

Chapter 2

Dissociated Neuronal Networks Coupled to Micro-Electrode Arrays Devices

In this chapter, I will outline some basics about neurons and how they can communicate with each other (see Sect. 2.1). In the following section, I will report some brief notes about different kinds of in vitro neuronal preparations (see Sect. 2.2). Then I will present an overview about the state-of-the-art of electrophysiological techniques focusing on in vitro applications (see Sect. 2.3). Then I will dedicate three paragraphs to the description of Micro-Electrode Arrays (MEA) technique (see Sects. 2.4–2.6), which is the main tool exploited in the context of this thesis. Finally I will use an entire section for an overview of MEA applications (see Sect. 2.7).

2.1 Neurons and Synapses

Although the human brain contains a thousand different types of neurons, they all share the same basic architecture (Fig. 2.1). 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 define 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 et al. 2000).

A neuron has four morphologically defined regions: soma, dendrites, axon and presynaptic terminals. The soma is the metabolic centre of the cell, it contains the nucleus and the endoplasmic reticulum. The dendrites branch out from the soma and are the main apparatus for receiving incoming signals from other nerve cells. The axon extends away from the cell body and it is the main conducting unit for carrying signals to other neurons. A single neuron can communicate with another one thanks to synapses. The synapse is the point at which two neurons communicate and involves a presynaptic cell, which transmits a signal (i.e. axon), and a postsynaptic cell, receiving the signal (i.e. dendrites). The presynaptic terminal is a specialized

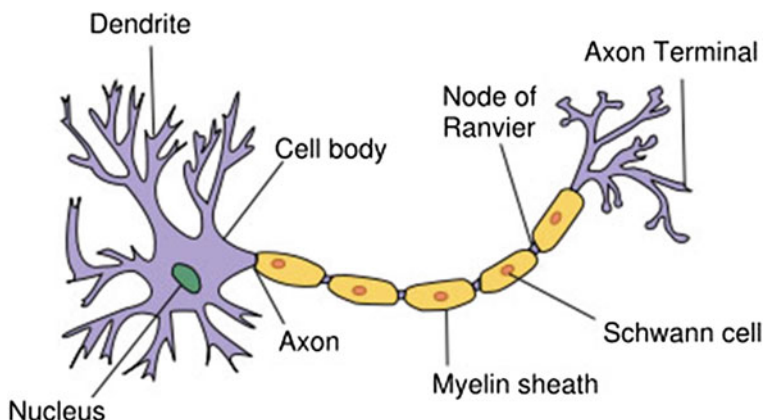


Fig. 2.1 Simplified sketch of a neuron

cell compartment in which the electrical signals (i.e. action potentials) traveling through the axon are transduced in a chemical signal (i.e. neuro-transmitter release) captured by the postsynaptic receptors present in the dendrites. This chemical signal is again translated in an electrical one, affecting the postsynaptic neuron's membrane potential, and thus accomplishing the synaptic transmission.

At rest, all nerve cells maintain a difference in the electrical potential (V) on either side of the plasma membrane: this is called the resting membrane potential (Fig. 2.2) and in a typical neuron it is about -65 mV ($V = V_{\text{intracellular}} - V_{\text{extracellular}}$). The difference in electrical potential when the cell is at rest results from two factors: the unequal distribution of electrically charged ions, in particular the positively charged Na^+ and K^+ ions and the negatively charged aminoacids and proteins on

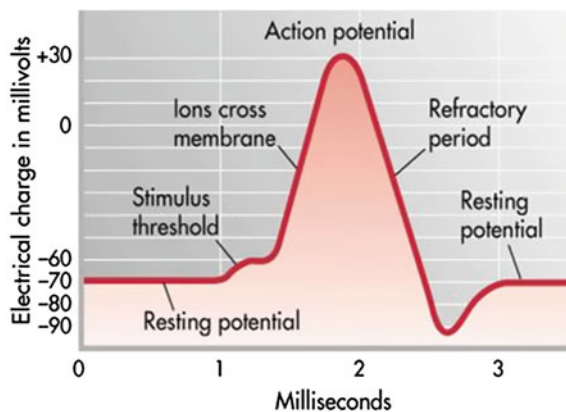


Fig. 2.2 Scheme of action potential generation

either side of the cell membrane, and the selective permeability of the membrane to just one of these ions, K^+ .

In order to send a signal, in the presynaptic cell an action potential has to be generated. The action potential is composed of a rising phase (depolarization), followed by a falling phase bringing the membrane potential V down to a hyperpolarization phase. This shape is due to the activity of voltage-controlled channels of the membrane that modify the Na^+ and K^+ ion permeability as a function of the membrane potential. The phases of the action potential are reported in Fig. 2.2.

Briefly, when the presynaptic neuron receives an input (synaptic or sensory, mediated by receptors) an influx of Na^+ or Ca^{2+} ions depolarizes the membrane: when the depolarization exceeds a critical threshold, the voltage-gated Na^+ —permeable ion channels are open and V rapidly increases towards positive values. When V reaches its peak, the voltage-gated Na^+ channels close and, at the same time, the voltage-gated K^+ channels open, leading to a strong outflux of K^+ ions that re-establishes a negative membrane potential. The action potential is an all-or-none

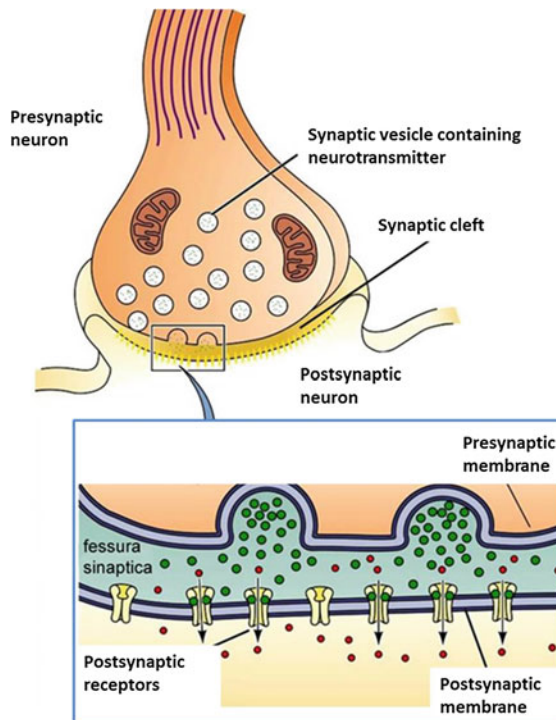


Fig. 2.3 Model of a synapse. Released of the neurotransmitters from the vesicles in the presynaptic neuron; neurotransmitters diffusion across the tight space that separates the presynaptic and postsynaptic terminals (i.e. synaptic cleft); neurotransmitters binding with the corresponding receptors in the membrane of the postsynaptic neuron

signal: this means that while stimuli below the threshold will not produce a signal, all stimuli above the threshold produce the same signal.

When an action potential reaches a pre-synaptic neuron's terminal, it stimulates the release of a chemical transmitter from the cell, as the neuron's output signal. The transmitters are held in vesicles, as it is shown in the sketch of Fig. 2.3. In the Central Nervous System (CNS) the major excitatory transmitter is L-glutamate, while the major inhibitory transmitter is γ -aminobutyric acid (GABA) (Kandel et al. 2000). After the transmitter is released from the presynaptic neuron, it diffuses across the tight space that separates the presynaptic and postsynaptic terminals (i.e. synaptic cleft) and it binds the corresponding receptors in the membrane of the postsynaptic neuron (Fig. 2.3). The binding of transmitter to receptors causes the postsynaptic cell to generate a synaptic potential. Whether the synaptic potential has an excitatory (i.e. depolarizing) or inhibitory (i.e. hyperpolarizing) effect will depend on the type of receptors in the postsynaptic cell, not on the particular neurotransmitter (Kandel et al. 2000).

2.2 In Vitro Preparations

In the early 1900s, the first studies in neurobiology employing tissue cultures were performed by Harrison (1907, 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 and Goslin 1998). These studies paved the way to the use of tissue cultures' techniques to address biological problems (Carrell and Burroughs 1910). In the following decades, in vitro culturing began to gain a more prominent and important position in neurobiology. Today, tissue culture is an integral part of modern neurobiology.

Two main methodologies of in vitro preparations are usually used: slice cultures and dissociated neuronal networks. Even if dissociated neuronal networks are the preparation used in all the experiments presented in this work, however, a brief description of the slice cultures preparation will be provided.

2.2.1 *Slice Preparations*

Brain slices are thin hundreds micrometers slices (200–500 μm) of brain regions containing about 10^3 neurons.

Brain slices are freshly cut from in vivo developed animal brains. These kinds of cultures are called acute slices. Organotypic cultures are explanted from relatively young animals (e.g. rats, 1–3 weeks old) and allowed to attach to an appropriate substrate and cultured in a rich medium. These cells continue to differentiate and

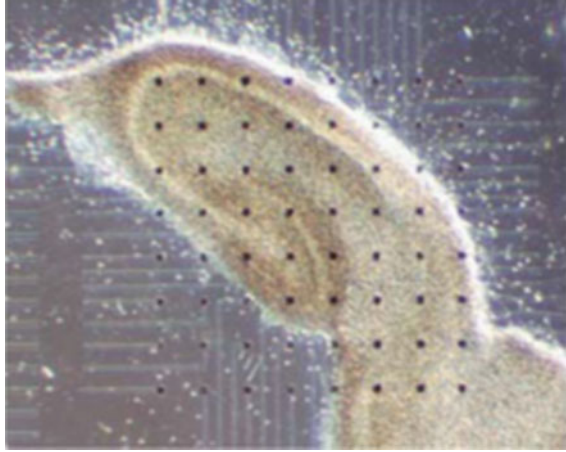


Fig. 2.4 Hippocampal slice from an 11-day-old rat, cultured and maintained on MED64 probes (Alpha Med Scientific Inc., Japan)

develop in culture, extending processes and forming new synapses and they can be maintained for months, in appropriate controlled conditions.

The main peculiarity and advantage of using this preparation consists in the partial preservation of the morphological and functional original structure of the intact brain. Brain slices allow recording from semi-intact neural circuits, with the advantages of mechanical stability and control over the extracellular environment. These preparations are used for a wide variety of studies, like synaptic plasticity and development, network oscillations, intrinsic and synaptic properties of defined neuronal populations (Kettenmann and Grantyn 1992). Many neuroscientists use brain slices coupled to Micro-Electrode Arrays devices (MEAs) instead of dissociated cultured neurons (Eytan and Marom 2006; Chialvo 2007) since they maintain the topology of the original tissue. An example of a brain slice coupled to MEA devices is reported in Fig. 2.4.

2.2.2 *Cultured Neuronal Networks*

At the beginning of the 20th century, the demonstration that the tissues could survive and grow outside the body and that complex aspects of neuronal maturation, such as synaptogenesis and myelination, could occur not only *in vivo* but also *in vitro* (Peterson and Murray 1955; Murray et al. 1977) opened new possibilities to study the nervous system creating a widespread interest from the scientific community and capturing the attention of many neuroscientists.

Since cultured neurons remain healthy for long periods (months), they represent an ideal substrate for investigating brain properties at the network and circuit, i.e.

parts of network, level. Thanks to the continuous technological improvements and with increasing experience, it has become possible preparing dissociated cell cultures from virtually every region of the nervous system and from many different animal species.

Generally, neurons in dissociated cultures appear to retain their individual identities and their morphological and physiological properties correspond closely to the characteristics of the cell populations in the original tissue (Kriegstein and Dichter 1983). Although synaptic interconnections grow randomly in culturing conditions and the morphology of the neuronal networks is not maintained with respect to *in vivo* condition, dissociated cultures represent a reasonable, simplified model of the brain. In fact, cultured neurons replicate *in vivo* synaptic events, at different scale levels, ranging from single synapses to complex circuitry. At the level of single synapse, both excitatory and inhibitory transmissions were observed and the kinetics of receptors, ion channels and neurotransmitters release were similar to the ones measured in *in vivo* conditions. At single-cell scale level, these analogies include action potentials and different types of modulatory currents. Finally, complex mechanisms, like long-term potentiation (LTP) which is interesting because it is considered the synaptic analogy of learning, have been observed and characterized in neuronal micro-cultures.

2.2.2.1 Preparation and Growth

In this thesis I made use of primary cultures of dissociated neurons (hippocampal and cortical) from rat embryos. In what follows, I briefly describe the procedure used to obtain and maintain the cultures.

Hippocampal and cortical neurons were dissociated from E18 Sprague Dawley rats (Charles River Laboratories, Milano). The procedure was approved by the European Animal Care Legislation and by the guidelines of the University of Genova. The day before the plating, MEAs (Multi Channel System MCS GmbH) are sterilized at 120 °C in the oven. The sterilized material is therefore exposed to the coating treatment with adhesion protein Laminin (L-2020 Sigma) and Poli-Lysin (P-6407 Sigma) at 0.05 µg/ml overnight in incubator at 37 °C. The adhesion factors are removed and the treated glass surfaces are washed with sterile water. MEAs are left to dry inside the laminar hood.

From each embryo, hippocampi and cortices were removed and placed into ice cold Hank's balanced salt solution. The tissue was then dissociated in 0.125 % of Trypsin/Hank's solution containing 0.05 % of DNase (D-5025 Sigma-Aldrich) for 15–18 min at 37 °C. The supernatant solution was removed and the enzymatic digestion was stopped by adding 10 % fetal bovine serum (FBS) in Neurobasal medium for 5 min. Medium with FBS was removed and replaced with Neurobasal medium supplemented with B27, 1 % Glutamax, gentamicin 10 µg/ml (Gibco Invitrogen). Cells were then plated directly onto the area of MEA electrodes at the final concentration of about 1200 cells/µl. The obtained 2D cultures were maintained in a humidified CO₂ atmosphere at 37 °C for 3–4 weeks. Half of the media was replaced

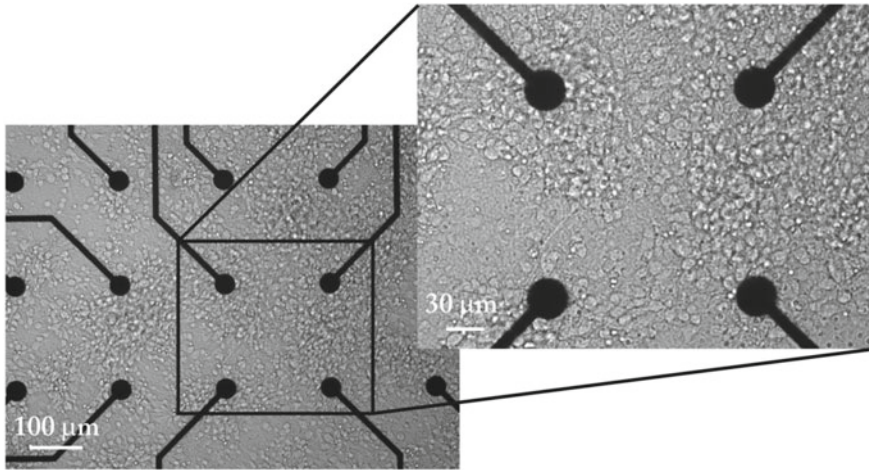


Fig. 2.5 Image of a neuronal network cultured over an MEA for 24 days in vitro at two magnifications (10× and 20×, calibration bars: 100 and 30 µm)

once a week. No antimitotic drug was added to prevent glia proliferation, since glial cells are essential elements for a healthy development of neuronal networks. 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 spike. In Fig. 2.5 an image of a dissociated neuronal network coupled to MEA is shown.

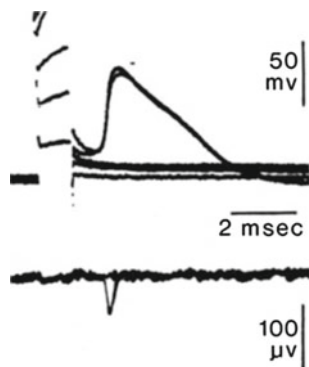
2.3 Electrophysiology Techniques

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.

The intracellular technique allows the direct measurement of the potential difference across the membrane. The action potential has been described considering the ionic flows across the cellular membrane. This kind of electrophysiological measurement needs two measuring points, one in the cell and the second outside and requires the breaking of the membrane.

Alternatively, utilizing an extracellular technique, 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. 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

Fig. 2.6 Intracellular oscilloscope traces are at the top. Action potential signals are seen for the largest two. Below are extracellular recordings made simultaneously with the intracellular ones. The two below baseline are from the action potentials, and are too similar to resolve. From Pine (1980)



results from a resistive drop in the medium between the reference electrode and the recording electrode.

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 (Fig. 2.6).

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 pulse to the extracellular electrode. 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 et al. 2006).

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 1 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.

Conventional methods (i.e. patch clamp) allow to record neuronal signals from only few cells at time in an invasive way. MEAs, on the contrary, allow recording extracellular neurons signals simultaneously from hundred electrodes at the time in a non-invasive way.

2.4 MEA for Network Electrophysiology

Thomas et al. described the first Micro-Electrode Arrays in 1972. It consisted of platinized gold microelectrodes (two rows of 15 electrodes each, spaced 100 μm apart) embedded onto a glass substrate and passivated by photoresist. This device allowed to record field potentials from spontaneous contracting sheets of cultured chick cardiomyocytes, but it was not able to record activity from each single cell.

Five years later, Guenter Gross and his collaborators proposed the idea of a MEA, without knowledge of the previous work (Gross et al. 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 a MEA were reported by Pine in 1980 (Fig. 2.6): 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 μm square and 250 μm apart), platinized and insulated with silicon dioxide (Pine 1980). 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 1980s, many studies employing MEAs for different purposes followed. Some exploited these new tools to investigate either the network activity of cultures of dissociated neurons (Gross et al. 1982) or of hippocampal slice preparations (Wheeler and Novak 1986). Soon, it was clear that large invertebrate neurons were the most suitable to be plated on top of MEAs (Regehr et al. 1989). They can be easily identified thanks to their size and location in the ganglia, can be dissected out, and can be used with other identified neurons to form simple networks in culture that replicate some 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 et 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 et al. 1994).

At the beginning of the 1990s, the combination of a MEA (for stimulation) and voltage sensitive dyes (for recording) was exploited to allow the detection and measurement of subthreshold synaptic potentials, otherwise impossible by recording extracellular electrical signals (Chien and Pine 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 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 et al. 1972), network development and plasticity are the most interesting questions that can be addressed by using MEAs.

Nowadays the MEA technique offers a useful experimental approach for in vitro electrophysiological investigations. MEA technology and culture methods, in parallel, have continuously improved during these years. To date, MEAs find several applications in many research fields, such as neuroscience, pharmacology, physiology, biophysics and cardiac electrophysiology.

2.5 MEA Technology

The current technology typically provides MEAs formed by 60–256 electrodes, 10–30 μm in diameter spaced at 100–500 μm . MEAs consist of microfabricated electrodes embedded in a biocompatible insulation substrate (e.g., polyamide or silicon nitride/oxide) which prevents short circuits with the electrolyte bath, forming a sort of wired Petri dish. The electrodes, typically made of Au, Indium-Tin Oxide (ITO), Titanium Nitride (TiN), or black platinum, must be biocompatible, long-term lasting, and preferably should have low impedance (less than 500 K Ω at 1 kHz) for low thermal noise. Cells adhesion on the MEA is promoted by covering them with

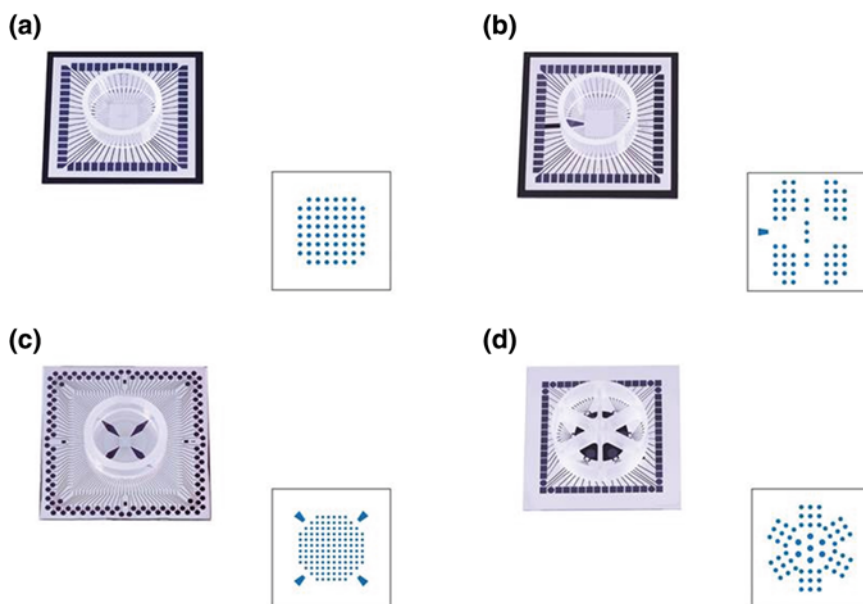


Fig. 2.7 MEA devices and electrodes layouts. **a** Standard 60MEA: 60 electrodes, electrode grid 8×8 , electrode spacing 100 μm and electrode diameter 10 μm . **b** 60-4QMEA: 60 electrodes, electrode grid $4 \times (1 \times 4 + 1 \times 5 + 1 \times 4) + \text{center line } 1 \times 7$, electrode spacing 200 μm inside the quadrants, 1000 μm between the quadrants, electrode diameter 30 μm . **c** 120MEA: 124 electrodes, electrode grid 12×12 , electrode spacing 200 μm , electrode diameter 30 μm . **d** 60-6WellsMEA: 54 electrodes, electrode grid 3×3 in each area, electrode spacing 200 μm , electrode diameter 30 μm

laminin and poly-L-lysine or collagen (Robinson et al. 1993; Jimbo et al. 1999). In Fig. 2.7 four different types of MEA devices are presented.

Since neurons are directly cultured on the MEA, the distance between the cells (adhering to a surface containing the electrode) and the electrode typically ranges from 10 to 100 nm (www.multichannelsystems.com, Reutlingen, Germany). The electrode size is a compromise regarding both biological and electrical considerations. Indeed, the electrode should be as small and close as possible to the cells in order to obtain information from localized point. At the same time, the electrode should have a sufficient surface to detect electrical signals with an acceptable signal to noise ratio.

The fabrication of MEAs is based on the thin-film technology (Elshabini-Riad and Barlow 1998) and is realized in a clean-room. Photolithography, i.e., the process of transferring geometric shapes from a mask to the surface of a silicon wafer, is used to make MEAs.

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 three complete acquisition systems based on MEAs: the MED System developed originally by Panasonic and now developed and manufactured by Alpha MED scientific (www.med64.com, Osaka, Japan), the MEA System by Multi Channel Systems (www.multichannelsystems.com, Reutlingen, Germany) and the 3-Brain system (www.3brain.com).

Other companies, such as Ayanda-Biosystems (www.ayanda-biosys.com, Lausanne, Switzerland), have only developed the microelectrode devices, for several different applications (cultures, slices, cardiomyocytes, retinal cells, pharmacological screening, etc.). Finally, Plexon (www.plexoninc.com, Dallas, USA) developed hardware and software tools to be used in conjunction with third parties micro-devices.

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

2.5.1 *Standard MEA*

The experimental set-up used to perform the experiments analyzed in this thesis, was based on the MEA System (Multi Channel Systems, MCS, Reutlingen, Germany). They offer, together with the MEAs, complete workstations for electrophysiological investigation based on micro-electrode arrays.

The device utilized in this thesis consists of 60 round planar micro-electrodes made of TiN/SiN (see Fig. 2.8a), with a diameter of 30 μm and spaced at 200 μm . The electrodes are arranged in a 8×8 matrix configuration with the four electrodes at the corners disconnected (Fig. 2.8c). In order to record the extracellular signals,

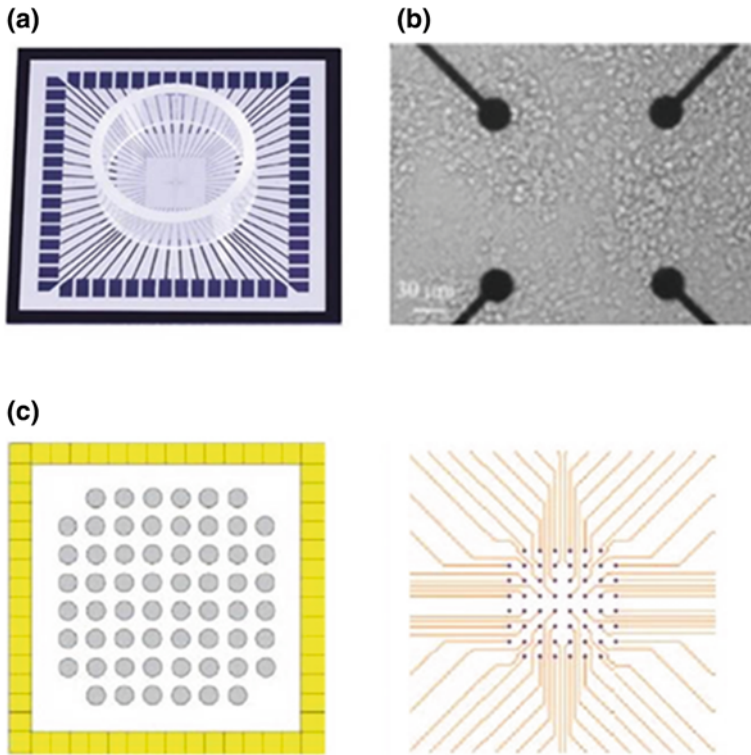


Fig. 2.8 Standard 60MEA. **a** Image of a typical MCS MEA. **b** Optical images of a neuronal network over an MEA at 14 days in vitro. **c** 8×8 layout of an MCS MEA (electrodes size of $30 \mu\text{m}$ and inter-electrode spacing of $200 \mu\text{m}$)

MEAs need a reference electrode respect to which evaluating the voltage potential. Some arrays, presenting an internal reference electrode, do not need to hole the led protecting the culture (differently from what happens by using the external reference) which exposes neurons more easily to infections. For this reason, internal references are usually used during experiments on developmental studies in order to maintain the condition of the culture as much healthy as possible during the whole developmental stage.

A glass/plastic ring is placed at the center of the array, and it allows to contain the culture medium. In this way, when placed in an incubator, the culture can survive for several weeks (Fig. 2.8b).

2.5.2 Experimental Set-up

The Multi Channel Systems set-up is made up of the following components contained inside a Faraday cage in order to reduce electromagnetic interferences. During this thesis I utilized two different amplifiers. All the components are presented in Fig. 2.9.

- **MEA 2100 Amplifier**

Extending the MultiChannel Systems MEA-product family, the MEA 2100-System follows the tradition of high-quality, low-noise amplifiers. It is the complete setup for extracellular recordings from MEAs. It includes data acquisition computer with software, interface board, MEA-headstage with integrated stimulation, MEAs. It is connected via only one MCS High Speed cable to the interface board, which offers various digital and analog in-/outputs for synchronization with other instruments. The main advantage of MEA-2100-System is the flexibility. Multichannel systems offers various contact units for the MEA headstage. It is possible to decide whether to work with one or two 32-electrodes MEA, one or two 60-electrodes MEA or one 120-electrodes MEA. The flexibility of the MEA-2100-System is also reflected in the possibility to connect two MEA-headstages to one interface board. This way, you can record from up to 240 channels. The MEA 2100 Amplifier is reported in Fig. 2.9a.

- **MEA 1060 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 ($165 \times 165 \times 19$ 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 ($\times 1200$, 12-bit resolution, 10 Hz to 3 kHz) of $\pm 3 \mu\text{V}$, which is well within the ± 5 to $10 \mu\text{V}$ 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. The MEA 60 Amplifier is reported in Fig. 2.9b.

- **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.

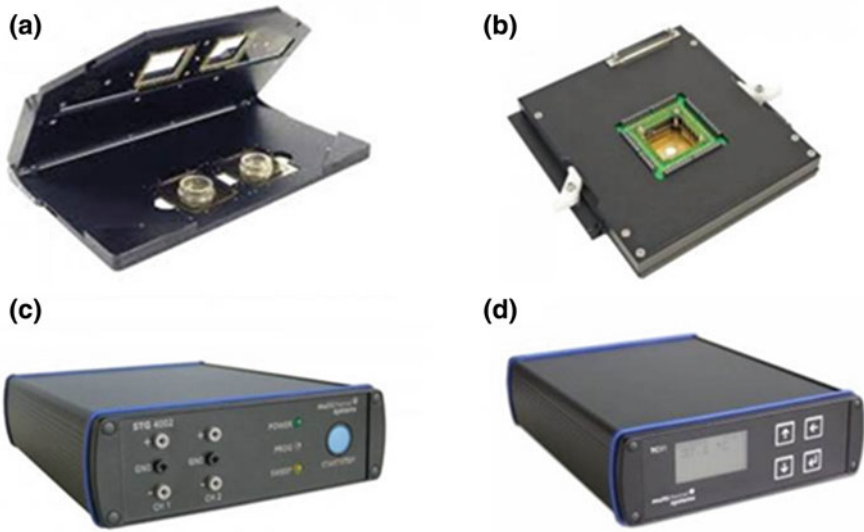


Fig. 2.9 Setup components. **a** MEA 2100 amplifier. **b** MEA 1060 amplifier. **c** Stimulus generator. **d** Temperature controller

- **MCS Stimulus Generator (STG1080)**

The MCS stimulus generator is a general-purpose stimulator which generates pulses to be delivered to stimulating electrodes (2 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 PCI-based acquisition board 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. The STG stimulus generator is reported in Fig. 2.9c.

- **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.

- **Temperature controller**

The MCS Temperature Controller 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 min, depending on the recording system configuration. The Temperature controller is reported in Fig. 2.9d.

2.6 Increasing Spatial Resolution with MEAs

Current MEAs provide typically 60–256 electrodes with 100–500 μm inter-electrode spacing. As the recorded signals originate from the cells in close proximity to the electrodes, and as the distribution of the cellular networks is random, the number of recording sites, i.e. the spatial resolution, is therefore limited. As an example, a culture of 50,000 neurons coupled to 50 electrodes presents an undersampling of the network activity by a factor of 10^3 .

Although a typical spatial resolution (inter-electrode spacing) of 100 μm is adequate for studying the overall network activity, it is not enough to exploit the potential capability of MEAs: in particular, it does not provide electrophysiological data at a multi-level spatial resolution for correlating network, cellular and sub-cellular activities. It is necessary to plate cells at a relatively high density (about 2000 cells/ mm^2) 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 (Imfeld et al. 2008). The availability of this feature is particularly suited for unraveling the fundamental properties of brain tissue, whose activity arises from signal integrations and propagations at synaptic, cellular and population levels.

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.

To achieve a high spatial resolution, Berdondini et al. (2009b) developed an high-density 60 microelectrode MEA (HD-MEA). Four different MEA layouts, (22 and 30 μm electrode diameters, 20 and 10 μm spaced) were designed. The layout of the array is divided into 4 clusters of 15 high-density microelectrodes each. The advantage of this configuration relies on the possibility of investigating interconnected neuron sub-population, both on a local network basis (i.e., considering the high-density clusters) and on a whole network basis (considering the four separated high-density clusters).

The previously described HD-MEAs offer the advantage to increase the spatial resolution with respect to the standard and commercial devices, but they are limited in terms of amount of electrodes. For studying signal propagation along neuronal networks it is essential to increase the recording sites of some order of magnitude. To achieve a high spatial resolution a CMOS technology-based solid state Active Pixel Sensor (APS) array, featuring 4096 pixels, has been developed (Fig. 2.10a) (Berdondini et al. 2005, 2009a; Imfeld et al. 2008). The APS-MEA core is a specific integrated circuit, which implements an array of 4096 electrodes (organized in a 64×64 grid) with corresponding amplification, addressing and multiplexing functions (Imfeld et al. 2008; Berdondini et al. 2009a).

The use of CMOS-based devices can overcome some limitations of passive MEAs, in particular for performing measurements at a high spatial and temporal resolution. The simultaneous recording from all electrodes requires the front-end

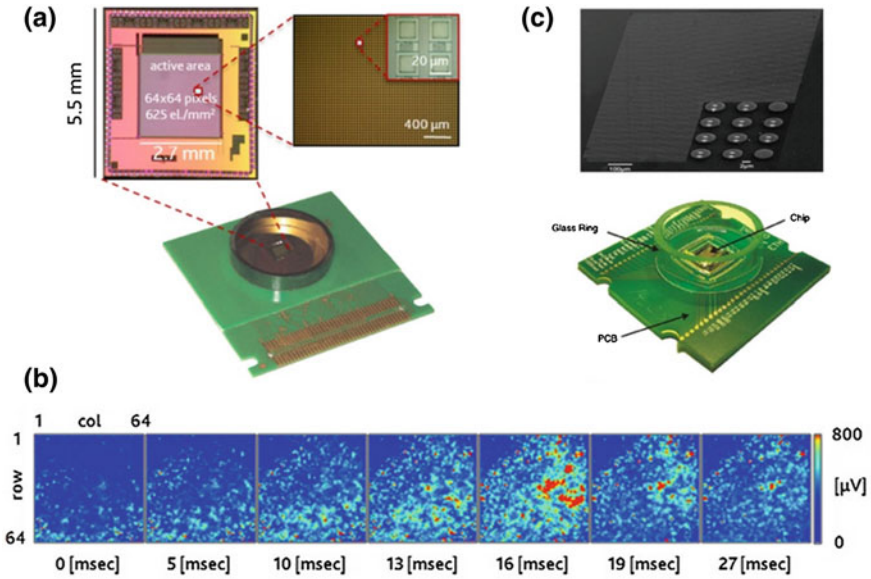


Fig. 2.10 High density devices. **a** CMOS technology-based solid state Active Pixel Sensor (APS) array (Berdondini et al. 2009a). **b** Activity recorded by 4096 electrodes of the APS device. **c** CMOS based device composed by 11,011 metal electrodes and 126 channels (Imfeld et al. 2008)

amplifiers being placed in each pixel (recording site), which, due to area constraints, entails rather high noise levels. Instead of scanning the entire electrode array, the approach presented by Frey and coworkers (Frey et al. 2007, 2009) provides a reconfigurable electrode/readout-channel routing to select an arbitrary subset of electrodes for recording and stimulation (Fig. 2.10b). The system is composed by 11,011 metal electrodes and 126 channels, each of which comprises recording and stimulation electronics. In another approach (Imfeld et al. 2008), an active MEA chip was described with a pitch of 42 μm of 4096 metal electrodes (subset of 126 channels for recording) on a CMOS chip with local filtering and amplification. In another work (Lambacher et al. 2010), Lambacher and co-workers reported a significant progress of neuronal recording by multi-transistor array (128×128 sensors) chips with EOMOS transistors.

2.7 MEA Applications

During these years I was also involved in neuro-pharmacological and synaptic plasticity studies. For this reason an overview of this type of MEA application are reported in the following paragraphs.

2.7.1 *Neuro-Pharmacological Applications*

In recent years the need of efficient neuropharmacological and neurotoxicological testing *in vitro* is increasing, as there are new directives to restrict animal use for laboratory tests (Johnstone et al. 2010). New experimental strategies based on alternative methods, in which the use of time, materials, and animals is reduced and refined or animal use is completely replaced, are required.

Thus, *in vitro* assessment of neurophysiological function could be used to screen chemicals for potential neuroactive or neurotoxic effects (Defranchi et al. 2011). To date, one of the most promising tools for neuropharmacological tests is the Micro-Electrode Array (MEA). MEA technology has been recognized as a standard experimental approach for *in vitro* long-term electrophysiological and neuropharmacological investigations (Gross et al. 1977; Gramowski et al. 2004). The pioneering works by Gross et al. (1977, 1992) demonstrated the possibility to use dissociated neuronal networks coupled to MEAs as a first prototype of cell-based biosensor. This system showed both high sensitivity to neuroactive and neurotoxic compounds and reproducible results (Gramowski et al. 2000). The biocompatibility of the used materials (i.e. titanium nitride for the electrodes and glass for the substrate) and the not invasive nature of the extracellular measurement, make this system a perfect candidate to routinely record and evaluate the dynamics of the network behavior, both in spontaneous condition and under chemical manipulation (Martinoia et al. 2005; Chiappalone et al. 2006), either on short or long time-scales. Cultured neuronal networks respond to neurotransmitters and their blockers in a similar way as the *in vivo* situation (Streit 1993; Gramowski et al. 2000; Martinoia et al. 2005), providing an excellent tool to study how pharmacological compounds can influence the electrophysiological behavior (Gross et al. 1977; Morefield et al. 2000; Keefer et al. 2001; Xia and Gross 2003; Parviz and Gross 2007). Moreover, by using MEA systems (i.e. MEA 2100 amplifier) and devices that allow activity

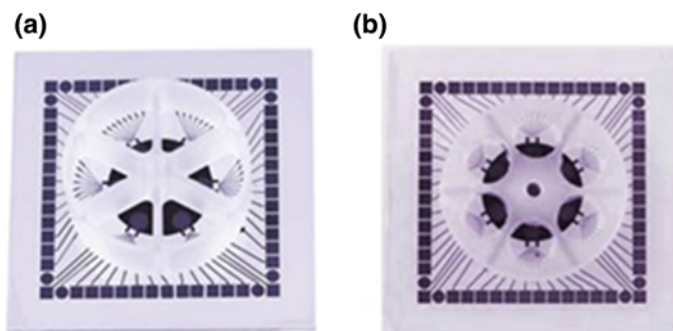


Fig. 2.11 MEA devices used for neuro-pharmacological investigations. 60-6Well MEA. MEA devices with 6 separated wells (9 recording channels plus 1 internal reference electrode for each compartment). **a** Six wells triangle chambers and **b** six wells round chambers

recordings from different cultures simultaneously (i.e. MEA devices with different wells reported in Fig. 2.11), high-throughput measurements are also possible.

In Chap. 6 a detailed report of two works I actively participated and in which MEA technology is used for neuro-pharmacological tests is reported. In the first paper (Frega et al. 2012) I evaluated the effect of ionotropic glutamate agonist (AMPA, NMDA) on the network activity. In the second paper (Colombi et al. 2013) I studied the effect of Antiepileptic drugs (CBZ and APV) on the spontaneous or induced-epileptic (i.e. after bicuculline treatment) network activity.

2.7.2 *Plasticity Studies*

Long Term Potentiation (LTP) and Long Term Depression (LTD) of synaptic strength are the most studied among the modifications that during processes, for instance, of learning and/or memory, synapses can undergo and that significantly depart from the background noise. Homosynaptic or Hebbian activity-dependent mechanisms are used primarily for learning and for short-term memory; on the other hand, heterosynaptic or modulatory input-dependent mechanisms are responsible for long-term memory and lead to transcription and to synaptic growth.

LTP and LTD were demonstrated in many experimental preparations in vitro and in vivo. Indeed, the great majority of the experimental works related to synaptic modifications concerns in vitro slices or dissociated cultures where defined synaptic pathways can be identified.

However, many studies on synaptic plasticity primarily focus attention on changes in the Excitatory Post Synaptic Potential (EPSP), rather than on changes in the firing behavior. In this respect, experimental investigations on the electrophysiological behavior shown by neuronal assemblies represent a fundamental step towards understanding the universal mechanisms of brain coding, learning and memory.

As reported in the literature, in order to understand such universal mechanisms, it is important to investigate how plasticity develops at network level. To address this issue, it is possible to use dissociated cortical cultures coupled to MEA devices, allowing to non-invasively stimulate the experimental preparations and to extracellularly record the output signals.

During these years of PhD I was involved in an Italian project (San Paolo Project) and I investigated neuronal events underlying the persistence of long-term memory, focusing on the role of structural changes at synaptic level. Experimental results showed differences in the electrophysiological activity of the cortical neuronal network after the application of a tetanic stimulation, denoting a potentiation, with respect to the level of ongoing activity initially shown by the cultured network (Chiappalone et al. 2008). It has been recently demonstrated how the effects induced by such protocols are long-lasting (i.e., hours), denoting a phenomenon of Long-Term Plasticity.

Following this line, I performed also experiments using dissociated hippocampal and cortical neuronal networks from a transgenic mouse model for Alzheimer disease. Compared to the control condition (wild-type mice), the results were ambiguous and up to now we could not draw any clear conclusion from that study. In some cases, we obtained no potentiation of the network activity after the application of a tetanic stimulation. In other cases, a network electrophysiological activity decrement is recorded 2 h after the tetanus delivery denoting a possible indication of late long term depression.

References

- Banker G, Goslin K (1998) Culturing nerve cells. MIT Press, 2nd edn. p 9, 10
- Berdondini L, van der Wal PD et al (2005) High-density electrode array for imaging in vitro electrophysiological activity. *Biosens Bioelectron* 21(1):167–174
- Berdondini L, Imfeld K et al (2009a) Active pixel sensor array for high spatio-temporal resolution electrophysiological recordings from single cell to large scale neuronal networks. *Lab Chip* 9(18):2644–2651
- Berdondini L, Massobrio P et al (2009b) Extracellular recordings from locally dense microelectrode arrays coupled to dissociated cortical cultures. *J Neurosci Methods* 177(2):386–396
- Carrell A, Burroughs M (1910) Cultivation of adult tissues and organs outside of the body. *JAMA* 55:1379–1381
- Chialvo DR (2007) The brain near the edge, in cooperative behavior in neural systems—Ninth Granada Lectures. Springer, Granada (Spain)
- Chiappalone M, Bove M et al (2006) Dissociated cortical networks show spontaneously correlated activity patterns during in vitro development. *Brain Res* 1093(1):41–53
- Chiappalone M, Massobrio P et al (2008) Network plasticity in cortical assemblies. *Eur J Neurosci* 28:221–237
- Chien CB, Pine J (1991) Voltage-sensitive dye recording of action potentials and synaptic potentials from sympathetic microcultures. *Biophys J* 60(3):697–711
- Colombi I, Mahajani S et al (2013) Effects of antiepileptic drugs on hippocampal neurons coupled to micro-electrode arrays. *Front Neuroeng* 6:10
- Defranchi E, Novellino A et al (2011) Feasibility assessment of micro-electrode chip assay as a method of detecting neurotoxicity in vitro. *Front Neuroeng* 4:6
- Elshabini-Riad A, Barlow FD (1998) Thin film technology handbook. McGraw-Hill
- Eytan D, Marom S (2006) Dynamics and effective topology underlying synchronization in networks of cortical neurons. *J Neurosci* 26(33):8465–8476
- Fejtl M, Stett A et al. (2006) On micro-electrode array revival: its development, sophistication of recording and stimulation. *Advances in network electrophysiology using micro-electrode arrays*. Springer, New York
- Frega M, Pasquale V et al (2012) Cortical cultures coupled to micro-electrode arrays: a novel approach to perform in vitro excitotoxicity testing. *Neurotoxicol Teratol* 34(1):116–127
- Frey U, Sanchez-Bustamante CD et al (2007) Cell recordings with a CMOS high-density microelectrode array. *Conf Proc IEEE Eng Med Biol Soc* 2007:167–170
- Frey U, Ebert U et al (2009) Microelectronic system for high-resolution mapping of extracellular electric fields applied to brain slices. *Biosens Bioelectron* 24(7):2191–2198
- Fromherz P, Offenhausser A et al (1991) Neuron-silicon junction: a Retzius cell of the leech on an insulated-gate field-effect transistor. *Science* 252:1290–1293
- Gramowski A, Schiffmann D et al (2000) Quantification of acute neurotoxic effects of trimethyltin using neuronal networks cultured on microelectrode arrays. *Neurotoxicology* 21(3):331–342

- Gramowski A, Jugelt K et al (2004) Substance identification by quantitative characterization of oscillatory activity in murine spinal cord networks on microelectrode arrays. *Eur J Neurosci* 19(10):2815–2825
- Gross GW, Rieske E et al (1977) A new fixed-array multi-microelectrode system designed for long-term monitoring of extracellular single unit neuronal activity in vitro. *Neurosci Lett* 6:101–105
- Gross GW, Williams AN et al (1982) Recording of spontaneous activity with photoetched microelectrode surfaces from mouse spinal neurons in culture. *J Neurosci Methods* 5(1–2):13–22
- Gross G, Rhoades B et al (1992) Neuronal networks for biochemical sensing. *Sens Actuators* 6:1–8
- Harrison R (1907) Observations on the living developing nerve fiber. *Anat Rec* 1:116–118
- Harrison R (1912) The cultivation of tissues in extraneous media as a method of morphogenetic study. *Anat Rec* 6:181–193
- Imfeld K, Neukom S et al (2008) Large-scale, high-resolution data acquisition system for extracellular recording of electrophysiological activity. *IEEE Trans Biomed Eng* 55(8):2064–2073
- Jimbo Y, Tateno Y et al (1999) Simultaneous induction of pathway-specific potentiation and depression in networks of cortical neurons. *Biophys J* 76:670–678
- Johnstone AF, Gross GW et al (2010) Microelectrode arrays: a physiologically based neurotoxicity testing platform for the 21st century. *Neurotoxicology* 31(4):331–350
- Kandel E, Schwartz J et al (2000) Principles of neural science, 4th edn. McGrawHill, pp 5–8, 14
- Keefer EW, Norton SJ et al (2001) Acute toxicity screening of novel AChE inhibitors using neuronal networks on microelectrode arrays. *Neurotoxicology* 22(1):3–12
- Kettenmann H, Grantyn R (1992) Practical electrophysiological methods: a guide for in vitro studies in vertebrate neurobiology. Wiley-Liss Inc., New York, p 12
- Kriegstein AR, Dichter MA (1983) Morphological classification of rat cortical neurons in cell culture. *J Neurosci* 3(8):1634–1647
- Lambacher A, Vitzthum V et al (2010) Identifying firing mammalian neurons in networks with high-resolution multi-transistor array (MTA). *Appl Phys A* 102:1–11
- Martinoia S, Bonzano L et al (2005) Electrophysiological activity modulation by chemical stimulation in networks of cortical neurons coupled to micro-electrode arrays: a biosensor for neuropharmacological applications. *Sens Actuators B Chem* 108(1–2):589–596
- Meister M, Pine J et al (1994) Multi-neuronal signals from the retina: acquisition and analysis. *J Neurosci Methods* 51(1):95–106
- Morefield SI, Keefer EW et al (2000) Drug evaluations using neuronal networks cultured on microelectrode arrays. *Biosens Bioelectron* 15(7–8):383–396
- Murray MR, Barber JH et al (1977) Introduction of recording booklets in general practice teaching. *Med Educ* 11(3):192–196
- Parviz M, Gross GW (2007) Quantification of zinc toxicity using neuronal networks on microelectrode arrays. *Neurotoxicology* 28(3):520–531
- Peterson ER, Murray MR (1955) Myelin sheath formation in cultures of avian spinal ganglia. *Am J Anat* 96(3):319–355
- Pine J (1980) Recording action potentials from cultured neurons with extracellular microcircuit electrodes. *J Neurosci Methods* 2(1):19–31
- Pine J (2006) A history of MEA development. In: Taketani M, Baudry M (eds) *Advances in network electrophysiology using micro-electrode arrays*. Springer, New York
- Regehr WG, Pine J et al (1989) Sealing cultured invertebrate neurons to embedded dish electrodes facilitates long-term stimulation and recording. *J Neurosci Methods* 30(2):91–106
- Robinson HPC, Kawahara M et al (1993) Periodic synchronized bursting in intracellular calcium transients elicited by low magnesium in cultured cortical neurons. *J Neurophysiol* 70(4):1606–1616
- Streit J (1993) Regular oscillations of synaptic activity in spinal networks in vitro. *J Neurophysiol* 70(3):871–878

- Thomas CA Jr, Springer PA et al (1972) A miniature microelectrode array to monitor the bioelectric activity of cultured cells. *Exp Cell Res* 74(1):61–66
- Wheeler BC, Novak JL (1986) Current source density estimation using microelectrode array data from the hippocampal slice preparation. *IEEE Trans Biomed Eng* 33(12):1204–1212
- Xia Y, Gross GW (2003) Histiotypic electrophysiological responses of cultured neuronal networks to ethanol. *Alcohol* 30(3):167–174

Neuronal Network Dynamics in 2D and 3D in vitro
Neuroengineered Systems

Frega, M.

2016, XIII, 148 p. 97 illus., 43 illus. in color., Hardcover

ISBN: 978-3-319-30236-2