISI) was delivered every 3s for 10 min.

2) *Experiment2 (E2): Cultures ID68-72*

a) *Days of experiment:* 10.

b) *Stimulation2:* train of 5 biphasic pulses cathodic-first (60 μA peak, 100 μs phase, 50ms ISI) was delivered every 3s for 8 min.

3) *Experiment3 (E3): Cultures ID73-77*

a) *Days of experiment:* 11.

b) *Stimulation3:* train of 5 biphasic pulses cathodic-first (40 μA peak, 100 μs phase, 50ms ISI) was delivered every 3s for 8 min.

In every experiment, two pairs of electrodes with no logical connections between them were selected using connectivity diagrams based on cross-correlation. In every stimulation session these steps were followed:

1) Spontaneous activity was recorded for 2 min after a recovery period.

2) Cultures were then stimulated through the two pairs of electrodes using the corresponding stimulation protocol.

3) Spontaneous activity was recorded for 2 min after the stimulation.

D. *Analysis Performed*

We observed the spontaneous activity of the cultures before and after the stimulation experiments, as well as their evoked response to the applied stimulus. Extensive burst analysis, post-stimulus time histograms and functional connectivity were the main analysis performed to the registered data.

Correlation and information theory-based methods are used to estimate the functional connectivity [16, 17] of in-vitro neural networks: Cross-correlation, Mutual Information, Transfer Entropy and Joint Entropy. Such methods need to be applied to each possible pair of electrodes, which shows spontaneous electrophysiological activity. For each pair of neurons, the connectivity method provides an estimation of the connection strength (one for each direction). The connection strength is supposed to be proportional to the value yielded by the method. Thus, each method is associated to a matrix, the Connectivity Matrix (CM), whose elements (X, Y) correspond to the estimated connection strength between neuron X and Y.

High and low values in the CM are expected to correspond to strong and weak connections. By using such approach, inhibitory connections could not be detected because they would be mixed with small connection values. However, non-zero CM values were also obtained when no apparent causal effects were evident, or no direct connections were present among the considered neurons.

In our experiments, *Connectivity maps* offered a visualization of the connectivity changes that occur in the culture. Connectivity maps were generated using the connectivity matrix (CM) obtained after applying the analysis and Cross-Correlation or Mutual Information. By setting thresholds in the CM, it is possible to filter out some small values that may correspond to noise or very weak connections. In consequence, these maps show the strongest synaptic pathways, and can be used for visualizing the neural weights dynamics, and validate the achieved learning.

IV. RESULTS

The low-frequency current stimulation used in this study had an impact on the electrophysiological responses of the cultures, as previous studies had reported [13]. Raster plots showed that all of the stimulations provided induce changes in the firing frequency of the cultures.

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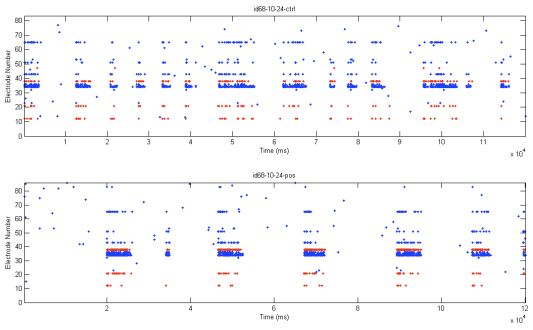

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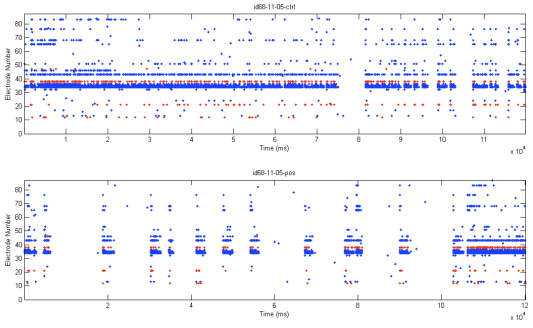

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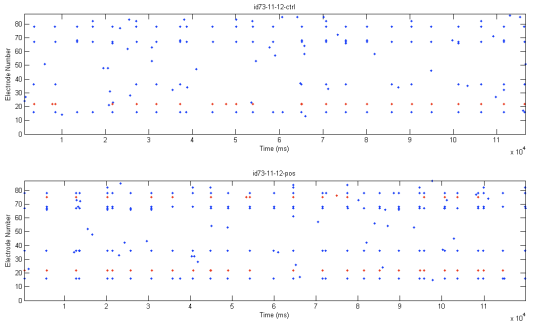

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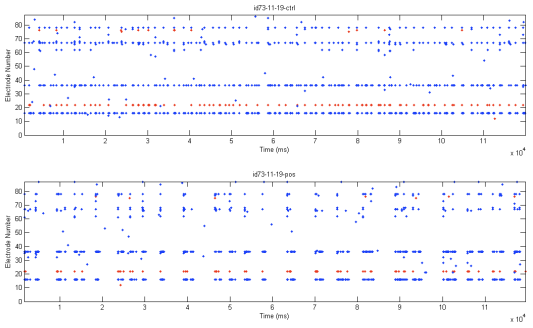
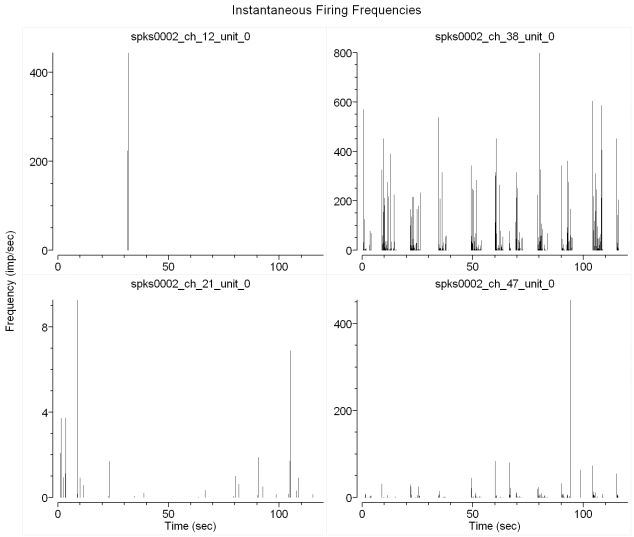


(d)

Figure 2. Raster plots extracted from cultures of experiments E2 and E3. (a) (21DIV) and (b) (32DIV) belong to ID68 from E2, (c) (30DIV) and (d) (37DIV) belong to ID73 from E3. Each figure is divided in two graphs, which show the spiking activity of the culture before and after stimulation. Raster plots show a change in the spiking activity, changing from a uniform activity before stimulation to a more concentrated activity after stimulation. This result is emphasized after the third week in vitro due to the maturing occurred in the cultures.

Furthermore, we can observe some kind of reorganization in the firing activity, from a uniform spiking activity to a discrete spiking activity. After the third week in vitro, the bursting activity becomes more frequent and robust and this effect is much more evident than during the first weeks (Figure 2).

The change on the spiking activity of the cultures can also be seen clearly observing the instantaneous firing frequencies (Figure 3) and the interspike intervals (Figure 4) over the time. Instantaneous firing frequency graphs shows that stimulated electrodes start firing in more separated period of times after stimulation but each firing period last longer.



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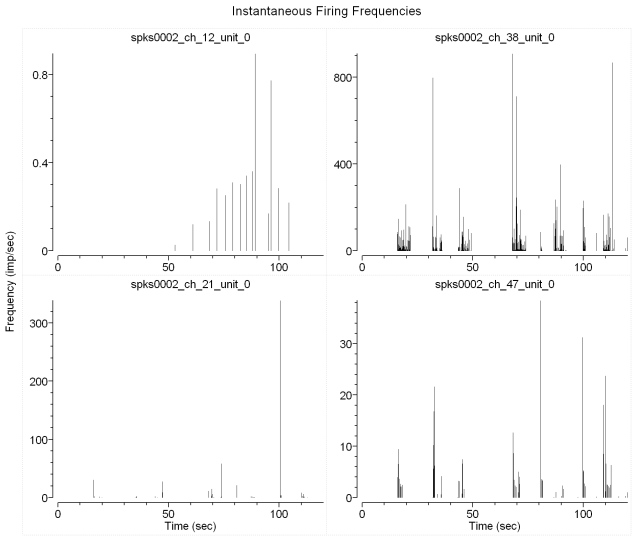

(b)

Figure 3. Instantaneous firing frequencies of stimulated electrodes in the culture ID68 (22DIV) from E2 before (a) and after (b) stimulation. A change in the spiking periods can clearly be seen, which are less in quantity and longer in duration. This culture only created a connection between the second pair of electrodes (38, 47), which had an impact on the instantaneous firing frequencies. Connected electrodes fire in the same firing periods, whereas not connected electrodes had no firing relation.

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In addition, interspike intervals graphs show the previous results in the spiking periods but also it can be seen that the ISI decrease both in value and dispersion. Both effects are related to the stimulation, which modulates the firing capabilities of the cultures.

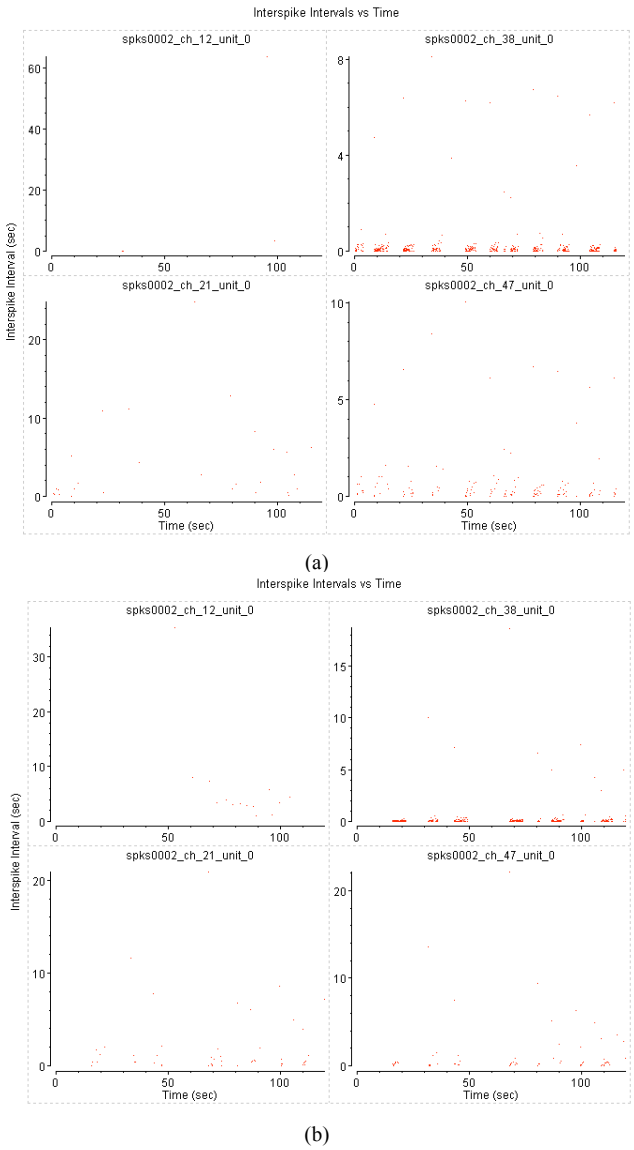

Figure 4. Interspike intervals on recordings from stimulated electrodes in the culture ID68 (22 DIV) from E2 before (a) and after (b) stimulation. Spiking periods have change after stimulation (b). ISI has decreased both in value and dispersion after stimulation.

Connectivity diagrams based on cross-correlation between electrodes showed some kind of connections reorganization after stimulations, concentrating them in a few electrodes. Furthermore, adjacent physical or logical connections in the connectivity graph following Hebb's law appeared in some pairs of stimulated electrodes (Figure 5).

Electrodes with created connections between them can distinctly be detected with the instantaneous firing frequencies graphs. Figure 3 showed two pair of stimulated electrodes (12, 21 and 38, 47) before and after the stimulation session. The firing periods of the electrodes from the second pair follow exactly each other, whereas the firing periods of the first pair of electrodes do not match. Furthermore, the electrodes of the second pair change both the firing periods after stimulation. This features indicates that there exits a strong connection between them.

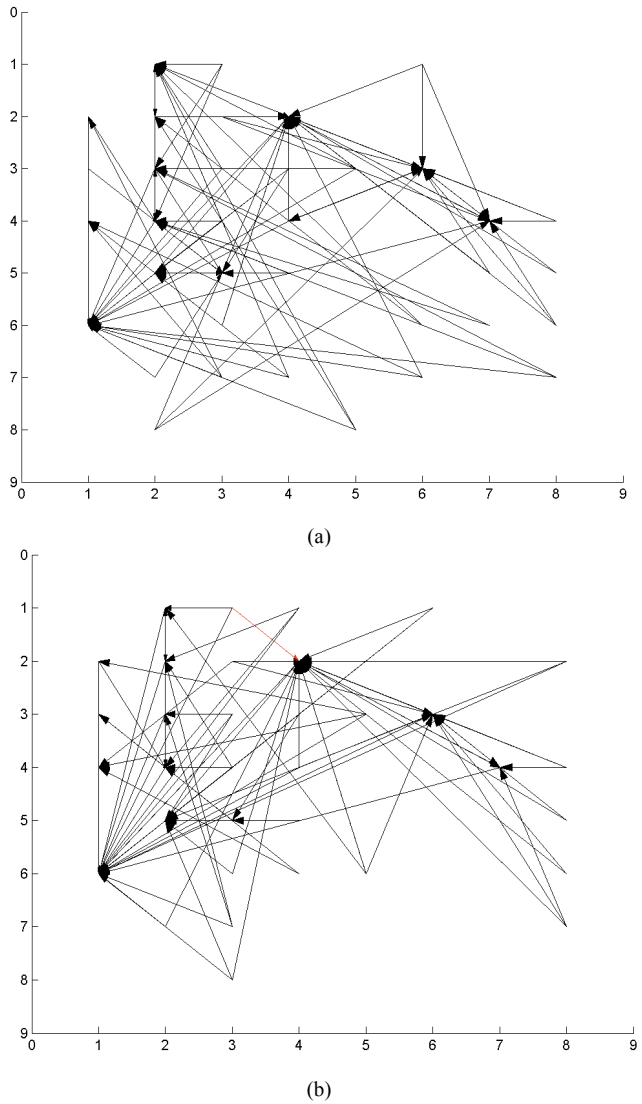

Figure 5. Connectivity graphs based on cross-correlation between electrodes. The graph belong to the culture ID48 (E1) at 25 DIV. Pair of electrodes 31, 42 and 52, 53 were stimulated with low-frequency current stimulation with 50 μA biphasic pulses. (a) No logical connections were observed before stimulation. (b) A connection (red arrow) between electrodes 31 and 42 has appeared.

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All of the cultures of E1 created a connection between the paired stimulated electrodes, whereas 60% of the cultures of E3 and only 20% of the cultures of E2 showed that connection. In some cases, the connection was intermittent, lasting one to several days. In others, a persistent connection was created. Finally, some cultures did not create any kind of connections. In this way, Hebbian tetanization created ad-hoc permanent or transient logical connections by modifying the efficiency of the paths between the selected electrodes. We speculate that the failed cultures may be caused by a not-homogeneous culture growth between the electrodes or by the neurobiological properties of the connections as will be confirmed using histological techniques in future works. In this case, using low-frequency current stimulation with 50 μA biphasic pulses provided the best results for creating connections following Hebb's law.

V. CONCLUSIONS

Learning in biological neural cultures is a challenging task. Different authors have proposed different methods for inducing a desired and controlled plasticity over the biological neural structure. Low-frequency stimulation has brought good results to researchers enhancing bursting activity in cortical cultures.

In this paper, we have shown that using this kind of stimulation it is possible to create adjacent physical or logical connections in the connectivity graph following Hebb's Law and such connections induce changes in the electrophysiological response of the cells in the culture, which can be observed in the different analysis performed. Furthermore, low-frequency stimulation induces changes using different values of current amplitude and stimulation time. Persistent and synchronous stimulation of relevant adjacent electrodes may be used for strengthen the efficiency of their connectivity graph. These processes may be used for imposing a desired behaviour over the network dynamics. In this work, a stimulation procedure is described in order to achieve the desired plasticity over the neural cultures, and shaping in this way the functional connectivity of the neural culture.

In future works, we will use different kind of electrical stimulations, such as tetanic stimulation, and try to find what are the optimal parameters of every stimulation that induce persistent changes in the cultured network. These induced connections will be used for driving a robot using Braitenberg's principles.

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