Master Thesis in Bioengineering

INVESTIGATING COMPUTATIONAL PROPERTIES OF A NEUROROBOTIC CLOSED LOOP SYSTEM

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Abstract

This work arises as an attempt to increase and deepen the knowledge of the encoding method of the information by the nervous system. In particular, this study focuses on computational properties of neuronal cultures grown in vitro. Through a neuro-robotic close-loop system composed of either cortical or hippocampal cultures (plated on micro-electrode arrays) on one side and of a robot controlled by the cultures on the other side, it has been possible to analyze novel experimental data. The collected results were related to the ability and the way through which the neuronal cultures process incoming information in order to drive the robot in an arena with obstacles. A system of variable frequency stimulation has been used as an input signal to the cultures in order to encode the distance of the robot from obstacles. The neuronal activity generated in response to these stimuli has been analyzed and divided into several features (spike train, bursts, isolated spikes). Starting from these features, reconstructions of the robot sensory signal have been computed in order to evaluate the amount of information about the position of the robot represented in the culture. Finally, a new experimental protocol has been developed: in its most recent implementation, it is able to consider the different features of the signal and to give all of them a different weight in terms of speed and control of the robot. The results have been presented and discussed. In particular, in terms of coding performance, a significant difference has been noted between isolated spikes and bursts and between the two types of cultures used (hippocampal and cortical).
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Introduction

In biological systems, high level cognitive functions, like learning, memory and processing of information emerge from the orchestrated activations of large neuronal assemblies. These functions are present in different organisms, regardless of their complexity and anatomical structure. But the wiring of the brain is also based on continuous interactions with the surrounding environment.

The induction of mechanisms of neural plasticity, particularly in sensory-motor integration, is one of the primary processes bringing to network modifications and, consequently, to reaction to the external world. Because of their complexity, nowadays biological structures cannot be fully translated into artificial systems. For these reasons, neuro-scientific research has been developing alternative approaches when trying to ‘emulate’ intelligent behaviors or when investigating them.

One of the most relevant aspects in neuro-robotics is the possibility to study how to extract the essential information embedded in a biological system, while maintaining a satisfactory level of neuronal complexity capable to support simple behaviors. In order to exploit a neural substrate (i.e. capable of mimicking basic brain functions) for artificial applications, hybrid model systems, where a set of living neurons are coupled with a robotic system, have been a possible choice in the recent past (Demarse, Wagenaar et al. 2001, Martinoia, Massobrio et al. 2004, Mussa-Ivaldi, Alford et al. 2010). Such developed artificial systems were used to study sensory-motor feedback loops and plastic modifications of the neuronal substrate with respect to a specific robotic task (e.g., obstacle avoidance, light following).

A system similar to those previously proposed in literature has been designed, implemented and tested at the Istituto Italiano di Tecnologia, with a certain degree of success. A small robot, either virtual or real, moves in an arena containing obstacles, while collecting information on the environment through its proximity sensors. Such information is transmitted, through a specific ‘coding box’, to a culture of dissociated neurons and the recorded electrophysiological signals are in turn used to drive the robot, through a specific ‘decoding box’, to correctly navigate within the arena.

An experimental setup like the one briefly described above, capable of real-time closed-loop experiments on in-vitro cell assemblies, is a powerful tool for the investigation of information
processing in neural systems, since it provides a straightforward mean to evaluate hypotheses on coding and decoding of information.

The present thesis project goes exactly towards this direction: provide a step forward in the investigation of the information processing mechanisms of a generic neuronal assembly, trying also to understand the advantages and disadvantages of such an experimental model. In particular, the contribution of different features of the recorded signals (i.e. spikes vs bursts) has been thoroughly investigated in the course of this project. Appropriate software modules were developed to this end. In particular, we have developed a real time burst detection able to recognize during an experiment the different features of the recorded signals. So the effects of spikes and bursts on wheel speeds can be independently controlled.

In our experiments, we made use of cortical and hippocampal cultures plated on micro-electrode arrays as ‘neural controller’ of our neuro-robotic platform. Then, we asked two main questions. First, we asked which features of the observed spike trains (e.g. spikes, bursts, doublets, burst events) may be used to reconstruct significant portions of the input signal, coded as a low-frequency train of stimulations, through optimal linear reconstruction techniques. We also wondered whether preparations of cortical or hippocampal cells might present different coding representations. In light of the obtained results, we developed a different experimental protocol and performed new experiments. In this context, we asked whether different features of the signal (in particular, spikes and burst) significantly influenced the adopted decoding scheme and, consequently, the performances of the robot in the arena.

The present thesis is organized in 5 Chapters.

**Chapter 1** describes the state of art of in-vitro neuronal preparations, of different techniques for recording and stimulating those experimental models.

**Chapter 2** presents the materials and methods which have been used during the entire thesis work. In particular, it shows the functioning and composition of a MEA-based system, the neurorobotic architecture and the data analysis algorithms.

**Chapter 3** describes in details the Graphical User Interface developed in the course of the present thesis, named HyBrain Tools. This software, developed in the MatLab environment, collects all the information and scripts used in this work, both for data analysis and the experimental phase.
Chapter 4 shows the results related to the implementation of a linear reconstruction algorithm in order to understand what are the features of the recorded signals from which we can better reconstruct the input time-varying stimulation train.

Chapter 5 shows the actual implementation of different decoding schemes during a set of closed-loop experiments. The preliminary results for these experiments, tightly connected to the results presented in Chapter 4, are presented.
Chapter 1

In vitro electrophysiology of neuronal networks: a historical perspective

1.1 Introduction

Humans are vastly superior to other animals in their ability to exploit their physical environment. The enormous complexity and variability of the world surrounding us is received by the nervous system through a sophisticated array of sensory receptors: the brain’s job consists in organizing the incoming sensory information in perceptions and elaborating an appropriate behavioral response. This task is accomplished by the brain using nerve cells (i.e. neurons) and the connections between them (i.e. synapses). Individual nerve cells, the basic units of the brain, are relatively simple in their morphology: although the human brain contains a thousand different types of neurons, they all share the same basic architecture. The complexity of human behavior depends less on the specialization of individual nerve cells and more on the fact that a great number of these cells form precise anatomical circuits. Hence, the capability of the nervous system to produce different actions in response to complex sensory stimuli derives from the way neurons are connected with each other and with sensory receptors and muscles, rather than single-cell specialization (Kandel, Schwartz et al. 2000). In summary, four basic features of the nervous system responsible of the generation of behavior can be identified:

1. The mechanisms by which neurons produce signals.
2. The patterns of connections between nerve cells.
3. The relationship of different patterns of interconnection to different types of behavior.
4. The means by which neurons and their connections are modified by experience.

In this introductory chapter, we report some brief notes about the methods neuroscientists use to investigate the function of neurons and synapses through different kinds of in vitro neural preparations. Finally, we dedicate an entire section to the description of state-of-the-art electrophysiological techniques, focusing on in-vitro applications and going from patch-clamp to microelectrode arrays (MEAs), which are the main tool exploited in the context of this thesis.
1.2 In vitro neural preparations

In the early 1900's, the first studies in neurobiology employing tissue cultures were performed by Ross Granville Harrison (Harrison 1907, Harrison 1912): he examined the outgrowth of fibers from fragments of frog and chick neural tube cultured in drops of clotted lymph or plasma and demonstrated for the first time that nerve fibers arise as outgrowths from individual nerve cell bodies (Banker 1998).

These studies opened the way to the use of tissue cultures' techniques to address biological problems and attracted many followers (Carrell 1910). In the following decades, together with the development of new approaches to cell culture methods (i.e. development of cloned cell lines of neuroblastoma cells and novel techniques to culture dissociated neurons, (Augusti-Tocco and Sato 1969)) and the advancement of technology (e.g. microscopy techniques, (Zernike 1934)), in vitro culturing began to gain a more prominent and important position in neurobiology.

Today, tissue culture is an integral part of modern neurobiology: nearly one third of the papers that currently appear in Neuron use nerve cell cultures as an important method (Banker 1998).

1.2.1 Cultures of dissociated neurons

When cells from the embryonic brain of animals are dissociated and placed into culture, neurons that have completed division in situ will extend processes, form synapses with one another and become electrically active. Differently from most types of tissues, even if tissue is removed at a time of active neurogenesis, it is rare to observe cells that divide in culture and subsequently acquire a neuronal phenotype. Such kind of cultures is referred to as primary cultures, because they are prepared from cells taken directly from the animal: the cells divide or not depending on the original tissue, acquire differentiated characteristics and ultimately die, but for the next experiment another animal has to be sacrificed in order to obtain new tissue and prepare new cultures. Alternatively, it is possible to use continuous cell lines, mostly derived from tumor cells (e.g. mouse neuroblastoma tumor C-1300) (Augusti-Tocco and Sato 1969): these cells can be sub-cultured repeatedly and express a reasonably stable phenotype.

Comparing cell lines vs. primary cultures is not the aim of this short introduction, not to mention the different fields of application. Briefly, one of the drawbacks of using cell lines is that they often do not express some key aspects of neuronal differentiation (e.g. development
of axons and dendrites, formation of synapses, etc.), although they share a great many of the individual characteristics of differentiated neurons (e.g. neurotransmitters, ion channels, receptors and other neuron-specific proteins) (Banker 1998).

In this thesis, for electrophysiology purpose, we made use of primary cultures of dissociated neurons from rat embryos, plated at a relatively high density (1500 cells/µl) onto planar MEAs. In what follows, we briefly describe the procedure used to obtain and maintain the cultures. Neuronal cultures are obtained from cerebral cortices and hippocampus of embryonic rats, at gestational day 18 (E18). The cerebral cortices and hippocampus of 4-5 rat embryos are dissected and then exposed to chemical (0.125% trypsin solution for 20 minutes at 37 °C) as well as mechanical dissociation (through flame-narrowed Pasteur pipettes). The resulting tissue is re-suspended in Neurobasal medium (Invitrogen, Carlsbad, CA, USA), supplemented with 2% B27 (Brewer, Torricelli et al. 1993, Brewer 1997) and 1% Glutamax-I (both Invitrogen) at the final concentration of about 1500 cells/µl. Cells are then plated onto the substrates, pre-coated with adhesion promoting molecules (first laminin, 50 µg/ml, and second poly-D-lysine, 100 µg/ml, both from Sigma-Aldrich), at the estimated density of 60,000 cells/device. The cultures are maintained in MEA devices, each containing 1 ml of nutrient medium (i.e. serum-free Neurobasal medium supplemented with 2% B27 and 1% Glutamax), in a humidified incubator having a controlled atmosphere (5% CO2, balance air) at 37°C. No antimitotic drug, that prevents glia proliferation, was added in our cultures, (Nedergaard 1994, Pfrieger and Barres 1997, Araque, Parpura et al. 1999) because of the essential role played by glial cells in the nervous system.

Figure 1.1: Image of a cortical network cultured over an MEA for 24 days in vitro at two magnifications (10x and 20x, calibration bars: 100µm and 30µm).
Half of the medium is replaced once a week until the 4th week in vitro and twice a week afterwards. The cultures can be kept in healthy conditions for several weeks and after 3-4 weeks in vitro they reach a mature developmental stage, characterized by quasi-synchronous array-wide bursts, mixed with isolated random spikes (Van Pelt, Corner et al. 2004, Chiappalone, Bove et al. 2006). In Fig. 1.1, a network of rat cortical neurons at 24 DIV over an MEA is shown at different magnifications.

1.2.2 Brain slice preparation

Instead of dissociate cells from the neural tissue, an alternative approach can be adopted that allows to partially keep the 3D structure of the intact brain in vitro: slices of brain can be cut from different areas - hippocampus alone or together with entorhinal cortex, cerebellum, cortex, striatum, etc. - and can be kept alive for several hours in various media (acute slices) or maintained in culture for days/weeks (organotypic slice cultures). Brain slices allow recording from semi-intact neural circuits, with the advantages of mechanical stability and control over the extracellular environment. These preparations are often used for a wide variety of studies, including synaptic plasticity and development, network oscillations, intrinsic and synaptic properties of defined neuronal populations, and many others (Kettenmann 1992).

Depending on the experimental needs several variants of the technique have been developed and are being used. One distinction between the variants relates to how thick the slices have been cut. The thin slice technique was developed to allow visualization of individual cells in slices less than 250 µm, while the thick slice technique is used in experiments where connectivity and maintenance of normal dendritic structure are crucial for the study.

The culturing of organotypic brain slice cultures have been based on earlier work on explants cultures derived from different anatomical region reviewed by Crain (Crain 1976) and became an increasing popular tool with the development of the roller-tube technique in 1981 by Gähwiler (Gahwiler 1981) and later the interface cultures in 1991 by Stoppini and Yu, 2006 (Stoppini, Buchs et al. 1991, Noraberg, Kristensen et al. 1999, De Simoni and Yu 2006); Hippocampal/cortical slice cultures have also been coupled to planar MEAs (Egert, Schlosshauer et al. 1998, Jahnsen, Kristensen et al. 1999, Beggs and Plenz 2003), thus allowing long-term extracellular measurements of defined neuronal circuits.
1.3 State-of-the-art of recording and stimulation techniques

Different techniques exist for measuring and evoking the electrophysiological activity of in vitro neuronal networks: a first distinction can be made between intracellular and extracellular techniques.

- **Intracellular technique**: previously, the action potential - the main feature of nerve cells' electrophysiological activity - has been described considering the ionic flows across the cellular membrane: the direct measurement of the potential difference across the membrane needs two measuring points, one in the cell and the second outside. This kind of electrophysiological measurement is called intracellular technique and requires the breaking of the membrane.

- **Extracellular technique**: alternatively, one can place the first measuring point outside the cell, but very close to the membrane, and the second one, the reference, far away from the cell. As described above, when an action potential occurs, the intracellular and extracellular ionic concentrations are both modified by the membrane transport properties: the extracellular changes are localized near the membrane and currents entering or leaving a neuron generate voltage signals at the electrode nearby. This results from a resistive drop in the medium between the reference electrode and the recording electrode. This measurement technique has been referred to as extracellular technique.
Intracellularly and extracellularly recorded signals are very different. Not only the amplitude of extracellular signals is lower than that of intracellular ones (20 - 200 µVpp for a typical action potential of 100 mVpp measured intracellularly), but also the shape is different. Models of the neuron-microelectrode junction have been developed (Martinoia, Massobrio et al. 2004) in order to better understand and correctly interpret the extracellular traces recorded from neuronal populations. A coupling stage taking into account several parameters of the junction allows reproducing the low-pass filtering effect introduced by the extracellular measurement.

The main variables involved in the signal transduction are: the sealing resistance $R_{\text{seal}}$, depending on the relative area of the microelectrode covered by the cell and the distance between the cell and the microelectrode; the cell membrane-electrolyte capacitance $C_{\text{hd}}$, modeling the polarization layers of the electrolyte solution in front of the cell and in front of the microelectrode.

A neuron can be excited intracellularly by injecting a current directly into it, but also an extracellular stimulation can be realized by applying a voltage or a current to the extracellular electrode. Application of voltage to the electrodes charges the capacity of the electrical double layer of the metal-electrolyte interface. This leads to fast, strong, but transient, capacitive currents with opposite sign at the rising and falling edges of voltage pulses resulting in transient hyperpolarization and depolarization of cellular membranes (Fromherz and Stett 1995, Stett, Barth et al. 2000). This is similar to the effect of brief biphasic current pulses commonly used for safe tissue stimulation (Tehovnik 1996).

In both cases, however, membrane polarization of the target neurons is primarily affected by the voltage gradient generated by the local current density and tissue resistance in the vicinity of the cells (Fejtl 2006).

### 1.4 The patch-clamp technique for single-cell electrophysiology

In vitro electrophysiology has seen the majority of its development using glass pipette electrodes. Neurophysiologists have studied single-cell properties, ion channels, drugs’ effects and synaptic signaling with these electrodes (Kettenmann and Grantyn 1992).

The electrodes are made by pulling a glass tube into a fine capillary at one end (less than µm in diameter) and filling it with a saline solution, whose composition matches with either the composition of the cytoplasm or of the bath solution, depending on the chosen configuration.
Finally an electrode, typically platinum or Ag/AgCl, electrically contacts the solution to the measuring circuit. Glass pipettes are used in different configurations: intracellular, extracellular or patch-clamp. The patch-clamp technique was proposed for the first time by Erwin Neher and Bert Sakmann in the late 1970s and they won the Nobel Prize in Physiology or Medicine in 1991 for this work. This technique is a refinement of voltage clamping and was developed in order to record current flow from single ion channels. The electrodes used for patch are distinct from the sharp microelectrodes used to impale cells in traditional intracellular recordings, in that they are sealed onto the surface of the cell membrane, rather than inserted through it. In some experiments, the micropipette tip is heated in a micro-forg to produce a smooth surface that assists in forming a high resistance seal with the cell membrane. After leaning the pipette against the cell membrane, a small amount of suction is applied to the patch pipette in order to increase the tightness of the seal between the pipette and the membrane. The high seal lowers the electronic noise and allows recording very small currents across the patch of membrane under the pipette, including those produced by ion channels with very small conductance (Kandel, Schwartz et al. 2000).

Several variations of the basic technique can be applied, depending on what the researcher wants to study. The inside-out and outside-out techniques are called excised patch techniques, because the patch is excised (i.e. removed) from the main body of the cell. They are different in which side of the membrane’s patch is exposed to the external medium. In the cell-attached configuration the electrode is sealed to the patch of membrane and the cell remains intact, allowing to record currents through single ion channels in that patch of membrane. Whole-cell recordings, in contrast, involve recording currents through multiple channels at once, over the membrane of the entire cell. The electrode is left in place on the cell, but more suction is applied to rupture the membrane patch, thus providing access to the intracellular space of the cell. Cell attached and both excised patch techniques are used to study the behavior of individual ion channels in the section of membrane attached to the electrode. Differently, whole-cell patch allows the researcher to study the electrical behavior of the entire cell.

1.5 Microelectrode arrays for network electrophysiology

In 1972, Thomas at al. published the first paper describing a planar MEA for use in recording from cultured cells (Thomas 1972). In their introduction, they underlined that “the most interesting questions to be asked of such cultures are those dealing with the development and
plasticity of electrical interactions among the cultured elements tissues or single cells” (Thomas 1972). They also clearly stated that the “exploration of these questions would be greatly facilitated by a convenient nondestructive method for maintaining electrical contact with an individual culture, at a large number of points, over periods of days or weeks”. More than 30 years ago, they charted a course for the years to come, identifying which directions of investigations to pursue and also delineating possible ways.

The MEA that was developed had two rows of 15 electrodes each, spaced 100 µm apart, and was intended for experiments with cultured chick dorsal root ganglion neurons. The array was on glass, with gold electrodes and leads over an adhesion layer, insulated with photoresist. The electrodes were plated with platinum black to reduce the impedance of their connection to the culture medium (Robinson 1968) and were 7 µm square (see Fig. 1.3). Although initial experiments were unsuccessful, turning to dissociated chick myocytes they found it possible to record robust electrical signals (200-1000 µV in amplitude). Five years later, in 1977, with a very similar introduction to that of Thomas at al., Guenter Gross and his collaborators proposed the idea of an MEA, without knowledge of the previous work (Gross 1977). They showed recordings from an isolated snail ganglion laid over the electrodes, with single action potentials having amplitudes up to 3 mV, depending upon the cell size.

The first successful recordings from single dissociated neurons using an MEA were reported by Pine in 1980 (see Fig.1.4): he succeeded in recording from a network of rat superior cervical ganglion neurons, cultured for up to three weeks over an MEA with 32 gold electrodes (two parallel lines of 16 electrodes each, 10 square and 250 µm apart), platinized and insulated with silicon dioxide (Pine 1980).

Figure 1.3: Schematic diagram of the MEA structure (Thomas 1972)
He also used the same MEA for stimulating neurons with a voltage pulse of 0.5 V and duration of 1 ms. These three works put a milestone for the upcoming work and marked the beginning of in vitro network electrophysiology using MEAs.

In the 1980’s, many studies employing MEAs for different purposes followed. While some were looking for alternative (and promising) new technical solutions (i.e. Field effect transistor-based MEAs, first proposed in 1981 by Jobling et al. (Jobling, Smith et al. 1981)), some others exploited these new tools to investigate either the network activity of cultures of dissociated neurons or of hippocampal slice preparations (Wheeler and Novak 1986). Soon, it was clear that large invertebrate neurons were the most suitable to be placed on top of MEAs (Regehr, Pine et al. 1989): they are easily identifiable by their size and location in ganglia, can be dissected out, and can be used with other identified neurons to form simple networks in culture that replicate some or all of their connections in vivo. MEAs can provide a means for long-term noninvasive communication with such networks for stimulation and recording, much superior to conventional electrodes (Pine 2006).

Differently, at the end of the 1980s, Meister at al. coupled an explanted salamander retina to an MEA (and later on retinas from newborn ferrets and cats) and they could record spontaneous and evoked by light stimulation bursts of activity (Meister 1989, Meister 1994).
At the beginning of the 1990’s, the combination of an MEA (for stimulation) and voltage sensitive dyes (for recording) was exploited to allow the detection and measurement of sub-threshold synaptic potentials, otherwise impossible by recording extracellular electrical signals (Chien 1991). At the same time, Fromherz and his collaborators investigated the use of a field effect transistor (FET) to record action potentials from large Retzius cells of the leech (Fromherz, Offenhausser et al. 1991), and this began a series of investigations in the Fromherz lab aimed at understanding the FET-neuron interface.

As originally foreseen by Thomas and collaborators (Thomas 1972), network development and plasticity are the most interesting questions that can be addressed by using MEAs. These are the main goals pursued in the 1990’s by groups in Japan at the Matsuhita and NTT laboratories, led by Taketani and Kawana: they fabricated 64-electrode MEAs for use in slice experiments (Oka, Shimono et al. 1999) and with cultures of dissociated cortical neurons (Maeda, Robinson et al. 1995) (see fig. 1.5). For slices, a rocking device was developed for keeping cultured organotypic slices alive over many weeks, so that their development could be observed over time (Kamioka 1997). For cortical cultures, experiments probed plasticity of connections as a result of titanic stimulation (Jimbo, Tateno et al. 1999).

At the same time, Pine and collaborators were looking for new technical solutions to improve the coupling between neurons and microelectrodes, through a neuro-cage approach (Maher 1999). Several of these studies, which provided promising preliminary results, have continued in the period from 2000 to the present. More recently, much of the attention of the scientific community has been focused on describing the ubiquitous spontaneous bursting activity present in almost all of in vitro preparations (Beggs and Plenz 2003, Wagenaar, Madhavan et al. 2005, Eytan and Marom 2006, Wagenaar, Pine et al. 2006, Pasquale, Massobrio et al. 2008, Raichman and Ben-Jacob 2008). Moreover, innovative protocols for inducing network plasticity have been presented in the literature, partially inspired by the work of Jimbo and collaborators (Shahaf and Marom 2001, Chiappalone, Massobrio et al. 2008). Since the 1990’s (Gross 1992, Gross 1995), neuronal networks grown on planar MEAs have been considered as a promising tool for drug screening and neurotoxicity studies (Gramowski, Schiffmann et al. 2000, Keefer 2001, Gramowski, Jugelt et al. 2004): this approach has proven to be useful in quantifying changes in the network activity in response to different neuro-active compounds (Martinoia 2005, Martinoia, Bonzano et al. 2005).
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Figure 1.5: The first 64-electrode MEA. (A) A transparent electrode array substrate. The recording area consists of two blocks of 32 embedded electrodes, separated by 500 mm. Electrode terminals had an area of 30 x 30 mm, and the distance between neighboring terminals was 180 mm (center to center). Each site is identified by row and column number. (B) Network of cortical neurons cultured on the substrate. The four dark squares are platinized substrate electrodes (Jimbo, Tateno et al. 1999).

Figure 1.6: (A) Block diagram of the NeuroBIT project’s bi-directional neural interface. (B-G) A 60-s portion of a typical closed-loop experiment: recorded spike trains (B) and instantaneous firing rates (C) from the 16 output sites, motor commands (D) and, on the right, the corresponding activity of the IR sensors (E), the average left and right sensor activity (F) and the corresponding pattern of stimulation (G) (Cozzi 2005)
Finally, the Potter group in Atlanta and, at the same time, the research group in Genova, led by Martinoia have developed systems for re-embodying cultured networks: they connected the culture in a closed-loop with an artificial body, either simulated on the computer (Demarse, Wagenaar et al. 2001) or represented by an actual robot (see fig. 1.6). The main goal of this approach was to provide the neuronal network “natural” inputs from the outside, in order to overcome one of the essential limitations of the in vitro methodology, i.e. the lack of a bi-directional communication with the surrounding environment. Research is still going on in this direction, exploiting the robot-culture closed-loop architecture as a platform for studying plasticity and learning (Bakkum 2004, Martinoia, Massobrio et al. 2004).

1.6 MEAs vs. traditional methods - advantages and limitations

During the last 30 years, the invention and the continuous improvement of planar multi-electrode array devices have made possible experiments unthinkable before the 1970’s, exploring network-level phenomena (i.e. network physiology). At the same time, MEAs allow experiments that can also be performed with traditional non-MEA instrumentation, but are enhanced by the use of multi-electrode devices. However, although modern MEAs can in general perform all the major types of traditional experiments (involving either dissociated cell cultures or slices), they are not always the best choice for a particular application (Whitson 2006). Determining MEA applicability requires careful consideration of their specific strengths and weaknesses.

From a traditional perspective, their major strengths include:

1. The ability to gather data from multiple sites in parallel as if running multiple experiments in a single culture/slice.
2. The ability to change stimulation and recording sites very quickly among those available in the array.
3. The ability to do away with the need to place multiple electrodes individually by hand.
4. The ability to increase culture sterility (either for dissociated cells or organotypic cultures).

Conversely, limitations include:

1. Smaller amplitude recordings (in most cases), as compared to traditional instrumentation because the electrodes are not inserted directly inside the cell.
2. Lesser independent mobility of the electrodes, since they are arranged in a fixed pattern.

Only by exploiting the 2-D structure of MEAs it is possible to perform a spatiotemporal analysis of activity propagation patterns: for example, the use of MEAs allowed capturing the 2-D functional anatomy of the hippocampus (Shimono, Brucher et al. 2000), by using a technique called 2-D current source density (2-D-CSD) analysis. This technique, made possible by 2-D MEAs, provides a clear picture of the concentration of currents into and out of neuronal regions, and allows identifying neuronal circuits in a brain’s portion. Similarly, the availability of several recording points to monitor the network’s activity is essential to evaluate the effects of plasticity protocols on the whole neuronal system (Jimbo, Tateno et al. 1999, Chiappalone, Massobrio et al. 2008) defining long-term network power (LTNP) or long-term network depression (LTND).

Considering the technological advances of the modern electronic industry (i.e. ever-increasing electronics miniaturization and machines’ storage and computation capabilities), quite recently there has been a strong effort towards the production of high-density MEAs (Berdondini, Imfeld et al. 2009, Frey, Egert et al. 2009). In fact, one of the main limitations of currently commercially available MEAs is the low number of recording points when compared to the total number of cells in the network. Moreover, due to the high inter-electrode distance (on the order of hundreds of µm for standard MEAs), it is necessary to plate cells at a relatively high density (about 2000 cells/mm²) to get a good covering of the electrodes. These issues have led to the search for an array with a very high number of embedded microelectrodes, whose size (and distance) is comparable to that of a neuron.
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Materials and Methods

2.1 Introduction

Nowadays, thanks to advances in electronic technology, commercial systems based on the microelectrode array (MEA) methodology are readily available. Actually, many neuro-engineering laboratories all over the world are using this approach to study the laws which underlie the behavior of neuronal networks. Nowadays, thanks to advances in electronic technology, commercial systems based on the microelectrode array (MEA) methodology are readily available. Actually, many neuro-engineering laboratories all over the world are using this approach to study the laws which underlie the behavior of neuronal networks (Eytan and Marom 2006, Pine 2006, Wagenaar, Pine et al. 2006, Chiappalone, Massobrio et al. 2008).

In the previous chapter, we outlined the history of MEA electrophysiology, starting from the very first applications to the most recent developments. Here, we would like to give further technical details about commercially available MEAs and the required experimental set-up.

2.2 Microelectrode array technology

MEAs are made of cell-sized electrodes (10-100 µm diameter) placed onto a glass substrate. The electrodes, typically made of Au, Indium-Tin Oxide (ITO), Titanium Nitride (TiN), or black platinum, must be bio-compatible, long-term lasting, and preferably should have low impedance (less than 500 k at 1 kHz) for low thermal noise. The MEA non-sensitive surface and electrode leads are coated with bio-compatible insulators (e.g. polyamide or silicon nitride/oxide) which prevent short circuits with the electrolyte bath. These insulators, again coated with adhesion-promoting molecules, such as polylysine and/or laminin, allow and help the neuron coupling to the device surface. The low impedance of the electrodes, and the choice of a correct voltage range to avoid the generation of neurotoxic redox complexes, enable using them to deliver external stimuli. The fabrication of MEAs is based on the thin-film technology (Elshabini-Riad 1998) and is realized in a clean room, using standard photolithographic techniques.
The rapid success met by MEAs in the neuroscience research field moved some electronic companies to develop commercial systems to perform electrophysiological measurements using MEAs. At the present, there are on the market at least two complete acquisition systems based on MEAs: the MED System produced by Panasonic (www.med64.com, Osaka, Japan) and the MEA System produced by Multi Channel Systems (www.multichannelsystems.com, Reutlingen, Germany). Other companies, such as Ayanda-Biosystems (www.ayanda-biosys.com, Lausanne, Switzerland), and Plexon (www.plexoninc.com, Dallas, USA) have only developed the microelectrode devices, for several different applications (cultures, slices, cardiomyocytes, retinal cells, pharmacological screening, etc.). Fig.2.1 shows four samples produced by Multi Channel Systems, Panasonic and Ayanda. 

All the experimental results involving standard commercial devices presented in this thesis were obtained from recordings performed on MEAs manufactured by Multi Channel Systems. The following sections deal with the description of this experimental set-up.

### 2.2.1 MCS microelectrode array design

Multi-Channel Systems (MCS) provides different types (electrode size and inter-electrode spacing) of MEAs. The MEAs used in this work consist of 60 at round electrodes made of TiN. Tracks and contact pads are made of titanium or ITO, and the insulation material is silicon.
nitride (Si3N4). The electrodes are positioned in an 8x8 layout grid (the four corner electrodes are not present). ITO contact pads and tracks are transparent to allow a perfect view of the specimen under the microscope. Electrode diameters of either 10 µm or 30 µm are available, with an inter-electrode distance of 30, 100, 200 or 500 µm (see Fig. 2.2). In this study we used only arrays of 30 µm electrodes, spaced 200 µm.

Figure 2.2: (A) Image of a typical MCS MEA, (B) 8x8 layout of an MCS MEA: a is the electrodes’ size (e.g. 30 µm) and d is the inter-electrode spacing (e.g. 200 µm). (C) and (D) are optical images of a neuronal network over an MEA at 14 days in vitro (20x magnification).

Latest generation MEAs are equipped with an internal reference electrode used to minimize the possibility of pollution that should be caused by the introduction of an external reference electrode. A glass ring is placed at the center of the devices, surrounding the recording area, and it allows to contain the culture medium. In this way, when placed in an incubator, the culture can survive for several weeks. There are various factors which may contribute to the gradual decline in the health of the culture, e.g. contamination by airborne pathogens or increase in the osmotic strength of the medium due to evaporation. For these reasons, we made use of flexible polydimethylsiloxane (PDMS) lids in order to reduce the effects of these external factors (see Fig. 2.3) (Blau 2009).
PDMS is a biocompatible and transparent polymer that can be modulated and cured in a template (made of polytetrauorethylene (PTFE/FEP) or poly (methyl methacrylate) (PMMA)) to get a proper shape: the peculiarity of PDMS is that it is permeable to gases (e.g. CO2, whose concentration at 5% is needed to maintain the medium's pH), but it greatly reduces evaporation, limiting the variation of the medium’s osmolarity (Blau 2009). Moreover, the PDMS lids prevent possible contamination by pathogens, thus allowing to record the same culture out of the incubator more than once. Finally, they can also include biocompatible tubing for continuous medium perfusion or an external Ag/AgCl reference electrode, preserving culture sterility.

2.2.2 The MCS MEA60 System

The Multi-Channel Systems MEA60 set-up is made up of the following components, usually contained within a Faraday cage in order to reduce electro-magnetic interferences (see also Fig. 2.4):

Figure 2.4: Multi-Channel Systems set-up Multi Channel Systems set-up: (A) MEA1060 amplifier, (B) MCS Stimulus Generator, (C) PCI-based acquisition card, (D) temperature controller.
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- **Amplifier**: An amplifier stage for multi-electrode recording has to meet two main requirements:
  - eliminating the cables connecting the electrodes and;
  - coping with the interference (cross-talk phenomenon) among channels.

The MEA1060 60-channel amplifier has a compact design (165×165×19 mm) and, due to the surface-mounted technology (SMD) of pre- and filter-amplifiers, the complete circuit and amplifier hardware was built into a single housing: this ensures optimal signal-to-noise ratio of the recording, because no further cables are necessary other than a single SCSI-type cable connecting the amplifier to the data acquisition card. This results in an overall low noise level of the complete amplifier chain (×1200, 12-bit resolution, 10 Hz to 3 kHz) of ±3 µV, which is well within the ±5 to 10 µV noise level of the MEA TiN electrode. Hence, the MEA sensor is placed directly inside the amplifier and settled so as to fit the standard microscopes.

- **Temperature controller**: The MCS temperature controller (TC02) uses a Proportional Integrative Derivative (PID) based technology. The MEA temperature can vary in the range from room temperature to +50°C. The set-point temperature is reached within a range of 30 s to 5 minutes, depending on the recording system configuration.

- **PCI-based acquisition board**: Standard PC technology is used as the backbone of high-speed multi-channel data acquisition. The data acquisition card is based on PCI-bus technology and allows the simultaneous sampling of up to 128 channels at a sampling rate of 50 kHz per channel. It is possible to set the input voltage range from ±400 mV to ±4 V in the data acquisition software and this allows to use the full 12-bit resolution bandwidth for signals of any amplitude. Three analog channels and a digital I/O port are accessible, allowing the simultaneous acquisition of analog data, such as current traces from a patch clamp amplifier or temperature together with the MEA electrode data. The digital I/O port features trigger IN/trigger OUT functionality.

- **Acquisition software**: The MC Rack software allows to record simultaneously the electrophysiological activity from the 60 electrodes of the MEA, and monitor the raw data in a real-time mode. Different parameters can be extracted from the data streams and the results can be plotted, saved, and exported to other programs for further analysis.
• **Stimulus generator:** The MCS stimulus generator (STG2004) is a general purpose stimulator which generates pulses to be delivered to stimulating electrodes (up to 4 for this model and up to 8 for upgraded models). Complex stimulus waveforms (both current and voltage) of arbitrary duration are designed by using the provided MCS stimulus software and then stored in the stimulus generator connected to the MEA. Stimuli are “tailored” by the user by specifying the desired pulse waveform defining parameters into a worksheet. The pulse waveform is then displayed and the stimulus protocol is downloaded to the stimulus generator via a serial communication port. The stimulus generator operates in both voltage and current mode, and it is equipped with separate voltage and current outputs for each channel.

In an improved version of the MEA60 System, up to four amplifiers, each hosting an MEA device, can be connected to the data acquisition card and 120 out of a potential 240 MEA electrodes can be monitored simultaneously. This allows the parallel recording of more than one sample, particularly useful if one wants to compare the effects of the same experimental protocol on different cultures at the same time. This scalable MEA60 System has been designed by MCS to answer the ever-growing demand in basic research and pharmaceutical applications for automation of experimental and data analysis procedures. In our lab at the Istituto Italiano di Tecnologia (IIT), we used an MEA120 System to record up to two cultures of dissociated neurons at the same time and double the number of monitored samples. More details on the complete MEA60 System are available in (Whitson 2006) and references therein. Further technical specifications and data sheets can be found on www.multichannelsystems.com.

**2.2.3 Custom fittings for the MCS MEA60 System**

*Perfusion control system*

The standard MEA electrophysiology set-up can be also combined with a perfusion system. Generally, a continuous flow of artificial CSF (ACSF), saturated with 95% O\(_2\) and 5% CO\(_2\), is necessary for maintaining acute slices functional during the MEA recording, while a continuous perfusion of nutrient medium is not strictly necessary for culture maintenance during the experiment. Nevertheless, a perfusion control system, either automated or manual, can be also used to perfuse cultures at extremely low rates (≈100 µl/h) for very long-term recordings (on the order of days/weeks) to stabilize the network's activity (Eytan and Marom 2006) or to deliver/wash-out drugs during pharmacological tests.
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*Figure 2.5: Custom recording chamber for MEA recordings. (A) Image of the recording chamber, highlighting the main features, and (B) image of the same chamber during normal operation over an MEA1060 amplifier.*

**Recording chamber**

Within the incubator the temperature (37°C), the relative humidity (90-95%) and the CO₂ concentration (5%) are controlled so as to maintain the culture medium pH ≈ 7.4 and the osmolarity ≈ 230 mOsm. In fact, large variations in these parameters strongly affect the network activity and, if permanent, can cause the culture death. Hence, the optimal recording conditions would be provided by the incubation environment, but actually it is not possible to store the MCS MEA1060 amplifier in a highly humidified atmosphere, as that of the incubator. This is the reason why we designed and realized a custom recording chamber, which, coupled to the use of a PDMS lid, allows the recording of cultures in safe conditions. The custom chamber (shown in Fig. 2.5) consists of a metallic box, sized 13×13×4 cm (W×L×H), heated on the topside through planar high-power ceramic resistors (*BI Technologies*, www.bitechnologies.com, Fullerton, CA, USA) and providing an inlet for a constant gas flow. In fact, the chamber is connected through PTFE/FEP tubing (*Legris*, www.legris.com, Rennes, France) to a gas cylinder containing a mix of 5% CO₂ - 20% O₂ - 75% N₂, practically the composition of air with 5% CO₂: a constant slow flow of this gas mix into the metallic box covering the MEA during the experiment has been demonstrated to prevent the medium’s pH to drift towards more basic values (up to 9 - 10 pH units) (Brewer, Boehler et al. 2009a, Brewer, Boehler et al. 2009b). Moreover, using this recording chamber, the MEA device containing the culture is not only heated bottom up by the amplifier’s warming plate driven by the MCS temperature controller, but also top down through the ceramic resistors heating the metallic box. In fact, when supplied with a suitable voltage, they constantly heat the conducting metallic surface of the chamber so as to provide a surrounding environment for the MEA whose temperature (≈ 37°C) is homogeneous.
This, together with the PDMS lid and with the fact that the gas is made bubble in water before being fed into the incubator, strongly reduces the evaporation and maintain the medium’s osmolarity constant during the experiment. The recording chamber features a hole on the topside, to let the observation of the culture either using a microscope or to the naked eye, and a lateral hole, to let optional perfusion tubing in or out of the box. These holes are hermetically sealed during normal operation, to limit leakage and keep the 5% CO\textsubscript{2} concentration and temperature as constant as possible inside the chamber (Pasquale 2009).

### 2.3 The neuro-robotic architecture

![Block diagram of the neuro-robotic architecture](image)

*Figure 2.6: Block diagram of the neuro-robotic architecture. From left to right: (i) the network module, constituted by a network of living neurons coupled to a Micro Electrode Array; (ii) a computer which hosts the developed software tool (i.e. HyBrain) which manages the communication between the biological and the artificial part; (iii) the robotic module composed by a robot, either real or virtual, with sensors and actuators navigating into a circular arena with obstacles.*

The neuro-robotic architecture developed in the lab at the Istituto Italiano di Tecnologia includes several different elements (Fig. 2.6):

- a network module, constituted by a neuronal culture over a MEA (i.e. the “brain” of the neuro-robotic loop);
- a computer, equipped with a data acquisition board, which hosts the developed software able to manage all the devices included in the architecture;
- a stimulation unit, which is able to handle two different stimulation patterns;
the stimulation signals are programmed via software and they are defined by their frequency, amplitude and stimulation site;

- a robotic module, characterized by a small robot (either physical or virtual) with sensors and wheels able to move inside a circular arena with obstacles.

These different modules are synchronized and managed by a custom developed software named HyBrain which runs in the Windows environment (Mulas 2010, Tessadori J 2012).

Through this software, it is possible to control the parameters of the neuro-robotic experiments, namely the coding, decoding and learning schemes and all the required data processing. The three modules (network, robotic and interface) are described here.

2.3.1 Network module

Neuronal preparation

Dissociated neuronal cultures were prepared from cortices of 18-day old embryonic rats (pregnant female rats were obtained from Charles River Laboratories). Culture preparation was performed as previously described in 1.2. Recordings were performed on cultures between 25 and 35 DIVs.

Microelectrode arrays

Microelectrode arrays (Multichannel Systems, MCS, Reutlingen, Germany) consist of 59 TiN/SiN planar round electrodes (30 μm diameter; 200 μm center-to-center inter-electrode distance) arranged in an 88 square grid excluding corners. One recording electrode is replaced by a larger ground electrode. Each electrode provides information on the activity of the neural network in its immediate area. A microware connects each microelectrode of the MEA to a different channel of a dedicated amplifying system with a gain of 1100. The amplified, analogic, 60-channel data is then conveyed to the data acquisition card which samples them at 10 kHz per channel and converts them into digital, 12 bit data.

2.3.2 Robotic module

The robot, either virtual or physical, is basically a two-wheeled sensor platform: six infrared sensors are mounted on the robot at different angles, providing information about the distance of surrounding objects in different directions, whereas the speed profile of each wheel determine the direction and velocity of the robot itself. The arena consists of an
enclosed space containing several different round obstacles in random positions and the robot.

A typical experiment with the virtual robot is shown in Fig. 2.7: the robot is moving in a 400×400 pixels circular arena, where dark green pixels represent obstacles or arena walls, whereas light green pixels are free for the robot to move in. The robot (small pink circle in the upper right) is collecting information about its environment through its six sensors: each black line departing from the robot represents the line of sight of a different sensor; their angles are fixed with respect to the robot heading (in this case, 30°, 45° and 90° on both sides of the robot direction), while the length of each line is equal to the distance from the robot center to the closest obstacle in the sensor direction.

Figure 2.7: The robotic module: the simulated robot (pink circle) with its sensors (black lines departing from the robot) within the virtual arena. Each green circle represents an obstacle of different diameter. The red line represents the trajectory followed by the robot.

This distance defines the reading of the sensor: the output is 0 if the robot is in direct contact with an obstacle, 1 if the closest obstacle is at the maximum distance possible (the diameter of the arena, in this case). The three sensor readings on each side are averaged to provide the neuronal network with a single value per side. In the case shown in Fig. 2.7, the robot is performing an obstacle-avoidance task, as can be inferred by the red trajectory. The speed of a wheel is inversely proportional to the average of the sensor readings on the same side, thus the robot turns away from close obstacles. The task shown above is achieved through a simple algorithm devised by Braitenberg (Braitenberg 1984) in this application neither information is lost nor there is any significant delay between sensor data collection and motor command execution. Obtaining a behavior as close as possible to this one is the goal of the coding-decoding-learning process implemented here. During experiments, collisions with obstacles
or walls are unavoidable: following such an event, the robot moves back to a previous position in its path, at a fixed distance from the obstacle location.

2.3.3 Interfacing the network and the robotic module

Decoding scheme

Although many different decoding schemes are possible, so far the only one implemented has been a frequency rate based algorithm (Adrian 1928, Rieke 1997). For this scheme, only one feature of the recorded signals is useful: the frequency of spikes at each location. Two groups of electrodes (i.e., two sub-population of neurons) on the MEA are selected and defined as the “output areas” (fig. 2.8).

![Figure 2.8: Schematically illustration of the input/output electrode configuration in the MEA device: the orange and light blue electrodes are selected as inputs, i.e. they are used to deliver stimuli as a function of the sensors readings from the robot. The red and dark blue sets of electrodes are instead used as control regions for the wheels: the speed of the left wheel will be a function of the average activity detected on the red electrodes, while the right wheel will be controlled by the blue ones.]

The number of spikes occurring over that area in a 0.1 s window constitutes the basis for calculating the motor signal for the corresponding wheel. In the current architecture, a linear relation is implemented between wheel speed and motor signal: if no spikes are detected in a time window, the corresponding wheel turns at a set minimum speed, increasing linearly with the number of detected spikes, up to a defined maximum rate. A low-pass filtering effect is added by taking into account previous samples, in order to smooth robot movements.

For each wheel, the speed is therefore defined as:

\[
\omega_i = \begin{cases} 
  \frac{f_{i,t} + f_{i,t-1}}{2(f_{i,\text{MAX}}(\omega_{i,\text{MAX}} - \omega_{i,\text{min}}) + \omega_{i,\text{min}})} & \text{for } f_i < f_{i,\text{MAX}} \\
  f_{i,\text{MAX}} & \text{for } f_i \geq f_{i,\text{MAX}}
\end{cases}
\]  

(1)
where subscript $i$ denotes wheel side, $\omega$ is the wheel speed and $f_{i,t}$ is the averaged firing rate over all the electrodes corresponding to the $i$-th recording area at time sample $t$. $\omega^{\text{MAX}}$, $\omega^{\text{min}}$ and $f^{\text{MAX}}$ are parameters set by the experimenter before the start of the experiment.

**Coding scheme**

Likewise, the coding scheme is linear and rate based: a group of electrodes is defined as an “input area” and assigned to the sensors on the left or the right side of the robot body (fig. 2.8).

Each sensor provides a reading, normalized to 1 for an object in direct contact with the robot and 0 for an object at the far end of the designed arena (while this behavior is nearly ideal for the virtual robot, it is far from so in the case of the physical robot). The readings from the sensors on the same side of the robot are then averaged and coded back to the corresponding sensory area. As mentioned before, the coding is linear and frequency based: a fixed stimulus is delivered at the sensory area at a frequency directly proportional to the averaged, same-side sensors readings.

The stimulation rate for each input region is determined as:

$$s_i = (s^{\text{MAX}}_i - s^{\text{min}}_i) r_i + s^{\text{min}}_i$$

(2)

where $s_i$ is the stimulation rate of the $i$-th input area and $r_i$ the average of all the sensor readings on the corresponding side of the robots, whereas $s^{\text{MAX}}_i$ and $s^{\text{min}}_i$ are user-set parameters fixing the maximum and minimum stimulation rate.

**Learning protocol**

In order to progress towards the desired behavior, it is necessary to define a learning rule that allows a modification of connectivity between input and output areas by rewarding “good behavior”, while discouraging “bad behavior”. According to (Rieke 1997), a 20 Hz stimulation should strengthen the synaptic connections of receiving neurons.

This observation has been used to define the learning rule in the current implementation of the software (Chiappalone, Massobrio et al. 2008, Tessadori, Bisio et al. 2012): following each robot hit with an obstacle, a 2 second-long, 20 Hz stimulation is delivered to the corresponding input area. The rationale for this choice is that hits are usually caused by poor
correlation between stimulation in an input area and detected activity in the corresponding output area: a strengthening of the synaptic connection should correct the problem. However, to the end of the study of the decoding, this protocol of learning has not been used.

2.4 Data Analysis Algorithms

2.4.1 Spike Analysis

Spike detection algorithm is a crucial step to analyze electrophysiological signals coming from neuronal networks. Several algorithms have been developed by researchers, and described in literature, ranging from simple hard-threshold to more sophisticated signal classifications (Demarse, Wagenaar et al. 2001, Marom and Shahaf 2002, Chapin 2004). The algorithm implemented and used in this work, belongs to the family of the threshold-based detection algorithm (Perkel, Gerstein et al. 1967). Briefly, it resorts to three parameters the user has to define: differential threshold (DT), peak lifetime period (PLP), and refractory period (RP). The differential threshold, defined separately for each recording electrode, is set according to the standard deviation of the biological and thermal noise of the signal. The peak lifetime and the refractory period are related to the minimum gap between two consecutive spike events. The algorithm computes the Relative Maximum/Minimum (RMM) by using an inspection buffer. When the RMM is a minimum, the algorithm looks for the nearest maximum within the peak lifetime window, and vice versa. If the difference between the two found RMMs (differential value) overcomes the absolute threshold and there is not any other RMM within the refractory period, the spike is identified and its timestamp is stored. (Novellino 2006, Maccione, Gandolfo et al. 2009).

Mean Firing Rate

The Mean Firing Rate (MFR) can be evaluated as the ratio between the number of recorded spikes \( N \) and the temporal window of observation \( T \), i.e.,

\[
MFR = \frac{\sum_{s=1}^{N} (\delta(t - t_s)) dt}{T} = \frac{N}{T} \quad (3)
\]

The MFR is used to characterize the electrophysiological activity level of the network: if an electrode presents an MFR < 0.2 spikes/s, it is considered not active and discharged from further analyses.
**Inter-Spike-Interval**

The Inter-Spike-Interval (ISI) distribution provides a statistical estimate of the firing probability of the subsequent spike relative to a reference spike. Mathematically, it can be expressed as:

\[
ISI(\tau) = \left(\frac{1}{N-1}\right) \sum_{s=1}^{N-1} \delta(t_{s+1} - t_s - \tau)
\]  

(4)

where \(N\) is the total number of the spikes, \(\tau\) is the bin width and \(t_s\) is the temporal instant of the spike.

**Post-Stimulus Time Histogram**

The Post-Stimulus Time Histogram (PSTH) represents the impulse response of each site of the neural preparation to the electrical stimulation. In this work, the PSTHs were calculated by taking 600 ms time windows from the recordings which follow each stimulus. Then, the number of spikes occurring in a 4 ms bin is counted, and the obtained histogram was normalized by dividing this measure by the number of stimuli and the bin size (Rieke 1997). As Fig. 2.9 shows, the spiking response of an electrode is usually composed of an early phase, ending within several decades of milliseconds (i.e., 0 – 100 ms), and a late phase which could last for several hundred milliseconds (i.e., 100 – 500 ms). Changing the stimulation site, it is possible to obtain different responses: from the pure early response up to the pure delayed response, with a great number of intermediate combinations of these two extremes.
2.4.2 Burst Analysis

Burst Detection

The burst detection algorithm is based on two main parameters. Since bursts are made of several closed spikes, an $\text{ISI}_{\text{max}}$ value is defined to state that a spike belongs to a burst. Moreover, since bursts are spaced hundreds milliseconds, an $\text{IBI}_{\text{min}}$ is defined to distinguish two consecutive bursts. Assuming $\text{ISI}_{\text{max}} = \text{IBI}_{\text{min}}$, and applying the aforementioned criteria to the spike train, the Burst Train (BT) can be obtained as follows (Chiappalone 2005):

$$ BT(t) = \sum_{b=1}^{M} A_b \cdot \pi \left( \frac{t - t_b - \frac{T_b}{2}}{T_b} \right) $$

where $T_b$ is the burst duration, $t_b$ is the burst onset time, $M$ the burst number, and $A_b$ the burst amplitude. Bursts are identified and their features saved (i.e., duration, rate, etc.) (Chiappalone 2005).

*Figure 2.9: Post Stimulus Time Histogram (PSTH). The early phase (0-100 ms), and the late phase (100 - 500 ms) are pointed out.*
**Mean Bursting Rate**

The *Mean Bursting Rate* (MBR) computes the number of bursts *per* minute, and it is defined as:

\[
MBR = \frac{M}{T} \quad (6)
\]

where \( M \) is the burst number, and \( T \) the temporal window of observation.

**Inter-Burst-Interval**

The *Inter-Burst-Interval* (IBI) is the dual of the ISI calculated for the bursting activity. It is defined as:

\[
IBI(T_b) = \frac{1}{M-1} \sum_{b=1}^{M-1} \delta(t_{b+1} - t_b - T_b) \quad (7)
\]

The IBI represents the temporal interval between two consecutive bursts.
Chapter 3

HyBrainTools

3.1 Introduction

HyBrainTools is a Graphical User Interface (i.e. GUI) developed in MatLab to allow an easy and intuitive analysis of data collected during the experiments in a real or virtual environment. It has been conceived to collect all the functions used for analysis of the neuro-robotic data in a single interface, in order to process and display the information on the different sessions of the performed experiments. Through the user-friendly GUI, it is possible to run the scripts for the type of action the user wants to perform on the data.

Figure 3.1: screenshot of the main interface of HyBrainTools
HyBrainTools mainly consists of three parts:

- A top panel, dedicated to the loading of data to be analyzed;
- A lower left panel with options for data analysis and visualization;
- A lower right panel, dedicated to the running of MatLab scripts.

### 3.2 Load Panel

This panel allows loading one or more paths in order to access the files the user wants to analyze. The *LoadDataFile* button opens a file browser that allows selecting the path of the directory containing the experimental data to be analyzed.

In particular, each experiment directory contains three data files in comma-separated values (CSV) format:

- **RobotData.csv** contains data on robot movements. The parameters saved in this file are:
  - Sensor 1 and 2: information about the distances from obstacles detected by the robot sensors in different time instants.
  - X and Y position: information about the robot position within the arena at different time instants;
  - Left and right wheel speeds at different time instants;
  - Hit: binary entry of impact instants between robot and obstacles. In the case of virtual experiments, it has the value of 1 for time instants before the robot would hit an obstacle. In the case of real experiments, it has a value of 1 in time instants where an hit is detected (both wheels stop running or sensor readings saturate).
  - Time: precise timing information of each log entry.
- **SpikeData.csv** contains time instants of spike detections, divided in 60 channels;
- **StimuliData.csv** includes time instants of feedback stimulus delivery and the delivering channel.

After the user has done his selection, the pathname for that particular experiment appears in the scroll box below and the user can add more folders by repeating the previous operation. Finally, the *Remove* button allows deleting a selected pathname from the list.
3.3 Tools Panel

This panel contains the necessary tools for the visualization and processing of data stored at the end of an experiment, and allows access to the following functions:

*Plot exp data*: this button will plot a graph from data pre-selected in the checkbox list. The data that can be plotted are:

- **Mean Firing Rate (MFR)**: it is obtained by calculating the firing rate (FR) of each single channel and their average over all electrodes of the MEA used for controlling one of the wheels; it is expressed as spikes/second.

  Through a specific function recalled by the *Activity rates* button, it is possible to store in the hard-disk the different MFRs which have been calculated and to classify them by their date.

- **Wheel Speed (WS)**: the speed of the robot wheels. This is represented with a decimal value and it ranges between a minimal value of 0.1 and a maximal value of 1. Those are arbitrary values: in the case of the physical robot, 1 corresponds to the maximum allowed speed. In the case of the virtual robot, we establish a maximum speed in pixel per second (it can be modified according to the experimental protocol) and the wheel speed is expressed, in both cases, as a fraction of its maximum value.

- **Exp Wheel Speed (EWS)**: the expected speed of the robot wheels. This is calculated from the MFR signal of one of the two areas (right or left) filtered with the same function used in the close-loop experiment to obtain the wheel speeds from the observed activity. Barring timing errors in the close-loop architecture, this should provide the value of the speed of the robot wheels that we expect from a given MFR. The EWS is useful if compared with the WS in order to confirm that the coding system is actually working as for specification.

- **Sensors**: the signal from position sensors of the robot, oscillating between 0 and 1. The closer the robot gets to an obstacle, the more this value increases, while sensor readings tend to 0 as the robot gets farther and farther from obstacles.

- **Stimuli**: the signal from the stimulation. It is binary and it can thus assume the values 0 and 1, where the positive values indicate the instant of delivery of a stimulation pulse.

*Cross-correlation*: this button allows the user to create a general table containing the Pearson coefficients between the data vectors described above. The data are different depending on
the side to which they refer (right or left). The Pearson coefficient is a measure of correlation between signals, where a value of 0 indicates that signals are totally uncorrelated and 1 linearly proportional signals.

Reconstruction: In order to evaluate the amount of signal information encoded in the neural activity, with this function it is possible to estimate the amount of sensory input that can be recovered from the spike train recorded at each site through an optimal linear filtering approach. More specifically, the linear filter $h(\cdot)$ was computed by applying Welch’s averaged periodogram method with windows of 2 s, a 75% overlap between windows and the use of a Hamming window on a train of stimuli or spikes. The sensory signal $s(t)$ is reconstructed from the train $y(t)$ as:

$$s(t) = s_{est}(t) + n(t) = \int_{-\infty}^{+\infty} h(\tau) y(t - \tau) d\tau + n(t) \quad (8)$$

where $s_{est}(t)$ represents the reconstructed signal, $s(t)$ the original one and $n(t)$ is the residual, i.e. unmodeled portion of $s(t)$. $h(\cdot)$ is constructed to be the average of the linear filters that minimize the variance of $n(t)$ in each of the 2 s observation windows.

Coding performance of each reconstruction has been evaluated by comparing the variance of the original signal with that of the residual. Namely, in the coding fraction is defined as:

$$CF = 1 - \left( \frac{\sigma_n}{\sigma_s} \right)^2 \quad (9)$$

This parameter is stored in the selected pathname for all recording channels.

In the pictures below, it is possible to see some screenshots of the potential analysis of this panel.
In figure 3.2, a comparison between measured wheel speeds (in green and black for the left and right wheels, respectively) and its expected counterpart (in red and blue, for the left and right wheels, respectively) is displayed. In this particular experiment, the graph shows that each couple of curves concerning one of the two wheels present almost identical WheelSpeed and ExpWheelSpeed values and we can clearly see that the measured wheel speed is an almost perfect match of the expected one.
In figure 3.3, it is possible to see the comparison between mean firing rates (yellow and red) and wheel speeds data. It is useful to perform this comparison for a double check on the correctness of the values relating to the speed of the wheels passed in input to the robot. In fact, as can be noted, the MFR has the same trend as WheelSpeed even if its values are significantly higher. This is also confirmed by the corresponding graph of the cross-correlation between the parameters.

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**Figure 3.4: screenshot of a table that contains the Pearson coefficients between all parameters analyzed.**
In figure 3.4, it is possible to observe a table displaying the computed Pearson’s coefficients between different data sets related to the two different areas controlling the wheels of the robot.

In particular, we can see how the values of maximum correlation between the WheelSpeed and ExpWheelSpeed confirms that the speed of the wheels of the robot is exactly what is expected from our encoding and decoding system.

The data between the SensorsL and SensorsR highlight a clear distinction between the two sensory areas of the robot that receive and encode two different signal forms.

Finally, the correlation between the left and right stimulus signals and their corresponding sensor signals point out that we are effectively providing the correct feedback signal to the culture.

### 3.4 Experiment Protocol Panel

Within this panel we have implemented all the functions needed for the correct preparation of an experiment in all the steps leading to the selection of the best channels to be used later during the navigation of the robot.

During an experiment, after the first step of registration of the spontaneous activity of the culture, the user has to select the channels on which the connection map will then be computed.

To do this, the user can use the appropriate function *Spontaneous Connectivity Map* that will automatically select the most active and interconnected channels among the 60 available.

After the connection map has been performed on the channels which were previously selected, the user has to perform two operations of data processing through two specific functions:

* **Convert preprocessing data**: this function imports in MatLab the text files created by HyBrain after the connection map, making them compatible for successive analysis.

* **Compute PSTH&Latency**: this function allows computing the response to the stimuli (PSTH area and latency) of all channels by inserting the appropriate parameters for artifact blanking. The function computes the number of spikes in the considered window and after blanking of stimulation artifacts. With Plot PSTH, it is also possible to plot the histograms of single
channels individually or with all the others (i.e., a multiple plot in which the PSTH of all the electrodes are plotted together).

All the generated plots are saved in both .fig and .jpg formats in the folder “..._PSTHresults”.

The last step before the robot navigation is the selection of the best-connected channels. This can be done through the function `stim rec channels` on this panel: an algorithm has been implemented to identify the best input-output channel selection.

The first step of this algorithm is a screening of channels whose average responses to stimulation must exceed a certain threshold in order to be considered acceptable. Then the algorithm provides a selection of the recording channels whose responses satisfy the defined criteria of amplitude and separation.

In particular, it is necessary that the selected recording channels provide an intense enough response to be discerned from spontaneous activity (amplitude) and different response to stimulation from the two different stimulation channels (separation).
Chapter 4

Closed loop experiments: input reconstruction analysis

4.1 Introduction

One unresolved issue in neuroscience is related to the coding of sensory information within the central nervous system. Strong evidence exists that different features of the spike train (e.g. bursts, isolated spikes) might be coding different aspects of the sensory signal (Metzner, Koch et al. 1998, Reinagel, Godwin et al. 1999, Martinez-Conde, Macknik et al. 2002, Krahe and Gabbiani 2004).

In order to approach this important scientific issue, we took advantage of the neuro-robotic setup developed in our lab (Tessadori, Bisio et al. 2012) to generate sensory data, code it as a stimulation train and deliver it to a neural culture. Two different preparations have been used in the experiments: cortical and hippocampal neurons. Data has been analyzed in order to determine the amount of information about the sensory signal that can be correctly reconstructed from different features of the spike train induced by the stimulation.

4.2 Experimental protocol

The typical experimental protocol followed so far consists of a four-step procedure:

1. Monitoring of the spontaneous activity of the culture;
2. Test stimulus from a set of electrodes in order to generate the map of the effective connection (i.e. connection map) and thus deciding which electrodes can be used as INPUT and OUTPUT of our culture;
3. 20-minutes long robot run;
4. Evaluation of the robot's performances on the basis of specific navigation's parameters.

Before the beginning of the experimental session, spontaneous activity of the network is subject to observation, in order to determine, by empirical observation, which electrodes are the most likely candidate as “input” sites (i.e. sites from which stimulation must be delivered). Typical features to look for in this phase are an adequate mean firing rate (i.e. number of
spikes per second) and patterns of activity not synchronous with other regions. The best candidates (usually a set of 8-10 sites) are then selected for the second step of the experiment. The second step is aimed at generating the map of the effective connection (i.e. connection map) of the channels involved. It is this step that allows us to make an intelligent choice of the input-output areas (Novellino, D’Angelo et al. 2007). In order to obtain a reactive behavior, we need the network to respond soon after the feedback stimulation, that is, we need input-output pathways characterized by a relatively early (up to 50 ms) and sustained response meaning a high strength in the effective connectivity (Shahaf and Marom 2001). If the network reacts to the sensory feedback and the evoked electrophysiological response is characterized by a relatively long activation phase (up to 200-300 ms), the robot would not be able to react to the presence of an obstacle in 100 ms (i.e., the delay among successive serial communications between the system and the robot). This is one of the reasons why we need to accurately select the input-output pathways, beside the fact that only low-frequency stimulation can be delivered for not fatiguing the culture (Shahaf and Marom 2001, Eytan, Brenner et al. 2003). We need the stimulus-evoked response to be fast, prolonged, reliable, and therefore effective for the entire duration of the experiments.

As already said, the general aim is to have a robot that follows a specific task on the basis of the stimulated electrophysiological activity shown by the neuronal culture.

To test the response to stimulation from different sites in different areas of the neuronal network, trains of 50 electrical stimuli are delivered (1.5V peak to peak-extracellular stimulation, 500 s, and duty cycle 50%). This procedure is repeated from at least 5 arbitrarily chosen microelectrodes (Wagenaar 2004). The post-stimulus time histogram (PSTH) (i.e., the average number of spikes obtained in response to a stimulus, at different latencies) is then used for quantifying the strength of connections between a specific stimulating sites and all the other recording sites. It is the impulse response (in terms of instantaneous firing rate) to a single test stimulus. A typical PSTH has been shown in fig. 4.1.
The algorithm for the selection of the output (motor) and input (sensory) sites supplies the I/O pairs corresponding to maximum selectivity and it is based on network effective functional interconnectivity. The ideal case is described in the following: given two (or more) stimulating channels (e.g., S1 and S2) and two groups of recording sites (e.g., R1 and R2), the strength of the connectivity S1-R1 and S2-R2 is “high” and simultaneously, the strength of the connectivity S1-R2 and S2-R1 is “low” (i.e., good selectivity in input-output pairs). The described scheme guarantees to a certain degree, that the responses in the two (groups of) recording sites are different on the basis of the stimulating electrodes. Of course the above is an ideal situation and, since the mean connectivity of the network is high, also due to the high density of plated cells, it is hard to get highly specific responses in the input-output pathways.

The methodology that we used to make a selection of the pathways is the “selectivity map” or “connectivity map” (see fig. 4.2). Each dot represents the PSTH area at a specific recording site given that there was a stimulation from a couple of stimulating sites. All the possible input-output combinations are explored and only the pathways producing responses lying more distant from the diagonal (i.e., closer to the axis) are selected. Those specific pathways (of...
sensory-motor activations) can be then conveniently utilized for driving the robot and for implementing simple reactive behaviors (e.g. obstacle avoidance).

During the third step, the robot is left free to roam the arena with the rules described above. If the starting hypotheses hold true, this will progressively drive the network towards the desired condition of reliable and specific evoked responses.

Finally, we collect and storage the data on the robot’s performances.

4.3 Signal processing

4.3.1 Reconstructions

The first aim of the analysis on spike trains was that of defining which features provide the largest amount of information about the input sensory signal. Furthermore, we wanted to find out whether significant differences could be found in the information content of different firing modalities of cortical and hippocampal cultures. For each analyzed channel, one reconstruction has been generated from each of several observed features. In particular, the following reconstructions have been performed:
• *all spikes:* the entire spike train of a channel was used to reconstruct the relative sensory signal;

• *doublets:* the train used for reconstruction consisted of the first spike in each detected doublet;

• *bursts:* the train used for reconstruction consisted of all the spikes belonging to all detected bursts;

• *burst events:* the train used for reconstruction consisted of the first spike in each detected burst;

• *isolated spikes:* the train used for reconstruction consisted of all the spikes not belonging to any burst;

• *burst events + isolated spikes:* the train used for reconstruction consisted of the first spike in each burst and all those not belonging to any burst;

• *other spikes in burst:* the train used for reconstruction consisted of all the spikes following the first within each burst;

• *linear reconstruction:* this reconstruction was obtained as a linear combination of the reconstructions from the points 4, 5 and 7 (further details are provided below).

Two more reconstructions have been generated starting from the complete spike train, but to match a different sensory signal: in the first case, reconstruction of the “opposite” sensory channel is attempted (i.e. the sensory input not associated with the recording channel); in the second case, the considered sensory signal is the sum of the two actual inputs. Furthermore, for each experiment, a reconstruction of the sensory signal was generated starting from the corresponding stimulation train.

### 4.3.2 Linear combination

In order to evaluate whether different features of the spike train code for different characteristics of the input signal, the reconstructions obtained from complete spike trains have been compared against a linear combination of reconstructions obtained from three subsets of the spike train, namely burst events, following 6 spikes within bursts and spikes outside of bursts. The multiplication coefficients have been chosen so as to minimize the reconstruction error of the linear combination. If bursts were reliably generated as a response to a specific signal feature, the reconstruction error should be significantly smaller on linear
combination reconstructions rather than that observed on reconstructions from the complete spike train.

4.3.3 Simulated data
Simulated data has been generated to validate the reconstruction process and test its robustness to different kinds of “disturb”. We generated a sensory signal through the convolution of one of the recorded stimulation trains with a Gaussian kernel of a width compatible with the filter generation (the value of the kernel has to be zero or close to zero at the end of the considered window), thus granting perfect match (CF>99%). This train-signal pairing will be referred to as “mixed data” in the following, since the event train is recorded, but the signal is simulated. A second train was generated as a point-process in which each time sample has a probability of being an event. This probability is computed so that the average number of events per time unit is the same as that in the recorded train. The matching signal has also been generated through convolution as described above (this train-signal pair will be referred to as “simulated”).

The different kinds of disturb introduced either a temporal jitter or changed the number of stimuli, by adding or removing to the original signal. In the first case, the timing of each stimulus was moved forward or backward in time by a random number of samples selected from a uniform distribution with a given maximum. Removal of stimuli was performed in a similar way, with each stimulus presenting a fixed probability of being removed. In the third kind of disturb, each non-stimulus time point in the stimulation train had a fixed probability of being converted to a stimulus.

4.4 Database and Statistics
We performed experiments on 15 different cell cultures (4 cortical and 11 hippocampal). In each experiment, two electrodes were selected to deliver the stimulation train, while 16 channels were monitored and recorded. Half of these were associated with the left input channel, the other half with the right one. Datasets were thinned by removing experiments displaying a CF of sensory signal from stimuli train less than 80% on either input channel or an average CF computed on all the channels less than 10%. The remaining dataset (i.e. the one actually used in this work) included 3 experiments on cortical and 3 experiments on hippocampal preparations, for a grand total of 96 analyzed spike trains. Furthermore, single
spike trains have been removed if they obtain CFs less than 5% when reconstructing either sensory signal.

Data within the text are expressed as mean ± standard error of the mean (se). Statistical tests were employed to assess the significant difference among different signal’s features. The normal distribution of experimental data was assessed using the Kolmogorov-Smirnov normality test. We then performed the one-way ANOVA test for multiple comparison and p values < 0.05 (indicated with an ‘*’ in the figures) were considered significant. The mean comparison was performed through the post-hoc Bonferroni test. Statistical analysis was carried out by using OriginPro (OriginLab Corporation, Northampton, MA, USA).

4.5  Results

In order to evaluate the performances in reconstructing the input sensory signal of cortical and hippocampal neurons, we used a neuro-robotic set-up constituted by a virtual robot bidirectionally connected to a MEA coupled to a neuronal assembly. Raw data were first acquired and then a spike detection procedure was applied. The result of this algorithm produced a spike train (second plot from the top in Fig. 4.3), which was in turn subdivided into several different features. The elements extracted from each spike train were doublets and bursts. Bursts were in turn divided into burst events (the first spike of each burst) and subsequent spikes. Isolated spikes are here defined as all those spikes that are not part of a burst. An example of the features just described is shown in Fig. 4.3.

First, we tested the reconstruction algorithm on mixed, simulated and recorded train-signal pairings under different conditions in order to evaluate its robustness. As it is possible to observe from the graphs in Fig. 4.4, the coding fraction is above 80% in the case of recorded stimulation train and signal (“recorded” trace in the graphs), while it gets virtually to 100% in the mixed data pairing. On the other hand, the reconstruction based on the simulated pairing is missing most of the information present in the original signal, with the maximum CF around 30%: although the mean rate of events is the same in all three cases, in the simulated train, the positioning of the events is random. This leads to imperfect reconstruction of the linear filter, as events closer than the length of the window of the periodogram will lead to partly overlapping responses.
Reconstruction performances are very robust to jitter (Fig. 4.4A), since displacements under 10 ms (100 samples) cause a loss of less than 10% of CF, while maximum jitter has to be increased to more than 100 ms (1000 samples) to effectively destroy the information content of the recorded signal and even more than that in the simulation from recorded data. On the other hand, addition or removal of events has a very different effect on the reconstruction of a simulated or recorded signal (Fig. 4.2B and C): in the first case addition or removal of around 10% of the events has an almost negligible impact on the computed CF, while in the latter less than 3% of events changed result in the impossibility of recovering the correct sensory signal. In Fig 4.5, for a sample channel, most of the reconstructions described above are shown in the case of a hippocampal and a cortical culture. Panels B and C of Fig. 4.5 show the performances obtained from the experiments that satisfied the conditions described in the Methods section. Spike trains usually provide the best reconstruction performances among all the considered features and, in general, the CF for spike trains and linear reconstructions is roughly the same, both for cortical and hippocampal cells. This implies that the division of the spike train into
burst events, other burst spikes and isolated spikes is not helpful in recovering the information content of the spike train.

Figure 4.4: (A) Effect of jitter on coding fraction. For each point, 25 reconstructions have been computed for each signal-stimulation train pairing, in which each event of the train was moved from its original position a random number of samples in either direction. Shown on the graph are the median values of the 25 repetitions. (B) Effect of event removal on coding fraction. For each point, 25 reconstructions have been computed for each signal-stimulation train pairing, with each event in the train having the same fixed chance of being removed. Median values of the 25 repetitions are shown in the graph. (C) Effect of event addition on coding fraction. For each point, 25 reconstructions have been computed. Each non-event sample of the train had the same probability of being changed into an event (10% fraction of added samples indicates that each non-event point had a probability of being changed into an event so that, on average, the output train would have 10% more events than the input train). Median values of the 25 repetitions are shown in the graph.
Figure 4.5: (A) The two set of graphs show, from top to bottom, one of the two input sensory signals delivered to the culture; reconstruction performed from the corresponding train of stimuli; from the complete set of spikes on a channel; from the train of doublets; from all the spikes belonging to bursts; from spike events (i.e. only the first spike in a burst); from spikes not belonging to bursts; from burst events and isolated spikes; from the best linear combination of reconstructions from burst events, other spikes within bursts and isolated spikes. The left set of graphs is generated from data recorded on a hippocampal culture, while the data from the right set is from a cortical culture. On the right side of each graph, the corresponding CF is indicated as a percentage. (B) Box plots of reconstruction performances, expressed as coding fractions, on all channels recorded from hippocampal cultures. Thresholds for exclusion of a channel from the dataset are explained in detail in the Materials and Methods section. (C) Box plots of reconstruction performances, expressed as coding fractions, on all channels recorded from cortical cultures. Thresholds for exclusion of a channel from the dataset are the same as for plot B.
The reconstruction performance has also been used to evaluate the ability of different preparations to process the information of two spatially separated input channels at the same time. In the setup of the neuro-robotic experiments, eight recording channels were associated with one of the two sensory inputs (Methods for details). In this work, we are evaluating a posteriori the selectivity of the representation of each recording channel by scoring the reconstructions of the associated sensory input, the opposite sensory input and that of a signal equal to the sum of both inputs from the spike train observed. Fig. 4.6 shows these results. Specifically, we reported the performance of each channel in reconstructing its associate stimulus and the opposite one (left panel), a comparison in the performances obtained in the reconstruction of the associated/opposite stimuli against those of the sum of stimuli (middle/right panel). While results from hippocampal cultures are rather spread in the parameter space, 9 data relative to cortical cultures tend to cluster along the bisector of both graphs. Spike trains from hippocampal cultures present better performances in the reconstruction of both associate and opposite signals, whereas recordings on cortical cultures tend to code more precisely the sum signal than either the associate or opposite ones.

![Figure 4.6: Comparison of CFs computed from the complete spike trains of channels in the reconstruction of the two input signals. The coordinates of each point represent the CF obtained when reconstructing one input signal (first graph: ‘same’-’opposite’ signals; second graph: ‘same’-’sum’ signals; third graph: ‘opposite’ – ’sum’ signals). Color codes the cell type of the culture each recording is from: red points plot data from cortical cultures, green points from hippocampal ones. ‘same’ signal is the sensory signal the recording channel is associated with (see text for details), ‘opposite’ is the other sensory signal and ‘sum’ is the sum of both input signals.](image)
4.6 Discussion

We made use of a hybrid neuro-robotic platform to investigate the basis of sensory coding of cortical and hippocampal cultures plated on micro-electrode arrays. First, we tested the chosen reconstruction technique on simulated data and we further asked whether and how reconstruction performances are affected by different kinds of data loss and/or corruption (see Fig. 4.3). For all datasets (i.e. recorded, mixed and simulated data) we found that the exact positioning of events within the train is not critical for signal reconstruction, as the addition of jitter to the event train does not cause significant loss of performance until very large values. This result was partially expected, since the employed coding scheme is rate-based. On the other hand, removal of events from the train causes a very quick degradation of performance, as does the addition of events in random positions or the loss of spectral information, as the simulated dataset indicates (Fig. 4.3). Translating these observations to spike trains, it turns out that the single most important property of the neural preparation is the reliable generation of spikes following stimulation and the absence of interfering spontaneous activity, as even a low number of spikes uncorrelated with the sensory signal are sufficient to disrupt reconstructions: if a stimulus does not generate responses, no estimation of sensory data can be performed in the corresponding interval.

On the other hand, uncorrelated spontaneous activity will degrade the quality of the linear filter. These observations are indicative of the findings from the other experiments performed: subdividing the spike train into finer features (in contrast to what reported by Cozzi and co-workers (Cozzi, D’Angelo et al. 2006)) does not improve the reconstruction performance. In our preparation, there does not seem to be any relationship between features of the sensory signal (such has sharp changes or high values for long periods of time) and the chance of occurrence of bursts: burst events score constantly lower than other trains and even linear reconstructions are not significantly different from those obtained from the complete spike train (Fig. 4.4). It is worth noting, though, that in Cozzi et al. bursts were defined as a group of at least 2 spikes closer than 100 ms, whereas in our work we considered bursts groups of at least 5 spikes, the commonly accepted definition of burst (Chiappalone 2005, Turnbull, Dian et al. 2005, Leondopulos, Boehler et al. 2012). Doublets were introduced in this work to provide a more direct comparison to the work of Cozzi and co-workers (Cozzi, D’Angelo et al. 2006). It is possible to observe, furthermore, that even the best reconstructions from spike trains rarely exceed a CF of 40%, but are closer to the performance obtained on simulated data in Fig. 4.5. This is ascribable to the fact that recorded spike trains do not have
the same peaked spectral content as stimulation trains, therefore they constitute suboptimal candidates for reconstruction. In particular, a better reconstruction could be obtained by designing suitable, different windows for the computation of the linear filter in each experiment. On the other hand, in our work, a difference between the behavior of cortical and hippocampal neurons can be noted. When preparations were subjected to two different sensory inputs at the same time, cortical preparations were generally unable to differentiate between the two, but the activity recorded on different channels was rather a function of the sum of the two channels (i.e. responses are not segregated). Hippocampal cultures showed a much more varied behavior, ranging from very selective responses to a behavior close to that observed for cortical preparations (Fig. 4.6). In a previous work (Tessadori, Bisio et al. 2012), it was shown that the use of a confinement mask to create interconnected sub-populations of neurons improves the segregation of responses to different input signals, thus correlating structural modularity to functional segregation. Here, we can speculate that our hippocampal cultures spontaneously tended to cluster, thus reproducing a modular architecture, whereas cortical ones are likely to reproduce in vitro a more homogeneous network.
Chapter 5

Closed loop experiments: testing different decoding schemes

5.1 Introduction

Classical computational architectures are still underperforming when compared to biological systems of similar size and energy consumption in terms of learning, memory and information-processing. A likely cause for this shortcoming is the lack of a developmental phase in silicon-based technology (Clark 1997, West-Eberhard 2003, Gilbert 2009, Bongard 2011), during which wiring of the nervous system changes according to a continuous interactions with the surrounding environment. While biological structures are still, at large, too complex to be fully translated into artificial systems, it is possible to realize hybrid systems to study specific information processing aspects, such as sensory-motor feedback loops and plastic modifications.

We realized an ‘embodied robot’ by interfacing a mobile sensor platform with a population of hippocampal neurons, cultured over Micro Electrode Arrays (MEA), as reported in the previous chapters. Strong evidence exists that different features of the spike train (e.g. bursts, isolated spikes) might be coding different aspects of the sensory signal (Metzner, Koch et al. 1998, Reinagel, Godwin et al. 1999, Martinez-Conde, Macknik et al. 2002, Krahe and Gabbiani 2004). In our experiments, we provided information on the external environment through stimulation to our robot and we tested several control algorithms for the robot based on different features of the resulting spike trains in order to provide a ‘behavioral’ meaning to their occurrence. We then tried to link the observed behavior to the different features in the underlying spike trains.
5.2 Experiment Protocol

This protocol is different from the previous one in the last two phases: the phase of random robot run in arena and the evaluation of the robot’s performances on the basis of specific navigation’s parameters.

Differently from the previous protocol, this kind of experiment involved three arenas: one with a straight path, another with a right turn, and the last one with a left turn. The turns are smooth and resemble a section of a two dimensional annulus. Each arena has only the ends of the annular segment as the exit points. The walls serve as the only obstacles that the robot sees (see fig.5.1).

![Figure 5.1](image_url)

*Figure 5.1: The robotic task consists of correctly navigating three different types of arena, presented randomly: a straight path (A), a right (B) or a left turn (C). The pink circle represents the robot.*

The robot is initially positioned at the center of one of the exits of the arena. It is expected to navigate out of the arena through the opposite exit, for the run to be counted as a successful one. As soon as the robot exits through one of the two open ends, it is presented with a new arena.

The choice of arena is randomly made from the above mentioned set of three possible arenas; in particular, for each control condition, the robot was presented 10 repetitions of each track in a random order. Each of the five robot runs performed had a different increment in wheel speed due to the presence of spikes or bursts. These parameters can be set by using a simple interface shown in Fig. 5.2.

Several different events have been tested as candidates for control: we analyzed the contributions of simple spike trains, bursts (groups of at least five spikes separated in time by no more than 80 ms), isolated spikes (spikes not belonging to a burst) and two differently
weighted combinations of the latter two (fig. 4.1 of the previous chapter illustrates the different considered features).

Especially for the combinations we thought, burst events have been used (the first spike of a burst) instead of bursts themselves because they were considered more adequate for a comparison with the isolated spikes.

Figure 5.2: screenshot of the HyBrain interface which was used to set the contribution parameters for the various events.

5.3 Database and Statistics

We performed experiments on 5 different cell cultures (all hippocampal cultures), ranging from 30 to 50 DIV. At the beginning of each experiment, the state of health of the culture was initially verified by using also a microscope, if we believed that a morphologic analysis of the culture itself was useful.

Data within the text are expressed as mean ± standard error of the mean (se). Statistical tests were employed to assess the significant difference among different signal’s features. The normal distribution of experimental data was assessed using the Kolmogorov-Smirnov normality test. We then performed the one-way ANOVA test for multiple comparison and p
values $< 0.05$ (indicated with an ‘*’ in the figures) were considered significant. The mean comparison was performed through the post-hoc Bonferroni test. Statistical analysis was carried out by using OriginPro (OriginLab Corporation, Northampton, MA, USA).

5.4 Results

The parameter that we selected to evaluate the performance of the neuro-robotic system was the rate of successful trials. The tools for the off-line data processing aimed at the analysis of the behavior of the neuro-robotic system were developed using Matlab.

Fig. 4.4 shows the success rates of the robot runs averaged over all experiments conducted. A total of 7 experiments were considered in the evaluation of these results. The x-axis represents the five different robot runs. The “H” and “L” in the labels stand for “high” and “low” respectively.

Therefore the labels corresponding to each of the five sets of robot runs may be summarized as follows ($\Delta \omega$ stands for an increment in the wheel speed):

- **Spikes**: $\Delta \omega_s = 0.1$ and the other values of $\Delta \omega$ which were set equal to zero.
- **Burst Spikes**: $\Delta \omega_b = 0.1$ and the other values of $\Delta \omega$ which were set equal to zero.
- **Isolated Spikes**: $\Delta \omega_{is} = 0.1$ and the other values of $\Delta \omega$ which were set equal to zero.
- **Isolated Spikes H and Burst Events L**: $\Delta \omega_{is} = 0.1$, $\Delta \omega_{be} = 0.01$ and the other values of $\Delta \omega$ which were set equal to zero.
- **Isolated Spikes L and Burst Events H**: $\Delta \omega_{is} = 0.05$, $\Delta \omega_{be} = 0.02$ and the other values of $\Delta \omega$ which were set equal to zero.

We have to notice that, in order to keep the right proportions of contribution, we chose to consider a different order of magnitude between isolated spikes and burst events.

The decay rates of both the spikes and bursts were fixed at a constant value, for each set of robot run: $d_s = d_b = 1$.

Success rates present a high degree of variability depending on the controlling feature: control with bursts tend to result in a robot with little to no control, with success rates highlighting very poor results regardless of track shape.

In general, a similar result holds true for control with complete spike trains, which lead to good success rates at most on one shape of the track (i.e. the robot can only turn in one direction or proceed straight ahead). The remaining control conditions prove better at achieving guidance of the robot, with a significant number of positive trials in two or all three the possible tracks (Fig. 5.3A).
In order to explain this result, we compared the ratio of the number of events recorded on the two motor areas (i.e. left and right), observing that such ratio could vary from ~100 in the case of bursts (i.e. one motor area is displaying 100 times more bursts than the other) to ~1 in the case of isolated spikes (i.e. outside of bursts, almost the same number of spikes is detected on both motor areas in almost all experiments), as reported in Fig. 5.3B.

In Fig. 5.4, we highlighted this relationship in a scatterplot: all the experiments with one of the “simple” controls (i.e. where only one parameter was involved) are plotted as dots on the graph. On the X-axis is represented the logarithm of the ratio of the number of events detected on one motor area with the number of events detected on the other, while the Y axis represents the success rate achieved in that particular trial.
Figure 5.4: Scatter plot of success rates in all the experiments over the ratio of event rates in different motor areas, expressed in logarithm. Regardless of the event considered for control, the average success rate observed decreases rather swiftly as the number of events recorded in each motor area gets more and more unbalanced. In particular, the number of bursts may vary significantly between the two motor areas (green dots and interpolation line), whereas spike number and isolated spike number tend to remain closer (respectively, red and blue dots and lines).

5.5 Discussion

In these set of experiments, we tested several decoding algorithms based on different signal features, while we kept the coding paradigm constant, linear and rate based. We found out that, in these conditions, one of the major obstacles to the robot control was the fact that our stimuli did not elicit a constant number of events. Bursting activity, in particular, seemed to be almost independent from stimulation: regions of the culture that tended to burst did so independently of the input signal. This, in turn, led to very diverse numbers of events being recorded from the two motor areas and basically to a failure in control. On the other hand, all the control paradigms that removed bursts or downplayed their weight achieved a much higher degree of success. This result partly agrees with other studies (Cozzi, D'Angelo et al. 2006), even this is the first time that different decoding schemes are applied and tested in a closed-loop experimental environment.

Further investigations will be necessary in order to better understand the role of burst in neuronal networks. In particular, bursts could either contribute to representing input
information in a more complex manner than what considered in this framework or their occurrence could be tied to spontaneous patterns of activity within the network and not affected by the delivery of stimulation.
Chapter 5 | Closed loop experiments: testing different decoding schemes
Conclusions

The goal of the present thesis is to investigate computational capacities of neuronal networks and how they represent information about the external world. In order to do this, we used a hybrid neuro-robotic platform developed at the Neural Interface and Network Electrophysiology (NINE) lab of the Italian Institute of Technology.

The first phase of the work consisted in the development of a series of tools in MatLab to analyze the collected data from different experiments. Thanks to this software, it was possible to implement a burst detection algorithm which was able to divide spike trains from neuronal activity recorded during the experiments according to different signal features (bursts, doublets, isolated spikes).

Once the different features of the signal were obtained, we proceeded with computing reconstructions of the sensorial signal coming from the robot, in order to assess the quality of the implemented coding system. In particular, the parameter of coding fraction was defined: this enabled us to provide a numeric evaluation of the reconstruction quality.

With this analysis, significant differences were observed when comparing reconstructions computed from isolated spikes with those from bursts: the first were significantly better than the second.

Moreover, we noted a significant relationship between the type of culture used and its ability to differentiate between spatially distinct stimuli. In this sense, hippocampal cultures seem to be better at discriminate spatially different stimuli than cortical ones.

In the light of these results, the second phase of this work was carried out. The software of the close-loop system management (HyBrain) was optimized and an upgrade for the real time detection of bursts was realized.

With the software fully upgraded, an experimental phase has been carried out to study the effect of several decoding algorithms based on different features of the signal. In particular, we distinguished between bursts and isolated spikes and we gave a different weight to these events. In practice, it was possible, for example, for a spike detected as part of a burst to have a small contribution to the speed of the controlled wheel, whereas an isolated spike might have a much stronger impact.
Two important results were obtained during these eight months of work:

- A difference in the ability of hippocampal and cortical cultures to discriminate between spatially distinct stimuli has been noticed.
- Different features from the spikes train have been defined and distinguished in real time. This lead to different decoding strategies that resulted in diverse robot behaviors.

The results have been illustrated in two articles, which were submitted and accepted in two important conferences in the neuroscience field.

As future developments, we propose a detailed analysis on the incidence of the interplay between spontaneous and evoked activity: in our experiments we have repeatedly observed that responses to stimulation fluctuate significantly and we would like to understand the relationship between past culture activity and response to stimulus.


References


