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Human Pluripotent Stem Cell-Derived Neuronal Networks: Their Electrical Functionality and Usability for Modelling and Toxicology

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1. Introduction

Micro electrode array (MEA)-based platforms have been used to study neuronal networks for decades. The used cells have, for the most part, been rodent primary neurons. The gained knowledge has indeed increased the understanding of neuronal network development and maturation both *in vitro* and *in vivo*. If aiming to understand the development of human brain, however, the used cell type should preferably be of human origin due to difficult interpolation from the rodent cell data. In addition, the development of functional human neuronal networks would open up a new era for, e.g., toxicology testing, drug screening and disease modelling.

The use of MEA with bioelectrically active cells was first reported by Thomas et al. 1972. Gross et al. (1977; 1979) extended the applicability of the MEA platform to long-term recordings of neuronal cells and Pine (1980) reported the first recordings from dissociated neurons. Interestingly, although the solutions related to the MEA fabrication and design were similar in the above mentioned papers, all three groups came to their conclusions independently demonstrating the drive and need to record neurons *in vitro*. MEAs have been successfully tested in several species and cell types using organotypic and primary dissociated cell cultures. Due to their ease of use, the long-term survival of the cultures and vast possibilities of manipulation of the culture, MEAs provide an attractive tool to explore basic neuroscience as well as for pharmacology and toxicology.

Functional neuronal networks have been derived from mouse embryonic stem cells (Evans & Kaufman 1981; Martin 1981), but the invention of culturing human embryonic stem cells (Thomson et al., 1998) and human induced pluripotent stem cells (Takahashi et al., 2007) have enabled the researchers to build up human cell-derived neuronal cells and networks (Ban et al., 2006; Carpenter et al., 2001; Heikkilä et al., 2009; Karumbayaram et al., 2009; Nat et al., 2007). These studies have proved that human pluripotent stem cell-derived neuronal cells are indeed capable of forming functional neuronal networks which most likely resemble the networks in human brain. These human-derived neuronal networks have increased and will further increase the understanding of human brain development and functions. More importantly, with these networks it is possible to model the “normal” brain

by using human embryonic stem cell-derived neuronal cells and various diseases can be modelled when using patient specific induced pluripotent stem cell-derived neuronal cells. In addition, these cell types are suitable for drug screening and neurotoxicological studies. This chapter will shortly introduce the neuronal differentiation of both human embryonic stem cells and human induced pluripotent stem cells. Also, MEA platforms will be discussed concentrating especially on the use of MEAs with human pluripotent stem cell-derived neuronal networks. Finally, some future aspects will be discussed.

2. MEA technology

The structure of the MEAs is simple. They consist of a glass slide that has wire electrodes photo-etched onto it. Each electrode is insulated from each other (e.g. by silicon nitrate) and only the tip of the electrode is coated with conductive material, such as titanium, platinum, or indium-tin oxide. Typically, the electrode size is 10 – 30 μm and they are aligned in a grid with 50 – 500 μm separation from the centres of the electrodes (Johnstone et al., 2010; Pine 2006), MEAs measure the potential difference between a common ground electrode and the measurement electrodes. Because the measured voltage differences between the ground and the measurement electrodes are small and the locations of the electrodes are static, the reduction of noise and the amplification of the recorded signals are crucial. Often, the whole MEA chip is placed inside of the amplifier to ensure minimal distance between the electrodes and the amplifiers.

To make hydrophobic MEA surface more appealing to neurons, the surface is often coated with different protein solutions like extracellular proteins (e.g. laminin, fibronectin, polylysines). Surfaces can be also pre-treated with Poly(ethyleneimine) (PEI) that enhances the coating procedure (Heikkilä et al., 2009; Illes et al., 2007; Wagenaar et al., 2006).

Cells grown on MEAs can be continuously perfused with fresh medium, but because this is a cumbersome and laborious task, the medium is usually replaced on a weekly basis. Different laboratories have different routines for medium replacement depending on the available staff and type and amount of the cells used in the experiments. Sensitive cells, such

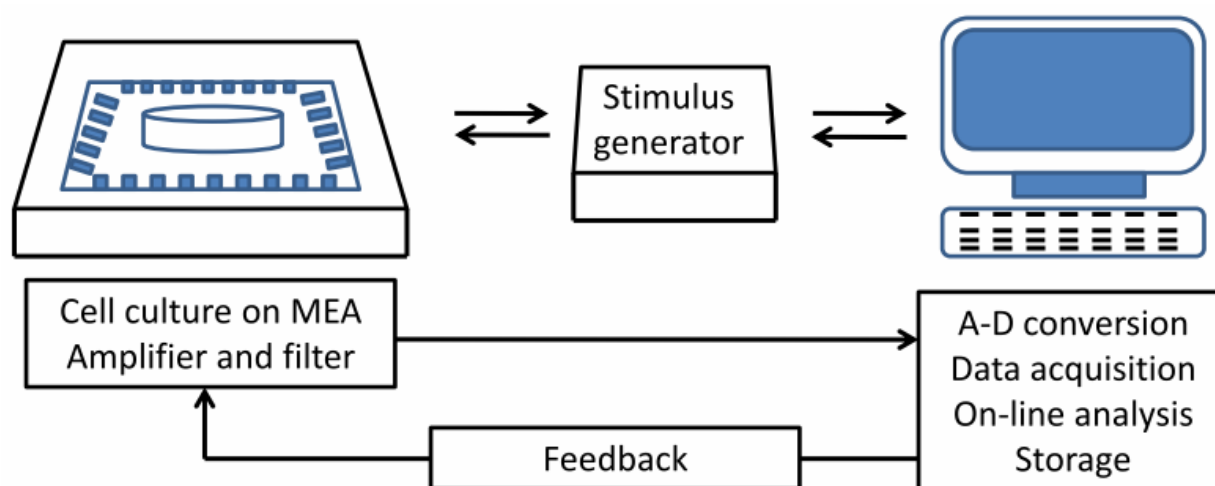


Fig. 1. A typical MEA setup. The Array is located inside an amplifier that is connected to an analog to digital converter on a computer. Bidirectional information transfer allows timing of the stimulations and more sophisticated study designs.

as hESC-derived neuronal cells, require fresh medium at least three times a week (Heikkilä et al., 2009). Typically, only one third or half of the medium is replaced in order to maintain more stable culturing conditions. Change of full medium would generate a massive change in the cellular microenvironment due to differences in the ionic and osmotic concentrations in the fresh and used growth media. Frequent change of the growth medium requires continuous handling of the MEAs and exposes the cultured neurons to an additional stress and contaminants in the laboratory. Thus, there is always a trade-off between the optimal medium composition and risk of contamination. After the introduction of the semi-permeable transparent hydrophobic membrane (Potter & DeMarse 2001) that seals the MEA from the surroundings allowing only gas exchange it has been possible to record and maintain neuronal cultures on MEAs for months or even years without evaporation or contamination problems (Heikkilä et al., 2009; Illes et al., 2009; Potter & DeMarse 2001). These long and stable experiments are a prerequisite e.g. for chronic toxicology experiments.

2.1 Functional MEA studies

One of the first functional MEA studies was performed by Corner & Ramkers (1991) who evaluated the activity changes of maturing neurons grown on MEAs using tetrodotoxin and picrotoxin in long-term culture. After inclusion of the stimulation electrode into the MEA design (Gross et al., 1993), the applicability of MEAs to conduct functional studies improved significantly. Modern imaging methods, such as gated neurotransmitters, calcium imaging and voltage sensitive dyes (Mennerick et al., 2010) have further enhanced the usability of the MEA platform.

Although neurons grown on a MEA chip form 2-dimensional networks that lack physiological input from the environment, they form functional spontaneously active networks that typically develop in 3 phases. First, activity is detected as single spiking that can reflect both axonal and dendritic signalling in the developing network. Second, as network matures, it starts to express a train-like spiking activity that can further mature into burst-like activity that is considered as mature signalling activity of the network (Heikkilä et al., 2009; Wagenaar et al., 2006). The described types of signalling are represented in Figure 2. These functional networks can interact with the environment by selectively responding to the artificial cues presented by the stimulation apparatus. Jimbo and co-workers demonstrated that repetitive stimulation reinforces the synchronicity on MEA grown networks (Jimbo et al., 1998). However, firing of the neurons is pathway-specific and stimulation may either promote or suppress the network firing activity depending on the stimulation location (Jimbo et al., 1999). Stimulations can also be used to



Fig. 2. Development of signalling in MEA. (A) First, after the neuronal cells have grown few days on MEA, first signals are detected. They are individual single spikes. (B) Then, about after 1-2 weeks of culturing spiking activity matures into training phase. There are several spikes organised together. (C) Finally, bursting activity occurs. Burst is a complex signal package that happens in several channels at the same time.

tune network firing activity. Shahaf & Marom (2001) introduced a method of learning by stimulus removal on MEA grown dissociated cortical neuronal networks. It seems that MEA grown neurons can communicate in an activity-dependent manner that resembles the behaviour of intact neurons *in vivo*. On MEAs, this communication typically occurs in bursts of synchronous spikes, whose pathways (Jimbo et al., 1999), timing (Shahaf & Marom 2001), type (Wagenaar et al., 2006) and initiation (Eytan & Marom 2006) can be considered as variables for systemic activity changes.

2.2 MEA analyses

Most commonly, routines of MEA research follow the paths of decades of research in *in vivo* and *in vitro* electrophysiology. Due to the ease of use of MEAs and the benefits of dissociated culturing, the speed of the experiments is, however, much faster. Furthermore, due to elevated control over experimental conditions and variables in culture, the reproducibility of the experiments is more reliable and large sample sizes easily attainable. Raw data is usually high-pass filtered to extract spikes from the raw data. Additional sorting may be applied to obtain unit data. Although many laboratories have in-house MATLAB scripts to do the task, commercial softwares also exist. Traditional scatter plots, time histograms and time-frequency analyses are commonly used as analysis tools for MEA data. As in psychology and medicine, stimulus averaged methods are common for triggered data. On a network scale, separation of activity regimes (Tanskanen et al., 2005), analysis of bursts (Mazzoni et al., 2007) and pattern clustering (Madhavan et al., 2007) are challenging the traditional estimations of network cross-correlations. Synfire chains (Abeles 1982; Tetzlaff et al., 2002), avalanches (Beggs & Plenz 2003; 2004) and unitary events (Gruen et al., 2002; Gruen 2009) are emerging as computational tools for more complex MEA network analysis. MEA data presentation can be found in Figure 3.

3. Human pluripotent stem cells and their neural differentiation

Short-term *in vitro* culturing of the inner cell mass of human blastocysts was first reported on 1994 (Bongso et al., 1994), but the successful isolation, culturing and characterization of human embryonic stem (hES) cells was reported 4 years later (Thomson et al., 1998). These cells are referred as human pluripotent stem cells due to their capability to form every cell type of the human body. After the discovery, hES cell research was intensively conducted for almost 10 years before remarkable progress took place with the invention of human induced pluripotent stem (hiPS) cells. These cells are formed from somatic cells by viral reprogramming (Takahashi et al., 2007; Yu et al., 2007) and they closely resemble hES cells by their characteristics. Thus, currently term human pluripotent stem cells includes both hES and hiPS cells (Figure 4). Most of the work cited here have been conducted using hES cells and their neural derivatives.

Originally hES cells were cultured in their undifferentiated stage on mouse embryonic fibroblasts as feeder cells (Thomson et al., 1998) but shortly after also on human foreskin fibroblasts (Hovatta et al., 2003). Several feeder-free systems have also been studied with success on maintaining hES cells in undifferentiated form (Benzing et al., 2006; Gerrard et al., 2005; Hakala et al., 2009). There are clear indications, however, that hES cells cultured without feeder cells exhibit more abnormalities caused by suboptimal culture conditions and enzymatic passaging in long-term cultures (Imreh et al., 2006; Mitalipova et al., 2005).

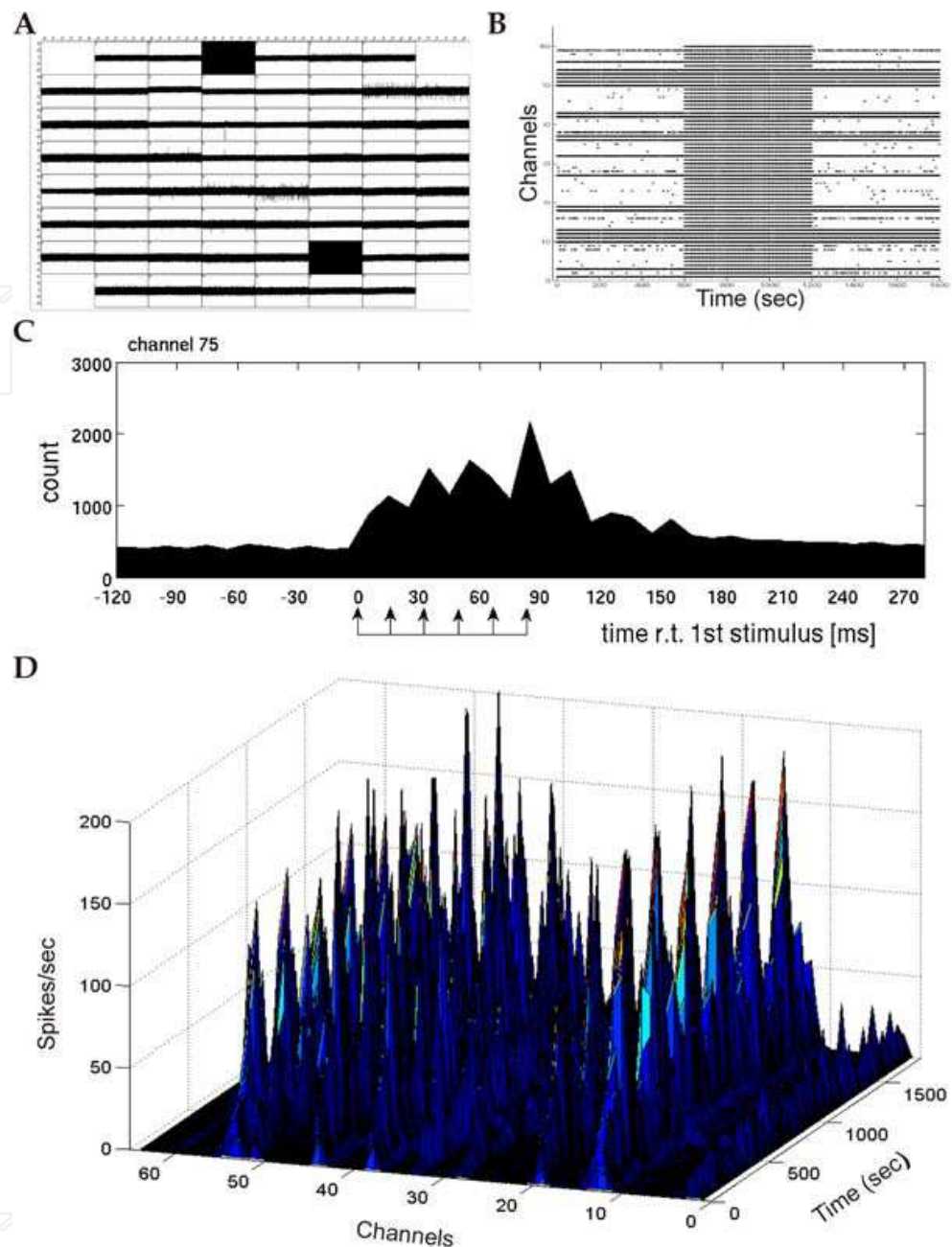


Fig. 3. MEA data and its analysis. A) Typical recorded MEA signal streams on 60 electrodes. Each small window represents a signal from one MEA electrode. Note the noisy electrodes 41 and 67 which appear all black. B) MEA activity plotted as a channel wise scatter plot. Each vertical line represents an electrode and each dot marks a spike recorded on that electrode. The easiest way to separate spikes is to draw a threshold based on standard deviation of the signal (e.g. $5 \times \text{SD}$) and accept all crossings of the threshold as spikes. Note the stimulus responses and artefacts present on all the channels from 600 s to 1200 s. C) Peri-stimulus time histogram of the selected channels in B. This histogram sums stimulus aligned responses and thus presents an average response to a given stimulus. Arrows below the figures show the timing of individual stimulus in a train of stimuli. Note the elevation of the spike rate in response to repetitive stimulus. D) Rate plot of the whole MEA recording. This 3D plot clarifies the parameters of time, electrode channels and spikes/sec in a single figure.

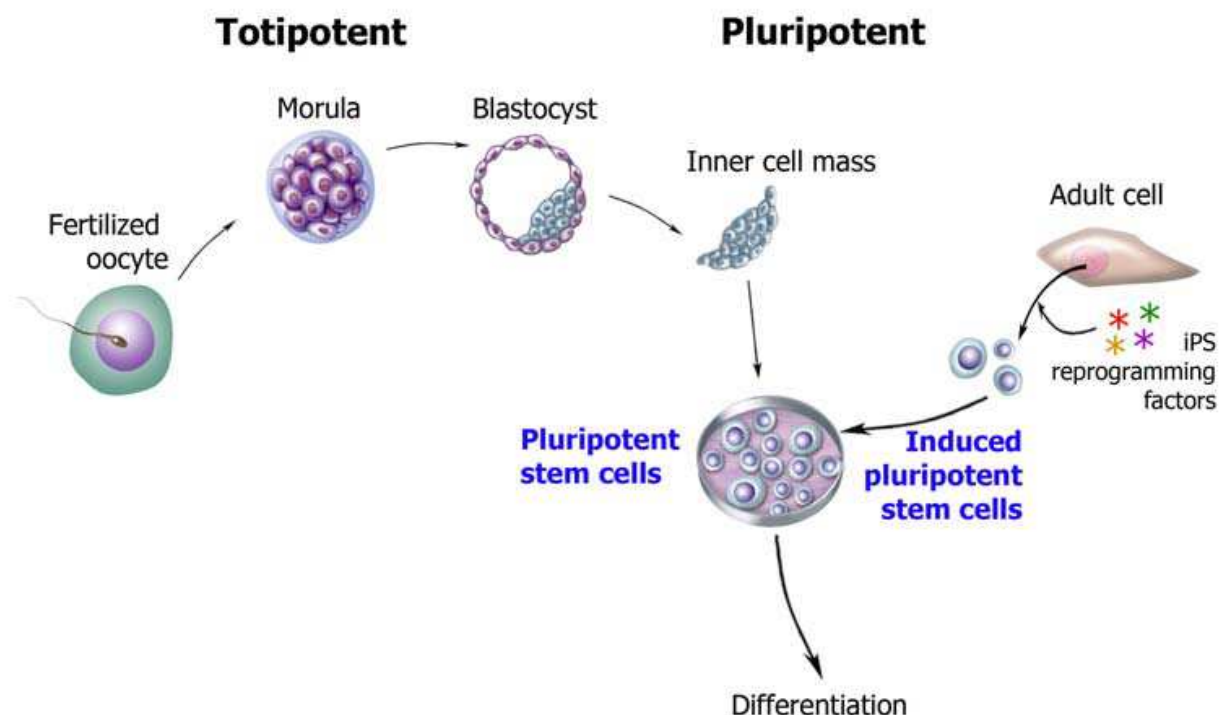


Fig. 4. Stem cells. Stem cells can be divided in groups in accordance to their differentiation capacity. Embryos in zygote and morula stages are defined as totipotent. In blastocyst stage the inner cell mass is capable of producing the three germ layers and primordial germ cells, thus defined as pluripotent embryonic stem cells (ESCs). Adult cells can be re-programmed to produce embryonic stem cell-like pluripotent cells (iPS cells). Original images prepared by Cathrine Twomey from the National Academies *Understanding stem cells: An Overview of the Science and Issues*, <http://www.nationalacademies.org/stemcells>.

Thus, the culture conditions of hES cells have been systematically improved towards containing only human or synthetic components. Similarly, hiPS cells need to be cultured on top of a feeder cell layer to avoid the spontaneous differentiation of these cells. This aspect has been widely studied due to ultimate aim to elude the use of feeder cells altogether.

Undifferentiated human pluripotent stem cells can not be used for disease modelling or regenerative medicine as such due to their massive teratoma formation capacity (Adewumi et al., 2007; Skottman et al., 2007; Thomson et al., 1998). Indeed, neuronal differentiation of human pluripotent stem cells has been widely studied. The first articles on neural differentiation of hES cells were published 2001 (Carpenter et al., 2001; Reubinoff et al., 2001; Zhang et al., 2001) and of hiPS cells 2009 (Chambers et al., 2009; Karumbayaram et al., 2009). All of these protocols relied on embryoid body (EB) formation and/or further replating of the cells on appropriately coated surfaces in neural differentiation medium. Regardless of the differentiation methods used, these studies all showed that neural progenitors, specific neuronal cells, astrocytes and, to a lesser extent, oligodendrocytes could be produced. Since then, several methods and protocols for neural differentiation of hES and also hiPS cells have been published. Some groups utilize co-culture method with e.g. PA-6 stromal cells (Aberdam et al., 2008; Pomp et al., 2008) whereas other groups differentiate the cells with medium including minimum amounts of animal material (Lappalainen et al., 2010; Nat et al., 2007) or in totally defined animal-component free medium (Erceg et al., 2008; Yao et al., 2006).

Methodologically hES cells can be differentiated towards neural lineages using adherent and suspension culture systems or their various combinations with the help of specific factors facilitating differentiation. These factors include basic fibroblast growth factor (bFGF) (Benzing et al., 2006; Lappalainen et al., 2010), bone morphogenic protein signalling blocker noggin (Gerrard et al., 2005; Itsykson et al., 2005; Li et al., 2008) or retinoic acid (Baharvand et al., 2007; Erceg et al., 2008). Thus, the production of neural progenitors and specific neuronal phenotypes from hES cells appears to be possible with many methods, growth factors and inducing agents. The functionality of the produced neuronal cells has not, however, been taken into consideration on a large scale in relation to the differentiation protocol. hiPS cells have been neurally differentiated with the same methods as hES cells but the electrical functionality of hiPS cell-derived neuronal cells has so far been studied only with patch clamp technology (Karumbayaram et al., 2009).

4. MEA and pluripotent stem cell-derived neuronal networks

In neural applications MEA setup has traditionally been used with rodent primary cells. With pluripotent stem cell-derived neural cells the research has been conducted just over a decade but for now only a few groups have reported the usage of MEA with neuronal networks derived from pluripotent stem cells (Ban et al., 2006; Heikkilä et al., 2009; Illes et al., 2007). To date, little is known about the electrical properties of the pluripotent stem cell-derived neuronal cells, their maturation, and network development and function.

The first articles describing the measurement of murine ES cell-derived neuronal networks were published 5 years ago (Ban et al., 2006; Illes et al., 2007). Both of these studies showed that the cells were capable of forming spontaneously active networks. Further, the activity developed from single spikes into more complex trains and bursts over time (Ban et al., 2006; Illes et al., 2007). The first study reporting successful measurement of human ES cell-derived neuronal networks with MEA was reported two years later (Heikkilä et al., 2009). This is a vital step in human research (Table 1) due to possible interspecies differences of developed neuronal networks. Especially with toxicological and drug studies, the specific pathways play important role in evaluating results and the translation between different species have to be taken into account. For example, mouse embryonic stem cell-derived neuronal cells need JAK/STAT pathway activation whereas for the human ones activin/nodal and FGF signalling are important. These differences may cause large variation to results in drug responses. Thus, it might be meaningful to test toxicological effects directly on human cells (Hardingham et al., 2010). Indeed, we recently performed a neurotoxicological evaluation of methyl mercury in human neuronal cell-based MEA platform (Ylä-Outinen et al., 2010). In this study we reported that at subcytotoxic levels methyl mercury caused changes in functionality of neuronal networks whereas no alterations were detected with standard molecule biological analyses (Ylä-Outinen et al., 2010).

MEAs with ES cell-derived neuronal networks can also be used for studying the fundamental properties of these networks by pharmaceutical modulation. It has been shown that these networks, from human or mouse origin, contain functional glutamate and GABA receptors as they respond to AMPA/kainate, NMDA and GABA_A receptor blockers (Heikkilä et al., 2009; Illes et al., 2007). These results are similar to results from rodent dissociated primary cultures (Kamioka et al., 1996; Corner & Ramakers 1991). Thus, human cell-based MEA platforms offer intriguing possibilities to conduct large scale *in vitro* drug screening and testing thereby reducing the need to use experimental animals. In addition, usage of human cells makes the translation into clinical setting more direct.

Research field	Benefits of using MEA networks from human origin
Basic research	Cell-cell signalling Cellular and network connectivity Receptor expression and functionality
Developmental studies	Time course of development of cell types and networks Developmental disorders Intervention in development
Toxicological studies	Study of toxicological mechanisms On-site-sampling Continuous screening Acute and chronic responses
Drug screening	High throughput Standardized environment Animal-free testing
Regenerative medicine	Functionality of cell grafts Verification of uniform graft quality Graft-host tissue interactions

Table 1. Research fields that benefit from the information gained with human cell-based MEA measurements.

Dissociated neuronal networks have capacity and plasticity to learn *in vitro*. This has been shown by electrical stimulation of neuronal populations on MEA (e.g. Shahaf & Marom 2001). Also, mES cell-derived neuronal networks have the ability to process information as shown by Ban and co-workers (Ban et al., 2006). They varied the intensity of the stimulus and showed that the neuronal networks responded to the intensity change. We have gained similar results with hES cell-derived neuronal cells (Heikkilä et al., 2009).

The electrophysiological properties of hiPS cell-derived neuronal cells have not been extensively studied. So far, a few groups have described hiPS cell-derived neuronal cell functions with patch clamp on a single cell level (Karumbayaram et al., 2009; Swistowski et al., 2010). The ability of these cells to form spontaneously active networks has been somewhat questionable. Here, for the first time we show that neuronal cells derived from normal hiPS cell line, indeed, form functional neuronal networks. This functionality was evidenced on MEAs already two days after cell plating. The activity evolved from single spikes to complex burst patterns within four weeks similarly to hES cell-derived counterparts as shown in Figure 5. In future, neurological diseases with genetic background can be modelled with patient-specific hiPS cell lines as already published with e.g. inherited metabolic disorders in liver (Rashid et al., 2010) or long QT-syndrome in heart with patch clamp (Moretti et al., 2010). Because neuronal networks rely on electrical communication, especially the modelling of neurological diseases would crucially benefit from MEA platform. MEA-based disease models have already been introduced. For example, Otto et al. show that mES cell-derived neuronal networks respond by lowered signalling when cultured in cerebrospinal fluid of brain trauma patients (Otto et al., 2009).

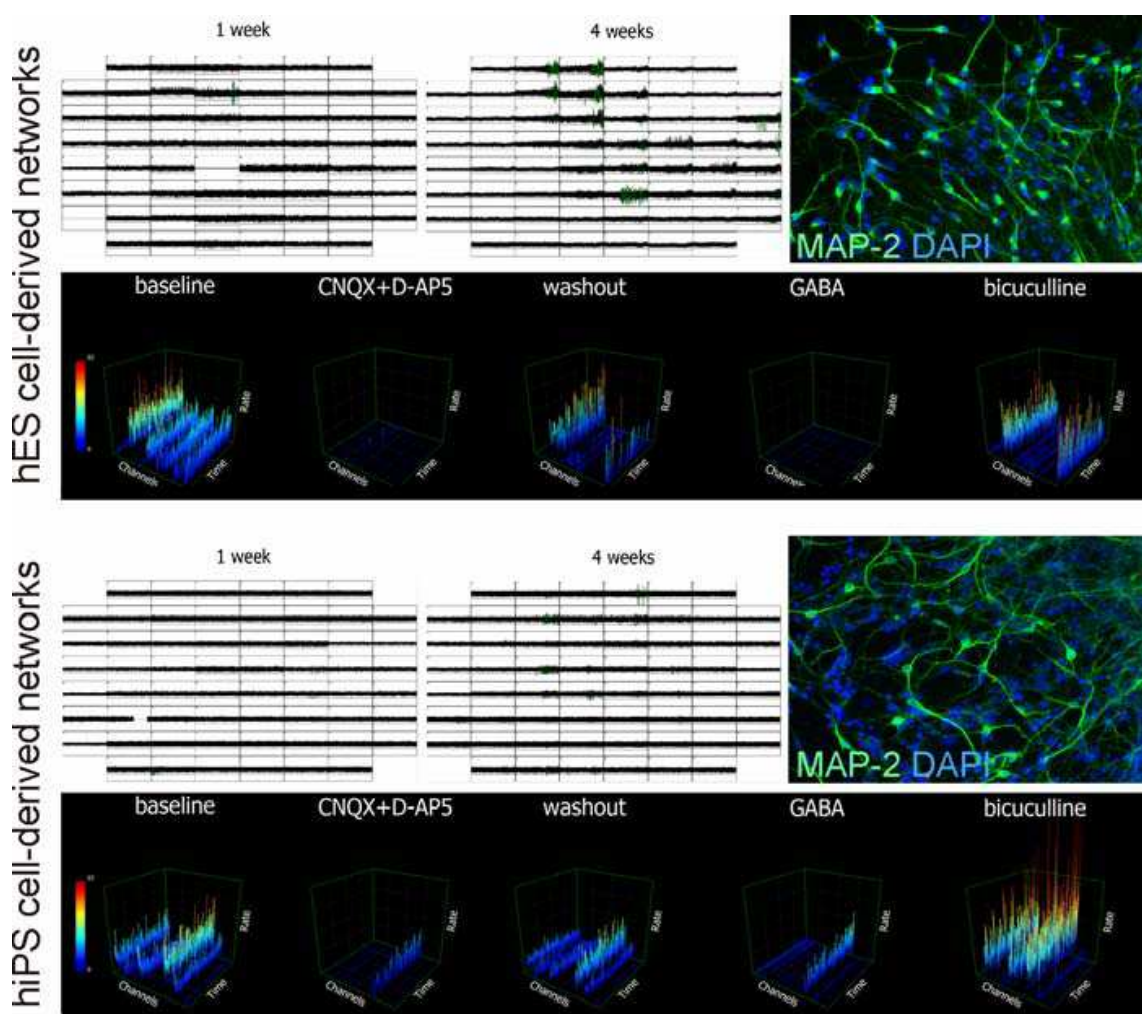


Fig. 5. Comparison of electrical and molecular properties of hES and hiPS cell-derived neuronal networks. Array wide plots of spontaneous activity of hES and hiPS cell-derived neuronal networks on MEA. After one week single spiking and training activity is occurring in both hES and hiPS cell-derived cultures. After four weeks both cultures show developed bursting activity. The cells are verified as neuronal cells with immunostaining. 3D-histograms present the responses of the hES and hiPS cell-derived neuronal networks to pharmaceuticals. First, the baseline activity was measured. Thereafter, the addition of 30 μM CNQX and 20 μM D-AP5 suppressed the activity. The activity of the networks reappeared when CNQX and D-AP5 were washed out. GABA-addition (100 μM) blocks the signalling whereas 30 μM bicuculline restored the signalling again. In 3D-histograms recording time [s] is represented by the y-axis and recorded channels in x-axis. The spike rate, spikes/second, is displayed on the z-axis.

5. Future perspectives

MEA-based platforms have been fairly satisfactorily established, yet several challenges still remain. For example, the large variation between individual MEA plates after cell seeding as well as the poor repeatability of connectivity in network development are areas that require improvement. One of the fruitful new approaches to reduce trial variability is a cell-cage (Erickson et al., 2008) where single cells on a network can be individually monitored. Future of

MEA technology will be 3-dimensional when moving towards stem cell technologies and cell therapies. It is essential to construct more *in vivo*-like environment where the whole culture can be screened in 3D. Similar approach is also important for toxicology and pharmacology to mimic tissues as accurately as possible. The current research has used mainly young, heterogeneous neuronal networks and in the future more efforts should be put into producing and measuring specific, well-characterized neuronal subtypes such as glutaminergic, GABAergic or dopaminergic neurons. This approach would bring MEA platforms closer to clinical relevance. For example, controlled co-cultures of various neuronal subtypes and glial cells would offer a tailored tool for clinical pharmacology and toxicology.

Also more relevant, standardized and more robust data analysis protocols should be developed. In addition to existing analysis protocols, companies, such as NeuroProof GmbH (Rostock, Germany), have developed protocols where hundreds of different parameters are evaluated from the functional networks (Schröder, et al., 2010). In practice, it seems that analysis focusing on different parameters on overall spiking activity and burst amount, duration and mean frequency are quite good indicators of networks' responses to different treatments (Hardingham, 2010), but these protocols are not standardised between laboratories. Clearly there is need for new kinds of analysis platforms as thousands of substances in EU area only should be screened for toxicological effects. Thus, MEA-based human cell platforms can offer new tools to take end point testing from animals (LD50, lethal dose for 50%) to cells (EC50, effective concentration in 50%) for testing more subtle and sensitive end points, such as alterations in neuronal activity patterns. Importantly, as there is a great need to reduce, replace and refine animal experiments, these functional level platforms will help to achieve this important goal. This goal requires that standardization and validation are seen as critical development points for the reproducibility and reliability of the MEA technology.

6. Conclusions

As MEA platforms have been shown to enable more accurate and refined analyses of for example developmental biology and neurotoxicity, the use of MEAs is expected to expand in the future. Some issues still, however, remain and efforts should be concentrated on improving e.g. the repeatability of the measurement platforms and setups.

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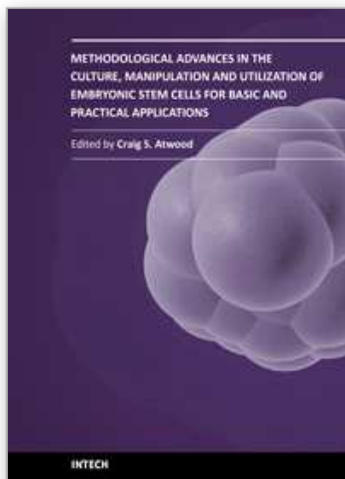
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