Novel aspects of neuronal differentiation in vitro and monitoring with advanced biosensor tools.

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Abstract

Neuronal differentiation is a very complex and sophisticated cellular process that encompasses the development of mature neurons and their specialization. In this review we will focus on the novel and less well-known aspects of neuronal differentiation. Cell lines, to which some pro-differentiation drugs are added, have been widely used because of their convenience in terms of cost-efficiency, ease of use and reproducibility. After a brief overview of these systems, this review focuses on the new pharmacological aspects of differentiation related to mitochondrial changes and cellular redox homeostasis. A number of different parameters are commonly evaluated to assess neuronal differentiation. These include neurite length, differential gene expression, mitochondrial mass, free radical levels, enzyme induction and others. However, the classical techniques used to detect neuronal differentiation (such as immunochemistry, flow cytometry and gene expression analysis) are time-consuming or dependent on the subjective view of the researcher. On the other hand, emerging novel, miniaturized biosensor technologies have the potential to revolutionize the study of neuronal differentiation, by detecting neuron-derived electrical signals and differentiation markers, such as shape or attachment in a non-invasive and high throughput fashion. These state-of-the-art technologies are being extensively reviewed. Emphasis is given to progress, made in the field of integrated systems (including impedance sensing, microfluidics and associated nanotechnologies), neuronal differentiation in 3-D cultures and the identification of novel agents controlling neuronal cell fate.

Keywords: biosensors**,** cellular models, micropatterning, free radicals, microfluidics, mitochondrial biogenesis, neuronal differentiation, three-dimensional culture.

Introduction

Neuronal differentiation is a complex process that encompasses the development of new neurons and their specialization. During the development of the central nervous system (CNS), highly coordinated programs generate a huge number of different subtypes of neurons, which then assemble to form functional neuronal circuits. The fundamental goal in developmental neuroscience is to understand the control of this system. This knowledge is not only important for the elucidation of natural neuron development, but also for the elucidaton of the origin of neurological diseases and may culminate in the development of new regenerative strategies to treat those diseases [1]. The complexity of the developmental process makes it necessary to use advanced techniques for the detection of important physicochemical parameters, signaling pathways and morphological features. In this review we will describe the state of these cutting-edge techniques which have been particularly developed for developmental neurobiology.

Cellular models in developmental neurobiology

Models are necessary for each research activity, in which the effect of natural variability on experimental observations must be reduced. The aim is to handle as less degrees of freedom as possible and that the only variable is the one that the researcher wants to induce in the system. Among the available cell models for neuronal differentiation, a distinct group of cell lines has been adopted by the scientific community, probably because they are an unlimited source of a considerable number of cells and because each cell line comes from the same clone, which means a high reproducibility of the cell line-specific traits [2]. Cultured neuroblastoma cells are commonly used for the screening of drugs which potentially alter neuronal growth, they are simple and easy to culture, cheap and available, and they are genetically modifiable. Moreover, neuroblastoma is the most common cancer of children's nervous system, so any progress in understanding the physiology of these cells has a medical relevance [3].

Primary cultures have been widely used as neural differentiation models [2]. Although their growth patterns in vitro resemble closely the physiological developmental process, the complexity of obtaining the cells from an in vivo source, the difficulty to obtain pure neuronal cultures and the intercultural variability makes primary cell lines less than the golden standard in developmental neurophysiology. Another possibility is the use of stem cells that will differentiate to mature neurons. Stem cells can be passaged and induced to differentiation in the appropriate stage of their growth cycle [4]. Although such experiments are very promising, the ethical concerns around stem cell research and the possible variability between passages and individuals are factors to take in account. Depending on the subject of study, the benefits of using cell lines can outweigh their potential disadvantages [3]

Among the cell lines commonly used to test differentiation, PC12, a rat pheochromocytoma cell line stimulated with NGF is the one used by the majority of researchers [5]. The reason for this is the ease of visualization of the changes produced in these spherical cells when they differentiate to neurons and produce axons and dendrites [6]. Other stimuli have been demonstrated to induce differentiation in PC12 cells like db-cAMP [7], PACAP [8-11], forskolin [12, 13], estrogens [14], hypoxia [15, 16], heat-shock [5] and even microwave irradiation [17].

However, other cell lines i.e. neuroblastoma cell lines have been used with the same purpose, with murine Neuro2a (N2a), human SK-N-SH and SH-SY5Y being the most common among them. The P19 embryonic carcinoma cell type has been used as a differentiation model due to the fact that cells are pluripotent, being able to differentiate in vitro into a wide range of cellular types including neurons, after the addition of all-trans retinoic acid. So far they resemble the behavior of stem cells [18]

Some of the cell lines differ in their responses to different stimuli. For example, N2a cells differentiate in response to db-cAMP and serum deprivation [7, 19, 20]. Human neuroblastoma and P19 cells do differentiate in response to retinoic acid while serum deprivation is an apoptotic stimulus for them [18]. Different agents can promote differential gene expression in the same cell line and exhibit synergistic effects on neurite outgrowth [21]. It should also be pointed out that the same agents can cause the opposite effect in different types of neurons [22], as dissimilar as differentiation or death depending on the cell line [23].

It is important to consider cell lines just as models, which have limitations when it comes to predicting what their physiological response to some compounds will be. However, cell lines preserve the basic cellular physiological mechanisms, allowing researchers to use them as an endless source of material for pharmacological screening. A very detailed and extensive description of the different models used for the detection of differentiation can be found in the review by Radio et al [2], as well as references for stimuli depending on the cell line and substances that modify their differentiation. Researchers in the field of neurobiology should take into account the advantages and disadvantages of each cellular model.

It is known from drug screening that most of the candidate drugs that enter Phase I trials fail to reach the bedside. This is due to the fact that the models commonly used to assess the pharmacological characteristics of one drug are not representative of the physiological reality. The most common cellular models are two dimensional models, in which cells coming from a certain cell line or tumor are grown on flat flask surfaces [24]. It is known that those cells do not express part of their natural phenotype [25] i.e. gene expression and protein/enzyme transcription, due to this mechanical alteration and the absence of extracellular matrix (ECM) and surrounding cells [26, 27]. That makes 2D models limited in the context of usefulness to predict the cellular responses of real organisms in vivo. However, whole organisms and

organs, organ slices, spheroids or simply ECM gels with embedded cells have risen as a new potential model for drug screening, with a higher predictive power.

3D models have opened a new window to the future of developmental neuroscience, since they better resemble the physiological conditions in vivo. Within normal tissues, cells live in a 3D environment, i.e. they relate to other cells in the three dimensions of the space and with the extracellular matrix. All these interactions are lost in classical 2D cultures. On the contrary, 3D models can simulate inter- and extra-cellular environments, for example by mimicking the conditions of nerve regeneration with a collagen sponge populated with Schwann cells and fibroblasts [28], or developing new matrices which could be transplanted for nerve regeneration [29].

The use of 3D models as an intermediate step between classical 2D cell culture and animal research will have a strong impact on the number of animals sacrificed for experimental purposes [24], due to the fact that more non-efficient compounds will be screened out and less will proceed to the animal experimentation phase.

Of course, as 3D culture models are not so extensively used within the scientific community. Comparative studies between already existing 3D matrices [29, 30], as well as between 2D and 3D environments [27, 29, 31-33], are required. Also necessary is the development of new techniques for monitoring the activity of neuronal cells in a 3D environment [31], and the development of novel scaffolds with increased biocompatibility and convenience of use[34], either natural, engineered [25] or gel-free [35]. An example of neuroblastoma cells differentiating in a 3D collagen-laminin gel matrix is shown in figure 1. As a quite recent example, three dimensional neural networks were developed on borosilicate glass spheres. Those beads are packed in layers with a hexagonal symmetry, making it possible for neurons to connect with other layers and establish synapses. Moreover, as the beads are transparent, it is possible to stain cells with a fluorescent dye or transfect them with a GFP and visualize those connections between neurons from different layers by fluorescence microscopy or simply by optical imaging. The axonal growth can be directed as desired with the use of chemical attractants or differentiating mediators such as cAMP [36].

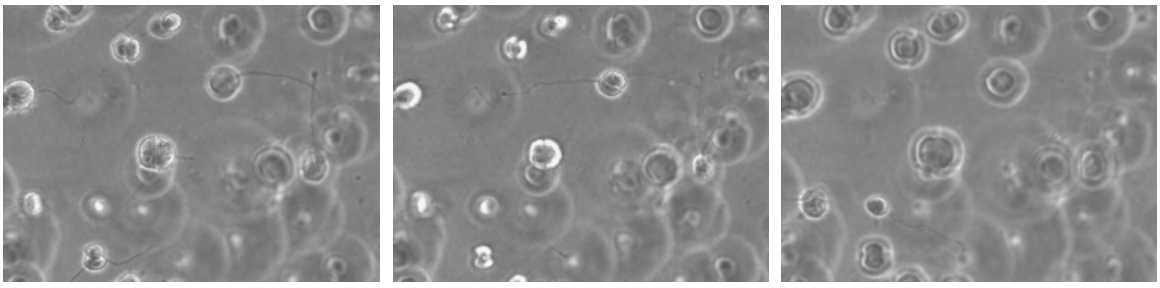

Figure 1: Differentiated N2a neuroblastoma cells embedded in a 3D matrix. Three pictures of different focus planes show the three dimensional growth of the dendrites

Differentiation, nitric oxide, mitochondrial biogenesis and free radicals

There are some very well known pathways of neuronal differentiation such as the pathway followed by retinoic acid [37]**,** NGF through TrkA receptors or PACAP through PAC1 receptors [38]. However, other mechanisms seem to be involved in the differentiation process, although they are not so often referred in literature. Among them, the link between mitochondrial biogenesis, free radicals and differentiation is a main focus of this review.

Nitric oxide (NO) is a volatile free radical with two contradictory roles in developmental neurobiology. On the one hand, a disregulation of NO homeostasis by an aberrant increase of this small diffusible molecule promotes neural cell apoptosis. On the other hand, small increases in the NO levels (through the cGMP pathway) have been reported in cells under differentiation [39]. The regulation of transcription factors is a key mechanism in the effect of NO on neuronal functions. Expression of a large number of genes is altered upon neuron exposure to NO, mainly through the activation or inhibition of transcription factors such as CREB, n-Myc, c-fos, c-jun, or zinc-finger transcription factors in a cGMP-dependent way or through nitrosative conformational modification of proteins [40].

A very elegant study on eNOS- and nNOS-deficient mice demonstrated the correlation between the nNOS activity, which is responsible of NO production, and mitochondrial biogenesis through the transcriptional coactivator PGC-1α, NRF-1 and Tfam under hypoxia conditions [16]. Actually, PGC-1α seems to be the master regulator of mitochondrial biogenesis, acting as a co-activator with NRF-1, NRF-2 and Tfam. It can be activated by NO, HIF1α/β or AMPK [41]. Activation of PGC-1α has been found in cells from other tissues under differentiation. Specifically, pharmacological activation of the cAMP or PPAR pathway has been observed to induce differentiation and promote PGC-1α dependent mitochondrial biogenesis in adipocytes [12].

PGC-1α is a transcriptional coactivator, which transduces several physiological stimuli into specific metabolic programs, most frequently by stimulation of mitochondrial activity. The activation of mitochondrial biogenesis by PGC-1α depends on the coactivation of ERRa, NRF-1, and NRF-2. For an extensive review of nuclear activators of mitochondrial biogenesis it is recommended to read Scarpulla´s work on the subject [42].

PGC-1α promoter contains some highly conservative consensus cis-elements that are critical for its transcription. MEF2 and CRE binding sites have been the most well studied. MEF2 is the responsible for

the autofeedback regulation of the PGC-1α transcription while CRE can increase transcription in response to cAMP/PKA and p38 MAPK pathways [43]

PGC-1α and PGC-1β are strongly induced by ROS. They regulate a complex ROS defense system, being part of the ROS homeostatic cycle. In particular they induce SOD1, SOD2 and catalase, among others. PGC-1α acts in a dual way, both stimulating mitochondrial electron transport, and suppressing ROS levels. Thus serves as an adaptive set-point regulator, which provides a precise balance between metabolic requirements and cytotoxic protection from ROS [44].

ROS can act as second messengers in the differentiation process. ROS have been found to mediate another differentiation mechanism promoted by semaphorin A. The suppression of mitochondrial free radicals by a non-apoptotic rotenone concentration or N-acetyl-L cysteine abolished its differentiating effect [45]. Figure 2 is a schematic review of crosstalk among differentiation, mitochondrial biogenesis, the production of free radicals (including NO) and signaling agents (such as cAMP).

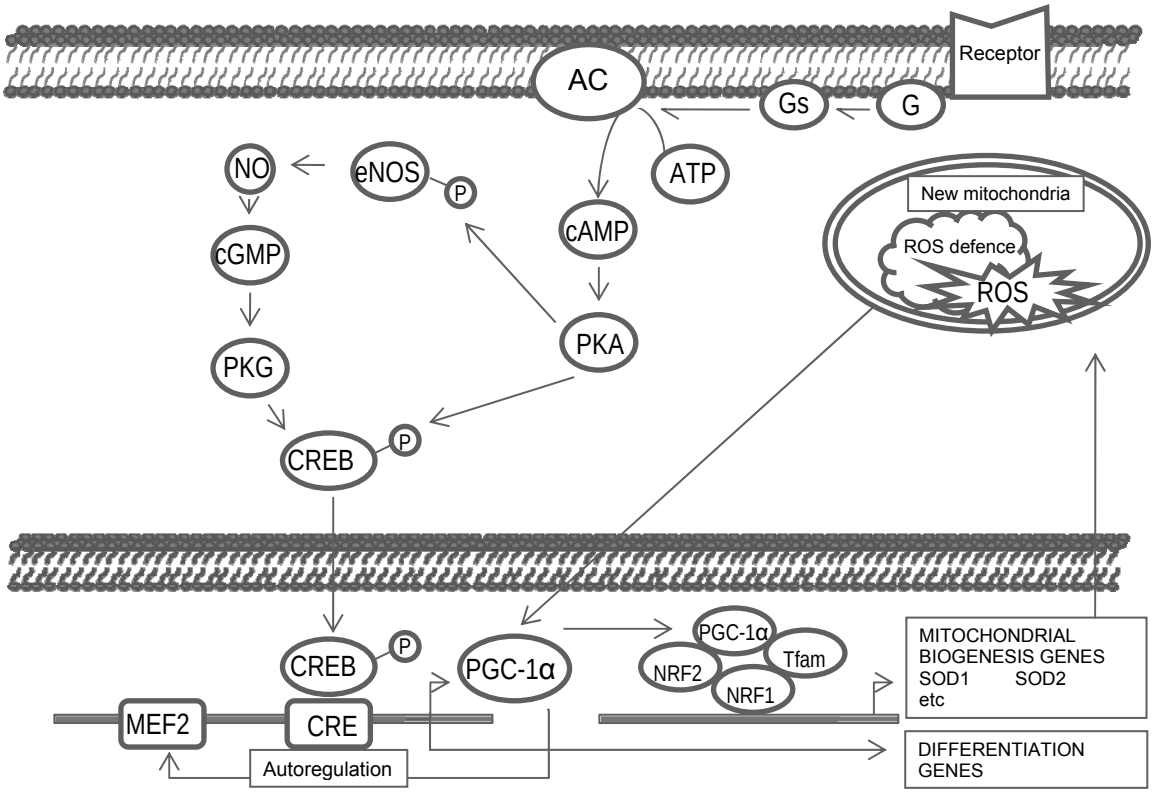

Figure 2: Schematic representation of the crosstalk among differentiation, mitochondrial biogenesis, ROS, cAMP and NO. Nitric oxide (NO), Endothelial Nitric Oxide Synthase (eNOS), Cyclic Guanosine Monophosphate (cGMP)**,** Protein Kinase G (PKG), cAMP Response Element-Binding Protein (CREB), Protein Kinase A (PKA), Cyclic Adenosine Monophosphate (cAMP), Adenylate Cyclase (AC), Adenosine-5'-triphosphate (ATP), G-protein (G), Reactive Oxygen species (ROS), Myocyte Enhancer Factor-2 (MEF-2), cAMP Response Element (CRE), Peroxisome Proliferator-

Activated Receptor Gamma Coactivator 1alpha (PGC-1α), Nuclear Respiratory Factor 1 (NRF1), Transcription Factor A (Tfam), Nuclear Respiratory Factor 2 (NRF2).

Quercetin, a natural flavonoid, known for its antioxidant and anti-inflammatory effect, has recently been demonstrated to use PGC-1α to increase the mitochondrial mass in muscle and brain and increase exercise tolerance in mice [46]. Resveratrol, another natural flavonoid, has been demonstrated to promote mitochondrial biogenesis, differentiation of N2a cells and to be neuroprotective, through the activation of AMPK [47].

There is evidence that mitochondrial biogenesis is not only activated by PGC-1α. It was found that ischemia can induce rapid mitochondrial biogenesis, in which the number of mtDNA copies was increased in a PGC-1α-independent mechanism [48].

One very recent study demonstrated that mitochondrial biogenesis is parallel to neuronal differentiation in a PC12 model which overexpressed the transcription factor NeuroD6. They found not only an increase in mitochondrial mass, but also a spatial redistribution of mitochondria toward the dendrites, together with a rearrangement of actin and microtubules. This increase in mitochondrial mass is dependent on the microtubule network integrity [6]. Previous works by this group had already shown that NeuroD6 is able to initiate by itself the differentiation of PC12 cells, linking the mitochondrial survival pathway, differentiation, and cell cycle arrest [49]. Moreover, they found a parallel increase in the expression of differentiation and mitochondrial proteins in a genome-wide microarray study [50]. This group's work provides with a good picture of the link between differentiation and mitochondrial biogenesis.

In an extensive proteomic analysis of the P19 differentiation cell model an increase in mitochondrial biogenesis, corresponding with the differentiation process, was found. It was accompanied by cell cycle proteins down-regulation and cytoskeletal remodeling proteins up-regulation [18]

All these findings suggest the important role of mitochondria during differentiation. Mitochondria are the energy-producing organelles. Probably, a higher energy demand during and after differentiation triggers mitochodriogenesis and the mitochondrial migration to the dendrites. In this context, free radicals would act as the messengers of this biogenesis process, which involves the activation of transcription factors and gene expression.

Mitochondrial biogenesis and apoptosis

Mitochondrial biogenesis, paradoxically, has also been found in cells under apoptosis. Actually, some of the mechanisms related to differentiation and apoptosis seem to be shared. Aberrant mitochondrial biogenesis and enhanced oxidative stress have been observed in a neuroblastoma cell line after the

induction of apoptosis by methamphetamine. The use of vitamin E as antioxidant was able of reverse those effects [51]

Nevertheless, some questions still remain open: is mitochondrial biogenesis a result of the increased energy demand after differentiation or apoptosis? Is, on the contrary, differentiation a result of a complex process which is mediated by mitochondrial biogenesis? Or are they just parallel processes? Which is the role of free radicals in this system, cause or effect? The physiological answer to those questions remains to be elucidated.

Conventional techniques to measure differentiation

Conventional techniques can be classified as semi-quantitative (or non-calibrated), quantitative (or calibrated) and biochemical. Quantitative and semi-quantitative techniques are based on cell morphology i.e. if the cells are presenting neurites [2]. Multiple parameters can be measured such as the number of cells and neurites, the number of neurites per cell, the identification of axons and neurites, the number of cells measured per optical field, the number of fields measured per culture well, the number of secondary tertiary neurites, neurite length, soma area, etc [3].

One option in order to increase the contrast for clearer imaging is cell fixation and staining, for instance with coomassie brilliant blue [52], crystal violet, Ramón y Cajal's silver-nitrate staining technique, or by attaching antibodies for later immunohistochemistry or flow cytometry analysis. Although fixation has some disadvantages such as the possible cell shrinkage, it allows the staining with antibodies for the detection of biochemical markers, allows the storage of samples, the imaging of fine neurites and their tips and allows the collection of data from a huge amount of samples at the same time point [3].

Biochemical techniques are based on the detection of some proteins that are overexpressed, or some genes that undergo a higher transcription only when the cell enters the differentiation process. For example, neurofilaments or Tau can be detected by immunoblotting or inmunocytochemistry with the help of a fluorescence microscope [2] or mRNAs encoding proteins of interest detected by RT-PCR.

Another very useful technique for fluorescence detection is flow cytometry, which not only allows to detect anything that is stained with a fluorescent dye or fluorescent antibodies at a single cell level [18], but also makes cell cycle analysis possible [53]. During differentiation, the cell cycle must change, as far as cells stop growing and enter the differentiation process. Usually cancer cells, as neuroblastoma or PC12, are continuously dividing, thus their cell cycle would be that of dividing cells. When differentiation starts, division gradually stops and cells differentiate to a concrete cell type. Cell cycle arrest and differentiation have a fateful relationship [54]. They are highly coordinated and interactive processes, governed by cell

cycle genes and transcription factors, which decide the neural cell fate [15, 55]. Withdrawal from the cell cycle accompanied by a reorganization of the cytoskeleton and an up-regulation of mitochondrial proteins was found in a quantitative proteomic analysis [18]. The complex interplay between cyclins, CDKs, cyclin kinase inhibitors, transcription factors, etc. is responsible for the expression of lineage-specific genes and thus it will control the neural cell fate [56].

Detection of cell cycle proteins has also been used for the description of neuronal differentiation [18]. The increase or decrease of some cyclins indicates in which phase of the cell cycle the cells are, i.e. if they exit the cell cycle and differentiate. Those proteins can be detected by immunoblotting, by immunocytochemistry, or even have their mRNAs detected by RT-PCR [53]. Moreover, new technical approaches have been developed to detect mitochondrial biogenesis [57]

Morphological studies can be automated with a camera and software that detects and counts neurites [58] or can be done manually by the researcher. Without automation high throughput screening does not present itself as a possibility. Therefore, a big step in developmental neuroscience has been the development of cameras and software adapted to the necessities of this discipline. With the aid of these tools, the same work can be done in much less time while the researcher can program the software, interpret the results and continue her/his research in a time-efficient manner. A practical review of the techniques that may be used for cell imaging, including advice on how to choose the appropriate software, and charge-coupled device cameras at an affordable price for two- and three-dimensional imaging, as well as suggestions on how to get successful images and choose the appropriate model of study, was made by Connolly et al. [3].

In the field of the three-dimensional imaging, confocal microscopy rises as the most extended technique able to deal with problems associated with 3D structures. Other, less common techniques are revised by Pampaloni et al. [24]. Among them two-photon and multiphoton microscopy, optical coherence tomography, optical projection tomography, confocal theta fluorescence microscopy, stimulated emission depletion fluorescence microscopy, or single plane illumination microscopy and light-sheet-based microscopes [59] seem to be the most promising for 3D culture analysis.

Although a considerable improvement has been made in terms of automation and resolution, classical techniques are still the researchers' favourite choice, even though they depend on their subjective appreciation, require bulky and expensive equipment and are not suitable for high throughput screening.

Novel biosensors and advanced techniques

It is possible to improve the cost- and time-efficiency of neuronal differentiation studies. Novel biosensors and advanced techniques offer a wide spectrum of possibilities. Microelectrode arrays (MEAs) and impedance analysis offer the possibility to non-invasively on-line monitor the electrical behavior of electrogenical cells, while micropatterning techniques and microfluidics permit the manipulation of the microenvironment around the cell, thus allowing a deeper study of physiological processes.

Microelectrode arrays (MEAs)

MEAs are planar substrates in which an array of microelectrodes is embedded. MEAs are able to measure extracellular action potentials from electrogenic cells and tissues [60]. In contrast to the classical patch-clamp technique, which allows only single cell measurements, which is quite expensive and time-consuming, MEAs offer the possibility of performing electrophysiological recordings in whole neuronal networks, thus making it possible to monitor intercellular signaling. Another advantage is the possibility to perform on-line and non-invasive electrical measurements for long time periods, for example during the differentiation process. It offers even the chance to monitor electrical activity of subcellular domains such as axons [61]. MEAs appear to be a non- invasive technique that has been successfully used in the study of neuronal synaptic plasticity, long term potentiation on acute slices and the development and regeneration of organotypic co-cultures [62].

MEAs are suitable for tissue studies such as detection of spontaneous excitatory spike firing on slices [63]. The creation of a CMOS-based microelectrode system with more than 11.011 electrodes opens up the possibility of performing extracellular electrophysiological recordings in brain slices, and of detecting simultaneously 126 selected recording sites on one slice [64]. The addition of three-dimensional tip-shaped electrodes [65] on a MEA instead of the planar ones provides the advantage of allowing the deeper insertion of electrodes in tissue slices or 3D structures and the detection of larger signals [66]

The electrical response of a whole neural network can be detected by extracellular recordings of single cells with MEAs. The temporal network activity progression from stem cells to a mature neuronal network has been studied with the help of MEAs during several weeks, due to the non-invasive fashion of this technique. Synchronization of spikes during the natural development, the effect of neuroactive drugs on developed networks [67], as well as the effect of different trophic factors on stem cell differentiation were studied [4].

A micropatterning strategy with poly-D-lysine on the surface of a MEA showed the electrical activity of neurons forming a reproducible triangular axonic network, which was maintained during several weeks.

The main advantage of this strategy is the appropriate adhesion of the neurons to the electrodes, giving rise to a better signal to noise ratio [68]. By developing an array of coated microwells, single neurons can attach to single wells and form networks in an ordered manner, thus facilitating statistical analysis and increasing throughput of the MEA [60].

The selection of the appropriate coating substrate is of huge importance, since different coatings can give rise to different degrees of fasciculation and thus different electrophysiological activity [61]. Moreover, it was possible to detect neuron to electrode contacts (hybrid synapses) on the surface of a MEA, the morphological and functional characteristics of a growth cone, and measure the impedance of the contact area [69]. The avoidance of coating material was achieved by using carbon nanotube-based electrode arrays. Carbon nanotubes were assembled in islands with a extremely rough surfaces, which play the double role of selectively anchoring the neurons and facilitating a precise stimulation and recording of extracellular membrane potential [70]. Carbon nanotubes can be easily used as coating material for metal electrodes. They have been demonstrated to reduce noise, to have a high biocompatibility and to improve the electrode-electrolyte interface [71].

Electrical stimulation and directed local electroporation of concrete axons have been achieved by using a MEA in combination with thickened microelectrodes with vertical sidewalls separated by the appropriate distance to host an axon [72]. By combining patch clamp detection of intracellular membrane potential and the application of voltage pulses through the electrodes of a MEA, it could be observed that it is possible to controllably electroporate neurons with the underlying electrodes of the MEA, which could be very useful for transfection purposes. Moreover, local stimulation of a subcellular fraction and the subsequent cellular signal spreading was observed by combining MEA with fluorescence monitoring of intracellular calcium [73].

Of course, the possibility of acquiring simultaneous recordings makes MEAs a time- and cost-efficient solution for neuronal studies [73]. Moreover, fabrication costs can be reduced to a minimum by simplifying fabrication procedures [74], materials [75] and increasing quantities of the devices produced.

Electrical impedance spectroscopy

Impedance is an important parameter used to characterize electronic circuits. Impedance (Z) is generally defined as the total opposition a device or circuit offers to the flow of an alternating current at a given frequency.

Impedance detection is a very useful tool for the measurement of cell's electric properties. Actually, cells can be introduced into an electronic circuit to form an electrochemical cell. When applying an AC voltage

perturbation, the current flows through all the components of the circuit, including cells. Impedance (the cells' opposition to the current), which will be a sum of the component's contributions, can be on-line monitored [76]. From a theoretical point of view, cell cytoplasm and extracellular space can act as conductive media, which are isolated from each other by the cell membrane. In other words, cytoplasm and extracellular space behave like resistive components, while the membrane behaves as the capacitive component. Electrical impedance spectroscopy can quantify macroscopic parameters related to tissue environment, based on the inherent electrical properties of cells [77].

There are impedance sensors that are implantable, they can record single neuron spikes and refractory times [78] and detect the reactivity of the brain tissue where they are inserted. The more cells attached to the electrode the bigger the magnitude of the impedance [79]. An alginate 3D cellular model developed for the simulation of this tissue reactivity in vitro, demonstrated to resemble in vivo conditions in terms of mitochondrial function and cell division, which combined with impedance spectrometry, is a good model to better understand the mechanisms by which the reactive response occurs [80].

In basic neurodevelopmental research impedance biosensors have emerged as a very powerful tool for the on-line detection of cellular properties such as shape, attachment, differentiation and cell death [81]. An impedimetric biosensor was able to discriminate the responsiveness of PC12 cells to differentiation induced by NGF, dexametasone and forskolin in a two-dimensional 8-well cell culture biochip [13]. Impedance spectroscopy has been demonstrated to be a useful tool for the study of differentiation, since it is able to distinguish between different electrical characteristics depending on the differentiation degree of the cells [82]. There are examples of applications of impedance sensors in three dimensional cell cultures. Electrical impedance sensing can be applied to the determination of culture growth, toxicity, membrane integrity [77]**,** response to neurotransmitters in three-dimensional neuronal cultures [83], the differentiation of neuroblastoma cells in three-dimensional cultures [84] or the effect of drugs on spheroids [81]. Impedance spectroscopy has a wide range of applications, since it does not require the presence of electrogenic cells to perform the electric measurements. On the contrary, every cell is susceptible of impedimetric analysis. Of special interest is the potential use of this technique for the study of stem cell viability [85], development and differentiation [86].

Micropatterning

Micropatterning of substrates allows us to choose the exact position where cells attach. For example, by patterning neuronal cells within a poly(dimethylsiloxane) (PDMS) hexagonal array of adhesion nodes, it is possible to standardize the distance between neighboring cellular nodes. Neurite outgrowths connect the cellular nodes and develop an axonal network in which the length of the neurite interconnections is standardized. This simple approach can reduce the effort for high throughput screening [87]. Other

micropatterning techniques have been used for neuroblastoma cell attachment, such as inkjet printing i.e. printing a polyethyleneimine (PEI) solution onto an albumin substrate using a modified commercial inkjet printer [88]. E-beam lithography on biocompatible hydrogels allowed the generation of micropatterns in which PC12 cell could differentiate and extend their neurites throughout the channels. This approach makes it possible to control the number, shape and length of the neurites [89]. Migration, outgrowth and differentiation of human stem cells could be observed thanks to the creation of a micropatterned poli-L lysine squared surface. Microstructured PDMS surfaces were used as stamps for the printing of Petri dishes with the poli-L-lysine [90].

Advances in the field of micropatterning give us the advantage of seeding the cells on the desired sites, and only on them. These advances give us a chance to explore the differentiation of single neurons and synapse formation, depending on their spatial distribution [91, 92]. By micropatterning it could be observed that there are important parameters in stem cell differentiation, like colony size and embryoid body size, which are usually neglected. Those parameters are easily modifiable with this technique [93]. Micropatterning is a very promising technique for the creation of three dimensional scaffolds for cell entrapment, with respect to nutrient diffusion and control of tissue orientation [94].

Depending on the substrate used, micropatterning can directly influence the degree of differentiation. For example, micropatterned polystyrene was observed to positively influence the differentiation of adult hippocampal progenitor cells [95]. Thus micropatterning is a very useful tool in developmental neuroscience, as far as it makes it possible to guide neuronal differentiation by directing cell protrusion outgrowth, to monitor developmental processes and to explore the influence of different cell arrangements and culture conditions on cell interactions and developmental processes [96].

Microfluidics

Microfluidic devices are usually defined as miniaturized versions of their macro-scale counterparts, systems in which a laminar flow perfuses some tiny channels, where the actual experiment is performed. Microfluidic devices offer a lot of advantages for sample handling, reagent mixing, separation and detection. They are ideal for the analysis of hard-to-obtain or high-cost substances and for parallelization. They have been widely used in methods requiring sophisticated equipment like flow cytometry, but also less sophisticated ones, like electrophoresis, dielectrophoresis, proteomics, immunoassays, sample preparation for mass spectrometry, RT-PCR improvement, etc [97]

The potential applications of microfluidic devices in neurobiology are vast. Cells can be seeded within the microfluidic channels or perfused to the system. Especially in neuronal differentiation there is a wide spectrum of applications that have already been developed and many challenges for the future development

in this field. Some examples are shown in figure 3. For example, it is possible to control the axon outgrowth and its direction. By using a microfluidic device with a microchannel, a microvalve, and a nano-hole array, one can control the spatio-temporal administration of NGF to a PC12 cell line and thus control neurite outgrowth in a very precise manner [98]. A microfluidic device composed of two separated chambers has been demonstrated to effectively separate neurons from their axons for further proteomic and RNA analysis. This chip has other potential applications, such as facilitating research in nerve injury and axon regeneration [99]. Another compartmentalized microfluidic platform has also been demonstrated to effectively separate cell soma from axons, thus making possible to study axon to glia interactions [100]

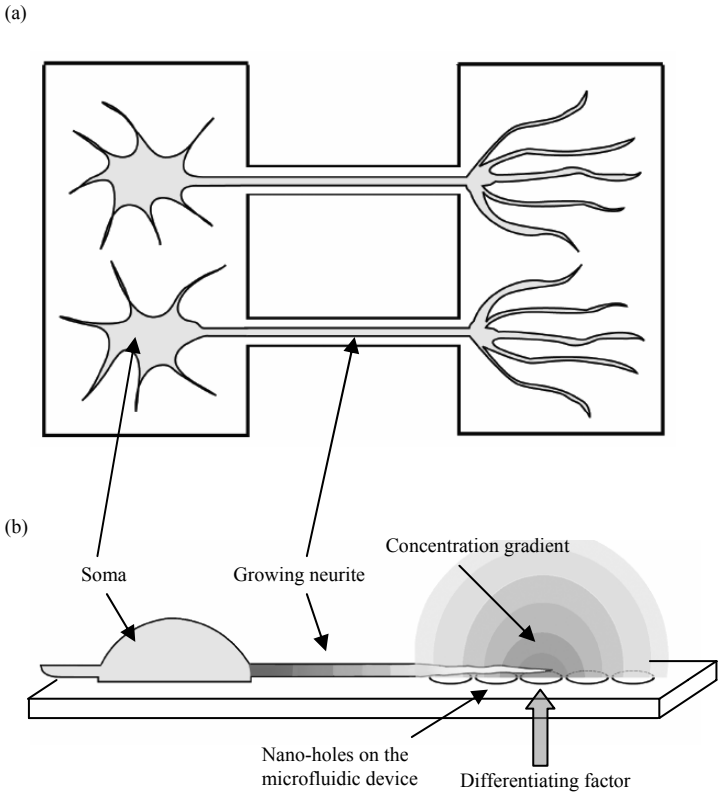

Figure 3: Schematic representation of two examples of possible applications of microfluidics for the study of neuronal differentiation. (a) Microfluidics allows the separation of axons and dendrites. (b) Concentration gradients of differentiating factors allow the guidance of axons.

A microfluidic device designed for the generation of gradient concentrations, without the generation of significant shear stress on the cells, is a powerful tool for the investigation of the mechanisms behind the growth cone guidance. I this device cells can live in the microfluidic channel and respond to the gradients in a similar way to that presented in physiological conditions [101]. Microfluidic devices can be combined with MEA or impedance technology to get the best of both. The cellular nutrient requirements or stimulation protocols can be satisfied with the help of a microfluidic system while maintaining the small

sample volume of the biosensor and avoiding the modification of the cell´s extracellular environment. Constant medium supply allows the performance of long term experiments while parallelization of channels facilitates the comparison between samples and controls [84]. MEAs can get extracellular simultaneous recordings of a large number of cells in the network while a microfluidic device supplements drugs or nutrients to a certain point of the network [102]. The design of narrow and long microtunnels with integrated electrodes allows the recording of single axon spikes and its propagation [61].

The appearance of nanostructures will revolutionize the research and treatment of neurodegenerative diseases, since many applications, such as the use of microrobots for axonal guidance, present huge advantages compared to conventional methods [103].

Conclusions and challenges for the future

The study of neuronal differentiation has experienced a rapid progress in the last few years, which did not correspond to the technical advances usually employed in research laboratories. Classical 2D cell line cultures are very useful since they represent a cheap source of unlimited material for drug screening, but they fail to predict the actual response of a physiological tissue. 3D models resemble in a better manner the physiological situation and also introduce a new challenge to drug screening, since techniques able to deal with the third dimension are very rare. The use of 3D models for drug screening would reduce the number of compounds that fail in clinical trials. Novel biosensors and advanced techniques emerge as a revolution in the field of developmental neuroscience, since they can overcome these difficulties. They offer the possibility of automation, single cell and even subcellular measurements, 3D cell culture and tissue slice testing, they can be manipulated to generate a desired environment, decide the shape, the gradient concentration, use extremely low reactive volumes, etc, and all at a low cost. Of course every biosensor application needs to be appropriately tested. It is very important that all the biosensor's materials in contact with cells are biocompatible. Biocompatibility tests based on adhesion, spreading, proliferation, metabolic activity or gene expression are needed to get reliable results [104]. The researcher should decide which application is more suitable for her/his particular research. A new interdisciplinary science is emerging, the future science of neurodevelopmental biosensing.

List of Abbreviations

AC:	Adenylate Cyclase
AMPK	AMP-Activated Protein Kinase
ATP	Adenosine-5'-triphosphate
cAMP:	Cyclic Adenosine Monophosphate
cGMP:	Cyclic Guanosine Monophosphate

CMOS	Complementary Metal–Oxide–Semiconductor
CNS:	Central Nervous System
CRE	cAMP Response Element
CREB:	cAMP Response Element-Binding Protein
db-c-AMP:	Dibutyryl Cyclic Adenosine Monophosphate
ECM:	Extracellular Matrix
eNOS:	Endothelial Nitric oxide synthase
ERRa	Estrogen Related Receptor a
G:	G Protein
HIF1α/β	Hypoxia-Inducible Factor
MEA	Microelectrode array
MEF-2	Myocyte Enhancer Factor-2
mRNA:	Messenger Ribonucleic Acid
NGF:	Nerve Growth Factor
nNOS:	Neuronal Nitric Oxide Synthase
NO:	Nitric Oxide
NRF	Nuclear Respiratory Factor
PAC1:	PACAP Type 1 Receptor
PACAP:	Pituitary Adenylate Cyclase-Activating Peptide
PDMS	Poly(dimethylsiloxane)
PEI	Polyethyleneimine
PGC-1β	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1beta
PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1alpha
PKA:	Protein Kinase A
PKG	Protein Kinase G
PPAR	Peroxisome Proliferator-Activated Receptors
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SOD	Superoxide Dismutase
Tfam:	Transcription Factor A
TrkA:	Neurotrophic Tyrosine Kinase Receptor Type 1

Conflict of Interest

This work was founded by the EU Marie-Curie Research Training Network project “CellCheck: On-chip cell handling and analysis” reference: MRTN-CT-2006-035854 project. No conflicts of interest exist.

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References

[1] Briscoe, J.; Novitch, B.G. Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. *Philos. Trans. R. Soc. London, Ser. B*, **2008**, *363*(1489), 57-70.

[2] Radio, N.M.; Mundy, W.R. Developmental neurotoxicity testing in vitro: Models for assessing chemical effects on neurite outgrowth. *Neurotoxicol.*, **2008**, *29*(3), 361-376.

[3] Connolly, G.P. Cell imaging and morphology - Application to studies of inherited purine metabolic disorders. *Pharmacol. Ther.*, **2001**, *90*(2-3), 267-281.

[4] O'Shaughnessy, T.J.; Liu, J.L.; Ma, W. Passaged neural stem cell-derived neuronal networks for a portable biosensor. *Biosens. Bioelectron.,* **2009**, *24*(8), 2365-2370.

[5] Read, D.E.; Herbert, K.R.; Gorman, A.M. Heat shock enhances NGF-induced neurite elongation which is not mediated by Hsp25 in PC12 cells. *Brain Res.*, **2008**, *1221*, 14-23.

[6] Kathleen Baxter, K.; Uittenbogaard, M.; Yoon, J.; Chiaramello, A. The neurogenic basic helix-loop-helix transcription factor NeuroD6 concomitantly increases mitochondrial mass and regulates cytoskeletal organization in the early stages of neuronal differentiation. *ASN NEURO*, **2009**, *1*(4), 195-211.

[7] De Girolamo, L.A.; Hargreaves, A.J.; Billett, E.E. Protection from MPTP-induced neurotoxicity in differentiating mouse N2a neuroblastoma cells. *J. Neurochem.*, **2001**, *76*(3), 650-660.

[8] Ravni, A.; Bourgault, S.; Lebon, A.; Chan, P.; Galas, L.; Fournier, A.; Vaudry, H.; Gonzalez, B.; Eiden, L.E.; Vaudry, D. The neurotrophic effects of PACAP in PC12 cells: control by multiple transduction pathways. *J. Neurochem*., **2006**, *98*(2), 321-329.

[9] McIlvain, H.B.; Baudy, A.; Sullivan, K.; Liu, D.; Pong, K.; Fennell, M.; Dunlop, J. Pituitary adenylate cyclase-activating peptide (PACAP) induces differentiation in the neuronal F11 cell line through a PKA-dependent pathway. *Brain Res.*, **2006**, *1077*, 16-23.

[10] Pisegna, J.R.; Oh, D.S. Pituitary adenylate cyclase-activating polypeptide: a novel peptide with protean implications. *Curr. Opin. Endocrinol., Diabetes & Obesity*, **2007**, 14(1), 58-62.

[11] Wang, G.; Qi, C.; Fan, G.H.; Zhou, H.Y.; Chen, S.D. PACAP protects neuronal differentiated PC12 cells against the neurotoxicity induced by a mitochondrial complex I inhibitor, rotenone. *Febs Lett.*, **2005**, *579*(18), 4005-4011.

[12] Bogacka, I.; Ukropcova, B.; McNeil, M.; Gimble, J.M.; Smith, S.R. Structural and functional consequences of mitochondrial biogenesis in human adipocytes in vitro. *J. Clin. Endocrinol. Metab*., **2005**, *90*(12), 6650-6656.

[13] Slaughter, G.E.; Hobson, R. An impedimetric biosensor based on PC 12 cells for the monitoring of exogenous agents. *Biosens. Bioelectron.*, **2009**, *24*(5), 1153-1158.

[14] Merot, Y.; Ferriere, F.; Debroas, E.; Flouriot, G.; Duval, D.; Saligaut, C. Estrogen receptor alpha mediates neuronal differentiation and neuroprotection in PC12 cells: critical role of the A/B domain of the receptor. *J. Mol. Endocrinol*., **2005**, *35*(2), 257-267.

[15] Pacary, E.; Tixier, E.; Coulet, F.; Roussel, S.; Petit, E.; Bernaudin, M. Crosstalk between HIF-1 and ROCK pathways in neuronal differentiation of mesenchymal stem cells, neurospheres and in PC12 neurite outgrowth. *Mol. Cell. Neurosci*., **2007**, *35*(3), 409-423.

[16] Gutsaeva, D.R.; Carraway, M.S.; Suliman, H.B.; Demchenko, I.T.; Shitara, H.; Yonekawa, H.; Piantadosi, C.A. Transient hypoxia stimulates mitochondrial biogenesis in brain subcortex by a neuronal nitric oxide synthase dependent mechanism. *J. Neurosci*., **2008**, *28*(9), 2015-2024.

[17] Inoue, S.; Motod, H.; Koike, Y.; Kawamura, K.; Hiragami, F.; Kano, Y. Microwave irradiation induces neurite outgrowth in PC12m3 cells via the p38 mitogen-activated protein kinase pathway. *Neurosci. Lett*., **2008**, *432*(1), 35-39.

[18] Watkins, J.; Basu, S.; Bogenhagen, D.F. A quantitative proteomic analysis of mitochondrial participation in P19 cell neuronal differentiation. *J. Proteome Res.*, **2008**, *7*(1), 328-338.

[19] De Girolamo, L.A.; Billett, E.E. Role of extracellular-regulated kinase and c-Jun NH2-terminal kinase in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurofilament phosphorylation. *J. Neurosci. Res.*, **2006**, *83*(4), 680-693.

[20] Flaskos, J.; McLean, W.G.; Fowler, M.J.; Hargreaves, A.J. Tricresyl phosphate inhibits the formation of axon-like processes and disrupts neurofilaments in cultured mouse N2a and rat PC12 cells. *Neurosci. Lett.*, **1998**, *242*(2), 101-104.

[21] Ng, Y.P.; Wu, Z.G.; Wise, H.; Tsim, K.W.K.; Wong, Y.H.; Ip, N.Y. Differential and Synergistic Effect of Nerve Growth Factor and cAMP on the Regulation of Early Response Genes during Neuronal Differentiation. *Neurosignals*, **2009**, *17*(2), 111-120.
[22] Mattson, M.P.; Taylorhunter, A.; Kater, S.B. Neurite Outgrowth in Individual Neurons of a Neuronal Population Is Differentially Regulated by Calcium and Cyclic-Amp. *J. Neurosci.*, **1988**, *8*(5), 1704-1711.
[23] Kuenzi, P.; Kiefer, S.; Koryakina, A.; Hamburger, M. Promotion of cell death or neurite outgrowth in PC-12 and N2a cells by the fungal alkaloid militarinone A depends on basal expression of p53. *Apoptosis*, **2008**, *13*(3), 364-376.
[24] Pampaloni, F.; Reynaud, E.G.; Stelzer, E.H.K. The third dimension bridges the gap between cell culture and live tissue. *Nat. Rev. Mol. Cell Biol.*, **2007**, *8*, 839-845.
[25] Wang, L.; Wu, Z.Z.; Xu, B.Q.; Zhao, Y.P.; Kisaalita, W.S. SU-8 microstructure for quasi-three-dimensional cell-based biosensing. *Sens. Actuators, B*, **2009**, *140*(2), 349-355.
[26] Birgersdotter, A.; Sandberg, R.; Ernberg, I. Gene expression perturbation in vitro - A growing case for three-dimensional (3D) culture systems. *Semin. Cancer Biol.*, **2005**, *15*(5), 405-412.
[27] Li, G.N.; Livi, L.L.; Gourd, C.M.; Deweerd, E.S.; Hoffman-Kim, D. Genomic and morphological changes of neuroblastoma cells in response to three-dimensional matrices. *Tissue Eng.*, **2007**, *13*(5), 1035-1047.
[28] Gingras, M.; Beaulieu, M.M.; Gagnon, V.; Durham, H.D.; Berthod, F. In vitro study of axonal migration and myelination of motor neurons in a three-dimensional tissue-engineered model. *Glia*, **2008**, *56*(3), 354-364.
[29] Lin, P.W.; Wu, C.C.; Chen, C.H.; Ho, H.O.; Chen, Y.C.; Sheu, M.T. Characterization of cortical neuron outgrowth in two- and three-dimensional culture systems. *J. Biomed. Mater. Res. Part B*, **2005**, *75B*(1), 146-157.
[30] Cullen, D.K.; Lessing, M.C.; LaPlaca, M.C. Collagen-dependent neurite outgrowth and response to dynamic deformation in three-dimensional neuronal cultures. *Ann. Biomed. Eng.*, **2007**, *35*(5), 835-846.
[31] Mao, C.; Kisaalita, W.S. Characterization of 3-D collagen hydrogels for functional cell-based biosensing. *Biosens. Bioelectron.*, **2004**, *19*(9), 1075-1088.
[32] O'Connor, S.M.; Stenger, D.A.; Shaffer, K.M.; Ma, W. Survival and neurite outgrowth of rat cortical neurons in three-dimensional agarose and collagen gel matrices. *Neurosci. Lett.*, **2001**, *304*(3), 189-193.
[33] Desai, A.; Kisaalita, W.S.; Keith, C.; Wu, Z.Z. Human neuroblastoma (SH-SY5Y) cell culture and differentiation in 3-D collagen hydrogels for cell-based biosensing. *Biosens. Bioelectron.*, **2006**, *21*(8), 1483-1492.
[34] Wu, Z.Z.; Zhao, Y.P.; Kisaalita, W.S. A packed Cytodex microbead array for three-dimensional cell-based biosensing. *Biosens. Bioelectron.*, **2006**, *22*(5), 685-693.
[35] Ong, S.M.; Zhang, C.; Toh, Y.C.; Kim, S.H.; Foo, H.L.; Tan, C.H.; van Noort, D.; Park, S.; Yu, H. A gel-free 3D microfluidic cell culture system. *Biomaterials*, **2008**, *29*(22), 3237-3244.
[36] Pautot, S.; Wyart, C.; Isacoff, E.Y. Colloid-guided assembly of oriented 3D neuronal networks. *Nat. Meth.*, **2008**, *5*(8), 735-740.
[37] Lovat, P.E.; Lowis, S.P.; Pearson, A.D.J.; Malcolm, A.J.; Redfern, C.P.F. Concentration-Dependent Effects of 9-Cis Retinoic Acid on Neuroblastoma-Differentiation and Proliferation in-Vitro. *Neurosci. Lett.*, **1994**, *182*(1), 29-32.
[38] Vaudry, D.; Stork, P.J.S.; Lazarovici, P.; Eiden, L.E. Signaling pathways for PC12 cell differentiation: Making the right connections. *Science*, **2002**, *296*(5573), 1648-1649.
[39] Contestabile, A.; Ciani, E. Role of nitric oxide in the regulation of neuronal proliferation, survival and differentiation. *Neurochem. Int.*, **2004**, *45*(6), 903-914.
[40] Contestabile, A. Regulation of transcription factors by nitric oxide in neurons and in neural-derived tumor cells. *Prog. Neurobiol.*, **2008**, *84*(4), 317-328.
[41] Onyango, I.G.; Lu, J.; Rodova, M.; Lezi, E.; Crafter, A.B.; Swerdlow, R.H. Regulation of neuron mitochondrial biogenesis and relevance to brain health. *Biochim. Biophys. Acta, Mol. Basis Dis.*, **2010**, *1802*(1), 228-234.
[42] Scarpulla, R.C. Nuclear activators and coactivators in mammalian mitochondrial biogenesis. *Biochim. Biophys. Acta, Gene Struct. Expression*, **2002**, *1576*(1-2), 1-14.

[43] Liang, J.; Yang, Y.; Zhu, X.; Wang, X.; Chen, R. Down-expression of PGC-1alpha partially mediated by JNK/c-Jun through binding to CRE site during apoptotic procedure in cerebellar granule neurons. *J. Neurosci. Res.*, **2010**, *88*(9), 1918-1925.
[44] St-Pierre, J.; Drori, S.; Uldry, M.; Silvaggi, J.M.; Rhee, J.; Jager, S.; Handschin, C.; Zheng, K.N.; Lin, J.D.; Yang, W.L.; Simon, D.K.; Bachoo, R.; Spiegelman, B.M. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell*, **2006**, *127*(2), 397-408.
[45] Schwamborn, J.C.; Fiore, R.; Bagnard, D.; Kappler, J.; Kaltschmidt, C.; Puschel, A.W. Semaphorin 3A stimulates neurite extension and regulates gene expression in PC12 cells. *J. Biol. Chem.*, **2004**, *279*(30), 30923-30926.
[46] Davis, J.M.; Murphy, E.A.; Carmichael, M.D.; Davis, B. Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **2009**, *296*(4), R1071-R1077.
[47] Dasgupta, B.; Milbrandt, J. Resveratrol stimulates AMP kinase activity in neurons. *Proc. Natl. Acad. Sci. U. S. A.*, **2007**, *104*(17), 7217-7222.
[48] Yin, W.; Signore, A.P.; Iwai, M.; Cao, G.D.; Gao, Y.Q.; Chen, J. Rapidly Increased Neuronal Mitochondrial Biogenesis After Hypoxic-Ischemic Brain Injury. *Stroke*, **2008**, *39*(11), 3057-3063.
[49] Uittenbogaard, M.; Chiaramello, A. The basic helix-loop-helix transcription factor Nex-1/Math-2 promotes neuronal survival of PC12 cells by modulating the dynamic expression of anti-apoptotic and cell cycle regulators. *J. Neurochem.*, **2005**, *92*(3), 585-596.
[50] Uittenbogaard, M.; Baxter, K.K.; Chiaramello, A. NeuroD6 Genomic Signature Bridging Neuronal Differentiation to Survival Via the Molecular Chaperone Network. *J. Neurosci. Res.*, *88*(1), 33-54.
[51] Wu, C.W.; Ping, Y.H.; Yen, J.C.; Chang, C.Y.; Wang, S.F.; Yeh, C.L.; Chi, C.W.; Lee, H.C. Enhanced oxidative stress and aberrant mitochondrial biogenesis in human neuroblastoma SH-SY5Y cells during methamphetamine induced apoptosis. *Toxicol. Appl. Pharmacol.*, **2007**, *220*(3), 243-251.
[52] Sachana, M.; Flaskos, J.; Alexaki, E.; Glynn, P.; Hargreaves, A.J. The toxicity of chlorpyrifos towards differentiating mouse N2a neuroblastoma cells. *Toxicol. in Vitro*, **2001**, *15*(4-5), 369-372.
[53] Georgopoulou, N.; Hurel, C.; Politis, P.K.; Gaitanou, M.; Matsas, R.; Thomaidou, D. BM88 Is a Dual Function Molecule Inducing Cell Cycle Exit and Neuronal Differentiation of Neuroblastoma Cells via Cyclin D1 Down-regulation and Retinoblastoma Protein Hypophosphorylation. *J. Biol. Chem.*, **2006**, *281*(44), 33606-33620.
[54] Tabata, K.; Yamazaki, Y.; Okada, M.; Fukumura, K.; Shimada, A.; Sun, Y.; Yasukawa, K.; Suzuki, T. Diarylheptanoids Derived from Alpinia officinarum Induce Apoptosis, S-Phase Arrest and Differentiation in Human Neuroblastoma Cells. *Anticancer Res.*, **2009**, *29*(12), 4981-4988.
[55] Cremisi, F.; Philpott, A.; Ohnuma, S. Cell cycle and cell fate interactions in neural development. *Curr. Opin Neurobiol.*, **2003**, *13*(1), 26-33.
[56] Galderisi, U.; Jori, F.P.; Giordano, A. Cell cycle regulation and neural differentiation. *Oncogene*, **2003**, *22*(33), 5208-5219.
[57] Lentz, S.I.; Edwards, J.L.; Backus, C.; McLean, L.L.; Haines, K.M.; Feldman, E.L. Mitochondrial DNA (mtDNA) Biogenesis: Visualization and Duel Incorporation of BrdU and EdU Into Newly Synthesized mtDNA In Vitro. *J. Histochem. Cytochem.*, *58*(2), 207-218.
[58] Ramm, P.; Alexandrov, Y.; Cholewinski, A.; Cybuch, Y.; Nadon, R.; Soltys, B.J. Automated Screening of Neurite Outgrowth. *J. Biomol. Screen.*, **2003**, *8*(1), 7-18.
[59] Keller, P.J.; Pampaloni, F.; Stelzer, E.H.K. Life sciences require the third dimension. *Curr. Opin. Cell Biol.*, **2006**, *18*(1), 117-124.
[60] Kang, G.; Lee, J.-H.; Lee, C.-S.; Nam, Y. Agarose microwell based neuronal micro-circuit arrays on microelectrode arrays for high throughput drug testing. *Lab Chip*, **2009**, *9*(22).
[61] Dworak, B.J.; Wheeler, B.C. Novel MEA platform with PDMS microtunnels enables the detection of action potential propagation from isolated axons in culture. *Lab Chip*, **2009**, *9*(3), 404-410.
[62] Hofmann, F.; Bading, H. Long term recordings with microelectrode arrays: Studies of transcription-dependent neuronal plasticity and axonal regeneration. *J. Physiology-Paris*, **2006**, *99*(2-3), 125-132.
[63] Ma, Y.L.; Weston, S.E.; Whalley, B.J.; Stephens, G.J. The phytocannabinoid Delta(9)-tetrahydrocannabivarin modulates inhibitory neurotransmission in the cerebellum. *Brit. J. Pharmacol.*, **2008**, *154*(1), 204-215.

[64] Frey, U.; Egert, U.; Heer, F.; Hafizovic, S.; Hierlemann, A. Microelectronic system for high-resolution mapping of extracellular electric fields applied to brain slices. *Biosens. Bioelectron.*, **2009**, *24*(7), 2191-2198.
[65] Dimaki, M.; Vazquez, P.; Olsen, M.H.; Sasso, L.; Rodriguez-Trujillo, R.; Vedarethinam, I.; Svendsen, W.E. Fabrication and Characterization of 3D Micro- and Nanoelectrodes for Neuron Recordings. *Sensors*, **2010**, *10*(11), 10339-10355.
[66] Kopanitsa, M.V.; O Afinowi, N.; Grant, S.G.N. Recording long-term potentiation of synaptic transmission by three-dimensional multi-electrode arrays. *BMC Neurosci.*, **2006**, *7*.
[67] Illes, S.; Fleischer, W.; Slebler, M.; Hartung, H.P.; Dihne, M. Development and pharmacological modulation of embryonic stem cell-derived neuronal network activity. *Exp. Neurol.*, **2007**, *207*(1), 171-176.
[68] Jungblut, M.; Knoll, W.; Thielemann, C.; Pottek, M. Triangular neuronal networks on microelectrode arrays: an approach to improve the properties of low-density networks for extracellular recording. *Biomed. Microdevices*, **2009**, *11*(6), 1269-1278.
[69] Bieberich, E.; Guiseppi-Elie, A. Neuronal differentiation and synapse formation of PC12 and embryonic stem cells on interdigitated microelectrode arrays: Contact structures for neuron-to-electrode signal transmission (NEST). *Biosens. Bioelectron.*, **2004**, *19*(8), 923-931.
[70] Shein, M.; Greenbaum, A.; Gabay, T.; Sorkin, R.; David-Pur, M.; Ben-Jacob, E.; Hanein, Y. Engineered neuronal circuits shaped and interfaced with carbon nanotube microelectrode arrays. *Biomed. Microdevices*, **2009**, *11*(2), 495-501.
[71] Gabriel, G.; Gomez, R.; Bongard, M.; Benito, N.; Fernandez, E.; Villa, R. Easily made single-walled carbon nanotube surface microelectrodes for neuronal applications. *Biosens. Bioelectron.*, **2009**, *24*(7), 1942-1948.
[72] Chang, W.C.; Sretavan, D.W. Single cell and neural process experimentation using laterally applied electrical fields between pairs of closely apposed microelectrodes with vertical sidewalls. *Biosens. Bioelectron.*, **2009**, *24*(12), 3600-3607.
[73] Braeken, D.; Huys, R.; Loo, J.; Bartic, C.; Borghs, G.; Callewaert, G.; Eberle, W. Localized electrical stimulation of in vitro neurons using an array of sub-cellular sized electrodes. *Biosens. Bioelectron.*, *In Press.*
[74] Xiang, G.X.; Pan, L.B.; Huang, L.H.; Yu, Z.Y.; Song, X.D.; Cheng, J.; Xing, W.L.; Zhou, Y.X. Microelectrode array-based system for neuropharmacological applications with cortical neurons cultured in vitro. *Biosens. Bioelectron.*, **2007**, *22*(11), 2478-2484.
[75] Morales, R.; Riss, M.; Wang, L.; Gavin, R.; Del Rio, J.A.; Alcubilla, R.; Claverol-Tinture, E. Integrating multi-unit electrophysiology and plastic culture dishes for network neuroscience. *Lab Chip*, **2008**, *8*(11), 1896-1905.
[76] Lisdat, F.; Schafer, D. The use of electrochemical impedance spectroscopy for biosensing. *Anal. Bioanal. Chem.*, **2008**, *391*(5), 1555-1567.
[77] Kyle, A.H.; Chan, C.T.O.; Minchinton, A.I. Characterization of three-dimensional tissue cultures using electrical impedance spectroscopy. *Biophys. J.*, **1999**, *76*(5), 2640-2648.
[78] Mercanzini, A.; Cheung, K.; Buhl, D.L.; Boers, M.; Maillard, A.; Colin, P.; Bensadoun, J.C.; Bertsch, A.; Renaud, P. Demonstration of cortical recording using novel flexible polymer neural probes. *Sens. Actuators, A*, **2008**, *143*(1), 90-96.
[79] Williams, J.C.; Hippensteel, J.A.; Dilgen, J.; Shain, W.; Kipke, D.R., Complex impedance spectroscopy for monitoring tissue responses to inserted neural implants. *J. Neural Eng.*, **2007**, *4*(4), 410-423.
[80] Frampton, J.P.; Hynd, M.R.; Williams, J.C.; Shuler, M.L.; Shain, W. Three-dimensional hydrogel cultures for modeling changes in tissue impedance around microfabricated neural probes. *J. Neural Eng.*, **2007**, *4*(4), 399-409.
[81] Kloss, D.; Fischer, M.; Rothermel, A.; Simon, J.C.; Robitzki, A.A. Drug testing on 3D in vitro tissues trapped on a microcavity chip. *Lab Chip*, **2008**, *8*(6), 879-884.
[82] Dalmay, C.; Cheray, M.; Pothier, A.; Lalloue, F.; Jauberteau, M.O.; Blondy, P. Ultra sensitive biosensor based on impedance spectroscopy at microwave frequencies for cell scale analysis. *Sens. Actuators, A*, **2010**, *162*(2), 189-197.
[83] Valero, T.; Jacobs, T.; Moschopoulou, G.; Naumann, M.; Hauptmann, P.; Kintzios, S. Electrical impedance analysis of N2a neuroblastoma cells in gel matrices after ACh-receptor triggering with an impedimetric biosensor. *Procedia Chem.*, **2009**, *1*, 734–737.

[84] Valero, T.; Moschopoulou, G.; Kintzios, S.; Hauptmann, P.; Naumann, M.; Jacobs, T. Studies on neuronal differentiation and signalling processes with a novel impedimetric biosensor. *Biosens. Bioelectron.*, **2010**, *26*(4), 1407-1413.
[85] Ehlers, A.; Stempin, S.; Al-Hamwi, R.; Lampen, A. Embryotoxic effects of the marine biotoxin okadaic acid on murine embryonic stem cells. *Toxicon*, **2010**, *55*,(4), 855-863.
[86] Hildebrandt, C.; Büth, H.; Cho, S.; Impidjati; Thielecke, H. Detection of the osteogenic differentiation of mesenchymal stem cells in 2D and 3D cultures by electrochemical impedance spectroscopy. *J. Biotechnol.*, **2010**, *148*(1), 83-90.
[87] Frimat, J.-P.; Sisnaiske, J.; Subbiah, S.; Menne, H.; Godoy, P.; Lampen, P.; Leist, M.; Franzke, J.; Hengstler, J.G.; Thriel, C.v.; West, J. The network formation assay: a spatially standardized neurite outgrowth analytical display for neurotoxicity screening. *Lab Chip*, **2010**, *10*, 701-709.
[88] Yamazoe, H.; Tanabe, T. Cell micropatterning on an albumin-based substrate using an inkjet printing technique. *J. Biomed. Mater. Res. Part A*, **2009**, *91A*(4), 1202-1209.
[89] Dos Reis, G.; Fenili, F.; Gianfelice, A.; Bongiorno, G.; Marchesi, D.; Scopelliti, P.E.; Borgonovo, A.; Podesta, A.; Indrieri, M.; Ranucci, E.; Ferruti, P.; Lenardi, C.; Milani, P. Direct Microfabrication of Topographical and Chemical Cues for the Guided Growth of Neural Cell Networks on Polyamidoamine Hydrogels. *Macromol. Biosci.*, *10*(8), 842-852.
[90] Ruiz, A.; Buzanska, L.; Gilliland, D.; Rauscher, H.; Sirghi, L.; Sobanski, T.; Zychowicz, M.; Ceriotti, L.; Bretagnol, F.; Coecke, S.; Colpo, P.; Ross, F. Micro-stamped surfaces for the patterned growth of neural stem cells. *Biomaterials*, **2008**, *29*(36), 4766-4774.
[91] Liu, Q.Y.; Coulombe, M.; Dumm, J.; Shaffer, K.M.; Schaffner, A.E.; Barker, J.L.; Pancrazio, J.J.; Stenger, D.A.; Ma, W. Synaptic connectivity in hippocampal neuronal networks cultured on micropatterned surfaces. *Dev. Brain Res.*, **2000**, *120*(2), 223-231.
[92] Angela, K.V.; Lars, L.; Wolfgang, K.; Andreas, O. Micropatterned Substrates for the Growth of Functional Neuronal Networks of Defined Geometry. *Biotechnol. Progr.*, **2003**, *19*(5), 1562-1568.
[93] Bauwens, C.L.; Peerani, R.; Niebruegge, S.; Woodhouse, K.A.; Kumacheva, E.; Husain, M.; Zandstra, P.W. Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. *Stem Cells*, **2008**, *26*(9), 2300-2310.
[94] Papenburg, B.J.; Vogelaar, L.; Bolhuis-Versteeg, L.A.M.; Lammertink, R.G.H.; Stamatialis, D.; Wessling, M. One-step fabrication of porous micropatterned scaffolds to control cell behavior. *Biomaterials*, **2007**, *28*(11), 1998-2009.
[95] Oh, J.S.; Recknor, J.B.; Recknor, J.C.; Mallapragada, S.K.; Sakaguchi, D.S. Soluble factors from neocortical astrocytes enhance neuronal differentiation of neural progenitor cells from adult rat hippocampus on micropatterned polymer substrates. *J. Biomed. Mater. Res. Part A*, **2009**, *91A*(2), 575-585.
[96] Buzanska, L.; Ruiz, A.; Zychowicz, M.; Rauscher, H.; Ceriotti, L.; Rossi, F.; Colpo, P.; Domansk-Janik, K.; Coecke, S. Patterned growth and differentiation of human cord blood-derived neural stem cells on bio-functionalized surfaces. *Acta Neurobiol. Exp.*, **2009**, *69*(1), 24-36.
[97] Weigl, B.H.; Bardell, R.L.; Cabrera, C.R. Lab-on-a-chip for drug development. *Adv. Drug Deliver. Rev.*, **2003**, *55*(3), 349-377.
[98] Nakashima, Y.; Yasuda, T. Cell differentiation guidance using chemical stimulation controlled by a microfluidic device. *Sens. Actuators, A*, **2007**, *139*(1-2), 252-258.
[99] Wu, H.-I.; Cheng, G.-H.; Wong, Y.-Y.; Lin, C.-M.; Fang, W.; Chow, W.-Y.; Chang, Y.-C. A lab-on-a-chip platform for studying the subcellular functional proteome of neuronal axons. *Lab Chip*, **2010**, *10*(5), 647-653.
[100] Hosmane, S.; Yang, I.H.; Ruffin, A.; Thakor, N.; Venkatesan, A. Circular compartmentalized microfluidic platform: Study of axon-glia interactions. *Lab Chip*, **2010**, *10*, 741-747.
[101] Wang, C.J.; Li, X.; Lin, B.; Shim, S.; Ming, G.-l.; Levchenko, A. A microfluidics-based turning assay reveals complex growth cone responses to integrated gradients of substrate-bound ECM molecules and diffusible guidance cues. *Lab Chip*, **2008**, *8*(2), 227-237.
[102] Kraus, T.; Verpoorte, E.; Linder, V.; Franks, W.; Hierlemann, A.; Heer, F.; Hafizovic, S.; Fujii, T.; Rooij, N.F.d.; Koster, S. Characterization of a microfluidic dispensing system for localised stimulation of cellular networks. *Lab Chip*, **2006**, *6*(2), 218-229.
[103] Modi, G.; Pillay, V.; Choonara, Y.E.; Ndesendo, V.M.K.; du Toit, L.C.; Naidoo, D. Nanotechnological applications for the treatment of neurodegenerative disorders. *Prog. Neurobiol.*, **2009**, *88*(4), 272-285.

[104] Stangegaard, M.; Wang, Z.; Kutter, J.P.; Dufva, M.; Wolff, A. Whole genome expression profiling using DNA microarray for determining biocompatibility of polymeric surfaces. *Mol. Biosyst.*, **2006**, *2*(9), 421-428.