Extracellular Electrical Stimulation-based *in Vitro* Neuroscience

A Minireview of Methods and a Paradigm Shift Proposal

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***Abstract*—Biological neuronal cells communicate using neurochemistry and electrical signals. Electrical stimulation (ES) is utilized to study neuronal cells and networks. Currently, ES is applied and responses observed in an open-loop fashion, which does not resemble natural network I/O. We hypothesize that real-time closed-loop full-duplex (simultaneous two-way) paradigms could provide deeper insight in natural neuronal networks, helping to understand our brains and to control neuronal network states to cure diseases. We present a minireview of ES-based extracellular *in vitro* neuroscience, our first long-term closed-loop ES experiment results as the proof-of-feasibility of the method, and our paradigm-shifting proposal of dialogical bio-ICT paradigms.**

***Keywords*—bio-ICT interaction; biological neuronal networks; closed-loop control; DSP; electrical stimulation; open-loop control; real-time**

I. Introduction

Networking and electrical activity are the hallmarks of neuronal cells [1][2]. In addition to other cell biological methods, electric potential measurements and electrical stimulation (ES) are used to study neuronal cell and network structures, properties, development, and functions. 'Stimulation' may affect a single cell or a cell population in a response-eliciting manner, and a 'response' is a measurable phenomenon, such as an action potential (AP) in a few milliseconds after stimulation or, e.g., a change in overall electrical activity or network formation. ES affects neurons and neuronal networks, and may also affect glial cells, such as astrocytes, which support neurons and their functions in many ways [3][4][5]. A neuronal network consists of at least these two types [6] of cells. The effects of ES on neuronal networks and astrocytes are not yet well known. The relations of neurons, astrocytes [5] and brain networks, and the mechanisms behind synaptogenesis, central nervous system regeneration and activity-dependent plasticity are also not yet fully understood.

Microelectrode array (MEA) technology [7][8] offers long-term recordings of neuronal network function and insight in the complex neuronal processes, such as network development and pathophysiological conditions. Here, we provide a minireview of the field of ES of neuronal systems, and the results of our first long-term closed-loop feedback ES experiment with neuronal cultures on MEAs. With this paper, our aims are to 1) coin "dialogical" bio-ICT paradigms, 2) raise interest in biomedical bio-ICT interaction system development among the circuits and systems community, and 3) provide a proof-of-feasibility of long-term real-time closed-loop *in vitro* ES experiments.

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II. Minireview of the Field

A. Stimulation Modalities

Several modalities can be used to stimulate neuronal cells: electrical currents and fields [9], electromagnetic fields [10], chemicals [11], incl. gasses [12], light [13], temperature [14], and mechanical means [15]. Here, we concentrate on ES, which is the most utilized modality besides chemical stimulation.

B. Electrical Stimulation in Vivo, in Vitro, and in Silico

ES of neuronal cells can take place *in vivo*, (in a living creature), *in vitro* (in a dish), or *in silico* (in a computational simulation). *In vivo* stimulation of human neuronal cells [16] is used in medical settings for therapeutic effects, such as deep brain stimulation for Parkinson's disease [17] and epilepsy [18], and electric shock therapy for depression [19]. *In vivo* animal brain stimulation experiments are conducted, e.g., to study brain diseases, to find cures, and to unravel functions of the brain.

In vitro studies are conducted using acute brain slices and cultured neuronal cells, organoids and organotypic cultures. Intracellular electrophysiology of single neuronal cells can be performed using patch clamp technique [20][21], in which a tiny glass pipette electrode is brought to contact with the cell membrane. This gives information, e.g., about synaptic potentials or ion channels, which largely contribute to the electrical activity of neurons [1]. Extracellular neuronal network function and characterization studies can be done using *in vitro* MEAs [7][8][21][22], often utilizing ES.

In silico studies are conducted by simulating neuronal cells and networks [23] in a computer. Such simulations can be based on cell biology, cellular networks, or phenomenology. Phenomenological simulations are aimed at reproducing observed natural phenomena without necessarily modeling the underlying biology. Also, the effects of ES can be simulated [24].

C. Current in Vitro MEA Systems and ES Experiments

In general, two general types of *in vitro* MEAs exists: MEAs with up to 256 galvanic microelectrodes (MEs)[1] embedded on planar substrate (e.g., glass or printed circuit board), and capacitive CMOS MEAs with hundreds or thousands of MEs[2]. Exemplary specifications: A MEA system can measure voltages between a reference electrode and each ME at 5–50 kHz sampling rate with 14 bits/sample. Analog bandwidth can range from 0–25000 Hz, often limited to 3–3000 Hz. The systems capture APs of approx. 2 ms duration, and if desired, slow frequency field potentials, which may be analyzed separately. The measurement equipment/software is usually able to filter measured data and detect APs and AP bursts online, and produce AP time stamp data, but more complex analyses is usually performed offline.

At least one commercially available MEA system, the MEA2100-System by MCS, contains an embedded DSP (Texas Instruments TMS320C6454) for real-time closed-loop feedback stimulation (FS). The manufacturer's software can be used to run FS experiments with simple user-defined feedback logic based on the detected APs [25]. The DSP with access to data and stimulation hardware can be programmed by the user for more advanced feedback logic, other functions and peripheral I/O.

ES is applied usually in a form of short rectangular voltage or current pulses [26] or sequences [27] of pulses. Such experiments are used, for example, to probe the electrical responses of cells and networks, to alter networks or their activity, e.g., in long-term potentiation and depression experiments [28], to test neurotoxicity of (potential) drugs and chemicals [29], or to steer stem cell fate and differentiation [30][31].

Closed-loop FS paradigms [7][32][33] have also appeared for extracellular electrical bio-ICT interaction, e.g., for robot control realized as animats [34][35] in which an *in vitro* neuronal network on a MEA is embodied with a robot. However, these have been mostly toy demonstrations and so far the neuroscientific value of such paradigms has been quite limited.

D. Current in Vitro MEA Signal Analysis and ES Experiments

Current *in vitro* MEA analyses include AP and burst statistics [36], raster plots [37], and stimulation response analysis, such as post-stimulus histograms [38]. Network connectivity analyses [39][40] have been proposed. For examples of analysis methods from AP detection to connectivity analysis, see [22].

III. Closed-loop Stimulation Experiment Example

Here, we present the methods and results of our first long-term real-time FS experiment with the MEA2100-System.

A. Materials and Methods

Approx. 100'000 commercial primary rat cortex neurons (RCN) (Gibco) were thawed and plated on each of six laminin-coated 60-electrode MEAs (60MEA200/30iR-ITO, MCS), and cultured according to the manufacturer's instructions. Half of the medium was replaced after each recording session. Before recordings, the MEAs rested for five minutes in the preamplifier to let the state of the cells stabilize. Thereafter, electrical activity was recorded for three or five minutes. Recordings were made using MEA2100-System approx. every second day.

Four MEAs underwent monopolar FS during the measurements and two remained as unstimulated controls from which spontaneous activity was recorded. An observed and an ES ME were selected for each stimulated MEA based on apparent connectivity between the observed and ES MEs. Apparent connectivity was determined by visually observing synchronous AP activity for possible functional connectivity and microscopy for possible physical connectivity. The same observed and ES MEs were used for the entire experiment. Data was measured at 10 kHz sampling rate before, during and after ES for each ES-MEA, and from the unstimulated MEAs. FS was initiated upon an AP train of three spikes in 500 ms at an observed electrode. Stimulation consisted of a train of 100 400 µs long square pulses of ±1000 mV in amplitude. One of the unstimulated control MEAs underwent FS at four months. Immunostaining and fluorescent imaging for protein expression analysis was performed at 4.5 months to reveal neurons and glial cells.

For this paper, we analyzed the recordings made at one, four and eight weeks, and four months using Multi Channel Analyzer (MCS). AP detection was done by thresholding at five times the standard deviation of the signal. An occurrence of at least four APs in 50 ms was considered an AP burst with at least 100 ms between the bursts. Fig. 1. was produced with Matlab, statistical analyzes (Fig. 2) were done with Microsoft Excel and IBM SSPS Statistics, and the images for Fig. 3 were processed with Fiji (ImageJ) and CorelDRAW X7.

B. Results

Representative MEA measurements before and after FS are shown in Fig. 1a and Fig. 1b, respectively. The results in Figs. 1–3 demonstrate that the cells stayed active for several months of culture and FS, and that FS affected cell or network behavior.

RCNs started showing electrical activity after a week *in vitro*, after which the FS was initiated based on the apparent connectivity. After 4 weeks of culture, FS caused an increase in the burst counts, but after 8 weeks, burst count decreased after FS (Fig. 2a). Possible reasons include too intensive stimulation causing decreased cell viability, cultures reaching different equilibrium state causing the stimulation response to change, and degradation of stimulation electrodes. Increased/decreased burst counts at all MEs during FS via one ME indicated that neurons had formed comprehensive neuronal networks through which depolarization could spread. Spreading of stimulation-enhanced activity was observed (Fig. 2a). After each FS session, spontaneous activity recovered well even after four months of culture (Fig. 2b). Furthermore, most MEAs showed statistically significant decrease in burst duration during FS ($p < 0.05$) but increase in post-stimulation burst duration ($p < 0.05$). In addition, applying FS at 4 months to the previously unstimulated MEA (MEA1,

[1] E.g., the MEA2100-System (32, 60, 120 or 256 MEs and an embedded digital signal processor (DSP)) by Multi Channel Systems MCS GmbH (MCS) (https://www.multichannelsystems.com/products/mea2100-systems/), and MEA64-Basic (64 MEs) by Alpha MED Scientific Inc. (http://www.med64.com/products/med64-basic/). Both companies offer also multiwell MEA systems for faster handling of larger sample sizes.

[2] E.g., the CMOS-MEA5000-System (4225 measurement MEs and 1024 stimulation sites, https://www.multichannelsystems.com/products/cmos-mea5000-system) by MCS, and BioCam X (4069 measurement MEs and 16 stimulation sites, https://www.3brain.com/biocamx.html) by 3Brain AG.

Fig. 2b) caused increased activity during FS and post-stimulation, alike what had happened with the long-term FS MEAs at four weeks but not anymore at 4 months.

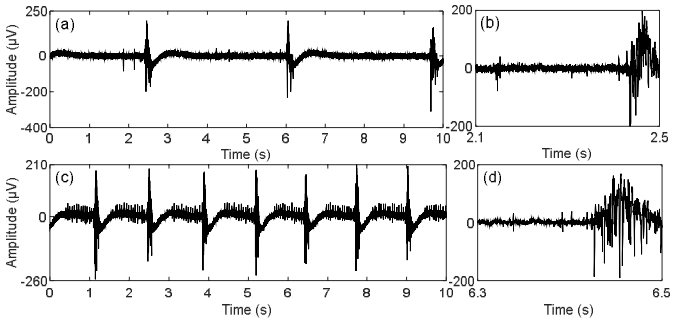

Fig. 1. Representative electrical activity recordings from one electrode at 4 months *in vitro* during spontaneous basal activity (pre-stimulation) ((a), with detail in (b)), and after FS (post-stimulation) ((c), with detail in (d)). Most MEAs showed significantly prolonged burst durations after FS during post-stimulation recordings, and both spike and burst counts increased before settling back to basal electrical activity.

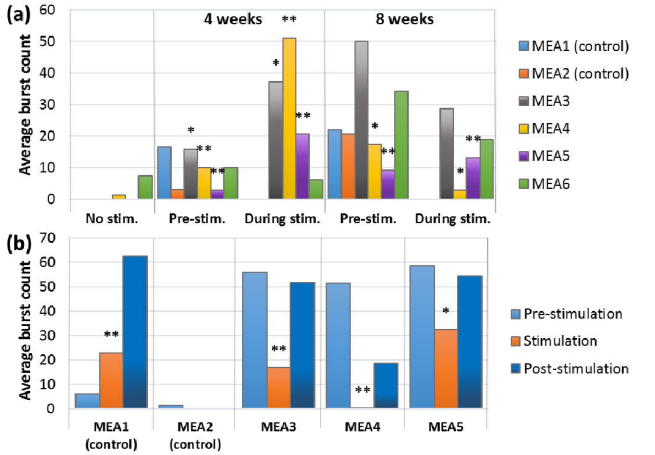

Fig. 2. Average burst counts in the entire recordings of the stimulated and unstimulated MEAs. Statistical analyzes were done separately for each MEA between the pre-stimulus, during stimulation (not for the control MEAs), and post-stimulus measurements (statistical significances: * $p < 0.05$, ** $p < 0.005$). (a) Burst counts after 1, 4, and 8 weeks *in vitro* and FS (no FS for control MEAs). After week 1 *in vitro*, only two MEAs showed activity. After 4 weeks, all MEAs were active and during FS burst counts increased almost in all cases. After 8 weeks, almost all stimulated MEAs showed decreased burst counts during FS compared to the corresponding spontaneous pre-stimulation burst counts. (b) To determine activity recovery after FS, post-stimulation activity was recorded for 3 minutes. Even at 4 months, spontaneous activity was high pre- and post-stimulation. Burst counts decreased during FS for all MEAs except for MEA1 (the previously unstimulated control). All MEAs exhibited reinstated or even increased spontaneous post-stimulation activity.

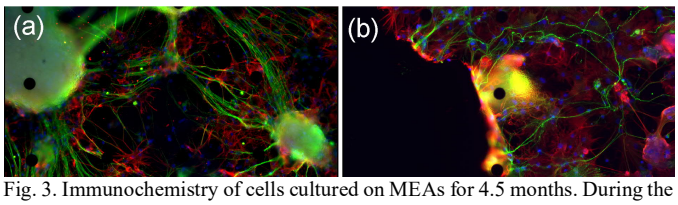

Fig. 3. Immunochemistry of cells cultured on MEAs for 4.5 months. During the experiment, neurite networks (green, Tuj1+) supported by glial cells (red, GFAP+) were formed both in the representative unstimulated MEA (a) and FS-MEA (b). On the ES side of the MEA (b), cell viability was decreased, and neuronal networks were missing on a part of the MEA area. At late stages of the experiment, both neurites and glial cells were present on the edges of the stimulated MEAs, but networks were fewer and glial cells were in worse condition compared to the unstimulated MEA (a). (a)–(b) Black round dots are MEs with the inter-ME distance of 200 µm.

Protein expression analysis revealed that both stimulated and unstimulated cells expressed neuron-specific protein Tuj1, as well as glial cell-specific protein GFAP (Fig. 3), i.e., both neurons and glial cells were present and alive after 4.5 months of culture and ES. On the ES-MEAs, neuron and astrocyte viability had decreased on electrode area (Fig. 3b).

IV. Technological Requirements for New FS Paradigms

Relatively simple real-time closed-loop bio-ICT paradigms can be realized with the DSPs embedded in the current measurement systems, like in the MCS MEA2100-System. The ICT specifications for future dialogical bio-ICT paradigms could be derived from the data rate (e.g., a CMOS MEA with 10000 channels sampled at 25 kHz, 14 bits/sample, i.e., approx. 3 Gbps), the required response time (e.g., similar to synaptic latency in the cortex of 0.2–6 ms [41]), and the desired signal analysis, e.g., such as described in [22][42]. The actual dialogical analysis methods are still unknown, and the MEA technology itself will likely develop to provide new data on synaptic and ion channel activity and cellular signaling. This may set new requirements on the real-time FS ICT systems.

V. Conclusions and Discussion

We have presented a feasibility study of long-term *in vitro* neuronal FS experiments, and a minireview for ES-based neuroscience. Since most of the ES experiments are relatively short-term, this paper adds to our knowledge on the effects of long-term ES of neuronal cultures on MEAs. Most of the current ES-based systems and applications noted are open-loop, or at least not real-time, and the I/O, alike in the brain between the brain regions and outside world, is in general missing. Our working hypothesis is that ES-based neuroscience and applications could benefit from real-time closed-loop full-duplex ES paradigms.

The current MEA systems have potential to be the platforms of the first next generation FS-based neuroscience paradigms. Next, to further our understanding of our brains, novel (probably artificial intelligence-based) analysis algorithms and dedicated ICT solutions are needed to take neuroscience to a new level. We believe that the next paradigm shift in neuroscience can be brought about by real-time closed-loop full-duplex neuronal system analysis and bio-ICT interaction paradigms, ***the dialogical bio-ICT paradigms***.

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