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Departamento de Ciências Médicas

José Carlos Barreiro Mateus

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Odete Abreu Beirão da Cruz e Silva, Professora Auxiliar com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro.

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Dedico esta dissertação à minha família pela educação, apoio e amor que me deram ao longo de todo este percurso.

o júri

presidente

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palavras-chave

Eletrofisiologia, medições extracelulares, MEA, revestimento, doença de Alzheimer, Aβ, linha celular SH-SY5Y, diferenciação neuronal, rede neuronal

resumo

O cérebro humano armazena, integra e transmite informação recorrendo a milhões de neurónios, interconetados por inúmeras sinapses. Embora os neurónios comuniquem entre si através de sinais químicos, a informação é codificada e conduzida sob a forma de sinais elétricos. A neuroeletrofisiologia foca-se no estudo deste tipo de sinalização. Tanto abordagens intra, como abordagens extracelulares são usadas em investigação, mas nenhuma detém tanto potencial em *screening* de alto débito e na descoberta de fármacos, como medições extracelulares baseadas em matrizes de multi-elétrodos (MEA). MEAs medem a atividade neuronal, tanto em *in vitro* como em *in vivo*. A sua principal vantagem é a capacidade de medir atividade elétrica a partir de vários locais simultaneamente.

A doença de Alzheimer (DA) é a doença neurodegenerativa mais comum e uma das principais causas de morte em todo o mundo. É caracterizada por emaranhados neurofibrilares e agregados de péptidos amilóides (Aβ), que conduzem à perda de sinapses e em última instância, à morte neuronal. Atualmente, não existe cura e os tratamentos disponíveis apenas retardam a sua progressão. Os ensaios *in vitro* com MEA permitem uma seleção rápida dos compostos neuroprotectores e neurotóxicos. Portanto, as medições com recurso a MEA são de grande utilidade na investigação básica e clínica da DA.

O principal objetivo desta tese foi otimizar a formação de redes neuronais SH-SY5Y em MEAs. Estas podem ser extremamente úteis para instalações que não têm acesso a culturas neuronais primárias, mas também podem economizar recursos e facilitar a obtenção mais rápida de resultados para aquelas que têm acesso.

Compostos mediadores de adesão provaram afetar a morfologia, viabilidade e a exibição espontânea de atividade elétrica das células. Além disso, as células SH-SY5Y foram diferenciadas com sucesso e demonstraram efeitos agudos sobre a função neuronal após a adição de Aβ. Este efeito sobre a sinalização elétrica foi dependente da concentração dos oligómeros de Aβ.

Os resultados aqui apresentados permitem concluir que a linha celular SH-SY5Y pode ser diferenciada com sucesso em MEAs devidamente tratados e pode ser usada para avaliar os efeitos agudos do Aβ sobre a sinalização neuronal.

keywords

Electrophysiology, extracellular recordings, MEA, coating, Alzheimer's disease, Aβ, SH-SY5Y cell line, neuronal differentiation, neuronal network

abstract

The human brain stores, integrates, and transmits information recurring to millions of neurons, interconnected by countless synapses. Though neurons communicate through chemical signaling, information is coded and conducted in the form of electrical signals. Neuroelectrophysiology focus on the study of this type of signaling. Both intra and extracellular approaches are used in research, but none holds as much potential in high-throughput screening and drug discovery, as extracellular recordings using multielectrode arrays (MEAs). MEAs measure neuronal activity, both *in vitro* and *in vivo*. Their key advantage is the capability to record electrical activity at multiple sites simultaneously.

Alzheimer's disease (AD) is the most common neurodegenerative disease and one of the leading causes of death worldwide. It is characterized by neurofibrillar tangles and aggregates of amyloid-β (Aβ) peptides, which lead to the loss of synapses and ultimately neuronal death. Currently, there is no cure and the drugs available can only delay its progression. *In vitro* MEA assays enable rapid screening of neuroprotective and neuroharming compounds. Therefore, MEA recordings are of great use in both AD basic and clinical research.

The main aim of this thesis was to optimize the formation of SH-SY5Y neuronal networks on MEAs. These can be extremely useful for facilities that do not have access to primary neuronal cultures, but can also save resources and facilitate obtaining faster high-throughput results to those that do.

Adhesion-mediating compounds proved to impact cell morphology, viability and exhibition of spontaneous electrical activity. Moreover, SH-SY5Y cells were successfully differentiated and demonstrated acute effects on neuronal function after Aβ addition. This effect on electrical signaling was dependent on Aβ oligomers concentration.

The results here presented allow us to conclude that the SH-SY5Y cell line can be successfully differentiated in properly coated MEAs and be used for assessing acute Aβ effects on neuronal signaling.

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A	AD	Alzheimer's Disease
	ADDL	Aβ-derived Diffusible Ligand
	AICD	APP Intracellular Domain
	ALS	Amyotrophic Lateral Sclerosis
	ApoE	Apolipoprotein E
	AP	Action Potential
	APP	Amyloid Precursor Protein
	ATP	Adenosine Triphosphate
	Aβ	β-Amyloid peptide
B	BACE1	β-site APP-Cleaving Enzyme 1
	BDNF	Brain Derived Neurotrophic Factor
C	C83	C-terminal fragment with 83 amino acids
	C99	C-terminal fragment with 99 amino acids
	CNS	Central Nervous System
	CSF	Cerebrospinal Fluid
D	DMSO	Dimethyl Sulfoxide
	DNA	Deoxyribonucleic Acid
E	EAP	Extracellular Action Potential
	ECM	Extracellular Matrix
	EEG	Electroencephalography
	EPSP	Excitatory Postsynaptic Potential
F	FAK	Focal Adhesion Kinase
	FAD	Familial Alzheimer’s Disease
	FBS	Fetal Bovine Serum
	fMRI	Functional Magnetic Resonance Imaging
G	GABA	Gamma-Aminobutyric Acid
H	hESC	Human Embryonic Stem Cell
	HTS	High-Throughput Screening
I	IAP	Intracellular Action Potential
	iPS	Induced Pluripotent Stem Cell
	IPSP	Inhibitory Postsynaptic Potential
	iR	Internal Reference Electrode
	ISI	Interspike interval

L	LFP	Local Field Potential
M	MCI	Mild Cognitive Impairment
	MEA	Multi- or Microelectrode Array
	MEM	Minimal Essential Medium
	MRI	Magnetic Resonance Imaging
N	ND	Neurodegenerative Disease
	NFT	Neurofibrillary Tangles
	NGF	Nerve Growth Factor
	NeuN	Neuronal Nuclei
	NP	Neuritic Amyloid Plaque
O	ON	Overnight
	OS	Oxidative Stress
P	PBS	Phosphate Buffered Saline
	PD	Parkinson's Disease
	PDL	Poly-D-lysine
	PEI	Polyethyleneimine
	PLL	Poly-L-lysine
	PSEN	Presenilin
R	RA	Retinoic Acid
	RAR	Retinoic Acid Receptor
	qRT-PCR	Real Time Quantitative PCR
	ROI	Region of Interest
	RPM	Revolutions per minute
	RT	Room Temperature
	RXR	Retinoid X Receptor
S	sAPP	Soluble APP-fragment
	SNR	Signal-to-Noise Ratio
	SP	Senile Plaque
T	T-Control	Temperature Controller
	TiN	Titanium Nitride
	TKI	Tyrosine Kinase Inhibitor
U	UV	Ultraviolet

1. Introduction

1.1 Neuron

The nervous system regulates all aspects of body function. In order to accomplish this massive task, it must communicate fast and efficiently with all the structures involved, from the receptors to the effectors. The communicative role of the nervous system is carried out by nerve cells, or neurons (1,2). The human brain is the control center that stores, computes, integrates, and transmits information recurring to about 10^{11} neurons. These are interconnected by some 10^{14} synapses, the junction points where two or more neurons communicate. Glial cells occupy the spaces between neurons and help maintain and modulate neurons functions (1,3).

Despite the multiple types and shapes of neurons, all nerve cells share many common properties (2). Neurons' main function is to communicate information, which they do by two methods: electrical and chemical signals. Electrical signals process and conduct information within neurons. The electrical pulse that travels along neurons is called action potential (AP), and information can be encoded as the frequency at which APs are fired. Chemical signals (neurotransmitters) transmit information between neurons. Taken together, the electrical and chemical signaling of the nervous system allows it to detect stimuli, integrate and process the information received, then generate an appropriate response to the stimulus (3).

Neurons' communicative functions demand unique cell structures. Fundamental physiological properties such as excitability, conductivity and secretion require specialized structures such as dendrites, axons and synaptic vesicles containing neurotransmitters, respectively.

1.1.1 Structure

A representation of a single neuron and its main distinctive structures is shown in Fig. 1.

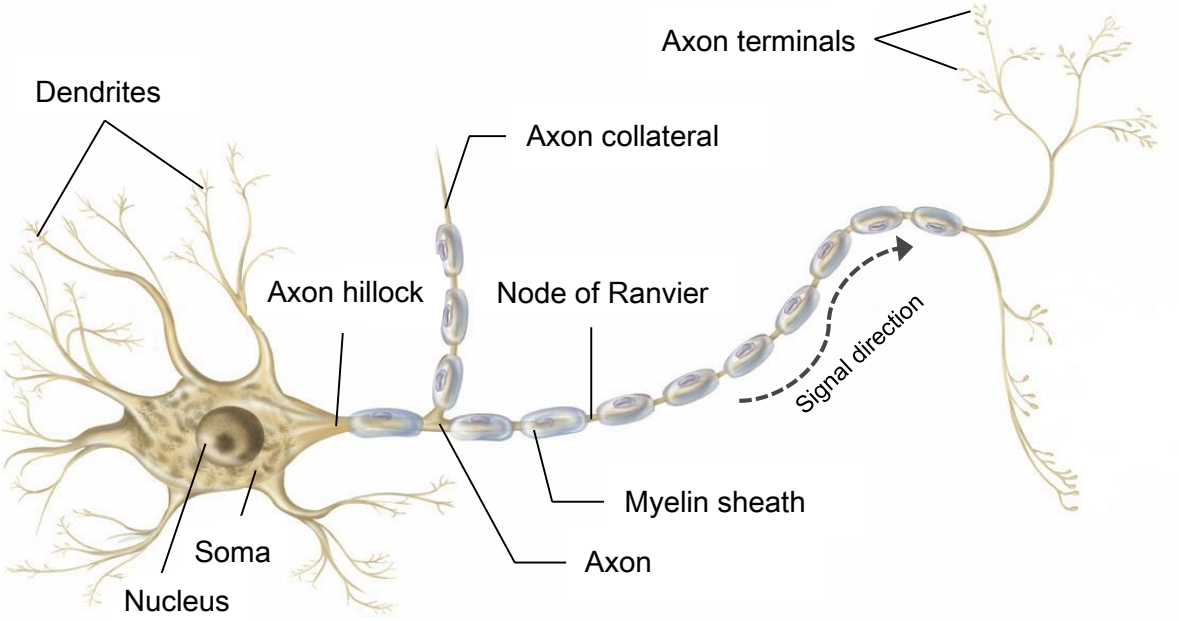


Figure 1 – Neuron. *Schematic representation of a multipolar neuron. Note that the electrical signal conduction occurs in the soma-axon terminals direction [adapted from (2)].*

The central part of the neuron, the soma or cell body, contains the nucleus, which is the site of most of the protein synthesis. The neuronal cell body processes the incoming signals via a direct spatial and temporal integration of the membrane potential shifts. The postsynaptic branches that originate from the cell body are called dendrites and are responsible for reception of excitatory or inhibitory input through the synapses (2,3). The axon hillock constitutes the interface between the cell body and the axon, as well as the "trigger zone" for APs generation. Growing outward from the axon hillock, the axon is a long extension that carries important subcellular components within the neuron and conducts the electrical signal to the next cell (1,3). The length of an axon can vary between 1 µm and 1 m, depending on the type of the nerve. Although the axon is relatively unbranched for most of its length, it may give rise to a few branches (axon collaterals) along the way. Still, most brain neuron's axons branch extensively at their distal end (terminal arborization). Here, each branch ends in a terminal button (axon terminal) that forms a synapse with another cell (2).

The axon can be coated by several sequential myelin sheaths. The myelin sheaths are formed in the central nervous system (CNS) by oligodendrocytes and in the peripheral nervous system (PNS) by Schwann cells. Small gaps are located between these myelin sheaths, called nodes of Ranvier. In myelinated axons of the same diameter, the nerve impulse conducts about ten times faster than in axons without myelin sheaths (2).This occurs because an AP current propagation is saltatory, as it jumps from node to node across the neuronal membrane (1).

Neurons contain the organelles found in most other cells, including the endoplasmic reticulum, Golgi apparatus, mitochondria, and a variety of vesicular structures. Yet, in comparison, these organelles are often localized in distinct regions of the cell. In addition to the distribution of organelles and subcellular components, neurons are in some measure different in the specialized fibrillar or tubular proteins that constitute the cytoskeleton (1). For example, neuron's microtubules extend along the axons due to their role in vesicular and subcellular components transport to the axon cytoplasm (axoplasm) and the axon terminals (1,2).

1.1.2 Electrical Signals

Neurons generate and conduct electrical signals that transmit information to other cells. These electrical signals may be produced in response to external stimuli. For example, receptor potentials are due to the activation of sensory neurons by external stimuli, such as pressure, light, sound, or heat. Yet, another type of electrical signal is associated with communication between neurons at synaptic contacts. Activation of these synapses generates synaptic potentials, which allow transmission of information between neurons. Both receptor and synaptic potentials are different types of graded potentials and trigger APs (also referred to as "spikes" or "impulses") (1,3).

On the basis of all these electrical signals are ion fluxes (mainly Na^+ and K^+) caused by the cell membrane's selective permeability to different ions, and the heterogeneous distribution of these ions across the membrane (1,3). These two facts depend on two different kinds of proteins in the neuronal membrane. The selective permeability is due largely to ion channels, proteins that allow only certain kinds of ions to cross the membrane in the direction of their concentration gradients. The ion concentration gradients are established by proteins known as active transporters, which

move ions into or out of the cell against their concentration gradients using energy in the form of adenosine triphosphate (ATP) (1).

A neuron receives its incoming signals in the form of neurotransmitters that originate in the presynaptic neuron. These are stored in synaptic vesicles and are released onto the synaptic cleft. Neurotransmitters induce synaptic potentials at the postsynaptic dendrites that lead to changes in membrane potential, which vary in size and decrease over time and space. These impulses are incremental and may be excitatory or inhibitory. Whether a neuron interacts in an inhibitory or excitatory way is determined by the synapses and depends on the type of the neurotransmitters it exchanges with the postsynaptic cells. An inhibitory neurotransmitter, like gamma-aminobutyric acid (GABA), produces inhibitory postsynaptic potentials (IPSPs), while an excitatory neurotransmitter like glutamate generates excitatory postsynaptic potentials (EPSPs). The magnitude of a synaptic potential is determined by the strength of the stimulus (1,3).

Like all excitable cells, neurons, have an inside negative voltage or electric potential gradient across their plasma membranes - the resting membrane potential [around -70 millivolts (mV)]. The resting membrane potential is the state when no signal is in transit, therefore this potential is reversed when an AP occurs. As neurotransmitters bind to the receptors of the postsynaptic cell, they trigger a depolarization of the resting membrane potential. If a certain membrane potential is surpassed – the threshold potential (ranging from –40mV to –55mV) – an AP is elicited (1,3). The signal is passed on over the dendrites to the cell body and from there to the axon hillock. The axon hillock contains the highest concentration of ion channels anywhere in the neuron, which reduces the threshold potential strongly and renders it a trigger for APs (1,3). An AP is only elicited at the axon hillock if the threshold potential is exceeded. Therefore, this process is often described as the “all-or-nothing law”: either there is an AP or there is none (1). At the beginning of the AP, Na^+ voltage-gated ion channels open as the membrane reaches the threshold potential. Na^+ ions rapidly move (influx) into the axon causing membrane depolarization. When the AP reaches its peak, Na^+ voltage-gated ion channels close and the K^+ voltage-gated ion channels begin to open. K^+ ions then exit (efflux) the axon and membrane repolarization occurs. As an AP travels down the axon, this change in polarity between the outside and the inside of the cell is constantly created (3). An AP plot showing the variations in membrane potential on a neuronal membrane site during AP conduction can be seen in Fig. 2A.

The best way to observe and measure APs is to use an intracellular microelectrode to measure the electrical potential across the neuronal membrane. A typical microelectrode is a piece of glass tubing, with an opening of less than 1 µm diameter, filled with a good electrical conductor such as a concentrated salt solution (1,4). A detailed understanding of the AP came only after the invention of the voltage clamp technique by Kenneth Cole in the 1940s. This device controls the membrane potential at any desired level by placing two microelectrodes inside the cell. Therefore, the voltage clamp technique indicates how membrane potential influences ionic current flow across the membrane (1). This method gave Hodgkin, A. and Huxley, A. (1952) the key insights that led to their model for AP generation (5). An AP may be “artificially” elicited by passing electrical current across the neuronal membrane. If current of the opposite polarity is delivered, so that depolarization occurs and the threshold potential is surpassed, an AP occurs (1).

Although APs can vary somewhat in duration, amplitude and shape, they are typically treated as identical stereotyped events in electrophysiological studies (6). If the brief duration of an AP (about 1ms) is ignored, an AP sequence ("spike train"), may be characterized simply by a series of all-or-none point events in time. The lengths of interspike intervals (ISIs) between two successive spikes in a spike train vary immensely (1,6,7). There is an ongoing debate on whether neurons use rate coding ("frequency coding") or temporal coding ("precise timing") to convey information (7,8).

When recording electrical activity of multiple neurons, intracellular techniques, such as voltage clamp, fail to measure the majority of occurring signal transmission. Field potentials, which represent the activity of very large groups of neurons, are routinely recorded along the scalp using electroencephalography (EEG) technology (9). In turn, this naturally occurring brain electrical activity produces magnetic fields, which can be measured by magnetoencephalography (MEG) (10). MEG, EEG and its variants are used in clinical practice to diagnose epilepsy, sleep disorders, coma, encephalopathies, brain tumors and brain death (9,10). However, their application in neurodegenerative diseases (NDs) research is very limited. In order to study such diseases at a molecular level, different recording techniques are needed.

On a minor scale, a local field potential (LFP) is an electrophysiological signal generated by the summed electric current flowing from multiple nearby neurons within a small volume of nervous tissue. It refers to the electric potential produced across the local extracellular space around neurons by APs and graded potentials, and varies as a result of synaptic activity (11). There is a lot of controversy about the sources of LFPs (11,12), still a synchrony of APs from many neurons seems to participate in their generation (12). LFPs can be measured recurring to extracellular recording techniques such as multielectrode arrays (11,13).

The electrical nature of neuronal activity makes it possible to detect signals on electrodes at a distance from the source, but not without limitations. It is essential to determine the recording capabilities and limits of the device used and to understand how the neuronal signal is transduced into a recorded digital form (12). A schematic comparison between an AP sign recorded by intracellular and extracellular electrodes can be seen in Fig. 2B.

Unlike the stereotypic uniformity of intracellularly recorded APs, extracellular recording renders traces that vary markedly in shape. They depend on the signal source, cell type and geometry, its developmental stage and with it the type, ratio and density of expressed (channel) proteins (14).

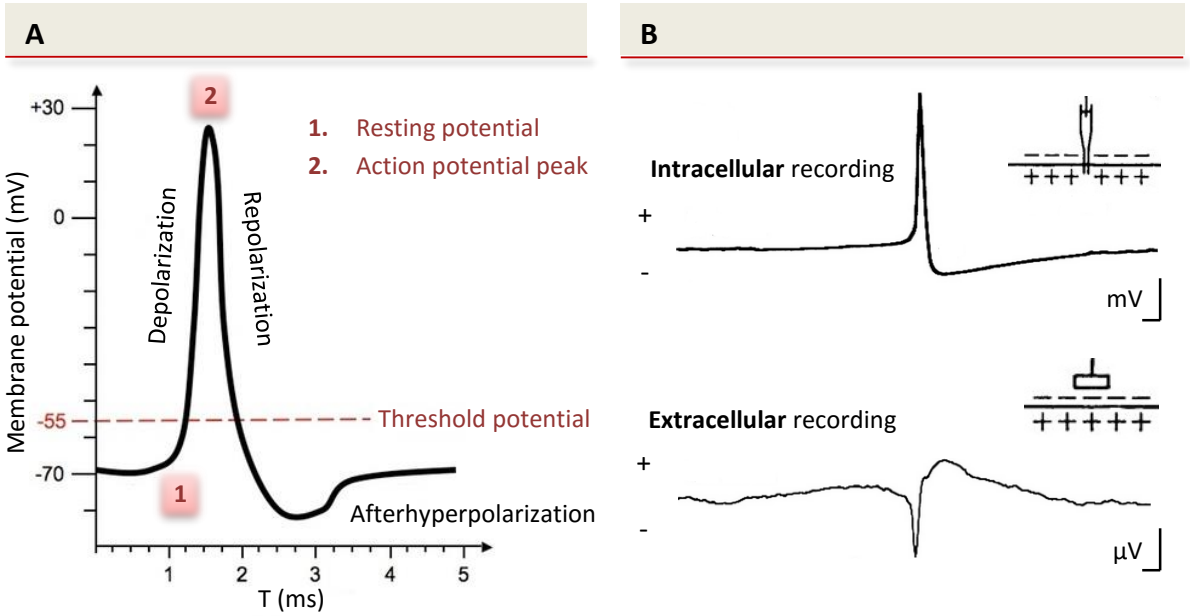


Figure 2 – Action potential recordings. (A) *Typical AP plot shows its various phases as the AP passes a neuronal membrane site. Afterhyperpolarization or refractory period prevents an AP from traveling back and the elicitation of a new AP on the site during a few milliseconds (ms).* ***(B)*** *Schematic comparison between the same AP sign recorded by intracellular and extracellular electrodes. Note that intracellular and extracellular recorded voltages are in the millivolt (mV) and microvolt (µV) ranges, respectively. The shape of the extracellular action potential (EAP) matches qualitatively the intracellular action potential (IAP), but varies along the extracellular space according to temporal distribution of membrane current and due to electrode/membrane distance.*

1.2 Electrophysiology – Applications in Neurosciences

Electrophysiology is the study of the electrical properties of biological cells and tissues. It involves measurements of voltage change or electric current on a wide variety of scales, from single-ion channel proteins to whole organs. In neuroscience, electrophysiology is considered to be the "gold standard" for investigating neuronal signaling (15). The primary strength of neuroelectrophysiology is its combination of time resolution and sensitivity, allowing the precise determination of the temporal pattern of neuronal signals over many orders of magnitude, with high signal-to-noise ratio (SNR) (15,16). By using electrodes to record electrical signals associated with ion fluxes across neuronal membranes, neuroelectrophysiology allows direct access to studying neuronal communication (15).

Only a concerted effort of many neurons make up what it is commonly experienced by humans. In particular, higher brain functions such as associative learning, memory acquisition and retrieval, and pattern and speech recognition depend on many neurons acting and communicating synchronically (17). Furthermore, pathophysiological conditions such as Alzheimer's disease (AD), epilepsy, or other neurological impairments relate to extensive neuronal injuries (18,19). Therefore, in order to understand these pathologies a multi-unit approach is necessary.

The available methodologies for the recording of neuroelectrophysiological activity include: (a) intracellular recordings and stimulation by sharp or patch electrodes, (b) extracellular recordings and stimulation by multielectrode arrays (MEAs), (c) other methods such as functional magnetic

resonance imaging (fMRI), EEG, MEG and electrocorticography, designed to record activity from very large scale neural populations (7,11). A schematic comparison between the different means of recording neuronal electrical activity can be seen in Fig. 3.

Sharp electrode intracellular recording techniques and Patch-Clamp led to considerable progress over the last decades in the understanding of electrophysiological processes at the single channel, single synapse and single neuron level (9). However, *in vivo* or *in vitro*, it is technically difficult to record from and stimulate more than three cells using standard intracellular microelectrodes, and those cells typically die within a few hours (20). Consequently, understanding of electrophysiological processes at the neuronal network level is still in its beginning. In large part due to the technical difficulties of recording electrical activity from large numbers of neurons simultaneously and for prolonged periods of time (9,17). Ideally, these recordings should be entirely non-invasive, long-term stable, economically and temporally efficient and highly reproducible (9).

Thomas et al. (1972) first found that electrical activity can be recorded extracellularly with a multi-electrode device (21). Despite the temporal distance, network electrophysiology has only recently started to make significant contributions to the understanding of complex brain operations and functions (17). These recent advances have been the result of progress in electronic technology, as well as advances in the computational methods required to store and analyze the enormous amount of data generated (9,17). Since then, multi- or microelectrode array (MEA) systems have been on the edge of *in vitro* extracellular recording (17).

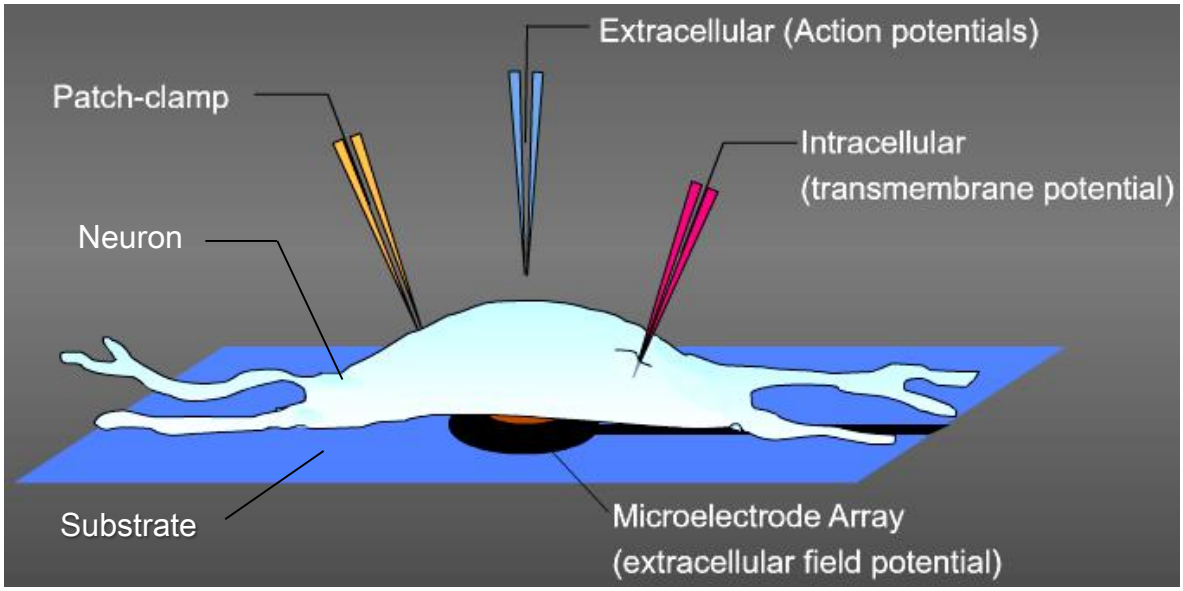


Figure 3 – Neuron-electrode interface in different neuroelectrophysiological techniques. *Contact comparison in extracellular and intracellular approaches. Planar microelectrode arrays (MEAs) can record both extracellular/local field potentials (LFPs) and extracellular action potentials (EAPs). Please note that the image is not to scale – single MEA's electrodes are usually larger than neurons' cell bodies [adapted from (22)].*

In vivo experiments using primates would obviously yield results with the highest certainty of being applicable to humans (9). But the successes obtained with *in vivo* recordings have come from the easily accessible areas of the brain such as the motor, sensory, and visual cortices (17). Although it

is possible to attain deep brain recording, the difficulty in data interpretation increases dramatically as these structures interact with multiple regions of the brain and are influenced by the behavioral state of the animal (17). The multiple factors that influence a nervous system make controlling *in vivo* networks an immense task, but properly controlled cell cultures provide an excellent model for controlling aspects of the experiment that could not be controlled in the living animal (9,17).

The need to reduce the time, cost and numbers of animals used in contemporary toxicity tests is also contributing to a paradigm shift. *In* vitro assays, cellular and alternative species models, as well as predictive computational methods that incorporate knowledge about toxicity pathways, will change neurotoxicity testing (23). Although a number of neurophysiological methods could be utilized to address the mentioned needs, one in particular – *in vitro* MEA recordings - may provide a highly effective neurophysiological method that could be used for predictive toxicity testing (9,23).

1.2.1 Intracellular Recording - Patch-Clamp

In 1976, Erwin Neher and Bert Sakmann first used the patch-clamp technique to record single-ion channels currents from frog skeletal muscle (24). The patch-clamp was an improvement upon the previously used voltage clamp method (1,5). Subsequent refinements, such as the "giga-seal" (25), led to techniques for high resolution recording of current. Since then, patch-clamp recording has been established as the reference method for measuring electrical activity in the form of APs at the cellular level (9).

While conventional intracellular recording involves impaling a cell with an electrode, patch-clamp recording takes a different approach (see Fig. 3). Patch-clamp recording uses a glass micropipette as a recording electrode, and another electrode in the bath around the cell, as a reference ground electrode (24). The micropipette tip is sealed onto the surface of the cell membrane and a gentle suction is applied through to draw a piece of the cell membrane (the “patch”). The suction applied helps forming an electric seal – the “giga-seal” - with resistances of 10-100 GΩ. The high resistance of a "giga-seal" reduces the background noise of the recording and allows a patch of membrane to be voltage-clamped without the use of microelectrodes (25). This configuration is called the "cell-attached" mode and it has been used for studying the activity of ion channels present in the patch of membrane (17,25).

If enough suction is applied, the small patch of membrane in the micropipette tip can be displaced, leaving the electrode sealed to the rest of the cell (25). This is called the "whole-cell" mode and allows very stable intracellular recording. Unfortunately, after a while, as the intracellular fluid of the cell mixes with the solution inside the micropipette, any properties of the cell that depend on soluble intracellular contents are altered, impairing subsequent research (9,17,25).

1.2.2 Extracellular Recording – Multielectrode Array (MEA)

Since its introduction 43 years ago (21), that micro- or multielectrode array (MEA) technology and the related culture methods for electrophysiological cell and tissue assays have been continually improved, while marking their way in scientific literature (13). Throughout the years there has been a great variety of explorations of the possibilities provided by MEA technology. Some have led to new understanding, while others have built technologies that promise new future knowledge (17). For example, Schnitzer, M. and Meister, M. (2003) used MEA technology to find that groups of retina ganglion cells fire synchronously and that such groups may account for more than 50% of all the spikes recorded from the retina (26). These patterns conveyed messages about the visual stimulus far different from what had been inferred from studies of single ganglion cells (17,26). On a completely different approach, Bakkum, D. et al. (2004) connected a MEA system to a robotic arm and utilized the recorded neural activity to control the robotic arm activity. They viewed this as a new research paradigm to study learning, memory, and information processing in real time (27).

There are two general classes of microelectrode arrays (MEAs): implantable MEAs, used *in vivo*, and non-implantable MEAs, used *in vitro* (17). Examples of implantable or *in vivo* MEAs are polytrodes or neural probes. Recently, Wei, W. et al. (2015) introduced a novel implantable dual-model MEA probe that can simultaneously measure glutamate levels, LFPs and spike activity across multiple spatial locations in the rat brain (28). Non-implantable or *in vitro* MEAs generally incorporate microelectrodes in a substrate forming a cell culture dish or medium chamber (12). Therefore, non-implantable MEA systems are intended for non-invasive extracellular recordings of different applications that include brain, heart, and retina slices, cultured slices, dissociated neuronal cell cultures and cell lines (7,17,29–32).

From here on, whenever referring to MEA systems and MEAs, non-implantable MEA systems and planar MEAs will be the subject. Concerning terminology, “MEA” is used to refer to the culture dish, while “system” refers to the MEA and all required components to operate a recording, such as the data acquisition hardware and software. The term “array” refers to the actual area that serves as culture chamber and includes the transducer elements.

MEAs allow the targeting of several sites in parallel for synchronized recording and stimulation of electrophysiological activity (7,17,19,29,33). By simultaneously measuring spontaneous and evoked (through stimulation) electrical activity, MEAs provide an excellent approach to studying the spatio-temporal patterns of neuronal signaling (9,29,33,34).

MEA systems enable simultaneous and long-term recordings of LFPs and extracellular action potentials (EAPs) at millisecond time scale (12,13). These incorporate stimulators, so microelectrodes are also used for extracellular electrical stimulation by applying either current or voltage impulses (17). Stimulation through non-implantable MEAs has been used to elicit spiking activity in, at least, dissociated cultures, brain slices, and isolated retina (35). Research has found that in most cases negative current pulses in fact excite neurons to fire APs (17).

Cell lines or primary cell preparations are cultivated directly on the arrays. Freshly prepared slices can be used for acute recordings, or can be cultivated as organotypic cultures (7–9,17,33,34). Dissociated neuronal cell cultures have been used in many studies of network physiology due to

their superior accessibility compared to *in vivo* models, in terms of electrical recording and stimulation, pharmacological manipulation and imaging (8).

MEA systems have been used to evaluate the plasticity of neuronal networks (20,34,36,37), neuronal diseases (7,19,31,38,39), and drug responsiveness (23,36,39,40). With respect to MEA applicability, careful consideration of their strengths and weaknesses is required. Their major strengths, when compared to more traditional methods such as Patch-Clamp, include (16,17):

1. Data is recorded from multiple electrodes in multiple sites simultaneously;
2. Simultaneous extracellular capture and elicitation of electrophysiological activity;
3. Stimulation and recording sites may be rapidly changed within the array;
4. Controls can be set up within the same experiment (taking advantage of the many available electrodes);
5. No need to place multiple electrodes individually.

On the other hand, major limitations are (16,17):

1. Smaller amplitude recordings, as the electrodes are not inserted in the tissue (signal amplitudes are on the order of tens of μV only);
2. Pre-determined recording and stimulation sites (electrodes are arranged in a fixed pattern);
3. Sensitivity to fluid level fluctuations under interface conditions is greater;
4. Non-transparent conductors partially obstruct transparency;
5. Inverted microscopes that use high power lenses are not able to image through standard MEAs due to their thickness (~1 mm).

Some of these disadvantages have been addressed using different configurations (17,32). For example, as high-quality signals depend on close contact between electrodes and tissue, perforated MEAs that apply negative pressure to openings in the substrate (suctioning) have been designed (17,32). "Thin"-MEAs (with approximately 180 μm) have been created using cover slip glass, allowing them to be used with high-power lenses (32).

MEAs are not adapted to the detection and measurement of subthreshold synaptic potentials. For a synaptic potential of 10 millivolts, the expected recording near a cell body will be much less than one tenth that for an AP, because it will be generated by a diffuse outward capacitative current from the cell body, neighboring dendrites or axon hillock (17). For studies of network development and plasticity this is a serious limitation (17), as it is conceivable that significant signaling between neurons is mediated by subthreshold potentials and is thus undetectable by MEAs (7). Consequently, neurons that do not fire APs during a recording session are not 'visible' to extracellular electrodes. In some brain areas, 90% of the neurons are not spiking or are firing occasionally at very low rates of <0.16 spikes per second (9). Intracellular recordings of synaptic potentials from such neurons could reveal information as to the role of this 'silent majority' in information processing (17,35).

For extracellular recordings, "spikes" are commonly considered to be the signal from a presumed AP and are identified as voltage signals that exceed a threshold. During an AP, the initial rapid Na^+ ion influx creates a sink and results in a large negative spike in the EAP. Afterwards, the slow K^+ efflux produces a source that renders a small positive spike (see Fig.2B). EAPs are usually around

tens to hundreds of microvolts in amplitude and <2 ms in duration. EAPs can be identified when microelectrodes are placed at the proximity (~100 µm) of the spike origin, usually at the soma or near the axon hillock (12). Moreover, aside from assessing spike activity, MEAs measure LFPs. LFPs are evaluated by the signal content in the low-frequency band of the recorded signal (<300 Hz), while EAPs are analyzed after filtering the LFP out (300–3000 Hz) (12). LFPs are more useful when recording *in vivo*, as monolayer cultures have weak LFPs (33).

Multi Channel Systems (MCS, Reutlingen, Germany) is currently the market leader in commercially available MEA technology (13,17). This company provides electrophysiological tools alongside MEA systems, including Patch-Clamps and *In Vivo* ME-systems (13).

1.2.2.1 Equipment Overview

Concisely, *in vitro* MEA systems record, amplify, and analyze spontaneous and/or evoked signals from biological samples (32). If an analysis of these samples and its transfer properties is to be performed, the electrical characteristics of the main components of the sample and the entire system have to be considered (12,17), namely:

1. Neuron signal sources spread of ionic current across the extracellular space;
2. Contact between the neuron and the microelectrodes;
3. The substrate and the embedded microelectrodes;
4. The external hardware connected to the microelectrodes.

MEA systems typically consist of MEAs, a MEA amplifier, a temperature controller and a data acquisition computer (29). A simplified pathway showing which parameters are involved in shaping the recorded signal can be seen in Fig. 4.

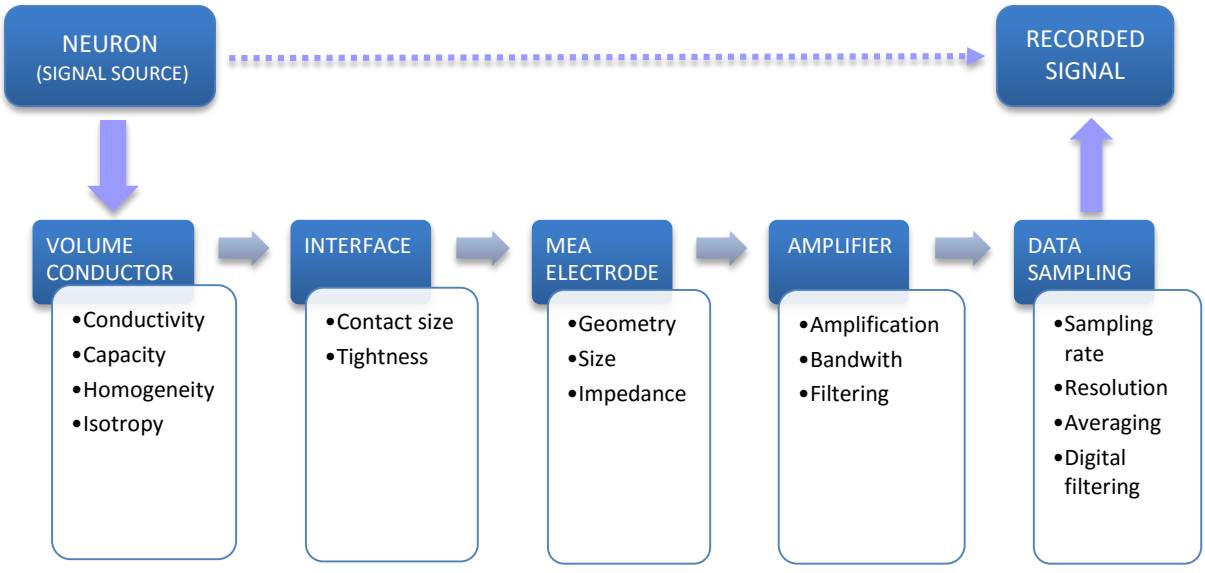


Figure 4 – The recorded signal pathway and the parameters that influence its shape. *The neuron electrical activity is transformed by different parameters across the components of the MEA system toward the recorded signal. Noise sources may vary across the chain, with the most common being biological interferences, electrode's contacts or malfunction and hardware [adapted from* (12,17)*].*

The analysis of EAPs and LFPs usually assumes a purely homogeneous, isotropic (uniform in all directions), ohmic (charge flows easily between conductors) culture medium (volume conductor) based on the volume conductor theory (Kirchoff's current law) and Ohm's law (12). Therefore, the difference in waveforms of a signal recorded at different locations in the tissue is considered to be mainly due to each neuronal source and its distance to the recording microelectrode (12,33).

The neuron-electrode interface and MEA design are the most sensitive and influential parts of a MEA system recording. MEAs serve as the culture chamber and comprise the actual recordings site, therefore this will be addressed in detail below.

At the core element of a classical MEA system is the MEA amplifier, where the electrophysiological signals are recorded (9,29). These are analyzed recurring to a data acquisition computer equipped with specific software. During the experiment, data can be filtered in order to separate events of interest. After the experiment the raw data can be saved, reviewed and adjusted (17,29). For example, spike detection adjustment, peak-peak amplitude comparison, or different signal frequencies separation may be performed re-running the data countless times (17,29).

1.2.2.2 Multielectrode Arrays (MEAs)

The recording process starts at the MEAs. MEAs consist of an arrangement of spatially distributed extracellular electrodes that are embedded in a biocompatible substrate (9,17,29). The MEA substrate is usually glass for simultaneous light microscopy and is covered with an insulation material, which exclusively exposes the electrode spots to the cells and shields the conductors from the culture medium (9,17).

The closer the cells are to the electrodes, the better the signal transduction (17). A number of previous studies have demonstrated that the distance, the strength and the stability of neuron-electrode contact is important for viable impedance recordings of both spontaneous and evoked signals (14,41,42). MEAs' materials tend to become hydrophobic during storage, which prevents attachment of the cells (43). Therefore, coating of MEAs with various biochemical adhesion factors is used for improving the attachment and growth of cell cultures or cultured slices (32,43,44). Coating the substrate and the electrodes does not only determine the tightness of the cell-electrode junction (14,45), but it also modulates substrate biocompatibility (14,43), biostability (14,43,44) and cell differentiation (14,41,43,44,46).

MEAs are equipped with a ring, which serves as a culture chamber (29). Culture chamber lids that incorporate a thin transparent Teflon membrane can be applied in order to enable long-term culturing (17,20,33). This membrane has no pores, thus prevents infection. At the same time it is selectively permeable to oxygen and carbon dioxide, but relatively impermeable to water vapor, which reduces medium evaporation. This characteristic has enabled neuronal cultures to exhibit spontaneous electrical activity after more than a year in culture (20).

MEAs differ in electrode number [16-10.000], electrode material (titanium nitride, gold, platinum, aluminum), electrode diameter [5-50 μm], spacing between electrodes [10-700 μm], and geometry (electrodes layout) (12,17,29,32,47). A variety of electrode geometries and materials have been proposed, manufactured, tested and provided for a wide variety of applications (7,8,17,29,34).

MCS focus on designs centered on square or hexagonal arrays of 60 titanium nitride (TiN) electrodes with 10-30 µm of diameter and 100-200 µm of spacing between electrodes, which are considered "standard" and used by several researchers (8,9,17). TiN allows for design of small electrodes with a low impedance and an excellent SNR (29,32). The advantage of 30 µm diameter electrodes is their low impedance and low noise level, while 10 µm electrodes enable recording from single neurons (29,32). For recording from cultured neurons, a medium spatial resolution with a spacing of 200 µm is generally sufficient (17,32). As the electrodes are 200 µm spaced from each other, it can be estimated that the signals recorded at one electrode are independent from signals recorded at the neighboring ones (48).

1.2.2.3 General Applications and Different Perspectives

Given that neural systems use distributed codes to process and store information much of their dynamics is missed without a multi-unit approach. MEAs provide a mean to record electrical activity from many neurons non-destructively, facilitating real-time and multi-point measurements (8,17,20,34,36). Even though the neuronal networks one can analyze with this methodology are relatively very small, they are large enough for basic intercellular communication and information processing to occur (9).

MEA studies have described fundamental properties of network activity patterns (8,9,17,20,26,34), plasticity (30,34,36) and learning *in vitro* (34); but have also shown promise from a clinical perspective through pharmacological testing (7,36,40,49–51), disease modelling (7,17,19) or even as a diagnostic tool (52). Neuronal diseases such as epilepsy (19) and AD (39,52–54) have been studied using MEAs.

The use of cultured neuronal networks has allowed researchers to investigate neuronal activity in a much more controlled environment than would be possible in a live organism (9). Using MEAs researchers have found important evidence about the mechanisms behind learning, memory and plasticity. MEA electrophysiological recording and stimulation can take place either across the network or locally, and the network development can be visually observed using microscopy techniques. Moreover, chemical analysis is easily accomplished compared to an *in vivo* setting (8,17,55). Unfortunately, cultured neuronal networks are by definition disembodied cultures of neurons. Because they lack a body, these cultures cannot express behavior, and are cut off from all sensory input. Thus, neurons are influenced and may respond in ways that are not biologically normal (55). For example, patterns that resemble epileptic seizures and typically involve the entire network firing rhythmically in synchrony have been described (55–57).

Although neurotoxicity of various chemicals is currently tested solely with *in vivo* methods due to the lack of proper, validated *in vitro* cell models, the MEA system has proven to be suitable for neurotoxicological screening (7,17,40). Ylä-Outinen et al (2010) studied the neurotoxicity of methyl mercury chloride to human embryonic stem cell (hESC)-derived neuronal cell networks and found decreases in the electrical signaling, as well as alterations in the pharmacologic response of hESC-derived neuronal networks in a delayed manner. Those alterations could not be detected with real time quantitative PCR (qRT-PCR), immunostainings, or proliferation measurements (40).

Pre-clinical pharmacological testing is one of the most important potential applications of the MEA system (7,17,50). There has been a great evolution in this field, particularly in cardiac pharmacology. For example, MEA technology has proven to be a sensitive and reliable technology to assess drug-induced functional alternation of human cardiomyocytes by the ion channel blockers and tyrosine kinase inhibitors (TKIs) (50). In fact, human induced pluripotent stem (iPS) cell-derived cardiomyocytes are frequently used to characterize the electrophysiological effects of drug candidates for the prediction of QT interval prolongation and proarrhythmic potential (51). The use of MEA-cultured cell lines may avoid unexpected toxicity in subsequent clinical drug testing (50,51).

MEAs have also been considered as a diagnostic tool for neurological diseases. Gortz, P. et al (2013) utilized MEAs to determine whether cerebrospinal fluid (CSF) alterations of individuals with AD have distinct neurofunctional properties that may distinguish it from that of individuals with mild cognitive impairment (MCI). The network electrical activity suppression correlated significantly with the degree of cognitive decline (52).

In the area of robotics different perspectives have been explored. Ferrández et al (2011) used the data obtained with human neuroblastoma cultured cells (SH-SY5Y cells) to define stimulation patterns, modulating the neural activity for controlling an autonomous robot (30). Another group connected a MEA-cultured neuronal network to a computer on which an animal's body was simulated (Animat), with the purpose of studying the process of learning and memorizing. Imaging and recording the neurons involved in the learning process “while the learning is happening”, could enable the studying of the links between electrical activity and morphology (34,56).

Across the years different applications have surged, often using standard MEAs coupled to other structures (7,30,49,58). For instance, MEAs have been coupled to glass co-culture systems consisting of two cultivation chambers interconnected by microchannels. In order to study signaling transmission between motor neurons and muscle fibers, this setup allowed stimulation of adherent neuronal cells in one chamber and measurement of action potentials induced in myotubes on the other (58).

1.2.2.4 Neuronal Cell lines Culturing on MEAs

Although rarely, neuronal cell lines have been used in MEA experiments. The easy availability of cell lines and their fast growth can be very useful in MEA experiments requiring high-throughput screening (HTS). For example, Teppola et al (2008) stated that human neuroblastoma cell networks may replace primary animal cell cultures in various electrophysiological experiments in the future, for example as a tool for toxicity and drug testing (44). A few examples where cell lines have been employed are discussed below.

Gortz et al (2008) cultured neurons derived from the human NT2 cell line in order to study its neuronal network properties (59). In this experiment, cells were pre-treated with retinoic acid (RA) for 6-7 weeks to induce neuronal differentiation. After trypsinization, the purified neurons were plated in a cell density of 1.0×10^6 cells/cm^2 on poly-D-lysine (1 mg/ml) and laminin (13 µg/ml)-coated MEAs (59).

Ariano et al (2008) used GT1-7 cells, a neuronal cell line showing spontaneous action potentials firing, to test if diamond-based electrodes would be suitable for the fabrication of stable MEAs. The time courses of the recorded signals were in good agreement with those recorded by means of conventional MEAs and patch-clamp from single cells (60).

Fernekorn et al (2008) coupled MEAs to glass co-culture systems consisting of two cultivation chambers interconnected by microchannels. In one chamber NG108-15 cells were cultured, and on the other C2C12 myogenic cells. This setup enabled signaling transmission between motor neurons and muscle fibers, by stimulation of adherent neuronal cells in one chamber and measurement of the action potentials induced in myotubes on the other (58).

Takayama et al (2011) RA-induced differentiation of P19 embryonal carcinoma cells on MEAs. P19-derived neuronal networks showed quite similar network properties to those of primary cultured neurons, exhibiting synchronized periodic bursts (61).

Ferrández et al (2011) used the data obtained with SH-SY5Y cells cultured on MEAs to define stimulation patterns that controlled an autonomous robot. This group found no advantages in coating with PEI solely, culturing 80.000-120.000 neuroblastoma cells over the uncoated MEA substrate (30).

Jahnke et al (2009) used MEAs to detect the pathological risk potential of hyperphosphorylated tau in the SH-SY5Y cell line. MEAs surfaces were coated with 0.5 mg/ml laminin and differentiation was induced with 20 nM staurosporine (41).

1.3 SH-SY5Y cell line

SH-SY5Y cell line derives from the original SK-N-SH cell line, from which it was thrice cloned in 1978 (62,63). SK-N-SH cell line had been previously isolated from a bone marrow biopsy taken from a four year-old female with neuroblastoma (62,64). The SK-N-SH cell line contains cells with three different phenotypes: neuronal (N-type), Schwannian (S-type), and intermediary (I-type) (65). N-type cells are considered immature nerve cells, S-type cells are multipotent precursors to Schwann cells, melanocytes and glial cells and I-type cells are intermediate with respect to N- and S-type cells in terms of morphology and biochemical markers (66). The SH-SY5Y cell line is a comparatively homogeneous neuronal cell line, mainly composed of N-type cells (63,66,67). Though, S-type cells remain present due to the ability of cells to transdifferentiate between cell phenotypes (66).

Since they are tumor derived cell lines, SH-SY5Y cells continuously divide and can provide the required quantity of cells for different experiments, without exhibiting a large variability. Therefore, this human neuroblastoma cell line has been extensively used as a neuronal cell model since the early 1980's as these cells possess morphological, functional and biochemical properties of primary neurons (46,67,68). Furthermore, SH-SY5Y cells proliferate in culture for long periods without contamination, a prerequisite for the development of a reliable *in vitro* cell model (67).

SH-SY5Y cells are also characterized by noticeable sensitivity to oxidative stress (OS), a pivotal contributor to progressive NDs such as AD, but also Parkinson's disease or amyotrophic lateral

sclerosis (ALS) (69). The neuronal properties and sensitivity to OS make this neuroblastoma cell line an excellent model to study neurological pathologies (69,70).

Undifferentiated SH-SY5Y cells are characterized morphologically by neuroblast-like, non-polarized cell bodies with few, truncated processes (63,71). These cells tend to grow in clusters and may form clumps in the central region of a cell mass. Cultures contain both adherent and floating cells, some studies suggest that the floating cells are more likely to adhere and differentiate into N-type cells upon treatment than the adherent cells present in undifferentiated cultures (71). N-type phenotype is characterized by relatively reduced cell growth and the formation of distinct neurites (neuritogenesis) (72).

Both undifferentiated and differentiated SH-SY5Y cells have been used for *in vitro* experiments that require neuron-like cells (71,73), despite undifferentiated SH-SY5Y cells being typically locked in an early neuronal differentiation stage, characterized biochemically by the low presence of neuronal markers (70). Therefore, the proliferative SH-SY5Y cells do not represent a suitable *in vitro* model for studying the molecular and cellular mechanisms underlying neuronal pathophysiology. However, upon induced-differentiation, there is formation and extension of neurites, synaptophysin-positive functional synapses, induction of neuron-specific enzymes, neurotransmitters, and neurotransmitter receptors (46,67,71,74,75).

SH-SY5Y cell line has brought several benefits to the field of neuroscience research. This *in vitro* model enables large-scale expansion prior to differentiation, when cells stop proliferating and become a stable population (67). This is achieved with relative ease and low cost when compared to primary neuronal cultures (71). As SH-SY5Y cells are human-derived, they express human-specific proteins and protein isoforms that would not be present in rodent primary cultures (71). Moreover, there are no ethical concerns, associated with primary human neuronal culture, as these cells originate from a cell line (62,63).

1.3.1 Differentiation of SH-SY5Y cells

Differentiation of SH-SY5Y cell line into a neuronal-like cell line is required to mimic the intracellular environment of a neuronal cell. Upon differentiation, SH-SY5Y cells possess more biochemical, structural, morphological, and electrophysiological similarity to neurons (46,64,67,74,75). In fact, MEA recordings of undifferentiated SH-SY5Y cells have a very low SNR (30), but differentiated cultures have shown electrophysiological responses similar to standard neurons, such as APs generation (30,41,76).

Depending on treatment, SH-SY5Y cells can be differentiated towards different mature phenotypes (cholinergic, adrenergic, or dopaminergic) (70,71,74). The most commonly used differentiation agent is RA (71). Nevertheless, growth factors such as brain derived neurotrophic factor (BDNF) (75), nerve growth factor (NGF), and neuregulins (77) are also extensively used and frequently combined (74,75). Therefore, the differentiation method selected should be determined by the desired phenotype following differentiation, as well as for the reduction of non-target effects on experimental pathways (71).

In order for SH-SY5Y cells to undergo optimal differentiation both adhesion and growth factor receptors should be stimulated simultaneously (46,72,74). In fact, the interface between the cell and its environment, especially the extracellular matrix (ECM), has a profound effect on cell phenotype and fate. Therefore, much research has been invested into altering the properties of non-biological substrate surfaces that are supposed to come in contact with cells or tissue (14).

ECM is a collection of soluble proteins secreted by adherent cells that plays a key role in tissue homeostasis, cell attachment, growth, proliferation, differentiation, morphology, polarization, directional motility, migration and cell spreading (14). Laminins are a major type of glycoprotein present in the ECM in the developing brain and stimulate neurite outgrowth in many neuronal cells *in vitro*. An optimization protocol of SH-SY5Y cells differentiation reported that laminin coating induces more differentiation when compared to other ECM proteins coatings, such as collagen or fibronectin. Laminin coating induced higher levels of focal adhesion kinase (FAK) expression and longer neurites (46).

Pre-treatment with RA followed by three-dimensional culturing in an extra ECM gel combined with several factors, such as BDNF, NGF, neuregulin β1 and vitamin D3 has been reported to generate SH-SY5Y differentiated cells with "unambiguous resemblance to adult neurons" (74).

As mentioned before, MEAs are coated with various biochemical adhesion factors in order to improve the attachment and growth of cell cultures. Among these are ECM proteins, such as laminin or fibronectin (13,44). MEAs coating with ECM proteins is often combined with a pre-treatment with polyethyleneimine (PEI) or poly-D-lysine (PDL) (20,61,78,79). Still, Ferrández, J. M. et al. (2011) cultured SH-SY5Y cells on MEAs and found no advantages in pre-treating with PEI, when comparing with no covered plates (30). Another group cultured SH-SY5Y cells on MEA plates treated with PDL, poly-L-lysine (PLL), PEI with laminin, and laminin alone. Additionally, in order to differentiate the cells, they were treated with RA and cholesterol for 7 days. The results showed that the cells attach with all of the used coating agents, but there was less cell growth with PEI+laminin coating than with no coating (44). However, the maturation, morphology, and distribution of the cells was more neuron-like with PEI+laminin than with any other treatment (44,79).

Patch-clamp studies have shown an increase in spontaneous electrical activity in differentiated SH-SY5Y cells. Development of a resting membrane potential as well as expression of Ca^{2+} channels is dependent on cellular differentiation (80). Moreover, untreated cells exhibit scarce spontaneous electrical activity, but upon RA-induced differentiation there is an increase in spontaneous electrical activity. This indicates that SH-SY5Y cells form functional synapses when differentiated (76).

1.3.2 Retinoic acid (RA)-induced Differentiation

The most commonly implemented and best-characterized method for induction of neuronal differentiation in SH-SY5Y cells is through addition of RA to the cell culture medium (71). Numerous lines of evidence have indicated that SH-SY5Y cells are able to acquire neuron-like phenotypes with RA treatment (46,67,73,74,81,82). After treatment with RA, cells arrest in the G1-phase of the cell cycle, DNA synthesis is inhibited and growth inhibition can be detected already at 48 h after treatment (64,82). SH-SY5Y cells differentiate primarily to a cholinergic phenotype in response to

RA treatment (71). While cells may also differentiate toward a dopaminergic phenotype, controversy exists in the literature over whether dopaminergic markers present in undifferentiated cells significantly increase during RA treatment (64,71,75).

RA is a vitamin A derivative known to possess powerful growth-inhibiting and cellular differentiation-promoting properties (71,75). RA induces transcriptional activation by binding to two families of nuclear receptors: the RA receptors (RARs) family, and the retinoid X receptors (RXRs) family (65). Typically, RA is administered at a concentration of 10 µM for a minimum of 3–5 days in serum-free or low serum medium to induce differentiation (46,71,75). It has been documented that neuroblastoma cells have to be differentiated for at least 7 days for experimental applications (73,83).

Many differentiation protocols for the SH-SY5Y cell line involve usage of RA as the only differentiation factor (82). Differentiation is in turn often appreciated on the basis of morphological changes and arrest of proliferation. It is unclear if cells differentiated in this way accurately exhibit neuronal characteristics without a detailed molecular analysis (46,71,82). Neuronal markers such as synaptophysin (71,73), neuronal nuclei (NeuN) (71,73), have been shown to increase following RA-induced differentiation.

RA-induced differentiation of SHSY5Y cells has been reported to confer SH-SY5Y cells higher tolerance to neurotoxins by altering survival signaling pathways. Therefore, undifferentiated cells have been considered as more appropriate for studying neurotoxicity or neuroprotection in disease research (73). Furthermore, 10 µM RA-differentiated cells were shown to be significantly more resistant against Aβ 1-40 and Aβ 1-42 aggregate toxicity than undifferentiated cells treated similarly (84).

1.4 Alzheimer's Disease (AD) – Problem and Molecular Basis

As of 2014, nearly 36 million people worldwide have AD (85). AD is the most common form of dementia, accounting for 60 to 80 percent of the cases (86,87). It is a progressive disease characterized by a deterioration of memory and other cognitive domains that lead to death within 3 to 9 years after diagnosis (87). In its early stages, memory loss is mild, but with late-stage AD, individuals lose their autonomy and do not respond to the environment (86). Currently, there is no cure and the drugs available can only delay the progression of the disease (85,88).

Although, the brain changes of AD may begin 20 or more years before symptoms appear, the principal risk factor is age (85,89). The incidence of the disease doubles every 5 years after 65 years of age (87). Even though age is the greatest risk factor, AD is not a normal part of aging and advanced age alone is not sufficient to cause the disease (85,87). Up to 5 percent of people with the disease have early onset AD, developing in 30-60 years old individuals (88). Genetic evidence indicates that inheritance of mutations in several genes causes autosomal dominant familial AD (FAD), while the presence of certain alleles of other genes, particularly apolipoprotein E (ApoE)-ε4 gene, are significant risk factors for putative sporadic disease (85,90). Possible risk factors with some association for AD include gender (female) (85,88), lack of education (91), head trauma (92),

memory deficit with severity of any extent, small hippocampal volume (88), diabetes mellitus, insulin resistance, high cholesterol, hypertension, reduced exercise, and obesity (93).

AD is a chronic ND with known pathophysiological mechanisms, mostly affecting medial temporal lobe and associative neocortical structures (89). Neuritic amyloid plaques (NPs) caused by extracellular deposition of the β-amyloid (Aβ) peptide in brain tissues; neurofibrillar tangles (NFT) derived from the hyperphosphorylation of the microtubule-associated Tau protein; and OS induced by impaired metabolic pathways and metals represent the hallmarks of the disease (18,89).

The most influential theory ("Amyloid Hypothesis") for the primary cause of AD is the overproduction and/or impaired clearance of Aβ peptides derived from amyloid precursor protein (APP), especially the markedly toxic 42-amino acid containing Aβ 1-42 (18). In fact, AD is characterized by the histological findings of NP deposits comprised primarily of fibrillar and ß-sheet rich aggregates of Aβ (9).

1.4.1 β-Amyloid (Aβ) peptide – The "Amyloid Hypothesis"

Aβ was first sequenced from the meningeal blood vessels of AD patients and individuals with Downs syndrome in 1984 (18,94). Shortly after, the same peptide was recognized as the primary component of the NPs characteristic of AD pathophysiology (95). Since then, the "amyloid hypothesis" has been widely accepted as a primary cause of the neurodegeneration observed in AD. This hypothesis considers Aβ as a toxic factor that impairs neuronal function and leads to cell death (18).

Aβ is produced normally throughout life by the intramembrane proteolysis of APP (18). APP is a ubiquitously expressed membrane glycoprotein that is encoded by a single gene on the chromosome 21q21. Though multiple isoforms exist, APP_{695} is the predominant isoform in neuronal cells (96). APP is processed by secretase enzymes mainly resulting in the release of the ectodomain of APP, the production of APP intracellular domain (AICD), and the generation of several Aβ peptide fragments (90). There are two pathways for processing APP: an amyloidogenic pathway and a non-amyloidogenic, constitutive secretory pathway (96–98). In the amyloidogenic pathway APP is cleaved by β–secretase (BACE1), discarding its soluble ectodomain (sAPPβ). The remaining 99-amino acid long membrane-bound residue (C99) is then cleaved by γ-secretase, producing AICD and different types of Aβ peptides (18,96). Depending on the exact site of γ-secretase cleavage the Aβ produced may have 40 or 42 aminoacids. Alternatively, in the non-amyloidogenic pathway APP is sequentially cleaved by α-secretase and γ-secretase. Cleavage by α-secretase originates the soluble APP-fragment (sAPPα) and a 83-amino acid membrane-bound residue (C83). C83 is further cleaved by γ-secretase releasing the AICD and a P3 peptide, which are rapidly degraded (96). A representation of both APP processing pathways and their resulting fragments can be seen in Fig. 5.

Under physiological conditions, APP is preferentially metabolized in the non-amyloidogenic pathway and there is equilibrium between Aβ production and clearance from the brain (89). In AD there is an imbalance between Aβ production and clearance. Aβ species are released as monomers

that progressively aggregate into dimmers, trimers, oligomers, protofibrils and fibrils, that deposit and originate NPs (89,96). Despite their similarities, Aβ 1-42 is more prone to aggregation and fibrilization, rendering the most neurotoxic Aβ peptide (87).

Synaptic terminals are thought to be a major source of APP that gives rise to Aβ (96). Thus, synapses release Aβ and are, in turn, damaged by elevated levels of Aβ peptides (90). Their accumulation into NPs triggers several harmful events that disrupt neuronal homeostasis, such as: mitochondrial dysfunction, activation of OS and inflammatory cascades, impaired neurotrophic support and response to injury, decreased neuroplasticity and neurogenesis, hyperphosphorylation of Tau protein, apoptosis, and abnormalities in calcium metabolism. Furthermore, these events are subject to positive feedback, amplifying neurotoxicity and culminating with neuronal death (89).

In vitro investigations in cultured neurons have found that Aβ oligomers bind exclusively and rapidly to synaptic terminals, altering both pre- and postsynaptic structures and affecting excitatory, but not inhibitory nerve terminals (99). Changes in the microenvironment of neurons are sensitively and immediately translated into activity changes, which can be directly monitored using, for example, MEA technology (9).

Recent findings on the physiological roles of Aβ challenge the way the "amyloid hypothesis" portrays Aβ peptides. According to Cárdenas-Aguayo, M. C. (2014), Aβ-peptides might help enhancing synaptic plasticity and memory at appropriate concentration levels. Moreover, recent studies have shown that Aβ may be vital for neuronal development, plasticity, and survival due to its integral membrane interactions and neurogenic properties (97).

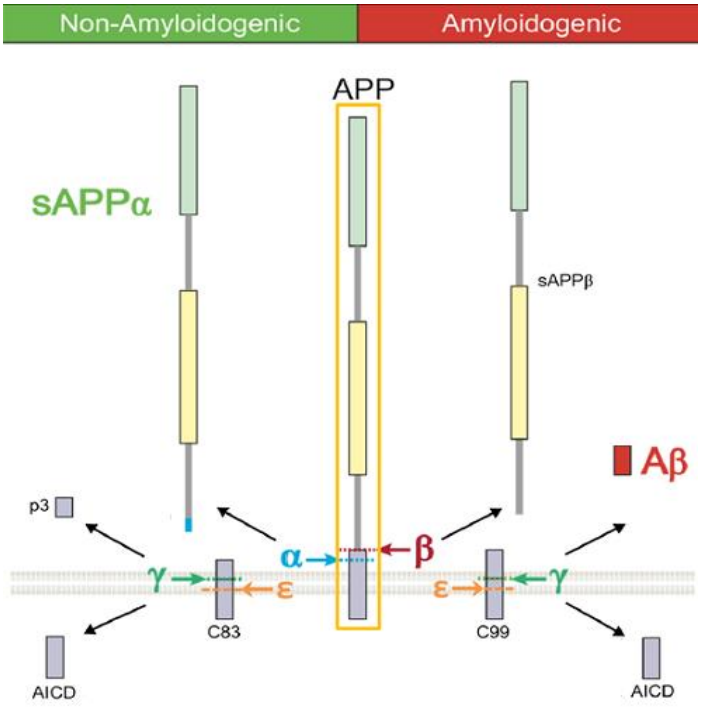


Figure 5 – APP processing. *Amyloid precursor protein (APP) can be cleaved via two mutually exclusive pathways – the non-amyloidogenic and the amyloidogenic. Different fragments result from each pathway. Though various fragments of APP processing, including Aß, may have roles in normal brain physiology, imbalance between production and clearance lead to pathology [adapted from (99)].*

1.4.2 MEA application in AD

Most *in vitro* functional electrophysiological studies on the effects of Aβ on neurons have been carried out using the patch-clamp technique (39). This technique enables the acquisition of detailed information concerning Aβ effects at the ion channel level, but it is very low throughput and difficult comparative to extracellular electrophysiological techniques like MEA (17,39). Development of this method and its applications could have a high impact on drug development in AD (39,100).

The use of MEAs as a research and diagnostic tool for NDs has not been explored extensively (9). As MEA-supported neuronal culture has proven to be a useful model to study synaptotoxicity *in vitro* (39,54), electrophysiological analysis of neuronal activity alterations can surely be valuable in both AD's basic and clinical research (7,9). Besides, both the addition of neuro-harming and neuroprotective substances is well controllable (9). Recent findings even assessed MEAs usefulness as a differential diagnosis tool in AD (52).

MEAs have been used before to monitor the impact of amyloid oligomers (39,54,101) and tau proteins (38) on hippocampal neurons isolated from rat embryos (39), mouse embryos (54), rat pups (101) and mice adults (38,53). Furthermore, Jahnke et al (2009) used MEAs to detect the pathological risk potential of hyperphosphorylated tau in the SH-SY5Y cell line (41).

Varghese et al (2010) utilized MEAs to create a high-throughput screening method for antagonists of the functional toxicity caused by Aβ 1-42 oligomers to embryonic rat hippocampal neurons. In this study Aβ had a pronounced effect on the spontaneous firing, even at concentrations in the nanomolar range. Treatment with Aβ stopped spontaneous activity completely and the time for cessation was concentration dependent. Furthermore, MEAs made it possible to screen a significantly higher number of cells for Aβ and drug effects in a much shorter amount of time than patch-clamp would require (39).

Benilova et al (2009) studied acute synaptotoxicity caused by Aβ oligomers in hippocampal neurons isolated from mouse embryos. Treatment with 1-20 μM concentrations of Aβ 1-42 oligomers altered firing rate in a concentration-dependent manner. Aβ 1-42 oligomers, but not fibrils, rapidly and significantly inhibited synaptic activity, supporting the data simultaneously obtained by other electrophysiological methods. The lethal concentration (20 μM) caused immediate silencing of the network, while the effect of sub-lethal low uM concentrations (<2 μM) was observed after overnight (ON) treatment. In order to verify specificity of the Aβ 1-42 oligomers effect, anti-Aβ antibodies were added to neuronal cultures prior to Aβ 1-42 oligomers and injury was prevented (54).

Hoppe et al (2013) studied synaptotoxicity caused by Aβ oligomers in organotypic hippocampal slice cultures of rat pups (6 to 8-day-old). After 14 days *in vitro*, cultures were treated with Aβ 1-42 oligomers (2 μM) and an inhibitor of Aβ oligomerization, curcumin (10 μM) for 24 and 48 hours. Extended exposure to Aβ (48 hours) was necessary to induce significant neuronal death, as cotreatment with curcumin prevented cellular damage. While treatment with Aβ for 24 hours did not cause significant neuronal loss, it exerted effects upon functional synaptic transmission (101).

Chong et al (2011) used MEAs to examine the early synaptic effects of amyloid oligomers and protein tau in AD transgenic mice and mouse models. Acute hippocampal slices were prepared and

analyzed under the MEA. They demonstrated that MEA recording is adequate and reliable to monitor early and region-specific defects inflicted by amyloid and tau proteins in AD transgenic mouse models, separately and combined (38).

Kuperstein et al (2010) utilized MEAs to analyze the influence of the Aβ 1-42:Aβ 1-40 ratio as a driver of acute synaptic alterations and posterior neuronal death. Small alterations in the ratio dramatically affected the biophysical and biological properties of the Aβ mixtures affecting their aggregation kinetics, the morphology of the resulting amyloid fibrils and synaptic function. According to this study the relative ratio of Aβ peptides is more crucial than the absolute amounts for the induction of neurotoxicity (53).

Charkhkar et al (2015) found that Aβ 1-42 oligomers, but not monomers, significantly reduce network spike rate in primary neuronal MEA cultures. Immediately after administration, 5 μM Aβ 1-42 oligomers significantly reduced the normalized spike rate by 60%, but oligomer concentrations at 1 μM and 200 nM failed to alter it in a statistically significant manner (100).

A number of compounds that may be able to protect neurons from amyloidogenic toxicity have been proposed (9), but few have been tested on MEAs. Among those proposed are included: curcumin (39,102); peptide-based compounds, such as KLVFF and LVFFA (103); hemin and related porphyrins (104). Until now, only curcumin treatment has been tested on MEAs (9,39,101).

Curcumin, a polyphenol from curry spice (101), has been reported to disaggregate Aβ aggregates, as well as prevent fibrils and oligomers formation (102), and to improve memory in animal models of AD (105). Reversal of Aβ harmful effects have also been reported on *in vitro* studies carried out on MEA (39,101). As already mentioned, Varghese et al (2010) plated embryonic rat neurons and demonstrated that Aβ1-42 functional toxicity could be reproduced using MEAs (39). Partial functional activity was recovered by administration of curcumin, but it was more effective in inhibiting the effect of Aβ when it was coadministered with it as opposed to the experiments in which it was applied 24 hours after Aβ exposure. The recovery of spontaneous firing frequency obtained with curcumin treatment on MEAs was comparable to results obtained with patch-clamp using similar experimental paradigms (39). Hoppe et al (2013) exposed organotypic hippocampal slice cultures of rat pups to Aβ1–42 and studied the neuroprotective effects of curcumin through MEA recordings of spontaneous neuronal activity. Curcumin counteracted both harmful effects of Aβ: the initial synaptic dysfunction and the later neuronal death (101).

Peptide-based compounds that mimic Aβ structure, such as KLVFF and LVFFA, may have potential therapeutic benefit. These derivatives have been constructed using the “Aβ binding element” and have reduced β-amyloid aggregation *in vitro* (103).

Hemin and related porphyrins have been shown to inhibit β-amyloid aggregation (104). However, recent findings support the hypothesis that the binding of hemin to the amyloid peptide is a key event in the early stages of AD, as Aβ-hemin complexes enhance oxidation and polymerization of neurotransmitters (106). Either way, MEA analysis could help clarify Aβ-hemin complexes relevance.

2. Aims

The main aim of this thesis was to optimize a neuronal cell culture model for obtaining reliable MEA recordings. Such model should render extracellular recordings of spontaneous electrical activity and enable the evaluation of the acute Aβ effects on such cultures.

In vitro MEA assays enable rapid screening of neuroprotective and neuroharming compounds. As a consequence, accelerate the drug development process and may help to find new potential therapeutics. From a basic research point of view, for example, such experiments help to understand neuronal networks signaling dynamics.

This thesis purports to offer clear instructions for the culture and maintenance of SH-SY5Y neuronal networks on MEAs. SH-SY5Y cells may be differentiated into a mature neuron-like phenotype, but have rarely been used in MEA studies. A protocol depicting SH-SY5Y cells culturing on MEAs and its successful differentiation into a mature neuronal network may be extremely useful for laboratories that do not have access to primary neuronal cultures. Moreover, cell lines usage in MEA studies may save resources and facilitate obtaining faster high-throughput results.

AD is one of the leading causes of death in developed countries. It is a ND characterized by a gradual and progressive decline in memory, executive function and ability to perform daily activities. This cognitive decline and subsequent morbidity are caused by a neuronal impairment thought to be triggered by Aβ's neurotoxicity.

The impact of Aβ on cell lines cultured on MEAs has not been assessed. Here, we aim to develop a model that may enable the evaluation of this impact.

Concisely, the main specific objectives were to:

- Optimize MEA coating and manipulation for SH-SY5Y cell culturing;
- Evaluate whether SH-SY5Y cell culture is a suitable model for extracellular recordings of spontaneous electrical activity;
- Follow neuronal differentiation by electrical activity measurements and morphological analysis;
- Evaluate the acute effect of Aβ oligomers on the neuronal network electrical activity.

3. Methods

3.1 MEAs

MEAs were obtained from Multi Channel Systems (MCS, Reutlingen, Germany). For the recording experiments four standard MEAs (60MEA200/30iR-Ti-gr) containing 60 electrodes in an 8 by 8 grid arrangement were used. The TiN electrodes diameter is 30 µm and the distance between electrode centers is 200 µm. One of the electrodes (electrode no. 15) is larger and functions as the ground or internal reference electrode (iR). The contact pads and tracks are opaque. A macro and a microscopic view of this standard MEA can be seen in Fig. 6A and Fig. 6B, respectively.

An additional distinct MEA (60MEA200/10iR-ITO) was used for imaging purposes and comparison needs. This MEA equally contains 60 electrodes separated by 200 µm in an 8 by 8 grid, with one being an iR. However, the titanium nitride electrodes diameter is just 10 µm and the contact pads and tracks are transparent.

Sealed MEA culture chambers (ALA-MEA-MEM) were also obtained from MCS. These culture chambers were applied to the MEAs in order to prevent contamination during recordings and medium evaporation throughout incubation. They are made of polytetrafluoroethylene Teflon and have a clear membrane (fluorinated ethylene-propylene Teflon) stretched across the top.

When handling MEAs, there are several aspects that must be taken into account in order to obtain reliable recordings. Moreover, as the MEA substrate and the electrodes are extremely fragile, some precautions are imperative. With the appropriate care, MEAs can be reused multiple times (43). For handling purposes, each MEA was placed in a standard 100 mm sterile polystyrene Petri dish. Complete information on how MEAs were manipulated during and in-between experiments work can be consulted in annex 7.1.

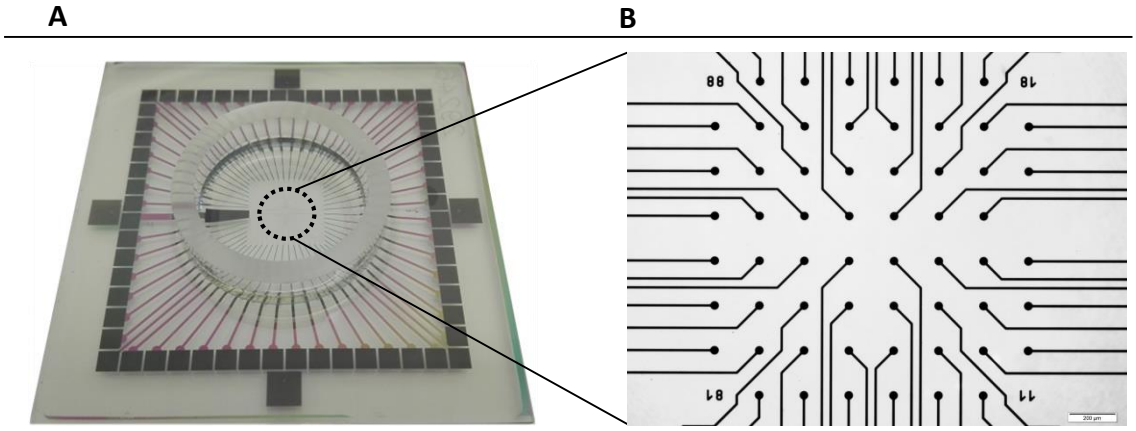


Figure 6 – Standard multielectrode array (MEA). (A) *60MEA200/30iR-Ti-gr MEA. Note that the glued glass ring forms the culture chamber (array). Tracks and contact pads disperse along the MEA substrate outside the array.* ***(B)*** *MEA's recording area is comprised of 60 electrodes arranged in a 8x8 layout grid. Electrodes and tracks appear as black dots and lines, respectively. Bright-field photograph at a magnification of 40×.*

3.1.1 Optimization

No guidebook or protocol exists in the literature for the culture and maintenance of SH-SY5Y cells for use with MEA technology. In fact, neuronal cell lines have been seldom used in MEA studies and no document describes clearly the problems most commonly encountered in cell culture. Most often, protocols found in the literature involve culture of neuronal dissociated cultures (20) of the cortex (33) or hippocampal (107) regions of pre- or post-natal rat central nervous systems or acute hippocampal rat slices (48). A protocol for iCell® Neurons culture, human induced pluripotent stem cell-derived neurons, has been provided by Cellular Dynamics International, Inc. (108). A comprehensive protocol of MEA recordings of human epileptic postoperative cortical tissue also exists (19).

In order to form functional neuronal networks for MEA recordings the first important step was to optimize cell growth on MEAs (20,44). Moreover, MEAs need to be sterilized and suffer surface treatment prior to any cell seeding. So as to achieve reliable recordings, several proceedings had to first be optimized. More information on how MEAs were sterilized and prepared for reuse can be consulted in annex 7.1.2. Several different MEA culture settings were tested, including: with and without MEA's surface pre-treatment (coating), different cell plating densities, with or without RA-induced cell differentiation, as well as different serum concentrations in the differentiation medium.

The surface of new MEAs is hydrophobic, thus preventing the attachment of the (hydrophilic) cells. Therefore, when preparing MEAs for use it was crucial to ensure that the surface was hydrophilic enough for cell adhesion. Coating of MEAs is used for improving the attachment and growth of cell cultures or cultured slices (14,43,44). It is extremely important that induced cell-surface adhesion be greater than naturally occurring cell-cell adhesion to avoid large aggregates of clumped cells. These cell clumps are problematic as they tend to detach from the MEA surface (17).

Even surface treated-MEAs tend to become hydrophobic again during storage, especially when dry. To maintain a hydrophilic surface, when not in use, arrays were immersed in sterile distilled water and stored at 4 °C in the dark (33,43).

3.1.1.1 Coating

Maintaining cells in culture is essential for studying their physiological properties. Cell culturing is dependent on the growth surfaces and cells must adhere to the electrode substrate in order to establish the best connection with the electrodes material. An optimization of the MEA coating associated with an SH-SY5Y differentiation protocol was performed. The objective was to find the best alternative amongst the various coating treatments by following the cell growth, morphology, level of differentiation and viability. The various combinations of MEA coatings and differentiation medium parameters tested are shown in section 3.2.2.2 in Table 1.

SH-SY5Y cells were cultured and differentiated for 7 days on MEAs without coating and various coating agents alone and combined. These coating agents were: PEI, PDL and laminin. Combinations of PEI+laminin and PDL+laminin were also tested. A Teflon lid was applied to the MEA in order to prevent evaporation of the coating agents during incubation.

3.1.1.1.1 Polyethyleneimine (PEI) treatment

PEI surface-treatment has been successfully used in MEA's dissociated cell (33) and cell line cultures (14,44). It has proven to enhance cell maturation (44) and induce less clustering of cells (33) when compared to poly-lysine coated MEAs (33,44). Unfortunately, PEI forms a uniform layer that can become easily detached from the surface (43).

This synthetic adhesion-mediating compound (a polycation) changes the charge on the glass substrate surface from negative to positive (33). The cell membrane's extracellular side has a net negative charge due to a dense negatively-charged network of proteoglycans, glycolipids and glycoproteins. By giving the glass substrate surface a positive charge, cell/substrate adhesion is enhanced. As polycations are synthetic molecules, they do not stimulate biological activity in the cells cultured on them (14).

After MEA sterilization and careful observation under an optical microscope (see annex 7.1.2), 500 µl of 0.1% PEI dissolved in sterile distilled water (from a 50% w/v PEI stock solution) was pipetted onto the MEA in order to cover the whole array. Then, the MEA was stored at 4 °C ON. The day after, the remaining solution was aspirated and the MEA was thoroughly rinsed with sterile distilled water and allowed to air-dry under the laminar flow cabinet. It was necessary to thoroughly rinse off unbound PEI from the arrays before using them, as the high pH (~9.5) of the solution is extremely cytotoxic, dramatically affecting the cell culture's viability (14,43). After PEI surface-treatment, each MEA was exposed to UV-light for 1 hour.

3.1.1.1.2 Poly-D-lysine (PDL) treatment

Poly-lysine is another synthetic polycation that functions as an adhesion-mediating compound. Thus, poly-lysine surface-treatment increases the number of positively-charged sites available for cell binding (14). Both polymers of D- and L-lysine are used to coat substrates to promote cell attachment (45,109). However, PDL, unlike PLL, is not easily digested by proteases released by cells in culture, hence it is less cytotoxic (14).

After MEA sterilization and careful observation under an optical microscope (see annex 7.1.2), 500 µl of 0.1% PDL solution dissolved in phosphate buffered saline (PBS) was pipetted onto the MEA in order to cover the whole array. Then, the MEA was stored at 4 °C ON. The following day, excess PDL was aspirated. As with PEI, thorough rinsing with sterile distilled water following the treatment is mandatory and was performed. Should the PDL be allowed to air-dry, without thorough rinsing, evaporation would form a cytotoxic crystal precipitate ring. In fact, simple aspiration of the PDL solution from the cell culture surface does not remove enough solute to prevent this precipitation (14,43). After PDL surface-treatment, each MEA was exposed at UV-light for 1 hour.

3.1.1.1.3 Laminin treatment

The ECM can be regenerated in an organism, but less efficiently in cell culture. Laminin is an ECM glycoprotein that has active domains for collagen binding, cell adhesion, heparin binding, and neurite outgrowth fragment (14). In addition to adhesion promotion (33), laminin is thought to play a key role in neuronal proliferation, migration, myelination, neurite outgrowth, and tissue survival both *in vivo* and *in vitro* (46,110).

Laminin coating is stable for several uses of the MEAs and does not have to be removed after use (43). Despite this, MEA's laminin coating was performed after every sterilization step. First, 400 µl of 10 µg/ml laminin (0.001%) diluted in PBS (from a 1 mg/ml stock solution) was pipetted onto the center of the array in order to cover the whole recording area and surroundings. Then, the MEA was incubated at 37 °C for 1 hour at room temperature (RT). Finally, excess laminin was aspirated, the MEA was rinsed with PBS and allowed to air-dry.

3.1.1.1.4 PEI + laminin and PDL + laminin treatments

In MEA cultures, polycations are often combined with proteins from the ECM such as laminin (14). Combinations of polycations and laminin have been proven to improve the attachment and growth of cell cultures, when compared to polycations alone (14,44). Relative positioning and thickness of the coating agents used, extracellular components and MEA's substrate can be seen in Fig. 7.

The two-step surface treatment was carried as mentioned before, but taken together. Concisely, the arrays were coated with PDL and PEI and incubated ON at 4 °C, after which they were thoroughly rinsed with sterile distilled water. The second step of surface adhesion promotion involved the use of laminin and was carried out just before culturing on the array. After UV-light sterilization, laminin was added for 1 hour at RT.

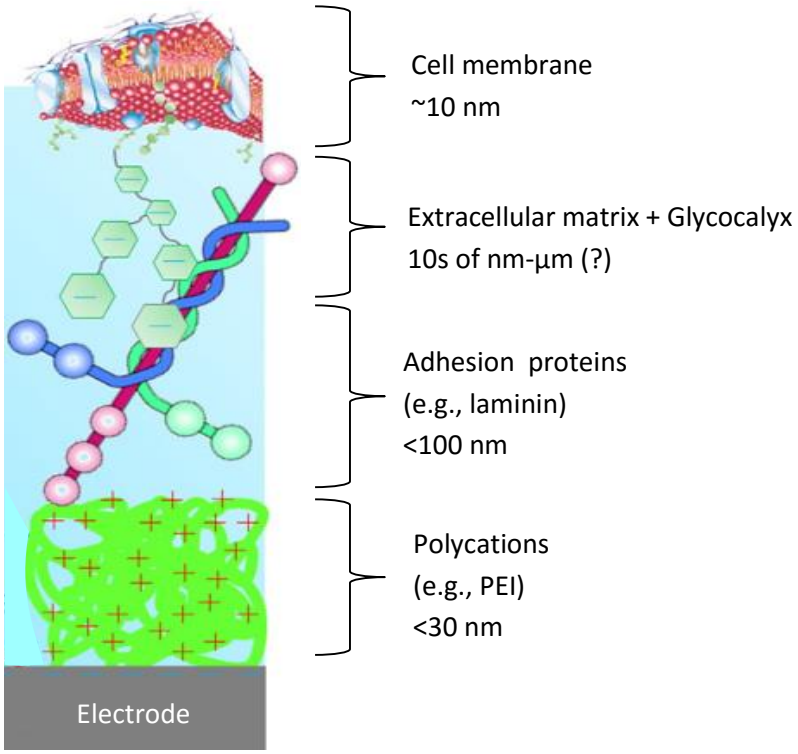


Figure 7 – Neuron-electrode distance after MEA coating. *Relative positioning and thickness of coating agents, extracellular matrix (ECM), other glycolipid and glycoprotein membrane components (glycocalyx) and MEA surface. Positively charged polycations and adhesion proteins promote negatively charged extracellular components adhesion. Due to its heterogeneity and plasticity, the extracellular space between the cell and the substrate/electrode is hard to calculate. In fact, according to recent findings the negatively charged glycocalyx may be orders of magnitude thicker than previously thought (14). Thus, the electrode-cell membrane distance may be between 100s of nm and a few µm [adapted from (14)].*

3.2 Cell Culture

For every experiment, the SH-SY5Y cell line (ATCC® CRL-2266™) was used. This neuroblastoma cell line was chosen since it is human derived and can be differentiated towards a more mature neuronal-like phenotype.

SH-SY5Y cells grow as clusters of neuroblastic cells with multiple, short, fine cell processes (neurites). Typically, they aggregate, form clumps and float, as they grow as a mixture of floating and adherent cells. Their proliferation kinetics resemble that of many tumor cell lines well with a population doubling time of approximately 48 hours (111). They continue to divide after the monolayer is confluent, tending to grow over each other. Therefore, cells were passaged to approximately 70% confluence on the MEAs and the experimental procedure was then carried out.

All procedures involving cell culture manipulation were performed under a class II air flow cabinet.

3.2.1 Growth and Maintenance of SH-SY5Y cell culture

Before use, SH-SY5Y cells were cryopreserved in liquid nitrogen (-196ºC) with a cell-freezing medium comprised of complete growth medium supplemented with 5% (v/v) dimethyl sulfoxide (DMSO). Complete growth medium was a 1:1 mix of minimal essential medium (MEM) and F12 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (200 mM stock solution), sodium bicarbonate, sodium pyruvate and 1% of antibiotic/antimycotic.

To use, cells were quickly thawed using a water bath at 37ºC and then collected into a conical tube. The culture medium was added dropwise (to avoid cell lysis) and re-suspended to dilute the DMSO present in the cell-freezing medium. Cells were centrifuged at 1000 rpm for 3 min and the supernatant removed. The pellet was re-suspended in complete growth medium and cells were then seeded in a 100 mm culture plate.

Cell cultures were maintained in an incubator at 37ºC with 5% CO_2 and 95% humidity. Complete growth medium was replaced in three-day intervals until cell passage or culture on MEAs. For every cell splitting, old medium was aspirated and cells were washed 3 times with 4 ml pre-warmed PBS to keep the pH approximately constant. Then, 2 ml of 0,05% trypsin-EDTA solution was added and the culture was incubated at 37 °C for 2 minutes in order to re-suspend cells adherent to the cell culture dish. After checking under an optical microscope whether cells were completely detached, 6 ml of fresh complete medium was added to the trypsinated cells in order to inactive the trypsin. Then, cell suspension was transferred to a 15 ml vial and centrifuged at 1000 RPM for 3 minutes. After the supernatant had been discarded, cells were carefully resuspended in fresh complete medium and seeded at the desired cell density.

3.2.2 SH-SY5Y cell culture on MEAs

After subculturing and MEA's preparation (sterilization and surface treatment), SH-SY5Y cells were seeded on the arrays. Optimization of the cell density on the arrays was performed. Therefore, 40.000, 60.000, 80.000 and 100.000 viable cells were seeded and differentiated for 7 days on MEAs.

RA-induced differentiation was also optimized, while concomitantly searching for the most indicated MEA surface treatment.

3.2.2.1 Optimization of Cell Density

Literature divergences and exploratory experiments discrepancies led to testing the effect of cell plating density on MEA recordings.

SH-SY5Y cells have a reported saturation density greater than 10.000 cells/mm^2 (111), however differentiation protocols diverge from as low as 35 cells/mm^2 (83) to a few 100s of cells/mm^2 (46,75) at initial seeding. An appropriate cell density for RA-induced differentiation has been defined as 70 to 210 cells/mm^2 (72). Ferrández et al (2011) cultured between 80.000 (~280 cells/mm^2) and 120.000 (~420 cells/mm^2) SH-SY5Y cells on identical MEAs, though cultures did not undergo differentiation treatment (30). Teppola et al (2008) cultured SH-SY5Y cells at a cell density of 50 cells/mm^2 and induced differentiation on MEAs with RA and cholesterol (44).

Based on literature and previous experiments the following cell censities were tested: 40.000 (~140 cells/mm^2); 60.000 (~210 cells/mm^2); 80.000 (~280 cells/mm^2) and 100.000 (~350 cells/mm^2) viable cells per MEA.

Before seeding, cell counting was performed using an adapted Trypan Blue protocol (112) as described below. Then, cells were seeded in MEAs, which had been previously treated with PEI and laminin.

3.2.2.1.1 Cell Counting

In order to count cells for plating, a Trypan Blue protocol was followed. This dye exclusion method is based on the principle that live (viable) cells do not take up impermeable dyes (like Trypan Blue), whereas dead (non-viable) cells are permeable and take up the dye. It is important to not expose cells to Trypan Blue for extended periods of time, as it is cytotoxic and viable cells may be affected. Once exposed to Trypan Blue, cells can be counted with a hemocytometer under an optical microscope.

The hemocytometer is the most used type of cell counting chamber. It consists of a thick glass microscope slide with a rectangular indentation that typically creates two separate chambers. Each chamber contains a gridded area, with divisions separated into 9 large squares. Each square has a surface area of 1 mm^2 and the depth of the chamber is 0.1 mm. Therefore, each square of the hemocytometer, with cover slip in place, represents a total volume of 0.1 mm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the cell concentration per ml of the original aliquot may be calculated.

Firstly, the hemocytometer and the coverslip were cleaned using 70% ethanol and the coverslip affixed gently. Cells were subcultured and harvested as mentioned in 3.2.1. Subculture's 1 ml of cell suspension was aliquoted to an eppendorf. Subsequently, 10 µl of the aliquoted cell suspension and 10 µl of 0.4% Trypan Blue solution were combined in a 1:1 dilution (dilution factor of 2). The solution was mixed thoroughly and allowed to stand for 1 minute. Then, 10 µl of Trypan Blue-cell suspension mixture was added to each chamber of the hemocytometer, filling by capillary action

(no over or underfilling each chamber). Under an optical microscope, viable and non-viable cells were counted in each chamber's five squares (one central and one on each corner).

The total number of cells per ml, total number of viable cells per ml and percentage of viable cells were calculated, as necessary.

The total number of cells per ml is given by the following formula:

$$Number\ of\ cells\ per\ ml = \left[\frac{Total\ cell\ count}{Number\ of\ squares\ counted}\right] \times Dilution\ factor \times 10^4$$

The total number of viable cells per ml is given by the following formula:

$$Number\ of\ viable\ cells\ per\ ml = \left[\frac{Number\ of\ viable\ cells}{Number\ of\ squares\ counted}\right] \times Dilution\ factor \times 10^4$$

The percentage of viable cells per ml of culture is given by the following formula:

$$\%\ of\ viable\ cells = \left[1 - \frac{Number\ of\ blue\ cells\ (non-viable)}{Number\ of\ total\ cells}\right] \times 100$$

3.2.2.1.2 Cell Seeding

Before MEA recording experiments took place, 40.000, 60.000, 80.000 and 100.000 viable cells were plated on different MEAs and differentiated along a 7-day period. The objective was to choose the cell density that produced the most viable and neuron-like culture. In order to monitor each culture, phase-contrast photographs were taken every 3 days and 3-minute recordings were acquired on the final day.

Viable cells were subcultured, harvested and counted as mentioned before in section 3.2.1 and 3.2.2.1.1, respectively. MEAs were prepared as mentioned in annex 7.1 and coated with PEI and laminin prior to cell seeding.

A dilution was calculated so that the desired number of cells for plating was in a final volume of 20-40 µl. Therefore, a minimum final concentration of 1000 cells per µl and a maximum of 5000 cells per µl was used. Cells were then seeded at a total suspension volume of 1 ml in complete growth medium.

3.2.2.2 Optimization of Cell Differentiation

When SH-SY5Y cells are suspended in solution, connections previously established are destroyed. Therefore, differentiation treatment must be carried out once cells are attached onto the array.

In order to differentiate SH-SY5Y cells into neuronal-like cells, cultures were treated with 10 µM RA. Several variations of the RA-induced differentiation conditions are found in the literature. Different serum and/or RA concentrations have been used in the differentiation medium (46,71), though RA is typically administered at a concentration of 10-20 µM. This protocol diversity led to a decision of testing different combinations of fetal bovine serum (FBS) concentration in the differentiation medium. Remaining constituents of the differentiation medium were the same as described in section 3.2.1 plus 10 µM RA.

Serum-free (0%) and low serum (1% and 3%) media were administered concomitantly with 10 µM RA along a 7-day period. Therefore, complete growth medium was aspirated and replaced by the differentiation medium the day after cell seeding. Differentiation medium was replaced every 3 days, totaling 3 additions per culture. The various combinations of MEA coatings and cell culture medium parameters tested can be seen in Table 1.

Table 1 – Combination of MEA cell culture conditions for optimization of the SH-SY5Y cell line differentiation. *Different MEA coatings and differentiation medium conditions were tested alone and combined. Ticks represent tested conditions.*

	SH-SY5Y CELLS	**NON-DIFFERENTIATED**	**DIFFERENTIATED**		
	CULTURE MEDIUM	10% FBS	0% FBS + 10 µM RA	1% FBS + 10 µM RA	3% FBS + 10 µM RA
MEA COATING	**Without coating**	✓	✓		✓
	0.1% **PEI**	✓	✓		✓
	0.1% **PDL**				✓
	0.001% **Laminin**	✓	✓		✓
	0.1% **PEI+** 0.001% **Laminin**	✓	✓	✓	✓
	0.1% **PDL+** 0.001% **Laminin**				✓

Abbreviations: MEA, multielectrode array; PEI, polyethyleneimine; PDL, poly-D-lysine; FBS, fetal bovine serum; RA, retinoic acid.

Phase-contrast photographs were taken at three separate times during the 7-day differentiation treatment. Furthermore the evaluation of neuronal differentiation was accompanied by electrical activity measurements in order to determine whether differentiated SH-SY5Y cells had the ability to generate spontaneous activity.

3.3 MEA Recordings of SH-SY5Y cell culture's Electrical Activity

3.3.1 MEA System Set Up

The MEA2100-System was used for recording and analysis of the MEA's SH-SY5Y cell cultures electrical activity. This MEA system is a versatile *in vitro* recording system manufactured by MCS (47,78). It consists of several components, including: headstage (equipped with integrated amplifier, stimulator and analog-to-digital converter), interface board, data acquisition computer, temperature controller (T-control), as well as a perfusion cannula and peristaltic perfusion pump which were not used in the course of these experiments (78).

A included T-control unit guarantees a stable and precise temperature control over a wide temperature range, from ambient temperature up to 105 °C (32). The T-control is connected to an internal heating element and a Pt-100 temperature sensor incorporated in the headstage. During recordings, MEAs contact with the internal heating element. Thus, the heating element guaranteed constant temperature conditions for the MEA cell culture.

Very small unnoticed vibrations could increase noise or render unspecific "signals". Therefore, recordings were performed with the headstage placed on top of a completely stable platform proper for long-term electrophysiology experiments – "Scientifica SlicePlatform". In turn, an up-right microscope was mounted on the fixed platform and allowed the optical monitorization of the region of interest (ROI) during recordings.

More information on how the MEA system was set-up can be seen in annex 7.2. The complete MEA system set up can be seen in Fig. 8.

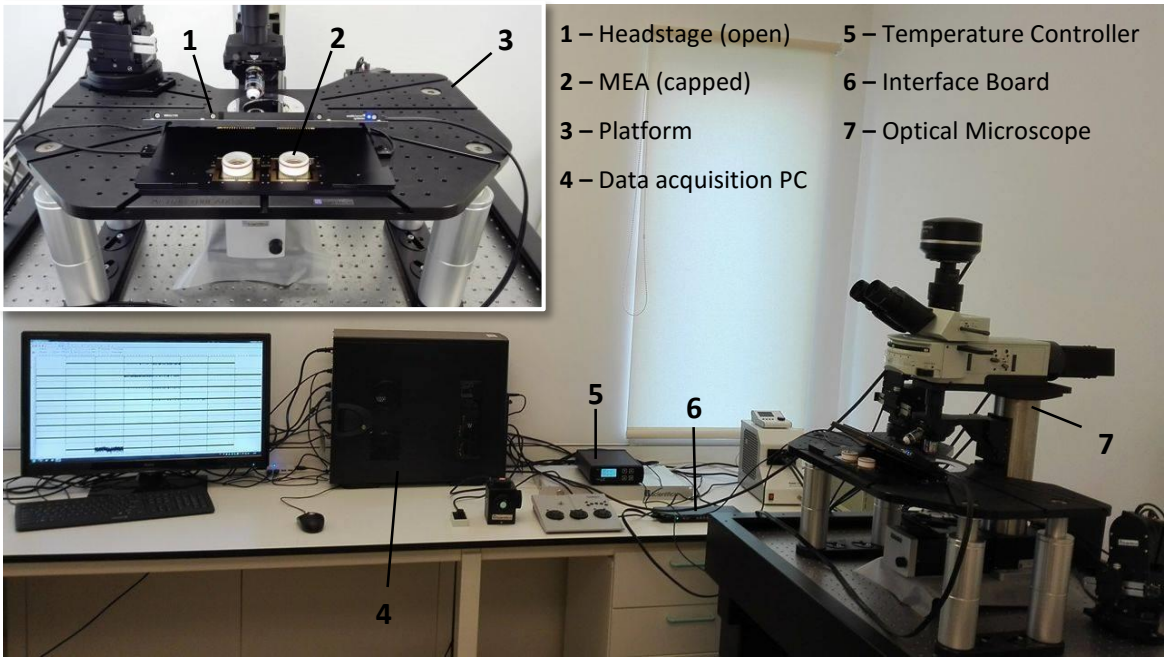


Figure 8 – MEA system set up. *Microscope controllers are in the photograph but were not subtitled.*

3.3.2 Recording Procedures

Though long-term recordings (spanning several days/months) are possible, these render enormous amounts of data and demand logistical and practical conditions which were impossible to attain. Maintaining ideal cell culture conditions for prolonged periods of time were especially limitating factors. Short-term recordings range from a few minutes to 1-2 days but still yield enough data and are more indicated for certain types of studies. In fact, similar studies have employed short-term recordings of pre-, during and post-Aβ 1-42 oligomers addition (39,54,100).

In this study, short-term recordings (3 to 33 minutes) were made. Though short-term, recordings required standard and optimized procedures. The next sections describe the recording procedures.

3.3.2.1 Preparation

Dirt on the MEA's contact pads or on the headstage's contact pins leads to bad contact and electrical noise. Therefore, before any recordings took place, pins and contact pads were cleaned by gently wiping with 70% ethanol.

Neuronal electrical activity is dependent on ideal environmental conditions including temperature and pH (33). Thus, the T-control unit was formerly set to 37 °C in order to mimic body temperature conditions and diminish the incubator-MEA system shifting shock.

When SH-SY5Y cell culture's electrical activity was to be recorded, MEAs were transported from the storing incubator to the MEA system, while inside the Petri dish. The bottom of the dish was kept parallel to the ground and quick movements were avoided, as jarring the MEA could detach the cell culture from the substrate. Then, the MEA was removed from the dish and placed on the MEA2100-System headstage/amplifier.

3.3.2.2 Recording

After placing on the headstage, cell cultures were left do adapt for 1 minute before recording took place. This minute also allowed for the stabilization of medium fluxes caused by transport. Even though gas conditions were different from the incubator-controlled environment, previously placed Teflon lids helped reduce evaporation and contamination.

The recording area of each MEA consisted of 59 recording electrodes and 1 iR disposed along an area measuring roughly 2.5 mm^2. As each array measured approximately 283 mm^2, each recording area accounted for less than 1% of the total cell culture area. This ROI was visually assessed during recordings by an Olympus up-right microscope. A bright-field photograph taken during recordings can be seen in Fig. 9A.

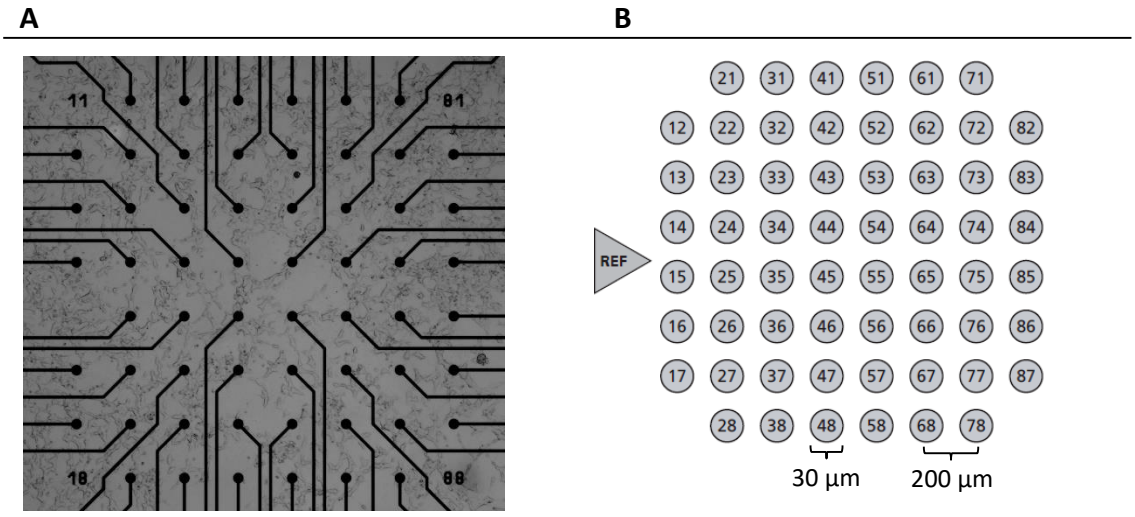


Figure 9 – Recording area during MEA recordings. ***(A)*** *Bright-field photograph of a SH-SY5Y cell culture recording area at a magnification of 40×. Note that the numbering in each corner functions as orientation.* ***(B)*** *Standard electrode numbering scheme. In the 8x8 layout grid, the numbering of MEA electrodes follows the standard numbering scheme for square grids: the first digit is the column number, while the second digit is the row number. Note that in 60MEA200/30iR-Ti-gr MEAs, electrodes diameter is 30 µm and inter-electrode centers distance is 200 µm [adapted from (78)].*

MC_Rack software is included with each MEA system provided by MCS. Here, tools can be chosen for displaying data, for digital filtering, for extracting spikes out of raw data, for analyzing the slope/amplitude of an evoked response, and/or calculating the spike rate. Simultaneous recording of multiple MEAs is possible. MEA's cultures electrical activity can be recorded independently of each other, creating their own data streams (32,78).

MC_Rack (4.5.16 version) was used to record and analyze MEA's SH-SY5Y cell cultures electrical activity. Extracellular signals were recorded and processed by a 120-channel, 0.1 Hz-10 kHz band-pass filter-amplifier data acquisition system at 25 kHz sampling rate per channel. The amplification stage functioned as an operational amplifier with a fixed gain of 2. The sampling rate (25 kHz), signal range (± 2500 mV) and bandwidth (1 Hz to 3 kHz) were adjusted via software control.

Recordings lasted for, at least, 180 s (3 min). Most often two MEAs were subject to experiment simultaneously. In order to do so, two MC_Rack independent instances were initiated on the data acquisition computer. When scheduled recording time finished, cell cultures were removed by trypsinization and MEA cleaning and sterilization ensued.

3.3.2.2.1 Aβ 1-42 Oligomers Addition

In order to evaluate the effects of Aβ on SH-SY5Y cell's electrical activity, MEA cultures were treated with different concentrations of Aβ 1-42 oligomers during recordings. The goal of this work was to determine an acute effect of Aβ 1-42 oligomers on SH-SY5Y cell cultures firing dynamics.

Synthetic Aβ 1-42 (Genic Bio) was dissolved in water to prepare 1 mM stock solution. Prior to cell culture exposure to the Aβ peptides, an aggregation step took place. This was achieved by incubating Aβ 1-42 for 48 hours at 37°C with PBS (1x) at a concentration of 100 μM.

Recordings without Aβ addition lasted 180 s. In cases where Aβ was to be added, the medium was rapidly substituted by medium containing the desired Aβ concentration after initial equivalent 180 s recordings. In two subsequent experiments, Aβ was added after previous medium exchange under a laminar flow cabinet (30 minutes before experiment). Different cell cultures were exposed to 0.1, 1 and 10 μM Aβ 1-42 oligomers. The whole procedure was recorded and microscopically monitored, so as to identify if cells detached during Aβ addition. Recordings continued for 1800 s (30 min) totaling 1980 s. A control cell culture was subject to medium exchange with only PBS (1x) instead of Aβ dissolved in PBS. This was to ensure that the results obtained were not due to PBS, which was used in the aggregation step, but due to Aβ 1-42.

3.3.3 Data Analysis

MC_Rack software is included with each MEA system provided by MCS. Here, tools can be chosen for displaying data, for digital filtering, for extracting spikes out of raw data, for analyzing the slope/amplitude of an evoked response, and/or calculating the spike rate. Simultaneous recording of multiple MEAs is possible. MEA's cultures electrical activity can be recorded independently of each other, creating their own data streams (32,78).

Spikes are usually extracted from the raw data using an amplitude threshold. The threshold is usually set as multiple (as 3 times) of the baseline noise level. The choice of the threshold represents a compromise between missing spikes if a high threshold is used (Type II error), or getting false positives due to noise surpassing a low threshold (Type I error).

An automatic threshold is preferable and can be set as a multiple of the standard deviation (SD) of the signal. Therefore, the SD of each electrode data trace was used to estimate its spike detection threshold. A time interval of 500 ms was used to calculate the SD. Taking into account previous MEA studies using SH-SY5Y cell line (30,44), spike detection threshold was set at a triple noise level (-3) in order to identify spikes among the noise. Only values above this threshold were extracted as spiking activity. Recordings of every single electrode (n) lasted for, at least, 180 s. Spikes above threshold were extracted from raw data and parameters, such as spike rate (Hz), were calculated separately in Graph Prism 6. All statistical analysis was performed in either GraphPad Prism 6 or Microsoft Excel.

Analysis was purely quantitative, not qualitative. Spike sorting algorithms are an advanced way of analyzing qualitatively extracted data. Spike sorting helps distinguish superimposed spikes, or false positives from data (6). However, there are no established algorithms for analyzing data obtained with SH-SY5Y cells. Thus, data was not analyzed qualitatively and it is prone to the two types of errors mentioned.

4. Results & Discussion

4.1 SH-SY5Y cell culture on MEAs

SH-SY5Y neuroblastoma cells have been employed in neuroscience investigation for the past decades. Despite its potential, SH-SY5Y cell line has rarely been used in MEA studies. We believe that a lack of consensual protocols in culturing methods is one of the main reasons for such disuse.

In cell cultures, normal attachment, growth, and development are dependent on attachment factors and ECM components. Though some cells are able to synthesize such components, others require an exogenous source, particularly when grown in serum-free medium. Surface-treating MEAs is mandatory, but has not been deeply explored in relation to SH-SY5Y cell line culturing. Cell plating density affects cell culture viability and subsequent differentiation treatment, however SH-SY5Y cell plating numbers vary immensely, even in similar studies. Furthermore, there is no available protocol for SH-SY5Y cells RA-induced differentiation on MEAs. These specificities led to a necessity to optimize the various steps for a MEA's SH-SY5Y cell culture protocol.

Ultimately, the main objective of these experiments was to evaluate whether an optimized SH-SY5Y cell culture could be a suitable model for reliable *in vitro* MEA recordings.

4.1.1 MEA Coating

The goal of this experiment was to find the best alternative amongst various coating agents by following the cell growth, morphology and spontaneous electrical activity exhibition. In order to do so, SH-SY5Y cells were cultured and differentiated with conditioned medium (3% FBS + 10 μM RA) for 7 days on MEAs without coating and various coating agents alone and combined. These coating agents were: PEI, PDL and laminin. Combinations of PEI+laminin and PDL+laminin were also tested.

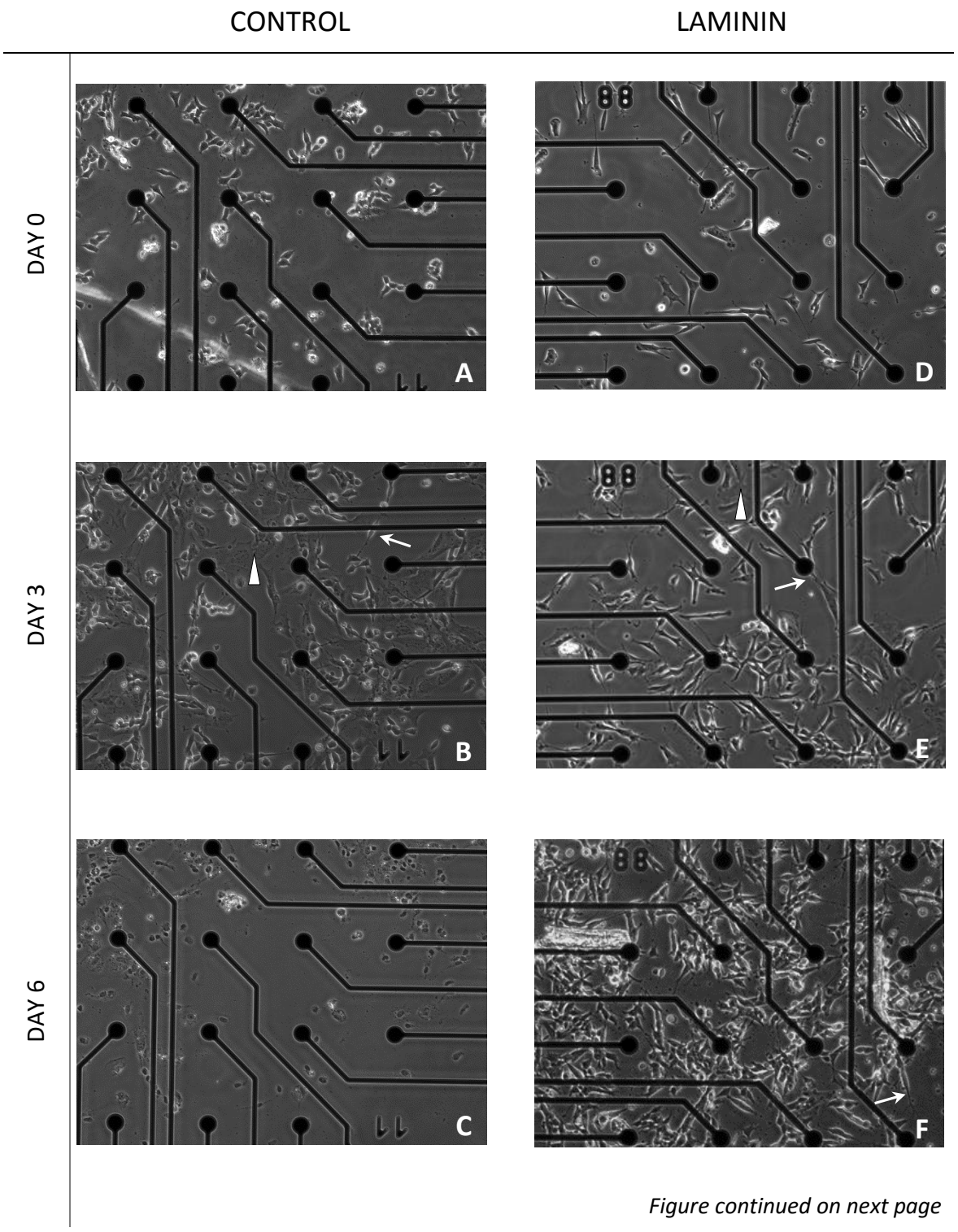
MEAs are hydrophobic, thus difficult cell-substrate adhesion. Polycations, such as PDL or PEI, change the substrate charge and facilitate cell adhesion. Moreover, laminin acts as adhesion-mediating compound but has also been shown to enhance neuronal differentiation (46,110). To date, no comprehensive study had focused on the effects of different MEA coatings in SH-SY5Y cells morphological differentiation, as well as electrophysiological activity. Teppola et al (2008) had tested PDL, PLL, PEI and laminin but with no correlation to cell cultures electrical activity (44). Coated MEAs are expected to have improved neuron-electrode contact and increased seal resistance. Therefore, higher SNR is predictable.

SH-SY5Y cell cultures are comprised of the two main distinct phenotypes: S- and N-type. These are easily distinguished under a microscope as the S-type phenotype is more epithelial-like with no or underdeveloped processes, whereas the N-type is more neuronal-like with pyramidal bodies and long processes. N-type cells tend to adhere weakly to the substrate. However, neuronal differentiation and neuritogenesis of SH-SY5Y cells have been proven to be regulated via cell-substrate mechanical interactions (72).

Although each recording area accounts for less than 1% of the total cell culture area, cell culture viability and spontaneous electrical activity depend on the surroundings, not only the recording area. Therefore, when treating MEAs it was important to homogeneously expose the array to the coating agents. Pipetting at least 400 μl of solution was enough to cover the whole array during incubation.

4.1.1.1 Morphological Evaluation

To evaluate how the different MEA coatings affected cell distribution and morphology during differentiation treatment, phase-contrast photographs were taken 8 hours after each medium exchange. Representative cell culture sections from each MEA are presented in Fig. 10.



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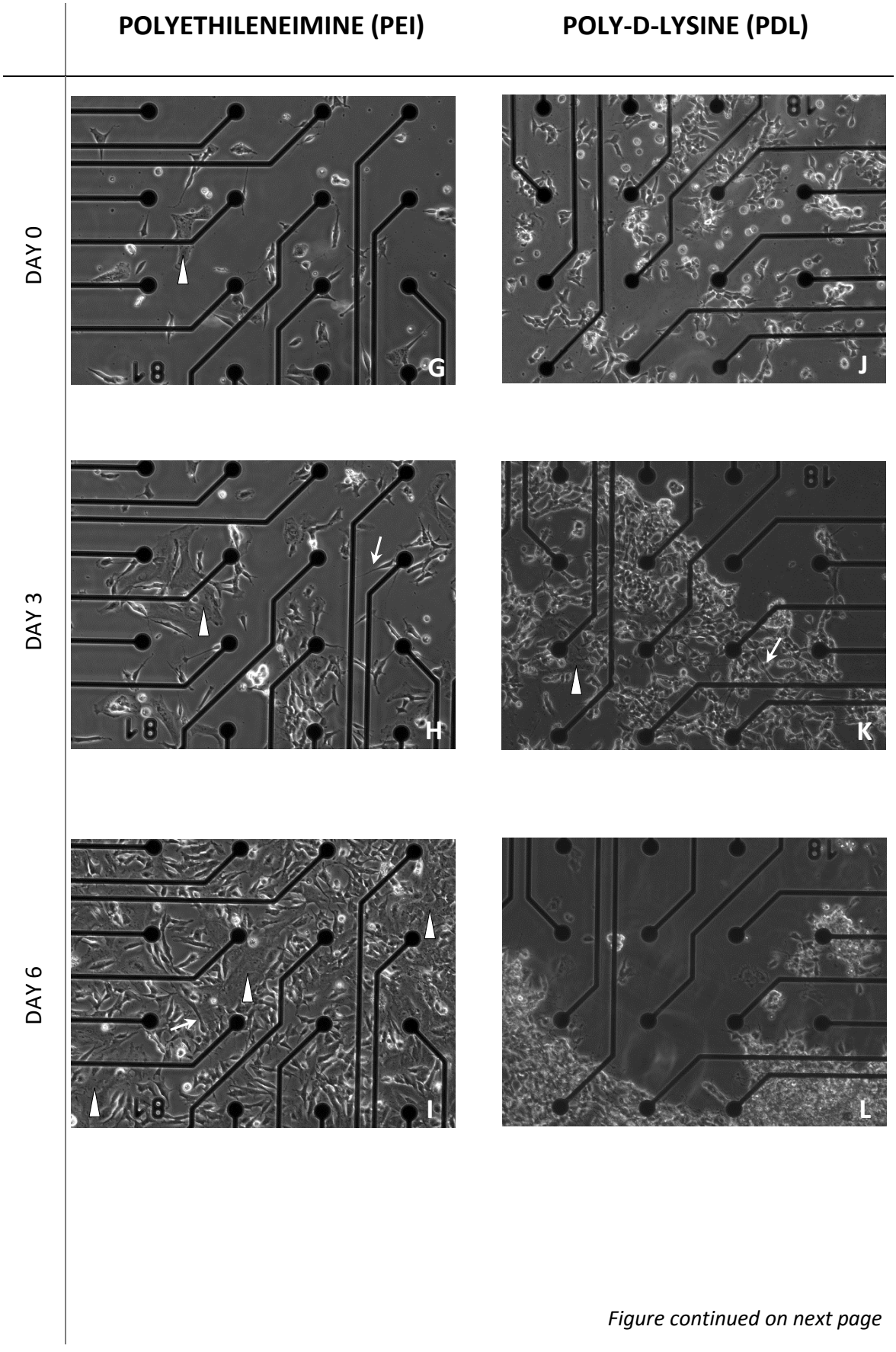

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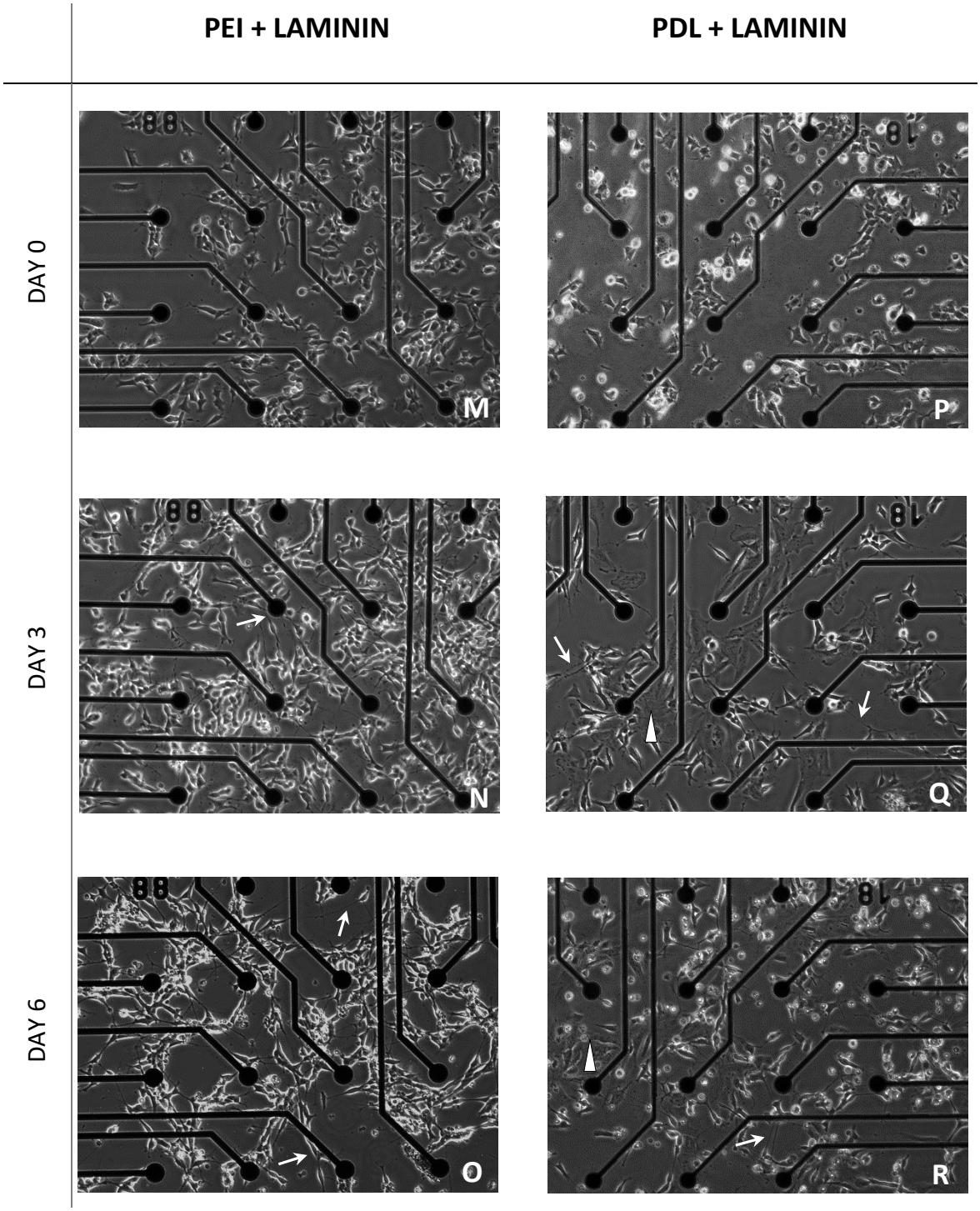


Figure 10 – Phase-contrast photographs of SH-SY5Y differentiated cells in coated MEAs. *Control culture was grown on MEA without surface treatment (no coating). A; D; G; J; M; P represent control; laminin; PEI; PDL; PEI + laminin and PDL + laminin coated MEAs, respectively, at the first day of differentiation treatment (day 0). B; E; H; K; N; Q represent control; laminin; PEI; PDL; PEI + laminin and PDL + laminin coated MEAs, respectively, at the fourth day of differentiation treatment (day 4). C; F; I; L; O; R represent control; laminin; PEI; PDL; PEI + laminin and PDL + laminin coated MEAs, respectively, at the seventh day of differentiation treatment (day 6). White arrows indicate extended neurites, while white triangles signpost S-type subpopulations. All photographs show a magnification of 100×.*

From the results here presented it was possible to observe that 32 hours after initial seeding, SH-SY5Y cells had adhered in all MEA substrates. However, there were already differences in cell distribution, viability and differentiation.

Uncoated MEA (control) showed unevenly distributed cells (Fig. 10A), when compared to the other MEAs. This has been previously reported in SH-SY5Y cell line MEA culturing (44) and negatively affects networks formation. Moreover, PDL (Fig. 10J) and PDL+laminin-coated (Fig. 10P) MEAs presented many floating cells. As these non-adherent and spherical cells are dead cells, PDL-coated MEAs seemed to provide a cytotoxic environment for the cells. Such cytotoxicity could have been caused by insufficient rinsing following surface treatment, as polycations have high pH. However, PEI-coated MEAs underwent the same preparation and did not present comparable quantities of dead cells.

Interestingly, laminin-coated (Fig. 10D) and PEI-coated (Fig. 10G) MEAs already exhibited cells with developed processes and short neurites. However, this was not so for PEI+laminin-coated MEA (Fig. 10M). Although laminin is known to enhance neurite extension (46), PEI alone has been reported as not being sufficient for optimal growth (44).

At day 3 (72 h after), cultures had developed in different ways. In spite of this, all cell cultures exhibited some degree of cellular differentiation and network formation.

Laminin (Fig. 10E), PEI (Fig. 10H), PEI+laminin-coated (Fig. 10N) MEAs exhibited higher neurite extension than other coatings. PDL+laminin-coated MEA (Fig. 10Q) displayed less dead cells than at day 0, but undifferentiated S-type small populations began to appear. This also happened in the PEI-coated MEA (Fig. 10H). A large clump of cells detached in the PDL-coated MEA (Fig. 10K) during medium exchange, indicating weak cell-substrate adhesion with this type of coating.

On the final day (day 6), MEA cultures had completely different outcomes. After 7 days of differentiation, it was possible to observe that most cultures were not optimal.

Uncoated MEA (Fig. 10C) had no remaining living cells, as most detached during medium exchange or had already died. Laminin-coated MEA (Fig. 10F) had an increased proliferative rate and cells exhibited shorter neurites on day 6 than at day 3. PEI-coated MEA (Fig. 10I) had achieved cell density saturation due to incontrollable proliferation, mainly by S-type subpopulations. PDL-coated MEA (Fig. 10L) cells had almost completely detached, after forming a clump. PDL+laminin-coated MEA (Fig. 10R) presented many floating cells and an underdeveloped network. PEI+laminin-coated MEA (Fig. 10O) had formed a mature network, as cells exhibited extended neurites and interconnected along the array.

It has previously been reported that PEI+laminin coating induces a more neuron-like than the other treatments. PDL+laminin has also been shown to induce more growth of S-type cells, when compared to PEI+laminin (44). These results corroborate such findings, as PEI+laminin-coated MEA exhibited the most mature neuronal network among tested conditions.

This study underlined the need for optimizing other parameters such as cell plating density and differentiation medium. Cell density proved to be critical to the final outcome of a neuronal

network, as not plating the exact cell numbers may have led to pronounced differences in cell culture differentiation and viability. Furthermore, the used conditioned medium (3% FBS + 10 μM RA) failed to contain S-type subpopulations growth. In the PEI-coated MEA (Fig. 10I), S-type cells eventually overgrew N-type cells.

RA has growth-inhibiting and differentiating properties. So, when exposed, SH-SY5Y cells should choose to differentiate rather than divide. However, 3% serum concentration in the medium could be sufficient to induce cell proliferation, despite RA presence. Moreover, long RA differentiation treatments have promoted proliferation of S-type cells, while short-term treatments induced differentiation the N-type cells (71).

The results indicate that the viability and distribution of the SH-SY5Y cells are similar on MEAs coated with laminin alone or combined with PEI. Despite this, cells plated in the PEI+laminin-coated MEA exhibited a more mature network further along the differentiation treatment. Thus, the present study opted for PEI + laminin coating for further experiments. Furthermore, detailed attention was employed with respect to the cell plating density and the chosen differentiation treatment.

4.1.1.2 Electrical Activity

To evaluate whether coating MEAs with different adhesion-mediating compounds correlated with spontaneous electrical activity differences, short-term MEA recordings were taken.

MEA recordings were made on the final day (day 6) of the differentiation treatment, after taking the phase-contrast photographs (day 6). Recordings were carried through and analyzed recurring to MC_Rack software.

Recordings of spontaneous electrical activity lasted for 180 s. Pairs of MEAs were subject to experiment simultaneously. Upon experiment completion, trypsinization and MEA cleaning removed MEA cell cultures, and sterilization was carried out.

Peak detection was applied to the raw data to better visualize data traces. A 2-second example of output from MC_Rack can be seen in Fig A. The SD of each data trace was used to estimate its detection threshold, which was established at triple noise (-3). Electrodes that picked up-threshold electrical activity during the experiment time-course were considered as “active electrodes”. These active electrodes were “visible” on a separate overlayed data stream, as can be seen in Fig. 11B and Fig. 11C. The total number of active electrodes per coated MEA can be seen in Fig. 11D.

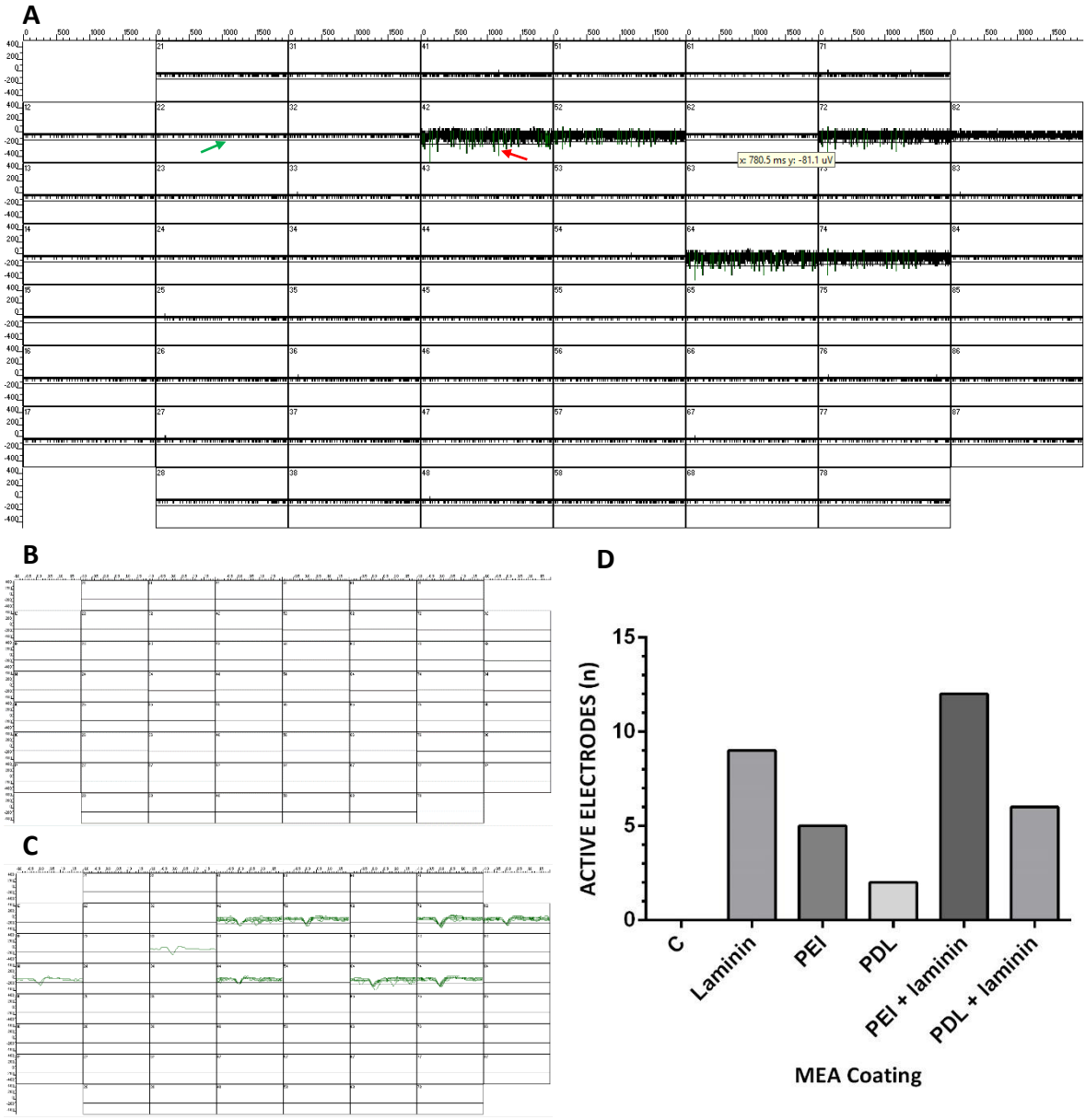


Figure 11 – MEA recordings of SH-SY5Y cell cultures with different coating treatments. (A) *2-second display of a recorded data stream corresponding to the laminin-coated MEA. Each square represents one electrode. Y-axis and X-axis expressed in µV and ms, respectively. Green arrow indicates detection threshold (SD method; -3) bar. Red arrow denotes up-threshold electrical activity. "Green" spikes surpassed the detection threshold and were extracted from raw data.* ***(B)*** *Display of overlayed extracted data corresponding to the uncoated MEA (control).* ***(C)*** *Display of overlayed extracted data corresponding to the laminin-coated MEA.* ***(D)*** *Graph represents the total number of active electrodes (i.e. ones from which signals were recorded) per 3-minute recordings of differently coated MEAs.*

Active electrodes were visible after spike detection using the SD method (Fig. 11A). Detected spikes were separated from raw data and displayed in overlayed data streams (Fig. 11B and Fig 11C). As an example, Fig. 11C shows 9 active electrodes and corresponds to the laminin-coated MEA. The

same procedure was applied to the remaining raw data. This simple analysis enabled the detection of immediate differences.

As can be seen in Fig. 11D, laminin-coated MEAs exhibited more active electrodes. Therefore, the role of laminin in neuronal differentiation (46) seemed to correlate with increased spontaneous electrical activity. PEI+laminin-coated MEA (Fig. 10O) was clearly the MEA with most active electrodes, as anticipated by microscopical evaluation. These results are in accordance with previous studies (14,44). Laminin-coated MEA (Fig. 10F) and PDL+laminin-coated MEA (Fig. 10R) were the 2nd and 3rd with more active electrodes.

As expected, uncoated MEA (control) did not present any active electrode (Fig. 10B). Remaining attached cells were either nonviable or did not have any developed processes (Fig. 10C). PDL-coated MEA (Fig. 10L) presented mostly inactive electrodes, as expected. Most of the electrodes were not covered up by cells, unlike the two which picked up-threshold signals (data not shown).

S-type cells do not have neurites and are relatively electrically inactive (76). This helps explain why PEI-coated MEA (Fig. 10I) presented less active electrodes, despite its greater cell density. As S-type subpopulations overgrew N-type cells, electrical function was reduced. This result underlines the importance of restricting S-type proliferation in culture.

The morphological and spontaneous electrical activity evaluations combined, led to selecting PEI + laminin coating as standard procedure in SH-SY5Y cells culturing on MEAs. However, such evaluation could have been influenced by different cell plating densities and non-optimized differentiation procedures. Therefore, optimization of such factors was carried on.

4.1.2 Cell Plating Density

The effect of cell density on MEA recordings and cell culture viability was assessed by seeding different total numbers of cells and following their differentiation along a 7-day period. Phase-contrast photographs were taken at day 0, day 3 and day 6, while MEA recordings were performed at the final day. Initial seedings were of 40.000 (~140 cells/mm^2); 60.000 (~210 cells/mm^2); 80.000 (~280 cells/mm^2) and 100.000 (~350 cells/mm^2) viable cells per MEA. MEAs were previously coated with PEI and laminin double-step treatment.

As cell density increases, the number of electrodes covered up by cells should also increase. On the other hand, if cell density is excessive, cells may lack the physical space to extend neurites during differentiation and form mature neuronal networks. Although the number of covered electrodes could be assessed by microscopically monitoring the culture, whether this cell-electrode proximity transduced into up-threshold electrical activity had to be tested recurring to recordings. A simple way of determining whether cell density affected network activity was to count the number of electrodes which detected up-threshold activity during the 3-minute recordings. These electrodes were considered as active electrodes.

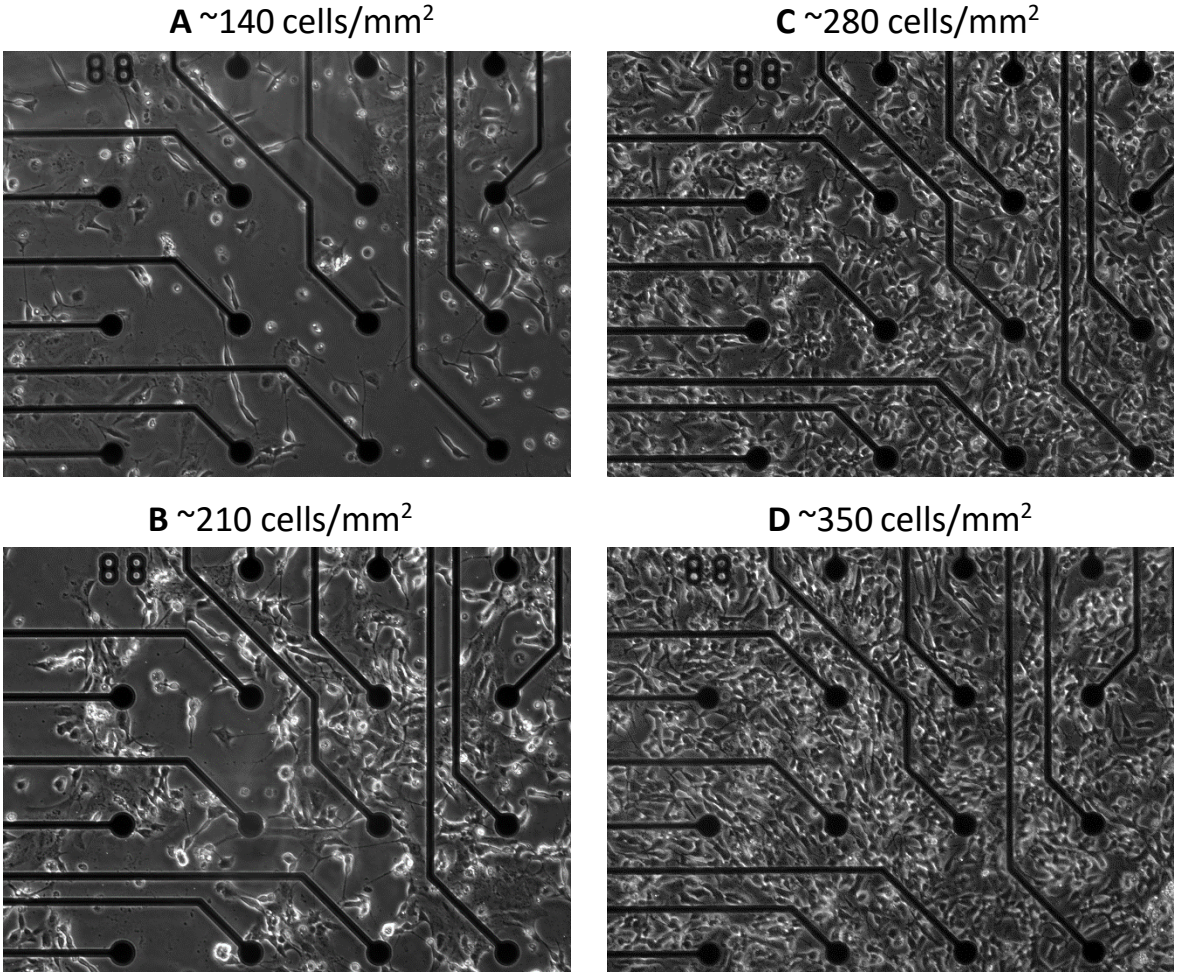


Figure 12 – Phase-contrast photographs of SH-SY5Y cells at different cell plating densities. *Photographs were taken after 7-day differentiation treatment. A; B; C; and D represent sections of MEAs where 40.000; 60.000; 80.000 and 100.000 cells were seeded, respectively. All photographs show a magnification of 100×.*

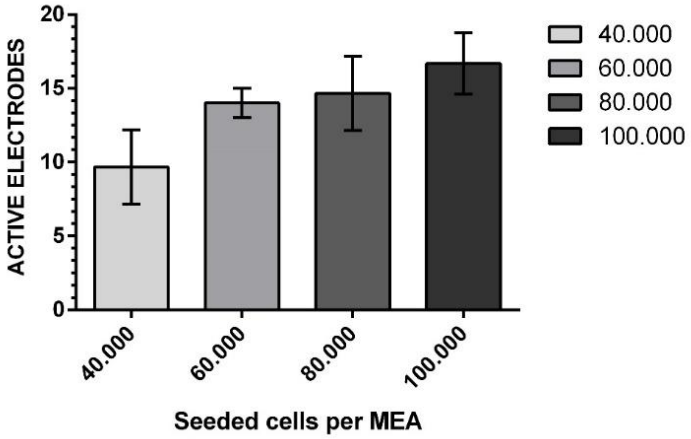


Figure 13 – Cell density effect on the number of electrodes activated. *Graph represents the number of active electrodes (i.e. ones from which signals were recorded) per 3-minute recordings of cell cultures with different cell plating densities, after 7-day differentiation treatment. All values are expressed as mean, error bars represent standard deviation (±SD), n=3.*

Brain neurons are typically grown in culture above a density of 300 cells/mm^2 (113). However, dissociated neuronal cultures have been plated on MEAs in densities ranging from 20 cells/mm^2 to 1000 cells/mm^2 (33). In turn, SH-SY5Y cells have been plated on MEAs in densities ranging from as low as 50 cells /mm^2 (44) to 420 cells/mm^2 (30).

From the results here presented, one can deduce that cell plating density plays an important role in SH-SY5Y network formation. From a morphological point of view, plating 60.000 cells produced the more neuronal-like networks (Fig. 12B). At this density, cells had enough space to develop extended neurites, but still covered most electrodes. In contrast, plating 80.000 and 100.000 cells resulted in saturated networks, where cells did not appear morphologically differentiated (Fig. 12C and Fig. 12D).

In most MEA cultures (n=12) not all electrodes were covered up by cells. Additionally, those that were, often distanced from the cell body or the axon hillock, where it would be more probable to record electrical activity (12). This helps explain why, from a total of 59 recording electrodes, most often, less than 30% picked up-threshold activity.

A minimum of 7 electrodes, at the lowest number of seeded cells (40.000), and a maximum of 18 electrodes, at the highest number of seeded cells (100.000), were activated. Cell density affected the number of electrodes which picked up cell electrical activity, but this effect was more pronounced from the lowest number of seeded cells (40.000) to the rest, than in between them (60.000;80.000;100.000).

Undifferentiated SH-SY5Y cells exhibit a very low SNR (30,76). Even after differentiation treatment, a substantial part of a culture can be composed of undifferentiated cells and/or S-type cells. This compromises the network spontaneous electrical activity recordings. Moreover, the spike detection threshold was set at triple noise level (-3). Such threshold may be too strict, rejecting data that would be extracted with lower thresholds. However, it reinforces extracted data strength.

Generally, the total number of active electrodes increased in relation to cell plating density. Still, the increase at 80.000 and 100.000 plated cells was not statistically significant in relation to plating 60.000 cells. Therefore, taking into account the morphological analysis, cell plating 60.000 SH-SY5Y cells per MEA was chosen for the following experiments.

4.1.3 Cell Differentiation

Differentiation of SH-SY5Y cells is required to mimic the intracellular environment of a mature neuronal cell. Upon differentiation, SH-SY5Y cells possess more biochemical, structural, morphological, and electrophysiological similarity to neurons (46,71,76). Furthermore, when SH-SY5Y cells are suspended in solution, connections previously established are destroyed. Therefore, the differentiation treatment must be made once cells are attached onto the array. Induction of differentiation by RA treatment helps solve this problem by forcing the creation of a new network.

Based on the literature, coating agents have an impact on SH-SY5Y cell morphology, growth and viability (44,46). For example, plates coated with 10 µg/ml laminin have been proven to enhance neurite outgrowth in SH-SY5Y cells (46). The results here presented corroborate such findings on MEA substrates. Nonetheless, substrate alterations, alone, do not induce optimal cell differentiation (72). Both adhesion and growth factor receptors should be stimulated simultaneously when differentiating SH-SY5Y cells (46,72).

Past experiments underlined the need to optimize a differentiation medium and a treatment time-course that would systematically render mature neuronal-like SH-SY5Y cell networks. An optimized protocol should favor a N-type population-dominant culture, concomitantly increasing network's spontaneous electrical activity.

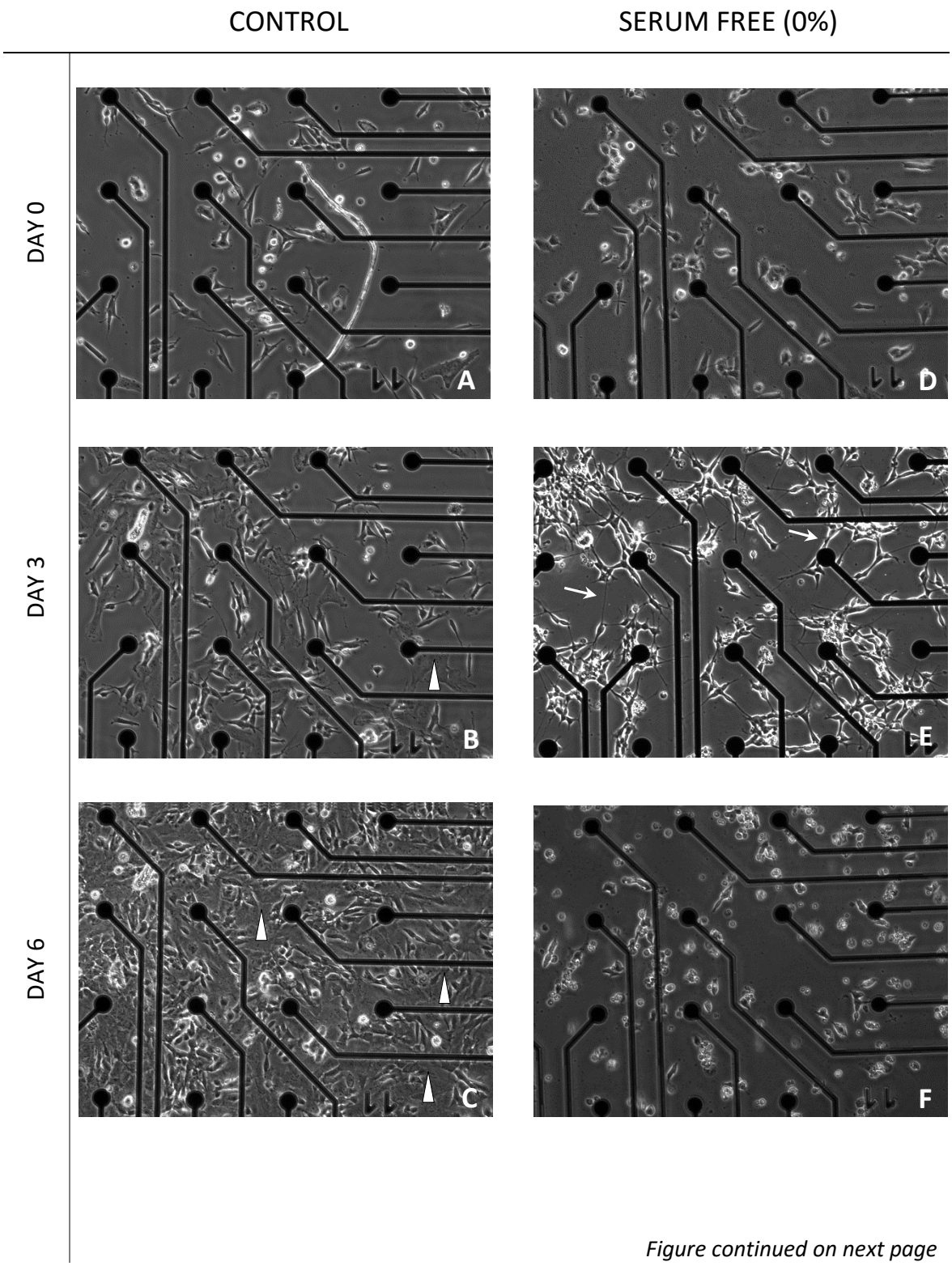
Moreover, precise electrophysiological and compound-effect investigations require the use of culture medium with well-defined effects. Thus, serum use in culture medium needs to be thoroughly analyzed. Serum-free medium is often considered a more appropriate choice during electrophysiological recordings of pharmacology and toxicology, since albumin (major component of FBS) is a non-specific binding agent (113). However, serum is an essential nutritional component of cell culture medium and plays a vital role during the growth and development of *in vitro* cultures.

In the course of this experiment, 60.000 SH-SY5Y cells were plated on PEI+laminin-coated MEAs in complete growth medium (supplemented with 10% FBS). Medium was completely aspirated 24h after plating. Then, differentiation was induced during a 7-day treatment with 3 separate additions of conditioned media (at day 0; day 3 and day 6). Combinations of 10 µM RA with serum-free (0%) and low serum (1% and 3%) media were tested.

Phase-contrast photographs were taken every 3 days in order to follow morphological differentiation and network formation along treatment. Furthermore, the evaluation of neuronal differentiation was accompanied by 180 s MEA recordings to determine how SH-SY5Y cells ability to generate spontaneous electrical activity evolved over the differentiation treatment.

4.1.3.1 Morphological Evaluation

To evaluate how the different conditioned media affected cell morphology and network formation along differentiation treatment, phase-contrast photographs were taken 8 hours after each medium exchange. Representative cell culture sections from each MEA are presented in Fig. 14.



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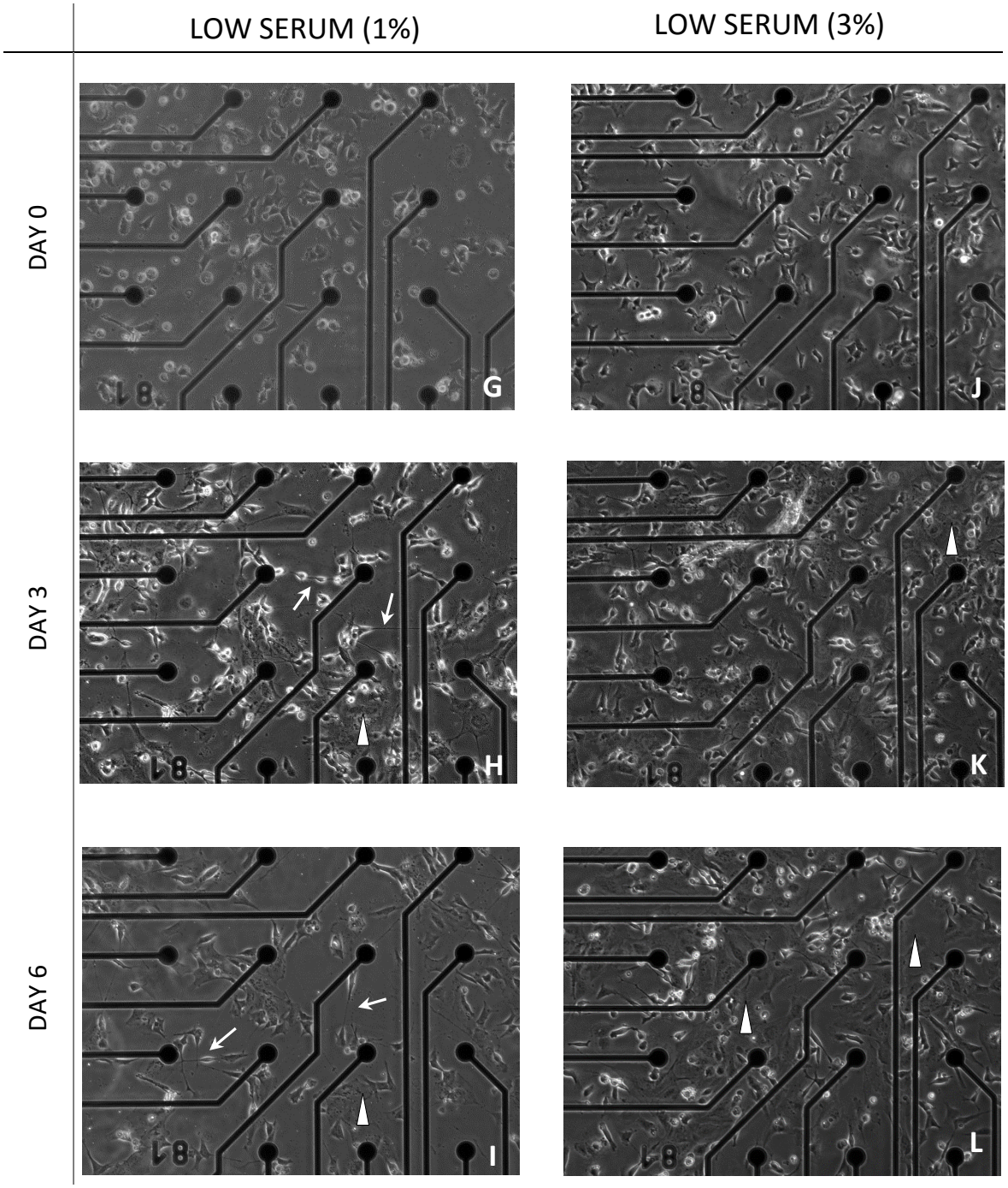

Figure 14 – Phase-contrast photographs of SH-SY5Y cells grown in different media. *Control culture did not undergo differentiation treatment with conditioned medium. A; D; G; J represent cell cultures in complete growth medium (10% FBS); serum-free medium (0% FBS); low serum medium (1% FBS) and low serum medium (3% FBS), respectively, at the first day of treatment (day 0). B; E; H; K; represent cell cultures in complete growth medium (10% FBS); serum-free medium (0% FBS); low serum medium (1% FBS) and low serum medium (3% FBS), respectively, at the fourth day of differentiation treatment (day 4). C; F; I; L; represent cell cultures in complete growth medium (10% FBS); serum-free medium (0% FBS); low serum medium (1% FBS) and low serum medium (3% FBS), respectively, at the seventh day of differentiation treatment (day 6). White arrows indicate extended neurites, while white triangles signpost S-type subpopulations. All photographs show a magnification of 100×.*

From the results here presented it was possible to observe that 8 hours after the first addition of conditioned medium (day 0), cells had minor differences in morphology.

Interestingly, undifferentiated cell culture (control) presented cells with some developed processes (Fig. 14A), unlike any other culture. This may have been due to the fact that, although medium was replaced, the medium composition did not change. Therefore, control culture did not suffer the initial negative impact that a severe change in medium composition caused. At this point, low serum medium (1%) presented the culture with more dead cells (Fig. 14G). Although this could have been caused by the lack of serum in medium, the same was not observed in the culture exposed to serum-free medium (Fig. 14D).

At day 3 (72 h after), cultures had differentiated and formed networks with different degrees of complexity. However, S-type cells began to proliferate in cultures treated with low serum media.

Serum-free medium had induced the formation of a complex network composed of N-type cells (Fig. 14E). Small clumps of cells with extended neurites spread homogeneously along the array. In the low serum medium (1%), some extended neurites were evident, but S-type cells still remained present (Fig. 14H). In the other low serum medium (3%), cells were not well differentiated, as no network formation was visible (Fig. 14K). At this point, the culture was already mainly composed of S-type cells.

After 7 days of differentiation treatment (day 6), it was possible to observe that cultures were not as optimal as before.

The undifferentiated culture had reached a point of cell density saturation, with almost no space in-between cells (Fig. 14C). The S-type population was dominant. The culture treated with serum-free medium presented mostly dead cells (Fig. 14F), while cultures treated with low serum media were composed mainly of S-type cells (Fig. 14I and Fig. 14L).

As expected, cells grown in complete growth medium proliferated unrestrictedly. On the contrary, cells grown in conditioned media, generally, differentiated rather than divided. Apparently, medium without serum did not provide enough conditions for maintaining a differentiated culture for more than 4 days, as most cells died between the 4^{th} and the 7^{th} day of the differentiation treatment. However, the culture treated with this medium did not have S-type cells. Such subpopulation was not restricted with low-serum media, especially at 3% FBS. Although differentiated cells were still present at the 7^{th} day in treatment with 1% FBS, remaining neurites appeared fragmented and generally not as viable as at the 4^{th} day (day 3).

4.1.3.2 Electrical Activity Evolution

Patch-clamp studies have shown that spontaneous electrical activity increases in differentiated SH-SY5Y cells (76). In fact, untreated cells exhibit scarce spontaneous electrical activity, but upon RA-induced differentiation there is an increase in activity. Moreover, a previous MEA study utilizing undifferentiated SH-SY5Y cells had reported low SNR (30).

In order to analyze whether differentiated cells exhibited increased spontaneous electrical activity with the methodology here presented, recordings were made at three separate times during differentiation treatment. These MEA recordings were made immediately before taking the phase-contrast photographs (day 0; day 3; day 6). This experiment also aimed to distinguish between cultures treated with the different conditioned media.

Neuroplasticity is associated with changes in the amplitude of synaptic potentials. However, in extracellular recordings, amplitude depends massively on the neuron-electrode distance. As MEAs are not adapted to recording synaptic potentials, such kind of analysis was not performed in this experiment. Unless these changes reached detection threshold, recordings were 'blind' to those critical events.

Recordings of spontaneous electrical activity lasted for 180 s. Pairs of MEAs were subject to experimentation simultaneously. A simple way of determining whether different conditions affected overall activity would be to count the total number of extracted spikes in the time window (180 s). This is problematic, as the number of covered electrodes varies in-between cultures. Even though cell plating density was the same, undifferentiated cells proliferated until saturation. Moreover, even MEAs containing the exact same number of cells, most often, do not have the same number of covered electrodes due to cells' random distribution nature. Therefore, cultures with more active electrodes could have an increased total count of extracted spikes, disregarding increased spontaneous electrical activity in individual electrodes.

In order to solve this problem, total spike counts from 3 active electrodes from each MEA were averaged. Cells migration kinetics during treatment, obliged to changing the electrodes chosen for analysis in-between day 0; day 3 and day 6 recordings. The evolution of the total spike counts can be seen in Table 2 and Fig. 15.

Table 2 – Total spike count during differentiation treatment with different media. *The number of active electrodes analyzed (n) is indicated for each condition.*

Conditioned Media	**Total spike count per active electrode [mean ±SEM *(n)*]**		
	Day 0	Day 3	Day 6
Control	28.5 ±6.5 *(2)*	133.0 ±23.5 *(3)*	60.0 ±18.7 *(3)*
Serum-free (0%)	30.0 ±3.0 *(2)*	679.3 ±105.0 *(3)*	- *(0)*
Low serum (1%)	36.0 *(1)*	452.3 ±63.2 *(3)*	237.3 ±29.0 *(3)*
Low serum (3%)	47.0 ±14.0 *(2)*	192.3 ±52.4 *(3)*	82.7 ±10.5 *(3)*

Abbreviations: SEM, standard error of mean

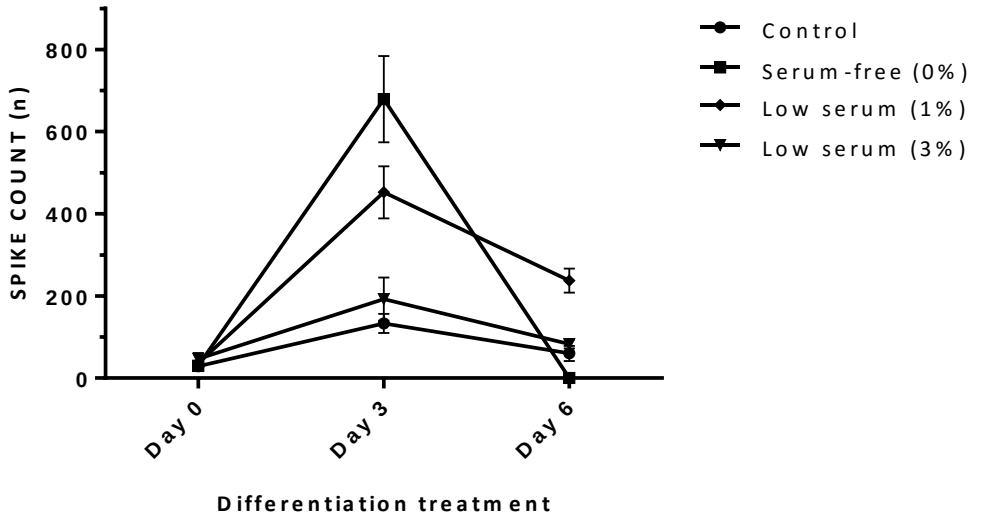

Figure 15 – Total spike count along differentiation treatment with different media. *Data is expressed as means ±SEM and represents the number of extracted spikes per active electrode in 180 s.*

From the results here presented one can conclude that RA-induced differentiation increases SH-SY5Y cells spontaneous electrical activity exhibition. All tested conditioned media increased the total spike count in some measure.

At the 1st day of treatment (day 0), differences were not statistically significant. Detected spikes were very sparse and very few electrodes detected up-threshold activity. Low serum (1%) only presented one active electrode.

At the 4th day of treatment (day 3), cultures had formed mature networks and exhibited extremely increased activity. Particularly, the culture treated with serum-free (0%) with 679.3 ±105.0 detected spikes along the 180 s. Culture treated with serum-free (3%) medium did not show relevant differences in comparison to the complete growth medium (10%) treated culture.

At the 7th day of treatment (day 6), activity had decreased in all conditions. Due to cell death, there were no active electrodes left in the serum-free (0%) medium-treated culture. At this point, low serum (1%) medium-treated culture was the one that totaled more spike events (237.3 ±29.0). However, its activity had reduced significantly since the 4th day of treatment (452.3 ±63.2).

Generally, exhibition of spontaneous electrical activity correlates with the morphological evaluation. Even when many cells covered the electrodes (Fig. 14C and Fig. 14L), this increased cell density did not correlate with increased activity. We believe that the proliferation of S-type cells in such cultures negatively affects recordings, as these cells are electrically inactive.

Serum-free (0%) medium seems to have contained S-type cells proliferation and induced the formation of mature network early on treatment. At the 4th day of treatment, cells exposed to a serum-free medium presented shorter ISIs (data not shown) and increased spiking activity. At this point, SH-SY5Y cells presented the highest spike count among treatments. However, such conditioned medium could have been too limiting for cell viability in more prolonged periods of time. Eventually, cells died between the 4th and the 7th day of differentiation treatment.

Most SH-SY5Y cell differentiation protocols include an initial phase (usually 24h) with 10% FBS present in medium, followed by at least three days of differentiation treatment with low or serum-free medium. However, these well-defined protocols do not take into account electrophysiological specificities. Low serum (1% and 3%) culture media did not show significant improvements in morphological differentiation, nor in spiking activity when compared to serum-free medium (0%). Moreover, such supplemented media could compromise inferences taken from Aβ addition, due to incontrollable interactions between peptides. Taking into account these factors combined and cell viability during the whole treatment, a 4-day differentiation treatment with serum-free medium was chosen for the final experiment.

Dissociated neuronal cultures demand increased efforts and expertise than SH-SY5Y cells manipulation. Furthermore, once cells are plated onto MEAs they still need 2-3 weeks of maturation before recordings take place (33,100). The methodology here proposed enables the formation of networks exhibiting spontaneous activity after 4 days in culture. Such short-cut can be very useful for a wide range of applications. However, this model comes with serious relative limitations as cultures did not survive for prolonged periods of time. Albeit this shortcoming, the proposed model may still be used for assessing acute effects in short-term experiments.

Relating morphology of networks maintained in the cell culture to general neuronal health and predicted electrophysiological is challenging. Knowing when cultures are mature enough for experimental use is a common problem faced by researchers. Here, total spike counts and firing rates changed markedly during the culture differentiation treatment. Although, spontaneous electrical activity exhibition seems to be closely related to the age of the network, more detailed studies are needed to establish the optimal day(s) for MEA recordings with the SH-SY5Y cell line.

4.2 Aβ Addition to SH-SY5Y MEA Cultures

Aβ 1-42 oligomers, but not monomers, have been shown to produce significant reductions in neuronal spontaneous electrical activity of cultured neuronal networks. Benilova et al (2009) showed that 20 μM Aβ 1-42 oligomers can immediately suppress spike activity on MEAs (54), while Kuperstein et al (2010) showed that 1 μM can rapidly and persistently depress spike activity on patch-clamp in cultured hippocampal neuronal networks (53). Recently, Charkhkar et al (2015) demonstrated that 5 μM significantly inhibited spiking activity. However, exposure to 0.2 μM and 1 μM failed to alter the spike rate in a statistically significant manner (100).

Spike rate is an important component of neural coding. As it is prone to alteration by electrical and chemical stimuli, the frequency of APs provides a quantitative evaluation of overall cell electrical activity (54). Charkhkar et al (2015) stated that 5 μM Aβ 1-42 oligomers reduced the normalized spike rate by approximately 60% from the baseline, immediately (100).

The initial toxic impact of these Aβ oligomers is synaptic in nature, however this can spread into the cells leading to neuronal cell death (53). Though, short-term recordings cannot give information on this development, they contribute to identifying acute Aβ effects on neuronal networks.

In order to test whether differentiated SH-SY5Y MEA culture's electrical activity was influenced by Aβ, cultures were exposed to 0,1; 1 and 10 μM Aβ 1-42 oligomers. Aβ was added after previous medium exchange under a laminar flow cabinet (30 minutes before experiment). Recordings time-course totaled 1980 s, from which 1800 s were after Aβ addition. Results from two independent experiments were utilized in this analysis.

The total number of spikes per second (spike rate) was calculated. Active electrodes were identified and spike rates (Hz) for each electrode were computed individually, as seen in Fig. 16. Mean spike rates (before and after Aβ addition) from 6 active electrodes (3 from each experiment) were considered in the analysis of each condition. These were calculated in 3-minute intervals, so as to follow alterations during the whole recording (see Fig. 18).

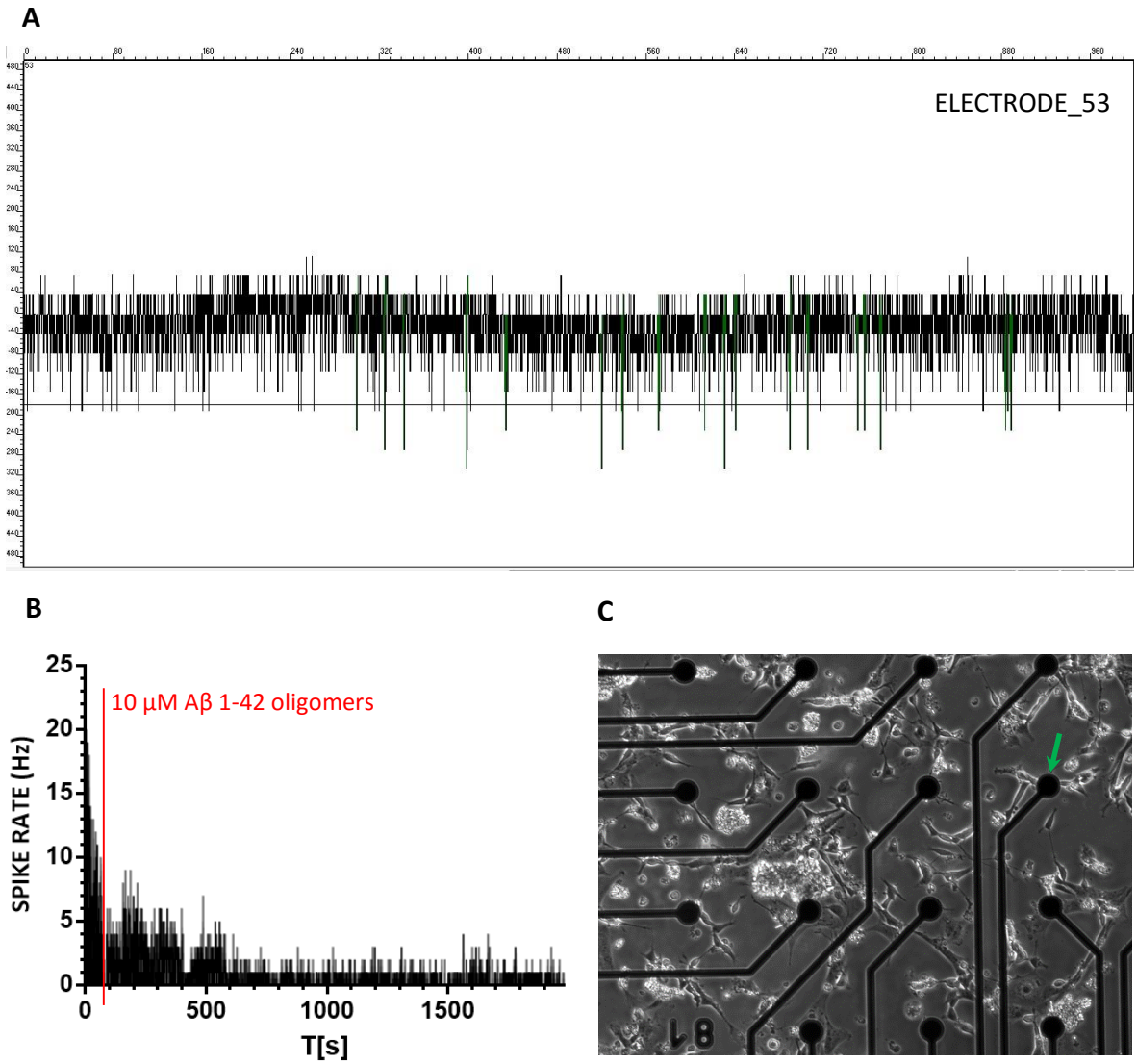

Figure 16 – Single recording of spontaneous electrical activity before and after Aβ addition. (A) *1 s display of recorded data stream corresponding to "electrode 53" at 14 seconds into recording session. Y-axis and X-axis expressed in µV and ms, respectively. Spikes extracted by detection threshold are enlighten in green.* ***(B)*** *Graph depicts the spike rate (spikes/sec) recorded at "electrode 53" during whole recording (1980 s). Red line represents the time point of 10 µM Aβ 1-42 oligomers addition (at 180 s). The 2 seconds post-Aβ addition were excluded from analysis as the addition fluxes caused widespread noise in all recording electrodes. Mean spike rate (Hz) was 4.61 ±4.15 SD before Aβ addition and 0.78 ±1.18 SD after.* ***(C)*** *Phase-contrast photograph of the cell culture after Aβ addition. Green arrow indicates recording electrode no. 53.*

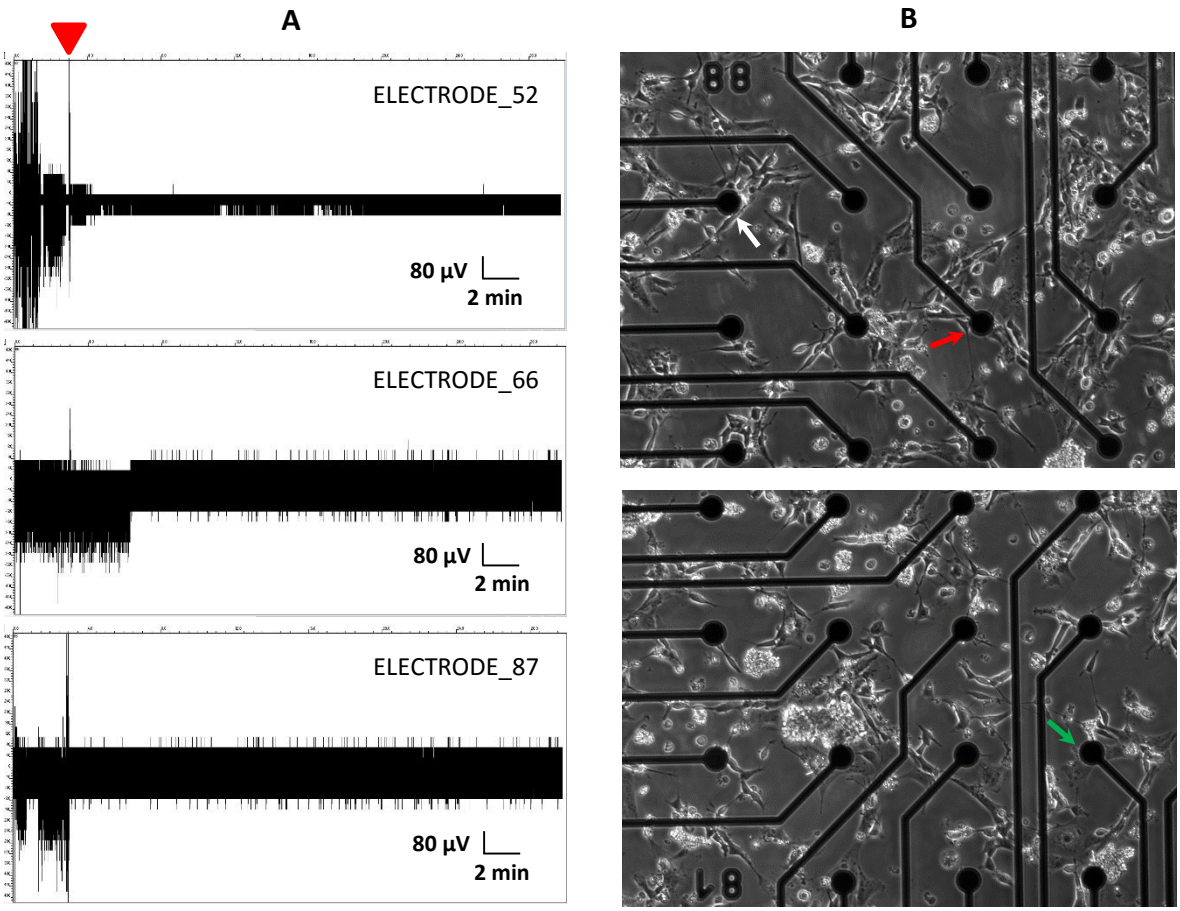

Figure 17 – Longterm data display of spontaneous electrical activity before and after Aβ addition. (A) *30-minute display of recorded activity in 3 active electrodes. Y-axis and X-axis expressed in µV and mins, respectively. Red triangle indicates the time point of 10 µM Aβ 1-42 oligomers addition (at 180 s).* ***(B)*** *Phase-contrast photograph of cell culture sections after Aβ addition. White, red and green arrows indicate recording electrodes no. 87; 66 and 52, respectively.*

As it can be seen in Fig. 16 and Fig. 17, addition of 10 µM Aβ 1-42 oligomers visibly decreased spike rate and overall spiking activity in electrode no 53 and electrodes no 52; 66 and 87, respectively. These findings are comparable with previous work demonstrating that a dose of 5 µM caused an immediate reduction in spike rate (100), whereas a lethal dose of 20 µM caused immediate silencing of the network (54) in primary neuronal cultures.

Large precipitates can be seen in Fig. 16C and Fig. 17B, these increased in correlation with Aβ concentration but were not present in the control culture. They were most probably caused by the synthetic Aβ formulation and their effect on cells was unknown.

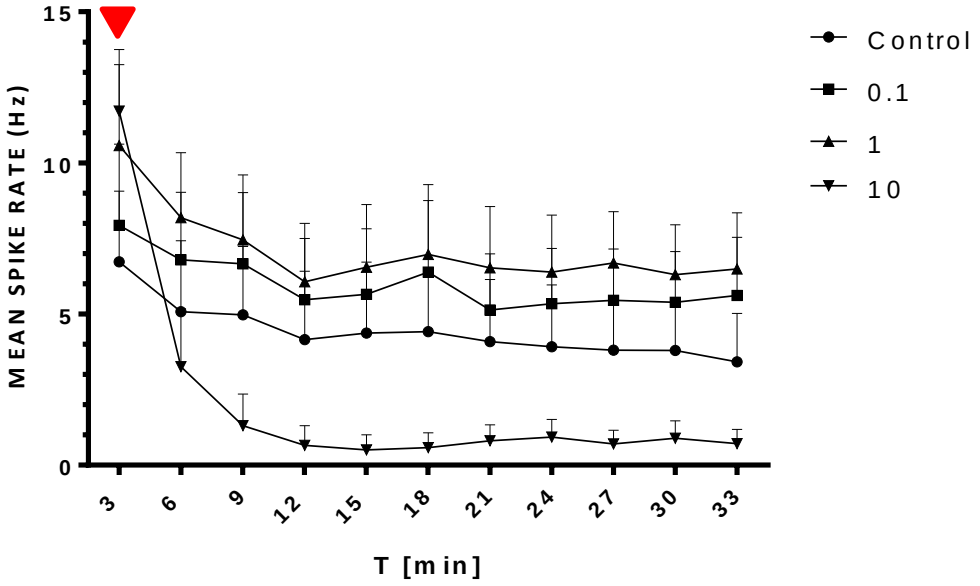

Figure 18 – Spike rate before and after 0.1, 1 and 10 µM Aβ 1-42 oligomers exposure. *The number of spikes per second was extracted from 6 active electrodes (3 from each independent experiment) in each condition. Spike rates (Hz) were averaged in 3-minute intervals. Red triangle indicates the time point of addition. Results are expressed as means and error bars represent ±SEM.*

The effect of sublethal low µM range concentrations (<2 µM) has previously only been observed after ON treatment (54). The concentrations here tested of 0.1 µM and 1 µM showed no significant acute effect on network activity. These results are also in agreement with previous studies, where 0.2 µM and 1 µM failed to immediately alter spike rate in a statistically significant manner (100).

However, 10 µM oligomers addition visibly decreased spike rate quasi-immediately. In half of the considered electrodes (n=3), activity was completely suppressed after addition and did not recover significantly during the experiment time-course. In fact, spontaneous electrical activity exhibition was significantly decreased in all the active electrodes analyzed for such concentration.

In all tested conditions, the mean spike rate exhibited a tendency to reduce markedly after solution addition and less pronouncedly along the experiment. However, this decrease was very augmented in the active electrodes where cells were exposed to 10 µM oligomers. Such cells exhibited very sparse spiking activity after Aβ addition, if any. These results indicate that the highest concentration tested deeply decreased spiking activity in the SH-SY5Y cell cultures.

Past results have suggested that inotropic glutamate receptors, AMPA/kainate and NMDA, are both involved in the net effects of Aβ 1-42 oligomers. However, immediate effects on network activity are probably mainly AMPA/kainate receptor mediated, as when exposed to a competitive antagonist of AMPA/kainate receptors, the subsequent treatment with 5 µM Aβ42 oligomer resulted in immediate significant inhibition (100). In recent years, new generation optical fluorescent probes have been tested in synchronization with MEA studies. Optimized fluorescent probes for visualizing glutamate neurotransmission (114) could help unravel Aβ acute effects on

the synapse function. The same line of thought can be applied to other molecules involved in signal transmission, such as calcium.

The main goal of this work was to determine an acute effect of Aβ 1-42 oligomers on SH-SY5Y cell cultures firing dynamics. For the highest concentration (10 μM), Aβ 1-42 oligomers markedly reduced spiking activity for the recording length. In most active electrodes, spontaneous electrical activity exhibition was suppressed quasi-immediately. Beside the differences in the cell types or experimental design, oligomers seem to strongly influence network behavior.

5. Conclusion

The objective of this thesis was to develop a SH-SY5Y cell culture model for obtaining reliable extracellular recordings. Seeding SH-SY5Y cells and forming a neuronal network on surface-treated MEAs could provide an effective model for a wide range of applications in neurosciences, including biosensors for neuroprotective and neuroharming compounds. The results here presented show the feasibility of using differentiated SH-SY5Y cells in MEA recordings. Particularly, to investigate the electrophysiological acute effects of neurodegenerative peptides, such as Aβ.

MEAs coating, cell plating density and differentiation treatment markedly influenced cell distribution, viability, morphological differentiation and electrophysiological properties. The methodology we propose enables the formation of networks exhibiting spontaneous activity after 4 days in culture. Alhtough cell cultures did not survive for prolonged periods of time, differentiatied SH-SY5Y cell cultures could still be useful for short-term experiments or assessing acute compound effects. Research fields where investigators may lack cell culture expertise, such as robotics, could also benefit from a simpler neuronal model with a well-defined growth and maintenance protocol.

Coating MEAs in a double-step treatment of PEI and laminin enhanced neuronal differentiation and increased the number of electrodes which picked up-threshold activity when compared to other coating treatments and uncoated MEAs. Cell plating density was optimized so as to achieve a balance between enough cells for relevant activity exhibition and enough spare space for neurite extension and network formation. Cell plating 60.000 cells per MEA led to the formation of the most neuronal-like networks. Differentiation treatment with 10 µM RA and serum-free conditioned medium proved to induce mature network formation at the 4^{th} day of treatment and spike rate levels comparable to primary neuronal cultures. Total spike counts and firing rates changed during the culture development with marked day differences. Spontaneous electrical activity exhibition seems to be closely related to the age of the network and more detailed studies are needed to establish the optimal day for recordings.

In the future, the SH-SY5Y cell line can be used to explore other differentiation treatments. Although RA-induced differentiation is the most common treatment, compounds such as BDNF have been shown to induce better biochemical differentiation. The work here presented explored morphological and quantitative spiking activity differences, future studies could explore concomitantly molecular alterations recurring, for example, to coupled fluorescence analysis and the new generation of optical probes. Recent years have been marked by coupled analysis of neuroelectrophysiology and fluorescent dye molecules. Voltage or molecule sensitive dyes could help unravel compound effects on single cell and network behavior.

Addition of 10 µM Aβ 1-42 oligomers visibly decreased spike rate and overall spiking activity, as expected. However, the concentrations of 0.1 µM and 1 µM showed no significant acute effect on network activity. These results prove that Aβ exerts a modulatory effect on differentiated SH-SY5Y cells spontaneous electrical activity. In the future, overnight incubation at such concentrations could be tested to sustain differences in spontaneous activity. Simultaneous incubation of Aβ oligomers with anti-oligomers antibodies could help understand whether those effects are Aβ-mediated or due to network ageing or stress.

The single-recording experiments here presented extract information and permit one to draw conclusions from representative, temporally limited snapshot timelines. This approach bears the

risk of missing crucial moments that may help our understanding of physiological events. One way of circumventing this problem would be resorting to an incubator-independent cell-culture perfusion platform, thus prolonging the recordings time-course.

The electrophysiological properties of the neuroblastoma cultures were analyzed by recording the spontaneous activity of the network. *In vitro* neuroblastoma networks show spontaneously firing. These firing rates change during the culture development with marked day differences and the global rate is closely related to the age of the network. However, recordings of SH-SY5Y have the disadvantage of having a very low SNR and reduced active electrodes in comparison to mature primary neuronal cultures.

Although SH-SY5Y cell line is widely used as an *in vitro* model for AD and the existence of evidence that these cells can have an electrophysiological behavior similar to neurons, until now there have been no MEA studies using SH-SY5Y cells for investigation on AD. This cell line culturing on MEAs and its successful differentiation into a mature neuronal network can be extremely useful for laboratories that do not have access to primary neuronal cultures. Moreover, its usage may save resources and facilitate obtaining faster high-throughput results. The work developed in this thesis led to obtaining SH-SY5Y cell cultures exhibiting relevant spontaneous electrical activity in 4 days.

Obtaining a viable and reliable MEA cell culture is the hardest component in a MEA experiment. SH-SY5Y MEA cultures are often fastidious and delicate, but thanks to their simplicity and accessibility they may provide a powerful model for studying neuronal activity. We propose that differentiated SH-SY5Y cells could be used to assess how Aβ acutely modifies the synaptic function throughout the neuronal network.

6. References

1. Purves D, Augustine GJ, Fitzpatrick D, Hall WC. Neuroscience. 5th ed. Vascular. 2012. 759 p.

2. Saladin K. Saladin: Anatomy & Physiology: The Unity of Form and Function. 3rd ed. The McGraw-Hill Companies. 2003. 447 p.

3. Lodish H, Berk A, Kaiser CA, Krieger M, Bretscher A, Ploegh H, et al. Molecular Cell Biology. 7th ed. 2013. 1247 p.

4. Ogden D, Stanfield P. Patch clamp techniques for single channel and whole-cell recording. Currents. 1981;2:53–78.

5. Schwiening CJ. A brief historical perspective: Hodgkin and Huxley. J Physiol. 2012 Jun 1;590(Pt 11):2571–5.

6. Bakkum DJ, Radivojevic M, Frey U, Franke F, Hierlemann A, Takahashi H. Parameters for burst detection. Front Comput Neurosci. 2013 Jan;7(January):193.

7. Spira ME, Hai A. Multi-electrode array technologies for neuroscience and cardiology. Nat Nanotechnol. Nature Publishing Group; 2013 Feb;8(2):83–94.

8. Wagenaar D a, Pine J, Potter SM. An extremely rich repertoire of bursting patterns during the development of cortical cultures. BMC Neurosci. 2006 Jan;7:11.

9. Meyer J, Wolf B. Assays for diagnostic and therapeutic systems. Technischen Universität München; 2009.

10. Stufflebeam SM, Tanaka N, Ahlfors SP. Clinical applications of magnetoencephalography. Hum Brain Mapp. 2009 Jun;30(6):1813–23.

11. Destexhe A, Bedard C. Local field potential [Internet]. Scholarpedia. 2013 [cited 2015 Feb 4]. Available from: http://www.scholarpedia.org/article/Local_field_potential

12. Obien MEJ, Deligkaris K, Bullmann T, Bakkum DJ, Frey U. Revealing neuronal function through microelectrode array recordings. Front Neurosci. Frontiers; 2015 Jan 6;8.

13. MCS. Multi Channel Systems - Inovations in Electrophysiology [Internet]. 2015 [cited 2015 Apr 20]. Available from: http://www.multichannelsystems.com/

14. Blau A. Cell adhesion promotion strategies for signal transduction enhancement in microelectrode array in vitro electrophysiology: An introductory overview and critical discussion. Curr Opin Colloid Interface Sci. 2013;18(5):481–92.

15. Scanziani M, Häusser M. Electrophysiology in the age of light. Nature. 2009 Oct 15;461(7266):930–9.

16. Difato F, Schibalsky L, Benfenati F, Blau A. Integration of optical manipulation and electrophysiological tools to modulate and record activity in neural networks. Int J Optomechatronics. 2011;5(October 2011):191–216.

17. Baudry M, Taketani M. Advances in Network Electrophysiology - Using Multielectrode Arrays. Springer; 2006. 478 p.

18. Hardy J, Selkoe DJ. The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. Science (80-). 2002 Jul 19;297(5580):353–6.

19. Dossi E, Blauwblomme T, Nabbout R, Huberfeld G, Rouach N. Multi-electrode Array Recordings of Human Epileptic Postoperative Cortical Tissue. J Vis Exp. 2014 Oct 26;(92):1–9.

20. Potter SM, DeMarse TB. A new approach to neural cell culture for long-term studies. J Neurosci Methods. 2001 Sep 30;110(1-2):17–24.

21. Thomas J et al. A Miniature Microelectrode Array to Monitor the Biolectric Activity of Cultured Cells. Exp Cell Res. 1972;74:61–6.

22. Werner L. Introduction to the MEA2100-System. Multi Channel Systems; 2015.

23. Johnstone AFM, Gross GW, Weiss DG, Schroeder OH-U, Gramowski A, Shafer TJ. Microelectrode arrays: a physiologically based neurotoxicity testing platform for the 21st century. Neurotoxicology. Elsevier B.V.; 2010 Aug;31(4):331–50.

24. Neher E, Sakmann B. Single-channel currents recorded from membrane of denervated frog muscle fibres. Nature. 1976;260.

25. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved Patch-Clamp Techniques for High-Resolution Current Recording from Cells and Cell-Free Membrane Patches. Eur J Physiol. 1981;85–100.

26. Schnitzer MJ, Meister M. Multineuronal firing patterns in the signal from eye to brain. Neuron. 2003;37:499–511.

27. Bakkum DJ, Shkolnik AC, Ben-Ary G, Gamblen P, DeMarse TB, Potter SM. Removing Some "A" from AI: Embodied Cultured Networks. Embodied Artif Intell. 2004;3139(2004):130–45.

28. Wei W, Song Y, Wang L, Zhang S, Luo J, Xu S, et al. An implantable microelectrode array for simultaneous L-glutamate and electrophysiological recordings in vivo. Microsystems Nanoeng. Nature Publishing Group; 2015 May 28;1.

29. MCS. Extracellular Recording with Microelectrode Arrays for All Applications. Reutlingen: Multi Channel Systems; 2010.

30. Ferrández JM, Lorente V, Garrigós J, Fernández E. Image Coding for Robotic Guidance Using Neuroblastoma Cultures. Majlesi J Electr Eng. 2011;5(1):12–20.

31. Jimbo Y, Tateno T, Robinson HPC. Simultaneous Induction of Pathway-Specific Potentiation and Depression in Networks of Cortical Neurons. Biophys J. 1999;76(February):670–8.

32. MCS. Extracellular Recording with Microelectrode Arrays for all Applications. Reutlingen: Multi Channel Systems; 2014.

33. Hales CM, Rolston JD, Potter SM. How to culture, record and stimulate neuronal networks on micro-electrode arrays (MEAs). J Vis Exp. 2010 Jan;(39):1–7.

34. Potter SM, Lukina N, Longmuir KJ, Wu Y. Multi-site two-photon imaging of neurons on multi-electrode arrays. 2001;4262:104–10.

35. Wagenaar D a., Pine J, Potter SM. Effective parameters for stimulation of dissociated cultures using multi-electrode arrays. J Neurosci Methods. 2004;138:27–37.

36. Odawara a, Saitoh Y, Alhebshi a H, Gotoh M, Suzuki I. Long-term electrophysiological activity and pharmacological response of a human induced pluripotent stem cell-derived neuron and astrocyte co-culture. Biochem Biophys Res Commun. Elsevier Inc.; 2014 Jan 24;443(4):1176–81.

37. Odawara A, Gotoh M, Suzuki I. A three-dimensional neuronal culture technique that controls the direction of neurite elongation and the position of soma to mimic the layered struure of the brain. Soc Biomater. 2014;23630.

38. Chong S-A, Benilova I, Shaban H, De Strooper B, Devijver H, Moechars D, et al. Synaptic dysfunction in hippocampus of transgenic mouse models of Alzheimer's disease: a multi-electrode array study. Neurobiol Dis. Elsevier Inc.; 2011 Dec;44(3):284–91.

39. Varghese K, Molnar P, Das M, Bhargava N, Lambert S, Kindy MS, et al. A new target for amyloid beta toxicity validated by standard and high-throughput electrophysiology. PLoS One. 2010 Jan;5(1):e8643.

40. Ylä-Outinen L, Heikkilä J, Skottman H, Suuronen R, Äänismaa R, Narkilahti S. Human cell-based micro electrode array platform for studying neurotoxicity. Front Neuroeng. 2010 Jan;3(September):1–9.

41. Jahnke H-G, Rothermel A, Sternberger I, Mack TG a, Kurz RG, Pänke O, et al. An impedimetric microelectrode-based array sensor for label-free detection of tau hyperphosphorylation in human cells. R Soc Chem. 2009;9:1422–8.

42. Asphahani F, Thein M, Veiseh O, Edmondson D, Kosai R, Veiseh M, et al. Influence of cell adhesion and spreading on impedance characteristics of cell-based sensors. Biosens Bioelectron. 2008;23:1307–13.

43. Multi Channel Systems. MEA Handling. 2014.

44. Teppola H, Sarkanen J-R, Selinummi J, Pettinen A, Jalonen T, Linne M-L. The Effects of Different MEA Coating Agents on Growth and Differentiation of Human SH-SY5Y Neuroblastoma Cells. 6th International Meeting on Substrate-Integrated Micro Electrode Arrays. Reutlingen; 2008. p. 26–7.

45. Suzuki M, Ikeda K, Yamaguchi M, Kudoh SN, Yokoyama K, Satoh R, et al. Neuronal cell patterning on a multi-electrode array for a network analysis platform. Biomaterials. Elsevier Ltd; 2013;34(21):5210–7.

46. Dwane S, Durack E, Kiely P a. Optimising parameters for the differentiation of SH-SY5Y cells to study cell adhesion and cell migration. BMC Res Notes. BMC Research Notes; 2013 Jan;6(1):366.

47. MCS. Versatile in vitro recording system : MEA2100-System. Reutlingen: Multi Channel Systems; 2014.

48. Steidl E-M, Neveu E, Bertrand D, Buisson B. The adult rat hippocampal slice revisited with multi-electrode arrays. Brain Res. 2006 Jun 22;1096(1):70–84.

49. Suzuki I, Yasuda K. Detection of tetanus-induced effects in linearly lined-up micropatterned neuronal networks: application of a multi-electrode array chip combined with agarose microstructures. Biochem Biophys Res Commun. 2007 May 4;356(2):470–5.

50. Xian H, Blanco C, Lam J, Brock M, Snodgrass R. Development of Improved hESC-CM Based High-Throughput Assays for Cardiotoxicity. p. 48.

51. Asakura K, Hayashi S, Ojima A, Taniguchi T, Miyamoto N, Nakamori C, et al. Improvement of acquisition and analysis methods in multi-electrode array experiments with iPS cell-derived cardiomyocytes. J Pharmacol Toxicol Methods. 2015 Apr 22;75:17–26.

52. Görtz P, Siebler M, Ihl R, Henning U, Luckhaus C, Supprian T, et al. Multielectrode array analysis of cerebrospinal fluid in Alzheimer's disease versus mild cognitive impairment: a potential diagnostic and treatment biomarker. Biochem Biophys Res Commun. 2013 May 3;434(2):293–7.

53. Kuperstein I, Broersen K, Benilova I, Rozenski J, Jonckheere W, Debulpaep M, et al. Neurotoxicity of Alzheimer's disease Aβ peptides is induced by small changes in the Aβ42 to Aβ40 ratio. EMBO J. 2010 Oct 6;29(19):3408–20.

54. Benilova I, Kuperstein I, Broersen K, Schymkowitz J, Rousseau F. MEA Neurosensor , the Tool for Synaptic Activity Detection : Acute Amyloid- β Oligomers Synaptotoxicity Study. 2009;42:314–6.

55. Potter SM, Wagenaar D a., Madhavan RMR, DeMarse TB. Long-term bidirectional neuron interfaces for robotic control, and in vitro learning studies. 25th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (IEEE Cat No03CH37439). 2003. p. 3690–3.

56. DeMarse TB, Wagenaar DA, Blau AW, Potter SM. The Neurally Controlled Animat: Biological Brains Acting with Simulated Bodies. Aut Robot. 2001;11(3):305–10.

57. Potter SM, Wagenaar D a, Demarse TB. Closing the Loop : Stimulation Feedback Systems for Embodied Mea Cultures. Data Process. 2005;78:215–42.

58. Fernekorn U, Fischer M, Klett M, Augspurger C, Hiebl B, Schober A, et al. MEMS based examination platform for neuro-muscular communication in a co-culture system coupled to a multi-electrode-array MacroNano ® Center for Innovation Competence , Dept . for Microfluidics and Biosensors. The Nanotechnology Conference and Trade Show. 2008. p. 227–30.

59. Görtz P, Fleischer W, Rosenbaum C, Otto F, Siebler M. Neuronal network properties of human teratocarcinoma cell line-derived neurons. Brain Res. 2004 Aug 20;1018(1):18–25.

60. Ariano P, Lo Giudice A, Marcantoni A, Vittone E, Carbone E, Lovisolo D. A diamond-based biosensor for the recording of neuronal activity. Biosens Bioelectron. Elsevier B.V.; 2009;24:2046–50.

61. Takayama Y, Saito A, Moriguchi H, Kotani K, Jimbo Y. Ensemble recording of electrical activity in neurons derived from P19 embryonal carcinoma cells. Electron Commun Japan. 2011;94(4):8–16.

62. Biedler JL, Helson L, Spengler BA. Morphology and Growth , Tumorigenicity , and Cytogenetics of Human Neuroblastoma Cells in Continuous Culture Morphology and Growth , Tumorigenicity , and Cytogenetics of Human Neuroblastoma Cells in Continuous Culture1. Cancer Res. 1973;2643–52.

63. Biedler JL, Roffler-tarlov S, Schachner M, Freedman LS. Multiple Neurotransmitter Synthesis by Human Neuroblastoma Cell Lines and Clones Multiple Neurotransmitter Synthesis by Human Neuroblastoma Cell Lines and Clones. Cancer Res. 1978;3751–7.

64. Gordon J, Amini S, White MK. General overview of neuronal cell culture. Methods Mol Biol. 2013 Jan;1078:1–8.

65. Joshi S, Guleria R, Pan J, DiPette D, Singh US. Retinoic acid receptors and tissue-transglutaminase mediate short-term effect of retinoic acid on migration and invasion of neuroblastoma SH-SY5Y cells. Oncogene. 2006 Jan 12;25(2):240–7.

66. Bell N, Hann V, Redfern CPF, Cheek TR. Store-operated Ca(2+) entry in proliferating and retinoic acid-differentiated N- and S-type neuroblastoma cells. Biochim Biophys Acta. 2013 Mar;1833(3):643–51.

67. Hong-rong X, Lin-sen H, Guo-yi L. SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. Chinese Med J. 2010;123(8):1086–92.

68. Recio-pinto E, Lang FF, Ishii DN. Insulin and insulin-like growth factor II permit nerve growth factor binding and the neurite formation response in cultured human neuroblastoma cells. Neurobiology. 1984;81(April):2562–6.

69. Schaeffer V, Patte-Mensah C, Eckert a, Mensah-Nyagan a G. Selective regulation of neurosteroid biosynthesis in human neuroblastoma cells under hydrogen peroxide-induced oxidative stress condition. Neuroscience. 2008 Feb 6;151(3):758–70.

70. Gilany K, Van Elzen R, Mous K, Coen E, Van Dongen W, Vandamme S, et al. The proteome of the human neuroblastoma cell line SH-SY5Y: an enlarged proteome. Biochim Biophys Acta. 2008;1784(7-8):983–5.

71. Kovalevich J, Langford D. Considerations for the Use of SH - SY5Y Neuroblastoma Cells in Neurobiology. In: Amini S, White MK, editors. Neuronal Cell Culture: Methods and Protocols. Totowa, NJ: Humana Press; 2013. p. 9–21.

72. Nam K-H, Jamilpour N, Mfoumou E, Wang F-Y, Zhang DD, Wong PK. Probing mechanoregulation of neuronal differentiation by plasma lithography patterned elastomeric substrates. Sci Rep. 2014;4:6965.

73. Cheung YT, Lau WKW, Yu MS, Lai CSW, Yeung SC, So KF, et al. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology. 2009;30:127–35.

74. Agholme L, Lindström T, Kågedal K, Marcusson J, Hallbeck M. An In Vitro Model for Neuroscience: Differentiation of SH-SY5Y Cells into Cells with Morphological and Biochemical Characteristics of Mature Neurons. J Alzheimer's Dis. 2010;(20):1069–82.

75. Encinas M, Iglesias M, Liu Y, Wang H, Muhaisen A, Cen V, et al. Sequential Treatment of SH-SY5Y Cells with Retinoic Acid and Brain-Derived Neurotrophic Factor Gives Rise to Fully Differentiated , Neurotrophic Factor-Dependent, Human Neuron-Like Cells. J Neurochem. 2000;

76. Juarez EH, Sackmann C, Granseth B, Hallbeck M. THE ELECTROPHYSIOLOGICAL PROPERTIES OF DIFFERENTIATED SH-SY5Y CELLS USED TO UNDERSTAND THE EFFECT OF PROPAGATION OF NEURODEGENERATIVE PROTEINS. Alzheimer's Dement J Alzheimer's Assoc. Elsevier; 2015;10(4):P644.

77. Gerecke KM, Wyss JM, Carroll SL. Neuregulin-1beta induces neurite extension and arborization in cultured hippocampal neurons. Mol Cell Neurosci. 2004 Dec;27(4):379–93.

78. MCS. MEA2100-System Manual. Reulingen: Multi Channel Systems; 2014.

79. Teppola H, Selinummi J, Sarkanen J-R, Pettinen A, Jalonen TO, Yli-Harja O, et al. Automated image analysis of neuroblastoma cell networks cultured on microelectrode array plates. Frontiers in Neuroinformatics. Stockholm; 2008.

80. Desai A, Kisaalita W. Human Neuroblastoma (SH-SY5Y) Cell Culture and Differentiation in 3-D Collagen Hydrogels for Cell-based Biosensing. 2004.

81. Lopes FM, Schröder R, da Frota MLC, Zanotto-Filho A, Müller CB, Pires AS, et al. Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain Res. Elsevier B.V.; 2010 Jun 14;1337:85–94.

82. Constantinescu R, Constantinescu AT, Reichmann H, Janetzky B. Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y. J Neural Transm. 2007;72:17–28.

83. Sarkanen JR, Nykky J, Siikanen J, Selinummi J, Ylikomi T, Jalonen TO. Cholesterol supports the retinoic acid-induced synaptic vesicle formation in differentiating human SH-SY5Y neuroblastoma cells. J Neurochem. 2007;102:1941–52.

84. Cecchi C, Pensalfini A, Liguri G, Baglioni S, Fiorillo C, Guadagna S, et al. Differentiation increases the resistance of neuronal cells to amyloid toxicity. Neurochem Res. 2008 Dec;33(12):2516–31.

85. Alzheimer ' s Disease Facts and Figures [Internet]. Alzheimer's Association; 2014 [cited 2015 Mar 29]. Available from: http://www.alz.org/downloads/Facts_Figures_2014.pdf

86. What Is Alzheimer's? [Internet]. 2015. Available from: http://www.alz.org/alzheimers_disease_what_is_alzheimers.asp

87. Querfurth HW, Laferla FM. Alzheimer's Disease. N Engl J Med. 2011;329–44.

88. Salawu F, Umar J, Olokoba A. Alzheimer's disease: A review of recent developments [Internet]. Ann Afr Med. 2011 [cited 2015 Mar 29]. Available from: http://www.annalsafrmed.org/text.asp?2011/10/2/73/82057

89. De-Paula VJ, Radanovic M, Diniz BS, Forlenza O V. Alzheimer's Disease. Protein Aggregation and Fibrillogenesis in Cerebral and Systemic Amyloid Disease. 2012. p. 329–52.

90. Stephen Waxman. From Neuroscience to Neurology. 2005. 547 p.

91. Schmand B, Smit JH, Geerlings MI, Lindeboom J. The effects of intelligence and education on the development of dementia. A test of the brain reserve hypothesis. Psychol Med. 1997;27:1337–44.

92. Fleminger S, Oliver DL, Lovestone S, Rabe-Hesketh S, Giora a. Head injury as a risk factor for Alzheimer's disease: the evidence 10 years on; a partial replication. J Neurol Neurosurg Psychiatry. 2003;74:857–62.

93. Martins IJ, Hone E, Foster JK, Sünram-Lea SI, Gnjec a, Fuller SJ, et al. Apolipoprotein E, cholesterol metabolism, diabetes, and the convergence of risk factors for Alzheimer's disease and cardiovascular disease. Mol Psychiatry. 2006;11:721–36.

94. Glenner GG, Wong CW. Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun. 1984;120(3):885–90.

95. Masters CL, Simms G, Weinman N a, Multhaup G, McDonald BL, Beyreuther K. Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A. 1985;82(June):4245–9.

96. O'Brien RJ, Wong PC. Amyloid precursor protein processing and Alzheimer's disease. Annu Rev Neurosci. 2011;34:185–204.

97. Cárdenas-aguayo MC, Silva-lucero MC, García U. Physiological Role of Amyloid Beta in Neural Cells : The Cellular Trophic Activity. Neurochemistry. 2014.

98. Zhang H, Ma Q, Zhang Y, Xu H. Proteolytic processing of Alzheimer's β-amyloid precursor protein. J Neurochem. 2012;120:9–21.

99. Morris GP, Clark I a, Vissel B. Inconsistencies and controversies surrounding the amyloid hypothesis of Alzheimer's disease. Acta Neuropathol Commun. 2014;2:135.

100. Charkhkar H, Meyyappan S, Matveeva E, Moll JR, Mchail DG, Peixoto N, et al. Amyloid beta modulation of neuron network activity in vitro. Brain Res. 2015;(November):1–9.

101. Hoppe JB, Haag M, Whalley BJ, Salbego CG, Cimarosti H. Curcumin protects organotypic hippocampal slice cultures from Aβ1-42-induced synaptic toxicity. Toxicol Vitr. Elsevier Ltd; 2013;27(8):2325–30.

102. Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, et al. Curcumin inhibits formation of amyloid β oligomers and fibrils, binds plaques, and reduces amyloid in vivo. J Biol Chem. 2005;280(7):5892–901.

103. Takahashi T, Mihara H. Peptide and protein mimetics inhibiting amyloid β-peptide aggregation. Acc Chem Res. 2008;41(10):1309–18.

104. Howlett D, Cutler P, Heales S, Camilleri P. Hemin and related porphyrins inhibit β-amyloid aggregation. FEBS. Federation of European Biochemical Societies; 1997;417(2):249–51.

105. Ishrat T, Hoda MN, Khan MB, Yousuf S, Ahmad M, Khan MM, et al. Amelioration of cognitive deficits and neurodegeneration by curcumin in rat model of sporadic dementia of Alzheimer's type (SDAT). Eur Neuropsychopharmacol. Elsevier B.V. and ECNP; 2009;19(9):636–47.

106. Neumann B, Yarman A, Wollenberger U, Scheller F. Characterization of the enhanced peroxidatic activity of amyloid β peptide-hemin complexes towards neurotransmitters. Anal Bioanal Chem. 2014;406:3359–64.

107. Multi Channel Systems. MEA Application Note : Primary Hippocampal Neurons from Rattus norvegicus. 2005. p. 12.

108. Cellular Dynamics International I. iCell® Neurons - Multielectrode Array System Application Proar Dynamicstocol. 2012. p. 1–6.

109. Sitterley G. Poly-lysine | Sigma-Aldrich [Internet]. 2008 [cited 2015 Sep 30]. Available from: http://www.sigmaaldrich.com/technical-documents/articles/biofiles/poly-lysine.html

110. Iorio V, Troughton LD, Hamill KJ. Laminins: Roles and Utility in Wound Repair. Wound Heal Soc. 2015 Apr 1;4(4):250–63.

111. SH-SY5Y (ATCC ® CRL-2266 ™). American Type Culture Collection; 2014. p. 4–6.

112. Louis KS, Siegel AC. Cell viability analysis using trypan blue: manual and automated methods. Methods Mol Biol. 2011 Jan;740:7–12.

113. Sellstrom A, Jacobson S. Modern Techniques in Neuroscience Research. Springer Science & Business Media; 2012. 1328 p.

114. Marvin JS, Borghuis BG, Tian L, Cichon J, Harnett MT, Akerboom J, et al. An optimized fluorescent probe for visualizing glutamate neurotransmission. Nat Methods. 2013;10(2):162–70.

7. Annexes

7.1 MEAs Handling

In this section one can find more info on how MEAs were manipulated, conserved and kept sterilized during and in-between experiments.

7.1.1 Precautions

Due to its fragility, MEAs were maintained inside a sterile 100 mm Petri dish at all times. MEAs are glass made and any crack in its substrate renders them unusable. Concerning this, any MEA manipulation was made on a flat and completely stable surface. Placing the Teflon lid and pipetting onto the MEA were especially delicate steps. It was vitally important that no solid object (e.g., pipette tips) ever touched the array as it could irreversibly damage the electrodes.

When needed, MEAs were gently transported while keeping the bottom of the dish parallel to the ground. Transferences from the storage incubator to the recording system were especially critical, as quick movements with the MEA or shaking could detach the cell culture from the substrate.

7.1.2 Sterilizing and Reusing

Prior to cell culturing, MEAs have to be sterilized with standard methods such as immersion in 70% ethanol, UV-light exposure, dry-heat sterilization, or vapor autoclavation (43,78). Furthermore, any work with an open MEA (without Teflon lid) must take place in a standard cell culture laminar flow cabinet to minimize the risk of contamination.

After unsatisfactory results with a 70% ethanol sterilization procedure (cell cultures were often inviable and ethanol could fix cell debris), vapor autoclavation was chosen as standard. Therefore, in order to sterilize the MEAs, each MEA was rinsed with sterile distilled water and allowed to dry under a laminar flow cabinet. Then, MEAs were carefully inspected for cleanliness, intact insulation and cell debris from previous experiments under an optical microscope. While inside a borosilicate glass Petri dish, MEAs were vapor autoclaved at 121 °C for 20 minutes. Typically, MEAs were allowed 2 – 3 hours for cooling and 1 day for complete drying. When not needed for immediate use, arrays were immersed in sterile distilled water and stored at 4 °C in the dark (to prevent growth of algae).

When reusing MEAs that had cell cultures on, the organic matter was completely removed first. Therefore, 500 µl of 0.05% trypsin was added to the array and the MEA was incubated at 37°C for 30 minutes. The array was then rinsed with distilled water and inspected under an optical microscope. If cellular matter still remained, the trypsin step was repeated. When the array was completely clean, the sterilization step proceeded.

7.2 Setting up the MEA System

In order to set up the MEA2100-System correctly, various system manuals were consulted. When preparing such delicate experiments, one must be extremely cautious in order to obtain reliable recordings. Therefore, settings were formerly and thoroughly tested.

MEA2100-System Manual (2014) states that all system devices must be placed on a stable and dry surface, where air can circulate freely and the devices are not exposed to direct sunlight. The data acquisition computer, the interface board and the temperature controller were all connected via power supply to a power outlet of the same electrical system. The headstage was connected via an eSATAp cable to the interface board. In turn, the interface board was connected via an USB high speed 2.0 cable to the data acquisition computer. The internal heating element of the MEA2100 headstage was also connected to the temperature controller, which in turn connected with the interface board. Consequently, the interface board transmitted all information (data recorded with the headstage and set temperature) to the data acquisition computer letting complete control over the experiment.

The MEA socket in the base plate featured a resistive heating element and a Pt-100 temperature sensor. An external T-control unit (Multi Channel Systems, HC-1) kept the temperature of the socket surface at less than or equal to 36.5°C.

MC_Rack software was installed in the data acquisition computer. This software let complete control over pre, during and post-experiment parameters.

MEAs are not symmetrical. When placing the MEA on the headstage, the MEA chip should be on the right side viewed from the front. MEAs have one big iR that should be placed with reference electrode to the left side in the amplifier. Otherwise, the MEA layout does not match with the pin layout of the channel map in MC_Rack.

7.3 List of Materials

7.3.1. Devices

- Hera cell CO2 incubator (Heraeus);
- Safety cabinet Hera safe (Heraeus);
- Inverted optical microscope (LEICA);
- Up-right optical microscope (Olympus);
- Hemacytometer (Sigma-Aldrich);
- Bath SBB6 (Grant);
- Culture Plates (Corning);
- 60MEA200/30iR-Ti-gr MEAs (Multichannel Systems);
- ALA-MEA MEM (ALA Scientific Instruments);
- Ultra-stable platform (Scientifica);

MEA2100-System (Multichannel Systems) set up:

- MEA2100-HS2x60 headstage for 2 x 60-electrode MEAs;
- Interface Board;
- T-control unit;
- Data acquisition computer (LG);
- MC-Card.

Software:

- MC_Rack;
- GraphPad Prism version 6.0;
- Microsoft Excel.

7.3.2 Reagents and Solutions

Complete growth medium 10% FBS MEM:F12 (1:1):

• MEM (Gibco, Invitrogen)	4.805 g
• F12 (Gibco, Invitrogen)	5.315 g
• NaHCO3 (Sigma)	1.7 g
• Sodium Pyruvate (Sigma-Aldrich)	0.055 g
• 1% Antibiotic/Antimycotic (AA) mix (Gibco, Invitrogen)	10 mL
• 10% FBS (Gibco, Invitrogen)	100 mL
• L-Glutamine (200 mM stock solution)	2.5 mL

Adjust to pH 7.4 and to a final volume of 1000 mL in dH_2O. Sterilize by filtering through a 0.2 µm filter and store at 4ºC.

Other consumables:

- PBS (Sigma-Aldrich);
- Ethanol;
- Trypsin-EDTA solution (Sigma-Aldrich);
- Poly-D-Lysine (Sigma-Aldrich);
- Laminin (Sigma-Aldrich);
- Polyethileneimine (Sigma-Aldrich);
- DMSO (Fisher Scientific);
- Synthetic Aβ 1-42 (Genic Bio).