SUBSTRATE ARRAYS OF MICROELECTRODES FOR *IN VITRO* ELECTROPHYSIOLOGY

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1. INTRODUCTION

Substrate arrays of microelectrodes (MEAs) are microfabricated devices that can be electrochemically coupled to portions of brain tissue maintained *in vitro* (i.e., either cultures of dissociated neurons or brain tissue slices), because the electrically excitable cellular components in these experimental preparations are comparable in size with the MEA-engineered microstructures (see Figs. 1 and 2). These arrays are usually made of up to 100 independent microelectrodes, each with a diameter of a few tens of micrometers, embedded in a planar biocompatible substrate and spatially distributed over an area of a few square millimeters (see Figs. 3 and 4) (1). MEAs are employed as the bottom of an ad hoc electrophysiological recording chamber, or as a kind of Petri dish for cell cultures, in the standard settings of *in vitro* cellular electrophysiology. Thanks to appropriate electronic instrumentation, such as low-noise multichannel preamplifiers and stimulus isolators, it is possible to extracellularly and noninvasively detect and stimulate the distributed electrophysiological activity of a neuronal population *in vitro*, either acutely coupled on top of the MEAs (see Fig. 1, right panel), or growth on it during several weeks and cultured under sterile physiological conditions (see Fig. 2).

The advantages of the MEAs can be better appreciated when compared with the current state-of-the-art network-level electrophysiological approaches *in vitro*. These consist of simultaneous intracellular/patch-clamp access to the membrane voltage from visually identified single neurons, on manual or semiautomatic placement of several glass pipette electrodes (e.g., 5–10) in close contact with the somatic membranes (2). However, the maximal number of electrodes, thus of cells, is severely restricted by space constraints below the stage of an upright microscope and by the availability of very expensive (e.g., piezoelectric) multi-micromanipulator units. More importantly, such an approach is highly invasive and requires the experimenter to possess considerable skill for a quick and precise micromanipulation. Although superior in terms of the quality of the electrophysiological recordings, patch-clamp electrodes dialyze the intracellular compartments with the pipette solution, altering the physiological cytosolic concentrations, and they always irreversibly damage the neuronal membrane, which makes it extremely difficult to keep stable and reliable recording conditions for more than a few hours, posing serious limits to the experimental issues that can be investigated. On the contrary, MEAs offer the unique opportunity to monitor and stimulate the temporal electrochemical activity of a neuronal network with a much higher spatial resolution, noninvasively and over a longer time horizon (i.e., up to several months with cultured neurons). Although only an extracellular detection of neuronal spiking activity is usually possible [but see (3)], MEAs further opened the way to the long-term study and identification of the impact of metabolic and homeostatic neuronal mechanisms, affecting and modulating the spatial patterns of the network electrical activity, as well as of the development and plasticity of synaptic connections.

2. MEAs FABRICATION

Several research groups today are using MEAs as a standard tool for neurophysiological investigations[1]. However, it was only after a pioneering period at the beginning of the 1980s, during which MEAs design and fabrication were exclusively achieved by researchers with access to nanotechnological facilities (i.e., clean-rooms), that substrate arrays and integrated multichannel hardware began to be commercially available[2].

As already mentioned, the technological processes involved in the fabrication of MEAs have been mainly derived from the microelectronic technological processes that, over the last several decades, evolved into reliable and precise industrial automated methods. Such technologies, oriented to the large-scale production, and particularly advanced in the field of microprocessors fabrication, basically consist of a multilayered bi-dimensional manipulation of substrate materials and semiconductors (see Fig. 3). A similar paradigm is conceptually similar to the fabrication of (macroscopic) electronic printed board circuits in discrete electronics.

For biocompatibility reasons, the substrate materials employed in the fabrication of MEAs are usually glass, quartz, or silicon. Individual leads are made of gold or indium-tin oxide and are able to carry electrical signals, linking external amplification/stimulation electronics to the microelectrode-electrolyte interface (see Figs. 1–4). The deposition of the bi-dimensional substrate leads, independently contacting each microelectrode, follows micro-photolithographic procedures, after the fabrication of a photographic *mask*, in a very similar way to what is requested for the design of VLSI electronic components. Leads are coated with an insulating layer (e.g., silicon oxide, silicon nitride), preventing the electrical shunting of the signals to the bath solution and further allowing the cells to be intimately coupled to the MEAs, after surface coating by adhesion-promoting molecules (e.g., laminin, polylisin, cellulose nitrate, etc.). The microelectrode active area, left exposed by the insulating layer, is usually

[1]Other types of excitable cells and tissues, such as *syncytia of cardiac miocytes*, are also being actively studied.
[2]See for example: *http://www.multichannelsystems.com/neuro*; *http://www.panasonic.com/medical_industrial/MEDSystem*; *http://www.ayanda-biosys.com*; *http://www.cnns.org*.

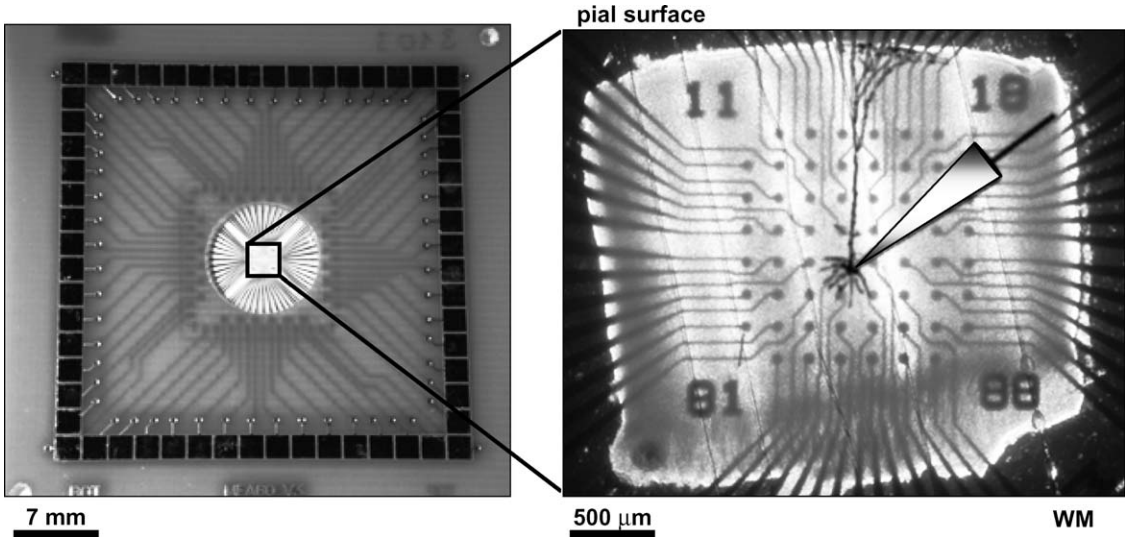


Figure 1. Substrate arrays of microelectrodes (MEAs) are suitable to be coupled to acute as well as organotypic brain tissue slices: (left) The MEA is made of a small microfabricated glass substrate attached to a standard *pcb* board, which provides the physical interface to external electrophysiological stimulation/recording equipment. Acute parasagittal slices of rat brain neocortex (right) are prepared according to standard methods, maintained under healthy conditions *in vitro*, and placed on the top of the MEAs. The location of the pial surface and of the white matter are indicated. Standard infrared interference contrast (*DIC*) videomicroscopy can be implemented, thanks to optical properties of the glass substrate, making it possible to combine MEAs to traditional glass electrode techniques (e.g., patch-clamp in the *whole-cell* configuration). The sketch of a glass pipette, patching a layer V pyramidal neuron, has been superimposed to the image of the cortical slice. This MEA has been developed at the Swiss Federal Institute of Technology of Lausanne (EPFL), Switzerland.

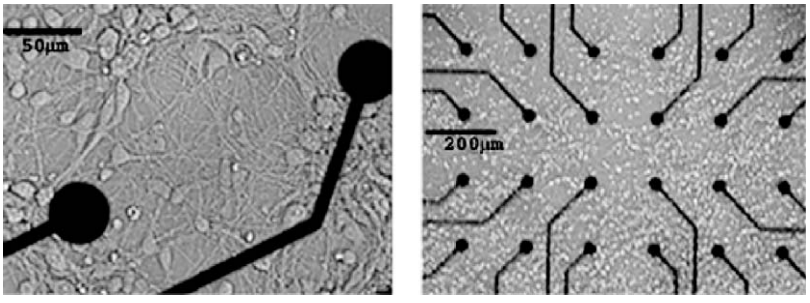


Figure 2. Cartical neurons dissociated from rat embryos can be cultured on arrays of planar microelectrodes. Cells grow and "randomly" develop neurites and synaptic connections over several weeks *in vitro*, organizing into a bi-dimensional network. Electrical signals can be detected extracellularly by each microelectrode, and mainly result from the combined emission of action potentials by a few neurons (usually up to three) covering the same microelectrode.

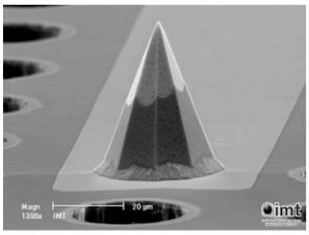
treated by platinum coating to fabricate a metallic electrode (Fig. 4).

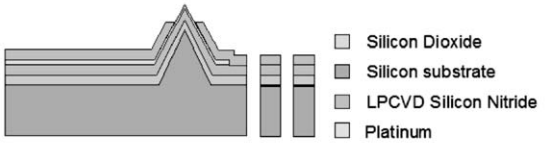
A few examples of MEA devices, microfabricated on a silicon substrate, are reported in Figs. 3 and 4, showing planar and three-dimensional micromachined MEAs for different applications. Planar arrays are suitable to be coupled to dissociated neurons when employed as cell-culture dishes (Figs. 2 and 4), while more sophisticated three-dimensional structures and perforated substrates are commonly used with acute and organotypic brain tissue slices (Figs. 1 and 3).

Finally, as attempted by several laboratories, silicon substrates can also be exploited as a semiconductor, fabricating arrays of a special kind of transducers, implementing ion-sensitive field-effect transistors (IS-FET) or insulated gate field-effect transistor (IG-FET) (4–6). Moreover, thanks to the compatibility with standard CMOS fabrication technology, besides difficulties developing from worse signal-to-noise ratio, compared with passive metallic microelectrodes, the same approaches can lead to the development of high-density arrays (i.e., with thousands of independent recording sites), with integrated signals multiplexing and processing capabilities.

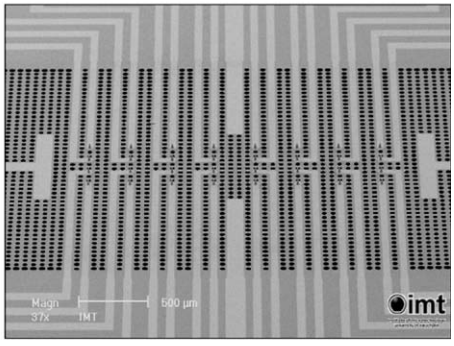
3. COMPLEMENTARY TECHNIQUES: OPTICAL IMAGING

It is easy to foresee that, in the next years, *in vitro* network neurosciences will take advantage of the simultaneous combination of MEAs and complementary techniques. In fact, MEAs do not represent the only possible technological way to overcome the poor spatial resolution of the traditional *in vitro* electrophysiology (1,7). Beyond the intracellular and whole-cell patch-clamp recording techniques, in the last few years, impressive advances in

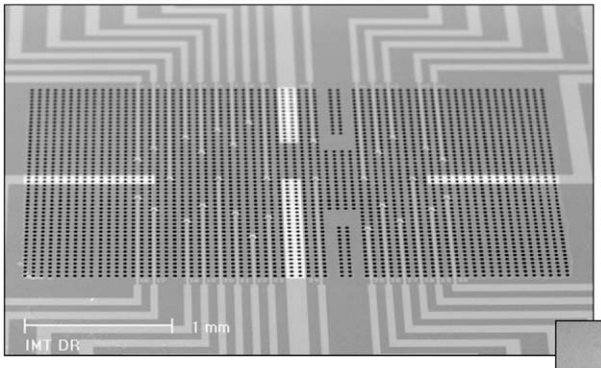


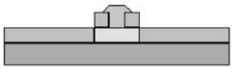


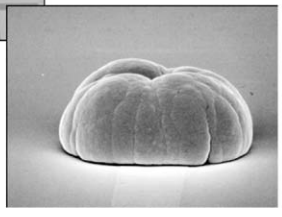
height	47 μm
microelectrode size	cone with circular base of 10 μm in diameter and 15 μm in height



(a)







height	20 μm
microelectrode size	40 μm x 40 μm x 20 μm

(b)

Figure 3. Examples of (a) Pt-tip and (b) Pt-hillock microelectrodes, micromachined on a perforated silicon substrate. The corresponding electron-microscopy images, the sketch of the cross sections and the geometrical features of the microelectrodes are reported. These arrays have been developed at the Institute of Microtechnology, University of Neuchatel, Switzerland, for brain tissue slices electrophysiology (picture kindly provided by L. Berdondini).

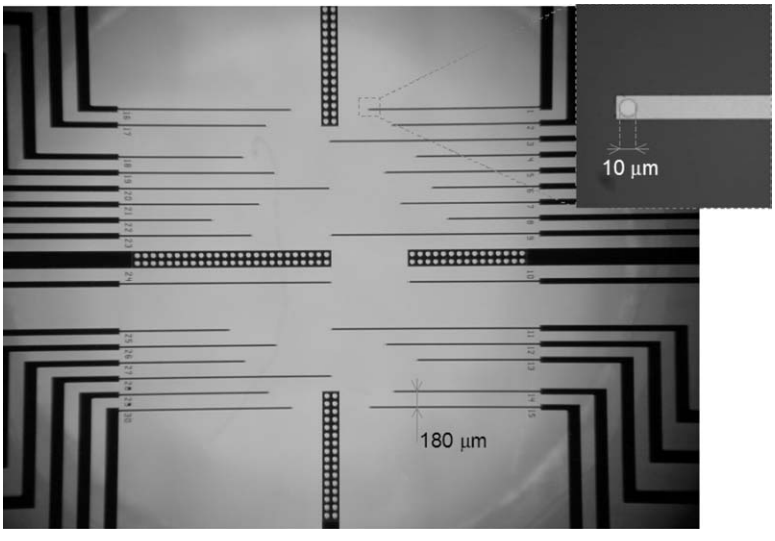


Figure 4. Example of an array of 30 planar microelectrodes, microfabricated on a silicon substrate, designed and developed at the Institute of Microtechnology, University of Neuchatel, Switzerland (picture kindly provided by L. Berdondini).

the optical imaging techniques and confocal microscopies occurred. Such approaches are usually combined with the use of calcium- and voltage-sensitive dyes, together with photodiode arrays or special high-sensitivity CCD cameras. Actually, once the individual cells of a population (i.e., both brain slices and dissociated cell cultures) are filled with these dyes *in vitro*, they emit fluorescent signals when excited by an appropriate wavelength light stimulation, as a function of the local intracellular free calcium concentration or electrical field (8). However, when the temporal resolution is of concern, these techniques are still one or two orders of magnitude slower than electrophysiological recordings. These approaches are, of course, extremely invasive and have several disadvantages related to low signal-to-noise ratios and phototoxicity. More importantly, by the optical techniques, it is usually not possible to deliver a distributed, patterned stimulation of the electrical activity in a network, as the signal transduction is, by definition, one-way [but see in the literature the light-activated chemically-caged neuroactive compounds (9,10)].

4. BRAIN TISSUE SLICES COUPLED TO MEAs

As mentioned in the Introduction, brain tissue slices can be coupled to MEAs for recording as well as stimulation purposes, acutely or in organotypic long-term tissue cultures. Compared with the cultures of dissociated neurons, slices have the advantage of retaining the same cytoarchitectural details and phenotypes of the *in vivo* brain microcircuits. Furthermore, the biophysical properties of single neurons and synapses, developing as a consequence of the expression and distribution of voltage-gated protein ion channels, are fully retained *in vitro*.

Until very recently, by coupling MEAs to hippocampal, cortical, and cerebellar brain tissue slices, it was possible to detect electrical signals that are mainly related to the simultaneous activation of a large number of closely located neurons, resulting in the so-called slow field-potential activity. Today, several groups are successful in also recording the individual action potentials (see Fig. 5), occurring on a much faster time scale (i.e., a few milliseconds), similarly to what is detected by conventional extracellular tungsten electrodes (11), which can be achieved by means of special three-dimensional microelectrode layouts, employed in the fabrication of the MEAs. In these devices, microelectrodes are microscopical tips, whose arrangement provides an intimate mechanical coupling to the tissue slice: It is thought that the shape of the individual microelectrodes helps in overcoming the outer cell layers of the slice, damaged during the cutting procedures, thus reducing the distance between the microelectrode and the active cells and improving considerably the signal-to-noise ratio.

Although the use of MEAs with acute brain tissue slices is still in its infancy and represents a new research field, several interesting applications have already been proposed. For instance, it has been experimentally shown that a spatially distributed electrical stimulation, delivered through the MEA, induces an *in vivo-like* sustained *background synaptic activity* in acute cortical slices (12). This activity mimics a physiological barrage of asynchronous postsynaptic currents, reproducing what is experienced by cortical cells in the intact cortex and affecting the single-cell discharge properties (4,13). Furthermore, distributed activity-dependent network plasticities, as well as the generation and spatial propagation of epileptiform electrical activity, can be induced and investigated in hippocampal slices (14), where stimulation and recording of the activity of neuronal microcircuits may lead to novel insights on the spatially distributed storage of information under physiological as well as pathological conditions.

Finally, it is possible to simultaneously combine the traditional electrophysiological techniques with MEA recording and stimulation (see Fig. 1). Taking advantage of the (transparent) optical properties of the glass-substrate MEAs, the infrared interference contrast microscopy (DIC) required by whole-cell patch-clamp technique in brain tissue slices, can be implemented. It is therefore possible to image living cells and micro manipulate the pipette electrodes accordingly, ultimately compensating for the compromise between the increased spatial resolution, provided by the MEA, and the poor (extracellular) access to the single-neuron electrical activity. As a consequence, it is routinely possible to monitor and control the intracellular membrane voltage of a few cells in great detail, while recording or stimulating the activity of an entire neuronal population, in which such cells are embedded.

5. NETWORKS OF DISSOCIATED NEURONS CULTURED ON MEAs

According to standard procedures, neurons can be enzymatically dissociated from the hippocampus, the cortex, and the spinal cord and plated over the MEAs surface. In such a way, cells can be kept in a healthy condition for a very long time, and cultured *in vitro* under a highly controlled physical-chemical environment (15). Under such conditions, neurons grow, develop, and establish functional synaptic connections, organizing into bi-dimensional networks so that their electrical activity can be detected by the MEA microelectrodes (16,17). Compared with the acute preparations, cultured neurons may not express a physiological set of membrane ion channels and, of course, the topological arrangement of the synaptic connection does not replicate the *in vivo* microcircuits. Nevertheless, the cultures of dissociated neurons offer the unique opportunity of observing and influencing the development and the electrical activity of relatively large neuronal networks (i.e., 10^4–10^5 neurons) *in vitro*, from days up to several weeks or even months (see Figs. 6–8) (18). Similarly to what was discussed for the brain tissue slices, it is possible to characterize the network discharge patterns, as well as the activity-dependent plasticities of the synaptic coupling between cells, exploiting, in particular, the lack of network topology and taking advantage of the enhanced spatial resolution offered by the MEAs.

Although such *in vitro* networks should be considered only as an artificial nervous system, deprived of any

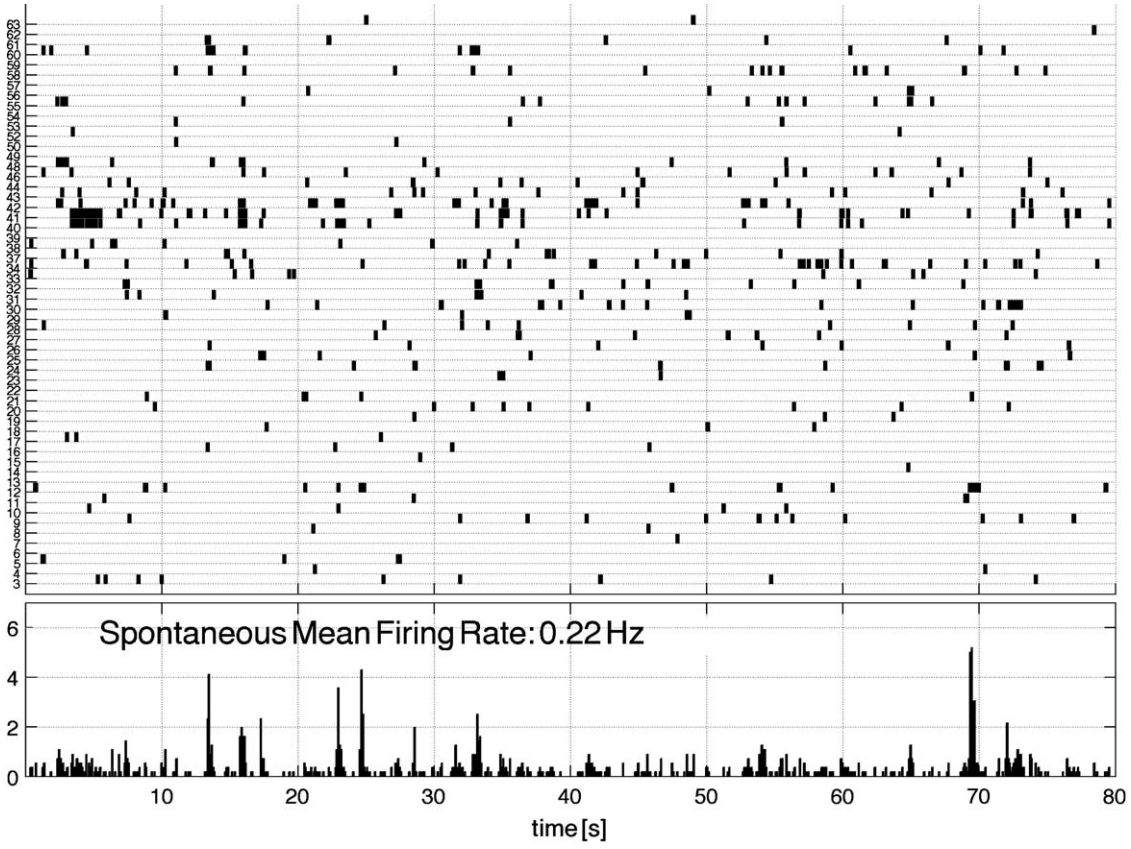


Figure 5. Simultaneous recording of the spiking activity from up to 60 microelectrodes in an acute slice of rat brain somatosensory cortex (see Fig. 1, right). The top panel indicates, for each recording channel, the times of occurrence of individual action potentials detected by the microelectrodes of the array as small vertical lines. Such an activity develops spontaneously under artificial cerebro-spinal fluid extracellular solution (*ACSF*). The population mean firing rate (bottom panel) was estimated by counting all the events, detected within a 10 *ms* sliding time window. The network is characterized by a very low spontaneous firing rate with rare bursts of activity, as opposed to the 5 – 10 *Hz* asynchronous activity observed in the intact cortex of behaving animals. Such a difference is mainly related to the network deafferentation and the lack of a substantial fraction of cortico-cortical afferents, a consequence of the brain slicing procedure.

sensory-related input and motor-related output, they represent a reduced ideal framework to explore and test hypotheses and mathematical models of the emergent activity patterns, expected to emerge in biological neuronal networks. Moreover, because any progress in the quantitative analysis of such phenomena is relevant for the understanding of the strategies employed by an *in vivo* nervous system to represent and process information (19,20), the use of MEAs represents an unparalleled research tool.

In particular, the *in vitro* generation of rhythmic electrical activity, spontaneously developing in cultured neuronal populations, has been extensively approached by several authors, taking advantage of MEAs (Fig. 6) (17,21,22). Such coordinated patterns, which can be, to some extent, modified by means of pharmacological and electrical stimulation (22), can be monitored with respect to the spatial and temporal features, with the aim of dissecting the cellular mechanisms that underlie the similar collective network activities *in vivo*.

In addition, such networks represent a novel kind of biosensor, whose applications range from biochemistry to whole-animal experiments, providing quantitative information on the neurophysiological responses to chemicals, drugs, and toxins (23–25) or to localized electrical stimulating waveforms (Figs. 7 and 8) (26,27).

6. MEAs AND NETWORK-LEVEL *IN VITRO* PHARMACOLOGY

The possibility of long-term monitoring and characterizing the spatial features of the electrical activity of an *in vitro* neuronal network proposes the use of the MEAs as a novel tool for studying the acute and chronic consequences of the delivery of neuroactive substances. In the simplest experiment, a substance can be bath-applied and the spontaneous and evoked activity of the network compared with control conditions and monitored over time. Although, in this case, substances are not delivered in a

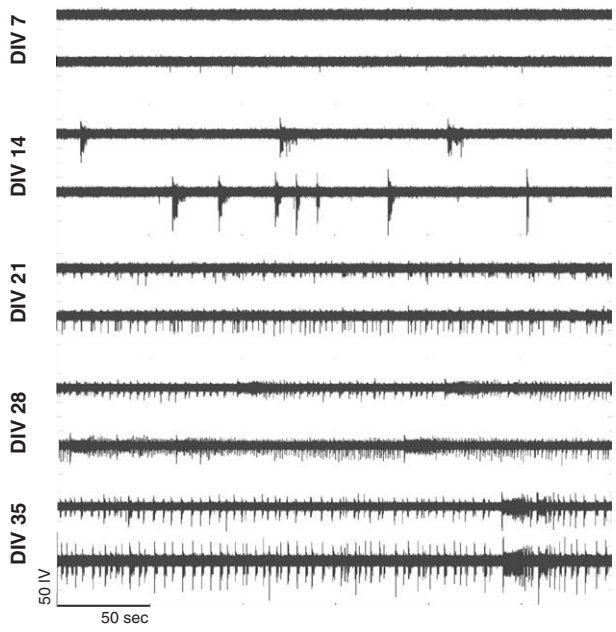


Figure 6. Cultures of dissociated cortical neurons, at different stages of development (i.e., *DIV*, days *in vitro*), show distinct patterns of activity. The raw extracellular voltage traces, simultaneously detected by two selected MEA microelectrodes, are reported. Spontaneous low-rate spiking activity start around the end of the first week in culture and increases as the synaptogenesis progresses *in vitro*. The random spiking (*DIV* 7) turns into a sporadic network *bursting* around *DIV* 14, and later, after the third week in culture, it evolves into a more synchronized activity pattern across the network.

spatially localized way, the spatial features of the ongoing network electrical activity, resulting from the neuron-to-neuron synaptic interactions, can be characterized and quantified. The biophysical effect of a particular substance on individual receptors and subcellular pathways, known from previous biochemical and molecular biology studies, for instance, can be related to its network-level influence, similarly to what is performed on living animals but with an unparalleled resolution.

For instance, it is possible to relate the physiological and pathological properties of glutamatergic excitatory synaptic transmission to the spontaneous collective activity that emerges in cultured networks of cortical and spinal cord neurons *in vitro*, which is possible by bath-applying *APV* (*D-2-amino-5-phosphonovalerate*), a competitive antagonist of the *NMDA* (*N-methyl-D-aspartate*) receptors, and *CNQX* (*6-cyano-7-nitroquinoxaline-2,3-dione*), an antagonist of nonNMDA receptors, specific blockers of the postsynaptic receptors. Such a pharmacological modulation is known to result in a strong change of the network recurrent activity and excitability, which can be quantified by the MEA recordings in terms of the lack of an organized collective spontaneous network bursting and of a different sensitivity to additional (e.g., electrical) stimuli, which, under control conditions, require the active recruitment of recurrent excitatory synaptic interactions (22).

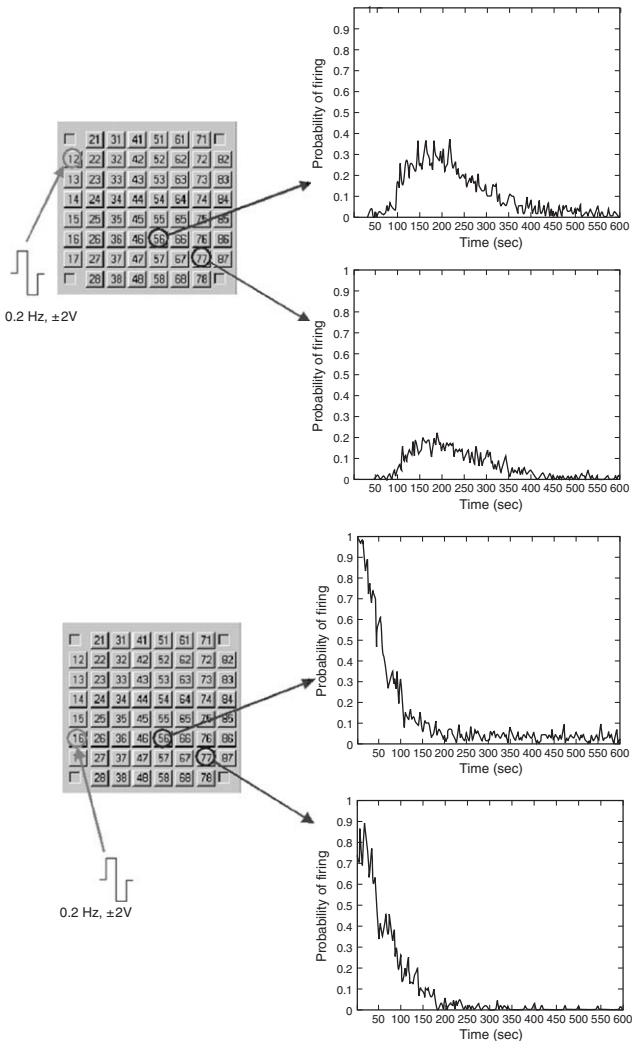


Figure 7. The peri-stimulus time histograms of the network spiking responses (right traces), evoked by electrical stimulation and detected at two recording sites, has been chosen as observable to examine the synaptic pathways in a cultured network. A *delayed* evoked response is apparent (upper panels), when the stimulus is delivered at the location indicated as "12. " A change in the location of stimulus delivery considerably modifies the evoked activity pattern, and only an *early* response is obtained (lower panels).

Finally, more sophisticated micromachined MEAs, which include microfluidics and nanoactuators, are expected to provide even more insights, as they can restrict the delivery of the same drugs at a specific spatial target (e.g., an identified subpopulation of neurons), with a precise temporal concentration profile, mimicking physiological and pathological conditions (e.g., the localized release of neuromodulator peptides).

7. MEAs AND THE ELECTRICAL STIMULATION OF THE NERVOUS SYSTEM

It is well known that neuronal networks activity can be modulated by electrical stimulation (19,27), as sometimes

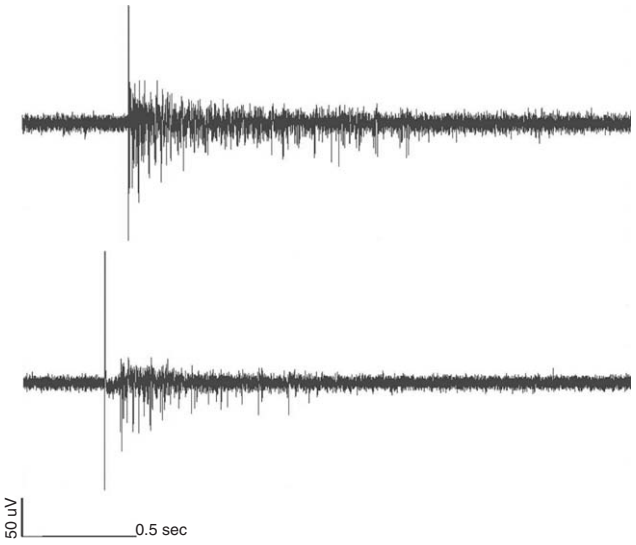


Figure 8. Extracellularly detected spiking activity detected by the same microelectrode during electrical stimulation from two alternative stimulating sites: The evoked burst of activity, which follows the stimulation artifact (i.e., the overshooting biphasic line), differs in terms of the response onset and duration.

employed in clinics for the treatment of epilepsies, motor impairments, as well as pathological motor tremors (28). Several *in vitro* experiments have shown that stimulation-induced persistent modifications in the network activity reflect changes in the synaptic efficacy (19,27), which is largely considered as a cellular correlate of learning, memory, and developmental plasticity (16,26,29). By delivering electrical stimuli through MEA microelectrodes, it is possible to characterize the activity evoked by brief stimuli on developing cultured neuronal networks. For instance, stimuli consisting of trains of monopolar biphasic pulses at low frequency (e.g., 0.2 Hz), delivered through several independent spatial locations, reliably modify the network spiking activity, which can be quantified by a Peri-stimulus time histogram (PSTH) (30), that is usually composed by an early or by a late discharge response component (see Figs. 7 and 8).

Recent results show remarkable differences in the electrophysiological activity of the network, under different stimulation paradigms, so that it is possible to induce plastic changes in the network and to *force* the network to shift toward desired collective dynamical states (29), which is an important experimental evidence attesting to the existence of an *adaptive learning*, retained even in *in vitro* neurobiological systems.

Additionally, when the distribution of the time intervals between successive spontaneous collective bursts of activity (i.e., the Inter-Burst-Intervals) is considered, the repetitive electrical stimulation can, in some cases, entrain the spontaneous rhythm, locking it around the stimulus frequency.

8. CONCLUSIONS

As introduced and exemplified in the previous sections, substantial contributions to the investigations of the auto-organization properties of the nervous system and the cellular basis of the elementary form of behavior (i.e., motor/rhythmic patterns generation and basic mechanisms of plasticity and learning) are coming from the novel experimental approach employing substrate arrays of microelectrodes.

In particular, its long-term, noninvasive, and distributed character is of paramount importance for the network-level neurosciences and neuroengineering, which suggests future development directions and trends, in the context of basic and applied research, under the general perspectives of a deeper understanding of the way the central nervous system represents, processes, and stores information. As far as the *information technology* field is concerned, this will undoubtedly lead to substantial sources of inspiration for the design of artificial neuromorphic systems (31,32). However, such achievements will be particularly crucial for the design and development of new brain-machine interfaces and neuroprostheses, such as the recent implantable artificial retina on-a-chip (33). Such devices necessarily include a bi-directional physical interface, usually interacting with the neuronal tissue by means of electrical signals, according to a multisite strategy, similarly to the MEAs. Therefore, the development of new signal-processing techniques, the interpretation of multichannel extracellular recordings, and the definition of appropriate strategies and microtechnologies for the electrochemical stimulation of *in vitro* reduced preparations represents the most important contribution of the MEA research for the future neuroprosthetic clinical applications.

Acknowledgments

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