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**Title of the Chapter:**
**Advances in Human Stem Cell-Derived Neuronal Cell Culturing and Analysis**

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## Abstract

This Chapter provides an overview of the current stage of human *in vitro* functional neuronal cultures, their biological application areas, and modalities to analyze their behavior. During last ten years, this research area has changed from being practically non-existent to one that is facing high expectations. Here, we present a case study as a comprehensive short history of this process based on extensive studies conducted at NeuroGroup (University of Tampere) and Computational Biophysics and Imaging Group (Tampere University of Technology), ranging from the differentiation and culturing of human pluripotent stem cell (hPSC)-derived neuronal networks to their electrophysiological analysis. After an introduction to neuronal differentiation in hPSCs, we review our work on their functionality and approaches for extending cultures from 2D to 3D systems. Thereafter, we discuss our target applications in neuronal developmental modeling, toxicology, drug screening, and disease modeling. The development of signal analysis methods was required due to the unique functional and developmental properties of hPSC-derived neuronal cells and networks, which separate them from their much-used rodent counterparts. Accordingly, a line of microelectrode array (MEA) signal analysis methods was developed. This work included the development of action potential spike detection methods, entropy-based methods and additional methods for burst detection and quantification, joint analysis of spikes and bursts to analyze the spike waveform compositions of bursts, assessment methods for network synchronization, and computational simulations of synapses and neuronal networks.

## 12.1 Introduction to human stem cells and neuronal differentiation

Human pluripotent stem cells (hPSCs) are defined by their capacity to self-renew and differentiate into derivatives of all three germ layers: the endoderm, mesoderm, and ectoderm. These cells also follow *in vivo* developmental principles and can be directed to differentiate into all cell types in the human body. hPSCs are divided into human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hIPSCs). hESCs are derived from the inner cell mass of blastocyst-stage embryos (Thomson et al. 1998), whereas hIPSCs are derived from somatic cells using specific transcription factors, such as *Oct4*, *Sox2*, *klf4*, *c-myc*, *Nanog*, and *lin28*, to induce the pluripotent state (Takahashi et al. 2007; Yu et al. 2007). Thus, hIPSCs enable the generation of a variety of cell types that represent an individual's unique genetic background, including known genetic cause(s) of disease(s).

Neural differentiation of hPSCs was first described in 2001 (Carpenter et al. 2001; Reubinoff et al. 2001). These protocols were based on the embryoid body (EB) formation step prior to adherent cell culturing in neural differentiation-inducing media. Since then, a vast number of differentiation protocols directing neural development from hPSCs have been published, many of which use the principle of EB formation (Erceg et al. 2009; Zirra et al. 2016). EBs are 3D cell aggregates that produce a mixed population of differentiating cells, including neural cells. In adherent culture conditions, the cells differentiating towards neural lineage (i.e., neural precursor cells (NPCs)) produce radially arranged structures called rosettes, mimicking neural tube formation *in vivo* (Fig. 1.1). Rosettes can be selectively isolated for further culturing in order to enhance the purity of the produced neural population (Muratore et al. 2014). The 3D aggregates can also be formed directly in neural induction media. In this case, the aggregates are considered to produce mostly cells committed to the neural lineage and are thus termed neurospheres (Nat et al. 2007). Lately, culturing methods for the creation of human brain organoids, which at least partially mimic human brain tissue, have been developed (Kawada et al. 2017; Kelava et al. 2016). Further development of differentiation methods has enabled capturing of cells in the neuroepithelial or NPC stage, where they are committed to neural lineage but can be still efficiently expanded and further differentiated into variety of different neural cells (Brafman et al. 2013; Falk et al. 2012). In 2009, an efficient neuronal differentiation method, fully based on adherent cell culture conditions, was introduced (Chambers et al. 2009). The key element in this protocol was the inhibition of BMP signaling with Noggin and Activin/Nodal/transforming growth factor beta (TGFβ) signaling by small molecule SB431542. The aim was to inhibit the formation of endodermal and mesodermal derivatives in the early stage of

differentiation. This principle, termed “dual-SMAD inhibition”, has since become extensively utilized in neuronal differentiation protocols. Current neuronal differentiation protocols often combine different culturing methods with temporally defined combinations of patterning factors and growth factors, aiming for the production of increasingly specified neuronal populations (Kirkeby et al. 2012; Maury et al. 2015; Paşca et al. 2015). Prolonged neural differentiation of hPSCs induces a neuroglial switch in the population (Lappalainen et al. 2010; Paavilainen et al. 2018). The differentiation capacity of radial glial NPCs shifts towards astrocytes and oligodendrocytes, representing *in vivo* development, where the generation of glial cells is initiated later than that of neurons (Martynoga et al. 2012) (Fig. 12.1). Initially, hPSC-derived glial cells (especially astrocytes) were mainly generated as side products of neuronal differentiation, but later, targeted differentiation protocols were developed for also glial cell types (Douvaras et al. 2014; Krencik et al. 2011; Pawlowski et al. 2017; Roybon et al. 2013).

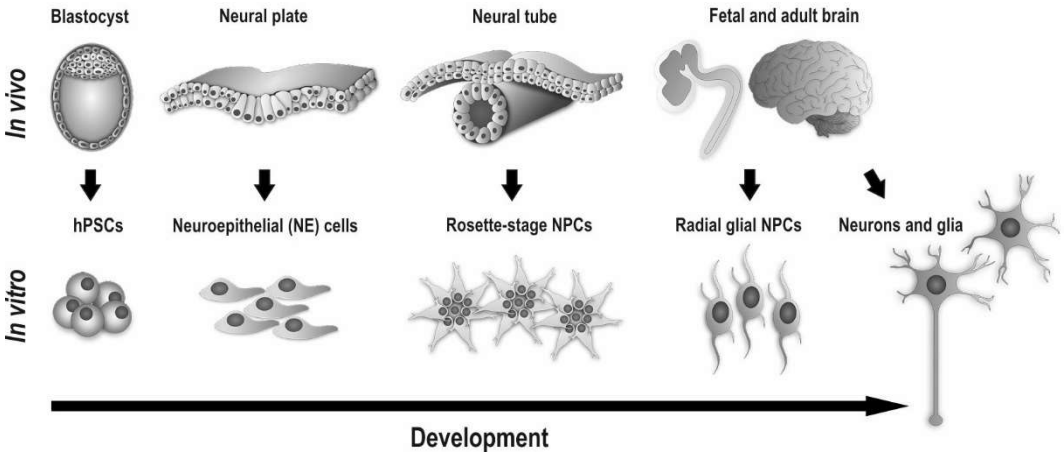


Fig. 12.1. Neural development *in vivo* and *in vitro*. Neural tube formation induces the transition of neuroepithelial cells into NPCs. NPCs further acquire the phenotype of radial glial precursor cells and give rise to differentiating neurons in the central nervous system through asymmetric cell division. They also form long radial processes to the outer surface of the neural tube, guiding the migration of newly born NPCs and neurons. (Martynoga et al. 2012). Although all the developmental stages are often not separated *in vitro*, neural differentiation typically proceeds from neuroepithelial cells to NPCs, which is followed by final maturation into neurons and glial cells. (Reprinted from (Hyysalo 2017a; adapted from Mertens et al. 2016) with permission from academic dissertation.)

Recently, hPSC-derived neural cells *in vitro* have been exploited in developmental and toxicological studies, drug discovery and disease modeling. Traditionally, these studies have been performed using

animal models or primary cell cultures due to the inaccessibility and technical limitations concerning the use of primary human cells and tissues (Markou et al. 2009) (Fig. 12.2). Although models based on animals (mainly rodents) are important and vastly utilized tools, these models have some drawbacks, and fundamental species-specific differences hinder the extrapolation of results from rodents to humans. At the genomic level, for example, despite the large number of orthologous genes between human and mouse, there are human-specific genes or functions of genes associated with various diseases (Miller et al. 2010). At the cellular level, differences between human and mouse brains are found in, for example, glial cell populations, as characteristics such as the astrocyte to neuron ratio and astrocyte complexity are both distinctly higher in the human brain (Nedergaard et al. 2003). The species also vary in several aspects of embryonic development, and human neurons require considerably longer morphological and electrophysiological maturation times than their mouse counterparts, both *in vivo* and *in vitro* (Suzuki and Vanderhaeghen 2015). Thus, although many neurodegenerative diseases can be modeled in rodents, pharmacological responses can be strikingly different between humans and rodents (Athauda and Foltynie, 2015). Furthermore, the polygenic and multifactorial nature of many diseases prevents the replication of an entire disease phenotype in animal models.

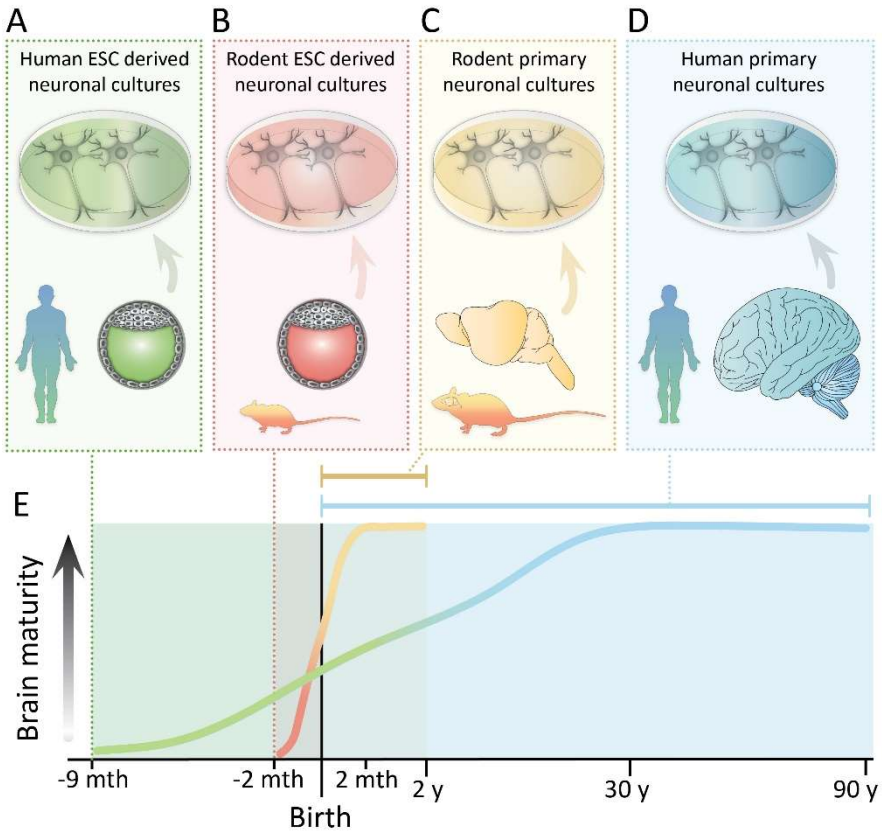

Fig. 12.2. Neuronal *in vitro* cultures. Traditionally, neuronal *in vitro* cultures have been created from rodent (typically mouse or rat) (C) embryonic or post-natal brain tissue and are called primary cultures, which are the most vastly used and explored cultures. (B) Mouse embryonic stem cells (mESCs) have also been used to create rodent neuronal cultures. (D) Human neuronal cultures have been created from aborted human fetal brain tissue, adult human brain after surgical operations, or postmortem tissue. (A) Since 2001, human neuronal cultures have been established from human ESCs. More recently, similar type of human neuronal cultures have been established from human IPSCs. Even though *in vitro* neuronal cultures in principle are considered similar to *in vivo* cultures, there are many source-dependent intrinsic properties that make them different from one another. Species-dependent characteristics of brain development (e.g., time scale, cellular composition, structural organization, maturation stage at birth, maturation time after birth) influence the properties of *in vitro* cultures. In (E), time dependent human brain development and maturation is shown with green to blue line whereas for rat it is shown with red to orange line. Expected lifespan is shown with blue line for humans and with orange line for rat. Time line (x-axis) is not in scale. For these species-dependent characteristics, ESC-derived neuronal cultures are quite different between humans and rodents. For example, the differentiation of all major neural cell types (neurons, astrocytes and oligodendrocytes) is much faster in mouse ESC-derived than in their human counterparts. As more and more work is performed with human PSC-derived neuronal cultures, these results are often compared to rodent primary neuronal cultures. In this case, in addition to species-dependent differences, the developmental stage of the cells is also very different. This difference is a very important aspect to consider, especially in functional studies (Odawara et al., 2014)

In conclusion, hPSC-derived, neural cell-based *in vitro* models hold remarkable potential for extending our knowledge of the developmental bases of human evolution and disease and developing therapeutic applications. While human *in vitro* models hold certain benefits over animal-based models, some challenges still exist. These challenges include, for example, a lack of understanding of comprehensive maturation processes of differentiated cells and thus relevant modeling of late-onset neurodegenerative diseases (Avior et al. 2016). In addition, novel research techniques will be developed to overcome these hindrances in the future.

## 12.2 Functionality of hPSC-derived neuronal networks

Typically, *in vitro* cell cultures are studied with various highly advanced techniques, ranging from genetic screenings to metabolomics. In addition, functional analysis is a specific requirement in neuronal cell culture studies, although this requirement has been addressed less frequently than other characteristics. Recently, the importance of functional analysis has been better recognized. Functional characterization includes variety of methods, such as patch clamp, fast calcium imaging, and microelectrode arrays (MEAs). Of these methods, MEAs are an interesting tool as they provide repeatable, non-invasive, network-level assays that can be performed in high throughput. Since 2009, hPSC-derived neuronal networks have been shown to develop spontaneous activity in MEAs (Heikkilä et al. 2009). Thereafter, these cells have been proven to be suitable for functional neurotoxicity studies (Ylä-Outinen et al. 2010). Importantly, hIPSC-derived neuronal cells are able to develop into spontaneously active networks that are similar to their hESC counterparts (Hyysalo et al. 2017c; Schutte et al. 2018; Toivanen et al. 2017). Recent studies, however, have shown that hPSC-derived networks develop and express unique functionalities that are not directly comparable to those in rodent primary cultures (Mäkinen et al. 2018, Paavilainen et al. 2018).

## 12.3 Human stem cell-derived neuronal networks: from 2D to 3D cultures

The traditional way to culture neuronal networks is 2D cultures on the bottom of a culture well (Fig. 12.3A). This approach is practical for functional studies, since MEAs can be easily embedded on the well bottom, and cells are easy to observe in monolayer or thin cultures. In addition, in 2D cultures, neurons are randomly oriented and form free connections between each other. This method has been regarded as a standard approach in rodent primary cultures and in hPSC-derived neuronal cells (Heikkilä et al. 2009; Lappalainen et al. 2010; Odawara et al. 2014; Ylä-Outinen et al. 2010).

During brain development, neuronal cells are guided by chemical and structural cues and form highly organized 3D structures *in vivo*. To mimic better this situation *in vitro*, both structural and 3D support might need to be provided (Hopkins et al. 2015). These goals can be achieved by structural guidance devices that provide cues and limited growth areas for the cells (Fig. 12.3B) or by 3D cell culture scaffolds (Fig. 12.4C). Structural guidance devices (Fig. 12.3B, more closely described in Section 12.4) can be fabricated in many ways, e.g., they can be microfluidistic devices, oriented nano/micro

scale fibers, grooves, or chemical patterns. All these devices can guide cell or neurite migration or limit cell growth area, providing more organized and structured 2D neuronal networks. For example, these devices can guide unidirectional axonal growth, providing a model for axon bundles (Hyysalo et al. 2017b; Park et al. 2006). In addition to these guidance devices, models that more closely resemble *in vivo* structures can be created with 3D culture systems (Fig. 12.3C, more closely described in Section 12.5). These models include organoids, hydrogel scaffolds, and engineered structural 3D scaffolds (Hopkins et al. 2015; Kawada et al.2017; Kelava et al. 2016; Shuler et al. 2014). Organoids or spheroids are cell aggregates that are formed spontaneously from stem cell-derived cell masses during differentiation. Cells, cell to cell interactions, and cell to extracellular matrix (ECM) interactions thus form 3D structures. So far, the most advanced brain organoids have been shown to contain cortical structures, mimicking the human cortex. However, the uncontrolled growth of brain organoids creates a challenge for their use in studies and standard analysis (Kelava et al. 2016). Hydrogel scaffolds provide practical scaffolds for hPSC-derived neuronal cells since their elastic and mechanical properties mimic native brain ECM. Another way to produce 3D structures is to mimic guidance cues in 3D. With 3D printing, molding or additive method structures, which are made of harder material than hydrogel scaffolds, can be engineered (Hopkins et al. 2015). These scaffolds provide growth cues but usually lack cell-ECM interactions.

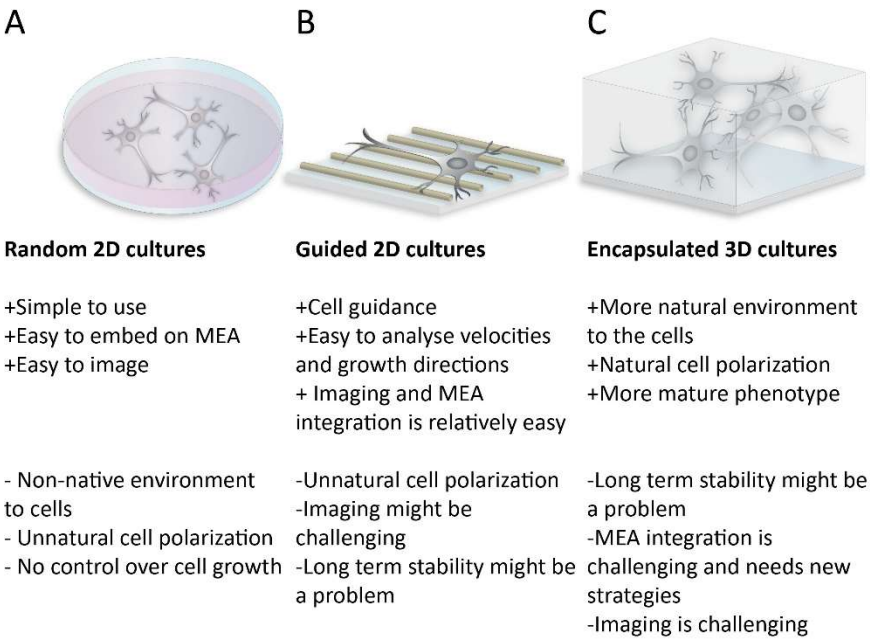


Fig. 12.3. Different strategies in the formation of stem cell-derived neuronal cultures. The pros (+) and cons (-) of each culture type are listed below. (A) Normal random 2D cultures are the most commonly used culture format and are suitable for many applications; however, they have some drawbacks. (B) Guided 2D devices are proposed for cell or axon guidance and allow different analysis

strategies since cells are unidirectional and cells or cell parts (like axons) can possibly be restricted. (C) Encapsulated 3D cell cultures, where cells are growing inside a biomaterial scaffold or form organoids, provide a more natural environment for the cells, but at the same time, they lead to more challenges in functional and imaging analyses.

### 12.4 Applications of 2D human stem cell-derived neuronal networks

Currently, human neuronal networks are increasingly used as *in vitro* models and their functional measurements give unique information about human neural development, how their properties compare to animal cells, and human diseases and disorders (Canals et al. 2015; Odawara et al. 2014, Seidel et al. 2017; Ylä-Outinen et al. 2010). Particularly, their usability has been recognized in the areas of (developmental) neurotoxicity, disease modeling and drug screening (Canals et al. 2015; Hopkins et al. 2015; Johnstone et al. 2010; Pamies et al. 2016; Tukker et al. 2016). Lately, their usability for studying very early human neuronal network development has been proven (Mäkinen et al. 2018).

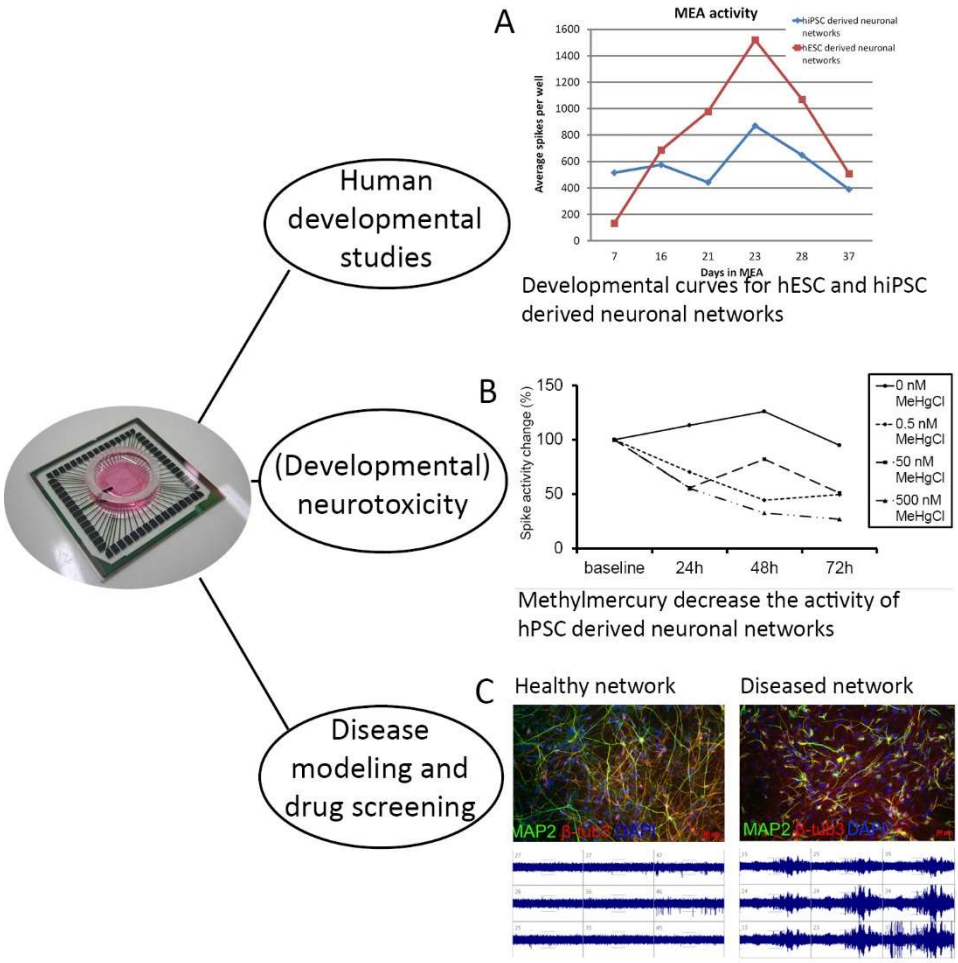


Fig. 12.4. Application areas for human pluripotent stem cell (hPSC)-derived neuronal networks on MEA. (A) HPSCs offer a tool to study species-specific development while also providing new important basic research information about human cells. Activity in MEA is shown for hESC and hIPSC-derived neuronal networks from culturing days 7 to 37. The red line represents hESC-derived neuronal networks, and the blue line represents hIPSC-derived neuronal networks. (B) Neurotoxicological platforms offer human cell-based methods to study toxicity at a functional level. An activity curve from hPSC-derived neuronal networks exposed to low doses of methyl mercury is shown. Even at low concentrations, a clear effect on activity is seen in MEA. (Reprinted from (Ylä-Outinen et al. 2010) with permission.) (C) Disease modeling in dish and *in vitro* drug screening have experienced a new era with hIPSC technology. hIPSCs can be used to reproduce the diseased phenotype in dish. Healthy neuronal network (left side) and diseased cells (right side, from patient suffering from epilepsy with genetic background) are cultured in MEA. Both networks express normal neuronal markers in immunostaining, but clearly, abnormal MEA activity is seen in the diseased networks. (Unpublished data courtesy of Meeri Mäkinen (NeuroGroup, BioMediTech and

Faculty of Medicine and Life Sciences, University of Tampere) and Riikka Äänismaa (Research Programs Unit, Molecular Neurology, Biomedicum-Helsinki, University of Helsinki, Helsinki).)

### *12.4.1 Developmental modeling*

Human neuronal development can be studied *in vitro* using hPSC-derived neuronal networks and using MEA as a continuous, network-level functionality measurement tool (Broccoli et al. 2014) (Fig. 12.4A). Human cell-based *in vitro* models provide unique data on human early development, which is otherwise impossible to study in detail. It has been found that human neuronal networks form similar networks to rodent derivatives (Heikkilä et al. 2009) but still possess unique properties (Mäkinen et al. 2018). The development and maturation of human neuronal networks takes much longer than that of rodent cultures (Odawara et al. 2016b, Paavilainen et al. 2018). In addition, human networks have some prominent features. One of these features is network variability (Mäkinen et al. 2018; Paavilainen et al. 2018, Ylä-Outinen et al. 2010). Human PSC-derived neuronal networks show cell to cell variability at their differentiation stage, specifically in GABA system development or cell composition (Mäkinen et al. 2018). Additionally, batch to batch and cell line to cell line variations influence the "neurality" or purity of the produced cultures (Toivonen et al. 2013). This variability naturally influences functionality, causing differences in activity patterns, the number of active electrodes, the time scale of functional maturation, and burst parameters, which prevents their direct comparison to primary rodent cultures (Mäkinen et al. 2018; Paavilainen et al. 2018). These variations cause new challenges to MEA analysis (discussed more closely later). All in all, human neuronal networks develop into bursting phase networks (Heikkilä et al. 2009; Paavilainen et al. 2018) and also develop network bursting activity at later time points (Odawara et al. 2016b). They respond to basic pharmacological treatments mostly as expected according to rodent data (Heikkilä et al. 2009; Odawara et al. 2016a, Paavilainen et al. 2018) but still possess specific characteristics, such as varying response to bicuculline (Mäkinen et al. 2018). In these cultures, bicuculline can have no effect, a silencing effect or an activating effect (Heikkilä et al. 2009; Odawara et al. 2016b, Paavilainen et al. 2018). Most likely, this difference in effects is caused by immaturity and different maturation stages, which are even found in the same populations (Mäkinen et al. 2018).

### *12.4.2 Neurodevelopmental toxicity and neurotoxicological applications*

Human neuronal tissue is unique, e.g., drug responses can differ from those seen in animal tissues, and thus, there is a huge need for toxicological platforms built on human neuronal cells (Fritsche et

al. 2018; Johnstone et al. 2010; Kasteel and Westerink 2017; Tukker et al. 2016). HPSC-derived neuronal networks on MEA platforms are an excellent approach for these studies. In particular, human developmental neurotoxicity is an area that is otherwise challenging to study (Bal-Price et al. 2010; Fritsche et al. 2018). Nevertheless, the stabilization of neuronal networks and minimization of variations are requirements for reliable testing platforms; however, in the case of human cultures, these goals have been challenging to achieve. As previously mentioned, hPSC-derived cells are commonly more immature than primary rodent cortical cells (Kasteel and Westerink 2017), which leads to differences in typical MEA signaling, and in some cases, in drug responses (Heikkilä et al. 2009; Kasteel and Westerink 2017; Odawara et al. 2014). Thus, both experimental setups (including analysis time points) and analysis (for example, burst detection) need to be optimized (Kapucu et al. 2012; Ylä-Outinen et al. 2010). In simplest terms, this optimization means an optimized culture period that is much longer than that of primary rodent cells (Heikkilä et al. 2009; Odawara et al. 2016b; Ylä-Outinen et al. 2010). Moreover, cell culture medium supplements, growth factors, cell density, and culturing conditions might also need to be considered (Kreutzer et al. 2012; Kreutzer et al. 2017; Paavilainen et al. 2018). These variabilities also increase the need for special requirements and solutions in MEA analysis, which we will discuss later in this Chapter.

Despite all these challenges, human neural cell-based MEA platforms have already been applied for testing the known toxicity of methylmercury (Ylä-Outinen et al. 2010). We demonstrated that MEA is a sensitive method, showing sub-acute toxic effects prior to phenotypical changes (Fig. 12.4B). In addition, the effect of well-known sodium channel blocker tetrodotoxin (TTX) was compared between human and rodent cultures on MEA (Kasteel and Westerink 2017). This study showed that interspecies differences are low in the case of TTX and that this information can be used for risk assessment purposes. These rare cases prove that human networks on MEA are applicable to toxicological studies, but there is a long way to go for robust, high-throughput human cell-based platforms in this field.

### *12.4.3 Disease modeling and drug screening*

HiPSCs provide a new way to study diseases *in vitro*. Cells carrying the genetic mutation that causes symptoms in the patient can be first induced into stem cells and then further differentiated into neuronal cells and cultured on MEA plates (Canals et al. 2015; Avior et al. 2016; Odawara et al. 2014). Using this strategy, it is possible to identify abnormal signaling in patient cells. For example,

cells from patients who are suffering severe epilepsy with a genetic background can be cultured in an MEA dish, and the signaling of these cells shows abnormal, seizure-like activity (Fig. 12.4C).

There is also a great need for drug screening platforms. HPSC-derived cells, especially induced pluripotent derivatives, create new possibilities for patient- and disease-specific drug screening *in vitro* (Avior et al. 2016. With hIPSC technology, cell lines from patients with various neurological diseases (including Amyotrophic lateral sclerosis, Alzheimer disease and Parkinson disease) have been established, differentiated towards neuronal cells and studied for drug screening purposes (Kasteel and Westerink 2017; Lee et al. 2017; Xie et al. 2017; Zirra et al. 2016). Still, only a limited number of these cells have been cultured on MEA platforms and studied at a functional network level. Odawara et al. (2014) demonstrated the usability of human neuronal networks on MEA as suitable for this purpose. However, cell maturation took several months, thus decreasing the usability and increasing the costs of human cell-based platforms for drug screening purposes. There is still an unmet need for higher throughput, more standardized platforms and overall better quality of drug screening and toxicity platforms (Johnstone et al. 2010; Kasteel and Westerink 2017).

## 12.5 Applications and advances of guidance devices for human stem cell-derived neuronal networks

As discussed earlier, *in vivo* brain tissue is highly organized, and for some *in vitro* platform purposes, it is important to try to mimic this organization. The organization and alignment of neuronal cell growth can be achieved with chemical or mechanical cues, patterns or topographical cues (Fig. 12.5). Axonal guidance or separation of somas and neuronal cell processes can be achieved with these cues. MEA analysis usually benefits from guidance, since signaling is more organized and homogenous than in freely formed networks (Toivanen et al. 2017). This characteristic is important, especially in a human neuronal network context, where signaling might be variable and only some of the cells are participating in detectable MEA signaling.

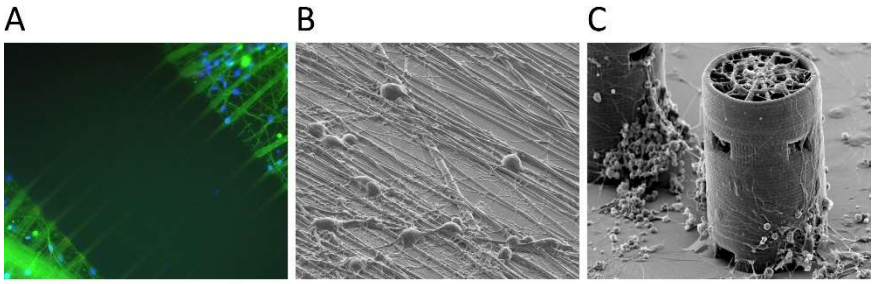


Fig. 12.5. Strategies to guide neuronal cells or axons. (A) Microfluidistic restriction devices are widely used to limit cell growth or restrict the axons from cell somas. Here, microfluidistic tunnels are used to separate two neuronal networks (labeled against MAP-2 in green and DAPI in blue) from each other, allowing only axons to migrate through tunnels. (Image courtesy of Anssi Pelkonen (NeuroGroup, BioMediTech and Faculty of Medicine and Life Sciences, University of Tampere).) (B) Fibers and topographical cues are used to guide and orientate cell growth. Here, hPSC-derived neuronal cells are growing along submicron-scale electrospun fibers. (C) Different microfabrication techniques can be used to build guidance scaffolds for neurons. Here, two-photon polymerized structures are used as a growth scaffold for hPSC-derived neuronal networks. (Image courtesy of Sanna Turunen (BioMediTech and Faculty of Biomedical Sciences and Engineering, Tampere University of Technology) and Tiina Joki (NeuroGroup, BioMediTech and Faculty of Medicine and Life Sciences, University of Tampere).)

Specifically, for example, axonal velocity experiments need some structural guidance. Microfluidic devices provide a means to guide, restrict and isolate the growth of neuronal cells or cell parts, such as somas or axons. With these devices, a variety of different *in vitro* models can be created, for example, to study myelination, schizophrenia or epilepsy in a way that better mimics *in vivo* structures. It has also been shown that a limited growth area increases hPSC-derived neuronal network activity (Kreutzer et al. 2012; Toivanen et al. 2017). A limited growth area increases the success rate of network formation and detected signaling (Kreutzer et al. 2012), whereas a limited cell culture liquid volume seems to increase the chances of detecting activity in neuronal networks (Toivanen et al. 2017). Various 2D guidance devices have been used with rodent neuronal cells, but there are fewer reports on utilizing them with human cells, indicating that there may be species-related challenges in using human neuronal cells with these devices.

## 12.6 Applications and advances of 3D human stem cell-derived neuronal networks

Neuronal cells are shown to mature into a more *in vivo*-like morphology in hydrogel scaffolds (Ylä-Outinen et al. 2014) or in brain organoids (Kelava et al. 2016). Thus, 3D cultures might be beneficial when the aim is to create mature cells and *in vivo* mimicking platforms (Hopkins et al. 2015). Plenty of different materials have been tested for the 3D growth of human neuronal networks (Edgar et al. 2017; Karvinen et al. 2018; Koivisto et al. 2017; Ylä-Outinen et al. 2014) (Fig. 12.6). The first challenge in 3D cultures is choosing the right materials. A suitable 3D scaffold material for *in vitro* models should be cytocompatible, should offer structural and chemical support so that the neuronal cells can spread and form networks, and should be stable enough to give cells sufficient time to mature (Hopkins et al. 2015; Ylä-Outinen et al. 2014). Studies have shown that with good supportive materials, human neuronal cells can form dense interconnected networks in 3D (Koivisto et al. 2017; Ylä-Outinen et al. 2014) (Fig. 12.6). The needed cell amount is, however, huge for 3D cultures compared to traditional 2D cultures, making it a limiting factor in some cases. Another important limiting factor is challenges in the analysis of 3D networks. Imaging of 3D networks is more difficult, requires specific equipment and is much slower than 2D imaging. Further, 3D functional measurements have not yet been reported.

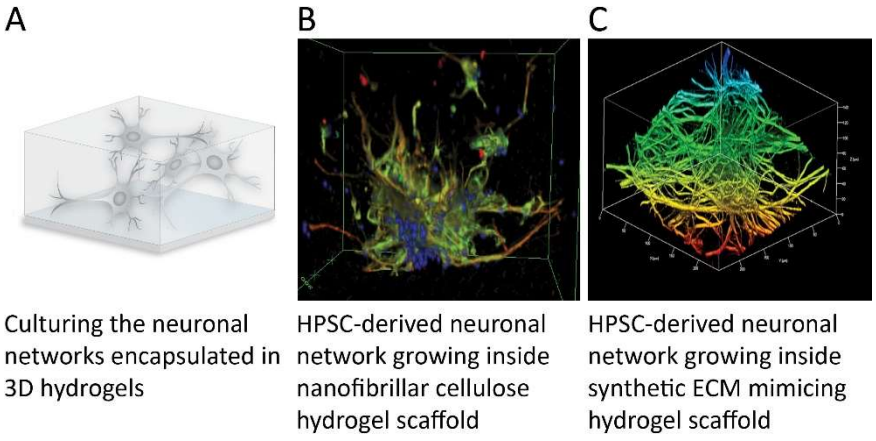


Fig. 12.6. 3D culturing of neuronal cells. (A) Concept of 3D culture. Network formation of hPSC-derived neuronal cells (B) on the nanofibrous cellulose material Growdex® (Image courtesy of Tiina Joki, Laura Ylä-Outinen, Lauri Paasonen, Susanna Narkilahti and UPM Biochemicals.) and (C) on synthetic ECM mimicking PuraMatrix® (Image courtesy of Tiina Joki.).

Little is known about the electrophysiological characteristics of 3D cultured human neuronal networks. Functional activity has been detected with planar 2D MEAs beneath 3D encapsulated networks (Ylä-Outinen et al. 2014). There is a clear need for electrophysiological measurements in 3D environments for more complex, *in vivo*-like developmental, toxicity and disease modeling (Hopkins et al. 2015). This goal is, however, very challenging; how do we stabilize the network in 3D? How do we realize MEA electrodes in 3D? What is sufficient spatial resolution in 3D cultures and how do we ensure sufficient neuron density in 3D so that most electrodes can detect signals? In Table 12.1, some potential modalities are listed to overcome these challenges. For electrophysiological measurements, MEA electrodes used in *in vivo* measurements can be applied *in vitro* to obtain data within hydrogel scaffolds. Calcium imaging has already been applied in 3D imaging but has only been used for rodent networks (Broguiere et al. 2016). Additionally, patch clamping from 3D neuronal networks is possible but challenging (Simão et al. 2015; Xu et al. 2009). All of these strategies are applicable to human neuronal networks, and with intensive optimization, these strategies could be the basis of new, powerful human cell *in vitro* platforms.

Table 12.1. Potential modalities for functional electrophysiological measurements in 3D.

| | 3D flexi-MEA | 3D Utah/Michigan array type MEAs | Calcium imaging | Patch clamp |
|---|---|---|---|---|
| Scale | network level | network level | single cell/network level | single cell level |
| Time-scalability | can be placed after cell seeding and immobilized during the | can be placed after cell seeding and immobilized during the whole culturing period | only used for one time point | only used for one time point |

| | | | | |
|---|---|---|---|---|
| | whole culturing period | | | |
| | allows long term measurements | allows long term measurements | end-point analysis | end-point analysis |
| Sensitivity | low detection rate | low detection rate | high detection rate | high detection rate |
| Spatial resolution | limited spatial access | good spatial access | good spatial access (depending on microscope) | very limited spatial access |
| | low/mid spatial resolution | low/mid spatial resolution | good spatial resolution | good spatial resolution |
| Temporal resolution | very good temporal resolution | very good temporal resolution | low temporal resolution | good temporal resolution |

## 12.7 Electrophysiological signal analysis and computational modeling

As we have seen earlier in this Chapter, the maturation process of hPSCs to neuronal cells has many degrees of freedom. Variations in electrical activity during maturation and network formation and in the developed neuronal networks in general are larger than those in primary cultures (Fig. 12.2). Moreover, the encountered signal characteristics are often different from those encountered with acute brain slices or dissociated cultures of animal neuronal cells of non-stem cell origin. This difference calls for the development of new, more adaptive signal-based electrophysiological signal analysis methods. The main philosophy of the work has been to develop methods that tune themselves

based on signal measurements, i.e., according to neuronal activity, and rely minimally (or preferably not at all) on a priori set parameters.

In addition to the need for new methods, a hypothesis in our work has been that MEA field potential measurements of neuronal activity carry more information than what can be assessed by the commonly used spike and burst analysis methods alone. With this need and hypothesis in mind, the following signal analysis tasks have been investigated and advanced:

1. Neuronal action potential detection
2. Adaptive burst detection and analysis
3. Joint analysis of bursts and sorted spikes
4. Network connectivity/synchronization analysis
5. Computational modeling and simulation

These MEA signal analysis approaches and the resulting new information are collected in Fig. 12.7.

Neuronal action potential spike detection is the basis of all analysis methods operating on spikes. To facilitate cell culture- and noise condition-optimized spike detection, an objective signal analysis-based thresholding method was developed. Bursts are generally considered to indicate network activity. For a wide range of developing hPSC-derived neurons and other neuronal systems with greatly varying spiking statistics, the burst detection algorithm needs to be based on signal dynamics, and such methods were indeed developed. To extract more information from the internal dynamics of network bursts, a method for the joint analysis of bursts and spike waveforms was developed to analyze the spike type compositions of bursts. One way to assess neuronal networks is through the mathematical complexity of MEA signals; for this approach, a spectral entropy-based method was modified for MEA signals, and demonstrated to be beneficial. Finally, to assess the development of functional neuronal connectivity and network synchrony, the correlated spectral entropy method (CorSE) was developed. Most of these developments have been described by Kapucu (2016b).

Computational modeling can be considered as a signal analysis method in the sense that it aims to provide explanatory models of neuronal network behavior. In addition, models can be used to build hypotheses of network behavior. In simulations, network models and their resulting MEA signals are known, so they provide excellent data for the validation of signal analysis methods. On the other hand, computational models can be used to predict the effects chemical alterations or exposure on neuronal networks and the effects of diseases.

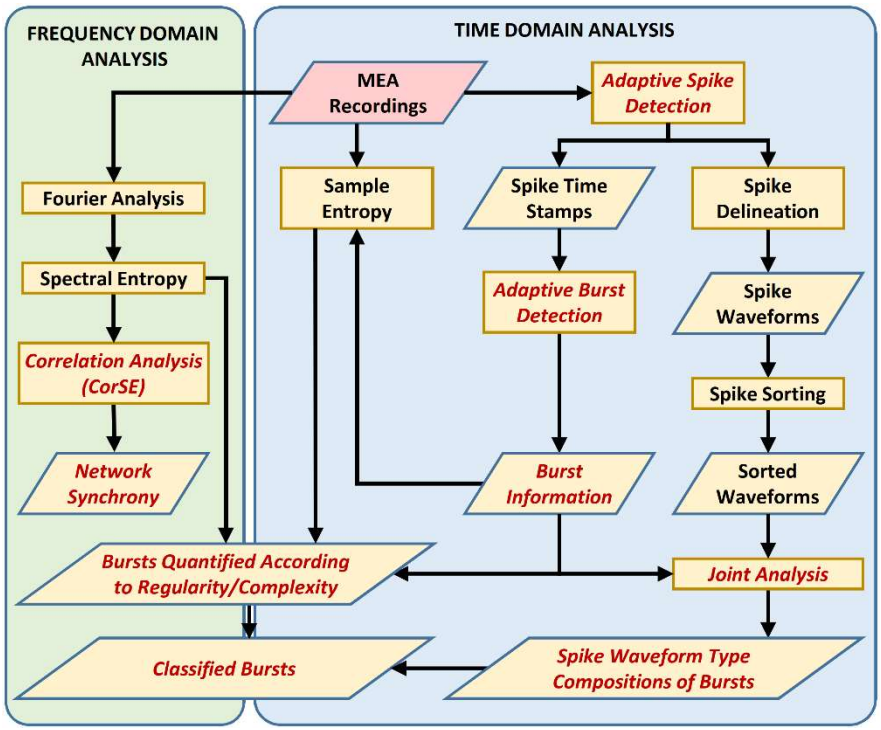


Fig. 12.7. MEA recording analysis methods (rectangles) and data (parallelograms) considered here. Only the most important data are shown. The developed methods and novel data are indicated by red text. All shown inputs to the methods are not necessarily always used, but different data can be used by the same method block in different analyses. (Adapted from (Kapucu 2016b).)

### *12.7.1 Adaptive thresholding-based spike detection*

Spike detection (Lewicki 1998; Wilson and Emerson 2002) is still often performed by mere thresholding: any signal reaching above a threshold is interpreted as a spike. Thresholding is computationally very efficient and can be performed online during measurement. Commonly, the standard deviation (STD) of measurement noise or STD of a measured signal is estimated, and the threshold is set to a multiple (e.g., 4 to 8 times) of the STD. Some thresholding methods use only either negative or positive thresholds, and thus detect only negative or positive spikes, whereas other methods utilize symmetric positive and negative thresholds to capture spikes of both polarities. In general, thresholds are set by convention by an expert operator, assessing measurements visually and aiming to maximize the number of detected real neuronal spikes while minimizing the number of spurious, i.e., false spikes detected due to noise. During measurement, the actual number of spurious spikes remains unknown. Spuriousness is not considered in the analysis, but all detected spikes are taken as true spikes. Spike waveform analysis might reveal spurious spikes, but it is usually not utilized. Additionally, thresholds set this way are always subjective.

We developed a simple objective measure for this approach. The working hypothesis of the developed spike detection method is that contributions from noise and spikes should be identifiable in spike count histograms, given that there is a sufficient number of spikes reaching sufficiently beyond the noise. The algorithm illustrated in Fig. 12.8A realizes this principle (Tanskanen et al. 2016). First, a spike count histogram is formed. To enhance the potentially useful features of the histogram, a smoothed gradient of the histogram is calculated. Next, the extrema of the smoothed gradient are found. The local minimum closest to the global maximum, appearing at an amplitude smaller than that of the global maximum, is taken as the negative threshold for spike detection. The positive threshold is found at the local maximum closest to global minimum appearing at an amplitude larger than that of the global maximum (Fig. 12.8A). Finding the thresholds for an *in vitro* measurement from human brain tissue samples (Roopun et al. 2010) is shown in Fig. 12.8B, and the signal with the found thresholds is shown in Fig. 12.8C.

Matlab (MathWorks, Natick, MA) implementation of the basic method is freely available (Tanskanen 2017). Planned future updates include adaptive threshold setting in a running window and baseline drift suppression. Traditional thresholding methods fail if the spiking is too dense compared to the signal sampling frequency; dense spiking results in the thresholds being set too high. The proposed method may also be beneficial in these cases, although the biological relevance of the spike detection results is yet to be demonstrated.

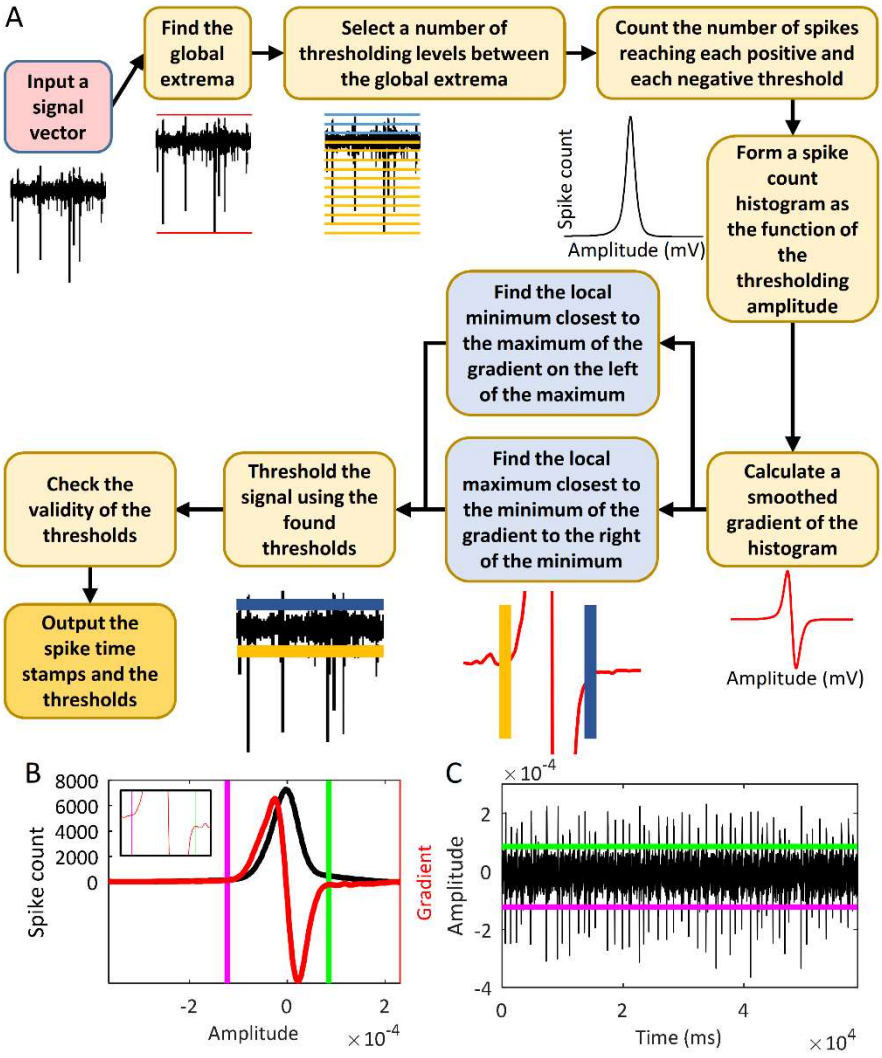


Fig. 12.8. (A) The proposed thresholding algorithm. (B) Spike count histogram (black), smoothed gradient of the histogram (red), and the found negative (magenta) and positive thresholds (green), with a detailed view of the gradient features in the inset. The gradient axis spans from -200 to 200. (C) An exemplary measurement from *in vitro* human brain tissue (black), and the automatically found thresholds. (©2016 IEEE. Reprinted, with permission, from (Tanskanen et al. 2016). Adaptations for clarity only.)

### *12.7.2 Adaptive burst detection and burst analysis*

For conventional rodent brain slices and primary neuronal cultures, in general, spike statistics remain quite stable during experiments. Traditional spike signal analysis methods with a priori set (often *ad hoc* selected) parameters work fine with such cultures. Interspike interval (ISI) is a simple and computationally efficient metric. Bursts are typically detected (Chiappalone et al. 2005; Wagenaar et al. 2006; Mazzoni et al. 2007) by the fulfillment of at least two conditions: a fixed minimum number

of spikes and a fixed maximum ISI in a burst. ISI histograms have been employed to assess general firing characteristics, e.g., by Christodoulou and Bugmann (2001). Since ISI histograms are challenging to read in their raw form, logarithmic scale ISI histograms have been used. Sometimes, they can reveal the differences between firing characteristics of individual and burst spikes (Selinger et al. 2007; Pasquale et al. 2010). In addition, several adaptive methods have been proposed for burst detection, such as the method by Pasquale et al. (2010), and they can also be applicable in analyzing measurements from hPSC-derived neuronal cultures. Recently, eight adaptive methods were thoroughly reviewed and compared by Cotterill et al. (2016). Adaptive methods are crucially important for detecting bursts recorded from dynamic cultures.

hPSC-derived neuronal cells and networks develop and evolve during culturing, and their spiking statistics vary greatly during this time (as discussed earlier in detail). Some studies have shown that such neuronal cultures exhibit not only very dynamic firing statistics but also highly varying bursting behavior (Heikkilä et al. 2009), with bursts lasting from milliseconds to seconds. In addition, bursts formed by a few or tens of spikes are seen frequently in these networks (Heikkilä et al. 2009; Kapucu et al. 2012) (see, Fig. 12.9). Thus, in the analysis, methods based on a priori fixed parameters tend to fail, and burst detection should be done based on the signals themselves.

To overcome the limitations of previous burst detection methods and successfully detect bursts under dynamic firing statistics, we proposed a general analysis framework based on the cumulative moving average (CMA) of ISI histograms (Kapucu et al. 2012) (Fig. 12.9). The general characteristics of bursting activity can be observed as a peak and followed by a gradual decay in the ISI histogram, which can be associated with a Poisson distribution with a signal dependent mean (Christodoulou and Bugmann 2001; Chen et al. 2009). Skewness is a measure of the asymmetry of a distribution. Skewness is an important feature of ISI histograms and can be utilized to detect bursts with different ISI distributions. The CMA of an ISI histogram shows the overall trend of the ISI histogram and allows the definition of an ISI value that marks a critical point in the histogram. In the CMA-based method described here, the maximum of the CMA curve is found, and the skewness of the ISI histogram is estimated. The ISI threshold for burst detection is set at the ISI corresponding to $\alpha$ times the maximum of the CMA curve (Fig. 12.9A). See (Kapucu et al. 2012) for a possible mapping between $\alpha$ and skewness. Similarly, an ISI threshold can be defined for burst relate spikes (Fig. 12.9B-D), i.e., for the pre-burst spikes and the spikes in the burst tails, by defining another fraction ($\alpha_2$) of the maximum CMA value.

The CMA-based burst detection method can be applied on single channel recordings (Kapucu et al. 2012) or tuned to network-wide spiking characteristics, which can provide more stable and comparable measures, e.g., for long term studies (Välkki et al. 2017). After delineating the bursts, burst cut-outs and burst statistics can be obtained for further analysis. The detected bursts can be quantified, e.g., by conventional parameters and metrics, such as burst duration and frequency and the number of spikes in bursts, by the developed entropy-based algorithms (Kapucu et al. 2015b) and by assessing the spike type compositions of the bursts (Kapucu et al. 2016d) (Fig. 12.10). Recently, the CMA method has been validated in hPSC works (Hyysalo et al. 2017c; Toivanen et al. 2017; Paavilainen et al. 2018; Mäkinen et al. 2018) and thus is routinely use in the analysis of hPSC-derived networks.

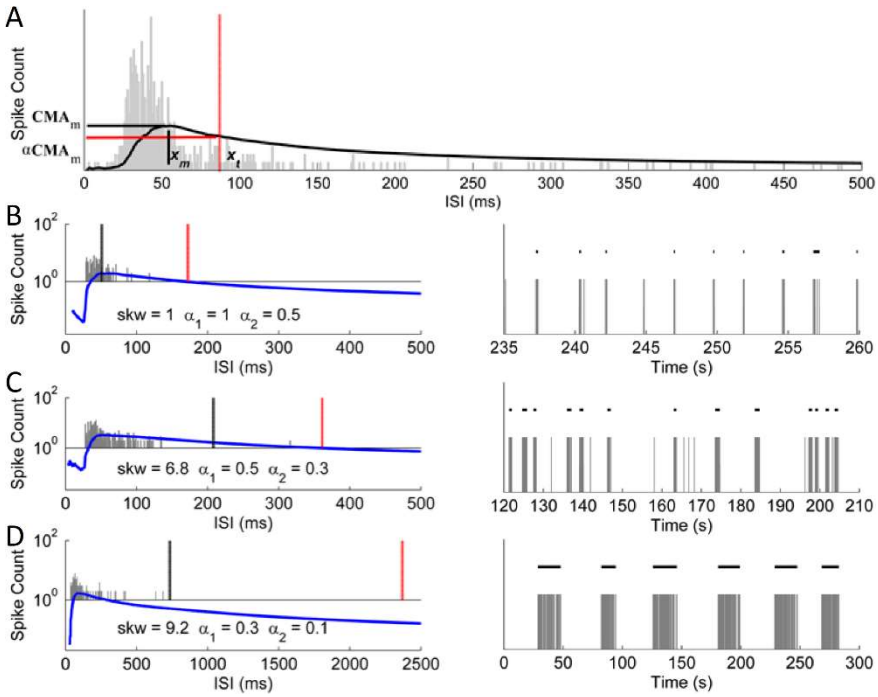


Fig. 12.9. Selecting the ISI threshold for burst detection using a CMA curve, along with exemplary bursts and their ISI characteristics. (A) The ISI histogram (gray vertical bars), and the corresponding CMA curve (black). The maximum of the CMA curve ($CMA_m$) is reached at the ISI $x_m$, and the ISI threshold $x_t$ for burst detection is set at the ISI corresponding to the CMA value closest to $\alpha \cdot CMA_m$. The ISI threshold for burst detection is marked with the red vertical line. (B)-(D) Setting burst detection ISI thresholds (left panels) for MEA measurements from three differently behaving hESC-derived neuronal networks, and the detected spikes and bursts (right panels). Left panels: ISI histograms (gray vertical bars), CMA curves (blue), skewness values (skw) and the corresponding $\alpha_1$ and $\alpha_2$ for calculating burst and burst-related spike detection ISI thresholds, along with the burst and burst-related spike detection ISI thresholds (black and red vertical lines, respectively). Right panels:

the detected spikes (vertical lines) and bursts (horizontal lines/dots above the spike indicators). (Reprinted from (Kapucu et al. 2012) under CC BY-NC 3.0 license.)

### *12.7.3 Joint burst and spike analysis: spike type compositions of bursts*

Waveform-based spike sorting and burst detection are often used in MEA signal analysis. By associating each burst spike with a spike type by spike sorting, it was possible to obtain the spike type compositions of the bursts. This observation resulted in a new method: joint analysis of bursts and spike waveforms (Kapucu et al. 2016d), which is schematically presented in Fig. 12.10A. In joint analysis, each spike in a burst is associated with a spike type according to the sorting results of the spike in question. During an experimental paradigm, the results of joint analysis provide the spike type compositions of bursts, which is a different type of information than what mere burst detection or spike sorting-based analysis alone can provide. Sample simulation results are shown in Fig. 12.10 (Kapucu et al. 2016d), where spike waveforms resulting from spike sorting are shown (Fig. 12.10B), along with a burst and its spikes associated with the spike waveforms (Fig. 12.10C). This method can be implemented with any waveform-based spike sorting and burst detection methods that are suitable to analyze the signal at hand.

A change in the spike type composition of bursts during an experimental paradigm indicates that something changed in the neuronal cells or in the functional networks participating in the burst generation. Such experimental paradigms include those with multiple pharmacological applications or other chemical alterations, electrical stimulation, or network maturation. Any experiments in which bursts can be observed before and after an alteration or at different time points can be subject to joint analysis. Joint analysis may reveal changes in bursts even though there might have been no observable changes in the traditional burst metrics, such as the number of spikes in the bursts or the number or duration of the bursts. For the advancement of neuroscience, it would be preferable that methods capable of extracting more information from the measurements, such as joint analysis, were utilized more extensively. However, these new methods need be validated, the biological relevance of the new information must be shown, and the methods must be adopted in everyday use. For joint analysis, these tasks are the next steps to be taken. A Matlab implementation of the method is freely available (Kapucu 2015a).

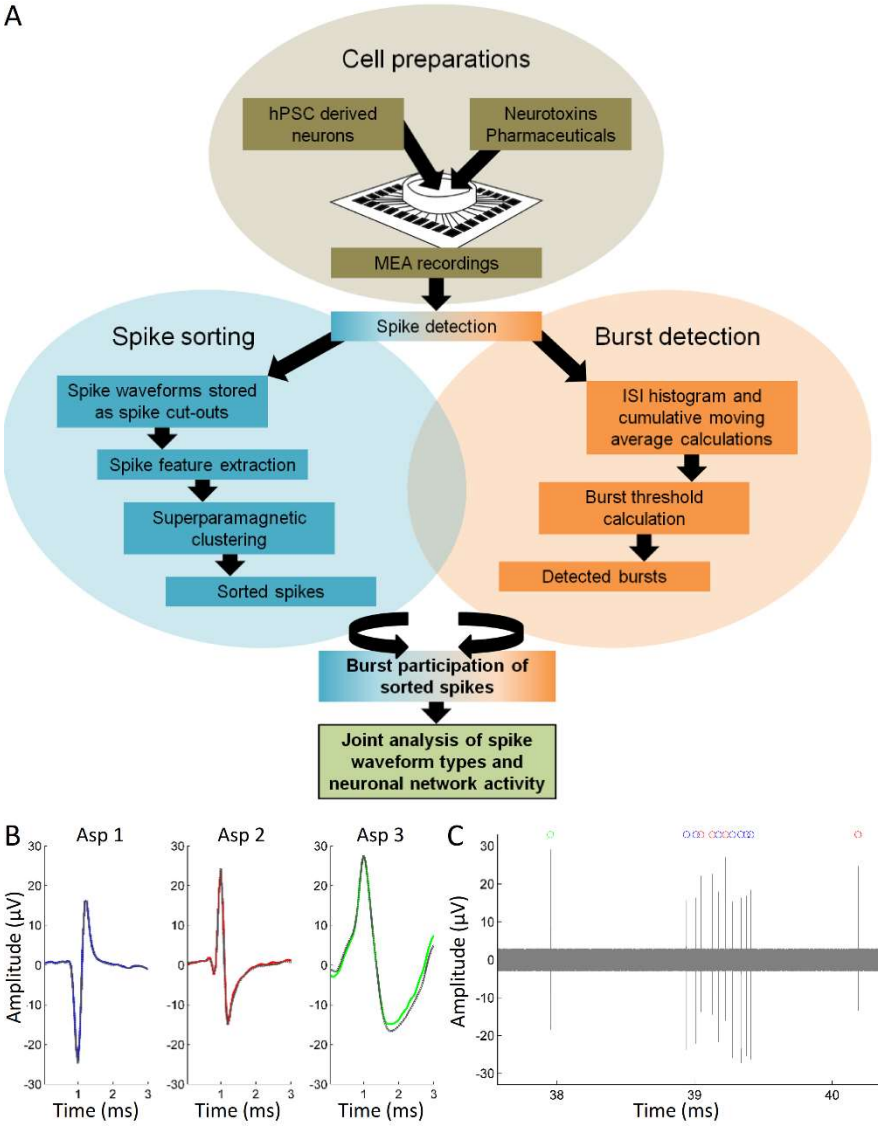


Fig. 12.10. (A) A schematic presentation of joint analysis. The joint analysis framework consists of spike sorting and burst detection, followed by analysis of the participation of spikes of different types in bursts. (B) Simulated spike sorting results with three spike waveforms, ASp 1 (blue), ASp 2 (red), and ASp 3 (green), along with the corresponding average waveform (gray). (C) Joint analysis result: a sample of the simulated signal with the sorted spikes indicated by circles with colors corresponding to the particular spike type (B). (Reprinted from (Kapucu et al. 2016d) with permission from Elsevier.)

### *12.7.4 Entropy-based burst assessment and network analysis*

Entropy is a measure of disorder or uncertainty, and it has been widely employed in signal analysis in many fields. In neuroscience, entropy-based methods have been used to study, for example,

complex physiological systems or the uncertainty of neuronal behavior at the brain level (Burggren and Monticino 2005) and information transfer between different neuronal populations or locations in a neuronal system (Garofalo et al. 2009; Ito et al. 2011). Complex system analysis methods have also been used to detect events of neuronal origin in epilepsy or even in complex decision tasks (Puthanmadam and Hyttinen 2015a; Puthanmadam et al. 2015b; Puthanmadam 2016). Here, entropy was found to be lucrative for assessing neuronal networks and their function based on MEA measurements. Entropy can be calculated to produce either one single entropy value for an entire MEA signal or for a section of it (such as an MEA signal during a burst). Alternatively, entropy can be calculated in a running window (with the window length only a small fraction of the length of the MEA signal of interest), producing an entropy signal for the entire MEA signal, or for example, for only the duration of a previously detected burst. Entropy-based methods were proposed for three different uses: 1) to detect bursts based on changes in the entropy signal of an MEA recording, 2) to evaluate the information content of bursts and thus to classify or quantify bursts, and 3) to assess functional connectivity, i.e., spatial network relations.

The applicability of entropy measures for burst detection was demonstrated with bursts detected in MEA measurements from dissociated rat cortical cell cultures (Fig. 12.11AB) (Kapucu et al. 2015b). An exemplary result is shown in Fig. 12.11, where an MEA signal is shown with bursts detected by the automatized burst detection method (Fig. 12.11A), along with the corresponding sample entropy (SmE) and spectral entropy (SE) signals (Fig. 12.11B). In the case seen in Fig. 12.11AB, the bursts are obvious and easily detectable, and SmE and SE could be used to quantify them. Self-similarity, quantified by SmE, and spectral uniformity, quantified by SE, were found to be promising metrics for quantifying bursts for further classification (Kapucu et al. 2015b). For the data at hand, both entropy measures were good at detecting changes in neuronal recordings during bursts. SmE was found more sensitive to the burst duration and number of spikes in bursts for the signals at hand. SmE also showed more potential for classifying presumably different kinds of bursts than SE.

In our further experiments with hPSC-derived neurons, it was demonstrated that SE also has the potential to detect bursts with burst spikes that are buried in noise. An MEA signal measured from hPSC-derived neurons with clearly observable bursts and the corresponding SE signal are shown in Fig. 12.11C and E, respectively, whereas a signal with no visually detectable bursts is shown in Fig. 12.11D with the corresponding SE signal in Fig. 12.11F, which clearly indicates probable bursts buried in noise. While entropy-based methods have shown clear potential for burst detection and quantification, their biological relevance in these applications should still be stringently validated.

Entropy-based measures can also be employed to reveal spatial network relations (Kapucu et al. 2016c, 2016e, 2017a, 2017b). Kapucu et al. (2016e) showed that spatial network relations can be revealed by time-variant SE signals, and the correlated spectral entropy method (CorSE) was developed for this application. SE quantifies the uniformity, or complexity, of the frequency spectrum distribution of a signal, such an MEA measurement. In CorSE, an SE signal is formed for each MEA signal measured via an electrode. Next, correlations between all SE signal pairs are calculated. Correlation between two SE signals quantifies how similarly the uniformities of the frequency spectra of the two MEA signals change in time. If the correlation between the two SE signals is sufficiently high, the network locations at the electrodes are considered to be functionally connected. A benefit of this method is that it is independent of spike timings at the two considered network locations; to observe functional connectivity, it is sufficient that the complexities of the two signals change similarly as the network functions. CorSE could be particularly beneficial in tracking the development of maturing neuronal networks. Work using CorSE in analysis of the functional networking dynamics of hPSC-derived neuronal networks during maturation is ongoing (Tanja Hyvärinen, Emre F. Kapucu, et al. unpublished). This work is expected to demonstrate the biological relevance of the results of CorSE. A Matlab implementation of CorSE is freely available (Kapucu 2016a).

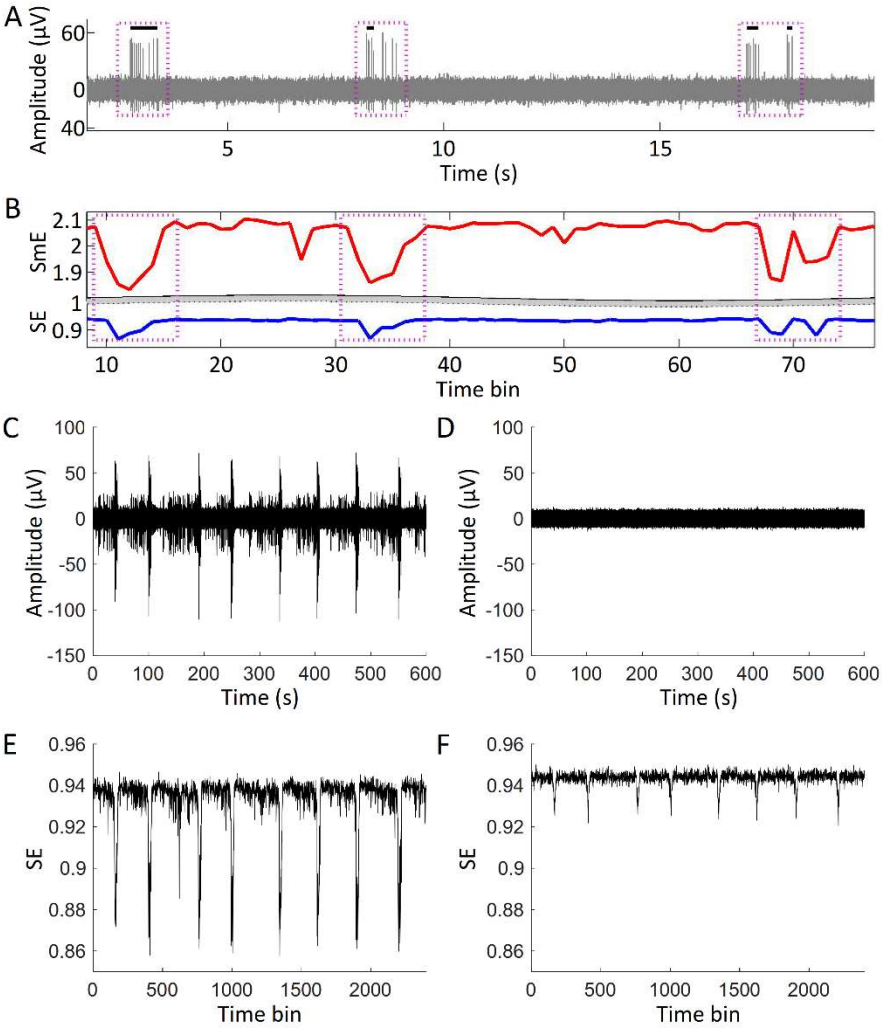


Fig. 12.11. (A) A signal with bursts detected with the automatized burst detection algorithm. Detected bursts are labeled with black horizontal lines above the signal. (B) SmE (red) and SE (blue) signals calculated for the signal seen in (A). The purple boxes delineate the changes in the SmE values. The boxes in (B) correspond to the boxes in (A) over time. (C) and (E) Exemplary MEA signals measured from hPSC-derived neurons (Data courtesy of Tanja Hyvärinen (NeuroGroup, BioMediTech and Faculty of Medicine and Life Sciences, University of Tampere). (D) and (F) The SE signals for the MEA signals in (C) and (E), correspondingly. ((A) and (B) ©2015 IEEE. Reprinted, with permission, from (Kapucu et al. 2015b).)

### *12.7.5 Computational modeling and neuronal network simulations*

Approaches to computationally model and simulate neuronal cells and networks range over several orders of magnitude spatiotemporally, from single ion channels and receptors via cell membrane and single synapses to neuronal microcircuits and brain connectivity (Reeke 2016; Gerstner et al. 2012).

Neuronal network models have been built with different models of biological neurons or their observed functionality. Lenk et al. (2016a) modeled the behavior of developing hESC-derived neuronal networks using a phenomenological network model based on the INEX cell model (Lenk 2011). The spiking characteristics and appearance of bursts during network maturation seen in MEA measurements were successfully modeled by increasing model connectivity, which reflected the formation of new synapses and connections between neurons (Lenk et al. 2016a).

There is growing evidence that astrocytes play an important role in neuronal information processing. It is known that astrocytes form networks and that a single astrocyte may participate in numerous synapses (Bushong et al. 2002). The astrocytes have not been, however, usually included in computational neuronal network models. Thus, the functioning of astrocytic-neuronal systems at a single synapse and at cellular microcircuit or network levels was studied and simulated.

Pre- and postsynaptic neurons and an astrocyte form a so-called tripartite synapse (Araque et al. 1999; Perea et al. 2009). Neuron-astrocyte models have been recently reviewed by Oschmann et al. (2018). Lenk et al. (2016b) modeled a tripartite synapse by augmenting the INEX model (Lenk 2011) with a presynapse-astrocyte communication model and additional refinements, which is schematically presented in Fig. 12.12A. The tripartite synapse model is based on biological knowledge of the synapse, whereas the developed network models were phenomenological, i.e., aimed at reproducing the phenomena observed in real MEA measurements. The astrocyte-augmented INEX model was used in a network simulation to produce virtual MEA recordings; the simulated network was able to reproduce the effects of different amounts of released astrocytic GABA on network activity. A snapshot of the neuronal-astrocytic network simulation is shown in Fig. 12.12B.

Spatially, synapse simulations can be considered to be point-like, i.e., 0D, whereas the simulations of neuronal networks on planar MEAs correspond to 2D networks. Related to neuronal-astrocytic networks, calcium wave propagation in a geometrical model of a single astrocyte was studied by Khalid et al. (2017). Microcircuit models can be extended to 3D to model *in vitro* cultures in hydrogels and scaffolds and *in vivo* microcircuits in the brain. The extension from 2D to 3D neuronal network models (Lallouette et al. 2014; Vornanen et al. 2014; Bosi et al. 2015) can be achieved, e.g., by modifying neuronal or neuronal-astrocytic connectivity and action potential propagation delay distributions accordingly.

Simulations are a flexible tool to study the effects of the properties of neuronal cell and networks and their alterations on their observed function. For these investigations, computational models need to be validated with real measured data. Phenomenological INEX-based models have been

demonstrated and successfully compared with a few sets of MEA measurements. The tripartite synapse model should still be validated with MEA data. Next, the models should be used to demonstrate their power in practical applications.

Fig. 12.12. (A) Schematic illustration of the interplay between a presynapse, a postsynapse, and an astrocyte. The presynaptic neurotransmitters affect the astrocyte, which in turn modulates both pre- and postsynaptic terminals. Legend: 1) synaptic basal release probability; 2) amount of calcium bound to the sensors; 3) available neurotransmitters; and 4) amount of neurotransmitter released. (B) A snapshot of neuronal-astrocytic network simulation with astrocytes (blue dots), astrocyte connections (blue lines), excitatory neurons (green dots), and inhibitory neurons (red dots) on a virtual MEA. The neuronal connections are not shown. (©2016 IEEE. Reprinted, with permission, from (Lenk et al. 2016b).)

## 12.8 Conclusions and discussion

hPSC have changed and opened up new research and application areas that in the long run, can have a significant impact on many fields, ranging from basic developmental science to an understanding of human tissue development, valid *in vitro* testing platforms for toxicology and drug screening, and finally, disease modeling and even regenerative medicine. Especially for neuroscience, these cells provide an invaluable source for producing neural cells, the building blocks of our brains, in a dish, and thus provide a tool to study the most complex organ closer than ever before. During the last few years, the use of hPSC-derived neuronal cells has received much interest, particularly in neurodevelopmental toxicity and disease modeling. At the same time, we have started to realize that *in vitro* cultured human neuronal cells and networks, and especially network functionality, is different from their rodent counterparts, which are considered the gold standard. Taking their biological differences into account, the differences between rodent and human cultures should not be very surprising. This finding does, however, increase our challenge beyond the difficulties encountered with traditional culturing and analysis methods. In this Chapter, we provided an overview of the current state of art of culturing hPSC-derived neuronal cells from traditional 2D to evolving 3D systems and noted specific needs that require novel approaches and the development of new analysis methods. As the demand to utilize these cells in numerous applications is continually increasing, we hope that our work identifies ways to further extend this research area.

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